RNA Interference in Ageing Research – A Mini-Review

Nadège Minois^{a, c} Peter Sykacek^b Brian Godsey^b David P. Kreil^b^aResearch Institute of Molecular Pathology/Institute of Molecular Biotechnology, and ^bChair of Bioinformatics, Boku University, Vienna, Austria; ^cSchool of Biology, University of St Andrews, St Andrews, UK

Key Words

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Abstract

Background: The search for genetic mechanisms affecting life-span and ageing represents an important part of ageing research, especially since the discovery of single-gene mutations with dramatic effects on these traits. Due to its relative ease of use and its power to specifically target arbitrary genes, RNA interference (RNAi) has rapidly been adopted as a technique for silencing gene expression. The feasibility of genome-wide RNAi screens potentially much simplifies the identification of novel ageing-related genes. **Objective:** In a review of applications of RNAi in ageing research with a focus on the model organisms *Caenorhabditis elegans* and *Drosophila melanogaster* and discussing recent technical developments, we aim to highlight the current and future impact of this technology in the field. **Method:** We show how RNAi has successfully been used to complement classic mutant studies. Moreover, we discuss the novel opportunities and challenges of an application of RNAi in genome-wide screens in *D. melanogaster*, which has become possible with the recent availability of a comprehensive transgenic RNAi library for the fly. We highlight, in particular, how the flexible control of RNAi induction can support the study of dynamic processes like ageing through specific experiments and the development of matching computational methods. In an overview of complementary approaches we discuss the challenge of extracting insight from the high-dimensional

measurement datasets that are required for the study of dynamic effects and interaction dependencies. **Conclusion:** RNAi has emerged as a powerful tool for the study of ageing, allowing the further characterization of the roles of specific genes in the ageing process as well as the efficient identification of new genes implicated. RNAi has contributed to our understanding of age-related diseases especially by making genes amenable to manipulation for which mutants were not easily available. Recent developments enable genome-wide screens with unprecedented temporal and spatial control of RNAi induction. Specific RNAi time-course experiments provide an opportunity for the analysis of high-resolution gene expression profiles capturing the dynamics of ageing-relevant processes and gene interactions. Research exploiting new avenues opened by the growing RNAi toolbox will considerably contribute to the next steps in researching the genetics of ageing and age-related diseases.

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Introduction

Life-span is partly under genetic control and, to understand ageing, we need to understand its genetic mechanisms. Established theories of the evolution of senescence assumed that life-span was under the control of many genes with small effects [1], by accumulation of deleterious mutations acting late in life when natural selection is absent because the organism has already produced its offspring (mutation accumulation) [2], or by selection of genes beneficial in early life even if they have deleterious

Fig. 1. a General mechanism of RNAi by siRNAs. dsRNAs are processed by enzymes such as Dicer into siRNAs, which are then uploaded into the RISC (RNA-induced silencing complex). There, the siRNAs can bind complementary sequence regions in endogenous mRNAs and lead to their degradation or the ‘silencing’ of the targeted mRNA. **b** The schema used in the construction of a genome-wide RNAi library in *D. melanogaster* [55].

effects late in life (antagonistic pleiotropy) [3]. It was shown subsequently that even single-gene mutations can lead to dramatic extensions of life-span [e.g., 4], which has opened the way to a new field of research in the genetics of ageing, in which the effects of single genes on life-span, ageing, or age-associated diseases are being studied.

In this context, we here review recent applications of RNA interference (RNAi) in ageing research, with an emphasis on invertebrate model organisms. Novel opportunities created by recent technical advances such as a genome-wide transgenic RNAi library in *Drosophila melanogaster* are discussed.

RNAi is a widespread and evolutionarily conserved mechanism found in many eukaryotes that contributes to gene regulation via short interfering RNA (siRNA) or microRNA (miRNA), as reviewed by Barstead [5]. Both types of small RNAs will bind and regulate matching endogenous mRNAs. Although RNAi mainly triggers

down-regulation of targeted genes, miRNAs have also been shown to enhance gene expression [6].

