

A putative GDP–GTP exchange factor is required for development of the excretory cell in *Caenorhabditis elegans*

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The *Caenorhabditis elegans* excretory cell extends tubular processes, called canals, along the basolateral surface of the epidermis. Mutations in the *exc-5* gene cause tubulocystic defects in this canal. Ultrastructural analysis suggests that *exc-5* is required for the proper placement of cytoskeletal elements at the apical epithelial surface. *exc-5* encodes a protein homologous to guanine nucleotide exchange factors and contains motif architecture similar to that of FGD1, which is responsible for faciogenital dysplasia. *exc-5* interacts genetically with *mig-2*, which encodes Rho GTPase. These results suggest that EXC-5 controls the structural organization of the excretory canal by regulating Rho family GTPase activities.

INTRODUCTION

One of the central questions in animal development is how the formation of organ structure during development is controlled. Genetic approaches in model organisms such as the nematode *Caenorhabditis elegans* have been useful in providing new insights into the molecular mechanisms controlling organ structure formation.

The *C. elegans* excretory system is believed to regulate osmoregulation and waste elimination, analogous to the renal organs of higher animals. The excretory cell, and its associated gland, duct and pore cells, form the *C. elegans* renal system (Chitwood and Chitwood, 1974; Nelson *et al.*, 1983; Broeks *et al.*, 1995). The excretory cell is a large ectodermal cell located beneath the posterior pharyngeal bulb, and it extends tube-like

canals dorsally on both sides during embryogenesis (Hedgecock *et al.*, 1987). Upon reaching the lateral epidermis, these canals bifurcate and grow anteriorly and posteriorly for nearly the length of the animal, creating an H-shaped canal system (Figure 1A). The longer, posterior canal reaches epidermoblast V3 by the time of hatching, and V6 by the end of the first larval stage (L1), thus completing its active outgrowth. Active outgrowth at the tip of the canal accounts for less than one-third of the adult canal length. The remaining growth occurs along the length of the canal, as the hypodermal cells, to which the canal is connected by gap junctions, expand. The *C. elegans* excretory cell provides a unique system to study the formation of organ structure in a unicellular epithelium.

In this study, we show that the *exc-5* gene is required for the formation of the excretory canal structure. *exc-5* encodes a protein homologous to guanine nucleotide exchange factors (GEFs) and contains motif architecture similar to that of FGD1, which is responsible for faciogenital dysplasia or Aarskog–Scott syndrome (Pasteris *et al.*, 1994). Our results suggest that EXC-5 controls the structural organization of the excretory canal by regulating activity of Rho family GTPases.

RESULTS AND DISCUSSION

To understand how the formation of the canal structure is determined, mutants with abnormal excretory canals were isolated using the Tc1 mutator strain NL917. One isolate, *km508*, exhibits extremely large septate cysts, formed predominantly at

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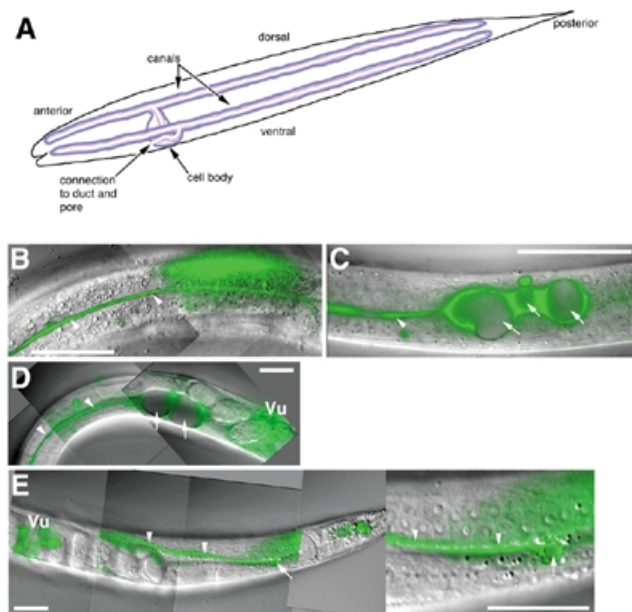


