INVESTIGATING HOW FHOD-FAMILY FORMIN PROMOTES Z-LINE ORGANIZATION AND STRIATION FORMATION IN C. ELEGANS STRIATED MUSCLES

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Approved ___________________________

(Sponsor’s signature)

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Abstract

Investigating how FHOD-family formin promotes Z-line organization and striation formation in C. elegans striated muscles

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Striated muscles are composed of basic structural and functional units called sarcomeres. The assembly of these sarcomeres is a well-studied process among vertebrates and multiple lines of evidence suggest formins as regulators of sarcomere assembly. Formins are regulators of unbranched actin networks and thus were ideal candidates to test for the initiators of thin filament assembly. We examined how Caenorhabditis elegans formins, FHOD-1 and CYK-1 regulate striated body wall muscle (BWM) growth. We found that FHOD family-related, FHOD-1 was the only formin that promoted BWM growth in a cell autonomous manner. However, the DIAPH-family related CYK-1 effect on BWMs was rather indirect. Interestingly, both these formins did not function as thin filament initiators. Our focus was to investigate the mechanisms of how FHOD-1 regulates striated muscle development. Loss of FHOD-1 however caused disorganized Z-lines in BWMs. Dense bodies (DBs) are analogous to Z-lines and are also similar to integrin-based focal adhesions. They are often arranged in rows that appear parallel in wild-type animals. We investigated how the loss of FHOD-1 affects the distribution, arrangement and morphology of the DBs. We found that loss of FHOD-1 led to the accumulation of non-parallel striations and FHOD-1 was enriched at the sites of new DB assembly as well as at sites where non-parallel striations would intersect. FHOD-1 supports the orientation of new striations. We also found that DBs from worms
that lack FHOD-1 were fragile and were not able to withstand prolonged contractions.

We interpret that FHOD-1 could regulate the actin dynamics or act as a linker to bundle actin filaments that are a part of this unique DB-associated cytoskeletal system, which provides structural integrity to the DBs.
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Absence of FHOD-1 but not acute loss of CYK-1 partially disrupts sarcomere structure in embryonic BWM.

FHOD-1 promotes BWM cell growth and proper DB organization in a cell-autonomous manner, whereas CYK-1 does not.
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FHOD-1 supports DB structural integrity against muscle contraction

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Abbreviations

BWM, body wall muscle
CGC, *Caenorhabditis Genetics Center*
CMT, Charcot-Marie tooth disease
CYK, cytokinesis (diaphanous family) formin
DAAM, disheveled-associated activator of morphogenesis proteins
DAD, diaphanous autoregulatory domain
DB, dense body
DCM, dilated cardiomyopathy
DD, dimerization domain
DIAPH, diaphanous proteins
DID, diaphanous inhibitory domain
ECA, extrachromosomal array
ECM, extracellular matrix
EM, electron microscopy
F-actin, filamentous actin
FH1, formin homology-1
FH2, formin homology-2
FHOD, formin homology-2 domain-containing
FMN, formin proteins
FMNL, formin-like proteins
FSGS, Focal segmental glomerulosclerosis
G-actin, globular actin
GBD, GTPase binding domain

GFP, green fluorescent protein

GRID2IP, glutamate receptor inotropic delta 2-interacting proteins

HCM, hypertrophic cardiomyopathy

INF, inverted formins

mCH, mCherry

MHC, myosin heavy chain

MIP, maximum intensity projection

MLC, myosin light chain

MWHF, multiple winged-hairs formins

NGM, nematode growth medium

PHCF, pleckstrin homology domain-containing formins

RNAi, RNA interference

TIRF, total internal reflection fluorescence

Z-LAC, Z-line associated cytoskeleton
Chapter 1: Introduction
Chapter 1 contributions

Sumana Sundaramurthy wrote this chapter to serve as an introduction to both her research presented in Chapters 2 and 3 and the overall implications elucidated in Chapter 4.
Overview

Formins are a family of regulators of actin dynamics in cells. The FHOD-family formins have been well-studied for their involvement in striated muscle development. But their precise mechanism in muscle development is unknown. We are using the body wall muscles (BWMs) of the simple nematode, Caenorhabditis elegans to understand how the only FHOD-family formin, FHOD-1 regulates BWM development. FHOD-family formins have been implicated in human cardiomyopathies. In this study we are investigating how FHOD-1 affects Z-line development and function. Our results could provide hints as to how FHOD-family formins could function in general, which could be used to understand their mechanism in the mammalian system.

Actin cytoskeleton

Actin is one of the most abundant proteins in eukaryotic cells. With a molecular weight of 42kDa, actin is well known as one of the core components of the cytoskeleton network in cells (Cooper, 2000; Michelot & Drubin, 2011). The cytoskeleton consisting of actin and its associated proteins is organized into bundles and filament networks. Distinct actin-based networks appear in specific cellular structures like the lamellipodia, filopodia, stress fibers, contractile rings, microvilli, growth cone, stereocilia, and sarcomeres (Figure 1-1) (Cooper, 2000). These actin-based cellular structures are responsible for specific cellular functions like cell motility, muscle contraction, maintaining cell shape, cellular contractility, cargo transport, mechanoelectrical transduction, and cell division. Actin is often highly dynamic in these intricate structures, and it exists in two different states in cells: the monomeric, globular actin (G-actin) and filamentous actin (F-actin) that forms the core of the actin filaments.
Figure 1-1. Types of actin-based networks in cells. Distinct actin-rich networks that aid in specific functions, appear in different cell types. Actin-based networks appear in (A) lamellipodium of a motile cell, (B) filopodia of a fibroblast, (C) contractile rings of dividing cells, (D) neural growth cone, (E) cell cortex (pink filaments), (F) sarcomere in a myofibril of a muscle cell (light red filaments), (G) adhesion belt of an enterocyte, (H) stereocilia of a hair cell, (I) microvilli of a brush cell. Created with BioRender.com.
F-actin is made up of a chain of monomeric actin subunits, which is formed by the polymerization of G-actin (Pollard & Borisy, 2003; Pollard, 2016). The two states are reversible as F-actin can depolymerize or disassemble back into G-actin. The polymerization of G-actin to F-actin occurs in three phases: a nucleation phase, an elongation phase, and a steady phase (Figure 1-2). The nucleation phase involves the formation of a stable ‘actin nucleus’ that is comprised of three actin monomers. The stable nucleus then becomes polarized where it has a rapidly growing end called the barbed (plus ‘+’) end that has faster dynamics, where the actin monomers are rapidly added. The opposite end called the pointed (minus ‘-’) end has slower dynamics due to the relatively slow net growth compared to the barbed end due to the faster rate disassembly at the pointed end (Figure 1-2) (Pollard, 2016). Filament growth or addition of monomers occurs at both these ends, but it is faster at the barbed end. Growth of filaments occurs via the elongation phase, which is often facilitated by barbed end actin regulators such as formins. Critical concentration is the minimum concentration of G-actin monomers that is required for net polymerization to occur. Above the critical concentration, the rate of polymerization is higher than the rate of depolymerization at any given end of a filament. The critical concentration of pointed ends is higher than that of the barbed ends. Muscle cells have a high concentration of actin when compared to other cells, which results in net polymerization at both pointed and barbed ends. During the steady state phase, the actin filament dynamics enter a state of equilibrium where the polymerization at the barbed end and monomer disassembly from the pointed end is balanced and maintained by the critical concentration of G-actin monomers in the cytosolic pool (Cooper, 2000; Pollard, 2016). This steady state actin assembly and
**Figure 1-2. Actin dynamics.** There is a lag in the formation of stable nucleus, which is the rate-limiting step during actin polymerization. Once the nucleus becomes polarized, it starts growing rapidly at the barbed (plus ‘+’) during the elongation/growth phase. The pointed (minus ‘-’) end dynamics are slower due to the relatively slow net growth compared. Once the critical concentration of the cytosolic G-actin pool is reached, the steady-state kicks in where the net growth of the filament is zero. The filaments enter a state of equilibrium where the polymerization at the barbed end and monomer disassembly from the pointed end is balanced, called ‘treadmilling’. Created with BioRender.com
disassembly is called ‘treadmilling’ (Figure 1-2). The steady pool of actin monomers must be present for the polymerization to continue beyond the elongation phase, and this is often aided by actin-binding proteins like profilin. The mechanism of regulation between these highly dynamic G-actin and F-actin states are controlled by various nucleation promotion, elongation, depolymerization factors, and actin-binding proteins like formins, spire, Arp2/3, Wiskott-Aldrich Syndrome Protein (WASP), cofilin, and profilin (Pollard, & Borisy, 2003; Pimm, Hotaling & Henty-Ridilla, 2020; Chesarone and Goode 2009). Despite the involvement of myriad factors, actin is assembled into specific networks, meant for an intended function in cells. Among the various groups of actin regulators, one of the largest groups are formins, which are notable for their ability to regulate nucleation and elongation of unbranched actin networks.

**Formins**

Formins are one of the highly conserved groups of proteins that can regulate both actin and microtubule networks (Chesarone, DuPage, and Goode 2010). The regulation of actin nucleation and elongation by formins is orchestrated with the help of two highly conserved domains: the formin homology 1 (FH1) and formin homology 2 (FH2) domains (Figure 1-3). These domains play critical roles in formin-dependent regulation of actin dynamics. The FH2 domains dimerize and nucleate actin filaments by recruiting actin monomers to form a stable nucleus for filament assembly. The FH2 dimer stays associated with the growing or barbed end of the actin filament and facilitates elongation of actin filaments by the addition of G-actin monomers to the growing filament (Kovar & Pollard, 2004; Moseley et al., 2004; Pring, Evangelista, Boone, Yang, & Zigmond, 2003; Pruyn et al., 2002; Sagot, Rodal, Moseley, Goode, & Pellman, 2002). The FH1 domain
Figure 1-3. Formin and formin-mediated actin assembly. (A) Domain map of a typical formin, containing GTPase-binding domain (GBD), diaphanous inhibitory domain (DID), dimerization domain (DD), formin homology 1 (FH1) domain, formin homology 2 (FH2) domains, and a diaphanous autoregulatory domain (DAD). (B) Autoinhibition is a result of the intramolecular interaction between DID and DAD domains of formins, making them inactive. (C) Autoinhibition is usually released due to the interaction between the GBD and GTP, which leads to an active dimer. (D) Formin dimers aid in the stabilization of the actin nucleus via their FH2 domains, where it remains associated at the barbed end of the filament. FH1 domain recruits profilin-bound actin and aids in the elongation of the actin filament. Created with BioRender.com
has multiple proline-rich sites that help in the binding of profilin-bound actin which is then used as a substrate for elongation at the barbed end (Figure 1-3) (Chang, Drubin, & Nurse, 1997; Evangelista et al., 1997; Imamura et al., 1997; Kovar, Harris, Mahaffy, Higgs, & Pollard, 2006; Romero et al., 2004). The activity of formins is often regulated by autoinhibition due to the association between the formin’s diaphanous inhibitory domain (DID) and diaphanous autoregulatory domain (DAD), which blocks FH2-actin interactions (Figure 1-3) (Li, F & Higgs, 2003; Breitsprecher & Goode, 2013). This autoinhibition is often broken by various pathways, one of which is the interaction between the GTPase-binding domain (GBD) in formins and the Rho-GTPases. It is impressive how tightly these interactions are controlled, by targeting formins in different families with specific Rho-GTPases (Kühn & Geyer, 2014).

**Formin families in different model systems**

Based on phylogenetic analysis of FH2 domains, animal formins can be grouped into nine families (Higgs & Peterson, 2005; Pruyne, 2016). These are the Formin homology domain-containing proteins (FHOD), Diaphanous-related formins (DIAPHs), Disheveled-associated activator of morphogenesis proteins (DAAMs), Formin-related proteins (FRL/FMN), FMN, Inverted formins (INF), Multiple Wing Hairs-related formins (MWHF), glutamate receptor ionotropic delta 2-interacting proteins/delphilins (GRID2IPs) and pleckstrin homology (PH) domain-containing formins. Invertebrates like *Caenorhabditis elegans* and *Drosophila melanogaster* have just six formins, belonging to five and six different families respectively. They are formins, FHOD-1, CYK-1, DAAM-1, EXC-6, FRL-1 and INFT-2 in worms and FHOS, DIA, FORM3, CAPU, DAAM and FRL in fruit flies (Liu et al. 2010; Mi-Mi et al. 2012; Pruyne 2016). However, placental
mammals have fifteen different formin genes that encode proteins that can be classified into seven major families (DIAPH, FHOD, DAAM, INF, FRL/FMNL, FMN, and Delphilin). It is amazing how invertebrates like *C. elegans* can sustain with just six formins, whereas humans need fifteen formins for effective function. Despite the presence of fifteen formin genes with possible redundant roles, even loss or changes to even a single formin gene leads to diseases that require clinical care. Formin families which are associated with diseases are more extensively studied due to their clinical significance.

**Clinical significance of formins with special focus on striated muscle development**

Mutations in the inverted formin gene, *inf2* are associated with Focal segmental glomerulosclerosis (FSGS), a kidney epithelial disease, and Charcot-Marie Tooth (CMT) disease, a neuropathy along with FSGS (Brown et al., 2010; Boyer et al., 2011). DIAPH formins are associated with nonsyndromic deafness, blood disorders, and ovarian failure (Lynch et al., 1997). Loss of the DIAPH formin, DIAPH1 causes microcephaly (Ercan-Sencicek et al., 2015). Truncated mutants of the formin, FMN2 causes nonsyndromic intellectual disability (Law et al., 2014). A few formin families are well studied due to their role in striated muscle development and mutations in their genes have led to various muscle pathologies.

Among the various studies, an RNA interference (RNAi) against formins in cultured neonatal mouse cardiomyocytes, is one of the early studies that implicated the importance of DAAM, FMNL, and DIAPH-family formins in myofibril organization (Rosado et al., 2014). Loss of DAAM in *Drosophila* greatly affects indirect flight muscles (IFMs), which have thin myofibrils with reduced thin, thick filaments with
abnormal Z-discs and M-lines (Molnár et al., 2014). Conditional knockout of DAAM1 in mice led to non-compaction cardiomyopathy. But simultaneous knockout of both DAAM1 and DAAM2 led to stronger myopathy with a disrupted sarcomere structure and reduced cardiac function (Ajima et al., 2015).

The FHOD-family formins in mammals, FHOD3 and FHOD1, have been of particular interest to researchers in the cardiac research community. Functional variants of the FHOD-family formin, FHOD3 have been associated with cardiovascular diseases like, hypertrophic (HCM) and dilated cardiomyopathies (DCM) (Arimura et al., 2013; Hayashi et al., 2018; Ochoa et al., 2020, 2018; Wooten et al., 2013). Studies in model systems like cultured mouse and rat cardiomyocytes suggest that FHOD-family formins regulate striated muscle development by affecting myofibrillogenesis (Taniguchi et al., 2009; Iskratsch et al., 2010; Rosado et al., 2014). Knockout of FHOD3 in mice led to embryonic lethality at day 11.5 in utero. These embryos died due to poorly developed hearts that failed to form mature myofibrils. Their heart muscles had malformed cardiac myofibrils, with irregular and immature Z-lines (Kan-O et al., 2012). The critical role of FHOD3 during cardiac myofibril assembly (myofibrillogenesis) is found to be dependent on its ability to interact with actin (Fujimoto et al., 2016; Kan-O et al., 2012). Further, conditional knockout of FHOD3 in adult mice showed that FHOD3 is required for the maintenance of cardiac function in both normal as well as pathological conditions like cardiomyopathies (Iskratsch et al., 2010; Ushijima et al., 2018). Studies in human induced pluripotent stem cell-derived cardiomyocytes suggest that FHOD3 regulates myofibrillogenesis (Fenix et al., 2018). The functional conservation of FHOD-family formins on striated muscle development can be well appreciated with comparative studies.
done in invertebrate model systems. In *Drosophila*, the absence of the only FHOD-family formin, FHOS, affects myofibrillogenesis and leads to defective indirect flight muscles (IFMs) (Shwartz, Dhanyasi, Schejter, & Shilo, 2016). Complete loss of FHOS abolished sarcomere organization, while early and late knockdown had varying defects. An early knockdown led to thin, irregular myofibrils with immature Z-discs while a late knockdown led to suppression of new thin filament incorporation into sarcomeres. Using *Caenorhabditis elegans*, we have shown that the only FHOD-family formin in worms, FHOD-1, promotes striated muscle development (Mi-Mi et al., 2012; Mi-Mi & Pruyne, 2015; Sundaramurthy, Votra, Laszlo, Davies, & Pruyne, 2020). The body wall muscles (BWMs) of *fhod-1* mutants are thin with fewer sarcomeres and have characteristic disorganized Z-lines that appear irregularly spaced. The functional similarity of the FHOD-family formins in striated muscle development among different model systems opens a wide range of opportunities for researchers to decipher its function. It is not surprising that formins have a major role in striated muscle development. Striated muscles not only contain a major pool of actin, but it is also the home of unbranched actin filaments, which form the core component of their contractile machinery.

**Types of vertebrate muscles**

Based on structure and function, muscle tissues are grouped into 3 main types: skeletal, cardiac, and smooth muscles. As the name suggests, the skeletal muscle is specialized to function along with our skeleton system and is responsible for locomotion and movement, whereas the cardiac muscles are responsible for the functioning of the heart (Henderson et al. 2017). Skeletal muscles are under the voluntary control of our body but both cardiac and smooth muscles are under involuntary control. Smooth
muscles are involved in several functions and they occur in many organs like blood vessels, stomach, and intestines. The contraction of smooth muscles is based on the contraction of thick and thin filaments, where the latter are attached to dense bodies. They are often referred to as non-striated due to the lack of striated appearance in the light microscope. However, both skeletal and cardiac muscles are referred to as striated muscles, as they appear as ‘cross striations’ under a light microscope. This appearance is due to the presence of a highly organized cytoskeleton network in these muscles, which are composed of individual contractile units called ‘sarcomeres’. However, skeletal muscles are arranged in regular parallel bundles and multinucleated whereas cardiac muscles have branches and are connected using intercalated discs. Both muscle types are made up of cells called myofibers (Figure 1-4). The muscle fibers contain bundles of organelles called myofibrils that enclose the contractile lattice. Muscle fibers are one of the structures, first documented by the famous Dutch microscopist, Antonie Von Leeuwenhoek in the 17th century as a ‘banded pattern’.

**Sarcomere: structure and mechanism of action**

A sarcomere is bordered by Z-lines at both ends that anchor the F-actin-based thin filaments, which are also associated with other proteins like tropomyosin and troponin (Figure 1-4) (Clark et al. 2002; Henderson et al. 2017). The thin filaments are interdigitated by the thick filaments containing bipolar filaments of myosin II, which are held together at the M-line. Titin is considered as a third filament system in the sarcomere, running from Z-line to M-line and it functions as a molecular spring. The sarcomere components can be differentiated into various bands based on the polarizing light properties when viewed under a microscope. The ‘I-band’ (for isotropic) is
Figure 1-4. Components of a sarcomere. The sarcomere is the basic structural and functional unit of the contractile lattice. The contractile machinery of the vertebrate muscles, containing repeated sarcomeric units, are enclosed in organelles called myofibrils within a muscle cell (muscle fiber). A sarcomere is bordered by the Z-lines that attach the actin-rich thin filaments, which interdigitate with myosin-rich thick filaments attached at the M-line. Besides actin, thin filaments are also made up of tropomyosin and troponin. A third filament system consists of titin, which acts as a molecular spring that extends from Z-line to M-line. Created with BioRender.com.
composed of the region of thin filaments that are not superimposed by thick filaments (Figure 1-5). The ‘A-band’ (for anisotropic) represents the entire thick filament region. The ‘H-band’ represents only the thick filaments that are not superimposed by thin filaments. The first groundbreaking papers which led to the molecular basis of muscle contraction described changes in muscle contraction based on these bands using interference, phase contrast, and electron microscopy techniques (Huxley & Niedergerke, 1954; Hanson & Huxley 1953). These observations led to the sliding filament theory and the idea of the cross-bridge cycle.

Sliding filament describes the mechanism of muscle contraction as thick and thin filaments slide past one another during muscle contraction while maintaining their lengths constant (Huxley & Niedergerke, 1954; Hanson & Huxley 1953). The cross-bridge cycle further describes the sequence of molecular events that occur between actin and myosin during muscle contraction. The weak binding of myosin heads extending from the thick filaments to the actin within the thin filament is referred to as a cross-bridge. An action potential triggers the release of calcium ions from the sarcoplasmic reticulum, which binds troponin C. This binding leads to a conformational change which further results in the exposure of the myosin-binding sites on actin that were covered by tropomyosin. The release of inorganic phosphate from the previous hydrolysis of ATP allows the myosin heads to tightly bind to exposed sites on actin, which were previously covered by tropomyosin, leading to a ‘power stroke’. This leads to the contraction of muscle by shortening the sarcomere length. The binding of new ATP to myosin leads to a low-energy conformation that disrupts the actomyosin interaction, breaking the cross-bridge. Once the ATP attached to the myosin heads is hydrolyzed and the cross-bridge is
formed, the cycle continues. This cycle and the basic sarcomere structure are conserved among striated muscles of all species including the striated muscles of *C. elegans*.

**Caenorhabditis elegans BWMs as a model to study formins**

The BWM of *C. elegans* has many vertebrate homologs (Benian & Epstein, 2011; Moerman & Williams, 2006). It is also well known for its simplicity and has only six formins: FHOD-1, CYK-1, DAAM-1, EXC-6, INFT-2, and FRL-1 (Mi-Mi et al. 2012; Pruyne 2017). Loss of one of the key formins in BWM development, FHOD-1, does not lead to lethality/severe muscle phenotype unlike that observed in mammalian systems (Fenix et al., 2018; Kan-o et al., 2012; Taniguchi et al., 2009), which is useful in examining FHOD-1’s role in sarcomere assembly. It provides access to many powerful genetic tools which aid in the creation of strains with fluorescently-tagged sarcomeric proteins (Gieseler et al., 2016). The optical transparency and thinness of the worm make it possible to use immobilized whole worm for studying *in vivo* protein dynamics and even perform whole-animal drug treatments. Apart from this, there are other general advantages for using worms, the ability to look at many progeny (due to large brood size), short generation time, easy genetic manipulation, rapid-outcrossing, and the rapid transgenic rescue of mutants. The BWMs of *C. elegans* provide an attractive model for investigating the role(s) of formins in striated muscle development, but the model has few features that are different from their vertebrate counterparts.

**Comparison of sarcomere arrangement in vertebrate striated muscle and invertebrate *C. elegans* striated BWM**

Vertebrate skeletal muscle and an invertebrate muscle such as the BWM of *C. elegans* are analogous to each other and both have well-defined sarcomeres. However,
their muscle cells do differ in their structures and arrangement of sarcomeres. Vertebrate muscle cells are cylindrical, with sarcomeres arranged inside specialized elongated organelles called myofibrils, whereas the BWM cells are spindle-shaped cells, which have sarcomeres arranged right underneath the plasma membrane (Figure 1-5) (Mi-Mi & Pruyn, 2015). Vertebrate sarcomeres are arranged at an angle of 90° with respect to the longitudinal axis of the cell, making them appear cross striated, whereas in C. elegans sarcomeres are obliquely striated where they are arranged at an angle of 6° with respect to the longitudinal axis of BWM cell. Both M-lines and Z-lines are attached to the plasma membrane in BWM cells, and they are considered integrin-based adhesion complexes (Figure 1-5). However, their Z-lines are similar to costameres (Figure 1-6) that appear at the periphery of myofibril Z-lines, where they anchor the myofibrils to the plasma membrane of the muscle cell. Vertebrate Z-lines and M-lines within the myofibrils are indirectly attached to the plasma membrane through the costameres (Figure 1-5).

Components of striated muscle cytoskeleton

Actin filaments are the core components of thin filaments, which are capped at pointed and barbed ends by tropomodulin and CapZ, respectively (Clark et al. 2002; Henderson et al. 2017). There are six different actin genes encoding actin isoforms (skeletal α-actin, cardiac α-actin, smooth α-actin, smooth γ-actin, cytoplasmic β-actin, and cytoplasmic γ-actin) in humans (Perrin & Ervasti, 2010). The core of thin filaments contains either α-skeletal or cardiac α-actin. There is tight control over the expression of these actin isoforms, which are critical for normal muscle structure and function. Mutations in the actin gene lead to various myopathies (Nowak et al.1999, Ilkovski et al.,
Figure 1-5. Organization of sarcomeres in vertebrate striated muscle and *C. elegans* BWM. The basic components of both sarcomeres appear similar. The I-band containing two half actin filament regions from adjacent sarcomeres separated by the Z-line and the A-band containing the thick filaments attached at the M-lines appear similar. Sarcomeres in vertebrate myofibrils are cross striated, where they are arranged at an angle of 90° with respect to the longitudinal axis of the cell and sarcomeres in *C. elegans* are obliquely striated where they are arranged at an angle of 6°, right below the BWM cell membrane. In BWMs, both Z-line (known as dense body) and M-lines are attached to the plasma membrane. Vertebrate Z-lines in general are and M-lines not attached to the sarcolemma, except the peripheral Z-lines that are attached indirectly via the costameres. Image adapted from Mi-Mi and Pruyne, 2015.
2001, Olson et al. 1998). However, *C. elegans* have five actin isoforms (ACT-1 thorough ACT-5) and ACT-5 is the only one that is considered as non-muscle actin (Ono, & Pruyne, 2012).

Tropomyosin and troponin associated with the actin filament control the interactions between actin and myosin. Tropomyosins contain two α-helical chains and appear as coiled homodimers surrounding the actin filaments stabilizing them. There are four different tropomyosin genes in humans (TPM1, 2, 3, 4) and each of them encodes several splice isoforms of tropomyosins (Clark et al. 2002). Specific isoforms of tropomyosin are associated with actin filaments that are functionally different and the expression ratio of different isoforms are critical and are found to be based on cell type (Gunning et al, 2015). Recent evidence also suggests that formins can dictate the tropomyosin isoform specificity for growing actin filaments (Gunning et al., 2015). Tropomyosin isoforms are associated with HCM, DCM, and nemaline myopathy in humans (Michele & Metzger 2000; Donner et al. 2002; Olson et al. 2001). *C. elegans* has one tropomyosin gene, *lev-11*, and has multiple isoforms (LEV-11A, C, D, E, T, O) that occur via alternate splicing (Watabe, Ono, & Kuroyanagi, 2018). The long LEV-11 isoforms are considered muscle tropomyosins and the short ones are considered non-muscle isoforms. Troponins are heteromeric protein complex comprising of three regulatory units, troponin C, I, and T. Troponin C is the main regulatory unit that binds to calcium ions, troponin I binds to the myosin-binding sites on actin, inhibiting their interaction and troponin T is considered to interact with tropomyosin and troponin C to regulate the conformational change (Clark, 2002). There are several reports that associate...
troponin mutations to impaired cardiac function and HCM (Hoffman et al. 2001, Seidman & Seidman 2001).

Titin, considered as a third filament system of the sarcomere, is the largest protein in the body, about 1 µm in length. It acts as a molecular spring, running between the Z-line to M-line, and is responsible for the elasticity of the sarcomere (Trombitás et al., 1995). Titin also provides structure, flexibility, stability to the sarcomere and provides binding sites for numerous sarcomeric proteins and is also considered as the sarcomeric ruler. Vertebrate thin filaments have nebulin which is often considered as the fourth filament system next to titin and it runs along the length of thin filaments, anchoring at the Z-line (Clark et al., 2000). Both titin and nebulin have been associated with different myopathies like DCM and nemaline myopathy, respectively (Gerull et al. 2002; Pelin et al. 1999). *C. elegans* have titin but they do not have any nebulin homologs (Flaherty et al, 2002).

The bipolar myosin filaments in the thick filaments consist of the conventional class II myosins from the myosin superfamily. Myosin II contains two heavy chains (MHC) and four light chains (MLC) (Henderson et al. 2017). There are occurrences of multiple MHC and MLC isoforms, and their expression is tightly regulated in striated muscles (Weiss & Leinwand 1996, Reggiani et al. 2000). The myosin heads which bind actin in the thin filaments to form cross-bridges, are localized to a region that is often referred to as the ‘C-zone’. It also contains other thick filaments associated proteins like myosin binding protein-C and H. Thick filaments are cross-linked at the M-line by transverse, M-bridges and contain additional proteins like Myomesin. Mutations in MHC lead to hereditary myosin myopathies with highly variable clinical features (Oldfors,
In *C. elegans*, mutants of MHC A (MYO-3) and MHC B (UNC-54) (Miller, Ortiz, Berliner, and Epstein, 1983) have reduced thick filaments whereas *myo-3* mutants were more severe with a paralyzed phenotype. The M-lines in BWMs are attached to the plasma membrane and are adhesion-based structures. BWMs also have paramyosin, an invertebrate-specific headless myosin (Kagawa et al., 1989).

