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Understanding Cardiac Sarcomere Assembly With Zebrafish Genetics

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Abstract

Mutations in sarcomere genes have been found in many inheritable human diseases, including hypertrophic cardiomyopathy. Elucidating the molecular mechanisms of sarcomere assembly shall facilitate understanding of the pathogenesis of sarcomere-based cardiac disease. Recently, biochemical and genomic studies have identified many new genes encoding proteins that localize to the sarcomere. However, their precise functions in sarcomere assembly and sarcomere-based cardiac disease are unknown. Here, we review zebrafish as an emerging vertebrate model for these studies. We summarize the techniques offered by this animal model to manipulate genes of interest, annotate gene expression, and describe the resulting phenotypes. We survey the sarcomere genes that have been investigated in zebrafish and discuss the potential of applying this in vivo model for larger-scale genetic studies.

Keywords

cardiomyopathy; genetics; myofibrillogenesis; sarcomere; zebrafish

Introduction to Sarcomere Assembly

The sarcomere is the basic contractile unit of myofibrils in striated muscles, including cardiac and skeletal muscles (Clark, McElhinny et al. 2002). When viewed with transmission electron microscopy (TEM), sarcomeres appear as semicrystalline structures, with substructures of 2 filamentary systems, ie, thick and thin filaments. The electron-dense A-bands are the myosin-based thick filaments, whereas the least electron-dense I-bands are the actin-based thin filaments. M-lines are located in the middle of A-bands and anchor the bipolar thick filaments. In the middle of the I-bands, Z-discs define the border of 2 neighboring sarcomere units, anchoring the end of the thin filaments and titin filaments, the latter being the third filament system in a sarcomere (Pyle and Solaro 2004; Frank, Kuhn et al. 2006). Titin is the largest known protein, spanning half of a sarcomere as a single molecule. The N-terminus of titin anchors in the Z-disc, and its C-terminus extends into the M-line (Tskhovrebova and Trinick 2003; LeWinter, Wu et al. 2007). The energy-consuming movement between the myosin-based thick filament and actin-based thin filament is the

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molecular basis for active contraction, whereas the Titin filament functions as a molecular spring and contributes to passive contraction.

Mutations in sarcomeric genes have been shown to cause many human cardiac diseases. For example, inheritable hypertrophic cardiomyopathy (HCM) has been found to be associated with more than 1,400 mutations in 11 or more genes encoding cardiac sarcomere proteins (Seidman and Seidman 2001; Seidman and Seidman 2011; Maron and Maron 2013). Thus, HCM has been described as a disease of the sarcomere. Disturbance of the sarcomeric structure, including myofibrillar disarray or Z-disc streaming, is a crucial pathologic step in the development of heart failure by various mechanisms (Knoll, Hoshijima et al. 2003). To understand the molecular mechanism of this step, fundamental knowledge of sarcomere assembly is necessary. The basic mechanisms obtained from studying de novo assembly, which can be done in experimentally accessible model systems, are expected to facilitate understanding of the pathogenesis of human diseases.

As a complicated multistep process, sarcomere assembly, also termed *myofibrillogenesis*, has fascinated scientists for more than 100 years. Recent extensive studies suggest several models of myofibrillogenesis (Sanger, Kang et al. 2005; Boateng and Goldspink 2008). One model emphasizes the existence of a stress fiber–like structure, composed of nonmuscle proteins in the cell periphery that functions as a reusable template for the recruitment of sarcomeric proteins to form myofibrils (Dlugosz, Antin et al. 1984). A second model hypothesizes that actin filaments attached to Z-bands (I-Z-I brushes) and A-bands form independently and are subsequently stitched together by Titin to form myofibrils (Schultheiss, Lin et al. 1990; Holtzer, Hijikata et al. 1997; Ojima, Lin et al. 1999). A third model emphasizes the formation of premyofibrils along the cell membrane; these have a short mini-sarcomere structure that includes Z-bodies, attached actin filaments, and miniature A-bands (Rhee, Sanger et al. 1994). Premyofibrils are composed of sarcomeric proteins, except for mini A-bands, which are composed of nonmuscle Myosin II filaments. The addition of titin and overlapping muscle myosin II filaments to Z-bodies transforms premyofibrils to nascent myofibrils (Rhee, Sanger et al. 1994; Dabiri, Turnacioglu et al. 1997; Du, Sanger et al. 2003; Sanger, Wang et al. 2009). The addition of Myosin-binding protein C and M-band proteins, the alignment of the thick filaments into A-bands, and the loss of nonmuscle Myosin II leads to the formation of mature myofibrils (Sanger, Chowrashi et al. 2002; Sanger and Sanger 2008; Sanger, Wang et al. 2010). These models describing the different aspects of sarcomere assembly are supported by varying levels of in vivo and in vitro evidence. The first and second models are based solely on fixed and antibody-stained muscle cells. The premyofibril model is based on immunofluorescence experiments and by the assembly of mature myofibrils, as revealed by sarcomeric protein fused to fluorescent protein in transfected living muscle cells (Dabiri, Turnacioglu et al. 1997; Sanger, Chowrashi et al. 2002; Stout, Wang et al. 2008; Liu, Chakkarapani et al. 2013).

In recent years, the study of myofibrillogenesis has attracted more attention through the identification of many new sarcomeric components and because previously known proteins were found to localize to the sarcomere (Sanger, Wang et al. 2009). The identification of these proteins suggests that the sarcomere not only provides a structural basis for contraction but also acts as a docking station for signaling molecules. The statement is exemplified by

recent research on the Z-disc substructure that consists of Titin, Actin, and α-Actinin (Pyle and Solaro 2004; Frank, Kuhn et al. 2006). In addition to Actin, Nebulin and Obscurin were later found to also bind to Titin (Kontrogianni-Konstantopoulos and Bloch 2005; Linke 2008). Many proteins were detected in the Z-disc that bind to α-Actinin, including FATZ/calsarcin/myozenin, Myotilin, Actinin-associated LIM Protein (ALP), ZASP/Cypher/Oracle, Muscle Lim protein (MLP), Nebulette/Lasp-2, Myospryn, Myomaxin, Myopalladin, Enigma homologue (ENH), CLP36/Elfin/CLIM1, and Zyxin (Durham, Brand et al. 2006; Frank, Kuhn et al. 2006; Huang, Brand et al. 2006; Linke 2008). These sarcomeric proteins typically interact with more than one partner to form an intricate protein network. Meanwhile, several Z-disc components bind to membrane proteins and link the Z-disc to membrane structures such as the transverse-tubule, costamere, and sarcoplasmic reticulum, whereas others bind to signaling molecules such as Calcineurin, Protein kinase C (PKC), Protein kinase D (PKD), and RhoA (Frank and Frey 2011; Knoll, Buyandelger et al. 2011; Bennett 2012). Transcription factors or cofactors such as Cardiac ankyrin repeat protein (CARP) and MLP are also found within the Z-disc and may translocate to the nuclei and regulate gene transcription (Pyle and Solaro 2004; Frank, Kuhn et al. 2006; Hoshijima 2006). In summary, the discovery of these new proteins indicates that the sarcomere assembly process is much more complicated than originally thought. To unify different models of sarcomere assembly and to decipher the relationship between sarcomere structural changes and pathogenesis in the heart, a systematic genetic study of the sarcomeric genes, especially in vivo, is needed.

