THE ART AND DESIGN OF GENETIC SCREENS

The art and design of genetic screens: RNA interference

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Abstract | The remarkable gene knockdown technique of RNAi has opened exciting new avenues for genetic screens in model organisms and human cells. Here we describe the current state of the art for RNAi screening, and stress the importance of well-designed assays and of analytical approaches for large-scale screening experiments, from high-throughput screens using simplified homogenous assays to microscopy and whole-animal experiments. Like classical genetic screens in the past, the success of large-scale RNAi surveys depends on a careful development of phenotypic assays and their interpretation in a relevant biological context.

Forward genetics
The selection of mutants displaying a phenotype after random mutagenesis. The mutated gene is identified by positional cloning or by a candidate-gene approach.

Reverse genetics
Genetic analysis that begins with a gene sequence followed by targeted inactivation. The function of the gene is inferred from the resulting phenotype.

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RNAi has become a widely used method for studying gene function. Since its discovery in *Caenorhabditis elegans*[1], generating loss-of-function phenotypes by depletion of the corresponding transcript has facilitated functional studies of genes in various organisms, from the classical genetic model systems of *C. elegans* and *Drosophila*, to human cells and organisms such as planaria and mosquitos for which genetic techniques were not available.

RNAi is an endogenous cellular process by which messenger RNAs are targeted for degradation by double-stranded (ds) RNA of identical sequence, leading to gene silencing[1,2]. Initially used to knock down the function of individual genes of interest, the technology was harnessed in several organisms on a global scale with the production of RNAi libraries to silence most of the genes in their genomes, allowing genome-wide loss-of-function screening (FIG. 1).

The promise and pace of RNAi screening has attracted many researchers but its practical application involves acquiring new technical expertise. Here we discuss RNAi screening parameters that are of vital importance for a successful screen, paying particular attention to assay design and data analyses. We will not discuss the mechanism of RNAi nor the design of RNAi constructs, as these are covered elsewhere[3,4].

RNAi screens versus classical genetic screens

Many of the methodologies that are used in RNAi screening — such as how to establish phenotypic assays, perform large-scale screens and select candidate hits — are similar to those used in 'classical' genetic screening approaches. The phenotypes to be scored might differ, for example, cell-based screens might require automation and new analysis methods (FIG. 2). However, when applied across the genome, an RNAi screen is essentially a forward genetics screen using a reverse genetics technique, and thus has similar promises and constraints.

Past successes of classical genetic screens have been in large part due to clearly defined and easily scorable phenotypes that were then traced to mutations in relevant genes. Prime examples include screens for vulval development defects in *C. elegans* and eye development phenotypes in *Drosophila*, which together identified most of the major components of the Ras signalling pathways (see REF. 5 for a review). Advances in genetic screening technology in model organisms, such as the use of transposon-mediated mutagenesis and somatic clonal analysis, have led to an increasingly powerful repertoire of tools for gene identification[6–8]. However, a genetic screen generates a set of mutants for which the molecular lesion is not known, and identifying the mutated gene is often cumbersome and time consuming. In addition, some genetic screening methods such as transposon-mediated mutagenesis suffer from target bias.

Two major advantages of RNAi screens over classical genetic screens are that the sequences of all identified genes are immediately known and lethal mutations are easier to identify because it is not necessary to recover mutants (TABLE 1). These features affect post-screening analyses, allowing more sophisticated data analyses on identified (and missed) genes. RNAi screens of functional subgroups based on sequence or other criteria are also possible, and sequence can be taken into account when choosing genes to study in detail. However,

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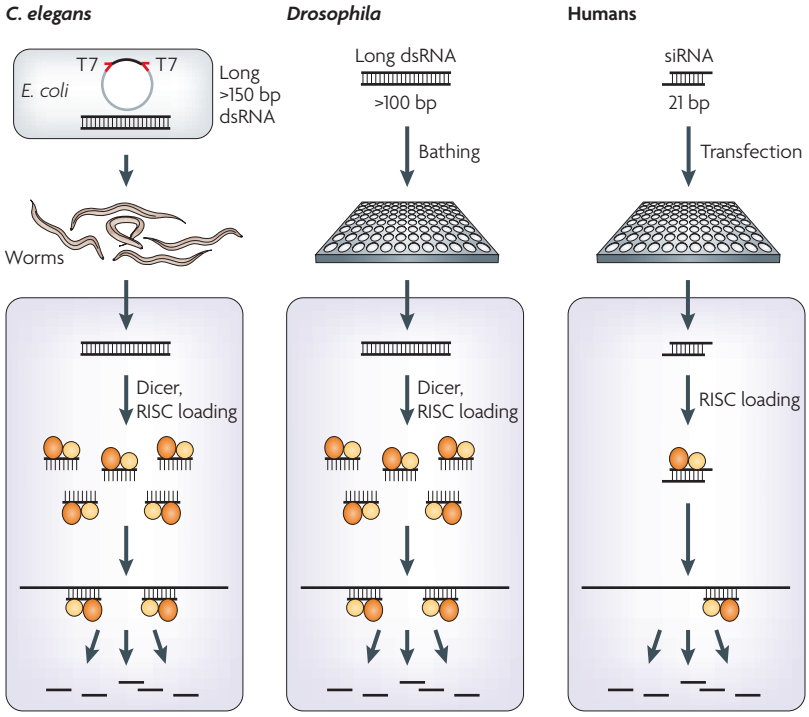

Figure 1 | **Approaches for genome-wide RNAi screens in different organisms.** Overview of RNAi screening approaches used in different organisms. Long double-stranded (ds) RNAs are introduced into *Caenorhabditis elegans* (by ingestion of expressing *Escherichia coli*) or *Drosophila* cells (by bathing) and are intracellularly diced into small-interfering RNAs (siRNAs). This leads to highly efficient knockdown because many different siRNAs are generated from each dsRNA. Introduction of siRNAs into human (or vertebrate) cells requires transfection. RNAi screens in human cells usually require multiple independent siRNAs, either in individual wells or delivered as pools. Other methods for human cells include viral transduction of hairpin expression constructs or endoribonuclease-derived siRNAs (esiRNAs), essentially pool of extracellular diced long dsRNAs. RISC, RNA-induced silencing complex; T7, bacteriophage T7 promoter.

RNAi also has disadvantages, such as the variability and incompleteness of knockdowns and the potential nonspecificity of reagents. In addition, whereas classical genetic screens can identify alleles that uncover regulatory mechanisms, RNAi is purely a loss-of-function technique targeting the mature message (TABLE 1). Below, we describe general considerations in RNAi screen design using examples from different systems.

Design of RNAi screens

Defining the goal. The first step in designing a good screen is to have a clear goal, as it will affect the design of the overall screen. For example, if the process to be analysed is well characterized, a directed screen might be designed to find missing components. This was done to identify the long sought-after gene encoding vitamin K epoxide reductase, an important drug target, by an RNAi screen in human cells that was focused on a set of genes in a particular chromosomal region[9]. Another goal might be to provide a broad overview of the types of genes involved in a less well-understood process, as was done in a *C. elegans* genome-wide screen for genes involved in endocytosis[10].

Synthetic phenotype
The phenotype that is seen only when two gene products are simultaneously inhibited, for example, in double mutants or using RNAi or drugs.