Z-lines border sarcomeres at both ends and they not only serve as anchor sites for thin filaments but also others like titin (Figure 1-6) and nebulin, in vertebrates (Clark et al. 2002; Henderson et al. 2017). The spectrin-family protein, α-actinin is the major component of Z-line and it cross-links thin filaments. Vertebrates have four α-actinin genes and some of them are known to have redundant functions. α-actinin also interacts with LIM domain proteins (ALP), FATZ (filamin, α-actinin and telethonin binding protein of the Z-disc). Studies have also revealed that loss of α-actinin is not essential for the initial assembly of the invertebrate sarcomeres (Fyrberg et al., 1988; Moulder et al., 2010). This has led researchers to propose that the interactions within the densely packed Z-line not only help in stabilizing the Z-line but also help in maintaining some of its function even in the absence of one of its components. The primary goal of Z-line is to transduce force from sarcomeres within the myofibrils to the membrane-associated complexes distributed along the peripheral sarcomere ends, called costameres (Figure 1-6). Costameres consists of the dystrophin-glycoprotein complex (DGC) and integrin-based adhesion system (Henderson et al., 2017). Dystrophin with the help of γ-actin (cytoplasmic actin) and various other proteins, help in the transduction of the force from the sarcomere Z-line to the sarcolemma and ECM (Figure 1-6). Mutations in the largest
Figure 1-6. *C. elegans* dense bodies are analogous to both vertebrate Z-line and costamere. Vertebrate sarcomeres are tightly packed in myofibrils found in muscle cells, where the thin filaments are attached to Z-lines. Bundles of myofibrils are found in a muscle cell and their sarcomeres are linked to the plasma membrane by a costamere complex. They serve as structures that help in transmitting the force from the sarcomere to the sarcolemma and extracellular matrix (ECM). Costameres are made of a highly complex network of proteins and for simplicity, only a few of them are represented here, dystrophin, integrins, vinculin, talin, and γ-actin. DBs in BWMs serve as both Z-lines and costameres in *C. elegans*. DBs serve as attachment sites for thin filaments, which are also linked together by ATN-1 (α-actinin). DBs are also attached to the plasma membrane at the base, acting as a structure that helps in transmitting the forces from the actomyosin contraction to the ECM. The base of the DB consists of many proteins and for simplicity, only few of them are represented here: PAT-2/3, DEB-1, TLN-1 and DYS-1. The DBs are often referred to as adhesion-based structures. Created with BioRender.com.
human gene encoding dystrophin causes Duchenne muscular dystrophy (DMD), which is characterized by progressive muscle degeneration and weakness (Aartsma-Rus et al., 2017). Worms have dystrophin-like protein (DYS-1), and their mutants show slight muscle degeneration, moderate cell death, and hyperactivity with exaggerated head bending phenotype (Bessou et al., 1998). DYS-1 localizes to regions similar to vertebrate muscles, at the BWM plasma membrane and around the Z-line analogs, which are called dense bodies (Brouilly et al., 2015).

**C. elegans dense body, the Z-line, and costamere of BWMs**

The Z-lines in *C. elegans* are attached to the plasma membrane of BWMs and they are known as dense bodies (DBs), which serve as both Z-lines and costameres (Figure 1-6, 1-7B, C) (Lecroisey, Ségalat, & Gieseler, 2007). DBs act as structures of thin filament anchorage and help in the efficient transduction of force from sarcomere contractions to the hypodermis to create effective locomotion. They are integrin-based adhesion structures and are considered to be analogous to vertebrate focal adhesions complexes (Moerman & Williams, 2006). They appear as finger-like projections that project into the cell body from the plasma membrane. α/β-integrins (PAT-2/3) occur at the plasma membrane and they interact with the ECM and other DB proteins like vinculin (DEB-1) and talin (TLN-1) (Gieseler et al., 2016; Lecroisey, Segalat, et al., 2007). Thin filaments are cross-linked at the DB by α-actinin (ATN-1) (Moulder et al., 2010). The *C. elegans* DBs have been used as a model system to better understand the function of its vertebrate protein homologs like focal adhesion proteins (Gieseler et al., 2016; Lecroisey, Segalat, et al., 2007; Moerman & Williams, 2006). Studies from a wide range of model systems have proposed that formins regulate striated muscle assembly. The striated
BWMs of *C. elegans* can be used to better understand the role(s) of formins in the vertebrate striated muscle system.

Integrin-based adhesion structures play an important role during sarcomere assembly (myofibrillogenesis) in vertebrates. Myofibrillogenesis is described as a three-step process: first is the formation of premyofibrils, next is the formation of nascent myofibrils, and then finally, the conversion of nascent myofibrils to mature myofibrils (Dabiri et al., 1997; Sanger et al., 2005, 2015). Assembly of sarcomeric components begins at the edge of the muscle cell with the formation of integrin-based adhesion structures called Z-bodies, associated with stress fiber-like premyofibrils. Premyofibrils are made of mini-sarcomeres with Z-bodies containing α-actinin attached to thin filaments and myosin filaments containing non-muscle myosin IIA and/or IIB. In nascent myofibrils, Z-Bodies form beaded structures, and the non-muscle myosin starts getting replaced by muscle myosin II. The maturation of these nascent myofibrils occurs by the transformation of beaded Z-bands to continuous Z-bands, completion of muscle myosin II replacement, and clear appearance of M-line. Sarcomere assembly is not that well understood in *C. elegans* compared to vertebrate systems.

**BWM development and sarcomerogenesis in *C. elegans***

The BWM of *C. elegans* does not arise from myoblast fusion, unlike their vertebrate counterparts. The BWMs develop during embryogenesis. After gastrulation, the embryo develops into various stages called pre-bean, bean, comma, 1.5-fold, 2-fold, 3-fold, and 4-fold named after the changing shape of the embryo, based on its elongation. The embryo starts twitching inside the eggshell during mid-embryogenesis when the first sarcomeres are assembled in the BWM cells (Hresko, Williams & Waterson, 1994;
Moerman & Williams, 2006; Altun & Hall, 2009). Sarcomerogenesis begins as myoblasts start to form around 290 min after first cell cleavage (Hresko, Williams, & Waterston, 1994). At this stage, there is an accumulation of structural components like vinculin, integrin, myosin A and B. Then around 310 min, muscle cells start to migrate into 4 quadrants and polarization begins, where the muscle components start to localize to membranes at the muscle cell edges. By 350 min, the comma stage, myoblasts have completed migration to dorsal and ventral quadrants, and muscle components have polarized to the cell edges towards the hypodermis. At 420 min these cells flatten to form the 1.5-fold embryogenic stage where the contractile lattice, basement membrane, and hemidesmosome components interact and organize into bands to form sarcomeres. At 450 min, around the 1.75-fold stage is when the first sarcomeres start to assemble to form the striations. First, there is an initial accumulation of Z-line and M-line components followed by assembly of thin and thick filaments. The embryo hatches as L1 larvae, after developing into a 4-fold embryo. However, mutants with a loss of function allele of certain genes (myo-3, deb-1) that are critical for sarcomere assembly, arrest at two-fold stage and hatch as paralyzed and arrested at two-fold stage (Pat) larvae. This is because the embryos are unable to move in the eggshell and are arrested at the 2-fold developmental stage. Phenotypes like ‘pat’, ‘let’ (lethal), ‘unc’ (uncoordinated), ‘dpy’ (dumpy), have been used to identify and characterize phenotypes and functions associated with the gene in *C. elegans*.

**Overview of BWM structure**

A newly-hatched L1 larva has 81 BWM cells with two sarcomeres per BWM cell, where their thick filaments are about 5 µm long (Mackenzie, Garcea, Zengel, & Epstein,
1978; Moerman & Williams, 2006). In the presence of food, L1 larvae develop into L2, L3, L4 larval, young adult, and adult stages. The larvae with 81 BWMs develop additional BWM cells resulting in a total of 95 BWM cells in adults, where their thick filaments are about 10 µm long. The BWMs cells are arranged in two pairs of longitudinal bundles (dorsal and ventral), running from head to tail, and are located in four quadrants (Figure 1-7 A). The F-actin-rich thin filaments are closely aligned and appear as ‘striations’. BWM cells can be identified by analyzing the slight change in the orientation of the striations and the ECM gap between cells. Both DBs and M-lines in BWMs are basal attachment structures that are integrin-based (Altun & Hall, 2009). In a strain expressing PAT-3::GFP (Plenefisch, Zhu, & Hedgecock, 2000) and ATN-1::mCh, PAT-3 localizes to DBs (arrows) and M-lines (M, arrowheads) (Figure 1-7 D). It also appears at the attachment plaques (A), which appear at the end of half I-bands in between two BWM cells (Figure 1-7 C). ATN-1 occurs only in DBs (presumptive mature DBs). PAT-3 appears closer to the cell membrane and ATN-1 appears deeper into the cell. From the top view of the BWMs, DBs appear as punctate structures, M-line appears like somewhat of a continuous line and attachment plaques appear as ‘zipper-like’ structures. BWM cells have rows of DBs that appear as ‘striations’. These DB striations are punctate rather than very closely spaced F-actin-rich think filaments that appear as banded ‘striations’. BWM cells can also be identified by analyzing the slight change in the orientation of the DB striations and the presence of attachment plaque between cells.

Creation of transgenic lines for the study

The powerful genetic tools in *C. elegans* make it an attractive model system to use for the study of striated muscle development. In this study, we have created strains expressing
Figure 1-7. *C. elegans* BWM views showing thin filaments and DBs. (A) Worm stained with fluorescently labeled phalloidin to show F-actin-rich BWMs. Dorsal view of adult worm showing BWMs (BWMs run from head to tail, but this image has pharynx in view obstructing the BWM at the head region). Scale bar, 35 μm. Higher magnification shows F-actin-rich thin filaments arranged as striations in each BWM cell. Scale bar, 6 μm. (B, C) Maximum intensity projections (MIPs) through BWM contractile lattice of worm, expressing PAT-3::GFP and ATN-1::mCh. (B) Scale bars, 27 μm. (C) Higher magnification shows the outline between two BWM cells is shown (dashed lines). PAT-3 occurs at DBs (arrows), along M-lines (M, arrowheads), and at attachment plaques (A). ATN-1 occurs only in DBs. BWM cells have rows of DBs that appear as striations. Scale bars, 6 μm. (A) was acquired using wide-field fluorescent microscopy and (B), (C) and (D) were acquired using SP8 confocal microscopy.
non-integrated transgenes, a mutant with a loss of function allele, strain expressing stable transgene, and strains expressing an endogenous protein that was tagged at its original locus. We have used microinjection to create strains with non-integrated transgenes to produce an extrachromosomal array (ECA) (Stinchcomb et al., 1985; Mello et al., 1991). The microinjected solution usually contains a mixture of plasmids containing the gene of interest and reporter(s) of choice. The injected plasmids get linearized, and the DNA concatenates into large arrays. These ECAs usually are heritable, and they are inherited based on principles of non-mendelian inheritance. Inheritance of ECAs is often variable between strains and in most cases their expressions are mosaic. Although the creation of ECAs and screening is easier than other methods, the mosaic inheritance of the ECAs is a caveat. In many cases, ECAs contain thousands of copies of the transgenes, which could lead to overexpression or even mislocalization. We often create multiple strains containing ECAs to analyze the effect of a particular transgene. To prevent this caveat, we used microparticle bombardment, to create strains that contained stably integrated transgenes. The technique works by insertion of the transgene at a random locus using gold particles. The transgene is bound to gold particles, and they are bombarded into worms using a bolistic bombardment instrument (Praitis et al., 2001). Strains created using this technique not only are more likely to have a stably integrated transgene, but they also have a lower copy number and are often concentrated at one locus. The disadvantages are that this technique takes a longer time, and the random insertion could sometimes lead to disruptions in other genes.

One of the most powerful tools that could overcome the caveats of the above techniques is CRISPR/Cas9, which can be used to edit the genome at a precise location
(Kim et al., 2014; Dickson and Goldstein, 2016). Two strains used in this study were created with reporters inserted at their respective endogenous loci using the CRISPR/Cas9 technique. In general, short guide RNAs are designed specific to the DNA cut site and they are introduced along with the Cas9 enzyme. Repair templates are created based on whether an insertion (in this case our reporter mCherry or GFP tag) or deletion is needed. We also created a loss of function, daam-1 mutant that contained a novel FH2-null daam-1 allele, which was generated using homologous recombination guided repair after targeting the protein’s functional domain. For this, we injected a mixture containing plasmid containing gene encoding Cas9, the short guide RNA, and the repair template into unc-119 mutants, and transformants were further screened for selection. Researchers have improved the efficiency of getting transformants using the CRISPR/Cas9 technique in worms, where they have been injecting Cas9 protein instead of plasmids directly to induce breaks. These techniques allowed us to test cell autonomous roles of FHOD-1 and CYK-1 in the first part of the study (Sundaramurthy et al, 2020) and it also enabled us to create strains expressing fluorescently-tagged DB proteins, which have been used extensively in the second part of the study.

The functional similarities among the FHOD-family formins in different model systems and C. elegans provide us with an opportunity to uncover the role(s) FHOD-1 plays during striated muscle development. The results from this study could give us a comparative model that would lead to a better understanding of the role(s) FHOD-family formins might regulate during vertebrate sarcomere assembly.
Dissertation overview

The overall goal of this research is to examine the processes that formin regulates during striated muscle development. By using the simple *C. elegans* as a model, we hope that our research could provide insights as to how formins could function in the mammalian system. In Chapter 2, we investigate the cell autonomous and non-cell autonomous roles of FHOD-1 and CYK-1. In Chapter 3, we are examining how FHOD-1 affects DB distribution, function along with contractility-based morphology. In Chapter 4, we present our discussion and various models for the FHOD-1 role in DB development. Appendix 1 is characterizing the localization of a potential interactor of FHOD-1, short tropomyosin, LEV-11(short).

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Chapter 2: FHOD-1 is the only formin in *Caenorhabditis elegans* that promotes striated muscle growth and Z-line organization in a cell autonomous manner.
Chapter 2 contributions

Chapter 2 was previously published:


Sumana Sundaramurthy developed, designed, performed all experiments not mentioned below, analyzed the data, and wrote the paper. Arianna Laszlo performed the temperature shift experiments on larvae, adults and analyzed data corresponding to Figure 2-1.

SaraBeth Votra made worm strains, DWP199, DWP202, DWP204, DWP206, DWP208, DWP209, DWP211, DWP213 and performed initial BWM cell width measurements. Tim Davies isolated the worm strain, JCC389 and made worm strain, JCC955. David Pruyne created worm strains, DWP219, DWP222, oversaw research, provided input with experimental design, and edited the paper.
Abstract

The striated body wall muscles of Caenorhabditis elegans are a simple model for sarcomere assembly. Previously, we observed deletion mutants for two formin genes, fhod-1 and cyk-1, develop thin muscles with abnormal dense bodies (the sarcomere Z-line analogs). However, this work left in question whether these formins work in a muscle cell autonomous manner, particularly since cyk-1(Δ) deletion has pleiotropic effects on development. Using a fast acting temperature-sensitive cyk-1(ts) mutant, we show here that neither post-embryonic loss nor acute loss of CYK-1 during embryonic sarcomerogenesis cause lasting muscle defects. Furthermore, mosaic expression of CYK-1 in cyk-1(Δ) mutants is unable to rescue muscle defects in a cell autonomous manner, suggesting muscle phenotypes caused by cyk-1(Δ) are likely indirect. Conversely, mosaic expression of FHOD-1 in fhod-1(Δ) mutants promotes muscle cell growth and proper dense body organization in a muscle cell autonomous manner. As we observe no effect of loss of any other formin on muscle development, we conclude FHOD-1 is the only worm formin that directly promotes striated muscle development, and the effects on formin loss in C. elegans are surprisingly modest compared to other systems.
Introduction

Striated muscles, found widely across the animal kingdom (Clark, McElhinny, Beckerle, & Gregorio, 2002), are so-called due to the appearance of striations composed of regularly repeating contractile units, called sarcomeres. Each sarcomere is bordered by Z-lines that anchor actin-based thin filaments, while myosin-based thick filaments interdigitate between thin filaments and are anchored at the M-line at the sarcomere center. Besides these major cytoskeletal components, the contractile lattice is made up of additional proteins that help maintain structure and function (Henderson, Gomez, Novak, Mi-Mi, & Gregorio, 2017).

Evidence from multiple model systems demonstrates formins contribute to the assembly of sarcomeres. Formins are stimulators of actin assembly that function largely through two conserved Formin Homology (FH) domains, the FH1 and FH2. When dimerized, FH2 domains nucleate actin filaments from monomers, and remain associated with the growing barbed-end of the filament, protecting the end from inhibitors of elongation, like capping proteins (Pruyne et al., 2002; Sagot, Rodal, Moseley, Goode, & Pellman, 2002; Pring, Evangelista, Boone, Yang, & Zigmond, 2003; Kovar & Pollard, 2004; Moseley et al., 2004). The proline-rich FH1 domain recruits profilin bound to actin monomers to allow monomer addition to the barbed ends (Chang, Drubin, & Nurse, 1997; Evangelista et al., 1997; Imamura et al., 1997; Romero et al., 2004; Kovar, Harris, Mahaffy, Higgs, & Pollard, 2006).

Based on sequence homology, animal formins are grouped into nine families (Higgs & Peterson, 2005; Pruyne, 2016). One member of the FHOD-family, FHOD3, is the best-studied vertebrate formin for its effects on muscle development. Variants of the
fhod3 gene have been associated with occurrence of hypertrophic and dilated cardiomyopathies in humans (Arimura et al., 2013; Wooten et al., 2013; Hayashi et al., 2018; Ochoa et al., 2018), while its knock out in mice results in lethality at embryonic day 11.5 due to cardiac insufficiency, with failure to form mature myofibrils in cardiac muscle (Kan-O et al., 2012). This crucial role for FHOD3 depends on its ability to interact with actin (Fujimoto et al., 2016). Studies with human induced pluripotent stem cell-derived cardiomyocytes and cultured rat cardiomyocytes confirm FHOD3 regulates myofibrillogenesis (Taniguchi et al., 2009; Iskratsch et al., 2010; Fenix et al., 2018).

Studies in Drosophila indirect flight muscles (IFMs) have shown complete absence of fly FHOD-family formin FHOS abolishes sarcomere organization, while early knockdown yields thin, irregular myofibrils with dispersed and rudimentary Z-discs, and late knockdown prevents thin filament elongation or new thin filament incorporation (Shwartz, Dhanyasi, Schejter, & Shilo, 2016).

DAAM-family formins have also been implicated in muscle development. In Drosophila IFMs, and to a lesser extent heart and larval body wall muscle, absence of DAAM leads to thin myofibrils with reduced F-actin and thick filament content, and abnormal Z-discs and M-lines (Molnár et al., 2014). In mice, conditional DAAM1 knock out led to non-compaction cardiomyopathy, while simultaneous knock out of DAAM1 and DAAM2 caused stronger myopathy, severely reduced cardiac function, and disrupted sarcomere structure (Ajima et al., 2015). An RNA interference (RNAi)-based study of cultured neonatal mouse cardiomyocytes confirmed the importance of DAAM1 for myofibril organization, and also implicated FMNL- and DIAPH-family formins FMNL1,
FMNL2, and DIAPH3 (Rosado et al., 2014). While these studies suggest formins promote sarcomere organization, it is not clear what roles they play.

Our work with the simple model nematode *Caenorhabditis elegans* also implicates formins in promoting sarcomere formation in striated muscle. The worm has two pairs of body wall muscles (BWMs) that extend the length of the animal in dorsal and ventral positions. Each BWM cell consists of a cell body overlying a spindle-shaped myofilament lattice that is adherent to a basal lamina. Distinct from cross-striated vertebrate or fly muscle, BWM is obliquely striated with striations oriented at a 6˚ angle with respect to the longitudinal axis of the cell. The majority of thin filaments are anchored at dense bodies (DBs), which are analogous to vertebrate Z-discs, whereas thick filaments are attached to M-lines (Moerman & Williams, 2006; Gieseler, Quadota, & Benian, 2017). In BWM, all DBs and M-lines are also attached to the plasma membrane, and based on protein composition, DBs also resemble costameres and focal adhesions (Lecroisey, Ségalat, & Gieseler, 2007).

Many sarcomeric proteins and interactions are conserved between *C. elegans* BWM and mammalian striated muscle (Benian & Epstein, 2011). But unlike mammals, where fifteen genes represent seven of the nine animal formin families, *C. elegans* has only six formin genes (*fhod-1, cyk-1, daam-1, frl-1, exc-6* and *inft-2*) representing five families (Pruyne, 2016). Due to this simplicity, *C. elegans* BWM serves as a good model to study roles of formins in the development and function of striated muscle.

We previously characterized BWM defects in mutants for five worm formin genes (Mi-Mi, Votra, Kemphues, Bretscher, & Pruyne, 2012; Mi-Mi & Pruyne, 2015). Among these, only loss of FHOD-family FHOD-1 and DIAPH-family CYK-1 resulted in
BWM defects. Based on the tendency of formins to promote assembly of long, unbranched actin filaments in vitro, an attractive model was that FHOD-1 and CYK-1 initiate assembly of the actin-based thin filaments, with the prediction that absence of both formins should prevent thin filament formation. The deletion alleles fhod-1(tm2363) and cyk-1(ok2300), referred to hereafter as cyk-1(Δ) and fhod-1(Δ), eliminate part or all of their respective FH2 domains, making them putative nulls for formin-mediated actin assembly. Either fhod-1(Δ) or cyk-1(Δ) individually results in thin BWM cells with fewer striations per cell, while mutants for both formin genes have even thinner BWM cells, suggesting overlapping roles in muscle (Mi-Mi et al., 2012). However, BWM in those double mutants still have thin filaments organized in sarcomeres (Mi-Mi & Pruyne, 2015), inconsistent with our initial prediction.

A complication to that analysis was that CYK-1 is also essential for cytokinesis during embryonic development (Swan et al., 1998). To circumvent this requirement, cyk-1(Δ) was maintained in a heterozygous background. Homozygous cyk-1(Δ) mutants that arose as one quarter of the progeny from cyk-1(Δ)/+ parents completed embryogenesis due to inheritance of maternally-provided CYK-1 protein/mRNA (Mi-Mi et al., 2012). This also left open the possibility maternally inherited CYK-1 supported some sarcomere assembly. Another complication was cyk-1(Δ) has pleiotropic effects on development (Mi-Mi et al., 2012), allowing the possibility that muscle effects were secondary to other primary defects. Elimination of CYK-1 by RNAi yielded the same phenotypes as cyk-1(Δ) (Mi-Mi et al., 2012), but slow onset also allowed for residual CYK-1 in developing muscle, and pleiotropic effects again complicated interpretation.
Recent isolation of a fast acting temperature-sensitive cyk-1 allele (Davies et al., 2014), and the generation here of strains with mosaic expression of wild-type formin, have allowed us to re-examine this question. We find here CYK-1 plays a non-cell autonomous role in promoting muscle growth, likely during early embryogenesis, leaving FHOD-1 the only formin to play a cell autonomous role in BWM development. We also observe that in contrast to vertebrate and insect muscle, worm BWM is strikingly resilient to loss of endogenous formin.

Materials and Methods

Worm strains and growth conditions

Worms were grown using standard protocols (Brenner, 1974) at 20°C, except for experiments involving temperature-sensitive worms, for which growth was at 16°C prior to temperature shifts. Age-synchronized populations were obtained in one of two ways. By one method, adult worms were treated with 1:2 ratio 5 M NaOH to reagent grade bleach to liberate embryos, which were then washed with M9 medium (Ausubel et al., 2002), and allowed to develop until the proportion at two-fold stage had peaked (Fig. 2-3, 2-4, 2-5, 2-S3, 2-S4), or until hatching into starvation-arrested L1 stage larvae (Fig. 2-1, 2-2, 2-8, 2-9, 2-S1,2- S4). Alternatively, adults were allowed to lay eggs on plates for ~4-8 hrs and then removed, resulting in semi-synchronized progeny (Fig. 2-6, 2-7, 2-S2, 2-S5,2- S6,2- S8).

For long temperature shift experiments (Fig. 2-1, 2-2, 2-S1), starvation-arrested L1s were introduced to food (Escherichia coli OP50) at permissive (16°C) or restrictive (26°C) temperature for 0-72 hrs before being prepared for fluorescence microscopy. For short temperature shift experiments (Fig. 2-4, 2-S3, 2-S4), embryos in M9 were
transferred to 16°C or 26°C water baths for 30 min before preparation for fluorescence microscopy.

For complete genotypes of strains used in this study, see Table 1. N2 (wild-type Bristol worms) and RW1596 [myo-3(-); gfp::myo-3] were supplied by the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). JCC389 [cyk-1(ts)] and JCC955 [cyk-1::gfp] were gifts from Julie Canman (Columbia University, New York, NY; Davis et al., 2014; Davies et al., 2018). GS7933 [Ex[cyk-1::gfp]] was a gift from Daniel Shaye (University of Illinois at Chicago, Chicago, IL; Shaye & Greenwald, 2016). XA8001 [fhod-1(Δ)], DWP8 [cyk-1(Δ)/+], DWP9 [fhod-1(Δ); cyk-1(Δ)/+], DWP10 [fhod-1(Δ); fhod-1::gfp], DWP22 [cyk-1(Δ); cyk-1::gfp], and DWP28 [eri-1(-)] were obtained previously (Mi-Mi et al., 2012). Parentage of strains produced for this study are in Table 1.

Novel loss-of-function allele daam-1(ups39) was generated using CRISPR/Cas9-mediated gene editing to target TAATTGACCCGAGACGCTAT near the start of an FH2-coding exon of daam-1. Homologous recombination-guided repair was directed using a template constructed as follows. Nucleotides 22,974 - 24,147 of daam-1 was amplified by PCR with appended upstream KpnI cloning site, and downstream EcoRI site, in-frame stop codon, LoxP, and SalI cloning site (primers GGTACCGAGCGATTGCAAAAGAGCTGGA and GTCGACATAACTTCGTATAATGTATGCTATACGAAGTTATTCAGAATTCGGTGATCTGGAAATGAAGTTGTATAC), and nucleotides 24,198 - 25,339 of daam-1 were amplified and appended with upstream BamHI cloning site and LoxP, and downstream NotI cloning site (primers GGATCCATAACTTCGTATAGCATACATTATACGAGT
Table 2-1. *Worm strains used in this study.* Strain names, genotypes, figure(s) in which they appear, and source.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source†</th>
<th>Figure(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DWP008</td>
<td>cyk-1(ok2300)/+ III</td>
<td>Mi-Mi et al., 2012</td>
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<td>DWP009</td>
<td><em>fhod-1(tm2363)</em> I; cyk-1(ok2300)/+ III</td>
<td>Mi-Mi et al., 2012</td>
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<td>DWP010</td>
<td><em>fhod-1(tm2363)</em> I; qaIs8001[<em>fhod-1::gfp mini-unc-119(+)]</em></td>
<td>Mi-Mi et al., 2012</td>
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<td>DWP022</td>
<td><em>cyk-1(or596ts)</em> III</td>
<td>XA8001 x JCC389</td>
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<td>DWP008 x mT1/+</td>
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<td>DWP153</td>
<td>mT1/+ II; cyk-1(ok2300)/mT1[dpy-10(e128)] III</td>
<td>DWP008 x mT1/+</td>
<td>2-2, 2-6, 2-S5</td>
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<tr>
<td>DWP154</td>
<td><em>fhod-1(tm2363)</em> I; mT1/+ II; cyk-1(ok2300)/ mT1[dpy-10(e128)] III</td>
<td>DWP009 x mT1/+</td>
<td>2-2</td>
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<td>DWP156</td>
<td><em>fhod-1(tm2363)</em> I; stEx30[myo-3p::gfp::myo-3 rol-6(su1006gf)]</td>
<td>XA8001 x RW1596</td>
<td>2-4, 2-5, 2-S3</td>
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<td>JCC389 x RW1596</td>
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<td>DWP068 x RW1596</td>
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<td>DWP199</td>
<td><em>fhod-1(tm2363)</em> I; upsEx136[*fhod-1(+) myo-2p::gfp myo-3p::gfp rab-3p::gfp]</td>
<td>XA8001</td>
<td>2-6, 2-7, 2-S6</td>
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<td>DWP206</td>
<td><em>fhod-1(tm2363)</em> I; upsEx143[myo-2p::gfp myo-3p::gfp rab-3p::gfp]</td>
<td>XA8001</td>
<td>2-6, 2-7, 2-S6</td>
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<td>DWP208</td>
<td>+/mT1 II; cyk-1(ok2300)/mT1[dpy-10(e128)] III; upsEx145[cyk-1(+) myo-2p::gfp myo-3p::gfp rab-3p::gfp]</td>
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<td>DWP209</td>
<td>/mT1 II; cyk-1(ok2300)/mT1[dpy-10(e128)] III; upsEx146[cyk-1(+)] myo-2p::gfp myo-3p::gfp rab-3p::gfp</td>
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<td>DWP213</td>
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<td>DWP219</td>
<td>daam-1(ups39) V</td>
<td>This study</td>
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<td>DWP222</td>
<td>fhod-1(tm2363) I; daam-1(ups39) V</td>
<td>DWP219 x XA8001</td>
<td>2-S2</td>
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<td>GS7933</td>
<td>unc-119(ed3) pha-1(e2123ts) III; arIs195[exc::LifeAct::gfp unc-119(+)]; arEx2357[cyk-1::gfp hygrR(pIR98) pha-1(+)(pBS)]</td>
<td>Shaye &amp; Greenwald, 2016</td>
<td>2-S8</td>
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<td>JCC389</td>
<td>cyk-1(or596ts) III</td>
<td>Davies et al., 2014</td>
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<td>Davies et al., 2018</td>
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<td>N2</td>
<td>Bristol wild type</td>
<td>CGC‡</td>
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<td>RW1596</td>
<td>myo-3(st386) V; stEx30[myo-3p::gfp::myo-3 rol-6(su1006gf)]</td>
<td>Campagnola et al., 2002</td>
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<td>Mi-Mi et al., 2012</td>
<td>2-1, 2-2, 2-6, 2-7, 2-S1, 2-S2, 2-S6</td>
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</tbody>
</table>

‡Reference for initial isolation, or parental strains for crossing or transgenesis in this study.

