Novel exc Genes Involved in Formation of the Tubular Excretory Canals of Caenorhabditis elegans

Hikmat Al-Hashimi,1 Travis Chiarelli,2 Erik A. Lundquist, and Matthew Buechner3

ABSTRACT

Regulation of luminal diameter is critical to the function of small single-celled tubes, of which the seamless tubular excretory canals of Caenorhabditis elegans provide a tractable genetic model. Mutations in several sets of genes exhibit the Exc phenotype, in which canal luminal growth is visibly altered. Here, a focused reverse genomic screen of genes highly expressed in the canals found 18 genes that significantly affect luminal outgrowth or diameter. These genes encode novel proteins as well as highly conserved proteins involved in processes including gene expression, cytoskeletal regulation, and vesicle transport. In addition, two genes act as suppressors on a pathway of conserved genes whose products mediate vesicle movement from early to recycling endosomes. The results provide new tools for understanding the integration of cytoplasmic structure and physiology in forming and maintaining the narrow diameter of single-cell tubules.

Many mutants have been discovered that affect the length, guidance of outgrowth, or lumen diameter of the excretory canals. An initial set of such identified “exc” mutants, in which the canal lumen is abnormally wide, contains cysts, or is short, were mapped (Buechner et al. 1999), and found to include multiple alleles of some exc genes, but only single alleles of others. The frequency of mutations suggested that additional genes should have mutable excretory lumen effects. Studies by multiple laboratories indeed found alleles of other genes with Exc phenotypes (Khan et al. 2013; Kolotuev et al. 2013; Armenti et al. 2015; Gill et al. 2016; Forman-Rubinsky et al. 2017; Lant et al. 2018). Almost all of the original exc genes have now been cloned (Suzuki et al. 2001; Berry et al. 2003; Fujita et al. 2003; Pratist et al. 2005; Tong and Buechner 2005; Mattingly and Buechner 2011; Shaye and Greenwald 2015; Grussendorf et al. 2016; Al-Hashimi et al. 2018), and found to affect multiple well-conserved cell processes, including ion transport, formation of cytoskeletal structures, and vesicle recycling pathways. The initial screen sought primarily non-lethal genetic effects, but several of the subsequently identified genes were lethal when null.

RNAi studies have been particularly useful in determining roles of excretory canal genes where the null allele is lethal, such as the gene encoding the NHR-31 nuclear hormone receptor (Hahn-windgassen and Van gilst 2009), the ABI-1 Abelson-Interactor (McShea et al. 2013), and the PROS-1 transcription factor (Kolotuev et al. 2013). In addition, null mutations in genes that affect the patency of the neighboring excretory duct cell (e.g., LET-4 (Mancuso et al. 2012) and LPR-1 (Forman-Rubinsky et al. 2017)) are lethal.

In order to identify other genes affecting the process of tubulogenesis and tubule maintenance in the excretory canals, we undertook a targeted genomic RNAi screen to identify excretory canal genes that exhibit lumen alterations (“Exc” phenotypes) when knocked down. This screen
confirmed or identified 18 genes preferentially expressed in the canals that showed effects on lumen and/or outgrowth of the excretory canals, including 10 genes with no prior known phenotypic effects on the canals. In addition, two knockdowns suppressed effects of mutation of the exc-5 guanine exchange factor gene affecting canal endosomal recycling, and therefore represent potential regulators of vesicle transport needed for single-cell tubulogenesis.

MATERIALS AND METHODS

Nematode genetics

C. elegans strains (Table 1) were grown by use of standard culture techniques on lawns of Escherichia coli strain OP50 (a streptomycin-resistant derivative of strain OP50) on nematode growth medium (NGM) plates (Sulston and Hodgkin 1988). All strains were grown and evaluated for canal phenotypes at 20°C. Worms observed in this study were young adults or adults.

Each nematode strain (wild-type N2, and exc-2, exc-3, exc-4, exc-5, and exc-7) was crossed to strain BK36, which harbors a chromosomal insertion of a canal-specific promoter driving cytoplasmic GFP expression (Pscribed-gfp). Strains were then sensitized for RNAi treatment by crossing them to mutant strain BK540 (a strain carrying an exc-7 mutation). The Ahringer RNAi bacterial library (Kamath et al. 2003) was utilized for this study. Bacterial clones used are listed in Supp. Tables S1 and S2, available on Figshare. Overnight cultures were prepared by inoculating bacteria in 5 ml LB + ampicillin (100 μg/ml) + tetracycline (12.5 μg/ml), and cultured at 37°C for 16 hr. In order to induce the bacteria with IPTG, overnight cultures were moved to fresh media, incubated at 37°C with rotation until cultures reached an O.D.600 in a range from 0.5 to 0.8. IPTG was then added to the culture to a final concentration of 95 μg/ml along with ampicillin at 100 μg/ml. The cultures were then incubated with rotation at 37°C for ninety minutes followed by re-induction with IPTG and ampicillin, and another ninety minutes of incubation at 37°C with rotation. Finally, IPTG and ampicillin were added one last time just prior to using these bacteria to seed NGM in 12-well plates and Petri dishes. Plates were then incubated at room temperature for 24 hr in order to dry. 3-4 L2 worms were added to the plates, and their F1 young adult progeny were evaluated for phenotypes in the excretory canals. Each set of genes tested was induced together with induction of the sid-1 negative control strain BK541 and of two positive control strains: a plate of bacteria induced to knock down dpy-11 (which affects the hypoderm but not the canals) (Brenner 1974) (Figure 1D); a plate of bacteria induced to knock down exc-1, which affects predominantly the canals and amphid sheaths but does not substantially affect viability (Grussendorf et al. 2016) (Figure 1E); and a plate of bacteria induced to knock down erm-1 (Figure 1F), which causes severe defects in excretory canal length and lumen diameter, as well as similar lethal defects in the intestine (Khan et al. 2013), as a test that the RNAi induced partial as well as full knockouts. Induction was considered successful and plates were screened only if worms grown on the control plates showed the appropriate phenotypes in at least 80% of the surviving progeny.

For each tested gene, the induced bacteria were seeded on one 12-well plate and one 60 mm plate. Two or three L2 nematodes were placed on the bacterial lawn of each well, and screened for phenotypes in the 4th, 5th, and 6th days of induction. Each gene was tested via RNAi treatment of twelve different strains of worms, shown in Table 1, while the sole 60 mm plate was used for further analysis of animals with wild-type canals (strain BK540, Table 1) grown on
Table 1 List of strains used in this study, with genotype descriptions

| STRAIN | GENOTYPE | DESCRIPTION | REFERENCE |
|--------|----------|-------------|-----------|
| BK36   | unc-119(ed3) III; qpls11 [unc-119; P_vha-1::gfp] | N2 with integrated GFP marker expressed in excretory canal cytoplasm | Mattingly and Buechner (2011) |
| BK540  | nrf-3(pk1426) II; qpls11 [unc-119; P_vha-1::gfp] | RNAi-sensitized strain expressing GFP in canals | This study |
| BK541  | sid-1(pk3321) II; qpls11 [unc-119; P_vha-1::gfp] | Systemic RNAi-impaired strain expressing GFP in canals | This study |
| BK542  | exc-2(rh90) X; qpls11 [unc-119; P_vha-1::gfp] | exc-2(rh90) expressing GFP in canals | This study |
| BK543  | exc-3(rh207) X; qpls11 [unc-119; P_vha-1::gfp] | exc-3(rh207) expressing GFP in canals | This study |
| BK544  | exc-4(rh133) qpls11 [unc-119; P_vha-1::gfp] | exc-4(rh133) expressing GFP in canals | This study |
| BK545  | exc-5(rh232) IV; qpls11 [unc-119; P_vha-1::gfp] | exc-5(rh232) expressing GFP in canals | This study |
| BK546  | exc-7(rh252) II; qpls11 [unc-119; P_vha-1::gfp] | exc-7(rh252) expressing GFP in canals | This study |
| BK547  | BK540; exc-2(rh90) X | exc-2(rh90) expressing GFP in canals in RNAi-sensitized background | This study |
| BK548  | BK540; exc-3(rh207) X | exc-3(rh207) expressing GFP in canals in RNAi-sensitized background | This study |
| BK549  | BK540; exc-4(rh133) I | exc-4(rh133) expressing GFP in canals in RNAi-sensitized background | This study |
| BK550  | BK540; exc-5(rh232) IV | exc-5(rh232) expressing GFP in canals in RNAi-sensitized background | This study |
| VC20239| exc-15(E89K) mutation | “million mutation” strain homozygous at ~3-6 loci | Thompson et al. (2013) |
| VC20363| H09G03.1(P155) mutation | “million mutation” strain homozygous at ~3-6 loci | Thompson et al. (2013) |
| VC20573| H09G03.1(G67R) mutation | “million mutation” strain homozygous at ~3-6 loci | Thompson et al. (2013) |
| VC40373| exc-13(C44Y) mutation | “million mutation” strain homozygous at ~3-6 loci | Thompson et al. (2013) |
| VC40556| T19D12.9(G61X) mutation | “million mutation” strain homozygous at ~3-6 loci | Thompson et al. (2013) |
| VC40788| C09F12.3(P41S) mutation | “million mutation” strain - Died at thaw; not used | Thompson et al. (2013) |

Microscopy

Living worms were mounted on 3% agarose pads to which were added 0.1μm-diameter Polybead polystyrene beads (Polysciences, Warrington, PA) to immobilize the animals (Kim et al. 2013). Images were captured with a MagnaFire Camera (Optronics) on a Zeiss Axioskop microscope equipped with Nomarski optics and fluorescence set to 488nm excitation and 520nm emission. Adobe Photoshop software was used to combine images from multiple sections of individual worms and to crop them. Contrast on images was uniformly increased to show the excretory canal tissue more clearly.

