RESEARCH NOTE

Mutations in *Caenorhabditis elegans* actin, which are equivalent to human cardiomyopathy mutations, cause abnormal actin aggregation in nematode striated muscle [version 1; peer review: 1 approved, 3 approved with reservations]

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Abstract

Actin is a central component of muscle contractile apparatuses, and a number of actin mutations cause diseases in skeletal, cardiac, and smooth muscles. However, many pathogenic actin mutations have not been characterized at cell biological and physiological levels. In this study, we tested whether the nematode *Caenorhabditis elegans* could be used to characterize properties of actin mutants in muscle cells *in vivo*. Two representative actin mutations, E99K and P164A, which cause hypertrophic cardiomyopathy in humans, are introduced in a muscle-specific *C. elegans* actin ACT-4 as E100K and P165A, respectively. When green fluorescent protein-tagged wild-type ACT-4 (GFP-ACT-4), is transgenically expressed in muscle at low levels as compared with endogenous actin, it is incorporated into sarcomeres without disturbing normal structures. GFP-ACT-4 variants with E100K and P165A are incorporated into sarcomeres, but also accumulated in abnormal aggregates, which have not been reported for equivalent actin mutations in previous studies. Muscle contractility, as determined by worm motility, is not apparently affected by expression of ACT-4 mutants. Our results suggest that *C. elegans* muscle is a useful model system to characterize abnormalities caused by actin mutations.

Keywords

actin, aggregates, cardiomyopathy, sarcomere, myofibrils

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Introduction

Actin is an essential component of the cytoskeleton in both muscle and non-muscle cells. A number of mutations in the six human actin genes cause a wide range of diseases in various tissues (Despond & Dawson, 2018; North & Laing, 2008; Rubenstein & Wen, 2014). In muscles, actin, together with myosin, generates contractile forces, and therefore, alterations in contractile and/or structural properties of actin can cause muscle malfunction. Mutations in skeletal muscle α -actin (*ACTA1*) cause congenital myopathies, including nemaline myopathy and intranuclear rod myopathy, in which skeletal muscle exhibits abnormal accumulations of sarcomeric components (Clarkson *et al.*, 2004; Laing *et al.*, 2009; North & Laing, 2008; Ono, 2010). Many of these cytoskeletal abnormalities can be reproduced by expression of mutant actins in cultured non-muscle or muscle cells (Bathe *et al.*, 2007; Costa *et al.*, 2004; Domazetovska *et al.*, 2007; Vandamme *et al.*, 2009a; Vandamme *et al.*, 2009b) or in transgenic mice (Lindqvist *et al.*, 2013; Ravenscroft *et al.*, 2011). By contrast, mutations in cardiac α -actin (*ACTC1*) cause hypertrophic and dilated cardiomyopathies (Mogensen *et al.*, 1999; Olson *et al.*, 1998). Biochemical studies indicate that these cardiomyopathy mutations of actin alter its properties to generate contractile forces (Despond & Dawson, 2018). However, abnormalities in sarcomeric or cytoskeletal structures have not been reported when the mutant actins are expressed in cultured cells (Muller *et al.*, 2012; Vang *et al.*, 2005) or transgenic mice (Song *et al.*, 2010; Song *et al.*, 2011).

In this study, we used the nematode *Caenorhabditis elegans* as a model to examine effects of cardiomyopathy mutations in actin. The body wall muscle of *C. elegans* is obliquely striated muscle with a number of functional and structural similarities to vertebrate striated muscles (Ono, 2014). Four actin genes are expressed in *C. elegans* muscle (Files *et al.*, 1983; Stone & Shaw, 1993), and they are 95% identical to human cardiac and skeletal muscle α -actins (Ono & Pruyne, 2012). Since all known residues that are mutated in human cardiomyopathies are conserved in *C. elegans* actins, we selected two representative hypertrophic cardiomyopathy mutations and tested whether these pathogenic mutations perturb the properties of actin in *C. elegans* muscle *in vivo*. We found that the mutant actins were incorporated into sarcomeres and also accumulated in abnormal aggregates, suggesting that *C. elegans* muscle is a unique model system to characterize pathogenic actin mutations.

