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Review

Applications of High-Throughput RNA Interference Screens to Problems in Cell and Developmental Biology

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ABSTRACT

RNA interference (RNAi) in tissue culture cells has emerged as an excellent methodology for identifying gene functions systematically and in an unbiased manner. Here, we describe how RNAi high-throughput screening (HTS) in Drosophila cells are currently being performed and emphasize the strengths and weaknesses of the approach. Further, to demonstrate the versatility of the technology, we provide examples of the various applications of the method to problems in signal transduction and cell and developmental biology. Finally, we discuss emerging technological advances that will extend RNAi-based screening methods.

MUCH of what we know today about the molecular mechanisms underlying fundamental cellular and developmental processes in higher eukaryotes can be traced back to a genetic screen in a model organism. Over the years, the development and application of new genetic tools have constantly provided new means to discover gene function. Recently, this has been best illustrated with the application of RNA interference (RNAi) to functional genomics, which relies on the ability of double-stranded RNAs (dsRNAs), small interfering RNAs (siRNAs), or small hairpin RNAs to silence a target gene through the specific destruction of that gene's mRNA (Fire *et al.* 1998; Elbashir *et al.* 2001; reviewed in Echeverri and Perrimon 2006).

Here, we focus on the application of RNAi to high-throughput screening (HTS) in Drosophila tissue culture cells. This systematic and unbiased approach to gene discovery was made possible with the breakthrough discovery by Hammond *et al.* (2000) and Clemens *et al.* (2000) that the addition of long dsRNAs to Drosophila cells can elicit a potent and specific RNAi effect. Further, the completion of the Drosophila genome sequence in 2000 (Adams *et al.* 2000) made it feasible to extend this method to the genomewide scale. These developments created an opportunity to investigate the function of every gene in tissue culture assays (cell-based assays), where each assay can be designed to address a specific biological question. RNAi HTS in Drosophila tissue culture is borrowed from systematic RNAi screens carried out earlier in *Caenorhabditis elegans*. RNAi reagents were delivered either by feeding bacteria expressing a specific dsRNA to animals or after dsRNA injection into embryos (Fraser *et al.* 2000; Gonczy *et al.* 2000; Sonnichsen *et al.* 2005). Similar genomewide libraries are being implemented in mammalian cell culture systems (Echeverri and Perrimon 2006; Moffat and Sabatini 2006).

In each organism or system, RNAi-based screening is being used to identify gene function rapidly, systematically, and in an unbiased manner. Because full genomes can be screened, two different philosophies have emerged behind performing such genomewide screens. The first one is centered on "gene discovery" and stems from the same rationale invoked in more traditional genetics screens and, as such, is concerned mainly with the identification of one or several important missing components in a biological process. The second one is more in tune with a "systems biology" approach. Rather than focusing primarily on a small number of genes, the goal here is to organize large sets of genes into functional subnetworks that carry out and modulate a specific program or response, with the overall goal of predicting the biological response of this network to perturbation or stimulation.

RNAi HTS METHODS

Building on the finding that long dsRNA added to Drosophila cells results in specific gene expression knockdown (Clemens *et al.* 2000; Hammond *et al.* 2000), a number of laboratories, including our own, applied the approach to perform genome-scale functional screens in Drosophila cell-based assays (Kiger

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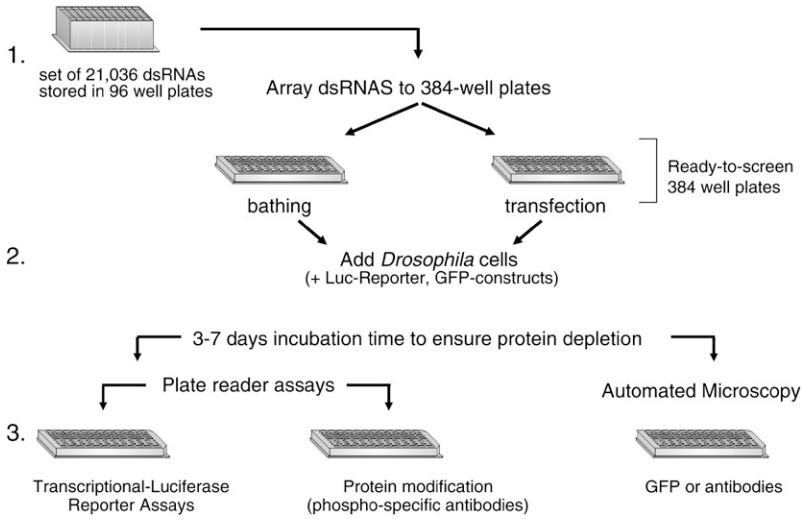

Figure 1.—RNAi HTS platform. The three main steps in RNAi HTS are depicted: (1) arraying of the library to 384-well plates; (2) addition of Drosophila cells (10,000–40,000/well) and treatment with dsRNAs for 30 min in the absence of serum (bathing method) or with transfection reagents together with other DNA constructs (if needed); and (3) automated detection step (most commonly performed with a plate reader or an inverted epifluorescence microscope) to quantitate or monitor the effects of the dsRNAs in each well.

et al. 2003; Lum *et al.* 2003; Boutros *et al.* 2004; Foley and O'Farrell 2004; Armknecht *et al.* 2005). In our laboratory, we assembled a genomewide collection of dsRNAs targeting all known Drosophila genes (Boutros *et al.* 2004). We then developed a robust experimental platform (Armknecht *et al.* 2005) and built a suitable infrastructure necessary to run large-scale RNAi screens in Drosophila cells. As this technology attracted a lot of outside interest, we made it available to the rest of the scientific community and opened the Drosophila RNAi Screening Center (DRSC) (see http://flyrnai.org) at Harvard Medical School (Flockhart *et al.* 2006).

