FGF and PI3 kinase signaling pathways antagonistically modulate sex muscle differentiation in *C. elegans*

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Accepted 31 August 2004

Development 131, 5381-5392
Published by The Company of Biologists 2004
doi:10.1242/dev.01423

Summary

Myogenesis in vertebrate myocytes is promoted by activation of the phosphatidyl-inositol 3′-kinase (PI3 kinase) pathway and inhibited by fibroblast growth factor (FGF) signaling. We show that hyperactivation of the *Caenorhabditis elegans* FGF receptor, EGL-15, similarly inhibits the differentiation of the hermaphrodite sex muscles. Activation of the PI3 kinase signaling pathway can partially suppress this differentiation defect, mimicking the antagonistic relationship between these two pathways known to influence vertebrate myogenesis. When ectopically expressed in body wall muscle precursor cells, hyperactivated EGL-15 can also interfere with the proper development of the body wall musculature. Hyperactivation of EGL-15 has also revealed additional effects on a number of fundamental processes within the postembryonic muscle lineage, such as cell division polarity. These studies provide important in vivo insights into the contribution of FGF signaling events to myogenesis.

Key words: FGF receptor, EGL-15, PI3 kinase, Signal transduction, Muscle differentiation, M lineage, Myoblast, SM, Sex muscles

Introduction

The development of functional muscles involves a series of distinct biological events, including fate specification, migration, proliferation and differentiation. Extensive analyses have demonstrated that these processes are highly regulated by multiple signaling pathways. In cultured myoblasts, stimulation of the fibroblast growth factor (FGF) receptor activates the MAP kinase cascade to negatively regulate muscle differentiation (Clegg et al., 1987; Gredinger et al., 1998; Spizz et al., 1986; Templeton and Hauschka, 1992; Vaidya et al., 1989). By contrast, activation of the insulin receptor stimulates the phosphatidyl-inositol 3′-kinase (PI3 kinase) signaling pathway to enhance myoblast differentiation (Kaliman et al., 1998; Kaliman et al., 1996; Tamir and Bengal, 2000). Additionally, biochemical analyses have shown that these receptors regulate myogenesis via a network of signaling molecules that include both the p38 and ERK MAP kinases, SHP-2, PKC, $G\alpha_l$, mTOR and FOXO transcription factors (Arden and Biggs, 2002; Cabane et al., 2003; Gredinger et al., 1998; Kontaridis et al., 2002; Li et al., 1992; Shu et al., 2002). The many roles of these signaling molecule necessitates in vivo analyses, where their interactions can be observed within a developmental context.

C. elegans is an excellent model organism in which to study the complex interplay of signaling pathways that regulate myogenesis. Post-embryonic muscles are derived from a single blast cell, the M mesoblast (Sulston and Horvitz, 1977). In hermaphrodites, M undergoes several rounds of cell division in the first larval stage to give rise to 14 body wall muscles (BWMs), two dorsally positioned coelomocytes and two sex myoblasts (SMs). The SMs, which are born in the posterior body region of the animal, migrate anteriorly during the second larval stage to flank the precise center of the developing gonad, the site of the future vulva and uterus. Midway through the third larval stage, the two SMs undergo three rounds of cell division to produce a total of 16 cells. During the fourth larval stage, these cells differentiate into the mature egg-laying muscles, alternatively known as the sex muscles. The sex muscles comprise two types of uterine muscles, um1s and um2s, and two types of vulval muscles, vm1s and vm2s. These cells assume highly reproducible morphologies and positions (Sulston and Horvitz, 1977), and they express molecular markers characteristic of differentiated muscle cells, including F-actin and other components of the contractile machinery, as well as other markers more specific to the sex muscles (Harfe et al., 1998a; Moerman and Fire, 1997).

FGF signaling plays an important role in the development of the hermaphrodite sex muscles by helping to guide the migrating SMs. The FGF-like ligand EGL-17 serves to attract the SMs to their precise final positions (Burdine et al., 1998). The receptor for this chemoattractant is encoded by *egl-15*, the sole FGF receptor gene in *C. elegans* (DeVore et al., 1995). *egl-15* is alternatively spliced to generate two isoforms termed EGL-15(5A) and EGL-15(5B). The 5A isoform is required for SM chemoattraction, while the 5B isoform is required to mediate an essential function of EGL-15 (Goodman et al., 2003). Mutations that perturb SM chemoattraction, either by eliminating the EGL-17 FGF or the EGL-15(5A) isoform, cause the SMs to be severely posteriorly displaced (Burdine et al., 1998; Goodman et al., 2003). Even when severely mispositioned, the SMs still proliferate normally and generate cells that appear to differentiate properly (I.E.S., unpublished).

Owing to their abnormal positions, however, these muscle cells are unable to attach and function properly, resulting in an egg-laying defective (Egl) phenotype.

Hyperactivating EGL-15 has provided an important approach for understanding the role of EGL-15 in *C. elegans* development and physiology (Kokel et al., 1998; Selfors et al., 1998). EGL-15 can be hyperactivated by mutational inactivation of *clr-1*, a gene encoding a receptor tyrosine phosphatase that negatively regulates EGL-15 activity (Kokel et al., 1998). *clr-1* mutants accumulate fluid within the pseudocoelomic cavity, resulting in a translucent or clear (Clr) appearance. A similar Clr phenotype results from directly hyperactivating EGL-15 via transgenic expression of the *egl-15(neu*)* construct (Kokel et al., 1998). This construct replaces the transmembrane domain of EGL-15 with that of oncogenic Neu, presumably causing constitutive dimerization of EGL-15. A temperature-sensitive mutation in *clr-1* has been extremely useful in helping to define a canonical FGF signaling pathway in *C. elegans* (Kokel et al., 1998; Schutzman et al., 2001; Selfors et al., 1998) as well as other functions of this pathway (Szewczyk and Jacobson, 2003). The usefulness of transgenic *egl-15(neu*)*, however, has been limited, as the constitutive Clr phenotype that it confers prohibits the establishment of heritable transgenic lines. By suppressing the Clr phenotype conferred by transgenic *egl-15(neu*)*, we have been able to assess other effects of hyperactivated FGF signaling on *C. elegans* development. Using this approach, we have discovered that EGL-15 hyperactivation can block sex muscle differentiation. This effect is consistent with the ability of FGF to inhibit vertebrate myoblast differentiation and can be used to identify signaling components that influence myogenesis in vivo.

Materials and methods

Genetic manipulations

C. elegans strains were maintained according to standard genetic protocols as described by Brenner (Brenner, 1974). All work used strains derived from *C. elegans* var. Bristol, strain N2 (Brenner, 1974), and standard genetic manipulations (Herman, 1988) were used for all strain construction. The *soc-2(n1774rf)* mutant allele was used throughout this work (Selfors et al., 1998). The *soc-2/sur-8* gene encodes a putative scaffolding protein that functions as a positive regulator of RAS signaling (Li et al., 2000; Selfors et al., 1998; Sieburth et al., 1998). Use of a *soc-2* mutant provides maximal suppression of the Clr phenotype but ensures that certain aspects of EGL-15 signaling remain intact: (1) use of a hypomorphic mutation in *soc-2* ensures that some amount of SOC-2/SUR-8 signaling remains; (2) even in the complete absence of SOC-2/SUR-8, significant RAS activity remains (J. Schutzman and M.J.S., unpublished); and (3) components of the activated receptor signaling complex that act upstream of SOC-2/SUR-8 can remain fully activated. The *hlh-1(cc450)* mutation was used as a null allele of the *hlh-1* gene (Chen et al., 1992).

Constructs and germline transformation

Transgenic arrays were generated using standard germline transformation techniques (Mello et al., 1991). Plasmids generated using PCR were confirmed by sequence analysis. Integrated strains were backcrossed at least four times prior to analysis.

Control strains

ccIs4251 is an integrated array of the pSAK2 plasmid (Fire et al., 1998), which expresses GFP under the control of the $P_{myo\text{-}3}$ myosin heavy chain promoter. *ccIs4251* is used as a control integrated array for the pSAK2 germline transformation marker. *ayIs29* was isolated as a spontaneous integrant of an array carrying the *egl-15* genomic rescuing fragment NH#112 (5 ng/μl) (DeVore et al., 1995), the $P_{myo\text{-}2}$*::GFP* transformation marker pJKL449.1 (5 ng/μl) and pGEM-5Z (70 ng/μl).

egl-15(neu)*

Germline transformants of NH#420 (20 ng/μl) (Kokel et al., 1998) were isolated in a *soc-2* background using pSAK2 (5 ng/μl) as a co-transformation marker. Two independent genomic integrants, *ayIs15* and *ayIs16*, were isolated using a standard gamma irradiation protocol (1251.3 rads) (Mello and Fire, 1995). *egl-15(neu*)* animals display multiple pleiotropies. Some animals display a mild uncoordinated phenotype that is most prominent in the adult, and rare animals display a multi-vulva phenotype.

