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Genetic evidence for pax-3 function in myogenesis in the nematode *Pristionchus pacificus*

Buqing Yi, Dan Bumbarger, and Ralf J. Sommer*

Department for Evolutionary Biology, Max-Planck Institute for Developmental Biology, Spemannstrasse 37, D-72076 Tübingen, Germany

*Author for correspondence (email: ralf.sommer@tuebingen.mpg.de)

SUMMARY PAX3 is a member of the PAX3/7 subfamily of the paired box proteins. In vertebrates, Pax3 is essential for skeletal myogenesis by activating a cascade of transcriptional events that are necessary and sufficient for skeletal myogenesis. Four related basic helix-loop-helix transcription factors, MyoD, Myf5, Mrf4, and Myogenin, are targets of PAX3 and serve as myogenic regulatory factors. Although the role of Pax3 in myogenesis is well studied in vertebrates, little is known about invertebrate PAX-3 proteins and myogenesis. Here, we took advantage of viable alleles of *pax-3* in the nematode satellite model organism *Pristionchus pacificus* to investigate the function of PAX-3 in myogenesis. Two strong reduction-of-function alleles of *Ppa-pax-3* show severe muscle-derived abnormalities and phalloidin staining indicates a disruption of body wall muscle patterning. Furthermore, we identified a myogenic regulatory factor-related gene *Ppa-hlh-1*/MyoD and a serum response factor-related gene *Ppa-unc-120*. Expression of both genes in *Ppa-pax-3* mutant animals is down regulated suggesting that *Ppa-pax-3* acts upstream in the regulatory network. Together, our results provide the first genetic evidence for a conserved function of PAX-3 in myogenesis between vertebrates and nematodes.

INTRODUCTION

Pax genes encode transcription factors that have important and highly conserved roles during animal development (for a review see Chi and Epstein 2002). PAX proteins contain up to three highly conserved protein domains: an N-terminal paired domain (PD) and a C-terminal homeodomain (HD), both of which are involved in DNA-binding, as well as an octapeptide. At the sequence level, the presence or absence of the HD and/or the octapeptide and the sequence similarities within the PD, allow the subdivision of Pax proteins into four specific subfamilies (Fig. 1A) (Chi and Epstein 2002).

The Pax3/7 subfamily is defined by the mammalian Pax3 and Pax7 gene products and is the only subfamily that contains all of the three protein domains (Fig. 1A). In the mouse Pax3 is essential for skeletal myogenesis, which is required for the specification of embryonic muscle progenitors and it initiates a cascade of transcriptional events that are necessary and sufficient for skeletal myogenesis (Buckingham and Relaix 2007). Critical in this cascade are the myogenic regulatory factors (MRFs) MyoD, Myf5, Mrf4, and Myogenin, a set of four related basic helix-loop-helix (bHLH) transcription factors (Buckingham and Relaix 2007; Charge and Rudnicki 2004; Wang et al. 2008; Tapscott 2005). A recent study shows that in muscle progenitor cells, expression of Pax3, or Pax7 dominant—negative constructs inhibits the expression of Myf5, MyoD, and Myogenin, and thereby, prevents differentiation. These results indicate that Pax3 and Pax7 are (i) required for myogenic differentiation, (ii) they function to maintain expression of myogenic regulatory factors, and (iii) they promote the expansion of muscle cell populations (Collins et al. 2009).

Although Pax genes are highly conserved throughout the animal kingdom, little is known about the function of Pax3/7 genes in myogenesis in invertebrates. In *Drosophila melanogaster*, three genes belong to the Pax3/7 subgroup, *paired*, *gooseberry*, and *gooseberry neuro* (Noll 1993). While the function of Pax3/7 genes in *Drosophila* embryogenesis and neurogenesis has been studied in great detail, there is no report on a potential role of Pax3/7 genes in *Drosophila* myogenesis. In contrast, a recent study of the Pax1/9 class gene *Pox meso* indicated a crucial role of this gene in the regulation for somatic myogenesis in *Drosophila* (Duan et al. 2007).

In the nematode *Caenorhabditis elegans* myogenesis can be studied with single cell resolution and its genetic and genomic regulation can be investigated. For example, the genome of *C. elegans* contains five bonafide Pax genes (Hobert and Ruvkun 1999). *vab-3* belongs to the Pax6 subfamily and is involved in head and tail development (Chisholm and Horvitz 1995; Zhang and Emmons 1995). *egl-38* is a member of the Pax2/5/8 subfamily and regulates uterine and tail development (Chamberlin et al. 1997). The highly related gene *pax-2*

