Letter to the Editor

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Cautionary note on the use of *Caenorhabditis elegans* to study muscle phenotypes caused by mutations in the human *MYH7* gene

Alejandro Gil-Gálvez[1], Pilar Carbonell-Corvillo[2], Carmen Paradas*,[2] & Antonio Miranda-Vizuete*,[1]

*[1]Redox Homeostasis Group, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, 41013 Sevilla, Spain; [2]Neuromuscular Unit, Department of Neurology, Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, 41013 Sevilla, Spain; *Author for correspondence: cparadas@us.es; amiranda-ibis@us.es*

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ABSTRACT

Mutations in the human *MYH7* gene, encoding a slow skeletal muscle/β-cardiac myosin heavy chain, cause different types of myopathies. The nematode model *Caenorhabditis elegans* has frequently been employed to study the molecular and physiological consequences of *MYH7* mutations in muscle function by introducing mutations into the *unc-54* gene, the worm *MYH7* ortholog. We report here that the *C. elegans* model is not appropriate for such studies if they involve expression of the UNC-54 protein (wild-type or fused to green fluorescent protein) above endogenous levels.

KEYWORDS:

Caenorhabditis elegans ● mutation ● *MYH7* ● myosin ● overexpression ● UNC-54

In 1974, Sydney Brenner published his groundbreaking work on the genetic characterization of *Caenorhabditis elegans* [1]. Since then, this nematode has consolidated as a powerful model organism, not only generating key findings in many different biological disciplines but also serving as a valuable preclinical model for several human diseases, including neuromuscular disorders [2–5]. In his pioneering study, Brenner identified and characterized several classes of mutant based on their visible phenotypes, with the largest gene class being defined by mutants with movement defects, generically designated as 'uncoordinated' (the 'unc' phenotype) [1]. Mutations in one such gene, *unc-54*, were found to cause body wall muscle cell defects that resulted in worm paralysis [1,6]. Subsequent work demonstrated that *unc-54* encodes one of the four skeletal muscle myosin heavy chain proteins in this organism [1,6]. *unc-54* is one of the most extensively studied genes in *C. elegans*, with hundreds of mutants available and phenotypes spanning from very mild motility defects to lethality, due to the requirement of a functional UNC-54 protein for muscle thick filament assembly [7–9].

unc-54 is the worm ortholog of the human myosin gene *MYH7*, mutations in which cause an important group of muscular diseases with variable clinical and morphological expression depending on the mutated isoform and the type and location of the mutation [10]. Slow skeletal muscle/β-cardiac myosin heavy chain, encoded by the *MYH7* gene, is expressed in cardiac muscle and all slow skeletal muscle fibers [11]. *MYH7* mutations are an established cause of hypertrophic or dilated cardiomyopathy [12], Laing distal myopathy [13] and myosin storage/hyaline body myopathy [14] as well as additional myopathy subtypes.

Laing distal myopathy is an autosomal dominant disease commonly characterized by slowly progressive distal weakness, typically involving ankle dorsiflexor muscles and long toe extensor muscles, causing steppage and the typical 'hanging big toe' sign [13]. However, highly variable clinical and histological phenotypes have been widely reported [15,16], and the same mutation can cause different clinical pictures (age of onset, weakness pattern and severity), even within the same family.

Due to the high amino acid sequence conservation between worm UNC-54 and human MYH7 proteins (51% identity and 71% similarity) and its transparency throughout the life cycle, the *C. elegans* model has frequently been used to recreate the phenotypic consequences of *MYH7* mutations in muscle cell function and morphology. In some cases, transgenic animals expressing an *unc-54* gene harboring a human *MYH7* mutation are used to evaluate worm motility phenotypes [17]. In other cases, the human *MYH7* mutation is incorporated in a transgene expressing an UNC-54::GFP (green fluorescent protein) fusion protein. This tagged transgene can be employed to visualize *in vivo* the impact of mutations on the integrity of worm muscle cells by labeling the myofilament distribution along the longitudinal cell axis, and to assess the effect of the mutation on worm motility [18,19]. We followed this second approach to further characterize a newly identified heterozygous *MYH7* founder mutation causing Laing distal myopathy in two apparently unrelated families from southern Spain in which a c.4679G>C transversion generates a p.R1560P amino acid change in the mid-rod region of the MYH7 protein (Figure 1A) [20]. The arginine residue at position 1560, mutated to proline in the families analyzed in this study, is conserved in worm UNC-54 and is located

