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Review

Genetic analysis of myosin II assembly and organization in model organisms

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Abstract. Myosins are a large family of actin-based motor proteins that are involved in a variety of cellular processes. Class II, or conventional, myosins are organized into a number of multi-component structures such as muscle thick filaments, non-muscle filaments and the actomyosin ring during cell division. A number of conditions must be met for the proper assembly and organization of myosin II-containing structures, including the correct stoichiometry of myosin and its associated proteins, and the conformation and regulation of the myosin molecule itself by molecular chaperones and protein kinases. In this review we discuss the use of model organisms in the genetic analysis of the assembly and organization of myosin-containing structures.

Key words. Myosin II; genetics; assembly; proteins.

Introduction

Myosins are actin-based motors that play critical roles in a variety of cellular processes, including cytokinesis, cellular trafficking, phagocytosis, maintenance of cell shape and muscle contraction [1, 2]. At least 18 classes of myosins have been identified to date, with each class showing a distinct function [3, 4]. Conventional myosins, also known as class II, make up the largest and most extensively studied group. The rest of the myosins are referred to as unconventional, with classes I and V being the best characterized to date.

All myosins are composed of a heavy chain with a well-conserved N-terminal head domain, followed by a 'neck' region usually containing one or more IQ motifs, and a C-terminal class-specific tail. The head domain is responsible for ATP hydrolysis and binding to F-actin, whereas the neck region binds light chains and calmodulin through IQ motifs. Tail domains of the various classes of myosin vary greatly in sequence and length and are thought to play a major role in class-specific functions [2]. Members of the myosin II class are hexameric proteins that are composed of two heavy chains, two essential light chains, and two regulatory light chains (fig. 1A). Each myosin II molecule has a two-headed structure due to the dimerization of the heavy chains. Proteolysis of myosin II can be used to cleave the protein into its individual domains. Most proteases cleave myosin II at the base of the neck, generating heavy meromyosin (HMM) containing the head and neck region, and light meromyosin (LMM), a rod-like tail fragment composed of an α-helical coiled coil. The ability to form filaments through the self-association of this rod-like tail is unique to class II myosins. HMM can then be further proteolytically digested into the neck region (subfragment 2 or S2) and two single heads (subfragment 1 or S1).

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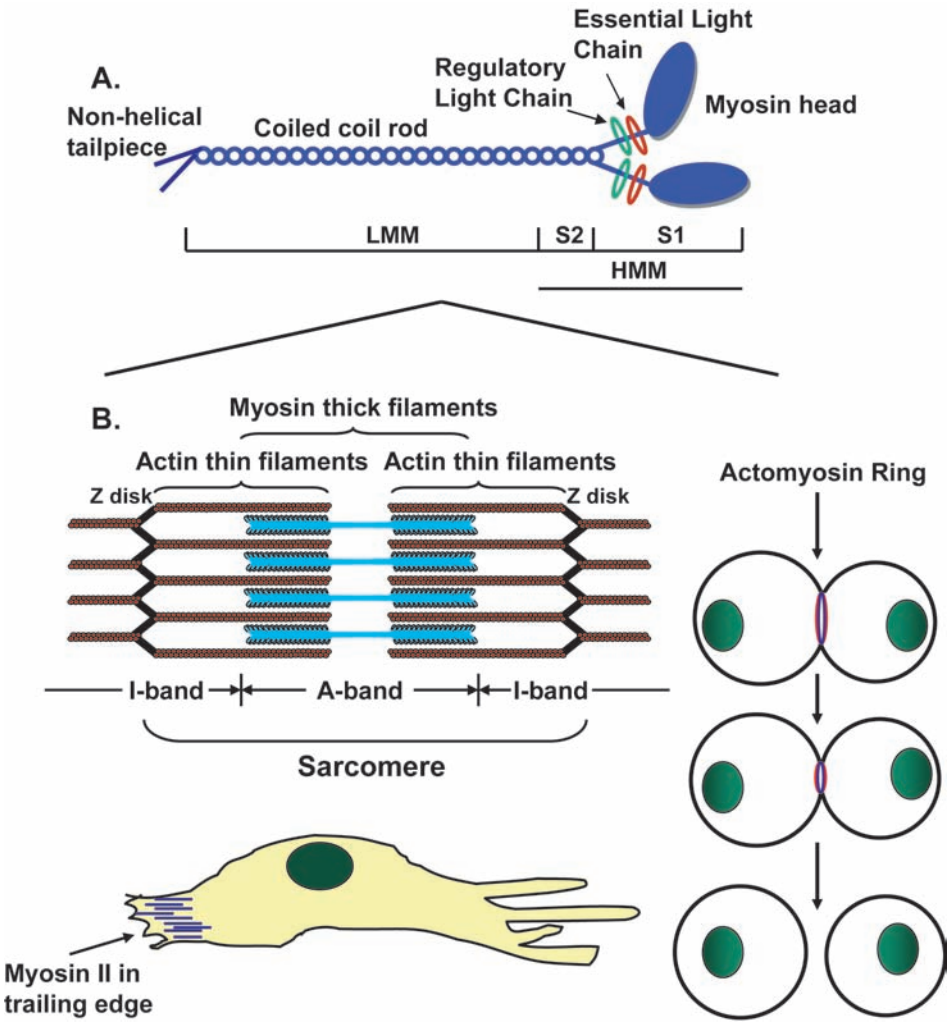

Figure 1. Myosin II-containing structures. (*A*) Myosin II is a hexameric complex of two heavy chains and two pairs of essential and regulatory light chains. The coiled-coil rod allows for dimerization of the heavy chains and assembly into filamentous structures. Myosin can be enzymatically cleaved into heavy and light meromyosin (HMM and LMM, respectively). HMM can then be further cleaved into subfragments 1 and 2 (S1 and S2, respectively). (*B*) Myosin II is found in a number of assembled structures, including muscle thick filaments, non-muscle filaments such as those in the posterior cortex of a migrating cell, and the actomyosin ring during cell division.

