

Dissecting mitosis by RNAi in *Drosophila* tissue culture cells

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ABSTRACT

Here we describe a detailed methodology to study the function of genes whose products function during mitosis by dsRNA-mediated interference (RNAi) in cultured cells of *Drosophila melanogaster*. This procedure is particularly useful for the analysis of genes for which genetic mutations are not available or for the dissection of complicated phenotypes derived from the analysis of such mutants. With the advent of whole genome sequencing it is expected that RNAi-based screenings will be one method of choice for the identification and study of novel genes involved in particular cellular processes. In this paper we focused particularly on the procedures for the proper phenotypic analysis of cells after RNAi-mediated depletion of proteins required for mitosis, the process by which the genetic information is segregated equally between daughter cells. We use RNAi of the microtubule-associated protein MAST/Orbit as an example for the usefulness of the technique.

INTRODUCTION

In many species, cellular uptake of double-stranded RNA (dsRNA) induces a potent and specific gene silencing, a phenomenon known as RNA interference or RNAi. Gene silencing through RNAi was first discovered after the introduction of dsRNA into *C. elegans* and it was demonstrated that gene expression was suppressed very efficiently in a homology-dependent manner (1). RNAi seems to act in a post-transcriptional pathway, targeting RNA transcripts for degradation, and is related to the previously known phenomenon of co-suppression in plants (for reviews on the detailed mechanism of RNAi see refs. 2-5). With the advent of whole genome sequencing, RNAi has become a quick and powerful tool for the genome-wide study of gene function (6-9).

Following the development of RNAi methods in *C. elegans*, RNAi was then shown to be highly effective in *Drosophila* embryos (10-11). Subsequent studies demonstrated that RNAi could efficiently knock down the levels of specific proteins in several *Drosophila* tissue culture cell lines (12). More recently, the discovery of short small interfering RNAs has led to the wide-scale application of this approach to a wide variety of mammalian cell lines (13) and *S. pombe* (R. Allshire, personal communication), however *S. cerevisiae* appears to have lost this pathway.

In *Drosophila* tissue culture cell lines, RNAi has been used successfully in the study of mitosis and cytokinesis (14-20) and the method has become a prominent tool in *Drosophila* cell cycle research. The advantages of using *Drosophila* cell lines for RNAi studies of mitosis include the sequenced and well annotated genome with a relatively high genetic conservation with humans (21), the favourable cytogenetics for the study of chromosomal

processes (*Drosophila* has only 4 chromosomes), the existence of a great number of reagents to visualize the mitotic apparatus, the ability to use a PCR approach to obtain dsRNAs rather than the necessity to purchase expensive oligonucleotides or construct complex vectors (as in mammalian cells), and the ease of cell culture, with cells grown at 25°C without any supplementary requirements for CO₂ and at relatively low cost. Additionally, RNAi could be used to simultaneously knock down the levels of expression of more than one gene, therefore providing a useful tool for the dissection of signalling pathways. Due to these reasons, one can predict a great boom of RNAi-based genomic screenings using *Drosophila* tissue culture cells in the upcoming years (22).

However, interpretation of results after RNAi in *Drosophila* tissue culture cells can be complex since the penetrance of RNAi is not absolute, these cells are hard to synchronize, live cell analysis is not yet well developed and the resulting phenotypes can often be heterogeneous. Here we describe a detailed methodology for the careful phenotypic interpretation after specific gene inactivation by RNAi in *Drosophila* tissue culture cells, with particular emphasis on one gene involved in mitosis. The gene in cause, encodes for a conserved microtubule-associated protein called MAST/Orbit that, during mitosis, is localized to the mitotic spindle, centrosomes and kinetochores, ending up accumulating in the central-spindle region and ultimately concentrating at the midbody (23-24). Mutations in *mast* show severe mitotic abnormalities including the formation of mono- and multi-polar spindles organized by clusters of centrosomes (23). The use of RNAi in *Drosophila* tissue culture cells was of great help in the dissection of the mitotic role of MAST/Orbit, namely its unexpected role in the behavior of kinetochore microtubules (17, 25).

