CLONING AND CHARACTERIZATION OF *EXC-9*, A *CAENORHABDITIS ELEGANS* CRIP HOMOLOGUE THAT REGULATES TUBULAR STRUCTURE

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ABSTRACT

Forming and maintaining tubular structure is fundamental to organismal development. The excretory canal cell of *C.elegans* forms a single-cell epithelial tubule, which provides a simple model for tubular structure study. The EXC proteins regulate maintenance of the apical (lumenal) cytoskeleton of the excretory canal. Loss of exc gene function allows formation of fluid-filled cysts in the excretory canal. *exc-9* mutants exhibit short and cystic canals compared to wild-type worms. *exc-9* mutants also exhibit tail defects in hermaphrodites and ray defects in male.

By SNP mapping, cosmid rescue, and RNAi experiments, we proved that F20D12.5 encodes *exc-9*. EXC-9 is a homologue of the mammalian intestinal LIM-domain protein CRIP. *exc-9* is highly expressed in the canal and tailspike, it is also expressed in some other cells, including UTSE, DTCs, and ALM neurons. Promoter regions important for *exc-9* expression were studied. It was found that EXC-9 functions cell-autonomously and the free N-terminus of the protein is required for unextended canal phenotype.

Overexpression of *exc-9* constructs in an N2 background sometimes causes an "unextended canal" phenotype, in which the canal forms a large cell body filled with lumen with proper diameter, but has no canals along the length of the animal. Since canal extension is sensitive to expression levels of *exc-9*,

injection of the construct also caused unextended canal phenotype in *exc-9* mutants. Similar unextended canal phenotype was also found in animals showing high levels of *exc-5* expression.

I used the unextended canal phenotype to examine epistasis of EXC-9 function with that of other EXC proteins. EXC-9 appears to function upstream of EXC-5 to regulate cytoskeletal formation at the apical surface (possibly via CDC-42); and EXC-9 in turn may depend upon EXC-2 and EXC-4 function.

A second well conserved CRIP homologue B0496.7 is highly expressed in multiple valves of *C.elegans*. It functions similarly to EXC-9 when ectopically expressed in the excretory canal. Expression of the mouse homologue CRIP in the canal failed to rescue *exc-9* mutants, but with some modifications, mouse CRIP can function similar as EXC-9.

Preliminary data of cloning of *exc-2* is reported in this work too.

Dedicated to:

My family

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LIST OF ABBREVIATIONS

AA	Amino Acid
cDNA	Complementary DNA
CRHP	Cysteine rich heart protein
CRIP	Cysteine rich intestinal protein
CRP	Cysteine rich protein
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
DTC	Distal tip cell
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
Kb	kilo base pairs
KD	kilo dalton
PAGE	Polyacrylamide gel electrophoresis
PKD	Polycystic kidney disease
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNAi	RNA interference
SDS	Sodium dodecyl sulfate

SK3 channel	small conductance calcium-activated potassium 3 channel
SNP	single nucleotide polymorphis

Chapter 1

Introduction

1.1 Introduction to *Caenorhabditis elegans*

C. elegans is a soil dwelling nematode that has been used as a model organism for more than 40 years. In 1965, Sydney Brenner chose *C. elegans* as a research model (Brenner, S. 1974), and began the era of *C. elegans* research. Since then, due to its transparency, short life cycle, small size, invariant cell lineage, fully sequenced genome and many other advantages, many scientists use it as their research model to study behavior, development, genetics, and cell biology.

There are two sexes of *C. elegans*: male (XO) and self-fertilizing hermaphrodites (XX). A hermaphrodite worm can produce about 300 progeny in its lifetime (Riddle, D.T., Blumenthal, T. et al. 1997). From a fertilized egg to a fully developed adult worm will take approximately two days. If supplied with enough food, the total lifespan for a worm is two to three weeks (Riddle, D.T., Blumenthal, T. et al. 1997). Male frequency in a typical wild-type population is 0.1%. When males mate with hermaphrodites, hermaphrodites will produce a 1:1 ratio of male and hermaphrodite cross-progeny (Riddle, D.T., Blumenthal, T. et al. 1997).

C. elegans adult worms are about 1 millimeter in length and can be cultured on *E. coli* covered agar in a petri dish. The dish can be checked under a dissecting microscope to examine obvious phenotypes. Internal structure can be easily detected in vivo using a compound microscope because the worms are

transparent (Brenner, S. 1974).

The complete genome sequence and predicted gene structure are available on wormbase (www.wormbase.org). Wormbase provides this information in order to find possible target genes for cloning, and also helps to make reverse genetics feasible in *C. elegans.*

All these advantages make *C. elegans* a great model for research.

1.2 Epithelial structure

An epithelium is a tissue composed of a layer of cells. Epithelia line both the outside and inside cavities of the body, and separate the inside of an organism from the outside and also form barriers between two adjacent compartments. Functions of epithelial cells include protection (*e.g.* skin), absorption (*e.g.* intestine), and secretion (*e.g.* kidney).

Cell junctions are abundant in epithelial tissues, and are essential for selective permeability between two different environments on both sides of the barrier. All epithelial structures have polarity (Fig. 1.2). The apical surface faces the lumen or outside of the organism; the basal surface faces the basal lamina; the lateral surface is between the cells. The apical surface is important for absorbing nutrients in the intestine and secreting waste in the kidney. There are specializations on the apical surface of epithelial cells, *e.g.* microvilli, that increase the surface area, and cilia, that move or detect substances. The

basolateral surface has junctions to maintain the polarity. Tight junctions make adjacent cells adhere to each other. They confine transport proteins to the appropriate membrane domains by acting as a barrier. Gap junctions allow cells to communicate with one another. Small molecules can pass through connexons. The keratin intermediate filament networks connect adjacent cells through desmosomes and to the basal lamina through hemidesmosomes (see Fig.1.1).

In *C. elegans,* the major epithelial tissues are: hypodermis, intestine, gonad, seam cells, excretory system and tail spike.

1.3 Tubular structure

Biological tubular structure is responsible for transport of vital fluids such as blood and liquid waste. The formation of these tubes is critical for the majority of organisms. In humans, most major organs such as kidney, lung and circulatory system are composed primarily or exclusively of tubes (Lubarsky, B. and Krasnow, M.A. 2003).

There are multi-cell tubes and single-cell tubes. The process of folding epithelial sheets is one method of forming multi-cell tubes. Hollowing of a single cell leads to the formation of a single-cell tube. Although they look and function differently, there are common aspects for both types of tubular structure: the inner lumen surface is apical-characteristic, and the outer surface of the tube is

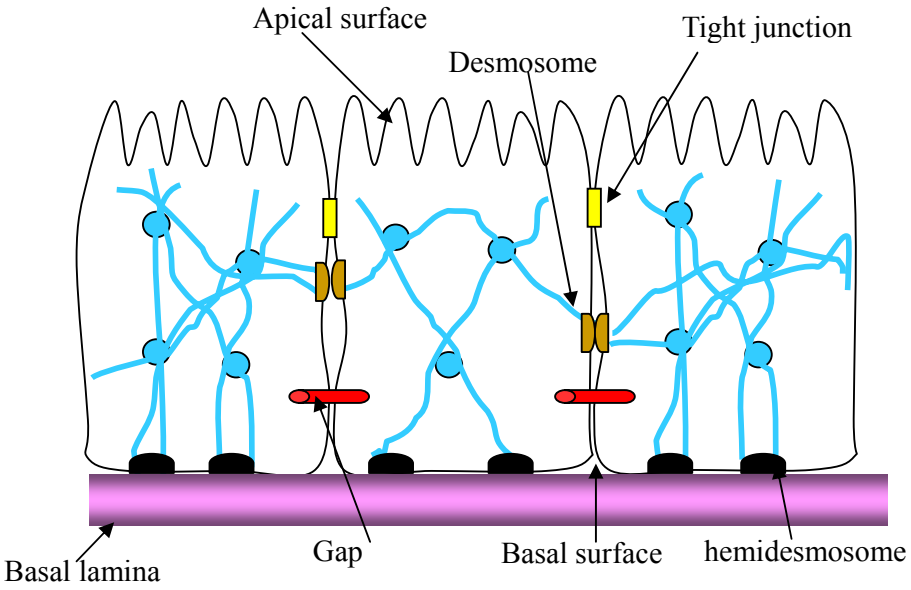


Figure1.1 Diagram of epithelium cell with different kinds of junctions. The junctions help epithelial structure to maintain the polarity.

basal-characteristic (Buechner, M. 2002; Hogan, B.L. and Kolodziej, P.A. 2002; Lubarsky, B. and Krasnow, M.A. 2003).

A recent model suggests that although tubes are different from each other, there is a common pathway for tubulogenesis. During the process of cell cord hollowing, cell hollowing, or cavitation for tubular formation, the apical surface is expanded by means of vesicle fusion after the determination of the apicobasal polarity of the tube (Lubarsky, B. and Krasnow, M.A. 2003). This conservation of mechanism implies that findings from the study of simple tubular networks on invertebrate model organisms may apply to the complex tubular organs of vertebrates.

1.4 The *Caenorhabditis elegans* excretory system

The excretory cell of *Caenorhabditis elegans* provides a genetic model of tubular development. The excretory cell is the largest cell in *C. elegans* and also a major epithelium structure in the worm. It functions to regulate osmolarity and internal hydrostatic pressure (Nelson, F.K., Albert, P.S. et al. 1983; Buechner, M. 2002).

The whole excretory system is composed of excretory cell, gland, duct, and pore cell. The excretory canal cell body is located on the ventral side of the posterior bulb of the pharynx. Two tubes form and extend right and left from the cell body to the lateral side of the worm. These two tubes bifurcate

when they reach the lateral side and grow both anteriorly and posteriorly; the four tubes then form an H-shaped structure. These processes make a seamless unicellular tube closed at its four endings (Nelson, F.K., Albert, P.S. et al. 1983; Nelson, F.K. and Riddle, D.L. 1984; Buechner, M. 2002). The excretory canal is connected with the excretory duct; the collected fluid in the canal will pass through the duct to be excreted to the outside from the excretory pore cell. Fluids and wastes are collected into the arm of excretory cell and then excreted to the outside of the worm through the excretory duct. The function of the excretory canal is analogous to that of the kidney (Nelson, F.K., Albert, P.S. et al. 1983; Nelson, F.K. and Riddle, D.L. 1984; Buechner, M. 2002).

The excretory system is required for worms to survive (Nelson, F.K. and Riddle, D.L. 1984; Buechner, M., Hall, D.H. et al. 1999). After laser ablation of the excretory cells, the worms died in several hours (Nelson, F.K. and Riddle, D.L. 1984), likely due to the inability to get rid of liquid waste.

1.5 *exc* mutants

There are several genes that affect the formation of excretory canals. Mutations in thee genes disrupt the formation of the apical surface of the excretory canals; fluid-filled cysts are formed in the canal lumen in these mutants. These mutants are called *exc* mutants, which stands for “excretory canal abnormal” (Buechner, M., Hall, D.H. et al. 1999). Several *exc* genes

have been cloned. A putative guanine nucleotide exchange factor, EXC-5, might work upstream of CDC-42 to direct new cytoskeleton to the apical membrane of the excretory canal (Gao, J., Estrada, L. et al. 2001; Suzuki, N., Buechner, M. et al. 2001). The *sma-1* gene encodes β_H-Spectrin, which helps maintain the apical membrane structure (Praitis, V., Ciccone, E. et al. 2005). *Let-653* encodes a secreted mucin that help to protect the exposed surface *(Jones, S.J. and Baillie, D.L. 1995).* EXC-7 binds to the 3'UTR of the *sma-1* mRNA, and is a nematode homologue of the *Drosophila* ELAV protein (Fujita, M., Hawkinson, D. et al. 2003). EXC-4 is a chloride intracellular channel locates on the apical surface of the excretory canal. How EXC-4 regulates the apical membrane structure of the excretory canal is not clear (Berry, K.L., Bulow, H.E. et al. 2003).

1.6 *exc-9* and *exc-2*mutants

exc-9 and *exc-2* are two genes that are also important for canal apical surface maintenance. Mutations in either gene cause strong canal defects. Knowing the identity of these genes will help us to understand the whole pathway better.

In my dissertation, I have mainly focued on the study of *exc-9* cloning and characterization. EXC-9 was found to be a single-LIM-domain protein. The mammalian homologue of EXC-9 is called CRIP (Cysteine rich intestinal

protein). I showed that EXC-9 functions with several other EXC proteins to regulate the excretory canal structure. I also found out that the other *C.elegans* CRIP homologue could function in the similar way as EXC-9.

Chapter 2

Cloning of the *exc-9* gene

2.1 *exc-9* mutants show a highly penetrant cystic phenotype

exc-9 is a mutant named after its excretory canal defect. Several obvious phenotypes of the *exc-9 (n2669)* mutants have been characterized. All the defects are recessive.

The most significant phenotype of *exc-9* worms is the abnormal excretory canal. The *exc-9* mutants have wider and shorter canals, sometimes with septate fluid-filled cysts (Fig. 2.1C and 2.1D), while canals of wild-type N2 worms have fine lumena that run from head to tail (Fig. 2.1A and 2.1B). The canal phenotype is not quite consistent from worm to worm, but it is 100% penetrant in homozygous animals (See Table 2.1), and it can be observed as early as the 2-fold stage. 13% of *exc-9* mutants show very large cysts (Fig. 2.1D). Due to impairment of this function, the worms with severe canal defects appear to have excess fluid and are sick.

In addition to the canal defects, 40% of the *exc-9* hermaphrodite mutants show tail defects (Fig. 2.1F) compared to the long pointed tailspike of wild-type worms (Fig. 2.1E). Since the tailspike also is an epithelial structure and needs to maintain long projection shape, it might share some similar pathways of epithelial morphogenesis with the excretory canal.

The tails of adult males are structurally different from hermaphrodites. They don't have a tail spike, instead, form the sensory rays in the male tail that are still epithelial tissue. In the *exc-9* male mutants, approximately 40% of the

Table 2.1 *exc-9* cystic phenotype.

Genotype	% cystic	N (canal number)
Wild type (N2)	0	100
exc-9(n2669)	100	104
exc-9(gk395)	100	119
exc-9(n2669)/exc-9(gk395)	100	67
+/ exc-9(n2669)	0	72
+/ exc-9(gk395)	0	56
exc-2(rh90)/ exc-9(n2669)	0	45
exc-4(rh133)/ exc-9(n2669)	0	55
exc-5(rh232)/ exc-9(n2669)	0	50

"% cystic" shows the percentage of canals with abnormal excretory canal apical structure of the corresponding worms. "N" stands for number of canals examined since two branches from the same excretory cell body develop independent from each other.

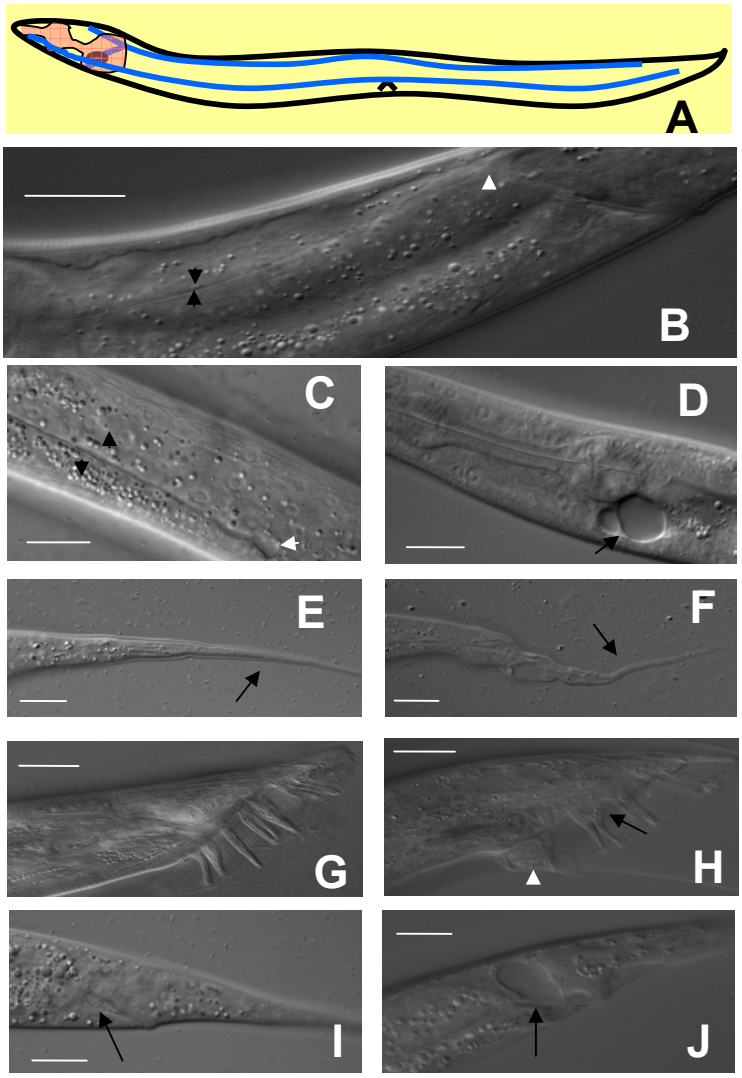

A
B
C
D
E
F
G
H
I
J

Figure 2.1 *exc-9* mutant phenotypes. Anterior is to the left. Dorsal is on the top. (A) Schematic drawing of excretory canals in wild-type worm. Blue line stands for canal tubes and orange part resemble the pharynx. (B) DIC micrograph of canal lumen in wild-type young adult. Black arrowheads indicate the canal lumen. The canals go all the way to near the tail of the worm as indicated by white arrowhead. (C, D) *exc-9 (n2669)* mutants show cystic and shortened canals compared to wild-type worms. Some mutants have small cysts (C) and some mutants have a more severe cystic phenotype (D). This phenotype is 100% penetrant. Black arrowheads indicate the lumen diameter and white arrowhead indicates the canal end in (C). Black arrow points to the large cyst in (D). (E) Tail whip in wild-type hermaphrodite tapers smoothly to a point. (F) Tail whip of *exc-9 (n2669)* animal has poorly formed tip. (G) Wild-type male has 9 sensory rays at one side of the tail, while *exc-9 (n2669)* animal (H) has malformed rays (indicated by white arrowhead) and fused rays (indicated by black arrow). (I) Wild-type hermaphrodite has small clear region next to the anal opening (black arrow). (J) Black arrow indicates huge clear region around anal opening in *exc-9 (n2669)* mutant. Tail defects in *exc-9 (n2669)* animals are about 40% penetrant. Scale bar: 25μm.

worms show fewer rays or have an abnormal ray structure (Fig. 2.1H). *exc-9* male worms show less mating efficiency. Since the male rays are essential for sensing hermaphrodites, the less mating efficiency might be due to the abnormal ray structure.

In addition to these phenotypes, *exc-9* worms also show larger vacuole area around the anus. Compared to the small clear region around the wild-type worm anus (Fig. 2.1I), 40% of the *exc-9* mutants show a large clear region. This phenotype is similar to the swollen vacuoles of *vha-8* mutants (Ji, Y.J., Choi, K.Y. et al. 2006). The mechanism for this phenotype is not understood.

2.2 *exc-9* mapping

Prior to my work, *exc-9* was mapped to chromosome IV, between *bli-6* and *fem-3 (Buechner, M., Hall, D.H. et al. 1999)*. A Snip-SNP (single nucleotide polymorphism) mapping strategy was used to narrow down the region further (see Materials and Methods for details). Double-mutant strains were constructed between *exc-9 (n2669)* and *bli-6 (sc16)* for the left side mapping (made by Matthew Buechner); and between *exc-9 (n2669)* and *fem-3 (q22)* for the right side mapping. Then the double-mutants were crossed with the wild-type Hawaiian strain CB4856 to get recombinants for SNP site testing. By means of SNP mapping, I narrowed the region of *exc-9* in between SNP sites on cosmids C06A6 and B0218, reducing the region of interest from 2.7 million base-pair to 340 kb

(Table 2.2 and Table 2.3). Only thirteen cosmids available from the *C. elegans* sequencing consortium contain all the DNA covering in this region (Figure 2.2).

Since RNAi is efficient in *C. elegans* to cause the post-transcriptional depletion of homologous endogenous RNA (Montgomery, M.K., Xu, S. et al. 1998), RNAi was chosen to identify *exc-9* at the beginning instead of Cosmid preparation and injection.

Some candidate genes in the region were tested by means of RNAi to determine if they are *exc-9* (Listed in Table 2.4). Injection of *sph-1* dsRNA plus a control marker, pCVC01, a repoter construct that expresses GFP in the canal (Oka, T., Yamamoto, R. et al. 1997), gave a cystic phenotype (5 out of 19 glowing progeny that have pCVC01 from injected N2 worms). However, the control injection of pCVC01 alone found to cause cystic phenotype (7 out of 25 glowing progeny of PCVC01 injected N2 worm show abnormal canal), so an alternative marker pRF4, neomorph copy of the *rol-6* gene, was tested. The results indicated that pRF4 is a good injection marker to use for the canal phenotype because none of the 26 roller progeny from pRF4-injected N2 worms gave abnormal canals, and none of the 19 roller progeny from pRF4-injected *exc-9 (n2669)* showed improved canals.

The rest of the RNAi experiments were carried out using pRF4 as injection marker. None of the tested dsRNAs of candidate genes caused abnormal canal phenotype.

Table 2.2 *Exc-9 non-Bli-6* SNP data.

Recombinant	SNP sites tested			
	R05G6:2917	C06A6:2004	C49H3:3770	T09A12:14845
1	N2	CB4856	\	\
2	N2	CB4856	\	\
3	N2	CB4856	\	\
4	N2	N2	CB4856	CB4856
5	N2	CB4856	CB4856	CB4856
6	N2	CB4856	\	\
7	N2	CB4856	\	\
8	N2	CB4856	\	\
9	N2	CB4856	\	\
10	N2	CB4856	\	\
11	N2	CB4856	\	\
12	N2	CB4856	\	\
13	N2	CB4856	\	\
14	N2	CB4856	\	\

On the top are the SNP sites used to test the *Exc-9 non-Bli-6* recombinants. N2 means the digestion pattern is the same as N2 worms and CB4856 stands for the same pattern as CB4856. "\" means the strain was not tested for the specific SNP site.

Table 2.3 *Exc-9 non-Fem-3* SNP data.

Recombinant	SNP sites tested			
	F20D12:32689	B0218:15870	T09A12:14845	D2096:39721
I	\	N2	CB4856	CB4856
II	\	N2	CB4856	CB4856
III	\	N2	CB4856	CB4856
IV	\	N2	CB4856	CB4856
V	\	N2	N2	CB4856
VI	\	N2	CB4856	CB4856
VII	N2	CB4856	CB4856	CB4856
VIII	\	N2	CB4856	CB4856

On the top are the SNP sites used to test the *Exc-9 non-Fem-3* recombinants. N2 means the digestion pattern is the same as N2 worms and CB4856 stands for the same pattern as CB4856. "\" means the strain was not tested for the specific SNP site.

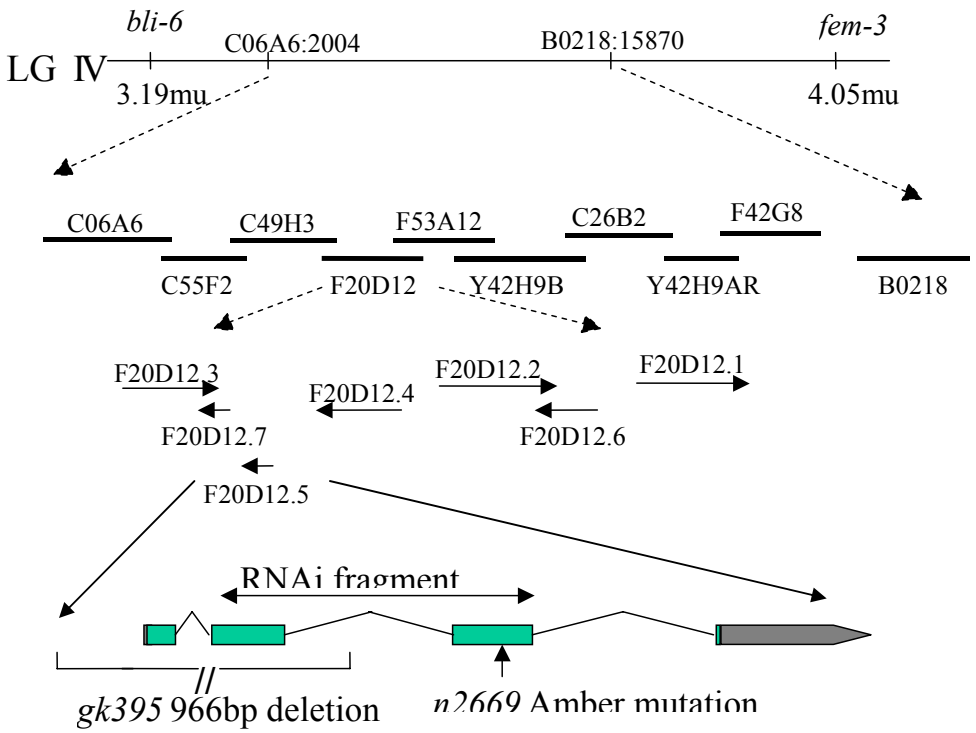


Figure 2.2 Genetic map of *exc-9* cloning and *exc-9* gene structure. *exc-9* was mapped in between *bli-6* and *fem-3* on linkage group IV. By SNP mapping, the region was narrowed down between SNP sites C06A6:2004 and B0218:15870. There are 13 cosmids in this region. Injection of cosmid F20D12 can rescue *exc-9* phenotype. There are seven genes in this cosmid and knockdown of gene F20D12.5 in wild-type background showed a similar phenotype as *exc-9* mutants. The green boxes stands for the *exc-9* (F20D12.5) exons and the lines in between stand for the introns. The mutations in *exc-9* alleles and RNAi fragment design are indicated in the gene structure.

