Facilitation of Endosomal Recycling by an IRG Protein Homolog Maintains Apical Tubule Structure in *Caenorhabditis elegans*

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ABSTRACT Determination of luminal diameter is critical to the function of small single-celled tubes. A series of EXC proteins, including EXC-1, prevent swelling of the tubular excretory canals in *Caenorhabditis elegans*. In this study, cloning of *exc-1* reveals it to encode a homolog of mammalian IRG proteins, which play roles in immune response and autophagy and are associated with Crohn's disease. Mutants in *exc-1* accumulate early endosomes, lack recycling endosomes, and exhibit abnormal apical cytoskeletal structure in regions of enlarged tubules. EXC-1 interacts genetically with two other EXC proteins that also affect endosomal trafficking. In yeast two-hybrid assays, wild-type and putative constitutively active EXC-1 binds to the LIM-domain protein EXC-9, whose homolog, cysteine-rich intestinal protein, is enriched in mammalian intestine. These results suggest a model for IRG function in forming and maintaining apical tubule structure via regulation of endosomal recycling.

KEYWORDS tubulogenesis; trafficking; endosomes; IRG; immunity-related GTPase

SMALL tubules are fundamental structures found in a wide range of tissues in multicellular organisms (Lubarsky and Krasnow 2003; Sigurbjornsdottir *et al.* 2014). Luminal diameter regulation occurs after initial tubule formation; mutations exist in a range of species in which tubes form initially, but cannot maintain their luminal diameter, and change shape over times ranging from minutes for some *Caenorhabditis elegans* mutants to decades for some forms of Schwann

cell degradation (Patzko and Shy 2012). The mechanisms of maintenance of tube diameter as animals age and grow are still relatively unknown. Mechanosensitive channels on primary cilia projecting into the lumen regulate luminal diameter in multicellular tubes such as blood vessels, nephrons, and biliary ducts (Ware *et al.* 2011; Martinac 2014), but narrower single-celled tubules such as those of myelinating Schwann cells do not express cilia on their luminal surface (Yoshimura and Takeda 2012).

The *C. elegans* excretory canal cell provides a tractable genetic model for investigating maintenance of luminal diameter in a seamless single-celled tube (Buechner 2002; Sundaram and Buechner 2016). The excretory canal cell is born in midembryogenesis, forms a hollow lumen, and extends both leftward and rightward to the lateral surface, where each side branches and extends canals both anteriorward and posteriorward to form an "H"-shaped structure (Chitwood and Chitwood 1974; Sulston *et al.* 1983) (Figure 1A). Once formed, the excretory

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Figure 1 Phenotypes of mutants in *exc-1*, encoded by C46E1.3. (A) Diagram showing position of excretory canals (blue) and amphid sheaths (green) in wild-type *C. elegans*; lateral view (anterior on left, dorsal on top). Apical (luminal) surface of excretory canals is shown in red, lumen in white. Lumen connects in the cell body (gray, containing nucleus), to the excretory duct cell (not shown). Canals extend both anteriorward and posteriorward from cell body. (B–J) Micrographs of lateral views of canals. (B–D) Canal marked by stable cytoplasmic *P_{vha-1}::gfp* construct *qpls11* in wild-type animal (B) and in *exc-1(rh26)* animals showing variable effects with mild (C) or severe (D) effects on tube diameter and length. Wide arrows indicate excretory cell body; hollow arrowheads indicate position of vulva at center of animal; narrow arrows indicate fluid-filled cysts. Bars, 100 μ m. (E and F) Micrographs of *exc-1(rh26)* mutant microinjected with rescuing amplified fragment of fosmid WRM0636cA10 containing the promoter and coding region of C46E1.3 DNA and containing the canal cytoplasmic GFP marker *qpls11*. (E) Both anterior and posterior canals are visible on either side of the canal cell body. (F) Posterior canal extends full-length to the tip of the animal and is of normal diameter, indicated between two solid arrowheads. (G) Wild-type animal injected with dsRNA corresponding to the sixth exon of C46E1.3 phenocopies the *exc-1(rh26)* canal, with large cysts (arrows) evident. (H) Injection of transcriptional construct containing 1.6 kb upstream of transcriptional start site linked to *gfp* shows expression in excretory canal cell (wide arrow) and amphid sheath cell, including cell body and amphid sheath (hollow arrows). (I and J) Injection of translational construct of *exc-1::gfp* shows expression concentrated near the apical surface near the canal lumen, and shortens canal length, with normal-diameter folded lumen apparent at terminal end of posterior canal. J is the same as I, sharpened and contrast enhanced to show regions of strongest expression. In E–J, arrowheads show normal-diameter tubule; narrow arrows indicate fluid-filled cysts; hollow arrows indicate amphid sheaths. Bars, 10 μ m. (K–N) Position of EXC-1 relative to marked apical surface in young adult animal. K shows confocal green fluorescence from injected *exc-1::gfp*; L is red fluorescence from injected *erm-1::mCherry* in same section; and M is DIC (contrast enhanced) of same section, with apical edges of lumen marked in red. N shows average brightness of fluorescence along horizontal lines within area boxed in K–M, starting outside of canal on the dorsal side and continuing ventrally. Position of lumen derived from DIC image is labeled in graph.

canals must continue to grow along with the animal. The diameter is tightly controlled, as the lumens taper toward their closed-tip distal ends and expand as the animal ages (Nelson *et al.* 1983; Sundaram and Buechner

2016). The luminal membrane is surrounded by a thick terminal web similar in appearance to that of intestinal cells and is rich in vacuolar ATPase (Nelson *et al.* 1983; Oka *et al.* 1997).

In a series of *exc* mutants, the excretory canals form normally initially, but lose the ability to regulate luminal diameter; the terminal web breaks or becomes separated from the apical membrane, and the lumen swells into fluid-filled cysts (Buechner *et al.* 1999). Cloned *exc* genes encode cytoskeletal proteins and proteins that anchor the terminal web to the apical membrane (Göbel *et al.* 2004; Praitis *et al.* 2005; Kolotuev *et al.* 2013; Shaye and Greenwald 2015), regulate ionic and fluid movement in the canal lumen (Berry *et al.* 2003; Liegeois *et al.* 2007; Hisamoto *et al.* 2008; Khan *et al.* 2013), and regulate movement of messenger RNA (mRNA) and endosomes within the cell (Fujita *et al.* 2003; Tong and Buechner 2008; Mattingly and Buechner 2011). Similar defects in single-cell tube maintenance have also been described for the *C. elegans* excretory duct cell (Stone *et al.* 2009; Sundaram and Buechner 2016), which initially forms as an autocellular sealed cell whose lumen connects to the seamless excretory cell lumen (Nelson *et al.* 1983).

The *EXC-5* guanine exchange factor (GEF) regulates endocytic recycling (Gao *et al.* 2001; Mattingly and Buechner 2011). Alleles of *exc-5* show epistatic effects on *exc-9* mutants, which suggests that *EXC-5* works downstream of the *EXC-9* LIM-domain protein to regulate movement between early and recycling endosomes (Mattingly and Buechner 2011). In *exc-5* mutants, early endosome markers accumulate in regions of the canal prior to formation of cysts, while recycling endosome markers are largely absent in cystic regions. The *EXC-5* GEF is homologous to the mammalian FGD family, including human FGD4, which is essential for maintaining Schwann cell structure and is the locus of Charcot-Marie-Tooth syndrome type 4H (Delague *et al.* 2007; Stendel *et al.* 2007; Fabrizi *et al.* 2009). *EXC-9* is homologous to mammalian cysteine-rich intestinal proteins (CRIPs), whose biochemical function is unclear (Birkenmeier and Gordon 1986; Cousins and Lanningham-Foster 2000; Lanningham-Foster *et al.* 2002; Tong and Buechner 2008). These two proteins appear to function in a pathway to help recycle apical surface material essential for small tubes to adapt to growth and bending (Mattingly and Buechner 2011).

Mutants in the *exc-1* gene exhibit canal phenotypes similar to those of *exc-5* mutants (Buechner *et al.* 1999). In addition, overexpression of *exc-5* prevents cyst formation and causes “convoluted tubules” in both *exc-1* mutants and *exc-9* mutants (Tong and Buechner 2008; and data in Table 2), while overexpression of *exc-9* had no effect on the cystic canals of *exc-1* mutants (Tong and Buechner 2008). Finally, *exc-1*; *exc-5* double mutants show the same phenotype as *exc-5* single mutants (Buechner *et al.* 1999) and *exc-9*; *exc-1* and *exc-9*; *exc-5* doubles show the same phenotype as *exc-9* single mutants (Tong and Buechner 2008). These genetic interactions suggest that *EXC-1* is an intermediary in a single pathway between *EXC-9* and *EXC-5*.

