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Genome-wide high-throughput screens in functional genomics

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The availability of complete genome sequences from many organisms has yielded the ability to perform high-throughput, genome-wide screens of gene function. Within the past year, rapid advances have been made towards this goal in many major model systems, including yeast, worms, flies, and mammals. Yeast genome-wide screens have taken advantage of libraries of deletion strains, but RNA-interference has been used in other organisms to knockdown gene function. Examples of recent large-scale functional genetic screens include drug-target identification in yeast, regulators of fat accumulation in worms, growth and viability in flies, and proteasome-mediated degradation in mammalian cells. Within the next five years, such screens are likely to lead to annotation of function of most genes across multiple organisms. Integration of such data with other genomic approaches will extend our understanding of cellular networks.

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Abbreviations

dsRNA	Double-stranded RNA
HTS	High-throughout Screen
RNAi	RNA Interference
shRNA	Short hairpin RNA
siRNA	Short interfering RNA

Introduction

Geneticists assign biological functions to genes by manipulating the genetic materials of model organisms, such as yeast, worms, flies and mice. This has historically taken place within the paradigm of forward genetic screens, in which genomes are semi-randomly mutated and phenotypes of interest are scored. This approach has been enormously successful in identifying thousands of genes that are related to all aspects of organism biology, from basic cellular machinery of growth and differentiation, to behavior and lifespan. Importantly, the task of identifying the genes themselves has become increasingly faster over the years, because of the development of various screening and molecular mapping techniques (reviewed in [1]). Regardless of the success of the forward genetic approach to functional genomics, the field is experiencing a paradigm shift, due to the recent availability of the full genome sequences of many organisms. This information, when combined with powerful new technologies to knock out gene function, has led to the development of several high-throughput approaches to functional genomics. Here, we describe the most recent advances in high-throughput screens (HTS) in functional genomics (see Table 1 for summary).

Yeasts and worms lead the pack

Gene function can be inferred by removing a particular gene from an organism or cell and observing the behavior or phenotype. Performing this in a systematic, genome-wide manner is possible, in theory, now that full genomes have been sequenced. However, it is only in the budding yeast *Saccharomyces cerevisiae* that deletion of every gene has been technically feasible, as a result of the high efficiency of homologous recombination and small genome size.

S. cerevisiae has traditionally been extremely useful for the study of many conserved cellular functions. This has now been accelerated by the availability of a gene deletion library of approximately 96% of known open reading frames [2,3]. Determination of the role of a gene was accelerated by pooling deletion strains and tracking their abundance under competitive growth conditions (e.g. different types of media) using genetic 'barcodes' that are included on each strain [2]. In addition, other phenotypes, such as cell shape [2] or centromere cohesion [4], have been screened individually through microscopy (see also Update). Furthermore, this library was used to construct 'synthetic genetic arrays', which allow systematic analysis of double-mutant phenotypes with a particular gene of interest, for example, synthetic lethality [5]. The library can also be used to address biological problems other than initial gene annotation, such as identifying putative drug targets. Several recent studies have taken advantage of the differential growth response of deletion strains to a particular drug, and followed their relative abundance using the barcodes [6,7•,8].

Many biological processes, such as intercellular signaling cascades, are not conserved in yeast; hence, the exploitation of animal genetic model systems. Although there are efforts underway to develop mutations in every gene, for

Table 1

Genome-wide functional genetic screens.

	Organism			
	Yeast (*S. cerevisiae*)	Worms (*C. elegans*)	Flies (*Drosophila*)	Mammals (human and mouse cells)
Knockdown methodology	Gene deletion	Long dsRNA	Long dsRNA	siRNA, shRNA
Knockdown introduction	Strains previously constructed by homologous recombination	Feeding bacteria Soaking Injection	Cell bathing Cell transfection Embryo injection	Cell transfection Cell infection
Recent assays	Centromere cohesion Drug target identification	Embryonic lethality Fat accumulation Transposon silencing	Growth and viability Cell morphology Hedgehog signaling Cardiogenesis	p53 signaling Proteasome-mediated degradation NFκB
References	[2,4,6,7•,8]	[23••,25,26]	[33•,36,37•,38••]	[42••–44••]