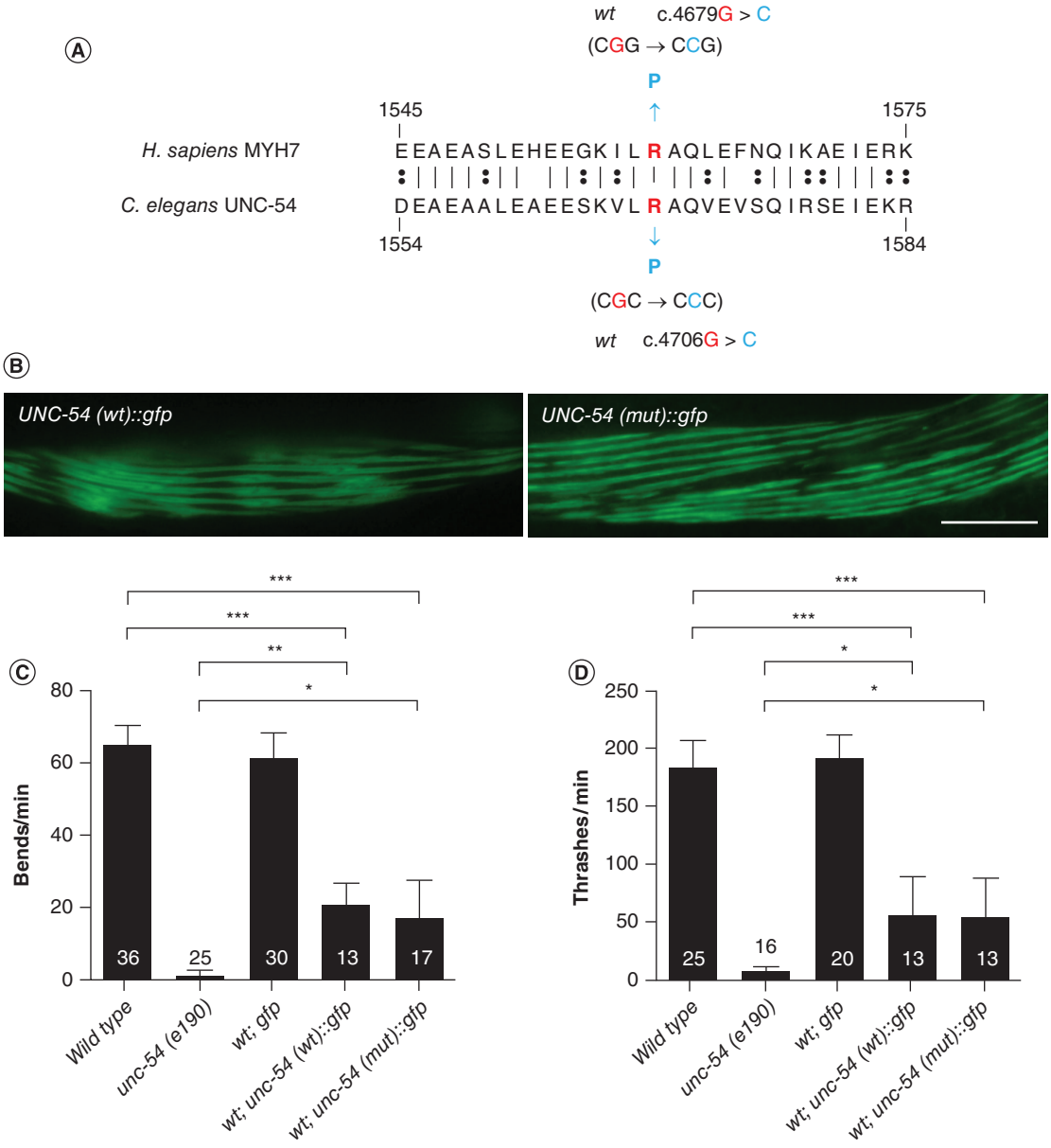

Figure 1. Functional analysis of the human c.4679G>C *MYH7* mutation in *C. elegans*. (A) Schematics of the amino acid residue change between the human MYH7 protein and the orthologous *C. elegans* protein UNC-54. Numbers indicate the amino acid residue of the respective proteins. The wild-type arginine residue is depicted in red and the change to proline is highlighted in blue; the corresponding mutations are shown in parentheses with the same color code. **(B)** Fluorescence microscopy images showing a normal myofibril distribution in *C. elegans* transgenic worms expressing UNC-54(*wt*)::GFP and UNC-54(*mut*)::GFP fusion proteins. Scale bar 20 μm. **(C, D)** Quantification of muscle function performance, measured as the numbers of body bends in solid medium **(C)** and the numbers of thrashes in liquid medium (D). Data are the mean ± SD from two independent experiments and the total number of individuals scored is indicated at the base of the column for each genotype (*p < 0.05; **p < 0.01; ***p < 0.001 by unpaired, two-tail *t*-test). GFP: Green fluorescent protein; mut: Mutation; SD: Standard deviation; wt: Wild type.

at position 1569 (Figure 1A). Previous studies have shown that introducing proline residues in the MYH7 protein interferes with myosin self-assembly [18,21]. Moreover, most *MYH7* mutations associated with distal myopathy are located in the mid-rod region of the protein, within exons 32–36 [22], and this is where the c.4679G>C mutation lies [20]. To test whether the p.R1460P change also causes such alteration, we expressed in worm muscle cells an UNC-54::GFP fusion protein with a corresponding proline change to that identified in the patients (UNC-54(*mut*)::GFP) (Figure 1A). Because the human mutation has an autosomal dominant inheritance pattern, we generated transgenic animals expressing UNC-54(*mut*)::GFP in a wild-type genetic background; worms expressing GFP alone and UNC-54(*wt*)::GFP fusion protein in the same wild-type background served as controls. Transgenic UNC-54(*mut*)::GFP animals were indistinguishable from wild-type, GFP or UNC-54(*wt*)::GFP control worms in terms of their body morphology (not shown) and they had normal muscle cell

morphology and longitudinal distribution of the myofilaments (Figure 1B), consistent with the mild alteration seen in patients' muscle biopsies [20].

