ABSTRACT

Title of Document: DECIPHERING THE SECRET OF SARCOMERE ASSEMBLY AND DISEASES USING THE ZEBRAFISH MODEL SYSTEM: REGULATION OF MYOFIBRILLOGENESIS BY SMYD1B AND ITS PARTNERS

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Myofibrillogenesis is a process of precise assembly of sarcomeric proteins into the highly organized sarcomeres which are essential for muscle cell differentiation and function. Myofibrillogenesis requires proper folding and assembly of newly synthesized sarcomeric proteins. Mutations of the sarcomeric proteins are known to cause skeletal and cardiac muscle diseases.

smyd1b is a skeletal and cardiac muscle-specific gene which encodes two alternatively spliced isoforms, *smyd1b_tv1* and *smyd1b_tv2*. Knockdown of *smyd1b* (tv1 and tv2) expression resulted in zebrafish larvae without locomotion and heart contraction. Thick filament assembly was significantly disrupted in *smyd1b* knockdown embryos. Yeast Two-Hybrid study showed that Smyd1 associates with another muscle-specific protein—skNAC, however, skNAC function in muscle cells is unknown.

In order to expand the understanding of *smyd1b* function and study the working mechanism, I further characterized the function of Smyd1b and its partners including skNAC and Hsp90α1 during muscle development, and carried out mechanistic studies using zebrafish as a model system.

Our findings show that: 1) In addition to the thick filament, *smyd1b* plays an important role in the assembly of thin and titin filaments, as well as Z-line and M-line. 2) Knockdown of *smyd1b* has no effect for heart tube formation; however, it disrupts the myofibril assembly of the cardiac muscle that causes the heart defect. 3) Smyd1b_tv1, but not Smyd1b_tv2 can be localized on the M-line of sarcomeres. 4) Ser225 on Smyd1b_tv1, which is a potential phosphorylation site, is important for the M-line localization of Smyd1b_tv1. 5) Knockdown of *smyd1b* causes the upregulation of *hsp90α1* and *unc45b* gene expression. 6) *hsp90α1* plays an important role for myofibril assembly. 6) Knockdown of *smyd1b* or *hsp90α1* causes the reduction of myosin protein accumulation. 7) Smyd1b_tv1, but not Smyd1b_tv2 associates with the myosin chaperones Hsp90α1 and Unc45b. 8) *sknac* is required for

the thick and thin filaments assembly. 9) Knockdown of *sknac* causes the reduction of myosin protein accumulation.

These studies provide us an in-depth characterization of *smyd1b* and its partners' function and expands the mechanistic understanding of how *smyd1b* fulfils its vital role in myofibrillogenesis. Most importantly, this study provides new insights to help us understand the complex process of myofibrillogenesis and sarcomere diseases.

DECIPHERING THE SECRET OF SARCOMERE ASSEMBLY AND DISEASES USING THE ZEBRAFISH MODEL SYSTEM: REGULATION OF MYOFIBRILLOGENESIS BY SMYD1B AND ITS PARTNERS

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Dissertation submitted to the Faculty of the Graduate School of the

University of Maryland, College Park, in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

2009

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Acknowledgements

I would like to express my sincere appreciation to all my friends and colleagues who have helped me in the completion of this work, special sincere gratitude and appreciation to my advisor and mentor, Dr. Shao Jun Du. Dr. Du taught me how to be a scientist, supported me and guided me through the past five years of my Ph.D. He was always patient to assist me in numerous ways, including how to design and conduct an experiment, how to write a manuscript and how to make a presentation. What I learned from him will benefit me for life.

I am very grateful to my committee members, Dr. William Randall, Dr. Shengyun Fang, Dr. Rachel Brewster, Dr. Rosemary Jagus, Dr. Wenxia Song and Dr. Chen-Ming Fan. They gave me guidance and invaluable scientific insights to allow me to make a constant progress for my project. I sincerely appreciate their time and effort. Special thanks to Dr. William Randall and Dr. Shengyun Fang for their help and guidance to me to finish some of my work in their labs.

I want to extend my thanks to the past and present Du lab members Dr. Pep Rotllant, Dr. Yue-Hong Bian, Dr. Rebecca Petre and Dr. Elena Bernick, who gave me big support and kind help; to the past and present members of Dr. Zohar's lab, Dr. Wei Xia and Dr. Eytan Abraham who help us to maintain the zebrafish facility; to the friendly COMB community who gave me help and happiness. I will miss you all.

Finally, and most importantly, I would like to thank my husband Haishan. His endless love, support and help made this jounery possible and joyful. I am forever grateful to my loving and supporting parents, who have given me encouragement and support to pursue my studies.

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Abbreviations

AP	Anteroposterior
bHLH	Basic helix-loop-helix
BTS	N-benzyl-p-toluene sulphonamide
CHIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
CtBP	C-terminal binding protein
DM	Differentiating medium
dpf	Days post fertilization
ECM	Extracellular matrix
Ef1α	Elongation factor 1α
FGF	Fibroblast growth factor
FMHC	Fast muscle heavy chain
GM	Growth medium
HATs	Histone acetyltransferases
Hh	Hedgehog
HDAC	Histone deacetylase
HDGF	Hepatoma derived growth factor
hpf	Hours post fertilization
HMTase	Histone methyltransferase
Hsp	Heat-shock protein
IP	Immunoprecipitation
LMB	Leptomycin B

MEF2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
miRNA	Micro ribonucleic acid
MO	Morpholino
MRFs	Myogenic regulatory factors
MYND	Myeloid-Nervy-DEAF-1
NAC	Nascent polypeptide-associated complex
NLS	Nuclear localization signal
PBST	Phosphate buffered saline
qPCR	Quantitative polymerase chain reation
RT-PCR	Reverse transcriptase polymerase chain reaction
SET	Su(va)-39, enhancer of zeste and trithorax
Shh	Sonic hedgehog
SMHC	Slow muscle heave chain
Smyd1b_tv	Smyd1b transcription variant
SRF	Serum response factor
TGF-β	Transforming growth factor β
Tg	Transgenic
Unc45	Uncoordinated 45
VEGFR1	Vascular endothelial growth factor receptor 1
WT	Wile type
ZF	Zebrafish

CHAPTER 1: GENERAL INTRODUCTION

1. Skeletal muscle origin and somite formation

The muscle progenitors originate from the paraxial mesoderm which lies on either side of the axial mesoderm along the embryo's axis. Specification of paraxial mesoderm is controlled by distinct signals such as fibroblast growth factor (FGF), Wnt signal transduction components, from the anterior and posterior regions of the embryos (Kimelman & Griffin 2000).

Once the anteroposterior (AP) axis of the embryos forms; the paraxial mesoderm would undergo a process of segmentation to establish the characteristic metameric body plan of vertebrates. Segmentation is closely coupled with somite formation in bird and mammalian embryos. Somites form by epithelialization of the paraxial mesoderm along either side of the neural tube (Engel et al 2004). However, in fish and frog, the embryos develop more rapidly than avian and mammalian embryos. Paraxial mesoderm in these embryos becomes segmented without epithelialization and somite formation (Pownall et al 2002).

The segmentation and somite formation are coupled to the gene regulatory program that initiates expression of regulatory genes for specification of muscle progenitors. The gene regulatory program is initiated by the inductive signals from the surrounding tissue of the myogenic progenitors (Boryck and Emerson, 1999).

2. Molecular control of muscle differentiation

Myoblast differentiation is a complex process which is regulated by the well-known transcriptional networks involving myogenic regulatory factors (MRFs) and myocyte enhancer factor 2 (MEF2), as well as growth factors such as fibroblast growth factor (FGF) and signaling molecules such as sonic hedgehog (Shh). The discovery of miRNA as a previously unrecognized component of this complex process starts to add an entirely new layer of complexity at the post-transcriptional level to our understanding of how muscle gene expression is regulated (Chen et al 2009).

1) Muscle differentiation regulated by transcription factors

The myogenic regulatory factors are well studied for their regulatory functions in the specification and differentiation of muscle progenitors in vertebrate and invertebrate embryos. MRFs are an evolutionarily conserved family of four bHLH (basic helix-loop-helix) transcription factors including MyoD, Myf5, Myogenin, and MRF4 (Davis et al 1987, Emerson 1990). MRF expression is highly restricted to skeletal muscles in vertebrate and invertebrate embryos (Pownall et al 2002).

Myf5- and *MyoD*- expressing myoblasts are a renewable source of progenitors that proliferate in the embryos at sites of myogenesis. The daughter cells of the progenitors undergo G1-specific cell-cycle withdrawal to initiate myocyte differentiation. *MyoD* expression persists in newly formed differentiated muscle

fibers, while *Myf5* expression is shut down during differentiation (Engel et al 2004). The expression pattern indicates their functions in myoblast differentiation.

MRFs coordinates with MEF2 to control the transcriptional activation of genes encoding contractile and scaffold proteins. Vertebrates have four mef2 genes, mef2a, b, c, and d. These are members of the MADS domain family of transcription factors (Molkentin & Olson 1996, Naya et al 1999). Accumulation of contractile proteins is controlled by the coordinated transcriptional activation of muscle genes including isoforms for all of the functionally related muscle-specific myofibrillar proteins as well as proteins involved in synapse formation and calcium signaling.

2) Muscle differentiation regulated by growth factors and signaling molecules

Growth factors and signaling molecules have been reported to play key roles to control myoblast proliferation and differentiation. FGF2 is a key growth factor regulator of myoblast proliferation and an inhibitor of differentiation in cell culture (Clegg et al 1987). The TGF-β ligand GDF-10 (also known as Myostatin) also has a role in the control of muscle differentiation. Mice and cattle mutant for GDF-10 have significantly increased muscle mass (McPherron et al 1997, McPherron & Lee 1997). Shh also is shown to control muscle proliferation and differentiation in the limb. Study shows that retroviral misexpression of Shh in limb progenitors increases muscle growth likely by promoting myoblast proliferation to control the initiation of differentiation (Bren-Mattison & Olwin 2002, Duprez et al 1998). However, it remains to be determined of how FGF, GDF-10 and Shh signaling are coordinated to control myogenesis.

3) Muscle differentiation regulated by microRNAs

microRNAs (miRNAs) are RNAs which are about 22 nucleotides long that control gene expression at the post-transcriptional level through various regulatory mechanisms. Those regulatory mechanisms include messenger RNA (mRNA) deadenylation, translation, and decay of target mRNAs (Bushati & Cohen 2007, Filipowicz et al 2008). A subset of miRNAs are either specifically or highly expressed in muscles, providing an opportunity to understand the post-transcriptional level regulation of gene expression in muscles by miRNAs (Chen et al 2006, Lagos-Quintana et al 2002, Wienholds et al 2005).

Studies have shown that the serum response factor (SRF) and MEF2, which are known for their regulation on muscle differentiation, can both regulate the expression of two pairs of related muscle-specific miRNAs genes: miR-1-1 and miR-133a-2, and miR-1-2 and miR-133a-1 (Chen et al 2006, Liu et al 2007, Niu et al 2007, Rao et al 2006). A third pair of the skeletal muscle-specific miRNAs, miR-206 and miR-133b are controlled by MEF2, MyoD, myogenin and other regulators (Kim et al 2006, Rao et al 2006, Rosenberg et al 2006). miR-1 promotes muscle differentiation by repressing the expression of histone deacetylase 4 (HDAC4) which can repress MEF2 activity to inhibit muscle differentiation. However, miR-133 reduces protein levels of SRF and results in enhancing the proliferation of myoblasts and inhibiting myoblast differentiation (Chen et al 2009). The identification of these previously unrecognized regulators provides new insights on the molecular mechanisms that underlie muscle development.

3. Muscle maturation and muscle fiber type

The postmitotic daughters of myoblasts are called myocytes. During muscle development, the mononucleated myocytes fuse to form multinucleated skeletal muscle cells termed myotubes. There are two waves of mononucleated cell proliferation which result in the formation of primary and secondary myotubes. They share a common basal lamina and are coupled by gap junctions (Engel et al 2004). The myotube maturation is a process that the centrally positioned nuclei move to the periphery. The primary and secondary myotubes lose their interconnecting junctions and gain their own basal lamina to become independent muscle fibers (Ling et al 1992, Rubinstein & Kelly 1981, Takekura et al 1993).

Vertebrate skeletal muscles are typically composed of slow- and fast-twitch fibers that differ in their morphology, gene expression profiles, contraction speeds, metabolic properties and patterns of innervation. In mammalian embryos, positions of fiber-type-specific myogenic precursor cells are unknown. During myogenesis, how muscle precursors are induced to mature into distinct slow- or fast-twitch fiber types is inadequately understood (Liew et al 2008).

In zebrafish, slow and fast muscle precursors occupy separate positions within the embryo and their origin has been characterized (Bessarab et al 2008, Devoto et al 1996, Ingham & Kim 2005). Slow muscle precursors (adaxial cells) are the most medial cells in the segmental plate and they are located on either side of the notochord (Devoto et al 1996). Upon somite formation, adaxial cells elongate and migrate to the surface of the myotome forming a superficial layer of mononucleated

slow MyHC-positive fibers. Cells of the segmental plate located laterally to the adaxial cells develop into fast muscle (Devoto et al 1996). After the differentiation program of the slow muscle cells is underway, myoblasts committed to the fast-twitch fate fuse with each other and mature into arrays of syncytial fibers that ultimately constitute the bulk of the myotome (Groves et al 2005, Hamade et al 2006, Liew et al 2008, Roy et al 2001, Srinivasan et al 2007).

The relative simplicity of cell lineages within the zebrafish myotome makes it an excellent system to dissect the genetic pathways that specify the fates of the distinct muscle cell-types. Studies have shown that loss-of-function mutations in the Hedgehog (Hh) signaling pathway eliminate the specification of slow myoblasts (Barresi et al 2000, Lewis et al 1999). Conversely, ectopic Hh can transform somitic cells located in a more lateral position away from the midline, which normally adopt the fast-twitch fate, into slow-twitch muscles (Blagden et al 1997, Du et al 1997, Hammerschmidt et al 1996).

Compared with slow muscle development, less is known about signals that promote development of fast muscle. The mammals Six1 homolog Six1a, which is a homeodomain transcription factor is implicated to be required for the onset of fast muscle differentiation in zebrafish (Bessarab et al 2008). The medial to lateral wave of fast muscle fiber is shown to be induced by slow muscle cells migration (Henry & Amacher 2004).

4. Myofibrillogenesis and sarcomere assembly

During muscle cell differentiation, contractile proteins are coordinately expressed. The newly synthesized proteins must assemble together into highly complex myofibrils. This process is called myofibrillogenesis which requires the ordered synthesis and assembly of the sarcomeric proteins. One muscle cell typically contains bundles of myofibrils, each consisting of many sarcomeres, the basic functional contractile unit of muscle. Sarcomere is a highly organized structure. The initial step of myofibrillogenesis, also called myofibril assembly, is the formation of a regular array of sarcomeres. The sarcomeres later grow in width and in some cases in length, and eventually align and attach to each other and the sarcolemma. The details of this process are not well understood. An important question in muscle differentiation is how muscle cells produce these myofibrils with such regular arrays of sarcomeres (Sparrow & Schock 2009).

1) Structure of the sarcomere

A sarcomere is defined as the segment between two neighbouring Z-lines (or Z-discs, or Z bodies), in which thin filaments are anchored and crosslinked by dimeric actin binding α-actinin molecules (Clark et al 2002, Frank et al 2006). Thin filaments consist of actin and the tropomyosin–troponin complex. The head of myosin in thick filaments binds to the actin of thin filaments to produce contraction. Thin filaments are polar and their plus and minus ends are capped by CAPZ and tropomodulin, respectively. Vertebrate skeletal muscle thin filaments additionally contain nebulin, a

long protein that regulates the length of thin filaments (Sparrow & Schock 2009). M-line is localized in the middle of the sarcomere which crosslinks and anchors the thick filaments. The region that the thick filaments occupy is called the A‑band, whereas the region in which thin filaments do not overlap with thick filaments is known as the I‑band. The interactions between actin and myosin generate contractile forces and the sarcomeres shorten owing to the sliding of the two filament systems. The giant protein titin spans the half sarcomere with their amino termini anchor at the Z-line and their carboxyl termini anchor at the M-line (Labeit & Kolmerer 1995, Trinick 1996). Titin anchors at the Z-line, closes to the thin filament, it crosses the I-band and then binds along thick filament as far as the M-line. The I-band region of titin forms an elastic element, which connects the Z-line to the thick filaments (Sparrow & Schock 2009).

Myofibril termini attach to the skeleton at myotendinous junctions or are connected end to end at intercalated discs in cardiac muscle. Heterodimeric integrins, which connect thin filaments to extracellular matrix (ECM) ligands, are the main structural and functional components of myotendinous junctions. Peripheral myofibrils are also laterally anchored to the ECM at the level of the Z-disk in both vertebrates and invertebrates (Danowski et al 1992, Hudson et al 2008, Pardo et al 1983). These adhesion sites are termed costameres, and, as with myotendinous junctions, they consist of many components that are typically found in integrin adhesion sites (Ervasti 2003, Pardo et al 1983, Quach & Rando 2006).

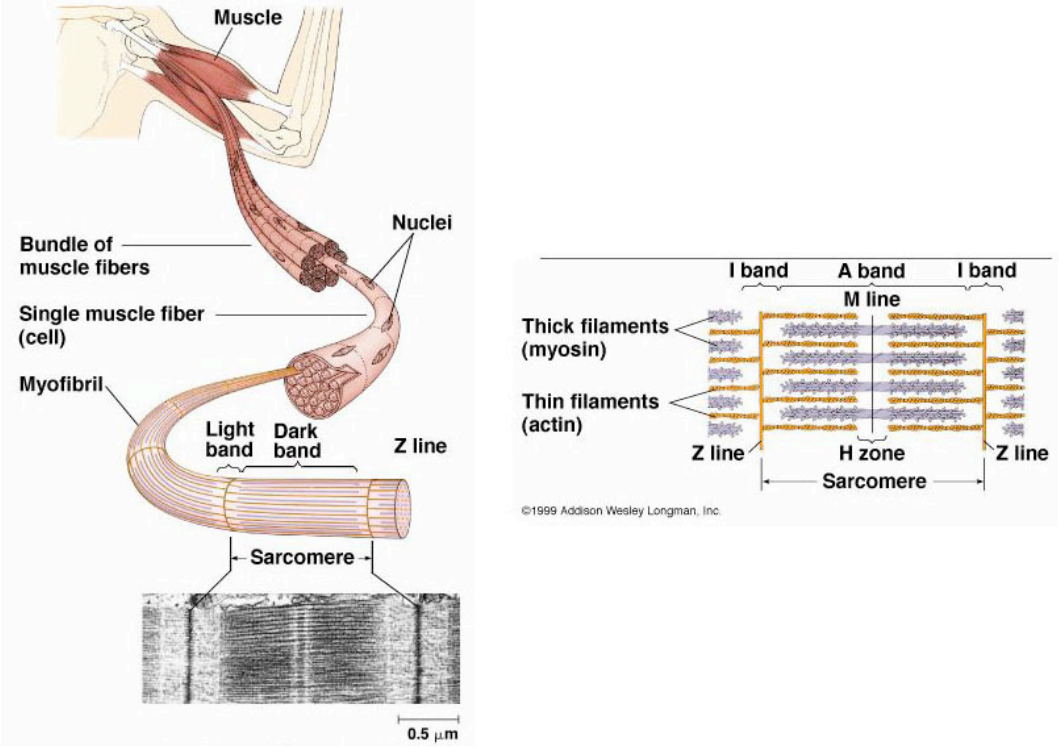


Figure 1. Structure and organization of the skeletal muscle and sarcomere.

The skeletal muscle consists of many muscle fibers. Each muscle fiber is composed of a lot of myofibrils. The basic unit of the myofibril is called sarcomere which is composed of hundreds of highly organized filamentous proteins. (http://themedicalbiochemistrypage.org/muscle.html)

2) Current models for sarcomere assembly

Currently there are three prevalent models for the myofibrillogenesis process. While all these models agree with one point of view that the first step of myofibrillogenesis occur in association with the cell surface or sarcolemma (Engel et al 2004), it still remains to be discovered for the specific assembly events which lead to a whole mature myofibril.

Premyofibril model of myofibrillogenesis

The main theory for this model is that there are three sequential structures exist during myofibrillogenesis, which are premyofibrils, nascent myofibrils and mature myofibrils (Du et al 2003, Rhee et al 1994). In this model, premyofbrils are considered to consist of minisarcomeres with the Z-bodies containing muscle alpha-actinin as the boundaries. The actin filaments interdigiate with nonmuscle myosin Π filaments and the barbed ends of the actin filaments are anchored on the Z-bodies. With the alignment and growth of the premyofibrils, titin and muscle myosin Π appear in fibrils. The fibrils of this stage are termed nascent myofibrils. With the transformation of Z-bodies to Z-lines or Z-bands and the alignment of muscle myosin Π into A-bands, the mature sarcomere structure is set up. M-band proteins may be responsible for the final alignment of the thick filaments (Sanger et al 2005). Most of the studies for this model were done in living avian cells, first from cardiac muscle

cells and then extend to skeletal muscle cells. More recently, this group confirmed their model in zebrafish embryos (Sanger et al 2009).

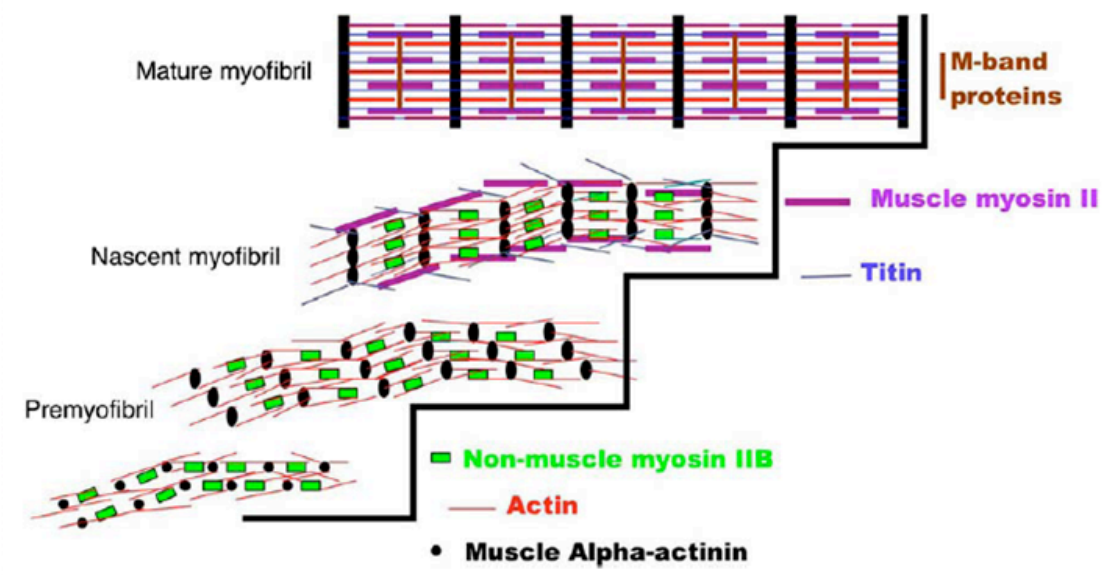


Figure 2. The premyofibril model of myofibril assembly.

Assembly begins premyofibrils composed of minisarcomeres that contain sarcomeric proteins in the α-actinin enriched z-bodies and thin filaments of actin. Nonmuscle myosin II filaments are present in the minisarcomeres of the premyofibrils. Z-bodies in adjacent fibrils begin to align in nascent myofibrils, forming beaded Z-bands that gradually become linear Z-bands or Z-lines in mature myofibrils. Titin molecules and muscle myosin II thick filaments are also present in nascent myofibrils. The thick filaments in the nascent myofibrils are not aligned, but are in an overlapped relationship. M-band proteins are recruited to the mature myofibrils; thick filaments become aligned into A-bands, while nonmuscle myosin II proteins are absent (Sanger et al 2005).

Template model of myofibrillogenesis

The main theory of this model is that at the early stage of myofibrillogenesis, there are stress fibers or stress-line structures which serve as a temporary template for the elements necessary to make one myofibril (Dlugosz et al 1984). The studies for this model were conducted on fixed and stained cell. However, later studies in living muscle cells showed that the stress fiber-like structures in muscle cells contains muscle-specific proteins (Almenar-Queralt et al 1999, Rhee et al 1994). In addition, those structures can response to inhibitors of polymerization which are not shown by stress fibers (Sanger et al 1990).

Independent subunit assembly model of myofibrillogenesis

The third model for myofibrillogenesis proposes that the I-Z-I bodies (which contain actin filaments, titin and Z bodies) and thick filaments assemble independently of one another. Titin plays an important role in joining them together and promote their assembly into mature myofibrils (Epstein & Fischman 1991, Holtzer et al 1997, Lu et al 1992). This model was first proposed from the studies in cardiac muscle cells, and then extended to skeletal muscle cells (Holtzer et al 1997, Lu et al 1992).

All these models focus on the morphogenesis of the sarcomere structure and only a rough structure has been set up. It remains undetermined of how most of the sarcomeric proteins are assembled and organized, as well as of how myofibrillogenesis is regulated by different kinds of factors.

5. Molecular regulation of myofibrillogenesis

During myofibrillogenesis, there are different levels of regulation to make it possible to set up and maintain the highly organized structure of the myofibrils. It was discussed earlier that the expression of contractile and scaffold proteins are regulated by transcription factors such as MRFs and MEF2. In addition, histone modification in transcriptional regulation of muscle gene expression has become another hot topic in recent years. Moreover, translational and post-translational regulations are very important for the folding and assembly of the myofibril proteins.

1) Myofibrillogenesis regulated by histone modification

Histone modifications such as histone acetylation and histone methylation are common reversible posttranslational modifications on histone tails that modulate chromatin structure and gene transcription, both positively and negatively. Many studies have shown that histone modifications play important roles in muscle-specific gene expression and muscle cell differentiation (Cirillo & Zaret 2004, Lee et al 2004, McKinsey et al 2002, Rupp et al 2002).

Histone acetylation is a dynamic process controlled by the antagonistic actions of two large families of enzymes − the histone acetyltransferases (HATs) and the histone deacetylases (HDACs) (Haberland et al 2009). Function for HDACs has been described during skeletal muscle development. The transcription factor MEF2 is a target for calcium signalling in skeletal muscle and is a key regulator of the slow myofiber phenotype. This function of MEF2 is mediated through its regulation by

class IIa HDACs (Potthoff et al 2007). In addition, a highly conserved MEF2-binding site in the proximal promoter of the HDAC9 gene has been shown to drive the expression of HDAC9, which establishes a negative feedback loop in which MEF2 drives the expression of its own repressor. This feedback loop is thought to provide robust and fine-tune to the gene programming controlled by HDAC9 and MEF2 (Haberland et al 2007).

Histone methylation is carried out by a class of enzymes called histone methyltransferase (HMTase). SET domain-containing family members have been well studied for their regulation on histone methylation (Cheng et al 2005, Rea et al 2000). More and more studies have shown the function of SET domain-containing proteins during muscle development. Previous study has reported that the histone methyltransferase, Ezh2, is a negative regulator of muscle differentiation, and its expression is down-regulated during myogenesis (Caretti et al 2004). Studies from our group have demonstrated that a muscle-specific SET domain-containing protein Smyd1b can work as an HMTase to control myofibril assembly (Tan et al 2006).

Although these studies have shown that histone modification enzymes control the expression of myofibril proteins, it remains to be determined of how they control the myofibril assembly, whether they are involved directly or through their target genes.

2) Myofibrillogenesis regulated by molecular chaperones

The myofibrils are highly organized macromolecular structures. Myofibrillogenesis demonstrates the need for intricately protein quality control to

ensure the assembly of macromolecular structures such as the sarcomere. The synthesis, assembly and organization of sarcomeric proteins during myofibrillogenesis require the temporal and spatial regulation by specialized molecular chaperones (Kim et al 2008).

Hsp90 and myofibrillogenesis

Although the precise folding and assembly of myosin has not been well studied, it is widely known that chaperone-mediated myosin folding is an integral part of myofibril assembly (Hutagalung et al 2002). Efficient folding and assembly of myosin isoform is possible only when it is expressed in muscle cells, suggesting that unique components from muscle cells are required for myosin folding and assembly (Chow et al 2002). Heat-shock proteins (Hsps) are molecular chaperones for huge numbers of proteins and Hsp90 alone may be able to interact with more than 400 different proteins (Zhao et al 2005). *In vitro* studies in myocytes indicated that Hsp90 forms a complex with newly synthesized myosin proteins and is involved in myosin folding and assembly. Inhibition of Hsp90 function by geldanamycin blocked myofibril assembly and triggered accumulation of myosin folding intermediates in C2C12 myocytes (Srikakulam & Winkelmann 2004). Hsp90α is expressed strongly in developing somites and skeletal muscles during zebrafish development, but the *in vivo* function of Hsp90α in vertebrate development has not been revealed (Sass et al 1999).

Unc45 and myofibrillogenesis

UNC-45 functions both as a molecular chaperone and as an Hsp90 cochaperone for myosin folding and assembly during myofibrillogenesis (Etard et al 2007). Genetic studies in *C. elegans* showed that UNC-45 played an essential role for myosin assembly. Mutations in UNC-45 resulted in paralyzed animals with severe myosin disorganization (Barral et al 1998, Epstein & Thomson 1974). In vertebrates, two *unc45* isoforms have been identified: a general cell ubiquitous isoform named *unc45a* and a striated muscle-specific isoform named *unc45b*. Knockdown or mutation of *unc45b* resulted in zebrafish embryos with no locomotion and a loss of myosin filaments in trunk muscles (Etard et al 2007, Wohlgemuth et al 2007).

Myofibrillogenesis and other molecular chaperons

Besides Hsp90, other heat-shock proteins can also work as molecular chaperones for muscle proteins. For example, the small heat-shock protein αB-crystallin exerts chaperone activity on a variety of filamentous and cytoskeletal proteins such as actin and the intermediate filament protein desmin (Bennardini et al 1992, Singh et al 2007). Two more molecular chaperones, GimC (also known as Prefoldin) and TRiC (TCP-1 Ring Complex, also known as CCT), are also known to play a synergistic role in the process of actin folding (Kim et al 2008). During actin translation, GimC can capture nascent actin polypeptide chains and help actin folding (Hansen et al 1999). GimC also works as a co-chaperone for the general chaperone TRiC. Partially folded actin is handed over from GimC to TRiC to mediate the subsequent maturation of actin. (Siegers et al 1999, Vainberg et al 1998).

6. Mutations of sarcomeric proteins cause sarcomere diseases

Mutations of the sarcomeric proteins are known to cause skeletal and cardiac muscle diseases. The diseases vary in severity from paralysis at birth, to mild conditions compatible with normal life span (Laing & Nowak 2005). Deciphering the precise relationships among striated muscle components often reveals candidate molecules for myopathies (the genetic lesions for which had not been identified) (Clark et al 2002). It has been known that human skeletal myopathies are associated with mutations in at least 20 sarcomeric proteins (Bonnemann & Laing 2004, Laing & Nowak 2005). Similarly 300 dominant mutations in sarcomeric proteins have been identified to be associated with human cardiomyopathies (Bashyam et al 2003, Morita et al 2005, Redwood et al 1999). Because of the nature of these mutations, familial hypertrophic cardiomyopathy (familial HCM or FHC) is known as a "disease of the sarcomere" (Thierfelder et al 1994). Mutations in myosin, a molecular motor, are responsible for both skeletal myopathies and cardiomyopathies (Fananapazir et al 1993, Laing & Nowak 2005). The identification of the disease genes allows more accurate diagnosis, including prenatal diagnosis, and to enhanced potential for prognosis, genetic counseling and developing possible treatments for these diseases (Laing & Nowak 2005).

Current knowledge on the relation between contractile protein dysfunction and the muscle disease pathogenesis has failed to decipher the mechanistic links between the gene mutations of sarcomere proteins and skeletal myopathies (Tajsharghi 2008). Strong efforts have been made to understand the myofibrillogenesis in both normal muscle development and muscle myopathy. Those efforts have benefited a lot from the use of model systems such as cultured myocytes from chick, rat and mouse, and from animal model systems including Drosophila, *C. elegans*, rodents and zebrafish. (Chien 2000, Gordon & Hoffman 2001, Marian & Roberts 2001, Seidman & Seidman 2001, Towbin & Bowles 2002, Tubridy et al 2001).

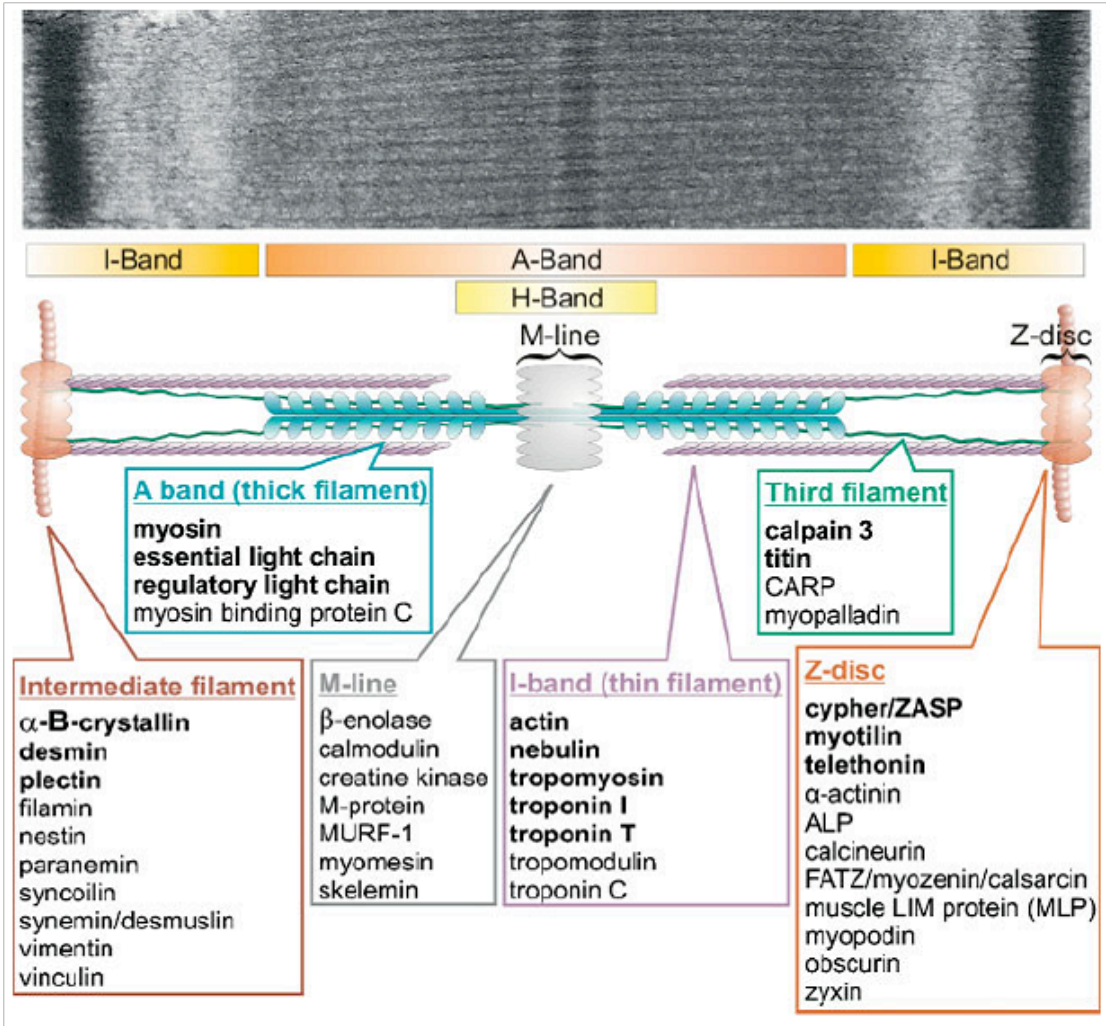


Figure 3. EM picture of a single sarcomere.

Underneath this is a schematic diagram showing the major compartments of the sarcomere. Proteins known for each compartment are listed. Those knownto be mutated in human skeletal muscle diseases are highlighted in bold. Those proteins not yet associated with human diseases are in plain text (Laing & Nowak 2005).

7. Zebrafish as a model to study muscle development and myofibrillogenesis

The zebrafish (Danio rerio) is a small tropical freshwater fish, a cyprinoid teleost, which originated in rivers in India. They are easy to maintain in aquariums. The emergence of using zebrafish as a model organism for modern biological investigation began with the pioneering work of George Streisinger and colleagues in the early 1980s (Streisinger et al 1981), who recognized many of the virtues of this experimental system. These include its short generation time, the large numbers of eggs produced by each mating, and the fact that, because fertilization is external, all stages of development are accessible (Briggs 2002). Now the zebrafish sequencing project is close to completion and it has been more and more widely used in forward and reverse genetics and embryology based on the gene similarity between the zebrafish and human being. Zebrafish genes share on average of more than 75% similarity to human genes. The characterization of many mutants from the large-scale mutagenesis performed to date has clearly and repeatedly demonstrated that mutations in zebrafish orthologues of human disease genes produce phenotypes similar to human disease states.

1) Zebrafish as a model in general muscle development

Zebrafish is an excellent model to study striated muscle development because it offers many advantages over other model systems. First, fish embryos develop

externally, simple patterns of motility and behavior can be observed during embryonic and early larval stages. Second, zebrafish embryos have a relatively simple muscle system, fast and slow muscles are clearly separated from each other, and specific muscle cell types are easily recognized. Third, myogenesis in zebrafish embryos begins relatively early during development, the first spontaneous muscle contractions occur at 18 hours post-fertilization (hpf) (Felsenfeld et al 1990). By 24hpf, functional embryonic myofibers are well developed and mechanical stimuli induce a wiggle reaction. Sarcomeres can be clearly visualized by anti-myosin antibody staining. Fourth, growing collections of zebrafish mutants with defects in early muscle development provide a rich resource for studying the roles of candidate genes in muscle development. Fifth, the transparent nature of the zebrafish embryos plus reliable transgenic technology provides a powerful tool to analyze the regulation of gene expression in developing embryos. Sixth, zebrafish embryos can tolerate absence of blood flow because the oxygen inside the zebrafish body is delivered by diffusion rather than by the cardiovascular system. This makes it possible to study the skeletal and cardiac muscle defects in zebrafish embryos with heart failure (Granato et al 1996). Finally, the morpholino (MO) antisense technique that has been successfully used to knock down gene expression in zebrafish can be designed to knock down specific isoform of mRNA transcripts generated by alternative splicing, which is difficult to do with gene knockout approach in mice (Ekker & Larson 2001).

2) Zebrafish as a model in myofibrillogenesis

Zebrafish is an ideal model system to study myofibril assembly. The optical clarity of the zebrafish body and well-defined sequence of embryonic muscle formation make it possible to observe the live muscle cell development with fluorescence *in situ* to determine how the sarcomeres are sequentially formed. In addition, the temporal progression of myofibril formation can be followed in a single fish in myotomes that form sequentially (Sanger et al 2009). Recent studies have been done in zebrafish to reveal how the sarcomeres are set up and how the myofibrillogenesis is regulated by all kinds of factors ranging from sarcomeric proteins to molecular chaperones.

Zebrafish as a model to study the function of sarcomeric proteins

Zebrafish genome can be easily manipulated by either gain-of-function method using mRNA injection or transgenic technology, or by loss-of-function experiments using morpholino knockdown technology (Nasevicius & Ekker 2000). Those technologies and large-scale mutagenesis screenings have been widely used in functional studies of sarcomeric proteins.

