LET-756/FGF is Implicated in the Control of *C. elegans* Body Size

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Abstract: LET-756 is one of the two fibroblast growth factors (FGF) of *Caenorhabditis elegans*. We have previously shown that worms homozygote for the *let-756* null allele *(s2887)* die at larval stage and that homozygotes for the partial loss-of-function allele (*s2613*) are viable but shorter than wild-type animals. We now show that expression of *let-756* in any region of the *s2887* worm can rescue the lethal phenotype but with variations in body size depending on the site of expression, harmonious growth being achieved by *let-756* expression in body wall muscles. Local action of *let-756* was also observed in mosaic animals homozygotes for the *s2613* allele. Our results support a paracrine activity for LET-756 exerting its effect locally on body size whether by acting as an actual growth factor or by acting on EGL-15/FGFR expressing hypodermal cells thereby regulating fluid balance.

INTRODUCTION

In vertebrates, Fibroblast Growth Factors (FGFs) are involved in various developmental and pathological processes (for review see [1]), usually in combination with other regulatory growth factors, such as members of WNT and TGF superfamilies. In humans, 22 FGFs are distributed in seven families [2]. FGFs activate transmembrane FGF tyrosine kinase receptors (FGFR). Activated FGFRs dimerize, are phosphorylated, and signal through cytoplasmic proteins. Coreceptors, such as heparan sulphate proteoglycans, are required for proper membrane signaling. A special family of FGFs, the fibroblast homologous factors (FHF), has different functions and signaling pathways [3]. Some FGFs may signal through a direct nuclear pathway since FGFs or both FGFs and FGFRs have been identified in the nucleus (reviewed in [4]).

Only a few FGFs have been found in protostomian species whose sequences are available. In the worm *Caenorhabditis elegans*, two FGFs, EGL-17 and LET-756, and one FGFR, EGL-15, have been described [5-6; for review 7]. An EGL-17 mutant has a defect in migration of sex muscles, and consequently in vulva function and egg laying [8, 9]. LET-756 is essential for worm development [10]. It regulates axonal growth [11], fluid homeostasis [12], muscle protein degradation [13] and muscle membrane extension [14].

In *C. elegans*, three different pathways determine body length: a TGFβ pathway, involving *dbl-1, sma-2, sma-3, sma-4, lon-1* and *lon-2* genes, a spectrin pathway involving *sma-1, spc-1* and *unc-70* and a calcineurin pathway involving sensory neurons and muscle cells expressing *tax-6* and

cnb-1 [15-19]. An underlying ectodermal cell layer called the hypodermis makes cuticle, an exoskeleton of collagenous extracellular matrix (ECM). The hypodermis surrounds the body of the animal [reviewed in 20]. Proteins implicated in body length act on or are expressed in hypodermal cells. Thus, the hypodermis is the primary site where growth is regulated.

LET-756/FGF partial loss-of-function mutants are of the same size as wild-type (wt) animals at hatching, but grow more slowly during larval stages and are about half the length of, and are thinner than wt worms in adulthood [10]. Reduction in body size could be due to reduced cell number, reduced cell size or fluid loss. Because no difference in the number of nuclei in wt and *let-756 (s2613)* mutants has been found [10], the small body size phenotype could be due to a reduction in size of some or all cells of the animal or to body fluid loss.

To document the effect of *let-756* on body size we performed rescue experiments of both the partial loss-of-function allele (*s2613*) and the null allele (*s2887*), followed by a morphometric analysis of the body size of the rescued animals. To determine which site of expression was important for rescue, *let-756* was expressed in various areas of the body.

MATERIALS AND METHODOLOGY

Nematode Culture and Transformation Rescue Experiments

C. elegans Bristol (N2) strain nematodes were cultured using standard techniques [21]. *let-756* rescuing activity was assayed by injecting tester DNA at 50 ng per μl into gravid *s2887* strain hermaphrodites, establishing transformed lines and scoring the Dpy progeny for a viable phenotype. The *s2887* allele used in these experiments was generated on a *dpy-17(e164) unc-32(e189)* chromosome after UV irradiation [22]; the *sDp3*, a portion of chromosome III, balances the lethal phenotype of the *s2887* strain. The rescue activity was tested for the presence in the progeny of live Dpy ani-

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mals. Dpy animals were genotyped for the presence of the inversion in the *let-756* locus characteristic of the *s2887* strain by PCR on single worm, as described in [23]. The strains *dpy-17(e164) unc-32 (e189)* and *unc-32 (e189)* used as control, were obtained from the Caenorhabditis Genetics Center (CGC).

The hypomorph FF628 *let-756(s2613) unc-32(e189)* strain was isolated by D. Thierry-Mieg from BC4253 and outcrossed an additional 7 times. Homozygous animals are viable and fertile, but slow growing, transparent and small. These animals were coinjected with P*let-756*::*let-756* and the pPD93.97 plasmid which contains P*myo3*::*GFP*. Fluorescent cells contain arrays bearing both plasmids and mosaic animals were analyzed.

Fusion of Tissue Specific Promoters and *let-756::gfp* Coding Region

The promoters used in muscle-specific expression of *let-756::gfp* were *myo-2* (pharyngeal muscles), *myo-3* (body muscles) and *egl-17* (sex muscles) [24, 8]. The constructs driving the coding region of *let-756::gfp* and *let-756^R318^::gfp* under the control of P*let-756* promoter have already been reported [25]. The P*myo-3*::*let-756::gfp* expression construct was made by ligating the PCR product containing the *let-756* cDNA in the *Xba*I-*Bam*HI digested pPD93.97. The *Xba*I-*Eco*RI fragment containing the *let-756::gfp* fusion was then excised and inserted in the *Nhe*I-*Bgl*II digested pPD96.48 vector to obtain P*myo-2*::*let-756::gfp* expression construct. The full length *let-756* genomic DNA under the control of *egl-17* promoter (NH#469) was a gift from M. Stern (Yale, USA).

