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Stefano Derossi March 10th, 2024

HUM-7, an unconventional myosin with a RhoGAP domain, is required for assembly of integrin adhesion complexes in muscle

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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By Stefano Derossi

In vertebrate striated muscle (skeletal and cardiac), much of the force of muscle contraction is transmitted to the outside of the cell via costameres, which are muscle-specific integrin adhesion complexes (IACs). Costameres attach myofibrils located at the perimeter of the muscle cell to the muscle cell membrane and overlying extracellular matrix, and occur at each sarcomeric Z-disk. In the striated muscle of *C. elegans*, IACs reside at 3 locations—the bases of the sarcomeric M-lines and dense bodies (Z-disks) and at the muscle cell boundaries (MCBs). Each IAC consists of the heterodimeric transmembrane protein integrin and many proteins associated with it both intra- and extracellularly. The MCBs contain only a subset of proteins found at dense bodies. In a screen for mutants with defects in the MCB, we identified the gene *pix-1*, which encodes a RacGEF (guanine nucleotide exchange factor) (Moody et al., 2020) and the gene *rrc-1*, which encodes a RacGAP (GTPase activating protein) (Moody et al., 2024). During RacGAP screening, we also found that *hum-7* mutants have defective MCBs. The HUM-7 protein is predicted to contain an RA (Ras association) domain, a myosin head domain, 4 consecutive IQ domains, 2 C1 (phorbol ester/diacylglycerol binding) domains, and a RhoGAP domain. Based on the sequence of its myosin motor domain and the presence of the other domains, HUM-7 is a class IX unconventional myosin. Two deletion mutants of *hum-7* show less accumulation of IAC components at the MCB, similar to *pix-1* and *rrc-1*. Two missense mutations in myosin head region show MCB defects, but one missense mutation in the RhoGAP domain did not show the defect in MCB, providing preliminary evidence that the myosin motor may be the domain critical for HUM-7's MCB function. PIX-1 is still localized to MCBs in several *hum-7* mutants, suggesting the HUM-7 may not be a component of the PIX signaling pathway. We prepared an antibody against HUM-7, and localized HUM-7 in muscle cells. We found that anti-HUM-7 antibodies localize between dense bodies and at the MCB but flanking the location of IAC components in body wall muscle cells. Transgenic rescue experiments are underway to determine which domain of HUM-7 more definitively is critical for its muscle function. Since most unconventional myosins deliver cargo along actin tracks, we hypothesize that HUM-7 functions as a "truck" to deliver IAC components towards the MCB and between dense body regions. As a first step to test this hypothesis, we performed yeast 2 hybrid assays to determine if the C-terminal half of HUM-7 interacts with any of the known components of the MCB. However, the results were negative. We are currently testing the hypothesis that an intermediary protein might be involved in attaching the HUM-7 motor to possible IAC cargo.

HUM-7, an unconventional myosin with a RhoGAP domain, is required for assembly of integrin adhesion complexes in muscle

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Table of Contents

Introduction

Muscle functionality and integrity are fundamental to the locomotion and overall health of organisms, from humans to *Caenorhabditis elegans*. At the heart of muscle biology lies the intricate structure of myofibrils, composed of repeated units called sarcomeres, which are essential for muscle contraction and force generation (Clark et al., 2002). Despite the vast evolutionary distance, the basic principles of muscle organization and the critical roles of myofibrils and sarcomeres show remarkable conservation between humans and *C. elegans* (Moerman and Fire, 1997; Ono, 2014; Gieseler et al., 2017). This conservation extends to the molecular machinery involved in force transmission, particularly the integrin adhesion complexes (IACs), which anchor muscle cells to the extracellular matrix, ensuring effective force transduction and structural integrity (Qadota et al., 2017).

Our laboratory has contributed significantly to the understanding of IACs in *C. elegans*, unveiling a complex network of proteins that localize to key structural sites within muscle cells—dense bodies and M-lines—reminiscent of the Z-discs and M-lines in vertebrate striated muscles (Gieseler et al., 2017). These discoveries were facilitated by molecular cloning of mutationally-defined genes, mutational analysis, extensive yeast 2-hybrid screens, immunolocalization techniques, and biochemical analysis, revealing that proteins such as CPNA-1(copine), PAT-6 (α-parvin), and PAT-4 (ILK) are integral to both dense bodies and M-lines, while others like UNC-89 (Obscurin) and ATN-1 (α-

actinin) exhibit site-specific localizations, underscoring the specialized functions of IACs in muscle architecture and dynamics (Qadota et al., 2017; Gieseler et al., 2017).

Central to our inquiry was our recognition that muscle cell boundaries (MCBs), regions lying between adjacent spindle-shaped body wall muscle cells, also contain IACs, and are composed of a subset of proteins normally localized to dense bodies (Qadota et al., 2017). At the MCB, the IACs of each adjacent cell are anchored to a thin layer of extracellular matrix, that lies between the cells. Thus, in body wall muscle, which is used for whole worm locomotion, IACs are found at 3 locations—the base of M-lines, the base of dense bodies, and at MCBs. Each site is likely involved in the transmission of force generated by the sarcomeres; M-lines and dense bodies transmitting most of the force of muscle contraction to the extracellular matrix and cuticle of the worm to allow bending and thus locomotion, and MCBs in transmitting force laterally between muscle cells. Intriguingly, our screening of 574 mutant worm strains identified the gene, *pix-1*, as being required for the assembly or maintenance of the MCB (Moody et al., 2020). The protein PIX-1 contains a GEF (guanine nucleotide exchange factor) domain required for activating the small GTPase Rac, and antibodies localize PIX-1 to MCBs, M-lines and dense bodies. The activated Rac then binds to an effector protein kinase called PAK, and phosphorylation of still unknown protein substrates is in some way required for the assembly of IACs (Moody et al., 2020). Although the PIX pathway had been reported to be required for the development of the nervous and immune systems in mammals, and for neuroblast migration, germ cell formation and morphogenesis of epidermal cells in *C. elegans*, Moody et al. (2020) was the first report of the PIX pathway being important for muscle in any organism. Highlighting the potential clinical

importance of this pathway, collaboration between the Benian lab, and the lab of Jennifer Kwong (Dept. of Pediatrics, Emory University), has led to the recent discovery that heart-specific knock out of the mouse ortholog of PIX-1 called β-PIX leads to dilated cardiomyopathy and early death (Shoemaker, Benian and Kwong, unpub. data).

