**Drosophila UNC-45 accumulates in embryonic blastoderm and in muscles, and is essential for muscle myosin stability**

Chi F. Lee¹, Girish C. Melkani¹, Qin Yu¹, Jennifer A. Suggs¹, William A. Kronert¹, Yoko Suzuki¹, Lori Hipolito¹, Maureen G. Price², Henry F. Epstein³ and Sanford I. Bernstein¹,*

¹Department of Biology and the Molecular Biology Institute, San Diego State University, San Diego, CA 92182, USA
²Department of Neurology, Baylor College of Medicine, Houston, TX 77730, USA
³Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555, USA

*Author for correspondence (sbernst@sciences.sdsu.edu)

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**Summary**

UNC-45 is a chaperone that facilitates folding of myosin motor domains. We have used *Drosophila melanogaster* to investigate the role of UNC-45 in muscle development and function. *Drosophila* UNC-45 (dUNC-45) is expressed at all developmental stages. It colocalizes with non-muscle myosin in embryonic blastoderm of 2-hour-old embryos. At 14 hours, it accumulates most strongly in embryonic striated muscles, similarly to muscle myosin. dUNC-45 localizes to the Z-discs of sarcomeres in third instar larval body-wall muscles. We produced a dunc-45 mutant in which zygotic expression is disrupted. This results in nearly undetectable dUNC-45 levels in maturing embryos as well as late embryonic lethality. Muscle myosin accumulation is robust in dunc-45 mutant embryos at 14 hours. However, myosin is dramatically decreased in the body-wall muscles of 22-hour-old mutant embryos. Furthermore, electron microscopy showed only a few thick filaments and irregular thick–thin filament lattice spacing. The lethality, defective protein accumulation, and ultrastructural abnormalities are rescued with a wild-type dunc-45 transgene, indicating that the mutant phenotypes arise from the dUNC-45 deficiency. Overall, our data indicate that dUNC-45 is important for myosin accumulation and muscle function. Furthermore, our results suggest that dUNC-45 acts post-translationally for proper myosin folding and maturation.

**Key words:** Chaperone, Drosophila, Muscle, Myofibril, UNC-45

**Introduction**

Myosins are molecular motors that function in cellular processes from cytokinesis and vesicle transport to cell motility. Currently, there are at least 24 classes of myosin (Foth et al., 2006), each with its own unique characteristics. Structurally, myosin molecules have globular heads and rod-like tails. The globular head contains the motor domain, including the ATPase and actin binding sites. The tail can be used to form coiled-coil dimers or attachments to substrates. Even though the different types of myosin are involved in different cellular functions, they have similar overall structure in the motor domain.

Myosin II is a major component of muscle thick filaments and is indispensable in muscle contraction. To facilitate the investigation of its function, researchers have attempted to synthesize muscle myosin in vitro. Functional cardiac (Sweeney et al., 2003) and smooth muscle (Trybus, 1994) myosin isoforms have been produced using insect cells, but in vitro expression of skeletal muscle isoforms has not been routinely performed without using a myogenic cell line (Chow et al., 2002) or its lysate (Srikakulam and Winkelmann, 1999). Because functional α-helical tails of rabbit skeletal muscle myosin can be synthesized in *Escherichia coli* (Atkinson and Stewart, 1991), it appears that some factor(s) in the myogenic cell line facilitates the folding of skeletal muscle myosin globular heads into the correct conformation. To gain an understanding of myosin folding, we have investigated the function of a recently characterized myosin chaperone UNC-45.

From its first description in a temperature-sensitive *Caenorhabditis elegans* mutant (Epstein and Thomson, 1974), to recent data supporting its role in facilitating myosin degradation (Landsverk et al., 2007), UNC-45 has been shown to be important for myosin maturation, thick filament assembly and muscle function. The discovery of a muscle-specific isoform of UNC-45 in vertebrates (Price et al., 2002) further underscores the importance of UNC-45 in muscle. *C. elegans* mutants of UNC-45 show movement defects and decreased thick filament formation (Barral et al., 1998), and morpholino knockdown of UNC-45 in zebrafish results in paralysis and cardiac dysfunction (Wohlgemuth et al., 2007). RNA interference (RNAi) knockdown of UNC-45 in *Drosophila* embryos results in wild-type body-wall muscle patterning, yet these muscles do not contract (Estrada et al., 2006).

