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The minus-end actin capping protein, UNC-94/tropomodulin, regulates development of the *Caenorhabditis elegans* intestine

Elisabeth Cox-Paulson¹, Vincent Cannataro¹, Thomas Gallagher¹, Corey Hoffman¹, Gary Mantione¹, Matthew McIntosh¹, Malan Silva¹, Nicole Vissichelli¹, Rachel Walker¹, Jeffrey Simske², Shoichiro Ono³, and Harold Hoops¹

¹Department of Biology, SUNY College at Geneseo, 1 College Circle, 332 Integrated Science Center, Geneseo, New York

²Rammelkamp Center for Education and Research, Case Western Reserve University School of Medicine, MetroHealth Medical Center, Cleveland, Ohio

³Department of Pathology, Emory University, Whitehead 105N, Atlanta, Georgia

Abstract

Background—Tropomodulins are actin capping proteins that regulate the stability of the slow growing, minus-ends of actin filaments. The *C. elegans* tropomodulin homolog, UNC-94 has sequence and functional similarity to vertebrate tropomodulins. We investigated the role of UNC-94 in *C. elegans* intestinal morphogenesis.

Results—In the embryonic *C. elegans* intestine, UNC-94 localizes to the terminal web, an actin and intermediate filament rich structure that underlies the apical membrane. Loss of UNC-94 function results in areas of flattened intestinal lumen. In worms homozygous for the strong loss-of-function allele, *unc-94(tm724)*, the terminal web is thinner and the amount of F-actin is reduced, pointing to a role for UNC-94 in regulating the structure of the terminal web. The non-muscle myosin, NMY-1, also localizes to the terminal web; and we present evidence that increasing actomyosin contractility by depleting the myosin phosphatase regulatory subunit, *mel-11*, can rescue the flattened lumen phenotype of *unc-94* mutants.

Conclusions—The data support a model in which minus-end actin capping by UNC-94 promotes proper F-actin structure and contraction in the terminal web, yielding proper shape of the intestinal lumen. This establishes a new role for a tropomodulin in regulating lumen shape during tubulogenesis.

Keywords

tubulogenesis; terminal web; actomyosin contractility

Introduction

Tubes are a central architectural feature of many tissues. In humans, these tissues include glands, the urogenital and gastrointestinal tracts, the respiratory tract and the vascular system. A better understanding of tubulogenesis is essential for understanding diseases and birth defects that affect tubes, such as polycystic kidney disease and neural tube defects, and also for advancing tissue engineering.

The *C. elegans* intestine is an outstanding model system for investigating tube formation. This structure consists of twenty clonally derived cells that form a simple unbranched tube without associated smooth muscles (Leung et al., 1999). The cells are arranged mostly as bilaterally symmetric pairs with a central lumen. The lumen of the *C. elegans* intestine, like that of some kidney cells (reviewed in Lubarsky & Krasnow, 2003) and most capillaries (Egginton & Gerritsen, 2003), forms through a cord hollowing mechanism that involves organization of cells into a cord followed by formation of a fluid filled space at the apical surface. The cell divisions and rearrangements that generate the *C. elegans* intestine have been well studied (Leung et al., 1999).

Microarray analysis has revealed over eight hundred genes that show the same expression pattern as known endoderm-specific transcription factors (Kim et al., 2001), though only a handful of these have been characterized. In particular, studies have revealed an important role for the actin isoform, *act-5* (MacQueen et al., 2005), and several actin associated proteins in intestinal development. One of these is the ezrin-radixin-moesin family protein, ERM-1, which has been proposed to have a role in regulating the apical actin cytoskeleton of the intestine (Göbel et al., 2004; Van Furden et al., 2004). *erm-1* loss-of-function results in an abnormally twisted lumen, giving it a “pearls on a string” appearance. This phenotype is enhanced by loss of the apical cytoskeletal component, SMA-1/ β_H spectrin (Göbel et al., 2004), raising the possibility that ERM-1 may anchor the actin-spectrin cytoskeleton to the membrane during lumen morphogenesis. Furthermore, the plus-end actin capping protein, EPS-8, also has a role in *C. elegans* intestinal morphogenesis, with lumen expansions and abnormally long microvilli observed with *eps-8* loss-of-function (Croce et al., 2004). Actin is known to be involved in tubulogenesis in other organisms as well, with documented roles in regulation of cell polarity and lumen initiation (reviewed in Rodríguez-Fraticelli et al., 2011).

The demonstrated importance of actin and some actin-associated proteins in tubulogenesis suggests that other actin-binding proteins may be important in this process as well. Tropomodulins are ~40kD proteins that have two actin capping domains (reviewed in Fisher and Fowler, 2003). The N-terminal half contains binding sites for actin and tropomyosin, which co-polymerizes along and stabilizes F-actin (Cooper, 2002). The C-terminal half contains five leucine rich repeats and a second actin binding domain. Vertebrate tropomodulins cap tropomyosin coated actin filaments with high affinity ($K_d = 0.05$ nM) and can also cap F-actin with weak affinity ($K_d = 100 - 200$ nM), when tropomyosin is not present or not in register with the end of the actin filament (Weber et al., 1999). Vertebrate tropomodulin isoforms can also bind to monomeric actin and most have actin nucleating activity (Yamashiro et al., 2010).

Vertebrates have four widely expressed tropomodulin isoforms (Tmod1-4) that are conserved across species. The functions of vertebrate tropomodulins have been most extensively studied in striated muscle, where they regulate F-actin lengths in sarcomeres. Tropomodulins also regulate the actin-spectrin cytoskeleton in erythrocytes and intestinal endothelial cells, the actin cytoskeleton in lens epithelial cells, and the lamellipodial actin network of migrating endothelial cells (reviewed in Fischer & Fowler, 2003; Weber et al., 2007). Studies of Tmod1 knockout mice underscore the importance of tropomodulins in mammalian development. Tmod1 null mice arrest during embryogenesis with defects in heart and vasculature development (Chu et al., 2003; Fritz-Six et al., 2003). Hearts of Tmod1 null mice form nascent myofibrils, but they do not become striated, resulting in failure of the heart to beat. Tmod1 knockout mice have other defects, including abnormal heart tube looping, failed outgrowth of the right ventricle, and defects in blood vessel development (Chu et al., 2003; Fritz-Six et al., 2003).

C. elegans has one highly conserved tropomodulin homolog, *unc-94/tmd-1*, and a more divergent homolog, *tmd-2*. UNC-94 has minus-end F-actin capping activity (Yamashiro et al., 2008), and is an important factor for regulation of muscle sarcomere assembly (Stevenson et al., 2007; Yamashiro et al., 2008) and epidermal adherens junctions (Cox-Paulson et al., 2012). The work presented here demonstrates a new role for UNC-94 in regulation of the apical actin cytoskeleton during *C. elegans* intestinal development. We have found that UNC-94 regulates terminal web F-actin levels, which is crucial for maintaining proper shape of the intestinal lumen.