We report here the cloning of *exc-1* and find that it encodes a large protein with two GTPase-like domains both homologous to those of the mammalian IRGC protein (Bekpen *et al.* 2005), a member of the IRG (immunity-related

GTPase) family of proteins (Martens and Howard 2006; Petkova *et al.* 2012) involved in autophagy and response to intracellular parasites (Gazzinelli *et al.* 2014). Loss of *exc-1* causes effects on endosome marker expression similar to those found for *exc-5*. Yeast two-hybrid assays show that wild-type *EXC-1* binds directly to *EXC-9*, and epistasis experiments confirm that *EXC-1* operates downstream of *EXC-9* but upstream of *EXC-5*. Our results support a model whereby these three *EXC* proteins function coordinately in a pathway to regulate the recycling of apical luminal membrane in response to signals that form and maintain the shape of narrow tubules.

Materials and Methods

Nematode genetics and genetic mapping

C. elegans strains (Table 1) were maintained by use of standard culture techniques on lawns of *Escherichia coli* strain BK16 (a streptomycin-resistant derivative of strain OP50) grown on NGM plates (Sulston and Hodgkin 1988). Strains were kept at 20° and phenotypic analyses were carried out on L4 larvae or young adults. The strains and alleles *RB5001 C46E1.3(ok5478)* and *SP1713 dyf-11(mn392)* were supplied by the *Caenorhabditis* Genetics Center, Minneapolis. *exc-1(ok5478)* was generated by the *C. elegans* knockout project (Flibotte *et al.* 2010).

By means of complementation tests and deficiency mapping, *exc-1* was previously mapped to the right end of the X chromosome to a region of 282 kb between genes *jud-4* and *dyn-1* (Buechner *et al.* 1999). Double strand RNA (dsRNA) constructs were produced via PCR amplification of *N2* DNA corresponding to a region of ~300 bp in size to exons in determined genes and the n transcribed by use of the MEGA-script T7 kit (Life Technologies, Grand Island, NY). Plasmid and RNA interference (RNAi) were injected together with plasmid pCV01, which expresses GFP in the excretory canal cell under control of the promoter of *vha-1*. Injection mixtures were microinjected into worms as described (Mello and Fire 1995). Injection of dsRNA corresponding to >30 genes in this region did not cause progeny of injected wild-type animals to develop fluid-filled canal cysts, while microinjection of dsRNA corresponding to the predicted gene *C46E1.3* into wild-type worms phenocopied *exc-1(rh26)* mutants (Supplemental Material, Table S1). A corresponding fragment amplified from fosmid *WRM0636cA10* (Source BioScience, Cambridge, UK) via PCR with primers 5' CCACGTCAGAAAAGAAATATTCTCGCTCCG 3' and 5' TCACATGTTTCATCAAGCGGAAAAGTTCACT 3' contained 1.6 kb upstream through 0.5 kb downstream of predicted gene *C46E1.3*. The amplified region was ligated into pCR-XL-TOPO vector (Life Technologies) and microinjected at 25 ng/μl into *exc-1(rh26)* mutants together with the canal marker plasmid pCV01 (containing a *P_{vha-1}::gfp* construct) at 50 ng/μl. Progeny of the injected animals were rescued, as they exhibited wild-type canal morphology.

Table 1 Strains used for this study

Strain	Genotype	Description	Reference
N2		Wild type	Brenner 1974
NJ51	<i>exc-1 (rh26)</i>	Nonsense mutation	Buechner <i>et al.</i> 1999
BK36	<i>unc-119(ed3); qpls11[unc-119; P_{vha-1}::gfp] I</i>	Wild type with integrated GFP marker expressed in excretory canals	Tong and Buechner 2008
BK258	<i>exc-1(rh26); qpls11[unc-119; P_{vha-1}::gfp] I</i>	NJ51 crossed to BK36, with integrated GFP marker in an <i>exc-1</i> mutant background	This study
NJ731	<i>exc-5(rh232)</i>	<i>exc-5</i> deletion	Suzuki <i>et al.</i> 2001
MT6984	<i>exc-9(n2669)</i>	Nonsense mutation	Tong and Buechner 2008
—	<i>nsls53 [P_{vap-1}::dsRED::unc-119]</i>	Wild type with integrated dsRED marker expressed in amphid sheath	Procko <i>et al.</i> 2011
BK473	<i>exc-1(rh26); nsls53[P_{vap-1}::dsRED::unc-119]</i>	<i>nsls53</i> strain crossed to NJ51, with integrated dsRed amphid sheath marker in an <i>exc-1</i> background	This study
RB5001	<i>exc-1(ok5478)</i>	Nonsense mutation	Flibotte <i>et al.</i> 2010
SP1713	<i>dyf-11(mn392)</i>	Nonsense mutation	Starich <i>et al.</i> 1995
VJ552	<i>fgEx41[P_{erm-1}::erm-1::mCherry; rol-6(su1006)]</i>	Labels ERM-1 at apical surfaces of excretory canals and intestine	Khan <i>et al.</i> 2013
Strains with integrated canal cytoplasmic markers and expressing integrated mCherry-labeled marker genes			
BK280	BK36; <i>qpls102[P_{exc-9}::mCherry::chc-1]</i>	Labels CHC-1: clathrin-coated pits	This study
BK282	BK36; <i>qpls95[P_{exc-9}::mCherry::eea-1] X</i>	EEA-1: early endosomes	This study
BK281	BK36; <i>qpls99[P_{exc-9}::mCherry::rab-5] IV</i>	RAB-5: early endosomes	This study
BK283	BK36; <i>qpls100[P_{exc-9}::mCherry::rab-7]</i>	RAB-7: late endosomes	This study
BK284	BK36; <i>qpls98[P_{exc-9}::mCherry::glo-1]</i>	GLO-1: lysosomes	This study
BK286	BK36; <i>qpls101[P_{exc-9}::mCherry::rme-1] X</i>	RME-1: recycling endosomes	This study
BK285	BK36; <i>qpls97[P_{exc-9}::mCherry::rab-11.1] V</i>	RAB-11.1: recycling endosomes	This study
BK287	BK36; <i>qpls103[P_{exc-9}::mCherry::GRIP]</i>	GRIP: Golgi apparatus	This study
BK288	BK36; <i>qpls96[P_{exc-9}::mCherry::cdc-42]</i>	CDC-42: cytoplasm	This study
<i>exc-1</i> deletion strains with integrated cytoplasmic marker and expressing integrated mCherry-labeled markers			
BK290	BK258; <i>qpls102[P_{exc-9}::mCherry::chc-1]</i>	CHC-1: clathrin-coated pits	This study
BK292	BK258; <i>qpls95[P_{exc-9}::mCherry::eea-1] X</i>	EEA-1: early endosomes	This study
BK291	BK258; <i>qpls99[P_{exc-9}::mCherry::rab-5] IV</i>	RAB-5: early endosomes	This study
BK293	BK258; <i>qpls100[P_{exc-9}::mCherry::rab-7]</i>	RAB-7: late endosomes	This study
BK294	BK258; <i>qpls98[P_{exc-9}::mCherry::glo-1]</i>	GLO-1: lysosomes	This study
BK296	BK258; <i>qpls101[P_{exc-9}::mCherry::rme-1] X</i>	RME-1: recycling endosomes	This study
BK295	BK258; <i>qpls97[P_{exc-9}::mCherry::rab-11.1] V</i>	RAB-11.1: recycling endosomes	This study
BK297	BK258; <i>qpls103[P_{exc-9}::mCherry::GRIP]</i>	GRIP: Golgi apparatus	This study
BK298	BK258; <i>qpls96[P_{exc-9}::mCherry::cdc-42]</i>	CDC-42: cytoplasm	This study

DNA constructs and sequence analysis

The 1.6-kb region predicted to contain the *exc-1* promoter was amplified from N2 DNA, with the upstream primer 5' GCGTCGGATCCTCCTAAAAAATTCAGTTGAA 3' and primer 5' CCGCCGGATCCTCATCAAAAATTTATTATCC 3' at the transcription start site. This PCR-amplified product was then cut with *Bam*HI and ligated into the backbone of plasmid L3691 to make construct pBK101.

The mRNA from N2 animals was isolated via the Magnetic mRNA Isolation Kit (New England Biolabs, Ipswich, MA), reverse transcribed to complementary DNA (cDNA) with Finnzymes' Phusion RT-PCR Kit (New England Biolabs), and PCR amplified with primers to various sections of the *exc-1* gene. Primers to the 5' end of the coding region: 5' CACCATGGGACACAAAACCTC 3' and 5' CTACCCACTTCTCCACAAAATCC 3'; to the first Ras-like domain: 5' CACCGGATTTTGTGGAAGAAGTGGGT 3' and 5' CTAGTTTCTCGGTTTTCTGCGT3'; to center: 5' CACCCTCCTAAC

AAAAAGCGAC 3' and 5' CTATCTTCCTCCAATAAATCCG; and to the second Ras-like domain and 3' end of the coding region: 5' CACCACATGCTTCAACTACGGA 3' and 5' TCAATGAATCCGGCTGTATCTAG 3' were used.