Fig. 1. The *exc-5* gene is required for formation of excretory canals. (A) Diagram showing the position of excretory canals. The apical surface is shown in pink, the basolateral in blue. (B–E) The excretory canal (arrowheads) is visualized using *sek-1::GFP* expression. The photos show posterior excretory canals. Cysts are shown by arrows. Vu, vulva. (B) *unc-119(e2498)* with *sek-1::GFP* and pDP#MM016B (*unc-119*) (Maduro and Pilgrim, 1995). (C) *exc-5(km508)* with *sek-1::GFP* and pRF4 (*rol-6^d*). (D) *exc-5(rh232)* with *sek-1::GFP* and pRF4 (*rol-6^d*). (E) *exc-5(rh232); mig-2(gm38)* with *sek-1::GFP* and pRF4. The right panel shows the excretory canal at high magnification. The cyst (arrow) is much smaller than that in *exc-5(rh232)* single mutants. Scale bars, 20 μ m.

the termini of shortened canals (Figure 1C). We mapped the *km508* allele to the right arm of linkage group IV using STS mapping (Williams *et al.*, 1992). Among the genes in this region lies *exc-5*, previously isolated in a screen for mutations with tubulocystic defects in the excretory canal (Buechner *et al.*, 1999). The similarity in phenotype and map position suggested that the *km508* mutation might be located in the *exc-5* gene. Consistent with this observation, *km508* failed to complement *exc-5(rh232)*.

Electron microscopy analysis revealed that the canals in wild-type animals are embedded in the hypodermis and are spread extensively along the basement membrane that separates the hypodermis from the pseudocoelomic space (Figure 2A). The central lumen is surrounded by electron-dense material, presumably actin-based cytoskeleton (Figure 2B) (Nelson *et al.*, 1983). In a large *exc-5* cyst, the electron-dense cytoskeletal material appears disrupted, and no longer evenly surrounds the lumen. In addition, the lumen swells immensely (Figure 2C). These defects are consistent with a defect of the cytoskeleton at the apical membrane required for organizing cell shape, and raise the possibility that the EXC-5 protein is required for the proper placement of cytoskeletal elements at the apical epithelial surface.

We cloned the *exc-5* gene by transposon tagging. This gene corresponds to the predicted *C33D9.1* gene identified by the *C. elegans* sequencing consortium. A 13.7-kb genomic DNA fragment that contains both regulatory and coding sequences of *C33D9.1* was found to rescue the *exc-5(km508)* mutant phenotype. Analysis of the *exc-5* cDNA indicated that the *exc-5* gene has 15 exons (Figure 3A) and encodes a single protein of 826 amino acids (Figure 3B). To confirm that we had cloned the correct gene, we sequenced the genomic DNA of three *exc-5* mutant alleles: *km508*, *rh232* and *n2672* (Figure 3). *km508* has a Tc1 transposon insertion at Cys758. The *rh232* mutation is a deletion that removes at least the first 12 exons of *exc-5* and thus is presumably a null allele. *n2672* is a nonsense mutation occurring at Trp604.

To determine the expression pattern of EXC-5, we generated a transgene, called *exc-5 5'::GFP*, carrying a predicted *exc-5* promoter fused to the coding region for green fluorescent protein (GFP). *exc-5 5'::GFP* drives GFP expression in the excretory canal (see Figure 4B). We also constructed a gene fusion containing the *exc-5* upstream regulatory sequence and its entire coding region fused to the coding region for GFP. This transgene, *exc-5::GFP*, rescued the *exc-5* phenotype, suggesting that it was functional and expressed in all cells that require *exc-5* activity. GFP fluorescence was observed in the excretory canal (Figure 4A), consistent with the role this protein plays in the formation of canal structure. In addition, *exc-5* expression was also seen in parts of pharyngeal muscles, rectal epithelial cells, and several head and tail neurons.

