RNAi screens to identify components of gene networks that modulate aging in *Caenorhabditis elegans*

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Abstract

Our understanding of the genetic mechanisms of organismal aging has advanced dramatically during the past two decades. With the development of large-scale RNAi screens, the last few years saw the remarkable identifications of hundreds of new longevity genes in the roundworm *Caenorhabditis elegans*. The various RNAi screens revealed many biological pathways previously unknown to be related to aging. In this review, we focus on findings from the recent large-scale RNAi longevity screens, and discuss insights they have provided into the complex biological process of aging and considerations of the RNAi technology will continue to have on the future development of the aging field.

Keywords: *aging; RNAi screen; gene networks; C. elegans; lifespan*

INTRODUCTION (AGING RESEARCH DURING PRE-RNAi ERA)

Studying the phenomenon of aging and trying to extend lifespan have been long-standing interests of mankind. For a long time, environmental changes had been the only interventions that people have attempted to increase lifespan. With the development of molecular biology, genetics and genomics approaches, great efforts have been taken to search for the genetic components that affect organism lifespan. However, the nature of lifespan studies present one major obstacle to move this field rapidly, that is, the longitudinal monitoring of the life of a population takes time. For our most interested species, *Homo sapiens*, it will probably take two generations to monitor one generation and do all the proper analyses. So it makes great sense to use short-lived model organisms, e.g. mouse, fly, roundworm and yeast, to identify the genetic basis and mechanisms of aging since many important cellular processes are highly conserved among species. Among these, research using the roundworm *Caenorhabditis elegans* present many groundbreaking findings because it is amenable to genetic manipulation, its genome is known, and the wild-type worm has a very short lifespan (2–3 weeks) comparing to many other model organisms [1, 2].

The first forward genetics screen using *C. elegans* to isolate long-lived mutants only identified eight mutant strains [3]. It led the author to conclude that 'specific life span genes are extremely rare or, alternatively, life span is controlled in a polygenic fashion'. The eight mutants either constitutively form dauer (a developmentally arrested and long-lived larval stage in response to harsh environment), or are defective in chemotaxis, or have defects in pharyngeal pumping that limits food intake [3]. However, the first single gene locus affecting lifespan (*age-1*, a gene later found to encode a PI3 kinase) was not identified until 5 years later [4, 5].

During the last two decades, aging research has advanced rapidly with many genetic mutants isolated in several longevity pathways. Here we will briefly review some of these longevity mutants (see details reviewed elsewhere [6–9]) and focus on the new

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insights that RNAi technology brought into the existing aging gene network.

In 1993, mutations in the *daf-2* gene (encoding the insulin/insulin-like growth factor-1 receptor) were discovered to live twice as long as wild-type [10, 11]. *daf-2* mutants constitutively form dauer during development [12]. Both the dauer and lifespan phenotype of the *daf-2* mutants require a functional *daf-16* gene (encoding a forkhead transcription factor FOXO), mutations in which cause short lifespan and defective dauer formation [10, 13–15]. When bound by insulin like peptides, DAF-2/insulin-like growth factor I receptor activates the PI3 kinase (AGE-1), which in turn activates AKT-1/2 and SGK-1 kinases in a PDK-1 kinase-dependent manner [5, 16–18], which eventually phosphorylate DAF-16/FOXO and sequester it in the cytoplasma [19–21]. DAF-18, the ortholog of human tumor suppressor PTEN (a PIP3 phosphatase), antagonizes AGE-1, to affect DAF-16/FOXO localization and its downstream gene expression to influence aging and other biological processes [22]. This *daf-2*/IIS longevity pathway is conserved among species. For example, mutations in the insulin receptor in both flies and mice extend animal lifespan [23–25]. Interestingly, specific polymorphic alleles of human IGF-1R (insulin growth factor -1 receptor) as well as FOXOs are associated with long-lived people [26–29], suggesting an evolutionary conserved mechanism.

