THE ART AND DESIGN OF GENETIC SCREENS

The art and design of genetic screens: RNA interference

Michael Boutros* and Julie Ahringer[‡]

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.

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 identification⁶⁻⁸. 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,

*Division of Signalling and Functional Genomics, German Cancer Research Center and CBTM. University of Heidelberg, Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany. *The Gurdon Institute and Department of Genetics, University of Cambridge, Tennis Court Road. Cambridge CB2 1QN, UK. e-mails: m.boutros@dkfz.de: ja219@cam.ac.uk doi:10.1038/nrg2364 Published online 3 June 2008

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.

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 region⁹. 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 endocytosis¹⁰.

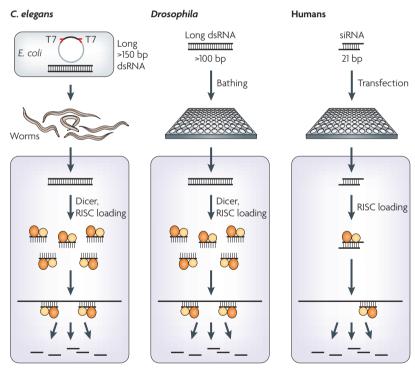


Figure 1 | Approaches for genome-wide RNAi screens in different organisms. Overview of RNAi screening approaches used in different organisms. Long double-stranded (ds) RNAs are introduced into Caenorhabditis elegans (by ingestion of expressing Escherichia coli) or Drosophila cells (by bathing) and are 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.

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 investigated¹³.

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

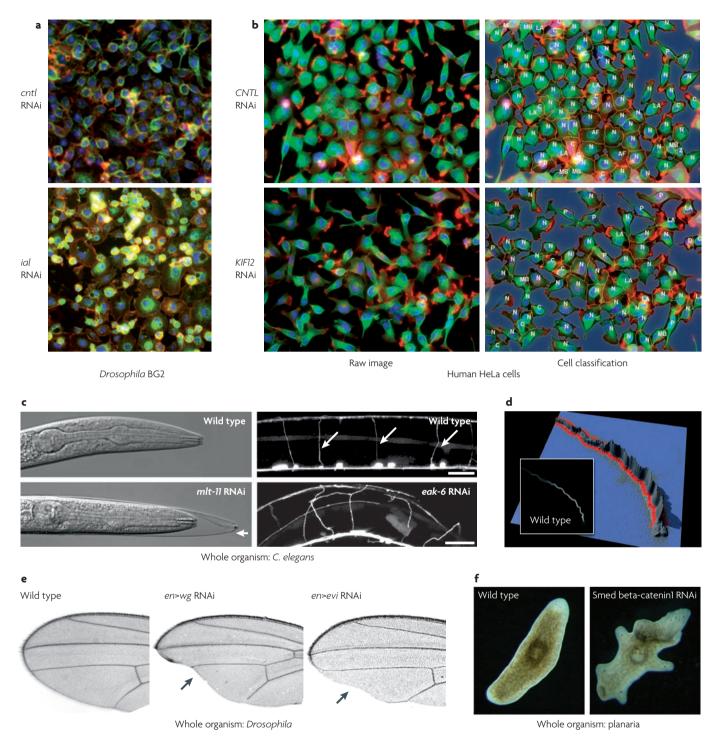


Figure 2 | Examples of RNAi phenotypes. Changes in morphology following RNAi treatment. a | Drosophila BG2 cells, a neuronal cell line, showing RNAi against a control (cntl) and Ipll-aurora-like kinase (ial) (photo courtesy of B. Baum, University College London, UK). b | Human cells, both unprocessed 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). \mathbf{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). \mathbf{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). \mathbf{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.

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 smallscale '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 reagents¹⁴.

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

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 retesting 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 gene¹⁴.

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 hits¹⁸. The application of standardized (and automated) analysis routines is particularly important when comparing large-scale RNAi data sets to find different and common phenotypes.