Canal Measurements

Effects on excretory canal length were measured and analyzed as described (Tong and Buechner 2008). Canal length was scored by eye on a scale from 0.0-4.0: A score of (4.0) was given if the canals had grown out to full length; canals that extended halfway past the vulva (midbody) were scored as (3.0); at the vulva (2.0); canals that ended halfway between the cell body and the vulva were scored as (1.0); and if the canal did not extend past the cell body, the canal was scored as (0.0). Lengths between these waypoints were visually estimated. For knockdowns where fluid-filled cysts were evident, cyst size was rated as large (cyst diameter at least half the width of the animal), medium (one-quarter to one-half animal width), or small (up to one-quarter animal width).

For statistical analyses, canals were binned into three categories for length (scores 0-1, scores 1.5-3.8, and score 3.9-4), and the results analyzed via a 3x2 Fisher’s Exact Test (www.vassarstats.net). A p-value at or below 10^-6 was regarded as strong statistical significance that disruption of the gene caused an excretory canal defect, as per earlier studies on the canals (Tong and Buechner 2008).
### Table 2 Ahringer clones causing RNAi effects on GFP-labeled canal length in RNAi-sensitized animals

| Gene   | # canals examined in later tests | % mutant canals | Avg. mutant canal length | Std. Dev. of mutants | p-value vs. wild-type |
|--------|---------------------------------|-----------------|--------------------------|----------------------|-----------------------|
| Strain BK540 Control: wild-type canals | 100              | 0%              | 4.0                      | ±0.0                 | —                     |
| ceh-6  | Knockdowns exhibiting effects with high statistical confidence: | | | | |
| K02B12.1 | large fluid-filled cysts | 33             | 100%                     | 1.1                  | ±0.35                 | 6.7x10^{-24} |
| T25C3.1 | large fluid-filled cysts | 63             | 68%                      | 3.3                  | ±0.39                 | 2.7x10^{-24} |
| C10G6.1 | medium-sized fluid-filled cysts | 40        | 100%                     | 1.1                  | ±0.37                 | 5.7x10^{-26} |
| Y53C12A.4 | medium-sized fluid-filled cysts, vesicles along swollen cytoplasm | 20       | 100%                     | 1.1                  | ±0.22                 | 3.4x10^{-28} |
| F41E7.1 | medium-sized fluid-filled cysts | 34       | 97%                      | 1.7                  | ±0.52                 | 1.4x10^{-30} |
| T05D4.3 | medium-sized fluid-filled cysts | 53       | 79%                      | 2.6                  | ±0.59                 | 9.4x10^{-28} |
| C01B12.3 | swollen luminal tip | 36       | 28%                      | 3.4                  | ±0.31                 | 5.8x10^{-7}  |
| Y59A8B.23 | swollen tip, vesicles, convolutions | 76       | 57%                      | 3.5                  | ±0.26                 | 1.6x10^{-20} |
| F44E7.6 | swollen tip with vesicles | 125      | 66%                      | 3.1                  | ±0.27                 | 3.0x10^{-30} |
| K11D12.9 | vesicles along swollen cytoplasm | 36       | 100%                     | 1.4                  | ±0.47                 | 9.5x10^{-34} |
| T08H10.1 | vesicles along swollen cytoplasm | 41       | 27%                      | 3.5                  | ±0.15                 | 4.3x10^{-7}  |
| C03G6.5 | vesicles along swollen cytoplasm | 79       | 19%                      | 3.5                  | ±0.32                 | 1.2x10^{-6}  |
| F11H8.4 | vesicles along swollen cytoplasm | 77       | 77%                      | 3.1                  | ±0.28                 | 2.8x10^{-34} |
| ZK770.3 | vesicles along swollen cytoplasm | 46       | 96%                      | 2.7                  | ±0.50                 | 2.2x10^{-35} |
| Y8G1A.2 | vesicles along swollen cytoplasm | 50       | 80%                      | 2.8                  | ±0.51                 | 3.3x10^{-29} |
| C37E2.5 | vesicles along swollen cytoplasm | 40       | 30%                      | 3.3                  | ±0.31                 | 7.7x10^{-8}  |
| Y59A8B.23 | vesicles along swollen cytoplasm | 59       | 93%                      | 2.6                  | ±0.51                 | 2.0x10^{-38} |
| F35H10.4 | beads, vesicles, swollen cytoplasm | 78       | 59%                      | 2.5                  | ±0.79                 | 7.7x10^{-22} |
| dhhc-2 | Knockdowns exhibiting effects with lower statistical confidence: | | | | |
| Y47H9C.2 | cytoplasmic beads with vesicles | 57       | 21%                      | 3.5                  | ±0.21                 | 2.3x10^{-6}  |
| T19D12.9 | swollen tip, vesicles, convolutions | 83       | 26%                      | 3.3                  | ±0.47                 | 1.3x10^{-6}  |
| Y53F4B.31 | swollen tip with vesicles | 26       | 27%                      | 3.4                  | ±0.38                 | 7.8x10^{-6}  |
| C46F11.2 | cytoplasmic beads, convolutions | 73       | 5%                       | 3.4                  | ±0.30                 | 0.030       |
| H09G03.1 | vesicles along swollen cytoplasm | 179      | 7%                       | 3.3                  | ±0.46                 | 4.7x10^{-4}  |
| C09F12.3 | vesicles along swollen cytoplasm | 88       | 8%                       | 3.3                  | ±0.22                 | 4.3x10^{-3}  |
| exc-5 (gp110) | large cysts at canal tips | 118      | 100%                     | 1.35                 | ±1.35                 | 1.3x10^{-18} |
| sxu-2 | Knockdowns exhibiting suppression effects on exc-5: | | | | |
| F12A10.7 | suppresses exc-5 mutant cysts | 224      | 95%                      | 2.25                 | ±0.65                 | 1.3x10^{-18} |
| C53B4.1 | suppresses exc-5 mutant cysts | 207      | 97%                      | 2.26                 | ±0.73                 | 1.7x10^{-14} |

Knockouts of genes found to have effects on C. elegans excretory canals. Thick lines separate knockdowns that showed effects with high confidence from knockdowns with lower confidence, and knockdowns that exerted suppression effects on exc-5 mutants. Thin lines separate genes by the most common phenotype exhibited in knockdowns, and correspond to panels in Figures 2, 3, 4, 5, and 6, respectively. Asterisks indicate genes previously demonstrated to have effects on the excretory canals. *p*-value was determined via 3x2 Fisher’s exact test (see Materials & Methods), with 1x10^{-6} viewed as determining high significance (exc-13 was included as being close to this threshold, plus showing effects in “million mutation” animals).

Even with multiple observations, knockdown of five putative novel exc genes yielded a lower statistical confidence in the ability of RNAi knockdown to affect canal structure (Tables S1 and S2, available on FigShare). For these genes, “million mutation” strains (Thompson et al. 2013) containing homozygous point mutations in these genes were requested from the Caenorhabditis Genetics Center (Minneapolis, MN), and canals of young adults observed (Table S2). The selected strains contain mutations causing missense or nonsense mutations in the desired genes (as well as similar mutations in a few other genes). Observations of canal defects in two of these strains supported the conclusions made from finding canal defects in knockdowns for two of the genes, now named exc-13 and exc-15, but still provided ambiguous results for knockdowns of two other genes (Table S2). exc-13 is therefore listed as a novel exc gene, even though its statistical significance (*p* = 1.2x10^{-6}) is not quite at the cutoff of 1.0x10^{-6} listed above. For a fifth gene, C99F12.3, the “million mutation strain” VC40788 showed substantial embryonic lethality and could not be supplied by the Caenorhabditis Genetics Center, which reported little obvious evidence of gross defects such as edema from lack of canal function within the dead animals. Photographs of affected canals of strains with only moderate statistical support for canal effects are provided in Supplemental Figure S1.