Table 2.4 *exc-9* candidate genes in the refined region.

Name	Gene	Identity
C49H3.1	*gcy-8*	receptor-type guanylyl cyclase
C49H3.8	--	actin-like protein
F42G8.11	*sph-1*	synaptic vesicle channel protein
B0218.8	--	contains similarity to Pfam domain PF00059 (Lectin C-type domain short and long forms)

The genes with possible role in cytoskeleton organization were selected as RNAi target to test whether they are *exc-9*.

2.3 Cosmid rescue

The cosmids in the refined region were purified and injected into *n2669* worms to see if they could rescue the *exc* mutant phenotype. Plasmid pRF4 was used as co-injection marker.

For the injection of cosmid F20D12, *n2669* with roller phenotype showed almost perfect canal phenotype (Fig. 2.3A, Table 2.5).

For the injection of other cosmids in the region into *n2669,* none of the progeny with roller phenotype showed perfect canals (See Table 2.5).

2.4 Identifying the *exc-9* gene

Cosmid F20D12 was shown possibly to contain *exc-9,* but there are seven genes in this cosmid. To find out which gene is *exc-9,* RNAi experiments were done for each of the genes. pRF4 was used as co-injected as marker (see Materials and Methods for details). Only when injected with double-stranded RNA complementary to the F20D12.5 gene (Fig.2.2) into wild-type worms, did roller progeny show an abnormal excretory canal phenotype similar to *exc-9* mutants (6 out of 23 roller worms) (Fig. 2.3B). In some roller progeny, the tail structure was affected, showing similar deformed tails as do *exc-9* mutants (Fig 2.3C). This indicates that F20D12.5 is the gene that encodes *exc-9.*

To confirm this, I tested whether smaller fragments from the cosmid that contain only F20D12.5 could rescue *exc-9* or not. Cosmid F20D12 was cut into

Table 2.5 Cosmid preparation and injection.

Cosmid	Enzyme*	Roller progeny	rescue
C06A6	ApaLI	\	\
T06C10	PstI	\	\
C55F2	PstI	\	\
C49H3	XbaI	\	\
F20D12	FspI	30	5 out of 30
C18F3	XbaI	\	\
C26B2	EcoRV	\	\
F42G8	SacI,EcoRV, XbaI	15	No
Y7B5 (including F42G8, B0218)		7	No
F35H10	XhoI	10	No

* "Enzyme" is referring to the enzymes used to cut the cosmids in the *exc-9* region to test whether the cosmids are intact or not. The numbers for the "roller progeny" are the numbers of progeny with injection marker.

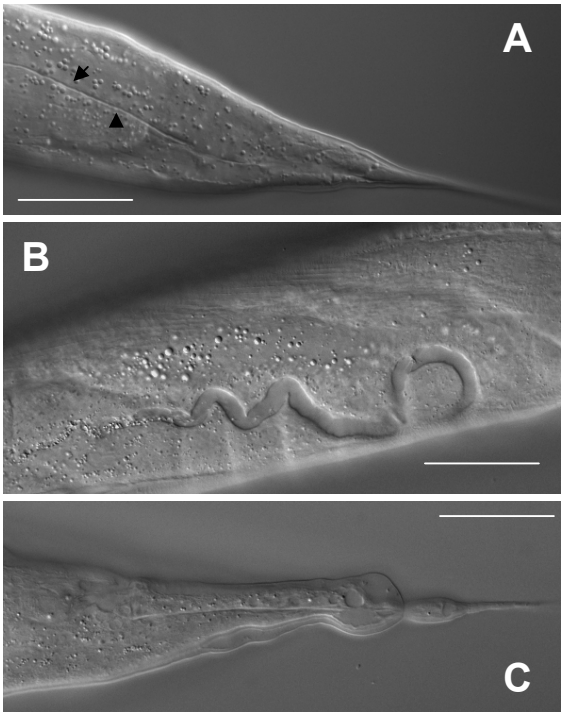


Figure 2.3 F20D12.5 encodes *exc-9*. (A) Canal in progeny of *exc-9 (n2669)* animal microinjected with F20D12.5 cosmid DNA. Canal lumen is restored to normal diameter (black arrowheads), and extends all the way to the tail. The tail whip phenotype is normal too. dsRNA (364 bp) corresponding to the *exc-9* 2nd and 3rd exons was injected into wild-type worms. Progeny shows wider canals (B) and malformed tail whips (C), similar to the phenotype of *exc-9(n2669)* mutants. Scale bar: 50 μ m.

three pieces using restriction enzyme FspI. Injection of a 11Kb fragment that contains the whole F20D12.5 coding region with promoter into *n2669* worms improved canal defects in the mutant background (4 out of 17 worms). A PCR fragment containing 2.2Kb of promoter and predicted coding region of F20D12.5 was made and also injected into *n2669* mutants. This construct rescued the *exc-9* mutant canal phenotype as well (4 out of 21 worms).

To determine where the mutation is in the F20D12.5 gene, I amplified this gene from *n2669* worms and sequenced through the predicted *exc-9* coding region. I found a C to T base-pair change in exon 3 on the sense strand, which is predicted to create an amber mutation (Fig. 2.2). This mutation would make the protein 30% shorter, and destroy the only identified conserved domain, LIM domain, in the EXC-9 protein.

We obtained a knockout strain VC976 (*gk395*) from the CGC with a large deletion of gene F20D12.5. The first two exons and part of the promoter of gene F20D12.5 is deleted in this allele (Fig. 2.2). Worms with allele *gk395* shows a similar extent of excretory canal defects as does allele *n2669*, and *gk395* failed to complement allele *n2669*; the heterozygotes had similar canal defects as did the two parental alleles (see Table 2.1). These data confirm that the gene F20D12.5 is *exc-9*, and indicates that allele *n2669* is likely a null allele, as is *gk395*.

2.5 cDNA analysis of *exc-9*

5'-RACE and 3'-RACE were done to determine the complete mRNA sequence of *exc-9*. I confirmed that the predictions of the coding region from Wormbase is accurate and found that an SL1 leader sequence is spliced onto 5'end of the *exc-9* mRNA.

2.6 Tissue-specific expression of *exc-9*

Another way to confirm the cloning data is the expression pattern of the cloned gene. To determine the expression pattern of *exc-9*, a GFP (Green Fluorescent Protein) reporter construct was made. The transcriptional GFP reporter for *exc-9* expression, called P*exc-9*::gfp (pBK104) was made by fusing 2.2kb of *exc-9* upstream sequence to the 5' end of the *gfp* coding region (Fig.2.4). The Fire vector pPD95.75 was used as backbone of this construct.

This construct was injected into wild-type worms to generate transgenic line carrying the extrachromosomal array of pBK104. The transgenic line showed expression of GFP in the excretory cell, the UTSE (uterus seam cell), tail spike, distal tip cell, ALM neuron, and PLN neuron (Fig.2.5). We also detected intestinal expression of GFP.

A translational GFP construct was made as well. 2.2kb upstream region of *exc-9* plus the whole *exc-9* genomic coding region (exons and introns, but no stop codon) was fused to the 5' end of *gfp* coding region in the backbone of pPD95.75. The coding region of *exc-9* is in frame with that of GFP (Fig. 2.4).

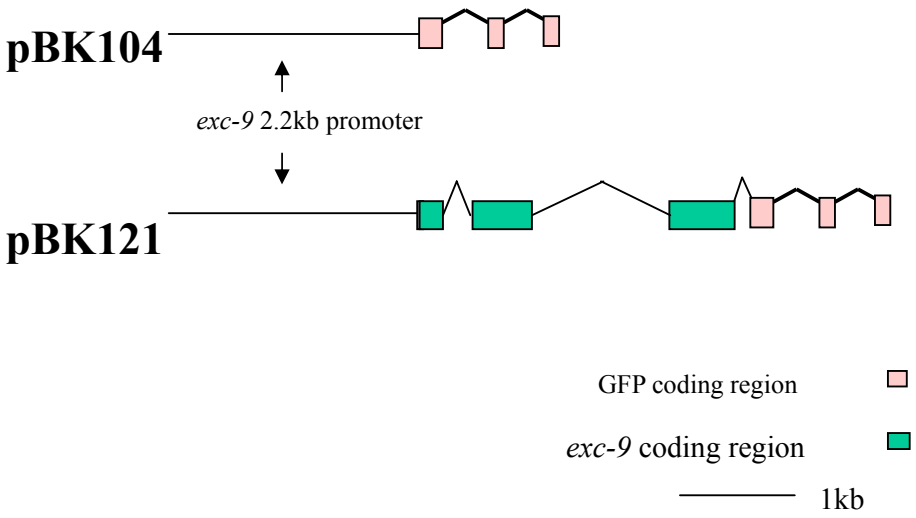


Figure 2.4 *exc-9* GFP construct structures. pBK104 is the transcriptional construct, and pBK121 is the translational construct. Both constructs have the 2.2 kb *exc-9* proter. pBK104 has the *gfp* coding region following the promoter. The promoter is followed by *exc-9* coding region and in-framed *gfp* coding region in pBK121.

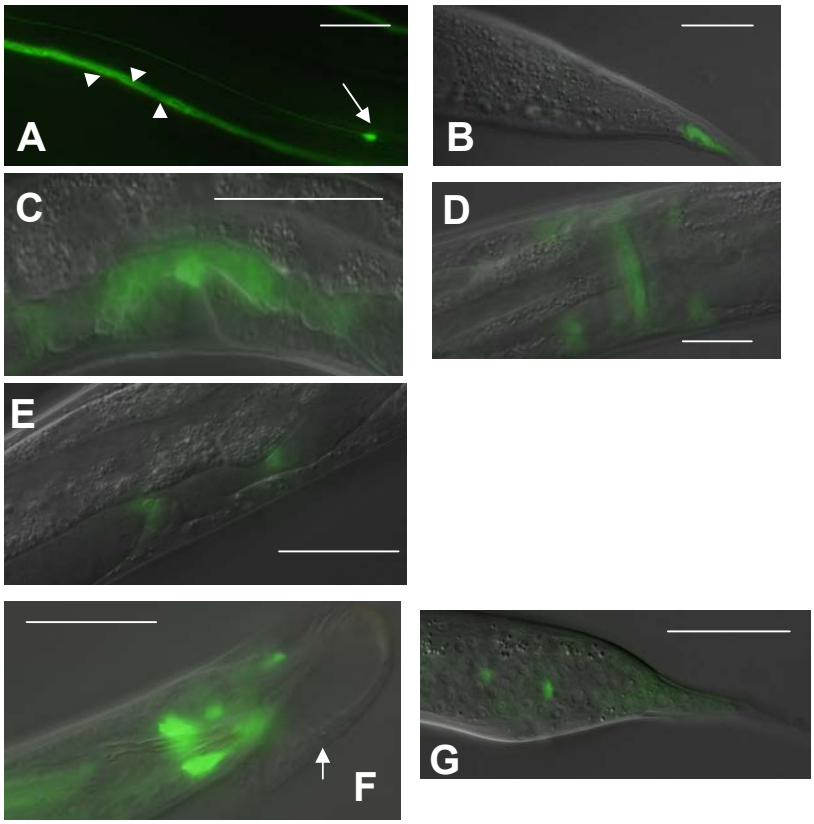


Figure 2.5 exc-*9* expression pattern. *exc-9* GFP reporter construct with the 2.2kb promoter region was found in the excretory canals (A) (as indicated by white arrowheads), and tail whip (B) that are affected in the mutants. *exc-9* is also expressed in the anchor cell (C), uterine seam cell (D), distal tip cells(E), and ALM neurons (A) indicated by white arrow. The GFP expression is not found in the male sensory rays indicated by white arrow (F), but is expressed in the male tail whip at an early stage of development (G). Anterior is to the left in all the pictures. Scale bars, 50 μm.

This construct is termed P*exc-9*::*exc-9*::*gfp* (pBK121). This construct was also introduced into wild-type background by means of injection, yielding a transgenic line carrying an extrachromosomal array of pBK121. This line showed the same expression pattern of GFP as did the transgenic line carrying the transcriptional construct pBK104.

To determine whether this translational construct was capable of rescuing the *exc-9* mutant phenotype, the construct was injected into *exc-9 (2669)* mutant worms. All the progeny with excretory canal GFP expression showed an improved canal phenotype (See Chapter 3, Table 3.4, Fig. 3.6 and Fig. 3.7).

Since both excretory canal and tail spike are affected in *exc-9* mutant background, and both of them showed *exc-9* expression, we wondered whether UTSE is affected in the *exc-9* background since it has EXC-9 expression as well. This examination was done by the laboratory of Dr. Wendy Hanna-Rose (Penn. State University, personal communication), who found that the UTSE does not appear to be affected in *exc-9* mutant background.

2.7 Expression pattern in male worms

The expression of the construct in male worms is similar to that in the hermaphrodite. The GFP expression was found in the excretory canal and neurons. Since male worms do not have a UTSE, it was not surprising that no GFP expression was found in the midsection of the body.

As mentioned earlier, male *exc-9* mutants show ray defects. Based on hermaphrodite expression pattern, I thought that the transcriptional construct expression would be found in the ray structure, just as EXC-9 expressed in the hermaphrodite tail spike.

To my surprise, EXC-9::GFP expression was not found in the ray structure. In early stages before the male tail developed completely, the tail structure of male is similar to that of the hermaphrodite. At those stages, GFP expression could be detected in the tail spike (Fig. 2.5G). When the tail was fully developed, however, the GFP expression disappeared from the region (Fig. 2.5F). This result suggests that ray structure maintenance once the rays are formed does not require the presence of EXC-9. But it is required to be in the tail to give some signals for proper male tail development in early stages.

2.8 Summary

exc-9 mutant worms show 100% penentrant canal defect. I used Snip-SNP mapping strategy narrowed down the region of this gene. By cosmid rescue, RNAi injection, complementation test, sequecing mutant and examing the expression pattern of the gene, I confirmed that gene F20D12.5 is *exc-9*.

Chapter 3

Characterizing the EXC-9 protein

3.1 *exc-9* encodes a single LIM domain protein

exc-9 encodes a small 85AA (amino acid)-protein. By using BLAST, CRIP (Cysteine rich intestinal protein) was found as the most closely related homologue. Mouse CRIP1 and EXC-9 share 62.8% identity and 72.1% similarity. The mouse CRIP1 is highly expressed in the intestine, and named because of this. Human CRIP1 is also known as CRHP (Cysteine rich heart protein) or CRP1 (Cysteine rich protein1). It has high expression levels in both intestine and heart in humans. This human homologue shows 74.4% identity and 62.8% similarity to EXC-9. In addition to the CRIP1 protein, mammals also have another LIM-domain-containing protein called CRIP2. This protein resembles two CRIP1 proteins that are stitched together. There also is a leech homologue that shows high similarity to EXC-9. Each of these homologues show high similarity in the N-terminal conserved LIM domain, but they all have shorter C-terminus than does EXC-9, although the C-terminus still show homology among these proteins. In addition to all these homologues in other organisms, there is another CRIP homologue in nematode. The locus of the corresponding gene in *C. elegans* is B0496.7. Compared to other EXC-9 homologues, protein B0496.7 shares much higher similarity in the unique C-terminus with EXC-9; overall it shows 81.2% identity and 88.2% similarity (Fig. 3.1 and Fig. 3.2).

The mouse homologue was shown to be important for Zn transport in

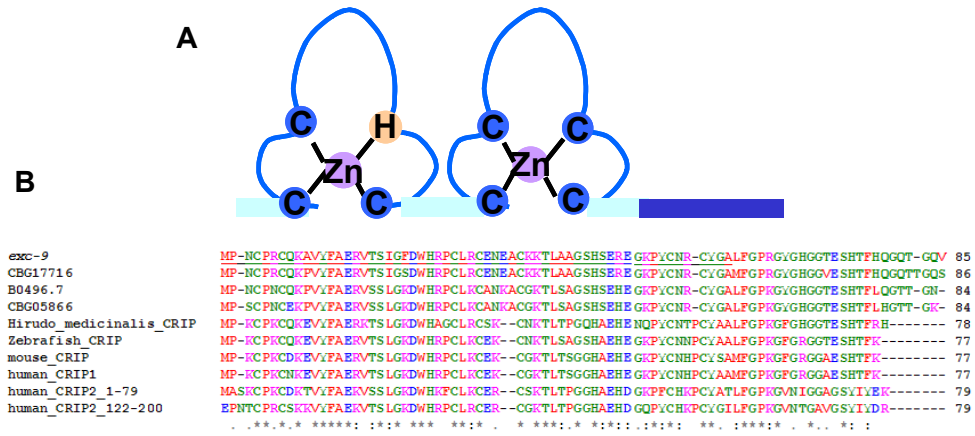


Figure 3.1 *exc-9* encodes the *C. elegans* CRIP homologue. (A) Schematic drawing of EXC-9 protein. (B) Clustal-W alignment of the EXC-9 protein to homologues. There is a paralogue B0496.7 in *C. elegans* that shows as high a similarity to *exc-9* as do other CRIP proteins. The underlined part is the LIM domain of EXC-9 protein. CBG proteins are the proteins from *C. briggsae.*

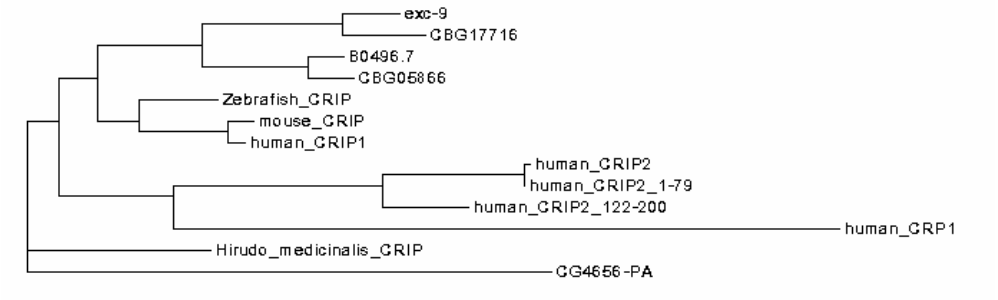


Figure 3.2 Phylogenetic tree of EXC-9.

intestine by *in vitro* experiment and is negatively regulated by metallothionein (Escobar, O., Sandoval, M. et al. 1995). Coursins and coworkers showed that mouse CRIP affects the immune response when overexpressed (Cousins, R.J. and Lanningham-Foster, L. 2000; Lanningham-Foster, L., Green, C.L. et al. 2002). CRIP in leech (*Hirudo Medicinalis*) stimulates neuron regeneration when overexpressed (Emes, R.D., Wang, W.Z. et al. 2003; Blackshaw, S.E., Babington, E.J. et al. 2004). Human CRIP was shown to have elevated expression in breast cancer cells (Ma, X.J., Salunga, R. et al. 2003), but is down-regulated in esophageal squamous cell carcinoma (Yamashita, K., Upadhyay, S. et al. 2002).

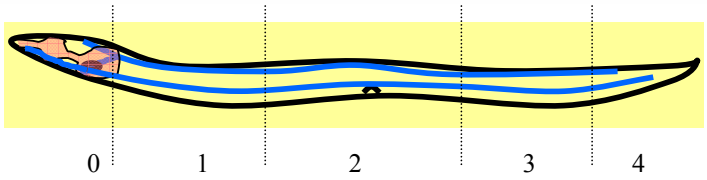
The homologues of EXC-9 didn't give obvious information about how EXC-9 regulates the apical surface of the excretory canal.

3.2 EXC-9 functions cell-autonomously

As mentioned in Chapter 2, injection of pBK121 (P*exc-9*::*exc-9*::*gfp*) into the *exc-9* background improves the canal phenotype, makes the canal longer and cysts shorter. Since the construct is an extrachromosomal array, the GFP expression is not as stable as are integrated arrays. Occasionally, the GFP expression in some tissues will be lost in the progeny. For the glowing worms that lost the construct's expression in the canal, 45% of them showed large cysts (n=20), but none of the worms with canal expression of GFP showed any large cysts (n=41, Table 3.4). The result suggests that EXC-9 is required to be present

Table 3.1 Canal phenotype of *exc-9 (gk395)* worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)		1		1		2
S (small)		28	9	2		33
M (medium)		52	9	1		52
L (large)		16				13
%		82	15	3		n=119



Length of the posterior canal are shown by showing the location of ending point. There are five catergories as shown in the figure above. Cyst size is also categorized as five levels: "N" for no cyst, "T" for tiny cyst of which the diameter is almost the same as canal diameter, "S" for small cyst that has diameter less than one quarter of the worm diameter, "M" for medium cyst of which the diameter is less than half of the worm diameter but larger than half of that, "L" for large cyst that has diameter larger than half of the worm diameter. Total number of canal screened is shown in the right bottom cell of the table. All the canal phenotype data table in the thesis are shown in the same way.

Table 3.2 Canal phenotype of F1 generation of *exc-9 (gk395)* injected with pBK122.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)						
S (small)		7	8	15	1	66
M (medium)		5	9	2		34
L (large)						
%		26	36	36	2	n=47

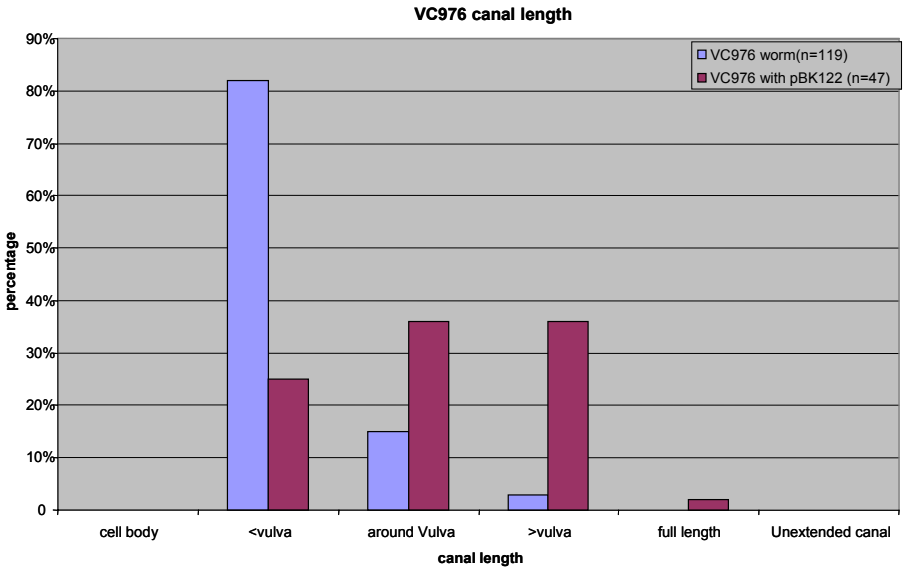


Figure 3.3 Canal ending point in *exc-9 (gk395)* worms with or without pBK122.

Diagramed using the data from Table 3.1 and Table 3.2.

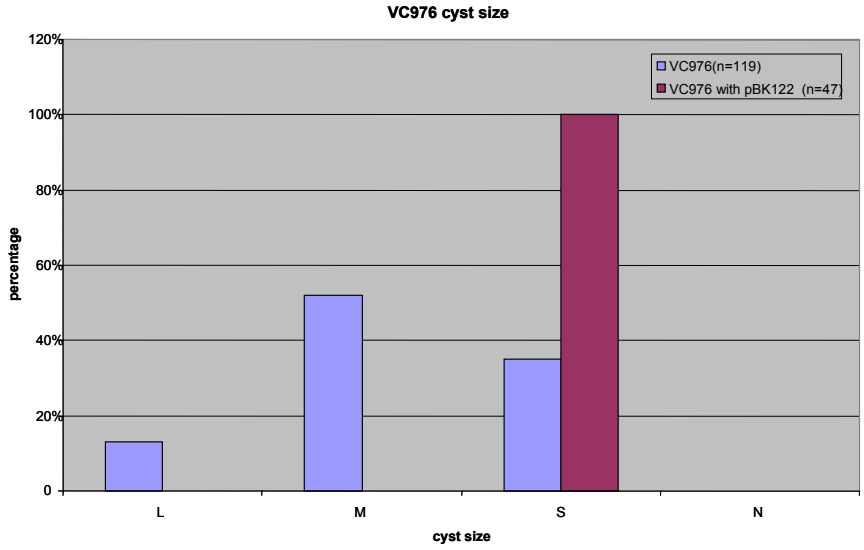


Figure 3.4 Canal cyst size in *exc-9 (gk395)* worms with or without pBK122.

Diagramed using the data from Table 3.1 and Table 3.2.

in the canal to maintain the tubular structure.

To determine whether canal expression of *exc-9* is sufficient for rescue of the *exc-9* mutant phenotype, I made a construct (pBK122) that expresses *exc-9* mainly in the canal. This construct has the *vha-1* promoter that drives gene expression mainly in the excretory canal. The genomic coding region of *exc-9* was fused downstream of the *vha-1* promoter and followed by GFP coding region in-frame (Fig. 3.10). The injection of this construct into the *exc-9* background showed GFP expression in the canals but not in the tailspike, and it rescued the *exc-9* cystic canal phenotype, but not the tail spike phenotype. 64% of the glowing worms still exhibited various extent of tail defects (n=47). Since this construct is not expressed in the tail spike, it is consistent with the rescue result.

In summary, EXC-9 protein function cell-autonomously in the canals. It is required to be expressed in the canal and expression solely in the excretory cell is sufficient to maintain the small diameter of the canals.