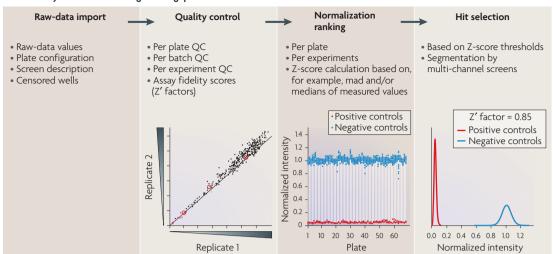
Microscopy-based imaging screens have their own technical challenges. These screens generate a large amount of data, easily exceeding a terabyte per screen. Open-source software packages have been developed for automated image analysis, such as <u>CellProfiler</u>¹⁹ and <u>EBImage</u>.

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, Wormbase²⁰ and the RNAi database (RNAiDB)21 have taken up the task. For Drosophila, the FLIGHT²², Genome RNAi²³ and Drosophila RNAi Screening Center²⁴ 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.

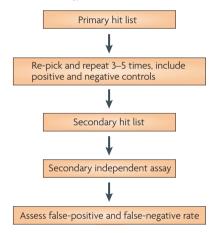
RNAi screening in C. elegans

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.

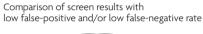
a Data analysis of cell-based high-throughput screens

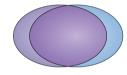


b Confirmation strategy



c Inter-screen comparisons





Comparison of screen results with high false-positive or high false-negative rate

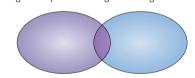


Figure 3 | **Analysis and validation approaches. a** | Data analysis workflow for high-throughput RNAi screens. After import of raw data (which can be large for microscopic screens), data is analysed to calculate quality control (QC) parameters, such as outliers between replicate measurements, plate 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. \mathbf{b} | Validation strategies to confirm the hit-list selection in secondary assays. \mathbf{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 maturation³⁶. 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.

Box 1 | RNAi feeding in Caenorhabditis elegans

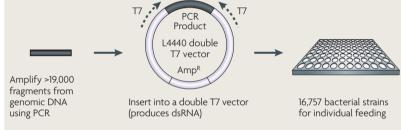
To carry out RNAi by feeding in *Caenorhabditis elegans*, a bacterial strain that expresses double-stranded (ds) RNA corresponding to sequence of the mature transcript of an individual gene is generated and fed to worms, leading to a systemic RNAi effect^{26,29}. Typically, dsRNAs are 200–2,000 bp long; dsRNAs shorter than 150 bp are less effective⁸⁹.

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 ORFs⁹⁰. 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}.



 $\mbox{Amp}^{R},$ 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 <u>par-2</u>, and of dynein heavy chain <u>dhc-1</u>, respectively^{39,40}.

Vulval development has been subjected to numerous classical forward genetic suppressor, enhancer and synthetic screens⁴¹. 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 genetics⁴². While screening for suppressors of the synMuv phenotype, Cui *et al.* also implicated many new essential genes in the process⁴³.

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

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 movement⁴⁷. 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 proteins⁴⁰. 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 apicobasolateral 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 <u>Elegenics</u> 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.

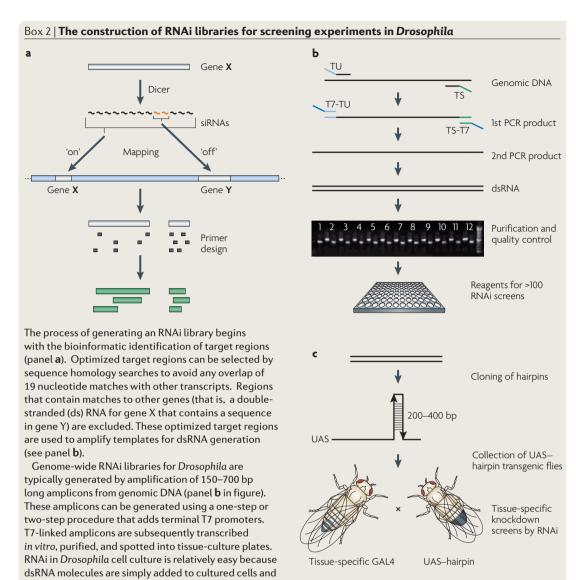
RNAi screening in Drosophila

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 genomewide 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.