Studies have been done in zebrafish to discover the function of titin during myofibrillogenesis. Titin is the biggest known protein and is the third most abundant protein of vertebrate striated muscle, after myosin and actin (Tskhovrebova & Trinick 2003). It has been proposed that titin provides a scaffold for the assembly of thin and thick filaments (Gregorio et al 1999). Two titin orthologs have been found in zebrafish, *ttna* and *ttnb* (Seeley et al 2007). Loss of function study has shown that

ttna is required for later steps of sarcomere assembly, including the assembly of Z-discs and A-bands, but not for early steps such as the assembly of Z-bodies and nonstriated myosin filaments (Seeley et al 2007). This differs from other studies in which cultured cells were used (Person et al 2000, van der Ven et al 2000).

Obscurin/obscurin-MLCK is another giant sarcomere-associated protein with multiple isoforms. Obscurin intimately surrounds the myofibrils at the level of the Z disk and the M line (Kontrogianni-Konstantopoulos et al 2003). The interactions between obscurin with titin and small ankyrin-1 suggest an important role in myofibril assembly (Bang et al 2001, Kontrogianni-Konstantopoulos et al 2004, Kontrogianni-Konstantopoulos et al 2006, Russell et al 2002, Young et al 2001). Depletion of *obscurin* using morpholino antisense oligos resulted in diminished numbers and marked disarray of skeletal myofibrils. The Z band architecture of the individual sarcomeres was intact with normal patterns of α-actinin staining, however, thick filament organization was not well preserved and there was diminished M band localization of obscurin (Raeker et al 2006). This suggested that obscurin was incorporated into the sarcomere after the Z bodies were set up.

Zebrafish as a model to study the regulation of myofibrillogenesis

MEF2 is an important transcription factor that controls the expression of contractile and scaffold protein during myofibrillogenesis. Knockdown of *mef2s* in zebrafish severely disrupted thick filament assembly and caused aggregation of thin filament. The actin, a-actinin, titin Z-line region and tropomyosin showed as puncta in *mef2* morphants, suggesting myofibrillogenesis was stuck in a pre-myofibril stage

(Hinits & Hughes 2007). This study is consistent with the premyofibril model (Wang et al 2005).

As it was mentioned before, molecular chaprones can regulate myofibrillognesis by control the folding and assembly of the sarcomeric proteins. Some of those studies were conducted in zebrafish embryos with mutations or gene depletion using morpholino oligos (Etard et al 2007, Wohlgemuth et al 2007).

A recent study has been done for the regulation of sarcomere assembly by microRNAs. The muscle-specific miRNAs, miR-1 and miR-133 have been shown to regulate actin organization. Knockdown of either miR-1 or miR-133 had a mild effect in the actin band; however, knockdown of both disrupted actin organization. No effect was detected on actinin organization (Mishima et al 2009). It remains to be determined of how miRNAs regulate myofibrillogenesis.

8. smyd1b in myofibrillogenesis

1) The nomenclature of smyd1

In our lab, we are interested in a gene called *smyd1b*. It is also known as *skm-Bop* and was first identified as an unknown gene in the opposite transcription orientation from the CD8b gene (Hwang & Gottlieb 1995). It was named Bop after CD8b opposite. *Smyd1* (Bop) was first discovered in cytotoxic T cells and adult heart and skeletal muscle (Hwang & Gottlieb 1997). *Smyd1* belongs to the Smyd (SET and MYND domain) family that represents a subfamily of SET domain containing

proteins with unique domain architecture. This family of proteins is defined by a SET (su(va)-39, enhancer of zeste and trithorax) domain that is split into two segments by an MYND (myeloid-Nervy-DEAF-1) domain, followed by a cysteine-rich post SET domain (Rea et al 2000).

In zebrafish, there are two copies of *smyd1* genes because of the gene duplication during evolution. One of them is called *smyd1a* and the other one is called *smyd1b*. Each of them has two alternatively spliced isoforms. The gene we are studying is *smyd1b* and the two isoforms are termed *smyd1b_tv1* and *smyd1b_tv2*. The only difference between these two isoforms is that Smyd1b_tv1 has 13 amino acids more than Smyd1b_tv2. Generation of *smyd1b_tv1* and *smyd1b_tv2* by alternative splicing appeared to be conserved during evolution because identical isoforms have been cloned in chicken and mouse embryos (Gottlieb et al 2002).

2) Functional studies of smyd1b

smyd1 is known to be required for skeletal and cardiac muscle development. Functional studies by gene knockout have clearly demonstrated an important role for *Smyd1* in cardiac muscle formation in mouse embryos. *Smyd1* null mutant mice failed to develop a right ventricle and had excessive extracellular matrix that resulted in embryonic lethality at approximately day E10.5 (Gottlieb et al 2002).

In order to determine the function of *smyd1b* in skeletal muscles, our group studied the *smyd1b* expression and function in zebrafish embryos. Because zebrafish embryos can tolerate the absence of blood flow since their oxygen is delivered by diffusion rather than by the cardiovascular system, so this made it possible to study

smyd1b function during skeletal muscle development without heart beating. Knockdown of *smyd1b_tv1* and *smyd1b_tv2* expression in zebrafish caused severe defects in skeletal and cardiac muscles. The embryos could not swim and had no heartbeat. Molecular and cellular analyses revealed that thick filament myosin structure was highly disorganized in *smyd1b* morphant embryos (Tan et al 2006).

3) Gene regulation of smyd1b

smyd1b is specifically expressed in cardiac and skeletal muscles and this tissue-specific expression pattern is controlled by the -500 bp promoter region of *smyd1b* in zebrafish embryos. Multiple E-boxes (MRF binding site) were found in this region, which were shown to be important for the skeletal muscle-specific expression (Du et al 2006). In mouse, a -637 bp *Smyd1* regulatory region was identified to be required for *Smyd1* expression (Phan et al 2005). Within this regulatory region, a single MEF2 consensus-binding site was found as the MEF2C target to regulate *Smyd1* expression in the anterior heart field. In addition, three E-boxes surrounding the essential MEF2 site were indentified to bind MyoD and control Smyd1 expression in skeletal muscle (Phan et al 2005). Recently, Hepatoma-derived growth factor (HDGF) has been shown to repress human SMYD1 gene expression through interaction with a transcriptional corepressor C-terminal binding protein (CtBP), but no functional study has been done for this regulation (Yang & Everett 2009).

4) Subcellular localization of Smyd1b

The subcellular localization of Smyd1 was detected during myogenesis in C2C12 myoblasts undergoing differentiation using immunofluorescence (Sims et al 2002). Polyclonal mouse anti-Smyd1 antibody was used in this study. Twenty-four hours after the onset of differentiation, Smyd1 was present in both the nucleus and cytoplasm of myoblasts, while at 96 h following the switch to differentiation medium, Smyd1 displayed high levels of cytoplasmic staining in myotubes, whereas the nuclear staining was either absent or very low compared with the staining in the cytoplasm (Sims et al 2002). This indicates that Smyd1 may have function in both the nucleus and the cytoplasm at different stages of muscle cell differentiation.

5) Mechanistic studies of smyd1b

Smyd1b has two conserved functional domains, the MYND domain and the SET domain. The MYND domain was predicted based on its primary sequence which has two putative, non-DNA-binding zinc-fingers (Gross & McGinnis 1996). It is known as a protein-protein interaction domain and it has been implicated in the recruitment of HDACs (Gelmetti et al 1998, Lutterbach et al 1998, Wang et al 1998). The SET domain is known for its histone methyltransferase activity, which is involved in permanent silencing of transcription or, in some cases, activation of transcription (Jenuwein 2001, Nakayama et al 2001, Rea et al 2000, Schotta et al 2002).

Molecular analysis revealed that expression of Hand2, a transcription factor essential for right ventricular development, is down regulated in Smyd1 mutant

embryos. The presence of both MYND and SET domains in Smyd1 suggests a possible role for Smyd1 in chromatin remodeling and transcription regulation during muscle cell differentiation. Consistent with this hypothesis, Smyd1 associates with HDAC proteins and represses gene transcription in a HDAC dependent manner (Gottlieb et al 2002).

Like other SET domain containing proteins which have been shown to have histione methyltranferase activity (Nakayama et al 2001, Rea et al 2000). *In vitro* study indicated that Smyd1b is also a histone methyltransferase (Tan et al 2006), but it remains unknown about the *in vivo* working mechanism of *smyd1b* during myofibrillogenesis.

6) Smyd family members

Smyd proteins are highly conserved during evolution. Human and mouse genome each contains five annotated Smyd proteins (Thompson & Travers 2008). Like other SET domain containing proteins, three of Smyd family members including Smyd1, Smyd2 and Smyd3, have been identified as histone methyltranseferase *in vitro* (Abu-Farha et al 2008, Hamamoto et al 2004, Tan et al 2006). With the identification of non-histone target for other SET containing proteins, Smyd proteins have also been shown as non-histone methyltrasnferase. Smyd2 has been shown to methylate P53 on lysine 370 to inhibit P53 transcriptional activity (Huang et al 2006). In addition, Smyd3 has recently been identified as a methyltransferase for VEGFR1 (Kunizaki et al 2007). Smyd1 has been shown to work as a histone methyltransferase

in vitro, whether it also has non-histone protein methyltransferase activity *in vivo* is totally unknown.

9. Smyd1b interacting proteins

Yeast Two-Hybrid analyses revealed that Smyd1 interacts with another cardiac and skeletal muscle-specific protein skNAC, which is a muscle-specific isoform of αNAC (nascent polypeptide-associated complex). NAC is a heterodimeric complex that binds the newly synthesized polypeptide chains in the cytoplasm as they emerge from the ribosome. NAC has two subunits, one is called α NAC and the other one is called β NAC. Like a typical chaperone, NAC interacts with unfolded polypeptide chains independent of their amino acid sequence. But what ribosome-bound NAC exactly does *in vivo* is poorly understood and controversial (Bukau et al 2000, Rospert et al 2002, Wiedmann et al 1994). While examining the expression pattern of *α NAC* in adult murine tissue, Yotov and colleagues detected *α NAC* in all tissues postnatally. Interestingly, a larger size transcript was identified in skeletal muscle and heart. Cloning of the cDNA corresponding to this 7.0-kb mRNA revealed that it arises from differential splicing-in of a 6.0-kb exon giving rise to a proline-rich isoform of *α NAC* that was termed *skNAC* (skeletal NAC) (Yotov & St-Arnaud 1996).

The 220-kD skNAC protein was specifically expressed in cardiac and skeletal muscle. It was only detected in differentiated myotubes but not in myoblasts (Yotov & St-Arnaud 1996). At the same time, *skNAC* was found to display transcriptional

activating capacity. The murine myoglobin promoter was identified as a natural promoter responsive to *skNAC*. In contrast, *αNAC* was not active in stimulating transcription from reporter constructs; neither did *αNAC* competitively inhibit transcriptional activation by *skNAC* (Yotov & St-Arnaud 1996). Other studies showed that *skNAC* is involved in the repair process of injured muscle cells, which confirmed a specific role of the large splicing variant *skNAC* in muscle development (Munz et al 1999).

Double immunofluorescence of C2C12 myoblasts undergoing differentiation showed that Smyd1 and skNAC can be colocalized in the nucleus in the early stage of myoblast differentiation, and both of them had a translocation from nucleus to cytoplasm during myoblast differentiation (Sims et al 2002).

Structural mapping revealed that the interaction between Smyd1 and skNAC required an intact MYND domain and amino-terminal S sequences of Smyd1 in mammalian muscle cells (Sims et al 2002). The Smyd1 interaction domain of skNAC was found in a carboxyl terminal region of skNAC. The sequence of the Smyd1 intercating motif was mapped as PPLIP which was encoded by sequence near the end of the skNAC-specific exon-3. Deletion of the PPLIP motif abolished the interaction between skNAC and Smyd1 (Sims et al 2002). The consensus *PXLXP* motif has been previously shown to be essential for the interaction of the closely related MYND domain of the transcriptional corepressor protein and other proteins (Ansieau & Leutz 2002). The known function of *skNAC* is limited and its *in vivo* function is still a mystery. The study of skNAC will give us new insights for its function and will also help us to understand the working mechanism of Smyd1.

Project hypothesis, objectives and organization

The overall goal of my research was to advance the understanding of the function of Smyd1b and its interacting proteins including skNAC and Hsp90α1 and to study their working mechanism during myofibrillogenesis using zebrafish as a model system.

To achieve this goal based on the available knowledge, we stipulated the following five hypotheses. Each hypothesis was tested by the following detailed objectives and methods:

Hypothesis 1: *smyd1b* may also be required for other sarcomeric protein assembly besides the thick filament myosin in skeletal muscle and knockdown of *smyd1b* may also affect the overall sarcomere assembly of cardiac muscles.

To test this hypothesis, our objectives were: 1) to determine whether the thin filament, Z-line, M-line and titin were affected in skeletal muscle of *smyd1b* knockdown embryos; 2) to detect whether the sarcomere assembly of cardiac muscles was affected in *smyd1b* knockdown embryos.

To achieve these objectives, we have: 1) detected the assembly of the thin filament using actin as a marker, the Z-line assembly using α-actinin as a marker, the M-line assembly using myomesin as a marker and titin assembly in skeletal muscle

with *smyd1b* knockdown; 2) detected whether the early development of the heart—the heart looping was affected by *smyd1b* knockdown; 3) detected the sarcomere assembly of the cardiac muscle using myosin as a marker for the thick filament and actinin as a marker for the Z-line.

Hypothesis 2: Smyd1b may have a sarcomeric localization and work directly on sracomere assembly.

To test this hypothesis, our objectives were: 1) detect the sarcomere localization of both Smyd1b_tv1 and Smyd1b_tv2 during muscle development; 2) map the exact sarcomere localization; 3) detect whether Smyd1b_tv1 and Smyd1b_tv2 show different or same sarcomere localization.

To achieve these objectives, we have: 1) detected the sarcomere localization of both Smyd1b_tv1 and Smyd1b_tv2 in the myoblast, myotube and muscle fibers; 2) detected the localization of Smyd1b_tv1 on the sarcomere using double staining with actinin or myomesin antibody; 3) mutated several amino acid residues one by one to see which one is important for the sarcomere localization of Smyd1b_tv1.

Hypothesis 3: Smyd1b interacting protein skNAC may have similar function as Smyd1b during myofibrillogenesis.

To test this hypothesis, our objectives were: 1) to clone *sknac* in zebrafish; 2) to determine the temporal and spatial expression pattern of *sknac* mRNA during zebrafish development; 3) to knockdown the endogenous *sknac* and to determine the phenotype on muscle development.

To achieve these objectives, we have: 1) cloned the cDNA sequence of *sknac* and analyzed the gene structure during evolution; 2) checked the expression pattern of *sknac* using both RT-PCR and in-situ hybridization; 3) knocked down the *sknac* in zebrafish using morpholino oligos and characterized the phenotype.

Hypothesis 4: *hsp90α1* may play an important role during myofibrillogenesis.

To test this hypothesis, our objectives were: 1) to determine whether expression of *hsp90α1* was affected in *smyd1b* knockdown embryos; 2) to determine the phenotype of *hsp90α1* knockdown on the sarcomere assembly.

To achieve these objectives, we have: 1) compared the mRNA level of *hsp90α1* in the control and *smyd1b* knockdown embryo; 2) knocked down *hsp90α1* using morpholino oligos and detected the effect of *hsp90α1* on the sarcomere assembly using myosin, actin, actinin and myomesin as markers for the thick filament, thin filament, Z-line and M-line, correspondently.

Hypothesis 5: Smyd1b may work together with Hsp90α1 and Unc45b to control myofibrillogenesis.

To test this hypothesis, our objectives were: 1) to determine whether Smyd1b can specifically interact with Hsp90α1 or Unc45b; 2) to determine the critical subcellular localization of Smyd1b in myofibril assembly.

To achieve these objectives, we have: 1) analyzed the interaction between Smyd1b and Hsp90α1, or Smyd1b and Unc45b using Co-Immunoprecipitation; 2) determined the subcellular localization of Smyd1b in mouse myoblast C2C12 cells;

3) demonstrated that the cytoplasmic localization is critical for Smyd1b function in myofibrillogenesis.

CHAPTER 2: M-LINE LOCALIZATION OF SMYD1B_TV1 IN SARCOMERES IS DETERMINED BY A SERINE RESIDUE AT POSITION 225

1. Abstract

smyd1b is a member of the *smyd* family that contains the highly conserved SET and MYND domains. *smyd1b* encodes two alternatively spliced isoforms, *smyd1b_tv1* and *smyd1b_tv2*. These two isoforms differ by a 13 aa insertion encoded by a *smyd1b_tv1*-specific exon 5. Both *smyd1b_tv1* and *smyd1b_tv2* are expressed in skeletal and cardiac muscles and play a key role in myofibril assembly during myogenesis. Knockdown of *smyd1b* (tv1 and tv2) expression resulted in paralyzed zebrafish larvae without heart contraction. Myofibril assembly was significantly disrupted in *smyd1b* knockdown embryos. To better understand the mechanism of action in sarcomere assembly, we analyzed the subcellular localization of Smyd1b_tv1 and Smyd1b_tv2 in transgenic zebrafish expressing a myc-tagged Smyd1b_tv1 or Smyd1b_tv2, respectively. We showed that Smyd1b_tv1 and Smyd1b_tv2 are primarily localized in the cytosol of myoblasts and myotubes of early stage zebrafish embryos. However, in differentiated myofibers, Smyd1b_tv1 and Smyd1b_tv2 showed distinct pattern of subcellular localization. Smyd1b_tv1 was strongly localized on the M-line of sarcomeres whereas Smyd1b_tv2 showed a diffuse pattern of distribution in the cytosol. The Ser225 located within the Smyd1b_tv1-specific 13 aa insertion appears to be critical for the M-line localization. Mutation of Ser225 to alanine abolished the sarcomeric localization of Smyd1b_tv1

in myofibers. However, replacing Ser225 with theronine or aspartic acid had no effect, suggesting that modification by phosphorylation may be involved in the sacromeric localization of Smyd1b_tv1.

2. Introduction

smyd1, also known as Bop, is a member of the *smyd* family. This family is characterized by the presence of the conserved SET and MYND domains involved in protein methylation and protein-protein interaction, respectively (Gelmetti et al 1998, Gross & McGinnis 1996, Hwang & Gottlieb 1995, Hwang & Gottlieb 1997, Lutterbach et al 1998, Rea et al 2000, Wang et al 1998). Five members of the *smyd* family have been identified in vertebrates, including *Smyd1* through *5* (Sun et al 2008). Members of the *smyd* family play several important roles in development and cancer. *Smyd1* is required for the development of skeletal and cardiac muscles (Gottlieb et al 2002, Tan et al 2006). *Smyd2* and *Smyd3* have been shown to be involved in cancer cell proliferation (Hamamoto et al 2004, Huang et al 2006).

Smyd1 is specifically expressed in skeletal and cardiac muscles of mouse and zebrafish embryos (Hwang & Gottlieb 1995, Hwang & Gottlieb 1997, Tan et al 2006). Knockout studies revealed that *Smyd1* plays a key role in cardiomyogenisis in mouse embryos (Hwang & Gottlieb 1995, Hwang & Gottlieb 1997). In zebrafish, there are two *smyd1* duplicates, *smyd1a* and *smyd1b*. Both of them are expressed in muscle cells during development. Knockdown of *smyd1b* expression resulted in paralyzed zebrafish larvae with highly disorganized myofibrils in skeletal and cardiac muscles (Tan et al 2006).

The molecular mechanism by which *smyd1b* control the myofibrillogenesis is not clear. It has been reported that *smyd1* represses gene transcription *in vitro* in an HDAC (histone deacetylase) dependent fashion (Gottlieb et al 2002). *In vitro* biochemical analysis indicated that Smyd1b could methylate histone H3 proteins in vitro (Tan et al 2006). Smyd1 appears to undergo translocation from the nucleus to the cytoplasm during myoblast differentiation into myotube and myofibers. Smyd1 protein is initially localized in the nucleus of C2C12 myoblasts and translocated to the cytosol after myoblast differention into myotube and myofibers *in vitro* (Sims et al 2002). The biological significance of this translocation is unknown. Moreover, it is not clear whether this Smyd1 translocation also occurs during muscle development *in vivo*.

Molecular analysis revealed that *smyd1b* encodes at least two mRNA transcripts, *smyd1b_tv1* and *Smyd1b_tv2,* that are expressed in skeletal and cardiac muscles (Hwang & Gottlieb 1995, Hwang & Gottlieb 1997, Tan et al 2006). *smyd1b_tv1* and *Smyd1b_tv2* are generated by an alternative splicing of exon 5 encoding 13 aa (Hwang & Gottlieb 1995, Hwang & Gottlieb 1997, Tan et al 2006). Functional analysis via ectopic expression revealed that both *smyd1b_tv1* and *smyd1b*_tv2 could rescue the *smyd1b* knockdown phenotype in zebrafish (Tan et al 2006), suggesting that they may have similar biological activity. However, it is not clear whether *smyd1b_tv1* and *smyd1b_tv2* each have their own unique functions and subcellular localization patterns.

To better understand the mechanistic action of s*myd1b*, we analyzed the subcellular localization *smyd1b_tv1* and *smyd1b_tv2* during muscle development in

zebrafish embryos and in adult skeletal muscles. The data showed that Smyd1b_tv1 and Smyd1b_tv2 are primarily localized in the cytosol of myoblasts and myotubes of fish embryos at early stage. However, in mature myofibers of late stage embryos, a sarcomeric localization was evident for Smyd1b_tv1 but not for Smyd1b_tv2. Double immunostaining revealed that Smyd1b_tv1 was localized on the M-line of sarcomeres. The M-line localization appeared to be determined by the Ser225 located within the Smyd1b_tv1-specific 13 aa insertion. Mutation of the Ser225 to alanine abolished the sarcomeric localization of Smyd1b_tv1. However, replacing the Ser225 with theronine or aspartic acid had no effect, suggesting that post-translational modification by phosphorylation may be involved in the sacromeric localization of Smyd1b_tv1.

3. Materials and methods:

1) Synthesis of Morpholino-modified antisense oligos for splicing blockers

The splicing blocker (E9I9-MO) of *smyd1b* was based on the sequence of splicing donor at the exon-9 and intron-9 junction (Tan et al 2006). The *hsp90α1* translation blockers (ATG-MO) were targeted to sequence near the ATG start codon.

Sequence of E9I9-MO: 5'-CGTCACCTCTAGGTCTTTAGTGATG-3'

Sequence of *hsp90α1* ATG-MO: 5'- CGACTTCTCAGGCATCTTGCTGTGT- 3' (Du et al 2008).

2) Morpholino microinjection in zebrafish embryos

Morpholino antisense oligos were dissolved in 1×Danieau buffer (Nasevicius & Ekker 2000) to a final concentration of 0.5 mM. 1-2 nl of the *smyd1b* E9I9-MO or *hsp90α1* ATG-MO was injected into zebrafish embryos at the 1-2 cell stages.

3) Immunostaining of cryostat sections and whole mount zebrafish embryos

Skeletal muscles were dissected from adult WT mouse or adult Smyd1b_tv1 and Smyd1b_tv2 transgenic zebrafish at 90 dpf. The muscle tissues were fixed in 4% paraformaldehyde for 1 h at room temperature. The fixed samples were washed with 1×PBS-Tween (PBS, 0.1% Tween) 2×10 min, and then soaked in 30% sucrose for 2 h. The samples were transferred into an embedding chamber filled with OCT cryostat embedding medium (Tissue Tek). The embedding chambers were frozen on dry ice, and the frozen blocks were cut on a cryostat at -20℃ to produce 15 mm sections. Sections were transferred to subbed slides and allowed to dry completely at 37℃ for 1 h. Sections were rehydrated in PBS-Tween, and non-specific binding was blocked using 10% goat serum in PBS-Tween for 10 min. Sections were then incubated overnight at 4℃ in primary antibodies diluted in PBS-Tween. They were then washed with PBS-Tween for 5×5 min and incubated with the appropriate fluorescence-labeled secondary antibodies for 1 h at room temperature. Sections were coverslipped in 50% Vector shield (Invitrogen) and observed under fluorescence microscopy (Axioplan-2, Zeiss).

Immunostaining with whole-mount zebrafish embryos was carried out as previously described (Tan et al 2006).

The following antibodies were used for both cryostat sections and whole mount zebrafish embryos: anti-myc antibody (9E10, DSHB), anti-α-actinin (clone EA-53, #A7811, Sigma), anti-MHC for slow muscles (F59, DSHB), anti-myomesin (mMaC myomesin B4, DSHB). Secondary antibodies were FITC or TRITC-conjugates (Sigma).

4) BTS (N-benzyl-p-toluene sulphonamide) treatment

BTS was used at a final concentration of 50 μm to treat WT embryos from 2 dpf for 24 h. The treated and control embryos were fixed in 4% paraformaldehyde for 1 h at room temperature. Whole mount antibody staining was done as previously described (Tan et al 2006). The following primary antibodies were used: anti-myc antibody (9E10, DSHB), anti-MHC for slow muscles (F59, DSHB), anti-myomesin (mMaC myomesin B4, DSHB). Secondary antibodies were FITC or TRITC-conjugates (Sigma).

5) Analysis of protein expression by Western blot

smyd1b transgenic embryos or *smyd1b* transgenic embryos injected with morpholino (50 embryos each) were dechorinated manually at 24 hpf. The embryos were washed with 1 ml of PBS and crushed gently to remove the yolk by pipetting with a glass pipet in 0.5 ml of PBS. The embryos were collected by a quick spin at

3000 rpm for 1 min. The embryonic extract was washed once with 0.5 ml of PBS and solublized in 100 μl of 2×SDS loading buffer (0.125 M Tris-Cl pH 6.8, 4%SDS, 20% Glycerol, 0.2 M DTT, 0.02% Bromophenol Blue). Two micrometers of DTT (1M) and 2 μl of PMSF (100mM) were added to the protein extract to reduce protein degradation. The proteins were denatured by boiling for 5 min and analyzed on a SDS-PAGE of 7.5%. Proteins from 5-10 embryos were loaded on each lane of the SDS-PAGE. Proteins from the SDS-PAGE were transferred onto a PVDF membrane (Immobilion-P, Millipore) by electrophoresis. Immunodetection of myc-tagged proteins was carried out with their primary antibody anti-myc antibody. Anti-α-Tubulin (Clone B-5-1-2, Sigma) antibody was used as a loading control. The primary antibody incubation was followed by corresponding peroxidase-conjugated secondary antibodies.

6) Mutagenesis:

To generate *Smyd1b_tv1* constructs with mutations in the exon 5, Ser217 and Thr221 were mutated to alanine residues by PCR using primers S217A+T221A-f and S217A+T221A-r. Ser225 was mutated to alanine, thronine or aspartic acid by PCR using primers S225A-f and S225A-r, A225T-f and A225T-r, or S225D-F and S225D-F, respectively. The mutagenesis was carried out on *smyd1b-smyd1b_tv1*^{myc} DNA constructs by using QuikChange site-directed mutagenesis kit (Stratagene).

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’

4. Results

1) Characterization of Smyd1b_tv1 and Smyd1b_tv2 subcellular localization during muscle development in zebrafish embryos.

In zebrafish, *smyd1b* encodes two alternatively spliced isoforms, *smyd1b_tv1* and *smyd1b_tv2*, that are specifically expressed in muscle cells and play important roles in myofibril assembly during muscle development (Tan et al 2006). Knockdown of *smyd1b_tv1* and *smyd1b_tv2* resulted in defective myofibers with disorganized myofibril assembly. Previous studies have shown that Smyd1 undergoes a translocation from nucleus to cytoplasm during myoblast differentiation *in vitro* (Sims et al 2002). However, it is not clear whether this translocation occurs during muscle development *in vivo*, or if these two isoforms have similar or distinct subcellular localization in muscle cells during muscle development.

To better understand *smyd1b* function in myofibril assembly, we analyzed the subcellular localization of Smyd1b_tv1 and Smyd1b_tv2 during muscle cell differentiation using transgenic zebrafish embryos that expressed a myc-tagged Smyd1b_tv1 or Smyd1b_tv2 under the control of the muscle-specific *smyd1b*

promoter. *smyd1b_tv1* or *smyd1b_tv1* transgenic zebrafish embryos were fixed at several development stages that contain myoblast, myotube and myofiber. The subcellular localization of Smyd1b_tv1 or Smyd1b_tv2 at these stages was determined by anti-myc antibody staining.

The results showed a dynamic change of Smyd1b_tv1 localization during muscle cell differentiation. In myoblats and myotubes of early stage zebrafish embryos, similar subcellular localization of Smyd1b_tv1 and Smyd1b_tv2 was observed (Figure 4 A-D). Both Smyd1b_tv1 and Smyd1b_tv2 appeared to be primarily localized in the cytosol of myoblats and myotubes at early stage. However, in myotubes and myofibers of late stage zebrafish embryos, Smyd1b_tv1 and Smyd1b_tv2 showed distinct pattern of subcellular localization. Sarcomeric localization was detected for Smyd1b_tv1 but not for Smyd1b_tv2 in myofibers of zebrafish embryos (Figure 4 C-F).

The sarcomeric localization of Smyd1b_tv1 appeared in a progressive fashion from anterior to posterior myotomes. Smyd1b_tv1^{myc} first exhibited sarcomere localization in myotome 1-3 in the early stage embryos at 20 hpf or 22 hpf (Figure 4 A, C). Within a defined myotome, a dynamic pattern of Smyd1b_tv1 sarcomere localization was also observed. Smyd1b_tv1^{myc} first exhibited sarcomere localization in muscle pioneer cells located in the middle region of the myotome in the early stage embryos at 20 hpf or 22 hpf (Figure 4 A, C). The majority of muscle cells in the dorsal and ventral region of the myotome did not show any sarcomeric localization until 26 hpf (Figure 5 C). By 48 hpf, sarcomeric localization of Smyd1b_tv1 was detected in all myofibers in most of the myotome (Figure 4 E), suggesting that

sarcomeric localization of Smyd1b_tv1 appeared in a progressive fashion during muscle cell differentiation.

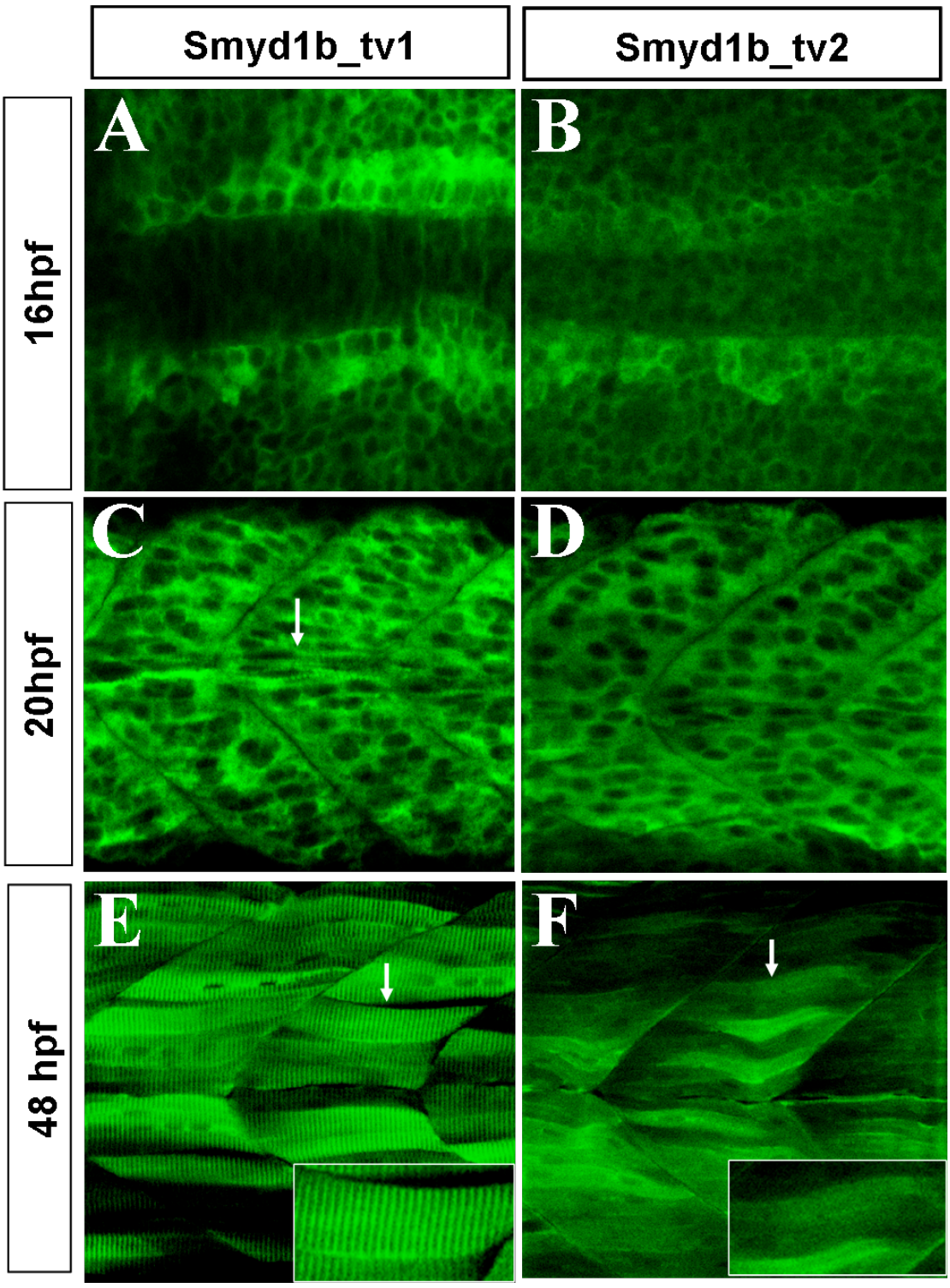


Figure 4. Smyd1b_tv1myc and Smyd1b_tv2myc show different sarcomere localization pattern during early muscle devlopment.

A-B. Whole-mount immunostaining with anti-myc antibody show the cytosolic localization of Smyd1b_tv1 (A) or Smyd1b_tv2 (B) in myoblasts of the *smyd1b_tv1*^{myc} (A) or *smyd1b_tv2*^{myc} (B) transgenic fish embryos at 16 hpf. C-D. Whole-mount immunostaining with anti-myc antibody show the cytosolic localization of Smyd1b_tv1 (C) or Smyd1b_tv2 (D) in myotubes of the *smyd1b_tv1*^{myc} (C) or *smyd1b_tv2*^{myc} (D) transgenic fish embryos at 20 hpf. The arrow shows the sarcomere localization of Smyd1b_tv1 in muscle pioneer cells located in the middle region of the myotome. E-F. Whole-mount immunostaining with anti-myc antibody show the cytosolic localization of Smyd1b_tv1 (E) or Smyd1b_tv2 (F) in muscle fibers of the *smyd1b_tv1*^{myc} (E) or *smyd1b_tv2*^{myc} (F) transgenic fish embryos at 48 hpf. The arrows indicate the enlarged the fibers to show the detail.

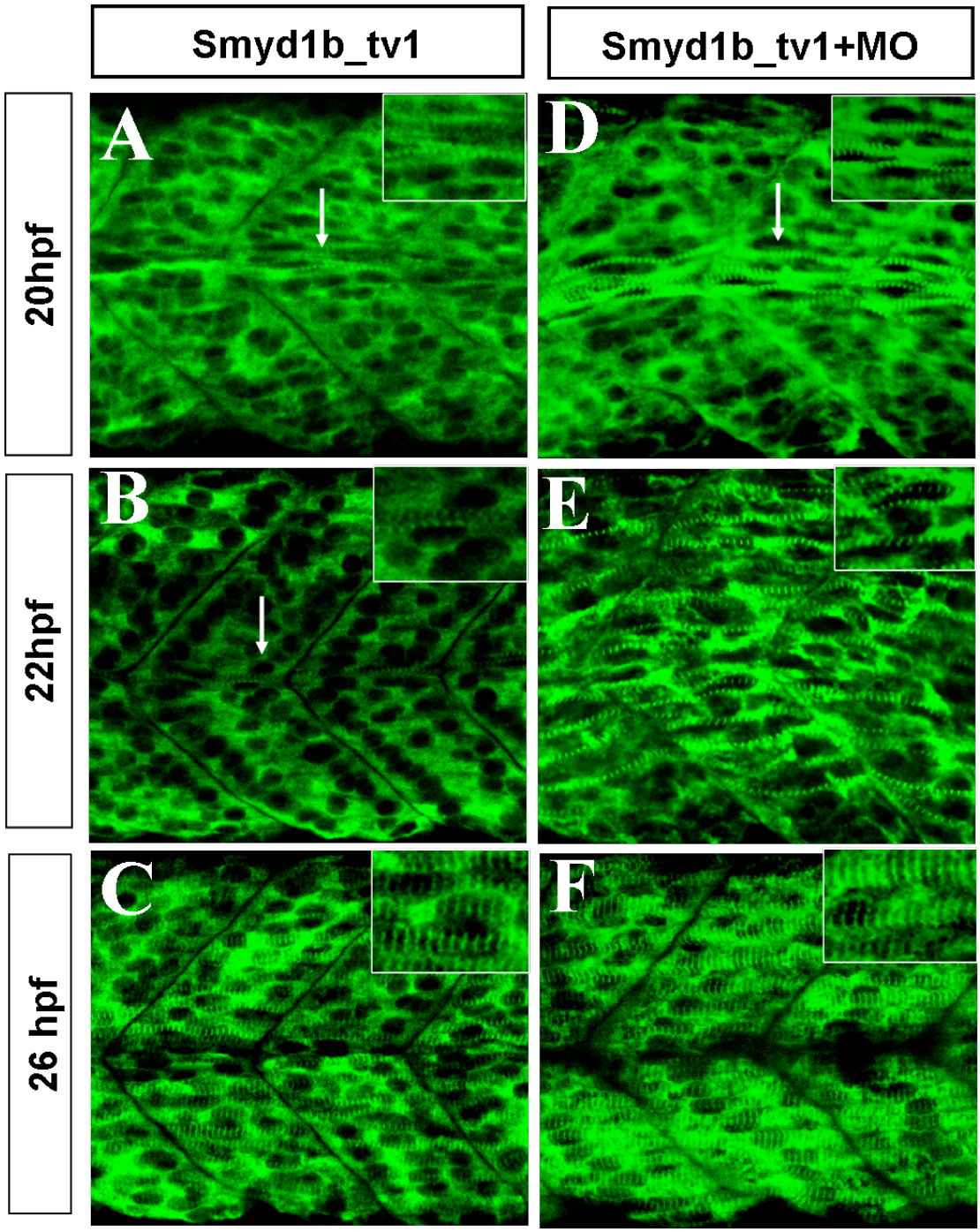
2) Knockdown of endogenous smyd1b advances the timing of sarcomeric localization of Smyd1b_tv1^{myc} in zebrafish embryos

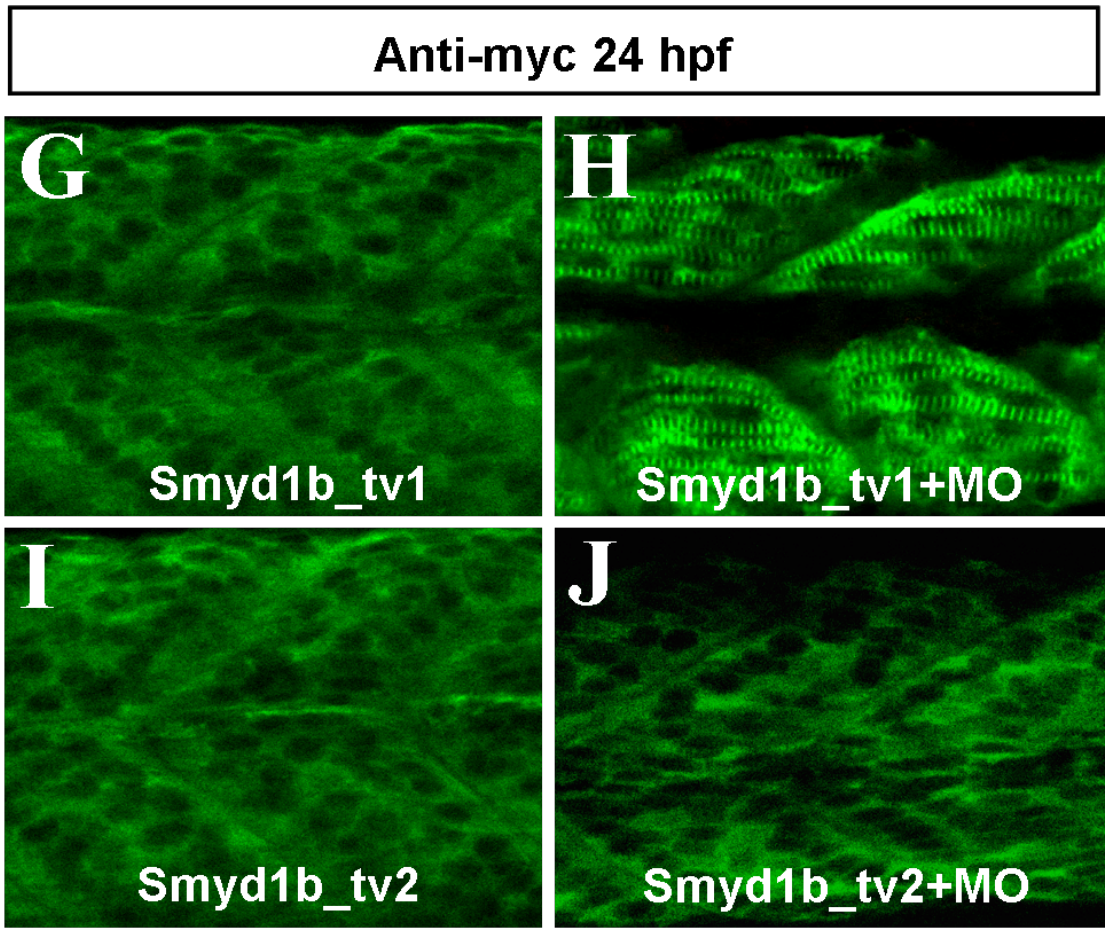
Considering that the sarcomeric localization of Smyd1b_tv1 was detected in transgenic zebrafish expressing a myc-tagged Smyd1b_tv1, we decided to test whether knockdown of the endogenous *smyd1b* could affect the subcellular localization of ectopically expressed myc-tagged Smyd1b_tv1. We have shown that the *smyd1b_tv1^{myc}* transgene could functionally replace the endogenous *smyd1b* in knockdown assays (Tan et al 2006). A splicing antisense oligo (E9I9-MO) targeted to *smyd1b* exon-9 intron-9 junction was injected into *smyd1b_tv1* or *smyd1b_tv2* transgenic zebrafish embryos at 1 or 2 cell stage to knock down the expression of endogenous *smyd1b* during development. The E9I9 splicing MO could knock down the expression of endogenous *smyd1b* (both *tv1* and *tv2*), but had no effect on the expression of the *smyd1b_tv1^{myc}* or *Smyd1b_tv2^{myc}* transgene because the transgene was constructed using the *smyd1b* cDNA which does not require splicing for expression.