Results

UNC-94 localizes to the intestinal terminal web

unc-94 encodes two isoforms (*unc-94a* and *unc-94b*) that have a domain structure similar to vertebrate tropomodulins (Fig. 1A), and their amino acid sequences are 41% identical (53% similar) to human Tmod1 (Cox & Zhogbi, 2000; Yamashiro et al., 2012). Previous reports have shown that a transcriptional reporter for *unc-94b*, but not for *unc-94a* is active in the adult intestine (Stevenson et al., 2007). To better understand the role of UNC-94 in embryogenesis, we examined its subcellular localization via immunostaining. We found that UNC-94 was expressed in the embryonic intestine and colocalized with F-actin in the intestinal terminal web, and was also present both diffusely and in puncta in the cytoplasm of intestinal cells and other tissues (Fig. 1B). The diffuse cytoplasmic signal may represent TMD-1 bound to G-actin, and it is unclear what the cytoplasmic puncta are exactly, but they could be transport vesicles.

unc-94 loss-of-function results in localized areas of increased lumen diameter in the embryonic intestine

Next the intestinal defects caused by *unc-94* mutations were documented. The *unc-94* gene encodes two isoforms (*unc-94a* and *unc-94b*) that differ in the 5' UTR and first exon. Three mutant alleles have been characterized (Fig. 1A). *su177* has a point mutation in the first exon of *unc-94a*, resulting in loss of *unc-94a* expression, but normal expression of *unc-94b* (Stevenson et al., 2007). *sf20* and *tm724* are putative null alleles. *sf20* has a point mutation

that results in a premature stop codon (Stevenson et al., 2007); and the *tm724* allele has a 695 base pair deletion and 17 bp insertion that is predicted to result in a frame shift after the third exon (Yamashiro et al., 2008). The effect of these *unc-94* alleles on body wall muscle development has been determined (Stevenson et al., 2007; Yamashiro et al., 2008), but their roles in other tissues have not been explored.

The intestinal lumens of embryos from *unc-94* mutants were examined via phalloidin staining, which labels F-actin in the terminal web (Fig. 2). *C. elegans* embryos elongate 4-fold the length of the egg shell before hatching, and maximum intestinal lumen diameter was measured for embryos at the 3 - 3.5 fold stage. Measurements were performed on 3 - 3.5 fold embryos because the areas of intestinal expansion were not typically present before this stage. Confocal microscopy was used to visually assess the intestinal lumen region that had the widest diameter, and measurements were performed using ImageJ. Measurements were only performed on embryos with a clear view along the transverse axis of the intestine. *unc-94(sf20)* and *unc-94(tm724)*, but not *unc-94(su177)* mutants often exhibited localized areas of increased lumen diameter (Fig. 2A). The observation that *unc-94(su177)* mutants, which produce UNC-94b but not UNC-94a, had normal intestinal lumen diameters, indicates that UNC-94b is most important for regulating intestinal lumen shape.

unc-94(sf20) and *unc-94(tm724)* mutant embryos had median maximum intestinal lumen diameters of $2.1 \pm 0.6 \mu\text{m}$ and $2.2 \pm 0.8 \mu\text{m}$, in comparison to $1.8 \pm 0.4 \mu\text{m}$ for wild-type embryos; which was a statistically significant increase of 17% and 22%, respectively (Fig. 2A, B; $p < 0.001$ for both). The range was $2.9 \mu\text{m}$ (from $0.8 - 3.7 \mu\text{m}$) for wild-type embryos, $3.5 \mu\text{m}$ (from $1.5 - 5.0 \mu\text{m}$) for *unc-94(sf20)* embryos, and $3.6 \mu\text{m}$ (from $1.1 - 4.7 \mu\text{m}$) *unc-94(tm724)* embryos. Thus the range was larger for the tropomodulin mutant embryos, with higher minimum and maximum values. 49% of *unc-94(sf20)* mutant embryos and 52% of *unc-94(tm724)* had maximum intestinal lumen diameter measurements above $2.2 \mu\text{m}$ (1 S.D. above the mean for wild-type) versus 14% for wild-type. In summary, the intestines of both *unc-94(sf20)* and *unc-94(tm724)* embryos tend to have localized regions that exhibit increased lumen diameter.

Subsequently, to avoid the need to phalloidin stain embryos, lumen diameter was scored with the apical membrane marker, *erm-1::gfp*. *unc-94(tm724); erm-1::gfp* embryos exhibited a similar statistically significant increase in maximum intestinal lumen diameter in comparison to wild-type; *erm-1::gfp* embryos ($p < 0.001$, Fig. 2B). The median lumen diameter was $1.9 \pm 0.4 \mu\text{m}$ for wild-type (range = $2.6 \mu\text{m}$, from $1.1 - 3.7 \mu\text{m}$) and $2.2 \pm 0.8 \mu\text{m}$ for *unc-94(tm724)* embryos expressing *erm-1::gfp* (range = $3.7 \mu\text{m}$, from $1.4 - 5.1 \mu\text{m}$). 44% of *unc-94(tm724); erm-1::gfp* embryos had lumen diameters $> 2.3 \mu\text{m}$ (1 S.D. above the mean for wild-type, *erm-1::gfp* embryos) versus 15% for wild-type *erm-1::gfp*.

The regions of widest lumen diameter for both wild-type and *unc-94* mutant embryos almost always occurred in the anterior half of the intestine. The location of widest lumen diameter was determined using z-stacks taken through the intestines of embryos expressing *erm-1::gfp*. The majority of both wild-type (30 out of 33 embryos) and *unc-94(tm724)* embryos (27 out of 30 embryos) were found to have their maximum intestinal lumen diameter occur in the anterior half of the intestine. Furthermore, 77% of the *unc-94(tm724)*

embryos had the widest point within the first two intestinal rings (23 embryos out of 30 total). This suggests that UNC-94, which is present throughout the intestine (Fig. 1B), is most critical for regulating lumen diameter in the anterior region.

Next, a potential role for TMD-2 in intestinal development was assessed. To accomplish this, lumen diameter was measured in putative null *tmd-2(ok3417)* embryos, which have an 800 bp deletion in the *tmd-2* gene (Fig. 1A), and it did not differ statistically from wild-type (Fig. 2B). Furthermore, inhibiting both TMD-2 and UNC-94, by performing *unc-94(RNAi)* into *tmd-2(ok3417)*, yielded an average maximum intestinal diameter that was not statistically different from *unc-94(RNAi)* on its own (Fig. 2B). This suggests that UNC-94, but not TMD-2, is involved in intestinal development.

***unc-94* loss-of-function alters 3D lumen shape in embryonic and adult *C. elegans* intestines**

Next, confocal microscopy and image processing of *erm-1::gfp* expression were used to examine the 3D intestinal lumen shape of wild-type and *unc-94(tm724)* embryos. The 3D models show that the intestines of wild-type embryos had relatively round lumens, whereas the *unc-94(tm724)* embryos had areas of lumen flattening (Fig. 2C, Supplemental Movies 1, 2). In flattened areas, the lumen diameter was expanded in the transverse axis, but decreased in the dorsal-ventral axis. Intestinal length, surface area, and volume were quantified for 3 - 3.5 fold stage embryos using 3D models. There was not a statistically significant difference in the intestinal surface area or volume for *unc-94(tm724)* mutants in comparison to wild-type (Table 1). However, there was a small, but statistically significant difference in length along the anteroposterior axis, with *unc-94(tm724)* embryos having a mean intestinal length 8% less than that of wild-type embryos (76.0 μ m versus 82.7 μ m, Table 1). Thus, we can conclude that the changes in lumen shape (i.e. areas of flattening and slight decrease in length) observed in *unc-94(tm724)* embryos are not accompanied by changes in lumen volume or amount of apical membrane. Additionally, we determined that intestinal lumen circumference is increased in *unc-94(tm724)* embryos, which can account for why intestinal length is slightly decreased, despite no difference in volume or surface area. The median circumference at the anterior intestine was 20% greater for *unc-94(tm724)* embryos, (n=20 for wild-type, n=19 for *unc-94(tm724)*, $p < 0.01$); and the median circumference in the middle of the intestine was 16% greater for *unc-94(tm724)* embryos ($p < 0.05$).

