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What Can We Learn About Human Disease from the Nematode C. elegans?

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Abstract

Numerous approaches have been taken in the hunt for human disease genes. The identification of such genes not only provides a great deal of information about the mechanism of disease development, but also provides potential avenues for better diagnosis and treatment. In this chapter, we review the use of the non-mammalian model organism *C. elegans* for the identification of human disease genes. Studies utilizing this relatively simple organism offer a good balance between the ability to recapitulate many aspects of human disease, while still offering an abundance of powerful cell biological, genetic, and genomic tools for disease gene discovery. *C. elegans* and other non-mammalian models have produced, and will continue to produce, key insights into human disease pathogenesis.

Keywords

Caenorhabditis elegans; genetic screens; genomic screens; RNAi; GFP

1. Introduction

The choice of model organism for study is a balance in trade-offs. While humans clearly are best in terms of mimicking human disease, there are practical and ethical limits to investigating disease in people. Other mammals, most notably mice, have proved very useful for modeling and studying human disease, but mice are limited in both how well they recapitulate some diseases and the ability to study them in rapid fashion. With the advent of tools like RNA interference (RNAi) and CRISPR/Cas9 genome editing, as well as more classical biochemical techniques, cell line studies have been very fruitful in identifying signaling pathways, for example, but are limited in that overall organismal physiology is generally not present in cell culture.

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Non-mammalian model organisms such as the fruit fly *Drosophila melanogaster*, the zebrafish *Danio rerio*, and the nematode *Caenorhabditis elegans* serve as a happy medium [1–5], allowing for ease of study while still having the physiology present in a whole animal and the ability to recapitulate at least some aspects of human disease. These and other model organisms have played key roles in human disease gene discovery. In the current review, we focus on the use of *C. elegans* as a non-mammalian model for human disease gene discovery. We first provide a brief introduction to *C. elegans* biology and the history of *C. elegans* research. Then we describe the key genetic and genomic techniques that have made *C. elegans* such a powerful research model. Using this background information, we illustrate two approaches that have been taken to identify human disease genes in *C. elegans*. In the first set of examples, we discuss how *C. elegans* disease models have been used for *de novo* discovery of human disease genes and pathways. In the second set of examples, we show how human disease genes have been engineered into *C. elegans* to develop models of human disease; these disease models have in turn been used to facilitate discovery of other genes that modulate that same human disease.

2. C. elegans Overview

"You have evolved from worm to man, but much within you is still worm."

-Friedrich Nietzsche, Thus Spoke Zarathustra

2.1. What Is *C. elegans* Anyway?

Caenorhabditis elegans is a free living transparent nematode worm [6,7] (Fig. 1). *C. elegans* starts out as an egg; when these eggs hatch, the nematodes pass through four larval stages before reaching adulthood. The *C. elegans* life cycle is relatively short, taking about three days for the animals to develop, and with an overall lifespan of about two to three weeks. Adults contain only 959 somatic nuclei and grow to be about a millimeter in length. Despite this small size, *C. elegans* has many of the organ systems present in more complex organisms, including a digestive system, nervous system, musculature, and reproductive system. These small nematodes also exhibit complex behaviors. *C. elegans* will move toward things they like and away from things they do not like. The nematodes also eat, excrete, and mate.

C. elegans exists as either of two sexes, a hermaphrodite or a male. The existence of self-fertile hermaphrodites has great advantages for the study of development, because mutant stocks that would be unable to mate (such as paralyzed animals) are still able to self-fertilize. Moreover, healthy hermaphrodites produce hundreds of progeny, allowing the generation of large stocks quickly. When males are present, hermaphrodites can cross-fertilize. Thus, the presence of both sexes coupled with the relatively short life cycle allows for rapid genetic crosses.

C. elegans is only three cells in radius, with an outer epidermal layer, a middle muscle layer, and a central intestinal layer, with nervous system, reproductive system, and others tissues in between. The small size, transparent nature, and invariant cell lineage in *C. elegans* led to an unprecedented view of development in this animal. The full juvenile and adult cell lineages

were reported more than 30 years ago [8,9], and more recently, the entire wiring diagram of the nervous system has been determined [10]. In principle, if a cell is moved a few microns or a single neuronal connection is altered by some genetic manipulation, it should be possible to sort that out in *C. elegans*.

In the wild, *C. elegans* eats bacteria present in its environment [11]. In the laboratory, *C. elegans* typically is maintained on small petri dishes seeded with lawns of *E. coli* [12]. These bacteria are nonpathogenic and serve as a food source. Because of their small size, nematode manipulations are performed using a dissecting microscope. Individual nematodes can be moved from plate to plate using a small platinum wire "pick," allowing investigators to isolate individual hermaphrodites for self-fertilization and the generation of large populations, or allowing investigators to set up crosses between the sexes. The small size of *C. elegans* means hundreds or thousands of animals can be maintained inexpensively on an individual dish. When the animals use up all the food, they will starve, and can be maintained as starved populations for months. For long-term storage of stocks, nematodes can be frozen and kept in frozen vials for decades at −80°C or in liquid nitrogen.

