

 TECHNOLOGIES AND TECHNIQUES

RNAi screening comes of age: improved techniques and complementary approaches

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Abstract | Gene silencing through sequence-specific targeting of mRNAs by RNAi has enabled genome-wide functional screens in cultured cells and *in vivo* in model organisms. These screens have resulted in the identification of new cellular pathways and potential drug targets. Considerable progress has been made to improve the quality of RNAi screen data through the development of new experimental and bioinformatics approaches. The recent availability of genome-editing strategies, such as the CRISPR (clustered regularly interspaced short palindromic repeats)–Cas9 system, when combined with RNAi, could lead to further improvements in screen data quality and follow-up experiments, thus promoting our understanding of gene function and gene regulatory networks.

Genetic screens

Identification of the organisms or cells that display a mutant phenotype of interest following large-scale disruption of genes; for example, by using mutagens.

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RNAi is an endogenous cellular process, first identified in *Caenorhabditis elegans* and conserved in most eukaryotic species, which involves targeted transcript cleavage and degradation following binding of a sequence-specific siRNA¹. For more than 15 years, researchers have harnessed RNAi activity as a research tool by introducing into cells or whole organisms RNAi reagents (such as synthetic siRNAs, endoribonuclease-prepared siRNAs (esiRNAs)) or siRNA precursors (such as short hairpin RNAs (shRNAs) or long double-stranded RNAs (dsRNAs))^{2–6} (FIG. 1) that are designed to target endogenous mRNA transcripts. Importantly, RNAi has enabled high-throughput gene silencing (knockdown) in cells and organisms, as this had been a challenge with classical genetic approaches. At its best, RNAi screening combines the power of genetic screens with phenotypic assays — the use of which had previously been limited, at least in cultured cell lines, to small-molecule screens. RNAi screening has made it possible to identify new genes, or gene networks, that are involved in a wide variety of biological processes^{2,3}, including assays relevant to signal transduction, cell viability, cell or organelle morphology, organelle or protein localization and/or function, drug resistance, and responses of host cells to pathogens (for reviews, see REFS 5, 7–10).

To facilitate large-scale screens, a number of genome-wide RNAi libraries comprised of one or more types of RNAi reagents were developed by academic

and commercial entities, with new libraries emerging as our understanding of the most effective strategies for the design and delivery of RNAi reagents improved (for information about available libraries and technological improvements to reagents, see REFS 4, 6, 7, 11–14). Readers unfamiliar with RNAi screens are referred to past reviews on assay development and optimization^{2,3,7,15,16}, high-throughput cell-based pooled format RNAi screens and arrayed format RNAi screens^{2,15,17}, *in vivo* screening^{4,12,14,18,19}, and screen data analysis^{2,7,20}. So far, hundreds of large-scale, cell-based RNAi screens have been carried out in *Drosophila melanogaster*, mouse and human cells. RNAi has also been used for large-scale *in vivo* screening in *C. elegans* and *D. melanogaster* (reviewed in REFS 2, 12, 14, 19), as well as Planaria^{21–23}, trypanosomes²⁴ and mice. Furthermore, a number of databases are now available that support the browsing and analysis of results from large-scale RNAi screens (BOX 1).

The initial burst of excitement about RNAi was somewhat tempered by the finding that RNAi screens, like all screening approaches, are associated with false discovery (false-positive and false-negative results). For RNAi, the most prominent concern is false positives that are due to sequence-specific off-target effects (OTEs)^{20,25} (FIG. 2). The availability of RNAi data sets (BOX 1) has made a number of meta-analyses possible, including those that aim to compare on-target findings and/or OTEs between

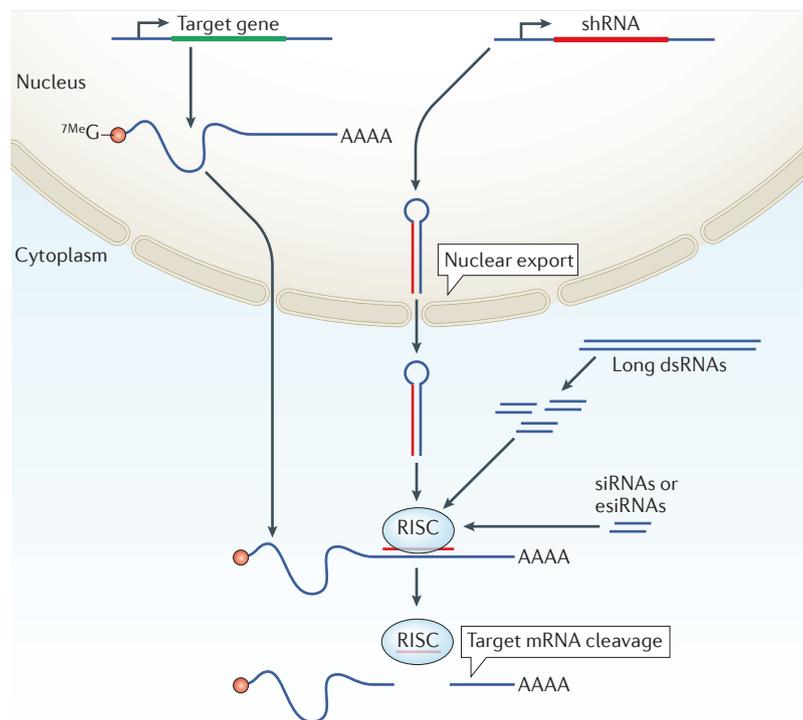


Figure 1 | Gene silencing by RNAi. RNAi reagents can be introduced into cells through different routes: siRNAs or endoribonuclease-prepared siRNAs (esiRNAs) can be transfected into mammalian (and other) cells; short hairpin RNAs (shRNAs) can be virally transduced into mammalian (and other) cells; double-stranded RNAs (dsRNAs) in solution can be applied to *Drosophila melanogaster* cells resulting in their uptake; dsRNAs (*D. melanogaster* or *C. elegans*) or shRNAs (*D. melanogaster* or mice) can be expressed from transgenic constructs; dsRNAs can be microinjected (*C. elegans*, *D. melanogaster* and some non-model insects); and *Escherichia coli* expressing dsRNAs can be fed to living animals (*C. elegans* or Planaria). Once in the cells, reagents such as dsRNAs are recognized by DICER (not shown), which processes them into siRNAs of 21–23 nucleotides in length. The synthetic or endogenously processed siRNA molecules are then incorporated into the RNA-induced silencing complex (RISC) and mediate gene silencing through target mRNA cleavage (if perfect sequence complementarity exists between the target mRNA and the siRNA) or translational interference (if the complementarity is partial; not shown).

