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RNAi in Cultured *Drosophila* Cells

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1. Introduction

Double-stranded RNA (dsRNA)-mediated interference, or RNAi, has emerged as an effective technique to phenocopy the loss of function of a given gene product. With this tool researchers can study the functions of individual molecules in living cells and elucidate the mechanisms that regulate cell division. For example, many molecules that are important for regulating mitosis and for controlling the assembly of the mitotic spindle are mutated in different cancer cell types (for a review, *see ref.* ¹). Functional analysis *in vivo* of molecules that play a role in mitosis is best implemented by a genetic analysis. For this, genetically malleable organisms such as *Drosophila*, *Caenorhabditis elegans*, yeast, and other micro-organisms have been extremely useful. Whereas genetic analysis usually requires a long-term effort, RNAi provides a rapid method for the reverse genetic analysis of gene product function and can be exploited to great advantage. In the era of sequenced genomes, this technique provides a valuable tool for functional genomics. Here, a detailed procedure for RNAi in *Drosophila* cells in culture is presented.

RNA interference was first described using *C. elegans* (2), although the phenomenon has been described in plants as posttranscriptional gene silencing (PTGS) (3) and as “quelling” in *Neurospora* (4). Furthermore, RNAi has been demonstrated on a number of organisms (2–9). For *Drosophila*, RNAi has been accomplished by injection of dsRNA into early syncytial cleavage stage embryos (6). Subsequently, heritable RNAi has been achieved in *C. elegans* and *Drosophila* using transgenic dsRNA “hairpin”-generating constructs (10–13). An important advance came when RNAi was demonstrated with cultured *Drosophila* cells (7).

More recently, RNAi has been applied successfully to vertebrate cells in culture using short interfering RNAs (siRNAs) (14,15). For this, the first step in the cellular response mechanism to dsRNA (*see below*) has to be bypassed, because full-length dsRNAs produce nonspecific effects in vertebrate cells (16–19).

RNAi-mediated interference occurs by a posttranscriptional mechanism that targets mRNA homologous to the dsRNA that is introduced for destruction (for reviews, *see refs.* 20–22). Using *Drosophila* embryo and S2 cell extracts, the mechanisms for RNAi are being elucidated (16,23). In these extracts, dsRNA is cleaved into 21- to 25-bp siRNAs with 5' phosphates, 3' hydroxyl groups, and contain two to three nucleotide 3' overhangs. dsRNA cleavage is mediated by Dicer, an ATP-dependent RNaseIII family RNase (24). siRNAs assemble into an approx 360-kDa complex called RNA-induced silencing complex (RISC) in *Drosophila* extracts (25). The siRNAs then unwind in an ATP-dependent manner (23,25). The single-stranded siRNAs in the RISC complex provide the homologous targeting to mRNA (23,26), enabling degradation by the RNase associated with RISC. Furthermore, Argonaute proteins are components of RISC (27) with homologs in plants, fungi, and *C. elegans* that are required for RNAi in those organisms (28–30). The degraded target mRNA appears to then be cycled into new siRNAs that repeat the process in an RNA polymerase-dependent cycle of mRNA degradation and siRNA production (31). The complete mechanism for RNAi has not been elucidated.

A teleological explanation for the existence of a mechanism to destroy mRNAs in response to homologous dsRNA has been proposed (32). It has been suggested that RNAi evolved as a mechanism to combat invading dsRNA viruses or to inhibit the activity of retrotransposons. Moreover, there is at least one gene in *Drosophila*, *Stellate*, that is regulated by dsRNA-mediated gene silencing in the testis (33).

This chapter describes the application of RNAi to cultured *Drosophila* cells, with a particular emphasis on the imaging of the cytoskeleton and chromosomes in affected cells. Materials and methods are provided to enable the researcher to implement the design and production of dsRNA from polymerase chain reaction (PCR) templates, the culture of *Drosophila* cells and their treatment by RNAi, the analysis of target protein depletion by Western blotting, and the fixation and treatment of cells for microscopic imaging. The depletion of centrosomin (Cnn) a centrosomal protein that is required for mitotic centrosome assembly and function (34³⁷) from S2 cells is presented for example, but the technique is widely applicable to different targets and cell lines (7^{17,38}). Importantly, *Drosophila* cells in culture readily take up exogenous dsRNA, and there is no need to use carriers or transfection methods like those required with mammalian cell culture (7). Thus, RNAi holds great promise for the analysis of protein function in living cells.