### Reagent and Data Availability

All nematode strains used in this study are listed in Table 1. Bacterial clone numbers tested, and summary of test results are presented in Tables S1 and S2, available on Figshare. Gene names exc-10 through exc-15 and sxu-1 and sxu-2 have been registered with Wormbase (www.wormbase.org). Sensitized exc mutant strains are available upon request, and may be made available through the Caenorhabditis Genetics Center (CGC), University of Minnesota (cgc.umn.edu), pending acceptance to that repository. Other strains are available upon request. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7710362.

### RESULTS

A focused RNAi screen for new exc mutations

A study of genomic expression in *C. elegans* was previously undertaken by the Miller lab (Spencer et al. 2011). In that study, lists of genes highly
expressed in various tissues, including 250 genes preferentially expressed in the excretory canal cell, were made public on the website WormViz (http://www.vanderbilt.edu/wormdoc/wormmap/WormViz.html). Of the corresponding strains in the Arrhingher library of bacteria expressing dsRNA to specific C. elegans genes (Kamath et al. 2003), 216 grew well, and were tested for effects on the various C. elegans strains (Table S1, S2).

The excretory canal cell has some characteristics similar to those of neurons: long processes guided by netrins and other neural guidance cues (Hedgecock et al. 1987), as well as early expression of the gene EXC-7/Hur/ELAV (Fujita et al. 2003), and so was considered potentially refractory to feeding RNAi (Calixto et al. 2010). We crossed strain BK36 (Figure 1B), containing a strong canal-specific integrated gfp marker, to a mutant in the rrf-3 gene (pk1426) in order to increase sensitivity to RNAi (Simmer et al. 2002) to create strain BK540. In addition, we also crossed the same gfp marker and rrf-3 mutation to excretory canal mutants exc-2, exc-3, exc-4, exc-5, and exc-7 (except that exc-7 was not RNAi-sensitized; see Materials and Methods). This was done in order to determine if the tested gene knockdowns interacted with known exc genes affecting excretory canal tubulogenesis, since double mutants in some exc genes (e.g., exc-3; exc-7 double mutants (Buechner et al. 1999) exhibit more severe canal phenotypes than either mutant alone.

We demonstrated the effectiveness of the treatment by performing successful knockdowns of canal-specific and -non-specific genes in these strains. Control knockdowns of dpy-11 resulted in short worms with normal canal phenotypes (Figure 1D), while knockdown of exc-1 caused formation of variable-sized cysts in a short-erened excretory canal, with no other obvious phenotypes (Figure 1E). Knockdown of the ezrin-moesin-radixin homolog gene erm-1 (Göbel et al. 2004; Khan et al. 2013) also caused severe malformation of the canals visible in 80% of surviving treated worms (Figure 1F). A deletion mutant of this gene is often lethal due to cystic malformation of the intestine as well as the canals (Göbel et al. 2004), while our treatment allowed many animals to survive to adulthood and reproduce. This result is consistent with our RNAi treatment causing variable levels of gene knockdown (Timmons and Fire 1998) in the excretory canals.

Of the 212 non-control genes tested, 182 caused no obvious phenotypic changes to the canals of BK540 worms, and 4 gave very low numbers (less than 5) of animals with mild defects (Supp. Tables S1, S2). Knockdown of 24 genes caused noticeable defects in the development of the excretory canals in at least 5 worms. These genes were subsequently retested via feeding RNAi at least once, which confirmed an RNAi effect for 18 of the tested strains (Table 2), with statistically lower frequency of effect observed for knockdowns of the other 6 genes (Supp. Table S2, Supp. Fig. S1). Canal length was rated according to a measure shown in Figure 1A, in which lack of extension past the excretory cell body was rated 0, extension to the animal midbody marked by the position of the vulva was measured as 2, and full extension was rated as 4. The average canal length of affected animals was characteristic for the gene knocked down (Table 2).

For this RNAi screen, several caveats apply to the reported results. The effects of feeding RNAi can be highly variable based on the strength of induction of bacterial transcription (Hull and Timmons 2004). This use of feeding RNAi knockdown, however, does allow observation of gene effects where knockouts have been reported to be lethal. For example, null mutations in the canal-expressed gene ceh-6 are lethal due in part to loss of expression of a wide range of channels and transporters both in the canals and in the rectal epithelium (Burglin and Ruvkun 2001; Armstrong and Chamberlin 2010). In the present screen, however, we found many viable ceh-6 knockdown animals, 100% of which contained strong luminal defects in the canals, and this gene was therefore used as a positive control for assessing whether RNAi knockdown occurred in the excretory canals. A corollary of this variability is that the statistical results reported here represent a minimum effect upon canal morphology caused by knockdown. Each gene noted (Tables 2 and 3) represents the results of an initial test where at least five viable animals display shortened canal lumens, followed by further tests where the number of multiple affected canals are reported in young adults. The length of the lumen in wild-type young adult canals is highly invariable (4.0+/−0.0), so even small numbers of shortened canals can represent a significant effect upon lumen formation. Finally, other cells and tissues affect canal outgrowth and morphology; mutations in basement membrane proteins affect canal length and direction of outgrowth (Hedgecock et al. 1987; Schmidt et al. 2009; Mcshea et al. 2013), while mutations affecting patency of the neighboring excretory duct cells can also cause excretory edema (Mancuso et al. 2012; Gill et al. 2016; Forman-Rubinsky et al. 2017). The mutations reported here show an Exc phenotype, and all genes are highly expressed within the excretory canal cell, but further study of each gene and its product is needed to determine the time of action and to confirm the location of action within the excretory cell.

**Excretory Canal Phenotypes**

In knockdown animals, the posterior canals did not extend fully to the back of the animal (Table 2, Figure 1E, 1F). The length of the canal lumen was often the same as the length of the canal cytoplasm, but in many cases the visible lumen (seen as a dark area in the center of the GFP-labeled cytoplasm) was substantially shorter than the length of the canal cytoplasm.

In addition to effects on canal length, the shape and width of the canal lumen and/or canal cytoplasm was affected by specific gene knockdown. We present knockdown results according to the most-frequently observed phenotype seen for specific gene knockdowns, but knockdown of a gene often yielded different phenotypes in different individuals.

**Cystic canals**: Two gene knockdowns, of ceh-6 and of T25C8.1 (which will be referred to as exc-10), primarily resulted in the formation of large fluid-filled cysts (Figure 2A, 2B), similar to those seen in exc-2, exc-4, and exc-9 mutants (encoding an intermediate filament, a CLC chloride channel, and a LIM-domain protein involved in vesicle-trafficking (Berry et al. 2003; Tong and Buechner 2008; Al-Hashimi et al. 2018)). The homeobox gene ceh-6 encodes a well-studied POU-domain transcription factor that defines expression of many genes in the canal and rectal epithelium, and is regarded as a master transcription factor for ion channels and transporters in these tissues (Burglin and Ruvkun 2001; Armstrong and Chamberlin 2010). Null mutants die early in the first larval stage. The knockdown animals were viable, but had very short canals with large fluid-filled cysts. The effect of ceh-6 knockdown could reflect the expected lower transcription levels of canal transporters and the excretory aquaporin aqp-8 (Mah et al. 2007), as well as the possibility of direct effects on other known or novel exc genes.

Knockdowns in egal-1, mop-25.2, F41E7.1 (exc-11), and T05D4.3 (exc-12) exhibited small-to-medium-sized cysts (Figure 2C-F). In these animals, cystic regions of the lumen often appear to consist of a series of hollow spherical domains, which may be connected or separate from each other along the lumen length.
### Table 3 Identity of tested genes and encoded proteins that affect excretory canal morphology

