Published in final edited form as:
Pediatr Res. 2009 January ; 65(1): 10–18. doi:10.1203/PDR.0b013e31819009b0.

Modeling Molecular and Cellular Aspects of Human Disease using the Nematode *Caenorhabditis elegans*

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Abstract

As an experimental system, *Caenorhabditis elegans*, offers a unique opportunity to interrogate *in vivo* the genetic and molecular functions of human disease-related genes. For example, *C. elegans* has provided crucial insights into fundamental biological processes such as cell death and cell fate determinations, as well as pathological processes such as neurodegeneration and microbial susceptibility. The *C. elegans* model has several distinct advantages including a completely sequenced genome that shares extensive homology with that of mammals, ease of cultivation and storage, a relatively short lifespan and techniques for generating null and transgenic animals. However, the ability to conduct unbiased forward and reverse genetic screens in *C. elegans* remains one of the most powerful experimental paradigms for discovering the biochemical pathways underlying human disease phenotypes. The identification of these pathways leads to a better understanding of the molecular interactions that perturb cellular physiology, and forms the foundation for designing mechanism-based therapies. To this end, the ability to process large numbers of isogenic animals through automated work stations suggests that *C. elegans*, manifesting different aspects of human disease phenotypes, will become the platform of choice for *in vivo* drug discovery and target validation using high-throughput/content screening technologies.

Keywords

C. elegans; serpin; necrosis; cell death; disease

The discovery of disease-related genes for simple Mendelian disorders and polygenic traits by positional cloning, linkage analysis or genome-wide association studies has increased at a frenetic pace (1). Although these strategies may identify genes encoding domains with recognizable functions (e.g., kinase domain) or folds belonging to a well-characterized protein family (e.g., G protein-coupled receptor), the relationship between mutations and disease phenotypes frequently are inapparent until genetic functions are placed within the context of molecular systems. Since cellular metabolism is comprised of an integrated network of biochemical pathways that fluctuate in response to environmental and constitutional cues, the precise positioning of disease-related genes within those pathways most proximal to their site of action is not a straightforward task. Identifying disease-related genes and their interacting partners is more than an academic exercise, as mechanism-based therapeutic strategies are based frequently on modulating the activities of different components of molecular pathways

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This article contains supplementary materials, which can be found online at www.pedresearch.org.

rather than those of the mutant genes themselves. Thus, a better understanding of genes and their interacting partners forms the foundation for more meaningful translational research.

Fortunately, a mutant gene, even if its function is unknown, can be used to gain an entrée into its biological system. *In vitro*, biochemical approaches including affinity chromatography, co-immunoprecipitation and liquid chromatography coupled to mass spectrometry are used to isolate interacting proteins (2,3). *In vivo*, genetic techniques employing yeast two-hybrid analysis, crosses between different mutants, transgene insertion and forward and reverse genetic screens have been instrumental in defining the components of biochemical systems associated with disease-related genes and their phenotypes. Humans, however, are poor subjects for these types of genetic studies as we lack inbred populations with homogeneous genetic backgrounds, reproduce inefficiently with small brood sizes, require generation times that far exceed the average NIH grant cycle, demonstrate wide variations in gene penetrance and expressivity and are unable to control consistently for confounding environmental variables. Moreover, manipulating human germline DNA is deemed unethical and arranging nuptials to study genetic interactions in our progeny is generally frowned upon in most societies. To circumvent these issues, an expanding group of model organisms is being exploited to make inferences about human biology that are based partially upon the extent of evolutionary relatedness at the genomic level. These organisms, initially dubbed the “Security Council of Genetic Organisms” by Gerald Fink, have grown to include additional members and collectively have provided crucial insights into most aspects of modern cellular and molecular biology (Table 1) (4). Although each model genetic organism possesses distinct advantages, they share three common features: 1) the ability to conduct sexual crosses for determining modes of inheritance, complementation groups and recombination frequencies; 2) a sequenced genome that is linked to a molecular toolbox containing genomic and cDNA clones and reagents to manipulate these sequences and 3) techniques for generating germline DNA transformations and mutations (4).

Convinced that the “classical problems of molecular biology had been solved and that the future lay in tackling more complex biological problems,” Sydney Brenner in the mid 1960s sought to establish an experimental platform that would facilitate the study of the “new, mysterious and exciting” fields of development and the nervous system (5). Buoyed by the success of discovering the mechanisms of DNA replication and gene regulation by using genetics and biochemistry to interrogate a simple a prokaryotic model system (bacteria and their phage), Brenner reasoned that similar technologies applied to a microbiological-like, multicellular organism (i.e., an organism of modest complexity) would help determine the molecular mechanisms for tissue organization and function underlying metazoan life. “Having once been discovered, their applicability to the higher forms of life could be tested afterwards.” In 1974, Brenner published his landmark paper describing methods for isolating and mapping disease-related genes in the mutagenized free-living nematode, *Caenorhabditis elegans* (6).

C. elegans resources

For *C. elegans*, becoming a charter member of the Council was no accident. Sydney Brenner identified several experimental traits that have made *C. elegans* the organism of choice for studying molecular aspects of cell biology *in vivo*. These and additional advantages include: 1) small size (adults are ~ 1 mm in length) that facilitates ease of propagation, manipulation (animals are reared on agar plates or liquid media seeded with bacteria, usually *E. coli*) and long-term storage (stocks can be frozen in glycerol), 2) transparency at all stages of development, which allows for visualization of all cells by differential interference contrast (DIC) and various forms of fluorescence microscopy, 3) a short generation cycle (~ 3 days) and lifespan (~ 3 weeks), 4) sexual dimorphism with self-fertilizing hermaphrodites and cross-fertilizing males leading to large brood sizes of ~ 300 and ~ 1000 F_1s, respectively, 5) a

complete and invariant cell lineage map (from zygote to adult) for the 959 and 1031 somatic cells of the adult hermaphrodite and male, respectively, 6) organ-based physiology with developmental specification of a nerve ring and nerve cords, intestine, muscle, hypoderm, secretory-excretory system, gonads and uterus, 7) complete connectivity maps for the 302 and 391 neurons comprising the adult hermaphrodite and male nervous systems, respectively, 8) complex behavioral traits such as mechano- and chemosensation, avoidance of noxious stimuli and thermotaxis, 9) experimental techniques for generating transgenic animals; ablating single cells by laser photolysis; conducting forward and reverse genetic screens by chemical mutagenesis and RNAi, respectively; flow sorting animals by both size and fluorescence; SNP mapping of phenotypic traits and drug screening using small molecule libraries, 10) a completely sequenced genome harboring ~19700 coding sequences and ~ 1300 non-coding RNAs, 11) repositories of genomic and cDNA clones, 12) a stock center for mutant animals including ongoing efforts to include null mutants for every gene and 13) a wealth of free on-line resources (e.g., WormBook) and databases (e.g., WormBase) (Table S1, online at www.pedrsearch.org).

