Fibulin-1C and Fibulin-1D splice variants have distinct functions and assemble in a hemicentin-dependent manner

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Accepted 20 July 2005

Development 132, 4223-4234
Published by The Company of Biologists 2005
doi:10.1242/dev.02007

Summary

Fibulins are a family of extracellular glycoproteins associated with basement membranes and elastic fibers in vertebrates. Conservation of the fibulin-1 gene throughout metazoan evolution includes fibulin-1C and fibulin-1D alternate splice variants, although little is known about variant specific functions that would justify this striking structural conservation. We have therefore investigated the structure, localization and loss-of-function phenotype specific to both fibulin-1 variants in *C. elegans*. We find that fibulin-1C has specific roles during pharynx, intestine, gonad and muscle morphogenesis, being required to regulate cell shape and adhesion, whereas fibulin-1D assembles in flexible polymers that connect the pharynx and body-wall-muscle basement membranes. The assembly of fibulin-1C and fibulin-1D in multiple locations is dependent upon the presence of hemicentin, a recently described extracellular member of the immunoglobulin superfamily. We suggest that the distinct developmental roles and hemicentin-dependent assembly for fibulin-1 splice variants demonstrated here may be relevant to fibulin-1 and possibly other fibulin family members in non-nematode species.

Keywords: Extracellular matrix, Basement membrane, Hemidesmosome, *C. elegans*, Cell adhesion

Introduction

Fibulins are a family of five extracellular glycoproteins associated with basement membranes and elastic fibers in vertebrates (Argraves et al., 2003; Timpl et al., 2003). Defects in fibulin family members result in connective tissue disorders that include macular degenerative diseases and Cutis Laxa (reviewed by Chu and Tsuda, 2004). Fibulin-1 is a prominent component of skin, lung and cardiovascular tissue, and is essential for the morphology of endothelial cells lining capillary walls and the integrity of small blood vessels (Kostka et al., 2001).

The structure of vertebrate fibulin-1 gene products has revealed the presence of conserved alternate splice forms, fibulin-1C and fibulin-1D; however, little is known about the specific functions that would justify their conservation throughout metazoan evolution. Specific roles have been suggested for fibulin-1C in tumor progression (Moll et al., 2002) and for fibulin-1D in tumor suppression, synpolydactyly and giant platelet syndrome (Qing et al., 1997; Debeer et al., 2002; Toren et al., 2003). These observations, in addition to data showing that fibulin-1C binds to the basement membrane glycoprotein nidogen at 30-fold higher affinity than does fibulin-1D, suggest that these two splice variants have distinct functions (Sasaki et al., 1995).

C. elegans has a single fibulin gene that is a highly conserved ortholog of vertebrate fibulin-1. Mutations in *C. elegans* fibulin-1 result in gonad morphogenesis defects, but can also suppress gonad morphogenesis defects associated with GON-1 and MIG-17 ADAM metalloproteases (Hesselson et al., 2004; Kubota et al., 2004). One model based on these studies suggests that fibulin-1 and GON-1 act in opposition to one another in the respective inhibition or promotion of tissue expansion (Hesselson et al., 2004).

Structural conservation of the nematode fibulin-1 gene extends to the presence of exons encoding the alternate isoforms fibulin-1C and fibulin-1D (Barth et al., 1998). To investigate the functions of individual fibulin splice variants, we have determined the structure, localization and loss-of-function phenotype specific to each. We present data indicating that these two splice variants have distinct roles during *C. elegans* morphogenesis. Fibulin-1C regulates cell shape and adhesion within developing pharynx, gonad, intestine and muscle tissues, whereas fibulin-1D assembles in flexible polymers that connect the pharynx and body-wall-muscle basement membranes. In addition, both variants are dependent on another extracellular matrix (ECM) protein, hemicentin, for assembly at hemidesmosome-mediated, mechanosensory neuron and uterine attachments to the epidermis. In *C. elegans*, hemicentin assembles into line-shaped structures that are adhesive and flexible (Vogel and Hedgecock, 2001). Although little is known about the distribution or function of the two vertebrate hemicentin orthologs, a mutation in human hemicentin-1 was recently implicated in age-related macular degenerative disease (Schultz et. al., 2003).

We suggest that the association between these two conserved ECM proteins is likely to be conserved in non-nematode species, and that fibulin splice variants have distinct but complementary roles in tissue assembly and organization that are likely to have been conserved in other species as well.

Materials and methods

Isolation of the deletion allele

The deletion allele *fbl-1(hd43)* was isolated from a frozen library of EMS-mutagenized worms, containing 2,000,000 mutagenized genomes consisting of 50,000 pools (40 genomes each), using a poison-primer PCR approach (Edgley et al., 2002). A nested set of primers was designed to isolate a deletion near the N terminus of the gene. The primary screen was done on metapools of 4000 genomes/PCR reaction and one candidate deletion was identified. The mutation was balanced over *dpy-20(e1282) unc-24(e138)* in heterozygous hermaphrodites after backcrossing six times with wild-type males.

Isolation and characterization of full-length fibulin-1C and fibulin-1D cDNAs

After reverse transcription of RNA isolated from mixed stages, cDNA from *fbl-1*C and *fbl-1*D was cloned using primers specific for the 5′ and 3′ ends of the predicted coding regions. After sequencing full-length clones, forward primers specific for exon 5C (5′-ATTGA-TGAGTGTGCCACACTG-3′) or exon 5D (5′-CGTAACGAATGT-TTAACCCGC-3′) were used in combination with reverse primers specific for exon 14 (5′-CTATCGAATCTTCATGAGCGG-3′) and exon 16 (5′-TTAAAATGGATACTTTGAAAC-3′) to amplify reverse transcribed cDNA in order to confirm the structure of sequenced transcripts and to detect the presence of other splice variants.

Constructs and plasmids

PCR fragments were generated with *Pfu* Turbo DNA polymerase (Stratagene), using cosmid DNA as templates. Cosmids F56H11 and T05A1 were used to amplify all fibulin constructs. The *fbl-1* rescuing construct was amplified from nucleotide 13,748 in F56H11 to nucleotide 3154 in T05A1. Fibulin promoter GFP constructs used 5′ regulatory sequences F56H11:13,748-15,683. Y47D3B was used to amplify the *sbp-1* regulatory region from nucleotide 16,731 to nucleotide 18,775. Y73C8B was used to amplify the *lag-2* promoter region, from nucleotide 6,395 to nucleotide 8,415. The *unc-54* promoter was obtained from vector pPD30.38. GFP and YFP coding sequences were obtained from vectors pPD113.37 and pPD132.112, respectively. GFP was inserted, in separate constructs, after nucleotide F56H11:15,892 in exon 2, after nucleotide T05A1:1,039 in exon 14, and after nucleotide T05A1:2,023 in exon 16. Stop codons and reading-frame shifts were introduced after nucleotide F56H11:17,565 to inactivate *fbl-1*C, or after nucleotide F56H11:16,872 to inactivate *fbl-1*D. All constructs were cloned in pGEM vector plasmid (Promega).

Transgenic rescue and expression studies

Transgenic lines were made by microinjection. All constructs were injected at a concentration of 40 ng/μl, together with pRF4 (a plasmid containing a *rol-6* dominant mutation) at 100 ng/μl.

Confocal, DIC and fluorescence microscopy

DIC and fluorescence images were obtained with an Olympus BX51 light microscope and a Magnafire camera. Confocal images were obtained with a Zeiss LSM 510 META microscope.

Electron microscopy

Adult hermaphrodites were fixed and embedded for serial thin-section electron microscopy according to Hall (Hall, 1995).

