

GON-1 and Fibulin Have Antagonistic Roles in Control of Organ Shape

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Summary

Most developing organs are surrounded by an extracellular matrix (ECM), which must be remodeled to accommodate growth and morphogenesis. In *C. elegans*, the GON-1 ADAMTS metalloprotease regulates both elongation and shape of the developing gonad [1]. Here, we report that either human ADAMTS-4 or ADAMTS-9 can substitute for GON-1 in transgenic worms, suggesting functional conservation between human and nematode homologs. We further identify fibulin (FBL-1), a widely conserved ECM component [2], as critical for gonadal morphogenesis. FBL-1 is expressed in nongonadal tissues but is present at the surface of the elongating gonad. A fibulin deletion mutant has a wider than normal gonad as well as body size defects. We find that GON-1 and fibulin have antagonistic roles in controlling gonadal shape. Depletion of *fbl-1*, but not other ECM components, rescues *gon-1* elongation defects, and removal of *gon-1* rescues *fbl-1* width defects. Therefore, the GON-1 protease normally promotes tissue elongation and expansion, whereas the fibulin ECM protein blocks these key morphogenetic processes. We suggest that control of organ shape by GON-1 and fibulin in *C. elegans* may provide a model for similar cellular processes, including vasculogenesis, in humans.

Results and Discussion

Elongation of the *C. elegans* gonad is controlled by migration of the distal tip cell (DTC), a specialized leader cell that guides the developing gonad as it extends, reflexes, and finally generates a U-shaped gonadal “arm” [3]. Normal DTC migration requires GON-1, a secreted metalloprotease of the ADAMTS family (for “a disintegrin and metalloprotease with thrombospondin repeats”) [1]. Human ADAMTS homologs include several well-characterized and clinically important members: ADAMTS-1, -4, -5, and -13 [4] (Figure 1A). To determine whether GON-1 and human ADAMTS proteins have conserved functions, we tested two of them for rescue of a *gon-1* null mutant. ADAMTS-4 (*aggrecanase-1*) was selected because of its small size (Figure 1A), relevance

to human disease [5], and biochemical characterization [6–8]; ADAMTS-9 was tested because of its close sequence and architectural similarities to GON-1 (Figure 1A).

To test the function of human ADAMTS proteases in the *C. elegans* gonad, we used the *lag-2* promoter (*lag-2 5'*) to drive transgene expression in distal tip cells. In wild-type animals, gonads are elongated and U-shaped by the fourth larval stage (L4), with DTCs at the distal end (Figure 1B); in contrast, *gon-1(0)* L4 gonads are not elongated [9], and they have enlarged and rounded DTCs near their site of birth (0% elongated arms, $n > 100$) (Figure 1C). However, either of two human ADAMTS transgenes, *lag-2 5'::ADAMTS-4* or *lag-2 5'::ADAMTS-9*, could rescue gonadal elongation in *gon-1(0)* mutants (ADAMTS-4: 18% elongated arms, $n = 96$, Figure 1D; ADAMTS-9: 19% elongated arms, $n = 244$, not shown). The extent of *gon-1(0)* rescue was variable for both human proteases, and most rescued gonads were only partially elongated. To determine whether a more distantly related extracellular protease might similarly rescue *gon-1(0)* elongation, we used *lag-2 5'::MIG-17::GFP*, a secreted ADAM family protease [10]; however, MIG-17 did not rescue *gon-1(0)* mutants (0% elongated arms, $n = 84$). We conclude that the nematode and human ADAMTS homologs have conserved activities.

How might ADAMTS metalloproteases promote DTC migration? One possibility is that they remodel the ECM. To address this idea, we explored the functional relationships of GON-1 and selected ECM components. To determine whether depletion of individual ECM components could rescue *gon-1* elongation defects, we employed either mutations or RNA interference (RNAi) to decrease the level of individual ECM proteins. We examined DTC migration in *gon-1(0)* mutants after reducing collagen type IV $\alpha 2$, laminin- α B, nidogen (entactin), perlecan (heparin sulfate proteoglycan), fibulin, or hemicentin, and we identified one *gon-1* suppressor (Figure 2A). Whereas DTCs do not migrate in *gon-1* mutants [1], they were able to migrate and extend gonadal arms in *gon-1(0); fibulin-1(RNAi)* animals (20%, $n > 100$) (Figure 2A). In contrast, depletion of other ECM proteins did not rescue DTC migration in *gon-1(0)* mutants (Figure 2A). Therefore, *gon-1* suppression appears specific to fibulin.