MATERIALS AND METHODS

Cell lines

RNAi has been shown to be effective in a wide variety of *Drosophila* tissue culture cells, including the S2, Dmel2, Kc and BG2-C6 lines (see for example ref. 15 and 26). Importantly, the penetrance of the RNAi appear to depend on the cell line used, with S2 cells thus far yielding the best results in our experiments (Fig. 1D).

S2 cells can be grown in plastic flasks at 25°C in Schneider's *Drosophila* medium (Gibco) supplemented with 10% FBS. For the RNAi experiment, the cells must be in exponential growth. This can be achieved by diluting the cells 1/5 into new media every 3-4 days.

Preparation of dsRNA

Drosophila cells respond to the presence of large dsRNAs by shutting down protein translation, but mammalian cells do not. This greatly simplifies the process and lowers the cost of RNAi in *Drosophila* cells, as it is not necessary to purchase custom oligonucleotides or construct complex vectors. Instead, the first

step towards to specifically knocking down the expression of a *Drosophila* protein of interest by RNAi is to synthesize a dsRNA fragment of ~700 bp (although we have also succeeded using smaller or larger fragments). For this purpose, we designed ~18mer sequence specific oligos to amplify a PCR product of ~700 bp from the cDNA of interest that is cloned into a plasmid. In order to generate the dsRNA from the PCR products, these primers must incorporate a 5' T7 RNA polymerase minimum binding site (Table 1).

The successful choice of the optimal sequence fragment seems to be random, however we have found that starting in the 5'-UTR covering the codon for the first methionine may in some cases improve the specific silencing of the protein of interest.

Table 1: Primer sequences used for the preparation of the PCR product used for *in vitro* RNA transcription of MAST/Orbit. The sequence corresponding to the T7 RNA polymerase-binding site is underlined.

Primer	Sequence
Forward	<u>TAATACGACTCACTATAGGG</u> GAAGGACGAATAGACATT
Reverse	<u>TAATACGACTCACTATAGGG</u> TCCTGTTTGACCTGGTCG

We then prepare 10-12 PCR reactions, each containing 0.5-1 ng of template plasmid DNA containing the cDNA of interest, 1 μM of each primer, 2.5 mM of dNTP mix, 2.5 U of Taq polymerase (Boehringer), enzyme buffer supplemented with Mg²⁺ according to the manufacturer and water for a final volume of 100 μl/PCR reaction. We use the following PCR program: 94°C for 2 min (hot start), add Taq polymerase and then do 30 cycles of 94°C – 30 s; 55°C – 60 s; 72°C – 60 s, followed by 72°C for 10 min. The PCR products are purified using the PCR Clean-Up kit (MoBio) according to the manufacturer's instructions (3 PCR reactions can be run through each column). We test the efficiency of the PCR by electrophoresis by running 1 μl of the pure PCR product on a 1% agarose gel (Fig. 1A). The purified DNA is then quantified by checking the optical density at 260 nm and adjusted to a concentration of 150 ng/μl or higher. This will be used as the template for preparation of dsRNA *in vitro*. For this purpose we normally do 10 reactions per RNA using the MEGAscript T7 kit (Ambion) with the following modification to the manufacturer's instructions: instead of the recommended incubation time for transcription at 37°C, we usually get better yield using longer incubation times of at least 6 h. The reactions are then pooled into a single tube and the RNA precipitated with LiCl (provided with the kit) and followed by 2 washes in 70% ethanol. The pellet is visible as a sloppy whitish precipitate and, after air-drying, is then re-suspended in 100 μl of nuclease-free water. The RNA concentration can be determined as before and should be 2-3 μg/μl or higher.

Another reliable alternative to prepare the RNA is to clone the DNA template into transcription vectors like pSPT18 and pSPT19 (Boehringer), which already contain the promoter sequence for T7 RNA polymerase, and *in vitro* transcribe both sense and anti-sense mRNA. To denature secondary structures,

the RNA is then heated for 30 min in a beaker containing about 200 ml of water at 65°C, and then left to slowly cool down to room temperature to form the dsRNA duplexes. Next, 1 µl is tested by electrophoresis in a 1% agarose gel (Fig. 1B). The dsRNA can finally be stored at -20°C for several months.