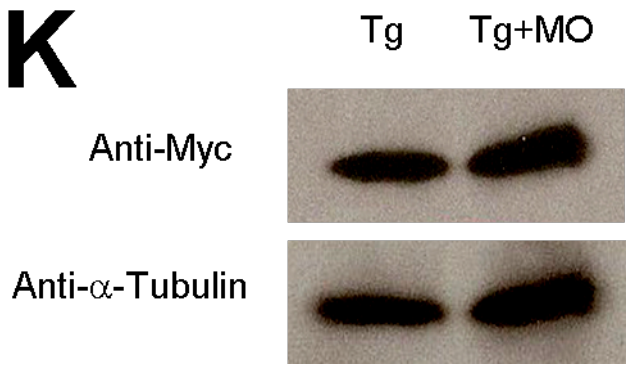
The expression and subcellular localization of Smyd1b_tv2^{myc} or Smyd1b_tv1^{myc} was analyzed in the E9I9-MO injected embryos by anti-myc antibody staining at 24 hpf (Figure 5 I, J) or 20 hpf (Figure 5 A, D), 22hpf (Figure 5 B, E), 24 hpf (Figure 5 C, F) and 26 hpf (Figure 5 G, H) respectively. The results showed that the E9I9-MO injection in Smyd1b_tv2^{myc} transgenic embryos did not change the cytosol localization of Smyd1b_tv2^{myc} (Figure 5 I, J). However, knockdown the expression of the endogenous *smyd1b* advanced the timing of Smyd1b_tv1

sarcomeric localization. The knockdown embryos showed an earlier appearance of sarcomeric localization relative to the uninjected transgenic control. Without knockdown of the endogenous *smyd1b_tv1*, the exogenous *smyd1b_tv1* cannot be localized on the sarcomere until 26 hpf (Figure 5 A-C). In contrast, the morpholino injected embryos showed much earlier sarcomeric localization. At 20 hpf, Smyd1b_tv1^{myc} showed sarcomeric localization only in muscle pioneer cells (Figure 5 D). While at 22 hpf, Smyd1b_tv1^{myc} showed clear localization in naïve sarcomeres (Figure 5 E). The sarcomeric localization of Smyd1b_tv1 appeared to be correlated with sarcomere formation in myofibers as revealed by anti-actin and anti-myosin antibody staining. This staining also revealed progressive sarcomere formation in the medial myotome, which then expanded both dorsally and ventrally (Figure 5 L-Q). Collectively, these results suggest that Smyd1b_tv1 may play a regulatory role in myofibril assembly and sarcomere formation.

To determine whether the early sarcomeric localization was caused by changes of Smyd1b_tv1^{myc} expression resulting from knockdown, we compared the protein expression of Smyd1b_tv1^{myc} in E9I9-MO injected and control transgenic embryos by western blot analysis. Western blot was done using 24 hpf *smyd1b_tv1^{myc}* transgenic embryos either with or without E9I9-MO injection. The result showed that injection of E9I9-MO did not affect the expression of the *smyd1b_tv1^{myc}* transgene (Figure 5 K). Similar levels of Smyd1b_tv1^{myc} protein expression were detected in control or E9I9-MO injected embryos, suggesting that the earlier appearance of Smyd1b_tv1^{myc} sarcomeric localization was not due to changes of its protein expression.


Smyd1b_tv1
Smyd1b_tv1+MO
20hpf
22hpf
26 hpf
A
B
C
D
E
F


Anti-myc 24 hpf
G
Smyd1b_tv1
H
Smyd1b_tv1+MO
I
Smyd1b_tv2
J
Smyd1b_tv2+MO


K
Tg
Tg+MO
Anti-Myc
Anti-α-Tubulin

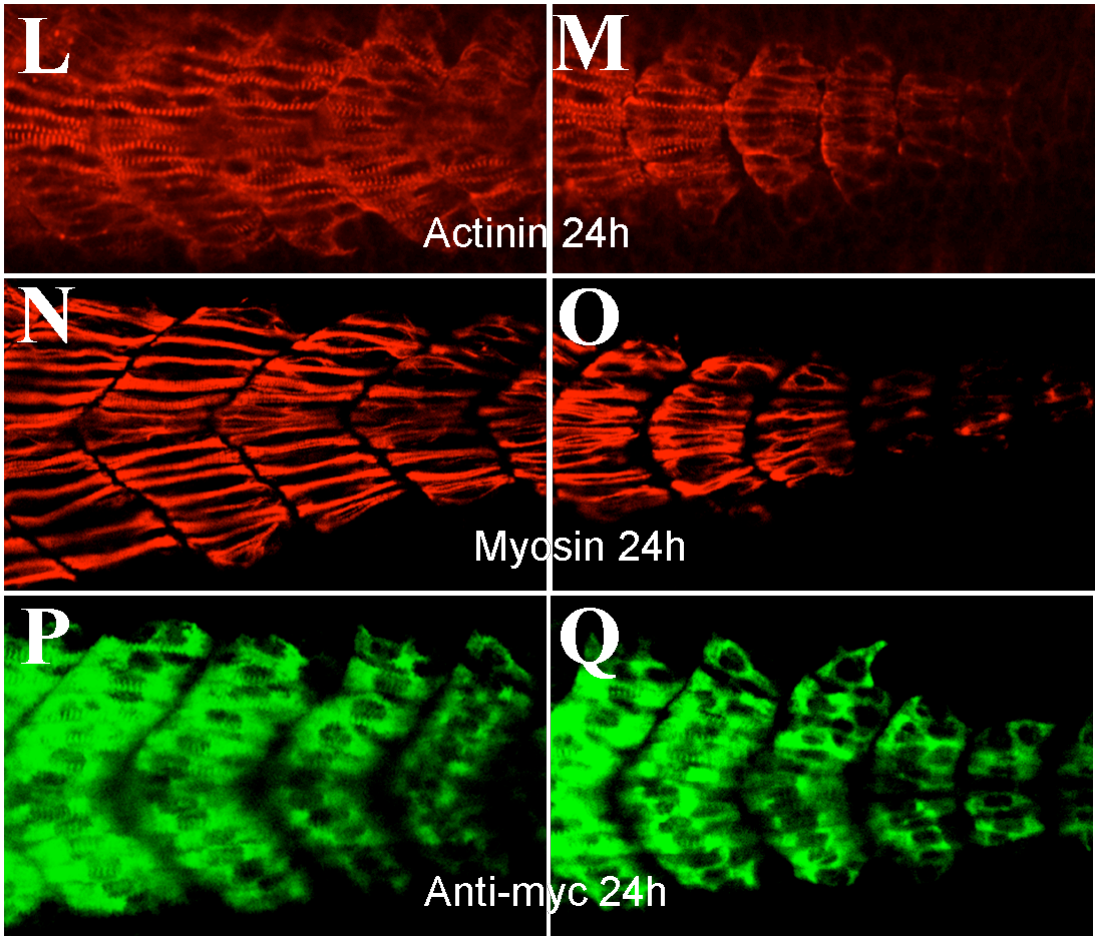


Figure 5. Knockdown of endogenous Smyd1b advances the timing of sarcomeric localization of Smyd1b_tv1myc in zebrafish embryos.

Knockdown of endogenous smyd1b advances the timing of sarcomeric localization of Smyd1b_tv1^{myc} in zebrafish embryos. A-C. Immunostaining using anti-myc antibody show the sarcomere localization of Smyd1b_tv1 at different development stages in *smyd1b_tv1^{myc}* transgenic fish embryos. At 20 hpf (A) and 22hpf (B) stages, Smy1b_tv1 show sarcomere localization only in muscle pioneer cells. Smy1b_tv1 starts to show clear sarcomere localization on the whole myotome from 26 hpf (C). D-F. Immunostaining using anti-myc antibody show the sarcomere localization of Smyd1b_tv1 at different development stages in *smyd1b_tv1^{myc}* transgenic fish

embryos with endogenous *smyd1b* gene knockdown. At 20 hpf (D) stages, Smy1b_tv1 show sarcomere localization in muscle pioneer cells and their adjacent cells. Smy1b_tv1 starts to show clear sarcomere localization on the whole myotome from 22 hpf (E) and at 26 hpf (F), the sarcomere localization is pretty strong. G-H. Immunostaining using anti-myc antibody show the sarcomere localization of Smyd1b_tv1 at 24 hpf in *smyd1b_tv1*^{myc} transgenic fish embryos without (G) or with (H) endogenous *smyd1b* gene knockdown. I-J. Immunostaining using anti-myc antibody show the sarcomere localization of Smyd1b_tv2 at 24 hpf in *smyd1b_tv2*^{myc} transgenic fish embryos without (I) or with (J) endogenous *smyd1b* gene knockdown. K. Western blot show that compared with the control, the level of myc-tagged Smyd1b_tv1 transgene protein level is not changed in *smyd1b-smyd1b_tv1*^{myc} transgenic embryos with morpholino injection. L-Q. Immunostaing using anti-actinin (L, M), anti-MHC (N, O) and anti-myc (P, Q) shows progressive sarcomere formation in the medial myotome, which then expanded both dorsally and ventrally.

3) Smyd1 is localized on the sarcomere in adult mouse skeletal muscles

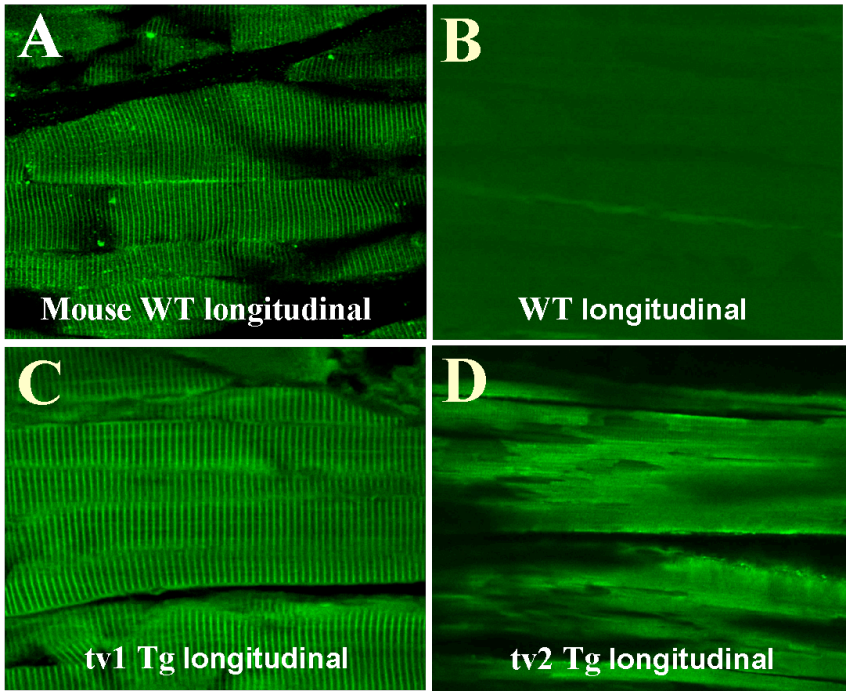
We have illulstrated distinct sarcomeric localization of Smyd1b_tv1 and Smyd1b_tv2 in transgenic zebrafish embryos. To confirm the sarcomere localization of the endogenous Smyd1b in skeletal muscles, we analyzed the subcellular localization of Smyd1 in mouse skeletal muscles. An anti-mouse Smyd1 polyclonal antibody was used in this analysis. The result showed a clear sarcomeric localization of Smyd1b in mouse skeletal muscles (Figure 6 A). This antibody recognizes both Smyd1b_tv1 and Smyd1b_tv2 in mouse. As a result it could not reveal the specific subcellular localization of each isoform (Smyd1b_tv1 and Smyd1b_tv2). As this antibody does not recognize zebrafish Smyd1b, it was not used in zebrafish study.

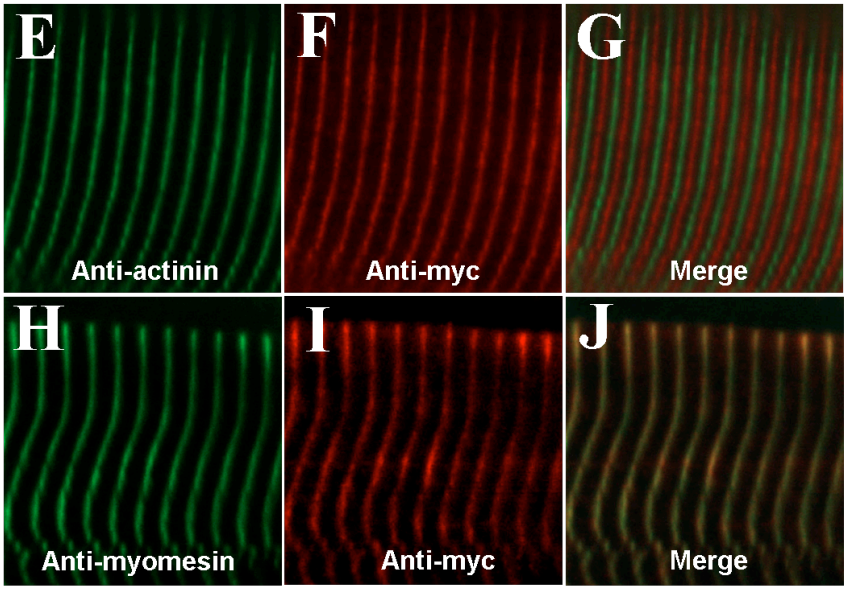
4) Smyd1b_tv1 is localized on the M-lines of sarcomeres in adult skeletal muscles

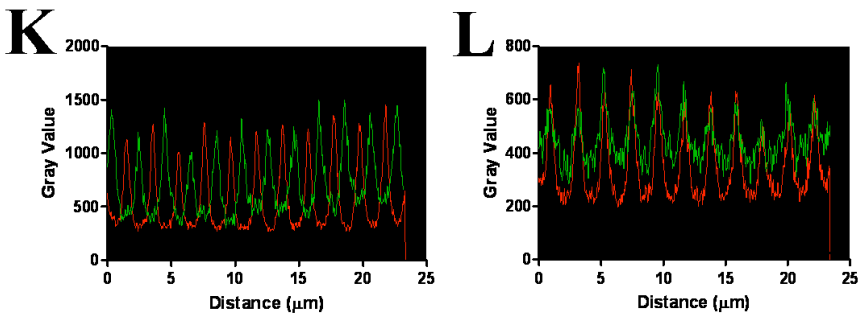
To determine subcellular localization in adult skeletal muscles of transgenic zebrafish, we analyzed the *smyd1b_tv1*^{myc} and *smyd1b_tv2*^{myc} subcellular localization in transgenic zebrafish at 90 dpf. Anti-myc antibody staining was performed on longitudinal sections of skeletal muscles of transgenic or WT zebrafish. The result indicated that Smyd1b_tv1 had a clear sarcomeric localization (Figure 6 C), whereas Smyd1b_tv2 did not show up on the sarcomere (Figure 6 D). No signal can be detected on muscle sections from the WT zebrafish (Figure 6 B).

To better characterize the sarcomeric localization of Smyd1b_tv1^{myc} in skeletal muscles, we carried out a double immunostaining with M- and Z-line specific

antibodies. Skeletal muscles were dissected from adult transgenic zebrafish at 90 dpf. Double staining was performed with longitudinal muscle sections using anti-myc antibody together with anti-myomesin or anti-a-actinin antibodies labelling the M-line and Z-line, respectively. The result showed that Smyd1b_tv1^{myc} is colocalized with myomesin on the M-line (Figure 6 H-J, L), but not with a-actinin on the Z-line (Figure 6 E-G, K).


A
Mouse WT longitudinal
B
WT longitudinal
C
tv1 Tg longitudinal
D
tv2 Tg longitudinal


E
Anti-actinin
F
Anti-myc
G
Merge
H
Anti-myomesin
I
Anti-myc
J
Merge


K
2000
1500
1000
500
0
Gray Value
0
5
10
15
20
25
Distance (μm)
L
800
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200
0
Gray Value
0
5
10
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20
25
Distance (μm)

Figure 6. Smy1b_tv1 can be localized on the M-line of the sarcomere in adult muscle fibers.

A. The longitudinal cryostat sectioning shows the sarcomere localization of the endogenous Smyd1 in adult mouse muscle tissue using anti-Smyd1 polyclonal antibody. B-D. The longitudinal cryostat sectioning shows the immunostaining using anti-myc antibody in adult WT zebrafish (B), *Smyd1b_tv1*^{myc} transgenic zebrafish (C) and *Smyd1b_tv2*^{myc} transgenic zebrafish muscle tissue (D). E-G and K. Double immunostaining using both anti-α-actinin and anti-myc antibodies shows that α-actinin can not be colocalized with Smy1b_tv1 ^{myc}. H-I and L. Double immunostaining using both anti-myomesin and anti-myc antibodies shows that myomesin can be colocalized with Smy1b_tv1 ^{myc}.

5) The Smyd1b-tv1 sarcomeric localization was disrupted in Hso90a1 knockdown embryos

We have known that knockdown of *Hsp90α1* severely affects the organization of the thick and thin filaments as well as the Z-lines and M-lines (Du et al 2008). In contrast, treating zebrafish embryos with BTS (N-benzyl-p-toluene sulphonamide), an inhibitor of myosin and actin interaction, specifically disrupts the organization of thick and thin filament without a significant effect on the M-line and Z-line organization (Kagawa et al 2006, Pinniger et al 2005). To confirm the M-line localization of Smyd1b_tv1, and to test whether Hsp90a1 knockdown and BTS treatment might have different effect on the Smyd1b_tv1 sarcomeric localization, we analyzed the subcellular localization of Smyd1b_tv1 in *Hsp90α1* knockdown or BTS treated zebrafish embryos. Smyd1b_tv1 transgenic zebrafish embryos were injected with *hsp90α1* ATG-MO at 1-2 cell stage. The Smyd1b_tv1 localization and sarcomere organization was determined by antibody staining using anti-myc (Figure 7 G-I), anti-MHC (F59) (Figure 7 A-C), or anti-myomesin antibodies (Figure 7 D-F). The result showed that *Hsp90α1* knockdown abolished the sarcomeric localization of Smyd1b_tv1 (Figure 7 I). In contrast, BTS treatment which disrupted the organization of myosin and actin filaments but not the M- and Z-lines, had little effect on the sarcomeric localization of Smyd1b_tv1 (Figure 7 B, E, H). Together, these data indicate that the sarcomeric localization of Smyd1b_tv1 depends on the organization of M-line structure.

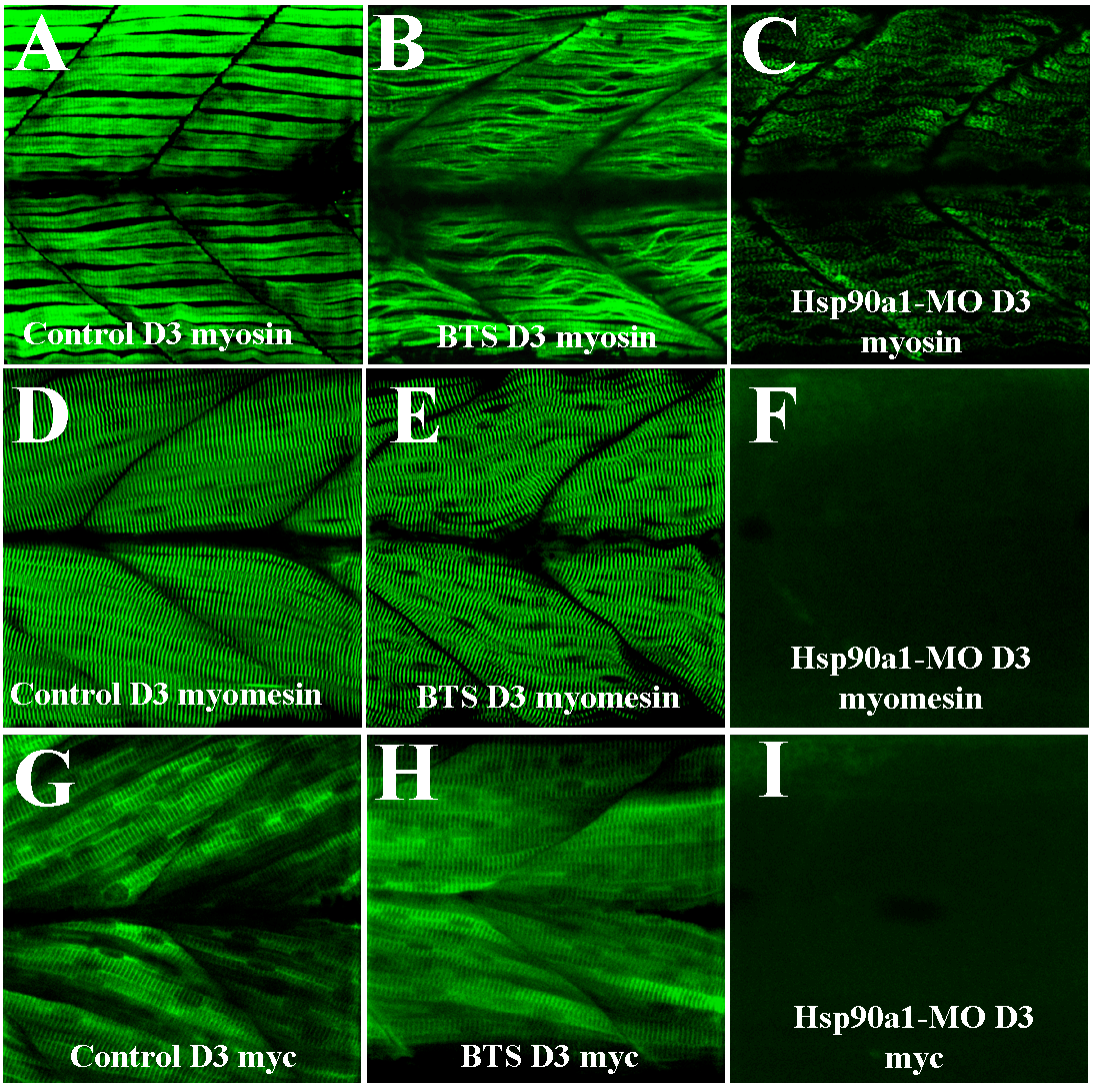


Figure 7. The sarcomeric localization of Smyd1b_tv1 depends on the organization of M-line structure.

A-C. F59 staining show the organization of slow muscle myosin in *Smyd1b_tv1*^{myc} transgenic embryos (A), *Smyd1b_tv1*^{myc} transgenic embryos with BTS treatment (B) and *Smyd1b_tv1*^{myc} transgenic embryos with *hsp90α1* ATG-MO injection (C) at 3 dpf. D-F. Immunostaining using anti-myomesin antibody show the organization of myomesin in *Smyd1b_tv1*^{myc} transgenic embryos (D), *Smyd1b_tv1*^{myc} transgenic embryos with BTS treatment (E) and *Smyd1b_tv1*^{myc} transgenic embryos with

hsp90α1 ATG-MO injection (F) at 3 dpf. G-I. Immunostaining using anti-myc antibody show the organization of Smyd1b_tv1 in *Smyd1b_tv1*^{myc} transgenic embryos (G), *Smyd1b_tv1*^{myc} transgenic embryos with BTS treatment (H) and *Smyd1b_tv1*^{myc} transgenic embryos with *hsp90α1* ATG-MO injection (I) at 3 dpf.

6) The sarcomeric localization of Smyd1b_tv1 requires Serine 225 within the Smyd1b_tv1-specific 13 aa insertion

The distinct subcellular localization of Smyd1b_tv1 and tv2 suggests that amino acid residues within the Smyd1b_tv1-specific 13 aa insertion may be involved in the sarcomeric localization. To identify the key amino acid residue(s) that required for the sarcomeric localization, we compared the protein sequence of the 13 aa sequence among Smyd1b_tv1 orthologs from several vertebrate species. Several conserved amino acid residues were identified within this region, including three potential phosphorylation sites at Ser217, Thr221 and Ser225 (Figure 8 A).

To test directly whether these conserved residues are required for the sarcomeric localization of Smyd1b_tv1, single or double mutation was made at the three positions by replacing them with alanine. The mutant constructs were injected into zebrafish embryos at 1 or 2 cell stage. Expression of myc-tagged mutant proteins was directed in muscle cells using the *smyd1b* muscle-specific promoter. Muscle specific expression of the mutant proteins was clearly detected in myofibers of the injected zebrafish embryos by anti-myc antibody staining (Figure 8 B-E). The subcellular localization of mutant proteins was carefully examined in the expressing myofibers. The results showed that substitution of Ser217 and Thr221 with alanine had no effect on the sarcomeric localization of Smyd1b_tv1 (Figure 8 B). However, substitution of Ser225 with alanine completely disrupted the sarcomeric localization of Smyd1b_tv1 (Figure 8 C). In contrast, mutating Ser225 to theronine had no effect

on the sarcomeric localization of Smyd1b_tv1 (Figure 8 D). Together, these results indicate the Ser225 is required for the sarcomeric localization of Smyd1b_tv1.

Serine and theronine are potential sites for post-translational modification by phosphorylation. To determine whether phosphorylation of Ser225 could be involved in sarcomeric localization of Smyd1b_tv1, we mutated Ser225 to aspartic acid. It has been reported that substitution of serine residues with aspartic acid mimics serine phosphorylation (Leger et al 1997, Saad et al 2007). The S225D mutant construct was generated by replacing the Ser225 with aspartic acid. The subcellular localization of myc-tagged S225D was analyzed in zebrafish embryos by anti-myc antibody staining. The results showed that replacing Ser225 with aspartic acid did not alter the sarcomeric localization of Smyd1b_tv1 (Figure 8 E). Collectively, these data indicate that post-translational modification by phosphorylation may be involved in the sarcomeric localization of Smyd1b_tv1.

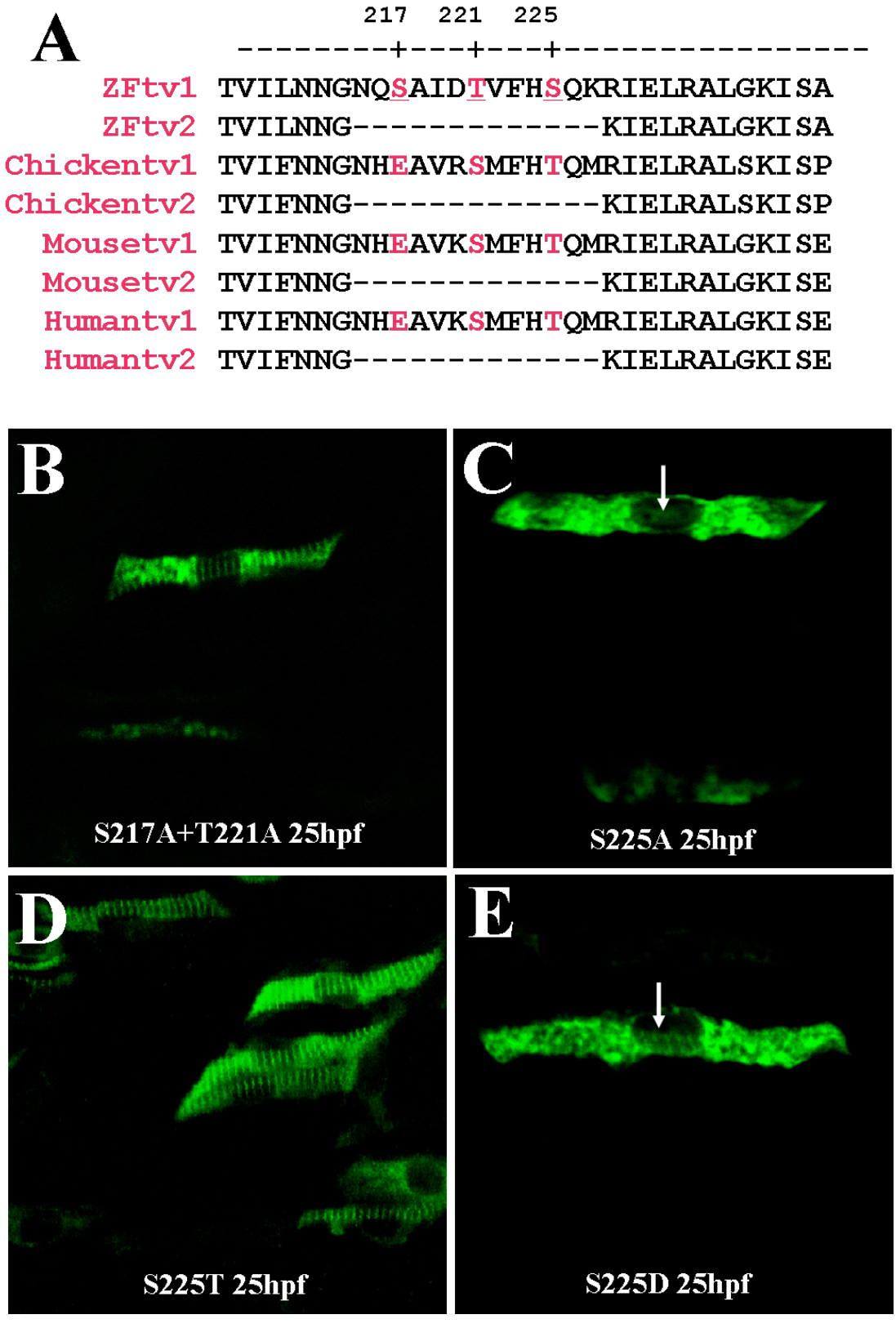

A
217 221 225
ZFtv1 TVILNNGNQSAIDTVFHSQKRIELRALGKISA
ZFtv2 TVILNNG----------KIELRALGKISA
Chickentv1 TVIFNNGNHEAVRSMFHTQMRIELRALSKISP
Chickentv2 TVIFNNG----------KIELRALSKISP
Mousetv1 TVIFNNGNHEAVKSMFHTQMRIELRALGKISE
Mousetv2 TVIFNNG----------KIELRALGKISE
Humantv1 TVIFNNGNHEAVKSMFHTQMRIELRALGKISE
Humantv2 TVIFNNG----------KIELRALGKISE
B
S217A+T221A 25hpf
C
S225A 25hpf
D
S225T 25hpf
E
S225D 25hpf

Figure 8. The sarcomere localization difference between Smyd1b_tv1 and Smyd1b_tv2 was caused by the Serine 225 of Smyd1b_tv1.

A. Comparison of Smyd1b_tv1 and Smyd1b_tv2 from different organisms, the potential post-translational modification sites are highlighted. B. Immunostaining using anti-myc antibody indicates that Smyd1b_tv1^{myc} with S217A+T221A mutations still shows sarcomere localization. C. Immunostaining using anti-myc antibody indicates that the sarcomere localization of Smyd1b_tv1^{myc} is deprived by S225A mutation (C). D-E. Immunostaining using anti-myc antibody indicates that the lost sarcomere localization of Smyd1b_tv1^{myc} can be retrieved by A225T mutation (D) or A225D mutation (E).

5. Discussion

In this study, we analyzed the subcellular localization of Smyd1b_tv1 and Smyd1b_tv2 during muscle development using transgenic zebrafish models expressing a myc-tagged Smyd1b_tv1 or Smyd1b_tv2. A primary cytosolic localization of both Smyd1b_tv1 and Smyd1b_tv2 was found in myoblasts and myotubes of early stage zebrafish embryos. However, in differentiated myofibers of late stage zebrafish embryos; a distinct pattern of subcellular localization was detected for Smyd1b_tv1 and Smyd1b_tv2 in skeletal muscles. Smyd1b_tv1 was predominantly localized on the M-line of sarcomeres whereas Smyd1b_tv2 showed a diffused pattern of distribution throughout the cytosol. The Ser225 located within the Smyd1b_tv1-specific 13 aa insertion appears to be critical for the M-line localization.

1) Cytosolic localization of Smyd1b_tv1 and Smyd1b_tv2 in myoblasts

It has been reported that in C2C12 myoblasts, Smyd1 is localized in the nucleus of myoblats and undergoes a nucleus to cytoplasm translocation to the cytoplasm during myoblast differentiation (Sims et al 2002). In this study, we showed that Smyd1b_tv1 and Smyd1b_tv2 are primarily localized in the cytosol of myoblasts and myotubes of early stage zebrafish embryos. Very little nuclear localization could be detected in myoblasts and myotubes of zebrafish embryos *in vivo*. The discrepancy between these two studies is not clear. There are several possible explanations. First, different antibodies in these two studies detected different Smyd1 proteins. In the

C2C12 culture studies, anti-mouse Smyd1 polyclonal antibodies were used to detect the endogenous Smyd1. In contrast, in this study we used the anti-myc antibody to detect the ectopically expressed myc-tagged Smyd1b. The myc-tagged Smyd1b may not exhibit similar subcellular localization as endogenous Smyd1b. However, arguing against this possibility, we showed that the myc-tagged *smyd1b* could functionally replace the endogenous *smyd1b* in a rescue assay (Tan et al 2006). Alternatively, the cultured mouse C2C12 cells may be different from myoblats cells in zebrafish embryos. C2C12 myoblast cells in culture may not fully resemble the development of muscle cells in zebrafish embryos *in vivo*. The nuclear localization was reported in cultured C2C12 myoblasts *in vitro*, whereas in this study, we directly analyzed the subcellular localization of Smyd1b_tv1 and Smyd1b_tv2 in developing zebrafish embryos *in vivo*. Nuclear localization may be transient and occur at low levels in zebrafish embryos that is below the sensitivity of detection by immunostaining. Consistent with this possibility, we expressed zebrafish Smyd1b_tv1 in C2C12 cells by DNA transfection, and a weak nuclear localization was detected. The nuclear localization only became prominent after addition of the nuclear export blocker LMB (Leptomycin B) was added in the cell cuture (Li et al., unpublished data). Collectively, these studies argue that Smyd1b may exhibit a dynamic subcellular localization and nucleus to cytoplasm translocation during muscle cell differentiation, and moreover Smyd1b_tv1 and Smyd1b_tv2 show distinct subcellular localization in sarcomeres of zebrafish skeletal muscles.

2) Smyd1b_tv1 is localized on the M-line of sarcomeres

We showed in this study that Smyd1b_tv1 and Smyd1b_tv2 have different subcellular localization in myofibers. The Smyd1b_tv2 is primarily localized in the cytosol of myotube and myofibers, whereas Smyd1b_tv1 is localized on the M-line of sarcomeres in myofibers. The sarcomeric localization appeared in a progressive fashion, correlating with the differentiation and maturation of myofibers in zebrafish embryos. Although the different subcellular localization of Smyd1b_tv1 and Smyd1b_tv2 was detected using transgenic zebrafish, it is unlikely that this difference is caused by an artifact from the specific transgenic line because similar difference in sarcomeric localization was detected in zebrafish embryos injected with *smyd1b_tv1* or *smyd1b_tv2* DNA constructs. In such a transient expression assay, hundreds of injected zebrafish embryos were analyzed and no sarcomere localization of Smyd1b_tv2 was detected. These data suggest that the Smyd1b_tv1-specific sarcomere localization was an intrinsic property of Smyd1b_tv1, not an artifact from the specific transgenic line. It should also be noted that within a single zebrafish embryo, different degrees of sarcomeric localization was observed in myofibers expressing different levels of ectopic Smyd1b_tv1. A better sarcomeric localization was observed in myofibers with low or modest levels of expression. Myofibers with high levels of protein expression failed to reveal the sarcomeric localization. The reason for this difference is not clear.

To our knowledge, this is the first report showing the different subcellular localization of Smyd1b_tv1 and Smyd1b_tv2 in the sarcomere of zebrafish skeletal muscle fibers. These studies could not be performed with monoclonal and polyclonal

antibodies against Smyd1b_tv1 or Smyd1b_tv2. No isoform-specific antibody is available and it may be difficult to generate due to the small difference (13 aa) between these two proteins. The transgenic lines thus provide useful models to study the regulation of subcellular localization of Smyd1b_tv1 or Smyd1b_tv2, and their specific biological function during muscle development *in vivo*.

