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Genome-wide RNAi screens in *Caenorhabditis elegans*: impact on cancer research

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Genes linked to human cancers often function in evolutionary conserved pathways, and research in *C. elegans* has been instrumental in dissecting some of the pathways affected, such as apoptosis and Ras signalling. The advent of RNA interference (RNAi) technology has allowed high-throughput loss-of-function analyses of *C. elegans* gene functions. Here we review some of the most recent genome-wide RNAi screens that have been conducted and discuss their impact on cancer research and possibilities for future screens. We also show that genes causally implicated in human cancers are significantly more likely to have a *C. elegans* homologue than average, validating the use of *C. elegans* as a cancer gene discovery platform. We foresee that genome-wide RNAi screens in *C. elegans* will continue to be productive in identifying new cancer gene candidates and will provide further insights into cancer gene functions.
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Introduction

Cancer has been described as a multistep process requiring six essential alterations in cell physiology: (i) self-sufficiency in growth signals, (ii) insensitivity to antigrowth signals, (iii) evasion of apoptosis, (iv) limitless replicative potential, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis (reviewed in Hanahan and Weinberg, 2000). To study these principles of malignant growth, numerous models are being used. The mouse model is one of the best because of the similarities between mouse and human tumours. However, it has disadvantages such as cost and space, and transgenic technologies are time consuming and tedious. An alternative is to use other model organisms such as *Xenopus*, *Drosophila*, or *Caenorhabditis elegans*. These model organisms have contributed importantly to our understanding of basic biological phenomena that are at the heart of the six alteration principles proposed by Hanahan and Weinberg.

In this review, we will focus on *C. elegans* and how it can contribute to cancer gene discovery. Although *C. elegans* does not form tumours *per se*, other types of mutant phenotypes are clearly relevant for cancer research. As a few examples, there are mutants where cell division is unregulated (e.g. *cki-1*, a cycline kinase inhibitor (Kostic *et al*., 2003) and *glp-1*, a Notch-like gene (Berry *et al*., 1997)), where cells that should undergo apoptosis fail to die (e.g. *ced-3* (Ellis and Horvitz, 1986), *ced-9* (Hengartner *et al*., 1992), and *cep-1 TP53* (Derry *et al*., 2001; Schumacher *et al*., 2001)), and where the genome is unstable, generating spontaneous mutations (e.g., *msh-2* and *msh-6*, mismatch repair genes; Tijsterman *et al*., 2002). For each of these phenotypes and for many others, some of the mutations causing the defects identify genes linked to cancer in humans. Screening for *C. elegans* genes disrupting processes relevant to cancer and concentrating research in these areas should further our understanding of the basic processes underlying cancer.

C. elegans as a model in the post-genomic era

Among multicellular model organisms, *C. elegans* can be considered a pioneer of the post-genomic era (Sternberg, 2001). *C. elegans* was the first metazoan to have its genome sequenced, its genome annotation is of remarkable accuracy, and the *C. elegans* database Wormbase presents data to the world in a user-friendly format. About 60% of *C. elegans* genes have similarity to a human gene (Harris *et al*., 2004). A real breakthrough for increasing the usefulness of *C. elegans* as a model for identifying and studying conserved gene functions was the discovery of RNA interference (RNAi). Previously, classical genetics was the most efficient way of identifying gene functions in *C. elegans* and it did so in a remarkable manner, being acknowledged by the 2002 Nobel Prize in Physiology and Medicine to Sydney Brenner, Robert Horvitz, and John Sulston. Nevertheless, gene identification through forward genetics is still labour intensive.

Fire and Mello discovered that injection of double-stranded RNA (dsRNA) into worms leads to specific degradation of the corresponding mRNA (Fire *et al*., 1998). Soon afterwards, it was shown that either soaking worms in dsRNA solution or feeding worms bacteria engineered to produce dsRNA could also elicit a robust RNAi response (Tabara *et al*., 1998; Timmons and Fire, 1998). Of these three methods (injection, soaking,

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feeding), RNAi by feeding has the advantage that, once a bacterial strain is made, it is a permanent reagent that can be reused and replicated. The availability of the genome sequence, together with the discovery of RNAi and the advances in dsRNA delivery, made possible the building of an RNAi feeding library to inactivate most *C. elegans* genes (Fraser *et al*., 2000; Kamath *et al*., 2003).

