The C. elegans F-spondin family protein SPON-1 maintains cell adhesion in neural and non-neural tissues

Wei-Meng Woo1,*,†, Emily C. Berry1,2,*, Martin L. Hudson1, Ryann E. Swale1, Alexandr Goncharov2,3 and Andrew D. Chisholm1,2

The F-spondin family of extracellular matrix proteins has been implicated in axon outgrowth, fasciculation and neuronal cell migration, as well as in the differentiation and proliferation of non-neuronal cells. In screens for mutants defective in C. elegans embryonic morphogenesis, we identified SPON-1, the only C. elegans member of the spondin family. SPON-1 is synthesized in body muscles and localizes to integrin-containing structures on body muscles and to other basement membranes. SPON-1 maintains strong attachments of muscles to epidermis; in the absence of SPON-1, muscles progressively detach from the epidermis, causing defective epidermal elongation. In animals with reduced integrin function, SPON-1 becomes dose dependent, suggesting that SPON-1 and integrins function in concert to promote the attachment of muscles to the basement membrane. Although spon-1 mutants display largely normal neurite outgrowth, spon-1 synergizes with outgrowth defective mutants, revealing a cryptic role for SPON-1 in axon extension. In motoneurons, SPON-1 acts in axon guidance and fasciculation, whereas in interneurons SPON-1 maintains process position. Our results show that a spondin maintains cell-matrix adhesion in multiple tissues.

KEY WORDS: Spondin, Extracellular matrix, Cell adhesion, Morphogenesis, Axon guidance

INTRODUCTION

The morphogenesis of epithelial organs and organs is profoundly dependent on the extracellular matrix (ECM), especially the specialized forms of ECM known as basement membranes (BMs) (Miner and Yurchenco, 2004). Active remodeling of BMs by tissues is essential for many developmental events, and aberrant cell-ECM interactions underlie tumorigenesis and metastasis of epithelial tissues (Larsen et al., 2006). Developing axons often grow over BM substrates, and BM components play central roles both in tissue morphogenesis and in axon guidance (Hinck, 2004). Yet, rather than a passive scaffold for tissue morphogenesis, the ECM can be regarded as an active participant in tissue morphogenesis and cell signaling (Nelson and Bissell, 2006).

C. elegans embryonic epidermal morphogenesis is an example of an organogenesis process that involves multiple interactions between an epithelial sheet, underlying muscle, and an intervening BM (Chisholm and Hardin, 2005). In late embryogenesis, epidermal cells undergo coordinated shape changes that lead to embryo elongation, converting the ovoid embryo into a worm-shaped larva. Forces for elongation are generated within the epidermis by actomyosin-based contraction of circumferential actin bundles (Priess and Hirsh, 1986). Nevertheless, epidermal elongation is also critically dependent on the BM, indicating the importance of cell-cell interactions in coordinating the development of embryonic tissues.

Epidermal elongation beyond the 2-fold stage requires underlying muscle (Williams and Waterston, 1994). Mutants defective in body muscle function are paralyzed and arrest at the 2-fold stage of elongation – the Pat phenotype. The need for body muscle function in epidermal elongation may reflect a role for muscle in organizing the epidermal cytoskeleton. Body muscles lie underneath dorsal and ventral epidermis, and adhere to adjacent epidermis via the BM, which allows force transmission from muscle to epidermis. The muscle-epidermal BM itself also promotes the organization of muscle and of the overlying epidermis. Importantly, different ECM components contribute to distinct aspects of epidermal morphogenesis. The earliest BM component to be deposited, laminin, is required for assembly of the myofilament lattice and the localization of dense bodies. Animals that lack laminin completely arrest in early embryonic elongation with defective muscle morphogenesis (Huang, C. C., et al., 2003; Kao et al., 2006). The BM proteoglycan Perlecen/UNC-52 is essential for assembly of the myofilament lattice (Rogalski et al., 1993), whereas type IV collagen has a later role in muscle-epidermal attachment (Guo et al., 1991).

Other BM components, such as nidogen (NID-1) or type XVIII collagen (CLE-1), are not required for embryonic morphogenesis, but play crucial roles in axon outgrowth, guidance and synaptogenesis (Ackley et al., 2001; Kang and Kramer, 2000). Similarly, the laminin receptor dystroglycan (DGN-1) is not required in embryonic morphogenesis, but functions in axon guidance (Johnson et al., 2006). Integrin signaling is required for multiple aspects of neuronal development, including cell migration, axon fasciculation (Baum and Garriga, 1997) and guidance (Point et al., 2002). These findings underscore the role of BM as a central scaffold for the developing nervous system.

Spondins are a conserved family of ECM proteins that were originally identified as axon guidance factors in the vertebrate spinal cord (Klar et al., 1992). In vertebrates, spondins have context-dependent effects on axon outgrowth and cell migration, and can promote neuronal differentiation (Schubert et al., 2006). Despite intensive analysis, the mechanisms by which spondins affect cell behavior and their in vivo roles remain poorly understood. Here, we report that SPON-1, the sole C. elegans member of the spondin family, is essential for embryonic morphogenesis. We show that SPON-1 promotes muscle-epidermal adhesion and is required for the completion of epidermal elongation. In the nervous system, SPON-1

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promotes axon fasciculation and guidance, and continuously maintains neural architecture. These results are the first demonstration of an essential role for spondins in development.