Techniques to Study Sarcomere Assembly in Zebrafish

Early studies of myofibrillogenesis were conducted using an in vitro cell culture system. Despite the convenience of high-resolution imaging and easy molecular manipulation, significant limitations of this system have been recognized. For example, 3-dimensional analysis of cell-cell communication that is crucial for sarcomere assembly is difficult to be recapitulated faithfully. Instead of examining de novo sarcomere assembly, a process of reassembling sarcomere components has been studied (Gregorio and Antin 2000; Rudy, Yatskievych et al. 2001; Russell, Curtis et al. 2010). The mouse has been the major whole-animal model for genetic studies of sarcomere assembly because of the elegant physiology analysis tools available. However, its in utero development means that embryos are less accessible for phenotyping the sarcomere assembly processes. A functional circulation system is crucial for survival; therefore, secondary defects of a dysfunctional circulation system complicate the interpretation of genetic studies in mice. To address these shortcomings, other animal models have been explored for sarcomere assembly studies, including the chicken and quail ex vivo culture systems (Rudy, Yatskievych et al. 2001; Du, Sanger et al. 2003; Du, Sanger et al. 2008). However, despite the gain of embryonic accessibility, the absence of genetic tools in these avian models prevents systematic genetic studies of sarcomere assembly.

In the past 2 decades, the zebrafish (*Danio rerio*) has become a popular model organism for studying embryogenesis (Driever, Stemple et al. 1994; Ingham 1997; Grunwald and Eisen 2002), including cardiac development (Staudt and Stainier 2012; Tu and Chi 2012) and myofibrillogenesis in skeletal muscle (Sanger, Wang et al. 2009; Raeker, Shavit et al. 2014).

The advance of novel gene manipulation techniques and the experimentally accessible embryos of zebrafish offer several advantages for studying sarcomere assembly. First, zebrafish embryos are transparent and develop ex utero. Myofibrillogenesis occurs in a single layer of cardiomyocytes, which is ideal for live observation, immunostaining, and fluorescent-tagged imaging (Schoenebeck and Yelon 2007). Second, a collection of genetic tools has been developed in zebrafish to conduct either gain-of-function or loss-of-function studies. Third, zebrafish mutant embryo can survive for several days without a functional circulation system. Because of their small size, oxygen diffuses into their bodies in quantities sufficient to support early development (Burggren and Pinder 1991). This unique feature is beneficial for studying sarcomeres because disruption of sarcomere assembly typically results in severe defects in the cardiac system. Below, we summarize major experimental techniques offered by zebrafish to study sarcomere assembly.

Techniques Used to Generate Loss-Of-Function Mutants

Mutagenesis Screening—The completion of 2 large-scale mutagenesis screens in 1996 was a milestone needed to establish zebrafish as a new vertebrate model (Haffter, Granato et al. 1996; Stainier, Fouquet et al. 1996). Using N-ethyl-N-nitrosourea (ENU) as a chemical mutagen, approximately 695 stable mutants were identified, of which 58 had cardiac phenotypes (Chen, Haffter et al. 1996; Stainier, Fouquet et al. 1996). Positional cloning identified causative genes for *pickwick/pik* and *silent heart/sil* as *titin* and *cardiac troponin T*, respectively (Sehnert, Huq et al. 2002; Xu, Meiler et al. 2002), and this opened the door for genetic studies of cardiac sarcomere assembly using the zebrafish model.

In addition to chemical mutagens, virus-based vectors have been explored as a method of conducting insertional mutagenesis screens. Because of the exogenous DNA of viral vectors, the cloning of the insertion loci is efficiently conducted by using polymerase chain reaction (PCR)–based techniques. Indeed, among 525 mutants with recessive embryonic lethal mutations identified from a large-scale virus-based screen, the majority have been cloned (Amsterdam, Burgess et al. 1999; Amsterdam, Nissen et al. 2004).

The other insertional mutagen that has been successfully developed is based on transposon vectors (Kawakami, Takeda et al. 2004). The higher cargo capacity of transposon-based vectors allows transgene of multiple DNA fragments, including promoters, splicing donors, and genes encoding fluorescent proteins. The pGBT-RP2.1 (RP2) vector is highly effective, with a typical knock-down efficiency of more than 97% (Clark, Balciunas et al. 2011; Ding, Liu et al. 2013). More than 350 mutant fish lines have been generated and cloned (Clark, Argue et al. 2012). In addition to RP2, other transposon-based systems have been reported, including the Flip-trap (Trinh le, Hochgreb et al. 2011) and FlEx-based conditional gene-trap mutagenesis systems (Ni, Lu et al. 2012), which generate conditionally mutated alleles that allow temporal- and spatial-specific manipulation of the gene. Most of these mutants and those generated via ENU and the virus are deposited in the Zebrafish International Resource Center (http://zebrafish.org/zirc/home/guide.php) or distributed via their own web-based platform (www.zFishbook.org; http://kawakami.lab.nig.ac.jp/ztrap/; http://www.fliptrap.org/static/index_new.html) (Varshney, Huang et al. 2013; Varshney, Lu et al. 2013) to enable sharing of these genetic resources with the research community.

Morpholino Technology—Morpholinos are modified antisense oligonucleotides that effectively knock down targeted gene expression by either blocking translation or splicing pre-mRNA. Zebrafish embryos develop ex utero from a single fertilized cell; therefore, morpholinos can be injected at the single-cell stage, which allows effective distribution to every cell to achieve whole-body knock down (Nasevicius and Ekker 2000). Morpholino technology has been widely used in loss-of-function studies, including many sarcomeric genes. However, we note that morpholinos are a transient gene knock-down technology. The dilution of morpholinos after multiple rounds of cell division causes them to lose their knock-down capacity after approximately 5 days postfertilization (dpf). Similar to other antisense technologies, the injection of morpholinos, especially at higher doses, leads to nonspecific adverse effects (eg, activation of p53 and induction of apoptosis) that complicate interpretation of the phenotype. Therefore, the specificity of morpholinos needs to be carefully determined, usually by injecting a second morpholino to confirm the phenotype, by conducting rescue experiments with co-injection of mRNA, or by partially eliminating secondary defects with co-injection of a morpholino against p53 (Robu, Larson et al. 2007).