| Gene    | Clone       | Protein Class                                      | Short Description of Known or Inferred Protein Function                                            | References                                                                 |
|---------|-------------|---------------------------------------------------|--------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| ceh-6*  | K02B12.1    | Transcriptional and post-transcriptional factors  | homeobox transcription factor                                                                     | (Burglin and Ruvken 2001; Armstrong and Chamberlin 2010)                  |
| ceh-37* | C37E2.5     | Otx homeobox transcription factor                  |                                                                                                  | (Lanjuin et al. 2003; Hench et al. 2015)                                   |
| egal-1  | C10G6.1     | Egalitarian exonuclease, regulates dynin            |                                                                                                  | (Kipros and Pagano 2000)                                                 |
| fbxa-183| F44E7.6     | F-box protein, possible effects on RNA              |                                                                                                  | (Fridolfsson et al. 2010)                                                |
| exc-14  | K1D12.9     | RING finger, possible E3 ubiquitin ligase           |                                                                                                  | (Kaneko et al. 2016)                                                     |
| mxt-1   | Y1B10A.8    | translation regulation                              |                                                                                                  | (Peter et al. 2015)                                                      |
| cyk-1*  | F11H8.4     | Cytoskeletal proteins and regulators                | Diaphanos formin                                                                                 | (Severson et al. 2002; Shaye and Greenwald 2016)                          |
| inx-12* | 2K770.3     | Transporters, channels, and receptors              | innexin gap junction protein                                                                     | (Altun et al. 2009; Hall 2017)                                           |
| inx-13* | Y8G1A.2     | innexin gap junction protein                        | innexin gap junction protein                                                                      | (Altun et al. 2009; Hall 2017)                                           |
| vha-5*  | F35H10.4    | Vacular ATPase component                           | vacular ATPase component                                                                         | (Oka et al. 2001; Liégeois et al. 2006; Hahn-Windgassen and Van Gilst 2009) |
| best-3  | C01B12.3    | Bestrohin chloride channel                         | Bestrophen chloride channel                                                                      | (Strauß et al. 2014)                                                    |
| exc-11  | F41E7.1     | Vesicle movement regulators                        | Na+/H+ solute carrier (SLC9 family)                                                               | (Fuster and Alexander 2014)                                              |
| gck-3*  | Y59A8B.23   | Vesicle movement regulators                        | Germinal center WNK kinase protein                                                                | (Falin et al. 2009; Gagnon and Delpire 2012)                              |
| mop-25.2* | Y53C12A.4 | Enzymatic activities                               | Scaffolding for endocytic recycling                                                              | (Lant et al. 2018; Sun et al. 2018)                                      |
| exc-10  | T25C8.1     | Enzymatic activities                               | Sedoheptulose kinase                                                                             | (Phompthukul et al. 2001; Wamelink et al. 2008)                           |
| exc-15  | T08H10.1    | Enzymatic activities                               | Aldo-keto reductase                                                                              | (Barski et al. 2008)                                                     |
| exc-12  | T05D4.3     | Unknown function                                   | Nematode-only transmembrane protein                                                               |                                                                           |
| exc-13  | C03G6.5     | Nematode conserved-domain protein                   | Nematode conserved-domain protein                                                                  |                                                                           |
| suex-1  | F12A10.7    | Caenorhabditis-only glycine-rich protein            | Caenorhabditis-only glycine-rich protein                                                           |                                                                           |
| suex-2  | C53B4.1     | Solute carrier (SLC22 family)                      | Solute carrier (SLC22 family)                                                                     |                                                                           |

Protein class shown known function, or function of closest homologs, of products of genes where knockouts affected morphology on *C. elegans* excretory canals. Thick line separates knockdowns that caused defects in canal morphology, and knockdowns that suppressed effects of exc-5 mutation. Thin lines separate genes by class of protein encoded. Asterisks indicate genes previously demonstrated to have effects on the excretory canals.

Large vesicles and swollen cytoplasm: The largest group of knockdown animals showed generally normal-diameter lumen surrounded by accumulation of larger vesicles outside the lumen and swollen canal cytoplasm (Figures 3, 4). In some cases, the swelling occurs primarily at the distal tip, and appears to be caused by accumulation of a convoluted lumen folded back on itself, while in other knockdowns this swelling reflect accumulation of a large number of vesicles at the end of the lumen. A combination of these structures also appears in many animals. Reflecting the variable effects of RNAi knockdown, some animals knocked down in *ceh-37* (in which knockdowns generally caused less extreme effects, see below), and in a gene mentioned above, *map25.2*, sometimes showed a highly convoluted lumen primarily at the distal tip (Figure 3A, 3B), possibly reflecting weaker knockdown than in other examples where the entire lumen was affected. Knockdown of other genes, *best-3* and *gck-3*, showed a similar effect (Figure 3C, 3D). Knockdowns of *fbxa-183* (Figure 3E) show clear and dramatic accumulation of large vesicles at the swelling at the tip of the lumen. A few knockdowns of *gst-28* gave similar but less reproducible effects (Fig. S1).

In other cases, the swollen cytoplasm and presence of vesicles was distributed along the length of the canals (Figure 4). Knockdown of the gene *K11D12.9* (to be referred to as *exc-14*) exhibited an extraordinary increase of vesicles in the cytoplasm of the canal, terminating in a large irregular swelling at the terminus of the canal (Figure 4A, 4A’). In these animals, the short lumen of the canal appears relatively normal in diameter, but is surrounded by cytoplasm that puffs out at the basal side of the cell. GFP labeling of the cytoplasm showed a thick layer of fluorescence surrounding the lumen suggestive of normal canaliculi excluding larger vesicles, and which is surrounded by a cytoplasm packed with vesicles of variable size.

Some animals knocked down for the *fbxa-183* gene (Figure 4B) also exhibited large vesicles surrounding an irregular lumen, (in addition to those *fbxa-183* knockdowns with large cysts discussed above). Knockdowns of *T08H10.1* (to be referred to as *exc-15*) similarly exhibited large numbers of these variable-sized vesicles in the canal cytoplasm (Figure 4C). The enlarged lumen was also apparent in DIC micrographs of animals of the “million-mutation” strain VC20239 that contains a substitution mutation of this gene (Figure 4C’).

Finally, knockdown of two other genes, *C03G6.5* (*exc-13*) and *cyk-1* (*CyD, 4E*) caused the appearance of large cysts or vesicles appearing at the basal surface of the canals in just a few seemingly random spots along the length of the canals, as well as at the distal tips of the canals. A few of the “million-mutation” animals containing a point mutation in *exc-13* also showed an enlarged shortened canal lumen, although large cysts along the canal length were not visible (Figure 4D’, Table S2). Similar but less reproducible effects were seen for knockdowns of *H09G03.1* and in a few of the “million-mutation” animals containing substitution mutations of the *H09G03.1* gene (Fig. S1, Table S2).

A very narrow canal “tail” completely lacking a visible lumen often extends substantially past the end of the lumenated portion of the canal in several of the knockdown animals (marked by arrows in...
Figure 3A, 3C, 3D, S1B). This tail follows the path of wild-type canal growth, and in rare instances even reaches the normal endpoint of the canal. In wild-type animals, the lumen and tip of the canal grow together and reach the same endpoint (Buechner et al. 1999), with a widening suggestive of a growth cone at the tip of the growing canal in the embryo and L1 stage (Fujita et al. 2003). The tip of the canal is enriched in the formin EXC-6, which mediates interactions between microtubules and actin filaments and may mediate connections between the canal tip and end of the lumen (Shaye and Greenwald 2015). The results here are consistent with the idea that canal lumens grow and extend separately from the growing basal surface that guides cytoplasmic outgrowth (Kolotuev et al. 2013).

**Periodic cytoplasmic swellings:** Knockdown of some genes caused animals to exhibit shorter canals with periodic swellings of the cytoplasm (Figure 5) rather than luminal distension. These swellings, also called “beads” or “pearls,” are commonly seen in wild-type animals with rapidly growing canals at the L1 stage, and in animals under osmotic stress (Kolotuev et al. 2013). These sites were hypothesized in that study to be locations of addition of membrane to allow the canal to continue to grow together with the animal. The knockdown animals here were measured in young adulthood, after the period of rapid growth, and so presence of these beads may reflect osmotic stress caused by partial loss of the encoded proteins. Knockdown of the inx-12 or inx-13 genes (Figure 5A, 5B), which encode innexins highly expressed in the canals (and in the adjacent CAN neurons), gave rise to these structures. Innexins form the gap junctions of invertebrates (Hall 2017), and the excretory canals are rich in these proteins along the basal surface, where they connect the canal cytoplasm to the overlying hypodermis (Nelson et al. 1983). Null mutants in either of these two genes results in early larval rod-like swollen lethality consistent with excretory cell malfunction (Altun et al. 2009). The knockdown phenotype here further suggests that these proteins regulate balancing of ionic content to allow normal canal growth.

A similar phenotype is seen in animals knocked down for ceh-37 or mxt-1, and, with lower statistical confidence, for knockdown of dhhc-2 (Figure 5C, 5D, Fig. S1). The dhhc-2 knockdown animals exhibited somewhat enlarged dark spots consistent with the presence of many variably enlarged vesicles within the beads, and the beads themselves show more variable size and placement than for the other knockdowns in this class.

