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Spaceflight results in increase of thick filament but not thin filament proteins in the paramyosin mutant of *Caenorhabditis elegans*

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Abstract

We have investigated the effect of microgravity during spaceflight on body-wall muscle fiber size and muscle proteins in the paramyosin mutant of *Caenorhabditis elegans*. Both mutant and wild-type strains were subjected to 10 days of microgravity during spaceflight and compared to ground control groups. No significant change in muscle fiber size or quantity of the protein was observed in wild-type worms; where as atrophy of body-wall muscle and an increase in thick filament proteins were observed in the paramyosin mutant *unc-15(e73)* animals after spaceflight. We conclude that the mutant with abnormal muscle responded to microgravity by increasing the total amount of muscle protein in order to compensate for the loss of muscle function.

key words: Spaceflight, Microgravity, Myosin heavy chain, Paramyosin, *C. elegans*, Mutation

1. Introduction

Gravity is an essential force for organisms living on the earth. Although we are not normally aware of this force, major biological changes in a number of organ systems occur in microgravity (Fitts et al., 2000; Turner, 2000). One of the most affected is the neuromuscular system. Weightlessness induces atrophy of skeletal muscle (Riley et al., 1987; Widrick et al., 1999) and cardiovascular deconditioning (Philpott et al., 1990; Antonutto and Prampero, 2003). Weightlessness also reduces functional capacity in limb skeletal muscle of animals including humans (Ilyina-Kakueva et al., 1976; Widrick et al., 1999; Fitts et al., 2000). In vertebrate skeletal muscle, the greatest changes are observed in the limb antigravity muscles, such as soleus (Riley et al., 1987; Widrick et al., 1999; Harrison et al., 2003). Major changes in these muscles alter the expression of muscle protein isoforms, mainly myosin heavy chains (MHCs). After microgravity exposure, the fibers that express slow type MHCs are decreased and those that express fast type MHCs are increased in soleus slow muscle (Caiozzo et al., 1996; Harrison et al., 2003). At the molecular level, distinct β-MHC promoter sequences mediate β-MHC expression in response to overload and unloading in mice (McCarthy et al., 1999). Furthermore, weightlessness also results in conversion from the slow to the fast isoform of troponin I mRNA in mice (Criswell et al., 1998). From these results, it appears that the isoform-type conversion resulting from weightlessness occurs at the level of transcription.

Caenorhabditis elegans has mainly two muscle tissues: pharyngeal muscle for feeding and body-wall muscle for locomotion. They correspond to the cardiac and skeletal muscles of vertebrates. The body-wall muscle of *C. elegans* has a striated pattern but is not fused as it is in vertebrate skeletal muscle (Moerman and Fire, 1997). *C. elegans* has no fast- and slow-type isoforms as is found in vertebrates, but has four different genes encoding MHCs; two for body-wall, *myo-3* and *unc-54,* encoding MHC A and B, and two for pharyngeal; *myo-2* and *myo-1,* encoding MHC C and D, respectively (Miller et al., 1986; Ardizzi and Epstein, 1987). Each muscle displays a different localization pattern and quantity of thick filaments. MHC A forms the central region, and MHC B is incorporated into either end of the thick filament as a major component of body-wall muscle (Miller et al., 1983; Epstein et al., 1985). Paramyosin, an invertebrate-specific protein having very similar amino acid composition of the rod part to MHC of vertebrates, is located at the core of the thick filament and is encoded by *unc-15* in the worm (Epstein et al., 1985; Kagawa et al., 1989). The *unc-15* gene is expressed in all muscle cell types, and mutants in this gene have an uncoordinated movement, Unc, phenotype (Ardizzi and Epstein, 1987; Waterston et al., 1977). Previous studies have investigated the sites of mutation and correlated them with abnormal thick filament assembly in *unc-15* animals (Gengyo-Ando and Kagawa, 1991). Mutant *unc-15(e73)* animals, which have a Glu342 to Lys substitution, form abnormal thick filaments but produce normal levels of paramyosin protein the same size as that of wild-type (Waterston et al., 1977; Gengyo-Ando and Kagawa, 1991). The tropomyosin

(TM) gene *tmy-1*/*lev-11* of *C. elegans* encodes four isoforms by alternative splicing, and two types of the four isoforms, TM I/II and TM III/IV are expressed in body-wall, pharyngeal and gut muscles, respectively (Kagawa et al., 1995; Anyanful et al., 2001).