The basic experimental design for HTS adopted in many experimental settings involves three major steps (Figure 1): (1) gene-specific dsRNAs are arrayed into 96- or 384-well assay plates using robotics, (2) cells are uniformly and rapidly dispensed into the plates using a liquid dispenser, and (3) after the appropriate incubation time, cells are subjected to individual treatments in a highly parallel fashion, fixed, or directly processed for the assay readout. The above experimental platform allows for a flexible screening methodology, where each step can be optimized to suit a wide range of assays, and is usually performed in 384-well plates to maximize the high-throughput nature of the screens (Armknecht *et al.* 2005; Echeverri and Perrimon 2006). Many of the screens performed to date rely on the use of either a plate reader (or a variation thereof, the automated fluorometric imaging plate reader) (Vig *et al.* 2006) to detect fluorescence or luminescence signals or high-throughput wide-field (Kiger *et al.* 2003) and confocal microscopy (Pelkmans *et al.* 2005) (Figure 2).

Cell lines for cell-based assays: The design of cell-based assays is often dictated by the characteristics of available cell lines, and whenever possible, has taken advantage of their different attributes. Importantly, cell lines can be manipulated to expand the range of possible assays through either transient or stable transfection of DNA constructs. The most commonly used cell lines in Drosophila RNAi HTS are S2 (and its derivative, S2R+) (Yanagawa *et al.* 1998) and K_c, which are of embryonic hemocyte origin. Their chief advantage is that they require simple bathing in serum-free medium to take up dsRNA efficiently (Clemens *et al.* 2000; Armknecht *et al.* 2005) and can be readily infected by a variety of viruses or bacteria (Agaisse *et al.* 2005; Cherry *et al.* 2005; Philips *et al.* 2005). Clone 8 cells are another popular cell line as these cells are more amenable for studying certain pathways (*e.g.*, Hedgehog signaling) (Lum *et al.* 2003; Nybakken *et al.* 2005), as they are derived from the wing-disc epithelium (Yanagawa *et al.* 1997). Although clone 8 cells show poor uptake with the bathing method, they are readily transfectable using standard lipid-based dsRNA transfection methods (Lum *et al.* 2003; Armknecht *et al.* 2005). Many other Drosophila cell lines of various origins can also be used for RNAi applications and are available from the Drosophila Genomics Research Center (http://dgrc.cgb.indiana.edu/).

Transcriptional and enzymatic reporter screens: Many assays are based on transcriptional reporters expressing various luciferases (*e.g.*, Lum *et al.* 2003; Baeg *et al.* 2005; DasGupta *et al.* 2005; Muller *et al.* 2005; Nybakken *et al.* 2005), detection of enzymatic activity (*e.g.*, Boutros *et al.* 2004; Bard *et al.* 2006), or fluorescent dyes (Vig *et al.* 2006; Zhang *et al.* 2006) whose overall chemiluminescence or fluorescence output is rapidly measured using a plate reader. The generation of numerical readouts for each condition in each well tested makes it possible to employ various statistical treatments to rigorously identify dsRNAs that are the true positives.

Antibody- and microscopy-based screens: A powerful application of antibody-based screens involves the use of protein-modification specific antibodies (such as anti-phospho-tyrosine/serine or methyl-lysine antibodies) directed against central components of a signaling