Results

The *fb1-1* gene encodes a protein with two splice forms

Full-length cDNAs for fibulin-1C (*fb1-1*C) and fibulin-1D (*fb1-1*D) were cloned following reverse transcription of mixed stage *C. elegans* mRNA, and PCR using primers designed to amplify the fibulin-1C and fibulin-1D sequences predicted by Barth et al. (Barth et al., 1998). The sequence of the two clones corresponded to the sequences presented in the earlier studies with one exception. The sequence of the fibulin-1D cDNA

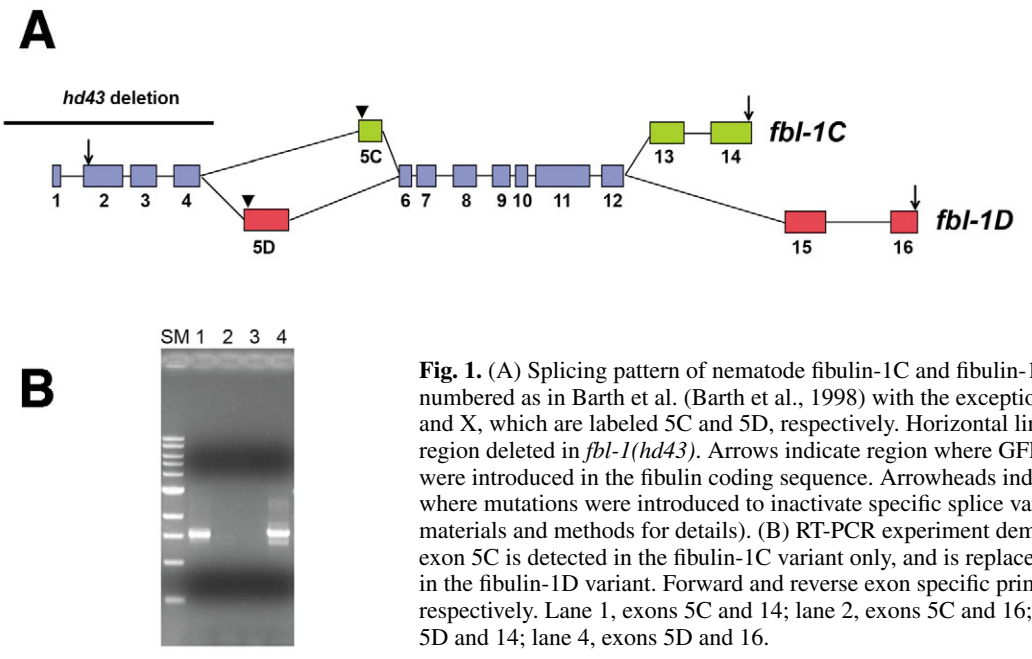

Fig. 1. (A) Splicing pattern of nematode fibulin-1C and fibulin-1D. Exons are numbered as in Barth et al. (Barth et al., 1998) with the exception of exons 5 and X, which are labeled 5C and 5D, respectively. Horizontal line indicates region deleted in *fbl-1(hd43)*. Arrows indicate region where GFP or YFP tags were introduced in the fibulin coding sequence. Arrowheads indicate region where mutations were introduced to inactivate specific splice variants (see materials and methods for details). (B) RT-PCR experiment demonstrating that exon 5C is detected in the fibulin-1C variant only, and is replaced by exon 5D in the fibulin-1D variant. Forward and reverse exon specific primers are listed respectively. Lane 1, exons 5C and 14; lane 2, exons 5C and 16; lane 3, exons 5D and 14; lane 4, exons 5D and 16.

(GenBank accession number AY851363) revealed that predicted exon 5 (F56H11:17,559-17,699) was absent and had been replaced by nucleotides F56H11:16,866-17123, designated exon 'X' in the earlier study (Barth et al., 1998). This splicing substitution replaces the second EGF domain with an unusual, large EGF (Fig. 1A). Using primers specific for exons 5 and X (Fig. 1B), we were not able to detect exon 5 in the fibulin-1D transcript, and we were not able to detect exon 'X' in the fibulin-1C transcript. Based on this data, in addition to the data on variant specific inactivation and localization presented below, we have renamed exons 5 and X, respectively, as exons '5C' and '5D' (see Fig. 1). Both C and D variants encode protein products with a signal sequence followed by three anaphylatoxin modules, nine EGFs and a fibulin C-terminal module.

Fibulin alleles, loss-of-function phenotype

A deletion mutant, *fbl-1(hd43)*, was identified in a PCR-based screen of a *C. elegans* mutant library (see Materials and methods). The deleted region includes nucleotides F56H11:15,472 to 16,582, removing sequences that encode the initiator methionine, the signal sequence, three anaphylatoxin modules and the first EGF, in addition to 209 base pairs of 5′ sequence upstream of the initiator methionine codon (Fig. 1A). The mutant animals are likely to be molecular nulls, sharing several penetrant phenotypic defects in cell adhesion and morphology that are rescued completely in mutant animals containing a fibulin transgene that includes nucleotides F56H11:13,748 to T05A1:3,154 (see below and Table 1).

Gonad

Gonadal leader cells undergo stereotypical migrations in developing larvae that determine the shape of the mature gonad (Hedgecock et al., 1987). The gonad is simultaneously pulled by the leader cell and pushed by proliferating germ cells inside the gonad. In *fbl-1(hd43)* L4 hermaphrodites, the migration of the leader cells, known as distal tip cells (DTCs), ceases as they begin to pass between the body-wall hypodermis and the intestine during their dorsal migration (Fig. 2A,B). The gonad and surrounding basement membrane expands and eventually ruptures as germ cells trapped in the proximal region of the gonad continue to proliferate. As a result, germ cells leak into the pseudocoelomic space and the *fbl-1* hermaphrodite is nearly sterile, producing rare broods of one to two progeny (Table 1).

In wild-type *C. elegans* male gonads, the distal tip cells are stationary and the migratory leader cell found at the proximal end of the gonad is the linker cell. In *fbl-1(hd43)* mutant males, linker cell migration appears to be unaffected, and gonad morphogenesis proceeds normally from early L2 larval stage through early L4. However, in late L4 males, the seminal vesicle ruptures and separates from the vas deferens, severing the pathway between testis and cloaca (Fig. 2C,D). As a result, *fbl-1(hd43)* adult males are sterile.

Pharynx

DIC examination of the pharynx of *fbl-1* mutants reveals that it is asymmetrical and may not function properly for feeding because animals are also small (about 60% of the wild-type body length) and grow slowly. Pharyngeal defects appear to be relatively mild in earlier larval stages, but increase in severity in adults, with the pharynx appearing increasingly asymmetric and also appearing wider and shorter than in wild type (Fig. 2E,F). Electron microscopy reveals that the shape of the basal surface of pharyngeal cells is deformed, particularly around pharyngeal muscle cells (Fig. 3C,D). It is likely that the defects originate during pharyngeal morphogenesis or that they result from mechanical tension caused by a contraction of pharyngeal muscle that distorts a weakened pharyngeal basement membrane.

Body-wall muscle

Although *hd43* animals are lethargic, they are able to make coordinated movements when prodded lightly. Examination of body-wall muscle attachments with a functional GFP-integrin β *pat-3* reporter (Plenefisch et al., 2000) reveals that integrin-based attachments of body-wall muscle to the epidermis,

Table 1. Rescue of fibulin-1 (*hd43*) mutant phenotypes by N-terminal GFP::*fbl-1* translational fusion constructs

Construct	Size	Gonad morphology (brood size)	Pharynx morphology	Muscle attachment	Intestine morphology	ALM(PLM) attachment	Uterine attachment	Flexible tracks
fbl-1C,D	+	+ (240±50)	+	+	+	+	+	+
fbl-1C	+	+ (220±40)	+	+	+	+/–	+	–
fbl-1D	–	– (0±0)	–	–	–	+/–	+/–	+
sbp-1::fbl-1C,D (intestine)	+	+ (235±10)	+	+	+	+/–	+/–	–
lag-2::fbl-1C,D (distal tip cell)	–	– (0±0)	–	–	–	–	–	–
unc-54::fbl-1C,D (muscle)	–	– (0±0)	–	–	–	–	–	+

For brood sizes, three to five broods were scored. For other phenotypes, more than 20 transgenic animals were compared with wild-type or *fbl-1(hd43)* mutant animals by DIC microscopy and by assembly of appropriate protein markers (e.g. GFP-hemicentin or GFP-integrin βpat-3). If all animals were identical to wild type, they were scored '+', if identical to mutant, '–'. If results were mixed or if some transgenic animals were judged to be only partially rescued, they were scored '+/–'.