Primary screen assay. A robust and specific assay is the most important element of a successful RNAi screen. Its development is usually the most time-consuming aspect, requiring repetitive work and careful attention to detail while minor changes in parameters are tested and optimized, but the time spent in assay development is rewarded in the results of the final screen.

Like any good genetic screen, an RNAi screen needs an assay that is specific for the biological process being investigated. Unfortunately, often the ease of the assay is inversely proportional to its specificity. Cell lethality is probably the easiest phenotype to score, but it does not give much information about a gene's function. By contrast, an assay in which the function of synapses is directly measured using electrophysiological techniques is specific, but also laborious and probably not feasible on a genome-wide scale. Often, large-scale RNAi screens have to find a compromise between specificity and practicality.

Fortunately, there is a rich history of genetic screens in *C. elegans* and *Drosophila* that can be applied to whole-animal RNAi screening. Although some classical screens might already have been extensively performed, it can still be useful to repeat them using an RNAi screen because a different range of hits can be found. In particular, lethal genes or those with weak effects are often missed in classical genetic screens. Because an RNAi screen involves identifying which reagents induce the phenotype rather than recovery of the mutant, these are easy to spot using this approach.

The range of whole-animal assays that can be used is vast. These can be simple visual assays of morphological defects, changes in the expression of GFP reporters, synthetic phenotypes, sensitivity or resistance to drugs or small molecules, or any other assay that gives a reproducible output. Biological processes that are difficult or impossible to access in cell culture, such as organ function or organ formation and behaviour, can be probed using whole-animal RNAi screening. Processes that occur at the level of single cells are also amenable to RNAi screening.

Cell culture-based screens open up new avenues for high-throughput screening and are particularly suitable for dissecting basic cellular processes. In contrast to whole-animal assays, cell-based phenotypes are comparatively reductionist and particular care has to be taken to choose the appropriate biological context. The simplest cell-based assay is a homogeneous or bulk-cell assay, in which the phenotypes of many cells are averaged across each well in a microtitre plate. An example of this type of assay is testing for viability by ATP production, as measured by the activity of ATP-dependent luciferase[11,12]. At the other extreme are imaging screens, in which an image of each well (or spot) is taken and then individual cells are scored, potentially with many phenotypic descriptors. Time-lapse imaging has recently been adapted to RNAi screens, allowing dynamic processes such as mitosis to be investigated[13].

Positive and negative controls should be selected to develop the primary screen assay in order to achieve high signal with the positive controls and low noise with the

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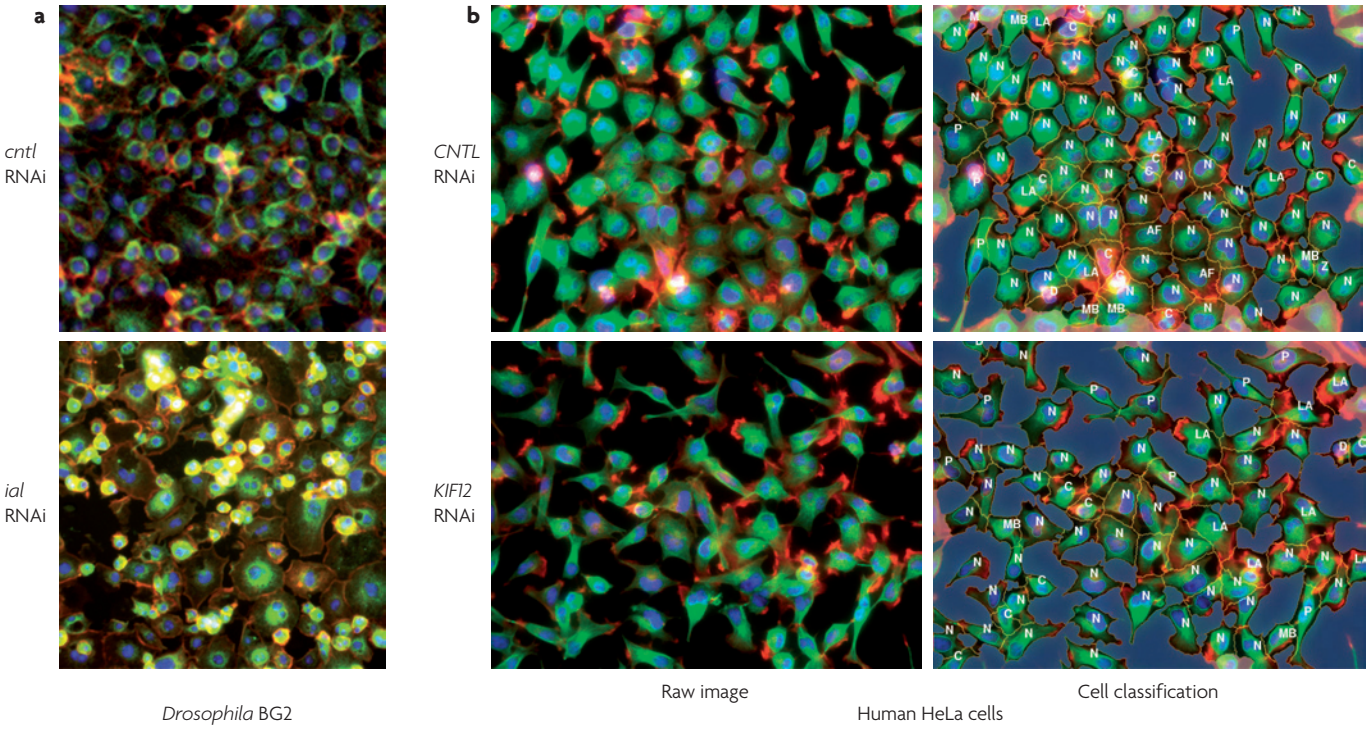

Figure 2 | **Examples of RNAi phenotypes.** Changes in morphology following RNAi treatment. **a** | *Drosophila* BG2 cells, a neuronal cell line, showing RNAi against a control (*cntl*) and IpIl-aurora-like kinase (*ial*) (photo courtesy of B. Baum, University College London, UK). **b** | Human cells, both unprocessed images (left panels) and images after automatic segmentation and cell classification with EBImage (right panels, different letters correspond to different cell classes) are shown following RNAi against a control (*CNTL*) and kinesin family member 12 (*KIF12*) (photo courtesy of F. Fuchs, German Cancer Research Center, Heidelberg, Germany and O. Sklyar, European Bioinformatics Institute, Cambridge, UK). **c**–**f** | Examples of whole-organism phenotypes. **c** | *Caenorhabditis elegans*. Left: molting defect induced by RNAi of molting defective (*mlt-11*) (photo courtesy of A. Frand, University of California, Los Angeles, USA). Right: branching defect induced by RNAi of enhancer of *akt-1* null (*eak-6*) (photo courtesy of H. Huttner, Simon Fraser University, Burnaby, Canada). **d** | Captured (inset) and processed image of a fluorescent apical membrane marker (image courtesy of S. Hoepfner and M. Zerial, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). **e** | *Drosophila* wing-margin defects induced by transgene RNAi hairpins directed against components of the wingless (*wg*) pathway, including *wg* and *evi* (evenness interrupted, also known as wntless) expressed under the control of *en*-GAL4 (photos courtesy of T. Buechling, German Cancer Research Center). **f** | Planaria multi-head phenotype after injection of double-stranded RNA against beta-catenin (photos courtesy of T. Adell and E. Sali, Universitat de Barcelona, Spain). *en*, engrailed.

