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Unusually effective microRNA targeting within repeat-rich coding regions of mammalian mRNAs

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MicroRNAs (miRNAs) regulate numerous biological processes by base-pairing with target messenger RNAs (mRNAs), primarily through sites in 3′ untranslated regions (UTRs), to direct the repression of these targets. Although miRNAs have sometimes been observed to target genes through sites in open reading frames (ORFs), large-scale studies have shown such targeting to be generally less effective than 3′ UTR targeting. Here, we show that several miRNAs each target significant groups of genes through multiple sites within their coding regions. This ORF targeting, which mediates both predictable and effective repression, arises from highly repeated sequences containing miRNA target sites. We show that such sequence repeats largely arise through evolutionary duplications and occur particularly frequently within families of paralogous C2H2 zinc-finger genes, suggesting the potential for their coordinated regulation. Examples of ORFs targeted by miR-181 include both the well-known tumor suppressor RB1 and RBAK, encoding a C2H2 zinc-finger protein and transcriptional binding partner of RB1. Our results indicate a function for repeat-rich coding sequences in mediating post-transcriptional regulation and reveal circumstances in which miRNA-mediated repression through ORF sites can be reliably predicted.

[Supplemental material is available for this article.]
Bartel 2009), suggesting that targeting might be substantial if a gene contained many sites in its coding region, as could arise from coding-sequence repeats. Repeats of various types are prevalent within many human genes. Particularly widespread are simple classes of repeats, such as microsatellites, which have been shown to cause important phenotypic effects through action at both the nucleotide and protein levels (Mirkin 2007). Of interest in the present study are repeated sequences of greater complexity, a striking case of which occurs within the C2H2 class of zinc-finger genes. These genes have undergone extensive expansion over the course of vertebrate evolution and constitute the largest group of human transcription factors (Ding et al. 2009). Typically, C2H2 genes contain a significant number of tandemly repeated C2H2 amino acid domains, each of which coordinates a zinc ion and has the potential to bind DNA in a sequence-specific manner (Wolfle et al. 2000). The emergence of highly repeated amino acid domains creates instances of highly repeated short nucleotide sequences in a large fraction of such genes.

Here, we show that the ORFs of many repeat-rich genes contain strikingly large numbers of target sites of particular miRNAs. Moreover, these genes with many sites are frequently strongly repressed. For seven miRNA families, this type of targeting appears to be extensive. In the most notable cases, four miRNA families (miR-23, miR-181, miR-188, and miR-199) have seed sites that match repeated sequences within C2H2 zinc-finger genes. Three others (miR-370, miR-766, and miR-1248) have seed sites that match simpler repeats. Effective targeting of coding-region repeats is highly predictable, and, due to the large number of target sites within a single ORF, down-regulation observed in reporter assays can be stronger than that of many 3’ UTR targets. For the C2H2 class of zinc-finger genes, targeting is shared among paralogous genes, suggesting the potential for their coordinate regulation. miRNAs of both RB1 and its accessory protein, RBAK, are targeted by miR-181 primarily through ORF sites, suggesting an unappreciated role in carcinogenesis for miR-181, which has previously been implicated in hematopoiesis and in tumorigenesis through its regulation of NFκB activity (Chen et al. 2004; Iliopoulos et al. 2010), and underscoring the potential importance of ORF sites in understanding miRNA biology.

Results

miR-181 represses mRNAs with repeated ORF sites

While re-analyzing previously published microarray data from miR-181a transfection in HeLa cells (Baek et al. 2008), we observed that some of the most strongly down-regulated miRNAs contained miR-181 seed sites in their coding regions. We found this result intriguing because, although miRNA targeting has been observed in ORFs, it usually confers only subtle repression. Further investigation revealed that the enrichment for strong down-regulation was largely confined to a group of miRNAs containing numerous miR-181 sites (Fig. 1A,B). Genes that contained a single 8mer site in their coding region were only slightly, though statistically significantly, down-regulated (mean log2 fold-change: \(-0.07; p < 2 \times 10^{-3}\); Mann-Whitney U-test) and significantly less so than those with a single 8mer site in their 3′ UTR (mean log2 fold-change: \(-0.07\) vs. \(-0.17; p < 0.01\); Mann-Whitney U-test). However, those genes with an increasing number of ORF sites showed increasingly strong down-regulation. In particular, the 52 genes with at least four 8mer ORF sites were nearly all repressed and, even when restricted to the subset of 18 of 52 genes with no 3′ UTR sites, showed significantly stronger repression than those genes with a single 8mer 3′ UTR site (mean log2 fold-change: \(-0.51\) vs. \(-0.17; p < 5 \times 10^{-3}\); Mann-Whitney U-test). Many of these genes had even more than four 8mers sites as well as equally large numbers of 7mer sites (Supplemental Table 2). Perhaps most surprising, considering the strikingly large number of sites, was not that such genes were strongly down-regulated, but that genes should have so many sites to a single miRNA. Indeed, miR-181 was exceptional in this regard (Fig. 1C). Whereas for most miRNAs, few or no genes contained large numbers of 8mer sites, for miR-181 there were many such genes.

