MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms

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MicroRNAs (miRNAs) are endogenously encoded small noncoding RNAs, derived by processing of short RNA hairpins, that can inhibit the translation of mRNAs bearing partially complementary target sequences. In contrast, small interfering RNAs (siRNAs), which are derived by processing of long double-stranded RNAs and are often of exogenous origin, degrade mRNAs bearing fully complementary sequences. Here, we demonstrate that an endogenously encoded human miRNA is able to cleave an mRNA bearing fully complementary target sites, whereas an exogenously supplied siRNA can inhibit the expression of an mRNA bearing partially complementary sequences without inducing detectable RNA cleavage. These data suggest that miRNAs and siRNAs can use similar mechanisms to repress mRNA expression and that the choice of mechanism may be largely or entirely determined by the degree of complementarity of the RNA target.

Considerable evidence now indicates that small noncoding RNAs can play a major role in regulating eukaryotic gene expression (reviewed in refs. 1 and 2). Of particular interest are a class of ≈ 22 -nt RNAs that can be further subdivided into small interfering RNAs (siRNAs) and microRNAs (miRNAs) (3). siRNAs are derived from long, double-stranded RNAs that are transcribed endogenously or introduced into cells by viral infection or transfection (3–6). siRNA duplexes are produced by processing of these longer double-stranded RNAs by the unusual Dicer ribonuclease (7, 8), and one strand of the duplex is then incorporated into a ribonucleoprotein complex, the RNA-induced silencing complex (RISC) (3, 9, 10). The siRNA component guides RISC to mRNA molecules bearing a homologous antisense sequence, resulting in cleavage and degradation of that mRNA (9, 10). This process is termed RNA interference (11). Preformed, synthetic siRNAs can also participate in RNA interference when introduced into human cells by transfection (12).

In contrast to siRNAs, miRNAs are encoded within the host genome as one arm of an ≈ 70 -nt RNA stem-loop structure termed a pre-miRNA (3, 13–15). Like siRNAs, mature miRNAs are dependent on Dicer for appropriate processing (16–18) and are also incorporated into a ribonucleoprotein complex (19). Although it remains unclear whether the protein components of this miRNA complex are identical to those present in RISC, evidence has been presented arguing that three proteins, termed eIF2C2, Gemin4, and Gemin3, are present in both complexes (20).

Although >100 different miRNAs have now been identified, their functions remain largely unknown. However, two miRNAs encoded by the nematode *Caenorhabditis elegans*, termed *let-7* and *lin-4*, have been shown to play an important role in nematode development by inhibiting the translation of target mRNAs in a stage-specific manner (21–23). This translational inhibition is dependent on target sequences, found in the 3' UTR of these mRNAs, that display partial homology to *let-7* or *lin-4*. Specifically, unlike the target sequences for siRNAs, these miRNA targets contain central mismatches that would be predicted to give rise to an RNA bulge on miRNA binding (21, 23, 24).

An important question is whether the fate of mRNAs targeted by siRNAs and miRNAs are necessarily distinct or whether the

observed differences instead reflect the degree of homology to the RNA target sequence. Evidence derived from the *C. elegans* system suggesting that different host gene products are required for siRNA-mediated RNA interference vs. miRNA-mediated translational repression (17, 25) suggests that siRNAs and miRNAs may not be functionally identical. Conversely, evidence obtained in plants, documenting the miRNA-mediated destruction of endogenous mRNAs bearing fully homologous RNA targets (26–28), would indicate that siRNAs and miRNAs may indeed be functionally interchangeable. In this article, we demonstrate that human miRNAs are able to induce the degradation of mRNAs bearing fully complementary target sites when produced endogenously or overexpressed. Conversely, an artificial siRNA is shown to induce the translational repression of an mRNA bearing bulged target sites. These *in vivo* data support the hypothesis that siRNAs and miRNAs may be functionally interchangeable, at least in cultured human cells.