This RNAi feeding library consists of 16 757 bacterial strains, kept as glycerol stocks, each designed to produce dsRNA to target an individual *C. elegans* gene; there is currently a bacterial strain for 86% of genes. Using this library, *C. elegans* was the first metazoan to have most of its genes subjected to a loss-of-function analysis. This tripled the number of genes associated with loss-of-function phenotypes, underlying the power of the RNAi library for rapidly finding gene functions and encouraging users of other model organisms to generate similar tools. The library can also be used for candidate-based approaches where only subsets of genes (e.g. 100–2000) are subjected to analysis. RNAi by feeding is versatile and can be performed in liquid culture in 96-well format, reducing the screening labour (Nollen *et al*., 2004). The bottleneck of genome-wide RNAi screens is the assessment of phenotypes. In many cases this will require a human scorer, and it is worth investing time to make the assay as easy and rapid as possible. Automated visual systems are starting to be used for screens where the assay is life versus death, and these should be amenable for assays of altered reporter expression as well (e.g. GFP). A genome-wide RNAi screen in liquid culture where the assay is rapid and unambiguous (life versus death or GFP+ versus GFP−) can be carried out manually by one person in about a month.

Apoptosis

Apoptosis is a genetically regulated form of cell death that is triggered by different stimuli. It is used to discard misbehaving cells, maintain homeostasis, and eliminate cells during development. Misregulation of apoptosis can lead to a range of pathological conditions, including cancer. Therefore, understanding the process and regulation of apoptosis is important for medical research.

During *C. elegans* development, 131 cells undergo programmed cell death to produce the final 959 somatic cells (Sulston and Horvitz, 1977). In addition, during oogenesis, about half of all germ cells are eliminated by apoptosis (Gumienny *et al*., 1999). As in other systems, apoptosis in *C. elegans* requires a caspase (CED-3) and an Apaf-1 homologue (CED-4), and is blocked by a Bcl-2-like protein (CED-9) (reviewed in Liu and Hengartner, 1999). DNA damage (such as γ-radiation) causes an increase in germline cell deaths, and this requires the *C. elegans* p53 orthologue *cep-1* and the checkpoint gene *hus-1* (Derry *et al*., 2001; Schumacher *et al*., 2001). Two RNAi screens for genes involved in apoptosis have already been carried out. In the first, the authors screened for an increase in germline apoptosis; germline corpses are easily detectable by acridine orange staining