The EXC-5 protein contains, in order, a Dbl-pleckstrin homology (DH-PH) domain, a cysteine-rich evolutionarily conserved zinc-finger motif termed a FYVE domain, and a second PH domain (Figure 3C). This type of tandem DH-PH domain is conserved among GEFs that act on members of the Rho GTPase family (Cerione and Zheng, 1996). The structural organization of EXC-5 is strikingly similar to that of mammalian FGD1 and Frabin (Figure 3C). FGD1 was determined to be the locus responsible for faciogenital dysplasia or Aarskog–Scott syndrome, a multi-systemic developmental disease affecting skeletal and urogenital systems (Pasteris *et al.*, 1994). Frabin was identified as an F-actin-binding protein (Obaishi *et al.*, 1998). FGD1 and Frabin act as GEFs for the Rho family GTPase Cdc42 (Olson *et al.*, 1996; Zheng *et al.*, 1996; Nagata *et al.*, 1998; Obaishi *et al.*, 1998; Umikawa *et al.*, 1999). Taken together, these results suggest that EXC-5 may function as a GEF for the *C. elegans* Rho family GTPases.

A number of Rho family members have been identified in *C. elegans*, including MIG-2, CED-10/RAC-1, RAC-2, CDC-42 and RHO-1 (Chen *et al.*, 1996a,b; Zipkin *et al.*, 1997; Reddien and Horvitz, 2000). Among them, activated and null mutations in the *mig-2* gene have been identified (Zipkin *et al.*, 1997). To test whether EXC-5 could act as a GEF for GTPases, we examined the genetic interaction between *exc-5* and *mig-2*. Animals with the activated *gm38* allele of *mig-2* exhibit normal canal structure (data not shown). We constructed *exc-5(rh232); mig-2(gm38)* double mutants. Activated MIG-2 partially suppressed the *Exc* phenotype; these animals have longer posterior canals and much smaller cysts than do *exc-5(rh232)* single mutants (Figure 1D and E). This supports the possibility that EXC-5 functions as a GEF for Rho family GTPases in the formation of canal structure. However, unlike *exc-5* loss-of-function mutants, the *mig-2(mu28)* null mutant