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Table 1 | **Classical genetic screens versus RNAi screens**

Classical genetic screen	RNAi screen
• Gain-of-function alleles can be isolated, which can uncover regulatory mechanisms	RNAi-mediated knockdown results in a reduced level of wild-type product
• Tissue-specific alleles can be recovered	
• Insights into structure–function relationships can be obtained from point mutations	
Every gene should be mutable using this approach	Not every gene is susceptible to RNAi — some tissues are resistant and genes encoding proteins with long half-lives are hard to knock down effectively
The cloning stage is laborious	The gene sequence is known immediately
Maternal-effect genes with zygotic requirement are hard to identify	Can introduce double-stranded RNA at different developmental stages, bypassing earlier requirements
Mutations usually affect single genes	Multiple genes with shared sequence can be knocked down, thereby uncovering redundancy
Mutant alleles are heritable	Knockdown is usually not heritable, except when the silencing construct is expressed as a transgene(s)

negative ones; where possible, positive controls should encompass a range of strengths to develop an assay that can identify both weak and strong hits. Selecting only strong positive controls will give a biased assessment of the assay quality. The positive and negative controls will give important information on the reproducibility, robustness and ease of the assay — an assay that is successful when used on a small number of genes might not be amenable to being scaled up. Further optimization, by altering parameters such as time, cell line, equipment and genetic background, is important for improving signal-to-noise ratio.

Although the aim is to be able to clearly say whether each gene tested scores as positive, there is usually a spectrum in the strength of the phenotype observed. Therefore a scoring strategy needs to be developed, which can be qualitative — such as yes, maybe, no or not scorable — or quantitative; the latter is common in homogeneous and imaging assays. The positive controls of different strengths will help to determine the range of signals that can be detected. Typically, the cut-off point at which a gene is scored as positive is determined by analysing the data from the whole screen.

The pilot screen. Once the assay is developed, a small-scale 'pilot' screen of a few hundred random genes plus the positive and negative controls is usually done before undertaking a whole-genome screen. This will ensure that the hit rate is not too high and that the screen can be feasibly carried out on a large scale. For example, if 10% of random genes score as positive in the pilot screen, the assay is probably not very specific and should be redesigned. Likewise, if the positive controls are not reproducibly detected, the assay might not be sensitive enough. Bottlenecks in screening can also be identified during the pilot screen (such as lack of incubator space, or time needed for microscopy or aliquoting). In addition, scoring parameters can be finalized or sometimes altered if unexpected phenotypes are seen. At the conclusion of the pilot screen, the assay should be running under the exact conditions that will be used during the genome-wide screen.

The genome-wide screen. Primary screens are usually conducted in duplicate or more to increase the confidence of positives and to avoid the false negatives that arise in large-scale screening. All candidate hits are normally retested in the original assay, to confirm that they are positive. The end result of the primary screen will be a list of reagents that reproducibly score as positive in the primary assay. In experimental systems in which false positives caused by off-target effects are common, such as when using small interfering RNAs (siRNAs) in mammalian cells or long dsRNAs in *Drosophila* cell culture, it is advisable to retest hits using independent, non-overlapping RNAi reagents[14].

It is important to have a detailed screening schedule of every step in the process and to keep track of the screen parameters: date, time of day, person setting up the assay, person scoring the screen, lot numbers of reagents used, equipment used, temperature variations, drying of plates and possible contaminations. Additional parameters are described in an ongoing community effort to define the minimum information for an RNAi experiment (MIARE). This information can be helpful for detecting alterations in signal that might be due to technical variability and that can then be resolved by troubleshooting.

Secondary validation. Most, or all of, the positive hits identified in the primary screen after validation are likely to be 'real', but not necessarily for an interesting biological reason. Secondary screens will reveal which genes are of particular relevance. For example, one class of hits from screens for genes that are required for the expression of a particular GFP or luciferase reporter might be members of the general transcription machinery.

Two types of secondary assay are valuable for distinguishing between specific and nonspecific positives. First, the relevance of the positives can be tested by probing the process in a different way; this might involve an assay that is more laborious but more specific than the genome-wide one. Second, an assay could test the candidates directly for nonspecificity. In a case in which loss of GFP reporter expression was

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the primary assay, testing for loss of expression of an unrelated reporter gene would assay for nonspecificity. Secondary assays might also use a different biological system from the primary one, providing independent evidence for the involvement of a candidate gene in a particular process. For example, an *in vivo* animal assay might be used as a secondary assay after a cell-based primary assay in *Drosophila*.

Finally, it is extremely important to verify the final list of hits by sequencing positive clones or by re-testing with sequence-independent reagents. Rescue experiments — for example, using RNAi-insensitive transgenes encoding the gene of interest — can be used to further demonstrate the involvement of a particular gene[14].

Analysis of screening results. How the primary and secondary screen results are analysed depends on the type of assay and screening mode. Qualitative data, which is more common in whole-animal screens, will be analysed differently from quantitative cell-based data. But there are some general properties that distinguish the results of RNAi screens from classical screens.

Because weak mutants are often discarded, a classical genetic screen usually yields a set of mutants that each displays a strongly penetrant phenotype. An RNAi screen will yield a set of genes with a large range of scores. Weak but genuine phenotypes can be caused either by partial knockdown of a gene with a strong effect (similar to a hypomorphic mutation) or a strong knockdown of a gene with a weak effect. For quantitative assays in particular, a rigorous procedure that includes statistical analyses of the genome-wide data is important for assessing the significance of hits.

If the genome-wide screen used a qualitative primary assay, then secondary assay results are often used for most of the analyses and for defining the final gene list. It is common for the secondary assays to provide some simple quantitative data, such as percentage of animals that show a given phenotype. In this case, a simple statistical test could be applied to define the cut-off point for positives. In cases in which quantitative data are not available, the investigator's expert knowledge of the biological system can be used to make an informed decision, sometimes incorporating information from several assays. It might also be useful to define both high- and low-confidence lists.

The false-positive and false-negative rates can be used to evaluate the success of an RNAi screen. In theory, such calculations might seem straightforward, but, in practice, they can be difficult. The false-negative rate is usually estimated by measuring the hit rate for known positive genes. If the positive-control genes are strong hits, this only estimates the rate of identifying strongly positive genes. Comparing reproducibility between duplicate or triplicate wells can also provide information on false-negative rates. The false-positive rate of the primary screen can be estimated using the results of the secondary assays. In a well-designed and well-controlled screen, the final false-positive rate should be negligible.

Large-scale quantitative data sets require more sophisticated analyses, including the design of analysis workflows and their integration into all parts of the screening procedures (FIG. 3). Other key requirements are assessing the reproducibility between technical replicates, identifying the outliers that often occur in high-throughput experiments, and integrating different measurements such as multiplex reporter-gene assays. Several analytical approaches have been developed for the analysis of high-throughput screens[15,16]. One that is specifically developed for RNAi screens is the cellHTS software package, based on R and Bioconductor, which capture both workflow and data analyses[17].

An additional complexity arises in siRNA experiments when analysing phenotypes of different strengths that are induced by independent siRNAs. Statistical tools that build scores based on multiple independent measurements might ultimately help to discriminate true from false hits[18]. The application of standardized (and automated) analysis routines is particularly important when comparing large-scale RNAi data sets to find different and common phenotypes.