Methods

Worm culture

Worms were cultured following standard methods (Stiernagle, 2006). Wild-type *C. elegans* strain N2 was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN) and used in this study.

Transgenic strains

An expression vector for GFP-ACT-4(wild-type: WT) was constructed by inserting ACT-4 cDNA at the *EcoRI*-*NheI* sites of pPD118.20 (provided by Andrew Fire, Stanford University) in-frame with the 3'-end of the GFP coding sequence. Briefly, first-strand cDNAs were reverse-transcribed from total RNAs

from the N2 strain using oligo-dT by a Maxima H⁺ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The ACT-4 cDNA with added *EcoRI* and *NheI* sites in the primer sequences was amplified from the pool of cDNAs by polymerase chain reaction using *Pfu* DNA polymerase (Agilent Technologies), digested with *EcoRI* and *NheI*, and ligated with pPD118.20 that had been cut with *EcoRI* and *NheI*. Expression vectors for GFP-ACT-4(E100K) and GFP-ACT-4(P165A) were generated by site-directed mutagenesis using a QuickChange Site-directed Mutagenesis Kit (Agilent Technologies). Sequences of the inserts were verified by DNA sequencing. Transgenic nematodes were generated by microinjection of DNA vectors into the distal gonads as described previously (Mohri *et al.*, 2006). Transgenic worms were selected by expression of GFP as observed by fluorescence microscopy, and the transgenes were maintained as extrachromosomal arrays. Strains used in this study are ON16, *ktEx6[Pmyo-3::GFP::ACT-4(WT)]*; ON209, *ktEx154[Pmyo-3::GFP::ACT-4(E100K)]*; and ON212, *ktEx157[Pmyo-3::GFP::ACT-4(P165A)]*.

Western blot

Ten adult worms were suspended in 15 μ l SDS lysis buffer (2% SDS, 80 mM Tris-HCl, 5% β -mercaptoethanol, 15% glycerol, 0.05% bromophenol blue, pH 6.8), heated at 97°C for 2 min, homogenized briefly by sonication, heated again at 97°C for 2 min, and subjected to SDS-PAGE (12% acrylamide gel). The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was blocked in 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and incubated for 1 hr with anti-actin mouse monoclonal antibody (C4, MB Biomedicals, catalog # 08691001; RRID:AB_2335127) at a 1:3000 dilution. The membrane was washed with PBS-T, treated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution) (Pierce/Thermo Scientific, catalog #31430) for 1 hr, and washed with PBS-T. The reactivity was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposure to X-ray films. Finally, the membrane was stained with 0.1% Coomassie Brilliant Blue R-250 (National Diagnostics) in 50% methanol and destained in a solution containing 10% acetic acid and 50% methanol to visualize total proteins (Welinder & Ekblad, 2011). The blots were scanned by an Epson Perfection V700 scanner at 300 dpi., and band intensity was quantified using ImageJ 1.47v.

Worm motility assay

Worm motility was determined by counting swinging motions of worms for 30 seconds in M9 buffer as described (Epstein & Thomson, 1974; Ono *et al.*, 1999).

Fluorescence microscopy

Fixation and staining of worms with rhodamine-phalloidin were performed as described previously (Ono, 2001). GFP was observed by its own fluorescence. Specimens were observed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope with a CFI Plan Fluor ELWD 40x (Dry; NA 0.60) objective. Images were captured by a SPOT RT monochrome CCD camera (Diagnostic Instruments) and processed by IPLab

4.0 imaging software (BD Biosciences) and Adobe Photoshop CS3.

Molecular graphics

Molecular graphics in [Figure 1A](#) were generated using PyMol 2.1.0 (Schrödinger), and texts added using Adobe Photoshop CS3.

Statistical analysis

The data used in [Figure 1C](#) were analyzed by Student's t-test using SigmaPlot 14.0 (Systat Software, Inc.). The data used in [Figure 1G](#) were analyzed by one-way ANOVA with Turkey test using SigmaPlot 14.0. The data used in [Figure 1H](#) were analyzed by one-way ANOVA with pairwise multiple comparison using the Student-Newman-Keuls method using SigmaPlot 14.0.

