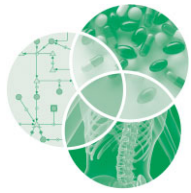
Focus Article



# Where gene discovery turns into systems biology: genome-scale RNAi screens in *Drosophila*

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Systems biology aims to describe the complex interplays between cellular building blocks which, in their concurrence, give rise to the emergent properties observed in cellular behaviors and responses. This approach tries to determine the molecular players and the architectural principles of their interactions within the genetic networks that control certain biological processes. Large-scale loss-of-function screens, applicable in various different model systems, have begun to systematically interrogate entire genomes to identify the genes that contribute to a certain cellular response. In particular, RNA interference (RNAi)-based high-throughput screens have been instrumental in determining the composition of regulatory systems and paired with integrative data analyses have begun to delineate the genetic networks that control cell biological and developmental processes. Through the creation of tools for both, *in vitro* and *in vivo* genome-wide RNAi screens, *Drosophila melanogaster* has emerged as one of the key model organisms in systems biology research and over the last years has massively contributed to and hence shaped this discipline.  *WIREs Syst Biol Med* 2011 3 471–478 DOI: 10.1002/wsbm.127

## INTRODUCTION

Increasingly used over the last years, the term 'systems biology' denotes current endeavors and concepts in biosciences to understand biological systems in their entity rather than their isolated parts.[1] This holistic approach not only aims to understand the interactions between components within a system but also aspires to decipher how a system as a whole responds to perturbations.[2] This perspective thus provides a contrasting yet complementary vision to that of the classical reductionist paradigm. Ultimately, both strive to understand the wiring of biological systems during development and homeostasis and to predict the responses by an organism, at the level of genes and proteins, upon environmental and genetic alterations.

Classical forward genetic screens have been exceedingly powerful in identifying genes that contribute to a specific phenotype. These screens rely on the generation of random mutations and the subsequent identification of the gene(s) responsible for the observed defect in the biological process at study. This approach has proven to be an excellent tool for gene discovery but has typically resulted in the characterization of only a small set of genes out of these screens due to the labor-intensive process of mapping the mutation responsible for a specific phenotype. Similarly, biochemical methods have mainly been employed in the context of 'single gene studies' and detailed molecular characterization of gene functions has thus been amenable to only a subset of genes implicated in a specific biological process. In contrast to these 'single gene-centered studies', recent technological advances have facilitated systems biology approaches, enabling researchers to systematically and quantitatively measure and perturb biological networks. Most notable are experimental techniques that monitor changes in the abundance of a multitude of transcriptional and translational products in parallel, and methods for systematic depletion or overproduction of system components. Along with these experimental strategies,

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statistical, mathematical and computational methods have empowered systems biologists, permitting more facile integration of data with models, ultimately generating a better comprehension of the complexity and architectural principles of biological networks.

## *DROSOPHILA* AND SYSTEMS BIOLOGY

The goal to study genomes on their whole scale has sparked efforts in various model organisms to generate novel tools and collections of reagents to systematically interrogate gene function. These reagent collections include a full-genome knockout collection in yeast,[3,4] genome-wide RNA interference (RNAi) libraries for cell culture-based screens in *Drosophila*,[5] mouse, and human cells (reviewed in Ref 6), and tools for *in vivo* genome-wide RNAi screens in *Caenorhabditis elegans*[7,8] and *Drosophila*[9] (Table 1). In addition, numerous large-scale protein complex purification[10–12] and protein localization studies[13] shed light on the organizational principles of the protein–protein interaction network. Besides genome-wide loss-of-function screens, these data provide another platform for systematic data integration and network analysis. As a number of recent articles have extensively reviewed genome-scale loss-of-function studies in these various organisms,[6,14] we have decided to focus our review on how the available repertoire of genetic tools in *Drosophila* makes this particular model organism an attractive choice for systems biology for both *in vitro* and *in vivo* studies.