Pooled format RNAi screens
Screens in which pools of reagents are introduced together into the cell population; for mammalian cell screening this is typically carried out with the goal of integrating just one of the short hairpin RNAs into each cell. Positive reagents are identified by comparing the abundance of any given reagent in the cell population before and after selection.

screens^{26–30}. These studies have explored overlap among gene sets or pathways identified in related screens, which has helped to improve estimates of false discovery rates³⁰; they have also revealed ‘frequent hitters’ — that is, genes that frequently score as positive hits across different assays, such as genes involved in ubiquitous processes that might exert relevant but relatively indirect effects, perhaps most notably genes encoding components of the ribosome or proteasome²⁶; and they have provided new information regarding the specificity and relevance of primary screen hits^{27,28,31}. Moreover, new experimental approaches and the use of novel genome-engineering systems to validate RNAi results are allowing better and faster identification of OTEs. Conversely, RNAi screening in mammalian cells has paved the way for innovation in related areas, including the use of microRNA mimics (miRNA mimics) and inhibitors (BOX 2), the use of RNAi or mutagenesis in three-dimensional (3D) mammalian culture systems (BOX 3) and the development of *in vivo* disease models in mice.

In this Review, we discuss state of the art RNAi screening, with an emphasis on new experimental and bioinformatics approaches to data validation, screening reagents and systems. We also discuss the intersection between RNAi screening and complementary approaches such as CRISPR–Cas9-mediated genome editing (FIG. 3).

Strategies for improving RNAi results

Sequence-specific OTEs occur when RNAi reagents bind to RNAs other than their intended target owing to partial complementarity (FIG. 2). It is fairly straightforward, using sequence alignment, to identify the subsets of OTEs that occur due to extended regions of complementarity between RNAi reagents and genes other than the target, such as regions common to the target gene and its paralogues. As gene annotations change (for example, following the identification of new alternative splice forms or the extension of the 5’ or 3’ untranslated region (UTR)), the interpretation of what constitutes on-targets and off-targets can also change, as can other relevant predictions, such as whether a reagent might target all isoforms of a gene. Improved approaches are now available for the re-annotation of RNAi reagents (for example, see UP-TORR¹³ and GenomeRNAi²⁶), thus facilitating the identification of reagents that no longer meet quality standards. However, eliminating reagents with extended complementarity from the library is not sufficient to fully address sequence-specific OTEs. To help address these concerns, new and improved approaches to identifying OTEs using bioinformatics, as well as experimental strategies for limiting OTEs, have recently been developed and successfully applied.

Addressing off-target effects with bioinformatics.

miRNAs, which are encoded by endogenous genes, are short transcripts that bind mRNAs, particularly in their 3’ UTRs, and inhibit their translation^{32,33}. This is mediated by incomplete complementarity between the miRNA and its target. Importantly, translational inhibition through incomplete complementarity is mechanistically independent from mRNA cleavage in the canonical RNAi pathway, which depends on extended sequence complementarity. Most sequence-specific OTEs are thought to occur when RNAi reagents function like miRNAs when incorporated into the RNA-induced silencing complex (RISC). In such cases, target recognition is not mediated by the binding of the full length of the siRNA to the 3’ UTRs and to other regions of the target mRNA (FIG. 2a), but is mediated by the binding of only a short seed region in the siRNA (nucleotide positions 2–8) (FIG. 2b). A recent report suggested that in three siRNA screens carried out in human cells, the majority of the primary hits could be attributed to seed region binding miRNA-like OTEs³⁴. This highlights the importance of addressing OTEs and validating primary screen results, as discussed below. Most commercial siRNA libraries incorporate chemical modifications to the siRNA seed region to help reduce OTEs (as reviewed in REF. 35), but data analysis and experimental

Box 1 | Databases for browsing and analysing RNAi screen data

Although no one database has been accepted as the established repository for RNAi data, several public databases have been developed as resources for sharing data from RNAi screens (see the table). RNAi data made public in this way can be used to help annotate gene function, be integrated with other large-scale data sets to investigate or provide support for new hypotheses, and provide helpful information to improve RNAi reagent design. To be most useful, RNAi data sets deposited in public repositories should include complete sequences for all RNAi reagents used, as well as detailed documentation of experimental and data analysis protocols and results.

Database	URL	Organisms	RNAi reagents
FLIGHT	http://flight.icr.ac.uk/	<i>Drosophila melanogaster</i>	Double-stranded RNA (dsRNA)
FlyRNAi.org	http://www.flyrna.org/index.html	<i>D. melanogaster</i>	dsRNA
GenomeRNAi	http://genomernai.org/	Human and <i>D. melanogaster</i>	dsRNA, siRNA and short hairpin RNA (shRNA)
PubChem BioAssay	http://www.ncbi.nlm.nih.gov/pcassay	Human, rat and <i>D. melanogaster</i>	siRNA, shRNA and dsRNA
RNAiDB	http://www.rnai.org/	<i>Caenorhabditis elegans</i>	dsRNA
WormBase	http://www.wormbase.org	<i>C. elegans</i> and other nematodes	dsRNA

follow-up of screen results are essential to identify false-positive hits due to OTEs. Because seed regions are short, any particular seed sequence might be present in hundreds of transcripts, making it difficult to predict computationally potential off-targeted transcripts using seed sequence alignments, or to design RNAi reagents with seeds that might result in fewer off-targets. Software tools that can be used to identify seed-dependent OTEs in RNAi data sets include Genome-wide Enrichment of Seed Sequence matches (GESS)²⁷ and Haystack²⁸. GESS and Haystack use two different strategies to identify potential off-target transcripts and both produce their best results when larger (genome-wide) data sets are used. Publicly accessible tools exist for the analysis of data sets by these methods: [Online GESS](#)³⁶ and [Haystack](#)²⁸.