Having observed the impact of miR-181 on a large class of endogenous transcripts, we aimed to confirm the direct, miRNA-mediated repression of a number of specific genes. To do so, we generated reporter proteins in which firefly luciferase was fused in-frame to the C terminus of the protein product of each of five genes: ZNF573, ZFP37, ZNF20, ZNF791, and RBAK (Fig. 1D). These genes contained nine, eight, six, five, and nine miR-181 8mer sites and two, seven, two, one, and ten miR-181 7mer sites, respectively. Repression by miR-181a was evaluated by comparing normalized luciferase values from cells cotransfected with miR-181a to those with miR-23a, a noncognate miRNA. For each gene tested, we observed significant and robust repression by miR-181a (\(p < 10^{-6}\); Mann-Whitney U-test; Fig. 1D). To confirm that the measured signal in each case was coming from the full-length fusion proteins, we disrupted the reading frame register by inserting or deleting one nucleotide in the zinc-finger coding sequence of the miRNA (within a few hundred nucleotides of the start codon), upstream of both the miRNA sites and the firefly luciferase sequence. As expected, these frame shift mutations significantly reduced luciferase activity for each fusion construct (Supplemental Fig. 1).

To confirm that the observed repression was directly mediated by the ORF sites, we generated a ZNF20-luciferase mutant in which each of the six miR-181 8mer and two miR-181 7mer seed sites within the ORF were mutated with two synonymous point substitutions. Compared with this mutated construct, the wild-type ZNF20 reporter was significantly and specifically repressed by miR-181a (\(p < 10^{-6}\); Fig. 1E). The repression attributed to these sites increased from 2.5-fold to 6.3-fold when the fragment containing the sites was incorporated as part of the reporter 3′ UTR (Supplemental Fig. 2A). This difference between the efficacy of ORF sites and 3′ UTR sites was significant (\(p < 10^{-10}\)) and consistent with the general observation of ORFs being more refractory to miRNA targeting than are 3′ UTRs (Grimson et al. 2007).

Similarly, we tested whether the repression of RBAK, observed in our luciferase assays (Fig. 1D) and in the previous microarray study (Baek et al. 2008), was directly mediated by its miR-181 sites. In addition to its 19 ORF sites (nine 8mers and 10 7mers), the 3′ UTR of RBAK contains a conserved 7mer-m8 site and a poorly conserved 7mer-A1 site (Friedman et al. 2009). A panel of constructs was generated, expressing either the RBAK ORF with a C-terminal luciferase tag or the RBAK 3′ UTR following the luciferase reporter (Fig. 1F). Although the 3′ UTR sites mediated statistically significant repression (1.3-fold; \(p = 0.0009\)), the ORF sites gave far stronger repression (3.2-fold repression; \(p < 10^{-6}\)). As with ZNF20, the repression mediated by these ORF sites increased significantly when they were incorporated as part of the reporter 3′ UTR (\(p < 10^{-4}\); Supplemental Fig. 2B). When both the ORF and 3′ UTR sites were monitored in combination, 3.3-fold repression was observed (\(p < 10^{-6}\); Supplemental Fig. 3), which was not significantly different from that observed with the ORF sites alone (\(p = 0.59\)). Although further experiments will be required to understand why additional repression was not observed in the combined reporter, these results indicate that the repression of RBAK...
by miR-181a was direct and that the majority of the miR-181a-mediated repression of RBAK was due to targeting through its ORF sites.