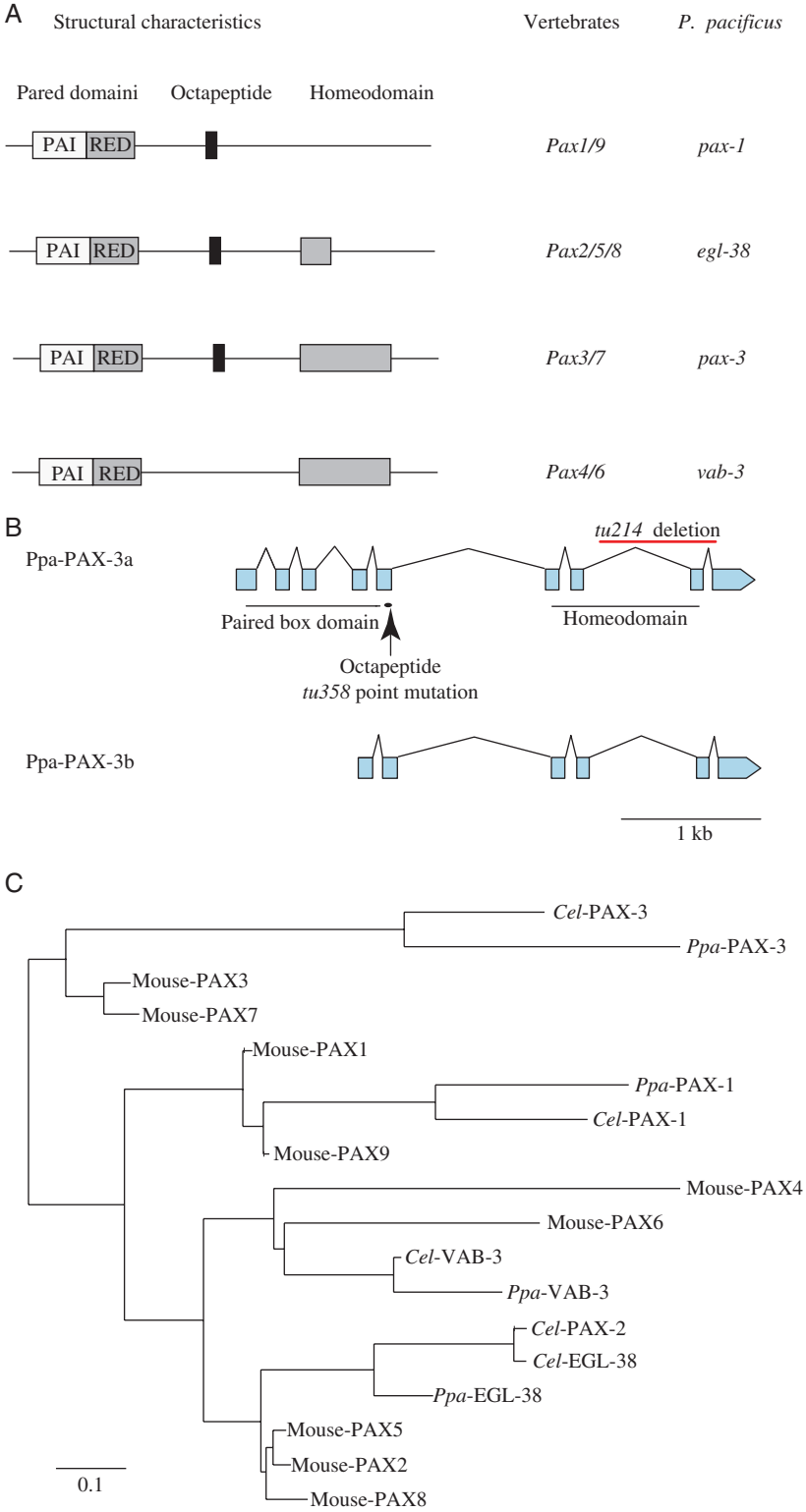

Fig. 1. Schematic representation of Pax gene and protein structure and phylogenetic relationship of Pax genes from *P. pacificus*, *C. elegans* and mouse. (A) PAX proteins can contain three conserved protein domains: an N-terminal paired domain (PD), a C-terminal homeodomain (HD) and an octapeptide. The presence or absence of the HD and/or the octapeptide and the sequence similarities within the PD, allow the subdivision of Pax proteins into four specific subfamilies. (B) *Ppa-pax-3* gene structure. Introns are indicated by closed triangles. The breakpoints of the C-terminal deletion of *tu214* are indicated by arrowheads. The mutation of *tu358* results in an amino acid replacement from His to Arg and is indicated by an arrow. The PD domain is boxed, the octapeptide is underlined and the HD is high lighted. (C) All *P. pacificus* Pax genes show a domain composition that is identical to the corresponding *C. elegans* genes. Phylogenetic analysis using the protein sequence of the PD domain of all *P. pacificus*, *C. elegans*, and mice Pax genes indicates that PAX-3, PAX-1, and VAB-3 of *P. pacificus* form 1:1 orthologs with the corresponding *C. elegans* proteins. The *P. pacificus* genome contains one gene encoding an EGL-38/PAX-2 protein, whereas the *C. elegans* genome contains two such genes.

(K06B9.5) results from a recent gene duplication (Wang et al. 2004). The *pax-3* (F27E5.2) gene is the only gene present in the *C. elegans* genome that contains all three protein domains, PD, HD, and octapeptide. Thus, the protein domain architecture of PAX proteins is conserved between nematodes and vertebrates. Mutations in the *Cel-pax-3* gene are early larval lethal and sterile. Although a potential role of *Cel-pax-3* in myogenesis has not been investigated genetically, RNAi

experiments suggest a role of *Cel-pax-3* in locomotion (http://www.wormbase.org).

When myogenesis in *C. elegans* and other invertebrates is compared with vertebrate systems, many apparent divergent features are observed at the molecular level. Most prominently, only one homolog of MRFs has been identified in *C. elegans* and other invertebrates, whereas vertebrates have four MRF encoding genes. *hlh-1* in *C. elegans* (Krause et al. 1990), *nau* in *D. melanogaster* (Michaelson et al. 1990; Paterson et al. 1991), *sum-1* in *sea urchins* (Venuti et al. 1991), and *amd-1* in the ascidian, *H. roretzi* (Araki et al. 1994) all represent single copy genes. While a subset of MRFs appears to be both necessary and sufficient for vertebrate skeletal myogenesis, mutants lacking the single MRF-related factor *nau* or *hlh-1*/*CeMyoD* are still able to specify and differentiate striated muscle (Chen et al. 1992; Balagopalan et al. 2001).

Instead, a recent study defined an extreme case of transcriptional redundancy of early body wall muscle development in *C. elegans*. The *C. elegans* serum response factor (SRF)-related gene *Cel-unc-120*, a MADS-box transcription factor, and the bHLH molecule *Cel-hnd-1* were shown to act redundantly with *Cel-hlh-1*/CeMyoD to control muscle specification (Fukushige et al. 2006). Whereas the *C. elegans* studies convincingly show that these three genes comprise a functionally robust "muscle module," the factors acting upstream remained largely elusive. In particular, given the fact that *Cel-pax-3* mutants are early larval lethal it is unknown if PAX-3 acts upstream in the developmental control of myogenesis. The first viable genetic mutant in a nematode *pax-3* gene has been isolated in *Pristionchus pacificus*, a species that has been established as a satellite organism to *C. elegans* (Fig. 1B) (Hong and Sommer 2006; Yi and Sommer 2007).

P. pacificus is amenable to forward and reverse genetic analysis and provides a platform to identify genetic and molecular alterations that control evolutionary changes of developmental processes (Sommer 2009). *P. pacificus* propagates as a self-fertilizing hermaphrodite and has an integrated genome map, including a genetic linkage map and a physical map (Hong and Sommer 2006). A whole-genome sequencing project has recently been finished indicated that the *P. pacificus* genome is substantially larger than the one of *C. elegans* and contains substantially more genes (Dieterich et al. 2007). For example, the *P. pacificus* is the first nonparasitic nematode that contains cellulases and other glycosylhydrolases and cellulase activity has been observed in the supernatant of mixed stage cultures (Dieterich et al. 2007). Although many *P. pacificus* gene predictions have no sequence similarity to known proteins in databases, the transcription factor modules, and signal transduction machineries are highly conserved at the sequence level between *P. pacificus* and *C. elegans*. For many transcription factors, homology relationships can be determined and 1:1 orthologs can be identified between the two species.

