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Kar Men Lee
Western Michigan University

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Thesis Chair: Dr. Pamela Hoppe

Committee Member: Dr. Todd Barkman

Abstract

This research investigates the unexpected significance of Myosin A in the organization of M-line protein UNC-89/obscurin within striated muscle cells of the nematode *Caenorhabditis elegans* (*C. elegans*). The study employs immunofluorescence techniques to examine the spatial and temporal dynamics of UNC-89/obscurin and Myosin A during embryonic development. The sarcomere, a fundamental unit in muscle cells, is governed by the 'sliding filament model,' where the M-line serves as a crucial anchor for Myosin A. This research seeks to understand the relationship between muscle function in humans and *C. elegans*, utilizing the latter's simple anatomy and genetic tractability. The investigation focuses on UNC-89, a protein analogous to human obscurin, and its unexpected dependence on Myosin A for proper localization and organization. The study involves two main aspects: first, examining the impact of UNC-89/obscurin on Myosin A organization in *unc-89* mutant embryos, and second, elucidating the role of Myosin A in UNC-89/obscurin organization in *pat-10* mutant embryos. Unexpectedly, UNC-89 appears to rely on Myosin A during later stages of embryonic development, emphasizing a dynamic relationship between these proteins. The experimental approach includes antibody staining of embryos, confocal microscopy, and comparative analysis of staining patterns in wild-type and mutant embryos. Results reveal distinct staining patterns in *unc-89* mutants at the 3-fold stage, suggesting a crucial role for UNC-89/obscurin in Myosin A organization during this developmental phase. In contrast, *pat-10* mutants, despite lacking muscle contraction and body elongation, exhibit organized UNC-89/obscurin and Myosin A, highlighting the unique contribution of Myosin A to UNC-89 organization. This research contributes valuable insights into the molecular mechanisms governing muscle organization in *C. elegans*, emphasizing the significance of UNC-89/obscurin and Myosin A interdependence. The

findings open more possibilities for further exploration of the genetic and molecular pathways influencing muscle development and may have implications for understanding muscle-related conditions in humans.

Acknowledgements

I express my deepest gratitude to Dr. Pamela Hoppe, my thesis chair, for her unwavering guidance, mentorship, and invaluable insights throughout the entire research process. Her expertise and encouragement have been instrumental in shaping this study. I am thankful to Dr. Todd Barkman, the committee member, for his constructive feedback, thoughtful suggestions, and scholarly input that have significantly enriched the quality of this research. Special thanks to the entire Hoppe Lab for fostering a collaborative and intellectually stimulating research environment. The guidance from Hao Peng in utilizing the confocal microscope has been crucial to the success of this project. I extend my appreciation to the Lee Honors College for their generous funding support, which has been instrumental in the successful completion of this research project. I am grateful to the Department of Biological Sciences at Western Michigan University for providing the necessary resources and facilities for conducting this research. Lastly, I would like to express my gratitude to my family and friends for their unwavering support, understanding, and encouragement throughout this academic journey. Their belief in me has been a constant source of motivation. This research would not have been possible without the collective contributions of these individuals, and for that, I am sincerely thankful.

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Introduction

Sarcomere structure and muscle contraction

The sarcomere (Figure 1) constitutes the fundamental structural unit present in all striated muscle cells across various tissues and organisms. Comprising parallel myosin and actin filaments, sarcomeres play a pivotal role in the contraction of muscle tissues through the mechanism described by the 'sliding filament model' (Riddle et. al., 1997). According to this model, the shortening of sarcomeres is driven by the sliding movement of actin filaments past myosin filaments, facilitated by the formation of cross-bridges through the binding of myosin motor domains to actin filaments (Riddle et. al., 1997). This process of sarcomere shortening translates into muscle contraction. At the core of the sarcomere structure lies the M-line, a critical component that serves as an anchor for myosin filaments. The M-line's structural integrity is indispensable for the proper organization of striated muscle tissue, which, in turn, plays a crucial role in the locomotion of nematodes. At the M-line sits the UNC-89/obscurin, UNC-82/NUAK, UNC-96 and UNC-98/ZnF, all of which contribute to the thick filament attachment to the M-line. In addition to these proteins, there are approximately 30 other proteins that contribute to contraction reaction leading to movement, most of which are prevalent in myosin and actin (Schorr et al., 2023).