Caloric (dietary) restriction (CR) is a well-documented environmental intervention that extends lifespan in many organisms (yeast, worms, flies, rodents and primates) [30–34]. Worms with mutations in the gene *eat-2* have defects in pharyngeal pumping, experience reduced food intake and thus live longer [31, 35]. Several genes known to be involved in metabolic regulation have also been implicated in CR-induced longevity. Genetic interaction analyses indicate that some components of the nutrient sensing Target of Rapamycin (TOR) pathway acts in the same pathway as CR to affect lifespan in yeast, worms and flies [35–38]. The activity of the NAD^+-dependent protein deacetylase Sir2 is also required for CR-induced longevity in yeast and flies [32, 39]. Interestingly, over-expression of Sir2 gene extends lifespan in yeast, worms and flies [40–42]. However, whether *C. elegans sir-2.1* is involved in CR signaling is still not clear. Mutations in the worm *clk-1* gene (a component of the electron transport chain, ETC) that affects ubiquinone biosynthesis extend lifespan and do not further extend the CR-mimic *eat-2* mutant lifespan [31]. A fascinating observation was made that loss of one copy of the mouse ortholog of *clk-1* ($mclk1^{+/-}$) also promotes animal longevity [43]. Whether mclk1 mediates CR-induced lifespan extension in mice is unknown.

A handful of genetic mutants involved in the functions of mitochondria are found to affect aging. In addition to *clk-1*, several other mutants with defects in mitochondrial ETC function, including *isp-1* and *lrs-2* mutants, also live long [44–46]. These mutants have other pleiotropic phentoypes including slow growth and reduced brood size, and their lifespan extension is not *daf-16*-dependent. The mechanism through which mitochondrial mutants affect lifespan is unclear although changes in transcriptional and metabolic profiles are likely involved [47, 48] (see more discussion below).

Interestingly, almost all of the known long-lived mutants present other phenotypes in addition to prolonged longevity. In fact, most of these longevity genes were initially identified/characterized based on these other associated phenotypes, and were subsequently recognized to also participate in longevity modulation. Previous forward genetic screens directly looking for lifespan changes [3, 49] were far from saturation, which is evident by the discovery of many other longevity mutants through surrogate phenotypes. More importantly, forward genetic screens involve a laborious gene-cloning phase, which is especially problematic for aging mutants because of the population nature and long-time frame of lifespan assays. Thus, a genome-wide unbiased screen for all classes of genes that affect longevity was and still is an intimidating task using forward genetic methods.

USING RNAi TO STUDY GENE FUNCTION IN *C. elegans*

RNAi (RNA interference) is a conserved cell defense mechanism induced by double-stranded RNA (dsRNA) to degrade RNA in a sequence-specific manner [50]. RNAi has been observed in all eukaryotes, from yeast to mammals. It has become a powerful 'gene silencing' technology to specifically and efficiently inhibit the expression of any gene for which sequence is available. It has been rapidly adopted as a tool for reverse genetics in eukaryotic systems. With technological advances,

RNAi can now be carried out in a high-throughput manner. It allows for unbiased, systematic screens using RNAi to knock down a large number of genes one by one to search for the gene whose reduced expression gives rise to a particular phenotype of interest. Large-scale RNAi screens have opened up a new era of functional genomics, allowing for uncovering of gene functions using strategies not previously amenable to classical forward genetic screens.

dsRNA can be introduced to *C. elegans* by microinjecting the dsRNA into the intestinal cavity, soaking worms in dsRNA solution, or feeding with bacteria expressing dsRNA targeting specific genes as food source, because RNAi can be transmitted through gut to somatic tissues and germline [51, 52]. Feeding RNAi provides the easiest and most cost-effective approach to deliver dsRNA in worms. Once the construct containing the target gene sequence is made, it can be maintained and propagated indefinitely. Feeding RNAi can be optimized to have the same if not more effectiveness as injecting RNAi for many genes [53]. As a result, feeding RNAi is ideal for genome-scale screens.