Results

We constructed an expression vector for GFP-tagged ACT-4, an actin isoform that is expressed in the body wall muscle ([Stone & Shaw, 1993](#)), under the control of the *myo-3* promoter (*Pmyo-3*) ([Okkema et al., 1993](#)). The ACT-4 sequence was fused to the C-terminus of GFP with a 9-residue linker sequence (SPQALEFSS) to minimize the interference of actin function by GFP ([Aizawa et al., 1997](#)). We selected two missense mutations, E99K and P164A in human cardiac α -actin ([Despond & Dawson, 2018](#); [Olson et al., 2000](#)), that dominantly cause hypertrophic cardiomyopathy. The E99K mutation weakens actin-myosin interaction ([Bookwalter & Trybus, 2006](#)) and increases the critical concentration of actin ([Mundia et al., 2012](#)). In a transgenic mouse model, E99K increases calcium sensitivity of the thin filaments and causes abnormal heart functions ([Song et al., 2011](#)). In contrast, the effect of P164A mutation remains unclear. Although P164A causes alteration in protein folding *in vitro* ([Vang et al., 2005](#)), an equivalent mutation in yeast actin does not change its basic biochemical properties ([Wong et al., 2001](#)). *C. elegans* ACT-4 is 95% identical in amino acid sequence to human cardiac α -actin, and E99 and P164 are conserved as E100 and P165, respectively ([Figure 1A](#)). Therefore, we introduced E100K and P165A mutations in GFP-ACT-4 and examined their effects on the sarcomeric structures in *C. elegans* body wall muscle.

Establishment of transgenic strains and expression quantification

We established at least three independent transgenic strains for each of the transgenes, GFP-ACT-4(wild-type: WT), GFP-ACT-4(E100K), and GFP-ACT-4(P165A), and examined expression levels of GFP-ACT-4 variants by western blot. We selected one strain each, which expressed the GFP-ACT-4 variants at similar levels ([Figure 1B, C](#)) for further analysis. Western blot analysis using anti-actin antibody showed that all the GFP-ACT-4 variants were expressed at much lower levels than endogenous actin in total worm lysates ([Figure 1B](#)). The level of GFP-ACT-4(WT) was roughly estimated by densitometry to be lower than 10% of that of total endogenous actin, although strong saturated signals for endogenous actin made precise quantification difficult. Considering that body wall muscle is the major tissue expressing actin as a sarcomeric component, the expression level of GFP-ACT-4 should be still much less

than that of endogenous actin within the body wall muscle cells. Raw uncropped western blots, alongside all other raw data, are available on Figshare ([Hayashi et al., 2019](#)).

Subcellular localization of GFP-ACT-4 variants and motility of worms

GFP-ACT-4(WT) was incorporated into sarcomeres in body wall muscle cells ([Figure 1D](#)). Staining of F-actin in fixed animals with rhodamine-phalloidin showed a nearly identical localization pattern to GFP-ACT-4(WT) ([Figure 1D](#)). Motility of the worms expressing GFP-ACT-4(WT) (81.5 ± 7.5 beats/30 sec, $n = 20$), as determined by beating frequency in liquid, was slightly slower than that of wild-type worms with no transgene (94.8 ± 11 beats/30 sec, $n = 20$), suggesting that GFP-ACT-4 (WT) has a weak negative effect on contractility of the body wall muscle.

