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Structural Analysis of Human Argonaute-2 Bound to a Modified siRNA Guide

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Supporting Information

ABSTRACT: Incorporation of chemical modifications into small interfering RNAs (siRNAs) increases their metabolic stability and improves their tissue distribution. However, how these modifications impact interactions with Argonaute-2 (Ago2), the molecular target of siRNAs, is not known. Herein we present the crystal structure of human Ago2 bound to a metabolically stable siRNA containing extensive backbone modifications. Comparison to the structure of an equivalent unmodified-siRNA complex indicates that the structure of Ago2 is relatively unaffected by chemical modifications in the bound siRNA. In contrast, the modified siRNA appears to be much more plastic and shifts, relative to the unmodified siRNA, to optimize contacts with Ago2. Structure–activity analysis reveals that even major conformational perturbations in the 3′ half of the siRNA seed region have a relatively modest effect on knockdown potency. These findings provide an explanation for a variety of modification patterns tolerated in siRNAs and a structural basis for advancing therapeutic siRNA design.

siRNA therapeutics hold tremendous therapeutic potential to treat unmet medical needs, and several siRNA drugs are progressing in the clinic with excellent promise.[1−5] Chemically modifed siRNA is necessary to improve the pharmacokinetic properties, and numerous nucleic acid modifications have been developed to improve the properties of siRNA therapeutics.[6−8] In general, chemical modifications lead to reduced potency compared with parent unmodified siRNAs.[6,7] However, optimization of the placement of modifications in each strand can lead to derivatives with both improved potency and stability.[9−11] Commonly used modifications include 2′-*O*-(2-methoxyethyl) (2′-*O*-MOE), 2′-fluoro (2′-F), and 2′-*O*-methyl (2′-*O*-Me) sugar modifications and phosphorothioate (s) backbone modifications (Figure 1). Additionally, synthetic siRNA containing a metabolically stable (*E*)-5′-vinylphosphonate (5′-VP) modification (Figure 1) is 5−10-fold more potent than siRNA containing natural phosphate in mice.[12]

Generally, siRNAs are administered as small RNA duplexes containing two-nucleotide 3′ overhangs.[2] Upon entering a target cell, the duplex is loaded into the protein Ago2. One RNA strand (termed the "passenger") is removed and degraded, while the other strand (termed the "guide") is retained in Ago2.[13,14] Ago2 uses the guide strand to identify and cleave complementary target mRNAs.[15] How commonly used guide RNA modifications impact interactions with Ago2 is not known.

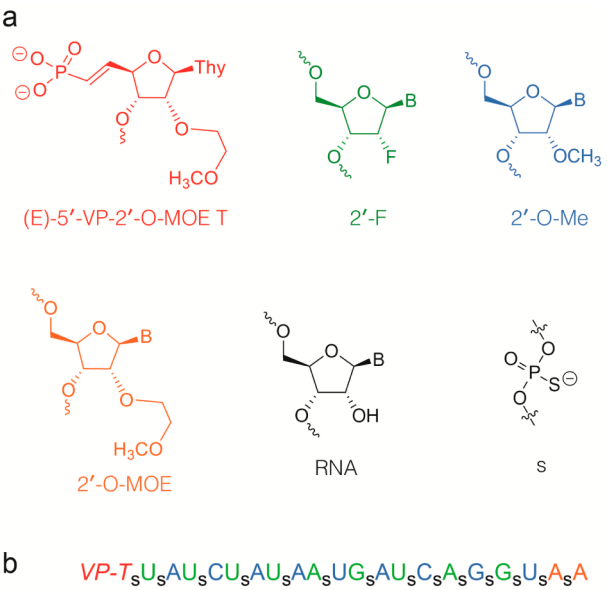

Figure 1. (a) RNA modifications used in this study. (b) Sequence and structure of modified siRNA. Legend: blue, 2′-*O*-Me; green, 2′-F; orange, 2′-*O*-MOE; *VP-T*, 2′-*O*-MOE-thymidine-(*E*)-5′-vinylphosphonate; s, phosphorothioate. All backbone linkages are as phosphodiesters except those indicated with s.