Experimental approaches addressing off-target effects. The C911 RNAi reagent controls, which can be generated for any RNAi reagent by replacing bases 9–11 with their complement bases (hence the name)³⁷, are experimental tools that enable specific concerns about seed-based OTEs to be addressed (FIG. 2b). A C911 control has the same siRNA seed region (bases 2–8) as the original RNAi reagent, but perfect complementarity with the intended target gene is destroyed. C911 versions of false-positive siRNAs maintain their phenotype when assayed, whereas C911 versions of true-positive siRNAs do not³⁷. The C911 control strategy should also be informative for shRNA experiments, as the endogenously processed shRNAs also have the potential to cause seed sequence-mediated miRNA-like effects. Because C911 controls are easily designed for all RNAi reagents, for example, by using the online [C911 calculator](#), it is feasible to test many RNAi hits using this strategy. A related strategy is to test seed region controls — RNAi reagents that have been designed with the seed sequences of the RNAi hits, but also with randomized nucleotide sequences outside of the seeds³⁴.

The most common and straightforward experimental strategy to validate RNAi screen hits is to test multiple RNAi reagents for each gene, as different reagents will have different seed sequences. For siRNA screens, seven or more independent reagents per gene might be assayed; for pooled shRNA screens, some investigators screen libraries using more than 15 constructs per gene^{38,39}. The greater the number of independent RNAi reagents per gene that reproduce the desired phenotype, the higher the confidence that the gene is a true hit in a screen. This is not always feasible, however, as it is not possible to design multiple independent RNAi reagents for some genes. Moreover, a potential caveat to this strategy is that potent reagents might be fairly rare, as suggested by the results of a large-scale study of shRNA effectiveness⁴⁰. In the study, fluorescence protein-encoding sensors with shRNA binding sites were used to monitor knockdown effectiveness. In total, 20,000 shRNAs targeting nine transcripts were assayed using the sensors, and fewer than 2,000 remained following enrichment for target-specific shRNAs. Moreover, shRNAs conferring robust knockdown constituted less than 3% of the total tested⁴⁰. If this proves true for most RNAi reagents, then even when large numbers of reagents are tested, only a small subset might score as hits.

A definitive experimental strategy for testing the specificity of an RNAi reagent is to show that the knockdown phenotype can be rescued by the expression of an RNAi-resistant version of the targeted gene (FIG. 2c). However, this has not been routinely carried out, probably because of the effort that was required until recently to design and produce such RNAi-resistant constructs, and because the interpretation of rescue experiments is complicated when rescue constructs are expressed at non-physiological levels²⁰. Several groups have used homologous genes from related species expressed in bacterial artificial chromosomes (BACs) or similar genomic fragments to rescue RNAi knockdown,

Arrayed format RNAi screens

Screens in which each reagent (or a small pool of reagents directed against the same gene) is introduced separately into cells.

miRNA mimics

Short, double-stranded RNAs that, when introduced into cells, mimic endogenous microRNAs by activating post-transcriptional repression of target genes.

Seed region

Base pairs 2–8 of the fully processed siRNA. It can mediate microRNA-like effects even in the absence of perfect complementarity between the remainder of its sequence and that of the transcript.