Since its discovery in *Caenorhabditis elegans* in the late 1990s [7], RNAi has been embraced by the research community as a research tool, using the ability of siRNAs to down-regulate the expression of target genes [8]. A gene-specific dsRNA is processed into siRNAs about 21 bp long by enzymes such as Dicer2 in *Drosophila*. These enzymes also separate the two strands of the siRNA and one of them is preferentially uploaded into the RNA-induced silencing complex (RISC) that mediates the degradation or silencing of mRNAs with complementary sequences to the siRNA (fig. 1a). The speed at which RNAi was integrated into the geneticist toolbox is probably a reflection of its relative ease of use compared to other knock-down methods. Geanacopoulos [9] provides an accessible review. Practically, RNAi can be induced in several ways. Soaking cells or *C. elegans* worms in siRNA

or dsRNA solution already suffices for its incorporation into the host cells. A more efficient way of inducing RNAi is through an appropriate expression vector for a gene-specific double-stranded hairpin. Cloned bacteria synthesizing this dsRNA can be fed to a target organism like *C. elegans*. On absorption, the dsRNA triggers RNAi in the host. Injecting an expression vector into an embryo leads to expression of the dsRNA directly in the target organism and thus induces RNAi. Genes affected can be predicted by thermodynamic models of target mRNAs and structural constraints. Programs implement these methods to design gene-specific dsRNAs for RNAi [e.g., 10]. Because the technique is amenable to large-scale high-throughput screening, RNAi has become an invaluable new tool for the rapid identification of genes involved in particular phenotypes of interest, including changes in life-span and ageing.

Applications of RNAi in Ageing Research

Investigation of Known Mechanisms of Ageing

Several genetic mechanisms have already been implicated in earlier studies of ageing, identifying the importance of the insulin/insulin-like growth factor pathway, the TOR pathway, SIR genes, and of mitochondria. RNAi brings a novel efficient tool supporting further investigations of their roles in regulating ageing and life-span, especially in organisms in which no knock-out or knock-down mutants are easily available. Consequently, RNAi is being applied in an increasing variety of ageing-related studies.

The inhibition of the insulin-like signalling pathway is known to increase the life-span of many organisms, as does dietary restriction, but the existence of an interaction between this pathway and the effects of dietary restriction is still under debate [11]. In *Drosophila*, the only study that directly investigated the interaction concluded that the insulin-like signalling pathway and dietary restriction worked by overlapping mechanisms [12]. Indirect studies also suggested a possible interaction, showing that the insulin-like signalling pathway modulated both life-span and metabolism [e.g., 13]. In *Drosophila*, the first actors in the insulin-like pathway are the insulin-like peptides (ilp), which activate the pathway. In a recent study, Min et al. [14] have shown that *ilp5* was down-regulated in diet-restricted wild-type flies. In order to investigate whether dietary restriction increased life-span by way of *ilp5* down-regulation, the authors tested the effect of dietary restriction on flies with RNAi knock-down of *ilp5*

in the medial secretory neurons where ilps are synthesized. Although the level of *ilp5* was now constantly low independent of diet, dietary restriction still increased life-span, suggesting that the mechanisms of dietary restriction may be independent of the insulin-like pathway.

Another mechanism that has been implicated in ageing is the accumulation of damage to molecules, cells, and tissues. The modulation of pathways involved in oxidative damage defence or DNA damage repair has been used widely to study the role of accumulated damage in ageing. The superoxide dismutase enzymes (SODs), for instance, are responsible for the transformation of superoxide into oxygen and hydrogen peroxide, a key step in the defence against oxidative stress. While the cytoplasmic Cu-ZnSOD (SOD1) had been studied in great detail, no mutants of the mitochondrial MnSOD (SOD2) were available. RNAi knock-down offered the first possibility of testing SOD2 involvement in oxidative stress and ageing. In *Drosophila*, ectopic down-regulation, although without major impact on development, led to hypersensitivity to oxidative stress (paraquat exposure) and a drastic decrease in life-span [15]. The decrease in life-span was later confirmed in a *Sod2* null mutant [16].

CKU-70 and CKU-80 Ku form a heterodimer with roles in double-strand break repair (DSB), telomere maintenance, cell cycle arrest, and tumour suppression, all mechanisms involved in ageing. Using RNAi in *C. elegans* to down-regulate one of the two subunits in wild-type worms at a time, McColl et al. [17] showed that the down-regulation of either subunit triggered an increased sensitivity to methyl-methane-sulfonate but not to UV, two different agents of DNA damage. Moreover, *cku-70* RNAi increased thermotolerance in wild-type worms and life-span in an RNAi-sensitive strain. The Ku dimer also seemed to interact with the insulin-like signalling pathway, as *cku-70* RNAi amplified the changes in life-span and stress resistance in *daf-2* and *daf-16* worms, mutants in the insulin-like signalling pathway increasing and decreasing life-span, respectively.