TILLING Technology—Targeting induced local lesions in genomes (TILLING) technology enables the identification of stable gene-specific point mutants in zebrafish using high-throughput screening (Wienholds, van Eeden et al. 2003; Moens, Donn et al. 2008; Kettleborough, Bruijn et al. 2011). Building on its success in zebrafish screening, ENU mutagenesis can be used to generate a randomly mutagenized zebrafish library that will be sufficient to cover most genes in the genome. Gene-specific primer pairs are used to screen mutations in the targeted gene via PCR using DNA templates generated from either sperm or fin. This technology has been successfully implemented in large research centers, including the Sanger Institute in the UK and the Fred Hutchinson Cancer Research Center. More than 11,000 mutants covering 45% of the genome have been generated and are available to the research community (Kettleborough, Busch-Nentwich et al. 2013).

TALENs and CRISP-Cas9 Technology—The lack of genetic tools to generate targeted gene knock-outs in a typical laboratory had been a bottleneck in the zebrafish model for many years. The recent development of zinc-finger nucleases (ZFN) (Meng, Noyes et al. 2008), transcription activator–like effectors nucleases (TALENs), and clustered, regularly interspaced, short palindromic repeats (CRISPR)–associated (Cas) systems effectively addressed this challenge. These 3 systems all contain endonucleases that incorporate a sequence-specific DNA-binding domain to recognize and cleave a precise genomic locus. To generate mutations in a targeted gene using TALENs technology, 2 TALEN constructs that recognize sequences surrounding the targeted locus are generated and co-injected, allowing the FokI nuclease from the 2 constructs to form an activated dimer and induce DNA double-strand breaks in the targeted locus (Christian, Cermak et al. 2010; Campbell, Hartjes et al. 2013). In most cases, the double-strand breaks are repaired via the nonhomologous end-joining mechanism, an intrinsically error-prone process that often results in small insertions or deletions (or both) at the target site that disrupt gene function. The high efficiency of TALEN in producing loss-of-function mutants has been demonstrated in zebrafish (Huang, Xiao et al. 2011; Sander, Cade et al. 2011; Dahlem, Hoshijima et al. 2012). Moreover, double-strand breaks can also be repaired by a homologous

recombination–based mechanism. Using either short single-strand oligonucleotides or long double-strand DNA molecules as templates, sequence tags and gene replacements can be introduced into a specific genomic locus (Bedell, Wang et al. 2012; Wefers, Meyer et al. 2013). Further development of these genome-editing tools is being actively researched and will significantly affect zebrafish as a model for genetic studies of sarcomere assembly.

Techniques for Studying Gene Overexpression

Transient Injection—Similar to *Xenopus*, ectopic expression of a gene can be achieved by the injection of either DNA or mRNA into zebrafish embryos at the single-cell stage. These transient injection assays can be used to conduct rescue experiments, to prove the specificity of morpholinos, to reveal subcellular expression of the protein of interest (if tagged with a fluorescent protein), and to determine the consequences of overexpressing a mutated sarcomeric gene. DNA with a circular or linear format can be injected. Cardiac-specific expression can be achieved by cloning a cardiac-specific promoter before the gene of interest. Several heart-specific promoters have been identified, including a promoter from *cmlc2* that drives cardiomyocyte-specific expression (Huang, Tu et al. 2003), a promoter from *titin* that drives both cardiomyocyte and somite expression (Yang and Xu 2012), and a promoter from *vmhc* or *amhc* that drives chamber-specific expression (Zhang and Xu 2009; Zhang, Han et al. 2013). Among these promoters, the *titin* promoter drives earliest-onset gene expression in cardiomyocytes, which is important for studying earlier stages of sarcomere assembly. Of note, DNA plasmid injections typically lead to mosaic expression. This unique feature can be leveraged to conduct rescue experiments because the surrounding cells without ectopic gene expression serve as ideal controls (Huang, Zhang et al. 2009). In contrast to DNA injection, mRNA injection leads to ubiquitous expression in the whole zebrafish embryo.

Transgenic Technology—Transgenic technology has been widely used in mice for functional studies of sarcomeric genes, especially genes with disease-causing mutations (Robbins 2000). In zebrafish, the transgenic rate was significantly improved after the introduction of transposon-based vectors such as *Sleeping Beauty* (*SB*) (Davidson, Gratsch et al. 2009), *Tc3* (Raz, van Luenen et al. 1998), and *Tol-2* (Kawakami, Takeda et al. 2004). When zebrafish embryos are co-injected with a Tol2-based vector and transposase-encoding mRNA, more than 50% of the F0 offspring are founders, making zebrafish possibly the easiest vertebrate model for generating a stable transgenic line.

To facilitate the cloning process, a recombination-based cloning system such as the Tol2Kit can be modified (Kwan, Fujimoto et al. 2007). Various promoters can be cloned into the 5′ entry vector, and fragments encoding various fluorescent proteins or other sequence tags can be cloned into a 3′ entry vector. After the gene of interest is cloned into the middle entry vector, 4-fragment recombination can be achieved by incubating a 5′-entry clone, a middle clone, a 3′-entry clone, and a Tol2-containing destination vector. A single cloning step with a recombinase can produce the final construct that is ready for injection. This versatile, recombination-based system can be used to swap different promoters and different tags to study sarcomeric genes of interest.

Techniques for Annotating Gene and Protein Expression

Define the mRNA Expression Pattern via In Situ Hybridization—Whole-mount in situ technology can be used to reveal the tissue-specific expression pattern of a gene of interest and to show the onset of gene transcription during cardiogenesis. Although the former is crucial to determine homologs or isoforms that function in the heart vs somites, the latter aids interpretation when different sarcomeric proteins appear in cardiomyocytes and participate in the sarcomere assembly process (Thisse and Thisse 2008). Because of their high fecundity and ex utero development, zebrafish embryos are readily available in large quantities for whole-mount in situ experiments.

Define Protein Expression via Immunostaining—Immunostaining is one of the most important experimental tools available to assess the sarcomere assembly process. Whole-mount embryos can be stained to label the heart, but poor penetration of antibodies prevents generation of high-quality images. To address this technical challenge, we developed a protocol in which the heart is dissected from the body and then stained on the surface of a microscope slide (Yang and Xu 2012). The improvement of antibody penetration allowed us to define different stages of sarcomere assembly in a developing zebrafish heart (Huang, Zhang et al. 2009).

Define Protein Subcellular Expression via Fluorescence-Tagged Imaging Technology—As detailed later in this article, a panel of antibodies against mammalian sarcomeric proteins has been confirmed to work in zebrafish. However, antibodies for many other sarcomere proteins are not yet available in zebrafish. An alternative approach is to generate fluorescent protein–tagged fusion proteins that reflect the assembly behavior of the endogenous proteins. This approach has been shown to work effectively in cell culture systems (Dabiri, Turnacioglu et al. 1997). In zebrafish, green fluorescent protein (GFP)–tagged Actn2 or Actn3 have been reported to faithfully show Z-disc localization of actinin proteins (Lin, Swinburne et al. 2012; Yang and Xu 2012). Moreover, a *Tg(cmlc2:Cypher-EGFP)* transgenic line was generated to assess the Z-disc assembly of Cypher.

