Piwi-interacting RNAs (piRNAs) are a class of small noncoding RNAs that bind Piwi proteins to silence transposons and to regulate gene expression. In Drosophila germ cells, the Aubergine (Aub)-Argonaute 3 (Ago3)-dependent ping-pong cycle generates most germline piRNAs. Loading of antisense piRNAs amplified by this cycle enables Piwi to enter the nucleus and silence transposons. Nuclear localization is crucial for Piwi function in transposon silencing, but how this process is regulated remains unknown. It is also not known whether any of the components of the nuclear pore complex (NPC) directly function in the piRNA pathway. Here, we show that nucleoporin 358 (Nup358) and Piwi interact with each other and that a germline knockdown (GLKD) of Nup358 with short hairpin RNA prevents Piwi entry into the nucleus. The Nup358 GLKD also activated transposons, increased genomic instability, and derailed piRNA biogenesis because of a combination of decreased piRNA precursor transcription and a collapse of the ping-pong cycle. Our results point to a critical role for Nup358 in the piRNA pathway, laying the foundation for future studies to fully elucidate the mechanisms by which Nup358 contributes to piRNA biogenesis and transposon silencing.

piRNAs\(^3\) are 23–26-nucleotide (nt)–long small RNAs that bind Piwi proteins and function to silence transposable elements and maintain genome integrity (1–5). In Drosophila germline, Piwi, Aubergine (Aub), and Argonaute 3 (Ago3) proteins bind piRNAs. piRNA biogenesis occurs in the “nuage,” an RNA-rich perinuclear granule in the cytoplasm. Most piRNAs are generated from genomic loci called piRNA clusters that contain truncated repetitive elements (6–8). Most of the piRNA clusters can produce piRNA precursors from both DNA strands, with their transcription dependent on an HP1 homolog called Rhino (9–11). piRNA biogenesis involves primary and secondary pathways. During primary piRNA biogenesis, long single-stranded antisense transcript is processed by Zucchini (Zuc), Armitage, and Gasz into mature piRNAs that are then loaded onto either Piwi or Aub (12–15). Primary piRNAs loaded onto Aub enter the secondary piRNA pathway where antisense piRNA–Aub and sense piRNA–Ago3 complexes cofunction in a “ping-pong” cycle to amplify piRNAs (7, 16, 17). Antisense piRNAs amplified by Ago3 are loaded onto Piwi (18).

Loading of primary and secondary piRNAs acts as a licensing step for Piwi to enter the nucleus and silence transposons (19–26). A seamless network needs to exist between nuclear pore complex (NPC) and the nuage to ensure that Piwi–piRNA complexes assembled in the nuage translocate into the nucleus and silence transposons. A role for NPC components, if any, in the piRNA pathway is not known. In this work, we show that nucleoporin 358 (Nup358) interacts with Piwi and that germline knockdown (GLKD) of Nup358 prevents Piwi’s entry into the nucleus. The Nup358 GLKD also activated transposons, increased genomic instability, and derailed piRNA biogenesis. Thus, we provide evidence for a direct role of an NPC component in piRNA pathway.

**Results and discussion**

To investigate the biochemical basis for Piwi function, we took advantage of biochemical fractionation performed earlier where we had identified Hsp70/Hsp90 – organizing protein homolog (Hop) and Tudor-SN as Piwi-interacting proteins in early embryos (32, 33). In addition to Hop and Tudor-SN, we discovered Nup358 as a potential interacting partner of Piwi as revealed by peptides unique to Nup358 in MS (Fig. 1A). A coimmunoprecipitation assay using ovary lysates confirmed that Piwi and Nup358 interact with each other in vivo (Fig. 1B, cf. lanes 2 and 4). Because of its interaction with Piwi, we decided to characterize the potential role of Nup358 in the piRNA pathway. Nup358 (also known as RanBP2) (27) is a large nuclear pore protein that along with Nup88 (nbo) (28) and Nup214 form the cytoplasmic filaments of NPC. Nup88 and Nup214 (29) mediate the localization of Nup358 to the NPC, and the absence of either Nup88 or Nup214 drastically reduces
Nup358 localization to the NPC (30). Nup358 is a highly modular protein that binds Ran via its four Ran-binding domains (31) (Fig. 1C). Also, Nup358 has a putative protein–protein interaction tetratricopeptide-repeat (TPR) domain in its N-terminal region, one zinc finger domain that interacts with RanGDP, and an FG-repeat region that is characteristic of nucleoporin proteins.