Many myosins are found in large, multi-component structures containing a variety of proteins. The genetic approach has proven to be an invaluable tool for studying myosin-containing structures, their regulation and assembly, and the role of myosin-interacting proteins. The availability of screening methods and analysis of mutations according to phenotype allow for the identification of genes involved in formation of myosin-containing structures. The use of a variety of different organisms in the study of conventional class II myosin highlights its numerous functions. *Caenorhabditis elegans* and *Drosophila melanogaster* continue to provide new insights through genetic dissection of striated muscle development and thick filament organization. Genetic experiments in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have determined the role of myosin and associated proteins in the formation of the actomyosin ring during cell division. Similarly, in *Dictyostelium discoideum* genetic analyses have revealed a role for phosphorylation in myosin motor function and myosin filament regulation. This review focuses on the organization of myosin, in particular that of class II. We will discuss the regulated organization and assembly of myosin II into structures such as muscle thick filaments, non-muscle filaments and the actomyosin ring (fig. 1B). The role of different myosin domains, requirement for proper stoichiometry and availability of accessory proteins, as well as regulation through phosphorylation will be highlighted.

Regulated levels of myosin II are required for proper structural organization

Class II myosin is a major component of thick filaments in muscle. *C. elegans* and *Drosophila* contribute the method of genetic dissection and in vivo development to the study of muscle structure. While the basic protein components of the contractile units of these organisms are similar, a number of differences are observed in overall organization, including assembly and the presence of accessory proteins.

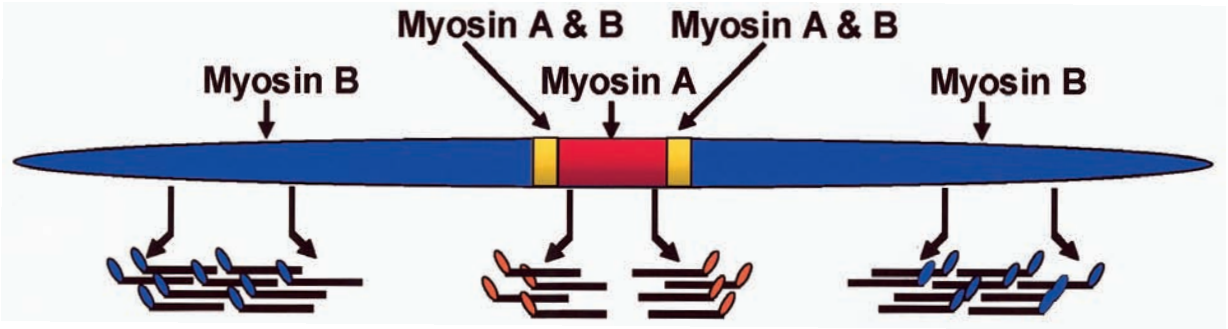

Figure 2. Organization of myosin heavy chains in *C. elegans* thick filaments. Myosin A is located in the central 1.8-μm region of the thick filament and is flanked on either side by myosin B at the polar arms of the thick filament. Both myosins can be found in a region of overlap on either side of the central myosin A region. Myosin A packs in an anti-parallel fashion to produce a central bare zone in the center of the filament. Myosin B packs in a parallel fashion to form the polar regions. Worms lacking myosin A arrest at the embryonic two-fold stage and do not elongate. In contrast, worms deficient in myosin B are viable but severely paralyzed, with a reduced number of thick filaments consisting entirely of myosin A.

C. elegans has four different muscle myosin heavy-chain (MHC) genes that each encode a different myosin II heavy chain [4, 5]. Heavy chains A and B are primarily expressed in body-wall muscle, while heavy chains C and D are found only in the pharynx [6]. The genes encoding the four isoforms A, B, C and D are *myo-3*, *unc-54*, *myo-2* and *myo-1,* respectively [7]. All *C. elegans* body-wall muscle cells contain myosin A and myosin B [8, 9]. These myosins are primarily homodimeric with respect to their heavy chains, and have a differential distribution within the thick filament (fig. 2) [8, 10, 11]. Myosin B represents approximately 80% of the body-wall muscle myosin and is localized at the polar arms of the thick filament, whereas myosin A composes approximately 20% of the body-wall muscle myosin and is restricted to a central ~1.8-μm region. Two small junctional regions on either side of the central region contain both myosins [8]. The bipolar central region contains the site at which initiation of the thick filament is thought to occur. In this region, myosin A molecules associate in an antiparallel fashion. Myosin B is then added in a parallel fashion to flanking sides of this central region. The distribution of the two MHCs implies that they have distinct roles during the assembly and maintenance of a thick filament.

Genetic analysis also supports the differing roles of myosin A and myosin B in the assembly and organization of *C. elegans* thick filaments. Worms that lack myosin A die during embryonic development at the two-fold stage. Analysis of these embryos reveals improper muscle development with severely impaired thick filament assembly, indicating a role of myosin A in the initial assembly of the thick filaments [12]. In contrast, mutants that lack myosin B are viable but severely paralyzed, exhibiting an uncoordinated phenotype [4]. These worms have a reduced number of thick filaments containing only the myosin A isoform, indicating that myosin A dimers are therefore capable of parallel packing in addition to the antiparallel arrangement seen in the central region of the thick filament [13, 14]. This has been substantiated by the fact that increasing the copy number of the myosin A gene, *myo-3*, can rescue thick filament defects and movement in myosin B null animals [15–17]. The inverse is not true; overexpression of myosin B cannot rescue myosin A null animals [12]. This indicates that the function of myosin A is essential and cannot be rescued by myosin B. Levels of myosin B also appear to be important for correct muscle development since wild-type worms containing high copy numbers of *unc-54* have defects in muscle structure and disorganized arrangements of myosin A and B [15].