Additional miRNA seeds match repeated coding-region motifs

To find other miRNAs that might affect ORF targets similarly, we searched for all 8mers highly repeated within human ORFs, and for each 8mer we counted the number of nonoverlapping occurrences within each ORF. Because four ORF sites mediated robust repression by miR-181, we chose this as a threshold, and for each 8mer recorded the number of genes with four or more instances of that 8mer (Fig. 2A). The majority (77%) of 8mers did not occur four or more times in any coding region, and the vast majority (97%) occurred four or more times in only five or fewer coding regions. At the tail of the distribution, 334 8mers appeared at least four times in at least 25 genes, among which were seven miRNA 8mer seed families (Table 1). For each of the seven corresponding miRNA seed families, we compiled potential target sets containing genes with at least four 8mer sites (Supplemental Tables 1–7).

Based on the sets of predicted targeted genes, these miRNA families split into two main groups (Fig. 2B). For four of the miRNAs (miR-23, miR-181, miR-188, and miR-199), the target sets were almost entirely C2H2 zinc-finger genes, and sites in these genes mostly occurred within tandemly repeated C2H2 amino acid domains. For the other three annotated miRNAs (miR-370, miR-766, and miR-1248), the predicted targets were more varied, and sites largely occurred within highly prevalent amino acid pairs or triplets, though in some cases they occurred within long stretches of simple...
nucleotide repeats. Because of their limited conservation, low expression levels and questionable status as authentic miRNAs (Chiang et al. 2010; Kozomara and Griffiths-Jones 2011), two of these three miRNAs (miR-766 and miR-1248) were not considered further.

The most common form of the repeated C2H2 amino acid domain is XCX[2]CX[12]HX[3]H (X represents any amino acid) (Emerson and Thomas 2009). Typically, C2H2 zinc-finger genes contain many tandem repeats of the finger motif (8.5 on average in humans), which are connected by a specific linker sequence most commonly of the form TGEKPY (Emerson and Thomas 2009). The 8mer sites for the four miRNA families each occurred within specific amino acid realizations at specific locations in this motif (Fig. 2C). Because of the large numbers of paralogous motifs within a single gene, the number of seed sites can be large. A typical example is the gene RBAK, which contains 14 C2H2 domains, eight miR-181 8mers and 10 miR-181 7mers (Fig. 2D).

Predicted ORF targets are subject to miRNA-mediated repression

From the list of predicted miR-23 targets (Supplemental Table 1), we chose three genes (ZNF225, ZNF486, and ZNF85) for experimental follow-up. Fusion constructs with a C-terminal luciferase tag were made as before, and disruption of the reading frame by a nucleotide insertion substantially decreased luciferase expression, confirming that the majority of signal came from the full-length protein (Supplemental Fig. 1). The constructs were transfected in the presence of miR-23a or a noncognate miRNA, miR-181a (Fig. 3A). For all three targets, normalized luciferase values were significantly reduced in the presence of miR-23a when compared with those with the noncognate miRNA (p < 10^{-6}). The magnitudes of repression were particularly notable because miR-23 has high target abundance and thus generally weaker targeting efficacy (Arvey et al. 2010).

We extended this analysis to predicted targets of either miR-199 or miR-370. ZNF20 and ZNF791, experimentally supported targets of miR-181 (Fig. 1), also contain multiple seed matches to miR-199 and were examined for their response to miR-199a (Fig. 3B). ZNF791, which contains six miR-199 8mer sites and one 7mer site, was significantly repressed (p < 10^{-5}). Similarly, in the case of miR-370, we probed the response of two targets, IVL and HDAC5, both of which were significantly repressed (p < 10^{-7} and p = 0.009, respectively; Fig. 3C). When we generated a mutant IVL construct, wherein all miR-370 sites were disrupted, we observed significant, direct repression by miR-370 (p < 10^{-4}; Fig. 3D). In contrast, the luciferase control was either unaffected by these miRNAs or, in the case of miR-199a, significantly less repressed than the ZNF791-luciferase fusion (p = 0.0003; Supplemental Fig. 4). Taken together, although individual ORF sites are less effective than 3’ UTR sites (Grimson et al. 2007), these results indicate that highly repetitive