In summary, these little animals have many of the organs and exhibit many of the behaviors present in mammals. Moreover, they offer the ability to study diseases in the context of a whole, living, and intact organism, which is not possible in isolated cells. This has been particularly fruitful in the many diseases that affect behavior and the nervous system as described below. Roughly 30–60% of genes in *C. elegans* have orthologs or strong homologs in mammals [13,14], suggesting that what is discovered about gene function in these small nematodes may be directly applicable to human development and disease.

2.2. Key Discoveries in *C. elegans*

The modern era of *C. elegans* research began over 50 years ago when Sydney Brenner first proposed using *C. elegans* to investigate developmental biology and neurobiology [15,16]. Three of the notable discoveries that earned *C. elegans* researchers Nobel Prizes included the award to Sydney Brenner, Robert Horvitz, and John Sulston in 2002 for their discoveries related to development and the cell death machinery [17–19]; Andrew Fire and Craig Mello in 2006 for their discovery of RNA interference (RNAi) [20]; and Osamu Shimomura, Martin Chalfie, and Roger Tsien in 2008, for the discovery of Green Fluorescence Protein (GFP) [21,22] and the demonstration that it could be a useful tool in other organisms including *C. elegans* [23]. Other key discoveries include the identification of microRNAs by Victor Ambros, Gary Ruvkun, and colleagues [24,25]. For a more complete list of key discoveries, see [15].

3. The *C. elegans* Toolbox

The small size, rapid life cycle, and amazing genetic and genomic tools available have made *C. elegans* a premier model organism for many purposes. We outline some of these tools here.

3.1. Construction of Transgenic Nematodes

The *C. elegans* germline initially develops as a multinucleate syncytium prior to membranes forming around each germ cell. Thus, DNA injected into the hermaphrodite gonad can be captured by numerous germ cells, making microinjection much easier than in other systems. DNA captured in this way will form extrachromosomal arrays that are semi-heritable [26,27]. Selectable markers can then be used to maintain stable transgenic lines, and the DNA can be integrated into the genome if desired [28,29]. In addition to direct microinjection, microparticle bombardment coupled with selection methods has been developed to generate stable nematode transgenic lines [30,31]. More recently, sophisticated CRISPR/Cas9-based genome engineering strategies have enabled rapid and precise gene editing, thus facilitating the generation of animals bearing targeted point mutations, deletions, insertions and complex chromosomal rearrangements [32,33].

The ease of *C. elegans* transgenic construction has served many purposes. Transgenic arrays can be used to restore gene function to “rescue” mutant phenotypes, greatly facilitating the cloning of mutated genes. Another common use for transgenic animals is the construction of GFP reporter strains. Promoter-GFP fusions can be used to determine where in the organism a particular gene is expressed. Protein-GFP fusions can be used for subcellular localization studies, and to quantify protein expression levels in live animals.

3.2. Genetic Tools and Forward Genetics in *C. elegans*

C. elegans is a diploid organism whose genome contains six chromosomes: five autosomes and one sex chromosome. XX animals are hermaphrodites; XO animals are males. The rapid lifecycle allows for quick genetic screens and crosses. Classical forward genetic screens used mutagens such as ethyl methanesulfonate (EMS) to randomly generate mutations in the nematode germ line [34–36]. F_1 hermaphrodite progeny that are heterozygous for these mutations can then be allowed to self-fertilize to isolate F_2 homozygous mutants of interest. If the homozygous mutant animals are self-fertile, they can be maintained as a homozygous stock. If the homozygous mutant animals are lethal or sterile, the screen can be engineered to recover heterozygous siblings to maintain the mutant stocks [37].

The ability to visualize *C. elegans* on a dissecting microscope or in more detail using a compound microscope equipped with differential interference contrast (DIC) optics allows for easy identification of mutant animals. Many classical mutants with visible phenotypes such as Unc (uncoordinated movement) or Dpy (dumpy shaped animals) were isolated by mutagenesis and visual screening for morphological or behavioral phenotypes [38]. More recently, screens have been performed for worms with altered levels or location of GFP expression, altered movement, or altered learning, and almost anything else *C. elegans* researchers can imagine. There are numerous mapping strategies to determine the identity of the mutant genes ranging from crosses with strains carrying known genetic markers, SNP mapping strains, strains carrying deletion chromosomes, or balancer chromosomes [39,7]. Once the mutation is mapped to a region where a candidate gene is found, the wild type copy of the locus can be injected into animals in an attempt to rescue the mutant phenotype. Alternatively or additionally, RNAi can be delivered to the animals in an attempt to phenocopy the mutant phenotype. The candidate locus also can be sequenced to identify

mutations, although more and more frequently whole genome sequencing is being used to identify the causative mutation [40,41]. To simplify mapping and mutation identification, transposon-mediated mutagenesis is also an option in *C. elegans* [42,35].

