

Genomic Screening with RNAi: Results and Challenges

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Annu. Rev. Biochem. 2010. 79:37–64

First published online as a Review in Advance on
April 1, 2010

The *Annual Review of Biochemistry* is online at
biochem.annualreviews.org

This article's doi:
10.1146/annurev-biochem-060408-092949

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0066-4154/10/0707-0037\$20.00

Key Words

bioinformatics, cell biology, *Drosophila*, high-throughput screening

Abstract

RNA interference (RNAi) is an effective tool for genome-scale, high-throughput analysis of gene function. In the past five years, a number of genome-scale RNAi high-throughput screens (HTSs) have been done in both *Drosophila* and mammalian cultured cells to study diverse biological processes, including signal transduction, cancer biology, and host cell responses to infection. Results from these screens have led to the identification of new components of these processes and, importantly, have also provided insights into the complexity of biological systems, forcing new and innovative approaches to understanding functional networks in cells. Here, we review the main findings that have emerged from RNAi HTS and discuss technical issues that remain to be improved, in particular the verification of RNAi results and validation of their biological relevance. Furthermore, we discuss the importance of multiplexed and integrated experimental data analysis pipelines to RNAi HTS.

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HIGH-THROUGHPUT RNAi SCREENING

RNA interference (RNAi) is an RNA-dependent gene-silencing process that is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA (dsRNA) molecules. In response to endogenous or exogenously introduced dsRNAs, the RNAi machinery knocks down (i.e., reduces but does not eliminate) the RNA targets of dsRNA in a sequence-specific manner (1, 2). The burgeoning of the RNAi field, recognized in its importance with a Nobel Prize to Andrew Fire and Craig Mello in 2006, has led to exciting new research in several areas. First, fundamental new biological insights have been obtained from the study of the genesis and function of small RNAs of 21–28 nucleotides (nt) in length that include microRNAs (miRNAs) and endogenous short interfering RNAs (endo-siRNAs) (recent

reviews include References 3–6), as well as the cellular responses to RNA viruses (recent reviews include References 7 and 8). Second, much effort is ongoing regarding the potential use of RNAi-inducing reagents as therapeutics (see recent reviews in References 9–14). Third, RNAi is being harnessed as a molecular tool for gene- and transcript-specific knockdown of mRNA levels, facilitating large-scale study of gene function in a wide variety of cells, tissues, and organisms, including *Caenorhabditis elegans*, *Drosophila*, mammalian cells, the flatworm *Planaria*, and *Arabidopsis* (see early examples and reviews in References 1, 15–29).

The pairing of RNAi technologies with cDNA and genomic sequence data has made it possible to construct genome-scale libraries of RNAi reagents for performing RNAi high-throughput screens (HTSs) in a wide variety of cell types (30). As such, RNAi allows in many systems the type of systematic functional analyses that were previously practical for only a relatively small set of genetically tractable model organisms. Arguably, the most important impact in this regard has been the ability to perform genome-scale cell-based RNAi HTS in mammalian cells. Indeed, RNAi screening in mammalian cells has already led to a large number of results with important biomedical implications (see **Table 1** and below), including the identification of novel oncogenes and potential targets for the development of therapeutic treatments (recent reviews include References 11, 31–34).

Even in well-established genetic model systems, such as *C. elegans* and *Drosophila*, RNAi screening has had a profound impact, increasing the scope and pace at which gene interrogation can proceed. In *C. elegans*, RNAi screens are performed in vivo, usually following feeding of bacteria that express dsRNAs (29, 35, 36). In *Drosophila*, RNAi screening can be done either in vivo using transgenes that express RNAi reagents or in cell culture (reviews include References 28 and 29). Conveniently, the RNAi approach itself facilitates rapid transfer of information learned in model organisms to

RNA interference

(RNAi): RNA-dependent gene silencing controlled and initiated by short double-stranded RNA molecules that can reduce levels of the mRNA target

HTS: high-throughput screen

Table 1 Results of genome-scale,^a cell-based RNAi high-throughput screens in mammalian or *Drosophila* cells

Cell type	Screen type	Reagent	Primary hits	Secondary hits ^b	Field of study	References
Human cells						
HeLa	Plate reader & imaging	esiRNA	275	37	Cell division	120, 121
U2OS	Imaging	siRNA	1,152	18	Cell cycle	114
NCI-H1155	Plate reader	siRNA	87	6	Cancer biology	103
NIH3T3	Pooled	shRNA	15	3	Stress resistance	43
293T	Plate reader	siRNA	—	295	Host-pathogen interactions	79
293T, HeLa, MCF-7	Pooled	shRNA	30	8	Cell death	42
DLD1	Plate reader	siRNA	740	268	Signal transduction	50
HEK293	Pooled	shRNA	13,140	21	Cell adhesion	40
HeLa	Imaging	siRNA	305	124	Host-pathogen interactions	180
HeLa	Plate reader	siRNA	530	23	Signal transduction	156
HeLa-derived TZM-b1	Plate reader	siRNA	386	273	Host-pathogen interactions	78
HeLa P4/R5	Plate reader	siRNA	931	232	Host-pathogen interactions	80
Jurkat	Pooled	shRNA	11	5	Cancer biology	46
MCF-10A ^c	Pooled	shRNA	201	166	Cancer biology	45
MNT-1	Plate reader	siRNA	98	35	Pigmentation	142
RDG3	Imaging	siRNA	—	171	Stress resistance	133
BJtsLT	Pooled	shRNA	100	37	Cancer biology	126
DLD-1	Pooled	shRNA	368	83	Cancer biology	48
Huh7/Rep-Feo	Plate reader	siRNA	236	96	Host-pathogen interactions	181
Jurkat	Pooled	shRNA	252	7	Host-pathogen interactions	47
Huh 7.5.1	Imaging	siRNA	521	262	Host-pathogen interactions	182
Mouse cells						
NIH 3T3	Pooled	shRNA	—	28	Cancer biology	104
L929	Plate reader	siRNA	666	432	Cell death	122
B16-F0	Pooled	shRNA	78	22	Cancer biology	41
Oct4-Gip ESCs	FACS ^d & imaging	esiRNA	296	21	Stem cell biology	109
Oct4-Gip ESCs	FACS	siRNA	148	104	Stem cell biology	107
<i>Drosophila</i> cells						
Kc167, S2R+	Imaging	dsRNA	438	—	Viability	97
S2	Imaging	dsRNA	—	121	Host-pathogen interactions	164
Clone 8	Plate reader	dsRNA	238	213	Signal transduction	152
Clone 8	Plate reader	dsRNA	509	96	Signal transduction	147
Kc167	Plate reader	dsRNA	—	90	Signal transduction	149
S2	Plate reader	dsRNA	474	121	Signal transduction	190
S2	Imaging	dsRNA	—	86	Host-pathogen interactions	167
S2	Imaging	dsRNA	305	~190	Host-pathogen interactions	169
S2	Imaging	dsRNA	210	112	Host-pathogen interactions	176
S2	Plate reader	dsRNA	—	14	Host-pathogen interactions	163, 191
S2	Plate reader	dsRNA	1,133	284	Protein secretion	139

(Continued)

Table 1 (Continued)