UNC-45 is composed of three domains: an N-terminal tetratricopeptide repeat (TPR) motif, a central domain, and a C-terminal UCS domain (Fig. 1A). The UCS domain is named after the three proteins (UNC-45, Cro1 and She4p) discovered to contain the homologous domain that was subsequently found to interact with myosin (Barral et al., 1998; Barral et al., 2002; Toi et al., 2003). The central domain of UNC-45 has an unknown function but its sequence is approximately 40% conserved between *C. elegans* and humans. The TPR domain has been found to interact with heat shock protein 90 (Barral et al., 2002; Mishra et al., 2005; Etard et al., 2007; Liu et al., 2008), which led to the notion that UNC-45 is a co-chaperone for heat shock protein 90. In-depth reviews of UNC-45 function have been published previously.
Developmental expression of UNC-45 in Drosophila

We determined dUNC-45 expression in the yellow white (yw) genotype (wild-type background) using western blotting analysis for developmental expression and immunofluorescence confocal microscopy for localization. A polyclonal antibody was generated in rabbits using bacterially expressed full-length, His-tagged dUNC-45 as the immunizing agent. The antibodies were highly specific on western blots (Fig. 2A) when probed against whole fly lysates. The third lane is bacterially expressed recombinant dUNC-45. It contains a His tag, which accounts for its slightly larger size. The protein size ladder is given on the right. (B–D) Developmental expression of dUNC-45. (B) Early (EE) and late stage embryos (LE). (C) First instar (1L), second instar (2L), and third instar larvae (3L). (D) Early pupae (EP), late pupae (LP), 2-hour-old adult (2hA), and 1-day-old adult (1dA). Equivalent amounts of protein were loaded in each lane.

localization was similar to that of muscle myosin, which is expressed strongly in skeletal muscles such as body-wall muscle and pharyngeal muscle. Non-muscle myosin localized to non-muscle tissues, as did some dUNC-45 (particularly to ectoderm). UNC-45 was also found in muscle-containing gut. However, dUNC-45 staining intensity was much brighter in the skeletal muscles. Lateral views of 14-hour-old embryos showed intense dUNC-45 fluorescence in the body-wall muscle, similar to that of skeletal muscle myosin (Fig. 3G). Furthermore, dUNC-45 localized to other skeletal muscle examined, including embryonic dorsal tube, adult body-wall muscle, adult dorsal tube and adult indirect flight muscle (C.F.L., unpublished results).

dUNC-45 subcellular localization was also investigated using immunofluorescence confocal microscopy. Third instar larval body-wall muscle was used to examine dUNC-45 sarcomeric localization because embryonic body-wall muscles do not show a distinct sarcomeric structure at 14 hours of age, and antibodies do not penetrate older embryos due to cuticle formation. Because both muscle myosin and dUNC-45 antibodies were generated using rabbits as host species, co-staining was not feasible. Instead, dissected third instar yw larvae were co-stained with dUNC-45 antibodies, α-actinin antibodies (Z-disc-specific) and phalloidin (for actin and I band visualization). The result of the triple staining indicated that dUNC-45 is localized to the Z-discs (Fig. 4). It colocalized with α-actinin staining and bisected phalloidin staining.

Mutant analysis

To discern the effects of mutating unc-45 in Drosophila, a mutant line (Tom34EY03034) from the Bloomington Stock Center was examined. Tom34EY03034 is an unc-45 allele created by insertion of a P element in the promoter region, upstream of the translation start site (Fig. 1B). Tom34EY03034 allele homozygotes display third larval instar lethality. Because the coding region of Drosophila unc-45 (dunc-45) is not disrupted, it is conceivable that functional protein could still be synthesized, albeit in lower amounts than normal, which could impede our mutant phenotype analysis. Western blotting of 22-hour-old homozygous Tom34EY03034...
embryos confirmed the presence of the full-length protein (Fig. 5A, lane 2).

To create an unc-45 null allele, which might have a more severe phenotype, a P element mobilization procedure was performed. Potential dUNC-45 null lines were identified by the absence of specific genetic marker phenotypes (see Materials and Methods) and confirmed by western blotting and DNA sequencing. Analysis of 22-hour-old homozygous embryos showed that line 33 (Tom34EY03043–33 or T-33) was dUNC-45 null (Fig. 5A, lane 3).