To visualize how siRNA modifications impact interactions with Ago2, we determined crystal structures of Ago2 bound to a modified siRNA and of Ago2 bound to an unmodified siRNA of the same length and nucleotide sequence. The modified siRNA contained a 5′-VP, several phosphorothioate linkages, and 2′-MOE, 2′-F and 2′-*O*-Me sugar modifications (Figure 1) and was synthesized according to a previously reported procedure.[13]

Ago2 samples were loaded with either a modified or unmodified siRNA targeting the PTEN gene, and the resulting complexes were purified and crystallized as described

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previously.[16,17] Diffraction data from both crystal forms were refined against protein atoms in the original Ago2 structure (PDB ID 4OLA) (Table S1). The conformation of Ago2 in complex with the modified siRNA was essentially identical to that of the unmodified complex (root-mean-square deviation of 0.303 Å for 735 equivalent Cα atoms), indicating that the modifications did not substantially alter the structure of Ago2 (Figure 2).

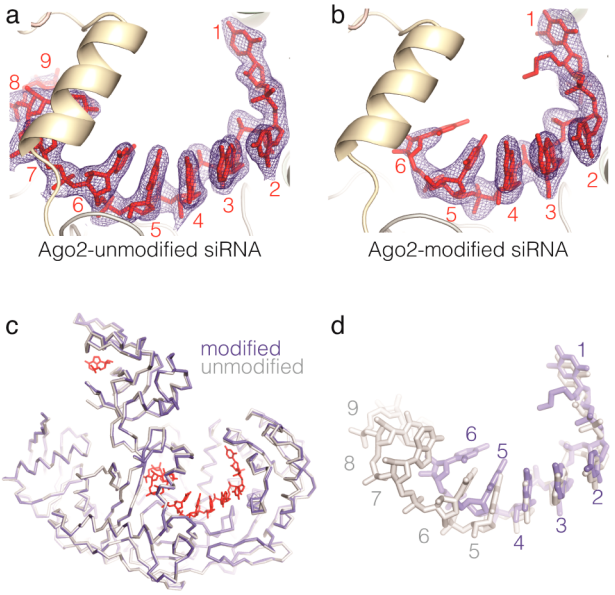

Figure 2. Structures of Ago2−siRNA complexes: (a) unmodified siRNA and (b) modified siRNA guides bound to Ago2. $2F_o - F_c$ electron density maps surrounding the siRNAs are displayed as wire meshes. (c) Superposition of the Ago2 Cα backbones from the unmodified and modified structures. Unmodified siRNA is shown in red. (d) Superposition of the unmodified and modified siRNAs. Nucleotides are numbered from the 5′ ends.

Inspection of the electron density maps revealed unambiguous density for nucleotides 1−9 and 21 of the unmodified siRNA and nucleotides 1−6 of the modified siRNA (Figure 2). Discontinuous electron density corresponding to the central and 3′ regions of the modified siRNA was also observed but could not be modeled with confidence. The well-ordered regions of both siRNAs follow a similar trajectory relative to Ago2, with the 5′ ends extending from the MID domain into the Ago2 central cleft. However, in contrast to the protein atoms in the two structures, there are pronounced differences between the modified and unmodified siRNA conformations (Figure S1).

As predicted from modeling studies,[13] the 5′-VP group of the modified siRNA bound the 5′-phosphate binding pocket at the interface of the Ago2 MID and PIWI domains[16,18−21] (Figure 3). The double-bond character of the 5′-VP group restricts rotation around the C5′−C6′ bond, and thus, the 5′-VP cannot perfectly mimic the unmodified 5′-phosphate (in which the ε dihedral angle is ∼140°). This difference appears to be accommodated by a minor shift (∼1 Å) in the position of nucleotide 1, which allows the 5′-VP to maintain the major interactions observed between Ago2 and the unmodified 5′-phosphate. Specifically, the 5′-VP oxygen atoms are within hydrogen bond/salt linkage distance (≤3.2 Å) of the Y529 hydroxyl and the K566, K533, and K570 ε-amines. The