Cell type	Screen type	Reagent	Primary hits	Secondary hits ^b	Field of study	References
S2	FACS	dsRNA	488	—	Cell cycle and/or cell size	115
S2	Imaging	dsRNA	—	—	Signal transduction	130
S2	FACS	dsRNA	66	23	RNA biology	157
S2	Plate reader	dsRNA	75	4	Ion transport	129
S2R+	Plate reader	dsRNA	138	7	RNA biology	158
S2R+	Plate reader	dsRNA	1,168	331	Signal transduction	144
S2R+	Imaging	dsRNA	699	—	Signal transduction	131
S2R+	Imaging	dsRNA	1,500	27	Signal transduction	132
S2	Imaging	dsRNA	90	24	RNA biology	162
Kc167	Plate reader	dsRNA	81	47	Cell death	123
S2	Plate reader	dsRNA	47	1	RNA biology	159
S2*	Plate reader	dsRNA	18	5	Chromatin regulation	140
S2R+	Imaging	dsRNA	346	—	Signal transduction	145a
S2	Imaging	dsRNA	162	54	Host-pathogen interactions	173
DL1	Plate reader	dsRNA	176	110	Host-pathogen interactions	178
Kc167	Imaging	dsRNA	526	—	Lipids	82
Kc167	Plate reader	dsRNA	265	120	Transcription and/or translation	141
Primary neurons	Imaging	dsRNA	336	104	Neural outgrowth	136
S2	Plate reader	dsRNA	821	152	Mitochondria	138
S2	Plate reader	dsRNA	—	—	Phagocytosis	128
S2	Imaging	dsRNA	—	—	Centrioles and/or centrosomes	119
S2	Imaging	dsRNA	847	227	Lipids	83
S2	Imaging	dsRNA	292	133	Cancer biology	117
S2	Imaging	dsRNA	23	—	Transcription and/or translation	85
S2-derived RZ-14	Plate reader	dsRNA	177	—	RNA biology	160
S2R+	Imaging	dsRNA	119	39	Centrioles and/or centrosomes	118
S2R+	Imaging	dsRNA	133	72	RNA biology	161
S2R+	Imaging	dsRNA	~500	1	Mitochondria	137
S2R+	Plate reader	dsRNA	303	173	Circadian rhythms	125
Clone 8	Plate reader	dsRNA	~100	11	Signal transduction	155
S2	Plate reader	dsRNA	218	116	Host-pathogen interactions	37
Kc167	Plate reader	dsRNA	996	202	Cell cycle and/or cell size	116
S2R+	Plate reader	dsRNA	42	33	Cell death	124
S2R+	Imaging	dsRNA	—	—	Signal transduction	154
S2R+	Imaging	dsRNA	15	7	Cell cycle and/or cell size	134

^aFor this summary, we defined genome-scale with a cutoff of approximately 5000 genes (mammalian cell screens) or at least 70% of the genome (*Drosophila* cell screens).

^bHere, we use secondary hits to refer to the largest set of primary hits that passed an additional test verifying the result at the reagent level (retest after re-synthesis or with another assay or cell type) or in most cases, at the gene level (retest with another reagent or single reagents from a pool, for example). In some cases, only a subset of primary hits were tested in secondary assays. For most reports, only a small number of genes (typically, one to five) were confirmed with a rigorous test, such as rescue of the RNAi effect with a cDNA, or were confirmed at the level of biological significance with another type of assay or an in vivo analysis.

^cAdditional cell types tested with smaller shRNA pools.

^dFACS, fluorescence-activated cell sorter.

mammalian studies, by making it possible to design and execute RNAi knockdown of homologous genes in mouse or human cells (or in another relevant system, such as mosquito vectors of mammalian diseases; see, for example, Reference 37) subsequent to performing the large-scale screen in *Drosophila* or another model organism.

Because many aspects of RNAi screening have been reviewed previously, we have focused this review primarily on results of genome-scale cell-based screens in *Drosophila* and mammalian cells (Table 1). Following a discussion of the technical aspects of RNAi HTSs, we discuss in more detail what has been learned from the results of the large number of screens performed to date, including issues of false discovery, specific genes, and pathways newly implicated in various processes, and discuss how researchers are working toward systems-wide understandings of various biological processes. Where relevant, we refer to other sources for further reading on specific subtopics.

PERFORMING HIGH-THROUGHPUT, CELL-BASED RNAi SCREENS

The effects of RNAi can be compared with reduction-of-function (hypomorphic) genetic approaches. When the normal function of a gene is required for a given function, RNAi knockdown may lead to a phenotype detectable in an assay that tests that function, either directly or indirectly. As such, RNAi facilitates both small-scale studies and HTSs. With HTSs (see Figure 1a,b), a large number of gene functions are interrogated concurrently, such that one can, at least in theory, begin to isolate multiple members of a functional pathway as well as implicate new genes in a given biological function, process, complex, or behavior. A cell-based RNAi HTS, as discussed here, is typically done in one of two formats: a pooled format, in which the library is introduced at random into cells (Figure 1a), or an arrayed format, in which single genes are targeted by reagents in individual wells of a microtiter plate (Figure 1b)

(recently reviewed in References 32, 38, 39). Each of these approaches has significant advantages and disadvantages, and both have been successfully applied to the investigation of a number of different biological questions.

Pooled Format Screening

With a pooled screen (Figure 1a), the RNAi reagent library [for mammalian cells, a viral-encoded short hairpin RNA (shRNA) library is typical] is introduced into cells en masse and at random, such that any given cell will contain approximately one gene-specific RNAi reagent. The screener may then perform a selection, in which only cells resistant to some treatment will survive [alternatively, a method such as fluorescence-activated cell sorter (FACS) can be used to isolate the specific subset of cells that are positive in the assay], followed by polymerase chain reaction (PCR) amplification of the RNAi reagents present in surviving cells and by sequencing to determine the identity of those reagents. The presence of a specific RNAi reagent after selection suggests that knockdown of the corresponding gene confers resistance to the treatment (see, for example, References 40–45).

Alternatively, the researcher may treat one or more subset of cells (or different cell types) differently, either before or after subjecting the cells to the pooled RNAi library (depending on the assay), creating a “reference set” and one or more “experimental sets” of cells. Subsequently, a molecular method, such as PCR amplification or microarray analysis, is used to detect which RNAi reagents are present in each set (via detection of the RNAi-inducing sequence itself or a unique molecular “barcode” that identifies each reagent). This makes it possible to determine which RNAi reagents are under- or over-represented in the experimental set(s) as compared with the reference set (see, for example, References 45–48).

In general, pooled approaches are more likely to be feasible in a standard lab setting than are arrayed screens, which require liquid handling automation and specialized

False discovery:

experimental findings that cannot later be verified (i.e., false-positive results) or should be identified but are not (i.e., false-negative results)

RNAi reagent

library: long or short dsRNAs designed to induce RNAi knockdown of specific genes (e.g., sets of dsRNAs, shRNAs, siRNAs, or esiRNAs)