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* genes⁶³. This library is distributed through the <u>Vienna Drosophila RNAi Center</u>. 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.

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 Ca²⁺ entry, the authors used time-resolved change in fluorescence of a Ca²⁺ 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-wide⁵⁹.

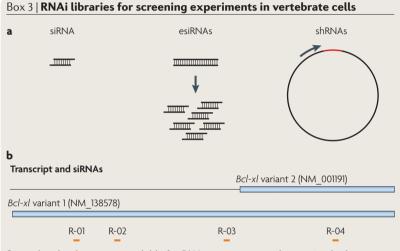
RNAi screening in vivo. In contrast to *C. elegans*, whole-animal screening approaches are technically more difficult in *Drosophila*. RNAi by feeding or soaking is not feasible, although RNAi by injection can be done in embryos⁶⁰. Small injection screens have been performed for genes that are involved in nervous-system development⁶¹ and embryonic cellularization⁶².

REVIEWS

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.



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 effects⁹⁷. 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 largescale 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 Kc₁₆₇, 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 constructs⁷⁰. 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 above⁷¹. 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 response⁷².

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 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 barcode⁷³⁻⁷⁶. 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 siRNAs⁷⁸.

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) pathway⁷⁹. 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 groups⁸⁰⁻⁸², 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 falsenegative 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 generated85. 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 screened86. A. gambiae cell lines are available and might be amenable for future genome-scale screens87. An RNAi-by-injection protocol has also been developed for the wasp Nasonia vitripennis88. 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.

