For additional information on DSB track clustering, we probed the γH2AX-CD tracks for Rad51, a protein involved in DSB repair by homologous recombination (*1*). The linearity of the tracks of Rad51 (Fig. 3A) and Mre11 foci (Fig. 2D) observed upon exposure to α particles demonstrates colocalization of the DNA repair proteins with DSBs. Rad51 (Fig. 3, B to E) and avidly bound Mre11 (Fig. 2, B and C) were concentrated mainly in small foci located at the interior or at the periphery of the γH2AX-CD. Moreover, we very rarely observed more than one repair protein focus in an individual γH2AX-CD. Mre11 was present in Subgroups Ia and Ib and Group II tracks in ~80% of the cells, which suggests that Mre11-mediated DSB processing occurred in all track types. By contrast, Rad51 localized to Subgroup Ib and Group II tracks but was absent from tracks that displayed clustering only (Subgroup Ia; Table 1). Possibly, DSB processing in these clusters proceeds through a Rad51-independent pathway. This is consistent with our observation that the cells containing this track morphology are in the G1 phase of the cell cycle in which Rad51 foci do not form (*21*).

To assess the influence of other DSB repair proteins on γH2AX-CD clustering, we introduced DSB tracks in Chinese hamster ovary cells deficient in one of two mechanistically distinct DSB repair pathways, homologous recombination and nonhomologous DNA end joining (*8*). Formation of both clustering (Subgroups Ia and Ib) and nonclustering (Group II) tracks was observed in these mutant cells, suggesting that the initial movement and adhesion of DSB-containing chromosome domains occurs upstream or independent of DSB repair.

Our results suggest why experiments using partial irradiation of cells with ultrasoft x-rays produced apparently immobile DSBs (*5*). Nuclei in those experiments absorbed a local dose of irradiation that was more than two orders of magnitude higher than that produced by an α-particle track. Every DSB resulting from the local x-irradiation would be embedded in a dense cloud of adhesive γH2AX-CDs that would prevent the DSB-containing chromosome domains from detectably moving. The use of lower doses delivered by local α irradiation reveals that the positions of DSB-induced γH2AX-CDs are not necessarily fixed and can move to cluster together. The clusters contain multiple γH2AX-CDs and multiple foci of the Mre11 and/or Rad51 DSB repair proteins, which supports the notion that distant DSBs can be juxtaposed. Gathering of multiple DSBs has also been observed in *Saccharomyces cerevisiae* cells (*22*). However, for yeast cells it is suggested that in S phase, multiple DSBs are recruited to repair centers containing a high concentration of repair proteins (*22*). In mammalian cells, juxtaposition of multiple DSBs occurs primarily in G1 phase, and the individual breaks already contain repair proteins. Juxtaposition of multiple DSBs, even though conserved among eukaryotes, seems potentially dangerous, because malfunctioning of repair could generate genomic rearrangements. Although chromosome translocations could also arise from the interaction of a single broken chromosome with a nondamaged chromosome (*23*), our demonstration that DSB-containing chromosome domains are mobile and can interact supports the breakage-first theory to explain the generation of translocations between two broken chromosomes.

