The UNC-45 chaperone mediates sarcomere assembly through myosin degradation in *Caenorhabditis elegans*

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Myosin motors are central to diverse cellular processes in eukaryotes. Homologues of the myosin chaperone UNC-45 have been implicated in the assembly and function of myosin-containing structures in organisms from fungi to humans. In muscle, the assembly of sarcomeric myosin is regulated to produce stable, uniform thick filaments. Loss-of-function mutations in *Caenorhabditis elegans* UNC-45 lead to decreased muscle myosin accumulation and defective thick filament assembly, resulting in paralyzed animals. We report that transgenic worms overexpressing UNC-45 also display defects in myosin assembly, with decreased myosin content and a mild paralysis phenotype. We find that the reduced myosin accumulation is the result of degradation through the ubiquitin/proteasome system. Partial proteasome inhibition is able to restore myosin protein and worm motility to nearly wild-type levels. These findings suggest a mechanism in which UNC-45–related proteins may contribute to the degradation of myosin in conditions such as heart failure and muscle wasting.

Introduction

Myosins are actin-based motors that play essential roles in a variety of cellular processes, including cytokinesis, cellular trafficking, phagocytosis, maintenance of cell shape, and muscle contraction (Berg et al., 2001). Myosin-based movement results from a precise cycle of the myosin head binding and releasing ATP and actin. During this process, the myosin head transitions through multiple folding conformations.

Molecular chaperones appear necessary for de novo folding and structural maintenance of the myosin head. Expression of the myosin motor domain in bacteria results in misfolding (McNally et al., 1988). In vertebrate systems, the chaperonin containing TCP-1 (CCT), as well as molecular chaperones Hsp90 and Hsc70, are necessary but not sufficient in the folding of striated muscle myosin (Srikakulam and Winkelmann, 1999; Srikakulam and Winkelmann, 2004).

Evidence from a variety of experimental systems indicates that myosins use specialized chaperones during their activity, folding, and assembly. Mutations in UNC-45/Cro1p/She4p(Dim1p) domain (UCS) proteins lead to phenotypes related to defects in myosin folding and assembly (Hutagalung et al., 2002). Decreased UCS domain protein function in fungal mutants produces myosins defective in actin:ATP transduction (Wesche et al., 2003; Lord and Pollard, 2004). In *Caenorhabditis elegans*, null *unc-45* alleles lead to embryonic arrest of body wall muscle development (Venolia and Waterston, 1990), and temperature-sensitive mutations lead to a paralyzed or uncoordinated phenotype at the restrictive temperature with marked disorganization of myofibrils (Epstein and Thomson, 1974; Barral et al., 1998). In vitro, UNC-45 exerts chaperone activity on the myosin head and acts as a cochaperone that specifically binds Hsp90 (Barral et al., 2002).

UNC-45 has recently been shown to be a substrate of an E3/E4-multiubiquitination complex containing CHN-1 (the *C. elegans* homologue of CHIP) and UFD-2 (Hoppe et al., 2004). *chn-1*–null worms are viable and morphologically normal. However, UNC-45 overexpression leads to an uncoordinated phenotype in these worms, suggesting that increased levels of UNC-45 may cause muscle defects. Previous studies have

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Abbreviations used in this paper: Lof, loss-of-function; MHC, myosin heavy chain; UCS, UNC-45/Cro1p/She4p(Dim1p) domain; UPS, ubiquitin/proteasome system; Ya, young adult.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 177, No. 2, April 23, 2007 205–210
http://www.jcb.org/cgi/doi/10.1083/jcb.200607084