‡Caenorhabditis Genetic Center (University of Minnesota, Minneapolis, MN)
TATAACTGCACAATAATGCTCTCTCAAGC and GCAGCGCCGCTCTACCCACCTACCTACACGC. These were sequentially cloned into pJKL702 (a gift from Jun Kelly Liu, Cornell University, Ithaca, NY) to flank its unc-119 mini-gene to create the homologous recombination-guided repair template. We generated and isolated transgenic lines from HT1593 [unc-119(-)] animals, and subsequently excised the integrated unc-119(+) from the daam-1 locus by expression of Cre recombinase, all as described by others (Dickinson & Goldstein, 2016). The resultant post-excision daam-1(ups39) encodes an in-frame stop codon near the start of its FH2-coding sequence, and a 1-nt frame shift due to the LoxP site, and is thus predicted encode a non-functional formin. DWP219 [daam-1(-)] was generated by crossing daam-1(ups39) into an unc-119(+) background, using EcoRI-mediated cleavage as a diagnostic for ups39.

To generate worms with mosaic expression of fhod-1, young adult XA8001 hermaphrodites were microinjected with a mixture of 50 ng/μL pRS315-fhod-1(+) (Mi-Mi et al., 2012), 100 ng/μL rab-3p::gfp plasmid, 50 ng/μL myo-3p::gfp plasmid, and 25 ng/μL myo-2p::gfp plasmid. Two independent strains (DWP199, DWP202) were isolated from progeny of different injected parents based on mosaic expression of GFP in in BWM. Two control strains (DWP204, DWP206) with mosaic transgene inheritance but lacking fhod-1(+), were produced similarly, but replacing pRS315-fhod-1(+) with pRS315 (Sikorski & Hieter, 1989). To isolate animals with mosaic expression of cyk-1, DWP153 [cyk-1(Δ)/+] worms were microinjected with 50 ng/μL pRS315-cyk-1(+) or pRS315, and 100 ng/μL rab-3p::gfp plasmid, 50 ng/μL myo-3p::gfp plasmid, and 25 ng/μL myo-2p::gfp plasmid, to generate test strains ng/μL myo-2p::gfp plasmid, to
generate test strains (DWP208, DWP209) and control strains (DWP211, DWP213) as above.

**RNAi**

RNAi-mediated knockdowns were performed by the standard feeding technique (Wang and Barr, 2005). Briefly, *E. coli* HT115 was transformed with control knockdown vector L4440 (Timmons & Fire, 1998) or *cyk-1* knockdown vector L4440-*cyk-1* (Mi-Mi et al., 2012), and grown overnight at 37˚C in 2xYT (Ausubel et al., 2002) with 12.5 µg/ml tetracycline, 100 µg/ml ampicillin. Cultures were diluted 1:100 in 2xYT, grown 3 hrs 37˚C, and induced 3-4 hrs with 0.4 mM IPTG at 37˚C before concentrating five-fold and seeding onto agar. Age-synchronized L1 worms were introduced and grown 3-4 days at 25˚C, then washed as adults using M9 to remove progeny, and introduced to freshly induced bacteria to continue RNAi knockdown for 5 days total.

**Staining for fluorescence microscopy**

F-actin stain was as previously described (Mi-Mi et al., 2012), as was immunostain (Finney & Ruvkun, 1990), except for omission of spermidine-HCl from the initial buffer for both protocols, and threefold higher initial methanol concentration (75%) for immunostain. For the specific case of *cyk-1* mosaic worms (Fig.2-6C), poor transmittance of *cyk-1* ECAs prevented harvesting large batches of transgenic animals. Instead, individual animals were picked and stained directly on glass slides, as previously (Hegsted, Wright, Votra, & Pruyne, 2016), with staining performed overnight. Monoclonal primary antibody MH35 (anti-ATN-1) generated by R.H. Waterston (Francis & Waterston, 1985) was a gift from Pamela Hoppe (Western Michigan University, Kalamazoo, MI), polyclonal rabbit anti-GFP was a gift from Anthony Bretscher (Cornell
University, Ithaca, NY), polyclonal affinity-purified anti-CYK-1 (DPMSP1) was generated previously (Mi-Mi et al., 2012), mouse anti-GFP and secondary antibodies (Texas red-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse, or reverse fluorophore/species) were purchased (Rockland Immunochemicals, Pottstown, PA). Antibody dilutions were 1:200 DPMSP1, 1:10⁴ MH35, 1:10³ mouse anti-GFP, 1:200 rabbit anti-GFP, and 1:500 for secondary antibodies.

**Microscopy and image analysis**

Wide field fluorescence images (Fig. 2-1, 2-4, 2-5, 2-6, 2-9 B, 2-9 C, 2-S2, 2-S4 A, B, 2-S5, 2-S7 A) were acquired using an Eclipse 90i Upright Research Microscope (Nikon, Tokyo, Japan) at room temperature (~25°C) with a CFI Plan Apochromat 40x/NA 1.0 oil immersion objective, or a CFI Plan Apochromat violet-corrected 60x/NA 1.4 oil immersion objective, with a Cool-SNAP HA2 digital monochrome charge-coupled device camera (Photometrics, Tucson, AZ) driven by NIS-Elements AR acquisition and analysis software (version 3.1; Nikon, Tokyo, Japan). Confocal images (Fig. 2-2, 2-3, 2-7, 2-8, 2-S4 C, 2-S7, 2-S8 B, 2-S8 C) were obtained on an SP8 Laser Scanning Confocal Microscope (Leica, Wetzlar, Germany) driven by LAS X Software (version 2.2.0, build 4758; Leica, Wetzlar, Germany), and using an HCX Plan Apochromat 63x/NA 1.4 oil lambda objective. Maximum intensity projections and XZ cross-sections were generated from confocal stacks using LAS X Software or ImageJ (version 2.0.0-rc-65/1.51g) (Schneider, Rasband, & Eliceiri, 2012). Images were linearly processed to enhance contrast and false-colored in Photoshop CS4 or CC 2018 (Adobe, San Jose, CA).

BWM and muscle cell widths were measured based on phalloidin stain, and total body width was measured based on body autofluorescence in the FITC channel using
NIS-Elements AR, as previously (Mi-Mi et al., 2012). Embryonic developmental stages were visually identified based on embryo shape, and BWMs were identified by GFP::MYO-3 (see results, Fig. 2-3, 2-4, 2-5). In animals with mosaic expression of soluble GFP in muscle cells, the FITC-channel was used to identify adjacent green and non-green muscle cells, followed by measurement of cell width based on phalloidin stain or analysis of DB organization based on ATN-1 immunostain (Fig 2-6, 2-7, 2-S6). To examine BWM cells in homozygous cyk-1(Δ) progeny of heterozygous cyk-1(Δ)/+ parents (Fig. 2-6), such progeny were identified by presence of protruding vulva and/or absence of embryos from the gonad (Mi-Mi et al., 2012). For examination of CYK-1 immunostain after cyk-1(RNAi) (Fig. 2-9), animals negative for anti-CYK-1 germline stain were selected as verification of efficient knockdown of CYK-1. Fluorescence images for comparison between cyk-1(RNAi) and control (Fig. 2-9) were consistently acquired at 500 ms exposures. All quantitative analyses were performed while blinded to the strain genotype, except for analysis of strains examined for Fig. 2-6 C (cyk-1 mosaic analysis), which due to poor propagation of cyk-1-containing ECAs across generations, were collected, stained, and analyzed as transgenic individuals became available over several weeks.

**Fast Fourier transform (FFT)**

Anti-ATN-1 stain intensity profiles were obtained for approximately eight DB-containing striations in one GFP-positive (ECA-containing) muscle cell and one adjacent GFP-negative (ECA-lacking) muscle cell each in ten mosaic animals of each strain (as seen in Fig. 2-7), using Freehand Line tool in ImageJ. There are relatively few DBs per striation, and their spacing is somewhat variable, producing a weak signal after FFT. To
amplify this, intensity profiles were concatenated at peak maxima for ATN-1 (i.e. the last peak maximum of one profile was joined to the first peak maximum of the next). FFT was performed and amplitude spectra obtained using MATLAB (R2019a Update 2). Analyses were performed while blinded to strain genotypes.

**Western blots**

Whole worm lysates were obtained from age-synchronized young adults by washing worms off agar and separating from *E. coli* before concentrating in 1.7 mL tubes to a 1:1 worm to M9 slurry. Reducing sample buffer (2X) was directly added to samples before boiling 3 min, disruption 30 sec with tissue homogenizer (VWR International, Radnor, PA), boiling 3 min, and pelleting 15 sec. To break up genomic DNA, samples were pulled through an insulin syringe eight times before loading for SDS-PAGE. For purposes of normalization, samples were subject to preliminary SDS-PAGE and Coomassie brilliant blue stain. Images were acquired using a Bio-Rad ChemiDoc MP imager (Bio-Rad, Hercules, CA), and intensities of stain for total lanes were compared using Image Lab software (Bio-Rad, Hercules, CA). For western blot analysis, proteins in normalized samples were resolved by SDS-PAGE and transferred to nitrocellulose (Bio-Rad, Hercules, CA). Blots were blocked in 10% milk/TBST (50 mM Tris-HCl, pH 8.3, 150 mM NaCl, 0.3% Tween 20) and incubated 5 hrs with DPMSP1 diluted 1:200 in TBST/1% milk, 5 hrs. Blots were washed and incubated 1 hr in goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Rockland Immunochemicals, Gilbertsville, PA) diluted 1:3000 in TBST/1% milk, before washing and treating with enhanced chemiluminescence substrate (ThermoFisher Scientific, Waltham, MA).
Images were acquired using a Bio-Rad ChemiDoc MP imager, and processed with Image Lab and Photoshop CS4.

**Statistical analyses**

Numerical data are expressed as mean ± standard error of the mean or one standard deviation, as defined in the text. Graphs were made in Excel:windows (version 14.7.2; Microsoft Corporation, Redmond, WA). For results where two groups are compared (Fig. 2-6), data were analyzed using a student t-test. For results from three or more groups, data were analyzed using Analysis of Variance, followed by a Least Significant Difference *post hoc* test. P-value < 0.05 was considered to be statistically significant.

**Data availability statement**

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

**Results**

**Post-embryonic CYK-1 loss leads to minimal BWM developmental defects.**

The vast majority of BWM growth and sarcomere assembly occurs after embryogenesis, when worms grow ~200-fold in size from L1 stage larvae to adults. If formins were required for sarcomere assembly, we would expect their absence to very significantly impact muscle growth during this period. To test the contribution of CYK-1 without the complications incurred by *cyk-1(Δ)* or *cyk-1(RNAi)*, we utilized the fast acting temperature-sensitive allele *cyk-1(or596ts)*, whose product supports embryonic development at a permissive temperature of 16°C, but becomes inactive within 5 min
after shift to 26°C (Davies et al., 2014). We combined cyk-1(ts) with fhod-1(Δ) to allow complete elimination of CYK-1 and FHOD-1 function during post-embryonic development. Age synchronized L1 stage larvae of wild-type, cyk-1(ts), fhod-1(Δ), or the combined fhod-1(Δ); cyk-1(ts) background, were shifted to either permissive or restrictive temperature and allowed to develop. Worms were collected every 12 hours and stained with fluorescently labeled phalloidin to visualize filamentous actin (F-actin) to track BWM growth. Prior to temperature shift, approximately 10% of the cyk-1(ts) and fhod-1(Δ) single mutants and 20% of the fhod-1(Δ); cyk-1(ts) double mutants failed in body elongation during embryogenesis, an effect related to formin functions in the epidermis, as described previously for fhod-1 and cyk-1 (Vanneste, Pruyne, & Mains, 2013; Refai, Smit, Votra, Pruyne, & Mains, 2018). As these larvae died soon after hatching, they were excluded from analysis. For remaining animals, total body width (as a proxy for overall body size), BWM width, and individual BWM cell widths (based on F-actin stain) were measured (Fig. 2-1, Fig. 2-S1).

At permissive temperature, cyk-1(ts) mutants grew normally, whereas at restrictive temperature, they laid no eggs and often developed protruding vulvas, phenotypes previously observed in cyk-1(Δ) mutants (Mi-Mi et al., 2012). As expected, BWM widths of all strains increased at permissive temperature as the animals grew, but more slowly in fhod-1(Δ) animals due to slower growth of individual muscle cells (Fig.2-1S1 A, B). In cyk-1(ts) mutants, we also observed narrower BWMs and narrower individual muscle cells compared to wild type at permissive temperatures, although this effect was less than for fhod-1(Δ) animals (Fig. 2-1S1 A, B), suggesting the cyk-1(ts) allele may be partially non-functional at 16°C. At the restrictive temperature, muscle cells
Figure 2-1. Post-embryonic CYK-1 loss causes minimal BWM defects. Larvae hatched at permissive temperature (16°C) were grown at restrictive temperature (26°C) for up to 72 hrs before staining with fluorescently-labeled phalloidin to show F-actin. (A) Dorsal views of BWM in worms of indicated genotypes grown 60 hrs. Double arrow shows lateral width of one BWM. Scale bar, 20 µm. (B) BWM widths, (C) individual muscle cell widths, and (D) total body widths were measured. Graphs depict average of the means of three experiments (n = 25 animals per strain per experiment, one body, two BWMs, and four muscle cells per animal). Error bars indicate standard error of the mean (SEM). Statistical significance p < 0.05 is indicated for: (a) wild type vs fhod-1(Δ); (b) wild type vs double mutant; (c) cyk-1(ts) vs fhod-1(Δ); (d) cyk-1(ts) vs double mutant; (e) all comparisons except wild type vs cyk-1(ts); (f) all comparisons except fhod-1(Δ) vs double mutant; (g) wild type vs cyk-1(ts); (h) fhod-1(Δ) vs double mutant; (all) all comparisons; or (n.s.) differences were not significant for any comparison, p ≥ 0.05. (E) Calculated ratio of (B) average BWM width to (D) average total body width. (F) Plot of BWM width versus total body width for 75 individual worms of each genotype grown at restrictive temperature 48 hrs, with linear trendlines shown. Wild-type and cyk-1(ts) animals show nearly identical linear relationships between total body and BWM width, suggesting the smaller BWM size of cyk-1(ts) animals may be a secondary consequence of smaller body size. Approximately linear relationships between BWM and total body sizes for fhod-1(Δ) and fhod-1(Δ); cyk-1(ts) animals were similar to each other, but with smaller muscle-to-body ratios than wild type.
**Figure 2-S1. cyk-1(ts) mutants grown at permissive temperature.** Embryos were hatched and grown for up to 72 hrs at permissive temperature (16°C), at which growth is approximately half the rate as at 26°C (Fig. 1). Samples were collected every 12 hrs, stained with fluorescently-labeled phalloidin, and measured for (A) BWM width, (B) individual muscle cell width, and (C) total body width. We observed modestly reduced BWM, individual muscle cell, and total body widths in cyk-1(ts) mutants compared to wild type at permissive temperatures. Graphs depict the average of the means of three experiments ± SEM (25, 50, or 100 width measurements for total body, BWM, and muscle cells, respectively, were made from n = 25 animals per strain in every experiment). Statistical significance p < 0.05 is indicated for: (a) wild type vs fhod-1(Δ), wild type vs double mutant, cyk-1(ts) vs fhod-1(Δ), and cyk-1(ts) vs double mutant; (b) wild type vs cyk-1(ts); (c) wild type vs double mutant; (d) fhod-1(Δ) vs double mutant; or (n.s.) differences were not significant for any comparison, p ≥ 0.05.
in *fhod-1(Δ)* mutants again grew slower than wild type, as expected (Fig. 2-1 A-C).

Surprisingly, BWM and muscle cells of *cyk-1(ts)* were only minimally reduced for growth compared to wild type, while those of *fhod-1(Δ); cyk-1(ts)* double mutants were similar to *fhod-1(Δ)* mutants (Fig. 2-1 A-C). These effects were much weaker than we had observed for *cyk-1(Δ)* (Mi-Mi et al., 2012).

We considered formins might affect overall body growth, and that reduced BWM might simply reflect smaller body size. Indeed, absence of FHOD-1 very modestly reduces body growth, and *cyk-1(Δ)* animals are significantly smaller than wild type (Mi-Mi et al., 2012; Refai et al., 2018). Thus, we measured the overall body widths of worms grown at 26°C (Fig.2-1 D, 2-S1 C), and determined the ratios of the BWM widths to total body. This average ratio was fairly constant for each strain throughout growth, and was similar between *cyk-1(ts)* and wild type (Fig. 2-1 E). Ratios for *fhod-1(Δ)* and *fhod-1(Δ); cyk-1(ts)* double mutants were also similar to each other, but proportionately smaller than wild type at all ages (Fig. 2-1 E). To determine whether this relationship held for individual worms, as opposed to average results for a population, we plotted BWM and total body widths for individual animals grown at 26°C, and observed nearly identical linear relationships between muscle and body size for wild-type and *cyk-1(ts)* animals, while those for *fhod-1(Δ)* and double mutants were different from wild type, but similar to each other (Fig. 2-1 F). These data confirm absence of FHOD-1 disrupts BWM growth to a greater extent than overall growth, whereas the modest BWM growth defects of *cyk-1(ts)* animals might be a consequence of reduced overall body size.

Our previous work suggested three of the remaining four formins in *C. elegans* (EXC-6/INFT-1, INFT-2, and FRL-1) make no obvious contribution to BWM
development (Mi-Mi et al., 2012). We had also observed an FH2-targeting deletion of the formin gene daam-1 caused no obvious synthetic defects in combination with fhod-1(Δ) (Mi-Mi et al., 2012), but careful analysis was not performed due to a deletion of thirteen genes very tightly linked to daam-1 that caused additional growth defects (Mangio, Votra, & Pruyne, 2015). Using CRISPR/Cas9-based genome editing, we generated a novel FH2-null daam-1 allele, ups39. On inspecting BWM in age-synchronized adult daam-1(ups39) mutants and fhod-1(Δ); daam-1(ups39) double mutants, we observed no effect of absence of DAAM-1 in either background (Fig. 2-1S2). Our current data suggest FHOD-1 might be the only formin that specifically supports BWM growth during post-embryonic development in C. elegans.

**Constitutive absence of FHOD-1 or CYK-1, but not post-embryonic loss of CYK-1, disrupts dense body (DB) organization in adult BWM.**

DBs serve as sarcomere Z-lines in BWM, and are structurally and compositionally similar to vertebrate focal adhesions and costameres (Lecroisey et al., 2007). The most prominent feature of these complex structures is an actinin (ATN-1)-rich portion that extends into the contractile lattice to anchor thin filaments (Gieseler et al., 2017). By transmission electron microscopy (TEM), DBs in wild-type BWM appear as distinct electron-dense finger-like protrusions, while in fhod-1(Δ) mutants they appear as multiple thinner electron-dense strands (Mi-Mi & Pruyne, 2015). Correlating with this, immunostain for ATN-1 reveals DBs as discrete puncta in wild-type BWM, but appears partially dispersed in fhod-1(Δ) mutants (Fig. 2-2).

By TEM, cyk-1(Δ) and fhod-1(Δ); cyk-1(Δ) mutant BWMs have less to almost no electron-dense material at some locations where DBs are otherwise expected (Mi-Mi &
Figure 2-S2. Absence of DAAM-1 did not cause any gross BWM defects. Dorsal views of adult worms of the indicated genotypes stained with fluorescently-labeled phalloidin to observe F-actin. Scale bar, 20 µm. There were no gross BWM defects observed due to absence of DAAM-1 compared to wild type, or with a combined absence of DAAM-1 and FHOD-1 compared to absence of FHOD-1, alone.
Figure 2-2. Constitutive absence of FHOD-1 or CYK-1, but not post-embryonic loss of CYK-1, disrupts DB organization. Maximum intensity projections (MIP) of dorsal views and reconstructed side views (XZ projection) of DBs in age-synchronized adult worms after ATN-1 immunostain. Scale bar, 2 µm. DBs appear as discrete puncta in wild-type BWM and partially dispersed in fhod-1(Δ) mutants. DBs of cyk-1(Δ) and fhod-1(Δ); cyk-1(Δ) mutants possess areas of stronger stain (arrows) and extremely faint stain (arrowheads). DBs of cyk-1(ts) appear as puncta, and those of fhod-1(Δ); cyk-1(ts) mutants appear partially dispersed.
Pruyne, 2015). Consistent with this, ATN-1 immunostain of adult cyk-1(Δ) mutants and fhod-1(Δ); cyk-1(Δ) double mutants revealed a variable staining. Some BWM regions showed prominent wild-type-appearing puncta in cyk-1(Δ) mutants, or fhod-1(Δ)-like dispersed structures in cyk-1(Δ); fhod-1(Δ) double mutants (Fig. 2-2, arrows), while other regions had no observable ATN-1-containing structures (arrowheads). Regions of absent staining were observed only in worms with cyk-1(Δ) in their genetic background, and we still observed diffuse background stain in these regions, suggesting this does not reflect failure of antibody penetration.

Because our results with cyk-1(ts) suggested postembryonic loss of CYK-1 does not affect BWM cell growth, we examined whether post-embryonic loss of CYK-1 had a similar lack of effect on DB morphology. L1 larvae were grown to adulthood at the restrictive temperature before immunostaining for ATN-1. In contrast to cyk-1(Δ) mutants, DBs in cyk-1(ts) mutants consistently appeared wild-type, with no areas lacking ATN-1 stain (Fig. 2-2). DBs of fhod-1(Δ); cyk-1(ts) mutants consistently appeared partially dispersed, as in fhod-1(Δ) mutants, again with no deficient areas (Fig. 2-2). Our data suggest that constitutive absence of either FHOD-1 or CYK-1, but not post-embryonic loss of CYK-1, results in abnormal DB morphology.

**Absence of FHOD-1 but not acute loss of CYK-1 partially disrupts sarcomere structure in embryonic BWM.**

Lack of major BWM effects after post-embryonic loss of CYK-1 led us to hypothesize that the more severe defects in cyk-1(Δ) mutants might be due to CYK-1 deficiency during embryogenesis. To test this, we used the cyk-1(ts) to induce acute CYK-1 loss during embryonic myogenesis. To allow identification of BWM in embryos,
we crossed into the formin mutants GFP-tagged muscle myosin II heavy chain A (GFP::MYO-3) (Campagnola et al., 2002), which is expressed in BWM.

Development of embryonic BWMs is reflected in characteristic changes in appearance of muscle components (Fig. 2-3) (Hresko, Williams, & Waterson, 1994). After gastrulation, C. elegans embryos develop through stages called pre-bean, bean, comma, 1.5-fold, 2-fold, and 3-fold, named after the changing shape of the embryo. At 290 min after first cell division, equivalent to a pre-bean stage, myoblasts appear at lateral positions and accumulate diffuse myosin. By 350 min, the comma stage, myoblasts complete migration to dorsal and ventral quadrants, and muscle components start to polarize at the cell edges towards the hypodermis (Fig. 2-3, Fig. 2-4 A). At 420 min, the 1.5-fold stage, muscle cells flatten and F-actin and myosin assemble into the contractile lattice, with a polarized accumulation at cell-cell junctions between myoblasts (Fig. 2-3). At 450 min, the 2-fold stage, functional sarcomeres have formed (Fig. 2-3), and muscle-muscle and muscle-hypodermis junctions assemble. In stages older than 2-fold, sarcomeres become more neatly organized into striations (Fig. 2-3, Fig. 2-5 A).

To examine embryonic myoblasts, we obtained pools of mixed-stage embryos by bleaching adult worms grown at permissive temperature, liberating embryos from the adult bodies. We allowed this asynchronous population to continue developing at permissive temperature until the percentage of embryos at the two-fold stage peaked in each individual pool. Embryos were maintained a further 30 min either at permissive or restrictive temperature before staining with fluorescent phalloidin.

We assigned embryos to two broad age categories: early embryonic stages encompassing comma to 1.5-fold, and late embryonic stages including 2-fold and older.
**Figure 2-3. Development of the myofilament lattice in wild-type embryonic BWM.** Maximum intensity projections (MIP) of embryos expressing GFP::MYO-3 (a BWM marker) and stained for F-actin, show BWM development at embryonic stages comma, 1.5-fold, 2-fold, older than 2-fold, and L1 larva. At comma stage, traces of F-actin appear as polarization of muscle components begins (white arrows). Polarization continues through the 1.5-fold stage, where F-actin and GFP::MYO-3 strongly accumulate (blue arrows). The first sarcomeres form just prior to the 2-fold stage with appearance of defined striations (yellow arrows). Stages older than 2-fold (older embryo, L1 larva) feature neatly aligned striations (green arrows) in BWM. Scale bars, 20 µm.
Figure 2-4. Simultaneous absence of FHOD-1 and CYK-1 delays polarization of sarcomere components in early embryonic BWMs. GFP-myosin-expressing embryos grown at permissive temperature were shifted to restrictive temperature 30 min before staining for F-actin. (A) Polarization of sarcomere components (white arrows) is normally observable in early embryos (comma to 1.5-fold stage), although not always (white arrows). Scale bar, 20 µm. (B) Quantification shows BWM in a majority of wild-type and single formin mutant embryos polarizes (normal), while polarization has not yet occurred in a majority of fhod-1(Δ); cyk-1(ts) double mutants. Data are averages of the means of three experiments ± SEM (n = 35 early or 70 late embryos, per strain per experiment). (*) indicates p < 0.01. Differences in all other pairwise comparisons were not statistically significant (p > 0.05). Also visible in (A), F-actin accumulates in nuclei (arrowheads) of embryos bearing cyk-1(ts) (see Fig. 2-S4).
Figure 2-5. Absence of FHOD-1 causes frayed F-actin striations in late embryonic BWM. (A) In later stage embryos (2-fold and older) grown at permissive temperature and shifted to restrictive temperature for 30 min, fluorescent phalloidin stain typically reveals neat F-actin-rich striations (blue arrows), but striations appear frayed (green arrows) in a subset of embryos. Scale bar, 20 µm. Insets show higher magnification of boxed areas. (B) Quantification shows higher frequency of frayed striations (and lower frequency of neat-appearing striations) correlates with fhod-1(Δ), irrespective of cyk-1. Data are averages of the means of three experiments ± SEM (n = 35 early or 70 late embryos, per strain per experiment). (*) indicates p < 0.01. Differences in all other pairwise comparisons were not statistically significant (p > 0.05).
Based on the timing of muscle development, we expect younger embryos would normally have undergone initial polarization of F-actin and myosin during the temperature shift. Consistent with this, a majority (~80%) of wild-type, fhod-1(Δ), and cyk-1(ts) early embryos had polarized BWMs. However, only 35% of fhod-1(Δ); cyk-1(ts) early embryos did so, with the remainder being nonpolarized (Fig. 2-4 A, B), indicating cyk-1 and fhod-1 make a redundant contribution to early polarization of sarcomeric components.

Surprisingly, fhod-1(Δ); cyk-1(ts) embryos maintained at permissive temperature had similar percentages of non-polarized BWM (Fig. 2-S3 A), suggesting this defect is not triggered by acute loss of CYK-1 function, but again consistent with cyk-1(ts) being partially non-functional at 16˚C.

On reaching later stages (2-fold stage and older), embryos of all strains had striations of F-actin and myosin, whether or not they were shifted to restrictive temperature, suggesting absence of polarization in early embryos was temporary. However, these F-actin-rich striations appeared frayed in some late embryos (Fig. 2-5 A). Again, the proportion varied by genotype, with roughly 75% of wild-type or cyk-1(ts) older embryos appearing normal, whereas only ~10-15% fhod-1(Δ) or fhod-1(Δ); cyk-1(ts) embryos did so, the remaining 85-90% having frayed F-actin (Fig. 2-5 B, Fig. 2-S3 B). Thus, absence of FHOD-1 but not acute loss of CYK-1 perturbs sarcomere organization in late embryogenesis.

