Analysis of nuclear RNA interference in human cells by subcellular fractionation and Argonaute loading

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RNAi is well known for its ability to regulate gene expression in the cytoplasm of mammalian cells. In mammalian cell nuclei, however, the impact of RNAi has remained more controversial. A key technical hurdle has been a lack of optimized protocols for the isolation and analysis of cell nuclei. Here we describe a simplified protocol for nuclei isolation from cultured cells that incorporates a method for obtaining nucleoplasmic and chromatin fractions and removing cytoplasmic contamination. Cell fractions can then be used to detect the presence and activity of RNAi factors in the nucleus. We include a method for investigating an early step in RNAi, Argonaute protein loading with small RNAs, which is enabled by our improved extract preparations. This protocol facilitates the characterization of nuclear RNAi, and it can be applied to the analysis of other nuclear proteins and pathways. From cellular fractionation to analysis of Argonaute loading results, this protocol takes 4–6 d to complete.

INTRODUCTION

RNAi is a regulatory mechanism in which cellular proteins associate with small RNAs. The resultant ribonucleoprotein complexes recognize complementary sequences within cellular RNA targets to control gene expression[1]. Although it was first recognized in *Caenorhabditis elegans*[2], Elbashir *et al.*[3] demonstrated that RNAi could also be programmed with small RNAs to function in mammalian cells. Since that discovery, RNAi in the mammalian cytoplasm has become a ubiquitous research tool and has been widely recognized as an important mechanism for controlling the expression of endogenous proteins.

Transcription and splicing are crucial processes that involve RNA and occur in cell nuclei. If the cellular RNAi machinery that drives recognition in the cytoplasm also operates in the nucleus, transcription or splicing might also be affected by RNAi. For example, recognition of a sequence near a splice site might alter splicing. Recognition of a nascent transcript near a gene promoter or enhancer region might affect transcription. Such events would represent a previously unappreciated layer of biological control and imply that microRNAs (miRNAs) have a much wider range of cellular roles.

Several groups have made experimental observations suggesting that RNAi factors might be involved in regulating gene expression in the nucleus of mammalian cells. These include observations of RNA-mediated inhibition of transcription[4–9], activation of transcription[10–13] and control of alternative splicing[14,15]. Transcriptional activation and silencing has been reported to be dependent on RNAi factors such as Argonaute[9,12,16,17] and GW182 (ref. 12).

Surprisingly, given the broad potential of nuclear RNAi to affect gene expression in mammalian cells, relatively little research on the topic has been published, and few researchers have entered the field. Our impression from informal discussions at meetings and from anonymous peer review led us to conclude that a significant number of RNA biologists believed that RNAi factors are either not present or not active in mammalian cell nuclei. This belief has helped discourage research on nuclear RNAi in mammalian cells and dampened the chances for significant progress.

Motivated by this unofficial consensus among many RNA biologists that nuclear RNAi was, at best, an uncertain phenomenon, we re-examined published evidence. Some studies had concluded that RNAi factors are inactive in mammalian cell nuclei[18,19]. Localization studies had shown the presence of RNAi factors in cytoplasmic p-bodies or associated with the endoplasmic reticulum (ER)[20,21]. In contrast, other data suggested the presence or activity of RNAi factors in cell nuclei[17,22–27]. As noted above, RNAi factors appeared to be involved in RNA-mediated control of transcription and splicing. These conflicting data were insufficient to conclusively determine whether the necessary RNAi factors were also nuclear and, if so, whether they were active.

Here we describe an improved protocol for obtaining nuclear fractions from cultured human cells and an *in vitro* assay for investigating Argonaute loading activity using these nuclear fractions. By combining these methods with complementary approaches, we have shown that RNAi factors are present and active in the human cell nucleus, but that loading of Argonaute-2 (Ago2) occurs in the cytoplasm[28]. This protocol will be useful for investigating RNAi activity in human cell nuclei. Our approach to obtaining cleaner nuclei and subnuclear fractions will also facilitate biochemical investigation of other nuclear processes where rigorous exclusion of organelle contamination, such as the ER, is necessary.

Protocol development

We concluded that it was necessary to re-examine the techniques used to evaluate nuclear localization and activity of RNAi factors. We immediately identified a key technical challenge. Any experiment designed to clarify whether or not a cellular activity exists in cell nuclei must build a strong case that nuclear extracts are free of cytosolic or cytoplasmic organelle contamination[29]. This is especially true for RNAi studies, as Ago proteins are known to be associated with the ER[21]. The ER is attached to the outer nuclear membrane and it can be difficult to dissociate[30]. The implications of ER contamination for interpreting Ago localization have been previously noted[29].

We found that standard methods often do not adequately or reliably remove ER proteins from purified nuclei[28]. These include protocols where complicated sucrose cushions are used to separate nuclei from other cellular organelles or large cell debris[31,32]. To optimize nuclei isolation, we took advantage of the ability of nonionic detergents to strip membrane proteins from the ER while keeping the nuclear membrane intact[28,31]. Differential centrifugation speeds were also explored for the separation of nuclei from contaminating organelles and cell debris without resorting to sucrose cushions. We developed the protocol by systematically varying the identity and concentration of the detergent, nuclei washing conditions and centrifugation speeds used to separate and wash nuclei. To evaluate purifications, we examined nuclei purity using fluorescence microscopy to detect ER integral membrane protein and western blot analysis to detect ER components and other cytoplasmic contaminants such as mitochondria. The resulting protocol removes ER proteins and other cytoplasmic contaminants while keeping nuclei intact.

The presence of RNAi factors in cell nuclei does not address whether they will be active. To answer this question, we used our nuclei purification protocol to obtain extracts suitable for biochemical studies. These studies include sequencing of small RNAs bound to Ago2 and assays to monitor Ago2-mediated cleavage, Dicer cleavage and small RNA loading of Ago2 (ref. 28). As no published protocols were found for directly evaluating *in vitro* loading of Ago proteins in cell extracts, a key early step in RNAi, we developed our own. It is important to directly assess Argonaute loading of small RNAs, as this step in RNAi is distinct from target RNA engagement and cleavage, and the activities of these steps may not directly correlate. By using our loading assay, we demonstrated that RNAi programming via Ago2 loading was deficient in nuclear extracts owing to the absence of the known loading factors C3PO and Hsp90 and its co-chaperones. Ago2 loading only occurred in the cytoplasm, suggesting a novel layer of RNAi regulation in the nucleus[28].

Applications of the method

The need for nuclear preparations free of ER protein contamination and other cytoplasmic contaminants is shared by many experimental approaches. In addition, there is often a need to simultaneously assay RNA, protein and enzyme activities from these fractions and to physically separate soluble nuclear and insoluble chromatin-associated nuclear fractions. Our subcellular fractionation approach is flexible and we have demonstrated its

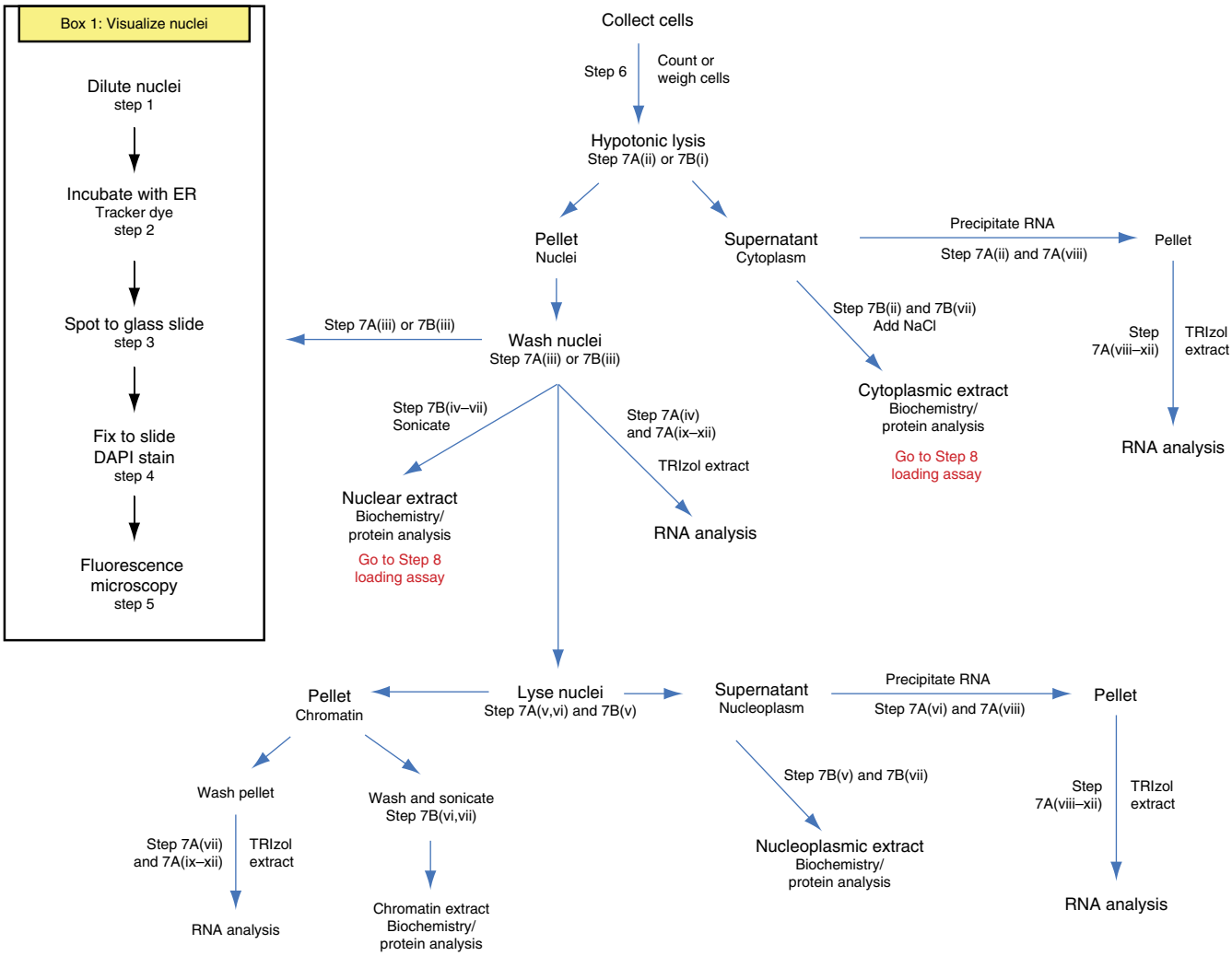

Figure 1 | Schematic of cell fractionation and extract preparation steps.