MATERIALS AND METHODS

Isolation of spon-1 mutants and molecular cloning

All strains were generated from Bristol N2, and were grown under standard conditions (Brenner, 1974) unless stated. We used the mutations emb-9(g23ts cg46), pat-3(st564), ina-1(gn144), dig-1(ky188), unc-71(ju156), zng-4(kg34) and egl-15(m484). We used the GFP-expressing balancer mnl1 mls14 (Edgley and Riddle, 2001) to balance spon-1 lethal alleles.

We isolated the mutations ju438, ju402 and ju430ts in a screen for EMS-induced mutants displaying defective elongation and lumpy epidermal morphology (Mei Ding, W.-M. and A.D.C., unpublished). Two F1 progeny of mutagenized parents were picked and placed onto a plate, and their F2 broods scored for malformed L1 larvae. e2623 was isolated by Jonathan Hodgkin (personal communication). nc30 was isolated in screens for mispositioned ventral nerve cords (Shioi et al., 2001).

Genetic mapping placed spon-1 between dpy-10 and unc-4 on chromosome II. SNP mapping placed ju430 between pkP2148 and pkP2150, an interval of 420 kb. A 5.6 kb DNA fragment that contains ~1.4 kb 5’ DNA sequence (primer sequences available on request). This DNA was used to prime PCR from wild-type embryos, and PCR products were run on agarose gels to determine if they were from wild-type or mutant backgrounds. To tag SPON-1 at the N terminus with Venus YFP, we used a modular pRF4 vector kit), such that GFP was fused in-frame at residue 756 of SPON-1, generating the expression construct pCZ695. Then, we ligated this PCR fragment into pPD95.75 (Fire lab -Stu’ Bam’ HI) DNA sequence (primer sequences available on request). This DNA was used to prime PCR from wild-type embryos, and PCR products were run on agarose gels to determine if they were from wild-type or mutant backgrounds.

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Generation of spon-1 reporter genes

We used duplex PCR to generate Pspgon-1-GFP reporters, using 3.3 kb of 5’ DNA sequence (primer sequences available on request). This DNA was injected into wild-type animals at ~20 ng/μl with pRF4, generating juEx592 and juEx593. To make the SPON-1::GFP C-terminal fusion pCZ697, a 5-kb BamHI-Stul fragment of pCZ695 was subcloned into pPD95.75 (Fire lab vector kit), such that GFP was fused in-frame at residue 756 of SPON-1, truncating the protein after TSR4. pCZ697 was injected into spon-1(ju430)/mnl1 mls14 animals at 1 ng/μl, with pRF4 as a marker. All 17 transgenic lines failed to rescue ju430. An outcrossed version of transgenic line juEx734 was used in immunostaining experiments.

To tag SPON-1 at the N terminus with Venus YFP, we used a modular strategy (Hudson et al., 2006). We injected SPON-1::GFP at 10 ng/μl with Ptxs-3-RFP (50 ng/μl) into spon-1(ju402)/mnl1 mls14 hermaphrodites. Rescued ju402 homozygotes were selected on the basis of the absence of mls14, yielding transgenic lines juEx1111 and juEx1112. To express SPON-1 in pharyngeal muscles, we used the myo-2 promoter from pPD118.3. Pmyo-2::GFP:SPON-1 was injected into spon-1(ju402)/mnl1 mls14 animals at 50 ng/μl with Ptxs-3-RFP. Viable ju402 homozygous transformants were selected, yielding lines juEx1302-1304.

<table>
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<tr>
<th>Allele, temperature</th>
<th>n</th>
<th>Embryonic lethality</th>
<th>Larval lethality</th>
<th>Total lethality</th>
<th>Vab adults</th>
<th>non-Vab adults</th>
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<td>1116*</td>
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<td>1.1%</td>
<td>4.4%</td>
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<td>148†</td>
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<td>39.9%</td>
<td>91.2%</td>
<td>8.8%</td>
<td>0%</td>
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<td>635†</td>
<td>44%</td>
<td>66%</td>
<td>100%</td>
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<td>na</td>
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<tr>
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<td>75.4%</td>
<td>96.6%</td>
<td>3.4%</td>
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<td>Pmyo-2-GFP::SPON-1†, 20°C</td>
<td>285†</td>
<td>66.3%</td>
<td>33.7%</td>
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<td>spon-1(ju402), Pmyo-2-GFP::SPON-1, 20°C</td>
<td>789*</td>
<td>0.4%</td>
<td>0.2%</td>
<td>0.6%</td>
<td>0%</td>
<td>99.4%</td>
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* Determined from complete broods of homozygous strains.
† Progeny of ju430 homozygotes grown at 15°C and shifted to 25°C as young adults.
‡ Progeny of heterozygous balanced strains. spon-1 mutants were identified as non-GFP-expressing animals segregating from spon-1(mnl1 mls14).
§ Transgene juEx1302: na, not applicable.
Antibody generation and immunostaining