Table 1. Annotated miRNAs with repeated coding-region sites in many genes

<table>
<thead>
<tr>
<th>Family</th>
<th>miRNAs</th>
<th>8mer site</th>
<th>miRNA conservation</th>
<th>Genes with ≥4 sites</th>
<th>Target family</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-23</td>
<td>miR-23a/b</td>
<td>AATGTGAA</td>
<td>Vertebrates</td>
<td>43</td>
<td>C2H2 zinc fingers</td>
</tr>
<tr>
<td>miR-181</td>
<td>miR-181a/b/c/d</td>
<td>TGAATGTA</td>
<td>Vertebrates</td>
<td>75</td>
<td>C2H2 zinc fingers</td>
</tr>
<tr>
<td>miR-188</td>
<td>miR-188-3p</td>
<td>TGTTGGA</td>
<td>Mammals</td>
<td>210</td>
<td>C2H2 zinc fingers</td>
</tr>
<tr>
<td>miR-199</td>
<td>miR-199a/b-5p</td>
<td>ACACGGA</td>
<td>Vertebrates</td>
<td>82</td>
<td>C2H2 zinc fingers</td>
</tr>
<tr>
<td>miR-370</td>
<td>miR-370</td>
<td>CAGCGAGA</td>
<td>Mammals</td>
<td>25</td>
<td>Varied</td>
</tr>
<tr>
<td>miR-766</td>
<td>miR-766</td>
<td>GCTGGAGA</td>
<td>Human</td>
<td>27</td>
<td>Varied</td>
</tr>
<tr>
<td>miR-1248</td>
<td>miR-1248</td>
<td>AAGAAGGA</td>
<td>Human</td>
<td>34</td>
<td>Varied</td>
</tr>
</tbody>
</table>

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ORFs containing many miRNA sites can generally be subject to significant and, in some cases, substantial repression by the cognate miRNA.

miRNAs target multiple paralogous families of C2H2 zinc-finger genes

Having observed the extent and, in some cases, surprising magnitude of targeting arising from ORF repeats, we considered the evolutionary processes giving rise to this phenomenon. Our focus was on C2H2 zinc-finger genes because these formed the most dramatic and frequent instances of this phenomenon. Among the predicted targets, most C2H2 genes (>80%) contained the general transcriptional repressor KRAB domain (Fig. 4A). KRAB-containing C2H2 genes display particularly interesting patterns of evolution, having high rates of gene duplication and loss as well as a dramatic expansion over the course of vertebrate and mammalian evolution (Huntley et al. 2006). To understand the role that these duplications played in forming miRNA target sets, we collected sequences of all KRAB domains annotated in human and used these to create a multiple alignment of KRAB domains. From this alignment, the inferred phylogeny of KRAB-containing genes provided a context for considering the four miRNA target sets (Fig. 4B; Supplemental Fig. 5). For this analysis, we again defined each target set as those genes containing at least four 8mer sites for the given miRNA.

The analysis revealed that the four miRNAs target multiple clades, each of which has emerged from significant expansions through gene duplication. Combined over the four miRNA families, the target sets covered the majority of KRAB-containing C2H2 genes with varying amounts of overlap between each set (Fig. 4C). Whereas the miR-188 target set spanned nearly the entire phylogeny, the other three miRNAs each targeted more specific subfamilies of genes. For instance, the miR-23 targets were nearly all members of one clade: the recently duplicated ZNF91 subfamily, which has undergone a significant expansion in the primate lineage (Hamilton et al. 2006).

Sequence analysis suggested that two duplication processes contributed to the creation of such extensive repeat-containing subfamilies. In one process, individual C2H2 domains were duplicated multiple times within a single zinc-finger gene. In a second process, genes were duplicated to form an extensive gene subfamily with high nucleotide similarity among many members. Due to the intragenic duplication process, the sequences of individual zinc fingers within a gene were far more similar to each other than expected by chance. To verify the importance of this effect, we implemented a randomization procedure for nucleotide sequences within C2H2 domains that preserved amino acid sequences and average codon usage over all domain instances. Even when fixing the observed amino acid sequences, real instances of C2H2 domains from within the same gene showed significantly higher nucleotide similarity than expected by chance (Supplemental Fig. 6). When C2H2 domains with randomized sequences were mapped back to genes, far fewer of these genes contained large numbers of miRNA sites than did the real genes (Fig. 5A). It was tempting to speculate that the similarity in nucleotide sequences across C2H2 domains within a gene represented an additional selective pressure to maintain miRNA seed sites in these genes. We observed marginal evidence for this model but could not detect such an effect at high confidence (data not shown).