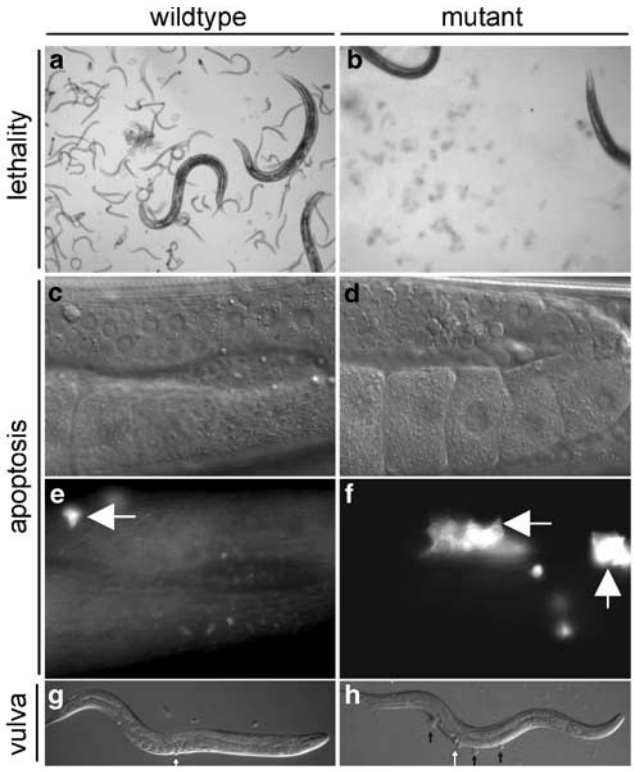

Figure 1 Examples of *C. elegans* phenotypes used for genome-wide RNAi screening. Growth, apoptosis, and vulval phenotypes are shown, with wildtype on the left and mutant on the right. (**a**, **b**) Growth /Lethality assay. Wells from an RNAi screen for lethality carried out in 96-well liquid culture format. (**a**) Wildtype adult worms put into the bacterial culture and their progenies are visible. (**b**) In a well where embryonic lethality was induced by RNAi, no progeny are visible. (**c–f**) Germline apoptosis assay. Germline corpses are visible after live staining with acridine orange. Sections of gonads viewed with DIC optics (**c** and **d**) or under fluorescence (**e** and **f**). (**c** and **e**) Wildtype adult hermaphrodite gonad with one corpse (arrow). (**d** and **f**) *ced-9* mutant gonad with excess corpses (arrows). (**g** and **h**) Multivulval assay. (**g**) A single wildtype vulva or (**h**) multiple vulvae protrusions that can be detected under the dissecting scope; this animal has a gain-of-function mutation in let-60 Ras. (**g** and **h**) White arrows point to the normal vulva and in (**h**) the black arrows indicate the ectopic vulvae

followed by fluorescent microscopy (Figure 1c–f). After screening 16 757 genes, 21 were discovered where an RNAi knockdown causes excess deaths (Lettre *et al*., 2004). Of these, 16 were previously unknown, including *pmk-3*, a p38 MAPK homologue, and *bmk-1*, a homologue of the BimC kinesin-like motor protein involved in spindle formation. Even though the screening procedure identified just a few of the expected known genes, it succeeded in finding new candidates. Of the 21 genes identified, 16 require p53 and a functional DNA damage response pathway to activate germ cell death; these genes might act in DNA repair or genome stability. In fact, the screen identified homologues of RAD50 and RAD51 which are required for DNA repair and meiotic recombination (de Jager and Kanaar, 2002; Sung *et al*., 2003). Of the five p53 independent genes found, one is *ced-9*, a regulator of physiological germ cell death (Gumienny *et al*., 1999), suggesting that the other four might also play a role in that process.

A second group used a candidate-based functional genomic screen for apoptotic DNA degradation in *C. elegans* (Parrish and Xue, 2003). Using INTERPRO and PFAM motifs to find genes corresponding to deoxyribonucleases, ribonucleases, cyclophillins, and topoisomerases, they found 77 candidates that were analysed by a terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labelling (TUNEL) assay. RNAi of nine of these candidates were positive in that assay and all of them have a human homologue. Candidate-based approaches that combine bioinformatics with RNAi technology, such as those described above, are very powerful. They can also be very rapid, since most of the selected candidates can be provided by the existing RNAi library, avoiding the step of generating reagents. Past studies of apoptosis have shown that *C. elegans* research has played a leading role, and future investigation into this process looks promising.

C. elegans cancer genes

The screens described above took advantage of a well-characterized biological process relevant to cancer (apoptosis). An alternative method for finding genes relevant to cancer is to study *C. elegans* homologues of human cancer genes. Futreal *et al.* (2004) recently compiled a list of 291 genes causally implicated in human cancer by conducting a census of the literature (listed at http://www.sanger.ac.uk/genetics/CGP/Census/). We took the subset of 61 of these cancer genes where germline mutations have been found and searched for *C. elegans* homologues. Strikingly, 80% have a putative worm homologue (Table 1), and a mutant or RNAi phenotype has been reported for 53% of these (the total set of cancer genes yielded similar results). These genes can be used as starting points for conducting RNAi screens for new cancer genes. For example, mutants of the mismatch repair genes *msh-2* and *msh-6* have mutator activity in *C. elegans* and an assay for genome instability was developed where these mutants scored positive (Tijsterman *et al.*, 2002). Pothof *et al.* (2003) then used this assay in a genome-wide RNAi screen and identified 61 genes required for genome stability (van Haaften, this issue); these are excellent cancer gene candidates. Indeed, *C. elegans* homologues of two of the human cancer genes in Table 1, the DNA mismatch repair proteins PMS2 and MLH1, were identified in that screen. Similar approaches could be used for other genes on this list. For example, screens could be designed to find genes with similar knockdown phenotypes or to find genes that show a synthetic phenotype with the *C. elegans* cancer genes. Mammalian counterparts of these genes could then be studied in human cells or mice. This type of approach will potentially be a powerful and fruitful way to systematically identify new cancer genes.