We raised antibodies against peptides corresponding to residues 499-547 in TSR2 and purified the antisera according to standard procedures. The antisera recognize recombinant SPON-1 C-terminal proteins in lysates (not shown). Whole-mount immunofluorescence was as described (Finney and Ruvkin, 1990), except that fixation was extended to 3.5-4 hours, an optimization for ECM antigens (J. R. Crew and J. M. Kramer, personal communication). Monoclonal supernatants MH3 (anti-PERLAC), and MH25 (anti-αPAT-2 integrin; Developmental Studies Hybridoma Bank, University of Iowa) were used at a dilution of 1:200; DMS-6 (anti-MHC A) (Miller et al., 1993) was diluted 1:200. Phalloidin staining was as described (Ding et al., 2003). To detect GFP, we used monoclonal 3E6 (Invitrogen, Carlsbad, CA) or a rabbit polyclonal (A11122) at 1:500 to 1:1000. Raw or purified anti-SPON-1 antisera were diluted 1:100 for embryo staining and 1:800 for mixed stage staining, unless stated. For anti-SPON-1 immunostaining, we used pCZ695 arrays juEx696 and juEx698, both in ju402 background. We acquired images on Zeiss Axioplan 2 or LSM 510 confocal microscopes.

RESULTS

Mutations in spon-1 disrupt morphogenesis and affect a C. elegans spondin

To identify genes required for muscle-epidermal interactions, we screened for mutations causing late arrest in elongation and muscle detachment. From a screen of approximately 8000 haploid genomes, we identified three mutations (ju438, ju402 and ju430ts) that cause highly penetrant defects in epidermal elongation and muscle attachment. We found that these mutations were allelic to vab-13(e2623), found in screens for morphogenetic mutants (J. A. Hodgkin, personal communication), and ven-3(nc30), isolated in a screen for mutations defective in fasciculation of the ventral nerve cord (Shioi et al., 2001). All five mutations affect the same gene, here renamed spon-1; e2623 mutants are slightly dumpy and are egg-laying defective (Egl); they display a variably abnormal (Vab) epidermal morphology that is more pronounced in early larvae (Fig. 1B,C). Other spon-1 alleles cause fully penetrant embryonic or early larval lethality with aberrant epidermal morphology and detached body muscles (Table 1, Fig. 1E,F). ju348 and ju402 are nonconditional lethals, and ju430 is a temperature-sensitive allele that resembles e2623 at 15°C and is inviable at 25°C. In penetrance, the five alleles rank from strongest to weakest: ju402> nc30> ju348> ju430ts> e2623. In addition to the epidermal elongation, muscle attachment and axon guidance defects described here, spon-1 mutations also disrupt development of the excretory canal and of epidermal seam cells (Shioi et al., 2001).

By mapping and transformation rescue, we identified the gene affected by these mutations as being F10E7.4 (Fig. 2A), which encodes a member of the F-spondin family of ECM proteins. Spondins are defined by the spondin domain, of unknown function, and are classified into two subfamilies, the F-spondins and the Mindins that resemble

SPON-1 is synthesized in body wall muscles and localizes to specific basement membranes

To understand how SPON-1 functions, we first assayed the activity of the spon-1 promoter. Pspon-1-GFP reporters were expressed exclusively in body wall muscles from early elongation onwards (Fig. 3A-C). We constructed translational GFP fusions in which SPON-1 was tagged with GFP either at the N terminus following the signal sequence, or after the fourth TSR (see Materials and methods). N-terminal SPON-1::GFP transgenes rescued all phenotypes of spon-1(ju402); C-terminal GFP fusions showed similar localization but did not rescue spon-1 mutants, suggesting that C-terminal TSRs are required for the morphogenetic function of SPON-1. Rescuing SPON-1::GFP by expression from the comma stage onwards (Fig. 3D, J) and showed localization to embryonic BM (Fig. 3I). In larvae and adults, SPON-1::GFP donor. The ju348, e2623 and ju430 ts mutants cause missense alterations in TSRs 2,3 and 4, respectively (Fig. 2D), suggesting that these TSRs are crucial for SPON-1 function.
Fig. 3. SPON-1 is synthesized in muscle and localizes to basement membranes. (A) Ventral view of an adult midbody showing that Pspon-1-GFP (juEx593) is expressed in body wall muscles (arrows, two muscle quadrants). (B) DIC optics showing the muscle quadrants (arrows) on the ventral side near the vulva. (C) Embryonic expression of Pspon-1-GFP from the comma stage onwards. (D-H) SPON-1::GFP localization visualized by anti-GFP immunostaining. (D) In 1.5-fold stage embryos SPON-1::GFP is localized to muscle quadrants (arrowhead). (E) In juEx1111 larvae, SPON-1::GFP localizes at muscle dense bodies (inset, large arrowhead) and at M lines (inset, small arrowhead). (F) Colocalization of SPON-1::GFP (anti-GFP) and the dense body component vinculin (MH24 immunostaining) in 1::GFP expressed under the control of the pharynx-specific myo-2 gene (juEx1302) localizes to muscle BMs (arrowhead) in 3-fold stage embryos (anti-GFP immunostaining). (J/K) Immunostaining with anti-SPON-1 antibodies of animals of genotype spon-1(ju402); juEx698[spoon-1(+)]. (I) Dorsal view of a 1.5-fold stage embryo stained with anti-SPON-1 (red), and anti-Myotactin (MH46) and anti-AJM-1 (MH27) (both green), which mark muscle-epidermal attachments and epidermal adherens junctions, respectively. SPON-1 is in muscle cells, beneath Myotactin staining. (K) Larva stained with anti-SPON-1 (red) and anti-MHC (green) to show body muscles. SPON-1 expression was detected in or adjacent to body muscles (arrow) and the excretory canal (arrowhead). Scale bars: 10 μm.