For control experiments, it is best to perform the RNAi experiment with an irrelevant dsRNA, in order to control for effects of the exposure of the cells to dsRNA. In our laboratory, we have typically done this by using dsRNA corresponding to a fragment of a human intron, and chosen at random (15). Alternatively, the use of dsRNA against a coding region of a protein known not to be involved in mitosis could also help to discard unspecific effects.

RNAi experiment

For a typical RNAi experiment, we diluted exponentially growing S2 cells in Schneider's *Drosophila* medium (Gibco) without serum and placed them in 6-well plates (chamber diameter 35 mm) at a final concentration of 10⁶ cells/ml/well. Two wells were set up for each time point, one for the RNAi experiment and one for the control. As a first step, it is advisable to titrate the minimum amount of dsRNA required for maximum depletion of the protein of interest. If too little dsRNA is added the RNAi is ineffective, however too much dsRNA may be toxic to cells as it was described in zebrafish where it can cause non-specific defects during development (27). In general, it is desirable to perform the experiments using a dsRNA concentration near the upper end of the tolerated range, as increasing the amount of dsRNA added to the culture can cause the depletion to occur at earlier time points during the experiment. We typically tested a range from 15 µg to 50 µg of dsRNA per well, and observed that addition of 30 µg was usually sufficient for complete depletion of the proteins of interest without causing obvious toxic side effects to the cells. Of course, this kind of experiment requires high sensitivity by the experimenter in order to distinguish between a real phenotype from a side effect, which can be helped by comparison with known mutant phenotypes when these are available.

After addition of the dsRNA to the cells, the dish was swirled to allow uniform distribution. In order to cut down possible pipeting errors or contamination, the dsRNA could be added directly to the cells before distributing them into individual wells. Concomitant with the addition of the dsRNA, cells were serum starved for 1 h at room temperature. This promotes incorporation of the dsRNA by an as-yet unknown mechanism. Subsequently, 2 ml of Schneider's *Drosophila* medium supplemented with FBS were added to each well and the cells returned to the incubator at 25°C.

To prepare protein extracts for analysis, cells from both RNAi and control experiments were collected at each time point and counted using a haemocytometer. The cells were then pelleted by centrifugation at 1,500 rpm for 4 min, re-suspended directly into an appropriate volume of Laemmli sample buffer sufficient to enable the loading of 10⁶ cells/lane on the gel, lysed by

sonication, boiled for 5 min and stored at -20°C. At the end of the experiment the collected samples for each time point were subjected to SDS-PAGE. The efficiency of silencing was determined by immunoblotting with a specific antibody (Fig. 1C) using the ECL detection system (Amersham). Quantitation was performed by visual comparison of signal intensity relative to that of a titration series as described next.

Antibody characterization

RNAi is not an absolute technique. Not all cells silence the gene in question, and silencing can occur to different extents in adjacent cells in the population. It is therefore important, if at all possible, to have a specific antibody recognizing the protein to be silenced. This, of course, is a drawback in large-scale RNAi studies, where one explanation for the absence of a phenotype must always be that the particular RNAi approach was not effective. In extraordinary circumstances where antibodies are not available, the effectiveness of RNAi can be determined by preparing stable cell lines expressing the protein of interest fused to green fluorescent protein (GFP). Success of the RNAi procedure can then be monitored by following the loss of GFP signal.

The first step towards assessing the effectiveness of the RNAi is to titrate the antibody against a range of cell concentrations. This is done by immunoblotting against cell extracts where each lane contains proteins from a differing number of cells. For example, we typically electrophorese a range of concentrations of protein extracts from between 10⁴ and 5 x 10⁶ cells per lane. This enables us to determine the minimum number of cell equivalents that can be detected by the antibody in question, and when compared with the signal obtained after RNAi, enables us to estimate the degree of silencing obtained.

To monitor the protein levels after addition of dsRNA in the cell culture, we collected samples every 24 h, which is roughly the doubling time for S2 cells (21 h in the case of Dmel2 cells). Usually, 10⁶ cells for each time point are enough for antibody detection, but this may need to be adjusted depending on the protein of interest. Also indispensable is a loading control that is typically an abundant structural protein like actin or tubulin, for which commercial antibodies are available (Sigma).

