CRM1 mediates nuclear-cytoplasmic shuttling of mature microRNAs

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Drosha-processed microRNAs (miRNAs) have been shown to be exported from the nucleus to the cytoplasm by Exportin 5, where they are processed a second time to generate mature miRNAs. In this work we show that miRNAs also use CRM1 for nuclear-cytoplasmic shuttling. Inhibition of CRM1 by Leptomycin B results in nuclear accumulation of miRNA guide sequences. Nuclear to cytoplasmic transport can be actively competed by synthetic small interfering RNAs, indicating that this pathway is shared by different classes of processed small RNAs. We also find that CRM1 coimmunoprecipitates with Ago-1, Ago-2, Topo2α, EzH2, and Mta, consistent with a role of Argonautes and small RNAs in chromatin remodeling.

competition | nucleus | cytoplasm | transport

MicroRNAs (miRNAs) are implicated in numerous biological processes. Through partial or full complementarities with their targets, these short RNAs can modulate cellular gene expression and have a critical role in development as well as in some diseases. Studies on their mechanism of action have exposed unforeseen complexities, so the roles of miRNAs in the regulation of gene repression are not yet completely understood. miRNAs are transcribed in primary miRNA containing (pri-miRNA) transcripts that can be several kilobases long. With the exception of a minor class of miRNAs called mirtrons, which are processed by the RNA splicing machinery (1), the majority of miRNAs are processed from the primary transcripts into precursor miRNA (pre-miRNAs) within the nucleus by a complex containing the RNase III enzyme Drosha and its partner DGCR8 (2). The pre-miRNAs are transported to the cytoplasm by the nuclear karyopherin Exportin-5 in a Ran-GTP-dependent manner and processed a second time by the RNase III family member Dicer to generate an approximately 20- to 25-nt duplex, one strand of which is incorporated into the RNA-induced silencing complex (RISC) (2). A member of the Argonaute family of proteins, Ago-2, has been identified as the catalytic core of this complex. The miRNA guide sequence directs base pairing to the 3′UTR of its mRNA target and guides target specific repression of protein synthesis (2). It is conventionally accepted that once the premiRNA is processed in the cytoplasm, the guide sequence remains in this cellular compartment. In this work, we show that in addition to Exportin 5, another karyopherin, CRM1 (XPO-1, Exportin-1), is part of the RNA interference pathway. CRM1 allows the nuclear-cytoplasmic shuttling of the miRNA guide sequences in a complex containing RNA Helicase A (RHA) and Argonaute proteins. Synthetic small interfering RNAs (siRNAs) also use this shuttling pathway and compete with endogenous miRNAs for CRM1. Although the function of endogenous miRNAs in the nucleus is poorly understood, we find that CRM1 coimmunoprecipitates with nuclear proteins such as Topo2α, EzH2, as well as Ago1 and Ago2, suggesting chromatin remodeling as a possible miRNA function.

Results and Discussion

It is known that nuclear Drosha-processed pre-miRNAs depend on the karyopherin Exportin-5 for export and cytoplasmic localization (3). The active strand of the miRNA is then incorporated into RISC, forming an active complex that is able to impair translation of the corresponding mRNA target(s). It is generally accepted that this process occurs in the cytoplasm. However, earlier reports have shown that RNA interference can also occur in the nuclear compartment (4, 5). The small nuclear 7SK RNA (4) and a nuclear retained mutant myotonic dystrophy protein kinase (DMPK) transcript (5) were shown to be susceptible to siRNA targeting. Moreover, it has been shown that siRNAs complementary to regions of specific cellular promoters can repress or activate gene expression (6, 7). Based on these and other published findings, it is expected that siRNAs and possibly miRNAs have access to the nuclear cellular compartment. Because these results cannot be explained by the Exportin 5 pathway, we looked for other potential transport factors that could be involved. Given that CRM1 has the broadest substrate range of all transport factors and is known to transport many different shuttling proteins and different classes of cellular RNAs, including the small structured U snRNAs and protein particles (snRNPs) (8–10), we speculated that it could function in the RNAi pathway by shuttling miRNAs between cellular compartments.