Microscopy and Statistical Analysis

The method used for immunofluorescence experiment was essentially as described by Ruvkun and Giusto [26]. Animals were fixed in a mixture of 1% paraformaldehyde, 20% methanol, permeabilized in 1% triton. Fixed animals were then incubated overnight with a 1:400 dilution of anti-GFP antiserum (Roche), washed extensively and then incubated for another two hours in a 1:500 dilution of the anti-mouse antibody conjugated to Texas Red (Molecular Probes). After extensive washing, the animals were mounted in DAPI-containing glycerol solution. In some experiments, DiI (Molecular Probes) dye filling was performed on living preparations of worms as previously described [27] to identify chemosensory neurons [28].

To analyze phenotypes, worms were collected on agar pads, anesthetized with levamisol and observed on a Zeiss LSM 510 laser scanning microscope equipped with Normasky optics. The number of *s2887* transformed strains analyzed was: 2 for the P*let-756*::*let-756::gfp* construct, 3 for P*let-756*:: *let-756^R318^::gfp*, 3 for P*myo-3*::*let-756::gfp*, 4 for P*myo-2*::*let-756::gfp* and 2 for P*egl-17*::*let-756*. Three *s2613* strains transformed by P*let-756*::*let-756::gfp* were analyzed. In the wild type context, 3 5 [rol] strains were compared to 2 strains transformed with P*let-756*::*let-756::gfp* and two strains transformed with P*let-756*:: *let-756^R318^::gfp*.

To assess body size in transgenic animals, cultures were synchronized by bleaching gravid animals. Eggs were allowed to grow for 90 hours in the adult stage. Selected plates containing adult rescued [dpy] animals were washed off in M9 medium and fixed in a mixture of 1% paraformaldehyde, 20% methanol. Body sizes (length and width at the vulva) were measured on 100 individual animals using LSM 510 software. Non-parametric Mann-Whitney u test was used. Differences were considered as significant when p values were < 0.01.

RESULTS

LET-756 Expression

To determine where *let-756* is required to insure worm viability, we examined the cells that express a GFP reporter from ~ 4 kb of promoter and enhancer sequences of *let-756* (P*let-756*::*gfp)*. Animals carrying this transgene express GFP in many cells throughout development and in the adult, as reported by us and others [7, 11, 14].

The major site of *let-756* expression was found in descendant cells of mesodermic precursors (muscle cells). However, some cephalic neurons, CAN cells, and the two glandular cells G1 and G2 (Fig. **1A**) were also positive.

Because LET-756 is secreted [23], we expected to find the FGF at some distance from its site of synthesis. We compared the sites of expression to those of LET-756 action as revealed by the localization of a LET-756::GFP fusion. We used a construct where identical *let-756* upstream sequence drives the LET-756::GFP fusion protein. We found accumulation of LET-756::GFP mainly in body wall muscle cells and incidentally in vulva, sphincter and pharyngeal muscle cells. However, under microscope observation only few animals were positive for LET-756::GFP despite the fact that they were perfectly rescued; in addition, the fluorescence was often lost with time in rescued strains although the rescuing ability was not.

To correlate protein level expression with rescue capacity we performed immunofluorescence staining of the rescued animals with anti-GFP antibody. Immunodetection of GFP is more sensitive than the observation of its direct fluorescence when exposed to UV light and thus more suitable to detect small amounts of protein. Immunostaining was seen in all rescued individuals, even in the absence of the GFP direct fluorescence, indicating that only animals containing a small number of arrays were able to survive and that these arrays did not contain enough copies of GFP to allow visualization by direct fluorescence.

After GFP immunostaining, animals displayed accumulation of fluorescence in a punctuate pattern. It was not possible to discriminate between a localization of LET-756 at the cell membrane on the cytoplasm side or, more likely, extracellularly on the basement membrane of body wall muscle cells (Fig. **1B,C**) and around the pharynx (Fig. **1D**), in regions where collagen [29] and UNC-52/perlecan [30] accumulate.