In addition to classical forward genetic screens, many researchers have used modifier screens with great success [34–36]. In this case, researchers start with a strain carrying a mutation that induces a phenotype and then mutagenize the animals to isolate mutant animals harboring suppressor or enhancer mutations. For example, one could start with a mildly uncoordinated animal, mutagenize, and screen visually using the dissecting microscope for suppressors that restore normal movement. These modifier mutations can then be genetically separated from the original mutation to determine if the modifier mutation has a phenotype on its own.

The ability to perform rapid genetic crosses also makes *C. elegans* an excellent system to perform genetic epistasis studies to place novel mutations in known genetic pathways [36].

3.3. Genomic Tools and Reverse Genetics in *C. elegans*

The discovery of RNAi opened up a whole new world for researchers in all fields including investigators studying *C. elegans*. Because there is no interferon response in *C. elegans*, long dsRNAs are not toxic to the nematode. Thus, long dsRNAs rather than siRNAs can be delivered to *C. elegans* with a concomitant increase in efficiency and specificity of knockdown. *C. elegans* RNAi screens generally do not suffer from the off-target effects that have plagued mammalian screens. The method of dsRNA delivery in *C. elegans* is also unique. Andy Fire and colleagues demonstrated the *E. coli* that are engineered to express dsRNA can be fed to *C. elegans*, resulting in knockdown of the target gene [43]. Taking advantage of this technique of RNAi feeding, the Ahringer and Vidal labs have generated two genomic RNAi bacterial feeding libraries that cover most of the *C. elegans* genome [44,45]; each bacterial strain enables the specific RNAi knockdown of a single gene, allowing for rapid and simple genome-wide screening. In these genomic RNAi screens, one simply feeds the bacteria to the nematodes, one bacterial strain at a time, and monitors for the occurrence of the phenotype of interest. Additionally, mutations that enhance RNAi-mediated knockdown have been identified and used to increase the sensitivity of these RNAi screens [46,47].

While RNAi is an invaluable tool, ultimately it is important to be able to monitor the effect of mutation of genes of interest. Unlike RNAi gene knockdowns, mutations allow for less heterogeneous effects. Mutations also can cause unique effects in gene function, such as gain of function or dominant-negative effects. Several labs that make up the *C. elegans* knockout consortia have been isolating thousands of knockout mutations available to the community of *C. elegans* researchers [48,49]. Likewise, the *Caenorhabditis* Genetics Center (CGC) is a stock center that provides ready access to these mutations and the myriad of other mutations that have been isolated and shared by the *C. elegans* research community. More recent targeted transposon insertion [50], and CRISPR/Cas9 genome editing [32,33] approaches have further enhanced the ability to perform reverse genetics in the nematode by enabling the introduction of almost any change in any gene in the genome.

4. Identifying Novel Human Disease Genes in *C. elegans*

In the next two sections, we outline several representative examples of human disease gene identification in *C. elegans*. We apologize to researchers whose work could not be included due to space limitations. Rather than aiming to be comprehensive, our goal is to be illustrative. These specific examples have been chosen to illustrate (1) the advantages of the techniques available in *C. elegans* to facilitate disease gene discovery and (2) some of the follow-up studies in mammals that have been performed. For *de novo* disease gene discovery, we outline various genetic and genomic screens for regulators of innate immunity, obesity, and aging (Subheadings 4.1–4.3). For human disease model studies in *C. elegans*, we outline the investigation of various neurodegenerative diseases (Subheading 5).

4.1. Innate Immunity

Infectious and inflammatory diseases are among the leading causes of death throughout the world. Infectious diseases account for 5 of the top 10 causes of death in the developing world [51]. In developed countries, the top three leading causes of death are heart disease, cancer, and COPD [52]. A key factor common to these three diseases is chronic inflammation [53–56]. This illustrates the importance of proper regulation of innate immunity and inflammation. While a robust innate immune response is essential in our pathogen-rich world, this response must be tightly regulated to prevent inflammatory disease. The identification of genes that regulate innate immunity has led to the identification of numerous genes that affect infectious or inflammatory disease [53,54,57–62].

C. elegans has emerged as a key model system for the discovery of innate immune genes [63–65]. For decades, *C. elegans* researchers cultured *C. elegans* on petri dishes containing lawns of nonpathogenic *E. coli*. However, Ausubel and colleagues discovered that by simply replacing this *E. coli* lawn with any of a number of human pathogens, the bacteria would infect and kill *C. elegans* [66–68]. Since then, pathogenesis models have been developed for Gram negative and positive bacteria, fungi, and viruses [69–72]. *C. elegans* lacks migratory immune cells and does not have an adaptive immune response. The nematode innate immune response is composed of the production of antimicrobial peptides and compounds that fight infection [73]. Importantly, the induction of antimicrobial production in the presence of pathogens is mediated by conserved signaling pathways including MAP kinase cascades [74]. However, there also are differences, most notably the absence of an NFκB homolog in *C. elegans*. Many investigators have now used *C. elegans* to study host-pathogen interactions.