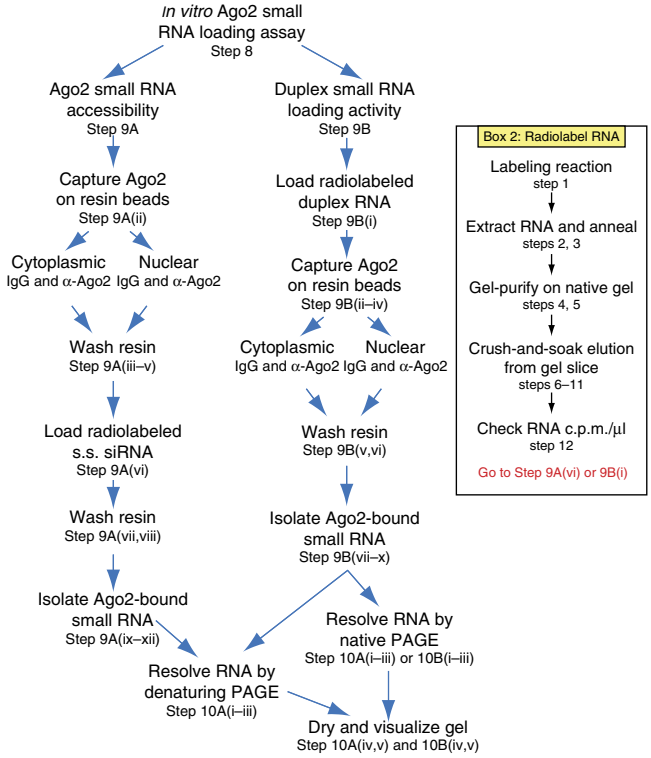

Figure 2 | Schematic of the *in vitro* Ago2 loading assay. s.s. siRNA, single-stranded siRNA.

application for checking RNA levels by quantitative PCR (qPCR), RNA cleavage products by 5′-rapid amplification of cloned ends (RACE), RNA-protein interactions by sequencing, protein levels by western blotting and enzyme activities by *in vitro* biochemical assays[28].

In this protocol, we present fractionation techniques for multiple end-point assays and demonstrate their use in a direct biochemical assay, Ago2 small RNA loading. Although we focus on RNAi factors, this approach is readily adapted for RNA and general protein isolation, and we describe protocols to obtain fractions for these purposes. These protocols should find wide application for a variety of experimental investigations of function within the nuclear compartment of mammalian cells.

Overview of the procedure

A schematic of the major end points in the cell fractionation and extract preparation is provided in **Figure 1**. Briefly, cells are collected and weighed or counted, and they are then lysed to generate cytoplasmic extract and nuclei. Nuclei are washed and directly lysed by sonication or incubation with TRIzol reagent, or are further fractionated to collect soluble nuclear (nucleoplasmic) or insoluble nuclear (chromatin-associated) fractions. From each fraction, protein or RNA can be isolated, or fractions can be used for biochemical assays. A schematic of the Ago2 loading assay is shown in **Figure 2**. The accessibility of Ago2 for binding small RNA is evaluated by incubation of radiolabeled siRNA with isolated Ago2. Ago2 duplex RNA loading activity is assessed by incubation of duplex small RNA with extract, followed by immunoprecipitation of Ago2. Radiolabeled RNA that co-purifies with Ago2 represents Ago2-bound and Ago2-loaded siRNA, which is then extracted and resolved by gel electrophoresis for visualization.

Collection of tissue culture cells (Steps 1–6). We have successfully used adherent HeLa, T47D, A549 and untransformed fibroblast cells for the protocols described here[28]. On the basis of our experience, we expect that these protocols will be applicable to a wide variety of mammalian tissue culture cells. It is expected that the user will have some experience with tissue culture and existing protocols for how to grow and maintain healthy cell cultures. For the protocol described here, we recommend HeLa cells as they are straightforward to culture. HeLa cells are cultured in DMEM supplemented with 5% (vol/vol) FBS and 0.5% (vol/vol) nonessential amino acids (NEAA). Cells are grown in sterile incubators at 37 °C in 5% CO_2.

The time required to grow a sufficient number of cells varies from 3 to 7 d, depending on the number of cells initially seeded. For the production of sufficient quantities of cell extract, seeding and growth of cells in multiple large flasks, such as ten or more 15-cm dishes, is recommended. To maximize cell numbers, cells are collected when they are close to 90% confluency. However, some tissue culture cell types are more sensitive to crowding and may need to be collected sooner, depending on future applications of the extract. For experiments such as *in vitro* Ago2 loading and RNAi factor localization, cell confluency did not appear to markedly affect our results.

Subcellular fractionation (Step 7 and Box 1). Gentle pipetting is key to successful fractionation, so as not to lyse nuclei during the initial cell lysis; this should be followed by low-speed centrifugation during wash steps to separate cytoplasmic debris and organelles from the isolated nuclei. The varying properties of different cell types mean that some protocol optimization may be required. For example, fibroblasts are larger cells, and therefore they have larger nuclei and a stronger cell membrane compared with HeLa cells. Accordingly, we have found that efficient fibroblast lysis required more pipetting and an additional 5 min of incubation on ice. However, during nuclei washing, lower speeds of 100*g* were sufficient to pellet and wash nuclei. We also observed that T47D cell nuclei usually need to be spun at higher speeds, up to 300*g*, for efficient washing.

Nuclei purity can be directly visualized by fluorescence microscopy using a DAPI stain (nuclear chromatin) and ER Tracker dye (ER integral membrane protein)[28]. Visualization of nuclei integrity and efficient ER protein removal is an important quality control checkpoint when beginning these experiments for the first time (**Box 1**). After washing, nuclei are incubated with ER Tracker dye, and then they are diluted and spotted to glass coverslips and set with mounting medium containing DAPI. For imaging, a confocal or wide-field microscope with blue and red fluorescence capability is sufficient. Once nuclei have been thoroughly washed, they are disrupted to make nuclear extract. Extracts should be kept on ice and flash-frozen in liquid nitrogen for storage at −80 °C. Extract quality is typically assessed by western blot analysis of subcellular marker proteins.

The subcellular fractionation protocol described here is quite flexible and can be adapted for various downstream assays. Here we detail how to obtain cytoplasmic, nuclear, nucleoplasmic and chromatin fractions and to either isolate RNA or protein

for analysis, or to make extracts suitable for biochemical study. Analysis of RNA levels or identity often needs to be performed in replicate. Therefore, it is important to count cells and to make aliquots with equivalent numbers for each sample or treatment.

During the isolation of RNA, both nuclei and chromatin can be directly dissolved in TRIzol reagent. However, cytoplasmic and nucleoplasmic fractions are in liquid phase. To efficiently extract RNA, both RNA and protein are first precipitated, and then the pellet is dissolved in TRIzol and heated. As an alternative to precipitation, TRIzol designed for liquid samples or phenol may be used to directly extract RNA. However, the efficiency of these alternatives has not been tested. A heating step is necessary, especially for the chromatin sample, to completely dissolve the precipitate or chromatin. Base-pairing of genomic DNA is not effectively denatured in TRIzol, so heating releases bound histones, denatures the hybridized strands and efficiently releases associated RNA. Sonication of the chromatin pellet is not recommended, as it will substantially increase levels of DNA contamination in downstream assays. Subsequent analysis of RNA must include a DNA removal step, such as DNase treatment, to ensure analysis of RNA only.

Preparation of subnuclear extracts for biochemical assays may require further processing for subsequent use. Nucleoplasmic fractions contain 1 M urea. Many enzyme assays will be sensitive to chaotropic agents such as urea, and the extract may need to be dialyzed or diluted beforehand. Chromatin fractions will contain fragmented genomic DNA from sonication, which may inhibit some activity assays. This DNA can be removed by treatment with DNase. If chromatin fractions are being prepared for western blot analysis, they can be directly boiled in SDS loading buffer rather than sonicated. After cooling, genomic DNA will reanneal but extracted protein can be recovered, largely free of any genomic DNA, by filtering through spin-filtration columns, as the genomic DNA will stick to the spin filter and will not flow through. If DNA is not removed, bands on the gel will tend to smear. The filtering step helps remove DNA and results in sharper gel images.

Radiolabeling RNA (Box 2). Although 5′-end radiolabeling of RNA is a standard procedure, we described our method here because it requires special considerations, including the differential labeling of the siRNA guide strand, annealing to the passenger strand and purification on a native gel, high specific activity labeling, and precipitation with an uncommon reagent. Differential labeling of the guide strand is achieved by labeling it before mixing it with the passenger strand. The success of the subsequent Ago2 loading assay in cell extracts requires purified siRNA of very high specific radioactivity, on the order of $0.5–2 \times 10^6$ c.p.m. per loading reaction. Key aspects of this method are the use of [γ]-^{32}P ATP with high specific activity, gel purification on a native gel to preserve native duplex siRNA structure, and a highly efficient precipitation method after gel purification using lithium perchlorate ($LiClO_4$) in acetone. Gel purification of radiolabeled RNA of low quantity requires visualization and orientation of the RNA bands, as they cannot be stained. To do this, we describe the simple use of radioactive dye spotted asymmetrically at the corners of a gel, followed by exposure to a film, which can then be slid underneath the gel to identify bands and extract them.