2. Materials

2.1. DNA Templates for Making dsRNA

1. Oligonucleotide primers for PCR.
2. Thermostable DNA polymerase (e.g., Clontech Advantage2 PCR reagent).
3. 10X buffer for PCR.
4. 10X Deoxyribonucleotide triphosphate (dNTP) mixture (solution containing 2 mM each of dATP, dGTP, dCTP, and dTTP).
5. PCR purification kit (Qiagen).
6. RNase-free water.
7. Thermal cycler.

2.2. Synthesis of dsRNA

1. T7 PCR template in water at approx 0.1 µg/µL.
2. Ambion MEGAscript T7 kit (cat no. 1334), or Promega Ribomax large-scale RNA Production System-T7 (cat. no. P1300).
3. RNase-free water.

2.3. Culture and Treatment of Cells

1. Live culture of *Drosophila* S2 cells (ATCC CRL-1963).
2. 100 × 20-mm cell culture dishes.
3. Sterile 15- or 50-mL conical centrifuge tubes.
4. Fetal bovine serum (FBS) (Hyclone or GIBCO). Heat treat at 65°C for 30 min prior to use (*see Note 1*).
5. Culture medium: M3 + BPYE (Bacto-peptone, yeast extract). Per liter: Mix 39.4 g Shields and Sang M3 powder (Sigma-Aldrich) and 0.5 g KHCO₃ into 800 mL deionized water. Mix until dissolved, then bring pH to 6.6 with HCl. Add 1 g

Yeastolate (yeast extract, cell culture grade; Sigma-Aldrich), 2.5 g Bacto-peptone and deionized water to a final volume of 1 L. Filter sterilize; store at 4°C (*see Note 2*).

6. M3 + BPYE + 10% FBS
7. Multiple well flat-bottomed Cluster-6 plates, or 60-mm culture dishes.

2.4. Western Blotting

1. 30:0.8 Acrylamide: bisacrylamide.
2. 1.5 M Tris-HCl, pH 8.8.
3. 0.5 M Tris-HCl, pH 6.8.
4. 10% (w/v) Sodium dodecyl sulfate (SDS).
5. 25% Ammonium persulfate (APS) (store at 4°C for up to 1 mo).
6. TEMED.
7. Protein minigel apparatus (e.g., Bio-Rad Protean system).
8. Gel transfer apparatus (e.g., Bio-Rad minigel transfer system).
9. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis buffer: 25 mM Tris base, 192 mM glycine, 0.1% SDS.
10. 5X SDS-PAGE loading buffer: 350 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue.
11. Gel transfer buffer: 25 mM Tris base, 192 mM glycine, 20% methanol.
12. TBS-T: 50 mM Tris-HCl, pH 7.2, 120 mM NaCl, 0.1% Tween-20, autoclaved.
13. Blocking Solution: 5% Nonfat dry milk in TBS-T.
14. Horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson IRL).
15. Chemiluminescence detection reagent (ECL, Amersham, or Supersignal West Pico, Pierce).

2.5. Cell Fixation and Staining

1. Glass slides (untreated) with approx 10-mm-diameter wells (e.g., Fisher cat. no. 12-568-20, or PGC Scientifics cat. no. 60-5453-24).
2. Coplin jars.
3. Humid chamber for slides.
4. Poly-L-lysine solution (MW > 300,000, Sigma-Aldrich P1524), 1 mg/mL.
5. 10X Phosphate-buffered saline (PBS): 18.6 mM NaH₂PO₄, 84.1 mM Na₂HPO₄, 1.75 M NaCl, pH 7.4.
6. PBS: Dilute 10X PBS stock to 1X with water.
7. 10% Solution of saponin (Sigma-Aldrich). Store aliquots at -20°C.
8. 100 mg/mL bovine serum albumin (BSA, fraction V) in PBS + 0.02% sodium azide. Store at 4°C.
9. Methanol at -20°C.
10. Primary antibodies (e.g., anti- α -tubulin mouse monoclonal DM1A [Sigma-Aldrich]).