The *C. elegans* fibulin gene, *fbl-1*, is most closely related to human *FBLN-1*, which encodes an ECM protein implicated in vasculogenesis [11, 12]. Both *C. elegans* and human FBL-1 proteins are composed of three anaphylotoxin (AT) repeats, nine EGF-like motifs, and either of two C-terminal fibulin motifs to generate FBL-1C or FBL-1D isoforms (Figure 2B; [2]). To assess *fbl-1* function, we isolated *fbl-1(q750)*, a 1.9 kb deletion that removes the FBL-1 protein's C-terminal half, including four EGF repeats, the entire C-specific fibulin motif, and part of the D-specific fibulin motif (Figure 2B). This mutant appears to be a strong loss-of-function allele (see Experimental Procedures).

As predicted by *fbl-1(RNAi)* (see above), the *fbl-1* dele-

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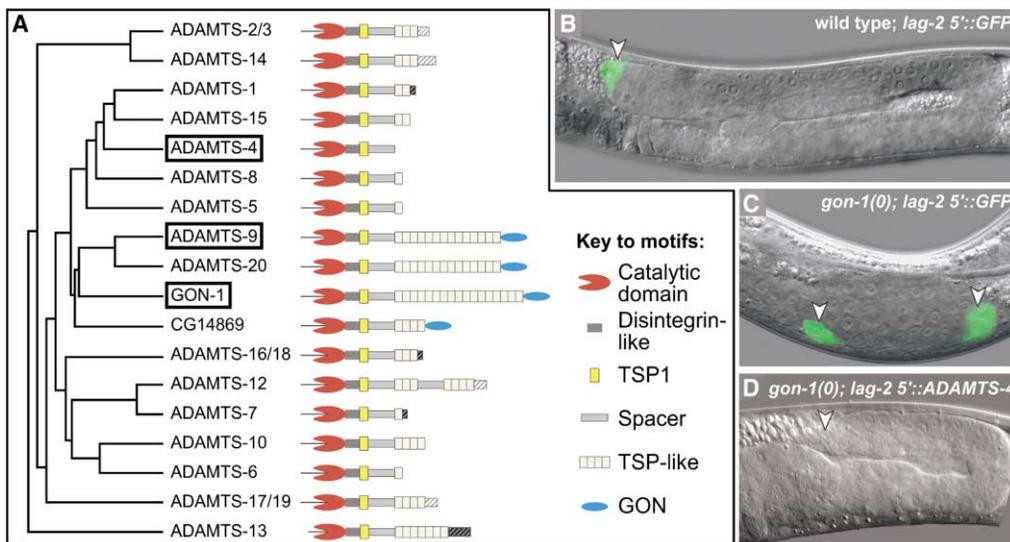


Figure 1. Human GON-1 Homologs Can Rescue *gon-1(0)* Gonadal Elongation Defect
(A) Phylogenetic tree and motif structures of ADAMTS homologs (adapted from [25]).
(B–D) L4s; genotype including transgene in figure; DTC marked by arrowhead.
(B) DTC at tip of U-shaped gonad.
(C) DTC near site of birth in disorganized gonad.
(D) DTC at end of elongated gonad with U-shape.

tion was a *gon-1* suppressor. In third larval-stage *gon-1* mutants, no DTC migration was seen (Figure 2C), but in *fb1-1 gon-1* double mutants of the same stage, all DTCs migrated and all gonads were elongated (Figure 2D). The *fb1-1* deletion is therefore a stronger *gon-1* suppressor than *fb1-1(RNAi)*. In addition, the *fb1-1* deletion suppressed other *gon-1* gonadogenesis defects: *gon-1* single mutants lack organized somatic gonadal structures [9], but *fb1-1 gon-1* double mutants possessed both recognizable uteri and spermathecae (Figure 3B). We conclude that the presence of fibulin blocks DTC migration in *gon-1* mutants.