3) Nucleus to sarcomere translocation

The nuclear to sarcomeric translocation has been noted with several proteins expressed in muscles. Transcription factor NFATc, for example, exhibits a dual localization in the nucleus and the Z-line of skeletal muscle cells. Its nuclear translocation is activity dependent in mature fibers and also is developmentally regulated (Liu et al 2001). The translocation could be involved in the response of muscle cells to mount efficient physiological response to muscle stress, load requirements, and/or stretch. In addition, several other proteins also show the dual nuclear and sarcomeric localization. Sarcomeric components such as human C-193 (Chu et al 1995), rodent cardiac ankyrin repeat protein (Zou et al 1997) and cardiac adriamycin-responsive protein (CARP) (Jeyaseelan et al 1997) have all been shown to localize to the I-band and the nucleus. Two titin-interacting proteins, the muscle-specific calpain p94 and MURF-1, also may participate in linking nuclear and sarcomeric functions (McElhinny et al 2002, Theriault et al 1999, Zeng et al 1998).

Protein phosphorylation is a common post-translational modification involved in the regulation of protein subcellular localization and shuttling of proteins between nucleuses and cytoplasm. It has been shown that the phosphorylated NFATc proteins

reside in the cytosol in an inactive state. Upon dephosphorylation by the $Ca2^{+}$-dependent phosphatase calcineurin, NFATc proteins translocate to the nucleus and become activated. (Beals et al 1997, Luo et al 1996). An NFATc mutant bearing S→A mutations is constitutively localized in the nucleus independent of calcium stimulation ((Beals et al 1997). Here we showed that the M-line localization of Smyd1b_tv1 requires Serine 225. Substitution of Ser225 to alanine abolished the sarcomeric localization of Smyd1b_tv1. In contrast, replacing Ser225 with theronine had no effect on its sarcomeric localization. It appears that phosphorylation of Ser225 is required for the subcellular localization. Substitution of Ser225 with aspartic acid did not significantly affect the sarcomeric localization of Smyd1b_tv1, suggesting that the sarcomeric localization of Smyd1b_tv1 is regulated by protein phosphorylation.

CHAPTER 3: SMYD1B_TV1 WORKS TOGETHER WITH MYOSIN CHAPERONES TO CONTROL MYOFIBRIL ASSEMBLY

1. Abstract

Smyd1b is a member of the Smyd family, which is specifically expressed in skeletal and cardiac muscles. Smyd1b plays a key role in myofibril assembly. Knockdown of *smyd1b* results in complete disruption of thick filament organization in skeletal muscles of zebrafish embryos. To better characterize *smyd1b* function and its mechanism of action in myofibrillogenesis, we have characterized the myofibril defects in skeletal and cardiac muscles of *smyd1b* knockdown zebrafish embryos. The results showed that in addition to thick filament defect, knockdown of *smyd1b* caused significant disruption of other sarcomeric structures including the thin and titin filaments, as well as M- and Z-lines in skeletal muscles of zebrafish embryos. Moreover, the sarcomere assembly of the cardiac muscles was also affected in *smyd1b* knockdown embryos. Smyd1 knockdown zebrafish embryos exhibited a significant increase in *unc45b* and *hsp90α1* expression. Both of them are myosin chaperones expressed in muscle cells. Functional study of *hsp90α1* indicated that *hsp90α1* also plays a vital role in myofibrillogenesis. Knockdown of *hsp90α1* or *smyd1b* caused reduction of myosin accumulation. Biochemical analysis revealed that Smyd1b_tv1 associates with myosin chaperone Hsp90α1 and Unc45b. Together, these data support the idea that Smyd1b_tv1 may work together with myosin chaperones to control sarcomere assembly during myofibrillogenesis.

2. Introduction

Myofibrillogenesis, the process of sarcomere assembly, is critical for muscle cell differentiation and contraction. Myofibrillogenesis involves hundreds of sarcomeric proteins assembled into a highly organized sarcomere, the basic contractile unit in striated muscles. The sarcomere is divided into four major compartments—the Z-line, I band, A band and M-line. The Z-line anchors the thin (actin) filaments of the I-band. The M-line anchors the thick (myosin) filaments of the A band. The assembly (and disassembly) of these multiprotein complexes follows ordered pathways, which are regulated at the transcriptional, translational and posttranslational levels. Disruption of these pathways in myofibril assembly is implicated in muscle diseases (Ehler & Gautel 2008).

More and more evidence suggests that correct folding and assembly of myofibrillar proteins into sarcomeres require auxiliary proteins (Barral & Epstein 1999, Srikakulam & Winkelmann 1999). Genetic studies in *Caenorhabditis elegans* and biochemical analyses *in vitro* indicated that chaperone-mediated myosin folding is an integral part of myofibril assembly during muscle development (Hutagalung et al 2002).

Molecular chaperones Hsp90 and Unc45 have been implicated in myosin folding and assembly in striated muscles (Srikakulam & Winkelmann 2004). *hsp90α* and *unc45* are strongly expressed in developing somites and skeletal muscles during development (Sass et al 1999). Biochemical studies revealed that Hsp90 and Unc45 form a complex with newly synthesized myosin proteins (Sass et al 1999, Srikakulam

& Winkelmann 2004). Knockdown or mutation of *hsp90α1* or *unc45* in embryonic *C. elegans* or zebrafish resulted in paralysis with severe myofibril disorganization in muscle fibers (Barral et al 1998, Barral et al 2002, Du et al 2008, Epstein & Thomson 1974, Etard et al 2007, Wohlgemuth et al 2007). All these studies indicated that both *hsp90* and *unc-45b* play vital roles during sarcomere assembly.

We showed recently that *smyd1b*, a member of the Smyd family, plays a vital role in cardiogenesis and myofibrillogenesis (Gottlieb et al 2002, Tan et al 2006). *smyd1b*, also known as *skm-Bop*, is specifically expressed in skeletal and cardiac muscles (Hwang & Gottlieb 1995, Hwang & Gottlieb 1997). There are two copies of *smyd1* in zebrafish, *smyd1a* and *smyd1b*. *smyd1b* has two alternatively spliced isoforms, *smyd1b_tv1* and *smyd1b_tv2*. Targeted deletion of *Smyd1* in mice is lethal. Embryos die at around embryonic day 10.5 due to the disruption of the cardiomyocyte maturation and the right ventricle formation (Gottlieb et al 2002). Knockdown of *smyd1b* (*tv1* and *tv2*) resulted in paralysis with disorganized thick filament assembly in slow muscles of zebrafish embryos (Tan et al 2006). Smyd1b function in other sarcomeric structures and its mechanistic action are unknown.

In this study, we have characterized the myofibril defects in skeletal and cardiac muscles of *smyd1b* knockdown zebrafish embryos. We demonstrated that in addition to thick filament defect, knockdown of *smyd1b* caused significant disorganization of thin and titin filaments, as well as M- and Z-lines in skeletal muscles of zebrafish embryos. Moreover, the cardiac muscles were also disrupted in *smyd1b* knockdown embryos. Disruption of myofibril organization by *smyd1b* knockdown resulted in increased expression of myosin chaperones, *unc45b* and

hsp90α1. Functional studies of *hsp90α1* indicated that *hsp90α1* has similar function as *smyd1b* and knockdown of either one caused the reduction of myosin accumulation. Biochemical analysis revealed that Smyd1b_tv1 associates with myosin chaperones Hsp90α1 and Unc45b. Together, these data support the idea that Smyd1b_tv1 may work together with myosin chaperones to control sarcomere assembly during myofibrillogenesis.

3. Materials and methods:

1) Morpholino and DNA microinjection in zebrafish embryos

Morpholino antisense oligos were dissolved in Danieau buffer (Nasevicius & Ekker 2000) to a final concentration of 0.5 mM. Zebrafish embryos were injected at the 1 or 2 cell stage with 2 nl of MO as described (Tan et al 2006). DNA microinjection was carried out as described (Du et al 1997).

2) Production of smyd1-Smyd1b_tv1^{myc} and smyd1-smyd1b^{myc} Transgenic Zebrafish

smyd1b-smyd1b_tv1^{myc} and *smyd1b- smyd1b_tv2^{myc}* minigenes were constructed by using cDNA encoding the myc-tagged Smyd1b_tv1 or Smyd1b_tv2 cloned after the 5.3-kb zebrafish *smyd1b* promoter and its 5′ flanking sequence. The transgenic fish were raised as described (Tan et al 2006).

3) Immunostaining of whole mount fish embryos

Immunostaining was carried out using whole mount zebrafish embryos as previously described (Tan et al 2006) with the following antibodies: anti-α-actinin (clone EA-53, #A7811, Sigma), anti-MHC for fast muscles (F310, DSHB), anti-myomesin (mMaC myomesin B4, DSHB), anti- α-actin (Ac1-20.4.2, Progen), Anti-α-Tubulin (Clone B-5-1-2, Sigma) and anti-titin (Clone T11, #T9030, Sigma). Secondary antibodies were FITC or TRITC-conjugates (Sigma).

4) Immunostaining of the fish heart

Forty-eight hpf zebrafish embryos, both WT and morpholino injected, were fixed in 4% paraformaldehyde in PBS for 1hr at room temperature, followed by washing with PBST 3×10min. The fixed embryos were then incubated in 1mg/ml collagenase for 45 minutes, followed by washing with PBST. The embryos were incubated in blocking buffer (5% goat serum in BDP) for 1hr at room temperature. The primary antibodies were suspended in BDP. The embryos were then incubated overnight at 4°C in these antibodies. On the second day, the embryos were washed with PBST 3×10min, followed by incubation in the secondary antibody (either anti-IgG1-FITC or anti-IgG2b-TRITC, 1:100 in BDP) at room temperature for 1hr. Then the embryos were washed with PBST 3×10min. The embryonic fish heart was dissected and observed using fluorescence microscopy (Axionplan-1, Carl Zeiss, Germany).

5) Cell culture and immunostaining

Murine skeletal myoblasts of the C2C12 line were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U each penicillin/ streptomycin) in 5% CO_2 at 37°C. When the cells had reached 80% confluence, the DNA construct *cmv-smyd1b_tv1/b*^{myc} or *cmv-smyd1b_tv2/b*^{myc} was transfected into the cells using the Lipofectamine reagent, according to the instructions of the manufacturer. Twenty four hours after transfection, cell differentiation was induced by changing the culture medium to a differentiating medium (DM). This DM contained fresh DMEM supplemented with 2% horse serum and antibiotics. All culture reagents were purchased from Invitrogen. C2C12 cells, grown on separate coverslips, were fixed at different stages with 4% paraformaldehyde for 15 min and washed with PBS. The cells were treated with monoclonal anti-myc antibody (9E10) at a 1:2000 dilution for 1 h and then exposed to FITC-conjugated secondary antibodies. In addition, cell nuclei were stained for 10 min using Hoechst 33258 solution (1 μg/ml; Sigma) and observed using fluorescence microscopy (Axioplan-1; Zeiss).

6) Construction of smyd1-2NLS-smyd1b_tv1myc

Two primers were designed to get *smyd1-2NLS-smyd1b_tv1*^{myc}. The sequences for these primers are:

NLS-smyd1b^{myc}-f: 5-GAATTCACCTCCAAAGAAGAAGCGAAAGGTAATGGAGTTTGTGGAAGTTTTTGATTC-3;

2NLS-smyd1b^{myc}-f: 5-ACC ATG GCT CCA AAA AAG AAA CGT AAG GTA CCT CCA AAG AAG AAG CGA AAG-3.

The first two rounds of PCR were done using one of the two primers together with the reverse primer *smyd1b-myc-r*: 5-CTA ATT CAG GTC CTC TTC AGA GAT GAG CTT CTG CTC CTT CCT GCG GAA CAG GTT CTT-3, to obtain the full length *2NLS-smyd1b_tv1*^{myc}. *smyd1-smyd1b_tv1* construct was used as template. This fragment was cloned into linearized *smyd1b/SmaI* vector.

7) Analysis of protein expression by Western blot

Wild type or MO-injected zebrafish embryos (50 embryos each) were dechorinated manually at 24 hpf. The embryos were washed with 1 ml of PBS and crushed gently to remove the yolk by pipetting with a glass pipet in 0.5 ml of PBS. The embryos were collected by a quick spin at 3000 rpm for 1 min. The embryo extract was washed once with 0.5 ml of PBS and solublized in 100 µl of 2×SDS loading buffer (0.125 M Tris-Cl pH 6.8, 4% SDS, 20% Glycerol, 0.2 M DTT, 0.02% Bromophenol Blue). DTT and PMSF were added at the final concentration of 1nM to the protein extract to reduce protein degradation. The proteins were denatured by boiling for 5 min and analyzed on a SDS-PAGE of 7.5%. Proteins from 5-10 embryos were loaded on each lane of the SDS-PAGE gel. Proteins from the gel were

transferred onto a PVDF membrane (Immobilion-P, Millipore) by electrophoresis. Immunodetection of MHC, α-tubulin, α-actin or myc-tagged proteins was carried out with their primary antibodies and followed by corresponding peroxidase-conjugated secondary antibodies.

8) Real time PCR analysis

Real time PCR was carried out using 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR reaction was carried out using the standard SYBR Green PCR Mater Mix (Applied Biosystems). Standard curves of cDNA samples were constructed using 10 fold serial dilutions. The relative levels of gene expression were compared based on the normalized value of the endogenous control *ef-1α*. Real time PCR was carried out using the following primers.

zfhsp90α1-P6: agccagacttcggtgaatcaa

zfhsp90α1-P7: ttctctctgtttctcaatgtaaa

zfmyhz2-P4: gctcacctaccagactgagga

zfmyhz2-P5: actcagcaatatcagcacgct

zfsmyhc1-P4: gctcacctaccagactgagga

zfsmyhc1-P5: catcttgttgacctgagattca

zfunc45b-P4: gctgcaaggaggtccaagaca

zfunc45b-P5: gatcatcagcatccagcatgt

zfef1a-P3: cttcaacgctcaggtcatcat

zfef1a-P4: acagcaaagcgaccaagagga

zsmyd1-RT-F: atctgaacgtgtctgcaga

smyd1-P6: tcttccggcaccttgactccatcc

9) Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was carried out using digoxigenin-labeled antisense probes as previously described (Du & Dienhart 2001a). The Plasmid *Hsp90α1-P* was digested with *NcoI* and transcribed with Sp6 RNA polymerase to synthesize digoxigenin-labled antisense RNA probes. Antisense probes against zebrafish slow myosin heavy chain 1 or fast muscle myosin heavy chain 2 were synthesized by Sp6 RNA polymerase from *NcoI* or *SphI* linearized *pGEM-smyhc1* or *pGEM-myhz2* plasmid, respectively. The zebrafish *unc-45b* antisense probe was synthesized with Sp6 RNA polymerase from plasmid *pGEM-unc45b* linearized with *BamHI*.

10) Immunoprecipitation and immunoblotting

HEK 293 cells were seeded at 2.5×10^5 per well in 6-well plates one day before transfection. Four micrograms of each indicated plasmid were transiently transfected by calcium phosphate precipitation. Cells were harvested 24 h after transfection and lysed in 1× cell lysis buffer (10 mM Tris -HCl pH 7.5, 150 mM NaCl,

1 mM EDTA, 1 mM EGTA and 0.2% Nonidet P-40) with 1× protease inhibitor mixture (Sigma P2714). For immunoprecipitation (IP), 300 μg of total protein were incubated with 30 μl of prewashed Anti-FLAG M2 Affinity Gel (Sigma A2220) at 4°C for 2 h in a total volume of 600 μl containing 4% glycerol in 1× cell lysis buffer. The beads were washed 3 times in 1× cell lysis buffer before processing for SDS/PAGE and immunoblotting.

11) Heat shock and cold shock of zebrafish embryos

For the heat shock experiment, 50 wild type zebrafish embryos at 24 hpf were treated for 15 min from 28.5°C to 38°C, then 38°C for 30 min, and then another 15 min from 38°C to 28.5°C. For the cold shock experiment, 50 wild type zebrafish embryos at 24 hpf were treated for 30 min at 4°C. For real-time PCR, total RNA of the heat-shocked or cold-shocked embryos, as well as WT control embryos was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the first strand cDNA synthesis kit (Life Sciences). For *in situ* hybridization, the heat-shocked or cold-shocked and WT embryos were fixed in 4% paraformaldehyde.

4. Results

1) Knockdown of smyd1b expression disrupted sarcomere formation in skeletal muscles

We have previously demonstrated that *smyd1b* plays an important role in the organized assembly of thick filaments in skeletal muscles of zebrafish embryos (Tan et al 2006). Knockdown of *smyd1b* completely disrupts the assembly of myosin thick filaments in slow muscles of zebrafish embryos (Tan et al 2006). However, the effects of *smyd1b* knockdown on other sarcomeric structures are not clear. Moreover, it is not known if *smyd1b* is required for myofibrillogenesis in fast muscles, the major muscle type in fish. To clarify these questions, we examined the organization of thin filaments and Z-line at earlier stage in slow muscles; thick filaments, titin filaments and M-lines in fast muscles at later stage in *smyd1b* knockdown zebrafish embryos. Results show that knockdown of *smyd1b* completely disrupts the organization of thin filaments in skeletal muscles at 24 hpf. Compared with the control-MO injected embryos, the *smyd1b* knockdown embryos have little or no organized thin filaments in slow muscles (Figure 9 A, B). Similarly, the sarcomeric localization of the Z-line protein α-actinin was also affected, but not as severe as the thin filament (Figure 9 C, D). At 3 dpf, the sarcomere localization of the thick filament myosin, the giant protein titin and the M-line protein myomesin were detected in the fast muscles, the result indicated that knockdown of *smyd1b* also disrupted the organization of thick filament, titin filament and M-line in fast muscles (Figure 9 E-J).

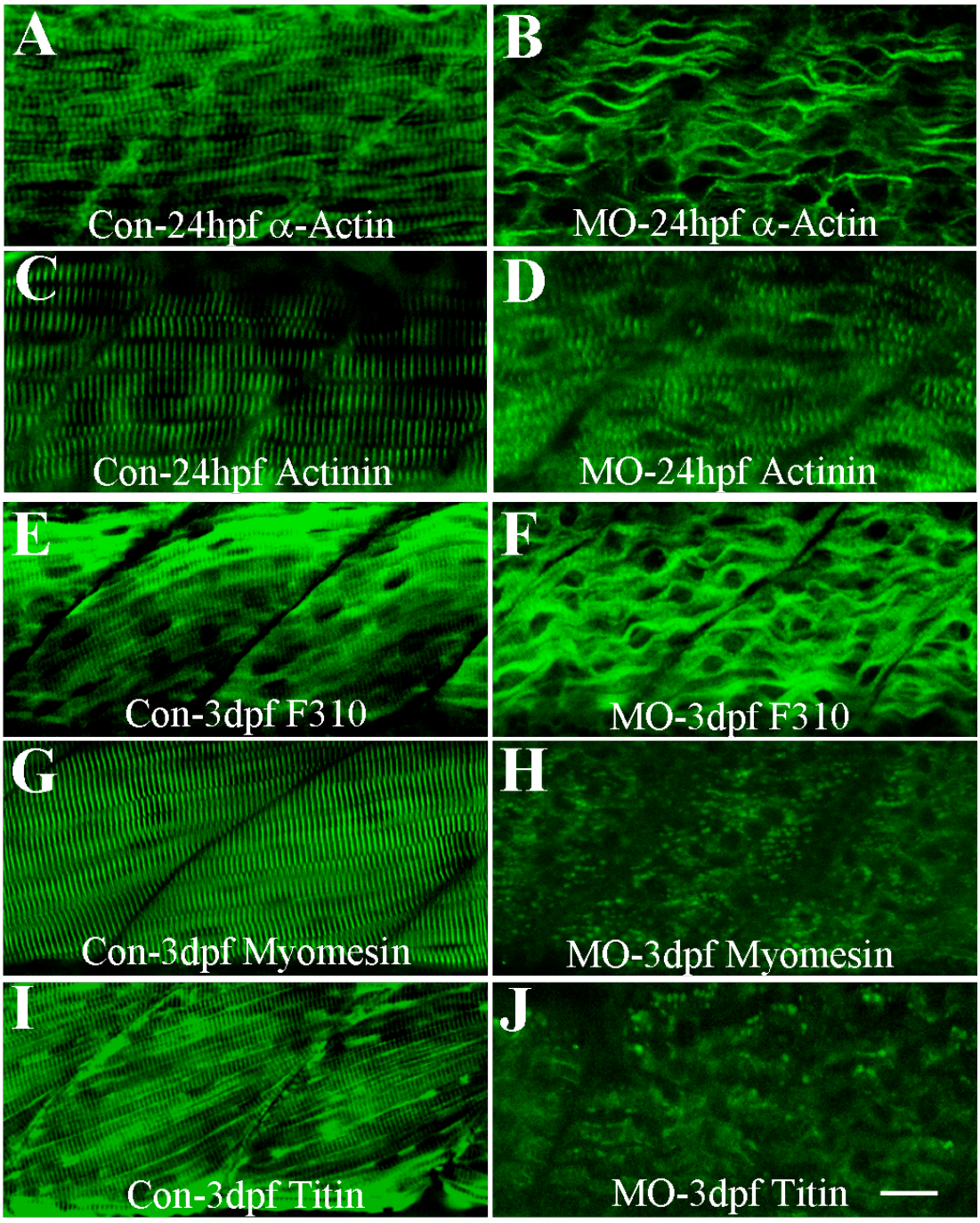


Figure 9. Knockdown of smyd1b expression results in defective thin filament, Z-line, M-line and titin assembly.

A and B. Anti-α-actin antibody (Acl-20.4.2) staining shows organization of thin filaments in slow muscle fibers of control-MO (A) or ATG-MO (B) injected embryos

at 24 hpf. C and D. Anti-actinin antibody (EA-53) staining shows Z-line structure in control-MO (C) or ATG-MO (D) injected embryos at 24 hpf. E and F. Anti-MHC (F310) antibody staining (lateral view) shows thick filaments in fast muscle fibers of control (E) or ATG-MO (F) injected embryos at 3 dpf. Scale bar: 20 μm. G and H. Anti-myomesin antibody (mMaC Myomesin B4) staining shows M-line structure in control-MO (G) or ATG-MO (H) injected embryos at 3 dpf. I and J. Anti-titin (T11) antibody staining shows the sarcomeric localization of titin in fast muscle fibers in control-MO (I) or ATG-MO (J) injected embryos at 3 dpf.

2) smyd1b-tv1^{myc} transgene can rescue the muscle defect

To confirm the specificity of the sarcomeric phenotype from *smyd1b* knockdown, we performed a rescue experiment using a transgenic zebrafish line expressing a myc-tagged Smyd1b_tv1. *smyd1*-MO was injected into transgenic embryos and their non-transgenic siblings at the one or two cell stages. Expression of the *smyd1b_tv1* transgene could not be knocked down by the splicing MO. The transgene was constructed using the *smyd1b_tv1* cDNA which does not require splicing for expression. The injected embryos were analyzed by double staining with anti-myc and anti-actin antibodies. The results showed that the *smyd1b* transgene could completely rescue the defective organization of thin filaments in zebrafish embryos (Figure 10 A, B). Similarly, the transgene could also rescue the structural defects of the Z-line, the thick filament, M-line and the giant protein titin as revealed by immunostaining with the anti-α-actinin, anti-MHC (F310), anti-myomesin and anti-titin antibodies, respectively. (Figure 10 C-J). Together, these data indicate that *smyd1b* is required for myofibril assembly of both thick and thin filaments, as well as the organization of Z-line, M-line and titin in all skeletal muscles. Thus *smyd1b* is essential for myofibrllogenesis of all key sarcomeric structures.

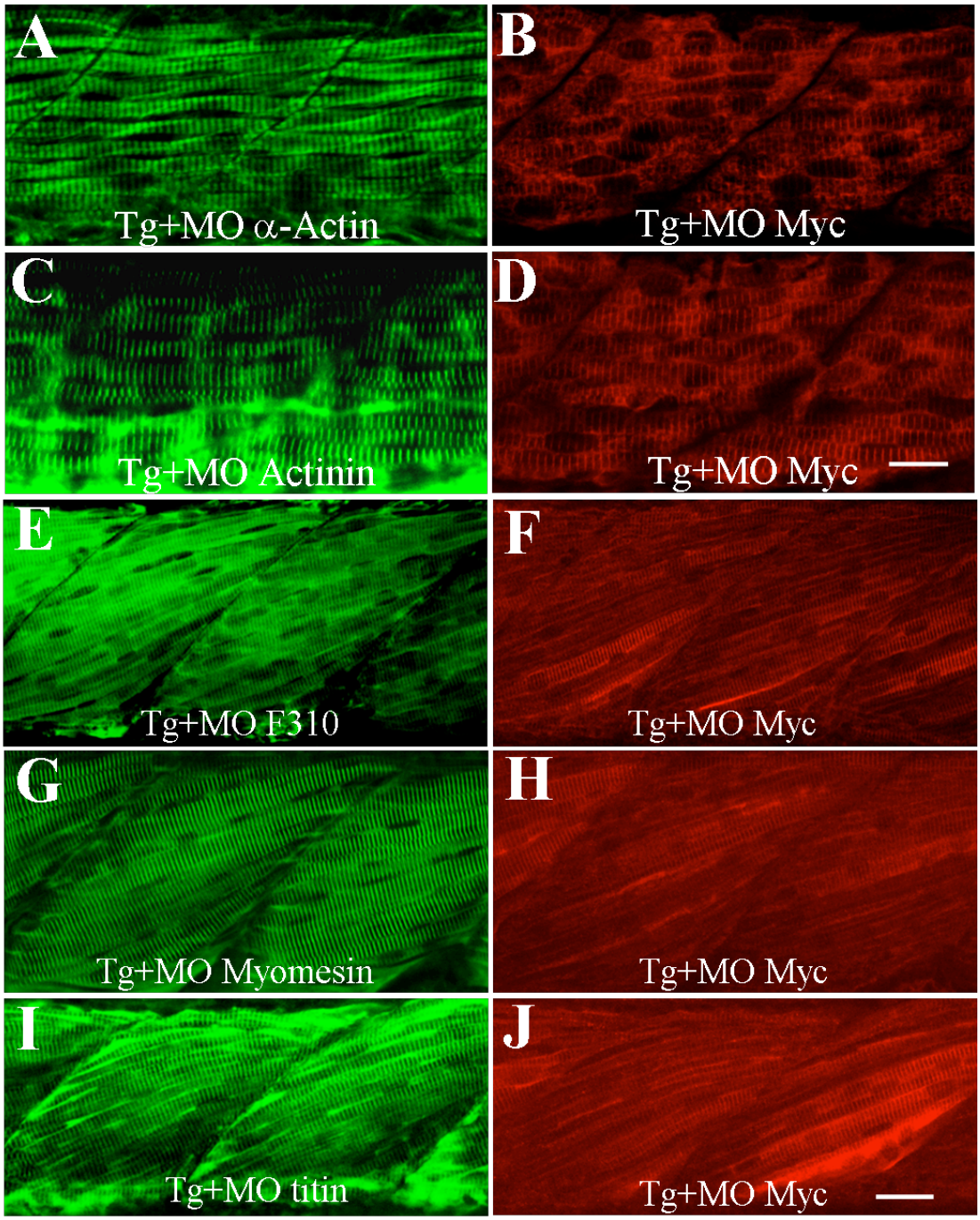


Figure 10. smyd1b_tv1myc transgene rescues the disruption in skeletal muscles of zebrafish embryos.

A and B. Anti-α-actin and anti-myc (9E10) antibody double staining shows that *smyd1b_tv1*^{myc} transgene (B) can rescue the disruption of actin in ATG-MO injected

embryos at 24 hpf (A). C and D. Anti-actinin and anti-myc antibody double staining shows that *smyd1b_tv1*^{myc} transgene (D) can rescue the disruption of actinin in ATG-MO injected embryos at 24 hpf (C). (Scale bar: 20μm.). E and F. Anti-MHC (F310) and anti-myc antibody double staining shows that *smyd1b_tv1*^{myc} transgene (F) can rescue the disruption of the thick filament in ATG-MO injected embryos at 3 dpf (E). G and H. Anti-myomesin and anti-myc antibody double staining show that *smyd1b_tv1*^{myc} transgene (H) can rescue the disruption of the M-line in ATG-MO injected embryos at 3 dpf (G). I and J. Anti-titin and anti-myc antibody double staining shows that *smyd1b_tv1*^{myc} transgene (J) can rescue the disruption of titin assembly in ATG-MO injected embryos at 3 dpf (I). (Scale bar: 25μm.).

3) Knockdown of smyd1b disrupts filament assembly in cardiac muscles

The *smyd1b* morpholino injected zebrafish embryos have no heartbeat (Tan et al 2006). It has been shown that *Smyd1* knock out in mouse embryos is lethal because heart development was inhibited (Gottlieb et al 2002). It is still unknown how *smyd1b* can affect heart development in zebrafish embryos. To detect whether *smyd1b* can affect the early heart development of zebrafish embryos, the myosin antibodies MF20 and S46 were used for double immunostaining. MF20 recognizes a sarcomeric myosin heavy chain epitope found in both the ventricle and atrium (Stainier & Fishman 1992). The monoclonal antibody S46 recognizes an atrium-specific sarcomeric myosin heavy chain epitope in zebrafish (Stainier & Fishman 1992). *smyd1b* knockdown embryos showed no difference in heart tube formation compared with control embryos (Figure 11 A-F). Therefore, heart failure in the morphant embryos was not caused by a defect in the heart formation

To further determine whether the sarcomere assembly in the cardiac muscles was affected similarly to skeletal muscles, the slow muscle myosin antibody F59 was used for the whole mount immunostaining. Hearts was dissected from both morphant and the control embryos. The results showed that thick filament myosin assembly was totally disrupted in the heart of *smyd1b* knockdown embryos. The muscle cells in the morphant were big and round (Figure 11 G, H). Anti-α-actinin staining also showed that the Z-line protein α-actinin was disorganized and less sarcomeres were observed compared with the control (Figure 11 I, J). These studies indicate that *smyd1b* is

required for cardiac muscle assembly and knockdown of *smyd1b* causes collapse of the sarcomereic structure in cardiac muscles.

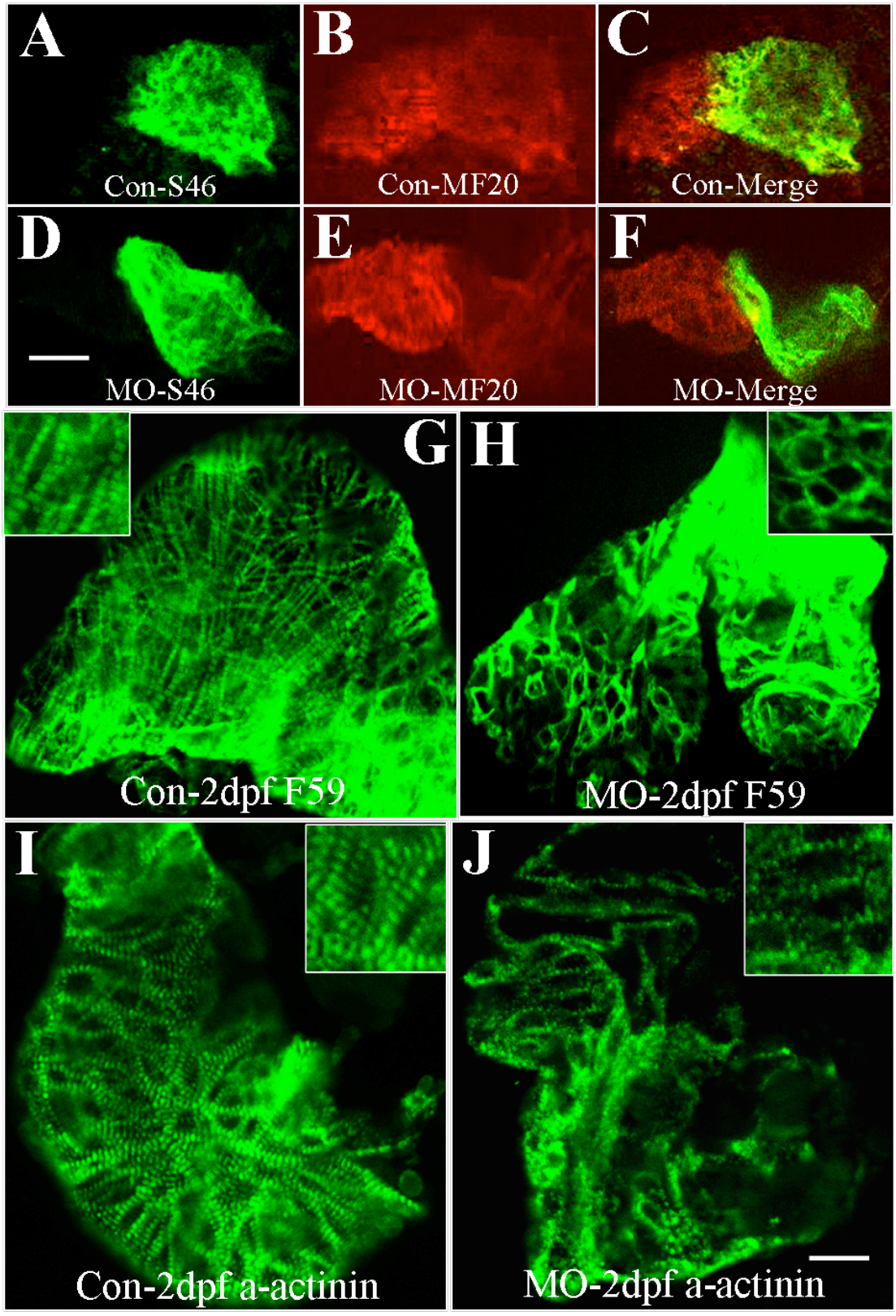

A
Con-S46
B
Con-MF20
C
Con-Merge
D
MO-S46
E
MO-MF20
F
MO-Merge
G
Con-2dpf F59
H
MO-2dpf F59
I
Con-2dpf a-actinin
J
MO-2dpf a-actinin

Figure 11. Immunostaining shows disorganized thick filaments and Z-lines in cardiac muscles of smyd1 knockdown embryos.

A-C. S46 (A) and MF20 (B) double staining (C) shows the atrium and ventricle formation in control-MO injected embryos at 2 dpf. D-F. S46 (D) and MF20 (E) double staining (F) shows the normal atrium and ventricle formation in ATG-MO injected embryos at 2 dpf. (Scale bar: 30μm.). G and H. Anti-MHC (F59) staining shows thick filaments in control-MO (G) and ATG-MO injected (H) embryos at 2 dpf. I and J. Anti-α-actinin staining shows Z-lines in control-MO (I) and ATG-MO injected (J) embryos at 2 dpf. (Scale bar: 15μm.).

4) Knockdown of smyd1b resulted in upregulation of hsp90α1 and unc45b gene expression

It has been shown that members of the Smyd family are methyltransferases that methylate histone proteins in vitro (Brown et al 2006, Hamamoto et al 2004, Tan et al 2006). Histone methylation has been implicated in regulation of gene expression. To test whether knockdown of *smyd1b* may affect gene expression involved in myofibrillogenesis, we carried out a cDNA microarray analysis comparing the expression profile of 20,000 genes in *smyd1b* knockdown and control zebrafish embryos at 24 hpf. The data revealed that 12 genes were upregulated by 2 to 6 fold in *smyd1b* knockdown embryos compared with the control. Strikingly, 10 of them represent members of the heat shock protein family (Table 1). In contrast, over 200 genes were found to be down regulated in smyd1b knockdown embryos and 18 of them may be related to muscle development (Table 1). However, no clear trend (common pathway) could be noted among those down regulated genes.

Among these upregulated *hsp* genes, *hsp90α1* is especially interesting. It has been shown that *hsp90α1* is specifically expressed in muscle cells of zebrafish embryos. *In vitro* studies indicated that Hsp90 forms a complex with newly synthesized myosin protein and is involved in myosin folding and assembly in developing myocytes (Sass et al 1999, Sass et al 1996, Srikakulam & Winkelmann 2004). To validate the results from the microarray analysis, we analyzed *hsp90α1* gene expression in *smyd1b* knockdown embryos by *in situ* hybridization and

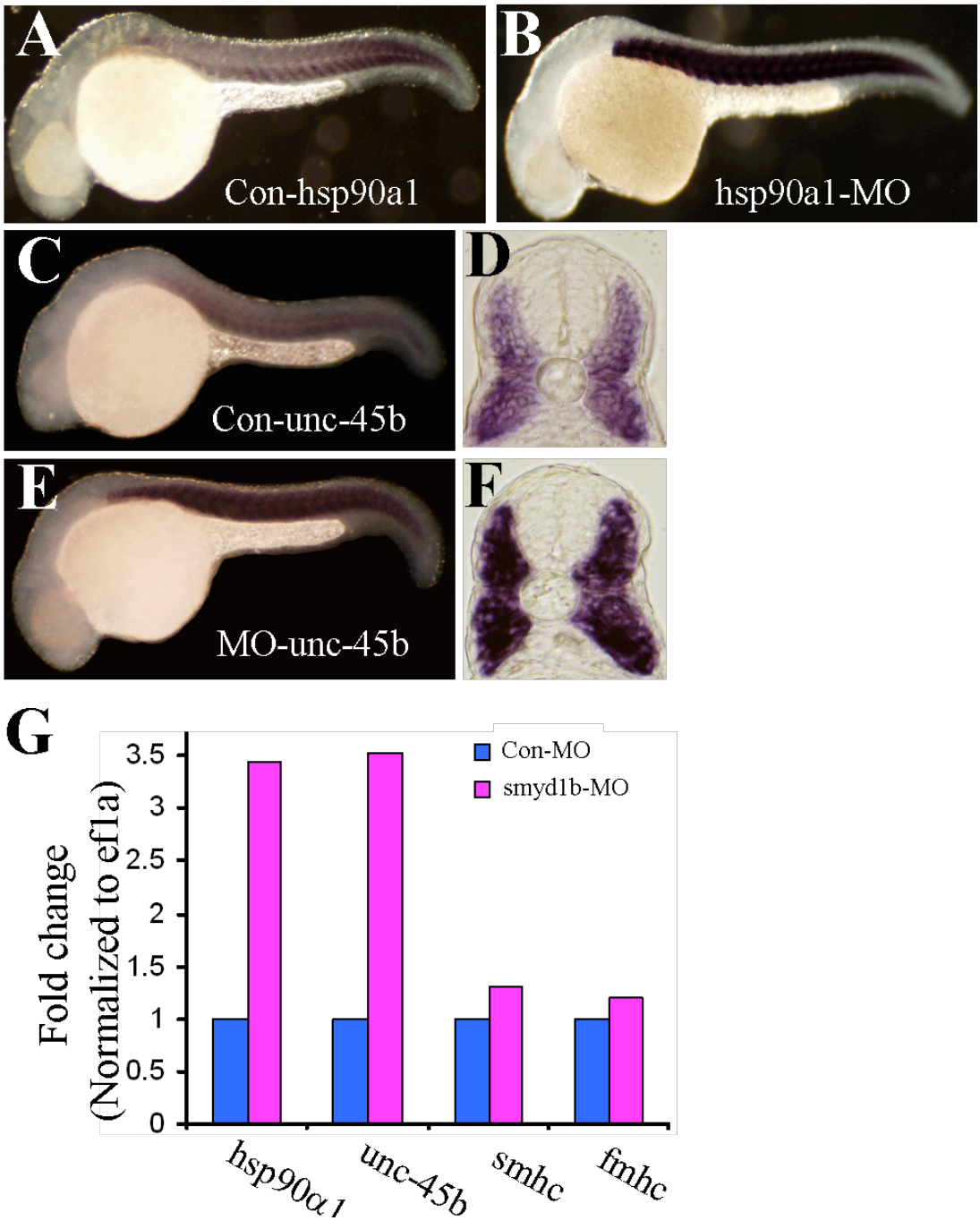
quantitative RT-PCR. The results confirmed that knockdown of *smyd1* significantly upregualted *hsp90α1* gene expression by 3.4 folds (Figure 12 A, B, G).