In order to verify cyk-1(ts) was behaving as a loss of function allele under these test conditions, we also maintained populations of embryos at restrictive temperature overnight before fixation and staining with fluorescent phalloidin. We observed in some fhod-1(Δ); cyk-1(ts) L1 larvae a temperature-sensitive detachment of the pharynx from
Figure 2-S3. Embryonic cyk-1(ts) mutants at permissive temperature. Embryos expressing GFP-myosin were maintained at permissive temperature before staining with fluorescently-labeled phalloidin to observe F-actin. Graphs show percentage of embryos with normal BWMs for (A) early embryos and (B) late embryos, based on the phenotypic categorization shown in Fig. 4 and Fig. 5. Graphs depict the average of the means of three experiments ± SEM (n = 35 or 70 embryos for early and late embryos, respectively, per strain per experiment). (*) indicates p < 0.01. All other differences in pairwise comparisons were statistically insignificant (p > 0.05).
the mouth (38% of animals at 26°C, versus 13% at 16°C, n = 100 animals per condition) (Fig. 2-S4 A), a phenotype we have previously noted with fhod-1(Δ); cyk-1(Δ) mutants or after RNAi against cyk-1 on fhod-1(Δ) mutants (Mi-Mi et al., 2012). In contrast, we never observed this in wild-type or cyk-1(ts) animals (n = 100 animals per strain per condition), and very rarely in fhod-1(Δ) animals (3% of animals at 16°C, 4% at 26°C, n = 100 animals per condition). Thus, cyk-1(ts) replicated cyk-1 loss-of-function phenotypes previously observed. Unexpectedly, we also observed accumulation of F-actin in many nuclei of cyk-1(ts) and fhod-1(Δ); cyk-1(ts) embryos at permissive and restrictive conditions (Fig. 2-4 A, arrowheads, Fig. 2-S4 B, C), but the significance of this is not clear. Overall, our data suggest FHOD-1 promotes proper F-actin organization in late embryonic BWM sarcomeres, while CYK-1 is redundant with FHOD-1 in a non-essential role only during initial stages of sarcomerogenesis.

**FHOD-1 promotes BWM cell growth and proper DB organization in a cell-autonomous manner, whereas CYK-1 does not.**

Despite the fact cyk-1(Δ) mutants exhibit significant BWM cell size and DB defects, our results here suggested CYK-1 makes minimal contributions to embryonic or post-embryonic BWM sarcomere assembly. One potential explanation for this discrepancy is that CYK-1 might promote BWM development indirectly by playing a role in some other tissue that affects BWM. Further, it remained formally possible FHOD-1 also works in a similar indirect manner. Thus, we tested whether re-expression of FHOD-1 or CYK-1 could support BWM development in a muscle cell autonomous manner in fhod-1(Δ) and cyk-1(Δ) mutants, as would be expected if either directly contributes to sarcomere assembly.
Figure 2-S4. Detached pharynx and nuclear actin phenotypes observed in cyk-
I(ts) mutants. (A) Wild-type and fhod-1(Δ); cyk-I(ts) double mutant L1 larvae stained
with fluorescently-labeled phalloidin showing normal F-actin-rich pharynx and a
pharynx that has detached from the mouth (white arrow), respectively. Scale bar, 20
µm. (B) Early stage embryos of wild-type and cyk-I(ts) grown under restrictive
conditions before being stained with fluorescently-labeled phalloidin show cyk-I(ts)
embryos have F-actin-rich round bodies (yellow arrows), while no such staining is
observed in wild-type embryos. Scale bar, 20 µm. (C) Confocal images show round F-
actin bodies in cyk-I(ts) embryos are nuclei based on counter-staining with DAPI
(arrows). Scale bar, 4 µm.
We had previously demonstrated \textit{fhod-1(+)} genomic sequence with an encoded C-terminal GFP tag (denoted \textit{fhod-1::gfp}) partially restores BWM growth in \textit{fhod-1(\Delta)} animals when integrated at an exogenous genomic site (Mi-Mi et al., 2012). We had also observed genomic integration of \textit{cyk-1::gfp} derived from genomic \textit{cyk-1(+)} sequence rescues normal body size and partially rescues fertility of homozygous \textit{cyk-1(\Delta)} animals (Mi-Mi et al., 2012). We phalloidin stained age-matched wild-type, \textit{cyk-1(\Delta)}, and transgene-rescued \textit{cyk-1(\Delta)}; \textit{cyk-1::gfp} adult animals, and confirmed this exogenous \textit{cyk-1} also restores normal BWM growth to \textit{cyk-1(\Delta)} mutants (Fig. 2-S5).

To test for whether BWM rescue occurs in a cell autonomous manner, we took advantage of the ability of \textit{C. elegans} to host extrachromosomal arrays (ECAs) that tend to be inherited in a mosaic manner (Mello, Kramer, Stinchcomb, & Ambros, 1991). When plasmids are microinjected into the worm gonad, they are linearized and concatenated into ECAs. ECAs can be inherited through multiple generations of progeny, but often variably among cells within an embryo to produce mosaic animals.

To induce mosaic \textit{fhod-1} expression, we microinjected homozygous \textit{fhod-1(\Delta)} worms with plasmid bearing \textit{fhod-1(+)} genomic sequence. To avoid potential interference from tags, we utilized the original untagged gene from which the GFP-tagged version had been created (Mi-Mi et al., 2012). To facilitate identifying transgenic BWM cells in transformed animals, we co-injected a plasmid encoding free GFP expressed from the muscle-specific \textit{myo-3} promoter. Concatenation of these plasmids into a single ECA ensured BWM cells expressing free GFP had also inherited \textit{fhod-1(+)} (Fig. 2-6 A). For analysis, we selected two independent transformant lines. To generate controls for the effects of harboring an ECA, we also injected \textit{fhod-1(\Delta)} animals with a
Figure 2-S5. *cyk-1::gfp* rescues BWM width of *cyk-1(Δ)* mutants. (A) Dorsal views of adult worms stained with fluorescently-labeled phalloidin to observe F-actin. Scale bar, 20 µm. (B) Measurement of BWM widths (as shown in A, white double arrows) demonstrates normal BWM size is restored in *cyk-1(Δ)* animals with a *cyk-1::gfp* transgene integrated into the genome at an ectopic site. Shown are the means for two independent experiments ± SD (n = 11 animals for each genotype for each experiment, with 2 BWM width measurements per animal). (*) indicates p < 0.01. Differences in all other pairwise comparisons were not statistically significant (p > 0.05).
Figure 2-6. FHOD-1 plays a muscle cell autonomous role in promoting BWM cell growth whereas CYK-1 does not. Homozygous *fhod-1(Δ)* or heterozygous *cyk-1(Δ)/+* transgenic lines were selected for mosaic inheritance of extrachromosomal arrays (ECAs) containing wild-type *fhod-1* or *cyk-1*, respectively, along with a muscle-expressed *gfp* gene. Similar control transgenic lines were selected for mosaic inheritance of ECAs constructed from non-formin DNA and the GFP marker. Two independently isolated lines of each genotype were selected, together with non-transformed wild-type and mutant controls, for analysis. (A) Fluorescent phalloidin stain of a worm with mosaic GFP expression allows comparison of cell widths of transgenic GFP-positive BWM cells (black arrow) and adjacent non-transgenic GFP-negative BWM cells (white arrow). Scale bar, 50 µm. (B) For *fhod-1(Δ)* adults bearing ECAs *control#1* and *control#2* without *fhod-1(+)*, there was no significant effect of ECA presence on muscle cell size. Conversely, for *fhod-1(Δ)* adults bearing ECAs *fhod-1(+)#1* and *fhod-1(+)#2* with *fhod-1(+)*, transgenic BWM cells (green bars) were significantly wider than non-transgenic cells in the same animal (blue bars). (C) For analysis of *cyk-1*, ECA-bearing homozygous *cyk-1(Δ)* adult progeny of *cyk-1(Δ)/+* parents were examined. ECAs *cyk-1(+)#1* and *cyk-1(+)#2* with *cyk-1(+)* had no significant effect on widths of transgenic BWM cells (green bars) compared to non-transgenic cells (blue bars), and were similar in size to those in non-transformed *cyk-1(Δ)* adults. Graphs show averages ±SEM  (*n* = 30 worms for each genotype, harvested and stained over 3 independent sessions for (B) *fhod-1* mosaic analysis, or seven independent sessions for (C) *cyk-1* mosaic analysis). (*) indicates *p < 0.01*. Differences in all other pairwise comparisons were not statistically significant (*p > 0.05*).
mixture of non-formin DNA and the GFP-encoding plasmid, and isolated two transformant lines. Similarly, to induce mosaic cyk-1 expression, we injected untagged genomic cyk-1(+) or non-formin DNA, together with gfp-coding plasmid. However, as cyk-1(Δ) homozygotes are sterile, this was done into heterozygous cyk-1(Δ)/+ worms.

Synchronized adult mosaic worms were stained with phalloidin, and widths of individual adjacent GFP-positive (transgenic) muscle cells and GFP-negative (non-transgenic) muscle cells were measured (Fig. 2-6 A). As expected, there were no significant differences between GFP-positive and GFP-negative cells of control strains in either background (Fig. 2-6 B, C). However, in fhod-1(Δ) mutants with mosaic fhod-1 expression, fhod-1(+) bearing GFP-positive muscle cells were significantly wider than non-GFP neighbors (Fig. 2-6 B), demonstrating FHOD-1 can promote muscle cell growth in a cell autonomous manner.

To test whether cyk-1 similarly promotes muscle cell growth, we identified homozygous cyk-1(Δ) progeny of heterozygous cyk-1(Δ)/+ parents by the presence of protruding vulva and/or absence of embryos from the gonad, and examined BWM cells in these animals after phalloidin stain. In contrast to fhod-1, widths of GFP-positive muscle cells expressing cyk-1(+) and GFP-negative cells not expressing cyk-1(+) were the same as each other, and as in non-transformed cyk-1(Δ) worms (Fig. 2-6 C), suggesting CYK-1 cannot promote BWM growth in a muscle cell autonomous manner.

Considering these results, we wanted to determine if FHOD-1 could also rescue DB organization in a cell autonomous manner. To test this, we immunostained the same mosaic fhod-1(Δ) strains with anti-GFP and anti-ATN-1, and quantitatively analyzed the regularity of DB spacing along striations by performing fast Fourier transform (FFT) on
anti-ATN-1 intensity profiles. Consistent with a fairly regular DB spacing in wild-type animals, amplitude spectra for their DBs showed a clustering of peaks near frequencies 0.8-1.0 µm⁻¹, whereas irregularly spaced ATN-1 in fhod-1(Δ) mutants correlated with spectra with no particular favored frequency (Fig. 2-7, 2-S6). As expected, in fhod-1(Δ) strains transformed with non-formin DNA, DBs of GFP-positive (transgenic) and GFP-negative (non-transgenic) BWM cells were irregular in shape and spacing, identical to non-transgenic fhod-1(Δ) mutants, and their amplitude spectra showed no favored frequency (Fig. 2-7, Fig. 2-S6). In contrast, DBs in GFP-positive fhod-1(+)expressing cells in the two fhod-1 mosaic strains were regularly spaced, similar to wild type, and their spectra peaks clustered near 0.8-1.0 µm⁻¹ (Fig. 2-7, Fig. 2-S6). Notably, neighboring GFP-negative (non-transgenic) cells in these same animals resembled those of non-transformed fhod-1(Δ) mutants, confirming FHOD-1 promotes proper DB organization in a muscle cell autonomous manner.

**FHOD-1 is enriched near sarcomeres in growing BWM cells throughout development, whereas CYK-1 does not localize to the BWM contractile lattice.**

Our results so far indicated FHOD-1 contributes to BWM sarcomere organization throughout embryonic and postembryonic development. Previously, we had observed FHOD-1 localizes in a diffuse pattern in BWM during late embryogenesis after F-actin rich sarcomeres had assembled (older than 2-fold stage), and becomes enriched at BWM cell edges from mid larval development until early adulthood (Mi-Mi et al., 2012). Considering evidence here for FHOD-1 function during earlier embryonic muscle development, we re-examined its distribution in embryos and young larvae expressing a
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Figure 2-7. FHOD-1 promotes proper DB morphology in a cell autonomous manner. Wild-type, non-transformed \textit{fhod-1(\Delta)}), and \textit{fhod-1(\Delta)} worms with mosaic inheritance of ECAs (from Fig. 6) were immunostained for ATN-1 and GFP. As shown in maximum intensity projections (MIP) of dorsal views from age-synchronized adults, ATN-1-positive DBs are punctate and regularly spaced in wild-type BWM, or \textit{fhod-1(\Delta)} BWM cells that inherit a \textit{fhod-1(+)-containing ECA} (yellow arrows), whereas DBs appear partially dispersed and irregular in \textit{fhod-1(\Delta)} BWM cells that inherit either no ECA or an ECA lacking \textit{fhod-1(+)} (blue arrows). DB spacing was analyzed by performing fast Fourier transform (FFT) on concatenated intensity profiles of ATN-1 immunostain along approximately eight striations within single BWM cells (\(n = 10\) animals per strain, one ECA-containing BWM cell and one BWM cell lacking an ECA per animal for transgenic lines; one BWM cell for non-transformed animals). Amplitude spectra for wild-type cells or \textit{fhod-1(\Delta)} cells that inherited a \textit{fhod-1(+)-containing ECA} show clustering of peaks 0.8-1.0 \(\mu m^{-1}\), while spectra peaks did not cluster near any particular frequency for \textit{fhod-1(\Delta)} cells that inherited no ECA or an ECA lacking \textit{fhod-1(+)}. Scale bars, 5 \(\mu m\). Similar results were obtained for two additional replicate experiments (Fig. 2-S6).
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**Figure 2-S6. FHOD-1 plays a cell autonomous role in rescuing DB morphology.**

DB spacing was quantitatively analyzed for two independent sets of animals by performing FFT on concatenated intensity profiles of ATN-1 immunostain along all striations within single BWM cells (one ECA-containing BWM cell and one non-ECA-containing BWM cell per animal for transgenic lines, and one BWM cell for control strains; n = 10 animals per strain), similar to as done for Fig. 2-7. Amplitude spectra for DBs in wild-type BWM cells or BWM cells that inherited a *fhod-1(+)*-containing ECA show clustering of peaks near a frequency of 0.8-1.0 µm⁻¹, whereas amplitude spectra peaks did not cluster near any particular frequency for *fhod-1(Δ)* BWM cells that inherited no ECA, or inherited an ECA lacking *fhod-1(+).*
rescuing FHOD-1::GFP, after staining with fluorescently-labeled phalloidin to identify BWMs. Expanding on our previous results, we observed FHOD-1::GFP in BWMs of animals from embryonic 1.75 fold stage through L1 (Fig. 2-8). FHOD-1 localized diffusely in younger stage embryonic BWM when sarcomeric components start to polarize and assemble into sarcomeres, and gradually appeared more punctuate at the edges of the F-actin-rich contractile lattices from later embryogenesis through early larval development, when sarcomeres mature. Similar patterns of fluorescence were visible in live embryos and larvae expressing FHOD-1::GFP (Fig. 2-S7). Thus, FHOD-1 is present through all stages of BWM development when sarcomere assembly is occurring.

Our results here also suggest CYK-1 plays no direct role in sarcomere formation, and that strong BWM defects in cyk-1(Δ) mutants reflect indirect contributions. This was surprising in light of previous evidence CYK-1 localizes to DBs, and thus we also re-examined CYK-1 localization. We had previously observed that when expressed from multiple copies of a transgene on an ECA, CYK-1::GFP appears along striations as puncta that resembled DBs (Mi-Mi et al., 2012). However, that transgenic line lost the unstable ECA before we were able to compare the CYK-1::GFP puncta to a bona fide DB marker. We had also previously examined the homozygous cyk-1(Δ) worms bearing cyk-1::gfp integrated in the genome that partially rescued fertility and fully rescued body morphology and BWM growth (Mi-Mi et al., 2012; Fig. 2-S5). In that case, we had not observed any BWM-associated GFP fluorescence (Mi-Mi et al., 2012), but had speculated the transgene might only be partially functional based on its limited ability to rescue fertility.

To avoid these issues, we examined here GFP fluorescence in a strain in which
Figure 2-8. Distribution of FHOD-1::GFP in developing BWM. FHOD-1::GFP-expressing embryos and larvae of indicated stages were stained to visualize F-actin.

FHOD-1::GFP localizes diffusely in early (1.75-fold) embryonic BWM, with gradual development of a punctuate appearance that becomes prominent in larval (L1) BWM. Note, in addition to staining F-actin striations in muscle (arrows), phalloidin decorates F-actin bundles in the epidermis (arrowheads). Scale bars, 20 μm.
Figure 2-S7. FHOD-1::GFP in developing BWM. Live FHOD-1::GFP visible in older embryos and L1 larvae is essentially identical in appearance in BWM (white arrows) as in fixed animals shown in Fig. 2-8. Arrowheads indicate autofluorescent granules in the intestine of the non-transgenic larva. Scale bars, 20 µm.
the endogenous cyk-1 locus had been tagged with gfp using CRISPR-Cas9, resulting in a cyk-1::gfp that is functional for its essential role during embryogenesis (Davies et al., 2018). As expected, we observed CYK-1:: GFP localized in the germline similar to endogenous CYK-1 (Severson, Baillie, & Bowerman, 2002; Fig. 2-S8 A). BWM appeared grossly normal in these animals, and we did not observe CYK-1::GFP at any structure in BWM, including DBs (Fig. 2-S8 B). We also examined a strain expressing CYK-1::GFP from an independently isolated ECA (Shaye & Greenwald, 2016), and observed punctate GFP-positive structures in BWM, but these were not positioned along the muscle I-bands or with any regularity, indicating these were not DBs (Fig. 2-S8 C). Rather, these might represent aggregates that arose due to CYK-1::GFP over-expression from the ECA.

We had also previously observed anti-CYK-1 stains DBs in BWM (Mi-Mi et al., 20120). Considering our negative results here, we tested the specificity of our previous CYK-1 immunostain. To eliminate CYK-1, we performed cyk-1(RNAi) for 5 days on a strain with enhanced sensitivity to RNAi due to a mutation in the exoribonuclease gene eri-1 (Pavelec et al., 2009). As a control to verify the efficacy of cyk-1(RNAi), we also treated the strain in which endogenous cyk-1 had been tagged with gfp (Davies et al., 2018). Consistent with efficient knockdown, cyk-1(RNAi)-treated worms were sterile. Western blot analysis using the same anti-CYK-1 from our previous study showed reactive bands close to predicted molecular weights for CYK-1 (arrows) and for CYK-1::GFP (arrowheads) were eliminated from cyk-1(RNAi)-treated animals (Fig. 2-9 A). Immunostain of adults with that same antibody decorated the germline in controls (arrowheads) but not cyk-1(RNAi) animals (Fig. 2-9 B). In contrast, strong immunostain
Figure 2-S8. GFP-tagged CYK-1 localizes to the gonad but does not localize to DBs in BWM. (A) Worms with endogenous cyk-1 tagged with GFP were treated with control RNAi or cyk-1(RNAi) for 5 days. CYK-1::GFP is present in the germline in control animals but absent in cyk-1(RNAi) animals. (B, C) Maximum intensity projections (MIP) of dorsal views of phalloidin-stained adult worms expressing CYK-1::GFP (B) from the endogenous cyk-1 gene tagged with GFP or (C) from an ECA. (B) Worms where the endogenous cyk-1 gene is tagged show no CYK-1::GFP localization to DBs or any enrichment at all in BWM. (C) Worms with the ECA show punctuate CYK-1::GFP in F-actin-rich BWM, possibly due to over-expression, but no localization of CYK-1::GFP to a pattern resembling DBs. Scale bars, 10 μm.
Figure A: Western blot analysis comparing RNAi sensitive strain and CYK-1::GFP strain under different RNAi conditions. The graph shows the protein bands at various molecular weights (200 kDa, 116 kDa, 97 kDa, 66 kDa, 45 kDa, 31 kDa) and indicates the percentage of RNAi (100%, 50%, 25%, 12.5%, 100%, 50%, 25%, 50%) for each strain.

Figure B: Images comparing control and cyk-1(RNAi) conditions. The images show a significant difference in morphology and structure.

Figure C: Textured images labeled 'CYK-1'.
**Figure 2-9. cyk-1(RNAi) does not alter DB-associated CYK-1 immunostain.** (A) Western blot for CYK-1 on dilutions (expressed as percentages) of extracts from adult animals treated for control or cyk-1(RNAi). Sample loads were approximately normalized based on previous whole lane protein determined by Coomassie Brilliant Blue staining. In RNAi-sensitive cyk-1(+) animals, bands at approximately 160 kDa and 170 kDa (arrows) for CYK-1 were lost after cyk-1(RNAi) but not control RNAi for 5 days, as were bands of approximately 187 kDa and 197 kDa (arrowheads) for CYK-1::GFP from animals in which endogenous cyk-1 was tagged with gfp. Unaffected bands recognized by this polyclonal anti-CYK-1 were presumed non-specific. (B) CYK-1 immunostain decorates the germline in control animals (arrowheads) but not after cyk-1(RNAi), whereas (C) anti-CYK-1 stain of DBs is similar between control and cyk-1(RNAi) animals, suggesting DB-associated stain is non-specific. Scale bars, 10 μm.
of DBs in adult BWM using with this anti-CYK-1 (counter-stained for MYO-3 to identify BWM, not shown) was not lost in cyk-1(RNAi)-treated animals (Fig. 2-9 C). Thus, DB stain in BWM from anti-CYK-1 is likely non-specific. The nature of this DB-associated antigen is unclear, but we think it is unlikely to be another formin based on our previous inability to detect cross-reactivity of this antibody to other worm formins (Mi-Mi et al., 2012). These results suggest CYK-1 does not localize to DBs or any other sarcomeric structure in BWM, consistent with this formin playing no direct role in promoting sarcomere formation.

**Discussion**

Formin contributions to sarcomere organization during striated muscle development have been documented across various model systems, but their precise roles in this are unclear (Sanger et al., 2017). The striated body wall muscle (BWM) of *C. elegans* is a powerful system to explore this due to the worm's smaller formin complement, which avoids potential redundancy due to multiple isoforms of each formin family in vertebrates. Despite this simplicity, there is remarkable similarity between sarcomeres of BWM and vertebrate cardiac muscle (Benian & Epstein, 2011).

A compelling hypothesis has been that formins initiate thin filament assembly owing to the tendency of many formins to assemble long, unbranched actin filaments associated with tropomyosin. We aimed to test that hypothesis using *C. elegans*, starting from our initial observations that simultaneous reduction of activity of two worm formins, FHOD-1 and CYK-1, profoundly stunts BWM development (Mi-Mi et al., 2012). Worms simultaneously bearing deletion alleles fhod-1(Δ); cyk-1(Δ) produce small BWM cells with a highly reduced number of sarcomeres, a phenotype that might be
considered consistent with formins initiating thin filament assembly. However, the requirement of CYK-1 for embryonic cell divisions made necessary the maternal rescue of cyk-1(Δ) (i.e. inheritance of CYK-1 protein/mRNA from a parent), complicating this earlier analysis. Recent isolation of a conditional cyk-1(ts) (Davies et al., 2014) provided means to more fully eliminate CYK-1 activity after the critical window during embryogenesis had passed, without the complication of maternal inheritance.

Contrary to our expectations, analysis of cyk-1(ts) mutants suggested CYK-1 plays, at best, a very minor role in BWM growth during larval development. Muscle cell growth is driven by the assembly of additional sarcomeres, the majority of which occurs during larval development. Despite this, maintenance of developing cyk-1(ts) mutant larvae at a temperature where CYK-1 is non-functional caused almost no change in muscle cell growth, particularly when controlled for effects on overall body growth (Fig. 2-1). Even when cyk-1(ts) was paired with fhod-1(Δ), rates of muscle cell growth were essentially identical to larvae bearing just fhod-1(Δ).

Similarly, BWM Z-line defects of cyk-1(Δ) mutants were not recapitulated in cyk-1(ts) animals. In electron micrographs, wild-type dense bodies (DBs, the BWM Z-line analogs) appear as electron-dense finger-like projections, but many DBs in cyk-1(Δ) mutants appear deficient in electron-dense material (Mi-Mi & Pruyne, 2015). Correspondingly, DBs of wild-type animals appear as ATN-1 (α-actinin)-rich puncta by immunostain, but many regions of cyk-1(Δ) BWM lack apparent ATN-1-stained DBs (Fig. 2-2). Conversely, DBs of cyk-1(ts) mutants grown at a restrictive temperature throughout larval development appear normal (Fig. 2-2), again despite most DBs having assembled during the restrictive period. FHOD-1 absence also affects DB morphology
(discussed below), but pairing of cyk-1(ts) with fhod-1(Δ) did not produce a more severe phenotype than fhod-1(Δ), alone (Fig. 2-2).

We suspected these differences in phenotypes between cyk-1(Δ) and cyk-1(ts) mutants might be due to differences in the timing of CYK-1 loss. That is, through temperature control we restricted loss in cyk-1(ts) mutants to post-embryonic development, whereas CYK-1 loss presumably begins earlier in cyk-1(Δ) mutants with depletion of maternal product. The more severe cyk-1(Δ) effects might therefore imply that CYK-1 contributes to BWM formation earlier in development. However, using the cyk-1(ts) we were unable to find evidence for a significant CYK-1 role in embryonic BWM sarcomere formation. We did observe that compared to wild-type or single mutant strains, a larger percentage of fhod-1(Δ); cyk-1(ts) double mutant embryos were delayed in the initial accumulation of F-actin and myosin at the start of sarcomere formation (Fig. 2-4). However, as sarcomeres are present in two-fold stage fhod-1(Δ); cyk-1(ts) embryos, we reasoned this early polarization defect is probably temporary, and cyk-1(ts) had no effect on the appearance of sarcomeres in older embryos (Fig. 2-4).

One caveat to interpreting results using the cyk-1(ts) is that it is possible the temperature-sensitive CYK-1 protein does not actually lose function in BWM. For example, the mutant protein might somehow be stabilized in BWM cytoplasm, or its role in BWM may rely on a biochemical activity or protein-protein interaction distinct from those required for cytokinesis. Thus, we used the alternative approach of examining cyk-1(Δ) mutants, which should lack all FH2-dependent functions of CYK-1, and which do exhibit strong BWM defects. We confirmed genomic integration of a cyk-1 transgene fully rescues BWM defects of cyk-1(Δ) animals (Fig. 2-S5), demonstrating those defects
are specific to loss of CYK-1. However, when a similar cyk-1 transgene was inherited in a mosaic pattern in cyk-1(Δ) animals, we failed to observe cell autonomous rescue of BWM growth (Fig. 2-6 C). Even more, surprising we also saw no evidence of non-cell autonomous rescue of BWM cell growth in these mosaic cyk-1-expressing animals. That is, we might have expected all BWM cells of mosaic cyk-1 animals would be larger than in cyk-1(Δ) controls lacking the transgene. However, we observed no difference in BWM cell size between those strains (Fig. 2-6 C). We therefore suggest that cyk-1 function may be required simultaneously in many cells, which would occur only rarely in a mosaic strain, or that it may be required in a tissue where expression of non-integrated transgenes is weak, such as the germline (Mello et al., 1991).