The sirtuins are another gene family recently surveyed by RNAi for its role in ageing. Over-expression and down-regulation of the *Sir2* gene were known, respectively, to increase and decrease life-span in many organisms. RNAi was used in *Drosophila* to target three *sir* and *sir-like* genes from classes of the large sirtuin family that had so far not been examined for their roles in ageing. Similarly to *Sir2*, down-regulation of these genes decreased female life-span in all except one line and, moreover, caused total or partial developmental lethality [18]. Complementary over-expression studies are required to demonstrate their poten-

tial roles in ageing. It is noteworthy, however, that null mutants for *Sir2* were reported to be viable, in contrast with the results of the RNAi study, in which the deleterious effects might have been due to off-target knock-downs.

Studies had shown that the down-regulation of the mitochondrial electron transport chain in *C. elegans* increased life-span. In contrast, mutations in humans lead to diseases and short life-spans. By feeding *C. elegans* with various concentrations of *E. coli* synthesizing dsRNA against four genes encoding mitochondrial proteins (*atp-3*, *nuo-2*, *isp-1*, *cco-1*) and *frh-1* (frataxin), Rea et al. [19] could show that mild down-regulations of these genes increased life-span whereas stronger down-regulations decreased life-span, thus resolving the paradox observed in the previous studies. This work highlights the power of being able to vary the amount of RNAi knock-down by adjusting the dose of active vector fed.

It is becoming apparent that the response to RNAi treatment – a phenotype or a lack of it – always has to be considered in the context of both the level and specificity of the down-regulation achieved. Depending on the level of knock-down, different phenotypes may be observed, as illustrated by the study above. In particular, a phenotype may only be seen at certain knock-down levels. A standardization of RNAi protocols and construct design will be critical in obtaining results that can be compared across studies and between laboratories.

Furthering an Understanding of Age-Related Diseases

In recent years, invertebrate models have become increasingly prominent in the study of age-related diseases, and associations with genetic disorders. *C. elegans* could, for instance, be established as a model for Werner syndrome (WS) when it was shown that specific RNAi knock-down of a WS protein homologue could reproduce phenotypes of the disease such as DNA damage and premature ageing [20]. Bates et al. [21] used a *C. elegans* model of Huntington's disease and addressed, using mutants and RNAi for genes for which no mutants are available, the role of histone deacetylases in huntingtin polyglutamine toxicity. Down-regulation of various histone deacetylases could either reduce (for *hda-3*) or increase neurodegeneration induced by polyglutamine accumulation (e.g., for *hda-1*, *hda-4*, *hda-2*). The employed RNAi constructs had been maximized for specificity to avoid unspecific confounding effects. The unique role of *hda-3* was also confirmed using a deletion mutant.

Ubiquilin 1 (*Ubqln1*) has recently been associated with an increased risk of late-onset Alzheimer's disease. Its links with the disease were studied by silencing or over-

expressing the *Drosophila* *Ubqln* orthologue [22]. Down-regulation in the nervous system shortened life-span and triggered age-dependent neurodegeneration. Furthermore, silencing *dUbqln* enhanced, and over-expressing it rescued a rough-eye phenotype induced by over-expression in the eye of *presenilin*. *dUbqln* also interacted with the amyloid precursor protein.

The potential therapeutic use of RNAi has early been recognized. By RNAi, the pathogenic expression of a mutated gene can be silenced. In a mouse model of amyotrophic lateral sclerosis, Ralph et al. [23] tested a vector particularly suitable for RNAi in the nervous system aiming to down-regulate a mutant form of the exogenic human SOD1 causing the disease. Mice injected with a vector containing the RNAi construct had delayed onset and lesser severity of motor impairment, and enjoyed a longer life-span due to the increased survival of motor neurons.

A particular strength of RNAi is how well it scales to larger studies. Comprehensive libraries of dsRNA, siRNA, and *E. coli* strains synthesizing dsRNA have been established. Also, viral vector-based libraries for mammalian cells are rapidly being developed. These tools potentially allow any gene to be targeted, making efficient large screens possible.

RNAi libraries can also be used to screen for candidates interacting with genes involved in age-related diseases. Such screens have been carried out to identify genes interacting with α -synuclein, a protein whose misfolding and aggregation play a major role in the development of the age-related Parkinson's disease. In a *C. elegans* model over-expressing GFP-fused human α -synuclein and co-expressing the chaperone torsinA in body wall muscles, Hamamichi et al. [24] were looking for genes protecting against α -synuclein misfolding. For 20 of the 868 tested candidates with potential links to the disease, RNAi knock-down from larval stage L3 led to increased α -synuclein-specific misfolding. Seven of these genes were further tested for their effects on neurodegeneration, with five of them shown to protect from dopaminergic neuron degeneration when over-expressed. These genes code for proteins involved in trafficking, scaffolding, autophagy, and include one uncharacterized protein. Kuwahara et al. [25] created a transgenic worm over-expressing human α -synuclein pan-neuronally and crossed it to an RNAi-sensitive worm strain. Out of 1,673 genes with synaptic functions and roles in the nervous system, ten produced growth and/or motor abnormalities on RNAi knock-down specifically in combination with α -synuclein expression, identifying them as interaction partners. Four of these genes were involved in the endo-