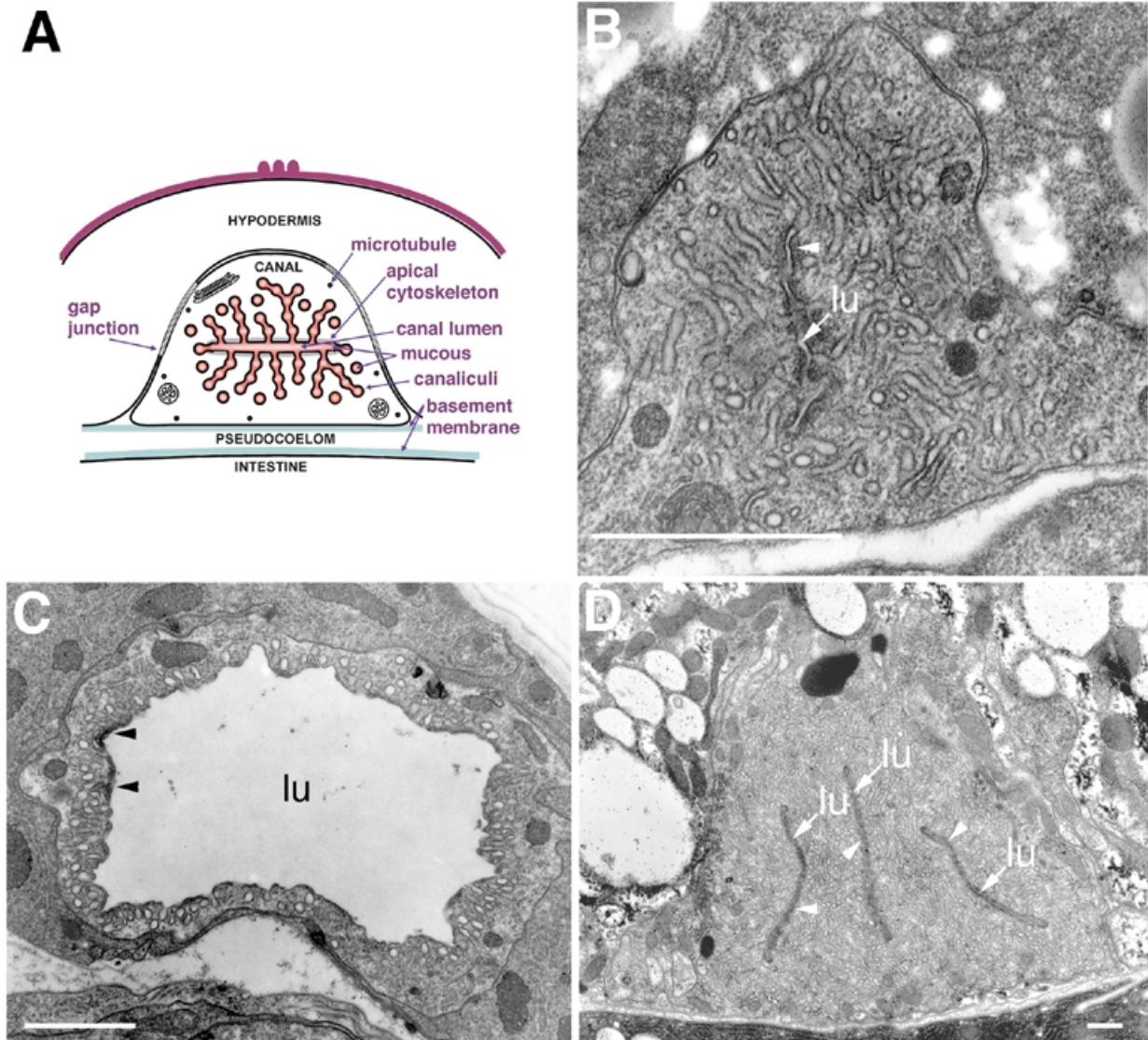


Fig. 2. Electron micrographs of excretory canals. (A) Schematic section showing an excretory canal. (B) Cross-section of the posterior canal in wild-type animals. A band of electron-dense material (arrowhead) decorates the cytoplasmic face of the membrane surrounding the lumen (lu; arrow) scale bar, 1 μ m. (C) Cross-section of a large canal cyst in *exc-5(rh232)* animals. Apical electron-dense material (arrowheads) is irregularly distributed, and no longer evenly surrounds the lumen. The lumen (lu) swells immensely scale bar, 1 μ m. (D) Cross-section of short canal in wild-type animals overexpressing *exc-5::GFP*. Three lumina (lu; arrows) are visible and they are evenly surrounded by electron-dense materials (arrowheads). The canaliculi are much longer than normal. All panels show transverse views, with lateral side at the top. Scale bar, 0.5 μ m.

animals have normal excretory canals (data not shown), suggesting that there are additional GTPase targets for EXC-5 activity that control the structural organization of the excretory canal.

To examine the effect of EXC-5 overexpression on canal structure formation, we generated transgenic animals carrying a high copy array of the *exc-5 5'::GFP* or *exc-5::GFP* genes. Whereas wild-type animals carrying *exc-5 5'::GFP* have longer posterior canals that reach a point in V6 at the L1 stage (Figure 4B), the canal in animals overexpressing *exc-5::GFP* stops at a point between V1 and V2 (Figure 4C). Even at the L4 stage, animals overexpressing *exc-5::GFP* have drastically shortened canal

extension, which contains a convoluted tubule. Furthermore, *exc-5::GFP* was found to be expressed at the apical surface of the cell (Figure 4D). Mosaic animals expressing *exc-5::GFP* only in the excretory cells show a shortened canal phenotype also seen in mosaic animals deficient in canal expression of the basement membrane-binding β 1 integrin (data not shown). GFP fluorescence was visible only in the center of the cell body as streaks representing convolutions of the lumen (Figure 4E). Mosaic animals that express EXC-5::GFP in cells other than the excretory canal show normal canal structure. This suggests that overexpression of EXC-5 acts in a cell-autonomous manner in the formation of abnormal canal structure. Electron microscopy