CRM1 transport is sensitive to Leptomycin B (LMB) (11), which modifies a cysteine residue in the central conserved region (12). We thus used this drug to monitor its effect on miRNA transport. Nuclear-cytoplasmic fractionations of RNA harvested from untreated cells or cells treated with LMB showed that reducing CRM1 function results in increased accumulation of miRNA guide sequences in the nucleus (Fig. 1). Mir16 (Fig. 1) behaved analogously to mir30 and mir19 in two different cell lines (HEK293 and HCT116). Without LMB treatment, the pre-mir16 was mostly located in the nucleus, while the processed guide sequence was mostly in the cytoplasm (Fig. 1*A*, pre-mir16 and mir16). However, when LMB was added to the cell cultures, the mir16 guide sequence was almost equally distributed between the cytoplasm and nucleus (Fig. 1*A*). In contrast to mir16, mir29b normally accumulates in the nucleus, because it contains a nuclear localization/retention sequence (13). As expected, the nuclear-cytoplasmic fractionation showed mir29b mostly in the nuclear fraction. In similarity to mir16, the level of the mir29b guide sequence increased in the nucleus after LMB treatment (Fig. 1*A*, mir29b). Because CRM1 is an essential cellular transport factor, it is not feasible to completely block its function without causing cellular toxicity. Although LBM-mediated reduction of CRM1 transport does not yield a radical phenotype, quantitation of four independent experiments indicated a reproducibly consistent 50% nuclear accumulation of the processed miRNA sequence after LMB treatment (Fig. S1).

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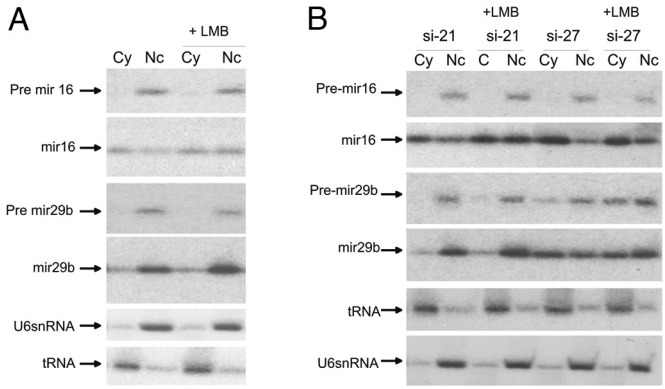

Fig. 1. Nuclear and cytoplasmic fractionations indicate that mature miRNAs are shuttled through the nucleus. The nuclear (Nc) and cytoplasmic (Cy) fractionations of HCT116 parental cells (*A*) or HCT116 transfected with a 21-mer (si-21) or 25/27-mer (si-27) siRNA (*B*) with or without 6 h of LMB treatment (20 ng/mL). The RNA fractions were electrophoresed in a denaturing acrylamide gel, blotted onto a nylon membrane, and hybridized with probes complementary to mir16 or mir29b. Both the premiRNAs (pre-mir16 and pre-mir29b) and the corresponding guide sequences (mir16 and mir29b) are shown. Probes specific for U6 snRNA (nuclear) and tRNA (cytoplasmic) were used to determine the relative purities of the fractions.

