Identification and characterization of genes involved in cilia development in the nematode, Caenorhabditis elegans

Author: Michael Joseph Reardon

Persistent link: http://hdl.handle.net/2345/30

This work is posted on eScholarship@BC, Boston College University Libraries.

Boston College Electronic Thesis or Dissertation, 2008

Copyright is held by the author, with all rights reserved, unless otherwise noted.
Boston College

The Graduate School of Arts and Sciences

Department of Biology

IDENTIFICATION AND CHARACTERIZATION OF GENES INVOLVED IN CILIA DEVELOPMENT IN THE NEMATODE, *CAENORHABDITIS ELEGANS*

a dissertation

by

MICHAEL JOSEPH REARDON

submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

March 2008
Abstract

Identification and Characterization of Genes Involved in Cilia Development in the Nematode, Caenorhabditis elegans

Michael Joseph Reardon

Dissertation Advisors: John Wing, Ph.D., Stephen Wicks, Ph.D.

Molecular biology and genetics, single nucleotide polymorphism genetic mapping, phenotypic assays including behavioral assessment, and fluorescent microscopy of GFP-tagged proteins were used to study ciliary defects in the nematode Caenorhabditis elegans. Mammalian cilia are multifunctional. Some of the physiological roles in which they are involved include sensing developmental signaling molecules and ligands as well as creating flows of mucus and cerebrospinal fluid that function as flow meters and mechanosensors. Due to the multifunctional nature of cilia, it is not surprising that many human diseases can be caused by ciliary defects. Bardet-Biedl Syndrome is a rare genetic ciliopathy characterized by retinal degeneration, polydactyly, obesity, cystic kidneys, mental retardation, and many other ailments. We have identified osm-12/bbs-7 to be a C. elegans homologue of human BBS7, a gene known to cause Bardet-Biedl Syndrome when mutated. With the help of Michel Leroux’s group, I showed the BBS-7 protein to be localized to the base of cilia and to undergo intraflagellar transport along the ciliary axoneme. Our findings suggest that BBS-7 plays a role in the assembly and/or functioning of the IFT complex. I also performed a mutagenesis and phenotypic screen for animals defective in the uptake of Dil into a subset of their ciliated neurons in order to identify new components involved in ciliogenesis and IFT. I describe an extended bulked
segregant analysis (BSA) mapping methodology, which can save time and resources by filtering out alleles of previously known genes without performing time-consuming interval mapping. In addition, I identified one of the 11 dye-filling defective alleles from the screen to be a novel allele of *dyf-3*, which encodes a protein required for sensory cilia formation.
# TABLE OF CONTENTS

## CHAPTER 1: INTRODUCTION

- Caenorhabditis elegans as a model organism for genetic analysis .............................................. 1
- Isolation of genetic mutants and two-point mapping ................................................................. 3
- Three-point mapping in C. elegans .......................................................................................... 7
- Chemosensation and the C. elegans nervous system ............................................................... 10
- Ciliary defects and the intraflagellar transport system ........................................................... 12

## CHAPTER 2: MATERIALS AND METHODS

- Nematode strains and culture ................................................................................................. 18
- Synchronizing cultures of C. elegans ..................................................................................... 19
- Worm lysis ............................................................................................................................ 19
- DNA sequencing .................................................................................................................... 21
- Mutagenesis .......................................................................................................................... 22
- Bulked segregant analysis ...................................................................................................... 23
- Extended BSA ....................................................................................................................... 25
- Interval mapping ..................................................................................................................... 25
- Generation of osm-12::gfp partial translational fusion construct ........................................ 26
- osm-12 mutation detection primers ....................................................................................... 29
- Generating a dyf-3 rescue construct ..................................................................................... 30
- Microinjection and DNA transformation .............................................................................. 31
- Dye-filling assays ................................................................................................................... 32
- Odorant assays ....................................................................................................................... 32
- Locomotion assays ................................................................................................................. 33
- Longevity assays .................................................................................................................... 34
- Pharyngeal pumping rate assays ........................................................................................... 35
- Total RNA extraction and isolation ...................................................................................... 35
- RNA formaldehyde gel electrophoresis ................................................................................ 36
- Reverse transcriptase PCR ..................................................................................................... 37

## CHAPTER 3: LOSS OF C. ELEGANS BBS-7 PROTEIN FUNCTION RESULTS IN CILIA DEFECTS AND COMPROMISED INTRAFLAGELLAR TRANSPORT

- Introduction .......................................................................................................................... 39
  - The connection between Bardet-Biedl Syndrome and cilia ............................................... 39
- Results ................................................................................................................................. 40
  - Identification and Characterization of osm-12/bbs-7 ....................................................... 40
  - Additional collaborative results further characterizing the involvement of osm-12/bbs-7 along with bbs-8 in intraflagellar transport ....................................................... 45
- Discussion ............................................................................................................................ 46

## CHAPTER 4: MAPPING AND PHENOTYPIC CHARACTERIZATION OF NOVEL DYE-FILLING MUTANTS

- Introduction .......................................................................................................................... 62
  - Sensory perception and Longevity ................................................................................... 62
- Results ................................................................................................................................... 64
  - Genetic screen for novel genes involved in ciliogenesis .................................................... 64
  - Chromosome mapping of screened Dyf strains using single nucleotide polymorphisms ... 65
  - Additional phenotypic analysis of screened Dyf strains ................................................... 70
- Discussion ............................................................................................................................ 73

## CHAPTER 5: ISOLATION AND CHARACTERIZATION OF A NOVEL DYF-3 ALLELE

- Introduction .......................................................................................................................... 99
TABLE OF FIGURES

FIGURE 1. IFT COMPONENTS ARE CLASSIFIED INTO “MODULES” BASED ON TRANSPORT PROFILES. 51
FIGURE 2. SCHEMATIC OF THE osm-12(n1606)/bbs-7 OPEN READING FRAME. ................................. 52
FIGURE 3. osm-12(n1606)/bbs-7 MUTATION DETECTION REACTIONS. .............................................. 53
FIGURE 4. ISO-AMYL ALCOHOL CHEMOTAXIS ASSAY. ........................................................................ 54
FIGURE 5. LOCOMOTION ASSAY. ........................................................................................................ 55
FIGURE 6. osm-12(n1606)/bbs-7 MUTANTS CONTAIN DEFECTIVE CILIARY STRUCTURES. ................. 56
FIGURE 7. BBS-7 LOCALIZES MAINLY TO THE TRANSITION ZONES AND AXONEMES OF CILIA. ....... 57
FIGURE 8. BBS-7 IS EXPRESSED EXCLUSIVELY IN CILIATED NEURONS. ......................................... 58
FIGURE 9. X-BOX TRANSCRIPTION FACTOR BINDING SITE ALIGNMENTS. ............................................ 59
FIGURE 10. osm-12(n1606)/bbs-7 EXPRESSION REQUIRES THE RFX-TYPE TRANSCRIPTION FACTOR DAF-19. 60
FIGURE 11. og007 IS LINKED TO THE X CHROMOSOME AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~1.89 CM. .................................................................................. 84
FIGURE 12. og008 IS LINKED TO THE X CHROMOSOME AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~8 CM. ......................................................................................... 85
FIGURE 13. og018 IS LINKED TO CHROMOSOME I AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~12 CM. ....................................................................................... 86
FIGURE 14. og019 IS LINKED TO CHROMOSOME IV AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~4 CM. ..................................................................................... 87
FIGURE 15. og020 IS LINKED TO CHROMOSOME I AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~12 CM. ....................................................................................... 88
FIGURE 16. og021 IS LINKED TO CHROMOSOME I AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~1 CM. ......................................................................................... 89
FIGURE 17. og022 IS LINKED TO CHROMOSOME IV AND HAS BEEN IDENTIFIED AS A NOVEL ALLELE OF Dfy-3 ............................................................................................................. 90
FIGURE 18. og023 IS LINKED TO THE X CHROMOSOME AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~2 CM. ..................................................................................... 91
FIGURE 19. og025 IS LINKED TO THE X CHROMOSOME AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~8 CM. ..................................................................................... 92
FIGURE 20. og026 IS LINKED TO CHROMOSOME V AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~7 CM. ..................................................................................... 93
FIGURE 21. og027 IS LINKED TO THE X CHROMOSOME AND SHOWS THE HIGHEST LINKAGE AT A GENETIC DISTANCE OF ~8 CM. ..................................................................................... 94
FIGURE 22. ISO-AMYL ALCOHOL CHEMOTAXIS ASSAY. ........................................................................ 96
FIGURE 23. ADULT LIFESPANS OF NOVEL Dyf STRAINS. ....................................................................... 97
FIGURE 24. PHARYNGEAL PUMPING ASSAY. ......................................................................................... 98
FIGURE 25. NARROWING DOWN THE GENETIC INTERVAL OF og022 THROUGH INTERVAL MAPPING USING SINGLE NUCLEOTIDE POLYMORPHISMS. .......................................................... 112
FIGURE 26. dfy-3(og022) CONTAINS A MUTATION IN A CONSERVED SPLICE DONOR RESIDUE........ 113
FIGURE 27. dfy-3 GENE STRUCTURE AND Dфy-3 PROTEIN ALIGNMENT. .......................................... 114
FIGURE 28. ALIGNMENT OF C04C3.5b cDNAs AMPLIFIED FROM N2 RNA AND dfy-3(og022) RNA. ............................................................................................................................. 115
FIGURE 29. dfy-3(og022) REVERSE TRANSCRIPTASE PCR. .............................................................. 116
FIGURE 30. dfy-3(og022) RESCUE. ........................................................................................................... 117

TABLES

TABLE 1. EXPAND LONG RANGE DNA POLYMERASE THERMAL CYCLE CONDITIONS. .................. 61
TABLE 2. A LIST OF SNP-SNPs USED IN DETERMINING CHROMOSOME LINKAGE. ......................... 81
TABLE 3. EXTENDED BULKED SEGREGANT ANALYSIS. ......................................................................... 82
TABLE 4. DISTRIBUTION OF GENES CAUSING CILIARY DYE-FILLING DEFECTS. ......................... 83
TABLE 5. GENETIC LOCATIONS OF NOVEL Dyf ALLELES. ................................................................. 85
TABLE 6. SNP MARKERS USED IN INTERVAL MAPPING og022. .......................................................... 111
<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>MEANING</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS</td>
<td>Bardet-Biedl Syndrome</td>
</tr>
<tr>
<td>BSA</td>
<td>Bulked Segregant Analysis</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CI</td>
<td>Chemotaxis Index</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl Methanesulfonate</td>
</tr>
<tr>
<td>FUDR</td>
<td>Fluorodeoxyuridine</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>IFT</td>
<td>Intraflagellar Transport</td>
</tr>
<tr>
<td>ILP</td>
<td>Insulin-like Peptide</td>
</tr>
<tr>
<td>NMG</td>
<td>Nematode Growth Media</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PKD</td>
<td>Polycystic Kidney Disease</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>snipSNP</td>
<td>SNP that alters a restriction enzyme cut site</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to dedicate my thesis work to my late grandfather, Joseph F. Ginese. I would have never been able to accomplish so much without my grandfather’s undying love and support. I would like to thank my grandmother, Elizabeth J. Ginese, along with my loving parents, stepfather, sister, family, and friends who have believed so strongly in my abilities throughout the years. I would like to thank Thomas Chiles whose dedication to seeing me reach my goals has propelled me through my degree. I am grateful to my mentor John Wing, who has provided an essential support structure during the last year of my thesis work. I would like to thank my committee members, Charles Hoffman, Marc-Jan Gubbels, Marc Muskavitch, and the late Mohammed Shahabuddin, for all their guidance and for meeting with me so frequently over the last year. I appreciate the work of our collaborators Michel Leroux at Simon Fraser University and Oliver Blacque at the University College Dublin who assisted us with the bbs-7 research. I am thankful for the technical help that Piali Sengupta and Kristin Ma provided me. I am grateful for the love, support, and help with statistical analysis that my partner Kathryn Holthaus so selflessly gave. I would like to give thanks to Michael Piatelli for his friendship and advice over the years. I am grateful for the present and former members of my lab, including Stephen Wicks, Mark Audeh, Sarah Fenerty, and Colin Huguenel. Finally, I would like to thank GOD for providing me with opportunities to succeed.
Chapter 1: Introduction

*Caenorhabditis elegans* as a model organism for genetic analysis

*Caenorhabditis elegans* is a free-living soil nematode that feeds mainly on bacteria. In 1965, Sydney Brenner chose *C. elegans* as a model organism to study concerted genetic, ultrastructural, and behavioral aspects of development and function in a simple nervous system. Today there are over 600 laboratories investigating the biology of this microscopic nematode (Edgely, 2007). The philosophy behind these large-scale research efforts is that the analysis of mutants through an assortment of different methods will lead to a greater understanding of animal development and behavior. Key elements that make *C. elegans* an excellent experimental system for genetic analysis include small genome size, short life cycle, transparent cuticle, and ease of manipulation and cultivation in the laboratory (Wood, 1988).

*C. elegans* has a reproductive life cycle of about 3 days under optimal conditions (Wood, 1988). There are two sexes, hermaphrodites and males. Since hermaphrodites produce both sperm and oocytes they are able to reproduce by self-fertilization. Males arise spontaneously at low frequencies and are able to fertilize hermaphrodites. Juvenile worms hatch and develop through four larval stages interrupted by molts. An adult hermaphrodite that has not mated is fertile for about 4 days during which it can lay around 300 eggs. Adult hermaphrodites contain 959 somatic nuclei and adult males contain 1031. The size of the haploid genome is $8 \times 10^7$ nucleotide pairs contained within six linkage groups corresponding to six haploid chromosomes (five autosomes and a sex
chromosome). Hermaphrodites are diploid for all six chromosomes, while males are diploid for the five autosomes but have only one X chromosome. There are an estimated 17,800 distinct genes that encode the information necessary for the development and function of this diploid organism (Edgely, 2007). Both hermaphrodites and males have a transparent cuticle throughout their life cycle and they maintain their shape through internal hydrostatic pressure (Wood, 1988).

*C. elegans* is easily maintained and manipulated under typical laboratory conditions. They are grown on nematode growth media (NGM) agar plates or in liquid culture with the *Escherichia coli* strain OP50 as a source of food. Individual adult worms are approximately 1 mm in length and can be handled using a platinum wire pick and easily observed under a dissecting microscope. Individual strains can be stored indefinitely by gradually cooling (~1°C/min.) freshly starved young larvae (L1-L2 larval stage) to -80°C (Wood, 1988).

Gene names are designated by a three letter code that usually is a phenotypic abbreviation (such as *dyf* for dye-filling defective) or acronym-like (such as *sur* for suppressor of ras). A hyphen and number that signify the order in which the mutation was discovered follow this three-letter code. The entire gene name is italicized (Fay, 2006a).

*Caenorhabditis elegans* rapid generation time and hermaphroditic lifestyle make it an excellent model system for the isolation and characterization of genetic mutants.
Isolation of genetic mutants and two-point mapping

One of the goals of our lab is to isolate mutant strains and genetically map their mutations. Well-designed genetic screens allow researchers to identify mutations that disturb particular biological processes (Jorgensen et al., 2002). In *Caenorhabditis elegans*, visible recessive mutations can be identified using an F2 screen. A mutagen such as ethyl methanesulfonate (EMS) is used in these screens to induce mutations in the sperm and oocytes of wild-type hermaphrodites. These mutagenized animals are grown for two generations to produce homozygous mutants. Their progeny are screened for a mutant phenotype of interest and then individually separated to determine whether this phenotype is transmitted to the next generation. Using standard concentrations of EMS, the frequency of mutations at any particular locus is approximately one null mutation for every 2,000 copies of the gene that is analyzed in the screen. EMS, C₃H₆O₃S, produces random mutations in genetic material by guanine alkylation, which leads to nucleotide substitutions. Once a stable mutant strain of interest is obtained, a researcher must locate the open reading frame that contains the causal molecular lesion (Jorgensen et al., 2002).

Our lab uses single nucleotide polymorphisms (SNPs) to map experimentally derived mutations in *Caenorhabditis elegans*, and the first step in this process is to determine which linkage group carries the causal gene of interest. Before high-throughput methods for two-point mapping using SNPs became popular, mutations in genes of interest were localized to individual linkage groups through two-point mapping using morphological marker mutations.
(Wicks et al., 2001; Fay, 2006b). In this methodology the mutant strain is crossed with a strain containing two adjacent chromosomal markers and F1 hermaphrodites are singled. To single a worm means to transfer it alone to an empty plate. In the situation where the mutation being mapped lies on the same/homologous chromosome as the genetic markers and is flanked by these markers, we observe three phenotypes in the F2 generation: wild type, mutant (representing the gene of interest), and the double mutant (representing the two morphological markers). We effectively never see the triple mutant because it would require a very rare double recombination event. Also, if an F2 mutant (representing the gene of interest) or double mutant (representing the two morphological markers) were singled, they would fail to segregate the triple mutant. This pattern of segregation is evidence that the mutation of interest lies on the same chromosome as the two morphological markers and that it lies close to or in between these markers.

On the other hand, in the situation where the mutation being mapped lies on a different chromosome than the genetic markers we see that 1/16 of the F2 progeny are triple mutants. In this case, another possibility is that the mutation resides on one of the chromosomes ends and the loci appear to be on separate chromosomes because a recombination event occurred between the genetic markers. This discrepancy can usually be resolved by scoring more animals. When the mutation is located at some distance to one side of the genetic markers one must take into account that the probability of recombination between loci increases with distance. When a recombination event occurs between the
mutation and markers, two recombinant chromosomes are formed. One contains all three mutations in cis and the other is completely wild type. These rare recombinant chromosomes occur by chance and more often than not pair up with a non-recombinant parental chromosome in a fertilized zygote. In this case, an F2 animal displaying your mutant phenotype can give rise to triple mutants, and one must be careful not to unwisely assume that the presence of such a phenotype means that your mutation is on a separate chromosome from the genetic markers being used (Fay, 2006b).

Genetic distance is calculated based on frequency of recombination. If your mutation is 5.0 map units away from one of the genetic markers then 5% of the gametes produced by the heterozygote will contain a chromosome in which a crossover event occurred within this interval. Considering this distance, F2 progeny will inherit two non-recombinant parental chromosomes 90.25% of the time, two recombinant chromosomes 0.25% of the time, and one non-recombinant and one recombinant chromosome 9.5% of the time. Suppose that we’re mapping a dyf mutation relative to a known unc (uncoordinated) mutation. In this example the two mutations are 5 map units from one another and the chance of obtaining a recombinant versus a non-recombinant chromosome are the same as stated above. To calculate this map distance, Unc animals are cloned from F2 progeny generated from the parental cross between the dyf strain being mapped and the unc genetic marker strain. At this point, the frequency at which these Unc animals give rise to Dyf Unc animals versus Unc only animals must be determined. After calculating the frequency of progeny with different
combinations of recombinant and non-recombinant chromosomes or by simply assessing the percentage of Unc progeny and the percentage of these Unc progeny that can give rise to Dyf Unc progeny, you would find that approximately 25% of the F2 progeny are Unc and approximately 9.54% of these Unc progeny can give rise to Dyf Unc progeny in the F3 generation (Fay, 2006b).

An often more efficient and less time consuming methodology for assigning a mutation to a particular chromosome involves the use of a geographically isolated strain of *C. elegans* and SNPs it contains in relation to the reference N2 strain. Mutagenesis is typically performed on the wild type reference N2 strain originally isolated in Bristol England. A reproductively isolated strain from a Hawaiian island, called CB4856, displays a uniformly high density of SNPs, in the form of single base pair substitutions (4,670 predicted), when compared to the N2 sequence (Wicks et al., 2001). Millions of years of evolutionary drift caused by geographical separation has led to many differences, in the form of DNA polymorphisms, between these two genomes (Fay et al., 2006d). Of the 4,670 predicted SNPs, an estimated 3,457 alter restriction endonuclease cut sites allowing them to be detected as restriction fragment length polymorphisms (RFLPs). These SNPs are referred to as ‘snip-SNPs.’

A variation on a rapid mapping strategy fit for qualitative monogenic traits called bulked segregant analysis (BSA) is used along with a subset of snip-SNPs to facilitate positional mapping and cloning of a mutation of interest. In this procedure, CB4856 is crossed into the N2 derived strain containing the mutation being mapped, and exclusion of CB4856 alleles of a subset of snip-SNPs in
mutant progeny is evaluated. A total of 50 phenotypically mutant and 50 phenotypically wild-type animals from the F2 generation of the cross between CB4856 and the mutant strain are pooled separately and lysed to collect their DNA. Primers were designed to amplify 24 snip-SNPs relatively equally spaced throughout the \textit{C. elegans} genome (4 per chromosome) (Wicks et al., 2001).

More recently, a method for chromosome mapping has been developed that utilizes 48 snip-SNPs, all of which alter a \textit{DraI} restriction endonuclease site (Davis et al., 2005). Each snip-SNP is amplified from each of the two bulked lysates. Each product is then digested and the relative quantities of N2 to CB4856 alleles for each snip-SNP in each bulked lysate is ascertained. Linkage to a given marker is calculated as a unitless Map Ratio for that marker. Equal representation of the biallelic markers in each lysate is expected if the mutation is not linked to that particular snip-SNP. In this case, the Map Ratio would be close to one. Linkage of a recessive locus in the N2 background to a particular snip-SNP would present a Map Ratio considerably below one (Wicks et al., 2001).