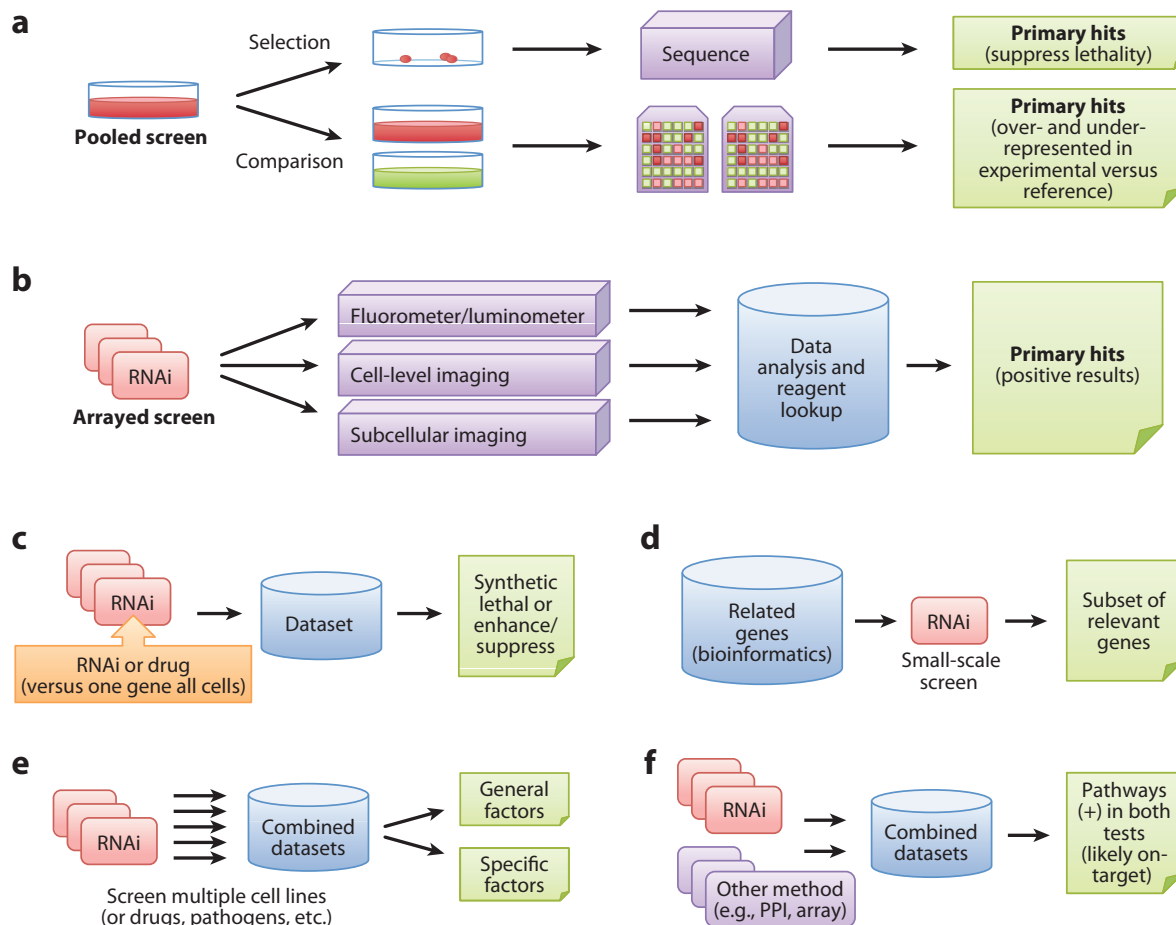


Figure 1

Approaches to high-throughput cell-based RNAi screening. (a) Pooled RNAi high-throughput screen (HTS) approach. (b) Arrayed RNAi HTS approach. (c) Modification of a pooled or arrayed approach via prior addition of a treatment, such as RNAi against a single gene in all cells or treatment with a small molecule. (d) Identification of related genes via informatics-based analysis (e.g., all kinases or genes previously implicated in a specific pathway or complex), followed by screening with reagents directed against the identified subset of genes. (e) A HTS with one assay type using multiple cell lines, the same cell line with multiple pathogens, or a similar multiplexed approach, followed by data integration to identify specific and general factors. (f) Parallel RNAi HTSs and an additional experimental high-throughput genomic or proteomic approach, followed by data integration to identify high-confidence hits. Abbreviations: PPI, protein-protein interaction; + sign, positive result.

assay readout instruments (see below). However, pooled approaches have the disadvantage that deconvolution of positive results can require a specialized and potentially costly approach (namely, microarrays to detect the RNAi reagents). Moreover, it is not currently feasible to use a pooled approach in conjunction with a high-content image-based cell assay (i.e., a microscopy screen), and there is at least some risk

that representation of the library will not be uniform, creating a difference between the theoretical and the actual number and proportion of reagents tested in a given screen. Nevertheless, the approach has been successfully applied for a number of HTSs that have yielded interesting results, including a number of recent studies in human cells (Table 1) (see for example References 46, 48). Finally, it should be

noted that, with a pooled approach, the time in cell culture after introduction of the library may be on the order of several days or weeks, whereas shorter incubations are more typical for arrayed format screens.

Arrayed Format Screening

The arrayed format is the more flexible format for RNAi HTSs as each unique RNAi reagent (or unique set of reagents, such as a small pool of independent siRNAs targeting a single gene) occupies a unique well in a microtiter plate, such as a 96- or 384-well plate, facilitating a wide variety of manipulations and readouts (**Figure 1b**). Detection of the assay is typically done via measuring colorimetric, fluorescence, or luminescent readouts at the total well level (plate-reader screens) or via measuring fluorescent readouts at the cellular or subcellular level using imaging. Examples of cell-based assays performed in arrayed formats include detection of responses to an external stimulus (e.g., a stress, drug treatment, pathogen, signaling ligand, or metabolic substrate), such as via a transcriptional reporter; changes in the expression, modification, and/or subcellular localization of a protein; cell death, cell-cycle arrest, or other changes related to cell survival, metabolism, and/or division; changes in cellular or organelle size and/or morphology; and changes in transport and/or accumulation of an ion, metabolite, and/or biomolecule (30).

As a large number of individual assay plates must be screened to reach genome scale with an arrayed screen, screening in this format typically requires a fairly large total volume of assay reagents (e.g., media, antibodies, and dyes) and automated equipment (e.g., for liquid handling automation and assay readouts). However, arrayed screens have the advantage that, after the assay, one can easily determine which cells were treated with which specific RNAi reagents by simply looking up the identity of the reagent in a given well using a database or spreadsheet. Notably, arrayed screens also have the advantages that multiple, related phenotypes can be assayed in a single screen (e.g., via detection

of multiple antibodies and/or fluorescent dyes) and one can have high confidence that all RNAi reagents in the library are tested in the screening assay (30).

Innovative and Multiplexed Screening Approaches

Researchers are increasing the complexity and usefulness of screening using the approaches outlined in **Figure 1c-f**, such as via multiplexed and/or combinatorial approaches. The most accessible of these techniques for individual laboratories may be incorporation of bioinformatics analysis at genome scale to identify a subset of candidate genes, followed by experimental testing with a corresponding subset of reagents (**Figure 1d**). For example, extensive screening has been done using sublibraries grouped by biochemical function (e.g., all human kinases). An increasing number of screens start with another type of bioinformatics-based approach to defining a candidate gene list, such as a literature-based analysis, followed by a small-scale screen. Moreover, integration of RNAi data with other “omics” approaches, such as protein-protein interaction maps, genetic interaction networks, and RNA-profiling experiments, can provide additional insight into relationships among the components of a network (49, 50).

RNAi Reagents

The specific RNAi reagent for knockdown is likely to be different for different types of cells, organisms, and assays, and reagent libraries are evolving as we learn more about RNAi mechanisms and rules for specific and effective design of RNAi reagents (recent reviews include those in References 12, 51, 52). The four types of RNAi reagent that are typically used for cell-based HTSs are dsRNAs, siRNAs, shRNAs, and endoribonuclease-prepared siRNAs (esiRNAs). In general, short dsRNA segments [~21 base pairs (bp), in the form of siRNA or shRNAs] are typical for mammalian systems, as longer segments can induce

a nonspecific interferon response (30, 32, 38, 53–55). Longer segments (~500 bp) are typical for model systems that lack an interferon response, such as *C. elegans* and *Drosophila* (30, 36, 54–59). Once inside the cell, dsRNAs are processed by the endogenous RNAi machinery to generate small dsRNA segments (typically 20–22 bp) with a characteristic 2-bp 3' overhang, the active agent for RNAi (recently reviewed in Reference 51).

Delivery to Cells

The appropriate delivery systems also differ for different cell types. Common delivery systems include viral transduction for shRNAs; lipid-mediated transfection or electroporation for shRNAs, siRNAs, esiRNAs, or dsRNAs (30, 32, 38, 52–54); or simply mixing cells with dsRNA in solution for most *Drosophila* cells, an approach referred to as “bathing” (29, 30, 54, 56–58).

Analysis and Follow-Up Studies

Subsequent to the primary screen, the resulting data are analyzed to identify positive results, “hits.” As mentioned above, for pooled screens, this typically involves identifying the set of reagents that conferred resistance or those that are under- and/or overrepresented in the experimental set(s) as compared with the reference. Analysis of arrayed screens can involve application of specialized image analysis software or custom programs, as well as various methods of statistical analysis (60). RNAi screening has learned much from applying what was developed for statistical analysis of other methods, in particular for cell-based small-molecule screens, and much progress has been made. For example, several approaches to data normalization, establishment of appropriate thresholds for cutoffs, replicate tests, and other criteria have been established (60–68).

Important factors to consider in RNAi HTSs include (a) performing at least one replicate test in the primary screen, (b) including an appropriate type and number of “no treatment”

and other controls, (c) a thoughtful array of the library and controls (e.g., randomized), (d) an early assessment of data quality to detect plate- or well-level problems such as dispensing errors, (e) data normalization, and (f) setting appropriate cutoff values for significant results (60–63, 66). Despite the recent improvements in addressing all of these factors during RNAi HTSs, subsequent data analysis, and follow-up tests, false discovery remains a significant and difficult problem to address. Statistical and experimental approaches can help to minimize the problem (60, 69). Sources contributing to false discovery are described in **Table 2**; methods of verification of RNAi HTS results at the level of the reagent, assay, gene, or biological process are described in **Table 3**.