Isoform specific neu* constructs

The *egl-15(5B*)* construct (NH#1033) was generated by replacing the extracellular coding sequence of NH#420 with the corresponding sequence containing a nonsense mutation in exon 5A from NH#872 (Goodman et al., 2003). The *egl-15(5A*)* construct (NH#1034) was generated similarly, using NH#873 as a source of a nonsense mutation at the first codon of exon 5B (Goodman et al., 2003). Germline transformants of NH#1033 (20 ng/μl) were isolated in a *soc-2* background using pSAK2 (5 ng/μl) as the co-transformation marker. Germline transformants of NH#1034 (20 ng/μl) were isolated in an N2 background using pSAK2 (5 ng/μl) as the co-transformation marker and crossed into a *soc-2(n1774)* background. Two independent integrated NH#1033 arrays, *ayIs17* and *ayIs18*, were isolated using a standard gamma irradiation protocol (1247 rads) (Mello and Fire, 1995). Two independent integrated NH#1034 arrays, *ayIs21* and *ayIs27*, were generated using 50 μg/ml TMP and 350 uJ(×100) UV light (Scott Clark, personal communication).

The effect of extrachromosomal *egl-15(5A*)* arrays appears to differ somewhat from that of integrated arrays. Both types of arrays behave similarly with regard to their effects on the Clr phenotype, failing to confer a Clr phenotype in a *soc-2(+)* background. In contrast to the strongly penetrant Egl phenotype conferred by integrated *egl-15(5A*)* arrays, several extrachromosomal arrays expressing *egl-15(5A*)* display only a partially penetrant Egl phenotype. The behavior of the integrated *egl-15(5A*)* arrays appears to be bona fide, as multiple integrated arrays display the identical 100% penetrant Egl phenotype, including those isolated from different extrachromosomal arrays that were generated in independent experiments. The reduced penetrance of the Egl phenotype in animals bearing extrachromosomal arrays may result from the mosaicism of the extrachromosomal arrays as well as in part to eliminating the EGL-15(5B*) contribution.

Tissue-specific expression of egl-15(neu*)

$P_{hlh\text{-}1}$ (Krause et al., 1990), $P_{unc\text{-}54}$ (Okkema et al., 1993), $P_{unc\text{-}14}$ (Ogura et al., 1997) and $P_{aex\text{-}3}$ (Iwasaki et al., 1997) were used to express EGL-15(neu*) in a variety of tissue types. Construction of the $P_{unc\text{-}54}$ (NH#1181), $P_{unc\text{-}14}$ (NH#1094) and $P_{aex\text{-}3}$ (NH#1190) plasmids have been described by Huang and Stern (Huang and Stern, 2004). The $P_{hlh\text{-}1}$ constructs were generated by PCR amplification of genomic DNA and include the region from –2960 to –1 bp 5′ to the start ATG. The PCR product was cloned upstream of the *egl-15* genomic sequence (NH#150) to generate $P_{hlh\text{-}1}$*::egl-15* (NH#1201), upstream of *egl-15(neu*)* (NH#526) to generate $P_{hlh\text{-}1}$*::egl-15(neu*)* (NH#1198), and upstream of the kinase-defective *egl-15(neu*K672A)* (NH#527) to generate $P_{hlh\text{-}1}$*::egl-15(neu*K-A)* (NH#1199).

Germline transformants expressing each construct (20 ng/μl), except $P_{unc\text{-}14}$*::egl-15(neu*)*, were generated in an N2 background using pJKL449.1 (5 ng/μl) as a co-transformation marker. $P_{unc\text{-}14}$*::egl-*

15(neu)* expressed in an N2 background results in a Clr phenotype that precludes the isolation of stably transmitted lines. Therefore, $P_{unc\text{-}14}$*::egl-15(neu*)* lines were established in a *soc-2* background. Heritable lines expressing $P_{hlh\text{-}1}$*::egl-15(neu*)* were established by selecting GFP^+ mosaic animals that were normal in appearance. Transgenic animals that maintained the array in the germline segregated some progeny that display a severe lumpy-dumpy phenotype.

Serotonin response assay

The serotonin response assay followed standard procedures (Schafer and Kenyon, 1995), and was modified by culturing young adult animals on unseeded 2% agar plates with 5 mM 5-hydroxytryptamine creatinine sulfate monohydrate (ICN Biomedicals) for 1 hour. Animals were subsequently removed from serotonin plates, and the number of eggs laid/animal was scored. At least 12 animals were assayed per data point, and the mean and standard deviation were calculated.

Egg-laying index

Animals were synchronized by L1 arrest and then fed for 44 hours. At the late L4 Christmas tree stage, individual animals were cloned and allowed to lay eggs, which were counted as larvae 48 hours later (number of laid eggs). After 24 hours, gravid adults were then removed, lysed in 20% bleach and the eggs remaining in the uterus were counted (number of unlaid eggs). The egg-laying index= (number of laid eggs)/(number of laid + number of unlaid eggs).

Rhodamine-phalloidin staining

Animals were synchronized by L1 arrest followed by feeding for 60 hours. Young adults were then harvested by washing three times in M9 and fixed in 3.7% formaldehyde in 0.1M Na_2HPO_4 for 3 hours with gentle rocking. Animals are washed three times in PBS, dehydrated in ice-cold acetone for 2 minutes, and washed three times in PBS. Packed worms (30 μl) were then stained with rhodamine-conjugated phalloidin (Molecular Probes) at 1:50 dilution for 3 hours at 20°C in the dark. Worms were repeatedly washed for 30 minutes in PBS. Both $P_{myo\text{-}3}$*::GFP* expression and rhodamine-phalloidin staining can be used to monitor the differentiation, morphology and position of the vulval muscles (Moerman and Fire, 1997); contractile fibers in the uterine muscles appear more loosely organized and cannot be reproducibly monitored by either of these two methods.

Anti-EGL-15 staining

Young adults were harvested for each strain and stained following the standard protocol of Finney and Ruvkun (Finney and Ruvkun, 1990). Two affinity-purified anti-EGL-15 primary antibodies (Pop, Crackle) were used at a 1:10 dilution; Alexa Fluor 546-conjugated anti-rabbit antibody (Molecular Probes) was used as a secondary antibody at a 1:250 dilution. EGL-15 antibodies detect the sixteen undifferentiated cells of the SM lineage, the differentiated vm1s, several neurons in the head and the hypodermis during L1-L3 stages. A more detailed analysis will be described elsewhere.

Identification of M lineage defects

A combination of DIC microscopic examination and the integrated P_{twist}*::GFP* marker, *ayIs6*, was used to assess the development of the early M lineage, the sex myoblasts (SMs) and the dorsal coelomocytes (dccs). P_{twist}*::GFP* is expressed in all undifferentiated cells of the M lineage (Harfe et al., 1998b) and was used to score the positions of undifferentiated cells in the early M lineage (L1 stage). Coelomocytes were scored by DIC. The final positions of the SMs were scored as previously described (Thomas et al., 1990). SM distributions are represented as box-and-whisker plots (Moore and McCabe, 1993) as described by Goodman et al. (Goodman et al., 2003).

Photomicroscopy

All micrographs were collected using a Hamamatsu C4742-95 digital camera mounted on a Zeiss Axioskop microscope that contains an internal 0.63× reduction lens. Images were collected as a *z*-series of 0.5 μm sections, merged into a single composite image and false colored using OpenLab software. Images were then cropped and scaled using Adobe Photoshop.

Results

Hyperactivation of EGL-15 disrupts sex muscle development

To circumvent the Clr phenotype caused by *egl-15(neu*)*-containing transgenes in a wild-type background, transgenic arrays containing *egl-15(neu*)* were generated in a *soc-2(n1774)* background. Interestingly, animals obtained from four independent lines were dramatically Egl. To characterize this phenotype, two independent integrated arrays were generated from these extrachromosomal arrays and designated *ayIs15* and *ayIs16* [*egl-15(neu*); $P_{myo\text{-}3}$::GFP*]. Both lines continue to display the Egl phenotype (Fig. 1; Fig. 2A; Table 1). These arrays confer a dominant Clr phenotype when not suppressed by *soc-2* (Fig. 1; Table 1), indicating that the EGL-15(neu*) product expressed from these arrays remains constitutively active.