**Three-point mapping in \textit{C. elegans}**

After the mutation being mapped has been assigned to a broad interval on a chromosome we narrow the genetic interval through interval mapping using a higher density of snip-SNP and sequencing SNP markers (Davis et al., 2005). This methodology is a variation on classical three-point mapping using morphological markers (Fay, 2006c). To describe classical three-point mapping, let’s consider an example where we map a mutation in a \textit{dyf} gene that has been
narrowed down to a broad interval on chromosome I. We will cross the strain carrying this mutation with a strain containing a mutation in a known *unc* gene as well as a mutation in a known *dpy* (dumpy) gene. These two genes have been previously mapped to chromosome I and their exact location is documented. We will isolate two classes of recombinant F2 progeny from this cross, Unc non-Dpy and Dpy non-Unc. Each genome pertaining to one of these phenotypes has experienced a recombination event between the two morphological markers. By calculating the percentage that each of these two classes produces the Dyf phenotype, we can determine if the *dyf* mutation lies to the left, right, or in between the two markers. If the *dyf* mutation lies to the left of both markers (giving us the order: *dyf*, *unc*, *dpy*), then with the exception of gametes carrying two recombinant chromosomes (which is a negligible percentage) all Dpy non-Unc recombinant animals will give rise to \( \frac{1}{4} \) Dpy Dyf progeny, \( \frac{1}{2} \) Dpy, and \( \frac{1}{4} \) Unc Dpy. Unc non-Dpy recombinant animals will give rise to only Unc and Unc Dpy (\( \frac{1}{4} \) Unc, \( \frac{1}{4} \) Unc Dpy). This pattern could also mean that *dyf* lies to the right of *unc*, but it would have to be very close and between *unc* and *dpy* because the frequency of obtaining the *unc dyf* recombinant chromosome is extremely low. The reverse outcome is true if the *dyf* mutation lies to the right of both morphological markers. In order to determine how far the *dyf* mutation lies from one of the genetic markers, the markers need to be flanking the *dyf* mutation. In this case, we calculate the ratio of Dpy non-Unc animals that give rise to Dyf to those that do not and compare this to the ratio of Unc non-Dpy that give rise to Dyf to those that do not. The genetic location of the unknown *dyf* mutation is
given by this ratio. Recombinants can be saved for finer mapping by crossing the recombinant strains into a strain that is homozygous for another morphological marker that is located between the first two markers used. Let’s say the *unc* gene and *dpy* gene flank the *dyf* gene being mapped. In addition, an *egl* (egg-laying defective) mutant line is available, and this gene is located between the *unc* gene and *dyf* genes based on the location indicated by the ratio value obtained from the initial three-point mapping attempt. Recombinants can be crossed into the *egl* strain and progeny screened for Unc non-Dyf animals. To determine a more precise map location for our *dyf* mutation, we must consider the percentage of Unc non-Dyf progeny that receive the *egl* mutation compared to the percentage of Unc non-Dyf progeny that do not (Fay, 2006c).

A variation of this classical approach utilizes individual F2 recombinants from a cross between the N2 derived mutant strain being mapped and the Hawaiian isolate CB4856, and assays the DNA of the progeny of each recombinant for a large number of SNPs within the interval established through two-point mapping (Davis et al., 2005). In this technique, the progeny (F3 generation) of each F2 recombinant homozygous mutant are separately lysed allowing each recombinant to be scored as a population. A high density of snip-SNPs or sequencing SNPs are chosen within the narrowed interval and then primers are designed to amplify each of these markers. Each of these markers is amplified and digested or sequenced for each recombinant population (about 200 recombinant populations at least). The products shown after gel electrophoresis or the sequences read (if we are dealing with SNPs that do not alter restriction
endonuclease cut sites) tell us whether that particular animal was homozygous N2, heterozygous N2/CB4856, or homozygous CB4856 at each SNP. From this information, we can find mutation-carrying N2 chromosomes that have recombined with CB4856 to the left or right of the mutation, in effect narrowing down the interval containing the mutation. At points near the mutation, only one of the two copies of the mutation-carrying chromosome will have recombined with CB4856 (Davis et al., 2005).

**Chemosensation and the *C. elegans* nervous system**

The hermaphrodite’s nervous system is made up of 302 neurons and 56 glial and associated support cells, whereas the male contains 381 neurons and 92 glial and support cells (Chalfie et al., 1988). The nervous system mediates the response of the animal to stimuli such as chemical changes in the environment, mechanical stimuli, changes in osmolarity, and changes in temperature (Chalfie et al., 1988). The 302 neurons in the adult hermaphrodite are divided into 118 classes. About half of these neurons are located in the head of the animal, encircling a central, fibrous network of neuronal processes called the nerve ring. Most sensory neurons are bilaterally symmetrical and located in the head of the animal. Most interneurons are also bilaterally symmetrical and make up all neurons that are not designated as sensory or motor neurons (Bargmann et al., 1998).

Of the 302 neurons, 64 function in chemosensation as they contain various sensory receptors and are components of specialized sense organs called sensilla.
Sensilla contain one or more ciliated nerve endings, extending from one of the 64 chemosensory neurons, along with a socket cell and a sheath cell. The cilia are comprised of a ciliary axoneme, that contains nine doublet microtubules in the periphery with a variable number of inner singlet microtubules, and extends from a basal body-like transition zone. Socket cells connect the sensillum to the hypodermis and the sheath cells are glial-like in that they envelope the endings of neurons. The cilia of certain classes of sensilla gain access to the environment through a hole in the overlying cuticle. There are two amphid sensilla in the head along with six inner labial sensilla. These, along with two phasmid sensilla in the tail, function in chemosensation. Ciliated endings that fail to penetrate the cuticle are thought to mediate mechanosensation (Chalfie et al., 1988).

Chemosensation is important for the worm to properly mate, find food, avoid harmful conditions, and to develop properly. Each left-right pair of chemosensory neurons is similar in structure and forms its own class based on cilium and axon morphology and synaptic targets. G protein-coupled receptor signaling mediates chemosensation on a molecular level. Divergent members of the G protein-coupled receptor family seem to be functionally involved in the ability of *C. elegans* to respond to dozens of different chemicals (Troemel et al., 1995). As many as four different receptor genes encoding receptors that bind different chemicals can be expressed on a single type of chemosensory neuron (Troemel et al., 1995). The microtubule-rich sensory cilia are located at the tip of most chemosensory neurons and contain high concentrations of sensory transduction molecules (Ward et al., 1975; Ware et al., 1975).
Sensory neurons involved in chemotaxis towards volatile compounds (i.e., iso-amyl alcohol) as well as lifespan determination include AWA and AWC (Bargmann, 2006). AWB has been shown to function in avoidance behavior triggered by volatile compounds. ASE is responsible for chemotaxis towards water-soluble compounds (i.e., Na\(^{+}\) and Cl\(^{-}\)). Other sensory neurons like ASG, ASJ, and ASK are also involved in lifespan determination (Bargmann, 2006).

Many *C. elegans* genes that function in all ciliated sensory neurons contain a transcription factor binding site, known as an X box, in their proximal promoter (Swoboda et al., 2000). The X box is typically located about 100 bp upstream of the start codon. The RFX-type (Regulatory Factor binding to the X box) transcription factor that binds the X box is DAF-19 (Swoboda et al., 2000). Swoboda et al. 2000 have shown *daf-19* to be expressed in ciliated sensory neurons during ciliogenesis and that loss of *daf-19* leads to the absence of cilia. Therefore, DAF-19 is required for cilia formation.

**Ciliary defects and the intraflagellar transport system**

*C. elegans* sensory mutants can have defects in cilia formation or in the connection between the nerve endings and the sensory pore (Bargmann, 2006). Mutants with defective cilia can also have chemosensory defects if their cilia are not properly contacting the environment in which they are meant to sample (Culotti et al., 1978; Lewis et al., 1977). These mutants are phenotyped through a dye-filling assay in which inability to take up a fluorescent lipophilic membrane dye (DiI:}
1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) indicates that the ciliated endings of animals being assayed are not reaching the external environment. Strains displaying this dye-filling defective phenotype are designated Dyf (Starich et al., 1995). N2 worms exposed to the lipophilic dye, DiI, will take up the dye into the amphid neurons AWB, ASH, ASJ, ASK, ADL, and ASI, as well as the phasmid neurons PHA and PHB (Hedgecock et al., 1985; Starich et al., 1995).

Dye-filling defective phenotypes are most likely caused by a deficiency in the intraflagellar transport system (IFT). Deficits in the IFT system can cause defects in cilia morphogenesis due to the fact that proteins required for the development and maintenance of ciliated endings are transported up and down the ciliary axoneme through IFT.

IFT was first observed in the bi-flagellate green alga *Chlamydomonas moewusii* through observations of a paralyzed flagellar mutant (Kozminski et al., 1993; Sloboda, 2002). Through differential interference contrast microscopy, various sized particles were viewed moving from the flagellar base to the tip (anterograde) at approximately 2 μm/sec and from the tip to the base (retrograde) at roughly 3.5 μm/sec. These particles are referred to as IFT rafts and can be separated into two size classes (IFT-A and IFT-B) through sucrose gradient ultracentrifugation (Kozminski et al., 1993; Sloboda, 2002).

In *C. elegans*, IFT works in the assembly, maintenance, and proper functioning of ciliated endings on the dendritic extensions of chemosensory neurons (Scholey et al., 2004). These endings are made up of a ciliary axoneme
that nucleates from a transition zone, which includes a modified centriole called a basal body. The initial portion of the ciliary axoneme is comprised of doublet microtubules and is referred to as the middle segment (Ou et al., 2007). The ciliary axoneme of many amphid channel sensory neurons terminates with a distal segment composed of singlet microtubules (Ou et al., 2007).

Anterograde IFT in *C. elegans* is accomplished by two separate kinesin-II complexes (Signor et al., 1999a). Heterotrimeric kinesin-II is similar to sea-urchin kinesin-II which contains two heterodimerized N-terminal motor subunits and one non-motor subunit linked to their C-terminal cargo-binding ends (Cole et al., 1993). The second kinesin-II complex is homodimeric OSM-3-kinesin (Cole et al., 1993). These two motor proteins form a complex with additional IFT particles to traffic cargo up the ciliary axoneme.

Retrograde IFT in *C. elegans* is made possible by the motor protein dynein. Dynein was first identified in sea urchin embryos and shown to be up-regulated in response to deciliation (Gibbons et al., 1994). The gene *che-3* was found to code for a cytosolic dynein heavy chain required for ciliogenesis (Wicks et al., 2000). Much of the subunit composition of *C. elegans* IFT-dynein is not known, however, a dynein light intermediate chain required for retrograde IFT has been found to be encoded by the gene *xbx-1* (Schafer et al., 2003).

It is thought that the IFT machinery is assembled and subsequently loaded onto the ciliary axoneme via transitional fibers originating from the basal body/transition zone located at the base of the cilium (Deane et al., 2001). It is believed that both heterotrimeric kinesin-II and homodimeric Osm-3-kinesin
move the same IFT particles along the doublet microtubules of the middle segment of the ciliary axoneme (Ou et al., 2007). IFT particle reorganization occurs at the tip of the middle segment. At this point Kinesin-II undergoes a “turnaround” along with its associated IFT particles and is moved in a retrograde fashion by IFT-dynein. At the tip of the middle segment, OSM-3-kinesin continues along with its cargo to the distal singlet microtubules and undergoes its own turnaround at the distal tip where it is recycled by IFT-dynein (Ou et al., 2007). Development and maintenance of the middle segment doublet microtubules is dependent upon both anterograde motors working together. Assembly of the distal singlet microtubules is dependent upon only OSM-3-kinesin (Evans et al., 2006; Pan et al., 2006). Evans et al. 2006 suggests that cilia morphology and function can be partially attributed to changes in action of IFT kinesins. This is hypothesized based on their findings that both kinesins function in a completely redundant manner to build the cilia on AWC neurons (Evans et al., 2006). AWC neurons lack distal singlet microtubules that OSM-3-kinesin motors are responsible for building (Evans et al., 2006). Rates of IFT observed in ciliary mutants where OSM-3-kinesin and kinesin-II are no longer acting together, show OSM-3-kinesin moves along microtubules at 1.3 μm/s and kinesin-II moves along microtubules at 0.5 μm/s which suggests that the intermediate rate of movement up the middle segment microtubules in a wild-type animal is 0.7 μm/s because both motors are working in conjunction (Snow et al., 2004; Ou et al., 2005). Pan et al. 2006 have observed that IFT particle subcomplexes A and B move together along the middle segment doublet microtubules at a single rate of
0.7 \mu m/s. They also show that mutations in two other ciliary genes (bbs-7/8) separate the two anterograde motors so that they travel at their respective speeds, suggesting that BBS-7/8 are involved in holding IFT subcomplex A and B together (Pan et al., 2006). bbs-7 and bbs-8 were given the descriptive designation “bbs” because these genes are homologous to human genes that when mutated lead to Bardet-Biedl Syndrome.

Recently, Ou et al. 2007 grouped known C. elegans IFT proteins into distinct “modules” based on similar transport profiles. Analysis of protein transport in IFT and ciliogenesis was accomplished through a time-lapse fluorescence microscopy assay. This assay made it possible to observe the motility of tagged IFT machinery proteins in live worms (Orozco et al., 1999; Signor et al., 1999b; Snow et al., 2004; Ou et al., 2005). GFP-tagged IFT proteins were observed in bbs mutant backgrounds to observe if their velocities suggested an association with the kinesin-II/IFT-A subcomplex (0.5 \mu m/s along middle segment) or the OSM-3/IFT-B subcomplex (1.3 \mu m/s along middle and distal segments) (Ou et al., 2007). Known IFT components have been divided into the following modules: IFT-particle subcomplex A, IFT-particle subcomplex B, BBS protein complex, accessory motor complex, and IFT cargo complex (Figure 1) (Ou et al., 2007). Loss of function of IFT-A components block retrograde IFT driven by IFT-dynein (Signor et al., 1999; Wicks et al., 2000). In contrast, loss of function of IFT-B components block anterograde IFT resulting in truncated middle segments (Scholey, 2003). The BBS protein complex is involved in the connection between IFT complex A and IFT complex B. bbs mutants display an
IFT transport defect where IFT complex A/heterotrimeric kinesin-II and IFT complex B/OSM-3-kinesin are separated and destabilization occurs (Ou et al., 2007). *bbs* double and triple mutants have identical transport defects, suggesting that individual BBS protein functioning depends on the proper functioning of other BBS proteins and that these proteins function in the same process (Ou et al., 2007). As described above, heterotrimeric kinesin-II and OSM-3-kinesin cooperate to construct the middle segment doublet microtubules of the ciliary axoneme, whereas OSM-3-kinesin is alone responsible for anterograde movement up the distal singlet microtubules (Evans et al., 2006; Pan et al., 2006). OSM-3-kinesin has been found to dock to IFT particles through an accessory motor complex comprised of DYF-1, DYF-13 and possibly additional components that help build distal segment microtubules (Ou et al., 2007). Components of the IFT cargo complex are not well categorized, but are thought to include structural proteins that make up the axoneme and ciliary membrane along with signaling and regulatory molecules (Ou et al., 2007).
Nematode Strains and Culture

All strains of *Caenorhabditis elegans* (N2: Bristol wild-type; CB4856: Hawaiian wild-type isolate; *daf-19*(m86)II*; *dpy-20*(e1282)IV; *osm-12/bbs-7*(n1606)III; *bbs-8*(nx77)V; *unc-33*(e1261)IV; *dyf-3*(m185)IV; *dyf-3*(og022)IV; *odr-1*(n1936)X; *age-1*(hx546)II; WX729(og007); WX730(og008); WX733(og018); WX734(og019); WX735(og020); WX736(og021); WX738(og023); WX740(og025); WX741(og026); WX742(og027); WX1257 *dyf-3*(og022)IV ogEx049(*dyf-3(*)+ + pRF4(*rol-6*(su1006)))**; WX295 *osm-12*(n1606)III ogEx050(*osm-12(*)+ + pRF4(*rol-6*(su1006)))**; WX311 ogEx001(*osm-12::gfp + pRF4(*rol-6*(su1006)))**; WX312 og052 (*osm-12*(n1606)III, *dpy-20*(e1282)IV); WX310 og051 (*daf-19*(m86)II, WX311 (ogEx001))***; MX38 *osm-12*(n1606)III, *bbs-8*(nx77)V, sEx11009(*dpy-5(*)+ + gcy-5p::gfp)****; MX27 *dpy-5*(e907) nxEx27(*dpy-5(*)+ + *bbs-8::gfp)****; MX23 *dpy-5*(e907) nxEx23(*dpy-5(*)+ + *bbs-1::gfp)****; MX78 *dpy-5*(e907) nx25(*dpy-5(*)+ + *bbs-7::gfp)****; MX63 *bbs-8*(nx77)V Ex[che-2::gfp + *rol-6*(su1006)]**; MX77 *osm-12*(n1606)III, *bbs-8*(nx77)V Ex[che-2::gfp + *rol-6*(su1006)]**; *grown at 15°C, **maintained by picking Rol animals, ***grown at 15°C and maintained by picking Rol animals with reduced GFP expression, ****maintained by picking nonDpy animals) were maintained at approximately 21°C on nematode growth media (NGM) Petri plates seeded with the *E. coli* strain OP50, unless otherwise stated (Brenner, 1974). All strains were handled, crossed,
frozen, and cleaned according to methods described by Sulston and Hodgkin (Sulston et al., 1988).

**Synchronizing Cultures of *C. elegans***

Bleached eggs, obtained from a plate containing gravid adults, were aseptically transferred to about 5 ml of M9 buffer (3 g KH$_2$PO$_4$, 6 g Na$_2$HPO$_4$, 5 g NaCl, 1 ml 1M MgSO$_4$, H$_2$O to 1 liter, sterilize by autoclaving) in a 15 ml conical tube and allowed to incubate overnight at 20°C on a rotator. Animals were collected in a pellet by centrifugation at 1,500 rpm for 1 minute. Most of the supernatant was aspirated in order to remove any dauer pheromone accumulated during starvation. The worm pellet was transferred to a NGM plate seeded with the *E. coli* strain OP50 to allow the arrested L1 animals to resume development (Stiernagle, 2006). For less exact synchronizations, two-dozen gravid adults can be placed on a seeded NGM plate and allowed to lay eggs for 2-3 hours before removing the adults.

**Worm Lysis**

DNA was isolated from individual worms by placing single worms into a PCR tube containing 2.5 μl of a 1::50 solution of Proteinase K in Single Worm Lysis Buffer (SWLB) [50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl$_2$, 0.45% IGEPAL CA-630 (or NP40) (detergent), 0.45% Tween-20, and 0.01% (w/v) gelatin]. The PCR tubes were placed in a thermal cycler and subjected to the following thermal cycle conditions: 60 min. at 60°C, 15 min. at 95°C, and
store at 4°C. Typically, after DNA isolation, genomic DNA was used as a template for amplification by PCR.

To isolate large amounts of DNA for long-term use and storage, a hard lysis DNA isolation protocol was followed. Worms of a single strain were washed off a 9 cm Petri plate containing gravid adults into a microcentrifuge tube using M9 buffer. After spinning the collected worms down into a pellet using a tabletop microcentrifuge, all but about 100μl of the supernatant was removed from the tube. 400μl of hard lysis buffer (0.2M NaCl, 0.1M Tris-HCl pH 8.5, 50mM EDTA, 0.5% SDS) and 10μl of Proteinase K (10mg/ml) were added to the worm pellet and gently agitated. The mixture was incubated at 65°C for 30 minutes while being mixed every two minutes at the beginning. Another 10 μl of Proteinase K was added, and the mixture was incubated for an additional 30 minutes at 65°C. Then 5 μl of RNAse A (10 mg/ml) was added and the mixture was incubated for 30 minutes at 37°C. 500 μl of phenol was added to the mixture before gently agitating and rotating the mixture for another 30 minutes. The mixture was centrifuged at 14,000 rpm for 5 minutes. The aqueous phase was transferred to a new microcentrifuge tube. 500 μl of phenol/chloroform/iso-amyl alcohol (25::24::1) was added to the aqueous phase, mixed gently, and rotated for 30 minutes. The mixture was centrifuged for 5 minutes and then the phenol/chloroform/iso-amyl alcohol extraction was repeated. The mixture was centrifuged for 5 minutes and again the aqueous phase was transferred to a new tube. 500μl of chloroform was added to the tube and mixed gently for 30 minutes. The mixture was centrifuged for another 5 minutes and the aqueous
phase transferred to another tube before 1ml of 96% ethanol (room temperature) was added to the tube, mixed and left overnight for DNA precipitation to occur at room temperature. The next morning the DNA was spun down for 10 minutes at 14,000 rpm. The pellet was then washed with 70% ethanol and left to air dry. Once dry, the pellet was resuspended in 200 µl of water and left on the bench to dissolve for 5 hours. Running 1 µl of the isolated genomic DNA on a 1% agarose gel with a concentration standard was used to assess DNA concentration. The DNA was subsequently used in downstream applications.

**DNA Sequencing**

All DNA samples to be sequenced were either sent out to MWG-Biotech or read in-house on a Beckman CEQ 8100 Sequencer after performing the following protocol.

For sequencing off of PCR products, samples were run on a 1% agarose gel and DNA was extracted from the agarose using a Qiaex II Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol. The concentration of purified PCR product was established by comparison to a concentration marker when both samples were run on a 1% agarose gel. Each sequencing reaction contained 50 fmol of purified PCR product, 4 µl of DTCS Quick Start Master Mix (CEQ Dye Terminator Cycle Sequencing with Quick Start Kit, Beckman Coulter, Fullerton, California), 0.5 µl of 10 µM sequencing primer, and enough H2O to obtain a final volume of 10 µl. The thermal cycle conditions are as follows (steps are separated by commas beginning with step 1): 20 sec. at 96°C, 20 sec. at 50°C, 4 min. at
60°C, cycle steps 1-3 35 times, and store at 4°C. After the thermal cycle, 0.6 μl of glycogen (supplied in CEQ Dye Terminator Cycle Sequencing with Quick Start Kit), and 2 μl of freshly prepared 1.5 M NaOAc + 50 mM EDTA was added to stop each reaction. Each sample was ethanol-precipitated by adding 60 μl of ice cold 95% ethanol before spinning down in a centrifuge at 16,000 rcf (relative centrifugal force). The ethanol was aspirated without disrupting the DNA pellets. The pellets were washed twice with 200 μl of ice cold 70% ethanol, allowed to air dry, and then resuspended in 35 μl Sample Loading Solution (supplied in CEQ Dye Terminator Cycle Sequencing with Quick Start Kit) before being analyzed on the Beckman CEQ 8100 Sequencer.