HUMAN-DISEASE RELATED GENES ARE WELL-CONSERVED IN *C. elegans*

The number of human disease-related genes that share at least modest homology ($E < 10^{-10}$ on BLASTP searches) with *C. elegans* genes ranges from 40–75% (7–12). However, this degree of relatedness may still be an underestimate as the rapid divergence of non-essential domains and exon shuffling may impair the ability of the BLASTP algorithm to detect high-scoring segment pairs between orthologous genes (13). For example, the *C. elegans* orthologue of vertebrate *P53*, *cep-1*, which also contains the five signature domains and residues commonly mutated in human malignancies, was detected only after using a combination of the squid P53 as the query, PSI-BLAST and the Block Maker tool (14). Moreover, even if a human disease-related gene does not have an orthologue in *C. elegans*, there is still a high likelihood that a homologous gene, a protein domain or the constituents of an associated biochemical pathway (especially if it encompasses a core cellular function like signal transduction, synaptic transmission or membrane trafficking) are conserved to the extent that this model system can be exploited to lend insight into human pathobiology (15,16).

A compendium of the *C. elegans* genes that are orthologous to human disease related genes is found in several excellent reviews (7–9) and partially summarized in Table S2 (online at www.pedreserach.org). Since a comprehensive update is beyond the scope of this review, we will focus on several experimental systems that underscore the importance of using this simple metazoan model, in combination with powerful genetic technologies, to enhance our understanding of fundamental human disease processes and to lead to new mechanism-based therapies.

THE REGULATION OF CELL DEATH PATHWAYS

Apoptosis

The apoptosis form of programmed cell death has been recognized as a normal developmental and tissue homeostatic process for over a century (17). More recently, apoptosis has been recognized as a cellular response associated with different pathologic conditions including, infection, tissue degeneration, malignancy and immunity (reviewed in (18)). In an extraordinary series of morphological studies, Sulston and colleagues showed that *C. elegans* development follows a stereotypical pattern. The adult hermaphrodite is comprised of 959 somatic nuclei after the loss of 113 and 18 cells in the embryonic and early larval (L2) stages of development, respectively (19,20). These programmed cell deaths are invariant in terms of morphology, cell type and timing. Dying cells complete the process 1–2 hours after mitosis and are eliminated by phagocytosis. Cells die autonomously and not secondary to

engulfment, as a forward genetic screen using ethyl methane sulfonate (EMS) as the mutagen showed the persistence of embryonic cell corpses in two different phagocytosis mutants, designated *ced-1* and *ced-2* for *ce*ll *d*eath abnormal (21). To isolate genetic mutants in which the programmed cell deaths would be disrupted (suppressed), *ced-1* hermaphrodites were mutagenized also with EMS and their F_2 progeny screened for the persistence of cells destined to die (22). Mapping of these suppressor mutants, led to the isolation of loss-of-function mutations (lf) in two genes, *ced-3* and *ced-4* (Fig. 1A). Subsequently these genes were found to be homologous to human caspases and apoptotic protease-activating factor-1 (APAF-1), respectively (23,24).

Egg laying in adult hermaphrodites is under neuronal control. Two hermaphrodite specific neurons (HSNs) innervate the vulval muscles and laser ablation of the HSNs leads to an egg-laying defect (25). During male development, the HSNs die, suggesting that their cell fate is under genetic control associated with sex determination. In a mutagenesis screen for hermaphrodites with egg-laying defects (Egl), the inappropriate loss of HSNs was detected in some animals and was associated with a gain-in-function (gf) mutation in what is now known as a pro-apoptotic BH3-only (*BCL2* homology domain-3) gene, *egl-1* (25). Transcription of *egl-1* is active in males and suppressed in hermaphrodites. The *egl-1*(gf) is due to the loss of a DNA binding site for a negative transcriptional regulator, TRA-1, thereby allowing for EGL-1 expression in the hermaphrodite.

In another mutagenesis screen for genes that altered programmed cell death, a gf mutant, *ced-9* (gf), was found to block nearly all developmental cell deaths (26). After *ced-9*(gf) was crossed with the *egl-1*(gf) mutant, HSN cell death was blocked. This data suggested that CED-9 functions downstream of EGL-1. In contrast, a lf mutation of *ced-9*(lf) resulted in ectopic cell deaths and embryonic lethality. Through a series of genetic crosses, *ced-4*(lf) and *ced-3*(lf) mutations blocked the ectopic cell deaths associated with the *ced-9*(lf) mutation, suggesting that these proteins function downstream of CED-9 (Fig. 1A). Biochemical and structural studies confirmed these findings and show that CED-9 localizes to the outer mitochondrial membrane where it binds a CED-4 dimer (reviewed in (27,28)). Binding of EGL-1 results in a conformational change in CED-9 and the release of CED-4. The released CED-4 dimers associate and bind four pro-CED-3 molecules, which are processed into the active catalytic form of the peptidase.

Conservation of the core apoptotic machinery in *C. elegans* was extended to mammals by showing that human BCL2 blocks ectopic cell deaths in *ced-9*(lf) mutants (29). The conservation of the apoptotic pathway(s) among metazoans was extended to at least 28 genes involving the initiation, execution and elimination (engulfment) phases of the process (28). Taken together, these findings were the first to show how a precise knowledge of cell fates combined with a series of mutagenesis studies and genetic crosses between different types of mutants led to a seminal finding: apoptosis is a carefully regulated genetic process. These studies in *C. elegans* programmed cell death led to the awarding of the 2002 Nobel Prize in Medicine to Sydney Brenner, John E. Sulston and H. Robert Horvtitz (27).