To identify the underlying cause of the egg-laying defect, *soc-2; ayIs15* animals were assessed for the functional and structural integrity of the components of the egg-laying system. Exogenous serotonin can be used to stimulate egg laying in worms that lack neurological input from the HSN motoneurons, provided they maintain a normal vulva and functional egg-laying muscles (Schafer and Kenyon, 1995). *soc-2; ayIs15* animals fail to lay eggs even in the presence of exogenous serotonin, indicating a defect downstream from the neurological input from the HSNs (data not shown). Examination of *soc-2; ayIs15* animals using Nomarski microscopy indicated that the vast majority of animals have normal vulval structure. Therefore, vulval morphology defects are unlikely to be the source of the highly penetrant Egl defect.

Abnormalities in the sex muscles could also account for a serotonin-resistant egg-laying defect. The distinctive architecture of the vulval and body wall muscles can be identified in wild-type animals using rhodamine-phalloidin, which binds to the highly organized filamentous actin cables of *C. elegans* muscles (Fig. 3A,B) (Moerman and Fire, 1997). These cell types can also be assessed based on their expression of the $P_{myo\text{-}3}$*::GFP* transformation marker. *myo-3* encodes one of two myosin heavy chain isoforms that are expressed in all non-pharyngeal muscles, including the body wall muscles and the vulval muscles (Ardizzi and Epstein, 1987; Dibb et al., 1989; Miller et al., 1983). In contrast to wild-type animals, no sex muscle staining is observed in *soc-2; ayIs15* animals (Fig. 3C; Table 2) despite a normal pattern of rhodamine-phalloidin staining for the body wall musculature. Consistent with this observation, *soc-2; ayIs15* animals express $P_{myo\text{-}3}$*::GFP* at normal levels in the body wall muscles, but lack the strong $P_{myo\text{-}3}$*::GFP* expression normally observed in the vulval muscles (Fig. 3C; Table 2). Thus, *soc-2; ayIs15* animals appear to be unable to lay eggs because of the lack of a normal set of sex muscles.

Isoform contribution to the *ayIs15* phenotype

The two EGL-15 isoforms contribute to distinct functions of

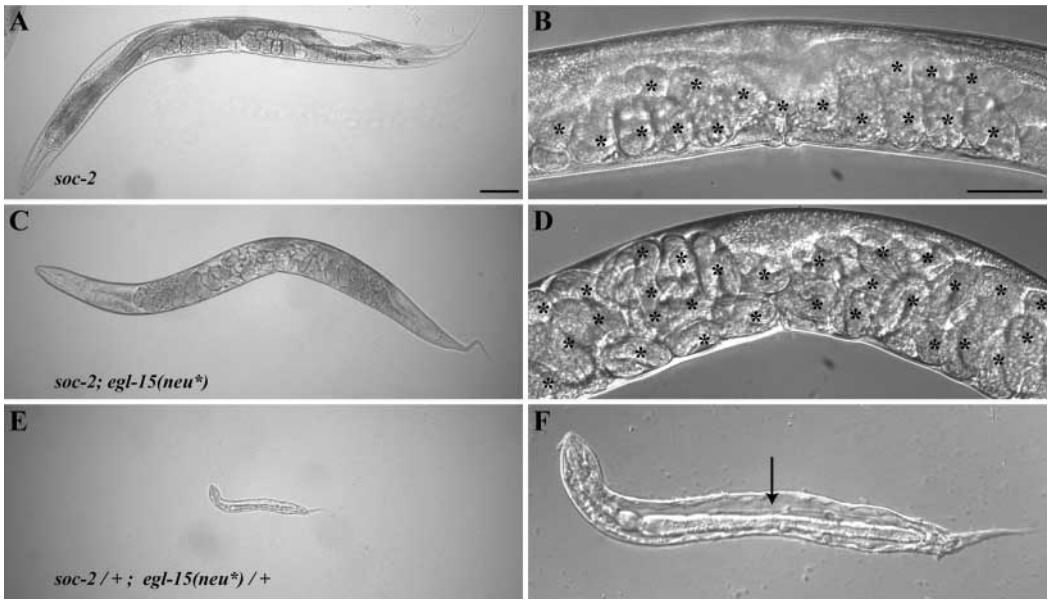


Fig. 1. Phenotypic effects of hyperactivated EGL-15. (A,B) *ccIs4251 [P_{myo-3}::GFP]; soc-2(n1774)* animals are egg-laying proficient, and animals display a normal number of embryos in the uterus. (C,D) *soc-2(n1774); ayIs15 [egl-15(neu*); P_{myo-3}::GFP]* animals display a severe Egl phenotype, with an increased number of eggs retained in the uterus. (E,F) *soc-2 + / + mIs11; ayIs15/+*. EGL-15 remains hyperactivated in these integrated lines as indicated by the dominant Clr phenotype revealed when the recessive *soc-2* suppressing mutation is heterozygous. *ccIs4251/+; soc-2 + / + mIs11* animals do not display a Clr phenotype (data not shown). *mIs11* [P_{myo-2}::*GFP*] was used to identify heterozygous cross progeny. All animals were photographed 30 hours after late L4. Scale bar: 100 μm in A,C,E; 50 μm in B,D,E. *, eggs. Arrow indicates clear fluid in the pseudocoelomic space.

EGL-15: for example, SM chemoattraction requires the EGL-15(5A) isoform, while the 5B isoform is necessary for viability (Goodman et al., 2003). To determine the contributions of the individual isoforms to the phenotypic consequences of EGL-15 hyperactivation, we generated constructs that eliminate expression of one of the two-hyperactivated isoforms. The 5A* construct expresses only hyperactivated EGL-15(5A), while the 5B* construct expresses only hyperactivated EGL-15(5B). We characterized extrachromosomal and integrated transgenic lines expressing the single isoforms and compared them with the lines expressing both hyperactivated EGL-15 isoforms (*ayIs15* and *ayIs16*).

Consistent with the normal functions of the isoforms, EGL-15(5B*) was found to be primarily responsible for the Clr phenotype of *egl-15(neu*)*, while EGL-15(5A*) showed a stronger effect on the egg-laying muscles. EGL-15(5B*) arrays confer a robust Clr phenotype in a *soc-2(+)* background, similar to that observed for intact *egl-15(neu*)* (Table 1). Similar effects were seen using either extrachromosomal or integrated 5B* arrays (*ayIs17* and *ayIs18*). When these arrays were expressed in a *soc-2* background, the Clr phenotype was suppressed, as in *ayIs15* (Table 1). These animals were also significantly Egl, although distinguishable from the *soc-2; ayIs15* Egl phenotype; *5B** animals retain some egg-laying capability (Fig. 2B) and have differentiated sex muscles, as determined by rhodamine-phalloidin staining and P_{myo-3}*::GFP* expression (Fig. 3D; Table 2).

By contrast, expression of EGL-15(5A*) alone can result in animals that completely lack differentiated sex muscles, but fail to develop a Clr phenotype. Wild-type animals expressing *egl-15(5A*)* from extrachromosomal arrays appear non-Clr, as do two independently isolated integrated lines (*ayIs21* and *ayIs27*; Table 1). Both integrated arrays confer a completely penetrant Egl phenotype identical to that of *soc-2; ayIs15* (Fig. 2B, Tables 1, 2). Similar to *soc-2; ayIs15* animals, *ayIs21* and *soc-2; ayIs27* animals fail to generate sex muscles that express P_{myo-3}*::GFP* or stain with rhodamine-phalloidin (Fig. 3E; data not shown). This constellation of phenotypes is consistent with the 5A isoform playing an important role specifically in the M lineage and with the ability of the 5A* isoform to interfere with the development of differentiated sex muscles.