**Mutagenesis**

Synchronized L1 animals were allowed to develop to the L4 stage (~37 hours at 20°C). *C. elegans* are mutagenized at the L4 stage because at this stage the pool of germ cells in each animal is large but still mostly mitotic, allowing several rounds of replication to occur between the mutagenic treatment and gamete formation. Well-fed, synchronized L4 animals were washed off NGM plates with M9 buffer into a microcentrifuge tube and subsequently washed to remove excess bacteria before the mutagenic treatment. A 0.1 M solution of ethyl methanesulfonate (EMS) was made by adding 0.02 ml of liquid EMS to 2 ml of M9 buffer. The mutagen, EMS, causes point mutations (GC-to-AT transitions) and occasionally small deletions (Anderson et al., 1984). The mixture is gently agitated until the EMS is dissolved and then 2 ml of the synchronized L4 worms
in M9 are added to the solution to give a final EMS concentration of 50 mM. The suspension was incubated at 20°C in a 15 ml conical tube with gentle agitation for 4 hours. After treatment, worms are washed with M9 buffer to remove traces of EMS and then allowed to recover overnight on a seeded NGM plate. Those animals that survived the treatment were picked onto individual plates for selfing. The F2 progeny of these worms were dye-filled using DiI and screened for animals unable to uptake this lipophilic dye into a subset of their amphid and phasmid ciliated-sensory neurons. Those animals found to be dye-filling defective were singled and given strain designations (Sulston et al., 1988).

**Bulked Segregant Analysis**

Bulked Segregant Analysis (BSA) was used to assign mutations to individual chromosomes. Using this method, *osm-12(n1606), dyf-3(og022)* and ten other novel alleles/mutations (*og007, og008, og018, og019, og020, og021, og022, og023, og025, og026, and og027*) were localized to one of six chromosomes that make up the genome of *C. elegans*. BSA is used in place of the standard two-point mapping with genetic (morphological) markers. Single nucleotide polymorphisms (SNPs) existing between two geographical isolated strains of *C. elegans* are used across the genome as markers in order to exhibit linkage of our mutation of interest to other known loci. CB4856, a *C. elegans* isolate from a Hawaiian island, has been shown to contain a uniformly high-density of polymorphisms when compared with the reference Bristol N2 strain (Wicks et al., 2001). A subset of these polymorphisms, called “snip-SNPs,”
modify restriction enzyme recognition sites and can therefore be detected as restriction fragment length polymorphisms or RFLPs.

N2-derived mutant animals were crossed with CB4856 animals. Two bulked lysates were isolated from groups of 50 phenotypically wild type or 50 phenotypically mutant F2 young adults. Four snip-SNP markers were chosen from each of the six chromosomes. These markers are approximately equally spaced out along each chromosome (Table 2). Each snip-SNP marker chosen altered a DraI restriction enzyme recognition site. A small region around each snip-SNP was amplified from each of the bulked lysates and the resulting product was digested with DraI. Standard PCR reagent concentrations were used. The thermal cycle conditions are as follows: 2 min. at 95°C, 15 sec. at 95°C, 1 min. at 58°C, 45 sec. at 72°C, cycle steps 2-4 35 times, 5 min. at 72°C, and store at 4°C.

The digested amplicons were run on a 2% agarose gel. The relative quantity of CB4856 to N2 alleles for each marker in each bulked lysate was determined by measuring the optical density of each band with the National Institutes of Health (NIH) developed, Java-based image processing and analysis software ImageJ. Measure of linkage to a given snip-SNP was calculated as a unitless Map Ratio for that particular snip-SNP. One would expect unlinked biallelic markers to be about equally represented in each lysate. These markers would therefore have a Map Ratio close to one. Linkage of a recessive locus, in the Bristol N2 background, to a given marker would display a Map Ratio significantly below one.
Extended BSA

Many genes involved in cilia formation and maintenance in *C. elegans* have been identified (Perkins et al., 1986). Extended bulk segregant analysis uses a higher density of snip-SNPs, located in close proximity to genes that are known to have a role in cilia development, with the aim of filtering out novel alleles of already characterized genes without having to perform interval mapping.

Extended BSA mapping was done on mutants that mapped to either chromosome I or X according to standard BSA results. Extended BSA is carried out in the same manner as standard BSA, except more snip-SNP markers are used that may use different enzymes than DraI (Table 3).

Interval Mapping

Once BSA has localized a mutation to a specific chromosome, the relevant interval is narrowed down through a process known as interval mapping. This process differs from BSA in that the genotype of individual mutant animals, rather than the genotype of pooled mutant and pooled wild-type animals, is established. Also, each mutant DNA template is assayed for many SNPs within the interval for which linkage has been determined.

Like BSA, CB4856 males are crossed into the mutant strain of interest (isolated from Bristol N2). 96 mutant animals, from the F2 generation of this cross, are singled onto NGM plates seeded with OP50 and are allowed to lay self-fertilized embryos. Once the F3 progeny have matured for each recombinant strain, the worms are washed off their respective plate using M9 buffer and
collected in one of 96 collection microtubes provided within the Qiagen DNeasy 96 Tissue Kit. The protocol supplied within this kit was followed to isolate DNA from each strain. Templates derived from the self-progeny of a homozygous mutant allow each mutant recombinant to be scored as a population instead of a single animal. The acquired DNA templates were assayed using a number of SNP primer pairs located around the interval of interest on the chromosome determined to carry the mutation. Since each template was derived from the progeny of a single F2 animal, it was feasible to establish whether that animal was homozygous Bristol N2, homozygous CB4856, or heterozygous Bristol N2/CB4856 at each SNP marker. Standard PCR reagent concentrations were used. The same thermal cycle conditions used for BSA were used for these reactions. These data will identify “mutant of interest” containing Bristol N2 chromosomes that have recombined with CB4856 DNA to the left or right of the mutant loci. Each worm contains two “mutant of interest” containing chromosomes; however within regions near the mutation, most often only one chromosome is recombinant.

Interval mapping was utilized in cloning dyf-3(og022). Table 6 displays information on the SNP markers and primer pairs used in the mapping of dyf-3(og022).

**Generation of osm-12::gfp Partial Translational Fusion Construct**

Expression of the OSM-12/BBS-7 protein was elucidated by microinjection of an osm-12::gfp partial translational fusion construct into wild
type animals. This construct was generated using a rapid, PCR-based protocol to create GFP fusions described by Oliver Hobert (2002). This protocol involves fusing two primary PCR amplicons using a set of nested primers. The first primary amplicon was amplified using a forward primer located 1,541 base pairs upstream of Y75B8A.12 (osm-12) (osm12upL: 5’-atctacagaaattccgccg-3’) and a reverse primer located in the first exon of osm-12, 69 base pairs 3’ from where the start codon begins (osm-12GFPlink: 5’-tcccgggatatcatcgcgtGTGTTCTTTTCTCTCCTATCCCGACGG-3’). This reverse primer contains an overhang that is complementary to the beginning of the second primary amplicon, which will be amplified from the gfp vector pPD95.77. The product of these two primers is 1,655 base pairs and since there are many repeat sequences in this region, the product was amplified using the high fidelity DNA Polymerase AccuTaq LA (Sigma-Aldrich). This reaction contained 5 µl of supplied 10X reaction buffer, 2.5 µl of dNTPs (10 mM for each nucleotide), 1 µl of dimethyl sulfoxide (DMSO), 2 µl of the forward primer (10 µM), 2 µl of the reverse primer (10 µM), 0.5 µl of AccuTaq, 32 µl of dH2O, and 1 µl of genomic template. The thermal cycle conditions are as follows: 30 sec. at 98°C, 10 sec. at 94°C, 20 sec. at 62°C, 20 min. at 68°C, cycle steps 2-4 30 times, 10 min. at 68°C, and store at 4°C. The second primary amplicon, namely gfp plus the unc-54 3’ UTR from the vector pPD95.77, was amplified using the forward primer gfp1fuser-new (5’-gactctagagttccgccgga-3’) along with the reverse primer gfp2fuser (5’-gacctctaaaccccaaccttc-3’) and was 1,740 base pairs in length. This reaction contained 2.1 µl of 10X reaction buffer, 0.42µl of dNTPs (10 mM for
each nucleotide), 0.84 μl of the forward primer (10 μM), 0.84 μl of the reverse primer (10 μM), 3 μl of sucrose/cresol red, 0.11 μl of Taq DNA polymerase (NEB), 12.8 μl of dH2O, and 1 μl of plasmid template. The thermal cycle conditions are as follows: 2 min. at 94°C, 30 sec. at 94°C, 30 sec. at 54°C, 2 min. & 30 sec. at 72°C, cycle steps 2-4 35 times, 7 min. at 72°C, and store at 4°C. The concentration of each of the two PCR amplicons was estimated by running 1 μl of each product on a 1% agarose gel along with a concentration standard. Each PCR reaction was then diluted to approximately 10-50 ng/μl and then 1 μl of each diluted reaction was combined in the fusion PCR reaction. For the fusion reaction, a forward primer was designed 56 base pairs 3’ from the beginning of the forward primer (osm12upL) used to amplify the first primary amplicon. This primer is osm12upL2gfp and its sequence is 5’-atgaagcccagctgaaag-3’. This primer was used along with the reverse primer, gfp3fuser (5’-gcatctgctcatcaatgtg-3’), which is located 35 base pairs 3’ in relation to the antisense strand from the beginning of the reverse primer (gfp2fuser) used to amplify the second primary amplicon. These nested primers, osm12upL2gfp and gfp3fuser, were used in the fusion reaction to amplify the complete 3,283 base pair osm-12::gfp product. Due to the complexity of the fusion reaction, the high fidelity, long range DNA polymerase mix, Expand (Roche), was used. The first five cycles of the PCR were conducted without the nested primers since the first primary amplicon contains an overhang that is complementary to the second primary amplicon, allowing the complementary sequences to fuse and act as primers for the remainder of the product. In order to achieve exponential
amplification, the nested primers were added for the rest of the thermal cycles (thermal cycle explained in the table below). In order to keep the concentrations of the reagents in the reaction constant, two master mixes were made (one without primers and one with primers). During the first five cycles the reaction contained 2.5 μl of Expand buffer 1, 3.5 μl of dNTPs (10 mM for each nucleotide), no primers, 0.38 μl of Expand Long Template Enzyme Mix, 17.38 μl of dH2O, and 1 μl of each primary amplicon dilution as a template. After the first five cycles, the thermal cycler was paused and the following mix was added to the reaction before restarting the cycler: 2.5 μl of Expand buffer 1, 3.5 μl of dNTPs (10 mM for each nucleotide), 1.5 μl of the forward primer (10 μM), 1.5 μl of the reverse primer (10 μM), 0.38 μl of Expand Long Template Enzyme Mix, and 14.38 μl of dH2O. This added up to a final reaction volume of 49.5 μl. Table 1 shows cycle conditions. Once the PCR was completed the reaction was run on a 1% agarose gel. The appropriately sized band was excised from the gel using a Qiaex II Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol. The DNA was subsequently prepared for microinjection.

**osm-12 Mutation Detection Primers**

Individual worms containing the *osm-12(n1606)* nonsense mutation [G → A mutation at nucleotide 2,899 of *osm-12(n1606)*] were identified by means of two PCR reactions designed to amplify wild-type (wt), *osm-12(+)*, or mutant (mut) *osm-12(n1606)* genomic DNA fragments. For clarification purposes, *osm-12* is transcribed 5’ to 3’ relative to the antisense strand, but the primers that will
be described were designed in relation to the sense strand. Two forward primers were designed whose 3’-most nucleotide would rest 2,899 base pairs downstream of the *osm-12* start codon, exactly where the *n1606* mutation is located. Both primers are identical with the exception of the 3’ most nucleotide. One of the forward primers (og1-Forward-WT: 5’-ccgacgttgtatcaatttcc-3’) will complement in the presence of wild-type DNA and along with the reverse primer Mike-og1-DU (5’-attgggcagtcattagatgg-3’), will produce a 545 base pair amplicon from wild type DNA. The other forward primer (og1-Forward-mut: 5’-ccgacgttgtatcaatttct-3’) will complement in the presence of the *osm-12(n1606)* mutation and along with the reverse primer Mike-og1-DU, will produce a 545 base pair amplicon from *osm-12(n1606)* DNA. The two reactions run separately on the same DNA template will show that template to be either homozygous wild type, homozygous mutant, or heterozygous in relation to the *osm-12(n1606)* allele. 545 base pair bands in both reaction lanes denotes heterozygosity, whereas a 545 base pair band shown only in the reaction utilizing the og1-Forward-WT primer denotes homozygous wild type DNA and a 545 base pair band shown only in the reaction utilizing the og1-Forward-mut primer denotes homozygous *osm-12(n1606)* DNA.

**Generating a *dyf-3* Rescue Construct**

The largest form of *dyf-3* was amplified using the forward primer Dyf-3ForA (5’-ttttctattgggacaggtaagg-3’) along with the reverse primer Dyf-3RescueRev (5’-atagcctgattagggcgtc-3’). *dyf-3* is transcribed on the antisense strand, therefore the forward primer is in the 3’ UTR and the reverse primer is in
the 5’ UTR. The primers should amplify a 3956bp product including the C04C3.5b (dyf-3) ORF, 798bp of 5’ UTR, and 685bp of 3’ UTR. Standard Taq DNA polymerase reagent conditions were used and thermal cycle conditions were as follows: 2 min. at 94°C, 30 sec. at 94°C, 30 sec. at 57.2°C, 4 min. at 72°C, repeat steps 2-4 35 times, 7 min. at 72°C, and store at 4°C.

**Microinjection and DNA Transformation**

Injection pads were made by placing a drop of 2% agarose in water onto a 24x50 mm coverslip and then flattening the agarose by dropping a second coverslip on top. After 10 seconds the coverslips were slid apart and the one that contained the thin agarose pad was allowed to dry overnight at room temperature.

“Glass 1BBL w.FIL 1.0 mm 4 IN” filaments, item #1B100F-4 from World Precision Instruments, Inc., were pulled into microinjection needles using the Kopf needle/pipette puller Model 750, from David Kopf Instruments, Tujunga, CA. The settings used were: heat1=5 AU, heat2=0 AU, sol=5 A, delay=0 sec, sol=0.1 sec. Needles were only kept if pulled in 10-12 seconds.

DNA injection mixes should contain 1-100 µg/ml of transgene and up to 100 µg/ml of a dominant marker gene like rol-6 contained within the injectable plasmid pRF4. Loading, mounting, and breaking the needle in addition to scope setup, microinjection and recovery were completed with the help of Michael R. Koelle’s “microinjecting worms” protocol (Koelle 1994).
Dye-Filling Assays

A subset of ciliated sensory neurons in the amphid and phasmid organs of *C. elegans* can be fluorescently stained by soaking animals in the lipophilic membrane dye DiI (1,1’-Dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate), [Abs (max): 549 nm; Em (max): 565 nm] as described by Hedgecock (1985). Mixed populations of live animals were soaked in a 15 μM solution of DiI in M9 buffer and rotated for 1 hour. The animals were then washed three times with deionized H₂O to remove excess dye. Worms were allowed to settle before worm pellets were collected and dispensed onto unseeded NGM Petri plates. Assayed worms were viewed and scored under a UV microscope equipped with a Texas Red filter.

Odorant Assays

Assays were performed on 9 cm Petri plates containing the following chemotaxis media: 1.6% agar, 5 mM potassium phosphate (pH 6.0), 1 mM CaCl₂, 1 mM MgSO₄, and 10 mM sodium phosphate (pH 7.2) (Bargmann et al., 1991). Two points were marked at opposite ends of the plate, each 2 cm from the edge. 1 μl of 1 M NaN₃, which is used to anesthetize the worms, is applied to each point. 1 μl of a 1:100 dilution of iso-amyl alcohol (attractant) in 95% ethanol was spotted at one of these points, and 1 μl of ethanol was spotted at the other point as a control. Young adult worms were washed three times with M9 buffer and once with deionized water before being applied to a “start” point on the plate 4.3 cm from each of the other two points. After using a wick to remove excess liquid
from the plate the worms were allowed to separate across the plate for 1 hour.

The chemotaxis index was calculated using the equation \( A-B/A+B+C \), where \( A \) equals the number of worms within 2 cm of the attractant, \( B \) equals the number of worms within 2 cm of the ethanol counter-attractant, \( C \) equals all space on the plate outside a 2 cm radius of each of the three points, and \( A+B+C \) equals the total number of worms included in the assays results (Section D encompasses a 2 cm radius around the “start” point and is not included in the total, see Figure 4). p-values were calculated to determine statistical significance using a threshold alpha level of 0.05. Microsoft Excel was used to calculate means, standard deviations, standard error of the mean, and two-tailed Student’s t tests.

**Locomotion Assays**

Assays were performed at room temperature on 9 cm Petri plates containing the following chemotaxis media: 1.6% agar, 5 mM potassium phosphate (pH 6.0), 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), and 10 mM sodium phosphate (pH 7.2) (Bargmann et al., 1991). A “start” point was marked at the center of the plate along with three concentric circles radiating outward from this point. The circles had radii of 1 cm, 2 cm, and 3 cm and were denoted as quadrants A, B, and C, respectively (Quadrant D represented the area from outside the largest circle to the edge of the plate) (see Figure 5). 2 µl of concentrated worm pellet, washed three times with M9 buffer and once with deionized water, was applied to the “start” point. Worms were allowed to separate across the plate for 25 minutes. Once the assay was complete, worms were placed at 4°C for approximately 20
minutes to anesthetize them before scoring. Only young adult and adult worms were scored after the assay. p-values were calculated to determine statistical significance using a threshold alpha level of 0.05. Microsoft Excel was used to calculate means, standard deviations, standard error of the mean, and two-tailed Student’s t tests.

**Longevity Assays**

Longevity assays were carried out as previously described with certain alterations (Kenyon et al., 1993). Assays were performed at room temperature (~21°C). In order to synchronize populations, approximately two-dozen gravid adults were placed onto a 9 cm seeded NGM plate and allowed to lay eggs for 2-3 hours before being removed. The day eggs were laid and adults were removed was counted as Day 0. When these worms reached adulthood, the plates were treated with the chemical 5-fluoro-2’-deoxyuridine (FUDR), which has been shown to prevent progeny production without significantly affecting life-span. 500 ml of a fresh dilution of 650 µg/ml FUDR was administered to cover each assay plate containing 9.5 ml of seeded NGM agar making the final concentration of FUDR in each plate 34.2 µg/ml. Plates were left ajar to allow excess liquid to evaporate. Plates were stored in a Rubbermaid container to prevent excess drying. Worms were scored daily beginning on Day 8. When a worm no longer moved, pumped, or responded to physical prodding then it was scored as dead and removed from the plate. Worms that crawled up the side of the plate and desiccated were not scored. p-values were calculated to determine statistical
significance using a threshold alpha level of 0.05. Microsoft Excel was used to calculate means, standard deviations, standard error of the mean, two-tailed Student’s t tests, and 75th percentiles.

**Pharyngeal Pumping Rate Assays**

Pumping rates were observed at 600x using a dissecting microscope. Approximately a dozen worms were placed on a 6 cm plate with seeded NGM agar and allowed to acclimate to their new surroundings for about 1 hour at room temperature.

Individual worms were observed for one minute wherein the number of contractions of the terminal bulb was counted (Chow et al., 2006). Five worms were scored per strain assayed. p-values were calculated to determine statistical significance using a threshold alpha level of 0.05. Microsoft Excel was used to calculate means, standard deviations, standard error of the mean, and two-tailed Student’s t tests.

**Total RNA Extraction and Isolation**

Animals from the strain of interest are grown on 20 separate 9 cm plates until gravid adults largely populate each plate and the *E. coli* lawn is almost entirely consumed. Worms are then washed from each plate using M9 buffer and pooled in a 50 ml conical vial. Worms are allowed to settle before much of the supernatant is removed. The remaining pellet is stored at -80°C for an hour to aid in the homogenization. To extract total RNA from our worm pellet, I utilized the
Qiagen RNeasy® Mini Kit (Cat. No. 74104) following the specifications for “Purification of Total RNA from Animal Tissue.” To homogenize our pellet, I used a mortar and pestle. The specific protocol was entitled “Purification of Total RNA from Animal Cells Using Spin Technology.” After step 5 of this protocol I performed an optional DNase digestion using the Qiagen RNase-Free DNase Set (Cat. No. 79254). After the RNA was eluted from the column, 1μl of RNasin was added to each sample before storing the RNA at -80°C for future use.

**RNA Formaldehyde Gel Electrophoresis**

Before beginning the procedure, all glassware and equipment, including gel casting trays, were treated with RNaseZAP® from Ambion (Cat. No. AM9780) to remove any RNase contamination. 82 ml DEPC-treated H2O, 10 ml 10x MOPS Buffer, and 1 g agarose were microwaved in a 250 ml Erlenmeyer flask until the solution appeared homogeneous. 5 μl of ethidium bromide was added and gently swirled into the mixture. Next, 17.6 ml of 37% formaldehyde was added to the flask and gently mixed under a fume hood. The solution was poured into a casting tray and allowed to polymerize for 1 hour. During this time, the RNA sample was prepared in a 1.5 ml microcentrifuge tube by combining 5 μl of RNA, 15 μl of loading buffer, and 1 μl of ethidium bromide. The mixture was incubated at 65°C for 15 minutes and then on ice for 2 minutes. A solution of 1X MOPS buffer in DEPC-treated H2O was used in the gel chamber as a running buffer. Once the gel was submerged in running buffer, the comb was removed from the gel, and 20 μl of each prepared sample was loaded into a separate well.
In addition, an RNA ladder was added to a separate well to determine molecular size. The gel was run at 70 V until the loading dye had traveled 2/3 the length of the gel, and then the gel was removed and visualized under UV light.