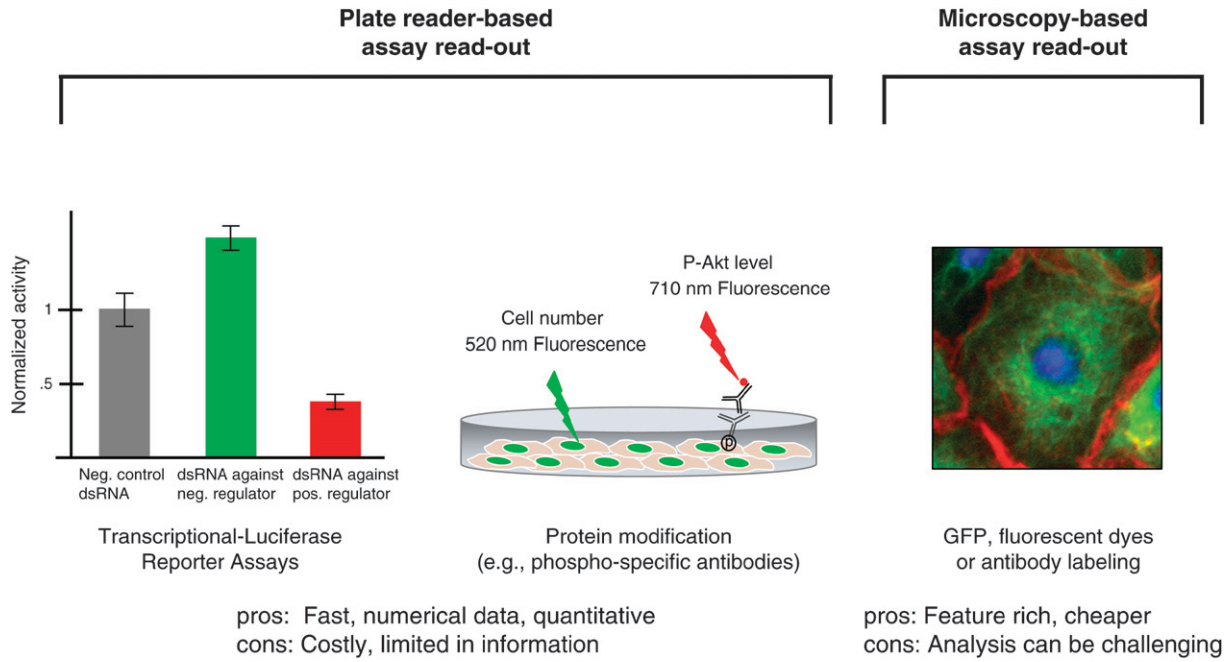

Figure 2.—Common plate reader or microscopy readouts scored in cell-based assays. Plate-reader readouts: firefly luciferase expressed off a promoter sensitive to activation of a particular signal transduction pathway (*e.g.*, Hh or Wg) is measured in each well. Normalization for cell number is achieved by dividing the firefly luciferase activity with that measured for Renilla luciferase typically expressed off a constitutive promoter [*e.g.*, Pol III (Nybakken *et al.* 2005), actin 5C (Baeg *et al.* 2005), or copia (Lum *et al.* 2003)] insensitive to the signaling pathway. Protein modifications in response to activation of a signaling pathway or a specific stage of the cell cycle recognized by specific fluorescently coupled antibodies serve as excellent endogenous readouts that can be easily measured in plate readers (Friedman and Perrimon 2006) or by the Aerius imaging system (see text). Cell numbers (as measured with a variety of DNA fluorescent dyes) are used to normalize for each well the fluorescence value corresponding to the specific binding of the antibody to its epitope. Microscopy readouts: Cells in each well are imaged live or fixed and processed to visualize particular proteins, cytoskeletal structures, organelles, or subcellular compartments. GFP fusion proteins, fluorescently coupled antibodies (primary or secondary antibodies), and fluorescent dyes are routinely used to label the structure(s) of interest (Kiger *et al.* 2003; Eggert *et al.* 2004; Feske *et al.* 2006). The automated microscope has several filters that allow the acquisition of different fluorophores, enabling the capture of multiple features (high-content imaging). The cellular features are analyzed either by eye or through the use of commercially available or open source software packages.

pathway or of a cellular response. Provided that the antibody is specific, such screens are highly quantitative and can be performed using either a plate reader to measure overall levels of fluorescence emitted by the fluorescently coupled antibody (Friedman and Perrimon 2006) or a laser-based plate reader that excites and scans in the far red the emission from appropriately conjugated antibodies (LI-COR Aerius automated infrared imaging system). Microscopy-based screens are arguably the most informative as a variety of cellular features can be visualized simultaneously for any sign of alteration in response to RNAi (see Figure 3 for specific examples). Such visualization typically relies on antibody staining, where a protein or structure of interest is labeled with a primary or secondary fluorescently conjugated antibody. However, cellular compartments or structures (*e.g.*, Golgi, mitochondria, nuclei, actin filaments) can be selectively labeled with either fluorescently labeled dyes or GFP proteins tagged with the appropriate localization tag (Kiger *et al.* 2003; Gwack *et al.* 2006). Indeed, two visual host/pathogen screens took advantage of engineered strains of bacteria that expressed GPF constitutively (Agaisse *et al.* 2005) or after entering phagosomes in infected cells (Philips *et al.* 2005). GFP signals were recorded by automated microscopy (Agaisse *et al.* 2005; Philips *et al.* 2005) and quantified using image analysis software (Philips *et al.* 2005). In this way, any change in GFP signal in response to a particular dsRNA can be easily identified.

Other screening strategies: Other detection methodologies and genome-scale dsRNA libraries (see Echeverri and Perrimon 2006 for a list of available Drosophila dsRNA libraries) have been used. Several large-scale RNAi screens that were performed in 48- or 96- well plates have relied on the use of flow cytometry to measure particular cell responses or assign cells to a particular stage of the cell cycle (Ramet *et al.* 2002; Bjorklund *et al.* 2006; Ulvila *et al.* 2006). Using this approach, Björklund *et al.* (2006) went on to identify pathways regulating cell size and cell cycle progression in Drosophila S2 cells. RNAi-treated cells were simultaneously analyzed by FACS for six distinct phenotypes (G_1 arrest, G_2/M transition, cell death, cytokinesis, and cell size in G_1 or G_2). This multi-parameter analysis identified 488 candidate genes involved in cell cycle