11. DAPI (4', 6-diamidine-2'-phenylindole) or TOTO-3 DNA dye (Molecular Probes).
12. Fluorescent secondary antibodies (e.g., fluorescein isothiocyanate [FITC]-conjugated donkey anti-mouse [Jackson ImmunoResearch Laboratories]).
13. Clear nail polish.

2.6. Imaging

1. Mountant: 0.05% *p*-Phenylenediamine, 0.1 M Tris, pH 8.8 in 90% glycerol. Store at -20°C shielded from light. Solution will turn brown over time. Make fresh every 6 mo.
2. Microscope with 600-1000× magnification (confocal microscope is preferred).
3. Filter sets for FITC, tetramethylrhodamine (TRITC) (or Cy3 or Texas Red), and Cy5 (for TOTO-3) (*see Note 3*).

3. Methods

3.1. Preparation of Templates by PCR To Be Used for In Vitro Transcription

1. Design oligo. Oligonucleotides should be designed against cDNA or exon sequences of the gene of choice, preceded by the T7 promoter sequence: TAATACGACTCACTATAGGGGA (the underlined G is the transcription start site for T7 polymerase) (Fig. 1A). Design primers to produce templates of 700-1000 bp in length, although shorter dsRNAs also appear to work (17). In general, the target sequence of the primer should be 18-24 nucleotides in length. Programs such as Oligo (Molecular Biology Insights), MacVector (Oxford Molecular Group), or Primer3 (free on the WWW) (39) can be used to design primers that fit guidelines for PCR effectiveness such as GC content and predicted T_m.
2. Amplify template by PCR. Mix: In a 0.5 mL tube mix 10 µL Advantage 2 PCR reagent (Clontech), 10 µL of 2 mM dNTP mix, 0.2 µM each primer, 50 ng cDNA or 1 µg of genomic DNA, and water to a final volume of 100 µL (*see Note 4*). Amplify in a thermal cycler for 30 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 1 min.
3. Analyze 5 µL of the reaction by agarose gel electrophoresis. The PCR product should appear as a single band of the expected size on the gel (*see Note 5* and Fig. 1B).
4. Purify the PCR product using a PCR purification kit (Qiagen). Collect the DNA in RNase-free water (included in Ambion MEGAscript kit) (*see Note 6*).
5. Quantify the PCR product using a spectrophotometer or by using the ethidium bromide spot method (40).
6. Adjust the concentration of PCR DNA to 0.1 µg/µL with RNase-free water.

3.2. Production of dsRNA (Briefly, from the Ambion MEGAscript Kit Protocol)

1. Mix 10 µL PCR DNA (1-2 µg), 16 µL nucleotide triphosphate mix, 6 µL RNase-free water, 4 µL of 10X reaction buffer, and 4 µL enzyme mix in a 0.5- or 1.5-mL tube to a final volume of 40 µL.
2. Incubate at 37°C for 5 h.
3. Add 1 µL DNase; incubate at 37°C for 15 min.
4. Precipitate RNA: add 50 µL RNase-free water, 10 µL 3.0 M sodium acetate pH 5.2, 250 µL 95% ethanol, and place at -20°C for >15 min.

5. Centrifuge for 15 min in microfuge at 4°C. Discard the supernatant.
6. Wash the pellet with 1 mL ice-cold 70% ethanol, centrifuge for 5 min.
7. Remove as much of the wash solution as possible and suspend the pellet in 100 µL RNase-free water. Perform this and subsequent handling of the dsRNA in a sterile hood. Repeated vortexing may be necessary to dissolve the RNA pellet.
8. Quantify the RNA concentration by ultraviolet (UV) absorbance with a spectrophotometer. Begin by diluting your samples 1:100-1:200 to obtain a reading in the linear range. To calculate yield, assume 1 A₂₆₀ unit corresponds to 40 µg/mL [A₂₆₀ × dilution factor × 40 = µg/mL dsRNA].
9. Analyze the integrity of dsRNA by agarose gel electrophoresis of the sample (3-5 µg).
10. Store the dsRNA solution at -20°C.