Unlike *C. elegans* and vertebrates which express muscle-specific myosin from different genes, *Drosophila melanogaster* only has one muscle myosin heavy chain gene (*Mhc)* that gives rise to multiple heavy chain isoforms by alternative RNA splicing [18–20]. Of the 19 exons that make up the *Mhc* gene, 5 are alternatively spliced and one is either excluded or included. The alternative splicings occur in a development stage- and tissue-specific manner with at least 14 different isoforms expressed during development [21–23]. Isoform usage also differs among the various muscle types in *Drosophila* [18].

Expression of the *Mhc* gene is essential for both larval and adult muscle function, as homozygous nulls die before hatching without any type of muscular movement [24]. Lethality results from a complete lack of thick filament formation, indicating the absolute requirement for Mhc in thick filament assembly of *Drosophila*.

As discussed for *C. elegans*, the amount of wild-type muscle myosin is important for proper muscle function in the fly. Heterozygous *Mhc* nulls have nearly a 50% decrease in levels of Mhc protein, which leads to an overall decrease in thick filaments. The effect of this decrease is variable among different muscles [24, 25]. The larval intersegmental muscle, which does not form

a regular array of myofilaments, is not affected by the decrease in thick filaments. However, the *indirect flight muscle (IFM)* and the tergal depressor of the trochanter muscle (TDT), which both contain a more organized lattice, are more sensitive to the loss of thick filaments. Transverse sections of indirect flight muscles show that the hexagonal array of interdigitating thick and thin filaments in the center of myofibrils appears similar to wild type but becomes increasingly disorganized towards the periphery [25]. This defect appears to be a result of myosin in the thick filaments being unable to interact properly with actin in the thin filaments. Flies containing the *Mhc36B* mutation, which is specific for the loss of Mhc in flight muscles, contain no flight muscle thick filaments and have myofibrils that consist only of thin filament arrays. The inverse is also true; flies that are homozygous nulls for actin in flight muscles have myofibrils consisting of thick filaments. However, those thick filaments are disorganized [26]. These results indicate that thick and thin filaments in the muscle cells of *Drosophila* arise independently, but proper interaction between thick and thin filaments is required for correct muscle function. In fact, the phenotype of double heterozygotes of both myosin and actin genes is closer to wild type than that of either single heterozygote [26]. This suggests that the correct stoichiometry between myosin-containing thick filaments and actin-containing thin filaments is required for proper sarcomere assembly and maintenance. Similar to the situation in *C. elegans*, overexpression of the *Mhc* gene also causes morphological defects in muscle structure. Transgenic flies with four copies of the *Mhc* gene have excess thick filaments at the periphery of myofibrils and have defects in both flying and jumping ability [27]. Increasing the gene copy number to greater than four results in lethality, indicating that correct myosin heavy chain levels are critical for development in *Drosophila.*

The importance of having correct levels of class II myosin in formation of the actomyosin ring during cell division has also been shown through deletion and overexpression studies in the fission yeast *Schizosaccharomyces pombe*. *S. pombe* contains two class II myosins encoded by *myo2* and *myp2*. While both myosins are involved in formation and maintenance of the actomyosin ring, they appear to have different functions. Myo2p is essential for viability and cytokinesis, whereas Myp2p is required for cytokinesis only under certain stress and temperature conditions [28–30].

Recent studies have proposed that *S. pombe* contains organized myosin II structures prior to actomyosin ring formation [31]. Myo2p is observed in progenitor spots that are then assembled into the ring structure [31, 32]. These spots appear to originate from the actomyosin ring of the previous mitotic event and may exist as a template for assembly during the next round of cell division. However, the possibility that these spots arise de novo has not been entirely ruled out. These myosin-containing spots are essential for proper cell division. Deletion of *myo2* results in spores that are able to germinate and grow, but are unable to divide, and die with an elongated or branched morphology. Overexpression of *myo2* also alters cell division. Cells fail to form a proper actin ring, and actin accumulates in the myosin spots, suggesting that it is being sequestered there by the overproduction of myosin [28, 33]. These results again highlight the importance of proper stoichiometry of myosin protein into assembled structures; if the required amount of myosin is not available, the actomyosin ring does not form at all.

The second class II myosin involved in cell division in *S. pombe*, Myp2p, appears to play a role in maintaining the integrity of the actomyosin ring once formed, as well as facilitating constriction of the ring. In cells lacking Myp2p, the actomyosin ring persists until separation, while in wild-type cells the ring disappears before separation, suggesting that a class II myosin may also play a role in facilitating the disassembly as well as assembly of the actomyosin ring [30].

In the budding yeast *Saccharomyces cerevisiae,* cell division is carried out by the concerted actions of actomyosin ring and septum formation. Unlike *S. pombe*, *S. cerevisiae* only contains one class II myosin, MYO1. The requirement for MYO1 during cell division has been controversial. Previous studies have shown that deletion of MYO1 eliminates the actomyosin ring, but does not lead to lethality. A variety of phenotypes have been observed in *myo1*Δ, from severe defects in cytokinesis and cell separation to only minor growth defects [34–36]. This variation appears to be dependent on the genetic background of the yeast strain used to create the deletion. As is true for most model organisms, there is no single wild-type strain of *S. cerevisiae* commonly used throughout genetic studies. Instead, a number of different strains, or their derivatives, are used. These strains have different properties, such as unidentified mutations, that can affect the outcome of an experiment. Deletion of MYO1 in the commonly used strain, W303a, is lethal. However, MYO1 deletion in a different genetic strain, BF264-Du, is not. The latter strain divides by the formation of multiple aberrant septa, most likely caused by an unknown suppressor mutation that has arisen in that strain [37]. These results show that while class II myosin is required for cytokinesis under normal conditions, mechanisms can arise that facilitate cell division in the absence of class II myosin function.