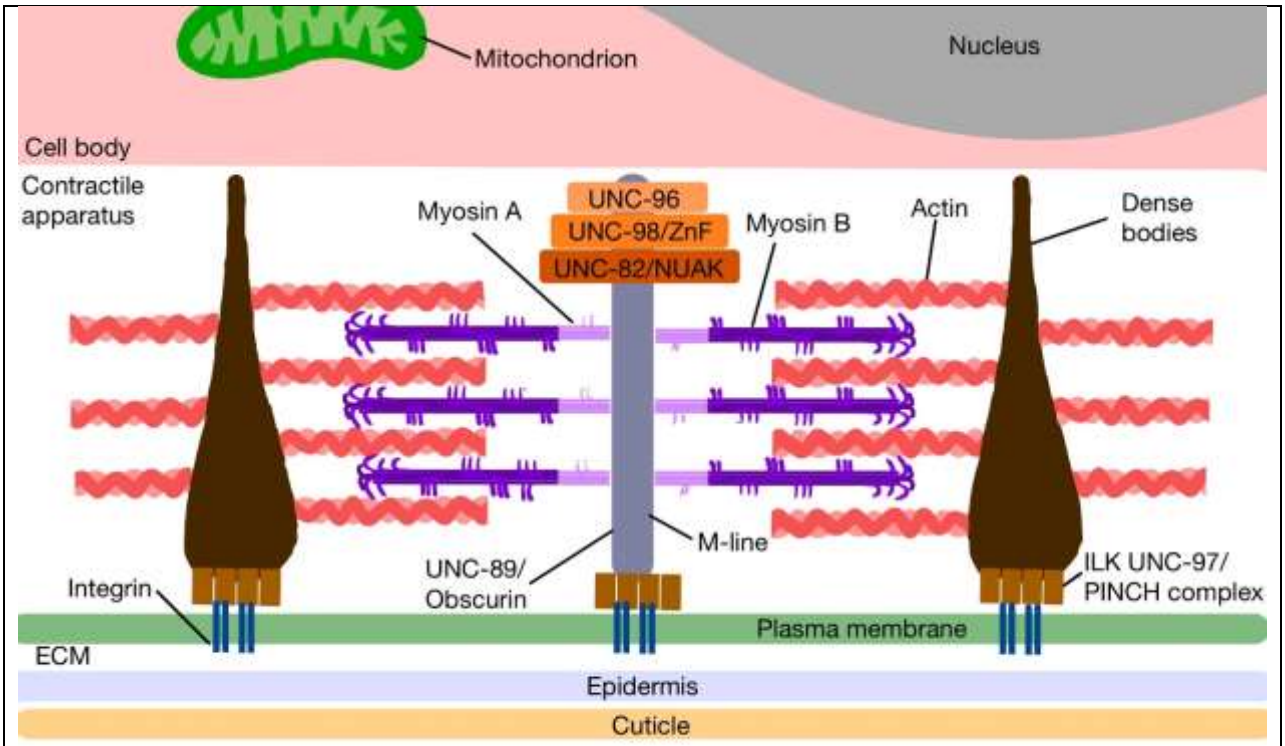

Figure 1. Diagram of a sarcomere in *C. elegans* body-wall muscle showing M-line proteins including UNC-96, UNC-98/ZnF, UNC-82/NUAK and UNC-89/obscurin. These proteins are localized at the M-line, suggesting a functional role in organization of myosin at the M-line. Contractile apparatus is attached to plasma membrane through integrin-mediated attachments at the M-line and dense bodies.

Identifying the link between muscle of humans and C. elegans

Muscle is a body tissue containing cells that contract to produce a movement. Muscle function is important due to its integral role in facilitating daily activities, posture, and exercise. Beyond its immediate impact, muscle strength is a crucial factor in maintaining bone health, thereby influencing overall mortality (García-Hermoso et. al., 2018). Because of this, there is a significant interest in uncovering genetic bases for improved muscle mass and strength with age

(Lesanpezeshki et. al., 2021). Leveraging genetics as a powerful investigative tool has proven instrumental in comprehending the intricate processes of muscle development and contraction. Genetic screens, in which mutant strains exhibiting altered muscle structure or function were isolated, have significantly contributed to unraveling the roles of individual molecules, providing valuable insights into muscle-related conditions such as Duchenne's Muscular Dystrophy (Ellwood et al., 2021). The decline in muscle size and strength associated with aging, resulting from a decrease in both the size and quantity of individual muscle fibers, is linked to the heightened frailty observed in the elderly (Lesanpezeshki et. al., 2021). Additionally, with the economic repercussions of an aging population on healthcare systems becoming increasingly significant, there is an amplified necessity to explore the genetic foundation of muscle strength (Lesanpezeshki et. al., 2021).

C. elegans is a free-living nematode with a simple anatomy composed of a mouth and brain at the anterior end and tail at the posterior end. The outer layer has a protective epidermis which is referred to as the "skin" with an underlying muscular layer (Alberts et. al., 2002). Embryogenesis in wild-type *C. elegans* exhibit muscle contraction during 1.5-fold, followed by organization of sarcomeres and attachment structures during after 1.5-fold stage (Hresko et. al., 1994). The structural and functional similarities between the body wall muscle of *C. elegans* and human skeletal and cardiac muscle make the nematode an advantageous model organism for studying the genetics of muscle. The distinctive c-shaped thrashing of *C. elegans* in liquid or its sinusoidal movement on semi-solid surfaces both necessitate properly functioning body wall muscles (Sawa & Korswagen, 2023). The nematode's optical transparency further facilitates research, enabling the observation of live animals using techniques like fluorescence microscopy or polarized light microscopy to study muscle structure (Sawa & Korswagen, 2023).

UNC-89/obscurin

UNC-89, a protein localized at the M-line in *C. elegans* muscle is approximately 900,000 Da in size (Qadota et al., 2020). UNC-89 shares homology with its human counterpart, obscurin (Qadota et al., 2020). Obscurin, recognized as a kinase, is abundantly present at the M-line, where it is postulated to function as a scaffolding protein, facilitating the assembly and stabilization of thick filaments (Spooner et al., 2012). Collaborating with other proteins, obscurin plays a vital role in cytoskeletal remodeling, contributing to the establishment and maintenance of sarcomere organization in mammals. Obscurin in mammals are localized to either the M-line or the Z-disk (Qadota et al., 2020). UNC-89 in *C. elegans* only exhibits localization to the M-line and has been implicated in critical roles relating to myofilament assembly and organization (Spooner et al., 2012). *C. elegans* with disrupted UNC-89/obscurin exhibit a deficiency in M-line structures or display disorganized muscle sarcomeres (Qadota et al., 2020).