References and Notes

1. B. Elliott, M. Jasin, *Cell. Mol. Life Sci.* **59**, 373 (2002).
2. J. R. Savage, *Science* **290**, 62 (2000).
3. A. S. Serebrovski, *Am. Nat.* **63**, 374 (1929).
4. K. Sax, *Cold Spring Harbor Symp. Quant. Biol.* **9**, 93 (1941).
5. B. E. Nelms, R. S. Maser, J. F. MacKay, M. G. Lagally, J. H. Petrini, *Science* **280**, 590 (1998).
6. M. N. Nikiforova, J. R. Stringe, R. Blough, M. Medvedovic, J. A. Fagin, Y. E. Nikiforov, *Science* **290**, 139 (2000).
7. R. M. Anderson, D. L. Stevens, D. T. Goodhead, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12167 (2002).
8. Materials and Methods are available as supporting material on *Science* Online.
9. G. W. Barendsen, *Nature* **193**, 1153 (1962).
10. W. R. Holley, I. S. Mian, S. J. Park, B. Rydberg, A. Chatterjee, *Radiat. Res.* **158**, 568 (2002).
11. B. Jakob, M. Scholz, G. Taucher-Scholz, *Radiat. Res.* **159**, 676 (2003).
12. G. L. Dianov, P. O'Neill, D. T. Goodhead, *BioEssays* **23**, 745 (2001).
13. E. P. Rogakou, C. Boon, C. Redon, W. M. Bonner, *J. Cell Biol.* **146**, 905 (1999).
14. O. A. Sedelnikova, E. P. Rogakou, I. G. Panyutin, W. M. Bonner, *Radiat. Res.* **158**, 486 (2002).
15. J. R. Chubb, S. Boyle, P. Berry, W. A. Bickmore, *Curr. Biol.* **12**, 439 (2002).
16. C. Wyman, R. Kanaar, *Curr. Biol.* **12**, R446 (2002).
17. M. de Jager, J. van Noort, D. C. van Gent, C. Dekker, R. Kanaar, C. Wyman, *Mol. Cell* **8**, 1129 (2001).
18. O. K. Mirzoeva, J. H. Petrini, *Mol. Cell. Biol.* **21**, 281 (2001).
19. J. Kobayashi *et al.*, *Curr. Biol.* **12**, 1846, (2002).
20. G. S. Stewart *et al.*, *Cell* **99**, 577 (1999).
21. S. Tashiro, N. Kotomura, A. Shinohara, K. Tanaka, K. Ueda, N. Kamada, *Oncogene* **12**, 2165 (1996).
22. M. Lisby, U. H. Mortensen, R. Rothstein, *Nature Cell Biol.* **5**, 572, 2003.
23. G. Ludwikow *et al.*, *Int. J. Radiat. Biol.* **78**, 239 (2002).
24. O. K. Mirzoeva, J. H. Petrini, *Molec. Cancer Res.* **1**, 217 (2003).

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Supporting Online Material

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Materials and Methods
Table S1
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Nuclear Export of MicroRNA Precursors

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MicroRNAs (miRNAs), which function as regulators of gene expression in eukaryotes, are processed from larger transcripts by sequential action of nuclear and cytoplasmic ribonuclease III–like endonucleases. We show that Exportin-5 (Exp5) mediates efficient nuclear export of short miRNA precursors (pre-miRNAs) and that its depletion by RNA interference results in reduced miRNA levels. Exp5 binds correctly processed pre-miRNAs directly and specifically, in a Ran guanosine triphosphate–dependent manner, but interacts only weakly with extended pre-miRNAs that yield incorrect miRNAs when processed by Dicer in vitro. Thus, Exp5 is key to miRNA biogenesis and may help coordinate nuclear and cytoplasmic processing steps.

MicroRNAs (miRNAs) are ~22-nucleotide (nt) single-stranded molecules that control post-transcriptional gene expression in eukaryotes. Several hundred distinct miRNAs exist in animals and plants (*1–5*), but few of their precise functions and mechanisms of action are known. miRNAs can silence gene expression through inactivation or degradation of mRNAs (*6–13*).

The biogenesis of miRNAs in mammalian cells involves both nuclear and cytoplasmic processing (*14*) catalyzed by ribonuclease III (RNase III)–like endonucleases that recognize double-stranded RNAs. First, nuclear Drosha cleaves long primary transcripts releasing 60- to 70-nt pre-miRNAs (*15*), which can be folded in silico into stem-loop hairpins. Then, cytoplasmic Dicer processes pre-miRNAs into ~20- to 22-nt duplexes bearing two nucleotide single-stranded 3′ extensions (*15–17*) (Fig. 1). Generally, only one strand of the duplex serves as the mature miRNA. Because the pre-miRNAs generated in the

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nucleus require further processing in the cytoplasm, nuclear export represents a critical, but hitherto uncharacterized, step in the biogenesis of miRNAs.

We studied three model human pre-miRNAs (Fig. 1A). Pre-miR-31 (57 nt) is the likely immediate precursor of miR-31, on the basis of sequence analyses of pre-miRNAs produced in vivo (*9*, *18*) or in vitro (*15*). Pre-miR-31(71) and pre-miR-22(85) are putative precursor forms with extended stem structures (*1*, *16*).