Because of the technical ease of RNAi knockdown, *C. elegans* is one of the first multi-cellular organisms in which large-scale RNAi screens were widely exploited to explore gene functions. Ahringer and colleagues constructed the first whole-genome bacterially-expressed dsRNA library targeting >16 000 of *C. elegans* genes, which revolutionalized the *C. elegans* research field [54, 55]. Vidal and colleagues subsequently constructed an independent bacterially-expressed dsRNA library corresponding to >11 000 ORFs [56]. The two libraries together cover >87% of the *C. elegans* genes. This review will focus on recent findings using large-scale RNAi screens in *C. elegans* to uncover new insights into aging biology (also see ref. [57]).

UNBIASED GENOME-SCALE RNAi SCREENS FOR LONGEVITY GENES IN *C. elegans*

In 2005, our group and the Kenyon group each independently completed the first genome-wide RNAi screens for longevity genes using the genomic RNAi library targeting >16 000 of the *C. elegans* genes [58, 59]. The primary screen for both studies was semi-high throughput. Both groups took the approach of feeding small population of worms with individual RNAi bacteria starting at either L1 worms (larval stage 1) or embryos for easy synchronization (see Table 1). The worms were allowed to develop and age on the RNAi bacteria, and when the worms fed on the control bacteria carrying empty vectors were all dead, the remaining RNAi plates were screened to identity those that still had live worms, which were considered 'positive' clones for follow-up retests. Thus, the first round of screen of both studies identified RNAi knockdowns that extend the maximal lifespan of the worms.

In total, we identified 90 RNAi knockdowns and the Kenyon group identified 29 RNAi knockdowns (~0.5% and 0.1% of the >16 000 clones, respectively) that consistently cause a lifespan extension phenotype [58, 59] (see Table 1). In both cases, among the life extending RNAi clones, only a couple correspond to genes previously known to increase lifespan when inactivated, while the majority correspond to new genes not previously implicated in longevity. This also indicates that among the genes already known to affect longevity based on previous studies, only a small fraction were rediscovered in either of the genome-scale RNAi screens. More surprisingly, the two genome-scale RNAi screens uncovered a very small number of overlapping genes (only three). These results indicate that while both screens intended to be comprehensive, the data indicated that both screens were far from saturation. The reasons are likely due to the high variability of RNAi efficiency, different strains and ways to inhibit fertility, and the scoring of only maximal lifespan leading to a high-rate of false negatives (see ref. [60] and Table 1 on this issue). Interestingly, despite the large non-overlapping sets of genes, when one compares the classes of genes recovered from the two screens, there is a very good agreement. Both candidate longevity gene sets are highly enriched for genes involved in energy production/mitochondria function, metabolism, and endocrine signaling. These findings highlight the importance of these biological processes in lifespan modulation.

A first major point learnt from these two RNAi screens is that many genes, in the order of hundreds or more (considering the screens were far from saturated), can be manipulated to cause an extension of lifespan. This indicates specific lifespan genes are not rare, in contrast to previous thinking based on the

