Methods 68 (2014) 82–88

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

RNAi screening in Drosophila cells and *in vivo*

Stephanie E. Mohr

Drosophila RNAi Screening Center, Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, United States

ARTICLE INFO

Article history:
Available online 24 February 2014

Keywords:
RNAi
Functional genomics
High-throughput screening
Drosophila
Cell-based assays
In vivo assays

ABSTRACT

Here, I discuss how RNAi screening can be used effectively to uncover gene function. Specifically, I discuss the types of high-throughput assays that can be done in Drosophila cells and *in vivo*, RNAi reagent design and available reagent collections, automated screen pipelines, analysis of screen results, and approaches to RNAi results verification.

1. Introduction

1.1. RNAi as a research tool in Drosophila

As a research tool, RNA interference (RNAi) harnesses an endogenous activity to reduce or "knock down" RNA levels in a sequence-specific manner. The availability of RNAi as a method has opened the door to functional genetic screening in new model systems and contexts (reviews include [1–5]). For *Drosophila*, RNAi made it possible to perform full-genome screens in cultured cells and in specific tissues *in vivo* using systematically designed and generated libraries of RNAi reagents, an important supplement to classical genetic screening [6]. For Drosophila cells, the active RNAi reagent is double-stranded RNA (dsRNA), typically 50–500 bp in length, which can be synthesized *in vitro* in 96-well format or larger volumes [7,8]. For *in vivo* studies, transgenic fly stock collections were initially based on long dsRNA hairpin-encoding constructs introduced into flies via P-element transposon-based transgenesis [9]. The field subsequently moved to using short hairpin (shRNA)-encoding constructs in optimized vectors and introduced into flies using site-directed approaches, resulting in improved expression and knockdown, including in the germline [10].

RNAi is frequently used in Drosophila for low-throughput studies, such as when a large amount of relatively uniform material is needed (e.g. cell-based RNAi knockdown) or to study the effects of disruption of one or a few genes in a specific stage or tissue (e.g. using the Gal4-UAS system *in vivo*). RNAi is also contributing to development of Drosophila as a tool for personalized medicine as reviewed in [11]. One of the most powerful aspects of RNAi, however, is that it can be applied at genome scale. The focus of this chapter will be on large-scale studies. Nevertheless, many of the same approaches also apply to small-scale RNAi studies. In addition, many of the approaches presented here for RNAi screens are also relevant to other types of screens in cells or *in vivo*, e.g. over-expression [6] or small molecule screens. RNAi and small molecule screens can be performed in parallel as reviewed in [12] and exemplified by [13]. In the past, classical genetic and small molecule screening approaches helped inform best practices for RNAi screening. Similarly, we can expect that what we have learned from RNAi will influence approaches to new technologies for screening, e.g. genome engineering-based screening technologies [14].

1.2. Information flow in RNAi screens

Although the specific reagents and assays used for cell-based vs. *in vivo* RNAi screens are different, the two approaches share similarities in terms of information flow (Fig. 1). Before embarking on a screen on a particular topic, several decisions have to be made, including what assay to perform, what controls to use, and whether or not a full-genome library is appropriate – and if not, what subset of genes will be included in the screen. Researchers typically rely on pre-existing library collections for screens and thus, decisions regarding reagent design have largely been made for them. Nevertheless, reagent design, "coverage" (i.e. the number of unique reagents per gene), the frequency of updates to the li-

Abbreviations: dsRNA, double-stranded RNA; GFP, green fluorescence protein; OTEs, off-target effects; qPCR, quantitative real-time PCR; RNAi, RNA interference; shRNA, short hairpin RNA.