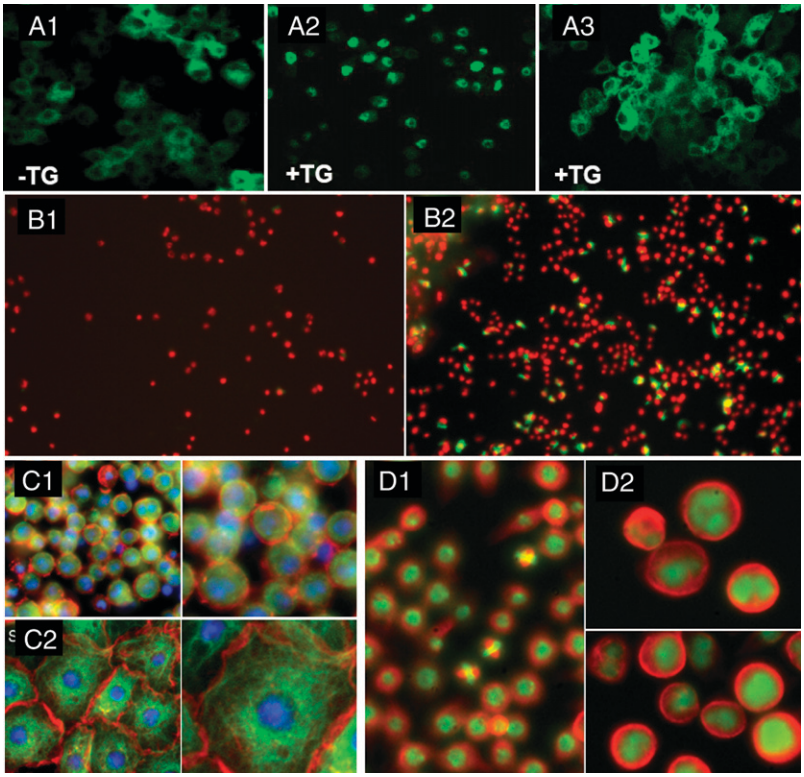

FIGURE 3.—Example of visual cell-based assays. (A) Translocation of a nuclear factor of activated T cells (NFAT)–GFP fusion transcription factor (in green) to the nucleus in response to the opening of Ca^{2+} channels evoked by thapsigargin (TG). (A1) In the absence of TG, NFAT–GFP is cytoplasmic. (A2) Addition of TG to the cells leads to the rapid translocation of NFAT–GFP to the nucleus. (A3) Addition of dsRNA targeting *olf186-F* abrogates the translocation of NFAT–GFP in response to TG (FESKE *et al.* 2006) (photographs are courtesy of Y. Gwack). (B) Cell-based assays that monitor the intracellular proliferation of a pathogen. *Mycobacterium fortuitum* engineered to express the GFP protein from the *pmap24* promoter (only active when the bacteria has been internalized in the phagosome compartment) was added to S2 cells. (B1) Little GFP signal is detected 6 hr after infection as most of the bacteria have not entered the phagosome compartment. (B2) Successful infection by the bacteria and robust growth are observed 24 hr after addition of the pathogen to the cells. The GFP signal is quantitated using automated image analysis software to monitor the effect of the various dsRNAs on infection and growth of the pathogen (PHILIPS *et al.* 2005). S2 nuclei are shown in red and bacteria in green (photographs are courtesy of J. Philips). (C) Round K_c cells (C1) and spread-out S2R+ cells (C2), shown in low and higher magnifications, can be used to study various aspects of cell morphology and adhesion (KIGER *et al.* 2003). Tubulin (green) was visualized with FITC-conjugated antitubulin antibody, actin (red) with tetramethyl rhodamine isothiocyanate (TRITC)–phalloidin, and DNA (blue) with DAPI (photographs are courtesy of A. Kiger). (D) A simple DNA stain can be used to screen for genes involved in cytokinesis. (D1) Untreated Drosophila Kc167 cells. (D2) Cells exposed to dsRNA targeting Aurora B kinase for 4 days. The cells are shown at low and higher magnifications. Cytoplasm (red) is stained with *N*-hydroxysuccinimide–tetramethylrhodamine and DNA (green) with Hoechst dye (photographs are courtesy of U. Eggert).

regulation, ubiquitination, vesicular and nuclear transport, and regulation of four ligand-induced signaling pathways [Wnt/Wg, MAPK, FKBP12-rapamycin-associated protein/target of rapamycin, and just another kinase (JAK)/signal transducers and activators of transcription (STAT)]. Coupled with high-resolution imaging microscopy, this approach was quite powerful as it allowed multiple parameters to be analyzed simultaneously (BJORKLUND *et al.* 2006). One limitation of monitoring cell cycle progression through DNA content only is the fact that many Drosophila cell lines are polyploid. Finally, a smaller-scale screen has been performed on RNAi cell microarrays where dsRNAs at first spotted at high density on a microscope slide to which cells are subsequently added (WHEELER *et al.* 2005).

CONSIDERATIONS WITH THE TECHNOLOGY

A major advantage of the RNAi HTS platform is that it is fast, versatile, and it enables screening in an unbiased manner. However, there are a number of important considerations to keep in mind regarding this evolving methodology. All high-throughput approaches have experimental noise from both technical and biological artifacts. The vast number of dsRNAs present in genomewide collections means that the primary screen can be performed at best in duplicate because of high cost, which limits the ability to gauge reproducibility of the data with confidence. Thus, in most cases, data analysis methods are used to aid in the extraction of robust data sets. Further, variations in the level of gene knockdown may affect the ability to assay the function of some genes. RNAi is a method of "gene knockdown" and not "gene knockout." Thus, while a hypomorphic state could be advantageous in cases where genes are associated with cell lethality, RNAi may fail to identify genes whose activities need to be completely eliminated to show a phenotype. In addition, the time required to achieve optimal knockdown may not be the same for all genes analyzed. In general, a 3-day RNAi treatment is chosen for most screens. However, if the protein encoded by the targeted gene has a long half-life, a 3-day treatment may be too short to cause a measurable phenotype. Conversely, when RNAi is rapid, secondary effects through feedback regulation may be scored. Finally, the large number of candidate genes (usually in the hundreds) typically implicated in these screens makes it virtually impossible to confirm every single hit through careful biochemical or genetic validation experiments.