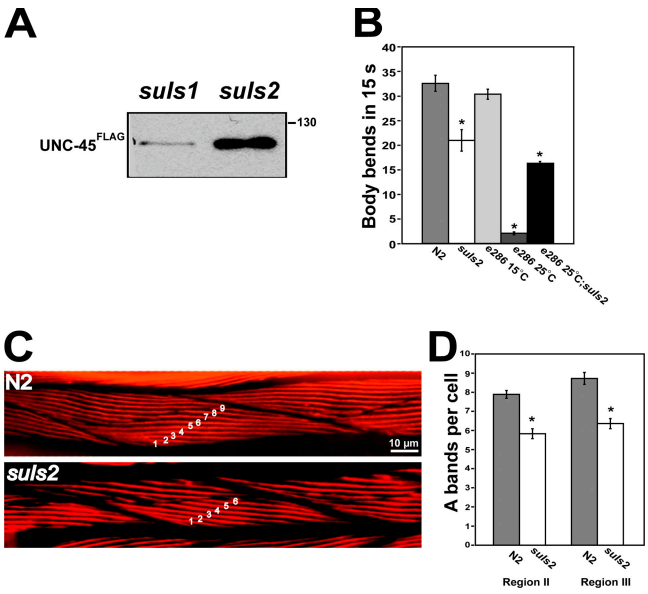

Figure 1. **Overexpression of UNC-45^{FLAG} results in decreased thick filament assembly and a mild paralysis phenotype.** (A) Expression levels of UNC-45^{FLAG} protein by two different integrated lines. Two integrated lines overexpressing UNC-45, *suls1*, and *suls2* were analyzed for UNC-45^{FLAG} levels by Western blots using a mAb to the C-terminal FLAG tag on transgenic UNC-45. Each lane contains 10 worms total. (B) UNC-45^{FLAG} overexpression results in a mild paralysis phenotype. Motility in liquid was measured at 20°C for N2 and *suls2* grown at 20°C, *unc-45(e286)* grown at 15 and 20°C, and *suls2;unc-45(e286)* grown at 25°C . N2 and *suls2* worms showed a mean of 33 and 21 body bends, respectively, over 15 s. The UNC-45^{FLAG} transgene expressed in *suls2* was able to rescue the motility defect of *unc-45(e286)* at 25°C from a mean of 2 to 16 bends in 15 s. *, $P < 0.001$ versus N2 wild type. (C) *suls2* worms have decreased number of MHC-containing A bands in body wall muscle cells. Ya worms were labeled with an anti-MHC A mAb to reveal the presence of A bands. (D) Quantitation of the myosin assembly defect of *suls2* worms. The number of A bands per cell in regions I and II, posterior to the pharynx (Mackenzie et al., 1978b) of N2 and *suls2* worms, was counted. In region II, N2 worms have a mean of 7.8 A bands per cell, whereas *susl2* worms have a mean of 5.8. In region III, N2 and *suls2* worms have 8.7 and 6.3 A bands per cell, respectively. *, $P < 0.0001$ versus N2 wild type. Error bars indicate SD.

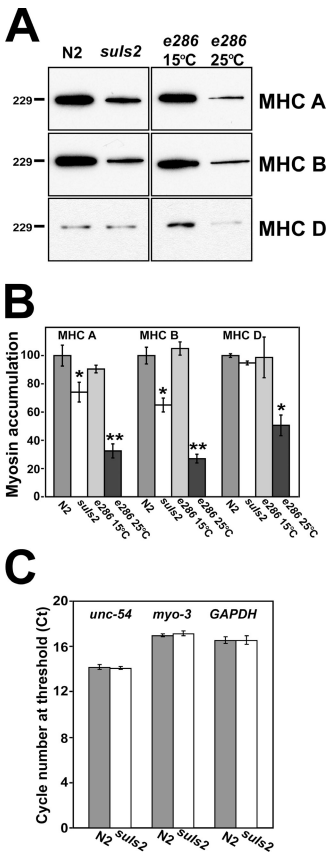

Figure 2. **Overexpression of UNC-45^{FLAG} results in diminished accumulation of body wall myosins.** (A) *C. elegans* worms overexpressing UNC-45^{FLAG} or harboring an UNC-45 Lof mutation have decreased levels of body wall muscle MHC. Individual worms from N2, *suls2*, and *unc-45(e286)* strains at either the permissive (15°C) or restrictive (25°C) temperatures were isolated at the Ya stage. Western blots against MHC A, B, and D show that only body wall muscle MHC A and B are affected in transgenic UNC-45^{FLAG} worms, whereas MHC D is also decreased in *e286* mutant worms. (B) Quantification of MHC levels in worms overexpressing UNC-45^{FLAG} and in UNC-45 Lof mutants. Analysis of Western blots performed in triplicate show that *suls2* worms have 30% less MHC A and B than N2 wild-type worms. Note that this decrease is not as great as that of *e286* mutants, which have 70% less body wall MHC than wild type. *, $P < 0.5$; **, $P < 0.01$, versus N2 wild type. (C) MHC mRNA levels between N2 and *suls2* remain unchanged. The threshold cycle (Ct) was set as the fractional cycle number at which the real-time fluorescence signal of the reaction could be measured above background. The cycle at which N2 wild type and *suls2* crossed the threshold was within one cycle each for *unc-54*, *myo-3*, and *GAPDH*. Error bars indicate SD.