cytic pathway. It is noteworthy that although the two studies were similarly looking for genes affecting α -synuclein misfolding and aggregation, they identified different sets of genes, with no overlap. This may be explained by the different strains of worms tested, screening for different phenotypes, and some differences in the sets of candidate genes examined. This highlights the difficulties of comparing or combining results of multiple screens. Eventually, however, such systematic studies can be expected to yield new therapeutic targets for age-related diseases with a strong genetic susceptibility.

Large-Scale Screens

The suitability of RNAi for comprehensive high-throughput screening can be exploited in large-scale screens for genes that increase life-span on RNAi knock-down. The first such screens have been discussed and reviewed in detail elsewhere [e.g., 26, 27]. In summary, about 150 genes have been identified that increased life-span when down-regulated by RNAi. These spanned a variety of genes, affecting mitochondrial function, metabolism, signalling, protein-protein interaction, and the regulation of gene expression. Only three earlier identified genes were recovered but not by all of the screens. In general, there was little overlap between genes found in the different screens. In only two of four large-scale RNAi screens there were three genes jointly identified. The differences observed may reflect the choice of screening methods, the employed bacterial-RNAi library, and also variations in the relative efficiency of RNAi. RNAi by feeding, in particular, can yield highly varying levels of down-regulation. Some screens exploit worms that carry mutations rendering them more sensitive to RNAi, also overcoming the wild-type's insensitivity to RNAi in the nervous system. Recently, a screen of 16,757 genes with such RNAi-sensitive strains identified 115 that reproducibly increased life-span by at least 10% on knock-down [28]. These genes predominantly had mitochondrial and metabolic functions. Of these genes, 18 were characterized further, showing that there was no strong association between life-span extension and a slower accumulation of age pigment (lipofuscin) or an increased stress resistance. This highlights the phenotype specificity of the screen. A screen for extended life-span will thus not necessarily uncover genes playing a role in the ageing process.

RNAi libraries also facilitate large-scale interaction screens. Triggering RNAi by feeding in a mutant strain for a gene of interest, Samuelson et al. [29] have screened 15,718 genes for a decrease of life-span in *daf-2* mutants on RNAi knock-down of the candidate. Of the 159 genes

identified, many also decreased life-span in wild-type worms and *daf-2;daf-16* mutants, albeit to a lesser extent. Thirty-nine genes shortened life-span specifically in *daf-2* mutants. Tracking general fitness by measuring the decline of activity and age pigment accumulation with age, they could show that only nine genes induced general sickness, whereas the others seemed to specifically accelerate the ageing process. Many of these genes had roles in trafficking and vesicle sorting.

Stress resistance has often been correlated with ageing. A screen of ~6,000 RNAi clones covering the genes on chromosomes III and IV in an RNAi-sensitive strain of *C. elegans* identified 608 clones conferring paraquat resistance [30]. Out of these, 84 also increased life-span, with only four of them already known from previous studies. The identified genes were involved in mitochondrial function, cell structure and signalling, cell-cell and protein-protein interactions, gene expression, protein turnover, and metabolism. Despite testing RNAi-sensitive strains, RNAi knock-down of only 14% of genes increased both paraquat resistance and life-span, indicating the specificity of RNAi screens for different complementary aspects of ageing, which have to be considered in the compilation of a full picture.

So far, large-scale screens in *C. elegans* have yielded about 400 genes increasing life-span on RNAi knock-down. While many of these genes could be mapped to known modulators of longevity, e.g., genes of mitochondrial function or involved in metabolism, almost no candidate genes have since been validated in independent studies, and the biological significance of most implicated genes is still unclear. Moreover, with accurate life-span assessments being very labour-intensive, many studies instead employ a rough measure like maximum life-span in their primary screens. While the resulting higher false-negative rates could explain the discrepancies between different screens, even the positive candidates require more detailed scrutiny. On the one hand, it seems surprising that, despite no selection for longevity, so many genes would individually have a strong effect on life-span. On the other hand, considering the high false-negative rates, many authors interestingly argue that many more genes increasing life-span are to be discovered. We consequently think that a lot more effort and time will be required to make sense of the accumulated results of rapidly increasing numbers of large-scale screens in ageing research. Improved means for interpreting results from complementary screens will be particularly critical due to the rapid growth of domains accessible to study by large-scale RNAi screens that can be expected. For ex-

ample, future developments of large-scale RNAi screens in vertebrates will allow an examination of additional important aspects of ageing, such as mechanisms of brain ageing and the potential role of inflammation.