Microscopy-based imaging screens have their own technical challenges. These screens generate a large amount of data, easily exceeding a terabyte per screen. Open-source software packages have been developed for automated image analysis, such as CellProfiler[19] and EBImage.

Prospects and challenges. The software solutions that are currently available for quantitative analysis of high-throughput data are an important step forward, but there are still enormous challenges in analysing and integrating the data. In particular, the public availability and storage of large-scale data sets remain problematic. In contrast to sequence and microarray data sets, the RNAi field has not yet converged on a public repository. In the *C. elegans* field, Wormbase[20] and the RNAi database (RNAiDB)[21] have taken up the task. For *Drosophila*, the FLIGHT[22], Genome RNAi[23] and *Drosophila* RNAi Screening Center[24] databases contain data from published screens as well as tools for annotation and integration of phenotypic data with other data sources. No equivalent mammalian RNAi database currently exists. This, in addition to a database of RNAi data and reagent documentation across organisms, would be an extremely valuable resource.

RNAi screening in *C. elegans*

Multiple methods can be used for RNAi in *C. elegans*, including injection or soaking of dsRNA, the use of transgene hairpins, and RNAi by feeding bacterial strains that have been engineered to express dsRNAs[1,25–29]. Although highly effective RNAi injection and soaking screens have been carried out[30–32], we focus here on the use of RNAi by feeding for genome-wide screens. This is usually the method of choice because of the availability of libraries, the low cost, and the ease of application and scaling (BOX 1). No *C. elegans* cell lines currently exist, so cell-based screens are not yet possible.

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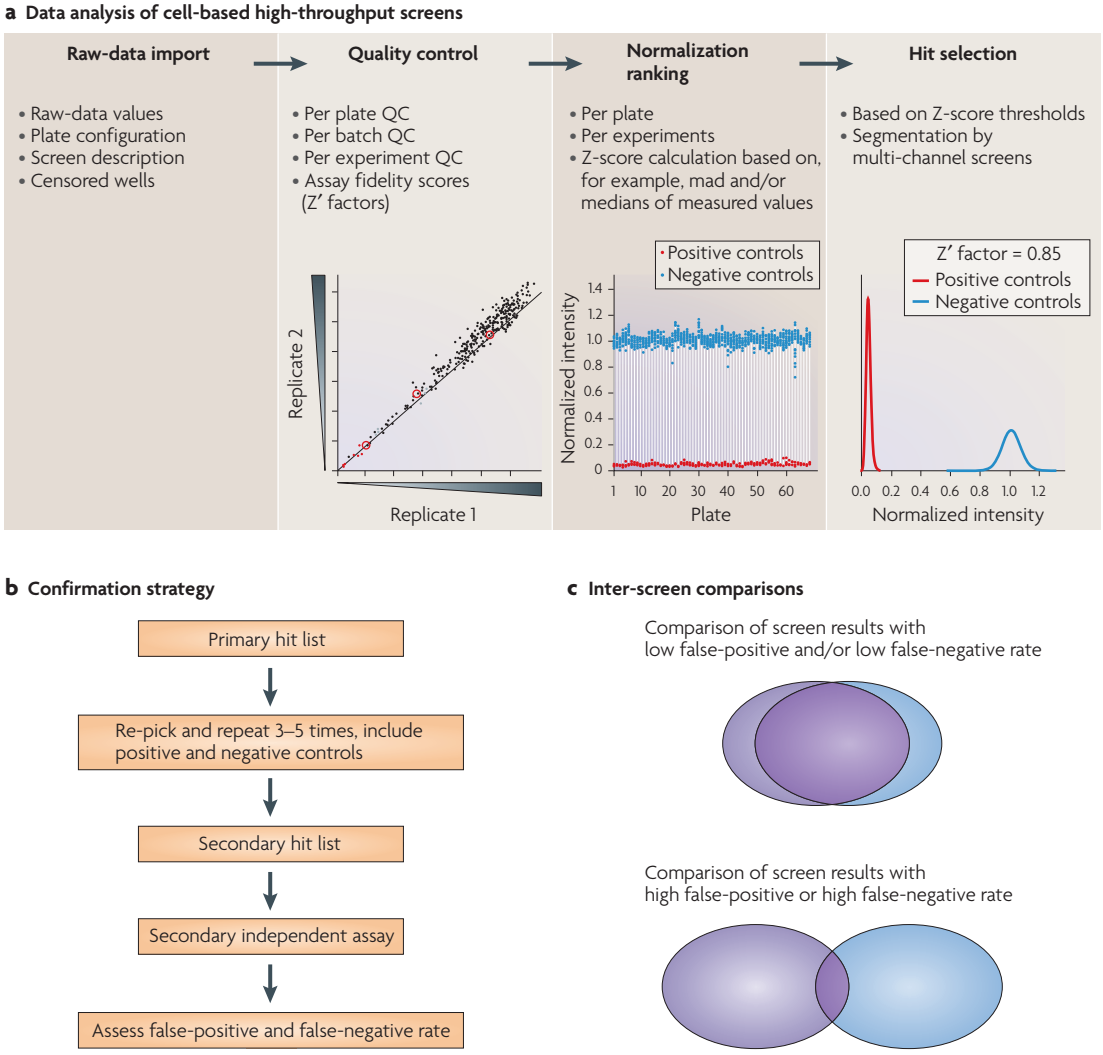

Figure 3 | **Analysis and validation approaches. a** | Data analysis workflow for high-throughput RNAi screens. After import of raw data (which can be large for microscopic screens), data is analysed to calculate quality control (QC) parameters, such as outliers between replicate measurements, plate effects and other artefacts, and to flag the measurements for downstream analysis. Quantitative phenotypic data is then normalized to a standard and scored according to predefined criteria such as the significance of the phenotypes compared with positive or negative controls, or the rank of hits. The overall quality of the screen and potential noise in large data sets can be quantified, for example, with Z′ factors, as a measure of the statistical power of the assay to discriminate between positive and negative hits[16] before hits are selected on the basis of predefined thresholds. **b** | Validation strategies to confirm the hit-list selection in secondary assays. **c** | False-positive and false-negative rates influence the overlap between screens (shown here as purple and blue ovals) done with different assays and/or reagents. Mad, median absolute deviaton.

Simple visible phenotypes. Screens need not be complicated to be effective. The first genome-scale RNAi screen in *C. elegans* assayed for obvious visible defects (for example, lethality, slow growth and morphological abnormalities) and connected ~1,700 genes to loss-of-function phenotypes[33,34]. Although it was successful for identifying essential genes and as a proof of principle of large-scale RNAi screening, the screen did not provide much specificity. However, a visible phenotype can be an extremely powerful assay if the phenotype scored has high specificity for a certain process. For example, Frand *et al.*[35] identified genes that are required for moulting by looking for animals trapped in old cuticle (FIG. 2c). Another good use of a visible phenotype assay was in a screen for genes that are involved in oocyte maturation[36]. When oocytes mature inappropriately, they pass through the spermatheca are laid unfertilized, so Govindan *et al.* simply screened for RNAi clones that induced the presence of laid oocytes on the agar plates.