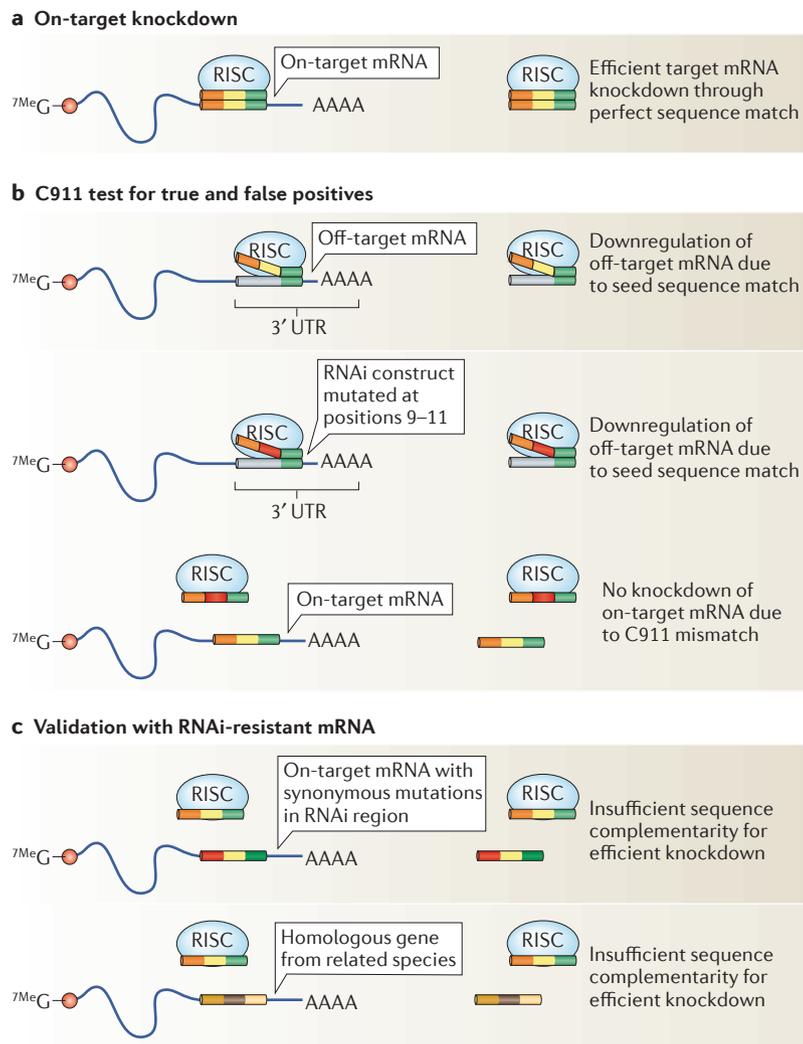


Figure 2 | Strategies for validating RNAi screen results. **a** | RNA-induced silencing complex (RISC)-incorporated siRNAs mediate target mRNA cleavage upon perfect sequence complementarity in either the coding region or the 3' untranslated region (UTR) of the mRNA (depending on the siRNA design). For the siRNA shown, the 5' seed region is in green, the middle region is in yellow and the 3' end is in orange. **b** | Testing for potential off-target effects of a given siRNA can be carried out using the C911 method³⁷. siRNA bases 9–11 are mutated while the seed region (bases 2–8) remains intact. This maintains off-target interactions mediated by seed region matches but perturbs on-target silencing. **c** | On-target specificity by phenotypic rescue can be demonstrated by the co-expression of RNAi-resistant versions of the target mRNA. Synonymous mutations in the siRNA-targeted region of the mRNA can be introduced to prevent RISC-mediated silencing while preserving function. Alternatively, a homologous gene from a related species that has sufficient sequence divergence in the siRNA targeting region to be RNAi resistant, but also sufficient similarity to elicit function, can be used to test on-target specificity of the RNAi construct.

for example, the use of a mouse homologue to rescue an siRNA phenotype in human cells⁴¹, or the use of a *Drosophila pseudoobscura* homologue to rescue a phenotype in *D. melanogaster*^{42,43}. This approach has the advantage that the homologous proteins are more likely to be expressed at physiological levels as they remain in their genomic context. However, this strategy only works when the homologous gene can functionally replace the tested gene.

Validating RNAi screen results with genome editing. The CRISPR–Cas9 system or other genome-editing approaches could be used to engineer RNAi-resistant versions of endogenous genes by introducing synonymous changes that abolish the RNAi target sequence, providing another means to assess potential OTEs. It is also possible to use genome-engineering approaches to create complete knockout (loss-of-function) alleles as follow-ups to functional screens (FIG. 3), providing a different type of validation of observed phenotypes. At least one recent study has reported the use of engineered knockouts to validate RNAi screen results⁴⁴. In the study, FAT1 was identified as a negative regulator of apoptosis in a genome-wide siRNA screen of a human glioblastoma cell line. CRISPR–Cas9-mediated knockout of FAT1 conferred sensitivity to death receptor-induced apoptosis, which was consistent with the screen result⁴⁴. It is conceivable that using the CRISPR–Cas9 system or other genome-engineering approaches to knock out non-essential genes will become a routine means of verifying RNAi screen results. The successful implementation of genome-editing technologies in several species⁴⁵ suggests that this will be a relevant tool for follow-up studies in many types of cell lines and model systems.

Genome-engineering approaches such as the CRISPR–Cas9 system are not without caveats. For example, careful attention must be paid to the design of genome-engineering vectors in order to maximize the chance of gene disruption and to minimize the potential for introducing DNA breaks in regions other than the target gene. A recent study demonstrated that, although some short guide RNAs (sgRNAs) can target Cas9 to thousands of ectopic sites in the genome, target cleavage only occurs at sites with extended complementarity⁴⁶. On the basis of these data, the authors proposed a two-step model for Cas9 binding and cleavage. Given the gaps in our overall understanding of how the CRISPR–Cas9 system functions in eukaryotic cells, it seems likely that we do not yet fully understand all the potential experimental problems of applying this technique. In addition, even when genome engineering can be used to induce effects that are strictly gene specific, RNAi knockdown, in which mRNA levels are typically reduced but not completely eliminated, might result in a weaker, incomplete or distinct phenotype compared with a gene knockout, which results in the full elimination of function. As a result, the two phenotypes might not appear identical in some cases even when both strategies are indeed exerting on-target effects⁴⁷. Nevertheless, when the RNAi and knockout phenotypes are concordant, the results will be of high confidence.

Parallel screening in multiple species. Another effective approach to validate RNAi results is to carry out related screens in different model systems, such as screening for related phenotypes using RNAi in *D. melanogaster* and mammalian cells (for example, see studies focused on dengue virus–host cell interactions⁴⁸, actin regulators⁴⁹ or androgen receptor function⁵⁰), or using RNAi in *D. melanogaster* cells and genetic screening in yeast (for example, see studies focused on the identification of genes required

Box 2 | Targeting non-coding RNAs

In recent years, screening strategies that were originally developed for targeting mRNAs with short hairpin RNA (shRNA) and siRNA have been applied to non-coding RNAs, notably using libraries of reagents that inhibit or mimic microRNAs (miRNAs). Several such libraries have been developed and commercialized, enabling functional high-throughput, unbiased screens to be performed. This format has facilitated the identification of miRNAs that contribute to a variety of diseases and physiological responses, including viral infection⁹⁵, breast cancer^{96,97} and drug treatment responses^{98,99}. A study involving both miRNA mimics and siRNA screening using the same assay, identified miRNAs involved in cisplatin resistance, as well as the kinases targeted by these miRNAs¹⁰⁰. Libraries of miRNA mimics are used more frequently than libraries of miRNA inhibitors, because inhibitors will only have an effect if the targeted miRNA is expressed during the assay.