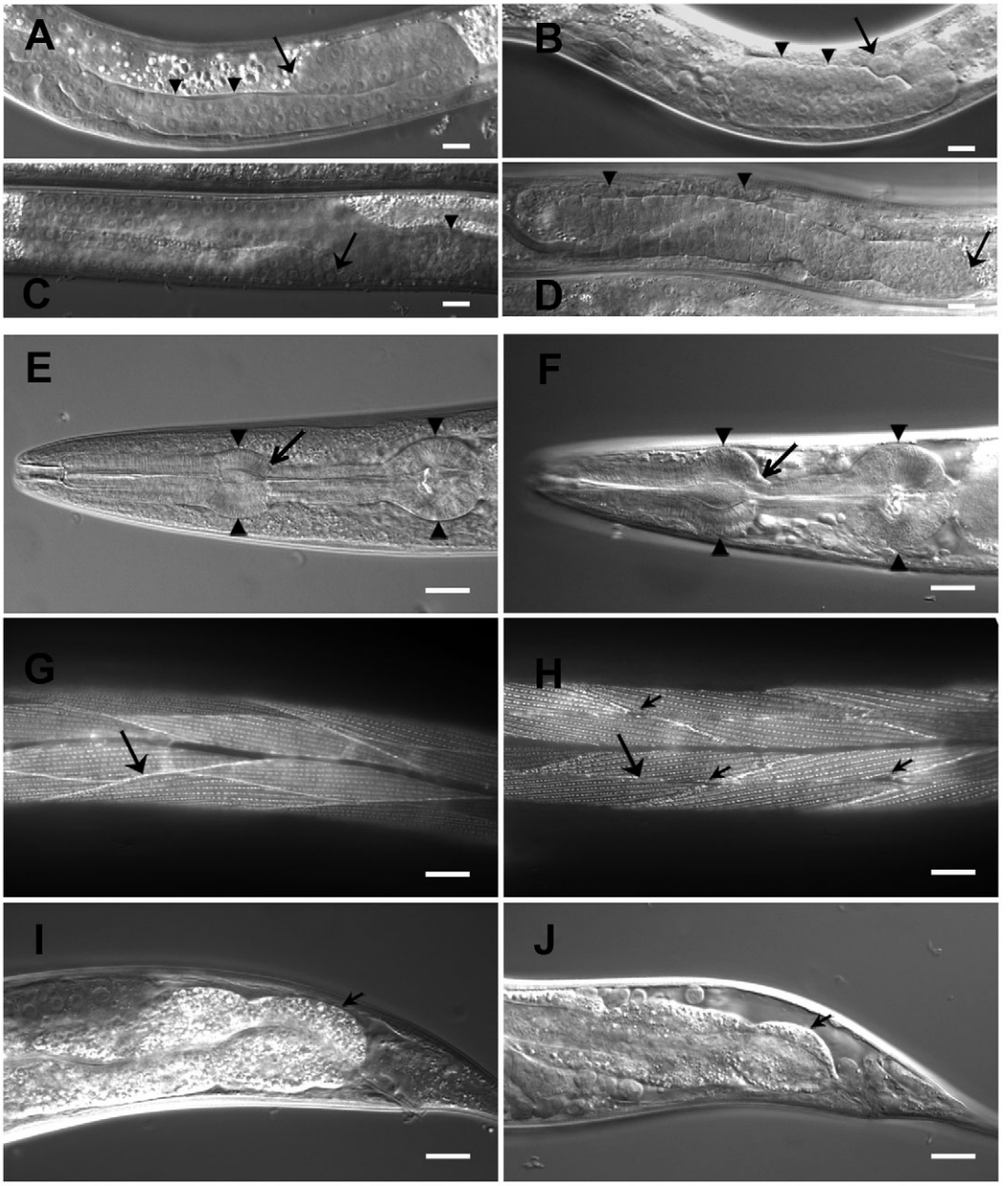

Fig. 2. Phenotypic defects in fibulin-1 (*hd43*) mutants. All panels except for G and H are oriented with dorsal up and anterior to the left. (A,B) Gonad migration in wild-type (A) and *fbl-1(hd43)* mutant (B) L4 hermaphrodites. Arrows indicate the position of the migrating distal tip cell; arrowheads indicate the width of the proximal gonad. (C,D) Gonad migration in wild-type (C) and *fbl-1(hd43)* mutant (D) L4 males. Arrows indicate the position of the stationary distal tip cell; arrowheads indicate the position of the seminal vesicle that is severed from the vas deferens and retracted to a dorsal anterior position in mutant. (E,F) Pharyngeal morphology in wild-type (E) and mutant (F) hermaphrodites. Arrows indicate the region of constriction and asymmetry in metacorpus; arrowheads show the abnormal width of the metacorpus and terminal bulb. (G,H) GFP-integrin β pat-3 distribution in wild-type (G) and *fbl-1(hd43)* mutant (H) body-wall muscle. Large arrow shows region of GFP-integrin β pat-3 accumulation at muscle-muscle junctions in wild-type animals only, and small arrows show the gaps at muscle-muscle junctions in the *fbl-1(hd43)* mutant. (I,J) Intestinal cells expand to fill posterior body cavity in wild-type (I) but not *fbl-1(hd43)* mutant (J) animals. Arrows in I and J indicate the basal surface of posterior intestinal cells. Scale bars: 10 μm.

including dense body organization and muscle cell polarity, which are defective in other ECM mutants (Rogalski et al., 1993; Huang et al., 2003), appear for the most part normal. However, gaps are found at nearly all (>95%) muscle-muscle junctions, and mutant cells have a compact, rhomboid shape in contrast to the elongated, spindle appearance found with wild-type muscle (Fig. 2G,H).

Intestine

Posterior to the gonad, intestinal cells fill the body cavity and are spread against the hypodermis in wild-type animals. In *fbl-1(hd43)*, the intestine appears to be shriveled and does not fill the body cavity (Fig. 2I,J). This defect appears to result from a defect in intestinal cell morphology; however, we cannot rule out the possibility that the phenotype is enhanced by

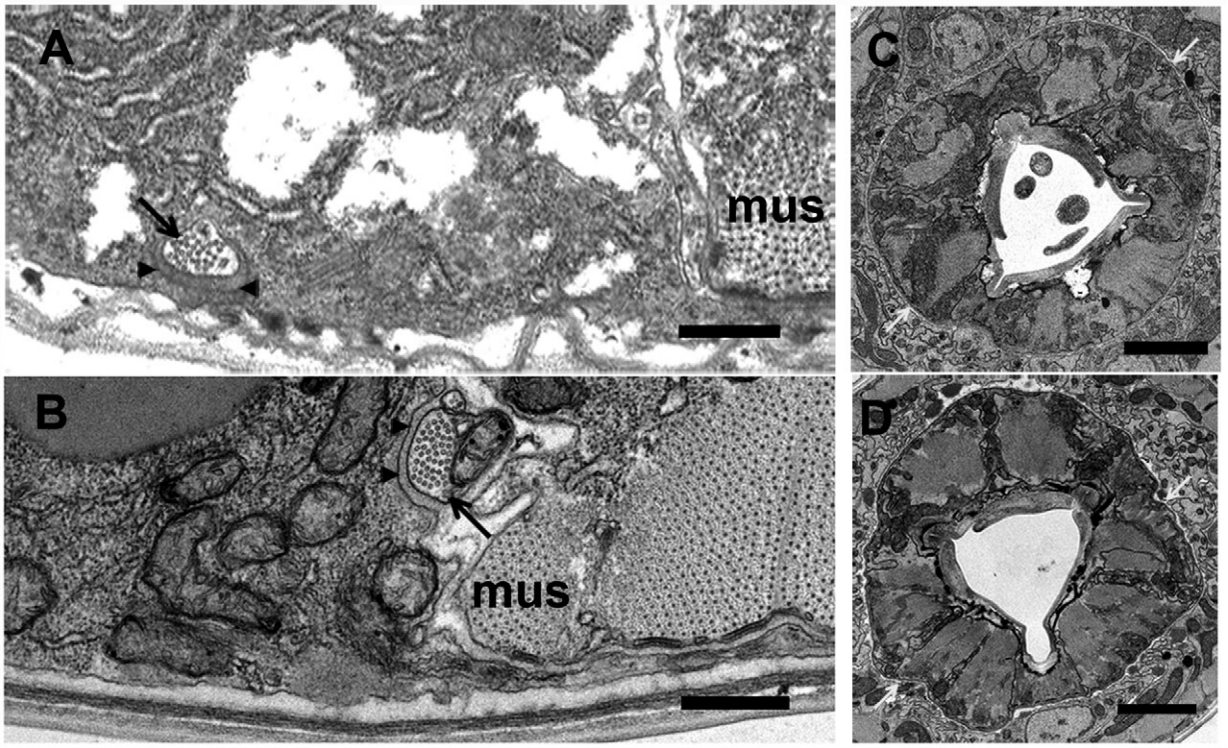

Fig. 3. Electron micrographs of mechanosensory neuron and pharyngeal defects in fibulin-1 (*hd43*) mutants. ALM mechanosensory neuron attachment in wild-type (A) and mutant (B) hermaphrodites. Arrows indicate position of axon; arrowheads indicate extracellular mantle. Also shown is the dorsal body-wall muscle (mus). (C,D) Electron micrographs of cross-sections through the pharynx of wild-type (C) and *fbl-1(hd43)* mutant (D) animals. Arrows indicate pharyngeal basement membranes that are distorted in mutant animals. Scale bars: 0.5 μm in A,B; 2 μm in C,D.

pharyngeal defects that reduce the ability of mutant animals to feed properly.

