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Characterizing the Role of the Actin-binding Protein, TMD-1/tropomodulin in C. elegans Excretory Cell Morphogenesis

Samantha Smith

Abstract

Tropomodulins are proteins widely expressed in all complex animals that help to regulate the shape of cells by modifying the cytoskeletal filament actin. *C. elegans* worms lacking the tropomodulin TMD-1 show defects in development of the excretory cell, which acts as a kidney for the worm. The excretory cell extends four canals out from the cell body so that the entire cell looks like a great letter H stretching the length of the worm. In *tmd-1* mutants, canals extend partially or not at all, and develop a dramatic crinkled appearance. The mechanism for this interference is unknown; however several possibilities are being explored. Excretory canals are often affected by mutations in genes, also needed in neuronal axon guidance. Knocking down one of these guidance proteins, MIG-10, produced a similar phenotype to that of the *tmd-1* mutants. However, further experiments did not uncover any neuronal guidance defects in *tmd-1* mutants. Instead, it is more likely that TMD-1 is essential for vesicle trafficking within the excretory canal. In this function TMD-1 may facilitate canal extension by providing membrane components to the growing canal tips. Insight into tropomodulin's role in single-celled tube formation can further our understanding of how these tubes form in many organisms.

Introduction

Tropomodulins are a family of proteins that regulate the minus end of actin filaments. In this capacity they are essential for many of the complex cellular processes in which actin is involved, including cell migration, adhesion, vesicle transport, and muscle contraction. The nematode *Caenorhabditis elegans* has two tropomodulin isoforms: TMD-1 and TMD-2. Their roles in the worm are not fully understood, but these roles are likely to be the same in vertebrates due to the large degree of homology between worm tropomodulins and vertebrate tropomodulins.

C. elegans are convenient model systems for developmental biology studies. First used for research by Sydney Brenner, they were chosen for their simple nervous system, short life cycle, and the ease with which they could be genetically modified (Brenner, 1974). Brenner induced mutations in the worms using ethanemethylsulfonate, and identified many of the mutant phenotype classes still used today, including uncoordinated (unc), dumpy (dpy), and small (sma). They are particularly useful for microscopy because they are transparent and their embryos can be preserved on slides. Their genome has been fully sequenced and the lineage of all of their cells has been determined. This information can be used to manipulate the worms genetically, as they are also capable of RNA interference. The *C. elegans* community is also an overwhelming benefit to this model system. Volumes of information have been compiled in online worm databases (including WormBook, WormAtlas, WormBase, and others). Additionally, thousands of mutant worm strains are available from the Caenorhabditis Genetics Center.

The work presented here demonstrates that the tropomodulin TMD-1 is important for formation of the *C. elegans* excretory cell. This is a giant, H-shaped cell that regulates the osmotic balance of the worm and excretes waste (WormAtlas, 2012). It does this by collecting waste or liquid into its lumen and then transporting it to the pore, where it empties into the

worm's surroundings. The excretory canal, as a single-celled tube with long processes, is an ideal simple model for both tubulogenesis and cell migration.

The excretory cell is first specified at the end of gastrulation (Buechner, 2002). Fluid-filled vesicles coalesce in the center of the cell and join to form the beginnings of the lumen (Fig. 1). The membranes of these vesicles become the apical membrane, which surrounds the lumen in the mature excretory cell. The outer membrane facing the outside of the cell is then specified as the basal membrane. These membranes become polarized by the different proteins that localize to them over the course of development. The mechanism by which the lumen forms in the excretory cell is a canonical example of the cell hollowing mechanism (Fig. 2). This is a conserved mechanism for the formation of single celled tubes in many organisms.

The excretory cell, located at the ventral midline of the worm, extends processes laterally until they span the width of the worm, and then the canals turn dorsally ((Buechner, 2002) Fig. 1). The canals do not grow entirely to the dorsal side of the worm, but stop about midway and extend processes anteriorly and posteriorly. The canals grow between the overlying hypodermis, or skin of the worm, and the hypodermal basement membrane. Adhesions form on these layers that anchor the canals as they grow (Buechner, 2002).

The width of the lumen and its distance from the basal membrane is tightly regulated. Twelve genes that affect the structure of the lumen have been identified, and are classified together as *exc* genes (Fujita et al., 2003). Several of these gene products are believed to interact with one another and to regulate different parts of the same processes. The *exc* mutant phenotypes reveal some of the processes necessary for shaping and maintaining this cell. *exc-5,* for example, codes for a guanine nucleotide exchange factor (Suzuki et al., 2001). A null mutation in *exc-5* causes large fluid-filled cysts to form in the lumen(Mattingly and Buechner, 2011). Conversely, when EXC-5 is overexpressed in nematodes, the apical and basal membrane of the canals polarize normally, but the basal membrane is defective (Tong and Buechner, 2008).

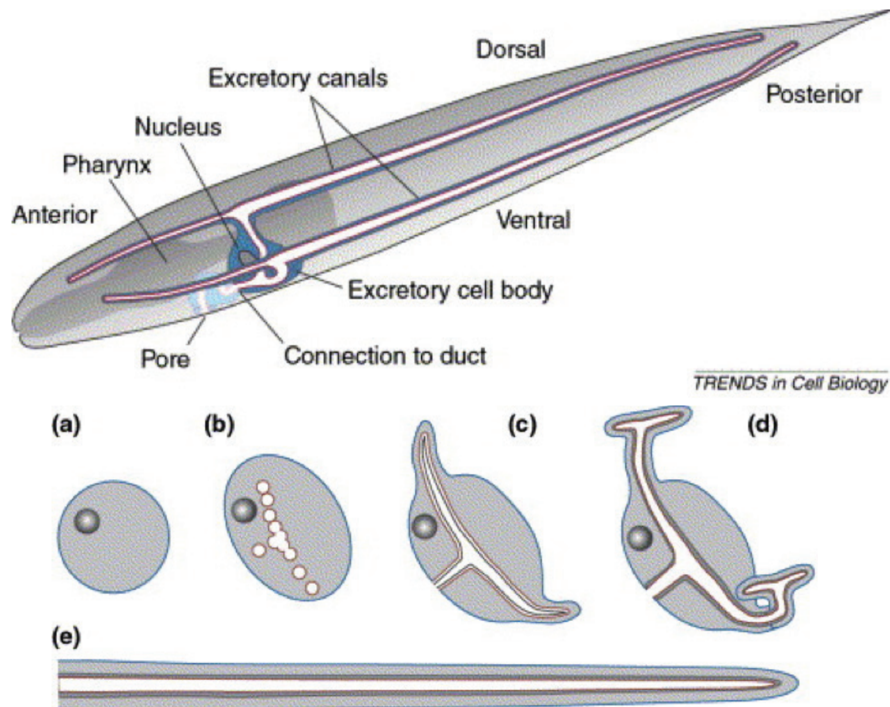

Figure 1: The excretory cell is shaped like a letter H, with 4 canals extending from a ventrally located cell body. The adult excretory cell (top) has polarized membranes, with the apical membrane surrounding the lumen and the basal membrane `facing the outside. The pharynx is shown as a gray shadow. When the excretory cell is first specified (a) it is shaped like a simple circle. Fluid filled vesicles coalesce to form the lumen (b), and the apical and basal membranes together grow outward towards the lateral