**Reverse Transcriptase PCR**

To generate complementary DNA (cDNA) from N2 and *dyf-3(og022)* RNA, I performed a reverse transcriptase PCR (RT-PCR). The Access RT-PCR System from Promega (Cat. No. A1260) was used for the RT-PCR reactions. Each reaction consisted of 10 μl AMV/Tfl 5x reaction buffer, 1 μl dNTP mix (10 mM concentration for each dNTP), 5 μl each of forward and reverse primer (1 μM final concentration of each primer in each reaction), 2 μl of 25 mM MgSO₄, 1 μl of AMV Reverse Transcriptase (5 U/μl), 1 μl of Tfl DNA Polymerase (5 U/μl), 2 μl of RNA sample, and brought up to a final volume of 50 μl with 23 μl of nuclease-free water. The first pair of primers was designed to amplify the same product in both genetic backgrounds. The forward primer Dyf-3.startFor2 (5’-ATGTCGTATCGTGAGCTGAG-3’) along with the reverse primer Dyf-3.bothRev2 (5’-CTTCGTGTGCGTCAAGGTT-3’) should amplify a 169 bp cDNA product off of N2 and *dyf-3(og022)* RNA. Dyf-3.startFor2 along with Dyf-3.N2Rev1 (5’-CGTACTCGTCCATATACTGA-3’) should amplify a 730 bp cDNA product off of N2 RNA and a 786 bp cDNA product off of *dyf-3(og022)* RNA. Dyf-3.startFor2 along with Dyf-3.d3Rev2 (5’-CTTTTTCAGGCTGGTGCCCT-3’) should amplify a 424 bp cDNA product off of *dyf-3(og022)* RNA and no product off of N2 RNA. Primers were designed to
amplify act-1 (encodes a C. elegans actin isoform) cDNA from both RNA samples as a positive control product. Act-1For (5’-CCAGGAATTCGCTGATCGTATGCAGAA-3’) along with Act-1Rev (5’-TGGAGAGGGAAGCGAGGATAGA-3’) should amplify a 132 bp product in both genetic backgrounds. For negative controls, the primers Dyf-3.startFor2 and Dyf-3.bothRev2 were used in reactions, which excluded the reverse transcriptase enzyme, for each sample of RNA. These negative control reactions should yield no products. The thermal cycle conditions are as follows: 45 min. at 45°C, 2 min. at 94°C, 30 sec. at 94°C, 1 min. at 60°C, 1 min. at 68°C, cycle steps 3-5 40 times, 7 min. at 68°C, and store at 4°C.
Chapter 3: Loss of *C. elegans* BBS-7 protein function results in cilia defects and compromised intraflagellar transport

Introduction

The connection between Bardet-Biedl Syndrome and cilia

Vertebrate cilia have a number of different functions. They make up the outer segments of rod and cone photoreceptor cells in the retina, generate force to propel sperm cells, and detect fluid flow through the kidneys. In addition to these functions, vertebrates have non-motile primary cilia located singly on the apical surface of most non-proliferating cells that are thought to be involved in sensing the extracellular environment (Pazour et al., 2002). Cilia and intraflagellar transport, the process that builds and maintains cilia, are essential to human development and health (Pazour et al., 2002). IFT and ciliary defects have been linked to human diseases such as polycystic kidney disease, retinal degeneration, situs inversus, and Bardet-Biedl Syndrome.

Bardet-Biedl Syndrome is a rare, genetically heterogeneous, autosomal recessive disorder with a pleiotropic phenotype that includes obesity, pigmented retinopathy, polydactyly, mental retardation, renal malformations, diabetes, and congenital heart defects (Katsanis et al., 2001). Defects in 12 human genes that affect basal bodies and cilia have been found to cause Bardet-Biedl Syndrome (Ou et al., 2007). The five known *C. elegans* homologues of human BBS genes, *bbs-1, bbs-2, bbs-3, bbs-7, and bbs-8*, have been shown to undergo IFT, and are expressed solely in ciliated cells due to the existence of a DAF-19 RFX
transcription factor binding site in their proximal promoters (Ou et al., 2007; Blacque et al., 2004; Fan et al., 2004). Our lab has cloned and characterized one of these bbs genes in *C. elegans* in order to investigate the possible link between the abnormal function of basal bodies and/or cilia and Bardet-Biedl Syndrome (Blacque et al., 2004).

**Results**

**Identification and Characterization of osm-12/bbs-7**

The mutant strain containing the *osm-12*(n1606) allele was initially generated and isolated during a mutagenesis and subsequent screen for animals defective in osmolarity avoidance. The uncloned *osm-12*(n1606) allele was identified as a candidate *bbs-7* mutant. This identification was based on the findings that the *bbs-7* gene was narrowed down to the *osm-12* critical interval, that the *bbs-7* gene contains an X box in its proximal promoter, and that defects in osmolarity avoidance are characteristic of ciliary abnormalities in *Caenorhabditis elegans* (Blacque et al., 2004). This gene is a homolog of the human BBS7 gene, which when mutated has been shown to cause the rare genetic disorder Bardet-Biedl syndrome (BBS) (Badano et al., 2003).

Sequencing the *bbs-7* gene (Y75B8A.12) from *osm-12*(n1606) mutant worms exposed a G → A nonsense, transition mutation at nucleotide 2,899 (Figure 2). Two PCR reactions were designed to distinguish between homozygous wild type, heterozygous *osm-12*, and homozygous mutant *osm-12* in samples of DNA from single animals (see Materials & Methods) (Figure 3). Figure 3 shows that a
product of 545 bp will only be visible from a wild type (+/+) worms DNA when the amplification utilizes wild type specific primers. DNA from a heterozygous animal, in regards to the osm-12 loci, will display a product of 545 bp when amplified using both the wild type and mutant specific primers. Lastly, DNA from a homozygous osm-12 mutant worm will only show a product of 545 bp when amplified using the osm-12 mutant specific primers (Figure 3). The n1606 allele contains a stop codon after only 32% of the coding region and therefore is assumed to be null. osm-12(n1606) animals were found to be phenotypically normal with regard to movement (Figure 5), development, and brood size (Blacque et al., 2004).

To measure the chemosensory function of sensory cilia in osm-12 mutants, I optimized a behavioral assay that quantifies the ability of the worms to chemotax toward the chemical attractant iso-amyl alcohol (Blacque et al., 2004) (Figure 4A). The cilia of two chemosensory amphid neurons, AWA and AWC, mediate a normal response to this volatile compound (Bargmann et al., 1993). Results of this assay show osm-12(n1606) mutants to be chemotaxis-defective toward iso-amyl alcohol (Figure 4). The mean chemotaxis index for N2 was 0.468 ± the S.E.M. 0.029. osm-12/bbs-7 has a significantly lower mean chemotaxis index of 0.083 ± the S.E.M. 0.028 [p < 0.05 at 5.33x10^-11, (Figure 4B)]. This phenotype was completely rescued in osm-12(n1606) mutants expressing wild-type bbs-7(+) (Blacque et al., 2004). Therefore, defects in chemotaxis towards the volatile compound iso-amyl alcohol, in osm-12(n1606) animals, are directly attributable to disturbances in the bbs-7 gene.
To further support the evidence that *osm-12(n1606)* animals do not gravitate towards iso-amyl alcohol because of a sensory defect, I conducted a locomotion assay to rule out motility defects in our mutant as a contributing factor (Figure 5). *osm-12* animals showed no statistically significant difference in mobility when compared to wild type N2 (p-values > 0.05 at 0.450 for region A, 0.564 for region B, 0.190 for region C, and 0.544 for region D). *osm-12* showed a statistically significant difference in mobility when compared to the locomotion defective *unc-33* strain (p-values < 0.05 at 0.005 for region A, 0.010 for region B, and 0.027 for region C) except when comparing the percentage of worms that reached the furthest region (p > 0.05 at 0.374 for region D). *unc-33(e1261)* worms never left quadrant A (Figure 5B).

Many strains that exhibit chemosensory defects also display structural abnormalities in their sensory cilia. In order to determine the structural integrity of cilia in the *osm-12(n1606)* mutant, a dye-filling assay was performed. The fluorescent, lipophilic, membrane dye (DiI) preferentially fills amphid head neurons and phasmid tail neurons by way of their exposed ciliated endings (Starich et al., 1995). Wild-type (N2) animals take up DiI into AWB, ASH, ASJ, ASK, ADL and ASI amphid neurons, and PHA and PHB phasmid neurons (Hedgecock et al., 1985; Starich et al., 1995). *osm-12(n1606)* worms subjected to a dye-filling assay were found to display little or no detectable DiI uptake, when compared to N2 controls (Figure 6). This strongly suggests a ciliary defect. The Dyf (dye-filling defective) phenotype was completely rescued with the wild type *bbs-7* gene (Figure 6).
In collaboration with Michel Leroux’s lab in Burnaby, B.C., I determined the cellular localization of GFP-tagged BBS-7. I generated a partial-translational fusion construct in which the \textit{gfp} gene was fused in frame to the first exon of Y75B8A.12 (thermal cycle conditions used in amplifying this construct are shown in Table 1). This construct contained 1,541 base pairs of upstream promoter sequence containing an X-box transcription factor binding site 107 base pairs from the translational start site (Ansley et al., 2003). I found OSM-12/BBS-7 expression to be restricted exclusively to all ciliated neurons and identical to the expression patterns of certain other known BBS proteins, as well as other IFT proteins that contain X-box transcription factor binding sites (Blacque et al., 2004) (Figure 7).

Figure 8A shows a close up of three bilaterally symmetrical amphid ciliated sensory neurons (ASK, ADL, and ASI) expressing my \textit{osm-12::gfp} construct. Expression of the partial-translational transgene in wild type (N2) worms generates GFP signals along the ciliary axonemes (denoted by cil) and at the ciliary transition zones (tz and arrowheads) of both amphid and phasmid ciliated neurons (Blacque et al., 2004) (Figure 7). The localization of the BBS-7::GFP protein overlaps with that of the IFT protein OSM-5 (Haycraft et al. 2001; Blacque et al., 2004) (Figure 7). In addition to OSM-5, the localization of the BBS-7::GFP protein overlaps with BBS-1, BBS-2, BBS-8 (Blacque et al., 2004), OSM-6 (Collet et al., 1998), XBX-1 (Schafer et al., 2003) and CHE-13 (Haycraft et al., 2003). Each of these proteins displays prominent localization along the ciliary axonemes and at the transition zones of ciliated neurons (Blacque et al.,
2004) (data not shown). Separate GFP fluorescence signals consistent with transition zones of other neurons were frequently observed closer to the anterior end of the worm (Blacque et al., 2004) (Figure 7, denoted by an asterisk in B). These include inner and outer labial neurons. Consistent with OSM-5::GFP, the BBS-GFP fusion proteins, including BBS-7 (Figure 7C and 7F), showed some localization in the cell bodies (cb; Figure 7 and 8) and the neuronal dendrites (den). Little cell body or dendritic staining was observed in the case of OSM-5::GFP. These data suggest that, along with other BBS proteins, the *C. elegans* BBS-7 protein functions mainly at the ciliary transition zones and axonemes.

*osm-12/bbs-7* expression is regulated by the RFX-type transcription factor DAF-19. 107 bp 5’ of the transcriptional start site of Y75B8A.12 (*osm-12/bbs-7*), there is a transcription factor binding site called an “X-box” where DAF-19 binds. The general X-box consensus sequence is 5’-GTTACCATGCAAC-3’ with certain variations at certain base pairs (Swoboda et al., 2000) (Figure 9). The X-box sequence located in the *bbs-7* promoter is 5’-GTTCATAGTAAC-3’ (Ansley et al., 2003) (Figure 9). Figure 9 also shows X-box sequences for several *C. elegans* genes expressed in ciliated neurons, as well as for most *bbs* genes (Ansley et al., 2003) (Figure 9).

I crossed our *osm-12(n1606)/bbs-7::gfp* expression construct strain with the *daf-19(m86)* mutant strain, and found that OSM-12::GFP neuronal expression was essentially abolished (Figure 10A). A fluorescent image of the *osm-12*(n1606)/bbs-7::gfp expression construct strain, taken with an identical exposure time as the image in Figure 10A, is shown in Figure 10B. OSM-12::GFP
expression in amphid ciliated neurons is extremely vibrant when compared to OSM-12::GFP expression in the \textit{daf-19(m86)} mutant background.

**Additional collaborative results further characterizing the involvement of \textit{osm-12/bbs-7} along with \textit{bbs-8} in intraflagellar transport**

In addition to our findings, our collaborators in the Leroux Lab performed time-lapse fluorescence microscopy assays in live \textit{C. elegans} to monitor the motility of GFP-tagged components of the IFT machinery (Blacque et al., 2004). They observed anterograde and retrograde movement of GFP-tagged BBS-1, -2, -7, and -8 in wild-type animals and reported that BBS proteins motility was comparable to GFP-tagged IFT proteins such as OSM-5, -6, XBX-1, and CHE-13 (Blacque et al., 2004) (data not shown). They also used the ASER neuronal cell marker, \textit{gcy-5p::gfp}, in \textit{osm-12/bbs-7} and \textit{bbs-8} mutant backgrounds to analyze cilium morphology. \textit{gcy-5p::gfp} produces diffuse GFP fluorescence throughout the cell body, dendrite, transition zone and cilia of ASER. Transition zones of these two mutants were indistinguishable from N2 transition zones; however, the ciliary structures of the two mutants were measured and determined to be significantly shorter than N2 (Blacque et al., 2004) (data not shown). Fragmented GFP fluorescence extending from the distal end of the shortened cilium was also observed in both mutants, along with an \textit{osm-12,bbs-8} double mutant.

Our collaborators also examined the IFT movement and localization of two IFT-B subcomplex proteins (OSM-5 and CHE-2) and one IFT-A subcomplex protein (CHE-11) in the \textit{osm-12} and \textit{bbs-8} mutant backgrounds. \textit{gfp} transgenes of
each IFT component were introduced into N2, \textit{osm-12} and \textit{bbs-8} animals. When \textit{osm-5::gfp} and \textit{che-11::gfp} constructs were expressed in the \textit{osm-12} or \textit{bbs-8} mutants, GFP-tagged proteins displayed atypical localization when compared to N2 controls (Blacque et al., 2004) (data not shown). On the other hand, the CHE-2::GFP IFT protein appeared to show normal localization and IFT movement in \textit{osm-12} and \textit{bbs-8} mutant backgrounds when compared to N2 controls with the exception of some GFP accumulation at the midpoint and distal ends of axonemes (Blacque et al., 2004) (data not shown). CHE-2::GFP movement was observed anterior to the mid-ciliary spot in both \textit{bbs} mutants. To determine the extent of the IFT defects, levels of GFP-tagged IFT component movement were quantified in \textit{osm-12} and \textit{bbs-8} backgrounds. OSM-5::GFP and CHE-11::GFP proteins had considerably decreased levels of IFT movement in the two \textit{bbs} mutants when compared to N2 controls, whereas the CHE-2::GFP protein showed only faintly compromised IFT levels (Blacque et al., 2004) (data not shown). \textit{osm-12,bbs-8} double mutants had comparable IFT defects to those of the single mutants.

\textbf{Discussion}

We have identified, cloned and characterized the \textit{osm-12} gene, showing that it is identical to \textit{bbs-7}. Based on our findings and the findings of the Leroux Lab, we have determined that the BBS-7 protein predominantly functions at ciliary transition zones and along ciliary axonemes (Blacque et al., 2004). Movement of BBS-7::GFP fusion proteins along ciliary axonemes suggests that BBS-7 is associated with IFT rafts and therefore with IFT motor proteins. These
findings are consistent with known IFT-core complex proteins indicating that BBS-7, along with other BBS proteins, is not transported as particle-associated cargo but instead is a bona fide IFT protein associated with the kinesin or dynein motor complexes and/or IFT-A or -B subcomplexes (Blacque et al., 2004).

Figure 8A and 8B, and to a lesser extent in Figure 7F, show BBS-7::GFP expression in the cell bodies of ciliated neurons. This is thought to be largely nonspecific and due to transgene overexpression.

We have isolated an osm-12/bbs-7 mutant C. elegans strain. Based on phenotypic assays, expression patterns, and IFT motility assays using known GFP-tagged IFT proteins in a bbs-7 background, loss of bbs-7 functioning leads to defects in ciliary structure and function. bbs-7 mutants have abnormal cilium length and structure, do not respond normally to certain chemical attractants, and are defective in dye-filling (Blacque et al., 2004). When measuring the ability of osm-12/bbs-7 mutants to chemotaxis towards the volatile attractant iso-amyl alcohol, I report here a more pronounced defect than was reported in our manuscript. Future experiments may include subjecting osm-12/bbs-7 mutants to a battery of chemotaxis assays that test a number of different volatile and water-soluble attractants and repellents in order to more completely assess remaining sensory neuron functioning in mutant animals.

Locomotion assays were performed to make sure any chemosensory defect observed was not due to the inability of the mutant to travel at a speed comparable to that of wild type worms toward the chemical attractant. There was no statistically significant difference between osm-12 and unc-33 worms reaching
region D; however, in the absence of an anesthetizing agent \textit{osm-12} worms were able to move in and out of regions before the end of the assay, whereas \textit{unc-33} animals never left region A. There was no statistically significant difference between \textit{unc-33} and N2 worms reaching region D either (p > 0.05 at 0.266). The relevant significance values are displayed in regions A through C, when comparing N2 and \textit{osm-12} to \textit{unc-33}, where both strains are significantly more mobile when compared to the locomotion mutant. When comparing N2 to \textit{osm-12}, there were no significant differences in percentage of worms that reached each region. Therefore, \textit{osm-12} worms are capable of wild type mobility.

Analysis of cilium length in a \textit{bbs-7} mutant background compared to cilium length in the IFT-B subcomplex component \textit{osm-5(p813)} mutant background, shows that \textit{bbs-7} mutant cilia are closer to wild type length than IFT-B component mutant cilia and therefore \textit{bbs} genes may function differently than IFT-B component genes (Blacque et al., 2004). IFT-A component mutants, along with kinesin and dynein mutants, have been shown to have similar ciliary truncations to that of \textit{bbs-7} mutants; however, since retrograde motility was observed for CHE-2::GFP in a \textit{bbs-7} background, a characteristic not seen in IFT-A component or dynein mutants, \textit{bbs} genes seem to have divergent functioning from IFT-A and IFT motor genes (Blacque et al., 2004; Perkins et al., 1986; Wicks et al., 2000; Schafer et al., 2003).

The finding that the GFP-tagged IFT-B component CHE-2 undergoes IFT movement at normal velocities in an \textit{osm-12/bbs-7} mutant background, suggests that IFT motor proteins are functional and that the structural platform necessary
for IFT is intact in the absence of BBS-7 (Blacque et al., 2004). The finding that CHE-11 and OSM-5 have highly decreased levels of IFT in a bbs-7 mutant background indicate that CHE-11 and OSM-5 proteins may inefficiently incorporate into IFT particles at the transition zone located at the base of the cilium, before being loaded onto the ciliary axoneme (Blacque et al., 2004).

Previous research describes steps that occur before IFT protein transport along the ciliary axoneme. These steps include recruitment of all IFT components to the transition zone at the base of the cilium, assembly of a motor-IFT particle-cargo complex, docking of complex at the transitional fibers, and finally loading of intact complex onto the ciliary axoneme (Scholey, 2003). IFT protein targeting to the transition zone probably occurs before formation of an IFT particle complex. che-13 mutants (CHE-13 has been shown to be an IFT-B component) are defective in the recruitment of OSM-5::GFP to the base of the cilia indicating that proper CHE-13 function is necessary for the targeting of OSM-5 to the transition zone of cilia (Haycraft et al., 2003). Since CHE-2::GFP and CHE-11::GFP localize to the base of the cilia in a bbs-7 mutant background, it is thought that BBS-7 is not directly involved in targeting these IFT proteins to the transition zone. BBS-7 is most likely not involved in the direct loading of the IFT complex onto the ciliary axoneme, given that CHE-2-associated IFT particles undergo IFT in the osm-12/bbs-7 mutant background (Blacque et al., 2004).

A model was proposed in which BBS-7 and BBS-8 are involved in assembly of certain IFT components into the motor-IFT particle-cargo complex (Blacque et al., 2004). It appears as though loss of BBS functioning leads to
assembly defects between components of the IFT-A and -B subcomplexes resulting in compromised IFT. BBS proteins were hypothesized to assist in incorporating IFT proteins into the final motor-IFT particle-cargo complex at the base of cilia. BBS proteins could possibly act as molecular chaperones, as in the case of mammalian BBS6 (Katsanis et al., 2000), or they could allow incorporation into IFT particles by directly interacting with IFT proteins (Blacque et al., 2004). The latter theory is supported by BBS proteins displaying IFT-like movement, which could mean that they are part of the core or peripheral components of the motor-IFT particle-cargo complex (Blacque et al., 2004). Since CHE-11 and OSM-5 proteins are components of IFT-A and IFT-B subcomplexes, respectively, the BBS-7 protein appears to be involved in the assembly of components from both subcomplexes (Blacque et al., 2004).