Recent investigations conducted in the Hoppe Lab have yielded unexpected insights into the organizational dynamics of the UNC-89/obscurin protein, revealing a previously unrecognized dependence on myosin A. Employing antibody staining techniques, this experiment observed UNC-89 protein patterns in embryos expressing myosin A and those lacking it. At the 1.5-fold stage, preceding the onset of muscle cell contractile activity, embryos devoid of myosin A exhibited defective UNC-89 localization. In contrast, embryos expressing myosin A contained well-organized UNC-89 in distinct stripes with normal localization. After the 1.5-fold stage, when active muscle contraction commences, embryos rescued with a myosin A transgene exhibited the normal localization of UNC-89. The embryos without myosin A die as malformed L1 larvae with the Pat phenotype (paralyzed, arrested elongation at the two-fold stage) (William & Waterson, 1994). Pat mutants have a lethal phenotype characterized by

embryos that are unable to move and remain paralyzed during the elongation stage (William & Waterson, 1994). Development of tissues within Pat mutants continues after the two-fold stage although elongation arrests, as shown by formation of cuticle and well-formed pharynx (William & Waterson, 1994). UNC-89 distribution in Pat embryos lacking myosin A is highly defective, appearing as two lines of dots rather than in stripes. These findings suggest the importance of myosin A in organization of the M-line scaffolding protein UNC-89/obscurin within the contractile apparatus during the early stages of muscle cell development. The experiments in this report further delve into the interdependence between UNC-89/obscurin, an M-line protein, and myosin A in the organization of muscle sarcomeres in *C. elegans*. In addition, staining of *pat-10* mutant embryos, which lack the thin-filament component troponin was used to elucidate the specific contribution of myosin A in orchestrating UNC-89/obscurin. In *C. elegans*, *pat-10* mutants have no muscle function and are immobile. However, *pat-10* mutants have myosin A proteins that are nicely arranged.

Materials and Methods

Antibody Staining of Embryos

Embryos underwent fixation and staining procedures to characterize staining patterns based on their muscle phenotype. Both N2 and the UNC-89/obscurin mutant *unc-89* embryos were grown on large nematode growth medium (NGM) plates to generate ample gravid adults and embryos. Subsequently, worms were removed from the plates utilizing M9 buffer, transferred to a small glass tube, and centrifuged at setting 4 to form a pellet. This pellet was then carefully transferred to a 1.5 mL microcentrifuge tube and filled with M9. The worms underwent

incubation in a bleach solution composed of 600 µL H2O, 300 µL 5% sodium hypochlorite, and 100 µL 5N KOH. After centrifugation at setting 3, embryos were rinsed four times with M9.

Following the initial preparation, embryos were fixed using a formaldehyde solution consisting of 0.9 mL 16% EM grade paraformaldehyde and phosphate-buffered saline (PBS) with specified salt molarity for 10 minutes at room temperature. The formaldehyde solution was aspirated, and the pellet underwent two PBS washes. Subsequently, embryos were treated with -20 °C methanol for 10 minutes, followed by two PBS washes and one wash in PBS containing 0.5% Tween-20, with a 5-minute incubation.

For primary antibody incubation, the mouse anti-Myosin A monoclonal antibody 5.6 supernatant was used at a 1:50 dilution for N2 and *unc-89* mutant embryos. Concurrently, the anti-UNC-89 mouse monoclonal MH42 and mouse anti-Myosin A 5.6 were used at a 1:10 dilution and 1:50 dilution, respectively for *pat-10* embryos. The dilution mixture comprised 70% PBS with 0.5% Tween-20, 30% normal goat serum, and 1 µL 10x sodium azide. Each tube received 250 µL of the diluted primary antibody and underwent a 1-hour incubation at room temperature. Following this, embryos were subjected to four washes with PBS containing 0.5% Tween-20, with 5-minute incubations between each wash.

The secondary antibody, goat anti-mouse labeled with fluorophore M488, was applied at a 1:500 dilution, repeating the dilution and staining procedure employed for the primary antibody. After removing the secondary antibody, embryos underwent the washing steps as outlined for the primary antibody. The final wash was mostly removed before adding an equal volume of mounting media, composed of 90% glycerol, 20 mM Tris pH 8.0, and 0.2 M DABCO.

Imaging

Embryos were meticulously examined and captured using a Leica DM6 CS upright microscope, integrated with the STERLLARIS 5 confocal microscope platform. The image acquisition process was facilitated through the Leica Application Suite X (LAS X) software. Employing the 63x objective lens, immersion oil was introduced for optimal imaging.

The illumination of embryos was achieved through 488 nm lasers designed for Green Fluorescent Protein (GFP) visualization. The acquisition parameters for Alexa Fluor 488 were as follows: 488 nm for emission wavelength range, a 512 x 512 format, 400 Hz speed, 3.16 µs dwell time, 0.773/s frame rate, 2.5 zoom factor, and 1 arbitrary unit (AU) for pinhole size. For z-stack imaging, a comprehensive dataset comprising 85 slices was acquired, with a 25 µm z-step size. The resulting images had dimensions of 73.81 µm x 73.81 µm, with a pixel size of 144.44 nm x 144.44 nm.