Localized to dense bodies and M lines on muscle surfaces (Fig. 3E,F), as determined by colocalization with the dense body component vinculin (Barstead and Waterston, 1989). Dense bodies and M lines are sites of integrin-based adhesions (Francis and Waterston, 1991), and are also enriched for the BM components UNC-52/Perlecain (Rogalski et al., 1993) and EPI-1/αβ3 laminin (Huang, C. C. et al., 2003). We consistently detected SPON-1::GFP on BM surrounding the pharynx (not shown), and within coelomocytes (Fig. 3H). As coelomocytes endocytose extracellular proteins (Fares and Greenwald, 2001), we infer that SPON-1::GFP fusions are secreted. As the spon-1 promoter is not active in the pharynx, SPON-1 can move from sites of synthesis to sites of localization, like type IV collagen (Graham et al., 1997). Unlike type IV collagen, we did not detect SPON-1::GFP at intestinal or gonadal BMs. We also generated antibodies that detect SPON-1 in whole-mount immunostaining of animals overexpressing SPON-1, but not in wild-type animals. The pattern of anti-SPON-1 staining in overexpressing animals was similar to that of SPON-1::GFP, including expression in embryonic and larval muscles (Fig. 3I,K), the excretory canal (Fig. 3K, arrowhead), pharyngeal BM (Fig. 3G) and coelomocytes (not shown). We conclude that SPON-1 is secreted from muscle and incorporates into some, but not all, BMs, and that it is enriched at integrin-based adhesion sites.

The accumulation of SPON-1 on pharyngeal basement membrane suggested that SPON-1 is recruited to BM distant from the cells that synthesize it. To test whether SPON-1 can function distant from its sites of synthesis, we expressed SPON-1::GFP in pharyngeal muscles using the myo-2 promoter (Okkema et al., 1993). Such transgenes rescued the epidermal elongation and lethality of spon-1(ju402)-null mutants (Table 1). Pharyngeally expressed SPON-1::GFP specifically accumulated at muscle-epidermal BMs during late embryogenesis (Fig. 3I). These results show that SPON-1 can be produced by cells that do not normally express it, and that it translocates within the ECM to function in specific BMs.

**spon-1 mutants display late-onset defects in epidermal elongation and muscle attachment**

To determine when spon-1 functions in epidermal morphogenesis, we performed timelapse microscopy on spon-1 embryos. Most (18/19) spon-1(ju402) mutants developed normally past the 2-fold stage of elongation, then either elongated to the 3-fold stage and retracted, or did not reach the 3-fold stage. spon-1 embryos displayed muscle movements before arrest (Fig. 4E-H; see Movie 1 in the supplementary material): they twitched at 1.75-fold (~430 minutes) and kept twitching until 3-fold stage. However, only three out of 18 embryos displayed vigorous rolling movements similar to those of the wild type. Muscle movements slowed down between the 2- and 3-fold stages, and then stopped (paralysis). During this period of slower muscle twitching, the epidermis retracted and became uneven in shape (Fig. 4G,H). ju348 and ju430 mutations caused similar or slightly weaker elongation phenotypes than did ju402. Thus, although many spon-1 mutants had a body length comparable to that of a 2- or 2.5-
fold stage embryo, they usually elongated beyond this stage and then retracted. Compared with other extracellular matrix mutants, spon-1 most resembles mutants such as emb-9, which lack type IV collagen.

To address when spon-1 acts in epidermal elongation, we determined the temperature-sensitive period for spon-1(ju430) (Fig. 4R). When spon-1(ju430) embryos grown at the permissive temperature (15°C) were shifted to the restrictive temperature (25°C) at the 1.5-fold stage, 92-100% arrested as embryos or early larvae. When these embryos were shifted up after the 2-fold stage, lethality progressively dropped to levels observed in animals raised at the permissive temperature. We infer that SPON-1 acts directly in elongation after the 2-fold stage.