The initial intragenic duplication of individual C2H2 domains allowed for the founding nucleotide-sequence choice to be amplified. After repeated duplication, a gene would ultimately include either many sites or no sites at all, depending on the presence or absence of a target site within a founding C2H2 domain. Such duplication contributed to the modularity of miRNA target sets; even for subfamilies of genes with C2H2 domains containing similar amino acid sequences, genes from one subfamily often contain many target sites, whereas those from another contain almost none. For example, although miR-188 sites are prevalent within which the miR-188 seed site appears. Such domains within a gene represented an additional selective pressure to maintain miRNA seed sites in these genes. We observed marginal evidence for this model but could not detect such an effect at high confidence (data not shown).
Discussion

In animals, miRNAs target many genes through sites in their 3′ UTRs but cause only modest repression of most of these targets. Compared with this generally modest targeting within 3′ UTRs, most targeting within ORFs is substantially weaker still. Nonetheless, we have shown that significant numbers of substantially repressed ORF targets exist and that such targets can be easily identified by the presence of large numbers of sites to the same miRNA. Recently, a similar observation of ORF targeting was independently observed to occur in the case of a single miRNA (Huang et al. 2010). Our work, going significantly further, provides a systematic examination of this phenomenon and gives the first bioinformatic evidence and experimental verification of the widespread nature of this type of targeting. Indeed, we have found that this targeting involves multiple miRNAs and, likely, hundreds of genes. While some of the target sites we have identified show the potential for supplemental 3′ base-pairing to the miRNA, none exhibit the extensive complementarity required for cleavage of the miRNA. It therefore appears likely that the mechanism of repression for these genes is identical to that for most 3′ UTR targets.

Interestingly, although there are examples of both 5′ and 3′ UTRs containing repeats, none of the highly repeated motifs matched known miRNA seeds, which indicates that repeat-mediated targeting is largely ORF specific. Moreover, although repeats in coding regions are prevalent in numerous animal clades, the presence of miRNA sites within these repeats appeared to be vertebrate-specific. Here, we focused on those miRNAs with at least four sites in large sets of genes, but there were many other miRNAs with at least this many sites in a handful of genes (Fig. 1). In addition, many genes contained multiple ORF sites to multiple miRNAs. The eventual determination of expression patterns for these miRNAs at cellular resolution should enable prediction of additional targets in which ORF sites combine to achieve substantial repression.

The most striking cases of repeat-rich targeting occurred within the KRAB-domain C2H2 zinc-finger genes, which constitute the largest collection of human transcription factors. Our results indicate that a single miRNA can target entire families of these genes, thereby simultaneously regulating large numbers of evolutionarily related transcription factors. The targeting of such a large number of transcription factors by a miRNA has the potential to cause significant and widespread downstream effects. Through phylogenetic analysis we have shown how repeat-mediated targeting arises in these genes under an extensive evolutionary duplication process. Analysis of KRAB-domain-containing families indicates positive selection toward diversification of DNA-binding residues (Emerson and Thomas 2009). Hence, following duplication, individual genes frequently gain new downstream regulatory roles, while most members of the family retain the potential for upstream miRNA-mediated regulation. With a few exceptions, the functions of KRAB-domain genes remain unknown (Huntley et al. 2006). While a few show tissue-specific expression, most are widely expressed (Vaquerizas et al. 2009), and most predicted target genes appear to have expression patterns overlapping that of the corresponding miRNA (data not shown), suggesting that the targeting described here is likely to affect their in vivo expression.