Our previous studies have shown that *Ppa-pax-3* regulates cell death and cell survival of ventral epidermal cells and thereby affects vulva formation (Yi and Sommer 2007). However, we have not investigated if *Ppa-pax-3* also has an affect on myogenesis.

In this study, we took advantage of the available *Ppa-pax-3* strong reduction-of-function alleles to study the role of *pax-3* in myogenesis and the related transcriptional network. Analysis of the *Ppa-pax-3* mutant reveals severe disruption of body wall muscle pattern. Further molecular analysis suggests that *Ppa*-PAX-3 is involved in the regulation of the expression of *Ppa-hlh-1*/MyoD and *Ppa-unc-120*/SRF. The *Ppa-pax-3* mutant represents the first viable mutant of a single Pax3/7 homolog in invertebrates and our results provide first evidence that *Pax3/7* gene function in myogenesis is evolutionarily conserved between invertebrates and vertebrates.

MATERIALS AND METHODS

Nematode strains and cultures

Worms were grown on 5 cm NG agar plates seeded with OP50, an uracil requiring mutant of *E. coli* (Sommer and Sternberg 1996). *P. pacificus* PS312 (the wild type strain) is a derivative of an isolate from Pasadena, California and represents the wild-type strain used for genetic analysis. Mutant strains used in this study are *Ppa-pax-3(tu214)* and *Ppa-pax-3(tu358)* (Yi and Sommer 2007).

Quantitative PCR experiments

Synchronized *P. pacificus* cultures were prepared as described before (Rudel et al. 2005), then worms were grown on OP50 until a predominantly gravid population was present. These worms were washed off plates, bleached, separated from debris by sucrose floating, and embryos were harvested for RNA extraction. RNA was reverse transcribed in 20 µl total volume reaction (Invitrogen) with negative controls without reverse transcriptase included for each sample. Quantitative PCR was performed on a Roche LC480 Light cycler using the manufacturers SYBR green PCR mix. Primer concentrations were 0.5 mM. Expression of target gene was normalized relative to that of *Ppa-β-tubulin* and/or ribosomal protein *Ppa-rpl-14*. RNA analysis as shown in Fig. 5 was subjected to three full biological replicates.

Scanning electron microscopy

Young adult worms were washed three times in PBS and then fixed with 2.5% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide in phosphate-buffered saline, dehydrated in a graded series of ethanol, and critical-point-dried from CO_2. Finally the samples were sputter-coated with a layer of 10 nm gold/palladium (Bal-Tec MED 010) and examined at 20 kV accelerating voltage in a Hitachi S-800 field emission scanning electron microscope.

Transmission electron microscopy

Living worms were cryoimmobilized with a Bal-tec HPF 010 high-pressure freezer. They were transferred to custom processing

chambers which were placed in 2 ml microtubes (Sarstedt #72.694) containing a substitution medium consisting of 1% osmium tetroxide and 0.5% uranyl acetate in acetone. Samples were kept at −90°C for 48 h, at −60°C for 20 h, and at −40°C for 10 h in a Leica EM AFS2 freeze substitution unit. After washing with acetone the samples were infiltrated with Epon and polymerized at 60°C for 48 h. Ultra-thin sections obtained with a Reichert Ultracut S ultramicrotome, mounted on wide slot grids with pioloform support films and stained with 2% methanolic uranyl acetate for 30 min and 0.4% lead citrate for 3 min. Sections were viewed in a FEI Tecnai G2 Spirit electron microscope at 120 kV.

Phalloidin staining

The phalloidin staining was performed as described (Rudel et al. 2005) with small modification. Young adult worms were washed three times in M9 and then fixed in a solution of 2% paraformaldehyde (PFA) in PBS for 1 h on ice. The PFA solution was removed and the worms were washed in a fresh PBS solution for 15 min on ice. PBS was removed and the sample was extracted sequentially with graded series of acetone (−20°C) for 15 min. After removal of acetone, the sample was re-hydrated in PBS for 15 min and PBS was replaced with a fresh solution containing the dye of interest. To stain actin filaments, fluorescently labeled phalloidin was used at a final concentration of approximately 0.15 AM (AlexaFluorR 488 Phalloidin A-12379, Molecular Probes, Invitrogen, Karlsruhe, Germany). Worms were stained for two hours. Fixed worms were mounted in a drop of VectaShield mounting medium (H-1000, Vector Laboratories Inc., Burlingame, CA, USA) on a 5% agar in water pad. Samples were viewed on an Axioplan 2 microscope (Zeiss, Munich, Germany) using a Polychromator Illumination System (Visitron Systems GmbH, Puchheim, Germany). Pictures were taken using the Meta View program (version Meta Series 4.5, Visitron Systems GmbH) and a digital camera (MicroMax 5 MHz System, Princeton Instruments Inc., Trenton, NJ, USA).

Phylogenetic analysis

The program MUSLCE (Edgar 2004) was used for multiple alignments; Gblocks (Castresana 2000) for alignment curation; PhyML (Anisimova and Gascuel 2006); (Guindon and Gascuel 2003) for phylogeny reconstruction and TreeDyn (Chevenet et al. 2006) for tree drawings.