3.3 Overexpression of EXC-9 causes a new canal phenotype

Overexpression pBK121 (P*exc-9*::*exc-9*::*gfp*) causes a phenotype similar to that caused by overexpression of *exc-5* translational construct (Suzuki, N., Buechner, M. et al. 2001). After injection of pBK121 into *exc-9* worms, some of the progeny with brighter GFP expression showed normal diameter canals, but the canals didn't extend to the posterior part of the worms. Instead, the canals

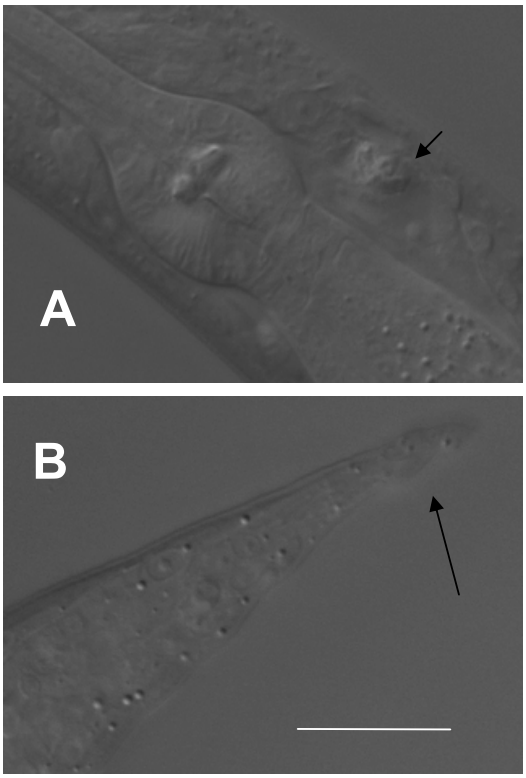


Figure 3.5 Over-expression of a translational *exc-9*::*gfp* fusion construct (pBK121) causes a new phenotype similar to the over-expression of an *exc-5* translational construct (Suzuki, N., Buechner, M. et al. 2001) in both *exc-9* and wild-type backgrounds; we call this phenotype an "unextended" canal. (A) In a wild-type worm containing the over-expression construct, the canal lumen diameter is normal, but the canals can't extend to the posterior. The lumen, indicated by black arrow, is rolled up inside the cell body. The tail whip is sometimes distorted also (B). The anterior is to the left in the pictures. Scale bar: 25μm

Table 3.3 Canal phenotype of *exc-9 (n2669)* worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)			1	4		5
S (small)	1	58	10	9		75
M (medium)		18		1		18
L (large)		2				2
%	1	75	11	13		n=104

Table 3.4 Canal phenotype of F1 generation of *exc-9 (n2669)* injected with pBK121

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	10	3		1		34
T (tiny)						
S (small)		5	14	4		56
M (medium)		3		1		10
L (large)						
%	24	27	34	15		n=41

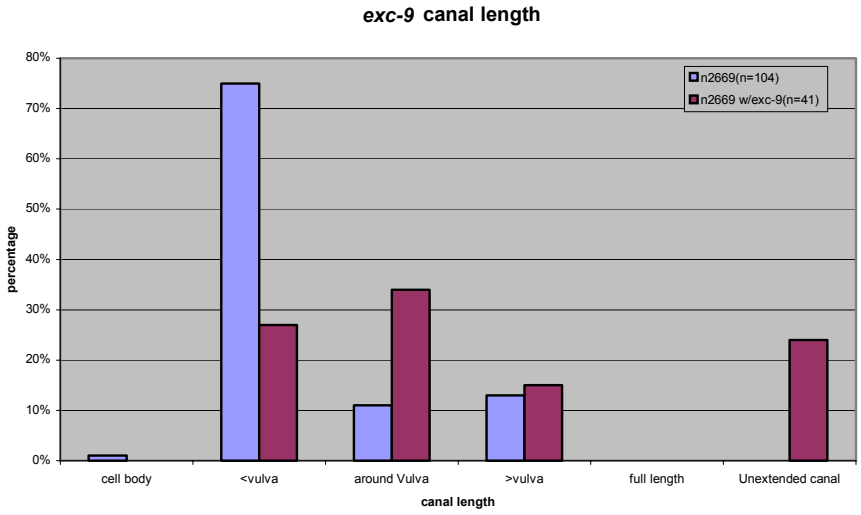


Figure 3.6 Canal ending point in *exc-9 (n2669)* worms with or without pBK121.

Diagramed using the data from Table 3.3 and Table 3.4.

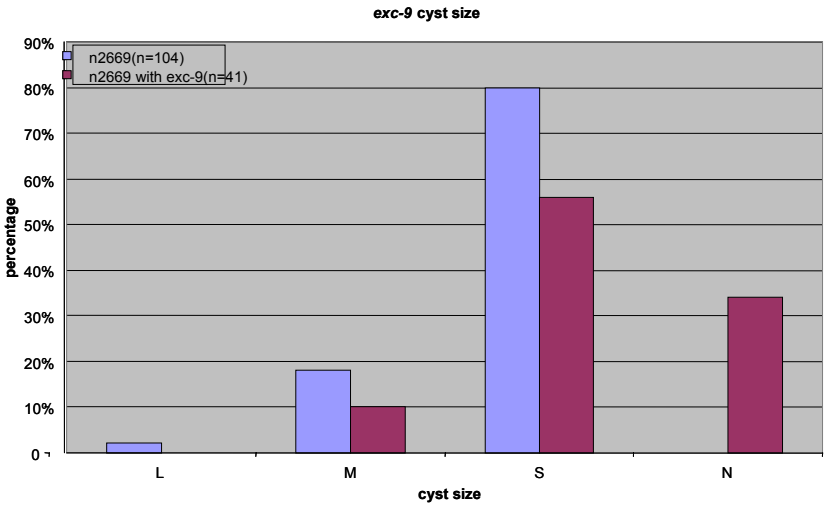


Figure 3.7 Canal cyst size in *exc-9 (n2669)* worms with or without pBK121.

Diagramed using the data from Table 3.3 and Table 3.4.

Table 3.5 Canal phenotype of N2 worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)					100	100
T (tiny)						
S (small)						
M (medium)						
L (large)						
%						n=100

Table 3.6 Canal phenotype of F1 generation of N2worm injected with pBK121.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	10				34	100
T (tiny)						
S (small)						
M (medium)						
L (large)						
%	23				77	n=44

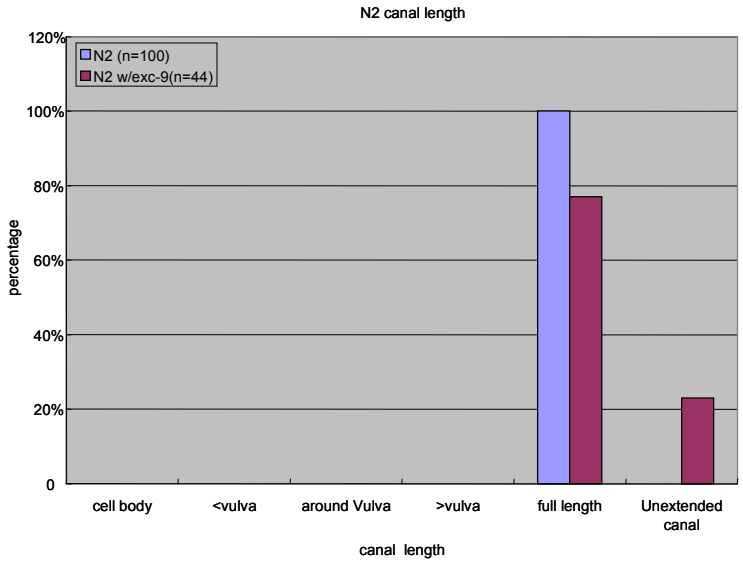


Figure 3.8 Canal ending point in N2 worms with or without pBK121. Diagramed using the data from Table 3.5 and Table 3.6.

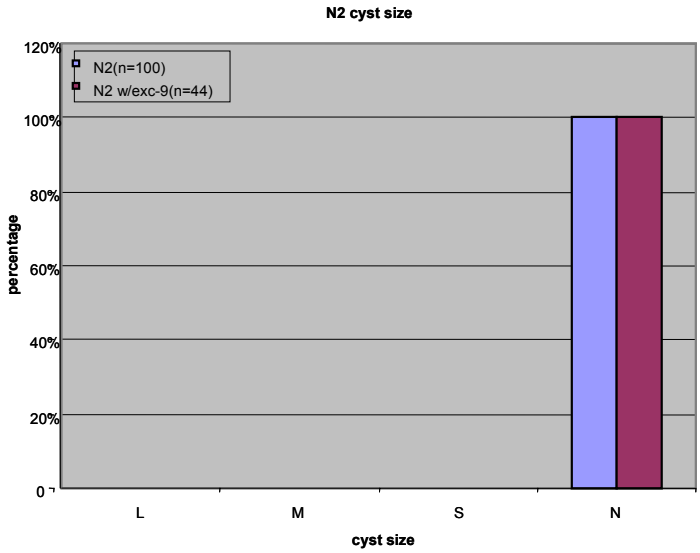


Figure 3.9 Canal cyst size in N2 worms with or without pBK121. Diagramed using the data from Table 3.5 and Table 3.6.

circled inside the canal cell body (Fig. 3.5A). I called this phenotype "unextended canal".

To determine if unextended canals occurred only in the *exc-9* background, the same construct was injected into N2 worms. A similar unextended phenotype was observed in these animals (Table 3.6, Fig. 3.8). N2 worms with unextended canal also occasionally had abnormal tail spike (Fig. 3.5B).

These results suggest that the canal extension and tail spike assembly are sensitive to the copy number of the *exc-9* gene. To ensure that this phenotype was caused by high levels of the EXC-9 protein, the transgenic N2 line with pBK121 was fed on a GFP RNAi feeding plate. As the level of EXC-9::GFP was reduced, animals with unextended canals were increasingly difficult to find. This result indicated that the unextended canals are caused by overexpression of *exc-9*.

3.4 A free N-terminus is essential for unextended canal phenotype

Injection of construct pBK122 (P*vha-1*::*exc-9*::*gfp*) rescued cystic canal defects of *exc-9* mutants, but the unextended canal phenotype was not observed in any of the glowing progeny (Table 3.4, Fig. 3.6). After inspection, I found out that the endogenous start codon of *vha-1* is in the plasmid pBK122, and it is in-frame with the *exc-9* coding region. The chimeric protein translated from this construct has eleven extra amino acids at the N-terminus (Fig. 3.10). These extra

amino acids may reduce EXC-9 activity and explain why no worms with unextended canal were found.

To prove this hypothesis, a new construct was made. The *vha-1* endogenous start codon was cut out from the pBK122 along with the *vha-1* promoter. Then the amplified *vha-1* promoter alone was ligated back to the same backbone. From this construct, the product should be a protein with the complete EXC-9 followed by GFP. This construct is named pBK130 (Fig. 3.10).

I injected this construct into *exc-9* mutants, and screened for canal phenotypes in the progeny. I found progeny with glowing canals showed unextended canal phenotypes (Table3.7, Fig. 3.11).

These results indicate that extra amino acids partially impaired the function of the EXC-9 N-terminus. Without the free N-terminus, EXC-9 protein could function to maintain the canal structure. But only when the free N-terminus is unblocked, the canals could respond to the excess amount of EXC-9 and fail to extend to the posterior, form the unextended canals.

3.5 EXC-9 and CSN-5 physically interact in a yeast two-hybrid assay

It is proposed that LIM domain proteins function by binding with other proteins (Kadrmas, J.L. and Beckerle, M.C. 2004). To find out the possible binding partner of EXC-9, a yeast two-hybrid assay was chosen to fulfill this goal.

The cDNA of *exc-9* was purified and sent to the Molecular Interaction

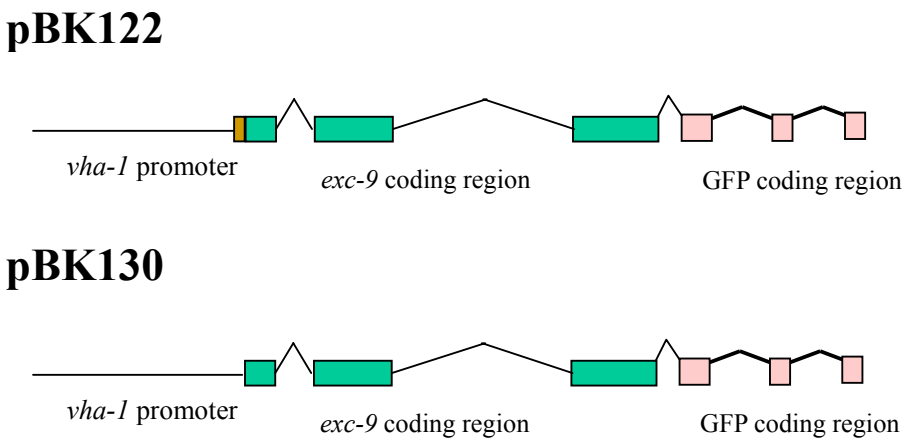


Figure 3.10 Structure of vector pBK122 and vector pBK130. both constructs have *vha-1* promoter and *exc-9, gfp* coding region. The difference is that pBK122 has eleven extra amino acids at the N-terminus start from *vha-1* endogenous start codon (brown block).

Table3.7 F1 generation of *exc-9* (gk395) animals injected with pBK130.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	6				3	21
T (tiny)		1	12	10	2	60
S (small)		1	2	4		17
M (medium)		1				2
L (large)						
%	14	7	33	33	12	n=42

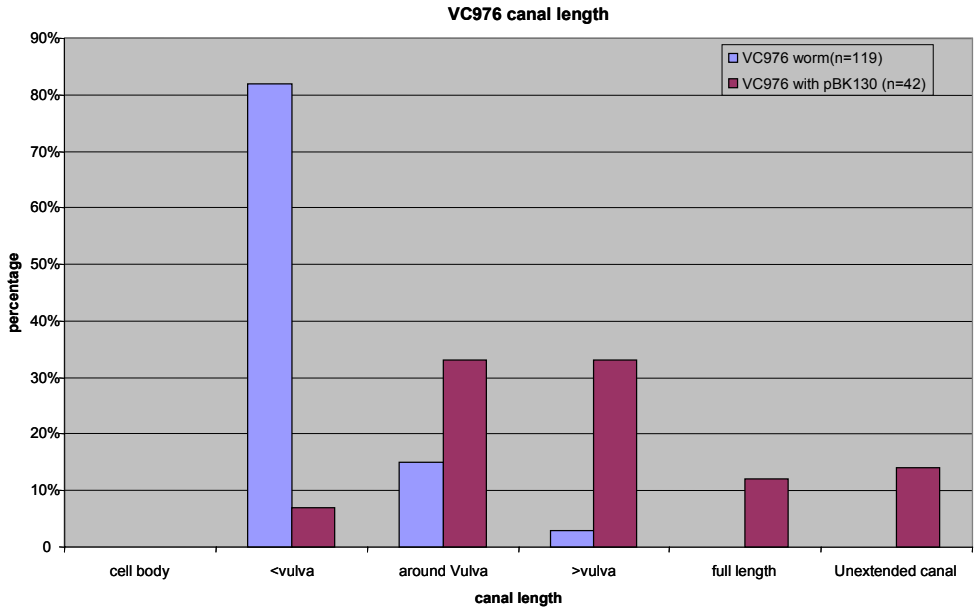


Figure3.11 Canal ending point in *exc-9 (gk395)* worms with or without pBK130. Diagramed using the data from Table 3.1 and Table 3.7.

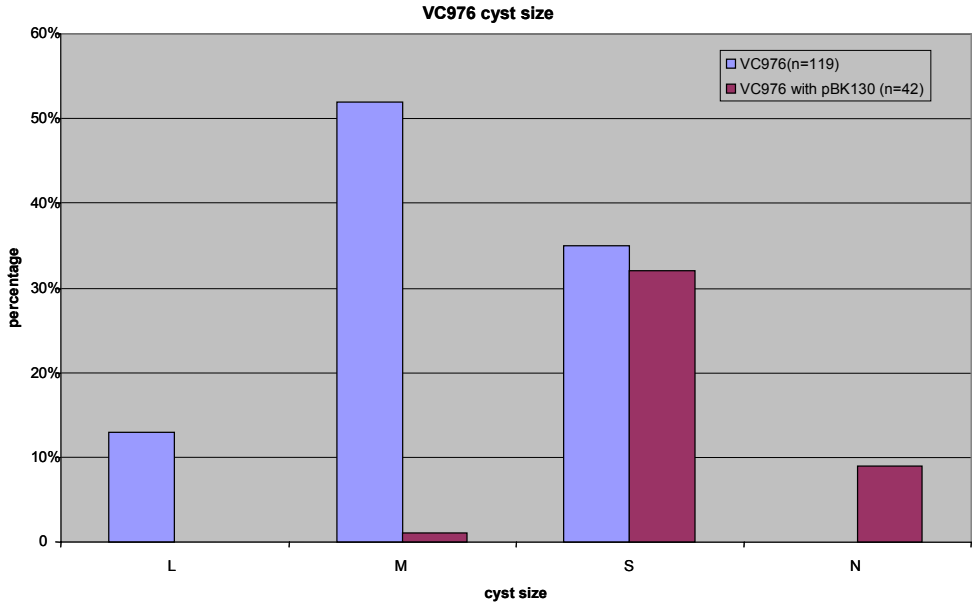


Figure3.12 Canal cyst size in *exc-9 (gk395)* worms with or without pBK130. Diagramed using the data from Table 3.1 and Table 3.7.

Facility of University of Wisconsin, Madison for a yeast two-hybrid assay with other *C. elegans* genes.

The *exc-9* complete cDNA was ligated into bait vector. The prey library was made from RNA isolated from mixed stage *C. elegans* hermaphrodites. The screen was conducted at low stringency to maximize the chance to find even weak interactors. Approximately 36 million clones were screened via mating. Five putative interactors were isolated with the *exc-9* bait. One of the colonies was eliminated as a false positive because of its ability to interact with the empty bait vector. After sequencing, all the other four validated prey turned out to be *csn-5*, which is a *C. elegans* COP-9 SigNalosome subunit family member. The COP-9 SigNalosome is involved in protein degradation.

A loss of function mutant strain of *csn-5* was then acquired from the CGC. The strain VC861 *csn-5 (ok1064) IV/ nT1 122*(IV; V) didn't show any canal defects (n>100).

To determine whether the lack of canal defects is because the strain is heterozygous, dsRNA complementary to *csn-5* was made and injected into N2 worms, with pRF4 as marker. None of the roller progeny showed any canal defects (n=35).

Another approach was to find out whether *csn-5* is expressed in the canal. The promoter of *csn-5* was amplified and ligated into the pPD95.75 vector. No GFP expression was detected in the canal or any other tissues with *exc-9*

expression.

The studies of *csn-5* in *C. elegans* suggest that CSN-5 does not work with EXC-9, which conflicts with the yeast two-hybrid data. Since CSN-5 shows strong affinity with EXC-9 in the yeast two-hybrid experiment, it is possible that these two proteins have potential to bind with each other. Because they don't have an overlapping expression pattern, they don't bind with each other *in vivo*.

3.6 EXC-9 antibody work

EXC-9 antibodies were made by Sigma-Genosys (The Woodlands, TX). One antibody is against a synthetic peptide from the mid region of the LIM domain, and the other is a synthetic peptide from the end of the LIM domain and part of the C-terminus region (Fig. 3.13).

Wild-type and mutant worm extracts were isolated from N2 and *exc-9 (n2669)* worms. A western blot was carried out. In the western blot, a difference was found between *exc-9* and N2 worm. N2 worms show a band at around 20KD, which is lacking or less abundant in the *exc-9* lanes (Fig. 3.14). This size is about double the size expected for EXC-9, which is 9.5KD.

We speculated whether this is because EXC-9 forms a homo-dimer through cysteine in the worm. Since the dimer will have a similar structure as mammalian CRIP2, the dimer might function similarly to CRIP2 as well. The reason I saw a dimer size instead of monomer size on the film might be

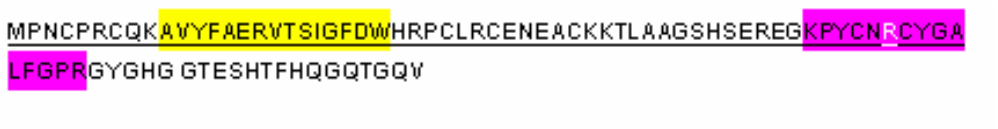


Figure 3.13 Antibody design of EXC-9. The underlined part is the LIM domain. Antibodies 14649 and 14650 are against the peptide highlighted in yellow. Antibodies 14651 and 14652 are against the peptide highlighted in magenta. The amino acid in white is mutated in allele *n2669*.

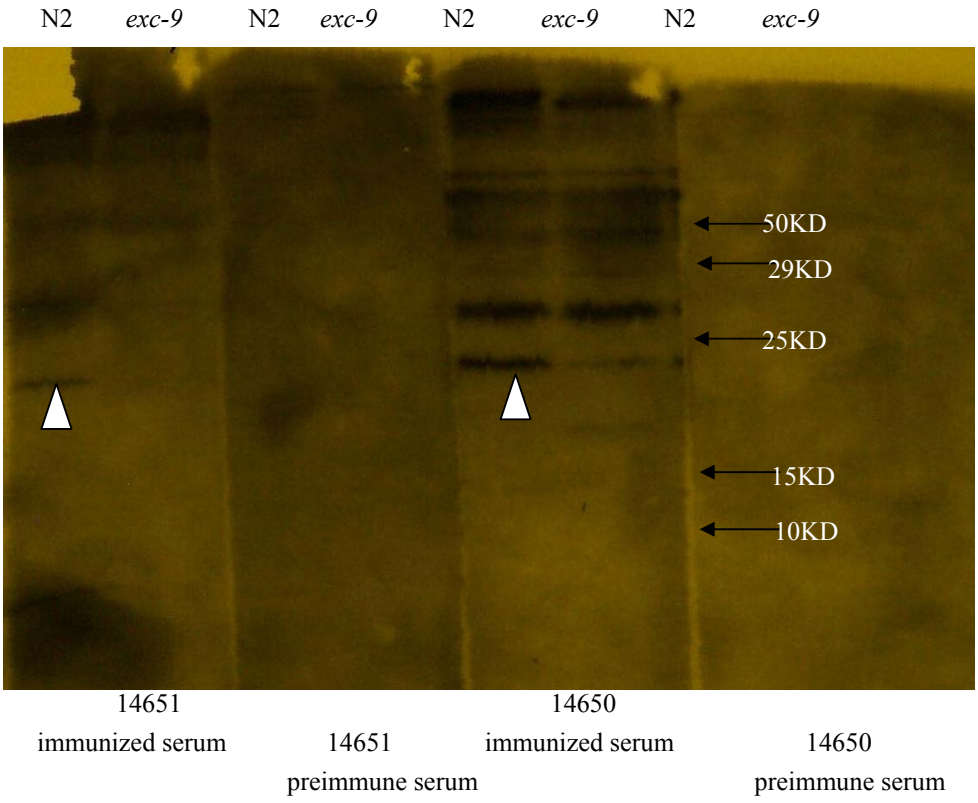


Fig 3.14 Western blot gel picture. White arrowheads indicate the different band in N2 lanes. The sample names are listed on the top and the seraum names are listed on the bottom of the blot.

because disulfide bonds were not broken completely in the SDS PAGE gel. Another possible reason is that these two antibodies are not specific enough. I performed the worm staining four times using these two antibodies on both N2 and *exc-9 (n2669)* worms. Intestinal staining was detected but none of the strains showed excretory canal specific staining. It didn't exclude the possibility that the canals are narrow and don't show antibody staining as well since SMA-1 antibody didn't stain the canal either (personal communication).

3.7 Conclusion

EXC-9 translational construct not only rescues *exc-9* mutant phenotype, it also causes a new phenotype called "unextended canal" when overexpressed. Canal specific expression of EXC-9 suggests that the protein function cell-autonomously and the unblocked N-terminus is important for the "unextended canal" phenotype.

CSN-5 was pulled out as a binding partner of EXC-9 in yeast two-hybrid assay. But study of mutant phenotype and expression pattern didn't support they bind to each other *in vivo*.

Chapter 4

Genetic interactions between *exc-9* and other *exc* genes

4.1 overexpression of *exc-9* in other *exc* backgrounds

As mentioned in Chapter 3, overexpression of an *exc-9* translational construct (pBK121) causes unextended canals in both *exc-9* mutant and wild-type backgrounds. We want to test whether this construct could cause a similar phenotype in the background of other *exc* mutants as well.

If this construct could cause similar phenotype in a mutant background, it is possible that EXC-9 works downstream of this protein; if not, the protein might function downstream of EXC-9 to control the canal diameter. If there is no reciprocal rescue, it is possible that the two proteins function in parallel pathways.

Since all the *exc* mutants have 100% penetrance canal defect, I checked whether the canal phenotype of those *exc* mutants got improved or not with the injection of *exc-9* translational construct.

4.2 Injection of pBK121 into *exc-4* background

EXC-4 is a *C. elegans* chloride intracellular channel homologue (Berry, K.L., Bulow, H.E. et al. 2003). It has been shown by means of heat-shock experiment that function of this gene in early stages of worm development is essential for normal canal structure (Berry, K.L., Bulow, H.E. et al. 2003). How EXC-4 regulates the excretory canal structure is not clear. It has been proposed that EXC-4 likely helps to acidify the fusing compartments, and in turn helps to

Table 4.1 Canal phenotype of *exc-4* (*rh133*) worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)						
S (small)	13	9				32
M (medium)	6	13				28
L (large)	20	8				40
%	57	43				n=69

Table 4.2 Canal phenotype of F1 progeny of *exc-4 (rh133)* injected with pBK121.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	6					
T (tiny)						
S (small)	3	10				57
M (medium)	1	5				26
L (large)		4				17
%	17	83				n=29

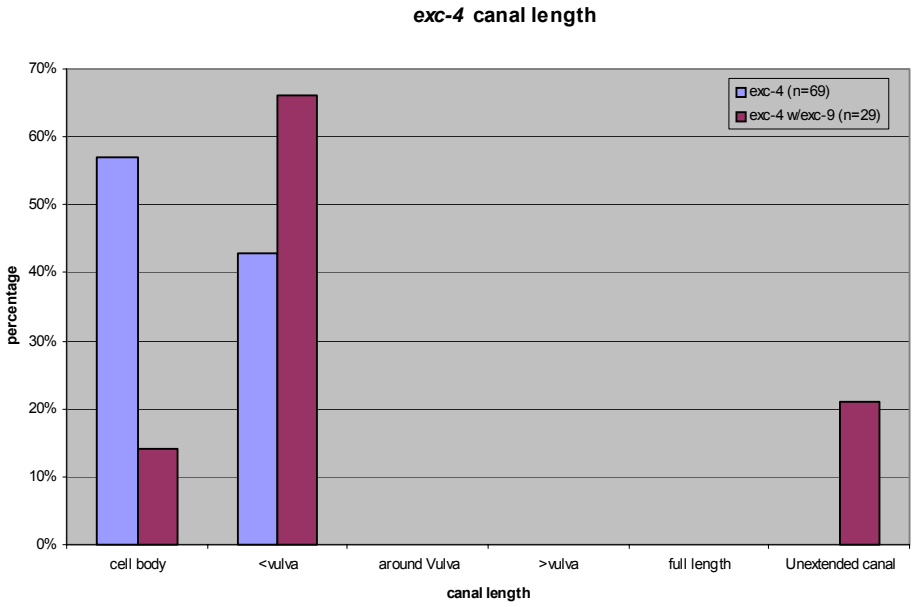


Figure 4.1 Canal ending point in *exc-4 (rh133)* worms with or without pBK121Diagramed using the data from Table 4.1 and Table 4.2.