It has previously been shown that Exportin 5, the primary export carrier for miRNAs (14) is a saturable export system in which Pol III expressed shRNAs compete with miRNAs for cytoplasmic export (15). Because our data support the involvement of CRM1 in the shuttling of mature miRNAs from the cytoplasm into the nucleus, we asked if siRNAs would compete with miRNAs for CRM1-mediated nuclear import. To address this possibility, we transfected two different forms of siRNAs into cells, 21-mer and 25/27-mer Dicer substrate RNAs (16, 17). The 25/27-mer dicer substrate RNAs go through an obligatory Dicer processing step before RISC entry, whereas siRNAs are capable of direct RISC entry without processing. The results in Fig. 1 show that the addition of LMB to the cell cultures with or without the added siRNAs resulted in enhanced nuclear accumulation of mature miRNAs. The effects of the 21-mer and 25/27-mer and LMB addition on the fractionation pattern of mir29b were strikingly different. This miRNA is normally more concentrated in the nucleus than the cytoplasm (13) (Fig. 1). The addition of the 21-mer siRNA did not change this pattern, but the addition of LMB resulted in more mir29b (Fig. 1*B*). Surprisingly the addition of the 25/27-mer alone resulted in a substantial change in the cytoplasmic versus nuclear levels, with mir29b being present in both compartments. The addition of LMB in the presence of the 25/27-mer resulted in an increased nuclear accumulation (Fig. 1*B*). Noteworthy is the pre-mir29b nuclear accumulation in the presence of the 21-mer and LMB alone, but a substantial localization in the cytoplasm with the addition of the 25/27-mer, which was further enhanced by including LMB with the 25/27-mer (Fig. 1*B*). These results suggest that the processing of pre-mir29b in the cytoplasm is in competition with the transfected 25/27-mer as is the nuclear import of the mature miRNA. Finally, the nuclear and cytoplasmic localization properties of the control U6snRNA and tRNA were not affected by the siRNAs or LMB (Fig. 1*B*). The data indicate that mature miRNAs shuttle between the cytoplasm and nucleus via a CRM1 mediated carrier.

It is well established that the pre-miRNAs are exported to the cytoplasm by Exportin 5, but the carrier by which miRNAs, such as 29b, shuttle back into the nucleus has not been previously determined. The ability of small RNA molecules to use two different export carriers has previously been shown for tRNAs, whose cytoplasmic export is occurring through exportin-t (18, 19) but can also be mediated by Exportin 5 (20, 21).

As a transport pathway for miRNAs, CRM1 would be expected to interact with RISC components. RNA helicase A (RHA) is a member of the DEAH-family of RNA helicases and is one of the components of RISC (22). RHA shuttles constantly between the nucleus and the cytoplasm (23), it contains three RNA binding domains that can displace 3′-tailed but not 5′-tailed partial duplex RNA molecules (24), and can bind to both double-stranded and single-stranded RNAs (24). RHA has been shown to interact with the Tap protein (also known as nuclear RNA export factor 1), which is thought to be an export receptor for cellular mRNA and structured RNAs (25, 26). Another DEAH-family RNA helicase, DDX3, that interacts with TAP (27) has also been shown to interact with CRM1 (28). Based on the fact that DDX3 can bind to both TAP and CRM1, we speculated that the DEAH family of RNA helicases can act on multiple import-export pathways and that RHA may also be able to interact with CRM1 in addition to TAP, especially in light of the fact that both proteins participate in the export of short-structured RNAs. Also noteworthy is the finding that CRM1 is a transport carrier for TNRC6B, also called the Ago hook (29). To investigate if RHA interacts with CRM1, we transfected 293 cells with pcDNA3fhis-RHA (kindly provided by Chee-Gun Lee), performed an immunoprecipitation (IP) using the anti-RHA antibody, and probed for the presence of CRM1 (Fig. 2*A*). The results show that RHA is able to coimmunoprecipitate CRM1, suggesting that these two proteins can be part of the same complex. Conversely, when we carried out the IP using an anti-CRM1 specific antibody, we were able to detect RHA (Fig. 2*A*), confirming the interaction between these two proteins. We then proceeded to investigate if this interaction can occur in RISC. We used HEK293 cells stably expressing Ago-2-Flag, or Flag alone, and performed an IP using the anti-Flag antibody. We then probed for Dicer, which served as our positive control as well as for RHA and CRM1 in the precipitated complex (Fig. 2*B*). The results show that CRM1 interacts with these RISC factors as well. To further confirm that these proteins are part of the same complex, we carried out the reverse experiment using anti-CRM1 antibodies to perform the coimmunoprecipitations followed by probing for Dicer, RHA, Ago-2, and Ago-1 (Fig. 2*C*). To test the RNA dependence of these protein interactions, we included parallel experimental samples with added RNase. The data show that these interactions require an RNA component (Fig. 2*C*). Based on these results, we went on to determine whether or not CRM1 could IP miRNA sequences (Fig. 2*D*). Using IPs with the anti-CRM1 antibody or with the control IgG nonspecific antibodies followed with a Northern analysis for cellular miRNAs mir16 and mir29b, we observed miRNA enrichment in the presence of specific CRM1 antibodies. This experiment was repeated three times with consistent results, demonstrating that CRM1 can interact (directly or indirectly) with miRNA guide sequences (Fig. 2*D*).