The shape of the intestinal lumen was also examined in adults expressing *erm-1::gfp*. By adulthood, the intestinal lumen in wild-type worms had an oval shape, in which the lumen was narrow along the dorsal-ventral axis and much wider along the transverse axis (Fig. 2D). In contrast, the lumens of *unc-94(tm724)* worms were rounder, due to being much wider in the dorsal-ventral axis than wild-type worms. This may be the result of a weakened terminal web and/or to constipation, potentially caused by impaired function of the body wall or enteric muscles involved in defecation. We found that the length of the defecation cycle was increased in *unc-94(tm724)* mutants. The time between defecation events was measured for three complete cycles and the average determined. The defecation cycle for wild-type worms is known to be 45 $\pm$ 3 sec at 20°C (Liu and Thomas, 1994). For 36 adult wild type worms observed at 22°C, the average defecation cycle was 47 $\pm$ 3 seconds. For

20 *unc-94(tm724)* worms, 12 worms were observed to have an average defecation cycle of 58 $\pm$ 11 seconds, whereas the rest were either completely or partially constipated (not defecating at all within 2 minutes or taking longer than 2 minutes for at least one of the observed cycles). Constipation may therefore be the cause of the uniformly expanded lumen shape in adult *unc-94(tm724)* mutants.

Abnormal shape of the intestinal lumen is likely to contribute to the slow growth of *unc-94(tm724)* worms, which take longer to reach adulthood and are shorter in length as adults. Specifically, *unc-94(tm724)* worms took 72 hours to reach adulthood (time at which half of worms have eggs), versus 54 hours for wild-type worms (n=24 for both). Also, at the first larval stage the lengths of *unc-94(tm724)* and wild-type worms were similar (about 255 μ m). However, at adulthood, *unc-94(tm724)* worms were 29% shorter in length compared to wild-type worms (964 $\pm$ 95 μ m for wild-type worms, n=24; 681 $\pm$ 77 μ m for *unc-94(tm724)* worms, n=24).

UNC-94 is required for excretory cell morphogenesis

In addition to marking the apical intestinal surface, ERM-1::GFP also localizes to the excretory cell apical surface. The excretory cell is a large H-shaped cell that has been proposed to regulate osmolarity and waste excretion (reviewed in Buechner, 2002). The excretory cell body is located near the posterior bulb of the pharynx, and it has two canals that extend anteriorly and two that extend posteriorly. The canals have a lumen at their center that is bordered by an apical membrane. While examining 3D intestinal shape in *unc-94(tm724); erm-1::gfp* worms, several excretory cell defects were observed, including incomplete extension of the apical membranes, abnormal shape of the cell body (white arrow, Fig. 2D), and canal cysts (red arrows, Fig. 2D). *unc-94(tm724); erm-1::gfp* embryos were also found to have defects in excretory cell body shape and extension of the apical canal membranes. We quantitated excretory cell apical membrane extension in 3.5 - 4 fold *unc-94(tm724); erm-1::gfp* embryos (n=99), and determined that 56% had no extension of the anterior or posterior excretory canal apical membranes, and in the remaining cases, there was only partial extension (in comparison to the amount of extension observed in similarly aged wild-type, *erm-1::gfp* embryos). Only 12% showed some apical membrane extension for one or both of the anterior canals; and 39% showed some apical membrane extension for one or both of the posterior canals. Therefore, UNC-94 is necessary for proper lumen morphogenesis in both the intestine and the excretory cell; however, its mechanisms of action in these organs may be distinct. We have focused our attention here on determining the role of UNC-94 in the intestine.

UNC-94 regulates F-actin levels in the intestinal terminal web

The intensity of F-actin staining at the apical surface of the embryonic intestine was determined for wild-type and homozygous *unc-94(tm724)* embryos at the 3 - 3.5 fold stage. Interestingly there was a strong reduction in levels of apical intestinal F-actin in *unc-94(tm724)* embryos in comparison to wild-type embryos (n=82 for wild-type, n=55 for *unc-94(tm724)*). Seven of the *unc-94(tm724)* embryos had no detectable F-actin in the intestine, despite having F-actin staining in the pharynx. For the *unc-94(tm724)* embryos

with detectable F-actin in the intestinal terminal web, there was a 28% reduction in fluorescence intensity in comparison to wild-type embryos ($p < 0.001$, Fig. 3A).

Intermediate filaments are also important components of the terminal web in the *C. elegans* intestine, and mammalian Tmod4 has been reported to bind filensin, an intermediate filament protein in lens epithelial cells (Fischer et al., 2003). In light of this, we examined the effect of *unc-94* loss of function on IFB-2, an intermediate filament protein found in the terminal web (Carberry et al., 2009). IFB-2 was visualized via immunostaining with the monoclonal MH33 antibody and fluorescence intensity was quantified using a similar approach to that used for actin quantification. There was not a statistically significant difference in the terminal web levels of IFB-2 in *unc-94(tm724)* mutants compared to similarly aged wild-type embryos (Fig. 3B). However, there was a slight increase in the number and brightness of IFB-2 puncta in the cytosol (boxed region in Fig. 3B). It is unclear whether this is material that has broken away from the terminal web, or if it may be IFB-2 in vesicles. Therefore, whereas UNC-94 has a major effect on regulating terminal web F-actin levels in the embryonic intestine, it does not have an effect on terminal web IFB-2 levels, although IFB-2 localization appears to be slightly disrupted.

Interestingly, transmission electron microscopy analysis indicated that the terminal web thickness of homozygous *unc-94(tm724)* worms at both the first larval stage and in adults was decreased in comparison to wild-type worms. Based on the data above, this could be a consequence of decreased F-actin content in this structure. For L1 larvae, the terminal web was 14.3% thinner in *unc-94(tm724)* worms. The terminal web was 0.070 ± 0.120 microns thick in wild-type L1s and 0.060 ± 0.008 microns thick in *unc-94(tm724)* L1s ($n=9$ for wild-type, $n=11$ for *unc-94(tm724)*, $p < 0.05$). In adults, the terminal web was 16.9% thinner in *unc-94(tm724)* worms. The terminal web was 0.109 ± 0.014 microns thick in wild-type adults and 0.090 ± 0.013 microns thick in *unc-94(tm724)* mutants ($n=8$ for both conditions, $p < 0.015$). Microvilli of normal structure were observed in both L1 and adult *unc-94(tm724)* worms (Fig. 4B).

***unc-94* loss-of-function does not disrupt cell junctions or cell polarity**

Next we determined if intestinal cell-cell junctions were affected in *unc-94(tm724)* mutants. We were particularly interested in doing this because UNC-94 has a documented role in reinforcing a subset of adherens junctions in the embryonic epidermis that are under stress during morphogenesis (Cox-Paulson et al., 2012). In the embryonic intestine, the adherens junctions and the more basal AJM-1 complex appeared to form normally in *unc-94(tm724)* mutants (Fig. 4A). Additionally, electron dense apical junctions with normal morphology were seen in adult *unc-94(tm724)* mutants via transmission electron microscopy (boxed regions in Fig. 4B). These results indicate that apical junctions are formed and maintained in the intestines of *unc-94(tm724)* mutants. This differs from the role of UNC-94 in the embryonic epidermis, where it is required for some apical junctions to maintain their integrity during development (Cox-Paulson et al., 2012). Furthermore, it can be concluded that intestinal cell polarity is not perturbed with *unc-94* loss-of-function, since normal microvilli were present at the apical surface, and ERM-1, IFB-2, and cell junction proteins all localized appropriately.

The *C. elegans* intestinal terminal web is contractile, and increasing actomyosin contractility can rescue the *tmd-1* loss-of-function phenotype in the intestine

The intestinal terminal webs of mice, rats and chickens contain myosin II (Bretscher and Weber, 1978; Drenckhahn et al., 1980; Herman and Pollard, 1981; Hirokawa et al., 1982; Mooseker, 1985; Heintzelman et al., 1994), and isolated brush borders have been shown to be contractile (Rodewald et al., 1976; Burgess, 1982; Keller et al., 1985). We found that the non-muscle myosin II isoform, NMY-1, localized to the intestinal terminal web in 3-fold and older *C. elegans* embryos (Fig. 5A). NMY-1 also localized normally in the intestinal terminal web of *unc-94(tm724)* embryos, and was present at levels similar to wild-type (Fig. 5A, B).