To investigate the effect of microgravity on *C. elegans* muscle, we used wild-type and *unc-15(e73)* animals. In this study, we investigated the fiber size of body-wall muscle and quantities of muscle contractile proteins in each of animal comparing results of ground control and spaceflight. We found significant changes in the size of muscle cells and the amount of thick filament protein in the *unc-15(e73)* animals. Thus, the mutant animals which had muscle filament defects were more sensitive to microgravity than the wild-type, and compensated for the loss in muscle function by increasing the amount of protein.

2. Materials and Methods

2.1. Microgravity exposure and animal care

The nematode *C. elegans* Bristol N2 is wild-type strain, and the mutant *unc-15(e73)* animals were obtained from the *Caenorhabditis* Genetics Center. These strains were divided into ground control (GC), laboratory control (LC), transportation control (TC), and spaceflight (SF) samples, respectively. The worms of the GC sample were packed similarly to the SF sample and cultured at Groupement Scientifique pour la Biologie et la Médecine Spatiales (GSBMS) in Toulouse (France) as a control. The worms of the other two samples were cultured under standard conditions at Toulouse for the LC sample, and transported to Baikonur (Kazakhstan) for the TC sample. Worms were cultured in the liquid medium, *C. elegans* Maintenance Medium (CeMM) (Szewczyk et al., 2003). For the GC and SF samples, worms were transferred into VIEW-PACK (Lawson Mardon Packaging Group Ltd., Shelbyville, KY, USA) at a concentration of 7000 adult worms/ml for wild-type and 2000 adult worms/ml for *unc-15(e73)* animals, in a total volume of 2.5 ml (Fig. 1). Each sample was cultured at 20˚C for the duration of the mission, the First International *C. elegans* Experiment (ICE-first) component of the Dutch Expedition for Life science Technology and Atmospheric research (DELTA) mission. Flight animals were flown on the Soyuz rocket (TMA-4) from April 19 to 29, 2004, with a total mission time of 10 days. During this time, worms were exposed to the

microgravity environment throughout development from egg to adult (Fig. 1). Immediately upon the return to earth, culture bags containing the worms were frozen.

In the experiments described below, young adult worms of the SF samples were compared to the GC samples, because the SF worms experienced microgravity during their entire development.

2.2. Western analysis

To remove CeMM, worms were washed with M9 buffer twice, and young adult animals were selected. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer to nitrocellulose filter were performed as described before (Laemmli, 1970; Towbin et al., 1979; Kagawa et al., 1995). Samples were prepared as follows; worms were transferred into 2 μl of phosphate buffer (20 mM), and equal volume of 2X Laemmli buffer was added. After 2 min boiling, they were sonicated for 1 min. For paramyosin and MHC detections, each sample was prepared from single worm of young adult stage and was applied on the 6% polyacrylamide gel as a single worm/lane. For actin and tropomyosin detections, 30 worms/lane were applied on the 12% polyacrylamide gel. We used the worms of young adult stage for adjusting the total protein amount per lane of polyacrylamide gel and averaged data of numbers of observations. This is important for concluding the correct answer of under microgravity condition. The antibodies used were as follows; anti-paramyosin polyclonal antibody,

R224 (Gengyo-Ando and Kagawa, 1991); anti-MHC B monoclonal antibody, 5-3; anti-MHC C monoclonal antibody, 9.2.1 (Miller et al., 1986); anti-slime mold actin polyclonal antibody (kind gift from K. Owaribe, Nagoya University); anti-tropomyosin antibody (Kagawa et al., 1995; Anyanful et al., 2001). As the secondary antibodies, horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Bioscience, Piscataway, NJ, USA) and horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Bioscience, Piscataway, NJ, USA) were used. Immunoreactive protein bands were detected using an ECL detection kit (Amersham Bioscience, Piscataway, NJ, USA)

Western blots with each antibody were performed over 3 trials, and detected bands were scanned into a Macintosh computer. The density of each protein band was calculated using the NIH image software, and normalized to the wild-type GC sample. Each band used for calculation is as follows; 18 - 20 paramyosin bands, 3 - 9 MHC B bands, 13 - 14 MHC C bands, 12 - 15 actin bands, and 12 - 15 tropomyosin bands from the GC and SF samples, respectively. The statistical significance of differences in the protein band density from the GC and SF samples was determined using a Student's *t*-test.