Results

Recent findings within the Hoppe Lab revealed that mutants of myosin A display an atypical UNC-89 staining pattern. Despite the protein's appropriate localization, the observed staining showed up as dots rather than the characteristic stripes. Since myosin A plays a pivotal role in muscle contraction and the organizational function of UNC-89/obscurin within muscle cells, the absence of myosin A disrupts the normal progression of muscle development, leading to a disorganized state of UNC-89/obscurin. These findings suggest a direct correlation between muscle contraction and the organization of the UNC-89/obscurin protein. To further elucidate the specific contribution of myosin A in orchestrating UNC-89/obscurin, the staining patterns of UNC-89/obscurin in *pat-10* mutant embryos should be examined. As these embryos exhibit defects in both body elongation and muscle contraction, analyzing the UNC-89/obscurin staining

patterns in this context will provide insights into the distinct role of myosin A in the organization of UNC-89/obscurin.

UNC-89/obscurin

To compare the myosin A staining pattern in wild-type embryos and *unc-89* mutant embryos, an immunofluorescence technique was applied. After antibody staining using mouse monoclonal antibody 5,6 and goat anti-mouse labeled with fluorophore MH488, the embryos were viewed under the confocal microscope and images were taken with 63x magnification. The images obtained for *unc-89* mutant embryos showed nice stripes with normal localization at 1.75-fold stage (Figure 2, B). The appearance of well-defined stripes in *unc-89* mutant is similar to that in the control group, wild-type N2 embryos (Figure 2, A). For the *unc-89* mutant embryos at the 3-fold stage, several lines of stripes could be observed based on the confocal images (Figure 2, D). This was compared to the control group which had two clearly defined stripes with short individual lines implying individual cells at the 3-fold stage (Figure 2, C).

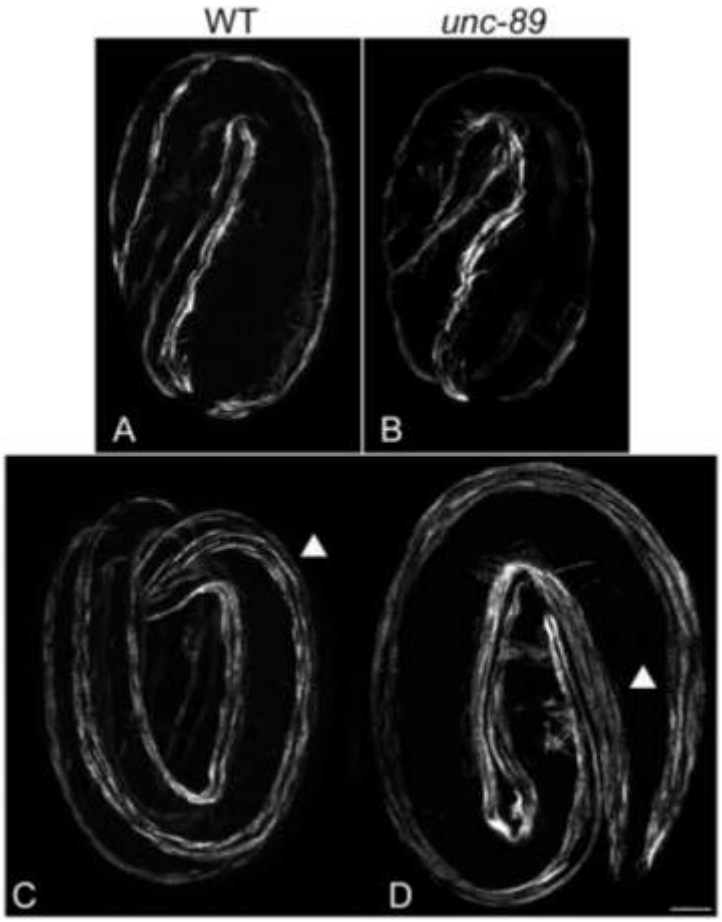

Figure 2: Localization of myosin A in *unc-89* mutant embryos is defective at the 3-fold stage but apparently normal at 1.75-fold stage. The first row (A-B) shows embryos at approximately the 1.75-fold stage. A ventral muscle quadrant of a wild-type N2 embryo stained with anti-myosin A (A) is compared to a ventral muscle of an *unc-89* mutant embryos with defective M-line (B). Clear, defined stripes of myosin A are seen in both embryos, with normal localization. The second row (C-D) shows embryos at approximately the 3-fold stage. Myosin A stripes in wild-type N2 embryos (C) are well-defined with two distinct stripes, especially at the head region (arrow in C). Myosin A in *unc-89* (D) is localized to defined stripes but the stripes have not coalesced into a single strong stripe in the head region when compared to wild-type N2

(arrow in D). For the 1.75-fold stage, embryos are shown in the same orientation. For the 3-fold stage, embryos are shown in different orientations.

The troponin mutant pat-10 has an organized UNC-89 pattern

To determine the contributing effects of myosin A on organization of UNC-89/obscurin, *pat-10* mutant embryos were stained with two different antibodies which stained for different proteins. *pat-10* mutant embryos were stained with mouse monoclonal antibody 5.6 to stain for myosin A and mouse monoclonal antibody MH42 to stain for UNC-89/obscurin. After antibody staining using mouse monoclonal antibodies and goat anti-mouse labeled with fluorophore MH488, the embryos were viewed under a confocal microscope and images were taken from 63x magnification. Both myosin A and UNC-89 had nicely arranged stripes with normal localization.