Our overall results, together with inability to detect CYK-1 in BWM sarcomeres (Fig. 2-9, Fig. 2-S8), suggest CYK-1 plays an indirect role in BWM development. That is, CYK-1 in some other tissue(s) indirectly promotes muscle development. Based on our cyk-1(ts) observations, we suggest this function likely occurs during early embryogenesis, possibly before myoblasts begin sarcomere formation. Consistent with this possibility, CYK-1 functions in a variety of tissues other than BWM, and cyk-1(Δ) or cyk-1(RNAi)-treated animals also exhibit defects in germline, epidermis, intestine, and excretory canal (Swan et al., 1998; Mi-Mi et al., 2012; Shaye & Greenwald, 2016; Gong et al., 2018). We also observed that embryos bearing cyk-1(ts) accumulate F-actin in many nuclei (Fig. 2-4 A, Fig. 2-S4 B, C), indicating loss of CYK-1 has effects throughout the body during early embryogenesis. Thus, we suggest BWM phenotypes of cyk-1(Δ) and cyk-1(RNAi)-treated animals are a secondary consequence of some function not directly related to sarcomere formation in BWM.
In contrast, FHOD-1 seems to promote BWM development and Z-line organization directly. FHOD-1 is initially diffuse in embryonic BWM cells, when sarcomeric components begin to accumulate at the cell membrane. Once the initial sarcomeres have formed, the formin appears as puncta at BWM cell edges, and more diffusely along sarcomere I bands (Fig. 2-7), remaining so until the end of BWM growth in adulthood, when localized formin is no longer detected (Mi-Mi et al., 2012). In embryos, lack of FHOD-1 results frayed-appearing F-actin in the newly formed sarcomeres (Fig. 2-5, Fig. 2-S4), suggesting a partial defect in thin filament anchorage and organization. In larvae lacking FHOD-1, additional sarcomere assembly in BWM cells is slow, resulting in smaller BWM cells (Fig. 2-1, Fig. 2-S1) (Mi-Mi et al., 2012). In contrast to CYK-1, mosaic expression of FHOD-1 in fhod-1(Δ) mutants promotes BWM growth in a cell autonomous manner (Fig. 2-6 B). We note rescue is not complete (Fig. 2-6 B, compare wild-type to GFP-positive fhod-1), but it is unclear whether this reflects an additional non-cell autonomous effect or just an artifact of gene expression from an ECA. FHOD-1 also promotes proper DB organization in a cell autonomous manner. That is, where DBs are of irregular size and spacing in fhod-1(Δ) adults (Mi-Mi et al., 2012), mosaic FHOD-1 expression restores regular, punctate DB organization in transgenic cells (Fig. 2-7, Fig. 2-S6). These results suggest the ability of FHOD-1 to promote BWM cell growth and proper DB organization are direct effects that may be functionally linked.

We have found no evidence any other formin contributes to BWM development in worms, either from individual formin gene mutations, or from pairing of fhod-1(Δ) with mutations in each of the remaining non-cyk-1 formins (Fig. 2-S2) (Mi-Mi et al., 2012). One caveat to this is possibility of a higher degree of redundancy among formins,
with simultaneous elimination of more (or all) worm formins resulting in a stronger BWM phenotype. However, absence of even hints of synthetic defects in BWM development between fhod-1(Δ) and other non-cyk-1 formin mutations makes this unlikely. Thus, we suggest fhod-1(Δ) BWM cells lack any formin activity that contributes directly to sarcomere formation. And as fhod-1(Δ) BWM cells contain abundant thin filaments, we think it unlikely that thin filament assembly in C. elegans BWM requires formins. It is possible this differs in vertebrate or fly muscle, and that formins assemble their thin filaments. However, vertebrate muscle contains non-formin actin nucleating factors that might initiate thin filament assembly, including leiomodins (Chereau et al., 2008; Yuen et al., 2014; Boczkowska, Rebowski, Kremneva, Lappalainen, & Dominguez, 2015) and a complex of N-WASP with nebulin (Takano et al., 2010). Worms lack unambiguous leiomodin and nebulin homologs, but they host related proteins, and other unknown actin nucleation factors could initiate their thin filament assembly.

A novel result from our study is that formins have only a modest effect on muscle development in the worm. Loss of DAAM-family formins significantly perturbs sarcomere organization in mammalian and insect muscle (Molnár et al., 2014; Ajima et al., 2015), but worm DAAM-1 appears dispensable (Fig. 2-S2). Absence of FHOD-1, the only C. elegans representative of FHOD-family, results in muscle defects much milder than the well-studied impacts of FHOD-family members in other systems (Taniguchi et al., 2009; Iskratsch et al., 2010; Kan-O et al., 2012; Shwartz et al., 2016; Ushijima et al., 2018; Fenix et al., 2018). For example, where fhod-1(Δ) worms exhibit reduced sarcomere assembly and partially defective Z-lines/DBs, knockdown of FHOD3 in
human induced pluripotent stem cell-derived cardiomyocytes blocks the maturation of stress fiber-like structures into sarcomere-containing myofibrils (Fenix et al., 2018), phenotypes that appear to recapitulate those of the fhod3−/− mouse heart (Kan-O et al., 2012). Moreover, where fhod-1(Δ) BWM is largely functional and mutant worms are fully viable, mice lacking FHOD3 die during embryonic development due to heart failure (Kan-O et al., 2012).

It is not immediately clear why worm muscle should be so resilient to formin loss as compared to other organisms. One possible explanation is the unique architecture of its contractile machinery. Sarcomeres in mammalian and Drosophila striated muscles organize into myofibrils, most of which are suspended in the cytoplasm away from the plasma membrane. Conversely, all C. elegans BWM sarcomeres are directly anchored to the plasma membrane and the underlying extracellular matrix through integrin-based adhesions. This likely provides significant mechanical reinforcement. This might, for example, permit DBs in worm muscle to tolerate structural defects that would be catastrophic for Z-lines in myofibrils. The mechanisms for precisely how mammalian FHOD3 or worm FHOD-1 promotes sarcomere formation are not understood, but the relative resilience of worm muscle to loss of its FHOD-family formin may prove to be an advantage in dissecting details of this process.

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Chapter 3: FHOD-1 promotes striation growth and supports dense body integrity against muscle contraction
Chapter 3 contributions

Sumana Sundaramurthy developed, designed, performed all experiments not mentioned below, analyzed the data, and wrote Chapter 3. David Pruyne oversaw research, provided input with experimental design and performed pluronic gel burrowing assay. Leila Lesanpezeshki performed muscle strength measurements using the NemaFlex platform. Ryan Littlefield created and provided the transgenic strain expressing ATN-1::mCh. Meaghan Geary counted ATN-1 striations in drug treatment experiments and Curtis Yingling counted non-parallel MYO-3 striations.
Abstract

FHOD-family formins are well known for their involvement in striated muscle development. In this study, we are investigating how the only FHOD-family formin in *C. elegans*, FHOD-1, regulates dense body (DB) assembly and function. In wild-type animals, DBs are punctate and neatly arranged in rows that appear as striations whereas loss of FHOD-1 leads to dispersed DBs. We found an increase in the accumulation of non-parallel striations in *fhod-1(Δ)* mutants. FHOD-1 was previously known to be enriched at the body wall muscle (BWM) cell edges. We observed FHOD-1 near growing striations and also at the intersection of these non-parallel striations suggesting that FHOD-1 regulated the formation or orientation of these DB striations. We also found that loss of FHOD-1 led to disruption in normal DB shape and the DBs in *fhod-1(Δ)* mutants were fragile and lacked any structural integrity to withstand prolonged contractions. Treatment of *fhod-1(Δ)* mutants with a contractility-enhancing drug exacerbated the DB phenotype. The presence of FHOD-1 around the DBs suggests that it supports the structural integrity of the DB and helps it withstand extreme contractile forces.
**Introduction**

Striated muscles are one of the most specialized cell types and they have a highly organized cytoskeleton network, which helps in the contraction and relaxation of muscles (Henderson, Gomez, Novak, Mi-Mi, & Gregorio, 2017). Contraction and relaxation occur due to the sliding of the thick and thin filaments within a basic contractile unit called sarcomere (Clark, McElhinny, Beckerle, & Gregorio, 2002). The thick filaments consist of bundles of bipolar myosin filaments, anchored at the M-line and they are arranged to interdigitate between thin filaments. The filamentous actin (F-actin) based thin filaments are also associated with tropomyosin and troponin and are anchored at the sarcomere border by Z-lines. Vertebrate muscles appear cross-striated where sarcomeres are arranged at an angle of 90° with respect to the longitudinal axis of the muscle cell, whereas in the striated muscles like that of *Caenorhabditis elegans*, sarcomeres are arranged at an angle of 6° with respect to the longitudinal axis of the cell, making them appear obliquely striated (Altun & Hall, 2009). In vertebrate muscles, sarcomeres are densely packed in organelles called myofibrils that are suspended in the cytoplasm of the muscle cell, and simple invertebrate systems like the body wall muscles (BWMs) of *C. elegans*, sarcomeres are arranged just below the plasma membrane of the BWM cells (Altun & Hall, 2005; Clark et al., 2002). Despite such contrasts, the muscle cytoskeletons of vertebrates and invertebrates are highly conserved with respect to homology among their sarcomeric proteins (Altun & Hall, 2005).

Formins belong to a highly conserved family of proteins and they are regulators of actin and microtubule networks in cells (Chesarone, DuPage, & Goode, 2010). Formins promote assembly and elongation of unbranched actin filaments via their two
conserved domains, Formin Homology (FH) domains, FH1 and FH2. Nucleation of actin filaments occurs via FH2 dimers, which remain associated with the growing barbed end of the actin filament (Kovar & Pollard, 2004; Moseley et al., 2004; Pring, Evangelista, Boone, Yang, & Zigmond, 2003; Pruyne et al., 2002; Sagot, Rodal, Moseley, Goode, & Pellman, 2002). The FH1 domains recruit profilin bound actin to further supplement the function of FH2 and increase the rate of filament elongation at the barbed ends (Chang, Drubin, & Nurse, 1997; Evangelista et al., 1997; Imamura et al., 1997; Kovar, Harris, Mahaffy, Higgs, & Pollard, 2006; Romero et al., 2004). Based on sequence homology, formins are grouped into nine families in the animal kingdom (Higgs & Peterson, 2005; Pruyne, 2016).

Among the nine formin families, the FHOD-family formins have been extensively studied for their role in striated muscle development. In mammals, there are two FHOD-family formins, FHOD1 and FHOD3. Functional variants of FHOD3 have been associated with the occurrence of hypertrophic and dilated cardiomyopathies (Arimura et al., 2013; Hayashi et al., 2018; Ochoa et al., 2020, 2018; Wooten et al., 2013). The first experiments which showed that FHOD-family formins regulate striated muscle development were done in cultured mouse and rat cardiomyocytes (Taniguchi et al., 2009; Iskratsch et al., 2010; Rosado et al., 2014). Knockout of FHOD3 exhibited lethality at embryonic day 11.5 in mice, due to malformed cardiac myofibrils with irregular and immature Z-lines (Kan-O et al., 2012). This critical role of FHOD3 during embryonic myofibrillogenesis was found to be dependent on its ability to interact with actin (Fujimoto et al., 2016; Kan-O et al., 2012). FHOD3 loss in adult mice showed that FHOD3 supports cardiac function in both normal and pathological conditions like
cardiomyopathies (Iskratsch et al., 2010; Ushijima et al., 2018). Studies using human induced pluripotent stem cell-derived cardiomyocytes and indirect flight muscles of *Drosophila* also suggest that FHOD-family formins regulate striated muscle development by affecting myofibrillogenesis (Fenix et al., 2018; Rosado et al., 2014; Shwartz, Dhanyasi, Schejter, & Shilo, 2016). Our lab has also shown that FHOD-1, the only FHOD-family formin in *C. elegans*, promotes striated muscle development (Mi-Mi & Pruyn, 2015; Mi-Mi et al., 2012; Sundaramurthy, Votra, Laszlo, Davies, & Pruyn, 2020). All these studies provide evidence that suggests FHOD-family formins regulate sarcomere assembly. Even though roles for FHOD-family formins in regulating striated muscle development seem to be conserved across species, their mechanism of action during sarcomere assembly is unclear. We will use the BWMS of the simple worm to understand how FHOD-1 regulates muscle development.

In *C. elegans*, the first sarcomeres are assembled at mid-embryogenesis during the two-fold embryonic stage, immediately after which the embryo starts twitching inside the eggshell (Altun & Hall, 2009; Hresko, Williams, & Waterson, 1990). A newly-hatched L1 larva has 2 sarcomeres per BWM cell, where their thick filaments are about 5 µm long (Moerman & Williams, 2006). The sarcomeres grow in width in due course of development and the sarcomeres in an adult worm have thick filaments that are about 10 µm long. Previously, we have shown that *fhod-1* mutants have thin BWMS with fewer sarcomeres and characteristic disorganized Z-lines that appear irregularly spaced (Mi-Mi & Pruyn, 2015; Mi-Mi et al., 2012; Sundaramurthy et al., 2020). We have also shown that FHOD-1 promotes BWM growth and Z-line development in a cell autonomous
manner, where re-expressing \textit{fhod-1} back into mutant BWMs led to the rescue BWM cell width and Z-line organization (Sundaramurthy et al., 2020).

In \textit{C. elegans}, dense bodies (DBs) serve as both Z-lines and costameres (Lecroisey, Ségalat, & Gieseler, 2007). The main role of DBs is to act as structures of thin filament anchorage that help in the efficient transduction of force from sarcomere contractions to the hypodermis to create effective locomotion. Computationally, DBs are integrin-based adhesion structures and are analogous to vertebrate focal adhesions complexes (Moerman & Williams, 2006). DBs project inwards into the cell body from the plasma membrane of BWM cells. \(\alpha/\beta\)-integrins (PAT-2/3) interact with the ECM and link other DB proteins like vinculin (DEB-1) and integrin-linked kinase/ILK (PAT-4) to the plasma membrane. Thin filaments project outwards from the DB and are anchored by \(\alpha\)-actinin (ATN-1). In vertebrates, sarcomere assembly begins at the spreading edge of the muscle cell with the formation of integrin-based adhesion structures (Z-bodies) associated with stress fiber-like premyofibrils (Dabiri, Turnacioglu, Sanger, & Sanger, 1997; Fenix et al., 2018; Sanger et al., 2005, 2017). In \textit{C. elegans}, as myoblasts start to form, first there is an accumulation of structural components like vinculin (DEB-1), integrin (PAT-2/3), myosin A and B (Hresko, Williams, & Waterson, 1994). The first steps in sarcomere assembly are dependent on integrins, which are found at the base of the DBs. Interestingly, FHOD-1 can be detected next to the dense body, partially overlapping ATN-1 and DEB-1, and is enriched in growing BWMs as bright bodies at the BWM cell edges, which fade away towards the internal DB striations (Mi-Mi et al., 2012; Sundaramurthy et al., 2020), suggesting that they could play a role in DB assembly.
In this study, we examined the effects due to the loss of FHOD-1 on the organization, distribution, assembly, and morphology of DBs. The creation of strains expressing fluorescently-tagged DB proteins, live imaging of immobilized worms, and paralytic treatment of animals allowed us to investigate the above characteristics. We also performed robust assays to test muscle function and examined DB striation length, distribution spacing, and morphology in wild-type and fhod-1(Δ) animals. We also found that DBs in fhod-1(Δ) animals lost their structural integrity against muscle contraction and discovered populations of FHOD-1 in BWMs that might be functionally distinct.

Materials and Methods

Worms and growth conditions

Worm strains were maintained on NGM plates with OP50-1 bacterial lawns for food, using standard protocols (Brenner, 1974). Age-synchronized populations of worms were obtained by one of two methods. For most experiments, adult worms were treated with a 1:2 ratio 5 M NaOH to reagent grade bleach to liberate embryos, which were then washed with M9 medium (Ausubel et al., 2004) and allowed to hatch into starvation-arrested L1 stage larvae. These were then introduced to food to allow them to develop synchronously. Alternatively, semi-synchronized progeny were obtained by allowing gravid adults to lay eggs on OP50-1/NGM plates for 4-6 hr and the adults were later removed.

Young adults and day 3 adults used for strength measurements were semi-synchronized population obtained using egg lays and they were collected at ~62 hr and ~134 hr, respectively, after egg-lay. For DB analysis, animals were age synchronized by bleaching and collected at the following time points. L1 stage larvae were hatched from
embryos incubated in M9 at 20°C. L2 larvae were collected after ~12 hr on NGM/OP50-1 plates, L4 larvae were collected after 44-46 hr, day 1 old adults after ~66 hr, day 3 adults after ~114 hr, and day 4 adults after ~114 hr. For complete genotypes of strains used in this chapter, see Table 3-1.

**Pluronic gel burrowing assay**

Pluronic gel burrowing assay was performed as described (Lesanpezeshki et al., 2019). Briefly, 26% (w/v) pluronic F-127 (Sigma-Aldrich, St. Louis, MO) suspension was made by dissolving in water at 4 °C overnight. The suspension was transferred to 14°C before conducting the experiment, to prevent gelation. 20–30 μL of suspension was added to a 35 mm culture dish and ~30 adult worms were added into the drop of suspension using a pick. The initial drop was allowed to solidify at room temperature, followed by the addition of pluronic suspension to a thickness of 0.76 cm. After ~10 min at room temperature, 20 μL of 10% OP50-1 was added to the top of the gel that had completely solidified. The number of animals that reached the surface of the gel was counted every 20 min for a total duration of 2 hr. The experiment was performed in triplicate.

**Muscle strength measurements using NemaFlex platform**

Muscle strength measurements were conducted using the NemaFlex platform as previously described (Hewitt et al., 2018; Rahman et al., 2018). Briefly, at least 30 animals were loaded individually into polydimethylsiloxane microfluidic chambers filled with M9 buffer, where they crawled through free-end deflectable micropillars with diameter, gap and height of 44 μm, 71 μm and 87 μm, respectively. The animals were imaged for 1 min at 5 fps at 20 ± 1°C using a Nikon Ti-E microscope with an Andor Zyla sCMOS 5.5 camera. Movies were analyzed using a custom-built image processing
Table 3-1: C. elegans strains used.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype</th>
<th>Source¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>DWP3</td>
<td>qaIs8001[fhod-1::gfp mini-unc-119(+)]</td>
<td>Mi-Mi et al., 2012</td>
</tr>
<tr>
<td>DWP10</td>
<td>fhod-1(tm2363) I; qaIs8001[fhod-1::gfp mini-unc-119(+)]</td>
<td>Mi-Mi et al., 2012</td>
</tr>
<tr>
<td>DWP20</td>
<td>fhod-1(tm2363) I; rhIs2[pat-3::HA::gfp]</td>
<td>Mi-Mi et al., 2012</td>
</tr>
<tr>
<td>DWP228</td>
<td>fhod-1(tm2363) I; atn-1(ftw35 atm-1::mCherry::ICR::GFPnls) V</td>
<td>RSL62 x XA8001</td>
</tr>
<tr>
<td>DWP229</td>
<td>rhIs2[pat-3::HA::gfp]; atm-1(ftw35 atm-1::mCherry::ICR::GFPnls) V</td>
<td>RSL62 x rhIs2</td>
</tr>
<tr>
<td>DWP230</td>
<td>fhod-1(tm2363) I; rhIs2[pat-3::HA::gfp]; atm-1(ftw35 atm-1::mCherry::ICR::GFPnls) V</td>
<td>DWP20 x DWP228</td>
</tr>
<tr>
<td>DWP231</td>
<td>qaIs8001[fhod-1::gfp mini-unc-119(+)];atn-1(ftw35 atm-1::mCherry::ICR::GFPnls) V</td>
<td>DWP3 x RSL62</td>
</tr>
<tr>
<td>RSL62</td>
<td>atm-1(ftw35 atm-1::mCherry::ICR::GFPnls) V</td>
<td>Ryan Littlefield</td>
</tr>
<tr>
<td>rhIs2</td>
<td>rhIs2[pat-3::HA::gfp]</td>
<td>Mi-Mi et al., 2012</td>
</tr>
<tr>
<td>N2</td>
<td>wild type</td>
<td>CGC²</td>
</tr>
<tr>
<td>XA8001</td>
<td>fhod-1(tm2363) I</td>
<td>Mi-Mi et al., 2012</td>
</tr>
</tbody>
</table>

¹Reference for initial isolation, or parental strains for crossing.

²Caenorhabditis Genetic Center (University of Minnesota, Minneapolis, MN)
software (MATLAB, R2016a; [https://github.com/VanapalliLabs/NemaFlex](https://github.com/VanapalliLabs/NemaFlex)). The muscle strength was calculated based on the maximum pillar deflection identified in each frame, using the Timoshenko theory for an elastic rod (Rahman et al., 2018). The maximum exertable force, $f_{95}$, was calculated as the 95th percentile of all maximal deflections for each animal and reported as muscle strength by averaging over the population tested.

**Drug treatments**

OP50-1 bacterial suspension was mixed with aqueous solutions of levamisole or muscimol to achieve a desired final concentration of the drug, 2.5 mM levamisole, or 10 mM muscimol (Brouilly et al., 2015). About ~0.25 mL of the mixture was uniformly spread on 35 mm culture plates and allowed to dry for 3-4 hours. Semi-synchronized L4 larvae were gently washed using M9 and seeded onto plates that had either 2.5 mM levamisole, 10 mM muscimol, or no drug (as control), and maintained for 24 hr at 20 °C before preparation for fluorescence microscopy.

**Staining for fluorescence microscopy**

F-actin stain for fluorescence microscopy was performed as previously described (Mi-Mi et al., 2012). Worms were immunostained for fluorescence microscopy as previously described (Sundaramurthy et al., 2020). Monoclonal primary antibody MH35 (anti-ATN-1) generated by Francis and Waterston (1985) was a gift from Pamela Hoppe (Western Michigan University, Kalamazoo, MI). Monoclonal antibody 5-6-s (anti-MYO3) generated by H.F. Epstein (Baylor College of Medicine, Houston, TX) was obtained through the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Secondary antibodies, FITC-conjugated goat anti-mouse and Texas red-conjugated goat anti-mouse were commercially obtained (Rockland Immunochemicals, Rockland Immunochemicals, Rockland Immunochemicals, Rockland Immunochemicals).
Pottstown, PA). Antibody dilutions used were 1:10⁴ MH35 (mouse), 1:10³ 5-6-s (mouse) and 1:500 for secondary antibodies.

**Fluorescence microscopy and image analysis**

Wide-field fluorescence images were acquired using an Eclipse 90i upright microscope (Nikon, Tokyo, Japan) with a CFI Plan Apochromat 40X/NA 1.0 oil immersion objective or CFI Plan Apochromat violet corrected 60x/NA 1.4 oil immersion objective with a Cool-SNAP HA2 digital monochrome charge-coupled device camera (Photometrics, Tuscon, AZ) at room temperature, driven by NIS-Elements AR acquisition and analysis software (version 3.1; Nikon, Tokyo, Japan). Images in Figure 3-5S2 were acquired using the wide-field microscope and images in other figures were acquired using the confocal microscope.

Confocal images were acquired using an SP8 Laser Scanning Confocal Microscope (Leica, Wetzlar, Germany) driven by LAS X Software (version 3.5.2, build 4758; Leica), and using an HCX Plan Apochromat ×63/NA 1.4 oil lambda objective. Confocal z-stacks of BWMs were collected at 0.1 µm intervals before deconvolution using Huygens Essential software (Huygens compute engine 18.10.0, Scientific Volume Imaging B.V.), Classic Maximum Likelihood Estimation deconvolution algorithm, with 40 iterations and a Signal/Noise ratio of 20. Maximum Intensity Projections (MIPs) were generated from deconvolved confocal z-stacks using LAS X Software or ImageJ (version 2.0.0-rc-65/1.51 g) (Schneider, Rasband, & Eliceiri, 2012). Images were linearly processed to enhance contrast and false-colored in Adobe Photoshop (version, 22.4.3/10, Adobe, San Jose, CA). Deconvolved confocal z-stacks were also used to construct 3D renderings using Imaris x64 software (version 9.2.1, Bitplane AG, Belfast, UK).
To image DB assembly in the BWMs of live animals, L4 larvae were immobilized on 3.5 µL polystyrene bead suspension (Polysciences, 2.5% by volume, 0.1 µm diameter), sandwiched between a coverslip and 10% agarose pad (w/v). Images were acquired every 10-30 min for about 90 min using an SP8 Laser Scanning Confocal Microscope (as described above).

For 3D form factor values, single DBs (4 DBs/animal) were cropped from the deconvolved z-stacks of BWMs expressing fluorescently-labeled DB proteins, PAT-3::GFP and ATN-1::mCh. 3D shape parameter called the 3D form factor was obtained using the 3D shape plugin (v 2.0) (Sheets et al., 2013) installed in ImageJ.

**Fast Fourier transformations**

ATN-1::mCh fluorescence intensity profiles were obtained for approximately 8 DB-containing striations in one muscle cell (or two muscle cells for L1 larvae) for 10 animals per strain and developmental stage using a four pixel-wide free-hand Line tool in ImageJ. FFT was performed as previously described (Sundaramurthy et al., 2020) and amplitude spectra were obtained using MATLAB (R2019a Update 2).

**Statistical analysis**

Data are expressed as mean ± SEM and graphs were made in Excel: Windows (version 21H1; Microsoft Corporation, Redmond, WA). Statistical analysis was performed using Wilcoxon rank-sum test for muscle strength measurements and for other results where two groups are compared, data were analyzed using a student t-test. $p > 0.05$ was considered not statistically significant.
Results

*fhod-1* mutants have defective locomotion and low muscle strength as young adults

Worm BWMs are essential for locomotion. Previously, we observed that while *fhod-1* mutants have dispersed DBs and small BWMs, they did not show any gross movement defects via standard crawling and thrashing assays (Mi-Mi et al., 2012). Studies have shown that stimulating animals to burrow through a dense three-dimensional medium similar to that of the worm’s natural environment, would challenge them to burrow and mimics conditions that could reveal their true capacity and their characteristics representative of their actual behavior (Frézal & Félix, 2015; Lesanpezeshki et al., 2019). We wanted to characterize the burrowing characteristics of *fhod-1(Δ)* mutants using the pluronic gel burrowing assay. Our data showed that *fhod-1(Δ)* adult worms took longer to burrow through a 26% (w/v) pluronic gel towards a food source, compared to their wild-type counterparts, suggesting that loss of FHOD-1 causes a defect in burrowing (Figure 3-1A).

To further examine the defect in burrowing, we performed muscle strength measurements using the NemaFlex platform, a micropillar-based force measurement system (Rahman et al., 2018). *fhod-1(Δ)* young adult worms were ~ 29% weaker than their wild-type counterparts (Figure 3-1B). As the effects of dystrophin loss on muscle strength in worms could be detected only from animals that had been adults for 3 days (day 3 adults) (Hewitt et al., 2018), we further wanted to investigate if the muscle strength in *fhod-1(Δ)* worms worsen as worms age. However, *fhod-1(Δ)* day 3 adults were not significantly weaker than wild-type day 3 adults (Figure 3-1B). This suggests that muscle strength in *fhod-1(Δ)* worms catch up to that of their wild-type counterparts.
Figure 3-1. Worms deficient in FHOD-1 have low muscle strength as young adults, but their muscle strength catches up as day 3 adults. (A) wild-type and fhod-1(Δ) adult worms were induced to burrow through 26% (w/v) pluronic gel via chemotaxis towards an E. coli food source, and the number of animals that crawled to the top of the gel were counted. fhod-1(Δ) worms took longer to burrow than their wild-type counterparts. Shown are the averages of the means of three experiments (n= 30 animals per strain, per experiment). (B) Young adult and day 3 adult wild-type and fhod-1(Δ) worms (n= 30 animals per strain) were subjected to strength measurements using the NemaFlex platform. Young adult but not day 3 adult fhod-1(Δ) worms were significantly weaker than their wild-type counterparts. Error bars indicate SEM. * p < 0.05, ** p < 0.01, n.s. not significant.
as day 3 adults and does not worsen in due course of development. This led us to investigate whether there are any structural changes that correlate with the improvement in muscle strength we observe between \textit{fhod-1(\Delta)} young and day 3 adults.

**Increase in \textit{fhod-1(\Delta)} BWM size partially correlates with the improvement in muscle strength**

Our previous work showed that FHOD-1 promotes BWM growth during postembryonic development (Sundaramurthy et al., 2020). To test how the loss of FHOD-1 affects muscle strength during adulthood, we first measured BWM widths and BWM cell widths (Figure 3-2). Worms were collected as L1 larvae, L4 larvae, day 1 adults, day 3 adults, and day 4 adults. These worms were stained with fluorescently labeled phalloidin to visualize filamentous actin (F-actin). As noted previously (Mi-Mi et al., 2012; Sundaramurthy et al., 2020), BWMs and BWM cells of wild-type and \textit{fhod-1(\Delta)} L1 larvae have similar widths. The BWM and BWM cell growth decrease significantly after day 3 of adulthood. However, in all the other developmental stages the BWM and cell widths of \textit{fhod-1(\Delta)} animals are significantly smaller than their wild-type counterparts (Figure 3-2B). Similarly, the BWM cell widths of \textit{fhod-1(\Delta)} animals are also significantly smaller than their wild-type counterparts (Figure 3-2C). However, the differences between \textit{fhod-1(\Delta)} and wild-type animals tend to get smaller as the animals age. The young adults used to measure muscle strength (Figure 3-1) correspond to an age between L4 larval stage and day 1 adults measured here (Figure 3-2). Thus, the increases in \textit{fhod-1(\Delta)} BWM and BWM cell widths with respect to wild-type partially correlate to the improvement in muscle strength between young adults and Day 3 adults (Figure 3-1). This partial but incomplete correlation led us to examine other structural components of
Figure 3-2. Increase in fhod-1(Δ) BWM size partially correlates with the increase in muscle strength. Worms were stained with fluorescently labeled phalloidin to show F-actin. (A) Dorsal views of L1 larvae and day 1 adults of wild-type and fhod-1(Δ) worms showing BWMs. Scale bars, 6 μm. (B) BWM widths (dashed double arrows in A) and (C) individual BWM cell widths (double arrows in A) were measured. The BWM and BWM cell widths of wild-type and fhod-1(Δ) are similar as L1 larvae. However, in all the other developmental stages (L4 larvae, day 1 adults, day 3 adults, and day 4 adults) the BWM widths and cell widths of fhod-1(Δ) are smaller than their wild-type counterparts. The increase in fhod-1(Δ) BWM and BWM cell widths with respect to wild-type, between L4 larval stage to day 3 adult stages partially correlates with the increase in muscle strength between fhod-1(Δ) young adults and day 3 adults (Figure 3-1). Shown are average of two experiments (n = 10 animals per strain per experiment, two BWMs, and four BWM cells per animal) with SEM error bars. * p < 0.05, ** p < 0.01, *** p < 0.001.
the BWM contractile lattice besides the F-actin rich thin filaments. Investigating other structural components provided us with clues as to why muscle strength improves in day 3 \( fhod-1(\Delta) \) adults.