A critical aspect of an RNAi HTS platform is the quality and specificity of the RNAi library in use. It is important to keep in mind that each library is based on

the best annotation at the time that it was assembled. As genome annotations are still evolving, any change needs to be incorporated in a library to keep it up to date. For instance, the first library used at the DRSC (Boutros *et al.* 2004) was designed to target all Drosophila genes predicted from two annotations: an early BDGP/Celera annotation (13,672 genes) (Adams *et al.* 2000) and one from the Sanger Center (20,622 genes) (Hild *et al.* 2003). Combining the two annotations resulted in a total of 21,306 nonredundant possible transcripts in the Drosophila genome, with 14,556 dsRNAs targeting annotations present in both the BDGP and Sanger Center sets and 6750 dsRNAs targeting Sanger annotations not found in the BDGP set (Sanger-only dsRNAs). However, many of the dsRNAs targeting the Sanger-only predictions were eventually removed from the DRSC RNAi library as Stolc *et al.* (2004) could confirm expression of only 291 of the Sanger-only predictions using a DNA oligonucleotide microarray approach, while Yandel *et al.* (2005) found that only 10% of the Sanger-only predictions were likely to correspond to genes containing introns.

In addition to requiring periodic updates to reflect changes introduced by more accurate and comprehensive annotations of the Drosophila genome, libraries need to be controlled for off-target effects (OTEs) potentially associated with the use of dsRNAs. Although this issue is familiar to investigators using siRNAs in mammalian systems (where it had been recognized early on) (Jackson *et al.* 2003), its extent had been underestimated in the Drosophila and *C. elegans* communities. However, two recent studies reported strong evidence for OTEs in Drosophila RNAi screens (Kulkarni *et al.* 2006; Ma *et al.* 2006). Ma *et al.* (2006) focused primarily on OTEs caused by the presence of trinucleotide CA[AGCT] or CAN repeats in some dsRNAs. Results from this study indicate that as few as five contiguous CAN repeats in dsRNAs can lead to toxicity or nonspecific effects in treated cells, resulting in likely false positives in certain types of screens (Ma *et al.* 2006). In the second study, the prevalence of OTEs in Drosophila RNAi screens was examined through a review of 30 genomewide screens (Kulkarni *et al.* 2006). The authors found that the presence of OTEs correlates with perfect homologies to genes (including but not restricted to CAN repeats) other than the intended targets that are present in some dsRNAs. A trend could already be observed with 17-nt perfect homology, but homologies of 19 nt or beyond were found to be highly significant. This analysis led to a complete overhaul of the library, where all dsRNAs predicted to have OTEs were replaced with new ones devoid of any homology to other genes (on the basis of a threshold of 19 nt). Regardless, one should keep in mind that we still do not fully understand all the rules governing OTEs in Drosophila cells. In fact, it has been shown in mammalian cells that short sequences in siRNAs identical to the seed region of microRNAs represent the major source of OTEs in mammalian cell-based screens (Birmingham *et al.* 2006). It is not known yet to what extent this mechanism is a concern in either *C. elegans* or Drosophila where long dsRNAs are used as RNAi reagents. Thus, because OTEs are particularly difficult to predict and to ensure data reproducibility, the recommended practice in the field is now to simply use multiple independent RNAi reagents against the same gene (Echeverri *et al.* 2006). In this regard, the practice of generating validation sets consisting of new dsRNAs that are distinct from any dsRNA tested in a primary screen should be encouraged. These dsRNAs should be devoid of predicted perfect homologies to nontarget genes and should correspond to every hit identified in a completed screen.

APPLICATIONS OF RNAi HTS TO GENE DISCOVERY

RNAi HTS in cell-based assays has proven to be a robust method for identifying gene function. Screens carried out in cell lines have covered a wide range of biological questions, including cell viability (Boutros *et al.* 2004), cell morphology (Kiger *et al.* 2003), cell cycle (Bjorklund *et al.* 2006), cytokinesis (Eggert *et al.* 2004), susceptibility to DNA-damaging agents, RNA processing, general (Bard *et al.* 2006) and specialized secretion, calcium stores (Feske *et al.* 2006; Vig *et al.* 2006; Zhang *et al.* 2006), factors influencing polyQ aggregation and toxicity, mitochondrial dynamics, circadian clock, cellular response to metals, hypoxia, phagocytosis (Ramet *et al.* 2002; Kocks *et al.* 2005), innate immunity (Foley and O'Farrell 2004; Gesellchen *et al.* 2005; Kleino *et al.* 2005), cell susceptibility to infection by viruses or other intracellular pathogens (Agaisse *et al.* 2005; Cherry *et al.* 2005, 2006; Philips *et al.* 2005) as well as most of the major signaling pathways (Lum *et al.* 2003; Baeg *et al.* 2005; DasGupta *et al.* 2005; Muller *et al.* 2005; Nybakken *et al.* 2005; Bartscherer *et al.* 2006; Friedman and Perrimon 2006; Gwack *et al.* 2006; Ma *et al.* 2006).

