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Fluid dynamics alter *Caenorhabditis elegans* body length via TGF-β/DBL-1 neuromuscular signaling

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Skeletal muscle wasting is a major obstacle for long-term space exploration. Similar to astronauts, the nematode *Caenorhabditis elegans* displays negative muscular and physical effects when in microgravity in space. It remains unclear what signaling molecules and behavior(s) cause these negative alterations. Here we studied key signaling molecules involved in alterations of *C. elegans* physique in response to fluid dynamics in ground-based experiments. Placing worms in space on a 1G accelerator increased a myosin heavy chain, *myo-3*, and a transforming growth factor-β (TGF-β), *dbl-1*, gene expression. These changes also occurred when the fluid dynamic parameters viscosity/drag resistance or depth of liquid culture were increased on the ground. In addition, body length increased in wild type and body wall cuticle collagen mutants, *rol-6* and *dpy-5*, grown in liquid culture. In contrast, body length did not increase in TGF-β, *dbl-1*, or downstream signaling pathway, *sma-4/Smad*, mutants. Similarly, a D1-like dopamine receptor, DOP-4, and a mechanosensory channel, UNC-8, were required for increased *dbl-1* expression and altered physique in liquid culture. As *C. elegans* contraction rates are much higher when swimming in liquid than when crawling on an agar surface, we also examined the relationship between body length enhancement and rate of contraction. Mutants with significantly reduced contraction rates were typically smaller. However, in *dop-4*, *dbl-1*, and *sma-4* mutants, contraction rates still increased in liquid. These results suggest that neuromuscular signaling via TGF-β/DBL-1 acts to alter body physique in response to environmental conditions including fluid dynamics.

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INTRODUCTION

An individual's physique is shaped over long periods by both external stimuli and locomotory gaits. Bone and muscle wasting are inevitable pathophysiological adaptations in microgravity, e.g., spaceflight, and with inactivity, e.g., in the bedridden.[1–4] The wasting of these tissues is a major obstacle for long-tern space exploration. Microgravity in particular markedly decreases mechanical loading and also results in drastic changes in fluid dynamics including hydrostatic forces. However, it is still unclear what signaling molecules and behavior(s) cause these pathophysiological adaptations.

Aquatic exercise is one of the best ways to achieve optimal body strength and to improve vigor. Such exercise involves the physical application of fluid dynamics, particularly hydrostatic forces and drag resistance accompanying liquid viscosity, and is effective not only in healthy individuals but also in bedridden patients.[5–10] Although many recent studies have evaluated flow dynamic parameters as physical stimuli, the mechanisms for perception of these stimuli and signal transduction from these stimuli to bone and skeletal muscle formation, enhanced physique and strength remain unclear.

Caenorhabditis elegans is a free-living nematode that is also a widely used laboratory animal. The body length can be altered via a highly conserved transforming growth factor-β (TGF-β)/DBL-1 Smad transcription factor signaling pathway.[11–16] *C. elegans* has at least two different locomotory gaits, one is displayed when swimming in liquid and the other when crawling on a surface.[17–21] The transition from the swimming gait to the crawling gait and vice versa is controlled by biogenic amines as a short-term adaptive response.[21] *C. elegans* also makes short-term adaptations to locomotion in response to gentle mechanical stimuli through a mechanosensory complex composed of the degenerin ion channels, MEC-4 and MEC-10, found in touch-sensitive neurons.[22–25] Worms also make long-term adaptive responses. For example, we reproducibly found that spaceflight induces reduced expression of some muscle genes,[26–29] including muscular thick filaments, other cytoskeletal elements, and mitochondrial metabolic enzymes. These gene expression changes appeared to be consistent with the changes in body length and fat accumulation during spaceflight.[29]

This study investigated alteration of muscular myosin and TGF-β gene expression in response to fluid dynamic properties (microgravity, viscosity/drag resistance, and depth of liquid culture). We also compared the relationship between the body physique established and the different moving behaviors displayed by worms cultured in liquid and on moisture agar surface, swimming

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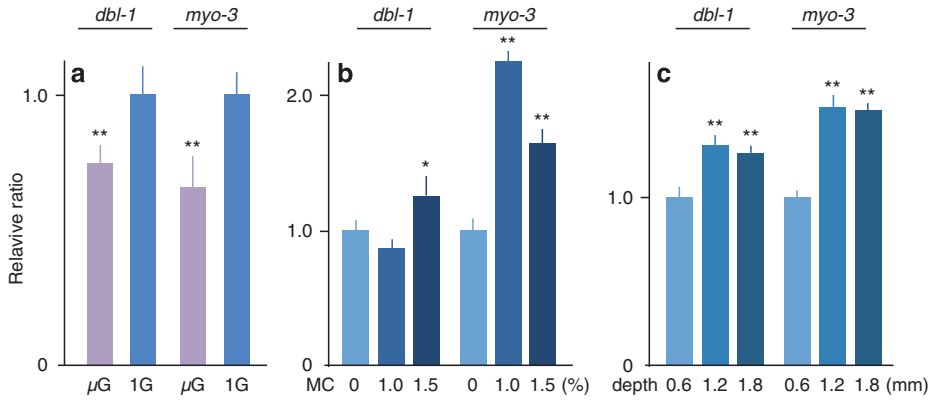