All three pre-miRNAs, synthesized in vitro (*19*), were processed by recombinant Dicer in vitro, albeit with different efficiencies, yielding 20- to 22-nt products (Fig. 1B). RNase T1 digestion of these processing products (fig. S1) showed that Dicer released RNA duplexes by cutting ~20 to 22 nt from the bottom of the stem (Fig. 1A), consistent with cleavage patterns of perfectly double-stranded RNA substrates (*20*). Notably, pre-miR-31, with the miR-31 sequence at its very 5′ end, yielded the correct mature miRNA, whereas the two extended pre-miRNAs did not.

Export of pre-miRNAs from *Xenopus* oocyte nuclei was rapid, with >90% of the RNAs accumulating in the cytoplasm within 30 min after nuclear injection (Fig. 2A, panel 1). All RNAs appeared to use the same saturable export pathway, as they competed specifically, in a dosage-dependent manner, for export of pre-miRNA but not for export of U1ΔSm or tRNA (panels 2 to 4; fig. S2A). Thus, pre-miRNA export is carrier-mediated but unlikely to use the nuclear export receptors CRM1 or Exportin-t (Exp-t) (*21*).

The affinities of most substrate-specific exportins for export cargos are greatly enhanced by complex formation with Ran guanosine triphosphate (RanGTP) (*21*). When RanGTP was depleted as a result of inhibition of the Ran guanine nucleotide exchange factor (*22*), export of pre-miRNA was greatly reduced (Fig. 2B), indicating that it is mediated by a RanGTP-binding export receptor.

All three pre-miRNA species were stable in the cytoplasm for several hours and were not processed into miRNA-sized products (*23*). This stability and lack of detectable processing suggests that oocyte cytoplasm is deficient in Dicer activity. Likewise, no RNA fragments resembling Dicer processing products were detected in the nucleus (*23*). However, when confined to the nucleus, both pre-miR-31(71) and pre-miR-22(85), but not pre-miR-31, gave rise to slightly shorter degradation products (Fig. 2, A and B).

Our competition experiments indicate that pre-miRNA uses an export receptor other than CRM1 or Exp-t. Exportin-5 (Exp5) (*24*) is a good candidate because it exports small, structured, minihelix-containing RNAs (*25*) and, less efficiently, tRNAs (*26*). As predicted, export of ^{32}P-pre-miR-31, which was competed by excess unlabeled pre-miRNA, was effectively restored by exogenous Exp5 but not by CRM1 or Exp-t (Fig. 2C, panels 3 to 5; fig. S2B). Thus, pre-miRNA export is specifically stimulated by Exp5.

To determine whether Exp5 can bind pre-miRNAs directly in the absence of adaptor proteins, we assayed for formation of complexes between ^{32}P-pre-miR-31 and recombinant Exp5 (Fig. 3A). As expected for an exportin/cargo interaction, the production of a stable complex was greatly enhanced by RanGTP and was competed by unlabeled pre-miR-31 (lanes 3 to 5). Complex formation was specific for Exp5, because neither Exp-t nor CRM1 bound pre-miR-31 (lanes 6 to 9). The direct binding of Exp5 to pre-miRNAs shows that adaptor proteins are not required for complex formation, but additional proteins might still promote interactions in vivo.

The Exp5 complex can accommodate any of the three pre-miRNAs, but with varying efficiencies (Fig. 3B). Pre-miR-31 was the strongest competitor of both complex formation (panels 2 to 4) and pre-miRNA export (Fig. 2A; fig. S2, A and B), and pre-miR-22(85) was the weakest. Unrelated small, structured RNAs competed poorly for Exp5 complex formation (Fig. 3B, panels 5 and 6).