Timed Knock-Downs and Dynamic Effects

A major advantage of RNAi is how well the timing of RNAi knock-down can be controlled. Being able to choose the onset of RNAi relative to the life cycle of an organism is of particular importance for studies of ageing. For instance, many genes have crucial roles during development and early interference may even be lethal. Knock-down of ~2,700 genes led to developmental lethality, larval arrest, or other abnormalities preventing the development of adult animals in *C. elegans* [31]. The role of such genes in ageing can still be studied by genetically inducing RNAi at a later developmental stage or by an external trigger. This is especially important for ageing research considering the antagonistic pleiotropy theory [3], according to which some genes with beneficial roles early in life could have deleterious effects later on and accelerate ageing. Indeed, for 64 genes essential during early development, RNAi knock-down in the L4 larval/young adult stage increased life-span by over 10% [32]. These genes were involved in mitochondrial function, protein synthesis, signalling, and gene expression. Remarkably, in another study, for 24 genes of 57 that caused larval arrest in constitutive knock-down (42%), an extended life-span was found in adult-specific RNAi knock-down [33]. The identified genes mainly had mitochondrial functions and roles in the regulation of mRNA translation. A considerable number of genes may thus exhibit antagonistic pleiotropy, and it is interesting to explore the consequences of disabling such genes only when they begin to have negative effects.

The ability of RNAi to trigger such a knock-down at known time points also allows the meaningful study of gene expression profile time-courses for an analysis of the regulatory interactions and the functional pathways implicated by a gene identified as relevant for ageing. In traditional knock-down experiments, only the steady-state long-term response of an organism can be seen. Often, initial effects may no longer be visible due to compensatory mechanisms creating redundancy, or the knock-down may be lethal. Timed RNAi knock-down, in contrast, permits an analysis of the immediate dynamic regulatory response to the loss of gene function, directly implicating interacting genes and pathways. In addition, long-term accumulative changes can be studied without confounding developmental effects. When enough time points are mea-

sured, moreover, dynamic changes over time can be observed with high sensitivity, examining processes like age-related break-down or implementation of compensatory mechanisms as well as threshold events as could be triggered by ageing-related accumulation of damage.

With high-dimensional time-course measurements increasingly employed [34–37], bioinformatics methods are of growing relevance, as the extraction of insight from these large datasets relies on the further development of matching computational analysis tools. Dynamic effects have long been modelled by differential equations. Due to the high numbers of parameters that need to be inferred even for simple sets of equations, these mechanistic models are best applied to systems where the key players and interaction partners are known. Typical analyses have studied 5–30 genes with 5–50 time points [37, 38]. The behaviour of these equation systems can be sensitive to noisy measurement signals. While *stochastic* differential equations directly model the noisy nature of expression data, they scale even less well to larger systems [39]. Dealing better with the high-dimensional nature of microarray data, methods from multivariate statistics have thus been applied to microarray time-course analysis [40, 41]. As discussed elsewhere, however, most tools do not exploit the consecutive nature of time points [42].

Recent work has suggested new approaches for identifying significant differences between expression profile time-courses that exploit temporal correlations. Functional representations of time dependencies, in particular, allow the joint analysis and comparison of time courses with different dynamics and measurements at non-aligned time points [43]. In one such approach, for each gene, Storey et al. [44] used smooth splines to model the common trend observed over time for an experimental group. Significant differences between groups were identified by comparing the respective trend models. In the same framework, experimentally observed trends over time could be compared to a ‘no change’ reference model, as was demonstrated for expression profiles of ageing kidney cortex. The method was also successfully applied in a study of ageing in *Drosophila* [36]. Supporting dependencies within each data series moreover allows longitudinal sampling, as in tracking ageing organisms of different genotypes or individual patients. Angelini et al. [45] similarly modelled gene expression time dependency for each experimental group by polynomial expansion but then took a hierarchical Bayesian approach, considering alternative noise models. Bayes factors were used to identify genes with trend models different from ‘no change’. Each of these methods showed considerable improve-

ments in sensitivity in comparison to standard multivariate microarray analyses. With alternative algorithms having different method bias, however, results from a variety of analyses must be considered for robust results. In situations where the limitation of false-positives is critical, an intersection of candidates from alternative methods can be carried forward. Conversely, if false-negatives are to be minimized, a union of candidates can be used.