shown that unassembled components of myofibrils are degraded through the ubiquitin/proteasome system (UPS) in muscle wasting conditions, including cancer and starvation (Koohmaraie, 1992; Solomon and Goldberg, 1996; Solomon et al., 1998; Acharyya et al., 2004). Our results suggest that enhanced levels of UNC-45 may promote nonnative myosin conformations, rendering them susceptible to degradation by the UPS.

Results and discussion

Wild-type levels of UNC-45 are critical for proper myofibril assembly

Loss-of-function (Lof) temperature-sensitive UNC-45 mutations result in severely paralyzed worms, with pronounced disorganization of the sarcomere (Epstein and Thomson, 1974; Barral et al., 1998). To investigate the consequences of increased UNC-45 levels in a wild-type background, we used the transgenic line, *suIs2* [$P_{unc\text{-}54}$::*unc-45*^{FLAG}], expressing UNC-45 under control of the strong muscle–specific *unc-54* promoter. In contrast to lines overexpressing UNC-45^{FLAG} from an extrachromosomal array, this line stably transmits the transgene from generation to generation. *suIs2* worms overexpress UNC-45 at ~10-fold greater amounts than that of the previously studied integrated line *suIs1* [$P_{unc\text{-}54}$::*unc-45*^{FLAG}] (Hoppe et al., 2004; Fig. 1 A).

Although not as pronounced as in Lof alleles, the increased concentrations of UNC-45 in *suIs2* also lead to abnormal thick filament assembly and a concomitant defect in movement. We measured body bend rates and found that *suIs2* worms were 36% slower than wild type (Fig. 1 B). However, this is not as severe as the temperature-sensitive *unc-45(e286)* mutants grown at the restrictive temperature, which is the result of diminished, rather than augmented, UNC-45 function. Transgenic UNC-45^{FLAG} was able to rescue the motility defects