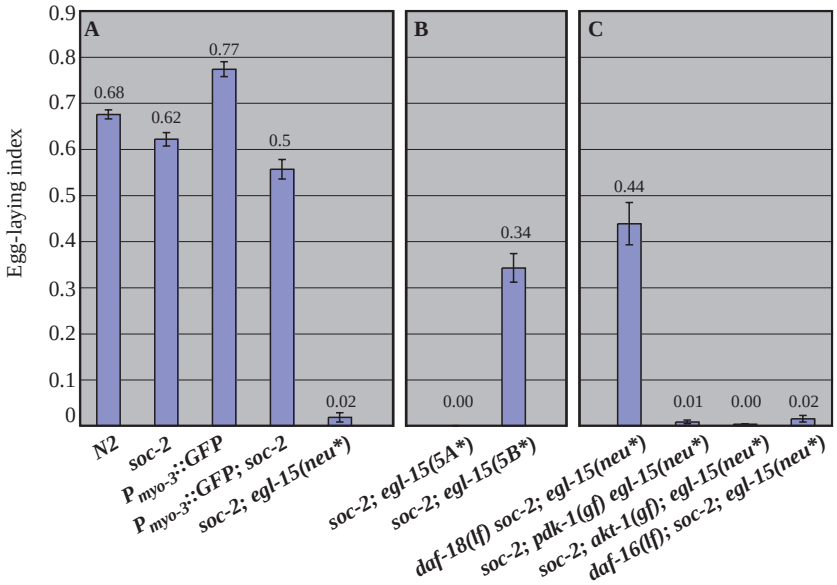


Fig. 2. Egg-laying index is an indicator of sex muscle functionality. The egg-laying index=(number of laid eggs)/(number of laid eggs + number of unlaid eggs). Mean and standard error for 30 animals are displayed.

Table 1. Summary of transgenic phenotypes

Integrated array	Transgenic *egl-15* products††	Phenotype† *soc-2(+)* background	Phenotype† *soc-2(n1774)*‡ background
–	–	Wild type	Wild type
ccIs4251§	–	Wild type	Wild type
ayIs29	A^+ B^+	Wild type	ND¶
ayIs15	A* B*	Clr	non-Clr, Egl
ayIs16	A* B*	Clr	non-Clr, Egl
ayIs17	– B*	Clr	non-Clr, Egl
ayIs18	– B*	Clr	non-Clr, Egl
ayIs21	A* –	non-Clr, Egl	ND¶
ayIs27	A* –	non-Clr, Egl	non-Clr, Egl

††+, wild-type *egl-15* construct; *activated *egl-15* construct.

†Clr, clear; Egl, egg-laying defective. A quantitative description of the Egl phenotype is shown in Fig. 2.

‡*soc-2(n1774)* suppresses the Clr phenotype of *ayIs15*, *ayIs16*, *ayIs17* and *ayIs18*.

§*ccIs4251* is a control integrated transgenic array expressing the co-transformation marker $P_{myo\text{-}3}$*::GFP*.

¶ND, not determined. The *ayIs29* and *ayIs21* transgenes integrated in close proximity to the *soc-2* locus, thereby precluding isolation of the *soc-2(n1774) ayIs29* or the *soc-2(n1774) ayIs21* recombinants.

egl-15(neu)* inhibits sex muscle differentiation

FGF signaling is known to inhibit muscle differentiation during vertebrate myogenesis (Itoh et al., 1996), suggesting that *egl-15(neu*)* might block the differentiation of the hermaphrodite sex muscles. To test whether the sex muscles were failing to differentiate, we used anti-EGL-15 antibodies to visualize undifferentiated SM descendants. These antibodies are only capable of detecting EGL-15 when expressed from transgenic arrays, which is probably due to the low-level expression of the endogenous receptor.

Wild-type *egl-15* transgenes express EGL-15 in the SMs and their descendants in the L3 and L4 larval stages. In the adult hermaphrodite, EGL-15 expression in the SM lineage becomes restricted to the type 1 vulval muscles, which display a characteristic morphology and position flanking the vulva (Fig. 4A,B). By contrast, adult animals expressing EGL-15(neu*) have many more cells that express EGL-15. The number and position of these cells is consistent with the number and position of the SM descendants observed by Nomarski microscopy (Fig. 6B; data not shown). The aberrant positions of these cells probably result from a combination of the prior defects in cell division polarity and SM migration (as described below). These cells have rounded cell bodies with projections that extend anteriorly and posteriorly (Fig. 4C,D), a morphology very distinct from that of differentiated sex muscle cells. The undifferentiated morphology of these cells, their failure to restrict *egl-15* expression, the weak expression of $P_{myo\text{-}3}$*::GFP*, and the absence of filamentous actin all indicate that these cells fail to differentiate correctly, resulting in a lack of differentiated sex muscles.

Hyperactivation of EGL-15 can affect body wall muscle development

To test whether hyperactivated EGL-15 could inhibit muscle differentiation in general or was restricted to inhibiting sex muscle differentiation specifically, we used the *hlh-1* promoter ($P_{hlh\text{-}1}$) to express *egl-15(neu*)* in muscle precursor cells. *hlh-1*, the *C. elegans* MyoD ortholog, is expressed in all non-pharyngeal muscle precursor cells and is predicted to be responsible for transactivating the genes required for development of mature muscle cells (Krause et al., 1990). *hlh-*

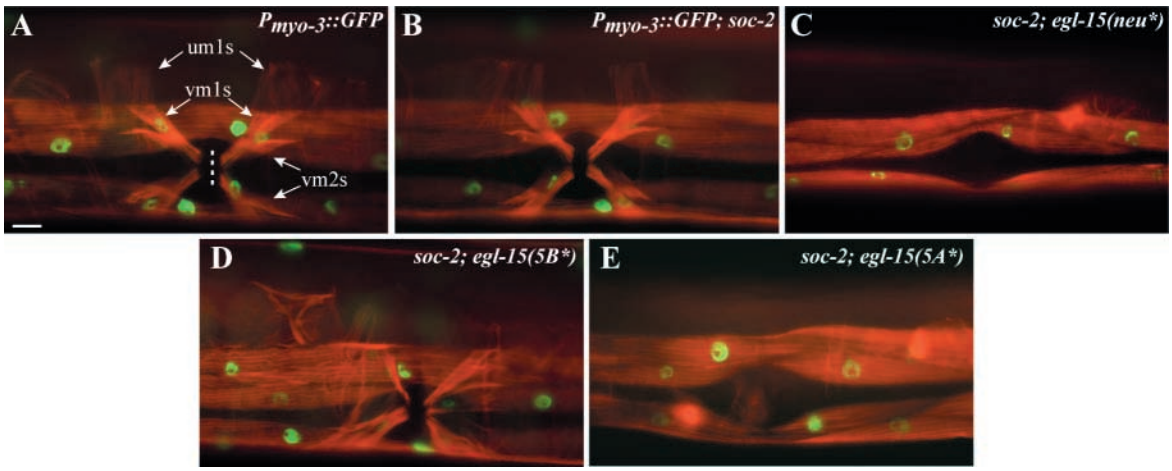


Fig. 3. Absence of sex muscles in *soc-2; ayIs15* [*egl-15(neu*)*] animals. Muscle cells are visualized with rhodamine-phalloidin and muscle nuclei with a $P_{myo\text{-}3}$*::GFP* reporter. Broad bands of body wall muscles can be seen just below the focal plane. Sex muscles are labeled in A. The control strains, *ccIs4251* [$P_{myo\text{-}3}$*::GFP*] (A) and *ccIs4251; soc-2(n1774)* (B), have wild-type sex muscles and body wall muscles. (C) *soc-2(n1774); ayIs15* [*egl-15(neu*); $P_{myo\text{-}3}$::GFP*]. SM descendants in these animals do not stain with rhodamine-phalloidin and fail to express $P_{myo\text{-}3}$*::GFP*. The rhodamine-phalloidin staining and the pattern of $P_{myo\text{-}3}$*::GFP* expression remains unchanged in the surrounding body wall musculature. (D) *soc-2(n1774) ayIs18* [*egl-15(5B*)*]; (E) *soc-2(n1774); ayIs27* [*egl-15(5A*)*]. All animals are positioned in a ventrolateral view with the head oriented towards the left. A broken line in A represents the position of the vulva. um1, uterine muscles 1; vm1, vulval muscles 1; vm2, vulval muscles 2. The um2s are very broad and contain loosely organized actin filaments. Using *ccIs4656* [P_{16Nde}*::GFP*], which can also be used to visualize both vms and ums in the absence of *ayIs15*, there are no observable sex muscles in an *ayIs15* background. The vulval musculature was scored because of the poor reproducibility of um visualization by either technique. Scale bar: 10 μm.