As the miRNAs that are involved in a biological process are often unknown before the screen, it can be challenging to identify positive and negative screen controls. Fortunately, the use of screening strategies for miRNAs similar to those used for mRNAs has enabled investigators to use siRNA controls in miRNA screens, selecting siRNAs that are known to elicit the desired phenotype or phenotypes of the screen as positive controls until corresponding miRNA reagent controls are identified. When analysing the results from miRNA reagent screens, potential hits usually have weaker phenotypes than siRNA screen hits, probably the consequence of functional redundancy among miRNAs. Therefore, it can be helpful to determine how many mimics of miRNA belonging to the same miRNA family elicit similar phenotypes. This provides an indication of true target mRNAs, the determination of which is an essential follow-up step.

Recently, RNAi libraries targeting long non-coding RNAs (lncRNAs) have been generated¹⁰¹. It is still unclear how effective siRNA libraries will be in knocking down lncRNAs, particularly those localized to the nucleus, and whether this experimental strategy will further our understanding of how lncRNAs influence cellular processes¹⁰².

for nucleolar size regulation⁵¹). High-throughput comparative analysis of phenotype conservation can identify genes and protein complexes that have been evolutionarily repurposed or that are part of more complex, redundant networks. This strategy has been used successfully to study the conservation of genetic interactions across species^{52,53}, as well as the conservation of mechanisms that control subcellular structures or features^{49,51}. Taking a comparative approach can also help to overcome species-specific limitations, such as incomplete genome coverage of screening reagents or sequence-specific OTEs. The hits that correspond to cellular processes and complexes for which gene ontology terms are consistently enriched in the data set of both species have a higher probability of being true positive hits. In addition, single genes that score as positive hits in both species can also be considered to be high confidence hits, as they have been independently confirmed by different screen reagents, methodologies and organisms.

Screening multiple phenotypes and genes

In cell-based RNAi screens, specific phenotypes can be characterized by screening for multiple features or parameters, such as by screening the same library on multiple cell lines or under different treatment conditions, and/or using assay readouts in which the phenotype monitored is comprised of multiple parameters. High-content imaging, such as standard or confocal fluorescence imaging of multiple cellular features, provides an opportunity to include hundreds of parameters in defining the phenotypes of interest, allowing the detection and quantification of cellular and subcellular

changes, as well as the classification of subphenotypes that might correspond to specific biological functions. Recent multi-parametric image-based screens have contributed to our understanding of several cell functions, including homologue pairing and cell morphology in *D. melanogaster* cells^{54,55}, endocytosis in human cells⁵⁶, epigenetic regulators of human colon cancer cells⁵⁷ and the responses of human macrophage primary cells to the pathogen *Mycobacterium tuberculosis*⁵⁸.

Single-cell analysis approaches are at the cutting edge of high-content, image-based analysis. Individual cells within a cell population might behave differently both as a consequence of intrinsic differences (for example, in cell cycle stage) and as a consequence of their unique microenvironment (for example, differences in local cell densities within a well of a micro-well plate) at the onset of or during a screen. Thus, the phenotypic responses of individual cells might differ. Moreover, in some cases, only a subset of cells might take up the RNAi reagent, such that cells with efficient knockdown will be interspersed with wild-type cells. The use of single-cell analyses to identify phenotypic differences among cells, as well as for filtering out wild-type-appearing cells within a population, can help to address these problems. A recent analysis of individual cell image data from several related cell-based RNAi screens provided direct evidence that the cell microenvironment affects RNAi reagent uptake and response⁵⁹. This approach suggests that it is feasible to differentiate phenotypes that are directly attributable to gene silencing from phenotypes that are attributable to indirect effects originating from changes in the microenvironment, such as increased cell growth that leads to a higher cell density⁵⁹. We anticipate that image-based screens will become less expensive and easier to develop and apply now that genome-engineering approaches can be used to create custom cell lines with fluorescent reporters or in-frame tags at endogenous loci (FIG. 3), circumventing the need for immunostaining, which can be more costly and result in higher screen result variability.

Even when effective and on-target knockdowns are achieved and the assay is robust, knock down of a single gene might not result in a discernable phenotype, for example, due to gene or pathway redundancy. Combinatorial RNAi screens, in which two genes are silenced simultaneously (double knockdowns), can be used to identify these phenotypes and uncover functional relationships between genes. The concept has been exemplified by the results of large-scale, combinatorial genetic studies in *Saccharomyces cerevisiae*^{60,61}. Combinatorial RNAi screens can also facilitate the identification of suppressive effects, in which the knock down of a gene eliminates or reduces a phenotype that is associated with the knock down of another gene, or the identification of synthetic effects, in which the knock down of two genes has a synergistic effect. These include screens querying all possible double knockdown combinations, for example, a recent screen of cell numbers and nuclear features in *D. melanogaster* that involved the pairwise knock down of 70,000 combinations of 93 genes involved in signal transduction, resulting in the identification of more than 600 potential interactions⁶².

