Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing

JENNIFER A. BRODERICK,[1] WILLIAM E. SALOMON,[2] SEAN P. RYDER,[2] NEIL ARONIN,[3] and PHILLIP D. ZAMORE[2,4,5]

[1]Program in Neuroscience, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA
[2]Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA
[3]Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA
[4]Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA

ABSTRACT

Small RNAs loaded into Argonaute proteins direct silencing of complementary target mRNAs. It has been proposed that multiple, imperfectly complementary small interfering RNAs or microRNAs, when bound to the 3′ untranslated region of a target mRNA, function cooperatively to silence target expression. We report that, in cultured human HeLa cells and mouse embryonic fibroblasts, Argonaute1 (Ago1), Ago3, and Ago4 act cooperatively to silence both perfectly and partially complementary target RNAs bearing multiple small RNA-binding sites. Our data suggest that for Ago1, Ago3, and Ago4, multiple, adjacent small RNA-binding sites facilitate cooperative interactions that stabilize Argonaute binding. In contrast, small RNAs bound to Ago2 and pairing perfectly to an mRNA target act independently to silence expression. Noncooperative silencing by Ago2 does not require the endoribonuclease activity of the protein: A mutant Ago2 that cannot cleave its mRNA target also silences noncooperatively. We propose that Ago2 binds its targets by a mechanism fundamentally distinct from that used by the three other mammalian Argonaute proteins.

Keywords: Argonaute; cooperativity; microRNA; miRNA; RNAi; siRNA; RNA silencing

INTRODUCTION

In plants and animals, small silencing RNAs such as small interfering RNAs (siRNAs) and microRNAs (miRNAs) provide the specificity determinants for Argonaute proteins. A small RNA guide bound to an Argonaute protein is called the RNA-induced silencing complex (RISC) (Hammond et al. 2000; Hannon 2002; Du and Zamore 2007; Matranga and Zamore 2007; Ghildiyal and Zamore 2009); binding of RISC to the 3′ untranslated region (UTR) of an mRNA silences its expression (Lee et al. 1993; Wightman et al. 1993; Olsen and Ambros 1999; Lai 2002; Doench et al. 2003; Grimson et al. 2007). Argonaute proteins are structural homologs of RNase H that typically cleave their target RNAs after the nucleotide paired to the tenth base of the small RNA guide (Elbashir et al. 2001a,b; Tolia and Joshua-Tor 2007). Cleavage requires three key amino acids—D, D, H—that form a magnesium-binding catalytic triad, which promotes nucleophilic attack by hydroxide on the phosphodiester bond (Kanaya et al. 1996; Haruki et al. 2000; Martinez and Tuschl 2004; Schwarz et al. 2004; Song et al. 2004; Rivas et al. 2005).

The human genome encodes four Argonaute paralogs—Ago1, Ago2, Ago3, and Ago4—and most cultured mammalian cell lines express all four proteins, albeit in different proportions (Meister et al. 2004). Of the four mammalian Argonautes, only Ago2 retains the ability to catalyze site-specific, small RNA-directed endonucleolytic target cleavage (Liu et al. 2004; Meister et al. 2004). Like Ago2, Ago3 contains an apparent catalytic triad, but unlike Ago2, it lacks endoribonuclease activity. For Ago1 and Ago4, there is no catalytic triad, explaining their lack of endoribonuclease activity (Meister et al. 2004; Rivas et al. 2005; Azuma-Mukai et al. 2008). Extensive, but not complete, complementarity between a small RNA guide and an mRNA is required for Argonaute-catalyzed target cleavage (Hutvágner and Zamore 2002; Schwarz et al. 2002; Haley and Zamore 2004; Liu et al. 2004; Meister et al. 2004; Rivas et al. 2005). In contrast, small RNAs with only partial complementarity to their target mRNAs, especially those bearing mismatches near the cleavage site, cannot direct endonucleolytic cleavage of their

target (Holen et al. 2002) but instead reduce the stability of the target mRNA (Guo et al. 2010) and, in some conditions, cause translational repression (Doench et al. 2003; Doench and Sharp 2004).

Experimental and computational analyses suggest that a single miRNA can regulate hundreds of genes, because a target mRNA need only pair with the seed sequence of a small RNA—comprising nucleotides 2 through 7 or 8—to recruit RISC and promote repression (Lewis et al. 2003; Doench and Sharp 2004; Rajewsky and Socci 2004; Brennecke et al. 2005; Krek et al. 2005; Lewis et al. 2005; Lim et al. 2005; Grimson et al. 2007; Baek et al. 2008; Selbach et al. 2008; Friedman et al. 2009). Multiple, partially complementary small RNAs, when bound to the 3′ UTR of a luciferase reporter target mRNA, may function cooperatively to repress its translation (Doench et al. 2003; Bartel and Chen 2004), and most mRNAs contain multiple potential miRNA-binding sites in their 3′ UTRs (Lee et al. 1993; Wightman et al. 1993; Reinhart et al. 2000; Abrahante et al. 2003; Lin et al. 2003; Bartel 2004; Grimson et al. 2007; Friedman et al. 2009). However, the molecular basis for cooperativity in small RNA silencing remains unknown.

Here, we show that both the nature of siRNA:mRNA target pairing and the identity of the Argonaute protein to which the small RNA is bound determine whether multiple target sites act cooperatively to recruit RISC. Small RNAs that pair perfectly to multiple target sites silenced noncooperatively when the small RNA guide acts through Ago2, whereas silencing directed by either perfectly or imperfectly pairing small RNAs bound to Ago1, Ago3, or Ago4 acted cooperatively to silence mRNA bearing multiple small-RNA-binding sites. Cooperativity required adjacent sites. Surprisingly, noncooperative silencing by perfectly pairing small RNAs bound to Ago2 did not require target cleavage, as catalytically inactive mutant Ago2 silenced essentially as well as wild type. Finally, we find that computationally predicted modes of miRNA:target pairing required far more small RNA to achieve repression than more extensively but still incompletely paired small RNA guides. We propose that cooperative binding of RISC to multiple adjacent sites, combined with high intracellular concentrations of miRNAs, allows robust regulation of mRNA targets by Ago1, Ago3, and Ago4.