RFVIFWS

- Fire, A. et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans Nature 391 806-811 (1998)
 - This is the landmark paper that started the field of RNAi
- Chapman, E. J. & Carrington, J. C. Specialization and evolution of endogenous small RNA pathways. Nature Rev. Genet. 8, 884-896 (2007).
- Dorsett, Y. & Tuschl, T. siRNAs: applications in functional genomics and potential as therapeutics. Nature Rev. Drug Discov. 3, 318-329 (2004)
- Hannon, G. J. RNA interference. Nature 418 244-251 (2002)
- Sundaram, M. V. The love—hate relationship between Ras and Notch. *Genes Dev.* **19**. 1825—1839 (2005). 5
- St. Johnston, D. The art and design of genetic screens: Drosophila melanogaster. Nature Rev. Genet. 3, 176-188 (2002).
- Jorgensen, E. M. & Mango, S. E. The art and design of genetic screens: Caenorhabditis elegans. Nature Rev. Genet. **3**, 356–369 (2002).
- Patton, E. E. & Zon, L. I. The art and design of genetic screens: zebrafish. Nature Rev. Genet. 2, 956-966 (2001).
- Li, T. *et al.* Identification of the gene for vitamin K epoxide reductase. *Nature* **427**, 541–544 (2004). 9 This paper demonstrates the highly effective use of a directed RNAi screen.
- Balklava, Z., Pant, S., Fares, H. & Grant, B. D. Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic. Nature Cell Biol. 9, 1066-1073 (2007).
- Boutros, M. et al. Genome-wide RNAi analysis of growth and viability in Drosophila cells. Science 303, 832-835 (2004) This paper details the first genome-scale RNAi screen in Drosophila cells.
- Whitehurst, A. W. et al. Synthetic lethal screen identification of chemosensitizer loci in cancer cells. Nature 446, 815-819 (2007).
- Neumann, B. et al. High-throughput RNAi screening by time-lapse imaging of live human cells. *Nature Methods* **3**, 385–390 (2006). This paper uses a combination of RNAi and timelapse imaging, which proves to be a powerful approach that allows dynamic processes to be investigated.
- Echeverri, C. J. *et al.* **Minimizing the risk of reporting** false positives in large-scale RNAi screens. *Nature* Methods 3, 777–779 (2006).
- Malo, N., Hanley, J. A., Cerquozzi, S., Pelletier, J. & Nadon, R. Statistical practice in high-throughput screening data analysis. Nature Biotechnol. 24, 167-175 (2006).
- Zhang, J. H., Chung, T. D. & Oldenburg, K. R. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen 4, 67–73 (1999). Boutros, M., Bras., L. P. & Huber, W. Analysis of cell-
- based RNAi screens. Genome Biol. 7, R66 (2006).
- Konig, R. et al. A probability-based approach for the analysis of large-scale RNAi screens. Nature Methods **4**, 847-849 (2007).
- Carpenter, A. E. et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 7, R100 (2006).
- Rogers, A. et al. WormBase 2007. Nucleic Acids Res. 36, D612-D617 (2008).
- 21 Gunsalus, K. C., Yueh, W. C., MacMenamin, P. & Piano, F. RNAiDB and PhenoBlast: web tools for genome-wide phenotypic mapping projects. *Nucleic Acids Res.* **32**, D406–410 (2004).
- Sims, D., Bursteinas, B., Gao, Q., Zvelebil, M. & Baum, B. FLIGHT: database and tools for the integration and cross-correlation of large-scale RNAi phenotypic datasets. Nucleic Acids Res. 34, D479-483 (2006).
- Horn, T., Arziman, Z., Berger, J. & Boutros, M GenomeRNAi: a database for cell-based RNAi phenotypes. Nucleic Acids Res. 35, D492-497 (2007).
- Flockhart, I. et al. FlyRNAi: the Drosophila RNAi screening center database, Nucleic Acids Res. 34. D489-D494 (2006).
- Tabara, H., Grishok, A. & Mello, C. C. RNAi in C. elegans: soaking in the genome sequence. Science 282, 430–431 (1998).
 Timmons, L. & Fire, A. Specific interference by ingested dsRNA. Nature 395, 854. (1998).
- Tabara, H. et al. The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell 99, 123-132 (1999).

- Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A. & Driscoll, M. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genet.* **24**, 180–183 (2000).
- Timmons, L., Court, D. L. & Fire, A. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene 263, 103-112 (2001).
- Gonczy, P. *et al.* Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. Nature 408, 331-336 (2000).
- Maeda, I., Kohara, Y., Yamamoto, M. & Sugimoto, A. Large-scale analysis of gene function in Caenorhabditis elegans by high-throughput RNAi. Curr. Biol. 11, 171–176 (2001).
- Sonnichsen, B. et al. Full-genome RNAi profiling of early embryogenesis in Caenorhabditis elegans. Nature 434, 462-469 (2005).
- Fraser, A. G. et al. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature 408, 325–330 (2000).
- Kamath, R. S. et al. Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature **421**, 231–237 (2003). References **33** and **34** describe the first genome
 - scale RNAi screen in C. elegans, which was also the first in any animal
- Frand, A. R., Russel, S. & Ruvkun, G. Functional genomic analysis of C. elegans molting. PLoS Biol. 3, e312 (2005)
- Govindan, J. A., Cheng, H., Harris, J. E. & Greenstein, D. Galphao/i and Galphas signaling function in parallel with the MSP/Eph receptor to control meiotic diapause in C. elegans. Curr. Biol. 16, 1257-1268 (2006).
- Pothof, J. et al. Identification of genes that protect the C. elegans genome against mutations by genome-wide RNAi, Genes Dev. 17, 443–448 (2003).
- Longman, D., Plasterk, R. H., Johnstone, I. L. & Caceres, J. F. Mechanistic insights and identification of two novel factors in the *C. elegans* NMD pathway. *Genes Dev.* **21**, 1075–1085 (2007).
- Labbe, J. C., Pacquelet, A., Marty, T. & Gotta, M. A genomewide screen for suppressors of *par-2* uncovers potential regulators of PAR proteindependent cell polarity in Caenorhabditis elegans. Genetics 174, 285-295 (2006).
- O'Rourke, S. M., Dorfman, M. D., Carter, J. C. & Bowerman, B. Dynein modifiers in *C. elegans*: light chains suppress conditional heavy chain mutants. PLoS Genet. 3, e128 (2007).
- Sternberg, P. W. & Han, M. Genetics of RAS signaling in C. elegans. Trends Genet. 14, 466-472 (1998).
- Poulin, G., Dong, Y., Fraser, A. G., Hopper, N. A. &Ahringer, J. Chromatin regulation and sumoylation in the inhibition of Ras-induced vulval development in Caenorhabditis elegans. EMBO J. 24, 2613-2623 (2005).
- Cui, M., Kim, E. B. & Han, M. Diverse chromatin remodeling genes antagonize the Rb-involved SynMuv pathways in C. elegans. PLoS Genet. 2, e74 (2006)
- Lehner, B., Crombie, C., Tischler, J., Fortunato, A. & Fraser, A. G. Systematic mapping of genetic interactions in Caenorhabditis elegans identifies common modifiers of diverse signaling pathways. Nature Genet. 38, 896–903 (2006).
- Byrne, A. B. et al. A global analysis of genetic interactions in Caenorhabditis elegans. J. Biol. 6, 8
 - References 44 and 45 use RNAi to explore genetic interactions on a large scale, a powerful approach that is likely to be increasingly used in the future.
- Dixon, S. J., Alexander, M., Fernandes, R., Ricker, N. & Roy, P. J. FGF negatively regulates muscle membrane extension in Caenorhabditis elegans. Development 133, 1263-1275 (2006).
- Kubagawa, H. M. *et al.* Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment in vivo. Nature Cell Biol. 8, 1143-1148 (2006).
- Parry, D. H., Xu, J. & Ruvkun, G. A whole-genome RNAi screen for *C. elegans* miRNA pathway genes. Curr. Biol. (2007).
- Pulak, R. Techniques for analysis, sorting, and dispensing of C. elegans on the COPAS flow-sorting system. Methods Mol. Biol. 351, 275-286 (2006).
- Clemens, J. C. et al. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc. Natl Acad. Sci. USA 97, 6499-6503 (2000).
- Baum, B. & Cherbas, L. Drosophila cell lines as model systems and as an experimental tool. Methods Mol. Biol. (in the press).