MEL-11/myosin phosphatase regulatory subunit and LET-502/Rho kinase are known to regulate actomyosin contractility in several epithelial tissues in *C. elegans*, including the hypodermis, spermatheca, and vulva (Wissmann et al., 1999; Piekny et al., 2000; Farooqui et al., 2012). Myosin phosphatases consist of three subunits (regulatory, catalytic, and a smaller subunit of unknown function) and inhibit actomyosin contraction by dephosphorylating myosin regulatory light chains. Rho kinases promote actomyosin contraction by phosphorylating myosin regulatory light chains and by inhibiting myosin phosphatases. We determined that a full length MEL-11::GFP fusion protein localized to the terminal web in 3-fold and older embryos (Fig. 5C). MEL-11 and LET-502 have previously been shown to co-localize with cell junction proteins in the intestines of younger, 2-fold stage embryos (Piekny et al., 2003); and up to this point, their functional roles in the intestine have not been documented.

Since there is less F-actin in the terminal web of *unc-94(tm724)* mutants, we reasoned that the regions of lumenal flattening may be due to abnormally low levels of actomyosin contraction in the terminal web. To test this, MEL-11 was partially depleted by feeding RNAi for 26–28 hours in wild-type and *unc-94(tm724)* worms expressing *erm-1::gfp*. This would be predicted to increase actomyosin contractility by increasing phosphorylation of regulatory myosin light chains. Partial depletion was necessary because strong loss of MEL-11 results in early embryonic lethality due to cytokinesis and/or enclosure defects (Wissmann et al., 1999; Piekny & Mains, 2002). Feeding RNAi for 26–28 hours reduced intestinal MEL-11::GFP levels by 74% (refer to Experimental Procedures). In wild-type worms, this RNAi treatment did not result in embryonic lethality, and maximum intestinal lumen diameter was normal (Fig. 5D). In *unc-94(tm724)* worms, *mel-11(RNAi)* increased the amount of early embryonic lethality; however, embryos that made it to the 3-fold stage of elongation exhibited rescue of maximum lumen diameter to wild-type levels (Fig. 5D). This result suggests that partial depletion of MEL-11 can rescue the wide lumen defect of *unc-94(tm724)* mutants. However, an important caveat is that RNAi effects can be variable, and we do not know exactly how much MEL-11 was expressed by the rescued embryos.

If the *unc-94(tm724)* flattened lumen phenotype is due entirely to lower levels of actomyosin contractility in the terminal web, then loss of *let-502*/Rho kinase function in wild-type embryos would be expected to yield a similar phenotype. To assess this, we examined the intestines of embryos from the HR0766 strain, which contain the

let-502(ca201) allele balanced by the translocation, hT2. *ca201* is a gain-of-function allele with a mutation in the kinase domain, and is likely to be dominant negative (Wissmann et al., 1997). Embryos homozygous for *ca201* do not elongate past the 1.5-fold stage (Wissmann et al., 1997); and those homozygous for hT2 exhibit early embryonic lethality due to aneuploidy. Therefore, only heterozygous *let-502(ca201)* embryos elongate to the 3-fold stage, and these were analyzed via phalloidin staining to visualize the intestinal lumen. Interestingly, *let-502(ca201)* heterozygotes exhibited areas of wider than normal lumen diameter ($2.4 \pm 0.7 \mu\text{m}$, $n=106$, $p < 0.05$ in comparison to wild-type), although these areas were not flattened (Fig. 5E, Supplemental Movie 3). Thus, although the *unc-94* flat lumen phenotype can be rescued by increasing actomyosin contractility, loss of actomyosin contractility does not precisely phenocopy *unc-94* loss-of-function.

UNC-94 does not nucleate actin filaments *in vitro*

Next we investigated how UNC-94 regulates F-actin levels in the terminal web. Tropomodulins have multiple effects on actin in different systems, and it is possible that UNC-94 may regulate F-actin levels in the intestinal terminal web by nucleating new actin filaments and/or by minus-end actin filament capping. UNC-94 has previously been shown to have minus-end actin capping activity; specifically, UNC-94 can inhibit cofilin-induced actin depolymerization from F-actin capped at the plus-ends with gelsolin (Yamashiro et al., 2008). The ability of UNC-94 to nucleate F-actin has not previously been explored, though it does have conservation of some sequences required for actin nucleation in mammalian tropomodulin isoforms (Yamashiro et al., 2012). As described in Experimental Procedures, the effect of UNC-94 on actin polymerization *in vitro* was examined with pyrene labeled G-actin, which exhibits enhanced fluorescence when it is incorporated into filaments. If actin nucleation is enhanced, the initial rate of actin polymerization would be accelerated. UNC-94 (0.4 and 0.8 μM) did not alter the initial rate of actin polymerization in this assay (Fig. 6), although equivalent concentrations of mammalian tropomodulins are reported to enhance actin nucleation (Yamashiro et al., 2010). The slight increase in polymerization observed at later time points is consistent with minus-end actin capping, which inhibits depolymerization from the minus ends. These results strongly suggest that UNC-94 lacks actin-nucleating activity. Thus UNC-94 is likely to exert its effects on terminal web F-actin levels by regulating lengths and/or stability of actin filaments via its minus-end capping activity and not by actin filament nucleation.

Discussion

The work presented here highlights a new role for the tropomodulin, UNC-94, in regulation of lumen shape during tubulogenesis. This adds to the list of key cellular processes that are regulated by tropomodulins, which includes muscle contraction, cell migration and cell shape regulation. Specifically, we have found that UNC-94 localizes to the terminal web in the embryonic *C. elegans* intestine, where it regulates F-actin levels. This, in turn, is necessary to support normal actomyosin contractility in the terminal web and to promote proper lumen shape.

Tropomodulins appear to have conserved, cell specific roles in regulating filamentous actin levels. Interestingly, similar to *unc-94* mutants, a decrease in F-actin is also seen in polarized mammalian epithelial cells treated with shRNA for Tmod3 (Weber et al., 2007). However, Tmod3 has the opposite effect on F-actin levels in migrating endothelial cells (Fischer et al., 2003). To account for this difference, it has been proposed that in polarized epithelial cells, Tmod3 may cap F-actin minus-ends and stabilize actin arrays; whereas in migrating cells, Tmod3 may sequester G-actin, making it unavailable for polymerization (reviewed in Yamashiro et al., 2012). In the *C. elegans* intestine it is most likely that UNC-94 regulates actin via minus-end capping, since it does not nucleate F-actin, nor interfere with its polymerization in biochemical assays. Thus in both mammalian epithelial cells and in the *C. elegans* intestine, tropomodulin appears to regulate levels of F-actin by minus-end actin capping.

This work also highlights the importance of actomyosin contractility in regulation of lumen shape. Isolated brush borders are contractile (Rodewald et al., 1976; Burgess, 1982; Keller et al., 1985); however, the role of actomyosin contractility in tubulogenesis is just beginning to be understood. There is evidence that actomyosin contraction actually inhibits lumen formation in 3D MDCK cell cultures, while not affecting maintenance of the lumen after it is formed (Ferrari et al., 2008; Rodríguez-Fraticelli, 2012). The work presented here shows that the *C. elegans* terminal web is indeed contractile, and that actomyosin contractility is an important regulator of lumen shape in this organism. However, as documented here, actomyosin contractility does not appear to inhibit lumen formation in the *C. elegans* intestine as it does in MDCK cells (Ferrari et al., 2008; Rodríguez-Fraticelli, 2012). This is somewhat surprising, since MDCK cells, like the cells of the *C. elegans* intestine, form their lumen via a cord-hollowing mechanism (reviewed in Lubarsky and Krasnow, 2003); and highlights that there can be molecular differences in the ways that any given lumen forming mechanism is executed.