All unique Nup358 peptides identified by MS were localized to the N-terminal region (Fig. 1C, black bar), suggesting that this region might interact with Piwi. To confirm this, we coexpressed the GST epitope–tagged first 200 amino acid residues of Nup358 (NNup358) and full-length HisG-Smt3-Piwi in *Escherichia coli* and pulled down GST-NNup358 using the epitope tags GST alone and His-G-Smt3 as negative controls. We then tested for the presence of Piwi by immunoblotting (Fig. 1D). We discovered that Piwi interacted with NNup358 but not with GST alone (Fig. 1D, cf. lanes 6 and 8). Furthermore, NNup358 interacted with Piwi but not with His-G-Smt3 (Fig. 1D, cf. lanes 7 and 8; also see Fig. S1A for full gel profile of Fig. 1D). Thus, consistent with our MS data shown in Fig. 1A, the first 200 amino acids of Nup358 are sufficient to interact with Piwi. Piwi and Nup358 interaction was further confirmed by a serial pulldown assay as shown in Fig. S1B.

We next knocked down Nup358 in the germline using the Nup358*HMS00865* RNAi line and tested whether Nup358 regulates Piwi function. As a control for Nup358 GLKD, we drove the expression of firefly luciferase gene located at the same genomic location as Nup358 shRNA. Nup358 GLKD using nos-Gal4 (nos>Nup358 RNAi), which expresses Nup358 shRNA throughout oogenesis, produced rudimentary ovaries (Fig. 2, A and B). In contrast, Nup358 GLKD using mat4-GAL-VP16 (mat>Nup358 RNAi), where Nup358 shRNA is expressed in stages 1–10 of oogenesis but not in the gerarium, produced ovaries with normal morphology (Fig. 2, A and B) despite ~88% reduction in steady-state levels of Nup358 mRNA (Fig. 2C) and significant loss of Nup358 protein (Fig. 2D). This suggests that Nup358 expression is essential in the gerarium but is dispensable for the development of stages 1–10 of oogenesis. However, mat>Nup358 flies were sterile and did not lay eggs (data not shown). Henceforth, Nup358 GLKD refers to mat>Nup358*HMS00865*. Nup358 GLKD led to the failure of Piwi to enter the nucleus in germline nurse cells (Fig. 2, E–G, cf. Piwi staining in upper and lower panels) but not in somatic follicle cells where Nup358 knockdown was not active (Fig. 2, E–G, lower panels, white arrows). A similar result was obtained for nos>Nup358*HMS00863* (Fig. S2), showing that the Piwi nuclear localization defect is not restricted to one shRNA line and one Gal4 driver. Although Piwi’s nuclear localization was affected in Nup358 GLKD ovaries, that for three other nuclear markers, heterochromatin protein 1a (HP1a) (Fig. 2F), RNA polymerase II (Fig. 2F), and Rhino (Fig. 2G), was not affected, showing that the nuclear import defect in Nup358 GLKD ovaries is not due to an impairment of general nuclear import pathways.

We next tested whether Nup358 is required for transposon silencing. mRNA from Nup358 GLKD and control ovaries was sequenced and analyzed (Fig. S3A). We noticed a ~2-fold increase in the number of reads that uniquely mapped to transposons in Nup358 GLKD ovaries when compared with control ovaries; however, there was no change in the number of reads that uniquely mapped to genes (Fig. 3A). To get deeper insights, we mapped sequenced reads to transposon consensus and gene mRNA sequences followed by quantification using the RSEM-EBSeq workflow as described earlier (34). The false discovery rate (FDR) was set at 0.1. Of the 126 different transposon fami-
Figure 3. Nup358 is needed for transposon silencing and germline genome stability. 

A, percentage of genome mapping reads that uniquely map to transposons and genes is shown. B, percentage of genes and transposons with at least 2-fold activation and FDR < 0.1. C, a bar plot indicating the extent of transposon activation (log2) in Nup358 GLKD ovaries when compared with control. Only transposons that were up-regulated at least 2-fold with FDR < 0.1 are shown. Black dotted line represents 2-fold change.

Figure 2. Nup358 is required for nuclear localization of Piwi. 