Necrosis

Necrosis has been defined as an accidental, catastrophic and uncontrolled form of cell death that occurs after overt energy failure, extreme metabolic embarrassment or after the failure of counter-regulatory systems (30). Moreover, once activated, necrosis was characterized as an accidental, chaotic, unregulated and irreversible process. Studies in *C. elegans* suggest this is not the case. Insight into the molecular pathogenesis of necrosis was first described in a model of mechanosensory neuronal degeneration involving gf mutations for genes encoding a subunit of the degenerin/epithelial Na^+ channel (ENaC) family (*deg-1*, *mec-4*, *mec-10*, *unc-8* and *unc-105*), a GTP binding protein subunit (*gsa-1*, a $G\alpha_s$) and a nicotinic acetylcholine receptor

(*deg-3*) (reviewed in (31)). Most of these mutations lead to hyperactivation of Na^+ or Ca^{2+} channels and contribute to cellular demise by perturbed ion flow, osmotic shifts or Ca^{2+} mediated excitotoxic injury (Fig. 1B) (32–34). Death follows a stereotypical pattern characterized by the formation of membranous whorls, vacuole formation, cytoplasmic and mitochondrial swelling, chromatin clumping, nuclear condensation and the occasional loss of plasma membrane integrity (32). Although features of the late-degenerative phases are reminiscent of apoptosis, cell death is independent of *ced-3* and *ced-4* (35). However, both apoptotic and necrotic corpses are eventually engulfed and both death pathways employ the same parallel sets of engulfment genes (*ced-1*, *−6*, *−7* and *ced-2*, *−5*, *−10*, *−12*) (35). Necrotic cell death of mechanosensory neurons requires a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) as death is blocked, in part, by lf mutations of the calreticulin (*crt-1*), inositol 1,4,5-trisphosphate (IP_3R/*itr-1*) or ryanodine receptor (RyR/*unc-68*) genes (36). The rise in $[Ca^{2+}]_i$ is thought to activate two members (*clp-1* and *tra-3*) of the calpain family (Ca^{2+} dependent, papain-like cysteine peptidases), which may activate the downstream aspartic peptidases, *asp-3* and *−4* (37). It is not known whether these peptidases kill cells directly or activate other downstream effectors.

The terminal phases of cellular death are a triggered by peptidase-inhibitor imbalance within the cytoplasm. Since a subset of the serpin superfamily of peptidase inhibitors (e.g., α1-antitrypsin (AT) and antithrombin) lack signal peptides, are highly expressed within the cytoplasm of metazoan cells, and neutralize lysosomal cysteine and serine peptidases; we hypothesized that some of these intracellular serpins regulate intracellular proteolytic pathways leading to cell death. This notion was confirmed using a reverse genetic approach in *C. elegans* (38). The intracellular serpin, SRP-6, exhibits a pro-survival function by blocking intestinal cell lysosomal disruption, cytoplasmic proteolysis and whole animal death induced by hypotonic shock, thermal stress, oxidative stress, hypoxia and cation channel hyperactivity (Fig. 2). This necrotic death phenotype was dependent upon calpains and lysosomal cysteine peptidases, two *in vitro* targets of SRP-6. Taken together, these findings suggest that multiple noxious stimuli converge upon a peptidase-driven, core stress response pathway that, in the absence of intracellular serpin regulation, triggers lysosomal-dependent necrotic cell death (Fig. 1C).

SRP-6 also protected animals from cell death after direct lysosomal rupture induced by the lysosomotropic and photo-sensitizing agent, acridine orange (38). Minutes after blue-light irradiation, nearly all of the intestinal cell lysosomes from both wild-type and *srp-6* null animals disintegrate. Not surprisingly, all of the *srp-6* null animals died. However, the majority of wild-type animals survived and reconstituted their lysosomal compartment. These findings show that even after massive lysosomal disruption, intestinal cells can survive providing that sufficient amounts of $serpins_{IC}$ are available to block collateral damage.

Based on these findings, necrosis appears to be evolutionarily conserved, orderly and a highly regulated peptidase-driven cell death pathway that is amenable to therapeutic intervention even after complete dissolution of the lysosomal compartment. Since intestinal epithelial cell necrosis is a common pathologic feature in many gastrointestinal disorders including inflammatory bowel disease, ischemic bowel disease, graft versus host disease, infectious gastroenteritis and necrotizing enterocolitis; the development of mechanism-based therapeutics that target different steps of the necrosis pathway could be used to treat a wide-array of disorders.

HUMANIZED WORMS: MODELING OF HUMAN DISEASE BY TRANSGENESIS

A practicing clinician might view the modeling of a human disease in a simple invertebrate by transgene insertion as experimental esoterica. If nematodes do not possess the complex organ-

based physiology of mammals, how can we be confident that heterologous transgene expression in *C. elegans* will provide any meaningful insight into the pathogenesis of a human disease phenotype? The answer will depend, in part, on how the question is framed and by recognizing that complex disease phenotypes in humans can be studied successfully by reducing complex molecular and cellular events into their component parts. For example, classical AT deficiency is a common autosomal recessive disorder (39). A single missense mutation renders the mutant protein (ATZ) prone to self-polymerization and aggregation (40). ATZ polymers/aggregates are poorly secreted and accumulate within the endoplasmic reticulum (ER) of liver cells (39). Since AT is the predominant serine peptidase inhibitor in extracellular fluids, decreased AT secretion results in a lf phenotype manifest by peptidase-inhibitor imbalance, connective tissue matrix destruction and susceptibility to chronic obstructive lung disease (41). In contrast, the accumulation of aggregation-prone ATZ in liver cells leads to a toxic gf phenotype characterized by liver failure and carcinogenesis (42). Clearly, modeling the lf phenotype in *C. elegans* is a difficult proposition. However, the toxic gf phenotype (i.e., the ER translocation defect observed in hepatocytes) could be modeled in *C. elegans* by driving ATZ transgene expression in different tissues (Pak, et al., unpublished).

