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Drug-target identification in *Drosophila* cells: combining high-throughout RNAi and small-molecule screens

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RNA interference (RNAi) and small-molecule approaches are synergistic on multiple levels, from technology and high-throughput screen development to target identification and functional studies. Here, we describe the RNAi screening platform that we have established and made available to the community through the *Drosophila* RNAi Screening Center at Harvard Medical School. We then illustrate how the combination of RNAi and small-molecule HTS can lead to effective identification of targets in drug discovery.

The success of *Drosophila* as a model system for developmental genetics, signal transduction and cell biology studies over the years has relied on the ease of growing this organism and the powerful genetic tools available. Although it is a 'driving horse' for basic research, the contribution of *Drosophila* to drug discovery has been modest and relatively untapped, which is surprising because small-molecule inhibitors often cross-react between species. Usually, small molecules function by interacting with a binding pocket in a protein and by making contacts with specific residues. Because many proteins are highly conserved across species, one would expect many specific small molecules to be also active in these species. This hypothesis is supported by our recent study to identify small molecules that inhibit cytokinesis in *Drosophila* cells in culture in which we showed that 64% and 48% of the drugs identified are also active in human HeLa cells and *Saccharomyces cerevisiae*, respectively (see below) [1].

To date, most of the few small-molecule studies in *Drosophila* have focused on neurobiological questions ranging from sleep studies [2] to neurological diseases (reviewed in [3]). For example, the potency of relevant small molecules has been evaluated in *Drosophila* models of Huntington's [4] Parkinson's [5] and Alzheimer's [6] diseases. In one example, Micchelli *et al.* demonstrated the bioactivity of the γ-secretase/presenilin (PS) inhibitor DATP in *Drosophila* after feeding the compound to larvae [6]. DATP-induced developmental defects are remarkably similar to those caused by a reduction of Notch activity, which is consistent with PS-dependent trans-membrane cleavage of the Notch receptor being required for pathway activation. The success of pharmacological studies in *Drosophila* models of neurological diseases is promising for small-molecule discovery in other disease areas, especially because all major, disease-relevant signaling pathways such as the insulin, epidermal growth factor, transforming growth factor β, Wnt, Hedgehog, JAK/STAT and Notch pathways are present in this organism, and >60% of the genes identified in human diseases have counterparts in *Drosophila* [7,8].

In addition, the use of *Drosophila* as a simple organism to study tumor development and metastasis *in vivo* has gained in acceptance [9]. In one study, flies were generated to ectopically express in the eye activated forms of the RET tyrosine kinase receptor modeled on mutations found in multiple endocrine neoplasia syndrome and papillary thyroid carcinoma. The resulting rough-eye phenotype is suppressed selectively and dose-dependently by feeding with the orally available tyrosine-kinase inhibitor ZD6474, which has been shown previously to inhibit oncogenic forms of RET [10]. Importantly, the concentrations of ZD6474 that suppress the phenotype cause no adverse toxicity and do not perturb normal development of the flies even though it was fed throughout all stages of fly development [11].

Active small molecules can be discovered by either screening chemical libraries for changes in particular activities in purified protein assays or for desired phenotypes in cell-based assays [12]. Each approach has advantages and limitations. *In vitro*, cell-free screens of purified proteins with enzymes such as kinases are relatively straightforward experimentally, but can identify promiscuous

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small-molecule inhibitors of other family members with highly conserved binding sites (e.g. kinase ATP-binding sites); these molecules might also be either cell-impermeable or toxic. By contrast, in cell-based screens, small molecules must be active in living cells and have some specificity to cause the desired phenotypic change.

Currently, phenotypic screens are less common in drug discovery because of their many challenges, particularly at the level of data analysis and, most importantly, target identification (because the desired phenotypic changes might be consistent with inhibition of any component of a particular pathway or process) [13]. Traditionally, small-molecule targets have been identified by either affinity methods or candidate-based approaches [13]. In affinity methods, a small molecule is tethered to a solid support over which a cell extract is passed to identify proteins that bind; the small molecule must be attached to a linker without changing its activity, which is not always possible. The candidate-based approach depends on an educated guess about the class of potential targets to be screened, but this is not always obvious. Here, we discuss how merging the information from RNAi and small-molecule screens can facilitate the identification of drug targets.