Methods

Plasmids and siRNAs. Plasmids pCMV-miR-30, pCMV-miR-21, and pBC12/cytomegalovirus (CMV)/ β -galactosidase (β -gal) have been described (29). pCMV-miR-30(B, bulge) is identical to pCMV-miR-30 except for two 3-nt mutations that change the central region of the predicted miR-30 pre-miRNA stem (see below). Indicator plasmids pCMV-luc-Target [Target being miR-30(B), miR-30(AB), miR-30(P), miR-30(AP), miR-21(B), miR-21(P), dNxt(B), dNxt(P), or random; AB, anti-miR-30 bulge; P, perfect; AP, anti-miR-30 perfect (Fig. 1A)] were made by combining oligos encoding two copies of the Target sequence and inserting them after the luciferase (*luc*) stop codon in pCMV-luc (29). At least a 2-bp separation was introduced between adjacent target sequences. All plasmids were sequenced to verify the inserted targets. A PCR-amplified chloramphenicol acetyl transferase (CAT) expression cassette (Fig. 1B) was then cloned into the unique *StuI* site present in each pCMV-luc-Target intermediate. The synthetic dNxt and dTap siRNAs were obtained from Dharmacon, annealed, and stored as 20 μ M stocks.

Transfections and Reporter Assays. Transfections were performed in triplicate in 24-well plates. FuGENE 6 (Roche) was used to transfect plasmids into 293T cells. Each well received 10 ng of pCMV-luc-Target-CAT, 8 ng of pBC12/CMV/ β -gal, and 400 ng of pCMV-miR-30 and/or pCMV-miR-21. For transfections involving both plasmids and siRNAs, Lipofectamine 2000 (Invitrogen) was used. Each well received 15 ng of pCMV-luc-Target, 8 ng of pBC12/CMV/ β -gal, 0.2 ng of pRL-CMV (Promega), and 40 pmol of the dNxt and/or dTap siRNA. From 36 to 44 h later, one well of cells was lysed and assayed for firefly luciferase and either CAT or *Renilla* luciferase (24). RNAs were isolated from the remaining two wells by using TRIzol Reagent

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Abbreviations: CAT, chloramphenicol acetyl transferase; miRNAs, microRNAs; RISC, RNA-induced silencing complex; siRNAs, small interfering RNAs; β -gal, β -galactosidase; CMV, cytomegalovirus; B, bulge; P, perfect; AP, anti-miR-30 perfect; AB, anti-miR-30 bulge.

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Fig. 1. Indicator construct design. (A) Sequences of the synthetic RNA targets used in this study and their predicted pairing with the miR-30, anti-miR-30, or miR-21 miRNA or the dNxt siRNA. Target sequences were either P complementary or were designed to form a central B. A random sequence, for which no complementary small RNA is known to exist, was used as a control. (B) Structure of the pCMV-luc-Target and pCMV-luc-Target-CAT indicator constructs. The Targets, represented by black boxes, are eight tandem repeats of one of the sequences shown in A. PA, polyadenylation signal.

(Invitrogen) or RNAeasy kits (Qiagen). Northern blotting was performed for at least two independent transfections, as described (29), using a probe derived from the *luc* ORF. The membranes were first hybridized with a *luc* probe, stripped, and then probed for β -gal mRNA.

Results

Previously, we have demonstrated that an indicator gene can be translationally repressed in human cells on overexpression of the human miR-30 miRNA, if the cognate mRNA bears four tandem copies of a bulged RNA target sequence in the 3' UTR (30). The similar indicator constructs used in this study are based on the firefly luciferase indicator gene and contain eight RNA target sites tandemly arrayed in the 3' UTR (Fig. 1B). This number is comparable to the seven target sites for the *lin-4* miRNA found in the *lin-14* mRNA 3' UTR in *C. elegans* (21, 24) and was chosen in the hope of maximizing the phenotype of low levels of endogenously expressed miRNAs. The introduced target sites were either perfectly (P) homologous to the miRNAs or siRNAs used in this study, or they contained a predicted central mismatch or B (Fig. 1A). An internal control is critical for the experiments described, and initial experiments therefore involved cotransfection of indicator constructs equivalent to pCMV-luc-Target (Fig. 1B) with a control plasmid encoding *Renilla* luciferase. In light of recent data suggesting that miRNAs can modulate the chromatin composition of genes bearing homologous DNA sequences (31), we also constructed a second set of analogous indicator constructs, termed pCMV-luc-Target-CAT, in which the *cat* gene was expressed from a cassette present on the same plasmid (Fig. 1B). Closely similar data were obtained by using either set of indicator plasmids.