Synthetic interactions

Most if not all oncogenes require cooperation with other factors. For example, activated forms of Ras cause cell-cycle arrest or apoptosis; however, if p53 is simultaneously inactivated, uncontrolled proliferation occurs. Hence, cancer could be classified as a type of synthetic disease. In a classical synthetic genetic interaction, single mutants of gene *A* or gene *B* do not show a phenotype, but an *A;B* double mutant does. Applying this to gene discovery, carrying out a synthetic genetic screen is an effective way to look for factors that cooperate together. As an example, one could carry out RNAi screens with a strain mutant for p53 (*cep-1*). After RNAi of each gene in this background, a number of synthetic phenotypes could be assayed (e.g., death, proliferation of the germline, over- or underexpression of a reporter, etc.). Homologues of positive candidates could then be tested for synthetic effects with p53 in human cells or transgenic mice. The value of this type of approach is illustrated by a recent screen for RNAi knockdowns that cause synthetic lethality with a mutant that generates double-strand breaks; several of the genes identified are needed for resistance to ionizing radiation (van Haaften *et al.*, personal communication).

The Ras and synthetic Multiple vulvae (synMuv) pathways

The RTK/Ras/Raf/MAPK signalling pathway is highly conserved and is used in many contexts during animal development; unregulated Ras signalling can lead to cancer. Work in model organisms has greatly contributed to our understanding of this pathway and its regulation (Wassarman *et al.*, 1995; Tan and Kim, 1999; Moghal and Sternberg, 2003). In *C. elegans*, Ras signalling has been most studied in the development of the vulva, the egg-laying organ, where Ras activity is needed for adoption of the vulval cell fate. In mutants where Ras signalling is hyperactivated, nonvulval cells are transformed to the vulval fate. This condition leads to ectopic vulval development, visualized by the presence of multiple vulvae (Figure 1g and h; Moghal and Sternberg, 2003). To prevent ectopic vulval development, Ras signalling is antagonized through the synMuv genes, which include components of Rb histone deacetylase (Lu and Horvitz, 1998; Ceol and Horvitz, 2001) and NuRD chromatin remodelling complexes (Solari and Ahringer, 2000; von Zelewsky *et al.*, 2000). In other systems, these complexes have transcriptional repressor activity (Harbour and Dean, 2000; Feng and Zhang, 2003), suggesting that Ras signalling is antagonized by transcriptional repression of vulval development genes (Fay and Han, 2000). The *C. elegans* vulva is an excellent system for studying the regulation of the Ras signal transduction cascade and of its antagonism by chromatin remodelling proteins.

Classical forward genetic screens have identified numerous regulators of Ras signalling (reviewed in Sternberg and Han, 1998). Many of these screens involved looking for enhancers or suppressors of altered Ras signalling. For example, an activating mutation in *let-60* RAS causes ectopic vulval development (Figure 1g and h); several genetic screens for suppressors of this