A screening platform for RNAi screening in *Drosophila* cells

Publication of the *Drosophila* genome sequence in 2000 [14] provided an unprecedented resource for functional genomic studies. In the context of drug discovery, the identification of new genes using RNAi to affect the activity of a signaling pathway provides new drug targets for that pathway. To analyze systematically the functions of the ~15 000 predicted genes, we established a high-throughput screen (HTS) platform to conduct RNAi screens in *Drosophila* cells in tissue culture in the 384-well plate format (Figures 1 and 2) [15–17]. This is available to the community through the *Drosophila* RNAi Screening Center (DRSC) (http://www.flyrnai.org) at Harvard Medical School. This approach is possible because, as first shown by Clemens *et al.* [18], the addition of long, double-stranded RNAs (dsRNAs) to *Drosophila* cells in culture either reduces or eliminates specifically the expression of target genes and, thus, efficiently phenocopies loss-of-function mutations. Once an appropriate cell-based assay has been established, genome-wide RNAi screens allow the identification of most of the genes that are involved in the process under investigation in a few weeks.

The first step in developing an appropriate RNAi screen in *Drosophila* cells is to ensure that the signal-to-noise and dynamic range of the assay read-out remain adequate during miniaturization to the 384-well plate format used in our RNAi HTS [17]. Once experimental conditions have been optimized, the screening procedure follows a basic experimental design, which we established at the DRSC [15,17]. This involves three main steps: (i) gene-specific dsRNAs from our collection stored in 96-well plates are arrayed into 384-well assay plates using robotics; (ii) cells are uniformly and rapidly dispensed into the 384-well plates using a MultiDrop liquid dispenser; (iii) after the appropriate incubation time, cells are subjected to individual treatments in a parallel fashion, and either fixed or directly processed for the assay read-out. The phenotypic output measured for each sample