The siRNA used in this protocol targets a sequence of the luciferase mRNA[3,28]. There are no special considerations when choosing an siRNA or duplex RNA for this method. However, standard siRNA designs should be used initially or as controls, such as a duplex RNA of 21 nt that contains 3′ dTdT overhangs[3]. This method can serve as a tool to evaluate the structural and sequence requirements of various RNAs for loading into Argonaute proteins.

Ago2 loading assay (Steps 8–10). For *in vitro* Ago2 loading assays, loading needs to be performed in the extract before Ago2 is subsequently captured with immunoaffinity resin. If Ago2 is bound to the resin first, duplex siRNA loading is inefficient. Thus, resin and antibody should not be mixed with the siRNA and extract together, but should only be added after an initial time of Ago2 loading in solution. It is important to centrifuge samples before the addition of resin to remove any precipitation that has occurred during incubation. Otherwise, the precipitated material will co-pellet with the resin in later steps and increase contaminating background levels during analysis.

During the wash steps, it can be difficult to prevent the loss of resin. Loss of resin will skew results, making comparison across samples less reliable. To avoid this potential problem, users may want to perform washes in small spin columns with a MWCO below that of the resin, such as 10 μm. If you are using this approach, do not spin over 2,000*g* for more than 30 s, and be sure to immediately add fresh buffer after centrifugation to prevent the resin from drying.

MATERIALS

REAGENTS

- Cells. We have successfully used adherent HeLa, T47D, A549 and untransformed fibroblast cells[28]. The PROCEDURE describes the use of HeLa cells
- Dulbecco's PBS (Sigma-Aldrich, cat. no. D8537)
- Trypsin-EDTA solution (0.1% (wt/vol); Sigma-Aldrich, cat. no. T4174)
- Tissue culture medium: DMEM (Sigma-Aldrich, cat. no. D5796)
- Non-essential amino acid solution (NEAA; 100×; Sigma-Aldrich, cat. no. M7145)
- FBS (Sigma-Aldrich, cat. no. F6178)
- Tris-HCl, pH 7.5 (Invitrogen, cat. no. 15567-027)
- Sodium chloride, 5 M (NaCl; Ambion, cat. no. AM9759)
- Magnesium chloride, 1 M ($MgCl_2$; Ambion, cat. no. AM9530G)
- Potassium chloride, 2 M (KCl; Ambion, cat. no. AM9640G)
- Sodium acetate, 3 M, pH 5.5 (Ambion, cat. no. AM9740)
- Glycerol (molecular biology grade; Fisher Scientific, cat. no. AC15892-2500)
- NP-40 or Igepal CA-630 nonionic detergent (Sigma-Aldrich, cat. no. 18896) ▲ **CRITICAL** This cannot be substituted with other detergents.
- Nuclease-free water (Sigma-Aldrich, cat. no. W4502)
- Protease inhibitor (PI), cocktail set I (Calbiochem, cat. no. 539131)
- Sodium fluoride (Sigma-Aldrich, cat. no. S-6776)
- Sodium orthovanadate (New England BioLabs, cat. no. P0758S)
- Paraformaldehyde (PFA) solution, 4% (wt/vol) in PBS (Santa Cruz Biotechnology, cat. no. sc-281692) ▲ **CRITICAL** Order or freshly prepare it and use it within 6 months.
- ER Tracker Red dye (Life Technologies, cat. no. E34250)

- Vectashield HardSet mounting medium with DAPI (Vector Laboratories, cat. no. H-1500)
- Liquid nitrogen (Airgas, NI NF240LT22)
- Synthetic siLuc siRNA guide strand (siLuc_as; IDT): 5′-rUrGrUrUrCrAr CrCrUrCrGrArUrArUrGrUrGrCTT-3′
- Synthetic siLuc siRNA passenger strand (siLuc_ss; IDT): 5′-rGrCrArCrAr UrArUrCrGrArGrGrUrGrArArCrATT-5′
- [γ]-^{32}P-ATP (7,000 Ci/mmol; MP Biomedicals, cat. no. 13502002)
- SUPERase-In RNase inhibitor (Ambion, cat. no. AM2696)
- T4 polynucleotide kinase (PNK; New England BioLabs, cat. no. M0201S)
- Redistilled phenol, pH 4.3 (Sigma-Aldrich, cat. no. P4682)
- TRIzol reagent (Life Technologies, cat. no. 15596-026)
- Chloroform:isoamyl alcohol (24:1; Fluka Analytical, cat. no. 25666)
- Acetone (Fisher, cat. no. A949-1)
- Ethanol (Pharmco-Aaper, cat. no. 111000200)
- Isopropanol (Sigma-Aldrich, cat. no. I9516)
- Acrylamide:bis-acrylamide solution (19:1; Bio-Rad, cat. no. 161-0154)
- 10× TBE (Ambion, cat. no. AM9863)
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- Xylene cyanol (Sigma-Aldrich, cat. no. X4126)
- Ammonium persulfate (APS) (Sigma-Aldrich, cat. no. A4418)
- Tetramethylethylenediamine (Bio-Rad, cat. no. 161-0801)
- Black India ink (Higgins, local art supply store)
- Yeast tRNA (Roche, cat. no. 10109517001)
- Lithium perchlorate ($LiClO_4$; Acros Organics, cat. no. 194715000)
- Protein G PLUS/protein A agarose (EMD Millipore, cat. no. IP05-1.5ML)
- α-Ago2 antibody (Abcam, cat. no. ab57113) ▲ **CRITICAL** Substituting with a different Ago2 antibody may alter the quality of the results.
- Mouse IgG antibody (Millipore, cat. no. 12-371)
- ATP (100 mM; Sigma-Aldrich, cat. no. A6559-25UMO)
- DTT (1 M; Life Technologies, cat. no. P2325) ▲ **CRITICAL** Freshly prepare DTT.
- EDTA, 0.5 M, pH 8.0 (Ambion, cat. no. AM9260)
- Urea (Sigma-Aldrich, cat. no. U5378)
- Phosphocreatine (1 M; Sigma-Aldrich, cat. no. P7936)
- Creatine kinase (4 U/μl; Sigma-Aldrich, cat. no. C3755)
- Deionized formamide (Sigma-Aldrich, cat. no. F9037)
- Anti-tubulin antibody (Sigma-Aldrich, cat. no. T5201)
- Anti-OxPhos antibody (Invitrogen, cat. no. A21351)
- Anti-calreticulin antibody (Cell Signaling, cat. no. 2891S)
- Anti–lamin A/C antibody (Abcam, cat. no. ab8984)
- Polyvinylpyrrolidone (PVP; Sigma-Aldrich, cat. no. PVP40)

EQUIPMENT

- Micro-pipettors (Gilson, Pipetman)
- Automatic pipettor (Eppendorf, Easypet)
- Disposable RNase-free plastic pipette tips
- Conical screw-top tubes (15 and 50 ml)
- Microcentrifuge tubes, 1.5 ml
- Refrigerated tabletop centrifuge (15 or 50 ml conical tube adapters)
- Room temperature benchtop centrifuge (1.5 ml tube rotor)
- Refrigerated benchtop centrifuge (1.5 ml tube rotor)
- Cell counter (Beckman-Coulter, Z1 Coulter particle counter)
- Digital scale
- Wide-field fluorescent deconvolution microscope (i.e., Deltavision) with blue and red filters
- Sonicator (Biologics, Model 150 V/T ultrasonic homogenizer)
- Microscope slides (Globe Scientific, cat. no. 1324)
- Premium cover glass (Fisher Scientific, cat. no. 12-548-A)
- Light microscope
- Variable temperature heating block
- Plexiglas shields
- Liquid and solid ^{32}P radioactive waste containers
- Geiger counter
- Scintillation counter
- Water-cooled PAGE apparatus (Owl Separation Systems, model P10DS)
- Denaturing polyacrylamide electrophoresis apparatus
- Electrophoresis power supply (Thermo Scientific, cat. no. EC 1000-90)
- Glass plates (19 × 20 × 0.3 cm and 16 × 20 × 0.3 cm) and gel spacers and combs (0.75 cm)
- Large binder clips (2 in wide; local business supply store)
- Plastic wrap (e.g., Saran Wrap)
- Clear tape (e.g., Scotch brand)
- Dark room
- Autoradiography film (Phenix Research Products, cat. no. F-BX810)
- Razor blades
- Bunsen burner
- Microcentrifuge tube rotator (room temperature (23 °C) and 4 °C; Thermo Scientific, Labquake)
- RNase-free mini-spin filtration columns (<10 μm; Pierce Spin Cups paper filter, Thermo Scientific, product no. 69700)
- Vortexer
- Microcentrifuge tube cap locks, 1.5 ml (Starlab, cat. no. I1415-1508)
- Whatman 3M paper
- Vacuum gel dryer (Bio-Rad, model 583)
- Phosphorimager screen and cassette
- Phosphorimager (Fujifilm, cat. no. FLA-5000)

REAGENT SETUP

Tissue culture medium for HeLa cells Mix 10 ml of 100× NEAA and 100 ml of FBS with 1 liter of DMEM medium in a sterile hood. Store the medium at 4 °C in the dark for up to 3 months.

Hypotonic lysis buffer (HLB) Final component concentrations of HLB are 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.3% (vol/vol) NP-40 and 10% (vol/vol) glycerol. Prepare the buffer using nuclease-free water. Store the buffer solution at 4 °C for up to 1 year.

Nuclear lysis buffer (NLB) Final component concentrations of NLB are 20 mM Tris (pH 7.5), 150 mM KCl, 3 mM $MgCl_2$, 0.3% (vol/vol) NP-40 and 10% (vol/vol) glycerol. Prepare the buffer using nuclease-free water. Store the buffer solution at 4 °C for up to 1 year.