Sveger followed the clinical course of 127 Swedish infants homozygous for the classical form of AT deficiency (43). Over a 40-year period, only 8% of the population at risk developed clinically significant liver disease. This epidemiological study demonstrates that there is wide variability in the liver disease phenotype associated with AT deficiency, and other genetic factors are likely to modulate liver disease susceptibility in this cohort of patients. Thus, enhancer and suppressor screens using the *C. elegans* model of AT deficiency could yield novel insights into disease susceptibility by identifying, for example, genes that regulate the cellular response to proteotoxic species and the overall disposition of ATZ.

Neurodegenerative diseases

Brain tissue from Alzheimer's disease patients harbor extracellular senile plaques and intracellular neurofibrillary tangles partially composed of β-amyloid peptide (Aβ) and TAU/MAPT (a microtubule-associated protein), respectively (44,45). Both human proteins have been expressed in *C. elegans* and used to model different aspects of the disease. The Link laboratory has generated transgenes that drive $A\beta_{1-42}$ peptide expression in body wall muscles or neurons (45). Young adults develop cytoplasmic Aβ inclusions in both cell types but only body wall muscle expression leads to progressive paralysis. Although Aβ-expressing animals do not appear to form extracellular plaques, the Aβ-containing intracellular inclusions are similar to those observed in patients with sporadic forms of Inclusion Body Myositis. Interestingly, transgenes expressing different $A\beta_{1-42}$ variants show that cellular toxicity, like those of the murine models, does not correlate with the intracellular accumulation of the dye-reactive fibrillar $A\beta_{1-42}$ species, but is related to the presence of soluble $A\beta_{1-42}$ oligomers (46,47). These studies show that the transgenic techniques for *C. elegans* are facile enough to probe for structure-function relationships *in vivo*. Transgenic animals can also be used to identify interacting proteins. MALDI-TOF mass spectrometry analysis of co-immunoprecipitates shows that several different types of chaperones, including two heat-shock proteins (HSP) of the HSP-70 family and three of the HSP-16 family associate with the $A\beta_{1-42}$ peptide (48). RNAi of an HSP-70 inhibitor or over-expression of the HSPs help ameliorate the disease phenotype and identified a potential therapeutic strategy for treating disorders associated with protein misfolding.

Aggregates of $A\beta_{1-42}$ have been considered a marker and possibly the initiator of cellular injury in patients with Alzheimer's disease. However, studies in *C. elegans* show that cellular dysfunction can precede the accumulation of intracellular Aβ inclusion bodies. To help resolve this issue, Dillon and colleagues took advantage of long-lived, insulin signaling pathway

mutants that show attenuated $A\beta_{1-42}$ toxicity (49). Animals with lf mutations of *daf-2* (an insulin-like receptor) demonstrate delayed ageing which is due, in part, to the de-repression of the transcription factors, DAF-16 (a forkhead family member) and HSF-1 (a heat-shock transcription factor). Subsequent loss of either of these factors reverses the protective effects observed in *daf-2*(lf) animals, but for different reasons. By combining biochemical techniques to investigate the nature of the $A\beta_{1-42}$ species in the genetic mutants, these investigators showed that soluble oligomers were the toxic intermediate and that DAF-16 and HSF-1 had different but complementary functions in their detoxification. Animals with an intact HSF-1 pathway targeted their soluble oligomers for degradation. However, if the HSF-1 pathway was blocked, the DAF-16 pathway reduced $A\beta_{1-42}$ toxicity by facilitating the assembly of less toxic high-molecular mass $A\beta_{1-42}$ aggregates. These studies suggest that the presence of $A\beta_{1-42}$ aggregates in patients of Alzheimer's disease may be more indicative of an adaptive cellular response to detoxify misfolded proteins rather than a marker of a toxic species *per se*. Thus, future therapies might be directed towards the elimination of toxic oligomers or the generation of high-molecular mass $A\beta_{1-42}$ aggregates.

Pan-neuronal expression of mutant (P301L or V337M) and, to a lesser extent, the wild-type form of human TAU in *C. elegans* leads to neuronal cell loss and progressive uncoordinated movement that simulates aspects of the pathology associated with Alzheimer's and frontotemporal dementia with Parkinsonism chromosome 17 type (FTDP-17) (44). One of the most advantageous aspects of modeling human diseases in *C. elegans* is that unbiased genetic screens can be used to identify components of the molecular pathway(s) associated with the disease phenotype. For example, an RNAi screen involving 16,757 genes identified ~75 genes that enhanced the disease phenotype and uncovered several candidate genes (e.g., peptidases, UPR signaling components, chaperones) that block TAU toxicity (50).

Huntington's disease, spinal and bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and the spinal-cerebellar ataxias including types 1, 2, 3 (Machado-Joseph disease), 6, 7 and 17 are all autosomal dominant neurodegenerative diseases hallmarked by the genomic amplification of CAG trinucleotide repeats. These repeats are translated into polyglutamine (polyQ) expansions within these different proteins (51). The expression of polyQ repeats above certain thresholds leads to toxic gf phenotypes marked by intranuclear or cytoplasmic inclusions, neuronal dysfunction and cell death. Although the mechanism of toxicity has not been firmly established, a combination of native protein dysfunction due to polyQ-induced protein misfolding, sequestration of associated proteins by the polyQ motif and the accumulation of cleaved polyQ aggregates appear to play a role (51). To help elucidate the mechanism of cell injury, transgenic lines expressing different numbers of polyQ repeats (*n* ranges from 2 to150) in different cell types have been established in *C. elegans* (52-56). Taken together, these studies show an age and threshold-dependent accumulation of intracellular inclusions that are associated with neuronal or body wall muscle dysfunction and, in certain cases, cell death. As in mammalian systems, a threshold of ~35-40 polyQ repeats in muscle, and possibly a slightly higher number in neurons, leads to cellular dysfunction. The threshold goes down with age and can be exacerbated by the presence of other metastable proteins encoded by the host genome. For example, the presence of unrelated temperature-sensitive mutants enhances the aggregation polyQ proteins (57). Similarly, the presence of polyQ proteins leads to the destabilization of temperature-sensitive mutant proteins even at their permissive temperatures. These studies suggest that polyQ proteins can lead to cellular dysfunction by perturbing the global balance of protein folding quality control systems (57).