sides of the worm (c). The canals extend anteroposteriorly once they have migrated about halfway towards the dorsal side of the animal. (From Buechner, M. Trends Cell Biol. 2002(12), 479-484.)

This causes the apical surface to crumple up inside the basal membrane as it does not have room to extend fully, which is characterized as the convoluted tubule phenotype. EXC-5 is utilized in the regulation of Rho GTPases which regulate organization of the cytoskeleton (Suzuki et al., 2001). The cytoskeleton is crucial in in the formation of the excretory cell and maintenance of its specialized structure. The cytoskeleton provides the means of transport for localizing specific proteins to the apical and basal membranes, which is how polarity is established. Actin has an additional cytoskeletal function in the reinforcement of the shape of the mature lumen. Actin and other associated proteins localize around the lumen to form a terminal web (WormAtlas). During development this must accommodate for the growth of the lumen lengthwise while preventing lateral swelling or collapsing. In *exc-5* mutants, polarity is established, but the cystic defects observed suggest that the terminal web is flawed.

Tropomodulins are a family of proteins that also regulate the cytoskeleton. They regulate the activity of actin by capping its slow-growing end, nucleating new filaments, and sequestering actin monomers (Fischer and Fowler, 2003; Fowler et al., 2003; Weber et al., 1994; Yamashiro et al., 2012). Actin filaments are composed of two strands of globular actin monomers that associate together to form a helical filament (Fig. 3). In some tissues, tropomyosin will co-polymerize with actin. These filaments are dynamic in their structure, depolymerizing and polymerizing rapidly and constantly. The two ends of actin filaments are polarized. At one end, actin monomers are associated with ATP, and at the opposite end monomers are associated with ADP instead. The ATP end is referred to as the barbed, or plus end, and the ADP end is the pointed, or minus end. Functionally, this polarity differentiates the two ends in their tendency to attract more monomers or to lose monomers. The barbed end is more likely to polymerize and the pointed end is more likely to depolymerize (Small et al., 1978).

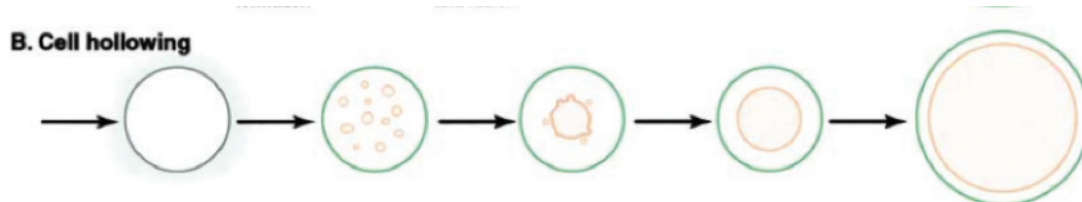

Figure 2: Cell hollowing involves the fusion of several fluid filled vesicles in a tubular cell to form a lumen within the cell. This is shown here in cross-section. The membrane surrounding the lumen is called the apical membrane, and the original cell membrane becomes the basal membrane. This process is conserved in many organisms. (Lubarsky and Krasnow, 2003)

Actin filaments are dynamic on their own, but their extensive regulation by dozens of proteins allows them to participate in such diverse cell processes as structural support, adhesion, vesicle transport, cell migration, and muscle contraction. Of the many of actin regulators that exist in eukaryotes, tropomodulins (Tmods) are the only well characterized family that caps the minus (or pointed) end of actin filaments (Yamashiro et al., 2012). Tropomodulins are found in almost all cell types in all metazoans (Yamashiro et al., 2012). Vertebrates have four isoforms of tropomodulin, called Tmods 1-4, and *C. elegans* have two: TMD-1 and TMD-2. The vertebrate tropomodulins, especially Tmod1, have been studied extensively in several cell types, but the *C. elegans* tropomodulins have only been studied in the body wall muscle (Stevenson et al., 2007; Yamashiro et al., 2008). However, due to homology in a number of conserved regions, findings from vertebrate tropomodulin studies can be tentatively assumed to apply to the *C. elegans* isoforms.

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Tropomodulins have two capping domains: the extended, unstructured TM cap domain and the densely packed LRR cap domain (Fischer and Fowler, 2003; Fowler et al., 2003; Yamashiro et al., 2012). The amino and carboxy halves of tropomodulin can each cap actin independently in truncated forms of tropomodulin, but the strongest capping activity occurs when both capping domains are active (Fig. 4, Weber et al., 1994; Yamashiro et al., 2012). Within the amino-terminal TM cap domain there is an actin binding site and two tropomyosin binding sites. Binding of tropomyosin at these sites causes an increase in the -helicity of the actin-binding site, which makes it far more active (Yamashiro et al., 2012). The carboxy-terminal LRR domain contains a TM-independent actin-binding site that alone can cap actin with a submicromolar affinity (Fowler et al., 2003).

Tropomodulin's binding to free actin filaments (without tropomyosin bound) is almost entirely accomplished by the actin-binding site in the LRR cap region with virtually no contribution by the TM-dependent actin-binding site (Fowler et al., 2003). Tropomodulin capping of TM-actin is much stronger than capping of free actin because it engages both the LRR and TM capping domains (Fowler et al., 2003; Weber et al., 1994). Some of this inhibition is caused simply by the association of tropomyosin, which alone slows elongation and depolymerization (Weber et al., 1994). Although tropomyosin may be working in concert with tropomodulin to slow polymerization, tropomodulin can cause complete arrest of depolymerization at concentrations of tropomyosin too low to cause an effect on their own (Weber et al., 1994).