3.3. RNAi Treatment of Cells (see Note 7)

1. Culture S2 cells to a density of $0.5\text{--}1.0 \times 10^6$ cells/mL in 100-mm dishes, 6-7 mL of culture per dish.
2. Suspend the cells by gently pipetting with a 10-mL pipet and transfer to a 15-mL conical tube. If using serum-free medium, suspend cells and skip to **step 8**.
3. Centrifuge for 2 min at 2000 rpm in a clinical centrifuge.
4. Suspend the cells in 10 mL of serum-free medium (M3+BPYE).
5. Centrifuge for 2 min at 650g in a clinical centrifuge.
6. Repeat **steps 4 and 5**.
7. Suspend the cells in 10 mL of serum-free medium.
8. Add 1 mL of cells into each well of a six-well cluster dish, or into a 60-mm culture dish (*see Note 8*). Alternatively, use 12-well dishes with 0.5 mL culture per well.
9. Add dsRNA to a final concentration of 40 nM (*see Note 9*) and mix well by swirling.
10. Incubate the cells and dsRNA for 1 h at room temperature.
11. Add 2 mL of medium with serum (M3 + BPYE + 10% FBS). Skip this step if using serum-free medium.
12. Examine cells daily. Passage as needed to maintain 30-80% confluence until d 4-9.
13. Wait an appropriate amount of time to examine the cells, which should be determined empirically in a time-course assay for each protein to be targeted (*see Note 10 and Fig. 2*).

3.4. Western Blot Analysis of RNAi-Treated Cells

Western blotting, like that shown for Cnn in Fig. 2, is recommended to assay the efficiency and time-course of decay for each target protein.

1. Remove aliquots of S2 cells at different time points and place in 1.5-mL tubes. Remove an aliquot of the cells at time zero (500 µL; *see Note 11*), before dsRNA addition, and remove aliquots every day (or other time increments thereafter), for several days.

2. Pellet cells by centrifugation in a microfuge. Discard the supernatant and suspend in 50 μ L 1X SDS-PAGE loading dye. Load 10 μ L of the sample onto SDS-PAGE gel following heating to 95-100°C for 5 min. Samples can be stored at -20°C or -70°C.
3. Pour an SDS-PAGE minigel (*see Note 12*). For the resolving gel, mix acrylamide (7-15% final, depending on the size of your protein), 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 1/1000 volume of 25% APS, 1/1000 volume TEMED. Pour gel immediately after the addition of TEMED, leaving about a 3-cm space for the stacking gel. Overlay with approx 100 μ L water. Let polymerize for 1 h. For the stacking gel, mix acrylamide (4%), 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 1/1000 volume of 25% APS, 1/1000 volume TEMED. Remove the overlay solution, then pour immediately and insert the comb. Let polymerize at least 30 min.
4. Separate the proteins by electrophoresis on an SDS-PAGE minigel.
5. Transfer to nitrocellulose membrane in gel transfer buffer using a cooled transfer chamber at 100 V for 1 h.
6. Place the membrane in 20 mL of blocking solution in a 9 $\times$ 9 cm square Petri dish or similar chamber. Incubate for 1 h at room temperature with gentle shaking.
7. Remove the blocking solution. Add primary antibody in 10 mL TBS-T and incubate for 1 h at room temperature (or overnight at 4°C).
8. Remove the antibody solution and wash the blot three times with 20 mL TBS-T for 5 min each.
9. Incubate with HRP-conjugated secondary antibody (1 : 10,000) in 10 mL TBS-T for 30 min.
10. Repeat **step 8**.
11. Treat the blot with chemiluminescence substrate reagent and expose to X-ray film for various times.

3.5. Staining of Cells

1. Treat the slides with poly-L-lysine as follows: Wash glass slides in water and wipe dry with a Kimwipe. Apply 50 μ L of 1 mg/mL poly-L-lysine into each well on the slide and let sit for 45 min. Wash slides with water three times in Coplin jars. Let slides dry (*see Note 13*).
2. Apply 50 μ L of cells to each well and let sit for 30 min.
3. Rinse cells briefly (2 s) in PBS and then place directly into -20°C methanol. For this, dip the slides into a Coplin jar containing PBS and place them into a Coplin jar with methanol that has been kept in the freezer. Incubate the slides in -20°C methanol for 10 min.
4. Remove the slides from -20°C and place into a Coplin jar with PBS. Rinse once with fresh PBS. The cells should appear as a film in the well. The cells should not be permitted to dry in any of the subsequent procedures.
5. Using a Kimwipe twisted into the shape of a probe, or using a cotton swab, blot the PBS from the region of the slide surrounding the well dry. This will prevent the antibody solution from spreading out from the well in subsequent procedures.
6. Apply the primary antibodies, diluted in PBS + 0.1% saponin + 5 mg/mL BSA, 50 μ L per well. If DNA dyes such as propidium iodide or TOTO-3 are to be used, RNase A can be added at this step at a concentration of 50 μ g/mL (*see Note 14*). We