Why does loss of *unc-94* function result in areas of flattened lumen versus the uniformly expanded lumen regions observed in *let-502/rho kinase* mutants? The reason for this is unclear. However, one possible explanation is that uniformly expanded regions may occur when terminal web actin does not anchor properly to apical junctions. Depletion of Arp2/3, ERM-1 or DLG-1 in *C. elegans* embryos results in areas of uniformly expanded intestinal lumen (Göbel et al., 1994; Bernadskaya et al., 2011). Among their other roles, Arp2/3 and ERM-1 have been shown to regulate membrane association of the apical junction protein DLG-1, which recruits F-actin to intestinal apical junctions during embryogenesis (Bernadskaya et al., 2011). Therefore, the uniformly expanded lumen regions observed in Arp2/3, ERM-1 and DLG-1 mutants could be a result of failed anchoring of actin to apical junctions. It is not known if LET-502/Rho Kinase plays a similar role, however, it is known to localize to intestinal apical junctions in 2-fold stage *C. elegans* embryos (Piekny et al., 2003). Loss of F-actin attachment to junctions could cause areas of uniformly widened lumen if the position of the junctions were to slip more basally, and/or if alterations to vesicle trafficking resulted in increases in apical membrane. In *unc-94(tm724)* mutants, AJM-1, which forms a complex with DLG-1 at apical junctions, localizes normally; and HMR-1/cadherin also shows proper localization. Thus, it could be that actin anchors

successfully to apical junctions in *unc-94(tm724)* mutants, but that improper structure of the terminal web results in a loss of tension, causing the observed regions of flattened lumen.

There is also the possibility that UNC-94 regulates lumen shape by alternative or additional mechanisms. For instance, the decreased terminal web actin levels caused by loss of UNC-94 could affect vesicle transport to the apical surface. Actin has a well-established role in regulating vesicle transport (reviewed in Lanzetti, 2007). Perhaps loss of *unc-94* function disrupts the terminal web actin structure, causing impaired delivery of structural or signaling molecules needed for proper lumen shape. UNC-94 could also have a more direct role in vesicle regulation, as it does have a punctate appearance in the intestinal cytosol that resembles vesicles. More simply, less actin in the terminal web may weaken its structure, and the lumen could collapse in regions due to failed mechanical support. Further investigation will be necessary to sort out these possibilities. In summary, the work presented here indicates that UNC-94 is an important regulator of lumen shape in the embryonic *C. elegans* intestine, and establishes a new role for a tropomodulin in regulation of tubulogenesis.

Experimental Procedures

Strains and Alleles

All *C. elegans* strains were maintained as described in Stiernagle, 2006. The Bristol N2 strain was used as wild-type. The following strains were kindly provided by those indicated: strains carrying the *unc-94(su177)* and *unc-94(sf20)* alleles were from Guy Benian (Emory University); the VJ402 strain (N2; *erm-1::gfp*) was from Verena Göbel (Massachusetts General Hospital and Harvard Medical School); and the HR0766 strain was from Paul Mains (Univ. of Calgary). The *unc-94(tm724)* strain was obtained from Shohei Mitani (National Bioresource Project for *C. elegans*, Tokyo Women's Medical University School of Medicine) and outcrossed six times. The GSC5 strain was generated by introducing *erm-1::gfp* into the homozygous *unc-94(tm724)* background.

The QQ155 strain was generated by injecting pJS588 (*mel-11::gfp*, a translational fusion) and pDPmm0168 (*unc-119+*) into *unc-119(ed3)*, generating *cvEx87*. pJS588 was generated by amplifying the *mel-11* genomic region (LGII: 9355193 to 9369242), and cloning into the PstI/BamHI site of pPD95.75. The resulting construct, pJS588, contains the entire *mel-11* genomic region including 2.7kb of the 5' end, 600bp downstream of F42A8.3 and 500bp of the 3' end, stopping at the 3' end of C06C3.3.

The RB2477 strain, which is homozygous for the *tmd-2(ok3417)* allele was obtained from the Caenorhabditis Genetics Center (Univ. of Minnesota), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). The *tmd-2(ok3417)* allele was sequenced and determined to contain a 788 bp deletion with an upstream flanking sequence of "AACCGAATTGTTTCTTGGGATGAAG", downstream flanking sequence of "ATAAAAATTGAAATTCTTGAAAAAT" and a 24 bp insertion: AGAACCGAATTGTTACTTGGGATA. All feeding RNAi was performed using clones from the *C. elegans* ORFeome feeding RNAi library, version 1.1 (Thermo Scientific).

Phalloidin and Antibody Staining

Phalloidin staining was performed as described in Cox-Paulson et al., 2012, except that embryos were treated with a bleach solution (0.25N KOH, 0.1% sodium hypochlorite) for 2 minutes and washed twice with double distilled water, instead of treated with chitinase. Alexa-labeled phalloidins were purchased from Molecular Probes.

Antibody staining was performed using a freeze-cracking approach as described in Costa et al., 1997, except that after the methanol fixation, the slides were incubated in 1x phosphate buffered saline with 1% Tween (PBST) with 1% bovine serum albumin for 30 minutes, and then washed three times for 10 minutes with 1xPBST before proceeding with antibody staining. The polyclonal rabbit anti-UNC-94 antibody was produced and affinity purified by the Proteintech Group, Inc. (Chicago, IL) using amino acids 144–401 of UNC-94b as the antigen (Stevenson et al., 2007). The MH27 antibody was purified from ascites fluid (Harlan Sprague Dawley, Madison, WI) from hybridoma cells obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by Univ. of Iowa). The MH33 antibody developed by R.H. Waterston was obtained from the Developmental Studies Hybridoma Bank (Univ. of Iowa). The HMR-1 antibody was kindly provided by J.D. Hardin (Univ. of Wisconsin). The NMY-1 polyclonal antibody was a generous gift from Alisa Piekny (Concordia University, Montreal). Secondary antibodies included: goat anti-rabbit fluorescein, goat anti-mouse DyLight 488, goat anti-mouse DyLight 549 (all from Jackson ImmunoResearch) and goat anti-mouse Alexa 633 (Molecular Probes).

Fluorescent Imaging and Analysis

Images and z-stacks of fluorescent samples were acquired with an EZ-C1 Nikon laser-scanning confocal microscope. Lumen width measurements were performed using the line function in ImageJ. In the text, all means for lumen width and other measurements are reported $\pm$ the standard deviation. For box plots, the box shows the interquartile deviation with a line at the median; the whiskers indicate values that fall within one and half of the interquartile range from the median; and the dots indicate outliers. Lumen length, volume, surface area, and circumference measurements were performed using Imaris software. Surface area measurements were determined from 3D models generated by tracing the outer edge of ERM-1::GFP or actin staining in z-stacks with an 0.6 μ m step size. Volume measurements were determined from 3D models generated by tracing the outer edge of the open lumen space in z-stacks with an 0.3 μ m step size. Circumference was determined in the second intestinal ring (anterior measurement) and in the fourth or fifth intestinal ring (middle measurement) using the models generated for surface area analysis. For statistical analysis, normality was determined using the Anderson-Darling test, and then appropriate statistical tests were performed (i.e. two-tailed t-tests for parametric data, and two-tailed Mann-Whitney U tests for non-parametric data).