Techniques for Annotating Mutant Phenotypes

Annotation of Sarcomere Assembly Defects via TEM and Immunostaining—TEM was the first technique used extensively to assess sarcomeric phenotypes in a zebrafish heart (Wanga, Eckberg et al. 2001; Sehnert, Huq et al. 2002; Xu, Meiler et al. 2002). We recommend treating hearts with relaxation buffer to ensure that the samples are imaged at the same contraction stage. Compared with TEM, immunostaining has been less frequently used in the phenotypic analysis of sarcomeric mutants, partially because of the penetration problems. In fact, immunostaining using antibodies against different sarcomeric substructures can yield more information on sarcomeric defects compared with TEM alone (Huang, Zhang et al. 2009; Yang and Xu 2012).

Annotation of Cellular Changes via Immunostaining and Transgenic Fish Lines—To access the morphometric changes of cardiomyocytes during cardiogenesis, the cell border of individual cardiomyocytes can be revealed by immunostaining using β-catenin or Zn5 antibodies. Transgenic fish lines with membrane-tagged fluorescent proteins have

also been used, including *Tg(titin:mGFP)* and *Tg(myl7:mkate-caax)* with membrane-tagged GFP to define cell membranes (Lin, Swinburne et al. 2012; Yang and Xu 2012). The nuclei of cardiomyocytes are revealed either by immunostaining using Mef2 antibody or transgenic fish lines using nuclei-tagged fluorescent protein, including *Tg(cmlc2:nDsRed)* (Rottbauer, Saurin et al. 2002). The proliferation of cardiomyocytes can be measured by immunostaining using antibodies against proliferating cell nuclear antigen (PCNA) or Phosphorylation of histone H3 (pH3) or transgenic fish lines such as *Tg(ccnb1:EGFP)* (Kassen, Thummel et al. 2008). Apoptosis can be assessed by either TUNEL staining or transgenic fish lines (UAS-secA5-YFP) (van Ham, Mapes et al. 2010).

Annotation of Cardiac Functions—As an in vivo vertebrate animal model, zebrafish has a major advantage because of its ability to quantify cardiac function changes that accompany the sarcomeric defects. Because of the transparency of fish embryos, the heartbeat of a living animal can be documented directly under a dissecting microscope. Cardiac function indices, including heart rate, ejection fraction, and myocardial wall velocity, can be calculated by analyzing video clips (Denvir, Tucker et al. 2008; Hoage, Ding et al. 2012).

Summary

Zebrafish are a mature vertebrate model with a collection of convenient genetic tools that enable the study of sarcomere genes via loss-of-function and gain-of-function manipulations. Mutants for most genes are already available for experimental investigation. The resultant phenotypes at the organ and cellular levels can be uncovered by using this whole-animal model for genetic studies of sarcomere assembly.

Zebrafish With Sarcomere Gene Mutations

The improved antibody-staining technique enables identification of the 6 stages of sarcomere assembly in a zebrafish heart (Figure) (Huang, Zhang et al. 2009; Yang and Xu 2012; Yang and Xu 2012). Of note, the timeline reflects the process in cardiac muscle, not skeletal muscle. During the first stage at approximately 10–12 somites (S), thin filaments appear as a perimembrane network, while thick filaments assemble independently into rodlets of various lengths. At the second stage (18 S), these thin and thick filaments assemble to form a rudimentary sarcomere structure, in which α-actinin integrates into Z-bodies, appearing as irregular spaced dots. The sarcomere at this stage resembles premyofibrils, as previously described (Dabiri, Turnacioglu et al. 1997). At the third stage (24 S), Z bodies form regular spaced dots, with shorter distance between neighboring dots (1.2 μm). The fourth stage corresponds to the initiation of heart contraction at 26 S. The distance between Z-bodies increases (1.9 μm) and becomes consistent, resembling nascent myofibrils. In this fourth stage, the Z-bodies fuse laterally to form longer Z-bands (1 to 2 dpf) at the fifth stage and then align with each other to form well-registered sarcomeres at the sixth stage at approximately 2 dpf. Genetic studies of *actn2* suggest the fifth stage as an intermediate step because depletion of this major Z-disc protein is required for lateral alignment but not lateral fusion of the Z-discs (Yang and Xu 2012). This 6-stage sarcomere assembly process provides the structural basis for the initiation of peristaltic contraction at

26 S and the switch to a much stronger and more coordinated contraction at 2 dpf. Accompanied with the sarcomere assembly process, the zebrafish heart transforms from a tube-like structure at 26 S into a 2-chambered heart with a ventricle and atrium. This baseline characterization of sarcomere assembly sets the stage for the interpretation of phenotypes in sarcomere mutants. Below, we review known sarcomeric genes with cardiac expression that have been studied in zebrafish.

Thin Filament

The thin filaments consist of polarized Actin polymers, Tropomyosin, and Troponin. The assembly process of thin filaments in a zebrafish heart is revealed by either immunostaining with an antibody against Tropomyosin (CH1) or by phalloidin staining of actin filaments (Huang, Zhang et al. 2009). The mutants and morphants described below have been generated and studied.

Actin—Zebrafish have at least 4 sarcomeric *actin* genes: *acta1a*, *acta1b*, *acta2,* and *actc1a*. Two *actin* mutants have been identified, one is the *cardiofunk* (*cfk*$^{-/-}$) mutant affecting *acta1b,* generated via ENU mutagenesis screening (Bartman, Walsh et al. 2004); the other is *s434*, which affects *actc1a* (Glenn, McKane et al. 2012). The R177H mutation in *cfk*$^{-/-}$ and the Y169S mutation in *s434* both affect polymerization of actin (Wen and Rubenstein 2003; Bartman, Walsh et al. 2004), but the sarcomere structure is differentially affected. The *cfk* mutant has no visible sarcomere defects, whereas the *s434* mutant has severe sarcomere defects, including loss and shortening of myofibrils. A further experiment indicated that *acta1b* and *actc1a* have partially redundant functions in the heart (Glenn, McKane et al. 2012). Both mutants show cardiac dilation, blood regurgitation, and cardiac cushion defects.

Troponin T—Calcium wave regulation of heart contractions is conferred by the troponin complex that consists of Troponin C, Troponin I, and Troponin T. Of the 3 *troponin T* genes in zebrafish (*tnnt1, tnnt2,* and *tnnt3*), *tnnt2a* is most likely the only homolog that is expressed in the heart. The mutant alleles of *silent heart* (*sih)* were identified from an ENU mutagenesis screen; these alleles affect either a splicing acceptor in intron 2 or the translation start site of the *tnnt2a* gene (Sehnert, Huq et al. 2002). In addition, 2 morpholinos against the splicing donor of either exon 12 (Huang, Zhang et al. 2009) or exon 13 (Becker, Deo et al. 2011) were reported to mimic the C-terminus truncation mutation in humans with cardiomyopathy.