HIGH-THROUGHPUT RNAi SCREEN RESULTS

Recognizing and Addressing False Discovery

Following the completion of the first full-genome screens in *Drosophila* and mammalian cells, it became apparent, from both comparative analysis of datasets and attempts to validate screen hits, that many primary screen hits were false positives attributable to off-target effects (OTEs) (70–73). Recognition of the problem, together with a better understanding of RNAi mechanisms, has prompted development of software tools for minimizing OTEs and better reagent libraries. Improved gene annotations, such the efforts of the ENCYCLOPEDIA OF DNA ELEMENTS (ENCODE) (74–76) and modENCODE (77) also contribute to improved reagent design. However, as we still lack a complete understanding of effective rules for reagent design and gene annotations continue to be revised, OTEs remain an issue. Intriguingly, even after improvements in library design (for a review, see Reference 52), overlap among screen hits from independent but related screens remains surprisingly low. For example, in multiple studies of human immunodeficiency virus (HIV) infection in mammalian

Table 2 Sources contributing to false discovery in RNAi high-throughput screens

Source	Contributes to	How to address during screening
Experimental noise inherent in large-scale studies	False positives and negatives	Use appropriate experimental controls, number of replicate tests, and statistical analyses for the specific screen performed
Bias inherent in the screen assay	False positives and negatives	Perform pilot tests to detect bias and flaws; correct for changes in cell number; include appropriate experimental controls; screen with multiple related assays
Off-target effects	False positives (and can obscure true positives)	Choose an optimized or verified RNAi reagent library; include more than one unique RNAi reagent per gene; learn from community annotation of RNAi reagents
Incomplete or incorrect gene models	False positives and negatives	Learn from community efforts to improve genome annotations
Potency of RNAi reagents	False negatives	Include more than one unique RNAi reagent per gene; choose an optimized or verified RNAi reagent library; work to improve reagent design
Knockdown causes weak phenotype not detected above a given cutoff	False negatives	Screen in a sensitized background; relax statistical cutoffs (at the cost of increasing false-positive discovery); increase the number of replicate tests to detect weak but repeatable results
The knockdown phenotype (e.g., cell death) obscures the screen assay phenotype	False negatives	Include all members of a pathway or complex in follow-up studies even if only a subset was identified in the screen; perform multiple screen assays; perform the screen assay in multiple cell lines.

cells, there is limited overlap among screen hits at the gene level (47, 78–81). Similarly, related screens performed in two different *Drosophila* cell types for components of the JAK/STAT signaling pathway resulted in only 25% overlap (82, 83). When analysis of either set of related screens was extended to the level of gene ontology or pathways, the results were more similar (81, 82).

Development of appropriate approaches to minimize false discovery rates remains a challenge as, to a large extent, minimizing false-positive results increases the number of false-negative results, and vice versa. The appropriate statistical cutoff applied to limit false discovery will vary depending on the tolerance for false discovery in one direction or the other, and the level of tolerance may be quite different for different screens. For example, if the ultimate goal is to identify the one gene (or a small set of genes) that confers a specific predicted gene activity, then a researcher might be fairly intolerant of false-negative results, for fear of tossing out the specific subset being sought. By contrast, for a screen aimed at studying a relatively

understudied process, one might be willing to sacrifice a fair number of false negatives in the interest of working from a limited set of statistically high-confidence hits. It is worth noting that the false discovery tolerances of the researchers who analyze an initial study may be in conflict with the tolerances of those who analyze the data in subsequent studies (e.g., meta-analyses), emphasizing the need for data reporting standards, including facilitating easy access to raw data (for reanalysis), transparency of analysis methods that were used to determine the reported hits, and standardized reporting formats to facilitate data integration. Efforts at standardization include MIARE (for Minimum Information about an RNAi Experiment, <http://www.miare.org>), and information about reagents and data is being collected at the Probe (<http://www.ncbi.nlm.nih.gov/probe>) and PubChem (<http://pubchem.ncbi.nlm.nih.gov/>) databases at NCBI (<http://www.ncbi.nlm.nih.gov>).

Nevertheless, data analysis is neither the sole contributor nor the sole answer to the problem of false discovery. For example, the gene-level

Table 3 Methods for experimental verification of RNAi screen results

Method	Examples	Rationale
Retest the reagents with the same assay	Test several replicates (including a re-synthesized or new batch of reagent); test single reagents in arrayed format after a pooled approach	Reagent-level verification
Retest with a related assay and/or different cell type	Switch the reporters in a dual-reporter assay; test a different cell line, marker, or antibody; test in a different cell line	Reagent-level verification
Retest with unique reagents	Test reagents designed to target different regions of the gene	Gene-level verification (confidence increases when more than one works)
Assay small molecule(s)	Test a known inhibitor of the gene product in the assay; test small molecules in parallel with RNAi and compare pathways implicated in each	Gene-level verification (correlation is suggestive of an on-target effect)
Determine mRNA or protein levels in the presence of the RNAi reagent	Q-PCR or immunoblotting ^a	Gene-level verification (correlation between knockdown and phenotype is suggestive of an on-target effect)
Rescue in the presence of the RNAi reagent ^b	Test rescue with a genomic fragment, cDNA, or open reading frame construct that evades RNAi knockdown	Gene-level verification (rescue demonstrates an on-target effect)
Pattern of gene expression of mRNAs corresponding to hits	Q-PCR or microarray in specific cell types, stages, and/or tissues	Gene-level verification (expression in relevant tissues or stages is suggestive of a relevant finding)
Pattern of expression of the proteins corresponding to hits	Immunoblotting in specific cell types, stages, and/or tissues	Gene-level verification (expression in relevant tissues or stages is suggestive of a relevant finding)
Subcellular distribution of proteins corresponding to hits	GFP-tagged construct or immunofluorescence	Gene-level verification (expression in relevant subcellular compartments is indicative of a relevant finding)
RNAi-induced phenotype in another species	Test effect of knockdown of homologs in mammalian cells as a follow-up to a nonmammalian cell screen	Gene-level verification (similar phenotype provides compelling evidence of a biologically relevant finding)
Correlation with a related disease or disorder	Map disease-associated regions, mutations, and amplifications	Gene- and pathway-level verification (disease association is indicative of a relevant finding)
Protein-protein interactions	Coimmunoprecipitation, mass spectrometry, yeast two-hybrid screen	Gene- and pathway-level verification (physical interactions among newly identified proteins or between new and established players are indicative of a relevant finding)
Genetic analysis in vivo	Test effects of mutations of gene hits in whole animals (same or different species than primary screen cells)	Gene and pathway-level verification [related phenotype provides compelling evidence of a relevant effect and can help refine the role(s) of the genes in specific pathways, events, or behaviors]

^aAbbreviation: GFP, green fluorescent protein; Q-PCR, Quantitative Reverse Transcriptase PCR.

^bThe “gold standard” approach to verification of an RNAi result at the gene level; similar to a genetic test for complementation.

differences among HIV screen datasets might suggest that different subsets of reagents in the different libraries used for those screens may have resulted in robust knockdown, such that the datasets cannot be compared in a straightforward manner. Consistent with this, there seems to be significant variability in the robustness of specific RNAi reagents for mammalian RNAi HTSs, including both among and between sets of siRNAs, shRNAs, and bifunctional or miRNA-like reagents (12). The specific cell type, assay, and biological process being tested are also relevant. For example, another source of false discovery was recognized via analysis of two different screens in *Drosophila* cells that both interrogate the JAK/STAT pathway but had little overlap among screen hits. Comparison of the screen datasets revealed that there can be inherent bias in the assays and/or specific biological functions being addressed (84). Moreover, even when there is robust knockdown, cells may respond in a manner that makes it impossible to assay the process of interest. For example, in a screen for factors involved in hypoxia, several nonessential TOR pathway components were isolated; however, as knockdown of PTEN results in cell death, a role for PTEN in hypoxia could not be addressed (85).