Overexpressed Human miRNAs Can Induce mRNA Cleavage. Although most miRNAs are expressed as single-stranded RNAs derived

Fig. 2. Biological activity of the miR-30 and anti-miR-30 miRNAs. (A) The level of expression of miR-30, anti-miR-30, and of miR-21 in mock-transfected 293T cells, or in 293T cells transfected with the indicated miRNA expression plasmids, was determined by primer extension. (B) The luc enzyme activities detected in 293T cell cultures transfected with the listed indicator and effector plasmids, as well as the pBC12/CMV/ β -gal control plasmid, were determined ≈ 40 h after transfection and then adjusted based on minor variations observed in the CAT internal control. These values are presented normalized to the culture transfected with pCMV-luc-random-CAT and pCMV-miR-21, which was arbitrarily set at 1. Average of three independent experiments with SD indicated. The number of nanograms of each miRNA expression plasmid transfected into each culture is indicated. (C) Parallel Northern analysis to detect the luc-reporter mRNA (Upper) and the control β -gal mRNA (Lower). Shown above Upper are the amounts of pCMV-miR-30 or pCMV-miR-21 transfected per culture. The level of luc enzyme activity detected for each indicator construct is given as a percentage of the level obtained on cotransfection with the pCMV-miR-21 control plasmid. Lane 1, RNA from mock-transfected 293T cells. The arrow indicates the position of the ≈ 1.8 -kb luc mRNA cleavage product.

from one arm of the pre-miRNA stem-loop structure, a small number of pre-miRNAs give rise to detectable levels of a miRNA derived from both arms (14, 19). One such miRNA is human miR-30, and its antisense form anti-miR-30, both of which have been detected in human cells (13, 19). Previously, we have reported that human 293T cells do not express detectable miR-30, but do express low levels of anti-miR-30 (Fig. 2A, lanes 1 and 3) (30). Transfection of 293T cells with pCMV-miR-30, which encodes the miR-30 pre-miRNA stem-loop structure contained within a longer transcript, results in overexpression of anti-miR-30 and in the production of readily detectable levels of miR-30 (Fig. 2A, lanes 2 and 4) (29, 30).

To assess the biological activity of these miRNAs, we transfected 293T cells with indicator constructs analogous to pCMV-luc-Target-CAT (Fig. 1B) containing eight copies of a target

miRNA was expressed at levels comparable to miR-30 in transfected cells (Fig. 3B and data not shown). Functional comparison of miR-30(B) with miR-30 showed that neither miRNA had a significant effect on the level of luciferase activity produced by a cotransfected pCMV-luc-random-CAT indicator construct (Fig. 3C). Although both pCMV-miR-30 and pCMV-miR-30(B) specifically inhibited luciferase expression from the cotransfected pCMV-luc-miR-30(P)-CAT and pCMV-luc-miR-30(B)-CAT indicator constructs, miR-30 was a more effective inhibitor of the former whereas the miR-30(B) miRNA was, as predicted, a more effective inhibitor of the latter (Fig. 3C). Northern analysis demonstrated, as also previously shown in Fig. 2C, that overexpression of WT miR-30 resulted in the production of a *luc* mRNA breakdown product in cells cotransfected with pCMV-luc-miR-30(P)-CAT (Fig. 3D, lane 3) but not in cells cotransfected with pCMV-luc-miR-30(B)-CAT (Fig. 3D, lane 5). In contrast, expression of the artificial miR-30(B) miRNA induced the production of the truncated *luc* RNA only on cotransfection with pCMV-luc-miR-30(B)-CAT (Fig. 3D, lane 6) whereas the *luc* mRNA encoded by pCMV-luc-miR-30(P)-CAT was unaffected (lane 4). In each case, the appearance of the truncated *luc* RNA species correlated with a drop in the level of the full-length *luc* mRNA (Fig. 3D, compare lanes 3 and 6 to lanes 4 and 5). Together, these data strongly suggest that the appearance of the ≈ 1.8 -kb *luc* mRNA cleavage intermediate indeed reflects the perfect complementarity of the overexpressed miRNA and its RNA target.

Cleavage of an mRNA by an Endogenous Human miRNA. Unlike miR-30, but like the majority of miRNAs, processing of the miR-21 pre-miRNA gives rise to only one stable mature miRNA (13, 29). Although miR-21 is expressed at detectable levels in 293T cells, this miRNA (but not its putative antisense partner) can be substantially overexpressed by transfection of 293T cells with the pCMV-miR-21 expression plasmid (Figs. 2A and 3B).