Importantly, RNAi screens have identified important components in a biological process that had eluded more conventional approaches. A perfect example is illustrated by three independent screens, which were largely designed to identify a long-sought transmembrane channel regulating calcium stores in eukaryotic cells. All three screens identified *olf186-F* as the gene encoding either the elusive CRAC channel or a component of it (Feske *et al.* 2006; Vig *et al.* 2006; Zhang *et al.* 2006). That all three screens, based on slightly different cell-based assays, identified the same component validates the notion that genomewide RNAi screens can be a powerful and specific approach for gene discovery. In a different line of investigation, Philips *et al.* (2005) sought to identify in a visual screen host factors that might interfere with Mycobacterium infection and intracellular growth in S2 cells. They identified a new

factor, Peste (Pes), a CD36 family member, which is required for uptake of mycobacteria (Philips *et al.* 2005). To address a related question, Kocks *et al.* (2005) used a clever combination of specific RNAi and RNA profiling, which led to the identification of *eater*, a gene encoding a scavenger receptor-like type I transmembrane protein mediating phagocytosis of bacteria. RNAi screens focusing on signaling pathways have also yielded new components that are important effectors in these pathways, such as Ihog (Yao *et al.* 2006) and PP2A (Hh pathway) (Nybakken *et al.* 2005), Evi (Wg pathway) (Bartscherer *et al.* 2006), PTP61F (JAK/STAT pathway) (Baeg *et al.* 2005; Muller *et al.* 2005), and dGCKIII (MAPK pathway). Kiger *et al.* (2003) performed a large-scale screen (targeting ~1000 genes) to identify genes that affected cell morphology when knocked down by their cognate dsRNAs. Using automated fluorescence microscopy to visualize actin filaments, microtubules, and DNA (see C1 and C2 in Figure 3), they classified phenotypes according to well-defined parameters that were applied to score each image by eye. Importantly, knockdown of genes known to be arranged within the same pathway resulted in similar phenotypic profiles or phenoprints. The authors took advantage of this property to predict where previously uncharacterized genes within a phenotypic cluster might be acting, leading them, for example, to assign the role for the citron kinase as a Rho 1 effector required for cytokinesis (Kiger *et al.* 2003).

APPLICATIONS OF RNAi HTS TO SYSTEM BIOLOGY

As exemplified by the various screens already performed, RNAi HTS in cell-based assays is an excellent method for identifying gene functions involved in cell signaling, cell biology, physiology, differentiation, and host/pathogen interactions. However, rather than focusing primarily on a few interesting new candidates, an exciting potential of RNAi HTS is to provide a global systems-wide understanding of the subcellular networks that carry out and modulate a specific program or response, such as the propagation and integration of input signals through a signaling network. The large number of genes, usually in the hundreds, identified in genomewide screens creates a challenge: it significantly increases the number of candidate genes that could be classified as *bona fide* effectors in a signaling pathway or biological process, but it could also lead to the inclusion of false positives as it is virtually impossible to confirm every single hit through biochemical or genetic validation experiments. Thus, the success of the approach relies on low experimental rates of false positives and false negatives. Providing that RNAi data sets have been curated to contain only high confidence hits, the information can be integrated with transcriptional profiling (RNA profiling), interactome data sets (proteome and genetic interactomes), and published literature (literature-mining tools) to support or confirm the connections made between components of a network (Gunsalus *et al.* 2005; Mukherji *et al.* 2006).

One of the especially promising applications of RNAi HTS is to expand our knowledge of the structure of signaling networks. Indeed, analysis of the proteome from either two-hybrid or mass spectrometry approaches has revealed that hundreds rather than tens of proteins are associated with a signaling network. This complexity in scale has been validated by recent studies of synthetic lethality screens in yeast (Tong *et al.* 2004), and most recently in *C. elegans* (Lehner *et al.* 2006), and thus illustrates our partial knowledge of the complexity of signaling networks. A recent screen, which took into account OTEs and controlled for them, delivered appropriate data sets that can be used for a systems biology approach. The screen for modulators of MAPK activity (Friedman and Perrimon 2006) monitored the state of phosphorylated Drosophila ERK (dpERK) as a marker of MAPK activation upon insulin stimulation. In this way, small but significant changes in MAPK signaling could be measured by following dpERK levels, providing the ability for the first time to reveal quantitative changes, which by themselves might not be enough to lead to a measurable phenotype *in vivo*. Similar to the findings of other signal transduction screens, the major known components were readily identified in this screen and >300 additional components were shown to affect the steady state of MAPK activity in RNAi-treated cells. Importantly, the list of genes showed a highly significant enrichment in genes encoding pathway components, in genes conserved across organisms utilizing this pathway, and finally in genes encoding orthologous proteins dynamically phosphorylated in HeLa cells following EGF stimulus (Olsen *et al.* 2006), suggesting the existence of extensive crosstalks among different signaling pathways. Ultimately, it will be important to project the information derived from such screens on data sets obtained from other "omics" approaches such as protein–protein interaction maps, genetic interaction networks, and RNA-profiling experiments, to strengthen the connectivity links among all these components, and to derive a comprehensive depiction of signaling networks, where the flow of information is highly dynamic and depends on extensive feedback loops and crosstalks among coexisting signaling pathways.

FUTURE DEVELOPMENTS OF THE TECHNOLOGY

Since the most important requirement for a successful RNAi HTS is a robust cell-based assay that can be processed in a high-throughput manner, it is an exciting time to think about where the major technological advances will come from in the next few years in this area.