### Cell Culture-Based RNAi Screens

RNAi has emerged as one of the key methodologies to interfere with gene function in a systematic manner. The availability of whole-genome sequences has permitted the design of genome-scale RNAi libraries in multiple organisms. RNAi relies on the ability of small interfering RNAs (siRNAs), long double-stranded RNAs (dsRNAs), or short hairpin RNAs (shRNAs) to degrade mRNAs and hence silence a specific target gene. RNAi-mediated loss of function generally results in a partial perturbation of gene function, similar to hypomorphic alleles. Compared to classical forward genetic screens that employ random mutagenesis for gene discovery, RNAi screens broaden the scope of loss-of-function examination to entire genomes. Because the identity of the knocked down gene is known, systematic RNAi approaches enable rapid assessment of whether 'hit lists' from large-scale screens are enriched for genes annotated as having similar molecular functions. Furthermore, this knowledge allows one to associate specific phenotypes to particular genes or groups of genes. By integrating protein–protein interaction data with RNAi screen-derived phenotypic data, these studies have begun to decipher the regulatory networks that underlie particular phenotypic classes and hence provide a 'genetic framework', wherein further biochemical analysis can determine the underlying molecular mechanisms responsible for certain terminal phenotypes.

**TABLE 1** | Resources for Systematic RNA Interference (RNAi) Experiments in *Drosophila*

| Study | Application |
|---|---|
| Boutros et al.[5] | Generation and initial use of a genome-wide RNAi library for cell culture-based screens |
| Dietzl et al.[9] | Generation and characterization of a genome-wide transgenic RNAi library |
| Ni et al.[15] | Generation of a transgenic RNAi library for a subset of 2043 neurally expressed genes |

Our laboratory has generated an infrastructure, the *Drosophila* RNAi Screening Center (DRSC), that is amenable for high-throughput *Drosophila* cell culture-based genome-wide RNAi screens.[5,14] RNAi constructs are typically spotted in an arrayed format, in which each well of a microtiter plate contains one individual RNAi construct. This format facilitates high-throughput screening such that the completion of a full-genome RNAi screen typically takes several weeks, using high-content imaging or a plate reader as detection methods for fluorescence- or luminescence-based reporter assays. The more than 100 full-genome screens that have been conducted at the DRSC to date have been recently reviewed by Mohr et al.[14]

These studies, over the last years, have greatly expanded and revised our understanding of numerous biological phenomena, such as, most notably, signal transduction cascades.[16] Over the past decades, classical genetic screens in various model organisms have identified a limited set of cellular signal transduction cascades. Within those cascades, a small number of canonical members were hypothesized to be responsible for a 'quasilinear' flow of information that transforms an input signal into a cellular response. Current concepts derived from large-scale analysis of these signal transduction cascades, however, question this architecture. Genome-wide loss-of-function screens have typically identified hundreds of hits that influence, modulate, and direct the cellular information flow upon stimulation. Collectively, these studies reject the notion that canonical signal transduction cascades function as strictly independent molecular branches and question the hierarchical transduction of a signal through a small set of core members. Systematic analysis of different signaling pathways suggests

  

that signal transduction cascades should rather be considered as signal transduction networks in which a plethora of components have a graded effect on the cellular output and share considerable molecular overlap with other networks.[17] These studies also suggest extensive feedback loops in signal transduction networks.[16,18]

Besides redefining the topological features of signal transduction networks, genome-scale RNAi screens in *Drosophila* have identified numerous novel players in their respective screens. A few notable examples include the transmembrane protein *evenness interrupted* (*evi*) required for Wingless secretion,[19] a Wnt/$\beta$-catenin signaling inhibitor named Bili,[20] *Drosophila moleskin* required for nuclear entry of transforming growth factor-$\beta$ (TGF-$\beta$)-activated Smads,[21] and CG5169/dGCKIII, a Ste20-like kinase, and the dPPM1 phosphatase required for receptor tyrosine kinase signaling through extracellular-signal-regulated kinases (RTK/ERK).[18] Surprisingly, although these studies queried different signaling cascades, there exists a considerable overlap in their 'hit lists'. While to some extent this may be explained by unspecific 'off-target' effects (OTEs) of the RNAi constructs or alternatively might stem from indirect general alterations of cell physiology, these overlaps might provide an interesting starting point to address signal transduction pathway cross-talk. A combinatorial cell-based RNAi screen recently reported an extensive phosphorylation network that underlies c-Jun N-terminal kinase (JNK) activity.[22] The combinatorial strategy, knocking down genes in a background sensitized for JNK activation using double RNAi treatment, not only enhanced the sensitivity of the assay but, in comparison with the single loss-of-function screen, also dramatically reduced the false-negative rate.