The fusion was observed in the nucleus and not in the nucleolus of CAN cells (Fig. **1E**), in agreement with the results obtained with Cos-1 transfected mammalian cells [25]. A construct that mimicked the partial loss-of-function allele, which we have previously denominated LET-756^R318^, fused to GFP and under the control of the same upstream sequence, was expressed in muscle cells. Surprisingly, it was also expressed in some neurons exhibiting peculiar endings unable to be filled by the DiI lipophilic dye (Fig. **1F**), which
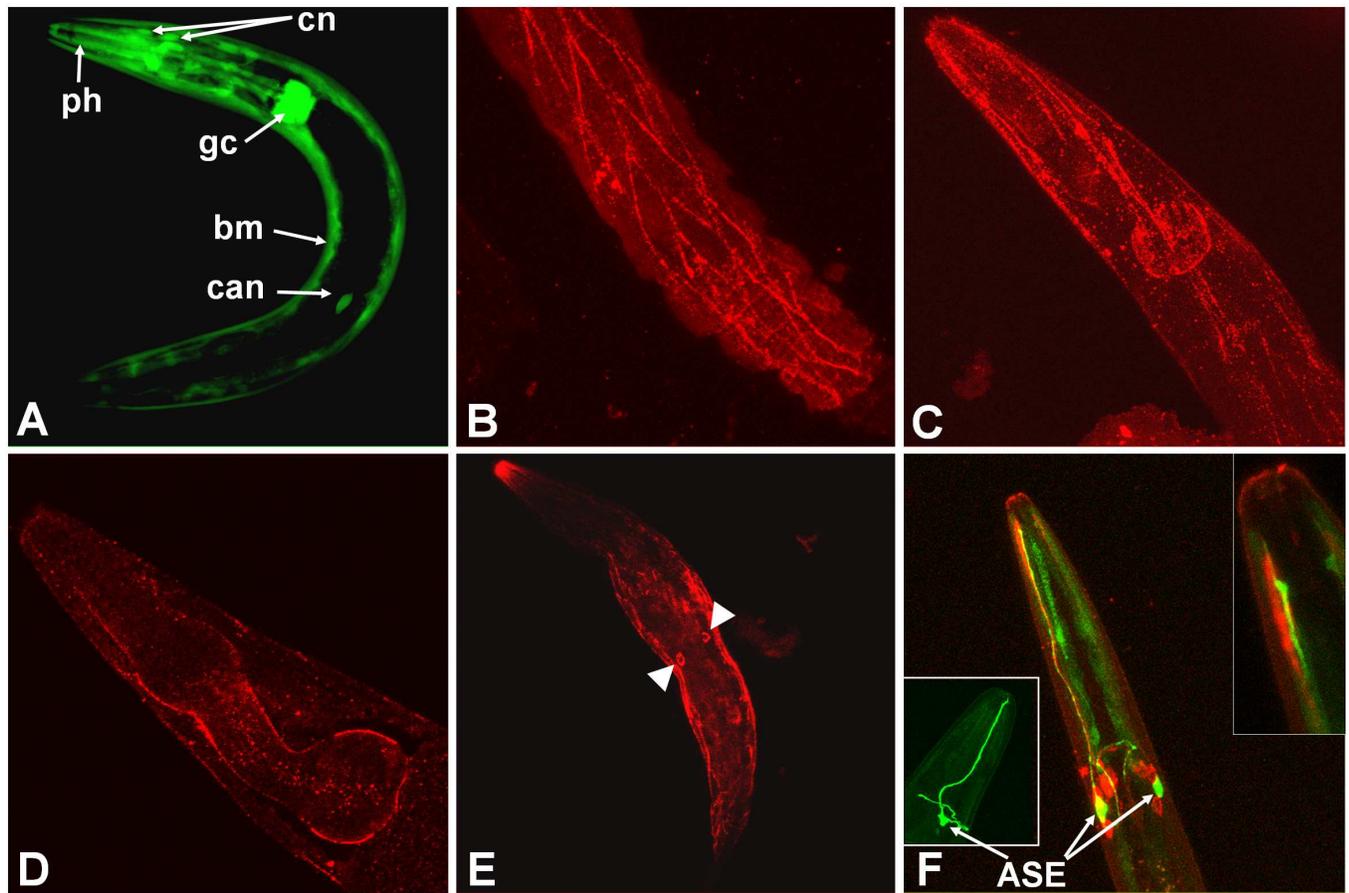
Fig. (1). LET-756 expression in wild type and *let-756^R318* transformed worms. (**A**) In this mosaic worm, GFP driven from the *let-756* promoter is expressed in body muscles (**bm**), pharynx procorpus (**ph**), cephalic neurons (**cn**), CAN cells (**can**), and G1 and G2 gland cells (**gc**). (**B – E**) worms were transformed by $P_{let-756}::let-756::gfp$, fixed and immunostained with anti-GFP antiserum. Accumulation of fluorescence is observed in a punctuate pattern at the junctions between body muscle cells (**C**) and around the pharynx (**D**). CAN cells are stained (**E**). (**F**) worms were transformed by $P_{let-756}::let-756^{R318}::gfp$; the construct was mainly expressed in neurons, which were identified as the ASE chemosensorial neurons since they do not fill with the lipophilic DiI dye and exhibit typical endings (magnified in the right insert).

we tentatively identified as the chemosensorial ASE neurons. We never observed this type of fluorescent cells when using the *let-756* promoter driving GFP. One possibility is that this staining reflects the specific translocation of the mutated protein in this cell type, but we do not have any definite explanation for this observation. Unlike cells with wt FGF, the nucleolus was also stained in these cells.

Lethality is Rescued by Tissue-Specific Expression of LET-756

let-756 is expressed mainly in various muscle cells. To determine which of these locations are essential for viability, we created transgenic animals expressing *let-756* in various muscles. Constructs were made with tissue-specific promoters and the *let-756::gfp* C-terminal fusion. For body wall muscle expression we used $P_{myo-3}::let-756$ [24] and for pharyngeal expression, we used $P_{myo-2}::let-756$ [24]. For sex myoblast expression, we used $P_{egl-17}::let-756$ [9], which drives expression specifically in primary fate vulval precursors and their descendants. These constructs were introduced in animals carrying the *let-756(s2887)* null allele [*dpy-17(e164) let-756(s2887) unc-32(e189)*] and animals were examined for both rescue activity and phenotype. In addition, we compared the rescuing activity of the wt LET-756

molecule with its truncated form LET-756^R318, which mimics the partial loss-of-function allele (*s2613*).

All types of tested ectopic muscle expression rescued the lethal phenotype: 7, 6 and 3 strains were obtained using *myo-3*, *myo-2* and *egl-17* promoters respectively, indicating that muscle expression is sufficient to insure viability.

