Caenorhabditis elegans Muscle
A Genetic and Molecular Model for Protein Interactions in the Heart
Guy M. Benian, Henry F. Epstein

Abstract: The nematode Caenorhabditis elegans has become established as a major experimental organism with applications to many biomedical research areas. The body wall muscle cells are a useful model for the study of human cardiomyocytes and their homologous structures and proteins. The ability to readily identify mutations affecting these proteins and structures in C elegans and to be able to rigorously characterize their genotypes and phenotypes at the cellular and molecular levels permits mechanistic studies of the responsible interactions relevant to the inherited human cardiomyopathies. Future work in C elegans muscle holds great promise in uncovering new mechanisms in the pathogenesis of these cardiac disorders. (Circ Res. 2011;109:1082-1095.)

Key Words: cardiomyopathies ■ Caenorhabditis elegans sarcomere structure ■ sarcomere assembly ■ model genetics

Cardiomyopathies are a diverse set of heart muscle diseases associated with mechanical and electric dysfunction and high risk of sudden cardiac death and cardiac failure. The two major clinical types of inherited cardiomyopathy are hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM)1–3 (Figure 1). Clinical histories indicate a positive family history in about 60% of HCM, and in 20% to 35% of DCM cases. The remaining primary HCM or DCM cases are strongly suspected to be unrecognized familial disease or new mutations. The most common inheritance pattern is autosomal dominant, but at a much lower frequency, autosomal recessive and X-linked recessive inheritance patterns are also observed. Over the past 20 years, more than 450 mutations in 20 genes have been discovered as causing HCM. Nearly all of the affected proteins are components of the sarcomere. The genes most commonly affected encode β-myosin heavy chain4,5 and myosin-binding protein C6,7 (accounting for at least 25% each), and troponin T, troponin I, tropomyosin, and regulatory myosin light chain (accounting for approximately 2–5% each).2,8 To date, mutations in more than 30 genes result in DCM. Most of these genes also encode sarcromeric proteins. Interestingly, different mutations in the same gene can result in either HCM or DCM. Currently, we only know the causative mutation for 50% of the cases of HCM, and 20% of the cases of DCM. Despite knowing that mutations in so many different sarcromere and sarcomere-associated proteins result in HCM and DCM, the exact molecular mechanisms by which these mutations result in clinical heart hypertrophy or dilatation are unknown.

The nematode Caenorhabditis elegans continues to be an excellent model for studying the organization, assembly and maintenance of the sarcomere. The major striated muscle of C elegans is found in the body wall and is required for the animal’s locomotion (Figure 2). Because of the small size of this nematode (1-mm-long adults), a heart and circulatory system are not required. Thus, C elegans is not suitable for studying a number of aspects of human cardiac function and disease including chamber development, fibrosis, hypertrophy, remodeling, and hemodynamics. Nevertheless, the close homology of proteins and structures of interest justify the study of nematode body wall muscle as a way to understand human heart muscle. Table 1 enumerates the advantages and disadvantages of C elegans as a model for normal and diseased human heart. In addition to being able to readily carry out mutational analysis in a whole organism, through both forward and reverse genetics, this nematode offers several advantages for studying muscle. These include its optical transparency, which allows evaluation of myofibrillar structure by polarized light, and localization of GFP-tagged proteins. In addition, its usual mode of self-fertilization allows propagation of muscle mutants that would otherwise be unable to mate.

C elegans offers a number of advantages as an experimental organism as compared with Drosophila, zebrafish, or the mouse. Each of these other organisms shares only a subset of these experimental capabilities. Also, it must be noted that none of the organisms used in genetic experiments offer an ideal model for the study of human heart disease. C elegans offers striated muscle. Drosophila and zebrafish exhibit only primitive heart-like structures that differ developmentally and...
physiologically from mammalian hearts. Even the mouse, although possessing a mammalian 4-chambered heart, being a small mammal, exhibits distinct patterns of sarcomeric gene expression and physiological characteristics in the heart from those of larger mammals, especially humans. An illustrative example is the difference in which myosin heavy chain gene is predominantly expressed in heart ventricles: in mice, fetuses express β-myosin and then this is supplanted by α-myosin in adults, whereas in humans, β-myosin is expressed at both fetal and adult stages.

Cellular and Molecular Architecture of C elegans Muscle
C elegans body wall muscle cells are divided among 4 quadrants that run down the length of the animal. The myofilament lattice is restricted to a narrow ~1.5-µm zone adjacent to the cell membrane along the outer side of the muscle cell (Figure 2E). Within most muscle cells, there are 9–10 A- and I-band pairs (Figure 2B). Polarized light microscopy was used to image body wall muscle at low (Figure 2A) and high (Figure 2D) magnification. The bright or birefringent material represents the thick filament containing A-bands and the dark material represents the thin filament containing I-bands. The high magnification/resolution image of Figure 2D shows that running down the middle of the I-bands are bright dashes; these represent cross sections of the Z-disk–like dense bodies.

The thin filaments are attached to the dense bodies (Z-disk analogs), and the thick filaments are organized around