Dictyostelium has also proven to be a useful model organism in analyzing the role of class II myosin during cell division. Three mechanisms of cell division have now been identified in *Dictyostelium* and can be defined as adherent cytokinesis, adhesion-independent cytokinesis and traction-mediated cytokinesis. Both adherent and adhesion-independent cytokinesis are linked to the

progression of the cell cycle, whereas traction-mediated cytokinesis is not. Adhesion-independent cytokinesis refers to cell division in suspension and requires the only class II myosin in *Dictyostelium*, *mhcA*. Myosin II null cells are unable to divide in suspension, yet can undergo successful division when adhered to a solid substrate [38]. This adhesion-dependent method of division does not require myosin II. Genetic analysis of mutants defective in these both of these methods of cell division showed that these processes are independent, but parallel pathways [39]. Adherent cells with mutations in both pathways have greater cytokinesis defects than the individual pathways alone yet are still able to proliferate, albeit inefficiently [39]. In these cells, cell division most likely occurs through traction-mediated cytokinesis. This method of cell division requires substrate adhesion and does not depend on myosin II. In traction-mediated cytokinesis, cells form multiple leading edges that are then torn into smaller fragments. These fragments usually contain a single nucleus; however, some cells are multinucleate, indicating that this method of cell division is not as efficient as the other two [40, 41]. These studies show that while myosin II is generally required for efficient cell division, under certain conditions some organisms have developed methods of cell division for which myosin II is not necessary.

All myosin domains are necessary for proper function

Genetic analysis has also shown that the entire myosin II molecule is necessary for proper organization of myosin-containing structures. While the entire coiled-coil rod is necessary and sufficient for assembly of myosin molecules, interaction of the myosin head with actin and other associating proteins, as well as the interaction of the neck region of myosin with light chains is important as well. The ability to create transgenic animals expressing different mutations and domains of the myosin molecule has lead to a further understanding of the interaction of myosin with other structural proteins.

The interaction of amino acid residues in the myosin rod domain plays a major role in assembly and structural organization of myosin filaments. Mutation and deletion studies in the rod domain have shown that specific sequences in this region of the myosin molecule are important for the proper assembly of thick filaments**.** In *C. elegans*, myosin A contains regions in its rod domain that are required for the proper initiation of thick filaments and cannot be substituted with the equivalent regions from the rod domain of myosin B [42]. The hydrophobicity of certain external residues in these regions of myosin A is higher than that of myosin B, mostly likely increasing the interaction of myosin A dimers packed in an antiparallel fashion. The lower hydrophobicity of these regions in myosin B dimers may not be stable enough to promote robust filament assembly in an antiparallel fashion. Recent evidence shows that the four terminal residues in the coiled-coil region of the myosin A rod are important for proper assembly of thick filaments in early development [43]. Constructs missing the non-helical tailpiece of myosin A are able to rescue the lethality of worms lacking myosin A and the double mutant lacking both myosins A and B. Localization of myosin and the onset of contraction during the early stages of development are not affected by this deletion [43]. In contrast, constructs that remove the tailpiece and the four terminal residues of the rod are able to rescue the loss of either myosin B or A, yet cannot rescue worms that are deficient in both isoforms. Embryos expressing only truncated MHCs have delayed onset of movement and myosin localization, and arrest at the embryonic twofold stage [43]. These results show the importance of having a fully functional myosin rod for thick filament development in *C. elegans*.

A number of mutations in the myosin heavy chain rod domain have also been identified in *Drosophila*. *Mhc^{6}, Mhc^{13} and Mhc^{19}* all contain mutations in the rod domain. However, unlike *C. elegans*, the assembly of thick filaments is not dramatically affected in these flies, although the stability of myofibrils is indeed altered. Myosin in these mutants is more susceptible to proteolysis, and structural abnormalities increase with age. IFMs of these mutants appear normal prior to muscle contraction. However, the ordered array of hexagonal packing is largely defective in adults, most likely because the thick filaments assembled from these mutant myosins are unable to withstand the amount of stress needed for contraction (fig. 3 A–C) [44]. *Mhc^{6}* and *Mhc^{13}* also show a decrease in the phosphorylation of flightin, an IFM-specific protein, during adult development suggesting that the defects observed in these mutants also affect the interaction of myosin heavy chain with other proteins.

While the rod domain is part of the structural backbone of thick filaments, it is the head domain of class II myosin that is responsible for the interaction with actin and generation of contraction. Over 40 different dominant mutations in *unc-54,* encoding myosin B, have been isolated in *C. elegans.* All of the dominant-negative mutations map to the head region of the myosin molecule in biologically important areas, namely, the ATP binding and actin binding sites [45]. Heterozygous worms containing these dominant mutations have mutant myosin B that fails to assemble into thick filaments, yet disrupts the assembly of wild-type myosin B and myosin A [46]. Surprisingly, this activity requires only low amounts of the mutant protein, as most of the mutant myosin B is unstable in vivo, and levels are often less than 2% of wild-type amounts [46]. The phenotype is worse than that of the myosin B null mutant, indicating that the paralysis is not caused