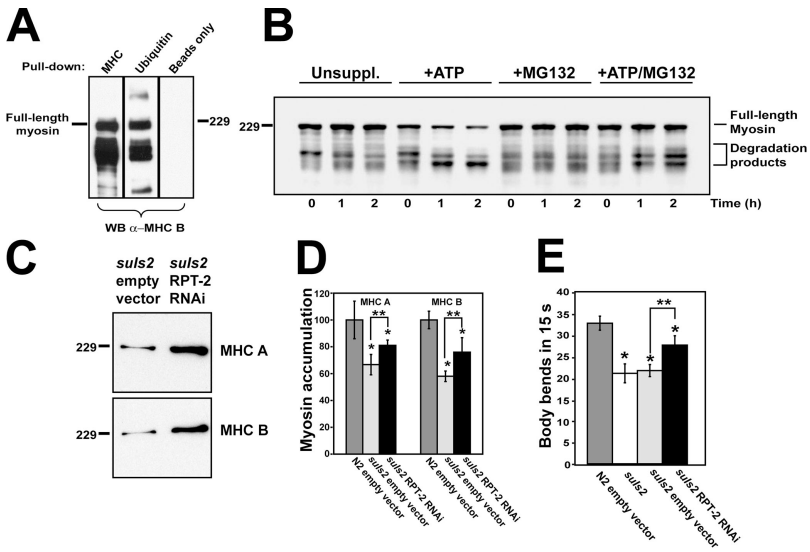

Figure 3. **The UPS is responsible for degrading myosin in worms overexpressing UNC-45^{FLAG}.** (A) *C. elegans* myosin is ubiquitinated in vivo. Pull downs from N2 wild-type worms using anti-MHC and antiubiquitin antibodies subsequently subjected to anti-MHC immunoblotting. Bands of slower mobility can be observed in the lane corresponding to the antiubiquitin pull down, which may correspond to multiubiquitinated myosin species. Bands of faster mobility observed in lanes of both pull downs may represent proteolytic fragments of myosin. (B) Degradation of myosin in *C. elegans* is dependent on ATP and is inhibited by the proteasome inhibitor MG132. Supernatants of worm lysates were incubated with no supplementation, ATP, MG132, or ATP/MG132 at room temperature. Samples were removed at the indicated time points. Western blots to detect MHC B were performed using mAb 28.2. (C) Partial inhibition of proteasomal function through RPT2 RNAi leads to restoration of both MHC isoforms in *suls2* worms. Western blots for MHC A and B were performed on *suls2* worms grown on *Escherichia coli* containing an empty vector or an RPT2 RNAi vector, as indicated. (D) Quantification of MHC levels in *suls2* worms after partial inhibition of the proteasome. Analysis of Western blots performed in triplicate shows that inhibition of the proteasome induces an increase in myosin levels in *suls2* worms. *, $P < 0.5$, versus N2 wild type; **, $P < 0.5$ *suls2* RNAi versus *suls2* empty vector. (E) Partial inhibition of proteasomal function results in limited rescue of mobility defects associated with UNC-45^{FLAG} overexpression. Inhibiting the proteasome in *suls2* worms increases the number of body bends in 15 s. *suls2*-empty vector– and *suls2*-RPT2-RNAi–treated worms displayed a mean of 21 and 27 body bends, respectively. *, $P < 0.001$ versus N2; **, $P < 0.001$ *susl2* RNAi versus *susl2* empty vector. Error bars indicate SD.

of *unc-45(e286)* at the restrictive temperature, indicating that it is functional (Fig. 1 B).

C. elegans young adult (Ya) wild-type muscle cells are ~120–150 μm in length with a mean of seven to nine myosin thick filament–containing A bands per cell (Fig. 1 C; Mackenzie et al., 1978a). Similarly staged *suIs2* worms demonstrated cells 108–130 μm in length that contained only five to six A bands (Fig. 1, C and D). Thus, UNC-45 overexpression in *suIs2* worms results in a decreased number of myosin-containing thick filaments. This phenotype arises specifically from increased levels of UNC-45^{FLAG}. Transgenic overexpression of the unrelated protein GFP under control of the *unc-54* promoter does not result in altered muscle cells (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1). Furthermore, integration of the UNC-45^{FLAG} transgene by itself is not responsible for the muscle phenotype, as the expression of UNC-45^{FLAG} as an extrachromosomal array also decreased the number of myosin-containing A bands in cells in which it was expressed (Fig. S1 B). Moreover, in rare instances in which individual muscle cells in *suIs2* worms expressed undetectable levels of UNC-45^{FLAG}, a wild-type phenotype was observed (Fig. S1 C).

Altered UNC-45 levels lead to decreased myosin accumulation

We next investigated whether the overall myosin content was decreased in these worms. Four distinct myosin heavy chain (MHC) isoforms are expressed in *C. elegans* as major components of the sarcomere (Miller et al., 1983; Dibb et al., 1989). MHC A and B are present in the body wall muscles, whereas MHC C and D are located in the minor pharyngeal muscles (Ardizzi and Epstein, 1987). To assess myosin levels, individual worms were analyzed for MHC A, B, and D. In contrast to MHC D, which was not affected because UNC-45^{FLAG} is specifically expressed in body wall muscle, both body wall MHC A and B were decreased in *suIs2* to ~70% of wild type (Fig. 2 A). Worms homozygous for the *e286* allele grown at the restrictive temperature resulted in decreased accumulation of all myosin isoforms examined (Fig. 2 A). This is expected, as endogenous UNC-45 is involved in pharyngeal as well as body wall muscle myosin assembly (Venolia and Waterston, 1990). Previous results showing that MHC A content was not substantially affected in *e286* mutant worms may be explained by the fact that the protein paramyosin was used for normalization. Paramyosin is also a major component of thick filaments, and *e286* worms at the restrictive temperature have a 45% mean decrease in number of thick filaments compared with worms at the permissive temperature (Barral et al., 1998). Paramyosin levels are also decreased in *suIs2* worms, indicating a loss of total thick filaments (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1).

Because our transgenic line carries multiple copies of the *unc-54* promoter, the possibility existed that the observed muscle phenotype was caused by its excessive binding of transcription factors. However, real-time RT-PCR verified that mRNA levels of both *myo-3* and *unc-54*, which encode MHC A and B, respectively, were not significantly different between N2 wild-type and *suIs2* worms (Fig. 2 C) with GAPDH mRNA as a control. These results indicated that the decrease in body wall myosin is not a result of diminished transcription.