Quantification of actin, IFB-2, and NMY-1 levels in the terminal web

To assess levels of F-actin in the intestinal lumen of 3-fold or older embryos, wild-type; *erm-1::gfp* and *unc-94(tm724); erm-1::gfp* embryos were labeled with Alexa-546 phalloidin. The terminal web was identified via ERM-1::GFP with wide-field fluorescent

microscopy (green channel). Then a confocal image (12-bit tonal range) was taken in the red channel (543 nm excitation). This technique was used to minimize photobleaching of the fluorescently labeled phalloidin. The same settings, including gain and pixel dwell, were used to collect all images. The maximum fluorescence intensity, on a scale of 0 (black) - 4095 (white), over three lines drawn through the intestinal lumen was determined using ImageJ and averaged. The average fluorescence intensity was calculated for each condition and used to determine the percentage of signal reduction. A similar approach was taken to quantify levels of IFB-2 and NMY-1.

Quantification of MEL-11 knockdown via feeding RNAi

mel-11(RNAi) was performed on the QQ155 strain for either 26 or 28 hours; and treatment with the empty feeding vector, L4440, was used as a negative control. Embryos were collected and stained with Alexa 546-phalloidin as described above. To assess the level of *mel-11* knockdown in the intestine, first, the intestinal lumen of 3-fold or older embryos was identified via phalloidin staining with wide-field fluorescence microscopy (red channel). Then a confocal image was taken in the green channel (488 nm excitation) to get an image of the MEL-11::GFP. The same settings, including gain and pixel dwell, were used to collect all images. For each experiment, 75 embryos were imaged for each condition. The average fluorescence intensity per embryo was calculated for each condition and used to determine the percentage of signal reduction, as described above for quantification of actin staining. 26 hour *mel-11(RNAi)* resulted in a 75.2% reduction in MEL-11::GFP signal, and 28 hour *mel-11(RNAi)* yielded 73.3% reduction.

Actin nucleation assay

Kinetics of actin polymerization from G-actin was measured essentially as described (Yamashiro et al., 2010). G-actin was purified from rabbit muscle acetone powder (Pel Freeze) as described (Pardee & Spudich, 1982) followed by gel filtration by Sephacryl S-300 in G-buffer (2 mM Tris-HCl, 0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM DTT, pH 8.0). Labeling of G-actin by pyrene was performed as described (Kouyama & Mihashi, 1981). Recombinant UNC-94 protein was purified as described (Yamashiro et al., 2008). Briefly, 40 mM G-actin was converted to Mg²⁺ATP actin by incubating with 50 mM MgCl₂ and 1 mM EGTA for 5 min on ice. Actin was pre-incubated with UNC-94 for 1 min, and diluted to 4 mM actin in polymerizing buffer (final 0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA, and 20 mM HEPES-NaOH, pH 7.5 at time 0). The pyrene fluorescence was monitored (excitation: 366 nm and emission: 407 nm) using an F-4500 fluorescence spectrophotometer (Hitachi High Technologies).

Electron Microscopy

For L1 worms, specimen preparation was performed using the conventional two-step fixation method as described in Hall, 1995 except that after ethanol dehydration, the specimens were transferred to acetone and embedded in a graded series of acetone Epon-Araldite mixtures. The Epon-Araldite was polymerized overnight at 60 °C. For adults, specimen preparation was performed in the Hall laboratory (Albert Einstein College of

Medicine) using microwave fixation as described in WormAtlas.org (<http://www.wormatlas.org/microwavefix.htm>).

For both L1s and adults, 60 nm thick sections were stained with 4% uranyl acetate and 0.15% lead citrate, and then viewed using a Morgagni transmission electron microscope. The thickness of the terminal web was determined at the base of microvilli, and at least four measurements were taken per worm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Bernadskaya YY, Patel FB, Hsu HT, Soto MC. Arp2/3 promotes junction formation and maintenance in the *Caenorhabditis elegans* intestine by regulating membrane association of apical proteins. *Mol Biol Cell*. 2011; 22:2886–2899. [PubMed: 21697505]
- Bretscher A, Weber K. Localization of actin and microfilament-associated proteins in the microvilli and terminal web of the intestinal brush border by immunofluorescence microscopy. *J Cell Biol*. 1978; 79:839–845. [PubMed: 365871]
- Buechner M. Tubes and the single *C. elegans* excretory cell. *Trends Cell Biol*. 2002; 10:479–84. [PubMed: 12441252]
- Burgess DR. Reactivation of intestinal epithelial cell brush border motility: ATP-dependent contraction via a terminal web contractile ring. *J Cell Biol*. 1982; 95:853–863. [PubMed: 7153249]
- Carberry K, Wiesenfahrt T, Geisler F, Stocker S, Gerhardus H, Uberbach D, Davis W, Jorgensen E, Leube RE, Bossinger O. The novel intestinal filament organizer IFO-1 contributes to epithelial integrity in concert with ERM-1 and DLG-1. *Development*. 2012; 139:1851–1862. [PubMed: 22510987]
- Carberry K, Wiesenfahrt T, Windoffer R, Bossinger O, Leube RE. Intermediate filaments in *Caenorhabditis elegans*. *Cell Motil Cytoskeleton*. 2009; 66:852–864. [PubMed: 19437512]
- Chu X, Chen J, Reedy MC, Vera C, Sung KL, Sung LA. E-Tmod capping of actin filaments at the slow-growing end is required to establish mouse embryonic circulation. *Am J Physiol Heart Circ Physiol*. 2003; 284:H1827–1838. [PubMed: 12543641]
- Cooper JA. Actin dynamics: tropomyosin provides stability. *Curr Biol*. 2002; 12:R523–525. [PubMed: 12176375]
- Costa M, Draper BW, Priess JR. The role of actin filaments in patterning the *Caenorhabditis elegans* cuticle. *Dev Biol*. 1997; 184:373–384. [PubMed: 9133443]
- Cox PR, Zoghbi HY. Sequencing, expression analysis, and mapping of three unique human tropomodulin genes and their mouse orthologs. *Genomics*. 2000; 63:97–107. [PubMed: 10662549]
- Cox-Paulson EA, Walck-Shannon E, Lynch AM, Yamashiro S, Zaidel-Bar R, Eno CC, Ono S, Hardin J. Tropomodulin protects alpha-catenin-dependent junctional-actin networks under stress during epithelial morphogenesis. *Curr Biol*. 2012; 22:1500–1505. [PubMed: 22771044]