As previously mentioned, it has been reported that RNA interference can occur in the nuclear compartment (4, 5). Moreover, it has recently been shown that although they are primarily localized in the nucleus (30), U6 sponge RNAs are effective in inhibiting miRNAs. In that study, the authors speculated that perhaps a sufficient amount of the U6 sponges localize to the cytoplasm where they interact with miRNAs. However, our data provide an alternate explanation, which is that the U6 sponges were titrating the mature miRNAs in the nucleus, thereby inhibiting their function. Processed miRNAs have also been detected in nucleoli (31). At this time the biological functions of nuclear trafficking of miRNAs is un-

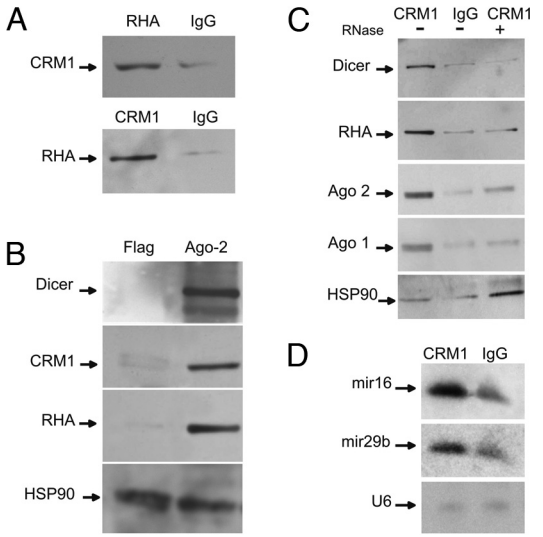

Fig. 2. Coimmunoprecipitations show association of CRM1 with RISC components. (*A*) Lysates collected from pcDNA3fhis-RHA transfected 293 cells were coimmunoprecipitated with an anti-RHA antibody (RHA; *Upper*) or with an anti-CRM1 antibody (CRM1; *Lower*) and probed with specific antibodies to detect CRM1 or RHA, respectively (indicated with arrows on the left side of the blots). Nonspecific antibodies of the same type and species of the anti-RHA or anti-CRM1 antibody (IgG) were used as nonspecific controls for the coimmunoprecipitations. (*B*) Lysates collected from Ago2-Flag (Ago-2) or the Flag nonspecific control expressing cell lines were coimmunoprecipitated with an anti-Flag antibody. The Western blot was probed with antibodies against Dicer, CRM1, or RHA. Small amounts of unrelated proteins are carried over during the procedure, thus we used an anti-HSP90 antibody to probe for HSP90 (indicated with an arrow on the left side of the blot), which served as loading control. (*C*) Lysates collected from Ago-2 expressing cell lines were coimmunoprecipitated with an anti-CRM1 antibody with (+) or without (−) RNase added to the reaction. IgG, nonspecific antibodies of the same type and species were used as a nonspecific control. The blot was probed with specific antibodies against Dicer, RHA, Ago-1, or Ago-2. An antibody against HSP90 was used as loading control. (*D*) Northern analysis of RNA yielded from lysates of cell lines stably expressing Ago-2 immunoprecipitated with an anti-CRM1 antibody (CRM1). IgG, nonspecific antibodies of the same type and species were used as a nonspecific control. Enrichment of mir16 and mir29b guide sequences is detected in presence of specific anti-CRM1 antibodies. A small amount of total RNA is nonspecifically carried through during the immunoprecipitation, thus a probe complementary to the endogenous U6snRNA (U6) was used as loading control.

clear. It is possible that miRNAs enter the nucleus to undergo modifications or to associate with nuclear localized proteins. It is also possible that miRNAs are involved in chromatin remodeling (6) or associate with target transcripts in the nucleus and are subsequently exported back to the cytoplasm with their targets.

