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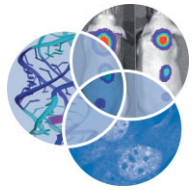

RNAi screening: new approaches, understandings, and organisms

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RNA interference (RNAi) leads to sequence-specific knockdown of gene function. The approach can be used in large-scale screens to interrogate function in various model organisms and an increasing number of other species. Genome-scale RNAi screens are routinely performed in cultured or primary cells or *in vivo* in organisms such as *C. elegans*. High-throughput RNAi screening is benefitting from the development of sophisticated new instrumentation and software tools for collecting and analyzing data, including high-content image data. The results of large-scale RNAi screens have already proved useful, leading to new understandings of gene function relevant to topics such as infection, cancer, obesity, and aging. Nevertheless, important caveats apply and should be taken into consideration when developing or interpreting RNAi screens. Some level of false discovery is inherent to high-throughput approaches and specific to RNAi screens, false discovery due to off-target effects (OTEs) of RNAi reagents remains a problem. The need to improve our ability to use RNAi to elucidate gene function at large scale and in additional systems continues to be addressed through improved RNAi library design, development of innovative computational and analysis tools and other approaches.

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INTRODUCTION

RNA interference (RNAi) is a conserved endogenous activity[1] that can be harnessed as a tool for functional genomics studies.[2–8] With RNAi, gene-specific reagents are introduced into cells, triggering 'knockdown' or reduction of gene function via sequence-specific degradation and translational interference of mRNA transcripts. RNAi screening provides a powerful reverse-genetic approach to large-scale functional analysis in cultured cells and in an increasing number of *in vivo* systems. Like genetic screening, RNAi screening allows for identification of genes relevant to a given pathway, structure or function via association of a mutant phenotype with gene knockdown. Like chemical screening, RNAi screening is amenable to miniaturization and automation, facilitating high-throughput studies. Because of, at least in part, the ease of delivery of RNAi reagents and resources available, *Caenorhabditis elegans*, *Drosophila* cells and mammalian cells have been the most-used systems for RNAi screening. Indeed, screens in these systems have already led to important new insights into a wide variety topics, including infectious disease, cancer, signaling, and aging.[2,3,6,8–16] Moreover, RNAi screening has benefitted from input from a variety of other fields, in particular engineering and computer science, for example, to improve methods for automated high-content image acquisition and analysis.[17]

Over the years, researchers have gained a better understanding of best practices for RNAi screening, both through performing screens and through study of endogenous RNAi pathways. In particular, recent improvements and refinements in methods for *in vivo* RNAi screening in *Drosophila* and mice have opened the doors to an increasing number of large-scale *in vivo* studies in those systems.[3] RNAi has been evolutionarily conserved and thus, it is being used to study an increasing number species for which functional genomics would otherwise not be feasible.[3,18–22] Despite all this progress, however, the problem of off-target effects and other sources of false discovery remain ongoing challenges. Improvements in reagent

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design, reagent delivery, assay design and data analysis have increased the quality of RNAi screen results in recent years. However, the picture remains complex in terms of understanding and addressing all possible sources of false positive and false negative results.[23,24] Despite these caveats, RNAi screening remains a powerful method-of-choice for genome-scale interrogation of gene function in an increasing number of systems, and the results of RNAi screens continue to provide new insights into diverse topics in biology and biomedicine. Below, we provide an overview of RNAi screening in cells and *in vivo*, focusing on new developments and results, as well as innovations stemming from interaction with other fields of study.

RNAi SCREENING IN CELLS

Why screen in cultured cells?

RNAi technology opened the doors to performing functional genomics in human cells and other types of cultured and primary cells. Cell-based RNAi screening builds upon established instrumentation, assays and other methods previously developed for chemical screening in cells. Overall, cell-based RNAi screening provides a relatively rapid and accessible platform for genome-scale functional studies.[2,4,5,7] A large number of RNAi screens has been performed in *Drosophila* and mammalian cultured cells.[2] More recently, researchers have developed methods for screening neuronal and muscle primary cells derived from dissociated *Drosophila* embryos,[25–27] as well as primary *Drosophila* haemocytes.[28] In addition, an increasing number of studies are being performed using mammalian stem cells (reviewed in Refs 29 and 30). The availability of transcriptome data for tissues, tumors and cell lines, made possible by next-generation sequencing technologies, is likely to shape choices and interpretation of cell-based RNAi screen data.[5,15,23,31] For example, transcriptome data may help us to understand the extent to which networks present in a cell line reflect what is happening *in vivo*, and detection of single-nucleotide polymorphisms (SNPs) can reveal mismatches to reagents that are relevant to the interpretation of results.

Reagent Libraries for Cell-Based Screening in *Drosophila* and Mammalian Cells

RNAi screening relies on the availability of genome-wide or other large-scale RNAi reagent libraries, with one or more unique RNAi reagent directed against each target gene. The appropriate RNAi reagent library for cell-based screening depends upon the cell type, approach and method of reagent delivery.[7] In *Drosophila* cells, the lack of an interferon response and ability of most cell types to take up the reagent in solution makes it possible to use *in vitro* synthesized long double-stranded RNA (dsRNAs) as the RNAi reagents.[2] Reagents in the form of small interfering RNAs (siRNAs), endoribonuclease-prepared siRNAs (esiRNAs) or small hairpin RNAs (shRNAs) are typical for mammalian cell screens,[2,4,7,32–34] as long dsRNAs can evoke nonspecific cellular responses that interfere with cell-based assays.

Design of effective and on-target RNAi reagents remains an ongoing challenge.[23,24] Useful tools for evaluation of RNAi libraries include NEXT-RNAi (http://www.nextrnai.org/).[35] A new approach was used recently to identify RNAi reagents conferring robust knockdown in mammalian cells.[36] The results of their analysis of 20,000 RNAi reporters suggest that shRNA reagents conferring robust knockdown are relatively rare and may help provide new insights into effective reagent design. Improved access to information about reagent designs and results may contribute to the ability to learn new rules for effective design in the future. In addition to sequence-based efforts to improve RNAi reagent design, some researchers are working to achieve robust knockdown by combining RNAi with other approaches, such as U1 interference.[37] Others are exploring the effects of adding various chemical modifications to siRNAs with the goal of developing more effective reagents.[24,38]

Arrayed Screening in Cultured Cells

With an arrayed screen, each RNAi reagent (or mini-pool of reagents, such as a set of independent siRNAs directed against a single gene) is contained in a separate well of a micro-well plate, such as a 96- or 384-well plate. Thus, following the experiment, the identity of the reagent can be determined by checking a database or spreadsheet that tracks which reagents were present in which wells. Researchers are using several different types of assays with arrayed screening approaches, facilitated by a number of different types of assay readout instruments. Further miniaturization of arrayed screens has been achieved through the use of microarray slides on which the siRNAs or other RNAi reagents have been printed, facilitating reverse transfection of reagents into cells.[39] Recent applications of this approach using *Drosophila* or mammalian cells have looked at signal transduction,[40] differentiation[41] and host–pathogen interactions.[42] The next frontier in arrayed screening may be the use of specially designed microfluidics instruments and micro-well platforms to facilitate single cell analyses.[43,44]

Assay Readouts for Arrayed Screening

Several types of cell-based assays are made possible by arrayed-format screening. For example, researchers can measure ATP levels, transcriptional activity and protein stability using 'plate-readers' (i.e., luminometers and fluorimeters) that measure whole-well intensity of luciferase or fluorescence readouts. To date, a large number of plate-reader screens have been performed in mammalian and *Drosophila* cultured cells.[2] As for other screening approaches, arrayed plate-reader screens can be combined with addition of a treatment such as a drug, infectious pathogen, or environmental stress in order to identify modifiers of the phenotype normally induced upon exposure to that treatment (see, e.g., Refs 45–48). Conversely, researchers are also sensitizing cells using RNAi treatment against one gene, followed by chemical screening.[49] A new approach known as real-time cell analysis (RTCA) has facilitated time-lapse screening.[50]

Resolution at the individual cell level is essential for some screen assays. Fluorescence-assisted cell sorting (FACS) has been used in arrayed-based screens to determine DNA content or the relative levels of two markers at the individual cell level (see, e.g., Refs 51–53). However, because of the relatively slow speed of FACS analysis in microwell formats as compared with some imaging approaches, researchers more often turn to imaging for cellular and subcellular resolution of various fluorescent-labeled dyes, probes or antibodies. Screen imaging instruments typically balance acquisition speed against image quality or resolution.[17] Relatively rapid, whole-well imaging can be achieved using instruments such as laser scanning cytometers. Alternatively, subcellular resolution can be achieved using epifluorescence or confocal screening microscopy.