A, illustration of Drosophila ovary with different stages marked. The extent of Gal4 expression dictated by nos-Gal4 and mat-Gal4 is indicated. B, ovary phenotypes of nos–Nup358 RNAi, mat–Nup358 RNAi, and wildtype. C, extent of Nup358 mRNA knockdown in mat–Nup358 RNAi ovaries. Act5c was used as an internal control. The histogram represents mean ± S.D. of five biological replicates. Each biological replicate had three technical repeats. The dashed line represents the Nup358 mRNA level in control ovaries. D, protein levels of Nup358 in control and Nup358 GLKD ovaries. E–G, confocal images of stage 2 egg chambers stained for Piwi, HP1a, RNA pol II, and Rhino. White arrows indicate somatic follicle cells where Nup358 RNAi is not active. Scale bars, 10 µm. Error bars represent S.D.
Transposon activation in piRNA pathway mutants leads to the induction of the DNA-damage response (37, 38). We examined this in the Nup358 GLKD and control ovaries using γ-H2Av staining that marks DNA double-strand breaks when the DNA-damage response pathway is activated. Nup358 GLKD led to a significant increase in γ-H2Av foci in germline cells of ovaries (Fig. 3E, cf. γ-H2Av staining pattern in control and Nup358 GLKD egg chambers), the site of active Nup358 RNAi.

Next, we analyzed whether Nup358 GLKD–induced transposon activation is due to impaired transcriptional silencing.  
Nup358 and piRNA pathway

ACCELERATED COMMUNICATION: Nup358 and piRNA pathway

Figure 4. Nup358 is needed for efficient piRNA biogenesis. A, a scatter plot indicating the levels of antisense piRNAs mapping to transposons. Germline and soma dominant candidate transposons are shown in red and blue, respectively. B, log₂-fold change in the number of 42AB and flamenco piRNAs. C, relative levels of Act5c and 42AB (log₂-fold change) in the nucleus when compared with the cytoplasm. A schematic of the method used is shown to the left. Histograms represent mean ± S.D. of two biological replicates with each biological repeat having three technical repeats. The dashed line represents relative levels of 42AB and 42AB transcripts in control ovaries. E, small RNA reads uniquely mapping to transposons and 42AB cluster were tested for 10-nt overlap between sense and antisense reads, and Z-score for 10-nt overlap (Z10) was plotted. Error bars represent S.D. of three biological replicates with each biological repeat having three technical repeats. The

families, we noticed that 38 (30%) transposon families were activated more than 2-fold (Fig. 3B); these results show that Nup358 is required for silencing transposons and that Nup358 GLKD–induced transposon activation is not due to global activation of transcription.

Both transcriptional and post-transcriptional mechanisms silence transposons. Because Nup358 GLKD prevented Piwi from entering the nucleus, we first tested whether transposon activation is primarily due to impaired transcriptional silencing. To this end, we performed global nuclear run-on sequencing (GRO-seq) (Fig. S3B) that measures nascent transcription levels across the genome (35, 36). Data were analyzed with the same stringency (FDR < 0.1) as mRNA sequencing (mRNA-seq). We noted that nascent transcription was activated by more than 2-fold at seven transposon families (compared with 38 in mRNA-seq) (Figs. 3D and S3C). Of these seven transposon families, five (accord, gypsy, blood, rover, and mdg3) were common to both mRNA-seq and GRO-seq, suggesting that these transposon families were activated by both transcriptional and post-transcriptional mechanisms; the remaining 33 transposon families were unique to mRNA-seq, implying failed post-transcriptional silencing. Based on these results, we conclude that Nup358 GLKD does induce transcriptional activation of transposons, perhaps due to retention of Piwi in the cytoplasm; however, transposon activation shown in Fig. 3C is predominantly due to failed post-transcriptional silencing.
ACCELERATED COMMUNICATION: Nup358 and piRNA pathway