Although SPON-1 does not appear to be essential for the early integrin-dependent assembly of the myofilament lattice, the localization of SPON-1 to integrin attachment sites suggested that SPON-1 might have a subtle or redundant role in integrin-mediated adhesion. We therefore used double-mutant analysis to test whether spon-1 interacts with integrin signaling. As animals lacking PAT-2 or PAT-3 arrest at the 2-fold stage, we focused on the α-integrin INA-1, which is not essential for embryonic elongation, but which colocalizes with PAT-3 in embryonic BMs (Baum and Garriga, 1997). Animals carrying the hypomorphic mutation ina-1(gm144) display normal epidermal elongation (not shown). spon-1(ju430) ina-1(gm144) double mutants were completely inviable (in contrast to either single mutant at 20°C) and arrested in early elongation (Fig. 4M-P), suggesting that SPON-1 and INA-1 may act redundantly in early elongation. Strikingly, spon-1/+ ina-1 animals resembled spon-1 homozygotes in that they showed a late elongation arrest (Fig. 4I-L). These results suggest that when integrin function is reduced, SPON-1 becomes dose dependent for embryonic elongation.

**SPON-1 maintains muscle attachment during later elongation and larval development**

The phenotype of spon-1 mutants suggests that, unlike integrins or Perlecan/UNC-52, SPON-1 was not essential for initial assembly of the myofilament lattice. Similarly, the late onset of epidermal elongation defects in spon-1 implies that it is not essential for the assembly of trans-epidermal attachments, as these are required for early elongation (Bosher et al., 2003; Woo et al., 2004). Instead, the late block in elongation appears to be a result of muscle detachment, which in turn leads to failure to maintain epidermal cell shape. We examined epidermal and muscle morphology by using the adherens junction protein AJM-1 to mark epidermal cell boundaries and body muscle myosin heavy chain (MHC) to mark muscle cells (Miller et al., 1983). MHC staining was normal in early elongation (Fig. 5A,B), but, as elongation progressed, we observed frequent gaps along muscle quadrants (Fig. 5C,D) due to muscle detachment. MHC organization in the remaining muscle was essentially normal. Muscle-associated PAT-3/β-integrin and UNC-52/Perlecan likewise became fragmented after the 2-fold stage because of muscle detachment (Fig. 5E-H). Epidermal actin bundles became misoriented following elongation arrest in regions where muscles had detached (Fig. 5I,J). Epidermal attachment structure components, including intermediate filaments and Myotactin, appeared normal before elongation arrest, but became discontinuous in arrested embryos (Fig. 5K,L). These results confirm that SPON-1 is not essential for early muscle or epidermal organization, but becomes essential in later muscle-BM-epidermal adhesion.

To test whether SPON-1 was required in later muscle adhesion, we analyzed post-embryonic muscle anatomy using GFP markers and electron microscopy. In addition to defective muscle-epidermal attachment, spon-1 mutants also displayed defects in muscle-muscle adhesion within a muscle quadrant (Fig. 5M,N). Muscle-muscle adhesion became progressively more defective during larval development and was suppressed by levamisole paralysis, indicating that spon-1 mutant muscles are pulled apart by the force of muscle contraction (Fig. 5O). Muscle ultrastructure in spon-1 mutant larvae
was disorganized; in particular, the BM between muscle and epidermis was thicker and invaginated into the muscle (Fig. 5P-R). We conclude that SPON-1 acts continuously throughout development to promote strong adhesion both at muscle-muscle and at muscle-BM interfaces.

**SPON-1 promotes axon fasciculation and guidance, and acts in parallel to ADAM UNC-71 in outgrowth**

F-spondin has context-dependent roles in axonal outgrowth and fasciculation in vertebrates (Bursten-Cohen et al., 1998; Tzarfati-Majar et al., 2001a). We therefore examined specific axon morphologies to determine the roles of SPON-1 in C. elegans nervous system architecture. As *spon-1* mutants arrest in embryogenesis, we examined the progeny of *spon-1* mutant animals for the weak allele *e2623* or the conditional allele *ju430*. *spon-1* mutants displayed widespread defects in axon guidance and fasciculation of motoneurons and PVQ interneurons, described below, as well as defects in migration, fasciculation and guidance of mechanosensory neurons (not shown).

*spon-1* mutants displayed extensive defasciculation of motoneuron processes within the ventral cord (Fig. 6A,B, Table 2). Motoneuron commissures also displayed defects in left-right choice of outgrowth (Table 2) and often deflected laterally upon reaching the subdorsal muscle quadrant, before eventually reaching the dorsal cord (Table 2A, Fig. 6C-E). To determine whether such guidance defects might be a secondary effect of muscle detachment, we examined motor commissure and muscle morphology simultaneously using the *trs10* marker. Of 20 commissures with dorsoventral guidance defects, only five were in regions of muscle detachment; conversely, in regions of muscle detachment, we infrequently saw normal commissural guidance (Fig. 6F). We conclude that dorsoventral guidance defects appear to arise independently of muscle detachment.