Within the last few years, there have been several reports indicating that siRNAs can trigger chromatin remodeling in higher eukaryotes, inducing both activation or repression of gene expression (6, 7). Because RNA helicase A plays important roles in transcription as a coactivator or corepressor, it opens the possibility that one of the functions of nuclear miRNAs could be related to chromatin remodeling. The chromatin remodeling function of siRNAs and their ability to induce histone modifications have been linked to polymerase II (Pol II) and polycomb proteins (7). Interestingly, RHA is a component of the holo-RNA Pol II complexes (32) and directly interacts with Topoisomerase 2α (33); this complex forms in the presence of an unknown RNA molecule (34). Topo 2α can also interact with the NURD complex (35), a potent repressor of gene expression. Using anti-Flag immunoprecipitation of protein complexes in cells expressing Ago-2 or Flag alone, we

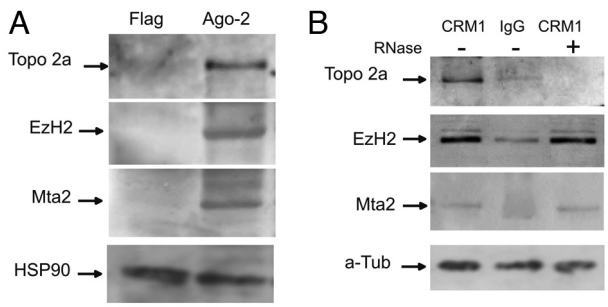

Fig. 3. Coimmunoprecipitations indicate interactions of RISC factors with chromatin remodeling factors. (*A*) Lysates collected from Ago2-Flag (Ago-2) or the Flag nonspecific control (Flag) expressing cell lines were coimmunoprecipitated with an anti-Flag antibody. The Western blot was probed with antibodies against Topo 2α, EzH2, and Mta2. An antibody against HSP90 was used as loading control. (*B*) Lysates collected from Ago2-Flag (Ago-2) expressing cell lines were coimmunoprecipitated with an anti-CRM1 antibody with (+) or without (−) RNase added to the reaction. IgG, nonspecific antibodies of the same type and species were used as a nonspecific control. The blot was probed with specific antibodies against Topo 2α, EzH2, and Mta2. An antibody against α-tubulin (α-Tub) was used as loading control.

find that Ago-2 can directly or indirectly interact with Topo 2α, EzH2 (the methyltransferase part of the polycomb repressive complexes), and Mta2, a component of the NURD complex (Fig. 3*A*). The reciprocal experiment, using an anti-CRM1 antibody, showed the same protein interactions with the Topo 2α being RNA-dependent (Fig. 3*B*). Our data show possible interactions of RISC factors with chromatin remodeling factors, but the biological significance of this observation warrants further investigation.

The nuclear import of processed guide sequences by CRM1 and the interaction of CRM1 with chromatin protein complexes raise important questions about the roles of small RNAs in this compartment and perhaps the nucleolus as well, adding another level of complexity to the functions of miRNAs.

Methods

Cell Lines and Transfection Conditions. Cells were grown in DMEM (Irvine Scientific) supplemented with 10% FCS (Irvine Scientific), 1 mM L-glutamine.