High-Content Image-Based Assays

The availability of high-throughput, high-content imaging instruments has made it possible to obtain simultaneous readouts of various different fluorescent or other visual markers. The sheer number and volume of images obtained in high-content image-base screening require the use of automated solutions to identifying the subset of RNAi reagents that generate the phenotype(s) of interest. Using image analysis software tools such as CellProfiler,[54] hundreds of different features can be extracted from screen image datasets and then used to define or identify phenotypes that are relevant to the topic being addressed (see Box 1). The state-of-the-art in image-based assays involves live cell and time-lapse screening (see, e.g., Refs 40,55–58). Although time-lapse screen imaging with live cells opens the door to new types of studies, these approaches also add significantly to acquisition time, increase the total volume of data that must be managed and stored, and add to the burden of image data processing and analysis. This problem has begun to be addressed with analysis tools such as CellCognition[59] and the development of start-to-finish automated platforms for sample processing, imaging, and analysis.[57]

BOX 1

CHALLENGES AND STRATEGIES FOR HIGH-CONTENT IMAGING IN RNAi SCREENING

Challenges for image-based screening include deciding on an instrument, storing and managing large datasets, and determining which features or parameters are most informative.[17,60–62] Deciding what to image can also affect results; for example, in a host–pathogen screen, the results differed when researchers imaged the virus versus imaging of host cell features indicative of infection.[63] Image analysts and statisticians help researchers make the most of screen image data, including through development of improved and new software tools such as Cell Profiler and CellCognition.[54,59] An approach that is growing in popularity and utility is multiparametric image analysis—that is, measurement of many different features, such as signal intensity, size, shape, and/or texture—which is sometimes done in combination with machine learning. Multiparametric analysis and machine learning are being used not just to identify predefined phenotypes but also to identify new phenotypes, limit false discovery, and place genes in networks based on shared phenotypes.[57,64–72] Determining which of the many available image analysis software tools might be appropriate for analysis of a specific dataset is another challenge. Establishment of benchmarking principles may help researchers not just to identify an appropriate analysis approach but also to tailor image acquisition parameters to best fit their downstream goals.[60] Efforts to standardize how we identify and describe subcellular features[73] may also have an impact on high-content screening, as standardizing terms can help facilitate cross-comparison of results from multiple screens.

Pooled Screening in Cultured Cells

Pooled screening provides a convenient method for screening large RNAi reagent collections, such as genome-wide mammalian shRNA libraries.[2,32] With

a pooled screen, the RNAi library is introduced into cells at random by DNA transfection or perhaps more commonly, by viral transduction, with the goal of introducing one RNAi reagent per cell. Pooled screen readouts depend upon comparison of two or more populations of cells and have contributed to our understanding of a number of topics, including cancer.[2,32] A related approach is to introduce library-transduced pools of cells into mice to perform what are known as *ex vivo* screens (see section on '*in vivo* Screening in Mice').

Deconvolution of the results of a pooled screen—that is, identifying the subset of RNAi reagents that are enriched and/or depleted in the experimental versus starting or control pools—is a key step. Deconvolution of pooled screen results is typically done using microarrays or next-generation sequencing to detect the total population of reagents present in each pool. Because of the technical challenge of deconvolving results obtained from very complex pools, researchers divide genome-scale collections into smaller pools, for example, six pools of ~13,000 unique reagents.[74,75] A newly developed microarray-based resource reportedly allows for deconvolution of pooled shRNA screens with up to ~90,000 unique shRNAs.[76]

Double-Knockdown Screens

Another area of innovation in cell-based screening is large-scale combined targeting of more than one gene, such as for detection of synthetic lethal interactions.[77] As we have learned from studies in yeast, combinatorial approaches can be particularly powerful in addressing issues of redundancy in genetic networks.[78] For *Drosophila* cell-based screens, RNAi reagents directed against two different genes can simply be combined in solution. Large-scale pairwise RNAi screens in *Drosophila* cells have provided insights into redundancy and connectivity of conserved signal transduction pathways.[79,80] One of these studies included rigorous analysis of all pairwise combinations among a large number of genes. Among other findings, the study showed that combinatorial RNAi can reveal results that could not have been predicted based on single gene analyses.[79] An alternative approach to reagent delivery for *Drosophila* cell-based or *in vivo* assays is expression of shRNAs targeting two different genes via a single transcript.[81] Researchers are exploring similar plasmid-based approaches, including expression of multiple hairpins from a single expression cassette, in mammalian cells.[82] Because of the large number of possible gene combinations, performing combinatorial RNAi screens in miniaturized formats such as using microarray slides might facilitate this type of screen.

RNAi SCREENING *IN VIVO*

Why Screen with RNAi *In Vivo*?

Many complex phenotypes cannot be reduced to a cell-based assay, thus requiring gene function to be directly analyzed *in vivo*. RNAi screening *in vivo* provides a relatively fast and straightforward route for screening a phenotype of interest in a tissue and stage-specific manner. Additionally, *in vivo* RNAi makes functional genomics studies possible in organisms for which classical genetic approaches have not been developed but for which genome or transcriptome sequences have become available—as gene annotations are necessary for the design of RNAi reagents.[3] Organisms for which RNAi-based approaches and libraries are now being developed include *Lepidoptera*[18] and other insects[22]; many types of ticks[83]; *Hydra*[84]; planarians[85]; a variety of plants[20,86]; and pathogens such as *Trypanosomes*[87,88] (see Box 2).

Because of species-specific differences in reagent uptake and endogenous RNAi pathways, what specific RNAi reagents and methods of delivery are most appropriate must be worked out for each species, with common methods including dsRNA injection and feeding.[19,83,97] Moreover, RNAi knockdown is systemic in some species—spreading from cell to cell—but not in others.[22] For the crop pest *Bactrocera dorsalis* (oriental fruit fly), one group observed knockdown of target genes upon short exposure to RNAi treatments and up-regulation of the genes following prolonged treatment,[96] emphasizing the need to carefully test the effects of RNAi treatments in each new species under study. The Lepidoptera community provides a nice example of how researchers can collaborate to try to improve RNAi methodologies in a related group of organisms, *i.e.* by sharing information and working to establish appropriate controls[18] (see http://insectacentral.org/RNAi).

