EXPLORING THE FUNCTION OF TRF-1, A TUMOR NECROSIS FACTOR (TNF) RECEPTOR ASSOCIATED FACTOR (TRAF), IN *C. ELEGANS* POLYCYSTIN-EXPRESSING SENSORY NEURONS

by

DIANALIZ SANTIAGO-MARTÍNEZ

A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Neuroscience

written under the direction of

Dr. Maureen M. Barr

and approved by

_____

_____

_____

_____

New Brunswick, New Jersey

January, 2013

ABSTRACT OF THE THESIS

EXPLORING THE FUNCTION OF TRF-1, A TUMOR NECROSIS FACTOR (TNF) RECEPTOR ASSOCIATED FACTOR (TRAF), IN *C. ELEGANS* POLYCYSTIN-EXPRESSING SENSORY NEURONS

By DIANALIZ SANTIAGO-MARTÍNEZ

Thesis Director:

Dr. Maureen M. Barr

TRAFs are adaptor molecules that function mainly in the immune system. Mammals have seven TRAFs that share a conserved C-terminal domain. The *C. elegans* genome encodes two TRAF genes, *trf-1* and *trf-2* (Y110A7A.2). In humans, mutations in the polycystin-1 (PC-1) or polycystin-2 (PC-2) ciliary mechanosensory complex cause ADPKD. In *C. elegans*, the polycystins LOV-1 and PKD-2 localize to cilia and are required for male sensory behaviors.

We find that *trf-1* is co-expressed with *pkd-2* in the male specific CEM, RnB, and HOB neurons and is required for male mating behaviors. We are interested in exploring a potential connection between immune recognition and mate recognition. We examined the mating behavior of mutant males defective in the Toll pathway including *tol-1* and *ikb-1*. We performed leaving, retention, response, and vulva location (Lov) behavior assays. Like *pkd-2* mutants, *trf-1(nr2014)* mutant males have response and Lov defects. In contrast, *tol-1* and *ikb-1* are like the wild-type. *trf-1* and *pkd-2* males are leaving assay (Las) defective, whereas *tol-1* is Las but statistically different from *pkd-2* and *trf-1*. *ikb-*

1 males are not Las. All mutant strains are normal for the retention assay, indicating that these males can sense the presence of a mate. We conclude that *trf-1* acts like *pkd-2,* suggesting they function in a similar pathway. The other TRAF gene, *trf-2,* is also mating defective and seems to act in the same pathway as *trf-1*. *trf-1* and *trf-2* act non-redundantly, since the double mutant does not yield a stronger phenotype.

To ascertain the site of TRF-1 action, we generated transgenic animals expressing TRF-1::GFP. TRF-1::GFP localizes to cell bodies, dendrites and axons of CEM, HOB and RnB neurons. TRF-1::GFP is detectable in sensory cilia. The mating defects were partially rescued in the mutant.

Genetic interactions between TRF-1 and components of the *C. elegans* PKD pathway (LOV-1, PKD-2, and KLP-6) showed no interaction in yeast two-hybrid experiments. Interestingly, TRF-1 showed interaction with EBAX-1/PQN-55, a substrate recognition subunit for cullin based E3 ligase complex. *Ebax-1/pqn-55* males were also shown to be response and location of vulva defective. *pqn-55* may act in the PKD pathway as well.

ACKNOWLEDGEMENT AND DEDICATION

I want to thank my advisor Maureen Barr for all her support and guidance, the Barr lab members for all their help and for being such a wonderful team, my committee members and the Rutgers *C. elegans* community for all their comments and suggestions, and the NIH for funding my research.

This thesis is dedicated to God, my family, and friends…

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I: INTRODUCTION

TRP Channels

The molecular basis of sensory transduction has been studied primarily based on two protein superfamilies: the transient receptor potential (TRP) channels and the DEGenerin/Epithelial Na^+ channel (DEG/ENaC) (Patapoutian et al., 2009; Wemmie et al., 2006). TRP ion channels are involved in sensing and transmitting a variety of external and internal stimuli, including but not limited to mechanical stress. TRP channels were initially identified in *Drosophila*. There have been 70 TRP channels in both vertebrates and invertebrates identified. In sea quirts, nematodes and fruitflies, 30, 24, or 16 different TRP channels have been identified, respectively. TRP channels are also expressed in yeast, and zebrafish have 60 TRP channel encoded genes. To date, there are 33 known mammalian TRP channels (Montell, 2001, Clapham, 2003).

TRP channels are nonspecific cation channels that generally consist of six putative transmembrane spanning segments (S1-6), with a pore-forming loop between S5 and S6, and the NH_2 and COOH termini located intracellularly (Hoenderop, 2003). Based on amino acid homologies, the TRP family is divided into seven subfamilies. In mammals, there are seven channels in the TRPC (canonical) subfamily, eight TRPM (melastatin), six TRPV (vanillinoid), one TRPA (ankyrin), three TRPP (polycystin, PC), and three TRPML (mucolipin) (Clapham, 2003). Channels of the TRPN (NOMPC) subfamily have been identified in worms, *Drosophila*, zebrafish, and frogs. TRP channels have a critical and diverse role in signal sensation and transduction. A number of TRP channels have been proposed to have mechanosensory properties as well. In this

thesis, the TRPP channel PKD-2 and polycystin signaling pathway in the nematode *Caenorhabditis elegans* are the main focus.

Polycystic Kidney Disease (PKD)

Polycystic kidney disease (PKD) is caused by various inherited human disorders that are major causes of end-stage renal disease in both children and adults. Autosomal dominant polycystic kidney disease (ADPKD) affects 1 in 1000 individuals and often results in end-stage renal failure. The phenotype for ADPKD, which usually presents in middle or late adulthood, is characterized by formation of renal tubule cysts as well as systemic effects on the cardio- and cerebrovascular systems, liver, and pancreas. Mutations in PKD1, also known as polycystin-1 or PC-1, are responsible for about 85% of cases, while the rest 5-10% of cases are caused by mutations in PKD2, also known as polycystin-2 or PC-2 (Wilson, 2004). PKD1 and PKD2 have been proposed to function together (Torres, 1998). In humans, PKD1 and PKD2 localize to cilia in renal epithelium (Newby et al., 2002; Yoder et al., 2002), where they have been proposed to function in intracellular traffic, fluid accumulation, ion transport, and as sensors of force.

Cilia and Ciliopathies

Cilia are hair-like structures found in eukaryotic cells (Gardiner, 2005). There are two types of cilia: motile and non-motile or primary cilia, which serve as sensory organelles. In humans, primary cilia are found on most cells of the body. *C. elegans* primary cilia of sensory neurons are studied in the Barr Lab (Bae et al., 2008). The cilium is made of a microtubule-based cytoskeleton called the axoneme. The

intraflagellar transport (IFT) plays an important role in ciliogenesis, the process of building the cilium (Rosenbaum and Witman, 2002). In humans, ciliary defects can give rise to genetic diseases called ciliopathies which include Polycystic Kidney Disease (PKD), Bardet-Biedl syndrome (BBS), nephronophthisis, Joubert syndrome, and Meckel-Gruber syndrome (Badano et al., 2006).

C. elegans

C. elegans is a transparent nematode, about 1 mm in length. *C. elegans* naturally comes in two sexes: self-fertilizing hermaphrodites and males. Males have a male soma and a germline that produces only sperm. Hermaphrodites, in the other hand, have a female soma but its germline produces sperm during the L4 stage and then during the rest of its adulthood produces only oocytes. In 1974, Sydney Brenner started using this nematode for molecular and developmental biology research (Brenner, 1974). Since then, *C. elegans* has become a powerful model organism. In 1998, sequencing of its entire genome was completed (The *C. elegans* Sequencing Consortium, 1998).

The hermaphrodite has 302 neurons and from those, 60 have cilia (White et al., 1986). The male has a total of 381 neurons and 52 are ciliated (Sulston et al., 1980). Scientists have explored the neural mechanisms for different *C. elegans* behaviors including mechanotransduction, chemotaxis, and male mating behavior.

C. elegans as a model for ADPKD

TRP channels have been identified in nematodes. In *C. elegans*, LOV-1 has been identified as the PKD1 homolog and PKD-2 is the PKD2 homolog (Barr and Sternberg,

1999; Barr et al., 2001). LOV-1 and PKD-2 form a channel/receptor complex (Fig. 1). In *C. elegans* the LOV-1 and PKD-2 TRPP complex localizes to ciliated male-specific sensory neurons and are required for male mating. *C. elegans* male mating behavior involves response, backing, turning, vulva location, spicule insertion, and sperm transfer (Fig. 2) (Liu and Sternberg, 1995). Most male-specific sensory neurons have been implicated in a mating behavior step. By cell ablations, the hook sensory neurons HOA and HOB were shown to be required for vulva location (Liu and Sternberg, 1995). The ray neurons are important for response. *lov-1* and *pkd-2* males are response and location of vulva (Lov) defective when they encounter a hermaphrodite, while other TRP mutants for example *osm-9* have normal response and vulva location (Barr and Sternberg, 1999; Barr et al., 2001). *lov-1* and *pkd-2* are expressed in the male cephalic (CEM) neurons, HOB and ray neurons (excluding ray 6), including the cilia of these sensory neurons (Barr and Sternberg, 1999; Barr et al., 2001). CEM neurons seem to be important for chemotaxis to hermaphrodites (Srinivasan et al., 2008; White et al., 2007). LOV-1 and PKD-2 proteins are enriched in sensory cilia and require intact cilia for their proper function (Qin et al., 2001).

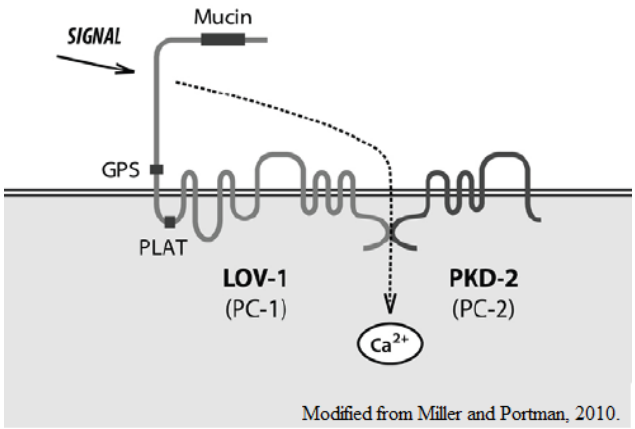


Figure 1. LOV-1 and PKD-2 form a channel/receptor complex.

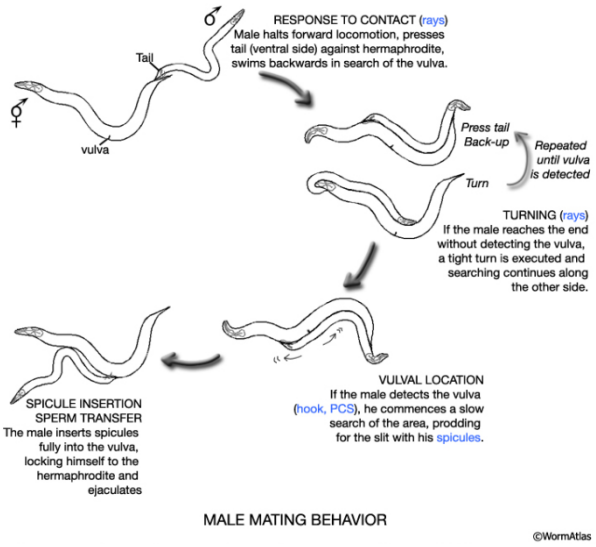


Figure 2. *C. elegans* mating behavior.