RESULTS

The *P. pacificus* genome contains a single member of all four subfamilies of Pax genes

To obtain the full complement of Pax genes of the nematode *P. pacificus*, we made use of the recently assembled draft of the *P. pacificus* genome and performed 5′ and 3′ RACE experiments (Dieterich et al. 2008). We identified four Pax genes, which differ from one another in their exact domain composition. Besides the previously identified *Ppa-pax-3* (Yi and Sommer 2007) we found three more Pax genes that show highest sequence similarity to *Cel-egl-38*, *Cel-pax-1*, and *Cel-vab-3*/*pax-6*, respectively (Fig. 1A). Interestingly, all four *P. pacificus* Pax genes show a domain composition that is identical to the corresponding *C. elegans* genes. Maximum-likelihood phylogenetic analysis of all *P. pacificus*, *C. elegans* and mice Pax genes show that three of the four *P. pacificus* genes form 1:1 orthologs with the corresponding *C. elegans* genes (Fig. 1C). *P. pacificus* contains one member of all four Pax gene subgroups, whereas *C. elegans* contains two members of the PAX2 family, *Cel-pax-2* and *Cel-egl-38*. Recent studies suggest that *Cel-pax-2* and *Cel-egl-38* result from a gene-duplication because *Caenorhabditis briggsae* has only one PAX2 family member (Wang et al. 2004). Similar to the situation in *C. briggsae*, we were unable to identify a second PAX 2/5/8 gene in *P. pacificus*, which might be due to its absence in the genome or the incompleteness of the genome draft. There is only one copy of a *pax-3* in the *P. pacificus* genome. *Ppa-pax-3* is special in that it encodes the only Pax protein that contains all three functional domains PD, OP, and HD (Fig. 1A). Thus, genome analysis suggests that *Ppa-pax-3* is a single copy gene. With the two available alleles *Ppa-pax-3* represents the first viable nematode *pax-3* mutant, providing the opportunity for a genetic investigation of PAX-3 function in nematodes (Yi and Sommer 2007).

P. pacificus pax-3 plays a role during embryogenesis and affects overall morphogenesis

Ppa-pax-3(*tu214*) is a long deletion mutant that misses the homeodomain, representing a strong reduction-of-function allele, *Ppa-pax-3*(*tu358*) contains an amino acid replacement of His to Arg in the conserved octapeptide (Fig. 1B). Interestingly, both of these alleles show a similar penetrance for the ventral epidermal cell death phenotype (Yi and Sommer 2007).

Ppa-pax-3 affects survival in both, embryonic and post-embryonic development. In *Ppa-pax-3*(*tu214*) homozygous mutants, approximately 23% of the progeny arrest as embryos and only 65% of the progeny mature to adult. In *Ppa-pax-3*(*tu358*), 15% of the progeny arrest during embryogenesis (Table 1). Many *Ppa-pax-3* mutant worms hatch with a Dumpy phenotype and nearly all mutant animals show movement defects. The average body length of

Table 1. *Ppa-pax-3* affects survival and body length

Genotype	Embryo lethal	Survival to adulthood (%)	Body length (mm)
(A) Wild-type PS312	0	100	0.82 ± 0.05
(B) *Ppa-pax-3*(*tu358*)	15%	70	0.67 ± 0.09
(C) *Ppa-pax-3*(*tu214*)	23%	65	0.72 ± 0.09

Embryo lethality and survival to adulthood were scored on >100 embryos for each genotype. Body length was measured on 20 late adult worms each, taking the length from the mouth to the tail. SD is given.

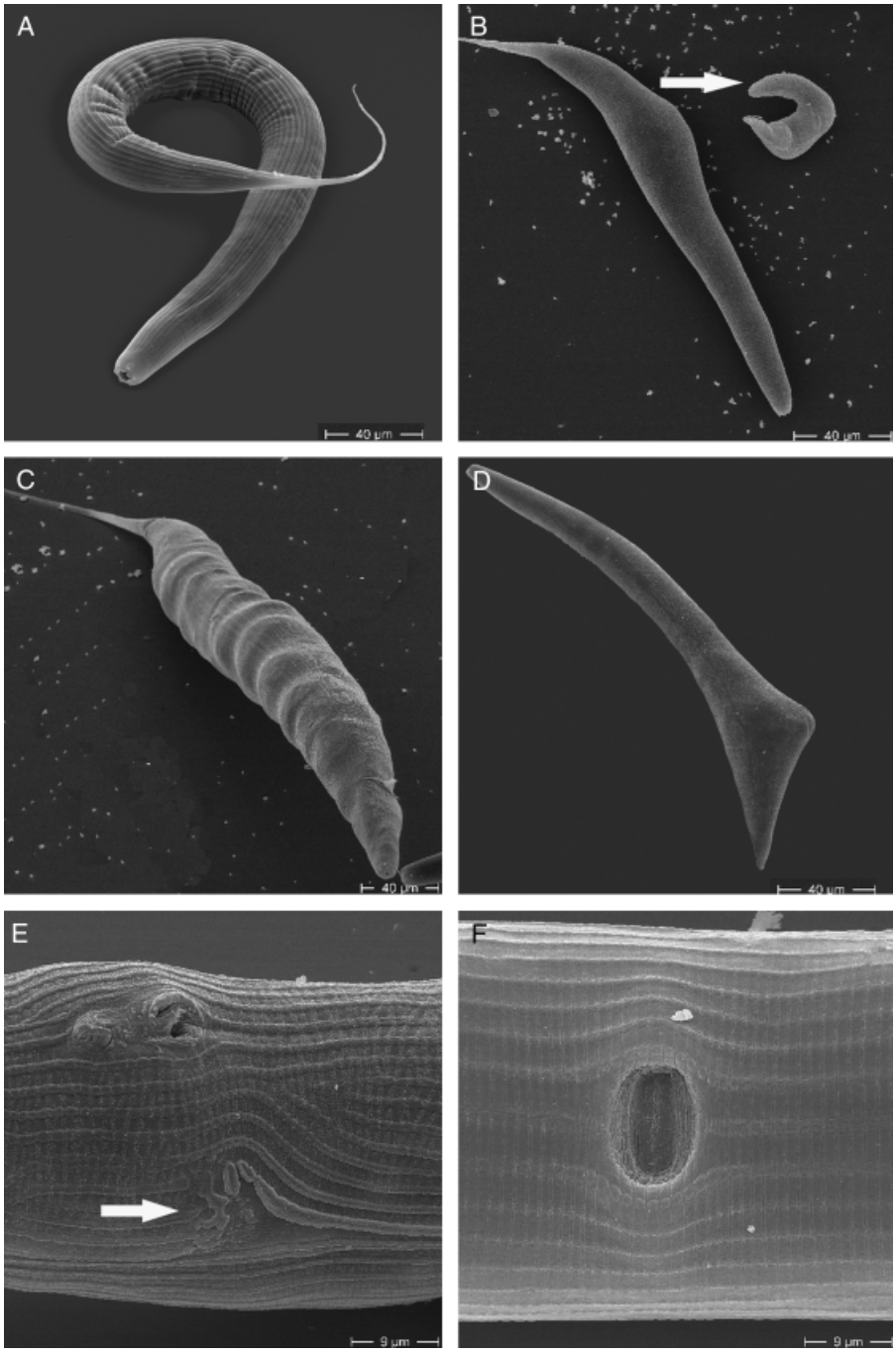

Fig. 2. Abnormal morphology and hypodermal defects reflect the disorganization of body wall muscle structures in *Ppa-pax-3* mutants. Scanning electron micrographs of young adult worms from wild-type (A and F) and *Ppa-pax-3* mutant (B–E). (A) *P. pacificus* wild-type adult hermaphrodite. (B–E) Typical *Ppa-pax-3* mutant animals. (B) The arrow points at an animal displaying the most severe, strong dumpy-like phenotype. (C and D) The surfaces of *Ppa-pax-3* mutant worms often appeared lumpy and uneven. (E) Arrow indicates the distorted cuticle pattern. (F) Surface structure of a wild-type animal in the region of the vulva.