Small GTPases like Rac, Cdc42 and RhoA, cycle between inactive, GDP-bound and active, GTP-bound states. GEFs like PIX-1 promote the replacement of GDP with GTP, thus activating the GTPase protein, but also require GAP proteins (GTPase activating proteins) to promote the GTPase activity of the small GTPase, thus forming GDP from GTP, and inactivating the small GTPase. The GAP for the PIX pathway had not been identified in any organism or cell type. Because of the cycling requirement of small GTPases, the loss of function phenotypes for GEFs and GAPs are often the same. With this hypothesis, previous graduate student Jasmine Moody, identified in *C. elegans*, 32 proteins containing GAP domains, 18 of which are expressed in muscle. By screening mutants in these 18 proteins, Jasmine was able to find that loss of function mutations in two such genes, *rrc-1* and *hum-7*, result in the same MCB defect as in *pix-1* mutants (Moody et al., 2024). The RRC-1 protein consists of SH3 and RhoGAP domains and is localized to the MCB, like PIX-1, and is very likely a member of the PIX pathway based on several criteria: (1) co-localization with PIX-1 to the MCB; (2) that the localization of PIX-1 depends on RRC-1, and the localization of RRC-1 depends on PIX-1; and (3) RRC-1 and PIX-1 can be demonstrated to exist in a protein complex (Moody et al., 2024). However, HUM-7 had not been extensively characterized until I joined the lab.

The HUM-7 protein is predicted to have the following domains: An RA (Ras association) domain, a myosin head domain, 4 consecutive IQ domains, 2 C1 (phorbol ester/ diacylglycerol binding) domains, and a RhoGAP domain. Based on the sequence of its myosin motor domain and the presence of the other domains, HUM-7 is a class IX unconventional myosin. I found that two deletion mutants of the *hum-7* gene show less accumulation of PAT-6 (α-parvin) at MCBs, similar to *pix-1* and *rrc-1* mutants. Since the HUM-7 contains myosin head and RhoGAP domains, I next examined which domain is important for HUM-7's function at the MCB. I investigated missense mutations in the myosin head and RhoGAP domains. Two of three missense mutations in myosin head region show the defect at MCBs, but the missense mutation in RhoGAP domain did not show the defect at MCBs. HUM-7 is likely not a component of the PIX pathway, since PIX-1 remains at the MCB in several *hum-7* mutant strains tested. I have prepared a full length cDNA for HUM-7 in wild type and mutant versions to be driven by a muscle specific promoter to use in transgenic rescue experiments. In the near future, the lab plans transgenic experiments to determine whether the myosin motor or the RhoGAP domain is required for the muscle function of HUM-7. I prepared an antibody against HUM-7, and localized HUM-7 in muscle cells. By confocal microscopy, anti-HUM-7 antibodies localize between dense bodies and at the MCB but flanking the location of IAC components in body wall muscle cells. Since nearly all unconventional myosins act as molecular motors to carry along F-actin tracts, vesicles or other cargo to a destination, my working hypothesis is that HUM-7 functions as a transporter of IAC components towards the MCB and between dense body regions. As a first step to

determine if HUM-7 carries IAC proteins to the MCB, I conducted yeast two hybrid assays testing the ability of the C-terminal, non-motor domain, region of HUM-7 to bind to known components of the MCBs. Results did not reveal any direct binding partners.

Results and Discussion

HUM-7 is a class IX unconventional myosin

Wormbase indicates that there are two isoforms of HUM-7, HUM-7a (1880 aa) and HUM-7b (1867 aa) generated by alternative mRNA splicing by inclusion or exclusion of one small exon. We used an on-line program, PFAM, to predict domains within the HUM-7 protein. PFAM predicts the following domains: An RA (Ras association) domain, a myosin head or motor domain, 4 consecutive IQ domains (each likely to bind calmodulin), 2 C1 (phorbol ester/diacylglycerol binding) domains, and a RhoGAP domain (**Figure 1**). Based on the sequence of its myosin motor domain and the presence of the other domains, HUM-7 is a class IX unconventional myosin.

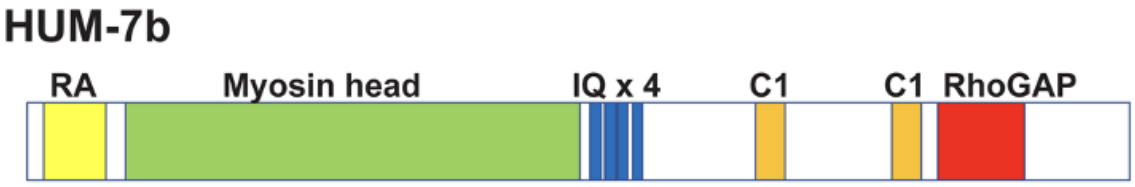

Figure 1. Schematic representation of predicted domains in HUM-7 and location of immunogen used to raise antibodies. Depicted are an RA (Ras association) domain (yellow), a myosin head (or motor) domain (green), 4 consecutive IQ domains (blue) that likely bind to calmodulin, 2 C1 (phorbol ester/diacylglycerol binding) domains

(orange), and a RhoGAP domain (red). Based on the sequence of its myosin motor domain and the presence of other domains, HUM-7 is classified as a class IX unconventional myosin.

Analysis of *hum-7* mutants preliminarily suggests the myosin motor is essential for the MCB function of HUM-7

I obtained 9 existing *hum-7* mutant alleles from the Caenorhabditis Genetics Center (CGC). Seven of these mutants, all missense or nonsense mutations, were derived from the Million Mutation Project (MMP), a collection of 2,000 adult-viable strains generated by chemical mutagenesis and subjected to whole genome sequencing, and the sequences deposited on WormBase (Thompson et al, 2013). Two of the mutants, *tm8236* and *ok3054*, are intragenic deletions. Since the typical way chemical mutagenesis is conducted for *C. elegans* creates lots of mutations in each mutant animal, on average, mutations in 400 genes, the mutant strains need to be outcrossed to wild type multiple times to remove most of the background mutations. If one selects for a given mutation, the background mutations are removed mostly by independent assortment of chromosomes, and to some extent, recombination. Typically, 5-6 outcrosses are standard in the field. **Figure 2** shows the location of the *hum-7* mutations in relation to the schematic of protein domains and indicates which mutants have been outcrossed and to which extent. Myself and other Benian lab members were engaged in outcrossing and this is also indicated in **Figure 2**. Note that 5 mutants in the myosin head were analyzed and three are missense mutations. Two of these missense

mutations, F319I and E583K, displayed abnormal MCBs, upon immunostaining for the IAC component PAT-6.