T-33 homozygotes display embryonic lethality, which could be rescued by a wild-type dunc-45 transgene but not by the Tom34EY03034 allele in the heterozygous complementation test (see Materials and Methods). Together, the data confirm that the lethal phenotype was specifically due to the dunc-45 knockout. DNA sequencing showed that T-33 contains a 1304 base pair (bp) deletion of the dunc-45 DNA from the promoter to the second exon (Fig. 1C), which includes the translation start site (details of the deleted region are presented in Materials and Methods); therefore, subsequent mutant characterization was carried out using this line.

To determine whether the T-33 allele disrupts dUNC-45 localization, we performed immunofluorescence microscopy of 14-hour-old embryos. Fig. 6A–C shows that embryos carrying at least one wild-type dunc-45 allele, identified by lacZ expression in the hindgut, had intense UNC-45 expression in the body-wall muscles, similar to the yw wild-type. Homozygous T-33 embryos showed barely detectable dUNC-45 staining in the body-wall muscles (Fig. 6F). Interestingly, the lack of dUNC-45 did not dramatically affect skeletal muscle myosin translation (Fig. 6E). The muscle myosin expression in the confocal images was assessed by comparing the staining levels of homozygous T-33 to other embryos within the same batch. Because each batch of embryos was stained in the same tube and imaged in parallel, qualitative assessment of fluorescence intensities suggested similar expression levels of muscle myosin in all the embryos. However, myosin did not remain stable during the normal period of myofibril assembly, in that myosin levels were greatly reduced in 22-hour-old homozygous T-33 embryos (Fig. 5B, lane 2). We examined the body-wall muscle ultrastructure of such embryos using transmission electron microscopy (TEM). Fig. 7 shows that 22-hour-old yw wild-type embryos exhibited regular thick and thin filament packing in transverse section (Fig. 7A) and regularly spaced thick filaments in longitudinal section (Fig. 7B). Homozygous T-33 embryos of the same age showed disrupted filament packing and a near absence of thick filaments (Fig. 7C,D). The muscle defects in the T-33 homozygous embryos were rescued by a wild-type dunc-45 transgene (Fig. 7E,F).

**Discussion**

Using Drosophila melanogaster wild-type and mutant flies, we examined UNC-45 localization and function. We found that dUNC-45 is expressed throughout development and its expression is maintained in adulthood (Fig. 2B–D), suggesting that it functions...
not only in myosin assembly and folding but possibly in muscle maintenance and/or repair.

dUNC-45 colocalized with non-muscle myosin in 2-hour-old embryos, prior to the expression of muscle myosin (Fig. 3, upper panels). At this cellularization stage, non-muscle myosin displays a cytosolic and peri-nuclear localization pattern (Young et al., 1991), as does dUNC-45. It is possible that at this early embryonic stage, both non-muscle myosin and dUNC-45 are distributed diffusely throughout the cytoplasm and that the immunofluorescence staining results do not indicate direct interaction. However, it is more likely that dUNC-45 interacts with non-muscle myosins, because this is consistent with findings in C. elegans, where UNC-45 and non-muscle myosin II colocalized at the cell cortex and were both needed for cytokinesis (Kachur et al., 2004; Kachur et al., 2008). Similarly, other UNC-45-like proteins such as She4p and Rng3 interact with myosin V and non-muscle myosin II, respectively as shown using genetic interaction, yeast two-hybrid, in vitro protein pull-down and in vivo mutant characterization experiments (Wong et al., 2000; Wescan et al., 2003). Furthermore, UNC-45 is maternally inherited in C. elegans (Venolia and Waterston, 1990). It is plausible that the Drosophila UNC-45 present at the blastoderm stage is maternally inherited as well.