2.3. Immunofluorescence microscopy

Animals were stained by the indirect immunofluorescence procedure of Garriga et al.

(1993). Paramyosin was visualized by R224 antibody staining (Gengyo-Ando and Kagawa, 1991). Antibody reaction was performed at 20˚C for 12 h. FITC-conjugated swine anti-rabbit IgG (F205, Dakopatts, Glostrup, Denmark) was used as secondary antibody and was treated at 37˚C for 2 h. For staining of actin filaments, rhodamine-conjugated phalloidin (Invitrogen, Eugene, OR, USA) was used at a final concentration of 10 μg/ml and was treated at 37˚C for 2 h. Stained worms were observed using a fluorescence microscope, Axiovert (Carl Zeiss, Germany). Images were captured with a CCD camera, Axiocam (Carl Zeiss, Germany). Double-stained images were merged using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA) on a Macintosh computer.

2.4. Width ratio of body-wall muscle cells

Muscle cells were treated with rhodamine-conjugated phalloidin to visualize actin filaments. Images were captured to the computer, and the width of individual muscle cells ($n > 25$) was measured with NIH image software. All measured cells were located near the vulva because these cells are comparatively flat and easy to identify. Width means the maximum length that is perpendicular to the filaments. Values were averaged and normalized by the value of wild-type N2 GC animal. The statistical significance of differences in the widths of GC and SF samples was determined using a Student's *t*-test.

3. Results

3.1. Microgravity effects on muscle structure

In CeMM, *C. elegans* grows much slower; 7 days in CeMM compared to 4 days on NG (nematode growth)-agarose plate growing to adult stage (Fig. 1; Szewczyk et al., 2003), and the body width is thinner than that of worm cultured on the NG-agarose plates, but had no significant difference in function and morphology has been reported (Fig. 2; Szewczyk et al., 2003). Wild-type N2 worms of both GC and SF samples had well organized body-wall muscle cells (Fig. 3a and c). To investigate whether or not muscle atrophy had occurred in *C. elegans* after spaceflight, we measured the width of body-wall muscle cells. In the case of wild-type N2, the width of body-wall muscle cells in GC and SF animals were almost same (Fig. 3e).

A muscle filament abnormal mutant, *unc-15(e73)* animal was also investigated. *unc-15(e73)* animals form abnormal thick filaments by the reason of an amino acid substitution of Glu342 to Lys, but produce normal levels of paramyosin protein of the same size as that of wild-type (Gengyo-Ando and Kagawa, 1991). Not only GC but also SF animals of *unc-15(e73)* had abnormal muscle structure. Thin filaments were deformed, and phalloidin labeled actin filaments were dotted (Fig. 3b and arrow in d). Mostly, paramyosin was not assembled into normal filaments but was aggregated as a para-crystal, but partially formed normal filaments were observed in some SF animals

(Fig. 3d arrowhead). On the other hand, the width of body-wall muscle cells was narrower in SF animals, a 24% decrease compared to the GC sample ($0.01 < P < 0.05$), suggesting that the spaceflight causes muscular atrophy in the animals of *unc-15(e73)* (Fig. 3e).

3.2. Protein amounts of paramyosin and myosin heavy chains

The amount of paramyosin in single young adult worms was measured with Western analysis (See Section 2). The density of the paramyosin band in each SF animal indicated that the amount was significantly increased in *unc-15(e73)* animals after spaceflight. The amount was twice that found in the GC samples ($0.01 < P < 0.05$) (Fig. 4a, b and Table 1). However, there was no significant difference in the wild-type animals between GC and SF paramyosin content (Fig. 4b and lane 1 in Table 1).

The amount of myosin heavy chains (MHCs) was determined using monoclonal antibodies as shown in Fig. 5 (See Section 2; Miller et al., 1986). We found that MHC B, a major component of body-wall muscle, was increased in each animal after spaceflight. A significant increase, about four times that of the GC sample, was observed in *unc-15(e73)* animals (Fig. 5a and lane 2 in Table 1). MHC C, the pharyngeal muscle type of MHC, was also increased in SF *unc-15(e73)* animals, about a 70% increase over the GC sample (Fig. 5b and lane 3 in Table 1).