Table 2. Activation of EGL-15 perturbs sex muscle differentiation

Genotype[†]	% Animals with differentiated sex muscles	% Animals with mispositioned-differentiated sex muscles	Sex muscle P_{myo-3}*::GFP* expression[‡]
N2	100	0	NA
ccIs4251; soc-2	100	0	+
soc-2; ayIs15 [egl-15(neu)]*	0	NA	+/–
soc-2 ayIs18 [5B]*	100	63	+
soc-2; ayIs27 [5A]*	0	NA	+/–
daf-18(lf) soc-2; ayIs15	100	100	+
soc-2; pdk-1(gf) ayIs15	68	63	+
soc-2; akt-1(gf); ayIs15	82.5	62	+
daf-16(lf); soc-2; ayIs15	20	20	+/–

Forty young adult animals were scored for differentiated sex muscles after being fixed and stained with rhodamine-phalloidin.

[†]Alleles used: *soc-2(n1774), daf-18(nr2037lf), pdk-1(mg142gf), akt-1(mg144gf)* and *daf-16(mg54lf)*. *ccIs4251; daf-18(lf), ccIs4251; daf-18(lf) soc-2, pdk-1(gf), akt-1(gf), daf-16(lf)* and *daf-16(lf); soc-2* animals all have differentiated sex muscles located in wild-type positions in 100% of the animals examined. The presence of the *ccIs4251* transgene or the *soc-2* mutation has no significant effects on sex muscle differentiation. The *daf-18(nr2037) soc-2(n1774)* double mutant has a mildly penetrant Egl phenotype and some subtle abnormalities in sex muscle morphology when stained with rhodamine-phalloidin.

[‡]+, strong GFP expression in the vm1s of all animals; +/–, weak GFP expression in poorly differentiated sex muscles, some animals with no detectable GFP expression. NA, not applicable.

1(0) mutants display a 'lumpy-dumpy' phenotype (Fig. 5A) that results from severely abnormal muscle development (Chen et al., 1994). Germline transformants expressing P_{hlh-1}*::egl-15(neu*)* also display the 'lumpy-dumpy' phenotype, phenocopying *hlh-1(0)* mutants (Fig. 5B). By contrast, control strains, in which P_{hlh-1} drives expression of wild-type EGL-15 or kinase-deficient EGL-15(neu*), display the lumpy-dumpy phenotype at dramatically reduced frequencies (Fig. 5C; Table 3). Furthermore, the lumpy-dumpy phenotype is not merely a consequence of broad expression of *egl-15(neu*)*, as pan-neural expression of *egl-15(neu*)*, using either the P_{aex-3} or P_{unc-14} promoter, does not confer any obvious phenotypes in transgenic animals (Table 3). Thus, hyperactive FGF signaling in muscle precursor cells appears to be capable of perturbing muscle development in general.

Interestingly, the timing of *egl-15(neu*)* expression during myogenesis appears to be crucial to its ability to interfere with muscle development. *unc-54* encodes a body wall muscle myosin heavy chain isoform (MacLeod et al., 1981), which is expressed only in differentiated muscle cells (Ardizzi and Epstein, 1987; Miller et al., 1983). Transgenic P_{unc-54}*::egl-15(neu*)* animals do not display the lumpy-dumpy phenotype (Table 3), despite expressing robust amounts of EGL-15(neu*) in muscle cells (data not shown). These results indicate that EGL-15(neu*) must be expressed prior to the commitment to a myogenic fate in order to influence myogenesis.

Hyperactivation of EGL-15 results in multiple M lineage defects

To better understand the developmental defects that result in the abnormal staining pattern observed in *soc-2; egl-15(neu*)* animals (Fig. 4C), we examined the sequence of developmental events from the divisions of the post-embryonic M mesoblast to the differentiation of the sex muscles. Multiple aspects of sex muscle development are affected by hyperactivation of EGL-15, including the polarity of the cell divisions in the early M lineage, cell fate determination events and SM positioning.

The earliest M lineage defect detected in *soc-2; ayIs15* animals was an abnormal number of M-derived cells in the four muscle quadrants. Cells in the M lineage were identified using a P_{twist}*::GFP* reporter (*ayIs6 [*P_{twist}*::GFP]*) (Harfe et al., 1998b). In wild-type animals, the first division of M is oriented along the dorsoventral axis. Each M daughter cell (M.x) then

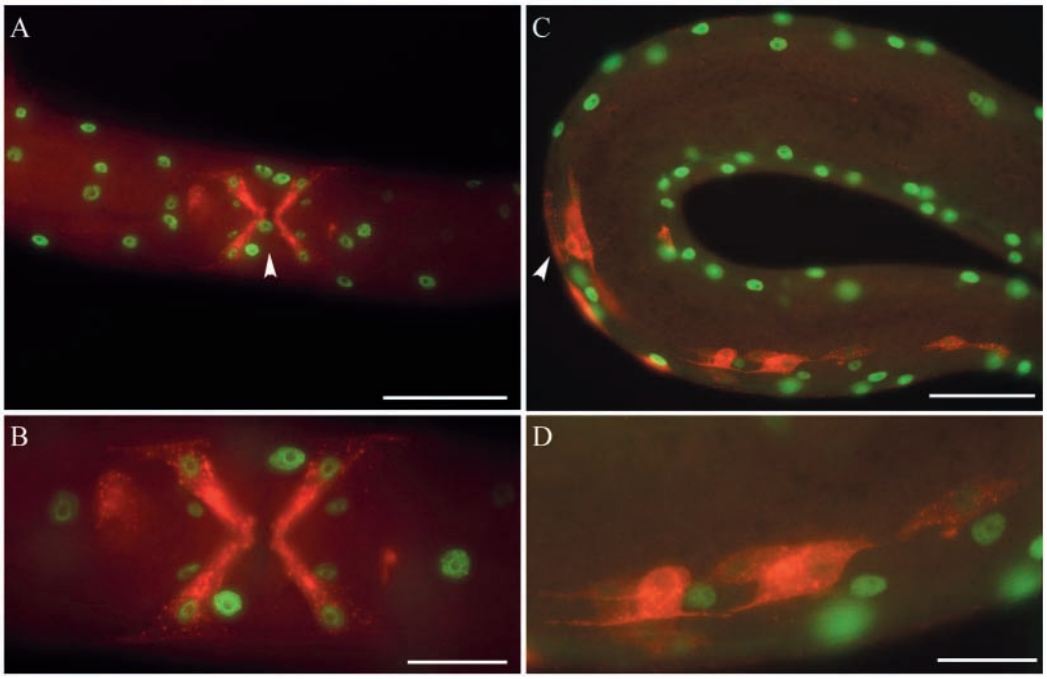


Fig. 4. Visualization of sex muscles with α-EGL-15 antibodies. (A,B) *ccIs4251; ayIs29* [*egl-15(+)*] animals display wild-type sex muscle morphology and positions. EGL-15 expression is restricted to the vm1s in differentiated hermaphrodite sex muscles. (C,D) SM descendants in *soc-2; ayIs15* animals. Red, α-EGL-15 immunoreactivity; green, muscle nuclei visualized using P_{myo-3}*::GFP* transgenic arrays. A,B, ventral view; C,D, lateral view. All animals are positioned with the tail oriented towards the right. Arrowheads indicate the position of the vulva. Scale bar: 50 μm in A,C; 10 μm B,D.