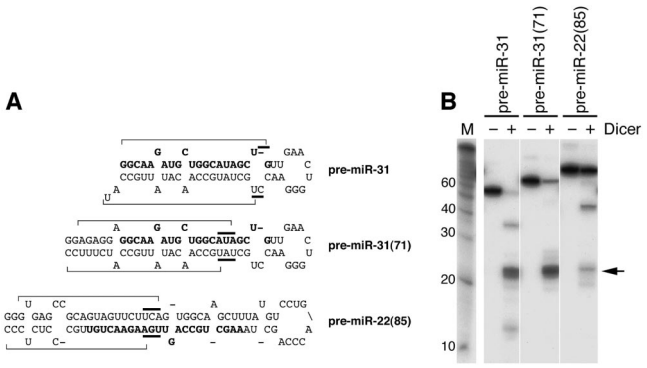

Fig. 1. Pre-miRNA substrates for nuclear export and processing. (**A**) Pre-miR-31 has the 5′ end of mature miR-31 and 2 nt 3′ overhang. Pre-miR-31(71) and pre-miR-22(85) contain extended duplexes predicted from human genomic sequences (*1*). Sequences of mature miRNAs are bolded. Thick bars and brackets indicate approximate in vitro Dicer cleavage sites. (**B**) Products of in vitro Dicer cleavage of ^{32}P-labeled pre-miRNA substrates. Arrow indicates ~22-nt products. See fig. S1 for detailed analyses.

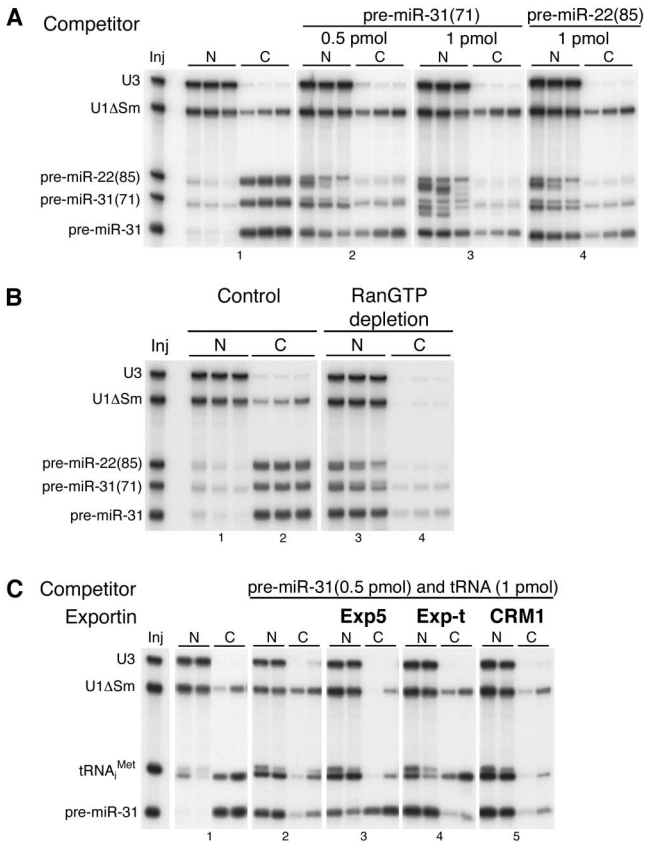

Fig. 2. Receptor-mediated, RanGTP-dependent pre-miRNA export. (**A**) A mixture of ^{32}P-labeled pre-miR-31, pre-miR-31(71), pre-miR-22(85), and U3 and U1ΔSm RNAs was injected into *Xenopus* oocyte nuclei (Inj). Export was monitored at 0.5, 1, and 2 hours in the absence (panel 1) or presence (panels 2 to 4) of unlabeled competitor pre-miRNAs. (**B**) Export of pre-miRNAs was monitored as in (A) in oocytes depleted of RanGTP upon preinjection of RanT24N. (**C**) Export of ^{32}P-labeled tRNA$_i^{Met}$ and pre-miRNA-31 (panel 1) was saturated by coinjection of unlabeled competitor RNAs (panels 2 to 5), and restoration of export was monitored after nuclear preinjection of recombinant export receptors (panels 3 to 5). RNA export was analyzed at 0.5 and 1 hour after RNA injection.