3.3. Protein amounts of actin and tropomyosin

Next, we evaluated the muscle thin filament proteins, actin and tropomyosin (TM). Anti-actin and anti-TM antibodies did not detect significant changes by Western analysis between GC and SF animals of each strain (Fig. 6). We investigated the amounts of body-wall and pharyngeal muscle TM isoforms. Anti-TM antibody also did not detect any changes on the amount of TMs after spaceflight (Fig. 6b).

We conclude that the quantities of thick and thin filament proteins are differently affected during spaceflight, and this result is only observed on the mutant animal with abnormal thick filament.

4. Discussion

4.1. Microgravity effect on C. elegans

In the defined media, CeMM, worms take 10 days to develop from egg to adult (Szewczyk et al., 2003; Fig. 1). Thus, over the duration of this space mission, the effect of microgravity on developing *C. elegans* could be observed. In wild-type animals, no morphological muscle alteration (i.e. muscle atrophy) (Figs. 2a, b and 3) and no muscle filament-protein quantitative change were observed (Figs. 4, 5 and 6). In contrast, *unc-15(e73)* paramyosin-gene mutants showed muscular atrophy of body-wall muscle (Fig. 3e) and dramatic changes in the amount of muscle thick filament protein (Figs. 4 and 5). In the laboratory, *C. elegans* is normally grown on NG-agarose plates, and experiences gravity perpendicular to its body-wall muscle. *C. elegans* has no antigravity muscle such as the soleus of vertebrates. Absence of an antigravity muscle suggests that perception of microgravity effects may not occur in *C. elegans*. Microgravity effects on non-antigravity muscle are difficult to observe in vertebrates (Jiang et al., 1992; Caiozzo et al., 1996). However, the microgravity effect on the animals of *unc-15(e73)* indicates that *C. elegans* does respond to gravity. This means that local tension heterogeneity in a mutant muscle filament stimulates protein-protein interaction followed by protein turnover including gene expression and filament assembly as discussed later.

4.2. Alteration of thick filament protein amounts after microgravity exposure

In the *unc-15(e73)* young adult animal after 10 days of exposure to microgravity, amounts of muscle thick filament proteins, paramyosin and MHCs, were changed (Figs. 4 and 5). Both body-wall muscle type MHCs and pharyngeal muscle type MHCs were increased after the spaceflight (Table 1). These indicate that the microgravity affects protein turnover rate including gene expression not only for body-wall muscle but also pharyngeal muscle. Some changes could occur in the pharynx although we could not observe pharyngeal muscle morphology.

The *e73* mutation in *unc-15* results in a single charge change Glu342 to Lys of paramyosin, that causes an increase in paramyosin affinity for itself during assembly (Gengyo-Ando and Kagawa, 1991). The mutant animal forms large para-crystalline multi-filaments in the muscle cells and shows an Unc phenotype (Waterston et al., 1977). From these results, we conclude that *unc-15(e73)* animals produce MHCs in order to compensate for muscle filament abnormality under microgravity. This conclusion is consistent with the previous report that a suppressor mutant of the *unc-15(e73)* animal maps on the MHC A gene, *sup-3*/*myo-3* (Riddle and Brenner, 1978; Brown and Riddle, 1985). MHC A molecules accumulated in *myo-3(e1407)* mutant are able to assemble into functional thick filaments with the mutant paramyosin produced in the *unc-15(e73)* mutant by the reason that interaction of these proteins are the first step

of thick filament assembly (Kagawa et al., 1989). As abnormally assembled thick filament poorly function in the stringent condition gene expression of thick filament proteins of the mutant worm could be stimulated in apace flight. In case we have to determine which step of the 10 days flight; to space, during space or back to earth is the main reason for changing the transcription rate in future.