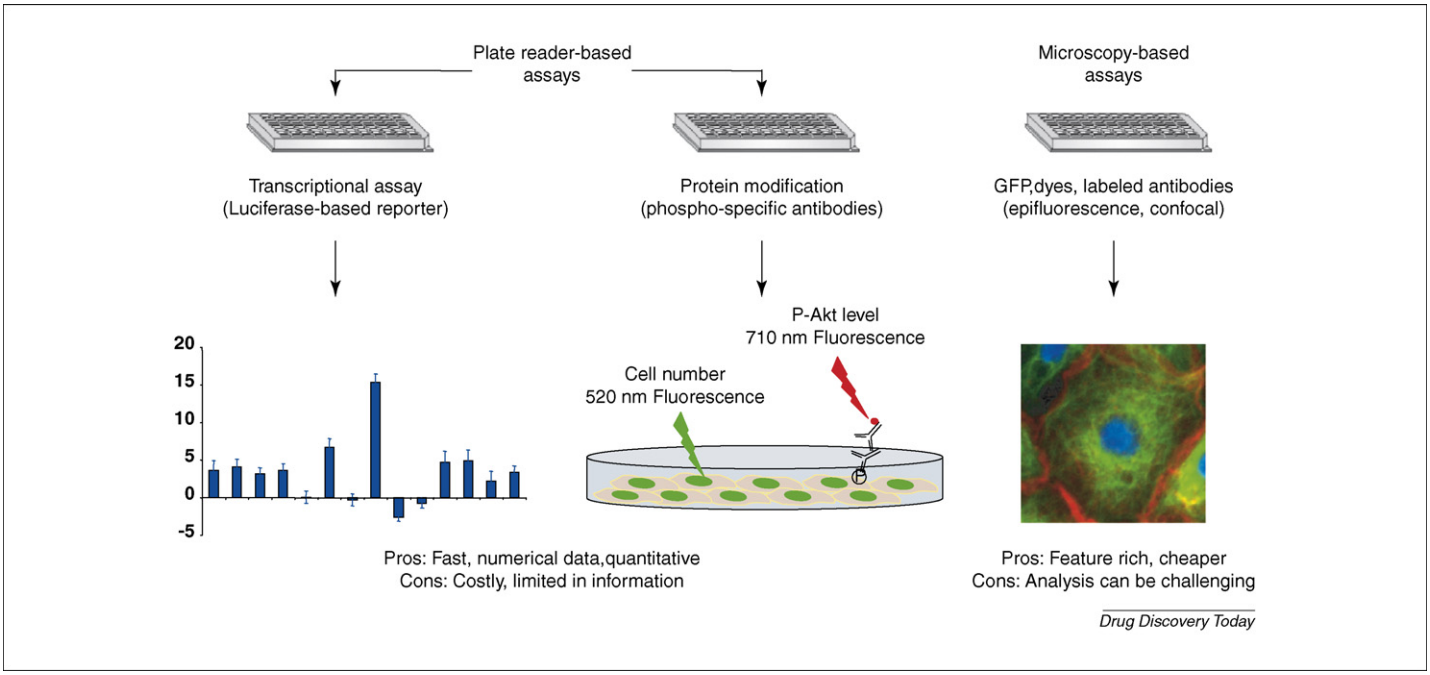

FIGURE 1

Screening platform for RNAi screening in *Drosophila* cells. Assays are developed in low-throughput to optimize signal-to-noise and dynamic range during miniaturization to 384-well plate format [17]. dsRNAs that target nearly every gene in the *Drosophila* genome are screened using either plate reader-based assays or microscopy-based high-content screening. After the appropriate incubation with dsRNAs (3–8 days, depending on the assay) cells are subjected to individual treatments in a highly parallel fashion, and either fixed or processed directly for the assay read-out. Plate-reader screens are fast and provide a quantitative set of data that is amenable to normalization and statistical analyses that are important for extracting robust, unbiased datasets. However, the information captures only a single output. Although visual screens are technically more cumbersome and their analysis is challenging and prone to individual bias, the information content is rich and conveys more accurately the overall consequence(s) of gene knockdown.