- Bartscherer, K., Pelte, N., Ingelfinger, D. & Boutros, M. Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. Cell 125, 523-533 (2006).
- DasGupta, R., Kaykas, A., Moon, R. T. & Perrimon, N. Functional genomic analysis of the Wnt-wingless signaling pathway. Science 308, 826-833 (2005).
- Kleino, A. et al. Inhibitor of apoptosis 2 and TAK1 binding protein are components of the *Drosophila* Imd pathway. *EMBO J.* **24**, 3423–3434 (2005).
- Muller, P., Kuttenkeuler, D., Gesellchen, V. Zeidler, M. P. & Boutros, M. Identification of JAK/STAT signalling components by genome-wide RNA
- interference. *Nature* **436**, 871–875 (2005). Ramadan, N., Flockhart, I., Booker, M., Perrimon, N. & Mathey-Prevot, B. Design and implementation of high-throughput RNAi screens in cultured Drosophila cells. Nature Protoc. 2,
- 2245–2264 (2007). Seto, E. S. & Bellen, H. J. Internalization is required for proper Wingless signaling in *Drosophila* melanogaster. J. Cell Biol. 173, 95-106 (2006).
- Friedman, A. & Perrimon, N. A functional RNAi screen for regulators of receptor tyrosine kinase and ERK signalling. *Nature* **444**, 230–234 (2006). Vig. M. *et al.* CRACM1 is a plasma membrane protein
- essential for store-operated Ca²⁺ entry. Science **312**, 1220-1223 (2006).
- Kennerdell, J. R. & Carthew, R. W. Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nature Biotechnol.* **18**, 896–898 (2000).
- Koizumi, K. et al. RNA interference screen to identify genes required for *Drosophila* embryonic nervous system development. Proc. Natl Acad. Sci. USA 104, 5626-5631 (2007).
- Pilot, F., Philippe, J. M., Lemmers, C., Chauvin, J. P. & Lecuit, T. Developmental control of nuclear morphogenesis and anchoring by charleston, identified in a functional genomic screen of Drosophila cellularisation. Development 133, 711-723 (2006).
- Dietzl, G. *et al.* A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature **448**, 151–156 (2007).
 - This paper and NIG-FLY describe the generation of libraries of transgenic Drosophila to allow whole animal RNAi screens.
- Bai, J., Hartwig, J. H. & Perrimon, N. SALS, a WH2-domain-containing protein, promotes sarcomeric actin filament elongation from pointed ends during Drosophila muscle growth. Dev. Cell 13, 828-842 (2007).
 - This paper shows that primary Drosophila cells can be used for RNAi screening, paving the way for more diverse screens in the future.
- Bai, J. et al. RNA interference screening in *Drosophila* primary cells for genes involved in muscle assembly and maintenance, Development 135, 1439-1449 (2008)
- Bischof, J., Maeda, R. K., Hediger, M., Karch, F. & Basler, K. An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proc. Natl Acad. Sci. USA* **104**, 3312–3317 (2007).
 Ni, J. O. *et al.* Vector and parameters for targeted
- transgenic RNA interference in Drosophila melanogaster. Nature Methods 5, 49-51 (2008).
- Ma, Y., Creanga, A., Lum, L. & Beachy, P. A. Prevalence of off-target effects in *Drosophila* RNA interference screens. *Nature* **443**, 359–363 (2006).
- Kulkarni, M. M. et al. Evidence of off-target effects associated with long dsRNAs in *Drosophila* melanogaster cell-based assays. Nature Methods 3, 833-838 (2006).
- Arziman, Z., Horn, T. & Boutros, M. E-RNAi: a web application to design optimized RNAi constructs. *Nucleic Acids Res.* **33**, W582–588 (2005).
- Echeverri, C. J. & Perrimon, N. High-throughput RNAi screening in cultured cells: a user's guide. Nature Rev. Genet. 7, 373-384 (2006).
- Elbashir, S. M. et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498 (2001).
- Berns, K. et al. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature **428**, 431–437 (2004). Paddison, P. J. *et al.* A resource for large-scale
- RNA-interference-based screens in mammals. *Nature* 428, 427-431 (2004). References 73 and 74 describe genome-scale RNAi screening in human cells.

- 75. Silva, J. M. et al. Profiling essential genes in human mammary cells by multiplex RNAi screening. Science **319**, 617–620 (2008).
- Schlabach, M. R. et al. Cancer proliferation gene discovery through functional genomics. Science 319, 620-624 (2008).
- 77. Moffat, J. et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* **124**, 1283–1298 (2006). Kittler, R. *et al.* Genome-scale RNAi profiling of cell
- division in human tissue culture cells. Nature Cell Biol. 9, 1401-1412 (2007).
- Berns, K. et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer, Cancer Cell 12, 395-402 (2007).
- Holen, T. Efficient prediction of siRNAs with siRNArules 1.0: an open-source JAVA approach to siRNA algorithms. RNA 12, 1620–1625 (2006). Jia, P., Shi, T., Cai, Y. & Li, Y. Demonstration of two
- novel methods for predicting functional siRNA efficiency. BMC Bioinformatics 7, 271 (2006).
- Koberle, C., Kaufmann, S. H. & Patzel, V. Selecting effective siRNAs based on guide RNA structure.
- Nature Protoc. 1, 1832–1839 (2006). Lohmann, J. U., Endl, I. & Bosch, T. C. Silencing of developmental genes in Hydra. Dev. Biol. 214, 211-214 (1999).
- Sanchez Alvarado, A. & Newmark, P. A. Doublestranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl Acad. Sci. USA* **96**, 5049–5054 (1999).
- Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R. & Sánchez Alvarado, A. Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev. Cell 8, 635-649 (2005).
- Vlachou, D., Schlegelmilch, T., Christophides, G. K. & Kafatos, F. C. Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. *Curr. Biol.* **15**, 1185–1195 (2005).