Irazoqui and colleagues took a variety of approaches to identify a novel innate immunity regulatory pathway conserved in *C. elegans* and mammals. They first monitored changes in *C. elegans* gene expression induced by infection with the Gram positive bacterial pathogen *S. aureus* [75]. They then used computational analysis of these data to determine that the *C. elegans* HLH-30 transcription factor (mammalian ortholog TFEB) target DNA sequence was overrepresented in the promoters of the genes whose expression was induced by *S. aureus*. To test if HLH-30 was involved in this response, they generated HLH-30-GFP transgenic nematodes and found that while HLH-30-GFP was present in both the nucleus and

cytoplasm in uninfected worms, all the HLH-30-GFP was present in the nucleus following infection [76]. They then used RNAseq to monitor *S. aureus*-induced gene expression changes in wild type and *hlh-30* mutant animals and discovered that much of the *S. aureus*-induced gene expression was dependent on the function of HLH-30; moreover, both HLH-30 and its target genes were required for full resistance to *S. aureus* [76]. This approach illustrates several advantages of the nematode system, including the ease of generating transgenic animals, localization of GFP fusions in the transparent nematode, the availability of a deletion mutant in *hlh-30*, and the availability of bacteria to deliver *hlh-30* dsRNA. Moreover, the identification of HLH-30/TFEB as a key innate immunity regulator was validated in mammalian cells. *S. aureus* infection in mammalian cell culture leads to redistribution of TFEB into the nucleus, and inhibition of TFEB weakens the *S. aureus*-induced pro-inflammatory response [75]. Other investigators have independently shown using knockout mice that TFEB affects innate immunity in mammals [77], providing further evidence of the validity of the *C. elegans* studies.

In a follow-up to these studies, Irazoqui and colleagues used a targeted RNAi screen in which they inhibited most of the kinases and phosphatases in the nematode genome. This targeted RNAi screening approach led to the identification a PLC-PKD-TFEB pathway regulating the nematode innate immune response [78]. They took advantage of the ease of nematode genetics to order the various genes into a pathway, and then went on to show that this signaling pathway functioned similarly in mouse macrophages [78]. This highlights the importance of the *C. elegans* approach. Similar RNAi screens in mammals would have been significantly more cumbersome and expensive, and it would have been much more complicated to perform the genetic epistasis studies to determine how these genes functioned in an ordered pathway. However, once these details were worked out in *C. elegans*, the confirmatory cell culture RNAi studies were much more straightforward.

We have used a slightly different strategy with similar results: using *C. elegans* as a rapid screening tool with follow-up studies in mammalian cells and mice. We used comparative genomics RNAi screens in *C. elegans* and mouse macrophages to identify innate immunity regulators, subsequently used *C. elegans* infection models to obtain *in vivo* validation of these RNAi data, and then used knockout mice to determine the effect of these genes in mammalian disease. We used the ease of generating nematode transgenics to generate 14 different antimicrobial-GFP reporter strains [79]. GFP expression in these lines could be monitored using fluorescence microscopy or by using the COPAS Biosort, a flow cytometer for *C. elegans* [80]. A key feature of the COPAS Biosort is that it can analyze nematodes in 96-well format, allowing for high-throughput screens. We used bacterial feeding RNAi to inhibit known innate immunity regulators in *C. elegans*, and found several antimicrobial-GFP reporters whose expression was regulated by these known pathways. This formed the basis for a genomic RNAi screen in which we screened for changes in antimicrobial-GFP levels in the presence of *E. coli*. To determine if the genes identified could regulate innate immunity in mammals, siRNAs targeting the mouse orthologs of these genes were delivered into mouse macrophage cell lines and the cytokine response induced by lipopolysaccharide (LPS) was monitored. Remarkably, 30–40% of the genes identified in *C. elegans* had an RNAi-induced defect in the innate immune response in mouse macrophages [81–83]. The ready availability of existing *C. elegans* knockouts allowed us to rapidly obtain *in vivo*

confirmation that these genes affected host defense. We found that 9 of 10 *C. elegans* knockouts tested had altered survival in the presence of the nematode and human pathogen *P. aeruginosa* [81–83]. Armed with the RNAi data in *C. elegans* and mouse macrophages, and *C. elegans* knockout data, we then tested four different mouse knockout lines and found that three of the four knockout mice exhibited an altered innate immune response when challenged with LPS ([83,84] and unpublished). Thus, our comparative genomics approach is an efficient method for finding novel innate immunity regulators.

There are several things worth noting about this approach. First, one of the complications of RNAi screens in mammalian cells is the high degree of false-positives due to off-target effects [85]. This is likely not a problem in *C. elegans* because of the use of long dsRNAs. Moreover, the screens in *C. elegans* and macrophages involved different methods of dsRNA delivery, different innate immune stimuli, and different immunological readouts. It seems highly unlikely that such different systems would coincidentally report similar results. Plus, the ability to obtain so many nematode mutants relatively rapidly and cheaply for *in vivo* validation would just not be plausible in mice. By the time these genes had passed all these tests, the efficiency of validating them *in vivo* in mice was very high. Mammalian follow-up studies focused on genes identified in these screens have led to the investigation of two pathways that regulate the maintenance but not the activation phase of innate immunity [86,84,87].