Reporter screens. Making use of reporter genes in RNAi screens has great power and versatility, and well-designed reporters can make a screen highly specific. One of the first screens of this type identified genes that are involved in genome stability. Pothof *et al.*[37] used a

Spermatheca
An organ of the hermaphrodite reproductive tract, which receives and stores semen.

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Box 1 | **RNAi feeding in *Caenorhabditis elegans***

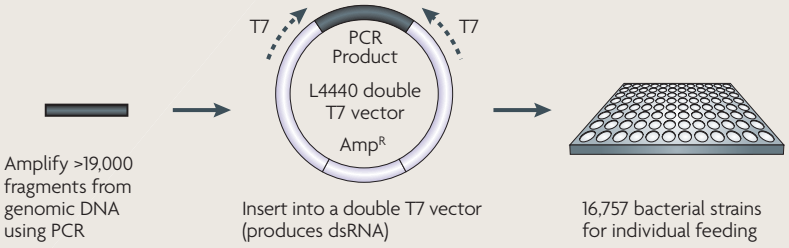
To carry out RNAi by feeding in *Caenorhabditis elegans*, a bacterial strain that expresses double-stranded (ds) RNA corresponding to sequence of the mature transcript of an individual gene is generated and fed to worms, leading to a systemic RNAi effect[26,29]. Typically, dsRNAs are 200–2,000 bp long; dsRNAs shorter than 150 bp are less effective[89].

Two RNAi feeding libraries are currently available. The first, which was made by cloning genomic DNA fragments, has 16,757 bacterial strains[33,34] (see figure). The second library has 11,511 strains expressing dsRNA corresponding to ORFs[90]. Together, the libraries currently target about 87% of genes; an effort is underway to create feeding strains for the remaining genes (J.A., unpublished observations).

Feeding is carried out either in 96-well liquid culture or on lawns of bacteria on agar plates[91,92]. Feeding in liquid is more rapid because liquid-handling devices can be used for all steps, and are ideal for population assays such as life versus death. Screens on agar plates are more time consuming but allow more detailed phenotypic scoring, for example, of morphological defects.

C. elegans can be subjected to RNAi at any stage and assayed later, or mothers can be treated and their progeny scored. The latter method is used for embryonic phenotypes to circumvent maternal effects and impermeability of eggshell to dsRNA.

The use of RNAi-supersensitive strains should also be considered. These are especially useful for screens involving the nervous system, in which RNAi by feeding is less effective[93–96]. However, these strains do have drawbacks: they all show temperature-sensitive sterility and lower brood sizes than the wild type[93,94,96].



Amp^R, ampicillin resistance gene. The arrows in the figure represent the orientation of the bacteriophage T7 promoters.

strain carrying an out-of-frame *lacZ* reporter gene, and screened for RNAi clones that induced its expression in patches in the animal, which would occur only if the reading frame was altered. Many of the positive RNAi clones also induced a mutator phenotype, supporting their role in genome stability. The use of fluorescent reporters has the benefit of allowing direct screening of live animals. Longman *et al.*[38] devised a clever GFP reporter screen for genes involved in nonsense-mediated decay (NMD), the process by which mRNAs with premature stop codons are recognized and degraded. They screened for RNAi clones that could activate expression of a synthetic GFP transgene that is normally silenced by NMD owing to a 3′ exon harbouring a premature stop codon. One benefit of this and of the above screen design is that positive clones cause gain rather than loss-of-reporter expression, preventing a background of knockdowns that lead to nonspecific inhibition of transcription.

Interaction screens. Genes that function in a particular process often show genetic interactions. Taking advantage of this property is an excellent way to find genes that are functionally related. Suppressor screens search for knockdowns that make a mutant phenotype less strong and can identify negative regulators. Enhancer screens — which search for genes that, when knocked down, exacerbate a phenotype — identify genes that act positively in the process of interest. Synthetic screens are a variant of enhancer screens; they usually start with a strain carrying a viable null mutation and then a search is performed for knockdowns that cause a phenotype only in combination with the starting mutant. This kind of screen can identify functionally redundant genes. Enhancer and synthetic screens suffer most from nonspecificity because general sickness can sometimes cause enhancement of a mutant phenotype. They therefore require more rigorous secondary assays for relevance. In addition, for both enhancer and synthetic screens, knockdowns must be tested in the absence of the starting mutation, to test whether they induce the phenotype on their own.

One of the easiest interaction screens is for suppressors of temperature-sensitive lethality. A homozygous mutant strain is first grown at the permissive temperature, and then subjected to RNAi at the restrictive temperature and screened for clones that overcome the lethality. Labbe *et al.* and O'Rourke *et al.* used this strategy to find suppressors of mutants of the cell polarity gene *par-2*, and of dynein heavy chain *dhc-1*, respectively[39,40].

Vulval development has been subjected to numerous classical forward genetic suppressor, enhancer and synthetic screens[41]. Repeating two of these using RNAi illustrates the complementarity of this approach to classical screening. An RNAi screen for synthetic multivulval (synMuv) genes identified nine new genes, most of which are essential for viability, potentially explaining why they had not yet been identified using forward genetics[42]. While screening for suppressors of the synMuv phenotype, Cui *et al.* also implicated many new essential genes in the process[43].

The above screens were designed to identify genes that were specific for the studied processes. In an alternative application of interaction screening, two groups performed large searches for genes that showed genetic interactions with different signalling pathways. Lehner *et al.*[44] identified 350 genetic interactions after testing 37 query genes against 1,750 targets. From this, they identified highly connected 'hub' genes that, when knocked down, modified multiple signalling pathways. Byrne *et al.*[45] uncovered 1,246 interactions after screening 11 target genes against 858 queries. Their analysis predicted functional modules that were connected to specific pathways. These RNAi screens illustrate an overall design that would be impossible using classical forward genetics — analysis of the complete set of hits derived from multiple RNAi screens revealed previously unknown connections.

Designing screens on the basis of previous screens or subsets of genes. Large-scale screens are excellent for an unbiased search for genes of interest. An alternative strategy that can be highly effective involves a smaller directed screen of a sub-library of RNAi reagents that is assembled using previous knowledge or data relating

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to the process in question. This approach also has the benefit of allowing a more technically challenging or time-consuming assay to be used, or more replicates to be done, thereby improving the robustness of results. In *C. elegans*, muscle 'arms' grow to regions of the nerve cords where they make synaptic contacts. To find genes that are involved in muscle-arm guidance, Dixon *et al.*[46] compiled a list of ~850 genes that had been shown to be required for animal movement or that had roles in guidance in other systems. Directly testing whether RNAi knockdown of any genes in this set caused muscle arm-guidance defects identified 23 genes as being involved in this process. Another example of a successful application of a small directed screen resulted in the identification of genes that are involved in sperm directional movement[47]. Starting with the knowledge that lipids are important for sperm movement, the authors found that among 35 lipid-synthesis or lipid-modifying genes, 6 were needed for correct sperm movement. Although clearly not exhaustive, screens of this type can quickly identify relevant genes for immediate study.