The UPS is responsible for decreased myosin accumulation in worms overexpressing UNC-45

To examine a role for the UPS in the degradation of endogenous myosin in *C. elegans*, we first tested whether we could detect ubiquitinated myosin species in whole worm lysates. We performed pull downs from wild-type worms with antiubiquitin and antimyosin antibodies and blotted them subsequently with

a myosin-specific mAb (Fig. 3 A). The ubiquitin mAb was able to pull down full-length myosin as well as species of slower mobility, consistent with polyubiquitinated myosin species. Several smaller myosin-immunoreactive bands were also detected in both pull downs, which may represent ubiquitinated proteolytic fragments of myosin, consistent with previous studies (Zdinak et al., 1997; Ikemoto et al., 2001; Acharyya et al., 2004; Du et al., 2004). Sarcomeric myosin can thus be ubiquitinated in *C. elegans*. Elevated ubiquitination would lead to accelerated degradation, which in fact was observed in lysates of *suIs2* and *unc-45(e286)* worms at 25°C when compared with N2 and *unc-45(e286)* worms at 15°C (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1).

Proteasome-dependent proteolytic degradation requires ATP and is inhibited by MG132 (Rock et al., 1994). We thus tested whether degradation of endogenous *C. elegans* myosin exhibited these properties. We incubated separate reactions containing crude wild-type worm extracts with no additions, ATP supplementation, MG132 supplementation, and a combination of both, and examined the levels of full-length myosin and its degradation products by immunoblotting (Fig. 3 B). The unsupplemented reaction exhibited constant levels of full-length myosin, whereas the reaction supplemented with ATP showed a clear reduction of full-length myosin over time. MG132 by itself had no effects on full-length myosin levels, but the observed ATP-dependent degradation was effectively blocked by addition of MG132. The proteasome is therefore capable of degrading myosin in *C. elegans*.

We next tested whether proteasome inhibition could restore the diminished myosin levels observed in the *suIs2* worms that overexpress UNC-45^{FLAG}. RNAi against the proteasomal subunit RPT-2 has been previously used to inhibit proteasome function in living *C. elegans* worms (Hoppe et al., 2004). Importantly, at the concentration used (see Materials and methods), RPT-2 RNAi did not result in complete inhibition of the proteasome, which allowed the nematodes to survive. When RPT-2 RNAi was fed to *suIs2* worms, we observed clear restoration of body wall myosin (Fig. 3, C and D). Furthermore, RPT-2 RNAi also significantly improved the mobility of *suIs2* nematodes (Fig. 3 E). Because UNC-45 itself is subject to degradation by the UPS, the previous observation that *unc-45* Lof mutants are rescued by RPT-2 RNAi (Hoppe et al., 2004) can be explained by increased levels of UNC-45, myosin, or both. Our current results show that endogenous myosin can be ubiquitinated in *C. elegans* and that proteasome inhibition can prevent its degradation, in both a wild-type background as well as that resulting from increased UNC-45 function.

Implications for mechanism and muscle disease

We have found that UNC-45 overexpression results in diminished myosin accumulation and assembly because of its increased degradation via the UPS. Lof mutants also show similar but enhanced defects. These results allow us to propose an idealized model in which precise levels of UNC-45 are critical for supporting adequate myosin folding and assembly into thick filaments (Fig. 4). As a myosin chaperone (Barral et al., 2002), UNC-45 binds to newly synthesized myosin motor domains that have not yet attained their full native structure. UNC-45 assists in folding of the motor domain so that it becomes competent for assembly. We hypothesize that myosin produced in the context of UNC-45 Lof mutations is less capable of attaining its native structure and, as a result, is degraded by the UPS, thus explaining its drastically reduced levels (Fig. 2 A) and aberrant assembly into thick filaments (Barral et al., 1998). Myosin synthesized in the context of excess UNC-45 may also be prevented from reaching the assembled state because of mass action and is then susceptible to degradation via the UPS. Because both endogenous and transgenic UNC-45 can associate with assembled myosin in vivo (Ao and Pilgrim, 2000; unpublished data), excess UNC-45 may also be capable of shifting the myosin equilibrium from the assembled state into a degradation-susceptible unassembled state. The interplay between UNC-45, myosin, and the UPS may be of relevance to similar concentration-dependent phenomena between chaperones and client proteins