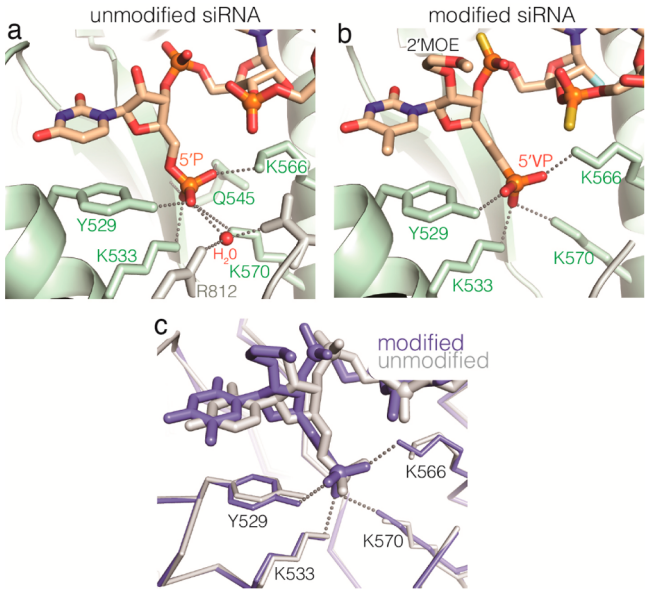

Figure 3. 5′-VP is accommodated by subtle repositioning of the 5′ nucleotide. (a, b) Close-up views of the (a) unmodified and (b) modified 5′ siRNA ends. The refined 5′-VP model lacks hydrogen bonds to the side chain of Q545, the C546 main-chain amine, and an ordered water molecule. (c) Superposition of the modified and unmodified 5′ nucleotides with surrounding protein atoms displayed.

repositioning of nucleotide 1 is subtle enough to preserve stacking interactions between the modified nucleotide 1 base and the Y529 phenolic ring. Therefore, although the 5′-VP is not identical to the unmodified siRNA, subtle repositioning of nucleotide 1 allows the majority of contacts to Ago2 to be retained.

Nucleotides 2−4 of the modified siRNA bound Ago2 in a conformation similar to their unmodified counterparts, indicating that the modifications incorporated into these nucleotides have a negligible impact on interactions with Ago2 (Figure 4). Specifically, phosphorothioate groups in the

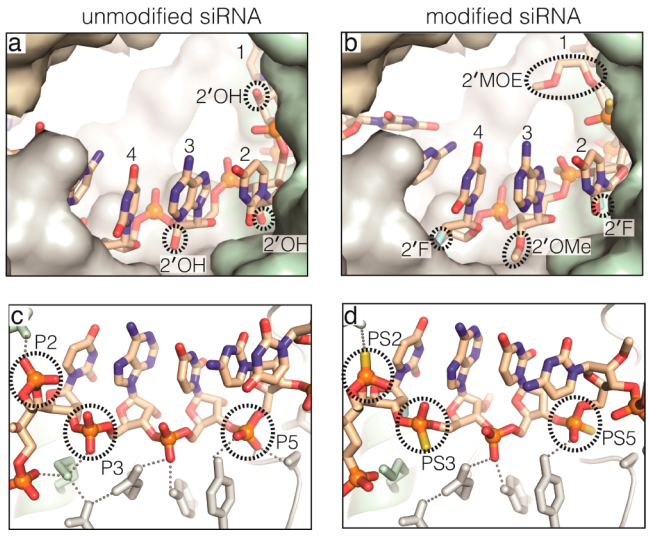

Figure 4. Modifications in nucleotides 2−4 do not impact interactions with Ago2. Views highlighting (a) and (b) the sugars and (c) and (d) the phosphate backbones in the unmodified and modified siRNA structures are shown.

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siRNA backbone (on nucleotides 2, 3, and 5) reside in the same positions as the equivalent phosphates in the unmodified siRNA. This may explain why phosphorothioates in the siRNA seed region (nucleotides 2−8) are not detrimental to silencing.[6,22] The sulfur atoms in the phosphorothioates could be modeled at either non-esterified position, indicating that Ago2 binds both enantiomeric forms at each PS position. The 2′-*O*-MOE modification on nucleotide 1 extends into the Ago2 central cleft without steric clash with the protein. Similarly, the 2′-*O*-Me group on nucleotide 3 fits into a narrow surface cleft between the Ago2MID and PIWI domains. The 2′-F atoms on nucleotides 2 and 4 occupy positions equivalent to the 2′-OH groups in the unmodified siRNA and do not directly contact Ago2. These findings may explain why an alternating pattern of 2′-*O*-Me/2′-F, with 2′-*O*-Me on the odd-numbered nucleotides, is well-tolerated in siRNAs.[23]

In contrast to nucleotides 2−4, the positions of modified nucleotides 5 and 6 deviate substantially from those in the unmodified siRNA (Figure 5). In the unmodified structure, the