Secondary assays. No matter how specific the primary screen seems to be, secondary assays are important for identifying the genes that are particularly relevant. Specificity and nonspecificity assays are both important. For example, O'Rourke *et al.* found that over half of their initial *dhc-1 ts* suppressor mutations were nonspecific because they also suppressed the lethal phenotype of unrelated temperature sensitive (*ts*) mutants, possibly by increasing the activity of temperature-sensitive proteins[40]. Parry *et al.*[48] applied a battery of secondary assays to primary hits found in a screen for microRNA (miRNA) function. Like the dynein screen described above, the primary screen was an interaction screen, this time for suppression of the vulval-bursting phenotype of a weak *let-7* miRNA mutant. The candidates were tested for general defects in vulval development (as a test for nonspecificity) and for specific effects on miRNA biogenesis or function. Direct tests of nonspecificity combined with tests of specificity greatly increase confidence that the resulting list of hits is relevant.

Future prospects. So far, all published *C. elegans* RNAi screens have been scored manually because it is currently many times faster, is often more accurate and it allows a broader range of phenotype output than automated scoring. However, automated scoring could bring benefits such as quantitative primary-screen data, which is usually lacking in manually scored screens.

The most difficult aspect of automated scoring of *C. elegans* is image analysis. Because greyscale images are hard to parse, screens using fluorescent reporters or stains are much more promising as they have a higher signal-to-noise ratio. This approach is being used in an RNAi screen for genes that are involved in apico–basolateral polarity, in which images of worms carrying fluorescent reporters are captured with a Cellomics Arrayscan HCS reader and analysed using dedicated software (S. Hoepfner and M. Zerial, personal communication) (FIG. 2d). The laboratory instrument company Elegenics produces an instrument that can capture both brightfield and fluorescent images of worms grown on agar plates and can count and size animals, although analysis of fluorescent images still has to be done manually or with user-designed software.

Another method for automating screens involves using the complex object parametric analyser and sorter (COPAS) biosort instrument, also known as the 'worm sorter'[49]. This is essentially a worm flow cytometer that can profile the size of worms and the distribution of fluorescence in two channels along their lengths. Two markers in single worms (for example, an assay marker and a control) or a mixed population each carrying a different reporter can be assayed in this way. Nevertheless, improvements to the speed of the procedure and to image analysis will be necessary to make automation widely viable.

RNAi screening in *Drosophila*

Cell-based RNAi screens. Clemens *et al.*[50] discovered that RNAi in cultured *Drosophila* cells can be triggered simply by adding *in vitro* generated dsRNAs to the cell culture medium (BOX 2). Because of the ease and rapidity of this method, and the availability of libraries, most *Drosophila* RNAi screens have been done using cell-based assays. Also, a number of well-characterized cell lines of embryonic and larval origin are available for RNAi in *Drosophila*[51]. Screening experiments can be performed for a small number of genes — for example, those in specific functional groups — or for genome-wide applications of libraries. Compared to cell-based screens in mammalian cells, *Drosophila* has the key advantages of relatively low genetic redundancy and high efficacy of RNAi reagents.

Cell-based assays can measure a diverse set of phenotypes, ranging from homogenous cell viability readouts to alterations in reporter-gene expression to high-content assays using automated microscopy. Luciferase reporters are especially amenable for screening, and have been used to identify genes that are involved in signalling pathways, including JAK–STAT, Wnt and the immune deficiency (IMD) pathways[52–56]. One drawback is that reporter gene and other homogenous assays suffer from 'biological nonspecificity' caused by the knockdown of essential factors that might indirectly influence reporter activity[53,57]. Some of these effects can be offset by cross comparing screen results and only selecting candidates that appear in one, but not multiple, pathway assays. However, this approach will miss bona fide components that act in multiple signalling pathways.

Another powerful approach involves measuring the immunofluorescence signal from a highly specific antibody after RNAi. Friedman *et al.*[58] devised such a screen by probing *Drosophila* cells with a phospho-specific antibody against mitogen-activated protein kinase (MAPK) in comparison to total MAPK protein levels. As a secondary assay, they retested candidates using different upstream stimuli and validated selected candidates using RNAi *in vivo*. Other screens use a

High-content assay
The phenotypic scoring of multiple detailed characters that are usually spatially or temporally resolved.

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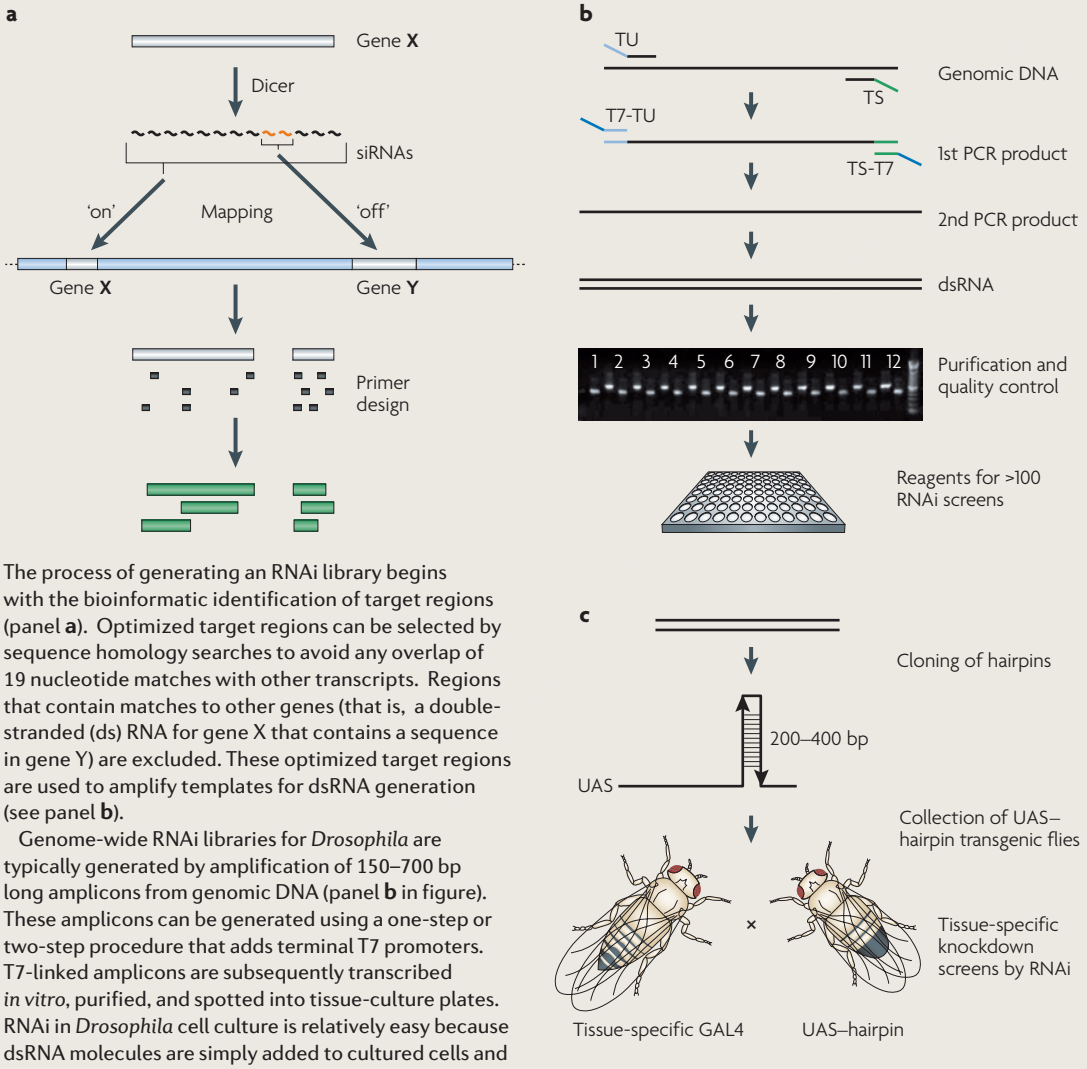
Patch-clamp assay
A technique for recording changes in electrical potentials of individual neurons. It is used as a way of recording neuronal activity.

relatively low stringency cut-off point in a first-pass screen coupled with a labour-intensive secondary assay. In a genome-wide screen to identify proteins required for Ca^{2+} entry, the authors used time-resolved change in fluorescence of a Ca^{2+} sensor to select approximately 1,500 candidates, and validated 27 of them in a second screen using a patch-clamp assay that would have been difficult to perform genome-wide[59].