Figure 1. Restoration of 1G on the International Space Station increases *myo-3* and *dbl-1* gene expression just as increasing liquid viscosity and depth of culture do in ground-based experiments. *myo-3* and *dbl-1* gene expression levels were monitored in liquid cultured, spaceflown wild-type animals (4-day adult) with or without 1G acceleration during the *C. elegans* RNAi space experiment (CERISE) in Japanese Experiment Module KIBO[30,31] (**a**). Wild-type animals were grown from the L1 larval stage for 4 days in different liquid viscosities with 1.0% (36.1 cSt) and 1.5% (123.3 cSt) methylcellulose (**b**). Wild-type animals were grown from the L1 larval stage for 4 days on OP50 nematode growth medium (NGM) agar submerged in indicated depth of OP50 NGM liquid medium (**c**). Alterations in *dbl-1* and *myo-3* gene expression were monitored by quantitative real-time PCR.

and crawling, respectively. Finally, we explored the hypothesis that neuromuscular signaling via TGF-β/DBL-1 modulates altered physique in response to fluid dynamic properties.

RESULTS

Fluid dynamic parameters modulate *myo-3* and *dbl-1* gene expression

In our *C. elegans* RNA interference space experiment (CERISE), L1 larvae stage animals were synchronously cultured to adulthood in liquid media for 4 days either in microgravity or a 1G centrifuge onboard the Japanese Experiment Module of the International Space Station.[30,31] The nematode L1 larvae were launched to the International Space Station onboard the Space Shuttle Atlantis, STS-129, on 16 November 2009. Cultures were initiated on 20 November 2009, frozen 4 days later, and the post-cultivation frozen samples were returned by the Space Shuttle Endeavour, STS-130, on 21 February 2010. Microarray expression analyses indicate that levels for muscular thick filaments, cytoskeletal elements, and mitochondrial metabolic enzymes decreased relative to parallel cultures on the 1G centrifuge (95% confidence interval ($P \leqslant 0.05$): accession number GSE71770 on GEO).[29] In addition, the body lengths of worms cultured in microgravity were slightly (~5.5%) but significantly decreased versus worms cultured in the 1G onboard centrifuge.[29] In this study, we confirmed that myosin heavy chain, *myo-3*, and TGF-β, *dbl-1*, gene expression were reduced, 60% and 70%, respectively, in microgravity versus in the centrifuge (Figure 1a). These observations suggest that body length reduction might be due to pathophysiological adaptations to microgravity caused by transcriptional repression of muscular genes and/or decreased TGF-β signaling, which is caused by decreased expression of *dbl-1*.

In ground-based experiments, in order to study the effect of a fluid dynamic parameter, drag resistance, on *myo-3* and *dbl-1* gene expression, wild-type worms were cultured for 4 days after the L1 larval stage under different liquid viscosities (1.0 cSt (0% methylcellulose), 36.1 cSt (1.0% methylcellulose), and 123.3 cSt (1.5% methylcellulose)). *myo-3* gene expression was significantly increased at 36.1 cSt viscosity and moderately increased at 123.3 cSt. *dbl-1* gene expression significantly increased modestly by ~20% at 123.3 cSt (Figure 1b). The growth rate and developmental timings were not significantly altered by viscosity, with all of the L1 larvae having developed to gravid, mature adult hermaphrodites at 4 days. In gravid adults, body length did not increase as much as expected for animals grown in 1.5% methylcellulose, perhaps suggesting that higher methylcellulose concentrations dehydrate the worms and/or inhibit digestion and absorption.

To study the effects of altering the depth of liquid cultivation, OP50 nematode growth medium (NGM) agar was covered with additional OP50 NGM liquid medium (0.6, 1.2, or 1.8 mm in depth). In the shallowest condition, the worms were covered completely in fluid and moving behavior changed to swimming. In gravid adults (day 4), both *myo-3* and *dbl-1* gene expression increased with increased depth of liquid culture (Figure 1c), with maximal observed expression achieved at 1.2 mm. The body lengths of worms cultured in different liquid depth were slightly but not significantly larger (0.6 mm: 1.35 ± 0.06 mm, 1.2 mm: 1.36 ± 0.07 mm, 1.8 mm: 1.37 ± 0.04 mm, $n = 21$ worms per group, $P > 0.1$). These results demonstrate that the nematode *C. elegans* can alter muscular and TGF-β gene expression in response to flow dynamic parameters. However, the gene expression and body length data for worms cultured at different depth suggests that the response to depth of culture is either saturated once worms are fully submerged or is an all-or-none response.