RESULTS

Experimental paradigm

At least three distinct regulatory mechanisms could explain the enhanced silencing of reporter mRNAs containing multiple miRNA-binding sites (Fig. 1). A *cooperative binding* model posits that the binding of a miRNA:Argonaute protein complex to one site increases the affinity of a second miRNA:Argonaute complex for an adjacent site (Fig. 1A). In this model, the binding of the first bulged siRNA would have a higher dissociation constant, K_D^A, than subsequent binding events, K_D^B and K_D^C; we predict that the amount of siRNA required to silence a reporter would decrease with an increasing number of target sites as cooperativity between bound Argonautes increases. Such cooperativity in small RNA-directed silencing might arise from direct interactions between adjacent Argonaute proteins. Alternatively, a pair of Argonaute proteins might be bridged by one or more additional proteins. In a *cooperative function*
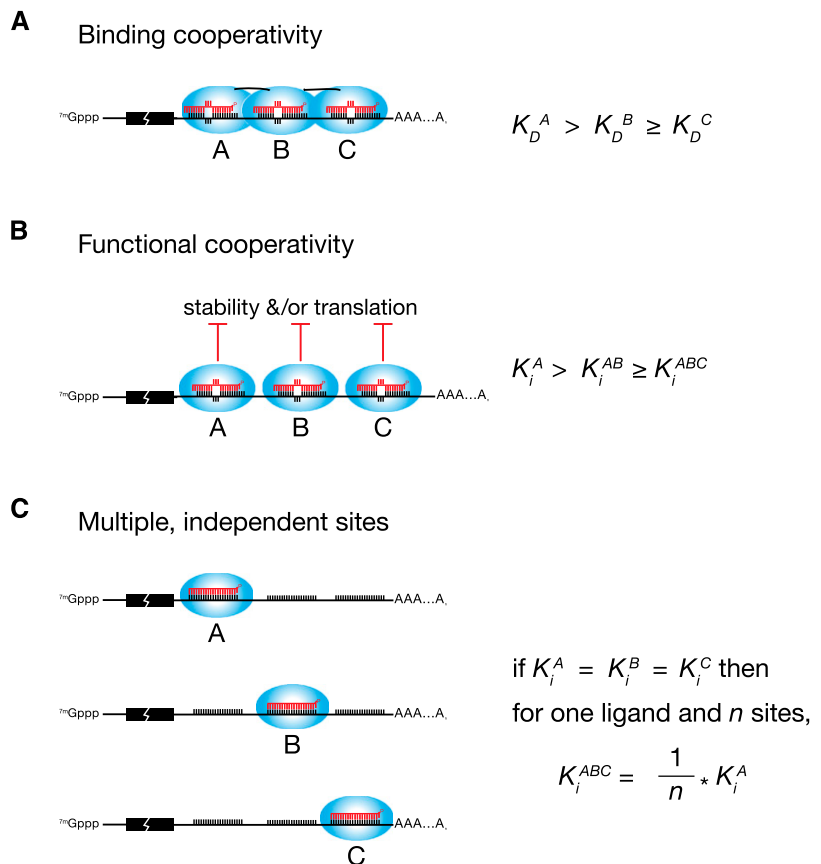

FIGURE 1. Potential sources of cooperativity in the repression of a target mRNA by the small RNA-directed Argonaute complex, RISC. (*A*) Cooperative binding. RISC binding at multiple target sites increases site occupancy by mutually stabilizing subsequent binding of RISCs. (*B*) Cooperative function. RISC binding at multiple sites may increase the likelihood that repressive factors, such as nucleases, are recruited to the mRNA. (*C*) Multiple independent sites. Each RISC functions independently, so the multiple sites increase the probability of repression but do not influence each other.

model, multiple miRNA:Argonaute complexes bind to the target mRNA independently, but the interaction of one miRNA:Argonaute complex could recruit binding proteins which block translation of the target mRNA or decrease the stability of the target (Fig. 1B). Historically, such protein targets of RISC have been envisioned to include components or regulators of the ribosome but more likely correspond to factors that promote accumulation of the target RNA in a P-body, where it would be degraded (Liu et al. 2005; Rehwinkel et al. 2005; Eulalio et al. 2007; Parker and Sheth 2007; Guo et al. 2010). In the *cooperative function* model, we predict that the presence of three bulged siRNAs on the target would have a lower inhibitory constant, K_i^{ABC}, than for the presence of two (K_i^{AB}) or one (K_i^A) bulged siRNA; the amount of siRNA required to silence a reporter would decrease with increasing number of target sites occupied by Argonautes and/or a protein factor X, until the concentration of Argonaute or factor X becomes limiting. Finally, in a *multiple independent sites* model, each miRNA:Argonaute complex binds and acts independently, but the presence of multiple miRNA-binding sites in the target increases its effective miRNA occupancy: I.e., the probability that the target mRNA is bound by at least one miRNA is increased by the presence of multiple sites (Fig. 1C). Such statistical effects cause the macroscopic binding constant, K, representative of all possible combinations of target mRNA with n sites where at least one site is occupied, to be determined by the statistical factors of identical microscopic binding constants, k, to give a fractional saturation of target: $[1/n]k$ (Cantor and Schimmel 1980). If we assume that the IC_{50} is governed by binding and that the microscopic binding constant for a single site is essentially identical to the macroscopic binding constant for the one-site target, then we expect the IC_{50} for the three-site target to be $1/3(IC_{50}$, one-site target).

In contrast to the cooperativity ascribed to miRNA-directed changes in mRNA stability or translation, small RNA-guided target cleavage—that is, RNAi—is thought to be noncooperative, with each RISC acting independently at each complementary site on the target mRNA. The presence of multiple, independent small RNA-binding sites in a target would increase its effective occupancy by RISC: the probability that the target mRNA is cleaved by at least one molecule of RISC is increased by the presence of multiple sites.

To evaluate the efficacy of silencing and the extent of cooperativity directed by a small silencing RNA bound at one or multiple sites on an mRNA, we established an experimental system comprising six *Renilla* luciferase reporter plasmids, each expressing an mRNA bearing one to six identical, adjacent target sites in its 3′ UTR (Fig. 2, left). We tested four siRNAs whose guide strands pair to different extents with the target sites (Fig. 2, top). The four siRNAs enabled evaluation of four siRNA:mRNA target RNA binding modes—perfect pairing, bulged pairing (mismatched at positions 9 and 10 of the guide strand),

seed pairing with supplemental 3′ pairing (matching the target at positions 2–8 and 13–16 of the guide strand), and seed-only pairing (paired only at positions 2–8 of the guide strand). Each siRNA duplex was designed to ensure preferential loading of its guide strand into RISC (Schwarz et al. 2003). Because all of our experiments comparing distinct modes of miRNA:target pairing employed a common target reporter mRNA, our strategy avoids differences in local target mRNA structure that might confound interpretation.

For each of the 24 reporter-siRNA combinations tested in HeLa cells, we calculated the concentration of siRNA required to achieve half-maximal silencing (IC_{50}) and the Hill coefficient (n^H), a measure of cooperativity, using dose-response data from at least 12 independent experiments, each evaluating silencing at ≥ 10 siRNA concentrations and spanning a 2000-fold concentration range. For each siRNA, we confirmed that the siRNA was inherently active by validating its ability to silence a *Renilla* luciferase reporter containing a single, fully complementary siRNA-binding site (Fig. 3). For the four siRNAs, the mean IC_{50} values $\pm$ standard deviation for the corresponding perfect, single-site reporter mRNA ranged from 0.27 ± 0.22 nM to 1.33 ± 0.78 nM, establishing that all four siRNAs were active.

Silencing by perfect pairing at multiple sites is not cooperative

Next, we targeted each reporter for silencing by a perfect siRNA to determine if increasing the number of target sites reduced the amount of siRNA needed to silence the reporter. Based on the *multiple independent sites* model, we anticipated that a reporter mRNA bearing more target sites would be more likely to recruit RISC and would, therefore, show a reduced IC_{50} for a fully complementary ("perfect") siRNA. Instead, our data suggest that RISC neither binds nor functions appreciably better when the target contained multiple sites (Fig. 2). In fact, reporter mRNAs bearing three ($IC_{50} = 0.75 \pm 0.93$ nM), four ($IC_{50} = 0.25 \pm 0.09$ nM), five ($IC_{50} = 0.41 \pm 0.33$ nM), or six ($IC_{50} = 0.30 \pm 0.14$ nM) perfect sites had essentially indistinguishable IC_{50} values and were silenced only slightly better than a reporter bearing a single perfect site ($IC_{50} = 0.63 \pm 0.25$ nM). None of the six reporters displayed positive cooperativity with the perfectly matched siRNA, with the Hill coefficients ranging from $n^H = 0.8 \pm 0.2$ (six-site reporter) to 1.2 ± 0.2 (four-site reporter). None of the Hill coefficients were significantly different from $n^H = 1$ (*P*-value > 0.05) (Supplemental Fig. S1). Moreover, when the same perfect siRNA was used to silence a reporter bearing three sites separated by 19 nt, the IC_{50} and the Hill coefficient were similar to the mRNA reporter with a single site ($IC_{50} = 0.37 \pm 0.39$ nM; $n^H = 1.0 \pm 0.2$) (Fig. 4).