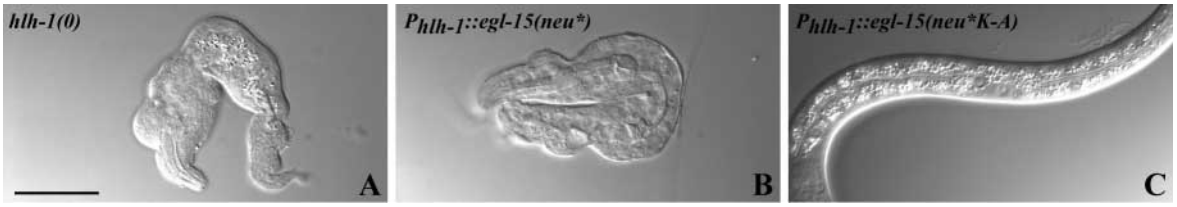
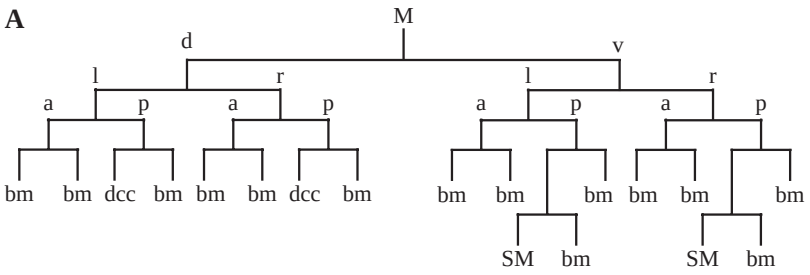


Fig. 5. Expression of *egl-15(neu*)* in muscle precursor cells inhibits muscle development. (A) *hlh-1(cc450)* homozygote; (B) $P_{hlh\text{-}1}$*::egl-15(neu*)* transgenic animal displaying a lumpy-dumpy phenotype; (C) $P_{hlh\text{-}1}$*::egl-15(neu*K-A)* kinase defective control transgenic animal. Transgenic EGL-15 expression was confirmed in all strains by immunohistochemical analysis (data not shown). All animals are photographed 14 hours after hatching. Scale bar: 50 μm.

divides transversely (left-right). The orientations of these first two divisions position a single M-derived muscle progenitor cell in each of the four-muscle quadrants. These cells subsequently divide along the anteroposterior axis to generate a total of 14 body wall muscles, two coelomocytes and two sex myoblasts (Fig. 6A) (Sulston and Horvitz, 1977). Because of the tight linkage between the *ayIs6* and *ayIs15* transgenes, the second *egl-15(neu*)* integrated array, *ayIs16*, was used for this analysis. In *soc-2; ayIs16* animals, the polarities of the early M-lineage divisions are often abnormal. This can be scored most easily for divisions of M.x or M.xx, where division polarity defects result in muscle quadrants lacking M-derived descendants. When *soc-2; ayIs16* animals are scored in this manner, the majority of *soc-2; ayIs16* animals display muscle quadrants lacking M-derived cells (Fig. 6B). A similar defect is seen in *5A** animals and, to a reduced extent, in *5B** animals. Despite the division polarity defect, the number of M descendants generated during the first larval stage still appears to be normal (data not shown).

Additional defects within the M lineage were also readily



B

Genotype	Transgenic *egl-15* products	% animals with dccs	% animals with quadrants lacking M-derived muscle cells
ccIs4251	none	100	0
soc-2	none	100	0
ccIs4251; soc-2	none	100	0
soc-2; ayIs16	5A* 5B*	0	90
soc-2; ayIs18	– 5B*	47	38
ayIs21	5A* –	0	87

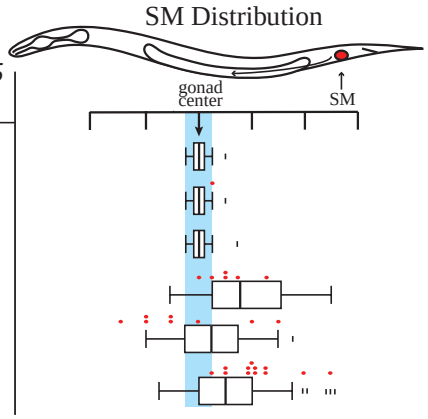


Fig. 6. Hyperactivation of EGL-15 results in multiple M lineage defects. (A) The cell lineage of the M mesoblast. M divides along the dorsoventral axis; the two M.x daughters divide along the left/right axis. The remaining divisions in the lineage are oriented along the anteroposterior axis. d, dorsal; v, ventral; l, left; r, right; a, anterior; p, posterior; bm, body wall muscle; dcc, dorsal coelomocyte; SM, sex myoblast (adapted from Sulston and Horvitz, 1977). (B) The effect of EGL-15 hyperactivation on SM positioning, presence of dorsal coelomocytes and orientation defects in early M lineage cell divisions. SM positions are scored with respect to the hypodermal Pn.p cell metric. SMs in wild-type animals are centered at P6.p, indicated by the arrow; the blue rectangle represents the positions of normal SMs. Each full SM distribution is represented by a box-and-whisker plot, where the box extends over the distribution of the central 50% of the SMs and the line within the box is at the median position. Whiskers extend anteriorly and posteriorly to data points up to 75% of the length of the box. SMs that fall outside the box and whiskers are represented by an individual hash mark (see Material and methods). Red circles indicate dorsally positioned SMs. *ayIs16* was used in place of *ayIs15* for technical strain construction considerations. A similar survey of SMs in *ayIs15* hermaphrodites gave a distribution consistent with that seen for *ayIs16*. At least 30 SMs were scored for each SM distribution and at least 30 animals were scored for dorsal coelomocyte and M-lineage cell-division defects.

Table 3. The effects of EGL-15(neu*) are limited to myoblast development

Promoter	Transgene	Expression pattern†	Number of lines displaying a lumpy-dumpy phenotype
egl-15	*egl-15*	M lineage, SM lineage,	0/5
	egl-15(neu)*	vm1s, IL neurons, hypodermis	0/4
hlh-1	*egl-15*	Muscle precursor cells	1/8
	egl-15(neu)*	Muscle precursor cells	5/5
	egl-15(neu) K-A*‡	Muscle precursor cells	0/6
unc-54	*egl-15(neu*)*	Differentiated muscle cells	0/8
unc-14	*egl-15(neu*)*	Neurons	0/9
aex-3	*egl-15(neu*)*	Neurons	0/12

†Immunohistochemical analysis has shown EGL-15 expression in at least one representative line for each type of transgene.
‡Kinase-defective mutation, where the lysine residue in the ATP-binding site has been mutated to alanine (K672A).

apparent in animals with hyperactive EGL-15 signaling. The two types of non-body muscle M descendants, the SMs and the dorsal coelomocytes, are sufficiently distinctive in their appearance and in their expression of the *ayIs6* marker that their presence can be scored independent of earlier division polarity defects. A dramatic absence of the distinctive M-derived coelomocytes was observed in animals bearing an *egl-15(neu*)* array (Fig. 6B), suggesting that hyperactivated EGL-15 signaling could interfere with their normal fate determination or differentiation. In addition, the normal precise positioning of the SMs was disrupted by EGL-15 hyperactivation. Both of these defects were apparent regardless of whether or not these cells were generated in their proper quadrants. Similar effects were also observed in *5A** animals and, to a reduced extent, in *5B** animals. The effects of *egl-15(neu*)* on multiple events that occur in the M lineage highlight the crucial role that FGF signaling can play during development.

Activation of PI3 kinase signaling can suppress the muscle differentiation defect of *egl-15(neu*)*

Biochemical and cell culture analyses have shown that insulin, which acts through a phosphatidyl-inositol 3′-kinase (PI3 kinase) signaling pathway, can stimulate myogenesis and oppose the inhibitory action of FGF (Kaliman et al., 1998; Kaliman et al., 1996; Milasincic et al., 1996; Tamir and Bengal, 2000; Tureckova et al., 2001). The inhibition of sex muscle differentiation by EGL-15(neu*) provides an in vivo system to test the involvement of the PI3 kinase signaling pathway in muscle differentiation in *C. elegans*. PI3 kinase phosphorylates PIP2 to generate PIP3, which in turn activates several downstream signaling components (Cantrell, 2001). This activity is antagonized by the lipid-phosphatase PTEN, regulating the amount of PIP3 generated (Leslie and Downes, 2002). The *C. elegans* PI3 kinase, AGE-1 (Morris et al., 1996), also functions within an insulin-signaling pathway and is opposed by the PTEN ortholog, DAF-18 (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998). Downstream components of this pathway include the serine/threonine kinases PDK and AKT, encoded by the *pdk-1*, *akt-1* and *akt-2* genes (Paradis et al., 1999; Paradis and Ruvkun, 1998).