Design of more sophisticated cell-based assays: In the context of signaling, RNAi HTS designed to capture

the activity of multiple readouts rather than to measure the activity of a single signaling pathway, as has been done so far, will be extremely informative with regards to the characterization of crosstalk and flow of information through signaling networks. For example, RNAi HTS that simultaneously monitors the state of two or more pathway activities would allow one to identify common regulators and crosstalk regulation. Emerging technologies such as multiplex FISH (Capodieci *et al.* 2005) and microsphere-based high-throughput gene expression profiling (based on the Luminex xMAP system) (Naciff *et al.* 2005) provide cost-effective methods to detect gene expression signatures composed of a subset of reporter genes of interest in a large number of samples. While the multiplex FISH approach can measure transcript levels of <15 reporter genes on a single-cell basis, the Luminex system can be employed to follow the levels of at least 100 genes simultaneously in cell extracts (Peck *et al.* 2006).

Similarly, multiplexing assays at the level of protein modification have great potential (Irish *et al.* 2004). For example, monitoring the phosphorylation state of both MAPK and another key kinase regulated in response to receptor tyrosine kinase activation (*i.e.*, Raf or MEK) would provide insights into the structure of the MAPK network, in particular of the feedback loops. The same kind of multi-sensors strategy can be applied directly to cell biological studies whereby the state of different cellular structures or cellular processes is monitored (*i.e.*, markers for different endosomal compartments). Similarly, in the context of the host/pathogen screens, screens can be designed whereby a single cell is infected by two different pathogens (*i.e.*, Mycobacterium and Listeria). Providing that each pathogen can be visualized independently, RNAi perturbations that affect selectively the uptake or growth of Mycobacteria but not of Listeria (or vice versa) would be readily identified.

Perhaps the most significant advances in RNAi HTS will come from high content screening (HCS). Cell-based HCS that rely on cellular phenotypes are becoming one of the preferred methods in RNAi HTS because they generate data sets that are rich in information. For example, the intensity and localization of fluorescent markers reflecting the number, size, and shape of cellular compartments can be analyzed quantitatively. Further, as described below, the use of primary cells offers ample opportunities to carry out cell morphology screens in a biologically relevant context, as diverse structures such as muscles, neuromuscular junctions, tracheal tubes, etc., can form in these cultures. Thus, many of the most interesting screens that will be performed in the future are likely to rely on microscopy. In the past few years, major advances have been made regarding the development of both conventional and confocal HTS microscopes, such that they now offer excellent image resolution and unparalleled image acquisition speed (see review by Carpenter and Sabatini 2004). One current limitation, however, is that the vast majority of commercial software packages use proprietary codes. As such, they perform quite well in focused applications that require an accurate cell or nuclei count, a measure of pixel intensities corresponding to well-defined objects, or an accurate numerical representation of how much of a protein localizes to specific subcellular compartments. However, they often fall short in the kind of flexibility and sophistication that many academic projects require. In this regard, major advancements in this field will come from the development of open source and sophisticated image analysis methods that are able to process quickly the large amount of images generated, *e.g.*, Cellprofiler (http://www.cellprofiler.org) (Carpenter *et al.* 2006).

Development of new cell lines and primary cells: Although advances in assay designs are poised to make major contributions to RNAi HTS, the success of the screens ultimately relies on the relevance of the cell types in which the questions are being studied. A significant caveat of using the established cell lines described earlier is that they have adapted physiologically to the culture medium, and most of the cell lines have departed from diploidy or may harbor activated signaling pathways. To resolve these issues and expand the repertoire of assays in more biologically relevant cells, RNAi HTS for axonal outgrowth and muscle integrity has been conducted by simply deriving cells from embryos that express a GFP marker in the cells of interest. The first screen focused on primary muscle cells (J. Bai and N. Perrimon, unpublished results). Muscle phenotypes were scored by visual inspection of microscopic images acquired using an automated inverted fluorescence microscope. A number of phenotypic classes (collapsed muscle, small or large muscles, disrupted sarcomere structures with no striation, actin filaments retracted or relaxed from the cell periphery) were identified. The second screen dealt with axonal outgrowth of neuronal cells, whereby the morphology of GFP-labeled neuronal cells in response to RNAi was recorded by automated microscopy and visually scored according to various criteria that included the extent of excessive branching, defasciculation, axon blebbing, cell loss, and reduced outgrowth (K. J. Sepp, P. Hong, S. B. Lizarraga, J. S. Liu, L. A. Mejia, C. A. Walsh and N. Perrimon, unpublished results).

Altogether, screens of muscle and neuronal cells are readily feasible and bode well for follow-up screens or other screen applications that require the use of primary cell cultures. For instance, assays can be devised whereby a specific gene (*e.g.*, a gene coding for a mutated form of a protein linked to a human disease) can be ectopically expressed in the cells of interest (Figure 4). If misexpression of that gene in the primary cells results in a phenotype, then an RNAi suppressor/enhancer screen can be conducted. Other promising HTS applications in primary cells include assays