Mechanosensory neurons

In wild-type larvae, four mechanosensory neurons induce assembly of an extracellular mantle, in addition to hemidesmosomes and intermediate filaments in adjacent epidermal tissue. Associated epidermal squamification causes mechanosensory axons to be anchored to the cuticle all along their length (Chalfie and Sulston, 1981). In *fbl-1(hd43)* mutants, ALM and PLM processes fail to induce the assembly of hemidesmosomes and intermediate filaments, and epidermal squamification. Instead, ALM and PLM processes remain in their juvenile position, adjacent to the muscle (Fig. 3A,B). This is similar to the phenotype seen in *him-4* and *mec-1* mutants (Chalfie and Sulston, 1981; Vogel and Hedgecock, 2001). However, unlike *him-4* and *mec-1*, mantle still accumulates between the surface of the mechanosensory neurons and the hypodermis in *fbl-1(hd43)* animals (Fig. 3A,B). In some *fbl-1(hd43)* animals, regions of normal attachment were visible, suggesting that the presence of fibulin is not an absolute requirement for hemidesmosome-mediated attachments. Despite the ultrastructural defect in hemidesmosome-mediated mechanosensory neuron attachment to the body wall, *fbl-1* mutants have a nearly normal response to light touch.

Uterine attachment

A complex junction forms where a multinucleate uterine cell, utse, attaches to epidermal seam cells. In *fbl-1* mutants, the assembly of the attachment at this site is defective as viewed by a defect in hemicentin assembly (Fig. 10I,J). In contrast to *him-4* mutants, where the uterus prolapses during egg-laying, few fertile eggs enter the uterus in *fbl-1(hd43)* animals. Because there is little or no egg-laying in most *fbl-1* animals, the mechanical stress on the uterus is reduced and uterine prolapse is rare.

Fibulin expression by intestine, muscle and hypodermal cells

A transcriptional fusion with the fibulin-1 5′ regulatory sequence (F56H11:13,748-15,683) driving expression of a GFP reporter was used to determine sites of fibulin synthesis. The same 5′ regulatory sequence was used in the fibulin constructs that rescue all of the phenotypes described below (Table 1). Expression was most dramatic in the anterior and posterior two to four intestinal cells, head and tail body-wall muscles, and in the hypodermal syncytium (Fig. 4). Intestinal expression is detected in late embryogenesis and gradually decreases in anterior intestinal cells until it is not detectable in adults, but it persists in the posterior intestinal cells well into adulthood. Hypodermal, and head and tail body-wall muscle, expression is also detected late in embryogenesis and persists into adulthood. In the hermaphrodite gonad, expression is seen in the spermatheca, starting in young adult hermaphrodites and remaining strong in older adults. Rare expression is also detected in three to five unidentified neurons in the nerve ring.

Fibulin splice forms have distinct localizations and functions

A transgene comprising the same 5′ regulatory sequence that was used to determine the sites of synthesis (Fig. 4) driving expression of a full-length fibulin gene can rescue each of the phenotypic defects described above (Table 1). A GFP or YFP tag was added to each fibulin-1 splice form at either the N or C terminal (see Materials and methods for a description of constructs). The splice form-specific constructs were expressed in *fbl-1(hd43)* mutant animals to determine localization and the ability of each splice form to rescue each mutant phenotype. Fibulin-1C with a GFP tag at either end localizes to male and hermaphrodite gonad, anterior and posterior intestine and pharyngeal basement membranes, body-wall muscle, GLR cells, uterine attachment and mechanosensory neurons (Fig. 5A-H). On body-wall muscle, fibulin-1C accumulates at dense bodies, but is most intense between muscle cells, particularly between anterior and posterior pairs of muscle cells. Fibulin-1C is detectable on the surface of each of these cell types from late embryonic stages onwards, with the exception of uterine attachments, where fibulin-1C::GFP becomes detectable as the uterine attachment develops in late L4. Fibulin-1C::GFP is able to completely rescue the size, pharynx, uterine and muscle attachment, and gonad migration phenotypes, and to partially rescue mechanosensory neuron attachment defects (see Table 1).

Fibulin-1D specific constructs localize on ALM/PLM mechanosensory neuron attachments and in flexible tracks connecting the pharyngeal and body-wall-muscle basement membranes. They also localize to posterior and anterior body-wall muscles and GLR cells from late embryos onwards (Fig. 6), and in uterine attachments beginning in L4 larvae. Fibulin-1D::GFP completely rescues flexible track structures in the absence of fibulin-1C, and partially rescues mechanosensory neuron and uterine attachments (Table 1).

Because we were not able to detect distal tip cells as a source of fibulin synthesis, this raised the possibility that fibulin might not be expressed by the migrating gonadal leader cell, but by its migration substratum. We expressed fibulin under the control of promoters specific for the muscle substratum (*unc-54*), intestine (*sbp-1*) and distal tip cells (*lag-2*) and found that only intestinal expression of fibulin completely rescues the

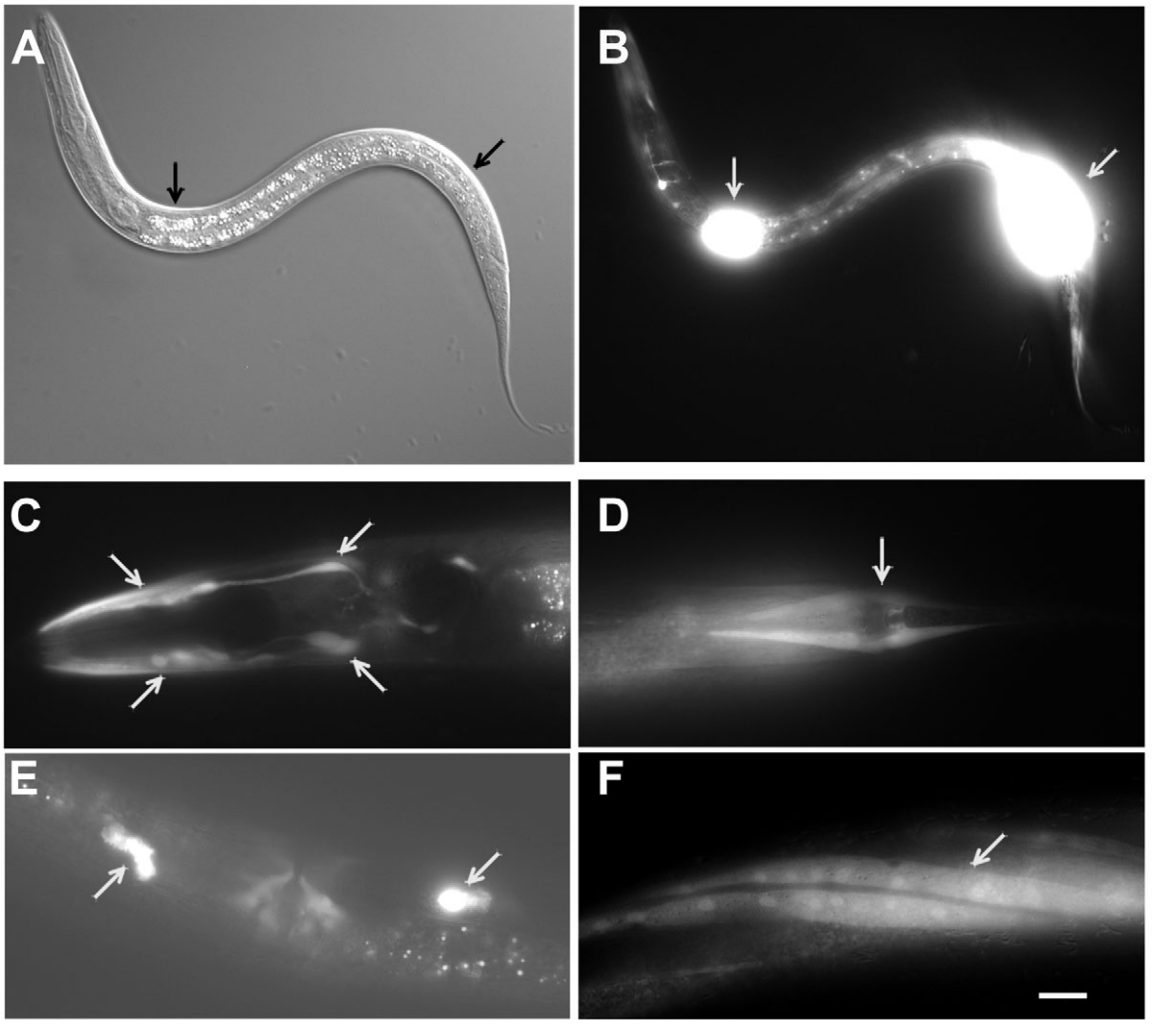

Fig. 4. Fibulin-1 is secreted by intestine, muscle and hypodermal cells. (A,B) DIC (A) and fluorescence (B) micrographs with arrows showing expression of GFP under the control of the fibulin promoter in intestine. (C-F) Fluorescence micrographs with arrows showing expression in the head (C) and tail (D) muscle, and the spermatheca (E) and hypodermis (F). Scale bar: 10 μm.

gonad morphology defect in addition to defects in intestine, pharynx and body-wall muscle; it also partially rescues mechanosensory neuron and uterine attachment defects (Table 1). Fibulin-1::GFP secreted from intestinal cells is detected on each of these tissues.