<table>
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<tr>
<th>Non-standard Abbreviations and Acronyms</th>
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<td>AMPK</td>
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<td>MyBP-C</td>
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Figure 1. Normal and cardiomyopathy hearts (A through C): A, hypertrophic; B, normal; C, dilated. Compare the left ventricular wall thickness and chamber volume of the normal heart (B) with the increased left ventricular wall thickness of hypertrophic cardiomyopathy (A) and with the thinner left ventricular wall thickness and increased left ventricular chamber volume of dilated cardiomyopathy (reprinted with permission of Cell Press from Seidman JG, Seidman C. The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. Cell. 2001;104:557–567). Light microscopy of tissue sections stained with hematoxylin and eosin from hypertrophic (D), normal (E), and dilated (F) cardiomyopathy patients. In the hypertrophic tissue, note the myocyte disarray (labeled) and interstitial fibrosis (whitish oval areas). In the dilated tissue, note the myocyte degeneration and large areas of interstitial fibrosis. (Reprinted with permission of Massachusetts Medical Society from Watkins H, Ashrafian H, Redwood C. Inherited cardiomyopathies. N Engl J Med. 2011;364:1643–1656.)
Figure 2. The body wall muscle of *C. elegans*. **A**, View of a live *C. elegans* by polarized light optics. One of the 4 quadrants of body wall muscle is in view; there are interlocking pairs of spindle-shaped cells (the boundary of one cell is marked by arrows). The parallel white lines running nearly parallel to the long axis of the worm are the thick filament–containing A-bands.122 (Reprinted with permission of Cell Press from Mackenzie JM, Garcea RL, Zengel JM, Epstein HF. Muscle development in Caenorhabditis elegans: mutants exhibiting retarded sarcomere construction. *Cell* 1978;15:751–762.) **B**, Higher magnification view of body wall muscle showing 1 muscle cell and portions of 3 other muscle cells, immunostained with antibodies to myosin. The parallel lines are the A-bands, roughly similar to the white lines viewed by polarized light in **A**. (Reprinted with permission of Rockefeller University Press from Landsverk ML, Li S, Hutagalung AH, Najafov A, Hoppe T, Barral JM, Epstein HF. The UNC-45 chaperone mediates sarcomere assembly through myosin degradation in Caenorhabditis elegans. *J Cell Biol.* 2007;177:205–210.) **C**, Portion of a body wall muscle cell immunostained with antibodies to UNC-112 (Kindlin), an integrin-associated protein localized to the bases of both M-lines and dense bodies (Z-disks). In this image, the cross sections of dense bodies appear as fat dots (indicated by arrow), and the M-lines appear as nearly continuous thinner lines (indicated by arrowhead). **D**, High resolution and magnification view of body wall muscle using polarized optics, in a plane identical to those in **A** through **C**. The dark bands are I bands containing thin filaments only. The bright dashes in the center of I bands are the dense bodies (Z-disk analogs), to which thin filaments are attached. The bright or white bands are A bands containing thick as well as thin filaments. L is the length of an individual thick filament. These filaments subextend an angle of 5.9° with the A-I interface. The
C. Limitations of

B. Advantages of

Figure 2 (Continued).

A. Advantages of C. elegans mutant analysis

Inexpensive and easy to culture

Fast generation time (3 d)

Large brood size (about 300)

Mutants can be frozen indefinitely and revived

Multiple successful mutagenesis strategies

Rapid out-crossing for removing secondary mutations

Fine structure mapping of mutations

Rapid mapping by deletion and deficiency arrays

Rapid and inexpensive correlation of mutations to the small genome sequence

High throughput screening by RNAi knockdowns in live nematodes

Rapid construction of transgenic and transformed nematodes

Rapid transgenic rescue of mutants

B. Advantages of C. elegans for studying heart muscle

Body wall muscle is striated like cardiac muscle

Extensive conservation of sarcomere structure and components

Body wall muscle required for locomotion but not viability

Optical transparency permits visualization of muscle structure in live animals

Self-fertilization allows propagation of mutants that are unable to mate

Purification of specific muscle proteins and myofilaments

C. Limitations of C. elegans as a specific heart disease model

No heart or vascular system

Body wall muscle is striated but not specifically cardiac

Body wall muscle expresses some specialized versions of myofilament arrays, membrane adhesion sites and their proteins distinct from their homologs in cardiac muscle

Body wall muscle dense bodies serve the function of both Z-disks and costameres

Body wall muscle does not undergo hypertrophy or remodeling as in the mammalian heart

M-lines. Moreover, all the dense bodies and M-lines are anchored to the muscle cell membrane and extracellular matrix, which is attached to the hypodermis and cuticle (Figure 2E). This allows the force of muscle contraction to be transmitted directly to the cuticle and allows movement of the whole animal.

Many components of C. elegans sarcomeres and their membrane–extracellular matrix attachment structures have been defined (Figure 2F). Nematode muscle M-lines and dense bodies not only serve the function of analogous structures in vertebrate muscle, but also are similar to the costameres of vertebrate striated muscle and the focal adhesions of nonmuscle cells in their anchorage to the plasma membrane and their major proteins (Figure 2F and Figure 3A and 3B).15–17

Over the past 7 years, it has been reported that multiple protein complexes link the muscle cell membrane to thick filaments at the M-line in C. elegans (Figure 3A). The cytoplasmic tail of integrin is associated with a complex of 4 conserved proteins [UNC-112 (Kindlin in mammals), PAT-4 (integrin linked kinase, ILK), PAT-6 (Actopaxin), and UNC-97 (PINCH)].18–20 UNC-97 links to myosin in thick filaments through 4 different complexes: via UNC-98, via LIM-9 (FHL) and UNC-96, via LIM-8, and via UNC-95 and LIM-8.21–23 Similar progress is being made in defining a dense body protein interaction matrix that helps explain linkage of the muscle cell membrane to thin filaments (Figure 3B).24

In C. elegans body wall muscle, all the M-lines and dense bodies are anchored to the muscle cell membrane (Figure 2E, plane 3). In human cardiac muscle, only those Z-disks and possibly M-lines that are located at the periphery of the muscle cell, are anchored to the muscle cell membrane via costameres. In addition, the intercalated disks act as terminal Z-disks for anchorage of thin filaments, and as strong intercellular junctions.

Most proteins of the C. elegans sarcomere (depicted in Figure 2F) were first identified through mutations in 2 major phenotypic classes. In the uncoordinated or “Unc” class, due to mutation in any one of ≈40 genes, animals develop into adults but they are slow moving or paralyzed.25,26 In the “Pat” class of mutants (paralyzed arrested at 2-fold) due to mutation in any one of 20 genes, embryos do not move within the eggshell and development arrests at the 2-fold stage.27,28 Mutations in several genes have loss-of-function Unc and null Pat phenotypes; examples include unc-45, unc-52, unc-97, and unc-112.