example, in the worm *Caenorhabditis elegans* (see *C. elegans* Gene Knockout Consortium; http://celeganskoconsortium.omrf.org/) or the fruitfly *Drosophila melanogaster* ([9,10]; Berkeley Drosophila Genome Project: Gene Disruption Project, http://www.bdgp.org/p_disrupt/index.html.), this process is incomplete and screening of these mutants in HTS may be laborious and inefficient. Within the past five years, however, RNA-mediated interference (RNAi), which allows sequence-specific degradation of gene transcript using double-stranded RNA (dsRNA; see [11,12] for review) in *Drosophila* and *C. elegans*, has opened up the possibility of performing loss-of-function screens with every known gene in a high-throughput and unbiased manner. Briefly, long stretches of dsRNA that are complementary to a given transcript, when introduced into cells or organisms, are digested into 21–23-nucleotide (nt) sequences by the Dicer family of ribonucleases; these short-interfering RNAs (siRNAs) are then targeted to the gene of interest through the RNAi-induced silencing complex, which leads to transcript degradation [11,12]. Since its introduction, RNAi technology has been adapted to full-genome HTS rapidly. As the process was originally described in worms [13], genome-wide screening was first developed in this organism.

Double-stranded RNA can be introduced in the worm through a variety of mechanisms, including injection, soaking, feeding bacteria expressing the dsRNA, or insertion of a transgene expressing a hairpin (inverted repeat) of the dsRNA (reviewed in [14]). The first three techniques have all been adapted to large-scale RNAi screens. Injection of dsRNA can be time-consuming but often generates more penetrant phenotypes [14]. This approach has been used systematically in the analysis of cell-division phenotypes of genes on the *C. elegans* third chromosome [15], and in the embryonic phenotype of genes that are expressed in the parental ovary [16], both using time-lapse video microscopy. Soaking of worms in dsRNA directed towards ~50% of predicted *C. elegans* genes has also been used to uncover phenotypic classes [17]; this effort used a cDNA library to produce dsRNA rather than gene predictions (as discussed below). Although this method might thus miss less abundant transcripts, it only includes *bona fide* expressed genes.

The availability of the complete annotated worm genome has also allowed entire classes of genes to be systematically identified. This has successfully been employed in screens for effects of RNAi to predicted G protein-coupled receptors on locomotion and reproduction [18], and to predicted DNases, ribonucleases, cyclophilins and topoisomerases on apoptotic DNA degradation [19]. Class-based screens allow investigators to focus on their genes of interest, while avoiding the time and expense of full-genome screening.

The goal of the full-genome RNAi functional genetic screens in *C. elegans* was recently realized through the efforts of Kamath, Fraser, Ahringer and colleagues [20,21••], who have developed a publicly available, full-genome (86%) collection of bacteria that can be induced to express each dsRNA and can then be fed to worms. This feeding library was originally described in a screen of general phenotypes for nearly every gene of the *C. elegans* first chromosome [22], but within the past year the 'full-genome' description of phenotypes has been published [21••]. The authors scored several classes of phenotypes in parents and their progeny, such as sterility, embryonic lethality, growth retardation or other post-embryonic effects. Roughly 10% of screened genes generated phenotypes, for which only one-third had a known phenotype. This work demonstrated the power and potential of genome-wide functional screening in both ability to assign function to new genes and speed of identification.

Different screen assays will necessarily identify different subsets of genes, as the recent use of this feeding library has demonstrated. Transposon silencing, protection from mutation, polyglutamine aggregation and fat aggregation assays have all successfully identified known and unknown members of these processes [23–26]. In addition, the original large-scale RNAi datasets of embryonic lethal genes have been sub-screened, in an attempt to look for

specific defects in pronuclear migration [27]. These studies demonstrate the wide range of possible readouts of gene function, dependent on the perspectives of the individual investigators.