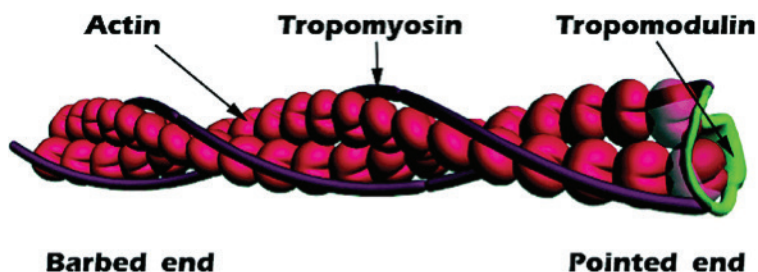

Figure 3: Tropomodulin caps the minus end of F-actin. Actin filaments are made up of two strands of globular actin monomers. There are two actin-capping domains on tropomodulin: one at the N-terminus (TM cap) and one at the C-terminus (LRR cap). Tropomyosin frequently co-polymerizes along actin filaments and stabilizes it. Tropomodulin's TM-binding site on its N-terminus associates with tropomyosin and allows stronger capping activity at the N-terminal actin-binding site in tropomodulin. The extended N-terminal half of the protein loops up and forward from the C-terminal half, which is more compactly folded and globular. (Kostyukova et al., 2006)

It is not known whether or not tropomyosin is associated with actin filaments in the excretory cell. However, actin is coated with tropomyosin in the intestine and pharynx of the worm (Anyanfu et al., 2001). As the intestine and excretory cell are both tubes in the worm and undergo similar developmental hollowing processes, this may indicate that tropomyosin is also present in the excretory cell. Even if this is the case, however, there may not always be tropomyosin associated with the entire length of the actin filament. If tropomyosin does not extend all the way to the pointed end, it will not be able to bind tropomodulin and tropomodulin's capping activity will resemble capping of a free actin (Fischer and Fowler, 2003). In addition to variable capping activities, tropomodulin has also been shown to be capable of sequestering actin monomers and nucleating new actin filaments (Fischer and Fowler, 2003; Fowler et al., 2003; Weber et al., 1994). These activities may also contribute to tropomodulin's effects *in vivo*.

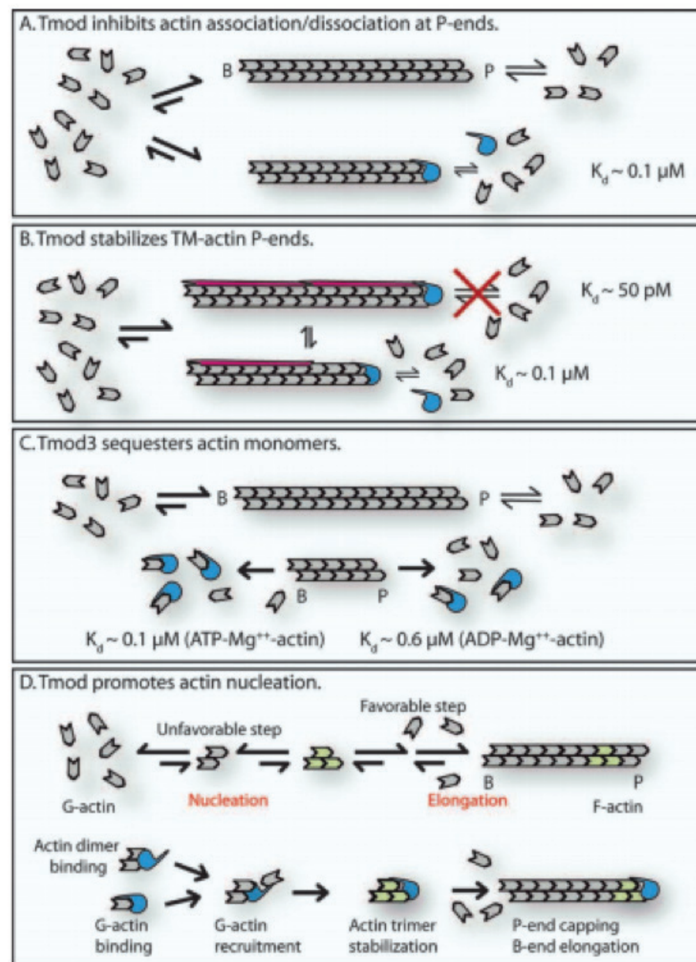

Figure 4: Tropomodulin caps actin pointed ends, binds to tropomyosin, can bind G-actin, and can nucleate actin filaments. A) Tropomodulin forms a leaky cap at the pointed end of free actin with a submicromolar affinity. (Weber et al., 1994). B) The presence of tropomyosin at the pointed end of actin filaments results in a strong capping affinity in the nanomolar range for tropomodulin. This can vacillate back to free-actin binding kinetics if tropomodulin dissociates from the pointed end and the filament polymerizes beyond tropomyosins. C) Vertebrate Tmods 2 and 3 have also been shown to sequester actin monomers, thus decreasing the concentration of G-actin available for polymerization (Yamashiro et al., 2012). D) Tmods 1-3 can nucleate new actin filaments. C. elegans isoforms lack this activity (Shoichiro Ono, personal communication). (Yamashiro et al., 2012)

Tropomodulins have diverse effects on actin in different tissues. In polarized epithelial cells, Tmod3 is found to localize to the lateral sides of cells and play a role in the structural support that gives epithelial cells their tall cuboidal shape (Weber et al., 2007). Without Tmod3, they become shorter and squatter, and significantly less F-actin and tropomyosin is found on the lateral sides of the cell. Tmod3 is believed to have a role in organizing the actin-spectrin network that structures these cells. In migratory endothelial cells, Tmod3 pointed end capping decreases the migration speed of cells and tends to decrease the polarization of the leading edge so that the cells adopt a more stationary morphology (Fischer et al., 2003). Further, Tmod3 tends to re-organize the actin cytoskeleton so that fewer barbed ends are positioned at the leading edge of lamellopodia. These two results are somewhat contradictory, in that Tmod3 seems to be upregulating the F-actin network in polarized epithelial cells and downregulating F-actin formation at the leading edge of migrating endothelial cells. These opposite effects suggest that Tmod3 may be executing different functions in each cell type. Indeed, it has been proposed that Tmod3 caps actin in polarized epithelial cells, stabilizing their structure, and sequesters actin monomers in migrating cells, which encourages depolymerization

(Yamashiro et al., 2012). The excretory cell is both a polarized structure and an extending one; thus it may be possible that tropomodulins have multiple roles in the excretory cell.