In contrast to the well-aligned and laterally fused thin and thick filaments in wild-type fish, only a few scattered myofibrils were noted in *sih* fish by ultrastructural analysis (Sehnert, Huq et al. 2002). More information regarding the sarcomere assembly defects has been revealed by immunostaining studies. Without Troponin T, both Tropomyosin and Troponin I are detached from the thin filaments. The striation of thin filaments, thick filaments, and Z-discs are all absent (Huang, Zhang et al. 2009). These data revealed an essential function of Troponin T in actin filament assembly in the first stage of sarcomere assembly, and the loss of Troponin T prevented the sarcomere assembly from progressing to the second stage, ie, the formation of premyofibrils. The C-terminus of Troponin T is indispensable for this function because both thin and thick filaments appear striated in embryos injected with the

morpholino against the exon 12 splice donor (*e12* morphants). The regular spaced periodic Z bodies can be detected, suggesting that the N-terminus of Troponin T is capable of driving sarcomere assembly to form premyofibril structures (Huang, Zhang et al. 2009).

The distinct sarcomere defects in *sih* and *e12* morphants explain the different phenotypes in cardiac function and morphologic changes. Although heart beating and blood circulation were not noted in *sih* embryos, weak contraction was still detectable in *e12* morphants. In contrast to normal ventricular chamber size in *sih* fish, *e12* morphant fish had significantly smaller ventricles. This suggests that Troponin T truncated at the C-terminus might exert dominant functions via activating unique signaling pathways in *e12* morphant fish (Huang, Zhang et al. 2009). Supporting this possibility, microarray studies of embryos injected with the morpholino against the exon 13 splice donor showed a phenotype that resembled HCM in human patients (Becker, Deo et al. 2011).

Troponin C—Among the 3 known *troponin C* homologs in zebrafish, only *troponin C1a* (*tnnc1a*) exhibits cardiac ventricle–specific expression; *tnnc1b* and *tnnc2* are expressed in skeletal muscles. Two mutations in the *tnnc1a* gene were reported in *silent partner* (*sil*) mutant fish that were identified from an ENU mutagenesis screen (Sogah, Serluca et al. 2010). The *sil* ^{m656} allele has a point mutation in the splicing donor in the second intron, and the *sil* ^{sk25} allele contains a deletion in this locus. Both mutations significantly attenuate *troponin C* expression and sequentially affect *ventricle myosin heavy chain (vmhc)* expression, resulting in consistent sarcomere defects. The sarcomere manifests as sparse and poorly organized structures under electron microscopy. Consistent with the ventricle-specific expression pattern of *tnnc1a*, depletion of Tnnc1a results in a silent ventricle but a beating atrium. Consequently, the ventricle becomes compacted and the atrium appears enlarged. Despite undetectable cardiac expression of *tnnc1b* in situ, co-injection of a *tnnc1b* morpholino and *tnnc1a* morpholino results in complete silence of both chambers, suggesting that *tnnc1b* has residual expression in the atrium and is responsible for sarcomere assembly in this chamber. Together, these genetic studies underscore a crucial function of *troponin C* in sarcomere assembly, presumably via supporting thin filament assembly. Of note, immunostaining studies that could reveal the precise function of Troponin C in sarcomere assembly have yet to be conducted.

Thick Filament

The assembly process of thick filaments in the zebrafish heart can be revealed by immunostaining using myosin-specific antibodies, including MF20 for the ventricle, S46 for the atrium, and F59 for both cardiac chambers (Berdougo, Coleman et al. 2003; Auman, Coleman et al. 2007; Huang, Zhang et al. 2009). The mutants and morphants described below have been generated and studied.

Myosin Heavy Chain—Several *myosin heavy-chain* homologs have been reported in the zebrafish genome Zv9. Mutants have been generated for *myh7* and *myh6*, also named *ventricle-specific myosin heavy chain* (*vmhc*) and *atrium-specific myosin heavy chain* (*amhc*), respectively. A nonsense mutation in *myh7* was found in the *half-hearted* (*haf*) mutant that results in truncated Vmhc (Auman, Coleman et al. 2007), and a nonsense

mutation in *myh6* was found in the w*eak atrium* (*wea*) mutant that results in the deletion of C-terminal amino acids of Amhc (Berdougo, Coleman et al. 2003). In both mutants, a significantly reduced myofibril number is noted, supporting the essential function of myosin in sarcomere assembly. Detailed studies of sarcomeric defects have not been conducted to date.

Consistent with sarcomeric defects in different chambers, contractility is completely lost in the ventricle of *haf* mutants and in the atrium of *wea* mutants. Interestingly, the cell size of ventricular cardiomyocytes is increased in *haf* mutants but reduced in *wea* mutants. Further studies have uncovered an important function of heart contraction in modulating heart morphogenesis (Auman, Coleman et al. 2007).

Essential Myosin Light Chain—By screening the expression of a panel of *essential myosin light chain* (ELC) homologs in zebrafish, *cardiac myosin light chain1* (*cmlc1*) was found to be specifically expressed in the heart. Genetic studies of *cmlc1* were conducted using morpholinos (Chen, Huang et al. 2008). The mutant *lazy susan* (*laz*), identified from an ENU mutagenesis screen, contains a nonsense mutation resulting in the truncation of 11 amino acids in the C terminus of Cmlc1 (Meder, Laufer et al. 2009).

In *cmlc1* morphants, myosin still assembles into rodlet structures, which integrate with thin filaments. Striated thin filaments and Z-discs can be observed by electron microscopy. However, the myofibrils appear narrow because lateral fusion does not occur. The length of individual sarcomeres becomes longer and irregular. Together, these observations suggest that ELC is not essential for the initial assembly of thick or thin filaments or for their initial interaction. Instead, ELC has an important role in the later lateral fusion step (stage 4). The 11 amino acids in the C-terminus are indispensable for this ELC function because the sarcomere structure appears normal in *laz* fish.

In both *laz* mutants and *cmlc1* morphants, cardiac contractility is severely reduced, suggesting an essential function of Cmlc1 and its C terminus in regulating heart beating. An enlarged ventricular chamber was noted in *cmlc1* morphants; however, a small and severely collapsed ventricular chamber was noted in *laz* mutant fish.

Regulatory Myosin Light Chain—Among several regulatory myosin light chain homologs, *cardiac myosin light chain 2* (*cmlc2*) is specifically expressed in the heart. In addition to a morpholino study, the function of *cmlc2* has also been studied in the *tell tale (tel)* mutant, which was identified in an ENU mutagenesis screen that affects a splice donor site and leads to early truncation of the Cmlc2 protein (Rottbauer, Wessels et al. 2006; Chen, Huang et al. 2008).