Unlike previous report using mice (Criswell et al., 1998), we did not find an increase in thin filament proteins (Fig. 6 and Table 1). This suggests that the transcriptional control of *C. elegans* contractile protein is different from that of mice, and that there are different transcriptional control responses to microgravity for thick and thin filament proteins in *C. elegans*. Recently we have determined that the *unc-27(e155)* animal, the null mutant of the major body-wall troponin I gene, produced UNC-27 only 20 % of the wild-type (Ruksana et al., 2005). As there are three body-wall type troponin I genes in the worm, two other troponin I genes can produce the product. In either case interacting molecules are controlled under a related gene expression control.

Another reason for thick filament protein concentrations is that the protein degradation rate was changed in the *unc-15(e73)* young adult animal after spaceflight. It is reported that the ubiquitin-dependent proteolytic pathway causes the microgravity induced muscular atrophy, and micro-array data indicates the up-regulation of the expression of ubiquitin ligases in the space-flown rat (Ikemoto et al., 2001; Nikawa et al., 2004). In our data, body-wall muscle of *unc-15(e73)* animal occurred atrophy after

spaceflight (Fig. 3e). If the function of ubiquitin-dependent proteolytic pathway in the animal of *unc-15(e73)* was elevated after spaceflight, muscle proteins were decreased. However, our Western analysis indicates that the protein bands of paramyosin and MHCs are increased after spaceflight (Figs. 4 and 5). Although we cannot rule out the possibility of protein degradation, we found that the increment of protein quantities was occurred after spaceflight.

We further assume the possibility that many proteins are up or down regulated in the animal and that they occur worm stage-dependent or not. Micro-array, etc technology could determine which and to what extent different proteins increase or decrease in amount.

4.3. Efficiency of a mutant animal model

The model animal of muscular dystrophy, *mdx* mouse, shows little clinical signs and no neurological signs of muscular dystrophy up to an age of 18 months, in spite of a nonsense mutation in the dystrophin gene (Sicinski et al., 1989; Vaillend et al., 1995). However, overloading experiments induce degeneration and fragility of skeletal muscle in *mdx* mouse (Vilquin et al., 1998; Yoshida et al., 1997). This indicates that the skeletal muscle of *mdx* mouse is sensitive to overloading. The results of this model animal experiment suggest that the symptoms of Duchenne muscular dystrophy could be milder under microgravity than under 1 G. Although there are many different systems between

vertebrate and invertebrate, our results of *unc-15(e73)* mutant animal are consistent with these reports. Since the spaceflight worms partially assembled paramyosin in body-wall muscle cells (Fig. 3d, arrowhead), the Unc phenotype of the mutant animals might display a milder phenotype than the ground control worms. In this study, we showed that *C. elegans* mutant of *unc-15(e73)* responded to microgravity and produced increased thick filament proteins. Under microgravity, animals have various biological changes in a number of organ systems. In muscle, these changes might be not detrimental but beneficial for patients with myopathy.

In any cases we have to conclude effects of spaceflight under different kinds of observations. In this study poor information was obtained by the reason of drastic fixation of the animals. Protein quantities were detected with Western analysis by using high specific antibodies. Unfortunately we can't do any observations on physiological study during spaceflight. It should be mentioned about that in the future experiment.

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Legends

Fig. 1. Growth profile of worms during the spaceflight in CeMM. Top vertical line represents the mission duration, and arrows indicate cultivation temperature during each day. Graph represents numbers and body length of wild-type animals corresponding to the mission duration. Open circles indicate body length of worms hatched at the day of sample preparation (4/13), and closed triangles indicate those hatched at the day of launch (4/19). This shows that eggs in worms grow to adult during ten days flight. The data of body length of worms in CeMM is referred to previous report (Szewczyk et al., 2003).

Fig. 2. The worms after spaceflight under a dissecting microscope. Anterior to the left, dorsal top. Young adult animals of wild-type N2 (a and c) and *unc-15(e73)* (b and d) are shown. Panel (a) and (b) are the GC, and panel (c) and (d) are the SF samples. The scale bar indicates 0.1 mm.