The cDNA corresponding to the *exc-1* gene was isolated and cloned into the plasmid pCV01 (containing *P_{vha-1}::gfp*), to make a translational construct that contained *P_{vha-1}::exc-1cDNA::gfp*. This construct was microinjected into worms at 25 ng/μl. Putative constitutively active (ca) (G250V and G255V) and dominantly negative (dn) (S257N) changes to the cloned *exc-1* gene (not linked to *gfp*) were produced through use of the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Stratagene, Santa Clara, CA). These constructs were microinjected at 25 ng/μl into both wild-type and *exc-1(rh26)* animals in combination with the canal marker plasmid pCV01 at 50 ng/μl. For location studies, this construct was microinjected at 10 ng/μl into strain VJ552, which expresses *erm-1::mCherry* at the luminal surface of the canal.

The predicted protein sequence of *EXC-1* was analyzed via BLAST on the PubMed servers and compared to human IRGC (GenBank no. EAW57222). The structures of *EXC-1*, and of individual domains of *EXC-1*, were predicted from the I-TASSER threading program (Roy *et al.* 2010) on the Zhang Lab server (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). Structure of the *EXC-1* GTPase-like domains were visually merged and compared via pairwise sequence alignments with the structure of the IRG GTPase domains via the Chimera Program server at University of California, San Francisco (Pettersen *et al.* 2004).

Microscopy

Live worms were mounted on 2% agarose pads with added 10 mM muscimol or 0.5% 1-phenoxy-2-propanol as an anesthetic, as described previously (Sulston and Hodgkin 1988). Images were captured with a MagnaFire Camera (Optronics) on a Zeiss Axioskop microscope with Nomarski optics. Images were also taken on an FV1000 laser scanning confocal microscope (Olympus), with lasers set to 488-nm excitation and 520-nm emission (GFP) or 543-nm excitation and 572-nm emission (mCherry). Images were captured via FluoView optics (Olympus) and analyzed with ImageJ software.

For electron microscopy, L4 larvae and young adults were cut in midbody and fixed immediately in buffered (100 mM Hepes, pH 7.5) 3% glutaraldehyde, followed by postfixation in buffered 1% OsO₄ (Sulston and Hodgkin 1988; Hall 1995). After encasement in 1% agar, samples were dehydrated and embedded in Polybed 812 resin (Polysciences). Serial sections, ~70 nm, were poststained in uranyl acetate followed by lead citrate. It should be noted that dehydration for this fixation can slightly shrink the size of the liquid-filled lumen, as compared to fixation via microwave or high-pressure freezing/freeze substitution, but allowed clear resolution of the canaliculi and supporting cytoskeleton.

Dye-filling assays

L4 and adult worms were exposed to the dyes DiI or DiO according to standard protocol (Hedgecock *et al.* 1985; Perkins *et al.* 1986) and observed via fluorescence microscopy. As control, *N2* worms take up both dyes, while the *dyf-11(mn392)* mutant is unable to absorb these lipophilic dyes into the amphid neural cell bodies (Starich *et al.* 1995; Bacaj *et al.* 2008).

Yeast two-hybrid assays

Yeast two-hybrid assays were performed according to recommended procedures for the DupLEX-A Yeast Two-Hybrid System (OriGene Technologies, Rockville, MD). The cDNA sequences of entire or fragments of wild-type *exc-1*, and versions of *exc-1* carrying either a putative ca or putative dn mutation in the first Ras-like domain, as well as cDNA sequences of *exc-5* and *exc-9*, were cloned into the Gateway entry vector pENTR (Life Technologies). The Gateway LR recombination was used to move the genes into pEG202 (modified to contain an LR recombination site, gift of B. Grant)

in order to fuse the protein to the LexA DNA-binding domain, and into pJG4-5 in order to fuse the protein to the activation protein acid-blob domain B42 fused to an HA tag.

Various forms of bait (pEG202) and prey (pJG4-5) plasmids were introduced into reporter strain EGY48, containing the *lacZ* reporter gene plasmid pSH18-34. To test for protein interactions and expression of the LEU2 reporter, transformants were grown on plates that contained minimal medium containing galactose and raffinose, but lacking uracil, histidine, tryptophan, and leucine, at 30° for 2–3 days. Controls of prey or bait plasmids alone showed no growth. As a positive control for the presence of all constructs, the strains were grown on medium lacking only uracil, histidine, and tryptophan (data not shown). The reactions were tested in all possible combination constructs of bait and prey plasmids.

Canal measurements

Measurement of excretory canal length and determination of cystic phenotype were carried out as described (Tong and Buechner 2008). Canal length was scored by eye on a scale from 0 to 4: A score of 4 was given if the canals had grown out to full length; canals that extended past the vulva but not full length were scored as 3 and at the vulva, 2; canals that ended between the cell body and the vulva were scored as 1; and if the canal did not extend past the cell body, the canal was scored as 0. If the canal failed to extend past the cell body, but the apical tubule remained narrow and traversed upon itself more than once, it was counted as a convoluted canal and scored as 4. Cyst size was measured by count and by assessment of size relative to normal canal width. Cysts wider than a normal canal up to one-quarter the diameter of the worm were scored as small; cysts between one-quarter diameter and half the diameter of the worm were scored as medium; and any cysts larger than half the diameter of the worm were scored as large. In cases where cyst diameter was ambiguous relative to worm size, photographs of the canal were analyzed via National Institutes of Health software ImageJ (<http://imagej.nih.gov/ij/>). Statistical analyses of canal length were conducted as previously described (Tong and Buechner 2008): Canals were binned into three categories for length (scores 0–1, scores 2–3, and score 4), and again for cyst size (none, small, and binned medium and large). The results were then analyzed via a 3 × 2 Fisher's exact test. *P*-values of 0.000001 (1×10^{-6}) or lower were regarded as strong statistical significance of an effect on the canal; scores between 0.000001 and 0.0001 were regarded as a partial effect.

Subcellular marker measurements

For analysis of effects of *exc* mutation on subcellular marker proteins, strains carrying different integrated subcellular markers (Mattingly and Buechner 2011) were crossed to *exc-1(rh26)* worms and to *exc-9(n2669)* worms. F₂ progeny expressing both the mutant phenotype and the fluorescent marker were isolated and maintained as a homozygous strain of both the subcellular marker and *exc-1(rh26)* or

Figure 2 Ultrastructure of *exc-1(rh26)* excretory canal. TEM of posterior distal canal in wild-type (A) and *exc-1(rh26)* mutant (B–E) young adult animals. (A) Wild-type distal canal shows canaliculi (C) in a subapical domain surrounding the apical surface of the central lumen (Lu). In addition, various microtubules and mitochondria, as well as other vesicles not coated with canaliculi material, surround the subapical domain. (B) *exc-1(rh26)* mutant canal in regions without cysts appears similar to wild type, although canaliculi may be somewhat enlarged. (C) In a region with enlarged lumen (Lu) and cyst evident (Cyst), there are many more vesicles in a greater range of diameters and irregular shapes. (D and E) Enlargements of C more clearly show electron-dense terminal web-like material (arrows) surrounding the cyst lumen where canaliculi remain attached to the lumen, but missing in regions (arrowheads) lacking such canaliculi. Ropy electron-dense material in E appears similar to terminal web-like material, but is no longer adjacent to cellular lumen nor canaliculi. C, canaliculi and tubules; ER, rough endoplasmic reticulum; Lu, large central lumen; M, microtubules; Mt, mitochondria. All figures contrast enhanced for clarity. All bars, 0.5 μ m.

exc-9(n2669) alleles. ImageJ was used to analyze the brightness and locations of the subcellular markers in cystic and noncystic areas of the excretory canals. *exc-1* mutant worms selected for analysis had a region of noncystic canal for comparison to the cystic region located at the ends of the tubules. A segmented line was created along the length of the tubule with line width sufficient to cover the largest cysts. A plot profile was recorded for this segmented line; from each value was subtracted the average value of a plot profile for a dark background outside the worm in that micrograph. To normalize the values, a region of noncystic, normal-looking canal was then assigned a brightness level of 100. *exc-9* mutant worms usually have large cysts throughout the canal length, so a section of normal canal diameter was not always present to use as a normalization control.

For determination of apical–basal location of *EXC-1* relative to apical *ERM-1*, animals containing relatively low levels of both genes (canal length and width were not affected) were analyzed. ImageJ was used to draw a straight line perpendicular to the length of the canal with wide line width spanning a section of canal with uniform diameter; a DIC image was used to determine position of the lumen. Fluorescence plot profiles were then recorded and analyzed as above.

Reagent and data availability

All strains used in this study are listed in Table 1. The *exc* mutant strains have been deposited with the *Caenorhabditis*

Genetics Center (CGC) (www.cbs.umn.edu/cgc) at the University of Minnesota. Marker strain BK36 for the excretory canal cytoplasm and wild-type strains marked for endosomal compartments in the excretory canal are also being deposited with the CGC. Other strains are available upon request.

