Supplemental Material

Supplemental Figures 1 to 7
**Supplemental Figure 1** Confirmation in three further cell lines (A-C) that Ago-2 limits shRNA activity (see Figure 1, D and F for details). All values were normalized to a control lacking shRNA and RNAi proteins (not shown, set to 100%). (D) Ago-2 expression from various promoters consistently enhanced shRNA activity in Huh-7 cells, as compared to a control (C, not expressing any Ago protein). Wildtype Ago cDNAs were used in this panel. AH, liver/hepatoma-specific ApoE/hAAT enhancer/promoter.
Supplemental Figure 3  Alignment of wildtype (wt) and codon-optimized (opt) Ago-3.
Supplemental Figure 4 Alignment of wildtype (wt) and codon-optimized (opt) Ago-4.
Supplemental Figure 5 Validation of the key role of human Ago proteins and of their competition for shRNA activity. Co-transfection of individual Ago proteins, or of equimolar (1x) or sub-molar (0.2x) amounts of Ago-2 with the other three human Ago proteins, into cells (A) or mice (n = 3 to 5, day 2) (B). Also added were hAAT expression and H25 shRNA plasmids. Note the dominant positive effect of Ago-2 on shRNA activity that could only be out-competed with the other Agos when they were delivered in excess. N; non-shRNA-transfected control. (C) Kinetics of competition and hAAT knockdown in murine livers (n = 2) hydrodynamically transfected with hAAT expression plasmid, H25 shRNA and the indicated Ago combinations. In all assays (A-C), the presence of exogenous Ago-2 markedly enhanced shRNA activity, validating and extending the data in Figure 1.
Supplemental Figure 6 Competition of wildtype and cleavage-deficient Ago-2 for shRNA activation (confirmation with an alternative reporter, see Figure 1, G and H for details). Huh-7 cells were co-transfected with a Firefly reporter, luc29 shRNA and the indicated Ago constructs (*+* indicates a four-fold excess of competitor).
Supplemental Figure 7 Further evidence for varying Ago-2 dependency of RNAi triggers. (A) Figure 2, B-D suggested that shRNAs against imperfect 3'UTR targets depend less on Ago-2 than those against perfect targets (ORF or 3'UTR), at least under transient conditions. This was substantiated here in Ago-2 knockout cells (MEF/Ago-2^-/-), where anti-ORF \(D_{57}:T_{P}^{-}\)-H25 was inactive, while anti-3'UTR \(D_{57}:T_{P}\) and \(D_{57}:T_{I}\)-sh122 were similarly potent (compare red and orange bars in the absence of Ago-2 ('CMV')). Ago-2 co-delivery enhanced \(D_{57}:T_{P}^{-}\)-H25 and \(D_{57}:T_{P}^{-}\)-sh122, but not \(D_{57}:T_{I}\)-sh122 activities. This was further confirmed with another reporter (Firefly, B). (C) For evaluation with miRNAs, we transfected Ago-2-positive (+) or -negative (-) MEFs with \(D_{1}\)-miR-122 expression plasmid or anti-ORF \(D_{57}\)-shRNA (Ren3, same results were obtained with two other shRNAs, Ren1/2), together with luciferase reporters tagged with perfect or imperfect miR-122 sites. The fact that \(D_{1}:T_{P}\) and \(D_{1}:T_{I}\) miRNA activity were conserved in Ago-2^-/- cells in the absence of exogenous Ago-2 ('/-CMV'), while the control shRNA was inactive (as noted before, A,B above), validated that miRNAs principally act independently of Ago-2. Notably, \(D_{1}:T_{P}\) miRNA activity was consistently enhanced upon Ago-2 co-delivery both in wildtype and Ago-2^-/- cells. This implied that with artificial perfect targets, high miRNA levels (estimated \(>10^5\) copies / transfected cell) can also saturate Ago-2. This is in line with our in vivo data in Figure 2G and was further validated in 293 cells inducibly expressing \(\sim25,000\) miR-122 copies (293hcr), where \(D_{1}:T_{P}\) activity also increased with Ago-2 expression (D, purple). Mutant cells with an inactive miR-122 served as negative control (not shown). Again validating the data in Figure 2G, \(D_{1}:T_{I}\) activity was always unaffected by ectopic Ago-2, suggesting no Ago-2 saturation with imperfect triggers against imperfect targets (C,D, green bars). Alas, as discussed in the main text, this combination is not therapeutically useful.