Based on additional data generated by the Leroux Lab on BBS-8, osm-12/bbs-7 and bbs-8 mutants, along with osm-12/bbs-7,bbs-8 double mutants, have the same phenotypes, which indicates that the proteins they encode function in a common cellular process (Blacque et al., 2004). Mutations in different human BBS genes cause clinically identical phenotypes, which is consistent with this data (Katsanis et al., 2001). Since the double mutant phenotype mimics that of each single mutant, the two proteins may form a complex in which each protein is just as necessary as the other with regard to the overall functioning of the complex (Blacque et al., 2004).
Figure 1. IFT components are classified into “modules” based on transport profiles. (Taken with permission from Ou et al., 2007) (A) C. elegans homologues of Chlamydomonas IFT components are shown. IFT-A and -B subcomplexes are transported along the middle segment doublet microtubules by kinesin-II and OSM-3-kinesin respectively. The BBS protein complex stabilizes the connection between IFT-A and -B subcomplexes and may be mediated by DYF-3. The accessory motor module involves DYF-1 and possibly DYF-13 and is required to activate the OSM-3-kinesin motor and/or its interaction with the IFT-B subcomplex. The IFT rafts deliver different kinds of potential cargo, which may include IFTA-2. (B) A schematic of the IFT pathway. Machinery assembles at the basal body/transition zone, then both anterograde motors move IFT particles together up the middle segment. At the tip of the middle segment, kinesin-II and associated particles are recycled by IFT-dynein and OSM-3-kinesin and associated particles continue up the distal segment until they reach the tip and are recycled by IFT-dynein. It is thought that both motors redundantly deliver cargo that build and stabilize the ciliary axoneme.
Sequencing of the n1606 allele found a G to A nonsense transition mutation at nucleotide 2899 of the predicted open reading frame Y75B8A.12. This gene was identified as osm-12; and after determining that this open reading frame was homologous to mammalian BBS7, it also became known as bbs-7. This allele is thought to be null because the mutation creates an early stop codon after only 32% of the coding region of the gene.

(Taken from Blacque et al. 2004)
Figure 3. *osm-12(n1606)/bbs-7* mutation detection reactions.

(Taken from Blacque et al. 2004) Two separate PCR reactions were designed to amplify wild type (wt), *osm-12(n1606)/+* heterozygotes, or homozygous mutant (mut) *osm-12(n1606)* genomic DNA fragments from single worms. PCR products of the appropriate size (545 bp) and with the appropriate primer pairs have been obtained from +/+, *osm-12(n1606)/+*, and *osm-12(n1606)/osm-12(n1606)* animals.
Figure 4. Iso-amyl alcohol chemotaxis assay.

(A) Schematic of assay plate showing attractant spot, counter attractant spot, and start point. Chemotaxis indexes were calculated by taking the number of worms in area A, minus the number of worms in area B, divided by the total number of worms to leave area D (A-B/A+B+C). (B) Chemotaxis indexes were calculated for 36 separate assays (18 for each strain). Approximately 50 - 200 worms were placed at the start position. Only worms able to leave the start circle were scored. Number of worms scored per assay ranged from 4 – 64. The mean chemotaxis index for N2 is 0.468 ± 0.029. osm-12/bbs-7 has a mean chemotaxis index of 0.083 ± 0.028. osm-12/bbs-7 animals have a statistically significant defect in chemotaxis toward iso-amyl alcohol when compared to N2 controls (p < 0.05 at 5.33x10^-11). Excel was used to calculate means, standard deviations, S.E.M.s, and two-tailed Student’s t tests.
Figure 5. Locomotion assay.

(A) Schematic of an assay plate showing three concentric circles radiating outward from a start point. The radii of the circles are 1 cm, 2 cm, and 3 cm along with the radius of the plate, which is approximately 4 cm. Together, the circles divided the plate into four regions. Worms were placed at the start point and allowed to move outward without any chemical influences. (B) After 25 minutes of movement, the total numbers of animals from each region were counted. Nine assays were performed (three per strain) and the number of animals per assay ranged from 13-113. *osm-12* animals showed no statistically significant difference in mobility when compared to wild type N2 (p-values > 0.05 at 0.450 for region A, 0.564 for region B, 0.190 for region C, and 0.544 for region D). *osm-12* showed a statistically significant difference in mobility when compared to the locomotion defective *unc-33* strain (p-values < 0.05 at 0.005 for region A, 0.010 for region B, and 0.027 for region C) except when comparing the percentage of worms that reached the furthest region (p > 0.05 at 0.374 for region D). Excel was used to calculate means, standard deviations, S.E.M.s, and two-tailed Student’s t tests.
Figure 6. *osm-12(n1606)/bbs-7* mutants contain defective ciliary structures.

(Taken from Blacque et al. 2004) *osm-12/bbs-7* animals are defective in the uptake of the lipophilic, fluorescent, membrane dye, Dil, into a subset of their amphid and phasmid ciliated sensory neurons. Animals with intact ciliated neurons, like the wild type strain N2, can incorporate Dil into a subset of ciliated neurons as shown in the figure. Occasionally, Dil staining can be seen in the *osm-12/bbs-7* mutant, but only after prolonged exposure times.
Figure 7. BBS-7 localizes mainly to the transition zones and axonemes of cilia.

(Taken from Blacque et al. 2004) (A,D) Schematics showing the position of cilia structures at the dendritic endings of amphid (A) and phasmid (D) neurons. (B,E) Fluorescent image of head region (B) and tail region (E) of N2 worms expressing GFP-tagged OSM-5, an IFT protein known to be expressed in transition zones and axonemes of sensory cilia (Haycraft et al., 2001). (C,F) BBS-7 protein is localized to transition zones (tz and arrowheads), as well as ciliary axonemes (cil), of amphid (C) and phasmid (F) sensory cilia. The asterisk indicates the ciliated endings of anterior positioned neurons such as IL1 and IL2. Dendrites are denoted by “den” and cell bodies are denoted by “cb.” All panels depict one set of amphid or phasmid neurons with the exception of the schematic in A which shows both sets of amphid channels.
Figure 8. **BBS-7 is expressed exclusively in ciliated neurons.**

*bbs-7::gfp* is expressed in ciliated neurons of wild-type worms. (A) *bbs-7::gfp* expression in three bilaterally symmetric amphid ciliated neurons. ASK and ASI are known to function in chemotaxis and ADL has been shown to function in avoidance and social feeding behaviors. DIC and fluorescent images were merged. (B) *bbs-7::gfp* expression in amphid (head) and phasmid (tail) neurons.
Figure 9. X-box transcription factor binding site alignments.

(Taken with permission from Ansley et al., 2003) Listed are *Caenorhabditis elegans* genes, including certain known *bbs* genes, which are expressed in ciliated neurons and have X-box transcription factor binding sites. Base pair numbers represent distances from X-box to start codon of respective gene.
Figure 10. *osm-12(n1606)/bbs-7* expression requires the RFX-type transcription factor DAF-19.

*osm-12(n1606)/bbs-7* contains an X-box transcription factor binding site, that binds DAF-19, 107bp from its translational start site. (A) An *osm-12(n1606)/bbs-7::gfp* expression construct strain was crossed with the *daf-19(m86)* mutant strain leading to essentially complete abolition of OSM-12::GFP expression as seen in this fluorescent image. (B) Fluorescent image of the *osm-12(n1606)/bbs-7::gfp* expression construct strain. OSM-12::GFP expression in amphid ciliated neurons is extremely vibrant when compared to OSM-12::GFP expression in the *daf-19(m86)* mutant background.
<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th># of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>2 min.</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>15 sec.</td>
<td>5X (no primers)</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>30 sec.</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>68°C</td>
<td>4 min.</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>10 sec.</td>
<td>10X (with primers)</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>30 sec.</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>68°C</td>
<td>4 min.</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>15 sec.</td>
<td>25X (with primers)</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>30 sec.</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>68°C</td>
<td>4 min. 20 sec. + 5 sec. cycle elongation for each successive cycle</td>
<td></td>
</tr>
<tr>
<td>Final Elongation</td>
<td>68°C</td>
<td>7 min.</td>
<td>1X</td>
</tr>
<tr>
<td>Storage</td>
<td>4°C</td>
<td>∞</td>
<td>1X</td>
</tr>
</tbody>
</table>

**Table 1. Expand long range DNA polymerase thermal cycle conditions.**

Cycle conditions used in the generation of an *osm-12::gfp* partial translational fusion construct.
Chapter 4: Mapping and phenotypic characterization of novel dye-filling mutants

Introduction

Sensory Perception and Longevity

To further characterize the Dyf strains isolated from my F2 screen I performed additional phenotypic assays. Defects in the uptake of a lipophilic dye into a subset of sensory neurons can be attributed to deficiencies in ciliated sensory neurons and IFT. If the IFT system is not functioning properly, then ciliated sensory endings could be compromised, which could lead to diminished sensory perception. It has been found that sensory perception can drastically influence lifespan; therefore, we subjected the Dyf strains to an aging assay (Antebi, 2007).

The role of the insulin/IGF-1 signaling pathway (IIS) in aging has been studied extensively in *C. elegans*. Downregulation of this pathway increases stress resistance and longevity across many species (Antebi, 2007; Kimura et al., 1997; Clancy et al., 2001; Holzenberger et al., 2003). In *C. elegans* it is thought that insulin-like peptides activate the DAF-2/insulin/IGF-1 receptor tyrosine kinase. This in turn triggers a PI3/AKT/SGK kinase cascade, which phosphorylates DAF-16/FOXO (Antebi, 2007; Lin et al., 1997; Hertweck et al., 2004; Paradis et al., 1998). Under optimal conditions the DAF-16/FOXO transcription factor is restricted to the cytoplasm. However, in response to decreased insulin/IGF-1 signaling, stress, or increased DAF-18/PTEN, inhibition
of which partially bypasses the need for DAF-2 signaling, DAF-16 enters the
nucleus (Lin et al., 2001; Ogg et al., 1998). Once in the nucleus, DAF-16 binds
and activates target survival genes, which promote longevity (Lin et al., 2001). C.
elegans mutants with sensory neuron defects have been shown to extend lifespan
by relocating the DAF-16 transcription factor to the nucleus (Apfeld et al., 1999).
The sensory cues involved in insulin-like peptide synthesis and release are
thought to be nutrients along with a number of attractants and/or repellents
(Antebi, 2007).

Caloric restriction has been shown to increase longevity in C. elegans. In
laboratory settings, the worm is fed a strain of E. coli known as OP50. Limiting
the amount of bacterial food when culturing animals constitutes caloric
restriction. In addition, certain feeding mutants that have slower rates of
pharyngeal pumping are calorically restricted. Caloric restriction induced
longevity has been shown to be mediated by the activity of the SKN-1/NRF
transcription factor in the ASI neurons (Bishop et al., 2007). I performed a
pharyngeal pumping assay to determine if any significant longevity phenotypes
among our Dyf mutants were due to caloric restriction.

Many Dyf mutants are chemotaxis defective due to defective ciliated
sensory endings. Sensory transduction molecules (i.e., olfactory receptors) are
abundant in sensory cilia and are essential for proper olfactory perception
(Bargmann, 2006). To further characterize our Dyf mutants, we performed
chemotaxis assays using the volatile attractant iso-amyl alcohol. The AWC
neurons mediate the response of the worm to iso-amyl alcohol (Roayaie et al.,
1998). The cilia of both AWC neurons form a large sheet of membrane and are enclosed within a sheath pocket that is continuous with the environment (Roayaie et al., 1998). Both the G alpha protein ODR-3 and the transmembrane guanylyl cyclase ODR-1 are required for wild type responses to all AWC-perceived odorants (Roayaie et al., 1998; L’Etoile et al., 2000).

Results

Genetic screen for novel genes involved in ciliogenesis

An EMS mutagenesis was performed on wild type N2 animals with the aim of identifying novel ciliogenesis and IFT components. I used a simple F2 screen to find mutants of interest. Mutagenized animals were screened using a dye-filling assay that tested the ability of the animals to uptake the fluorescent lipophilic membrane dye DiI into a subset of amphid and phasmid ciliated neurons. A set of 12 separate dye-filling defective strains were isolated from this screen, 11 of which were mapped to a linkage group using single nucleotide polymorphisms between N2 and the wild type isolate CB4856. The 12th strain was lost due to a fungal infection that greatly reduced the fecundity of the animals to the point where the strain was unsalvageable. The 11 isolated strains were given the following allele designations: og007, og008, og018, og019, og020, og021, og022, og023, og025, og026, and og027.
Chromosome mapping of screened Dyf strains using single nucleotide polymorphisms

Each isolated strain was linked to one of the six chromosomes, which make up the genome of *C. elegans*, by gene mapping using a single nucleotide polymorphism map (Wicks et al., 2001) (Table 2). The SNPs used in this mapping protocol exist between the wild type Bristol N2 strain and a wild type Hawaiian isolate strain known as CB4856. These SNPs are relatively evenly spaced throughout the *C. elegans* genome and are referred to as “snip-SNPs” because they modify restriction enzyme recognition sites. Since the Dyf mutants were derived from an N2 background, these snip-SNPs can be used in a mapping strategy where the highly polymorphic wild type *C. elegans* isolate, CB4856, is crossed into an N2 derived mutant strain and exclusion of CB4856 alleles of these snip-SNPs in mutant progeny is assessed with bulk segregant analysis. This protocol is a more rapid method of linking a mutation of interest to a specific chromosome than the classic two-point mapping using morphological markers methodology.

Ou et al. 2007 recently performed a similar mutagenesis and screen in which they isolated 47 independent dye-filling defective strains and linked 42 of them to their respective chromosome. They also compiled a relatively comprehensive list of known mutants that display amphid channel ciliary defects along with their respective locations on the genetic map (Ou et al., 2007). These genes are *dyf-1* through *dyf-6*, *dyf-8* through *dyf-12*, *che-2*, *che-10*, *che-11*, *che-13*, *che-14*, *mec-8*, *daf-6*, *osm-5*, *unc-6*, *unc-33*, *unc-101*, *unc-104*, and *unc-119*. 
I compared the chromosomal distribution of known genes and novel alleles from Ou et al. 2007 to the novel alleles that I isolated. I found a remarkably similar dispersal of loci responsible for Dyf phenotypes among the six chromosomes (Table 4). Table 4 shows that the highest percentages of known genes that display amphid ciliary defects (list from Ou et al., 2007) as well as novel Dyf alleles, from my screen and the published screen, are located on chromosomes X and I. Unlike the novel alleles, which have been mapped to a particular chromosome but not yet a specific open reading frame, the known genes represented in Table 4 have been localized to independent ORFs and therefore more accurately represent the distribution of genes among each chromosome. It has yet to be determined whether the novel alleles are located in a novel uncharacterized gene or a gene that has already been identified and characterized as causing a Dyf phenotype. Novel alleles that have yet to be assigned to an open-reading frame do not accurately represent this value due to the fact that they could be novel alleles of previously identified genes. For instance, che-3, a gene that encodes a dynein heavy chain and is located at 2.45 cM on chromosome I, contains 46 exons over approximately 20 kb of genomic DNA (12.3 kb of transcript) making it very vulnerable to mutagenesis due to its overall size and density of exons (Wicks et al., 2000). Therefore, it is likely that multiple novel alleles isolated from the screens in Table 4, which have been localized to chromosome I, are novel alleles of che-3. In fact, eight of the nine novel alleles assigned to chromosome I according to Ou et al. 2007 have been
narrowed to an interval on chromosome I that contains the *che-3* open reading frame.

I wondered if the screen for novel genes involved in ciliogenesis and IFT was saturated. The findings in Ou et al. 2007 suggest that there are more genes to be found that cause ciliary defects; however, it may also suggest that screening for novel ciliary genes may be approaching saturation. Based on this assumption, I designed an extended BSA protocol using a higher density of snip-SNPs on chromosomes X and I (Table 3). Each of these snip-SNPs was chosen based on its linkage to one or more of the known genes that cause amphid channel ciliary defects described by Ou et al. 2007. The causal gene from eight of the eleven strains I isolated is located on either chromosome X or I and eight and seven of the known genes involved in proper ciliary functioning according to Ou et al. 2007 are located on chromosomes X and I, respectively (Table 4). I chose these chromosomes because of the high frequency of Dyf genes linked to them. *wrt-6* is a 9th X-linked gene not included in the Ou et al. 2007 list that causes a Dyf phenotype due to what is thought to be a structural problem with the sheath and or socket cells and not necessarily the amphid cilia (Aspock et al., 2000). Before continuing with the time consuming task of interval mapping using SNPs to narrow down the genetic interval of each novel allele, an extended BSA can link a novel allele to an already characterized Dyf gene. Then, performing the less time consuming reactions it would take to sequence the known Dyf gene on the mutagenized chromosome could determine whether the novel Dyf allele is a novel allele of an already characterized Dyf gene. This could save substantial amounts
of time that would have been consumed performing interval mapping. Even if the novel allele proves not to be an allele of a characterized gene, only minimal amounts of time and resources could have been spent and there is a higher degree of assurance that the allele being mapped is located in a novel uncharacterized gene.

For example, *og007* was mapped to the X chromosome through chromosomal BSA mapping. Another round of BSA was performed utilizing a higher density and number of snip-SNP markers located on the X chromosome (Table 3, Figure 11) to see which known Dyf gene the *og007* allele is most highly linked to. The BSA methodology is described in more detail in Chapters 1 and 2. To obtain a numerical linkage value for the mutation, in relation to each SNP marker, I needed to assign numbers to individual DNA bands based on the intensity of each band when run on gel. A software program called ImageJ was used to measure the intensity of N2 and CB4856 (CB) bands on gel from each bulked lysate for each snip-SNP. ImageJ measures the optical density of an N2 and CB band from the phenotypically wild type bulked lysate (“WT” lanes on gels in Figures 11 through 21) and the phenotypically mutant bulked lysate (“Mut” lanes on gels in Figures 11 through 21). Bands in each lane of each gel are considered to be N2 or CB based on the presence or absence of a SNP from the CB4856 genetic background. Lengths of restriction enzyme-cut amplicons and the genetic background on which they belong are described in Table 3. A graphical peak, calculated by ImageJ, represents the optical density for each band. The area under each of the four peaks (one N2 and one CB band amplified from
each bulked lysate is selected) is measured and the following ratio is taken with
the numbers obtained: (N2 Mut/CB Mut)/(N2 WT/CB WT). Each numerator and
denominator represents one of the four areas. The value of this ratio is the linkage
value represented in the graphs in Figures 11 through 21. The larger the linkage
value the stronger the linkage of that particular allele at that particular genetic
location.

Figures 11 through 21 report mapping data obtained for all eleven novel
Dyf alleles. Extended BSA was used to analyze novel alleles that were mapped to
chromosome I or X through standard BSA (Figures 11-21, Table 5). These alleles
are og007, og008, og018, og020, og021, og023, og025, and og027. For example,
through extended BSA, og007 was shown to have the strongest linkage at -1.89
cM on the X chromosome (Figure 11). The snip-SNP at this location is strongly
linked to the unc-6 locus. Sequencing the unc-6 open-reading frame in the og007
genetic background would be necessary to determine if og007 is an allele of unc-
6. The novel Dyf alleles that were mapped to chromosomes other than I or X
through standard BSA are og019, og022, and og026. For example, BSA of og019
showed this mutation to have the strongest linkage to a snip-SNP marker at ~ -4
cM on chromosome IV (Figure 14). Interval mapping using a higher density of
SNP markers on chromosome IV is needed to assign the og019 mutation to a
specific open-reading frame. Mapping data for the remaining strains is
summarized in Table 5.
**Additional phenotypic analysis of screened Dyf strains**

Sensory cilia located at the tip of chemosensory neurons contain high concentrations of sensory transduction molecules (Ward et al., 1975; Ware et al., 1975). Many of these molecules are G protein-coupled receptors whose signaling mediates chemosensation on a molecular level. If cilia are not functioning properly, as in many dye-filling-defective mutants, then it is likely that certain sensory transduction molecules are also not functioning properly. We performed a chemotaxis assay that tested the ability of the novel Dyf strains to chemotax toward the volatile attractant iso-amyl alcohol (Figure 22). Sensory neurons involved in chemotaxis toward volatile compounds, like iso-amyl alcohol, include AWA and AWC (Bargmann, 2006).

ODR-1, a transmembrane guanylyl cyclase, is required for responses to all AWC-sensed odorants, including iso-amyl alcohol (L'Etoile et al., 2000), therefore, *odr-1* animals were used as a control along with wild type N2, which is known to chemotax towards iso-amyl alcohol. A chemotaxis index (C.I.) of 1 is defined as complete attraction, whereas a chemotaxis index of -1 is defined as complete repulsion. Results of the chemotaxis assays showed the following strains to have a statistically significant defect in sensing iso-amyl alcohol when compared to N2 controls: *odr-1, og007, og008, og018, og020, og021, dyf-3(og022), og023, og025, og026*, and *og027* (see Figure 22A for mean C.I.s ± S.E.M. and p-values). *og019* showed no statistically significant defect in chemotaxis towards iso-amyl alcohol (see Figure 22A for mean C.I. ± S.E.M. and p-value).
Mutations that cause defects in sensory cilia or defects in sensory signal transduction have been known to extend lifespan (Apfeld et al., 1999). Downregulation of the insulin/IGF-1 signaling pathway (IIS), known to be involved in aging in many species, increases stress resistance and longevity in *C. elegans* (Antebi, 2007; Kimura et al., 1997; Clancy et al., 2001; Holzenberger et al., 2003). The sensory cues responsible for insulin-like peptide synthesis and release are thought to be nutrients along with a number of attractants and/or repellents (Antebi, 2007). These sensory cues may not be able to be detected in mutants with defective sensory cilia. Therefore, we performed an aging assay on each screened Dyf strain (Figure 23).

I calculated and compared the mean lifespan, in days, of each novel Dyf strain to the mean lifespan of N2 (17.8±S.E.M. 0.3). *age-1* was assayed as a control because it has been previously shown to have a 40% longer mean lifespan when compared to wild type (Friedman et al., 1988). The wild type isolate CB4856 was also assayed as a control because it has been shown to have a similar lifespan to N2. The following Dyf strains, along with the *age-1* control (20.5±0.7), showed a statistically significant increase in mean lifespan when compared to N2 controls (17.8±0.3) (shown are mean lifespans in days with S.E.M.): *og008* (21.9±0.9), *og018* (21.0±0.9), *dyf-3(og022)* (22.2±0.7), *og023* (20.4±0.5), *og026* (22.4±0.5), and *og027* (25.4±0.4). *og007* (12.4±0.6) and *og025* (16.1±0.4) displayed a statistically significant decrease in mean lifespan when compared to N2 controls. Finally, *og019* (19.0±0.7), *og020* (16.9±0.2), *og021*
(17.1±0.3), and CB4856 (17.5±0.6) displayed no significant difference in mean lifespan when compared to N2 controls (p-values listed in Figure 23A).