One of the values of these *C. elegans* models resides in the ability to generate multiple transgenic lines with subtle variations in the transgenes. Similar approaches are being used to study different forms of superoxide dismutase in a model of amyotrophic lateral sclerosis (58), and α-synuclein mutants in a model of Parkinson's disease (59). The cost in terms of

manpower and resource utilization makes this strategy impractical in mice. Moreover, the ability to utilize these animals as a platform to search for disease modifiers by transcriptional profiling and both forward and reverse genetics and to screen for new drugs (*vide infra*) makes humanized *C. elegans* animals unique tools for human disease research.

C. elegans AS A MODEL SYSTEM TO STUDY HOST-PATHOGEN INTERACTIONS

Although *C. elegans* is a bacteriophagus organism, these animals are killed by a variety of human pathogens including gram-negative (*Pseudomonas aeruginosa*, *Salmonella enterica*, *Serratia marcescens*, *Burkholderia cepacia*) and gram-positive organisms (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*) as well as fungi (*Candida* species) (reviewed in (60-63)). Seminal studies conducted in the laboratories of Ausubel, Ewbank and others show unequivocally that many of the host resistance (e.g., antimicrobial peptides (AMPs), MAPK signaling cascades) and bacterial (and fungal) virulence factors (e.g., *Pseudomonas* exotoxin A, *Staphylococcus* V8 protease) that operate in *C. elegans* infection models are conserved in those of plants, arthropods and mammals. Thus, *C. elegans* infection models serve as a useful system for dissecting apart the molecular components of host-pathogen interactions.

P. aeruginosa is a virulent opportunistic gram-negative organism that causes severe sepsis, pneumonia and skin and soft tissue infections in patients with compromised immunity or barrier function (64). Chronic infections with mucoid strains of *P. aeruginosa* occur in patients with cystic fibrosis, which is intimately associated with an inexorable decline of pulmonary function (64,65). *P. aeruginosa* relies on a range of virulence factors including adhesins, secreted peptidases (LasA, LasB, alkaline protease and protease IV), exotoxin A and type III secretion system toxins (ExoU, ExoS, ExoT and ExoY) to infect its host (64,65). While there are significant anatomical, cellular and biochemical differences between the human lung and the nematode intestine (e.g., that the absence of an adaptive immune system), this latter system does provide the opportunity to identify 1) primordial virulence factors that *P. aeruginosa* employs to breech the most salient metazoan defense, the epithelial surface, 2) elemental host resistance factors used to repel potential pathogens and 3) new antimicrobial compounds active *in vivo* (*vide infra*).

Depending on the culture conditions and the bacterial strain, *C. elegans* fed on lawns of *P. aeruginosa* are killed by fast (phenazine toxin or cyanide-induced paralysis) or slow (intestinal infection) mechanisms. To identify new virulence factors, a library of 2400 *P. aeruginosa* PA14 Tn*pho*A insertion mutants were screened in a slow-killing assay by feeding the organisms to *C. elegans*. Eight mutants with attenuated killing were isolated. Five of these mutants (*lasR*, *lemA* and *mtrR* were known virulence factors while *ptsP* and open reading frame 338 were novel) also showed decreased virulence in a mouse full-thickness burn model (66). Thus, the use of high-throughput genetic systems provides a means to identify additional *P. aeruginosa* virulence factors that can be targeted for a therapeutic advantage.

Significant portions of the *C. elegans* pathogen sensing and stress-signaling pathways are conserved in flies and mammals. Since there are no Rel/NFκB family members and a single Toll-like receptor gene (*tol-1*) appears to participate in a pathogen avoidance pathway, but not in pathogen recognition *per se* (67,68), the induction of innate immunity in *C. elegans* differs somewhat from that of *Drosophila* and higher vertebrates. For example, humans employ a repertoire of Toll-like receptors (TLRs) and a core-signaling cassette using Rel/NFκB family members to activate the transcription of host defense molecules (reviewed in (69-71)). Using a forward genetics screen for genes that enhanced susceptibility to pathogens, *C. elegans* was shown to possess several well-defined MAPK signaling cassettes (including those associated

with PMK-1 (P38), KGB-1 (JNK) and MPK-1 (ERK)) that are crucial for mounting an innate immune defense (reviewed in (61)). These MAPK pathways also participate in the innate defenses of arthropods and mammals and are activated most likely by the MAPKKK, TAK-1. TAK-1 is activated in response TGF-β and Toll/TLR signaling. *C. elegans* also utilize TGF-β and insulin growth factor (Daf) signaling pathways to activate host resistance to pathogens (61). Although *C. elegans* does not harbor a Rel/NFκB signaling cassette, it does encode for several components of the cascade including TRF-1 (TRAF-1), PIK-1 (IRAK), IKB-1 (IKB) and TIR-1 (68,72). TOL-1 and TIR-1 are the only *C. elegans* proteins that contain a Toll-interleukin-1 receptor (TIR) domain (73,74). TIR domains are well-conserved in eukaryotic cells including plants and serve as binding sites for adapter proteins (e.g., MyD88, Mal) involved in host defense signaling (69-71,74). In response to bacterial challenge, *C. elegans* TIR-1 serves as an upstream activator of an MAPK signaling pathway (73-75). Interestingly, TIR-1 has its greatest homology in humans to SARM (73-75). Although SARM contains a TIR domain, it does not function in TLR signaling cascades either. It has not been determined whether SARM activates MAPK cascades in mammals. These findings suggest that the MAPK and TLR signaling pathways are linked biochemically and share several orthologous signaling proteins and transcriptional targets (e.g., AMP genes). Taken together, genetic mutant analysis, transcriptional profiling and biochemical studies in *C. elegans* show that MAPK signaling is one of the primordial, yet still essential, stress response pathways in eukaryotes and, in part, formed the basis of TLR signaling in metazoans (74). A better delineation of these signaling pathways should enhance our understanding of innate host resistance to a range of human pathogens.