Fig. 3. Muscle filaments and width of body-wall muscle cells. The muscle filaments of wild-type N2 (a and c) and *unc-15(e73)* (b and d) were visualized with anti-paramyosin antibody (green) and rhodamine-conjugated phalloidin (red). In each strain, GC (a and b) and SF (c and d) animals were treated. Each panel is a merged image of paramyosin and actin fluorescences. Arrows indicate deformed and dotted fluorescence of actin

filaments, and an arrowhead indicates partially formed normal filaments. (e) Width ratio of body-wall muscle cells measured from fluorescence images, and normalized by the value of wild-type GC animal. Values are means ± SE. n > 25 cells. Asterisk indicates significant difference from *unc-15(e73)* GC sample ($0.01 < P < 0.05$). The scale bar indicates 50 μm. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Paramyosin amount in single worms. (a) Western analysis using anti-paramyosin antibody. In each panel, left five lanes are GC and right five lanes are SF samples. Arrowheads indicate the band of paramyosin. (b) Amount of paramyosin in single worms. Western analysis was performed 4 trials as shown in (a). Values are means ± SE. Asterisk indicates significant difference from *unc-15(e73)* GC sample ($0.01 < P < 0.05$).

Fig. 5. Myosin heavy chain amount in single worms. Like Fig. 4, amounts of MHC B (a) and that of MHC C bands (b) of each sample were averaged. Values are means ± SE.

Fig. 6. Thin filament protein amounts. After 3 trials of Western analyses, amounts of actin (a) and that of TM III bands (b) of each sample were averaged. Values are means ± SE.

Table 1 Summary of microgravity effect in the muscle proteins

	Localization	Flight / Ground	
		Wild-type	*unc-15(e73)*
Thick filament			
Paramyosin	Whole muscle	1.09	2.03
MHC B	BWM	1.31	3.95
MHC C	PHX	0.89	1.68
Thin filament			
Actin	Whole muscle	0.88	0.88
Tropomyosin III	PHX	1.20	0.88