Fibulin assembly is dependent upon hemicentin

Localization of the C and D isoforms at uterine and mechanosensory neurons attachments, and the D isoform on flexible tracks, is reminiscent of the localization of hemicentin, an ECM protein with two vertebrate orthologs that was recently characterized in *C. elegans* (Vogel and Hedgecock, 2001). Examination of hemicentin::GFP and fibulin-1D::YFP distribution revealed that in a wild-type background, hemicentin and fibulin-1D co-localize in mechanosensory neuron and uterine attachments, and in flexible tracks (Fig. 7). Although the two proteins are clearly localized in the same structures, the extent of overlap is not complete (i.e. there are regions in all three structures with fibulin and no hemicentin, and vice versa).

To determine whether the assembly of either of the fibulin-1 splice variants found in these structures are dependent on hemicentin, we examined fibulin localization in the presence and absence of hemicentin. In a hemicentin null [*him-4(rh319)*] background, fibulin-1C and fibulin-1D are nearly undetectable at mechanosensory neuron and uterine attachments (Fig. 8B,D,

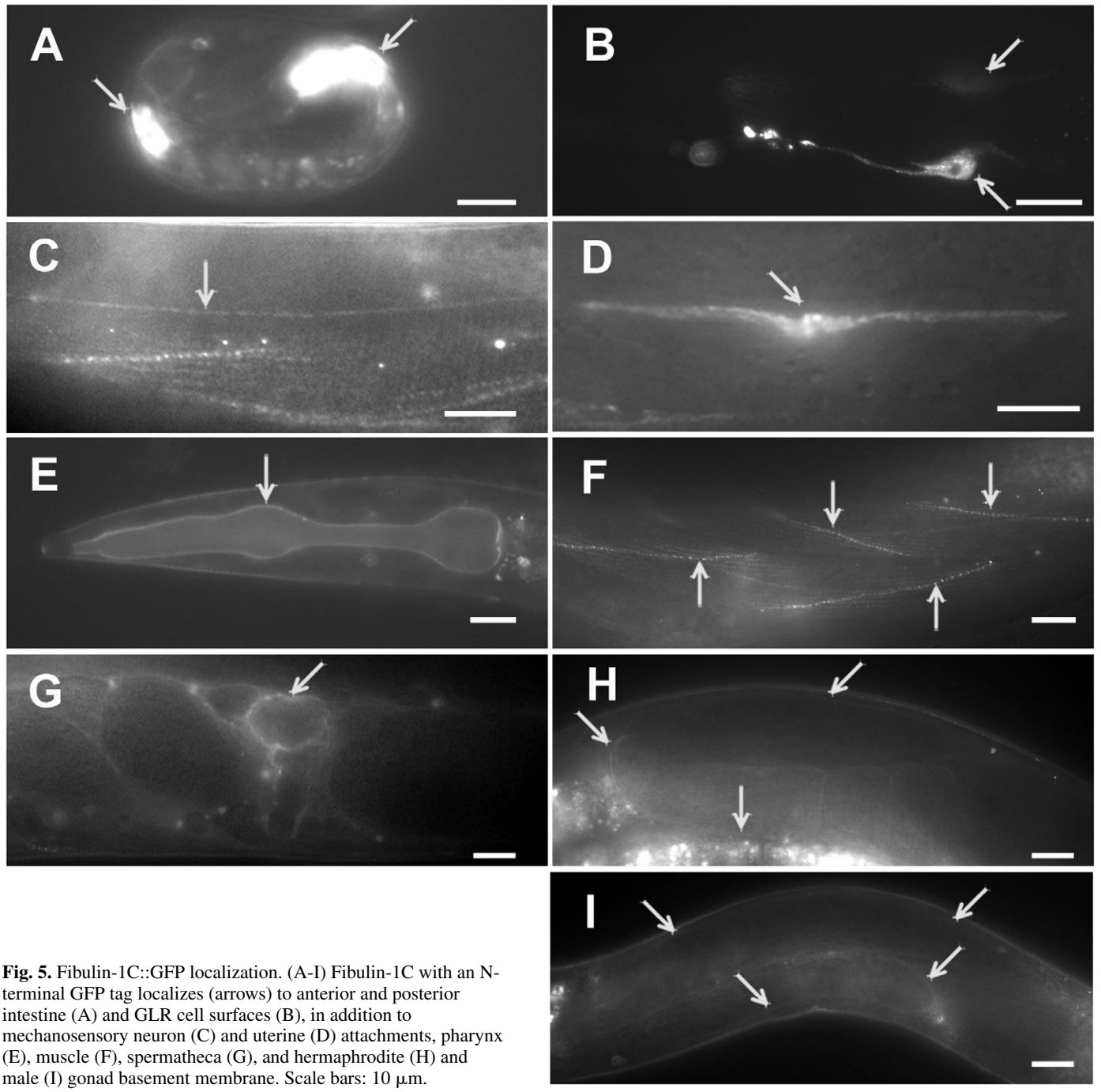

Fig. 5. Fibulin-1C::GFP localization. (A-I) Fibulin-1C with an N-terminal GFP tag localizes (arrows) to anterior and posterior intestine (A) and GLR cell surfaces (B), in addition to mechanosensory neuron (C) and uterine (D) attachments, pharynx (E), muscle (F), spermatheca (G), and hermaphrodite (H) and male (I) gonad basement membrane. Scale bars: 10 μm.