As revealed by TEM studies, only Z-bodies and immaturely assembled thin filaments are noted in *tel* mutants. In a cross-sectional view, the hexagonal lattice of thin and thick filaments is disrupted, and only scattered thin filaments are noted (Rottbauer, Wessels et al. 2006). An immunostaining study of both the *tel* mutant and *cmlc2* morphants showed that the early step of sarcomere assembly is not disturbed (Chen, Huang et al. 2008). Myosin forms irregular spaced dots or continuous lines along myofibril; however, Actin still forms

striated and periodic dots. Consequently, the sarcomere length in *cmlc2* morphants appears shorter, unlike the longer sarcomere in *cmlc1* morphants. Moreover, morpholino studies of *myosin light chain kinase* (*Cardiac-MLCK*) suggest that phosphorylation of *cmlc2* is crucial for its function in sarcomere assembly (Seguchi, Takashima et al. 2007).

Heart contraction is compromised in *cmlc2* morphants and *tel* mutants. In the *tel* mutant, cardiomyocytes in the ventricle and atrium are completely silent, and only the cardiomyocytes in the atrioventricular junction continue to contract rhythmically (Rottbauer, Wessels et al. 2006). Small ventricle chamber size was reported in *cmlc2* morphant fish, unlike the enlarged size in the *cmlc1* morphant (Chen, Huang et al. 2008).

Myosin-Binding Protein C3—Only one gene for *myosin-binding protein c3* (*mybpc3*) is present in zebrafish, and it is evolutionarily conserved with the human homolog. However, 3 different transcripts of *mybpc3* are dynamically expressed during embryogenesis, and all are expressed in the heart and slow muscle. Morpholinos have been used to knock down *mybpc3* expression (translational blockage). A specific cardiac phenotype was noted, but a detailed analysis of the sarcomere structure was not conducted. Both cardiac chambers, but especially the atrium, were enlarged. The ventricle wall thickness was reduced, and the ventricular diastolic relaxation velocity was reduced. The electrophysiologic phenotype in morphants, including ventricular action potential duration, resembles the phenotype in human patients with heart failure (Chen, Pai et al. 2013).

Titin Filament

In contrast to the single *titin* gene in mammals, the zebrafish genome contains 2 *titin* homologs that are localized in tandem on chromosome 9 (Seeley, Huang et al. 2007). Most of the functional domains are encoded by *ttna* and *ttnb*, except the Novex3 domain that is encoded only by *ttna*. Morpholinos against either *ttna* or *ttnb* cause specific embryonic lethal phenotypes, indicating that these genes are not functionally redundant (Seeley, Huang et al. 2007). The injection of a morpholino against the cardiac-specific N2B exon of *ttna* results in cardiac defects, whereas injection of a morpholino against the N2A exon of *ttna* results in somite phenotypes (Xu, Meiler et al. 2002). A group of mutants named *pickwick (pik)*, *herzschlag*, and *runzel* have been identified from ENU mutagenesis screens (Xu, Meiler et al. 2002; Steffen, Guyon et al. 2007; Myhre, Hills et al. 2013). A nonsense mutation was identified in the N2B domain of *ttna* from one of the *pik* alleles (Xu, Meiler et al. 2002). Due to the extremely large size of the *ttna* gene, mutations in *herzschlag*, *runzel* and other *pik* alleles have not been identified. From epitope mapping, the mutation in the *herzschlag* was suggested to be between exons 27 and 103, which presumably truncates the A-band rod domain of Ttna (Myhre, Hills et al. 2013).

Both *ttna* and *ttnb* are expressed in the heart; *ttna* is by far the earliest sarcomere gene that is transcribed in the heart, with a transcript detectable as early as the 5 S stage (using whole-mount in situ staining). This is earlier than the onset of the *vmhc* transcript at 10 S, the *cmlc1*/*cmlc2* transcripts at 15 S, and the *ttnb* transcript at 30 hpf (Seeley, Huang et al. 2007; Chen, Huang et al. 2008). Therefore, *ttna* likely has a more important role than *ttnb* in the heart. Indeed, significantly reduced myofibril number and disrupted sarcomere structure

were noted under TEM in *pikwick* mutants that affect *ttna*, but this was not observed in morphants affecting *ttnb*. Although Z-bodies and myosin filaments still form, they fail to laterally fuse and fail to form Z-discs and A-bands in *pikwick* fish (Seeley, Huang et al. 2007). These observations support the hypothesis that titin is required for sarcomere assembly, including the lateral fusion of Z-discs and A-bands. Of note, less severe sarcomeric phenotypes were noted in somites of the *herzschlag* mutant (Myhre, Hills et al. 2013). Stable null mutations are needed for both *ttna* and *ttnb* for more comprehensive genetic studies of *titin*.

As a consequence of the primary sarcomere defects, the cardiac contractility of *pickwick* fish is significantly reduced. Individual cardiomyocytes appear thin and stretched, resembling dilated cardiomyopathy (Xu, Meiler et al. 2002).

Z-Disc

The Z-disc defines the border of a sarcomere, where the thin filaments and Titin filaments are anchored. The Actinin antibody is widely used to mark the Z-disc, which conveniently defines different stages of sarcomere assembly in a zebrafish heart. The transformation of irregular spaced and shorter Actinin-immunopositive dots to periodically spaced dots at approximately 22 S reflects the advance of sarcomere assembly from premyofibrils (stage 2) to nascent myofibrils (stage 3) (Huang, Zhang et al. 2009). Later, the lateral expansion of Actinin-immunopositive Z-bodies to form longer Z-discs represents the advance of the sarcomere assembly from stage 3 to stage 5 (Figure). A large number of Z-disc genes have been studied genetically in zebrafish, as summarized below.

α-Actinin—As a major component of the Z-disc, α-Actinin binds a large number of proteins in the Z-disc, including Actin via its N-terminal binding domain and Titin via its central 4 spectrin repeats (Young, Ferguson et al. 1998; Clark, McElhinny et al. 2002). Together with its early appearance within the Z-disc, Actinin is postulated to function as a cross-linker, with an essential function in Z-disc assembly. Among the 4 α-*actinin* homologs in zebrafish (*actn1*, *actn2*, *actn3a*, and *actn3b*), only *actn2* is expressed in the heart (Holterhoff, Saunders et al. 2009; Gupta, Discenza et al. 2012; Yang and Xu 2012). Actn2 protein can be detected as irregular spaced dots at approximately 18 S in a developing zebrafish heart, before detection of 2 other Z-disc proteins, Tcap and Mlp (Zhang, Yang et al. 2009). Genetic studies of *actn2* have been conducted with several morpholinos (Holterhoff, Saunders et al. 2009; Yang and Xu 2012).

In *actn2* morphants, sarcomere assembly proceeds to stage 4. Well-defined thin filaments, thick filaments, and Z-discs can still be detected. However, Z-discs manifest as zigzagged lines or noncontinuous pieces. Therefore, genetic studies in zebrafish do not support the idea that Actn2 is required for Z-disc assembly. Rather than functioning in the early steps of sarcomere assembly, *actn2* appears to be specifically needed for the lateral alignment of Z discs.

