Minireview

RNA interference pinpoints regulators of cell size and the cell cycle

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Abstract

Cell-based genome-wide RNA interference screens are being used to address an increasingly broad spectrum of biological questions. In one recent screen, *Drosophila* cell cultures treated with double-stranded RNA were analyzed by flow cytometry, providing a wealth of new information and identifying 488 regulators of the cell cycle, cell size, and cell death.

The growth of an organism is the net result of a variety of processes, including changes in cell size, cell division and apoptosis. These processes are regulated by intricate, interrelated molecular networks, and their disruption can have major biological consequences. In particular, the relationship between changes in cell size and the cell cycle has long fascinated researchers. It is complex, poorly understood, and varies according to the organism, tissue type and developmental context. In yeast, large-scale genetic screens have uncovered many genes involved in cell growth and the initiation of DNA synthesis (S phase) [1,2]. It is now clear that yeast cells must grow to a certain minimal size before starting DNA synthesis, providing a 'cell size checkpoint' at the transition from the preceding G1 phase to S phase (the G1/S transition). Yeast is a unicellular organism, however, and there is increasing evidence that the relationship between cell growth and cell division may be different in metazoans.

Excitingly, recent technical advances in high-throughput RNA interference (RNAi) mean that large-scale screening approaches, somewhat analogous to the genetic screens in yeast, can now be applied to cultured metazoan cells. *Drosophila* hemocyte cell lines have emerged as popular cell systems for this experimental approach for a number of reasons. First, they are very amenable to RNAi mediated by double-stranded RNA (dsRNA): dsRNA molecules of more than 500 bp can be easily introduced into these cells and are rapidly processed into short interfering RNAs (siRNAs). Second, there are significantly fewer genes in *Drosophila* than in mammals, making the mammoth undertaking of a genome-wide screen a little less daunting. Finally, there is less genetic redundancy in *Drosophila* than in mammals, so depletion of just one gene is more likely to reveal a phenotype.

Genomic screens for the total complement of protein kinases (the kinome) and general genome-wide screens have been performed in *Drosophila* cell cultures using diverse readouts such as cell shape, resistance to bacterial infection and transcriptional activity [3-8]. Bjorklund *et al.* [9] have recently published one of the most comprehensive screens to date, in which they searched on a genome-wide scale for dsRNAs that alter cell size, cell-cycle distribution and cell death. The dataset they generated provides an excellent starting point for many new avenues of research. At the same time, this massive undertaking highlights some of the bioinformatic challenges associated with screens on this scale. For example, the data generated can be analyzed and presented in various ways to highlight the different phenotypic effects (see the supplementary data accompanying [9]).

The Taipale lab [9] used dsRNAs corresponding to 11,971 individual cDNAs to target the silencing of approximately

70% of known *Drosophila* genes. After 4 days culture, flow-cytometry profiles were generated for each dsRNA treatment in triplicate to provide information on the distribution of cells in different phases of the cell cycle as well as cell size. The simultaneous effect of each dsRNA on six different cellular phenotypes was recorded: the percentage of cells with a DNA content of 2*N* (percentage of cells in G1; 2*N* denotes cells in G1); 4*N* cells (percentage of cells in G2, the phase after the DNA has been replicated); less than 2*N* (percentage of dying cells); and greater than 4*N* (percentage of cells with defective cytokinesis); as well as the average cell size of the G1 population (G1 cell size), and the G2 population (G2 cell size). A dsRNA was considered a 'hit' if it changed one of these percentages relative to control cells by more than 5 standard deviations. The phenotypes of all the hits were then clustered using an unbiased approach, allowing the authors to identify groups of genes whose downregulation results in similar phenotypes. In many cases, genes with similar known functions clustered tightly together, but a number of new or unexpected groups of genes were also identified.

Identifying genes involved in cell-cycle progression

One major aim of the screen by Bjorklund *et al.* [9] was to identify genes involved in cell-cycle progression by screening for dsRNAs that alter the proportion of cells in different phases of the cell cycle. Although these data are informative in themselves, more can be learnt when they are combined with data on any simultaneous changes in cell number. This is best illustrated by an example. dsRNAs can increase the percentage of cells in G1 either by delaying progression from G1 to S phase or by accelerating progression through M phase (mitosis), and cell-number data can distinguish between these two possibilities. Cyclin E is known to promote the transition from G1 into S phase, and its depletion increased the proportion of cells in G1, presumably by delaying their progression into S phase. Such an effect would have been accompanied by a reduction in cell number. The protein kinase Wee1 inhibits progression through G2 and M phase, and its depletion also increased the proportion of cells in G1. In this case, however, the increased percentage of the population in G1 is likely to reflect accelerated progression through M phase, and would therefore be accompanied by an increase in cell number. Unfortunately, high-throughput flow cytometry does not allow the simultaneous collection of reliable cell-number data. The current dataset might be fruitfully exploited, however, by identifying the dsRNAs that altered cell-cycle distribution, and then carrying out a secondary screen of those dsRNAs to determine their effect on cell number.