Both GFP-ACT-4(E100K) and GFP-ACT-4(P165A) were incorporated into sarcomeres but also formed spherical aggregates in the cytoplasm of the body wall muscle cells ([Figure 1E, F](#)). Staining with rhodamine-phalloidin showed that sarcomeric organization of actin filaments were somewhat disorganized by expression of GFP-ACT-4(E100K) ([Figure 1E](#)) but not GFP-ACT-4(P165A) ([Figure 1F](#)). However, motility of the worms expressing GFP-ACT-4(E100K) or GFP-ACT-4(P165A) was not significantly different from that of wild-type worms ([Figure 1G](#)), suggesting that these actin mutants did not disturb muscle contractility. These aggregates resemble F-actin aggregates induced by inhibitors of actin dynamics ([Lázaro-Díéguez et al., 2008](#)). However, the aggregates of GFP-ACT-4(E100K) or GFP-ACT-4(P165A) were not recognized by rhodamine-phalloidin, a specific probe for F-actin ([Figure 1E, F](#)). In addition, we could not detect these aggregates by immunofluorescence using anti-actin monoclonal or polyclonal antibodies, even after attempts to expose antigens using guanidine hydrochloride ([Peränen et al., 1993](#)) or microwave ([Shi et al., 1991](#)), suggesting that the mutant forms of actin were present in an inclusion-body-like state and not readily accessible to the actin probes. Such aggregates were not detected in worms expressing GFP-ACT-4(WT) ([Figure 1D, H](#)), while variable numbers (0 - 36 per cell) of aggregates were found in worms expressing GFP-ACT-4(E100K) or GFP-ACT-4(P165A) ([Figure 1E, F](#)). In randomly selected worms ($n = 30$), GFP-ACT-4(E100K) (median = 9.5 aggregates per cell) induced significantly more aggregates than GFP-ACT-4(P165A) (median = 6.0 aggregates per cell) ([Figure 1H](#)). These aggregates were randomly located in the cytoplasm but not within the nucleus. Thus, we conclude that the missense mutations in ACT-4 induced the formation of abnormal cytoplasmic aggregates in muscle cells.

Discussion

Formation of actin aggregates by E99K (E100K in worm) or P164A (P165A in worm) mutation in actin has not been reported in human patients or other experimental systems. When cardiac α -actin mutants (E99K and P164A) are expressed in COS-7 cells, these actin mutants are not incorporated in the non-muscle actin cytoskeleton with no detectable aggregate formation ([Vang et al., 2005](#)). When E99K cardiac α -actin is

Figure 1. Effects of expression of GFP-ACT-4 variants in *C. elegans* body wall muscle. (A) Structure of porcine cardiac α -actin (Risi *et al.*, 2017) (Protein Data Bank accession number 5N0J) and *C. elegans* ACT-1 (Vorobiev *et al.*, 2003) (Protein Data Bank accession number 1D4X). ACT-1 is also expressed in *C. elegans* muscle and differs from ACT-4 by only one amino acid. Mutated residues (E99 and P164 in porcine cardiac α -actin; E100 and P165 in *C. elegans* ACT-1) are shown in yellow. Actin subdomains 1-4 are labeled as SD1-SD4. Molecular graphics were generated by PyMol (Schrödinger). (B) Western blot analysis of expression levels of GFP-ACT-4 variants. Total worm lysates (10 worms each) from wild-type without a transgene (WT) or with transgenes expressing GFP-ACT-4 variants were analyzed by western blot using an anti-actin antibody (top). Coomassie Brilliant Blue staining of the membranes after chemiluminescence detection (bottom) was used to normalize protein loading. Positions of GFP-ACT-4 (70 kDa) and endogenous actin (42 kDa) are indicated on the right. Representative molecular weight markers in kDa are indicated on the left. For each transgenic strain, three independently prepared lysates (#1-3) were analyzed. (C) Quantitative analysis of the Western blot (Dataset 1). Band intensity in arbitrary units (AU) of GFP-ACT-4 was normalized to intensity of total protein staining by Coomassie Brilliant Blue (Welinder & Ekblad, 2011) and plotted on the graph. GFP-ACT-4(WT) and each GFP-ACT-4 mutant were compared on the same western blot, and no significant differences were found by Student's t-test (ns) (n=3). (D-F) Localization patterns of GFP-ACT-4 (left) and F-actin (middle) in the *C. elegans* body wall muscle from worms expressing GFP-ACT-4(WT) (D), GFP-ACT-4(E100K) (E), and GFP-ACT-4(P165A) (F). Merged images (GFP in green and F-actin in red) are shown on the right. Bar, 20 μ m. (G) Worm motility of each strain was examined by beating frequency (beats per 30 sec) (Dataset 2). The results were analyzed by one-way ANOVA (n=20): ns, not significant (p>0.05); *p<0.05 p<0.01; **p<0.01; and ***p<0.001. (H) Number of GFP-ACT-4 aggregates per cell was counted (Dataset 3). The results were analyzed by one-way ANOVA (n=30) and significant difference was found between the data for GFP-ACT-4(E100K) and GFP-ACT-4(P165A) (**p = 0.006).