Regarding cardiac function, Actn2 deficiency attenuates the shortening fraction without disturbing the heart rate. The ventricle chamber size is significantly reduced, but this

phenotype can be rescued by cessation of contraction, supporting the importance of mechanical force in regulating heart morphogenesis (Yang and Xu 2012).

Cypher—Cypher has been shown to form a protein complex with Actinin and Titin and to function as a linker-strut supporting the Z-disc structure (Sheikh, Bang et al. 2007). Genetic studies in the *cypher* knock-out mouse uncovered severe Z-disc defects, including a wide and zigzagged Z line, suggesting the function of *cypher* in Z-disc maintenance (Zhou, Chu et al. 2001). A recent study in a *Drosophila* mutant confirmed the important role of *cypher* in adult Z-disc stability and pupal myofibril assembly (Katzemich, Liao et al. 2013). A *cypher* ortholog was identified in zebrafish (van der Meer, Marques et al. 2006). In total, 13 isoforms from this gene locus have been detected as a consequence of alternative splicing, and isoforms 2α, 3α, 3β, and 3γ are expressed in the heart. Morpholino studies were designed to knock down these isoforms of *cypher*. Consistent with sarcomere defects in mouse *cypher* knock outs, deformed somites and pericardiac edema were found in *cypher* morphant fish (van der Meer, Marques et al. 2006). In addition to reduced cardiac contraction, a reduced ventricle chamber size with a thinner ventricular wall was noted.

Obscurin—The *obscurin* gene encodes a giant sarcomere protein that is detected in both Z-discs and M-lines. Based on its interaction with Titin in sarcomeres, as well as an interaction with Small Ankyrin-1 in sarcolemma, Obscurin is hypothesized to have a role supporting sarcomere structure and aligning sarcomeres with the sarcoplasmic reticulum (Kontrogianni-Konstantopoulos and Bloch 2005). There is a single *obscurin* ortholog in zebrafish, and 2 morpholinos have been designed to knock down zebrafish *obscurin*. An ATG morpholino blocks the translation of full-length *obscurin*, whereas the other splicing-donor morpholino specifically deletes the RhoGEF domain at the C-terminus (Raeker, Su et al. 2006; Raeker, Bieniek et al. 2010). Full-length Obscurin is required for lateral alignment between adjacent myofibrils but not for the early steps of sarcomere assembly, as indicated by impaired lateral alignment of the adjacent myofibrils and disorganized sarcoplasmic reticulum but properly striated thin filaments, thick filaments, and Z-discs. Moreover, a normal and well-aligned sarcomere structure was detected in embryos injected with the splicing morpholino, suggesting that the structural property but not the RhoGEF activity of Obscurin is important for sarcomere assembly. Detailed analysis of the splicing donor morphant suggests a function of the RhoGEF domain in the formation of the intercalated disc. Despite different sarcomere structural defects, both morphants have similar cardiac morphologic and functional defects, including cardiac edema, reduced heart rate, and reduced shortening fraction.

Nexilin—Nexilin is a novel Z-disc protein that was discovered and genetically characterized in zebrafish. The injection of a translation-blocking morpholino results in sarcomere defects (Hassel, Dahme et al. 2009). Both thin and thick filaments still form but have a disrupted structure that frequently detaches from irregular spaced and blurred Z discs. Ruptured sarcomeres were increasingly noted when the fish developed from 2 dpf to 3 dpf, suggesting an essential role of Nexilin in the maintenance (rather than in the initial assembly) of the sarcomere. Further studies revealed a severe sarcomere disruption in

stretched skeletal muscle, suggesting a function of *nexilin* in protecting the sarcomere from mechanical trauma.

As a consequence of the sarcomere defects, *nexilin* morphants have dilated atriums and ventricles, as well as reduced shortening fractions. These phenotypes prompted the search of mutations in *NEXILIN* from human patients lacking mutations in known cardiomyopathy-causing genes. Indeed, mutations in *NEXILIN* have been found in patients with dilated cardiomyopathy; as such, *NEXILIN* was then defined as a novel causative gene (Hassel, Dahme et al. 2009). Z-disc defects similar to those observed in *nexilin* morphant fish were also noted in these human patients. Notably, overexpression of zebrafish *nexilin* with a human mutation also leads to Z-disc damage and heart failure in zebrafish embryos.

Cytoskeletal Heart-Enriched Actin-Associated Protein—Cytoskeletal heart-enriched actin-associated protein (Chap) is a newly identified Z-disc protein that can translocate to the nucleus (Beqqali, Monshouwer-Kloots et al. 2010). Two *chap* orthologs, *chap1* and *chap2,* are found in zebrafish and expressed in the heart. The *chap1* transcript is expressed at an earlier stage and has a higher expression level than the *chap2* transcript. A translation-blocking morpholino against *chap1* leads to severely disrupted Z-discs, as revealed by electron microscopy. Heart looping consequently is impaired at 32 hpf, and both the atrium and ventricle broaden at 48 hpf.

Core-Binding Factor β—Z-disc localization of the *core-binding factor β* (Cbfβ) has been determined in both skeletal and cardiac myocytes. Depletion of full-length *cbfβ* in morphant fish causes thinner myofibrils, misaligned Z-discs, and misaligned intercalated discs, indicating an essential role of *cbfβ* in sarcomere lateral alignment and lateral fusion (Meder, Just et al. 2010).

M-Line

The M-line is located in the middle of a sarcomere and anchors the bipolar myosin bundles to form thick filaments. In zebrafish, immunostaining with a Myomesin-specific antibody shows that the M-line is assembled at approximately 18 to 22 S, when thick filaments start to interact and assemble with thin filaments (Huang, Zhang et al. 2009). The initial irregular spaced dots of Myomesin along the myofibril become periodic at 26 S, when the heart begins beating. Zebrafish have 4 *myomesin* genes: *myom1a*, myom*1b*, *myom2*, and *myom3*. Knocking down *myom3* in slow muscle has little effect on sarcomere assembly (Xu, Gao et al. 2012). The specific *myomesins* expressed in the heart and their function in sarcomere assembly during zebrafish heart development remain unclear.

Zebrafish have 2 *SET and MYND domain–containing protein 1* genes (*smyd1a* and smyd1b). The *smyd1b* gene encodes an M-line protein that is expressed in the heart and in skeletal muscle (Just, Meder et al. 2011). Smyd1a has similar function as Smyd1b in slow muscle but not in cardiomyocytes (Li, Zhong et al. 2013). In addition to interacting with myosin in the M-line, Smyd1b also translocates into the nucleus to exert its function as a transcriptional suppressor. A nonsense mutation in zebrafish *flat line* (*fla*) leads to a truncated and unstable Symd1b protein. Sarcomeric defects, including premature sarcomere

I-Z-I structure and loss of thick filaments, were noted in *fla* mutants by using electron microscopy for ultrastructural analysis (Just, Meder et al. 2011). This sarcomere defect was also confirmed in fish embryos injected with a *smyd1b* ATG morpholino (Li, Zhong et al. 2013). As a consequence of the sarcomere defects, the heart stops beating in *fla* mutant fish.