BWM: body-wall muscle, PHX: pharyngeal muscle

Fig. 1 Adachi et al

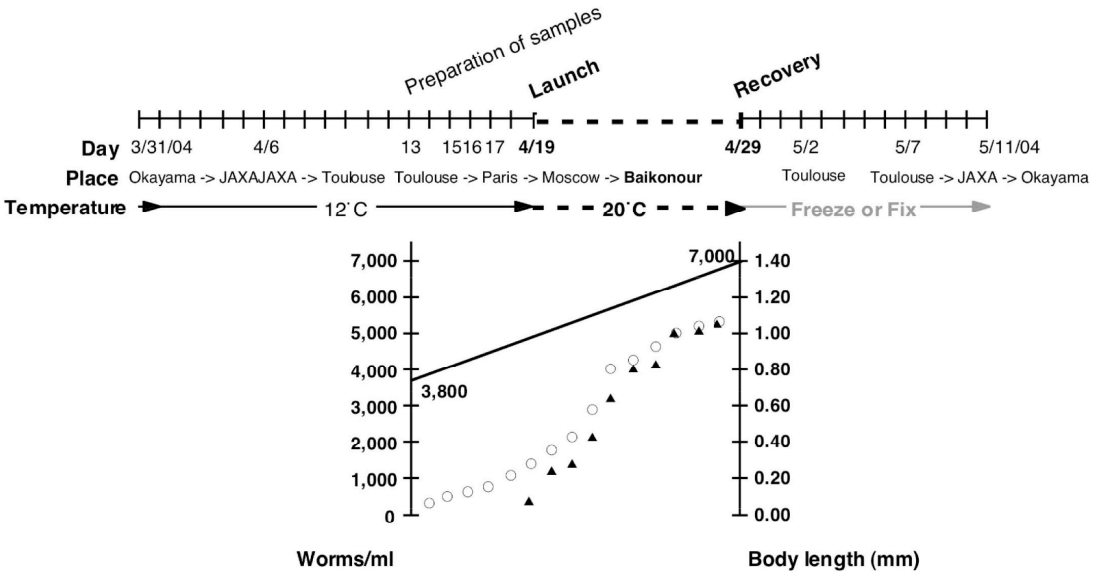

Fig. 2 Adachi et al

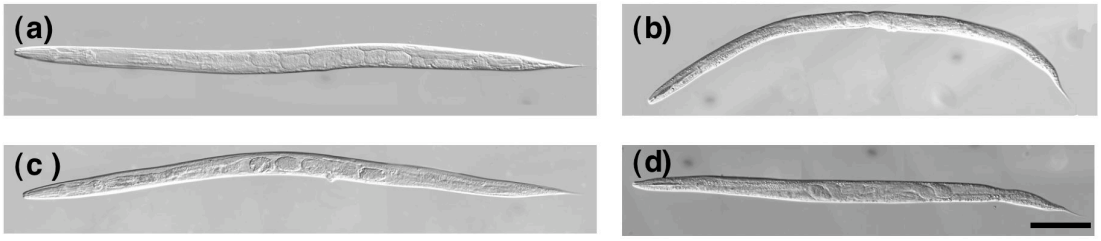

Fig. 3 Adachi et al