E-mail address: stephanie_mohr@hms.harvard.edu

http://dx.doi.org/10.1016/j.ymeth.2014.02.018
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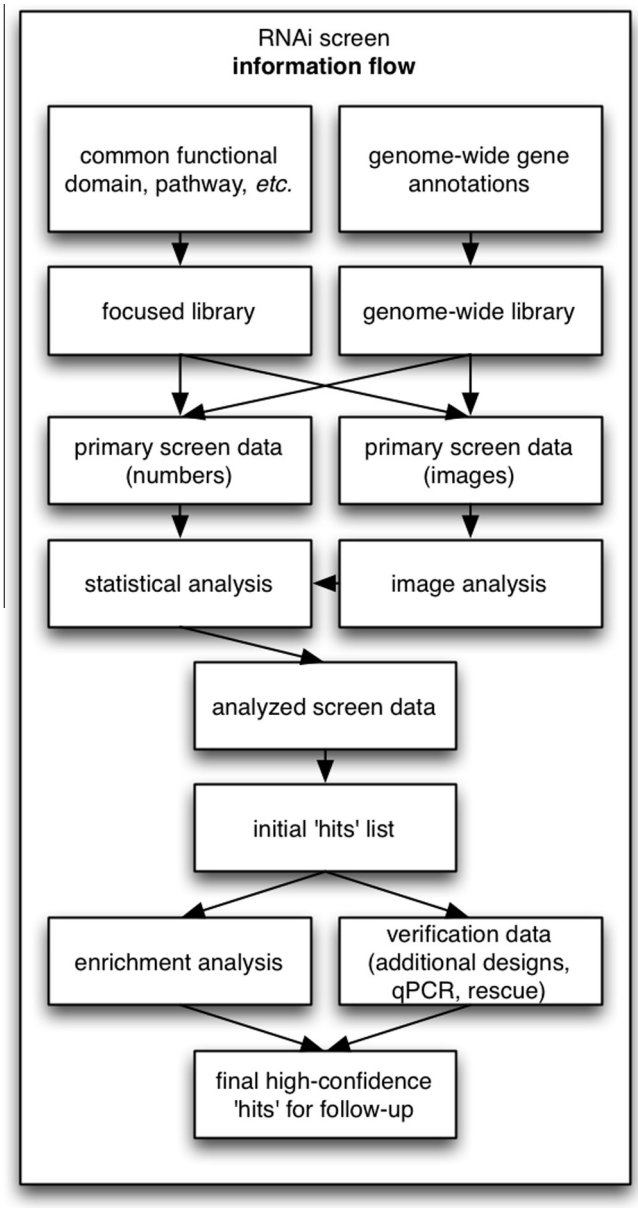

Fig. 1. Information flow typical for cell-based or *in vivo* RNAi screens. Prior to screening, gene annotation information is used for reagent design and identification. Additional information can be used to narrow the list of candidates, e.g. to a subset of genes that encode proteins that share a common functional domain (e.g. all kinases), genes previously implicated in a specific pathway or process, genes identified in another type of large-scale study. etc. Image data is typically reduced to numerical values via automated analysis. These values or raw values (e.g. from 'plate-reader' screens such as for assays based on luciferase readouts) are then analyzed statistically to separate outliers or 'hits' in the primary screen from non-hits. The statistically determined initial hits list is then (1) used to identify additional reagents (e.g. distinct designs targeting the same genes) and tests (e.g. additional phenotypic assays) appropriate for moderate-throughput experimental verification of the primary results, and (2) integrated and compared with existing knowledge. Some combination of experimental testing and bioinformatics is typically used to determine a 'high-confidence' list of candidates that are then subjected to additional validation (e.g. RNAi rescue) and more detailed follow-up studies.

brary and other aspects of library design should be taken into consideration, as these will affect the number of plates or fly crosses in the screen, the quality and scope of results, and how data are analyzed later. Moreover, as gene annotations change over time, reagent annotations should be revisited prior to making final decisions about what set of reagents is used, as well as post-screening.

During the screen itself, a large number of samples and volume of data must be managed, with accurate preservation of the relationship between specific reagents, their positions on plates or in fly vials, and corresponding phenotype data. The leading edge in information tracking during an RNAi screen (or other large-scale screen, such as a small molecule screen) is tracking of screening plates or fly vials using barcode labels. For automated screens, barcode readers can be integrated into the automation pipeline. For fly vials handled directly by researchers, hand-held readers can be used to scan barcodes, followed by entry of phenotype data or other information into a spreadsheet or database. Following a large-scale image-based screen, the images must be analyzed, ideally using an automated approach. Images and other readouts are typically reduced to numerical scores, followed by statistical analysis to identify 'hits' in the primary screen. Next, the initial hits list is processed, additional experiments are performed, and together these results are used to define a high-confidence set of genes for further follow-up. Keeping careful digital records throughout a cell-based or *in vivo* screen is an essential basic task. For cell-based screens, researchers typically have access to data management tools through the library provider. For *in vivo* screens, researchers more typically set up their own databases or spreadsheets to track results. If phenotypes are expressed in text (e.g. "lethal" or "up-turned wing") rather than auto-processed images or numbers, then standardized wording (i.e. a controlled vocabulary) should be established and used consistently in order to facilitate later analysis, interpretation and comparison.