**Variability of canal phenotypes:** Some knockdowns of ceh-37 resulted in regular beads along the canal length (Figure 5C), while others resulted in a swollen lumen (Figure 3A). Expression of ceh-37 is itself regulated by CEH-6 (Burglin and Ruvkun 2001), knockdown of which
caused large cysts (Figure 2A). The ultimate canal phenotype in all of these treated animals likely represents the degree of gene knockdown as well as the nature of the specific protein affected by knockdown. This hypothesis is supported from the results seen from RNAi-knockdown of the \textit{vha-5} gene (Figure 6). This gene encodes a protein of the membrane-bound V$_0$ subunit of the vacular ATPase (Oka et al. 2001). Mutations of this gene are lethal, and a point mutation led to strong whorls of labeled VHA-5 at the apical surface (Liégeois et al. 2006). Here, knockdown of \textit{vha-5} resulted in a wide range of canal phenotypes in different animals (Figure 6). Some animals exhibited beads surrounding a normal-diameter lumen (Figure 6A), similar to animals under osmotic stress, as in Figure 5. Other animals showed small septate cysts in the canal lumen, but the canal lumen overall was of near-normal diameter and the basal surface had mostly minor irregularities (Figure 6B), similar to animals knocked down for \textit{exc-11} (Figure 4C). Other \textit{vha-5} knockdown animals exhibited a similar luminal phenotype, but also showed large vesicles within a highly irregular-shaped cytoplasm (Figure 6C), similar to animals impaired in \textit{exc-13} or \textit{cyk-1} expression (Figure 6D, E). Finally, the most extremely affected \textit{vha-5} knockdown animals (Figure 6D) showed cysts throughout the lumen, a swollen terminus to the lumen, and a range of variable-sized vesicles or cysts that pack the entire swollen cytoplasm of the canals.

**Other Phenotypes**

While the focus of this RNAi screen centered on excretory canal morphology, a few other phenotypes were noted, including occasional effects on gonadal shape, fertility, and viability. In many \textit{exc} mutants, the shape of the normally smooth hermaphrodite tail spike (Figure 7A) is affected (Buechner et al. 1999), and similar strong results were reproducibly observed here for multiple RNAi knockdowns (Figure 7B-F). In addition to the knockdowns shown (for genes \textit{exc-11}, \textit{exc-14}, \textit{egal-1}, \textit{mop-25.2}, and \textit{inx-12}), tail spike defects were also seen in animals knocked down in genes encoding homeobox protein \textit{CEH-6}, vacular ATPase component \textit{VHA-5}, sedoheptulose kinase \textit{EXC-10}, aldo-keto reductase \textit{EXC-15}, and innexin \textit{INX-13} (data not shown). The tail spike is formed from the interaction of hypodermal tissue hyp10 with a syncytium of two other hypodermal cells that later undergo cell death (Sulston et al. 1983); it remains to be determined what features this structure has in common with the canals that require the same proteins.

**Suppressors of the Exc-5 Phenotype**

Finally, the RNAi screen was also carried out in animals carrying mutations in various \textit{exc} genes, to try to find genes that interacted to form more severe phenotypes. Previous interactions have found, for example, that \textit{exc-3}; \textit{exc-7} double mutants have a more severe canal phenotype than does either mutant alone (Buechner et al. 1999), and similar synergetic effects are seen for \textit{exc-5}; \textit{exc-6} double mutants (Shaye and Greenwald 2016). No such effects were detected in this screen, unfortunately. We conclude that the variability of knockdown strengths and resultant wide range of canal length in the starting strains prevented easy identification of severely affected animals at the initial screening step, so that such enhancer mutations could not be easily identified.

Knockdown of two genes, however, caused an unexpected phenotype: the restoration of near-wild-type phenotype from strongly cystic homozygous \textit{exc-5} (\textit{rh232}) animals (Figure 8) carrying a large deletion of almost all of the \textit{exc-5} gene (Suzuki et al. 2001). \textit{exc-5} encodes a guanine exchange factor (GEF) specific for CDC-42 (Gao et al. 2001; Suzuki et al. 2001), and mutants are defective in transport from early endosomes to recycling endosomes (Mattingly and Buechner 2011). \textit{EXC-5} is homologous to four human FGD proteins, including two that are implicated in Aarskog-Scott Syndrome (Facio-Genital Dysplasia) and Charcot-Tooth-Marie Syndrome Type 4H, respectively (Gao et al. 2001; Delague et al. 2007; Horn et al. 2012). The latter disease affects outgrowth of the single-celled tubular Schwann cells during rapid growth, and identification of mutations in suppressor genes therefore has the potential to increase understanding of this disease.

\textit{exc-5} null mutants are characterized by large fluid-filled cysts at the terminus of both anterior and posterior canals (Figure 8A). Knockdown RNAi of these suppressor genes, both by feeding and by direct dsRNA microinjection, yielded a large number of progeny exhibiting near-normal canal phenotypes (Figure 8B, 8C), with canal length extending near-full-length (Figure 8D). We will refer to this phenotype as \textit{Sux}, for \textit{S}uppressor of \textit{EX}cretory defects. In \textit{SUX} canals, no obvious septate cysts are evident, although parts of the canal lumen were slightly widened (Figure 8B, 8C).

**DISCUSSION**

**Proteins Encoded by the Knockdown Genes**

Several types of protein appear repeatedly as products of the genes with knockdown effects on the canals (Table 3). Significantly, knockdown of some proteins with similar functions appear in different phenotypic classes. This observation again suggests that the difference between large and smaller cysts, and between cyst formation and vesicle accumulation, may reflect the relative expression level of the proteins involved.

![Figure 3](image-url) **Figure 3** RNAi knockdowns causing swelling at end of lumen. GFP fluorescence images of swollen canals at termination of lumen caused by RNAi knockdown of genes (A) \textit{ceh-37}; (B) \textit{mop-25.2}; (C) \textit{best-3}; (D) \textit{gck-3}; (E) \textit{fbxa-183}; all images show regions of convoluted canals. Some additional areas in panels D and E appear as individual separated small cysts or large vesicles. Arrows: Cytoplasmic tail continuing past termination of lumen in panels A, C, and D.
rather than fundamentally different processes involved in preventing cyst formation or vesicle accumulation within the canals.

**Transcriptional and post-transcriptional regulation:** Since this screen focused on genes preferentially expressed within the canals, it is not surprising that transcriptional factors affecting canal expression were among the proteins identified. As noted above, the POU-domain transcription factor CEH-6 (Figure 2A) regulates transcription of many genes in the canal (Burglin and Ruvkun 2001; Armstrong and Chamberlin 2010), including the well-conserved OTX Homeobox gene *ceh-37* (Figure 3A), expression of which is restricted to the excretory canals in adults (Lanjuin et al. 2003; Hench et al. 2015). In addition, the EGAL-1 (Figure 2C) exonuclease is homologous to *Drosophila* Egalitarian, which degrades RNA (Fridolfsson et al. 2010), and the F-box protein FBXA-183 (Figure 3E, 4C) facilitates targeting substrates for E3 ubiquitinase-mediated destruction (Kipreos and Pagano 2000). EXC-14 (K11D12.9) (Figure 4A) encodes a protein containing a RING finger domain at the C-terminus, with BLASTP homology to the human CGRRF ubiquitin ligases that regulates ER stress (Kaneko et al. 2016). MXT-1 (Figure 5D) binds both to mRNA and to eukaryotic initiation factor 4E to regulate translation rate (Peter et al. 2015).

**Cytoskeletal Regulators:** Several cytoskeletal proteins are critical for canal structure. The ACT-5 actin, and EXC-2 and IFB-1 intermediate filaments are expressed predominantly in the canals and intestines, both tissues with a thick actin-based terminal web that restricts apical expansion (Macqueen et al. 2005; Kolotuev et al. 2013; Al-Hashimi et al. 2018). Mutants in the SMA-1 β-spectrin or the
ERMs—ERMs (ERM) ezrin/radixin/moesin—that bind actin to the apical membrane allow dilation of the canal into very large cysts (Mckewon et al. 1998; Göbel et al. 2004; Khan et al. 2013). The present screen confirmed the role of the CYK-1 Diaphanous homolog (Severson et al. 2002) in canal morphogenesis; this protein was previously found to interact with the EXC-6 homolog to regulate the EXC-5 guanine exchange factor (Mattingly and Buechner 2011; Shaye and Greenwald 2016). RNAi knockdown of cyk-1 here (Figure 4E) produced a stronger phenotype (shorter canals with large cysts on the basal side) than seen in the temperature-sensitive mutant used by the Greenwald laboratory, but not as strong an effect as was seen in double mutants of cyk-1 (ts) with exc-6 null mutants (Shaye and Greenwald 2016).

