**Figure legends**

**Figure 1. miRNA 3′-compensatory site features characterized using AGO-RBNS with programmed libraries.**

(A) Pairing requirements of 3′ supplementary (left), and 3′ compensatory (right) sites. 3′ supplementary sites (left) have at least six nucleotides of contiguous Watson–Crick pairing (blue) to the miRNA seed region (red), sometimes with an A opposite miRNA nucleotide 1, or an additional match to miRNA nucleotide 8, in addition to pairing (green) to miRNA 3′ end (yellow), typically centered around miRNA nucleotides 13–16. 3′-compensatory sites (right) contains fewer than six nucleotides of contiguous Watson–Crick pairing to the seed region (due to a mismatch, wobble, or single-nucleotide bulge) with additional pairing in 3′ region, typically centered around positions 13–16. N denotes any nucleotide, and vertical lines denote Watson–Crick pairing.

(B) Four independent features comprising the diversity of possible 3′-compensatory sites. These are 1.) the position of the target pairing within the miRNA 3′ end (top left), 2.) the extent of the target pairing, measured in the number of contiguous nucleotides pairing to the miRNA 3′ end, 3.) the offset between the seed pairing and 3′ pairing, where offsets of +1, 0, and −1 indicate one more unpaired target nucleotide, an equal number of target nucleotides, and one fewer unpaired target nucleotide, respectively, in comparison to the number of unpaired miRNA nucleotides separating the seed- and 3′- paired segments, and 4.) the position and identify of the mismatched target nucleotide within the seed pairing (bottom right).

(C) Programmed, miRNA-specific AGO-RBNS RNA libraries. The libraries contain an 8-nt region with all 18 possible single-nucleotide mismatches to nucleotides 2–7 for a particular miRNA (shown here for let-7a), downstream of 25 nucleotides of randomized sequence. Each library is generated by combining the transcription products of six synthetic DNA libraries that each contain a single mismatch position (pink). (D, not C; V, not U; and B, not A, N, any nucleotide).

(D) Enrichment of top 20 positional 8-nt *k*-mers in the highest concentration AGO-RBNS reaction performed with AGO2–let-7a and the let-7a-specific programmed library. *k*-mers are ranked by the sum of their top five enrichments at each position across the sequencing read. Left, *k-*mers are aligned by the CAAC motif shared by all 20 *k*-mers, with nucleotides that are not a Watson–Crick match to the miRNA sequence shown in red. The x-axis is numbered to indicate the position of the 3′-most nucleotide of the *k-*mer relative to the pairing position of the miRNA nucleotide 1 in the programmed region of the target sequence, such that an 8-nt *k*-mers at the 5′-most 3′-most positions preceding the programmed site in the target library correspond to positions 26 and 9, respectively. Above shows the pairing corresponding to the *k-*mer–position pair with the greatest enrichment.

**Figure 2.** **Let-7a relative affinity measurements of compensatory target sites.**

(A) Emperical cumulative distribution of relative *K*D values for 3′-compensatory sites aginst let-7a, spanning extents of pairing of 4 contiguous (goldenrod) nucleotides to 11 contiguous nucleotides (dark blue). Each relative *K*D value corresponds to a single position (e.g., nucleotides 13–16) and offset (e.g., +1), and is calculated by summing the read counts of all 18 possible seed-mismatch types at the programmed region of the library. The distribution of sites with an extent of 3′ pairing <4 consists the relative *K*D values of each of the 18 seed-mismatch types calculated separately.

(B) Relative *K*D values of 3′- compensatoy sites of let-7a sites varying both the position and extent of 3′ pairing. At the left, the pairing position with the greatest affinity is plotted for each extent of pairing, spanning the full series of offsets for which the relative *K*D of that pairing position was measured. Vertical lines indicate 95% confidence intervals. Thedashed horizontal line indicates the gometric mean of the 18 relative *K*D values of the seed mismatch sites, each calculated from reads with fewer than 4 nucleotides of complementarity to the miRNA 3′ end. At the right, each of the highest-affinity ranges of pairing is shown in the context of the seed (red) and 3′ supplemental (yellow) sequence of let-7a.