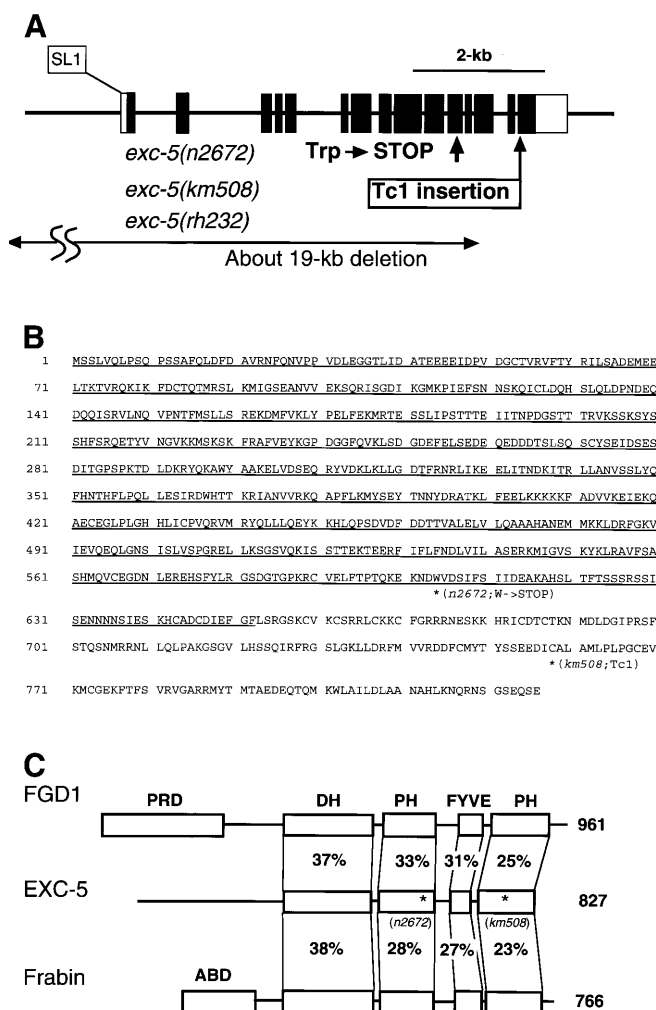


Fig. 3. The *exc-5* gene and protein. **(A)** The exon–intron structure of *exc-5*. This structure is deduced by comparing genomic and cDNA sequences. Boxes represent exons, lines represent introns, and black boxes represent the coding region. The positions and type of the three *exc-5* mutations are indicated. **(B)** Deduced EXC-5 protein sequence. Asterisks indicate the positions of two *exc-5* mutations: stop codon at Trp-604 (TGG-TGA) in *n2672*; Tc1 insertion at Cys758 in *km508*. The region that is deleted in *rh232* is underlined. The DDBJ/EMBL/GenBank accession No. for the EXC-5 sequence is AB060647. **(C)** Comparison of the domain organization among FGD1, EXC-5 and Frabin. The percentage identity compared with EXC-5 is indicated in each of the conserved domains. Asterisks indicate the positions of two *exc-5* mutations, *n2672* and *km508*. PRD, proline-rich domain; ABD, actin-binding domain.

analysis revealed that in animals overexpressing *exc-5::GFP*, the excretory cell body is much larger than normal and exhibits multiple lumina due to convolutions of the luminal surface within the canal (Figure 2D). However, the lumina have an apparently normal structure and are evenly surrounded by electron-dense materials. In addition, in EXC-5-overexpressing canals, the canals have lost some of their attachment to the basement membrane. These results suggest that EXC-5 overexpression causes the apical surface to form normally; tubule convolution results from the failure of canal extension, known to

require basolateral protein function (Hedgecock *et al.*, 1987; Gettner *et al.*, 1995). In contrast to *exc-5* overexpression, the activated *mig-2* mutation does not affect the canal structure. This suggests that EXC-5 may have another activity in addition to the activation of GTPases and that both activities may be required for the phenotypes induced by *exc-5* overexpression. In fact, Frabin possesses Cdc42-activating and F-actin-binding activities, both of which are necessary for microspike formation (Umikawa *et al.*, 1999).