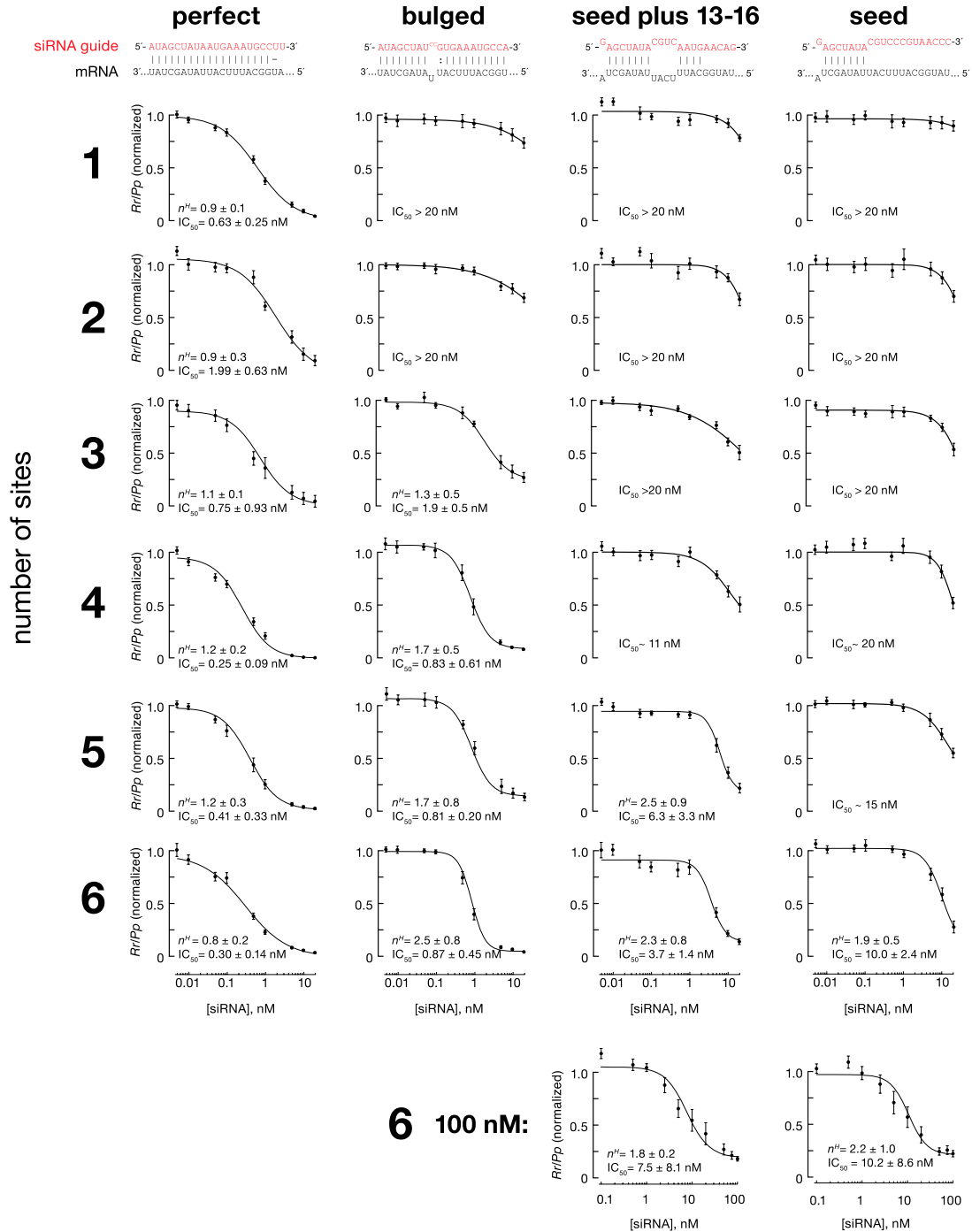In general, silencing by the perfect siRNA was well described by a sigmoidal curve with a Hill coefficient of

FIGURE 2. Extent of pairing and target site number determine both efficacy and cooperativity in small RNA-directed silencing in HeLa cells. Silencing of a *Renilla* luciferase reporter mRNA bearing 1–6 target sites in its 3′ UTR, relative to a firefly luciferase internal control, was determined at different siRNA concentrations. Pairing between the siRNA guide (red) to the 3′ UTR sites (black) is shown at *top*. IC_{50} and Hill coefficient (n^H) were calculated for each dose-response curve. Throughout this study, values are reported as mean ± standard deviation for IC_{50} values and n^H; error bars indicate standard error for ≥12 biological replicates. The curves correspond to the concentration-dependence of silencing expected for the mean IC_{50} and n^H values.

one, irrespective of the number of sites (Fig. 2, left). We conclude that, when guided by a perfectly pairing siRNA, each RISC acts independently from other RISCs that bind to nearby target sites.

Multiple bulged sites act cooperatively

Central internal loops—or "bulges"—typically block siRNA- or miRNA-directed target cleavage, the most potent post-
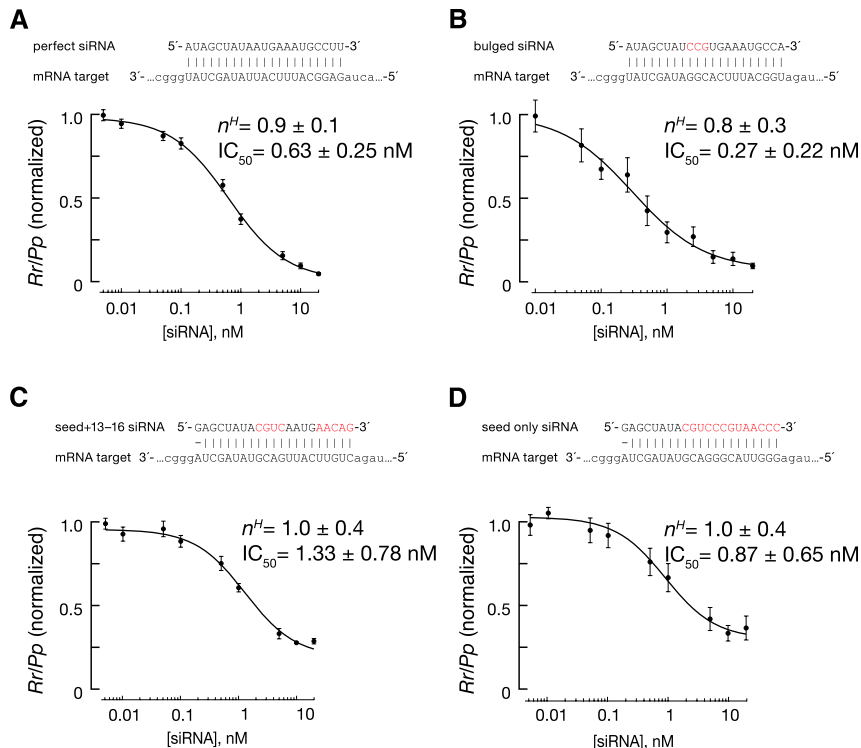
FIGURE 3. siRNA validation in HeLa cells. Each siRNA was functional in silencing a reporter containing a single perfect target site. (*A*) Perfect siRNA. (*B*) Bulged siRNA. (*C*) siRNA with seed plus supplementary 3′ pairing (nt 13–16). (*D*) siRNA with only seed pairing. The curves correspond to the concentration-dependence of silencing expected for the mean IC_{50} and n^H values (± standard deviation) calculated from three independent trials.