Modified Wuarin-Schibler buffer (MWS) Final component concentrations of modified Wuarin-Schibler buffer (MWS) are 10 mM Tris-HCl (pH 7.0), 4 mM EDTA, 0.3 M NaCl, 1 M urea, and 1% (vol/vol) NP-40. Prepare the buffer using nuclease-free water. Store the buffer solution at 4 °C for up to 6 months.

PI cocktail Prepare a 100× solution by dissolving lyophilized powder with 1 ml of nuclease-free water. Keep the solution on ice and store it at −20 °C for up to 6 months.

Phosphatase inhibitor solution, (PhI) (100×) PhI at 100× concentration contains 0.1 M sodium orthovanadate and 0.1 M sodium fluoride in nuclease-free water. Keep the solution on ice and store it at −20 °C for up to 1 year.

Denaturing polyacrylamide gel solution, 500 ml, 15% (wt/vol) Prepare stock solution by dissolving 210 g of urea in 50 ml of 10× TBE and 187.5 ml of 40% (19:1) acrylamide. Add 10 ml of glycerol and bring it up to 500 ml with nuclease-free water. Filter it through a 0.22-μm bottle filter. Store the solution at 4 °C in the dark for up to 6 months.

Native polyacrylamide gel solution, 500 ml, 15% (wt/vol) Prepare stock solution by mixing 187.5 ml of 40% (19:1) acrylamide, 50 ml of 10× TBE and 10 ml of glycerol. Bring the volume to 500 ml with nuclease-free water. Filter the solution through a 0.22-μm bottle filter. Store it at 4 °C in the dark for up to 6 months.

APS, 10% (wt/vol) Dissolve 1 g of APS in a final volume of 10 ml of nuclease-free water. Divide the solution into 0.5-ml aliquots in 1.5-ml microcentrifuge tubes and store them at −20 °C for up to 6 months.

Radioactive black India ink Mix 1 μl of fresh [γ]-^{32}P-ATP into 100 μl of black India ink. Vortex to mix, spin down the solution and store it behind a Plexiglas shield at room temperature. It can be used until radioactivity decays below useful signal (~1–3 months). It can be regenerated by the addition of fresh [γ]-^{32}P-ATP.

tRNA solution, 10 mg/ml Dissolve 5 mg of yeast tRNA in 0.5 ml of nuclease-free water. Phenol-chloroform extract and ethanol precipitate the RNA. Wash the RNA pellet with 70% (vol/vol) ethanol, air-dry the RNA pellet and dissolve it again in 0.5 ml of nuclease-free water. Store the solution at −20 °C for up to 2 years.

$LiClO_4$ in acetone, 2% (wt/vol) Dissolve 1 g of $LiClO_4$ in 50 ml of acetone. Store it at room temperature indefinitely.

RNA precipitation solution Mix 0.5 ml of 3 M sodium acetate (pH 5.5) with 9.5 ml of ethanol. Store the solution at −20 °C indefinitely.

IP_{EQ} buffer Final component concentrations of IP_{EQ} buffer are 20 mM Tris (pH 7.5), 0.15 M NaCl, 2 mM $MgCl_2$, 0.05% (vol/vol) NP-40 and 0.1% (wt/vol) PVP. Prepare the buffer using nuclease-free water. Store the buffer solution at room temperature for up to 1 year.

IP_{150} buffer Final component concentrations of IP_{150} buffer are 50 mM Tris (pH 7.5), 0.15 M NaCl, 4 mM $MgCl_2$ and 0.05% (vol/vol) NP-40.

Prepare the buffer using nuclease-free water. Store the buffer solution at room temperature for up to 1 year.

IP_{500} buffer Final component concentrations of IP_{500} buffer are 50 mM Tris (pH 7.5), 0.5 M NaCl, 4 mM $MgCl_2$ and 0.05% (vol/vol) NP-40. Prepare the buffer using nuclease-free water. Store the buffer solution at room temperature for up to 1 year.

RNAi buffer, 10× Final component concentrations of 10× RNAi buffer are 0.2 M Tris (pH 7.5), 0.8 M NaCl, 40 mM $MgCl_2$, 5 mM DTT, 5 mM EDTA and 0.2 M KCl. Prepare the buffer using nuclease-free water. Store the buffer solution at −20 °C for up to 1 year.

Native loading buffer, 5× Mix 1 ml of 10× TBE, 5 ml of glycerol and 4 ml of nuclease-free water. Add 2 mg of xylene cyanol and 2 mg of bromophenol blue. Mix well to dissolve the dyes. Store the buffer at room temperature indefinitely.

Formamide loading buffer Mix 1 ml of 10× TBE with 9 ml of formamide. Store the buffer at room temperature indefinitely.

PROCEDURE

Collection of tissue culture cells ● TIMING 1–2 h

! CAUTION All experiments should be performed in accordance with relevant guidelines and regulations.

1| Grow cells to <90% confluency. Wash the cells with 1× PBS at room temperature.

2| For nonadherent cells, proceed directly to Step 5. To detach adherent cells, add just enough trypsin-EDTA solution to lightly bathe the cells, and incubate them at 37 °C for 5 min.

3| Add several milliliters of full medium (containing 10% (vol/vol) FBS) and pipette the cells to stop trypsin proteolysis and to detach the cells and break up cell clumps.

4| Move the cell suspension to conical tubes on ice. Pool the dishes of cells as needed. Typical extract preparations start with a few to several 15-cm dishes, amounting to tens to hundreds of millions of cells. We recommend starting with ten dishes.

5| Centrifuge the cells at 500*g* at 4 °C for 5 min. Decant the supernatant and gently suspend the cells in ice-cold 1× PBS to wash them.

6| Count the cells (or weigh the cell pellet). If cells are counted, divide them into separate tubes at the desired number of cells and centrifuge at 500*g* at 4 °C for 5 min.

▲ CRITICAL STEP At this point, it is important to either count cells or weigh the cell pellet for accurate comparison of results across separate experiments.

▲ CRITICAL STEP Keep the cell pellets on ice to slow metabolic processes and to inhibit RNA or protein degradation.

Subcellular fractionation ● TIMING 2–4 h

7| Cells can be fractionated for at least three different purpose: RNA isolation, biochemical assays or protein analysis. This protocol describes how to prepare cytoplasmic, nucleoplasmic (soluble nuclear) and chromatin (insoluble nuclear) fractions for each of these particular purposes. Follow option A for the preparation of RNA from cellular fractions. Follow option B for the preparation of extracts for biochemical assays and protein analysis. This protocol does not provide downstream protocols for analysis of RNA or protein, but it does provide a biochemical assay to evaluate extracts prepared in option B.

(A) Isolation of RNA from cytoplasmic, total nuclear, nucleoplasmic and chromatin fractions

(i) For each sample, divide 10 million cells into aliquots in 1.5-ml microcentrifuge tubes. Centrifuge the cells at 500*g* at 4 °C for 5 min to pellet them. Decant the supernatant.

▲ CRITICAL STEP Keep the cell pellets on ice to slow metabolic processes and to inhibit RNA degradation.

(ii) Resuspend the cells by gently pipetting them up and down in 380 µl of ice-cold HLB supplemented with 100 U of SUPERase-In. Incubate the mixture on ice for 10 min. Vortex it briefly. Centrifuge the cells at 1,000*g* at 4 °C for 3 min. Carefully transfer the supernatant by pipette to a new tube and keep the pellet on ice. The supernatant is the cytoplasmic fraction. Immediately add 1 ml of RNA precipitation solution (RPS) and store it at −20 °C for at least 1 h and until Step 7A(viii).

▲ CRITICAL STEP HLB contains NP-40, a detergent that is crucial to the successful removal of ER contamination. As a negative control for ER contamination removal, consider instead lysing cells with no detergent or 0.5% (vol/vol) Tween-20, which will lyse the cell membrane but leave ER integral membrane proteins intact.

■ PAUSE POINT Fractions in RPS are stable at −20 °C overnight.

(iii) Wash the pellet from Step 7A(ii) (semipure nuclei) with 1 ml of ice-cold HLB three times by gently pipetting up and down and by centrifuging at 200*g* at 4 °C for 2 min.

▲ CRITICAL STEP Collect a sample of nuclei for analysis of nuclei purity, as described in **Box 1**. Also collect and analyze the samples for any controls set up at Step 7A(ii).

? TROUBLESHOOTING

Box 1 | Fluorescence microscopy of isolated nuclei ● TIMING 2–3 h

Visualization of isolated nuclei is a qualitative method for determining whether nuclei are intact and whether the purification procedure was sufficient to remove contaminants such as ER membrane protein from the surface of nuclei. This method complements the results of western blot analysis.

1. Dilute the nuclei aliquots taken at PROCEDURE Step 7A(iii) or Step 7B(iii) tenfold by adding 90 µl of PBS. Spin the aliquots at 500*g* for 1 min to pellet them.
2. Gently resuspend the nuclei in 10 µl of PBS containing 1 µM ER Tracker Red dye and incubate it on ice for 20 min.
3. Dilute 10-fold by adding 90 µl of 4% (wt/vol) PFA in PBS. Spot 5 µl of nuclei onto a glass slide and allow it to partially air-dry.
4. Add 1 drop of Vectashield HardSet mounting medium with DAPI and carefully place a coverslip on top. Allow the mounting medium to harden for 15 min at room temperature.
5. Visualize nuclei at 60× magnification with blue (DAPI) and red (TRITC) filters on a wide-field fluorescence microscope. Collect *z*-sections through the nuclei at 0.15-µm thickness. Deconvolute images by blind deconvolution. We use AutoQuant X3 (Media Cybernetics) for deconvolution, and we process stacked and pseudocolored images in ImageJ for visualization. Alternatively, nuclei can be visualized using a confocal microscope with appropriate DAPI and TRITC filters. *Z*-sectioning is optional for this analysis, but it enhances image clarity and assessment of nuclei purity. An example of nuclei imaged by this procedure is shown in **Figure 3b**.