Furthermore, the overlap in RNAi screens could originate from the impact of different signal transduction cascades on basic cellular machineries that regulate processes such as translation, mitosis, ribosome biosynthesis, protein degradation, or global transcriptional regulation. Multiple lines of evidence suggest a growth-promoting and mitogenic role for the RTK/ERK,[23] Dpp,[24] Janus kinase/signal transducers and activators of transcription (JAK/STAT),[25] target of rapamycin (TOR)[23] and Hippo[26,27] signaling networks during development. Thus, signal transduction networks might have similar effector molecules through which they exert their effect on cell growth. Similarly, signal transduction networks might globally influence chromatin, as recently shown for JAK/STAT signaling,[28] or rather these networks might be modulated by general transcriptional regulators. For instance, Polycomb group proteins have recently been implicated in both JAK/STAT and Notch signaling.[25,29] It will therefore be interesting to determine to what extent and through which molecular players these signaling networks regulate basic cellular machineries.

## *In Vivo* Genome-Scale RNAi Screens

To systematically interfere with gene function in a cell type-specific manner within a living animal, large-scale collections of transgenic RNAi lines have recently been generated. The three most comprehensive are the Vienna *Drosophila* RNAi collection (VDRC)[9] currently targeting 13327, the National Institute of Genetics (NIG-FLY) collection targeting 6000, and the Transgenic RNAi Project (TRIP)[15] collection currently targeting 2034 of the total 13,929 annotated protein-coding genes in *Drosophila*. These lines share a basic design principle that relies on the *UAS-GAL4* system to induce RNAi expression in a timely and spatially defined manner.[30] Similar to *Drosophila* cell culture-based approaches, RNAi is triggered by a long, dsRNA 'hairpin' expressed from a transgene, which was cloned as an inverted repeat. Besides long dsRNA-based constructs, a microRNA-based RNAi system has proven effective for RNAi in *Drosophila*.[31] This strategy might be a valuable alternative for future efforts to generate transgenic RNAi lines as the short gene-specific sequence allows more flexibility in construct design and potentially eliminates a majority of OTEs as a single siRNA species is generated. In addition, multiple independent and nonoverlapping short RNAi constructs can be generated per gene.

To date, these collections have been used in several *in vivo* genome-wide RNAi screens to systematically interrogate host–pathogen interactions,[32] metabolism,[33] muscle development,[34] and Notch signaling[29,35] (Table 2). Similar to cell culture-based RNAi screens, these studies have uncovered an unappreciated complexity in several developmental contexts and have proven to be excellent tools for gene discovery. A recent screen for heart function in *Drosophila*[36] has, e.g., identified NOT3 as a conserved regulator of heart function. Through integration of RNAi screen-derived phenotypic data and protein–protein interaction data, Neely et al. could show a requirement for the *Drosophila* CCR4–Not complex in heart function. Knockdown of the complex components *not1*, *not3*, *not4*, *UBC4*, and *Hsp83*, and to a weaker extent *not2* and CG8759, scored in the screen for *Drosophila* heart function. In-depth analysis of the *not3* RNAi phenotype showed a significant increase in systolic and diastolic diameters, contractile irregularities, and marked perturbation of the

  

**TABLE 2** | Large-Scale *Drosophila* RNA Interference (RNAi) Studies Discussed in the Text

| Study | Field of Study | Cell Type/ Tissue Analyzed | Number of Genes Screened |
|---|---|---|---|
| **Cell culture-based RNAi screens** | | | |
| Bakal et al.[22] | JNK signaling | BG-2 cells | Combinatorial kinome screen (17,724 combinations) |
| Bai et al.[37] | Muscle development | Embryonic primary cells | 1140 genes |
| Saj et al.[29] | Notch signaling | S2 cells | Genome wide |
| ***In vivo* RNAi screens** | | | |
| Cronin et al.[32] | Immunity (bacterial infection) | Ubiquitous (*HSP70-GAL4*) | Genome wide (10,689 genes) |
| Mummery-Widmer et al.[35] | Notch signaling | Notum specific (*pannier-GAL4*) | Genome wide (11,619 genes) |
| Neely et al.[36] | Heart function | Cardioblast specific (*TinCΔ4-GAL4*) | 6751 conserved genes |
| Pospisilik et al.[33] | Obesity/triglyceride levels | Ubiquitous (*HSP70-GAL4*) | Genome wide (10,489 genes) |
| Saj et al.[29] | Notch signaling | Wing specific (*engrailed-GAL4*), eye specific (*GMR-GAL4*) | 501 Notch pathway enriched genes identified in a cell culture-based primary screen |
| Schnorrer et al.[34] | Muscle development | Muscle specific (*Mef2-GAL4*) | Genome wide (10,461 genes) |

JNK, c-Jun N-terminal kinase.

myofibrillar organization. Interestingly, Neely et al. could directly translate their findings to mammalian species, as *not3* haploinsufficiency in mice results in an impairment of cardiac contractility. The relevance of this finding is further underlined by the identification of a common single nucleotide polymorphism (SNP) in the *not3* promoter that correlates with altered cardiac QT intervals in humans.[36] Hence, this and other *in vivo* RNAi screening studies exemplify how unbiased genome-wide RNAi screens in *Drosophila* can identify genes and molecular complexes relevant to human pathologies.