In the investigation of microarray profile data, a reduction of complexity is often required for visualization purposes and the application of more elaborate analysis methods that have not been designed to handle high-dimensional datasets directly. Common techniques of dimensionality reduction are based on traditional factor decomposition and clustering algorithms. Besides linear models like PCA, SVD, or ICA [46, 47], for sufficiently many measurements, more complex non-linear decomposition methods can be applied [48, 49]. These all transform the original measurements into a continuous component or factor space, with the hope that meaningful patterns can more easily be identified there, and that particular components or factors represent biologically relevant effects. In contrast, numerous clustering algorithms aim to identify discrete groups or states: for example, hierarchical or *k*-means like clustering, mixture density models, and Hidden Markov Models [50–53]. The above algorithms provide powerful abstractions. These, however, strongly depend on the underlying model and one cannot a priori know how well a model matches the specific biological system under investigation. Consequently, no particular algorithm can be expected to reliably provide biologically meaningful abstractions. Nevertheless, the tentative identification of an extracted factor with a biological process, or of a computed group with a biological state can, for example, help in formulating valuable hypotheses for follow-up experiments.

Dynamic Bayesian networks (DBNs) are often the method of choice when more fine-grained interactions are of interest, while not suffering the intractability of detailed mechanistic models for high-dimensional systems. The derived networks imply directed dependencies between individual genes. The reactions of genes to knock-down experiments as predicted by the networks can thus immediately be tested. As a result, this approach is particularly suitable for the study of dynamic processes like ageing by RNAi. Interaction topologies between up to a thousand partners for 5–20 point time series are feasible application scenarios [54], and the algorithms by Kim et al. [55] and Beal et al. [56] remain the state of the art in this area. Fully parameterized DBNs are inherent-

ly unidentified, i.e., certain transformations of the DBN coefficients give an infinite number of solutions which explain the expression profiles equally well. For converting the DBN into a unique directed gene interaction network, Rangel et al. [57] and Beal et al. [56] constructed a mathematical expression of the DBN coefficients, which itself is identified, and scanned for significantly non-zero interaction weights. The resulting network gives causal gene interactions that can be interpreted directly. In large networks of complex topologies, hub nodes have attracted particular interest. Hirose et al. [54] suggest focussing on hubs for which most interaction partners have plausible biological roles, as determined by associated gene ontology terms. Such a prioritization of specific interactions for validation in the laboratory increases the efficiency of the discovery process.

Although the difficulty of obtaining a full knock-down by RNAi for some targets can restrict the range of gene dosages testable, RNAi is in general well suited for studying dynamic system response. In contrast to plain knock-outs, the partial knock-downs that can be achieved with RNAi allow the biologically relevant investigation of interaction dynamics under physiological conditions. Moreover, many computational methods that remain tractable for high-dimensional expression data were designed for modelling linear system behaviour. Linear models are more likely to be a good approximation for gentle perturbances, whereas they are less well suited for capturing the non-linear responses potentially triggered by full knock-out experiments. There thus is a wider range of computational methods suitable for the quantitative analysis of partial RNAi knock-down experiments.

A Genome-Wide Transgenic RNAi Library for *Drosophila*

In a systematic effort, a comprehensive library of transgenic RNAi *Drosophila* lines has recently been constructed (lines available at vdr.ac.at [58]). This is the first genome-wide transgenic RNAi library available for a multicellular organism. Each RNAi construct consists of inverted repeats driven by an upstream activating sequence (UAS), cloned in a pMF3 vector. In total, 13,251 RNAi constructs targeting over 12,000 genes were injected in an isogenic w^{1118} strain to create 22,270 transgenic RNAi lines, covering 88% of predicted protein-coding genes. When crossed with a line carrying a GAL4 expression vector, in the progeny that carries both the GAL4 and the RNAi constructs, the GAL4 protein will bind to

the UAS and trigger down-regulation of the targeted gene (fig. 1b). Testing the library's efficiency, the level of target down-regulation was measured by RT-PCR for a set of 64 lines with an *Act5C-GAL4* driver. While observed effects ranged from none to almost complete down-regulation, more than half the lines showed at least a 50% decrease in target gene expression. In screening for lethality and adult morphological defects in lines for 432 positive controls (genes known to show a mutant phenotype), 48 negative controls (genes described as showing no specific visible mutant phenotype), as well as 499 random genes, about a third of the random genes produced a visible knock-down phenotype, while a false-negative rate of 35% and a false-positive rate below 2% were observed.

Opportunities and Specific Issues

Drosophila is the invertebrate model organism with the highest degree of homology to vertebrates, and the range of observable phenotypes in the fruit fly is larger than in any other system for which genome-wide RNAi is available. This makes the fly a powerful model organism for the study of genes and processes found in vertebrates, including conserved mechanisms of ageing relevant to human age-related disorders.