Indicator constructs analogous to pCMV-luc-Target-CAT, but containing eight copies of a P or B target specific for miR-21 (Fig. 1A), were constructed and their biological activity was analyzed. As shown in Fig. 4A, these constructs behaved similarly to the equivalent constructs analyzed in Fig. 2A, in that both the B and P target sites supported specific inhibition by the cotransfected pCMV-miR-21 effector plasmid, with the perfect indicator again being somewhat more responsive. Of note, the pCMV-luc-miR-21(P)-CAT indicator construct gave rise to a quite low level of luc enzyme expression even in the absence of a cotransfected effector plasmid, thus again suggesting inhibition by endogenous miR-21 (Fig. 4A, lane 7).

Analysis of mRNA expression by Northern blot analysis revealed readily detectable levels of the ≈ 1.8 -kb *luc* mRNA cleavage intermediate in cultures transfected with the indicator construct bearing the miR-21(P) target but not the miR-21(B) target (Fig. 4B, lanes 2, 4, and 5), as previously also seen with miR-30 (Figs. 2C and 3D). Importantly, however, this cleavage product was also readily detectable, albeit at a lower level, in pCMV-luc-miR-21(P)-CAT transfected cultures that were not cotransfected with pCMV-miR-21 (Fig. 4B, lane 6). The simplest explanation for this observation is that the endogenous miR-21 miRNA is responsible for cleavage of the miR-21(P) *luc* indicator mRNA within the fully homologous target sequence. In contrast, neither endogenous nor overexpressed miR-21 was able to induce mRNA cleavage when this target bore a central mismatch (Fig. 4C, lanes 2 and 3). Similarly, the low level of endogenous anti-miR-30 miRNA (Fig. 2A) also gave rise to a low level of cleavage of the mRNA encoded by the pCMV-luc-miR-30(AP)-CAT indicator construct in some experiments, although the resultant mRNA cleavage product was present at levels only barely above background (Fig. 2C, lane 12).

Fig. 4. Biological activity of the human miR-21 miRNA. (A) This experiment was performed as described in Fig. 2B. Data shown are the average of four independent experiments. (B) Parallel Northern analysis of *luc* (Upper) and β -gal (Lower) mRNA expression. The level of *luc* enzyme activity detected with each indicator construct is given as a percentage of the level obtained on cotransfection with the pCMV-miR-30 control plasmid. The arrow indicates the position of the ≈ 1.8 -kb *luc* mRNA cleavage product.

Inhibition of mRNA Translation by a Synthetic siRNA. Having established that both overexpressed and endogenous miRNAs can cleave target mRNAs, we next wished to ask whether synthetic siRNAs can inhibit mRNA function without inducing mRNA cleavage. To address this issue, we used two synthetic siRNAs specific for mRNAs encoding the *Drosophila* Nxt and Tap proteins. Although these reagents can inhibit dNxt and dTap protein and mRNA expression in transfected *Drosophila* S2 cells (ref. 32 and data not shown), these target nucleotide sequences are not conserved in the human *Nxt* and *Tap* genes.

Indicator constructs based on pCMV-luc-Target, bearing P or B target sequences homologous to the dNxt siRNA (Fig. 1A) were transfected into 293T cells along with either the dNxt or dTap siRNA (the latter as a negative control) and a β -gal expression plasmid. As shown in Fig. 5A, both the B and P dNxt target supported specific inhibition of luc protein expression on dNxt siRNA cotransfection, although the P target was again more responsive than the B target. Analysis of *luc* mRNA expression by Northern blot revealed a drop in the level of full-length *luc* mRNA and the appearance of the predicted truncated *luc* mRNA fragment in cultures transfected with the construct bearing the P dNxt target, even when inhibition of luc enzyme activity was only a relatively modest, ≈ 5 -fold (Fig. 5B, lanes 7 and 8). In contrast, an equivalent ≈ 5 -fold inhibition of the construct bearing the B dNxt target failed to give rise to any detectable truncated *luc* mRNA and indeed failed to significantly affect the level of expression the full-length *luc* mRNA (Fig. 5B, lane 5). We therefore conclude that the inhibition of luc enzyme expression seen with the indicator construct bearing the B dNxt targets is due not to cleavage and degradation of the target *luc* mRNA but rather to some form of translational inhibition.