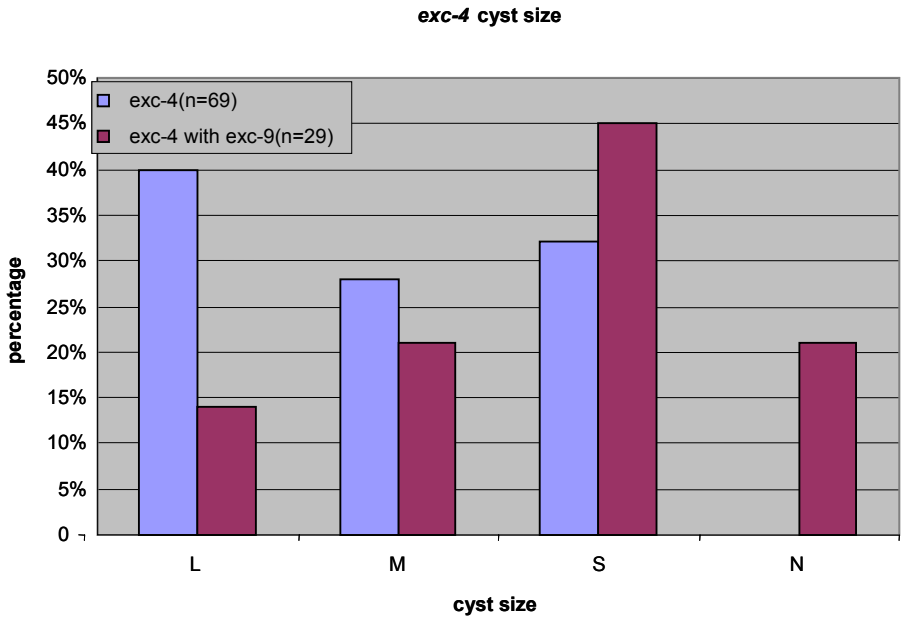


Figure 4.2 Canal cyst size in *exc-4 (rh133)* worms with or without pK121. Diagramed using the data from Table 4.1 and Table 4.2.

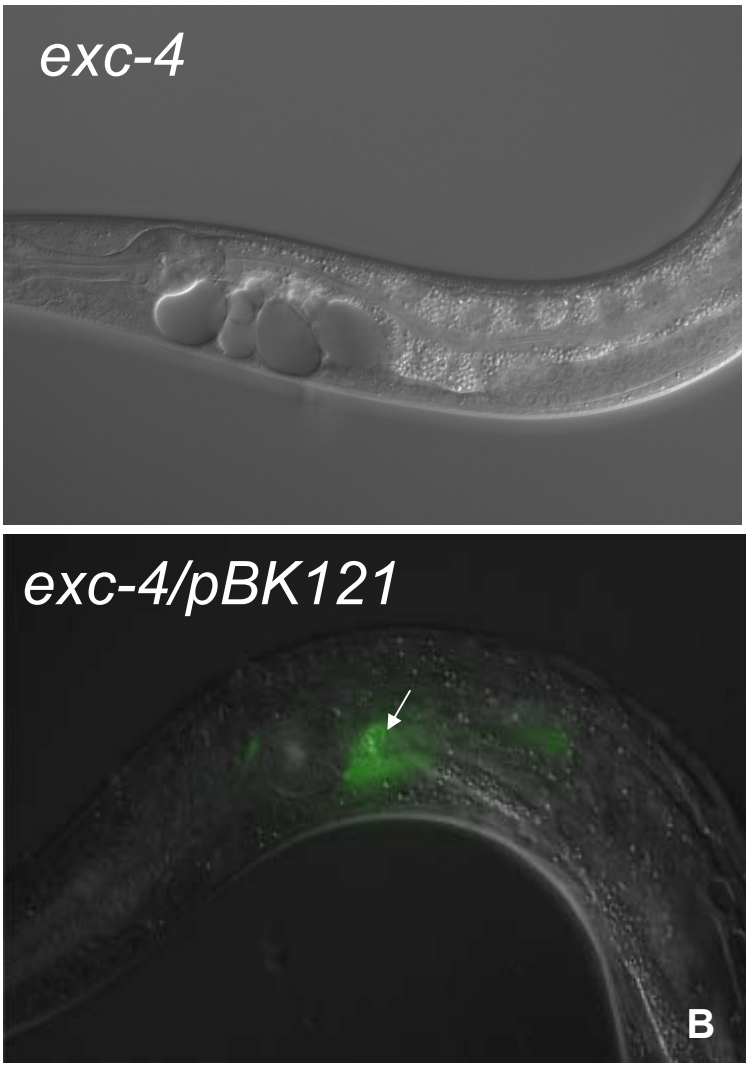


Figure 4.3 *exc-4* mutant worms with or without *exc-9* translational construct. (A) An *exc-4 (rh133)* mutant that showed large cysts next to the pharynx. (B) An *exc-4 (rh133)* with pBK121 (*exc-9::gfp*) construct showed unextended canal instead of cystic canal (indicated by white arrow).

maintain the canal structure, or it may control canal diameter by regulating water transport (Berry, K.L., Bulow, H.E. et al. 2003).

Injection of pBK121 into *exc-4 (rh133)* worms improved the canal defects of the mutants in F1 generation, and some worms with unextended canal phenotype were found (Table 4.1, Table 4.2 and Fig. 4.1, 4.2). In the transgenic line of *exc-4 (rh133)* worms carrying pBK121, almost 100% of the glowing worms showed unextended canals, as seen in Figure 4.3. The reason for this difference between F1 generation and stable line might be due to maternal effect in the transgentic line.

4.3 Interactions between *exc-5* and *exc-9*

EXC-5 is a putative guanine nucleotide exchange factor of CDC-42 (Gao, J., Estrada, L. et al. 2001; Suzuki, N., Buechner, M. et al. 2001). EXC-5 is a homologue of human FGD1, which plays an important role in skeletal formation (Gao, J., Estrada, L. et al. 2001). Overexpression of EXC-5 also causes the unextended canal phenotype (Suzuki, N., Buechner, M. et al. 2001). Since *exc-5* and *exc-9* mutants have similar phenotypes and overexpression of respective translational constructs caused a similar unextended canal phenotype, it is likely that these two genes work in the same pathway.

Injection of a *exc-9* translational construct into *exc-5 (rh232)* didn't create any unextended canal worms or improve the canal phenotype (Table 4.3 and Table

Table 4.3 Canal phenotype of *exc-5 (rh232)* worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)	1	1		2		5
S (small)	17	15	6			44
M (medium)	20	8				32
L (large)	17					20
%	63	28	7	2		n=87

Table 4.4 Canal phenotype of F1 progeny of *exc-5 (rh232)* injected with pBK121.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	2					1
T (tiny)						
S (small)	4	61	5	2	2	33
M (medium)	13	42	15			31
L (large)	11	59	9			35
%	13	72	13	1	1	n=225

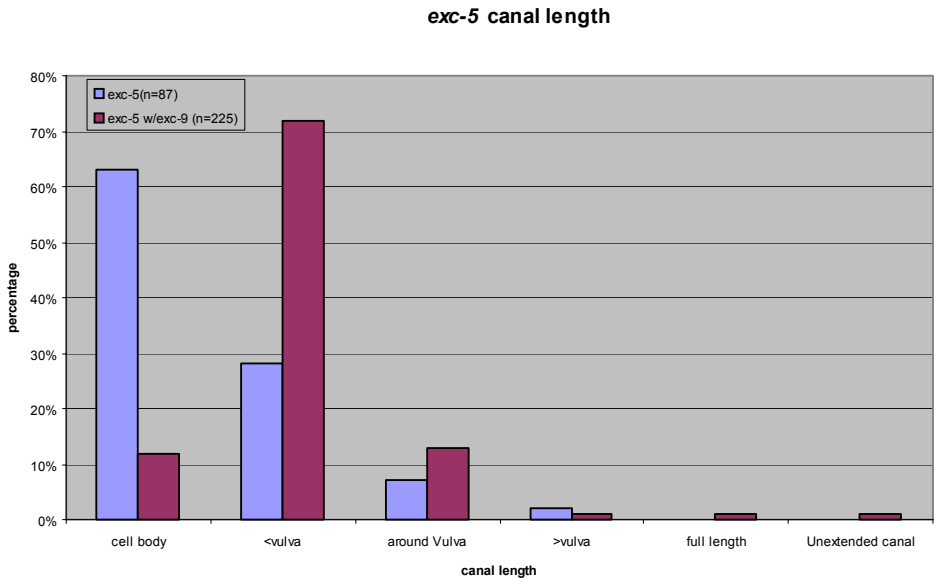


Figure 4.4 Canal ending point in *exc-5 (rh232)* worms with or without pBK121.Diagramed using the data from Table 4.3 and Table 4.4.

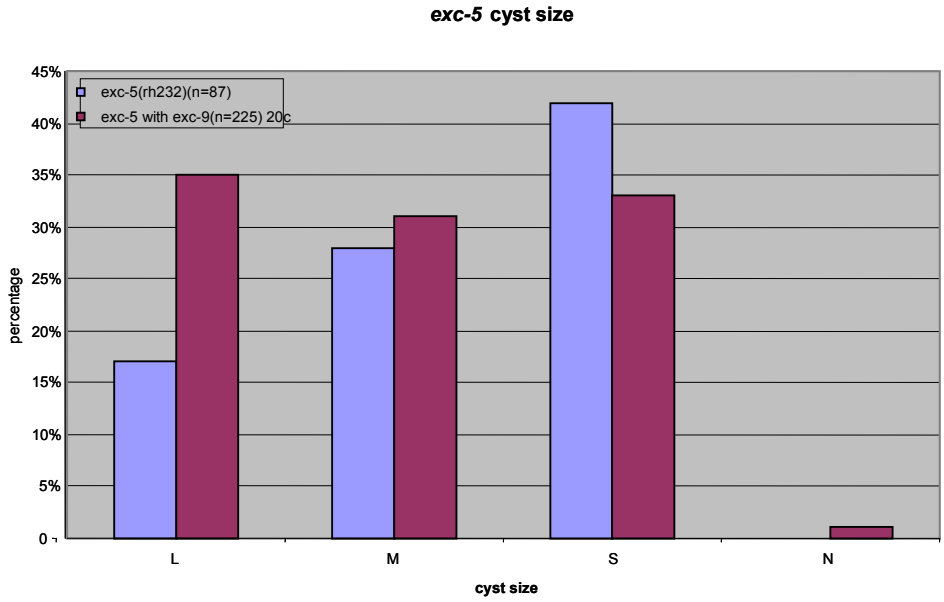


Figure 4.5 Canal cyst size in *exc-5 (rh232)* worms with or without pBK121.

Diagramed using the data from Table 4.3 and Table 4.4.

Table 4.5 Canal phenotype of *exc-9 (gk395)* worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)		1		1		2
S (small)		28	9	2		33
M (medium)		52	9	1		52
L (large)		16				13
%		82	15	3		n=119

Table 4.6 Canal phenotype of F1 progeny of *exc-9 (gk395)* injected with pBK121.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	12					17
T (tiny)		2	11	14	4	43
S (small)		9	12	8		40
M (medium)						
L (large)						
%	17	15	32	31	6	n=72

Table 4.7 Canal phenotype of F1 progeny of *exc-9 (gk395)* worms injected with P*exc-5::exc-5*::*gfp*.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	26		1			84
T (tiny)						
S (small)	4			1		16
M (medium)						0
L (large)						
%	94		3	3	0	n=32

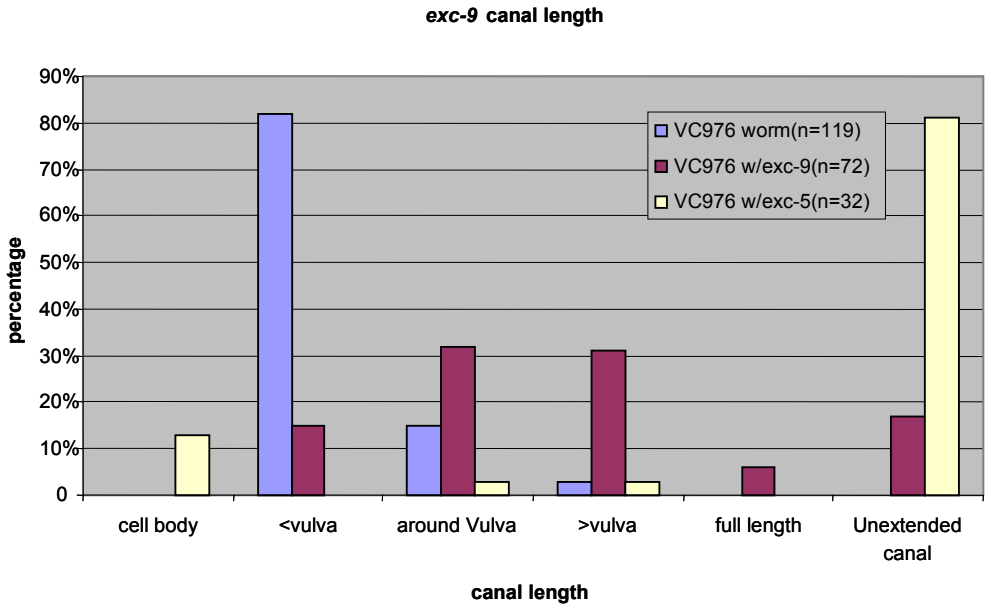


Figure 4.6 Canal ending point in *exc-9 (gk395)* worms with or without pBK121, and with P*exc-5::exc-5*::*gfp*. Diagramed using the data from Table 4.5, 4.6 and 4.7.

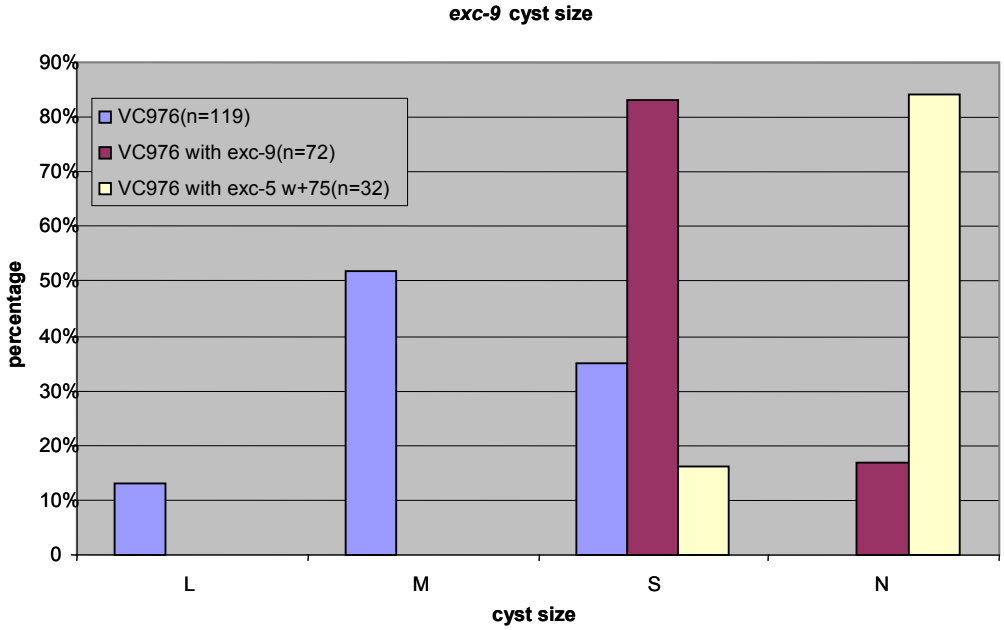


Figure 4.7 Canal cyst size in *exc-9 (gk395)* worms with or without pBK121, and with P*exc-5::exc-5*::*gfp*. Diagramed using the data from Table 4.5, 4.6 and 4.7.

4.4, Fig4.4 and Fig 4.5). On the other hand, injection of a *exc-5* translational construct into *exc-9 (gk395)* caused a high percentage of unextended canals (Table4.5, Table4.6, Table4.7, Fig4.6 and Fig 4.7).

4.4 Injection of pBK121 into *exc-2* background

exc-2 has not been identified. The *exc-2* mutant is one of the sickest *exc* mutants. Over 40% of the *exc-2* worms show large cysts in the canal, larval lethality was observed in *exc-2* mutants. Injection of pBK121 into *exc-2* mutants greatly improved the canal defects in the progeny. The percentage of worms with large cyst dropped dramatically (Table4.8 and Table 4.9; Fig.4.8 and Fig 4.9). These suggest that EXC-9 functions downstream of EXC-4.

4.5 *sma-1* worms with *exc-9*

The *sma-1* gene encodes β_H-Spectrin that is locate on the apical membrane of the excretory canal (Praitis, V., Ciccone, E. et al. 2005). To our surprise, *exc-9* construct could partially rescued *sma-1* mutants (Table4.10 and Table 4.11; Fig.4.10 and Fig.4.11). Indicates that *exc-9* plays a role downstream of *sma-1*.

4.6 Injection of pBK121 into *exc-1* worm

exc-1 is another uncloned gene. After injection with *exc-9* translational construct into parents, the progeny with glowing canals didn't show any improvements

Table 4.8 Canal phenotype of *exc-2 (rh90)* worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)						
S (small)		33	1			27
M (medium)	2	33	1			29
L (large)	2	52	1			44
%	3	95	2			n=125

Table 4.9 Canal phenotype of F1 progeny of *exc-2 (rh90)* injected with pBK121.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	14					12
T (tiny)			4			3
S (small)		23	48	8		68
M (medium)		11	5	2		15
L (large)		2				2
%	12	31	49	9		n=117

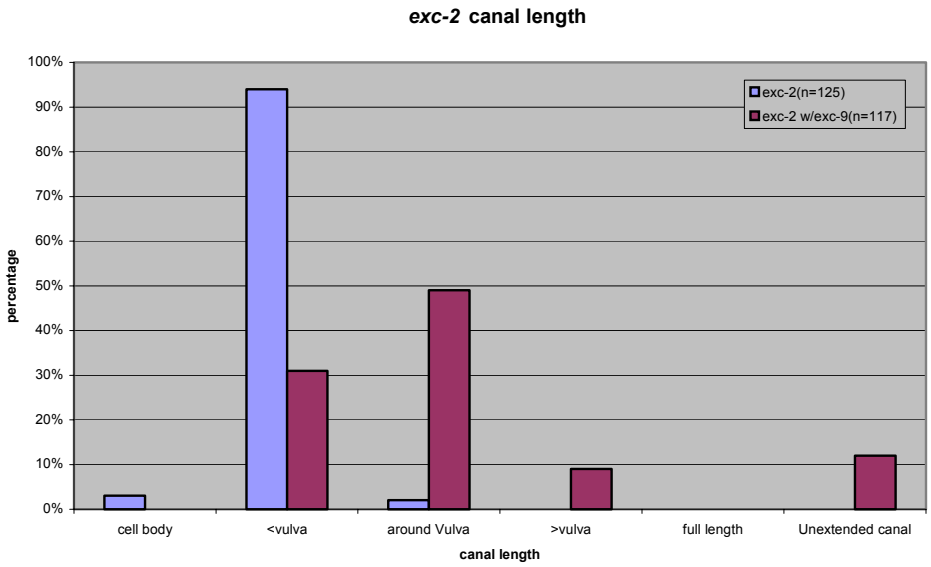


Figure 4.8 Canal ending point in *exc-2 (rh90)* worms with or without pBK121.

Diagramed using the data from Table 4.8 and Table 4.9.

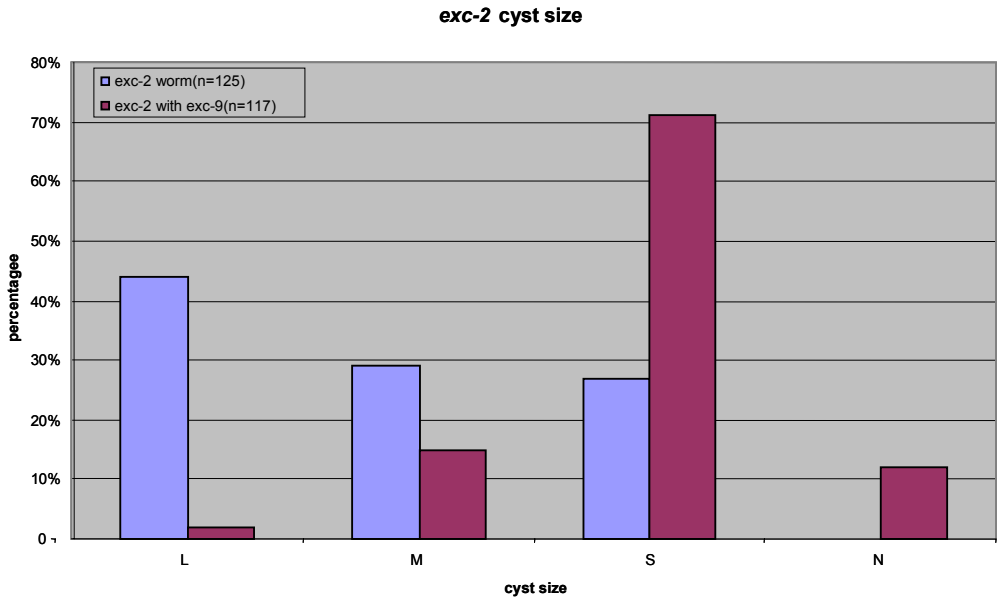


Figure 4.9 Canal cyst size in *exc-2 (rh90)* worms with or without pBK121.

Diagramed using the data from Table 4.8 and Table 4.9.

Table 4.10 Canal phenotype of *sma-1 (ru18)* worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)		5		1		11
S (small)		36	6	3		80
M (medium)		3	1	1		9
L (large)						
%		79	13	9		n=56

Table 4.11 Canal phenotype of F1 progeny of *sma-1 (ru18)* injected with pBK121.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)	26				1	20
T (tiny)		11	7	1		14
S (small)		62	18	3		63
M (medium)		2				2
L (large)		1				1
%	20	58	19	3	1	n=132

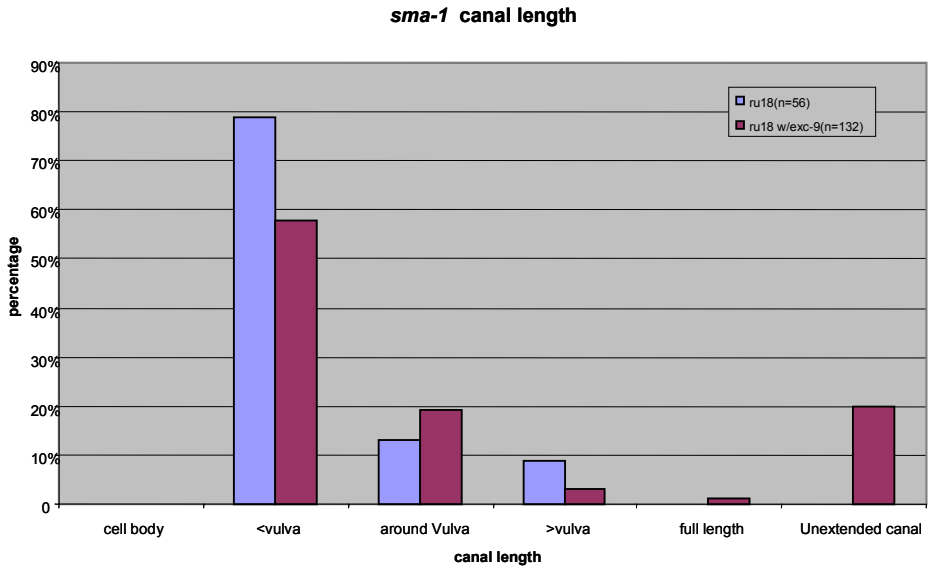


Figure 4.10 Canal ending point in *sma-1 (ru18)* worms with or without pBK121.

Diagramed using the data from Table 4.10 and Table 4.11.