(C) Affinity profile of the contribution of nucleotides 9–21 of let-7a. Each cell represents the fold-change between the relative *K*D of a particular position, extent, and offset of pairing with that of the geometric mean of 18 relative *K*D corresponding to the seed mismatch sites in the programmed library. Each row corresponds to a different starting beginning 5′ nucleotide of pairing, each column corresponds to a different ending 3′ of pairing, and each individual heatmap corresponds to a different offset. The two pairing diagrams indicate the fold-change in relative *K*D contributed by pairing to the 3′-supplemental region (left), in comparison to greatest fold change measured for let-7a, at the same offset. Gray boxes indicate pairing ranges were either too short (<4 nt) or too long (>11 nt) for relative *K*D values to be reliably calculated.

**Figure 3.** **Relative affinity measurements of compensatory target sites of miR-1 and miR-155.**

(A) Empirical cumulative distribution of relative *K*D values for 3′-compensatory sites aginst miR-1. Relative *K*D calculations and plot features are as in Figure 2A.

(B) Relative *K*D values of 3′-compensatory sites of miR-1 sites varying both the position and extent of 3′ pairing. Everything is as in Figure 2B.

(C) Empirical cumulative distribution of relative *K*D values for 3′-compensatory sites against miR-155. Relative *K*D calculations and plot features are as in Figure 2A.

(D) Relative *K*D values of 3′-compensatoy sites of miR-155 sites varying both the position and extent of 3′ pairing. Everything is as in Figure 2B.

**Figure 4. Distinct pairing position and offset preferences between different miRNAs**

(A–C) Pairing and offset preferences describing the differences in compensatory pairing between let-7a (A), miR-1 (B), and miR-155 (C). For each miRNA, the binding preferences are decomposed into a set of pairing-range (left) and offset (middle-left) parameters recapitulating the full distribution of *K*D fold-change (middle-right and right, and Figure 2C). The parameters were obtained by maximum-likelihood estimation with a nonlinear binding model. For both miR-1 (B) and miR-155 (C), two pairing diagrams indicate the fold-change in *K*D contributed by pairing to the 3′-supplementary region (upper panel) in comparison to greatest fold change measured for that miRNA at the offset with the greatest coefficient (lower panel).

(D) Predicted ∆*G* values for each of the pairing ranges for which pairing coefficients were calculated in A–C. For each pairing range, the reported ∆*G* value represents the average of that between the miRNA sequence beginning at nucleotide 9, and all sequences of the same length bearing contiguous Watson–Crick complementarity to the miRNA within that range and non-complementarity elsewhere.

(E) The relationship between the model-derived pairing coefficients (A, B, and C, left) and the predicted ∆*G* values (D) for all possible pairing ranges ≥4 nt and ≤11 nt, for all three miRNAs. In order to query this relationship without the confounding effects of length, pairing coefficient was divided by the geometric mean value of all pairing coefficients of the same length, and the predicted ∆*G* values were similarly normalized by subtracting the mean ∆*G* value over all pairings of the same length. The gray interval represents the 95% confidence interval of the relationship when fitting a linear model of the log-transformed pairing coefficients as a function of the ∆∆*G* values, and the dashed line represents the predicted thermodynamic relationship given by *K* = *e*−*G*/*RT*. The *r*2 reports on coefficient of determination between the log-transformed pairing coefficients and the ∆∆*G* values.

(F) Distinct seed-mismatch preferences for let-7a, miR-1, and miR-155. For all three miRNAs, these parameters were derived by maximum-likelihood estimation, fitting a nonlinear model to the *K*D fold-change values when further subdividing the read counts into all sixteen possible seed-mismatch types, for each of the *K*D fold-change values initially calculated (Figure 2C and Figure S2). The letter and number beneath each bar denote the position of pairing with respect to the miRNA as well as the target nucleotide identity. Wobble pairing configurations in which the G nucleotide is located in either the miRNA or the target are denoted in blue and red, respectively.

**Figure 5. AGO-RBNS performed with chimeric miRNAs demonstrates that both the range and offset preferences are independent of seed-mismatch preferences.**

(A) Sequences of native let-7a, native miR-155, a chimeric miRNA containing the seed of let-7a appended to nucleotides 9–23 of miR-155 (let-7a–miR-155), and a chimeric miRNA containing the seed of miR-155 appended to nucleotides 9–21 of let-7a (miR-155–let-7a).