*spon-1* mutants raised at semi-permissive temperatures had a normal number of D neuron commissures, suggesting SPON-1 is not essential for the outgrowth of commissures (Table 2). To test whether a further reduction in SPON-1 function could affect outgrowth, we examined the progeny of *spon-1* mutant animals shifted to the restrictive temperature as L4s, and found that 100% (60/60) of *trIs10* animals developed normal number of DD commissures reached the dorsal midline, as in the wild type (Fig. 6I-J). *spon-1* mutant animals also displayed a normal number of DD commissures (Fig. 6H). Although motoneuron outgrowth appeared normal in *spon-1* single mutants, *spon-1* mutations significantly enhanced the outgrowth defects of *unc-71* ADAM mutants (Huang, X. et al., 2003) (Table 2B). We infer that SPON-1 has a minor role in outgrowth that is masked by redundancy with other pathways, such as UNC-71.
Functions of a C. elegans spondin

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Fig. 6. SPON-1 is required for motoneuron fasciculation and dorsoventral guidance. (A-E) Motoneuron processes (juls76) in wild-type (A,C) and spon-1(ju430) mutant (B,D,E) L4s. (B) Defasciculation of D neuron ventral processes in ju430 mutants (arrowhead). (C) Motoneuron commissures in the wild type extend from ventral to dorsal on the right side. (D,E) Motor commissures in spon-1(ju430) mutants turn laterally (arrowhead) upon contacting the dorsal muscle quadrant (m, dotted line) and either extend and terminate subdorsally (D) or turn back to the dorsal midline (arrowhead, E). (F,G) Commisural guidance defects occur independently of muscle detachment (tris10); commissure guidance can be normal (arrowhead) in regions of body wall muscle detachment (F) and can be aberrant in regions of normal attachment (arrowhead, G). (H-J) D neuron outgrowth in wild type (juls76, H), in a ju430 juls76 embryo (I, progeny of animal shifted to 25°C as L4) and in a spon-1(ju402) arrested hatchling (j, ynl373 marker); all six D neuron commissures (DD1-6) extend dorsally. Scale bars: 10 μm.

Table 2. Motoneuron fasciculation and guidance defects in spon-1 mutants

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<th>Genotype, temp</th>
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<th>Ventral cord defasciculation (%)</th>
<th>Commissure handedness defect (%)</th>
<th>Dorsoventral guidance defect (%)</th>
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<td>43</td>
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<td>42</td>
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<td>56.1% (8.7%)**</td>
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<td>40</td>
<td>17.5% (2.5%)*</td>
<td>87.5% (15%)**</td>
<td>40% (4.1%)**</td>
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<tr>
<td>ju430ts 15°-25° upshift</td>
<td>37</td>
<td>16.2% (4.1%)**</td>
<td>43.2% (4.7%)**</td>
<td>2.7% (0.2%) ns</td>
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A Interaction between spon-1 and unc-71

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<th>Genotype</th>
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<th>Commissure handedness defect (%)</th>
<th>Dorsoventral guidance defect (%)</th>
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<td>N2</td>
<td>61</td>
<td>0%</td>
<td>0% (0.2%)</td>
<td>0%</td>
</tr>
<tr>
<td>unc-71(ju156)</td>
<td>45</td>
<td>73%</td>
<td>24% (5%)</td>
<td>0% (0%)</td>
</tr>
<tr>
<td>ju430ts</td>
<td>45</td>
<td>0%</td>
<td>11% (3%)</td>
<td>0% (0%)</td>
</tr>
<tr>
<td>ju430ts:ju156</td>
<td>48</td>
<td>100%***</td>
<td>44% (10%)</td>
<td>44% (9%)**</td>
</tr>
</tbody>
</table>

D morphology was scored using the Punc-25-GFP (juls76) marker in young adults (A), or in L1 larvae (B); only commissures of DD2-6 were scored in L1s. All defects are scored per animal (n) and per neuron/commissure (or per ventral cord segment for defasciculation). Data in B are all at 20°C. Tests of significance use the Fisher exact test (*P<0.05, **P<0.01, ***P<0.001).

Ventral cord defasciculation: the ventral nerve cord was divided into 10 sections between D neuron cell bodies. An animal is scored as defasciculated if two or more segments are defasciculated.

Commissure handedness defect is outgrowth of a commissure on the left side. The two anterior commissures (DD1/VD2) of the left side and most anterior commissure on the right side (VD3) were not scored.

Dorsoventral guidance defect is % animals with >1 commissure that fails to reach dorsal cord. About 8% of D neurons did not extend commissures from the ventral cord. We observed similar defects in A and B type motoneurons (not shown).