Table 1 *C. elegans* homologues of germline mutated human cancer genes

Human gene	*Description*	*Worm hit*	E-*value*	*Worm locus*	*RNAi/mutant phen*
APC	Adenomatous polyposis coli protein (APC protein)	K04G2.8b	1.90E−33	apr-1	Emb, Lva, Bmd
ATM	Serine-protein kinase	Y48G1BL.2	2.70E−62	atm-1	None
BLM	Bloom's syndrome protein	T04A11.6	2.00E−118	him-6	Him
BMPR1A	Bone morphogenetic protein receptor type IA precursor	F29C4.1a	4.60E−62	daf-1	Daf
BRCA1	Breast cancer type 1 susceptibility protein	C36A4.8	0.121(lit)	brc-1	Gro
BRCA2	Breast cancer type 2 susceptibility protein	T07E3.5	(lit)	—	None
CDH1	Epithelial-cadherin precursor (E-cadherin)	W02B9.1b	1.10E−21	hmr-1	Emb, Bmd, Dpy, Unc
CDK4	Cell division protein kinase 4	F18H3.5b	7.40E−58	cdk-4	Pvl, Dpy, Ste, Lva
CYLD	cylindromatosis (turban tumor syndrome)	F40F12.5	3.40E−53	—	None
ERCC2	TFIIH basal transcription factor complex helicase subunit	Y50D7A.2	0	—	—
ERCC3	TFIIH basal transcription factor complex helicase	Y66D12A.15	0	—	—
ERCC4	DNA-repair protein complementing XP-F cell	C47D12.8	5.90E−87	—	None
ERCC5	DNA-repair protein complementing XP-G cells	F57B10.6	1.80E−22	—	None
EXT1	Exostosin-1 (Putative tumor suppressor)	F12F6.3	3.00E−94	rib-1	None
EXT2	Exostosin-2 (Putative tumor suppressor protein E)	K01G5.6	7.90E−60	rib-2	None
FANCD2	Fanconi anemia, complementation group D2	Y41E3.9	6.50E−06	—	—
FH	Fumarate hydratase, mitochondrial precursor	H14A12.2a	0	fum-1	Emb, Gro
HRPT2	Hyperparathyroidism 2	F35F11.1	2.20E−48	—	—
MADH4	Mothers against decapentaplegic homolog 4 (SMAD 4)	R12B2.1	4.40E−39	sma-4	Dpy
MLH1	DNA mismatch repair	T28A8.7	1.00E−104	mlh-1	None
MSH2	DNA mismatch repair protein Msh2	H26D21.2	2.00E−111	msh-2	Mut
MSH6	DNA mismatch repair	Y47G6A.11	0	msh-6	Mut
NF1	Neurofibromatosis-related protein NF-1	ZK899.8h	7.10E−25	gap-2	None
NF2	Merlin (Moesin-ezrin-radixin-like protein)	C01G8.5a	1.00E−120	erm-1	Emb, Ste, Gro, Unc
NM_144606	folliculin isoform 1	F22D3.2	4.70E−15	—	None
PMS1	DNA mismatch repair protein	H12C20.2a	2.20E−41	pms-2	None
PMS2	DNA mismatch repair protein	H12C20.2a	8.00E−123	pms-2	None
PRKAR1B	cAMP-dependent protein kinase type I	R07E4.6a	3.00E−117	kin-2	Ste, Bli, Dpy, Gro
PTCH	Patched protein homolog 1	ZK675.1	1.10E−93	ptc-1	Emb, Lva, Unc, Egl
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase	T07A9.6	1.90E−46	daf-18	Daf
RB1	Retinoblastoma-associated protein (RB)	C32F10.2	1.90E−21	lin-35	Gro
RECQL4	ATP-dependent DNA helicase (RecQ4)	F18C5.2	1.90E−40	wrn-1	None
RET	tyrosine-protein kinase receptor ret precursor (C-ret)	F58A3.2b	1.50E−71	egl-15	Egl, Unc, Pvl
SBDS	Shwachman-bodian-diamond syndrome protein	W06E11.4	2.50E−74	—	None
SDHB	Succinate dehydrogenase	F42A8.2	2.00E−100	—	Emb, Gro, Lva, Bmd
SDHC	Succinate dehydrogenase cytochrome b560 subunit	T07C4.7	7.90E−16	mev-1	Emb, Ste, Gro
SDHD	Succinate dehydrogenase cytochrome B	F33A8.5	7.90E−07	cey-1	Emb, Lva
SMARCB1	SWI/SNF related, matrix associated, (hSNF5) (BAF47)	R07E5.3	6.80E−94	—	Emb, Egl, Slu
STK11	Serine/threonine- protein kinase LKB1	Y59A8B.14	1.20E−54	par-4	Emb
TP53	Cellular tumor antigen p53 (Tumor suppressor p53)	F52B5.5	0.037§ (lit)	cep-1	Him, Rad-R
TSC2	Tuberin (tuberous sclerosis 2 protein)	F53A10.2a	2.80E−14	—	None
TSHR	Thyrotropin receptor precursor	C50H2.1	8.50E−76	—	Emb
VHL	Von Hippel–Lindau disease tumor suppressor (pVHL)	F08G12.4	0.003	vhl-1	Heat-R
WRN	Werner syndrome helicase	F18C5.2	1.00E−107	wrn-1	None
WT1	Wilms' tumor protein	Y55F3AM.7	1.60E−28	—	None
XPA	DNA-repair protein complementing XP-A cells	K07G5.2	8.70E−39	xpa-1	Emb, Rad-R
XPC	DNA-repair protein complementing XP-C cells	Y76B12C.2	6.30E−68	—	None