***RNAi screening* in vivo.** In contrast to *C. elegans*, whole-animal screening approaches are technically more difficult in *Drosophila*. RNAi by feeding or soaking is not feasible, although RNAi by injection can be done in embryos[60]. Small injection screens have been performed for genes that are involved in nervous-system development[61] and embryonic cellularization[62].

Box 2 | **The construction of RNAi libraries for screening experiments in *Drosophila***



The process of generating an RNAi library begins with the bioinformatic identification of target regions (panel **a**). Optimized target regions can be selected by sequence homology searches to avoid any overlap of 19 nucleotide matches with other transcripts. Regions that contain matches to other genes (that is, a double-stranded (ds) RNA for gene X that contains a sequence in gene Y) are excluded. These optimized target regions are used to amplify templates for dsRNA generation (see panel **b**).

Genome-wide RNAi libraries for *Drosophila* are typically generated by amplification of 150–700 bp long amplicons from genomic DNA (panel **b** in figure). These amplicons can be generated using a one-step or two-step procedure that adds terminal T7 promoters. T7-linked amplicons are subsequently transcribed *in vitro*, purified, and spotted into tissue-culture plates. RNAi in *Drosophila* cell culture is relatively easy because dsRNA molecules are simply added to cultured cells and incubated for 24–96 hours (depending on the assay and cell type) before the phenotypes are assessed. This approach is feasible for small-scale experiments as well as large-scale screens using high-density tissue-culture plates. A single preparation of a library is usually sufficient for 100–300 screens. Two-step protocols have the advantage that the amount of T7-linked amplicons used as a starting material is relatively standardized and generates a similar amount of dsRNA after *in vitro* transcription. These amplicons can also be modified by using different adaptor primers in the second round in preparation for cloning (for example, hairpins), thereby increasing the versatility of the library.

Two libraries of transgenic flies have been generated and are being used for large-scale and smaller-scale screens (panel **c** in figure). The Dickson laboratory (IMP/IMBA, Vienna, Austria) constructed a library of 22,247 transgenic strains, covering 88% of the predicted 13,681 *Drosophila* genes[63]. This library is distributed through the Vienna *Drosophila* RNAi Center. These UAS–RNAi transgenes were prepared by amplification of a typically 200–400 bp gene fragment by PCR. A similar approach has been taken by the National Institutes of Genetics Fly Stocks. Both libraries are available and have become a valuable resource for the community.

siRNA, small-interfering RNA; TU, universal adaptor primer; TS, specific adaptor primer; UAS, upstream activator sequence.

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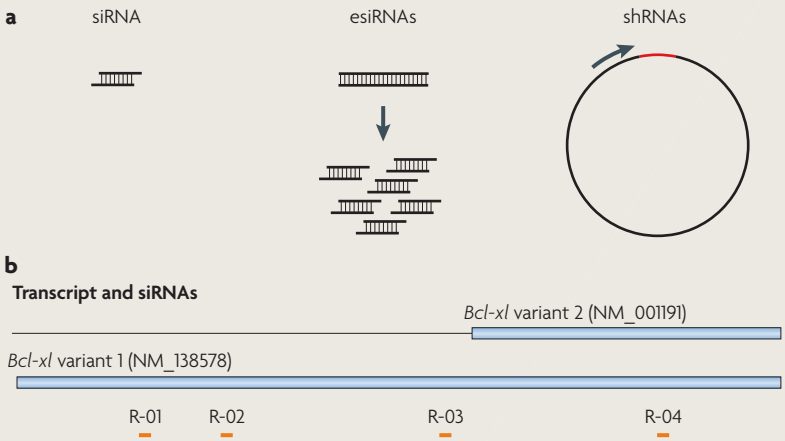
P element
Transposable element used in *Drosophila* for transformation and mutagenesis.

RNAi *in vivo* can also be effectively triggered by the expression of a hairpin dsRNA that contains a long (≥200 bp) inverted repeat. Expressing such transgenes under the control of a generic promoter containing the GAL4-responsive upstream activator sequence (UAS) element can target RNAi to any specific cells or tissues in the intact organism for which a suitable GAL4 driver line is available. Two libraries of transgenic flies harbouring UAS-driven RNAi hairpins have recently been produced on a genome-wide scale (REF. 63 and National Institutes of Genetics Fly Stocks (NIG-FLY); BOX 3). Although no genome-wide screens using them have yet been published, their availability means that whole-organism RNAi screens in *Drosophila* is now possible.

Box 3 | **RNAi libraries for screening experiments in vertebrate cells**



Several technologies are available for RNAi experiments in human (and other vertebrate) cells as shown in panel **a** in the figure. Small-interfering RNAs (siRNAs) are usually chemically synthesized on the basis of various public and commercial prediction algorithms. Recently, chemical modification of siRNAs has been described that decrease off-target effects[97]. Although many advances have been made in making siRNAs more efficient, a general problem has been the prediction (and avoidance) of off-target effects, in which siRNAs lead to unintended downregulation of unrelated genes with minimal sequence overlap. Multiple siRNAs per gene are therefore almost always required.

The generation of endoribonuclease-derived siRNAs (esiRNAs) is similar to the synthesis of double-stranded (ds) RNAs for *Drosophila*. Long dsRNAs synthesized by *in vitro* transcription of PCR products are 'diced' *in vitro* by Dicer or RNAse III to yield siRNAs that are then transfected into cells.

Large collections of vectors that transcribe short-hairpin RNAs (shRNAs) have been produced by several academic consortia in retroviral or lentiviral backbones that are suitable for transfection of even primary cells. These vectors often carry barcode labels, which can be quantitatively detected using microarrays. Such experimental set-ups facilitate an examination of changes in the population of shRNAs following, for example, a selection treatment.

It is important to note that genome coverage decreases over time for all libraries owing to ongoing genome annotation. Regular re-annotation of libraries is important to keep track of the target gene of each siRNA. In addition, siRNAs against the same gene might target different splice variants and give different results. Panel **b** of the figure shows four pre-designed siRNAs, targeting *Bcl-xl*, but only one siRNA hits both transcript variants (National Center for Biotechnology Information (NCBI) accession numbers NM_001191 and NM_138578). Annotation of siRNAs is important to allow the proper interpretation of phenotypic results. Schematic representations of ORFs are shown in blue; the siRNAs R-01 to R-04 in orange.