1.3. Key starting points for development of Drosophila RNAi screens

Below I present more details on cell-based and *in vivo* RNAi screening, as well as information about data analysis and integration, reducing false positive discovery and experimental validation. In addition, I would like to point out that a large number of protocols, software tools, databases, helpful tips, stock collections, etc. relevant to Drosophila RNAi are freely available online. A few good starting points include the Drosophila RNAi Screening Center (DRSC) website (www.flyrnai.org) [15], the GenomeRNAi database (www.genomernai.org) [16], the Vienna Drosophila RNAi Center (VDRC) (http://stockcenter.vdrc.at/control/main), the National Institute of Genetics (NIG)-Fly (http://www.shigen.nig.ac.jp/fly/nigfly/), and the Sheffield RNAi Screening Facility (http://www.rnai.group.shef.ac.uk/). I also maintain an informational blog on Drosophila RNAi and related topics (http://flyrnai.blogspot.com/). Additional relevant online tools, resources and references are presented below and in Tables 1 and 2.

2. Drosophila cell-based RNAi screening

2.1. Overview of cell-based screens

More than a hundred Drosophila cell-based RNAi screens have been performed since the approach was first put to use at genome scale more than ten years ago [1]. Nevertheless, we have only scratched the surface in terms of the plethora of single- and multi-parameter assays that could be performed in this context (see Section 2.2. and 2.3). Cell-based screens in general are usually performed in one of two formats. For pooled screens, RNAi reagents are introduced at random, cells positive in the assay are selected (e.g. survive some treatment) and the RNAi reagents responsible for the phenotype are then deconvolved, e.g. via next-generation sequencing. For arrayed screens, RNAi reagent(s) are individually located in wells of a micro-well plate (96- or 384-well plate), each well is assayed, and positive hits are simply looked up using a spreadsheet or database of plate-to-well mapping information.

Table 1
Selected online resources for Drosophila cell-based and *in vivo* RNAi screening.

Resource type/name	URL(s)	Reference(s)
General information, external links and data access		
Drosophila RNAi Screening Center (DRSC)	www.flyrnai.org	[15]
FlyBase resources list	http://flybase.org/static_pages/allied-data/external_resources5.html	[38]
Genome RNAi	www.genomernai.org	[16]
RNAi fly stock collections and information		
Bloomington Drosophila Stock Center	http://flystocks.bio.indiana.edu/	[80]
NIG-Japan	http://www.shigen.nig.ac.jp/fly/nigfly/	[81]
RNAi Stock Validation and Phenotypes (RSVP)	http://www.flyrnai.org/rsvp	
Transgenic RNAi Project (TRiP)	http://www.flyrnai.org/trip	
Vienna Drosophila RNAi Center (VDRC)	http://stockcenter.vdrc.at/control/main	[9]
Drosophila cultured cell lines and information		
Drosophila Genome Resource Center (DGRC)	https://dgrc.cgb.indiana.edu/Home	
Cell-based and in vivo fly RNAi collections search tool		
Updated Targets of RNAi Reagents (UP-TORR)	http://www.flyrnai.org/up-torr	[49]
Cross-species RNAi rescue		
DRSC RNAi rescue search tool	http://www.flyrnai.org/cgi-bin/RNAi_find_rescue_compl.pl	[74]
FlyFos collection search tool	http://transgeneome-old.mpi-cbg.de/index.php?id=42	[76]

Table 2
Selected publications relevant to Drosophila RNAi screen assays, analysis and validation.

Task/activity	Reference(s)
Cell-based assay development/optimization – general	[5,28,40]
Cell-based assay development/optimization – cell line selection	[7,17,18]
Cell-based assay development/optimization – plate-reader	[24,25,33]
Cell-based assays, high-content image-based – examples	[30–32]
Cell-based assays, double knockdown – examples	[34,35]
Analysis of cell-based screen data – overview	[5,7,36]
Full-genome *in vivo* screens – examples	[33,51–54]
Gal4-UAS system – reviews	[47,48]
Integration/enrichment of screen data	[70–72]
Minimizing false discovery	[25,40,41,61,62]

Although pooled screening is common for mammalian cell screens, introduction of single RNAi reagents and deconvolution of reagents corresponding to hits is not feasible with dsRNA as used for fly cells. Thus, all of the material presented below assumes an arrayed screening approach in which the library is provided in 96- or 384-well plates. Fig. 2 provides a summary workflow for cell-based RNAi screening. For any new cell-based screen, researchers must first define the appropriate cell type, assay, controls, reagent library and mode of reagent delivery. Additional considerations include cost reduction (such as replacing costly dyes or antibodies with fluorescent protein markers), optimization of readouts (such as defining an appropriate number of images per well for an image-based screen) and automation of routine steps.