expressed in the mouse heart, the mutant actin is incorporated in the cardiac thin filaments and causes disarray of cardiomyocytes but with no detectable aggregate formation (Song *et al.*, 2011). Thus, effects of these actin mutations appear to be dependent on cellular contexts. Formation of actin aggregates by these actin mutations might be specific to the nematode muscle. We also cannot exclude the possibility that the aggregate formation is artificially enhanced by the GFP tag. Nonetheless, we were able to detect actin aggregates because of the GFP tag and might not have been able to detect the aggregates if a fluorescent tag was absent. Abnormal protein aggregates have been reported in idiopathic dilated cardiomyopathy (Gianni *et al.*, 2010; Subramanian *et al.*, 2015) and cardiomyopathies caused by mutations in desmin (McLendon & Robbins, 2011; Sanbe *et al.*, 2004), filamin (Brodehl *et al.*, 2016; Reinstein *et al.*, 2016; Valdes-Mas *et al.*, 2014), α -B-crystallin (Vicart *et al.*, 1998), or phospholamban (Te Rijdt *et al.*, 2016). Whether transient or stable protein aggregates are formed in actin-linked cardiomyopathies remains to be investigated. Our observations suggest that the *C. elegans* might be a relevant model system to study certain types of cardiomyopathies.

Data availability

Figshare: Raw data - Mutations in *Caenorhabditis elegans* actin, which are equivalent to human cardiomyopathy mutations, cause abnormal aggregation in nematode striated muscle. <https://doi.org/10.6084/m9.figshare.c.4424546> (Hayashi *et al.*, 2019).

This collection contains the following underlying data:

- Uncropped western blots
- Unprocessed microscopy images
- Dataset 1–3 (containing western blot quantification, and raw data for worm motility and number of aggregates per cell)

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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Christophe Ampe

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This reports describe the expression of two mutant actins, associated with cardiac myopathy, in the body wall muscle of the model organism *Caenorhabditis elegans*. Expression of these mutants as N-terminally tagged GFP-variants at low to moderate expression levels (relative to the endogenous actin) disturbs the fine structure of body wall muscle and dot-like patterns of GFP-mutant protein are evident in contrast to the experiments with GFP-WT-ACT-4 (see also below).

On the whole the experiments are well conducted and properly documented. I have some suggestions regarding interpretation of the results which the authors should look into but this does not require further experimentation (i.e. only textual revision).

Expression of WT GFP-ACT4 results in a negative effect on worm motility. This is mentioned by the authors in the results (Subcellular localization of GFP-ACT-4 variants and motility of worms). Such an observation is not unprecedented in mammalian cells and is basically due to the GFP—moiety possibly interfering with cellular functions. This was further investigated in Agbulut *et al.* (2007¹) and it was shown that GFP on its own interferes with myosin-based contractility which is potentially also the case here. In this respect should the authors not interpret the worm motility relative to the GFP-ACT-4 control rather than relative to WT worms? I realise it is more difficult to reconcile disturbed muscle structure (D-F) and the expected dominant-negative phenotype with increased worm motility (G) but the authors indicate the motility of the worms is significantly different between mutant and WT-GFP actin variants and this should be commented on in the results or the discussion section.

In the discussion the authors interpret their results solely in terms of cardiomyopathy based on the origin of the mutations. However the experimental system used is body wall muscle and interestingly the cellular phenotype is more reminiscent of nemaline myopathy (dotted patterns and irregular fibers). Functional differences between alpha-cardiac actin and alpha-skeletal muscle actin in human are unclear because the proteins are highly similar (only 4 amino acids different

and these changes are even conservative) and differential phenotypes of disease mutants likely result from differential expression in the respective tissues (in agreement with the authors' statement: "Thus, effects of these actin mutations appear to be dependent on cellular contexts"). These ACTC1 mutants do not cause aggregates in COS cells (Vang *et al.*, 2005, mentioned in the discussion), therefore I suggest to also make the parallel with nemaline myopathy where dot-like patterns in cellular context of (fused) myoblasts are frequently documented.

The dots appear to be phalloidin-negative as judged by visual inspection (Figure 1D and 1E); perhaps this should be mentioned in the main text.