The selectivity of the interaction between pre-miRNA and Exp5 was demonstrated in total HeLa cell extract. Two complexes containing ^{32}P-pre-miR-31 were observed by electrophoretic mobility shift assay (EMSA) (Fig. 3C). The slower migrating complex (lane 3), which resembles the one formed with purified factors (lane 2), was supershifted by Exp5-specific but not unrelated control antibodies (lanes 4 and 5), demonstrating that it contained Exp5. The faster migrating complex (*) likely contains the nuclear antigen La, because its formation was competed by U6 small nuclear RNA (lane 7) and NL15 RNA (*23*), both of which bind La strongly (*27*, *28*). This complex does not contain Exp5 (lane 4) and probably is not involved in export. Formation of both complexes was competed by unlabeled pre-miR-31 (lane 6).

The highly specific interactions between Exp5 and pre-miRNAs implicate this exportin as an essential factor in miRNA biogenesis. If that is the case, depletion of cellular Exp5 by RNA interference (RNAi) should decrease the levels of mature miRNAs. Treatment of HeLa cells with three different Exp5-specific short interfering RNAs (siRNAs), but not control siRNAs against CRM1 or Exp-t, effectively reduced the levels of Exp5 (Fig. 4A, fig. S4). Cells depleted of Exp5 were also impaired for Exp5 function (Fig. 4B), as indicated by the nuclear accumulation of GFP-NLS-eEF1A (*29*). Normally, this reporter protein is exported to the cytoplasm by Exp5, using aminoacylated tRNA as an adaptor (*26*, *29*).

Upon depletion of Exp5 by RNAi for 48 to 72 hours, the levels of let-7a-1 and other mature miRNAs were reduced by 40 to 60% (Fig. 4C) (*23*). The residual levels of miRNA may indicate that low amounts of Exp5 are sufficient for continued pre-miRNA export (and hence miRNA production) or that mature miRNAs have long half-lives. The latter possibility is consistent with observations of comparable reductions of miRNA levels after 3 days of RNAi against Drosha or Dicer (*15*, *23*). Thus, our results demonstrate a direct and central role of Exp5 in miRNA biogenesis and offer a possible explanation for the extensive developmental defects observed in strains of *Arabidopsis thaliana* carrying loss-of-function mutations in the ortholog of Exp5, HASTY (*30*).

RNAs that interact with Exp5 all have a high degree of double-stranded character (*25*) (Fig. 1). The slightly shorter pre-miRNAs that accumulated in nuclei when export was saturated (Fig. 2A) were poorly exported even when excess Exp5 was provided (fig. S2B). It is unclear whether this inefficient export results from changes in RNA secondary structure or from loss (or gain) of terminal nucleotides or phosphate groups. Variant