Results

The *exc-1* mutation causes alterations in canal and amphid sheath structure

The *exc-1* gene was identified through a mutation that alters the shape and size of the excretory canal lumen (Buechner *et al.* 1999). Normally, the canal stretches the entire length of the organism with a narrow lumen that tapers from a maximum of $\sim 5 \mu$ m near the cell body to $<1 \mu$ m wide at the distal tips (Figure 1, A and B). In the *exc-1(rh26)* mutant, the canals exhibit a variable morphology, with canals terminating in fluid-filled cysts of varying size, from near-normal tubule diameter to cysts as wide as the entire animal (Figure 1, C and D). In many *exc-1* mutant animals, cysts are most evident at the distal tips of the canals and at the cell body. The length of the canals also varies considerably in *exc-1(rh26)* animals, with posterior canals stretching in some cases to near-normal length, while mutants with larger cysts tend to have much shorter canals, not reaching the vulva in the center of the animal.

The lumen of the wild-type excretory canal is surrounded by small canaliculi that appear, at the ultrastructural level, as

uniform-size vesicles or tubules that are often connected to each other and to the canal lumen (Nelson *et al.* 1983; Buechner *et al.* 1999; Kolotuev *et al.* 2013) (Figure 2A). Canalicular vesicles and canaliculi, unlike endosomes and vesicles, are surrounded by electron-dense material that is likely made up of vacuolar ATPase (Kolotuev *et al.* 2013). Regions of *exc-1* mutant canals where cysts are not evident appear normal in electron micrographs (Figure 2B). In regions of mutant canals where cysts are visible (Figure 2, C–E), canaliculi appear disordered and canalicular vesicles have less regular shapes (Figure 2D), although the number of vesicles appears normal. Serial thin sections starting at the large cyst show that its lumen is continuous with lumen in areas with normal lumen diameter. The diameter of canaliculi visible in cystic areas is less uniform than in wild-type animals, and the thick electron-dense material that normally surrounds the central lumen is disrupted, with cystic areas often lacking this material. In some thin sections, electron-dense material appears in ropy filaments in the cytoplasm (Figure 2E). Finally, there are many uncoated vesicles of various sizes among and surrounding the canaliculi.

Expression of *exc-1* was seen reliably solely within the canals and within the amphid sheath cells (Figure 1 and Figure 3). Sheath cell expression was not as strong as in the canal, but also persisted throughout the lifetime of the animal. Subsequent examination of *exc-1(rh26)* animals showed that these mutants exhibit occasional small cysts in the long processes of the amphid sheath cells, but these did not seem to prevent the amphid sheath from properly wrapping around the amphid neurons (Figure 3, B and D). Dye-filling assays (Perkins *et al.* 1986; Tong and Burglin 2010; Schultz and Gumienny 2012) using both DiO and DiI stains showed that the amphids were open to the outside and able to take up dye; therefore, the amphid channel is not occluded by the amphid sheath in *exc-1* mutants.

The *exc-1* gene was previously mapped between the *jud-4* and *dyn-1* genes on the right end of the X chromosome (Buechner *et al.* 1999), a region encompassing 31 predicted genes and covered by 11 fosmids (www.wormbase.org) (*Caenorhabditis elegans* Sequencing Consortium 1998). Microinjection of the PCR-amplified fragment of the predicted gene C46E1.3 from fosmid WRM0636cA10 rescued the *rh26* cystic phenotype (Figure 1, E and F), while microinjection of dsRNA corresponding to the predicted sixth exon of C46E1.3 into wild-type animals phenocopied *rh26* excretory canals (Figure 1G and Table 2). An extrachromosomal array containing 1.6 kb of the presumptive C46E1.3 promoter linked to *gfp* is strongly expressed both in the excretory canal cell and in the glial amphid sheath cells (Figure 1H). Expression within the canal cell begins soon after the cell is born and persists throughout the lifetime of the animal. A translational construct expressing C46E1.3 cDNA linked to GFP solely within the canal and head mesodermal cell (driven by the strong canal promoter *Pvha-1*) is strongly expressed within the canal cytoplasm (Figure 1, I and J). This canal-specific construct rescued the canal phenotype, which strongly sug-

Figure 3 *exc-1* mutation allows formation of small cysts in amphid sheath processes. (A–D) Animals stained with DiO (A and B) or DiI (C and D) to indicate opening of amphids to environment in L4 animals. A and C show wild-type controls; B and D show *exc-1(rh26)* animals. Fluorescence image in D (contrast enhanced) is overlaid on DIC image showing abnormal excretory canal cysts at distal end of anterior canal (arrow). (E–H) Expression of stable amphid glia-marking array *Pvap-1::dsRED* in a wild-type animal (E and G) and in an *exc-1(rh26)* mutant (F and H). Arrows show position of cystic enlargements in amphid sheath cell process. G and H show enlargement of boxed areas of E and F, respectively. All bars, 10 μ m. Fluorescence in all panels brightened to show narrow amphid sheath processes and cysts.

gests that the gene functions cell autonomously. Expression of the same rescuing construct in animals expressing the apical-specific protein ERM-1 linked to mCherry (Figure 1, K–N) showed that EXC-1 expression overlaps that of ERM-1, but is present throughout the canal cytoplasm (Figure 1N).

We cloned cDNA corresponding to the wild-type *exc-1* gene, and found that the sequence of this clone largely matches the computer-predicted structure on WormBase (www.wormbase.org, version WS247), with the exception

Table 2 Effects of *exc-1* expression on excretory canal phenotype

Strain/genotype	N ^d	% Conv. ^e	Canal lumen length ^a			Overall cyst size ^b			P-value ^c	
			% No growth + short	% Mid-length	% Full-size + Conv.	% None	% Small	% Med + large	Lumen length	Cyst size
N2 (wild type) animals	100	0	0	0	100	100	0	0	Compared to wild type:	
N2 injected with wt <i>exc-1</i>	26	69*	0	0	100	100	0	0	1.00	1.00
N2 injected with Pro-rich domain	38	0	0	0	100	100	0	0	1.00	1.00
N2 injected with <i>exc-1(dn)::gfp</i>	50	0	26	50	24	10	80	10	<0.000001*	<0.000001*
N2 injected with <i>exc-1(ca)::gfp</i>	50	0	0	12	88	100	0	0	0.0011	1.00
<i>exc-1(rh26)</i>	100	0	29	64	7	1	55	44	Compared to <i>exc-1(rh26)</i>	
<i>exc-1(ok5478)</i>	33	0	39	30	30	0	33	67	0.39	0.133
<i>rh26</i> injected with <i>exc-1::gfp</i>	66	94*	0	0	100	100	0	0	<0.000001*	<0.000001*
<i>rh26</i> injected with <i>exc-1(dn)::gfp</i>	50	0	12	82	6	0	82	18	0.0621	0.0004
<i>rh26</i> injected with <i>exc-1(ca)::gfp</i>	50	0	0	12	88	92	8	0	<0.000001*	<0.000001*
<i>rh26</i> injected with <i>exc-5::gfp</i>	28	46*	32	14	54	57	21	21	<0.000001*	<0.000001*
<i>exc-5(rh232)</i>	105	0	79	19	2	0	17	83	Compared to <i>exc-5(rh232)</i> :	
<i>rh232</i> injected with <i>exc-1::gfp</i>	57	0	79	21	0	0	35	65	0.719	0.0122
<i>exc-9(n2669)</i>	54	0	74	26	0	0	61	39	Compared to <i>exc-9(n2669)</i> :	
<i>n2669</i> injected with <i>exc-1::gfp</i>	41	26*	0	17	83	78	22	0	<0.000001*	<0.000001*

Conv., convoluted.

^a Canal lengths, as described in *Materials and Methods*; a convoluted canal was counted as having a full-length lumen.

^b Percentage of canals examined with cysts primarily of size denoted.

^c P-value from Fisher's exact test, probability of not being different from denoted comparison strain. Values in boldface type labeled with asterisk indicate significant differences.

^d No. of canals examined.