Along these same lines, two recent studies have established the musculature in *Drosophila* as a valuable system for studying gene function related to human disease. Owing to the syncytial nature of muscle fibers, this tissue is not amenable for clonal analysis frequently used to perform loss-of-function studies in *Drosophila*. The availability of robust methods to study myogenesis in primary cell cultures and the availability of muscle-specific transgenic *GAL4* lines have established *Drosophila* as a powerful system to study muscle biology and myopathies in a comprehensive and systematic manner. Bai et al. used primary cells to study *Drosophila* homologs of human genes associated with muscle disease and screen for novel regulators in muscle assembly and maintenance.[37] Nineteen out of 28 human disease genes showed abnormal muscle phenotypes in *Drosophila* primary muscle cells following RNAi knockdown. These data suggest that RNAi in *Drosophila* primary cells is a powerful way to annotate the phenotypes of disease-relevant genes. In addition, this strategy identified the conserved WH2 domain-containing protein sarcomere length short (SALS) as a regulator of sarcomeric actin elongation[38] and from a set of 1140, identified 49 novel potential regulators of late muscle differentiation.[37] With a similar goal of finding human muscle disease-relevant genes, Schnorrer at al. performed a full-genome *in vivo* RNAi screen using the muscle-specific *Mef2-GAL4* line. This screen implicated 2785 genes in muscle function in *Drosophila* for which a majority could be grouped into distinct phenotypic classes.[34] Overall, the screen is strongly enriched for genes that are associated with human muscle diseases and highlights the potential of unbiased genetic RNAi screens to identify genes relevant for human pathology. In conclusion, these approaches substantiate the value of *in vivo* and *in vitro* RNAi screens in *Drosophila* to study and identify human disease-relevant genes.

The utilization of different approaches to comprehensively map the genetic network of the same biological process has been exemplified by the studies of Mummery-Widmer et al.[35] and Saj et al.[29]. These two studies focused on Notch signaling, an evolutionary conserved signal transduction cascade implicated in a plethora of developmental and pathophysiological processes. The Notch signal transduction pathway has recently been extensively reviewed.[39] In brief, Notch is activated by binding to one of its ligands (Delta or Serrate in *Drosophila*) that induces a proteolytic cleavage sarcomere length short (SALS) in the release

  

of the intracellular domain of Notch. This domain acts as a transcriptional regulator by interacting with suppressor of hairless (Su(H)). Both groups undertook a genome-scale analysis of Notch signaling, identifying numerous novel candidates involved in this signal transduction cascade, but perhaps more importantly uncovering an unvalued complexity in the regulation of Notch signaling.

Mummery-Widmer et al. conducted a genome-wide *in vivo* RNAi screen using the *pannier-GAL4* line to induce RNAi in the fly notum. Over the years, the fly notum has emerged as an excellent model system to screen for genes required in Notch signaling. The specification and the subsequent asymmetric divisions of the sensory organ precursor cells are Notch dependent and therefore an increase or decrease in final bristle number on the notum is indicative of defects in Notch signaling (reviewed in Ref 40 and references therein). Mummery-Widmer et al. identified 177 putative Notch regulators and integrated the phenotypic information derived from the genome-wide RNAi screen with protein–protein interaction data to arrive at a Notch interaction map, which revealed important roles for particular biological processes and protein complexes in Notch signaling, such as nuclear import and the COP9 signalosome.[35]

The screening strategy used by Mummery-Widmer et al. is extremely labor intensive, as it requires monitoring every fly cross for often very subtle phenotypes on the notum. Saj et al. alternatively used a strategy that takes full advantage of the high-throughput approach of a cell culture-based RNAi screen. Saj et al. used a Notch::VP16 fusion protein that can activate the expression of luciferase under the control of Notch-responsive elements. This strategy allowed a rapid identification of a 'short list' of Notch regulators that were further screened and validated using *in vivo* transgenic RNAi.[29] As the study of Mummery-Widmer et al., Saj et al. identified several novel modules previously not implicated in Notch signaling, amongst which the identification of an interaction between Notch signaling and the metabolic network of pyruvate metabolism is one of the most notable ones.