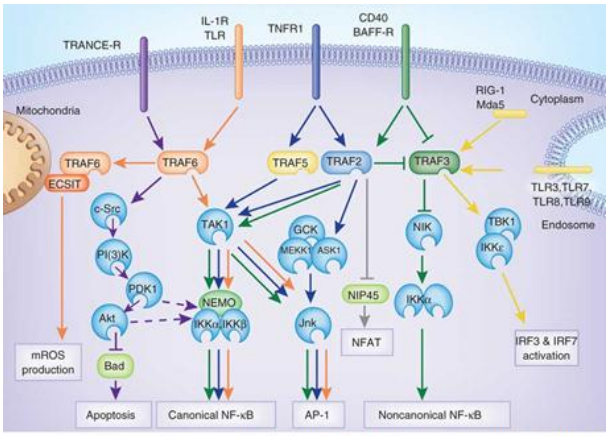
PKD Gene Battery

By studying mating behavior, *C. elegans* has been used to identify genes in the Polycystic Kidney Disease (PKD) pathway. *lov-1* and *pkd-2* are known to function in the PKD pathway and mutant males have mating defects. Additional genes have been shown to function in the PKD pathway in *C. elegans* (also known as the PKD gene battery): *klp-6* (a kinesin-3) (Peden and Barr, 2005), *cwp-1* to *–5* (co-expressed with polycystins) (Miller and Portman, 2010), *daf-19m* (male isoform of RFX transcription factor) (Wang et al., 2010), *cil-7* (ciliary localization gene 7) (Bae et al., 2008; Julie Maguire, unpublished), *F28A12.3* (TGF-β receptor/activin receptor type I/II) (Juan Wang, unpublished), and *trf-1* (tumor necrosis factor receptor associated factor (Traf) (Dianaliz Santiago-Martínez, unpublished)). These genes are all expressed in the male specific sensory neurons. *klp-6*, *cil-7* and *daf-19m* are required for PKD-2 expression or

localization. The other genes in the PKD pathway are co-expressed with *lov-1* and *pkd-2* but the function of most of them is still not known.

TRAFs and *trf-1*, The Tumor Necrosis Factor (TNF) Receptor Associated Factor (TRAF) Gene

Membrane-integrated receptors often transduce signals by the help of associated proteins. This is the case of the tumor necrosis factor receptor (TNFR) family of receptors and the Toll/interleukin-1 receptor (Toll/IL-1R) family. The tumor necrosis factor receptor associated factor (TRAF) family of proteins have been identified as the adaptors that associate with the cytoplasmic tail of these receptors. These adaptor molecules function mainly in the immune system (Fig. 3). In mammals, there are seven TRAFs that share a conserved C-terminal TRAF domain (Fig 4).



Oeckinghaus, A. Nature Immunology. 2011

Figure 3. TRAFs are adaptor molecules. TRAFs participate in different pathways and function mainly in the immune system.

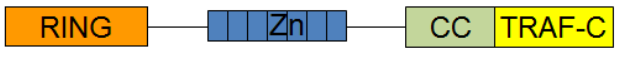


Figure 4. Domains of the mammalian TRAFs. Mammalian TRAFs have a RING domain, variable zinc finger repeats, a coiled-coil domain and a conserved TRAF-C domain.

trf-1 is a tumor necrosis factor (TNF) receptor associated factor (TRAF) homolog in *C. elegans*. *trf-1* has a paralog, Y110A7A.2, which is also a TRAF in *C. elegans*. Little is known about Y110A7A.2. *trf-1* is the gene of interest in my thesis work. The *trf-1* gene encodes a protein with a meprin-associated Traf homology (MATH) domain that may be involved in apoptosis (Aravind et al., 2001; Pujol et al., 2001). Since *trf-1* is co-expressed with *pkd-2* in the male-specific sensory neurons (personal communication, Drs. Maria Gravato-Noble and Jonathan Hodgkin, Oxford, UK), my project focused on exploring the function of TRF-1 in the *C. elegans* polycystin-expressing sensory neurons.

Innate Immunity and Toll Pathway

When exposed to pathogens, animals and plants respond quickly by inducing the expression of defense-related genes. The innate immune system provides this immediate defense against infection. The Toll pathway is a conserved signaling pathway important for innate immunity and has been well characterized (Valanne et al., 2011). In *Drosophila*, infection with the fungus *Beauveria bassiana* leads to the expression of the antifungal peptide drosomycin, whose induction depends on the nuclear import of Dif, a transcription factor of the Rel/NFκB family (Ip et al., 1993); this results from the activation of the *spätzle/Toll/tube/pelle/cactus* regulatory gene cassette (Lemaitre et al., 1996, Meng et al., 1999). This cassette has been initially shown to play an important role in *Drosophila* development. *C. elegans* does not seem to have an adaptive immune system and does not appear to have specialized immune cells. On the other hand, bacterial and fungal infections trigger a response in their system. *C. elegans* has some

structural homologs of components of the Toll pathway (Pujol et al., 2001).

Pujol et al., 2001, identified and characterized deletion mutants for four genes that may function in *C. elegans* Toll pathway: *tol-1*, *trf-1*, *pik-1*, and *ikb-1*, which are homologous to the *Drosophila Toll*, *dTraf1*, *pelle*, and *cactus* genes, respectively (Fig. 5). *tol-1* is important for *C. elegans* development and pathogen recognition (Pujol et al., 2001). TRF-1, but not IKB-1, might be required for the effects of TOL-1 in the immunity pathway (Tenor and Aballay, 2008).

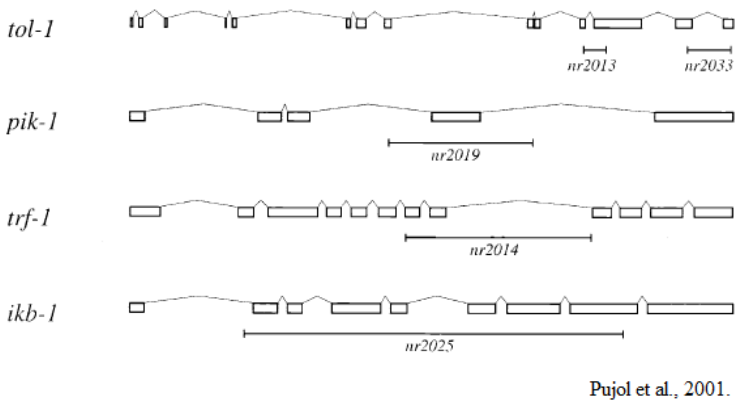


Figure 5. *C. elegans* Toll pathway genes. *tol-1*, *trf-1*, *pik-1*, and *ikb-1* are homologous to the *Drosophila Toll*, *dTraf1*, *pelle*, and *cactus* genes, respectively.

Thesis

For my thesis I explored the function of TRF-1, a Tumor Necrosis Factor (TNF) Receptor Associated Factor (TRAF), in *C. elegans* Polycystin-Expressing Sensory Neurons. In Chapter II, I characterize the *trf-1* gene, TRF-1 protein, expression and localization patterns and behavioral genetics studies. In Chapter III, I embark on TRF-1 structure-function analysis and protein interaction Yeast Two-Hybrid assay. In Chapter IV, I describe the initial characterization of the *trf-1* paralog, *trf-2*.

CHAPTER II: TRF-1

Introduction

The *trf-1* gene encodes a protein with a meprin-associated Traf homology (MATH) domain that may be involved in apoptosis (Aravind et al., 2001; Pujol et al., 2001). The tumor necrosis factor receptor associated factor (TRAF) family of proteins have been identified as the adaptors that associate with the cytoplasmic tail of TNF and Toll/IL-1 receptors. These adaptor molecules function mainly in the immune system. In mammals, there are seven TRAFs. *C. elegans* seems to have two TRAFs: *trf-1* and *trf-2* (For information on *trf-2*, see Chapter IV). TRF-1 has three important domains: RING, zinc finger TRAF, and MATH. These domains are important for binding to different proteins and receptors.

Since *trf-1* is co-expressed with *pkd-2* in the male-specific sensory neurons (personal communication, Drs. Maria Gravato-Noble and Jonathan Hodgkin, Oxford, UK), my project focused on exploring the function of TRF-1 in the *C. elegans* polycystin-expressing sensory neurons. In order to characterize *trf-1*, I looked at the expression of the gene and the subcellular localization of the protein. Since *trf-1* is co-expressed with *lov-1* and *pkd-2* in the male-specific sensory neurons, I performed behavioral genetics studies to determine if *trf-1* acts in the same pathway as *lov-1* and *pkd-2*. For the behavioral studies I did Response, Location of vulva, Leaving and Retention assays.

A. *trf-1* encodes a TRAF homolog in *C. elegans*

trf-1 is a tumor necrosis factor (TNF) receptor associated factor (TRAF) homolog in *C. elegans*. The *trf-1* gene encodes a protein with a meprin-associated Traf homology (MATH) domain that may be involved in apoptosis (Aravind et al., 2001; Pujol et al., 2001). This gene is on chromosome III at position 21.22 +/- 0.001 cM. *trf-1* has 2977 bp (unspliced + UTR), 1573 bp (spliced + UTR), and 462 amino acids. *trf-1* is male enriched. There are two mutant alleles of *trf-1*, *ok1721*, and *nr2014* (Fig. 6). For my thesis project I was working with *nr2014*. *nr2014* is a 896 bp deletion (25261-26156) of *trf-1* (F45G2.6). It is an intron to exon deletion.

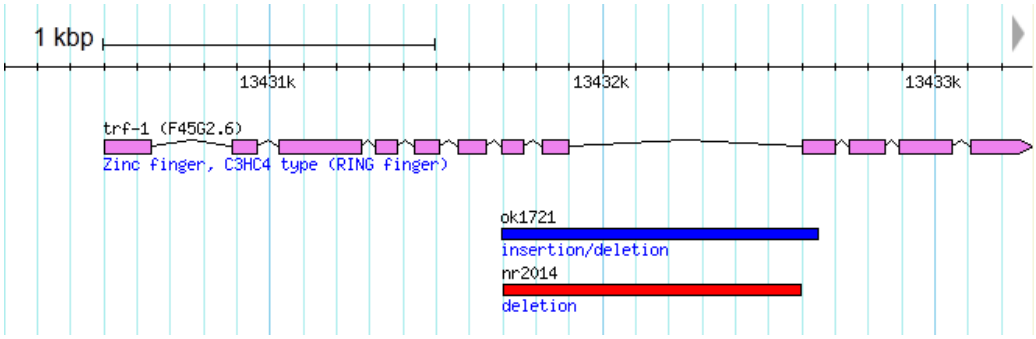


Figure 6. *trf-1* gene model and the two mutant alleles: *ok1721* and *nr2014*.

TRF-1 protein has three important domains: a Really interesting new gene (RING) domain at the N-terminus, a Tumor necrosis factor receptor associated factor (TRAF) zinc finger domain in the middle, and a Meprin-associated Traf homology (MATH) domain at the C-terminus (Fig. 7). A RING finger domain is a zinc finger type of structural domain that contains a $Cys_3HisCys_4$ amino acid motif that binds two zinc cations (Freemont et al., 1991; Lovering et al., 1993; Borden and Freemont, 1996). Many proteins that have a RING finger domain play a key role in the

ubiquitination pathway. The next domain in TRAFs is the zinc finger TRAF domain. This domain functions as interaction modules that bind DNA, RNA, proteins, or other small molecules. The final domain is the MATH domain. The MATH domain is necessary for self-association and receptor interaction. The *nr2014* deletion of *trf-1* cuts part of the N-terminus of the MATH domain (Fig.7).

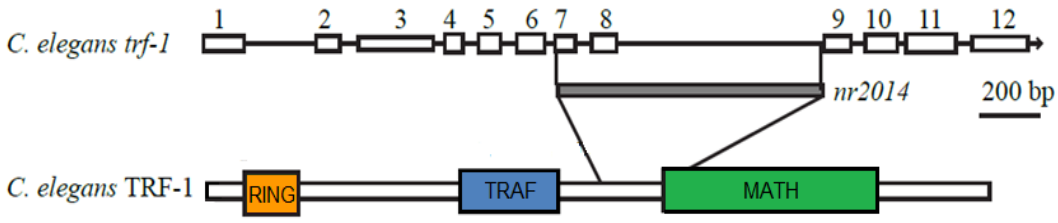


Figure 7. *trf-1* gene model and TRF-1 domain model. The *nr2014* allele is shown and the region it affects.

There are seven TRAFs in mammals. The best BLASTP match of *trf-1* and the human TRAFs is TRAF3 with a BLAST e-value of 7e-52 with an 82.9% length (www.wormbase.org) (Table 1). By using Muscle Multiple Alignment software, TRF-1 is 28.8% identical and 29.2% similar to hTRAF3 (Fig. 8). hTRAF3 functions in the signal transduction of CD40, a TNFR family member involved in the activation of the immune response. This protein is an important component of the lymphotoxin-beta receptor (LTbetaR) signaling complex, which induces NF-kappaB activation and cell death initiated by LTbeta ligation ("Entrez Gene: TRAF3 TNF receptor-associated factor 3").

The best BLASTP match to *trf-1* is the *C. elegans* Y110A7A.2 with an e-value of 1.1e-32 (Table 1). Y110A7A.2 (*trf-2*) is also a TRAF and is a *trf-1* paralog (See Chapter IV – *trf-1* paralog, *trf-2*). By using Muscle Multiple Alignment

software, TRF-1 is 21.4% identical and 26.2% similar to TRF-2 (See Fig. 26 from Chapter IV:TRF-1 Paralog, TRF-2).