Because of the similarities between neuron development and excretory cell development, the role of tropomodulins in neurons is worth exploring. Like developing neurons, the excretory canals must synthesize and transport new membrane, form new adhesions, and the canal termini must migrate to the far reaches of the worm body. Further, the excretory canals use guidance cues much the same way as axon growth cones do during development (Buechner, 2002; McShea et al., 2013). Tmod1 and Tmod2 are expressed in neurons, but Tmod1 is found associated with actin, whereas Tmod2 is spread throughout the cytoplasm (Fath et al., 2011). This differential localization could be due to the different affinities for tropomyosin between the two isoforms. Tmod2 was found to act as a damper on the time of neurite extension and the length of these extensions. The knockdown of Tmod2 causes a compensatory rise in Tmod1 levels in neurons; however, it is the lack of Tmod2, and not Tmod1's increased expression, that makes neurite outgrowth more active (Fath et al., 2011). Because Tmod2 can bind to actin monomers, it may be that Tmod2 decreases the rate of actin polymerization, and thus the outgrowth ability of neurites, by sequestering actin monomers. Although these findings have not been studied in *C. elegans*, it is likely that tropomodulin would regulate actin in a similar way, tending to decrease the rate of outgrowth in neurons and, perhaps, the excretory canals.

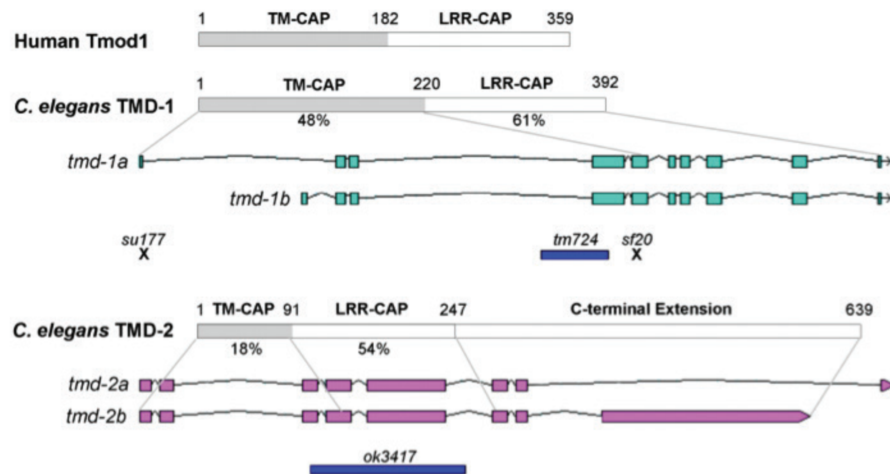

Figure 5: Structure of Human Tmod1, C. elegans TMD-1 and TMD-2 and position of mutations that were studied. Tropomodulins have two actin capping regions: a tropomyosin-dependent actin binding domain at the N-terminus (TM-CAP) and an actin binding domain at the C-terminus that contains leucine rich repeats (LRR-CAP). The percentage similarity of these domains in TMD-1 and TMD-2 to human Tmod1 is indicated below the domain (Yamashiro et al., 2012). The tmd-1 gene encodes two isoforms that differ in their first exon and 5' UTR. The protein structure of TMD-1a 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 indicated below the gene structures. Image taken from Abbi Paulson.

Here, we explored the mechanism for excretory canal malformation in *tmd-1 (tm724)* mutants (Fig. 5). This genetic deletion in the *tmd-1* gene yields failed apical surface extension, cysts, and dysregulated lumen width. A weak knockdown of *tmd-1* by RNAi results in generally successful lumen formation and canal extension. However, the presence of fluid-filled swellings both continuous and non-continuous with the lumen, suggests defects in vesicle transport. Notably, in *tmd-2* knockouts, small cysts form in their excretory cell lumens as well. A role for tropomodulin in vesicular transport has also been explored in the intestine, but not proven.

This role for tropomodulins has not yet been characterized. Tropomodulins have been shown previously to play a role in cell migration and the structure of specialized cells, both of which are relevant to the excretory cell. However, tropomodulin may be affecting the structure and extension of the excretory cell by the mechanism of vesicle transport rather than direct regulation of F-actin structures. Thus, the findings reported here could have far reaching implications for tubulogenesis studies or cell migration. If tropomodulin is indeed playing a role in vesicle transport that enables apical and/or basal surface extension, this role may be conserved in other tissue types or in other organisms.

Materials and Methods

C. elegans culturing and strains used

C. elegans were grown on plates containing nematode growth media (NGM) and fed OP50 bacteria according to standard practice described in Stiernagle 2006.

Strain	Description
N2	Wild type
VJ402	*erm-1::gfp* (apical surface marker)
TM724	*tmd-1* genetic deletion
GSC5	*tmd-1* genetic deletion, *erm-1::gfp*
GSC9	*tmd-2* genetic deletion, *erm-1::gfp*
BK36	*vha-1::gfp* (cytoplasmic marker)

Feeding RNAi

In *C. elegans* it is possible to target the expression of specific genes by simply feeding the worms containing double stranded RNA (dsRNA) corresponding to the target gene (Timmons and Fire, 1998). This activates the natural RNA degradation processes in the worm, and over the course of one or multiple generations the expression of the gene is reduced. A feeding RNAi library originally designed by the Vidal lab (Rual et al., 2004) was purchased from Open Biosystems (now ThermoScientific) and was used for all feeding RNAi experiments. The bacteria in this library were all HT115 (DE3), a strain of *E. coli*.

Feeding RNAi is a multi-day process. On the first day feeding RNAi media was made, autoclaved, and poured into 65mm petri dishes (see recipe below). Worms were bleached using bleach solution (see recipe below) to obtain embryos of approximately the same age as described in Stiernagle 2006. Bacterial cultures containing dsRNA corresponding to the target gene were inoculated. Feeding RNAi bacteria containing either an empty feeding vector (L4440) or a feeding vector for *cap-1* were used as negative and positive controls, respectively.

On day two, bacterial cultures were spread on the feeding RNAi plates and allowed to grow overnight. On day three, worms were added to the feeding plates and allowed to grow for either two days (single generational knockdown) or four days (for multi-generational knockdown). On the final day, worms and embryos were collected from the plates. Worms were bleached to release their eggs using bleach solution. Eggs were added to poly-L-lysine coated slides and fixed using a fix solution (see recipe below). After 20 minutes of fixing, eggs were washed with phosphate buffered saline and SlowFade Gold mounting media (Invitrogen) and

coverslips were added. Slides were stored at 4°C for imaging.