Flies and mammals go high-throughput

The development of full-genome functional screens in *C. elegans* has proceeded from initial identification of RNAi effects on small groups of genes, to proof-of-principle large-scale screens, and finally to full-genome, systematic, high-throughput functional genetic screens. Similar steps have been made within the past year in *Drosophila* and mammals. Unlike *C. elegans*, for which phenotypes are scored in the entire organism and are thus automatically 'biologically relevant', most of the efforts in both of these systems seek phenotypes in cell populations. Although these approaches cannot address multicellular developmental processes, many of these pathways can nevertheless be screened in cells with the appropriate reporters. However, rigorous *in vivo* validation is essential to confirm the relevance of RNAi phenotypes. For this reason, *Drosophila* are particularly useful as a model system: unlike those using worms, which do not have established cell-culture techniques, fly cell-based screens can be performed quickly, and, unlike those of mammalian cells, potential hits can be quickly validated in flies *in vivo* using large mutant collections and other fly genetic tools.

The possibility of using dsRNA to inactivate genes in *Drosophila* cells in culture was first explored several years ago [28]. Following these initial successes, multiple groups have subsequently demonstrated effects of dsRNA to a limited number of known genes on, for example, cytokinesis [29,30]. *Drosophila* cell lines can be either transfected with the dsRNA of interest or, in many lines, more conveniently bathed in medium with the dsRNA. Although some cell lines are refractory to dsRNA introduction through soaking and must be transfected [31•], the recent discovery of the SID-1 dsRNA transporter in *C. elegans* has suggested that ectopic expression of this protein in these cells permits dsRNA uptake from the medium [32].

Partial-genome RNAi screens in *Drosophila* were reported recently. The first of these, conducted by Ramet and coworkers [33], screened 1000 dsRNAs, synthesized from a random cDNA library, for effects on phagocytosis in S2 cells; they identified a key receptor for Gram-negative bacterial recognition in *Drosophila*. Lum and colleagues [31•] sought genes that are involved in the regulation of the Hedgehog (Hh) pathway, one of the major signaling cascades involved in animal development and disease. Using a collection of 43% of the fly genome, the authors identified several genes that resulted in reduced activity of a luciferase Hh transcriptional reporter. In a contrasting approach, Kiger *et al.* [34] used automated microscopy to score for morphological phenotypes among approximately 1000 signaling and cytoskeletal components in two different cell types. By developing a complex phenotype annotation method, the authors could then assign phenotypic signatures (or 'phenoprint') to groups of genes, presumably implying functional relationships. This type of 'phenotypic clustering' is similar to the transcriptional profiling of microarray data (see also Update). Finally, as in *C. elegans*, *Drosophila* embryos can be directly injected with dsRNA to score for *in vivo* phenotypes; a method that is useful for both cell-based screen hit validation and, although more laborious, direct screening. This has been demonstrated in a large-scale screen of 5800 genes for effects on cardiogenesis [35•].

Following these large-scale screens, the first full-genome functional RNAi screen in *Drosophila* has recently been published. With a collection of dsRNAs directed towards 91% of the predicted fly genome, Boutros and co-workers [36••] analyzed cell growth and viability using a luminescent readout of cellular ATP levels. A total of 438 dsRNAs resulted in a phenotype in duplicate screens, suggesting that these genes fulfill essential cellular functions. Importantly, 80% of these had not been identified from previous classical genetic screens. As with the first *C. elegans* full-genome screen, this report outlines the potential of using *Drosophila* cells to assay gene function across the entire genome.

Mammalian genetics has long relied on spontaneous mutations or the laborious process of knockout and transgenic methodology in mice to analyze gene function. RNAi initially appeared to be out of reach for functional analyses in mammalian cells. In contrast to flies and worms, the introduction of long dsRNA in mammalian cells induces a non-sequence-specific interferon response and shutdown of translation (reviewed in [11]); however, it was subsequently discovered that this response can be bypassed by the direct introduction of Dicer products, siRNAs of 21–23nt in length (mentioned earlier). Considering the enormous potential of this technology for understanding gene function in mammals, as well as therapeutic promise, there has been much effort towards understanding the best method for introducing siRNAs into cells and the ideal targeting region within the gene [11,37,38]. siRNA is commonly introduced through transfection of chemically synthesized siRNA or vectors expressing short hairpin RNAs (shRNAs), under the control of RNA polymerase III promoters.

Initial efforts in mammalian cells used smaller libraries that demonstrated the potential of high-throughput RNAi screening. One of the first large-scale siRNA screens in mammalian cells used a library of 510 genes to identify modulators of TRAIL (TNF-related apoptosis-inducing ligand) [39]. For this screen, individual siRNAs were transfected in a multiwell format into HeLa cells, and assayed for apoptosis using a vital dye.