Ageing is complex and can be influenced by many factors. When studying the effect of a gene on life-span and ageing, it is thus critical to consider the effects of other genes. Experimental and control groups should thus have the same genetic background. A genome-wide library of uniform genetic background consequently facilitates the comparative study of many genes in large-scale screens of genetically identical organisms differing only in the inserted RNAi construct and target knock-down.

Several age-related disorders, such as cardiac disease are organ-specific. A major advantage of *Drosophila* over other existing model systems is that genetic constructs can target RNAi to particular tissues or cell types, allowing appropriately specific studies [e.g., 58]. Systemic surveys of a gene's role in various tissues require a comprehensive genetic toolkit of corresponding tissue-specific drivers. With the recent development of a genome-wide RNAi library and the historically large collection of driver lines, the fruit fly *D. melanogaster* becomes an especially interesting host organism for large-scale RNAi screens, allowing strong control of both when and where RNAi is triggered. Roles specific to phases in the life cycle or particular tissues or cell types can thus be studied. The compiled RNAi library has this flexibility built in by way of the well-established UAS-GAL4 system [59]. GAL4 is a transcriptional activator from yeast and when it binds to

UAS it triggers the expression of the insert downstream of the UAS. In the transgenic RNAi flies, the insert expressed is the RNAi construct leading to the transcription of dsRNA. The sequence will be transcribed only in the cells expressing GAL4. There are thousands of GAL4 driver lines with varied patterns of GAL4 expression, ranging from targeting only a few cells to covering the whole organism depending on the promoter by which GAL4 is controlled, and different levels of induction can also be achieved. Consequently, RNAi can be triggered specifically in a complex tissue type like muscles or in particular classes of neurons [14, 58]. In contrast, RNAi in *C. elegans* is cell non-autonomous and neurons are particularly refractory to RNAi. Elegant systems have also been designed to express GAL4 in a temporally controlled manner or conditionally inducing GAL4, for instance by temperature triggers (e.g., *hs-GAL4*, GAL80ts), drugs like RU486 or tetracycline, or flip-out techniques [59].

Driver Characteristics. To take advantage of the many GAL4 driver lines available, alternative drivers can be employed for their respective characteristics, and it is thus advisable to test the efficiencies of alternative drivers in triggering a phenotype of interest in pilots before embarking on a large-scale screen.

Insertion Site. As with any P-element-based technique, the location of the insertion of the RNAi constructs in the lines of the library described in [58] is largely random. The location of the insertion site can induce variability in the expression of the transgene and can also lead to the misregulation of neighbouring genes. It is therefore important to test the effect of the insertion site, either by directly comparing the RNAi line (without GAL4 driver) with its parent strain (same genetic background, no RNAi construct), or by comparing RNAi lines targeting the same gene with different insertion site locations. If the phenotype varies across lines, further experiments are required. The insertion site of the transgenic construct can actually be controlled with a vector exploiting the recently developed insertion technique based on phiC31-targeted integration. Transgenic RNAi libraries using this technique are being built and will reduce insertion-site effects [60]. Considering the increased amount of genetic manipulation required, however, it might be harder to match the genetic background of controls and RNAi lines for these libraries.

Off-Target Effects. During the RNAi process, the long hairpin dsRNA is cut into siRNAs and, given their short sizes, these siRNAs can potentially be complementary to sequences of mRNAs of several genes, bind to them and thus trigger RNAi of several genes at a time. This effect is

typically responsible for false-positives in screens (reviewed for *Drosophila* cell-based screens in Moffat et al. [61]). The careful design of RNAi constructs and comprehensive target/off-target prediction are therefore critical and still constitute active fields of research [10, 62, 63]. In a genome-wide library [58], about 97% of all constructs were predicted to have a unique target, the remainder reflecting the high similarities of some genes. Secondary interactions with off-targets were predicted for 39% of the constructs, although for most of these (~80%) with less than 1% of the 19-mer siRNAs sequences. Consequently, overall off-target effects were expected to be low for the library, which is in line with the observed false-positive rate <2%. Positives can in general be verified using different RNAi constructs with non-overlapping dsRNAs targeting the same gene. Interestingly, in screens for phenotypes like slower ageing or increased life-span, where most random knock-downs will have deleterious consequences, off-target effects may actually contribute to the false-negative rate. The off-target knock-downs are then likely to generally weaken the flies, potentially leading to a wrong conclusion that the target knock-down had no positive effect on life-span, even though the specific down-regulation of the target had been beneficial.