4.2. Obesity

Obesity has become an epidemic in developed countries; more than 1/3 of adults in the USA are now obese [88]. Obesity is among the leading causes of preventable death and also affects many comorbidities such as Type 2 Diabetes [89]. The excess fat accumulation in obesity is caused by both genetic and environmental factors [90]. The ability to monitor fat accumulation in *C. elegans* coupled with the ease of RNAi screening in the nematode has led to a number of studies that identified genes that control fat accumulation [91–93]. In one study, McKay et al. [94] demonstrated that RNAi-mediated inhibition of genes known to affect fat accumulation in mammals, including SREBP and C/EBP homologs, led to arrested *C. elegans* development. Moreover, these animals did not accumulate fat [94], as assayed using Sudan Black or Nile Red staining. The authors reasoned that inhibition of other genes that affect fat production would likewise arrest larval development and would be lethal. The investigators used RNAi to inhibit 80 genes known to be larval-lethal when inhibited, and discovered that 10 gene inhibitions affected fat accumulation. They then used RNAi to verify that these genes affected mammalian cells as well [94]. Ashrafi et al. [95] used genome-wide RNAi screens followed by Nile Red staining to identify the full complement of genes that alter fat accumulation in *C. elegans*; these investigators identified 305 gene inactivations that reduced fat accumulation and 112 gene inactivations that increased fat accumulation. In another approach, a GFP reporter that localized to fat droplets was used as a screening tool to identify RNAi treatments that altered fat accumulation [96]. All these studies, and many others, demonstrate the ease of RNAi screening in *C. elegans* coupled with the effective readout tools available to study different diseases in a transparent organism.

4.3. Aging

The study of aging in *C. elegans* is unusual in that prior to these investigations, most researchers would not have even considered aging a disease that could be investigated and manipulated genetically. Thus, not only have *C. elegans* studies of aging been fruitful for finding potential human disease genes, but these studies also established that aging was a phenomenon that could be studied genetically in the first place.

As we grow older, we become increasingly frail and eventually die. Age is a major risk factor for a wide variety of diseases. These include almost all of the major neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, as well as cardiovascular disease, metabolic disease, and many cancers. Until recently, aging was not considered a genetically tractable phenomenon and instead was thought to result from the unregulated accumulation of all sorts of errors that together lead to the decay in function and death of the organism. As a result, our understanding of the mechanisms of aging was very poor. Over the last 25 years, however, our understanding of aging has been transformed by pioneering studies in *C. elegans*. Powerful genetics coupled with a relatively short lifespan of 20 days make *C. elegans* an excellent system to study aging. Its short lifespan makes it possible to conduct experiments that just are not practical in mice (mean life span of 2 years) or humans (mean lifespan of 80 years). In addition, its simple and inexpensive ease of manipulation makes it possible to assay the lifespan of hundreds or even thousands of worms. These studies have shown that aging is a regulated phenomenon that can be studied with the tools of molecular biology and genetics, and that many of the genes that regulate aging in nematodes also regulate aging in other organisms, including *Drosophila*, mice, and possibly humans.

The first forward-genetic screen for long-lived *C. elegans* mutants was conducted in the 1980s by Michael Klass [97]. This elegant genetic screen surmounted several technical challenges specific to *C. elegans* aging studies. Nematodes produce hundreds of progeny, and thus, parents will rapidly be lost among their progeny as they grow on small petri dishes. To measure the lifespan of a population of worms, one has to separate each worm from its progeny, typically by daily transfer to new petri plates until reproduction ends. This is a very cumbersome process. Moreover, once a mutant worm is deemed long-lived, one needs to obtain progeny to maintain a mutant line that can be studied; however, old worms are no longer fertile. Klass overcame these two challenges using a known temperature-sensitive spermatogenesis mutation. After mutagenesis, F_2 animals were each transferred singly to new "master" plates where they reproduced at the lower permissive temperature. Some of the F_3 progeny were grown at a high "restrictive" temperature, where they developed into animals that could not self-fertilize. Klass determined the lifespan of thousands of such cohorts to identify eight long-lived mutants. He re-isolated these mutants from their respective master plates that were maintained at the permissive temperature, since their siblings had the same mutations. Three of these mutations were subsequently mapped and shown to be in the same genetic locus, named *age-1* [98,99]. Remarkably, *age-1* mutant animals lived more than twice as long as wild-type control animals. These studies showed that mutations in a single gene could have a dramatic effect on the lifespan of a multicellular organism.