Over the years, C. elegans has proven to be a superb platform for discovery of new information about conserved sarcomere components [eg, myosin heavy chain, UNC-112 (kindlin)], and for discovery of absolutely new and yet conserved components of sarcomeres [eg, UNC-89 (obscurin) and UNC-45]. The first complete sequence of a myosin heavy chain from any organism was UNC-54 (MHC B).29 Its sequence analysis led to an explanation for not only how myosin rods assemble in a parallel manner along most of the thick filament shaft, but also accurately predicted the absolutely conserved pattern and spacing of myosin heads proj-

Table 1. C. elegans Muscle as a Model for Heart Muscle

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Figure 2 (Continued). A band is 1.0 μm wide. Since sine equals opposite over hypotenuse lengths, L is calculated to be 9.7 μm.

(Reprinted with permission of Cell Press from Mackenzie JM, Epstein HF. Paramyosin is necessary for determination of nematode thick filament length in vivo. Cell. 1980;22:747–755.) E, Depiction of part of one muscle cell in one of the body wall quadrants. The upper right shows a portion of the worm cut in cross section with body wall muscle quadrants in yellow. The enlargement shows the organization of filaments and membrane attachment structures in several planes of section. Plane 1 shows a single sarcomere in a view typical of cross-striated human muscle. A single sarcomere spans between 2 dense bodies. An M-line is found in the middle at which thick filaments are presumably cross-linked. Plane 2 shows plane of section found in parts A through D, when a worm is placed on a slide and viewed by fluorescent or polarized light microscopy. Plane 3 is a cross section through the worm and the muscle cell. Note that all the dense bodies and M-lines are attached to the muscle cell membrane and are ideally positioned to transmit the force of muscle contraction. F, Schematic indicating the location of most of the known components of the sarcomere and membrane attachment sites of nematode body wall muscle. Note that some proteins are found at both M-lines and dense bodies. These proteins are mostly membrane-proximal, such as UNC-52 (perlecan), PAT-3 (β-integrin), and PAT-4 (integrin linked kinase).
ecting from the thick filament surface. Mutations in \textit{unc-54}
myosin heavy chain that affect its synthesis, enzymatic
activity, and assembly, both recessive and dominant, \textsuperscript{30–32}
served as a foundation for interpreting the breakthrough
discoveries of the Seidmans that certain missense mutations
in \textit{H9252-cardiac myosin heavy chain cause many cases of
HCM}. \textsuperscript{4,5}
The sequence of twitchin\textsuperscript{33,34} showed for the first time that
an intracellular protein can contain Ig and Fn3 domains and
was the first complete sequence of a member of the titin
family of giant cytoskeletal proteins, being reported before
the sequence of vertebrate titin. The crystal structure of the
kinase domain of twitchin was the first crystal structure of a
member of the myosin light chain kinase family\textsuperscript{35} and was
confirmed in a similar structural determination of titin ki-
nase.\textsuperscript{36} The structure of twitchin kinase explained the “auto-
inhibition” of the myosin light chain kinase family of protein
kinases.

The essential features of UNC-89 as a signaling and
scaffolding giant crucial for A-band/M-line assembly were
described \textsuperscript{5 years before the vertebrate homolog obscurin.\textsuperscript{38,39}}
The conserved myosin head chaperone, UNC-45, was first
described in \textit{C. elegans}.\textsuperscript{40–44} Later, vertebrates were found to
have both a striated muscle specific and general cell
UNC-45.\textsuperscript{45}

Although kindlins were first identified in humans (and
recently implicated in inherited diseases of skin and leuko-
cytes/platelets), the first indication that they have a role in
integrin adhesion complexes came from studies in \textit{C. elegans}
on its ortholog, UNC-112.\textsuperscript{46} Studies in \textit{C. elegans}
demonstrated for the first time that kindlin (UNC-112) interacts with
integrin linked kinase (PINCH), and that these 2 proteins
cooperate in vivo.\textsuperscript{18}

\textbf{Strength of \textit{C. elegans} as a Model Genetic System}
Many genes required for muscle assembly or function in \textit{C.
\textit{elegans}} have been identified by forward genetic screens.
These screens were facilitated by the ease of performing
classical genetics in this organism: a fast generation time (3
days), large brood size per adult (\textsim 300), and the small size of
the animal and ease of culture permits large numbers of
animals to be screened for rare mutations (up to 10 000
animals can be grown on a standard-sized Petri dish).\textsuperscript{47}
Moreover, homozygotes can be readily recovered as the
standard mode of reproduction is self-fertilization. (Neverthe-
less, males can be obtained and used in genetic crosses.) As
with mutagenesis in any organism, the concentration of
mutagen is used such that the frequency of obtaining a
desired mutant is practical. However, this results in mutagen-
esis of not only a desired gene but many other genes in the
genome.\textsuperscript{48} To remove most of these background mutations,
the original mutant strain is crossed to wild-type, typically 5
to 7 times. The outcrossing procedure is excellent for remov-
ing mutations on other chromosomes but does not remove
mutations that are closely linked to the desired mutation. To
overcome this problem, multiple mutant alleles are obtained,
and both their phenotypes and phenotypes of heteroallelic
combinations are compared.

Individual mutants are placed into complementation
groups, and, by use of marker strains, mutants are assigned to
1 of 6 chromosomes (5 autosomes and X), and then 2 and 3
factor crosses are performed to genetically map the mutation
within a chromosome.\textsuperscript{49} Finer mapping is often accompli
d
using deficiency strains. There is a correlation between the genetic (recombination) and physical map, which exists in a set of overlapping cosmid and YAC clones available from the *C elegans* stock center. These clones are used in transgenic rescue experiments\textsuperscript{50} to map the mutant gene to a set of overlapping cosmids, 1 cosmid, and then, by use of restriction or PCR fragments, to a single protein coding gene. DNA sequencing for identification of the mutation and RNAi to phenocopy the mutant\textsuperscript{51} can then be used to verify that the gene, first defined through mutation, corresponds to a particular DNA segment.