Indirect immunofluorescence

Detection of the initial phenotype resulting from addition of dsRNA to the cell culture is an important goal of the RNAi experiment. However, this can often be very tricky, especially for proteins that have multiple roles in a cellular process, as is often the case for regulatory proteins such as kinases or phosphatases. Ideally, in order to minimize heterogeneity between different cell populations, a small aliquot of cells from each time point used for immunoblotting should be used for immunofluorescence analysis. Nevertheless, this problem can be minimized by collecting, from a parallel experiment, samples at several time points after addition of the dsRNA to the culture followed by analysis by immunofluorescence using appropriate antibodies

(again, samples must be collected from both RNAi and control experiments at each time point). For this purpose we repeated the same procedures described for the immunoblot, but using LAB-TEK permanox 2-chamber slides from Nalge Nunc (distributed by Gibco). Due to the size of the chambers, we used 5×10^5 cells/0.5 ml of media/chamber, and added half the amount of dsRNA used for the immunoblot experiment (in this case 15 μg /chamber), with one chamber used for the experimental and the other for the corresponding control. After serum starvation for 1 h, 1 ml of Schneider's *Drosophila* medium supplemented with FBS was added to each chamber and the slides were returned to the incubator at 25°C. As S2 cells grow in suspension it was necessary at each time point to centrifuge cells onto the slides for 15 min at 4,000 rpm at room temperature to render them adherent.

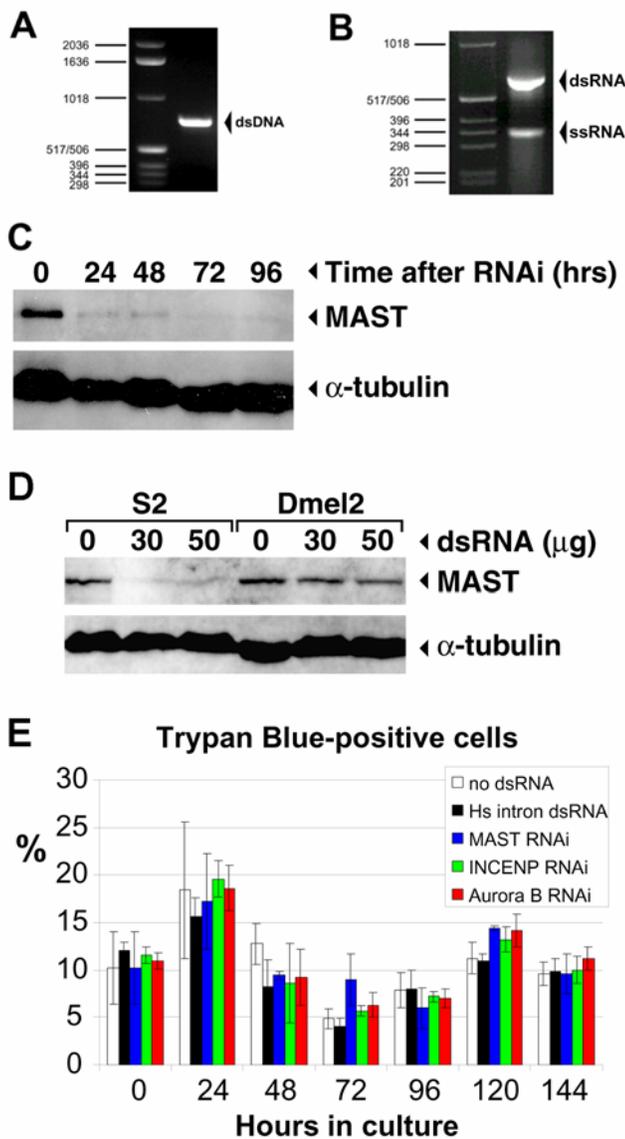


Fig. 1: Preparation of dsRNA and specific protein depletion. (A) PCR fragment of ~700 bp synthesized from MAST/Orbit cDNA. (B) Corresponding dsRNA obtained by *in vitro* transcription using the previous PCR fragment as template. The lower band corresponds to ssRNA that did not form duplexes. (C) Monitoring of protein levels upon addition of 30 μg of dsRNA to the cells by immunoblot using MAST polyclonal antibodies and anti- α -tubulin monoclonal

antibodies as a loading control. (D) Determination of the amount of dsRNA necessary to deplete MAST from S2 and Dmel2 cells. Protein levels were monitored 120 h after addition of dsRNA. It is clear that for the case of MAST, protein depletion is significantly more effective in S2 cells. (E) Addition of dsRNA to S2 cells did not cause a significant effect upon cell viability throughout the experiment.