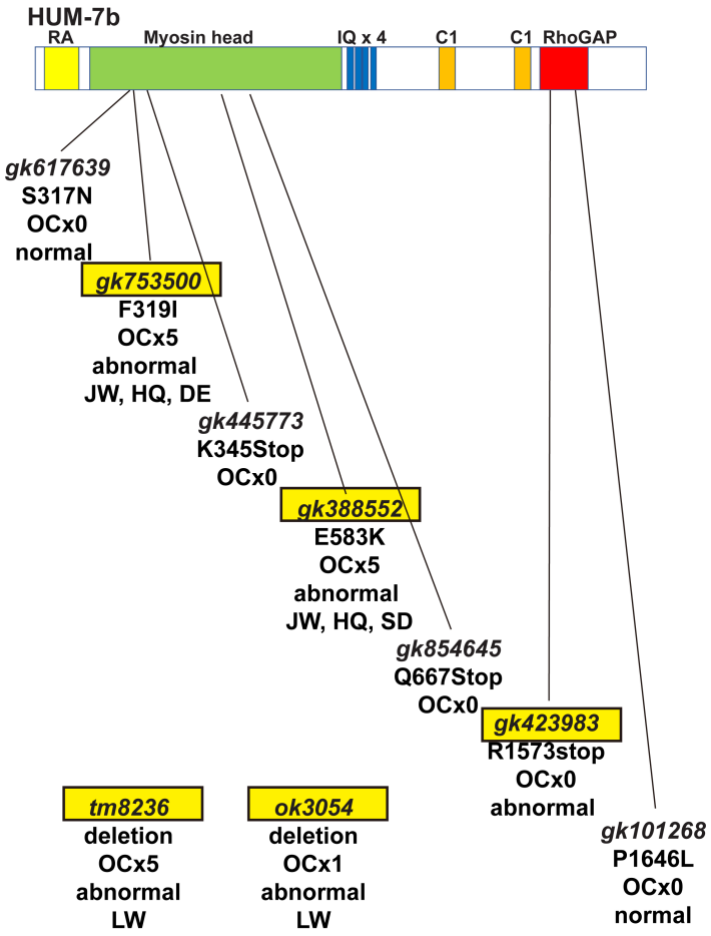

Figure 2. Location and nature of *hum-7* mutations. Shown is a schematic of the domain organization of the HUM-7 protein. Lines point to the approximate location of the mutation sites in the protein. Below the lines are indicated the allele numbers, types of mutations (missense, nonsense, deletion), number of times outcrossed to wild type (e.g. none (“OCx0”), 5 times (“OCx5”)), whether the MCB organization is normal or abnormal, and the lab personnel involved in the outcrossing (JW: Jordan Walter; HQ: Hiroshi Qadota; SD: Stefano Derossi; LW: Lauryn Worley; DE: Don Eaford).

Examples of immunostaining of some hum-7 mutants is shown in **Figure 3**. This is preliminary evidence that a functional myosin head is critical for the MCB function of HUM-7. It is also noteworthy that one missense mutation in the RhoGAP domain, P1646L, showed normal organization of the MCB, suggesting that perhaps the RhoGAP domain function is not required for HUM-7's function at the MCB. Although a second mutation in the RhoGAP domain does have an abnormal MCB, this mutation, *gk423983*, is a nonsense mutation, and thus cannot inform us as to whether the RhoGAP is critical since mRNAs with nonsense mutations are degraded by the "nonsense mediated decay" surveillance system, and thus very little truncated HUM-7 protein, would be expected. In the near future, each of these mutants will be outcrossed at least 5 times, and the immunostaining repeated before definitive conclusions can be made.

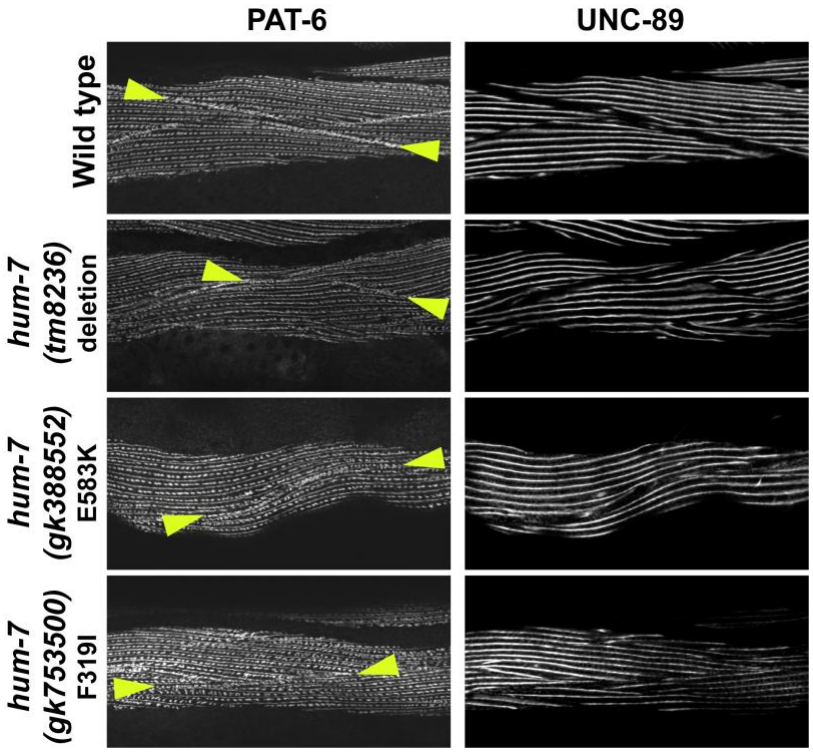

Figure 3. Co-immunostaining with antibodies to PAT-6 and UNC-89 shows lack of accumulation of PAT-6 at MCBs. Comparing PAT-6 and UNC-89 staining allow us to visualize muscle cell borders (darker regions in UNC-89). Arrowheads denote MCBs. Two point mutations (*gk388552* & *gk753500*) as well as a deletion mutation (*tm8236*) show little to no localization at MCBs, indicating that *hum-7* could play a role in accumulation IAC proteins at MCBs.