Worm Body Growth Depends on Spatial Expression of LET-756

Variations in body length and proportions were observed in the various rescued animals described above. To identify the action of spatially-restricted gene expression on body length and proportions we analyzed transgenic animals with wt *let-756* expression driven by both endogenous and heterologous promoters in the null allele (*s2887*) background. Three types of spatial expression patterns were studied: two spatially-restricted expression patterns (anterior and central regions of the worm body, corresponding to the expression of the transgene under the control of the heterologous promoters P_{myo-2} and P_{egl-17}, respectively) [24, 9] and a widespread expression pattern corresponding to the expression of the transgene under the control of the endogenous and P_{myo-3} promoters [24].

Table 1.   **Morphometric Analysis of the *let-756* Rescued *s2887* Allele**

Genotype	Transgene	Expression Sites	Number of Rescued Strains	Length (μm)	Anterior (μm)	Width (μm)	Length Width	Anterior Length	Number of Analysed Individuals
dpy-17(e164) unc-32(e189)	-	-	-	776 ± 17	408 ± 11	82 ± 2	9.46	0.53	96
dpy-17(e164) let-756(s2887) unc-32(e189)	$P_{let-756}$::*let-756*	muscles, neurons	> 20	1029 ± 27	603 ± 15	105 ± 3	9.80	0.59	93
dpy-17(e164) let-756(s2887) unc-32(e189)	$P_{let-756}$::*let756^{R318}*	muscles, neurons	5	822 ± 18	425 ± 11	79 ± 2	10.40	0.52	97
dpy-17(e164) let-756(s2887) unc-32(e189)	P_{myo-2}::*let-756*	pharynx	6	745 ± 19	520 ± 16	98 ± 3	7.60	0.70	140
dpy-17(e164) let-756(s2887) unc-32(e189)	P_{myo-3}::*let-756*	body muscles	7	920 ± 21	529 ± 16	93 ± 3	9.89	0.58	126
dpy-17(e164) let-756(s2887) unc-32(e189)	P_{egl-17}::*let-756*	sex myoblasts	3	898 ± 36	477 ± 18	109 ± 7	8.24	0.53	71

Data are presented by mean ± SE. Body lengths and widths were determined in adult hermaphrodites at 4 days after hatching.

Body sizes of animals were measured - without assessing the size of individual cells - in these different conditions. Individual total body length, mouth-to-vulva length, and width at the vulva are shown in Table **1**.

Expression of wt *let-756* from the *myo-3* promoter produced animals with the same characteristics as those rescued by using the *let-756* promoter (Fig. **2B**,**E**). MYO-3 is expressed in body wall muscles, which are distributed all along the body from head to tail. Therefore, LET-756 expression in body wall muscles is sufficient for full rescue of the lethal phenotype and insures the same body length development as expression driven by the proper promoter. The respective proportions of anterior and posterior body parts in strains rescued with $P_{let-756}$ and P_{myo-3} promoters being similar (Fig. **3**), the contribution of pharyngeal LET-756 expression seems to be minor.

Expression of LET-756 from the P_{myo-2} promoter, which drives expression exclusively in the pharynx musculature, led to shorter animals than the one described above. In addition, they exhibited a misplaced vulva due to a better development of the anterior (defined as the length from mouth to vulva) than the posterior part of the body (Fig. **2D**). These animals exhibited a ratio "anterior length over total" larger than control individuals or individuals with LET-756 or LET-756^{R318} expression driven by $P_{let-756}$ and P_{myo-3} (Table **1**).

Expression of LET-756 from the *egl-17* promoter led to animals with larger width size than control animals (Fig. **2F**). EGL-17 is expressed in sex muscles [9]. It is thus coherent that LET-756 expression driven by the *egl-17* promoter in the middle of the animal increased locally the size of the animals. In addition, the posterior length was also increased compared to control strain and animals rescued with the *let-756^{R318}* transgene. Sex muscles arise from a pair of bilaterally symmetrical sex myoblasts; these cells are born in the posterior body. During larval development of the transgenic animals, enough LET-756 vehiculated by migrat-

ing myoblasts may allow the growth of the posterior part of the animals.

Reduced Body Size Results from *let-756^{R318}* Transgene Expression

We have previously reported that no major morphological and/or behavior differences was observed between lines rescued with $P_{let-756}$::*let-756* and $P_{let-756}$::*let-756^{R318}* constructs [25], which mimic wt and partial-loss-of-function alleles, respectively. However, in light of our results on body size we analyzed the length and width of these transgenic animals.

As compared to [*dpy-17(e164) unc-32(e189)*] animals, which have only two copies of the wt *let-756* gene, animals from the *s2887* strain (null allele) rescued by *let-756* expressed under the control of its own promoter had enlarged body length mostly due to a better development of the anterior part of the animal (Table **1**, Fig. **2A**,**B**). In contrast, the body length of animals rescued by *let-756^{R318}* was smaller than the length of those rescued by the wt molecule, and was in the range of the body length of the control (*dpy-17 (e164) unc-32(e189)*) animals (Figs. **2C**,**3**). As already reported [29], we found a four-fold increase in the number of transgene copies in *let-756^{R318}* strains compared to strains rescued with the wt *let-756*. This increase in array copy number could be explained by different activities of the proteins encoded by transgenes on the tyrosine kinase FGFR/EGL-15, since both FGFs are secreted (23) or by the different subcellular localizations of the two molecules in the expressing cells [25].