In 14-hour-old embryos, dUNC-45 colocalized with muscle myosin (Fig. 3, lower panels). Muscle myosin expression starts at approximately 10 hours AEL, correlating with the onset of muscle fiber formation and patterning (C.F.L., unpublished results), where muscle fibers are present in the correct pattern but muscle striation is still not apparent. Because skeletal muscle myosin and dUNC-45 showed intense staining in the embryonic body-wall muscle, it appears that the major zygotic expression of dUNC-45 coincides with muscle myosin expression both spatially and temporally. Subcellularly, dUNC-45 localized to the Z-discs of the sarcomeres in the body-wall muscle of wild-type yw third instar larvae (Fig. 4). This result agrees with the observations of Etard and colleagues (Etard et al. 2008), who localized UNC-45 at the Z-discs of zebrafish under normal developmental conditions. We used a polyclonal antibody against wild-type dUNC-45, whereas an UNC-45-GFP chimera was used in the zebrafish study. It is possible that our polyclonal dUNC-45 antibodies recognize targets other than UNC-45. However, this is unlikely because immunofluorescence confocal microscopy staining using pre-immunized rabbit serum did not yield a specific staining pattern above background (C.F.L., unpublished results) and western blot analysis showed a single band of the correct protein size when using total adult fly lysate (Fig. 2A). Because UNC-45 contributes to myosin degradation when overexpressed in C. elegans (Landsverk et al., 2007), it might be important to regulate UNC-45 localization. Consequently, Z-disc localization might be required to prevent the degradation of normal, functional myosin. The Z-disc could sequester UNC-45, which is then released in response to muscle stress and/or damage, as has been shown to occur in zebrafish (Etard et al., 2008). The timeliness of Z-disc formation appears to be dependent upon UNC-45 function in Xenopus tropicalis (Geach and Zimmerman, 2010). Although dUNC-45 localization to the Z-disc is similar to that reported for zebrafish muscle, it is different to the localization observed in C. elegans. In the nematode muscles, UNC-45 is localized to the two ends of the A band, corresponding to the location of the myosin heavy chain B myosin isoform (Ao and Pilgrim, 2000). The reason for this discrepancy is unclear, but Kachur and Pilgrim speculate that C. elegans might employ an alternative strategy in keeping UNC-45 close to myosin by binding to the neck region of myosin (Kachur and Pilgrim, 2008). Interestingly, dUNC-45 staining in the Drosophila sarcomere appears to be wider and more diffuse than that of α-actinin (Fig. 4), which could suggest dUNC-45 mobility in and out of the Z-disc or the presence of peri-Z-disc components that anchor dUNC-45.

Analysis of T-33 dunc-45 mutant flies provides important insights into the function of UNC-45 during muscle formation. Western blot analysis showed a drastic decrease of dUNC-45 in the 22-hour-old homozygous embryos compared to wild-type yw embryos (Fig. 5A). Occasionally, a small amount of dUNC-45 could be seen in the T-33 line, which is probably the remnant of maternally inherited protein. Immunofluorescence confocal microscopy showed that 14-hour-old T-33 homozygous embryos barely stained

![Fig. 5. Western blot analysis of dUNC-45 and myosin expression in wild-type (yw), Tom34EY03034 and T-33 22-hour-old embryos. (A) Western blot analysis of dUNC-45 expression. Control is bacterially expressed recombinant dUNC-45. (B) Western blot analysis of myosin expression. Control is indirect flight muscle lysate. Results of the analysis show that dUNC-45 can still be detected in the Tom34EY03034 line but not in the T-33 line. Muscle myosin heavy chain is somewhat reduced in T-33 homozygotes, but is nearly absent from T-33 embryos. 10 μg of protein were loaded for each sample.](image)