? TROUBLESHOOTING

(iv) To directly isolate the total nuclear RNA from the nuclei pellet from Step 7A(iii), add 1 ml of TRIzol, vortex it briefly and store it at –20 °C until Step 7A(ix). Alternatively, to further fractionate nuclei into nucleoplasmic and chromatin-associated RNA fractions, proceed with Steps 7A(v–vii).

■ **PAUSE POINT** Fractions in TRIzol are stable at –20 °C overnight.

(v) Add 380 µl of MWS supplemented with 100 units of SUPERase-In to the pellet, vortex for 30 s and set the mixture on ice for 5 min.

▲ **CRITICAL STEP** Do not pipette the sample. It will stick to the inside of the pipette tip.

(vi) Vortex nuclei in MWS for 30 s, and then incubate them on ice for an additional 10 min. Centrifuge at 1,000*g* at 4 °C for 3 min. Carefully transfer the supernatant by pipette to a new tube and keep the pellet on ice. The supernatant is the nucleoplasmic fraction. Immediately add 1 ml of RPS and store it at –20 °C for at least 1 h and until Step 7A(viii).

■ **PAUSE POINT** Fractions in RPS are stable at –20 °C overnight.

(vii) Wash the pellet (chromatin) three times with 1 ml of ice-cold MWS by vortexing for 30 s and by centrifuging at 500*g* at 4 °C for 2 min. Add 1 ml of TRIzol to the chromatin pellet, vortex it briefly and store it at –20 °C until ready to proceed with Step 7A(ix).

■ **PAUSE POINT** Fractions in TRIzol are stable at –20 °C overnight.

(viii) For samples that have been incubated in RPS at –20 °C for >1 h (cytoplasmic and nucleoplasmic fractions from Step 7A(ii) and Step 7A(vi), respectively), vortex for 30 s and then centrifuge at 18,000*g* at 4 °C for 15 min. Discard the supernatant and wash the pellet by vortexing in ice-cold 70% (vol/vol) ethanol and centrifuging at 18,000*g* at 4 °C for 5 min. Discard the supernatant. Let the pellet partially air-dry. Add 1 ml of TRIzol to semidry pellets.

■ **PAUSE POINT** Fractions in TRIzol are stable at –80 °C for several weeks.

(ix) To all samples, which are now all in TRIzol, add 10 µl of 0.5 M EDTA and heat them to 65 °C with vortexing until the pellet is dissolved (~10 min).

! **CAUTION** Sample tube tops can pop open when TRIzol is heated. TRIzol is caustic, and proper lab safety procedures, including the wearing of eye protection, should be followed. Microcentrifuge tube cap locks should be used and samples should be watched closely.

▲ **CRITICAL STEP** Without heating in TRIzol, RNA may not be efficiently released from chromatin or insoluble RNA-protein complexes.

(x) Allow the samples to cool to room temperature, add 200 µl of chloroform:isoamyl alcohol (1:24), vortex for 30 s and then centrifuge the samples at 18,000*g* at room temperature for 10 min.

(xi) Pipette the aqueous supernatant to new tubes and add 1 volume of isopropanol to each tube. Incubate the mixture at –20 °C for >1 h, vortex it and then centrifuge at 18,000*g* at room temperature for 15 min.

(xii) Wash the RNA pellets with 70% (vol/vol) ethanol by vortexing and by centrifugation at 18,000*g* at room temperature for 5 min. Remove the supernatant and air-dry the pellet. Typical RNA yield ranges from 100 mg of cells are 50–100 µg, 25–75 µg and 15–30 µg for cytoplasmic, nucleoplasmic and chromatin-associated fractions, respectively.
▲ **CRITICAL STEP** RNA purified from nuclear and chromatin fractions will contain DNA contamination. Treatment with DNase may be required before downstream analysis. DNase treatment and downstream analysis of chromatin-associated RNA are not described here.
■ **PAUSE POINT** Purified and dry RNA is stable at –80 °C for long periods of time.

(B) Preparation of cytoplasmic, total nuclear, nucleoplasmic and chromatin fractions for biochemical assays or protein analysis

(i) Resuspend the cell pellet by gentle pipetting with 1 ml of ice-cold HLB for every 75 mg of cells or every 10 million cells. To this cell suspension, add PI cocktail and PhI solution to a final concentration of 1×. Incubate the cells on ice for 10 min. Vortex them briefly.
▲ **CRITICAL STEP** HLB contains NP-40, which is a detergent that is crucial to the success of ER contamination removal. As a negative control for ER contamination removal, consider lysing cells with no detergent or 0.5% (vol/vol) Tween-20, which will lyse the cell membrane but will leave ER integral membrane proteins intact.

(ii) Centrifuge the cell suspension at 800*g* at 4 °C for 8 min. Transfer the supernatant (cytoplasmic fraction) to a new tube on ice. Add enough 5 M NaCl to this cytoplasmic fraction to equal a concentration of 140 mM (final NaCl concentration is 150 mM), and mix well by gentle inversion. Keep the tube on ice until Step 7B(vii).
▲ **CRITICAL STEP** Keep the nuclei pellet on ice to slow the metabolic processes, to inhibit protein degradation and to maintain enzyme activity.

(iii) Wash the nuclei pellet from Step 7B(ii) four times by adding HLB, by pipetting and centrifuging at 200*g* at 4 °C for 2 min. To prepare total nuclear extract, carry out Step 7B(iv). Otherwise, proceed directly to Step 7B(v) to prepare nucleoplasmic and chromatin extracts.
▲ **CRITICAL STEP** These conditions work well for HeLa cells. Wash steps need to be optimized for different cell types. In general, low-speed spins for short periods are sufficient to wash most nuclei. The nuclei of some cell types can withstand vigorous pipetting, whereas others cannot. Centrifugation speeds can range from 100 to 300*g*.
▲ **CRITICAL STEP** Collect a sample of nuclei for analysis of nuclei purity, as described in **Box 1**.
? TROUBLESHOOTING

(iv) To prepare total nuclear extract, resuspend nuclei from Step 7B(iii) in 0.5 ml of ice-cold NLB for every 75 mg or 10 million cells of the original cell pellet. Add PI cocktail and PhI solution to the nuclei suspension to a final concentration of 1×. Divide the nuclei suspension into aliquots in 15-ml conical tubes at 2–4 ml/tube. Sonicate the nuclei three times at 20% power for 15 s in an ice bath with 2 min of cooling between each sonication. Sonication success can be checked by observing several microliters of lysed nuclei on a glass coverslip with a light microscope. Place the sonicated nuclei extract on ice until Step 7B(vii).
▲ **CRITICAL STEP** Oversonication can damage proteins and their native complexes. Sonication instruments vary in power and efficiency.
? TROUBLESHOOTING

(v) To prepare nucleoplasmic and chromatin extracts, resuspend nuclei in 0.5 ml of ice-cold MWS buffer for every 75 mg or 10 million cells of the original cell pellet. Add PI cocktail and PhI solution to the nuclei suspension to a final concentration of 1×. Vortex it gently and incubate it on ice for 15 min. Vortex the mixture again and centrifuge it at 1,000*g* at 4 °C for 5 min. Collect the supernatant as nucleoplasmic fraction and transfer it to a new tube. Place the nucleoplasmic extract on ice until Step 7B(vii).
▲ **CRITICAL STEP** Do not pipette the chromatin pellet. It will stick to the inside of the pipette tip.

(vi) Wash the chromatin pellet twice with MWS buffer by vortexing, by incubating on ice for 5 min and centrifuging at 500*g* at 4 °C for 3 min. Add 0.5 ml of ice-cold NLB to the chromatin pellet for every 75 mg or 10 million cells of the original cell pellet. Sonicate chromatin three times at 20% power for 15 s in an ice bath with 2 min of cooling between each sonication. Place the sonicated chromatin on ice until Step 7B(vii).
▲ **CRITICAL STEP** Oversonication can damage proteins and their native complexes. Sonication instruments vary in power and efficiency.
▲ **CRITICAL STEP** Chromatin extracts contain large amounts of DNA, which may need to be minimized or removed by DNase treatment for some downstream assays. DNase treatment and downstream analysis of the chromatin fraction are not described here.

(vii) Centrifuge the cytoplasmic, nuclear, nucleoplasmic and chromatin extracts in 1.5-ml microcentrifuge tubes at 18,000*g* for 15 min at 4 °C. Pool the supernatants of the respective samples in a new conical tube, invert the tube to mix, and divide the mixture into 1-ml volumes in 1.5-ml microcentrifuge tubes.

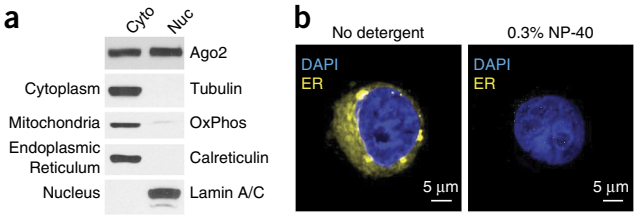

Figure 3 | Quality assessment of subcellular fractionation. (**a**) Western blot analyses of cytoplasmic (Cyto) and nuclear (Nuc) fractions. Western blot analysis is as described by Gagnon *et al.*[28]. (**b**) Fluorescence microscopy of isolated nuclei. Blue is DAPI stain, which binds chromatin DNA. Yellow is ER Tracker Red dye pseudocolored yellow, which binds sulfonylurea receptors on the ER membrane surface.

▲ **CRITICAL STEP** Typical protein concentrations can range from 3–6 mg/ml and 1–3 mg/ml for cytoplasmic and nuclear extracts, respectively.

■ **PAUSE POINT** Flash-freeze the samples in liquid nitrogen and store them at –80 °C. Extracts can be stored at –80 °C for several weeks.