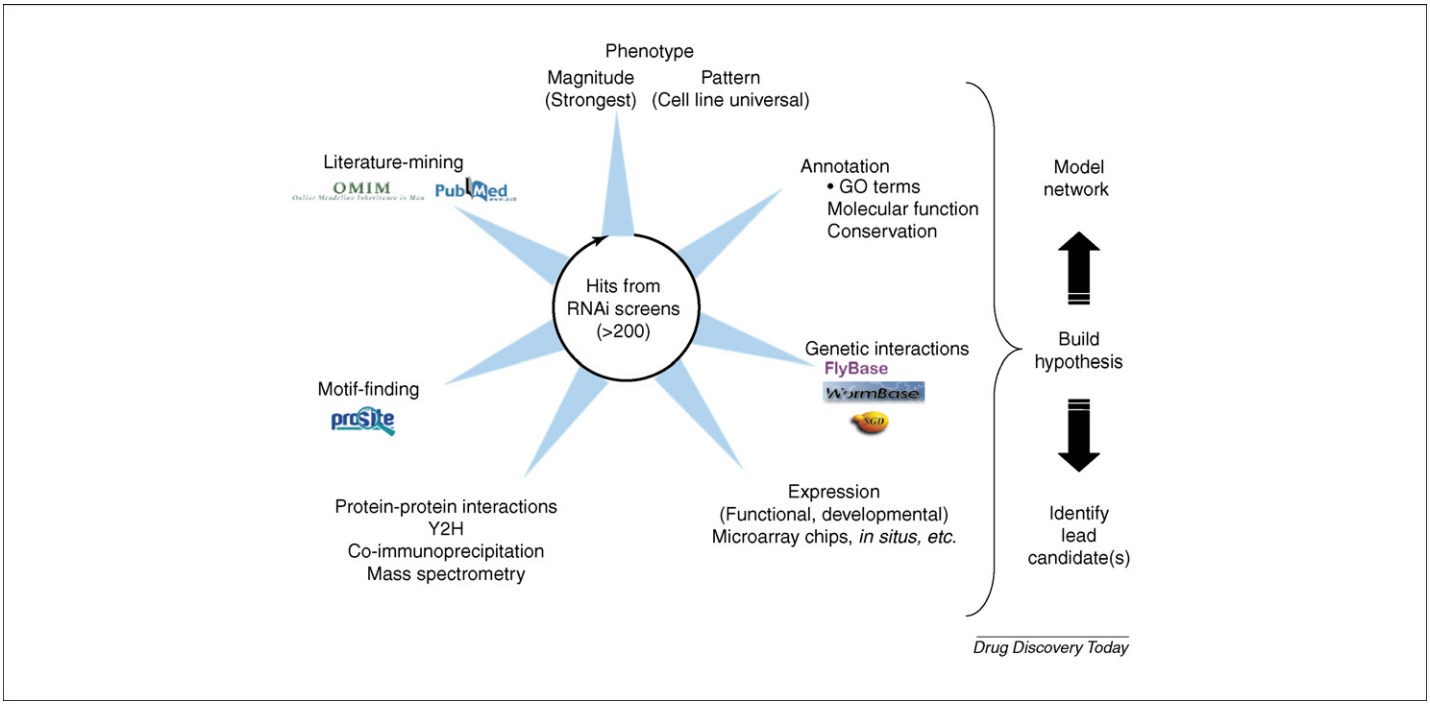

FIGURE 2

Mining data sets. Selecting which validated hits from secondary screens should be characterized further can involve multiple information sources (genomics, proteomics, organism-specific genetic-databases and literature databases) and differs with the overall goal of a given screen (gene discovery versus systems biology). Using the screening data itself, hits that are the strongest or are cell-type universal might be the most interesting to validate *in vivo*. Genes that are either highly conserved or with molecular functions linked to the process of interest can also be chosen. Networks of genetic or protein–protein interactions among hits and known components of the process of interest might implicate which genes are more closely linked to the given pathway (network building). Genes with particular binding or phosphorylation motifs linked to members of the pathway, or which show differential expression in functional microarray experiments might be components of feedback loops that control the pathway. Finally, new resources such as literature-mining applications might help identify connections between screen hits and generate testable hypotheses.

depends on the detection method; quantitative measurements tend to be acquired with a plate reader, whereas qualitative measurements are usually captured by automated microscopy (Figure 1). The incubation period with dsRNAs varies and needs to be optimized for each specific assay. A 3-day incubation period is standard in our experiments, but incubation with dsRNAs can be for much longer (up to 10 days) without deleterious cytotoxicity.

In cell-based assays that rely on transcriptional reporters [19–23], the overall chemi-luminescence output is measured rapidly using a plate reader and normalized to the number of cells present in the well, and various statistical analyses can be applied to the normalized data (Figure 1). A common application of visual screens involves the use of fluorescently-labeled antibodies to recognize cytoskeletal proteins or a given protein (Figure 1). Such antibodies have been used in our group to image either the cytoskeletal organization or monitor the infection load by the detection of a viral capsid protein of cells treated with dsRNAs [16,24]. Topics amenable to investigation include cytokinesis, cell morphology, signal transduction pathways and host factors involved in viral infection [1,16,24,25]. This approach can be generalized to any assay that relies on following the expression or localization of a protein for which there is a specific antibody or GFP-tagged protein [26–29]. Another powerful application of antibody-based screens involves the use of phospho-specific antibodies to monitor the activity of the relevant pathway under either basal or stimulated conditions (A. Friedman and N. Perrimon, unpublished). Provided that the specificity of the phospho-antibodies is well-defined, these screens are highly quantitative and can be performed by measuring the overall levels of fluorescence emitted by fluorescently-coupled secondary antibodies using either a standard plate-reader or with the Aerius platform (LI-COR Biosciences), a laser-based microscope/plate-reader hybrid (Figure 1).

Primary screen data must be normalized and corrected to minimize assay noise before analysis. As plate reader-based assays generate numerical data, various statistical analyses are normally performed to select appropriate values for the processed data above and below which a phenotype is declared significant. These values are chosen empirically to maximize the balance between sensitivity and selectivity in identifying hits (for a brief discussion, see Data Analysis section in [17]). Cell-based, high-content, visual screens that rely on cellular phenotypes hold tremendous potential for RNAi HTS because they generate datasets that are rich in information [30]. However, they are more difficult to evaluate because the raw data or phenotype is often either subjective (if analyzed by eye) or too challenging to measure and quantitate using commercially available image-analysis packages. Despite this limitation, we foresee that the field of image analysis will experience rapid, steady growth in the development of more powerful, sophisticated algorithms that can be applied to perform unbiased, quantitative image analysis of the phenotypes generated in RNAi screens. The use of such algorithms will produce numerical data that are amenable to statistical approaches and allow various methods to classify phenotypes, for example, using clustering approaches.