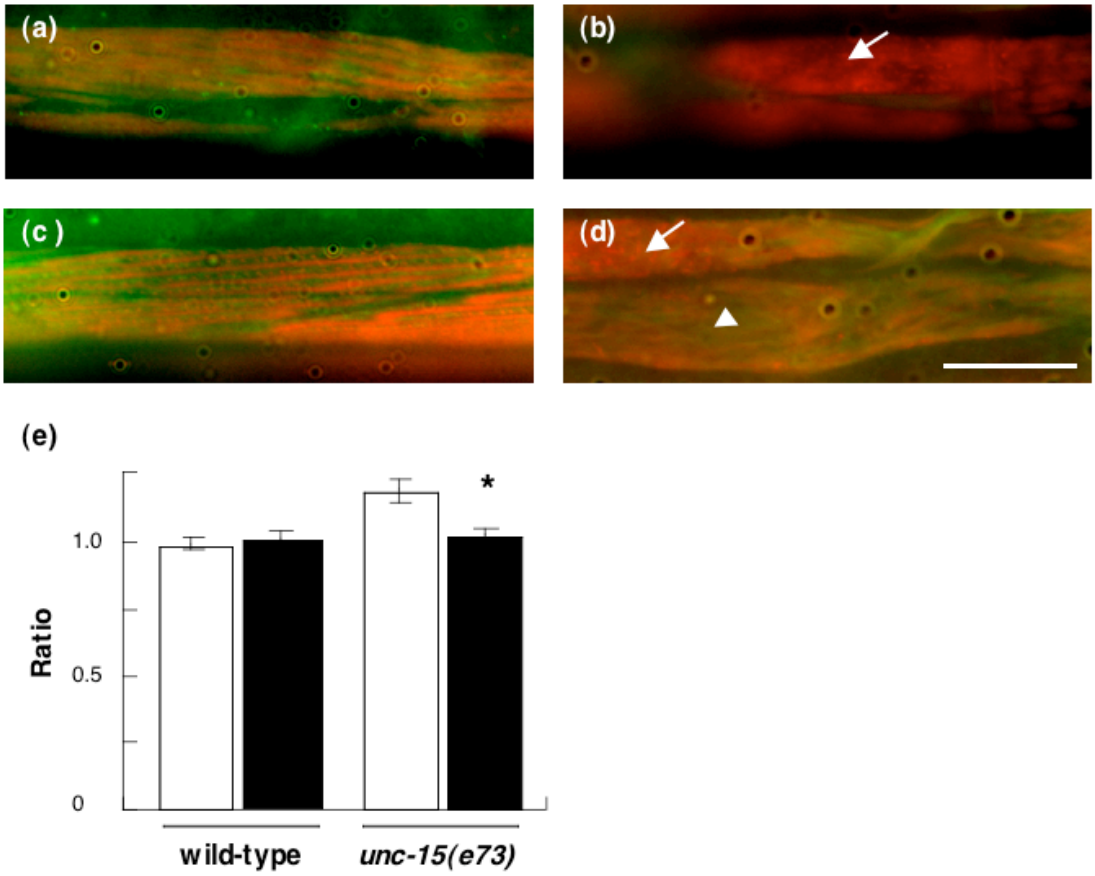

Fig. 4 Adachi et al

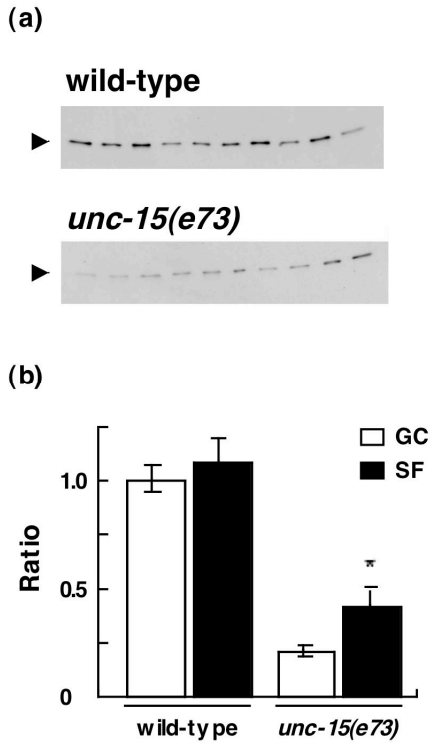

Fig. 5 Adachi et al

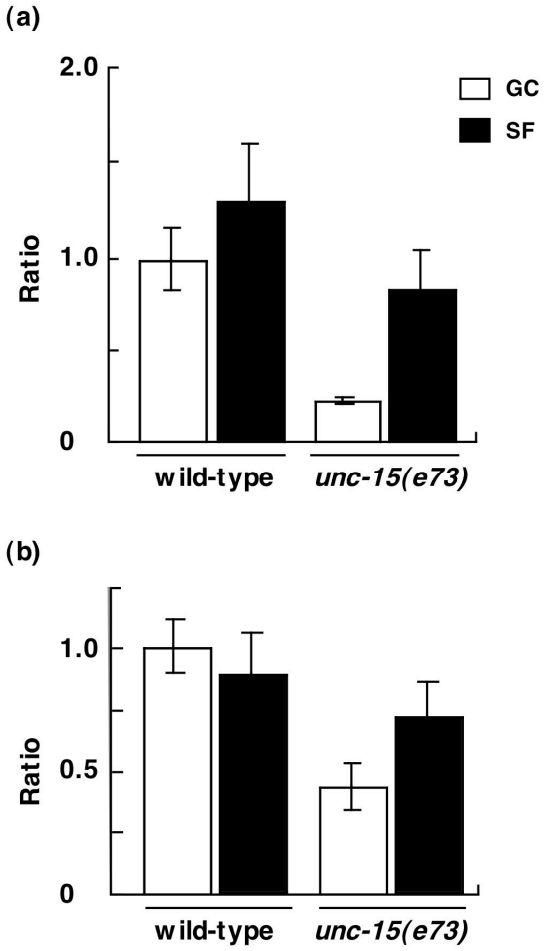
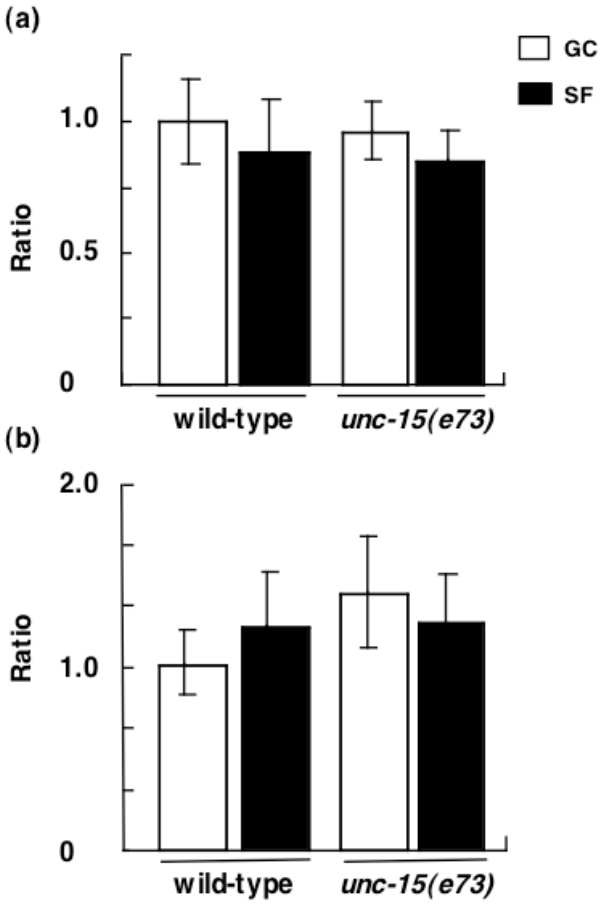
Fig. 6 Adachi et al