transcriptional silencing mechanism (Holen et al. 2002; Du et al. 2005; Dahlgren et al. 2008; Huang et al. 2009). Consistent with that view, effective silencing by a small RNA that forms a central bulge when paired to its target site required a higher concentration of siRNA than did the corresponding perfect siRNA, even when comparing multiple bulged sites to a single perfect site (Table 1; Fig. 2). In fact, we were unable to achieve half-maximal silencing of a *Renilla* luciferase reporter bearing one or two bulged sites even at 20 nM transfected siRNA. For a reporter bearing six bulged sites, the IC_{50} was nearly three times greater than that for perfect sites. Unlike silencing mediated by perfect sites, silencing via bulged sites showed positive cooperativity, with a Hill coefficient of 2.5 ± 0.8 ($P = <0.0001$ for six sites) (Table 1).

Seed matches and supportive pairing

Although bulged sites have been shown to effectively silence both reporter mRNAs and endogenous genes (Zeng et al. 2002; Doench et al. 2003; Doench and Sharp 2004), they rarely occur for natural miRNAs and their endogenous targets (Lewis et al. 2003; Vella et al. 2004; Yekta et al. 2004; Brennecke et al. 2005; Lewis et al. 2005; Grimson et al. 2007). Instead, miRNAs generally pair with the mRNAs they regulate at positions 2–8 of the guide strand—the seed

sequence (Lewis et al. 2003; Brennecke et al. 2005; Krek et al. 2005; Lewis et al. 2005). Additional base pairs between the mRNA and miRNA positions 13–16 (Grimson et al. 2007) and target adenosines flanking the seed match sequence at position 1 (t1A) and 9 (t9A) (Lewis et al. 2005) enhance the likelihood that a miRNA will regulate a putative mRNA target.

We tested seed-matched (t1A) sites with supplemental 3′ pairing and seed only (t1A) sites for their ability to regulate reporter mRNA bearing one to six siRNA-binding sites. A seed match plus supplemental 3′ pairing required far more siRNA to achieve silencing equivalent to the bulged sites (Fig. 2). For example, the bulged siRNA regulated the three-site reporter with an $IC_{50} = 1.9 ± 0.5$ nM, whereas half-maximal silencing for the same reporter with the siRNA pairing with both the seed and supplemental 3′ nucleotides could not be achieved even using 20 nM siRNA. With six sites in the reporter, the siRNA with seed plus supplemental 3′ pairing achieved an $IC_{50} = 3.7 ± 1.4$ nM. The seed siRNA was even less potent, reaching half-maximal silencing at a siRNA concentration of 10 ± 2.4 nM only for the reporter mRNA with six sites; the IC_{50} could not be reliably determined for mRNAs with fewer than six sites. Our data suggest that the intracellular concentration of functional miRISC exceeds the RISC concentration we achieved using transfected, synthetic siRNA duplexes.

Most studies of small RNA-directed silencing report the extent of repression ("fold-repression") for a single concentration of small RNA. To permit comparison of our data to those in the published literature, we used our data to calculate the observed "fold-repression" of the multiple-site reporters by the seed-only siRNA (Supplemental Table S1). Like Grimson et al. (2007) before us, we observe a ≥1.4-fold repression of targets bearing two or more small RNA-binding sites when using the seed-only siRNA. For three or more sites, the observed repression, which ranged from 1.8- to 4.8-fold, was significantly different from that predicted by a multiple, independent sites model ($0.002 ≤ P ≤ 0.04$) (Supplemental Table S1).

Cooperativity requires adjacent target sites

Silencing for bulged sites displayed positive cooperativity for all multiple-site reporter mRNAs for which we could
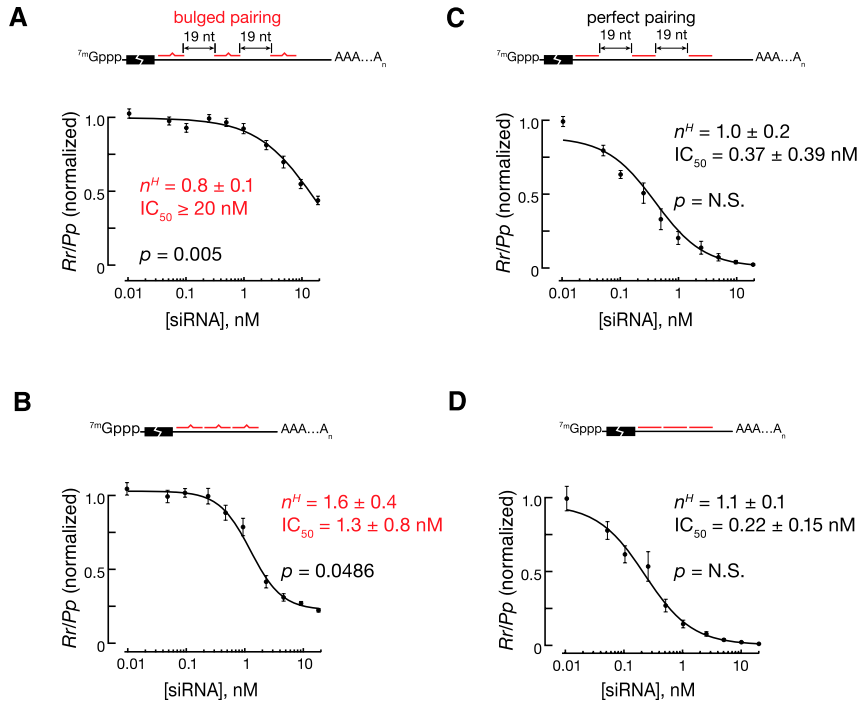
FIGURE 4. Cooperative binding of RISC requires adjacent target sites in HeLa cells. Three sites spaced 19 nt apart (*A*) require more siRNA to achieve half-maximal silencing, compared to three adjacent sites (*B*), and act noncooperatively. In contrast, a perfectly matched siRNA silences a three-site reporter with sites separated by 19 nt (*C*) or a reporter with three adjacent sites (*D*) with equal efficacy and without detectable cooperativity. The three adjacent-site experiments in this figure were performed independently from those in Figure 2. A one sample, two-tailed Student's *t*-test was used to calculate the P-values at 95% confidence for the Hill coefficients to determine if n^H was significantly different from the null hypothesis: $n^H = 1$ (i.e., noncooperative).

measure the IC_{50} and Hill coefficient. To test if the sites need to be adjacent in order to observe positive cooperativity, we altered the sequence of every other target site in the six-site *Renilla* luciferase mRNA to create a three-site reporter in which 19 nt separate each site targeted by the siRNAs (Fig. 4A). Silencing of this expanded three-site reporter mRNA by the bulged siRNA required >15-fold more siRNA and showed no evidence of cooperativity ($IC_{50} \geq 20$ nM; $n^H = 0.8 \pm 0.1$) (Fig. 4A) relative to the reporter mRNA

in which the three sites were adjacent ($IC_{50} = 1.3 \pm 0.8$ nM; $n^H = 1.6 \pm 0.4$) (Fig. 4B).