Secondary assays and data mining

Typically, a primary RNAi HTS produces 300–600 hits, a number that is not practical for detailed follow-up studies. Several criteria are used to trim the list of candidate hits to a manageable number. For example, hits corresponding to genes that are conserved between the *Drosophila* and mammalian systems can be prioritized. If large molecular machines (e.g. the ribosome or proteasome) are important in the process under study, a few components rather than all are selected for further analysis. Once the first pass at trimming the list of hits is complete, a smaller number of selected hits, usually around 200, are validated in secondary screens. This is achieved, preferably, by using a slightly different read-out in several cell lines, by varying experimental conditions, and by a combination of the above. At this stage, the number of validated hits isreduced to 100–150. The rationale to prioritize validated genes and their orthologs for further characterization *in vivo* in *Drosophila* or other organisms incorporates multiple data sources and differs with the overall goal of a given screen (Figure 2). In screens that emphasize 'gene discovery' (where screeners are after a particular gene that has eluded identification), the choice is straightforward, particularly if a candidate gene fulfills the characteristics of the missing component, based on either GO (gene ontology) annotation or other information available from genomic and/or genetic databases. If such candidates are not obvious, the screening data itself can be used to select hits. For example, when searching for new components of a 'canonical signaling pathway', the strongest hits that score in multiple different cell lines are the most logical class to validate *in vivo*. In cases where the pathway or biological process is fairly well understood or where there is little information to guide prioritization, the value of a genome-wide RNAi screen can reside in the global information it provides (a 'systems biology' view). In these cases, integration of various databases, ranging from literature mining to protein–protein and genetic interactions, will be attempted to indicate, build and confirm connections among the various hits validated in the secondary screens. Networks of genetic or protein–protein interactions between hits and known components of the process of interest might indicate which genes are more closely linked to the given pathway. Genes with particular binding or phosphorylation motifs linked to members of the pathway, or that show differential expression in functional microarray experiments might be components of feedback loops that control the pathway. Finally, new resources such as literature-mining applications might help find connections between screen hits and generate hypotheses that can be tested.

Advantages of conducting RNAi HTS in *Drosophila* cells

Conducting RNAi HTS in *Drosophila* presents several advantages over screening in mammalian cells. First, despite progress in the last few years, RNAi might never be as robust in mammalian cells as in *Drosophila* cells [31]. Second, the overall cost of an RNAi screen in *Drosophila* cells is, conservatively, four times less than that in mammalian cells. Third, reagents that target either the full human genome (siRNAs) or a significant portion of mouse and human genomes (shRNAs) are available only from commercial sources and academic consortia, respectively.

Regardless of these technical issues, it is clear that after optimization of the screening reagents, the next advances will be in the quality of the cell-based assays. Although the diversity of *Drosophila* cell lines lags far behind that of mammalian cell lines, *Drosophila* cells have shown a remarkable versatility in their use. For example, they have been used successfully to develop assays for host–pathogen interactions with infectious agents including *E. coli*, *Listeria* and *Mycobacterium* [26,28,32]. Although the normal host for the obligatory intracellular bacteria such as *Listeria* and *Mycobacterium* are vertebrate cells, they can be grown successfully in the hematopoietically-derived S2 and Kc *Drosophila* cells to conduct RNAi HTS screens to identify component involved in entry, proliferation and propagation of the bacteria [26,28]. Importantly, the homologs of candidates identified in the *Drosophila* screen were then validated in mammalian cells [28]. Furthermore, *Drosophila* cell-based assays can be designed to query the *Drosophila* genome for specific activities. For example, Ca^{2+} influx is strongly conserved between mammals and *Drosophila*, so three groups launched a screen to identify genes that are important in regulating store-operated Ca^{2+} entry. Using slightly different strategies, each group identified a crucial component necessary for the function of the Ca2+ release-activated Ca2+-channel (CRAC) pathway [27,33,34]. Significantly, this novel gene (named Orai 1, CRACM1 and *olf186-F*) was found to be mutated in patients suffering from a form of severe combined immune deficiency syndrome [27]. A powerful, general approach would be to run in parallel the same assay in *Drosophila* and mammalian cells and compare the two datasets to prioritize the hits for validation.