As it seems likely that all of the above-mentioned factors (i.e., cell type, assay design, experimental noise, assay design, and the choice of reagent library) contribute to false discovery, it is important to continue to learn how to best address them. If robustness of reagents is a major contributing factor, then improving RNAi libraries such that each reagent is effective (and, ideally, knockdown occurs at comparable levels gene to gene) may in turn improve RNAi HTS results. There is variability in the effectiveness of RNAi reagents designed using a single set of tools, such that, in the absence of gene-by-gene testing (see, for example, Reference 86), this is currently difficult to accomplish. Recent evidence that miRNA-like approaches result in more robust knockdown, however, as well as using specific strategies designed to address difficult-to-target genes, may contribute to im-

proved library design (12, 87). Moreover, problems related to pleiotropy might be overcome, at least in part, via screening in multiple cell lines, which are likely to have different essential requirements (see, for example, Reference 88).

Another approach to successfully overcome problems of false discovery is to combine RNAi with the results of other high-throughput methods, including overexpression screening, protein-protein interaction analysis, genomic analysis (e.g., mapping of disease-associated genomic regions or amplifications), and mRNA expression array data (see approaches outlined in **Figure 1d-f**). These include projects in which new experimental studies were done in conjunction with a screen (see, for example, References 50, 89–94). In addition, bioinformatics-based analyses have been applied to preselect a set of genes to be tested or identify a high-confidence set of primary or verified hits (see, for example, References 89, 90, 95, 96).

Both genome-scale RNAi HTSs (**Figure 1a,b**) and genome- or smaller-scale screens, combined with more integrated and/or multiplexed approaches (**Figure 1c-f**), have led to important insights into a number of biological topics. Particular progress has been made in general cellular functions, signal transduction, cancer biology, and host cell responses to infection by bacterial, fungal, eukaryotic, or viral pathogens. Below, we highlight key findings from a large number of RNAi HTSs performed in *Drosophila* or mammalian cells (see also **Table 1**).

Biological Findings from Screens

Despite the relative infancy of cell-based RNAi HTSs, particularly as compared with more classic genetic screens, the approach has already resulted in a large number of studies with significant impact in a wide variety of fields. Indeed, many screens have investigated basic cellular processes, including how cells survive, proliferate, and divide, and more specialized cellular functions, such as responding to specific viral, bacterial, and fungal pathogens; surviving

specific chemotherapeutic treatments; and generating pigment. Below and in **Table 1**, we summarize the results of many large-scale RNAi HTSs in mammalian and *Drosophila* screens published to date. Then, we discuss what has (and has not been) learned thus far about gene function and interactions at a system-wide level from the results of these studies.

Cell viability, proliferation, and cancer.

Cell proliferation and survival are fundamental processes that are particularly relevant to human diseases such as cancer. Moreover, routine methods for detecting cell number, viability, and/or basic metabolic readouts in an automated fashion are well established. Thus, it is perhaps not surprising that screens aimed at identifying genes required for cell proliferation and survival were among the earliest cell-based RNAi HTS studies (97). More recently, a number of kinome-wide and other studies have identified factors essential for survival and/or proliferation of a number of mammalian cell types, with a particular emphasis on the requirements of cancer cells (44–46, 88, 92, 98–101). Each of these studies implicates several genes in cell survival and/or proliferation in various cell types. Taken together, the results of these studies highlight the different dependencies that various cell types have for survival, even when the cell lines differ only in expression of a specific factor such as the HPV16 E7 oncogene or mammalian von Hippel-Lindau (*VHL*) tumor suppressor gene (98, 99, 102).

At least one of the studies of cancer cell proliferation or survival looked at requirements for response to tumoricidal drugs (46). This and similar studies aimed at identifying factors required for death and/or survival in the presence of a specific chemical, hormone, or other treatment have yielded informative results. The findings include (*a*) identification of a requirement for mammalian PTPN1, NF1, SMARCB1, and SMARCE1 for the response of chronic myelogenous leukemia cells to imatinib (Gleevec) (46), a small-molecule inhibitor of BCR-ABL; (*b*) the sensitivity of MCF-10A breast cancer cells to disruption of

DNA methyltransferase and proteasome activity (45); and (*c*) a role for *Drosophila* Sox14 as a positive regulator of hormone-induced cell death (95). A number of genes required for proliferation and/or viability of multiple cell lines were also identified in these studies, including more than 250 genes implicated as essential in 12 cancer cell lines (46), a set of 14 kinases implicated as essential across a diverse set of human cell types (88), and 19 genes essential in a set of three cancerous and one breast epithelial cell line (44).

Cancer biology. Extension of this type of approach to screening for drug sensitivity led to identification of factors that alter cellular responses to paclitaxel, a chemotherapeutic treatment used for breast cancer, and specifically implicated the genes *ACRBP*, *TUBGCP2*, and *MAD2* in various aspects of mitotic spindle assembly (103). In addition, a highly integrated approach that included not just cell-based RNAi HTSs to interrogate the kinome, but also overexpression and human genetic analyses, identified *IKBKE* as an oncogene in breast cancer (92). Furthermore, comparison of colorectal DLD-1 cancer cells with and without a mutant form of the oncogene *KRAS* point to sensitivity of Ras mutant cells to disruption of mitosis, including via disruption of ubiquitination and proteasome degradation of mitotic factors, in particular by way of perturbation of PLK1 (48). *KRAS* was also the subject of a genome-wide screen for epigenetic *Fas* silencing in K-*ras*-transformed mouse cells, which identified at least eight proteins (NPM2, TRIM66, ZFP354B, TSS, BMI1, DNMT1, SIRT6, and TRIM37) subsequently shown to bind *Fas* promoter regions (104). Another study identified genes that encode kinases such as CHK1 that, upon knockdown, can sensitize pancreatic cancer cells to treatment with the chemotherapeutic agent gemcitabine (105). Recently, RNAi screening of 30 patient samples (and 4 normal individuals for comparison) was applied to the study of leukemia and identified a number of genes associated with

patient-specific sensitivities to downregulation of specific tyrosine kinases (106).

Stem cell biology. RNAi HTS assays for viability and/or proliferation, in addition to other assays, have also been applied to stem cells with the goal of identifying factors required for self-renewal (107–110). The results of these studies (reviewed in Reference 111) implicate the transcriptional regulators Cnot2 and Trim28 in self-renewal or differentiation of mouse stem cells (107) and implicate Paf1C in maintaining stem cell identity (109). The results of stem cell studies also suggest a role for human EXT1 in erythroid burst or colony formation (BFU-E or CFU-E) (108). Comparison of results from Hu et al. (107) and Ding et al. (109) further underscore the idea that even with similar assays and cells, screen results differ, a finding that Subramanian et al. (111) suggest may be attributable to the use of different libraries, or it may be that neither screen achieves saturation. A preliminary OCT4 regulatory network has been constructed on the basis of integrated analysis of these studies and other high-throughput datasets (112).

Cell division, cell death, and the cell cycle. Whereas many of the above studies used pooled screens or simple viability assays to detect changes in cell proliferation or viability, additional studies have used specific reporters, cell-cycle FACS analysis, or image-based assay readouts to uncover specific phenotypes related to cell division, cell death, or related processes. Among the findings of these studies are cell cycle roles for *Drosophila* kinases with known or putative roles in the cytoskeleton and signal transduction (113); a number of human kinases, phosphatases, and proteins involved in proteolysis (114); and the COP9 signalosome and the Wg/Wnt, MAPK, TOR and JAK/STAT signaling pathways (115). A number of signaling networks, including the TOR pathway (in *Drosophila*, Tor pathway), were implicated in a screen for survival of *Drosophila* cells after treatment with a DNA damaging agent (116). RNAi HTS studies in *Drosophila* cells also point to

roles for a number of proteins in cell division and cell cycle events: HSET, a kinesin motor, was implicated in tumor-related multipolar mitoses (117); disruption of Polo or Centrosomin were found to block centrosome maturation (118). A number of proteins were implicated in centromere propagation, including at least three proteins shown to localize to centromeres, CAL1, CENP-C, and the mitotic cyclin CYCA (119); and the ubiquitin ligase SCF^{Slimb} was shown to regulate centriole duplication (96). Moreover, an early esiRNA-based screen in human HeLa cells identified several candidates involved in various aspects of mitosis and cytokinesis (120, 121).