Based on the antagonistic effects of FGF and insulin on myogenesis in vertebrates, increases in the activity of the PI3 kinase signaling pathway would be predicted to suppress the effects of hyperactivated EGL-15 and restore muscle differentiation to *soc-2; ayIs15* animals. Consistent with this hypothesis, loss-of-function mutations in *daf-18* and gain-of-function mutations in *pdk-1* and *akt-1* all suppress the sex muscle differentiation defect of *soc-2; ayIs15* animals (Fig. 7C-E; Table 2). Eliminating DAF-18/PTEN has the most dramatic suppression, restoring sex muscle differentiation in 100% of *daf-18(nr2037) soc-2; ayIs15* animals. The gain-of-function mutations *pdk-1(mg142gf)* and *akt-1(mg144gf)* have weaker, but still dramatic suppressive effects (68% and 82.5%, respectively). Suppression of the differentiation defect in all

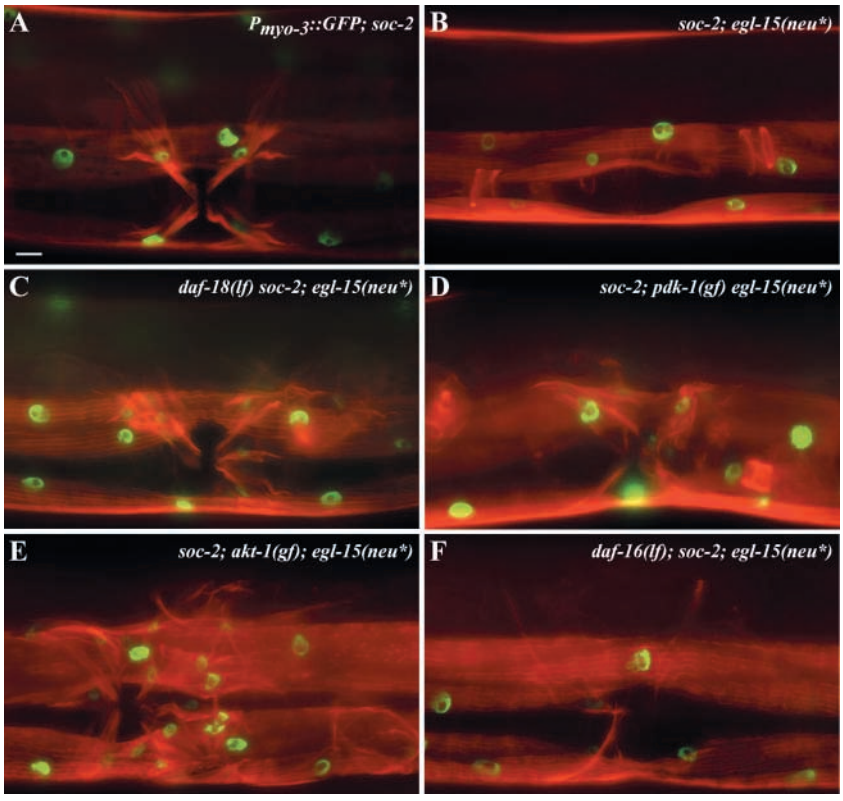


Fig. 7. Suppression of the EGL-15(neu*)-induced sex muscle differentiation defect by activating the PI3 kinase signaling pathway. (A) *ccIs4251; soc-2(n1774)*. (B) *soc-2(n1774); ayIs15*. (C) *daf-18(nr2037lf) soc-2(n1774); ayIs15*. (D) *soc-2(n1774); pdk-1(mg142gf) ayIs15*. (E) *soc-2(n1774); akt-1(mg144gf); ayIs15*. (F) *daf-16(mg54lf); soc-2(n1774); ayIs15.* All animals are positioned in a ventrolateral view with the head oriented to the left. Red, rhodamine-phalloidin staining; green, P_{myo-3}*::GFP* expression. Scale bar: 10 μm.

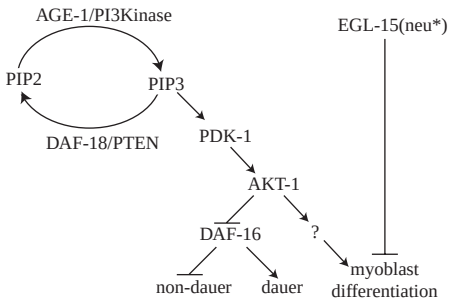


Fig. 8. Model for EGL-15 and PI3 kinase signaling pathways in muscle differentiation.

of these strains is indicated by restoration of sex muscle morphology, muscle actin filament structure and the robust expression of $P_{myo\text{-}3}$*::GFP* in the vulval muscles (Fig. 7).

Although the sex muscle differentiation defect is significantly suppressed by activating the PI3 kinase signaling pathway (Fig. 8), the abnormalities that remain indicate that some of the other processes affected by EGL-15(neu*) are not fully suppressed. Animals from all of the suppressed strains display differentiated muscles both at the vulva and at multiple aberrant positions (Fig. 7; Table 2). Consistent with these misplaced muscles being sex muscles, a similar distribution of cells express P_{16Nde}*::GFP* (*ccIs4656*), the sex muscle-specific reporter (Harfe et al., 1998a), in *ccIs4656*; *daf-18(nr2037) soc-2; ayIs15* animals (data not shown). The sex muscle abnormalities that persist cause these suppressed strains to remain predominantly egg-laying defective. Despite the continuing defects, the differentiated sex muscles in *daf-18(nr2037) soc-2; ayIs15* have significant functionality; *daf-18(nr2037) soc-2; ayIs15* animals lay many eggs prior to becoming Egl, as indicated by the increased egg-laying index of this strain (Fig. 2C). By contrast, the differentiated sex muscles in the *pdk-1(gf)* and *akt-1(gf)* backgrounds lack similar functionality (Fig. 2C).

The AGE-1/PI3 kinase signaling pathway serves to inhibit the DAF-16 forkhead transcription factor in its roles in the regulation of metabolism, dauer development and longevity in *C. elegans* (Lin et al., 1997; Ogg et al., 1997; Paradis and Ruvkun, 1998; Tang et al., 1999). In these processes, the activity of the PI3 kinase pathway is predominantly mediated by the regulation of DAF-16 (Lin et al., 2001; Tissenbaum and Ruvkun, 1998). To test whether promotion of myogenesis by the PI3 kinase pathway is mediated by inhibiting DAF-16, we characterized *daf-16(mg54); soc-2; ayIs15* animals in which DAF-16 is eliminated by the null mutation *daf-16(mg54)*. In contrast to the restoration of sex muscle differentiation by *daf-18(nr2037), pdk-1(mg142gf)* and *akt-1(mg144gf)*, eliminating DAF-16 fails to restore a similar extent of sex muscle differentiation (20%, as opposed to 100%, 68% and 82.5%, respectively). The pattern of $P_{myo\text{-}3}$*::GFP* expression and rhodamine-phalloidin staining remains unchanged in the vast majority of *daf-16(mg54); soc-2(n1774); ayIs15* animals (Fig. 7; Table 2). As complete loss of DAF-16 is significantly less effective than mutations that activate the PI3 kinase pathway, the effects of this pathway on sex muscle differentiation must involve an alternative mechanism that is not limited to the inhibition of DAF-16.

Discussion

Hyperactivation of the *C. elegans* FGF receptor, EGL-15, has provided an important tool for understanding the role of FGF signaling in *C. elegans* development (Kokel et al., 1998; Selfors et al., 1998). By circumventing the Clr phenotype caused by increased EGL-15 signaling, we have shown that hyperactivation of EGL-15 can also inhibit muscle differentiation. The severe inhibition of sex muscle differentiation leads to the dramatic egg-laying defective phenotype of *soc-2; egl-15(neu*)* animals. However, the ability of hyperactivated EGL-15 to block myogenesis appears to be more general in nature because its expression in precursors to the body wall muscles can interfere with their normal development as well.

The severe effect of hyperactivated EGL-15 on sex muscle development appears to be due primarily to the inhibition of muscle differentiation in this lineage. Although there are earlier defects within the M lineage, SMs are still generated in *soc-2; egl-15(neu*)* animals and undergo the normal number of rounds of proliferation to generate sex muscle precursors. SM descendants, however, fail to mature properly. Immunohistochemical analysis of wild-type EGL-15 expression revealed that EGL-15 is normally expressed in the undifferentiated cells of the SM lineage and becomes restricted to the four differentiated vm1s in the adult. In *soc-2; egl-15(neu*)* animals, however, many more cells express EGL-15 during adulthood. Furthermore, these cells remain morphologically immature, unlike differentiated muscle cells. These cells also fail to stain for F-actin and only weakly express $P_{myo\text{-}3}$*::GFP*, both molecular markers of differentiated muscle cells. Taken together, these data indicate that hyperactivation of EGL-15 represses sex muscle differentiation.