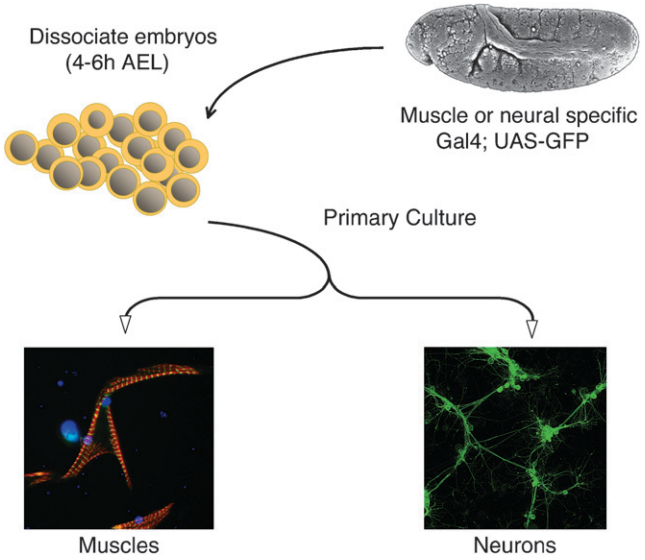

Figure 4.—Applications of primary cell cultures to RNAi HTS. Embryos (4–6 hr after egg laying) are mechanically dissociated by douncing and the cell suspension cultured in the presence of dsRNA (bathing method) (J. Bai and N. Perrimon, unpublished results; K. J. Sepp, P. Hong, S. B. Lizarraga, J. S. Liu, L. A. Mejia, C. A. Walsh and N. Perrimon, unpublished results). Phenotypes in these cultures are typically read after 7–10 days. Cell-based assays in primary muscle and neuronal cells can be easily engineered by expressing a gene of interest using an appropriate Gal4 driver, *e.g.*, *dmef2-Gal4* for muscle or *elav-Gal4* for neuronal cells. Primary cells can be visualized using specific antibody labeling or TRITC–phalloidin staining for muscle cells that reveals the sarcomeric structure of differentiated muscles or, as shown here for neurons, following the expression of GFP (*elav-Gal4; UAS-GFP*).

designed to determine the mode of action of a drug. If, for example, a small molecule induces neurite outgrowth, a suppressor/enhancer RNAi screen may identify components of the pathway that are modulated by the compound. Finally, culture of additional cell types also may be feasible. Indeed, there are reports in the literature that fat body and tracheal cells as well as various epithelial cells types can be cultured (Shields *et al.* 1975). In addition, Niki *et al.* (2006) recently reported their success at culturing germline stem cells from adult Drosophila ovaries, thus opening up the possibility of conducting RNAi screens in totipotent cells.

Novel RNAi screening platforms: Although RNAi HTS in multi-well plates have great applications, two emerging technologies offer new opportunities in Drosophila functional genomics: cell arrays and transgenic RNAi.

Cell arrays and their applications to synthetic phenotypic screens: As we have learned from yeast studies, many genetic interactions can be revealed by unbiased analysis of synthetic genetic interactions (see, for example, Tong *et al.* 2004). Although one can envision testing by RNAi HTS in 384-well plates all genomewide interactions between a specific gene and others (~15,000 pairwise combination), or alternatively, testing for all pairwise combinations in a set of 100 genes (4900 combinations), it is clearly not realistic on a larger scale. Miniaturization of the methodology offered by the RNAi-cell microarray method, whereby dsRNAs can be spotted at high density on glass slides (RNAi microarrays) and assayed in visual screens (Wheeler *et al.* 2004; Guertin *et al.* 2006), provides a solution as it reduces significantly the cost associated with the technology. In this system, cells are cultured on a glass slide printed at known locations with an array of 2–3 nl of salt solution droplets containing the RNAi reagent. Because each spot is only 200 μm in diameter, 6000 such spots can easily be printed on one standard microscope slide. Cells (80–200) that settle on the printed spots take up the dsRNA and induce over time, through RNAi, the degradation of the targeted mRNAs, resulting in a concomitant decrease in the encoded proteins. This technique requires very little reagents, lends itself well for streamlining assay conditions, and, since it is based on microscopy, allows for a variety of cellular phenotypes to be assayed (Wheeler *et al.* 2005).

In vivo validation using transgenic hairpin flies: In vivo validation of the results obtained from RNAi tissue culture screens is an important and necessary step to fully establish the relevance of the observation made from RNAi HTS. In addition to collections of existing Drosophila mutations and the arsenal of existing tools available to generate mutations (local excision of a *P* element inserted near the gene under investigation, homologous recombination, etc.) (Nagy *et al.* 2003; Venken and Bellen 2005; Beller and Oliver 2006), transgenic RNAi—whereby a hairpin construct against each gene of interest is expressed under upstream activating sequence (UAS) control (see, for example, Lee and Carthew 2003)—is emerging as the method of choice for *in vivo* validation. There are currently two major efforts to assemble large collections of transgenic flies for RNAi (http://www.imba.oeaw.ac.at/index.php?id=252 and http://www.shigen.nig.ac.jp/fly/nigfly/rnaiListAction.do?browseOrSearch=browse). One advantage of using the UAS/Gal4 for expression of hairpins used for knockdowns is the rich repertoire of cell-type-specific and heat-inducible Gal4 drivers available, which can be exploited to achieve spatially and temporally controlled RNAi knockdown of genes that may be essential and/or pleiotropic. Importantly, the generation of such lines would benefit from using the site-specific integration method (Groth *et al.* 2004) to target transgenes to loci that are highly inducible.

Concluding remarks: The power of model systems over the years has relied on the design of sophisticated methods to conduct genetic screens. The amazing advances in RNAi in the past few years have added an even more comprehensive set of tools to the already powerful armamentarium available to a geneticist. Although we

are already witnessing the impressive impact that RNAi has had on the field of developmental and cell biology, emerging improvements and technologies in RNAi-based screening methods will undoubtedly continue to extend our ability to access knowledge in unprecedented ways.

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