The upregulation of *hsp90α1* gene expression by *smyd1b* knockdown raises a question of whether *smyd1b* could be involved in stress response. To test this idea, we stressed zebrafish embryos with heat or cold shock. Expression of *hsp90α1* and *smyd1b* was subsequently determined in the stressed embryos. Although both heat and cold shock significantly increased *hsp90α1* expression, little or no change in *smyd1b* expression could be detected in heat- or cold-stressed embryos (Figure 12 H-N). Together, these data indicate that knockdown of *smyd1b* could create stress in zebrafish embryos; however, *smyd1b* itself is not directly involved in stress response.

It has been reported that Unc45 associates with Hsp90 and functions as a muscle-specific chaperone involved in myosin folding and assembly (Barral et al 2002, Etard et al 2007). To determine whether *unc45b* expression was also upregulated in *smyd1b* knockdown zebrafish embryos, we conducted in situ hybridization and quantitative PCR in the *smyd1b* morphant embryos. The result showed that *unc45* expression was significantly increased in *smyd1b* knockdown embryos compared with the controls (Figure 12 C-F). Together, these results indicate that disruption of myofibril organization by *smyd1b* knockdown increases myosin chaperone expression in skeletal muscles of zebrafish embryos.

Table 1. *smyd1b* cDNA microarray result of MO vs WT at 24 hpf.

Upregulated genes	**1. Danio rerio HSP70 mRNA for stress protein HSP70 (Hsp70A)** **2. Danio rerio inducible 70 kDa heat shock protein (hsp70) gene (Hsp70B)** **3. Danio rerio Hsp70 gene (Hsp70C)** **4. Danio rerio heat shock protein 90 (hsp90) mRNA** **5. Danio rerio heat shock cognate 70.II** **6. Danio rerio heat shock protein 47**
Downregulated genes	**1. Danio rerio DMbeta2a mRNA** **2. Danio rerio mesoderm posterior a (mespa)** **3. Danio rerio clone VH88 immunoglobulin heavy chain variable region** **4. Danio rerio ets related protein erm (erm)** **5. Danio rerio homeobox protein (hoxb10a) gene** **6. Danio rerio eukaryotic translation initiation factor 4e 1b(eif4e1b)** **7. Danio rerio SRY-box containing gene 21a (sox21a)** **8. Danio rerio Cecr1 (cecr1)** **9. Danio rerio zinc finger homeobox 1 (zfhx1)** **10. Danio rerio translocon-associated protein beta** **11. Danio rerio L-plastin** **12. Similar to Homo sapiens mRNA for alpha actinin 4** **13. Similar to Fugu rubripes beta-cytoplasmic actin2 gene** **14. 14.Similar to Homo sapiens TANK binding kinase TBK1 (TBK1)** **15. Similar to Homo sapiens deubiquitinating enzyme UnpES (UNP)** **16. Similar to Homo sapiens calbindin 2, 29kDa (calretinin)** **17. Smimilar to Oryctolagus cuniculus mRNA for calmodulin-dependent protein kinase** **18. Similar to Sus scrofa CYP51 gene for lanosterol 14 alpha-demethylase**


A
Con-hsp90a1
B
hsp90a1-MO
C
Con-unc-45b
D
E
MO-unc-45b
F
G
Fold change
(Normalized to ef1a)
3.5
3
2.5
2
1.5
1
0.5
0
Con-MO
smyd1b-MO
hsp90α1
unc-45b
smhc
fmhc

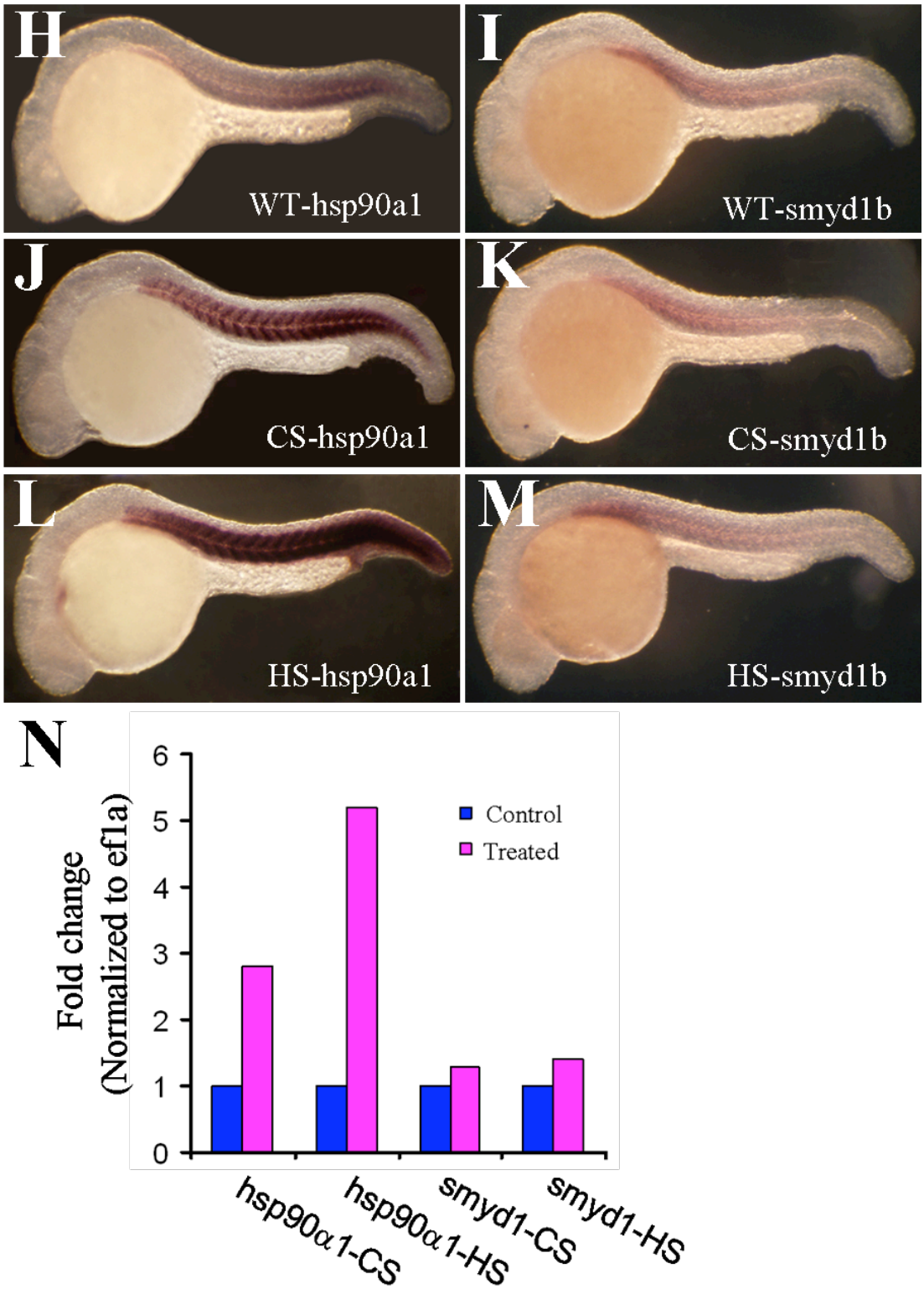


Figure 12. Knockdown of smyd1b causes the upregulation of hsp90α1 and unc45b gene expression.

A-B. *In situ* hybridization shows that there is an upregulation of *hsp90α1* gene expression in *smyd1b* knockdown embryo (B) than in control embryo (A). C-F. *In-situ* hybridization shows that there is an upregulation of *unc45b* gene expression in *smyd1b* knockdown embryo (E, F) than in control embryo (C, D). G. The upregulation of both *hsp90α1* and *unc45b* in smyd1b knockdown embryos is confirmed by qRT-PCR. H-M. *In situ* hybridization shows there is an increase of *hsp90α1* gene expression in cold (J) or heat shocked (L) embryos than in WT embryos (H), but there is no change of *smyd1b* gene expression (I, K, M). N. The expression level of both *hsp90α1* and *smyd1b* in the cold or heat shocked embryos and WT embryos is confirmed by qRT-PCR.

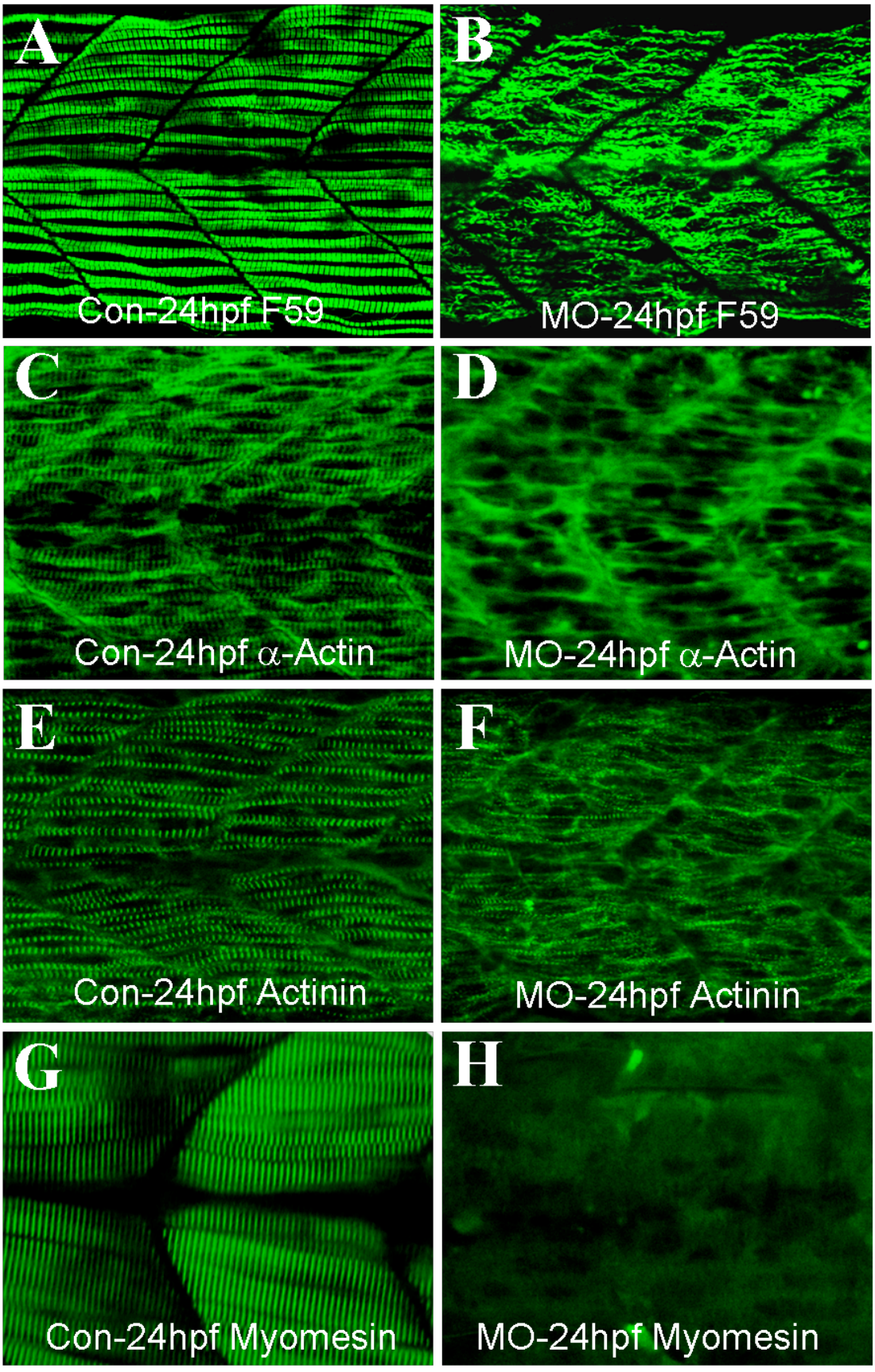
5) Knockdown of hsp90α1 disrupted myofibril organization in skeletal muscles but not in cardiac muscles of zebrafish embryos.

To determine whether *hsp90α1* also plays a role like *smyd1b* in muscle development *in vivo*, we knocked down *hsp90α1* expression in zebrafish embryos. The *hsp90α1* translational blocker ATG-MO was used to specifically target the sequence flanking the start codon of the *hsp90α1* transcripts. Studies from our lab has shown that the ATG-MO can specifically knockdown the expression of *hsp90α1* efficiently (Du et al 2008). Although the ATG-MO injected embryos appeared morphologically normal, they were unable to swim and failed to show any sign of skeletal muscle contraction in response to physical stimulation by touch.

To determine whether blocking *hsp90α1* expression might disrupt myofibril organization during myofiber maturation, the *hsp90α1* knockdown embryos were examined by immunostaining with an anti-MHC antibody F59. Knockdown of *hsp90α1* expression severely disrupted the sarcomere formation and thick filament organization in slow muscles. Very few sarcomeres could be detected in *hsp90α1* knockdown myofibers (Figure 13 B). In contrast, injection with the control MO had no effect on the sarcomere formation and myofibril organization (Figure 13 A). To test whether the thin filaments were also affected by *hsp90α1* knockdown, *hsp90α1* knockdown embryos were stained with anti-α-actin antibody. Compared with the control-MO injected embryos (Figure 13 C), *hsp90α1* knockdown embryos showed few or no thin filaments (Figure 13 D). To test whether other sarcomeric structures, such as the M and Z lines, were also affected in the *hsp90α1* knockdown of slow

muscles, we analyzed the localization of myomesin and α-actinin, the respective M- and Z-line-specific proteins, by antibody staining. Both M- and Z-line organization was significantly disrupted (Figure 13 E-H). Immunostaining with the anti-myomesin antibody confirmed that the M-line was disorganized in fast muscles of *hsp90α1* knockdown embryos (Figure 13 H). Together, these data indicate that *hsp90α1* is required for myofibril organization in both slow and fast muscles during muscle development in zebrafish embryos.

To determine whether the sarcomere assembly in cardiac muscles was also disrupted, immunostaining with anti-MHC (F59) and anti--α-actin antibody was done in both control and ATG-MO injected embryos. The result showed that although the skeletal muscle assembly was disrupted by ATG-MO, there was no effect on cardiac muscle assembly in the same zebrafish embryo (Figure 13 I-P). Both the thick and thin filament had normal organization in the cardiac muscle in ATG-MO injected muscle. This indicated that knockdown of *hsp90α1* had no effect on cardiac muscle assembly.


A
Con-24hpf F59
B
MO-24hpf F59
C
Con-24hpf α-Actin
D
MO-24hpf α-Actin
E
Con-24hpf Actinin
F
MO-24hpf Actinin
G
Con-24hpf Myomesin
H
MO-24hpf Myomesin

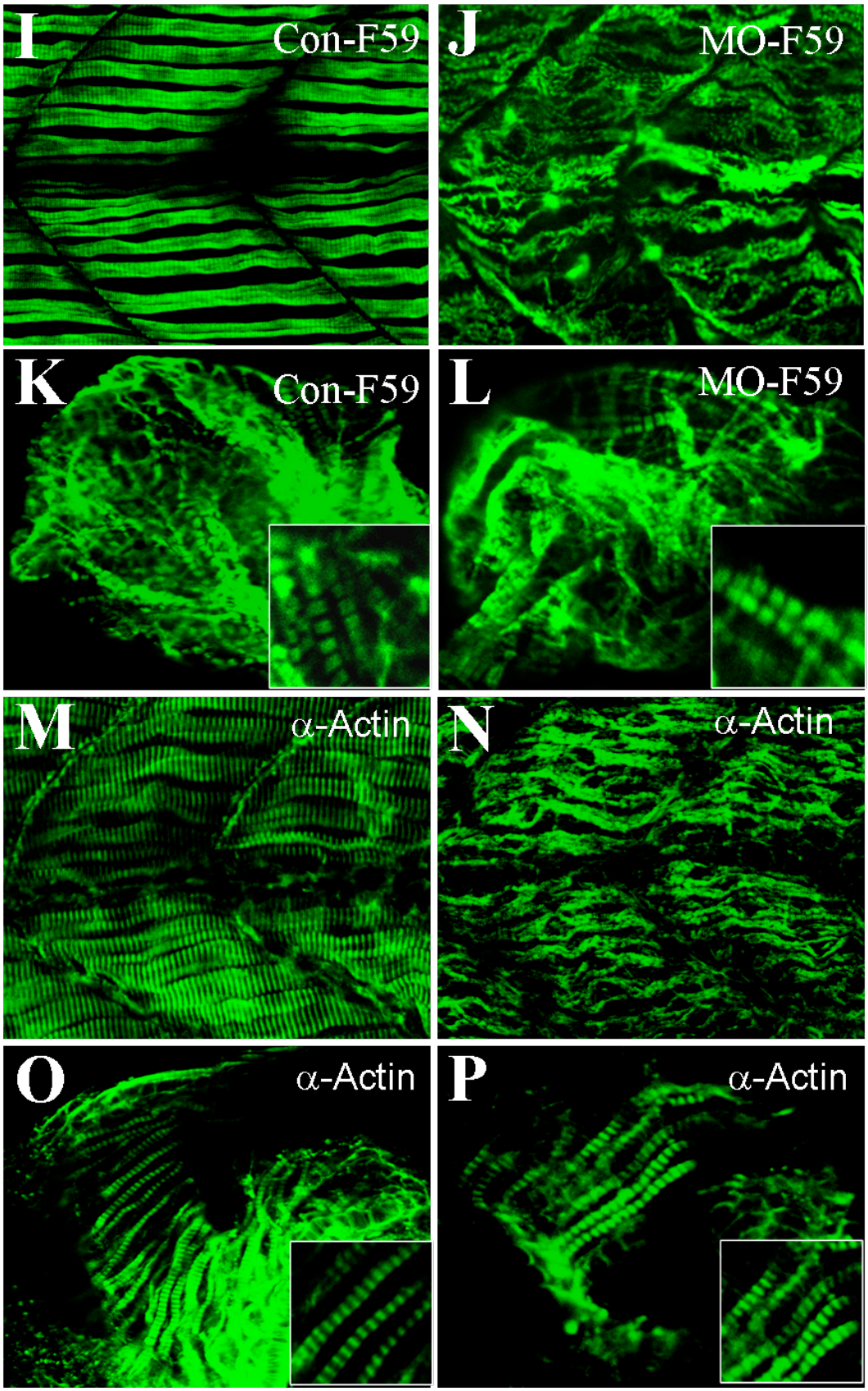

I
Con-F59
J
MO-F59
K
Con-F59
L
MO-F59
M
α-Actin
N
α-Actin
O
α-Actin
P
α-Actin

Figure 13. Knockdown of hsp90α1 expression resulted in myofibril disorganization in skeletal muscles of zebrafish embryos.

A and B. Anti-MHC antibody (F59) staining shows the organization of thick filaments in trunk slow muscles of control-MO (A) or *hsp90α1*-ATG-MO (B) injected embryos at 24 hpf. C and D. Anti-actin antibody staining shows the organization of thin filaments in control-MO (C) or *hsp90α1*-ATG-MO (D) injected embryos at 24 hpf. E and F. Anti-α-actinin antibody staining shows the organization of the Z-line in control-MO (E) or *hsp90α1*-ATG-MO (F) injected embryos at 24 hpf. G and H. Anti-myomesin antibody staining shows the organization of the M-line in control-MO (G) or *hsp90α1*-ATG-MO (H) injected embryos at 72 hpf. I-L. Anti-MHC antibody (F59) staining shows the organization of thick filaments in cardiac muscles of control-MO (K) or *hsp90α1*-ATG-MO (L) injected embryos at 72 hpf. M-P. Anti-actin antibody staining shows the organization of thin filaments in cardiac muscles of control-MO (O) or *hsp90α1*-ATG-MO (P) injected embryos at 72 hpf.

6) Smyd1b_tv1 associates with myosin chaperone Hsp90α1 and Unc45b

It has been reported that Hsp90α1 and Unc45b are chaperones that work together to control myosin folding and assembly (Barral et al 2002, Etard et al 2007). Knockdown or mutation of *hsp90α1* or *unc45b* resulted in defective myofibril organization in skeletal muscles of zebrafish embryos (Etard et al 2007, Hawkins et al 2008, Wohlgemuth et al 2007). Strikingly, the myofibril defects are similar to that observed in *smyd1b* knockdown zebrafish embryos. To test whether Smyd1b could physically interact with myosin chaperone Hsp90α1, we performed co-immunoprecipitation (Co-IP) analysis using HEK293 cells co-transfected with *cmv-hsp90α1*^{flag} and *cmv-smyd1b_tv1*^{myc} or *cmv-smyd1b_tv2*^{myc} DNA expression constructs. The result showed that Smyd1b_tv1 immunoprecipitated with a complex containing Hsp90α1 (Figure 14 A). This interaction appears to be isoform specific, because Smyd1b_tv2, an alternatively spliced isoform of Smyd1b, did not show any interaction with Hsp90α1 in the Co-IP assay (Figure 14 A). To test whether Smyd1b also interacts with Unc45b, we performed similar Co-IP in HEK293 cells co-transfected with *cmv-unc-45b*^{flag} and *cmv-smyd1b_tv1*^{myc} or *cmv-smyd1b_tv2*^{myc} constructs. Interestingly, similar results were obtained from the Co-IP assay with Unc45b. Unc45b interacts with Smyd1b_tv1 but not Smyd1b_tv2 (Figure 14 B). Together, these data indicate that Smyd1b_tv1 associates with Unc45b and Hsp90α1 and may work together with these myosin chaperones to control myofibril assembly.

Hsp90α1 is a well known chaperone required for proper folding of many client proteins (Zhao et al 2005). The association of Smyd1b_tv1 with Hsp90α1 could

be due to functional interaction between these two proteins. Alternatively Smyd1b_tv1 may be a Hsp90α1 client protein. To test these ideas, we analyzed Smyd1b_tv1 protein expression in *hsp90α1* knockdown zebrafish embryos. The result showed that *hsp90a1* knockdown had no effect on Smyd1b_tv1 protein accumulation in zebrafish embryos (Figure 14 C). These data argue against the idea that Smyd1b_tv1 being a client protein of Hsp90α1, suggesting that their interaction may be more functionally significant.

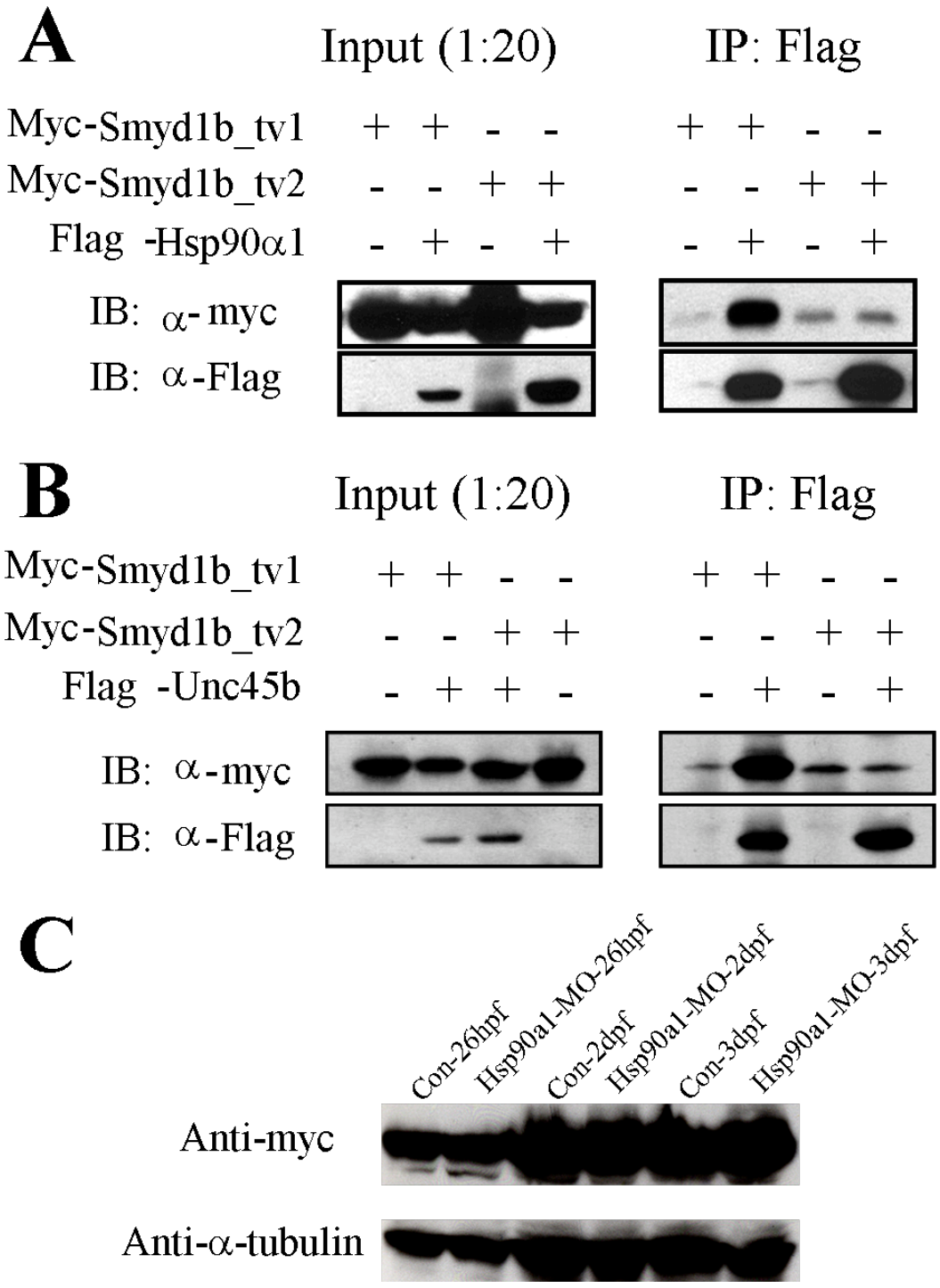


Figure 14. Co-immunoprecipitation shows Smyd1b_tv1, but not Smyd1b_tv2 interacts with myosin chaperones Hsp90α1 and Unc-45b.

A. Co-IP shows that myc-tagged Smyd1b_tv1 can be pulled down by the flag-tagged Hsp90α1 co-expressed in HEK293 cells. B. Co-IP shows that myc-tagged Smyd1b_tv1 can be pulled down by the flag-tagged Unc-45b co-expressed in HEK293 cells. C. Western Blot shows that there is no degradation of Smyd1b_tv1 in *hsp90α1* knockdown embryos at different development stages.

7) Knockdown of hsp90α1 or smyd1b resulted in reduced myosin protein accumulation

In vitro studies have suggested that Hsp90α associates with myosin proteins and is required for myosin folding and assembly in C2C12 myoblast cells (Srikakulam & Winkelmann 2004). To test whether knockdown of *hsp90α1* might disrupt myosin folding and result in myosin degradation, we analyzed the levels of myosin in *hsp90α1* knockdown embryos by western blot using the anti-MHC antibody (MF20). MHC protein levels were significantly reduced in *hsp90α1* knockdown embryos compared with wild type control (Figure 15 A). In contrast, expression of total actin appeared normal (Figure 15 A).

Data from the functional and biochemical analyses revealed that *smyd1b* plays a vital role in myosin folding and assembly in conjunction with *hsp90α1* and *unc45b*. It has been shown that knockdown or mutation of *hsp90α1* resulted in significant decline in the accumulation of myosin proteins (Du et al 2008, Hawkins et al 2008). To determine whether or not knockdown of *smyd1b* affects myosin accumulation in zebrafish embryos, we compared myosin protein levels in *smyd1b* knockdown and control zebrafish embryos at 24 hpf by western blot analysis. Compared with the control, there was a clear reduction of myosin protein levels in *smyd1* knockdown embryos (Figure 15 B). The expression of α-tubulin, serving as a loading control, was not affected (Figure 15 B). Together, these data indicate that *smyd1b* is required for myosin expression or stability.

To test whether this reduced protein accumulation was due to decreased gene transcription, we analyzed myosin mRNA levels by *in situ* hybridization and quantitative RT-PCR. The result showed little or no change of myosin mRNA levels in *smyd1b* knockdown embryos compared with control (Figure 15 C-J). Together, these data indicate that the reduced myosin accumulation was not due to decreased myosin mRNA expression; it is likely caused by myosin degradation.

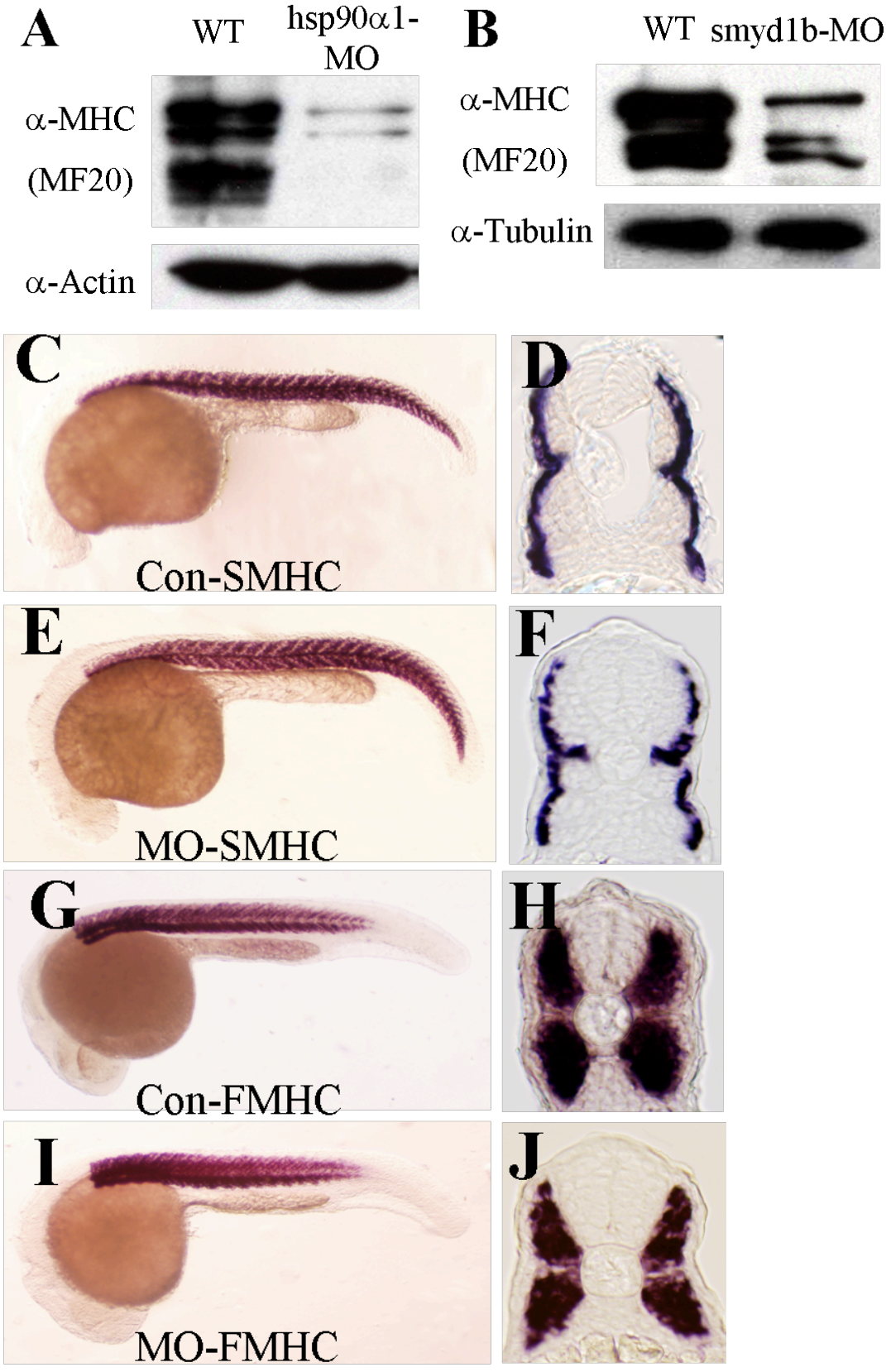

A
WT
hsp90α1-MO
α-MHC
(MF20)
α-Actin
B
WT
smyd1b-MO
α-MHC
(MF20)
α-Tubulin
C
Con-SMHC
D
E
MO-SMHC
F
G
Con-FMHC
H
I
MO-FMHC
J

Figure 15. Knockdown of hsp90α1 or smyd1b results in decreased levels of myosin proteins.

A**.** Western blot using MF20 shows decreased levels of myosin protein in *hsp90α1* knockdown embryos. B. Western blot using MF20 shows decreased levels of myosin protein in *smyd1b* knockdown embryos. C-F. *In-situ* hybridization (C-F) shows the mRNA levels of SMHC in control-MO (C and D) or ATG-MO injected (E and F) embryos at 24 hpf at different views. G-J. *In-situ* hybridization (G-J) shows the mRNA levels of FMHC in control-MO (G and H) or ATG-MO injected (I and J) embryos at 24 hpf at different views.

8) The cytosolic localization of Smyd1_tv1 is important for its function in myofibrillogenesis.

We have shown that zebrafish Smyd1b is primarily localized in the cytoplasm of myoblasts and sarcomeres of differentiated myofibers in fish embryos (Li et al. unpublished data). To detect whether Smyd1b shows the same distribution pattern in muscle cells, murine skeletal myoblasts of the C2C12 line was transfected with *cmv-Smyd1b_tv1/b*^{myc}. The expression of Smyd1b_tv1/b was then detected by anti-myc immunostaining. The result indicated that Smyd1b showed very clear cytoplasmic localization (Figure 16 A-C). It has been shown that Smyd1 is primarily localized in the cytoplasm of cultured myotubes (Sims et al 2002). Smyd1 appears to undergo a nucleus to cytoplasm translocation during C2C12 myoblast differentiation (Sims et al 2002). The biological significance of this translocation is not clear. In order to see whether zebrafish Smyd1b undergoes a similar translocation process, the cells were treated by Leptomycin B (LMB). LMB is a potent and specific nuclear export inhibitor (Julien et al 2003). Interestingly, the differentiated cells treated with LMB showed clear nuclear localization (Figure 16 D-F). This suggested that zebrafish Smyd1b has a transient nucleus to cytoplasm translocation during myoblast differentiation.

To determine whether the cytoplasmic or the nuclear localization of Smyd1b is more critical for its biological function in myofibrillogenesis, we generated a nuclear Smyd1b_tv1 containing two strong nuclear localization signal (NLS) at the N-terminus. The nuclear Smyd1b_tv1 showed a clear nuclear localization when

expressed in myofibers of zebrafish embryos (Figure 16 H, K). To determine whether nuclear localization of Smyd1b_tv1 alters its function in myofibril assembly, the nuclear Smyd1b_tv1 was used in a rescue assay in *smyd1b* knockdown zebrafish embryos. DNA constructs expressing the wild type or nuclear form of Smyd1b_tv1 were co-injected with *smyd1b* morpholino into zebrafish embryos. Compared with the DNA construct expressing the regular Smyd1b_tv1, the nuclear Smyd1b_tv1 showed less efficiency in the rescue assay (Table 2). In contrast to wild type Smyd1b_tv1 (which showed almost a 100% rescue), approximately 67% of the slow myofibers expressing the nucleaus and cytosol localization was rescued (Figure 16 G-I). 33% of the embryos that had a strong nuclear localization showed no rescue (Figure 16 J-L). This result indicated that the Smyd1b_tv1 nuclear localization reduced its activity in myofibril assembly, arguing that the cytoplasmic localization of Smyd1b_tv1 is important for its biological function in myofibrillogenesis.

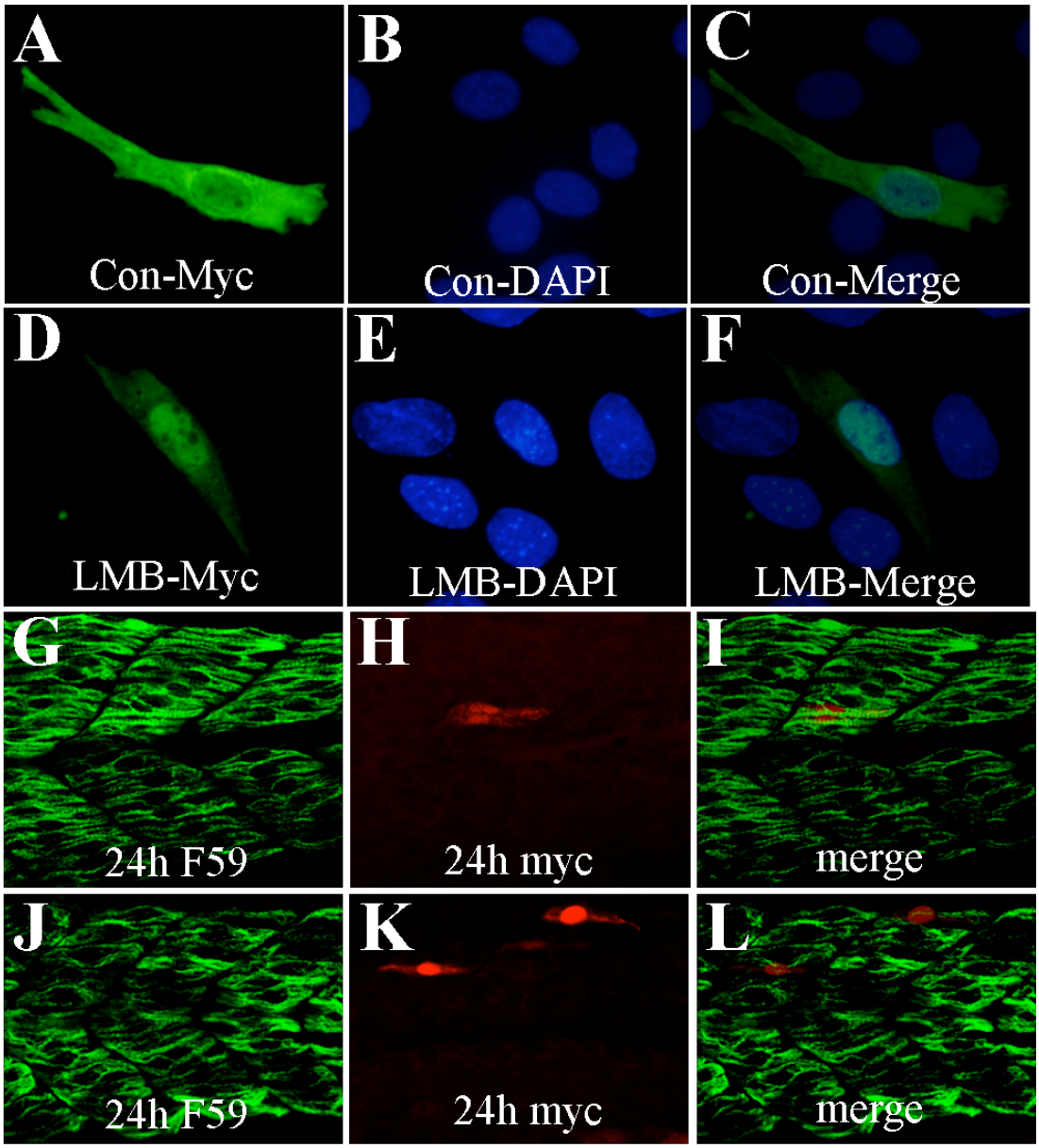


Figure 16. The cytosolic localization is important for Smyd1b_tv1 function.