To determine whether the mutation would cause more subtle motility phenotypes, we used the same transgenic worms to perform motility assays in both liquid medium and on seeded plates. The first is carried out by placing worms in a 5-μl drop of M9 buffer and counting the number of lateral swimming movements (thrashes) [23]; the second quantifies the worms' locomotion rate by counting body bends as they move on the bacterial lawn [24]. To our surprise, when performing the motility assay in liquid medium, we found that worms expressing UNC-54(*wt*)::GFP or UNC-54(*mut*)::GFP fusion proteins had a very erratic and irregular thrashing pattern compared with the robust, repetitive pattern showed by wild-type or GFP control animals (Supplementary Movies 1–5). The worms in solid medium on seeded plates also demonstrated motility deficits in UNC-54(*wt*)::GFP or UNC-54(*mut*)::GFP worms (not shown). In concordance with these observations, quantification of thrashes in liquid medium and body bends in solid medium showed that UNC-54(*wt*)::GFP or UNC-54(*mut*)::GFP fusion proteins decreased worm motility threefold, compared with wild-type or GFP-expressing control animals (Figure 1C & D). As the transgenes used in this study are high copy number, this result suggests that increased levels of UNC-54::GFP protein may negatively affect worm muscle cell functionality without impairing myosin filament assembly or overall cell morphology. Prompted by this apparent deleterious effect of the UNC-54::GFP protein *per se* – which would invalidate previous studies using the same experimental strategy – we revisited similar experiments done by other groups [17–19,25]. In all these studies, production of transgenic UNC-54 or UNC-54::GFP protein above wild-type levels effectively impairs motility in *C. elegans* [17–19,25]. Indeed, we found an older work reporting that very high levels of UNC-54 can even disrupt muscle structure [26]. It should be noted that although there has been some controversy about a possible inhibitory effect of GFP on myosin function [27–29], our data clearly show that GFP overexpression does not have any deleterious impact on worm motility (Figure 1C & D). Thus, worms expressing GFP can be used as controls in this type of experiment.