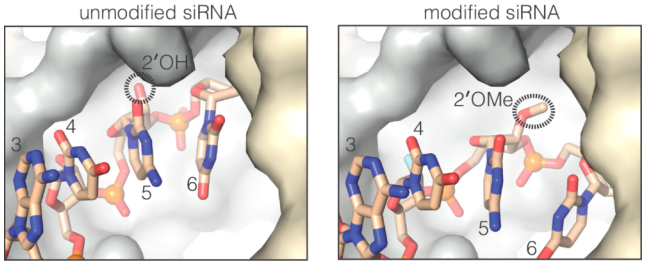

Figure 5. Repositioning of nucleotides 5−9 in the modified siRNA. The 2′-*O*-Me of nucleotide 5 shifts into the binding pocket of the ribose of nucleotide 6.

2′-OH of nucleotide 5 inserts into a small pocket on the surface of Ago2 and makes a hydrogen bond with the main-chain amide of I756.[18] In the modified siRNA, the 2′-*O*-Me of nucleotide 5 is instead inserted into a larger adjacent surface pocket, which is normally occupied by the ribose of nucleotide 6 in unmodified guide RNA structures.[18,20,21,24] Thus, the 2′-*O*-Me of nucleotide 5 appears to displace the ribose of nucleotide 6, leading to a major shift (∼6 Å) in the position of the modified siRNA compared to the unmodified one. The positional shift of modified nucleotide 6 also likely propagates to nucleotides 7−9, which are disordered in the modified siRNA structure.

Upon observing major conformational differences between modified and unmodified siRNAs in the region surrounding nucleotides 5 and 6, we wondered how these differences impact siRNA potency and what effects other modifications in this region might have on the efficiency of silencing by RNAi. To explore these questions, we measured knockdown of the PTEN gene in HeLa cells using variants of the modified siRNA with 2′ modifications at positions 5 and 6 (Table 1).[12,13] Strikingly, all possible combinations of 2′-F and 2′-*O*-Me at positions 5 and 6 led to similar levels of PTEN knockdown (Table 2). Moreover, siRNAs with unmodified nucleotides at position 5, position 6, or both were equivalently effective. In contrast, siRNAs including abasic or unlocked nucleotides at either position 5 or 6 were compromised.

Our results show how Ago2 binds a pharmacologically stable and potent siRNA containing extensive chemical modifications. Notably, modified nucleotides 1−4 bind in a conformation

Table 1. Sequences and Structures of siRNA Guide Strands[a]

siRNA No.	Guide Sequence
1	5′-*VP*-*T*$_s$U$_s$AU$_s$CU$_s$AU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
2	5′-P-T$_s$U$_s$AU$_s$CU$_s$AU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
3	5′-P-T$_s$U$_s$AU$_s$CU$_s$AU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
4	5′-P-T$_s$U$_s$AU$_s$CU$_s$AU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
5	5′-P-T$_s$U$_s$AU$_s$CUAU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
6	5′-P-T$_s$U$_s$AU$_s$CUAU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
7	5′-P-T$_s$U$_s$AU$_s$CUAU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
8	5′-P-T$_s$U$_s$AU$_s$X$_1$UAU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
9	5′-P-T$_s$U$_s$AU$_s$CX$_1$AU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
10	5′-P-T$_s$U$_s$AU$_s$X$_2$UAU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′
11	5′-P-T$_s$U$_s$AU$_s$CX$_2$AU$_s$AA$_s$UG$_s$AU$_s$C$_s$A$_s$G$_s$G$_s$U$_s$A$_s$A-3′

[a]Legend: blue, 2′-*O*-Me; green, 2′-F; orange, 2′-*O*-MOE; *VP-T*, 2′-*O*-MOE-thymidine-(*E*)-5′-vinylphosphonate; P, phosphate; s, phosphorothioate; X_1, Abasic; X_2, UNA. All backbone linkages are as phosphodiesters except those indicated with s.