mutant animals is approximately 20% shorter than that of wild-type animals (Table 1). In addition, their bodies appeared lumpy and uneven and random bumps are often observed (Fig. 2, B–D). Mutant animals with the most severe phenotype are strongly Dumpy and sterile and they die precociously (Fig. 2B). These phenotypes are similar to the phenotype described for *Cel-hlh-1*/CeMyoD mutants (Chen et al. 1994).

The *Ppa-pax-3* mutant shows severe disruption of the body wall muscles

The Dpy and movement phenotype of *Ppa-pax-3* mutant animals suggests defects in the formation of the body wall muscles. Locomotion in nematode is mediated by striated body wall muscles oriented in four quadrants longitudinally along the anterior/posterior body axis. Each muscle cell

contains approximately eight sarcomeres, which are composed of alternating bundles of thick filaments, containing myosin, and thin filaments, containing actin (Moerman and Fire 1977). In an attempt to identify specific abnormalities, we first examined the overall structure of muscle tissue by staining animals with phalloidin to visualize the actin-containing thin filaments of the body wall muscles. In wild-type worms, the actin network appeared straight and evenly stained (Fig. 3A). In *Ppa-pax-3* mutants in contrast, the actin network was poorly organized with patched sarcomeres (Fig. 3B). Some phalloidin staining extended outside the four muscle quadrants, which might correspond to muscle cells with abnormal morphogenesis (Fig. 3B).

To better characterize this muscle phenotype we used transmission electron microscopy. Wild-type worms displayed normal sarcomere organization and cellular composition (Fig. 3C). Muscle cells in the *Ppa-pax-3* mutant adults contained both thick and thin filaments, whereas sarcomeric organization was irregular and uneven (Fig. 3D). Cross-sections indicated that many myofilaments are wrongly oriented, often completely disorganized or they occurred along the antero-posterior axis of the animals (Fig. 3D). This phenotype is reminiscent of the muscle phenotype of *Cel-hlh-1* (Chen et al. 1994). Finally, both *Ppa-pax-3* mutants do not show a pharyngeal muscle phenotype, suggesting that *Ppa-pax-3* specially affects the development of striated muscles (supporting information, Fig. S1).

Ppa-hlh-1 and *Ppa-unc-120* show similar temporal expression patterns

In vertebrates, Pax3 controls myogenesis through its effects on the MRFs, Myf5, MyoD, and Mrf4 (Buckingham and Relaix 2007). We identified only one MRF-related gene in the *P. pacificus* genome. cDNA sequencing from 5′ and 3′ RACE experiments indicated that this *P. pacificus* gene shows high sequence similarity to *hlh-1* in *C. elegans*. Specifically, the conserved bHLH domain has an amino acid identity of 84% and a similarity of 92%. These two genes represent 1:1 orthologs and we have therefore named the *P. pacificus* gene