Localization of PIX-1 is not affected in *hum-7* mutants.

The lack of IAC assembly at MCBs is similar in *pix-1*, *rrc-1* and *hum-7* mutants. As mentioned above, PIX-1 and RRC-1 are components of the PIX pathway. We wondered if HUM-7 was also part of this pathway. To test this idea, I immunostained several *hum-*

7 mutants with antibodies to PIX-1. As shown in **Figure 4**, in wild type, PIX-1 localizes to the MCB, M-lines and dense bodies, as reported in Moody et al. (2020). PIX-1 localizes to these same locations in the *hum-7* mutants, including one deletion and two myosin motor missense mutations. This result suggests that HUM-7 may not be a component of the PIX signaling pathway.

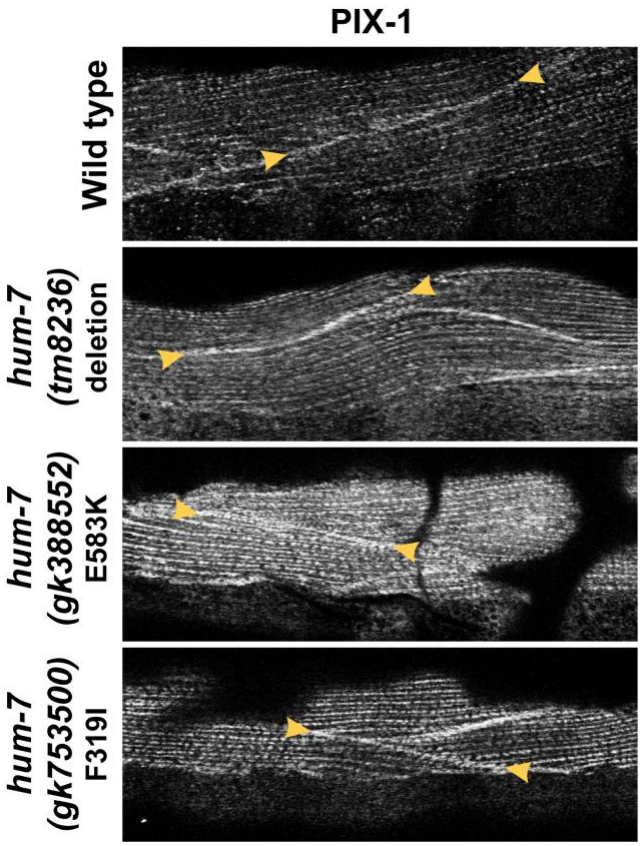

Figure 4. The localization of PIX-1 is not affected in hum-7 mutants. When *hum-7* mutants were stained with antibodies to PIX-1, normal accumulation of PIX-1 at MCBs was observed. Arrowheads point to MCBs. These results suggest that HUM-7 may not be part of the PIX-1 signaling pathway.

Antibodies generated to HUM-7 detect an appropriately sized protein (~207 kDa) by western blot

Rabbit polyclonal antibodies were generated to the N-terminal-most 165 residues of HUM-7, using as immunogen a GST fusion protein, and the antibodies were affinity-purified using an MBP fusion coupled to a column matrix. As indicated in **Figure 5**, a western blot in which total SDS-soluble protein extracts from wild type worms were separated by SDS-PAGE, blotted and reacted with anti-HUM-7, shows detection of a protein of the size expected for HUM-7, 207 kDa. Unfortunately, in addition, an ~47 kDa protein of unknown origin, perhaps either an alternatively spliced isoform or cross reaction to a bacterial protein, is also detected. Note that in some mutants (deletion tm8236, R1573Stop, K345Stop) the level of HUM-7 is reduced, and in one mutant (deletion ok3054) it is even increased. We had expected that the deletion mutants would have completely eliminated the presumptive HUM-7 protein. That the protein is not eliminated in these mutants suggests that the deletion is circumvented by alternative splicing. Another possibility is that our antibody is simply cross-reacting to another protein entirely. Soon, we hope to sort this out, by performing western blots on extracts from worms subjected to RNAi knockdown of *hum-7*, or using a CRISPR generated strain that expresses HUM-7-GFP.

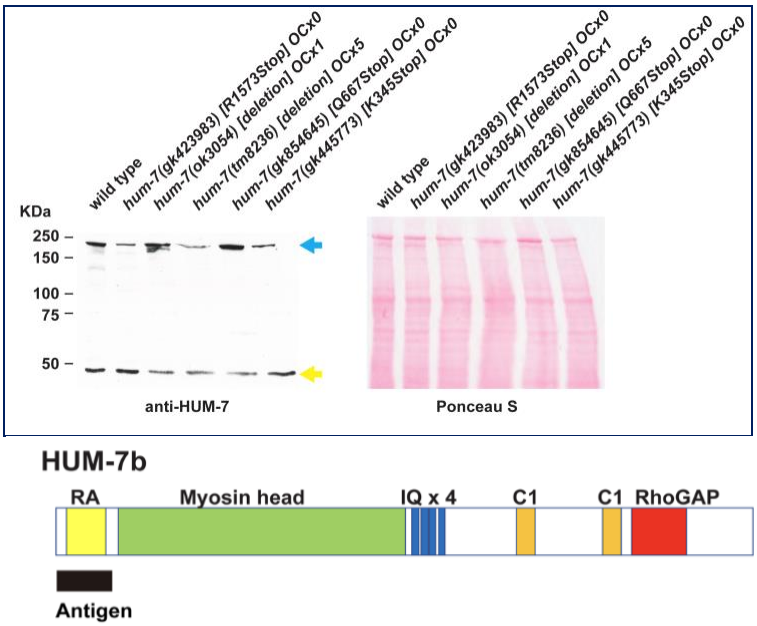

Figure 5. Antibodies to HUM-7 detect an appropriately sized (~207 kDa) protein by western blot. Rabbit polyclonal antibodies were generated to the indicated 165 residue region of HUM-7 as a GST fusion protein, and the antibodies were affinity-purified using an MBP fusion coupled to a column matrix. On the far left is a western blot in which total SDS-soluble protein extracts from the indicated strains were separated by SDS-PAGE, blotted and reacted with anti-HUM-7. Near left is shown the blot after Ponceau S staining indicating approximately equal loading of each sample. The blue arrowhead points to the presumptive HUM-7 protein based on its expected size (1880 aa). The yellow arrowhead point to a protein of unknown origin, perhaps either an alternatively spliced isoform or cross reaction to a bacterial protein. Note that in some mutants the level of HUM-7 is reduced, and in one mutant it is even increased.