Table 1: Unbiased genome-scale RNAi screens for longevity genes in *C. elegans*

References	Method					Genes interrogated	Longevity genes identified	Identification rate (%)	Some classes of longevity genes
	Strain	RNAi initiation	Inhibition of sterility	Primary screen	Follow-up screens				
Lee *et al*. [46]	N2	LI	FUDR	For increased lifespan	For increased lifespan	5,690 (Chr. I and II)	52 of 2 663 genes on Chr. I	1.8	Energy metabolism (mitochondria function ~15%), gene expression, protein processing, signal transduction
Hamilton *et al*. [58]	N2 (first) *rrf-3(pk1426)* (second)	LI	FUDR	For increased maximal lifespan	For increased lifespan	16 475	89	0.5	Metabolism (20%, including seven ETC genes), signal transduction (14.6%), gene expression (7.9%), protein turnover (6.7%), protein interaction (6.7%), cell structure (5.6%), cell surface (4.5%), reproduction (3.4%)
Hansen *et al*. [59]	*fer-15(b26); fem-1 (hc17)*, (first and second) (N2, second)	Embryo	Temperature-sensitive sterile	For increased maximal lifespan	For increased lifespan	16 757	29	0.1	Mitochondria ETC (41.4%)
Chen *et al*. [69]	N2	Late L4	FUDR	For increased lifespan	For increased lifespan	57 (essential for larval development)	24	42.1	Translation (42%), mitochondrial function (38%)
Curran and Ruvkun [66]	*eri-1 (mg366)*	L4/young adult	FUDR	For increased maximal lifespan	For increased lifespan (>10%)	2 698 (essential for embryo and larval development)	64	2.4	Gene expression (26.6%), signaling (23.4%), Protein synthesis (15.6%), mitochondria (12.5%), trafficking (6.3%)
Kim and Sun [79]	*rrf-3(pk1426)*	LI	None	For increased resistance to paraquat	For increased lifespan	~6 000 (Chr. III & IV)	84 (out of 608 with increased resistance)	1.4 (out of 6,000) 14 (out of 608)	Mitochondria (31%), cell surface (12%), cell structure (8.3%), signaling (8.3%), metabolism (8.3%), protein interaction (6%), protein synthesis 4.8%)

small number of longevity mutants identified through forward genetic screens (see 'Introduction' section).

A second major point is that genes involved in energy transformation (energy metabolism) are highly enriched (26 and 60% of genes with annotated functions, respectively) among new longevity genes identified from both RNAi screens [58, 59]. This is not surprising, considering nutritional interventions is currently the only consistent way to influence lifespan from yeast to primates [30–34].

Among the metabolic genes identified, a large number are involved in mitochondrial functions (8/17 and 12/15) [58, 59]. This corroborated with findings from two previous large-scale RNAi screens for longevity genes carried out using RNAi clones corresponding to genes on Chromosome I of the *C. elegans* genome [46, 61]. Among the 52 longevity genes identified in the study by Lee *et al*. [46], 15% are predicted to encode genes of mitochondrial function. As discussed in the 'Introduction' section, previous genetic analyses have revealed a few mitochondrial function genes whose loss-of-function mutations cause a long-lived phenotype [44–46]. However, prior to results from large-scale RNAi screens, it was not clear whether a general dampening of mitochondrial electron transport can increase lifespan, or whether genes of particular mitochondrial function play specific roles in aging. Results from the various large-scale RNAi screens favor the possibility that a decrease in mitochondrial function, which can be achieved through mutations or through RNAi knockdown of a large number of different ETC subunits, can all lead to lifespan increase.

Further analyses also based on RNAi technology have begun to elucidate how compromising mitochondrial oxidative phosphorylation extends worm lifespan. Rea *et al.* [62] demonstrated a threshold effect associated with these mitochondrial genes, in which a partial knockdown can lead to lifespan increase, but a severe knockdown can lead to detrimental effects. In addition, findings by both Dillin *et al.* [61] and Rea *et al*. [62] showed that mitochondrial ETC subunits need to be knocked down during development (before L3/L4 larval stage) to affect longevity. Intriguingly, the temporal window during which mitochondrial function needs to be altered to affect lifespan correlates well with the time during which mitochondrial expansion takes place in *C. elegans* (L3/L4 stage) [63].

Most of the long-lived genetic mutants or RNAi knockdown worms with defects in mitochondrial ETC subunits also show reduced fertility and slower development and growth rate, consistent with reduced ATP production in these worms [44–46, 61, 62]. Genetic interaction studies suggest that decreased mitochondrial oxidative phosphorylation acts independently of the IIS pathway to affect lifespan [44, 61], whether all the mutations and RNAi inactivation of mitochondrial ETC components trigger the same downstream response to influence aging is an active area of ongoing research. Recently, expression profiling of worms with mitochondria dysfunction (by RNAi or mutations) revealed significant expression changes of many metabolic genes [47, 48]. It will be interesting to further examine whether there is a 'signature' metabolic profile for long-lived mitochondria dysfunction animals.

It is worthy to note that the non-metabolic genes uncovered from both RNAi screens encompass components of many diverse cellular processes [58, 59]. This is also not surprising, as the longevity of animals can be influenced by a whole host of cellular processes, and it appears that tinkering many different biological pathways can cause lifespan increase.