Feeding RNAi Media (200 mL)

0.24g NaCl
0.8g Bacto Peptone
0.6g KH_2PO_4
0.1g K_2HPO_4
320 mL cholesterol
4g agar
Bring to 200 mL with ddH_2O

After autoclaving and media has cooled to 55°C, add:

200 μL 1M $CaCl_2$
200 μL 25 mg/mL ampicillin (filter sterilized)
2 mL lactose

Bleach solution (8 mL)

7.2 mL ddH_2O
0.4 mL NaOCl
0.4 mL 5M KOH

Fix solution (500 mL)

206 μL H_2O
10 μL 4mg/mL lysolecithin
10 μL 0.5M EGTA, pH 8
48 μL 1M PIPES, pH 6.8
25 μL 1M HEPES, pH 6.8
1 μL 1M $MgCl_2$
200 μL 10% paraformadehyde

Immobilizing worms for live imaging

Worms were anesthetized with 20mM levamisol to immobilize them for imaging without the necessity of fixation. Worms were mounted either on poly-L-lysine coated slides or gelatin-coated slides sealed with velap.

Immunostaining

Immunostaining to visualize neurons in *tmd-1* knockdown and wild type worms was performed on mixed stage worms. Antibody 6-11 B-1 (Sigma) was used to stain acetylated tubulin with GFP. 6-11 B-1 stains six touch neurons: the PVM (posterior ventral microtubule), ALML (anterior lateral microtubule, left), ALMR, AVM (anterior ventral microtubule), PLML (posterior lateral microtubule) and PLMR (Siddiqui, 1990). Protocol was modified from Siddiqui 1990.

Briefly, worms were washed off plates in M9 and fixed in 4% paraformaldehyde and 1X PBS for 30-40 minutes at 0°C. Worms were washed in M9, then added to gelatin coated slides. A second slide was pressed hard on top of the worms and left on dry ice for 30 minutes. The slides were then pried apart and immersed in 1:1 acetone: methanol for 4 minutes on dry ice. The worms were re-hydrated in an ethanol series (90%, 70%, 50%, 30%) for 20 minutes each at 0°C. Slides were then immersed in 0.1% Triton X-100 for 4-6 hours at 0°C. Excess Triton X-100 was wiped off the slides and 10 mL of primary antibody was added. The primary antibody incubated on the slides at 4°C in a humidified container for 8-10 hours. Slides were washed with phosphate buffered saline and then incubated with 10 of μL secondary antibody (Jackson Immunoresearch) at room temperature in humidified containers. Slides were washed and mounting media and coverslips were added and sealed with nail polish.

Imaging

All microscopy was performed with a Nikon EZ-C1 Confocal Microscope or a Zeiss Imager M1. Images were taken with Nikon EZ C1 software or AxioVision Rel. 4.6. Image processing was done with ImageJ 1.47a and Imaris versions 7.2.3 and 7.4.2. The scale bar in Figure 4C was calculated using Adobe Photoshop Elements.

Results

Previous work in the Paulson lab has shown that the *C. elegans* tropomodulin TMD-1 is essential for proper development of the excretory cell, although its precise function has not been elucidated. Worms

with a *tmd-1(tm-724)* mutation, which is a putative null allele (Yamashiro et al., 2008), do not produce TMD-1. Using a green fluorescent protein tag on the apical surface marker ERM-1, we have observed that the apical surface of the excretory cell in *tmd-1(tm724)* homozygotes is crinkled and displays failed extension of some of the canals (Fig. 1B). The lumens which do extend are also wider than in wild type (Fig. 1C) and show cysts (Fig. 1D). These defects begin within 1 hour of development and persist into adulthood (Fig. 2). The goal of the experiments presented here was to elucidate the mechanism by which tropomodulin facilitates extension of the apical and basal surfaces of the excretory canal.

Several hypotheses guided our experimentation. Two of these featured tropomodulin acting cell-non-autonomously, as tropomodulin has thus far not been found in the excretory cell by antibody staining. This result is not conclusive proof that tropomodulin is not present in the excretory cell, however, as the methanol treatment used during antibody staining destroys the cytoskeleton. It could be that tropomodulin is localized to the skeleton and is also removed by the methanol. Nonetheless, because we had no proof that tropomodulin was expressed in the excretory cell, we considered ways that tropomodulin could affect the cell from other tissues. In one hypothesis, tropomodulin would act as an aid to some sort of adhesion complex. The excretory canals form adhesions with the basement membrane upon which it extends; these are necessary for traction and forward motion as the canals extend away from the cell body. Tropomodulin might be involved in the regulated secretion of some basement membrane components by the hypodermis, without which adhesions would be compromised. In this case, I might expect the basal surface to appear as a bubble around the mass of apical membrane observed near the cell body. The apical surface would grow, but would be prevented from extending as usual by the fact that the basal membrane was not growing away from the cell body.

Another theory included tropomodulin as critical regulator in the release of a guidance cue. The excretory cell has been shown to rely on guidance cues to extend; the same cues, in fact, that axon growth cones use (Buechner, 2002). Additionally, tropomodulin has been shown to potentially prevent vesicle release in the *C. elegans* intestine (Hoffman, 2012). If this is also occurring in the tissues surrounding the excretory cell, it could be that the worm is missing a chemical gradient that guides the canals as they extend. In this case, the basal surface of the canals would grow and the canal width would not be disrupted, but the canals would not extend properly in a straight line because they would not have the guidance provided by the chemical gradient.

Our third hypothesis accounted for the fact that TMD-1 could be present in the excretory cell even if it did not appear by antibody staining. If this is the case, TMD-1 could still participate in adhesions and in guidance, but simply from within the excretory cell. Instead of affecting secretion of a basement membrane protein to which the excretory cell adheres, TMD-1 could regulate actin participating in the adhesion complex on the inside of the cell. Similarly, TMD-1 could participate in the interpretation of a guidance cue inside the excretory cell, rather than the secretion of that cue from the hypodermis.

We also hypothesized an entirely new role for TMD-1 as a regulator of vesicle transport. Actin is needed both for vesicle transport and vesicle fusion. The osmotic regulatory function of the excretory cell necessitates a large amount of vesicle transport, as does the outgrowth of the canals and lengthening of both apical and basal membrane. If actin is not available to facilitate vesicle transport, the outgrowth of the canals could be compromised.