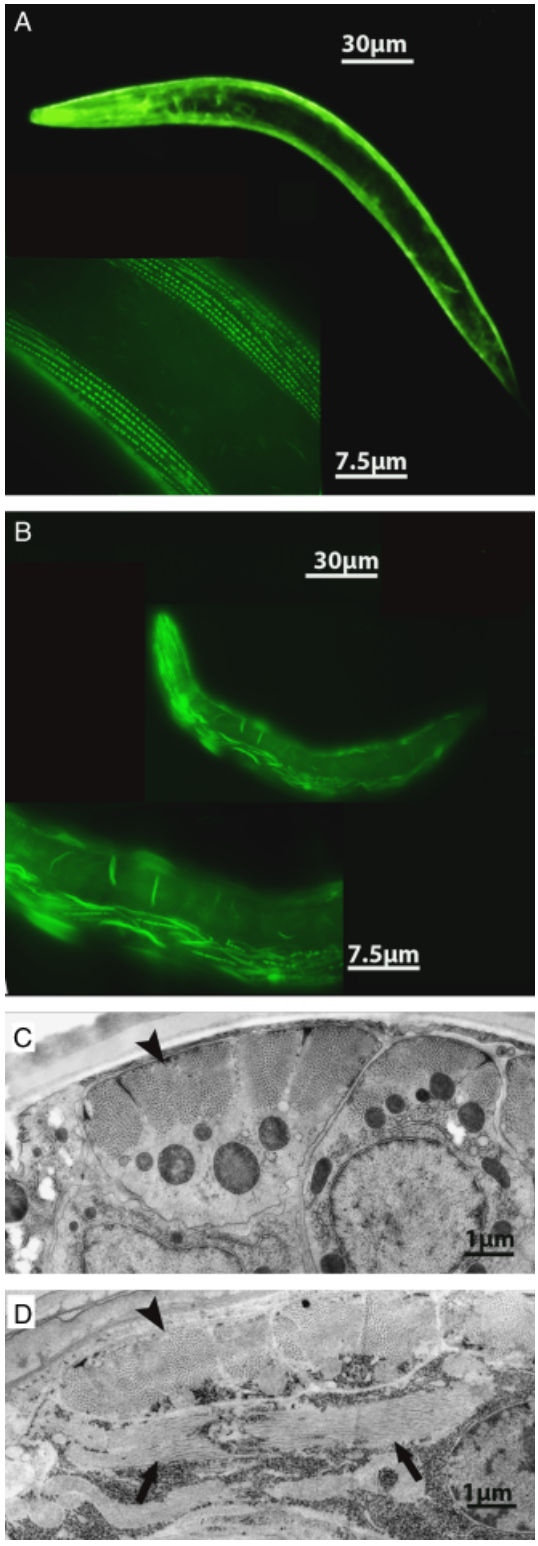

Fig. 3. Muscle structure in wild-type and *Ppa-pax-3(tu214)* mutant animals. Phalloidin staining (A and B) and transmission electron microscopy (C and D) of wild-type (A and C) and *Ppa-pax-3(tu214)* mutant animals (B and D). (A) *P. pacificus* wild-type animal shows a straight and evenly stained actin network. Inlet provides close-up view. (B) *Ppa-pax-3* mutant worm with a twisted actin network. Close-up view in inlet indicates that sarcomeres are poorly organized and some actin staining extends outside the four muscle quadrants. (C) Transmission electron micrsocopy of body wall muscle cells of a wild-type animal with normal sarcomere organization. The arrowhead points to a muscle cell in its appropriate positions directly beneath the hypodermis. (D) Transmission electron micrsocopy of a *Ppa-pax-3*(*tu214*) mutant animal shows misplacement and misorientation of muscle cells. The arrows point to internally misplaced muscle cells with abnormally oriented myofilaments. The arrowhead points to a muscle cell that is appropriately positioned beneath the hypodermis.

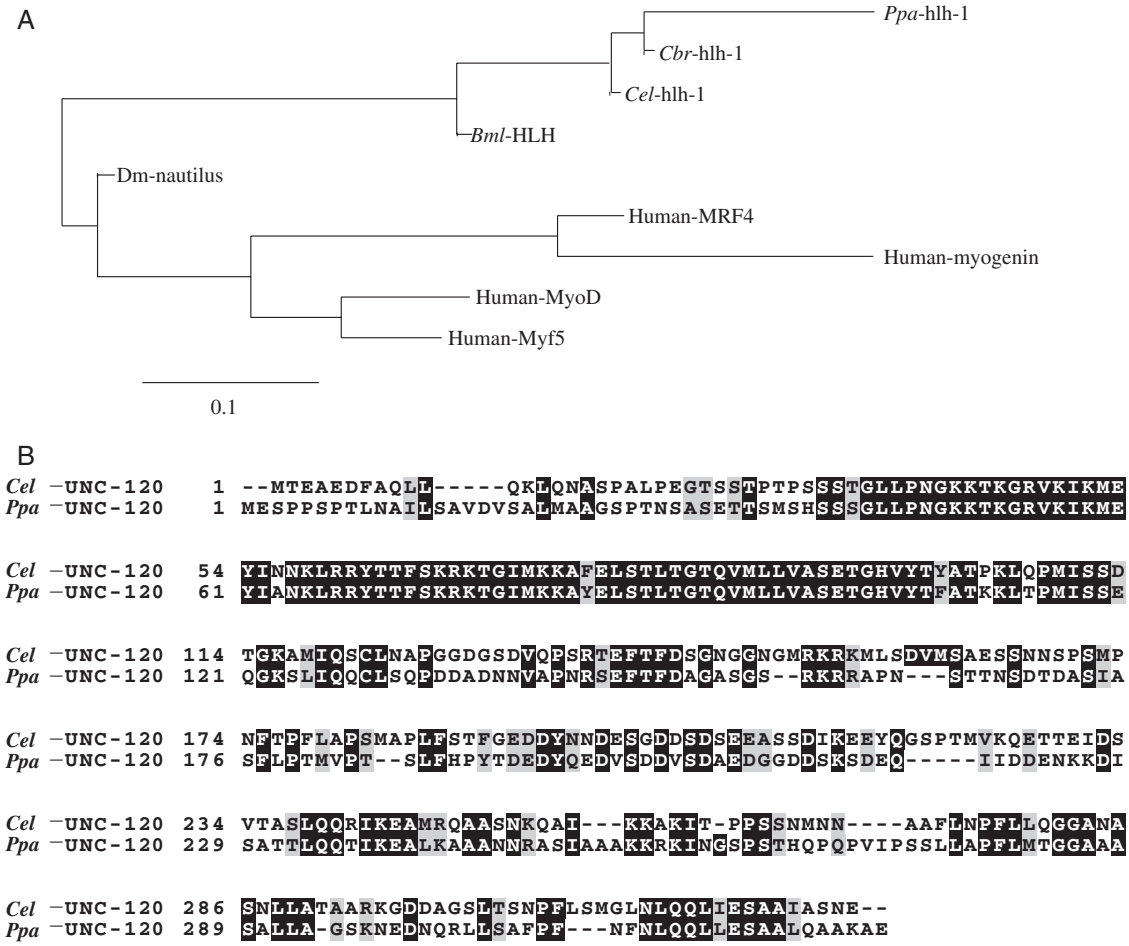

Fig. 4. Phylogeny of HLH-1 and UNC-120-like proteins. (A) Maximum-likelihood phylogenetic analysis of the bHLH domain of human MRFs and some MRF-related proteins from invertebrate species. Dm, *Drosophila melanogaster*; Cel, *C. elegans*; Cbr, *C. briggsae*; Ppa, *P. pacificus*; Bml, *Brugia malayi*. (B) Amino acid sequence alignment of *C. elegans* and *P. pacificus* UNC-120 proteins.

Ppa-hlh-1. Phylogenetic analysis indicates that *Ppa-hlh-1* groups together with all other known nematode MRF-related genes from *C. elegans*, *C. briggsae*, and the human parasite *Brugia malayi* (Fig. 4A). Another transcription factor known to be involved in myogenesis is SRF and its *C. elegans* ortholog *Cel-unc-120* (Fukushige et al. 2006). We cloned a *P. pacificus* gene that shows highest sequence similarity to *Cel-unc-120*. This gene has been named *Ppa-unc-120*/SRF and the encoded protein shows a 94% amino acid identity in the MADS box domain to *Cel*-UNC-120/SRF (Fig. 4B). Unfortunately, no *Ppa-hnd-1* gene was found in the draft of the *P. pacificus* genome. This might result from the incomplete *P. pacificus* genome sequence or rapid changes of the sequence of the *Ppa-hnd-1* gene, making it undetectable as a 1:1 ortholog.

To study the expression of *Ppa-hlh-1*, we have used quantitative RT-PCR experiments with synchronized embryos, J2, J3, J4, and adult animals. Specifically, we found *Ppa-hlh-1* to be expressed highest in the J2 larval stage, the stage with extreme muscle growth (Fig. 5A). *Ppa-hlh-1*/MyoD is highly expressed. Interestingly, *Ppa-unc-120*/SRF shows a very similar expression pattern to that of *Ppa-hlh-1*/MyoD (Fig. 5A). Both genes show their strongest expression in the J2 stage, whereas later larval stages show a reduced expression level (Fig. 5).