In theory, these data might reflect reduced target-site accessibility in the expanded three-site reporter (Brown et al. 2005; Ameres et al. 2007; Tafer et al. 2008). We view this as unlikely. First, both the adjacent and expanded three-site reporters were silenced equally well by a perfectly pairing siRNA ($IC_{50} = 0.37 \pm 0.39$ nM versus 0.22 ± 0.15 nM) (Fig. 4C,D). Second, antisense oligonucleotide-directed RNase H cleavage at each of the target sites occurred with similar rates (6.1, 6.0, and 6.6 nM min^{-1} for sites 1, 2, and 3, respectively) (Supplemental Fig. S2). Finally, the RNase H cleavage kinetics fit better to a model of independent action compared to a dependent, sequential model for cleavage at each site (Supplemental Fig. S2).

Silencing by the perfectly paired siRNA was noncooperative for both the expanded ($n^H = 1.0 \pm 0.2$) and original ($n^H = 1.1 \pm 0.1$) three-site reporter mRNAs (Fig. 4C,D). The observation that cooperative silencing by a small RNA requires that fewer than 19 nt separate the RISC-binding sites to promote efficient, cooperative silencing suggests that cooperativity springs from interactions between adjacent Argonaute-siRNA complexes, rather than cooperative recruitment of proteins involved in subsequent steps in repressing mRNA expression.

Ago2-RISC binding prevents cooperative silencing

A simple explanation for why Ago2 acts noncooperatively to silence a multiple-site reporter with a perfect guide is

TABLE 1. Concentration-dependence and cooperativity for distinct siRNA:target pairing modes using reporters bearing one to six siRNA-binding sites

Number of sites	Perfect		Bulged		Seed plus 13–16		Seed only	
	IC_{50}	n^H	IC_{50}	n^H	IC_{50}	n^H	IC_{50}	n^H
1	0.63 ± 0.25	0.9 ± 0.1	>20	N.D.	>20	N.D.	>20	N.D.
2	1.99 ± 0.63	0.9 ± 0.2	>20	N.D.	>20	N.D.	>20	N.D.
3	0.75 ± 0.93	1.1 ± 0.1	1.9 ± 0.5	1.3 ± 0.5	>20	N.D.	>20	N.D.
4	0.25 ± 0.09	1.2 ± 0.2	0.83 ± 0.61	1.7 ± 0.5	~11	N.D.	~20	N.D.
5	0.41 ± 0.33	1.2 ± 0.3	0.81 ± 0.20	1.7 ± 0.8	6.3 ± 3.3	2.5 ± 0.9	~15	N.D.
6	0.30 ± 0.14	0.8 ± 0.2	0.87 ± 0.45	2.5 ± 0.8	3.7 ± 1.4	2.3 ± 0.8	10.0 ± 2.4	1.9 ± 0.5

(N.D.) not determined. IC_{50} values (nM) and Hill coefficients (n^H) of the fitted curves are reported as mean values $\pm$ standard deviation for the IC_{50} and Hill coefficients for at least 12 trials.

that silencing reflects the endonucleolytic activity unique to mammalian Ago2. To test this idea, we evaluated silencing of the six-site reporter mRNA in three mouse embryonic fibroblast (MEF) cell lines derived from an Ago2 knockout mouse: Ago2$^{-/-}$ MEFs, Ago2$^{-/-}$ MEFs reconstituted with mouse Ago2, and Ago2$^{-/-}$ MEFs reconstituted with a mutant mouse Ago2 in which aspartic acid 669 was changed to alanine (D669A) (O'Carroll et al. 2007). The D669A mutant Ago2 cannot cleave an RNA target (Liu et al. 2004). In Ago2$^{-/-}$ MEF cells, the perfect siRNA and the bulged siRNA were both cooperative: $n^H_{perfect} = 1.6 \pm 0.4$; $P = 0.03$, and $n^H_{bulged} = 1.8 \pm 0.3$; $P = 0.006$ (Fig. 5A). These data suggest that Ago1, Ago3, and Ago4 bind cooperatively to a reporter mRNA bearing multiple small RNA-binding sites, irrespective of the nature of small RNA:target pairing.

As expected, repression mediated by a perfectly pairing siRNA was noncooperative ($n^H_{perfect} = 1.0 \pm 0.1$) in Ago2$^{-/-}$ MEFs reconstituted with overexpressed Ago2 (Figs. 5B, 6). In contrast, silencing directed by the bulged siRNA in the Ago2-reconstituted cells was cooperative ($n^H_{bulged} = 1.5 \pm 0.2$; $P = 0.02$) (Fig. 5B). To test whether the apparent cooperativity observed in reconstituted Ago2 MEF cells was caused by Ago2 overexpression, we measured silencing in

Ago1$^{-/-}$ MEF cells, which express far less Ago2 mRNA and protein than reconstituted Ago2$^{-/-}$ MEF cells (Figs. 5D, 6; Supplemental Table S2). (All four Argonautes are expressed in the HeLa line we used [Supplemental Fig. S3].) We detected no cooperativity for silencing by the perfect siRNA in the Ago1$^{-/-}$ MEFs ($n^H_{perfect} = 1.1 \pm 0.1$). However, silencing by the bulged siRNA was cooperative ($n^H_{bulged} = 1.7 \pm 0.2$; $P = 0.003$), suggesting that Ago2 is capable of cooperative silencing (Fig. 5D).

To test whether Ago3 or Ago4 contributes to the cooperativity that we observed for a bulged siRNA in the Ago1$^{-/-}$ MEFs, we used siRNAs to deplete Ago3 and Ago4 mRNAs before transfecting the reporter plasmids and bulged siRNA. Ago4 mRNA was reduced 50% compared to Ago1$^{-/-}$ MEF cells transfected with a control siRNA. By qRT-PCR, we detected Ago3 mRNA two threshold cycles after detection of Ago1 mRNA in the Ago1$^{-/-}$ MEF cells, indicating that its expression is probably functionally inconsequential in our analysis (data not shown). The level of Ago3 protein in Ago1$^{-/-}$ MEFs was very low, and our attempts to reduce it further by RNAi were unsuccessful (Supplemental Fig. S4A). Under these conditions, n^H for silencing by the bulged siRNA was not significantly different
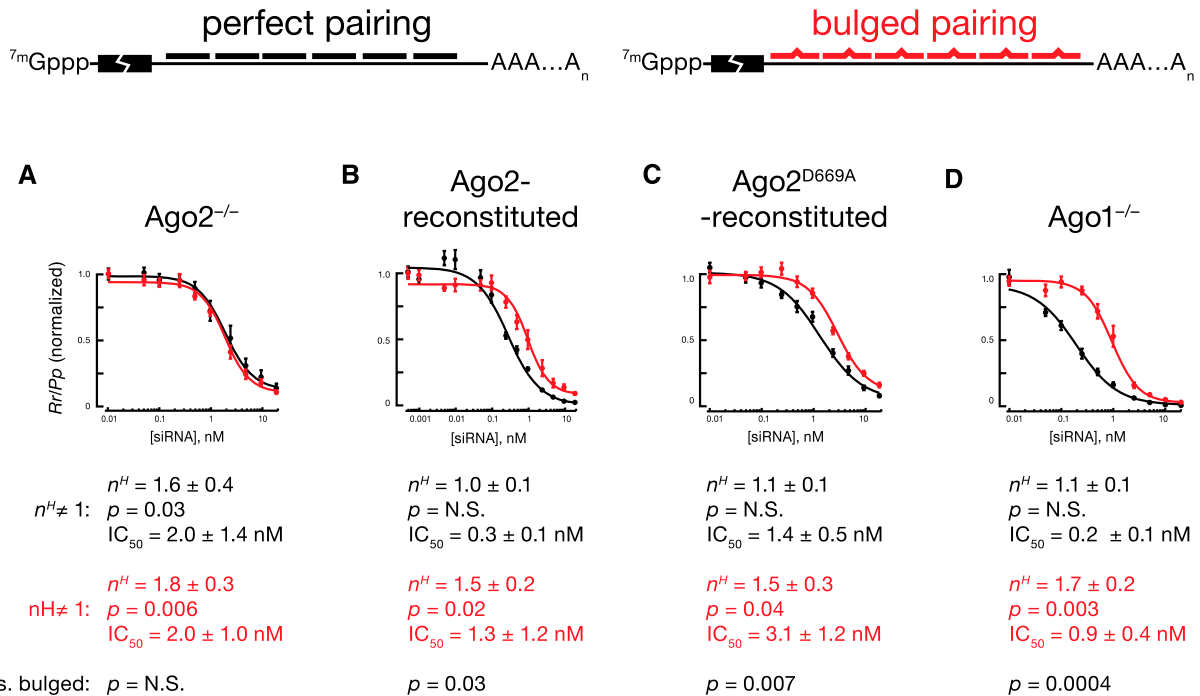
perfect pairing