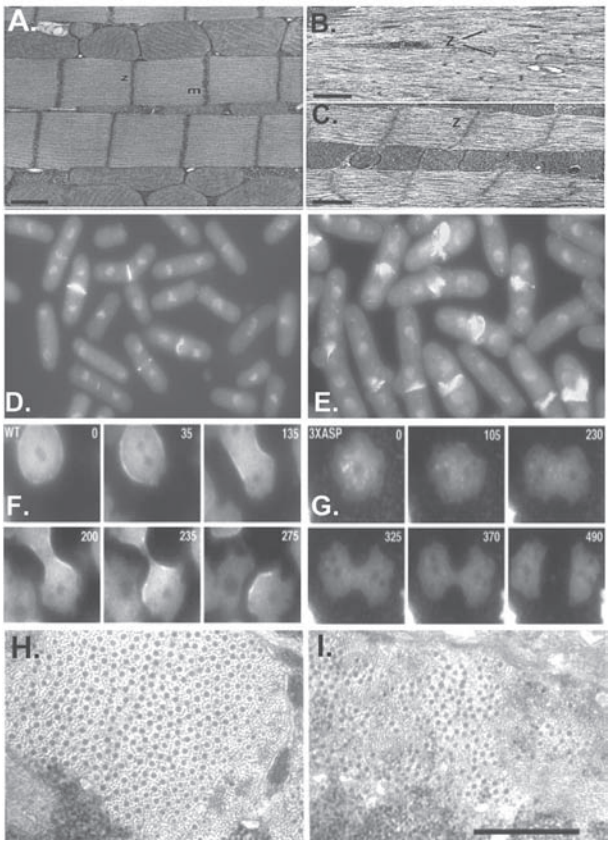

Figure 3. Effects of alterations in myosin II organization in organisms used for the study of myosin-containing structures. The *Mhc*^{13} mutation in the *Drosophila* myosin rod causes a disorganized myofibrillar structure. Longitudinal sections of wild-type (*A*) and *Mhc*^{13} (*B,C*) indirect flight muscles. m, M-line; z, Z-line. Bar, 1μm. *A–C* are reprinted from [43] with permission from Elsevier. The Myo2p-E1 mutation in *S. pombe* causes defects in cell division. Myo2p-E1 cells at the restrictive temperature (*E*) have defects in cell division and septum formation when compared with cells grown at the permissive temperature (*D*). *D* and *E* courtesy of M. Balasubramanian. Myosin unable to form thick filaments in *Dictyostelium* cannot localize to the cleavage furrow during cell division. (*F*) Green fluorescent protein-myosin in wild-type cells accumulates in the center of the cell prior to cell division and localizes to the posterior of the cell after separation. (*G*) GFP-3XASP-myosin is unable to form filaments and has a diffuse cytoplasmic localization. Elapsed time is in seconds. Bar, 5 μm. *F* and *G* are reprinted from [90] with permission from the American Society of Cell Biology via Copyright Clearance Center. Temperature-sensitive mutations in the *C. elegans* myosin chaperone UNC-45 cause myofibrillar disorganization. Electron micrographs of cross sections from *unc-45(e286)* mutant worms at the permissive temperature of 15 °C (*H*), and the restrictive temperature of 25 °C (*I*). Bar, 0.5 mm. *H* and *I* are reprinted from [105] by copyright permission of the Rockefeller University Press.

strictly by low levels of myosin B, which confirms the notion that the myosin head is necessary for the proper assembly and organization of the thick filament in *C. elegans*.

How the head region of the myosin molecule is involved in assembly or stability of thick filaments is not fully understood. In *Drosophila,* the head of the myosin molecule is not required for assembly of myosin molecules into thick filaments in IFMs. A transgenic myosin molecule lacking the entire head region gets incorporated into thick filaments and does not affect the incorporation of endogenous myosin [47]. However, the function of flight muscles is altered due to a variety of defects, including improper packing and length determination of the filaments. Taken together, these data indicate that while the myosin head per se is not necessary for assembly, its interactions with other components of the sarcomere affect assembly processes.

Mutations in the fungal myosin IIs also provide examples for the importance of having a fully functional myosin protein during the assembly of a contractile apparatus. Three temperature-sensitive alleles of Myo2p in *S. pombe* have been identified, *myo2p-E1 myo2p-S1* and *myo2p-S2*, and all display phenotypes that are similar to that of *myo2* null cells (fig. 3D,E). E1 and S1 mutants have single residue changes located in the cleft between the ATP and actin binding domains of the myosin head, whereas the S2 allele contains two mutations, one in the neck region and one in the myosin tail [48, 49]. The gene products of these mutations are expressed at wild-type levels, suggesting that they are stable under restrictive conditions, and have defects in structure or function rather than stability. Myo2p defective in its ATP binding site is still able to localize to the site of division, yet is not capable of forming proper actomyosin rings [50]. Spores are capable of germination and elongation, yet fail to divide and accumulate multiple nuclei before lysing. F-actin is also abnormally localized in these cells, indicating that the interaction of myosin with actin is important even before the ring is formed.

Additional proteins in myosin organization

While class II myosins are found in a number of different structures, these structures generally do not consist exclusively of myosin. Even though myosin is a major component of thick filaments, its assembly, organization and structural stability is also regulated by other proteins found in thick filaments. Paramyosin is an abundant protein in, and is unique to, thick filaments isolated from invertebrates [51]. In *C. elegans*, paramyosin is encoded by *unc-15* [52] and is around 35–40% identical in amino acid sequence to the rod domain of MHC [53]. Its structure is characteristic of an MHC rod containing an α-helical coiled-coil throughout most of its length [53]. Paramyosin shares similar rod sequences with myosin A, including the amount of hydrophobicity and glycine content, suggesting that they may assemble into thick filaments in a similar fashion [42]. This assembled paramyosin is thought to serve as a scaffold along which the two body-wall myosin isoforms assemble. In the absence of

paramyosin, the worm is paralyzed, and thick filaments have filament-like structures containing myosin. Myosin A is not affected; however, the synthesis, accumulation and assembly of myosin B are altered [13, 54]. Myosin B becomes coextensive with myosin A in the central region of the filament, indicating that myosin B indeed has the ability to assemble in an antiparallel fashion together with myosin A. The overall organization of *C. elegans* thick filaments and epigenetics of myosin A, myosin B and paramyosin point to a pathway in which the central myosin A-containing region forms first, followed by elongation of the paramyosin core, which is then followed by the addition of myosin B to the poles. This relationship is also observed when purified filaments are dissociated. Increasing salt concentrations applied to isolated thick filaments gradually solubilizes filament proteins from the poles inward to the center. Myosin B is solubilized first, followed by myosin A and approximately 70% of the paramyosin, leaving an insoluble region defined as the core containing myosin A and other core proteins [55]. This core is morphologically distinct from the myosin and other paramyosin substructures of the thick filament cortex, and is proposed to act as template for assembly of thick filament components [55, 56].