In vivo RNAi Screening in *C. elegans*

RNAi was first identified and characterized in *C. elegans*, and many *in vivo* RNAi screens have now been performed, leading to new understandings in diverse biological and biomedical topics.[3,11] In *C. elegans*, RNAi is systemic and heritable. Notable recent examples of genome-wide *C. elegans* RNAi screens include studies of aging and obesity.[13,98–101] Although methods for RNAi screening in *C. elegans* are well established, screening in this system is not

BOX 2

RNAi AS TOOL AND TREATMENT FOR DISEASE VECTORS, PARASITES, AND PESTS

RNAi makes it possible to do functional studies in species for which other genetic tools are not available.[3] Thus, it is now possible to study gene function directly in organisms that impact human health, including disease vectors (e.g., mosquitoes), parasites, pathogens, and crop pests.[19,21,22,83,89,90] Topics of particular interest for RNAi screening in health-relevant species include viability, fertility, innate immunity, and biocide resistance.[87,88,91,92] Improved genome-wide libraries were used recently in RNAi screens of the bloodborne pathogen that causes African trypanosomiasis (or sleeping sickness), *Trypanosoma brucei*.[87,88] A method called reciprocal allele-specific RNAi has been used to study variability in the ability of *Anopheles* mosquitoes to transmit the malaria parasite *Plasmodium*.[92] For species lacking gene annotations, next-generation sequencing is being used to perform large-scale identification of mRNA transcripts, followed by RNAi reagent design and production.[93] In addition to its use as a research tool, RNAi is also being explored as a method for pest population control, and in the case of disease vectors, for controlling infection of the vectors by disease agents.[19,22,90] Researchers are also testing expression of RNAi reagents in crop plants as a defense against infection.[94,95] Issues such as off-target effects, differences in endogenous RNAi pathways and activities, genetic variation within a species, a report of up-regulation of gene activity upon prolonged exposure to RNAi, and the potential of RNAi treatments to affect other species serve as important cautions as research efforts in these areas move forward.[95,96]

without caveats. For example, a recent study of the widely-used Ahringer feeding library suggests that a significant number of gene annotations attached to strains in the collection need to be updated, and that some of the bacterial strains in the collection do not express dsRNAs or express dsRNAs that do not correspond to *C. elegans* genes.[102]

Similar to cell-based screening, RNAi screening in *C. elegans* increasingly relies on the use of image-based and modifier screens. In one recent screen, for example, researchers fed the transparent nematodes vital lipid dyes and used stimulated Raman scattering (SRS) microscopy to visualize stored lipid droplets.[99,100] A sophisticated high-content imaging and analysis approach was recently used to describe a global network of essential genes.[70] A recent study of oxysterol-binding protein-related proteins is notable in that it was conducted as an enhancer screen in a quadruple-mutant background.[103] Another enhancer screen demonstrated that genes with redundant or partially redundant functions can be revealed using RNAi screening in sensitized backgrounds.[104] Moreover, although in *C. elegans* RNAi knockdown is less efficient in the nervous system than in other tissues, the use of sensitized backgrounds can facilitate screening for neuronal phenotypes.[105]

In vivo RNAi Screening in *Drosophila*

With a well-annotated genome and wealth of other molecular genetic tools behind it, *Drosophila melanogaster* is another popular model system for *in vivo* RNAi-based screens. Although there is a report of systemic spread of RNAi *in vivo* in *Drosophila* as a mechanism for antiviral immunity,[106] RNAi knockdown induced via injection or expression of dsRNAs acts cell-autonomously in *Drosophila*, facilitating tissue- and stage-specific studies.[3,107] Expression of long or short dsRNA hairpins via a transgene is a flexible and robust option. Thus, it has become the method-of-choice for RNAi in this species, and three groups have built genome-scale libraries for RNAi screening in *Drosophila* (reviewed in Ref 3).

The ability to induce RNAi in specific tissues and stages opens the door to screening not just in embryonic or larval stage animals but also in adults, even when knockdown in early stages is associated with lethality. Moreover, using shRNAs rather than long dsRNAs have made it possible to achieve robust knockdown not only in somatic cells but also in the germline.[108] Recently reported genome-wide RNAi screens *in vivo* in *Drosophila* are notable in focusing on medically-relevant topics, including pain perception, obesity, heart function, bacterial infections of the gut, neural stem cell self-renewal, and neurological disease.[109–114] *Drosophila* RNAi has also recently been used for relatively rapid *in vivo* follow up on ~500 gene candidates identified using a cell-based screen for regulators of Notch signaling.[115]

In vivo RNAi Screening in Mice

In addition to performing pooled screens by comparing differently treated cell populations grown in culture, researchers have also introduced pools

of shRNA-transfected cells into mice, an approach referred to as *ex vivo* screening. This approach combines the relative ease of introducing large-scale libraries into a pool of cultured cells with the advantages of placing cells in an *in vivo* context. The *ex vivo* screening approach has proved particularly useful for cancer-based studies, in which transduced cells can be assayed for their ability to contribute to tumor formation following introduction into the host animal (see, e.g., Refs 116 and 117).

RNAi screening *in vivo* directly in mice, such as via inducible expression of transgenes, is in early stages as compared with *in vivo* screening with *Drosophila* or *C. elegans*. Nevertheless, recent breakthroughs in reagent design and delivery suggest that high-throughput RNAi screening will soon be feasible in at least some cell types and tissues. Reports from Lowe and colleagues describe inducible constructs that can be introduced into embryonic stem (ES) cells, facilitating RNAi in ES cells or production of transgenic mice for *in vivo* RNAi.[118,119] An alternative approach based on infection with lentivirus facilitates *in vivo* RNAi in accessible tissues such as skin.[120,121] Lentiviral vectors specifically designed with *in vivo* approaches in mind have also been reported and should help facilitate *in vivo* approaches in mice in the future.[122]

OFF-TARGET EFFECTS IN RNAi SCREENS

The problem of false discovery in RNAi screens—that is, false positive and false negative screen results—is made particularly clear by the results of meta-analyses of multiple related screens in *Drosophila* or mammalian cells, which reveal poor reproducibility between or among related screens.[123,124] The lack of overlap appears to be due to both false positive and false negative results. Many sources of false discovery are inherent to high-throughput studies.[4,7,23,125] Problems like instrument errors or flawed assay designs can often be detected and addressed during assay development and optimization. Statistical noise is inherent to any large-scale study but can be kept to a minimum through conducting an appropriate number of replicate tests and applying appropriate statistical analyses (see section on 'Limiting False Discovery'). For image-based screens, image processing such as correcting for uneven illumination can be done prior to image analysis. Even after these sources of false discovery are addressed, however, problems of false discovery due to RNAi reagent design remain.

Contribution of RNAi Reagents to False Discovery

False positive results attributable to RNAi reagents include both sequence-independent and sequence-specific effects. Sequence-independent effects include invoking an interferon response and toxicity of the reagent delivery method,[125,126] as well as general disruption of the endogenous miRNA pathway.[127] Sequence-independent effects can often be addressed by using reagent and delivery methods that are appropriate to the specific cell type or organism being used in the study.[7] A recent study of *Drosophila* cell screens underscores the idea that choosing an appropriate number of reagents and replicates is important to limiting false negative results.[31]

Arguably the more challenging goal in effective reagent design has been to address sequence-specific false positive results, or OTEs. These are due to sequence-specific recognition of transcripts other than the intended target by the RNAi reagent, followed by entry into the RNAi or miRNA pathways. To limit sequence-specific OTEs, most design algorithms avoid regions in the target sequences that have 19 or more base pairs of contiguous nucleotide identity to another mature transcript, as a 19-mer is sufficient to induce RNAi knockdown of a target transcript. Application of the '19 based-pair rule' has resulted in a marked improvement in RNAi reagent libraries for mammalian and *Drosophila* cells as compared with early libraries.[128] Nevertheless, shorter perfect matches and imperfect matches to other mRNA sequences also contribute to OTEs.[23,129] In addition, a recent report suggests that at least in *Drosophila*, matches to intronic sequences might be relevant as well.[130] The same researchers have also shown that unique reagents against a gene can share common OTEs.[131] In the case of imperfect matches, the RNAi reagents appear to shuttle transcripts into the endogenous miRNA pathway. Consistent with this, analysis of the of the hits from one recent screen revealed that a large number of reagents that scored as positive in the primary screen were acting via miRNA-like effects on transcripts that encode TGF-β receptors.[132] This allowed the researchers to identify endogenous miRNAs that might be involved in TGF-β signaling. Nevertheless, the initial goal of uncovering new protein-coding genes involved in TGF-β signaling was not achieved, and this serves as an important caution for analysis and interpretation of similar screen results.