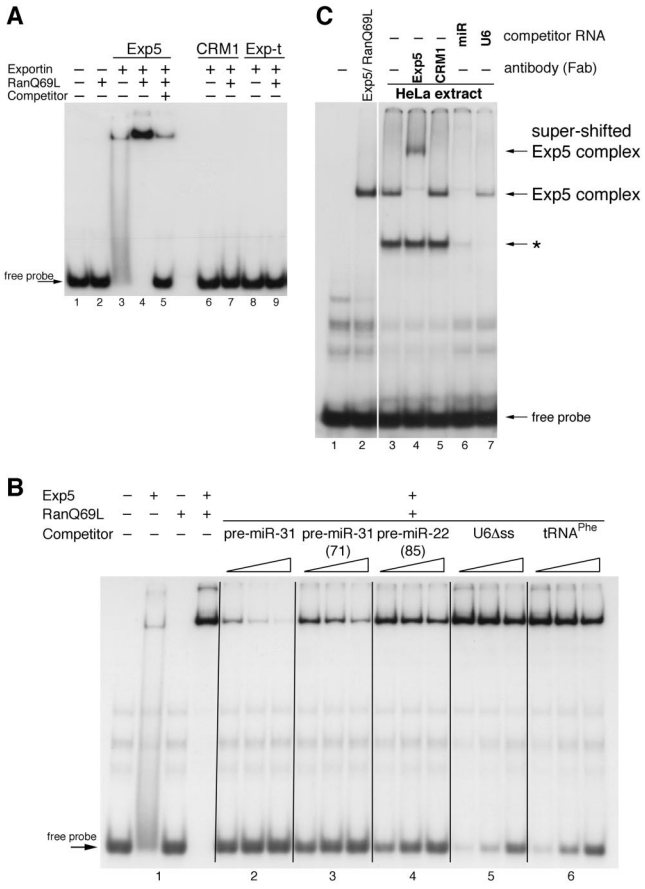

Fig. 3. Direct and specific binding of pre-miRNA-31 to Exp5. (**A**) Complexes formed between ^{32}P-pre-miR-31, recombinant 2z-tagged export receptors (2 μM), and RanQ69LGTP (5 μM) were analyzed by EMSA. Unlabeled competitor pre-miR-31 (lane 5) was present at 2.5 μM. (**B**) EMSA of ^{32}P-pre-miR-31 in the absence (panel 1) or presence (panels 2 to 6) of 1, 2, or 4 μM of the indicated unlabeled competitor RNAs. (**C**) EMSA of ^{32}P-pre-miR-31 incubated with HeLa cell extract plus RanQ69LGTP (lanes 3 to 7). Pre-miR-31/N-His-Exp5/RanQ69LGTP complex was a mobility marker (lane 2). Fab fragments of monospecific antibodies to human Exp5 or CRM1, or unlabeled competitor pre-miR-31 or U6Δss RNAs, were included as indicated (lanes 4 to 7).

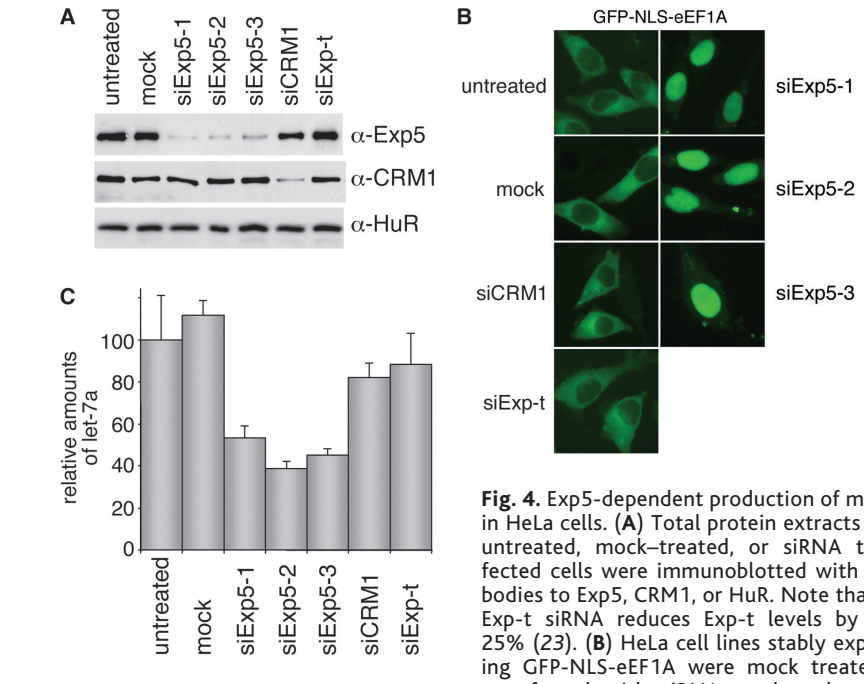

Fig. 4. Exp5-dependent production of miRNA in HeLa cells. (**A**) Total protein extracts from untreated, mock–treated, or siRNA transfected cells were immunoblotted with antibodies to Exp5, CRM1, or HuR. Note that the Exp-t siRNA reduces Exp-t levels by only 25% (*23*). (**B**) HeLa cell lines stably expressing GFP-NLS-eEF1A were mock treated or transfected with siRNAs and analyzed by fluorescence microscopy 48 hours later. (**C**) Total cellular RNAs from untreated, mock–treated, or siRNA transfected cells (52 hours) were assayed for levels of let-7a RNA and normalized to a constant amount of glyceraldehyde phosphate dehydrogenase mRNA with RNA Invader assays (Third Wave Technologies Inc., Madison, WI) (*19*). Error bars indicate the standard deviations of triplicate assays. Depletion of Exp5 resulted in similar reductions of let-7a RNA levels in eight independent RNAi experiments.

pre-miRNA-31 with an unpaired 5′ extension was greatly impaired for export and Exp5-binding (fig. S3, B and C) (*23*), indicating that the precise ends of pre-miRNAs generated in vivo by Drosha (*15*) may contribute both to efficient export and to correct cytoplasmic processing by Dicer (Fig. 1B) (*23*).