Prospects and limitations. A key limitation of RNAi screens in *Drosophila* has been the availability of cell lines of diverse tissue origins that are suitable for large-scale screening experiments. Although a range of cell lines have become available through stock centres, many cannot be used for screening because they show irregular growth behaviour, they cannot be transfected or they have a relatively low RNAi efficiency. Almost all of the screens published to date have been performed in cell lines of embryonic blood-cell origin (lines such as Kc_{167}, S2 and derivatives), which has limited the spectrum of phenotypes that can be scored. Recently, RNAi screening has become feasible in primary cells[64,65]. Establishing protocols for deriving primary cells from different tissues would allow more diverse screens as well as synthetic interaction screens using cells derived from flies with different genetic backgrounds.

The availability of transgene libraries for *Drosophila* should greatly expand the range of biological phenomena that can be explored using RNAi screens, as they make whole-animal screening possible. However, because each RNAi experiment involves using a different *Drosophila* strain, such a screen will involve a considerable investment of time. A further potential complication is that the expression of transgenes that are integrated using P elements depends on their integration site, which will influence knockdown efficiency. Technical advances such as site-specific integration could remedy this problem[66,67]. In the future, *in vivo Drosophila* RNAi screens will become an important genetic screening approach.

RNAi experiments in *Drosophila* can also suffer from off-target effects, leading to knockdown of genes with homologies to the introduced dsRNA[68,69]. The design and stringent quality control of long dsRNAs remains an important issue, both in small-scale and large-scale RNAi experiments. Several software tools have been developed that can identify the most common off-target sequences so that they can be excluded from RNAi constructs[70]. Also, second-generation libraries have become available that seem to have a higher target specificity and higher genome coverage (see the *Drosophila* RNAi screening center and the Genome RNAi database for sequence information).

RNAi screening in human cells

Several RNAi methods are suitable for large-scale RNAi screening in human cells: the use of synthetic siRNAs, vector-expressed short-hairpin RNA and endoribonuclease-derived siRNAs (esiRNAs) (BOX 3). All RNAi screens in human cells are cell based, essentially as described above[71]. Because long dsRNAs activate the interferon response, leading to apoptosis, RNAi in human cells must use siRNAs of 21–23 nucleotides, which evade the interferon response[72].

Genome-wide siRNA libraries that cover almost the complete human genome with several independent siRNAs are available from several commercial companies. Since the knockdown efficiencies of siRNAs vary, multiple independent siRNAs per gene are required. These can be screened individually or in pools. Pooling

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of siRNAs can reduce the costs of screening but it increases the likelihood that the phenotype is caused by an off-target effect of a single siRNA. This can be determined by retesting positives with multiple independent siRNAs. Although human siRNA libraries are expensive compared with libraries that are generated in-house for *Drosophila* and *C. elegans*, even a small aliquot is usually sufficient for hundreds of screens in a high-throughput format.

Several laboratories have developed vector-based short-hairpin RNA (shRNA) libraries that can be either transfected into cells or packaged as viruses, and transduced into cells that are difficult to transfect, such as primary cells. Because retroviruses and lentiviruses are stably integrated, these libraries are often screened in pools. Clonal cell populations of the desired phenotype can be selected and then the 'causative' shRNA identified. Alternatively, population-based barcoding screens can be performed in which cells are transduced with pooled viruses that contain shRNAs that can be uniquely identified with a molecular barcode. After phenotypic selection, enrichment of integrated shRNAs is measured using a microarray that contains sequences of the barcode[73–76]. Although pooling strategies are powerful, screen coverage is often difficult to assess.

Viral libraries have not been used as much as siRNAs for genome-wide screening of individual genes because of the difficulties in producing a high titre of virus on a large scale. Moffat *et al.*[77] recently described improvements to virus production that allowed them to individually screen 5,000 shRNA-producing viruses targeting about 1,000 human genes in a high-content imaging screen for mitotic progression.

Another approach for generating a large-scale RNAi library involves esiRNAs. In this approach, long dsRNAs are synthesized *in vitro*, diced, and transfected into cells — this is similar to the situation in *C. elegans* and *Drosophila* in which long dsRNAs are intracellularly diced into siRNAs (rather than diced *in vitro*). Such libraries have been shown to be highly efficient and have fewer off-target effects than siRNAs[78].

Synthetic screens. Similar to enhancer–suppressor screens in whole organisms, synthetic screens for modifiers of a particular chemical compound or double knockdown of other genes hold great promise for identifying genes in particular pathways. For example, Whitehurst *et al.*[12] screened a genome-wide RNAi library for enhancers of paclitaxel, a compound that inhibits the growth of microtubules and that is used as a cancer therapeutic. They found 87 genes that, when knocked down, sensitized a human lung-cancer cell line to paclitaxel-induced cytotoxity. Some of these gene products were predicted to act in the same protein complexes, indicating that they might work in a shared pathway. In a recent shRNA library-based barcode screen for resistance genes against Herceptin, an antagonistic epidermal growth factor receptor antibody now commonly used as a breast-cancer therapeutic, identified components of the phosphatase and tensin homologue (PTEN) pathway[79]. Synthetic screens should be broadly applicable to studying the effects of drugs, and could aid in identifying markers to predict treatment outcome.

Prospects and limitations. RNAi screens in vertebrate cells have the promise to shed light on many processes that are directly relevant to human health; however, important challenges remain. Improvements in the design rules for siRNAs have been published by a number of groups[80–82], but compared to RNAi in invertebrates, siRNAs usually have a lower efficiency and a higher number of off-target effects. In large-scale screening experiments, this can lead to high false-positive and false-negative rates. Validation strategies have to be carefully adapted for the particular experiment and library. One approach is to only consider and validate common hits that are identified using different libraries (or siRNAs). This would yield a shortlist of highly validated phenotypes, although it would greatly increase the false-negative rate. Because siRNA efficiency and specificity is still improving, these issues remain unresolved and recommendations of how many independent siRNAs should be used are evolving. It is certainly advisable to use independent siRNAs from different suppliers for validation experiments.

RNAi screening in other organisms

RNAi has become an important tool for genetic analyses in organisms for which classical genetics is not available. For example, loss-of-function analysis by RNAi is feasible using injection and feeding methods for planaria and *Hydra*[83,84], and a planaria library of 1,065 clones has been generated[85]. The regeneration capacity of these organisms makes them excellent systems for studying stem cells. Injection of dsRNA into *Anopheles gambiae* was used to identify genes that are required for innate immune responses against the malaria parasite, although so far only small gene sets have been screened[86]. *A. gambiae* cell lines are available and might be amenable for future genome-scale screens[87]. An RNAi-by-injection protocol has also been developed for the wasp *Nasonia vitripennis*[88]. Although whole-organism RNAi remains a technical challenge in many cases, any organism for which cell lines can be derived and transfected should be amenable to cell-based screening approaches.

Conclusion

RNAi screening is becoming part of the standard experimental repertoire. Through the distribution of public-domain libraries and the establishment of screening centres that can provide automation for cell-based screens, performing genome-wide RNAi screens is within the reach of most laboratories. However, we would like to emphasize that the novelty and speed of RNAi surveys should not distract investigators from the knowledge that a well-defined assay is crucial for a successful screen. In the future, we anticipate that, similar to genetic screens, the RNAi screen will only be the first step in the comprehensive analysis of biological phenomena — the end of the screen is the beginning of the experiment.

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