recommend using one combination of antibodies for all the samples on the same slide to prevent cross-contamination. For different antibody mixtures, use additional slides.

7. Incubate slides in a humid chamber for 1 h at room temperature, or overnight at 4°C. A simple humid chamber can be made by taking an empty pipet tip box, adding water into the box, and placing the slides onto the slotted tip holder.
8. Wash slides in a Coplin jar with three changes of PBS, 5 min each.
9. Apply secondary antibodies to the slides (*see Note 15*). First, blot the area around the wells dry as described in **step 5**. Add 50 µL of secondary antibodies, diluted in PBS + 0.1% saponin + 5 mg/mL BSA, into the wells. Incubate for 1 h at room temperature in the dark.
10. Wash as in **step 8**; then, blot the slides dry as in **step 5**.
11. Apply 4 µL of Mountant to each well. Overlay a cover slip slowly and at an angle to prevent the inclusion of air bubbles under the cover slip (*see Note 16*). Fix coverslip to the slide with clear nail polish.

3.6. Imaging Cells by Confocal Microscopy

1. High magnification with a 60× or higher objective is required to image S2 cells effectively. These objectives require immersion in oil or water (*see Note 17*).
2. For confocal microscopy, use multiple excitation lasers to image multiple fluorophors. There are a variety of configurations available; some include lasers that produce lines typically at 488, 568, and 647 nm (argon-krypton), or 488, 568, and 633 nm (argon, krypton, and helium-neon (RedHeNe), or 488, 543, and 633 nm (argon, GreenHeNe, RedHeNe). These should all be compatible with three-color imaging like that shown in **Figs. 3 and 4**, where the (excitation peak wavelength/emission peak wavelength [in nm]) for FITC (490/520), TRITC (541/572), and TOTO-3 (642/660) allowed separation of all three emission signals.
3. S2 cells are small, approx 10 µm thick. Therefore, when a z-series is collected, a large stack of images will not need to be produced. Steps of 0.5-1.0 µm may be adequate for most purposes.
4. If bleaching becomes a problem, one method is to set up the imaging using only one of the fluorescent signals (the more robust) to view the cell. Then, turn on the other lasers when the images are being captured. This strategy reduces the bleaching of weaker signals or sensitive fluorophors.

4. Notes

1. Fetal bovine serum is of the highest quality (mycoplasma, virus, bacteriophage, and endotoxin tested). Store serum at -20°C before heat treatment and at 4°C after heat treatment unless it will be stored for a long period, and in that case, store it at -20°C. We have used Hyclone and GIBCO brands of FBS.
2. S2 cells can also be cultured in commercially available Schneider's *Drosophila* medium (GIBCO) supplemented with 10% FBS, or adapted to CCM3, a synthetic medium supplied by Hyclone.
3. A variety of fluorophore conjugates are available commercially. Molecular Probes sells a set of secondary antibodies conjugated to a variety of "Alexa" fluorophores, which are more resistant to bleaching.