After mutagenesis, screens for muscle defective mutants can be carried out in the F1 or F2 generations. Because the body wall musculature is required for normal locomotion, one strategy has been direct observation for such mutants—pick up slow-moving animals and observe their body wall musculature by polarized light microscopy for defects in structure, or, more laboriously, pick up animals at random and observe by polarized light microscopy.\textsuperscript{25} Another strategy has been to enrich for mutants with slow movement: mutagenized worms were placed in the center of a large plate containing a ring of bacteria at the perimeter. After allowing worms to crawl out for several hours (worms chemotax to bacteria), the animals that remained in the center were screened for slow movement and by polarized light.\textsuperscript{26}

Other types of screens have searched for extragenic suppressor mutations of genes that confer paralysis or unusual movement: For example, several missense mutations in the head of one of the myosins (encoded by *unc-54*) were recovered as suppressors of the “twitching” phenotype of *unc-22* mutants.\textsuperscript{52} The gene encoding the splicing factor *sup-12* was isolated as an extragenic suppressor of the *unc-60* gene (which encodes ADF/cofilin).\textsuperscript{14,53} Finally, mutations in many different genes have been recovered as extragenic suppressors of the “rigid paralysis” of a particular allele of *unc-105*, which encodes a muscle-specific amiloride sensitive Na\textsuperscript{+} channel.\textsuperscript{54,55} These types of screens resulting in adult Unc genes have probably been saturated and as noted above total 40 genes. However, semiautomated and high-throughput methods for screening adults for mutants having abnormal localization of GFP-tagged proteins\textsuperscript{56} that may in the future use “computer vision” for pattern recognition, and newer methods for assessing nematode motility\textsuperscript{57,58} open the door to identifying additional mutant genes in the future.

Due to the attributes of *C elegans* as a model genetic system, often more sophisticated experiments on the physiology of mutant muscle proteins can be performed than can be performed in either mice or in humans (Table 1). Biochemical analyses of specific protein interactions can be performed in vivo. For example, Hoppe et al\textsuperscript{59} showed that the molecular chaperone UNC-45 requires the ubiquitination-proteasome system for regulation of its in vivo turnover, and Landsverk et al\textsuperscript{60} showed that UNC-45 mediates the turnover of the body-wall myosins by a similar mechanism. Both studies used specific mutations, rescue by transgenes, and RNAi knockdowns in live nematodes.

### Proteomes of Nematodes and Human Heart Are Conserved

As noted above, 20 genes, when mutated, result in the other major phenotypic class of muscle affecting genes, the Pat class. A genome-wide screen resulted in recovery of 16 such genes.\textsuperscript{27} A screen for the Pat phenotype of \textasciitilde 3000 muscle expressed genes by RNAi resulted in recovery of 4 new Pat genes.\textsuperscript{28} This same set of genes was subjected to RNAi and screened for defects in the organization of GFP tagged myosin: 104 new genes were identified. During the past decade, at least 10 new sarcomeric proteins have been identified using screens of a yeast 2-hybrid library with baits consisting of known nematode sarcomeric proteins.\textsuperscript{17} Homology to vertebrate muscle proteins identify at least another 2 dozen proteins. Thus, the 40 Unc, 20 Pat, 104 sarcomere-defective, 10 interacting, and 24 homologous proteins sum to a total of 198. Several Unc and several Pat genes have yet to be defined at the molecular level. As noted below, many human genes mutated in cardiomyopathy have homologous proteins/genes in *C elegans*. At least 7 of these nematode genes are expressed in muscle, but their function in muscle have not been studied (MLP-1, CAV-1, CAV-2, JPH-1, EYA-1, ACL-3, T07D3.6; Table 2). Undoubtedly, new genes will be identified in the future through new mutant hunts, RNAi and 2-hybrid screens, homologies, and so forth. Thus, at least 200 proteins are required for the assembly, maintenance or function of the sarcomere in *C elegans*, and we are still counting.

Most of the proteins mutated in cardiomyopathy have orthologs or homologs in *C elegans*. To date, mutations in at least 42 different genes/proteins have been reported to result in various types of HCM, DCM, or LVNC\textsuperscript{25,8,61} Of these 42 proteins, 35 have orthologs or homologs in the *C elegans* proteome and are expressed in nematode body wall muscle (Table 2). BLAST searches of the *C elegans* genome, and other criteria, failed to reveal homologs for 7 cardiomyopathy-causing genes (Table 3). That only 7 of the 42 known cardiomyopathy causing genes/proteins do not have counterparts in *C elegans* further highlights the validity of using *C elegans* to model human cardiomyopathies.

Table 4 lists 13 genes that we suggest are leading candidates for new genes involved in cardiomyopathies. Each of these *C elegans* genes, when mutant, results in body wall muscle phenotypes and encode proteins that have human homologs, but the human homologs have not yet been reported to be mutated in cardiomyopathies. All except UNC-45 are located at nematode muscle focal adhesions (colored yellow in Figure 3A and 3B). At least for 3 of them, loss of function of homologous proteins in zebrafish and mice result in abnormal heart development or cardiomyopathy. Two of these proteins, UNC-112 and UNC-97, are components of nematode muscle adhesion sites, based on integrin. The counterparts of these sites in human muscle are costameres and intercalated disks.

Although *C elegans* has only 1 kindlin called UNC-112, vertebrates have 3 kindlins. Kindlin-2 is the only kindlin expressed in the heart, and the protein is localized at intercalated discs and costameres.\textsuperscript{62} Mophilino-induced knockdown of Kindlin-2 in zebrafish results in severe abnormalities
In heart development: hearts are abnormally shaped with thin-walled chambers, weak contractions, and ultrastructurally disrupted intercalated disks and failure of attachment of myofibrils to membrane complexes.62

The vertebrate ortholog of UNC-97,63 is called PINCH. In mammals, there are 2 such proteins, PINCH1 and PINCH2. Mice that are doubly homozygous null for PINCH1 and PINCH2 specifically in the myocardium are viable at birth but develop dilated cardiomyopathy and die of heart failure within 4 weeks.64 The mutant hearts display disruptions of intercalated disks and costameres, together with fibrosis.