A few years later, Cynthia Kenyon's laboratory discovered that mutations in another gene, *daf-2*, could more than double *C. elegans* lifespan; moreover, the aged *daf-2* mutant animals remained youthful in appearance and mobility, even when all wild-type control animals had died [100]. The *daf-2* mutation was previously known to also affect the developmental decision to form dauer larvae [101]. Under unfavorable growth conditions of high-temperature, low food, and high population density, *C. elegans* develops into developmentally-arrested, stress-resistant, non-feeding dauer larvae; dauers can resume development into fertile adults once they encounter a more favorable environment [102]. *daf-2* mutant animals were known to inappropriately form dauer larvae at high temperature, but in an otherwise favorable growth environment. Kenyon and colleagues showed that at a low temperature where these mutant animals did not form dauers, they instead developed into fertile adults that were long-lived. A few years earlier, the Riddle lab [103,104,101] had performed several genetic screens and assembled a genetic pathway for the regulation of dauer formation. Kenyon and colleagues took advantage of this knowledge and asked whether a similar regulatory pathway existed for lifespan. They found that *daf-16*, a gene required for *daf-2* mutant animals to form dauer larvae, is also necessary for the increased lifespan of *daf-2* mutant adults [100]. Taken together, these finding demonstrated that aging is subject to regulation.

Subsequent studies have shown that *daf-2*, *age-1*, and *daf-16* are all part of a conserved insulin/IGF1 signaling pathway: *daf-2* encodes the worm's only ortholog of the human insulin and IGF1 receptor tyrosine kinases; *age-1* encodes a phosphoinositide 3-kinase (PI-3 kinase); and *daf-16* encodes a FOXO transcription factor that is negatively regulated by the *age-1* effector kinases AKT-1 and AKT-2 [105,106]. These genes are part of a well-conserved signaling pathway, raising the question of whether insulin/IGF1 signaling likewise regulated lifespan in other organisms. Subsequent studies in the fruit fly *Drosophila melanogaster* [106–110] and mice [111,112] showed that manipulation of the insulin/IGF1 signaling pathway can increase lifespan in fruit flies and mice. While these follow-up mouse studies were critical to demonstrate that these pathways were conserved in mammals, these studies highlight the practicality of forward genetic screens for lifespan in *C. elegans*, which would be a much more challenging in mice.

These remarkable studies prompted the question of whether similar mechanisms may regulate aging in humans [113,114]. Several candidate-based and unbiased association studies have since identified variants in the *daf-16* ortholog *FOXO3A* that are associated with exceptional longevity in humans from multiple ethnic origins [115–124]. In addition, mutations in the IGF1 receptor gene that cause diminished IGF1 signaling were found to be more prevalent in a cohort of Ashkenazi Jewish centenarians, compared to control individuals that do not exhibit exceptional longevity [125,126]. Taken together, these findings suggest that differences in human lifespan may result, at least in part, from the normal variation in signaling by the IGF1 receptor and its transcriptional effector *FOXO3A*.

Since the discovery of the regulation of lifespan by insulin/IGF-1 signaling, the study of aging in *C. elegans* has exploded, leading to the discovery of hundreds of genes that affect lifespan. These lifespan-determining genes have been identified by a combination of forward and reverse-genetic approaches. One of the most fruitful approaches has been to determine

the effect of each gene on lifespan by systematically knocking down each gene in the genome using RNAi. To date, three such genome-wide RNAi screens have been completed [127–129]. In addition, a genome-wide RNAi screen was performed to identify genes whose knockdown shortens lifespan in *daf-2* mutant animals [130], as well as numerous more targeted screens [131,132]. It likely will take many years until all these discoveries are replicated in mammalian systems, but investigators are already tackling the question of whether aging may be "druggable," potentially leading to an extension of "healthspan" and lifespan, and a delay in the onset of many age-related diseases [133,134].

5. Modeling Human Diseases in *C. elegans*

In contrast to the above approaches which involve screening *de novo* in *C. elegans* for genes that alter a phenotype that is involved in human disease, an alternate approach has been to artificially engineer the human disease into *C. elegans*, typically by expressing the human disease gene in the nematode. Animals engineered to exhibit the human disease are then used as tools to screen for suppressers or enhancers of the disease phenotype with the goal of finding additional gene targets that affect the disease in humans. While there are many examples of this approach, they are perhaps best exemplified by the study of neurodegenerative disorders in *C. elegans*, as outlined below.

5.1. Poly-Glutamine Repeat Diseases.

Trinucleotide repeat diseases are typically neurodegenerative or neuromuscular disorders caused by inheritance of a trinucleotide repeat (often greater than 30 repeats in length) in particular genes [135–139]. These trinucleotide repeats are formed by the expansion of unstable shorter triplet repeats present in the genome [135–139]. Some of the most studied triplet repeat disorders are caused by expansion of CAG repeats. These are the poly-Glutamine (polyQ) repeat diseases, which include Huntington's disease, spinocerebellar ataxias, and many others. Key questions about such disorders include how these unstable repeats expand in the genome, why there is apparently a threshold length for the repeat beyond which disease occurs, and how to develop possible treatments.