- Croce A, Cassata G, Disanza A, Gagliani MC, Tacchetti C, Malabarba MG, Carlier MF, Scita G, Baumeister R, Di Fiore PP. A novel actin barbed-end-capping activity in EPS-8 regulates apical morphogenesis in intestinal cells of *Caenorhabditis elegans*. *Nat Cell Biol.* 2004; 6:1173–1179. [PubMed: 15558032]
- Drenckhahn D, Steffens R, Groschel-Stewart U. Immunocytochemical localization of myosin in the brush border region of the intestinal epithelium. *Cell Tissue Res.* 1980; 205:163–166. [PubMed: 6988079]
- Egginton S, Gerritsen M. Lumen formation: in vivo versus in vitro observations. *Microcirculation.* 2003; 10:45–61. [PubMed: 12610663]
- Farooqui S, Pellegrino MW, Rimann I, Morf MK, Muller L, Frohli E, Hajnal A. Coordinated lumen contraction and expansion during vulval tube morphogenesis in *Caenorhabditis elegans*. *Dev Cell.* 2012; 23:494–506. [PubMed: 22975323]
- Ferrari A, Veligodskiy A, Berge U, Lucas MS, Kroschewski R. ROCK-mediated contractility, tight junctions and channels contribute to the conversion of a preapical patch into apical surface during isochoric lumen initiation. *J Cell Sci.* 2008; 121:3649–3663. [PubMed: 18946028]
- Fischer RS, Fowler VM. Tropomodulins: life at the slow end. *Trends Cell Biol.* 2003; 13:593–601. [PubMed: 14573353]
- Fischer RS, Fritz-Six KL, Fowler VM. Pointed-end capping by tropomodulin3 negatively regulates endothelial cell motility. *J Cell Biol.* 2003a; 161:371–380. [PubMed: 12707310]
- Fischer RS, Quinlan RA, Fowler VM. Tropomodulin binds to filensin intermediate filaments. *FEBS Lett.* 2003b; 547:228–232. [PubMed: 12860419]
- Fritz-Six KL, Cox PR, Fischer RS, Xu B, Gregorio CC, Zoghbi HY, Fowler VM. Aberrant myofibril assembly in tropomodulin1 null mice leads to aborted heart development and embryonic lethality. *J Cell Biol.* 2003; 163:1033–1044. [PubMed: 14657235]
- Göbel V, Barrett PL, Hall DH, Fleming JT. Lumen morphogenesis in *C. elegans* requires the membrane-cytoskeleton linker erm-1. *Dev Cell.* 2004; 6:865–873. [PubMed: 15177034]
- Hall DH. Electron microscopy and three-dimensional image reconstruction. *Methods Cell Biol.* 1995; 48:395–436. [PubMed: 8531736]
- Heintzelman MB, Hasson T, Mooseker MS. Multiple unconventional myosin domains of the intestinal brush border cytoskeleton. *J Cell Sci.* 1994; 107 (Pt 12):3535–3543. [PubMed: 7706404]
- Herman IM, Pollard TD. Electron microscopic localization of cytoplasmic myosin with ferritin-labeled antibodies. *J Cell Biol.* 1981; 88:346–351. [PubMed: 7193682]
- Hirokawa N, Tilney LG, Fujiwara K, Heuser JE. Organization of actin, myosin, and intermediate filaments in the brush border of intestinal epithelial cells. *J Cell Biol.* 1982; 94:425–443. [PubMed: 7202010]
- Keller TC 3rd, Conzelman KA, Chasan R, Mooseker MS. Role of myosin in terminal web contraction in isolated intestinal epithelial brush borders. *J Cell Biol.* 1985; 100:1647–1655. [PubMed: 3988804]
- Kim SK, Lund J, Kiraly M, Duke K, Jiang M, Stuart JM, Eizinger A, Wylie BN, Davidson GS. A gene expression map for *Caenorhabditis elegans*. *Science.* 2001; 293:2087–2092. [PubMed: 11557892]
- Kouyama T, Mhashi K. Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *Eur J Biochem.* 1981; 114:33–38. [PubMed: 7011802]
- Lanzetti L. Actin in membrane trafficking. *Curr Opin Cell Biol.* 2007; 19:453–8. [PubMed: 17616384]
- Leung B, Hermann GJ, Priess JR. Organogenesis of the *Caenorhabditis elegans* intestine. *Dev Biol.* 1999; 216:114–134. [PubMed: 10588867]
- Lu S, Symersky J, Li S, Carson M, Chen L, Meehan E, Luo M. Structural genomics of *Caenorhabditis elegans*: crystal structure of the tropomodulin C-terminal domain. *Proteins.* 2004; 56:384–386. [PubMed: 15211521]
- Lubarsky B, Krasnow MA. Tube morphogenesis: making and shaping biological tubes. *Cell.* 2003; 112:19–28. [PubMed: 12526790]
- MacQueen AJ, Baggett JJ, Perumov N, Bauer RA, Januszewski T, Schrieffer L, Waddle JA. ACT-5 is an essential *Caenorhabditis elegans* actin required for intestinal microvilli formation. *Mol Biol Cell.* 2005; 16:3247–3259. [PubMed: 15872090]

- Maduro MF, Rothman JH. Making worm guts: the gene regulatory network of the *Caenorhabditis elegans* endoderm. *Dev Biol.* 2002; 246:68–85. [PubMed: 12027435]
- Mooseker MS. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu Rev Cell Biol.* 1985; 1:209–241. [PubMed: 3916317]
- Ono S. Purification and biochemical characterization of actin from *Caenorhabditis elegans*: its difference from rabbit muscle actin in the interaction with nematode ADF/cofilin. *Cell Motil Cytoskeleton.* 1999; 43:128–136. [PubMed: 10379837]
- Ono S, Ono K. Tropomyosin inhibits ADF/cofilin-dependent actin filament dynamics. *J Cell Biol.* 2002; 156:1065–1076. [PubMed: 11901171]
- Pardee JD, Spudich JA. Purification of muscle actin. *Methods Cell Biol.* 1982; 24:271–289. [PubMed: 7098993]
- Piekny AJ, Johnson JL, Cham GD, Mains PE. The *Caenorhabditis elegans* nonmuscle myosin genes nmy-1 and nmy-2 function as redundant components of the let-502/Rho-binding kinase and mel-11/myosin phosphatase pathway during embryonic morphogenesis. *Development.* 2003; 130:5695–5704. [PubMed: 14522875]
- Piekny AJ, Mains PE. Rho-binding kinase (LET-502) and myosin phosphatase (MEL-11) regulate cytokinesis in the early *Caenorhabditis elegans* embryo. *J Cell Sci.* 2002; 115:2271–2282. [PubMed: 12006612]
- Piekny AJ, Wissmann A, Mains PE. Embryonic morphogenesis in *Caenorhabditis elegans* integrates the activity of LET-502 Rho-binding kinase, MEL-11 myosin phosphatase, DAF-2 insulin receptor and FEM-2 PP2c phosphatase. *Genetics.* 2000; 156:1671–1689. [PubMed: 11102366]
- Rodewald R, Newman SB, Karnovsky MJ. Contraction of isolated brush borders from the intestinal epithelium. *J Cell Biol.* 1976; 70:541–554. [PubMed: 783170]
- Rodriguez-Fraticelli AE, Auzan M, Alonso MA, Bornens M, Martin-Belmonte F. Cell confinement controls centrosome positioning and lumen initiation during epithelial morphogenesis. *J Cell Biol.* 2012; 198:1011–1023. [PubMed: 22965908]
- Stiernagle, T. WormBook, editor. Maintenance of *C. elegans*. The *C. elegans* Research Community, WormBook. 2006. <http://www.wormbook.org>
- Stevenson TO, Mercer KB, Cox EA, Szewczyk NJ, Conley CA, Hardin JD, Benian GM. *unc-94* encodes a tropomodulin in *Caenorhabditis elegans*. *J Mol Biol.* 2007; 374:936–950. [PubMed: 17976644]
- Van Furden D, Johnson K, Segbert C, Bossinger O. The *C. elegans* ezrin-radixin-moesin protein ERM-1 is necessary for apical junction remodelling and tubulogenesis in the intestine. *Dev Biol.* 2004; 272:262–276. [PubMed: 15242805]
- Weber A, Pennise CR, Babcock GG, Fowler VM. Tropomodulin caps the pointed ends of actin filaments. *J Cell Biol.* 1994; 127:1627–1635. [PubMed: 7798317]
- Weber A, Pennise CR, Fowler VM. Tropomodulin increases the critical concentration of barbed end-capped actin filaments by converting ADP·P_i-actin to ADP-actin at all pointed filament ends. *J Biol Chem.* 1999; 274:34637–34645. [PubMed: 10574928]
- Weber KL, Fischer RS, Fowler VM. Tmod3 regulates polarized epithelial cell morphology. *J Cell Sci.* 2007; 120:3625–3632. [PubMed: 17928307]
- Wissmann A, Ingles J, McGhee JD, Mains PE. *Caenorhabditis elegans* LET-502 is related to Rho-binding kinases and human myotonic dystrophy kinase and interacts genetically with a homolog of the regulatory subunit of smooth muscle myosin phosphatase to affect cell shape. *Genes Dev.* 1997; 11:409–422. [PubMed: 9042856]
- Wissmann A, Ingles J, Mains PE. The *Caenorhabditis elegans* mel-11 myosin phosphatase regulatory subunit affects tissue contraction in the somatic gonad and the embryonic epidermis and genetically interacts with the Rac signaling pathway. *Dev Biol.* 1999; 209:111–127. [PubMed: 10208747]
- Yamashiro S, Cox EA, Baillie DL, Hardin JD, Ono S. Sarcomeric actin organization is synergistically promoted by tropomodulin, ADF/cofilin, AIP1 and profilin in *C. elegans*. *J Cell Sci.* 2008; 121:3867–3877. [PubMed: 18984629]