Box 3 | RNAi screens in 3D cell cultures

Recent technological advances have enabled gene silencing in three-dimensional (3D) cell culture systems. Thus far, 3D RNAi screening has only been implemented on a small scale¹⁰³, but its adaptation for use in large-scale screening is plausible. The appeal of screening in three dimensions is the ability to produce phenotypes that are more physiologically relevant than those obtained in two dimensional (2D) cell cultures, as some aspects of tissue and tumour growth are not reproduced in two dimensions. To ensure the access of RNAi reagents to all cells in a 3D culture, they are typically introduced by viral infection or transfection of homogeneous 2D cell cultures before inducing 3D structure formation. As with all RNAi screens, the most appropriate reagent for the assay depends on the question being asked. If the length of the experiment is short (for example, up to 5 days), siRNAs can be used, as was recently demonstrated in a study to identify genes influencing the ability of breast cancer cells to grow in an anchorage-independent manner¹⁰⁴. The authors determined that the oestrogen receptor 1-positive MCF7 cells were inhibited by oestrogen receptor 1 knockdown, whereas the growth of HER2 (also known as ERBB2)-positive SK-BR-3 cells was suppressed by the knockdown of HER2. In addition, both cell lines exhibited reduced colony growth in soft agar in the presence of siRNAs targeting β -actin, a result not observed in standard 2D cultures. If the experiment requires a longer time course (greater than 5 days), stable expression of short hairpin RNA (shRNA) reagents is more appropriate in order to ensure target gene silencing throughout the course of the experiment. As with RNAi screens carried out in 2D cultures, various assay readouts are possible. High content image-based readouts are likely to preserve the most information because the different locations within a 3D structure can be analysed independently to determine how physical location and the 3D structure of the microenvironment (for example, hypoxic versus normoxic microenvironments) affect the observed phenotype.

Another approach to combinatorial screening involves using RNAi, small molecules or genetic alterations to generate a sensitized cell background, which is then used in a large-scale RNAi screen. Differences in the genes conferring lethality in various isogenic cell lines — for example, with or without the expression of a specific oncogene — were reported in two studies related to oncogenic RAS signalling^{63,64}. Similarly, some very large-scale RNAi screens have been carried out with the aim of uncovering cancer vulnerabilities through the identification of genes that are essential in various cancer cell lines. The results of these studies show promise for the identification of new drug targets for cancer therapy^{65–67}. In the future, genome-engineering approaches could be used to generate sets of related cell lines, differing only in a specific compromised cellular pathway or process (FIG. 3). This will allow specifically controlled parallel RNAi screens that might uncover synergistic or new effects caused by the perturbation of more than one gene.

RNAi screening in vivo

Various approaches have been developed to harness the power of large-scale RNAi screening in mammalian cells in contexts that simulate *in vivo* environments. The development of one such approach — 3D culture systems for screening — is discussed in BOX 3. Even more physiologically relevant are screens carried out in living animals, where the effects of gene silencing can be assessed in defined populations of cells in their proper physiological context.

RNAi screening in vivo in mice. Systematic loss-of-function screening is becoming increasingly feasible in mammalian systems. For example, *in vivo* shRNA screens in mice have identified bromodomain-containing protein 4 (BRD4) as a therapeutic target in acute myeloid leukaemia⁶⁸ and have identified novel regulators of oncogenic growth in a Hras^{G12V} mouse model of skin tumorigenesis⁶⁹. The approaches for carrying out *in vivo*

screens vary, including *ex vivo* transduction of shRNA pools into mouse cells, which are then transplanted into specific tissues and organs^{70,71}; direct viral infection into target cell populations in adult mice⁷²; or infection of cells during embryogenesis for tissue-specific silencing during animal development⁶⁹. The expression of shRNAs *in vivo* can be constitutive⁷² or inducible⁷³.

Achieving robust results from *in vivo* screening is dependent on multiple factors⁶⁹, including accurate shRNA quantification within each pool, a low per-cell transduction level of shRNAs (accomplished using low multiplicity of infection (MOI)), and testing of multiple shRNA reagents per gene. *In vivo* RNAi screening of multiple phenotypes requires less work than creating individual gene knockout lines. The results of *in vivo* RNAi experiments can then be translated to producing low-throughput knockout models for validation and follow-up studies. We note that the use of RNAi to make genetically engineered mouse models was recently reviewed elsewhere⁷⁴.

RNAi screening in vivo in other organisms. A number of genome-wide RNAi libraries and related resources are available for investigating a variety of topics by *in vivo* screening in other model organisms, in particular in *C. elegans* and in *D. melanogaster* (reviewed in REFS 12,14,18,19). RNAi screens have also been carried out in some non-model organisms, such as in trypanosomes (for example, a study on quorum sensing signalling²⁴) and Planaria (for example, studies on regeneration and stem cells^{21–23}, as well as on the identification of a conserved factor required for Wnt secretion⁷⁵), and have been proposed for others, such as parasitic nematodes⁴. Results from *in vivo* screens in flies and worms have frequently been translated to mammalian systems, and these data sets continue to be important for hypothesis generation and single gene mechanistic studies. For example, *straightjacket*, a *D. melanogaster* orthologue of the mammalian gene *Cacna2d3*, was identified in a genome-wide *in vivo* RNAi screen for mediators of heat

Multiplicity of infection (MOI). During a viral infection, the ratio of the number of infectious virions to the number of targeted cells.

nociception, and it was later shown that disruption of *Cacna2d3* in mice is associated with heat pain sensitivity⁷⁶. Other examples come from *C. elegans*, in which a number of RNAi screens have addressed cell biology processes related to neurodegenerative diseases; some genes identified in these screens have been studied in mammalian cells or in knockout mice (for a review, see REF. 14).

As is the case in cell-based screens, OTEs are relevant to interpreting genome-wide *in vivo* RNAi screens. The development of new resources, such as fly stock collections that harbour parts of the genomes of other species⁴³, can facilitate validation by RNAi resistance. In addition, in some cases, RNAi reagents that are known to have a high degree of on-target specificity to 'gene traps' might be used to bypass unwanted OTEs in other genes. In *D. melanogaster*, for example, GFP-trap fly stocks are available in which a GFP-tagged gene replaces the endogenous gene, and so validated RNAi reagents that target GFP can be used to knock down the GFP-fused mRNAs: this was shown to elicit highly reproducible phenotypes^{77,78}. Broad use of this method is currently limited to *D. melanogaster*, the only organism so far in which a number of homozygous viable GFP-trap stocks have been produced. Even in *D. melanogaster*, the number of GFP-trap stocks is small; however, a resource consisting of fly stocks designed for systematic generation of GFP (or other) tagged genes was recently established⁷⁹, suggesting that the approach might prove useful for larger gene sets in the future. In principle, fusion targeting might be used in any system in which endogenous genes can be tagged with GFP or any other sequence that can be effectively targeted by RNAi.