Another example that might be either a candidate gene or a genetic modifier for cardiomyopathy is the gene that encodes the myosin head chaperone, UNC-45. In zebrafish, morpholino-induced knockdown of the striated muscle specific UNC-45b results in abnormal cardiac development and function, including improper cardiac looping, lack of ventricular pumping and circulation, and cardiac edema.65 This

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<td></td>
<td></td>
<td>unc-22</td>
<td>Twitchin*</td>
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<td>HCM</td>
<td>cav-1, cav-2</td>
<td>CAV-1, CAV-2</td>
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<tr>
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<td>Vinculin/metavinculin</td>
<td>HCM</td>
<td>deb-1</td>
<td>Vinculin</td>
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<td>α-actinin 2</td>
<td>HCM, DCM</td>
<td>atn-1</td>
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<tr>
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<td>Junctophilin-2</td>
<td>HCM</td>
<td>jph-1</td>
<td>JPH-1</td>
</tr>
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<td>Obscurin</td>
<td>HCM</td>
<td>unc-89</td>
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</tr>
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<td>Lamin A/C</td>
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<td>DCM</td>
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<td>Four and half LIM protein-2 (FHL2)</td>
<td>DCM</td>
<td>lim-9</td>
<td>LIM-9</td>
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</tbody>
</table>

LVCN indicates left ventricular noncompaction cardiomyopathy; WPW, Wolff-Parkinson-White syndrome.

*Twitchin is a functional homolog of both myosin binding protein C and titin.

Not included: mutations in 6 proteins that result in arrhythmogenic right ventricular cardiomyopathy (ARVC).

in heart development: hearts are abnormally shaped with thin-walled chambers, weak contractions, and ultrastructurally disrupted intercalated disks and failure of attachment of myofibrils to membrane complexes.62

The vertebrate ortholog of UNC-97,63 is called PINCH. In mammals, there are 2 such proteins, PINCH1 and PINCH2. Mice that are doubly homozygous null for PINCH1 and PINCH2 specifically in the myocardium are viable at birth but develop dilated cardiomyopathy and die of heart failure within 4 weeks.64 The mutant hearts display disruptions of intercalated disks and costameres, together with fibrosis.

Another example that might be either a candidate gene or a genetic modifier for cardiomyopathy is the gene that encodes the myosin head chaperone, UNC-45. In zebrafish, morpholino-induced knockdown of the striated muscle specific UNC-45b results in abnormal cardiac development and function, including improper cardiac looping, lack of ventricular pumping and circulation, and cardiac edema.65 This

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Disorder</th>
<th>Gene</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAP</td>
<td>Telethonin</td>
<td>HCM, DCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYOZ2</td>
<td>Myozin 2</td>
<td>HCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
<td>HCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCN5A</td>
<td>Cardiac sodium channel</td>
<td>DCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td>Desmin</td>
<td>DCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMPO</td>
<td>Thymopalladin</td>
<td>DCM</td>
<td></td>
<td></td>
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<tr>
<td>MYPN</td>
<td>Myopalladin</td>
<td>DCM</td>
<td></td>
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</tr>
</tbody>
</table>

Table 3. Genes/Proteins Mutated in Humans That Cause Cardiomyopathy But Without Corresponding Genes/Proteins in Caenorhabditis elegans

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAP</td>
<td>Telethonin</td>
<td>HCM, DCM</td>
</tr>
<tr>
<td>MYOZ2</td>
<td>Myozin 2</td>
<td>HCM</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
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<tr>
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<td>Cardiac sodium channel</td>
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</tr>
<tr>
<td>DES</td>
<td>Desmin</td>
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<tr>
<td>TEMPO</td>
<td>Thymopalladin</td>
<td>DCM</td>
</tr>
<tr>
<td>MYPN</td>
<td>Myopalladin</td>
<td>DCM</td>
</tr>
</tbody>
</table>
result was confirmed by characterizing a stop codon mutation in the UNC-45b gene: these mutants show a lack of heart beating and disorganized myofibrils in cardiomyocytes.66 These results have recently been confirmed in the mouse, in which mutations in UNC-45b result in arrest of cardiac development and function (D. Chen, S. Li, R. Singh, S. Spinette, R. Seidlmeier, and H.F. Epstein, unpublished results). In contrast to the zebrafish and mouse studies on the UNC-45 phenotype, the authors were able to conclude that UNC-45 is most crucial during the remodeling that occurs during the formation of the adult heart. However, because some defects were seen when knockdown was initiated after the metamorphosis, the authors were able to conclude that UNC-45 is also required for cardiac myofibril maintenance during adulthood.

A final example is UNC-82,68 a member of the AMP-activated protein kinase (AMPK) family of protein kinases. Missense mutations in the gene PRKAG2, which encodes the γ regulatory subunit of AMPK, result in Wolff-Parkinson-White syndrome.69 Wolff-Parkinson-White syndrome is characterized by hypertrophic cardiomyopathy, ventricular preexcitation, and conduction abnormalities. AMPK is thought to play a major role in the synthesis and distribution of ATP through regulating carbohydrate and fatty acid metabolism70 and interacting with multiple signaling pathways.71 However, the phenotype of unc-82 loss of function mutants in C elegans, suggests that AMPK and related protein kinases may have an additional (or even alternative) function in the assembly or stability of the sarcomere. unc-82 mutations result in disorganized sarcomeres, especially in mislocalization of thick filament and M-line components.68 UNC-82 is localized to M-lines and is a candidate kinase that phosphorylates myosin heavy chain and paramyosin.