Cell death and circadian rhythms. A study in mammalian cells has identified a number of genes required for RIP1 kinase-mediated necroptosis and apoptosis, including a role for the BH3-only Bcl-2 family member Bmf in cell death receptor-induced necroptosis (122). Cell death was also a specific focus of screens in *Drosophila* cells (123, 124), which have implicated metabolic regulators such as Charlatan and ARD1 in caspase activation (123) and identified *Tango7* and its mammalian homolog PCID1 (EIF3M) as effectors of cell death (124). In a cell-based assay for light-induced degradation of cryptochrome, three genes, subsequently validated in vivo, were identified as required for cryptochrome degradation: *Drosophila* CG17735, *ssh*, and *Bruce* (125). A study of circadian clock components in human cells linked circadian clocks to cancer and cell death via identification of ARNTL, a putative regulator of p53 (126). In addition, a screen for human kinases and phosphatases involved in the circadian clock identified casein kinase 2 as a circadian clock component that can phosphorylate PER2 (127). The role of calcium in cellular functions has also been the focus of several cell-based RNAi HTS studies in *Drosophila* cells. These studies shed light on calcium channels and homeostasis, including identification of a link between Draper-mediated phagocytosis and calcium homeostasis, and helped to

identify the human immune deficiency-associated gene *Orai1* (128–132).

Cell death and stress responses. A number of screens have analyzed cellular responses to stress conditions. For example, a recent RNAi HTS study of conserved *Drosophila* genes assayed cellular responses to hypoxic conditions, leading to identification of Tor pathway components and *Protein tyrosine phosphatase 61F* as important for regulation of translation in response to hypoxia (85). In addition, a genome-wide study in mammalian cells that combined RNAi HTSs with expression analysis implicated TAF1 in regulation of apoptosis in response to genotoxic stress (42). In addition, a genome-wide pooled screen for mammalian genes, whose knockdown confers resistance to stress via the organic oxidant *tert*-butylhydroperoxide, linked retinol saturase, an enzyme that acts on vitamin A, to stress sensitivity (43). Finally, a “druggable genome”-wide image-based RNAi HTS allowed the simultaneous isolation of factors that disrupt formation of stress granules (SGs) and/or processing bodies (PBs) in response to arsenite treatment; components of the hexosamine biosynthetic pathway were implicated in SGs but not PB assembly, suggesting a role for *O*-linked *N*-acetylglucosamine modification in stress response (133).

Cell morphology, size, and adhesion. High-content image-based RNAi HTSs and other assays have also allowed a number of studies of cell morphology, cell size, and organelles, such as mitochondria. For example, an early screen in *Drosophila* cells identified several known and new genes with roles in cell shape and cytoskeletal regulation (27). A more recent report of factors involved in the control of cell size points to the Pvr, Ras, and Tor pathways as key growth regulators of cultured *Drosophila* cells (134). RNAi HTSs of the *Drosophila* kinome in multiple cell lines revealed general and cell type-specific genes required for cell morphology, including a role for *minibrain* (which encodes a homolog of the human DYRK1A

protein) in a central nervous system-derived cell line (135). Coincidentally, DYRK-family kinases were also identified in a screen for components downstream of the NFAT signaling pathway (131). A screen in primary *Drosophila* neurons revealed genes with roles in outgrowth-related processes in *Drosophila* and mouse cells, including the vesicle trafficking genes *Sec61-alpha* and *Ran* (136). Huang et al. (40) used the pooled approach to identify cells that remain attached after induction of detachment via expression of a mutant form of c-Abl tyrosine kinase. Among the genes identified was *IL6ST*, which was previously implicated in cell-cell adhesion of another human cell type (40). A three-dimensional culture system was used with a pooled approach to identify mouse genes involved in metastasis. Among these genes was *Gas1*; the human homolog of *Gas1* is frequently downregulated in human metastatic melanoma cells and tumor samples (41).

Mitochondria and mitochondrial disease. Image-based RNAi HTSs in *Drosophila* cells identified a protein required for mitochondrial fission in *Drosophila* and mammalian cells, Mff (137). A luciferase reporter of citrate synthase (CS) activity was useful to identify genes subsequently confirmed to have in vivo relevance to mitochondrial CS activity: *barren*, *CG3249*, *HDAC6*, *klumpfuss*, *Rpd3* (*HDAC1*), *smt3*, *Src42A*, and *vimar* (138). Small-scale RNAi and overexpression screening with the subset of human genes that encode E3 ubiquitin ligases identified the mitochondrial MULAN (90). Another study that integrated multiple approaches (namely, combining mass spectrometry and bioinformatics to identify genes that were subsequently assayed via small-scale RNAi) identified mouse *C8orf38*, which is associated with an early lethal complex I-linked inherited human disease (93).

Other cell biological processes. A number of screens in *Drosophila* and mammalian cells have looked at additional aspects of cellular and molecular biology. For example, cells stably expressing a signal sequence–fused horseradish

peroxidase were used to look for components required for protein secretion; four candidate proteins were shown to localize to the Golgi apparatus and seven to the endoplasmic reticulum (ER), and together the results led to identification of a number of previously uncharacterized “TANGO” genes (named for transport and Golgi organization) that should be interesting for further study (139). Image analysis was used in a genome-wide study of lipid droplets in *Drosophila* cells that implicated coat protein complex I (COPI) proteins also implicated in the Bard et al. study (139) and in host responses to infection (83). An RNAi HTS, with a set of 308 candidates identified in expression and literature analyses, led to identification of 20 genes implicated in homeostasis of cellular cholesterol levels, including *TMEM97* (89). Again in fly cells, full-genome RNAi HTS studies have identified genes involved in E2F repression, including *domino* (140), and genes required for SUMO-dependent transcriptional repression, which implicated a protein complex that includes MEP-1, Mi-2, and Sfm1b (141). Additionally, a recent genome-wide study in human melanocytes implicated a number of genes, including genes involved in tyrosinase expression and stability and in melanogenesis, which has relevance to a number of different human disorders (142).

Signal transduction. A number of screens that interrogated signal transduction have led to novel findings, including (a) identification of *Slpr2* in a screen for insulin signaling in adipocytes (143); (b) extensive analysis of pathways that intersect with JNK (49); (c) identification of novel components of the ERK (Map kinase) signaling pathway (144); (d) identification of kinase and phosphatase requirements in FOXO transcription factor regulation, which implicate protein kinase C (PKC) and glycogen synthase kinase 3 β in insulin signaling (145); and (e) identification of *Drosophila mole-skine* (*msk*) and implication of mammalian homologs of *msk* in TGF- β signaling (145a). Additionally, there have been at least three studies of Hedgehog (Hh) signaling, which implicate

Dally-like protein (Dlp), PP2A, Cdc21I, casein kinase 1 α , and other kinases in aspects of Hh signaling (146–148); at least two studies of the JAK/STAT pathway (reviewed in References 84 and 149); and a large number of studies of the Wnt signal transduction pathway (reviewed in References 150 and 151).

Signal transduction: Wnt pathway. The Wnt pathway screen results are interesting in that they show that repeated RNAi HTS-based investigation of a pathway can continue to yield new and relevant findings. An early full-genome screen in *Drosophila* identified several candidates for Wnt regulation (152), and a screen focused on *Drosophila* transmembrane protein-encoding genes identified a conserved factor involved in Wnt secretion encoded by *evenness interrupted* (*evi*) (153). Several subsequent studies identified proteins required for subcellular localization of Wnt pathway components. A visual screen to detect disruption of the normal membrane localization of Dishevelled (Dsh) revealed the importance of pH and charge in recruitment of Dsh by Frizzled (154). And a screen for negative regulators of Wnt signaling identified Bili, which inhibits recruitment of Axin to Lrp6 during Wnt pathway activation (155). An arrayed screen for Wnt-related factors in mammalian cells revealed a link between transcription factor 7-like 2 and colorectal cancer (156). More recently, the results of concurrent small-molecule and RNAi screens in colorectal cancer cells identified Bruton’s tyrosine kinase as an inhibitor of Wnt signaling that binds to the Wnt pathway component CDC73 (91). Additionally, a highly integrated and validated HTS RNAi screen in human DLD1 colon cancer cells led to identification of the nuclear chromatin-associated protein AGGF1, which has previously been implicated in human disease and was shown to be involved in β -catenin-mediated transcription in colon cancer cells (50).