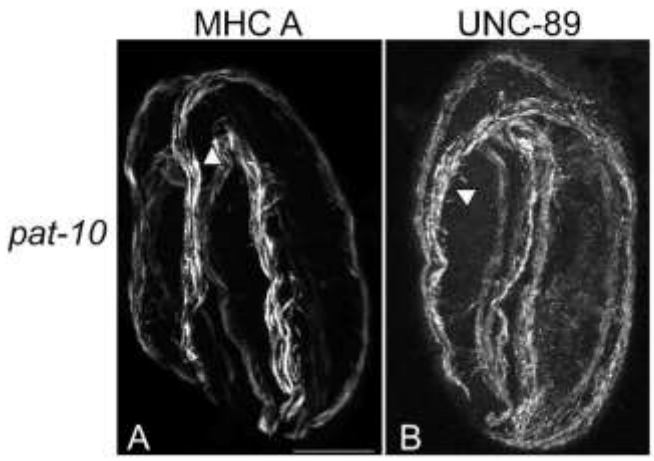

Figure 3: Myosin A and UNC-89 organization showed nice stirpes with normal localization in *pat-10* mutant embryos which lack muscle contraction and body elongation. This row (A-B) shows final staining patterns of embryos in the same orientation. Both myosin A signal (A) and

UNC-89 signal (B) showed distinct stripes organized along the ventral muscle quadrant (arrows in A and B).

Discussion

UNC-89/obscurin

In this experiment, *unc-89* mutant embryos were stained with mouse monoclonal antibody 5.6 to observe organization of myosin A in the presence and absence of UNC-89/obscurin. The objective of this experiment was to determine if organization of myosin A will be affected by loss of the UNC-89/obscurin protein. The hypothesis of this experiment was that myosin A requires UNC-89/obscurin to assemble at the M-line in body wall muscle.

Antibody specificity is the capability of an antibody to interact with a particular antigen and is important in immune response technique. Using immunofluorescence techniques, *unc-89* mutant embryos were fixed and stained before imaging using a confocal microscope. Wild-type animals were subjected to antibody staining as a positive reference, demonstrating the typical positioning of UNC-89/obscurin proteins at the M-line, where they coexist with myosin A. Both embryos were stained with mouse monoclonal antibody 5.6 diluted at 1:500 to detect myosin A. Antibody staining patterns of *unc-89* mutant embryos were compared with wild-type embryos at 1.75-fold stage and 3-fold stage to identify when the abnormalities first appear in *unc-89* mutant embryos. At the 1.75-fold stage, *unc-89* mutant embryos showed the beginning of myosin A assembly to the M-line along the ventral muscle quadrant. Proper arrangement and localization, similar to that of wild-type N2 embryos, implied that UNC-89 did not have a significant impact on ability of myosin A to organize at the M-line. At the 3-fold stage, *unc-89* mutant embryos

showed disorganized striped muscle patterns as compared to wild-type N2 embryos which exhibited individual stripes indicating individual muscle cells. Each stage was observed in 10 embryos that showed disorganized myosin A assembly to body wall in *unc-89* mutant embryos. This implied that *unc-89* mutant embryos showed abnormalities in organization of myosin A, as seen by lack of individualized, defined stripes at both stages. This observation was compared to wild-type N2 embryos with functional UNC-89/obscurin with myosin A arranged in individualized, defined stripes and normal localization to the M-line. This comparison indicates that UNC-89/obscurin is required by myosin A in later embryo development instead of early embryo development.

Pat-10

In this experiment, *pat-10* mutant embryos were stained with mouse monoclonal antibody 5.6 to observe staining patterns of myosin A and mouse monoclonal antibody MH42 to observe staining patterns of UNC-89/obscurin. The objective of this experiment was to determine if myosin A plays a definitive role in organization of UNC-89/obscurin without involvement of other factors such as muscle contraction and body elongation. The hypothesis of this experiment was that myosin A is the sole factor affecting organization of UNC-89/obscurin to the M-line.

Applying immunofluorescence techniques similar to that of *unc-89* mutant embryos and wild-type N2 embryos, *pat-10* mutant embryos were fixed and stained before imaging using a confocal microscope. Myosin A staining patterns of *pat-10* mutant embryos acted as a reference for how UNC-89/obscurin organization is affected by the of lack contractile activity and body elongation in a mutant where myosin A is relatively well organized. A set of *pat-10* mutant embryos were stained with mouse monoclonal anti-myosin 5,6 antibody diluted at 1:500 to detect myosin A while another set of said embryos were stained with mouse monoclonal anti-

UNC-89 MH42 antibody diluted at 1:10 to detect UNC-89. Myosin A staining patterns of *pat-10* mutant embryos were compared to UNC-89 staining patterns of said embryos at which developed past 2-fold stages to observe final staining patterns.

Results obtained showed both myosin A and UNC-89 arranged in definitive stripes. UNC-89 staining patterns of *pat-10* mutant embryos were compared to that of wild-type myosin A and myosin A mutants from previous research in Hoppe Lab. UNC-89 in myosin A mutant showed dots scattered along the muscle quadrant which is less organized than that of *pat-10* mutant embryos. This observation eliminated the possibility of contractile activity in wild-type myosin A affecting organization of UNC-89. As UNC-89 remains organized in *pat-10* mutant embryos despite defects in muscle contraction and body elongation, this suggests a direct link between myosin A and the organization of UNC-89. This implies that myosin A alone is sufficient for maintaining the organizing striping pattern of UNC-89.