Dietary restriction has been shown to increase longevity in *C. elegans*. Certain feeding mutants that have slower rates of pharyngeal pumping are calorically restricted. The feeding mutant, *eat-2*, has a slow rate of pharyngeal pumping and an extended lifespan, due to caloric restriction, when compared to wild type controls (Lakowski et al., 1998). *eat-2;daf-2* double mutants have been found to live even longer than either single mutant suggesting that *eat-2* extends lifespan independently of DAF-16 activity (Lakowski et al., 1998). This suggests that caloric restriction works through a mechanism separate from the insulin/IGF-1 signaling pathway to extend lifespan.

I performed a pharyngeal pumping rate assay to assess whether any of our Dyf strains were calorically restricted (Figure 24). I compared the average pumps/minute of the terminal pharyngeal bulb in our Dyf strains to N2 controls and found that *og007* and *og025*, along with the characterized pharyngeal pumping mutant *eat-1*, display a statistically significant decrease in pharyngeal pumping rate when compared to N2 controls (p-values = 0.006, 0.004, and <0.0001 respectively). *og023* has a significantly increased rate of pharyngeal pumping when compared to N2 controls. The remainder of the Dyf strains show no significant difference in pharyngeal pumping rate when compared to N2 controls (see Figure 24A for means ± S.E.M. and p-values).
Discussion

I performed a mutagenesis and subsequent screen for animals defective in the uptake of DiI into a subset of ciliated sensory neurons in order to identify novel genes involved in intraflagellar transport and ciliogenesis. Time constraints prevented us from accomplishing this feat; however, eleven independent strains defective in dye-filling were isolated and mapped to 1 of 6 chromosomes in *C. elegans*. I hypothesize that there are genes involved in IFT that have yet to be discovered in *C. elegans*, and I have yet to determine if one or more of these novel Dyf strains contains a mutation in a novel, uncharacterized IFT gene.

When screening the F2 progeny of mutagenized animals for a phenotype of interest, with the intent of discovering mutations in uncharacterized genes that are responsible for that phenotype, it is important to consider that many of the mutant strains isolated from the screen may be alleles of already characterized genes. I expanded on a rapid method for chromosome mapping using single nucleotide polymorphisms in *C. elegans*, established by Wicks et al., 2001, with the aim of filtering out novel alleles of characterized genes without performing time-consuming interval mapping. This methodology, which I am referring to as extended bulked segregant analysis, will only prove to be efficient if a large number of characterized (“known”) genes exist that when mutated result in the phenotype sought in the screen.

In the case of dye-filling mutants, the percent distribution of known genes involved in ciliary defects (Ou et al., 2007) among the six chromosomes is remarkably similar to the distribution of alleles isolated by Ou et al. 2007 as well
as our Dyf alleles. These data can be interpreted as suggesting that the screen for dye-filling defective mutants is approaching saturation. The list of amphid channel ciliary mutants described by Ou et al. 2007 is not complete and would have to be added to in order to account for all known ciliary mutants (i.e., bbs-7 and bbs-8 are not included in this list).

In a screen like this, after chromosome linkage has been established and before genetic intervals are narrowed through time-consuming interval mapping, extended BSA, which uses a higher density of snip-SNP markers on the chromosome of interest, can be performed in order to determine whether the mutation being mapped is an allele of a known gene. Since the majority of novel Dyf alleles, as well as known Dyf genes, are located on chromosomes X and I, I designed primers to amplify regions around snip-SNPs strongly linked to the known genes on these chromosomes. Since each snip-SNP marker is strongly linked to a known gene, the snip-SNP marker that the mutation being mapped is most strongly linked to will inform us of the known gene to which the mutation is most strongly linked. Next, the known gene can be sequenced in the mutant background to see if the known gene has been mutated. This extra step involves minimal time and resources and can save the trouble of performing extensive interval mapping. Ideally, if the mutation proves not to be an allele of the known gene, then the chances of it being a novel gene should increase dramatically.

The data amassed through extended BSA is preliminary in nature and has certain drawbacks. As seen in Figures 11 through 21, many primer pairs failed to amplify the desired product even after attempts to optimize reaction conditions.
Linkage of screened alleles to these genetic distances could not be analyzed. In addition, certain linkage values are exaggerated because of complete linkage, where either a CB band or N2 band is not detectable by the imaging software. An example of this can be seen in Figure 11 where a CB band cannot be seen in the mutant lane at -1.89 cM on the X chromosome. This could be due to the efficiency of the restriction enzymes used to cut the amplicons or possibly the imaging software is not sensitive enough to detect a baseline optical density. A third caveat is seen in Figure 16, where the linkage values do not display a smooth curve in the bar graph. *og021* is most strongly linked to the SNP at ~ -1 cM on chromosome I. A stronger linkage value would be expected at -0.74 cM than at ~12 cM or even 3.8 cM, which is not the case. Again, this could be due to the sensitivity of the ImageJ software or the efficacy of the restriction enzyme. It is necessary to manually close off the calculated peaks produced by ImageJ, in order for the software to calculate an area under the peak. This can lead to a large margin of error. Imaging software that can detect optical density with an increased sensitivity and that does not rely on human precision to complete calculations may enhance the mapping resolution achieved through extended BSA.

Another drawback of this mapping strategy is that it is only a viable option when complementation testing is problematic. In *C. elegans*, novel alleles of characterized genes can be filtered out through complementation tests. Once a mutation is linked to a chromosome through SNP mapping, the mutant strain carrying this mutation can be crossed with a strain carrying a mutation in a known
gene that causes the same phenotype and is located on the same chromosome.
Phenotypic analysis of cross-progeny will determine whether the novel mutation is an allele of the known gene. However, complementation tests are only advantageous when the phenotype being studied does not negatively affect the ability of the animal to mate. Many strains of *C. elegans* that display dye-filling defects also have difficulty sensing mating partners, and are therefore difficult to use in complementation tests. There are other mutant phenotypes that negatively affect mating. For example, certain Unc mutants are partially paralyzed and may have difficulty mating because of this locomotion defect. Unc mutants generated through mutagenesis and isolated through a screen for locomotion defective animals, would be good candidates for extended BSA mapping.

Our proof of principle for extended BSA is shown in Figure 17. After linking *og022* to chromosome IV through BSA, I interval mapped this allele and found it to be a novel allele of *dyf-3*. *dyf-3* is located at -4.42 cM on chromosome IV. Our BSA results are consistent with this distance as seen in Figure 17. *og022* is most strongly linked to ~ -4 cM which is the closest marker to *dyf-3*. Future work would include sequencing the known genes (described in the Results section and Figure legends) most strongly linked to each novel Dyf allele. If a novel allele did not prove to be an allele of a known gene after sequencing, then interval mapping would be employed to find the exact genetic position of the mutation.

More sensitive imaging software, a more comprehensive list of known Dyf genes, new amplification primers, and optimized reaction conditions would be tested to optimize extended BSA.
To further characterize the screened Dyf strains we performed additional
phenotypic assays. Dye-filling defects suggest cilia malformations or other kinds
of ciliary deficiencies; therefore, I hypothesized that dye filling-defective strains
are likely to have chemosensory defects. Sensory transduction molecules are
located on sensory cilia and mediate chemosensation. We assessed the ability of
each Dyf allele to chemotax toward the volatile attractant iso-amyl alcohol. Like
the known chemotaxis defective strain, *odr-1*, 10 of the 11 screened Dyf strains
displayed statistically significant defects in chemotaxis toward iso-amyl alcohol.
This suggests that the AWC neurons are not functioning properly in each of these
10 strains, since iso-amyl alcohol is an AWC-sensed odorant. DiI does not
normally dye-fill AWC neurons in wild-type animals; therefore, the dye-filling
assay cannot assess functioning of AWC neurons directly. Once the mutations in
these 10 alleles are mapped to a specific ORF, AWC-specific rescue can be
attempted by introducing wild-type versions of the causal genes under the
regulation of an AWC-specific promoter into the mutant animals. If these
constructs rescue the mutant phenotype, then functional defects in the AWC
neurons are most likely responsible for the mutant phenotypes and are probably
due to defects in the ciliated endings of both AWC neurons. *og019* showed no
statistically significant defect in chemotaxis suggesting that this strain obtains
residual chemosensory functioning, especially in regards to AWC-sensed
odorants. Future experiments would include additional chemotaxis assays using
other volatile and water soluble attractants and repellents to assess the functioning
of other amphid sensory neurons in dye-filling defective strains.
Since sensory transduction defects and ciliary defects are known to extend lifespan by causing DAF-16/FOXO to relocate to the nucleus where it turns on survival genes, I decided to perform an aging assay on the screened Dyf strains (Antebi, 2007). There are many known mutants with ciliary defects that also have been shown to be long-lived. These strains include *daf-19, che-2, che-3, che-11, che-13, osm-1, osm-5, osm-6*, and *daf-10* (Apfeld et al., 1999). Six of our eleven novel Dyf strains showed a statistically significant increase in mean lifespan when compared to N2 controls, suggesting that the insulin/IGF-1 signaling pathway is downregulated in these strains, causing the transcription factor DAF-16 to enter the nucleus where it turns on survival genes that promote prolongevity. Three of the eleven strains showed no statistically significant difference in mean lifespan when compared to N2 controls, suggesting that residual chemosensation in these Dyf strains supports the ability to respond to sensory cues that are responsible for releasing insulin-like peptides, which lead to DAF-16 being retained in the cytoplasm. One of these strains, *og019*, had a rather low 75th percentile value (20.5 days) when compared to the strain’s maximum lifespan (35 days). It turns out that all the *og019* worms died on day 23 except for one animal that lived until day 35. This strain may actually have a significantly decreased mean lifespan and should be assayed again. The remaining two strains displayed a statistically significant decrease in mean lifespan when compared to N2 controls, which could suggest that the causal gene in these mutant strains could be involved in any number of processes that when compromised leads to a decrease in lifespan. Not
surprisingly, there appear to more processes that when disturbed lead to decreased longevity than those that lead to increased longevity.

Future experiments would include crossing each long-lived Dyf strain with the long-lived *daf-2* strain to see if the double mutant lives even longer than either of the single mutants. If the double mutant lived longer than either single mutant then it would be likely that the Dyf allele extends lifespan through a different mechanism than insulin/IGF-1 signaling. Another way I could test whether my Dyf mutants are long-lived due to a downregulation of the insulin/IGF-1 signaling pathway is by amplifying and quantifying mRNA from genes involved in this pathway through quantitative real-time PCR combined with reverse transcriptase PCR. Changes in the levels of these transcripts due to a mutation in one of my Dyf strains strongly suggests that the mutation is located in a gene involved in this pathway.

A pharyngeal pumping rate assay was performed on each screened Dyf strain to determine whether the strain was calorically restricted. It is possible that animals with ciliary defects may have trouble sensing food and therefore slow the rate by which they pump their pharynx to obtain food. This could lead to dietary restriction, which is known to extended lifespan. *og007, og023, and og025*, along with the characterized pharyngeal pumping mutant *eat-1*, displayed statistically significant differences in pharyngeal pumping rate when compared to N2. However, I do not believe that caloric restriction plays any role in extending longevity in these three novel Dyf mutants. *og007 and og025* have significantly decreased rates of pharyngeal pumping but are significantly shorter lived than N2
controls. *og023* animals are significantly longer lived than N2, but as far as pharyngeal pumping rate is concerned, they have significantly increased rates, which is not consistent with caloric restriction.

In summary, these novel Dyf alleles can be used as a tool to further understand the connections between ciliary deficiencies, chemotaxis defects, and longevity. Also, more data is needed to assess the efficacy of extended BSA. However, once optimized, extended BSA may prove to be an attractive alternative to complementation testing.
<table>
<thead>
<tr>
<th>LGI</th>
<th>Cosmid/YAC N2 Digest</th>
<th>CB4856 Digest</th>
<th>Primer Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-27cM</td>
<td>F56C11 350bp, 150bp</td>
<td>500bp</td>
<td>ATGCCAGTGAAGAAGCAGGTCACATCCTGTTGATGAA</td>
</tr>
<tr>
<td>-6cM</td>
<td>W03D8 400bp, 130bp</td>
<td>530bp</td>
<td>TTTCTCACTTTTGCGCGTGTTGAGG</td>
</tr>
<tr>
<td>-1cM</td>
<td>D1007 322bp, 177bp</td>
<td>499bp</td>
<td>AAAATACCGGAAAGGTTTCCGTTGAGG</td>
</tr>
<tr>
<td>12cM</td>
<td>F58D5 450bp</td>
<td>300bp, 150bp</td>
<td>TCCGTCAGATTCCATTGGTTT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LGII</th>
<th>Cosmid/YAC N2 Digest</th>
<th>CB4856 Digest</th>
<th>Primer Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-16cM</td>
<td>T01D1 265bp, 109bp</td>
<td>374bp</td>
<td>CGAATTTCGAAATGACCCGACACAA</td>
</tr>
<tr>
<td>-5cM</td>
<td>F54D10 516bp</td>
<td>387bp, 129bp</td>
<td>TTGGAGGTTTATATCGGTTGAGG</td>
</tr>
<tr>
<td>1cM</td>
<td>T24B8 372bp, 122bp</td>
<td>494bp</td>
<td>TAAAGTCGACACTCCAGGAGGAC</td>
</tr>
<tr>
<td>14cM</td>
<td>F15D4 541bp</td>
<td>408bp, 133bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LGIII</th>
<th>Cosmid/YAC N2 Digest</th>
<th>CB4856 Digest</th>
<th>Primer Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-13cM</td>
<td>Y71H2B 367bp, 105bp</td>
<td>472bp</td>
<td>GAGAACACCCACCTGGTGAAGGAA</td>
</tr>
<tr>
<td>-1cM</td>
<td>F56C9 485bp</td>
<td>335bp, 150bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>4cM</td>
<td>Y39A1A 355bp, 171bp</td>
<td>526bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>10cM</td>
<td>Y41C4A 339bp, 153bp</td>
<td>492bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LGIV</th>
<th>Cosmid/YAC N2 Digest</th>
<th>CB4856 Digest</th>
<th>Primer Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15cM</td>
<td>Y41D4B 378bp, 155bp</td>
<td>533bp</td>
<td>CAAATTAAATATTTCAGG</td>
</tr>
<tr>
<td>-4cM</td>
<td>F42A6 296bp, 122bp</td>
<td>418bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>1cM</td>
<td>E03H12 375bp</td>
<td>297bp, 78bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>22cM</td>
<td>Y105C5B 316bp, 90bp</td>
<td>238bp, 90bp, 78bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LGV</th>
<th>Cosmid/YAC N2 Digest</th>
<th>CB4856 Digest</th>
<th>Primer Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-19cM</td>
<td>F36H9 309bp, 84bp, 79bp</td>
<td>388bp, 84bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>-7cM</td>
<td>Y61A9LA 300bp, 150bp</td>
<td>450bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>5cM</td>
<td>R10D12 500bp</td>
<td>350bp, 150bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>20cM</td>
<td>Y17D7B 325bp, 160bp</td>
<td>485bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LGX</th>
<th>Cosmid/YAC N2 Digest</th>
<th>CB4856 Digest</th>
<th>Primer Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15cM</td>
<td>F49H12 540bp</td>
<td>320bp, 220bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>-8cM</td>
<td>ZK470 445bp, 78bp</td>
<td>350bp, 95bp, 78bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>2cM</td>
<td>F11A1 406bp, 136bp</td>
<td>542bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
<tr>
<td>12cM</td>
<td>F46G10 320bp, 191bp, 34bp</td>
<td>545bp</td>
<td>TTTGCGGTTCTTCGCTTCGACAA</td>
</tr>
</tbody>
</table>

Table 2. A list of snip-SNPs used in determining chromosome linkage.

Most snip-SNPs and primer pairs are taken from Wicks et al., 2001. These snip-SNPs are used in a mapping strategy where the highly polymorphic wild type *C. elegans* isolate, CB4856, is crossed into an N2-derived mutant strain and exclusion of CB4856 alleles of these snip-SNPs in mutant progeny is assessed with bulk segregant analysis.
<table>
<thead>
<tr>
<th>Genetic Position (saip-SNP)</th>
<th>Adjacent Dyf Gene Position</th>
<th>Dyf Gene Adjacent</th>
<th>Enzyme</th>
<th>N2 Digest (bp)</th>
<th>CR4856 Digest (bp)</th>
<th>Primers (5’-3’)</th>
<th>Wormbase Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: ~-27</td>
<td></td>
<td></td>
<td>DraI</td>
<td>350, 150</td>
<td></td>
<td></td>
<td>pkP1054</td>
</tr>
<tr>
<td>1: ~-6</td>
<td></td>
<td></td>
<td>DraI</td>
<td>400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: ~-1</td>
<td></td>
<td></td>
<td>DraI</td>
<td>322, 177</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: -0.74</td>
<td>dyf-1</td>
<td>1: -0.43</td>
<td>SmaI</td>
<td>297, 259</td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>1: 0.61</td>
<td>che-14, dyf-10</td>
<td>1: 0.45, 1.56</td>
<td>DraI</td>
<td>153, 135</td>
<td></td>
<td></td>
<td>pkP1056</td>
</tr>
<tr>
<td>1: 3.80</td>
<td>dyf-5, mec-8</td>
<td>1: 3.76, 3.76</td>
<td>NsiI</td>
<td>387</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: 5.05</td>
<td>che-13</td>
<td>1: 5.05</td>
<td>HpaII</td>
<td>215, 94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1: ~-12</td>
<td></td>
<td></td>
<td>DraI</td>
<td>450</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: ~-15</td>
<td></td>
<td></td>
<td>DraI</td>
<td>540</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: -12.25</td>
<td>osm-5</td>
<td>X: -12.66</td>
<td>TaqI</td>
<td>484, 41</td>
<td>306, 178, 41</td>
<td></td>
<td>pkP6119</td>
</tr>
<tr>
<td>X: -9.81</td>
<td>wrt-6</td>
<td>X: -10.30</td>
<td>MseI</td>
<td>270, 215, 111, 74</td>
<td>381, 215, 74</td>
<td></td>
<td>pkP6103</td>
</tr>
<tr>
<td>X: ~-8</td>
<td></td>
<td></td>
<td>DraI</td>
<td>452, 78</td>
<td>350, 95, 78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: -1.89</td>
<td>unc-6</td>
<td>X: -2.01</td>
<td>BglII</td>
<td>363</td>
<td>277, 86</td>
<td></td>
<td>pkP6155</td>
</tr>
<tr>
<td>X: 1.45</td>
<td>dyf-8</td>
<td>X: 1.45</td>
<td>SspI</td>
<td>297, 224</td>
<td></td>
<td></td>
<td>pkP6127</td>
</tr>
<tr>
<td>X: 1.92</td>
<td>dyf-6, dyf-12</td>
<td>X: 1.88, 2</td>
<td>DraI</td>
<td>620</td>
<td>299, 318</td>
<td></td>
<td>CE6-173</td>
</tr>
<tr>
<td>X: ~2</td>
<td></td>
<td></td>
<td>DraI</td>
<td>406, 136</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: ~12</td>
<td></td>
<td></td>
<td>DraI</td>
<td>320, 191, 34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X: 22.08</td>
<td>daf-6</td>
<td>X: 21.49</td>
<td>HinfI</td>
<td>287, 255</td>
<td></td>
<td></td>
<td>pkP6167</td>
</tr>
</tbody>
</table>

**Table 3. Extended bulked segregant analysis.**

The above table contains the SNP markers, from chromosomes I and X, used for chromosome linkage mapping plus additional SNP markers that are closely linked to known genes described as having amphid channel ciliary defects when mutated (Ou et al., 2007). The purpose of this extended BSA protocol is to filter out novel alleles of already characterized genes, generated through mutagenesis and a subsequent phenotypic screen, by linking the causal gene to a SNP marker that is also linked to a known gene (in this case, one that causes a Dyf phenotype when rendered nonfunctional). This mapping strategy is most useful when the phenotype being screened for has been linked to many different cloned genes.
Table 4. Distribution of genes causing ciliary dye-filling defects.

Ou et al. 2007 isolated 126 independent strains defective in chemotaxis towards NaAc from 150,000 mutagenized haploid genomes. 47 of these were Dyf and 42 of the 47 were mapped to a linkage group using SNPs. These 42 novel alleles are assigned to their appropriate chromosome in the above table and labeled “Novel.” Ou et al. 2007 also compiled a list of known genes that cause Dyf phenotypes, which are labeled “Known.” The Dyf alleles isolated from our screen are labeled “Ours.” The percentages are similar between the two screens and the known list with regard to chromosomal distribution with the highest percentage of known genes and isolated alleles being linked to chromosomes X and I. This is the reason that these chromosomes were targeted for extended BSA.
Figure 11. *og007* is linked to the X chromosome and shows the highest linkage at a genetic distance of -1.89 cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the *og007* allele to the X chromosome. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing *og007* as being most strongly linked to the SNP marker at -1.89 cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is strongly linked to the *unc-6* locus, which displays a Dyf phenotype. Before further SNP mapping is performed, the *unc-6* open-reading frame could be sequenced in the *og007* genetic background to rule out *og007* as being a novel allele of *unc-6*. 
Figure 12. *og008* is linked to the X chromosome and shows the highest linkage at a genetic distance of ~ -8 cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the *og008* allele to the X chromosome. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing *og008* as being most strongly linked to the SNP marker at ~ -8 cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is linked to the *wrt-6* locus, which displays a Dyf phenotype. Before further SNP mapping is performed, the *wrt-6* open-reading frame could be sequenced in the *og008* genetic background to rule out *og008* as being a novel allele of *wrt-6*. 
Figure 13. \textit{og018} is linked to chromosome I and shows the highest linkage at a genetic distance of \(\sim 12\) cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the \textit{og018} allele to chromosome I. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing \textit{og018} as being most strongly linked to the SNP marker at \(\sim 12\) cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is linked to the \textit{unc-101} locus, which displays a Dyf phenotype. Before further SNP mapping is performed, the \textit{unc-101} open-reading frame could be sequenced in the \textit{og018} genetic background to rule out \textit{og018} as being a novel allele of \textit{unc-101}. 
Figure 14. \textit{og019} is linked to chromosome IV and shows the highest linkage at a genetic distance of \textasciitilde -4 cM.