2.2. Cell type selection

After choosing a topic of interest, an appropriate cell type should be selected. Many helpful suggestions and lists of commonly used cell lines have been presented previously [7,17,18] and additional updated information on Drosophila cultured cells is presented in another chapter [19]. The ideal candidate cell type for an RNAi screen would be similar to the tissue of interest, express relevant genes, amenable to growth in micro-well plates, responsive to RNAi, and otherwise appropriate for the assay (e.g. adherent to the assay plates, as is needed for most automated imaging systems). Many of these features are assay-specific and best tested directly. The availability of transcriptomic data for many cell lines from modENCODE [20] makes it possible to search for expression of genes of interest, which might help define an appropriate cell type. Historically, most screens have relied on a few common blood-like cell types (e.g. S2 cells). However, additional lines of different origins are available [19] and a new oncogene-based approach makes it possible to more easily establish new cell lines in the future [21]. This is important because cell lines of different lineages express different genes and exhibit different activities. Thus, as new cell lines become available, cell-type specific activities can be assayed and cell-specific components of known pathways might be identified. As an alternative to cell lines, primary cultures can be generated from embryos that express a fluorescent protein in the lineage of interest as described in [22,23] (see also www.flyrnai.org/DRSC-PCP.html). Drosophila cell lines can be obtained from the Drosophila Genome Resource Center (DGRC) (dgrc.cgb.indiana.edu) (Table 1). In some cases a screen is performed with a derivative cell line that stably expresses a reporter construct (see Section 2.3). Genome engineering techniques [14] open the door to production of specific mutant and reporter cell lines.

2.3. Cell-based assay development

Choosing an assay that will identify genes of interest, can be done in high-throughput mode, and has a good signal-to-noise ratio is critical to the overall success of a screen. Helpful information on assay development has been presented previously [4,5,8,18,24,25]. Major categories of past Drosophila cell-based RNAi screens include screens related to signal transduction and host-pathogen interaction screens [1,26] but many other topics have been covered and the possibilities are endless. Some assays are measured at the microplate well level, such that all cells in the well are assayed (e.g. total luminescence or total fluorescence per well). By contrast, image-based assays can be used to take measurements at the level of individual cells, facilitating complex multi-parameter readouts [2,4]. Assay readout instruments for high-throughput cell-based screens generally fall into three categories, (1) luminometers/fluorimeters, commonly called "plate readers," (2) laser scanning cytometers, which provide cell-level information but are not true microscopes, and (3) automated microscopes, which generate detailed images.

Previously published protocols for specific approaches provide helpful starting points for assay development [27–29]. Instructive recent examples of high-content image-based screens include screens based on antibody staining [30], green fluorescence protein (GFP) tagging [31], or fluorescence in situ hybridization [32]. Instructive examples of plate-reader screens include a luciferase