line dominant 42AB cluster (Fig. 4B). Consistent with germ-line-specific knockdown of Nup358, the number of piRNAs originating from soma dominant flamenco did not show any reduction. Based on these results, we conclude that Nup358 is critical for piRNA biogenesis and that Nup358 GLKD–induced transposon activation is due to impaired piRNA biogenesis. Nup358 binds NFX1–p15 dimers and functions in nuclear mRNA export (39). So, to test whether piRNA biogenesis defect is due to impaired nuclear export of piRNA precursors, we fractionated ovary lysates into cytoplasmic and nuclear fractions (Fig. 54C) and quantified relative retention of 42AB precursor in the nucleus (Fig. 4C). Although Act5c mRNA nuclear export was not affected, 42AB piRNA precursor was retained in the nucleus 1.23-fold. This retention is minor when compared with ~5-fold reduction in the number of piRNAs mapping to the 42AB cluster as shown in Fig. 4B, suggesting that the piRNA biogenesis defect is not due to impaired nuclear export. However, we noticed that Nup358 GLKD led to ~40% reduction in transcription of 42AB piRNA precursor (Fig. 4D). In comparison, we did not notice any change in the transcription of flamenco, a piRNA cluster active in the somatic cells where Nup358 RNAi was not active. In addition, we noticed a collapse of the ping-pong signature (Z_p) for both transposons and 42AB (Fig. 4E). These results show that Nup358 GLKD–induced defective piRNA biogenesis is due to a combination of decreased piRNA precursor transcription and collapse of the ping-pong cycle.

Recent genome-wide RNAi screens revealed that several components of the NPC are required for transposon silencing (13, 40); however, a direct role for any of the NPC proteins in the piRNA pathway remains elusive. In this work, we show that Nup358 interacts with Piwi and is required for the entry of Piwi, but not for other examined nuclear components of the piRNA pathway, into the nucleus. With recent work showing that Nup358 is dispensable for general nuclear import (41), our work shows points to a specific role for Nup358 in nuclear localization of Piwi. We predict that Nup358 sequesters Piwi near the nuclear envelope and in the nuage to facilitate efficient loading of piRNAs. Inefficient loading of piRNAs onto Piwi derails its nuclear translocation because loading of piRNAs acts as a licensing step for Piwi to enter the nucleus and silence transposons (19–26). We also show that transposon activation in Nup358 GLKD ovaries is primarily due to impaired post-transcriptional silencing. Post-transcriptional silencing of transposons is driven by Aub; and thus, lack of antisense piRNAs would impair Aub to efficiently scan and target transposon mRNAs as they traverse through the nuclear pore. Furthermore, because sense transcripts of active transposons act as precursors for piRNA biogenesis (42), impaired ping-pong between Aub and Ago3 in Nup358 GLKD ovaries would negatively affect utilization of transposon transcripts as precursors for piRNA biogenesis. We also show that Nup358 GLKD–induced impaired piRNA biogenesis is due to a combination of decreased piRNA precursor transcription and ping-pong cycle. With Nup358 GLKD having no effect on nuclear localization of RNA pol II and Rhino (Fig. 2) and recent reports that nuclear Piwi is dispensable for 42AB precursor transcription in ovaries (43, 44), it is intriguing that Nup358 GLKD affects piRNA precursor transcription. Results presented in this work point to a critical role for Nup358 in the piRNA pathway; and thus, this work lays the foundation for future mechanistic work that comprehensively characterizes how Nup358 is needed for piRNA biogenesis and transposon silencing.

Experimental procedures

Cytoplasmic lysate preparation and chromatography

Lysate preparation and chromatography were performed as described previously (32). Note that Nup358 was found to comigrate with Piwi in the same Superdex 200 fraction as Hop; a separate fractionation strategy was not repeated in this work. Mass spectrometry was performed at ProTech Inc., Phoenixville, PA.

Immunoprecipitation

To immunoprecipitate Nup358 as shown in Fig. 1B, whole-ovary lysate was prepared by homogenizing 20–25 pairs of manually dissected ovaries in ice-cold ovary lysis buffer (30 mM HEPES, 150 mM potassium acetate, 5 mM magnesium acetate, 5 mM DTT, 0.1% Nonidet P-40, 5% glycerol, cComplete Mini EDTA-free protease inhibitor mixture). The precleared lysate was incubated on ice for 1 h prior to centrifugation at 21,130 × g for 10 min. Supernatant was carefully collected, and 500 µl of lysate was incubated with 2 µg of α-Nup358 (Bethyl Laboratories, A301-797A) antibody overnight at 4 °C. This lysate-antibody mixture, 40 µl of SureBeads Protein A magnetic beads (Bio-Rad) were added and incubated for 3 h at 4 °C with gentle agitation. Beads were washed seven times with ovary lysis buffer. Each wash was performed for 5 min with end-over-end mixing at 4 °C. After the final wash, the beads were processed for immunoblotting.