Homology

Best BLASTP matches:

BLAST e-value	Species	Hit	Description	% Length
1.1e-32	*C. elegans*	Y110A7A.2	Y110A7A.2	57.6%
1.2e-54	*C. japonica*	CJA37635, isoform b	CJA37635, isoform b	29.0%
1.9e-30	*D. melanogaster*	Traf4-PC;	Flybase gene name is Traf4-PC;	83.8%
2.3e-92	*P. pacificus*	PP:PP48860	gene PPA15348	89.6%
2.4e-66	*S. purpuratus*	TR:H3J955	Uncharacterized protein	89.2%
2.5e-230	*C. remanei*	CRE-TRF-1	CRE-TRF-1	99.6%
4.3e-51	*M. musculus*	SW:Q60803	TNF receptor-associated factor 3	82.9%
4.5e-222	*C. brenneri*	CBN31542	CBN31542	98.1%
5.11e-121	*C. briggsae*	CBR-TRF-1	CBR-TRF-1	89.2%
7e-52	*H. sapiens*	TRAF3	Isoform 1 of TNF receptor-associated factor 3	82.9%

www.wormbase.org

Table 1. Homology analysis of *trf-1* and different species.

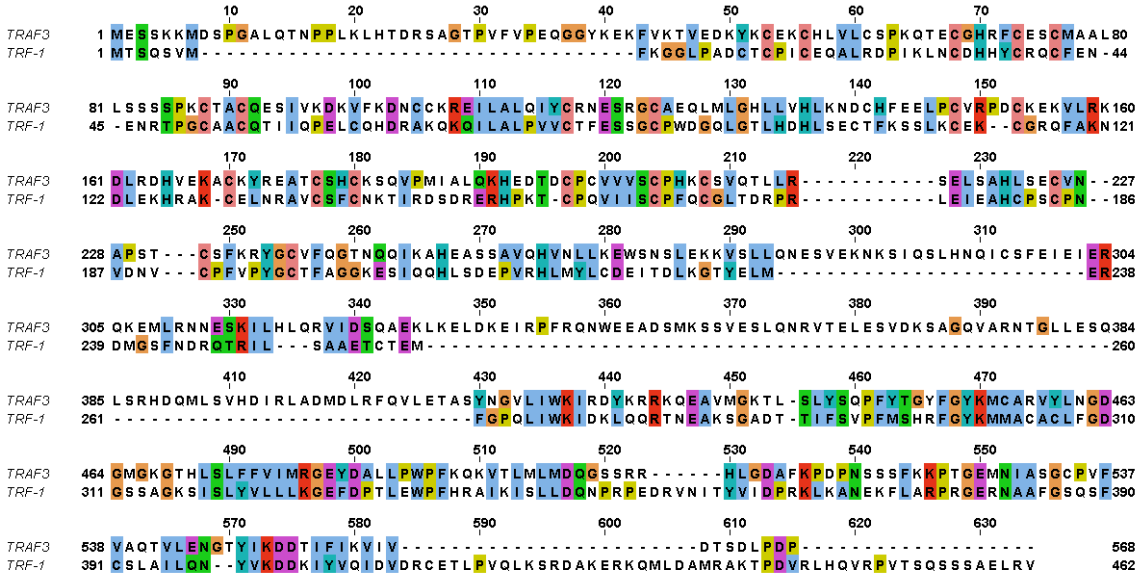


Figure 8. TRF-1 and hTRAF-3 alignment by Muscle Multiple Alignment.

B. *trf-1* is expressed in male-specific neurons

In order to determine the expression of *trf-1*, a transcriptional reporter was made. For brevity, the 2 kb promoter of *trf-1* was fused to GFP by PCR-Splicing by overlapping extension (SOE) (Hobert, 2002). The construct had pBx (rescues *pha-1* (wt)) as a cotransformation marker (Granato et al., 1994) and was injected to *pha-1;him-5* worms at a concentration of 10 ng/µl and cultured at 25°C to select for transformants. Stable lines were obtained and visualized under epi-fluorescence microscopy. *Ptrf-1::gfp* is expressed in the adult male CEMs in the head and in the HOB and RnBs (excluding ray 6) in the tail (Fig. 9). *trf-1* is not expressed in hermaphrodites. These results show that *trf-1* is expressed in the same neurons as *lov-1* and *pkd-2*.

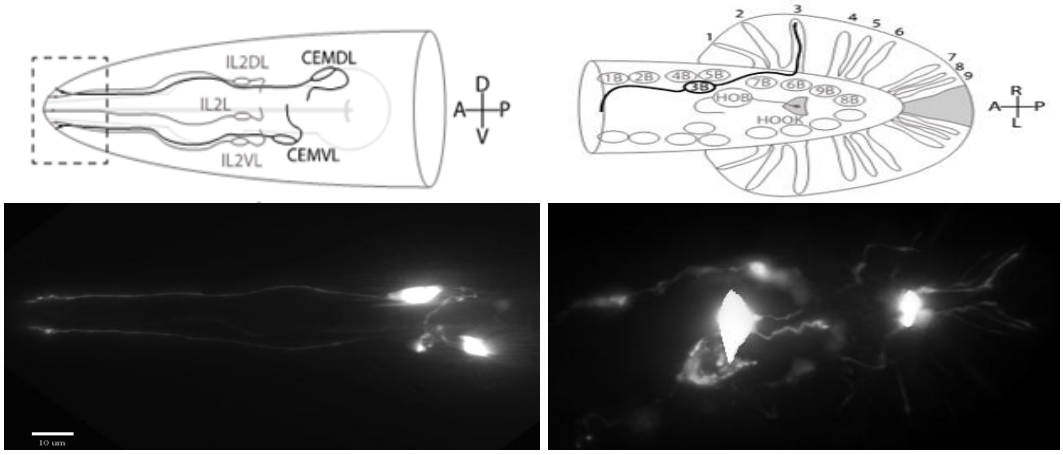


Figure 9. *Ptrf-1::gfp* expression in the adult male head and tail. Cartoon of *C. elegans* male-specific neurons in the head (Top, left) and the tail (top, right). *Ptrf-1::gfp* expression in the head (bottom, left) and tail (bottom, right).

In order to determine the subcellular localization of TRF-1, a translational reporter was made. For brevity, *trf-1* (wt) genomic DNA under the *trf-1* 2 kb promoter was fused to GFP. *Ptrf-1::TRF-1::GFP* and pBx cotransformation marker

pha-1(wt) were co-injected into *pha-1;him-5* worms at 10ng/µl and cultured at 25°C to select for transformants. Stable lines were obtained and visualized under epi-fluorescence microscopy. *Ptrf-1::TRF-1::GFP* is weakly expressed in the head CEMs and hook HOB, and is variable in the ray RnBs (Fig. 10 A and B). Expression in rays 3, 8 and 9 is most consistent in the initial transgenic line. TRF-1 localizes to the cell body of the neurons and to the dendrites and axons of the CEMs and RnB neurons but is excluded from the nuclei of these neurons. To select for a highly expressing line, Dr. Juan Wang outcrossed the stable line into *pha-1; him-5* and saw brighter expression and consistent expression in all rays, excluding ray #6 (Fig. 10 C and D). TRF-1 is also localized to the cilia of the CEMs and the rays (Fig. 10 C and D yellow inset).

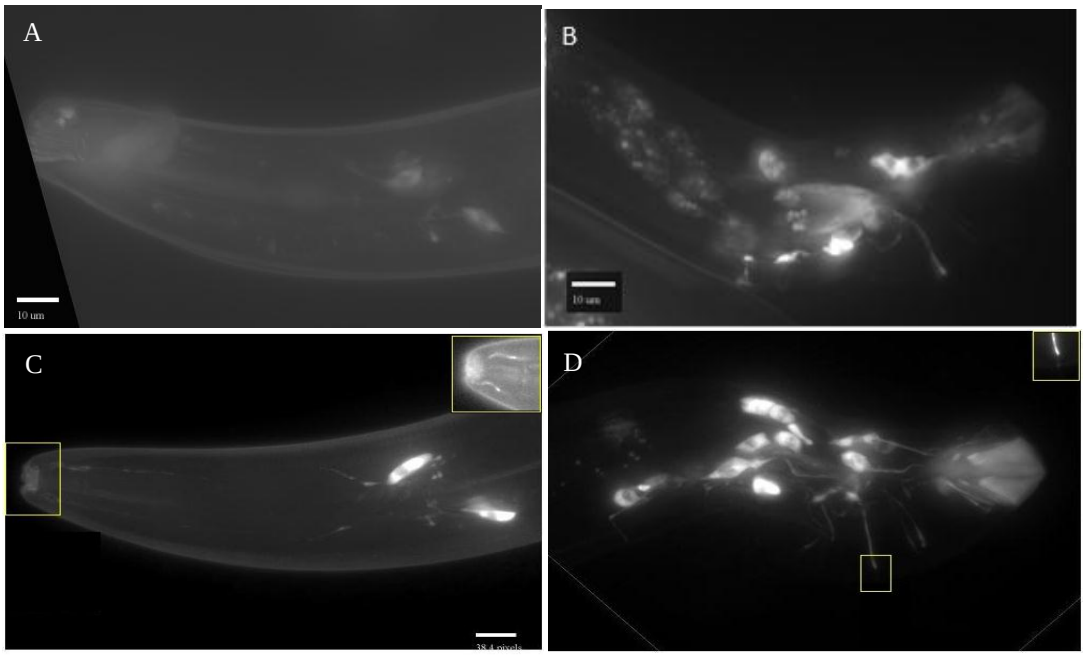


Figure 10. *Ptrf-1::TRF-1::GFP* (10 ng/µl injection) expression. Initial transgenic is dimly expressed in the CEMs in the head (A) and the hook HOB and in some rays (variable) (B). After outcrossing the line, expression is bright and consistent in the rays (excluding ray 6) (C and D). TRF-1 localizes to the cell body of the neurons and to the dendrites and axons of the CEMs and RnB neurons but is excluded from the nuclei of these neurons. TRF-1 is also localized to the cilia of the CEMs (yellow inset on C) and the rays (yellow inset on D).

Since the initial *Ptrf-1::TRF-1::GFP* transgenic line was injected at 10 ng/µl and exhibited dim and variable expression, I hypothesize that injecting at a lower concentration will give a brighter and stable expression. I injected the translational reporter at lower concentration (7 ng/µl) to *pha-1 trf-1;him-5* worms to see if the expression would be more stable. 10 ng/µl might have been too high and toxic for the worm and may explain why the expression was dim and variable. After injecting at a lower concentration, TRF-1::GFP transgenic males still showed variability in the rays, with rays 3, 8, and 9 being the most consistent (Fig. 11). Interestingly, injections at this lower concentration showed expression of neurons in the hermaphrodite (Fig. 12). Since there are a lot of neurons in the hermaphrodite head in that area, it was difficult to determine in which neurons TRF-1::GFP is being expressed. Two possible neurons are the CEP and the URX. The CEP are neurons of the cephalic sensilla that contain dopamine. URX is a ring interneuron. A cross with CEP and URX markers could be done to determine the identity of these neurons expressing TRF-1::GFP.

Injecting at a lower concentration did not solve the dim and variable expression of the translational reporter that I had hypothesized. However, injecting at 7 ng/µl showed expression in the hermaphrodite that was not seen when the translational reporter was injected at 10 ng/µl.

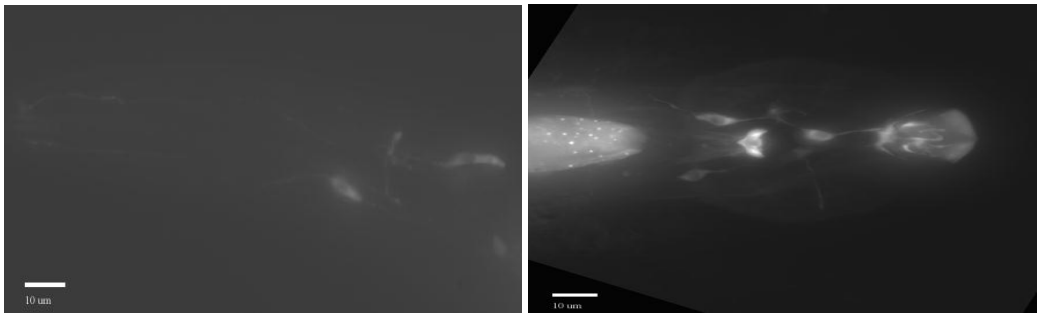


Figure 11. TRF-1::GFP expression at 7 ng/µl injections. TRF-1::GFP localizes to the male-specific neurons and is excluded from the nuclei of adult males. At this concentration, there is still variability in the rays, with rays 3, 8, and 9 being the most consistent (left, top and bottom).