Fig. 5. Inhibition of mRNA utilization by a synthetic siRNA. (A) Cultures were cotransfected with one of the three listed indicator plasmids together with the dNxt or dTap siRNA and the pRL-CMV and pBC12/CMV/β-gal internal control plasmids. The amount of each siRNA used is given in picomoles. Approximately 40 h after transfection, cultures were used for the dual luciferase assay or for RNA isolation. Firefly luc activities were adjusted for minor variations in the *Renilla* luc internal control and are presented normalized to the activity observed in the culture transfected with the pCMV-luc-random control plasmid and the dTap control siRNA, which was set at 1. Average of three independent experiments with 5D indicated. (B) Northern analysis of firefly luc (*Upper*) and β-gal (*Lower*) mRNA expression. The level of firefly luc enzyme activity detected for each indicator construct is given as a percentage of the level obtained with the dTap control siRNA. The arrow indicates the position of the ≈1.8-kb luc mRNA cleavage product.

Discussion

Using assays performed in cultured human cells, we present evidence demonstrating that endogenous human miR-21 miRNA, or overexpressed forms of the human miR-30 and anti-miR-30 miRNAs, can induce the cleavage of mRNAs bearing fully complementary target sites, a phenotype previously viewed as characteristic of siRNAs (Figs. 2–4). Conversely, we also demonstrate that a synthetic siRNA is able to down-regulate the expression of an mRNA bearing partially mismatched, B target sites, without inducing detectable mRNA cleavage or reducing mRNA expression levels (Fig. 5), an attribute previously viewed as characteristic of miRNAs (1). Together, these data indicate that miRNAs and siRNAs can interact identically with mRNA molecules bearing target sites of equivalent complementarity, i.e., in both cases perfect homology leads to mRNA

cleavage whereas a central bulge induces translational inhibition. These observations confirm and extend recent data documenting the specific cleavage of an artificial RNA target in a cytoplasmic extract containing the human miRNA let-7 (20) and demonstrating the translational inhibition, by an artificial siRNA, of an mRNA bearing a partially mismatched target site (33). Together, these observations are consistent with the hypothesis that mRNA degradation and mRNA translation inhibition by siRNAs or miRNAs are due to the same or very similar cellular ribonucleoprotein complexes, with the outcome being determined entirely by the level of complementarity of the mRNA target site. Conversely, mRNA degradation and translation inhibition may involve the recruitment of distinct ribonucleoprotein complexes, perhaps containing different members of the Argonaute protein family (34, 35). However, in that case these data would suggest that entry into these distinct complexes is comparably efficient for siRNAs and miRNAs, at least in human 293T cells.

Interpretation of the data presented in this article was greatly facilitated by the finding that the ≈2.3-kb luc mRNA encoded by the indicator constructs used gives rise to a stable ≈1.8-kb 5' breakdown product after siRNA- or miRNA-mediated cleavage at the introduced target sites. This RNA intermediate was invariably detected when a miRNA or siRNA encountered a fully complementary artificial target but was never seen when the target was designed with a central mismatch (Figs. 2C, 3D, 4B, and 5B). This RNA also differed from full-length luc mRNA in that only the latter was detectable by Northern analysis when a probe specific for sequences 3' to the introduced target sites was tested (data not shown). Although the stability of this mRNA fragment is clearly fortuitous, we note that others have previously detected the appearance of stable mRNA cleavage intermediates in cells treated with siRNAs (36, 37).

Although the data presented in this article demonstrate that miRNAs and siRNAs can inhibit mRNA expression by presumably identical mechanisms, it could be argued that siRNAs might still be more effective at RNA degradation than at translational inhibition, whereas miRNAs might display the converse activity. However, both for miRNAs and siRNAs we in fact observed significantly more effective inhibition of luc enzyme activity if the luc mRNA bore a fully complementary target and was therefore subject to RNA cleavage (Figs. 2B, 3C, 4A, and 5A). This could simply reflect the more efficient recruitment of miRNA- or siRNA-containing ribonucleoprotein complexes to higher affinity RNA-binding sites or could, as recently proposed, be due to the cooperative nature of translational inhibition by miRNAs (33). However, although RISC appears to function as a true RNA cleavage enzyme when presented with fully complementary RNA target sites (20), we speculate that target site mismatches that preclude cleavage, such as a central RNA bulge, may freeze RISC in place on the mismatched RNA target. In this manner, centrally mismatched RNA targets may reduce the effective concentration of their cognate RISC complex and thereby reduce the efficiency with which mRNA expression is inhibited.