^e Percentage of canals exhibiting a lumen that traversed itself at or near the cell body more than once.

that the second exon is shorter than predicted (Figure 4A); we do not know if this is a single splice variant, but injection of this clone fully rescued the *rh26* mutant. Sequencing of the *rh26* allele revealed a nonsense mutation in the 11th predicted exon (Figure 4A). Multiple variants of *C46E1.3* have been found by various whole-genome sequencing projects and by the *C. elegans* knockout project. The allele *ok5478* in strain *RB5001* (Flibotte *et al.* 2010) contains a nonsense mutation in the predicted 4th exon. This mutation also causes the canals to exhibit large cysts to the same extent as those of the *rh26* allele (Table 3), and *rh26* was previously shown to exhibit the same phenotype when placed in *trans* to a large deficiency (Buechner *et al.* 1999). *rh26* therefore appears to represent a null allele of this gene. Mutations in alleles *ok5960*, containing a substitution within a predicted intron, and in alleles *gk303482*, *gk303483*, *gk303484*, *gk303492*, *gk303494*, and *gk303495*, which contain missense mutations in the predicted protein sequence, caused no obvious effect to the excretory canals. Between genetic location, gene rescue, RNAi phenocopy, sequencing of mutant alleles, and expression pattern, we conclude that *C46E1.3* encodes *EXC-1*.

exc-1 encodes a homolog to mammalian IRG proteins

The *exc-1* gene encodes a large (736 amino acid) protein with a proline-rich N terminus (Figure 4A) and two regions that both show homology to the IRG family of mammalian proteins (Howard 2008). *EXC-1* is the sole nematode homolog to IRG proteins. These proteins bind to various internal membranes, including endosomes, Golgi, and autophagosomes, depending on the specific protein (Martens and Howard

2006; Pilla-Moffett *et al.* 2016). Both of the two *EXC-1* domains show the closest homology to the single Ras-like domain of the IRG protein (Figure 4B) (Bekpen *et al.* 2005), in that they clearly contain the characteristic GTP- and GEF/GAP-binding G1, G2, and G4 boxes of Ras, with lower homology to the G3 conformational change (switch II) domain and G5 guanine interaction domains. The first Ras-like domain has closer overall homology to IRG than does the second. Alignment via the TASSER program (Roy *et al.* 2010) of the two *EXC-1* Ras-like domains showed strong structural homology, with a root-mean-squared deviation (rmsd) from the crystal structure of IIGP (Ghosh *et al.* 2004) of 1.51 Å for the first Ras domain and 1.27 Å for the second. *EXC-1* lacks a homodimerization domain found in many mammalian IRG proteins (Ghosh *et al.* 2004); this domain might presumably be superfluous for *EXC-1*, as its two Ras domains are physically linked. The linker region of *EXC-1* contains a region of homology to a ubiquitous eukaryotic enzyme, porphobilinogen deaminase (Gill *et al.* 2009; Layer *et al.* 2010).

Since *exc-1* shows genetic interactions with *exc-5* and with *exc-9* (Tong and Buechner 2008; Mattingly and Buechner 2011), we measured interactions of these proteins via yeast two-hybrid assays. Wild-type *exc-1* was expressed on yeast plasmids pEG202 and pJG4-5, as were engineered mutants that contained the putative ca G249V, G254V or dn S257N forms of the first Ras-like domain of the protein. The mutations are homologous to similar mutants in the Ras-like domains of other IRG proteins (Kaiser *et al.* 2004; Hunn *et al.* 2008). No form of *EXC-1* showed interactions with *EXC-5* nor to endosomal proteins *RME-1* or *EEA-1* (data not shown). In

Figure 4 Structure of the *exc-1* gene and protein. (A) Structure of the *exc-1* coding region and encoded protein. Major domains of the protein are shown in color. Region in black (bases 15,477,167–15,477,093 of the X chromosome) is predicted to be part of the cDNA on WormBase (www.wormbase.org, release WS247), but was not found in the amplified cDNA. Another alternative splice in this region (15,477,149–15,477,088 on release WS249) was listed as a confirmed mid-L3 intron by the Waterston group for the ModEncode project (www.wormbase.org). Bracket shows area of cDNA used for dsRNA injections. Mutations of *rh26* and *ok5478* alleles are indicated. (B) Structural alignment of first Ras domain of EXC-1 (light blue) bound to GTP, as predicted by TASSER (Roy *et al.* 2010) to Ras domain of mouse Irga6 protein (dark blue) (Ghosh *et al.* 2004) shows overlap of backbone with rmsd of 1.51 Å. (C) Alignment of EXC-1 and Ras domain of human IRGC. Table shows the sequence of specific subdomains found in a consensus Ras protein, human IRGC, and the first and second Ras-homology domains of EXC-1. The colors of the domains in the diagram above the table are also shown in the structural model (B) and show close positional alignment. The putative G5 motifs of EXC-1 show greater sequence similarity to that of the consensus Ras protein than does the G5 motif of mammalian IRGC. (D) Yeast two-hybrid assays of entire gene and specific domains of *exc-1* to full-length *exc-9*. Diagram on left shows structure of encoded regions of *exc-1* in constructs on bait plasmids to scale, with positions of proline-rich and first and second IRG-like Ras-like domains indicated. Position of putative ca (star) and dn (X) domains of first Ras-like domain are indicated. Table on right shows results of hybridization to the same gene constructs in prey plasmids. At bottom are growth results using *exc-9* construct as bait, with interpreted results below.

addition, a BLAST search revealed no obvious homologs of EXC-1 or EXC-9 in *Saccharomyces cerevisiae*, and strains containing either bait or prey plasmid alone did not grow on selective media, so these proteins did not react with endog-

enous yeast proteins. In contrast, both the wild-type and putative ca forms of EXC-1 gave strong reactions to EXC-9 (Figure 4D), while the putative dn form failed to bind to EXC-9. We narrowed the location of interaction by testing

Table 3 Effects of marker expression on excretory canal phenotype

Strain/genotype	N ^d	% Conv. ^e	Canal lumen length ^a			Overall cyst size ^b			P-value ^c	
			% No growth + short	% Mid-length	% Full-size + Conv.	% None	% Small	% Med + Large	Lumen length	Cyst size
N2 (wild type) animals	100	0	0	0	100	100	0	0	Compared to wild type:	
Expressing: <i>mCherry::chc-1</i>	100	0	0	13	87	98	2	0	0.000081	0.25
<i>mCherry::eea-1</i>	100	0	0	0	100	98	2	0	1.00	0.25
<i>mCherry::glo-1</i>	100	0	0	1	99	100	0	0	0.50	1.00
<i>mCherry::GRIP</i>	100	0	0	0	100	100	0	0	1.00	1.00
<i>mCherry::rab-5</i>	86	0	7	47	47	85	15	0	<0.000001*	0.0000261
<i>mCherry::rab-7</i>	98	0	0	30	70	98	2	0	<0.000001*	0.24
<i>mCherry::rab-11.1</i>	100	0	0	29	71	94	6	0	<0.000001*	0.029
<i>mCherry::rme-1</i>	51	0	0	0	100	100	0	0	1.00	1.00
<i>exc-1(rh26)</i>	100	0	29	64	7	1	55	44	Compared to <i>exc-1(rh26)</i>	
Expressing: <i>mCherry::chc-1</i>	89	0	24	63	13	0	60	40	0.284	0.71
<i>mCherry::eea-1</i>	92	0	3	91	5	2	74	24	<0.000001*	0.0064
<i>mCherry::glo-1</i>	51	0	31	59	10	0	78	22	0.74	0.0086
<i>mCherry::rab-5</i>	104	0	27	64	9	1	68	31	0.90	0.085
<i>mCherry::rab-7</i>	53	0	40	36	25	11	49	40	0.00084	0.018
<i>mCherry::rab-11.1</i>	52	0	48	52	0	0	27	73	0.0183	0.0013
<i>mCherry::rme-1</i>	55	0	40	49	11	0	36	64	0.193	0.034
<i>exc-9(n2669)</i>	54	0	74	26	0	0	61	39	Compared to <i>exc-9(n2669)</i>	
Expressing: <i>mCherry::eea-1</i>	51	0	75	25	0	0	59	41	1.00	0.84
<i>mCherry::rme-1</i>	47	0	57	43	0	13	66	21	0.094	0.0060

Conv., convoluted.

^a Average canal length, from 0 to 4, as described in *Materials and Methods*.^b Percentage of canals examined with cysts primarily of size denoted.^c P-value from Fisher's exact test, probability of not being different from denoted comparison strain. Values in boldface type labeled with asterisk indicate significant differences.^d No. of canals examined.^e Percentage of canals examined that exhibited a lumen that traversed itself more than once.

the ability of **EXC-9** to bind to individual domains of the wild-type **EXC-1** protein. **EXC-9** bound well to the N-terminal proline-rich region, and to the first Ras-like domain, but did not bind to the C-terminal Ras-like domain nor to the region between the two Ras-like domains. These data suggest that **EXC-1** acts via binding of its N-terminal domains to **EXC-9** protein, but acts on **EXC-5** through an indirect mechanism.

The effects of varying expression level and of mutation of the first Ras domain on excretory canal morphology were measured (Figure 5 and Table 2). As noted above (Figure 1, E and F), expression of a low level of *exc-1* via its native promoter substantially rescued the mutant phenotype, significantly reducing the number and size of cysts while allowing the canal to extend much farther than in the mutant. Higher expression levels of this construct (as assayed via GFP fluorescence) caused a narrow convoluted tubule phenotype (Figure 5A, Table 3, and File S1) that has previously been observed at light and electron-microscopic levels for overexpression of *exc-9* and of *exc-5* (Suzuki *et al.* 2001; Tong and Buechner 2008; Mattingly and Buechner 2011). Wild-type canal lumens form and begin to grow out almost as soon as the cell is born and extend together with extension of the basolateral processes (Sulston *et al.* 1983; Suzuki *et al.* 2001). In convoluted tubules, a long lumen is visible within an enlarged cell body; this lumen has the narrow diameter of wild-type canal lumens rather than an enlarged cystic phe-

notype. We have interpreted this phenotype to indicate that the luminal surface is growing normally, but has no place to extend, so it forms normal-diameter canals folded up within the cell body. While folding prevents exact measurement of the luminal length, we have measured lumens that, if extended, would easily extend a great distance along the length of the animal. The convoluted tubule phenotype therefore represents a rescue of the apical (luminal) surface of the canals by overexpression of *exc-1*, *exc-5*, or *exc-9*, while at the same time extension of the basolateral surface is impaired.