C. elegans body length and muscle gene expression increase in liquid culture

Although altering the flow dynamic parameters increased *myo-3* and *dbl-1* expression (Figure 1), the magnitude of the significant changes in response to cultivation in liquid on the ground was small. In order to further explore whether and how *C. elegans* alters gene expression and body physique in response to different environmental stimuli and moving behavior, we measured the gene expression levels and the body length of wild-type worms grown either in fixed depth liquid medium or on a moist agar surface as described in Methods. Culturing occurred in parallel with L1 larvae hatched on agar plates and then cultured in liquid or on agar. The growth rate and developing timings were not significantly different between the culture conditions, with all of the larvae having developed into young adult hermaphrodites 3 days after the onset of culturing, as evidenced by the onset of egg production. However, the body length at day 4 (gravid adult stage) was significantly longer for animals cultured in liquid medium versus on moist agar (Figure 2a,b); this difference persisted at the later time points (5 and 6 days; Figure 2c). The

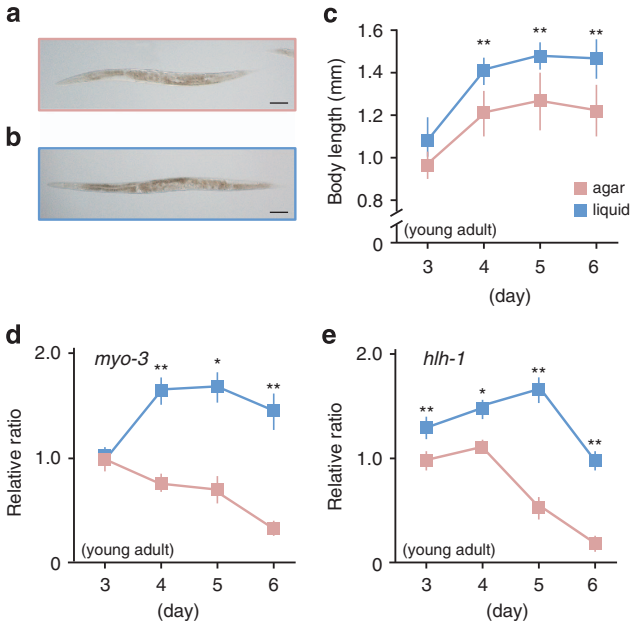

Figure 2. Alterations in body length and gene expression levels of *myo-3* and *hlh-1* in *C. elegans* grown under different culture conditions. Wild-type gravid adult hermaphrodite grown on moist agar plate (**a**, indicated in pink) or in liquid culture (**b**, indicated in blue), for 4 days starting from the L1 larval stage. (**c**) Body lengths were significantly increased by liquid versus agar culture. Alterations in *myo-3* (**d**) and *hlh-1* (**e**) gene expression levels were monitored by quantitative real-time PCR. Data points and error bars indicate the means ± s.d. ($n = 60$ worms per time point, $^{**}P \leq 0.01$).

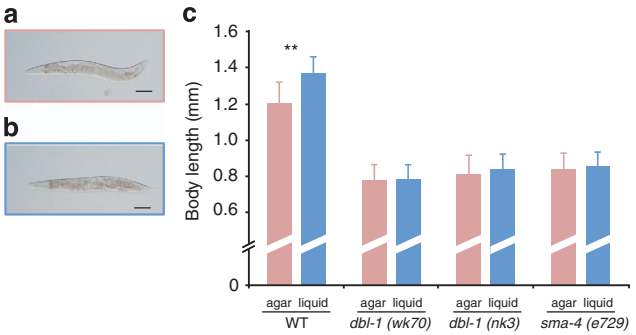

Figure 3. DBL-1 and its signaling pathway are required for *C. elegans* body length alterations in response to liquid culture conditions. *dbl-1(wk70)* gravid adult hermaphrodite grown on moist agar plates (**a**) or in liquid culture (**b**) for 4 days, starting from the L1 larval stage. (**c**) Body lengths of wild-type, *dbl-1(wk70)*, *dbl-1(nk3)*, and *sma-4(e729)* grown in liquid or on agar for 4 days were measured. Bars and error bars indicate the means and s.d. ($n = 30$ worms per condition; $^{**}P \leq 0.01$).

expression levels of a myosin heavy chain gene, *myo-3*, and its upstream transcription factor, *hlh-1*, were also significantly higher in animals cultured in liquid versus on agar (Figure 2d,e). Myosin protein expression levels were similarly increased by 1.6-fold for animals cultured in liquid versus on agar (1.24-fold in liquid and 0.78-fold on agar as compared with the relative ratio of a ribosomal protein).