**DB arrangement in larval and adult worms**

Our previous work had revealed the dispersed structure of \( fhod-1(\Delta) \) DBs (Mi-Mi et al., 2012; Mi-Mi & Pruyne, 2015). The dispersed structure and DB spacing were cell-autonomously rescued in ~ day 1 \( fhod-1(\Delta) \) adults re-expressing full-length \( fhod-1 \) in a mosaic manner (Sundaramurthy et al., 2020). Characterizing DB changes in all stages of development could help us understand why the DBs appear dispersed in \( fhod-1(\Delta) \) mutants and also understand why muscle strength improves in day 3 \( fhod-1(\Delta) \) adults.

The recruitment of DB proteins during sarcomere assembly is based on interdependence on one another (Hresko et al., 1990; Moerman & Williams, 2006). Examining loss of function mutants have led us to a proposed order of assembly among DB proteins as follows: \( \alpha/\beta \)-integrin (PAT-2/3), vinculin (DEB-1), UNC-112, integrin-linked kinase (PAT-4), \( \alpha \)-actinin (ATN-1) (Hresko et al., 1990; Gieseler et al., 2016; Moerman & Williams, 2006). Based on this order of assembly, we selected an early-assembling and a late-assembling DB marker, namely PAT-3 and ATN-1.

We used strains that expressed fluorescently-labeled DB proteins, PAT-3::GFP (Plenefisch et al., 2000) and ATN-1::Ch to visualize proteins in BWMs. BWMs have three integrin-based adhesion structures: DBs, M-line and attachment plaques. The M-lines are anchored to the plasma membrane in *C. elegans*, and the attachment plaques appear in between two adjacent BWM cells and anchor half I-bands. Age synchronized \( fhod-1 \) wild-type (DWP229) and \( fhod-1(\Delta) \) (DWP230) worms were fixed and imaged.
PAT-3 occurs at DBs (arrows), along M-lines (M, arrowheads), and at attachment plaques (A) (Figure 3-3A) (Plenefisch et al., 2000). ATN-1 occurs only in a subset of DBs and is absent along M-lines and attachment plaques (Moulder et al., 2010). DBs are arranged in rows that correspond to the striations of BWM (Figure 3-3). In general, all DBs have PAT-3 but only a subset of DBs contain ATN-1.

Each BWM cell in a *wild-type* L1 larvae has a single row of ATN-1 decorated DBs and five rows of PAT-3 rich structures. This includes two rows of M-lines, two rows of attachment plaques containing some PAT-3 only DBs and one row of DBs that contain both PAT-3 only and PAT-3 plus ATN-1. The rows of attachment plaques containing few DBs appear at the outer-most edges of the BWM cells, followed by two rows of M-lines and the row of DBs are located right at the middle of the BWM cell. However, in *fhod-1*\((\Delta)\) L1 larvae, there are occurrences of non-parallel and multiple DB rows (arrowheads) within a single cell (Figure 3-3B). *Wild-type* L4 larvae BWMs have multiple rows of DBs that are parallel, neatly aligned where each DB appears as a single distinct puncta. However, the BWM cells of *fhod-1*\((\Delta)\) L4 larvae have, on average, one row of DBs that is not parallel to the other striations (Fig.3-3, arrowhead), and most PAT-3 and ATN-1 containing DBs in *fhod-1*\((\Delta)\) animals appear partially dispersed (Fig.3-3, arrows) when compared to their *wild-type* counterparts.

*Wild-type* day 1 adult and day 4 BWMs have multiple rows of DBs similar to L4 larvae (Figure 3-4), parallel and neatly aligned, where each DB appears as a single distinct puncta. Most PAT-3 and ATN-1 containing DBs in adult *fhod-1*\((\Delta)\) animals appear dispersed (Figure 3-4, arrows) when compared to their *wild-type* counterparts. However, there is an increase in the size of DBs, M-lines, and attachment plaques due to
Figure 3-3. **DB organization in wild-type and fhod-1(Δ) larvae.** Maximum intensity projections (MIPs) of BWM contractile lattice from larvae expressing PAT-3::GFP and ATN-1::mCh. (A) The outline of two BWM cells (cell 1 and cell 2) are shown (dashed lines). PAT-3 occurs at DBs (arrows), along M-lines (M, arrowheads), and at attachment plaques (A). ATN-1 occurs only in DBs. (B) BWM cells in wild-type animals have DBs that appear as distinct puncta and are arranged in striations that are parallel. But fhod-1(Δ) BWM cells have DBs that appear partially dispersed (arrows) and there are regions where DB rows appear non-parallel (arrow heads). Scale bars, 6 μm.
<table>
<thead>
<tr>
<th>Day 4 embryos</th>
<th>wild-type</th>
<th>PAT-3::GFP</th>
<th>ATN-1::mCh</th>
<th>PAT-3::GFP/ATN-1::mCh</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 4 adults</td>
<td>wild-type</td>
<td>PAT-3::GFP</td>
<td>ATN-1::mCh</td>
<td>PAT-3::GFP/ATN-1::mCh</td>
</tr>
<tr>
<td>Dros 1/1</td>
<td></td>
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Figure 3-4. DB organization in wild-type and fhod-1(Δ) adults. MIPs of BWM contractile lattice from adults expressing PAT-3::GFP and ATN-1::mCh. Wild-type BWMs have multiple rows of DBs, parallel and neatly aligned, where each DB appear as a single distinct punctate. Most PAT-3 and ATN-1 rich structures in fhod-1(Δ) animals appear dispersed (arrows) when compared to their wild-type counterparts and they appear worse as the worms develops into day 4 adults when compared to day 1 adults. Scale bars, 6 μm.
accumulation of either PAT-3 or PAT-3 and ATN-1 through- out BWM development as day 4 adults (Figure 3-4). Although the \textit{fhod-1(Δ)} day 4 adults have more rows of DBs in comparison to \textit{fhod-1(Δ)} day 1 adult, there are more occurrences of non-parallel striations when compared to day 1 adults (Figure 3-4, arrowheads). The dispersed structures in \textit{fhod-1(Δ)} animals appear worse as the worms develop into day 4 adults from day 1 adults. The appearance of non-parallel striations and dispersed DBs in \textit{fhod-1(Δ)} animals suggest that an in-depth analysis of temporal distribution, organization, and morphology of DBs, could help us understand how changes in \textit{fhod-1(Δ)} DBs might correlate to the observed improvement in muscle strength (Figure 3-1B).

\textit{fhod-1(Δ)} worms have fewer DB striations that are shorter, and they appear more non-parallel than wild-type

Our data show that \textit{fhod-1(Δ)} animals have fewer striations in the early stages of development (except L1 larvae), but the number of striations catches up with the wild-type as day-4 adults (Figure 3-5B). Our data also show that \textit{fhod-1(Δ)} worms had shorter striations compared to wild-type (Figure 3-S1). During the early larval stage, \textit{fhod-1(Δ)} animals have a high number of shorter striations, which slightly overlap with striation lengths of wild-type counterparts. This is apparent in L1 larvae as the frequency distribution of lengths appears as two distinct peaks (Figure 3-S1). But as the animals develop, the striation length in \textit{fhod-1(Δ)} mutants catch up with wild-type lengths, creating partial overlapping frequency distributions. Our data indicate that the distribution of \textit{fhod-1(Δ)} mutants skew towards shorter lengths and that of wild-type skew towards the longer lengths (except in day 1 adults where the peaks seem to mostly overlap, Figure 3-S1).
Figure 3-5. BWMs of FHOD-1 deficient worms have fewer DB striations than wild-type worms and many are non-parallel. (A) MIPs of BWM contractile lattice from larvae and adults expressing ATN-1::mCh. Scale bars, 6 μm. (B) The number of DB-containing striations per individual BWM cell, and (C) the occurrence of non-parallel DB striations per individual BWM cell, were counted. 

fhod-1(Δ) animals have fewer striations in early developmental stages (except L1), but they have a significantly higher number of non-parallel DB striations (arrows) compared when compared to wild-type (arrowheads). Shown are the average of two experiments (n = 10 animals per strain per experiment, two BWM cells per animal) with SEM error bars. * p < 0.05, ** p < 0.01, *** p < 0.001.
<table>
<thead>
<tr>
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<td>Day 4 adults</td>
<td><img src="image11" alt="Graph" /></td>
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Figure 3-S1. *fhod-1(Δ)* worms have more striations that are short compared to wild-type. The lengths of PAT-3- or ATN-1-containing striations were measured for one experiment (n = 10 animals per strain, one BWM cell per animal). In early larval stage, *fhod-1(Δ)* animals have a higher number of shorter striations than their wild-type counterparts, but the lengths catch up as they reach adulthood.
The BWMs of *fhod-1(Δ)* animals also have a significantly higher number of non-parallel DB striations (arrows) at all ages (Figure 3-5A, C). However, there are very few non-parallel striations in wild-type BWMs (Figure 3-5A, arrowheads). The occurrence of such non-parallel striations was also observed with MYO-3 striations, where we observed a significantly higher number of non-parallel striations in *fhod-1(Δ)* animals (Figure 3-S2). The increase in the number of striations (Figure 3-5B) in *fhod-1(Δ)* mutants does not correlate with the improvement in muscle strength observed in *fhod-1(Δ)* day 3 adults by NemaFlex assay. But the increase in the number of non-parallel striations (Figure 3-5C) could play a role in compensating for the lost muscle strength in young adult animals. However, the increase in *fhod-1(Δ)* striation length (Figure 3-S1) partially correlates with the improvement of muscle strength observed in *fhod-1(Δ)* day 3 animals (Figure 3-1B).

**Loss of FHOD-1 has a greater impact on DB spacing in younger animals than in older animals**

The DB spacing is another criterion that could provide insight as to whether the arrangement of DBs might act as a player in muscle strength and if any of those changes correlate to the improvement in muscle strength observed in day 3 *fhod-1(Δ)* mutants (Figure 3-1B). As previously, we quantified the regularity of ATN-1-rich DB spacing along striations by performing fast Fourier transformation (FFT) on concatenated sets of ATN-1::mCh fluorescence intensity profiles along striations (Sundaramurthy et al., 2020). ATN-1::mCh DBs are punctate and regularly spaced in wild-type BWMs, but in *fhod-1(Δ)* the DBs appear partially dispersed and irregular (Figure 3-6, 3-S3, 3-3, 3-4). The amplitude spectra of DB spacing for wild-type animals show clustering of peaks characteristic at each developmental stage (Figure 3-6, 3-S3), from ~ 1.4 μm⁻¹ in L1.
Figure 3-S2. *fhod-1(Δ)* worms have more non-parallel MYO-3 striations than wild-type worms. (A) Dorsal views of age synchronized wild-type and *fhod-1(Δ)* adult worms, stained with anti-MYO-3. Scale bars, 50 μm. (B) The numbers of non-parallel DB striations per length of total striations imaged were counted. Wild-type BWMs have few non-parallel MYO-3 striations but *fhod-1(Δ)* animals have a significantly higher number of non-parallel MYO-3 striations (arrowheads). Shown is the average result of one experiment (n = 6 worms for wild-type, n = 13 worms for *fhod-1(Δ)* worms) with SEM error bars. **p < 0.01.
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<tr>
<th></th>
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<td>primary Frq = 0.63369</td>
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Figure 3-6. Loss of FHOD-1 has a greater impact on DB spacing in younger animals than in older animals. MIPs of BWM contractile lattice from larvae and adults expressing ATN-1::mCh. Scale bars, 4 μm. ATN-1::mCh DBs are punctate and regularly spaced in wild-type BWMs, but in fhod-1(Δ) the DBs appear partially dispersed and irregular. DB spacing along striations was analyzed by performing fast Fourier transform (FFT). In fhod-1(Δ) animals, the DB spacing is irregular in younger animals, but the spectral peaks reveal some spacing regularity in older animals.
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<td>![Graph 19]</td>
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</table>

- **L1 larvae**
  - Primary Frq = 0.68927
  - Frequency, cycles $\mu$m$^{-1}$

- **L2 larvae**
  - Primary Frq = 0.26681
  - Frequency, cycles $\mu$m$^{-1}$

- **Day 1 adults**
  - Primary Frq = 0.63369
  - Frequency, cycles $\mu$m$^{-1}$

- **Day 3 adults**
  - Primary Frq = 0.47804
  - Frequency, cycles $\mu$m$^{-1}$

- **Day 4 adults**
  - Primary Frq = 0.44469
  - Frequency, cycles $\mu$m$^{-1}$
Figure 3-S3. FHOD-1 impacts DB spacing in younger animals. MIPs of BWM contractile lattice from larvae and adults expressing ATN-1::mCh. Scale bars, 4 μm. DB spacing was analyzed for a second set of animals, as per Figure 3-6. In fhod-1(Δ) animals, the DB spacing is irregular in younger animals, but the spectral peaks appear to become regular in older animals.
larvae to around 0.55 μm−1 in day 4 adults, corresponding to an increase in spacing between DBs. The amplitude spectrum for wild-type L1 DBs was notable for having a peak near 1.4 μm−1 that corresponds to the spacing of DBs within a striation, as well as a second peak near 1.0 μm−1. However, this second peak is likely an artifact of the concatenation of profiles from individual striations, which each begins and ends with particularly dim DBs (Figure 3-6, 3-3). In young fhod-1(Δ) animals (L1 and L4 larvae), the spectral peaks do not cluster near any frequency, representing the irregularity of DB arrangement. But in older fhod-1(Δ) animals (day 3 and day 4 adults), the spectral peaks cluster at a little higher value than wild-type DBs, suggesting that the DBs become regular. DB spacing appears irregular in younger fhod-1(Δ) animals and it appears to become regular in older animals. This partially correlates with the improvement in muscle strength between young adults and day 3 adults.

**Loss of FHOD-1 has a strong impact on the morphology of DBs**

Investigating the 3D structure of DBs would not only allow us to comprehend the morphological changes that occur temporally; it could help us determine if any changes in the structure of fhod-1(Δ) mutant DBs correlate with the improvement in muscle strength we observed in fhod-1(Δ) day 3 adults. In wild-type DBs, PAT-3 appears as a globule at the plasma membrane and ATN-1 appears as a finger-like projection running deep into the cell (Figure 3-7, white arrows). In wild-type L1 larval DBs, PAT-3 overlaps significantly with ATN-1 (Figure 3-7, white arrows). Both PAT-3 and ATN-1 rich structures in wild-type and fhod-1(Δ) animals grow bigger compared to the DBs in younger animals.
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<tr>
<td>Day 1 adults</td>
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<tr>
<td>Day 3 adults</td>
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<tr>
<td>Day 4 adults</td>
<td><img src="image9" alt="Image" /></td>
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</tbody>
</table>

**PAT-3::GFP/ATN-1::mCh**
Figure 3-7. Loss of FHOD-1 affects the structure and morphology of the DBs.

Three-dimensional (3D) visualization of BWM contractile lattice from larvae and adults expressing PAT-3::GFP and ATN-1::mCh. In wild-type animals, the DBs have PAT-3 and ATN-1, which accumulate to form two intact and defined structures. PAT-3 appears as a globule at the plasma membrane and ATN-1 appears as a finger-like projection (white arrows), which run deep into the cell. However, in fhod-1(Δ) animals, both PAT-3 and ATN-1 are misshapen/deformed (cyan arrows) and sometimes appear as multiple structures, which worsen as the worms age. PAT-3 is also associated with the M-lines (white arrowheads), PAT-3 only DBs (immature DBs) and attachment plaques, at cell edges (cyan arrowheads in L1 larvae)
However, in *fhod-1(Δ)* animals, both PAT-3 and ATN-1-enriched shapes are misshapen/deformed (Figure 3-7, white arrows) and/or sometimes appear as multiple structures. In *fhod-1(Δ)* L1 larvae, we can observe variable structures, including those that appear to form duplicate DBs (Figure 3-7, cyan arrow, L1 larvae) or multiple structures as shown in day 4 adults (cyan arrows). Besides lacking the normal *wild-type* DB shape, *fhod-1(Δ)* DBs seem to collapse on one another (Figure 3-7, cyan arrow, day 1 adults). The 3D structures also revealed that ATN-1 containing region of the DB in the *fhod-1(Δ)* DBs appear shorter than their *wild-type* counterparts (Figure 3-8A).

DBs are three-dimensional (3D) structures in BWMs, which led us to use a 3D parameter to examine the shape of these structures. To quantify this morphological change in DB structure, we turned to a 3D shape descriptor called the form factor. Shape descriptors are dimensionless quantities that are often used to describe the shape of a structure, independent of its size. They represent the degree of deviation from an ideal shape and are often normalized whose values range between zero to one. One usually represents an ideal shape/symmetry like a sphere. The 3D form factor values for both PAT-3 (Figure 3-8B) and ATN-1 (Figure 3-8C) decreased with age in *wild-type* and *fhod-1(Δ)* DBs, but this is due to the change in shape. Based on 3D form factor analysis, *fhod-1(Δ)* DBs worsen morphologically with age, and this holds true for both PAT-3 and ATN-1. The 3D form factor trends away from the ideal value of 1 as the animals grow older in both *wild-type* and *fhod-1(Δ)*. However, the *fhod-1(Δ)* DBs have a significantly smaller 3D form factor value than their *wild-type* counterparts, indicating that *fhod-1(Δ)* DB shapes are significantly less spherical than *wild-type* DBs. These results also demonstrate that the degree to which *fhod-1(Δ)* DBs diverge morphologically from
Figure 3-8. Loss of FHOD-1 has a strong impact on DB morphology. (A) 3D visualizations of two DBs from BWM contractile lattice in larvae and adults expressing PAT-3::GFP and ATN-1::mCh. The DBs in fhod-1(Δ) animals are misshapen and appear much shorter than their wild-type counterparts. 3D form factor values were obtained for (B) PAT-3 and (C) ATN-1. The 3D form factor values suggest that fhod-1(Δ) DBs are significantly less compact than wild-type DBs. Shown are average of two experiments (n = ten animals per strain per experiment, four DBs in one BWM cell per animal) with SEM error bars. * p < 0.05, ** p < 0.01, *** p < 0.001.
wild-type increases with age, which could be due to the fragile nature of the fhod-1(Δ) mutant DBs.

FHOD-1 loss does not alter the location of DB assembly in growing BWMs

To understand the assembly of DBs, we first demonstrated the maturation of DB striations at BWM cell edges using live imaging. We observed accumulation of new ATN-1::mCh (Figure 3-9, 4-8, d-j) and additional (Figure 3-9, 1-3, a-c) ATN-1::mCh to pre-existing PAT-3::GFP- rich (Figure 3-9, 1-8, a-j) DBs, in five out of six BWM cells we examined. This accumulation of new and additional ATN-1::mCh can be observed at the cell-cell boundaries (Figure 3-9, 4-8 in cell 1) and cell edges (Figure 3-9, d-j in cell 2) after 90 min. However, this process is very slow, on the order of hours. The time required for a sarcomere to assemble is on the order of 8-10 hr (Mackenzie et al., 1976; Moerman & Williams, 2006). Based on this, the small amounts of ATN-1::mCh observed after 90 min is reasonable. This observation suggests that PAT-3 only DBs are immature and PAT-3-containing DBs accumulate ATN-1 to become mature DBs.

For most DB rows or striations in a BWM cell, we can observe a set of immature DBs, containing only PAT-3, at both ends of the striation. These immature DBs appear at both ends of the striation, while the mature DBs containing both PAT-3 and ATN-1, appear farther inside the cell as one follows DBs along the striation away from the cell edge.

This arrangement of immature and mature DBs is found in both wild-type and fhod-1(Δ) animals. Since DB maturation is very slow, on the order of hours, we used fixed animals to measure the span that is covered by immature PAT-3-only containing DBs from the BWM cell edges (Figure 3-10 A). The transition distance is the distance measured from the first PAT-3 only DB (p1) from the end of a striation to the first PAT-1/ATN-1
Figure 3-9. New DB striation assembly occurs mostly at BWM cell edges. (A) MIPs of BWM contractile lattice in live animal expressing PAT-3::GFP and ATN-1::mCh. At 0 min, shown are three ATN-1-positive DBs at the indicated position in cell 1 (DBs 1-3) and three in cell 2 (DBs a-c). At 90 min, the ATN-1-negative, PAT-3-positive DBs in cell 1 (DBs 4-8) and cell 2 (DBs d-j) have acquired small amounts of ATN-1, while DBs (2,3) in cell 1 and DBs (a-c) in cell 2 have accumulated additional ATN-1. (B) and (C) are higher magnification of boxed areas. Scale bars, 10 μm.
Figure 3-10. FHOD-1 loss does not impact the location of DB assembly. (A) MIP of BWM contractile lattice from animals expressing PAT-3::GFP and ATN-1::mCh. A zoomed view shows the transition distance (yellow arrows), measured from the first PAT-3-only DB (p1) in a striation, to the first PAT-1- and ATN-1-positive DB (p+a1). This distance reflects the length of the stretch of the putative immature DBs that are decorated only by PAT-3. (B) The transition distance was measured for four developmental stages. Shown are average of two experiments (n = 10 animals per strain per experiment, one BWM cell per animal). Error bars indicate SEM. * p < 0.05, n.s. not significant.
containing DB (p+a1). Transition distance was measured for 4 stages from L4 larvae to
day 4 adults (Figure 3-10B). The transition distance was not significantly different
between fhod-1(Δ) and wild-type in L4 larvae, day 1 adults, and day 3 adults. These
results suggest that FHOD-1 loss does not change the span of the region containing
immature PAT-3 only DBs in growing BWMs, and thereby causes no apparent change in
the conversion of immature to mature DBs. However, the transition distance was
significantly lower in fhod-1(Δ) day 4 adults, compared to wild-type animals. These
results do not completely rule out the possibility of FHOD-1 having another role in the
process of DB assembly.

**Prolonged muscle contraction exacerbates the fhod-1(Δ) phenotype in BWMs
causing severe DB dispersion**

We hypothesized that FHOD-1 supports Z-line integrity against contractile forces.
We wanted to test if DBs in fhod-1(Δ) mutants are strong enough to withstand muscle
contractions. To test this, we treated worms with either a muscle relaxant or a stimulant
to further examine if these treatments might lead to the rescue or exacerbation of
dispersed DB phenotype seen in fhod-1 mutants, respectively. Muscimol is a GABA
agonist, that activates γ-aminobutyric acid (GABA) receptors and helps in maintaining
the BWMs in a relaxed state (Brouilly et al., 2015; Butkevich et al., 2015). Levamisole is
a nicotinic acetylcholine receptor agonist that causes continuous stimulation of BWMs
and maintains them in a hyper-contracted state. We treated wild-type and fhod-1(Δ) L4
larvae with either 2.5 mM levamisole or 10 mM muscimol along with untreated control
for 24 hr. Worms treated with muscimol had a relaxed body shape (flaccid) during the
treatment and they were alive at the end of treatment. However, worms treated with
levamisole had a contracted body shape (taut) and a few *fhod-1(Δ)* worms, ~5%, were dead at the end of treatment.

*Wild-type* and *fhod-1(Δ)* worms treated with levamisole or muscimol showed a significant decrease in the number of ATN-1 striation count (Figure 3-S4) compared to untreated controls, suggesting that these treatments stunted BWM development. This effect was greater with levamisole. The BWMs in *wild-type* animals appeared normal with the DBs either contracted or elongated when treated with levamisole or muscimol, respectively (Figure 3-11). These DBs remained intact with both treatments. *fhod-1(Δ)* animals treated with muscimol show very minor improvement of DB dispersion in BWMs, but this was highly variable, suggesting that muscimol may not rescue DB dispersion in *fhod-1(Δ)* animals as expected (Figure 3-11). However, treatment with levamisole exacerbated the *fhod-1(Δ)* phenotype in BWMs and caused severe DB dispersion (Figure 3-11, arrows) compared to control animals. This suggests that FHOD-1 could support the integrity of DBs against contractile forces.

**Evidence for functionally distinct populations of FHOD-1 in BWMs**

Previously, we have shown that FHOD-1 can be detected in BWMs of all developmental stages in which the BWMs are growing (Mi-Mi et al., 2012; Sundaramurthy et al., 2020). We have observed FHOD-1 enriched bodies at the cell edges and fainter bodies were associated with the internal DB striations (Mi-Mi et al., 2012; Sundaramurthy et al., 2020). A strain expressing FHOD-1::GFP can significantly rescue BWM growth (Mi-Mi et al., 2012) and partially rescue DB morphology (Figure 3-S5). We created a strain expressing FHOD-1::GFP and ATN-1::mCh (DWP231). We observed FHOD-1::GFP bodies to be enriched at the lateral cell edges (white arrows), but
**Figure 3-S4.** Prolonged contracted/relaxed state stunts striation growth.

*Wild-type* and *fhod-1(Δ) L4 larvae, expressing PAT-3::GFP and ATN-1::mCh, were treated with 2.5 mM levamisole or 10 mM muscimol along with controls and collected as adults after 24 hours of treatment (Figure 3-11). ATN-1 decorated DB-containing striations per individual BWM cell were counted. *Wild-type* and *fhod-1(Δ) worms* treated with levamisole or muscimol showed a significant decrease in the number of ATN-1 striation count compared to untreated controls. Shown are averages of two experiments (n = 10 animals per strain per experiment, one cell per animal) with SEM error bars. **p < 0.01, ***p < 0.001.
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Figure 3-11. Prolonged muscle contraction exacerbates the \textit{fhod-1(A)} phenotype in BWMs causing severe DB dispersion. The first three columns of images are MIPs and the fourth column of images are 3D reconstructions of BWM contractile lattice in adult worms expressing PAT-3::GFP and ATN-1::mCh, maintained for 24 hr under the following conditions: untreated control, 2.5 mM levamisole, or 10 mM muscimol. Treatment with levamisole caused severe DB dispersion in \textit{fhod-1(A)} (arrows) but not in wild-type animals. However, muscimol treatment did not rescue DB dispersion in \textit{fhod-1(A)} when compared with untreated control. Shown are representative images from one of two experiments. Scale bars, 6 μm. 3D visualization shows PAT-3::GFP oriented at the bottom and ATN-1::mCh facing upward.
Figure 3-S5. FHOD-1::GFP partially rescues DB morphology in fhod-1(Δ) mutants. MIPs of BWM contractile lattice from adult wild-type, fhod-1(Δ) and fhod-1(Δ); fhod-1::gfp worms stained with anti-ATN-1. Scale bars, 10 μm.
were absent at attachment plaques at BWM cell-cell borders (Figure 3-12B). FHOD-1::GFP at the lateral edges are accumulated near ATN-1::mCh at the growing end of the striation, where new DBs are being formed (Figure 3-13A, B, C). The ATN-1 decorated DBs at the cell edges (#1 to #6) have more FHOD-1 associated with them than the ATN-1 decorated DBs that are deeper in the cell (Figure 3-13B, C). These FHOD-1::GFP rich bodies at the cell edges are apparent in growing BWMs, and are greatly reduced in day 3 adults (white arrows) when there is minimal further growth (Figure 3-12C). Further, the intensity of these bodies decreases with the animal’s age. Interestingly, in levamisole treated worms, whose BWM growth is severely stunted, we also saw a significant reduction in FHOD-1::GFP enriched bodies at the cell edges compared to control or muscimol treated animals (Figure 3-12D). This suggests that loss of FHOD-1 bodies from the cell edges in animals treated with levamisole could be due to the absence of striation growth (Figure 3-S4) or the stunted growth could be a result of loss of FHOD-1 bodies from the BWM cell edges. All these observations suggest that FHOD-1 bodies localized at BWM cell edges might promote striation formation and BWM cell growth. Interestingly, we also observed bright FHOD-1::GFP bodies at intersections of the rare non-parallel striations (Figure 3-12A, cyan arrowheads) (10 of 11 striation intersections in L4 larvae, 5 of 5 in day 1 adults). This suggests that FHOD-1 might regulate/control the appearance of such non-parallel branches.