Microinjection of the same *exc-1* construct into *exc-9* mutants also caused an enlarged excretory cell body with narrow convoluted tubules (Figure 5, B and C), but had no effect on the *Exc-5* phenotype of very large cysts at the distal ends of the canals (Figure 5, D and E), even when expressed at much higher levels as measured via GFP fluorescence. These results are consistent with other observations, suggesting that **EXC-1** acts in a common pathway downstream of **EXC-9** but upstream of **EXC-5** to regulate canal diameter (Tong and Buechner 2008) (Table 2).

Microinjection of genomic *exc-1* constructs containing putative ca or dn forms (homologous to similar mutations in Ras) of the first Ras domain caused phenotypes that indicate that these forms have biological function (Table 2). Injection of the dn *exc-1* constructs into wild-type worms (Figure 5F

Figure 5 Wild-type *exc-1* acts between *exc-9* and *exc-5* to regulate canal diameter. (A) High level of expression of *exc-1* (same construct as in Figure 1, E and F) rescues luminal diameter but shortens canal length so that no canal extension is evident. The canal cell body (arrow) is in normal position below posterior bulb of the pharynx (asterisk), but is enlarged due to presence of long normal-diameter luminal tube folded within the cell body. (B and D) Control uninjected *exc-9(n26669)* and *exc-5(rh232)* animals show large cysts in the cell body and in the distal tips of the canals. (C and E) Injection of high levels of the same *exc-1* construct as in Figure 4A caused similar convolution of normal-diameter canal tubules in *exc-9(n26669)* animals (C), but had no effect upon canal

and Table 2) caused their progeny to develop cysts, similar to the effects of RNAi to *exc-1*. Conversely, the putative ca *exc-1* construct rescued both the long length and narrow diameter of the *exc-1* mutants (Figure 5G). When injected into wild-type animals, no convoluted tubules were observed (Table 2), even when the fluorescence from the construct was visibly brighter (presumably indicating greater copy number of the gene in the array) than for constructs of wild-type *exc-1* that cause highly convoluted canals. It is interesting that injection of putative ca *exc-1* substantially rescues formation of the apical surface to restore canals to full length, while injection of wild-type *exc-1* also forms tubules of near-normal length, although often convoluted inside a shortened canal cell (indicating an induced defect in basolateral surface formation). These results indicate that putatively ca *EXC-1* is functional but has altered activity from that of wild-type *EXC-1*. We conclude that activation of the first *EXC-1* Ras domain is essential for full activity of the protein.

EXC-1 effects endocytic recycling from early to recycling endosomes

Since *EXC-1* is homologous to IRG proteins believed to affect internal membrane organization, and other *EXC* proteins also affect vesicular recycling (Martens and Howard 2006; Howard 2008; Mattingly and Buechner 2011), we examined the role of *EXC-1* in accumulation of vesicles. The *rh26* mutation was crossed into various strains expressing mCherry marker linked to subcellular compartment-specific proteins (Figure 6 and Table 3). In wild-type animals, the early endosome marker protein *EEA-1* and recycling endosome marker protein *RME-1*, labeled with mCherry, are highly expressed in the cell body, and exhibit discrete puncta throughout the canal (Figure 6, A and B) with some diminution in frequency of puncta toward the distal ends of the canal. The *exc-1(rh26)* mutant showed similar effects on markers to those found for *exc-5* mutants (Mattingly and Buechner 2011). The early endosome marker *EEA-1* accumulated greatly in areas around cysts (Figure 6, C and D), while the recycling endosome marker *RME-1* was severely depleted in cystic areas (Figure 6, E and F). No obvious effects were seen for other markers (Figure 7): cytoplasmic *CDC-42* (Figure 7, A and B) clathrin-coated pits (*CHC-1*) (Figure 7, C and D), late endosomes (*RAB-7*) (Figure 7, E and F), lysosomes (*GLO-1*) (Figure 7, G and H), or Golgi (*GRIP*) (Figure 7, I and J), although some accumulation around cysts was evident for another early endosomal marker, *RAB-5* (Figure 7, K and L). Similar accumulations of early endosome markers *EEA-1* and (to a

morphology in *exc-5(rh232)* mutants (E). (F) Microinjection into wild-type worms of a similar construct as in Figure 5A, but containing putative dn *exc-1* construct. Note normal-size cell body, with visible small- and medium-sized cysts toward the distal end of the canal. The canal did not reach the vulva. (G) Injection of a similar putative ca *exc-1* construct shows rescue of both the canal luminal diameter and canal length. Posterior half of animal is shown; canal stretched to wild-type length. All DIC figures contrast-enhanced for clarity. All bars, 10 μ m.

Figure 6 Cysts accumulate EEA-1 while losing RME-1 expression. Expression of fluorescent endosomal markers in (A and B) wild-type controls, in (C–F) *exc-1(rh26)* mutants, and in (G–J) *exc-9(n2669)* mutants. (A, C, and G) Canals expressing integrated array of $P_{exc-9}::mCherry::eea-1$ to mark early endosomes. (B, E, and I) Canals expressing integrated array of $P_{exc-9}::mCherry::rme-1$ to mark recycling endosomes. Animal in E additionally carries $P_{vha-1}::gfp$ to label entire canal cytoplasm, otherwise not visible surrounding cyst ($P_{vha-1}::gfp$ expression does not alter canal morphology). Arrowheads in all panels show diameter of normal-width canal. Arrows in A and B indicate typical punctate appearance of endosomes, while arrows in C and E indicate cyst at posterior terminus of canal. G–J show *exc-9* animals exhibiting partial amelioration from overexpression of *eea-1* or *rme-1*. G shows the canal extended well past the vulva (midpoint of the animal); arrow shows expression driven by P_{exc-9} in the uterine seam cell. Arrows in I show areas where the canals remain constricted and exhibit higher levels of RME-1 than do areas where the canal lumen has further expanded. D, F, H, and J show relative intensity of marker fluorescence normalized to average fluorescence from a length of normal-diameter canal in the same animal. Bars, 10 μ m. All figures are brightened to show position of puncta in normal-width canals; graphs were generated from original unbrightened images.

lesser extent) *RAB-5*, with loss of recycling endosome marker *RME-1* in areas of large cysts was previously described for *exc-5* mutants, again with no effects evident for other endosomal markers (Mattingly and Buechner 2011).

As *EXC-1* shows interactions with *EXC-9* via yeast two-hybrid assay (Figure 4D), we tried to examine the effect of

exc-9 mutation on location of labeled *EEA-1* and *RME-1*. Surprisingly, expression of these marker constructs appeared to ameliorate partially the phenotype of *exc-9* mutants (Figure 6, G–J and Table 3). Although the canals were similar in length and the largest cysts present were about the same size as in untreated *exc-9* animals (no significant differences

Figure 7 Other subcellular markers show no major effects in *exc-1* animals. Fluorescence of animals expressing stable integrated arrays of fluorescently labeled subcellular markers within the excretory canals, driven by moderate expression promoter P_{exc-9} . A, C, E, G, I, and K show expression in a wild-type background; B, D, F, H, J, and L show expression in *exc-1(rh26)* background. (A and B) *mCherry::cdc-42* primarily labels cytoplasm; (C and D) *mCherry::chc-1*, clathrin-coated pits; (E and F) *mCherry::rab-7*, late endosomes; (G and H) *mCherry::glo-1*, lysosomes; (I and J) *mCherry::GRIP*, Golgi; and (K and L) *mCherry::rab-5*, early endosomes. Arrows indicate position of large cysts in *exc-1* mutants. All bars, 10 μ m. Fluorescence brightened in all figures to show positions of puncta and image false-colored magenta to increase visibility.

shown in Table 3), the number of obvious cysts in each animal tended to be fewer, and the width of canals overexpressing *EEA-1* or *RME-1* appeared generally closer to that of wild-type canals (examples in Figure 6). The effect of *exc-9* mutation was not as pronounced on accumulation of *EEA-1* and exclusion of *RME-1* from cystic areas of *exc-9* animals.

Statistical analysis of the shape of canals also suggests that *EXC-1* acts on endosome trafficking (Table 3). Strong expression of mCherry-linked *RAB-7*, *RAB-11.1*, and especially *RAB-5* caused mild shortening of the canals and occasional formation of small cysts in some wild-type animals, but this effect was not evident in an *exc-1* mutant, which could suggest that overexpression of these proteins may have similar effects as loss of *EXC-1* function on maintenance of canal structure. The effect of RAB expression on cyst formation was much lower than the effect of *exc-1* mutation, however. In contrast, expression of mCherry-linked *EEA-1* had no effects on wild-type canals, but partially suppressed the *Exc-1* mutant phenotype so that canals grew longer (Table 3).