In a developing multicellular organism, dynamic reorganization of the cytoskeleton is essential for many cellular functions, including changes in cell shape, adhesion and motility. Rho family GTPases regulate cytoskeletal reorganization (Hall, 1998). Our results suggest that the EXC-5 protein is involved in the regulation of Rho family GTPase activity. *exc-5* loss-of-function mutations induce the formation of cysts in the excretory canal, whereas overexpression of *exc-5* causes convolutions of the apical surface within the canal. These results raise the possibility that EXC-5 facilitates the formation of the canal structure by regulating the polarized organization of the cytoskeleton on the apical surfaces through activation of Rho family GTPases. Therefore, elucidating the role of *exc-5* in *C. elegans* development should lead to a better understanding of the role GEF-GTPases play more generally in the formation of organ structure, and may shed light on the function of the FGD1 family of proteins involved in signal transduction.

METHODS

Mutant isolation and genetic mapping. *exc-5(km508)* was isolated as a mutant with abnormal excretory canals by visual screening of ~12 000 individuals of the *mut-7(pk204)* strain NL917. *km508* mapped between *stP44* and *stP4* on linkage group IV by PCR STS mapping (Williams *et al.*, 1992).

Cloning of *exc-5*. The genomic sequence corresponding to *exc-5* was identified by use of a transposon display method. The mutant-specific Tc1 insertion was found in the *C33D9.1* gene. The mixture of two cosmids, C33D9 and W07F6, was found to rescue *exc-5(rh232)*, as did a subclone containing only the *C33D9.1* gene. The 5' terminus of the *exc-5* mRNA was analyzed by use of the 5' RACE System for Rapid Amplification of cDNA Ends (Life Technologies). Molecular lesions were identified by direct sequencing of PCR products spanning the *exc-5* gene.

Analysis of canal defects. *Caenorhabditis elegans* hermaphrodites were observed using a Zeiss Axioplan microscope equipped with a Plan 63 objective and differential interference contrast optics. Images were captured with a Hamamatsu Photonics C5810 Color Chilled 3-CCD camera connected to a Macintosh G3 computer. To visualize the excretory canal, a construct expressing GFP under control of the *sek-1* promoter was coinjected with pRF4, which contains the *rol-6^d* gene (Mello *et al.*, 1991). The *sek-1* gene encodes MAP kinase kinase (Kawasaki *et al.*, 1999) and is expressed in excretory canals (Tanaka-Hino and Matsumoto, unpublished result).

Electron microscopy. L4 larvae and young adults were cut through the midbody and fixed immediately in buffered (199 mM HEPES pH 7.5) 3% glutaraldehyde, followed by post-fixation in buffered 1% OsO₄. After encasement in 1% agar, samples were dehydrated and embedded in Polybed 812 resin