Alternatively, the cells could be grown in 6 well plates as described for the immunoblot, and at each time point cytospun or left to adhere for 2 hours onto sterile poly-L-lysine treated slides (BDH). Cells were then immediately fixed and permeabilized and processed for immunofluorescence. Alternatively, after fixation, cells can be kept in PBS at 4°C up to one week and the complete set of cells from each time point processed at the same time for immunofluorescence (Fig. 2). In the case of MAST RNAi, the first visible abnormality that we observed was an increase in the mitotic index caused by the accumulation of cells in a prometaphase-like stage. Among these prometaphase cells, we observed two distinct populations, those with monopolar spindles (Fig. 2B and 2F) and those in which the spindle was bipolar but on which the chromosomes could not align at a metaphase plate (Fig. 2G). We defined these as the primary consequences due to MAST depletion. These results were confirmed independently by *in vivo* analysis of *mast* mutant embryos (17).

A number of other phenotypes could be observed at later time-points. These included the formation of polyploid cells with multiple centrosomes (Fig. 2C) and the appearance of anaphase-like cells with two distinct sets of segregated chromosomes separated on a bipolar spindle (Fig. 2D and 2H). These cells frequently display both centrosomes abnormally clustered at a single pole (Fig. 2D). After completion of the studies described here, Vale and co-workers published that S2 cells will adhere strongly and spread out on cover slips coated with concanavalin A (18). We have not yet tested the efficacy of RNAi in such adherent cells, but this is potentially a significant advance, at least with regard to the morphological analyses. However, we do know that this technique seriously compromise cytokinesis in S2 cells (P. Sampaio, personal communication).

Because not all cells are affected equally by the RNAi procedures, the culture at each time point is a mixture of cells in which the target protein has been depleted and cells in which it is still present. This makes description of the phenotype difficult, as some cells are affected and others are not. If cells are stained for the protein that is being targeted, then phenotypic analysis can be limited to those cells in which the treatment has been effective (i.e. in which the target protein is severely depleted or undetectable). This greatly simplifies the data analysis.

Growth curves and cell viability

A simple and quick assay to assess for cell viability in the RNAi experiment is to stain a small sample of cells at each time point with trypan-blue (Sigma). This method relies on the alteration of membrane integrity as determined by the uptake of dye by dead cells that turn out blue. In the case of MAST RNAi, when compared with controls and RNAi of other proteins (INCENP

and Aurora B), cell viability throughout the experiment typically did not change significantly (Fig. 1E).

The growth curves corresponding to the RNAi and control experiments must take into consideration only the number of viable cells, and the doubling time of the population is given by the slope of the best fit (semi-log scale) that can be calculated from the corresponding exponential equations.

Determination of the mitotic index and quantification of mitotic parameters

One can find many ways of determining the mitotic index of a cell population described in the literature, for example: number of mitotic cells per optical field, number of cells positive for phosphorylated histone H3 over the total number of cells or number of mitotic cells scored by direct visualization over the total number of cells. In our experiments, the mitotic index is calculated as the percentage of total cells that are in mitosis, and can typically be scored accurately by direct observation of 100-200 cells.