Experiment. Experiments with or without LMB (+/− LMB) were done in parallel. Approximately 20 h after transfection, the medium in all samples (+/− LMB) was replaced with a fresh aliquot containing 10 μg/mL cycloheximide. Cycloheximide treatment was performed for 1 h prior to adding the LMB (20 ng/mL) to one set of the experimental samples for an additional 5 to 6 h of incubation. Cycloheximide is used to inhibit translation before and during the LMB treatment, to facilitate discard of partially translated proteins from the cytoplasm that could increase nonspecific cytoplasmic signals. The experiments were repeated four times with consistent and reproducible results. Quantitation for nuclear miRNA accumulation after LMB treatment is shown in Fig. S1.

The 21-mer or 25/27-mer siRNAs are targeted against the EGFP coding sequence and were added as competitors to the transfection reactions (Fig. 1*B*) at a concentration of 5 nM using siQuest (Mirus) as described by the manufacturer.

Preparation of Cytoplasmic and Nuclear Fractions. Cells were briefly treated with Trypsin-EDTA and gently resuspended in DMEM. Cells were spun down in Falcon tubes for 5 min at 3,000 rpm in a Beckman tabletop centrifuge, washed once with PBS, and spun again for 5 min at 3,000 rpm. After completely aspirating the PBS, 800 μL hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) were added to each sample, the pellet was gently resuspended, transferred to a microfuge tube, and placed in ice for 2 min. Through the remaining steps of the protocol, samples were kept in ice and spun at 4 °C. Ten percent Nonidet P-40 was added to a final concentration of 0.4% (35 μL). Samples were inverted a few times and spun at 3,000 rpm for 7 min. Supernatants (450 μL; cytoplasmic fractions) were collected for process-

ing, and the remaining supernatants were discarded. Samples were quickly spun again (3,000 for 30 s) to remove any leftover supernatant, then the pellet (nuclear fraction) was gently resuspended in 500 μL hypotonic buffer and spun at 3,000 rpm for 2 min. This washing step was repeated three to four times. After removing the buffer following the last spin, the samples were briefly spun once again for few seconds to remove the remaining supernatant from the cells. Both the cytoplasmic and nuclear fractions were processed following the RNA STAT-60 (TEL-TEST B) protocol as recommended by the manufacturer.

Northern Analyses. Total RNA was isolated using RNA STAT-60 according to the manufacturer's instructions. Total RNA (30 μg) was fractionated in 7 M 8% PAGE and transferred onto a Hybond-$n+$ membrane (Amersham Pharmacia Biotech). ^{32}P-radiolabeled 21-mer probes complementary to the various miRNA guide sequences or the U6snRNA or tRNAarg controls were used for the hybridization reactions, which were performed for 16 h at 37 °C. Specific sequences of the probes are available upon request.

Preparation of Lysates for Coimmunoprecipitation. Cells were grown in complete medium to a confluency of 80–90% in a 10-cm dish. The media were removed, and the cells were washed twice with 5 mL cold PBS. In some instances, the cells were fixed by UV exposure for 4 min in a Stratalinker 2400 (Stratagene). The results were consistent with the nonfixed cells. One milliliter cold low salt lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 10 mM NaCl, 0.1% Nonidet P-40) with 1× protease inhibitors (Complete Mini 1 836 153; Roche Diagnostics) was added to the plates. Fifty units RNase inhibitor (rRNasin N2111; Promega) was also added to samples that were not to be treated with RNase A. Plates were kept in ice while cells were scraped and collected into microfuge tubes. Samples were incubated in ice for 30 min and then frozen in liquid nitrogen. The samples were subsequently thawed in cool water and drawn up and down in a narrow gauge needle (27G1/2 309602; Becton Dickinson) five to six times to ensure complete cell lysis. Cell debris was collected by spinning samples in a microfuge tube at 4 °C at top speed for 5 min. The supernatant/lysate was then transferred to a new tube. Lysates were assayed for protein concentration using a DC Protein Assay kit (500–0111; Bio-Rad Laboratories).