- Brown, A. E. & Catteruccia, F. Toward silencing the burden of malaria: progress and prospects for RNAi-based approaches. Biotechniques Suppl, 38-44 (2006).
- Lynch, J. A. & Desplan, C. A method for parental RNA interference in the wasp Nasonia vitripennis. Nature Protoc 1, 486-494 (2006)
- Parrish, S., Fleenor, J., Xu, S., Mello, C. & Fire, A. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol. Cell 6, 1077-1087 (2000).
- Rual, J. F. et al. Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. Genome Res. 14, 2162–2168 (2004). Ahringer, J. (ed.) Reverse Genetics, Wormbook
- [online], < http://www.wormbook.org/chapters/www introreversegenetics/introreversegenetics.html > (ed. The C. elegans Research Community, 2006).
- Lehner, B., Tischler, J. & Fraser, A. G. RNAi screens in Caenorhabditis elegans in a 96-well liquid format and their application to the systematic identification of genetic interactions. Nature Protoc. 1, 1617-1620 (2006).
- Kennedy, S., Wang, D. & Ruvkun, G. A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans. Nature* **427**, 645–649 (2004).
- Schmitz, C., Kinge, P. & Hutter, H. Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive Caenorhabditis elegans strain nre-1(hd20) lin-15b(hd126). Proc. Natl Acad. Sci. USA 104, 834-839 (2007).
- Sieburth, D. et al. Systematic analysis of genes required for synapse structure and function. Nature **436**, 510-517 (2005).
- Simmer, F. et al. Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Curr. Biol.* **12**, 1317–1319 (2002).
- Jackson, A. L. et al. Position-specific chemical modification of siRNAs reduces 'off-target' transcript silencing, RNA 12, 1197-1205 (2006).

Acknowledgements

We thank anonymous reviewers for comments. We are grateful to M. Zerial, E. Sali and H. Hutter for images and sharing unpublished data. J. A. is funded by a Wellcome Trust Senior Research Fellowship (054523). Work in the laboratory of M. B. is supported by grants from the European Commission, the Helmholz Association and the German Research Council.

DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query. fcgi?db=gene dhc-1 | par-2

FURTHER INFORMATION

The Ahringer laboratory: http://www.gurdon.cam.ac.uk/~ahringerlab

The Boutros laboratory:

http://www.dkfz.de/signaling

CellHTS software package: http://www.bioconductor.org/

packages/bioc/1.9/html/cellHTS.html

CellProfiler: http://www.cellprofiler.org
Drosophila RNAi Screening Center:

http://www.flyrnai.org

EBImage: http://www.bioconductor.org/packages/bioc/1.9/

html/EBImage.html

Elegenics: http://elegenics.com E-RNAi: http://e-rnai.dkfz.de FLIGHT: http://flight.licr.org

Flybase: http://flybase.net Genome RNAi: http://rnai.dkfz.de

MIARE (minimum information for an RNAi experiment):

http://www.miare.org

National Institutes of Genetics Fly Stocks (NIG-FLY):

http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp

RNAiDB (RNAi database):

http://nematoda.bio.nyu.edu:8001/cgi-bin/index.cgi RNAi Codex: http://codex.cshl.edu/scripts/newmain.pl

Vienna RNAi Screening Center: http://www.vdrc.at

Wormbase: http://www.wormbase.org

ALL LINKS ARE ACTIVE IN THE ONLINE PDF