The success of RNAi HTS relies heavily on the development of 'informative and biologically relevant' cell-based assays, as described above, such as those using *Drosophila* primary cells. In particular, we have shown that neurons and muscle cells isolated from *Drosophila* embryos are well suited for RNAi screens (K. Sepp, J. Bai and N. Perrimon, unpublished data).

Parallel chemical and genome-wide RNAi screens for cytokinesis

In many cases, the phenotype caused by small-molecule inhibition of a protein is similar to the RNAi depletion phenotype of the same protein. Thus, to determine whether RNAi HTS might facilitate the identification of drug targets, we integrated genome-wide RNAi screening with small-molecule discovery in a study of cytokinesis. Our rationale was that examples of shared phenotypes should predict the biochemical target of a small molecule because the identity of the gene affected by the dsRNA is known. Based on this premise, we conducted parallel chemical genetic and genome-wide RNAi screens in *Drosophila* cells to identify genes that are important for cytokinesis, the final step in cell division, [1]. To insure full comparability between the two datasets, we used the same staining and imaging conditions for both screens. We arrayed cells into optical-bottom, 384-well plates, treated them with either small molecules or dsRNAs, and then screened specifically for binucleate cells, which is a hallmark of failed cytokinesis, by staining whole cells and DNA. After capturing images by automated microscopy, we identified wells that contained an elevated number of binucleate cells using a combination of automated image analysis and visual inspection.

In these screens we isolated 50 small-molecule inhibitors of cytokinesis and identified 214 genes that participate in this process, including a novel gene (*borr*) in the Aurora B pathway. We

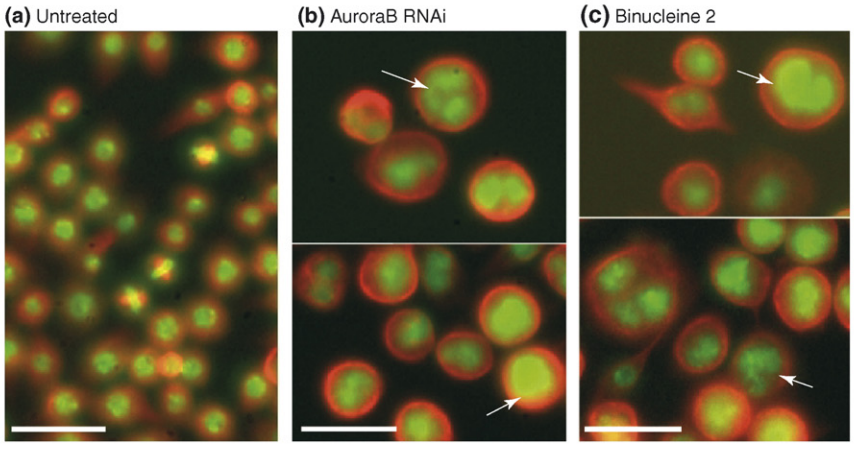

FIGURE 3

Comparison between small molecule and RNAi phenotypes. (a) Untreated *Drosophila* Kc_{167} cells. **(b)** Cells exposed to dsRNA targeting Aurora B kinase for 4 days. **(c)** Cells treated with 100 μM binucleine 2 for 24 h. Tubulin is in red, DNA in green. Note the similarities between the phenotypes of cells in (b) and (c), which are larger than untreated control cells (a), and include both bi-nucleate cells and cells with large diffuse DNA (see arrows). In addition, the increase in size is part of the phenotype caused by the *Aurora B* dsRNA and binucleine 2. All panels are imaged at the same magnification (scale bars, 20 μM).

characterized both RNAi and small-molecule phenotypes in detail using a bank of antibodies to important cytokinesis proteins. After systematically grouping the phenotypes into different categories, we compared the RNAi and small-molecule phenotypes within each group. The phenotypes caused by RNAi depletion of proteins from the Aurora B kinase complex and the small molecule binucleine 2 were identical (Figure 3), which indicates that the small molecule targets the Aurora B kinase pathway [1]. We confirmed that binucleine affects the Aurora B pathway by showing that it inhibits Aurora B cellular functions such as Histone H3 phosphorylation during mitosis. This finding is particularly satisfying because it validates our contention that a combination approach can enable rapid identification of a protein or pathway that is affected specifically by a small molecule.