EGL-15 hyperactivation also appears to be able to inhibit muscle development more generally, as expression of EGL-15(neu*) in body wall muscle precursor cells can also perturb the development of the body wall musculature. $P_{hlh\text{-}1}$*::egl-15(neu*)* transgenic animals display a lumpy-dumpy phenotype that phenocopies *hlh-1(0)* animals (Chen et al., 1994), both of which display abnormally developed body wall muscles. The common lumpy dumpy phenotype of these animals may reflect the negative regulation of HLH-1 by EGL-15 activation in body wall muscle precursor cells. Consistent with this hypothesis, FGF signaling can negatively regulate MyoD function via inhibitory phosphorylation by protein kinase C (Li et al., 1992). The restricted muscle defects observed in $P_{egl\text{-}15}$*::egl-15(neu*)* transgenic animals is probably due to high levels of *egl-15* expression being confined to muscle precursor cells within the M lineage. Although high levels of EGL-15 have not been observed in the body wall muscles, EGL-15 has been shown to play a role in protein degradation in differentiated body wall muscles (Szewczyk and Jacobson, 2003).

Not only has the analysis of *egl-15(neu*)* phenotypes provided insights into muscle development, it has also served to confirm and extend our understanding of additional roles of EGL-15 in *C. elegans*. FGF signaling through EGL-15 is thought to play an instructive role in guiding the migrating SMs to their precise final positions (Burdine et al., 1998; DeVore et al., 1995; Goodman et al., 2003). The disruption of proper migration events by hyperactivation of the normal signaling

pathway has often been used as evidence for an instructive role for the affected pathway (Duchek et al., 2001). The behavior of the SMs in *egl-15(neu*)* animals is consistent with an instructive role of EGL-15 in SM migration. Our results also fit the temporal series of events thought to lead to muscle differentiation. Expression of *egl-15(neu*)* under the control of the *unc-54* promoter fails to confer the same lumpy-dumpy phenotype in transgenic animals that is observed when the *hlh-1/CeMyoD* promoter is used. Only cells well along their commitment to a muscle fate begin to express the UNC-54/MYO-4 myosin heavy chain (Ardizzi and Epstein, 1987; Miller et al., 1983). Unlike uncommitted muscle precursor cells, these cells have apparently progressed sufficiently along the myogenic pathway so as to be insensitive to the inhibitory signal resulting from hyperactive EGL-15 signaling. Finally, analysis of *egl-15(neu*)* animals has revealed a potential new role for EGL-15 signaling in influencing cell division polarity in the early M lineage. In the wild type, the division planes for cells in the M lineage are spatially determined (Sulston and Horvitz, 1977). Hyperactivation of EGL-15 disrupts the polarity of these divisions, and the cells divide along improper division planes. It will be interesting to use *egl-15(neu*)* to probe the mechanisms that determine the polarities of these divisions.

A model for studying myogenesis

The phenotypes of *egl-15(neu*)* are due to dramatic constitutive activation of EGL-15, and, therefore, do not necessarily reflect the normal roles of EGL-15. Nonetheless, the effects of *egl-15(neu*)* on sex muscle differentiation are an accurate reflection of a normal role of FGF signaling during vertebrate myogenesis. In cell culture, activation of the FGF receptor inhibits myoblast differentiation (Clegg et al., 1987; Spizz et al., 1986; Templeton and Hauschka, 1992; Vaidya et al., 1989). This effect of FGF signaling is antagonized by the PI3 kinase signaling pathway, which functions to promote myogenesis. Overexpression and activation of the PI3 kinase signaling components PDK1 and AKT1 can result in myoblast differentiation even in the presence of an FGF signal that normally blocks muscle differentiation (Kaliman et al., 1996; Milasincic et al., 1996; Tamir and Bengal, 2000; Tureckova et al., 2001). In *C. elegans*, signaling through the PI3 kinase pathway can be increased either by loss of DAF-18/CePTEN or via gain-of-function alleles of *pdk-1* or *akt-1* (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Paradis et al., 1999; Paradis and Ruvkun, 1998). Similar to the antagonism between FGF and PI3 kinase signaling observed in cultured myoblasts, all of these mutations can suppress the sex muscle differentiation defect in *egl-15(neu*)* animals. Thus, the effects of EGL-15(neu*) on sex muscle differentiation parallel the effects of mammalian FGF receptor activation in cultured myoblasts and can serve as a good model for understanding the complex interplay of signaling pathways in myogenic processes.

The myogenic function of the PI3 kinase pathway in *C. elegans* appears to be mediated by an effector other than the DAF-16 transcription factor normally associated with PI3 kinase signaling in worms. Activation of PI3 kinase signaling using a null allele of *daf-18/CePTEN* can cause dramatic suppression of the inhibition of sex muscle differentiation because of *egl-15(neu*)*. PI3 kinase signaling normally inhibits the function of HNF-Forkhead transcription factors that transduce the effects of the upstream signaling pathway (Lin et al., 2001; Ogg et al., 1997; Tang et al., 1999). Thus, loss of these factors often mimics activation of the signaling pathway. The suppression of the muscle differentiation defect by *daf-18(nr2037)* is not mimicked by eliminating DAF-16, the forkhead transcription factor that functions as the target of PI3 kinase signaling in other known events in *C. elegans* (Lin et al., 2001; Ogg et al., 1997). Thus, our data suggest that other targets of the PI3 kinase pathway function in the process of sex muscle differentiation, and that signaling specificity in this pathway is likely to be derived from tissue specific expression of AKT-regulated factors. Likely candidates include the Foxo transcription factors (Arden and Biggs, 2002; Hope et al., 2003) and TOR/p70S6 kinase signaling pathways, which have been shown to be regulated by AKT and to be important for myoblast differentiation in vivo (Conejo et al., 2002; Hribal et al., 2003).

Separation of EGL-15 function

FGFs can stimulate many distinct biological processes, and multiple mechanisms are used to specify the consequence of their activation (Szebenyi and Fallon, 1999). FGF signaling in *C. elegans* regulates a number of processes (Burdine et al., 1998; DeVore et al., 1995; Szewczyk and Jacobson, 2003), and the mechanisms that determine which process is triggered are beginning to be revealed.

One major determinant of signaling specificity is tissue-specific expression. EGL-15 expression in the hypodermis regulates fluid balance (Huang and Stern, 2004), whereas expression in the M lineage controls a number of aspects of sex muscle development (Burdine et al., 1998). The restricted normal expression of *egl-15* to the M lineage apparently confines the dramatic effects of *egl-15(neu*)* to the sex muscles while leaving body wall muscle development intact. Even the timing of expression within a cell lineage is crucial for specifying potential outcomes: early expression of EGL-15(neu*) in muscle precursor cells has dramatic effects on myogenesis, while its expression in committed muscle cells does not.

Alternative splicing is a second important mechanism used to increase receptor diversity and potential for signaling specificity (Green et al., 1996). In *C. elegans*, *egl-15* is alternatively spliced to yield two isoforms, EGL-15(5A) and EGL-15(5B). These isoforms can be assigned to different physiological functions of EGL-15. The 5B isoform is required to maintain fluid homeostasis, while the 5A isoform is essential for chemotaxis of the M-derived sex myoblasts (Goodman et al., 2003). The effects of hyperactivated EGL-15 are also specified in large part by this alternative splicing event; hyperactivation of 5B results in the dramatic Clear phenotype, while hyperactivation of 5A has effects that are predominantly confined to the M lineage.

Differences in signaling pathway components or their activity thresholds (Tan and Kim, 1999) also can play a crucial role in the specification of signaling outcomes (Boilly et al., 2000). The effects of a *soc-2* mutation on the processes affected by EGL-15 hyperactivation is a potent illustration of the importance of this mechanism. The phenotypic specificity of a hypomorphic *soc-2* mutation revealed the effects of

hyperactive EGL-15 signaling on sex muscle development. Delineating the EGL-15 pathway used in myogenesis will be an important step towards a better understanding of EGL-15 signaling specificity mechanisms. Moreover, the amenability of the sex muscle phenotype to systematic genetic analysis could also provide a broader understanding of how the FGF pathway interacts with the other signaling pathways required for functional muscle formation.

We thank A. Fire and M. Krause for plasmids; T. Lo and P. Huang for generating germline transformants; A. Bennett for helpful discussions; D. Chase, P. Roy, S. Dixon and members of our laboratory for a critical reading of this manuscript. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH. I.E.S. was supported by NIH/National Institute of General Medical Sciences Medical Scientist Training Grant GM07205. This work was supported by NIH grant GM50504 awarded to M.J.S.

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