Three recent reports of large-scale RNAi screens in mammalian cells demonstrate progress towards the full-genome functional screen. The first of these has used transfection of vectors expressing short hairpin (sh)RNA directed towards 8000 individual genes [40••]; due to the partially unknown rules for siRNA potency, they used two shRNAs per gene. The authors identified several known and unknown regulators of NFκB luciferase reporter activation in response to TNF-α stimulation. The remaining two reports used retrovirus-based shRNA expression constructs for approximately 15 000 genes (10 000 human and 5000 mouse) and 8000 human genes, respectively [41••,42••]. Both groups used multiple shRNAs to each gene to maximize the knockdown probability. Paddison and colleagues [41••] tested their library to identify regulators of proteasome function; Berns and colleagues [42••] screened for modulators of p53-induced growth arrest. Following the success of yeast 'barcodes', both groups also used different barcoding strategies to track multiple shRNAs in pooled populations.

Problems and perspectives

The expected improvement, in the near future, of algorithms detailing the precise region within a given gene for targeting, as well as a better efficiency for RNAi delivery will permit the realization of full-genome RNAi screens in mammalian cells. Particularly important to clarify are the discoveries of 'off-target' responses. These include partially sequence-specific [43••] and non-sequence-specific interferon-based responses that can even be generated from siRNAs (both chemically synthesized and transfected in shRNA vectors) [44,45]. Recent reports suggest that particular 5′ modifications of siRNAs can circumvent both of these problems [46,47]. Although off-target responses were not addressed in the recent screening papers [40••,41••,42••], a phenotype produced by any siRNA in mammalian cells must be rigorously verified.

All RNAi-based functional screens suffer from the well-known drawbacks of the variable penetrance of the technology. The degree of transcript knock-down varies according to the cell type and gene of interest, leading to significant percentages of false negatives [48]. For example, *C. elegans* neurons appear to be particularly resistant to dsRNA-mediated knockdown, although the discovery of *rrf-3* mutants (of the RNA-dependent RNA polymerase family), which can sensitize worms to the effects of RNAi, might alleviate some of these false negatives [48,49]. Also, RNAi knockdown, unlike random mutagenesis, cannot generate temperature-sensitive or gain-of-function mutants; however, overexpression of genes in a systematic manner to generate gain-of-function phenotypes might be able to address this [50•], complementing loss-of-function screens. Another issue to address is whether pooling dsRNA or siRNA, as performed in several screens [31•,35•,41••,42••], is appropriate for all assays, particularly those that seek quantitative information on gene contribution to pathways on the genome level.

The central component of any HTS is the assay itself, which must be robust, accurate and have a high signal: noise ratio and dynamic range. It is clear from the early screens that a tremendous number of potential assays might be possible, particularly in cell-based approaches. These can rely on the significant technological improvements in multiwell plate readers and in automated microscopy and imaging algorithms. Given the investment that is required for each screen, 'multiplexing' assays with readouts of multiple variables and internal standardizations may improve the through-put and specificity of any given assay. Standardizing the output nomenclature, such as phenotypic annotation in *C. elegans* or *Drosophila* microscopy [34,51], will facilitate cross-assay and cross-species comparisons. Recent developments towards printing slides with whole-genome dsRNA, siRNA or cDNA collections and 'reverse transfecting' cells onto them will miniaturize the screening format, accelerating the screening process [52] (reviewed in [53]).

As the number of screens in multiple organisms increases rapidly, robust statistical tools are needed to generate inter- and intra-screen conclusions. This will be aided by the ability to perform many cell-based screens quickly and in replicate. Furthermore, genome-wide screens have the advantage that, in addition to individual gene annotations, they can, by their nature, generate conclusions on the genome level, such as chromosomal location of hits, implying evolutionary patterns in gene clustering, and contributions of classes of genes to particular phenotypes (e.g. [21••]). Understanding these results and comparing across organisms and assays will be a formidable challenge.

Considerable time and resources must be invested in generating the libraries of RNAi. Consequently, only a few such libraries will be available, making their public availability, either through distributable kits or centralized screening centers, essential. Thus, investigators with disparate interests can screen in their system of interest. Paradigms of this are the distributable gene-deletion kits in yeast and *C. elegans* feeding library (*Saccharomyces* Genome Deletion Project, http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html; *C. elegans* RNAi library, http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/Celegans.shtml).