Antibodies to HUM-7 localize between dense bodies and flank PAT-6 at MCBs.

Wild type body wall muscle was immunostained with rabbit antibodies to HUM-7 and rat antibodies to PAT-6. In **Figure 6**, the top row focuses on the region of the muscle cell containing M-lines and dense bodies. The yellow arrow points to an M-line; the yellow arrowhead points to a row of dense bodies. PAT-6 is known to localize to M-lines and dense bodies. Note that HUM-7 is localized between dense bodies. The lower row focuses on a region containing a muscle cell boundary (MCB). Note that, as indicated by the blue arrow, HUM-7 is localized near the MCB. However, this lies outside the region of PAT-6 localization. To obtain a more detailed view of HUM-7 localization at the MCB, I used structured illumination microscopy (SIM), a “super resolution” method, which has twice the resolution of confocal micscroscopy. As shown in **Figure 7**, a preliminary result shows that HUM-7 lies between the “zipper-like” structures labeled by PAT-6 at the MCB.

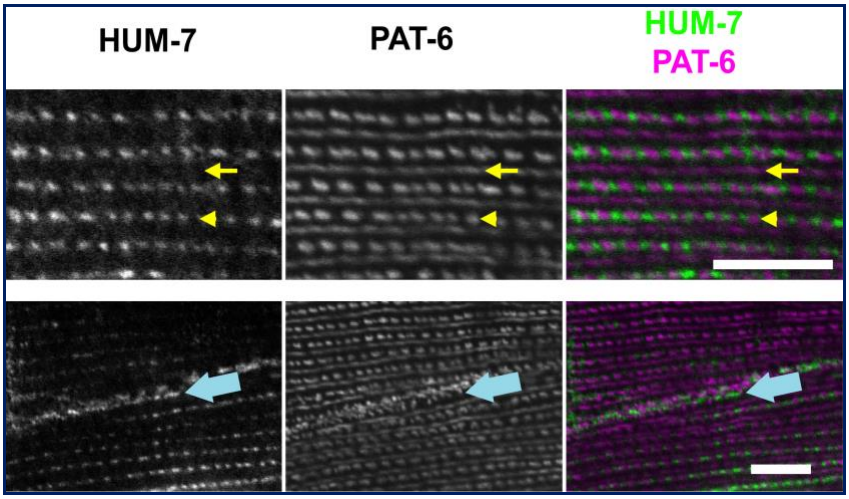

Figure 6. Antibodies to HUM-7 localize between dense bodies and flank PAT-6 at MCBs. Wild type body wall muscle was immunostained with rabbit antibodies to HUM-7 and rat antibodies to PAT-6. The top row focuses on the region of the muscle cell containing M-lines and dense bodies. The yellow arrow points to an M-line; the yellow

arrowhead points to a row of dense bodies. PAT-6 is known to localize to M-lines and dense bodies. Note that HUM-7 is localized between dense bodies. The lower row focuses on a region containing a muscle cell boundary (MCB). Note that, as indicated by the blue arrow, HUM-7 is localized near the MCB. However, this lies outside the region of PAT-6 localization. Scale bar,10 µm.

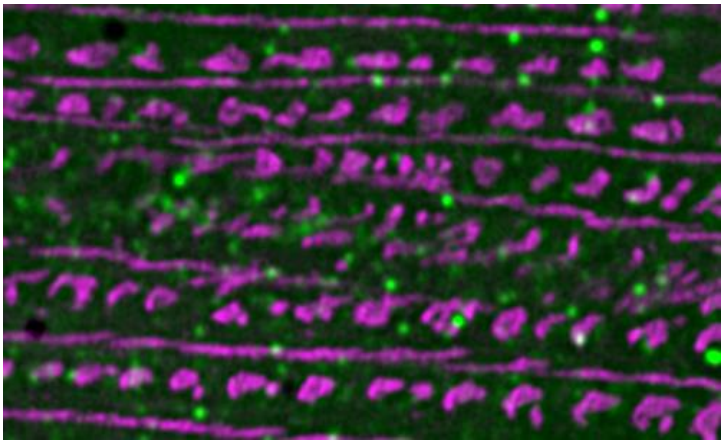

Figure 7. SIM imaging of HUM-7 and PAT-6 at MCB. Wild type body wall muscle was immunostained with rabbit antibodies to HUM-7 and rat antibodies to PAT-6, and images were obtained with a N-SIM super resolution microscope. Note that HUM-7 is localized between PAT-6 at MCBs.

Progress in determining which domain is required for HUM-7's function in muscle.