![Fig. 6. Immunofluorescence confocal micrographs of 14-hour-old T-33/lacZ or lacZ/lacZ embryos and homozygous T-33 embryos. (A–F) Embryos were probed with antibodies to lacZ (A,D), muscle myosin (B,E) and dUNC-45 (C,F). T-33/lacZ or lacZ/lacZ embryos were identified by lacZ expression in the hind-gut (A). Homozygous T-33 embryos do not show a detectable expression of lacZ (D). dUNC-45 cannot be detected in the body-wall muscle of the homozygous T-33 embryos (F). Muscle patterning and muscle myosin expression are not affected in the absence of dUNC-45 at this stage of embryogenesis (E). Scale bar: 75 μm.](image)
for dUNC-45 in their body-wall muscles (Fig. 6), which contrasts with intense staining in the heterozygous T-33 and wild-type embryos. This confirms the zygotic expression of dUNC-45 at this stage, which coincides with muscle myosin expression. Interestingly, in the absence of zygotically expressed dUNC-45, skeletal muscle myosin was still expressed and the muscle patterning appeared normal (Fig. 6E). The muscle myosin produced at this time might be folded by the remnants of maternally inherited dUNC-45 or not folded appropriately. By contrast, western blot analysis of 22-hour-old, only 8 hours older, homozygous dUNC-45 or not folded appropriately. By contrast, western blot analysis of 22-hour-old, only 8 hours older, homozygous dUNC-45 embryos showed a drastic decrease in muscle myosin (Fig. 5B). The body-wall muscles of 22-hour-old homozygous embryos exhibited a lack of sarcomeric structure, with only a few thick filaments visible in electron micrographs (Fig. 7C,D). Together, the data support the idea that the lack of dUNC-45 does not affect muscle myosin translation, but results in the accumulation of non-functional myosin that is later degraded, leading to embryonic lethality.

If dUNC-45 is not needed during myosin translation, then it must be needed post-translationally for proper myosin folding and/or stabilization. In vitro translation of chicken smooth muscle myosin in the presence of mouse UNC-45 also supports a post-translational association of the two proteins (Liu et al., 2008). However, a yeast UCS protein, Rng3, associates with myosin-encoding mRNA in polysomes in ribonucleoprotein immunoprecipitation–microarray experiments, which implies a co-translational involvement (Amorim and Mata, 2009).

Overall, we have demonstrated that dUNC-45 is essential for Drosophila skeletal muscle myosin stability, muscle function and viability. However, as a chaperone, UNC-45 could also function in muscle maintenance and protection during stress. Structural information (Lee et al., 2011) will greatly advance our knowledge about this protein by facilitating in vivo studies of specific UNC-45 site-directed mutants in Drosophila and other model organisms. Once the mechanism of action of UNC-45 is unraveled, it could serve as a therapeutic target for muscle and heart disease.

Materials and Methods

Fly lines

Drosophila yellow white (y, w) flies were used as a wild-type control line. The Tom34Δ57030/H11032, Sh UNC-45 mutant was obtained from the Bloomington Stock Center. It was crossed to a balancer line containing MKRS, Sh/TM3, y′, Ser to take advantage of the y′ and the Serrate markers, which resulted in the generation of Tom34Δ57030/TM3, y′, Ser. This line was used to create the dUNC-45 knockout T-33/TM3, y′, Ser line through P element mobilization by selecting for imperfect excision mutants with deletions in the genomic DNA regions of dunc-45 (Robertson et al., 1988). Briefly, virgin females of Tom34Δ57030/TM3, y′, Ser were crossed with males of the balancer line w*, CyO;Sp,CyO,TM2, U/sbΔ2-3, Sh containing the Δ2-3 transposase. The F1 male individuals with blotchy eye color, stubble bristles and curly wings (y, w; CyO/+; Tom34Δ57030Δ2-3, Sh) were selected and crossed with the yw; +; MKRS, Sh/TM3, y′, Ser balancer line to remove the transposase. The F2 male individuals with white eyes, wild-type cuticle coloration and serrated wings (y, w; +; Tom34Δ57030ΔTM3, y′, Ser) were selected and crossed with the MKRS, Sh/TM3, y′, Ser balancer line again to stabilize the line. A total of 23 potential hypomorphic dunc-45 jump-out lines were created and each was assessed by western blot analysis for dUNC-45 expression. Each line was placed in a Plexiglas box containing apple agar plates at 25°C to obtain fertilized eggs that produced homozygous 22-hour-old embryos, identified by yellow mouth hooks (y, w; +; Tom34Δ57030ΔTM3, y′, Ser). Western blotting analysis identified the T-33 line as a dUNC-45 knockout line. PCR and DNA sequencing of T-33 genomic DNA showed a 1304 bp deletion starting from 5′-CTCTCAATTTTATAA-3′ to 5′-ACGAGATGCAGATTGATC-3′. Furthermore, a piece of the P element (5′-CATGATGAAATAACATCATGTTAGCCACAG-3′) was left behind.