(viii) Perform western blotting analysis using standard methods to evaluate the extract quality and success; use antibodies that detect subcellular markers such as tubulin (cytoplasm), OxPhos (mitochondria), calreticulin (ER) and lamin A/C (nucleus)[28]. Typical results are shown in **Figure 3**. Successful extracts can be used for biochemical analysis in Step 8.

▲ **CRITICAL STEP** Note that nuclear, nucleoplasmic and chromatin fractions are half the volume of corresponding cytoplasmic extracts. This makes application in biochemical assays more convenient, as the cytoplasm usually has 2–4× more total protein than the nucleus. For western blot analyses, be sure to load half the volume of nuclear versus cytoplasmic extracts. This will give total protein of the same cell equivalents.

In vitro Ago2 loading assay ● TIMING 2–3 d

8| If the cell extracts were frozen, thaw them on ice. Centrifuge the cell extract at 18,000*g* for 10 min at 4 °C. Transfer the supernatant to a new tube. Keep it on ice to avoid degradation or inactivation of enzymes in the extract.

9| Perform the Ago2 loading assay according to either options A or B. Option A evaluates the accessibility of Ago2 for loading. Ago2 is first immunoprecipitated and then mixed with radiolabeled single-stranded siLuc guide RNA, prepared as described in **Box 2**. Single-stranded RNA cannot be directly mixed with extract, as it is rapidly degraded, even with the addition of RNase inhibitors. However, Ago2 can bind and load single-stranded small RNAs without the need for accessory proteins. Option B (**Fig. 4a**) determines the ability of the cell extract to efficiently load a duplex siRNA (prepared as described in **Box 2**) into Ago2, which requires accessory proteins in mammalian cells.

(A) Ago2 accessibility for small RNA loading

(i) Vortex Protein G PLUS/protein A resin to resuspend it, and then divide it into 40-µl aliquots in four 1.5-ml microcentrifuge tubes. Equilibrate the resin by adding 1 ml of IP_{EQ} buffer and rotating for 5 min at room temperature. Pellet the resin by centrifugation at 2,000*g* for 30 s at room temperature. Add 1 ml of IP_{150} buffer, rotate the tube for 5 min and centrifuge it again.

(ii) Prepare the following four reactions and rotate them at room temperature for 1 h.

	Volume (µl)				
Component	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	**Final**
Cytoplasmic extract	300	300	—	—	300 µl
Nuclear extract	—	—	300	300	
Anti-Ago2 (1 µg/µl)	2	—	2	—	2 µg
IgG antibody (1 µg/µl)	—	2	—	2	
Equilibrated resin from Step 9A(i)	40	40	40	40	40 µl

(iii) Centrifuge the resin at 2,000*g* for 30 s at room temperature.

(iv) Wash the resin three times by adding 0.5 ml of IP_{500} buffer, by rotating it for 5 min at room temperature and by centrifuging at 2,000g for 30 s at room temperature.

(v) Wash it one more time with 0.5 ml of IP_{150} buffer.

Box 2 | Preparation of radiolabeled siRNA ● TIMING 2 d

1. Prepare the following 30-µl reaction and incubate it at 37 °C for 2 h.

Component	Volume (µl)	Final
siLuc siRNA guide strand (100 µM)	2	200 pmol
[γ]-^{32}P-ATP	4	0.5 mCi
SUPERase-In (40 U/µl)	1	40 U
10× T4 PNK buffer	3	1×
T4 PNK enzyme (10 U/µl)	3	30 U
Nuclease-free water	17	—

! CAUTION ^{32}P is radioactive and poses serious health risks. Follow accepted safety procedures, such as Plexiglas shielding, when handling radioactive materials.

2. Phenol-chloroform extract radiolabeled RNA using standard methods.

■ PAUSE POINT Extracted RNA can be stored at –20 °C for a few days.

3. Divide radiolabeled siLuc guide RNA equally into two tubes. Add 120 pmol of the siLuc passenger strand RNA to one tube and heat it to 90 °C for 3 min. Remove it from the heating block and allow it to cool at room temperature for 15 min. This step generates a duplex siLuc siRNA from the half of the sample that was mixed with passenger strand RNA. One sample is used for the single-stranded RNA probe for Step 9A(vi) and the other as a duplex RNA probe for use in Step 9B(i).

4. Add 5 µl of 4× native loading buffer and resolve the samples on a native 15% (wt/vol) TBE-buffered 19:1 polyacrylamide gel. As an alternative to pouring a gel, native TBE-buffered gels can often be purchased commercially.

▲ CRITICAL STEP Use water cooling and low current to maintain native conditions and to prevent denaturation of the annealed siRNA duplex. Run the gel at 35 mA (the gel should stay below 30 °C) until the lower dye (bromophenol blue) band reaches 2/3 of the way down the gel.

5. Remove the gel from the apparatus. Open the gel cassette and lay a sheet of Saran Wrap over the gel. At three corners of the gel, spot 1 µl of radioactive black India ink and allow it to air-dry (~10 min). Cover the dried ink spots with clear tape.

! CAUTION The buffer in the anode tank (the positive electrode that contacts buffer at the bottom of the gel) will be radioactive owing to unincorporated [γ]-^{32}P-ATP. Discard it carefully in a designated liquid ^{32}P waste container. Use a Geiger counter to monitor contamination and cleanup.

6. Expose the gel in the darkroom to the autoradiography film placed on top of the gel. Expose it for 15–60 s and develop it.

7. Slide the film under the glass that the gel is on. Orient and align the spots at the three corners of the gel to identify the location of the RNA bands. Gently peel back the Saran Wrap and cut out the band with a flamed razor (RNase free).

8. Crush the gel slice with a 1-ml plastic pipette tip (RNase free and flame sealed at the tip), and pipette 300 µl of nuclease-free water down the side of the tip and into a microcentrifuge tube so as to collect all the gel bits; rotate at 4 °C for 4–16 h.

9. Cut ~2 mm off the end of a 1-ml pipette tip and use it to move the gel bits and solution to an RNase-free filter spin column (~10 µm cutoff). Spin at 2,000*g* for 2 min to collect the eluted RNA in solution. Discard the gel bits.

10. Add 10 µg of tRNA and split the sample evenly into two microcentrifuge tubes to accommodate the total volume required during precipitation. Precipitate the RNA from the solution by adding 9 volumes of 2% (wt/vol) $LiClO_4$ in acetone and incubating at –20 °C for >15 min.

! CAUTION $LiClO_4$ is a dangerous oxidizer. Handle it with caution.

▲ CRITICAL STEP The radiolabeled RNA is extremely dilute and it will not precipitate well by standard ethanol precipitation.

11. Spin the mixture at 12,000*g* for 10 min, wash the pellet with ice-cold acetone and spin it again at 12,000*g* for 2 min. Allow the pellet to air-dry at room temperature.

■ PAUSE POINT Dried RNA pellets can be stored at –20 °C for a few days.

12. Resuspend the RNA pellet in 30 µl of RNase-free water. Measure the radioactivity by scintillation counting of 1 µl (do not use scintillation fluid).

? TROUBLESHOOTING

■ PAUSE POINT Label side of the tube with the date and the c.p.m./µl value and store the RNA frozen at –20 or –80 °C. RNA radioactivity should range from several hundred thousand to a few million c.p.m./µl. The half-life of ^{32}P is 14.2 d.

(vi) Prepare the following 20-µl reaction and rotate it at 4 °C for 4 h.

Component	Volume (µl)	Final
Washed resin from Step 9A(v)	—	—
tRNA (10 µg/µl)	1.5	0.75 µg/µl
SUPERase-In (40 U/µl)	1.5	3 U/µl
ATP (100 mM)	0.3	1.5 mM
siLuc guide strand RNA (1 × 10^6 c.p.m./µl)	1	1 × 10^6 c.p.m.
10× RNAi buffer	3	1×
Nuclease-free water	12.7	—

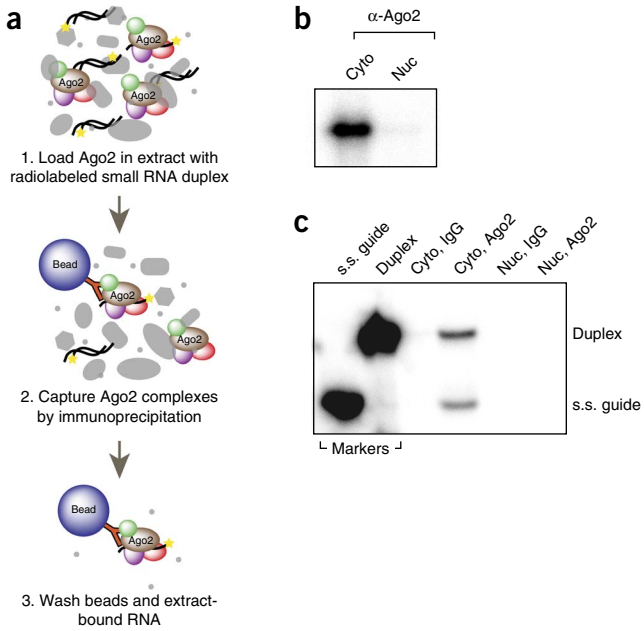

Figure 4 | *In vitro* Ago2 loading assay and typical results from Step 9B. (**a**) Illustration of *in vitro* Ago2 loading assay described in Step 9B. (**b**) Denaturing PAGE of Ago2-bound small RNAs from typical cytoplasmic (cyto) and nuclear (nuc) loading reactions. (**c**) Native polyacrylamide gel electrophoresis of small RNAs that co-purified with IgG or Ago2 antibody. Markers used are single-stranded guide siRNAs (s.s. guide) and duplex siRNAs (duplex) loaded in separate lanes to the left.

(vii) Wash the resin three times by adding 0.5 ml of IP_{500} buffer, rotating it for 5 min at room temperature and centrifuging it at 2,000*g* for 30 s at room temperature.

(viii) Wash it one more time with 0.5 ml of IP_{150} buffer.