### Interactions Between Membrane, Cytoskeleton, and Sarcomere Proteins Are Conserved Between Nematodes and Humans

#### MHC B and Twitchin in C elegans/MHC 7 and Titin in Human Heart

Twitchin is a 754 000–Da polypeptide encoded by the unc-22 gene and first described in C elegans.33,34,72 It consists of a single protein kinase domain (homologous to myosin light chain kinase and titin kinase domains), 31 Fn3, and 30 Ig domains. These are arranged into patterns, mostly Ig-(Fn3)2, which is similar to the arrangement of these domains in the A-band portion of human titin. Indeed, the pattern of organization of Ig and Fn3 domains is conserved between twitchin and human titin for the 16 domains surrounding the protein kinase domains.73

Antibodies localize twitchin to the outer portions of A-bands, colocalizing with MHC B.74 This localization is consistent with at least some portion of twitchin interacting with MHC B, although a biochemical experiment demonstrating this expectation has not been reported. Nevertheless, genetic evidence supports such an interaction. Even before unc-22 had been identified at the molecular level, an association of the unc-22 gene product and MHC B had been suspected: Only rare missense alleles of unc-54, residing in the head domain, were found to suppress unc-22 mutants; mutations in no other genes were identified despite exhaustive screening.52 In the unc-54 mutant, st273, thick filaments are severely disorganized. In unc-54(st273), clumps of MHC B, outside of normal A-bands, colocalize with clumps of twitchin.74

Finally, it should be pointed out that given its giant size and domain organization, there is a high likelihood that twitchin, like titin, is a scaffold interacting with multiple proteins, although the requisite studies have not yet been conducted. Nevertheless, progress is being made in finding binding partners for the similar giant Ig domain containing protein, UNC-89.75–77

In vertebrate muscle, titin polypeptides are 3 to 4 MDa, spanning half a sarcomere, about 1.2 μm in the relaxed state, with N-termini anchored at the Z-disk, and C-termini anchored at the M-line.78,79 The A-band portion of titin is associated with the thick filament shaft; it is estimated that there are 6 titin polypeptides per half-thick filament. The

### Table 4. Caenorhabditis elegans Genes/Proteins With Human Homologs That When Mutant Display Body Wall Muscle Phenotypes But Have Not Yet Been Implicated in Cardiomyopathy

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Phenotype</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>unc-52</td>
<td>Perlecan</td>
<td>Pat and UNC</td>
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<tr>
<td>pat-3</td>
<td>β-integrin</td>
<td>Pat</td>
<td></td>
</tr>
<tr>
<td>pat-2</td>
<td>α-integrin</td>
<td>Pat</td>
<td></td>
</tr>
<tr>
<td>unc-112</td>
<td>Kindlin</td>
<td>Pat and UNC</td>
<td>Knockdown in zebrafish—abnormal heart development</td>
</tr>
<tr>
<td>pat-6</td>
<td>Actopaxin</td>
<td>Pat</td>
<td></td>
</tr>
<tr>
<td>unc-97</td>
<td>PINCH</td>
<td>Pat and UNC</td>
<td>Knockout in mice—cardiomyopathy</td>
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<tr>
<td>cpxn-1</td>
<td>CPNA-1</td>
<td>Pat</td>
<td></td>
</tr>
<tr>
<td>pxl-1</td>
<td>Paxillin</td>
<td>Mislocalization of an M-line protein</td>
<td></td>
</tr>
<tr>
<td>scpl-1†</td>
<td>SCPL-1</td>
<td>Increased maximum bending</td>
<td></td>
</tr>
<tr>
<td>zyx-1†‡</td>
<td>Zyxin</td>
<td>Reduced maximum bending</td>
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</tr>
<tr>
<td>unc-45</td>
<td>UNC-45</td>
<td>Pat and UNC</td>
<td>Knockdown in zebrafish—disrupts heart sarcomeres</td>
</tr>
<tr>
<td>csn-5</td>
<td>CSN-5</td>
<td>Altered accumulation of M-line proteins</td>
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<tr>
<td>cpn-1</td>
<td>Protein</td>
<td>N</td>
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</tr>
</tbody>
</table>

**Table Notes:**
- †‡The gene also encodes the thick filament core protein γ-filagenin. A single mRNA encodes each protein by use of separate translational starts and reading frames that are partially overlapping and shifted by 1 base (A. Hutagalung and H.F. Epstein, unpublished data).

**Gene Protein Phenotype Notes**

- **Gene**
  - csn-5: CSN-5
  - pkn-1: Protein kinase N

- **Protein**
  - Pat and UNC
  - Pat
  - Pat
  - Pat and UNC
  - Pat
  - Pat
  - Pat and UNC
  - Pat
  - Mislocalization of an M-line protein
  - Increased maximum bending
  - Reduced maximum bending
  - Pat and UNC
  - Pat
  - N

- **Phenotype**
  - Altered accumulation of M-line proteins
  - Knockdown in zebrafish—disrupts heart sarcomeres
  - Knockout in mice—cardiomyopathy
  - Pat and UNC
  - Pat
  - Pat
  - Pat
  - Pat
  - Pat
  - Pat
  - Pat
  - Pat
  - Pat

- **Notes**
  - Knockdown in zebrafish—abnormal heart development
  - Knockout in mice—cardiomyopathy
  - Knockdown in zebrafish—disrupts heart sarcomeres
  - Mislocalization of an M-line protein
  - Increased maximum bending
  - Reduced maximum bending
  - Pat and UNC
  - Pat
  - N

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A-band part of titin is mainly composed of immunoglobulin (Ig) and fibronectin type 3 (Fn3) domains arranged in specific patterns that vary with the position along the thick filament. There is a single protein kinase domain near the M-line. The I-band part of titin forms an elastic connection between the end of the thick filament and the Z-disk. It provides restoring force to allow sarcomeres to maintain a set resting length. The I-band part of titin consists of many tandem copies of Ig domains, a PEVK domain, and specific for cardiac muscle, an N2B domain. The I-band region of cardiac titin can be modeled as a molecular spring,\(^\text{80}\) which may explain elastic recoil in early diastole.\(^\text{81}\)

Titin, with its enormous length, placement in the sarcomere, and its many Ig and Fn3 domains, can be regarded as a molecular scaffold and has been shown to interact with many different sarcomeric and signaling proteins.\(^\text{79}\) Myosin-binding protein C (MyBP-C), also consisting of multiple Ig and Fn3 domains, and mutated in many cases of cardiomyopathy, binds repeatedly along the titin A-band portion by interacting with the first Ig domain of an 11 domain super-repeat of Ig and Fn3 domains that is present in 11 copies.\(^\text{82}\) A-band titin and myosin are tightly associated via Fn3 domains of titin.\(^\text{83}\) Several groups have reported that A-band titin binds to the C-terminal most (\(\approx 20 \text{ nm}\)) section of light meromyosin that forms the shaft of the thick filament.\(^\text{84}–\text{86}\)