Thus, in all circumstances, LET-756 increased the animal body size in areas where it was expressed and body muscle cell expression was sufficient for ensuring normal body length. Quantification indicated that, as compared to [*dpy-17(e164) unc-3(e189)*] animals, the total length of all transgenic animals (except for P_{myo-2}::*let-756*) was significantly increased (p < 0.01) in a hierarchy $P_{let-756}$::*let-756* > P_{myo-3}::*let-*
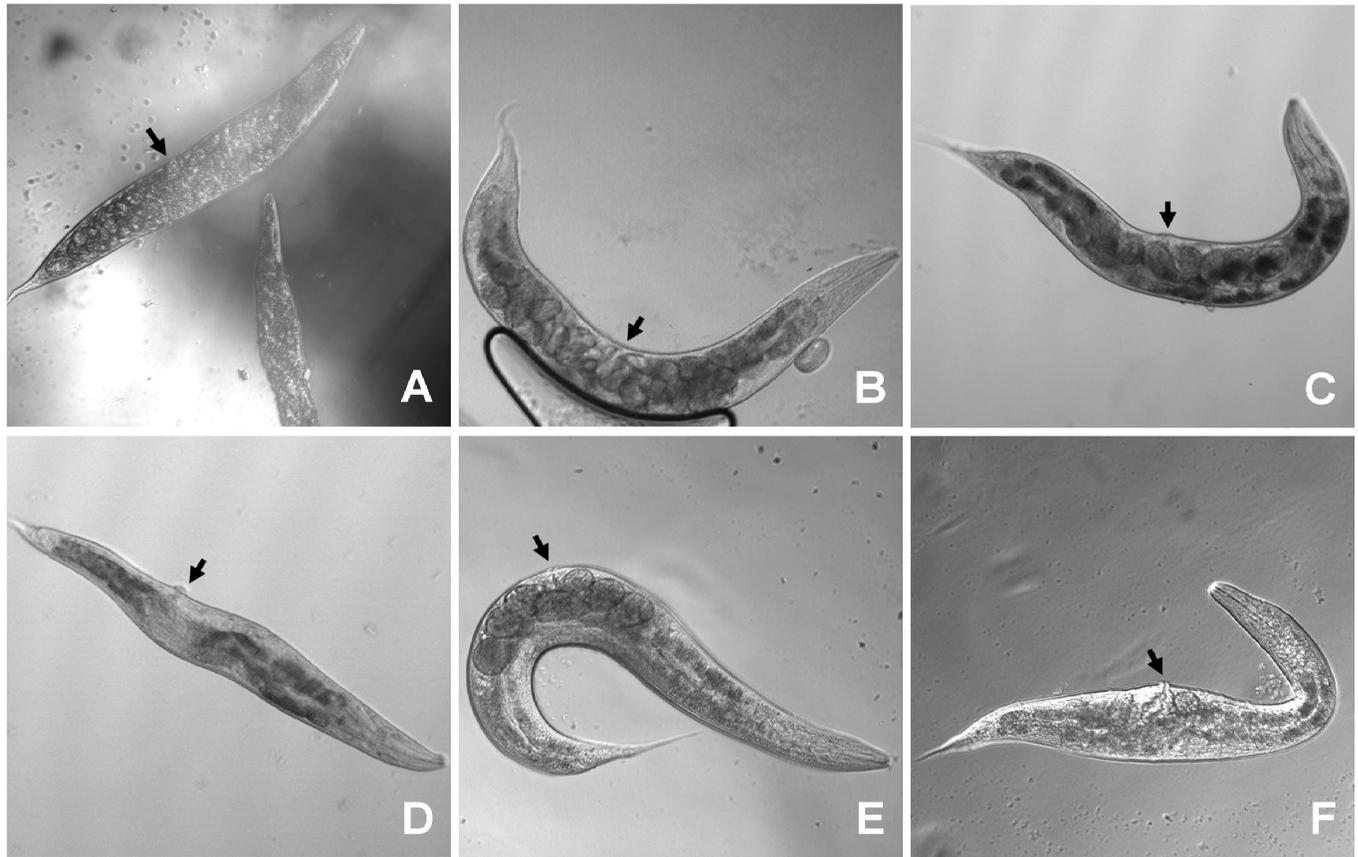
Fig. (2). Worms transformed with *let-756* under the control of tissue specific promoters were phenotypically different. Worms transformed by *let-756* (**B**) or *let-756^{R318}* (**C**) under the control of $P_{let-756}$ promoter are not different from the control [Dpy Unc] strain (**A**) although the former are bigger (see Fig. (**3**) for morphometric analysis). Worms transformed with *let-756* under the control of the P_{myo-2} promoter show reduction in the size of the posterior part of the body (**D**). Animals with *let-756* under the control of P_{myo-3} develop harmoniously (**E**). Animals with *let-756* under the P_{egl-17} promoter show enlargement of the body width at the vulva (**F**). Arrows point to the vulva.

$756 > P_{let-756}::let756^{R318} = P_{egl-17}::let-756 >$ no transgene $> P_{myo-2}::let-756$. In contrast, the anterior part of animals transformed with $P_{myo-2}::let-756$ was significantly ($p < 10^{-5}$) larger than untransformed worms or worms transformed with $P_{egl-17}::let-756$ or $P_{let-756}::let756^{R318}$ transgene. It was not different from worms transformed with $P_{myo-3}::let-756$ and smaller than those transformed with $P_{let-756}::let-756$. The posterior part of $P_{myo-2}::let-756$ was shorter than all the other transgenic or wild-type strains ($p < 10^{-7}$). Finally, the width of $P_{myo-2}::let-756$ and $P_{egl-17}::let-756$ transgenic strains was increased ($p < 10^{-4}$) as compared to all other animals (Fig. **3**).

let-756 Effect on Body Length Does Not Depend on Genetic Background

Some genes coding for cuticle collagens modify worm body length [20]. We were thus concerned by a possible effect of *dpy-17* (20), which was used as a balancer in the establishment of homozygote strain for *let-756* loss-of-function allele, and *rol-6* [31], a largely used coinjection marker of transformation, both encoding cuticle collagens.