The direct binding of RNAs to Exp5 allows aminoacylated tRNAs to function as export adaptors for eEF1A (Fig. 4B) (*26*, *29*). Because tRNAs can be exported by both Exp5 and Exp-t, whereas pre-miRNAs are exported only by Exp5 (Fig. 2C), tRNA can compete for export of pre-miRNA (fig. S2C), but not vice versa (fig. S2A).

The spatial separation, and hence sequential action, of Drosha and Dicer, which are localized in the nucleus and cytoplasm of mammalian cells, respectively, appears to promote the correct and efficient processing of precursors in the generation of mature miRNAs (*15*). Exp5, which functions in the middle of this pathway, may facilitate miRNA biogenesis by monitoring the integrity of pre-miRNAs and by promoting efficient release of pre-miRNAs from Drosha in the nucleus, where the level of RanGTP is high. Conversely, in the cytoplasm, where the level of RanGTP is low, Exp5 would release pre-miRNAs to Dicer for further processing.

References and Notes

1. M. Lagos-Quintana, R. Rauhut, W. Lendeckel, T. Tuschl, *Science* **294**, 853 (2001).
2. N. Lau, L. Lim, E. Weinstein, D. Bartel, *Science* **294**, 858 (2001).
3. R. Lee, V. Ambros, *Science* **294**, 862 (2001).
4. Z. Mourelatos *et al.*, *Genes Dev.* **16**, 720 (2002).
5. B. Reinhart, E. Weinstein, M. Rhoades, B. Bartel, D. Bartel, *Genes Dev.* **16**, 1616 (2002).
6. M. Carmell, Z. Xuan, M. Zhang, G. Hannon, *Genes Dev.* **16**, 2733 (2002).
7. E. Moss, R. Poethig, *Curr. Biol.* **12**, R688 (2002).
8. D. Dykxhoorn, C. Novina, P. Sharp, *Nature Rev. Mol. Cell Biol.* **4**, 457 (2003).
9. R. Lee, R. Feinbaum, V. Ambros, *Cell* **75**, 843 (1993).
10. D. Banerjee, F. Slack, *Bioessays* **24**, 119 (2002).
11. A. Pasquinelli, G. Ruvkun, *Annu. Rev. Cell Dev. Biol.* **18**, 495 (2002).
12. J. Carrington, V. Ambros, *Science* **301**, 336 (2003).
13. C. Kidner, R. Martienssen, *Trends Genet.* **19**, 13 (2003).
14. Y. Lee, K. Jeon, J. Lee, S. Kim, V. Kim, *EMBO J.* **21**, 4663 (2002).
15. Y. Lee *et al.*, *Nature* **425**, 415 (2003).
16. G. Hutvagner *et al.*, *Science* **293**, 834 (2001).
17. R. Ketting *et al.*, *Genes Dev.* **15**, 2654 (2001).
18. Y. Zeng, B. Cullen, *RNA* **9**, 112 (2003).
19. Materials and Methods are available as supporting material on *Science* Online.
20. H. Zhang, F. Kolb, V. Brondani, E. Billy, W. Filipowicz, *EMBO J.* **21**, 5875 (2002).
21. H. Fried, U. Kutay, *Cell. Mol. Life Sci.* **60**, 1659 (2003).
22. E. Izaurralde, U. Kutay, C. von Kobbe, I. Mattaj, D. Görlich, *EMBO J.* **16**, 6535 (1997).
23. E. Lund *et al.*, unpublished data.
24. A. Brownawell, I. Macara, *J. Cell Biol.* **156**, 53 (2002).
25. C. Gwizdek *et al.*, *J. Biol. Chem.* **278**, 5505 (2003).
26. A. Calado, N. Treichel, E. Müller, A. Otto, U. Kutay, *EMBO J.* **21**, 6216 (2002).
27. M. Terns, E. Lund, J. Dahlberg, *Mol. Cell Biol.* **12**, 3032 (1992).
28. C. Grimm, E. Lund, J. Dahlberg, *EMBO J.* **16**, 793 (1997).
29. M. Bohnsack *et al.*, *EMBO J.* **21**, 6205 (2002).
30. K. Bollman *et al.*, *Development* **130**, 1493 (2003).