(ix) Stop the reaction by adding the following components:

Component	Volume (μl)	Final
Nuclease-free water	40	—
EDTA (0.5 M)	0.5	1.8 mM
tRNA (10 μg/μl)	0.5	35 ng/μl
Phenol	80	—

(x) Vortex the mixture for 30 s, and then perform phenol extraction by centrifugation. Retain the top aqueous layer (transfer it to a new tube).

(xi) Precipitate the RNA by adding 9 volumes of 2% (wt/vol) $LiClO_4$ in acetone and incubating at –20 °C for >15 min.
! CAUTION $LiClO_4$ is a dangerous oxidizer. Handle it with caution.

(xii) Spin at 12,000*g* for 10 min at room temperature, wash the pellet with ice-cold acetone and spin it again at 12,000*g* for 2 min. Allow the pellet to air-dry at room temperature.
■ PAUSE POINT Dried RNA pellets can be stored at –20 °C for a few days.

(B) Ago2 duplex RNA loading activity assay

(i) Prepare the following four loading reactions and rotate them at room temperature for 1 h.

	Volume (μl)				
Component	Tube 1	Tube 2	Tube 3	Tube 4	Final
Cytoplasmic extract	300	300	—	—	300 μl
Nuclear extract	—	—	300	300	—
siLuc duplex siRNA (1×10^6 c.p.m./μl)	1	1	1	1	1×10^6 c.p.m.
ATP (100 mM)	3	3	3	3	1 mM
Phosphocreatine (1 M)	3	3	3	3	10 mM
Creatine kinase (4 U/μl)	3	3	3	3	12 U

(ii) While the loading reactions are rotating, prepare the resin for immunoprecipitation. Vortex Protein G PLUS/protein A resin to resuspend it, and then divide the resin into 40-μl aliquots in four 1.5-ml microcentrifuge tubes. Equilibrate the resin by adding 1 ml of IP_{EQ} buffer and rotating it for 5 min at room temperature. Pellet the resin by centrifugation at 2,000*g* for 30 s at room temperature. Add 1 ml of IP_{150} buffer, rotate it for 5 min and centrifuge it again.

(iii) Centrifuge the loading reactions from Step 9B(i) at 12,000*g* at room temperature for 5 min. Move the supernatant to a 1.5-ml microcentrifuge tube containing equilibrated resin (from Step 9B(ii)).

(iv) Add 2 µg of IgG antibody (control) into tubes 1 and 3, and add 2 µg of anti-Ago2 antibody into tubes 2 and 4 from Step 9B(iii). Rotate them at room temperature for 1 h.
(v) Wash the resin three times by adding 0.5 ml of IP_{500} buffer, by rotating it for 5 min at room temperature and centrifuging it at 2,000*g* for 30 s at room temperature.
(vi) Wash it one more time with 0.5 ml of IP_{150} buffer.
(vii) Stop the reaction by adding the following components:

Component	Volume (µl)	Final
Nuclease-free water	40	—
EDTA (0.5 M)	0.5	1.8 mM
tRNA (10 µg/µl)	0.5	5 µg
Phenol	80	—

(viii) Vortex for 30 s, and then perform phenol extraction by centrifuging for 5 min at 10,000*g* at room temperature. Retain the top aqueous layer (move to a new tube).
(ix) Precipitate RNA by adding 9 volumes of 2% (wt/vol) $LiClO_4$ in acetone and incubating at –20 °C for >15 min.
! CAUTION $LiClO_4$ is a dangerous oxidizer. Handle it with caution.
(x) Spin the mixture at 12,000*g* for 10 min, wash the pellet with ice-cold acetone and spin it again at 12,000*g* for 2 min. Allow the pellet to air-dry at room temperature.
■ **PAUSE POINT** Dried RNA pellet can be stored at –20 °C for a few days.

10| Resolve the samples by denaturation or by native gel electrophoresis. Denaturing gel electrophoresis (option A) will not distinguish duplex versus single-stranded guide RNA bound to Ago2, but it will reveal a single band for radioactive RNA bound to Ago2. Native electrophoresis (option B) conditions will separate duplex from single-stranded small RNAs, which may suggest formation of premature and mature RISC complexes, respectively.

(A) Assessing Ago2 loading by denaturing gel electrophoresis

(i) Cast a denaturing 15% (wt/vol) polyacrylamide gel. Mix the following components and then pour them into a gel cassette assembled from glass plates, spacers, binder clips and a comb. As an alternative to casting a gel, denaturing TBE-buffered gels can often be purchased commercially.

Component	Volume (ml)	Final (%)
15% (wt/vol) denaturing polyacrylamide gel solution	35	15
10% (wt/vol) APS	0.21	0.0006
TEMED	0.035	0.001

(ii) Prerun the gel at 40 mA with 1× TBE running buffer for 30 min without cooling. The gel should become very warm to the touch and should reach a temperature of ~45–50 °C for optimal denaturation and resolving conditions.
! CAUTION If denaturing gels become too hot, the glass plates will crack. Monitor the temperature and current to maintain a safe operating range.
(iii) Add 6–10 µl of formamide loading buffer to dried RNA from Step 9A(xii) or Step 9B(x), boil the mixture for 5 min at 90 °C, vortex it, centrifuge it at 2,000*g* 30 s and then load it onto a prerun gel. Run the gel at 40 mA until the bottom blue dye band (bromophenol blue) is 1/2–2/3 of the way down the gel. The RNA will run between the two blue dye bands on the gel.
(iv) Stop the gel, separate the glass plates and lay Saran Wrap over the top of the gel. Flip the gel over and peel the glass plate off so that the gel is now adhered to the Saran Wrap. Press a sheet of Whatman 3M paper to the gel and dry it in a vacuum gel dryer at 80 °C for 2 h.
(v) Expose the dried gel to a phosphorimager screen overnight. Develop the screen to visualize radioactive RNA that coeluted with Ago2 in the *in vitro* loading assay. Typical results for Step 9B samples resolved by native gel electrophoresis are shown in **Figure 4b**.
? TROUBLESHOOTING

(B) Assessing Ago2 loading by native gel electrophoresis

(i) Cast a native 15% (wt/vol) polyacrylamide gel. Mix the following components and then pour them into a gel cassette assembled from glass plates, spacers, binder clips and a comb. As an alternative to pouring a gel, native TBE-buffered gels can often be purchased commercially.

Component	Volume (ml)	Final (%)
15% (wt/vol) native polyacrylamide gel solution	35	15
10% (wt/vol) APS	0.21	0.0006
TEMED	0.035	0.001

(ii) Prerun the gel at 30 mA with 1× TBE running buffer for 30 min with cooling. The gel should stay cool with an ideal running temperature of <30 °C.

(iii) Add 6–10 µl of 1× native loading buffer to dried RNA from Step 9B(x), vortex it, centrifuge it at 2,000*g* for 30 s and then load it onto prerun gel. Run the gel at 35 mA until the bottom blue dye band (bromophenol blue) is 2/3–3/4 of the way down the gel. The RNA will run between the two blue dye bands on the gel.

(iv) Stop the gel, separate the glass plates and lay Saran Wrap over the top of the gel. Flip the gel over and peel the glass plate off so that the gel is now adhered to the Saran Wrap. Press a sheet of Whatman 3M paper to the gel and dry it in a vacuum gel dryer at 80 °C for 2 h.

(v) Expose the dried gel to a phosphorimager screen overnight. Develop the screen to visualize radioactive RNA that coeluted with Ago2 in the *in vitro* loading assay. Typical results for Step 9B samples resolved by native gel electrophoresis are shown in **Figure 4c**.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7A(iii), 7B(iii)	Nuclei do not pellet well	Cells are not completely lysed	Incubate the cells on ice longer and use more vigorous pipetting to resuspend cells in Step 7A(ii) or 7B(ii)
		Nuclei are smaller than typical	Centrifuge at higher speed or for longer duration
		Washes are incomplete	Wash additional times or with more vigorous pipetting during resuspension
7B(iv)	Nuclei are not completely lysed	Sonication is ineffective	Increase sonication time or sonicator power output
	Nuclear extract is foamy	Oversonication	Decrease sonication time or sonicator power output
	Nuclear extract is viscous	Genomic DNA is not sufficiently sheared	Increase sonication time or sonicator power output
Box 1	Nuclei are not oval or rounded	Nuclear membrane is damaged	Wash nuclei with fewer washes or pipette more gently in Step 7A(ii) or 7B(ii)
	DAPI staining outside of nuclei	Nuclear membrane damaged	Wash nuclei with fewer washes or pipette more gently in Step 7A(ii) or 7B(ii)
			Check to ensure that NP-40 concentrations are correct during reagent setup

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Signal from ER tracker dye	ER membrane proteins are not efficiently removed	Increase wash times, NP-40 concentration, or pipetting stringency in Step 7A(ii) or 7B(ii)
		Tracker dye concentration is too high	Check tracker dye concentration and ensure that nuclei are diluted after incubation
	No ER tracker signal from controls	ER tracker dye concentration is too low	Increase tracker dye concentration or incubation time
Box 2	RNA radioactivity is low	Inefficient labeling	Increase [λ]-^{32}P-ATP, PNK enzyme or reaction duration in step 1 of **Box 2**
		Inefficient gel extraction	Use more water during elution from gel in step 8 of **Box 2**
			Recover gel bits off the pipette tip by pipetting buffer down the tip step 8 of **Box 2**
			Add more tRNA and $LiClO_4$ in acetone to the RNA solution during precipitation in step 10 of **Box 2**
10A(v), 10B(v)	Bands are not visible	Inefficient siRNA loading	Add higher amounts of radioactive RNA to the loading reaction in Step 9A(vi) or 9B(i)
			Increase the extract volume or use more antibody and resin in Step 9A(ii) or 9B(ii–iv)
			Incubate the loading reactions longer in Step 9A(vi) or 9B(i, iv)
		Inefficient Ago2 immunoprecipitation	Increase the amount of antibody or resin during immunoprecipitation in Step 9A(ii) or 9B(ii, iv)
			Incubate the antibody and resin with extract longer in Step 9A(ii) or 9B(iii, iv)
			Reduce the wash duration or wash buffer NaCl concentration in Step 9A(vii) or 9B(v)
		Extract quality is poor	Optimize the fractionation in Step 7B
		Ago2 expression is low	Increase the extract volume during loading reactions in Step 9A(ii) and 9B(i)
	Bands are blurry or heterogeneous	Poor gel quality	Optimize the gel pouring conditions in Step 10A(i) and 10B(i) or purchase commercial gels
		Gel runs too hot or too fast	Reduce the current or improve cooling in Step 10A(ii, iii)
		Gel runs too cool or too slow	Increase the current in Step 10B(ii, iii)
		Gel runs too long	Reduce the run time in Step 10A(ii) or 10B(ii)
	Bands are visible in IgG controls	Inefficient resin washing	Wash longer, wash with more buffer, wash more times or wash with a higher NaCl concentration in the wash buffer in Step 9A(vii) or 9B(v)
		Overexposed phosphorimager screen	Reduce exposure time to the phosporimager screen in Step 10A(v) or 10B(v)