Expression of polyQ repeat proteins in *C. elegans* muscle [140] or neurons [141,142] recapitulates some aspects of human polyQ disease. In particular, some of these authors and others have found a similar threshold for the number of repeats that cause disease. Expression of roughly 35–40 repeats of polyQ-YFP were required to induce polyQ-protein aggregation and resulting muscle or neuronal dysfunction. The ability to monitor YFP-tagged polyQ-protein aggregation in this transparent organism allowed for straightforward modifier screens to monitor polyQ-induced aggregation or dysfunction. For example, Nollen et al. [143] used a genomic RNAi screen to identify 186 genes whose inhibition led to increased or earlier onset aggregation of Q35-YFP (polyQ protein with 35 Q repeats). These genes fell into five broad functional categories, including regulation of RNA metabolism, protein synthesis, protein folding, protein degradation, and protein trafficking. Similarly, candidate based-approaches have been used to identify modifiers of polyQ aggregation in *C. elegans*. For example, overexpression of the *C. elegans* homolog of the torsin gene suppressed polyQ aggregation [144]. Likewise, overexpression of ubiquitin suppressed

polyQ-induced toxicity in *C. elegans* and mammalian cells while inhibition of ubiquitin expression induced the opposite effect. [145]. The ease of such genetic and genomic studies in *C. elegans* coupled with the ability to monitor fluorescently tagged polyQ proteins in this transparent organism has made such studies very straightforward and powerful.

5.2. Alzheimer's Disease.

Alzheimer's disease is the sixth leading cause of death in the USA, affecting more than 5 million people in the USA and more than 35 million people worldwide [146,147]. As is the case for most age-dependent diseases, the incidence of Alzheimer's disease is expected to increase in the future. Despite intensive study, much about Alzheimer's disease remains a mystery, and no effective treatments have been developed. Much of the research focus centers on trying to understand the aggregation of proteins such as Tau or beta amyloid and the resulting effects on neurological function [146,147].

Several investigators have used overexpression of wild type or mutant Tau as a model for tauopathy in *C. elegans* [148,149]. Kraemer and colleagues [150] expressed wild type or mutant Tau in all nematode neurons; they observed that Tau aggregated in these animals and that Tau overexpression led to a moderate uncoordinated phenotype. They used this model as the basis for a genome-wide RNAi screen for enhancers of this uncoordinated phenotype [151]. The genes and pathways identified in this screen as potential modifiers of Tau-induced pathology were very similar to those identified in *Drosophila* screens, suggesting that they may be conserved regulators that might play a role in tauopathies and Alzheimer's disease [152]. In addition to their genomic RNAi screen, the investigators performed a forward genetic screen to identify mutations that suppress the Tau-induced uncoordinated phenotype. In this genetic screen, they identified mutations in *sut-2*, which suppressed the Tau aggregation, uncoordinated, and neurodegenerative phenotypes induced by Tau overexpression in *C. elegans* [153]. Moreover, overexpression of *sut-2* in nematodes exacerbated Tau-induced neurotoxicity, the opposite of the RNAi-induced phenotype [154]. The role of SUT-2 was not unique to *C. elegans*. Follow-up studies in mammalian cells demonstrated that (1) Tau overexpression increased expression of the mammalian homolog MSUT2, (2) MSUT2 RNAi in mammalian cells diminished aggregation of insoluble Tau, and (3) there is less MSUT2 present in the brain in autopsy samples from Alzheimer's disease patients [154]. Thus, these genomic and genetic modifier screens in *C. elegans* successfully identified key genes to investigate in the human disease.

Studies in *C. elegans* relevant to Alzheimer's disease are not limited to the investigation of Tau. For example, beta-amyloid-expressing models of disease have also been engineered in *C. elegans* [155–160]. Likewise, investigation of the nematode homologs of Presinilin 1 and 2, mutations in which cause early-onset familial Alzheimer's disease [161–163], led to the discovery that nematode and human Presinilin 1 regulates Notch signaling [164–166]. As an illustration of the power of genetic screens in *C. elegans*, our lab conducted a sensitized forward genetic screen in *C. elegans* to identify genes that function with the Presinilins. In this screen, we identified mutations in two novel genes (*aph-1*and *pen-2)* that enhanced the phenotype induced by mutation of *sel-12* (Presenilin) [167]. *aph-1* also was identified in a *C. elegans* genetic screen for enhancers of Notch signaling [168]. These genes were later shown

to be part of the evolutionarily-conserved γ-secretase protease complex, where they regulate the maturation of Presenilin [169], the catalytic component of this complex. This complex is involved in the proteolytic maturation or degradation of many transmembrane proteins, including the Amyloid Precursor Protein (APP), which is important in Alzheimer's disease pathogenesis, and the Notch receptor.

5.3. Parkinson's Disease

Parkinson's disease is second only to Alzheimer's disease as the most common neurodegenerative disease. Like Alzheimer's disease, Parkinson's disease usually, but not exclusively, is an age-dependent disease, with an incidence of roughly 1% in people over 65 rising to an incidence of 5% by age 85 [170–172]. The primary cause of Parkinson's disease is a loss of dopaminergic neurons in the substantia nigra region of the brain. This results in the neurological symptoms that are a hallmark of the disease, including tremor of the hands, legs, limbs, and jaw, muscle rigidity of the limbs and trunk, bradykinesia, and postural instability. A key histological feature of patients with Parkinson's disease is the accumulation of Lewy Bodies in the brain.