It is important to note that this approach assumes that small-molecule and RNAi phenotypes are identical. This is often, but not always, the case because the mechanisms of small molecule inhibition and RNAi depletion differ [31]. First, the kinetics of achieving inhibition are drastically different: it is nearly instantaneous with small molecules but can take hours and even days with dsRNAs. Second, a protein is still present when it is treated with a small molecule, but it is either absent or severely depleted in an RNAi experiment. Although the outcome is the same in most cases, it will differ if the small molecule inhibits only one function of a multi-domain protein or if it affects a scaffold protein. Nevertheless, this approach has added another option to address target identification.

Clearly, the approach of combining genome-wide RNAi and small-molecule screens to drug-target identification should be widely applicable, especially in mammalian cells where there have been major advances in recent years in cell-based RNAi HTS [31,35]. In addition to the direct comparison approach, another strategy that is likely to be useful in drug discovery are modifier screens whereby cells are 'mutated' (or sensitized) by RNAi and screened for small molecules that modify the RNAi-induced phenotype. Such combination screens have the potential to identify very specific, active small molecules. Conversely, screens for RNAi modifiers of small molecule-induced phenotypes might also be valuable. As genome-wide RNAi screens become more routine and less costly, these modifier screens might form an unbiased target-identification tool for small molecules of completely unknown function. They might also uncover novel connections between proteins targeted by small molecules and other proteins, so revealing potential therapeutic areas.

Finally, another major advantage of the *Drosophila* system is the versatility and ease of validation from cells in tissue culture to animal, because compounds can be tested in whole animals by either injection or feeding [6,11]. Furthermore, the many mutant strains available and the ease of creating transgenic animals allow detailed follow-up analyses of the effect of a small molecule in several genetic backgrounds. Of course, small molecules discovered in *Drosophila* cells might not always translate to humans, but the preliminary work is much more straightforward and cost effective than in mammalian cells.

Conclusion: perspective on the relevance of *Drosophila* as a drug-discovery 'tool'

As a tool for drug discovery, *Drosophila* has two major advantages over other systems: (i) the ease of genetic manipulation both *in vivo* through traditional techniques and in cell culture through RNAi; and (ii) the reduced redundancy of the *Drosophila* genome compared to mammalian systems. The latter is particularly important given the conservation of the major signaling pathways; as the focus of drug development shifts from inhibiting individual genes and proteins to targeting pathways [36], *Drosophila* is an attractive system for four major areas of drug discovery.

First, for nearly three decades now, *Drosophila* has been a powerful system for gene discovery and, thus, discovery of novel drug targets. Unbiased RNAi HTS, combined with quantitative readouts of either

pathways or processes, significantly extends this utility. Genes identified through RNAi screens, such as novel components of signaling pathways (e.g. Wingless/Wnt [20], Hedgehog [21,22] and JAK/STAT [19,23]), many of which are likely to be conserved, become attractive new targets for drug intervention. Second, as these novel cell-based assays are developed for RNAi screening, HTS of diverse chemical libraries in *Drosophila* cells provide a strong complement to traditional chemical screening either in mammalian cells or *in vitro*. Third, combining these two approaches – chemical and RNAi screening in *Drosophila* cells (see above) – allows rapid identification of novel drugs and their targets in a parallel fashion. Fourth, *Drosophila* remains a powerful system for studying the biological effects of existing drugs with known, highly conserved targets (e.g. rapamycin) [37]. Major signaling pathways have distinct, visible phenotypes. Thus, studies in *Drosophila* might identify off-target effects of promiscuous inhibitors of kinases, and provide a platform for screening for drug effect-modifying genes either *in vivo* or in cells.

Finally, traditionally the relevance of *Drosophila* to understanding human diseases has focused on the molecular basis of the misregulation of signaling pathways in cancer, but the fly is becoming a model of choice in studies of physiology, neurodegenerative disease and aging [8]. More recently, studies in *Drosophila* have provided fundamental insights into innate immunity [38] and fat metabolism [39], and these studies are a preview of the important contributions that will come from the humble fly.

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