Our planned approach is to test various mutants in the major domains, the myosin motor domain and the RhoGAP, for their ability to rescue the *hum-7* mutant MCB defect. Through considerable effort, I have succeeded to make a full length, 5,604 bp,

cDNA for HUM-7. This was accomplished by making 5 error-free PCR fragments, and then stitching them together using restriction enzyme sites (**Figure 8**). I have succeeded to make this cDNA for both a wild type *hum-7*, and a mutant *hum-7*, containing a missense mutation in the RhoGAP domain, R1564M, which is expected to eliminate RhoGAP activity. In addition, I am in the process of creating a *hum-7* cDNA carrying a mutation in the myosin motor domain, R481N, expected to eliminate myosin motor activity. The prediction about the R481N mutation was made by the Benian lab collaborator, Dr. Andres Obherhauser (UTMB), an expert on myosin protein structure. For the wild type and RhoGAP mutant versions, I have also added at the N-terminus an HA tag so that the transgenic HUM-7 can be detected by anti-HA to distinguish it from endogenous HUM-7. Finally, this HA-HUM-7 sequence was placed downstream of a *myo-3* gene promoter, which will permit it to be expressed in body wall muscle. Soon, I will create transgenic animals bearing these wild type and mutant *hum-7* constructs in wild type background and then cross them into the *hum-7* mutants. Rescue would be assessed by immunostaining the transgenic animals and checking for conversion of the MCB defect to a wild type looking MCB. If the wild type *hum-7* can rescue and either the myosin motor mutant or the RhoGAP mutant can rescue the MCB defect, then that would eliminate either of those domains as being important for muscle function. Given that two of our MMP mutants in the myosin motor show the MCB defect, our expectation is that the RhoGAP will rescue, but the myosin mutant will not rescue.

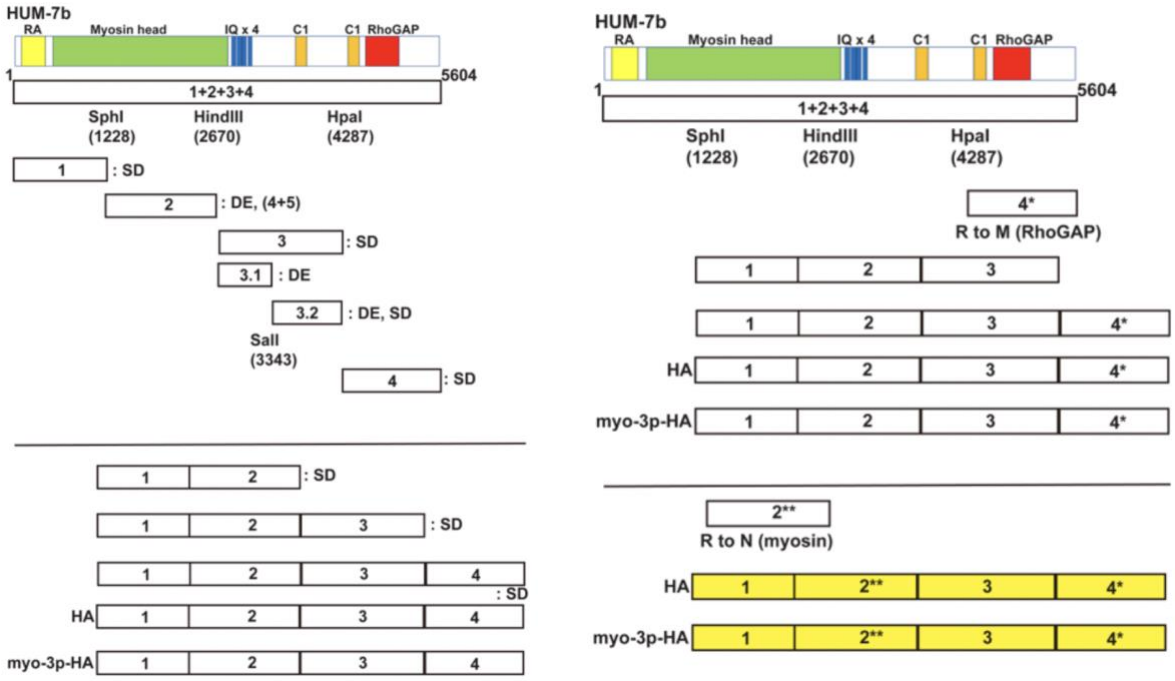

Figure 8. Schematic showing the steps involved in creating a full-length cDNA for wild type (left) and two mutant versions (right) HUM-7 to be used in transgenic rescue experiments. Clear indicates completed, yellow indicates in progress. SD and DE indicate “the cloners”, Stefano Derossi, and Don Eaford respectively.

Yeast Two-Hybrid Assays Reveal Lack of Direct Interaction Between HUM-7 and IAC Proteins known to be located at MCBs

There is a family of myosin proteins, consisting of at least 30 members (Mooseker and Cheney, 1995; Hartman et al., 2011). All myosins have a motor domain followed by a neck which contains binding sites for 2-6 calmodulins or myosin light chains, followed by a tail which varies in length and domain composition and distinguishes the myosin class. Conventional myosin, also known as muscle myosin or myosin II (class II) is the

type of myosin which forms thick filaments. Myosin II has a long tail (~1000 aa) which has a coiled-coil structure, and is essential for myosin II to form dimers, and then the rod portion of myosin (coil coil dimer), has the ability to form the shaft of the thick filament by electrostatic interactions of adjacent myosin rods. Many of the other classes of myosin also have coiled coil sequence/structure and these are also used to form dimers, although the dimerization regions are much shorter and none of these other myosin classes assemble into thick filaments. In fact, class IX myosin, including HUM-7, does not have any coiled coil sequence and has been shown to exist as a monomer. The major known function of the non-class II myosins or “unconventional myosins” is to act as a motor to carry cargo, usually in the form of vesicles along F-actin tracks to destinations. That is, they act as “trucks”. I hypothesize that HUM-7 is a truck that carries IAC components along actin tracks, to the MCB and/or between dense bodies (**Figure 9**). To begin to test this hypothesis, I have used the C-terminal half of HUM-7 to screen the known components of the muscle cell boundary (Qadota et al., 2017).