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Appendix A: *N2* embryo images

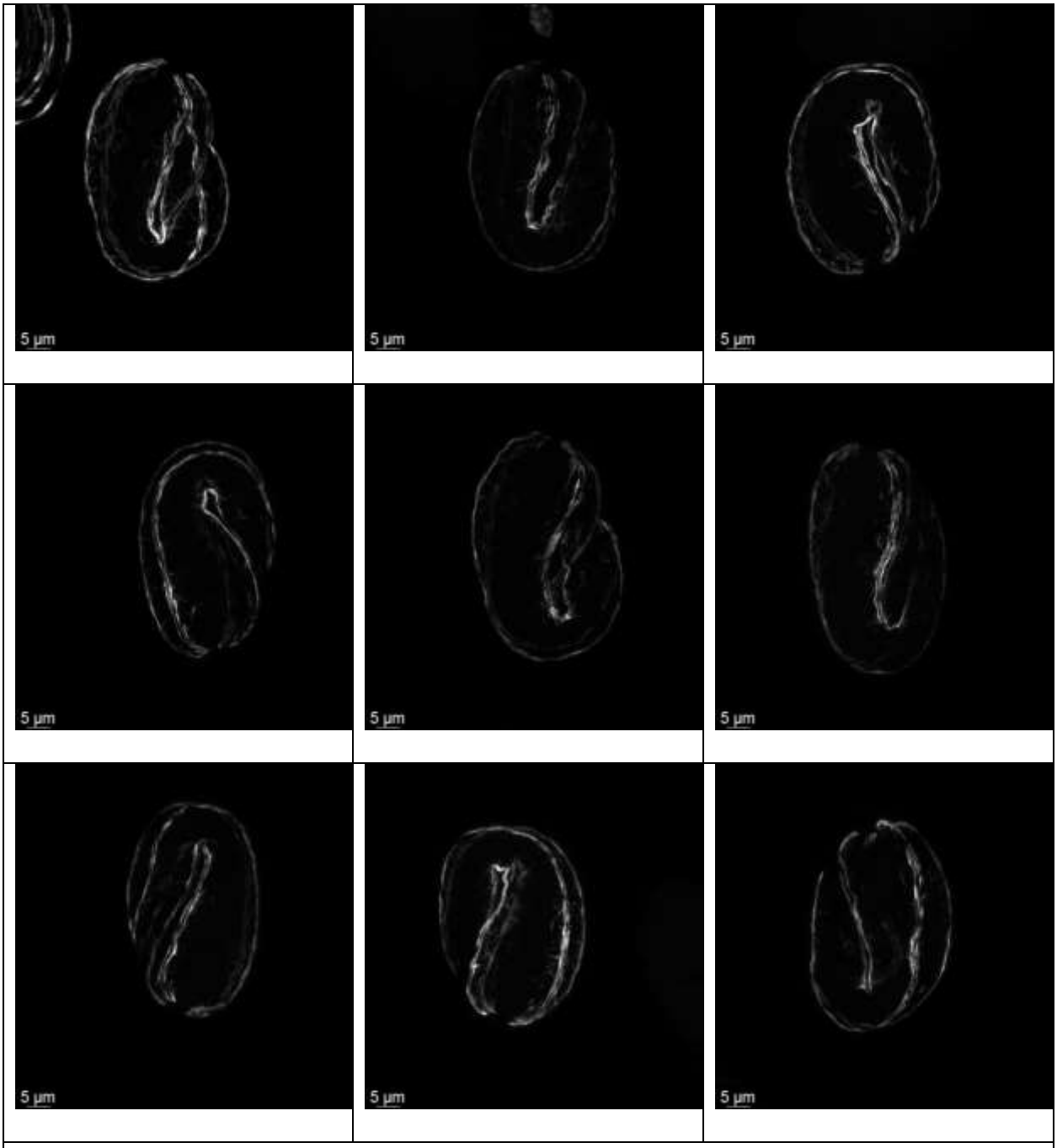

Figure 1. Images of 9 wild-type N2 embryos showing Myosin A stains at 1.75-fold stage.