One main advantage of using RNAi in a model organism such as *D. melanogaster* is that gene silencing can be restricted to specific tissues and developmental stages; for example, the use of the Gal4–UAS (Gal4–upstream activating sequence) system, in which the transcription factor Gal4 specifically binds the UAS enhancer and thus drives the expression of the cloned RNAi construct in a tissue-specific and spatiotemporal-specific manner¹², which circumvents the problems associated with studying genes and pathways that function in multiple tissues and developmental stages. This is in contrast to *C. elegans*, in which RNAi is usually systemic and tissue-specific gene silencing can be accomplished only through complex genetic manipulation^{80,81}. Recently, the expression of CRISPR–Cas9 in a tissue-restricted manner in *D. melanogaster* was shown to efficiently disrupt both alleles of the targeted fly gene in somatic tissues⁸². Although this approach provides, in principle, an alternative to analysing by RNAi phenotypes in somatic tissues in a targeted manner, it has limitations. For example, the approach is not 100% efficient and some sgRNA targeting will destroy the target site but will not cause a frameshift or other disruptive mutations (which will result in the production of a protein with wild-type function). As a consequence, gene disruptions will be present in only a subset of the cells in which the sgRNA is expressed (that is, present in only a subset of Gal4-expressing cells). Because gene

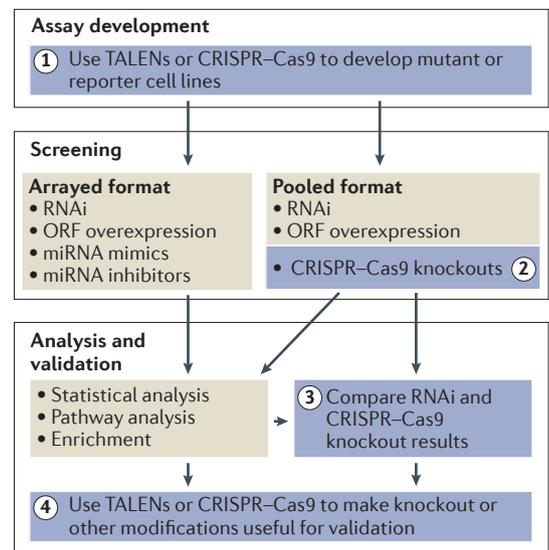


Figure 3 | Genome-engineering approaches offer new opportunities for assay development, screening and validation. A number of points of intersection exist between RNAi screening (or other types of large-scale screening, such as overexpression of open reading frame (ORF) clones, or microRNA (miRNA) mimics or inhibitors (BOX 2)) and genome-engineering technologies (step 1) such as TAL effector nucleases (TALENs) and the CRISPR (clustered regularly interspaced short palindromic repeats)–Cas9 systems (highlighted in blue). Genome engineering can be used to create robust, well-controlled assays (step 2) in cell lines and model organisms by introducing various mutations such as gene knockouts, disease-associated mutations and knock-in of selectable markers or in-frame fusions or reporter genes. CRISPR–Cas9-mediated knockouts (step 3) in mouse or human cells have been reported as an effective method for pooled-format screens. These can be performed in parallel to RNAi screens, followed by comparison of results from the two types of screens. Independently of the screening approach, genome engineering can be used to modify cells or organisms for follow-up studies of specific gene candidates (step 4). In this case, the CRISPR–Cas9 system or TALENs can be used to knock out genes identified in RNAi screens, with a concordance of the knockdown and knockout phenotypes providing a high degree of confidence in the results. They can also be used to create other types of modifications useful for follow-up studies, for example, transcriptional upregulation or downregulation, using modified forms of Cas9, or introducing fluorescence tags or reporters using a knock-in approach.

disruption is not labelled, it will be difficult to identify the subset of cells in which gene activity has been disrupted unless an antibody against the gene product exists. This is in contrast to tissue-specific Gal4–UAS-mediated RNAi, in which the expression of the RNAi reagent is induced in all cells that express Gal4 and thus knock down is uniform throughout the Gal4-expressing tissue. Regardless, tissue-specific CRISPR–Cas9 activity can be used for large-scale screens and may complement RNAi approaches in *D. melanogaster*. Using CRISPR–Cas9 to enable mosaic analyses may be more beneficial in organisms such as *C. elegans*, where RNAi is systemic.

Data analysis and integration

In addition to carrying out statistical analyses and experimental follow-up studies of RNAi screen data, the application of various bioinformatics approaches can greatly aid in distinguishing between high-confidence and low-confidence screen hits. One common approach is to analyse the data in aggregate using a gene-set enrichment or related algorithms, such as using the DAVID database (the database for annotation, visualization and integrated discovery) from the US National Institutes of Health⁸³, COMPLEAT⁸⁴ and other software to detect gene ontology terms, protein complexes or signalling pathways that are enriched in the screen hits compared to controls. These results can be used to confirm low-confidence hits, such as hits with borderline statistical scores or hits for which not all RNAi reagents targeting the gene were positive and, conversely, to rule out hits that are the sole representatives of a category, which might suggest that they are false positives. Pathway enrichment can also help to address false-negative hits, as genes that were not represented among the screen hits but that were members of the selected gene ontology category, protein complex or pathway could be added to the list of genes to be included in follow-up studies.