In *Drosophila*, several libraries are now available (Open Biosystems, http://www.openbiosystems.com/index.php; Eurogentec, http://www.eurogentec.be/). In addition, we have established a *Drosophila* RNAi Screening Center, to which researchers can apply to screen for their pathway of interest (Drosophila RNAi Screening Center, http://www.flyrnai.org). Making the data from screens publicly

available will permit validation of gene lists and data-mining by investigators with different perspectives; the early *C. elegans* efforts demonstrate this potential [54].

Making data publicly accessible will also hasten the convergence of diverse genomic approaches. It is clear that combining functional genomics with proteomics, expression genomics and chemical genetics will expand the conclusions of any single assay or approach (reviewed in [55,56]). For example, within the past year, three different genomic efforts have investigated the p53 signaling pathway: global gene profiling [57], large-scale RNAi-mediated knockdown [42••], and large-scale cDNA-based overexpression [50]. These separate approaches have all helped in deciphering the complex regulation of this pathway, but combining the datasets through novel computational algorithms might lead to more accurate network modeling and the prediction of system behavior. This approach towards understanding 'systems-level' information through genomics has recently been shown to be valid in *E. coli* metabolic pathway modeling [58]. Combining '-omic' approaches in practice, on a smaller scale, has also been successful in *C. elegans* and *S. cerevisiae*: candidates in protein–protein interaction maps of TGF (transforming growth factor)-β signaling in worms have been validated with systematic RNAi in various genetic backgrounds [59•]; germline-enriched genes were explored with protein–protein interaction, expression profiling and RNAi phenotypes in worms [60]; and chemical genetic and genetic interaction datasets were combined to suggest drug targets in yeast [61].

Conclusions

We can view full-genome functional genetic screens within the appropriate historical context of their predecessor, the traditional forward genetic screen. In *Drosophila*, for example, the first saturation mutagenesis screens analyzed simple, easily scored phenotypes, such as female sterility or embryonic lethal phenotypes, from zygotic or maternally-contributed genes (reviewed in [1]). As different phenotypes and gene classes were sought, screen complexity increased, leading to suppression/enhancement, clonal and overexpression analyses [1]. We anticipate that high-throughput functional reverse genetic screens will take a similar path. Initial screens will focus on simple phenotypes, such as cell viability [36••], morphology [34] or single signaling pathways [31•]. Screens within the next few years will build increasing layers of complexity in the uncovering of novel gene functions, by multiplexing assays with advances in automated microscopy, imaging, miniaturization and detection systems.

Update

Recent work has expanded the universe of genome-wide screens. For example, in *Drosophila*, a library of dsRNA to 7216 conserved genes has been developed and screened for regulators of innate immunity in S2 cells, using a microscopy-based assay [62]. The library of yeast haploid mutant strains has yielded genes that are involved in regulation of telomere length [63]. Lastly, Hartman and Tippery [64] recently described methods for understanding genetic and chemical-genetic interactions quantitatively using phenotypic clustering; although they used a small number of the yeast haploid deletion strains to model these interactions, their methods are adaptable and scaleable to full-genome screens in other organisms.

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Together with [41••] and [42••], this report was one of the first large-scale RNAi screens in mammalian cells, opening this system towards high-throughput loss-of-function genetic manipulation. The authors developed a transfectable shRNA expression vector, and screened their library of 8000 human genes for effects on NFκB activity, using a luciferase reporter.

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See annotation for [40••]. The authors scored their library (10 000 human genes and 5000 mouse genes) for effects on proteasome function, identifying several known members of this class of genes, and thus verifying their approach. In addition, they incorporated multifunctional shRNA cassettes that could be transported to other expression vectors, as well as genetic barcodes, permitting microarray tracking of pooled shRNA collections.

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See annotation for [40••]. Using a library of 8000 human genes, the authors identified suppressors of p53-mediated proliferation arrest. As in [41••], they used genetic barcodes (in this case, the 19nt siRNA targeting sequence itself) to track gene effects in pools of siRNA, increasing screen throughput.

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