Liquid culture alters *C. elegans* physique through TGF-β signaling

DBL-1 is a member of the TGF-β protein family. It, along with its signaling pathway, is a known regulator of *C. elegans* body length.[11–16] To determine whether DBL-1 was required for the observed body length alteration, we measured the length of *dbl-1* (*wk70*) and (*nk3*) mutants after culturing in liquid or on agar. We also measured *sma-4*(*e729*) mutants, as SMA-4 is a known downstream transcription factor component of the DBL-1 signaling cascade that controls the body size. In contrast to wild-type (Figures 2 and 3), these mutants' body lengths did not increase in liquid culture (Figure 3), implying that *dbl-1* and *sma-4* are required for the body length alteration in response to liquid culture.

Because previous work reported that DBL-1 controls the expression of several genes, including those encoding extracellular matrix-associated collagens, we next investigated whether liquid culture suppresses dumpy and roller phenotypes in collagen mutants. The body lengths of dumpy *dpy-5*(*e907*) and *rol-6*(*su1006*) mutants were markedly increased in liquid versus agar culture (Figure 4a–d). In addition, the frequency of the right-handed roller phenotype induced by *rol-6*(*su1006*) was reduced by liquid culture (Figure 4b). Taken together, the results displayed in Figures 1, 2, and 3 suggest that liquid culture alters *C. elegans* physique via activation of TGF-β/DBL-1 signaling (Figure 3), not only in terms of muscle myosin expression (Figure 2) but also in terms of extracellular matrix/collagen deposition (Figure 4).

UNC-8 degenerin channel and D1-like dopamine receptor DOP-4 are required for body length increase in liquid culture

In *C. elegans*, a degenerin/epithelial sodium ion channel composed of MEC-4 and MEC-10 functions as a mechanosensor for physical stimuli (e.g., touch).[22–25] To determine whether degenerin family members act as mechanosensors in response to fluid dynamic parameters, body length alterations in response to liquid culture was assessed in degenerin channel mutants (*mec-4*(*e1611*), *mec-10*(*e1515*), and *unc-8*(*e15*)). While both *mec-4*(*e1611*) and *mec-10*(*e1515*) displayed increased body length in response to liquid culture, *unc-8*(*e15*) showed no significant increase in body length (Figure 5).

The *C. elegans* transition between distinctive crawling and swimming gaits is controlled by the biogenic amines, dopamine, and serotonin.[21] Therefore, we measured body length alterations in animals with mutations in genes encoding the serotonin biosynthetic enzyme, *tph-1*(*mg280*), a serotonin/octopamine receptor family member, *ser-5*(*ok3087*), and the D1-like dopamine receptors, *dop-1*(*vs101*) and *dop-4*(*tm1329*). *dop-4*(*tm1329*) showed no culture-dependent alterations in body length (Figure 6), suggesting that DOP-4 is also required for physique alteration. In contrast, body lengths were significantly increased in liquid versus agar culture for *tph-1*(*mg280*), *ser-5*(*ok3087*), and *dop-1* (*vs101*) (Figure 6). We next investigated the impact of other factors on body length in the liquid culture system, starting with oxygen and nutrient sensing. To do this, we used animals with mutations in genes encoding hypoxia response factor, *hif-1*(*ia4*), an insulin/IGF-1-like peptide, *ins-7*(*ok1573*), and a key transcriptional regulator that acts downstream of insulin/IGF-1-mediated signaling, *daf-16*(*mu86*). In all cases, body length increased in response to liquid culture in these mutants (Figure 6). A ryanodine receptor *unc-68*(*r1161*)[32] mutant, which is sluggish and flaccid, also displayed increased body length in liquid culture (Figure 6). These results suggest that alteration of *C. elegans* physique in response to liquid culturing requires some neuromuscular signaling, but is largely distinct from oxygen and nutrient sensing.

TGF-β/DBL-1 signaling is required but swimming behavior is not sufficient for increasing body length

Finally, we assessed whether the increased contraction rates observed in swimming worms were involved in body length

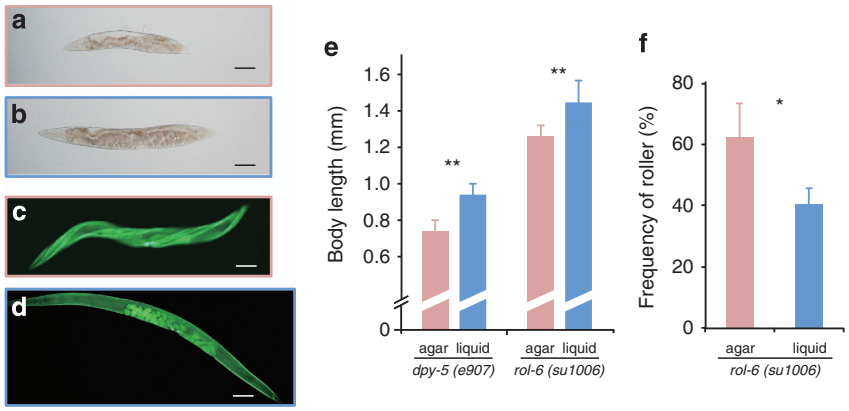

Figure 4. Dumpy and roller phenotypes are mitigated by liquid culture. *dpy-5(e907)* (**a**, **b**), and RW1596 *rol-6 (su1006)* and *Pmyo-3::GFP::myo-3* (**c**, **d**), animals were grown on moist agar (**a**, **c**) or in liquid culture (**b**, **d**) for 4 days, starting from the L1 larval stage. (**e**) Their body lengths were measured. (**f**) The frequency of the right-handed roller phenotype in RW1596 was counted. Bars and error bars indicate the means and s.d. ($n = 30$ worms per condition; $^*P \leq 0.05$, $^{**}P \leq 0.01$).