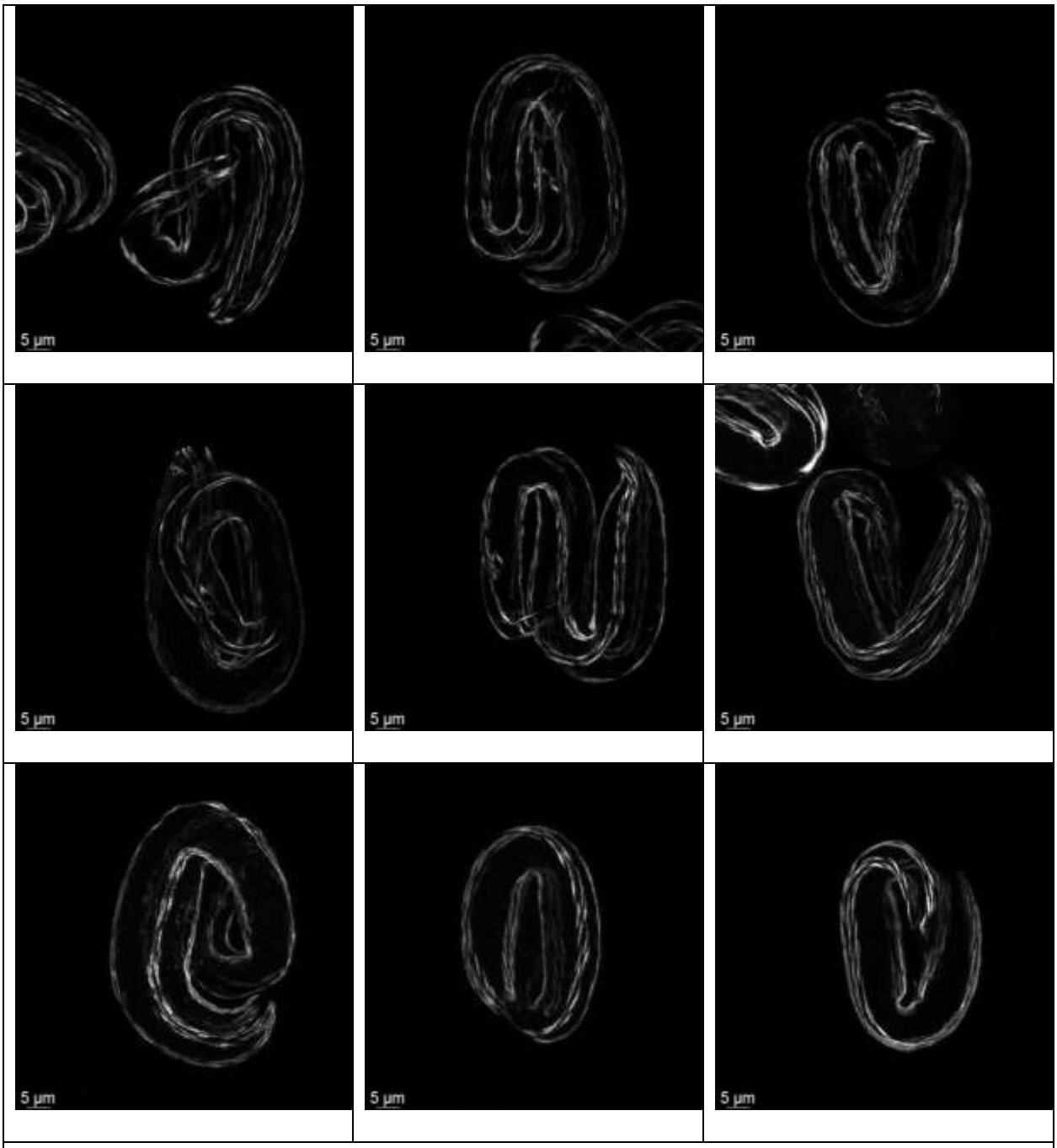

Figure 2. Images of 9 wild-type N2 embryos showing Myosin A stains at 3-fold stage.