To show that T-33 is an allele of Tom34Δ57030, the T-33 line (y, w; T-33/TM3, y′, Ser) was crossed with the original Tom34Δ57030 mutant line (Tom34Δ57030/TM3, y′, Ser). All of the 100 offspring examined showed the balancer markers black body
(y) and serrated wings (Ser), indicating that T-33/Tom34Δ78-151 is not viable and the mutations are allelic.

To identify homozygous 14-hour-old T-33 embryos, the T-33/TM3, y, Ser line was crossed with the TM3, lacZ-arg, Sh balancer (Gorman and Kaufman, 1995). The resulting line, T-33/TM3, lacZ-arg, Sh, with storable bristles and normal wings was used to obtain fertilized eggs for immunofluorescence confocal microscopy. Homozygous T-33 embryos showed an absence of lacZ staining in the hind-gut.

Antibodies, sera, and fluorescent probes

Polyclonal dUNC-45 antibody was obtained through Sigma-Genosys (St Louis, MO) using the standard antibody service without affinity purification. Bacterially expressed recombinant His-tagged full-length dUNC-45 (Mellkan et al., 2010) was used as the immunizing agent in two New Zealand white rabbits (GN-15577 and GN-15578). The pre-immune serum and third bleed serum from rabbit GN-15578 were used in this study. Rabbit anti-non-muscle myosin IIa and rabbit anti-muscle myosin antibodies were kindly provided by Dan Kiehart, Duke University, Durham, NC. Rabbit anti-tubulin antibody was a generous gift from Judith Saide, Boston University School of Medicine, Boston, MA. Other antibodies, sera and fluorescent probes were purchased from commercial sources: goat-anti-rabbit-HRP (Bio-Rad, Hercules, CA), goat-anti-rabbit-Cy2 (Chemicon, Temecula, CA), goat-anti-mouse-FITC (Sigma, St Louis, MO), mouse-anti-LacZ (Promega, Madison, WI), normal goat serum (Thermo Fisher, Waltham, MA) and phallolidin-TRITC (Sigma).

Protein electrophoresis and western blot analysis

Protein electrophoresis of Drosophila lysates and Western blotting were performed as described previously (Laemmli, 1970; Sambrook and Russell, 2001) using a Bio-Rad Mini-Protein II minigel apparatus and precast 4% stacking, 10% separating acrylamide gels. Protein quantification was done using advanced protein assay reagent (Cytoskeleton, Denver, CO). Aliquots of 10 μg of protein were loaded for each sample. dUNC-45 antibody was diluted 1:1000 and myosin antibody was diluted 1:1000. Each primary antibody was incubated with the embryos for at least 4 hours (maximum overnight) at 4°C. Secondary goat-anti-rabbit-Cy5 and goat-anti-mouse-FITC antibodies were diluted 1:500 and incubated with the larvae for 2 hours at room temperature. Stained embryos were stored in PBS with 50% glycerol. Vectashield (Vector Laboratories, Burlingame, CA) was added before imaging using a Leica DM IRBE confocal microscope. Phallolidin-TRITC (1:500) was added with the secondary antibodies to stain filamentous actin.

Drosophila yw third instar larvae were dissected on Sylgard 184 Silicone elastomer (K. R. Anderson, Morgan Hill, CA). Fixation and staining were performed as previously described (Molina and Cripps, 2001). Primary antibodies were used at the following dilutions: anti-dUNC-45 1:500, anti-skeletal muscle myosin 1:1,000, anti-actin 1:500. Each primary antibody was incubated with the larvae for at least 4 hours (maximum overnight) at 4°C. Secondary goat-anti-rabbit-Cy5 or goat-anti-mouse-FITC antibodies were diluted 1:500 and incubated with the larvae for 2 hours at room temperature. Stained larvae were then visualized as for embryos.

Electron microscopy

22-hour-old embryos of yw, T-33 mutant, and T-33 mutant rescued with a dunc-45 transgene, were isolated and prepared for TEM as described (Cripps et al., 1999). Fixatives and Embed812 resin were from Electron Microscopy Sciences (Fort Washington, PA); other reagents were from Sigma. Samples were examined with a FEI Tecnai 12 transmission electron microscope operating at 80 kV. Digital images were taken with a TVIPS (Tietz) TemCam-F214 high-resolution digital camera.

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