These hypotheses were explored using various experimental approaches. I compared the tropomodulin mutant apical surface phenotype to that of different gene mutants using RNAi. The genes tested have known roles in excretory canal development. The hope was that a phenocopy of the *tmd-1(tm724)* mutant would also be functioning in the same process or pathway as TMD-1, and this would give us a clue as to its mechanism. I also observed the development of neurons in *tmd-1(tm724)* mutants to determine whether tropomodulin also had a role there. If indeed TMD-1 was regulating the secretion of a guidance cue, it likely would affect

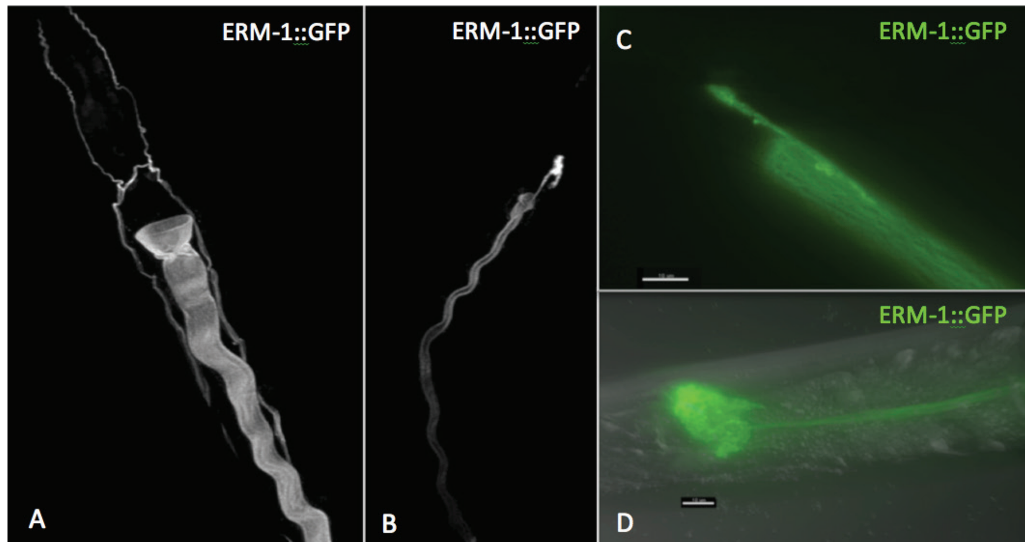

Figure 1: The tm-724 genetic deletion of tmd-1 causes failed extension of the apical surface of the excretory canals and lumenal defects. A) Wild-type worm expressing a GFP fused to the apical surface marker ERM-1. This marks the excretory canal lumen and the intestinal lumen. The lumen of the canals is uniformly narrow and in the bridge it is slightly thicker. Kinks in the canals are caused by the rol-6 marker used to distinguish worms with the fusion protein (Tong and Buechner, 2008). B) Genetic deletion of tmd-1 results in failed apical surface extension. Some canals extend partially or fully, or no canals extend. C) tmd-1(tm724) mutants show cysts in the excretory cell lumen. D) The apical surface has a crenelated appearance when it fails to extend. Width of the lumen is also increased abnormally in tmd-1 mutants. Images A and B are projections of confocal z-stacks generated by Matthew McIntosh, taken of L1 larvae. Images C and D are of worms between the L1 and adult stages. Bars=10 mm.

the neurons as well as the excretory cell. I visualized weak tropomodulin knockdown using a fluorescent marker for the entire excretory cell in order to inspect the development of the basal surface as well as the apical surface. The weak tropomodulin knockdown also provided insight into a phenotype partway between wild type and the dramatic failed extension of the *tmd-1(tm724)* mutants. This showed us the defects that still persisted even when the worms had a partial dose of TMD-1. Finally, I performed initial characterization of the *tmd-2* mutant excretory cell phenotype to gain a more fully rounded idea of the role of tropomodulins in the excretory cell.

The knockdown of mig-10 induces a crinkling and failed extension of the excretory cell similar to tmd-1(tm724) mutants

Several genes were identified from the literature to have a role in excretory cell development. Of these, *unc-52* and *mig-10* were tested to see if their mutation produced a comparable phenotype to that of *tmd-1(tm724)*. These genes were chosen to allow us to address our first two hypotheses. If TMD-1 is necessary for the secretion of basement membrane proteins, the *unc-52* mutant phenotype should be similar. UNC-52 is analogous to perlecan, an extracellular matrix proteoglycan (Rogalski et al., 2001). In *C. elegans* this is secreted by the hypodermis as a component of the basement membrane whose absence causes defects in canal and neuronal axon outgrowth (Buechner, 2002). Perlecan links to the actin cytoskeleton through associations with integrin adhesion complexes (Rogalski et al., 2001). If TMD-1 is instead regulating a guidance cue, then the *mig-10* mutant phenotype may resemble *tmd-1(tm724)* mutants. The *mig-10* gene encodes two proteins of unknown function, but which resemble Grb7 and Grb10, proteins used in vertebrates for signal transduction (Manser et al., 1997). Defects in *mig-10* cause aberrant migration of the CANs (canal associated neurons), as well as the hermaphrodite specific neurons (HSNs) and anterior lateral microtubule (ALM) neurons (Manser and Wood, 1990). MIG-10 has additionally been shown to be necessary for vesicle transport neurons near

synapses, and this role in vesicle transport may be related to its role in facilitating excretory canal and neuron migration (McShea et al., 2013). Vesicle transport provides necessary membrane components and proteins to the leading edge of the migrating cell.

unc-52 and *mig-10* were knocked down by feeding RNAi in worms expressing the apical membrane marker ERM-1::GFP. *unc-52(RNAi)* did not produce an abnormal apical surface phenotype. *unc*-52 may be one of the genes that cannot be knocked down by feeding RNAi.

mig-10(RNAi), however, produced a phenotype not identical, but similar to that of tropomodulin mutants. The apical surface of all four canals extended only partially and terminated in crinkled clumps (Fig. 2). The lumen width was consistent and without cysts in worms exhibiting this phenotype.