C. elegans AS A MODEL OF PARASITIC NEMATODES

The phylum, Nematoda, harbors the largest number of metazoans on earth. While many of the species are free-living, some cause significant human suffering by damaging crops and livestock. In addition, human parasitic nematodes infect ~2/5 of the world's population and cause debilitating diseases such as lymphatic filariasis/elephantiasis (*Brugia malayi* and *timori*, *Wuchereria bancrofti*), onchocerciasis/river blindness (*Onchocerca volvulus*), pinworm (*Enterobius vermicularis*), hookworm (*Necator americanus*, *Ancyclostoma duodenle*), trichinosis (*Trichinella spiralis*), ascariasis (*Ascaris lumbricoides*), strongyloidiasis (*Strongyloides stercoralis* and *fuelleborni*) and Guinea worm (*Dracunculus medinensis*) (76). Although a mass drug administration effort by the Global Programme and Alliance to Eliminate Lymphatic Filariasis has met with some success (77), concerns of evolving drug resistance may jeopardize these efforts (78).

Since human parasitic nematodes are not free-living, may require intermediate hosts (e.g., sandflies or mosquitoes) to complete their life cycles and frequently lack surrogate laboratory hosts or *in vitro* culture systems that allow long-term propagation; current laboratory-based approaches are limited in their ability to deliver new therapeutic modalities (e.g., RNAi), vaccine candidates or anthelmintics. Fortunately, similarities between genomes, anatomy, development, pharmacology and physiology have allowed certain aspects of the parasitic lifestyle to be modeled in *C. elegans* (79,80). Many of the drugs used to treat parasitic nematodes target the neuromuscular unit and are toxic to *C. elegans* (78). For example, benzimidazoles, which are a mainstay of current anthelmintic therapy by inhibiting β-tubulin, also lead to paralysis of *C. elegans.* A genetic screen for drug resistant *C. elegans*, using EMS or γ-irradiation as the mutagen, identified 28 different lines with mutations that mapped to one of three β-tubulin genes, *ben-1* (81). Subsequently, molecular analysis of drug resistance in *Haemonchus contortus* revealed amino acid variants at three different positions, 76, 200 and 368, in the β-tubulin isotype 1 allele, *tub-1* (82). The Phe200Tyr variant was most suspicious as this allele always segregated with the drug resistant strains and this exact codon position distinguishes *ben-1* as the only benzimidazole-sensitive β-tubulin in *C. elegans*. To confirm

that the Phe200Tyr mutation was the cause of drug resistance in *H. contortus*, *ben-1* null *C. elegans* transformed with the wild-type, but not the mutant, allele of *tub-1* restored drug responsiveness. These studies show how a combination of forward genetic screens and transgenic rescue in *C. elegans* was used to identify drug targets and study mechanisms of drug resistance in parasitic nematodes.

C. elegans AS MODEL FOR DRUG DISCOVERY

With the advent of the NIH Molecular Library Screening Network, which encompasses compound collections totaling hundreds-of-thousands of molecules and high-throughput screening (HTS) technologies, small molecule drug discovery campaigns are a realistic goal for a wide range of human diseases (83). Typically, these screening campaigns involve monitoring the effects of different compounds on cellular or molecular activities within individual wells of a 98-, 384- or 1536-well microplates. HTS has become more informative by the development of high-content screens that employ 1) fluorescent probes with different excitation and emission spectra, 2) automated fluorescent microscopy screening devices and 3) complex image capture and analysis algorithms to qualitatively, quantitatively, temporally and spatially assess drug effects on individual living cells within a single well of a microplate (84). More recently, HTS drug discovery screens have been adapted to using whole animals such as *C. elegans* and zebrafish embryos. Drug discovery screens with whole animals have the advantage of identifying compounds that modulate more complex phenotypes and for eliminating compounds with systemic toxicity early in the discovery process. The genetic tractability of *C. elegans* has the added advantage of alleviating one of the major bottlenecks of drug development, target validation (85). Kwok et al., exposed larval forms of *C. elegans* to ~14,000 different compounds, and identified ~300 that induced a variety of defects. One compound, nemadipine-A, caused growth and egg-laying defects (85). This compound belongs to the 1,4-dyhydropyridine (DHP) class of anti-hypertensives, which block the α_1 subunit of L-type calcium channels. To help identify the drug target, a suppressor screen was conducted by exposing 180,000 genome equivalents to EMS and isolating several dominant genetic suppressor mutants that were no longer sensitive to the drug. The mutant genes mapped to *egl-19*, the only *C. elegans* L-type calcium channel α_1 subunit. DNA sequence analysis showed that one of the missense mutations altered a conserved residue required for mammalian channels to bind DHPs. These studies confirmed that live whole animal screens using *C. elegans* could be used as a biologic platform for both drug discovery and target validation. Moreover, the abilities to: 1) create transgenic animals with different types of fluorescent fusion proteins, 2) partition staged animals with similar degrees of transgene expression into microplate wells using the COPAS *BIOSORT* (worm sorter), 3) automate reagent delivery by liquid handling robots and 4) monitor changes in overall animal size and internal florescence intensity/distribution in response to drug treatment using automated wide-field or confocal microscopes coupled to image capture and analysis software (Fig. 3), suggest that *C. elegans* will become the organism of choice for conducting HTS and high-content screening campaigns for gene (e.g., RNAi) and drug discovery for a wide range of human disease phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: The authors are supported by grants from the Cystic Fibrosis Foundation, The Hartwell Foundation and the National Institutes of Health (DK079806).