RNAi reagent design also contributes to false negative results, as some reagents do not result in robust knockdown of the target gene. In *Drosophila* one way to improve the efficiency of long dsRNAs is to co-express Dicer2 and the RNAi reagent,[133] as

over-expression of Dicer2 presumably improves the processing of the dsRNAs into siRNAs. Screening in sensitized strains to enhance the effects of RNAi is also done in *C. elegans*, such as for screens of the nervous system, wherein the feeding approach is less effective.[4] In the case of transgenic or viral delivery methods, robust expression of the active RNAi reagent can be critical to achieving robust knockdown, such that optimization of vector designs, delivery, chromosomal insertion sites, etc. can help reduce false negative discovery. The results of a recent study using a fluorescent protein sensor to detect effective shRNA sequences suggest that more can be learned about rules for effective RNAi reagent design, such that development of new and improved RNAi reagent libraries can be expected to continue.[36]

Limiting False Discovery

Prior to conducting a screen, it is important to establish what number of unique RNAi reagents per gene and what number of replicate tests will yield meaningful data, as well as carefully assessing pilot screen data to check for instrument error or other sources contributing to false discovery.[4,7,31,134,135] Subsequent to conducting a screen, a number of computational and experimental approaches can be used to limit false discovery. A set of best practice guidelines for statistical analysis of mammalian siRNA arrayed screen data has been put forward.[135] Additional statistical approaches specifically designed for RNAi reagents such as siRNAs have also been proposed.[136,137] Cut-off values can be chosen strictly based on analysis of the screen data or can follow an informed approach, such as using information about protein-protein interactions or functional networks to establish an appropriate statistical cut-off value for a particular screen.[138] After screen data are published, other groups should be able to reanalyze datasets to test the utility of new statistical approaches. This would be facilitated by general adoption of recently proposed minimal information about RNAi experiments (MIARE) standards (see http://miare.sourceforge.net/HomePage) and deposition of data into a centralized database such as NCBI PubChem (http://pubchem.ncbi.nlm.nih.gov/).

Other methods for limiting false discovery are appropriate for specific screen assays, cell types, topics, or approaches. For example, recent reports suggest that careful analysis of multiple parameters extracted from high-content image data can point to the subset of parameters that are most informative, limiting false positive results.[64,65] For some cells or systems, it is also possible to remove false positive results by comparing screen hits with transcriptome data. In this case, researchers make the underlying assumption that screen hits corresponding to genes known to be expressed in a given cell type or tissue are more likely to represent on-target true positive results than hits corresponding to genes for which there is no evidence that the gene is expressed.[31] Comparison with pathways or networks culled from the published literature or large-scale proteomics datasets is also proving to be an appropriate method for limiting false discovery.[139] In these cases, false positive results can be limited by excluding screen hits that are not supported in the orthogonal dataset, and potential false negative results can be addressed by adding genes to the list for secondary analyses, such as genes that did not show up as strong positives but are components of a given pathway or complex.[140]

Testing of two or more nonoverlapping RNAi reagents per gene is a general standard for initial verification of primary screen results. Increased confidence in cell-based RNAi results can also be achieved by testing for comparable effects *in vivo* in the same species or testing for comparable effects in cells or *in vivo* in another species (see for example Refs 115,119,141). Ultimately, the 'gold standard' test for an on-target effect is rescue, such as with a genomic fragment, cDNA or open reading frame construct designed to evade RNAi,[142–150] and confidence in screen data is further increased when results are confirmed using other molecular genetic methods.

CONCLUSIONS

RNAi has proven to be a powerful tool for systematic testing of gene function, including at genome-wide scale.[2–8] Indeed, the results of RNAi screens have already led to new understandings of gene functions and networks in the context of basic cell biology and biomedicine.[2,5,6] Topics under particularly intense study using RNAi screening include cancer biology and resistance to anticancer treatments, interactions between host cells and viral or bacterial pathogens, and basic cellular functions such as growth, division and metabolism.[2,6,8–11,14,16] Pooled screens, including *ex vivo* screens in mice, are having particular impact in understanding cancer.[32,116,117] Arrayed cell-based screens have also proved informative and recently, they have gained from the availability of increasingly sophisticated assay read-out instruments, such as for high-content imaging, as well as increasingly sophisticated analysis tools.[4,17,62]

Large-scale *in vivo* RNAi in model systems has led to new insights into topics like obesity and

aging, and, making the most of what can be done *in vivo* but not in cells, studies of complex behaviors such as nociception.[3,98,99,101,112] RNAi requires little more than transcript annotations, a reagent library, and a method of delivery of reagents and consequently, genome-scale *in vivo* RNAi screens are becoming possible in an increasing number of health-relevant species.[3] Improvements in the speed at which we can annotate transcripts—*i.e.* using next generation sequencing technologies—and the ease of RNAi reagent delivery in some systems suggest that we can expect rapid development of RNAi tools for an increasingly broad spectrum of organisms.[93] The many known and emerging caveats to interpretation of RNAi screen results, including but not limited to the enduring problem of OTEs,[23] should be better addressed as new reagent libraries, approaches and instrumentation are developed for established models and emerging organisms. Moreover, as our understanding of the biology behind RNAi effects continues to grow, we can continue to exploit that knowledge in the design of more robust and specific reagents, approaches and analysis tools. Thus, both the scope and the quality of results from RNAi screens can be expected to improve in the future.

REFERENCES

1. Ketting RF. The many faces of RNAi. *Dev cell* 2011, 20:148–161.
2. Mohr S, Bakal C, Perrimon N. Genomic screening with RNAi: results and challenges. *Ann Rev Biochem* 2010, 79:37–64.
3. Perrimon N, Ni JQ, Perkins L. In vivo RNAi: today and tomorrow. *Cold Spring Harbor Persp Biol* 2010, 2:a003640.
4. Boutros M, Ahringer J. The art and design of genetic screens: RNA interference. *Nature Rev Genet* 2008, 9:554–566.
5. Neumuller RA, Perrimon N. Where gene discovery turns into systems biology: genome-scale RNAi screens in Drosophila. *Wiley Interdiscip Rev. Syst Biol Med* 2010, 3:471–478.
6. Seyhan AA, Rya TE. RNAi screening for the discovery of novel modulators of human disease. *Curr Pharma Biotechnol* 2010, 11:735–756.
7. Falschlehner C, Steinbrink S, Erdmann G, Boutros M. High-throughput RNAi screening to dissect cellular pathways: a how-to guide. *Biotechnol J* 2010, 5:368–376.
8. Kassner PD. Discovery of novel targets with high throughput RNA interference screening. *Combin Chem High Throughput Screen* 2008, 11:175–184.
9. Cherry S. What have RNAi screens taught us about viral-host interactions? *Curr Opin Microbiol* 2009, 12:446–452.
10. Cherry S. Genomic RNAi screening in Drosophila S2 cells: what have we learned about host-pathogen interactions? *Curr Opin Microbiol* 2008, 11:262–270.
11. Wolters NM, MacKeigan JP. From sequence to function: using RNAi to elucidate mechanisms of human disease. *Cell Death Different* 2008, 15:809–819.
12. Prudencio M, Lehmann MJ. Illuminating the host - how RNAi screens shed light on host-pathogen interactions. *Biotechnol J* 2009, 4:826–837.
13. Ni Z, Lee SS. RNAi screens to identify components of gene networks that modulate aging in Caenorhabditis elegans. *Brief Funct Genom* 2010, 9:53–64.
14. Brognard J, Hunter T. Protein kinase signaling networks in cancer. *Curr Opin Genet Dev* 2011, 21:4–11.
15. Nijwening JH, Beijersbergen RL. Using large-scale RNAi screens to identify novel drug targets for cancer. *Drugs: Investig Drugs J* 2010, 13:772–777.
16. Westbrook TF, Stegmeier F, Elledge SJ. Dissecting cancer pathways and vulnerabilities with RNAi. *Cold Spring Harbor Symp Quant Biol* 2005, 70:435–444.
17. Conrad C, Gerlich DW. Automated microscopy for high-content RNAi screening. *J Cell Biol* 2010, 188:453–461.
18. Terenius O, Papanicolaou A, Garbutt JS, Eleftherianos I, Huvenne H, Kanginakudru S, Albrechtsen M, An C, Aymeric JL, Barthel A, et al. RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. *J Insect Physiol* 2011, 57:231–245.
19. Batista TM, Marques JT. RNAi pathways in parasitic protists and worms. *J Proteom* 2011, 74:1504–1514.
20. McGinnis KM. RNAi for functional genomics in plants. *Brief Funct Genom* 2010, 9:111–117.
21. Krautz-Peterson G, Bhardwaj R, Faghiri Z, Tararam CA, Skelly PJ. RNA interference in schistosomes: machinery and methodology. *Parasitology* 2010, 137:485–495.
22. Belles X. Beyond Drosophila: RNAi in vivo and functional genomics in insects. *Ann Rev of Entomol* 2010, 55:111–128.
23. Sigoillot FD, King RW. Vigilance and validation: keys to success in RNAi screening. *ACS Chem Biol* 2011, 6:47–60.