4. For PCR, substitute any thermostable DNA polymerase and reaction conditions with which you are familiar. We have found that the Clontech Advantage2 PCR enzyme/buffer mixture gives a high yield of product.
5. If the PCR produces multiple bands, the reaction conditions need to be optimized. See ref. ⁴¹, or the *Promega Protocols and Applications Guide* (free from Promega) for guidelines. If the yield is low, multiple reactions can be combined.
6. As an alternative, satisfactory results were obtained from PCR templates that were cleaned up by phenol/CHCl₃ and CHCl₃ extraction followed by ethanol precipitation and a wash with ice-cold 70% ethanol.
7. The methods for the preparation of culture media and the culture of *Drosophila* cells were all from ref. ⁴².
8. It is necessary to always include a control dsRNA in these experiments. Use something that should have no effect (like lacZ, green fluorescent protein [GFP], or bacterial plasmid vector sequences).
9. For a dsRNA of approx 700 bp in length, 40 nM corresponds to approx 15 µg of dsRNA in 1 mL of medium. If 15 µg of dsRNA does not effectively reduce the target mRNA, consider increasing the amount to 30 µg, as this increased amount appears to have no side effects on control cells.
10. When Cnn levels were measured following RNAi, the protein fell to low levels by d 3 and 4 and then began to rise again by d 7 (see Fig. 2). In one experiment, Cnn was knocked down four consecutive times (not shown). Following the initial RNAi, the procedure was repeated three more times on the culture every 4 d. This experiment was possible in this case, because Cnn is not required for cell viability. One can also add dsRNA to the culture every day to achieve depletion of the target protein (43). Half-lives vary widely among proteins. Wei et al. (38) showed that one protein (HSF) with a known half-life of 8-10 h was reduced dramatically 2 d following treatment, whereas the more stable β-tubulin protein was relatively less diminished in the same time period upon RNAi targeting. Thus, for more stable proteins, it might be necessary to implement a second dose of RNAi on d 4.
11. Five hundred microliters of the cell pellet from the culture at d 0 gives ample protein for one or two gel loadings. S2 cells double approx once every 24 h, so on d 2, take 250 µL of the culture, on d 3 take 125 µL, and so on. It may be necessary to supply fresh medium to the cells on d 3, and this dilution should be accounted for when taking the next aliquot.
12. Precast gels are commercially available from Invitrogen, Bio-Rad, and others. Make sure the company's gels will fit your system.
13. Slides can be prepared in advance and stored dry for at least 2 wk.
14. For triple labeling cells *in situ*, like those in Figs. 3 and 4, we generally use a combination of secondary antibodies that include FITC, TRITC, and Cy5. For DNA staining we use TOTO-3. Texas Red emission overlaps too much with Cy5 and TOTO-3. For microtubule staining, Sigma sells a FITC-conjugated version of the DM1A monoclonal that gives a robust signal.
15. When the secondary antibodies are applied, other dyes can also be incubated in the same mixture (TOTO-3, Rhodamine-Phalloidin, etc.). Thus, a third incubation step is not required following the application of secondary antibodies.
16. Slides can be stored at -20°C for 2 wk, and possibly longer, with retention of the fluorescent signals.

17. Because the cells need to be imaged under an immersion lens, it is important that there is not too much solution under the cover slip. Otherwise, the surface tension from the immersion fluid will cause the cover slip to move in the Z direction, distorting the image. Alternatively, the cover slip can be anchored to the slide with clear nail polish applied to the edges of the cover slip.

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References

1. Jordan MA, Wilson L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr. Opin. Cell. Biol* 1998;10:123–130. [PubMed: 9484604]
2. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806–811. [PubMed: 9486653]
3. Vaucheret H, Beclin C, Fagard M. Post-transcriptional gene silencing in plants. *J. Cell Sci* 2001;114:3083–3091. [PubMed: 11590235]
4. Cogoni C. Homology-dependent gene silencing mechanisms in fungi. *Annu. Rev. Microbiol* 2001;55:381–406. [PubMed: 11544361]
5. Cogoni C, Macino G. Post-transcriptional gene silencing across kingdoms. *Curr. Opin. Genet. Dev* 2000;10:638–643. [PubMed: 11088014]
6. Kennerdell JR, Carthew RW. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* 1998;95:1017–1026. [PubMed: 9875855]
7. Clemens JC, Worby CA, Simonson-Leff N, et al. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA* 2000;97:6499–6503. [PubMed: 10823906]
8. Brown SJ, Mahaffey JP, Lorenzen MD, Denell RE, Mahaffey JW. Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. *Evol. Dev* 1999;1:11–15. [PubMed: 11324015]
9. Hughes CL, Kaufman TC. RNAi analysis of *Deformed*, *proboscipedia* and *Sex combs reduced* in the milkweed bug *Oncopeltus fasciatus*: novel roles for *Hox* genes in the hemipteran head. *Development* 2000;127:3683–3694. [PubMed: 10934013]
10. Tavernarakis N, Wang SL, Dorovkov M, Ryazanov A, Driscoll M. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genet* 2000;24:180–183. [PubMed: 10655066]
11. Kennerdell JR, Carthew RW. Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nature Biotechnol* 2000;18:896–898. [PubMed: 10932163]
12. Piccin A, Salameh A, Benna C, et al. Efficient and heritable functional knock-out of an adult phenotype in *Drosophila* using a GAL4-driven hairpin RNA incorporating a heterologous spacer. *Nucleic Acids Res* 2001;29:E55–5. [PubMed: 11410678]
13. Kalidas S, Smith DP. Novel genomic cDNA hybrids produce effective RNA interference in adult *Drosophila*. *Neuron* 2002;33:177–184. [PubMed: 11804566]
14. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494–498. [PubMed: 11373684]
15. Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA* 2001;98:9742–9747. [PubMed: 11481446]