Coimmunoprecipitation. One to two milligrams cell lysate protein was used in each coimmunoprecipitation. The volume of each sample was adjusted up to 1 mL with low salt lysis buffer containing 1× protease inhibitors and 50 units/mL RNase inhibitor if appropriate for that sample. Salt concentration was adjusted to 150 mM NaCl. RNaseA was added to RNase treated samples. Anti-Crm-1 antibody (sc-74454, sc-7825; Santa Cruz Biotechnology, or ab3459; Abcam) or anti-Flag M2 (F 3165;Sigma) was added to the experimental samples. IgG, nonspecific antibodies of the same type and species, or peptide-blocked primary antibody were added to the negative control samples. Samples were rotated overnight at 4 °C. Fifty microliters 50% Protein A or Protein G agarose beads (16–156, 16–266;Millipore) was added to each tube the next day and rotated at 4 °C for 1 h. Beads were pelleted by spinning at 4 °C at 8,000 rpm for 1 min. The supernatants were removed, and pellets were washed three times with wash buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl). After the last wash the beads were resuspended in 2× SDS-PAGE sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 10% vol/vol glycerol, 0.002% bromophenol blue, and 5% 2-mercaptoethanol). Samples were boiled for 5 min and separated by SDS-PAGE. The coimmunoprecipitations were done a minimum of two times for each experiment with an identical outcome.

Immunoblotting. Separated proteins were transferred from the gel to a nitrocellulose membrane (Protran BA83; Whatman) with a semidry blotter. The membrane was incubated in blocking buffer (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, .05% Tween, 3% powdered milk solution, 3% BSA) for 1 h. Primary antibodies were diluted in blocking buffer and added to the membrane overnight at 4 °C with rotating or shaking. Horseradish peroxidase-conjugated secondary antibody diluted in the blocking buffer was added to the blots the next day, and the membranes were incubated for 1 h with shaking. ECL Plus Western Blotting Detection Reagents (RPN2132; GE Healthcare) were used as described by the manufacturer's protocol, and the resulting signal was detected by autoradiography. Primary antibodies used were: Anti-Ago1 (07–599; Millipore), anti-Ago1 (04–083; Millipore), anti-Ago2 (eIF2C2, sc-32659; Santa Cruz Biotechnology), anti-Dicer (ab14601; Abcam), anti-RNA Helicase A (ab26271; Abcam), anti-Ezh2 (39104; Active Motif), anti-Ezh2 (AC22; Cell Signaling), anti-MBD2 (39548; Active Motif), anti-MTA2 (ab50209; Abcam), and anti-Topo2α (sc-13058; Santa Cruz Biotechnology). The conjugated secondary antibodies used were: anti-mouse IgG-peroxidase (A3682; Sigma), anti-rabbit IgG-peroxidase (A0545; Sigma), and anti-goat IgG-peroxidase (A-5420; Sigma). Because a small amount of unrelated proteins is carried over during the procedure, based on the gel running condition, we used either an anti-SHP90 (SPA 840; Stressgen) or an anti α-tubulin (T5168; Sigma) antibody to probe for an unrelated protein that was used as loading control.