A-C. Anti-myc immunolabelling shows clear cytoplasmic localization of Smyd1b_tv1/2 in C2C12 cells under differentiation (A), DAPI shows the nuclear localization (B). D-F. Anti-myc immunolabelling shows both nuclear and cytoplasmic localization of Smyd1b_tv1/2 in C2C12 cells treated with LMB (D), DAPI shows the nuclear localization (E). G-L. Anti-myc and anti-MHC (F59) double

staining show that there is a rescue if Smyd1b_tv1 has both nuclear and cytoplasmic localization (G-I), whereas there is no rescue if Smyd1b_tv has a dominant nuclear localization (J-L).

Table 2. The addition of the two NLS decreased the activity of *smyd1b_tv1*^{myc}.

	smyd1b*-MO+ *smyd1b*-2NLS-*smdy1b_tv1-myc	***smyd1b*-MO+ *smyd1b-smdy1b_tv1-myc***
Rescued	**65 fibers**	**53 fibers**
Non-rescued	**22 fibers**	**0 fibers**
Percentage of rescue	**67%**	**100%**

5. Discussion

In this study, we have characterized the myofibril defects in skeletal and cardiac muscles of *smyd1b* knockdown zebrafish embryos. We demonstrated that in addition to thick filament defects, knockdown of *smyd1b* caused significant disorganization of the thin and titin filaments, as well as M- and Z-lines in skeletal muscles of zebrafish embryos. In addition, sarcomere assembly of the cardiac muscle was also disrupted in *smyd1b* knockdown embryos. Disruption of myofibril organization by *smyd1b* knockdown resulted in increased expression of the myosin chaperones *unc45b* and *hsp90α1*. Functional studies of *hsp90α1* indicate the importance of *hsp90α1* during myofibrillogenesis. A Biochemical analysis revealed

that Smyd1b_tv1 associates with myosin chaperone Hsp90α1 and Unc45b. Together, these data support the idea that Smyd1b_tv1 may work in a complex together with myosin chaperones to control sarcomere assembly during myofibrillogenesis.

1) smyd1b function in cardiac muscle myofibrillogenesis

Our study shows a vital function of *smyd1b* in cardiac muscle development. The morphants die 5 dpf due to lack of heart function. Targeted deletion of *Smyd1* in mice is lethal; the mouse embryos die at E10.5 because of heart malformation. This indicates that *Smyd1* is required for normal heart development in mouse (Gottlieb et al 2002). The *Smyd1* knockout study in mouse showed that this peptide is required for the expression of Hand2 in the precardiac. Additionally, *Smyd1* is necessary for maturation of cardiomyocytes and morphogenesis of the right ventricle (Gottlieb et al 2002). A study of *hand2* in zebrafish showed that the *hand2* mutant *han*^{s6} developed two small lateral clusters of myocardial cells that never fuse together. This differs from the wild type, where a single midline heart tube develops (Yelon et al 2000). While the wild type heart is clearly divided into two distinct chambers – an anterior ventricle and a posterior atrium – the *han*^{s6} myocardial tissue is primarily atrial (Yelon et al 2000). In our study we showed that knockdown of *smyd1b* does not affect heart tube formation and looping. This suggests that *hand2* may not be a target gene of *smyd1*, which is consistent with our microarray data, indicating *hand2* was not detected downregulated in *smyd1b* knockdown embryos. Here we showed that knockdown of *smyd1b* caused disorganization of sarcomeric proteins in the heart. This indicated that *smyd1b* is required for cardiac muscle sarcomere assembly.

2) Smyd1b_tv1 interaction with myosin chaperones

We showed by Co-IP that Smyd1b_tv1 associates with myosin chaperones Hsp90α1 and Unc45b. Hsp90α1 and Unc45b are specifically expressed in skeletal muscles of zebrafish embryos and have been shown to function as myosin chaperones involved in myosin folding and assembly. Knockdown or mutation of *unc45b* or *hsp90α1* resulted in paralyzed zebrafish embryos with a loss of myosin filaments in trunk muscles (Du et al 2008, Etard et al 2007, Hawkins et al 2008, Wohlgemuth et al 2007). The disruption of myosin thick filaments in *smyd1b* knockdown embryos is consistent with the idea that Smyd1b_tv1 may work together with Hsp90α1 and Unc45b to control myosin folding and assembly into sarcomeres.,

We showed that myosin protein levels were significantly reduced in *hsp90α1* or *smyd1b* knockdown zerafish embryos. However, we noted that in addition to thick filament defects, thin and titin filaments, as well as Z- and M-lines were also disrupted in skeletal muscles of smyd1 knockdown embryos. Results from these studies raise the question of whether some of the myofibril defects in *smyd1b* knockdown embryos were caused indirectly by myosin degradation and thick filament disorganization.

Several pieces of evidence suggest that the sarcomeric defects in *smyd1b* knockdown embryos may not result entirely from the disruption of myosin thick filament organization in skeletal muscles of the zebrafish embryo. We showed that knockdown of *smyd1b* disrupted titin organization. Titin is the largest known protein molecule in muscle fibers and spans half the length of the sarcomere. The N terminus

of titin is anchored in the Z-disc where actin filaments are anchored, whereas its C terminus extends into the M-band where myosin filaments are anchored (Labeit & Kolmerer 1995). It has been reported that titin plays a key role in myofibrillogenesis. Depletion of *titin* in zebrafish and mouse embryos disrupts the assembly of the A-band, Z- and M-line in both skeletal and cardiac muscles (Seeley et al 2007, Weinert et al 2006). Our recent studies show that knockdown myosin heavy chain in slow muscle affects the thin filament organization, but had little effect on Z-line organization (Codina et al unpublished). Therefore, although thick filament disorganization may affect the assembly of the thin filament, it is unlikely that thick filament defects could disrupt titin association at the Z line structure. Therefore, we argue that the disruption of the sarcomere structure in *smyd1b* or *hsp90a1* knockdown embryos was not solely caused by their effect on myosin.

3) Upregulation of hsp90α1 and unc45b from disruption of myofibril organization

In this study, we showed that knockdown of *smyd1b* resulted in upregulation of *hsp90α* and *unc45b* expression in zebrafish embryos. The mechanism underlying their increased expression is not clear. Hsp90α1 is a protein chaperone also involved in the stress response (Kampinga 2006). The upregulation of *hsp90α1* expression may be induced by a stress response to myofibril disorganization in *smyd1b* knockdown embryos. Consistent with this idea, it has been shown that disruption of myofibril organization by *unc45* mutation or knockdown also led to the upregulation of Hsp90α1 expression; and vice versa, upregulation of *unc45b* expression was noted in *hsp90α1* mutant or knockdown zebrafish embryos (Du et al 2008, Etard et al 2007).

However, unlike *hsp90α1* or *unc45b*, *smyd1b* does not appear to be involved in the stress response. Disruption of myofibril organization by *hsp90α1* or *unc45b* knockdown did not result in up-regulation of *smyd1b* gene expression. Moreover, we showed that stress from heat or cold treatment did not alter *smyd1b* expression in zebrafish embryos. Together these data indicate that although Smyd1b_tv1 associates with chaperone Hsp90α1 and plays a critical role in myofibril assembly, *smyd1b* and *hsp90a1* may have a different role in stress response.

Members of the Smyd family with the conserved SET domain have been implicated in protein methylation (Brown et al 2006, Hamamoto et al 2004). We have reported that Smyd1b can methylate histones in vitro (Tan et al 2006). Recent studies indicate that Smyd proteins could methylate both histone and non-histone proteins. More and more non-histone proteins have been identified as the substrate of SET domain containing proteins (Huang & Berger 2008). Lysine methylation is a common post-translational modification for muscle proteins. Several key sarcomeric proteins, such as myosin, α-actin, muscle creatine kinase, have been shown to be methylated at lysine residues by anti-methyl-lysine antibody staining (Iwabata et al 2005, Tong & Elzinga 1983). We hypothesize that the methyltransferase works together with the chaperone machinery for the target proteins. To date, no muscle-specific methyltransferase has been identified. It remains to be determined whether Smyd1b_tv1 functions as a muscle-specific methyltransferase that methylates sarcomeric proteins required for myofibril folding and assembly.

4) The cytosolic localization is critical for Smyd1b_tv1 function in myofibril assembly

Our studies indicate that the cytosolic localization of Smyd1b_tv1 is critical for its biological function in myofibril assembly. Nuclear localization of Smyd1b_tv1 reduced its activity in the rescue assay, which suggested an important function of Smyd1b_tv1 in the cytoplasm. The cytosolic function of Smyd1b_tv1 is consistent with the idea that Smyd1b_tv1 may function directly in methylation of myofibrillar proteins required for folding and assembly of myofibrils. It is also consistent with the subcellular localization of Smyd1b_tv1 in muscle cells of zebrafish embryos. We noted that Smyd1b_tv1 is primarily localized in the cytosol of myoblasts in early stage fish embryos and later on the sarcomeres of myofibers (Li et al., unpublished).

A Recent study showed that mammalian Unc45b is a cytosolic protein that forms a stable complex with Hsp90 in the cytosol, selectively binds the unfolded conformation of the myosin motor domain, and promotes motor domain folding (Srikakulam et al 2008). This suggests that Smyd1b_tv1 may form complex with Hsp90α1 and Unc-45b in the cytoplasm to control sarcomere assembly.

We can not rule out that *smyd1b* may also have a biological function in the nucleus. It has been shown that Smyd1 is initially localized in the nucleus of cultured myoblast cells (Sims et al 2002). Smyd1 appears to undergo a nucleus to cytoplasm translocation during myoblast differentiation into myotube (Sims et al 2002). The biological significance of this early nuclear localization and subsequent translocation is not clear. Smyd1b may be required for chromatin remodeling via histone methylation at an early stage of myoblast proliferation and differentiation. It has been

shown that histone modification plays a key role in muscle cell differentiation (Cirillo & Zaret 2004, Lee et al 2004, McKinsey et al 2001, McKinsey et al 2002, Rupp et al 2002). However, at later stage of muscle cell differentiation, Smyd1b may need to be translocated to the cytosol and play an important role in myofibril assembly. Although we did not observe a clear nucleus to cytoplasm translocation in muscle cells of zebrafish embryos, subcellular localization studies revealed that zebrafish Smyd1b could be localized in the nucleus of transfected C2C12 myoblasts in the presence of LMB, a nuclear exporter inhibitor. This is consistent with histone methylation activity of Smyd1b in vitro. Moreover, it is consistent with previous studies showing that Smyd1 represses gene transcription in a HDAC dependent manner (Gottlieb et al 2002). Together, these studies indicate that Smyd1b may have dual functions in both nucleus and cytoplasm during muscle development.

CHAPTER 4: SKNAC (SKELETAL NACA), A MUSCLE-SPECIFIC ISOFORM OF NACA (NASCENT POLYPEPTIDE-ASSOCIATED COMPLEX ALPHA), IS REQUIRED FOR MYOFIBRIL ORGANIZATION IN ZEBRAFISH EMBRYO

1. Abstract

Myofibrillogenesis, the precise assembly of sarcomeric proteins into the highly organized sarcomeres which are essential for muscle cell differentiation and function. Myofibrillogenesis requires proper folding and assembly of newly synthesized sarcomeric proteins. *sknac* (skeletal *naca*) is an alternatively spliced isoform of *naca* which encodes the nascent polypeptide-associated complex alpha polypeptide that binds to newly synthesized polypeptides emerging from the ribosome. *sknac* is specifically expressed in skeletal and cardiac muscles. However, little is known about the function of *sknac* in muscle development *in vivo*. To determine *sknac* function, we have isolated and characterized the *sknac* gene from zebrafish. Zebrafish *sknac* cDNA differs from *naca* by containing an extra large exon that encodes 815 amino acids. Knockdown of *sknac* expression by antisense oligos resulted in zebrafish embryos with skeletal muscle defects. The *sknac* knockdown embryos showed a paralyzed phenotype with little muscle contraction. In contrast, injection of a control oligos had no effect. Immunostaining and histological analyses revealed that *sknac* knockdown embryos contained disorganized thick and thin filaments. Western blot analysis reveraled that myosin protein levels were

significantly reduced. Collectively, these results demonstrate that skNAC plays a vital role in myofibril assembly and function during muscle cell differentiation.

2. Introduction

Muscle fibers are composed of myofibrils, one of the most complex and highly ordered macromolecular assemblies known. Each myofibril is made up of highly organized repetitive structures called sarcomeres, the basic contractile unit of striated muscle. Sarcomeres contain thick and thin filaments that are primarily composed of myosin and actin, respectively. The precise expression of myofibrillar proteins and their assembly into sarcomeres are critically important for muscle function. However, the regulatory mechanisms that lead to the formation of this highly ordered structure are not completely understood despite extensive investigation in the past few decades (Sanger et al 2002).

Myofibrillogenesis requires proper folding and assembly of newly synthesized sarcomeric proteins. Although several chaperone proteins, including Hsp90α and Unc45, have been implicated in the correct folding and assembly of myosin and myofibrillar proteins (Du et al 2008, Etard et al 2007, Hawkins et al 2008, Liu et al 2008, Srikakulam & Winkelmann 2004), others are required and remain to be identified. *sknac* (skeletal *naca*) is an alternatively spliced isoform of *naca* which encodes the nascent polypeptide-associated complex alpha polypeptide that binds to newly synthesized polypeptides emerging from the ribosome (Sims et al 2002, Wiedmann et al 1994). The Naca has been found in all eukaryotes from yeast to human (Shi et al 1995). In contrast, skNAC has only been reported in mouse (Yotov

& St-Arnaud 1996). *naca* and *sknac* exhibit different patterns of expression; *naca* is expressed ubiquitously whereas *sknac* is expressed specifically in muscle cells (Yotov & St-Arnaud 1996). While most studies focused on Naca (Lopez et al 2005, Moreau et al 1998, Wang et al 1995, Yotov & St-Arnaud 1996), little is known about the biological function of skNAC in vivo.

skNAC has been implicated in muscle cell differentiation because of its muscle-specific expression and increased levels of expression during muscle damage and repair (Munz et al 1999). Yeast two-hybrid analysis indicated that skNAC associates with Smyd1, a protein methyltransferase involved in cardiogenesis and myofibrillogenesis (Gottlieb et al 2002, Sims et al 2002, Tan et al 2006). However, the biological significance of this interaction is unknown and skNAC function in muscle development has not been demonstrated in vivo. This is likely due to the early embryonic lethality of *naca* gene knockout mice (Deng & Behringer 1995), and the lack of skNAC-specific knockout models.

To determine skNAC specific function in muscle cell differentiation in vivo, we isolated and characterized *sknac* from zebrafish, an animal model that has been successfully used to study myofibrillogenesis in vivo (Du et al 2008, Etard et al 2007, Hawkins et al 2008, Raeker et al 2006, Tan et al 2006). We showed that *sknac* transcripts are specifically expressed in developing somites and skeletal muscles of zebrafish embryo. Knockdown of skNAC expression by antisense oligos resulted in zebrafish embryos with skeletal muscle defects. The *sknac* knockdown zebrafish embryos showed little muscle contraction. Immunostaining with anti-myosin and anti-α-actin antibodies revealed disorganized thick and thin filaments. Western blot

analysis demonstrated that the levels of myosin heavy chain (MHC) were significantly reduced in *sknac* knockdown embryos. The myofibril defects appeared to be caused by specific knockdown of *sknac* because ectopic expression of Naca had no effect on myofibrillogenesis. Together, these data suggest that skNAC plays a vital role in myofibril organization and sarcomere assembly during myofibrillogenesis.

3. Materials and methods:

1) Isolation of sknac and naca cDNAs from zebrafish

Total RNA was extracted from 24 hours-post-fertilization (hpf) zebrafish embryos by using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA). A RACE cDNA library (5') was made from the purified total RNAs using a RACE cDNA kit (BD Biosciences). Zebrafish *naca* and *sknac* cDNA were cloned from the 5'RACE library using a 5' NAC-EcoRI primer which has an *EcoRI* site upstream of the start codon and a 3' NAC-XhoI primer which has a *BamHI* and an *XhoI* site downstream of the codon. The PCR products were purified and cloned into pGEM-T easy vector to generate *pGEM-naca* and *pGEM-sknac* plasmid, respectively.

NAC-EcoRI: 5'-GGAATTCCATGCCAGGCGAAGCCACAGAA-3'

NAC-XhoI: 5'-CCGCTCGAGCGGATCCCTACATCGTCAATTCCATAAT- 3'

2) Whole mount in situ hybridization

Whole mount in situ hybridization was carried out using digoxigenin-labeled antisense probes as described (Du & Dienhart 2001b). A *naca* antisense probe was

synthesized using the *pGEM-naca* plasmid as a template. *pGEM-naca* was linearized with *SalI* and transcribed with T7 RNA polymerase. This antisense probe hybridizes with both *naca* and *sknac* mRNA transcripts because the sequence covered by the probe is identical in *naca* and *sknac* mRNA transcripts. *Sknac* specific antisense probe was synthesized using the pGEM-NAC-EX3 plasmid as a template. *pGEM-skNAC-EX3* contains the 5' region of *skNAC* specific exon 3 sequence. It was generated by PCR from genomic DNA using skNAC-P1 and skNAC-P2 primers and cloned into pGEM-T easy vector. *pGEM-skNAC-EX3* was linearized with *SalI* and transcribed with T7 RNA polymerase.

skNAC-P1: 5'-gtgatggattacaccatgcaact-3'

skNAC-P2: 5'-actgctactcttccaaagcctg-3'

skNAC-P3: 5'-caggctttggaagagtagcagt-3'

3) Synthesis of morpholino-modified antisense oligos for splicing blockers

The *sknac* splicing blockers (E3I3-MO and I2E3-MO) were made based on the antisense sequence of splicing sites at the exon-3 and intron-3 junction, or the intron-2 and exon-3 junction, respectively. In addition, a control mopholino was made based on the sense sequence of the exon-3 and intron-3 junction. A p53-MO was purchased from Gene Tools and used as described (Robu et al 2007).

E3I3-MO: cagaaagagagatacCCGTGTCGTTCTTGATG

I2E3-MO: agccctttatttccattagCCACACCTGCTGCTGCAGCCC

Control MO: CATCAAGAACGACACGGgtatctctctttctg

4) Construction of cmv:naca^{myc}, cmv:sknac^{myc}, smyd1:naca^{myc} smyd1:sknac^{myc} and smyd1:sknac$^{c\text{-}myc}$ constructs

To generate DNA constructs expressing a myc-tagged Naca or skNAC, the *naca* or *sknac* coding sequence was released by *EcoRI* and *XhoI* digestion. The DNA insert of *naca* or *sknac* was then subcloned into *EcoRI* and *XhoI* site of the CS^{2+}-MT vector (Rupp et al 1994, Turner & Weintraub 1994). This vector contains a cmv promoter and an N-terminal myc-tag sequence to produce the plasmid *cmv:naca^{myc}* or *cmv:sknac^{myc}*, respectively. To generate the DNA construct expressing a N-terminal myc-tagged Naca or skNAC specifically in muscle cells, the *naca* or *sknac* coding sequence was released from the *cmv:naca^{myc}* or *cmv:sknac^{myc}* plasmid by *BamHI* digestion and cloned downstream of the zebrafish muscle-specific smyd1 promoter (Tan et al 2006). The resultant constructs were named *smyd1:naca^{myc}* or *smyd1:sknac^{myc}*, respectively. To generate a C-terminal myc-tagged skNAC, a new reverse primer (skNAC-myc) of *sknac* containing a myc-tag on the C-terminus was used to generate the *sknac^{cmyc}* by PCR. The PCR product was then subcloned after the zebrafish muscle-specific *smyd1* promoter to generate the *smyd1:sknac^{cmyc}* DNA construct.

skNAC-myc: 5′- ctaattcaggtcctcttcagagatgagcttctgctccatcgtcaattccataatagc-3′

5) Morpholino and DNA microinjection in zebrafish embryos

Morpholino antisense oligos were dissolved in Danieau buffer (Nasevicius & Ekker 2000) to a final concentration of 0.5 mM except E3I3-MO, which was 0.25 mM. Zebrafish embryos were injected at the 1 or 2 cell stage with 2 nl of MO as

described (Tan et al 2006). DNA microinjection was carried out as described (Du et al 1997).

6) Analysis of sknac specific expression in wild type or MO injected embryos by RT-PCR

Total RNA was extracted from zebrafish embryos at 0h, 3h, 6h, 9h, 12h, 14h 19h, 24h, 2d, 3d, 4d, 5d and 6d post fertilization. *naca* was amplified using primers NAC-5' and NAC-3', and *sknac* was amplified using primers skNAC-P1 and skNAC-P3. To determine the effect of E3I3-MO or I2E3-MO on *sknac* splicing, total RNA was extracted from WT or MO injected embryos at 24 hpf. Expression of *sknac* transcripts was analyzed by RT-PCR using *sknac* specific primers skNAC-P1 and skNAC-P3. *Elongation factor 1α (ef-1α)* was amplified as control.

NAC-5': 5'-ccagaatgccaggcgaagccacag-3'

NAC-3': 5'-ccatctacatcgtcaattccataa- 3'

skNAC-P1: 5'-gtgatggattacaccatgcaact-3'

skNAC-P3: 5'-caggctttggaagagtagcagt-3'

ef-1α-P1: 5'-gcatacatcaagaagatcggc-3'

ef-1α-P2: 5'-gcagccttctgtgcagactttg-3'

7) Immunostaining of whole mount fish embryos

Immunostaining was carried out using whole mount zebrafish embryos or tissue sections as previously described (Tan et al 2006) with the following antibodies:

anti-α-actinin (clone EA-53, #A7811, Sigma), anti-MHC for slow muscles (F59, DSHB), anti-MHC (F310, DSHB), anti-myomesin (mMaC myomesin B4, DSHB), and anti- α-actin (Ac1-20.4.2, Progen). 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, Hercules, CA).

8) Phalloidin-FITC staining

Zebrafish embryos were fixed at 24 hpf or 3 dpf in 4% paraformaldehyde for 1h at room temperature. The fixed embryos were washed 3 times for 10 minutes each with 1×PBST. To achieve a better permeation, 3 dpf embryos were treated with collagenase (1mg/ml, Sigma C-9891) for 75 min at room temperature, and then were washed 3 times for 10 minutes each with 1×PBST. All embryos were subjected to cold acetone treatment at -20 °C for 10 minutes. The embryos were washed 3 times for 10 minutes each with 1×PBST, and then stained with phalloidin-FITC at 10 μg/ml (Sigma P5282) for 40 minutes at room temperature. The embryos were washed 3 times with PBST, 10 minutes each and photographed using a confocal microscope (BIO-RAD Radiance 2100 Imaging Systems, Hercules, CA).

9) Histological analyses using thin plastic sections

Wild type and E3I3-MO injected zebrafish embryos were fixed at 3 dpf in 2.5% glutaraldehyde overnight at 4^{o}C. The fixed embryos were used for plastic section as described (Tan et al 2006).

10) Cell culture and immunostaing

Murine skeletal myoblasts of the C2C12 line were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U each penicillin/streptomycin) in 5% CO_2 at 37^{o}C. When the cells had reached 80% confluence, DNA construct pCS-skNAC^{myc} was transfected into the cells using the LipofectAMINE reagent according to the instructions of the manufacturer. Twenty four hours after transfection, cell differentiation was induced by changing the culture medium to differentiating medium (DM) which contains fresh DMEM medium supplemented with 2% horse serum and antibiotics. All culture reagents were from Invitrogen (La Jolla, CA).

C2C12 cells grown on separate coverslips were fixed at different stages with 4% paraformaldehyde for 15 min and washed with PBS. The cells were treated with monoclonal anti-myc antibody (9E10) at a 1:2000 dilution for 1 h and then exposed to FITC-conjugated secondary antibodies. In addition, cell nuclei were stained for 10 min using Hoechst 33258 solution (1 μ g/ml; Sigma, St. Louis, MO) and observed using fluorescence microscopy (Axionplan-1).

11) Western blot analysis

Wild type or MO-injected zebrafish embryos at 24 hpf (100 embryos each) were dechorinated manually and crushed gently to remove the yolk by triturating with a glass pipet. The embryos were solublized in 200 μl of SDS loading buffer (0.125 M

Tris-Cl pH 6.8, 4% SDS, 20% Glycerol, 0.2 M DTT, 0.02% Bromophenol Blue) containing PMSF (1mM) as a protease inhibitor. Samples (20 ul each; 10 embryos) were boiled for 5 min and the proteins were separated on a 7.5% SDS-PAGE. Proteins were electrophoretically transferred onto a PVDF membrane (Immobilion-P, Millipore). Immunodetection of α-tubulin and MHC was carried out with the anti-α-tubulin or anti-MHC (MF-20) antibodies, followed by incubation with peroxidase-conjugated secondary antibodies.

12) Real time PCR analysis

Real time PCR was carried out to analyze myosin gene expression using 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR reaction was carried out using the standard SYBR Green PCR Mater Mix (Applied Biosystems). Standard curves of cDNA samples were constructed using 10 fold serial dilutions. The relative levels of gene expression were compared based on the normalized value of the endogenous control ef-1α. Real time PCR was carried out using the following primers designed at the junctions of two adjacent exons to eliminate potential problem with genomic DNA contamination.

zfmyhz2-P4: gctcacctaccagactgagga

zfmyhz2-P5: actcagcaatatcagcacgct

zfsmyhc1-P4: gctcacctaccagactgagga

zfsmyhc1-P5: catcttgttgacctgagattca

zfef1α-P3: cttcaacgctcaggtcatcat

zfef1α-P4: acagcaaagcgaccaagagga

4. Results:

1) Isolation and characterization of zebrafish sknac

A full-length *sknac* cDNA was isolated from zebrafish embryos by RT-PCR. Two DNA products were generated by the PCR reaction. The shorter PCR product encodes a protein of 215 amino acids that shares high sequence identity with Naca from yeast to humans, suggesting that it is a *naca* ortholog. The longer product, the *sknac* isoform, encodes a protein of 1030 amino acids, considerably larger than the *naca* isoform (Figure 17 A). Sequence analysis indicates that *naca* and *sknac* are generated by alternative splicing of RNA transcripts from the same gene (Figure 17 A). *sknac* contains 9 exons, including the *sknac* specific exon 3, whereas *naca* shares 8 exons with *sknac* but omits the large exon 3.

To better characterize *sknac* structure and expression, we searched through a nucleotide blast for *sknac* transcripts and analyzed genomic sequences of several invertebrates and vertebrates. No *sknac* sequence could be identified in *Drosophila* or in *C. elegans* although they both contain the highly conserved *naca* transcripts. *sknac* appears to be unique to vertebrates. Analysis of potential transcripts using Gene Tool (Genetool, Bio Tools Incorporated) predicts a *sknac* spliced isoform in tetrodon and fugu. Sequence alignment revealed that the length of exon-3 varies dramatically among *sknacs* from mammals and predicted from different organisms (Figure 17 B). The skNAC protein in zebrafish is shorter than that in mice and humans, which are 2187 aa and 2078 aa, respectively. The size difference is exclusively attributed to exon 3, encoding a 815 aa peptide in zebrafish and a 1972 aa peptide in the mouse.

A sequence comparison of zebrafish and mouse revealed that the skNACs are highly conserved at the N- and C-terminal regions, containing the Naca sequence, with 96.3% identity. The sequence encoded by the *sknac*-specific exon 3 is less conserved. Nevertheless, exon 3 encodes a common proline rich region among the vertebrate sequences. Proline residues represent 17% and 23% of the amino acid sequence encoded by the exon 3 in zebrafish and human skNAC, respectively. In addition, exon 3 encodes several repetitive sequences. Zebrafish skNAC contains eight 28 aa repeats, while human skNAC contains eighteen 23 aa repeats. Except being proline rich, no conserved motif has been identified among these repetitive sequences.

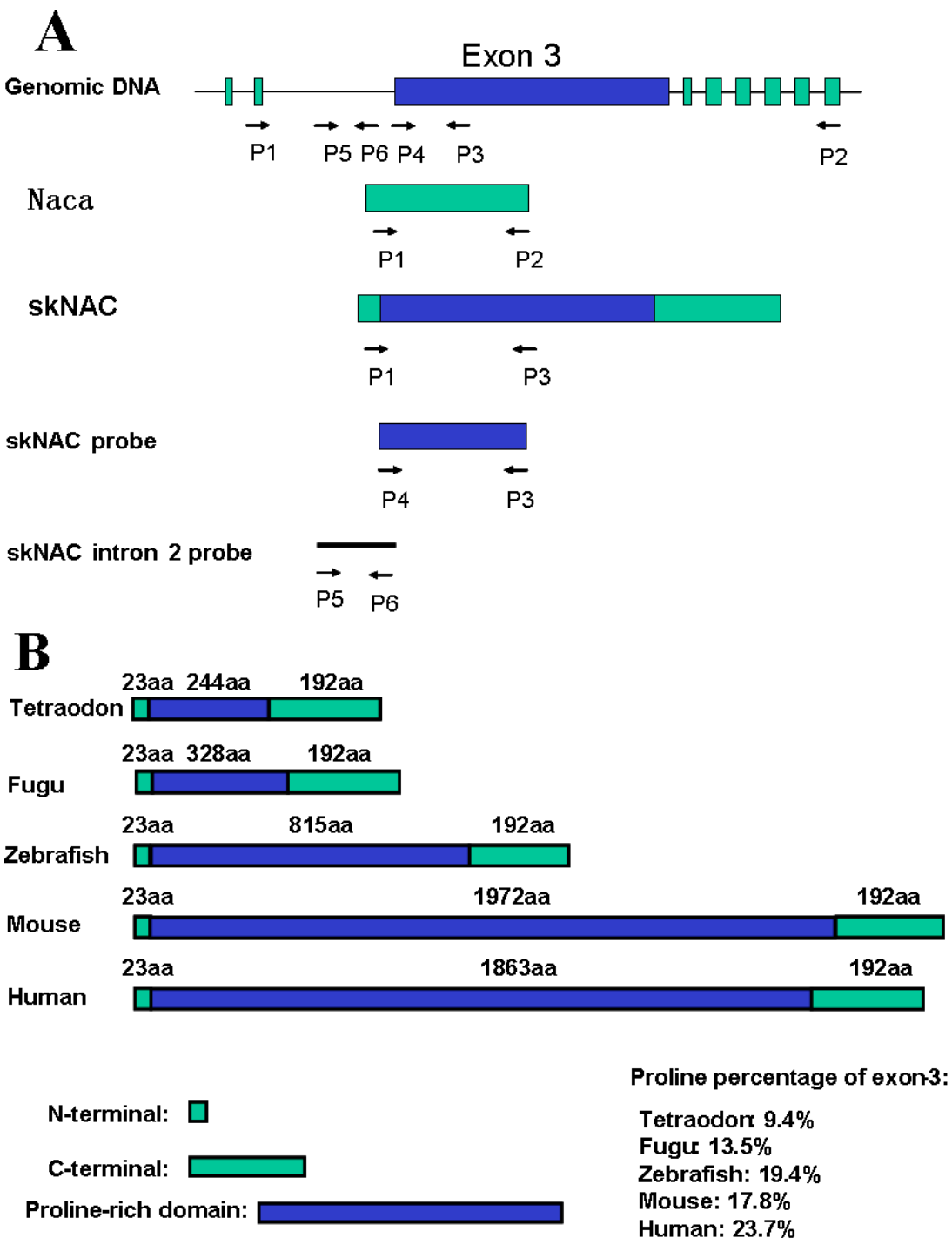


Figure 17. Alternative splicing of the zebrafish naca gene generates naca and sknac mRNA transcripts.

The zebrafish *naca* gene contains 9 exons including a large, alternatively spliced exon 3, specific for the *sknac* transcript expressed in cardiac and skeletal muscles. In

contrast, the *naca* transcript contains 8 exons in common with the *sknac* transcript but missing exon 3. The primers P1, P2, P3 and P4 were used for analyzing the expression of *naca* and *sknac* by RT-PCR, and the cloning of the DNA fragment for making specific probes against *naca* and *sknac*. B. Sequence comparison showing the size variation of *sknacs* from different organisms. Translated amino acid sequences are shown from known transcripts of zebrafish and mouse, and predicted transcripts from tetraodon, fugu, and human. The N-terminal and C-terminal sequences, shared with Naca, are highly conserved during evolution. However, the encoded sequences and the length of the muscle-specific exon 3 in *sknac* varies dramatically among different species. One common feature is the proline-rich content of the sequence, ranging from 9% in tetraodon to 23% in human.

2) Muscle specific expression of sknac in zebrafish embryos

The temporal and spatial expression of *naca* and *sknac* was determined in zebrafish embryos by RT-PCR and whole mount in situ hybridization (Figure 18 A). *naca* and *sknac* exhibited distinct patterns of expression. *naca* transcripts could be detected in fertilized eggs, suggesting that it was expressed maternally (Figure 18 A). In contrast, *sknac* expression was first detected around 12 hpf (Figure 18 A). The level of *naca* expression remained high in all embryonic and larval stages analyzed, from fertilization up to 6 days post-fertilization (Figure 18 A), whereas the levels of *sknac* expression increased significantly during somitogenesis and myogenesis between 19-24 hpf (Figure 18 A).

To determine whether *naca* and *sknac* have different spatial patterns of expression, we analyzed *naca* and *sknac* expression in zebrafish embryos by whole mount in situ hybridization (Figure 18 B-K). Two antisense probes were used: one was the full-length *naca* cDNA, capable of hybridizing with both *naca* and s*knac* RNA transcripts, the other was a *sknac*-specific probe derived from part of the exon 3 sequence. In situ hybridization using these two probes revealed two distinct patterns. The *naca* probe showed a ubiquitous pattern of expression in zebrafish embryos (Figure 18 B-F). In contrast, the *sknac* specific probe showed a tissue-specific expression in skeletal and cardiac muscles of zebrafish embryos (Figure 18 G-K).

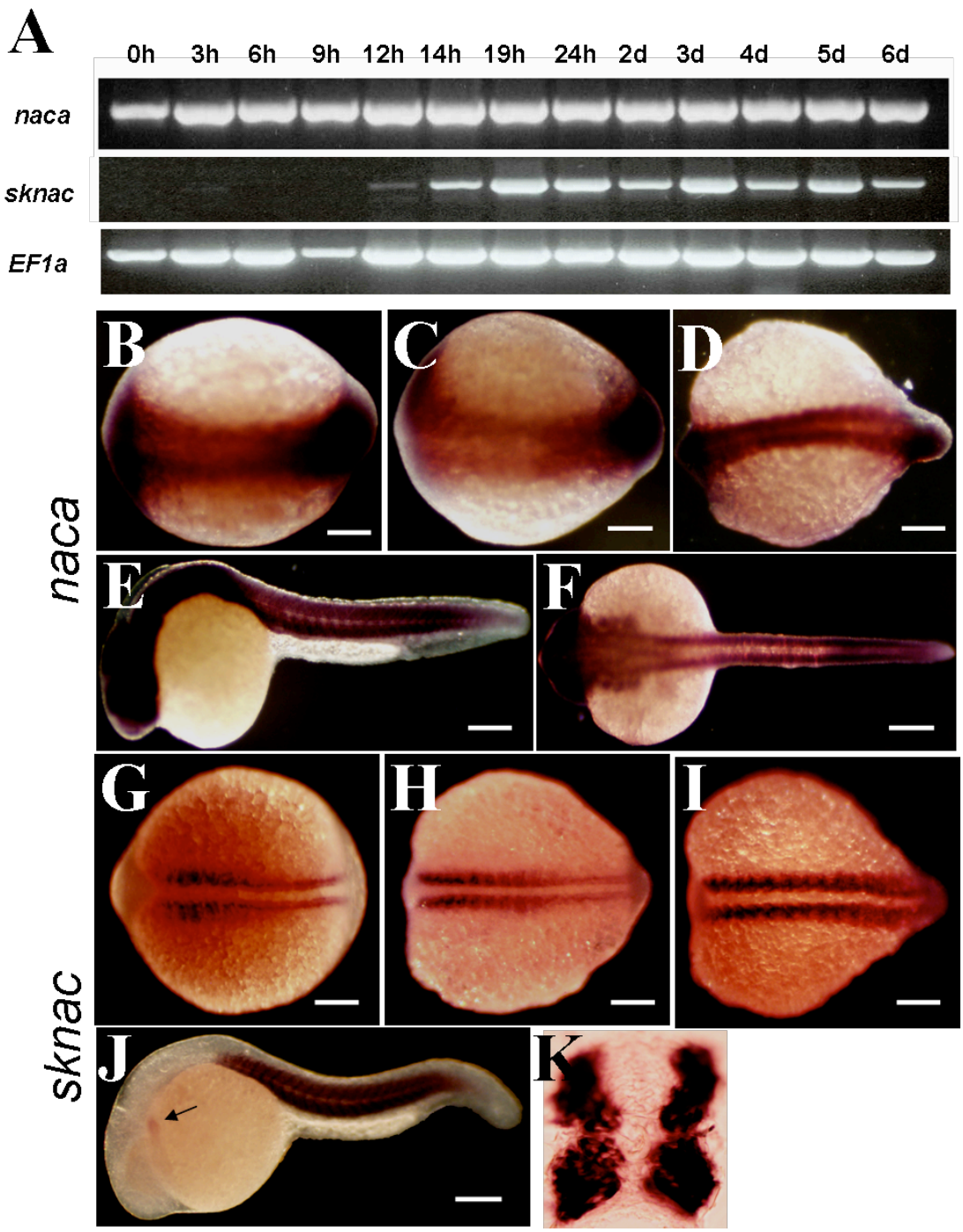


Figure 18. Temporal and spatial patterns of naca and sknac expression in zebrafish embryos.

A. RT-PCR results showing different temporal expression patterns of *naca* and *sknac* mRNAs in zebrafish embryos. *naca* is expressed both maternally and zygotically

during the 6 day development period analyzed here. In contrast, *sknac* is only expressed zygotically starting around 12 hpf. B-F. Whole mount in situ hybridization showing the spatial patterns of *naca* and *sknac* expression using a dig-labeled *naca* antisense probe. The following stages were analyzed: 12hpf (B), 14hpf (C), 16hpf (D), 24hpf (E,F). Dorsal (B-D,F) and side (E) views are shown. Scale bar: (B-D), 200 μm, (E,F), 400 μm. G-K. In situ hybridization showing the spatial patterns of *sknac* expression using a dig-labeled antisense probe from exon 3 sequence specific for *sknac* mRNA transcripts. The following stages were analyzed: 12 hpf (G), 14 hpf (H), 16 hpf (I), 24 hpf (J-K). Dorsal (G-I) and side (J) views are shown. The arrow in J indicates cardiac muscles. K is a view from a cross section showing the expression of *sknac* in skeletal muscles. Scale bar: (G-I), 200 μm, (J), 400 μm.

3) Knockdown of sknac expression resulted in skeletal and cardiac muscle defects

To characterize the function of skNAC in muscle development, we performed a knockdown analysis of *sknac* expression in zebrafish embryos. Two splicing blockers, I2E3-MO and E3I3-MO, were synthesized based on the DNA sequences at the 5' and 3' regions surrounding the muscle-specific exon 3 (Figure 19 A). The I2E3-MO or E3I3-MO was injected into zebrafish embryos at 0.5 mM or 0.25 mM, respectively. Their effect on *sknac* splicing was analyzed by RT-PCR (Figure 19 A-C). As shown in Figure 19 B, C, injection of either one of these two morpholinos blocked the splicing of *sknac* transcripts. Compared with the PCR results from un-injected embryos, expression of *sknac* transcripts was completely blocked in E3I3-MO injected embryos (Figure 19 B). The I2E3-MO appeared to be less effective than the E3I3-MO, but still knocked down *sknac* transcripts by approximately 70% (Figure 19 C). To determine whether *naca* splicing was affected in *sknac* knockdown embryos, we analyzed *naca* expression by RT-PCR. A strong *naca* expression was detected in *sknac* knockdown embryos (Figure 19 D), suggesting that knockdown of *sknac* by the morpholinos was selective.