Overexpression of *EEA-1* occurs prior to the formation of cysts in embryonic *exc-5* mutants, which strongly suggests that disruption of endosomal trafficking causes cyst formation (Mattingly and Buechner 2011). We examined the timing of *EEA-1* expression in *exc-1* mutants and found a similar effect (Figure 8). In embryonic *exc-1(rh26)* mutant animals,

accumulations of labeled *EEA-1* were apparent within the excretory canals 60–90 min before luminal cysts became visible at the light microscopic levels; no such build-up was seen in wild-type embryos expressing *EEA-1* (data not shown). These results support the hypothesis that abnormal build-up of *EEA-1*-labeled endosomes occurs prior to, and may therefore be the cause of, collapse of the lumen.

Discussion

EXC-1 as a model for mammalian IRG proteins

C. elegans exc-1 mutants exhibit severe deficiencies in maintenance of the narrow tubular excretory canals. The results presented here show that through gene rescue, expression pattern, RNAi phenocopy, and mutant sequence analysis, that *C46E1.3*, the closest *C. elegans* homolog to the vertebrate IRG proteins, encodes the *exc-1* gene.

The IRG (also called interferon-inducible GTPase) proteins are a large family of proteins related to the dynamin-like family, the guanine-binding protein (GBP) superfamily, and the Ras superfamily of guanine-binding proteins (Bekpen *et al.* 2005; Kim *et al.* 2012; Lilue *et al.* 2013). The IRG family was discovered in mice and includes a single IRGC protein and a large series of IRGA, IRGB, and IRGM proteins. The

Figure 8 Build-up of EEA-1-marked puncta precedes cyst formation in *exc-1* mutants. DIC (A, C, E, G, I, and K; contrast enhanced) and fluorescence (B, D, F, H, J, and L) images of typical *exc-1(rh26)* embryo and newly hatched L1 larva strongly expressing early endosome *mCherry::eea-1* marker. (A and B) Start of measurements at threefold stage, about 9 hr into normal 13-hr embryogenesis. No cysts are evident, and fluorescence is barely detectable in posterior canals (arrows). Bright spots in other areas of animal represent autofluorescence, especially within intestinal cells. (C and D) Thirty minutes after start of measurement. No cysts are evident, but fluorescence is easily detectable in posterior canals (arrows). (E and F) Sixty minutes after start. Animals have just hatched, marked EEA-1 is easily visible in locations (arrows) along posterior canal, but no cysts are evident. (G and H) Ninety minutes after start. Cyst (wide arrow) has become visible at distal tip of posterior canal, surrounded by marked EEA-1. (I and J) One-hundred twenty minutes after start. Marked EEA-1 has continued to accumulate at distal cyst. (K and L) One-hundred fifty minutes after start. Distal tip cyst has expanded in size, surrounded by fluorescently marked EEA-1. Build-up of EEA-1 was clearly observed in 9 of 11 embryos where cysts could be measured. Bars, 10 μ m.

number of vertebrate IRG genes varies considerably from one species to another and even within species and appears to be related to interactions with parasites (Lilue *et al.* 2013). IRGC is found in virtually all vertebrates as well as in many other animals, including nematodes. Humans have a single IRGM protein (with multiple alternative-splice forms) as well as an IRGC protein (Singh *et al.* 2006). *EXC-1* is the sole nematode homolog of this protein family.

IRG proteins in mice bind to and disrupt parasitophorous vacuoles in which parasites such as *Toxoplasma* and *Chlamydia* replicate (Kim *et al.* 2012). Many of the parasites, in turn, produce proteins to prevent IRG binding. The methods

by which IRG proteins are activated, their function, how they are activated, and even their GTP-turnover rate, are poorly understood, and vary considerably from one IRG protein to another. Interest in IRG proteins has increased recently, as human IRGM has been implicated in genome-wide association studies with Crohn's disease (Hoeftkens *et al.* 2013; Moon *et al.* 2013).

A common feature of all IRG proteins is an incomplete Ras-related GTP-binding domain that contains the core G1-G4 box sequences regulating binding to GTP and to GEF (Bourne *et al.* 1991), but with weaker homology to the Ras G5 box. The G1 boxes of *EXC-1* contain the canonical GKS motif (Figure 4C) found in most effector IRG proteins, rather than the GMS motif found in some IRG proteins with regulatory functions (Kim *et al.* 2012). Most mammalian IRG proteins contain homodimerization motifs in the center of the Ras-homology domain (Uthaiiah *et al.* 2003; Ghosh *et al.* 2004). This region is missing in *EXC-1*; instead, the *C. elegans* protein contains two separate Ras-homology domains within a single protein. A few mouse IRGB proteins are linked in tandem to contain two Ras-homology domains, but these retain the homodimerization motifs (Lilue *et al.* 2013), while the mouse Irga6 protein dimerizes in a head-to-head fashion (Schulte *et al.* 2016). Intriguingly, the two *C. elegans* Ras-like domains are separated by a region with homology to a central hinge domain of porphobilinogen deaminase (Louie *et al.* 1992; Song *et al.* 2009), a member of the PBP2 superfamily found in all creatures from bacteria to humans (Shoolingin-Jordan 1995). In many of these enzymes, the hinge is located between two ATPase domains which, when activated, bind to substrate. Binding and turnover of GTP and GDP has been difficult to detect in IRG proteins to date (Martens and Howard 2006), although some results suggest the rapid turnover of GTP in oligomerized Irga6 protein (Uthaiiah *et al.* 2003; Ghosh *et al.* 2004). Further studies will be needed to determine the substrate of *EXC-1* binding, and if GTP binding by both Ras-like domains is needed for turnover in *C. elegans EXC-1*.

The cellular function of IRG proteins is becoming better understood (Martens and Howard 2006; Petkova *et al.* 2012). The immune system stimulates activity of most vertebrate IRG proteins, such as the large family of mouse IRGM proteins involved in autophagosome formation to resist pathogen infection (Singh *et al.* 2006; Al-Zeer *et al.* 2009; Gazzinelli *et al.* 2014). IRGC, however, is not activated via immune response (Bekpen *et al.* 2005). In the excretory canal of *C. elegans exc-1* mutants, early endosomes accumulate and recycling endosomes are strongly affected, with no significant effects on late endosome levels detected; these observations suggest that autophagy is not at issue here. We conclude that the *EXC-1* protein, and by inference other IRGC proteins, act at a different step of endosomal trafficking than does human IRGM protein. The results presented here provide a possible model for investigating the function of IRG in endosomal formation and movement.

Figure 9 Model of possible role of EXC-1 in maintaining canal structure. Diagram shows conceivable mechanism of action of EXC-1 at the apical surface of growing canal. Thick terminal web is adjacent to apical (luminal) membrane. Canaliculi are made up of coated vesicular structure, with some vesicles connected to apical membrane and others not connected (Kolotuev *et al.* 2013). Apical membrane is presumably taken up into early endosomes; whether from apical surface directly or from canalicular vesicles is unknown. EXC-1 is inactive. In area of canal bending or canal growth, EXC-9 binds to the EXC-1 polyproline domain, either to activate EXC-1 directly or to allow EXC-1 to persist in the activated state. EXC-1 signals via other proteins to EXC-5. Signaling results in removal of EEA-1 label from early endosome to be transported to recycling endosome. Recycled material is then resupplied, although whether to apical surface directly or to canalicular vesicles is unknown.

EXC-1/IRG binds EXC-9/CRIP to stimulate EXC-5/FGD to maintain tubule diameter

Yeast two-hybrid data show that EXC-9 protein interacts strongly with both the N-terminal proline-rich domain and the first Ras-like domain of EXC-1, consistent with a model in which EXC-9 activates EXC-1. EXC-9 is a member of the CRIP family of small cytoplasmic LIM-domain protein-binding proteins, which are expressed particularly highly in the mammalian intestine, but are found in many tissues, including neurons in annelids (Emes *et al.* 2003) and in *C. elegans* (Tong and Buechner 2008). The function of these small LIM-domain proteins is poorly understood, but mouse mutants alter the ratio of T_H1 to T_H2 lymphoblasts in the gut (Cousins and Lanningham-Foster 2000).

Genetic results suggest that EXC-9, EXC-1, and EXC-5 act in a common pathway, where EXC-9 acts upstream of EXC-1 to stimulate the GEF EXC-5. EXC-5 is homologous to human FGD proteins found to stimulate CDC-42 (Zheng *et al.* 1996; Huber *et al.* 2008); here, EXC-5 presumably mediates disappearance or movement of early endosomes and appearance of labeled recycling endosomes to prevent cyst formation (Table 3) (Mattingly and Buechner 2011). Our results did not find a direct interaction of EXC-1 to EXC-5, which suggests that EXC-1/EXC-9 activates other factors that cause EXC-5 to mediate endosomal movement and/or conversion.