Immunoblotting

Ovary lysate preparation and Western blotting were performed as described earlier (34). In Figs. 1B and 2D, α-Nup358, α-Piwi, and α-Act5c (Cell Signaling Technology, 4967) antibodies were used at 1:2000, 1:5000, and 1:1000, respectively. In Fig. 1D, α-HisG (Thermo Fisher Scientific, R941-25) and α-GST (Cell Signaling Technology, 2622) were used at 1:5000 and 1:1000, respectively. Quantitation of Piwi enrichment in Fig. 1B was performed in Image Lab from Bio-Rad.

Plasmids

The HisG-Smt3-Piwi plasmid is described elsewhere (32). NNup358-expressing plasmid was created by first PCR-amplifying bp 1–3960 of Nup358 using “cactcatggttaaaacggaaaagaattg” and “cactcatggttaaaacggaaaagaattg” primers and LD24888 plasmid (Drosophila Genomics Resource Center, Bloomington, IN). The PCR amplicon was cloned into the pENTR/D-TOPO vector and then into pDEST15 using Gateway technology from Thermo Fisher Scientific to generate pDEST15-NNup358 plasmid. pDEST15 vector introduces a GST epitope tag at the N terminus of the NNup358 fragment. pGEX-4T1 plasmid was used to express the GST tag (Fig. 1D, lane 2). To express just the HisG-Smt3 tag (Fig. 1D, lane 3), a triple stop codon was introduced in HisG-Smt3-Piwi after HisG-Smt3 tag.

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In vitro interaction assays

Proteins shown in Fig. 1D were expressed in BL21-DE3 cells (New England Biolabs). Lysates were prepared in GST buffer: 1× phosphate-buffered saline (PBS) supplemented with 150 mM NaCl (total NaCl in the buffer is 300 mM), 0.2% Nonidet-P40, 1 mM DTT, and 10% glycerol. For pulldown experiments, 20 μl of a 50% slurry of GSH-Sepharose (Pierce, 16100) was added to the lysates and incubated at 4 °C for 1 h with gentle agitation. Beads were pelleted at 1000 × g for 5 min and then washed three times with 1 ml of GST buffer with end-over-end mixing for 5 min at 4 °C. After the final wash, the beads were processed for immunoblotting.

GLKD of Nup358

All fly stocks were maintained at 25 °C. Nup358 GLKD was induced by mating virgin females of Gal4-nos.NGT (Bloomington Drosophila Stock Center, 32563) with males from either Nup358HMS00803 (Bloomington Drosophila Stock Center, 33003) or Nup358HMS00865 (Bloomington Drosophila Stock Center, 34967). Alternatively, males from mate4-GAL-VP16 (Bloomington Drosophila Stock Center, 7062) were crossed with virgin females from Nup358HMS00865.

Quantitative PCR

Quantitative PCR was performed as described earlier (34) using the following primers: (a) AAGTTGCTGCTCTGGTT-GTCG and GCCACACGCAGCTCATTGTAG for Act5c, (b) CACTTCCATGACCCTTCCC and CCAGGCTCCGATT-TATCCGT for Nup358, and (c) CGGACCTTTTCTGCACT-GTA and TCAAGCCCTCAAACGTAATC for flamenco. Three sets of primer pairs were used to measure 42AB precursor: (a) CGTCCAGCCTACCTGATCA and ACTTCCCG-GTGAAGACTCCT, (b) CGCTGTTGAAGCAATTTGA and GAGACCTTGCTCAGTTGC, and (c) GTGGAGTT-GTGGTGCAAGGC and AGCCGTGCTTTATGCTTTC. 42AB primer sequences were obtained from an earlier publication (45). Data for 42AB piRNA precursor in Fig. 4, C and D, were an average from three different primer sets described above. Act5c was used as an internal control in Figs. 2C and 4D.

Statistical analysis

All quantitative PCR results in this work are presented as histograms representing mean ± S.D. All quantitative PCR experiments had at least three biological repeats except for Fig. 4C, which had two biological repeats with two independent nuclear–cytoplasmic fractionations. Each biological repeat had three technical repeats. Both mRNA-seq and GRO-seq had two biological repeats, and the FDR for differential expression analysis was set at 0.1.