- Yamashiro S, Speicher KD, Speicher DW, Fowler VM. Mammalian tropomodulins nucleate actin polymerization via their actin monomer binding and filament pointed end-capping activities. *J Biol Chem*. 2010; 285:33265–33280. [PubMed: 20650902]
- Yamashiro S, Gokhin DS, Kimura S, Nowak RB, Fowler VM. Tropomodulins: pointed-end capping proteins that regulate actin filament architecture in diverse cell types. *Cytoskeleton (Hoboken)*. 2012; 69:337–370. [PubMed: 22488942]

Key Findings

- UNC-94 localizes to the intestinal terminal web in *C. elegans* embryos, where it modulates F-actin levels.
- The *C. elegans* intestinal terminal web, like that of mammals, contains non-muscle myosin.
- UNC-94 supports actomyosin contractility in the intestinal terminal web, which is needed for proper lumen morphogenesis.

Figure 1.

Tropomodulin structure and UNC-94 localization in the embryonic *C. elegans* intestine. **A:** Structure of Human Tmod1, *C. elegans* UNC-94 and TMD-2 and position of mutations that were studied. Tropomodulins have two actin capping regions: a tropomyosin-dependent actin binding domain at the N-terminus (TM-CAP) and an actin binding domain at the C-terminus that contains leucine rich repeats (LRR-CAP). The percentage similarity of these domains to those in human Tmod1 is indicated below the domain (Yamashiro et al., 2012). The *unc-94* gene encodes two isoforms that differ in their first exon and 5' UTR. The protein structure of UNC-94a is shown, and lines indicate which regions of the gene encode the protein domains. The *tmd-2* gene also encodes two isoforms, with *tmd-2b* encoding a C-terminal extension that is not present in *tmd-2a*. The protein structure of TMD-2b is shown, and lines indicate which regions of the gene encode the protein domains. The location of mutations that were studied is shown below the gene structures. **B:** Embryo co-stained for UNC-94 (via antibody staining) and F-actin (via phalloidin staining). Arrow points to co-localization at the intestinal terminal web. In all images, the anterior of the intestine is positioned to the left.

Figure 2.

UNC-94 is required for proper intestinal shape in *C. elegans* embryos and adults. **A:** Embryos stained with phalloidin (3 - 3.5 fold stage). Red arrows indicate regions of abnormally wide intestinal lumen diameter. These are representative images and do not show the most extreme phenotypes observed. Bar = 10 microns. **B:** Box plots showing distribution of maximum lumen diameters for 3 - 3.5 fold *C. elegans* embryos of various genotypes. Black asterisks: statistically different from wild-type ($p < 0.001$ in a two-sided Student's t-test). Blue asterisk: statistically different from wild-type; *erm-1::gfp* control ($p < 0.001$ in a Mann-Whitney U test). Red asterisk: statistically different from *tmd-2(ok3417);L4440(RNAi)* ($p < 0.001$ in a Mann-Whitney U test) but not statistically different from *unc-94(RNAi)*. L4440 is the empty feeding vector used as a negative control. **C:** 3D reconstructions of 3-fold stage wild-type and *unc-94(tm724)* embryos expressing *erm-1::gfp*. Bar = 10 microns. Movies available in Supplemental Material. **D:** 3D reconstructions of adult wild-type (lateral view) and *unc-94(tm724)* (transverse view) worms expressing *erm-1::gfp*. Insets show 3D models of the intestinal surface in the boxed regions and are magnified. The anteroposterior (AP), dorsal-ventral (DV), and transverse (T) axes are indicated. White arrows point to the excretory cell body and red arrows point to cystic excretory canals. Bar = 50 microns.

Figure 3.

UNC-94 regulates F-actin levels in the intestinal terminal web, but does not regulate levels of the intermediate filament protein, IFB-2. **A:** Images of phalloidin stained 3 - 3.5 fold embryos and graph showing quantification of fluorescence intensity. Images on the left show a wild-type embryo (average fluorescence intensity = 1155 on a scale of 0–4095) and a *unc-94(tm724)* embryo (average fluorescent intensity = 816). **B:** Images of 3 - 3.5 fold embryos immunostained for IFB-2 with the monoclonal MH33 antibody and graph showing quantification of fluorescence intensity. Images on the left are from 3D reconstructions, and the arrowhead points to small cytoplasmic accumulations of IFB-2. In A and B, bars indicate the standard error of the mean. Asterisk indicates a statistically significance difference in comparison to the wild-type control ($p < 0.001$ in a two-sided Student's t-test).

Figure 4.

Loss of UNC-94 function does not compromise cell junctions. **A:** Top images show 2-fold embryos stained for HMR-1/cadherin to mark adherens junctions (green), and IFB-2 (blue) to mark the terminal web. Bottom images are of 2-fold embryos stained for AJM-1, a basal component of apical junctions in *C. elegans*. In the *unc-94(tm724)* embryo shown, there is greater distance between some of the junctions within the same intestinal ring, indicating that the lumen is wider in these regions. Asterisks mark the first intestinal ring. Bar = 10 microns. Enlargements of the HMR-1 and AJM-1 staining in the boxed regions are provided to the right of the originals. **B:** TEM images of adult *C. elegans* worms. Top images show electron dense apical junctions (AJ), and bottom images show microvilli (M) and the terminal web (TW).

Figure 5.

Increasing actomyosin contraction partially rescues the *unc-94(tm724)* mutant phenotype.

A: Wild-type and *unc-94(tm724)* *C. elegans* embryos immunostained for NMY-1 and IFB-2 (to mark the terminal web). **B:** Quantification of fluorescence intensity in wild-type and *unc-94(tm724)* *C. elegans* embryos immunostained for NMY-1. Bars show standard error of the mean. **C:** *C. elegans* embryo expressing MEL-11::GFP. **D:** Graph showing maximum lumen diameters of wild-type and *unc-94(tm724)* mutants treated with *mel-11*(RNAi) or with the empty feeding vector (*L4440*). Widths were measured at the widest part of the lumen; and the percentage of 3 - 3.5 fold embryos with the designated lumen widths is shown. **E:** 3D reconstruction of *let-502(ca201)* heterozygous embryo stained for F-actin. Red arrow points to an area where the intestinal lumen is expanded, but not flattened. Movie available in Supplemental Material. Bars = 10 microns.

Figure 6.

UNC-94 does not nucleate F-actin *in vitro*. Pyrene labeled G-actin at 4 μ M was pre-incubated with buffer only (black), or buffer with 0.4 μ M UNC-94 (red) or 0.8 μ M UNC-94 (green), and was polymerized at time zero by adding polymerization buffer. Kinetics of actin polymerization was monitored by pyrene fluorescence, which is increased upon polymerization.

Table 1

Intestinal length, surface area and volume in wild-type and *unc-94(tm724)* homozygotes expressing *erm-1::gfp*.

Genotype	Intestinal Length	Intestinal Surface Area	Intestinal Volume
wild-type	82.7 +/- 8.2 μm (n=22)	384.3 +/- 88.5 μm^2 (n=20)	107.6 +/- 52.6 μm^3 (n=20)
<i>unc-94(tm724)</i>	76.0 +/- 14.7 μm * (n=25)	389.0 +/- 110.7 μm^2 (n=20)	104.4 +/- 47.5 μm^3 (n=19)

* Statistically different from wild-type, $p < 0.01$, in a Mann Whitney U test.