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Fig. 7. SPON-1 maintains process position at the ventral midline. (A) PVQ axons in wild type (oy/s14). (B) In spon-1(ju430), PVQ1 crosses over to the right-hand VNC (open arrowhead), as in the wild type, and undergoes two ectopic crossovers (white arrowheads). (C) Midline crossing is more penetrant in spon-1 mutants than in l1s, is enhanced by shifting L1s from 15 to 25°C, and is suppressed by growth in 1 mM levamisole (***P<0.001). (D) Suppression of spon-1(ju430) midline crossing by zig-4 and egl-15 (at 22.5°C; the number of animals is shown in the column) and enhancement by dig-1 (at 20°C; **P<0.01). (E) Model for the interaction of SPON-1 and ZIG-4/EGL-15A pathways; a cross-section of ventral nerve cord is shown. SPON-1 in the BM (green) mediates adhesion of PVQ axons (blue) to their normal environment; ZIG-4 and EGL-15 (red) locally inhibit axon-BM adhesion at the midline. (F-J) spon-1(ju430ts) oy/s14 animals were shifted from 15°C to 25°C in the L1 stage and PVQ scored in young adults. The adults were sectioned in the region of PVQ crossing over posterior to the vulva; approximate levels of sections are shown in F. (G) Anterior to PVQR crossover (0 μm), presumably PVQR, has crossed over and fasciculated with the left-hand bundle. (J) At ~160 μm posterior, the left-hand bundle again contains three processes. The ventral hypodermal ridge (HYP green) and the right-hand ventral nerve cord (RVC, blue outline) appear normal in both crossover regions. Similar results were obtained for a second animal (not shown). Scale bars: in A, 10 μm; in G-J, 0.2 μm.

DIGUSSION

The founding member of the spondin family, F-spondin, was identified by its expression in the floor plate of the rat embryonic spinal cord (Klar et al., 1992). Since the discovery of F-spondin, a family of spondin-related genes has been defined, containing two subfamilies, F-spondins and the Mindins (Higashijima et al., 1997). Most animals encode at least one member of each subfamily. Genetic analysis of the spondins in other animals has been hampered by putative redundancy between family members, which are often expressed in overlapping patterns (Higashijima et al., 1997). For example, the loss of M-spondin function in Drosophila does not cause detectable phenotypes (Umemiya et al., 1997), and mindin mutant mice have a normal nervous system (He et al., 2004). By contrast, we find that the sole C. elegans spondin, SPON-1, is essential for embryonic development. The essential nature of SPON-1 function suggests that it may combine the functions performed separately by F-spondins and mindins in other animals.

Roles of SPON-1 domains

F-spondins contain a reelin domain, a spon domain and multiple TSRs. Our genetic and transgenic analysis suggests the TSRs of SPON-1 are crucial for its roles in morphogenesis and axon guidance. Three spon-1 alleles cause missense alterations in TSRs, including the strong allele ju348 and the weak allele e2623. These two mutations affect equivalent residues in the WXXW motifs of TSRs 2 and 3; as these residues are not highly conserved, TSR2 may be more critical for the embryonic morphogenesis function of SPON-1 than TSR3. The temperature-sensitive allele ju430 affects a highly conserved cysteine in TSR4, and could render SPON-1 thermostable as a result of disruption one of the disulfide bridges important in TSR tertiary structure (Tan et al., 2002). Although all of these mutations could cause a global disruption of SPON-1 folding, they suggest that the TSRs are required for SPON-1 function in vivo. TSRs 5 and 6 of vertebrate F-spondin mediate interactions with ECM proteoglycans; plasmin-mediated cleavage within TSRs 5 and 6 can release the rest of the protein from the ECM (Tzarfaty-Majar et al., 2001b). As the motifs in TSRs 5 and 6 that mediate proteoglycan interactions are not present in invertebrate spondins, SPON-1 may be less stably attached to the ECM than are vertebrate spondins, consistent with our findings that SPON-1 can accumulate on BMs far from its site of synthesis.

SPON-1 may act in integrin-mediated muscle-epidermal adhesion

Several lines of evidence suggest that SPON-1 acts either in integrin-mediated adhesion or in a closely related parallel process. SPON-1 is localized to integrin-containing sites and SPON-1 function becomes dosage sensitive when α-integrin INA-1 function is reduced, indicating that SPON-1 and INA-1