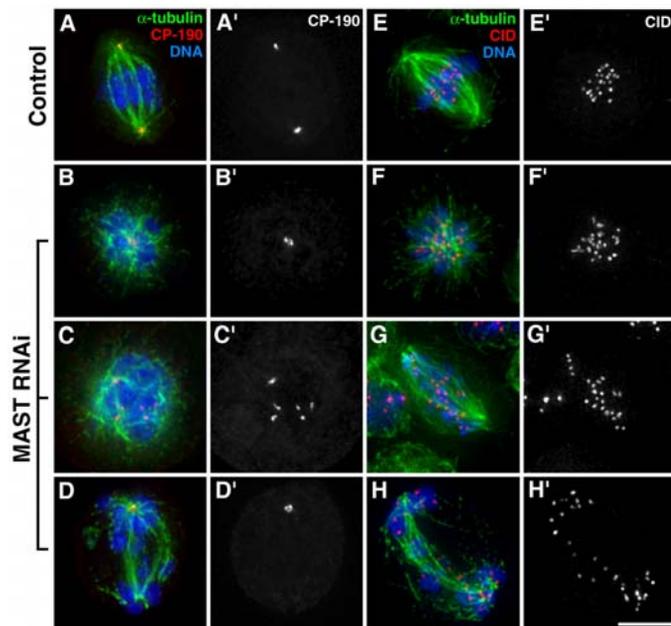


Fig. 2: Immunofluorescence analysis of mitotic S2 cells after RNAi. Chromosomes were stained with DAPI (blue), microtubules were stained with an anti- α -tubulin antibody (green), centrosomes were stained with an anti-CP-190 antibody and the centromeres were stained with an anti-CID antibody (both white or red in the merged images). (A, A' and E, E') Control metaphase cells showing a well organized bipolar spindle with one centrosome at each pole and with the chromosomes correctly aligned at the metaphase plate. (B, B' and F, F') 72 h after RNAi, cells form monopolar spindles with the centrosomes clustered in the centre and the centromeres dispersed in the monoaster. (C, C') Polyploid cell 96 h after RNAi showing multiple centrosomes. (G, G') Cell showing abnormal chromosome congression 72 h after RNAi. (D, D') Abnormal anaphase-like cells 120 h after RNAi showing a bipolar spindle where the centrosomes are clustered at only one of the poles. (E, E') Another anaphase-like cell where non-disjoined chromosomes have attempted to segregate towards opposite poles. Scale bar is 5 μ m.

We have used a combination of DNA and tubulin staining to accurately identify mitotic cells from prophase through telophase. In some cases, staining with an antibody to histone H3 phosphorylated on serine¹⁰ can be used to identify mitotic cells from prophase through anaphase, however RNAi of certain proteins (like INCENP and Aurora-B) causes a loss of phospho-H3 staining (14-15).

In some cases, it is desirable to assess the distribution of mitotic cells amongst the different phases of mitosis. This is informative if, for example as in the case of the INCENP or Aurora-B RNAi, cells have difficulty in aligning their chromosomes and accumulate in prometaphase (15). We have typically performed such analyses only on cells stained for the target protein, and limited our conclusions to cells in which this protein was below the level of detectability.

DISCUSSION AND PERSPECTIVES

While genetic analysis in *Drosophila* remains one of the most powerful methods to determine the function of a particular protein involved in cell division (reviewed in ref. 28), this is not always possible due to the lack of mutations for particular genes or due to the complexity of phenotypes observed with certain hypomorphic alleles. In those cases, RNAi in *Drosophila* tissue culture cells can be an excellent alternative for the study of gene function. It is important to note that analysis of fixed material can lead to ambiguous interpretations since each fixed sample represents a single time point in what is a highly dynamic process. Live cell imaging would be of great utility for the study of mitosis on RNAi-depleted cells. In fact, this is now routinely performed for RNAi experiments using human cells. In those cases, cells are transfected with siRNAs or suitable vectors, then blocked in S phase with elevated levels of thymidine. Upon release from the thymidine block, cells are filmed as they enter mitosis. Unfortunately, this approach has yet to be developed for *Drosophila* cultured cells, where so far *in vivo* analysis of mitosis has been restricted to embryos and primary cultures of larval neuroblasts (29-30).

This problem can be minimized for *Drosophila* cells by doing a time-course analysis after protein depletion by RNAi and monitoring the evolution of the phenotype using fixed material. This allows the investigator to follow the evolution of the phenotype as the cell cycle progress in the absence of a particular protein. In the example of MAST/Orbit mutant alleles, where there is a wide range of mitotic abnormalities, from monopolar spindles to highly polyploid cells with multiple asters, a time course analysis after depletion of MAST/Orbit by RNAi in tissue culture cells, allowed us to follow the evolution of the phenotypes in a null background. Our results (see ref. 17) clearly indicated that the first requirements of MAST/Orbit were in the normal congression of chromosomes into the metaphase plate and in the maintenance of spindle bipolarity, which contributed for a mitotic delay and eventual abnormal mitotic slippage without chromosome segregation and cytokinesis. These cells then must have undergone several rounds of DNA replication resulting in polyploidy. This phenotype could also be explained

by the observed role of MAST at the microtubule-kinetochore interface, which would have direct implications in the control of cell cycle progression by the spindle-assembly checkpoint (for reviews see ref. 31).