bulged pairing

FIGURE 5. Silencing in Ago2$^{-/-}$ MEFs or Ago2$^{-/-}$ MEFs reconstituted with mouse Ago2 or catalytically inactive, mutant Ago2^{D669A} or Ago1$^{-/-}$ MEFs. (A) In the absence of Ago2, silencing by a perfect site ($n^H = 1.6 \pm 0.4$; $P = 0.03$) is equally cooperative as a bulged site ($n^H = 1.8 \pm 0.3$; $P = 0.006$). (B) Mouse Ago2 expression restored noncooperative silencing by the perfect siRNA (black; $n^H = 1.0 \pm 0.1$); silencing directed by a bulged siRNA became less cooperative (red; $n^H = 1.5 \pm 0.2$; $P = 0.02$) than in the absence of Ago2 (red in A; $n^H = 1.8 \pm 0.3$). (C) Catalytically inactive mouse Ago2^{D669A} likewise restored noncooperative silencing by a perfect siRNA (black; $n^H = 1.1 \pm 0.1$), but silencing by the bulged siRNA (red; $n^H = 1.5 \pm 0.3$; $P = 0.04$), was cooperative. (D) In the absence of Ago1, silencing by the perfect siRNA was not cooperative (black; $n^H = 1.1 \pm 0.1$), but silencing by the bulged siRNA was cooperative (red; $n^H = 1.7 \pm 0.2$; $P = 0.003$). A one sample, two-tailed Student's *t*-test was used to calculate the *P*-values at 95% confidence for the Hill coefficients to determine if n^H was significantly different from the null hypothesis: $n^H = 1$ (i.e., noncooperative).
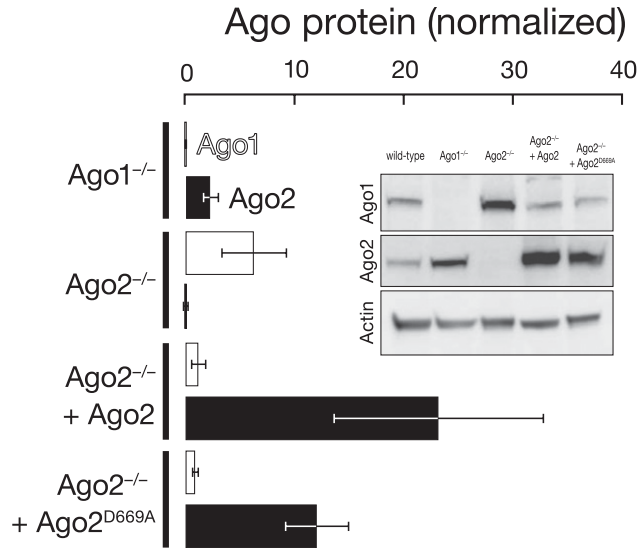
Ago protein (normalized)

FIGURE 6. Ago1 and Ago2 protein levels in MEF cells. Ago2 was detected by Western blotting using a rabbit anti-Ago1 antibody that recognizes both mouse and human Ago1 and a rabbit anti-Ago2 antibody that recognizes both mouse and human Ago2. Ago protein levels were normalized to actin, and the level of Ago protein in wild-type MEFs was set to 1. Data are mean ± standard deviation for three trials. Inset shows representative data from a single experiment.

from the null hypothesis (noncooperative binding), although the Hill coefficients for the bulged and perfect siRNAs were significantly different ($n_{bulged}^H = 1.8 \pm 0.5$ versus $n_{perfect}^H = 1.0 \pm 0.1$; $P = 0.03$) (Supplemental Fig. S4B).

As a final test of the idea that noncooperative silencing reflects target cleavage, we analyzed silencing directed by a perfectly pairing siRNA in Ago2$^{-/-}$ MEFs reconstituted with D669A mutant Ago2. In the cells reconstituted with catalytically inactive Ago2, the single-site reporter was not silenced by the perfect siRNA (Supplemental Fig. S5). Surprisingly, cells reconstituted with catalytically inactive Ago2 exhibited noncooperative silencing of the six-site reporter by a perfect siRNA ($n_{perfect}^H = 1.1 \pm 0.1$), while silencing by a bulged siRNA displayed positive cooperativity ($n_{bulged}^H = 1.5 \pm 0.3$; $P = 0.04$) (Fig. 5C). This unexpected result suggests that target cleavage per se is not required for noncooperative silencing mediated by Ago2. Rather, both the identity of the Argonaute protein and the nature of pairing between the small RNA and its target determine if RISC bound to multiple sites in the 3′ UTR of an mRNA can collaborate to generate cooperativity in silencing.

Only Ago2-RISC can repress a reporter with nonadjacent sites

In HeLa cells, the mRNA with the expanded target sites (Fig. 4A) was less efficiently silenced by a bulged siRNA than an mRNA in which the three sites were adjacent (Fig. 4B). We propose that RISCs bound to adjacent sites collaborate to achieve efficient silencing. Is Ago2 required to silence an

mRNA in which the small RNA binding sites cannot collaborate? We tested silencing of the expanded three-site reporter mRNA by the perfect and bulged siRNAs in the Ago2$^{-/-}$ MEFs. Silencing of the expanded three-site reporter was completely dependent on Ago2; little or no silencing was observed in the Ago2$^{-/-}$ MEFs for either type of small RNA:target pairing (Fig. 7C,D). In contrast, both perfect and bulged siRNAs cooperatively silenced the reporter bearing three adjacent small RNA-binding sites in the Ago2$^{-/-}$ MEFs (Fig. 7A,B). Notably, in the absence of Ago2, silencing by the perfect siRNA of the reporter containing three adjacent sites was highly cooperative ($n_{perfect}^H = 2.1 \pm 0.3$; $P = 0.007$). We conclude that, for widely spaced sites, only Ago2 can silence at the intracellular RISC concentration achieved at the highest amount of siRNA transfected, likely because, in the absence of cooperativity, the intracellular concentration of Ago1-, Ago3-, and Ago4-RISC is less than the K_D for target binding for these Argonaute:siRNA complexes.