We obtained the list of 61 human cancer genes with known germline mutations from the cancer gene census (http://www.sanger.ac.uk/genetics/CGP/Census/) and the corresponding protein sequences were compared to *C. elegans* wormpep 125 using BlastP. We found 42 matches with an *E*-value less than or equal to e-10 (§ refers to an *E*-value to the mouse homolog). The remaining 19 were checked manually for matches to *C. elegans* proteins using proteome and wormbase and manual BlastP, and reciprocal best matches with higher *E*-values kept. We attempted to annotate genes with no matches after this step using literature information (lit; see below). In total, we found matches for 47 of the 61 human genes. Human genes with no known *C. elegans* homologue are: CDKN2A, DDB2, FANCA, FANCC, FANCE, FANCF, FANCG, KIT, MEN1, MUTYH, NBS1, SUFU, TCF1 and TSC1. RNAi and mutant phenotype information was gathered from Wormbase. The phenotypes are Bli: Blister, Bmd: Body morphology defect, Daf: Dauer formation defective, Dpy: Dumpy, Egl: Egg-laying defective, Emb: Embryonic lethal, Gro: Growth rate retarded, Heat-R: Heat resistant, Him: High incidence of male, Lva: Larval arrested, Mut: Mutator, Prz: Paralysed, Pvl: Protruding vulva, Rad-R: Radiation resistant, Slu: Suggish, Ste: Sterile and Unc: Uncoordinated. In the 'RNAi/mutant phen' column, 'none' means an RNAi experiment has been performed and no defects were noted; '—' means that no mutant or RNAi information is available for that gene. Homologues found through literature searches (lit) are BRCA1 (Boulton *et al*., 2004), BRCA2 (Wong *et al*., 1997), and TP53 (Derry *et al*., 2001; Schumacher *et al*., 2001)

phenotype identified positive regulators of the pathway (Kornfeld *et al*., 1995; Sundaram and Han, 1995; Sieburth *et al*., 1998). Repeating the classic screens (e.g., suppressor or enhancer of activated Ras) using RNA interference will likely prove effective in identifying additional regulators, since, in a pilot RNAi screen of 1000 genes, three new suppressors of activated *let-60* Ras were found (AG Fraser and JA, unpublished). An advantage of the RNAi screening approach is that it can easily identify genes where loss-of-function mutants are unviable; these can be difficult to isolate in a standard genetic screen. Indeed, an RNAi screen for synMuv mutants identified 11 new genes, most of which are needed for viability, whereas only about 30% of

synMuv genes identified by genetics are essential; several of the new genes encode components of the sumoylation pathway, suggesting that sumoylation of one or more proteins has a role in antagonizing Ras signalling (GP and JA, unpublished results).

Cell-invasive behavior

Some cells have an invasive activity that allows them to travel through basement membranes, and this is important in several developmental contexts including blastocyst implantation and organogenesis. Metastatic cancer is associated with a loss of regulation over invasive activity. Recently, a *C. elegans* model system for studying cell invasiveness was developed (Sherwood and Sternberg, 2003). In making the connection between the gonad and the vulva, a gonadal cell called the anchor cell (AC) invades the basement membranes of vulval cells. The authors identified mutant conditions where only 20% animals showed invasion by the AC. By screening for reduced or increased invasiveness in this background, one might be able to identify genes that control invasive activity.

Conclusion

Cancer is a complex illness involving many pathways conserved in *C. elegans*. The development of RNAi technology as a tool for systematic screening in *C. elegans* has already been fruitful for identifying new gene functions relevant to cancer. Careful investigation of the new phenotypes observed can provide a deeper understanding of the underlying biology and give ideas for the design of new RNAi screens. As human cancer genes are more highly conserved than other genes on average, the new genes identified are also expected in many cases to have human counterparts. A drawback to RNAi screening is that it is a loss-of-function method – it cannot be used to find gain-of-function mutants, which in the past have been very informative in understanding cancer processes. Nevertheless, taken together, RNAi screening and phenotypic analysis tools in *C. elegans* are extremely powerful for uncovering new gene functions and driving new mechanistic hypotheses. It will be important to investigate these new hypotheses in both *C. elegans* and mammalian systems; collaborations between laboratories with different and complementary expertise should lead to a better understanding of the biological processes disrupted in cancer cells.

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