Abbreviations

Aβ, β-amyloid peptide
AMPs, antimicrobial peptides
AT, α1-antitrypsin
ATZ, Z-mutant of AT
$[Ca^{2+}]_i$, intracellular calcium concentration
EMS, ethyl methane sulfonate
gf, gain-of-function
HTS, high-throughput screening
HSNs, hermaphrodite specific neurons
HSP, heat-shock proteins
Lf, loss-of-function
polyQ, polyglutamine
TLRs, Toll-like receptors

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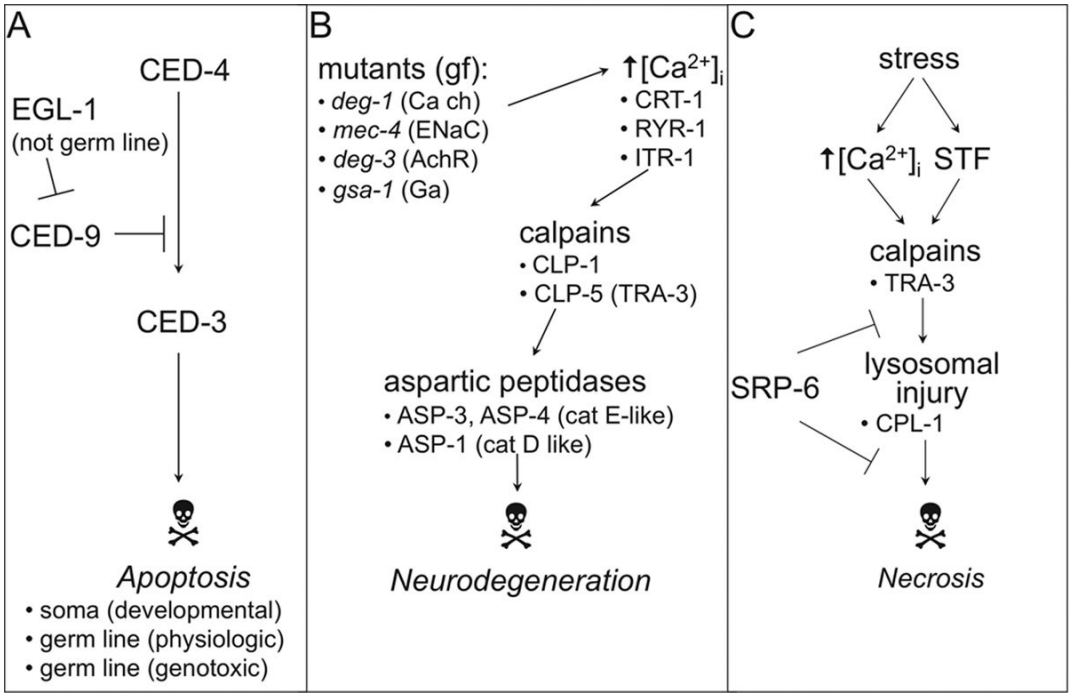

Figure 1.

C. elegans cell death pathways. *A.* The now classical core apoptosis pathway occurs in development and the germ line, but rarely in the adult soma. Upstream regulators block activation of the caspase, CED-3. *B.* The neurodegenerin pathway was first described in mechanosensory neurons and stimulated by mutations that elicit excitotoxic injury. A rise in $[Ca^{2+}]_i$ triggers peptidase activity that kills the cells over several hours. Although not pictured, lysosomal injury and acidification of the cytoplasm may contribute to injury. The necrosis pathway is triggered after different types of stress (hypotonic, thermal, oxidative, hypoxic) and is most evident within the intestinal cytoplasm. *C.* An intracellular serpin (SRP-6) protects the lysosome from stress-induced injury and also protects the cell itself from lysosome-induced damage. There is strong genetic, biochemical and morphological evidence for the conservation of these pathways in higher eukaryotes, including humans.