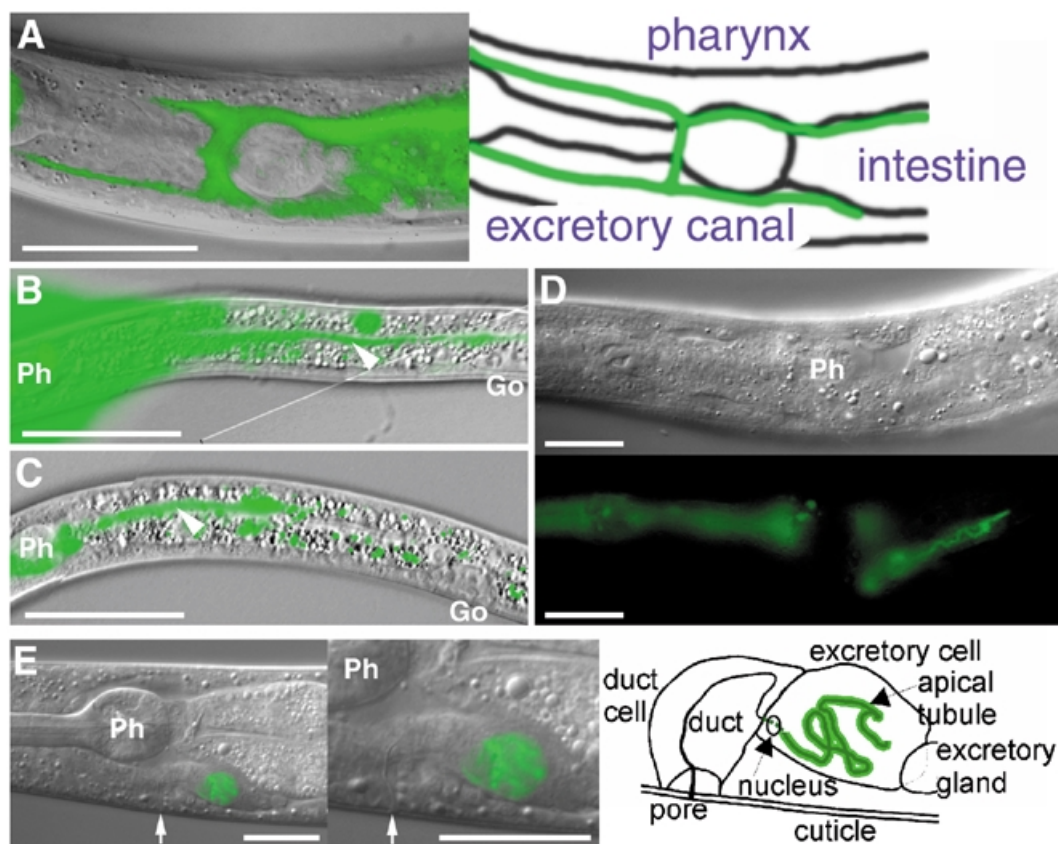


Fig. 4. Effect of *exc-5* overexpression on structure of the excretory canal. (A) Expression of *exc-5* is visualized using *exc-5::GFP* in *exc-5(km508)*. The photo shows the ventral view. The dotted fluorescence results from the autofluorescence of gut granules. (B) L1 *unc-119(e2498)* larva with *exc-5 5'::GFP* and pDP#MM016B (*unc-119*). The excretory canal is visualized using *exc-5 5'::GFP* expression. High fluorescence in pharynx (Ph) is overexposed to show expression extending throughout the narrow canal (arrowhead). Go, gonad. (C–E) The excretory canal is visualized using *exc-5::GFP* expression in *unc-119(e2498)* with *exc-5::GFP* and pDP#MM016B. (C) The photo shows a truncated posterior excretory canal (arrowhead) from a left lateral aspect in L1 larva. (D) The photos show a shortened posterior excretory canal in L4 larva. Upper and lower panels show Nomarski and epifluorescence images, respectively. The animal has a convoluted tubule in the excretory canal. *exc-5::GFP* is predominantly expressed at the luminal (apical) surface of the cell. (E) The photos show a large non-cystic cell body in a mosaic L4 larval animal expressing *exc-5::GFP* in the excretory canal. The middle panel shows the cell body at high magnification. The arrow indicates a pore connecting the excretory canals to the surface of the animal. GFP expression is seen at the apical surface of the cell. The right panel shows a diagram of the middle panel. Scale bars, 20 μ m.

(Polysciences). Serial sections (~70 nm) were post-stained in uranyl acetate followed by lead citrate.

Transgene experiments. To construct a plasmid that contains only the *C33D9.1* gene, the *Sall-PstI* fragment was cloned into the *Sall-PstI* site of the pBSII KS(-) vector. This construct contained 12.5 kb of the upstream sequence, coding regions, and all of the introns of the *exc-5* gene, as well as 1.1 kb of its downstream sequence. The plasmid containing only the *C33D9.1* gene was co-injected into *exc-5(rh232)* with a marker plasmid pEF1a, which contains the EF1a promoter fused to GFP (Kawasaki et al., 1999). To construct the *exc-5::GFP* protein fusion gene, the 12.6 kb fragment from the cosmid clones W07F6 and C33D9 was fused to a *Sall-BamHI* DNA fragment containing *GFP* plus the *unc-54 3' UTR* from pPD95.75 (from A. Fire) using appropriate synthetic oligonucleotides. This construct contained 7.2 kb of the *exc-5* upstream sequence and

5.3 kb of the *exc-5* coding region and all introns. The plasmid containing the *exc-5::GFP* fusion gene was coinjected with pRF4 into *exc-5(km508)*.

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