A third striking point revealed by the two genome-wide RNAi screens is that a large number of the newly discovered longevity genes are found to require *daf-16* activity to influence aging (24 out of 33 genes and 7 out of the 23 genes tested, respectively), despite the fact that their molecular annotations indicate they participate in diverse biological processes [58, 59]. These results indicate that many different biological pathways eventually converge upon DAF-16 to influence lifespan, underscoring DAF-16 as a major regulator for longevity determination.

RNAi SCREENS FOR THE MISSING LONGEVITY GENES

According to the design of the first two genome-wide RNAi screens, one can anticipate a set of longevity genes were missed other than those caused by experimental variability.

First, those 13% of genes not represented in the feeding RNAi library are obviously left out. With continuous efforts to improve the library coverage, this problem will be addressed [64]. Investigations using individual RNAi clones generated by a wide range of interested groups in the aging research community will also contribute to the effort

of identifying and integrating components of the aging gene network.

Second, those genes that when inactivated can increase worm mean lifespan, but not maximal lifespan, were false negatives since both genome-wide RNAi screens used maximal lifespan increase as the criteria for the initial high-throughput screens [58, 59]. This notion is supported by the findings that many *daf-16* regulated aging genes only affect mean, but not maximal lifespan [65]. Maybe this is part of the reason that the two genome-wide screens together identified only 112 longevity genes from >16 000 candidates (0.6%), while the earlier screen identified 52 genes out of just 2 663 genes on chromosome I (2%) [46]. Such a big difference is probably due to more frequent lifespan monitoring (every 5 days) in the smaller-scale screen than the high-throughput screen for only maximal lifespan extension. Evidently, the solution for this problem requires more careful and frequent lifespan scoring, which will increase the amount of work exponentially in genome-scale screens. At present, it may not be very practical. Automation of lifespan assay/scoring will be the answer. We are glad to know that the research community is working towards that direction, identifying aging biomarkers easy to monitor and developing new imaging techniques.

Third, those genes functioning in neurons to affect aging might be missed out since neurons are more refractory to RNAi than other tissues in *C. elegans* [53]. The field studying RNAi mechanism has already provided some solutions to this problem. One can use a mutant strain with enhanced RNAi efficiency, including in the neuronal cells, to increase the sensitivity of the screen. For example, the *eri-1* and *rrf-3* mutants have been shown to enhance susceptibility to neurons, using the RNAi feeding techniques, and do not affect the worm lifespan [66–68]. This approach was implemented in the RNAi screen of the essential genes described later [66]. Out of the 64 newly identified longevity genes, there was a clear enrichment of genes expressed in neurons (>12). It demonstrated the effectiveness of these mutants to study gene functions in all cell types, especially in neurons, using RNAi technique.

Fourth, those genes essential for worm survival and development were tossed out in the genome-wide screens for extension of adult lifespan since both screens used RNAi feeding starting from early development stage (L1 or embryo). To overcome this issue, two groups used a modified approach to screen the essential genes by initiating RNAi feeding post developmentally (at L4 or young adult stage, see Table 1) [66, 69]. From the ~2,700 essential genes, the Ruvkun lab identified 64 new longevity genes (2.4%) involved in protein synthesis, signal transduction, mitochondria function and gene expression/chromatin associated factors [66]. The Kapahi lab screened 57 genes known to be essential for larval development [55] and identified 24 novel longevity genes (42%) [69]. Among the 24 genes identified, most of them are involved in regulation of mRNA translation (42%) and mitochondrial functions (38%). The two essential-gene studies underscore another advantage of RNAi technology, which is that one can control when to start RNAi inactivation to resolve the temporal effects of gene knockdown.