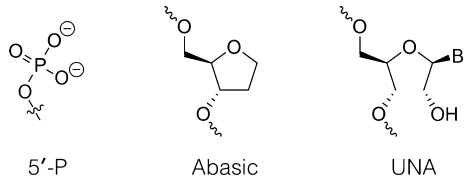

Table 2. IC_{50} Values for siRNAs Tested[a]

siRNA no.	position 5 chemistry	position 6 chemistry	siRNA IC_{50} (nM)
1	2′-*O*-Me	2′-F	2.3 ± 0.8
2	2′-*O*-Me	2′-F	1.2 ± 0.9
3	2′-F	2′-*O*-Me	2.4 ± 0.8
4	2′-F	2′-F	3.2 ± 0.7
5	RNA	2′-F	1.8 ± 0.9
6	2′-*O*-Me	RNA	2.2 ± 0.8
7	RNA	RNA	3.1 ± 0.6
8	Abasic	2′-F	7.0 ± 0.8
9	2′-*O*-Me	Abasic	12.6 ± 7.0
10	UNA	2′-F	7.2 ± 0.8
11	2′-*O*-Me	UNA	23.2 ± 7.1

[a]The siRNA duplexes were generated by pairing the guide strand with a complementary unmodified passenger strand (5′-ACCUGAUCA-UUAUAGAUAA-3′).

closely matching that of the unmodified siRNA. We suggest that this may be a critical feature of effective modified siRNAs because (1) the 5′ nucleotide serves as an anchor for siRNA binding[25] and (2) nucleotides 2−4 play a major role in initiating pairing to target RNAs.[26,27] Indeed, single-stranded (ss) siRNAs with (*Z*)-5′-vinylphosphonate, which cannot mimic the ε dihedral angle of the unmodified 5′-P, are significantly less active than ss-siRNA containing (*E*)-5′-vinylphosphonate in mammalian cells.[28] Moreover, bulky 2′-*O*-MOE modifications in nucleotides 2−4 dramatically reduce siRNA activity.[10] In contrast, the conformations of modified nucleotides 5−8 deviate substantially from those in the unmodified siRNA, revealing that strict structuring of the 3′ half of the seed region prior to target binding is not necessary for Ago2 function. Thus, these nucleotides are likely to be less sensitive to a wider range of chemical modifications. Consistent with this idea, 2′-*O*-MOE modifications are relatively well tolerated in siRNA nucleotides 6−8.[10] The combined results provide mechanistic insights into modified siRNA function and a structural basis for advancing the design of therapeutic siRNAs.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04454.

Crystallographic and refinement statistics, analytical data for guide strands, PTEN knockdown data, detailed methods, and supplemental references (PDF)

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Notes

The authors declare no competing financial interest.

Structures of the Ago2−unmodified siRNA complex and the Ago2−modified siRNA complex have been deposited in the Protein Data Bank (PDB IDs 5JS1 and 5JS2, respectively).

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■ REFERENCES

(1) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806.

(2) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494.

(3) Elbashir, S. M.; Lendeckel, W.; Tuschl, T. *Genes Dev.* **2001**, *15*, 188.

(4) Sehgal, A.; Barros, S.; Ivanciu, L.; Cooley, B.; Qin, J.; Racie, T.; Hettinger, J.; Carioto, M.; Jiang, Y.; Brodsky, J.; Prabhala, H.; Zhang, X.; Attarwala, H.; Hutabarat, R.; Foster, D.; Milstein, S.; Charisse, K.; Kuchimanchi, S.; Maier, M. A.; Nechev, L.; Kandasamy, P.; Kel'in, A. V.; Nair, J. K.; Rajeev, K. G.; Manoharan, M.; Meyers, R.; Sorensen, B.; Simon, A. R.; Dargaud, Y.; Negrier, C.; Camire, R. M.; Akinc, A. *Nat. Med.* **2015**, *21*, 492.

(5) Coelho, T.; Adams, D.; Silva, A.; Lozeron, P.; Hawkins, P. N.; Mant, T.; Perez, J.; Chiesa, J.; Warrington, S.; Tranter, E.; Munisamy, M.; Falzone, R.; Harrop, J.; Cehelsky, J.; Bettencourt, B. R.; Geissler, M.; Butler, J. S.; Sehgal, A.; Meyers, R. E.; Chen, Q.; Borland, T.; Hutabarat, R. M.; Clausen, V. A.; Alvarez, R.; Fitzgerald, K.; Gamba-Vitalo, C.; Nochur, S. V.; Vaishnaw, A. K.; Sah, D. W.; Gollob, J. A.; Suhr, O. B. *N. Engl. J. Med.* **2013**, *369*, 819.

(6) Braasch, D. A.; Jensen, S.; Liu, Y.; Kaur, K.; Arar, K.; White, M. A.; Corey, D. R. *Biochemistry* **2003**, *42*, 7967.