BSA SNP mapping (gel shown above), using the SNP markers in Table 2, linked the \textit{og019} allele to chromosome IV. Of the four SNP markers used in this mapping, \textit{og019} was most strongly linked to the SNP marker at \textasciitilde -4 cM (linkage value for each SNP shown in graph). Interval mapping using a higher density of SNP markers on chromosome IV would be a logical next step in assigning the \textit{og019} mutation to a specific open-reading frame.
Figure 15. \textit{og020} is linked to chromosome I and shows the highest linkage at a genetic distance of \(\sim 12\) cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the \textit{og020} allele to chromosome I. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing \textit{og020} as being most strongly linked to the SNP marker at \(\sim 12\) cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is linked to the \textit{unc-101} locus, which displays a Dyf phenotype. Before further SNP mapping is performed, the \textit{unc-101} open-reading frame could be sequenced in the \textit{og020} genetic background to rule out \textit{og020} as being a novel allele of \textit{unc-101}.
Figure 16. *og021* is linked to chromosome I and shows the highest linkage at a genetic distance of ~ -1 cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the *og021* allele to chromosome 1. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing *og021* as being most strongly linked to the SNP marker at ~ -1 cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is strongly linked to the *dyf-1* locus, which displays a Dyf phenotype. Before further SNP mapping is performed, the *dyf-1* open-reading frame could be sequenced in the *og021* genetic background to rule out *og021* being a novel allele of *dyf-1*. 
**Figure 17.** *og022* is linked to chromosome IV and has been identified as a novel allele of *dyf-3*.

BSA SNP mapping (gel shown above), using the SNP markers in Table 2, linked the *og022* allele to chromosome IV. Once chromosome linkage was established, interval mapping using a higher density of SNP markers on chromosome IV lead to the identification of *og022* as a novel allele of *dyf-3*. *dyf-3* is located at ~-4.42 cM on chromosome IV and the initial BSA mapping showed *og022* to be more highly linked to the SNP at ~-4 cM than the other three SNP markers used (linkage value for each SNP shown in graph). Linkage to the other three SNP markers weakened appropriately when considering the genetic distance these markers are from the location of the mutation. This served as a proof of principle experiment when considering the extended BSA protocol.
Figure 18. *og023* is linked to the X chromosome and shows the highest linkage at a genetic distance of ~ 2 cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the *og023* allele to the X chromosome. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing *og023* as being most strongly linked to the SNP marker at ~ 2 cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is strongly linked to the *dyf-12* and *dyf-6* loci, which both display Dyf phenotypes. Before further SNP mapping is performed, the *dyf-12* and *dyf-6* open-reading frames could be sequenced in the *og023* genetic background to rule out *og023* as being a novel allele of either *dyf-12* or *dyf-6*. 
Figure 19. \textit{og025} is linked to the X chromosome and shows the highest linkage at a genetic distance of \textasciitilde -8 cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the \textit{og025} allele to the X chromosome. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing \textit{og025} as being most strongly linked to the SNP marker at \textasciitilde -8 cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is strongly linked to the \textit{wrt-6} locus, which displays a Dyf phenotype. Before further SNP mapping is performed, the \textit{wrt-6} open-reading frame could be sequenced in the \textit{og025} genetic background to rule out \textit{og025} as being a novel allele of \textit{wrt-6}.
Figure 20. *og026* is linked to chromosome V and shows the highest linkage at a genetic distance of ~ -7 cM.

BSA SNP mapping (gel shown above), using the SNP markers in Table 2, linked the *og026* allele to chromosome V. Of the four SNP markers used in this mapping, *og026* was most strongly linked to the SNP marker at ~ -7 cM (linkage value for each SNP shown in graph). Interval mapping using a higher density of SNP markers on chromosome V would be a logical next step in assigning the *og026* mutation to a specific open-reading frame.
Figure 21. *og027* is linked to the X chromosome and shows the highest linkage at a genetic distance of ~ -8 cM.

BSA SNP mapping, using the SNP markers in Table 2, linked the *og027* allele to the X chromosome. Once chromosomal linkage was established, an extended BSA was performed (gel shown above) showing *og027* as being most strongly linked to the SNP marker at ~ -8 cM, when compared to the other markers used (linkage value for each SNP shown in graph). This particular SNP is strongly linked to the *wrt-6* locus, which displays a Dyf phenotype. Before further SNP mapping is performed, the *wrt-6* open-reading frame could be sequenced in the *og027* genetic background to rule out *og027* as being a novel allele of *wrt-6.*
<table>
<thead>
<tr>
<th>Strain #</th>
<th>Allele #</th>
<th>Genetic Location</th>
<th>Closest Known Dyf Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>WX729</td>
<td>og007</td>
<td>Linked to the X chromosome close to -1.89 cM</td>
<td>unc-6</td>
</tr>
<tr>
<td>WX730</td>
<td>og008</td>
<td>Linked to the X chromosome close to -8 cM</td>
<td>wrt-6</td>
</tr>
<tr>
<td>WX733</td>
<td>og018</td>
<td>Linked to chromosome I close to 12 cM</td>
<td>unc-101</td>
</tr>
<tr>
<td>WX734</td>
<td>og019</td>
<td>Linked to chromosome IV close to -4 cM</td>
<td>N/A</td>
</tr>
<tr>
<td>WX735</td>
<td>og020</td>
<td>Linked to chromosome I close to 12 cM</td>
<td>unc-101</td>
</tr>
<tr>
<td>WX736</td>
<td>og021</td>
<td>Linked to chromosome I close to -1 cM</td>
<td>dyf-1</td>
</tr>
<tr>
<td>WX737</td>
<td>og022</td>
<td>Linked to chromosome IV and identified as a novel allele of dyf-3</td>
<td>N/A</td>
</tr>
<tr>
<td>WX738</td>
<td>og023</td>
<td>Linked to the X chromosome close to 2 cM</td>
<td>dyf-12 and dyf-6</td>
</tr>
<tr>
<td>WX740</td>
<td>og025</td>
<td>Linked to the X chromosome close to -8 cM</td>
<td>wrt-6</td>
</tr>
<tr>
<td>WX741</td>
<td>og026</td>
<td>Linked to chromosome V close to -7 cM</td>
<td>N/A</td>
</tr>
<tr>
<td>WX742</td>
<td>og027</td>
<td>Linked to the X chromosome close to -8 cM</td>
<td>wrt-6</td>
</tr>
</tbody>
</table>

**Table 5. Genetic locations of novel Dyf alleles.**

Novel Dyf alleles were mapped to a linkage group using bulked segregant analysis and extended bulked segregant analysis. Only alleles linked to chromosome I or X through BSA were analyzed by extended BSA. The purpose of extended BSA is to filter out novel alleles of already characterized genes in order to prevent time-consuming interval mapping. “Known” Dyf genes refer to characterized genes that have been shown to cause a Dyf phenotype when mutated. “Known” Dyf genes are listed that are highly linked to each of the novel Dyf alleles. Sequencing the ORFs of each “known” gene in the genetic background of the novel Dyf mutation for which the gene is linked, will be necessary to determine if the novel mutation is an allele of that “known” gene.
Figure 22. Iso-amyl alcohol chemotaxis assay.

Dyf strains were assayed to assess their response to the volatile attractant iso-amyl alcohol. (A) When compared to N2 controls, ten of eleven novel Dyf strains, plus the known chemotaxis mutant *odr-1*, display statistically significant decreases in chemotaxis toward this attractant. Mean chemotaxis indexes, S.E.M.s, and p-values are reported. * denotes significant difference from N2. Each Dyf strain, except for *og019*, was shown to have a statistically significant defect in its response to the chemical attractant iso-amyl alcohol. Number of worms assayed per plate [number of worms that were able to travel outside of the start circle (area D)] ranged from 17 to 420 with an average of approximately 133. (B) Graphical representation of the data with error bars that illustrate +/- S.E.M. Excel was used to calculate means, standard deviations, S.E.M.s, and two-tailed Student’s t tests.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean±SEM</th>
<th>p-value (α=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>0.70±0.057</td>
<td></td>
</tr>
<tr>
<td><em>odr-1</em></td>
<td>-0.01±0.069</td>
<td>*0.0014</td>
</tr>
<tr>
<td><em>og007</em></td>
<td>0.07±0.055</td>
<td>*0.0014</td>
</tr>
<tr>
<td><em>og008</em></td>
<td>0.07±0.044</td>
<td>*0.0010</td>
</tr>
<tr>
<td><em>og018</em></td>
<td>0.22±0.092</td>
<td>*0.0121</td>
</tr>
<tr>
<td><em>og019</em></td>
<td>0.68±0.186</td>
<td>0.9416</td>
</tr>
<tr>
<td><em>og020</em></td>
<td>-0.05±0.024</td>
<td>*0.0003</td>
</tr>
<tr>
<td><em>og021</em></td>
<td>0.28±0.014</td>
<td>*0.0020</td>
</tr>
<tr>
<td><em>og022</em></td>
<td>0.13±0.019</td>
<td>*0.0007</td>
</tr>
<tr>
<td><em>og023</em></td>
<td>0.09±0.044</td>
<td>*0.0011</td>
</tr>
<tr>
<td><em>og025</em></td>
<td>0.08±0.035</td>
<td>*0.0008</td>
</tr>
<tr>
<td><em>og026</em></td>
<td>0.10±0.046</td>
<td>*0.0013</td>
</tr>
<tr>
<td><em>og027</em></td>
<td>0.16±0.041</td>
<td>*0.0016</td>
</tr>
</tbody>
</table>
**Figure 23. Adult lifespans of novel Dyf strains.**

(A) When compared to N2 controls, novel Dyf strains display significant differences in longevity. *og008, og018, og022, og023, og026, and og027, along with the known longevity mutant age-1, all are significantly longer lived than wild type N2. *og007 and og025 possess significantly shorter lifespans when compared to N2 controls. *og019, og020, and og021, along with the characterized wild type isolate CB4856, show no significant differences in lifespan when compared to N2 controls. * denotes significant difference from N2. (B) Survival curves of Dyf strains are shown. Percentage of animals remaining alive is plotted against the age of the animal in days. Total number of worms per assay per strain ranged from 23-107. Excel was used to calculate means, standard deviations, S.E.M.s, two-tailed Student’s t test, and 75th percentiles.

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Mean±SEM</th>
<th>75th Percentile</th>
<th>p-value (α=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>60</td>
<td>17.8±0.3</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CB4856</td>
<td>77</td>
<td>17.5±0.6</td>
<td>19</td>
<td>0.5</td>
</tr>
<tr>
<td>age-1</td>
<td>40</td>
<td>20.5±0.7</td>
<td>24.3</td>
<td>*0.0003</td>
</tr>
<tr>
<td>og007</td>
<td>29</td>
<td>12.4±0.6</td>
<td>14</td>
<td>*&lt;0.0001</td>
</tr>
<tr>
<td>og008</td>
<td>53</td>
<td>21.9±0.9</td>
<td>27</td>
<td>*&lt;0.0001</td>
</tr>
<tr>
<td>og018</td>
<td>51</td>
<td>21.0±0.9</td>
<td>26</td>
<td>*0.0003</td>
</tr>
<tr>
<td>og019</td>
<td>23</td>
<td>19.0±0.7</td>
<td>20.5</td>
<td>0.103</td>
</tr>
<tr>
<td>og020</td>
<td>52</td>
<td>16.9±0.2</td>
<td>18</td>
<td>0.038</td>
</tr>
<tr>
<td>og021</td>
<td>36</td>
<td>17.1±0.3</td>
<td>18</td>
<td>0.16</td>
</tr>
<tr>
<td>og022</td>
<td>81</td>
<td>22.2±0.7</td>
<td>27</td>
<td>*&lt;0.0001</td>
</tr>
<tr>
<td>og023</td>
<td>57</td>
<td>20.4±0.5</td>
<td>23</td>
<td>*&lt;0.0001</td>
</tr>
<tr>
<td>og025</td>
<td>74</td>
<td>16.1±0.4</td>
<td>19</td>
<td>*0.0022</td>
</tr>
<tr>
<td>og026</td>
<td>49</td>
<td>22.4±0.5</td>
<td>25</td>
<td>*&lt;0.0001</td>
</tr>
<tr>
<td>og027</td>
<td>107</td>
<td>25.4±0.4</td>
<td>28</td>
<td>*&lt;0.0001</td>
</tr>
</tbody>
</table>
Figure 24. Pharyngeal pumping assay.

Dyf strains were subjected to a pharyngeal pumping assay. Results showing defects in pharyngeal pumping could suggest that worms are undergoing caloric restriction, which is known to extend lifespan. (A) og007, og023, and og025, along with the characterized pharyngeal pumping mutant eat-1, display a statistically significant difference in pharyngeal pumping rate when compared to N2 (p-values = 0.006, 0.029, 0.004, and <0.0001 respectively). All other novel Dyf strains show no significant difference in pumping rate when compared to N2. Means, S.E.M.s, and p-values are reported. * denotes significant difference from N2. (B) Graphical representation of the data. Error bars illustrate +/- S.E.M. Pumps per minute in the terminal bulb of the pharynx were counted for five individual worms per strain. Excel was used to calculate means, standard deviations, S.E.M.s, and two-tailed Student’s t tests.
Chapter 5: Isolation and characterization of a novel dyf-3 allele

Introduction

DYF-3 is necessary for proper cilia morphogenesis in Caenorhabditis elegans

I mapped one of the mutant strains isolated from our F2 screen and found it to be a novel allele of dyf-3. The previously isolated alleles of dyf-3, m185 and mn331, have been shown to have defects in the uptake of fluorescent lipophilic dye into their ciliated sensory neurons (Murayama et al., 2005). Both alleles have mutations that lead to early translational stop codons. Murayama’s group used gfp constructs under the control of a promoter specific for certain sensory neurons or pairs of neurons in order to investigate cilium morphology of those neurons in a dyf-3(m185) background compared to a wild type background (2005). The amphid sensory neurons ADF, ASE, and AWB were found to have abnormal ciliated endings in the dyf-3(m185) mutant. In addition, empty amphid sockets were found in the mutant by analyzing a cross-section of the head using transmission electron microscopy (Murayama et al., 2005).

Through reverse transcriptase PCR of dyf-3 cDNA, it has been determined that dyf-3 encodes three proteins with different N-termini. Each protein contains two predicted coiled-coil motifs. Coiled-coil motifs often function in protein oligomerization and are found in many types of proteins. The largest form of DYF-3 is 38% identical to a cDNA clone conceptual translation product named KIAA0643, which has been isolated from human brain (Murayama et al., 2005). This similarity is based on amino acid sequence. A zebrafish screen identified 12
genes that when mutated can cause cysts in the glomerular-tubular region of the zebrafish larval pronephros (primitive kidney) (Sun et al., 2004). Two of these genes are known to be associated with human polycystic kidney disease (PKD), which has been linked to defects in primary cilia. Wormbase identified another of the 12 genes, *qilin/cluap-1* (clusterin-associated protein-1), as coding for a protein product that is 93.3% similar to the wild type DYF-3 protein product. The precise function of this zebrafish protein is unknown, but is thought to be involved in cilia development or in connecting cilia signals to downstream events (Sun et al., 2004). A *dyf-3::gfp* construct was used to determine where *dyf-3* functions. GFP expression was detected in eight pairs of amphid neurons, six inner labial neurons, and two pairs of phasmid neurons, that all are thought to function in chemosensation because their sensory cilia are exposed to the external environment (Murayama et al., 2005).

Wild type *dyf-3* cDNA, under the control of a *daf-7* promoter, was expressed solely in ASI neurons of *dyf-3* animals. *dyf-3* animals fail to take up the lipophilic dye, DiI, into any of their sensory neurons. However, *dyf-3* worms carrying the *daf-7p::dyf-3* construct display normal dye-filling in ASI neurons only, suggesting that *dyf-3* functions cell-autonomously with regard to dye uptake (Murayama et al., 2005). All known IFT-B subcomplex genes in *C. elegans* contain an X box and are regulated by the RFX-type transcription factor DAF-19 (Swoboda et al., 2000). *dyf-3* has an IFT transport phenotype and cilia truncation consistent with it being associated with the OSM-3-kinesin/IFT-B subcomplex (Ou et al., 2007).
Interestingly, there is a link between my work on *bbs-7/osm-12* and *dyf-3*. A protein-protein interaction between BBS-7 and DYF-3 was found by the *C. elegans* interactome project suggesting that DYF-3 may link the BBS protein module to the IFT-B subcomplex (Li et al., 2004; Ou et al., 2007). *dyf-3* contains an X box transcription factor binding site 75 bp-88 bp upstream from the start codon of the largest *dyf-3* open reading frame (ORF) (Murayama et al., 2005). Expression levels of a *dyf-3::gfp* construct in a *daf-19(m86)* animal are greatly reduced when compared to wild type. Based on these findings, DYF-3 is associated with the IFT-B subcomplex of the IFT system and is regulated by the RFX-type transcription factor DAF-19 (Murayama et al., 2005; Ou et al., 2007).

**RNA Splicing**

I isolated a novel allele of *dyf-3*, *(og022)*, from a mutagenesis and subsequent F2 screen for animals that failed to take up a lipophilic dye into a subset of their ciliated sensory neurons. I determined that our Dyf strain carried an allele of *dyf-3* by SNP mapping and sequencing the open reading frame (ORF) C04C3.5. I discovered a G to A transition mutation in the first base pair of the third intron of the largest *dyf-3* ORF. This lesion fell directly on a conserved nucleotide in the splice donor of the third intron (Karp, 2002).

Transcription of a gene produces a primary transcript that includes sequences that correspond to introns. These sequences must be removed by RNA splicing. The splicing machinery recognizes highly conserved nucleotide sequences located at the boundaries between exons and introns. The 5’ splice site
is located at the 5’ end of every intron and has the conserved sequence G/GU, where the slash denotes the exon/intron boundary. The 3’ splice site, located at the 3’ end of every intron, has the conserved sequence AG/G, where AG are the last two base pairs of the intron. These splice site sequences are located in the majority of eukaryotic pre-mRNAs. The conserved site at the beginning of each intron is called the splice donor and the site at the end is referred to as the splice acceptor (Karp, 2002).

The splicing process begins when each pre-mRNA intron becomes associated with a spliceosome. Spliceosomes consist of proteins and ribonucleoprotein particles called snRNPs (sn = “small nuclear”), which are composed of snRNAs bound to specific proteins. Spliceosomes are assembled once their constituent snRNPs bind to the pre-mRNA. The U1 snRNP is the first splicing component to contact the pre-mRNA. The sequence of its snRNA is complementary to the 5’ splice site of an intron, allowing it to attach to the pre-mRNA at this location. Once it has attached, the U2 snRNP can bind to a conserved sequence within the intron that becomes a branching point. This binding causes a specific adenosine residue within the branch site to bulge out of the helix. Next, the U5/U4/U6 trimer binds. RNA rearrangements occur within the snRNPs, breaking apart the U4/U6 base pairs and releasing U4 and allowing U6 to displace U1. Next, the conserved adenosine residue located at the branch point attacks the 5’ splice site of the intron, cutting the RNA backbone and forming a lariat structure. The spliceosome rearranges to bring together the flanking exons, allowing the 3’ hydroxyl group of the first exon to react with the
5’ end of the second exon. Once the exons have joined, the lariat and the U2/U5/U6 snRNP complex are released (Alberts et al., 2004; Karp, 2002).

Results

Mapping and identification of the og022 allele

og022 was isolated in a mutagenesis and subsequent screen for animals that fail to uptake DiI into a subset of their ciliated sensory neurons. In addition to being dye-filling defective, the strain containing the og022 allele has been shown to be chemotaxis defective with regard to iso-amyl alcohol (Figure 22), has a statistically significant increase in lifespan (Figure 23), and displays wild type rates of pharyngeal pumping (Figure 24). Of the 11 strains isolated from our Dyf screen, og022 was chosen to be interval mapped to narrow down its critical genetic interval.