Even though the two screens started with very different sized pools, they identified four common novel longevity genes [66, 69]. Furthermore, several common themes were uncovered through these two screens: (i) Because genes essential for development are more likely to be conserved during evolution, most of the positives from the two screens are conserved from yeast to human, providing a good handle for aging research in other organisms. (ii) Even though the careful lifespan monitoring probably contributed to the high identification rates of the two screens, it also pointed to the possibility that developmentally essential genes might have a higher chance of limiting adult lifespan. This would be consistent with the 'antagonistic pleiotropy' theory, which states the pleiotropic genes that are beneficial early in life can be deleterious or limiting for later life maintenance [70]. For example, the well-known longevity gene *daf-2* is essential for development, but weak mutation or RNAi inactivation of *daf-2* later in life promotes adult longevity [10, 71, 72]. (iii) Both of the essential gene screens identified gene classes highly enriched in mitochondria functions (similar as previous screens), as well as protein translation.

Genes involved in protein synthesis have been identified in previous longevity screens but not in high number, probably because protein synthesis is essential for development [58]. Other studies in *C. elegans* also revealed that inhibiting protein translation can extend worm lifespan [38, 73, 74]. But how they interact with other known longevity pathways may depend on which step of protein synthesis is compromised. Interestingly, deletion of genes

in the 60S ribosome in yeast also leads to extension of replicative lifespan [75]. One pathway that regulates protein synthesis is the TOR pathway [76]. Inhibiting TOR signaling by genetic manipulations or pharmacological interventions could extend animal lifespan in fruit flies and mice [36, 77], suggesting that a link between protein synthesis and aging might be conserved in higher organisms. Very recently, the Kapahi group reported that a translational repressor downstream of the TOR pathway is required for DR-mediated lifespan extension by enhancing mitochondrial activity in fruitfly [91], which implicated a close link between two important biological processes in aging modulation.

RNAi SCREENS FOR SPECIFIC PHENOTYPES RELATED TO AGING

Because longitudinal assays monitoring lifespan is time and labor consuming, a more efficient way to screen for new longevity genes is to base on a surrogate phenotype often associated with longevity that is also easier to monitor. For example, many long-lived mutants show increase of stress resistance [78]. Since stress response phenotypes can often be scored in a matter of hours or days, rather than weeks, one can use stress resistance augmentation as a criteria to screen for candidates of longevity genes. Kim and Sun carried out such a screen first to identify genes, when inactivated by RNAi, confer higher resistance to the superoxide-inducing agent paraquat [79] (see Table 1). They then retested the RNAi candidates for a lifespan extension phenotype. Among the ~6,000 RNAi clones (covering chromosome III and IV of *C. elegans*) they screened in the first step, 608 clones exhibited resistance to paraquat treatments, and of which, 86 produced >10% lifespan extension. Most of the genes identified in this screen were previously unknown to affect longevity. They are categorized into functional groups like cell signaling, cell-cell interactions, gene expression, protein turnover, metabolism, protein–protein interaction, mitochondria and cell structure. The higher positive rate compared to the two genome-wide screens [58, 59] could be due to one or more of the following: distinctive criteria for the primary screen (oxidative stress resistance versus maximal lifespan extension), lifespan scoring methods (mean versus maximal lifespan), strains used (RNAi-enhancing strain versus non-RNAi enhancing strains), etc. This screen further strengthens the notion that different methods/design of the RNAi screen could affect the outcome greatly. In addition, it showcased that using an aging-related phenotype for primary screen can highly enrich the longevity genes for the second round lifespan screen, from 1.4% (86/6000) to 14% (86/608).

As not all the long-lived mutants have enhanced stress resistance or resistance to the same types of stress [80], using different stress resistance or other aging-related phenotypes are likely to yield different longevity genes, which will be good avenues to identify different subsets of longevity genes.

RNAi SCREENS FOR NEW COMPONENTS OF THE GENE NETWORK THAT AFFECT AGING

To advance our understanding of aging, identification of new genes that affect lifespan is not enough. We need to keep incorporating the new members into the existing gene networks as well. The published genome-scale RNAi screens all did some classifications of the novel longevity genes by genetic epistasis or bioinformatics analyses (interaction and expression data) to categorize them into known or novel longevity pathways. One alternative approach is to directly search for new components in a known aging pathway.