However, 2 labs have reported binding of A-band titin to myosin S1 heads.\(^\text{87,88}\)

Currently, at least 19 different mutations, mostly stop codon and frame shift mutations in human titin, result in muscle disease.\(^\text{78,79}\) The most frequent disease is DCM (at least 8 mutations), but HCM also is represented, as are several skeletal muscle–specific diseases, including a late onset tibial muscular dystrophy, limb girdle muscular dystrophy 2J, and hereditary myopathy with early respiratory failure. All segments of titin have been hit by mutations that result in muscular dystrophy, limb girdle muscular dystrophy 2J, and immunostained with antibodies to twitchin. Twitchin is localized to most of the A-band, except for the central portion (indicated by \textit{arrow}). (Reprinted with permission of the American Society for Clinical Investigation from Yang Q, Sanbe A, Osinska H, Hewett TE, Klevitsky R, Robbins J. A mouse model of myosin binding protein C human familial hypertrophic cardiomyopathy. J Clin Invest. 1998;102:1292–1300.)

A-band part of titin associated with cardiomyopathy were described.\(^\text{89,90}\) Probably, the relatively small number of disease-causing mutations identified is due to the difficulty in sequencing such a large coding sequence and complex gene from individual patients. Also, given the huge size of the coding sequence (\(\approx 100 \text{ kb}\)), it is difficult to distinguish disease-causing missense mutations from polymorphisms. However, even polymorphisms may contribute to disease presentation and progression. Familial HCM and DCM are caused by mutations in many different, mostly sarcomeric proteins. One cardinal feature is that there is considerable phenotypic diversity between families having mutation in the same gene, attributed to variable expressivity and incomplete penetrance.

An excellent candidate for a cause of this diversity is polymorphism within the titin gene, given the fact that titin interacts with so many different proteins and is involved in sarcomere assembly and signaling. Given the advent of next-generation sequencing methods, there is good reason to expect that many more disease-causing titin mutations, including missense mutations, and disease-altering polymorphisms will be described in the near future.

\textbf{Twitchin Is Also a Homolog of MyBP-C}

Nematode twitchin is also a homolog of the vertebrate protein MyBP-C, which is frequently mutated in cardiomyopathy patients. Like twitchin and titin, MyBP-C consists almost entirely of Ig and Fn3 domains.\(^\text{92}\) The similarity between twitchin and MyBP-C is even more compelling at the functional level. First, each protein is localized to most of the A-band, except for the central region. This can be appreciated at the level of immunofluorescence\(^\text{57,91}\) (Figure 4). Furthermore, by immunogold EM, in skeletal muscle, MyBP-C is localized to a series of 7–9 transverse stripes, spaced at 43-nm intervals.\(^\text{54,95}\) A similar level of resolution for twitchin localization has not been achieved. Second, both MyBP-C and twitchin are estimated to have multiple molecules per thick filament, as compared with only 6 titin molecules per half-thick filament.\(^\text{96}\) MyBP-C is estimated to have 36 to 50 molecules per thick filament.\(^\text{97}\) Based on considering the domain organization of twitchin and electron microscopic images of rotary shadowed molecules (W. Sale and G. Benian, unpublished results), the contour length of twitchin is approximately 172 nm. Twitchin is localized throughout most of the A-band, except for the middle.\(^\text{74}\) Adult muscle thick

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\end{figure}
filaments are 9.7 μm long.\textsuperscript{13,98} Therefore, multiple twitchin molecules (≈30–60) are likely to interact with the shaft of the nematode thick filament.

Third, there is evidence that both MyBP-C and twitchin interact with both myosin and actin. As noted above, genetic analysis in \textit{C elegans} suggests an interaction between twitchin and myosin. The C-terminal most Ig domain of MyBP-C interacts strongly with myosin.\textsuperscript{99} Insight into the physiological function of twitchin has come from study of twitchin in mollusks. This has arisen by analysis of the “catch state” of smooth muscle in Mytilus.\textsuperscript{100} In the catch state, high tension is maintained for extended periods with a very low rate of ATP hydrolysis and allows bivalve mollusks to keep their shells firmly closed. The release of catch is associated with a cAMP-dependent phosphorylation of twitchin.\textsuperscript{100} Moreover, molluscan twitchin has been shown to bind to both myosin and F-actin, and there is additional evidence that the “catch state” involves twitchin forming a physical linkage between thick and thin filaments.\textsuperscript{101,102} MyBP-C has also been shown to interact with F-actin in vitro, via its N-terminal region.\textsuperscript{103–106} Recently, electron tomography of intact muscle has demonstrated that MyBP-C does bind to actin.\textsuperscript{107} In fact, MyBP-C was directly visualized as forming a physical bridge between thick and thin filaments, independent of myosin heads.

Because of the much larger sarcomeres in nematode muscle, there is no single polypeptide that spans the A and I bands like titin in vertebrate muscle. In \textit{C elegans}, the largest polypeptide predicted from sequence analysis and confirmed by Western blot is TTN-1, which has a molecular mass of 2.2 MDa.\textsuperscript{108,109} The N-terminus of TTN-1 is anchored at the dense body (Z-disk equivalent), and the molecule extends through the I-band and may reach the outer edge of the A-band. The domain organization of TTN-1 also is similar to the I-band portion of titin; there is a predominance of tandem Ig domains and several long regions composed of short tandem repeats that are likely to function as elastic elements. A polymer of 3 different molecules in nematode muscle might provide the function of a single molecule of vertebrate titin. Beginning at the M-line, perhaps 1 molecule of UNC-89 connects to a polymer of twitchin molecules (serving the function of A-band titin and MyBP-C) in the A-band; at the outer edge of the A-band, perhaps a part of one of the twitchin molecules interacts with the C-terminal portion of TTN-1, and then a single TTN-1 molecule (serving the function of I-band titin) extends through the length of the I-band and connects its N-terminus to the dense body.