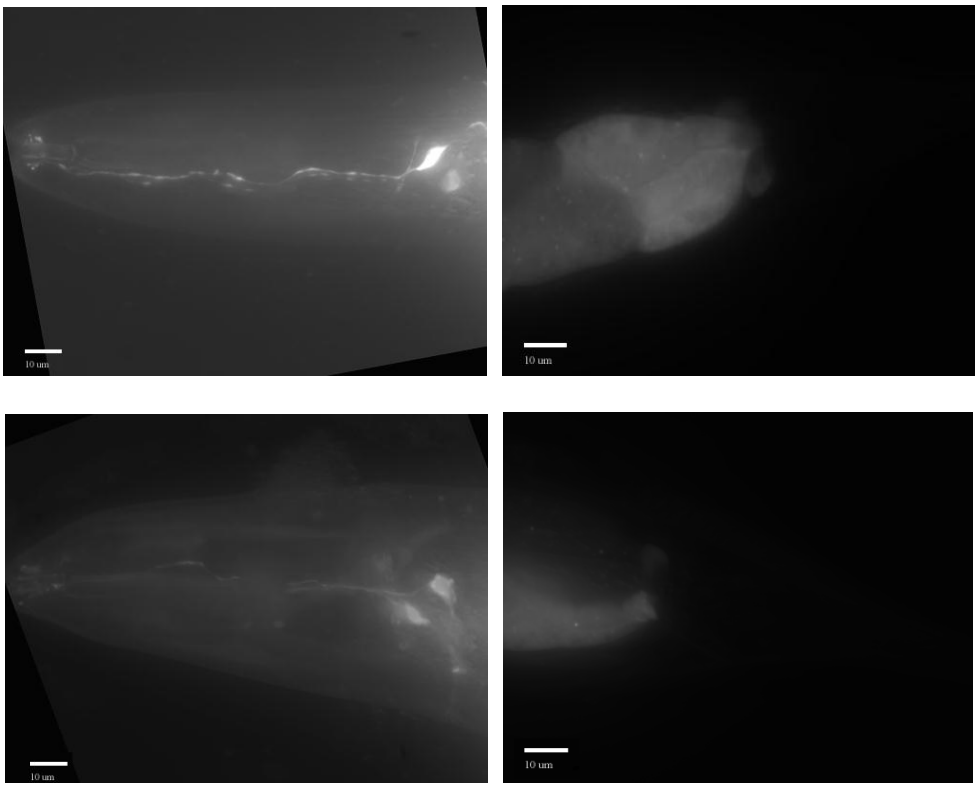


Figure 12. Two adult hermaphrodites expressing *Ptrf-1::TRF-1::GFP* (7 ng/µl injection). TRF-1::GFP localizes to some neurons in the head (left, top and bottom). The identity of these neurons needs to be determined. Expression in the tail is autofluorescence (right, top and bottom).

TRF-1::GFP is co-expressed with Pklp-6::TdTomato in the male-specific neurons

As mentioned in the Introduction, *klp-6* is also part of the PKD gene battery and is also expressed in the male-specific neurons. In addition to the male-specific neurons, *klp-6* is also expressed in the core IL2 neurons. Dr. Juan Wang determined the *trf-1* expression pattern by co-labeling TRF-1::GFP reporter with Pklp-6::TdTomato reporter. As shown in Fig. 13, *trf-1* is expressed in the male-specific neurons but is not expressed in the six sensory IL2 neurons that *klp-6* expresses. These results once again show that *trf-1* is expressed in the same neurons as *lov-1* and *pkd-2* and is a component of the PKD gene battery.

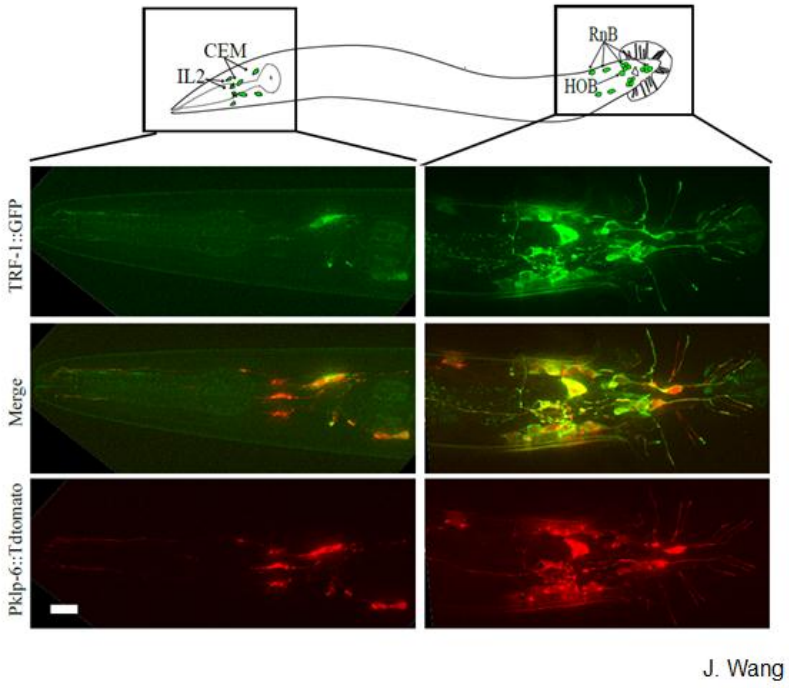


Figure 13. TRF-1::GFP is co-expressed with Pklp-6::TdTomato in the male-specific neurons. Top: TRF-1::GFP, Middle: Merge of TRF-1::GFP and Pklp-6::TdTomato, Bottom: Pklp-6::TdTomato. *trf-1* is expressed in the male-specific neurons but is not expressed in the six sensory IL2 neurons that *klp-6* expresses.

C. *trf-1* is required for male mating behaviors and acts in the PKD pathway

Different behaviors of *C. elegans* have been monitored to study different cellular pathways. *C. elegans* male mating behavior has been studied by the Barr lab to understand the genetics behind a polycystic kidney disease (PKD) pathway.

Response and Location of Vulva Assays

C. elegans male mating behavior involves response, backing, turning, vulva location, spicule insertion, and sperm transfer (see Fig. 2 from Chapter I - Introduction). I hypothesize that *trf-1* mutant males are mating defective in the response and location of vulva steps. For assaying mating behaviors I focused on the response and location of vulva steps because *lov-1* and *pkd-2* are response and location of vulva defective. Mating behavior assays were performed with adult, virgin males isolated at the L4 stage 24-hours prior to the assay. Young adult males were placed on a ~0.8–cm lawn of *E. coli* OP50 containing 20 *unc-31* hermaphrodites (that were also isolated as L4 24-hours prior to the assay) and observed for a four-minute period. *unc-31* hermaphrodites were used because they do not move and makes it easier to observe and study the male mating behavior. Response efficiency reflects the percentage of a population of males that successfully responded to adult, virgin *unc-31* hermaphrodite contact within four minutes. Location efficiency was calculated as a ratio of the number of successful stops at the vulva (full stop for ten seconds) to the number of total vulval passes by the male tail. Response efficiency

and location efficiency were analyzed by Kruskal-Wallis and Dunn's multiple comparison tests using Prism 5 Software.

trf-1(nr2014) acts in the same genetic pathway as *lov-1* and *pkd-2*.

As hypothesized, *trf-1* males exhibit response (Fig. 14) and Lov (Fig. 15) defects. *trf-1* males resemble *pkd-2* and *lov-1* mutants, and *trf-1* response and Lov defects are not statistically different from *lov-1* or *pkd-2*. *trf-1*; *pkd-2* and *lov-1*; *trf-1* double mutants are also response and Lov defective and are not statistically different than the single mutants. The *lov-1*; *trf-1*; *pkd-2* triple mutant showed the same behavior and is also not statistically different than the single mutants. These results show that *trf-1* acts in the same pathway as *lov-1* and *pkd-2*.

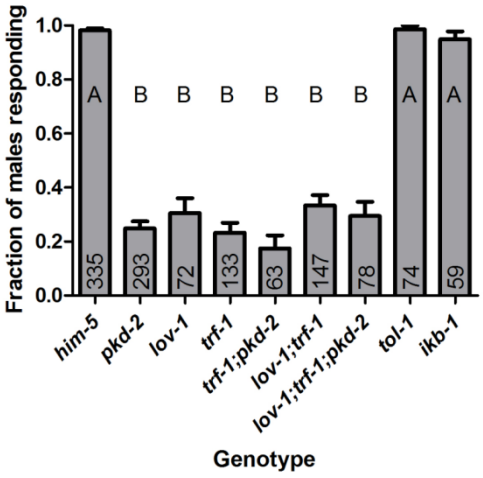


Figure 14. *trf-1* Response Assay. Fraction of males that respond to a hermaphrodite in a 4 min assay. Same letters represent a group that is not statistically different from each other.

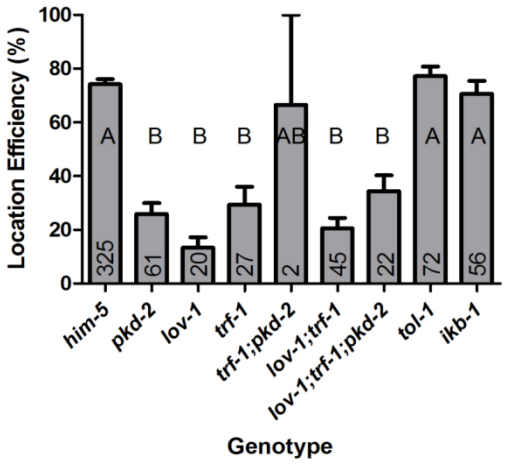


Figure 15. *trf-1* Location of vulva assay. Location efficiency was calculated as a ratio of the number of successful stops at the vulva to the number of total vulval passes by the male tail. Same letters represent a group that is not statistically different from each other.

Although *trf-1* has been implicated in innate immunity, it has an important role in male mating behavior. *tol-1* and *ikb-1*, two genes involved in innate immunity, were also assayed for response and vulva location. Both *tol-1* and *ikb-1* show normal response and location of vulva behaviors and are statistically different from *trf-1*, *pkd-2* and *lov-1*. Combined these results show that *trf-1* is acting in a polycystic kidney disease (PKD) pathway.

Rescue Experiments

Introduction of WT TRF-1::GFP into *trf-1* mutant partially rescues its mating defects

In order to determine if TRF-1::GFP is functional, a WT copy of TRF-1::GFP was introduced into the *trf-1* mutant strain, visualized for localization and assayed for response and location of vulva. I predict that the response and location of vulva defects of the mutant will be rescued after introducing a WT copy of TRF-1::GFP. TRF-1::GFP expression and localization was similar in wild-type and *trf-1* mutant males: cell body localization and nuclear exclusion in the CEMs in the head and HOB and RnBs (excluding ray 6) (Fig.16).

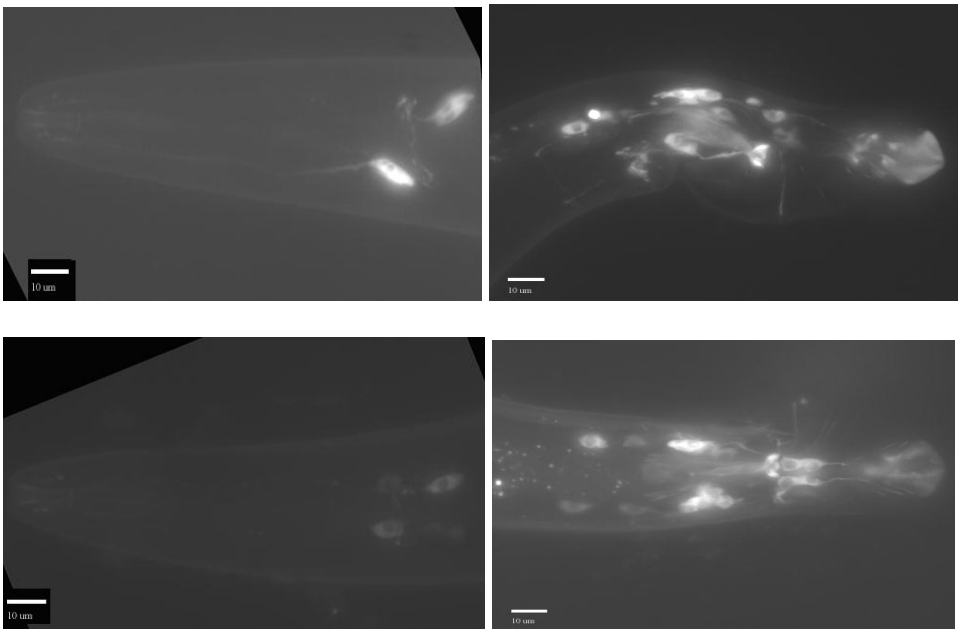


Figure 16. Two adult males that had WT TRF-1::GFP into *trf-1*. TRF-1::GFP expression and localization was similar in wild-type and *trf-1* mutant males: cell body localization and nuclear exclusion in the CEMs in the head and HOB and RnBs (excluding ray 6).