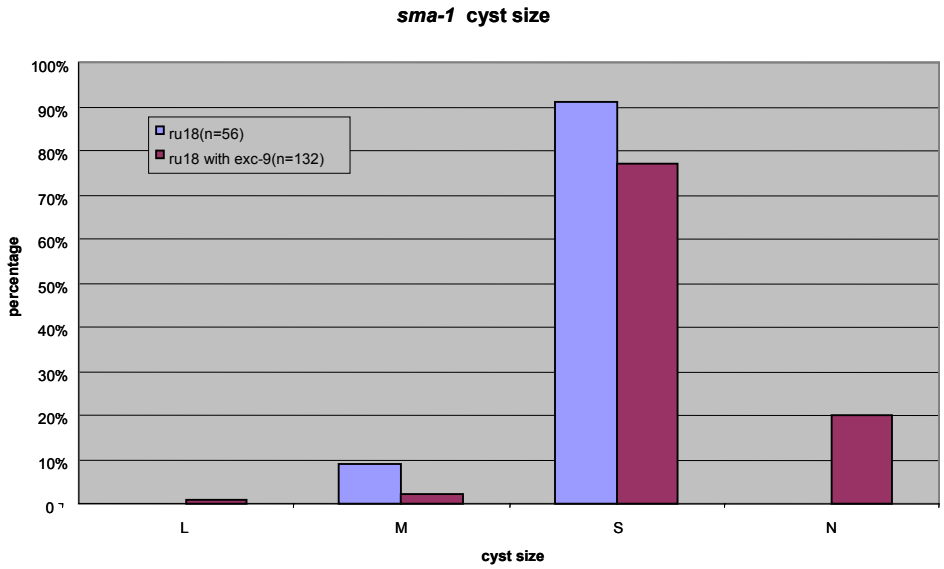


Figure 4.11 Canal cyst size in *sma-1 (ru18)* worms with or without pBK121.

Diagramed using the data from Table 4.10 and Table 4.11.

Table 4.12 Canal phenotype of *exc-1 (rh26)* worms.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)				2	3	11
T (tiny)				3	3	13
S (small)		3	3	15	1	48
M (medium)		3	2	1		13
L (large)		7				15
%		28	11	46	15	n=46

Table 4.13 Canal phenotype of F1 progeny of *exc-1 (rh26)* injected with pBK121.

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)				2	18	17
T (tiny)			3	12	7	18
S (small)	6	11	20	15	1	45
M (medium)	3	2	3			7
L (large)	8	6	2			13
%	15	16	24	24	22	n=119

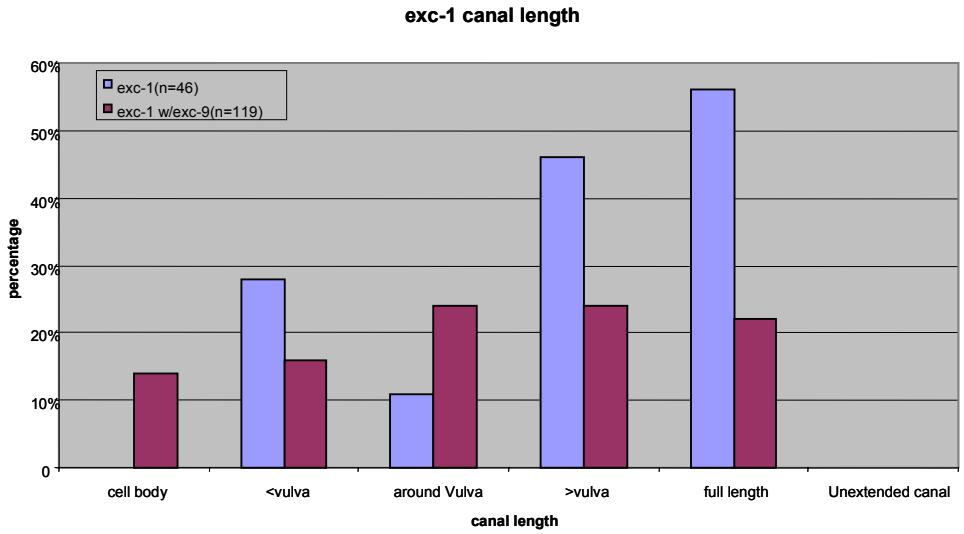


Figure 4.12 Canal ending point in *exc-1 (rh26)* worms with or without pBK121. Diagramed using the data from Table 4.12 and Table 4.13.

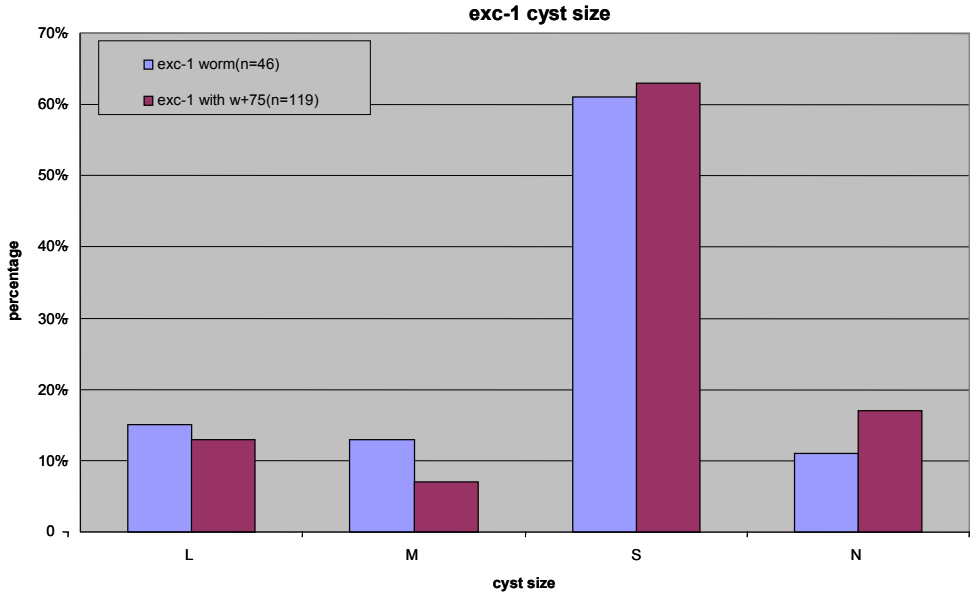


Figure 4.13 Canal cyst size in *exc-1 (rh26)* worms with or without pBK121. Diagramed using the data from Table 4.12 and Table 4.13.

(Table4.12 and Table 4.13; Fig.4.12 and Fig.4.13). It is possible that this gene is downstream of *exc-9,* so the mutants would not respond to the high level of EXC-9.

4.7 Summary

Injections of *exc-9* into different *exc* background were done to find out the possible pathway for canal structure regulation.

Injection of *exc-9* into *exc-4, exc-2,* and *sma-1* backgrounds could lengthen the canal, shrink the cysts in these mutants, and, sometimes, cause unextended canal as in wild-type worms. In *exc-1* and *exc-5* mutant worms, no obvious changes have been seen. On the other hand, overexpression of *exc-5* translational construct in *exc-9* mutant worms could partial rescue *exc-9* phenotype and sometimes cause unextended canal. All these indicate that *exc-9* might work downstream of *exc-2, exc-4* and *sma-1*, and works upstream of *exc-5* and *exc-1* to control the canal lumen formation (Summary in Table 4.14).

4.8 A possible pathway that regulates the excretory canal structure

sma-1 encodes β_H-Spectrin to maintain apical structure of excretory canal (Praitis, V., Ciccone, E. et al. 2005). Since overexpressed *exc-9* could partially rescue the phenotype of *sma-1* mutants, it is possible there are other structural elements downstream of *exc-9*. When *sma-1* is missing, overexpression of

Table 4.14 Effects of overexpression *exc-9* or *exc-5* translational construct in other *exc* background.

Worm strain	DNA construct	Lengthens canal /shrinks cysts (Apical improvement)	Unextended canal (Basal disruption)
N2	*exc-9*	\	++
N2	*exc-5*	\	+++
exc-9	*exc-9*	++	++
exc-2	*exc-9*	+	+
exc-4	*exc-9*	+	++
sma-1	*exc-9*	?	+
exc-1	*exc-9*	-	-
exc-5	*exc-9*	-	-
exc-9	*exc-5*	+	+++

"+" means had positive effects. More symbol means stronger effects.
"-" means the construct had no effect.
"?" means effect is not obvious.

"\" means no base to judge.

EXC-9 could upregulate these elements and help reinforce the apical surface of excretory canal.

exc-4 is a chloride intracellular channel that might help to maintain the ionic concentration and pPH in the canal (Berry, K.L., Bulow, H.E. et al. 2003). Overexpression of *exc-9* might be able to override the phenotype caused by *exc-4* mutation.

exc-5 is a putative guanine nucleotide exchange factor(Gao, J., Estrada, L. et al. 2001; Suzuki, N., Buechner, M. et al. 2001). The reciprocal experiments strongly support that *exc-9* is upstream of *exc-5*. EXC-9 might help stabilize EXC-5 or help localization of EXC-5.

exc-1 and *exc-2* are two uncloned genes. The overexpression data suggest that *exc-9* is downstream of *exc-2* but upstream of *exc-1*.

The EXC-9 protein works downstream of EXC-2 and EXC-4, but upstream of EXC-1, EXC-5 and CDC-42. This pathway helps to localize SMA-1 protein to the apical surface of excretory canal. When the worm has correct quantity of every protein in the canal, the canal can grow all the way to the tail and fine lumen of canal could be maintained properly. If worm lost one of the components in the pathway, cysts formed in the lumen, and elongation of canal did not occor properly. Having too much of one of the components will lead to proper formed apical surface, but the basal surface elongation got interrupted somehow and then form the unextended canal (Fig. 4.14).

All of these *exc* genes must work together to maintain the normal diameter of the excretory canal. Since all *exc* mutants show some extend defects in the tail formation, the same pathway might also be involved in tail shape formation.

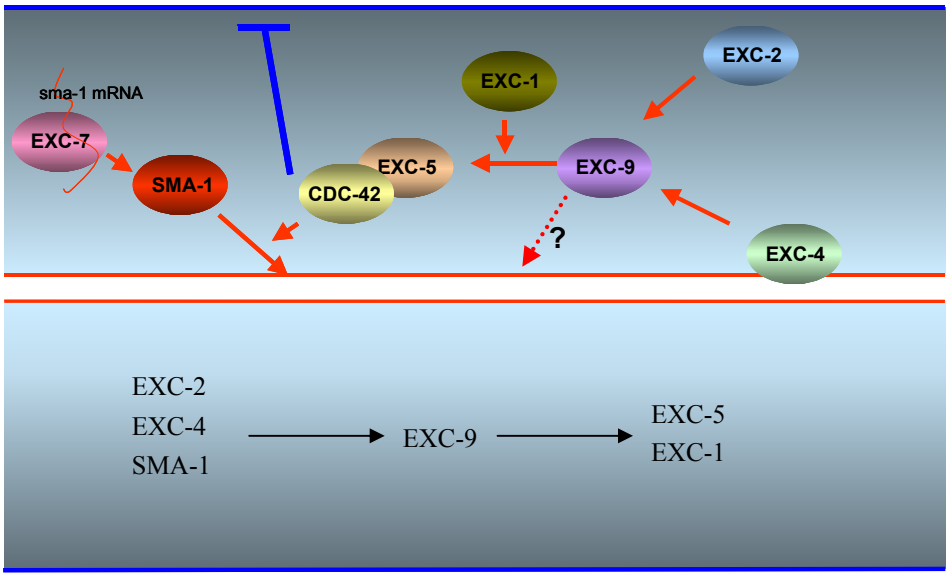


Figure 4.14. New model of *exc* genes regulate excretory canal structure. The figure pictured part of the excretory tube. Blue line stands for basal surface and red line stands for apical surface. In between the blue lines is the canal tube, and in between the red lines is the lumen of the canal. Blue arrow means suppress and red arrow indicates upregulate. EXC-9 might work downstream of EXC-2 and EXC-4, but upstream of EXC-1, EXC-5 and CDC-42 to help localize SMA-1 to the apical surface of excretory canal to reinforce the structure. EXC-9 might help other materials localize to the apical surface, thus it can partially rescue the *sma-1* phenotype. The EXC-9 pathway also play a role in regulating outgrowth of basal membrane. Overexpression of either EXC-5 or EXC-9 can prevent the outgrowth of basal membrane and causes unextended canal phenotype. The pathway is also shown as flow chart at the bottome of the figure.

Chapter 5

Excretory canal-specific promoters

5.1 Excretory canal-specific promoter

A promoter is a regulatory region of DNA that is normally located upstream of a gene. The promoter is not directly responsible for the protein production level from a gene, but provides information as to when and where the gene should be transcribed. Thus, it is important for the proper expression of the gene. It is possible that co-expressed genes share common regulatory elements. Identifying promoter elements for excretory canal expression will provide clues as to which genes are expressed in the canal.

The excretory canal of *C. elegans* is a long tubular epithelial structure. The apical membrane of this tissue faces the lumen that connects to the outer environment for excretion. The apical membrane organization depends on the proteins on the surface and in the cytoplasm. It is probable that most of these proteins, if not all, are transcribed and translated in this cell. In other words, most of the genes that control the apical structure of excretory canal are cell-autonomously. Because of this, identifying which genes are expressed in the canal will give us a candidate pool for possible *exc* genes.

Some of the excretory canal-specific promoter elements have been identified by Baillie's group (Zhao, Z., Fang, L. et al. 2005). In their work, three promoters were studied for the indispensable element for canal expression. These three genes did not show identical elements for expression regulation. One possibility is that there are more than three transcription factors that specify

the excretory canal, so the elements are distinct. Alternatively, there might be some common domains in some of those elements. Since many promoters have not been studied, it is difficult to rule out the possibility of common residues.

During the current study of the *exc* genes, two new regions important for respective gene expression in the excretory canal that might contribute to the prediction of canal expression genes that have canal expression were identified.

5.2 Promoter study of *exc-9*

During the expression study of *exc-9*, worms expressing the pBK104 construct (P*exc-9*::*gfp*) showed a "lumpy-dumpy" phenotype at low percentage (<10%) (Fig.5.1, Table 5.1). This is an embryonic lethality in which tissues form but fail to be shaped properly, and is found in mutants defective for *ceh-6* which also is highly expressed in the canal (Burglin, T.R. and Ruvkun, G. 2001). Most of the lumpy-dumpy worms caused by injection of P*exc-9*:: *gfp* exhibited a high level of GFP expression. In the transgenic line of pBK121 (*exc-9*::*gfp*), the similar phenotype and ratio was also observed. Because the common sequences are the *exc-9* promoter and the pPD95.75 backbone, one of these sequences must cause the lumpy-dumpy phenotype.

To determine if this phenotype is caused by pPD95.75 backbone, two injections were done. The constructs injected are similar to pBK104, and have the same pPD95.75 backbone, but with different promoter sequences. The

Figure 5.1 “lumpy-dumpy” worms caused by injection of pBK104, the transcriptional construct of *exc-9*. Anterior is to the left. GFP expressed in the canal. Buccal opening is clearly formed and gut granules are evident. Scale bar: 10 μm.

promoters are from genes B0496.7 and C03F11.1. For injections with these two constructs, none of the progeny with GFP expression showed the lumpy-dumpy phenotype (>3000 worms screened). Because of these results, the pPD95.75 backbone should not be the cause of the lumpy-dumpy phenotype.

The *exc-9* promoter then is the only possible cause for the lumpy-dumpy phenotype. The lumpy-dumpy phenotype is also seen in *ceh-6* mutants. Since *ceh-6* and *exc-9* are expressed in the same tissue and similar phenotype has been observed, it is possible that there are links between these two genes. A likely possibility is that CEH-6 is the transcriptional factor for *exc-9*, and a high copy number of the *exc-9* promoter will lead to depletion of CEH-6, to result in similar phenotype as the *ceh-6* mutant phenotype. The other possibility is that the *ceh-6* and *exc-9* genes share common transcriptional factors. Too many copies of *exc-9* promoter will compete with the *ceh-6* promoter for the transcriptional factors, and lead to low levels of CEH-6 produced. In turn, some other vital genes that depend on CEH-6 will not be transcribed properly.

To determine if the promoter of *exc-9* was the reason for the lumpy-dumpy worms, three more new transcriptional vectors were made to examine the effect of various length promoters of *exc-9*. The plasmid pPD95.75 was still used as the backbone for the vectors. The 2.2 kb promoter includes the entire region between *exc-9* and the next upstream gene. I divided this region into four pieces to make new transcriptional constructs. The lengths of the new promoters are 0.7

kb, 1.4 kb and 1.8 kb, respectively to the start codon of *exc-9.* The structures of all transcriptional vectors are listed in Figure 5.2.

For a stable line expressing construct pBK101 (with 0.7 Kb promoter), no canal GFP expression was detected. The expression in intestine and neurons was not disrupted. Similar to pBK104, the two constructs with larger promoter regions (1.4 Kb and 1.8 Kb) gave canal, UTSE, intestinal, and neuronal expression of GFP in the transgenic lines. There are two noticeable differences between the stable lines with the three longest promoter constructs. The first is that the N2 stable line with pBK102 (with 1.4 Kb promoter) showed weaker GFP expression level compared to the other two stable lines. The second is that no lumpy-dumpy worms were found on plates of the stable line with pBK102 (See Table 5.1).

One possible reason for the absence of lumpy-dumpy worms might be that there was less transcription of the injected construct in the stable line with pBK102. With less transcription, the GFP production will be less and lead to lower visible fluorescence. The reason of less transcribes might due to loss of the essential binding domain in the promoter since pBK102 has a shorter promoter compared to other constructs.

As seen in Table 5.1, pBK103 caused less occurrence of the lumpy-dumpy phenotype than did pBK104. It is possible that pBK104 could bind more transcriptional factors than did pBK103.

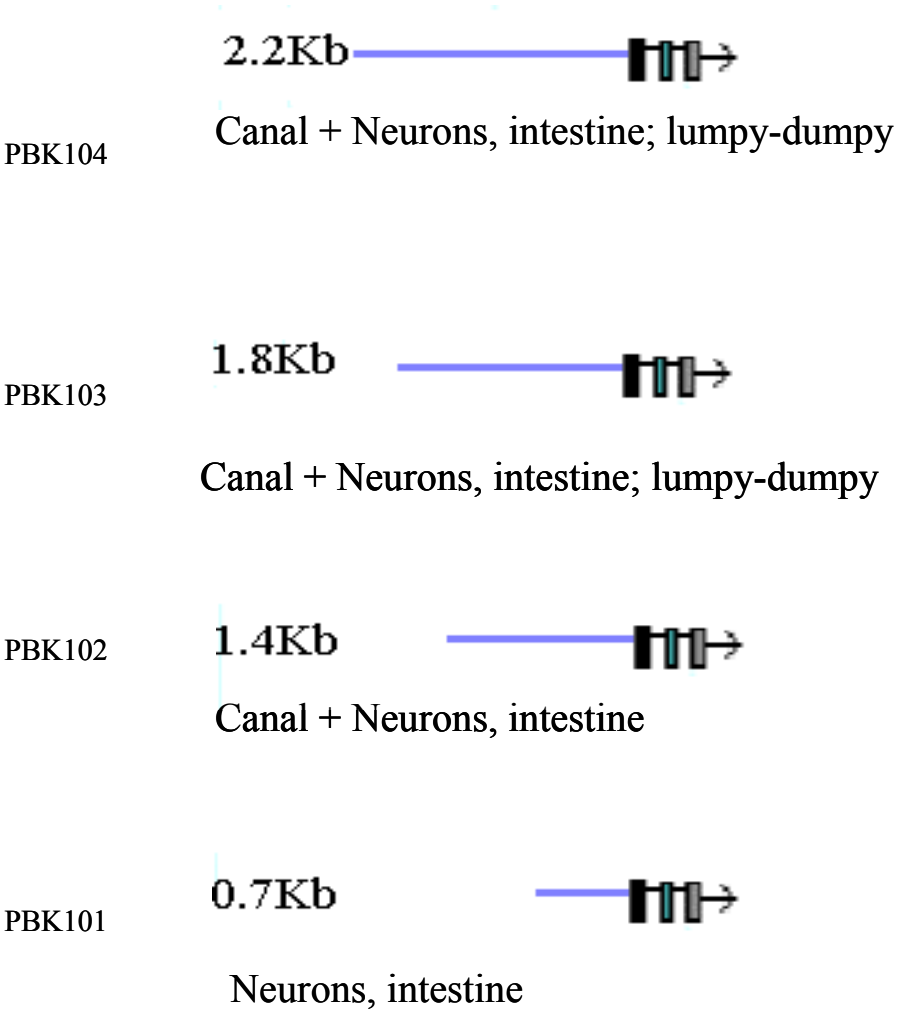


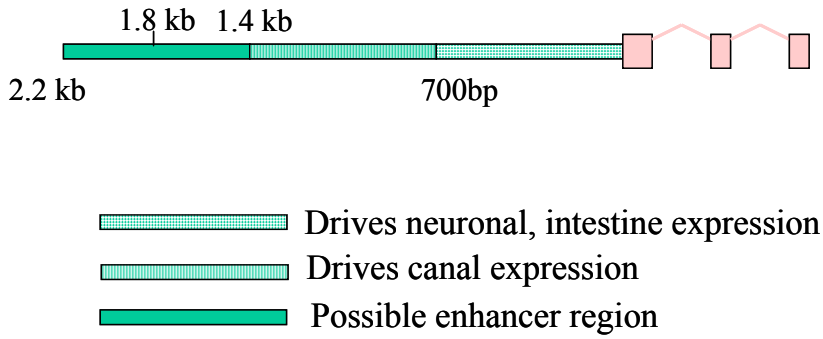
Figure 5.2 Structure of *exc-9* transcriptional constructs with different length promoters. The expression patterns are shown beneath the structure.

Table 5.1 Number of lumpy-dumpy worms found on the plate of stable lines with different *exc-9* transcriptional constructs.

Strain	Vector	Lumpy-dumpy worms
N2	pBK104	17
N2	pBK103	3
N2	pBK102	0

There are about 300 worms on each plate

.



Green boxes: promoter region of *exc-9*. Pink box: exons of GFP

Figure 5.3 Possible functions of different upstream regions of *exc-9* promoter based on the study of expression pattern of *exc-9* transcriptional constructs.

Based on these results, several possible functional domains of *exc-9* promoter were predicted (Shown in Fig. 5.3). The region between 0.7 kb and 1.4 kb upstream of *exc-9* is important for canal expression. Without this part, the gene downstream will not be expressed in the canal or in other tissues in which *exc-9* is normally expressed. But the 700 bp region is still capable of driving neuronal and intestinal expression of the gene. The next 400 bp upstream is important for stronger binding of transcriptional factors, and serves as an enhancer region. With this part, the expression level of the gene will be upregulated. But this expression also leads to the depletion of transcriptional factors from other genes. The 400 bp farthest upstream contains some more binding sites for the transcriptional factors. This is the reason that the stable line with pBK104 has the highest percentage of lumpy-dumpy worms.

5.3 Elements crucial for *vha-1* excretory canal expression

When I made construct pBK130 (P*vha-1*::*exc-9*::*gfp*), I used PCR to amplify the promoter region of *vha-1* and then ligated the promoter into the revised vector. *Taq* polymerase was used, so minor mistakes were introduced into the promoter. It turned out that those minor mistakes did matter. With these mistakes, the expression of GFP in the canal was interrupted.

All the promoters sequenced from two independent amplifications had one common mistake a two-basepair substitution in 269 bp upstream of the endogenous start codon of *vha-1*. It was a change of GC to TA. The possible explanation is that the mistake exists in the original PCV01(Oka, T., Yamamoto, R. et al. 1997) vector, but didn't interfere with the expression of GFP in the canal.

Because of this, I didn't take this mistake into account for interruption of expression.

One sequenced promoter with fewer mistakes was chosen for the new vector construct. It has two additional mistakes other than the substitution mentioned before: one is a transition from A to G at –1024 bp, the other is a four-basepair deletion from –877 to –874 bp, the sequence GTTG in the promoter is deleted in the amplified new promoter. The construct with this promoter sequence showed poor expression in the canal. Among 50 glowing F1 progeny from injection, only 11 of them showed canal expression, a percentage of just 22%. As the deletion is closer to the start codon, and the sequence was affected more, I believe that the deletion is the main reason for the new expression pattern.

In summary, the region around 870 bp upstream of the start codon is important for proper expression in the canal (Fig. 5.5). Changes in this region will result in loss of gene expression in the canal.

5.4 Summary

The promoter regions of canal specific genes were analyzed to identify the key element for canal expression. The two regions I found to be important for canal expression are not similar to the elements reported in previous work (Zhao, Z., Fang, L. et al. 2005), and nor does any similarity between these two regions exist.

Additional work on the canal specific genes' promoter will be useful to discover common control elements for canal expression.

The phenotype caused by overexpression of *exc-9* promoter suggests that

ceh-6 might be involved with *exc-9* transcription. Finding out whether *exc-9* has normal expression pattern in *ceh-6* mutant background will help us to determine whether *exc-9* is transcribed by *ceh-6* or these two genes share same transcriptional factors.

Chapter 6

Study of the EXC-9 homologues

6.1 Tissue-specific expression of B0496.7

As shown in Chapter 3, *exc-9* has a close homologue in *C. elegans,* B0496.7. To determine if this gene is expressed in the same tissue as is *exc-9,* the transcriptional construct pBK118 was created by fusing 1.1 kb upstream sequence of B0496.7 with the pPD95.75 backbone. The construct was injected into wild-type worms and was expressed in different tissues than was the *exc-9* GFP transcriptional construct. The construct is highly expressed in all the valves in the worm, including the pharyngeal-intestinal valve, the intestinal-rectal valve, and the spermatheca-uterine valve (Fig. 6.1). In addition to the valves, GFP was also found in the gonadal sheath cell, seam cells, vulva muscle and some neurons including the PVD neuron (Fig. 6.1).

Taken together, the B0496.7 reporter construct is expressed in different locations from *exc-9* (See table 6.1 for comparison). Most of the tissues with B0496.7 expression need to maintain accurate structure for proper function. It is possible that these two genes function in similar pathways, but in different tissues because of the promoter difference.