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Coordinated Activation of Hsp70 Chaperones

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Hsp70s are a ubiquitous family of molecular chaperones involved in many cellular processes. Two Hsp70s, Lhs1p and Kar2p, are required for protein biogenesis in the yeast endoplasmic reticulum. Here, we found that Lhs1p and Kar2p specifically interacted to couple, and coordinately regulate, their respective activities. Lhs1p stimulated Kar2p by providing a specific nucleotide exchange activity, whereas Kar2p reciprocally activated the Lhs1p adenosine triphosphatase (ATPase). The two ATPase activities are coupled, and their coordinated regulation is essential for normal function in vivo.

The chaperone function of an Hsp70 protein requires an intrinsic ATPase activity that is enhanced by a DnaJ-like partner and modulated by a nucleotide exchange factor (*1*). The yeast endoplasmic reticulum (ER) contains two distinct Hsp70s, Kar2p and Lhs1p, that are conserved throughout eukaryotes (*2–4*). Evidence suggests that these two chaperones functionally overlap because mutation of either one alone results in defects in common pathways, including protein translocation into the ER and protein folding in the ER lumen (*2*, *3*, *5–9*). Kar2p has been extensively studied, and its interactions with the J-partner, Sec63p, and the nucleotide exchange factor, Sil1p, have been examined (*10–15*). However, the biochemical role of Lhs1p has remained elusive, and no co-chaperones have been identified.

To understand the molecular role of Lhs1p, we sought to identify putative co-chaperones. The Kar2p ATPase is regulated by the co-chaperones Sec63p (*13*) and Sil1p (*14–16*), and therefore we investigated whether Lhs1p might also be stimulated by these same proteins. Kar2p exhibited a low basal activity that was stimulated about eightfold by the soluble J-domain of Sec63p (Fig. 1A). This activity was further enhanced to 14-fold basal activity by the addition of Sil1p (Fig. 1A). Unlike Kar2p, Lhs1p had no measurable ATPase activity and was unaffected by the presence of either the J-domain, Sil1p, or both in combination (Fig. 1A). Because Sil1p is the only known nucleotide exchange factor in the ER lumen, we also examined its ability to physically interact with Lhs1p. We incubated a native ER lumenal extract with immobilized recombinant Sil1p and found that Kar2p bound, as expected, but that Lhs1p did not (Fig. 1B). Thus, Lhs1p and Kar2p do not share a common complement of co-chaperones.

We next sought to identify candidate co-chaperones for Lhs1p by adapting the binding approach above. Recombinant Lhs1p was immobilized and incubated with a detergent-solubilized microsomal extract. A single major interacting species of 75 kD was observed (Fig. 1C) and identified as Kar2p by immunoblotting. This interaction was direct because purified recombinant Kar2p also bound immobilized Lhs1p (fig. S1). Native forms of Kar2p and Lhs1p also interacted in yeast microsomal extract, and this increased after cross-linking (Fig. 1D). No Sil1p was coprecipitated either in the presence or absence of cross-linker (Fig. 1D). Thus, we have found a specific interaction between Lhs1p and Kar2p that is labile, at least in vitro, but that can be trapped by photochemical cross-linking. Our results demonstrate that both Sil1p and Lhs1p can bind Kar2p but suggest that they do so in a mutually exclusive manner.

Does this interaction have any effect upon ATPase activity? When Kar2p (K) and Lhs1p (L) were combined, their overall ATPase activity was similar to that of Kar2p alone (Fig. 1E). However, the further addition of the J-domain (J) led to a total activity >30-fold over the basal level of Kar2p. The combined activity for LKJ

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