Conclusion

A panel of sarcomeric genes has been studied in zebrafish, generating a wealth of information on sarcomere assembly. Although studies of genes such as *cypher* supported conclusions reached in previous genetic studies of mouse and *Drosophila* models, studies of other genes such as *tnnt2*, *cmlc1*, *cmlc2*, and *actn2* were able to generate new knowledge on sarcomere assembly. The zebrafish is becoming an increasingly popular model to elucidate functions of novel sarcomere genes, as exemplified by studies of *nexilin, chap*, and *cbfβ*. Previously, TEM was the major and, in many cases, only technique to be used to assess sarcomeric defects, but immunostaining now is able to elicit greater detail regarding sarcomere-related phenotypes and will be an indispensable assay for future genetic studies. As an in vivo vertebrate model, the zebrafish offers a wealth of convenient genetic and phenotyping tools to elucidate gene function beyond sarcomere assembly, including cardiomyocyte morphology and cardiac function. Indeed, genetic studies of *cardiofunk, haf, wea*, and *actn2* mutants promote use of fish embryos as an in vivo model to interrogate the functional mechanism of mechanotransduction, ie, how contraction affects cardiomyocyte remodeling (Bartman, Walsh et al. 2004; Auman, Coleman et al. 2007; Yang and Xu 2012).

One challenge in generating null mutants in zebrafish for a particular sarcomere component is the coexistence of multiple homologs of several sarcomeric genes, including those encoding *actin*, *myosin*, and *titin*. To elucidate the functions of these sarcomeric components, compound mutants are needed, as was done for the 3 *tnnt2* homologs in zebrafish somites (Ferrante, Kiff et al. 2011). Of note, compound mutants that completely remove 1 of the 3 main filament systems (ie, thin, thick, and Titin filaments) are yet to be generated, but these will be pursued to further understand the de novo assembly of each filament and their interactions. The convenient morpholino technology will continue to provide additional insights into sarcomere assembly. However, because of its adverse effects at higher doses, the specificity of the null phenotypes of a morpholino is difficult to determine. Therefore, we anticipate that future genetic studies in zebrafish will be conducted using stable knock-out mutants.

In addition to analyzing the phenotypes of null mutations, zebrafish embryos can be a fruitful platform for investigating disease-causing mutations. As exemplified by genetic studies of *tnnt2, cmlc1, titin*, and *obscurin*, the functions of particular domains in a sarcomeric protein can be uncovered by comparing the phenotypes among mutants and morphants containing different truncation alleles (Xu, Meiler et al. 2002; Huang, Zhang et al. 2009; Meder, Laufer et al. 2009; Raeker, Bieniek et al. 2010). The feasibility of using fish embryos to study point mutations identified from humans has been proven (Hassel, Dahme et al. 2009; Sarparanta, Jonson et al. 2012). To study stable mutants, genetic tools such as TALENs and the Cas9 system are being developed and can be used to generate

knock-in mutants that mimic point mutations (Bedell, Wang et al. 2012; Campbell, Hartjes et al. 2013; Hruscha, Krawitz et al. 2013).

The completed zebrafish genome opens the door for a comprehensive analysis of genes involved in sarcomere assembly. Efforts are being coordinated to generate a near-complete collection of loss-of-function mutants for every zebrafish gene. Thousands of mutants have already been deposited in the Zebrafish International Resource Center, including mutants generated by ENU and virus-based mutagenesis screens and by transposon-based insertional screens. Moreover, mutants for approximately half the genome have been generated via TILLING technology at the Sanger Institute (Kettleborough, Busch-Nentwich et al. 2013). Mutants for most genes are expected to be available to the research community within a decade. Together with the maturing phenotyping tools, the zebrafish is emerging as a premier model for genetic studies of sarcomere assembly and will facilitate understanding of the relationship among gene mutations, sarcomere structural changes, and sarcomere-based cardiac disease.

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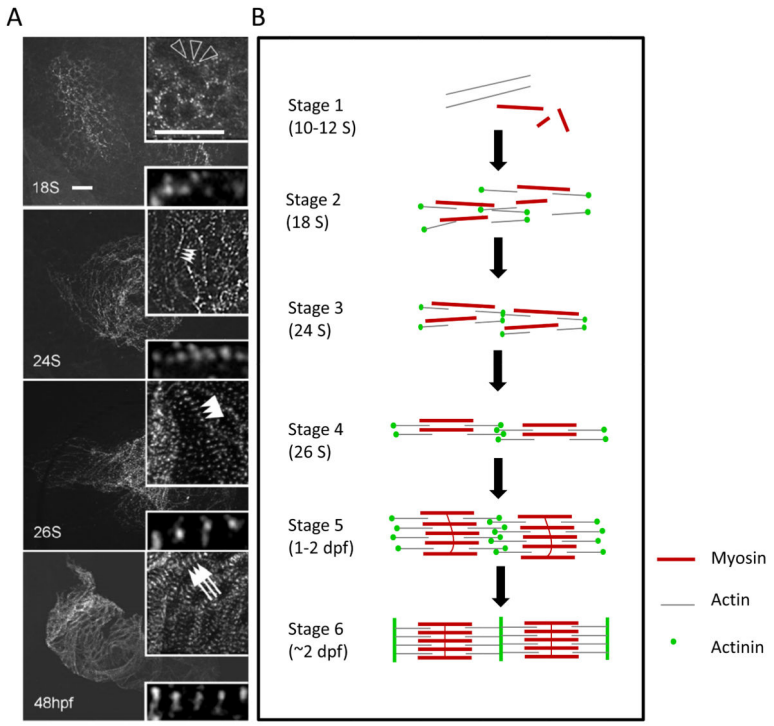

Figure. Sarcomere Assembly in a Zebrafish Heart

A, Immunostaining of a zebrafish heart using anti-Actinin antibody on embryos at the 18 somite (S), 24 S, 26 S, and 48 hours postfertilization (hpf) stages, respectively. Scale bar indicates 20 μm. Arrows and arrowheads indicate Z-bodies and Z-discs at different developmental stages. Images adapted from (Huang, Zhang et al. 2009). B, Schematic of the 6 stages of sarcomere assembly in the zebrafish heart. Stage 1, around 10–12 S, the actin filament is assembled along the cell membrane while the myosin filaments independently form rodlet structures. Stage 2, around 18 S, actin filaments and thick filaments are integrated where Actinin is randomly located as irregular spaced dots. Stage 3, around 24 S, the sarcomere becomes more organized, with consistent shorter length. Stage 4, around 26 S, the striated sarcomere becomes elongated. Stage 5, 1–2 days postfertilization (dpf), myofibrils are bundled through lateral fusion of adjacent myofibrils. Stage 6, around 2 dpf, mature sarcomeres appear after a lateral registration process, as reflected by the formation of well-aligned Z-discs.