Although genome-wide RNAi based screening may provide new insights of protein function in cells, its results should be interpreted with caution. It may be misleading to deduce the role of a particular protein in a large-scale experiment using standard conditions for all the genes, since different genes behave differently in response to RNAi treatment. Furthermore, the absence of a clear mitotic phenotype does not prove that the protein under study is not involved in the process. At an early stage in the analysis, this only indicates that the conditions used might not be adequate. Importantly, the results can vary significantly between different experiments, mostly due to the use of different batches of dsRNA who may have distinct penetrability. Apart from the phenotypes resulting from RNAi of a particular protein, control S2 cells also often showed a panoply of background mitotic abnormalities, such as bipolar spindles with irregular centrosome number at each pole, bipolar spindles with absence of centrosomes in one of the poles and monopolar spindles. Thus, the analysis of the control RNAi experiment can be very important in order to distinguish between specific effects arising from the RNAi, and background effects due to the baseline behavior of the cell line.

For the analysis to be convincing, a specific antibody that works in both immunoblots and immunofluorescence experiments is also a requirement. Without such an antibody, it is not possible to unambiguously correlate particular phenotypes with loss of the protein. This is probably the strongest disadvantage relatively to other methods of protein inactivation, namely those that interfere at the DNA level (reviewed in ref. 32). Nevertheless, when adequately performed, RNAi in *Drosophila* S2 cells is certainly a highly effective and rapid method for the study of gene function.

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PROTOCOLS

Protocol 1: Preparation of dsRNA

1. Design 18-mer sequence-specific oligonucleotides to make a PCR product of ~700 bp from the cDNA of interest covering the codon for the first methionine. Don't forget to incorporate the 5' T7 RNA polymerase binding site in your primers (TAATACGACTCACTATAGGG).
2. Prepare 10-12 individual PCR reactions on ice containing the following reagents:
 - 0.5-1 ng of template cDNA from a plasmid
 - 1 μ M of each primer
 - 2.5 mM of dNTP mix
 - Taq polymerase enzyme buffer
 - 2 mM MgCl₂ (if not included in the enzyme buffer)
 - water to a final volume of 100 μ l (take into consideration the volume of Taq polymerase that will be added afterwards)
3. Set up the following PCR program:
 - 94°C – 2 min
 - add 2.5 U of Taq polymerase to the PCR reaction
 - 94°C – 30 sec
 - 55°C – 60 sec
 - 72°C – 60 sec
 - 72°C – 10 min

} x 30 cycles
4. Purify the PCR products by pooling 3 reactions, and running them through each column from a PCR Clean-Up Kit (MoBio) according to the manufacturer's instructions.
5. Test 1 μ l of the clean product by electrophoresis on a 1% agarose gel and quantify the DNA by measuring Abs₂₆₀. The DNA yield can be calculated as follows:
$$\text{Abs}_{260} \times \text{dilution factor} \times 50 = \text{DNA conc. in } \mu\text{g/ml}$$
6. Use this DNA as a template for *in vitro* RNA synthesis with the MEGA script T7 Kit (Ambion) according to the manufacturer's instructions, except that the incubation time should be increased to at least 6 h.
7. Pool the reactions into a single tube and precipitate the RNA with LiCl according to the instructions included with the kit.
8. Carefully wash the pellet twice with 70% ethanol and let air-dry.
9. Re-suspend the pellet in 100 μ l of nuclease-free water and check the RNA concentration as before but using the following algorithm:
$$\text{A}_{260} \times \text{dilution factor} \times 40 = \text{RNA conc. in } \mu\text{g/ml}$$
10. Denature RNA secondary structures by heating at 65°C for 30 min in a beaker containing 200 ml of previously warmed water and then let it cool down to room temperature to make dsRNA duplexes.
11. Test 1 μ l of the dsRNA by electrophoresis in 1% agarose gel. It should run like DNA as a clean band of ~700 bp. Sometimes a lower band is also visible. This corresponds to ssRNA that did not make duplexes (see Fig. 1B).
12. Store the dsRNA at -20°C.