Appendix B: *unc-89* mutant embryo images

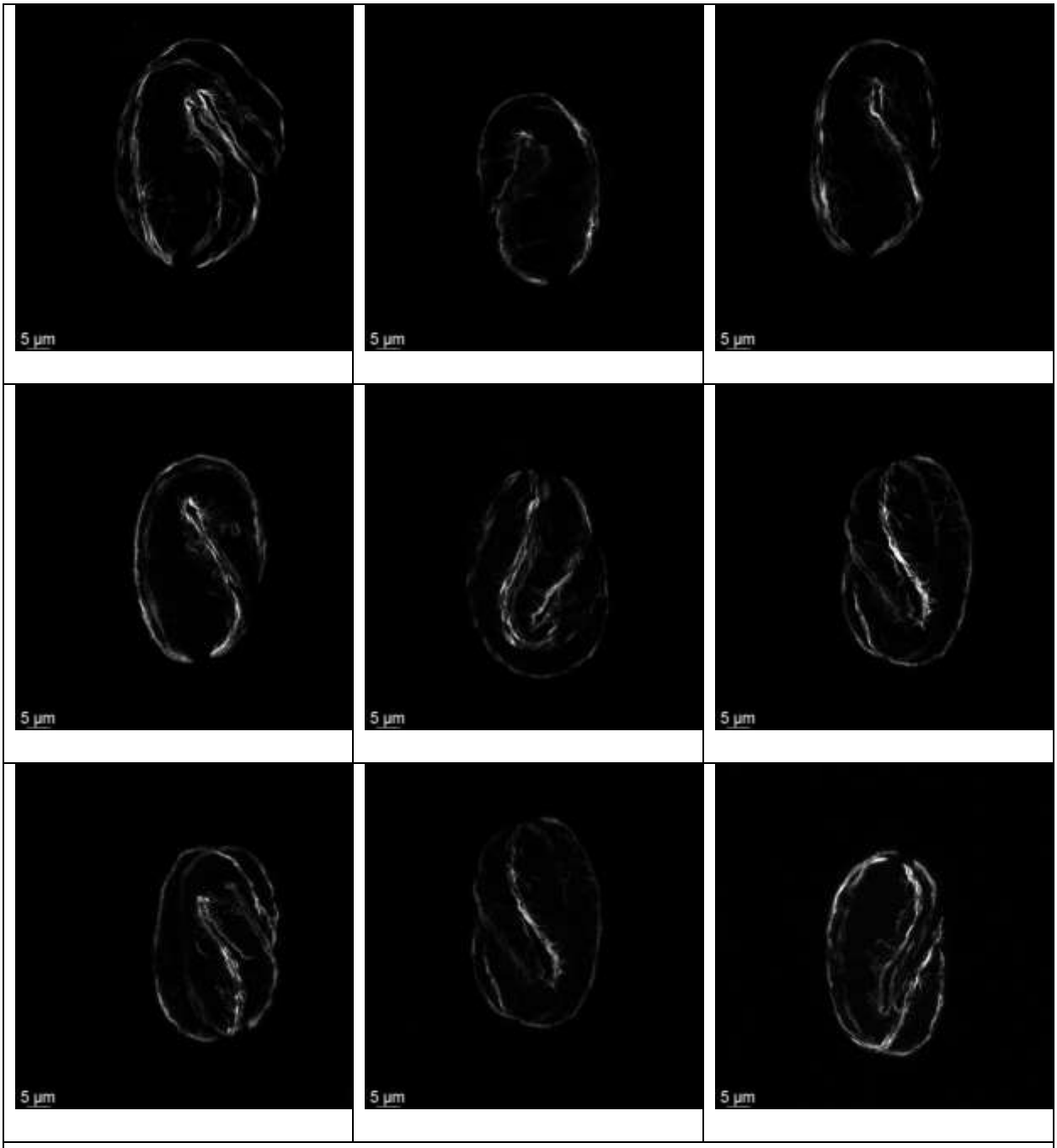

Figure 3. Images of 9 *unc-89* mutant embryos showing Myosin A stains at 1.75-fold stage.

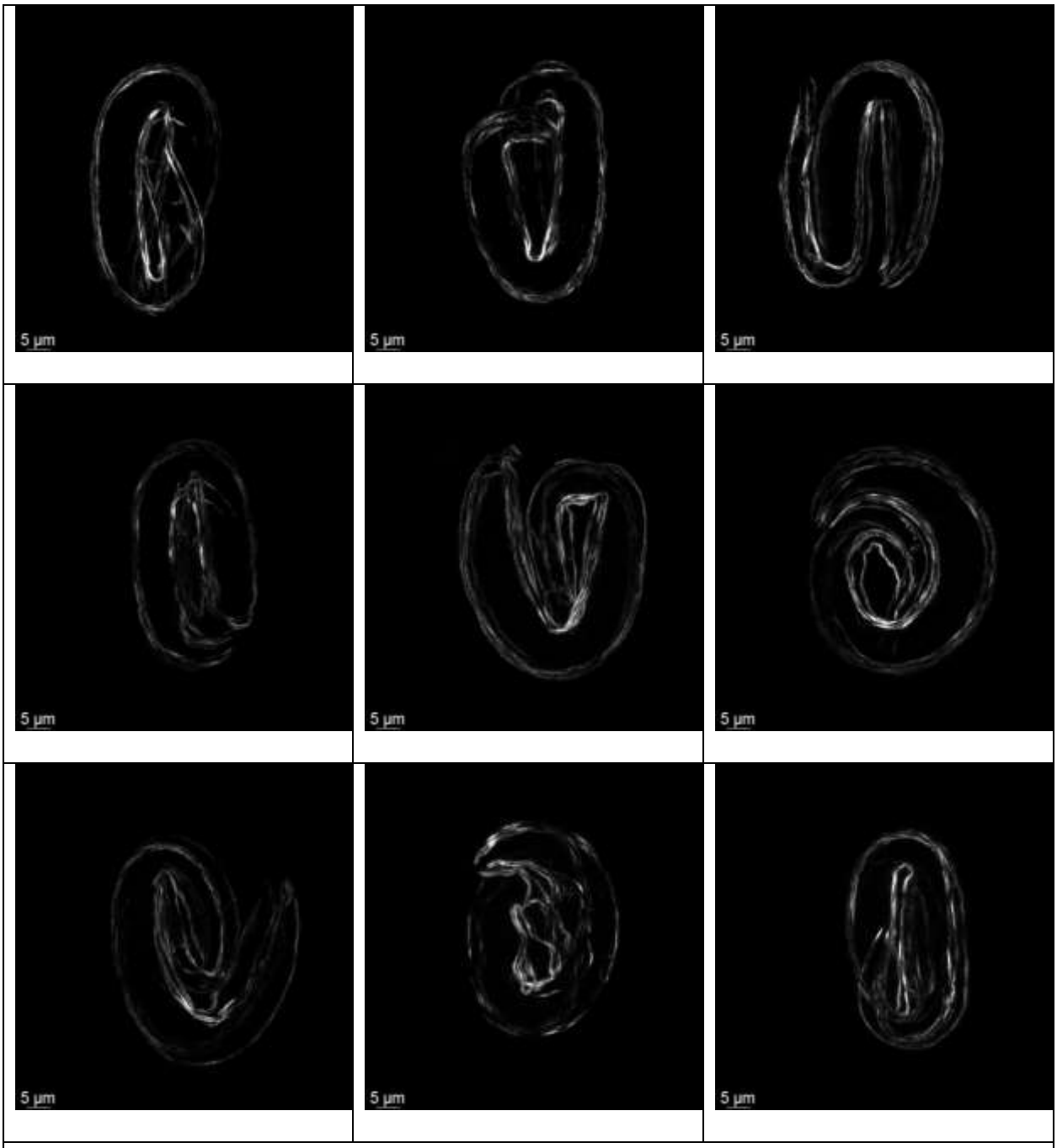

Figure 4. Images of 9 *unc-89* mutant embryos showing Myosin A stains at 3-fold stage.