FHOD-1 also appears in the internal striations, right next to the DBs as faint bodies (Figure 3-12B, cyan arrows). Previously, this was reported as FHOD-1 puncta that were fainter than bright puncta observed at cell edges. However, we found that the association of FHOD-1 with more internal, mature DBs appeared as a faint halo around
Figure 3-12. Evidence for functionally distinct populations of FHOD-1 in BWMs. (A-D) MIPs of BWM contractile lattice from larvae and adults expressing FHOD-1::GFP and ATN-1::mCh. (A) FHOD-1::GFP is enriched at the intersection of DB striations that appear non-parallel (cyan arrowheads). (B) FHOD-1::GFP bodies are found enriched at the lateral cell edges (white arrows) and also appear in the internal striations, as faint bodies next to DBs (cyan arrows). The FHOD-1::GFP rich bodies at the cell edges are (A, B) apparent in growing BWMs and (C) are greatly reduced in day 3 adults, the BWMs have minimal growth (white arrows). (D) L4 larvae were treated with 2.5 mM levamisole or 10 mM muscimol or no treatment for controls and collected as adults after 24 hr. Control and muscimol treated worms have FHOD-1::GFP rich puncta at the cell edges (white arrows), but worms treated with levamisole lose the FHOD-1::GFP rich bodies at the cell edges. Shown are representative images from two experiments. Scale bars, 10 μm.
the ATN-1 decorated DBs (white arrowheads) (Figure 3-13D). In the mature DBs, FHOD-1 could support maintaining the shape of the DB and contribute to its strength. This supports the hypothesis that FHOD-1 loss leads to loss of strength in DBs of fhod-1(Δ) mutants.

**Discussion**

Multiple studies from a wide range of model systems propose that FHOD-family formins regulate striated muscle assembly (Fenix et al., 2018; Iskratsch et al., 2010; Kan-o et al., 2012; Rosado et al., 2014; Shwartz et al., 2016; Taniguchi et al., 2009; Ushijima et al., 2018). The *C. elegans* BWMs and its proteins have often served as a model system that aided in the understanding of its vertebrate homologs like focal adhesion proteins (Gieseler et al., 2016; Lecroisey, Segalat, et al., 2007; Moerman & Williams, 2006). Deciphering the role(s) of the only FHOD-family formin in the simple *C. elegans* can lead to the path that might help in uncovering how FHOD-family formins function in vertebrate muscles. In this study, our key goal was to identify the processes FHOD-1 regulates in DB assembly and function.

**Effect of FHOD-1 loss on DB distribution, organization, morphology and its correlation to muscle strength**

Examining muscle physiology revealed that *fhod-1(Δ)* mutants had significantly lower muscle strength than their wild-type counterparts as young adults, but their muscle strengths were almost similar as day 3 adults. Changes or increases in BWM width (Figure 3-2B), BWM cell width (Figure 3-2C), striation length (Figure 3-S1) and DB spacing (Figure 3-6) between day 1 and day 3 *fhod-1(Δ)* mutants partially correlate with
Figure 3-13. FHOD-1::GFP distribution in the contractile lattice of BWMs. 3D visualization of BWM contractile lattice from L4 larvae expressing FHOD-1::GFP and ATN-1::mCh. (A) FHOD-1::GFP is enriched at the lateral edges between cell 1 and cell 2, particularly near ATN-1::mCh at the ends of striations. (B, C) Higher magnifications of region between cell 1 and cell 2 from A (C) and a region between two cells (B). Higher amounts of FHOD-1 are accumulated at the cell edges, where new striations containing ATN-1 decorated DBs are being formed. (D) Higher magnification show FHOD-1 appears as a faint halo (white arrowheads) which seems to enclose or surround ATN-1 rich DBs.
the improvement in muscle strength (Figure 3-1B). The BWM and BWM cell width have a direct relationship with muscle strength, where their sizes are often proportional to muscle strength. Although, there is not much increase in striations (Figure 3-5B) between day 1 and day 3 \textit{fhod-1}(\Delta) mutants, the increase of striation length (Figure 3-S1) in these animals does partially correlate to the improvement in muscle strength. DB spacing in \textit{fhod-1}(\Delta) mutants (Figure 3-6) strongly correlate with the improvement in muscle strength observed in day 3 adults. These results suggest that distribution and organization of striations are independent of FHOD-1 loss, between day 1 and day 3 adults. These changes could play a role in compensation and led to the improvement of muscle strength in day 3 \textit{fhod-1}(\Delta) mutants.

While investigating the changes in muscle structure of \textit{fhod-1}(\Delta) mutants, to understand the improvement in muscle strength, we should not overlook the fact that these measurements from the NemaFlex platform are based on 2D crawling (Rahman et al., 2018) and the improvement of muscle strength that we observed in day 3 \textit{fhod-1}(\Delta) mutants might not be the true reflection of the animal’s complete muscle function. This could be one of the reasons why although we observe worsening of DB morphology (Figure 3-8) and appearance of non-parallel striations (Figure 3-5C), they seem to not correlate with the muscle strength measurements. Allowing day 3 adult worms to burrow in a 3D environment mimicking their natural habitat and comparing them with day 1 adults might reveal interesting results (Lesanpezeshki et al., 2019, 2021), and this could correlate well with the muscle structural changes, we observe. However, NemaFlex platform-based strength measurements and pluronic gel burrowing assays report on
different aspects (2D and 3D locomotion) of muscle physiology (Lesanpezeshki et al., 2021).

**FHOD-1 regulates new DB striation assembly**

We have supporting evidence that DB assembly initiates at the cell edges in *C. elegans* BWM (Figure 3-9) similar to the initiation of sarcomere assembly in a vertebrate system (Sanger et al., 2005, 2017; White et al., 2018). Based on our detection of enriched FHOD-1 bodies in lateral cell edges of growing BWMs (Figure 3-12B, 3-13A) and occurrence of dispersed DBs in *fhod-1*(Δ) mutants, we hypothesized that FHOD-1 regulates DB assembly. There is a strong association of bright FHOD-1 bodies (arrows) with ATN-1 decorated DBs at the BWM cell edges where new DBs are being assembled (Figure 3-13, B, C). Also, the significant reduction of FHOD-1::GFP enriched bodies at the cell edges of levamisole treated animals (Figure 3-12D) with stunted growth (Figure 3-S4) and in day 3 adults (Figure 3-12C), strongly suggest that FHOD-1::GFP is always associated with growing striations.

Based on the close association of FHOD-1 rich bodies at lateral cell edges with newly formed ATN-1 positive DB, one possible mechanism for FHOD-1 would be its role in the conversion of immature DBs containing only PAT-3 to mature DBs containing both PAT-3 and ATN-1. We analyzed this by measuring transition distance, where a larger distance in *fhod-1*(Δ) compared to *wild-type* would suggest that the rate of DB maturation was slower in mutants. However, the transition distance was not significantly different between *fhod-1*(Δ) and *wild-type* in L4 larvae, day 1 adults, and day 3 adults, but it was significantly higher in *fhod-1*(Δ) day 4 adults. As the BWM growth is significantly reduced after day 3 adulthood (Figure 3-2), the reduction might not be
reflective of the DB maturation process in growing BWMs. However, this does not rule out the possibility that FHOD-1 could still affect DB assembly. *fhod-1(Δ)* mutants have shorter ATN-1 structures than *wild-types* (Figure 3-8) and here FHOD-1 might affect the DB assembly, where it contributes to the length of the DBs or even the accumulation of ATN-1.

Loss of FHOD-1 leads to an increase in the appearance of non-parallel striations in *fhod-1(Δ)* mutants (Figure 3-5) and it worsens with age. Interestingly, FHOD-1 rich bodies are enriched at the intersection of such non-parallel striations in *wild-type* worms (Figure 3-12A). This suggests that FHOD-1 could regulate the occurrence of such non-parallel striations. FHOD-1 could act as a check point allowing the appearance of only few non-parallel striations; however, its loss might trigger the formation of multiple such non-parallel striations leading to the phenotype observed in *fhod-1(Δ)* mutants (Figure 3-5). Our current model suggests that FHOD-1 promotes DB assembly, controls the alignment and growth of new DB striations and perhaps acts as a scaffold during the formation of DBs.

**FHOD-1 supports the structural integrity of DBs**

Another possible cause for dispersed DBs in *fhod-1(Δ)* mutants could be the loss of structural integrity of DB. Based on the 3D form factor, our data suggest that loss of FHOD-1 leads to age-dependent changes to DB shape (Figure 3-7, 3-8). Besides lacking normal *wild-type* DB shape, *fhod-1(Δ)* DBs seem to collapse on one another (Figure 3-7, cyan arrow, day 1 adults). We used paralytic agents to test if FHOD-1 supports the structural integrity of DB against contractile forces, as to whether prolonged contraction/relaxation would exacerbate/rescue the *fhod-1(Δ)* DB phenotype. Treatment
with muscimol did not cause any significant changes to \textit{fhod-1(\textLambda)} DB compared to no drug controls. This suggests that prolonged relaxation using muscimol could not rescue DB morphology in \textit{fhod-1(\textLambda)} mutants. However, prolonged treatment with levamisole caused severe deformity in DBs of \textit{fhod-1(\textLambda)} mutants but not in \textit{wild-type} animals (Figure 3-11). This suggests that DBs of \textit{fhod-1(\textLambda)} mutants are not strong enough to withstand prolonged muscle contractions. FHOD-1 besides being enriched at the lateral edges of the growing BWMs (Figure 3-12B, white arrows, Figure 3-13A, B. C, white arrows), faint bodies are found to be associated with DBs in the internal striations. On higher magnification, these faint bodies appear as halo, which seems to enclose/surround ATN-1-decorated DBs (Figure 3-13D, E, arrowheads). The evidence of FHOD-1’s association around mature ATN-1 decorated DBs adds to the previous model where FHOD-1 could act as a scaffold surrounding the DBs, either on its own or with other associated proteins as a DB supporting structure that help in maintaining the structural integrity of the DB.

The presence of faint FHOD-1 bodies around ATN-1 structures could mean that FHOD-1 can be considered as a structural component supporting the DB. FHOD-1 could likely be considered as one of the early markers during DB assembly. Our overall model is based on possible roles of FHOD-1: supports the growth of new DB striations, controls alignment of new striations, helps in directed growth of DB striation and remains associated with mature ATN-1-positive DBs to provide structural support (Figure 3-14). FHOD-1 appears around the DBs in \textit{wild-type} animals, but in \textit{fhod-1(\textLambda)} animals, DBs appear misshapen due to loss of structural integrity. FHOD-1 could act as a scaffold along with other associated proteins, particularly actin, to regulate these processes. It would be intriguing to uncover the molecular mechanisms that FHOD-1 regulates during
Figure 3-14. Schematic showing changes to DB due to loss of FHOD-1. DBs contain many structural components including, PAT-2/3 (α/β-integrin), DEB-1 (vinculin), TLN-1 (talin), and ATN-1 (α-actinin). FHOD-1 appears around the DBs in wild-type BWMs. Blue blobs are outlines of DBs. Loss of FHOD-1 leads to disorganized DBs and our data demonstrates that DBs in fhod-1(Δ) animals are fragile, possibly due to lack of structural integrity. The DBs in fhod-1(Δ) animals appear shorter and they are prone to contraction-dependent disorganization and deformity. Loss of FHOD-1 leads to a contraction-dependent loss of DB structural integrity.
striation assembly and maintenance.

Our data provide new insights into FHOD-1’s role in striation muscle development and suggests that FHOD-1 supports DB structure by acting as a scaffold, providing support to maintain the structural integrity of the DB. mammalian FHOD3 could play a similar role as well in cardiac myofibrils. The dispersed DBs in fhod-1(Δ) worms become highly disorganized due to prolonged contraction and a similar process occurs in FHOD3-deficient mice whose cardiac myofibrils appear to be severely disrupted only after contractions of the heart begins (Kan-O et al., 2012).

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Chapter 4: Discussion
Chapter 4 contributions

Sumana Sundaramurthy wrote Chapter 4 for this dissertation to serve as a discussion to both her research presented in Chapters 2, 3, and Appendix 1.
**Role(s) of formins in striated muscle development**

Striated muscle cells contain one of the most intricate but highly organized cytoskeletal networks among specialized cells, with high amounts of actin (Henderson et al. 2017). Thin filaments, which are rich in F-actin are one of the major structures of the sarcomere. F-actin-based thin filaments are one of the first few components to be assembled during sarcomere assembly, but the factors that initiate their assembly are unclear (Sanger et al., 2017; Ono, 2014). Formins are one of the key regulators of unbranched actin filaments in cells, where they nucleate, elongate, cap, and even bundle actin filaments (Breitsprecher & Goode, 2013). Multiple lines of evidence support formins as potential candidates that could initiate sarcomere assembly. One key piece of evidence was the association of formins near Z-lines, precursors of which are the first structures to be formed (Z-bodies) during vertebrate sarcomere assembly (Sanger et al., 2017). Various studies also discovered formin roles in striated muscle development, but their exact mechanism(s) were not known (Rosado et al., 2014; Taniguchi et al., 2009; Iskratsch et al., 2010; Kan-O et al., 2012; Kan-O et al., 2012; Fenix et al., 2018).

Previously, we had found that two *C. elegans* formins, FHOD-family, FHOD-1 and DIAPH-family, CYK-1 were localized, next to and on the dense body (DBs), which are integrin-based adhesions in body wall muscles (BWMs) (Mi-Mi et al., 2012). FHOD-1 appeared near the DBs and CYK-1 appeared on the DBs. DIAPH-family formin, mDia2 localized to Z-lines (Rosado et al., 2014). The localization of mammalian FHOD-family formins has been controversial. One group observed FHOD3 near the C-zone (Kan-O et al., 2012) and other groups found it near the Z-line (Iskratsch et al., 2010; Rosado et al., 2014). One group found mammalian FHOD1 appear at the costameres and
intercalated discs (Dwyer et al., 2014; Haj et al., 2015), whereas it was undetectable in mouse hearts (Sanematsu et al., 2019). These differences in localizations did not improve the chances of interpreting the role of FHOD-family formins. Among the six formins in *C. elegans*, only loss of CYK-1 and FHOD-1 resulted in defective BWM growth (Mi-Mi et al., 2012). We observed *fhod-1(Δ)* mutants had thin BWMs than wild-type animals and *cyk-1 (Δ)* mutants had thinner BWMs than *fhod-1(Δ)* mutants. Both these mutants had deletions in the critical actin interacting FH2 domains and were putative nulls for actin interaction. CYK-1 has been shown to nucleate and elongate actin filaments in vitro (Neidt, Skau, & Kovar, 2008). However, FHOD-1’s role is still unclear, but mammalian FHOD-formins, FHOD1, and FHOD3 have been shown to inhibit actin polymerization in vitro (Taniguchi et al, 2009, Schönichen et al, 2013).

The localization of formins near and to the integrin adhesions and their in vitro properties provided us with a strong rationale to examine FHOD- and DIAPH-family formins, as possible initiators of thin filament assembly during sarcomerogenesis. We wanted to test if complete loss of formins would terminate or at least reduce thin filament assembly. To prevent the redundancy among formin activity, we analyzed the double mutant, *fhod-1(Δ); cyk-1(Δ)* and we did not observe, complete loss of thin filaments but found they had much thinner BWMs than either of the single mutants (Mi-Mi et al., 2012). This could be due to the nature of our *cyk-1(Δ)* mutants, which were constitutive. *cyk-1* is an essential gene and is required for cytokinesis (Swan et al, 1998). Our homozygous *cyk-1(Δ)* mutants were derived from heterozygous parents as the homozygous mutants were lethal, although some of them reached adulthood due to maternal rescue but were sterile. The previously observed effects of CYK-1 loss in the
homozygous cyk-1(Δ) mutants may have been due to some residual CYK-1 due to maternal rescue. To observe the effects of complete loss of CYK-1 and prevent the issue of maternal rescue, we used a fast-acting temperature-sensitive (ts) conditional mutant, cyk-1(ts) (Davies et al, 2014), which is functional at permissive temperature and non-functional at the restrictive temperature.

We performed temperate shift experiments on age-synchronized L1 larvae to analyze the effects of both FHOD-1 and CYK-1 loss on thin filaments and BWMs (Sundaramurthy et al., 2020). Surprisingly, we did not see any disruption in F-actin-based thin filaments at restrictive temperature in the double mutant, fhod-1(Δ); cyk-1(ts), or cyk-1(ts) (Figure 2-1). However, we found fhod-1(Δ) mutants had a much thinner BWM than cyk-1(ts) or wild-type animals. The cyk-1(ts) mutants had slightly thinner BWMs than wild-type. The double mutant fhod-1(Δ); cyk-1(ts) worms seemed to have BWM widths slightly thinner than fhod-1(Δ) mutants. Even the minor reduction of BWM width in cyk-1(ts) seemed to be due to its small body size (Figure 2-1E). These results were intriguing when compared to our earlier results with cyk-1(Δ) mutants (Mi-Mi et al., 2012). We thought that our previous results in cyk-1(Δ) mutants occurred due to loss of CYK-1 much earlier during development. When we performed a temperature shift experiment on embryos to test if acute loss of CYK-1 affected embryonic sarcomerogenesis, we found that cyk-1(ts) mutants showed no long-term polarization defects in early embryos or frayed F-actin in late embryos (Figure 2-4, 2-5) (Sundaramurthy et al., 2020). However, a significant number of fhod-1(Δ) embryos showed increased frayed F-actin striations during late embryogenesis, suggesting FHOD-1 to be a significant player during BWM development in late embryos as well as during post-embryonic larval stages.
To test if the differences in BWM defects between cyk-1(Δ) or cyk-1(ts) were due to pleiotropic or secondary effects caused due to CYK-1 loss other tissue(s), we created mosaic strains expressing genomic untagged cyk-1 gene in cyk-1(Δ) mutant background (Sundaramurthy et al., 2020). We found that BWM cells expressing inherited extrachromosomal arrays (ECAs) containing genomic cyk-1 were about the same width as cells that didn’t inherit ECA (Figure 2-6), suggesting CYK-1’s role on BWM growth could be indirect. However, FHOD-1’s role seems to be direct with a significant rescue of BWM cell width (Figure 2-6) of cells that express fhod-1 ECA. More evidence from a strain expressing an endogenously-tagged version of CYK-1::GFP (Davies et al., 2018) showed that CYK-1 did not localize to DBs in BWMs (Figure 2-9S8). On re-examination, we found that our anti-CYK-1, which neatly decorated DBs (Figure 2-9, Figure 4-1) previously (Mi-Mi et al., 2012), was non-specific (Figure 2-9). This provided clarification as to why we observed such drastic differences in BWM phenotypes between cyk-1(Δ) and cyk-1(ts) animals, even though cyk-1(ts) animals exhibited loss of function phenotypes similar to cyk-1(Δ) animals. Our results also point out that CYK-1’s effect on BWMs is almost negligible based on the body width (Figure 2-1 E) and its effect on BWMs is most likely due to loss of CYK-1 in other tissue(s) like germline (Figure 2-S8) or even due to global CYK-1 loss during early embryonic development (Figure 2-S4). Experiments designed to express cyk-1 fused to different promoters that are expressed in specific tissues could help us determine the tissue source of CYK-1 that affects the BWMs.

Our initial hypothesis suggesting formins as initiators of thin filament assembly seems to be unfavorable at this point as we fail to see CYK-1 direct involvement with
Figure 4-1. Schematic of DBs showing initial and updated formin localization and changes due to loss of FHOD-1. DBs contain many vertebrate focal adhesion homologs: PAT-2/3 (α/β-integrin), DEB-1 (vinculin), TLN-1 (talin), ATN-1 (α-actinin). Pink blobs are outlines of DBs. FHOD-1 is localized near the integrin-based DB (Mi-Mi et al 2012). Re-examination of anti-CYK-1 localization revealed a non-specific localization of anti-CYK-1 to DBs, suggesting that CYK-1 has an indirect effect on BWM growth (Sundaramurthy et al, 2020). FHOD-1 is the sole contributor to BWM development, with a direct effect on BWM growth. Loss of FHOD-1 does not lead to loss of thin filaments but causes disorganized DBs. Created with BioRender.com.
BWMs and even with FHOD-1’s direct involvement, *fhod-1(Δ)* mutants do contain a significant number of thin filaments (Mi-Mi & Pruyne, 2015) packed in their contractile lattice. Based on multiple studies, leiomodins and the complex of N-WASP/nebulin might act as initiators of thin filament assembly in the vertebrate systems. Leiomodin-2 appears to nucleate actin filaments and controls the length of thin filaments in cardiomyocytes (Chereau et al., 2008; Tsukada et al., 2010). Leiomodin-3 has been suggested to regulate thin filament length, similar to leiomodin-2, but in skeletal muscles (Yuen et al., 2014). A complex of N-WASP and nebulin has been shown to nucleate unbranched actin filaments at the Z-lines, acting downstream of IGF-1 signaling and resulting in muscle hypertrophy (Takano et al., 2010). The appearance of leiomodins in cardiomyocytes after completion of sarcomere assembly and preferred actin filament assembly of N-WASP/nebulin complex on preformed skeletal myofibrils suggest that these two factors might not contribute to thin filament assembly during initial muscle formation. The thin filaments in BWMs are more likely initiated by some unknown actin nucleating factor as *C. elegans* lack homologs of leiomodins or nebulins. RNAi experiments designed to knock down potential actin nucleating factors that contain WH2/other actin interacting domains in *C. elegans* could provide us insight into factors that could contribute to the initiation of thin filament assembly (Ono, 2014). Our goal moved away from investigating the initiators of thin filament assembly to examining the role(s) of formins in striated muscle development.

The FHOD-family formins are the most well-studied formins for their role in striated muscle development (Iskratsch et al., 2010; Taniguchi et al., 2009; Kan-O et al., 2012; Fenix et al., 2018; Rosado et al., 2014; Shwartz et al., 2016) and their clinical
relevance to cardiomyopathies (Arimura et al., 2013; Hayashi et al., 2018; Ochoa et al., 2020, 2018; Wooten et al., 2013). Few characteristic features of \textit{fhod-1(\Delta)} mutant BWMs resemble traits that are similar to human DCM and transgenic mice that overexpress FHOD3 that is defective to binding actin, which are characterized by thin ventricle wall muscles and irregular Z-line (Kan-O et al., 2012; Benian, & Epstein, 2011; Wexler et al., 2009). With the functional similarities between the BWMs and vertebrate striated muscle and the conservation of FHOD-family formin function, it is possible that mammalian FHOD-family members could potentially show similar effects and have even similar molecular mechanisms. FHOD-1 is the only FHOD-family formin in \textit{C. elegans} and understanding the role of FHOD-1 could provide insights as to what role(s) FHOD-family formins regulate in mammals. Our data show that FHOD-1 could rescue BWM cell width in a cell autonomous manner (Figure 2-6), suggesting that FHOD-1 has a direct role in BWM growth. Previously, we observed disrupted DBs in BWMs (Mi-Mi & Pruyn, 2015; Figure 2-2). In this study, we were able to quantitatively, analyze DB spacing using FFT (Figure 2-7). Amplitude spectra of ATN-1 intensities suggested that the frequency peaks clustered around 1.0 µm^{-1} in wild-type animals. However, the DB spacing was aberrant in \textit{fhod-1(\Delta)} mutants and there was no clustering of frequency peaks observed. However, in BWM cells that expressed ECAs containing \textit{fhod-1} in the \textit{fhod-1(\Delta)} mutant background the peaks clustered similar to wild-type (Figure 2-7), suggesting a rescue of the DB spacing in the cells that express the ECA. These results suggest that FHOD-1 rescues DB organization and BWM cell width in a cell autonomous manner. FHOD-1 is the only key formin that contributes to striated BWM growth in \textit{C. elegans} (Sundaramurthy et al., 2020, Mi-Mi & Pruyn, 2015; Mi-Mi et al., 2012). However, loss
of FHOD-1 causes a more subtle effect on BWMs than its vertebrate homolog, FHOD3, where there is failure to form mature cardiac myofibrils and the entire contractile lattice appears highly disorganized (Taniguchi et al., 2009; Kan-O et al., 2012; Kan-O et al., 2012). This could be due to the difference in structures of the contractile lattice within the myofibrils and the BWMs.

The contractile lattice is packed in specialized organelles called myofibrils within the vertebrate muscle cell cytoplasm, where not all Z-lines are anchored to the plasma membrane (Clark et al. 2002). However, in BWMs, the contractile lattice appears right beneath the plasma membrane, where all DBs (Z-line analogs) and M-lines remain attached to the plasma membrane (Gieseler et al., 2016). The BWMs appear to be more resistant to FHOD-1 loss probably due to the direct attachment of contractile lattice to the plasma membrane. Other features could contribute to this resilience due to FHOD-1 loss where compensatory structures might appear due to support disorganized DBs in *fhod-1(Δ)* mutants. Mammalian FHOD3 and FHOD1 have been shown to regulate actin stress fiber formation and microtubule co-alignment in non-muscle cells (Thurston et al., 2012; Gasteier et al., 2005). Although the location of microtubule networks is unclear in BWMs, FHOD-1 could regulate microtubule networks in BWMs. Experiments could be designed to investigate how FHOD-1 loss affects the microtubule network. Loss of FHOD-1 could increase the amount of the microtubule network as a compensatory mechanism to support dispersed DBs in *fhod-1(Δ)* mutants. To investigate how FHOD-1 affects the overall BWM cell biology-mitochondrial network (Loveless et al., 2017), calcium dynamics (Martin et al., 2019) and muscle contraction rates (Hwang et al., 2016) could be examined in *fhod-1(Δ)* mutants.
FHOD-1 promotes DB striation distribution and organization

Our major phenotype due to loss of FHOD-1 is disorganized DBs (Figure 4-1) in fhod-1(Δ) mutants. The main function of the DB is not only to anchor thin filaments but also to be able to transduce force from the sarcomere to the hypodermis for effective locomotion (Lecroisey, Ségalat & Gieseler, 2007). Previously, thrashing assays suggested that fhod-1(Δ) mutant’s swimming ability was similar to wild-type animals (Mi-Mi, 2012). However, we believed that crawling and thrashing assays were not sufficient to characterize if fhod-1(Δ) BWMs were physiologically defective. To investigate this, we used robust assays like pluronic gel burrowing assay and NemaFlex platform (Lesanpezeshki et al., 2019; Rahman et al., 2018) to characterize the effect of FHOD-1 loss on 3D and 2D locomotion. fhod-1(Δ) mutants performed poorly in burrowing assays when compared to wild-type and this suggested that the mutants have a burrowing defect (Figure 3-1). We also wanted to examine if these animals behaved poorly in burrowing assay due to lower muscle strength that could be due to small BWMs and defective DBs. We observed a significant decrease in muscle strength in young adults of fhod-1(Δ) mutants, but the muscle strength of mutants improved as day 3 adults (Figure 3-1). We found that the BWM width (Figure 3-2) increase partially correlated to the improvement in muscle strength. This partial correlation led us to explore other structural components of BWMs like DBs, which could contribute to the improvement of muscle strength in day 3 fhod-1(Δ) animals. Additional experiments could be performed to determine the burrowing effectiveness of fhod-1(Δ) animals, particularly in young and day 3 adult animals to compare the muscle strengths and burrowing effectiveness.
We characterized the organization and distribution of DBs in the larval and adult stages in the due course of development. We used strains expressing fluorescently-tagged DB proteins to characterize the DBs of wild-type and fhod-1(Δ) mutants (Figure 3-3, 3-4) in larval and adult stages. One apparent feature that is noticeable by observing the DBs of wild-type and fhod-1(Δ) mutants was the aberrations in DB distribution, alignment, and structure of mutants. Examination of striations revealed that fhod-1(Δ) had a significantly fewer number of striations (except L1 larvae, Figure 3-5) and most striations were smaller (Figure 3-S1) compared to striations in wild-type BWMs. However, both the number of striations (after day 3) and length (after day 1) catch up to their wild-type counterparts, suggesting a partial correlation to the improvement seen in muscle strength (Figure 3-1 B). Quantitative analysis of DB spacing using FFTs also revealed a similar correlation to the improvement in muscle strength. There was clustering of frequency peaks in the amplitude spectrum of DBs in wild-type animals characteristic of each developmental stage (Figure 3-6, 3-S3), from ~ 1.4 μm⁻¹ in L1 larvae to around 0.55 μm⁻¹ in day 4 adults. These characteristic clustering of frequency peaks suggest that DBs are moving far apart in older animals, reflecting the increase in sarcomere size to accommodate more filaments in bigger BWM cells. This also reflects on the regularity of DB arrangement in wild-type BWMs. However, in younger fhod-1(Δ) mutants, there is no apparent clustering of frequency peaks in the amplitude spectrum of L4 animals, where the DB spacing appears highly irregular. But older fhod-1(Δ) animals, after day 1 adults, the amplitude spectrum shows some regularity in DB spacing, suggesting an improvement in DB organization.
DBs are analogous to vertebrate focal adhesions (Moerman & Williams, 2006). Vertebrate sarcomere assembly begins with the formation of integrin-based adhesion structures called Z-bodies at the spreading edge of the muscle cell (Sanger et al., 2005, 2017). FHOD-1 appears as bright bodies at BWM cell edges (Mi-Mi et al, 2012), suggesting that it could support the formation of the DBs. To test if FHOD-1 supports DB assembly, we first demonstrated DB assembly occurs at BWM edges, using live imaging to investigate the maturation of DBs. We observed new ATN::mCh accumulation to PAT-3-only DBs (DBs 4-8, d-j, Figure 3-9) and additional ATN::mCh to DBs that already contained ATN-1::mCh/PAT-3::GFP DBs (DBs 2,3, a-c, Figure 3-9). Bright FHOD-1 bodies at lateral cell edges (Figure 3-12B, 3-13A) and the strong association of bright FHOD-1 bodies with ATN-1 decorated DBs at the BWM cell edges where new DBs are being assembled (Figure 3-13, B, C), suggests that FHOD-1 is associated with growing BWMs. The data from levamisole treated FHOD-1::GFP animals where FHOD-1 disappears from the cell edges (Figure 3-12D), possibly a result of reduced striations (Figure 3-S4), also suggest that FHOD-1 is associated with growing DB striations. As a measure of the maturation of DBs, we analyzed ‘transition distance’ and observed no relative changes between wild-type animals fhod-1(Δ) animals, suggesting that FHOD-1 might not affect the location of ATN-1 addition during DB assembly (Figure 3-10). Only day 4 adults fhod-1(Δ) animals showed a significant reduction in transition distance which could be reflective of slower DB maturation in fhod-1(Δ) animals. However, this could be of less significance as the BWM growth is greatly diminished after the day 3 adult stage (Figure 3-2) and so is the drastic reduction of FHOD-1-rich bodies at BWM cell edges (Figure 3-12 C) on day 3 adults. We also noticed a relative decrease in the
amount of FHOD-1::GFP localized to the BWM edges as the animals reached adulthood, which correlates to the reduction in BWM growth rate at adulthood. Although our data here suggests that FHOD-1 might not support DB maturation, we are examining only one parameter here, the location of ATN-1 addition along the striation. So, this does not completely rule out the possibility that FHOD-1 could still support DB assembly, via other mechanisms. Live imaging of DB assembly in wild-type and fhod-1(Δ) mutants, expressing PAT-3::GFP and ATN-1::mCh could provide insight into how DB assembly is similar or different due to FHOD-1 loss.