Other minor points:

Introduction:

- In the two sentences: "Many of these cytoskeletal abnormalities can be reproduced by expression of mutant actins in cultured non-muscle or muscle cells (Bathe *et al.*, 2007; Costa *et al.*, 2004; Domazetovska *et al.*, 2007; Vandamme *et al.*, 2009a; Vandamme *et al.*, 2009b) or in transgenic mice (Lindqvist *et al.*, 2013; Ravenscroft *et al.*, 2011). By contrast, mutations in cardiac α -actin (*ACTC1*) cause hypertrophic and dilated cardiomyopathies (Mogensen *et al.*, 1999; Olson *et al.*, 1998)." a contrast is mentioned although the two items do not contrast each other.

Methods:

- Correct: Agilent.
- Figure 1A: upon printing (and in the 100% view option of PDF) these figures are difficult to interpret. I suggest to make them larger by rearranging this figure (quite some white space left and right).

References

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.**Reviewer Expertise:** actin cytoskeleton

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 01 April 2019

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James R. Bamburg

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Marcello Pignataro

Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA

The specific aim of this Research Note is to demonstrate that *C. elegans* can be used as a useful *in vivo* model to study mutations in actin associated with human myopathies, specifically in this case, cardiomyopathies. The authors have thus expressed only in the muscle tissue of *C. elegans* GFP-chimeras of wild type (WT) actin and two actin mutants (E100K and P165A) equivalent to human actin mutants E99K and P164A both of which are known to cause hypertrophic cardiomyopathy in humans. The authors then study by fluorescence microscopy the localization of the actin-GFP in the body wall muscle, the sarcomeric organization using fluorescent phalloidin to visualize F-actin, and worm motility. Their main findings are that all of the GFP-actin proteins incorporate into the sarcomere with only the E100K mutant causing sarcomeric disarray and that both mutant actins, but not WT, form GFP-positive aggregates that do not stain with fluorescent phalloidin or with an array of actin antibodies even after implementing antigen retrieval protocols. From that, the authors suggest the relevance of *C. elegans* to study actin-related cardiomyopathies. However, actin aggregates have not been detected in mouse heart from animals expressing the E99K mutant nor have aggregates been reported in human cardiomyopathies from subjects with either of the mutations used here. Thus, we are left to wonder, as indeed the authors have themselves, if the aggregates are specific to nematode muscle, if aggregate formation by the mutant actins is enhanced by the presence of the GFP-tag, and what other molecules are found in the aggregates that might provide a clue to their composition.

As the authors have clearly written in their introduction, there is a need for further

characterization of pathogenic mutations and expanding the available organisms to study specific diseases is always welcomed, as every model comes with their benefits and caveats. In spite of this, and the fact that the manuscript is well written and self-critical, there are some limitations in the studies presented that prevent us from approving its acceptance at this stage.

Although it is understandable why the authors choose to express the fluorescently tagged version of the actin mutants, expression of the untagged versions should also have been examined to see if the sarcomere disruption by the E100K occurs in the absence of the GFP tag. It is recognized that under these conditions it would not be possible to obtain ratios of the expressed transgene with the endogenous protein, but with a fluorescent protein expressed from a different promoter, it should be possible to obtain worms with the untagged actin mutants to determine its effect on sarcomere disruption. As recognized by the authors, the GFP tag could increase the propensity of the construct to oligomerize/aggregate. Whether this could be driven by GFP aggregation on its own is not clear. It has been reported that GFP linked via its C-terminus to a 16 amino acid "degron" peptide is not effectively degraded but forms aggregates very similar to the ones observed here when expressed in the body wall muscle of *C. elegans* (Link *et al.*, 2006¹). Since the aggregates observed in the current study have not been found to contain actin, one has to wonder if some cleavage has occurred to give rise to the same type of GFP-aggregates observed by Link *et al.* Why these do not form with the GFP-WT actin is unknown, but might reflect its greater stability to proteolysis. It would be useful to know if the number of aggregates correlate with total GFP intensity in a muscle cell.