*Fig. 7. SPON-1 maintains process position at the ventral midline. (A) PVQ axons in wild type (oy/s14). (B) In spon-1(ju430), PVQ1 crosses over to the right-hand VNC (open arrowhead), as in the wild type, and undergoes two ectopic crossovers (white arrowheads). (C) Midline crossing is more penetrant in spon-1 mutants than in l1s, is enhanced by shifting L1s from 15 to 25°C, and is suppressed by growth in 1 mM levamisole (***P<0.001). (D) Suppression of spon-1(ju430) midline crossing by zig-4 and egl-15 (at 22.5°C; the number of animals is shown in the column) and enhancement by dig-1 (at 20°C; **P<0.01). (E) Model for the interaction of SPON-1 and ZIG-4/EGL-15A pathways; a cross-section of ventral nerve cord is shown. SPON-1 in the BM (green) mediates adhesion of PVQ axons (blue) to their normal environment; ZIG-4 and EGL-15 (red) locally inhibit axon-BM adhesion at the midline. (F-J) spon-1(ju430ts) oy/s14 animals were shifted from 15°C to 25°C in the L1 stage and PVQ scored in young adults. The adults were sectioned in the region of PVQ crossing over posterior to the vulva; approximate levels of sections are shown in F. (G) Anterior to PVQR crossover (0 μm), presumably PVQR, has crossed over and fasciculated with the left-hand bundle. (J) At ~160 μm posterior, the left-hand bundle again contains three processes. The ventral hypodermal ridge (HYP green) and the right-hand ventral nerve cord (RVC, blue outline) appear normal in both crossover regions. Similar results were obtained for a second animal (not shown). Scale bars: in A, 10 μm; in G-J, 0.2 μm.*
have closely related functions in embryonic development. *C. elegans* expresses two integrin heterodimers, αPAT-2/βPAT-3 and αINA-1/βPAT-3 (Brown, 2000; Gettner et al., 1995). PAT-2 is a member of the RGD-binding subfamily of α-integrins and is likely to bind Perlecan. INA-1 is a member of the laminin-binding subfamily; it is expressed in embryonic BM but is not required for embryonic morphogenesis (Baum and Garriga, 1997; Poinat et al., 2002). As *spon-1 ina-1* double homozygotes arrest earlier in elongation than does either single mutant, *SPON-1*, at least in part, acts in parallel to INA-1. We speculate that *SPON-1* might function in a subset of both INA-1- and PAT-2-dependent adhesion processes. Interestingly, F-spondin has also been linked to integrin signaling (Terai et al., 2001), as has Mindin (Jia et al., 2005; Li et al., 2006). An important goal for the future is to determine whether spondins interact directly with integrins in vivo, or whether they indirectly influence integrin-mediated adhesion via candidate receptors, such as APP or ApoER2 (Ho and Sudhof, 2004; Hoe et al., 2005).

**Context-dependent roles of *SPON-1* in axonal development**

*SPON-1* has distinct roles in axon development depending on the neuron type. Such context dependence is reminiscent of the known roles for F-spondin in the vertebrate nervous system. F-spondin promotes the fasciculation of commissural axons after they cross the floor plate (Burstyn-Cohen et al., 1999), and repels the growth cones of motoneurons that do not cross the midline (Tzafart-Majar et al., 2001a). F-spondin also promotes the outgrowth of sensory and hippocampal neurons in vitro (Burstyn-Cohen et al., 1998; Burstyn-Cohen et al., 1999; Feinstein et al., 1999). In *C. elegans*, *SPON-1* promotes the fasciculation of motoneuron axons in the ventral nerve cord, and acts partly redundantly to promote motoneuron outgrowth. By contrast, *SPON-1* appears to prevent midline crossing (fasciculation) of PVQ interneurons.

The deflections of motor commissures at the muscle boundary in *spon-1* mutants suggest that *SPON-1* promotes the entry of growth cones into the muscle-epidermal BM. Defects in entry of the commissural growth cone at the dorsal muscle quadrant are also found in integrin mutants (Poinat et al., 2002), indicating that integrins act at this choice point. The similarity between *spon-1* and integrin defects in motor axon development is consistent with *SPON-1* promoting integrin-mediated adhesion or a closely related process in axon guidance.

Midline crossing defects could result from lack of repulsion from a midline repellent, or from lack of axonal adhesion to the normal environment (Hobert and Bulow, 2003). Because we have not seen differential localization of *SPON-1* at the ventral midline, we favor the interpretation that the inappropriate crossing of PVQs in *spon-1* mutants reflects a lack of adhesion. As *dig-1* and *spon-1* show additive effects on crossing over, these two ECM molecules may act in distinct adhesive pathways. The ZIG-4 and FGF/EGFR/EGL-15A pathways also maintain PVQ positions, but unexpectedly zig-4 and egl-15A mutations suppress *spon-1* midline crossing defects, suggesting that *spon-1* and zig-4/egl-15 maintain PVQ positions by opposing mechanisms. We speculate that PVQ maintenance involves a balance of adhesive and repulsive mechanisms. *SPON-1* could promote the normal adhesion of PVQs in their respective fascicles, so that a lack of *SPON-1* leads to defasciculation and flip-overs. Several models have been proposed for how ZIG-4 might prevent midline crossing (Hobert and Bulow, 2003). One possibility is that ZIG-4 interferes with axon-BM adhesion at the midline, so that axons are unable to cross the midline region. As depicted in Fig. 7E, *SPON-1* in the adjacent BM (green) may promote adhesion of PVQ axons (blue) to their appropriate locations, whereas extracellular ZIG-4/EGL-15 (red) might act locally to inhibit axon-BM adhesion at the midline. Thus, impaired axon-BM adhesion in *spon-1* mutants might be compensated for by the loss of the ZIG/FGFR anti-adhesive pathway. ZIG/FGFR signaling might directly inhibit *SPON-1* based adhesion, or could inhibit a parallel adhesion pathway. Our results underscore the view that the maintenance of process position in the nervous system involves a balance of multiple interacting forces.

In conclusion, our results demonstrate that an F-spondin-related protein promotes tissue adhesion in multiple contexts. *SPON-1* functions in parallel to integrin-mediated adhesion in embryogenesis, and is antagonized by other extracellular axon maintenance factors in post-embryonic growth. These results should be useful in elucidating the in vivo cellular receptors for spondins.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/16/2747/DC1

**References**