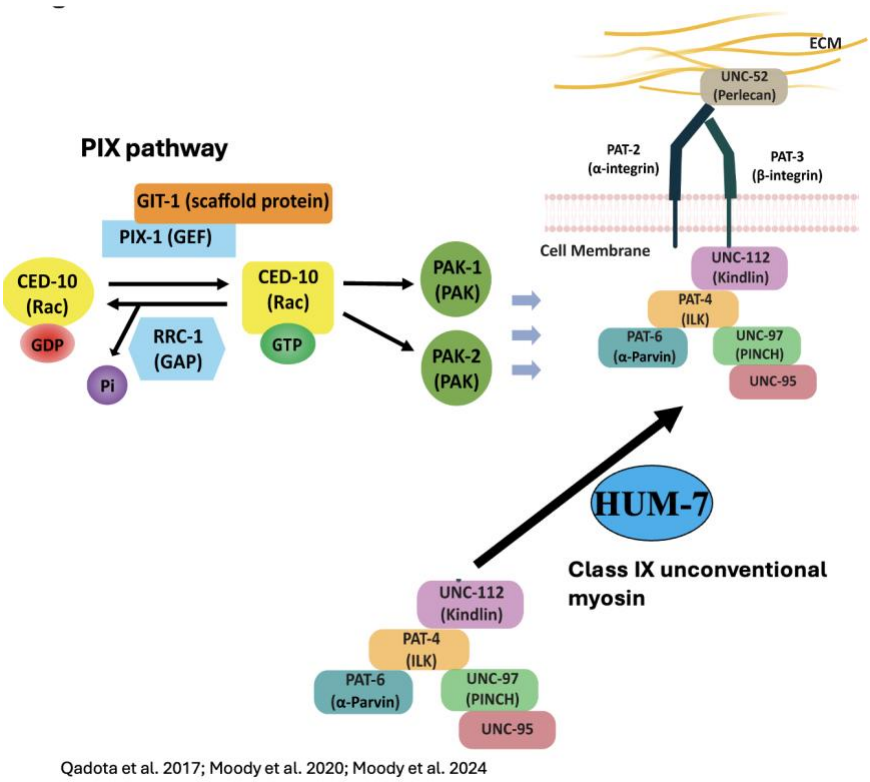

Figure 9. Model for the role of HUM-7 as a "delivery truck" to promote assembly of IACs.

The proteins tested in this assay included, but were not limited to, UNC-97, UNC-95, UNC-112, PAT-4, PAT-6, CeTalin(TLN-1), Cepaxillin(PXL-1), UIG-1, DEB-1, and CPNA-1. Negative controls included ATN-1 (α-actinin), which is not located at MCBs, and for each experiment, an empty vector. HUM-7 was used both as a bait and as a prey because some interactions are only found in "one direction".

Remarkably, my Y2H assay results indicated no significant growth on selective media for any of the protein pairs tested, suggesting a lack of direct interaction between the HUM-7 tail region and the chosen IAC components under the conditions of our assay. This was consistently observed across all protein pairs tested, including both the bait and prey configurations of the assay, with growth observed only on control plates confirming the presence of both plasmids within the yeast cells (**Figure 10**).

Strain: PJ69-4A		3/29/2024		
Bait	Prey	1/3ATMH	TMH	ATM+3AT
1 pGBDU-UNC-97	hum-7 3.2+4	+	-	+
2 pGBDU-UNC-97	C1	+	-	+
3 pGBDU-i7-207(UNC-95)	hum-7 3.2+4	+	-	-
4 pGBDU-i7-207(UNC-95)	C1	+	-	-
5 pGBDU-UNC-112	hum-7 3.2+4	+	-	-
6 pGBDU-UNC-112	C1	+	-	-
7 pGBDU-PAT-4	hum-7 3.2+4	+	-	-
8 pGBDU-PAT-4	C1	+	-	-
9 pGBDU-PAT-6	hum-7 3.2+4	+	-	-
10 pGBDU-PAT-6	C1	+	-	-
11 pGBDU-CeTalin(TLN-1)	hum-7 3.2+4	+	-	-
12 pGBDU-CeTalin(TLN-1)	C1	+	-	-
13 pGBDU-Cepaxillin(PXL-1)	hum-7 3.2+4	+	-	-
14 pGBDU-Cepaxillin(PXL-1)	C1	+	-	-
15 pGBDU-DEB-1	hum-7 3.2+4	+	-	+
16 pGBDU-DEB-1	C1	+	-	+
17 pGBDU-ATN-1 L	hum-7 3.2+4	+	-	-
18 pGBDU-ATN-1 L	C1	+	-	-
19 pGBDU-ATN-1 S	hum-7 3.2+4	+	-	+
20 pGBDU-ATN-1 S	C1	+	-	+
21 pGBDU-F9-2(HUM-6)	hum-7 3.2+4	+	-	-
22 pGBDU-F9-2(HUM-6)	C1	+	-	-
23 pGBDU-F6-1 (UIG-1)	hum-7 3.2+4	+	-	-
24 pGBDU-F6-1 (UIG-1)	C1	+	-	-
25 pGBDU-GX9-109(CPNA-1)	hum-7 3.2+4	+	+	+
26 pGBDU-GX9-109(CPNA-1)	C1	+	+	+
27 pGBDU-B15-101(CPNA-1)	hum-7 3.2+4	+	-	-
28 pGBDU-B15-101(CPNA-1)	C1	+	-	-

3/29/2024			
	4/4/2024		
Strain: PJ69-4A with pGBDU-hum-7 3.2+4			
Prey	1/3ATMH	TMH	ATM+3AT
1 pGAD-UNC-97	+	-	-
2 i7-207(UMC-95)	+	-	-
3 pGAD-UNC-112	+	-	-
4 pGAD-PAT-4	+	-	-
5 pGAD-PAT-6	+	-	-
6 pGAD-CeTalin(TLN-1)	+	-	-
7 pGAD-Cepaxillin(PXL-1)	+	-	-
8 pGAD-DEB-1	+	-	-
9 pGAD-ATN-1 L	+	-	-
10 pGAD-ATN-1 S	+	-	-
11 F9-2(HUM-6)	+	-	-
12 F6-1(UIG-1)	+	-	-
13 GX9-109(CPNA-1)	+	-	-
14 B15-101(CPNA-1)	+	-	-
15 pGAD-hum-7 3.2+4	+	-	-
16 pGAD-C1	+	-	-

Figure 10. Results of Y2H assays for HUM-7 tail region.