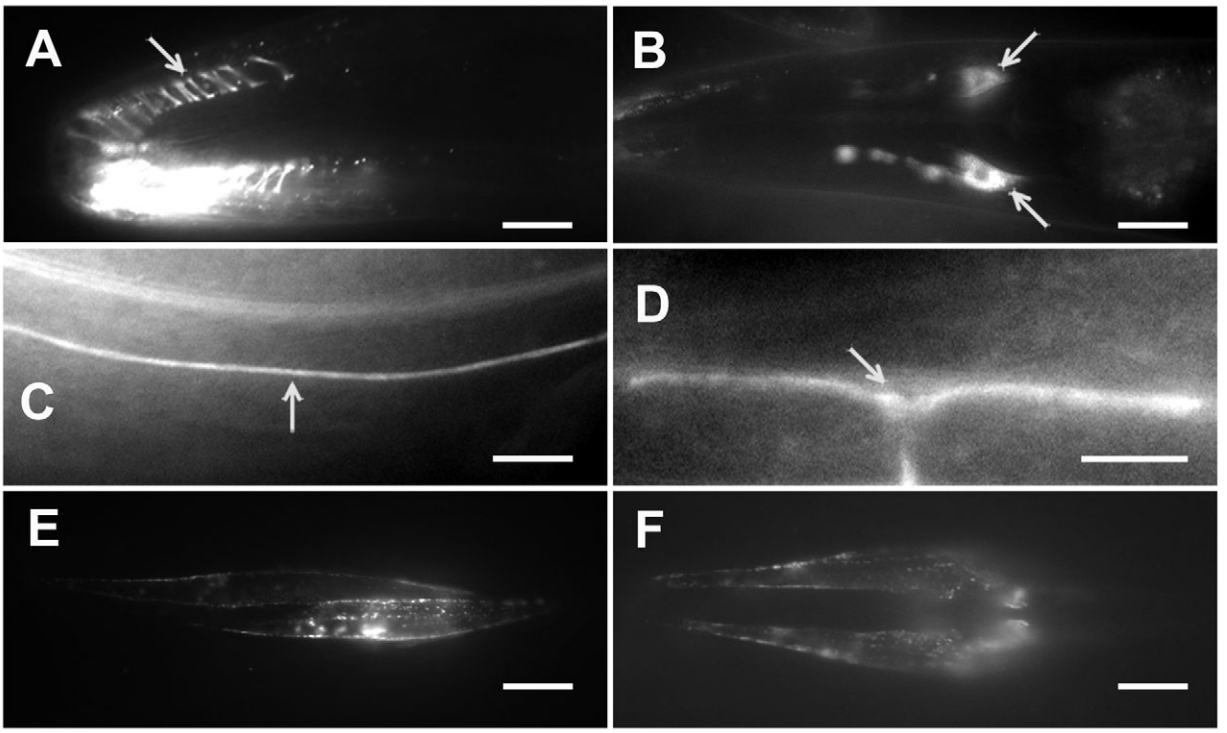

Fig. 6. Fibulin-1D::GFP localization. (A-F) Fibulin-1D with an N-terminal GFP tag localizes (arrows) to flexible tracks in the head (A) and GLR cell (B) surfaces, in addition to mechanosensory neuron (C) and uterine (D) attachments, and dorsal (E) and ventral (F) body-wall muscle in the tail. [Fibulin-1D with an N-terminal GFP tag also localizes to dorsal and ventral body-wall muscle in the head (data not shown).] Scale bars: 10 μm.

Fig. 9B,E), and fibulin-1D is undetectable in flexible tracks connecting pharyngeal and body-wall-muscle basement membranes (Fig. 8F). We also examined the assembly of fibulin-1 variants in the absence of another extracellular protein known to be required for mechanosensory neuron attachments, MEC-1 (Emtage et al., 2004). In *mec-1(e1066)* animals, fibulin-1 distribution on mechanosensory neurons is not detectably different from fibulin distribution in wild-type animals (cf. Fig. 9A,C with 9D,F).

We also examined hemicentin assembly in the absence of fibulin-1 and found that, in a fibulin-1 null (*hd43*) background, hemicentin::GFP is undetectable in flexible tracks (Fig. 10E,F), but is found on mechanosensory neuron and uterine attachments, although it does not form continuous solid lines

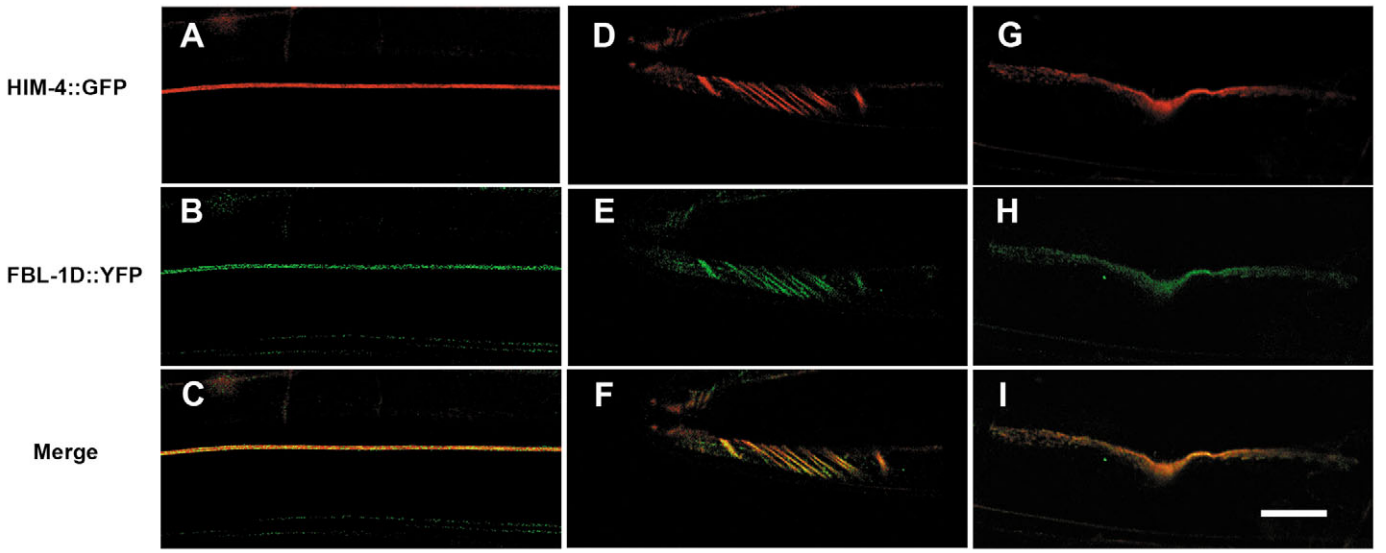

Fig. 7. Co-localization of fibulin-1D and hemicentin. (A-I) Hemicentin::GFP (A,D,G) and Fibulin-1D with an N-terminal YFP tag (B,E,H) co-localize (C,F,I) on ALM mechanosensory neurons (A-C), flexible tracks (D-F), and uterine attachments (G-I). Scale bar: 10 μm.

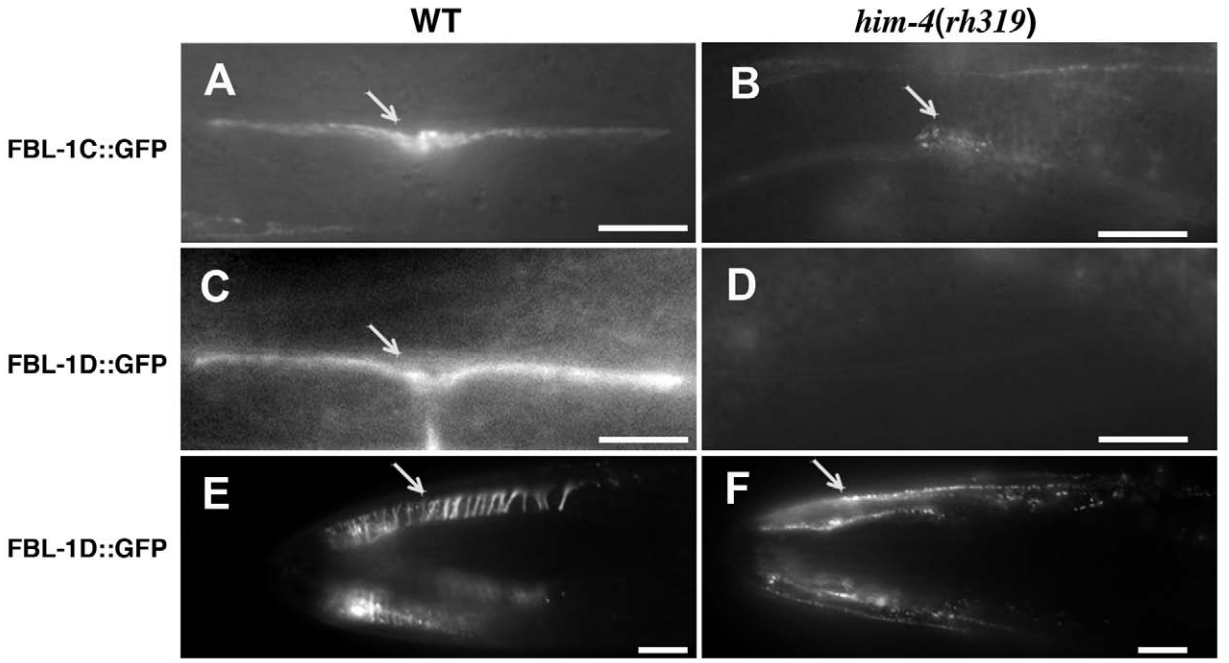

Fig. 8. Fibulin-1C and fibulin-1D assembly at uterine attachments and in flexible tracks is dependent on the presence of hemicentin. (A,B) Fibulin-1C with an N-terminal GFP tag localization (arrows) at uterine attachments in wild-type (A) and hemicentin null [*him-4(rh319)*] mutant (B) animals. (C-F) Fibulin-1D with an N-terminal GFP tag localization (arrows) at uterine attachments (C,D) and flexible tracks (E,F) in wild-type (C,E) and hemicentin null [*him-4(rh319)*] mutant (D,F) animals. Fibulin-1D is absent from flexible tracks in hemicentin mutants (arrows, E,F), but fibulin-1D assembly on body-wall muscle in the head is not affected. Scale bars: 10 μm.