Reconstituting the Ago2$^{-/-}$ MEFs with either wild-type or catalytically inactive mouse Ago2 rescued silencing of the expanded three-site reporter (Fig. 7E–J). Silencing showed no significant cooperativity for the perfect siRNA in MEFs reconstituted with wild-type ($n_{perfect}^H = 1.1 \pm 0.1$) or catalytically inactive Ago2 ($n_{perfect}^H = 1.2 \pm 0.2$). Silencing by the bulged siRNA was cooperative for both the wild-type Ago2-reconstituted ($n_{bulged}^H = 1.6 \pm 0.1$; $P = 0.02$) (Fig. 7F) and the catalytically inactive Ago2 MEFs ($n_{bulged}^H = 1.3 \pm 0.2$; $P = 0.03$) (Fig. 7H). Intriguingly, in the absence of Ago1, silencing of the expanded three-site reporter by the bulged siRNA was highly cooperative ($n_{bulged}^H = 2.5 \pm 0.2$; $P = 0.006$) (Fig. 7J).

DISCUSSION

In our assays, Ago2 noncooperatively silenced mRNAs bearing multiple, perfectly complementary small RNA-binding sites, even when its endoribonuclease activity was inactivated by mutation. This finding is surprising because we and others have assumed that endonucleolytic cleavage by Ago2 explained its lack of cooperativity in silencing when guided by a perfectly pairing siRNA. Clearly, a more complex explanation is warranted. We suggest that the Ago2 conformation associated with perfect small RNA:target pairing precludes protein:protein interactions, causing both nearby and adjacent binding sites to act independently. Alternatively, Ago2 protein, when guided by a small RNA that pairs extensively with its target mRNA, might be bound by proteins that prevent its association with factors promoting cooperativity.

Silencing via multiple small RNA-binding sites is likely always cooperative for Ago1, Ago3, or Ago4, irrespective of the type of pairing between the small RNA and its target. We suggest that these noncatalytic Argonaute proteins adopt a single conformation when bound by different
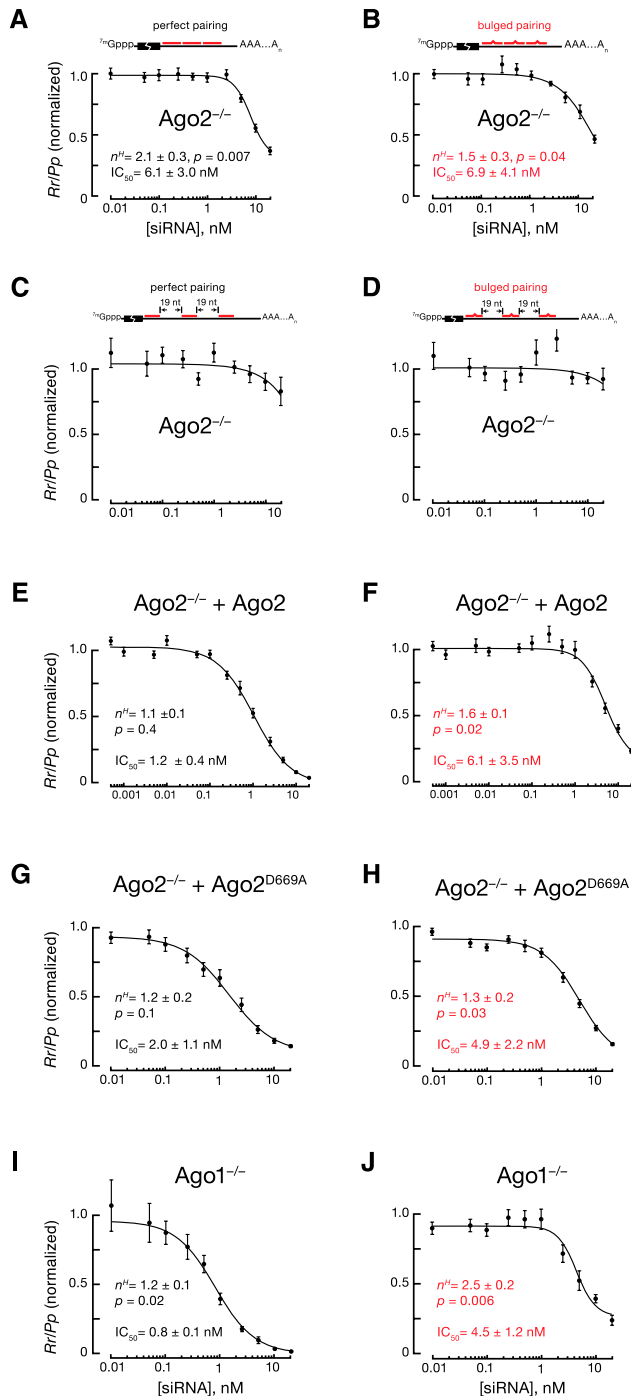
FIGURE 7. In the absence of Ago2, effective silencing requires adjacent sites. (*A,B*) Both perfect ($n^H = 2.1 \pm 0.3$; $P = 0.007$) and bulged ($n^H = 1.5 \pm 0.3$; $P = 0.04$) adjacent sites were silenced cooperatively in the absence of Ago2. (*C,D*) In Ago2$^{-/-}$ MEFs, three target sites spaced 19 nt apart did not silence the reporter. (*E,F*) Expressing mouse Ago2 in the Ago2$^{-/-}$ MEFs allowed three distant sites to silence the reporter. (*G,H*) Expressing catalytically inactive, mutant Ago2^{D669A} also allowed three distant sites to silence the reporter. (*I,J*) In the Ago1$^{-/-}$ MEFs the three distant sites silenced the reporter. A one sample, two-tailed Student's *t*-test was used to calculate the *P*-values at 95% confidence for the Hill coefficients to determine if n^H was significantly different from the null hypothesis: $n^H = 1$ (i.e., noncooperative).

modes to their mRNA targets or that the conformations produced by both perfect and bulged small RNAs are compatible with protein:protein interactions between adjacent RISC molecules. Intrinsic differences between Ago2 and Ago1, Ago3, and Ago4 may dictate the combination of Ago proteins capable of cooperative silencing.

Cooperativity versus statistical effects for closely apposed target sites

We find that multiple, imperfect binding sites need to be surprisingly close in order to mediate cooperative silencing. Our data fundamentally agree with previous reports but differ quantitatively in the precise inter-site distance that supports cooperativity (Grimson et al. 2007; Bartel 2004. We note that the precise inter-site distance that supports cooperative interactions may reflect the intracellular concentrations of Argonaute proteins and associated factors, as well as the local structure or sequence of the mRNA target. When testing silencing of a two-site reporter, Grimson et al. observed that a seed-matched siRNA transfected at 25 nM cooperatively silenced a reporter bearing two 3′ UTR target sites spaced 8–40 nt apart (counting the number of nucleotides between the 3′ end of the first site and the 5′ end of the second site); expanding the distance to 56 nt disrupted cooperative silencing (Grimson et al. 2007). We note that these authors defined cooperativity as an excess of silencing when the observed repression for a two-site reporter was compared to the product of the repression observed for each site acting alone. Enhanced silencing measured in this way may correspond to true cooperativity or may simply reflect the statistical effects[6] of multiple independent sites. Our Hill analyses distinguish between these two possibilities and suggest that RISCs bound to adjacent sites cooperate to confer greater silencing than would be expected from statistical effects alone.