Response and Location of vulva were assayed as previously described. Four out of six lines tested rescued the response defect but none of the six lines rescued the Lov defect (Table 2). These results show that TRF-1 partially recues the mating defects of *trf-1*. It is interesting that TRF-1::GFP is expressed in the hook HOB neuron, one of the neurons involved in location of vulva, yet fails to rescue the Lov defect. It seems that there is another factor, yet to be discovered, involved that does not allow the rescue of the Lov defect.

Response efficiency and location efficiency of wild-type, *trf-1* and transgenic lines		
Genotype	Response efficiency % ±SE (n=number of males)	Location efficiency % ±SE (n=number of males)
Wild-type	97.4±1.5 (78) *	78.6±3.8 (77) *
trf-1	20.5±4.8 (73)	14.3±4.7 (15)
TRF-1::GFP in WT	89.6±4.5 (48) *	64.5±5.3 (43) *
trf-1; TRF-1::GFP 1	66.7±6.5 (54) *	28.3±5.5 (36)
trf-1; TRF-1::GFP 2	53.3±9.3 (30)	12.5±7.2 (16)
trf-1; TRF-1::GFP 3	38.7±7.0 (49)	29.4±9.4 (19)
trf-1; TRF-1::GFP 4	52.2±7.4 (46) *	30.2±7.5 (24)
trf-1; TRF-1::GFP 5	52.5±6.6 (59) *	22.8±6.1 (31)
trf-1; TRF-1::GFP 6	70.2±6.7 (47) *	30.7±6.1 (33)

Table 2. Response efficiency and location efficiency of WT, *trf-1* and transgenic lines. Four out of six lines tested rescued the response defect but none of the six lines rescued the Lov defect. (*) means that they are wt.

Leaving and Retention Assays

In *C. elegans*, the male's exploratory (mate-searching) behavior resembles the motivated behaviors of vertebrates (Lipton et al., 2004). A WT isolated male on restricted food source will eventually leave the food and wander around the plate in search of a mate. On the other hand, if hermaphrodites are present, males do not leave the food source. Isolated mutant males that do not leave the food source are leaving assay defective (Las) (Barrios et al., 2012). I hypothesize that *trf-1* males are Las.

Mate-searching was assayed by measuring the rate at which isolated males leave a food spot in a Leaving Assay (Lipton et al., 2004, Barrios et al., 2008). L4 males were isolated the day before of the assay. For the assay, a single male was placed on a bacterial spot (20 µL of OP50) and the tracks made on the agar were

monitored for two, four, six, and 24 hours. 20 males were scored for each genotype and the assay was done blind. If the tracks reached outside or beyond a 3.5 cm radius circle (1 cm from the edge of the plate), the male was scored as a leaver (Fig.17). The leaving rate was calculated as the probability of leaving per hour. The probability of leaving per hour was estimated as the hazard obtained by fitting an exponential parametric survival model to the data using maximum likelihood. This was calculated using the software R (http://www.R-project.org). For the Retention assay, the procedure was the same as for the Leaving assay but for the exception that two *unc-51* hermaphrodites were placed on the bacterial lawn.

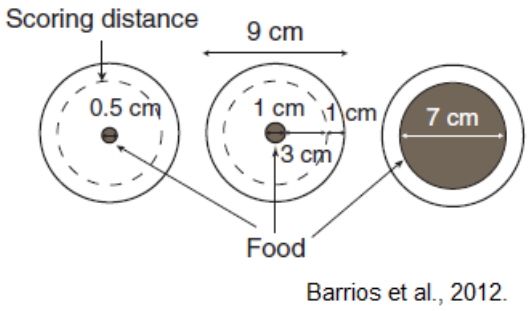


Figure 17. Scoring in the Leaving Assay. If the tracks reached outside or beyond a 3.5 cm radius circle (1 cm from the edge of the plate), the male was scored as a leaver.

***trf-1(nr2014)* is leaving assay defective (Las) but is WT in retention assays**

As hypothesized, *trf-1* males are Las, similar to *pkd-2* mutants (Fig. 18), meaning that *trf-1* males do not leave the food source to search for a mate. *trf-1; pkd-2* double mutant is also Las but to a lesser degree than the single mutants. It seems that *pkd-2* weekly suppresses the Las phenotype of *trf-1*.

I examined mutants in the innate immunity pathway. *tol-1* mutants males are also Las and not statistically different than the *trf-1;pkd-2* double mutant. It seems that *tol-1* is also playing a role in the mate-searching behavior. The other innate

immunity gene tested, *ikb-1* behaves like the WT. These results show that *trf-1* also plays a role in mate-searching behavior in addition to response and location of vulva in mating behavior. When a hermaphrodite is present (retention assay), *trf-1* males do not leave the food, indicating that they can sense the presence of a mate (Fig. 19).

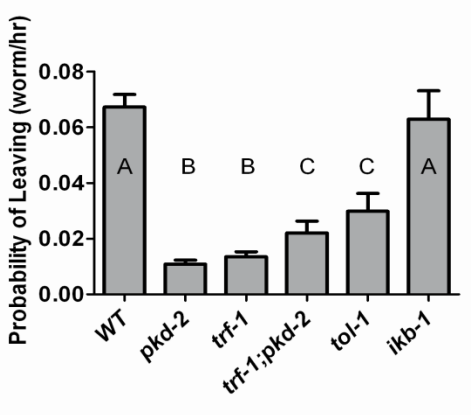


Figure 18. *trf-1* Leaving Assay. Isolated males were placed on a food source and the tracks made on the plate were observed. The leaving rate was calculated as the probability of leaving per hour.

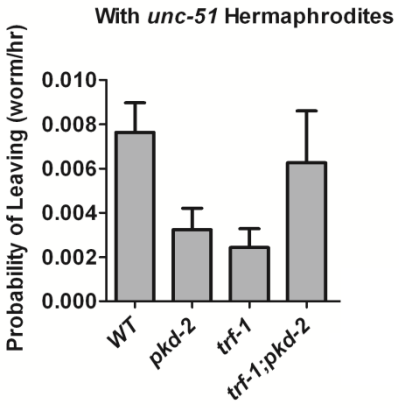


Figure 19. *trf-1* Retention Assay. Two *unc-51* hermaphrodites were placed in the food spot on the plate. The leaving rate was calculated as the probability of leaving per hour.

Conclusion

Ptrf-1::gfp is expressed in the adult male CEMs in the head and in the HOB and RnBs (excluding ray 6) in the tail. *trf-1* is not expressed in hermaphrodites. The initial transgenic *Ptrf-1::TRF-1::GFP* lines are weakly expressed in the head CEMs and hook HOB, and are variable in the ray RnBs (most consistent in rays 3, 8 and 9) when injected at 10 ng/µl. TRF-1 localizes to the cell body of the neurons and to the dendrites and axons of the CEMs and RnB neurons but is excluded from the nuclei of these neurons. When outcrossed into *pha-1; him-5, Ptrf-1::TRF-1::GFP* expression is brighter and

consistent in all rays, excluding ray #6. TRF-1 is also localized to the cilia of the CEMs and the rays. When the translational reporter was injected at a lower concentration (7 ng/µl), TRF-1::GFP was dimly expressed in the male-specific neurons and was variable in the rays. Interestingly, at a lower concentration, expression in some hermaphrodite neurons was observed. The identity of these neurons still needs to be determined. These results show that like *lov-1* and *pkd-2*, *trf-1* belongs to the PKD gene battery which are specifically expressed in the male CEM, RnB (not R6B), and HOB neurons; some of the genes in this battery are also expressed in the core IL2 neurons (not expressed in *trf-1*).

During mating, the male executes characteristic behavior steps in order to have successful sperm transfer. The CEMs seem to be important for chemotaxis to hermaphrodites, the tail ray RnBs are important for the initial response step and the hook HOB is important for location of vulva. *trf-1* mutant males are response and location of vulva defective. The *trf-1; pkd-2* and *lov-1; trf-1* double mutants are also mating defective and are not statistically different from the single mutants. The *lov-1; trf-1; pkd-2* triple mutant is not statistically different from the single mutants either. *tol-1* and *ikb-1* mutants of the innate immunity pathway have normal response and location of vulva. Introduction of WT TRF-1::GFP into *trf-1* mutant rescued response for four out of six tested lines. However, none of the tested lines recued location of vulva defects. It seems that there is another factor, yet to be discovered, involved that does not allow the rescue of the Lov defect.

The male's exploratory (mate-searching) behavior was also tested in a Leaving assay. A wild-type isolated male on food eventually leaves the food and wanders around the plate in search of a mate. *trf-1* mutant males do not leave the food source in search of

a mate, meaning that they has a Las phenotype. *pkd-2* is also Las. *tol-1* mutants, from the immunity pathway are also Las but to a lesser degree than *trf-1* and *pkd-2*. *tol-1* also seem to be having a role in mate-searching behavior. The other mutant from the innate immunity pathway, *ikb-1*, has normal mate-searching behavior. When hermaphrodites are present (Retention assay), a wild-type male does not leave the food source. *trf-1* mutant males present normal behavior in Retention assays meaning that they can sense the presence of a mate.

Together, these results show that *trf-1* is expressed in the same neurons as *lov-1* and *pkd-2* and has mating defects as well. *lov-1*, *pkd-2* and *trf-1* act together in response, location of vulva and in mate-searching behaviors.

CHAPTER III: TRF-1 STRUCTURE-FUNCTION ANALYSIS

Introduction

trf-1 is a tumor necrosis factor (TNF) receptor associated factor (TRAF) homolog in *C. elegans*. *trf-1* encodes a protein with a meprin-associated Traf homology (MATH) domain that may be involved in apoptosis (Aravind et al., 2001; Pujol et al., 2001). TRAFs have been identified as the adaptors that associate with the cytoplasmic tail of tumor necrosis factor (TNF) receptors. These adaptor molecules function mainly in the immune system. In mammals, there are seven TRAFs that share a conserved C-terminal TRAF domain (Fig 20, top). TRF-1 protein has three important domains: a Really interesting new gene (RING) domain at the N-terminus, a Tumor necrosis factor receptor associated factor (TRAF) zinc finger domain in the middle, and a Meprin-associated Traf homology (MATH) domain at the C-terminus (Fig. 20, bottom). A RING finger domain is a zinc finger type of domain that contains a $Cys_3HisCys_4$ amino acid motif that binds two zinc cations (Freemont et al., 1991; Lovering et al., 1993; Borden and Freemont, 1996). RING finger domain-containing proteins play a key role in the ubiquitination pathway (Deshaies and Joazeiro, 2009). The TRAF domain may function as interaction modules that binds DNA, RNA, proteins, or other small molecules. The MATH domain may be necessary for self-association and receptor interaction. The *nr2014* deletion of *trf-1* removes part of the N-terminus of the MATH domain.

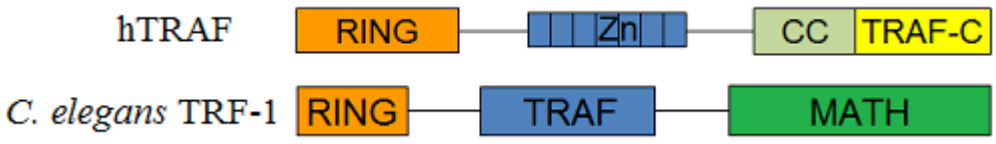


Figure 20. Human TRAF (top) and *C. elegans* TRAF (bottom) protein domains.

TRF-1 does not physically interact with components of the PKD pathway

Since *trf-1* is expressed in the same male-specific neurons as *lov-1* and *pkd-2*, and is part of PKD gene battery, I next wanted to test if TRF-1 physically interacts with components of the PKD gene battery by Yeast Two-Hybrid experiments. I hypothesize that TRF-1 interacts with components of the PKD pathway. For brevity, the TRF-1 cDNA clone was obtained from the Barr Lab ORFeome library and was cloned into the Yeast Two-Hybrid prey (pGADT7) and bait (pGBKT7) vectors by Gateway cloning system. Yeast Two-Hybrid is a protein-fragment complementation assay. In a Yeast Two-Hybrid assay, the transcription factor is split into two fragments: the binding domain (also known as bait) and the activating domain (also known as prey). Proteins of interest are fused to these domains. When the transcription factors (with the proteins of interest) come together and bind onto an upstream activation sequence, they induce the activation of downstream genes (Young, 1998).