Samuelson *et al.* [81] carried out the first genome-wide RNAi suppressor screen looking for genes when inactivated to suppress the long lifespan of a insulin/IGF-1 receptor *daf-2* mutant. As mentioned in the 'Introduction' section, *daf-2/daf-16* is one of the well-studied longevity pathways. This suppressor screen identified ~200 genes that, when inactivated, shorten the *daf-2* mutant lifespan [81]. To distinguish true progeric genes from those causing general sickness, and the *daf-2* pathway-specific genes from general progeric genes, the authors carried out detailed demographic analyses (e.g. measurement of the rate of aging), monitored molecular and behavior aging markers, and filtered the candidates with insulin signaling-specific tests. Among the ~100 genes found to be regulated by the *daf-2*/insulin pathway, ~4% have conserved DAF-16 binding sites, which suggests that they are likely to be direct targets of DAF-16; and about one-third of the genes when RNAi inactivated suppresses a known DAF-16 target gene expression,

which indicates that they act upstream of DAF-16 to influence lifespan. Interestingly, among the *daf-2*-specific suppressors, there is an enrichment of the components of the vesicular trafficking to lysosomes, suggesting that endocytosis to remove damaged cellular components might be required for lifespan extension by reduced *daf-2* signaling.

Another method to look for new factors in a specific pathway is to do a targeted screen (a candidate approach) based on existing knowledge of that pathway. For example, two recent screens were carried out to identify new kinases and phosphatases in the *daf-2*/insulin cascades [82, 83]. To identify new partners of the phosphatase DAF-18/PTEN, the antagonizer of the AGE-1/PI3 kinase [22], Masse and colleagues subjected a small RNAi library of ~265 kinases to screen for those which confer lifespan extension in a *daf-18*-dependent manner [82]. They found that SMG-1, a conserved serine–threonine kinase, is one of the kinases that acts parallel to *daf-2*/IGF-1, but converges on DAF-18/PTEN and DAF-16/FOXO. The second screen was based on the hypothesis that there is more than one phosphatase (DAF-18) to counter the many kinases known in the insulin-signaling pathway [83]. Padmanabhan and colleagues cherry-picked 60 Ser/Thr phosphatases to screen for gene inactivations that counteract the constitutive dauer phenotype of a *daf-2* temperature-sensitive mutation, which is an important phenotype related to aging [83]. They identified *pptr-1* (encoding the homolog of a subunit of the PP2A phosphatase complex) that acts parallel to *daf-2*, but dephosphorylate a highly conserved residue of AKT-1 to stimulate dauer formation, stress resistance and maintains normal lifespan. Both screens identified structurally and functionally conserved new components, which have important implications in future studies of human aging and aging-related diseases.

FUTURE CONSIDERATIONS

The development of genome-scale RNAi screening allows for unbiased search for longevity genes to an extent not previously possible using classical genetic means. A major advance stems from RNAi longevity screens in the past few years is the discovery of a large number of new genes whose manipulations can lead to lifespan increase or decrease phenotypes. A major challenge with these new discoveries is to elucidate how these various genes act to affect lifespan. Continuous discovery of new components of the gene networks that contribute to organism aging is definitely a pre-requisite for the understanding of the molecular mechanism. No doubt, some of the genes uncovered likely have indirect effects on lifespan, or they may have specific effects on nematode longevity but not other organisms. However, it is certain that many also likely represent key players in longevity determination, and have relevance to aging in diverse organisms, from yeast to mammals [84, 85]. The discovery of new candidate genes is a major step forward, and a major challenge for the future is to further characterize a large number of possible new longevity mechanisms.