As shown in Figure 17, og022 is most strongly linked to a snip-SNP marker located at approximately -4 cM on chromosome IV. This was found through chromosomal mapping using snip-SNP markers evenly distributed throughout the C. elegans genome (Wicks et al., 2001). Once I determined that og022 was linked to chromosome IV, I conducted interval mapping using a higher density of SNP markers between ~ -15 cM and ~ 1 cM on chromosome IV (Table 6). Even though BSA mapping most strongly linked og022 to ~ -4 cM, I chose a more broad interval for fine mapping to be confident that our causal gene is contained within the interval.
Unlike chromosomal mapping, interval mapping analyzes the genotypes of individual mutant animals, rather than the genotypes of pooled mutant and pooled wild-type bulked lysates, each containing genomes from many animals with different recombination patterns (Davis et al., 2005). In addition, the DNA of the mutants analyzed by interval mapping is assayed for many SNPs within the interval for which linkage has been determined (Davis et al., 2005). I used 11 SNP markers between ~ -15 cM and ~ 1 cM on chromosome IV to determine recombination patterns in approximately 288 phenotypically mutant recombinant progeny (derived from the F2 generation of a cross between the N2-derived og022 mutant strain and CB4856) to narrow down og022 to a region small enough to sequence candidate genes (Table 6). Analyzing the recombinant strains using this higher density of SNP markers narrowed the og022 genetic position to a 0.39 cM interval that contains 14 candidate genes (Figure 25). Among these 14 genes were characterized genes and bioinformatically predicted ORFs partially confirmed by cDNAs. The cloned and characterized gene dyf-3 was located in this region and was the most likely candidate to be the location of the og022 allele. The predicted ORF, C04C3.5, has been shown to be dyf-3 (Murayama et al., 2005). Additional data published on dyf-3 is summarized in the introduction to Chapter 5. C04C3.5 was sequenced in an og022 genetic background, which revealed a G to A transition mutation in the conserved splice donor site at the first base pair of the third intron (Figure 26). I had determined that og022 was in fact a novel allele of dyf-3. Figure 27A shows a schematic of the exon/intron structure of dyf-3 and points out the location of the og022 mutation.
Genetic characterization of the *dyf-3(og022)* allele

At this point, I hypothesized that the *og022* mutation caused intron 3 of C04C3.5b to be left in the *og022* transcript. To test my hypothesis I isolated total RNA from N2 and *dyf-3(og022)* animals and then amplified cDNA from each RNA sample using reverse transcriptase PCR with primers designed to amplify different regions of the C04C3.5b transcript (three different ORFs have been predicted for C04C3.5, of which only the b variant has been partially confirmed by cDNA with our results and by Marc Vidal’s ORFeome project). Results of our reverse transcriptase PCR are shown in Figure 29 and the location of the designed primer pairs are indicated in Figure 27A. The blue primers in Figure 27A were designed in the first and third exons (both before the *og022* mutation) to amplify a 169 bp cDNA amplicon from both N2 and *og022* RNA samples. The forward green primer is identical to the forward blue primer; however, the reverse green primer is located in the fifth exon. This primer pair was expected to amplify a 730 bp cDNA amplicon from the N2 RNA and a 786 bp amplicon from the *og022* RNA if the third intron was left in the *og022* transcript, and was (Figure 29). The forward red primer is also identical to the forward blue primer, but the reverse red primer is located in intron 3. This primer pair was expected to amplify a 424 bp cDNA amplicon from the *dyf-3(og022)* RNA and no product from the N2 RNA. Two controls were performed (positive and negative) and are described in the Figure 29 legend. Results of the reverse transcriptase PCR show that intron 3 of C04C3.5b is not spliced out after transcription of the *og022* allele due to a G to A
transition mutation in the conserved splice donor site at the first base pair of the third intron of \textit{og022} (Figure 29).

To acquire further evidence of the splicing defect in the \textit{og022} transcript, N2 and \textit{og022} cDNA amplicons (from the “green primer” lanes in Figure 29) were excised and isolated from the agarose gel before being sequenced using primers (Dyf-3.startFor2 and Dyf-3.N2Rev1; see Materials and Methods) located in exonic regions that flanked the third intron. The aligned cDNA sequences displayed in Figure 28 partially reflect the results of our sequencing reactions (our cDNA sequencing reactions did not confirm the presence of the last four exons of C04C3.5b; however, sequencing the complete C04C3.5 ORF from \textit{og022} genomic DNA revealed no genetic lesions in this region, suggesting that these exons would be intact in the \textit{og022} transcript). These data constitute further evidence that the \textit{og022} mutation led to the third intron of C04C3.5b failing to be spliced from the \textit{dyf-3(og022)} transcript, which is consistent with the amplicon sizes amplified by the “green primers” in Figure 29 and partially confirms the C04C3.5b predicted ORF. The non-excision of intron 3 introduces an early opal stop codon into the altered transcript, 34 base pairs from the beginning of intron 3. 67% of the \textit{dyf-3(og022)} ORF is not translated into protein due to the \textit{og022} mutation. Therefore, the two coiled-coil motifs predicted by Murayama et al. 2005 are absent from the mutant protein (Figure 27B).

To further establish that the mutation in C04C3.5b in the \textit{og022} genetic background is the sole genetic lesion in the \textit{og022} strain leading to a dye-filling defective phenotype, we amplified the C04C3.5b ORF, including 798 bp of 5’
UTR and 685 bp of 3’ UTR, from the genomic clone C04C3 and injected it along with the dominant marker plasmid pRF4 into og022 mutant animals. The plasmid pRF4 contains the dominant rol-6 mutant gene that when successfully injected into an animal and incorporated into the genome of the progeny of the animal, through means of an extrachromosomal array or physical integration into the genome, causes the progeny to move in a circle due to cuticle defects. This Rol phenotype is easily distinguishable, and Rol worms are analyzed for dye-filling defects, because co-injecting the pRF4 plasmid and the wild type C04C3.5b rescue construct will most always lead to both or neither being incorporated into the genome of the progeny, to assess rescue. Once transgenic animals were obtained through microinjection of these DNA samples, they were subjected to a dye-filling assay. Of 100 transgenic animals observed after being dye-filled (transgenic animals were detected by the presence of the Rol phenotype), 97 were dye-filling wild type in both amphid and phasmid channels. This was compared to a dye-filling assay performed on og022 mutants, where out of 100 animals observed only 4 appeared to be dye-filling wild type in amphid channels, and of these 4 only 1 showed dye-filling in phasmid channels. Since wild type C04C3.5b can rescue the Dyf phenotype of og022, we can conclude that the mutation in C04C3.5b in the og022 strain is responsible for the dye-filling defective phenotype observed in og022 animals (Figure 30). Since dyf-3 is C04C3.5, og022 is a novel allele of dyf-3.

The X-box RFX-type transcription factor binding site located 75-88 bp upstream from the start codon of dyf-3 (Murayama et al., 2005) has been
confirmed by sequencing the proximal promoter of C04C3.5b within a C04C3 genomic clone.

Discussion

Interval mapping of the og022 allele narrowed its genetic location to a 0.39 cM interval on chromosome IV, which contains 14 candidate genes. Of these 14 genes, dyf-3 was sequenced, in the og022 genetic background, first, primarily because it was the only gene in this interval that had been previously characterized as being involved in ciliogenesis (Murayama et al., 2005). dyf-3 animals had also been shown to be defective in the uptake of DiI into a subset of their ciliated neurons (Murayama et al., 2005).

og022 was found to contain a G to A transition mutation in a conserved splice donor site at the first base pair of intron 3 of the C04C3.5b ORF (dyf-3). Hence, og022 was shown to be a novel allele of dyf-3. The location of the mutation suggested a transcriptional splicing defect might impede functioning of the DYF-3 protein. Reverse transcriptase PCR and og022 cDNA sequencing confirmed that the third intron of C04C3.5b had been retained in the og022 transcript. This splice donor mutation is thought to cause the U1 snRNP spliceosome component to be unable to bind properly to the transcript. This could lead to a failure to form a fully functional spliceosome complex and therefore cause the third intron to remain in the transcript. Since all the other splice sites are intact in the og022 allele, the spliceosome components are thought to bind properly at the fourth intron, as well as all subsequent introns, so that these introns
are spliced out of the transcript. The addition of intron 3 to the mature transcript introduces an early opal stop codon located 34 bp downstream of the beginning of the intron. As a result, only 33% of the mutant mRNA transcript will be translated into protein. This mutant protein contains neither coiled-coil motif predicted by Murayama et al. 2005 and is therefore thought to be nonfunctional.

The multitude of PCR reactions, restriction digests, and gel electrophoresis required to analyze almost 300 separate recombinant DNA samples in interval mapping can be quite time consuming. Our BSA chromosome mapping results showed og022 to be most strongly linked to a SNP marker at ~ -4 cM on chromosome IV. It turns out that this SNP marker is very strongly linked to the dyf-3 gene (IV: -4.42 cM), which was identified as the gene mutated in og022 (through extensive interval mapping). If extended BSA mapping were performed before resorting to interval mapping, then og022 would have been identified as a novel allele of dyf-3 much earlier than it was.

Since dyf-3(og022) animals are chemotaxis-defective toward iso-amyl alcohol, are dye-filling defective, have an extended lifespan, and show no signs of caloric restriction (i.e., pharyngeal pumping rate is normal), it is possible that the extended lifespan is due to a defect in sensing one or more sensory cues that govern insulin-like peptide (ILP) synthesis and release. This would cause a downregulation of the insulin/IGF-1 signaling pathway, which normally leads to DAF-16 relocation to the nucleus where it can turn on survival genes. In order to further support this hypothesis, the nutrients, repellents, and/or attractants thought to be responsible for triggering ILP synthesis and release would have to be
identified along with receptors that bind these cues, and it would then have to be
determined whether \textit{dyf-3(og022)} animals can sense these compounds.

I confirmed the existence of an X-box transcription factor binding site 75-
88 bp upstream of the start codon of C04C3.5b. The sequence of this X-box (5’-
GTTTCT AT GGGAAC-3’) is consistent with 13 of 14 bases of the consensus X-
box sequence described in Figure 9 (Murayama et al., 2005). The presence of an
X-box sequence in the proximal promotor of \textit{dyf-3} would suggest that \textit{dyf-3} is
regulated by the RFX-type transcription factor DAF-19. Murayama et al. 2005
have shown this to be the case (see Chapter 5 introduction).

Wormbase identified homology between the zebrafish \textit{qilin/cluap-1}
(clusterin-associated protein-1) gene and \textit{C. elegans dyf-3}. The protein product
translated from the \textit{qilin/cluap-1} gene is 93.3\% similar to the wild type DYF-3
protein product. The function of this zebrafish protein is unclear, but is thought to
be involved in ciliation development because when this gene is mutated kidney
cysts (reminiscent of PKD) form in the zebrafish larvae (Sun et al., 2004). The
homology between \textit{qilin/cluap-1} and \textit{dyf-3} constitutes additional evidence that the
\textit{qilin/cluap-1} gene is involved in ciliation development.
<table>
<thead>
<tr>
<th>Position</th>
<th>Cosmid/ YAC</th>
<th>Enzyme</th>
<th>N2 Digest</th>
<th>CB4856 Digest</th>
<th>Primer Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV:~15cM</td>
<td>Y41D4B</td>
<td>Dral</td>
<td>378bp, 155bp</td>
<td>533bp</td>
<td>CACACACAAATACCTCCCAATACC CATTTAGAAATTACGTAGCTTTCG</td>
</tr>
<tr>
<td>IV:~7cM</td>
<td>Y54G2A</td>
<td>Dral</td>
<td>498bp</td>
<td>250bp, 248bp</td>
<td>ACTCCGGATCCTCACG CATTTAGAGAATTACTGTAGCTTTCG</td>
</tr>
<tr>
<td>IV:~4.56cM</td>
<td>F42A6</td>
<td>Dral</td>
<td>296bp, 122bp</td>
<td>418bp</td>
<td>TTATATCGGAGGAGGTTAGAGG</td>
</tr>
<tr>
<td>IV:~4.17cM</td>
<td>C04C3</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>AGACCAACGTATACGTACGTAGC Amp Prim Amp Prim ATCCCCAGCTGTTGGATATAGC</td>
</tr>
<tr>
<td>IV:~3.76cM</td>
<td>C09G12</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Amp Prim Amp Prim ATCCCAGTTGATACGTACGTAGC Seq Prim Seq Prim</td>
</tr>
<tr>
<td>IV:~3.64cM</td>
<td>C09G12</td>
<td>Rsal</td>
<td>164bp, 131bp, 50bp</td>
<td>295bp, 30bp</td>
<td>ATCCAGCTCAAAGTGTGCG TGCTACCCGATACCTGACC</td>
</tr>
<tr>
<td>IV:~3.44cM</td>
<td>M57</td>
<td>NlaIII</td>
<td>398bp, 167bp, 19bp</td>
<td>295bp, 167bp, 103bp, 19bp</td>
<td>TCTCCCTAATACCCATGCG TGGGATTTGCAATATAGG</td>
</tr>
<tr>
<td>IV:~3.33cM</td>
<td>B0212</td>
<td>AflIII</td>
<td>517bp</td>
<td>284bp, 233bp</td>
<td>CGTCATCACCCAAGTCTTC Seq Prim Seq Prim</td>
</tr>
<tr>
<td>IV:~2.97cM</td>
<td>Y37E11AL</td>
<td>PstI</td>
<td>427bp</td>
<td>213bp, 209bp</td>
<td>TCCGAGTGTGCGAGGAGG</td>
</tr>
<tr>
<td>IV:~2.23cM</td>
<td>M02B7</td>
<td>NruI</td>
<td>302bp, 226bp</td>
<td>528bp</td>
<td>CTGAGGATGCAAGAGAG</td>
</tr>
<tr>
<td>IV:~1cM</td>
<td>E03H12</td>
<td>Dral</td>
<td>375bp</td>
<td>297bp, 78bp</td>
<td>AAATGGAAGACTACCCAAA TGCTTGTAGCGTCTCCAG</td>
</tr>
</tbody>
</table>

Table 6. SNP markers used in interval mapping *og022*.

BSA chromosomal mapping linked *og022* to a broad interval on chromosome IV. Interval mapping using a higher density of snip-SNPs and sequencing SNPs within this interval on chromosome IV narrowed the *og022* critical interval down to 0.39 cM. This region contained 14 gene candidates of which, *dyf-3* was the most likely candidate and was sequenced in the *og022* genetic background. *og022* was found to be a novel allele of *dyf-3*. 
Figure 25. Narrowing down the genetic interval of og022 through interval mapping using single nucleotide polymorphisms.

Schematic of the og022 interval map showing approximate regions of recombination in 6 recombinant strains derived from a cross between the N2-derived og022 mutant strain and CB4856. Based on the strains phenotype and positional genotype, it is possible to narrow down the critical interval through exclusion of CB4856 alleles. A phenotypic designation of “Het” refers to a recombinant animal that produced both Dyf and WT progeny. The DNA of approximately 288 independent recombinant progeny were genotypically analyzed using a high density of snip-SNP and sequencing SNP markers from a broad interval on chromosome IV to narrow down the critical region of og022 to a 0.39cM distance containing 14 candidate genes. dyf-3 was the most likely candidate gene and was sequenced in the og022 genetic background. og022 was found to be a novel allele of dyf-3. Arrows point in the direction of the og022 allele in relation to a SNP marker.
Figure 26. *dyf-3(og022)* contains a mutation in a conserved splice donor residue.

A G to A transition mutation (relative to the sense strand) in the conserved splice donor site of the third intron leads to a failure to excise this intron during transcription. The above sequence is shown relative to the anti-sense strand and therefore displays the mutation as being C to T.
**Figure 27. dyf-3 gene structure and DYF-3 protein alignment.**

(A) Schematic of the dyf-3 (C04C3.5b) ORF showing the splice donor mutation located at the first base pair of intron 3 in the og022 allele. Color-coded arrows represent reverse transcriptase PCR primers used to amplify cDNA from N2 and dyf-3(og022) RNA. The blue primers are expected to amplify a cDNA product from both RNA templates. The red primers are expected to amplify a cDNA product from the dyf-3(og022) RNA but not the N2 RNA. The green primers are expected to amplify a larger cDNA product from the dyf-3(og022) RNA than from the N2 RNA because of intron 3 being left in the transcript in dyf-3(og022) RNA. (B) Amino acid sequence alignment of the wild type DYF-3 protein with the mutant DYF-3 protein. The addition of intron 3 to the mature transcript introduces an early opal stop codon; therefore, only 33% of the mutant transcript will be translated into protein. This mutant protein contains neither coiled-coil motif predicted by Murayama et al. 2005 and is therefore thought to be nonfunctional. The two predicted coiled-coil motifs are underlined in the wild type DYF-3 amino acid sequence.
Figure 28. Alignment of C04C3.5b cDNAs amplified from N2 RNA and dyf-3(og022) RNA.

The expected sequences of the cDNA amplicons generated from N2 and dyf-3(og022) RNA have been confirmed through sequencing of both products. The “left in” intron 3 is shown in black in the dyf-3(og022) cDNA sequence along with the mutation highlighted in turquoise.
Figure 29. *dyf-3(og022)* reverse transcriptase PCR.

Location of color-coded primer pairs is depicted in Figure 27A. As expected the blue primers amplified a 169 bp cDNA product from both N2 and *dyf-3(og022)* RNA, the red primers amplified a 424 bp cDNA product from *dyf-3(og022)* RNA and no specific product for N2 RNA, and the green primers amplified a 730 bp cDNA product from N2 RNA and a 786 bp cDNA product from *dyf-3(og022)* RNA. The blue primers were used to amplify each template without reverse transcriptase as a negative control. As expected, no specific products were amplified. As a positive control, each template was amplified using primers designed to amplify the protein actin (*act-1*). Actin is found in high concentrations in all eukaryotic cells (except for nematode sperm cells) and therefore is ideal to use as a positive control. As expected, a 132bp cDNA product was amplified off both N2 and *dyf-3(og022)* RNA.
Figure 30. *dyf-3(og022)* rescue.

The wild type C04C3.5b ORF, including 798 bp of 5’ UTR and 685 bp of 3’ UTR, was amplified and injected along with the dominant marker plasmid pRF4 into *og022* mutant animals to provide further evidence that *og022* is a novel allele of *dyf-3*. (A) After being exposed to DiI, transgenic *og022* mutant animals carrying an extrachromosomal array containing multiple copies of the rescue construct and dominant marker, displayed a Rol phenotype and were dye-filling wild type in amphid and phasmid channels. (B) *og022* animals not carrying the array were dye-filling defective in amphid and phasmid channels after exposure to DiI. Both worms display different degrees of intestinal fluorescence due to bacterial autofluorescence or DiI ingestion. Red lines represent the approximate outline of the cuticle of each worm.
Chapter 6: Discussion

Cilia are multifunctional in mammalian systems. They play roles in everything from sensing developmental signaling molecules and ligands to creating flows of cerebrospinal fluid and mucus that function as flow meters and mechanosensors (Marshall 2008). Most sensory cilia assume canonical structures. However, their shapes and structures can vary greatly. For instance, the AWC neurons in C. elegans have fan-like ciliated endings, whereas ADF and ADL are biciliated (Perkins et al., 1986). Since cilia have such a variety of different functions, it is not surprising that many human diseases can be caused by ciliary defects (Marshall 2008).

Symptoms can vary among individual diseases; however, ciliopathies tend to have certain symptoms that overlap. For example, patients with both Bardet-Biedl Syndrome and Oral-Facial-Digital Syndrome, two disorders resulting from defects in genes that code for protein products that localize to ciliary basal bodies, suffer from renal malformations and polydactyly (Marshall 2008). Retinitis pigmentosa as well as Bardet-Biedl patients suffer from retinal degeneration. Another ciliopathy, known as Meckel-Gruber Syndrome, is characterized by brain malformation, cystic kidneys, and polydactyly (Marshall 2008). Additional diseases resulting from ciliary defects include polycystic kidney disease and situs inversus. Cilia are complex organelles that can vary in structure and function in different tissue types. Therefore, defects in separate genes involved in ciliogenesis can lead to distinctly different symptoms. Assay development for
ciliary structure and function in different animal models can lead to a greater understanding of the complexity of cilia (Marshall 2008).

I study ciliary function in the nematode *Caenorhabditis elegans*. A microtubule-dependent motility process known as intraflagellar transport (IFT) is responsible for ciliogenesis and maintenance across many species. We have identified *bbs-7*, a *C. elegans* homologue of a human disease gene that when mutated causes the rare genetic disorder Bardet-Biedl Syndrome (Blacque et al., 2004). *C. elegans bbs-7* mutants display sensory defects due to truncated cilia located at the tip of sensory neuronal dendrites. With the help of Michel Leroux’s group, we showed that the BBS-7 protein is localized to the base of cilia and undergoes intraflagellar transport along the ciliary axoneme (Blacque et al., 2004). In addition, BBS-7 is necessary for the proper localization and motility of the IFT proteins OSM-5 and CHE-11, and to a lesser extent CHE-2. The accumulated data suggest that BBS-7 plays a role in the assembly and/or functioning of the IFT complex. In addition, these findings imply that symptoms of human Bardet-Biedl Syndrome may result from ciliary defects caused by compromised IFT function (Blacque et al., 2004). The *bbs-7* mutant provides a model system for examining the IFT process, BBS, and the link between ciliary defects and clinical symptoms such as obesity, mental retardation, and diabetes (Blacque et al., 2004).

More recent work by Ou et al. 2007 has grouped IFT components into modules based on IFT transport and phenotypic profiles. The BBS protein module, which contains the BBS-7 protein, has been shown to function in
stabilizing IFT particles (Ou et al., 2007). A defect in a single BBS protein will disrupt the assembly of the entire module preventing its incorporation into the IFT complex, which results in dissociation of the two kinesin motors along with their associated IFT subcomplex components (Ou et al., 2007).

I conducted a mutagenesis and phenotypic screen for animals defective in the uptake of DiI into a subset of their ciliated neurons in order to identify new components involved in IFT and ciliogenesis. We genetically mapped and performed a battery of phenotypic assays on 11 Dyf mutants isolated from my screen. I describe an extended BSA mapping methodology that can save time and resources when mapping novel mutant alleles, isolated through a screen in which phenotype has already been linked to many characterized causal genes, by filtering out alleles of already known genes without performing time consuming interval mapping. Most of the eleven isolated mutants are chemotaxis-defective and long-lived, which supports the hypothesis that inability to detect certain sensory cues can lead to prolongevity by possibly downregulating the insulin/IGF-1 signaling pathway (Antebi 2007).

One of the isolated Dyf alleles was determined to be a novel allele of dyf-3, which encodes a protein required for sensory cilia formation (Murayama et al., 2005). The DYF-3 protein is thought to function as an IFT-B subcomplex component based on mutant ciliary phenotype and IFT motility (Murayama et al., 2005; Ou et al., 2005b). Interestingly, the proteins encoded by the two major genes discussed in this manuscript, bbs-7 and dyf-3, have been found to interact by the C. elegans interactome project (Li et al., 2004). Consistent with these
findings, Ou et al. 2007 found that DYF-3::GFP, in a bbs mutant background, is moved uniquely by the OSM-3-kinesin motor along the middle and remaining distal segments of cilia. This finding shows that DYF-3 is associated with the OSM-3-kinesin/IFT-B subcomplex. Yeast two-hybrid and coimmunoprecipitation analysis would be necessary to confirm these interactions (Ou et al., 2007).
References


Troemel ER, Chou JH, Dwyer ND, Colbert HA, Bargmann CI. Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. *Cell.* 1995; 83: 207-218.