The *fb1-1* deletion mutant grows more slowly than normal and is smaller in overall body size than wild-type animals (not shown). Gonadogenesis begins normally in *fb1-1* mutants: Gonadal morphology appears normal in young (L1–L3) larvae, and DTCs follow a U-shaped trajectory (not shown). The first gonadogenesis defect was seen at the L4 stage. Whereas the width of a wild-type L4 gonad is about half that of the body cavity (Figure 3A), *fb1-1* L4 gonads are distended and expand to fill the width of the body cavity. Furthermore, DTCs do not migrate past the bulging proximal gonad in *fb1-1* mutants (Figure 3B), and in adults, germ cells are released into the body cavity (not shown), a defect also seen in *epi-1* mutants [13]. To investigate whether the DTC block was due to a migration defect or obstruction by the widened gonad, we examined gonadal elongation in males. In wild-type males, the elongating gonad reflexes earlier than in hermaphrodites, and the male DTC equivalent, called the linker cell (LC), moves past the reflexed portion of the gonad before L4. In contrast to hermaphrodites, *fb1-1* males have a typical J-shaped gonad, albeit wider than normal (not shown). By watching LC migration during development, we found it to migrate along

its typical route all the way to the posterior. Therefore, the DTC migration defect in *fb1-1* hermaphrodites is likely to be a secondary consequence of physical obstruction imposed by the widened gonad. We conclude that FBL-1 is crucial for determining the normal width of the gonad.

We next examined the relationship between *gon-1* and fibulin in double mutants. In contrast to *fb1-1* single mutants, gonadal width was essentially normal in *fb1-1 gon-1* double mutants (Figure 3C). However, the small body size and slow growth rate typical of *gon-1* mutants were not suppressed by removal of *gon-1*. To test the specificity of *fb1-1* suppression by *gon-1*, we asked whether loss of a different metalloprotease might act similarly. Specifically, we asked whether *mig-17(0)* mutants, which lack a metalloprotease critical for DTC pathfinding [10, 14], might suppress the *fb1-1* gonadal width defect. However, *fb1-1; mig-17* mutants were morphologically indistinguishable from *fb1-1* single mutants. We conclude that wild-type GON-1 protein is responsible for the abnormal gonadal widening in *fb1-1* mutants.

To investigate where *fb1-1* is expressed, we generated transcriptional and translational reporters, both using the same 4.5 kb putative *fb1-1* promoter (*fb1-1 5'*). All *fb1-1* defects were rescued by the translational reporter; *fb1-1(q750); fb1-1 5'::GFP::FBL-1* transgenic animals were fertile and achieved normal body size. Both reporters expressed GFP in head muscle cells (Figures 3D, 3E, and 3H), anterior intestinal cells (Figures 3D, 3E, and 3H), posterior intestinal cells (Figures 3F and 3G), and tail muscle cells (not shown). The GFP::FBL-1 fusion protein accumulated around the pharynx, apparently in its basal lamina (Figure 3H), and in the intestinal cytoplasm. The pharyngeal accumulation was not dependent on *gon-1* activity because we detected GFP::FBL-1 fusion protein around the pharynx in *gon-1(0)* mutants.