\section*{UNC-89 and RHO-1 in \textit{C elegans}/Obscurin and RhoA in Human Heart}

In \textit{C elegans}, \textit{unc}-89 mutants display a disorganization of the myofilament lattice, usually lack M-lines, and have decreased locomotion.\textsuperscript{25,110} \textit{unc}-89 encodes 6 major polypeptides, ranging in size from 156 000—900 000 Da.\textsuperscript{37,111,112} The largest of these isoforms consists of 53 Ig domains, 2 Fn3 domains, 2 protein kinase domains at its C-terminus, and SH3, DH, and PH domains at its N-terminus. Antibodies localize UNC-89 to the M-line. The human homolog is called “obscurin.”\textsuperscript{38,39,113} Various obscurin isoforms are located at either the M-line, A/I junction, or at the Z-disk and Z/I junction.\textsuperscript{114}

In other proteins, DH domains, usually followed by PH domains, are known to act as guanine nucleotide exchange factors (GEFs) for Rho family GTPases. The DH domain of UNC-89 has been shown to have exchange activity for RHO-1 (RhoA in \textit{C elegans}) but not for CED-10 (Rac), CDC-42 (Cdc42), or MIG-2 (RhoG).\textsuperscript{76} Partial knockdown of \textit{rho-1} in \textit{C elegans} adults showed a pattern of disorganization of myosin thick filaments similar to the pattern found in \textit{unc-89(su75)}, a mutant allele which is lacking all of the UNC-89 isoforms that contain the DH domain.\textsuperscript{76,111} It is proposed that the DH domain of UNC-89 activates RhoA for organization of myosin filaments in nematode muscle cells. This interaction is conserved for vertebrate obscurin.\textsuperscript{115} The DH domain of obscurin was shown to bind to RhoA, and RhoA was shown to colocalize with obscurin at M-lines in skeletal muscle. Overexpression of the DH domain in skeletal muscle was found to increase RhoA expression and, more importantly, activity, and also resulted in increased Rho-kinase and decreased expression of citron kinase (known RhoA effectors in other cell types). Intriguingly, contraction-induced injury altered the localization of RhoA from M-lines, to M-lines, Z-disks, and Z/I junctions, and resulted in an increase in RhoA activity.

It has long been known that the Rho GTPases are involved in the organization of actin filaments, especially in nonmuscle cells such as fibroblasts and epithelial cells.\textsuperscript{116} In smooth muscle, the activation of RhoA and its downstream effector Rho-kinase promotes muscle contraction. This occurs through Rho-kinase phosphorylating regulatory myosin light chains and by phosphorylating myosin light chain phosphatase (inactivating this phosphatase, which also results in higher levels of phosphorylation of myosin light chains). The studies cited above showing a functional interaction of UNC-89 or obscurin with RhoA in muscle indicates that RhoA also functions in the organization of striated muscle thick filaments, again probably through Rho-kinase. Earlier studies had shown that Rho and Rho-kinase promote cardiac hypertrophy,\textsuperscript{117} reperfusion injury after acute myocardial infarction,\textsuperscript{118} and remodeling in congestive heart failure.\textsuperscript{119} Perhaps in the future inhibition of the obscurin/RhoA interaction can be exploited as a treatment for these conditions.

Currently, only a single patient with HCM has been found to be heterozygous for a sequence variant in obscurin.\textsuperscript{120} Linkage analysis revealed a variation in one region (Ig58–Ig59) of obscurin that interacts with a Z-disk segment of titin, specifically an arginine to glutamine change in Ig58. This change was shown to reduce binding of obscurin to titin in vitro and to reduce the localization of obscurin to the Z-disk in transfected neonatal rat cardiomyocytes. Additional evidence implicates obscurin in cardiac hypertrophy: obscurin is upregulated transcriptionally during early stages of cardiac hypertrophy induced in mice by aortic constriction.\textsuperscript{121} Obscurin was identified only 10 years ago and it has a large coding sequence (≈25 kb). Thus, like titin, with modern sequencing technology, it is likely that additional cardiomyopathy causing mutations in obscurin will be found in the near future.
Future Directions
What can research in C elegans contribute further to our understanding of human cardiomyopathies? Three areas that appear most promising are (1) providing an environmentally controlled, genetically homogeneous in vivo medium for evaluating mutations of interest; (2) permitting a sensitive in vivo assay for testing the significance of specific protein-protein interactions; and (3) continued discovery of new proteins by genetics that prove to be either homologs or orthologs of human cardiac proteins.

The value of an environmentally controlled, genetically homogeneous in vivo medium cannot be underestimated. Most if not all, the mutations causing cardiomyopathies show considerable incomplete penetrance and variable expressivity. These characteristics arise from the considerable genetic diversity of humans, and, to some extent, environmental heterogeneity in which they live that act as modifiers of mutant gene expression. The standard C elegans N2 strain is genetically homogeneous because its self-fertilizing hermaphrodite sex permits clonal reproduction. Furthermore, the standard strain or any established mutants derived from it can be stored as larvae frozen in liquid nitrogen and recovered at will. New stocks are obtainable routinely from a central C elegans repository (the Caenorhabditis Genetics Center). Nematodes are maintained on a standard Escherichia coli strain on agar plates at controlled temperatures. These features make C elegans superior for genetics to any vertebrate or mammalian organism, and possibly even Drosophila.

The body wall muscle cells of C elegans provide a highly sensitive assay for studying protein-protein interactions. The sarcomeres and membrane attachments of C elegans muscle and human cardiomyocytes are exquisite molecular machines that depend on the functional interactions between their protein components. The tools of nematode motility function, polarized light and electron microscopic evaluation of muscle structure, and immunofluorescent microscopic localization of specific proteins have all well-developed applications in C elegans muscle. Polarized light and GFP-related fluorescence microscopy permit ready evaluations in live nematodes using standard equipment in the laboratory.

The study of mutant nematode lines continues to produce discovery of new proteins expressed in humans also and demonstrate the significance of loss-of-function of known proteins expressed in both species. In addition to in vivo analysis, current methods permit biochemical experiments and molecular analysis to be performed in lysates or purified fractions of wild-type and mutant nematodes. This area is likely to benefit from future advances in methodology that will expand our capabilities of in vitro work in C elegans and lead to a greater understanding of the protein complexes affected in human cardiomyopathies.

Acknowledgments
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None.

References


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