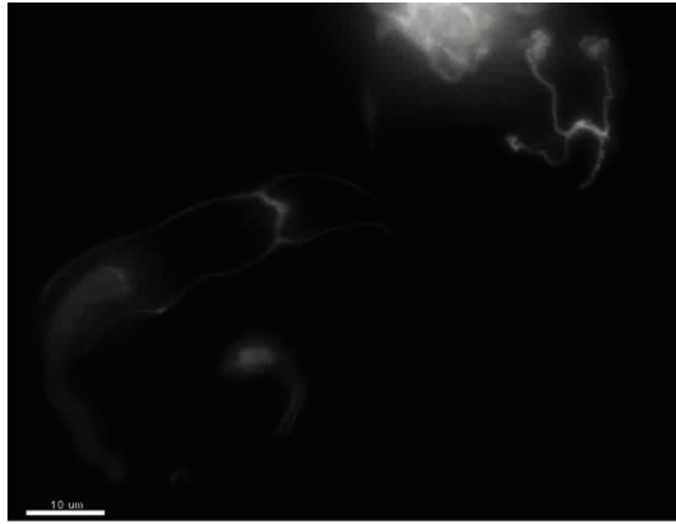

Figure 2: mig-10 RNAi causes the early termination of canals and the same crinkled apical surface phenotype seen in tmd-1 mutants. The phenotype was not fully penetrant; only one embryo in the picture is affected (right). Embryos that did show a loss-of-function phenotype showed partial canal extension in one or more canals and apical surface convolutions at the canal ends. These embryos were between the comma and three-fold stage. Bar = 10 mm.

A lack of neuronal defects in tmd-1(tm724) mutants decreases the likelihood that TMD-1 regulates the secretion or intake of a guidance cue.

The similarity between the *mig-10* and the *tmd-1* mutant phenotypes encouraged us to further pursue the guidance cue hypothesis. If TMD-1 was affecting the release of a guidance cue, it would be likely that these guidance cues would also be used in axon guidance and that in *tmd-1(tm724)* mutants neuronal defects would be present. The possibility of neuronal defects was supported by the observation of compromised worm coordination in tropomodulin mutants, although it was also possible that this was due to known defects in the body wall muscle (Stevenson et al., 2007; Yamashiro et al., 2008).

Antibody 6-11 B-1 was used to stain some of the neurons in the worm, including the ALM neurons, which are known to be affected by *mig-10*. Antibody staining was performed on the *tmd1 (tm724)* mutants. This experiment did not show any neuronal defects, suggesting that tropomodulin may not be regulating the release or intake of a guidance cue, despite its similarity with *mig-10* in its excretory cell mutant phenotype.

Weak knockdown of tmd-1 leads to successful extension of the excretory canals, but fluid-filled sacs are present in the cytoplasm.

After the neuron experiment I turned my attention to other hypotheses. The full visualization of the excretory canal would allow us to see the morphology of the entire excretory cell and not the apical membrane alone. This might provide clues as to the process that goes awry upon *tmd1 (tm724)* mutation. To this end, I performed *tmd-1 (RNAi)* on worms with a VHA-1::GFP cytoplasmic marker so as to visualize the entire excretory cell, not just the lumen. In wild type worms, the excretory canals showed small periodic swellings in the cytoplasm (Fig. 5A). The lumen width was narrow and uniform. However, a small number of the worms showed fluid filled sacs in the cytoplasm that were distinct from the nucleus (Fig. 5B). These may have been vesicles just endocytosed, waiting to be exocytosed, or parts of the lumen that had failed to fuse.

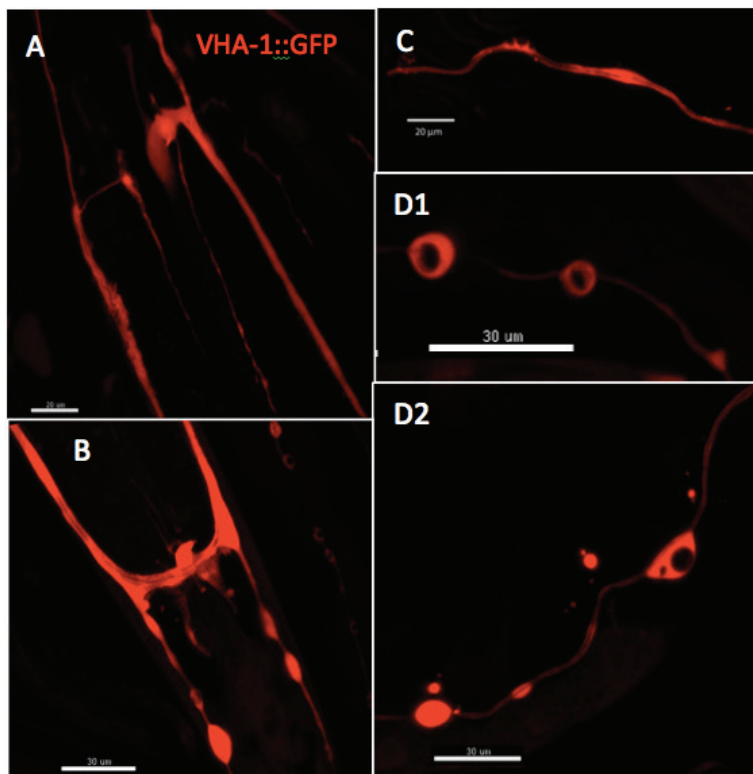

Figure 3: Weak tmd-1 knockdown by RNAi causes cysts to form in the excretory canals. A) The wild-type excretory canal is thicker at the bridge of the canal than elsewhere but the canal width changes gradually. Thickness at the cell body is to be expected to accommodate the nucleus and a higher concentration of organelles. B) tmd-1 knockdown causes a thickening in the bridge of the canal and swellings in the cytoplasm as compared to wild type. C) The wild type canal has only small cytoplasmic swellings and rarely has fluid filled sacs, which never reach the size observed in the tmd-1 knockdown worms. D1, D2) The excretory canals in tmd-1 knockdown worms show dramatic periodic swellings in the cytoplasm and fluid filled sacs. These sacs are sometimes separate from the lumen (D2) or part of a lumenal swelling (D1). These images were taken in worms ranging from L1 to adult. The fluorescent marker here (VHA-1::GFP) marks the cytoplasm.

In the *tmd-1* knockdowns these fluid-filled sacs were far more prevalent and larger in size. The swellings in the cytoplasm were correspondingly larger in the areas where the fluid-filled sacs were present. Some of these fluid filled sacs were distinct from the lumen, but some were continuous with the lumen (Fig. 5C, 5D). In these cases, the lumen was swollen and cystic. Despite these deformations, the lumenal width overall was narrow and consistent and the canals displayed full extension of both apical and basal surfaces. This phenotype most likely represented an incomplete tropomodulin knockdown by RNAi, and this can account for the differences in lumen formation here from the *tmd-1(tm724)* mutants.

tmd-2 mutation causes small cysts to form in the lumen of the excretory cell body

In an attempt to gain a more generalized idea of tropomodulin's role in the excretory cell, the role of TMD-2 was studied. GSC9 worms containing an apical surface marker and a deletion in the *tmd-2* gene were imaged. The lumen was found to extend normally in all worms, but some cysts were present in the lumens, especially in the cell body (Fig. 3). These cysts were not always circular and bulbous in appearance as were those noticed in the *tmd-1* mutants, but sometimes appeared as flaps protruding from the lumen. Thus, TMD-2 may also play a role in vesicle trafficking in the excretory cell.