The loss of paramyosin in *Drosophila* leads to a more severe phenotype than in *C. elegans*. Flies that are homozygous nulls for paramyosin die at a late embryonic stage and have disruptions in muscle morphology [57, 58]. Myosin-containing thick filaments are also reduced in number and have defects in structural integrity [57, 58]. These results indicate that, similar to *C. elegans*, paramyosin in *Drosophila* is involved in the proper assembly of thick filaments. Also, cells show defects in localization of both myosin and actin, suggesting that paramyosin may also affect the interaction of myosin with actin filaments.

Mutant analysis in *C. elegans* shows that the determination of the length and diameter of thick filaments correlates with paramyosin content [12]. The amount of paramyosin found in thick filaments of various species can be directly correlated to the length and diameter of those thick filaments and the muscle types in which they are found [59]. Fast-acting muscles such as the indirect flight muscles in *Drosophila* have shorter filaments and lower paramyosin content, whereas slow acting muscles such as those found in *C. elegans* have longer filaments due to higher paramyosin concentrations. Vertebrates do not produce paramyosin. The increased length of thick filaments due to the presence of paramyosin may lead to a greater actin-myosin linkage and thereby greater force per cross-sectional area. The rigidity of filaments may also be increased, hindering the frequency of muscle contraction required by more complex organisms.

The differences in length and diameter of thick filaments in invertebrates are mirrored by the diversity of thick filament components in different species. For instance, *C. elegans* thick filaments contain α-, β- and γ-filagenins, three thick filament core proteins that are differentially expressed during development and are also differentially localized along the length of the thick filament, possibly playing a role in assembly and stabilization [60, 61]. *Drosophila* possesses a number of proteins that are not found in *C. elegans*, including miniparamyosin [62], myosin rod protein [63], flightin [64, 65] and myofilin [66]. Flies that are homozygous null for flightin (fln^0) are viable but flightless, with abnormally long thick filaments that become unstable during contraction [65]. The phenotype of fln^0 is similar to that of Mhc^{13}: degeneration of the sarcomere only in adults, increased proteolytic degradation of myosin heavy chain, and a decrease in the accumulation of flightin protein [44, 65]. Flightin has also been shown to directly interact with the rod portion of the Mhc [67]. It has been suggested that flightin plays a role in determining the proper length of thick filaments during assembly by correctly organizing Mhc in the filament and maintaining the stability of the thick filament after assembly [65].

Similar to thick filament organization, the formation of the actomyosin ring during cell division involves a number of proteins in addition to actin and myosin. In *S. cerevisiae,* septins, members of a family of GTP-binding and filament forming proteins, are essential for cytokinesis and have been suggested as possible scaffolding sites for the components of the actomyosin ring. Assembly of septin filaments into a ring at the bud site in G1 phase occurs before the localization and ring formation of Myo1p in late G1/early S phase [68]. The localization of Myo1p to the bud site is dependant on septins since cells containing septin mutations do not contain detectable Myo1p rings [69, 70]. At cytokinesis, the septin ring splits into two separate rings one on either side of the neck [71]. Recent studies by Dobbelaere and Barral [72] have shown that the two septin rings function as a sort of diffusion barrier to compartmentalize the actomyosin ring. Similar septin rings have been observed in *S. pombe* as well. However, septins in *S. pombe* are not required for the localization of either Myo2p or Myp2p and appear at the division site only after the contractile ring has been fully assembled [73]. Instead, accumulation of myosin II at the site of cell division in *S. pombe* is dependent on the localization of Mid1p, a protein that exits the nucleus during mid-interphase and becomes concentrated in a broad band in the adjacent cellular cortex [73–75]. Mid1p is then joined by Myo2p and other members of the actomyosin ring complex [73, 76–79]. Similar to the loss of septins in *S. cerevisae, S. pombe* cells containing deletions of Mid1p do not assemble normal Myo2p rings [73].

Regulation of myosin assembly through phosphorylation

The spatial organization and function of myosin is often regulated though phosphorylation. As previously noted,

the proper assembly of Myo2p during cell division in *S. pombe* is dependant on Mid1p. Mid1p directly interacts with Myo2p most likely through a C-terminal tail region and requires the dephosphorylation of Ser1444 in that region [80]. The phosphorylation of this tail region plays an important role in cytokinesis since Myo2p that has this tail region removed does not localize to the site of cell division and constructs mimicking phosphorylation cannot rescue *myo2*Δ cells [80]. This suggests that during interphase Myo2p is phosphorylated, preventing its interaction with Mid1p. The onset of mitosis would then cause the dephosphorylation of Myo2p, allowing the two proteins to interact and the proper localization of Myo2p. However, the proteins involved in this regulation have not all been identified.