One ORF target of miR-181, RBAK, is reported to function as an RB1-associated transcriptional repressor (Skapek et al. 2000). Suggestive of coordinate regulation, RB1, which encodes the well-characterized tumor-suppressor protein (Hanahan and Weinberg 2000; Classon and Harlow 2002), also contains sites to miR-181 in both its ORF (two 8mers and one 7mer) and 3′ UTR (one non-conserved 7mer). Although no repression was observed for the 3′ UTR site, significant repression by miR-181a was observed for the RB1 ORF-luciferase fusion (1.4-fold; p < 10−6; Supplemental Fig. 7). This newly recognized miR-181 targeting of RB1 and RBAK had not been appreciated from previous analyses focusing only on 3′ UTR

Figure 4. MicroRNA targeting of paralogous C2H2 genes. (A) Diagram of domain structure of C2H2 zinc-finger genes. (B) The relationship between shared ancestry of KRAB-containing C2H2 genes and shared miRNA sites. The phylogeny inferred from alignment of the KRAB domains is shown, marking at the perimeter those with at least four 8mer sites to the indicated miRNA. (C) Overlap between the predicted targets of the indicated miRNAs.
sites and is intriguing when considering that miR-181 is up-regulated in some cancers and is important for maintaining cancer stem cells in hepatocellular carcinoma (Schetter et al. 2008; Cervigne et al. 2009; Ji et al. 2009). Even transient induction of miR-181b is sufficient to mediate an epigenetic switch to cancer, and inhibition of miR-181b reduces colony formation in several cancer cell lines, observations proposed to result from direct targeting of the tumor-suppressor CYLD (Iliopoulos et al. 2010). Because both RB1 and RBAP repress activation of E2F-dependent promoters and decrease DNA synthesis (Skapek et al. 2000), the ability of miR-181 to repress RB1 and RBAP might provide an additional mechanism by which this miRNA mediates transformation.

Our work also suggests a general role of coding-sequence repeats in post-transcriptional regulation. For transcription regulation, the accumulation and clustering of multiple transcription factor binding motifs has been used for some time to predict functional regulatory relationships (Hannenhalli 2008). Given the large number of post-transcriptional regulatory processes that exist beyond miRNAs (Garneau et al. 2007; Parker and Sheth 2007) and the vast extent of sequence repeats within many protein-coding genes, miRNAs might not be the only regulatory process utilizing this phenomenon.

## Methods

### Luciferase assays

HEK293 cells (ATCC) were plated in 24-well plates and transfected 24 h later using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Sigma) with 50 ng of Renilla luciferase control reporter plasmid pIS1 (Grimson et al. 2007), 400 ng of firefly luciferase reporter plasmid, and 25 nM miRNA duplex (Supplemental Table 9) per well. After 12 h, transfection media was replaced with DMEM containing 10% fetal bovine serum and penicillin-streptomycin. Cells were harvested 48 h post-transfection. Luciferase activities were measured using dual-luciferase assays (Promega), as described by the manufacturer. Four to five biological replicates, each with three technical replicates, were performed. Firefly activity was first normalized to Renilla activity to control for transfection efficiency, and then normalized values were analyzed as described previously (Grimson et al. 2007). If a mutant construct was generated, Renilla-normalized firefly values were normalized to the geometric mean of values from the reporter in which the miRNA sites were mutated. To control for variability in plasmid preparation, the value plotted for each construct was the geometric mean of normalized firefly values from transfections with the cognate miRNA divided by the geometric mean of the control transfections.
by that from transfections with the noncognate miRNA. To combine replicate values from independent experiments for which no mutant construct was generated, each Renilla-normalized firefly value was normalized to the geometric mean of values from transfections with the noncognate miRNA. Statistical significance was determined using the Mann-Whitney U-test. Plasmids (deposited at Addgene) were constructed as described (see Supplemental Methods).

**Gene sequences**

ReSeq sequences for human ORFs were downloaded from the UCSC Genome Browser (www.genome.ucsc.edu), version hg19, Feb. 2009. In cases of multiple transcript variants for a single gene, one variant was chosen at random as a representative. 3′ UTR sequences were downloaded from the TargetScan website (www.targetscan.org), version 5.1. For analysis of k-mers and generation of target sets, only nonoverlapping k-mers were considered, and all transcripts with coding region length >10 kilobases were excluded. Note that there was a single nucleotide difference between the RAkA clone used in luciferase assays and the ReSeq sequence, resulting in an additional 8mer in the clone sequence. In Figure 2 and Supplemental Table 2, we referred to the counts from the ReSeq sequence.

**Microarray data**

The microarray data examining the response of introducing miR-181a into HeLa cells (GSM302995) (Baek et al. 2008) were analyzed using Agilent Feature Extraction software. Log2 fold-change values for genes were obtained by taking the median value of log2 fold-change for all probes against that gene (excluding any probes not flagged by the Feature Extraction software as “Well Above Background”). Down-regulation of a group of genes was taken as the mean of log2 fold-changes across the genes, with errors in these means estimated using 100 bootstrap trials.