Due to early lethality of the *let-756* loss-of-function allele, we could not compare the size of rescued *vs* unrescued animals. We thus compared the ratio between body length and width in the various rescued strains, as a hallmark of the

Dpy phenotype. Dpy mutants are defined as small, fatty worms and thus display a larger width to length size ratio than wt animals. The body dimensions were increased in the rescued strains expressing the wt *let-756* transgene under the control of the $P_{let-756}$ or P_{myo-3} compared to the *dpy-1((e164) unc-3(e189)* reference strain, but, importantly, the width to length size ratio was not modified (Table **1**). Thus, overexpression of *let-756* cannot suppress the Dpy phenotype of the *dpy-17* mutant allele.

To eliminate unambiguously any effect of the *dpy-17* background on the LET-756-induced body length, we compared the consequence of *let-756* transgene expression under the control of its own promoter in the [*let-756(s2613) unc-32(e189)*] hypomorph as well in wt N2 animals. In the hypomorph, the *myo-3* marker was used as an indicator of which area of the body was transformed, and in wt N2 animals, the *rol-6* marker was used to identify transformed animals. Expression of LET-756 increased both length and width in the [*let-756(s2613) unc-32(e189)*] hypomorph in which the *dpy-17* mutation had being outcrossed (Table **2**). As compared to the hypomorph (Fig. **4A**), the rescued animals appeared larger and developed a normal number of eggs (Fig. **4B**). They did not look different from the *unc-32 (e189)* strain (Fig. **4C**), which contains two wt *let-756* al-
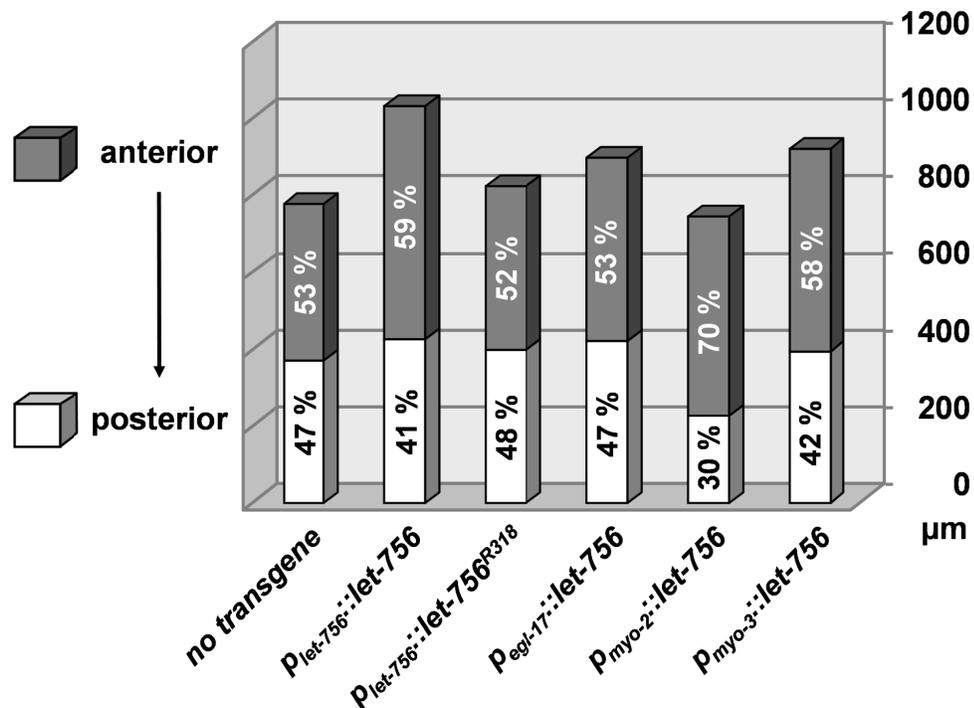
Fig. (3). Morphometric analysis. Anterior (shaded area) and posterior (unshaded) sizes of the different strains are represented (in μm) as well as their relative contribution in the total body length.

leles. However, in mosaic animals where LET-756 was expressed only in the anterior part of the animal, the posterior part looked scrawny and thinner than normal (Fig. **4D**). Reciprocally, animals where FGF expression occurred only in the posterior part of the animals exhibited a thinner anterior part (Fig. **4E**,**F**). Thus, the same phenotypes as those described in the worms carrying the loss-of-function allele *s2887* were observed in the animals carrying the partial loss-of-function allele *s2613*, showing that spatial expression of LET-756 increases body size independently of the genetic background.

Finally, we examined the effect of *let-756* overexpression in a wt context. We found that N2 animals injected with with $P_{let-756}::let-756$ or $P_{let-756}::let756^{R318}$ were not different in size (Table **3**) than those injected with $P_{let-756}::gfp$ although smaller than untransformed worms, due to the presence of *rol-6* (*su1006*) allele, the transformation marker gene. Thus, overexpression of LET-756 or LET-756^{R318} in a wt context did not result in an overgrowth phenotype. A threshold ligand amount expressed by endogenous alleles of the N2 worms and sufficient to saturate the EGL-15 receptors may explain size conservation.