**Ion transport:** Vacuolar ATPase is an important ion pump that generates proton gradients in excretory tissues from *Paramecium* to human (Wassmer et al. 2008), it is strongly expressed in the canals (Oka et al. 2001), and knockdown of vacuolar ATPase genes affects morphology of multiple tissues, including the canals (Liégeois et al. 2006; Hahn-Windgassen and Van gilst 2009), so it was expected to see strong effects from knockdown of the vha-5 gene within the canals (Figure 6). In addition, INX-12 and INX-13 (Figure 5A, 5B) encode innexins, key components of invertebrate gap junctions (Hall 2017). The excretory canals are rich in these proteins along the basal surface, where they connect the canal cytoplasm to the overlaying hypodermis (Nelson et al. 1983), as well as in the adjacent CAN neurons that are postulated to control excretory canal function (Manser and Wood 1990; Altun et al. 2009). Null mutants in either of these two genes results in early larval rod-like swollen lethality consistent with excretory system malfunction (Altun et al. 2009).

This screen found three additional genes that encode proteins likely to regulate canal cell ion content: best-3, exc-11, and the suppressor gene suex-2. BEST-3 (Figure 3C) is homologous to the mammalian chloride channel bestrophin, essential for Ca$^{2+}$ signaling in muscles, neurons, and eyes, and defective in retinal diseases (Strauss et al. 2014).

EXC-11 (F41E7.1) (Figure 2E) and SUEx-2 (C53B4.1) (Figure 8C) are both previously unstudied proteins with homology to members of the ubiquitous SLC (Solute Carrier) proteins found in a wide range of animals, including humans. EXC-11 appears to be a member of the SLC9 family (SLC9B subgroup) of Na(+)/H(+) antporters found in plasma membrane and endosomes, and might be expected to exchange the acidic protons of canalicul vesicles for sodium (Fuster and Alexander 2014), in order to increase luminal osmolality and draw excess body water into the canals to be excreted. SUEx-2 is a transporter that has been implicated in gonadal distal tip cell migration in a previous RNAi screen (Cram et al. 2006). Its closest homolog SLC22A1 encodes a 12-tm-domain integral membrane protein transporting organic cations (Nigam 2018) and expressed in the human liver and small intestine. The effect of knocking down this transporter implies that ionic milieu or lipid composition affects transport of vesicles mediated by EXC-5 signaling, but future work will be needed to determine the role that this transporter exerts on ionic content, and possibly on endosomal recycling in the developing excretory canal cell.

**Regulation of excretory cell vesicle transport:** In addition to SUEx-2, SUEx-1 (F12A10.7) (Figure 8B) also presumably regulates transport of endosomes within the excretory canals, which is the process disrupted through impairment of the EXC-5 guanine exchange factor. The small SUEx-1 protein (113 amino acids) is unique to *C. elegans*, expressed in the excretory canal cell and in some neural subtypes, with homology to genes in only a few other *Caenorhabditis* species. The C-terminal half of the protein contains a number of repeats of tri- and tetra-peptides GGY and GGGY.

Two other genes were identified here that likely affect transport of canal vesicles. MOP-25.2 (homolog of M OAuth embryo scaffolding Protein 25) (Figure 3B) acts as a scaffolding for endocytic recycling, and reduced the number of vesicles expressing the recycling endosome marker RAB-11 in a recent canal mutant screen (Lant et al. 2018). That screen searched for interactors of the protein CCM-3 (for Cerebral Cavernous Malformations), a homolog of a mammalian protein that regulates vascular integrity in the brain. Mutants in ccm-3 and other components of the CCM complex also show strong canal defects, though not as severe as those found for loss or reduction of MOP-25.2 function (Table 2).

The STE20-related kinase GCK-3 was also identified in our screen (Figure 3D). GCK-3 is homologous to cell-volume-regulating kinases (Falin et al. 2009; Gagnon and Delipire 2012), and phosphorylates to inactivate the voltage-gated intracellular chloride channel CLH-3 found in the excretory cell as well as in the egg-laying HSN neurons and enteric muscles (Denton et al. 2005; Miyazaki et al. 2012). Interestingly, in *Drosophila*, MOP-25.2 has also recently been found to regulate ion transport in the Malpighian Tubule GCK-3 through activation of WNK kinase signaling (Sun et al. 2018). The present knockdown results are consistent with these other findings and with the above observations and conclusions from the Derry laboratory screen.
(Lant et al. 2018) that MOP-25.2 likely acts in multiple pathways to regulate excretory cell shape.

**Enzymatic activities:** Two surprising genes found in this screen encode proteins with homology to enzymes involved in lipid and sugar metabolism. EXC-15 (T08H10.1) (Figure 4C) has strong homology to aldo-keto-reductase family 1 member B10, a human intestinal protein that may detoxify aromatic aldehydes and ketones, and is overexpressed in tumor tissues (Barski et al. 2008). In a previous RNAi screen, knockdown of the C. elegans gene slowed the defecation rate by about 20%, possibly through effects on mitochondrial stress (Liu et al. 2012). exc-10 (T25C8.1) (Figure 2B) encodes a carbohydrate kinase (with homology to sedoheptulose kinase, an enzyme of the glycolytic pentose phosphate pathway) of unknown function in nematodes. The human homolog SHPK has been linked to a lysosomal storage disease (Phornphutkul et al. 2001; Wamelink et al. 2008).

![Figure 6](image1.png)

**Figure 6** Knockdown of vha-5 leads to a wide range of phenotypes. (A-D) GFP fluorescence of four different worms exhibiting a range of excretory canal phenotypic severity in response to vha-5 knockdown. For each animal, the area boxed in red is enlarged below. (A) Periodic cytoplasmic swellings along lumen of canal. Arrows show visible lumen of normal diameter. (B) Small septate cysts in the lumen of the canal, surrounded by area of bright GFP fluorescence, and somewhat irregular diameter cytoplasm. (C) Lumen with septate cysts similar to 4B and surrounded by cytoplasm of more irregular diameter containing large cysts-vesicles. (D) Wider-diameter lumen with larger cysts, surrounded by cytoplasm filled with vesicles in a wide range of sizes.

![Figure 7](image2.png)

**Figure 7** Knockdown of some exc genes causes tailspike defect. DIC images of the narrow tail spike of adult hermaphrodite wild-type animal (A) and of adult mutants exhibiting RNAi knockdown for genes. Knockdowns of: (B) F41E7.1 (exc-11); (C) K11D12.9 (exc-14); (D) egal-1; (E) mop-25.2; (F) inx-12.
Novel proteins: EXC-12 (T05D4.3) (Figure 2F), EXC-13 (C03G6.5) (Figure 4D), and SUEX-1 (F12A10.7) (Figure 8B) proteins have no known function and no obvious homology except to other nematode proteins. SUEX-1 even appears to be unique to the Caenorhabditids. While the majority of proteins with strong effects on canal structure have close homologs in a wide range of eukaryotes, the discovery of these proteins implies that the universal structure of unicellular tubes can be modified or regulated for the specific phylum and family.

Source of Swollen Vesicles
As described above, many of the mutant phenotypes involve the accumulation of large vesicles in various regions of the excretory canals, and the source of these vesicles is an unanswered question from this study. Several types of vesicles are visible in electron micrographs of the excretory canals (Nelson et al. 1983). The canal lumen is surrounded by myriad strings of small vesicles that are each encased in a thick electron-dense coat that includes the vacuolar ATPase (Kolotuev et al. 2013). These vesicles remain separate from the lumen, or can attach to each other and the lumen to form small canalculus that allow pumping of protons into the lumen. In electron micrographs of many of the previously described exc mutants for which electron micrographs were taken (Buechner et al. 1999), the number and placement of canalicular vesicles around sections of the canal lumen varies greatly, but when present, the canalicular vesicles in these mutants, as for wild-type animals, have a diameter of about 80-110 nm.

Larger vesicles visible in the light microscope are marked by various fluorescently tagged Rab proteins (Tong and Buechner 2008) that have been used to delineate the complicated processes of secretion and endocytosis in cells of C. elegans by labeling early, recycling, and late endosomes (Sato et al. 2014). Labeled vesicles derived from these endosomes do not have an obvious thick protein coat, are larger than canalicular vesicles, and in electron microscopic images, are predominantly found outside the region of canalicular vesicles. In exc-1, exc-5, and exc-9 mutants, the early endosomes are greatly enlarged (Tong and Buechner 2008; 2011; Grussendorf et al. 2016). In the present study, knockdown of exc-14 clearly results in large vesicles that are excluded from a sub-apical domain surrounding the lumen (Figure 4A), as would be expected if canalicular vesicles are intact as in wild-type animals.

On the other hand, the vacuolar ATPase proteins, including VHA-5, are clearly highly enriched on canalicular vesicles, and knockdown of this gene results in the wide range of vesicular defects described in this study. Mutants affecting the small GTPase RAL-1 that regulates canalicular vesicle fusion appear to result in fewer but larger vesicles (Armenti et al. 2014). Mutants defective in CCM-3/PDCD10, a protein that resides in several complexes that regulate mammalian vascular integrity (preventing Cerebral Cavernous Malformations), also show variation in vesicle size, with larger vesicles with a thick coat that may contain vacuolar ATPase located farther from the luminal surface than are smaller vesicles closer to the apical surface (Lant et al. 2015; Pal et al. 2017). Significantly, mutants in mop-25.2 have recently been shown to affect the location of CCM-3 in the excretory canals (Lant et al. 2018).