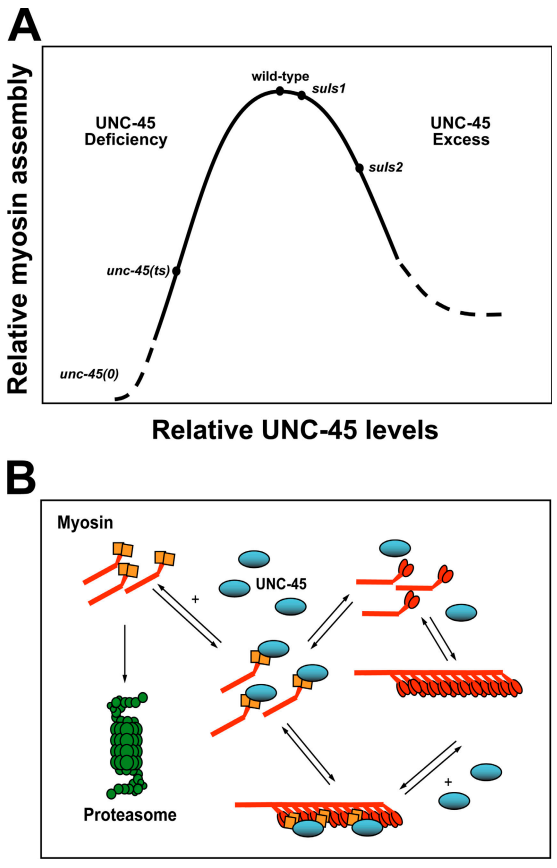

Figure 4. **Mechanism of UNC-45 chaperone–mediated proteasomal degradation of myosin.** (A) Myosin assembly as an idealized function of UNC-45 chaperone levels. The two are linked by control of myosin accumulation through its degradation. ts, temperature sensitive. (B) Proposed mechanism of UNC-45 chaperone–mediated myosin assembly, disassembly, and consequent degradation. All reactions represented by opposing arrows are reversible; myosin degradation represented by a single arrow is irreversible. By mass action, chaperone deficiency leads to a buildup of free, nonnative myosin, a substrate for the proteasome. Similarly, excess chaperone leads to a buildup of its complexes with myosin molecules and filaments. These complexes lead to increased levels of free, nonnative myosin and its proteasomal degradation.

capable of polymerization, such as those reported for Hsp104 and yeast prions (Chernoff et al., 1995).

Vertebrates contain two distinct genes encoding different UNC-45 isoforms. General cell UNC-45 is expressed in multiple tissues and appears necessary for various cytoskeletal functions, whereas striated muscle (SM) UNC-45 is specifically expressed in heart and skeletal muscle and may be necessary for sarcomere organization (Price et al., 2002). SM UNC-45 may function during sarcomere assembly in a mechanism similar to the one described here for *C. elegans* UNC-45. Alterations in human SM UNC-45 function or concentrations may be significant in hypertrophy, dilation, and failure of the heart, as well as in skeletal muscle wasting in a variety of human disorders.

Materials and methods

General *C. elegans* methods
Nematode strains were grown under standard conditions (Brenner, 1974). *suIs2* was created as previously described (Hoppe et al., 2004). Genomic integration of the $P_{unc\text{-}54}$::*unc-45*^{FLAG} extrachromosomal array was performed via gamma irradiation (Greenwald and Horvitz, 1980). The resulting worms were outcrossed five times. Strain BC10095 was obtained from D. Baillie (Simon Fraser University, Burnaby, Canada).

Immunoblotting
In Fig. 2 A, Fig. 3 C, and Fig. S2, Ya worms were hand-picked, placed in SDS sample buffer, and heated at 95°C for 10 min. Amounts corresponding to a single worm were loaded per lane. In Fig. S3, equal volumes of L4 Ya stage worms were lysed in 50 mM $NaPO_4$, pH 7.4, 200 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 5 mM DTT, and 5 mM ATP. Samples were resolved by SDS-PAGE and transferred to Hybond nitrocellulose (GE Healthcare). Blots were reacted with mAb 5-6 (anti-MHC A), 28.2 (anti-MHC B), 5-17 (anti-MHC D), 5-23 (anti-paramyosin; Miller et al., 1983), or anti-HDA-1 (Santa Cruz Biotechnology, Inc.) at 2, 5, 5.25, 5, and 2 μg/ml, respectively, for 1 h, followed by HRP-conjugated anti–mouse IgG or anti–rabbit IgG at 1:10,000 dilutions. Densitometry was performed using AlphaEaseFC software (Alpha Innotech).