Also, the absolute level of the expressed proteins relative to endogenous actin is problematic. As recognized by the authors themselves, their western blot was made with whole organism lysate and the signal of endogenous actin was near saturation. Although the contribution of actin from regions outside the body wall muscle is thought by the author's to be low, if it were possible to isolate only the body wall muscle, the values would be more meaningful. For the results to mimic human disease pathology, the authors need to try and reproduce the ratio between mutant and endogenous actin. As the authors demonstrate decreased worm motility when overexpressing total WT GFP-actin, it is clear that the expression of exogenous actin must be kept low to avoid potential toxic effects, although in this case it is unclear if the decreased motility arises from the presence of the GFP-tag.

While trying to explain the difference observed in their worm model with results from a previous paper (Song *et al.*, 2011) in which no aggregates were found with the E99K mutant, the first explanation given is "Thus, effects of these actin mutations appear to be dependent on cellular context." Such an explanation actually negates the main purpose of the paper. We would thus recommend to first try to resolve the issues that have been identified in this review, because, as interesting as the starting idea is, there are some flaws that render the results difficult to interpret.

Minor questions:

1. How do the authors explain the lack of correlation between sarcomeric disarray and worm motility?
2. Does the expression of any of the constructs lead to an increased mortality or to a reduced lifespan?

3. The nature of the aggregates that contain the GFP should be explored in more detail. Are they phase separated protein clusters such as one finds in stress granules and which can be identified by rapid diffusion within the structure if photobleached, or are these structured solid aggregates that might be able to be isolated, such as those from human from idiopathic dilated cardiomyopathy? The reason for their lack of actin staining needs to be identified since right now the title of the manuscript that states "human cardiomyopathy mutations, cause abnormal actin aggregation" cannot be used since no actin in the aggregates has been detected.

References

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cytoskeletal dynamics, especially actin biology.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reviewer Report 20 March 2019

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Amy Shaub Maddox

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Several human muscle pathologies result from mutations in actin genes. To understand the cellular effects of such alleles, Hayashi and colleagues introduced the relevant mutations from human diseases into a muscle-enriched actin gene in *C. elegans*, an animal with a simple body plan and behavior. They found that mutant actins localize to muscle fibers but also form aggregates. At least at the low expression level examined, mutant actins did not cause animal movement defects.

This is a clear manuscript with interesting potential. However, before it is suitable to be indexed, several points need to be addressed.

Major point:

1. The authors should comment on the significant decrease in body bends exhibited by worms expressing GFP-ACT-4(WT). Could this relate to lower expression of this transgene – did this phenotype scale with expression level among the various isolates? Could it relate to the ability of the (lowly expressed) transgene to incorporate into muscle (measure GFP/F-actin staining ratio)? Arguably the relevant comparisons are between mutant and WT transgene, and there was a statistically significant difference in body bends for both these comparisons, though this was an increase. Are there known perturbations that lead to more beats per second than controls?

Minor points:

1. The qualitative assay for aggregate formation is unlikely to have the dynamic range necessary to discern among conditions of different severity. Doing so would probably require a quantitative assay such as measuring the range of fluorescence intensity throughout regions of the image, or the proportion of the image occupied by pixels over some intensity threshold.
2. The E100K mutation appears to have severely disrupted muscle fiber organization – the 9-11 prominent F-actin bundles apparent in WT cells are replaced with many more partially-overlapping wispy structures. This effect should be related to the disarray noted by Song *et al.*, 2011 (Discussion 3rd sentence). The degree of subcellular morphological perturbation could be quantified with image analysis. It is interesting that despite this dramatic change, animal mobility is normal.
3. It would be nice to note somewhere the origin of 3' UTR (act-4).
4. "Turkey" should be "Tukey"; "Promotor" should be "promoter".

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: contractility, cell division, development, *C. elegans*, cytoskeleton

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 20 March 2019

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Michael Glotzer

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This short note describes the ability of two cardiomyopathy associated actin alleles to incorporate into body wall myosin filaments in *C. elegans*. The authors find that whereas transgenic, tagged wild-type control actin ACT-4 incorporates well into the muscle, the two mutant variants do so with lower efficiency and also accumulate as non-filamentous aggregates.

This report is technically sound and the conclusions are appropriately drawn from the evidence provided. In future studies it would be of interest to incorporate these mutations into the genome so that the relative dosage of the mutant protein would better reflect that seen in cardiomyopathy patients.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell biology, RhoA regulation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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