- Hutvagner, G. & Zamore, P. D. (2002) *Curr. Opin. Genet. Dev.* **12**, 225–232.
- Cullen, B. R. (2002) *Nat. Immunol.* **3**, 597–599.
- Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X., Dreyfuss, G., Eddy, S. R., Griffith-Jones, S., Marshall, M., et al. (2003) *RNA* **9**, 277–279.
- Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. (2000) *Nature* **404**, 293–295.
- Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. (2000) *Cell* **101**, 25–33.
- Elbashir, S. M., Lendeckel, W. & Tuschl, T. (2001) *Genes Dev.* **15**, 188–200.
- Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001) *Nature* **409**, 363–366.

- Knight, S. W. & Bass, B. L. (2001) *Science* **293**, 2269–2271.
- Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R. & Tuschl, T. (2002) *Cell* **110**, 563–574.
- Schwarz, D. S., Hutvagner, G., Haley, B. & Zamore, P. D. (2002) *Mol. Cell* **10**, 537–548.
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998) *Nature* **391**, 806–811.
- Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) *Nature* **411**, 494–498.
- Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. (2001) *Science* **294**, 853–858.

14. Lau, N. C., Lim, L. P., Weinstein, E. G. & Bartel, D. P. (2001) *Science* **294**, 858–862.
15. Lee, R. C. & Ambros, V. (2001) *Science* **294**, 862–864.
16. Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, I., Tuschl, T. & Zamore, P. D. (2001) *Science* **293**, 834–838.
17. Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G. & Mello, C. C. (2001) *Cell* **106**, 23–34.
18. Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J. & Plasterk, R. H. (2001) *Genes Dev.* **15**, 2654–2659.
19. Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M. & Dreyfuss, G. (2002) *Genes Dev.* **16**, 720–728.
20. Hutvagner, G. & Zamore, P. D. (2002) *Science* **297**, 2056–2060.
21. Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993) *Cell* **75**, 843–854.
22. Olsen, P. H. & Ambros, V. (1999) *Dev. Biol.* **216**, 671–680.
23. Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. & Ruvkun, G. (2000) *Nature* **403**, 901–904.
24. Wightman, B., Ha, I. & Ruvkun, G. (1993) *Cell* **75**, 855–862.
25. Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Thomas, L., Fire, A. & Mello, C. C. (1999) *Cell* **99**, 123–132.
26. Llave, C., Xie, Z., Kasschau, K. D. & Carrington, J. C. (2002) *Science* **297**, 2053–2055.
27. Kasschau, K. D., Xie, Z., Allen, E., Llave, C., Chapman, E. J., Krizan, K. A. & Carrington, J. C. (2003) *Dev. Cell* **4**, 205–217.
28. Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. (2003) *Genes Dev.* **17**, 49–63.
29. Zeng, Y. & Cullen, B. R. (2003) *RNA* **9**, 112–123.
30. Zeng, Y., Wagner, E. J. & Cullen, B. R. (2002) *Mol. Cell* **9**, 1327–1333.
31. Dernburg, A. F. & Karpen, G. H. (2002) *Cell* **111**, 159–162.
32. Wiegand, H. L., Coburn, G. A., Zeng, Y., Kang, Y., Bogerd, H. P. & Cullen, B. R. (2002) *Mol. Cell. Biol.* **22**, 245–256.
33. Doench, J. G., Petersen, C. P. & Sharp, P. A. (2003) *Genes Dev.* **17**, 438–442.
34. Schwarz, D. S. & Zamore, P. D. (2002) *Genes Dev.* **16**, 1025–1031.
35. Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K. & Saigo, K. (2003) *Curr. Biol.* **13**, 41–48.
36. Gitlin, L., Karelsky, S. & Andino, R. (2002) *Nature* **418**, 320–324.
37. Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E. & Prydz, H. (2002) *Nucleic Acids Res.* **30**, 1757–1766.