24. Sioud M. Promises and challenges in developing RNAi as a research tool and therapy. *Methods Mol Biol* 2011, 703:173–187.
25. Sepp KJ, Hong P, Lizarraga SB, Liu JS, Mejia LA, Walsh CA, Perrimon N. Identification of neural outgrowth genes using genome-wide RNAi. *PLoS Genet* 2008, 4:e1000111.
26. Bai J, Sepp KJ, Perrimon N. Culture of Drosophila primary cells dissociated from gastrula embryos and their use in RNAi screening. *Nat Protoc* 2009, 4:1502–1512.
27. Bai J, Binari R, Ni JQ, Vijayakanthan M, Li HS, Perrimon N. RNA interference screening in Drosophila primary cells for genes involved in muscle assembly and maintenance. *Development* 2008, 135:1439–1449.
28. Ulvila J, Vanha-Aho LM, Kleino A, Vaha-Makila M, Vuoksio M, Eskelinen S, Hultmark D, Kocks C, Hallman M, Parikka M, et al. Cofilin regulator 14-3-3{ζ} is an evolutionarily conserved protein required for phagocytosis and microbial resistance. *J Leukocyte Biol* 2011, 89:649–659.
29. Zou GM. RNAi in stem cells: current status and future perspectives. *Methods Mol Biol* 2010, 650:3–14.
30. Ding L, Poser I, Paszkowski-Rogacz M, Buchholz F. From RNAi screens to molecular function in embryonic stem cells. *Stem Cell Rev* 2011.
31. Booker M, Samsonova AA, Kwon Y, Flockhart I, Mohr SE, Perrimon N. False negative rates in Drosophila cell-based RNAi screens: a case study. *BMC Genom* 2011, 12:50.
32. Boettcher M, Hoheisel JD. Pooled RNAi screens—technical and biological aspects. *Curr Genom* 2010, 11:162–167.
33. Theis M, Buchholz F. MISSION esiRNA for RNAi screening in mammalian cells. *J Visual Exper : JoVE* 2010.
34. Theis M, Buchholz F. High-throughput RNAi screening in mammalian cells with esiRNAs. *Methods* 2011, 53:424–429.
35. Horn T, Sandmann T, Boutros M. Design and evaluation of genome-wide libraries for RNA interference screens. *Genom Biol* 2010, 11:R61.
36. Fellmann C, Zuber J, McJunkin K, Chang K, Malone CD, Dickins RA, Xu Q, Hengartner MO, Elledge SJ, Hannon GJ, et al. Functional Identification of optimized RNAi triggers using a massively parallel sensor assay. *Molecular cell* 2011, 41:733–746.
37. Goraczniak R, Behlke MA, Gunderson SI. Gene silencing by synthetic U1 adaptors. *Nat Biotechnol* 2009, 27:257–263.
38. Gaynor JW, Campbell BJ, Cosstick R. RNA interference: a chemist's perspective. *Chem Soc Rev* 2010, 39:4169–4184.
39. Wheeler DB, Bailey SN, Guertin DA, Carpenter AE, Higgins CO, Sabatini DM. RNAi living-cell microarrays for loss-of-function screens in Drosophila melanogaster cells. *Nat Meth* 2004, 1:127–132.
40. Lindquist RA, Ottina KA, Wheeler DB, Hsu PP, Thoreen CC, Guertin DA, Ali SM, Sengupta S, Shaul YD, Lamprecht MR, et al. Genome-scale RNAi on living-cell microarrays identifies novel regulators of Drosophila melanogaster TORC1-S6K pathway signaling. *Genom Res* 2011, 21:433–446.
41. Wu N, Castel D, Debily MA, Vigano MA, Alibert O, Mantovani R, Iljin K, Romeo PH, Gidrol X. Large scale RNAi screen reveals that the Inhibitor of DNA binding 2 (ID2) protein is repressed by p53 family member p63 and functions in human keratinocyte differentiation. *J Biol Chem* 2011, 286:20870–20879.
42. Genovesio A, Giardini MA, Kwon YJ, de Macedo Dossin F, Choi SY, Kim NY, Kim HC, Jung SY, Schenkman S, Almeida IC, et al. Visual genome-wide RNAi screening to identify human host factors required for trypanosoma cruzi infection. *PloS One* 2011, 6:e19733.
43. Lindstrom S, Andersson-Svahn H. Miniaturization of biological assays—overview on microwell devices for single-cell analyses. *Biochim Biophys Acta* 2011, 1810:308–316.
44. Huang Y, Agrawal B, Sun D, Kuo JS, Williams JC. Microfluidics-based devices: New tools for studying cancer and cancer stem cell migration. *Biomicrofluidics* 2011, 5:13412.
45. Gonsalves FC, Klein K, Carson BB, Katz S, Ekas LA, Evans S, Nagourney R, Cardozo T, Brown AM, Dasgupta R. Feature Article: from the cover: an RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. *Proc Natl Acad Sci U S A* 2011, 108:5954–5963.
46. Seyhan AA, Varadarajan U, Choe S, Liu Y, McGraw J, Woods M, Murray S, Eckert A, Liu W, Ryan TE. A genome-wide RNAi screen identifies novel targets of neratinib sensitivity leading to neratinib and paclitaxel combination drug treatments. *Mol Biosyst* 2011, 7:1974–1989.
47. Bauer JA, Ye F, Marshall CB, Lehmann BD, Pendleton CS, Shyr Y, Arteaga CL, Pietenpol JA. RNA interference (RNAi) screening approach identifies agents that enhance paclitaxel activity in breast cancer cells. *Breast Cancer Res: BCR* 2010, 12:R41.
48. Zhu YX, Tiedemann R, Shi CX, Yin H, Schmidt JE, Bruins LA, Keats JJ, Braggio E, Sereduk C, Mousses S, et al. RNAi screen of the druggable genome identifies modulators of proteasome inhibitor sensitivity in myeloma including CDK5. *Blood* 2011, 117:3847–3857.
49. Castoreno AB, Smurnyy Y, Torres AD, Vokes MS, Jones TR, Carpenter AE, Eggert US. Small molecules discovered in a pathway screen target the Rho pathway in cytokinesis. *Nat Chem Biol* 2010, 6:457–463.