like those seen in a wild-type background (Fig. 10A,B,I,J). Fibulin-1D alone is sufficient to restore hemicentin assembly in flexible tracks and mechanosensory neurons, and to partially restore hemicentin to uterine attachments (Fig. 10C,G,K). Fibulin-1C can restore hemicentin assembly on uterine attachments and can partially restore hemicentin on mechanosensory neurons (Fig. 10D,L). In locations where hemicentin and fibulin localization do not overlap, assembly of each protein is not affected by the absence of the other. Finally, *fbl-1(hd43)*; *him-4(rh319)* double homozygous animals display all of the phenotypes described for *fbl-1(hd43)* and *him-4(rh319)* single mutants; suppression, enhancement

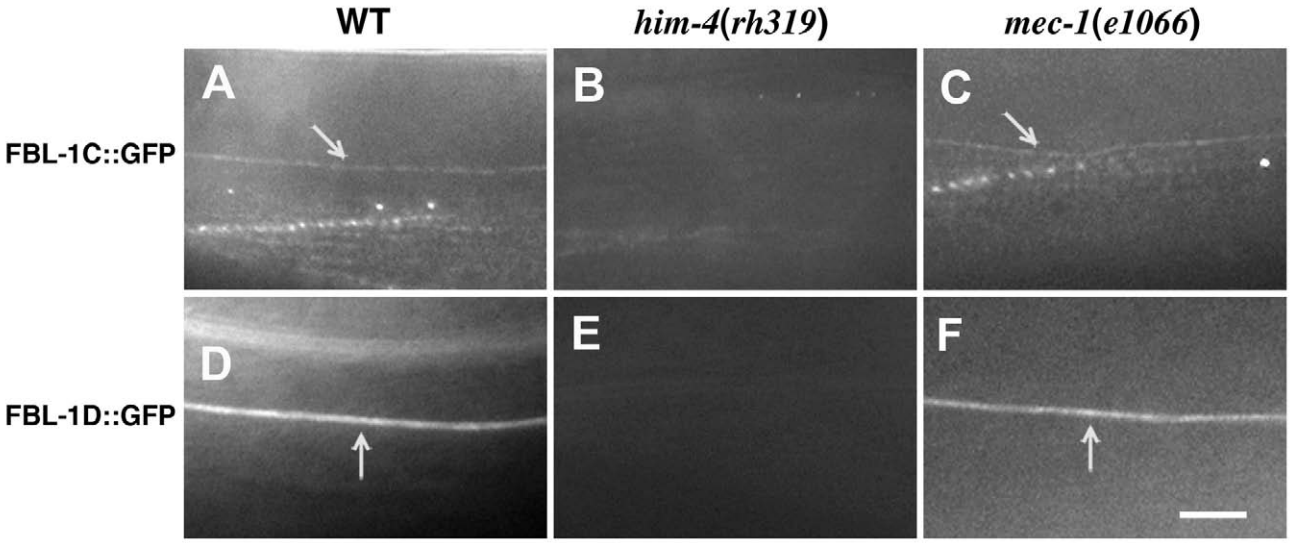

Fig. 9. Fibulin-1C and fibulin-1D assembly at mechanosensory neuron attachments is dependent on the presence of hemicentin but not on the presence of MEC-1. (A-C) Fibulin-1C with an N-terminal GFP tag localization (arrows) at mechanosensory neuron attachments in wild-type (A), hemicentin null [*him-4(rh319)*; B], and *mec-1(e1066)* mutant (C) animals. (D-F) Fibulin-1D with an N-terminal GFP tag localization (arrows) at mechanosensory neuron attachments in wild-type (D), hemicentin null [*him-4(rh319)*; E], and *mec-1(e1066)* mutant (F) animals. Scale bar: 10 μm.

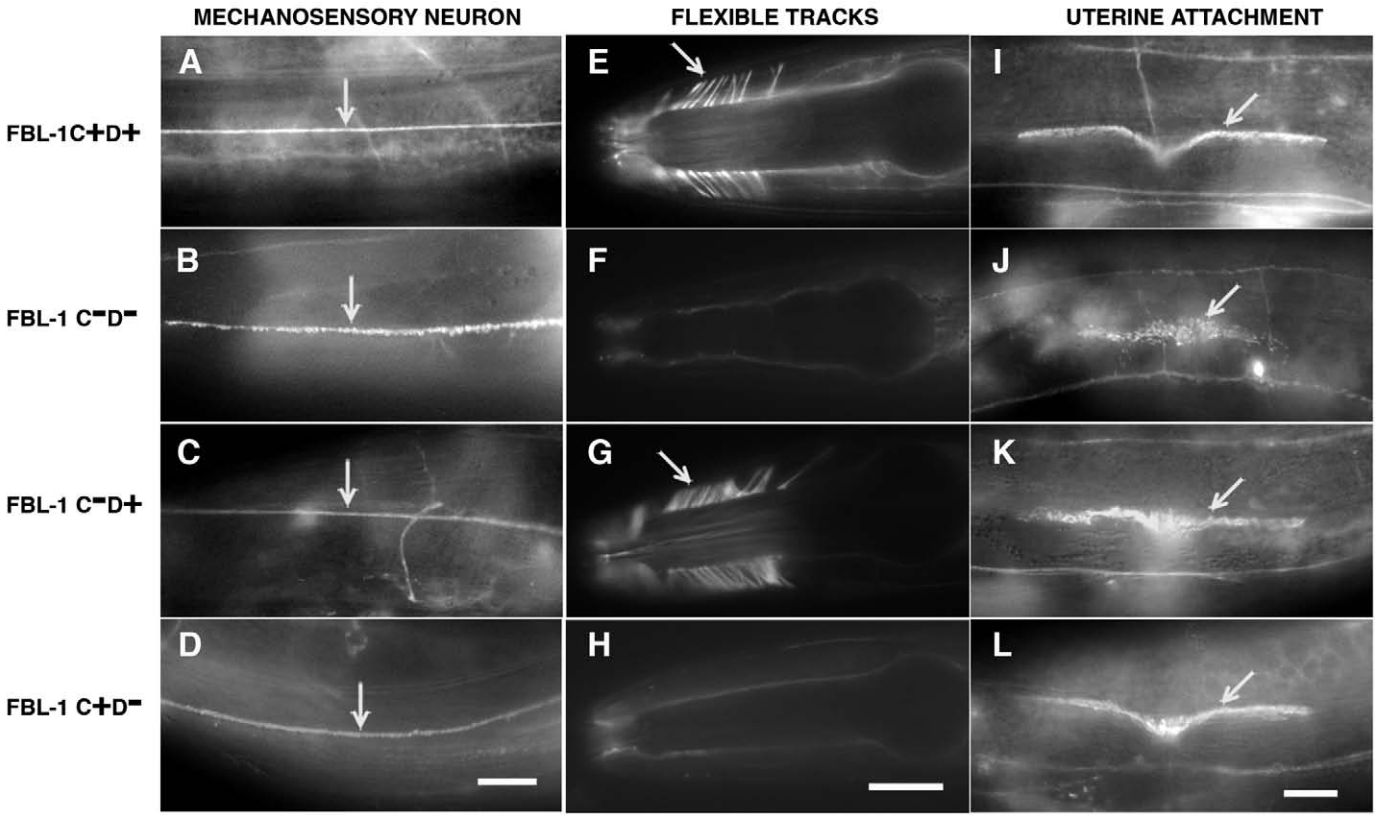

Fig. 10. Hemicentin assembly in some tissues is dependent upon fibulin-1C and/or fibulin-1D expression. (A-L) Hemicentin::GFP localization (arrows) at mechanosensory neuron attachments (A-D), flexible tracks (E-H) and uterine attachments (I-L) in wild type (A,E,I), fibulin-1 (*hd43*) mutants (B,F,J), and fibulin-1 (*hd43*) mutants carrying a wild-type fibulin-1D (C,G,K) or fibulin-1C (D,H,L) transgene are shown. Scale bars: 10 μm.

or novel phenotypes were not detected in double mutant animals.

Discussion

Fibulin-1 splice variants have distinct functions in *C. elegans* development

Structural conservation of the fibulin-1 gene extends to the retention of fibulin-1C and fibulin-1D splice variants. To assess possible functions unique to each fibulin isoform, we determined the localization of the individual splice variants with GFP or YFP tags and monitored the ability of each variant to rescue specific phenotypes in a fibulin null mutant background. Based on our findings, we conclude that the variants have distinct functions that have probably been retained throughout metazoan evolution. This is not entirely unexpected, as alternate splice variants with redundant functions are unlikely to be conserved in lineages phylogenetically separated by 600 million years.