As control (Table 3), TRF-1 was tested as Prey and the empty Gateway vector was the Bait. From this, there was no yeast growth on the TLH plate, meaning that TRF-1 does not autoactivate. When TRF-1 was used as Bait and the empty vector as Prey, there was no growth, meaning that it does not autoactivate in that plasmid. When TRF-1 was testes as Bait and an unrelated yeast protein, SNF4 was used as Prey, there was no yeast growth either, meaning that TRF-1 does not bind unspecifically. Together, these controls show that TRF-1 does not autoactivate in any of the vector and that it does exhibit non-specific interactions.

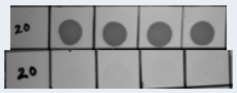
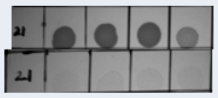
Prey	Bait	Interaction?	Comments	TL TLH
TRF-1	GWY	No	TRF-1 does not autoactivate	
GWY	TRF-1	No	TRF-1 does not autoactivate	
SNF4	TRF-1	No	Does not interact unspecifically	

Table 3. Yeast Two-Hybrid controls. TRF-1 with empty Gateway vector (GWY) and with and unspecific protein (SNF4).

For the Yeast Two-Hybrid experimentation, TRF-1 (full-length) was the Prey and as Bait I used PKD-2 C-terminal, PKD-2 N-terminal, LOV-1 C-terminal, and kinesin KLP-6 cargo binding domain. The PKD components in the Bait vector were previously made by Dr. Natalia Morsci. Of the PKD pathway proteins tested, PKD-2 C-terminal, LOV-1 C-terminal, and KLP-6 cargo binding did not show growth on TLH plates, meaning that they do not physically associate with each other (Table 4). There was growth on TLH and TLHA plates with the PKD-2 N-terminal but this result cannot be interpreted as a positive interaction because the PKD-2 N-terminal construct autoactivates when in the Bait plasmid (pGBKT7). TRF-1 does not seem to interact with components of the PKD pathway in the plasmid tested. Interactions of the proteins in the opposite plasmid could be done to determine if there is interaction between them or to confirm that they do not physically associate with each other.

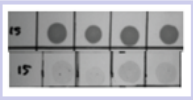
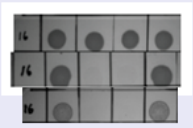
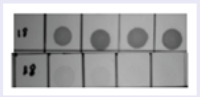
Prey	Bait	Interaction?	Comments	TL TLH
TRF-1	PKD-2 C-term	No		
TRF-1	PKD-2 N-term	?	PKD-2 N-term autoactivates in pGBKT7	 TLHA
TRF-1	LOV-1 C-term	No		
TRF-1	KLP-6 [352-928] (Cargo binding)	No		

Table 4. TRF-1 Yeast Two-Hybrid experiments with PKD pathway components.

TRF-1 specific domain constructs were made

Since there was no physical interaction with the full-length TRF-1 and the components of the PKD pathway tested, I divided TRF-1 into its domains (RING, TRAF, and MATH) and combination of domains (RING-TRAF and TRAF-MATH) (Fig. 21). These domain constructs were cloned into the Yeast Two-Hybrid Gateway pGADT7 vector (Prey). As future experiments, physical interactions of these domains with the PKD-2 pathway components can be tested by Yeast Two-Hybrid.

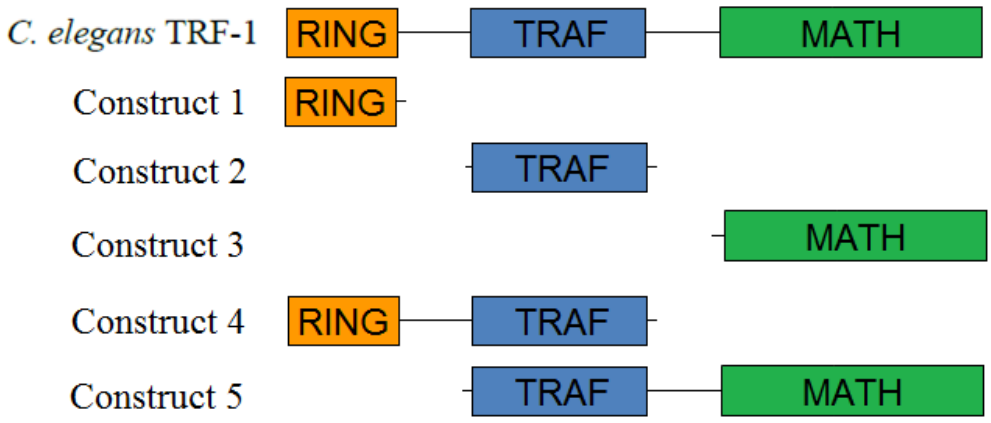


Figure 21. TRF-1 specific domain and domain combination constructs built.

TRF-1 interacts with Ebax-1/PQN-55

EBAX-1/PQN-55 belongs to a novel class of protein. Yishi Jin lab proposes EBAX-1/PQN-55 is a substrate recognition subunit of cullin based E3 complex. *pqn-55* mutants are defective in PKD-2::GFP expression (Bae et al., 2008; Nathan Schroeder, unpublished). Since TRF-1 has a RING domain and RING finger domain-containing proteins play a key role in the ubiquitination pathway, I next tested if TRF-1 interacts with EBAX-1/PQN-55 (a possible substrate recognition subunit of cullin based E3 ligase complex). I hypothesize that TRF-1interacts with EBAX-1/PQN-55. With Malan Silva, constructs for EBAX-1/PQN-55 were tested (obtained from Yishi Jin Lab) (Fig. 22). We tested the full-length (EBAX-1 FL) and specific domain constructs: Ebax-1 N2 (containing the BC-box, Cul2 box, and SWIM domain), EBAX-1 N1 (containing the BC-box, Cul2 box, SWIM domain, and the a domain), and EBAX-1 C1 (containing the b, c, and d domains).

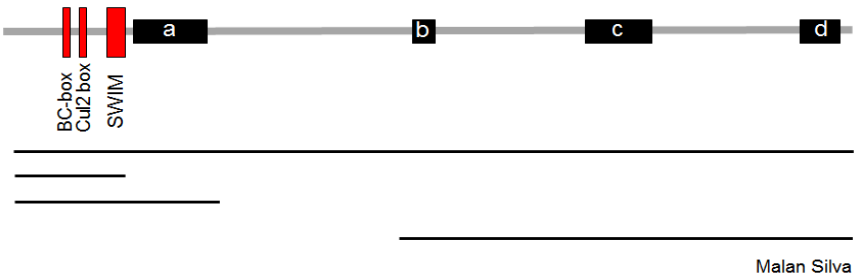


Figure 22. EBAX-1/PQN-55 protein domains and the constructs it was broken into.

For the Yeast Two-Hybrid, TRF-1 was the Prey and EBAX-1/PQN-55 constructs were the Bait. TRF-1 does not interact with the full-length EBAX-1/PQN-55, EBAX-1 N2, or the EBAX-1 C1 (Table 5). Interestingly, there was yeast growth on the TLH plate for TRF-1 and the EBAX-1 N1 (containing the BC-box, Cul2 box, SWIM domain, and the a domain). TRF-1 may interact with EBAX-1/PQN-55 through the N-terminus of EBAX-1/PQN-55. This interaction still needs to be repeated. We also need to determine whether EBAX-1/PQN-55 autoactivates. As a future experiment, the interaction can be tested with the proteins in the opposite vector to see if there is a positive interaction.

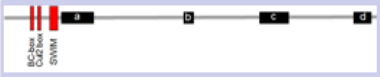
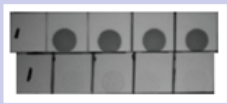
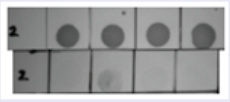
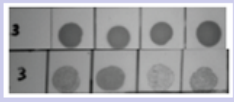
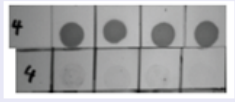
Prey	Bait	Interaction?	Ebax-1/PQN-55 Domain	TL TLH
TRF-1	Ebax-1 FL	No		
TRF-1	Ebax-1 N2	No		
TRF-1	Ebax-1 N1	Yes		
TRF-1	Ebax-1 C1	No		

Table 5. TRF-1 Yeast Two-Hybrid experiments with EBAX-1/PQN-55. Full-length and specific domains were tested.

pqn-55 males are mating defective

Since TRF-1 and EBAX-1/PQN-55 seem to be interacting, I next wanted to determine if *ebax-1/pqn-55 (ju699)* has mating defects. I hypothesize that ebax-1/pqn-55 males are response and location of vulva defective. Response and Location of vulva assays were performed as previously (See Chapter II: TRF-1). As hypothesized, *pqn-55 (ju699)* males are response defective and resemble the *trf-1* response defect (Fig. 23). The *trf-1; pqn-55* double mutant is also response defective and is not statistically different from the *trf-1* and *pqn-55* single mutants. In the location of vulva assay, *pqn-55* is Lov defective and is not statistically different from *trf-1* (Fig. 24). The *trf-1; pqn-55* double mutant in addition of being response defective, seem to have turning defects, which made it impossible to score Location of vulva. *trf-1; pqn-55* double mutant males would leave the hermaphrodite quickly after responding or after attempting to turn. Combined, these results suggest that *pqn-55* is also acting in the PKD pathway.

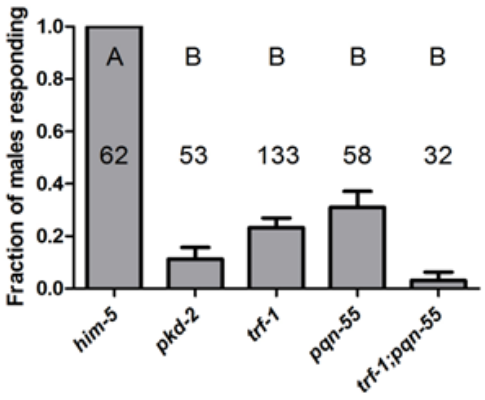


Figure 23. *pqn-55* Response Assay. Fraction of males that respond to a hermaphrodite in a 4 min assay. Same letters represent a group that is not statistically different from each other.

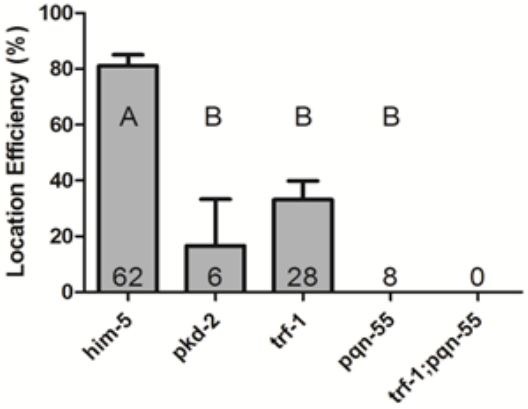


Figure 24. *pqn-55* Location of vulva assay. Location efficiency was calculated as a ratio of the number of successful stops at the vulva to the number of total vulval passes by the male tail. Same letters represent a group that is not statistically different from each other.

Conclusion

TRF-1 does not seem to interact with the PKD components LOV-1, PKD-2 and KLP-6 in Yeast Two-Hybrid Assays. As future experimentation, these interactions can be tested in the opposite vectors to see if there is interaction. Also, by testing interactions with the TRF-1 domain-specific constructs, specific domain interactions can be determined. TRF-1 might not physically interact with LOV-1 or PKD-2 but it could be an adaptor that physically associates with an unidentified protein that is physically interacting with LOV-1 or PKD-2.

TRF-1 seems to interact with EBAX-1/PQN-55. This interaction needs to be confirmed. If this is a true interaction, the TRF-1 domain constructs should be tested to determine what specific domains are interacting. I predict that TRF-1 is interacting with EBAX-1/PQN-55 through the RING domain since this domain seems to play a role in the ubiquitination pathway and EBAX-1/PQN-55 may be seem to be a substrate recognition subunit for E3 ubiquitin ligases (Yishi Jin lab, personal communication). TRF-1 and EBAX-1/PQN-55 might be part of an E3 ligase complex that ubiquitinates PKD-2 or ubiquitinates another protein that interferes with PKD-2 (Hu et al., 2007).

pqn-55 may act in the PKD pathway since mutant males have defects in PKD-2::GFP expression, response, and vulva location. *trf-1* does not seem to affect PKD::GFP expression. *trf-1; pqn-55* double mutant males are not statistically different than *trf-1* and *pqn-55* single mutants. *trf-1* and *pqn-55* have a role in response but role(s) in the PKD pathway still needs to be determined.