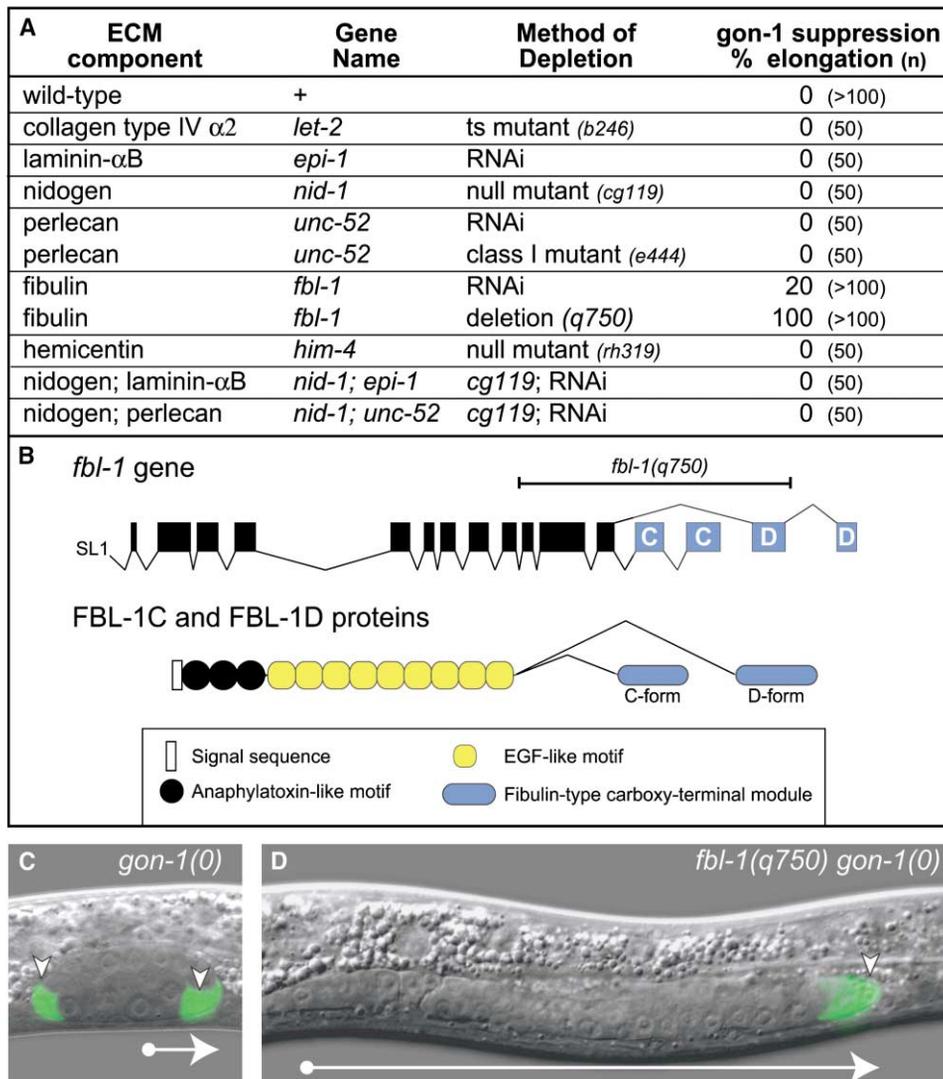


Figure 2. Suppression of *gon-1* Elongation Defect by Removal of *fibulin-1* (*fbl-1*)

(A) Summary of ECM depletion experiments. *epi-1*: [26]; *let-2*: [21]; *nid-1*: [27]; *unc-52*: [28]; *fbl-1*: [11]; and *him-4*: [29]. n represents the number of animals scored.

(B) Top: *fbl-1* gene structure, modified from [11]; exons marked by "C" and "D" encode "fibulin" motifs of FBL-1C and FBL-1D, respectively. Bottom: conserved motifs in FBL-1 protein.

(C and D) L3 larvae carrying *lag-2 5'::GFP*; DTC (arrowhead); the white arrow indicates extent of gonadal elongation. (C) shows a *gon-1(0)* single mutant, and (D) shows an *fbl-1(q750) gon-1(0)* double mutant.

We suspected that fibulin might associate with the gonadal ECM, but GFP::FBL-1 was not detectable there. To increase the sensitivity of the reporter, we replaced GFP with VENUS, a bright YFP variant [15]; this *fbl-1 5'::VENUS::RBL-1* transgene also rescued *fbl-1* defects, and its reporter protein was visible in a line surrounding the gonad during elongation (Figures 3I and 3J; not shown). In a parallel study, Nishiwaki and colleagues detected a similar pattern of *fbl-1* expression and also detected FBL-1C accumulation around dissected gonads ([16], this issue of *Current Biology*). We conclude that the *fbl-1* gene is expressed in nongonadal tissues and that FBL-1 protein becomes incorporated into basal laminae, including that surrounding the elongating gonad.