6.2 Phenotype of B0496.7 RNAi worms

Since there is no knockout allele of B0496.7, RNAi was carried out to find out the knockdown phenotype of B0496.7. dsRNA complementary to the coding region of B0496.7 was made. Although the two gene products are similar in sequence, the DNA sequences of coding region vary from each other significantly. The dsRNA made should be only complementary to the B0496.7 mRNA but not to the *exc-9* mRNA. This dsRNA was introduced into wild-type worms by means

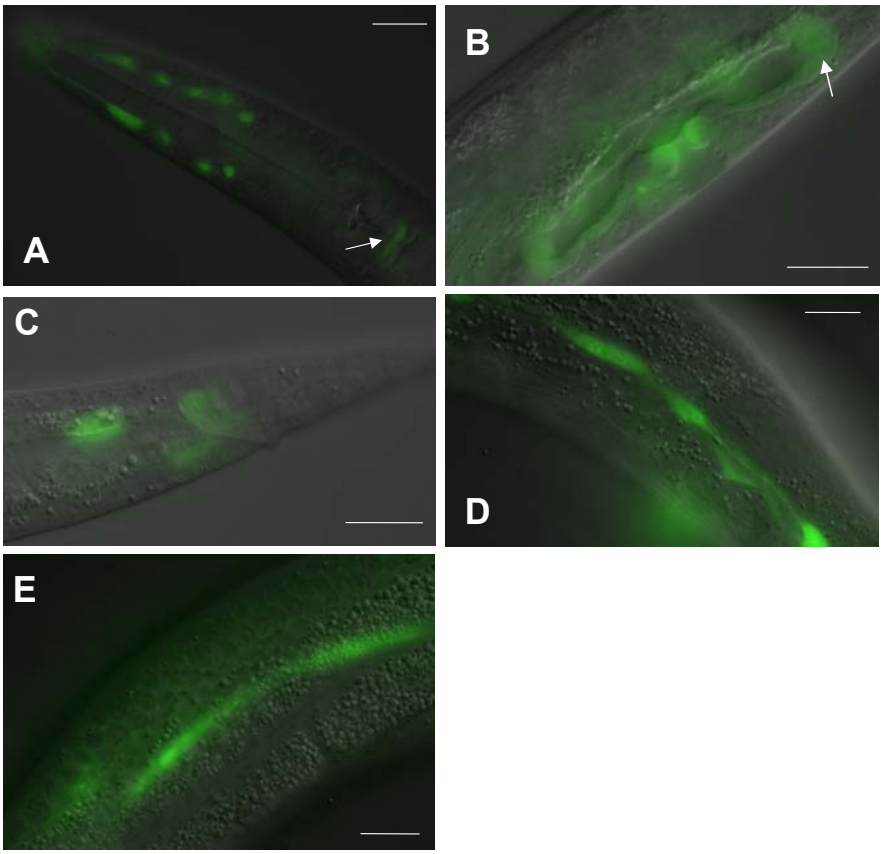


Figure 6.1 B0496.7 expression pattern. B0496.7 GFP reporter construct was found in several valve tissues including Pharyngeal-intestinal valve (indicated by white arrow in A), spermatheca-uterus (sp-ut) (B), and Rectal valve(C). The expression was also found in the seam cells (D) and gonadal sheath cells (E). Scale bars, 25 μ m.

Table 6.1 Comparison of expression of *exc-9* and B0496.7

Expression	*exc-9*	B0496.7
Excretory canal	+	-
Tail spike	+	-
Intestine	+	+
UTSE	+	-
Distal tip cell	+	-
ALM neuron	+	-
PLN neuron	+	-
Seam cells	-	+
Gonad sheath cell	-	+
pharyngeal-intestinal valve	-	+
intestine-rectal valve	-	+
spermatheca-uterine valve	-	+
vulva muscle	-	+
PVD neuron	-	+

“+” means the expression of GFP was found in the corresponding tissues.

“-” means the expression of GFP was not found in the corresponding tissues.

of injection with pRF4 (*rol-6*) as marker.

Three phenotypes were observed in the roller progeny (Fig.6.2). The first one is the lack of bacteria in the intestine even when food is abundant. This is likely due to the pharyngeal-intestinal valve was not being able to function properly, so the worms cannot eat. The second phenotype is that in some worms, the eggs were trapped in the uterus after fertilization. The reason might be that the spermatheca-uterine valve was not working. The other phenotype is a defecation problem of the worms which might be caused by a intestinal-rectal valve problem. These three phenotypes didn't appear in all worms. I repeated the injection 4 times, but only obtained 20 roller progeny, and all of them showed some extent of these defects. The low number of progeny with marker suggests that knockout this gene is lethal to the worms. Lethality may be the reason why no knockout allele has been isolated yet.

dsRNA of B0496.7 was also injected into *exc-9 (n2669)* worms to see the phenotype. No viable progeny with marker were observed from 200 injected P0 animals. Since knockdown of either of these two genes leads to sick worms, worms losing both genes might not be able to survive.

6.3 Functional interchangeability of B0496.7 and EXC-9

I speculated that *exc-9* and the *C. elegans* homologue B0496.7 have similar function. One proof is that both proteins bound to CSN-5 in the yeast two-hybrid assay. The binding between B0496.7 and CSN-5 was shown previously by another group (Li, S., Armstrong, C.M. et al. 2004).

The other thing I tried to determine was whether B0496.7 could rescue the

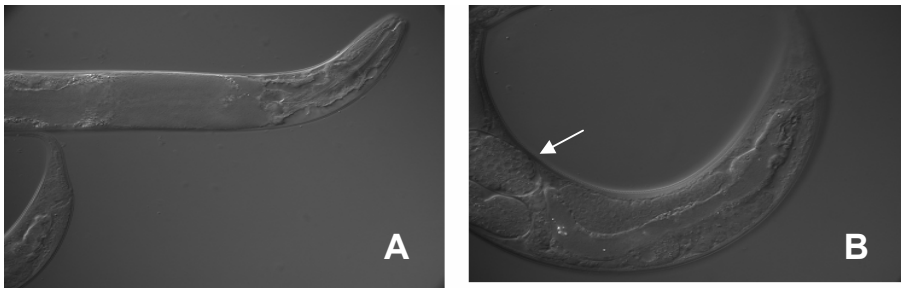


Figure 6.2 B0496.7 RNAi phenotypes. (A) Dying animal from RNAi injection. The worm did not have enough food, and interior began to degrade. Anterior is to the right. (B) An egg arrested in the spermatheca indicated by the white arrow. Anterior is to the left in this picture.

exc-9 phenotype when it is expressed in the same tissue as *exc-9*. I showed in chapter 3 that EXC-9 is cell-autonomous, so that expression of B0496.7 only in the canal should be able to rescue *exc-9* cystic canal phenotype if both genes have similar biochemical activity.

A new construct, pBK123, was created similar as pBK122 (P*vha-1*::11aa::*exc-9*::*gfp*). The only difference between these two constructs is the replacement of the *exc-9* genomic coding region with the B0496.7 genomic coding region. With this construct, the protein B0496.7 should be found mainly in the canal, and this protein will have eleven extra amino acids in the N-terminus.

The plasmid pBK123 was introduced into *exc-9 (gk395)* worm by means of injection. As we thought, the cystic canal phenotype of *exc-9* was largely improved in the glowing progeny (Table 6.2, Fig. 6.3 and Fig. 6.4), but rescue was not as efficient as pBK122 injection. This indicates that B0496.7 shares similar but not identical biochemical activity, or that the injections of pBK123 were less efficient.

The plasmid pBK123 still has extra amino acids in the N-terminus. Without that part, B0496.7 might be able to give unextended canal phenotype as EXC-9 with a normal N-terminus. This research will be carried out in the near future.

6.4 Study of mouse CRIP

Since B0496.7 could rescue the *exc-9* cystic canal phenotype when expressed in the canals, we asked if the vertebrate homologues also are able to rescue *exc-9*.

Table 6.2 Canal phenotype of F1 progeny of *exc-9* (gk395) injected with pBK123 (P*vha-1*::11aa::B0496.7::*gfp*).

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)		1	6	1		24
S (small)		7	12	1		59
M (medium)		3	3			18
L (large)						
%		32	62	6		n=34

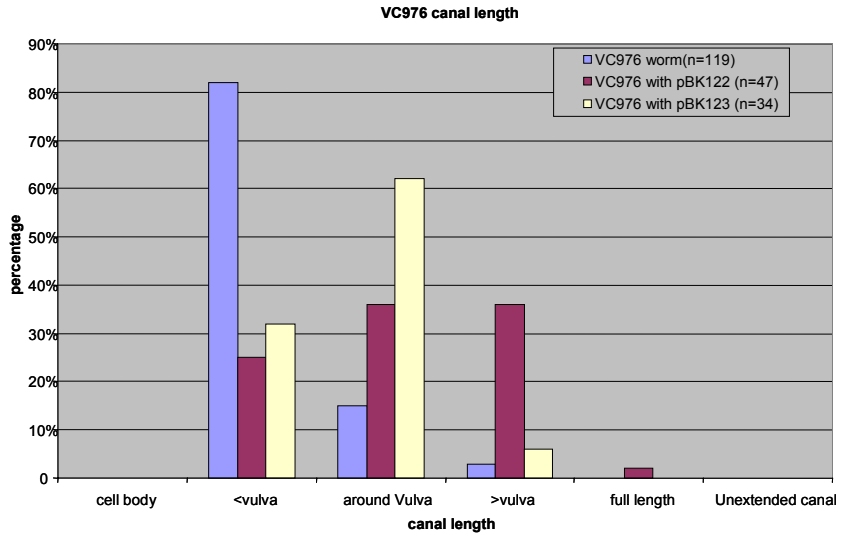


Figure 6.3 Canal ending point in *exc-9 (gk395)* worms with or without pBK122 (P*vha-1*::11aa::*exc-9*::*gfp*) or with pBK123 (P*vha-1*::11aa::B0496.7::*gfp*). Diagramed using the data from Table 3.1, Table 3.2, and Table 6.2.

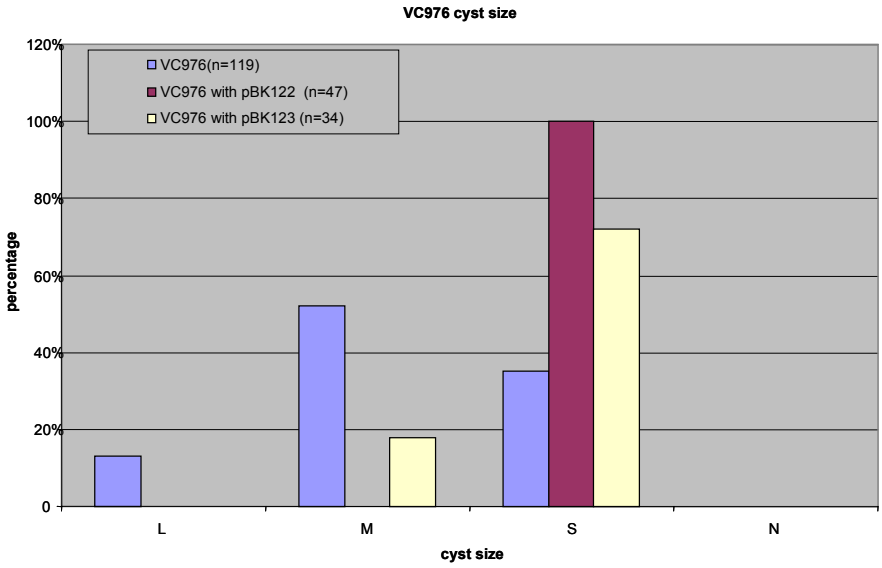


Figure 6.4 Canal cyst size in *exc-9 (gk395)* worms with or without pBK122 (P*vha-1*::11aa::*exc-9*::*gfp*) or with pBK123 (P*vha-1*::11aa::B0496.7::*gfp*). Diagramed using the data from Table 3.1, Table 3.2, and Table 6.2.

Compared to B0496.7, the amino acid sequence of mouse CRIP is more distant from that of EXC-9. Three major differences were found between the amino acid sequences of mouse CRIP and EXC-9 (Fig. 6.5). The first is that there is a shorter C-terminal tail in mouse CRIP. The second is that there are two additional amino acids in the LIM domain of EXC-9. The third one is that near the end of LIM domain, all vertebrate CRIP homologues have a proline, but none of the nematode homologues have it. Since proline has a unique structure that alters protein secondary structure and breaks α-helixes, the presence or absence of proline could result in a different orientation of the rest of the protein. The structural study of mouse CRIP already showed that the orientation of the C-terminus of this protein is different from that of CRP1 LIM domain, which has no proline in the corresponding place (Perez-Alvarado, G.C., Kosa, J.L. et al. 1996).

I made four constructs with different versions of the mouse CRIP coding region, as listed in Table 6.3. The backbone used was still pCV01, which contains the *vha-1* promoter linked to GFP, so the eleven amino acids on the N-terminus still existed.

Some of the constructs were introduced into the *exc-9* background separately to see whether any of them could rescue the cystic canal phenotype of *exc-9* (Table 6.3-Table 6.6; Fig. 6.6-Fig. 6.9).

Mouse CRIP by itself or with the addition of the nematode C-terminus did not rescue the cystic canal phenotype. On the other hand, the construct that lacks the extra proline but with the nematode tail did partially rescue the cystic canal phenotype. These results suggest that the proline does play a significant

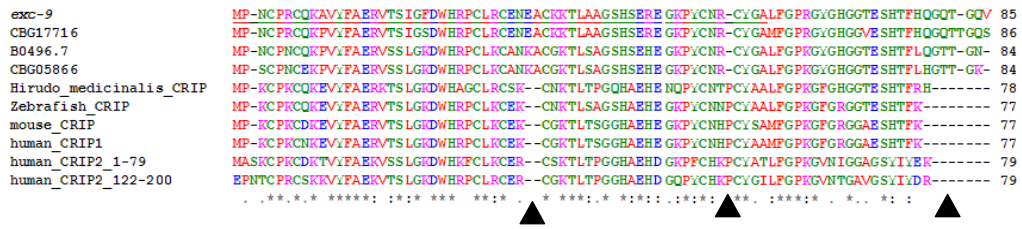


Figure 6.5 Alignment of the CRIP proteins. Major differences between nematode CRIP and vertebrate CRIP are indicated by black arrowhead.

Table 6.3 Coding regions in different mouse CRIP vectors.

Plasmid name	Coding region
pBK131	Mouse CRIP cDNA
pBK132	Mouse CRIP cDNA in frame with nematode C-terminus
pBK133	Mouse CRIP cDNA in frame with nematode C-terminus, without extra proline
pBK134	Mouse CRIP cDNA in frame with nematode C-terminus, with two additional nematode amino acids in LIM domain

Table 6.4 Canal phenotype of F1 progeny of *exc*-9 (n2669*)* injected with pBK131 (P*vha-1*::11aa::mouse CRIP::*gfp*).

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)		3	1			8
S (small)		23	3			49
M (medium)		13				25
L (large)		10				19
%		92	8			n=53

Table 6.5 Canal phenotype of F1 progeny of *exc-9* (*n2669*) injected with pBK132 (P*vha-1*::11aa:: mouse CRIP+tail::*gfp*).

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)						
T (tiny)			1			3
S (small)		10	10			63
M (medium)		5				16
L (large)	1	3	2			19
%	3	56	41			n=32

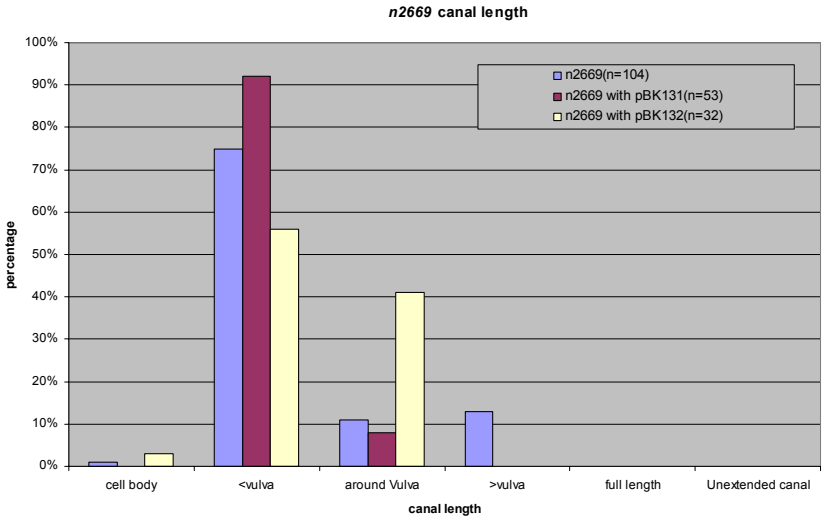


Figure 6.6 Canal ending point in *exc-9 (n2669)* worms with or without pBK131 (P*vha-1*::11aa:: mouse CRIP::*gfp*) or pBK132 (P*vha-1*::11aa:: mouse CRIP+tail::*gfp*). Diagramed using the data from Table 3.3, Table 6.4, and Table 6.5.

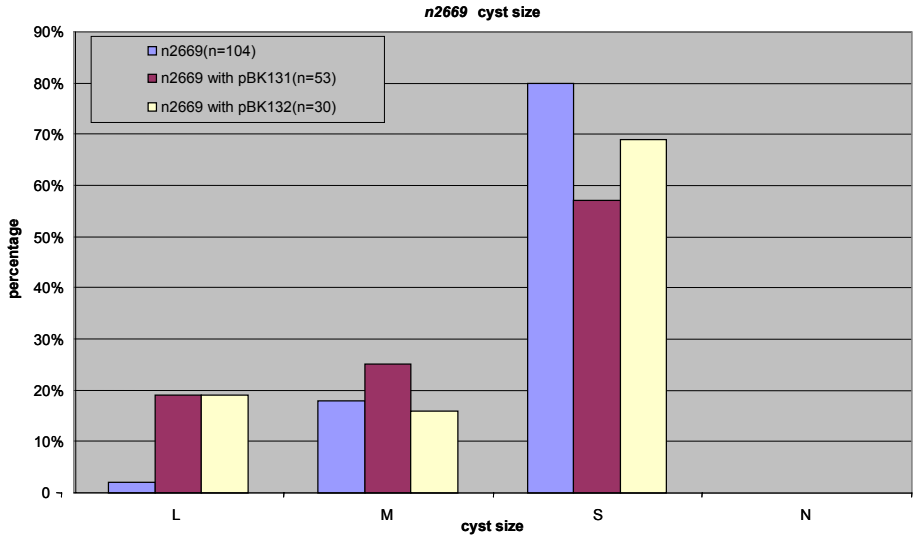


Figure 6.7 Canal cyst size in *exc-9 (n2669)* worms with or without pBK131 (P*vha-1*::11aa:: mouse CRIP::*gfp*) or pBK132 (P*vha-1*::11aa:: mouse CRIP+tail::*gfp*). Diagramed using the data from Table 3.3, Table 6.4, and Table 6.5.

Table 6.6 Canal phenotype of F1 progeny of *exc-9* (gk395) worms injected with pBK133 (P*vha-1*::11aa:: mouse CRIP+tail+pro::*gfp*).

Length of posterior canal / Size of largest cyst	0(cell body)	1(<vulva)	2(around V)	3(>vulva)	4(full length)	%
N (none)		1	4			7
T (tiny)		3	9	3	1	24
S (small)		14	20	3		54
M (medium)	1	3	5			13
L (large)		1				1
%	1	34	56	9	1	n=68

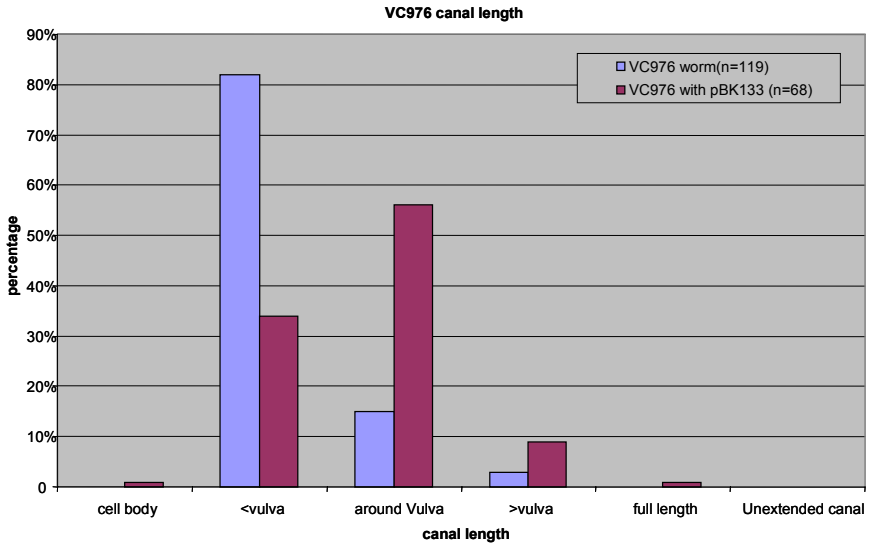


Figure 6.8 Canal ending point in *exc-9 (gk395)* worms with or without pBK133 (P*vha-1*::11aa:: mouse CRIP+tail+pro::*gfp*). Diagramed using the data from Table 3.1 and Table 6.6.

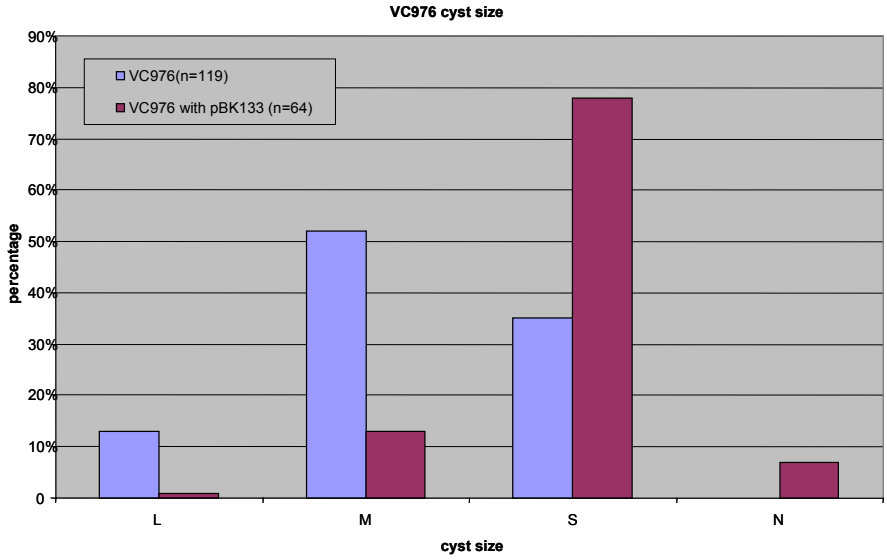


Figure 6.9 Canal cyst size in *exc-9 (gk395)* worms with or without pBK133 (P*vha-1*::11aa:: mouse CRIP+tail+pro::*gfp*). Diagramed using the data from Table 3.1 and Table 6.6.

functional role in the protein conformation. With or without this residue, the angle between the C-terminus and the LIM domain will be different. As a result, the orientation of any binding partners will likely be different as well. Since all these constructs have the extra amino acids on the N-terminus, the results did not preclude the possibility that with a free N-terminus, the mouse CRIP could cause the same unextended canal phenotype as EXC-9.

6.5 Summary

By studying the function of the homologues in the excretory canal, I determined that B0496.7 not only shares similar sequence but also similar function as does EXC-9. These two proteins function in distinct locations to fulfill a similar function.

The mouse CRIP failed to rescue the cystic canal phenotype when introduced into the excretory canal cell. After modification, partial rescue was observed. The lack of rescue by mouse CRIP might be due to the additional proline in the vertebrate CRIP, which alters the relative orientation of the LIM domain and the C-terminus tail, and then change the protein conformation. Without this proline, the mouse CRIP could function similarly to EXC-9 as well.

Chapter 7

exc-2 cloning

7.1 *exc-2* mutant

exc-2 is a recessive mutant with excretory canal defects similar to but more sever than those of *exc-9*. The canal phenotype is the main defect of *exc-2* (Fig. 7.1). 44% of *exc-2* mutants show cysts that large enough to be visible under the dissecting microscope. In some of the *exc-2* worms, because the abnormal canal structure interferes with the normal function of the canal, they are unable to get rid of liquid waste. As a result, variable amounts of lethality can be observed at all larval stages on *exc-2* plates.

Similarly to *exc-9, exc-2* mutants also show tail defects in both hermaphrodites and males.

exc-2 is located on chromosome X. It was mapped between *dpy-8* and *mec-2* previously (Buechner, M., Hall, D.H. et al. 1999). Since it was shown that *exc-9* overexpression can partially rescue *exc-2,* knowing the identity of *exc-2* will help us to understand the pathway that regulates the apical membrane organization of excretory canal.

7.2 Does C03F11.1 encode *exc-2*?

exc-2 was previously thought to be encoded by C03F11.1, a gene that encodes a small conductance calcium-activated potassium channel protein, based on RNAi results and one sequencing result. But no rescue attempt was carried out at that time (done by Kelly Homb).

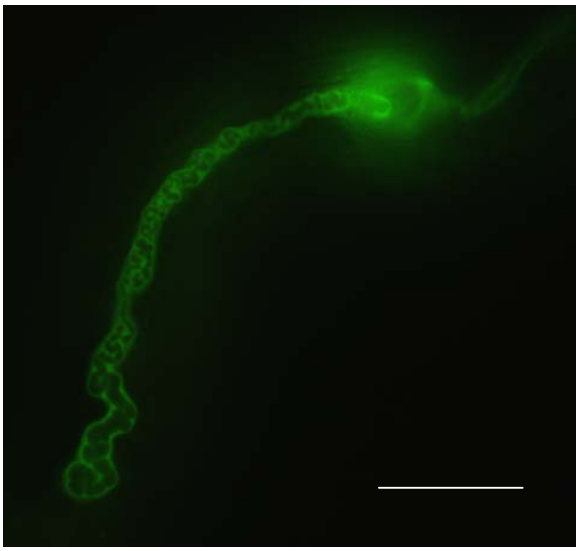


Figure 7.1 *exc-2* canal phenotype. This is a worm has better canal phenotype compare to most of the *exc-2* mutants. The head of the worm is to the right in this picture. Scale bar: 50µm

To test this result, several approaches were carried out. The first was to redo the RNAi experiment. The previous RNAi injection was done using pCV01 as injection marker. To ensure that the maker wouldn't interfere with the RNAi result, I used the marker pRF4 instead. 31 roller worms were found from injection, but none of them showed canal defect. The phenotype observed before might be caused by pCV01 instead of dsRNA of C03F11 since it has been shown that the pCV01 construct can interfere with canal structure. The second thing is to confirm the mutation in the gene. The gene C03F11.1 from *exc-2* mutant *rh90* was amplified three times for sequencing of the mutation. It turned out that none of the amplifications had the stop codon that the previous sequencing showed, and no other mutations have been found. The previous mistake could have been introduced by PCR. I also tried the rescue experiment to see whether the gene C03F11.1 encodes *exc-2*. I used different DNA constructs, including cosmid, and HindIII digestion fragments including C03F11.1 and C03F11.1 PCR products, to rescue the *exc-2* mutant phenotype. More than 15 progeny expressing the pRF4 were isolated from each injection, but none of them showed improved canals. The final thing I tried was to find out the expression pattern of C03F11.1. A transcriptional construct was made using the C03F11.1 promoter followed by the GFP coding region. It turned out that the GFP is highly expressed in the head neurons, but not in the excretory canals (Fig. 7.2). In sum, it is unlikely that C03F11.1 is the gene that encodes *exc-2*.