CHAPTER IV: TRF-1 PARALOG, TRF-2

Introduction

The tumor necrosis factor receptor (TNFR) family of receptors and the Toll/interleukin-1 receptor (Toll/IL-1R) family transduce signals by the help of TRAFs. The TRAF family of proteins have been identified as the adaptors that associate with the cytoplasmic tail of these receptors. These adaptor molecules function mainly in the immune system. In mammals, there are seven TRAFs that share a conserved C-terminal TRAF domain. In *C. elegans*, there are two TRAF genes: *trf-1* (See Chapters I and II – TRF-1) and Y110A7A.2 (*trf-2*). Y110A7A.2 is a paralog of *trf-1*. Since very little is known about Y110A7A.2, I initiated characterization of this gene.

trf-1 paralog, Y110A7A.2, is *trf-2*

trf-1 has a paralog (separated by a gene duplication event), Y110A7A.2. Y110A7A.2 encodes a TRAF-like protein in *C. elegans*. Herein, I will refer to Y110A7A.2 as the *trf-2* gene. *trf-2* is male soma enriched in *C. elegans*. *trf-2* is located on chromosome I at position -0.35 cM and has an insertion/deletion allele, *tm5167* (Fig. 25). The mutation site for *tm5167* is 53844/53845-AGA-54283/54284 (439 bp deletion + 3 bp insertion).

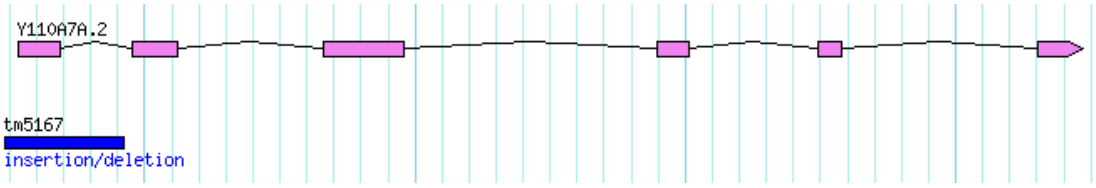


Figure 25. *trf-2* (Y110A7A.2) gene model and the mutant allele *tm5167*.

The function of *trf-2* is not known. As mentioned on the TRF-1Chapter (Chapter II), Y110A7A.2 has a homology e-value of 1.1e-32 (Table 1 from Chapter II: TRF-1) when compared to *trf-1*. By using Muscle Multiple Alignment software, TRF-1 is 21.4% identical and 26.2% similar to TRF-2 (Fig. 26). Since *trf-1* is also a TRAF and plays a role in *C. elegans* male mating behaviors, I examined the mating behaviors of *trf-2* (*tm5167*) males. I hypothesize that *trf-2* is also mating defective.

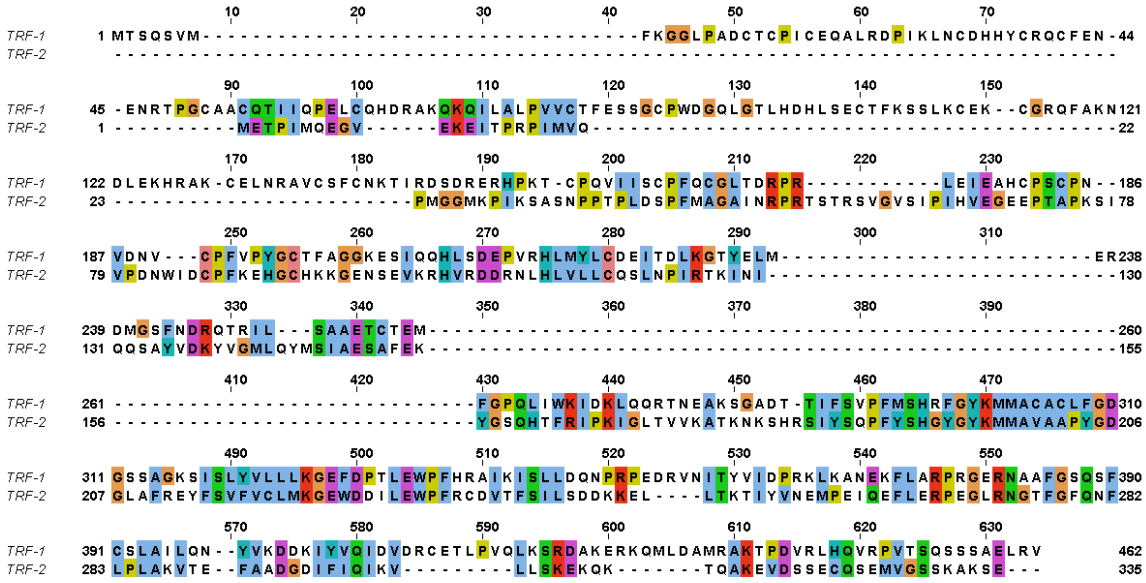


Figure 26. TRF-1 and TRF-2 alignment by Muscle Multiple Alignment.

trf-2 (*tm5167*) males are response and location of vulva defective

Response and Location of vulva were assayed as previously described in Chapter II: TRF-1. As hypothesized, *trf-2* males are response defective and are not statistically different than *trf-1*(Fig. 27). Interestingly, the *trf-2* response defect is statistically different than *pkd-2*. The *trf-1;trf-2* double mutant is also response defective and is not statistically different than the *trf-1* and *pkd-2* single mutants. In the location of vulva

assay, the *trf-2* single and the *trf-1; trf-2* double mutant are location of vulva defective and are not statistically different than *trf-1, trf-2* and *pkd-2* single mutants (Fig.28).

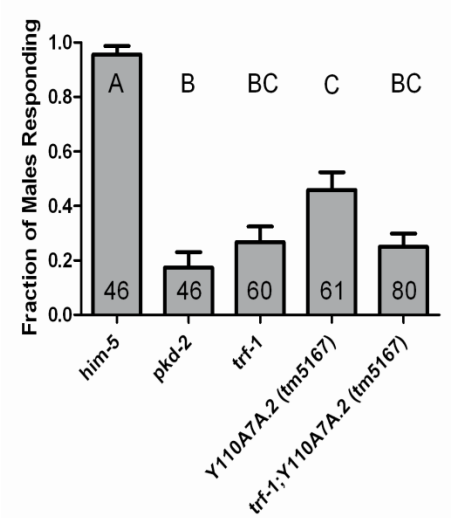


Figure 27. *trf-2* (Y110A7A.2) Response Assay. Fraction of males that respond to a hermaphrodite in a 4 min assay. Same letters represent a group that is not statistically different from each other.

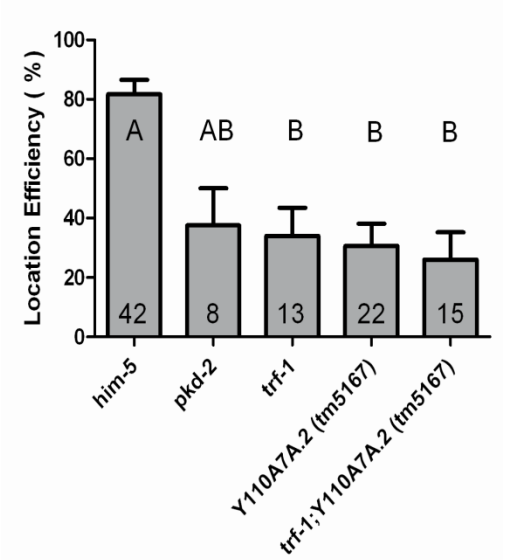


Figure 28. *trf-2* (Y110A7A.2) Location of vulva assay. Location efficiency was calculated as a ratio of the number of successful stops at the vulva to the number of total vulval passes by the male tail. Same letters represent a group that is not statistically different from each other.

Conclusion

I conclude that *trf-2* plays a role in *C. elegans* mating behaviors and seems to act in the PKD pathway. *trf-1* and *trf-2* act non-redundantly, since *trf-1; trf-2* double mutant does not yield a stronger phenotype. I predict that *trf-2* should be co-expressed with *trf-1* and *pkd-2* in *C. elegans* male-specific neurons. An important future direction is determining *trf-2* expression pattern and TRF-2 protein localization patterns.

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

trf-1 is the *C. elegans* TRAF gene. TRAFs function in the immune system but in *C. elegans* males, *trf-1* plays a role in mating behaviors. *Ptrf-1::gfp* is expressed in the adult male CEMs in the head and in the HOB and RnBs (excluding ray 6) in the tail. *trf-1* injected at a concentration of 10 ng/µl is not expressed in hermaphrodites. The initial transgenic *Ptrf-1::TRF-1::GFP* lines are weakly expressed in the head CEMs and hook HOB, and are variable in the ray RnBs (most consistent in rays 3, 8 and 9) when injected at 10 ng/µl. TRF-1 localizes to the cell body of the neurons and to the dendrites and axons of the CEMs and RnB neurons but is excluded from the nuclei of these neurons. When outcrossed into *pha-1; him-5*, *Ptrf-1::TRF-1::GFP* expression is brighter and consistent in all rays, excluding ray 6. TRF-1 is also localized to the cilia of the CEMs and the rays. When the translational reporter was injected at a lower concentration (7 ng/µl), the initial TRF-1::GFP transgenic line was dimly expressed in the male-specific neurons and was variable in the rays. Interestingly, at this lower concentration, expression in some hermaphrodite neurons was observed. The identity of these neurons still needs to be determined. A future experiment would be to outcross this line to see if the expression in males also gets brighter and consistent in the rays. This approach might also help in visualizing the neurons expressed in the hermaphrodite or could even reveal more neurons where TRF-1::GFP is being expressed. In males, *trf-1* plays in role in mating behaviors but in the hermaphrodite, *trf-1* could be playing a role in pathogen recognition and acting in an immune pathway with *tol-1*. The expression and localization results show that like *lov-1* and *pkd-2*, *trf-1* belongs to the PKD gene battery, which are

specifically expressed in the male CEM, RnB (not R6B), and HOB neurons; some of the genes in this battery are also expressed in the core IL2 neurons (not expressed in *trf-1*).

During mating, the male executes characteristic behavior steps in order to have successful sperm transfer. The CEMs seem to be important for chemotaxis to hermaphrodites, the tail ray RnBs are important for the initial response step and the hook HOB is important for location of vulva. *trf-1* mutant males are response and location of vulva defective. The *trf-1; pkd-2* and *lov-1; trf-1* double mutants are also mating defective and are not statistically different from the single mutants. The *lov-1; trf-1; pkd-2* triple mutant is not statistically different from the single mutants either. *tol-1* and *ikb-1* mutants of the innate immunity pathway have normal response and location of vulva. Introduction of WT TRF-1::GFP into *trf-1* mutant rescued response for four out of six tested lines. However, none of the tested lines recued location of vulva defects. It seems that there is another factor, yet to be discovered, involved that does not allow the rescue of the Lov defect.

The male's exploratory (mate-searching) behavior was also tested in a Leaving assay. A wild-type isolated male on food eventually leaves the food and wanders around the plate in search of a mate. *trf-1* mutant males do not leave the food source in search of a mate, meaning that they has a Las phenotype. *pkd-2* is also Las. *tol-1* mutants, from the immunity pathway are also Las but to a lesser degree than *trf-1* and *pkd-2*. *tol-1* also seem to be having a role in mate-searching behavior. The other mutant from the innate immunity pathway, *ikb-1*, has normal mate-searching behavior. When hermaphrodites are present (Retention assay), a wild-type male does not leave the food source. *trf-1*

mutant males present normal behavior in Retention assays meaning that they can sense the presence of a mate.

Together, these results show that *trf-1* is expressed in the same neurons as *lov-1* and *pkd-2* and has mating defects as well. *lov-1, pkd-2* and *trf-1* act together in response, location of vulva and in mate-searching behaviors.

Since *trf-1* is expressed in the same male-specific neurons as *lov-1* and *pkd-2*, and is part of PKD gene battery, I next tested if TRF-1 physically interacts with components (LOV-1, PKD-2 and KLP-6) of the PKD gene battery by Yeast Two-Hybrid experiments. In the combination tested with these components, TRF-1 does not seem to interact with them. As future experimentation, these interactions can be tested in the opposite vectors to see if there is interaction. Also, by testing interactions with the TRF-1 domain-specific constructs, specific domain interactions can be determined. TRF-1 might not physically interact with LOV-1 or PKD-2 but it could be an adaptor that physically associates with another protein that is physically interacting with LOV-1 or PKD-2.