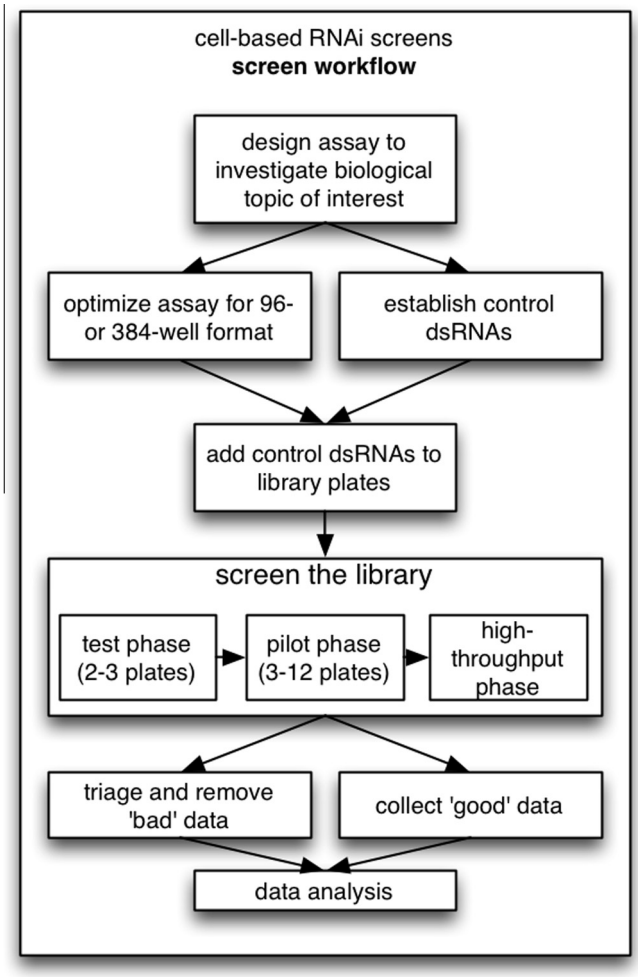

Fig. 2. Cell-based RNAi screen workflow. For cell based screening, once a topic and appropriate cell type has been selected, the next step is typically to develop and optimize an assay for 96- or 384-well format plates. Establishment of controls can occur concurrent with that step. The results of a Z-factor analysis with controls in the final plate format for screening can help determine if the signal-to-noise ratio is appropriate for a high-throughput screen. For the screen itself, a small-scale test phase is followed by a period of ramping up (pilot phase) and then the high-throughput screen itself. Data triage, such as making a heatmap view of plate-based data, can reveal 'bad' data (e.g. plate-level patterns indicative of pipeting errors) that should be excluded prior to analysis. Including a positive control such as dsRNA targeting the gene *thread* can help confirm that RNAi is working in the specific cells used for screening. This is particularly important for screens conducted over weeks, as cells sometimes become resistant to RNAi when they are passaged several times or become overcrowded.

reporter-based signaling study [33]. Instructive examples of double-knockdown assays, in which RNAi is used to create a sensitized background or uncover synthetic effects, have also been reported [34,35]. Past screens (see http://www.flyrnai.org/DRSC-PRY.html and www.genomernai.org) provide additional examples of the broad range of assays that can be used successfully for Drosophila cell-based RNAi screening. Moreover, based on past progress and new technologies, I expect that the number and scope of possible cell-based screens will continue expand. As new assays are developed, such as additional multi-parametric image-based assays or more sensitive and specific reporter assays, we can explore increasingly complex and specific phenotypes. New approaches to gene perturbation will also allow for generation of specific modified and/or tagged backgrounds for screening. In the case of engineered mutant backgrounds, researchers might choose to perform a screen in parallel in the unmodified and modified cells, leading to identification of context-specific results.

2.4. Cell-based assay optimization

Once a cell-based assay has been developed, and important next step is to optimize the assay, i.e. to improve the signal-to-noise, establish controls, make sure the assay is compatible with the available facilities, library and equipment, make sure the cells grow in the appropriate format (e.g. 384-well plates), reduce costs, and improve the signal-to-noise ratio. Above-mentioned publications helpful for assay development also provide input on optimization. Helpful advice on using statistical analysis to assess the signal-to-noise ratio (or "assay robustness") has been presented by others [36]. Z-factor analysis is arguably the most commonly used statistical measure of assay robustness [37]. Many RNAi reagents will have direct or indirect impact on cell growth, division and/or viability. If these are not taken into consideration in the assay, they can significantly contribute to false positive discovery [25]. Thus, it is important to have a 'built-in' strategy for separating general effects from specific positive phenotypes. Controlling for cell number is typically done by normalizing results with the experimental readout to results obtained with another, constitutively expressed readout (for plate-reader screens) or a cell count (for image-based screens). Additional advice on plate-reader assays has been presented previously [25].

2.5. Cell-based reagent libraries and dsRNA synthesis

A limited number of commercial and academic groups have generated genome-scale libraries for Drosophila cell-based RNAi screens (see "RNAi" under the header "Drosophila Material Resources" in the "All Resources" sub-section of the "Resources" tab on the FlyBase menu bar) [38]. Cell-based reagents from the DRSC and DKFZ are included in searches with the Updated Targets of RNAi Reagents (UP-TORR) tool from the DRSC (www.flyrnai.org/up-torr), which provides information about RNAi reagents from major public collections based on updated gene annotation information. A tool for evaluation of RNAi libraries has also been reported [39]. Library layout and replicate number will impact the screen outcome [40]. A comparison of similar screens performed with first-generation and updated libraries additionally highlights the impact of library composition on results [41]. Researchers can choose from genome-wide libraries or smaller focused libraries (e.g. targeting all kinases and phosphatases), or create custom libraries. Custom libraries can be created *de novo* based on new primer designs or, to save time and resources, based on existing PCR template collections. Detailed protocols for making dsRNAs (e.g. to create new libraries or establish controls) can be viewed or downloaded online (http://www.flyrnai.org/DRSC-PRS.html) and have been presented previously [7,8].