Microscopy
Immunofluorescence microscopy on whole mounts of Ya worms was performed as described previously (Finney and Ruvkun, 1990). Worms were reacted with rhodamine-conjugated mAb 5-6 (anti-MHC A), FITC-conjugated mAb 5-8 (anti-MHC B), or Cy3-conjugated M2 (anti-FLAG; Sigma-Aldrich). Images were visualized using a microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) with a 40×/0.75 plan-NEOFLUAR objective and acquired using a digital camera (AxioCam MRc5; Carl Zeiss MicroImaging, Inc.) and AxioVision 3.0 software (Carl Zeiss MicroImaging, Inc.) at room temperature. Fluorescence microscopy of strain BC10095 ($P_{unc\text{-}54}$::GFP) was visualized as described. Polarized light microscopy was performed as described previously (Epstein and Thomson, 1974), except that image acquisition was performed using the system described above.

Real-time RT-PCR
Total RNA was isolated from synchronized N2 and *suIs2* worms using an RNeasy mini kit (QIAGEN). Real-time RT-PCR was used to determine gene expression using the QuantiTech SYBR Green RT-PCR kit (QIAGEN) according to the manufacturer's suggestions. Triplicate C_T values were analyzed in Excel (Microsoft) using the comparative $C_T(\Delta\Delta C_T)$ method.

Motility assays
Individual Ya worms were placed in M9 buffer, and body bends were counted over 15 s (Epstein and Thomson, 1974).

RNAi
RNAi was performed by feeding nematodes the HT115 bacteria containing RPT2 double-stranded RNA in plasmid pPD129.36 (Hoppe et al., 2004) as described previously (Kamath et al., 2001) except that RNA transcripts were induced with 10 nM IPTG to ensure survival.

Myosin degradation assay
N2 worms were equilibrated and lysed in 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM $MgCl_2$, and 5 mM DTT. The homogenate was centrifuged at 5,000 *g* for 10 min at 4°C, and the supernatant was divided into four aliquots to which no supplementation, 2 mM ATP, and/or 100 μM proteasome inhibitor MG132 were added. Samples were kept at room temperature, and aliquots were removed at each time point, mixed with SDS-PAGE sample buffer, and immediately heated at 95°C for 10 min. Samples were resolved by SDS-PAGE followed by Western blots using 5 μg/ml mAb 28.2.

Pull-down assays
N2 worms were lysed and incubated with 100 μg mAb 28.2 or 200 μg anti-ubiquitin mAb (Santa Cruz Biotechnology, Inc.) bound to protein G Sepharose (GE Healthcare) for 2 h at 4°C. Pull downs were washed five times with lysis buffer, mixed with SDS sample buffer, and heated at 95°C for 10 min. Samples were separated by SDS-PAGE followed by Western blotting using 5 μg/ml mAb 28.2. Fivefold less volume of the MHC B pull down compared with the ubiquitin pull down was loaded to avoid saturation.

Online supplemental material
Fig. S1 shows that the *suIs2* phenotype arises specifically from UNC-*45*^{FLAG} overexpression. Fig. S2 shows that paramyosin levels are also decreased in *suIs2* worms. Fig. S3 shows that myosin accumulation is also decreased in whole worm lysates from *suIs2* and *unc-45(e286)* worms at the restrictive temperature. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1.

We thank Dr. N. Dharajiya for assistance with real-time RT-PCR, Dr. David Baillie for *C. elegans* strain BC10095, I. Ortiz for assistance in microscopy, and Dr. D.F. Boehning for critically reading the manuscript.

This work was supported by grants from the National Institutes of Health (RO1AR050051), the Muscular Dystrophy Association, and the Cecil H. and Ida M. Green Endowment to H.F. Epstein.

Submitted: 17 July 2006
Accepted: 5 March 2007

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