Ppa-pax-3 acts upstream of Ppa-hlh-1 and Ppa-unc-120

Given that the body wall muscle defects of *Ppa-pax-3* and *Cel-hlh-1* mutant animals are quite similar (Chen et al. 1994), we wanted to test if nematode *pax-3* and *hlh-1* might work in one signal pathway to regulate myogenesis in body wall muscles. To analyze if *Ppa-pax-3* is involved in the regulation of *hlh-1* expression, we analyzed the expression of *Ppa-hlh-1*/MyoD in *Ppa-pax-3* mutant and wild-type animals by quantitative RT-PCR. Considering the abnormal body wall muscle pattern in *Ppa-pax-3* mutants, we used the expression of two different internal standard genes, *Ppa-β-tubulin* and the ribo-

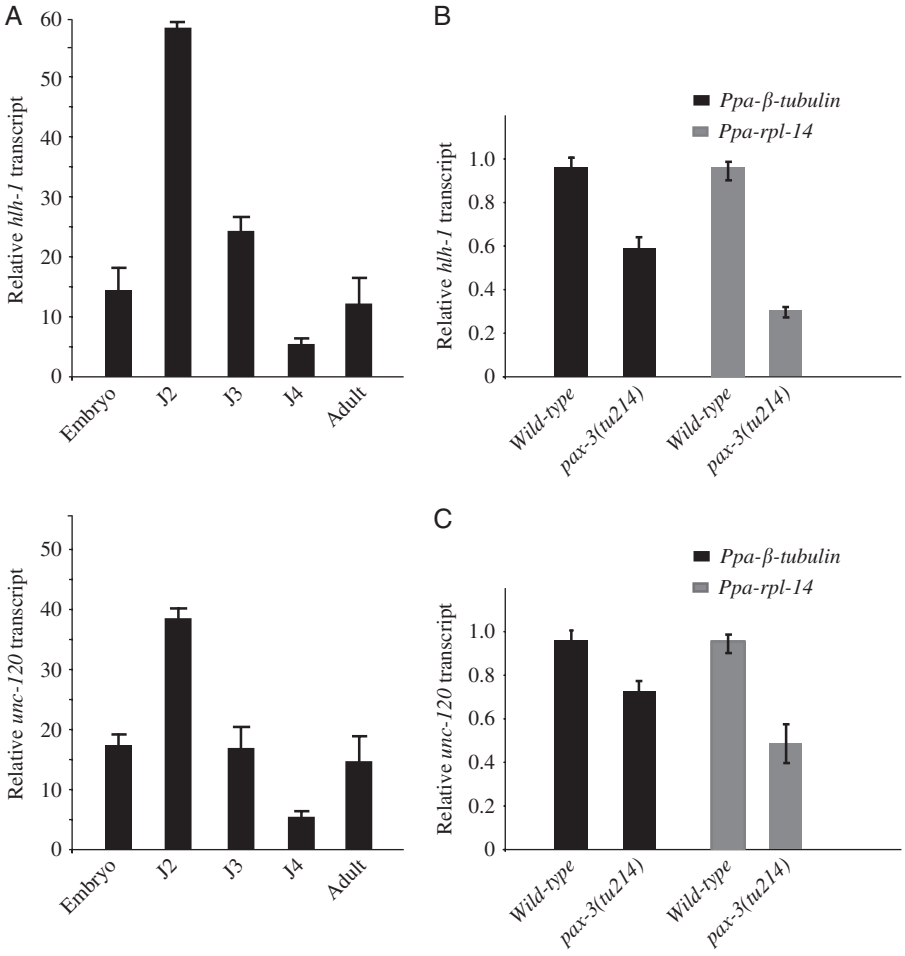

Fig. 5. *Ppa-hlh-1*/MRF and *Ppa-unc-120*/SRF transcript levels are reduced in *Ppa-pax-3* mutants compared with *P. pacificus* wild type. (A) *Ppa-hlh-1*/MRF and *Ppa-unc-120*/SRF transcript levels in staged *P. pacificus* wild-type animals. Transcript levels are given as arbitrary concentration unit ratios between targeted genes and *Ppa-β-tubulin*. RNA was prepared from 100 animals and experiments were carried out in triplicate. Error bars represent standard deviations. (B and C) Relative *Ppa-hlh-1*/MRF (B) and *Ppa-unc-120*/SRF (C) transcript levels in wild-type and *Ppa-pax-3(tu214)* mutant animals, with *Ppa-β-tubulin* (black bars) and *Ppa-rpl-14* (gray bars) as internal standard. Both *Ppa-hlh-1*/MRF and *Ppa-unc-120*/SRF transcript levels are decreased in mutant animals.

somal protein gene *Ppa-rpl-14* as reference to normalize the expression levels of the target gene. We used *Ppa-rpl-14* as a second internal control because *Ppa-β-tubulin* might itself be influenced in mutants affecting myogenesis. The relative *Ppa-hlh-1*/MyoD transcript level was reduced around 40% in *Ppa-pax-3* mutant when *β-tubulin* was used as the reference gene; whereas the expression of *hlh-1* was much more significantly reduced when *Ppa-rpl-14* was used as reference, with 70% reduction in *Ppa-pax-3* mutant compared with wild type (Fig. 5B). Interestingly, the expression of *Ppa-unc-120*/SRF is also reduced in *Ppa-pax-3* mutant animals (Fig. 5C). With *Ppa-β-tubulin* and *Ppa-rpl-14* as reference, *Ppa-unc-120* was 30% and 50% reduced, respectively. Together these results suggest that *Ppa-pax-3* acts upstream of *Ppa-hlh-1*/MyoD and *Ppa-unc-120*/SRF.

DISCUSSION

In this study we describe a critical role of *Ppa-pax-3* in the regulation of the development of body wall muscles. This is the first study describing a function of PAX-3 in myogenesis in any invertebrate. Together with other analyses, our studies suggest the conservation of a related transcriptional network between vertebrates and nematodes.