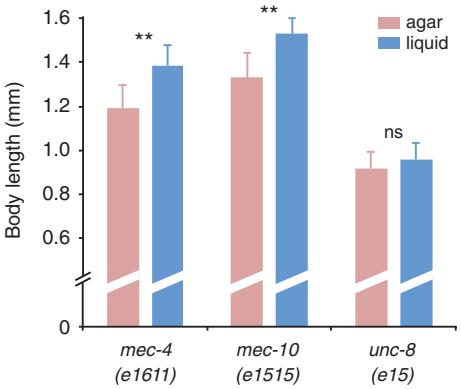

Figure 5. UNC-8 degenerin is required for *C. elegans* body length alterations. *mec-4(e1611)*, *mec-10(e1515),* and *unc-8(e15)* were grown for 4 days in liquid or agar culture, starting from the L1 larval stage. Body lengths were measured. Bars and error bars indicate the means and s.d. ($n = 30$ worms per condition; $^{**}P \leq 0.01$).

enhancement. The dorsal and ventral (DV) head bending cycle was counted in various mutants (Table 1): TGF-β signaling (*dbl-1* and *sma-4*); a degenerin channel (*unc-8*); a D1-like dopamine receptor (*dop-4*); and a ryanodine receptor (*unc-68*). *unc-8(e15)* often abnormally curled and did not alter the DV cycle in either liquid or on the agar plate, this corresponded with the mutant not displaying the normal increase in the cycle when swimming (Table 1). In contrast, other degenerin mutants, *mec-4* and *mec-10*, were not significantly different from wild type in movement or cycle increase in response to swimming movement (*mec-4 (e1611)*: 0.53 ± 0.15 Hz on agar and 1.56 ± 0.19 Hz in liquid; *mec-10 (e1515)*: 0.53 ± 0.19 and 1.53 ± 0.13 Hz, $P > 0.1$). These observations suggest that mutants that do not display an increase in body length (e.g., *unc-8*) also do not display an increase in DV cycle when swimming. However, the TGF-β signaling (*dbl-1* and *sma-4*) and D1-like dopamine receptor (*dop-4*) mutants, which also did not display an increase in body length, did, in fact, display and increase in DV cycle when swimming. Thus, although DV cycle increase when swimming is not sufficient for body length increase (e.g., *dbl-1*, *dop-4*, and *sma-4*), it may be required (e.g., *unc-8*).

To further assess the requirement for TGF-β signaling, we quantitatively measured the gene expression of TGF-β, *dbl-1* and its downstream signal, *wrt-4*.[33] As shown in Table 1, *unc-8(e15)* and *dop-4(tm1392),* whose body length did not increase in response to liquid culture (Figures 5 and 6), did not display induction of *dbl-1* or *wrt-4*. These results suggest that *unc-8* and *dop-4* act upstream of *dbl-1* to control the response to cultivation in liquid. Consistent with this, *dbl-1* and *sma-4* did display induction of *dbl-1*, presumably as upstream sensors are intact in these mutants, but did not display induction of *wrt-4*, thus confirming TGF-β signaling is required for *wrt-4* induction in response to liquid cultivation. In contrast, *unc-68(r1161)* increased body length and gene expression (Table 1, Figures 5 and 6). These results suggest that to extend body length in liquid culture: (1) TGF-β/DBL-1 signaling is essential, but (2) increasing the DV cycle by swimming behavior may be necessary but is not sufficient.

DISCUSSION

C. elegans is a free-living nematode found in soil and decaying vegetation, and it persists on particles with moist surfaces and in aquatic conditions. *C. elegans* exhibits at least two distinct locomotory gaits, swimming when in liquid and crawling when on surfaces.[17–21] Surface tension is required to retain *C. elegans* on moist surfaces and is predicted to be on the order of $10{,}000 \times G$.[28,34,35] Thus, the crawling gait on surfaces likely results from the larger forces caused by surface tension verses the flow dynamics in liquid.[20] The crawling gait is triggered by large external loads, and substantial muscle power is used to counter the external load and move the body. In support of this speculation, a continuous gait transition between undulations that resemble either swimming or crawling has previously been observed with increasing liquid viscosity.[20]

These observations, however, do not fully explain why *C. elegans* physique is significantly altered in liquid versus on agar. Gait transition, by necessity, must be a short-term adaptive response, whereas alteration of physique is usually a long-term adaptive response.