To further confirm the knockdown of *sknac* transcripts in zebrafish embryos, we performed a whole mount in situ hybridization using the *sknac* specific probe, corresponding to part of the exon 3 sequence, in E3I3-MO injected embryos. Compared with control embryos (Figure 19 E, G), E3I3-MO injected embryos showed little or no *sknac* expression (Figure 19 F, H). Together, these data indicate

that the splicing blocker is very effective in knocking down the splicing of *sknac* transcripts in muscle cells of zebrafish embryos.

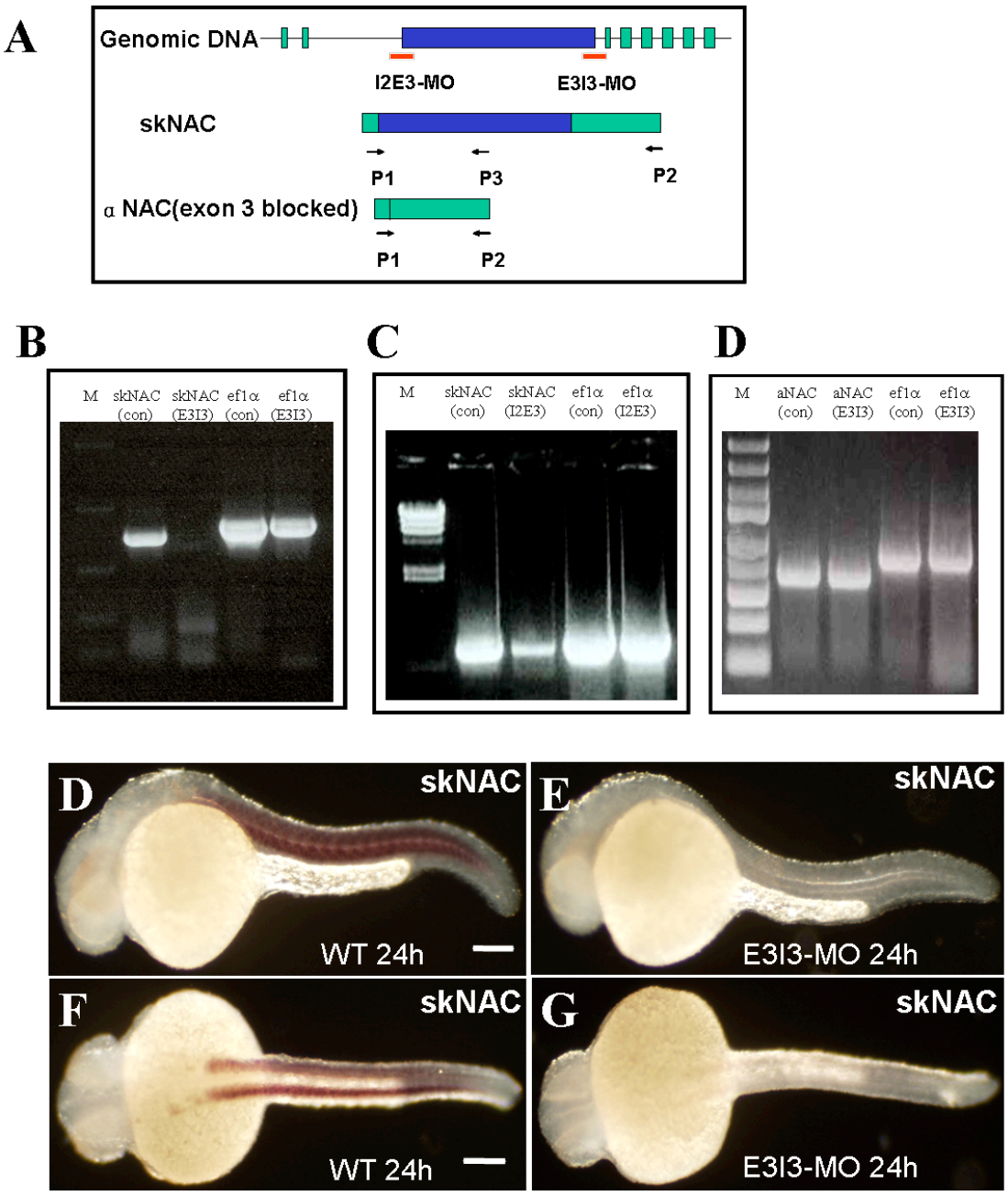


Figure 19. Knockdown of sknac transcripts using splicing blockers.

A. Locations of the morpholino splicing blockers I2E3-MO and E3I3-MO. I2E3-MO is targeted at the intron-2/exon-3 junction, while the E3I3-MO is targeted at the exon-

3/intron-3 junction. Both splicing blockers knocked down the splicing of *sknac* transcripts. B and C. RT-PCR results showing the decreased *sknac* mRNA levels induced by E3I3-MO (B) or I2E3-MO (C). E3I3-MO appears to be more efficient than I2E3-MO in knocking down *sknac* transcripts. *elongation factor 1α (ef1α)* was used as an internal control. D. RT-PCR results showing the normal expression of *naca* in E3I3-MO injected zebrafish embryos. E-H. In situ hybridization using a dig-labeled *sknac* specific antisense probe showing the lack of *sknac* mRNA transcripts in E3I3-MO injected embryos at 24hpf (F and H) compared with un-injected embryos (E and G). Scale bar: (D-G), 400 μm.

4) Knockdown of sknac expression blocks muscle contraction without any effect on myoblast specification and early differentiation.

To determine the morphological phenotype of the *sknac* knockdown embryos, the E3I3-MO or I2E3-MO injected embryos were examined every day for 4-5 days following the injection. The *sknac* knockdown embryos showed a paralyzed phenotype with little locomotion and muscle contraction. The morphant embryos also exhibited a clear heart edema on day 3 (Figure 20 C, D). These data suggest that knockdown of *sknac* expression may affect the normal function of skeletal and cardiac muscles in zebrafish embryos. The morphant embryos died around day 5 and 6.

To determine which step of muscle development was affected by *sknac* knockdown, we analyzed myoblast specification, differentiation and maturation in *sknac* knockdown embryos using several molecular markers. Compared with control embryos (Figure 20 E, G), expression of the myogenic regulatory genes *myod* and *myogenin* was normal in E3I3-MO injected embryos (Figure 20 F, H). Moreover, specification of slow and fast muscles also appeared normal (Figure 20 J, L). However, MHC expression levels appeared weaker in *sknac* knockdown embryos (Figure 20 L).

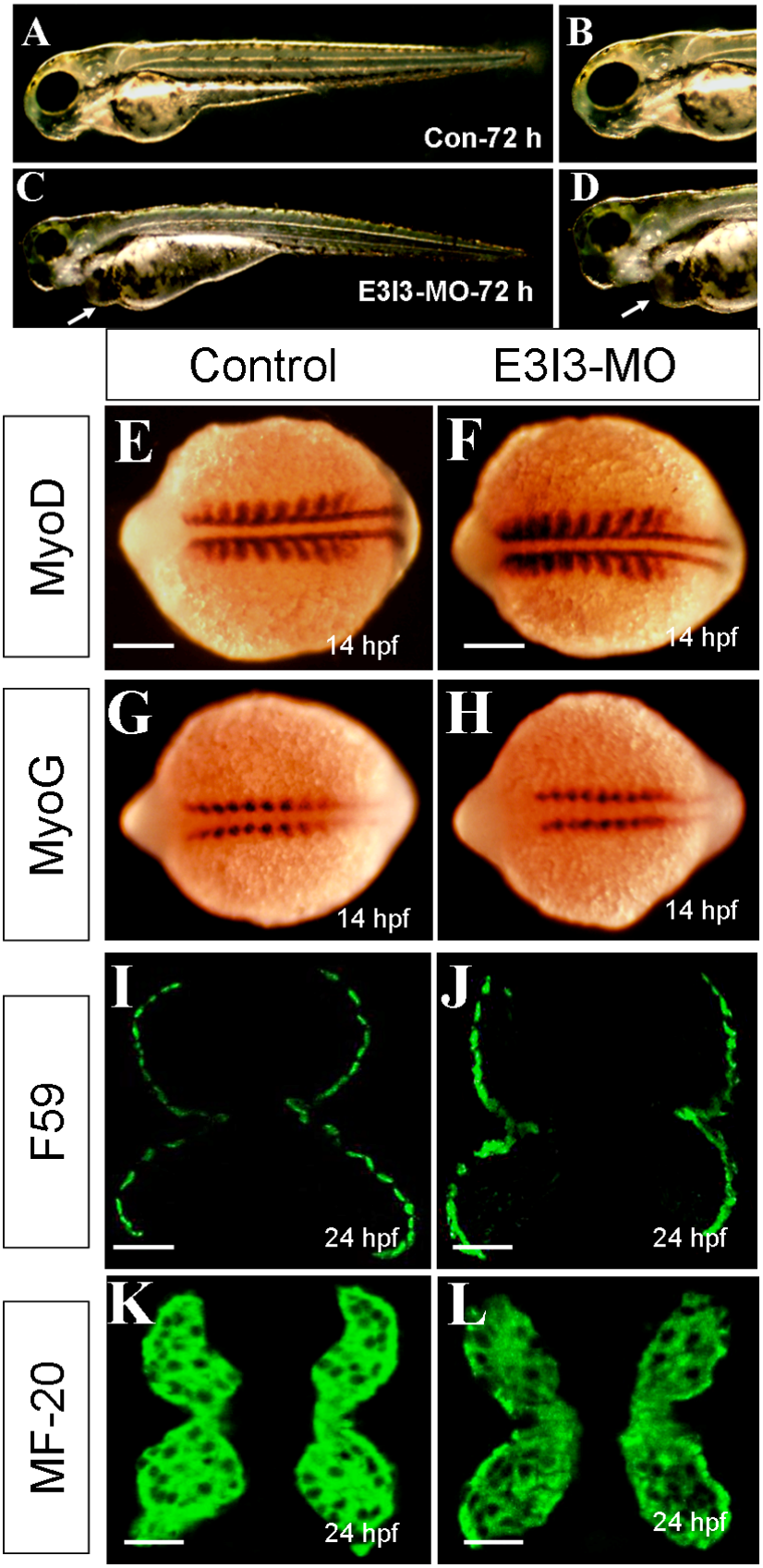

A
Con-72 h
B
C
E3I3-MO-72 h
D
Control
E3I3-MO
MyoD
E
14 hpf
F
14 hpf
MyoG
G
14 hpf
H
14 hpf
F59
I
24 hpf
J
24 hpf
MF-20
K
24 hpf
L
24 hpf

Figure 20. Knockdown of sknac expression has no effect on myoblast specification and slow and fast muscle fiber differentiation.

A–D) Morphological comparison of control-MO-injected (A, B) or sknac-E3I3-MO-injected embryos (C, D) at 72 hpf. A clear edema was detected in *sknac*-knockdown embryos (arrows). E-H) In situ hybridization shows expression of *myod* (E, F) and *myogenin* (G, H) in *sknac* knockdown (F, H) or control embryos (E, G) at the ten somite stage. Scale bar: 200 μm. I-J) Immunostaining with F59 antibody on cross sections showing normal distribution of slow muscle fibers at 24 hpf in control (I) and E3I3-MO (J) injected embryos. Scale bar: 50 μm. K-L) Immunostaining with MF-20 antibody on cross sections showing the distribution of all slow and fast muscle fibers at 24 hpf in control (K) and E3I3-MO (L) injected embryos. Scale bar is 50 μm.

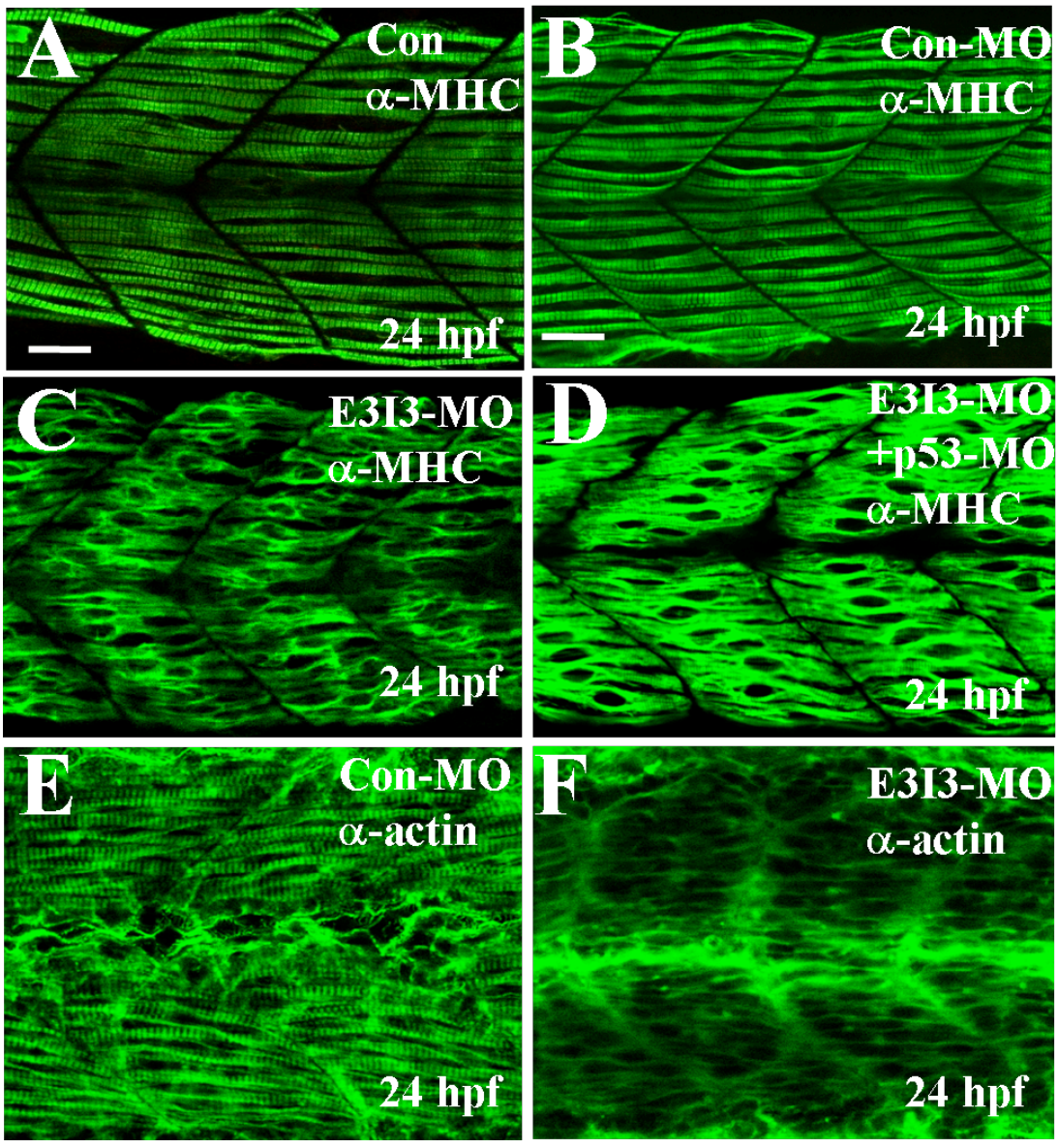
5) Knockdown of sknac expression disrupts thick and thin filament assembly in slow muscles

To determine whether knockdown of *sknac* expression might disrupt myofibril assembly during myofiber maturation, *sknac* knockdown embryos were closely examined for thick filament organization in slow muscles. Immunostaining with anti-MHC antibody (F59) showed that thick filament alignment was completely disrupted in slow muscles of E3I3-MO injected embryos (Figure 21 C, 85%, n=250). The *sknac* knockdown myofibers contain a large nucleus at the center, a characteristic of immature myofibers (Figure 21 C). Injection of I2E3-MO also resulted in disruption of thick filament organization although to a less degree compared with E3I3-MO. This is consistent with the lower efficacy of I2E3-MO in knockdown of *sknac* splicing compared with E3I3-MO. Injection of a control MO based on the sense sequence at the exon 3 and intron 3 junction did not result in any muscle phenotype (Figure 21 B).

To further rule out the possibility that the disorganized thick filament pattern was not caused by nonspecific morpholino toxicity, a p53 MO was co-injected with *sknac* E3I3-MO into zebrafish embryos. The p53 MO has been shown to reduce the non-specific effects of MO injection (Robu et al 2007). A clear myofibril defect was found in thick filaments of E3I3-MO and p53 MO co-injected embryos (Figure 21 D). In contrast, the thick filament defect was not observed in zebrafish embryos injected with p53 MO alone, confirming that the thick filament disorganization was not

caused by MO toxicity. Together, these data indicate that the skNAC may play an important role in thick filament organization and assembly in zebrafish embryos.

To test whether knockdown of *sknac* affected the thin filament organization in slow muscles, the *sknac* knockdown embryos were examined with anti-α-actin antibody. Compared with the control-MO injected embryos (Figure 21 E), *sknac* knockdown embryos showed few or no thin filaments (Figure 21 F). This was further confirmed by phalloidin staining (Figure 21 H). Together, these data indicate that skNAC was also required for the thin filament assembly. To test whether the sarcomeric structure Z-line was affected in the *sknac* knockdown embryos, we analyzed the localization of α-actinin by antibody staining. Interestingly, a sarcomeric localization of α-actinin was detected, suggesting that organization of Z-line was not severely disrupted (Figure 21 J). However, the *sknac* knockdown fibers appeared a little twisted compared with control (Figure 21 J). This is likely due to a secondary effect from the large central nucleus within the *sknac* knockdown fibers. Together, these data indicate that knockdown of skNAC might have a specific effect on the organization and assembly of both thick and thin filaments. This is consistent with previous *in vitro* studies showing that the basic framework of the sarcomere consisting of Z-line and M-line is maintained in isolated myofibrils stuck to glass slides when both thick and thin filaments are removed by gelsolin and potassium acetate treatment (Funatsu et al 1990, Funatsu et al 1993).


A
Con
α-MHC
24 hpf
B
Con-MO
α-MHC
24 hpf
C
E3I3-MO
α-MHC
24 hpf
D
E3I3-MO
+p53-MO
α-MHC
24 hpf
E
Con-MO
α-actin
24 hpf
F
E3I3-MO
α-actin
24 hpf

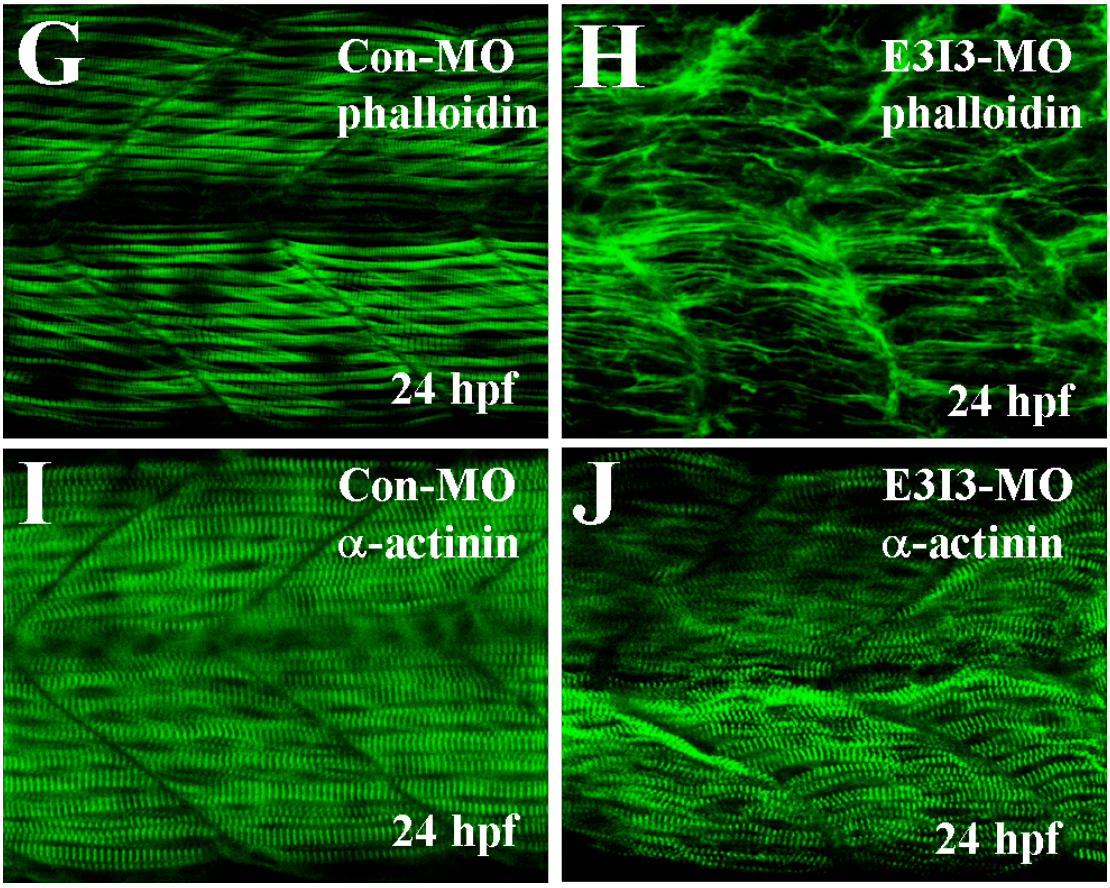


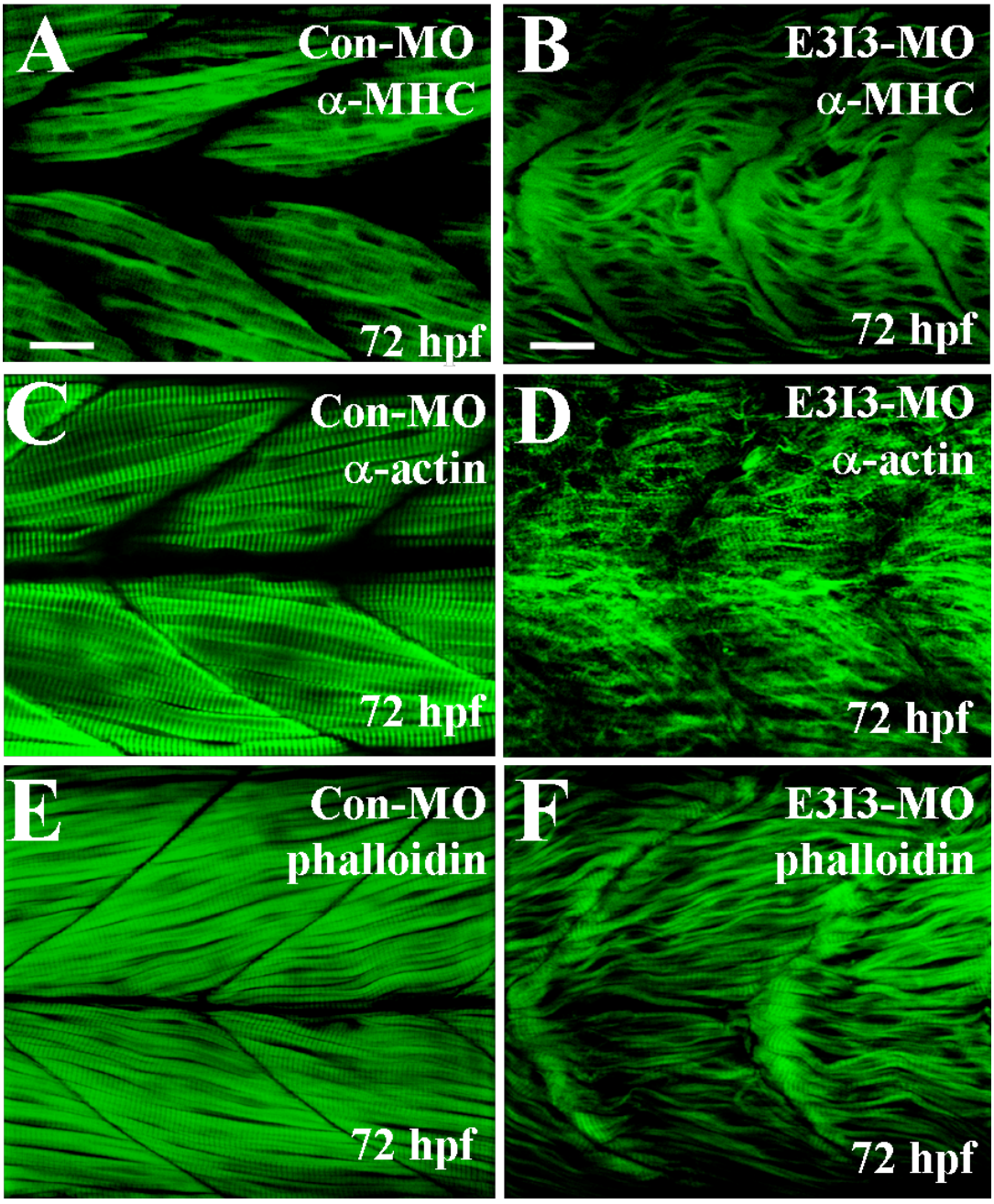
Figure 21. Knockdown of sknac expression resulted in defective thick- and thin-filament assembly in slow muscles of zebrafish embryos.

A, B) Anti-MHC (F59) antibody staining (lateral view) shows thick filaments in slow muscle fibers of control (A) or control-MO-injected embryos (B) at 24 hpf. Scale bar=20 μm. C, D). F59 staining shows defective thick-filament organization in slow fibers of E3I3-MOinjected (C) or E3I3-MO- and p53 MO-coinjected embryos (D) at 24 hpf. Note that higher gain was used in images C and D to show the disorganized thick filaments. E, F) Anti-α-actin antibody (Acl-20.4.2) staining shows organization of thin filaments in slow muscle fibers of control-MO-injected (E) or E3I3-MO-injected embryos (F) at 24 hpf. G, H) Phalloidin-FITC staining shows organization of thin filaments in slow muscle fibers of control-MO-injected (G) or E3I3-MO-injected

embryos (H) at 24 hpf. I, J) Anti-α-actinin antibody (EA-53) staining shows Z-line structure in control-MO-injected (I) or E3I3-MO-injected embryos (J) at 24 hpf.

6) Knockdown of sknac expression disrupts filament assembly in fast muscles

To determine whether fast muscles located in the deep region of myotome are also affected in *sknac* knockdown embryos, immunostaining was carried out in zebrafish embryos at 3 dpf when functional fast fibers are well developed. Fast fibers could be easily distinguished from slow fibers by their location and myofiber projection (Roy et al 2001). Unlike slow fibers that are localized in the superficial layer with a parallel projection to the midline structure, fast muscles are located within the deep myotome and project with a 20-30 degree angle with respect to the midline structure (Figure 22 A). Immunostaining with anti-MLC and anti-α-actin antibodies showed that knockdown of *sknac* disrupted thick and thin filament organization in fast muscles (Figure 22 B, D). The thin filament disorganization was further confirmed by phalloidin staining (Figure 22 F). Similar to slow muscles, there was little effect on the sarcomeric localization of α-actinin and myomesin in the Z-line and M-line in fast fiber although the fibers appeared to be twisted (Figure 22 H, J). Histological analyses further confirmed the results from the immunostaining. Multinucleated fast fibers contained few or no sarcomeres in the *sknac* knockdown embryos (Figure 22 L). Consistent with data from immunostaining in slow muscles, large central nuclei were also found in *sknac* knockdown fast muscles (Figure 22 L). Collectively, these results support a specific function of skNAC on the myofibrillogenesis of thick and thin filaments in skeletal muscles.


A
Con-MO
α-MHC
72 hpf
B
E3I3-MO
α-MHC
72 hpf
C
Con-MO
α-actin
72 hpf
D
E3I3-MO
α-actin
72 hpf
E
Con-MO
phalloidin
72 hpf
F
E3I3-MO
phalloidin
72 hpf

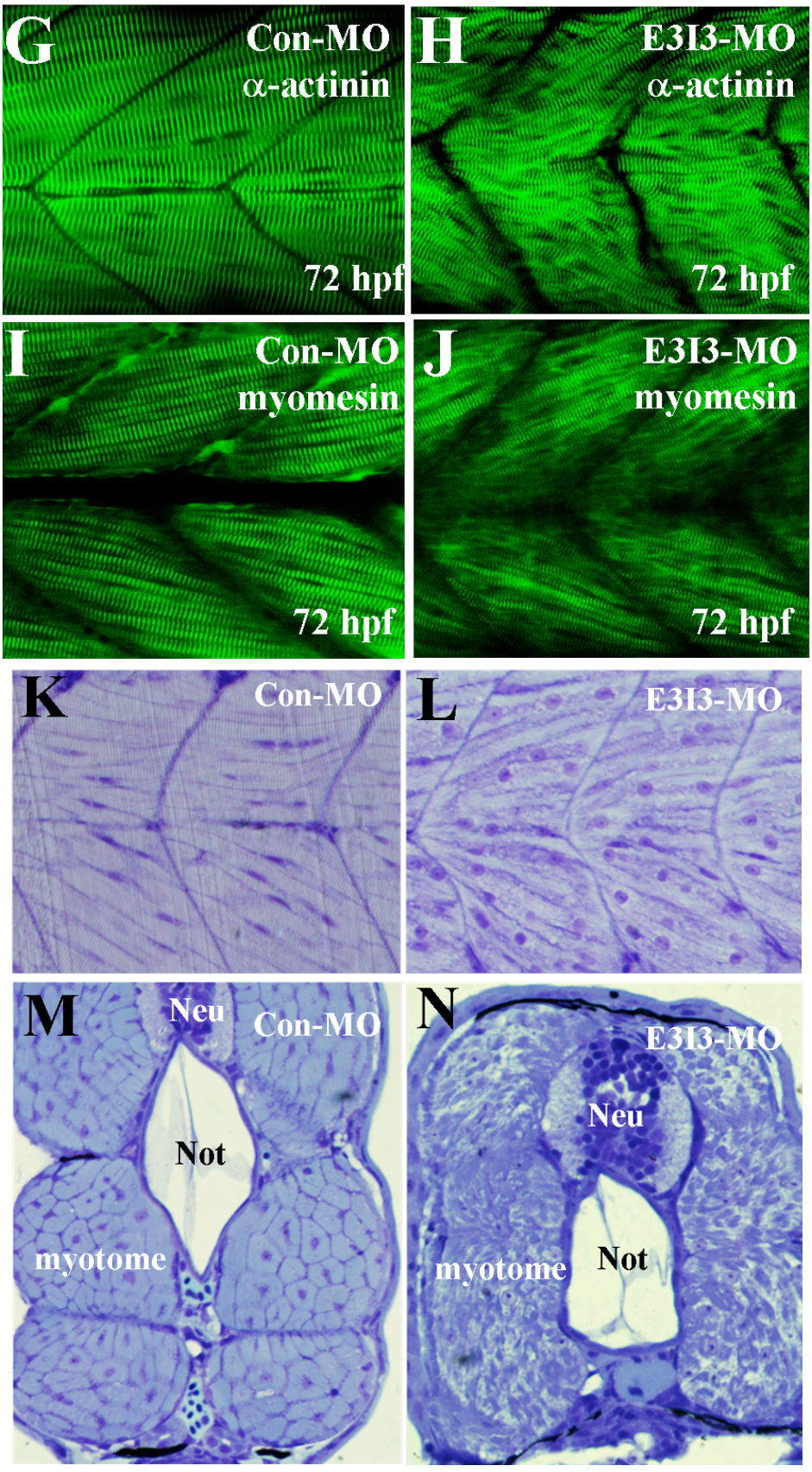

G
Con-MO
α-actinin
72 hpf
H
E3I3-MO
α-actinin
72 hpf
I
Con-MO
myomesin
72 hpf
J
E3I3-MO
myomesin
72 hpf
K
Con-MO
L
E3I3-MO
M
Neu
Con-MO
Not
myotome
N
E3I3-MO
Neu
myotome
Not

Figure 22. Knockdown of sknac expression resulted in defective thick- and thin-filament assembly in fast muscles of zebrafish embryos.

A, B) Anti-MHC (F310) antibody staining (lateral view) shows thick filaments in fast muscle fibers of control (A) or E3I3-MO-injected embryos (B) at 72 hpf. Scale bar=20 μm. C, D) Anti-α-actin antibody staining shows disorganized thin filaments in E3I3-MO-injected embryos (D) compared with control (C) at 72 hpf. E, F) Phalloidin-FITC staining shows disorganized thin filaments in E3I3-MO-injected embryos (H) compared with control (E) at 72 hpf. G, H) Anti-α-actinin antibody staining shows sarcomeric localization of actinin on Z-lines of fast muscle fibers in control-MO-injected (E) or E3I3-MO-injected embryos (F) at 72 hpt. I, J) Anti-myomesin antibody (mMaC Myomesin B4) staining shows M-line structure in control-MO-injected (G) or E3I3-MO-injected embryos (H) at 72 hpf. K–N) Toluidine blue staining on longitudinal (K, L) and cross (M, N) plastic sections of control-MO-injected (K, M) or E3IE-MO-injected embryos (L, N) at 72 hpf. Structures of neurotube (Neu), notochord (Not), and myotome are indicated.

7) Naca and skNAC shows different subcellular localization in muscle cells

To better understand skNAC function in myofibrillogenesis, we analyzed the subcellular localization of skNAC and Naca during muscle cell differentiation in zebrafish embryos. DNA constructs expressing a N-terminal myc-tagged Naca or skNAC were constructed after a muscle-specific *smyd1* promoter (Du et al 2006). The expression constructs were injected into fertilized zebrafish eggs. A typical mosaic, but muscle-specific, pattern of expression was clearly detected in muscle fibers of the injected embryos by anti-myc antibody staining (Figure 23 A-F). Interestingly, Naca and skNAC showed different subcellular localization in myoblasts and myotubes of zebrafish embryos. Naca was found in the nucleus (Figure 23 B, C) whereas skNAC was localized in the cytosol (Figure 23 D-F). Moreover, Naca exhibited a nuclear to cytoplasmic translocation during myotube differentiation into myofibers (Figure 23 A). Myoblasts and newly formed myotubes in the posterior somites contained mostly the nuclear Naca (Figure 23 B, C). In contrast, myofibers in the anterior somites contained mostly cytosolic Naca (Figure 23 A). The nuclear to cytoplasmic translocation was, however, not seen in skNAC expressing muscle cells (Figure 23 D-F). To rule out the possible interference of the N-terminal myc-tag on skNAC subcellular localization, another DNA construct expressing a C-terminal myc-tagged skNAC was injected into zebrafish embryos. A similar cytoplasmic localization was found in the skNAC expressing myoblasts and myotubes (Figure 23 G-I). The cytoplasmic localization of skNAC was further confirmed in C2C12 myoblast cells expressing a myc-tagged skNAC in cell culture (Figure 23 J-L). Together, these data

indicate that skNAc and Naca are localized differently in muscle cells and thus may have different functions in muscle development.

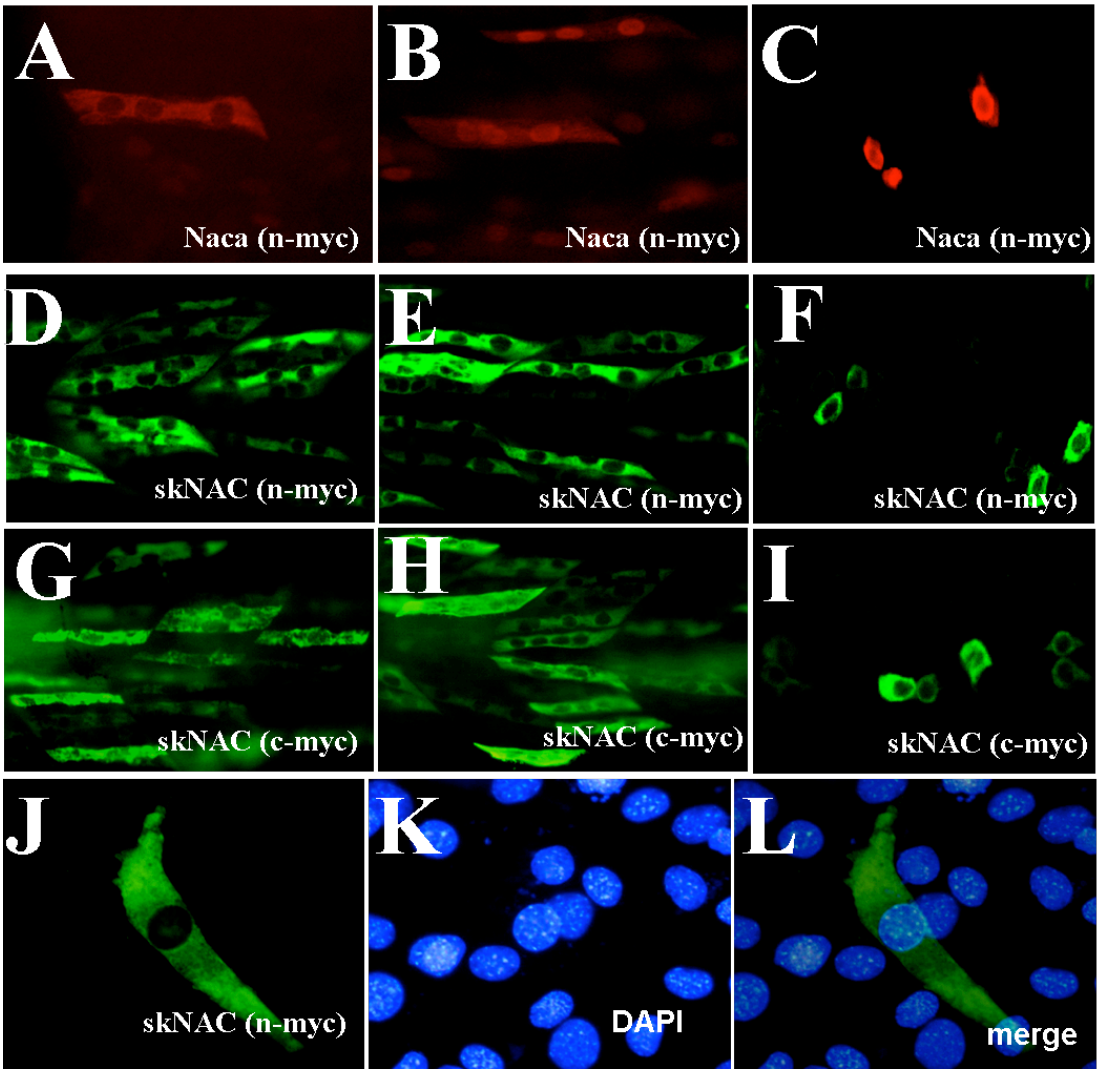


Figure 23. Subcellular localization of Naca and skNAC in myoblasts and myotubes of zebrafish embryos.

DNA constructs expressing a myc-tagged Naca or skNAC were injected into fertilized zebrafish embryos at 1–2 cell stages. Expression and subcellular localization of Naca or skNAC were determined by anti-myc antibody staining in zebrafish embryo at 18 and 24 hpf. A–C) Anti-myc antibody staining (TRITC

conjugated) showing nuclear (B, C) to cytoplasmic (A) translocation of Naca during muscle cell differentiation; myotubes in anterior (A) or posterior (B) myotome of a zebrafish embryo at 24 hpf; myoblast cells in posterior myotomes of 18-hpf embryos (C). D–F) Anti-myc antibody staining (FTIC conjugated) showing primary cytoplasmic localization of N-terminal tagged skNAC in myoblasts and myotubes; myotubes in anterior (D) or posterior (E) myotome of a zebrafish embryo at 24 hpf; myoblast cells in posterior myotome of 18-hpf embryos (F). G–I). Anti-myc antibody staining (FTIC conjugated) showing primary cytoplasmic localization of C-terminal-tagged skNAC in myoblasts and myotubes. Myotubes in anterior (G) or posterior (H) myotome of a zebrafish embryo at 24 hpf; myoblast cells in posterior myotome of 18-hpf embryos (I). J–L) Anti-myc (J) and DAPI (K) staining shows cytosolic localization of skNAC in C2C12 cells expressing a N-terminal myc-tagged skNAC; merged image also shown (L).