How might GON-1 and fibulin control morphogenesis? GON-1 is a metalloprotease, and one simple model is that GON-1 cleaves fibulin to remove its antagonistic activity and promote elongation. Such cleavage might degrade fibulin or process it to a form that does not interfere with elongation. To test this idea, we generated transgenic animals carrying tagged versions of fibulin; an N-terminally tagged GFP::FBL-1 transgene marked both FBL-1C and FBL-1D isoforms in one set of experiments (Figure 4A), and C-terminally tagged FBL-1::C-His and FBL-1::D-His transgenes marked FBL-1C and FBL-1D individually in a second set of experiments (not shown). All three tagged FBL-1 versions rescued *fbl-1(q750)* to fertility, but on Western blots, none was subject to detectable GON-1-dependent cleavage (Figure

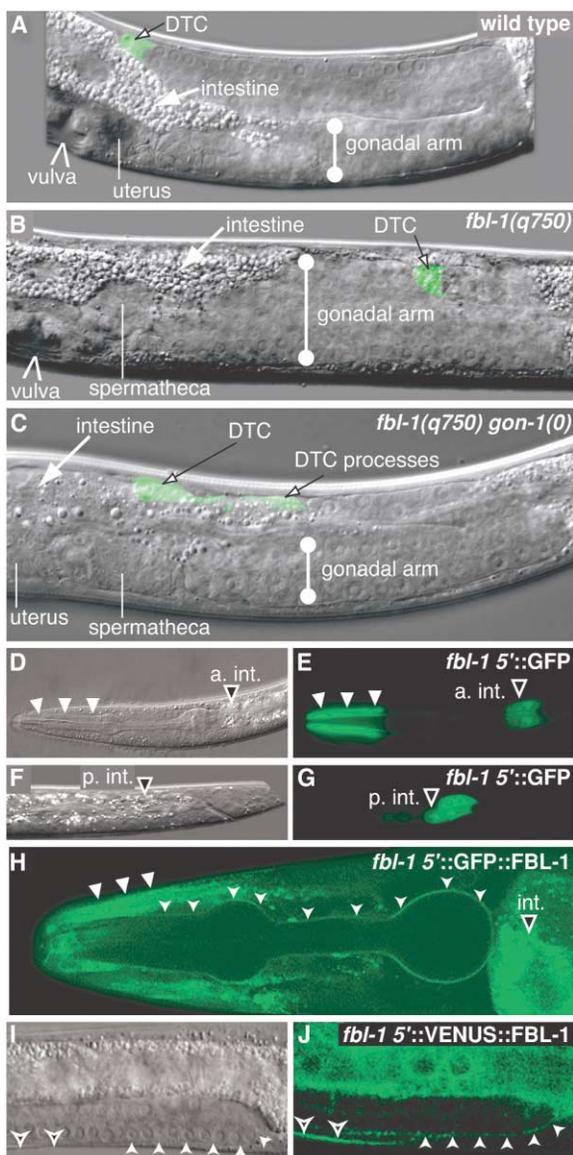


Figure 3. Fibulin Controls Gonadal Girth and Accumulates around Gonad during Elongation

(A–C) L4s; DTC expresses GFP; the white bar shows gonadal width. (A) Wild-type. The gonadal width is about half of the body cavity. (B) *fbl-1* single mutant. The gonad is wider, and DTC stops migration. (C) *fbl-1 gon-1* double mutant. The gonadal width is restored to about normal, and DTC migrates proximally. (D–G) L4s expressing *fbl-1 5'::GFP* transcriptional reporter. (D and E) Anterior part of animal. The white arrowheads indicate head muscle cells; the open arrowhead indicates an intestinal cell. (F and G) Posterior part of animal. The open arrowhead indicates an intestinal cell. (H–J) L4s expressing one of two FBL-1 translational reporters, which both rescue *fbl-1* mutants. (H) GFP::FBL-1 in ECM surrounding pharynx (small arrowheads) and in same cells as transcriptional reporter (same conventions as [D]). (I and J) VENU::FBL-1 in ECM surrounding gonad during elongation (small arrowheads); open arrowheads indicate background fluorescence on animal surface.