Immunostaining and microscopy

In Fig. 2, E–G, α-Piwi, α-RNA pol II (Abcam, ab 5131), α-Rhino, and α-HP1a (Developmental Studies Hybridoma Bank, C1A9) antibodies were used at 1:700, 1:700, 1:500, and 1:50, respectively. Because both α-HP1a and α-Piwi antibodies used in Fig. 2E were generated in mouse, we followed a serial immunostaining procedure where we stained ovaries with α-HP1a antibody on day 1 followed by Alexa Fluor secondary antibody on day 2. On day 3, after thorough washing, we stained the ovaries with α-Piwi antibody followed by Alexa Fluor secondary antibody on day 4. α-Rhino antibody was a kind gift from William Theurkauf (University of Massachusetts, Worcester, MA). C1A9 was deposited to the Developmental Studies Hybridoma Bank by L. L. Wallrath. All secondary antibodies (Alexa Fluor, Invitrogen) were used at 1:500. Confocal images were taken using a Zeiss LSM 880 NLO microscope and Plan-Apochromat 63×/1.40 oil differential interference contrast objective. Images were analyzed using Fiji (ImageJ) (46).

mRNA-seq and data analysis

Total RNA isolation from ovaries and mRNA-seq were performed as described earlier (34). In Fig. 3A, sequenced reads were mapped to dm6 genome using Bowtie 2 (47, 48), and the percentage of genome unique mappers that map to transposon consensus sequences and genes is plotted. In Fig. 3, B and C, sequenced reads were mapped to gene and transposon consensus sequences and analyzed using the RSEM-EBSeq workflow as described earlier (34). mRNA-seq was performed twice with two independent biological replicates.

GRO-seq and data analysis

GRO-seq was conducted as described before with the following changes (35, 36). RNA fragmentation was performed using the NEBNext Magnesium RNA Fragmentation Module (New England Biolabs) according to the manufacturer’s instructions. Polynucleotide kinase (PNK) treatment of RNA fragments was performed as described earlier (49). Briefly, 1 μl of RNase inhibitor (murine; New England Biolabs), 30 units of T4 PNK (New England Biolabs), 1 mM ATP (Thermo Fisher Scientific), and PNK Buffer (New England Biolabs) was added to purified RNA fragments, and the reaction was incubated at 37 °C for 60 min. To this reaction, 20 units of PNK and 10 mM EDTA were added and incubated at 37 °C for an additional 60 min. The enzyme was heat-inactivated by incubating the reaction mixture at 65 °C for 20 min. Libraries were prepared using a TrueSeq Small RNA Library Prep kit (Illumina) and sequenced using single-end 50-bp sequencing. GRO-seq was performed twice with two independent biological repeats. After sequencing, the 3′-adapter was removed from the sequenced reads using cutadapt, and processed reads fewer than 18 nt were discarded. Data were analyzed like mRNA-seq.

Small RNA-seq and data analysis

Cloning of small RNAs and deep sequencing were performed as described elsewhere (34). Sequenced reads were trimmed of the 3′-adapter using cutadapt and analyzed using picPipes. Trimmed reads from control and Nup358 GLKD were first analyzed by picPipes small RNA pipeline single-sample mode (19). Drosophila melanogaster genome (dm3) was used as the reference sequence. Then control and Nup358 GLKD were compared using picPipes small RNA pipeline dual-sample mode. Reads were normalized to the number of reads uniquely mapping to the genome.
Nuclear and cytoplasmic fractionation

50 pairs of *Drosophila* ovaries were manually dissected in ice-cold 1× PBS. Tissues were homogenized in 300 μl of ice-cold ovary lysis buffer using 20 strokes of loose and tight pestles. Homogenate was centrifuged at 300 × g for 5 min at 4 °C. Supernatant was carefully separated from the pellet. The pellet containing nuclei was suspended in 300 μl of ovary lysis buffer and used as the nuclear preparation. To clear tissue debris from the first spin, the supernatant was centrifuged again at 21,130 × g for 10 min at 4 °C, and the cleared supernatant was used as the cytoplasmic preparation. Efficiency of fractionation was analyzed using nucleus- and cytoplasm-specific markers as shown in Fig. S4C. Total RNA isolation was performed using TRIzol reagent (Invitrogen, 15596018).

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