7.3 SK3 channel study

During the study of C03F11.1, we wondered whether the failure of the

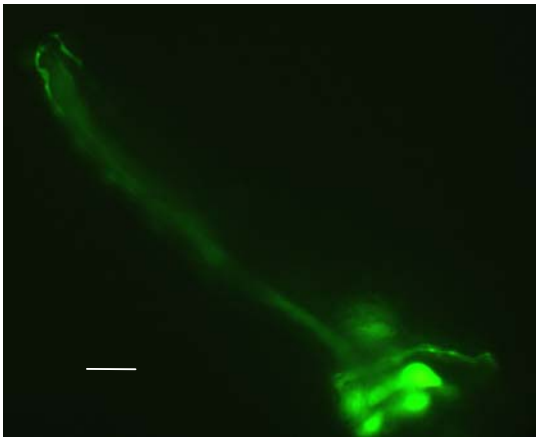


Figure 7.2 Expression of C03F11.1 reporter construct in ADF neuron, a amphid neuron. Scale bar: 10μm

RNAi experiment was due to redundant function of the homologues. In addition to C03F11.1, there are three other SK3 homologues in *C. elegans*. They are B0399.1, C53A5.5, and F08A10.1. They are members of the small conductance calcium-activated potassium channel group. The hypothesis was that this kind of potassium channel might function with EXC-4, a chloride intracellular channel (Berry, K.L., Bulow, H.E. et al. 2003), to regulate the ionic strength in the canal.

I did RNAi experiments on single genes and also combined four dsRNAs for injection to see the phenotype. More than ten progeny expressing the pRF4 were found from each injection, but none of them gave similar canal phenotype as found in *exc-2* animals. Some subtle effects were observed from one injection of B0399.1 dsRNA injection (Table 7.1). Those injection progeny displayed a pharynx-pumping defect. Most of the time, the pumping is at the same rate as in wild-type worms. But in affected progeny, the pharynx sometimes stops for a long time (usually longer than 30 seconds) and then resumes as normal. A similar phenotype was also observed in the knock-out strain of this gene.

It is possible that there are some other defects that I didn't notice.

7.4 RNAi screen for *exc-2*

Since the region in between *dpy-8* and *mec-2* is not too huge, I choose to do RNAi on every single gene in the region to find out *exc-2*. pBK104

Table 7.1 RNAi phenotype of the possible *exc-2* genes. The order of the genes in the table is according to the injection order.

Gene	Canal defect	Other noticeable defect
C03F11.1	No	No
B0399.1	No	Slow pharynx pumping
T03G11.2	No	No
T03G11.8	No	No
Y34B4A.4	No	No
C25F6.4	No	No
T03G11.1	No	No
T03G11.3	No	No
T03G11.4	No	No
T03G11.5	No	No
T03G11.6	No	No
C31H2.3	No	No
C31H2.4	No	No
H28G03.6	No	No
C41A3.2a	No	No
Y34B4A.2	No	No
Y34B4A.3	No	No
Y34B4A.5	No	No
Y34B4A.6	No	No
Y34B4A.7	No	No
Y34B4A.8	No	No
Y34B4A.9	No	No
Y34B4A.10	No	No
F32A6.3	No	Dead glowing eggs
C41A3.2B	No	No
C41A3.2	No	No
C41A3.1	No	No
C25F6.7	No	No
C25F6.6	No	No
C25F6.3	No	No
C25F6.2	No	Dead glowing eggs
C25F6.1	No	No
F55D1.1	No	No
F55D1.2	No	No

C54G7.1	No	No
C54G7.2	No	No
C54G7.3	No	No
C54G7.4	No	No
T23F2.1	No	Some unc progeny
H28G03.1	No	No
H28G03.3	No	No
F35C8.1	No	No
F35C8.2	No	No
T23F2.2	No	No
EGAP5.1	No	No
H28G03.2	No	No
H28G03.4	No	No
F32A6.2	No	No
F32A6.1	No	No
F32A6.5	No	No
C26B9.5	No	No
C26B9.3	No	No
C26B9.2	No	No
C26B9.1	No	No
C26B9.7	No	No
C26B9.6	No	No
F35C8.4	No	No
F35C8.6	No	No
F35C8.7	No	No
F35C8.5	No	No
F35C8.3	No	No
F35C8.8	No	No
C03F11.2	No	No
C03F11.3	No	No
C03F11.4	No	No
T23F2.3	No	No
T23F2.4	No	No
T23F2.5	No	No
C38C5.1	No	No
C53A5.5	No	No
F08A10.1	No	No

(P*exc-9*::*gfp*) was used as injection marker since it can label the excretory canal. More than ten progeny showing the GFP expression in canal were screened from every injection for the canal phenotype. The results are listed in Table 7.1. As shown in the table, none of the injections yielded progeny with cystic canals as did *exc-2* mutants. There are two plausible reasons. One is that the dsRNA design was not specific enough to break down the mRNA. The other possibility is that the region does not include *exc-2*.

7.5 SNP mapping of *exc-2*

SNP mapping was chosen to narrow down the region of *exc-2*. *dpy-8* was used as left marker for SNP mapping. A single Dpy-8 non-Exc-2 recombinant was isolated from the cross and its homozygous Dpa-8 non Exc-2 progeny were subjected to SNP sites testing. The chromosome to the left side of the recombination site should have the same pattern as N2 worms, and to the right side of the recombination break point should have the CB4856 pattern. Based on the digestion result of SNP site C54G7: 22992 (Table7.2, Fig. 7.3), which is the same pattern as N2 digestion pattern, it should be to the left of recombination site, and in turn to the left of *exc-2*. There are only three genes in between this position and *mec-2* location.

Some of the primers for dsRNA complementary to these three genes were remade, aiming to amplify a larger region and knock down the mRNA more

efficiently. RNAi experiments were done to knock down these three genes. It turned out that none of these RNAi experiments gave cystic canal progeny.

The SNP sites downstream of this site were tested for the digestion pattern. The digestion pattern showed that *mec-2* is not the boundary of *exc-2* (Table7.2, Fig. 7.3). *exc-2* should locate to the right of *mec-2* .

More recombinants are required to further narrow down the region of *exc-2*.

SNP site	comments
C54G7:22992	N2 Digestion pattern
F14D12 :39094	PCR didn't work
T21F4:13904	N2 Digestion pattern
W01C8:26415	Digestion didn't work
C54H2:23555	N2 Digestion pattern
F13D11:6838	CB4856 Digestion pattern

Table7.2 *exc-2* SNP mapping data.

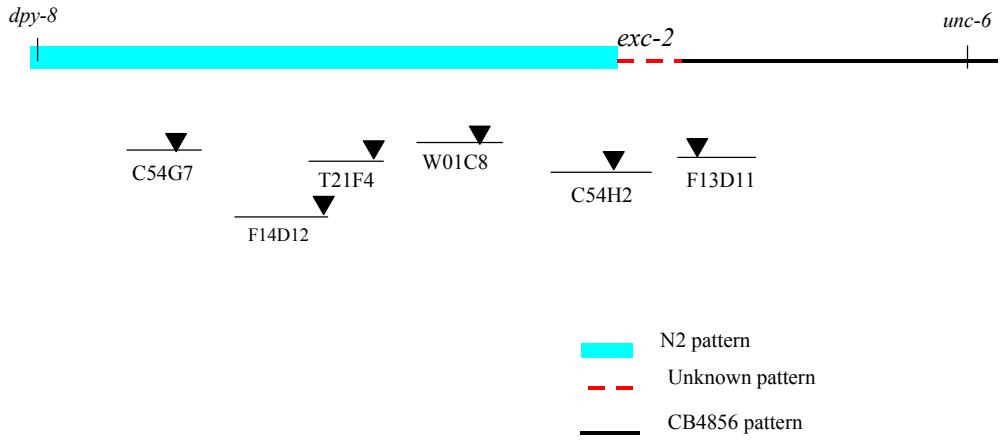


Figure 7.3 Schematic drawing of the Dpy non-Unc recombinant chromosome and SNP sites in the region. Black arrows point to the tested SNP sites position in the cosmid. SNP sites on cosmid C54G7, T21F4, and C54H2 turned out to have the same digestion pattern as N2 worms, they should be to the left of *exc-2*. SNP site on cosmid F13D11 showed same digestion pattern as CB4856. *exc-2* should be to the right of SNP site C54H2:23555, but to the left of *unc-6*.

Chapter 8

Conclusion and future directions

This thesis reports the cloning and characterization of *exc-9*. I showed that a novel single-LIM-domain protein is required for apical membrane maintenance of the shape of the excretory canals. In this chapter, I will discuss the implications from these results and also discuss the future experiments that could be done to better understand EXC-9 function and the pathway(s) that regulates the excretory canal apical organization.

8.1 *C. elegans* CRIP homologue is required for tubulogenesis

The *exc-9* mutant is the first CRIP mutant available for study. In this report, I demonstrate that EXC-9 is required to help maintain the apical cytoskeletal strutcture of the excretory canals. EXC-9 is located in the cytoplasm of the excretory canal tubes and the presence of EXC-9 in the canal is required and sufficient to maintain the apical structure of the excretory canal. How EXC-9 functions to maintain the tubular structure is not clear, but it likely functions by binding with other proteins. Overexpression of EXC-9 with an unblocked LIM N-terminus can lead to "unextended" canals, and that his phenotype might be due to the exposure of the LIM domain binding site to the partners.

These data provide information of CRIP function *in vivo*. It will be interesting to determine if the function of vertebrate homologues is conserved in similar developmental events. Since vertebrate homologues are located in the

intestine and heart, which also have tubular structures, the pathway in which EXC-9 functions might be conserved in vertebrates as well.

It is proposed that LIM-only proteins function by binding with other proteins (Kadrmas, J.L. and Beckerle, M.C. 2004). To prove that EXC-9 does work by binding with other proteins, some more experiments are required. The yeast two-hybrid experiment has been done, but we don't think that CSN-5 is a binding partner of EXC-9 *in vivo* since they have distinct expression pattern and don't show similar phenotype in the mutants. After a better antibody is acquired, immunoprecipitation will be a good experiment to try for finding binding partners of EXC-9.

8.2 The pathway that regulates the apical membrane organization in excretory canal

Since overexpression of EXC-9 causes unextended canal in wild-type and *exc-9* mutant backgrounds, I carried out injections of *exc-9* translational construct into different *exc* backgrounds to rule out a possible pathway that regulates the excretory canal apical membrane. Overexpression of EXC-9 can partially rescue *exc-2, exc-4,* and *sma-1* mutants, and cause unextended canal, but failed to rescue *exc-1* and *exc-5* mutants. Overexpression of EXC-5 causes unextended canal phenotype in *exc-9* mutants.

Based on these rescue data, it is possible that EXC-9 functions

downstream of EXC-2 and EXC-4, but upstream of EXC-1, EXC-5 and CDC-42 to organize the apical membrane of the excretory canal (See Fig. 4.14 for detail).

Since I only have the reciprocal rescue data for EXC-5 and EXC-9, it is possible that other genes are in parallel pathways that regulate canal structure. Cloning projects of *exc-1* and *exc-2* are being carried out in the lab now. The cloning data of these genes will be important for the reciprocal rescue results later.

8.3 Functional interchangeability of *C.elegans* CRIP proteins

As described, B0496.7 rescues the *exc-9* cystic canal phenotype when ectopically expressed in the *C. elegans* excretory canals. Thus, these two *C. elegans* CRIP homologues function in a similar way. Mouse CRIP cannot rescue the *exc-9* canal phenotype directly, but after modification, it can rescue the phenotype as well. It is possible that mouse CRIP still functions in a similar way as does EXC-9, but because of the conformation changes of the binding partners, the structure of mouse CRIP is slightly different from that of EXC-9. With the addition of nematode specific C-terminus and without the additional vertebrate specific proline, the conformations of mouse CRIP is similar to that of EXC-9.

Structural work of EXC-9 will help to find out the exact differences between EXC-9 and mouse CRIP. We can do either NMR or crystallography to solve the structure of EXC-9, or do the threading to fit the EXC-9 sequence with mouse CRIP tertiary structure.

8.4 Screens for suppressor allele

Finding suppressors of EXC-9 will help us to better understand the pathway that regulates the canal structure.

Overexpression of EXC-9 causes the unextended canal in wild-type and in some *exc* mutant backgrounds. In one stable line of *exc-4 (rh133)* with pBK121, the *exc-9* translational construct, almost 100% of glowing worms have unextended canals. If we can integrate the extrachromosomal array into the chromosome, we could use this allele for screening of EXC-9 suppressors. The suppressor should be able to make the glowing worms have longer canal or even cystic canal even in the presence of the pBK121 construct.

By doing this, we might be able to find more genes in the same or relevant pathway as *exc-9*.

Chapter 9

Materials and Methods

Worm Culture

Most worms were grown at 20°C using standard conditions (Sulston, J.E. and Hodgkin, J. 1988). Worms with *fem-3* mutation are sterile at high temperature (Hodgkin, J.A. 1986). The original *fem-3* strain was kept at 15°C; heterozygous *fem-3 exc-9* double mutants over Hawaiian chromosome were kept at 25°C to get *exc-9*-non-*fem-3* recombinants.

The wild-type strains were Bristol N2 and Hawaiian CB4856. Other strains includs:

LG I: NJ469 *exc-4 (rh133)*

BK36 *unc-119(ed3)*; *qpIs11*[*unc-119*;P*vha-1::gfp*].

LG IV: MT6984 *exc-9 (n2669)*

VC961 *exc-9 (gk395)*

BK9 *bli-6 (sc16) exc-9 (n2669)*

BK39 *exc-9 (n2669) fem-3 (q22)*

BK41 *exc-9 (n2669) unc-5 (e53)*

CB3824 *eDf19/unc-24 (e138) dpy-20 (e1282)*;

NJ731 *exc-5 (rh232);*

VC861 *csn-5(ok1064) IV/nT1[qIs51]* (IV;V).

LG V: AZ30 *sma-1 (ru18)*

DR466 *him-5 (e1490)*

LG X: NJ51 *exc-1 (rh26)*

NJ242 *exc-2 (rh90)*.

CB4856 was a gift from Dr. Lisa Timmons. VC961, VC861 and JK551 *fem-3 (q22) unc-5 (e53)* were obtained from the CGC (University of Minnesota).

SNP mapping

SNP, single-nucleotide polymorphism, mapping is based on the basepair differences between two wild-type alleles. When a double-mutant in the N2 background is crossed with a CB4856 wild type, recombinations will happen in the heterozygous F1 progeny (Fig.9.1). As seen in the figure, the chromosome to the left of the recombination site is the same as N2 chromosome, and the one to the right is the same as CB4856. If we can determine that one SNP site is the same pattern as N2, it should be to the left of the recombination site, then it has to be to the left of the *unc* gene.

CB4856 male line was obtained by picking spontaneous males onto new plates for crossing.

For the SNP mapping on the left side of *exc-9,* CB4856 males were crossed to BK9 (*bli-6 (sc16) exc-9 (n2669)*) hermaphrodites. *bli-6 exc-9*/CB4856 worms were isolated in the F1 generation. These worms are wild-type, as compared to the homozygous blistering self-progeny of the hermaphrodites. Since *bli-6* mutations are not 100% penetrant, all these worms were checked via DIC microscopy to make sure that the canals appeared wild-type. For the F2 and

later generations, I still kept the wild-type heterozygous worms for later isolation of recombinants. I screened the progeny for worms with blister phenotype but no canal defect. After letting the recombinants self-fertilize, I got the homozygous recombinants, and analyzed the progeny of the recombinants by means of single-worm PCR/restriction digestion for the SNP sites in between *bli-6* and *fem-3* (See Figure 9.1 for details). SNP sites with differences in sequence at restriction sites in either N2 or CB4856 worms were chosen to be tested. SNPs examined and primers used for amplification are listed in Table 9.1. Information regarding the location of the polymorphism and digestion pattern was found at the following site:

http://genome.wustl.edu/genome/celegans/celegans_snp.cgi

For the SNP mapping to the right side of *exc-9*, similar strategy was used. *exc-9 fem-3*/CB4856 worms were picked from a cross of CB4856 males and *exc-9 fem-3* hermaphrodites, and maintained at 15°C. The heterozygotes then were kept at 25°C to produce progeny. All homozygous *fem-3* worms will be sterile at this temperature. Only heterozygotes, segregating CB4856 and *Exc-9 non-Fem-3* worms, will be able to produce progeny. The worms with eggs were picked out to be examined for their canal phenotype. The ones with abnormal canals are the *exc-9*-non-*fem-3* worms, and were kept to get homozygous progeny and then analyzed for SNP sites (Fig. 9.3). The SNP site information is listed in Table 9.2.

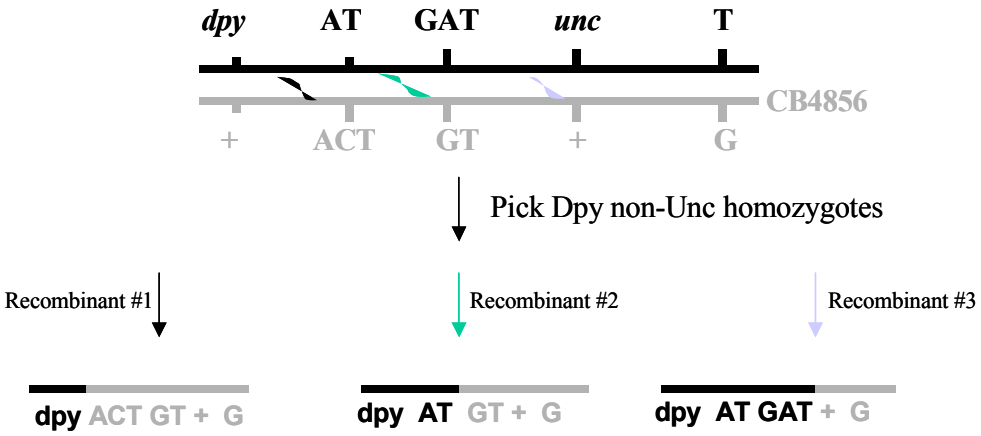


Figure 9.1 Schematic drawing shows how SNP mapping works.

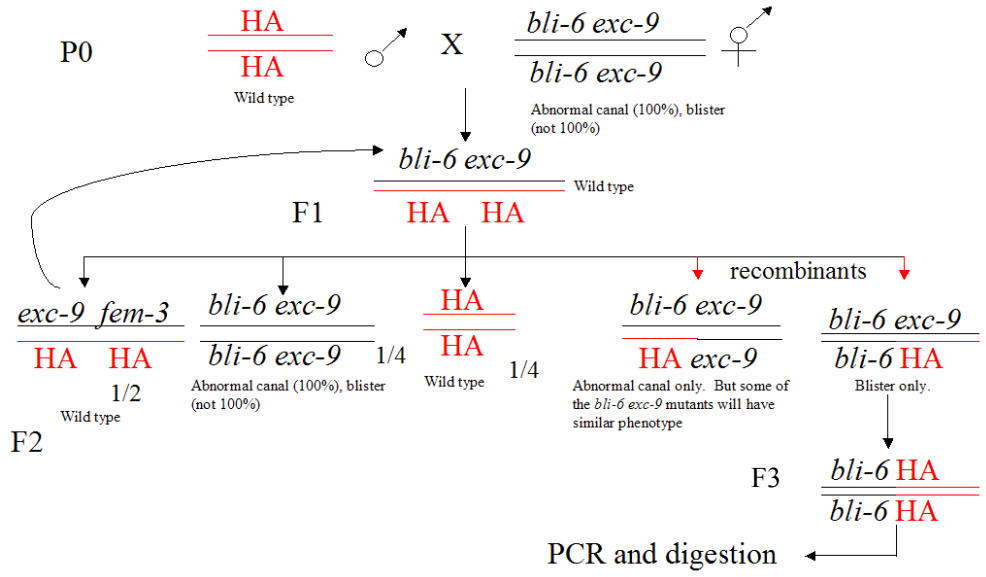


Figure 9.2 Strategy used for *exc-9* SNP mapping on the left side.

Table 9.1 Primers used for SNP mapping of *exc-9* from left side.

SNP site	5' primer (5'→ 3')	3'primer (5'→ 3')	Enzyme	Cutting strain
R05G6:2917	GGT GTT CAA ACA TGC GAC G	TTT GGA CGG ATA GCT ACA TAC G	PstI	CB4856
C06A6:2004	ATC GAT TGT CGA CTT GTC CG	ATG TGG TGC CGG AAA AGT TC	BfaI	N2
C49H3:3770	TTG CAG TTC GGA GTG TCT TAT G	TGG CTC GGT GCA AGT CTA TTG	DraI	N2
T09A12:14845	TCC GAA ACC AAT CGC CTA AG	AAT TCA GGT TCC TGC TTG CG	MlyI	CB4856

The enzyme used for digestion, and the strain whose DNA is cut by the enzyme are listed in the table.

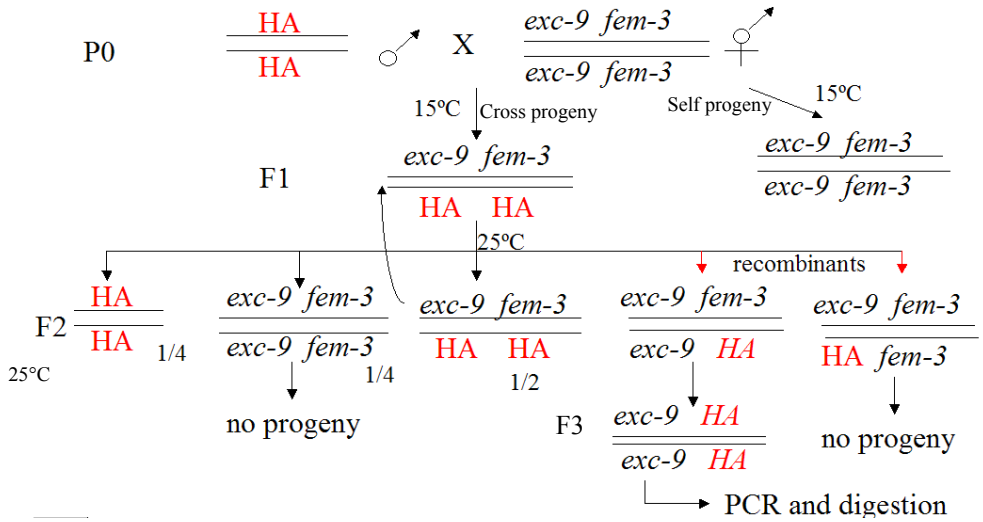


Figure 9.2 Strategy used for *exc-9* SNP mapping on the right side.

Table 9.2 Primers used for SNP mapping of *exc-9* from the right side.

SNP site	5' primer (5'→ 3')	3'primer (5'→ 3')	Enzyme	Cutting strain
F20D12:32689	TTT CCT TTG ACT AAT GTA ATG T	CAA AAT GGC TGA GAC TT	DraI	CB4856
B0218:15870	GTA ACT AAA TCT GAC GAG GA	ATC AGT TCC TCG TTG GTA A	AlwNI	CB4856
T09A12:14845	TCC GAA ACC AAT CGC CTA AG	AAT TCA GGT TCC TGC TTG CG	MlyI	CB4856
D2096:39721	ACG AAA AAT CAC AGA GCG GG	AAT CAA CAA CGG ACG ACG AG	EcoRI	N2

The enzyme used for digestion, and the strain whose DNA is cut by the enzyme are listed in the table

SNP mapping of *exc-2* was done in a similar way as for *exc-9*, *dpy-8* was selected as the marker on the left side. The primers used for *exc-2* SNP mapping are listed in Table 9.3.

Cosmid rescue

Bacteria containing cosmids in the region between cosmid C06A6 and B0218 were obtained from the Sanger center (http://www.sanger.ac.uk/). They were grown in the appropriate selective medium (Kan^R or Amp^R). Cosmid DNA was isolated using the DNA prep protocol. After purification, the cosmids were digested using restriction enzyme and fragments analyzed via gel electrophoresis to make sure that the cosmid structure is intact. After confirmation that the cosmid is intact, *exc-9* worms were injected with the cosmid at the concentration of 100μg/ml in water. Plasmid pCV01 was used as co-injection marker. Worm injections were performed using standard techniques (Mello, C. and Fire, A. 1995). Injection mothers were let recover for several hours before transfer onto fresh seeded plates. Progeny with glowing canals were examined under fluorescent dissecting microscope.

Digestion fragment and Single gene rescue

To prove that F20D12.5 is the gene encoding *exc-9*, two rescue injections were done.

Table 9.3 Primers used for SNP mapping of *exc-2*.