(7) Corey, D. R. *J. Clin. Invest.* **2007**, *117*, 3615.

(8) Deleavey, G. F.; Damha, M. J. *Chem. Biol.* **2012**, *19*, 937.

(9) Allerson, C. R.; Sioufi, N.; Jarres, R.; Prakash, T. P.; Naik, N.; Berdeja, A.; Wanders, L.; Griffey, R. H.; Swayze, E. E.; Bhat, B. *J. Med. Chem.* **2005**, *48*, 901.

(10) Prakash, T. P.; Allerson, C. R.; Dande, P.; Vickers, T. A.; Sioufi, N.; Jarres, R.; Baker, B. F.; Swayze, E. E.; Griffey, R. H.; Bhat, B. *J. Med. Chem.* **2005**, *48*, 4247.

(11) Chiu, Y. L.; Rana, T. M. *RNA* **2003**, *9*, 1034.

(12) Prakash, T. P.; Kinberger, G. A.; Murray, H. M.; Chappell, A.; Riney, S.; Graham, M. J.; Lima, W. F.; Swayze, E. E.; Seth, P. P. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 2817−2820.

(13) Schwarz, D. S.; Hutvagner, G.; Du, T.; Xu, Z.; Aronin, N.; Zamore, P. D. *Cell* **2003**, *115*, 199.

(14) Khvorova, A.; Reynolds, A.; Jayasena, S. D. *Cell* **2003**, *115*, 209.

(15) Liu, J.; Carmell, M. A.; Rivas, F. V.; Marsden, C. G.; Thomson, J. M.; Song, J.-J.; Hammond, S. M.; Joshua-Tor, L.; Hannon, G. J. *Science* **2004**, *305*, 1437.

(16) Schirle, N. T.; Sheu-Gruttadauria, J.; MacRae, I. J. *Science* **2014**, *346*, 608.

(17) Flores-Jasso, C. F.; Salomon, W. E.; Zamore, P. D. *RNA* **2013**, *19*, 271.

(18) Schirle, N. T.; MacRae, I. J. *Science* **2012**, *336*, 1037.

(19) Nakanishi, K.; Weinberg, D. E.; Bartel, D. P.; Patel, D. J. *Nature* **2012**, *486*, 368.

(20) Nakanishi, K.; Ascano, M.; Gogakos, T.; Ishibe-Murakami, S.; Serganov, A. A.; Briskin, D.; Morozov, P.; Tuschl, T.; Patel, D. J. *Cell Rep.* **2013**, *3*, 1893.

(21) Faehnle, C. R.; Elkayam, E.; Haase, A. D.; Hannon, G. J.; Joshua-Tor, L. *Cell Rep.* **2013**, *3*, 1901.

(22) Harborth, J.; Elbashir, S. M.; Vandenburgh, K.; Manninga, H.; Scaringe, S. A.; Weber, K.; Tuschl, T. *Antisense Nucleic Acid Drug Dev.* **2003**, *13*, 83.

(23) Czauderna, F.; Fechtner, M.; Dames, S.; Aygun, H.; Klippel, A.; Pronk, G. J.; Giese, K.; Kaufmann, J. *Nucleic Acids Res.* **2003**, *31*, 2705.

(24) Schirle, N. T.; Sheu-Gruttadauria, J.; Chandradoss, S. D.; Joo, C.; MacRae, I. J. *eLife* **2015**, *4*, e07646.

(25) Rivas, F. V.; Tolia, N. H.; Song, J. J.; Aragon, J. P.; Liu, J.; Hannon, G. J.; Joshua-Tor, L. *Nat. Struct. Mol. Biol.* **2005**, *12*, 340.

(26) Chandradoss, S. D.; Schirle, N. T.; Szczepaniak, M.; MacRae, I. J.; Joo, C. *Cell* **2015**, *162*, 96.

(27) Salomon, W. E.; Jolly, S. M.; Moore, M. J.; Zamore, P. D.; Serebrov, V. *Cell* **2015**, *162*, 84.

(28) Prakash, T. P.; Lima, W. F.; Murray, H. M.; Li, W.; Kinberger, G. A.; Chappell, A. E.; Gaus, H.; Seth, P. P.; Bhat, B.; Crooke, S. T.; Swayze, E. E. *Nucleic Acids Res.* **2015**, *43*, 2993.

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