DISCUSSION

Body size control is an oligogenic/polygenic character that depends on both nutrition-dependent and independent pathways. Studies in *Drosophila melanogaster* and *Caenorhabditis elegans* highlighted the insulin pathways as the principal player in controling nutrition-dependent growth rates [32 and 33 for reviews]. In the nematode worm, three nutrition-independent pathways (a TGFβ pathway, a spectrin pathway and a calcineurin pathway) determine body length by acting on the hypodermis, the primary site of growth regulation. The size of hypodermal cells and the composition of cuticle, the exoskeleton surrounding the body of the worm synthesized by hypodermal cells, are the main factors affecting body size.

In this study, we demonstrate that the FGF pathway is implicated in the control of the worm body size. Although *let-756* loss-of-function allele (*s2887)* is lethal, individuals carrying the partial loss-of-function allele (*s2613)* grow very slowly into small transparent, starved-looking adults. Explanations could be defects in cells that make cuticle or dysfunction of the pharyngeal musculature.

Table 2.   Morphometric Analysis of the *let-756* Rescued *s2613* Allele

Genotype	Transgene*	Number of Rescued Strains	Length (μm)	Anterior (μm)	Width (μm)	Number of Analysed Individuals
unc-32(e189)	-	-	1074 ± 29	571 ± 18	71 ± 2	37
let-756(s2613) unc-32(e189)	-	-	790 ± 11	395 ± 7	52 ± 1	52
let-756(s2613) unc-32(e189)	$P_{let-756}::let-756$	7	1080 ± 11	562 ± 6	78 ± 1	78

*The *myo-3::GFP* construct was coinjected as a marker.
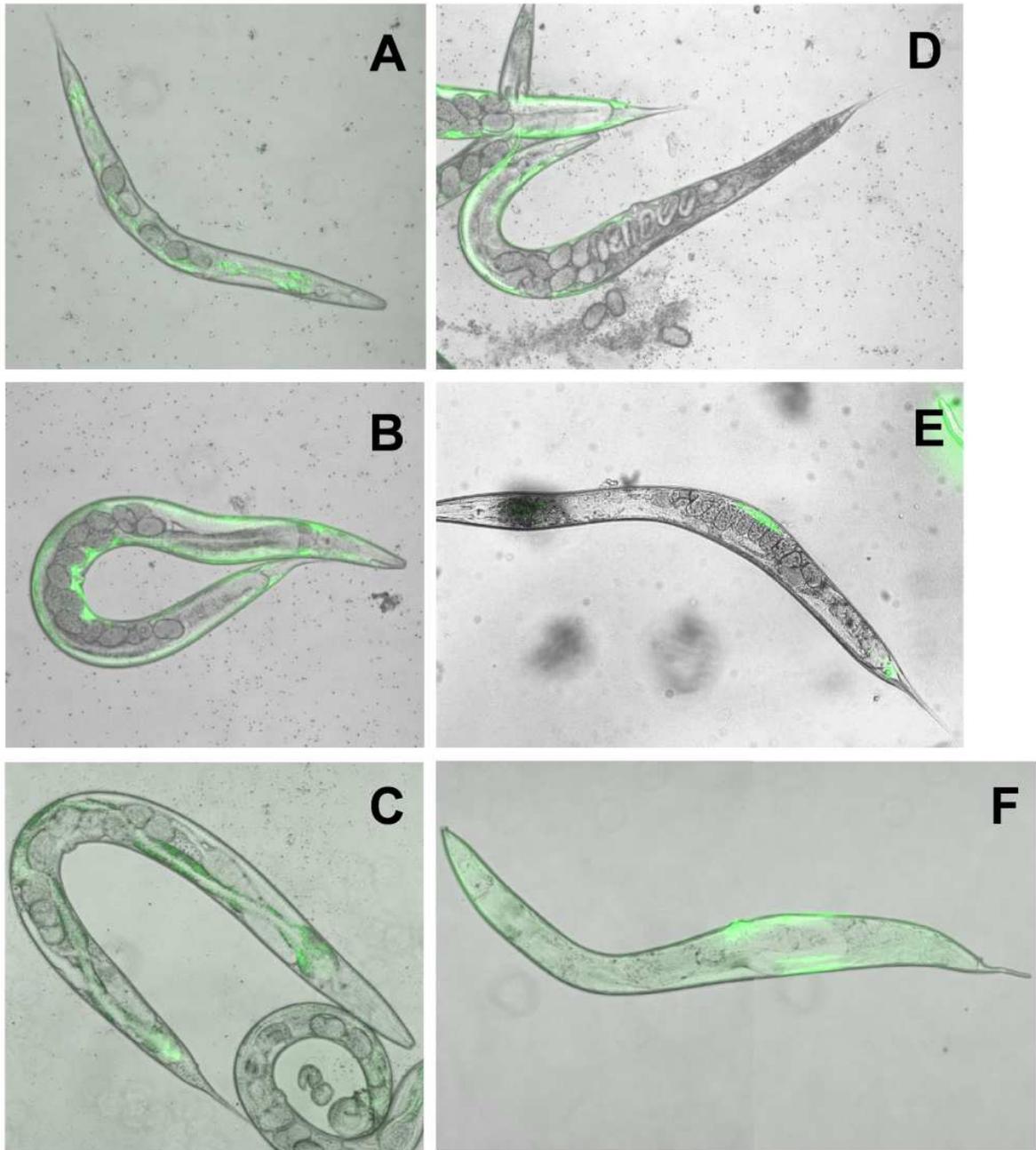
Fig. (4). Rescue of the hypomorph by *let-756* expressed under the control of its own promoter. *let-756(s2613) unc-32(e189)* worms (**A**) were transformed by the $P_{let-756}::let-756$ construct. (**B**) shows the phenotype of animals rescued by *let-756* expressed in all body muscles. These animals resemble the *unc-32* strain (**C**). Animals shown in (**D**) exhibit LET-756 expression in anterior muscles: development of the posterior part of the animal is impaired and reciprocally, animals shown in (**E**) and (**F**) exhibit LET-756 expression in posterior muscles and their anterior part is thinner than normal. The autofluorescence of the gut granules is visible in (**A**) and (**C**).