A number of genomic and candidate gene-based RNAi screens have been performed in *C. elegans* models of Parkinson's disease. These models have focused on overexpression of α-Synuclein, a key candidate Parkinson's disease protein. α-Synuclein is the main component of Lewy Bodies. It is overexpressed and often mis-expressed in the brain of Parkinson's disease patients, and mutations in α-Synuclein have been identified in some patients [173,174]. α-Synuclein is not present in *C. elegans*. Nematode researchers have taken advantage of this to overexpress α-Synuclein and screen for genes that affect α-Synuclein aggregation or cell function [175]. In two studies, YFP or GFP-tagged human α-Synuclein was expressed in nematode muscle using cell-type specific promoters. Aggregated α-Synuclein was monitored by the appearance of punctate fluorescent structures, and either a genomic RNAi screen [176] or an RNAi screen of 900 priority candidate genes (based on various bioinformatics approaches) [177] led to the discovery of numerous genes that affect α-Synuclein aggregation. Many of these genes, in turn, were found to serve a neuroprotective function.

RNAi screens focusing on neurons in *C. elegans* are more challenging because nematode neurons are somewhat resistant to RNAi. Thus, to study the effects of α-Synuclein expressed in neurons, Kuwahara et al. [178] took advantage of a mutation, *eri-1*, that enhances RNAi in *C. elegans*. They expressed human α-Synuclein in all nematode neurons in a strain carrying this *eri-1* mutation. Under these conditions, there was little gross effect on the animals. They then performed an enhancer RNAi screen targeting 1,673 prioritized candidate genes (genes known to affect the nervous system) to identify RNAi treatments that induced a visible phenotype such as uncoordinated movement or growth retardation. Ten candidate genes passed their screening criteria; four of these genes functioned in the endocytic machinery, implicating endocytosis in the pathogenesis of α-Synuclein.

6. Conclusion

The choice of models to investigate human disease is often a trade-off between how well the model mimics the human condition and how easy it is to manipulate the system. Invertebrate models such as *C. elegans* and *D. melanogaster* have been invaluable for the study of development, signaling pathways, and many other aspects of biology. In this chapter, we have outlined several examples that illustrate the ease of such *C. elegans* studies. Some of the features that have rendered *C. elegans* such a powerful research organism include the ease of genetics (forward genetic screening, transgenic animal construction, mutation mapping), cell biology (using GFP in a transparent organism with a fully-described and invariant cell lineage), genomics (RNAi and other techniques), modifier screens (enhancement and suppression), and the ability to mimic many human diseases. We also have highlighted how more and more frequently, follow-up studies in mammals have validated these nematode findings. The tools and ease-of-use of *C. elegans* and other "simple" model organisms continues to make them invaluable for research, and these organisms will continue to play an important role in our understanding of human disease and human disease gene discovery in the future.

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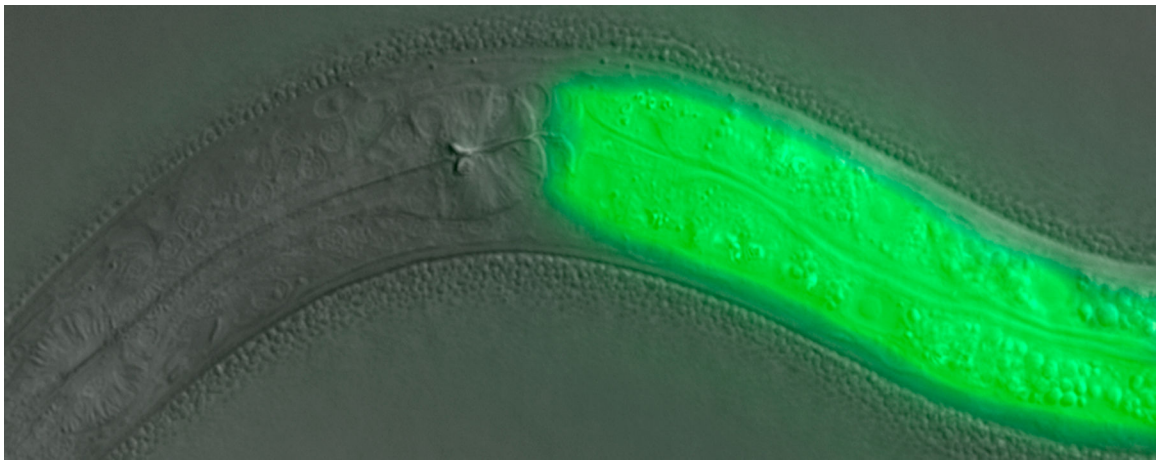

Figure 1.

Depicted is a *C. elegans* hermaphrodite carrying a *lys-7::gfp* transgene. In this animal, GFP expression is controlled by the gut-specific lysozyme-7 promoter. The image is an overlay of fluorescence and Nomarski images (images merged using Adobe Photoshop). Image adapted from Fig. 1 in [79]. Copyright © American Society for Microbiology, Molecular and Cellular Biology, 27, 2007, 5544–5553, doi:10.1128/MCB.02070–06.