Supplemental 3′ pairing reduces the amount of small RNA required to repress an mRNA

The amount of siRNA required to repress an mRNA target is determined by the number of small RNA-binding sites, the spacing of the sites, and the extent of complementarity beyond the seed sequence at each site. Compared to an mRNA in which the siRNA seed sequence alone paired with the small RNA-binding sites, an mRNA in which seed pairing was supplemented with additional 3′ base pairs

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[6]Statistical effects result from the simultaneous occupancy of multiple, independent binding sites of similar affinities even in the absence of cooperativity (Cantor and Schimmel 1980). This effect, with increasing site occupancy, causes a steep threshold response that appears nonadditive. In contrast, cooperativity results in the concerted loading of sites at a lower overall concentration of ligand (e.g., miRISC) and a sharp dose-response to a relatively small increase in ligand concentration.

required slightly less siRNA to achieve comparable repression, particularly for multisite target RNAs (Table 1; Fig. 2). These data reinforce the view that has emerged from previous computational analyses of miRNA target binding: 3′ supplemental pairing provides a small but measurable increase in the affinity of a small RNA for its target (Grimson et al. 2007).

Nonetheless, it is striking how much more siRNA is needed to regulate a target containing small RNA-binding sites with a seed-match only or a seed-match plus supplementary pairing, compared to a target containing sites that fully pair to the small RNA but for a central bulge. While our current data do not permit direct estimation of the binding affinity of a small RNA for a reporter mRNA within a cell, they suggest that one explanation for the remarkably high intracellular abundance of some miRNAs is that most miRNAs bind weakly to the mRNAs they regulate.

MATERIALS AND METHODS

Plasmids

Renilla luciferase vector pRL-TK (Promega) containing target sites for CXCR4 and a modified linker sequence (Doench et al. 2003) was mutated from TAG to CTC (lower case letters in the oligonucleotides) at nt 387–389 of the *Renilla* luciferase open reading frame to generate mismatches with seed positions 5, 6, and 7 of the siRNA guide strand by PCR-directed mutagenesis using DNA oligonucleotides:

5′-CTTGTTTGGCATTTCATTACtccTATGAGCATCAAGATAAG
ATC-3′ (sense),
5′-GATCTTATCTTGATGCTCATAGgagTAATGAAATGCCAAAC
AAG-3′ (antisense).

Mutagenesis was confirmed by sequencing, and then 5′ phosphorylated oligos containing the target sites and pairing to create appropriate ends were cloned into the *XbaI* and *ApaI* sites of the mutant pRL-TK. Supplemental Table S3 lists the sequences of the DNA oligonucleotides used to construct target sites. psiCheck2 (Promega) reporters were constructed by digesting psiCheck2 with *NheI* and *NotI* and inserting the 3′ UTR target site-containing *NheI–NotI* fragment from the mutant pRL-TK vectors. Supplemental Table S3 lists the oligonucleotide sequences used to generate plasmid reporters. Dual Reporter Luciferase assays were conducted using Dual Luciferase Assay Reagents (Promega) in a Veritas Microplate Luminometer (Turner Biosystems) according to the manufacturer's directions.

Cell culture and transfection

HeLa CCL2 cell cultures were maintained at 37°C and 5% CO_2 in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and 50 U/mL penicillin and streptomycin (Invitrogen). MEF cells were cultured in DMEM (Invitrogen) supplemented with 15% heat-inactivated FBS (Invitrogen), 50 U/mL penicillin and streptomycin (Invitrogen), 0.1 mM NEAA (Invitrogen), 2 mM glutamine (Invitrogen). Cells were seeded at a density of 0.1×10^6 cells per well in 24-well plates in DMEM

(Invitrogen) containing 10% heat-inactivated FBS (Invitrogen). Twenty-four hours later, cells were washed three times in 500 μL PBS (Invitrogen), and then 400 μL DMEM with serum was added to each well. *Renilla* luciferase plasmid (0.025 mg), firefly luciferase plasmid pGL3 (0.025 mg), and 20 nM siRNA were mixed with 99 μL DMEM and 1 μL DharmaFECT Duo (Dharmacon) per well. A control siRNA (CXCR4) was used to equalize the total amount of siRNA in each transfection. Cells are incubated with 0.5 mL final volume of DMEM plus serum containing 100 μL of transfection reagent nucleic acid mixture for 24 h.

siRNA annealing

Single-stranded guide and passenger siRNA strands (Dharmacon) (Supplemental Table S4) were annealed by incubating 10 μM of each strand in 500 μL annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h at 37°C.

Luciferase assays

Cells were washed once in 500 μL PBS and lysed in 100 mL of Passive Lysis Buffer (Promega) at room temperature for 20 min in 24-well plates. For each well, 10 μL lysate was read in triplicate using dual luciferase reagents (Promega) in a Turner Biosystems luminometer controlled by Veritas software (Turner). *Renilla* luciferase activity for each concentration of transfected siRNA was normalized to the corresponding firefly luciferase activity.

Data analysis

The individual biological replicates for normalized *Renilla* luciferase activity versus siRNA concentration were fit using Igor Pro 6.10 (Lake Oswego, OR) to the Hill equation to determine IC_{50} and n^H. Fitting was weighted using the standard error of each mean value. Throughout this study, the standard deviation is reported for mean IC_{50} and n^H values. The Hill coefficients from each replicate were subjected to the Student's *t*-test to determine *P*-values at 95% confidence using GraphPad Prism (La Jolla, CA).

Statistical testing

To test if the individual Hill coefficients from the replicates of each experiment followed a Gaussian distribution, data were subjected to the Kolmogorov–Smirnov, D'Agostino-Pearson omnibus, and Shapiro-Wilk normality tests. By all three tests, all Hill coefficient data was normally distributed. The *P*-values at 95% confidence were calculated using an unpaired, one sample, two-tailed Student's *t*-test (GraphPad Prism) to test whether n^H was significantly different from the null hypothesis that $n^H = 1$ (i.e., noncooperative). An unpaired, two-tailed Student's *t*-test with Welch's correction at 95% confidence, which does not assume equal variances, was used to test the significance of differences in n^H for a perfect and bulged siRNA. For non-normally distributed fold-repression data, we used the nonparametric Wilcoxon Signed-Rank test at 95% confidence to determine *P*-values.

Western blotting

Forty micrograms cell lysate in cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% v/v NP-40) containing Complete, mini, EDTA-

free protease inhibitor (Roche) were separated by 4%–20% HEPES-SDS-PAGE and transferred at 4°C in Tris-bicine buffer to nitrocellulose membrane overnight at 30 V. Membranes were blocked in 5% w/v milk-TBST (100 mM Tris Cl pH 7.5, 150 mM NaCl, 0.1% TWEEN 20) for 1 h and incubated overnight at 4°C with primary antibody diluted in 3% milk-TBST. Rabbit anti-human and mouse Ago2 antibody (Cell Signaling Technologies) (Li et al. 2010) or rabbit anti-human and mouse Ago1 antibody (MBL International) were diluted 1:1000 and rabbit anti-actin antibody (Bethyl Laboratories) was diluted 1:5000. After three 5-min washes in TBST, the membranes were incubated 1 h with secondary goat anti-rabbit HRP-conjugated antibody (GE Healthcare) diluted 1:10,000. After five 5-min washes in TBST, the membranes were incubated for 5 min in Super Signal West-Dura Extended Duration Substrate (Pierce). Chemiluminescent signal was recorded using an LAS-4000 (Fuji).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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