(B–C) Pairing and offset preferences describing the 3′ compensatory pairing of the let-7a–miR-155 (B) and miR-155–let-7a (C) chimeric miRNAs. Everything is as in Figure 4A.

(D) Comparison of the pairing (left) and offset (right) coefficients determined for the let-7a–miR-155 chimeric miRNA with that of miR-155. Each pairing coefficient (left) was divided by the geometric mean value of all pairing coefficients of the same length for that miRNA. Points are colored according to the extent of pairing as in Figure 2A–B, The offset coefficients (right) are colored from light blue to dark blue progressing from offsets of −4 to +16. Each *r* value reports on the Pearson correlation coefficient between either the pairwise log-transformed, length-adjusted pairing coefficients (left), or the offset coefficients (right), between the chimeric and the native miRNA.

(E) Comparison of the pairing and offset coefficients determined for the miR-155–let-7a chimeric miRNA with that of let-7a. Everything is as in (D).

(F) Comparison of the pairing and offset coefficients determined for the let-7a–miR-155 chimeric miRNA with that of let-7a. Everything is as in (D).

(G) Comparison of the pairing and offset coefficients determined for the miR-155–let-7a chimeric miRNA with that of miR-155. Everything is as in (D).

(H) Seed-mismatch preferences of the let-7a–miR155 (left) and miR-155–let-7a (right) chimeric miRNAs. Everything is as in Figure 4F.

(I) Correspondence of seed-mismatch preferences between chimeric miRNAs and their seed-native miRNAs. For let-7a–miR-155 (left) and miR-155–let-7a (right), the corresponding values from H are plotted against those of Figure F4, left and right, respectively. Everything else is as in Figure 4F. Each *r*2 value reports on the coefficient of determination between the chimeric and native miRNA–derived seed mismatch values.

**Figure 6. Let-7a variants sequences with permuted 3′ sequences demonstrate that pairing and offset preferences track with the position of relevant miRNA nucleotides.**

(A) Sequences of let-7a(−1), which contains a 3′ end permuted one nucleotide toward the 5′ end, native let-7a, and let-7a(+1),which contains a 3′ end permuted one nucleotide toward the 3′ end. The seed and 3′ supplementary regions of each miRNA are indicated in red and yellow, respectively. The permuted sequence shared between all three miRNAs is shaded in blue, and the A and U nucleotides that are rearranged to generate the variant let-7a sequences are in blue and purple, respectively.

(B–D) Pairing and offset preferences describing the 3′ compensatory pairing of the let-7a(−1) variant miRNA (B), let-7a (C), and the variant let-7a(+1) miRNA (D). Everything is as in Figure 4A.

(E) Cross-correlation of the offset coefficient series for either of let-7a(−1) (blue) or let-7a(+1) (red) with that of let-7a (B–C, middle-left), as a function of the number of displaced nucleotides between the variant-sequence miRNA series and the let-7a series.

(F) Seed-mismatch preferences of let-7a(−1) (left), let-7a (middle), let-7a(+1) (right). Everything is as in Figure 4F.

**Figure 7. The impact of mismatched, bulged, and deleted target nucleotides on 3′ compensatory pairing.**

(A) The effect of mismatched, bulged, and deleted target nucleotides on 3′ compensatory pairing for let-7a. At the top is a schematic depicting each of the highest-affinity ranges of pairing for let-7a, reproduced from Figure 2B. At the bottom left are plots corresponding to each of the pairing ranges shown above, allowing all four possible nucleotide identities at each of the internal positions within the site (e.g., positions 11–19 when considering pairing at positions 10–20). Those mismatches that couldn’t be unambiguously calculated due to overlap with another type of site (e.g., the mismatched U across from position 14 within the context of pairing at positions 10–20 is indistinquishable from 6mer-m8 seed site) are represented in gray. At the bottom right are plots corresponding to the effects of bulged or deleted target nucleotides on 3′ compensatory pairing for let-7a, for each of the pairing ranges shown above. “Del” represents the deletion of the target nucleotide pairing to that miRNA position within the target site. A bulged target nucleotide at position *n* corresponds to having a target nucleotide unpaired between those pairing to miRNA positions *n* – 1 and *n*.

(B) The effects of mismatched, bulged, and deleted target nucleotides for miR-1. Everything is as in A.

(C) The effects of mismatched, bulged, and deleted target nucleotides for miR-155. Everything is as in A.