16. Tuschl T, Zamore PD, Lehmann R, Bartel DP, Sharp PA. Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev* 1999;13:3191–3197. [PubMed: 10617568]
17. Caplen NJ, Fleenor J, Fire A, Morgan RA. dsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference. *Gene* 2000;252:95–105. [PubMed: 10903441]
18. Oates AC, Bruce AE, Ho RK. Too much interference: injection of double-stranded RNA has nonspecific effects in the zebrafish embryo. *Dev. Biol* 2000;224:20–28. [PubMed: 10898958]
19. Zhao Z, Cao Y, Li M, Meng A. Double-stranded RNA injection produces nonspecific defects in zebrafish. *Dev. Biol* 2001;229:215–223. [PubMed: 11133165]
20. Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet* 2001;2:110–119. [PubMed: 11253050]
21. Carthew RW. Gene silencing by double-stranded RNA. *Curr. Opin. Cell Biol* 2001;13:244–248. [PubMed: 11248560]
22. Zamore PD. RNA interference: listening to the sound of silence. *Nature Struct. Biol* 2001;8:746–750. [PubMed: 11524674]
23. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 2000;404:293–296. [PubMed: 10749213]
24. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 2001;409:363–366. [PubMed: 11201747]
25. Nykanen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 2001;107:309–321. [PubMed: 11701122]
26. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2002;101:25–33. [PubMed: 10778853]
27. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 2001;293:1146–1150. [PubMed: 11498593]
28. Fagard M, Boutet S, Morel JB, Bellini C, Vaucheret H. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proc. Natl. Acad. Sci. USA* 2000;97:11,650–11,654.
29. Catalanotto C, Azzalin G, Macino G, Cogoni C. Gene silencing in worms and fungi. *Nature* 2000;404:245. [PubMed: 10749199]
30. Tabara H, Sarkissian M, Kelly WG, et al. The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 1999;99:123–132. [PubMed: 10535731]
31. Lipardi C, Wei Q, Paterson BM. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell* 2001;107:297–307. [PubMed: 11701121]
32. Waterhouse PM, Wang MB, Lough T. Gene silencing as an adaptive defence against viruses. *Nature* 2001;411:834–842. [PubMed: 11459066]
33. Aravin AA, Naumova NM, Tulin AV, Vagin VV, Rozovsky YM, Gvozdev VA. Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the *D. melanogaster* germline. *Curr. Biol* 2001;11:1017–1027. [PubMed: 11470406]
34. Megraw TL, Kao LR, Kaufman TC. Zygotic development without functional mitotic centrosomes. *Curr. Biol* 2001;11:116–120. [PubMed: 11231128]
35. Raff JW. Centrosomes: central no more? *Curr. Biol* 2001;11:R159–R161. [PubMed: 11267881]
36. Megraw TL, Li K, Kao LR, Kaufman TC. The centrosomin protein is required for centrosome assembly and function during cleavage in *Drosophila*. *Development* 1999;126:2829–2839. [PubMed: 10357928]
37. Vaizel-Ohayon D, Schejter ED. Mutations in centrosomin reveal requirements for centrosomal function during early *Drosophila* embryogenesis. *Curr. Biol* 1999;9:889–898. [PubMed: 10469591]
38. Wei Q, Marchler G, Edington K, Karsch-Mizrachi I, Paterson BM. RNA interference demonstrates a role for nautilus in the myogenic conversion of Schneider cells by daughterless. *Dev. Biol* 2000;228:239–255. [PubMed: 11112327]