Another powerful bioinformatics approach is to integrate results from RNAi screens with the results of 'omics' studies based on other methods, such as proteome or transcriptome analyses, which have different strengths and caveats and thus can complement RNAi. Genes or proteins identified on the basis of multiple lines of evidence can be assigned to higher confidence categories. For example, a combined proteomic and RNAi approach was used to functionally annotate putative protein complexes related to Hippo signalling⁸⁵. The study identified at least one new component of the Hippo pathway, an α -arrestin protein family member, Leash, which is involved in the degradation of Yorkie, the fly orthologue of YAP1 (REF. 85). Importantly, whereas approaches such as gene ontology or pathway analysis are likely to bias the results towards what is already known from the literature, the integration of results from additional omics data sets or other functional screening approaches might help to uncover truly novel findings. It is therefore critically important to make complete and annotated screen data available to others in order to facilitate improved reagent and assay design, re-analysis of results, and integration of screen results with other studies⁸⁶. When presenting RNAi screen hit lists, it is the responsibility of each investigator to make their level of confidence in their screen hits explicit by referencing the specific experiments and the statistical approaches that led to their conclusions.

New methods of mammalian cell screening

An alternative strategy to RNAi screening in mammalian cultured cells has been to carry out transposon-based genetic screens in haploid mammalian cell lines. For example, a derivative of the KBM7 chronic myeloid leukaemia (CML) near-haploid mammalian cell line, together with gene-trap retroviruses that contain a strong splice acceptor site and a marker gene, could

be used to identify genes that are involved in specific biological processes, such as survival in response to TRAIL (tumour necrosis factor-related apoptosis-inducing ligand; also known as TNFSF10) and exposure to pathogens^{87,88}. These reports showed that systematic loss-of-function screens in cultured cells, which until recently were thought to be feasible only in yeast⁶¹, can be applied to mammalian cells. Similarly, a gene-trap retrovirus approach is being used to generate large-scale knockout collections of human cells, with more than 3,396 genes tagged to date⁸⁹.

With the demonstration that CRISPR-Cas9-based methods allow the efficient recovery of biallelic mutants in diploid cells⁹⁰, large-scale knockout screens are no longer limited to haploid cell lines. Indeed, genome-engineering approaches⁴⁵ not only offer new routes to assay development and validation of RNAi results (FIG. 3), but can also be used for high-throughput screening⁴⁷. Recently, two publications reported using genome-wide CRISPR-Cas9-based knockout libraries to carry out pooled-format screens in human cells. In one study, a screen of more than 70,000 unique sgRNAs (targeting ~7,000 genes, with ten sgRNAs per gene) was performed in the presence of the nucleotide analogue 6-thioguanine, and the four mismatch repair pathway components expected to score as positive in the assay were indeed identified, with four or more sgRNAs per gene scoring as positive hits⁹¹. In another study, researchers identified genes essential for the survival of cancer cells and pluripotent stem cells, as well as for resistance to the BRAF inhibitor vemurafenib⁹². A mouse large-scale pooled CRISPR-Cas9 knockout library was used in screens to determine the sensitivity of mouse cells to *Clostridium septicum* α -toxin or 6-thioguanine, resulting in the identification of four previously unknown gene candidates for sensitivity to these treatments⁹³.

In each of the three CRISPR-Cas9 screen studies discussed here, several unique sgRNAs targeting the same gene produced comparable phenotypes, suggesting that the CRISPR-Cas9 system has an efficient recovery of on-target hits⁹¹⁻⁹³. It is therefore plausible that CRISPR-Cas9-based screens will become an important complement to RNAi screens in the future, perhaps replacing a subset of pooled shRNA screens. As mentioned above, however, we do not yet fully understand how to design effective and on-target CRISPR-Cas9 reagents, and there can be biologically meaningful differences between gene knockdown and gene knockout. Presumably, some gene functions revealed by incomplete, RNAi-based gene disruption phenotypes would be missed in gene knockout screens⁴⁷. As is the case for RNAi, there is a need to carefully follow-up on results from CRISPR-Cas9-based screens, including carrying out rescue assays to confirm that the phenotypes observed result only from on-target genomic knockouts.

Concluding remarks

The availability of genome-wide RNAi screening platforms in several model organisms allows for systematic interrogation of gene function. Although many caveats apply to the design, analysis and interpretation

Gene-trap retrovirus approach

Screening using mutagenic retroviral integration into genes. The integrated retrovirus can be used as a trap for identifying the genes disrupted in each resulting phenotype.

of high-throughput RNAi studies, RNAi screens are clearly having an impact, perhaps most notably in the fields of cancer research, host–pathogen interactions and signal transduction. Recent developments in experimental controls and data analysis strategies to detect OTEs and to confirm on-target effects have provided increased confidence in the results obtained from large-scale RNAi screens. Multi-pronged approaches, such as performing related omics experiments in parallel with RNAi screening, or performing complementary screens in other systems, can also be used to generate high-confidence results and to promote network-level analysis.

Genome-engineering technologies intersect with RNAi in a number of ways, including in assay development, screening procedures and hit validation. However, these approaches also come with their own set of caveats, including the potential to introduce off-target DNA breaks or chromosomal rearrangements that might be difficult to detect. The ability to use various

TAL effector nucleases (TALENs) or Cas9 modifications as transcriptional repressors rather than as inducers of DNA breaks^{45,94}, should help to reduce such unwanted effects. Importantly, there are no reports to date of using CRISPR–Cas9 knockouts in arrayed screening formats, so at least in the near future RNAi is likely to remain the method of choice for studies that require high-content image-based (and other) arrayed-format screening assays.

It is generally accepted that high-throughput RNAi screen data sets are insufficient for high-confidence annotation of gene function (for example, see REF. 30). However, with careful attention to reagent and assay design, data analysis, data integration and follow-up experimental validation, large-scale RNAi screens can be successful at uncovering new genes, signalling pathways and gene networks involved in various biological processes, and will continue to be a valuable experimental tool in many research areas for years to come.

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Competing interests statement

The authors declare no competing interests.

FURTHER INFORMATION

C911 calculator: <http://rna.nih.gov/haystack/C911Calc.html>

Haystack: <http://rna.nih.gov/haystack/>

Online GESS: <http://flyrna.org/gess>

ALL LINKS ARE ACTIVE IN THE ONLINE PDF