The regulation of myosin through phosphorylation is also important for the variety of cellular process involving class II myosin in *Dictyostelium*. In addition to cytokinesis, myosin II in *Dictyostelium* also plays a role in motility and development. Myosin II null cells do not complete the normal cycle of development and have inefficient chemotaxis [81, 82]. To carry out all of its cellular functions in *Dictyostelium*, myosin II must constantly relocate. Myosin II accumulates in the cleavage furrow of dividing cells and at the posterior of the cell during migration, and is located throughout the cortex of vegetative cells [83, 84]. Myosin must be assembled as thick filaments to function properly during these cellular processes. Myosin molecules that have motor activity but cannot form filaments are unable to rescue the defects associated with myosin null cells [81, 83, 85]. Conversely, myosin molecules that can form filaments but do not have motor activity due to a lack of essential light chains have defects in contractile function. However, they are still transported to the cleavage furrow and contribute to cortical stiffness and constrained movement in aggregation streams [86, 87]. These filaments may play a structural role by cross-linking and organizing actin filaments. The control of filament assembly and disassembly in *Dictyostelium* is regulated primarily by phosphorylation of the myosin heavy chain. The ability of myosin II in *Dictyostelium* to assemble into filaments is inhibited by phosphorylation of three threonine residues near the C-terminus of the heavy chain [88, 89, 90]. Myosin molecules that have these sites mutated to alanine (3XALA), abolishing phosphorylation, can rescue the myosin null phenotype. In these cells, myosin is predominantly found as assembled filaments that are more stable than wild type [89, 91]. However, these filaments accumulate at the cleavage furrow during cytokinesis [92] and cells display defects in cell migration [93]. When these same sites are mutated to aspartate residues (3XASP), mimicking phosphorylation, filament assembly is impaired and the null phenotype cannot be rescued (fig. 3F,G) [89]. Recent studies from Hostetter et al. [90] have shown that the assembly and disassembly of myosin is sensitive to the overall charge on the C-terminal 68-kDa region of the myosin II tail. This C-terminal region contains clusters of charged residues, including a 196-amino acid charge repeat found only in the myosin tail of *Dictyostelium*. The assembly properties of this region alone are similar to that of full-length myosin either in the phosphorylated or unphosphorylated state. However, truncations smaller than this region have altered assembly regardless of the state of phosphorylation, indicating that the overall charge of this region is important for myosin assembly [90]. The position of the phosphorylation sites are near positive clusters of charges in this region, therefore a slight alteration of charge such as phosphorylation may have a major effect on assembly. These results highlight the dynamic nature of myosin thick filament assembly in *Dictyostelium* and how delicately the system of regulation is balanced.

Regulation of the phosphorylation of myosin heavy chain in *Dictyostelium* occurs through the activities of a group of myosin heavy chain kinases (MHCK) [94, 95] and at least one phosphatase [96]. The MHCKs A, B and C all contain a conserved protein kinase domain distinct from that of conventional kinases, and a WD repeat domain at the C-terminus that targets the proteins to myosin filaments. However, while MHCK A has a unique coiled-coil domain at its amino-terminus that drives oligomerization, MHCK B has a much shorter amino-terminus of unknown function, and MHCK C has no appreciable amino-terminus, with only 30 residues upstream from the kinase domain. MHCK C also contains stretches of low-complexity sequence rich in serine, asparagine, proline and glutamine or SNPQ between the catalytic domain and the WD repeat domain [84, 95, 94]. All of the MHCK null phenotypes are reminiscent of those seen in the expression of the non-phosphorylatable state of the myosin molecule. MHCK A null cells are viable, but have higher levels of assembled myosin II filaments and partial defects in cytokinesis, while overexpression of MHCK A leads to a decrease in assembled myosin [97]. Cells deficient in or overexpressing MHCK B also show an increase in assembled myosin or cell division defects, respectively, similar to the phenotypes observed in MHCK A mutants [98]. MHCK C null cells have a longer division time than wild type, most likely caused by the excessive accumulation of myosin filaments in the cleavage furrow [95].

The reason for multiple functionally similar MHCKs in *Dictyostelium* was elucidated by analyzing their differential localization. MHCK A is cytosolic during interphase but preferentially localizes to the actin-rich protruding edge during migration and cytokinesis [84, 99]. In contrast, MHCK C is found at the posterior of migrating cells and localizes to the cleavage furrow during cell division [84, 95]. MHCK B remains cytoplasmic throughout migration and cell division and appears to be

excluded from the poles and cleavage furrow [84]. The dependence on myosin II for localization also differs among the MHCKs. In the absence of myosin II, the localization of MHCK A and B does not change; however, MHCK C fails to localize to the cortex in interphase cells or the cleavage furrow during cell division [84]. These results suggest the MHCK A plays a role in preventing the assembly of myosin in the actin-rich region, MHCK B may play a role in maintaining the general turnover of myosin filaments throughout the cell, and MHCK C localization depends directly on myosin II and mediates its turnover in the cell cortex and cleavage furrow. Recent evidence has suggested that it is actin itself that increases the activity of MHCK A in the actin-rich cortical sites, facilitating the disassembly of myosin filaments in those regions. This suggests that actin may indirectly affect the assembly of myosin [100].

Chaperone proteins in myosin assembly

Little is known about the folding pathway of the motor domain of myosin. Various rod segments and myosin light chains are functional when recombinantly expressed in bacteria [101, 102]. However, fragments containing the myosin II head are not functional when recombinantly expressed and require additional factors [103, 104]. *C. elegans* UNC-45, the canonical member of the UCS-domain containing family of proteins [105], may provide such a function. UNC-45 in *C. elegans* is essential for proper muscle development. Lethal alleles of *unc-45* fail to develop past the two-fold embryonic stage and do not produce functional body wall muscle, similar to myosin A null animals [106]. Temperature sensitive alleles of *unc-45* lead to paralyzed animals with severe defects in thick filament organization (fig. 3H,I). Myosin A and B are scrambled along the filament instead of assembling into their distinct regions [107]. This defect appears to be developmentally regulated, as larvae grown at the permissive temperature of 15 °C will become paralyzed if switched to the restrictive temperature of 25 °C before sexual maturity [108]. Strikingly, the inverse is also true: animals grown at the restrictive temperature will develop normally if switched to the permissive temperature before adulthood. Once adulthood is reached, the phenotype of the worms cannot be reversed. The localization of UNC-45 also appears to be developmentally regulated. In early larvae, UNC-45 appears to be cytosolic, whereas in mature adults, the protein appears to be more abundant along assembled thick filaments, in a pattern similar to that of myosin B [109]. In vitro studies have shown that UNC-45 binds directly to and prevents the thermal aggregation of the myosin head. UNC-45 also binds the well characterized chaperone Hsp90 through its N-terminal TPR domain [110]. These studies indicate that UNC-45 may facilitate the proper folding of the myosin head during filament assembly. This function would most likely be necessary during larval stages of development when UNC-45 is cytosolic because, as previously noted, less than 2% of mutant myosin B is enough to completely disrupt thick filaments in *C. elegans* body-wall muscle development. The phenotype resulting from myosin B dominant-negative mutations may be a result of sequestering UNC-45 during thick filament assembly. The function of UNC-45 in the adult worm may be to ensure the properly folded state of myosin B is maintained once it has been assembled.