RNAi Efficiency. The least predictable characteristic of RNAi is its efficiency. The level and speed of down-regulation depend on both the target gene and the RNAi construct and will lead to corresponding differences in phenotype. Depending on the level of down-regulation of the same gene, one has observed no change, an increase, or a decrease of life-span [e.g., 19]. Sometimes RNAi efficiency can be modulated by technical changes to an experiment. Poor RNAi efficiency can be due to construct insertion at a poorly expressed locus, and a line with a different insertion site can thus give improved results. The level of down-regulation also depends on the strength of the promoter in the GAL4 driver used. GAL4 expression is thought to be temperature-dependent and higher temperatures may thus lead to stronger phenotypes. Increasing the level of Dicer 2 (Dcr2), an enzyme involved in the processing of dsRNA has been reported to improve RNAi efficiency, probably by boosting the natural RNAi mechanism [58, 60]. For instance, co-expressing Dcr2 with an eye-specific GAL4 driver enhanced RNAi effects, but also increased the rate of false-positives, probably by general amplification of endogenous RNAi. Similar to off-target effects, Dcr2 co-expression can lead to unspecific phenotypes, masking the true effect of the gene under study. Moreover, the frequency of developmentally lethal knock-downs was also increased [58]. Careful prelimi-

nary experiments are therefore required to determine if the benefits of a GAL4 driver coupled with Dcr2 outweigh its problems for a particular study. While a higher number of UAS sequences inserted in front of the RNAi construct has been reported to improve efficiency [60], this requires the creation of new RNAi lines.

Sex Differences. For unknown reasons, it has been reported that RNAi phenotypes tend to be stronger in male than in female flies [60]. In contrast, we have sometimes observed stronger effects of RNAi on life-span in females than in males [unpubl. data]. Finally, while some studies have reported sex-specific phenotypes in males [e.g., 22], the same effects have been observed in both sexes by others [18]. Consequently, both sexes should be investigated whenever possible and appropriate.

To summarize, systematic work with RNAi still holds a number of challenges, like the large-scale construction of specific constructs with minimal off-target effects or controlling for the effects of insertion sites. Recognizing the possibility of qualitatively different responses to different levels of down-regulation, moreover, certainly raises non-trivial questions. For many genes known to be of relevance to ageing, hypomorphic mutations increase life-span, whereas a complete loss of function of the same gene will decrease life-span. A modulation of knock-down levels is required to discern these effects, yet this has so far been beyond the scope of large-scale screening work. On the other hand, recent technical developments have much expanded the power of systematic RNAi knock-downs. Uniform libraries with a common genetic background providing well-matched controls for all RNAi lines are especially helpful in ageing research, which typically examines perturbations of potentially ageing-related processes relative to controls. Furthermore, the highly flexible UAS-GAL4 system offers a unique opportunity of temporally and spatially controlling RNAi induction. This is of direct relevance to the study of biological processes with varying effects on different age-groups (temporal control), or that are specific to certain organs or tissues (spatial control). The achievable fine-grained temporal control together with current developments in algorithms for computational analysis moreover allows the meaningful collection and study of dynamic data, permitting a direct investigation of regulatory interactions and the evolvement of ageing-related processes over time.

Conclusion

Allowing the controlled knock-down of practically any gene, RNAi has grown into a powerful tool for the study of gene function and the investigation of interaction networks. Its flexibility permits a wide variety of complementary experimental designs. Depending on the host organism, different approaches lend themselves to efficient large-scale screens or studies of dosage response. A genome-wide transgenic RNAi library for *Drosophila* now enables systematic screens in an invertebrate model closer to vertebrates, which seems particularly relevant to ageing research.

Like with other large-scale methods, technical particulars may affect outcome, and thorough pilots support the design of efficient larger screens. Screen results turn out to be highly specific to the chosen phenotype screened. Alternative aspects of ageing thus have to be examined in complementary studies. Besides steady-state screens for candidate genes, RNAi also much facilitates genome-wide quantitative perturbation studies of gene expression, and recent progress in the development of matching computational analysis tools means we can more and more take advantage of such data.

In summary, RNAi can be expected to considerably accelerate the identification of candidate genes affecting ageing and their potential interaction partners, leading to the subsequent further characterization of key players and their relevant interactions and dynamics in the ageing process.

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Note Added in Proof

While this manuscript was under review, Copeland et al. [64] reported on the knock-down of nuclear-encoded genes of the electron transport chain via the transgenic *Drosophila* RNAi library discussed above. For five genes an 8–19% increase in life-span was observed in females, and variable effects in males. For some genes, adult-specific and/or neuron-specific knock-down was sufficient for increased life-span. While thus illustrating several of the issues discussed in the present review, this recent study also well reflects the opportunities of applying RNAi libraries in ageing research.

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