The latter strategy represents a reasonable approach to bypass the labor-intensive full-genome *in vivo* RNAi screen to generate a short list relatively fast. Moreover, the composition of this short list can influence the design of *in vivo* secondary assays. This approach permitted Saj et al. to implicate 121 genes in the Notch signaling pathway that were inaccessible for examination at the adult stage in the Mummery-Widmer study due to an early lethality phenotype. Conversely, a full-genome screen, in a complex tissue, might yield cell type-specific regulators that can be missed when preselection through a cell culture-based screen is applied. Moreover, the broad expression of *pannier-GAL4* allowed Mummery-Widmer et al. to identify a wide range of phenotypes, as e.g., alterations in planar cell polarity, asymmetric cell division, and cell/ tissue growth.

## FUTURE DIRECTIONS

Systems biology has and will doubtlessly change our view of biological systems. Currently, the biggest challenge is to develop new experimental strategies that will further increase the quality and reliability of the datasets to largely eliminate false negatives and false positives. Initially unexpected, unspecific OTEs have been identified as one of the main sources of experimental noise in RNAi-based loss-of-function screens.[41,42] Molecularly poorly understood, certain RNAi constructs elicit unwanted silencing of additional genes besides the intended, primary target. Although improvements in RNAi construct design have reduced the number of predicted off targets in whole-genome libraries, a definitive assessment of the quality and specificity of a particular RNAi construct based solely on bioinformatics tools is not possible to date. Hence, RNAi phenotypes have to be experimentally validated. In the case of RNAi-based loss-of-function screens, the generation of multiple independent constructs per gene will be of great value to produce high confidence datasets. This strategy has been realized at the DRSC, as sublibraries like the *Drosophila* kinase and phosphatase, ubiquitin-related or transcription factor gene sets contain multiple RNAi constructs per gene. Combined with RNAi rescue systems, for both *in vitro* and *in vivo* applications,[43–45] RNAi phenotypes can be verified at a rapid pace. Several groups have developed systems to introduce or coexpress RNAi-insensitive constructs along with the RNAi construct of interest. Current approaches include cross-species RNAi rescue platforms[43,44] or *de novo* synthesis of RNAi-insensitive *D. melanogaster* genes that are based on synonymous changes in the codon wobble positions.[45] Availability of genome-scale collections of these rescue strains and constructs would most certainly eliminate many of the false positives from RNAi screen datasets. Besides rescue constructs, a comprehensive knowledge of cell type-specific gene expression would be of great value to assess the quality of screening results and to identify constructs with off targets. Efforts toward a comprehensive annotation of functional elements

  

in the *Drosophila* (and *C. elegans*) genome(s) are currently undertaken by the modENCODE consortium,[46] which is conceived as a 'community resource project'. These data will greatly improve our ability to interpret loss-of-function derived screening data and will help to decipher the principles of regulatory genetic networks that orchestrate different biological processes.

Besides these improvements in reagents, the main future challenge will be to better integrate data from different systems biology studies. The aforementioned studies of Bakal et al., Neely et al., Mummery-Widmer et al., and Saj et al., in addition to many others, represent interesting examples of the power of integrative data analysis. The integration of phenotypic data with protein–protein interaction information, protein localization, and posttranslational modification data dramatically increases our ability to interpret the complex genotype–phenotype relationships. With genome-scale protein localization and affinity purification studies, yeast geneticists have been at the forefront of comprehensive proteomics data generation[10,13]. Similar efforts have now begun in higher organisms. For instance, the availability of genetic tools for tagging genes at their endogenous loci[47] in *Drosophila* offers an opportunity to generate resources for large-scale proteomics analyses in flies. Similar efforts are also feasible in higher vertebrates. A recent paper by Hutchins et al.[48] reported the use of 'BAC TransgeneOmics'[49] to study the localization and interaction pattern of about 100 mitotic protein complexes in mammalian cell culture. This study provides a valuable complement to RNAi screens for mitotic defects,[50–52] as it exemplifies an experimental strategy for high-throughput molecular characterization and validation for RNAi screening results. Similar proteomics analyses of cellular networks, like that recently reported for autophagy,[53] or directed proteomics analyses of purified organelles[54] will be an invaluable counterpart to loss-of-function screens.

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