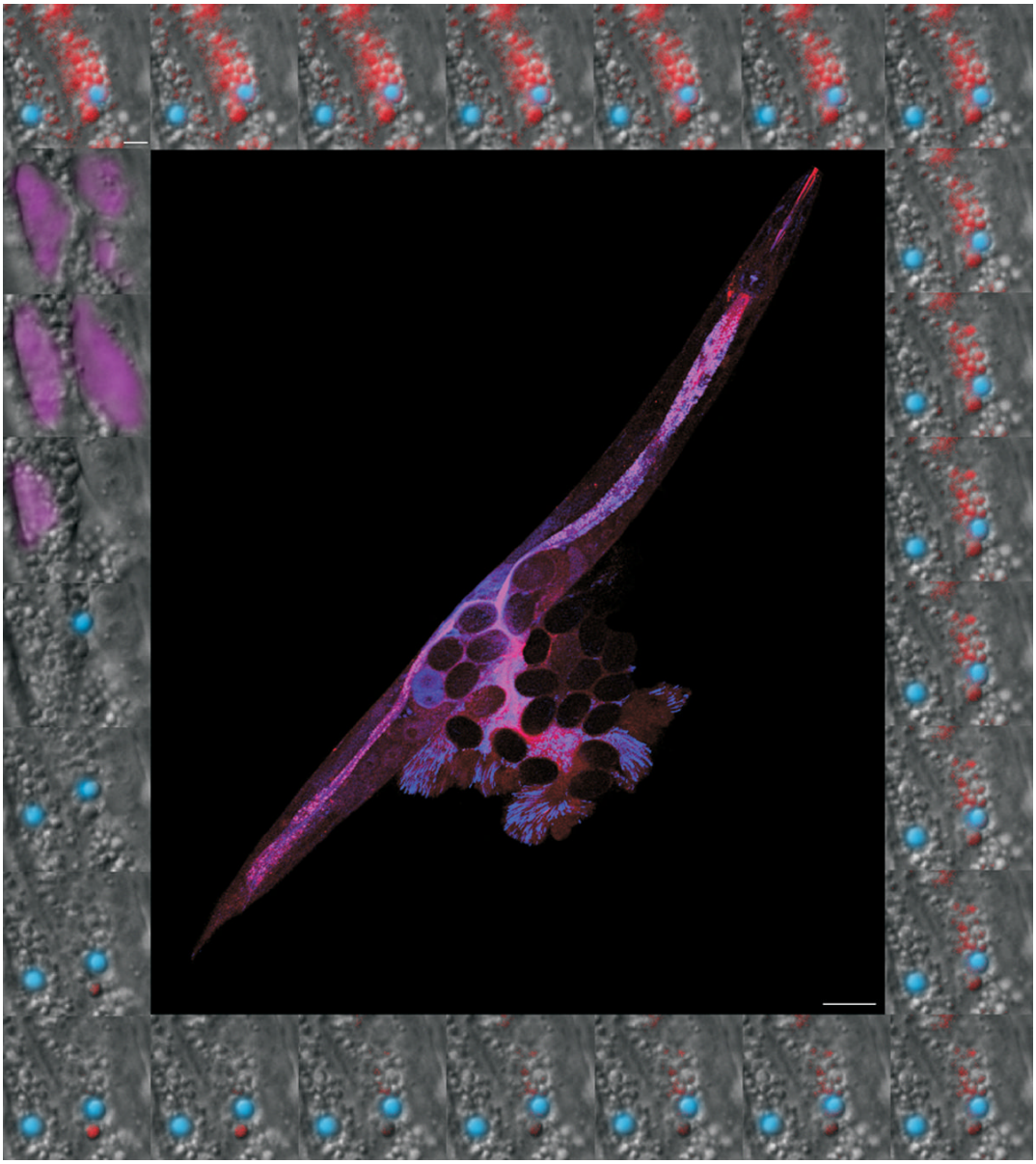

Figure 2.

Intestinal cell necrosis in *C. elegans*. (Center) Three dimensional image showing hypotonic shock-induced intestinal cell necrosis in *srp-6* null *C. elegans* stained with Lysotracker Red and the cysteine peptidase substrate, $(Z\text{-}FR)_2$-R110. Animals undergo extensive lysosome disintegration (red) and propagate a wave of cysteine peptidase activity (blue) across the cytoplasm. As an agonal event, adult worms often extrude their uteri and necrotic intestine through the vulva. Images were collected using an Olympus Fluoview1000 confocal microscope with 488 and 568 lasers. The micrograph is an overlay of fluorescent images taken over a z-series and merged. Volocity (Improvision) was used to psuedocolor and render the images. Background subtraction was performed using Canvas (Deneba). Scale bar = 50 μm. (Perimeter) Four-dimensional, time-lapsed images of intestinal cell necrosis occurring in live *srp-6* null *C. elegans* stained with Lysotracker Red. Merged DIC and fluorescent images start at upper left-hand corner and progress clockwise. Images were captured at 20-second intervals (total time ~10 minutes) after the initiation of hypotonic shock. Intestinal cells undergo

lysosomal rupture (red) and large vacuoles appear (purple) as intestinal cell necrosis progresses over time. Images were collected using a Zeiss Axioskop II Mot microscope using DIC optics at 250 × magnification. Fluorescent images were captured using excitation and emission wavelengths of 572 nm and 630 nm, respectively. Volocity and Canvas was used to pseudocolor and render the images. Scale bar (first frame) = 5 μm.

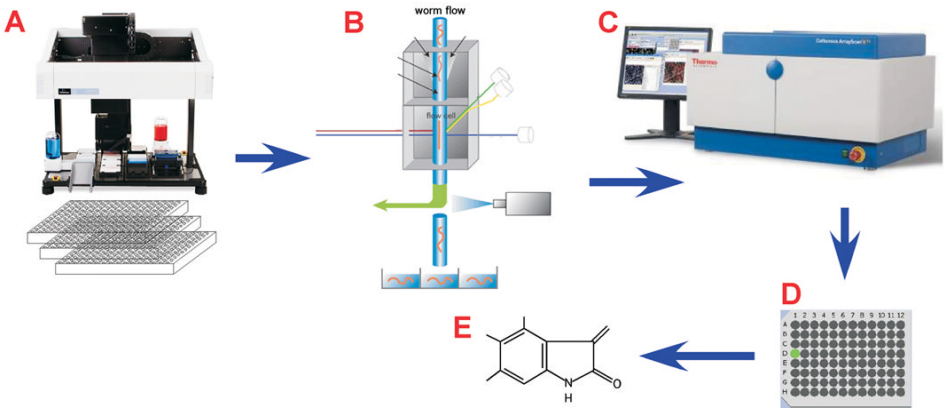

Figure 3.

Global strategy for high-throughput/high-content screening of lead compounds using *C. elegans*. *A*, compounds, culture media and *E. coli* are dispensed into multi-well (96- or 384-well) plates using a high-throughput liquid handler (Evolution-P3 ©2001–2008 Perkin Elmer Inc., all rights reserved, printed with permission). *B*, Animals of the desired size and/or fluorescence profiles are sorted into multi-well plates using a COPAS *BIOSORT* (Union Biometrica Inc., image used with permission). *C*, An automated high-throughput imaging system, ArrayScan R VTi HCS Reader (Thermo Fisher Scientific, images used with permission) captures images and identifies changes in phenotype of interest. *D*, Lead compounds are identified and further scrutinized to confirm positives and eliminate nuisance compounds. *E*, New compounds are synthesized to develop potential therapeutics.

Table 1

An expanded security council of genetic organisms

Organism	Description	Biological systems studied
Bacillus subtilis	a gram-positive bacterium	flagellar function, sporulation and differentiation, DNA replication
Escherichia coli	a gram-negative bacterium	gene regulation, antibiotic resistance, DNA transformation, molecular cloning, host-pathogen interactions
bacteriophages		site-specific DNA recombination, transcriptional activation and repression, lysogenicity
Saccharomyces cerevisiae	budding (baker's) yeast	DNA replication and repair, cell cycle control control, cell divisionMAP kinases, homologous DNA recombination, ERAD. two-hybird techniques
Chlamydomonas reinhardtii Caenorhabditis elegans	green alga nematode, roundworm	cell motility, response to light, energy metabolism development, sex determination, aging, cell death (apoptosis and necrosis), RNAi
Drosophila melanogaster Mus musculus	arachnid, fruitfly mammal, laboratory mouse	innate immunity, multiple developmental pathways cancer, aneuploidy syndromes
Arabidopsis thalania	plant, thale cress, mustard weed	photosensing, innate immunity
Rattus norvegicus Danio rerio	laboratory rat vertebrate, fish	QTLs, cardiovascular diseases vertebrate morphogenesis, hematopoiesis, cardiovascular development
Schizosaccharomyces pombe	fission yeast	cell cycle regulation