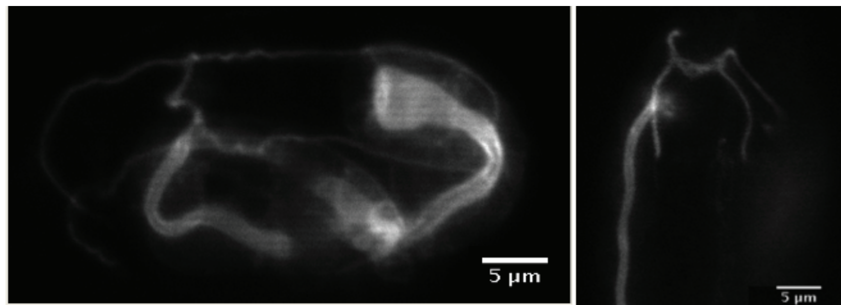

Figure 4: A genetic deletion in tmd-2 does not disrupt canal extension, but does cause small cysts to form in the bridge of the lumen. Lumen width in the canals is narrow and consistent. These worms were between the comma and three-fold embryo stages.

Discussion

The role of tropomodulin in excretory canal development has not yet been determined. However, these experiments provide some insight. Although we originally doubted the presence of tropomodulin in the excretory cell itself, we are now reconsidering. The methanol fixation in the antibody staining procedure destroys the cytoskeleton, and this is where tropomodulin would be present. Unfortunately, other attempts to visualize tropomodulin in the worm have been unsuccessful. The creation of a tropomodulin-GFP fusion protein has been reported for vertebrate isoforms, but all attempts with TMD-1 have failed. This may be due to the fact that most of tropomodulin's capping activity comes from the two ends, and the middle seems to merely link the two capping domains together. It may be possible to fuse a fluorescent tag to the middle of the protein. Another avenue for localizing tropomodulin is the development of a tissue-specific RNAi strain. I attempted this unsuccessfully using a strain with RNAi activity only in the hypodermis. In the future, I would like to attempt this once more by crossing a worm strain with hypodermal-specific RNAi capability and our strain carrying the *tmd-1(tm724)* mutation.

Because *mig-10* knockdown produces a mutant phenotype similar to the *tmd-1(tm724)* mutation, it is possible that they are performing similar functions. Both TMD-1 and MIG-10 organize the actin cytoskeleton (Stavoe and Colon-Ramos, 2012). MIG-10 acts downstream of the guidance cue netrin in axons, presumably to participate in cell signaling that aids in axon guidance and outgrowth (Stavoe and Colon-Ramos, 2012). This role in guidance was the reason that we believed TMD-1 might be necessary for neural development. However, more recent research suggests that MIG-10 is actually involved in vesicle trafficking, and this may be the mechanism for its effects on axon and excretory canal migration (McShea et al., 2013). This result, taken with our observations of a weak *tmd-1* knockdown, suggest that tropomodulin may also be regulating vesicle trafficking. Although tropomodulins are present in vertebrate neurons, TMD-1 does not appear to share this role. TMD-1 may have a different role in neurons or may not expressed there.

As tropomodulin has previously been shown to negatively regulate cell migration, it seems counterintuitive that in this situation it is facilitating excretory canal outgrowth. This suggests that its role in not to directly regulate the actin cytoskeleton at the leading edge of the canal, as occurs in migrating endothelial cells and neurons. Instead, it is more likely to be regulating processes that indirectly enable canal extension and apical surface maintenance. We hypothesized several roles for tropomodulin both in the excretory cell itself and in surrounding tissues. Because TMD-1 does not nucleate new actin filaments in *C. elegans*, our hypotheses featured tropomodulin in its actin-capping capacity, only. The hypothesis that seems most supported by our results is that tropomodulin is acting in vesicle transport needed to bring membrane components to the growing apical and basal membranes during canal extension.

Cell migration requires constant transportation of materials from the site of synthesis to the site of membrane expansion. Therefore defects in vesicle trafficking would certainly account for failed canal outgrowth. This explanation would also account for the observations of the weak tropomodulin knockdown and the *tmd-2* mutant phenotype. The fact that genetic deletion of *tmd-2* did not produce strong excretory canal defects suggests that TMD-2 is not the primary isoform used in the excretory cell. The small cysts observed in the lumen of the canal bridge could simply be a small manifestation of the same vesicle trafficking defect observed in *tmd-1* mutants. Weak knockdown of *tmd-1* by RNAi also manifests abnormalities that could be attributed to vesicle trafficking defects. Fluid filled sacs exist in the cytoplasm in larger size and greater numbers than in wild type worms. We have not yet characterized these sacs, but they appear as vesicles that have either failed to fuse with the lumen or be exocytosed. The lumen is occasionally cystic as well, which is reminiscent of the *exc-5* mutants previously described (Mattingly and Buechner, 2011). If tropomodulin is acting to stabilize actin structures involved in vesicle transport, fusion, or the integrity of the lumen, it could be that the actin filaments are far too dynamic in its absence to accomplish these processes.

To confirm or refute this hypothesis, I would like to cross the *tmd-1(tm724)* mutants with worms expressing the *vha-1* driven GFP so that the entire cytoplasm can be visualized in worms without any TMD-1. If TMD-1 is indeed causing defects in vesicle transport, I expect that the basal surface will also show failed extension and will form a large bubble around the crinkled apical membrane. I would also like to stain the excretory canals for actin to observe any cell-wide or localized changes in actin organization in *tmd-1* mutants. This may support the vesicle transport hypothesis or one of the other hypotheses proposed at the beginning of experimentation.

Tropomodulin has been previously shown to have widespread effects in diverse tissue types. It can act as a negative regulator of cell migration or an organizer of the actin cytoskeleton in specialized structures. Here, tropomodulin is facilitating the formation and maintenance of an apical lumen and the outgrowth of this lumen along the sides of the worm. Tropomodulin's role in the excretory canal is in need of further study. The high amount of homology between *C. elegans* TMD-1 and vertebrate Tmod isoforms makes this work highly translatable. Similarly, the fact that single celled tubes in higher organisms form by cell hollowing, the same mechanism used by the excretory cell, means that this process is likely to be affected by tropomodulins in other tissues in other organisms.

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