Crawling and swimming are quite different behaviors. *C. elegans* crawling on moist agar exhibits dorsoventral bends of an S-shaped posture with an average amplitude of 135° at a frequency of 0.5–0.8 Hz.[18,21] In contrast, swimming *C. elegans* display dorsoventral bends of a C-shaped posture with an average amplitude of 45° at a frequency of 1.7–2.1 Hz.[18,21] Moreover, *C. elegans* swims continuously for extended periods, 45 min more.[21] These quantitatively distinct behaviors in frequency, amplitude, and propagation of dorsoventral bends may elicit distinct long-term adaptive responses that alter gene expression to shape a physique most suited to the environment.

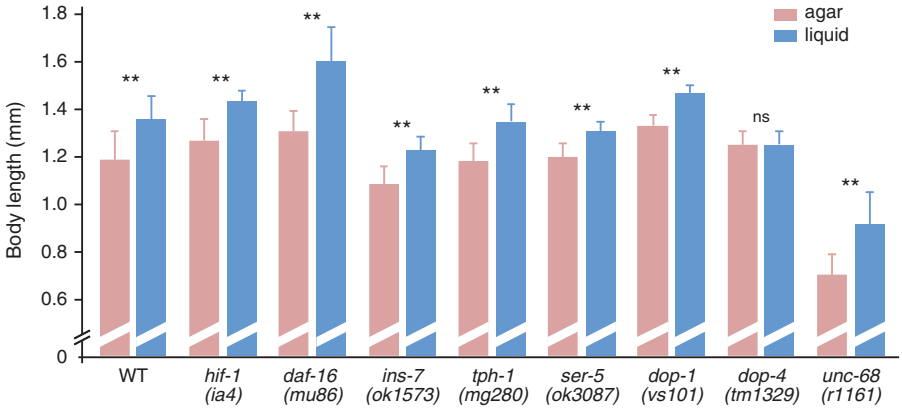

Figure 6. Several *C. elegans* mutants show wild-type body length increases in liquid versus agar culture. Of all the mutants tested, only *dop-4 (tm1329)* failed to increase its body length after 4 days in liquid culture ($n = 30$ worms per condition; $^{**}P \leq 0.01$).

Table 1. Crawling and swimming behaviors and *dbl-1* and *wrt-4* gene expression in *dbl-1*, *sma-4*, *unc-8*, *dop-4*, and *unc-68* mutants

Strains	*Moving behavior*		*Gene expression (liquid versus agar)*	
	Crawling (Hz)	*Swimming (Hz)*	dbl-1 *(fold change)*	wrt-4 *(fold change)*
N2	0.65 ± 0.18	1.66 ± 0.18	2.22 ± 0.08	9.24 ± 1.40
LT121 *dbl-1 (wk70)*	$0.41 \pm 0.10^{**}$	$1.40 \pm 0.25^{*}$	1.42 ± 0.02	0.36 ± 0.05
DR1369 *sma-4 (e729)*	$0.46 \pm 0.11^{**}$	1.57 ± 0.15	1.36 ± 0.07	0.47 ± 0.03
CB15 *unc-8 (e15)*	$0.23 \pm 0.07^{***}$	$0.27 \pm 0.16^{***}$	1.11 ± 0.01	0.60 ± 0.06
FG58 *dop-4 (tm1392)*	0.54 ± 0.17	$1.48 \pm 0.21^{*}$	0.89 ± 0.01	0.66 ± 0.03
TR2170 *unc-68 (r1161)*	$0.22 \pm 0.07^{***}$	$0.46 \pm 0.15^{***}$	1.48 ± 0.04	1.56 ± 0.11

Dorsal and ventral (DV) head bending cycle was counted for 30 s under stereo microscopy as contraction rate of swimming or crawling, just after tapping each culture plate. ($n = 10$ worms per condition; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

When cultured in liquid, body length and expression levels of the myosin heavy chain gene *myo-3* and its transcriptional activator, *hlh-1* (MyoD) increased versus when cultured on agar. These would appear to be a long-term adaptive response to growth in the liquid environment. A key signaling pathway already known to modulate *C. elegans* body length, the TGF-β/DBL-1 signaling pathway,[11–16] was both required for physique alteration in response to liquid cultivation and transcriptionally induced in response to liquid cultivation. The induction of *dbl-1* expression in response to liquid culture appears to have been due to fluid dynamics as *dbl-1* expression was induced both by increasing viscosity and depth of liquid culture (Figure 1). Intriguingly, *dbl-1* expression was induced by liquid culture on a 1G centrifuge onboard the International Space Station versus in space microgravity (Figure 1). It is possible that increased hydrostatic pressure on the worms cultured with 1G acceleration in space has impacted *dbl-1* expression. As we have not measured hydrostatic pressure, future experiments examining *dbl-1* expression alteration in response to hydrostatic pressure are needed.