TRF-1 seems to interact with EBAX-1/PQN-55, a substrate recognition subunit for E3 ubiquitin ligases (Yishi Jin lab, personal communication). This interaction needs to be confirmed. If this is a true interaction, the TRF-1 domain constructs should be tested to determine what specific domains are interacting. I predict that TRF-1 is interacting with EBAX-1/PQN-55 through the RING domain since this domain seems to play a role in the ubiquitination pathway and EBAX-1/PQN-55 seems to be a substrate recognition subunit for E3 ubiquitin ligases. TRF-1 with EBAX-1/PQN-55 might be part of an E3 ligase complex that ubiquitinates PKD-2 or ubiquitinates another protein that interferes with PKD-2.

Since TRF-1 and EBAX-1/PQN-55 seem to interact, I tested the response and vulva location of *pqn-55(ju699)* males. *pqn-55* may act in the PKD pathway since it has mating defects. *trf-1; pqn-55* double mutant males are not statistically different than *trf-1* and *pqn-55* single mutants. *trf-1* and *pqn-55* have a role in response but the role(s) in the PKD pathway still needs to be determined.

The *C. elegans* genome encodes two TRAFs: *trf-1* and *trf-2* (Y110A7A.2). Since *trf-1* is a TRAF that has a role in male mating behaviors, I tested if *trf-2* also has a role in mating behaviors by testing response and location of vulva. *trf-2* is response and location of vulva defective. *trf-2* plays a role in *C. elegans* mating behaviors and seems to act in the PKD pathway. *trf-1* and *trf-2* act non-redundantly, since the *trf-1; trf-2* double mutant does not yield a stronger phenotype. I predict that *trf-2* should be co-expressed with *trf-1* and *pkd-2* in *C. elegans* male-specific neurons. An important future direction is determining *trf-2* expression pattern and TRF-2 protein localization patterns.

My results show that TRF-1, a TRAF, is involved in *C. elegans* male mating behaviors and is part of the PKD pathway. TRF-1 seems to function downstream of LOV-1 and PKD-2 in the pathway; somewhere in the cytoplasm (Fig. 29). My Yeast Two-Hybrid experiment did not show physical interaction between TRF-1 and LOV-1 or PKD-2 in the plasmid tested, but we cannot rule out the possibility that they interact. TRF-1 seems to interact with EBAX-1/PQN-55 somewhere in the cytoplasm, downstream of LOV-1 and PKD-2 (Fig. 29).

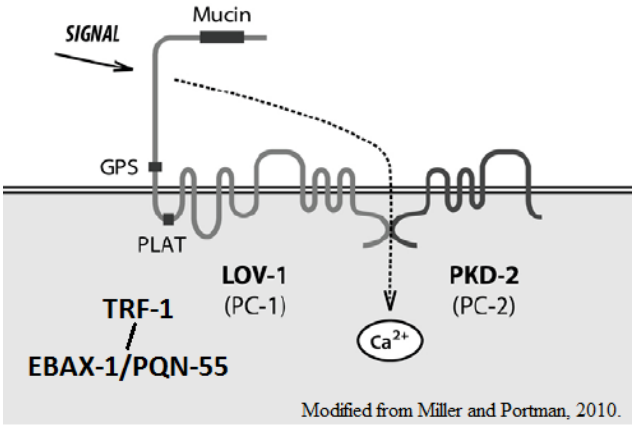


Figure 29. TRF-1 functions downstream of LOV-1 and PKD-2. TRF-1 interacts with EBAX-1/PQN-55 in the cytoplasm.

REFERENCES

Aravind, L., Dixit, V. M., & Koonin, E. V. (2001). Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. *Science, 291,* 1279-84.

Badano JL, Mitsuma N, Beales PL, Katsanis N (2006). "The ciliopathies: an emerging class of human genetic disorders". *Annual Review of Genomics and Human Genetics* 7: 125–48.

Bae, Y.K., Lyman-Gingerich, J., Barr, M.M., and Knobel, K.M. (2008). Identification of genes involved in the ciliary trafficking *of C. elegans* PKD-2. Dev. Dyn. 237, 2021–2029.

Barr MM, DeModena J, Braun D, Nguyen CQ, Hall DH, Sternberg PW. (2001). The Caenorhabditis elegans autosomal dominant polycystic kidney disease gene homologs lov-1 and pkd-2 act in the same pathway. Curr Biol. 4;11(17):1341-6.

Barr MM, Sternberg PW. (1999). A polycystic kidney-disease gene homologue required for male mating behaviour in C. elegans. Nature. Sep 23;401(6751):386-9.

Barrios A, Ghosh R, Fang C, Emmons SW, Barr MM. (2012). PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in C. elegans. Nat Neurosci. (12):1675-7682.

Barrios, A., Nurrish, S, Emmons, S.W. (2008). Sensory Regulation of *C. elegans* Male Mate-Searching Behavior. Current Biology 18:1865–71.

Borden KL, Freemont PS (1996). "The RING finger domain: a recent example of a sequence-structure family". *Curr. Opin. Struct. Biol.* 6 (3): 395–401.

Brenner, S. "The Genetics of CAENORHABDITIS ELEGANS" (1974). *Genetics* 77 (1): 71–94.

Clapham, D. E. (2003). TRP channels as cellular sensors. Nature, 426, 517–524.

Deshaies, R.J., and Joazeiro, C.A.P. (2009). RING Domain E3 Ubiquitin Ligases. Annu. Rev. Biochem. 78:399–434.

"Entrez Gene: TRAF3 TNF receptor-associated factor 3".

Freemont PS, Hanson IM, Trowsdale J (1991). "A novel cysteine-rich sequence motif". *Cell* 64 (3): 483–4.

Gardiner, Mary Beth (2005). "The Importance of Being Cilia". *HHMI Bulletin* (Howard Hughes Medical Institute) 18 (2).

Granato, M., Schnabel, H., and Schnabel, R. (1994). *pha-1*, A selectable marker for gene transfer in *C. elegans*. Nucleic Acids Res. *22*, 1762–1763.

Hao, L; Thein M, Brust-Mascher I, Civelekoglu-Scholey G, Lu Y, Acar S, Prevo B, Shaham S, Scholey JM (2011). "Intraflagellar transport delivers tubulin isotypes to sensory cilium middle and distal segments". *Nature Cell Biology* 13: 790–798.

Hobert, O., 2002 PCR fusion-based approach to create reporter gene constructs for expression

analysis in transgenic *C. elegans*. BioTechniques (32): 728–730.

Hoenderop, J. G., Voets, T., Hoefs, S., Weidema, F., Prenen, J., Nilius, B., et al. (2003). Homo- and heterotetrameric architecture of the epithelial Ca^{2+} channels TRPV5 and TRPV6. EMBO Journal, 22, 776–785.

Hu, J., Wittekind, S.G., and Barr, M.M. (2007). STAM and Hrs downregulate ciliary receptors and signaling in *C. elegans*. Molecular Biology of the Cell. 18(9):3277-89.

Ip, Y.T., Reach, M., Engstrom, Y., Kadalayil, L., Cai, H., Gonzalez-Crespo, S., Tatei, K., and Levine. (1993). Dif, a dorsal-related gene that mediates an immune response in *Drosophila* Cell, 75:753–763.

Lemaitre, B., Nicolas, E., Michaut,L., Reichhart, J.M., Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. Cell, 86:973–983.

Lipton J., Kleemann, G., Ghosh, R., Lints, R., and Emmons, SW. (2004). Mate searching in Caenorhabditis elegans: a genetic model for sex drive in a simple invertebrate. J Neurosci. 24(34):7427-34.

Liu KS, Sternberg PW. (1995). Sensory regulation of male mating behavior in Caenorhabditis elegans. Neuron. Jan;14(1):79-89.

Lovering R, Hanson IM, Borden KL, Martin S, O'Reilly NJ, Evan GI, Rahman D, Pappin DJ, Trowsdale J, Freemont PS (1993). "Identification and preliminary characterization of a protein motif related to the zinc finger". *Proc. Natl. Acad. Sci. U.S.A.* 90 (6): 2112–6.

Miller, R. and Portman, D. (2010). A latent capacity of the *C. elegans* polycystins to disrupt sensory transduction is repressed by the single-pass ciliary membrane protein CWP-5. Dis Model Mech. 3(7-8): 441–450.

Montell, C. (2001). Physiology, phylogeny, and functions of the TRP superfamily of cation channels. Science's STKE.

Newby LJ, Streets AJ, Zhao Y, Harris PC, Ward CJ, Ong AC. (2002). Identification, characterization, and localization of a novel kidney polycystin-1-polycystin-2 complex. J. Biol. Chem. 277:20763–73.

Oeckinghaus, A., Hayden, M.S., and Ghosh, S. (2011). Crosstalk in NF-κB signaling pathways. Nature Immunology 12, 695–708.

Patapoutian A, Tate S, Woolf CJ. (2009). Transient receptor potential channels: targeting pain at the source. Nat Rev Drug Discov. Jan;8(1):55-68. Review.

Peden, E. M. and Barr, M.M. (2005) KLP-6 is a Kinesin Required for Polycystin Ciliary Localization and Male Mating Behavior in *Caenorhabditis elegans.* Current Biology 5, 394-404.

Pujol, N., Link, E. M., Liu, L. X., Kurz, C. L., Alloing, G., Ray, K. P., Solari, R., Johnson, C. D., Ewbank, J. J., & M-W, T. (2001). A reverse genetic analysis of components of the Toll signaling pathway in Caenorhabditis elegans. *Curr Biol, 11*, 809-21.

Qin H, Rosenbaum JL, Barr MM. (2001). An autosomal recessive polycystic kidney disease gene homolog is involved in intraflagellar transport in *C. elegans* ciliated sensory neurons. *Curr. Biol.* 11:457–61.

Rosenbaum JL, Witman GB. 2002. Intraflagellar transport. *Nat Rev Mol Cell Biol.* 3,813-25.

Srinivasan, J.; Kaplan, F., Ajredini, R., Zachariah, C., Alborn, H.T., Teal, P., Malik, R.U., Edison, A.S., Sternberg, P and Schroeder, F.C. (2008). A blend of small molecules regulates both mating and development in Caenorhabditis elegans. Nature 454, 1115-1118.

Sulston, J.E., Albertson, D.G., and Thomson, J.N. (1980). The *Caenorhabditis elegans* male: Postembryonic development of nongonadal structures. Dev. Biol. *78*, 542–576.

Tenor, J.L. and Aballay, A. (2008). A conserved Toll-like receptor is required for *Caenorhabditis elegans* innate immunity. EMBO reports (9): 103–109.

The C. elegans Sequencing Consortium. (1998). Genome Sequence of the Nematode *C. elegans*: A Platform for Investigating Biology. Science 282 (5396): 2012-2018.

Torres VE . (1998). New insights into polycystic kidney disease and its treatment. Curr Opin Nephrol Hypertens. 7, 159-169.

Valanne S, Wang JH, Rämet M. (2011). The Drosophila Toll signaling pathway. J Immunol. 2011 Jan 15;186(2):649-56.

Wang J, Schwartz H.T., Barr M.M. (2010) Functional specialization of sensory cilia by an RFX transcription factor isoform. Genetics. 186(4):1295-307.

Wemmie JA, Price MP, Welsh MJ. (2006). Acid-sensing ion channels: advances, questions and therapeutic opportunities. Trends Neurosci. Oct;29(10):578-86. Epub 2006 Aug 7. Review.

White, J.Q., Nicholas, T.J., Gritton, J., Truong, L., Davidson, E.R., and Jorgensen, E.M. (2007). The sensory circuitry for sexual attraction in C. elegans males. Curr. Biol. 17, 1847–1857.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*: The mind of a worm. Philos. Trans. R. Soc. Lond. B Biol. Sci. *314*, 1–340.

Wilson, P.D. (2004). Polycystic kidney disease. N Engl J Med. 350:151-164.

X. Meng, B.S. Khanuja, Y.T. Ip. (1999). Toll receptor-mediated *Drosophila* immune response requires Dif, an NF- kappaB factor. Genes Dev, 13:792–797.

Yoder BK, Hou X, Guay-Woodford LM. (2002). The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. *J. Am. Soc. Nephrol.* 13:2508–16.

Young, K. (1998). "Yeast two-hybrid: so many interactions, (in) so little time.". *Biol Reprod* 58 (2): 302–11.

http://www.R-project.org

www.wormbase.org

www.wormbook.org