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1. Ruby JG, Jan CH, Bartel DP (2007) Intronic microRNA precursors that bypass Drosha processing. *Nature* 448:83–86.
2. Winter J, Jung S, Keller S, Gregory RI, Diederichs S (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 11:228–234.
3. Kim VN (2004) microRNA precursors in motion: Exportin-5 mediates their nuclear export. *Trends Cell Biol* 14:156–159.
4. Robb GB, Brown KM, Khurana J, Rana TM (2005) Specific and potent RNAi in the nucleus of human cells. *Nat Struct Mol Biol* 12:133–137.
5. Langlois MA, et al. (2005) Cytoplasmic and nuclear retained DMPK mRNAs are targets for RNA interference in myotonic dystrophy cells. *J Biol Chem* 280:16949–16954.
6. Kim DH, Saetrom P, Snove O Jr, Rossi JJ (2008) microRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci USA* 105:16230–16235.
7. Hawkins PG, Morris KV (2008) RNA and transcriptional modulation of gene expression. *Cell Cycle* 7:602–607.
8. Hamm J, Mattaj IW (1990) Monomethylated cap structures facilitate RNA export from the nucleus. *Cell* 63:109–118.
9. Jarmolowski A, Boelens WC, Izaurralde E, Mattaj IW (1994) Nuclear export of different classes of RNA is mediated by specific factors. *J Cell Biol* 124:627–635.
10. Fornerod M, Ohno M, Yoshida M, Mattaj IW (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 90:1051–1060.
11. Wolff B, Sanglier JJ, Wang Y (1997) Leptomycin B is an inhibitor of nuclear export: Inhibition of nucleocytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem Biol* 4:139–147.
12. Kudo N, et al. (1999) Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci USA* 96:9112–9117.
13. Hwang HW, Wentzel EA, Mendell JT (2007) A hexanucleotide element directs microRNA nuclear import. *Science* 315:97–100.
14. Yi R, Qin Y, Macara IG, Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* 17:3011–3016.
15. Yi R, Doehle BP, Qin Y, Macara IG, Cullen BR (2005) Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *RNA* 11:220–226.
16. Kim DH, et al. (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* 23:222–226.
17. Rose SD, et al. (2005) Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res* 33:4140–4156.
18. Arts GJ, Fornerod M, Mattaj IW (1998) Identification of a nuclear export receptor for tRNA. *Curr Biol* 8:305–314.
19. Kutay U, et al. (1998) Identification of a tRNA-specific nuclear export receptor. *Mol Cell* 1:359–369.
20. Bohnsack MT, et al. (2002) Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. *EMBO J* 21:6205–6215.
21. Calado A, Treichel N, Muller EC, Otto A, Kutay U (2002) Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. *EMBO J* 21:6216–6224.
22. Robb GB, Rana TM (2007) RNA helicase A interacts with RISC in human cells and functions in RISC loading. *Mol Cell* 26:523–537.
23. Tang H, Gaietta GM, Fischer WH, Ellisman MH, Wong-Staal F (1997) A cellular cofactor for the constitutive transport element of type D retrovirus. *Science* 276:1412–1415.
24. Lee CG, Hurwitz J (1992) A new RNA helicase isolated from HeLa cells that catalytically translocates in the 3′ to 5′ direction. *J Biol Chem* 267:4398–4407.
25. Gruter P, et al. (1998) TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol Cell* 1:649–659.
26. Tang H, Wong-Staal F (2000) Specific interaction between RNA helicase A and Tap, two cellular proteins that bind to the constitutive transport element of type D retrovirus. *J Biol Chem* 275:32694–32700.
27. Lai MC, Lee YH, Tarn WY (2008) The DEAD-box RNA helicase DDX3 associates with export messenger ribonucleoproteins as well as tip-associated protein and participates in translational control. *Mol Biol Cell* 19:3847–3858.
28. Yedavalli VS, Neuveut C, Chi YH, Kleiman L, Jeang KT (2004) Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. *Cell* 119:381–392.
29. Till S, et al. (2007) A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat Struct Mol Biol* 14:897–903.

30. Ebert MS, Neilson JR, Sharp PA (2007) microRNA sponges: Competitive inhibitors of small RNAs in mammalian cells. *Nat Methods* 4:721–726.
31. Politz JC, Hogan EM, Pederson T (2009) microRNAs with a nucleolar location. *RNA* 15:1705–1715.
32. Nakajima T, et al. (1997) RNA helicase A mediates association of CBP with RNA polymerase II. *Cell* 90:1107–1112.
33. Zhou K, et al. (2003) RNA helicase A interacts with dsDNA and topoisomerase IIalpha. *Nucleic Acids Res* 31:2253–2260.
34. Park SW, et al. (2008) Regulation of the catalytic function of topoisomerase II alpha through association with RNA. *Nucleic Acids Res* 36:6080–6090.
35. Tsai SC, et al. (2000) Histone deacetylase interacts directly with DNA topoisomerase II. *Nat Genet* 26:349–353.