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Minireview

Functional genomics of cell morphology using RNA interference: pick your style, broad or deep

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Abstract

Several new studies have used RNA interference to screen for protein functions affecting cell shape, mitosis and cytokinesis of *Drosophila* cells in culture. One broad survey of nearly 1,000 proteins and three studies focused on cytoskeletal and motor proteins have identified key proteins essential for these processes in animal cells.

Given complete genome sequences from a growing number of organisms, investigators are confronted with how, most efficiently, to complete the inventory of proteins that participate in complex cellular processes, such as cytokinesis, cellular motility or the establishment of asymmetric cell shapes. Such an inventory is the essential first step in beginning to think mechanistically about how any such system of interdependent parts functions as a whole. In the pre-genomic era, classical forward genetics (screening of mutants), biochemical isolation with reconstitution, and pharmacology provided laborious but definitive methods to connect genes with molecular functions, working on one protein at a time. Now, strategies can aim for broad or even complete coverage. Experiments with budding yeast led the way with four sorts of experiments: first, a complete set of deletion mutations, which showed that only 19% of the 6,200 genes are required for viability in the laboratory [1]; second, crosses between viable deletion strains, revealing synthetic interactions [2]; third, large-scale two-hybrid assays mapping out networks of protein-protein interactions [3]; and fourth, comprehensive tagging with green fluorescent protein (GFP) to visualize subcellular localization [4]. Such experiments are complicated in higher eukaryotes, where homologous recombination is more difficult to achieve.

Fortunately, RNA interference (RNAi) came to the rescue, providing a simple method for depleting specific mRNAs, and making it possible to assay for the effects of loss of protein function on a grand scale. Pioneering experiments with *Caenorhabditis elegans* examined the effects of systematically deleting mRNAs for most of the 2,769 genes on chromosome I [5] and 2,300 genes on chromosome III [6]. Both of these studies assayed for gross developmental and functional defects - supplemented in the chromosome III study with high-resolution time-lapse movies of the first two embryonic cell divisions. Both studies yielded phenotypes for about 13% of the genes screened, including most, but not all, of the genes on these chromosomes that were already known to give developmental phenotypes.

The *Drosophila* community has followed suit, with several new studies using RNAi to deplete mRNAs and proteins from tissue-culture cells [7-10]. The method is simple and efficient. Where antibodies were available for testing, the

levels of target proteins were found to be reduced by more than 90%, and sometimes more than 99%. These studies represent a range of styles, from a highly focused examination of the role of 26 microtubule motors in mitosis [7] to a much more ambitious screen of nearly 1,000 mRNAs for possible effects on cellular morphology and division [8]. Where they overlap in the genes studied, these studies generally agree on which depletions produce defects in cytokinesis or in the actin cytoskeleton in the lamellae of spread cells (Table 1).

When initiating such a screen, a decision must be made regarding breadth or depth, because for practical reasons the assays can be more detailed in a small screen than in a very broad one. For example, the relatively small size of the screen of microtubule motors enabled Goshima and Vale [7] to use time-lapse microscopy of cells expressing GFP-tubulin to identify subtle phenotypes. This kinetic information allowed them to detect, for example, that depletion of dynein caused the cells to delay at the metaphase-to-anaphase transition, before completing an otherwise normal mitosis. The other studies relied on fluorescence microscopy of fixed cells to reveal effects of protein depletion on cellular shape, the actin cytoskeleton, microtubules or the process of cytokinesis. Somma *et al.* [9] focused on eight proteins suspected to participate in cytokinesis. Kiger *et al.* [8] hand-picked a selection of 994 *Drosophila* genes, many known to be functional from mutagenesis studies and others suspected to be functional from work in other systems; their targets included not only cytoskeletal and motor proteins, but also a large number of cell-cycle, signal-transduction and receptor proteins. Rogers *et al.* [10] screened 90 targets known to be components of the actin cytoskeleton for defects in cytokinesis and/or extension of leading lamellae. In addition, Kiger *et al.* [8] examined two *Drosophila* cell lines with distinct morphologies: large, flat $S2R^+$ cells and small, round Kc_{167} cells. Some RNA depletions produced phenotypes in both lines, but others produced different phenotypes in the two lines or a phenotype in only one of the two lines. The other groups [7,9,10] tested S2 cell lines; and Rogers *et al.* [10] plated their S2 cells on concanavalin A, to promote spreading on the microscope slide.

These studies all agree on target RNAs that give cytokinesis phenotypes: those encoding anillin, citron kinase, cofilin, Diaphanous, myosin-II heavy chain, myosin-II regulatory light chain, Pavarotti/kinesin, Pebble Rho GTPase exchange factor (GEF) and Rho1 GTPase. Of the three groups that performed the studies, only Rogers *et al.* [10] found a cytokinesis phenotype with RNAi targeting profilin and cyclase-associated protein. The Rogers *et al.* [10] and Kiger *et al.* [8] screens also agree on the RNA depletions that cause defects in the actin-based lamella and alter cellular shape: Abl tyrosine kinase, Arp2/3 complex, capping protein, Cdc42, cofilin, cofilin phosphatase, cyclase-associated protein, profilin and Scar. The screens did not agree on whether lamellar or cell-shape defects were found with the depletion of the following 15 RNAs: Aip1, formin, anillin, Rho1 GTPase, Aurora kinase, cortactin, Nck, Rac2, Abelson-interacting protein, Enabled kinase, Rho kinase, PAK, Rho GEF (vav), phosphatidylinositol trisphosphate (PIP_3) phosphatase (Pten), and myosin essential light chain (unnamed open reading frame CG15780). In four of these cases, one of the labs did not test the RNA, but with depletion of the remaining 11 RNAs one lab found a lamellar defect whereas the other lab did not (Table 1).