In *C. elegans*, degenerin/epithelial Na^+ channel family members act as mechanosensors for assorted physical stimuli. Thus, we were curious if they might be sensing fluid dynamics and possibly effecting *dbl-1* expression. MEC-4 and MEC-10 form a heterocomplex of ion pore-forming subunits in touch-sensitive neurons[22–24] and this complex is essential for response to hypergravity.[25] However, our finding that body size increases in liquid culture even in the absence of the MEC-4/10 heterocomplex suggests that this complex is not required for altering physique in response to fluid dynamics. Instead, we found that other degenerin/epithelial Na^+ channel family members, specifically UNC-8 is required for altered physique in response to fluid dynamics (Figure 1). This suggests that they may be acting as fluid-responsive mechanoreceptors. UNC-8 is expressed in ventral cord motor neurons.[36–38] In addition, TGF-β/DBL-1, which dose dependently regulates *C. elegans* body size, is primarily expressed in motor neurons and the nerve ring;[12] the known site of *dbl-1* expression closely matches the known site of UNC-8 action. Therefore, the UNC-8 degenerin complex might sense physical stimuli from culture conditions and/or body posture alterations and/or tension generated by varying gaits, leading to the upregulation of DBL-1. The upregulated DBL-1 ligand then acts, via known mechanisms, on muscular and hypodermal cells to control body physique by facilitating the expression of muscle myosin and cuticle collagen.

As another possibility, swimming behavior could be necessary to alter body physique because *unc-8(e15)* mutants completely lose normal moving behaviors both in liquid and on an agar surface (Table 1). Last, as fluid dynamics, particularly hydrostatic forces and drag resistance accompanying liquid viscosity, enhance bone and skeletal muscle formation in less active people,[5–10] we were curious if this was also true of less active worms. Indeed, a sluggish and flaccid mutant, *unc-68*, displayed body length increase in response to liquid culture (Figure 6). This suggests not only that an activity-independent fluid dynamics effect, possibly buoyancy facilitated ease of mobility, appears to be evolutionarily conserved between *C. elegans* and man but also that *C. elegans* might be a suitable model for studying and combating the impact of inactivity on human muscle.

Recent work has elucidated that *C. elegans* employs biogenic amines (dopamine and serotonin) to control gait transition

between crawling and swimming,[21] a short-term adaptation. Dopamine is necessary to initiate and maintain crawling on land after swimming in water, and serotonin is necessary to transition from crawling to swimming behavior. We were curious if this short-term adaptation also had a role in long-term adaptation of physique. While mutations in *dop-1, ser-5*, and *tph-1* displayed wild-type body length increase in response to liquid culture, *dop-4* mutants did not display a length increase. This suggests that DOP-4 is required not just for the short-term gait adaptation but also for the long-term physique adaptation to liquid culture. DOP-4 is a D1-like dopamine receptor that is known to be involved in alcohol-induced disinhibition of certain behaviors, including foraging and crawling postures in water.[39] Thus, DOP-4 may function as a key component for *C. elegans* adaptation to aquatic environments participating in both short-term and long-term adaptive responses.

C. elegans growing in liquid, as in the wild, can be subject to hypoxia and limited nutrition. We therefore investigated whether these factors might contribute to increased body length in liquid culture. However, mutations in *hif-1*, *ins-7*, and *daf-16* all responded like wild type, suggesting that neither oxygen sensing nor nutrition sensing was contributing to the increased body length. This may not be surprising given that UNC-8 and/or DOP-4 appear to be sensing the fluid dynamics and that wild type and all other mutants tested developed at the same rate in either liquid or agar culture.

In conclusion, our results suggest that UNC-8 and/or DOP-4 may function as neuronal sensors/transmitters of fluid dynamic properties including viscosity/drag resistance and possibly hydrostatic pressure. It appears that activation of these neuronal sensors/transmitters by fluid dynamic properties increase expression of *dbl-1* to increase DBL-1 signaling causing an increase in body size and expression of muscle proteins.

MATERIALS AND METHODS

Nematode strains

C. elegans N2 Bristol strain was used as wild type. The mutant strains were as follows: BC15777 derivative: *dpy-5(e907)*; RW1596: *myo-3(st386), stEx30* [*myo-3p::GFP+rol-6(su1006)*]; LT121: *dbl-1(wk70)*; NU3: *dbl-1(nk3)*; DR1369: *sma-4(e729)*; CB1611: *mec-4(e1611)*; CB1515: *mec-10(e1515)*; CB15: *unc-8 (e15)*; ZG31: *hif-1(ia4)*; CF1038: *daf-16(mu86)*; RB1388: *ins-7(ok1573)*; GR1321: *tph-1(mg280)*; RB2277: *ser-5(ok3087)*; LX636: *dop-1(vs101)*; FG58: *dop-4(tm1392)*; and TR2170: *unc-68(r1161)*. These strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA).