4A; data not shown). In an attempt to force GON-1 cleavage of fibulin, we added another transgene expressing a heat shock-driven GON-1. This second transgene,

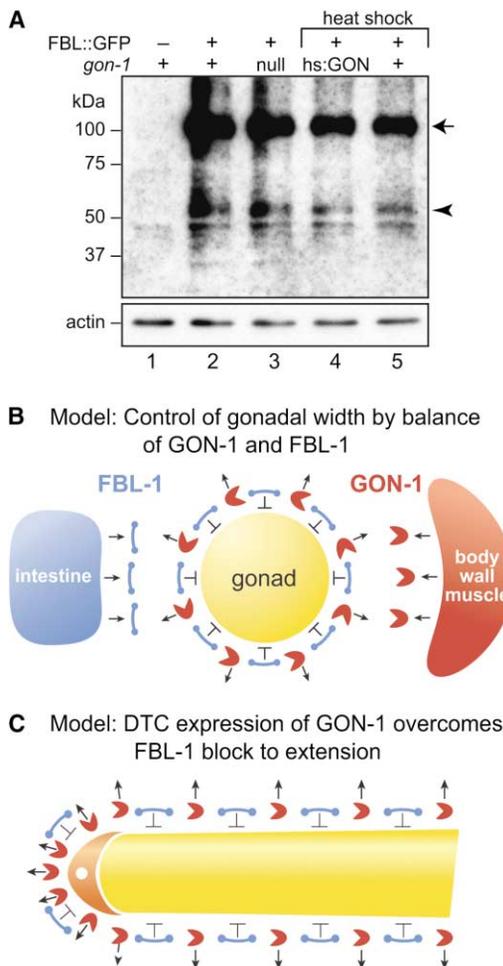


Figure 4. Molecular Basis of GON-1/FBL-1 Relationship: Tests and Models

(A) GFP::FBL-1 accumulates as major band of size predicted for full-length fusion protein (arrow) and smaller band in wild-type (lane 2), absence of GON-1 (lane 3), and excess GON-1 (lane 4). (B) Model for control of gonadal width by GON-1 and FBL-1. Transverse view. Both proteins are secreted by nongonadal tissues but are likely to function in gonadal ECM. GON-1 promotes gonadal expansion (arrows), and FBL-1 restricts gonadal expansion (bar). (C) Model for control of gonadal extension by GON-1 and FBL-1. Lateral view. Localized GON-1 expression by DTC shifts balance in favor of GON-1 to permit DTC migration and gonadal extension.

called *hs::GON-1*, makes functional GON-1, as assayed by *gon-1(0)* rescue. However, the tagged FBL-1 proteins were not cleaved after heat shock in animals carrying *hs::GON-1* (Figure 4A, lane 4). We did find that the full-length GFP::FBL-1 protein is processed into a smaller N-terminally tagged fragment predicted to contain the AT repeats and the first EGF module (Figure 4A); however, the generation of this fragment is independent of GON-1 activity (Figure 4A, compare lanes 2 and 3). These results do not exclude the possibility that GON-1 cleaves fibulin. Cleavage might occur in a minor fraction of total worm fibulin, for example in a gonad-specific manner, or cleavage products may be unstable and difficult to detect.

Wild-type GON-1 is required for gonadal widening in

fb1-1 mutants (see above), and, therefore, GON-1 is likely to cleave some substrate other than fibulin. Because human ADAMTS-4 and ADAMTS-9 can partially substitute for GON-1 (see above), their substrates might be relevant to GON-1. Aggrecan, brevican, and versican are the best-characterized ADAMTS-4 substrates [6, 17–19], but the *C. elegans* genome does not encode these proteoglycans [20]. Therefore, GON-1 and human ADAMTS proteases are likely to have additional substrates. Perhaps GON-1 and FBL-1 mediate their antagonistic effects by an indirect molecular mechanism. For example, GON-1 might cleave a protein that anchors FBL-1 within the extracellular matrix, and conversely, FBL-1 might antagonize GON-1 by forming interactions with other ECM components and thereby excluding GON-1 from its substrate. However, many mechanisms remain possible, including a direct interaction, and additional biochemical experiments are required to unravel the molecular basis of their antagonism.