Given the functional bias in the selection of the target RNAs for all of these *Drosophila* RNAi screens, it is striking how many individual proteins can be depleted with little or no effect on tissue-culture cells. The microscopic assays by Kiger *et al.* [8] revealed phenotypes such as changes in cell shape or the accumulation of multinucleated cells upon depletion of only 160 of the RNA targets (one third of which correspond to a gene lacking a previously characterized mutant allele). In the Rogers *et al.* screen [10] depletion of 66 of 90 known cytoskeletal targets had no effects on lamellar morphology or cytokinesis; these 66 include a number of overlaps with negative results from the Kiger *et al.* screen: Ciboulot, filamin, gelsolin, several myosins, tropomodulin, tropomyosin and Wiskott-Aldrich syndrome protein (WASP).

What is the explanation for the RNA depletions with no phenotype in these assays? Are these true negatives or false negatives? A trivial explanation is a failure of depletion, but the efficiency of depletion is impressive in the examples tested. Somma *et al.* [9], Rogers *et al.* [10] and Goshima and Vale [7] confirmed that S2 cells express most of the RNAs that they tested, ruling out another trivial explanation for a negative result, namely absence of the target. Kiger *et al.* [8] included many mRNAs encoding proteins with specialized functions in differentiated tissues such as muscle, nerve or germ cells, so some of their negative results are expected for S2 cells as they lack these functions.

Geneticists might tell us not to worry about the false negatives, since they are inevitable in any screen. But if one wants to reach the goal stated at the outset - a complete inventory of the proteins that participate in a complex cellular process - one cannot ignore the negatives. The highly focused test of microtubule motors in mitosis [7] provides some clues about the negative results. Goshima and Vale [7] targeted all 25 kinesins and the single dynein in the *Drosophila* genome. Their assay on live cells detected subtle phenotypes, such as the delay in metaphase caused by

Table 1

Screens for lamellar and cytokinesis phenotypes in cultured *Drosophila* cells

Phenotype:	Cytokinesis			Lamellar	Actin/shape
Cell type:	S2	S2	S2R, Kc_{167}	S2 cells	S2R, Kc_{167}
Study:	Somma *et al.* [9]	Rogers *et al.* [10]; Goshima and Vale [7]	Kiger *et al.* [8]	Rogers *et al.* [10]	Kiger *et al.* [8]
Protein (*gene*)*					
Myosin-II (*zipper*)	NT	+	-†	-	-†
Myosin RLC (*spaghetti squash*)	+	NT	+	NT	-
Profilin (*chickadee*)	-	+	-†	+	+†
CAP (*act up*/*capulet*)	NT	+	-	+	+
Cofilin (*twinstar*)	+	+	+	+	+
Actin interacting protein 1; Aip1	NT	+	NT	+	NT
Formin (*diaphanous*)	NT	+	+	-	+
Anillin (*scraps*)	+	+	+	-	+
Kinesin (*pavarotti*)	+	+	+	-	-
Rho1 GTPase	+	+	+	+	-
Rho GEF (*pebble*)	+	NT	+	NT	-
RacGAP; RacGAP50C	+	+	-	-	-
Syntaxin1A	+	NT	NT	NT	NT
Dynamin (*shibire*)	NT	NT	+	NT	-
Citron kinase	NT	+	+	-	-
Aurora kinase	NT	NT	+	NT	+
Arp2/3 complex	NT	-	-	+	+
Scar	NT	-	-	+	+
Cortactin	NT	-	-	-	+
Nck (*dock*)	NT	-	-	+	-
Cdc42 GTPase	NT	-	-	+	+
Rac1/2 GTPase	NT	-	-	-	+ (Rac2 only)
Capping protein	NT	-	-	+	+
Abl kinase	NT	-	-	+	+
Abelson-interacting protein (*abi*)	NT	-	-	+	-
Enabled kinase	NT	-	-	-	+
Rho kinase	NT	NT	-	NT	+
PAK	NT	- (PAK1)	-	-	+ (PAK3)
Cofilin phosphatase (*slingshot*)	NT	-	-	+	+
Rho GEF (*vav*)	NT	-	-	-	+
PIP_3 phosphatase (*pten*)	NT	NT	-	NT	+
Other myosins	NT	-	-	-	+ (uncharacterized ORF, *CG15780*)
Polo kinase	NT	NT	+‡	NT	+‡
LIM kinase	NT	-	-	-	-