One possible role of FHOD-1, where it could regulate the appearance of non-parallel striations. Wild-type (Figure 3-5 A) animals had rare occurrences of striations that appeared non-parallel to each other, but fhod-1(Δ) animals accumulated a significantly higher number of such non-parallel striations (Figure 3-5 C). Interestingly, FHOD-1 rich bodies were observed at the intersection of such non-parallel striations (Figure 3-12 A). This suggests that FHOD-1 rich bodies not only support the parallel alignment of striations, but they could also act as a checkpoint in controlling the appearances of such non-parallel striations, which is significantly higher in fhod-1(Δ) mutants (Figure 3-5 C). Loss of FHOD-1 leads to positioning defects in BWMs (Mi-Mi et al, 2012), and here FHOD-1 could also act as a scaffold to guide these DB striations to the right position and prevent the occurrence of such non-parallel striations.

Formins play a major role in actin dynamics and are associated with focal adhesions in non-muscle cells (Ciobanasu et al., 2012; Valencia et al., 2021). Mammalian FHOD1 has been shown to support actin polymerization at sites of integrin clusters and supports the maturation of focal adhesions in mouse fibroblasts (Iskratsch et al., 2013).
To understand the role of *C. elegans* FHOD-1 in BWMs, we could move to a model that could be related to focal adhesion assembly. Ultimately, FHOD-1 appears near integrin-based DBs, and they perform the function of both Z-lines and costameres in BWMs (Lecroisey, Ségalat, & Gieseler, 2007). FHOD-1 could act as a scaffold and regulate the assembly of DB components, or actin dynamics that are associated with the DBs or disassembly of DBs, or even aid in DB splitting. Live imaging of DBs in a strain expressing FHOD-1::GFP and ATN-1::mCh could provide insight into whether FHOD-1 would regulate DB assembly, DB disassembly, DB splitting (similar to focal adhesion splitting), which could lead to the appearance of non-parallel striations.

**FHOD-1 supports DB structural integrity against muscle contraction**

Although our transition distance suggests that FHOD-1 may not affect the location of ATN-1 addition during DB assembly, 3D views of DBs showed that *fhod-1(Δ)* mutants have shorter ATN-1-rich structures than *wild-types* (Figure 3-7, 3-8). The DBs appear misshapen in *fhod-1(Δ)* mutants compared to *wild-type* animals and the structures appear more misshapen as the *fhod-1(Δ)* animals age. These are reflected in the 3D form factor values for both PAT-3 and ATN-1 (Figure 3-8 B, C). Besides being misshapen, we noticed some DBs in *fhod-1(Δ)* animals collapsed from their normal orientation and even appear disrupted, suggesting that the DBs might lack structural integrity. We wanted to examine if FHOD-1 supports the structural integrity of DB against contractile forces. To test this hypothesis, we used paralytic agents to keep BWMs in a prolonged contracted state using levamisole or relaxed state using muscimol. We expected to see that prolonged contraction/relaxation might either exacerbate/rescue the *fhod-1(Δ)* DB phenotype. We did not observe a rescue of the DBs with muscimol.
treatment, but we did observe severe deformity of DBs in *fhod-1(Δ)* mutants, suggesting that these DBs were fragile, and were unable to withstand prolonged contractile forces due to treatment with levamisole. The notion that FHOD-1 supports the structural integrity of DBs gets stronger when we observe FHOD-1::GFP localize around ATN-1-rich DBs (Figure 3-13 D, E). Our current model suggests that FHOD-1 appears around DBs and it provides support to maintain its structural integrity. FHOD-1 could appear as a scaffold to support the DBs against the wear and tear of the contractile forces, or even be associated with other proteins to provide some rigidity to DBs. The amount of FHOD-1 in BWMs is inversely proportional to the developmental stage (i.e. there is more FHOD-1 in day 1 old adults than day 2 or day 3 adults). This correlates to the DB shape changes observed in wild-type animals (3D form factor, Figure 3-8) where the DB shape becomes slightly misshapen as animals age, suggesting FHOD-1’s role in the maintenance of DB integrity. Experiments designed to perturb the workload of animals could serve as a second test to test the structural integrity of the *fhod-1(Δ)* mutant DBs. Burrowing assays could serve as a good technique to test this, where adjusting the pluronic gel concentration and thickness of the gel could serve as a varying workload.

Fluorescent Recovery After Photobleaching (FRAP) of ATN-1::mCh in DBs, expressed by wild-type and *fhod-1(Δ)* animals showed interesting results. We observed a large pool of dynamic ATN-1::mCh in *fhod-1(Δ)* mutants compared to wild-type animals, in the order of (unpublished data). This could be due to ATN-1 in the *fhod-1(Δ)* mutant DBs being unstable due to disruption in protein networks in/around the DBs. FHOD-1 loss leads to a contraction-dependent loss of structural integrity in DBs, making them susceptible to severe deformity (Figure 4-2). An extension of this would be the formation
Figure 4-2. Loss of FHOD-1 leads to a contraction-dependent loss of DB structural integrity. Pink blobs are outlines of DBs. DBs contain many vertebrate focal adhesion homologs: PAT-2/3 (α/β-integrin), DEB-1 (vinculin), TLN-1 (talin), ATN-1 (α-actinin). FHOD-1 appears around the DBs rather than appearing punctate. Loss of FHOD-1 leads to disorganized DBs and our data demonstrates that DBs in fhod-1(Δ) animals are fragile, possibly due to lack of structural integrity. The DBs in fhod-1(Δ) animals appear shorter and they are prone to contraction-dependent disorganization and deformity. Created with BioRender.com.
of the secondary DB-like extensions from the primary DB as seen in fhod-1(Δ) mutants (blue arrows, Figure 3-7). The dynamic ATN-1 in fhod-1(Δ) mutants might act as a potential initiator for the formation of such structures. These observations lead to many intriguing molecular mechanisms of FHOD-1 that might support the structural integrity of the DB. This study could be expanded by additional FRAP experiments to understand the dynamics of FHOD-1::GFP and how other DB protein dynamics (SORB-1::GFP, CAP-1::GFP, DYS-1::GFP, ACT-4::GFP, GFP::ACT-5) are affected by FHOD-1 loss.

Cardiac myofibrils appear normal until embryonic day 8.5 (E 8.5) in both wild-type and fhod3 knock out mice, but the myofibrils appear severely disorganized in fhod3 knock out mice, at E 9.5 and the mice die due to cardiac failure by E 11.5 (Kan-O et al., 2012; Kan-O et al., 2012). In mice, cardiac contractions begin right around the E 8.5, which could be right before the appearance of severely disorganized cardiac myofibrils. This suggests that loss of FHOD3 might lead to the proper assembly of sarcomeres until E 8.5 and the newly assembled sarcomeres could be too weak to withstand cardiac contractions. Our model of how FHOD-1 supports DB integrity against muscle contraction could be applied to understanding the role of how its mammalian homolog, FHOD3 in striated muscle development.

DBs seem resilient to FHOD-1 loss in BWMs compared to their mammalian homologs like FHOD3. One of the key components that help in the stabilization of sarcomeres in the immunoglobulin (Ig) repeat-containing proteins like titin, which is called “the molecular spring” of the sarcomere (Henderson et al., 2017). C. elegans contain many Ig-containing proteins that belong to the titin/connectin family of proteins like TTN-1 (titin-like protein), KETN-1 (kettin), DIM-1 (myopallidin), and UNC-22
(twitchin) (Gieseler et al., 2016). Most of these proteins often help in the stabilization of the sarcomere, mainly F-actin-rich thin filaments. Kettin is a member of the titin-family of elastic proteins and is often associated with thin filaments in arthropods. *C. elegans*, KETN-1 is encoded by a separate gene and different from TTN-1 (titin-like protein in *C. elegans*) (Ono & Ono, 2005). RNAi against KETN-1 caused disorganization of actin filaments and even DBs when treated with levamisole in a contractility-dependent manner (Ono et al., 2006). This suggests that components that support the integrity of the sarcomere, often show contractility-dependent disorganization under a loss of function condition.

Even BWMs of *dim-1* mutants have dispersed DBs similar to *fhod-1*(Δ) mutants and the severity of dispersion increase with *dim-1* mutant’s age (Rogalski et al., 2003). DIM-1 appears around the DBs in a pattern similar to DYS-1 (dystrophin-like protein) (Brouilly et al., 2015) localization around DBs and like FHOD-1 to some extent (Figure 3-13). DIM-1 helps in maintaining BWM integrity and FHOD-1 could play a similar role by maintaining DB integrity in BWMs. Contractility-defective mutants could be used to test if these mutants could suppress the DB phenotype that is caused due to FHOD-1 loss. An RNAi screen to determine possible genetic suppressors of *fhod-1*(Δ) mutants could also be done. FHOD-1 could be one of the early DB markers, but its dependence on other DB proteins is unknown. An RNAi screen that targets against the well-known DB proteins (Moerman & Williams, 2006) could help determine the order of FHOD-1 appearance during DB assembly.
**FHOD-1 supports Z-line integrity via a unique DB-associated actin cytoskeletal network decorated with short tropomyosin**

During cardiac myofibrillogenesis, stress fiber-like premyofibrils containing Z-bodies, associate with non-muscle isoforms of myosin, actin, and even tropomyosin (Handel et al., 1991; Sanger et al., 2005). It is believed that the existence of such primitive structures with non-muscle isoforms acts as a template for the formation of mature myofibrils. Recent evidence using human induced pluripotent cardiomyocytes showed that the conversion of muscle stress fibers to sarcomeric actin filaments required FHOD3 (Fenix et al., 2018). Lei Mi-Mi, a former graduate student in our lab, performed immunoprecipitation experiments and identified a short splice isoform of tropomyosin (LEV-11) that co-immunoprecipitated with FHOD-1 from the muscle enriched extracts of only wild type but not fhod-1Δ mutants (unpublished data). Short isoforms of tropomyosin are non-muscle tropomyosins, whereas the long isoforms of tropomyosin are considered muscle tropomyosins (Watabe et al., 2018). An affinity-purified antiserum raised against the short isoforms of tropomyosin, anti-LEV-11(short) was not only able to recognize the purified protein of the expected molecular weight in western blots, but it also decorated regions adjacent to DBs (Figure A-1). This is different from the anti-LEV-11(long), which decorates thin filaments and recognizes the purified long LEV-11 protein. The appearance of FHOD-1 and LEV-11(short) around DBs suggests that there is a unique actin network that is decorated by LEV-11(short). The appearance of LEV-11(short) near the DBs is similar to FHOD-1 localization near DBs (Figure A-2). FHOD-1 and LEV-11(short) could be components of a unique DB-associated cytoskeleton network that could act as a scaffold during DB assembly.
Vertebrate striated skeletal muscles contain non-muscle (non-sarcomeric) tropomyosin isoforms adjacent to the Z-lines, which contain a unique Z-line associated cytoskeletal (Z-LAC) network (Kee et al., 2004; Vlahovich et al., 2008). Mammalian non-muscle isoforms of tropomyosin, Tm3 and Tm4, have been shown to localize to the Z-LAC in skeletal muscles. Tm4 is upregulated during muscle repair and regeneration during muscle dystrophic conditions, similar to γ-actin (a cytoplasmic/non-muscle actin) upregulation (Vlahovich et al., 2008). γ-actin is associated with the costameres in striated skeletal muscles (Sonnemann et al., 2006) and mammalian FHOD1 has been shown to localize to the costameres in striated cardiac muscles (Haj et al., 2015). Interestingly, FHOD1 has been shown to polymerize cytoplasmic actin but not muscle actin (Patel et al., 2018).

The association of FHOD-1 with actin filaments decorated with LEV-11(short), suggests the actin dynamics within the DB-associated cytoskeleton, could be regulated by FHOD-1. The actin decorated by LEV-11(short) near DBs could be a type of cytoplasmic actin isoform. Ultimately, DBs are costameres and Z-lines of BWMs, where the vertebrate skeletal muscle costameres (Sonnemann et al., 2006) are known to be associated with actin structures made of γ-actin isoform. *C. elegans* has 5 different actins (Ono & Pruyne, 2012). ACT-5 is considered non-muscle actin in worms, and it is found in the microvilli-containing cells of the excretory canal (MacQueen et al., 2005). Additionally, I have also observed GFP-tagged ACT-5 driven by the act-5 promoter in the BWMs (Szumowski et al., 2016). We predicted that the presence of ACT-5 in BWMs could be functionally related to cytoplasmic actin in cells. Our results suggest that FHOD-1 has been shown to inhibit polymerization of rabbit muscle actin (unpublished
data), but it could potentially polymerize a cytoplasmic actin like ACT-5, similar to mammalian FHOD1 (Patel et al., 2018). In vitro pyrene actin and total internal reflection fluorescence (TIRF) assays with different actin isoforms could provide insight into whether FHOD-1 prefers to polymerize non-muscle/cytoplasmic actin over muscle actin. Potential mutant variants targeting the FH2 domain of FHOD-1 could abolish this function, which could be created to test this in vitro. Further, strains containing these mutations (deletion/point) could be created using CRISPR and their DBs could be analyzed to determine if the polymerization activity of FHOD-1 is responsible for the DB phenotypes in fhod-1(Δ) mutants.

Anti-LEV-11(short) signal appears weaker in fhod-1(Δ) mutant BWMs, but it does not disappear (Figure A-3). The presence of these possible actin filaments decorated with LEV-11(short) in fhod-1(Δ) mutant BWMs, suggests that FHOD-1 could even support the DB-associated cytoskeleton network in an alternate/additional role, acting as a bundler for the LEV-11(short) decorated actin filaments. Our unpublished data that FHOD-1 acts as a bundler with rabbit muscle actin filaments in low-speed centrifugation assays and TIRF assays. This in vitro property of FHOD-1 is similar to mammalian FHOD1, where the N-terminal of FHOD1 was found to be required for the bundling activity (Schönichen et al, 2013). So the fhod-1(Δ) mutants don’t lose the filaments decorated with LEV-11 (short), but rather they have a very fragile network that is unable to effectively support DBs against contractile forces. In vitro, TIRF and low-speed centrifugation assays with different actin isoforms could provide insight into whether FHOD-1 prefers to bundle non-muscle/cytoplasmic actin over muscle actin. Potential mutant variants targeting the N-terminal domain of FHOD-1 could abolish this function,
which could be created to test this in vitro. Further, strains containing these mutations (deletion/point) could be created using CRISPR and their DBs could be analyzed to determine if the bundling activity of FHOD-1 is responsible for the DB phenotypes in *fhod-1(Δ)* mutants.

Based on the polymerization and bundling characteristic of FHOD-1, we have two potential molecular mechanisms of how FHOD-1 could act to support the structural integrity of the DB against contractile forces. FHOD-1 could act as a potential bundler as well as help in the polymerization of non-muscle actin. Both these roles could contribute to the structural integrity of the DB via the DB-associated cytoskeletal network. In our model FHOD-1 supports DB structural integrity by being a component of the DB-associated network, which contains actin filaments decorated with short tropomysins (Figure 4-3). FHOD-1 could be a component of a DB-associated cytoskeleton network that supports and contributes to the elastic nature of the DB. The contraction-dependent loss of DB integrity in *fhod-1(Δ)* mutants, due to reduction in LEV-11(short)-decorated actin filaments could be caused due to a possible dysregulation of actin filament dynamics/bundling activity loss by FHOD-1.

**Conclusions and future directions**

Our results present the first evidence that suggests the association of an FHOD-family formin to Z-LAC. We have also provided new insights on possible molecular mechanisms that FHOD-1 could regulate to support DB integrity against contractile forces. We even have a potential interacting partner which would help us understand the nature of the DB-associated cytoskeletal network or support structure if present. With this work, we have made progress in understanding how FHOD-family formins regulate
Figure 4-3. FHOD-1 is associated with a unique actin network decorated with short tropomyosin that could constitute the DB-associated cytoskeleton network.

DBs contain many vertebrate focal adhesion homologs: PAT-2/3 (α/β-integrin), DEB-1 (vinculin), TLN-1 (talin), ATN-1 (α-actinin). Pink blobs are outlines of DBs. FHOD-1 was found to interact indirectly with short tropomyosin, LEV-11(short), and this short isoform was found near DBs. There suggests that short tropomyosin decorated actin filaments are associated with FHOD-1 near DB in wild-type animals. FHOD-1 around DBs could be a part of a unique DB-associated cytoskeletal network that supports its structural integrity. Loss of FHOD-1 leads to a reduction in tropomyosin-decorated actin filaments. This suggests that there is a contraction-dependent loss of DB integrity due to lack of short tropomyosin decorated actin filaments in *fhod-1(Δ)* animals.

FHOD-1 potentially interacts with actin filaments decorated with short tropomyosin, where it could regulate the actin filament dynamics or even act as a bundler to these filaments. Created with BioRender.com.
striated muscle development. We have found that FHOD-1 is always associated with DBs in both new as well as mature DBs. Our results suggest that FHOD-1 plays a supportive role during new striation assembly where there are large accumulations of FHOD-1 present. We also found that FHOD-1 is primarily responsible for providing structural support to the DB and it protects it against contractile forces. Future directions for this project would be to investigate the precise molecular mechanism of how FHOD-1 would interact and if there is a possible DB-associated cytoskeletal network similar to that found in skeletal muscles (Vlahovich et al. 2008).

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Appendix 1: FHOD-1 associates with short tropomyosin as a structural component of the DB-associated cytoskeleton network
Appendix 1 contributions

Sumana Sundaramurthy developed, designed, performed all experiments not mentioned below, analyzed the data, and wrote Appendix 1 for this dissertation. David Pruyne oversaw research, provided input with experimental design. Chris Pellenz affinity purified the antibody DPMSP13, did preliminary antibody titrations for western blot and made RNAi constructs. SarahBeth Votra did preliminary immunostain for DPMSP13 and 10-42. Meaghan Geary performed western blot on RNAi samples.
**Introduction**

Using mass spectroscopic analysis, Lei Mi-Mi, a former graduate student from our lab identified a short splice isoform of tropomyosin that co-immunoprecipitated with FHOD-1, from the muscle enriched extracts of only wild-type but not fhod-1(Δ) mutant worms (unpublished). In *C. elegans*, there is only one tropomyosin gene called LEV-11, but this single gene produces many LEV-11 isoforms via alternate splicing (Watabe et al., 2018). There are long and short LEV-11 isoforms. The short isoforms of LEV-11 are generally considered non-muscle tropomyosins, whereas the long isoforms of LEV-11 are called the muscle tropomyosins since they decorate the sarcomeric thin filaments. In vertebrates, non-muscle tropomyosins were also identified in skeletal muscles, where they appeared at a unique Z-line associated cytoskeletal (Z-LAC) structure (Vlahovich et al., 2008; Kee et al., 2004). During cardiac myofibril formation, stress fiber-like premyofibrils are associated with non-muscle isoforms of myosin, actin and even tropomyosins (Handel et al., 1991; Sanger et al., 2005; Sanger et al., 2017). It is believed that the existence of such primitive structures with non-muscle isoforms act as a template for the formation of mature myofibrils.

We raised antibodies against the long and short LEV-11 isoforms. Our antibodies for LEV-11A, long tropomyosin isoform was raised in chicken and for LEV-11(short) was raised in guinea pigs. Among the *C. elegans* LEV-11 isoforms, that occurs due to alternate splicing. LEV-11C, LEV-11E, and LEV-11T are the known short isoforms (Watabe et al., 2018). We performed immunostain in animals using these antibodies and found
**Materials and methods**

**Staining for fluorescence microscopy**

Worms were immunostained for fluorescence microscopy as previously described (Sundaramurthy et al., 2020). Monoclonal primary antibody MH35 (anti-ATN-1) generated by Francis and Waterston (1985) was a gift from Pamela Hoppe (Western Michigan University, Kalamazoo, MI). DPMSP13, anti-LEV-11(short) was affinity-purified from crude serum. Mouse anti-GFP, Secondary antibodies, FITC-conjugated goat anti-mouse, Texas red-conjugated goat anti-chicken, Texas red-conjugated goat anti-guinea pig, Texas red-135 conjugated goat anti-mouse, Dylight conjugated goat anti-mouse were commercially obtained (Rockland Immunochemicals, Pottstown, PA).

Antibody dilutions used were 1:10 DPMSP13 (guinea pig), 1:10⁴ MH35 (mouse), 1:10³ anti-GFP, 1:100 10-42 crude serum for anti-LEV-11(long), and 1:500 for secondary antibodies. Confocal images were acquired using an SP8 Laser Scanning Confocal Microscope and deconvolved as described in Chapter 3.

**RNAi**

RNAi-mediated knockdowns were performed by the standard feeding technique (Wang and Barr, 2005), as previously described (Sundaramurthy et al., 2020). Two sets of L4440-lev-11(short) constructs were made. One construct would target exon 5a and another would target exon 5b. These constructs would target either lev-11c or lev-11e. For these experiments, a 1:1 mixture of transformed and induced bacteria containing constructs targeting lev-11c or lev-11e were used for RNAi treatment. Animals were treated for two generations before visualizing their DBs for any changes in their structure.
Western blots

Whole worm lysates were obtained from RNAi-treated worms and worms expressing GFP-tagged LEV-11 isoforms as previously described (Sundaramurthy et al., 2020).

Results and Discussion

Our antibodies raised against the short or long LEV-11 isoforms, recognize either only the short or long LEV-11 isoforms, respectively, but not both (Figure A-1 A). DPMSP13 was able to recognize purified protein (LEV-11C/E) but not LEV-11A. When we performed immunostain using DPMSP13 (anti-LEV-11 short), we observed that it localized near the DBs (yellow arrows, Figure A-1) similar to anti-FHOD-1 (Figure A-2, A). This pattern of staining is distinct from the long isoform of tropomyosin (LEV-11A) that appears enriched on the thin filaments (blue arrows, Figure A-1). In mammalian striated cardiac muscle, FHOD1 has been found at the costameres (Haj et al., 2015; Dwyer et al., 2014). Mammalian FHOD3 is required for the formation of precursors of sarcomeres in induced pluripotent cardiomyocytes (Fenix et al., 2018). Our localization of anti-LEV-11(short) near DBs (Figure A-1) and the appearance of LEV-11(short) with FHOD-1 (Figure A-2, A) suggests that FHOD-1 and LEV-11(short) could be components of a DB-associated actin network, similar to vertebrate Z-LAC (Vlahovich et al., 2008; Kee et al., 2004). This suggests that the unique DB-associated cytoskeleton network has actin filaments decorated with LEV-11(short), which could be regulated by FHOD-1. Formins appear to control the fate of tropomyosin isoforms by regulating actin filament dynamics (Gunning et al., 2015). We also found anti-LEV-11(short) localized near the DBs in fhod-1(Δ) as well (Figure A-2 B). However, the staining appears to be more diffuse in fhod-1(Δ) animals than in wild-type. This
Figure A-1. LEV-11(long) appears on thin filaments and LEV-11(short) appears near DB. 

(A) Western blots with purified LEV-11 proteins and extracts from worms expressing GFP-tagged LEV-11 isoforms. Anti-LEV-11(long) recognizes ~33 KDa bands, purified LEV-11A (long isoform), endogenous LEV-11(long) and GFP-tagged LEV-11A (~60 KDa), from worm extracts. Anti-LEV-11(short) recognizes ~29 KDa bands, purified LEV-11C and E (short isoforms), endogenous LEV-11(short) and GFP-tagged LEV-11C and E (~55 KDa), from worm extracts. 

(B) MIPs of BWM contractile lattice of L4 larvae. Anti-LEV-11(long) decorates thin filaments (blue arrows), whereas anti-LEV-11(short) appears near the Z-lines (yellow arrows). Scale bars, 4 µm.
Figure A-2. Loss of FHOD-1 leads to faint LEV-11(short) striations in BWMs.

(A) MIPs of BWM contractile lattice of L4 larvae expressing FHOD-1::GFP and stained for LEV-11(short). Anti-LEV-11(short) localizes near the Z-lines (yellow arrows) similar to FHOD-1. Second panel is the magnified view of the white box. Scale bars, 7 µm and 3 µm (magnified view). (B) wild-type BWMs have bright LEV-11(short) striations, whereas in fhod-1(Δ) mutants, the LEV-11(short) striations are fainter. Scale bars, 7 µm.
Figure A-3. RNAi against *lev-11(short)* does not disrupt DBs. (A) Worms expressing PAT-3::GFP and ATN-1::mCh were subjected to RNAi against *lev-11c* and *lev-11e* isoforms for two generations. The RNAi treatment does not impact the DBs. Scale bar, 6 µm. (B) Western blot with extracts from worms treated with control and *lev-11(short)* RNAi, worms expressing GFP-tagged LEV-11 isoforms and purified LEV-11 proteins. Anti-LEV-11(short) recognizes ~29 KDa band, purified LEV-11C and E (short isoforms), endogenous LEV-11(short) and GFP-tagged LEV-11C and E (~55 KDa), from worm extracts.
suggests that LEV-11(short) does not require FHOD-1 to localize near the DBs. The regulation of FHOD-1 in BWMs could be actin isoform-specific, similar to its mammalian homolog, FHOD1, where FHOD1 can polymerize only non-muscle/cytoplasmic actin but not muscle actin (Patel et al., 2018). Worms have five actin genes (Ono & Pruyne, 2012), among which, ACT-5 is considered as non-muscle actin (Macqueen et al., 2005). FHOD-1 might regulate the polymerization of ACT-5. I observed ACT-5 in BWMs using a strain that expressed GFP-tagged ACT-5 driven by its endogenous promoter. FHOD-1 could also act as a bundler and have a preference in bundling LEV-11(short) decorated actin filaments. Mammalian FHOD1 has been shown to bundle actin filaments (Schönichen, et al, 2013). A more detailed discussion of possible mechanisms of LEV-11 and FHOD-1 can be found in Chapter 4.

One of our hypotheses was that LEV-11(short) could play a role in the integrity of DBs. To test this, I performed RNAi against lev-11(short) on strain expressing PAT-3::GFP and mCh::ATN-1, for two generations. Surprisingly, the RNAi treatment against lev-11(short) c, e and t isoforms, did not disrupt the DBs. Western blot analysis of the RNAi-treated animals suggests that the RNAi might have not been effective as we did not observe any reduction in the amount of LEV-11(short) between control and RNAi-treated worm extracts (Figure A-3 B). Creating CRISPR-based mutants to disrupt lev-11(short) function in worms could help us determine the specificity of anti-LEV-11(short) and could even reveal if loss of LEV-11(short) has a similar phenotype like fhod-1(Δ) mutants.
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