2.6. Delivery of dsRNA to cells

Introducing RNAi reagents into cells is typically done using the "bathing" method, in which cells take up dsRNA from solution, or using a lipid-based cell transfection reagent. Protocols and information about both methods are presented online (http://www.flyrnai.org/DRSC-PRR.html) and have been published [7,8]. The bathing method has significant advantages over transfection, as transfection is costly, inefficient and can be toxic to cells. Thus, transfection is typically used only when there is another reason to do so (e.g. when a DNA reporter construct will be introduced concurrent with delivery of the dsRNA).

2.7. Cell-based screen pipeline

The high-throughput cell-based screen itself is typically broken down into a test phase, with appropriate controls to confirm that

things are working; a pilot phase, in which the screener begins to work with more plates; and a high-throughput phase during which plates are processed as rapidly as is practically possible without compromising quality (Fig. 2). Assay development and optimization typically takes months, even years, whereas the cell-based screen itself can typically be completed in weeks (in my experience, one person working full time on a screen in a facility with automated equipment can complete a genome-wide screen in five to ten weeks, with the difference in timing depending upon the specific assay being performed—plate-reader screens can usually be done more quickly than image-based screens, for example). Following the screen, data are collected and for image-based screens, automated image analysis solutions are used to reduce image data to numerical outputs [4]. It is useful to 'triage' data (i.e. make an assessment of data quality), such as by looking at a heat map of results values in plate format in order to detect systematic problems [36]. Common problems that might be detected this way include "edge effects," which suggest that cells in outer wells are less healthy than those in inner wells, or repeating patterns, which are usually attributable to automation or pipetting errors. Following data triage, 'bad' data are excluded from further analysis and 'good' data continue in the analysis pipeline. Approaches to data normalization and analysis are presented online (see "Documentation" at www.dkfz.de/signaling/cellHTS) and published previously [36,42–44]. Approaches to limiting false discovery and for integrating data from cell-based or *in vivo* RNAi screens with other datasets are discussed in Sections 4.1 and 4.2. The 'gold standard' RNAi verification test, RNAi rescue, is discussed in Section 5.

3. Drosophila *in vivo* screens

3.1. Overview of Drosophila in vivo screening

Drosophila *in vivo* RNAi screens use the Gal4-UAS approach to drive expression of the RNAi reagent in a specific stage or tissue. Methods for Drosophila *in vivo* RNAi screening have been presented previously [3]. In addition, a broader discussion of genetic approaches is presented in another chapter [6]. The possibilities for *in vivo* RNAi assays are extremely broad, ranging from early developmental processes [45] to activities like flight that are restricted to adults [46]. The Gal4-UAS approach and permutations (e.g. use of Gal80) have been reviewed previously (see for example [47,48]). Fig. 3 provides a summary workflow for *in vivo* RNAi screening.

3.2. Identifying appropriate reagents for in vivo RNAi

Changes to reagent design have improved the quality of fly stock collections and made it possible to knock down genes in the germline [10]. The UP-TORR tool provides one starting point for identification of publically available fly stocks for a given input gene list [49]. Genome-scale collections are available from the Transgenic RNAi Project (TRiP) through the Bloomington Drosophila Stock Center (BDSC), as well as at the VDRC and NIG-Japan (Table 1). The RNAi Stock Validation and Phenotype (RSVP) online resource (www.flyrnai.org/rsvp) makes it possible to view available knockdown data, including qPCR and phenotype information. FlyBase also curates information about RNAi fly stocks [38]. RNAi is commonly used *in vivo* for small-scale studies and focused screens. At least one group has performed a small-scale *in vivo* double-knockdown screen [50]. In addition, a number of full-genome studies have been reported [33,51–54]. Not all assay are dependent on transgenic RNAi; a large-scale screen in which dsRNA was injected into embryos, followed by time-lapse confocal microscopy, has also been reported [55].