● TIMING

Steps 1–6, collection of tissue culture cells: 1–2 h
Step 7, subcellular fractionation: 2–4 h
Steps 8–10, *in vitro* Ago2 loading assay: 2–3 d
Box 1, performing fluorescence microscopy: 2–3 h
Box 2, preparation of radiolabeled siRNA: 2 d

ANTICIPATED RESULTS

The overall aim of this procedure is to generate nuclear extracts from mammalian cells of high quality with minimal contamination from the cytoplasm or other cellular organelles, especially the ER. These extracts enable investigation of nuclear-specific processes, such as the presence and activity of RNAi. Extracts are then used to test Ago2 loading with small RNAs, an activity that we have found to be deficient in human nuclear extracts.

Western blot analysis

It is essential that the purity of subcellular fractionations be confirmed before any subsequent experiments and that efficient exclusion of potential cyoplasmic contaminants be demonstrated. When analyzed by western blot analysis, nuclear fractions produced by this protocol should show little or no evidence of cytoplasmic markers such as tubulin, ER markers such as calreticulin or mitochondrial markers such as OxPhos (**Fig. 3a**). The nuclear fraction should reveal the presence of nuclear-specific proteins such as lamin A/C or histone H3, whereas the cytoplasmic fraction should not contain these proteins. Abundant Ago2 should be seen in both cytoplasmic and nuclear fractions. Nucleoplasmic fractions should show a strong depletion of chromatin-associated proteins such as histone H3. It is important to load the protein from equal numbers of cells because the cytoplasm contains substantially more protein per cell. It will be impossible to compare fractions directly by western blotting if equal cell equivalents are not used.

Fluorescence microscopy

Fluorescence microscopy is a useful tool for assessing the quality of isolated nuclei[28]. Key indicators of high-quality nuclei include evidence of intact nuclear membrane and removal of membrane-associated ER proteins. Staining nuclei with DAPI should show no leakage of chromatin, which would appear as strings or wisps of blue staining outside of the oval nuclei. DAPI staining should also show sharp, well-defined boundaries. After isolation using 0.3% (vo/vol) NP-40 for washes and incubation with ER tracker dye, there should be little or no fluorescence from the tracker dye in contrast to abundant staining when NP-40 detergent is not used (**Fig. 3b**). There is no special requirement for confocal microscopy or other 3D imaging technologies other than the ability to measure fluorescence with blue (i.e., DAPI) and red (i.e., tetramethylrhodamine (TRITC)) filters.

In vitro Ago2 loading assays

For the loading of Ago2 with duplex small RNA (**Fig. 4a**), the first quality check is to observe a single band on a denaturing gel after immunoprecipitation of Ago2 from cytoplasmic extracts incubated with radiolabeled duplex (**Fig. 4b**). No band should appear in the samples immunoprecipitated with nonspecific IgG antibody when used. Running on a native, nondenaturing gel might be helpful in discriminating between duplex loading and passenger strand removal. If the passenger strand has not been completely removed, two distinct bands will be observed (**Fig. 4c**). The faster migrating band represents guide RNA bound to Ago2, whereas the slower migrating band represents duplex RNA bound to Ago2. In the cell lines used in our studies, Ago2 loading is observed in cytoplasmic extracts but not in nuclear extracts[28].

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1. Wilson, R.C. & Doudna, J.A. Molecular mechanisms of RNA interference. *Annu. Rev. Biophys.* **42**, 217–239 (2013).
2. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
3. Elbashir, S.M. *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
4. Morris, K.V., Chan, S.W., Jacobsen, S.E. & Looney, D.J. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**, 1289–1292 (2004).

5. Janowski, B.A. *et al.* Inhibiting gene expression at transcription start sites in chromosomal DNA with antigen RNAs. *Nat. Chem. Bio.* **1**, 216–222 (2005).
6. Castanotto, D. *et al.* Short hairpin RNA-directed cytosine (CpG) methylation of the *RASSF1A* gene promoter in HeLa cells. *Mol. Ther.* **12**, 179–183 (2005).
7. Ting, A.H., Schuebel, K.E., Herman, J.G. & Baylin, S.B. Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nat. Genet.* **37**, 906–910 (2005).
8. Napoli, S., Pastori, C., Magistri, M., Carbone, G.M. & Catapano, C.V. Promoter-specific transcriptional interference and *c-myc* gene silencing by siRNAs in human cells. *EMBO J.* **28**, 1708–1719 (2009).
9. Kim, D.H., Villeneuve, L.M., Morris, K.V. & Rossi, J.J. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat. Struct. Mol. Biol.* **13**, 793–797 (2006).
10. Li, L.C. *et al.* Small dsRNAs induce transcriptional activation in human cells. *Proc. Natl. Acad. Sci. USA* **103**, 17337–17342 (2006).
11. Janowski, B.A. *et al.* Activating gene expression in mammalian cells with promoter-targeted duplex RNAs. *Nat. Chem. Biol.* **3**, 166–173 (2007).
12. Matsui, M. *et al.* Promoter RNA links transcriptional regulation of inflammatory pathway genes. *Nucleic Acids Res.* **41**, 10086–10109 (2013).
13. Huang, V. *et al.* RNAa is conserved in mammalian cells. *PLoS ONE* **5**, e8848 (2010).
14. Liu, J., Hu, J. & Corey, D.R. Expanding the action of duplex RNAs into the nucleus: redirecting alternative splicing. *Nucleic Acids Res.* **40**, 1240–1250 (2012).
15. Allo, M. *et al.* Control of alternative splicing through siRNA-mediated transcriptional gene silencing. *Nat. Struct. Mol. Biol.* **16**, 717–724 (2009).
16. Janowski, B.A. *et al.* Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. *Nat. Struct. Mol. Biol.* **13**, 787–792 (2006).
17. Chu, Y., Yue, X., Younger, S.T., Janowski, B.A. & Corey, D.R. Involvement of argonaute proteins in gene silencing and activation by RNAs complementary to a non-coding transcript at the progesterone receptor promoter. *Nucleic Acids Res.* **38**, 7736–7748 (2010).
18. Vickers, T.A. *et al.* Efficient reduction of target RNAs by small interfering RNA and RNase H–dependent antisense agents: a comparative analysis. *J. Biol. Chem.* **278**, 7108–7118 (2003).
19. Zeng, Y. & Cullen, B.R. RNA interference in human cells is restricted to the cytoplasm. *RNA* **8**, 855–860 (2002).
20. Ikeda, K. *et al.* Detection of the argonaute protein Ago2 and microRNAs in the RNA induced silencing complex (RISC) using monoclonal antibody. *J. Immunol. Methods* **317**, 38–44 (2006).
21. Stalder, L. *et al.* The rough endoplasmatic reticulum is a central nucleation site of siRNA-mediated RNA silencing. *EMBO J.* **32**, 1115–1127 (2013).
22. Ando, Y. *et al.* Nuclear pore complex protein mediated nuclear localization of dicer protein in human cells. *PLoS ONE* **6**, E23385 (2011).
23. Doyle, M. *et al.* The double-stranded RNA binding domain of human Dicer functions as a nuclear localization signal. *RNA* **19**, 1238–1252 (2013).
24. Ohrt, T., Muetze, J., Svoboda, P. & Schwille, P. Intracellular localization and routing of miRNA and RNAi pathway components. *Curr. Top. Med. Chem.* **12**, 79–88 (2012).
25. Rudel, S., Flatley, A., Weinmann, L., Kremmer, E. & Meister, G. A multifunctional human Argonaute2-specific monoclonal antibody. *RNA* **14**, 1244–1253 (2008).
26. Till, S. *et al.* A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat. Struct. Mol. Biol.* **14**, 897–903 (2007).
27. Weinmann, L. *et al.* Importin 8 is a gene silencing factor that targets argonaute proteins to distinct mRNAs. *Cell* **136**, 496–507 (2009).
28. Gagnon, K.T., Li, L., Chu, Y., Janowski, B.A. & Corey, D.R. RNAi factors are present and active in human cell nuclei. *Cell Rep.* **6**, 211–221 (2014).
29. Holding, C. RNAi active in the nucleus? *The Scientist* http://www.the-scientist.com/?articles.view/articleNo/23231/title/RNAi-active-in-the-nucleus-/ (2005).
30. Hetzer, M.W. The nuclear envelope. *Cold Spring Harb. Perspect. Biol.* **2**, a000539 (2010).
31. Michelson, U. & von Hagen, J. Isolation of subcellular organelles and structures. *Methods Enzymol.* **463**, 306–328 (2009).
32. Greenberg, M.E. & Bender, T.P. Identification of newly transcribed RNA. *Curr. Protoc. Mol. Biol.* **78**, 4.10.11–14.10.12 (2007).