50. Zhang JD, Koerner C, Bechtel S, Bender C, Keklikoglou I, Schmidt C, Irsigler A, Ernst U, Sahin O, Wiemann S, et al. Time-resolved human kinome RNAi screen identifies a network regulating mitotic-events as early regulators of cell proliferation. *PloS One* 2011, 6:e22176.
51. Yoon JC, Ng A, Kim BH, Bianco A, Xavier RJ, Elledge SJ. Wnt signaling regulates mitochondrial physiology and insulin sensitivity. *Genes Dev* 2010, 24:1507–1518.
52. Slabicki M, Theis M, Krastev DB, Samsonov S, Mundwiller E, Junqueira M, Paszkowski-Rogacz M, Teyra J, Heninger AK, Poser I, et al. A genome-scale DNA repair RNAi screen identifies SPG48 as a novel gene associated with hereditary spastic paraplegia. *PLoS Biol* 2010, 8:e1000408.
53. Paul P, van den Hoorn T, Jongsma ML, Bakker MJ, Hengeveld R, Janssen L, Cresswell P, Egan DA, van Ham M, Ten Brinke A, et al. A genome-wide multidimensional RNAi screen reveals pathways controlling MHC class II antigen presentation. *Cell* 2011, 145:268–283.
54. Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL, Ljosa V, Rueden C, Eliceiri KW, Carpenter AE. Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. *Bioinformatics* 2011, 27:1179–1180.
55. Walter T, Held M, Neumann B, Heriche JK, Conrad C, Pepperkok R, Ellenberg J. Automatic identification and clustering of chromosome phenotypes in a genome wide RNAi screen by time-lapse imaging. *J Struct Biol* 2010, 170:1–9.
56. Schmitz MH, Held M, Janssens V, Hutchins JR, Hudecz O, Ivanova E, Goris J, Trinkle-Mulcahy L, Lamond AI, Poser I, et al. Live-cell imaging RNAi screen identifies PP2A-B55α and importin-β1 as key mitotic exit regulators in human cells. *Nat Cell Biol* 2010, 12:886–893.
57. Neumann B, Held M, Liebel U, Erfle H, Rogers P, Pepperkok R, Ellenberg J. High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat Meth* 2006, 3:385–390.
58. McKenna BK, Evans JG, Cheung MC, Ehrlich DJ. A parallel microfluidic flow cytometer for high-content screening. *Nat Meth* 2011, 8:401–403.
59. Held M, Schmitz MH, Fischer B, Walter T, Neumann B, Olma MH, Peter M, Ellenberg J, Gerlich DW. CellCognition: time-resolved phenotype annotation in high-throughput live cell imaging. *Nat Meth* 2010, 7:747–754.
60. Fuller CJ, Straight AF. Image analysis benchmarking methods for high-content screen design. *J Microsc* 2010, 238:145–161.
61. Shumate C, Hoffman AF. Instrumental considerations in high content screening. *Combin Chem High Throughput Screen* 2009, 12:888–898.
62. Niederlein A, Meyenhofer F, White D, Bickle M. Image analysis in high-content screening. *Combin Chem High Throughput Screen* 2009, 12:899–907.
63. Suratanee A, Rebhan I, Matula P, Kumar A, Kaderali L, Rohr K, Bartenschlager R, Eils R, Konig R. Detecting host factors involved in virus infection by observing the clustering of infected cells in siRNA screening images. *Bioinformatics* 2010, 26:i653–i658.
64. Collinet C, Stoter M, Bradshaw CR, Samusik N, Rink JC, Kenski D, Habermann B, Buchholz F, Henschel R, Mueller MS, et al. Systems survey of endocytosis by multiparametric image analysis. *Nature* 2010, 464:243–249.
65. Kummel A, Gubler H, Gehin P, Beibel M, Gabriel D, Parker CN. Integration of multiple readouts into the *z*' factor for assay quality assessment. *J Biomol Screen: Off J Soc Biomol Screen* 2010, 15:95–101.
66. Fuchs F, Pau G, Kranz D, Sklyar O, Budjan C, Steinbrink S, Horn T, Pedal A, Huber W, Boutros M. Clustering phenotype populations by genome-wide RNAi and multiparametric imaging. *Mol Syst Biol* 2010, 6:370.
67. Bakal C, Aach J, Church G, Perrimon N. Quantitative morphological signatures define local signaling networks regulating cell morphology. *Science* 2007, 316:1753–1756.
68. Yin Z, Zhou X, Bakal C, Li F, Sun Y, Perrimon N, Wong ST. Using iterative cluster merging with improved gap statistics to perform online phenotype discovery in the context of high-throughput RNAi screens. *BMC Bioinform* 2008, 9:264.
69. Nir O, Bakal C, Perrimon N, Berger B. Inference of RhoGAP/GTPase regulation using single-cell morphological data from a combinatorial RNAi screen. *Genome Res* 2010, 20:372–380.
70. Green RA, Kao HL, Audhya A, Arur S, Mayers JR, Fridolfsson HN, Schulman M, Schloissnig S, Niessen S, Laband K, et al. A High-Resolution C. elegans Essential Gene Network Based on Phenotypic Profiling of a Complex Tissue. *Cell* 2011, 145:470–482.
71. Lin C, Mak W, Hong P, Sepp K, Perrimon N. Intelligent Interfaces for Mining Large-Scale RNAi-HCS Image Databases. *Proceedings/Annual IEEE International Symposium on Bioinformatics and Bioengineering, 2007* , 2007, 1333–1337.
72. Jones TR, Carpenter AE, Lamprecht MR, Moffat J, Silver SJ, Grenier JK, Castoreno AB, Eggert US, Root DE, Golland P, et al. Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci U S A* 2009, 106:1826–1831.
73. Murphy RF. Communicating subcellular distributions. *Cytometry. Part A: J Int Soc Anal Cytol* 2010, 77:686–692.
74. Hurov KE, Cotta-Ramusino C, Elledge SJ. A genetic screen identifies the Triple T complex required for

DNA damage signaling and ATM and ATR stability. *Genes Dev* 2010, 24:1939–1950.

75. Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF, Wong KK, Elledge SJ. A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell* 2009, 137:835–848.
76. Ketela T, Heisler LE, Brown KR, Ammar R, Kasimer D, Surendra A, Ericson E, Blakely K, Karamboulas D, Smith AM, et al. A comprehensive platform for highly multiplexed mammalian functional genetic screens. *BMC Genom* 2011, 12:213.
77. Grimm D, Kay MA. Combinatorial RNAi: a winning strategy for the race against evolving targets? *Mol Therapy: J Am Soc Gene Therapy* 2007, 15:878–888.
78. Boone C, Bussey H, Andrews BJ. Exploring genetic interactions and networks with yeast. *Nat Rev Genet* 2007, 8:437–449.
79. Horn T, Sandmann T, Fischer B, Axelsson E, Huber W, Boutros M. Mapping of signaling networks through synthetic genetic interaction analysis by RNAi. *Nat Meth* 2011, 8:341–346.
80. Bakal C, Linding R, Llense F, Heffern E, Martin-Blanco E, Pawson T, Perrimon N. Phosphorylation networks regulating JNK activity in diverse genetic backgrounds. *Science* 2008, 322:453–456.
81. Haley B, Foys B, Levine M. Vectors and parameters that enhance the efficacy of RNAi-mediated gene disruption in transgenic Drosophila. *Proc Natl Acad Sci U S A* 2010, 107:11435–11440.
82. Lambeth LS, Van Hateren NJ, Wilson SA, Nair V. A direct comparison of strategies for combinatorial RNA interference. *BMC Mol Biol* 2010, 11:77.
83. de la Fuente J, Kocan KM, Almazan C, Blouin EF. RNA interference for the study and genetic manipulation of ticks. *Trend Parasitol* 2007, 23:427–433.
84. Lohmann JU, Endl I, Bosch TC. Silencing of developmental genes in Hydra. *Dev Biol* 1999, 214:211–214.
85. Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Sanchez Alvarado A. Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev Cell* 2005, 8:635–649.
86. Gilchrist E, Haughn G. Reverse genetics techniques: engineering loss and gain of gene function in plants. *Brief Funct Genom* 2010, 9:103–110.
87. Schumann Burkard G, Jutzi P, Roditi I. Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. *Mol Biochem Parasitol* 2011, 175:91–94.
88. Baker N, Alsford S, Horn D. Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. *Mol Biochem Parasitol* 2011, 176:55–57.
89. Rosso MN, Jones JT, Abad P. RNAi and functional genomics in plant parasitic nematodes. *Ann Rev Phytopathol* 2009, 47:207–232.
90. Li J, Todd TC, Lee J, Trick HN. Biotechnological application of functional genomics towards plant-parasitic nematode control. *Plant Biotechnol J* 2011. doi:10.1111/j 1467-7652.2011.00601.x. [Epub ahead of print].
91. Pinto SB, Lombardo F, Koutsos AC, Waterhouse RM, McKay K, An C, Ramakrishnan C, Kafatos FC, Michel K. Discovery of plasmodium modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae. *Proc Natl Acad Sci U S A* 2009, 106:21270–21275.
92. Blandin SA, Wang-Sattler R, Lamacchia M, Gagneur J, Lycett G, Ning Y, Levashina EA, Steinmetz LM. Dissecting the genetic basis of resistance to malaria parasites in Anopheles gambiae. *Science* 2009, 326:147–150.
93. Wang Y, Zhang H, Li H, Miao X. Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. *PloS One* 2011, 6:e18644.
94. Price DR, Gatehouse JA. RNAi-mediated crop protection against insects. *Trend Biotechnol* 2008, 26:393–400.
95. Auer C, Frederick R. Crop improvement using small RNAs: applications and predictive ecological risk assessments. *Trend Biotechnol* 2009, 27:644–651.
96. Li X, Zhang M, Zhang H. RNA interference of four genes in adult bactrocera dorsalis by feeding their dsRNAs. *PloS One* 2011, 6:e17788.
97. Huvenne H, Smagghe G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J Insect Physiol* 2010, 56:227–235.
98. Minois N, Sykacek P, Godsey B, Kreil DP. RNA interference in ageing research—a mini-review. *Gerontology* 2010, 56:496–506.
99. Wang MC, Min W, Freudiger CW, Ruvkun G, Xie XS. RNAi screening for fat regulatory genes with SRS microscopy. *Nat Meth* 2011, 8:135–138.
100. Bewersdorf J, Farese RV, Jr, Walther TC. A new way to look at fat. *Nat Meth* 2011, 8:132–133.
101. Partridge L. Some highlights of research on aging with invertebrates, 2010. *Aging Cell* 2011, 10:5–9.
102. Qu W, Ren C, Li Y, Shi J, Zhang J, Wang X, Hang X, Lu Y, Zhao D, Zhang C. Reliability analysis of the Ahringer Caenorhabditis elegans RNAi feeding library: a guide for genome-wide screens. *BMC Genom* 2011, 12:170.
103. Kobuna H, Inoue T, Shibata M, Gengyo-Ando K, Yamamoto A, Mitani S, Arai H. Multivesicular body formation requires OSBP-related proteins and cholesterol. *PLoS Genet* 2010, 6.
104. Sawyer JM, Glass S, Li T, Shemer G, White ND, Starostina NG, Kipreos ET, Jones CD, Goldstein B.

Overcoming redundancy: an RNAi enhancer screen for morphogenesis genes in caenorhabditis elegans. *Genetics* 2011, 188:549–564.

105. Poole RJ, Bashllari E, Cochella L, Flowers EB, Hobert O. A genome-wide RNAi screen for factors involved in neuronal specification in Caenorhabditis elegans. *PLoS Genet* 2011, 7:e1002109.
106. Saleh MC, Tassetto M, van Rij RP, Goic B, Gausson V, Berry B, Jacquier C, Antoniewski C, Andino R. Antiviral immunity in Drosophila requires systemic RNA interference spread. *Nature* 2009, 458:346–350.
107. Roignant JY, Carre C, Mugat B, Szymczak D, Lepesant JA, Antoniewski C. Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in Drosophila. *RNA* 2003, 9:299–308.
108. Ni JQ, Zhou R, Czech B, Liu LP, Holderbaum L, Yang-Zhou D, Shim HS, Tao R, Handler D, Karpowicz P, et al. A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nat Meth* 2011.
109. Cronin SJ, Nehme NT, Limmer S, Liegeois S, Pospisilik JA, Schramek D, Leibbrandt A, Simoes Rde M, Gruber S, Puc U, et al. Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. *Science* 2009, 325:340–343.
110. Neely GG, Kuba K, Cammarato A, Isobe K, Amann S, Zhang L, Murata M, Elmen L, Gupta V, Arora S, et al. A global in vivo Drosophila RNAi screen identifies NOT3 as a conserved regulator of heart function. *Cell* 2010, 141:142–153.
111. Pospisilik JA, Schramek D, Schnidar H, Cronin SJ, Nehme NT, Zhang X, Knauf C, Cani PD, Aumayr K, Todoric J, et al. Drosophila genome-wide obesity screen reveals hedgehog as a determinant of brown versus white adipose cell fate. *Cell* 2010, 140:148–160.
112. Neely GG, Hess A, Costigan M, Keene AC, Goulas S, Langeslag M, Griffin RS, Belfer I, Dai F, Smith SB, et al. A genome-wide Drosophila screen for heat nociception identifies $\alpha 2\delta 3$ as an evolutionarily conserved pain gene. *Cell* 2010, 143:628–638.
113. Fernandes C, Rao Y. Genome-wide screen for modifiers of Parkinson's disease genes in Drosophila. *Mol Brain* 2011, 4:17.
114. Neumuller RA, Richter C, Fischer A, Novatchkova M, Neumuller KG, Knoblich JA. Genome-Wide Analysis of Self-Renewal in Drosophila Neural Stem Cells by Transgenic RNAi. *Cell Stem Cell* 2011, 8:580–593.
115. Saj A, Arziman Z, Stempfle D, van Belle W, Sauder U, Horn T, Durrenberger M, Paro R, Boutros M, Merdes G. A combined ex vivo and in vivo RNAi screen for notch regulators in Drosophila reveals an extensive notch interaction network. *Develop Cell* 2010, 18:862–876.
116. Meacham CE, Ho EE, Dubrovsky E, Gertler FB, Hemann MT. In vivo RNAi screening identifies regulators of actin dynamics as key determinants of lymphoma progression. *Nat Genet* 2009, 41:1133–1137.
117. Bric A, Miething C, Bialucha CU, Scuoppo C, Zender L, Krasnitz A, Xuan Z, Zuber J, Wigler M, Hicks J, et al. Functional identification of tumor-suppressor genes through an in vivo RNA interference screen in a mouse lymphoma model. *Cancer Cell* 2009, 16:324–335.
118. Premsrirut PK, Dow LE, Kim SY, Camiolo M, Malone CD, Miething C, Scuoppo C, Zuber J, Dickins RA, Kogan SC, et al. A rapid and scalable system for studying gene function in mice using conditional RNA interference. *Cell* 2011, 145:145–158.
119. McJunkin K, Mazurek A, Premsrirut PK, Zuber J, Dow LE, Simon J, Stillman B, Lowe SW. Reversible suppression of an essential gene in adult mice using transgenic RNA interference. *Proc Natl Acad Sci U S A* 2011, 108:7113–7118.
120. Beronja S, Livshits G, Williams S, Fuchs E. Rapid functional dissection of genetic networks via tissue-specific transduction and RNAi in mouse embryos. *Nat Med* 2010, 16:821–827.
121. Williams SE, Beronja S, Pasolli HA, Fuchs E. Asymmetric cell divisions promote Notch-dependent epidermal differentiation. *Nature* 2011, 470:353–358.
122. Meerbrey KL, Hu G, Kessler JD, Roarty K, Li MZ, Fang JE, Herschkowitz JI, Burrows AE, Ciccia A, Sun T, et al. The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. *Proc Natl Acad Sci U S A* 2011, 108:3665–3670.
123. Muller P, Boutros M, Zeidler MP. Identification of JAK/STAT pathway regulators–insights from RNAi screens. *Semin Cell Dev Biol* 2008, 19:360–369.
124. Bushman FD, Malani N, Fernandes J, D'Orso I, Cagney G, Diamond TL, Zhou H, Hazuda DJ, Espeseth AS, Konig R, et al. Host cell factors in HIV replication: meta-analysis of genome-wide studies. *PLoS Pathog* 2009, 5:e1000437.
125. Echeverri CJ, Perrimon N. High-throughput RNAi screening in cultured cells: a user's guide. *Nature Rev Genet* 2006, 7:373–384.
126. Echeverri CJ, Beachy PA, Baum B, Boutros M, Buchholz F, Chanda SK, Downward J, Ellenberg J, Fraser AG, Hacohen N, et al. Minimizing the risk of reporting false positives in large-scale RNAi screens. *Nat Meth* 2006, 3:777–779.
127. Pan Q, de Ruiter PE, von Eije KJ, Smits R, Kwekkeboom J, Tilanus HW, Berkhout B, Janssen HL, van der Laan LJ. Disturbance of the microRNA pathway by commonly used lentiviral shRNA libraries limits the application for screening host factors involved in hepatitis C virus infection. *FEBS Lett* 2011, 585:1025–1030.
128. Kulkarni MM, Booker M, Silver SJ, Friedman A, Hong P, Perrimon N, Mathey-Prevot B. Evidence of off-target effects associated with long dsRNAs in Drosophila melanogaster cell-based assays. *Nat Meth* 2006, 3:833–838.

129. Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y, Baskerville S, Maksimova E, Robinson K, Karpilow J, et al. 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Meth* 2006, 3: 199–204.

130. Seinen E, Burgerhof JG, Jansen RC, Sibon OC. RNAi-induced off-target effects in Drosophila melanogaster: frequencies and solutions. *Brief Funct Genom* 2011, 10:206–214.

131. Seinen E, Burgerhof JG, Jansen RC, Sibon OC. RNAi experiments in *D. melanogaster*: solutions to the overlooked problem of off-targets shared by independent dsRNAs. *PloS One* 2010, 5:e13119.

132. Schultz N, Marenstein DR, De Angelis DA, Wang WQ, Nelander S, Jacobsen A, Marks DS, Massague J, Sander C. Off-target effects dominate a large-scale RNAi screen for modulators of the TGF-beta pathway and reveal microRNA regulation of TGFBR2. *Silence* 2011, 2:3.

133. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, et al. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* 2007, 448:151–156.

134. Ramadan N, Flockhart I, Booker M, Perrimon N, Mathey-Prevot B. Design and implementation of high-throughput RNAi screens in cultured Drosophila cells. *Nature Protocols* 2007, 2:2245–2264.

135. Birmingham A, Selfors LM, Forster T, Wrobel D, Kennedy CJ, Shanks E, Santoyo-Lopez J, Dunican DJ, Long A, Kelleher D, et al. Statistical methods for analysis of high-throughput RNA interference screens. *Nat Meth* 2009, 6:569–575.

136. Zhang XD. An effective method for controlling false discovery and false nondiscovery rates in genome-scale RNAi screens. *J Biomol Screen: Off J Soc Biomol Screen* 2010, 15:1116–1122.

137. Zhang XD, Lacson R, Yang R, Marine SD, McCampbell A, Toolan DM, Hare TR, Kajdas J, Berger JP, Holder DJ, et al. The use of SSMD-based false discovery and false nondiscovery rates in genome-scale RNAi screens. *J Biomol Screen: Off J Soc Biomol Screen* 2010, 15:1123–1131.

138. Kaplow IM, Singh R, Friedman A, Bakal C, Perrimon N, Berger B. RNAiCut: automated detection of significant genes from functional genomic screens. *Nat Meth* 2009, 6:476–477.

139. Collins MA. Generating 'omic knowledge': the role of informatics in high content screening. *Combin Chem High Throughput Screen* 2009, 12:917–925.

140. Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, Lieberman J, Elledge SJ. Identification of host proteins required for HIV infection through a functional genomic screen. *Science* 2008, 319:921–926.

141. Sessions OM, Barrows NJ, Souza-Neto JA, Robinson TJ, Hershey CL, Rodgers MA, Ramirez JL, Dimopoulos G, Yang PL, Pearson JL, et al. Discovery of insect and human dengue virus host factors. *Nature* 2009, 458:1047–1050.

142. Kondo S, Booker M, Perrimon N. Cross-species RNAi rescue platform in Drosophila melanogaster. *Genetics* 2009, 183:1165–1173.

143. Schulz JG, David G, Hassan BA. A novel method for tissue-specific RNAi rescue in Drosophila. *Nucleic Acids Res* 2009, 37:e93.

144. Langer CC, Ejsmont RK, Schonbauer C, Schnorrer F, Tomancak P. In vivo RNAi rescue in Drosophila melanogaster with genomic transgenes from Drosophila pseudoobscura. *PloS One* 2010, 5:e8928.

145. Kittler R, Pelletier L, Ma C, Poser I, Fischer S, Hyman AA, Buchholz F. RNA interference rescue by bacterial artificial chromosome transgenesis in mammalian tissue culture cells. *Proc Natl Acad Sci U S A* 2005, 102:2396–2401.

146. Stielow B, Sapetschnig A, Kruger I, Kunert N, Brehm A, Boutros M, Suske G. Identification of SUMO-dependent chromatin-associated transcriptional repression components by a genome-wide RNAi screen. *Mol Cell* 2008, 29:742–754.

147. Lassus P, Rodriguez J, Lazebnik Y. Confirming specificity of RNAi in mammalian cells. *Science's STKE: Signal Transd Knowled Environ* 2002, 2002:pl13.

148. Sarov M, Stewart AF. The best control for the specificity of RNAi. *Trend Biotechnol* 2005, 23:446–448.

149. Yokokura T, Dresnek D, Huseinovic N, Lisi S, Abdelwahid E, Bangs P, White K. Dissection of DIAP1 functional domains via a mutant replacement strategy. *J Biol Chem* 2004, 279:52603–52612.

150. Poser I, Sarov M, Hutchins JR, Heriche JK, Toyoda Y, Pozniakovsky A, Weigl D, Nitzsche A, Hegemann B, Bird AW, et al. BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. *Nat Meth* 2008, 5: 409–415.

FURTHER READING

BioMed Central RNAi Gateway, http://www.biomedcentral.com/gateways/RNAi.

Davidson BL, McCray PB Jr. Current prospects for RNA interference-based therapies. *Nat Rev Genet* 2011, 12:329–340.

Howard Hughes Medical Institute Biointeractive, RNA Interference: New Twist in the RNA World, http://www.hhmi.org/biointeractive/rna/rna_interference.

July 2005 NOVA Program on RNAi, http://www.pbs.org/wgbh/nova/body/rnai.html.

Nature Reviews RNAi Collection, http://www.nature.com/focus/rnai/index.html.

Nikon MicroscopyU Online Review Article, The Automatic Microscope, http://www.microscopyu.com/articles/livecellimaging/automaticmicroscope.html.