Despite the many ways in which large-scale RNAi screens have advanced the aging field, as eluded to several times in the text, the current RNAi technology is not without limitations. As we continue to depend on this versatile tool for future aging research, improvement of the RNAi technology and complementing it with other technologies promise to bring about even greater progress to the aging research field. Like any other technology, RNAi screens have their pros and cons comparing to classical forward genetic screen [64]. RNAi can only induce a knockdown of a gene target (like a loss-of-function mutation), while the classic genetic methods can produce gain-of-function, functional point mutations and complete null mutations. Weak RNAi efficiency may produce a false-negative result. However, for some genes, moderate reduction of function may reveal new phenotypes not observed by a severe mutation. In addition, certain tissues, like neurons, are more refractory to RNAi [53]. Furthermore, RNAi may have secondary targets comparing to the one-gene effect by classical mutational analyses. Researchers are continuously working towards more efficient RNAi technology, including optimization of the delivery method and the format and size of the dsRNA, which will improve the stability, efficacy and selectivity of gene silencing.

Additionally, focused screens or candidate approaches can improve the robustness and accuracy of the RNAi screen. Smaller number of candidates definitely makes it more practical to do more careful lifespan assays, which can decrease the false-negative rate. Several sub-libraries containing specific classes of genes (e.g. signaling molecules, transcription factors, etc.) have been derived from the genome-wide RNAi library (GeneService, UK), which are very

useful for the research community to look into specific biological functions or pathways that may affect aging. Other specific subgroups of RNAi can be easily cherry-picked from the available whole-genome RNAi libraries to tailor to the research interest of specific groups.

The ability to apply RNAi at different times of an animal lifespan has already revealed some temporal resolutions of gene functions [66, 69], one can imagine tissue-specific RNAi is likely to increase the sensitivity to identify genes that function differentially in different tissues and fine tune the information on their functions in aging [86]. Given that many important components of RNAi pathway are known, mutant and transgenic *C. elegans* strains allowing for tissue-specific RNAi are available and can be used for future analysis of longevity genes with some further improvement [87, 88]. This approach can help paint a much more detailed picture of aging gene network with spatial resolution.

The lifespan assay is still a very labor-intensive assay, the bottleneck of much of the aging studies. Automation of the assay (including the experimental set-up and the longitudinal assay) could potentially make large-scale lifespan measurement both more accurate and high throughput. In addition, thanks to the conservation of many longevity pathways, computational biologists are using the vast amount of information about the existing longevity genes that were largely identified through genome-scale RNAi screen to predict new longevity genes in various organisms with great success [84, 89].

Up to date, thanks to RNAi technology, over 300 *C. elegans* genes have been identified to affect aging. Many of them were classified genetically into several major longevity pathways. Interaction screens, like the *daf-2* suppressor screen [81], can help identify genes that are involved in specific pathways. However, the research community already realized that genetic epistasis analyses are not enough to sort through the ever-growing number of longevity genes and these pathways have more complicated cross talks than we expected. After all, aging is a very complex phenomenon. System biology approaches will be necessary to study the gene network that influence aging.

Finally, comparison of the gene network across various organisms will provide tremendous amount of new information on the evolution and conservation of aging mechanisms. Given the short lifespan and the availability of the genome-wide RNAi libraries for *C. elegans*, all the genome-scale RNAi screens for longevity genes were carried in nematodes so far. Longevity research in other organisms is mostly based on genes with high homology/conservation. Recently, the *Drosophila* RNAi library has improved its coverage and specificity dramatically for whole-animal screens [90]. We expect genome-scale RNAi screens for the fruit fly longevity genes will come forward in the near future, which will underscore conserved genes and pathways in lifespan modulation. Conservation among the invertebrate model organisms will highlight the molecular mechanisms most likely to be applicable to human longevity.

Key Points

- RNAi technology has facilitated unbiased genome-scale screens to identify new components of genetic networks that modulate aging.
- Genome-wide RNAi screens has identified hundreds of novel longevity genes in *C. elegans*, and many of which have homologs in diverse organisms.
- Genome-scale RNAi screens has revealed cellular processes that were previously not linked to aging.
- RNAi screens can be designed to identify longevity genes specific to particular genetic pathways, or to particular environmental conditions.
- Advancement of our understanding of aging requires improvement and incorporation of RNAi technology with many other tools to reveal additional new components and their functionality in the overall gene networks.

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