Table 1 *(continued)*

Phenotype:	Cytokinesis			Lamellar	Actin/shape
Cell type:	S2	S2	S2R, Kc_{167}	S2 cells	S2R, Kc_{167}
Study:	Somma *et al.* [9]	Rogers *et al.* [10]; Goshima and Vale [7]	Kiger *et al.* [8]	Rogers *et al.* [10]	Kiger *et al.* [8]
Protein (*gene*)*					
Ciboulot	NT	-	-	-	-
Coronin	NT	-	NT	-	NT
Filamin	NT	-	- (*jitterbug*)	-	- (*jitterbug*)
Gelsolin	NT	-	-	-	-
Tropomodulin (*sanpodo*)	NT	-	-	-	-
Tropomyosin	NT	-	NT	-	NT
Septin (*peanut*)	-	NT	NT	NT	NT
WASP	NT	-	-	-	-

+ indicates that a cytokinesis or lamellar phenotype was observed; -, no phenotype was observed; NT, not tested. *Protein names (sometimes more than one alternative) are given in roman type and gene names in italics. †In unpublished experiments, accumulation of actin and disruption of the cell monolayer was found with profilin RNAi and a cytokinesis phenotype was seen with *zipper* RNAi (A. Kiger, personal communication). ‡Mitotic phenotypes were observed.

depletion of the dynein heavy chain, which might have been reported as a false negative in a study with fixed cells. Depletion of any of eight single kinesins compromised formation of a bipolar spindle or the alignment of chromosomes on the metaphase plate, but depletion of multiple kinesins with related functions was required for severe misalignment of metaphase chromosomes. Loss of one kinesin (Pavarotti) compromised cytokinesis owing to disruption of the central spindle, but depletion of two other kinesins had no effect on cytokinesis in spite of the ability of mutations in the respective genes to have an effect. One-off depletion of 17 other kinesins had no detectable effect on cell division, and no single kinesin depletion slowed anaphase chromosome movements. Only 3 of the 26 depletions caused defects so substantial that the cells failed to find an alternative pathway to complete mitosis.

Thus, redundant function, alternative pathways and antagonistic actions are the rule rather than the exception in cell division and probably in many other vital processes. The frequent lack of phenotypes upon depletion of single proteins simply reflects the complexity of redundant molecular machines with multiple safeguards to protect function. Most negatives in the RNAi screens are therefore likely to be real negatives rather than false negatives, but this does not indicate lack of function.

The occurrence of proteins lacking RNAi phenotypes opens the door to combinatorial RNAi screens for synthetic interactions and the construction of protein interaction networks (as in yeast [3]). Double RNAi depletions have already revealed synthetic interactions between some kinesins [7] and between the signaling molecules Nck and Rac [10]. Other modifier screens are feasible; for example, Kiger *et al.* [8] show RNAi suppressor interactions between the Pten phosphatase and 20 kinases. The targets that do have positive RNAi phenotypes need to be followed up with targeted gene disruption and/or gene replacement, to investigate mechanisms. This has been the Achilles' heel for *Drosophila*, but fortunately, gene targeting is becoming more efficient [11].

Both focused and broad approaches to RNAi have strengths, so that the way that one screens is largely a matter of taste. As someone interested in the details of molecular mechanisms, I am inclined toward slow, deliberate screens with high-resolution assays in an effort to maximize the information extracted on the first try. Such 'second generation' screens should include verification of the expression of each target in controls and its depletion in test cells. On the other hand, one cannot deny the importance of broad depletion screens designed to give a comprehensive account of genes that contribute to cellular function, such as the shape and morphology assay of Kiger *et al.* [8]. Fortunately, the groups of Norbert Perrimon at Harvard Medical School and Renato Paro in Heidelberg, Germany have set up the 'Drosophila RNAi Screening Center' (DRSC) [12], with a library of RNAi reagents for all of the known open reading frames in the *Drosophila* genome and facilities for high-throughout screens. Support from the National Institute of General Medical Sciences makes this resource available to other investigators, and the DRSC is eager to collaborate with experts with sophisticated assays for various cellular activities.

Having struggled to compare the recent RNAi studies in *Drosophila* [7-10], I feel compelled to finish with an observation about gene names. The clever and amusing names that geneticists have assigned to mutations and/or genes are genuinely hampering communication. Even for someone familiar with a particular field, matching dozens of meaningless gene names with generic protein names has become impossible. FlyBase [13] is an indispensable resource that allowed me to sort out most of the nomenclature, but a trip to FlyBase or PubMed [14] should not be required to match *pavarotti* with a kinesin, *sanpodo* with tropomodulin or *zipper* with cytoplasmic myosin-II heavy chain. The three letter and number nomenclature used by the yeast community to name yeast genes is just as opaque. Although it is strenuously resisted ("our history will be lost"), it is past time for gene names to be converted as soon as practical to common usage in all of the genetic model organisms. Nomenclature also needs to be consolidated across the phylogenetic tree, as has been done for myosins [15] but not yet for kinesins, where some family members have five or more names. The insights promised by new technologies such as RNAi may be wasted if jargon impedes communication with many interested readers.

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