To determine the focus of lethality and to understand the defect in body size we analyzed which *let-756*-expressing cell was responsible for correct larval development. We focused on the role of different *let-756* expressing muscle cells in allowing post-embryonic development rescue and found that expressing *let-756* under the control of the *myo-3* promoter induced the same harmonious development of the animal as expressing the endogenous protein under the control of the proper promoter. Body length is controled by hypodermal cells, which make cuticle. Hypodermal cells express EGL-15, the FGF receptor. Most probably, LET-756, through paracrine expression in muscle cells, acts on the hypodermal cells, triggers EGL-15 signaling, which regulates fluid balance [12] and cuticle formation. Thus, muscle expression of LET-756 is sufficient to obtain a well developed worm and may be essential for proper development of the nematode *C. elegans*. It is worth noting that the promoter region of *let-756* contains a muscle-specific responsive element also present in the *myo-3* but not *myo-2* promoter [34].

Table 3.   Morphometric Analysis of the *let-756* Surexpression in Wild-Type Animals

Genotype	Transgene*	Number of Strains	Length (µm)	Number of Analysed Individuals
N2	-	5	1330 ± 15	42
N2	$P_{let-756}::GFP$	6	1157 ± 21	87
N2	$P_{let-756}::let-756$	11	1233 ± 13	47
N2	$P_{let-756}::let756^{R318}$	11	1243 ± 18	66

*The pRF4 construct containing the *rol-6* (*su1006*) was coinjected as marker.

Another explanation for the impaired growth of the *let-756* mutant could be a defect in the pharyngeal musculature, impairing the pumping or grinding of bacteria - the nematode food source - or in pharyngeal gland cells. The function of the latter cells is unknown; their anatomy indicates that they are secretory cells, pouring their content in the lumen of the pharynx. Pharynx and gland cells are two sites were LET-756 is expressed. Driving LET-756 expression in the pharyngeal musculature from the *myo-2* promoter confers viability to the null allele but the rescue looked incomplete since only the anterior part of the animal developed correctly. Thus, we can exclude a pharyngeal cell-autonomous action of LET-756 responsible for an either mechanistic or digestive problem of feeding. Interestingly, body width was increased in worms transformed by $P_{myo-2}::let-756$. However, these animals appeared very often [Egl], indicating that the limited available amount of LET-756 at middle body (resulting from the pharynx muscle cells expression) could interfere with EGL-15 activation and not allow proper sex myoblast positioning and/or progeny formation (uterine and vulval muscles). Thus, LET-756 may constitute, alone or in cooperation with other diffusible ligands, the gonad-independent attraction signal for sex myoblast cells described previously [9].

Body length was increased when *let-756* was expressed under the control of the *egl-17* promoter. This was mainly due to a well-developed posterior part of the animal. EGL-17, the other *C. elegans* FGF, is expressed in the developing gonad and vulva and acts as the chemoattractant that guides the migration of the sex myoblasts. During sex muscles migration from the posterior body, enough LET-756 vehiculated by migrating myoblasts may allow the growth of the posterior part of the animal. In addition, body width was increased indicating again that LET-756 increases body mass wherever it is produced.

Thus, any spatial rescue results in a local growth of the animal leaving the other part of the animal with a scrawny phenotype. This may result from either abnormal cell growth or from worm lacking sufficient fluid as described by Huang and Stern [12], as observed in the null or partial loss-of-function allele. We suggest that overexpression of LET-756 from a small number of cells - and herein from any cells of mesodermal origin - might be sufficient to regulate fluid balance and trigger EGL-15 activity on hypodermal cells.

Our observations suggest that wherever LET-756 is expressed and acts on its receptor on hypodermal cells, post-embryonic development is insured. The extracellular matrix components are essential players in the regulation of local concentration of growth factors and morphogens. Experimental observations showed that glypicans, cell-anchored heparan sulfate proteoglycans, bind growth factors such as FGF in *Drosophila* [35] and TGFβ in *C. elegans* [19], modulating their local concentration and protecting them from proteolytic degradation. We have observed accumulation of LET-756 around the pharynx and in the extracellular space of body muscle cells. Collagens are critical for the assembly and function of the extracellular matrix. It is noteworthy that we found COL-129, the product of a structural collagen gene, interacting with LET-756 in yeast two-hybrid screens [36].

Dixon *et al*. [14] reported that expression of LET-756 in a single cell is sufficient to rescue the lethality of *let-756(s2887)* null animals and that the rescued animals have a pronounced bulge near the site of expression. Our results indicate that even if the animals are capable of surviving when only one cell type is expressing LET-756, normal body growth is limited to the area where LET-756 can diffuse.

In summary, our data show that misexpression of LET-756, as in the partial loss-of-function allele, reduces the size of the animal as compared to wild-type. Reciprocally, LET-756 overexpression in the hypomorph or the null allele increases body size. Expression in body muscles is sufficient to allow the development of the null *let-756* allele probably by acting on hypodermal cells through EGL-15 activation. Our results support a juxtacrine/paracrine activity for LET-756 exerting locally its effect rather than through a large diffusion through the pseudocoelum.

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