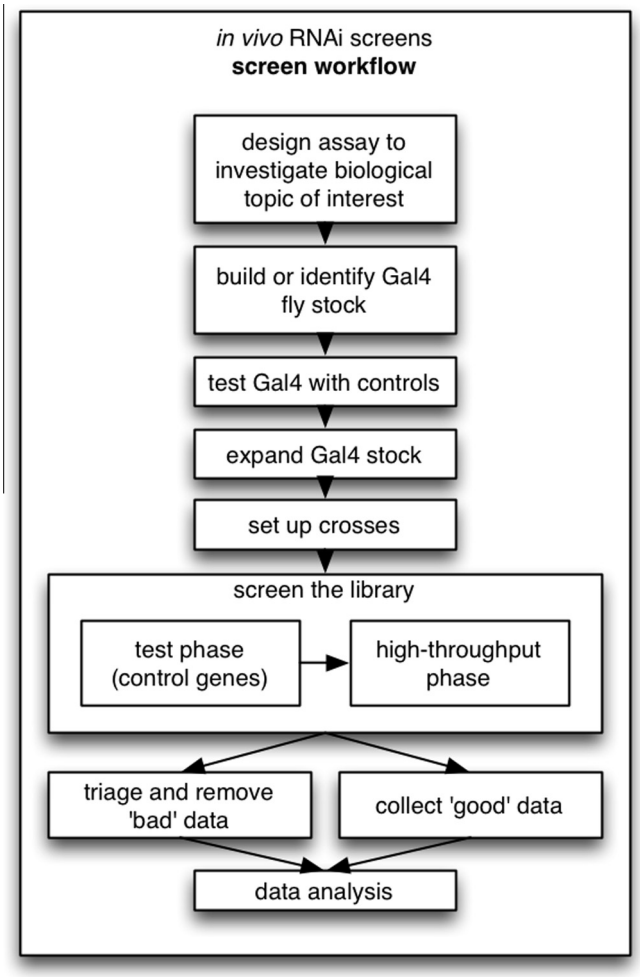

Fig. 3. *In vivo* RNAi screen workflow. The first step is typically to develop an assay that is compatible with the RNAi system, in which ideally, the assay is performed on F1 flies following cross of virgin females carrying an appropriate Gal4 driver to males that provide the RNAi transgene. Some assays, such as those requiring a specific genetic background, specific fluorescent protein markers, Gal80 control of expression, and so on, require building of one or more complex genetic background prior to the *in vivo* screen. Negative and positive control RNAi transgenic stocks should be established and included in the screen. Screening itself typically begins with a test phase followed by a high-throughput phase. For many screens, a subset of RNAi transgenes result in lethality prior to the stage at which the assay will be performed, such that a category of "lethal in combination with this Gal4 driver" (or for maternal screens, "no eggs") becomes one major category of screen 'hits' and "positive in the assay" another. After the high-throughput screen, failed crosses, inconclusive results, etc. ('bad' data) are removed and useful data is analyzed.

3.3. In vivo assay development, optimization and screening

The number and type of *in vivo* assays that can be performed is extremely broad and limited for the most part by throughput (it is much easier to screen for some phenotypes than others). Phenotypic assays are also discussed by [6]. Sequence non-specific effects have been reported for longevity assays [56], emphasizing the need for careful assay development. As for cell-based screens, it is critical to establish appropriate negative and positive controls. Ideally, negative controls would be in the same genetic background and include RNAi, such as an RNAi construct targeting an unrelated gene (e.g. *white*) or a gene not encoded by the Drosophila genome (e.g. GFP or luciferase). The time it takes to perform an *in vivo* screen depends for the most part on (1) the specific design of genetic crosses necessary to generate the experimental animals, (2) the assay itself (with increased time and effort required if the assay depends upon

dissected tissue, a large volume of that tissue or whole animal material, etc.), (3) the number of gene targets screened (i.e. full-genome or subset), and (4) reagent coverage (all available reagents or a subset). As mentioned in Section 3.2, due to the time-intensive nature of setting up crosses, dissecting tissues, etc., for many *in vivo* RNAi screens researchers limit the set of genes to be tested, for example to a group of related proteins (e.g. all kinases as described in [57]).

3.4. Increasing throughput and other innovations for in vivo screens

As compared with cell-based screens, relatively few *in vivo* studies incorporate automation, miniaturization or other strategies to improving throughput. Advances in things like automated video tracking (see for example http://towerlab.usc.edu/?q=USCVideoTrack) and higher-throughput approaches to staining [58] might be relevant in the future. Culturing of larvae in 96-well format has been used in small molecule screening [59]. As mentioned in Section 1.2, using barcode-labeled fly vials and a hand-held barcode reader, together with an associated spreadsheet or database, can make it easier to capture raw data in digital format during the high-throughput phase of a screen. Availability of new Gal4 lines opens the door to additional *in vivo* assays. At least two new collections are available, the Vienna Tiles collection (http://stockcenter.vdrc.at/control/vtlibrary) and the Janelia Farm Research Campus collection [60].