39. Rozen, S.; Skaletsky, HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S.; Misener, S., editors. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press; Totowa, NJ: 2002. p. 365-386.
40. Ausubel, FM. *Current Protocols in Molecular Biology*. Greene Publishing; Brooklyn, NY: 1987.
41. Roux KH. Optimization and troubleshooting in PCR. *PCR Methods Appl* 1995;4:S185–S194. [PubMed: 7580907]
42. Cherbas, C.; Cherbas, P. Cell Culture, in *Drosophila: A Practical Approach*. 2nd ed.. Roberts, DB., editor. IRL/Oxford University Press; Oxford: 1998. p. 319-338.
43. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 2001;294:1337–1340. [PubMed: 11598268]

Fig. 1.

Polymerase chain reaction (PCR) template DNA and dsRNA. **(A)** Diagram illustrating the scheme for producing T7 promoter-flanked templates by PCR. In *panel 1*, primers, designed with T7 promoter sequences at their 5' ends, are used to amplify a DNA fragment from genomic or cDNA sources. *Panel 2* shows the PCR product, with the T7 promoter flanks, to be used for transcription of dsRNA by T7 RNA polymerase. *Panel 3* illustrates the dsRNA product that is produced from the transcription of both strands of the template drawn in *panel 2*. **(B)** PCR amplification of a 938-bp segment of *cnn* cDNA with T7 flanking sequences (*lane 1*). Transcription from the PCR fragment with T7 polymerase yields a double-stranded RNA product that migrates slower on a gel than the double-stranded DNA template (*lane 2*). The sample in *lane 2* was treated with DNase I prior to loading. dsRNA samples typically produce a smear on an agarose gel like that shown in *lane 2*. DNA size markers (1-kb ladder) are shown in *lane 3*.

Fig. 2.

Time-course of Cnn protein levels following RNAi treatment. Protein samples were collected from control RNAi (**A**) and Cnn RNAi (**B**) S2 cells at 24-h time-points: d 0, *lanes 1 and 8*; d 1, *lanes 2 and 9*; d 2, *lanes 3 and 10*; d 3, *lanes 4 and 11*; d 4, *lanes 5 and 12*; d 5, *lanes 6 and 13*; d 7, *lanes 7 and 14*. No sample was collected on d 6. Prestained protein size markers (Bio-Rad Kaleidoscope) were loaded in the lane labeled M and are (from the top) 200 kDa, 120 kDa, 85 kDa, and 45 kDa. Lysate from approx 2.5×10^5 cells were loaded into each lane. Samples were electrophoresed on a single 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) minigel using the Bio-Rad Protean II system and a 15-well comb. The blot was probed with anti-Cnn and anti- α -tubulin antibodies (as a loading control). Cnn levels dropped perceptibly in the first 24 h and continued to drop until d 5, when the levels appear to rise again. By d 7, Cnn levels still had not returned to normal.

Fig. 3. Spindle assembly in cells depleted of Cnn by RNAi. S2 cells treated with control dsRNA (**A** and **C**) and *cnn* dsRNA (**B** and **D**) were fixed and stained for microtubules (anti- α -tubulin) (**A** and **B**) and Cnn (**C** and **D**). The cells were also stained for DNA, and the merged three-color images are shown in Fig 4. Note that in the cell depleted of Cnn by RNAi, there is no signal for Cnn detected at the spindle poles, which are consequently deficient in astral microtubules. Cells at different stages of the cell cycle are deficient in astral microtubules in Cnn RNAi cells (not shown). In these cells, the mitotic spindle is assembled via an alternate pathway that does not utilize centrosomes (34). The images shown were captured on a Leica TCS SP confocal microscope equipped with argon, krypton and He-Ne Red lasers. The images were collected as a Z-series about 6 μ m thick, and the maximum projection through the stack is shown.

Fig. 4.

Three-color merged image of cells depleted of Cnn by RNAi. Shown are S2 cells treated with control dsRNA (**A**) and *cnn* dsRNA (**B**) that were fixed and stained for microtubules (anti- α -tubulin) (green), Cnn (red), and DNA (TOTO-3, blue). For more details, *see* legend to Fig. 3. (*See* color plate 7 in the insert following p. 242.)