Silencing of genes encoding components of the small and large ribosomal subunits resulted in cellular phenotypes that clustered into three distinct groups. dsRNAs corresponding to one group of ribosomal proteins increased the percentage of cells in G1, decreased the percentage of cells in G2, increased the percentage of cells undergoing apoptosis and decreased both G1 and G2 cell size. It is tempting to speculate that these cells are impaired in their ability to synthesize proteins and progress more slowly through G1/S, perhaps because of reduced G1 cyclin synthesis. They are also impaired in their ability to grow, consistent with the known role of protein synthesis in cell growth; the increase in apoptosis may be due to a reduction in the translation of proteins necessary for cell survival. Depletion of the second group of ribosomal proteins seemed to cause a G1 arrest, as it resulted in an even more marked increase in the G1 population at the expense of the G2 population, with little effect on apoptosis and no effect on cell size. Finally, dsRNAs corresponding to a third group of ribosomal proteins increased apoptosis but had no effect on the cell cycle or cell size. While it is possible that the ribosomal proteins in these different groups have different functions, it is perhaps more likely that the different phenotypes simply reflect different efficiencies of RNAi. For example, slightly decreased ribosome function might result in a G1 arrest, whereas complete ablation of ribosome function might induce apoptosis irrespective of the cell-cycle phase. Thus, the group with a primarily apoptotic phenotype would contain the most effective dsRNAs, whereas the group with a cell-cycle arrest-like phenotype would contain the least effective dsRNAs. To test this hypothesis, one could treat *Drosophila* S2 cells with increasing amounts of dsRNA corresponding to representative ribosomal proteins from each of the three groups in an attempt to reproduce all three phenotypes.

Identifying genes that regulate cell size

Another aim of the screen was to identify genes whose downregulation alters cell size. Becuase cells grow as they progress through the cell cycle, dsRNAs that increase the proportion of cells in G2 will also increase the mean cell size of the entire cell sample without necessarily having an independent effect on cell growth. Bjorklund *et al.* [9] got round this problem by gating the flow-cytometry data and analyzing the size of cells in G1 and G2 separately. A number of genes were identified whose depletion increased both G1 and G2 cell size without having any effect on the distribution of cells in the cell cycle. In theory, these genes might represent proteins which, when depleted, allow increased growth, or proteins whose depletion delays cell-cycle progression without having an effect on growth or the distribution of cells in the cell cycle. These two possibilities could be distinguished by examining the effect of these dsRNAs on cell number. Intriguingly, many of these hits for increased cell size were identified by Computed Gene (CG) numbers only (the CG nomenclature refers to genes of unknown function). The way in which these genes affect cell size is hard to predict but may represent an untapped source of information. Some of these genes contain identifiable protein domains and have potential orthologs in other organisms;

thus, their functions may also be conserved. Some, for example, are homologous to known transcription factors; CG5684 and CG1884 are similar to components of the general transcriptional machinery, while CG1024 and CG18081 resemble zinc finger proteins.

The insulin signaling pathway leading to activation of the protein kinase Tor is a well known regulator of growth and can alter cell size in both *Drosophila* and mammals [10]. Activating insulin/Tor signaling increases cell and organism size, whereas inhibiting this pathway has the opposite effect. Despite the well known role of this signaling pathway in cell-size regulation, only one of its known components, Tsc1, was identified by Bjorklund *et al*. [9]. Tsc1 is a negative regulator of the insulin/Tor pathway, so its depletion is predicted to increase cell size. Although dsRNA-mediated RNAi silencing of the *Tsc1* gene did cause a modest increase in G1 and G2 cell size, it also caused defective cytokinesis, and hence *Tsc1* clustered with other genes whose downregulation gave a phenotype including defective cytokinesis. By searching through the data manually, Bjorklund *et al*. [9] found that dsRNA-mediated RNAi against eight other components of the insulin/Tor pathway gave weak but detectable phenotypes. Surprisingly though, they primarily affected the cell cycle rather than cell size. Activation of this pathway *in vivo*, through the mutation of negative regulators or the over-expression of positive regulators, decreases the proportion of cells in G1 and increases the proportion of cells in S phase and G2/M [11-13]. Conversely, inhibiting the pathway increases the proportion of cells in G1 [13]. The data provided by this RNAi screen are consistent with these observations: inactivation of the pathway reduced the proportion of cells in G1. It is, however, unclear why depletion of the proteins on this pathway had such a weak effect on cell size - modulation of the insulin pathway in *Drosophila* S2 cells by dsRNA-mediated RNAi can induce changes in cell size of more than 25% ([14] and M.J.C. and S.J.L., unpublished observations). Perhaps different culture conditions, variation among S2 cell lines, or different RNAi efficiencies are responsible for this difference in sensitivity.

Although increasing numbers of dsRNA-mediated RNAi screens with similar phenotypic readouts are being performed, there is relatively little overlap between the gene sets identified. This lack of overlap may result partly from false negatives due to low RNAi efficiency and the inherent problems associated with targeting stable proteins. In addition, false positives may have been generated by off-target effects. The dsRNA library used by Bjorklund *et al*. [9] was produced using full-length cDNA templates, so off-target effects (generated when a stretch of 21 bp or more in the dsRNA is identical to another transcript) may be substantial. No doubt, the ongoing generation of large, searchable databases containing data from different RNAi screens will become crucial to interpreting the results of these genomic approaches [8]. Correlation of the rich dataset generated by Bjorklund *et al*. [9] with related screens, both past and future, should help to clarify the roles of many molecular networks that act together to regulate growth, cell size and the cell cycle.

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