RNA biology: RNAi. In an interesting overlap of experimental approach and biological topic, several cell-based RNAi HTSs have

focused on RNA biology, including the study of RNAi mechanisms. Screens *in vivo* in *C. elegans* made early and notable contributions to this field, but screens in other systems have also contributed to our rapidly growing understanding of the mechanisms and control of RNAi, miRNAs, and other aspects of RNA biology. For example, studies in *Drosophila* cells include a screen for components required for uptake of dsRNA into cells, which revealed evolutionary conservation of the relevant genes in *C. elegans* (157), and a screen for factors that disrupt RNAi knockdown identified five previously described genes, including *AGO2* and *Hsc70-73* and two genes of unknown function (*CG17625* and *CG10883*) (158). A screen in *Drosophila* cells that focused on dissection of the miRNA pathways identified the conserved P-body component Ge-1 (159). Furthermore, multiple Argonaut-dependent pathways were interrogated in another screen, resulting in a new understanding of components shared among the pathways, with 54 genes in common for the siRNA, endo-siRNA, and miRNA pathways (160).

RNA biology: mRNA export and pre-mRNAs. Looking at different aspects of RNA biology, another *Drosophila* RNAi HTS addressed nuclear export of mRNA. The *Drosophila* PCI domain-containing protein (PCID2), which interacts with polysomes, was identified in the screen, and more generally, the list of genes identified in the screen emphasizes the links between mRNA export and other processes, including translation (161). A specific look at histone premRNAs in a *Drosophila* genome-wide RNAi HTSs using a low-content imaging approach identified factors previously known to be involved in mRNA cleavage and/or polyadenylation, i.e., zinc finger domain-containing and signaling genes as well as the histone variants H2Av and H3.3A/B (162).

Innate immunity and host-pathogen interactions. Innate immunity has been the focus of at least two RNAi HTSs in *Drosophila* screens,

leading to the identification of *LAP2*, implicating the IAP family in nonapoptotic pathways (163); a new protein with IAP-like functions, Defense repressor 1; and the conserved gene *sickie*, required for Relish activation (164). Perhaps the largest category of RNAi HTSs in *Drosophila* and mammalian cells is for investigations of host cell responses to infection by viral, bacterial, fungal, or other pathogens (recently reviewed in References 8 and 165). In these screens, researchers typically treated a responsive cell type with a pathogen, followed by detection of a readout related to the pathogen and/or the host cell response, such as changes in the extent of localization, internalization, or proliferation of the pathogen; host cell death; or a specific host cell transcriptional or other response to infection. Screening in *Drosophila* cells has the advantage of being relevant not only to the conserved aspects of human host cell response but also to the responses of other dipterans (e.g., biting flies and mosquitoes) that act as disease vectors (reviewed in References 8, 165, 166). And screening directly in human cells is beginning to provide insights into significant threats to human health such as HIV (47, 78–80).

Responses to bacterial pathogens. Early studies in *Drosophila* cells looked at host-pathogen interactions relevant to bacterial infection. The findings included identification of Peste, a CD36 family protein involved in uptake into a *Drosophila* macrophage-like cell type; implicated the ESCRT (endosomal sorting complex required for transport) machinery in infection by mycobacteria (167, 168); and identified several candidates for interaction with *Listeria* (169). The latter study was followed up by RNAi in human cells, using a small library of RNAi reagents targeting putative vesicle trafficking genes; among the findings were 18 genes involved in vesicular trafficking of *Listeria* in a manner independent of listeriolysin O (170). RNAi interrogation of the human kinome implicated the AKT1 pathway in growth of the bacterial pathogens *Salmonella typhimurium* and *Mycobacterium*

tuberculosis (171). Simultaneous RNAi knockdown of membrane trafficking components or knockdown of single components involved in ER-associated degradation affected *Legionella* replication in *Drosophila* cells (172). Genome-wide RNAi HTSs in *Drosophila* cells with another human disease-relevant infectious agent, *Chlamydia*, uncovered the importance of the multiprotein Tim-Tom complex, which is required for import of nuclear-encoded proteins into the mitochondria, in both *Drosophila* and mammalian cells (173).

Responses to other pathogens. Of course, RNAi HTSs have not been limited to bacterial pathogens. RNAi HTS interrogation of host cell-virus interactions has been the focus of several studies (see below). Additionally, RNAi HTSs in *Drosophila* cells demonstrated the feasibility of RNAi HTSs with the human fungal pathogen, *Candida albicans* (174). In addition, a kinome-wide RNAi HTS in human hepatoma cells was done to study the eukaryotic disease agent of malaria, *Plasmodium*. Following identification of candidates in the screen, candidates were tested in vivo by RNAi in mice, and the results support the idea that PKC ζ is involved in invasion of hepatocytes by *Plasmodium* sporozoites (175).

Viral pathogen studies in *Drosophila* cells. RNAi HTSs in *Drosophila* have contributed to our general understanding of host cell responses to infection by viruses and have led to identification of specific factors. For example, an early screen utilizing an internal ribosome entry site (IRES)-containing virus, *Drosophila* C virus, implicated a large number of *Drosophila* ribosomal proteins in *Drosophila* C virus growth, a finding shown to have implications for treatment and for human infection by the IRES-containing poliovirus (176). Recently, *Ars2* (for arsenite resistance gene 2) was identified as a key component required for viral immunity in *Drosophila* cells (177). A modified influenza virus that can infect *Drosophila* cells was similarly used in a full-genome study to look at the influenza life cycle in host

cells, and an extension of the results to human cells demonstrated the relevance of results with human homologs of several genes identified in the screen, including *ATP6V0D1*, *COX6A1*, and *NXF1* (178). Relevance to both human cells and a dipteran mosquito vector of human dengue virus (DENV), *Aedes aegypti*, was demonstrated after a full-genome screen with a DENV in *Drosophila* cells; specifically, 42 of the 82 genes identified in the *Drosophila* screen for which human homologs could be identified also acted as host factors for DENV in human cells (37). Additional studies have implicated the COPI coatmer in both viral replication and lipid homeostasis (82, 179). Thus, screening in *Drosophila* cells has rich potential for informing our understanding of general processes and specific genes involved in the interaction of viruses with their insect and human hosts, with likely implications for the development of therapeutic treatments.

Viral pathogen studies in mammalian cells.

Recent studies in human cells have looked at infections with West Nile virus (180) and hepatitis C virus (181, 182), and at least four studies have focused on HIV (47, 78–80). For West Nile virus, researchers identified roles in various stages of infection or replication for the ubiquitin ligase CBLL1 and the monocarboxylic acid transporter MCT4, and this research implicated the ERAD pathway for transport of misfolded pathways previously implicated in the flavivirus life cycle (180). The COPI and PI4KA were implicated in a genome-wide RNAi HTS for factors involved in hepatitis C virus replication (181). The results of RNAi HTSs with HIV are at once encouraging and cautionary. On the one hand, taken together, the results of the screens implicate common activities and pathways with putative roles in various aspects of HIV infection, including genes involved in nuclear transport, GTP binding, and protein complex assembly, and point to a large number of potential therapeutic targets (47, 78–81). On the other hand, the degree of overlap among the screens is fairly modest at the gene level (81). This may reflect various sources of

false discovery, including screen noise, timing of the assays, and methods of analysis (81), and shows that even large-scale screens fail to identify all genes required for a given process (i.e., the screens are not saturating). It also emphasizes the need to compare data among related screens and to combine standard RNAi HTSs with additional approaches, particularly when the goal of a study is to gain a systems-wide understanding of gene functions relevant to a given biological process or behavior.