**Phylogenetic reconstruction**

The sequences of all KRAB-A domain instances, as well as the UniProt identifier for the protein in which each domain occurred, were downloaded from the Pfam website (pfam.sanger.ac.uk), version 24, Oct. 2009. UniProt identifiers were mapped to the corresponding genes using conversion files from the HUGO Gene Nomenclature Committee (www.genenames.org). Of the resulting 311 genes containing KRAB-A domains, all but four (ZNF862, ZNF560, ZNF333, and ZFP28) contained a single copy of the domain. For those four genes, a single instance of the domain was chosen at random to use in the phylogenetic analysis. A multiple alignment was inferred on the amino acid sequences of the 311 KRAB-A domains, based on which a phylogenetic tree of the corresponding genes was reconstructed, using ClustalX software (version 2.0.12) under default parameter settings. Visualization of the tree and overlap with miRNA target sets was done using the Interactive Tree of Life software tool (itol.embl.de).

**Randomization of C2H2-domain sequences**

Instances of a C2H2 domain of the form XCX_{27}CX_{13}HX_{13}H, as well as the six residues N-terminal of this motif (to capture the linker sequence) were recorded from within all KRAB-containing genes. For each position in this 28-amino-acid motif, and each possible amino acid within this position, empirical codon usage frequencies combined across all C2H2 domains were calculated. For each instance of a C2H2 domain, randomized nucleotide versions of this C2H2 domain were generated by maintaining the amino acid sequence of each motif instance but randomly sampling with replacement from the empirical codon frequencies for each amino acid at each position in the domain. To generate C2H2 genes with randomized nucleotide sequences, the above randomization procedure was repeated for all of the C2H2 domains in the gene, and the randomized nucleotide sequences for each C2H2 domain were used to replace the true nucleotide sequences.

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The microarray data examining the response of introducing miR-181a into HeLa cells (GSM302995) (Baek et al. 2008) were analyzed using Agilent Feature Extraction software. Log2 fold-change values for genes were obtained by taking the median value of log2 fold-change for all probes against that gene (excluding any probes not flagged by the Feature Extraction software as “Well Above Background”). Down-regulation of a group of genes was taken as the mean of log2 fold-changes across the genes, with errors in these means estimated using 100 bootstrap trials.

**Phylogenetic reconstruction**

The sequences of all KRAB-A domain instances, as well as the UniProt identifier for the protein in which each domain occurred, were downloaded from the Pfam website (pfam.sanger.ac.uk), version 24, Oct. 2009. UniProt identifiers were mapped to the corresponding genes using conversion files from the HUGO Gene Nomenclature Committee (www.genenames.org). Of the resulting 311 genes containing KRAB-A domains, all but four (ZNF862, ZNF560, ZNF333, and ZFP28) contained a single copy of the domain. For those four genes, a single instance of the domain was chosen at random to use in the phylogenetic analysis. A multiple alignment was inferred on the amino acid sequences of the 311 KRAB-A domains, based on which a phylogenetic tree of the corresponding genes was reconstructed, using ClustalX software (version 2.0.12) under default parameter settings. Visualization of the tree and overlap with miRNA target sets was done using the Interactive Tree of Life software tool (itol.embl.de).

**Randomization of C2H2-domain sequences**

Instances of a C2H2 domain of the form XCX_{27}CX_{13}HX_{13}H, as well as the six residues N-terminal of this motif (to capture the linker sequence) were recorded from within all KRAB-containing genes. For each position in this 28-amino-acid motif, and each possible amino acid within this position, empirical codon usage frequencies combined across all C2H2 domains were calculated. For each instance of a C2H2 domain, randomized nucleotide versions of this C2H2 domain were generated by maintaining the amino acid sequence of each motif instance but randomly sampling with replacement from the empirical codon frequencies for each amino acid at each position in the domain. To generate C2H2 genes with randomized nucleotide sequences, the above randomization procedure was repeated for all of the C2H2 domains in the gene, and the randomized nucleotide sequences for each C2H2 domain were used to replace the true nucleotide sequences.
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