4. Limiting false discovery in cell-based or *in vivo* RNAi screens

4.1. General strategies for limiting false discovery

Large-scale studies are associated with reagent non-specific 'noise' that contributes to false discovery. Moreover, RNAi is associated with the possibility of reagent-specific off-target effects (OTEs), contributing to false positive discovery, as well as false negative results, e.g. due to a lack of robust knockdown. Others have previously reviewed or presented approaches to detecting and limiting the impact of false discovery, which include improvements to reagent design, improvements to statistical analyses, and comparison of primary 'hits' with transcriptomics data [61] [2,36,43,62–65]. For plate-reader screens, it can be useful to eliminate hits corresponding to severely reduced cell viability prior to further analysis, as exemplified in a signaling study [33]. Repeating the screen assay to confirm primary screen hits can eliminate some percentage of candidates. Performing additional RNAi-based secondary assays can also limit false positive discovery. Because secondary assays can be performed with a smaller number of candidates as compared with the large-scale screen, it becomes possible to test multiple related assays and more replicates, providing additional confidence at the RNAi reagent level, as well as to test more reagents per gene, increasing gene-level confidence in the primary screen results.

4.2. Integration of multiple datasets as a strategy for limiting false discovery

Integration of the analyzed RNAi screen results with data obtained using independent methods is an effective approach to further limiting false discovery, focusing on high-confidence candidates, and identifying functional networks [66]. To help accomplish this, RNAi screening can be performed in combination with transcriptomics or proteomics (e.g. [67–69]). Similarly, if orthologs of different species are hits in related assays, confidence increases in those hits. Thus, performing similar functional genomics screens in parallel, as was done at genome scale using cell-based Drosophila RNAi and yeast mutant strains in a recent study of nucleolar size [30], is an effective way to identify high-confidence, evolutionarily conserved candidates for further study. Arguably the most approachable method for data integration is comparison of RNAi screen data with the existing literature, such as via enrichment analysis. The underlying assumption with an enrichment approach is that if more than one member of the same pathway, process or complex are hits in the screen, confidence increases that those hits are valid. FlyMine [70], NIH DAVID [71], and COMPLEAT [72] are among the online resources that facilitate enrichment approaches and help screeners focus in on the highest confidence candidates, pathways and complexes for further analysis.

5. Verification of results from cell-based or *in vivo* RNAi screens

Verification of RNAi results is a significant bottleneck. Confirmation of knockdown efficiency via quantitative real-time PCR (qPCR) can provide supporting evidence for a relevant target gene-to-phenotype relationship. A method to assess protein depletion following RNAi in flies has also been reported [73]. Ultimately, however, verification of RNAi results rests on additional experimental analyses, such as rescue of the RNAi phenotype with an RNAi-resistant transgene and comparison of RNAi results with mutant phenotypes. Approaches to RNAi rescue reported for Drosophila include the use of non-*melanogaster* Drosophila species genome fragments, which can be similar enough in amino acid sequence to confer activity but divergent enough in nucleotide sequence to evade RNAi [74–76], and the use of synthetic or codon usage-altered constructs [77,78]. RNAi reagents can also be altered to maintain potential miRNA-like seed sequences while disrupting perfect match to the target, i.e. the "C911" approach, as has been reported for mammalian systems [79]. The new availability of genome engineering approaches should make it possible to create custom RNAi-resistant fly stocks and more easily generate mutant animals (for in-depth discussions of genome engineering methodologies and other genetic approaches see [14,6]). The ease of high-throughput RNAi screening in cells, as well as the ease of performing stage- and tissue-specific knockdown *in vivo*, together suggests that genome engineering approaches will not replace RNAi in the future but instead, will become an important additional tool in the Drosophila toolbox.

Acknowledgements

I extend my thanks to Norbert Perrimon, Liz Perkins, and the DRSC/TRiP staff for many helpful discussions. The Drosophila RNAi Screening Center and the Transgenic RNAi Project at Harvard Medical School and are supported by NIH NIGMS R01 GM067761, NIGMS R01 GM084947 and NCRR/ORIP R24 RR032668 to N. Perrimon. I also receive support from the Dana Farber/Harvard Cancer Center, which is supported in part by NCI Cancer Center Support Grant # NIH 5 P30 CA06516 to E. Benz.

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