COMBINATORIAL AND SENSITIZED SCREENS TO REVEAL FUNCTION

Combining multiple screening strategies provides an excellent means to identify genes that are relevant to particular cellular behaviors that may have been missed in single RNAi HTS assays (Figure 1*d-f*). For example, the same assay can be performed iteratively in the same cell type in a sensitized background (e.g., in which another gene has been targeted by RNAi or overexpressed in all cells); under different environmental conditions (e.g., via exposure of cells to a small-molecule inhibitor); or across different cell types (which can be compared with screening in very different genetic backgrounds). Such screens are conceptually similar to studies in yeast, whereby the viability of gene deletions has been tested on backgrounds where other genes have been deleted (synthetic lethality) or overexpressed, and in the presence of various small molecules. Indeed, systematic analysis of gene pairs has revealed that the number of gene pairs required for viability far exceeds the number of single genes that are essential (reviewed in Reference 183), suggesting that taking a similar approach in RNAi HTSs will help identify many new pairs or sets of genes with related functions.

Many groups have begun to utilize combinatorial approaches in cell-based RNAi screens, not only to comprehensively describe the components of particular signaling networks but also to attempt to understand how the pheno-

typic output of a signaling molecule or network is dependent on the surrounding context. For example, how activation of molecules, such as Ras, ERK, JNK, and PI3K, can lead to proliferation in one cell type but to differentiation, migration, or apoptosis in others is unclear. To this end, Bakal et al. (49) recently implemented a combinatorial approach to describe genes involved in the regulation of JNK activity across different genetic backgrounds. In that study, JNK activity was monitored in live *Drosophila* cells using a fluorescence resonance energy transfer-based reporter. Systematic targeting of all *Drosophila* kinases and phosphatases individually by RNAi resulted in the isolation of 24 genes that regulate JNK activity in normal growth conditions (5% of genes tested). However, by performing the same screen 12 subsequent times in the presence of a second RNAi, which targeted a particular “query” gene, 55 more kinase and phosphatase regulators of JNK activity (17% of genes tested) were identified. A striking aspect to these screens is the difference in both the number and identity of the genes that were isolated in any given condition. For example, in that study, although 11 genes were identified as JNK suppressors following single RNAi, 17 enhancer genes were isolated in *Rac1*-deficient cells, 54 in *slpr/MLK* deficient cells, and 3 in *hippo* deficient cells. Similarly, combinatorial and integrative approaches to study of the ERBB network in cancer cells have been discussed recently by Sahin and colleagues (184, 185). The results of these studies dramatically illustrate how the results of a single RNAi screen are dependent on genetic context. Even inhibition of a single additional gene in the same cell line can significantly alter the final hit list of an RNAi screen. This suggests that the results from any screen in a specific cell type must be interpreted with the caveats that the genes identified could be highly specific to the cell type that was screened and that multiple cell types should be screened iteratively in order to comprehensively identify genes involved in specific cellular behaviors.

Genes that are repeatedly isolated in many screens are likely to correspond to the core regulators of a particular process. For example, the JNK phosphatase-encoding gene *puckered* (186) was repeatedly isolated in more than one *Drosophila* screen (including in *Rac1*-, *MLK*-, and *hippo*-deficient cells), reflecting the central role of JNK phosphatase as a JNK regulator in the majority of tissues and organisms studied to date. The isolation of genes only in specific backgrounds should provide insight into how genetic interactions modulate phenotypic outputs. Similar observations have been made in recent screens for regulators of ERK activation downstream of epidermal growth factor activation, where a genome-wide screen in the Kc cell line isolated 1405 genes, the identical screen in the S2R+ line isolated 1101 genes, and 422 genes were common to both screens (144). Another example is from a recent screen for genes required for the viability of four different cancer lines, which identified 1057 genes across the lines, revealing both a core of 23 genes required for viability across lines, as well as genes uniquely required for viability in each case (44). Thus, iterative screening is essential to understand how regulatory networks are capable of dynamically rewiring to maintain cellular function in the face of large genetic and environmental fluctuations and how subtle sequence variations lead to overt phenotypic differences.

CONCLUDING REMARKS AND PERSPECTIVES

As demonstrated by the various findings described above, the application of RNAi HTSs to gene discovery has been extremely successful in interrogating important biological questions. Collectively, the results of RNAi HTSs reaffirm the ideas that many gene networks are involved in a given process (e.g., cell viability, signal transduction, host-pathogen interactions) and that the various pathways and complexes represented in screen results are highly interlinked and interdependent. Both the issue

of false discovery and the complexity of biological systems point to the importance of limiting false discovery rates. Improving RNAi reagent libraries, particularly for mammalian cell screens, is one important direction to follow toward limiting false discovery. However, there is no substitute for verification of results. Toward that goal, the development of improved, high-throughput methodologies for verification of RNAi results at the RNAi reagent and gene level, as well as for validation of RNAi results *in vivo*, will also be of significant benefit. Undoubtedly, the results of RNAi HTSs will continue to provide important contributions to biology and biomedicine in the future, as researchers both generate new screen data and perform reanalyses and meta-analyses of existing screen datasets, either alone or in conjunction with other studies. Although much of what has been learned is currently understood only at the level of individual genes or small functional networks or complexes, the scale and scope of studies made possible by RNAi HTSs, along with the emergence and refinement of other genomic, transcriptomic, and proteomic techniques, should also allow us to move toward system-wide understandings of gene networks in an increasing number of specific cell types, tissues, and organisms. In addition, RNAi screening may facilitate a shift toward genome-scale investigation in a larger number of different species, and in disease-related or similar studies, in the specific species of interest (or at the least, a closely related species) rather than in a distantly related model system. This is likely to be particularly true for organisms of specific industrial, agricultural, and biomedical importance. Thus, one can speculate that additional genome-scale collections may be generated in the future, such as for fungi of agricultural and industrial relevance (reviewed in Reference 18), parasitic nematodes (reviewed in Reference 187), and dipteran vectors of human disease [i.e., biting flies and mosquitoes (reviewed in References 165, 188, 189)].

SUMMARY POINTS

1. RNAi has emerged as a powerful method for genome-scale interrogation of gene function in a number of traditional and emerging model systems, including but not limited to *Drosophila* and mammalian cells.
2. The results of RNAi high-throughput screens (HTSs) are acutely sensitive to assay design and are subject to significant rates of false discovery, which can be addressed using various statistical, bioinformatics, and experimental approaches.
3. For high-throughput cell-based RNAi screens in *Drosophila* and human cells, primary hits (positive results) can be verified in high- or moderate-throughput modes to confirm the RNAi result; however, validation of the biological relevance of a given finding (i.e., via methods other than RNAi) remains principally a low-throughput process that would benefit from development of new technologies.
4. The results of high-throughput cell-based RNAi screens have led to discoveries in a wide variety of fields, with particular impacts in signal transduction, general cell biology, RNA biology, cancer biology, and host cell responses to infectious pathogens.

FUTURE ISSUES

1. Improved gene models, large-scale experimental verification of mRNA knockdown, new insights into effective RNAi reagent design, and the subsequent building of better genome-scale RNAi libraries are needed to support more effective RNAi screens.
2. Improved methods for RNAi reagent delivery, such as for difficult-to-transfect mammalian cell types, would open the door to screening in additional cellular contexts.
3. Continued efforts at standardization of data reporting, access to information about reagent design and efficacy, and availability of raw data from RNAi HTSs, along with the subsequent improved bioinformatics-based analyses would likely help inform our understanding of RNAi results.
4. New methods for large-scale verification and validation of screen results should be developed, and existing methods should be improved, made more affordable, and include development of genome-scale libraries for rescue experiments.
5. Screening pipelines should incorporate innovative, integrative, and multiplexed approaches, such as via concurrent RNAi and overexpression screens; screening of multiple cell lines, pathogens, drug treatments and/or assays; and capture of multiple assay or image readouts per screen.
6. Integration of high-quality results from other high-throughput datasets (e.g., microarray analysis, protein-protein interaction studies, genomic analyses) is needed to maximize the power of high-throughput approaches and gain a system-wide understanding of gene networks involved in various processes, events, and behaviors.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We are grateful to Matthew Booker and Dr. Caroline Shamu for thoughtful comments on the manuscript. S.E.M. is supported by R01 GM067761 from the National Institute of General Medical Sciences at the National Institutes of Health. C.B. was a Fellow of the Leukemia and Lymphoma Society and is currently at the Institute of Cancer Research. N.P. is an investigator of the Howard Hughes Medical Institute.

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