SNP site	Forward primer	Reverse primer	Enzyme	Cutting strain
C54G7:22992	5' - CCT CTT GTC TTC CGC TTA - 3'	5' - AGT AAG ACT ACA TAC TTT T - 3'	DdeI	CB4856
F14D12 :39094	5' - ACA TCA CAA TAG TGA TAT - 3'	5' - TTA GCT GAG AGT CTG TAG GC - 3'	CviKI	Both
T21F4:13904	5' - TTT AAG TAC GAC CAC GTG - 3'	5' - CGG TGT CAC TTG TGT CAG GG - 3'	MnlI	Both
W01C8:26415	5' - GTC AAG TTG CGA ATC GTC T - 3'	5' - ACG CCT CTT ATC CGT TTG AC - 3'	MnlI	Both
C54H2:23555	5' - TTA AAA GCT GGC TCT AGT GTT G - 3'	5' - AGC AAT TAT AGT GTC ATT GCC G - 3'	DraI	N2
F13D11:6838	5' - ATA CAG AAA CGC AAA TGA AA - 3'	5' - TTC ACA GAA TCA GAA AAT AA - 3'	CviKI	CB4856

The enzyme used for digestion, and the strain whose DNA is cut by the enzyme are listed in the table.

Cosmid F20D12 was digested using FspI into 3 pieces. The digestion fragments were run on a 1% crystal violet agarose gel (followed Invitrogen reagent instruction) and then the 11kb fragment was purified using Wizard® SV gel and PCR Clean-up system (Promega, Madison, WI). The 11Kb fragment contained the predicted *exc-9* gene and the 2.2kb region between *exc-9* and the next gene upstream. The *exc-9* gene was also PCR-amplified from N2 genomic DNA to get the 2.2kb upstream region plus the predicted coding region. These two DNA fragments were injected into *exc-9 (n2669)* mutants separately. pRF4 was used as co-injection marker.

RNAi

dsRNAs were synthesized for the genes of interest. The fragments complementary to the coding region of the gene were amplified by means of PCR first. All the primers for amplification have the T7 promoter at the 5' end. The dsRNAs were made using MEGAscript® kit from Ambion (Ausin, Texas). The synthesized dsRNA was used directly for injection with co-injection marker.

The injection progeny with marker were screened for abnormal canal phenotype.

The primers used for dsRNA synthesis for *exc-9* and *exc-2* are listed in Table 9.4 and Table 9.5.

Sequencing of mutant allele

Genomic DNA was isolated from *exc-9 (2669)* mutants by use of DNA prep protocol. The predicted gene region was amplified by use of PCR and then ligated into TOPO® TA cloning vector (Invitrogen, Carlsbad, CA). Every colony from the transformation plate with a positive insert has a different clone of PCR product. We purified DNA from 8 colonies derived from two independent PCR reactions and ligation/ transformation reactions. DNA sequencing of the amplified region was performed on both strands. All sequencing reactions were done by Idaho State University Molecular Research Core Facility (ISU MRCF).

5' RACE and 3'RACE

To find out the ends of *exc-9*, 5'RACE and 3'RACE were performed. mRNA was purified from N2 worms. The experiments were done following manufacturer's instructions; kits for RT-PCR and RACE are from Invitrogen, Calsbad, CA. The primers used are listed in Table 9.6 and Table 9.7.

Complementation test

Homozygous *gk395* males were obtained from mating N2 males with VC961 hermaphrodites and back-crossing the heterozygous F1 males to VC961. The *gk395* males were then crossed with BK41 *exc-9 (n2669) unc-5 (e53)*, to make cross progenys. The male cross-progeny were screened for canal

Table 9.4 RNAi primers used to synthesize dsRNA to test as apossible *exc-9* gene.

Gene	Forward Primer* (5' → 3')	Reverse Primer* (5' → 3')
C49H3.8	5'-TCT CAA TCC TAC ATC ATT CG-3'	5'-TAT CAG TCC AAT CAG CAG-3'
C49H3.1	5'-CCG CTG TTC ACG AC-3'	5'-AAA GCC GAC AAG TCC A-3'
F20D12.3	5'-CTC GGT GAA CTG GAT GAA GC -3'	5'-CTC CAT TAT GAG CAA ATC GT-3'
F42G8.11	5'-CCA GTC TCT CAA AAG TA-3'	5'-AAC ATC TCT CTC AAA AA-3'
B0218.8	5'-TGT CTC AGA CTA TTC CGT-3'	5'-TGG GAA AGT TGG CAT T-3'
C07G1.1	5'-CGG AAC GAC TTC AAG CA-3'	5'-CGG TTG TTT CTT TTG TGC-3'
F20D12.1	5'-ACT TGG GAT GCT GAT TGC GAT G-3'	5'-GTG CTG CGT TGG AAT GTC ACT CA-3'
F20D12.2	5'-ACA ACT ATC GCT TTC CGA CAC G-3'	5'-GCG ACC CTG CCG ATT GTG CG-3'
F20D12.4	5'-GTG AAG ATG AAG GTG CTC GTG-3'	5'-GCA CAG AAA TCG GTC ACT TT-3'
F20D12.5	5'-AAG TCA TTC GGA GGT TTG TTT AG-3'	5'-TCT TAC CTG TCC AGT TTG TC-3'
F20D12.6	5'-ACG ACA AGC GTA TTC AGC AA-3'	5'-ACA AGC GAA CAG GGA GTT GA-3'
F20D12.7	5'-TGG TTA TGA TTA CAA TGG GT-3'	5'-TAT CTG ACA GCA ATC CGC CAA TAA-3'

*All the RNAi primers have T7 promoter region at the 5' end. Sequence: 5'- GAT AAT ACG ACT CAC TAT AGG G-3'

Table 9.5 RNAi primers used to synthesize dsRNA to test as a possible *exc-2* gene

Gene	Forward Primer* (5'→ 3')	Reverse Primer* (5'→ 3')
B0399.1	AGG GTG ACG ACT TGC GAC TG	AAT CAG CGG TGT GGT TAC TG
C25F6.1	AGC CTG GGA AGA CTC AAC TTG T	AAA TGC TTC CTG AGC CAC CT
C25F6.2	TGA GGT TGC AGT CAA CGC AT	ATT CGG TCG TGT GGA ACG AT
C25F6.3	CGT TGA CAT GTG CGG CGT TAAA	ACA AGC GCG AAC CCA TCT ACA A
C25F6.4	TCT TCA GAA TGT CAA AAA AG	ATT TTC CGA ACA CTT TTA A
C25F6.6	TTC TAT GTA TGC CAG GCG GA	ACA GCA AGG AAG GGC TCG ATT T
C25F6.7	TGG AAT GAC AGA GCG TCT GGT T	ACA ACG CGA GTT TGT GCG TA
C31H2.3	AGC GAG TTC GAC ATT CGC AAG A	TTG ACA CCA GTG ACA TGA TCG TCC
C31H2.4	AAG CAG CCG CCG ATT GGT ATT T	TGT TGA ACT CCT GAG CCA CCA T
C38C5.1	A TG CTC CAA GAG CAG CAT GG	AGG AAT AGA ATT AGG ATG CA
C41A3.1	ACC AAA GCC CAT CAC TTG CT	TGG CCA ATG CGC TAG CAA TA
C41A3.2	ACT CCT TCA TCG TCG GAT TGT	TCT GAG AAG ATG TCG TTG GTA ACA C
C41A3.2A	AAG CCC GCA AGA AAC ACC AAC A	TCG GCA CAC AAT CGT GCT TT
C41A3.2B	AAG CCC GCA AGA AAC ACC AAC A	TCG GCA CAC AAT CGT GCT TT
C54G7.1	AAT CGG GCG TGC AGG AGA AAT	TGT GG ACC ACC TTG AAG AAG T
	A TG CGG CTG AAC TTA GCA ATT (new)	
C54G7.2	ACT GGG CCT GCT AAC AAC TAC T	CCA TGC AAA GTA ATA GTC ACA ACG G
C54G7.3	ATT GAT GTG TGT GTG GGT GGG A	TTT CTG CGT GCA GCC TCT T
C54G7.4	ATA GCA ACA TGC TTG CCC TC	CAA GTG TAT CAC GGT ATG CG
EGAP5.1	CGG ACT TGT GTT CTT CCT CA	CTA TAT CGT TCG GCC TGC GA
F32A6.3	ATC GGG CAA TCT AGC AGA CGT TGA	TGT CAG CAA ACT CAG TTC CCT CGT
F35C8.1	AAA TGA GTC AAC CTC CCG GAC	GAC GAT ACA CTG CTG ATT GTC CT
F35C8.2	ACC TCA AGG CGG AGA TGA AGA T	TGC GTG AAT TAC AGA GCA AGC G
F55D1.1	CGC AGC ATT GCC ATT GTA TG	TCA GTC ACG GAC TGT AGA ACC A
F55D1.2	CGG TTT GGT TAA TGA GTG TCT TCC	GCA TGA GGT GAA AGT GCA GGT A
H28G03.1	TGC AAG CTG CTA CTC AAC GGA A	AGT TGC TCG AAC AGG TCA CGT T
H28G03.2	ACA ACC AAT GCC TCA CCA CA	TGG CGC ATT GGA GAA TGC AA
H28G03.3	GGG TAT CAA CGG TGG AGT ATG A	GAG ACA TAT TGT TTG ATT CGT GG
H28G03.6	AGC ACT GCC AAG ACG GAA ACA T	ACG CAT TGA CGA TTT CAC GGC A
T03G11.1	TTG ATG GCA GTT TGC ACC AAG G	AGG GCA TCA TTC GGT TTC CAG T
T03G11.2	CTT ATT TTC AGT GGT CGT CT	TGA GTA GCC AAC TGC CAT AA
T03G11.3	AGG CGG CAA TTT GAA ATC CTC TGG	TGA ATC ATC TCG ACG AGC CCT T
T03G11.4	ACT GCC ACA AAT CCA CAA TGG G	GGA GAG TAC GTT TGA CCA CTC A
T03G11.5	ACA AGC AGC GTG GTG AGA TT	ACG TTG CCG TTG TTG TTG GT
T03G11.6	AAA CTT GTT CGC CGC ATC GT	TCG ATT GCG AGA AAC TTG GCG A
T03G11.8	CTC AAG ACA AAC CTT CCA G	ATG TTT TAG AAC TGG AA
T03G11.8	AAC TGG AAA CGT CGA GGT CA	ATT TGC TTC CCG TGA CGA GT
T23F2.1	TCA GCG CGC CAA GAA CGA AA	ATC TTC CAA GAC GGT TTC AGC TGG G
T23F2.2	ACT GAG CTT GTG GAT GCT CT	TGC GAA ACG TGA TCG ATG CT
Y34B4A.10	AAG TGC CTG TGA CAA CAT CCG A	TGC CAC TTC AGT CTG TTG GCT T
Y34B4A.2	TGC AGG CGA TAC GAG TTG AA	ATT GTC AGC TTG TGT GCC CAG T
Y34B4A.3	TCG ACA TGT TCA AAG AGG CGC T	GCG TTT GTC GGT TGA ATG CCA A
Y34B4A.4	CAG GTG TGT GAA GAA GAA GTG G	CTG GTA TGT ATC AAC TTG C
Y34B4A.5	ATT TGC GTG CGG AGC TGG ATT T	AAG ACT GCA ATC GTG CAG AAC AGC

Gene	Forward Primer* (5'→ 3')	Reverse Primer* (5'→ 3')
Y34B4A.6	AAA GTT TCA GGC GTT GTG GC	TGC TTG AAG GCA TCG CAA GT
Y34B4A.7	TTG TTG TCG GCA TTG CTC GT	AGA GTT GTC ACA GGC ATC CA
Y34B4A.8	TGC CTT GGA TGC TGG ACA AT	TGA GGC ACC GAT CAA TTG CAG A
Y34B4A.9	AGG ACC CAG TGG AAT GCG TAA A	TGC AAT CCA GCT CCA CAG ACA A
H28G03.4	TGC CCT GGG TCA AGA AGC AT	TGT GGC GCG CAA GTA GTT GA
F32A6.2	TCC TGA ACA TCG AAG CCA GA	CTT GAA ACA ACT TGC CAT TC
F32A6.1	GTG ATG ACA GAA TAC CTG AGT TG	CTA GAG TTT GCT CTC TCT TTG
F32A6.5	TGT GGG TGG TTT CTT ATG GGA C	CCA CGC TAA CTG TTG CAT TGC T
C26B9.5	ACT CGT TCT CGG AGC ACT TT	CAG CTC CAA ATT CCT TTG CCC
C26B9.3	TGT GGT GAT ATG CAA CCA GG	TAG TCA AAC GAC GGA AGA GCG
C26B9.2	GTG GCT ACA ATC CTC TTC CT	TGG TCC TTT GTA AGT CCG AGC A
C26B9.1	TGT TGG AGA ATG TAC TGC CG	CGT CGA TAA ATT GAC ACA GGC
C26B9.7	TGT TTG ATT ATA CAG AAC AAC CTG	TCT TTG ACT TCT GGA TCT TCG C
C26B9.6	GCC ATG GGT AAT GAT TGT CCC GTT	TGA CCG TCT CAA TGT AGG CT
F35C8.4	AGT CTC GAG GAA GAC TAC GA	TCC TCT TTG CCT TCT CTT T
F35C8.6	TCC CGT CCG CAT CAT TCT TT	TTG GTT TGT CTT GGC AGC GA
F35C8.7	TGG AGT ATT TGC CTT GTG GG	AGA ACA CTT CCA GAC ACC AG
F35C8.5	TGA CGT GTT TCT GTG CAA CG	TGT GAA ATC AAA GGC CAG GA
F35C8.3	CGT GAC GTC CGA ATT ATG TC	CGA TAG TTA ATC TTT CAG GAG CC
F35C8.8	ATG TTG TTG CAAATC CGC CC	TTG GCT GGA GGA AGA ACT TGG T
C03F11.2	TTA ACC GCA TCG GTC AGC AT	TCG GAA TAC TCA TAA CTC CCA
C03F11.3	TCC AGC TCA AAG CGC ATG TT	TGT GTT AAC CGT GCT GAC AGG T
C03F11.4	TAT GGG CTC AAC TAG AGG CA	CTC GCT AAT TTC ATC AAC TCT TG
T23F2.3	ACC TGC ACC GAC ATT CCA AA	AGG ATG ACG TAG CAA GCG TA
T23F2.4	AAG ATC CCA GTT AGT TCC A	AAC AGG CGT AGA TGA TTC CG
T23F2.5	TGC AAC TAT GGC TCT CAC TTG CAC	GCA TGA TAA GCT TAG TAG GCG AGG
C38C5.1	TGA GGC GTT ATA ATA CAC AAG T	GAC AAG AAG TCT CAC TTA CCT CC

*All the RNAi primers have T7 promoter region at the 5' end.

Sequence: 5'- GAT AAT ACG ACT CAC TAT AGG G-3'

The primers are listed in the order that the corresponding dsRNA was injected.

phenotype, and all of them showed abnormal canals.

Mapping of BK36

Strain BK36 has an integrated array in the chromosome. Knowing the location of this array will make future crosses easier. BK36 males were mated with available marker strains of different chromosomes. It turned out that the array is located on LG I (See Table 9.8 for details of the crosses).

Plasmid construction

All the inserts were amplified via PCR using *Taq* or *Pfu* polymerase. The inserts were ligated into pCR®-XL-TOPO® vector (Invitrogen, Carlsbad, CA) first for optimal yield. The inserts were then digested out from the vector by use of corresponding restriction enzymes; and purified after running on acrystal violet gel using using Wizard® SV gel and PCR Clean-up system (Promega, Madison, WI).

For all the constructs with pPD95.75 as backbone, the inserts were ligated into PstI and HindIII cutting sites. MscI and SacI sites were used to ligate inserts into the pCV01 backbone. EarI enzyme was used to modify the mouse CRIP sequence. Since the EarI recognition site will be cleft off after digestion, no new sequence will be introduced into the new constructs because of EarI. Plasmid pBK121 was sequenced to make sure that no mistake was made during the PCR

Table 9.6 Primers used for 5'RACE.

Primer name	Sequence
F20D12.5 racing1	5'-CTG TCC AGT TTG TCC TTG AT -3'
F20D12.5 racing2	5'-GGC CGT ATC CAC GTG GTC CG-3'
Abridged Anchor Primer	5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3'

Table 9.7 Primers used for 3'RACE.

Primer name	Sequence
C03F11.1 dT R	5'- GCA CAT TGA ACT GTA CGT TTT TTT TTT TTT TTT TT -3'
F20D12.5 3'race1	5'-GCG TTG CCA GAA AGC TGT GT-3'
F20D12.5 3'race2	5'- ATT GGT TTC GAC TGG CAT CG -3'
C03F11.1 Anchor R	5'-GCA CAT TGA ACT GTA CGA CG -3'

Table 9.8 Results of BK36 linkage crosses to markers on different chromosomes.

Linkage group	Dpy Unc non-glowing	Dpy Unc glowing	WT non-glowing	WT glowing
LGI (*dpy-5 unc-13*)	25	0	1	78
LGII (*dpy-10 unc-4*)	2	7	13	78
LGIII (*dpy-17 unc-32*)	14	18	20	70
LGIV (*unc-5 exc-9*)	4	6	9	18

The array is linked to *dpy-5 unc-13* on LGI.

reaction.

The plasmids are listed in Table 9.9. The primers used for amplification and sequencing are listed in Table 9.10.

Classification of *exc* phenotype and plotting

I divided the canal cyst size into five categories: none (N), tiny (T), small (S), medium (M) and large (L). "None" stands for a perfect canal with no cysts. Canals with some slightly wide regions were termed as canals with tiny cysts. When the cyst diameter was smaller than one quarter of the worm diameter, we called it a "small" cyst; when it is larger than one quarter of the worm diameter, but smaller than half the worm diameter, it is classified as "medium" size cyst; when the cyst diameter is larger than half of the worm diameter, it is termed as a "large" cyst.

For the canal length, five categories were used. "Cell body" means the canal didn't extend to the posterior at all, or slightly. "<vulva" represents the canal length in between cell body and vulva, which is located in the middle of the animal. Canals extending to the area close to the vulva are classified as "around vulva". If canals extended past the vulva but did not reach the full length, they are called ">vulva". Only the canals that went all the way near the tail are called "full length".

In this report, I record the canal phenotype according to these categories.

Table 9.9 List of all the constructs used in the study.

Plasmid	Type	Insert	Backbone
pBK101	Transcriptional	*exc-9* 0.7 kb promoter	pPD95.75
pBK102	Transcriptional	*exc-9* 1.4 kb promoter	pPD95.75
pBK103	Transcriptional	*exc-9* 1.8 kb promoter	pPD95.75
pBK104	Transcriptional	*exc-9* 2.2 kb promoter	pPD95.75
pBK118	Transcriptional	B0496.7 promoter	pPD95.75
pBK109	Transcriptional	C03F11.1 promoter	pPD95.75
pBK121	Translational	*exc-9* 2.2 kb promoter with whole coding region	pPD95.75
pBK122	Translational	*exc-9* coding region plus 11 AA on N-terminus	pCV01
pBK123	Translational	B0496.7 coding region plus 11 AA on N-terminus	pCV01
pBK130	Translational	New *vha-1* promoter	pBK122
pBK131	Translational	Mouse CRIP plus 11 AA on N-terminus	pCV01
pBK132	Translational	Mouse CRIP with nematode C-tail plus 11 AA on N-terminus	pCV01
pBK133	Translational	Mouse CRIP with nematode C-tail, without Proline, plus 11 AA on N-terminus	pBK132
pBK134	Translational	Mouse CRIP with nematode C-tail and 2 AA gap plus 11 AA on N-terminus	pBK132

Table 9.10 Primers used for amplification and sequencing for the construction of the plasmids.

Primer	Sequence (5'→ 3')	Purpose
F20D12.5 P0.7k R HindIII	AAG CTT CAA TAA TCC TCC GAA TGT TT	Amplify pBK101 insert
F20D12.5 P1.4k R HindIII	AAG CTT GTG ATG TAG TCT AAA CTT AC	Amplify pBK102 insert
F20D12.5P1.8 R HindIII	AAG CTT CGA ACT ATT TTA CCA TCC CA	Amplify pBK103 insert
F20D12.5 L HindIII	AAG CTT CAA CTT CGG GTC CTG GCA CGA	Amplify pBK104 insert
F20D12.5 R PstI	CTG CAG TGT TGG CTC TCT GAA ATG GA	Amplify *exc-9* promoter 3' end
F20D12.5 coding PstI	CTG CAG GGA ACC TGA AAA TGT TGA GA	Amplify pBK121
Exc-9 coding-L Msc I	TGG CCA CAT TTC AGA GAG CCA ACA	Amplify pBK122
Exc-9 Coding –R Sac I	GAG CTC GGA ACC TGA AAA TGT TGA GAA T	Amplify pBK122
B0496.7 coding-L Msc I	TGG CCA ATG CCA AAC TGT CCA AAT	Amplify pBK123
B0496.7 coding-R Sac I	GAG CTC GGA TTT CCA GTA GTT CCT TGA A	Amplify pBK123
mCRIP cDNA-L Msc I	TGG CCA ATG CCG AAG TGC CCC AAG T	Amplify pBK131 and pBK132
hCRIP cDNA-R Sac I	GAG CTC GGC TTG AAA GTG TGG CTC TC	Amplify pBK131
mCRIP cDNA tail-R Sac I	GAG CTC ACC TGT CCA GTT TGT CCT TGC TTG AAA GTG TGG CTC TC	Amplify pBK132
mCRIP cDNA P-down Ear I	CTC TTC TGC TAC TCC GCC ATG TTT G	Amplify pBK133
mCRIP cDNA P-up Ear I	CTC TTC GAGC AT GAT TGC AGT AGG GCT	Amplify pBK133
mCRIP cDNA gap-down Ear I	CTC TTC GAA GCA TGT GGA AAG ACA CTG ACC T	Amplify pBK134
mCRIP cDNA gap-up Ear I	CTC TTC GCT TTT TCT CAC ACT TCA GGC A	Amplify pBK134
Pvha-1 L Hind III	AAG CTT GCA TGC CTG CAG CC	Amplify pBK130
Pvha-1 R MscI	TGG CCA ACC TGA AAC ATC TGA GTG A	Amplify pBK130

The “unextended canal” will be “none” for cyst, and “cell body” for canal length.

When I plotted the diagram, I combined the tiny and small categories. And since the two tubes of the left and right excretory canals are two independent events, the number refers to the canal number instead of worm number. For the canal length diagrams, “cell body” is close to the intersection, and “full length” is far away. So the further away from X, Y axis intersection, the closer the canal length phenotype is to wild-type. This also applies to the diagrams of cyst size that have “large (L)” close to the intersection and “none (N)” far from intersection.

Alignment of EXC-9 protein sequence with that of homologues

Alignment is done on http://www.ebi.ac.uk/clustalw/ as described (Chenna, R., Sugawara, H. et al. 2003).

Primers used for B0496.7 RNAi

5’Primer: 5' - TAATACCACTCACTATAGGGTAAGTTGTTTTTTCAAGTT - 3'

3’ Primer: 5' - TAATACCACTCACTATAGGTTTCCAGTAGTTCCTTGA - 3'

Western blot and protein detection.

SDS/PAGE was performed following standard protocol, using pre-made NupageTM 4-12% Bis-Tris gel (1.0mmX10well) (Invitrogen) and XCell

SureLockTM gel box (Invitrogen).

For each lane, 30 μ g of sample was loaded. Kaleidoscope Prestained Standards (Bio-rad) was loaded and run along with protein samples as marker.

Protein bands were transferred from gel to PVDF membrane (Millipore) (presoaked in methanol followed by transfer buffer) by electrophoresis at 150mA for 4 hours. The presence of the transferred protein was estimated by visualization of the rainbow marker on the membrane.

Immediately after transfer, the membrane was blocked in blocking buffer (5% nonfat milk in 1XPBS) for overnight at room temp, then incubated with either purified 14650/14651 immunized serum or 14650/14651 preimmune serum (1:1000 dilution) in blocking buffer overnight, washed with blocking buffer four times, each time 5 minutes, followed by the incubation with secondary fluorescein-conjugated goat anti rabbit IgG (1:10000 dilution, ICN biomedicals, Inc. Aurora, OH) for 1 hr at room temp, followed by four 5 minutes PBS-tween washes.

The immunoreactive bands were detected by ECL^{TM} Western blotting detection reagents (Amershan Biosciences, UK) and protein bands were visualized from 13x18cm individually wrapped films by autoradiography.

Cell identification

GFP-positive cells were identified according to their position and

morphology (www.wormatlas.org; Sulston, J.E. and Horvitz, H.R. 1977).

Microscopy

Worms and embryos were observed by placing them on 2% agarose pads in M9 buffer with 1% 1-phenoxy-2-propanol added. For observation of active worms, 1-phenoxy-2-propanol was omitted. DIC and/or fluorescence microscopy were done (Sulston, J.E. and Hodgkin, J. 1988) with a Zeiss Axioskop Microscope and photographed with a MagnaFire Electronic Camera (Optronics). The images were assembled with Corel Photopaint.

Single-worm PCR

DNA from the recombinant progeny for SNP mapping was examined by use of single-worm PCR. To be sure, five worms were used for reaction instead of one.

Preparation for the PCR:

Put 13.5µl water into Eppendorf® tube, and pick 5 worms in to this liquid

Freeze in –80 °C freezer for 10 min

Thaw at room temperature

Add 1.5µl lysis solution (proteinase K included), vortex

Freeze in –80 °C freezer for 10 min again

65°C incubation 60 min (proteinase K digestion)

95°C incubation 10 min (inactivation of proteinase K)

Spin for 1 min and take supernatant for PCR reaction

PCR cycle

Step No.	Cycle temperature	Cycle time	Repeat
1	92°C	2min	1 cycle
2	92°C	35sec	
3	50°C -60°C depends on annealing temperature	35sec	
4	72°C	1min/kb of expected product length	Go back to step 2 39 times
5	72°C	10min	1 cycle
6	4°C	Forever	1 cycle

Worm lysis solution:

100mM Tris (PH 8.5)

100mM NaCl

50mM EDTA

1% SDS

1% β-mercaptoethanol

100μg/ml proteinase K

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