While we postulate that the large vesicles in these knockdown animals are generally of endosomal origin, final identification of the origin of canalicular vesicles as endosomally derived, apical-membrane-derived, or both, therefore awaits detailed study of mutants in these genes.

CONCLUSION
This RNAi screen was successful at identifying 18 genes (10 not implicated before) needed to form a normal lumen of the long excretory canals of C. elegans. These genes encode transcription and translation factors, innexins and other channels, and proteins involved in subcellular trafficking, among others. While these processes have been implicated previously in canal tubulogenesis, these proteins represent new
actors that could provide insights into how these cellular processes are integrated in single-cell tubulogenesis. Several proteins, such as sedoheptulose kinase, could introduce insight into previously unidentified processes involved in canal morphogenesis. Finally, two additional genes were identified as suppressors of exc-5 mutation; determining the function of these suppressor proteins has the potential to increase understanding of the role of FGD protein function during normal and in disease state single-cell tubulogenesis.

ACKNOWLEDGMENTS

H.A. was supported in part by KU Graduate Research Funds #2301847 and #2144091. E.A.L. was supported by National Institutes of Health grants NS0090945, NS0095682, NS0076063, and GM103638. Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). RNAi-refractive strain NLS321 sid-1(pk3321) was the gift of Lisa Timmons, U. Kansas. Some strains were created by the C. elegans Reverse Genetics Core Facility at the University of British Columbia, which is part of the international C. elegans Gene Knockout Consortium.

LITERATURE CITED

Al-Hashimi, H., D. H. Hall, B. D. Ackley, A. E. Lundquist, and M. Buechner, 2014 The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. Drug Metab. Rev. 46: 553–624. https://doi.org/10.1080/03602530802431439

Barski, O. A., S. M. Tipparaju, and A. Bhatnagar, 2008 The ald-o-keto reductase superfamily and its role in drug metabolism and detoxification. Drug Metab. Rev. 40: 553–624. https://doi.org/10.1080/03602530802431439

Barker, L. C., H. E. Bulow, D. H. Hall, and T. Strange, 2009 Unidentified Genes and Function through Coordinate Regulation of the Vacuolar ATPase. PLoS Genet. 5: e1000553. https://doi.org/10.1371/journal.pgen.1000553

Brenner, S., 1974 The genetics of Caenorhabditis elegans. Genetics 77: 71–94. http://www.genetics.org/content/77/1/71

Buechner, M., D. H. Hall, H. Bhatt, and E. M. Hedgecock, 1999 Cystic Canal Mutants in Caenorhabditis elegans Are Defective in the Apical Membrane Domain of the Renal (Excretory) Cell. Dev. Biol. 214: 227–241. https://doi.org/10.1006/dbio.1999.9398

Burklin, T. R., and G. Ruvkun, 2001 Regulation of ectodermal and excretory function by the C. elegans POU homeobox gene ceh-6. Development 128: 779–790.

Calixto, A., D. Chelur, I. Topalidou, X. Chen, and M. Chalfie, 2010 Enhanced neuronal RNAi in C. elegans using SID-1. Nat. Methods 7: 554–559. https://doi.org/10.1038/nmeth.1463

Cram, E. J., H. Shang, and J. E. Schwarzbauer, 2006 A systematic RNA interference screen reveals a cell migration gene network in C. elegans. J. Cell Sci. 119: 4811–4818. https://doi.org/10.1242/jcs.03274

Delave, V., A. Jacquier, T. Hamadouche, Y. Poitelon, C. Baudot et al., 2007 Mutations in FGD4 encoding the Rho GDP/GTP exchange factor FRABIN cause autosomal recessive Charcot-Marie-Tooth type 4H. Am. J. Hum. Genet. 81: 1–16. https://doi.org/10.1016/j.ajhg.2007.05.013

Denton, J., K. Nehru, Y. Xin, R. Morrison, and K. Strange, 2005 GCK-3, a newly identified Ste20 kinase, binds to and regulates the activity of a cell cycle-dependent CIC anion channel. J. Gen. Physiol. 125: 113–125. https://doi.org/10.1085/jgp.200409215

Falin, R. A., R. Morrison, A. J. Ham, and K. Strange, 2009 Identification of regulatory phosphorylation sites in a cell volume- and Ste20 kinase-dependent CIC anion channel. J. Gen. Physiol. 133: 29–42. https://doi.org/10.1085/jgp.200810080

Forman-Rubinsky, R., J. D. Cohen, and M. V. Sundaram, 2017 Lipocalins Are Required for Apical Extracellular Matrix Organization and Remodeling in Caenorhabditis elegans. Genetics 207: 625–642. https://doi.org/10.1534/genetics.117.300207

Fridolfsson, H. N., N. Ly, M. Meyerzon, and D. A. Starr, 2010 UNC-83 coordinates kinesis-1 and dynein activities at the nuclear envelope during nuclear migration. Dev. Biol. 338: 237–250. https://doi.org/10.1016/j.ydbio.2009.12.004

Fujita, M., D. Kawasaki, K. King, D. H. Hall, H. Sakamoto et al., 2003 The role of the ELAV homologue EXC-7 in the development of the Caenorhabditis elegans excretory canals. Dev. Biol. 256: 290–301. https://doi.org/10.1016/S0012-1606(03)00040-X

Fuster, D. G., and R. T. Alexander, 2014 Traditional and emerging roles for the SLCO Na+/H+ exchangers. Pflugers Arch. 466: 61–76. https://doi.org/10.1007/s00424-013-1408-8

Gagnon, K. B., and E. Delpire, 2012 Molecular physiology of SPAK and ORAI1: two Ste20-related protein kinases regulating ion transport. Physiol. Rev. 92: 1577–1617. https://doi.org/10.1152/physrev.00009.2012

Gao, J., L. Estrada, S. Cho, R. E. Ellis, and J. L. Gorski, 2001 The Caenorhabditis elegans homolog of FGD1, the human Cdc42 GEF gene responsible for faciogenital dysplasia, is critical for excretory cell morphogenesis. Hum. Mol. Genet. 10: 3049–3062. https://doi.org/10.1093/hmg/10.13.3049

Gill, H. K., J. D. Cohen, J. Ayala-Figueroa, R. Forman-Rubinsky, C. Poggioli et al., 2010 Integrity of Narrow Epithelial Tubes in the C. elegans Excretory System Requires a Transient Luminal Matrix. PLoS Genet. 12: e1006205. https://doi.org/10.1371/journal.pgen.1006205

Göbel, V., P. L. Barrett, D. H. Hall, and J. T. Fleming, 2004 Lumen morphogenesis in C. elegans requires the membrane-cytoskeleton linker erm-1. Dev. Cell 6: 865–873. https://doi.org/10.1016/j.dcell.2004.05.018

Gruusendorf, K. A., C. A. Trezza, A. T. Salem, H. Al-Hashimi, B. C. Mattingly et al., 2016 Facilitation of Endosomial Recycling by an IRG Protein Homolog Maintains Apical Tubule Structure in Caenorhabditis elegans. Genetics 203: 1789–1806. https://doi.org/10.1534/genetics.116.192559

Hahn-Windgassen, A., and M. R. Van Gilst, 2009 The Caenorhabditis elegans HNF4alpha Homolog, NHR-31, mediates excretory tube growth and function through coordinate regulation of the vacuolar ATPase. PLoS Genet. 5: e1000553. https://doi.org/10.1371/journal.pgen.1000553

Hall, D. H., 2017 Gap junctions in C. elegans: Their roles in behavior and development. Dev. Neurobiol. 77: 587–596. https://doi.org/10.1002/dneu.22408

Hedgecock, E. M., J. G. Culotti, D. H. Hall, and B. D. Stern, 2007 Genetics of cell and axon migrations in Caenorhabditis elegans. Development 134: 365–382.

Hench, J., J. Henriksen, A. M. Abou-Zied, M. Luppert, J. Dethlefsen et al., 2015 The Homeobox Genes of Caenorhabditis elegans and Insights into Their Spatio-Temporal Expression Dynamics during Embryogenesis. PLoS One 10: e0126947. https://doi.org/10.1371/journal.pone.0126947

Horn, M., R. Baumann, J. A. Pereira, P. N. Sidropoulos, C. Somandin et al., 2012 Myelin is dependent on the Charcot-Marie-Tooth Type 4H disease culprit protein FRABIN/FGD4 in Schwann cells. Brain 135: 3567–3583. https://doi.org/10.1093/brain/awt275

Hull, D., and L. Timmons, 2004 Methods for delivery of double-stranded RNA into Caenorhabditis elegans. Methods Mol. Biol. 265: 23–38.

Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin et al., 2003 Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 421: 231–237. https://doi.org/10.1038/nature01278

Kaneko, M., I. Iwase, Y. Yamasaki, T. Takai, Y. Wu et al., 2016 Genomewide identification and gene expression profiling of ubiquitin ligases for
endoplasmic reticulum protein degradation. Sci. Rep. 6: 30955. https://doi.org/10.1038/srep30955

Khan, L. A., H. Zhang, N. Abraham, L. Sun, J. T. Fleming, et al., 2013 Intracellular lumen extension requires ERM-1-dependent apical membrane expansion and AQP-8-mediated flux. Nat. Cell Biol. 15: 143–156. Erratum in: Nat. Cell Biol. 15: 335. https://doi.org/10.1038/ncb2656

Kim, E., L. Sun, C. V. Gabel, and C. Fang-Yen, 2013 Long-term imaging of Caenorhabditis elegans using nanoparticle-mediated immobilization. PLoS One 8: e53419. https://doi.org/10.1371/journal.pone.0053419

Kipreos, E. T., and M. Pagano, 2000 The F-box protein family. Genome Biol. 1: REVIEWS3002. https://doi.org/10.1186/ib-2000-1-5-reviews3002

Kolotuev, I. V., H. Hyenne, Y. Schwab, D. Rodriguez, and M. Labouesse, 2013 A pathway for unicellular tube extension depending on the lymphatic vessel determinant Prox1 and on osmoregulation. Nat. Cell Biol. 15: 157–168. https://doi.org/10.1038/ncb2662

Lanjuin, A., M. K. VanHoven, C. I. Bargmann, J. K. Thompson, and P. Sengupta, 2003 Otx/cod homeobox genes specify distinct sensory neuron identities in C. elegans. Dev. Cell 5: 621–633. https://doi.org/10.1016/S1534-5807(03)00293-4

Lant, B., S. Pal, E. M. Chapman, B. Yu, D. Witvliet et al., 2018 Interrogating the ccm-3 Gene Network. Cell Reports 24: 2857–2868.e4. https://doi.org/10.1016/j.celrep.2018.08.039

Lant, B., B. Yu, M. Goudreault, D. Holmyard, J. D. Knight, L. B. Agellon, and S. Hekimi, 2013 Long-term imaging of protein MIG-10 and is required in guided cell migrations and process outgrowth. In: C. elegans Germline Development by Regulating Vesicle Trafficking Cytoskeleton and Polarity. Curr. Biol. 27: 868–876. https://doi.org/10.1016/j.cub.2017.02.028

Peter, D., R. Weber, C. Kone, M. Y. Chung, L. Ebertsch et al., 2015 Mxelti proteins use both canonical bipartite and novel tripartite binding modes to form efl4E complexes that display differential sensitivity to 4E-BP regulation. Genes Dev. 29: 1835–1849. https://doi.org/10.1101/gad.269068.115

Phornphutkul, C., Y. Anikster, M. Huizing, P. Braun, C. Brodie et al., 2001 The promoter of a lysosomal membrane transporter gene, CTNS, binds Sp-1, shares sequences with the promoter of an adjacent gene, CARKL, and causes cystinosis if mutated in a critical region. Am. J. Hum. Genet. 69: 712–721. https://doi.org/10.1016/S0002-9293(01)02446-1

Qu, W., C. Ren, Y. Li, J. Shi, J. Zhang et al., 2011 Reliability analysis of the Ahringer Caenorhabditis elegans RNAi feeding library: a guide for genome-wide screens. BMC Genomics 12: 170. https://doi.org/10.1186/1471-2164-12-170

Sato, K., A. Norris, M. Sato, and B. D. Grant, 2014 C. elegans as a model for membrane trafficking. WormBook: 1–47. https://doi.org/10.1895/wormbook.1.77.2

Schmidt, K. L., N. Marcus-Gueret, A. Adeleye, J. Webber, D. Baillie et al., 2009 The cell migration molecule UNC-53/NAV2 is linked to the ARP2/3 complex by ABI-1. Development 136: 563–574. https://doi.org/10.1242/dev.016816

Severson, A. F., D. L. Baillie, and B. Bowerman, 2002 A Formin Homology protein and a prolin are required for cytokinesis and Arp2/3-independent assembly of cortical microfilaments in C. elegans. Curr. Biol. 12: 206–2075. https://doi.org/10.1016/S0960-9822(02)01355-6

Shaye, D. D., and J. Greenwald, 2015 The Disease-Associated Formin INF2/ EXC-6 Organizes Lumen and Cell Outgrowth during Tubulogenesis by Regulating F-Actin and Microtubule Cytoskeletons. Dev. Cell 32: 743–755. https://doi.org/10.1016/j.devcel.2015.01.009

Shaye, D. D., and J. Greenwald, 2016 A network of conserved formins, regulated by the guanine exchange factor EXC-5 and the GTPase CDC-42, modulates tubulogenesis in vivo. Development 143: 4173–4181. https://doi.org/10.1242/dev.141861

Sigurbjörnsdóttir, S., R. Mathew, and M. Leptin, 2014 Molecular mechanisms of de novo lumen formation. Nat. Rev. Mol. Cell Biol. 15: 157–168. https://doi.org/10.1038/nrm3871

Simmer, F., M. Tijsterman, S. Parrish, S. P. Koushika, M. L. Nonet et al., 2002 Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. Curr. Biol. 12: 1317–1319. https://doi.org/10.1016/S0960-9822(02)01041-2

Spencer, W. C., G. Zeller, J. D. Watson, S. R. Henz, K. L. Watkins et al., 2011 A spatial and temporal map of C. elegans gene expression. Genome Res. 21: 325–341. https://doi.org/10.1101/gr.114959.110

Strauß, O., C. Muller, N. Reichhart, E. R. Tamm, and N. M. Gomez, 2014 The role of bestrophin-1 in intracellular Ca(2+) signaling. Adv.
Sulston, J. E., and J. Hodgkin, 1988 Methods, pp. 587–606 in *The Nematode Caenorhabditis elegans*, edited by Wood, W. B. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Sulston, J. E., E. Schierenberg, J. G. White, and J. N. Thomson, 1983 The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev. Biol. 100: 64–119. https://doi.org/10.1016/0012-1606(83)90201-4

Sun, Q., Y. Wu, S. Jonusaite, J. M. Pleinis, J. M. Humphreys et al., 2018 Intracellular Chloride and Scaffold Protein Mo25 Cooperatively Regulate Transepithelial Ion Transport through WNK Signaling in the Malpighian Tubule. J Am Soc Nephrol. https://doi.org/10.1681/ASN.2017101091

Sundaram, M. V., and M. Buechner, 2016 The Caenorhabditis elegans Excretory System: A Model for Tubulogenesis, Cell Fate Specification, and Plasticity. Genetics 203: 35–63. https://doi.org/10.1534/genetics.116.189357

Suzuki, N., M. Buechner, K. Nishiwaki, D. H. Hall, H. Nakanishi et al., 2001 A putative GDP-GTP exchange factor is required for development of the excretory cell in Caenorhabditis elegans. EMBO Rep. 2: 530–535. https://doi.org/10.1093/embo-reports/kve110

Thompson, O., M. Edgley, P. Strasbourger, S. Flibotte, B. Ewing et al., 2013 The million mutation project: a new approach to genetics in Caenorhabditis elegans. Genome Res. 23: 1749–1762. https://doi.org/10.1101/gr.157651.113

Timmons, L., and A. Fire, 1998 Specific interference by ingested dsRNA. Nature 395: 854. https://doi.org/10.1038/27579

Tong, X., and M. Buechner, 2008 CRIP homologues maintain apical cytoskeleton to regulate tubule size in C. elegans. Dev. Biol. 317: 225–233. https://doi.org/10.1016/j.ydbio.2008.02.040

Wamelink, M. M., E. A. Struys, E. E. Jansen, E. N. Levchenko, F. S. Zijlstra et al., 2008 Sedoheptulokinase deficiency due to a 57-kb deletion in cystinosis patients causes urinary accumulation of sedoheptulose: elucidation of the CARKL gene. Hum. Mutat. 29: 532–536. https://doi.org/10.1002/humu.20685

Wassmer, T., I. M. Sehring, R. Kissmehl, and H. Plattner, 2008 The V-ATPase in Paramecium: functional specialization by multiple gene isoforms. Pflugers Arch. 457: 599–607. https://doi.org/10.1007/s00424-007-0417-x

Communicating editor: A. Walhout