How might GON-1/FBL-1 antagonistic activities differentially affect DTC migration and gonadal widening? A clue comes from their tissue expression. The *gon-1* gene is expressed by the gonadal DTC and nongonadal body wall muscles [1]; *fb1-1* is also expressed by nongonadal tissues, but not the DTC. Other *C. elegans* ECM components (e.g., collagen IV) accumulate in the gonadal ECM when expressed by nongonadal tissues [21], and FBL-1C similarly appears to accumulate around the gonad (this work; [16]). We suggest that the normal gonadal width results from a balance between GON-1 and FBL-1 activities, perhaps because both are produced in nongonadal sites and accumulate together at the gonadal surface (Figure 4B). In contrast, we know that DTC migration and gonadal extension rely on localized expression of GON-1 by the DTC [1]. This DTC expression could swing the GON-1/FBL-1 balance in favor of GON-1 and overcome the FBL-1-dependent block (Figure 4C).

We propose that control of gonadal shape by nematode GON-1 and FBL-1 may provide insight into the function and regulatory relationships of their mammalian homologs. Both GON-1 and FBL-1 are widely conserved proteins among metazoa, and we show here that human ADAMTS-4 and ADAMTS-9 can substitute for GON-1 in transgenic nematodes. Human FBLN-1 accumulates in the basement membrane surrounding endothelial cells [22], and murine fibulin-1 is essential for development of the vascular system [12]. Most *fibulin-1*(*-/-*) mice die shortly after birth, at least in part from vascular defects that include distended capillaries and a misshapen blood vessel endothelium. The vascular distension in *fibulin-1*(*-/-*) mice is reminiscent of gonadal widening in *fb1-1* mutant nematodes, suggesting that these cellular processes may be controlled by similar molecular mechanisms. Indeed, the fibulin-1 knockout mice also have renal and lung defects, suggesting that these mechanisms may extend to the generation of elongated tubules more broadly.

Experimental Procedures

Mutants and RNAi

The *gon-1*(518) and *mig-1*(k174) nonsense mutants were used as *gon-1*(0) and *mig-17*(0) respectively; other alleles are specified in

Figure 2A. All animals were grown at 20°C unless specified otherwise. The *fb1-1*(q750) deletion removes genomic DNA between positions F56H11:14083 and T05A1:1523; *fb1-1*(q750) cDNA analyses show that exon 9 is spliced to exon 16 and that the reading frame is shifted to generate a premature termination codon. RNAi of *unc-52* and *epi-1* was performed by feeding L1s with bacteria expressing dsRNA corresponding to each gene [23]. For disrupting *let-2* post-embryonically, L1s were shifted to 25°C to complete development. For depletion of *fb1-1*, dsRNA was injected into adult hermaphrodites, and progeny were scored.

Scoring Gonadal Elongation

In L4 hermaphrodites, gonads were scored either as not elongated, if they were disorganized and a rounded DTC was visible near the DTC site of birth, or as elongated, if a gonadal arm was visible with a migrating DTC at the tip.

Transgenes

All transgenes were constructed by standard procedures; details are provided in the Supplemental Data available with this article online. Control transgenes were *qls56* [24] and *Ex[lag-2 5'::MIG-17::GFP + mec-7::GFP]* [10].

Western Blot Analysis

Blots were prepared by standard procedures. Each lane contains 20 L4 worms, proteins were separated on 4%–20% gradient gels (Cambrex), and the blot was probed with primary monoclonal antibody JL-8 (Clontech).

Supplemental Data

Supplemental Experimental Procedures for this article are available online at <http://www.current-biology.com/cgi/content/full/14/22/2005/DC1/>.

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