Together, we conclude that the results of modeling the pathogenic effects of human *MYH7* mutations by evaluating the motility phenotypes of such mutations in worms expressing UNC-54 from high-copy transgenes should be viewed with caution. Regardless of whether they are integrated in the genome or maintained extrachromosomally, these transgenes may cause anomalous results by interfering with normal muscle cell function and should therefore be avoided. Effective alternatives to overcome this limitation could come from the use of *unc-54* single-copy transgenes or direct CRISPR/Cas9 editing of the endogenous *unc-54* gene. These approaches would more faithfully reproduce the *MYH7* mutations of patients in worms with *unc-54* wild-type gene dosage.

The *C. elegans* strains used in this study were N2, wild-type Bristol and CB190, *unc-54(e190)* I. Nematode strains were grown and maintained at 20°C on nematode growth medium agar plates seeded with *E. coli* OP50 as food source [30]. The pPD95.77 plasmid derivative containing the *unc-54* promoter plus the genomic ORF fragment that generates an UNC-54::GFP fusion protein [19], a kind gift from Prof. Andras Malnasi-Csizmadia, was used as bait to generate the mutant variant identified in this study with the following mutagenic primers: 5′-GAGCAAGGTTCTCCCCGCCCAGGTTGAAG-3′ (forward) and 5′-CTTCAACCTGGGCGGGGAGAACCTTGCTC-3′ (reverse). Mutagenesis was performed using the QuickChange II XL Site Directed Mutagenesis Kit (Stratagene) following manufacturer instructions and confirmed by sequencing in both orientations. Wild-type and mutated pPD95.77/*unc-54* plasmids were transformed into DH5α bacteria, purified using the NucleoSpin Plasmid kit (Macherey-Nagel) and injected into the gonad of wild-type worms at a concentration of 20 ng/μl. Transgenic progeny were identified by GFP expression in worm muscle cells and stably transmitting lines were used in the experiments. Transgenic animals were mounted in a 5-μl drop of 10 mM levamisole (Sigma) on a microscope slide with a 3% agarose pad covered with a 24 × 24 mm coverslip and the muscle cells' GFP was scored on an Olympus BX61 fluorescence microscope. To assay the motility, well-fed L4 animals were transferred to fresh seeded plates and incubated further for 24 h at 20°C prior to assaying their motility parameters. Then, worms were gently placed on seeded 60-mm plates (solid medium motility) or in a 5 μl drop of M9 buffer on a glass slide (liquid medium motility); after acclimation for 1 min, the number of bends (solid) or thrashes (liquid) were recorded during the following 2 min using a Leica M165FC stereoscope equipped with a CCD camera (Greenlight Solutions).

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2020-0012

Author contributions

A Gil-Gálvez performed all the experiments. P Carbonell-Corvillo and A Miranda-Vizuete designed the experiments. C Paradas and A Miranda-Vizuete wrote the manuscript.

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