Another UCS-domain-containing protein found in *S. pombe*, Rng3p, also appears to play a dual role in assembly and maintenance of the actomyosin ring during cell division. A complete lack of myosin containing progenitor spots is observed in cells containing mutations in Rng3p, indicating a role in the assembly of the myosin spot [111]. Rng3p is also necessary for the maintenance of the actomyosin ring once it is formed since rings formed in temperature-sensitive Rng3p mutants during permissive temperature are not maintained when cells are shifted to the restrictive temperature [111]. Interestingly, Rng3p is sequestered at the contractile ring of *myo2-E1* mutants even under permissive conditions. This localization is not seen with *myo2-S1* or *myo2-S2* mutants or any other cytokinesis mutants tested [111]. The explanation for this allele specific effect is not known. However, it does suggest an interaction between Rng3p and the myosin head similar to what is observed with UNC-45. Rng3p may transiently bind myosin under normal conditions, but become trapped due to a structural defect in the myosin molecule.

Recent evidence indicates that the Hsp90 homologue in *S. pombe*, Swo1p, is also involved in proper formation of the actomyosin ring and, like *C. elegans* Hsp90, interacts with a UCS-domain-containing protein. Temperature-sensitive mutations in *swo-1* have defects in cell division and actomyosin ring assembly [112]. Interestingly, genetic analysis and immunoprecipitation experiments indicate that Swo1p interacts with and directly binds to Rng3p even though, unlike *C. elegans* UNC-45, Rng3p does not contain a TPR domain. Like *rng3*, mutations in *swo-1* also show a genetic interaction specific to the *myo2-E1* mutation and Swo1p persists at the improperly formed actomyosin ring [113]. However, in vitro studies measuring actin-activated ATPase and in vitro motility activities of Myo2p purified from *S. pombe* indicate a requirement for Rng3p but not Hsp90 [114]. It has been proposed that Rng3p may play a role in the interaction of Myo2p and F-actin for proper actomyosin ring formation after their accumulation at the division site [113]. Wong et al. suggested that the interaction among Rng3p, Myo2p and F-actin is lost upon inactivation of Rng3p, which causes a collapse of the actomyosin ring [111]. It

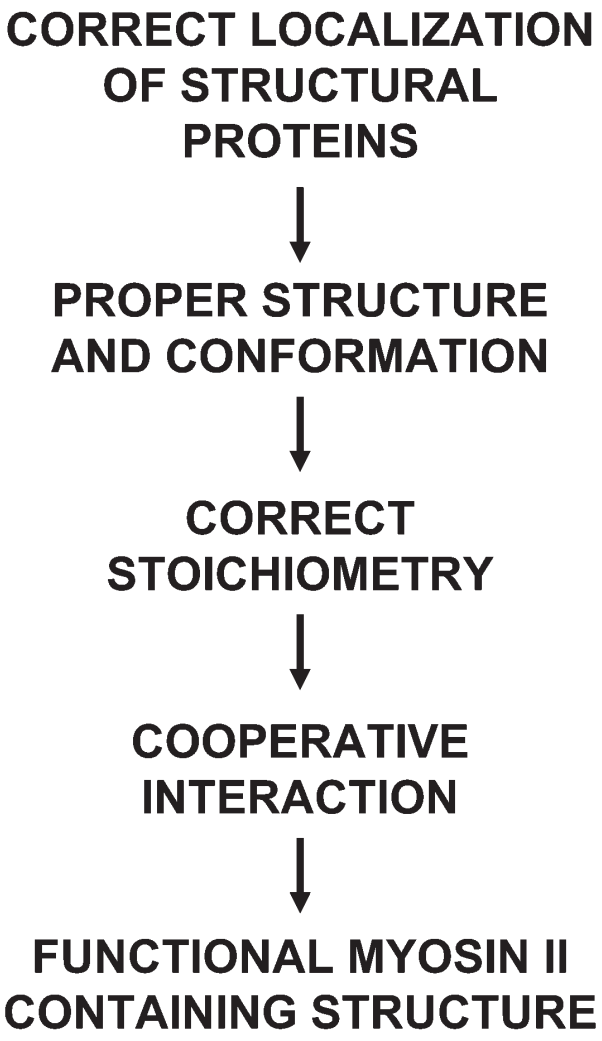

Figure 4. Requirements for properly assembled and organized myosin-containing structure.

is possible that the function of Swo1p is only required for formation of the ring, and Rng3p is necessary for both formation and stabilization of the ring once formed. These results are consistent with what is observed in differentiating C2C12 myocytes where the molecular chaperones Hsc70 and Hsp90 colocalize with intermediate states of myosin, but are not found in mature myofibrils [115].

Conclusions

Identification of the mechanisms and proteins involved in the assembly, organization and maintenance of class II myosin-containing structures is constantly progressing. From the results reviewed here, we conclude that a number of criteria must occur for the proper assembly of myosin structures. Molecules must first be properly localized to the site of structure formation, such as the actomyosin ring or a muscle cell thick filament. The concentration and conformation of native species is also of utmost importance. As we reviewed, increasing the concentration of proteins involved in these complexes to greater than native amounts can have just as deleterious an effect as protein deficiencies. These effects may be the consequence of sequestering other proteins necessary for proper assembly or trapping molecules in a conformation that does not favor assembly. Modifications such as phosphorylation also play a major role in regulation. Formation of organized myosin structures also requires the cooperative interaction of multiple protein components, all at proper stoichiometries to one another. The diversity of proteins involved in the formation of myosin-containing structures highlights the various functions of class II myosins. Genetic manipulation of experimental organisms, such as the ones discussed in this review, continue to provide insight into the assembly, organization and maintenance of organized myosin structures.

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