Protocol 2: Monitoring protein depletion after RNAi by immunoblot

1. Grow *Drosophila* S2 cells exponentially in T-flasks at 25°C in Schneider's *Drosophila* medium supplemented with 10% FBS for 4 days.
2. Check how many time points and how many wells will be needed for the experiment (2 wells per time point e.g. 0, 24, 48, 72, 96, 120, 144 hours: total of 14 wells).
3. Count viable cells by staining with trypan-blue (Sigma) using a haemocytometer and dilute enough cells in Schneider's *Drosophila* medium (Gibco) without serum to make up the total volume needed for the experiment for a final concentration of 10⁶ cells/ml.
4. Distribute 1 ml of media containing 10⁶ cells per each 35 mm well (this can be done using 6 well culture plates).

5. Add 30 μg of specific dsRNA to half of the wells, and an equivalent amount of control dsRNA to the control wells. Mix well by swirling and leave the cells at room temperature for 1 h to allow incorporation of the dsRNA.
6. Add 2 ml of Schneider's *Drosophila* medium supplemented with FBS to each well and put the cells back in the incubator at 25°C.
7. At each time point collect a small sample of cells (10 μl) from both RNAi and control experiments and count viable cells by staining with trypan-blue (Sigma). Then collect the remaining cells from each well and pellet by centrifugation at 1,500 rpm for 4 min.
8. Discard the medium and re-suspend the cells directly into the appropriate volume of Laemmli sample buffer to run 10^6 cells/lane of a gel.
9. Lyse the cells by sonication, boil for 5 min and store the protein extracts at -20°C.
10. At the end of the time course, subject every sample from each time point (RNAi and control experiment) to SDS-PAGE.
11. Transfer the proteins to nitrocellulose membranes and process for routine immunoblotting using specific primary antibodies to the protein of interest together with antibodies for a loading control (we use commercial anti-actin or anti-tubulin antibodies from Sigma). Secondary antibodies conjugated to horseradish peroxidase were detected by ECL (Amersham) according to the manufacturer's instructions.

Protocol 3: Phenotypic analysis after RNAi by immunofluorescence

1. Grow *Drosophila* S2 cells exponentially in T-flasks at 25°C in Schneider's *Drosophila* medium for 4 days.
2. Check how many time points and how many slide chambers will be needed for the experiment (1 slide = 2 chambers per time point e.g. 0, 24, 48, 72, 96, 120, 144 hours: total of 7 slides, 7 chamber for specific RNAi treated cells and 7 chambers for controls).
3. Count viable cells by staining with trypan-blue (Sigma) using a haemocytometer and dilute in Schneider's *Drosophila* medium (Gibco) without serum to make up the total volume needed for the experiment and for a final concentration of 10^6 cells/ml.
4. Distribute 0.5 ml of media containing 10^6 cells/ml into each chamber.
5. Add 15 μg of dsRNA to one of the chambers of the slide, and an equivalent amount of control dsRNA to the control wells. Mix well by swirling.
6. Leave the cells at room temperature for 1 h to allow incorporation of the dsRNA.
7. Add 1 ml of Schneider's *Drosophila* medium supplemented with to each chamber and incubate the slides at 25°C.
8. At each time point, re-suspend the cells by pipetting up and down and centrifuge the slide using appropriate racks for 15 min at 4,000 rpm and at room temperature.
9. Discard the medium and immediately fix the adherent cells using appropriate conditions for the antibodies to be used (we fix the cells using 4 % paraformaldehyde diluted in cytoskeleton buffer: 1.1 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 137 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 2 mM EGTA, 5 mM Pipes, 5.5 mM glucose, pH 6.1; see ref. 33). If necessary, permeabilize the cells after fixation using 0.1 % Triton X-100 (Merck) diluted in cytoskeleton buffer.
10. Cells can now be processed immediately for immunofluorescence or alternatively can be kept in PBS at 4°C up to one week, or in PBS:Glycerol (60%) at -20°C for several months.