All three of these proteins are expressed within the excretory canals and in a few other tissues that do not make direct contact with the canals, which suggests that these proteins all act cell autonomously. Several of the other expressed tissues also form either unicellular or multicellular epithelial tubes: EXC-1/IRGC in the amphid sheath (Figure 3), EXC-9/CRIP in the uterine seam cell (Tong and Buechner 2008), and EXC-5/

FGD in the pharyngeal muscles (Mattingly and Buechner 2011). It is likely that these proteins have other partners to be discovered in these tissues.

In *exc* mutants with large cysts, canals begin to form normally, but fail to maintain normal luminal diameter as the canal extends. The lumen is normally surrounded by a series of small canalicular vesicles of uniform size containing vacuolar ATPase and aquaporin, and which are believed to mediate canal function as well as growth (Göbel *et al.* 2004; Kolotuev *et al.* 2013). We have shown that in *exc-1* mutants, similar to previous results in *exc-5* mutants (Mattingly and Buechner 2011), early endosomes build up in the developing embryonic canals prior to breakdown of the cytoskeleton to form large fluid-filled cysts (Figure 8). It should be noted, however, that our data cannot distinguish whether EEA-1 build-up is the proximal event causing cyst formation, or if instead some other event (such as a very small cyst) occurs that causes both EEA-1 build-up and eventual cytoskeletal breakdown. Electron-dense terminal web-like material around canal lumen is also disrupted, with coated canalicular vesicles retained only in areas where terminal web-like material is still intact (Figure 2) (Buechner *et al.* 1999).

These data are consistent with a proposed model (Figure 9) in which EXC proteins regulate endosomal recycling to direct the cytoskeleton to maintain a narrow tubular structure. In this speculative model, EXC-9 would normally be activated by a signal (perhaps a change in cytoskeletal tension, change in osmolarity, thinning or breakage of apical cytoskeleton, or membrane stress) to activate cytoplasmic EXC-1, which then signals indirectly to the GEF EXC-5 to carry out early-to-recycling endosomal vesicular traffic to

mediate cytoskeletal rebuilding at that site. Loss of any of these proteins is hypothesized to prevent recycling of necessary membrane or membrane proteins needed to rebuild or maintain apical structure. IRG proteins show homology to dynamin-like proteins involved in vesicle fusion and budding (Williams and Kim 2014). It is tempting to speculate that *EXC-9*/CRIP represents a conserved activator of *EXC-1*/IRGC that carries out vesicle budding from early-to-recycling endosomes. That vertebrate CRIP is highly expressed in the gut also raises the tempting possibility that this protein may activate IRG function associated with Crohn's disease. Future testing of the proposed *EXC-9*/*EXC-1*/*EXC-5* pathway will require further testing of the mechanism by which *EXC-9* activates *EXC-1*, measurement of GTP turnover rates of *EXC-1*, determination of the identity of cargo in endosomes affected by *exc-1*, -5, -9 mutations, and dynamic tracking of the movement of individual endosomes within the excretory canals.

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Author contributions: M.B. and K.A.G. designed research; K.A.G. performed microinjections and most work with nematodes; A.T.S. and C.J.T. performed the yeast two-hybrid experiments; B.C.M. microinjected *exc-5* constructs; D.E.K. built constructs and analyzed cyst size in worms; D.H.H. conducted and analyzed electron microscopic experiments; V.G. and L.A.K. designed, H.A.-H. performed, and B.D.A. oversaw the use of confocal microscopy experiments; and M.B. and K.A.G. prepared the figures and wrote the manuscript.

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Supporting Information

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Facilitation of Endosomal Recycling by an IRG Protein Homolog Maintains Apical Tubule Structure in *Caenorhabditis elegans*

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Brian D. Ackley, and Matthew Buechner

Table S1. dsRNAs tested for ability to phenocopy *exc-1* mutants:

dsRNA	Predicted Gene	# Animals			
to clone:	(from wormbase.org)	Forward Primer	Reverse Primer	Injected	Result
ser-2	SERotonin/octopamine receptor family	TGCATACGAATTTGG	GAGTTACCGTATGCC	38	No canal cysts
nhr-91	Nuclear Hormone Receptor Family	GGACTGCCTAGACCG	TGAGATCTCGTCAGT	60	No canal cysts
glb-28	GLOBin Related	CTTAAAGTTCGCGTG	ATCTTGAATCGAAT	35	No canal cysts
Y15E3A.3	None	ATATCAGGAAATGAG	ATTCATATTAGAAAA	44	No canal cysts
Y15E3A.4	SLC Family solute carrier	AGCACCCACAAATGT	CAGAACTGGATACGA	45	No canal cysts
Y15E3A.5	None	CGAGTACCTCTTGAC	GCTCTTCAGATCAAT	43	No canal cysts
nfki-1	Protein phosphatase 1, regulatory subunit	TGGAAGAACGTGCTT	AATGCTTCTTGCAA	55	No canal cysts
C33A11.2	DRAM1 autophagy modulator	CTACACTATCGCAGT	TTTGCCACAAAGAAC	59	No canal cysts
tag-97	ELF3 transcription factor	ACGCGGGGTTTAACC	TCCTTGGGATTACAA	27	No canal cysts
psa-3	Phasmid Socket Absent	TCAGCAACACTACGC	TACTTGTTTAAAGAA	22	No canal cysts
pqn-36	Prion-like-(Q/N-rich)- domain-bearing protein	TTTTGGTTCTGGTTT	CTGGCTATCAAACGTG	25	No canal cysts
F39D8.3	None	GACGAACAGTATTTT	CTATTAATTTCTGTC	61	No canal cysts
nas-13	Nematode AStacin protease	CAATTCTCCCAACTA	CAGTCCACTTTTGG	27	No canal cysts

blos-8	human C10ORF32	TTAATTATTCGGCGG	ATTCAGATTGGATA	23	No canal cysts
flp-32	FMRF-Like Peptide	CCAATATTCCAGCCC	ACTAGCAAATTCAGA	25	No canal cysts
mocs-1	MOLybdenum Cofactor Sulfurase	CATTCTGACGTGAT	AGGAATATCTTCCAT	28	No canal cysts
nkat-3	Cysteine Conjugate- Beta Lyase	CATCGAGCCAGCCTA	CACACCCTGTTCTTA	34	No canal cysts
RO3A10.5	SEC14 Family	AGTGCGGAAAAACCG	GCTTTACTAATTCCA	48	No canal cysts
spr-1	G-Protein-Coupled Receptor	CTATGAAGCTTTGCT	CTGCCGCTGTCGCGC	42	No canal cysts
spr-1		GATTGCTCAACACAA	TATATCTAGTGTCAC	57	No canal cysts
gcy-36	Guanylyl CYclase	AAACCTATATGAAGA	TTCATTTCCACGTCC	26	No canal cysts
C46E1.3	GTP-Binding	TTTCTTTTCGAAGCC	AAAGCGACGAGGACC	63	Canal, glial cysts
H13NO6.2	None	AACTGAAAACCATCG	AGCGAACTCCAGTCC	41	No canal cysts
gob-1	Gut Obstructed: Trehalose Phosphatase	CTCTTCACCCGAGCA	TTTGAACCTCTAGGG	30	No canal cysts
suox-1	SULfite OXidase	GAACACAGTTCCATC	TACTGCTTTCGCAAT	40	No canal cysts
tbh-1	Tyramine Beta Hydroxylase	TTGCCCATGCAGCAA	CCAGCCGCTCTCGAG	25	No canal cysts
H13NO6.2	man FAM154	ATCAAACCTGCGCTGC	TGCATATTCACATT	43	No canal cysts
hke-4.2	SLC39A7 Zinc carrier	CGCTCATGTCATGA\G	CCTTAATGATCCAAG	56	No canal cysts
eat-20	EATing: abnormal pharyngeal pumping: <i>crumbs</i> homologue	AGACGAAGATATGAC	TTCCGTA CTGTTTC	67	No canal cysts
olrn-1	Olfactory LeaRNING defective	AATCCCCCAAAC TCG	GTTGATGGTTTGAGC	30	No canal cysts
Iron-1	Pharyngeal eLRR	TGTAATTAGCCGAGA	ACTAATTTGCGCTGG	15	No canal cysts

(extracellular Leu-Rich

Repeat ONLY)

File S1: Three-dimensional image of enlarged excretory canal with high level of *exc-1* expression. The canal cell forms a convoluted canal with a normal-diameter apical surface and an open lumen (dark regions) but fails to extend its basal surface, resulting in a larger canal cell body. Video was made from a stack of images taken with FV1000 Confocal microscope (Olympus) laser scanning confocal microscope with each image taken 0.41 μ m apart. Animal carries extrachromosomal array $P_{vha-1}::gfp$ labeling canal cytoplasm. Cell nucleus is located in lower left corner. (.mov, 467 KB)

Available for download as a .mov file at:

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.192559/-/DC1/FileS1.mov>