8) Overexpression of Naca or skNAC does not affect thick filament assembly

Blocking the splicing of *sknac* will result in production of *naca*. Previous studies have indicated that overexpression of Naca and skNAC altered myoblast differentiation *in vitro* (Yotov & St-Arnaud 1996). Interestingly, the effects appeared to be different for Naca and skNAC. Overexpression of Naca in C2C12 cells inhibited their differentiation and cell fusion. In contrast, overexpression of skNAC induced myoblast fusion to form gigantic, myosin heavy chain-positive, multinucleated myosacs (Yotov & St-Arnaud 1996).

To determine whether ectopic expression of Naca or skNAC affect myofibrillogenesis, zebrafish embryos injected with Naca or skNAC expression construct were analyzed by double staining. The double staining was performed with anti-myc antibody (9E10) and anti-myosin antibody (F59) antibodies. Organized thick filament assembly was clearly observed in myofibers with extopic expression of Naca or skNAC (Figure 24). These results indicate that overexpression of Naca or skNAC did not affect the myofibril assembly in zebrafish embryos, suggesting the muscle defect in skNAC knockdown embryos is not likely caused by overexpression of Naca.

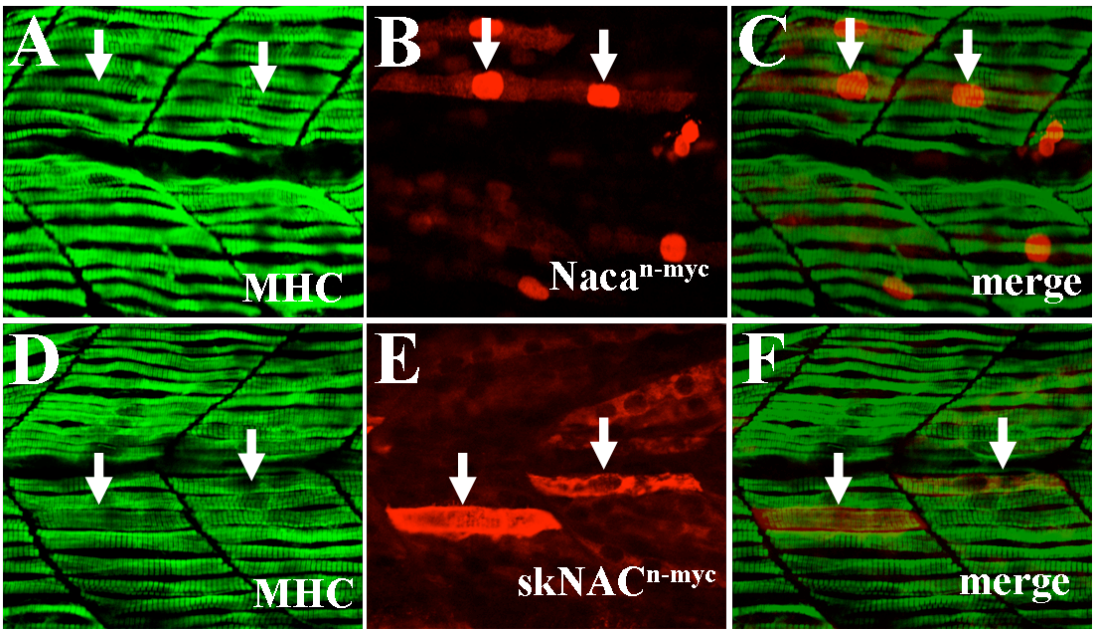


Figure 24. Overexpression of Naca or skNAC does not affect myofibril assembly in zebrafish embryo muscle cells.

A–C) Double staining with anti-MHC (A) and anti-myc (B) antibodies shows that expression of the naca transgene did not affect the thick-filament organization; merged image also shown (C). Arrows indicate myofibers with ectopic Naca expression. D–F) Double staining with anti-MHC (D) and anti-myc (E) antibodies shows that overexpression of skNAC did not affect the thick-filament organization; merged image also shown (F). Arrows indicate myofibers with ectopic skNAC expression.

9) Knockdown of sknac expression results in low levels of MHC protein accumulation in zebrafish embryos

Coordinated expression of myosin and other myofibrillar proteins is critical for myofibril assembly during myofiber maturation. To test whether myosin expression was altered in *sknac* knockdown embryos, we analyzed myosin expression at the RNA and protein levels. Western blotting analysis demonstrated that the MHC protein levels were significantly reduced in *sknac* knockdown embryos (Figure 25 A).

To test whether this was affected at the transcriptional or post-transcriptional level, we analyzed *myosin* gene expression whole mount in situ hybridization. The results showed that knockdown of *sknac* expression did not alter MHC gene expression at the RNA level (Figure 25 B-I). This was further confirmed by real time PCR (Table 3). Together, these data indicate that skNAC is required for translation and/or stabilization of myosin proteins. Knockdown of skNAC results in low levels of myosin expression, leading to defective thick and thin filament assembly.

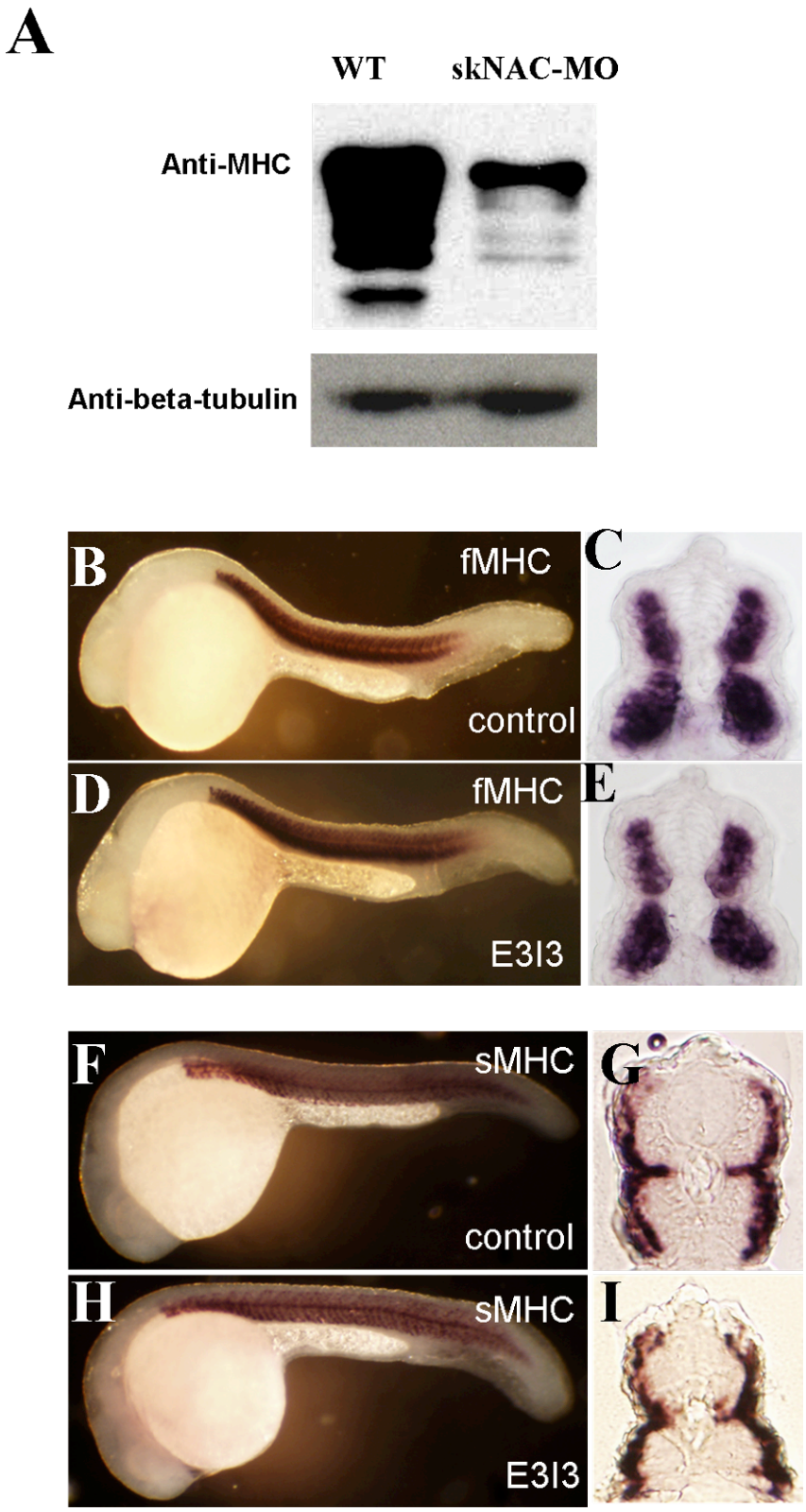

A
WT
skNAC-MO
Anti-MHC
Anti-beta-tubulin
B
fMHC
control
C
D
fMHC
E3I3
E
F
sMHC
control
G
H
sMHC
E3I3
I

Figure 25. Knockdown of sknac results in low levels of MHC protein accumulation.

A) Western blot analysis shows low levels of MHC protein accumulation in sknac-knockdown embryos at 24 hpf. β-Tubulin was used as the loading control. B–E) Whole-mount in situ analysis shows similar levels of MHC mRNA expression in fast muscle (fMHC) of control (B, C) or E3I3-MO-injected embryos (D, E) at 24 hpf. F–I) Whole-mount in situ analysis shows similar levels of MHC mRNA expression in slow muscle MHC (sMHC) of control (F, G) or E3I3-MO-injected embryos (H, I) at 24 hpf.

Table 3. Analysis of gene expression in skNAC knockdown and wild type embryos by qPCR.

	sMHC (Ct)	sMHC (Ct)	fMHC (Ct)	fMHC (Ct)	Ef-1a (Ct)	Ef-1a (Ct)
WT (cDNA) 1:10	23.28	23.31	25.81	26.56	15.11	15.10
skNAC E3I3-MO (cDNA) 1:10	23.94	23.38	26.88	26.16	14.92	14.88
WT (cDNA) 1:100	27.09	26.92	30.20	30.31	17.68	17.72
skNAC E3I3-MO (cDNA) 1:100	27.38	27.29	31.44	31.25	17.34	17.37
WT RT-cDNA	Undet	Undet	36.65	37.60	36.34	Undet
skNAC E3I3-MO RT-cDNA	Undet	Undet	37.64	37.33	Undet	Undet
Water Con	Undet	Undet	37.48	Undet	Undet	Undet

5. DISCUSSION

In this study, we have isolated and characterized *sknac*, the muscle-specific isoform of *naca*, from zebrafish and demonstrated that skNAC plays an important role in myofibrillogenesis. Knockdown of *sknac* expression resulted in defective myofibers with completely disorganized thick and thin filaments. The *sknac* knockdown embryos showed low levels of myosin protein accumulation. To our

knowledge, this is the first demonstration of skNAC function in muscle cell differentiation *in vivo*.

1) Characterization of sknac orthologue in zebrafish

Sequence analysis revealed that the zebrafish *naca* gene encodes two alternatively spliced mRNA transcripts, *naca* and *sknac*. Naca is highly conserved throughout evolution, with only 7 amino acid differences between zebrafish and mouse Naca. The expression of *naca* transcripts has been detected in species ranging from yeast to human. The production of *sknac* by alternative splicing, however, appears to be specific in vertebrates because no *sknac* transcripts have been reported in invertebrates. Gene Tool analyses failed to identify a large exon 3 in *naca* genes in *C. elegans* and *Drosophila*. Our identification of *sknac* from zebrafish thus represents the first *sknac* identified in lower vertebrates.

2) The size of sknac exon-3 evolves during evolution

Sequence comparison revealed that zebrafish *sknac* is significantly shorter than that found in mouse and human. The difference is due entirely to the size of exon 3. *sknacs* from mammals contain a large exon 3 (approximately 6 kb). In contrast, *sknac* from zebrafish contains an exon 3 of only 3 kb.

To better understand the size variation of exon 3 during evolution, we examined the sequences of several vertebrate organisms through a genome blast and analyses of the exon 3 sequence. An interesting observation was noted. Although the sequence and location of all 8 exons included in *naca* cDNAs are highly conserved

during evolution, the size and sequence of the *sknac* specific exon 3 changed dramatically during evolution. In lower vertebrates, including fugu, medaka and zebrafish, the size of *sknac* specific exon 3 is less than 3 kb, while the size increased significantly to 6 kb in mammals. The mechanism underlying this increase is unknown. Analysis by Gene Tool discovered that there are two *Alu* repeats in intron 2 and intron 5 in the human *naca* gene. In addition, one B1 repetitive sequence and two mouse B2 repetitive sequences were identified in intron 2 and intron 5 of the mouse *naca* genome. The mouse B1 and B2 repetitive sequences are short interspersed elements (SINEs) which are equivalent to primate *Alu* elements (Maraia, 1991). It has been shown that retrotransposition of *Alu* elements can insert novel sequences within or near genes, leading to altered transcriptional expression (Britten 1996, Norris et al 1995, Vansant & Reynolds 1995). Whether these repetitive sequences are involved in the expansion of the exon 3 sequence in mammalian *sknacs* is still unknown.

3) The skNAC-specific exon 3 encodes a proline rich sequence

The protein sequence encoded by the *sknac*-specific exon 3 is characterized by the rich contents of proline residues. Over 19% of the amino acid residues are prolines in the zebrafish *sknac* exon 3 coding region, and over 23% are proline residues encoded by human *sknac* exon 3. The position of proline residues are highly conserved between mouse and human exon 3 coding sequences. The biological significance of the proline rich region in skNAC is unknown. Interestingly, a proline rich sequence has been identified in the EH domain of EH-myomesin, an alternatively spliced isoform of myomesin that is specifically expressed in embryonic heart and

slow muscles (Schoenauer et al 2005). Like the skNAC-specific exon 3, the sequence of the EH domain is rather heterogeneous in sequence between different species. The EH domain has no defined secondary structure and is present in a mostly as non-folded state at physiological condition. This is very similar to the proline rich PEVK domain found in titin that has been suggested to function as an entropic spring (Schoenauer et al 2005). It is not clear whether the skNAC specific sequence is also present in a random coil conformation and involved in increasing the structural flexibility of skNAC protein.

It should be noted that the proline-rich protein domains have also been implicated in binding to the SH3 domain involved in many protein-protein interactions (Hiroaki et al 2001, Lim & Richards 1994, Yu et al 1994). Interestingly, the tail regions of myosins I, IV, VII, XII and XV contain a putative SH3 domain, and have been shown to interact with the C-terminal proline-rich region in Myosin VII (Wang et al 2007). Whether skNAC interacts with myosin proteins through the proline rich region remains to be determined.

4) skNAC functions in myofibrillogenesis

In this study, we demonstrated that skNAC has a specific function in muscle development that could not be replaced by *naca* transcript. This is consistent with the muscle-specific expression of *sknac* in muscle cells of zebrafish and mouse embryos (Sims et al 2002). It appears that skNAC plays an important role in organized assembly of thick and thin filaments. Knockdown of skNAC expression disrupted the organization of thick and thin filaments, but had little effect on the organization of Z-

disc and M-bands. This result is consistent with previous studies showing that there is a basic stabilizing structure within the sarcomere, consisting of α-actinin at the Z-disc, titin and an integrating molecule at the M-bands (Handel et al 1991, Komiyama et al 1993, Wang et al 1988). Together, these studies indicate that the organization of Z-disc and M-band may occur independently of thick and thin filaments.

The molecular mechanism by which skNAC controls myofibril organization is not clear at present. It has been demonstrated that Naca functions as a protein chaperone involved in folding of newly synthesized protein emerging from the ribosome (Wiedmann et al 1994). Given that skNAC is an alternatively spliced isoform of Naca and skNAC shares the same protein sequence as Naca in the N-terminal and C-terminal regions, skNAC may play a role in folding and assembly of newly synthesized proteins in muscles. Consistent with this hypothesis, we showed that knockdown of skNAC resulted in significant reduction of myosin protein levels in zebrafish embryos. Considering the high demand for protein synthesis in muscle cells, muscle cells may have a tissue-specific chaperone system to facilitate the folding and assembly of high levels of myofibrillar proteins during myofibrillogensis. Recent studies demonstrate that the heat shock chaperone system is very active in muscle cells and required for myofibrillogenesis in skeletal muscles (Du et al 2008, Hawkins et al 2008).

CHAPTER 5: GENERAL DISCUSSION

In this chapter, the major findings of this thesis will be summarized and the overall conclusions will be discussed. In addition, our understanding of the Smyd1b working mechanism will be addressed based on our current research .The future direction of Smyd1b mechanistic studies will also be discussed.

1. The function of smyd1b, sknac, hsp90α1 in myofibrillogenesis

Our study had shown that *smyd1b*, *sknac* and *hsp90α1* play vital roles in sarcomere assembly. Knockdown of any one of them by morpholino antisense oligos causes a similar, but not identical, phenotype. Table 4 summarizes the morphological phenotype and the sarcomere structure in *smyd1b*, *sknac* and *hsp90α1* knockdown embryos.

Table 4: Skeletal muscle sarcomere assembly

Morpholino	Phenotype	Sarcomeric protein assembly				Myosin expression (By WB)
		Thick filament (Myosin)	Thin filament (Actin)	M-line (Myomesin)	Z-line (Actinin)	
sknac-MO	No moving No heartbeating	Affected	Affected	Not affected	Not affected	Reduced
smyd1b-MO	No moving No heartbeating	Affected	Affected	Affected	Affected	Reduced
hsp90α1-MO	No moving Nomal heartbeating	Affected	Affected	Affected	Affected	Reduced

1) sknac and myofibrillogenesis

From Table 4 we can see that *sknac* is required for both thin and thick filament assembly, but not important for the Z-line and M-line assembly. It has been widely accepted that sarcomeric proteins are expressed and assembled in a defined sequence during muscle development. The first protein appearing on the sarcomere is desmin, an intermediate filament protein. Desmin is subsequently followed by titin and the I-Z-I complexes (Ehler et al 1999, Furst et al 1989). The organization of titin precedes the accumulation of actin and thick filaments (Handel et al 1991, Komiyama et al 1993, Wang et al 1988). This suggests that there is a basic stabilizing structure of the sarcomere composed of α-actinin at the Z-disk, titin, and an integrating molecule at the M-line (Ehler et al 1999). Our studies provide new evidence that the M-line can be assembled independently of the thick filament.

This is the first time that *sknac* has been cloned in zebrafish and the *in vivo* function has been identified. Little is known about the *sknac* working mechanism during sarcomere assembly. From our study in zebrafish, we hypothesized that *sknac* may work as a chaperone for either myosin or actin. This is based on the following facts:

1) *Naca* functions as a protein chaperone involved in folding of newly synthesized protein emerging from the ribosome (Wiedmann et al 1994). *sknac* includes all the sequence that *Naca* has, indicating they may share similar character;

2) skNAC has a large exon encoding proline-rich sequence. A Proline-rich domain is known as a protein-protein interacting domain of the SH3 domain and the

tail regions of myosins I, IV, VII, XII, and XV has been shown to contain a putative SH3 domain (Hiroaki et al 2001, Lim & Richards 1994, Wang et al 2007);

3) skNAC can only be detected in the cytoplasm under normal circumstances, which suggests a vital role in the cytoplasm.

Molecular chaperones appear necessary for *de novo* folding and structural maintenance of myosin and actin. Expression of the myosin motor domain in bacteria results in the misfolding of myosin (McNally et al 1988). In vertebrate systems, the chaperonin containing TCP-1 (CCT), as well as molecular chaperones Hsp90 and Hsc70, are necessary but not sufficient in the folding of striated muscle myosin (Srikakulam & Winkelmann 1999, Srikakulam & Winkelmann 2004). Recent studies from our lab and others demonstrated the important function of Hsp90α1 and Unc45 working as myosin chaperones in sarcomere assembly (Barral et al 2002, Du et al 2008, Landsverk et al 2007, Liu et al 2008, Wohlgemuth et al 2007). The correct folding and assembly of actin also requires chaperones. These include αB-Crystallin, a Small Heat Shock Protein has been shown to modulate actin filament dynamics *in vivo* (Singh et al 2007). Two more molecular chaperones, GimC (also known as Prefoldin) and TRiC (TCP-1 Ring Complex, also known as CCT), are also known to play a synergistic role in the process of actin folding (Kim et al 2008). Interestingly, TRiC has also been proposed to play an active role in the folding pathway of skeletal muscle myosin (Srikakulam & Winkelmann 1999). skNAC may also be one of the chaperones for myosin or actin or both to help their folding and assembly.

2) hsp90α1 and myofibrillogenesis

From Table 4, we can clearly see that *hsp90α1* is required for the assembly of actin and myosin, as well as the Z-line and M-line in the skeletal muscle. Anti-MHC and anti-myomesin immunoactivity is reduced dramatically in *hsp90α1* knockdown embryos, while anti-actin and anti-α-actinin immunoactivity is comparable with the control. This suggests a vital role of *hsp90α1* in the folding of both myosin and myomesin in the skeletal muscle.

Heat-shock proteins are molecular chaperones of a large number of proteins. Hsp90 alone may work as a chaperone and interact with about 400 proteins (Zhao et al 2005). We can imagine that myosin may not be the only target of Hsp90α1 in skeletal muscle; myomesin may also represent another target. In our study we showed a significant influence of *hsp90α1* on the M-line protein myomesin. To the best of our knowledge, this is the first time that the M-line protein myomesin has been reported to be affected by *hsp90α1*. This may provide us new insights of the working mechanism of *hsp90α1* during sarcomere assembly.

Although *in situ* hybridization showed clear expression of *hsp90α1* in the cardiac muscles, knockdown of *hsp90α1* didn't affect the function of the heart (Du et al 2008). In zebrafish genome, there are two highly related *hsp90α* genes, *hsp90α1* and *hsp90α2*. Sequence alignment revealed that *hsp90α2* shares more similarity with human and mouse Hsp90α and is likely the ortholog of Hsp90α in zebrafish (Du et al 2008). Hsp90α2 also shows a strong expression in cardiac and skeletal muscles. In addition, it can also be detected in the head and eye regions (Du et al 2008). It is

possible that Hsp90α2 has a redundant function in the heart to compensate for the loss of Hsp90α1 function.

3) smyd1b and myofibrillogenesis

Our study showed that Smyd1b_tv1 is localized on the M-line of the sarcomere. The M-line is the cytoskeletal structure that cross-links the myosin and titin filaments in the middle of the sarcomere. It is known to stabilize the thick filament lattice in the sarcomere during the contraction cycle (Agarkova & Perriard 2005). Although the structure of the M-line is quite complex, it is surprising that only a small number of M-line constituents have been identified to date (Clark et al 2002). Apart from the myosin tails and the C-termini of titin, there are only three closely related structural proteins detected at the M-line, myomesin, myomesin 3 and M-protein (Eppenberger et al 1981, Grove et al 1984, Labeit & Kolmerer 1995, Schoenauer et al 2008). Little has been known relating to the function of these M-line proteins and how the M-line contributes to the sarcomere assembly. Our study showed that knockdown of *smyd1b* disrupts the assembly of the Z-line, the myosin thick filament and the M-line. This is consistent with previous reports demostrating that M-line deletion of titin affected the maturation of the Z-line, myosin and the M-line (Musa et al 2006, Weinert et al 2006). The study of Smyd1b_tv1 will provide us some new insights on how the M-line is involved in the process of sarcomere assembly.

The localization study also shows that Smyd1b_tv1 has potential phosphorylation site which causes the different sarcomere localization between

Smyd1ba and Smyd1b_tv2. This suggests the post-translational modification of Smyd1b_tv1 may regulate its activity.

In addition to the skeletal muscle defect, our study also showed a dramatic effect of *smyd1b* knockdown on the cardiac muscle organization. This is the first time a clear function of *smyd1b* has been identified during cardiac muscle assembly. A *Smyd1b* knockout study in mouse has shown that *Smyd1b* is required for the expression of *hand2* in the precardiac. Additionally, *Smyd1b* is necessary for maturation of cardiomyocytes and morphogenesis of the right ventricle (Gottlieb et al 2002). Our study indicates that knockdown of smyd1b does not affect heart tube formation and looping. When compared with a previous study in the embryonic zebrafish indicaging that hand2 is important for ventricle formation (Yelon et al., 2002), we argue that hand2 may not be a target of smyd1b. This is consistent with our microarray data, showing that *hand2* expression was not affected in *smyd1b* knockdown embryos.

2. Smyd1b and skNAC

Yeast two hybrid analyses revealed interaction between Smyd1b and skNAC. This result was further confirmed by co-immunoprecipitation assays (Sims et al 2002). Mutagenesis analyses identified that this interaction requires the MYND domain of Smyd1b and the PXLXP motif within skNAC (Sims et al 2002).

Our study showed similar defects in thick and thin filaments in *smyd1b* and *sknac* knockdown embryos. The morphants are immobile and lack heartbeat prior to deatharound 5 dpf. Immunostaining using different markers demonstrated the similar

but not identical effect on sarcomere assembly between *smyd1b* and *sknac*. *smyd1b* can affect the assembly of the thick filament, thin filament, M-line, and Z-line. Knockdown of *sknac* only affects the thick and thin filaments. Both the interaction between Smyd1b and skNAC and the similar functions between them suggest that they may work together to control the assembly of thick and thin filaments. Moreover, Smyd1b may have its additional roles in the assembly of other sarcomeric structures that does not requires skNAC

Recent study revealed that skNAC can be methylated by Smyd1b and the direct interaction is necessary for the methylation to occur (Li Zhu's dissertation, 2006). This study also showed that both of the two splicing isoforms---Smyd1b_tv1 and Smyd1b_tv2--- can methylate skNAC *in vitro*, while only Smyd1b_tv2 can methylate skNAC *in vivo* (Li Zhu's dissertation, 2006). This is consistent with our study, showing that *sknac* and *smyd1b_tv2* exhibit the same temporal expression pattern during zebrafish muscle development, whereas *smyd1b_tv1* is expressed 5 hrs earlier than *sknac* and *smyd1b_tv2*.

3. Smyd1b interacts with myosin chaperones Hsp90α1, Unc45b

I demonstrated in these studies that knockdown of *smyd1b* resulted in a phenotype similar to that of *hsp90α1* knockdown during sarcomere assembly of skeletal muscles. Biochemical studies by Co-IP showed that Smyd1b_tv1 interacts with Hsp90α1, whereas Smyd1b_tv2 cannot. In general, Hsp90 binding proteins belong to two different protein families. One protein family members are the Hsp90 substrates, which are also known as client proteins. The other protein family members

are co-chaperones, which help Hsp90's action on substrates or mediate Hsp90-substrate recognition (Sbroggio et al 2008). Myosin is a substrate for Hsp90α1, so it belongs to the first group. Unc45b and other Hsp90 interacting protein such as melusin, a muscle-specific stress response protein, belong to the second group (Sbroggio et al 2008). We ruled out the possibility that Hsp90α1 is a chaperone for Smyd1b_tv1. It remains to be determined if Smyd1b_tv1 may work as a co-chaperone of Hsp90a1.

It should be noted that Hsp90 protein could be modified by methylation (Cimmino et al 2008). Interestingly, Hsp90α1 expression overlaps with Smyd1b_tv1 during muscle development (Sass et al 1996, Tan et al 2006). It remains to be determined whether Hsp90α1 may represent a methylation target of Smyd1b_tv1.

Heat shock proteins are well known as stress responsers whose expression can be enhanced by multiple stress stimuli, including heat shock and cold shock (Gething & Sambrook 1992). Our study showed that knockdown of *smyd1b* resulted in upregualtion of Hsp90a1 expression. However, unlike *hsp90α1*, *smyd1b* was not upregulated in either heat shocked or cold shocked embryos. Moreover, knockdown of *hsp90a1* expression did not result in increased *smyd1* expression. Together, these studies indicate that Smyd1b may not be involved in a general stress response as Hsp90α1 does.

Unc45b is a myosin co-chaperone specifically expressed in skeletal and cardiac muscles (Wohlgemuth et al 2007). Knockdown of *unc45b* in zebrafish results in paralysis and ventricular dysfunction, which is also very similar to the phenotype from *smyd1b* knockdown (Wohlgemuth et al 2007). Immunostaining indicates that in

addition to myosin defects, other sarcomeric structures such as thin filaments, the Z-line and M-line are also disrupted in *unc45b* knockdown embryos, a phenotype very similar to that of *smyd1b* or *hsp90α1* knockdown (Bernick and Du, unpublished data).

We demonstrated in this study that Smyd1b_tv1 could also interacts with Unc45b, suggesting that they may work together to control myobril assembly. It has been well documented that Unc45b and Hsp90α1 work together to control myosin folding and assembly. (Barral et al 2002, Liu et al 2008). Because Smyd1b_tv1 can bind to both one of them, it suggests that Smyd1b_tv1 may form complex with the myosin chaperone machinery to help myosin folding and assembly.

In addition to myosin thick filaments, our studies indicate that Smyd1b_tv1/Hsp90α1/unc45b complex may play a direct role in folding and assembly of other sarcomeric proteins. One particular candidate is the M-line protein myomesin. We showed that knockdown of *smyd1b*, *hsp90α1* or *unc45b* compleletely abolished the expression of myomesin. This is unlikely due to an indirect effect from disruption of myosin thick filaments because knockdown of myosin directly had little effect on myomesin expression and M-line organization (Codina and Du, unpublished).

The identification of Smyd1b_tv1 interacting protein Hsp90α1 and Unc45b, together with the functional study of Hsp90α1, links Smyd1b_tv1 with the sarcomeric protein chaperone machinery, which will lead us to a new direction to detect how Smyd1b_tv1 to fulfill its function. Hsp90α1 and Unc45 have been very well studied for their functions during myosin assembly, which will help to study the relationship between Smyd1b_tv1 and myosin, vice versa; the study of Hsp90α1 and Unc45's

function on other contractile components will also benefit from the mechanistic study of Smyd1b_tv1.

4. The proposed model of smyd1b working mechanism

In this study, we have demonstrated several important roles of *smyd1b* and its partners during skeletal and cardiac muscle development. Smyd1b belongs to a subfamily which contains a SET and a MYND domain. Members of this protein family are conserved from yeast to vertebrates. The human and mouse genomes each contain five annotated Smyd proteins (Thompson & Travers 2008). The SET domain has been identified as a histone methyltransferase domain in Smyd1, Smyd2 and Smyd3 (Abu-Farha et al 2008, Hamamoto et al 2004, Tan et al 2006).

More recently, nonhistone proteins have been found to be methylated by members of the Smyd family. Smyd2 has been shown to methylate p53 K370 to repress its activity both *in vitro* and *in vivo* (Huang et al 2006). In addition, recent study reported that VEGFR1 is a novel nonhistone target of Smyd3 histone methyltransferase(Kunizaki et al 2007). Smyd2 and Smyd3 have both nuclear and cytoplasm localization, which makes it possible for them to work as a histone methyltransferase to control gene expression in the nucleus and work as nonhistone mehtyltransferase to modify other proteins in the cytoplasm. In our study, we also showed the nuclear and cytoplasm localization of Smyd1b. This suggests Smyd1b may fulfill its function *in vivo* in the same way as Smyd2 and Smyd3.

It has been reported that cytoskeleton associated proteins often display dynamic distributions in cells and can participate in signal transduction cascades to

control gene expression (Clark et al 2002). In skeletal and cardiac muscles, several sarcomeric proteins have been shown to shuttle between the nucleus and the cytoplasm to fulfill their functions. They could associate with the sarcomere to maintain organization. They may also participate in the response of the muscle cells to the structural and functional changes of the contractile machinery. One example is the M-line protein myomesin-1, which was long thought as a cytoskeletal protein in the cytoplasm. Myomesin-1 has been found in the nucleus and may control gene expression (Price & Gomer 1993, Reddy et al 2008, Vinkemeier et al 1993). Another example includes the transcription factor NFATc, whose nuclear translocation is activity dependent in mature fibers and also is developmentally regulated (Liu et al 2001). NF-AT3 can be tethered to the Z-line and dephosphorylated by calcineurin. This activates NF-AT3 and allows it to enter the nucleus (Frey et al 2000, Olson et al 2000). Two titin-interacting proteins, the muscle-specific calpain p94 and MURF-1, the RNA-binding protein raver1, and the actin binding protein myopodin also participate in linking nuclear and sarcomeric functions and their dynamic localization coincident with different stages of muscle differentiation (Centner et al 2001, Huttelmaier et al 2001, McElhinny et al 2002, Sorimachi et al 1995, Weins et al 2001). These cases illustrate several potential mechanisms by which communication between the sarcomeres and the nucleus may be mediated by sarcomeric proteins. The sarcomeric proteins which also have roles inside the nucleus could make it possible for the muscle cells to have the ability to respond to mechnical stress and other physiological signals. This kind of communication may be critical for the

maintenance of the muscle cell homeostasis as well as muscle plasticity (Clark et al 2002).

Smyd1b may represent another example of this kind of protein which can transfer stress or other kind of signals from the contractile machinery to the nucleus. Smyd1b thus may play an important signaling role between the nucleus and the contractile components to maintain cytoarchitectural integrity of muscle cells. Based on all our studies as well as previous studies, we propose the following model of how Smyd1 may fulfill its biological function *in vivo*.

We propose that Smyd1b may have roles both inside the nucleus and in the cytoplasm. Smyd1b may work as a histone methyltransferase to control gene expression inside the nucleus during muscle cell differentiation. In addition, Smyd1b in the cytoplasm may modify proteins by methylation, regulating the activity of the target proteins. It has been demonstrated that several sarcomeric proteins, such as myosin and actin, are modified by methylation (Iwabata et al 2005, Tong & Elzinga 1983). Post-translational modification by methylation may be important for protein folding, assembly and stability.

We further hypothesize that Smyd1b_tv1 may share the same targets as Hsp90α1 and Unc45b because of their close association. Hsp90α1 may help the folding and assembly of these target proteins that are modified by Smyd1b_tv1. Moreover, we can not rule out the possibility that Hsp90α1 may be a target of Smyd1b_tv1. Methylation of Hsp90a1 by Smyd1b_tv1 may regulate its chaperone activity.

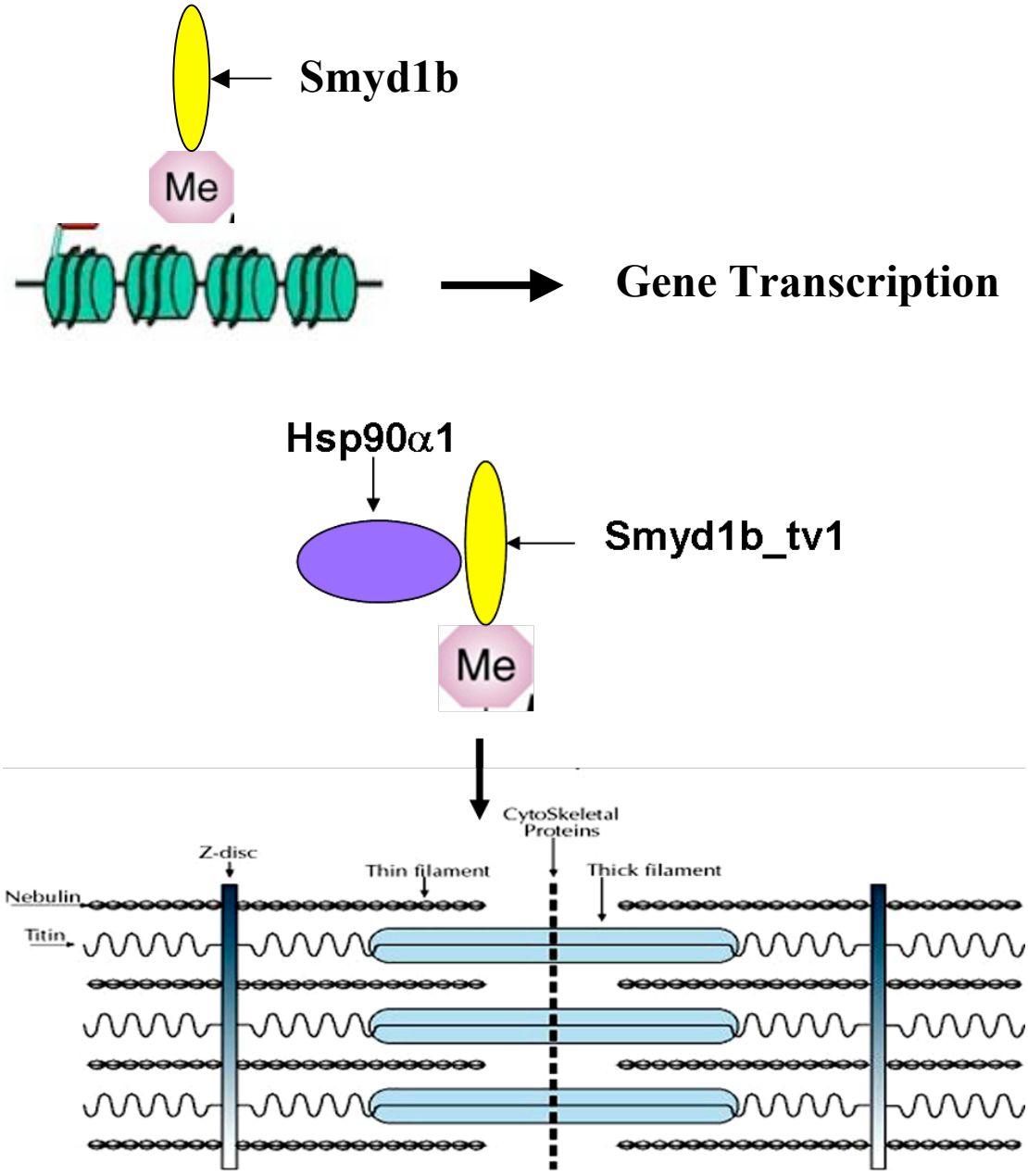


Figure 26. Final model of Smyd1b working mechanism.

Smyd1b may work as a histone methyltransferase to control gene transcription inside nucleus during early muscle differentiation or under stress at adult stage.

Within the cytoplasm, Smyd1b may work as methyltransferase for sarcomeric proteins or Hsp90a1. Studies have shown that skNAC is a target for Smyd1b_tv2. The interaction between Smyd1b_tv1 and Hsp90α1 may indicate Smyd1b_tv1 can

methylate Hsp90α1 or methylate other contractile components which need the help of Hsp90α1 for folding and assembly.

5. Future directions

As for future directions, it is important to further dissect the mechanistic action of Smyd1b and its partners. Epigenetic studies via CHIP (chromatin immunoprecipitation) would be used to identify the gene targets of *smyd1b*, and to determine whether altering the expression of these target genes would affect myofibril assembly. Equally important, proteomic analyses via Co-immunoprecipitation and western blot by anti-methyllysine antibody would be carried out to identify the protein targets of Smyd1b that are methylated by post-translational modification. Potential candidates include myosin, myomesin, and Hsp90a1. Together, these studies will further advance our mechanistic understanding of *smyd1b* function in myofibrillogenesis. Moreover, results from these studies will establish the basis for investigating the potential involvement of Smyd1b and its partners in cardiac and skeletal muscle diseases in human.

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