Regulation of *hlh-1* expression by PAX-3

Analysis of myogenesis in *C. elegans* revealed the unexpected redundancy of a muscle module consisting of *Cel-unc-120*, *Cel-hlh-1*, and *Cel-hnd-1* (Baugh and Hunter 2006; Fukushige et al. 2006). Due to the early larval lethality of *Cel-pax-3* mutants, a role of this gene as one potential regulator known from vertebrates has not been studied in detail. The data reported in this study on the satellite organism *P. pacificus* provide evidence for the function of PAX-3 in nematode myogenesis. First, phalloidin staining and transmission electron microscopy of muscle cells as well as the severe movement defects of *Ppa-pax-3* mutant animals revealed strong similarities to the phenotype of *Cel-hlh-1* mutant animals, which has been described in great detail

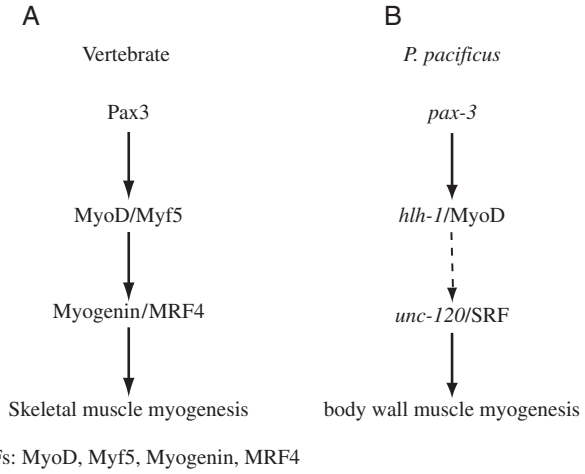

Fig. 6. A regulatory network of myogenesis in vertebrates and nematodes contains Pax3 to MRF as conserved modules. (A) Model of skeletal myogenesis in vertebrates. (B) Model for *Ppa-pax-3* function in myogenesis based on the data presented in this study for *P. pacificus*. Solid lines in the models do not necessarily represent direct regulation. While we draw a linear model, the data presented in this study do not completely rule out that PAX-3 regulates *Ppa-hlh-1*/MyoD and *Ppa-unc-120* in parallel.

(Chen et al. 1992; Chen et al. 1994). Second, expression profiling of *Ppa-unc-120* and *Ppa-hlh-1* in *Ppa-pax-3* mutant animals indicated that transcription of both genes is down regulated. Together, these findings suggest that myogenesis in vertebrates and nematodes is controlled by an—at least in parts—conserved regulatory module that involves Pax3 as an upstream regulator and among others, the MRF-like proteins HLH-1 (Fig. 6). Because no putative PAX binding sites are present in the promoter of *Ppa-hlh-1*, we assume this regulation to be indirect.

The existence of conserved genes in a regulatory network of myogenesis between vertebrates and nematodes does not rule out the likely existence of species-specific regulators. Already within the nematodes, such species-specific regulators are most likely to be present. Multiple genetic studies in *P. pacificus* on various aspects of postembryonic development and comparison to *C. elegans* revealed species-specific elements in transcription factor modules and signaling pathways (Sommer 2009). For example, the size of the vulva equivalence group is regulated by a transcriptional module in *P. pacificus* that consists of *Ppa*-HAIRY and *Ppa*-GROUCHO. While conserved in vertebrates and insects, the HAIRY/GROUCHO module does not even exist in *C. elegans*, although the downstream target of *Ppa*-HAIRY and *Ppa*-GROUCHO, the Hox transcription factor LIN-39 is a conserved regulator of the nematode vulva equivalence group (Chamberlin 2006; Schlager et al. 2006). Similarly, our previous studies on *Ppa-pax-3* in the regulation of programmed cell death in the ventral epidermis of hermaphroditic animals revealed the different composition of regulatory networks that share some key components (Yi and Sommer 2007). Therefore, studies reported by Fukushige et al. (2006) in *C. elegans* and those reported here for *P. pacificus* indicate the presence of a conserved core machinery of myogenesis. However, they do not suggest that the complete regulatory network will be shared among distantly related organisms.

An early separation of cardiac and striated muscle cell types?

There are two major muscle cell types in nematodes: striated body wall muscles used for locomotion and single-sarcomere pharyngeal muscles used for feeding (Epstein et al. 1974; Waterston 1988). Body wall muscles of nematodes are thought to be functionally equivalent to vertebrate skeletal muscles (for a review see Moerman and Fire 1977). Our analysis of *Ppa-pax-3* mutants suggests the absence of a pharyngeal muscle phenotype. This observation indicates the presence of other currently unknown regulators of nematode muscle development, which might act redundantly with *Ppa-pax3* or indepedently. In vertebrates, Pax3 specifically regulates skeletal muscle myogenesis (the body wall muscle equivalent), which would be consistent with the existence of other regulators involved in pharyngeal muscle development.

At the same time, the similarities in the *Ppa-pax-3* and the vertebrate Pax3 mutant phenotypes make it likely to suggest that already the last common ancestor of nematodes and vertebrates had at least two distinct contractile cell types, one cardiac-like, and one skeletal-like. We would speculate therefore, that the regulatory network involving Pax3 and MRF-like proteins only exists in the skeletal-like muscle.

Finally, it should be noted that whereas the strong reduction-of-function alleles of *Ppa-pax-3* cause severe disruption of the body wall muscle pattern, mutant animals still form body wall muscles. This finding suggests that *Ppa-pax-3* acts either in parallel with other pathways or that other genes function redundantly in the control of myogenesis. Two potential candidates known from *C. elegans* are *fozi-1* and *pal-1*. FOZI-1 is a transcription factor, which was shown to function in the specification of the striated body wall muscle fate in *C. elegans* (Amin et al. 2007). PAL-1 is a Caudal-related homeobox transcription factor, which is required genetically to activate key transcriptional regulators of myogenesis, including HLH-1/MRF and UNC-120/SRF (Baugh et al. 2005a, b; Fukushige et al. 2006; Yanai et al. 2008). More recent studies indicate that PAL-1 directly activates *hlh-1* in *C. elegans* (Lei et al. 2009). Therefore, further investigation is required to obtain a more complete picture of the myogenesis regulatory network in *P. pacificus*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Nomarski photomicrograph (A, C) and phalloidin staining (B, D) of pharyngeal muscle in wild-type animals (A, B) and *Ppa-pax-3* mutant animals (C, D) indicating that *Ppa-pax-3* mutants form normal pharyngeal muscles.