The absence of detectable interactions in our Y2H assay has several potential explanations. First, HUM-7 may not directly interact with a cargo, but only does so through an intermediary protein. To address this possibility, I will soon use the HUM-7 tail bait in a Y2H screen of an entire library of cDNA clones, representing most or all of the approximately 19,000 proteins expressed in *C. elegans*. Hopefully, an intermediary protein will be revealed. If so, it will be tested for its ability to also bind IAC components, again by the Y2H assay. In the future, any potential interactions will need to be confirmed by biochemical binding assays (e.g. ELISA, ITC, biolayer interferometry) using bacterially expressed recombinant proteins. Such assays will also provide Kd binding constants. Another possibility for our negative results, is that interaction between HUM-7 and its cargo depends on post-translational modifications (e.g. phosphorylation) which does not occur in yeast as extensively as it does in animal cells. If our library screen is negative, we can try to immunoprecipitated HUM-7 from worms, hopefully isolate a complex of proteins, and send them for mass spec for identification. However, I am aware that such analysis will only reveal whether HUM-7 exist in a protein complex, not whether HUM-7 interacts directly with such proteins. Finally, it should be noted that I have only performed the Y2H assays on proteins known to be located at the MCBs. Recall that HUM-7 is also located between dense bodies. This location is mysterious and has an unknown function, although it is possible that it contains binding sites for actin containing thin filaments. The Benian lab and others have discovered that there are a small set of proteins that are exclusively (e.g. kettin, LIM-8, and TTN-1) or partially located between dense bodies. In the near future, these proteins could also be tested for binding to HUM-7 by Y2H assays.

Materials and Methods

Sequence analysis

Nematode HUM-7 protein sequence was obtained from Wormbase. The domain organization for HUM-7 was predicted via PFAM.

Nematode strains and outcrossing

All nematode strains were obtained from the Caenorhabditis Genetics Center and grown on NGM plates using standard methods and maintained at 20° (Brenner, 1974). Outcrossing to wild type was done up to 5 times, and for each time, in the F2 generation *hum-7* homozygotes were identified by PCR and sequencing or by running PCR products on agarose gels.

Immunostaining, confocal and structured illumination microscopy

Adult nematodes were fixed and immunostained using the method described by Nonet et al. (1993) with further details provided in Wilson et al. (2012). The following primary antibodies were used at 1:200 dilution: anti-PAT-6 (rat polyclonal; Warner et al., 2013), anti-UNC-89(rabbit polyclonal EU30; Benian et al., 1996), anti-PIX-1 (rabbit polyclonal; Moody et al., 2020), and anti-HUM-7 (rabbit polyclonal described here). Secondary antibodies, also used at 1:200 dilution, included anti-rabbit Alexa 488, and anti-rat Alexa 594, purchased from Invitrogen. Images were captured at room temperature with a Zeiss confocal system (LSM510) equipped with an Axiovert 100M microscope and an

Apochromat x63/1.4 numerical aperture oil immersion objective. For SIM we used a Nikon N-SIM system in 3D structured illumination mode on an Eclipse Ti-E microscope equipped with a 100×/1.49 NA oil immersion objective, 488- and 561-nm solid-state lasers, and an EM-CCD camera (DU-897, Andor Technology). Super-resolution images were reconstructed using the N-SIM module in NIS-Elements software. For all the images, confocal and SIM, the color balances were adjusted by using Adobe Photoshop (Adobe, San Jose, CA).

Generation of a HUM-7 rabbit polyclonal antibody

We used PCR to amplify a 495 bp cDNA encoding the most N-terminal 165 aa of HUM-7 using as template the RB2 cDNA library (kind gift from Robert Barstead, Oklahoma Medical Research Foundation). This coding sequence was cloned into vectors pGEX-KK1 and pMAL-KK1, respectively to express glutahione S-transferase (GST) and maltose binding protein (MBP) fusion proteins in *E. coli*. The GST fusion was sent to Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, Pennsylvania) for generation of polyclonal antibodies in two rabbits. We then used the MBP fusion to affinity purify the antibodies. Methods for protein production and affinity purification were essentially as described in Moody et al. (2020).

Western blot

The method of Hannak et al. (2002) was used to prepare total protein lysates from wild-type, and *hum-7* mutant strains. Equal amounts of total protein were separated on a 7.5% polyacrylamide-SDS- Laemmli gel, transferred to nitrocellulose membrane, and reacted with affinity purified, E. coli-OP50-absorbed anti-HUM-7 at 1:200 dilution, and then reacted with goat anti-rabbit immunoglobulin G conjugated to HRP (GE Healthcare) at 1:10,000 dilution, and visualized by ECL. Prior to reaction with antibodies, total protein in each lane was detected by Ponceau S staining.

Cloning of a full-length cDNA for *hum-7*

Following the schematic shown in Figure 8, we amplified by PCR using the RB2 cDNA library, 5 segments containing the added restriction endonuclease cleavage sites, and then in steps, ligating together to create a single ~5.6 kb cDNA. For each fragment, we first cloned into pBluescript and by Sanger sequencing identified clones that were PCR-error-free. Sequence encoding a 3XHA tag was added to the 5' end, by cloning the full length cDNA into pKS-HA(Nhe) vector, excising the insert with the HA tag, and re-cloning into plasmid pPD95.86 (kind gift of Andrew Fire, Stanford University) that upon creation of a transgenic worm would express in body wall muscle from the *myo-3* gene promoter. Missense mutations for RhoGAP and the myosin motor were/are being introducing using PCR primers containing the mutations.

Yeast Two-Hybrid Assays

The yeast two-hybrid (Y2H) system was employed to investigate potential interactions between HUM-7 and various IAC proteins, focusing on the tail region of HUM-7. This region was selected based on its presumed role in cargo binding, hypothesizing that it might mediate interactions with IAC components. Methods for the Y2H assays were essentially as described in Miller et al. (2006). The PJ69-4A yeast strain was utilized for transformations of plasmids carrying the GAL4 activation domain (AD) or DNA-binding domain (BD). The HUM-7 tail region was amplified by PCR and cloned into the pGAD-C1 (AD) and pGBDU-C1 (BD) vectors. IAC proteins, including UNC-97, UNC-95, UNC-112, PAT-4, PAT-6, CeTalin(TLN-1), Cepaxillin(PXL-1), DEB-1, ATN-1 (L and S isoforms), F9-2(HUM-6), UIG-1, GX9-109(CPNA-1), and B15-101(CPNA-1), were similarly cloned into the pGBDU-C1 or -C2 (BD) vector and pGAD-C1 or pACT vector. Empty vector (pGAD-C1) served as negative controls.

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