Fibulin-1C appears to have specific roles in regulating the shape and adhesion of cells in the developing pharynx, intestine, body-wall muscle and gonadal tissue. Recent studies show that mutations in fibulin-1 suppress the gonad migration phenotypes seen in metalloprotease defective *gon-1* and *mig-17* mutants. They also suggest a functional interaction between fibulin-1 and ADAM metalloproteases (Hesselson et al., 2004; Kubota et al., 2004), and are consistent with a specific role for fibulin-1C in regulating the shape of the hermaphrodite gonad. Fibulin-1C is also necessary for the assembly of hemicentin, which is required for hemidesmosome-mediated uterine attachments. Although fibulin-1D is also found in this location, it can only partially compensate for the lack of fibulin-1C.

By contrast, fibulin-1D appears to have a single unique role in the assembly of the flexible, hemicentin-containing tracks found joining the pharynx and body-wall-muscle basement membranes. Although fibulin-1C is also found in the flexible tracks, fibulin-1D is necessary and sufficient for these structures; loss of fibulin-1C has no effect on these structures and cannot compensate for the lack of fibulin-1D in this location. Complete rescue of the mechanosensory neuron attachment defects in *fbl-1(hd43)* animals requires the presence of both fibulin-1C and fibulin-1D, suggesting that at some hemidesmosome-mediated attachment sites these two splice forms can function in tandem. Because both are required, it seems that their functions are distinct in this location as well. An alternate possibility is that the effect is a function of dose: either splice form may rescue alone if it is present in sufficient quantity. However, because we are already expressing each splice form at high levels in transgenic animals, this possibility seems less likely.

The source of fibulin for many tissues appears to be at or near the site of assembly. It was therefore unexpected to find that fibulin-1 expressed ectopically under the control of a distal

tip cell promoter did not rescue gonad morphogenesis or any other defects, and that expression of fibulin-1 under control of a muscle-specific promoter rescued assembly of flexible hemicentin tracks but did not rescue the muscle attachment (or any other) defects. Instead, we found that the fibulin-1 gene was able to rescue gonad migration and most other defects when expressed under the control of an intestine-specific promoter (Table 1). We observed that intestinally expressed fibulin::GFP was secreted and accumulated on the surface of intestine, gonad, pharynx and body-wall muscle cells. This pattern is identical to the pattern seen for fibulin-1C under control of its endogenous promoter. This suggests that fibulin-1C must assemble into a complex or be modified in some other way by intestinal cells for proper secretion, localization and/or function.

Fibulin-1 assembly is hemicentin dependent in some locations

In vertebrates, fibulin-1 (and other fibulins) interacts directly with a large number of ECM proteins, including nidogen and laminin (Argraves et al., 2003; Timpl et al., 2003). Although it is not known whether the interactions between fibulin-1 and hemicentin described here are direct or indirect, fibulin-1C and fibulin-1D splice variants require hemicentin for localization and assembly in multiple tissue locations, including flexible tracks, and mechanosensory neuron and uterine attachments. Conversely, hemicentin localization to mechanosensory neurons and uterine attachments is independent of fibulin-1. However, hemicentin assembled at these sites has a discontinuous and frayed appearance in the absence of both fibulin-1 isoforms. Therefore, one function of fibulin-1 at these sites appears to be to refine the hemicentin localized there into smooth continuous tracks. The lack of a complete overlap in the localization of hemicentin and fibulin in these locations suggests that there may be other proteins involved in the assembly of these structures, but it does not exclude the possibility that fibulins and hemicentin can interact directly. In locations other than uterine and mechanosensory neuron attachments and flexible tracks, hemicentin and fibulin-1 variants can assemble and function independently of one another.

MEC-1, a secreted protein composed of EGF and Kunitz repeats is an obvious candidate to interact with fibulin at mechanosensory neuron attachments (Emtage et al., 2004). Like hemicentin, MEC-1 is required for the assembly of mantle, hemidesmosomes and associated intermediate filaments at mechanosensory neuron attachments to the epidermis. Although we found that fibulin-1C and fibulin-1D assembly at these attachments is hemicentin dependent, loss of MEC-1 from these structures has no detectable effect on fibulin assembly. This indicates that fibulin assembly is dependent on the presence of hemicentin, but is not dependent on the presence of hemidesmosome-mediated attachments, mantle, MEC-1 or the large number of other proteins likely to be found in these structures. Conversely, MEC-1 is required for the assembly and function of mechanosensory channel complexes, whereas fibulin and hemicentin appear to have no direct role in the assembly and function of these complexes or in mechanotransduction, reinforcing earlier observations that mechanosensory neuron anchorages are not required for mechanotransduction (Vogel and Hedgecock, 2001; Emtage et al., 2004).

Relevance to vertebrate fibulins

Fibulin-1 is associated with basement membranes and elastic fibers in vertebrates. The fibulin-1 phenotypes seen in the blood vessels of mice where the fibulin-1 gene has been inactivated, including the dilated and irregular lumen and the disruption in endothelial morphology and cell-cell-contacts (Kostka et al., 2001), are reminiscent of the fibulin-1C specific defects in gonad, pharynx, intestine and muscle cell adhesion and morphology seen here. Based on this striking similarity, a reasonable speculation is that the blood vessel defects observed in the fibulin-1-deficient mouse are primarily due to loss of the fibulin-1C splice variant. It is tempting to speculate further that similar to *C. elegans* fibulin-1C, vertebrate fibulin-1C is preferentially distributed in basement membranes and fibulin-1D is preferentially distributed in elastic fibers. This model is consistent with data showing that fibulin-1C binds to the basement membrane glycoprotein nidogen with a 30-fold higher affinity than does fibulin-1D (Sasaki et al., 1995). However, specific protein localization data for vertebrate fibulin-1 splice variants have not yet been determined and will require variant specific reagents to discriminate between the fibulin-1 isoforms.

Nematode fibulin-1C and fibulin-1D variants differ not only in their C-terminal sequences, but also have a single alternately spliced EGF repeat. We do not know whether the functional differences between the two splice variants are a result of the different EGF, C-terminal modules, or a combination of both. Although alternate splicing of vertebrate fibulin-1D EGF repeats has not been reported, vertebrate fibulins 2 and 3 have single EGF repeats that are alternately spliced in a tissue-specific manner (Lecka-Czernik et al., 1995; Grassel et al., 1999). It is possible that after duplication of the ancestral, founding fibulin gene in the chordate lineage, alternately spliced exons encoding EGF repeats were lost in fibulin-1 but retained in fibulins 2 and 3, or that alternately spliced fibulin-1 transcripts exist that have not yet been reported in the literature. Either way, it is likely that the functional consequences of alternate EGF splicing observed in nematodes extends to vertebrate fibulins (Lecka-Czernik et al., 1995; Grassel et al., 1999).

Hemicentin and fibulin-1 are the only two proteins that have been localized to flexible tracks connecting the pharynx and body-wall-muscle basement membranes. The flexible tracks pivot, flex and stretch as the worm forages. It is possible that these tracks are unique structures specific to nematodes, a precursor to the elastic fibers that evolved further in the vertebrate lineage, or that they represent new structures yet to be described in vertebrates.

Fibulins and hemicentins share a region of high homology at the C terminus of both proteins that contains EGF and FC modules. Based on this homology, human hemicentin-1 has occasionally been included as an atypical member of the fibulin family and referred to as fibulin-6. In *C. elegans*, this region in the hemicentin protein appears to be involved in hemicentin track assembly (C.D. and B.E.V., unpublished) and may provide a potential mechanism for the interaction between fibulin and hemicentin. We speculate that the hemicentin-dependent assembly of fibulin-1 variants observed in some nematode tissues may have a broader relevance for hemicentin and fibulin orthologs in vertebrates. Although a relationship between the vertebrate orthologs of these genes has not been

demonstrated, a potential link is suggested by the observation that human hemicentin-1, fibulin-3 and fibulin-5 have recently been implicated in multiple forms of human macular dystrophy (Stone et al., 1999; Stone et al., 2004; Schultz et al., 2003).

We would like to thank Mike Sepanski, Paul Luther and Elise Shumsky for assistance with electron and light microscopy. We would also like to thank Drs Mon-Li Chu and Benjamin Podbilewicz for valuable discussions and Dr A. Fire for GFP cloning vectors. This work was supported by the National Institutes of Health (GM65184) and the American Heart Association. Some nematode strains used in this work were obtained from the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources.

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