Culture conditions

Thirty to fifty wild-type or mutant adult hermaphrodites were transferred onto a freshly prepared nematode growth medium (NGM) agar plate (ϕ6-cm plastic culture dish) with *Escherichia coli* strain OP50 spread over the surface as a food source. Adults were allowed to lay eggs for 4 hours at 20 °C; this yielded at least 500 eggs on each plate. Adults and the bacterial food source were washed off the plate by gentle pipetting with 2 ml M9 buffer three times. The remaining eggs were left for an additional 12 h at 20 °C at which point the hatched L1 larvae were collected in 500 µl M9 buffer. Sixty L1 larvae per condition were simultaneously cultured at 20 °C on either an OP50 NGM agar plate or in a 2 ml liquid NGM culture system containing *E. coli* OP50 ($OD_{600} = 1.0$; liquid depth = 0.8 mm) in a ϕ6-cm plastic culture dish. Three days after cultivation, wild-type and all other mutants tested in this study had grown to young adulthood, as evidenced by the onset of egg production; this was observed under both culture conditions. To prevent starvation, adult animals were picked using a ϕ0.2-mm platinum wire and transferred to new medium each day; transfers occurred for both culture conditions.

To study the effects of liquid viscosity on gene expression (see below), final concentrations of 1.0 and 1.5% methylcellulose in 2.0 ml of NGM liquid medium containing *E. coli* OP50 ($OD_{600} = 1.0$) were used. The kinetic viscosities, as measured by a viscometer Visoboy2 (LAUDA, Germany), were 1.0 cSt (mm^2/s) for the control OP50 NGM liquid, 36.1 cSt for the 1.0% methylcellulose, and 123.3 cSt for the 1.5% methylcelluose.

To study the effects of the depth of liquid culturing, OP50 NGM agar in ϕ6-cm plates were additionally covered with 1.5 ml (~0.6 mm in depth), 3.0 ml (~1.2 mm in depth), or 4.5 ml (~1.8 mm in depth) of NGM liquid medium containing *E. coli* OP50 ($OD_{600} = 1.0$). From L1 to adulthood, all animals are fully covered and showed swimming behavior even in the shallowest condition.

Measurements of body lengths

C. elegans body length was evaluated at young adulthood (3 days after starting as a L1 larvae) and the subsequent 3 days. Each day, a subset of cultured animals were fixed with 1% paraformaldehyde for 30 min at room temperature, and were imaged using a BX51 microscope and a DP71 camera (Olympus Optical, Tokyo, Japan). Body lengths were measured using CellSens image analysis software (Olympus). Each experiment was performed in triplicate with three independent samples (total $n = 60$ worms per time point). Statistical analysis was performed in MS Excel (Microsoft Co., Redmond, WA, USA). Statistical significance was set at $P < 0.05$, using a Student's two-tailed *t*-test.

Measurements of contraction rate of moving behavior

Each assay was conducted on 10, never starved, adult worms at 4 days from L1 larvae cultured in liquid or on moisture agar plate. DV head bending cycle was counted for 30 s under stereo microscopy as contraction rate of swimming or crawling, just after tapping each culture plate.

Gene expression analysis

Total RNA was isolated on the indicated day of cultivation from ~300 adult hermaphrodites using TRIzol (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR analysis was performed with a CFX96 Touch Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) and a SYBER ExScript RT-PCR Kit (TaKaRa Bio, Shiga, Japan). The expression level of elongation factor *eef-2* was used as an internal standard, and the relative ratio of gene expression for each gene was calculated as described.[40] The following primer sets were used to amplify *eef-2*, *hlh-1*, *myo-3*, *dbl-1*, and *wrt-4*: *eef-2* (forward) 5′-GAC GCT ATC CAC AGA GGA GG-3′ and (reverse) 5′-TTC CTG TGA CCT GAG ACT CC-3′; *hlh-1* (forward) 5′-GCT CGG GAA CGC GGT CGA-3′ and (reverse) 5′-GGA ATG CTC GCA ACG ATC CGC GA-3′; *myo-3* (forward) 5′-ACT CTC GAA GCC GAA ACC AAG-3′ and (reverse) 5′-TGG CAT GGT CCA AAG CAA TC-3′; *dbl-1* (forward) 5′-CAG TTT GGC TTC GAT TGC TC-3′ and (reverse) 5′-TGA AGC TGG TCC TCT GTC TG-3′; and *wrt-4* (forward) 5′-TGG ATG AGC TCG CAG TGG-3′ and (reverse) 5′-CTC CGT TGT CAA GTG TGA ATT CTA C-3′. Real-time PCR experiments were performed in triplicate for each biological sample.

Spaceflight experiment

We also measured expression levels of some genes in spaceflown wild-type 4-day-old adults from the CERISE.[30,31]

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CONTRIBUTIONS

AtsH designed research. SH, TH, KN, ZS, NH, and AkH performed gene expression analyses and microscopic observation. KF and AkH coordinated the CERISE flight experiment. SH, AkH, TH, KN, TE, NJS, and AtsH analyzed data, and wrote the paper.

COMPETING INTERESTS

The authors declare no conflict of interest.

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