MicroRNA-Directed Cleavage of *HOXB8* mRNA

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MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs, some of which are known to play important regulatory roles in animals by targeting the messages of protein-coding genes for translational repression. We find that miR-196, a miRNA encoded at three paralogous locations in the A, B, and C mammalian HOX clusters, has extensive, evolutionarily conserved complementarity to messages of *HOXB8*, *HOXC8*, and *HOXD8*. RNA fragments diagnostic of miR-196–directed cleavage of *HOXB8* were detected in mouse embryos. Cell culture experiments demonstrated down-regulation of *HOXB8*, *HOXC8*, *HOXD8*, and *HOXA7* and supported the cleavage mechanism for miR-196–directed repression of *HOXB8*. These results point to a miRNA-mediated mechanism for the posttranscriptional restriction of HOX gene expression during vertebrate development and demonstrate that metazoan miRNAs can repress expression of their natural targets through mRNA cleavage in addition to inhibiting productive translation.

Nearly 1% of the predicted mammalian genes encode microRNAs (miRNAs) (*1*–*3*). As previously reported for miR-10 (*1*), genes for miR-196 (*1*, *2*) map to homeobox (HOX) clusters (Fig. 1 and table S1). HOX clusters are groups of related transcription factor genes crucial for numerous developmental programs in animals (*4*). Mammals have four HOX clusters (HOX A to D) containing a total of 39 genes organized into 13 paralogous subgroups (Fig. 1) (*4*).

The miR-196 miRNAs have intriguing complementarity to sites in the 3′ untranslated regions (3′ UTRs) of HOX genes representing each cluster. With the exception of a single G:U wobble, pairing between miR-196a and the human *HOXB8* 3′ UTR is perfect. The functional importance of this miR-196 complementary site is supported by its conservation in the fish and frog *HOXB8* 3′ UTRs, despite the divergence of surrounding UTR sequences (Fig. 2A). This conserved, near-perfect pairing suggested that, like natural miRNA targets in plants (*5*–*7*) or engineered miRNA targets in animals (*8*, *9*), *HOXB8* mRNA is targeted for cleavage. MicroRNA-directed cleavage can be detected by using a modified form of 5′-RACE (rapid amplification of cDNA ends) because the 3′ product of this cleavage has two unusual properties: (i) a 5′ terminus that is a suitable substrate, without further modification, for ligation to an RNA adaptor using T4 RNA ligase and (ii) a 5′ terminus that maps precisely to the nucleotide that pairs with the tenth nucleotide of the miRNA (*5*, *7*). Accordingly, we used this method to examine whether *HOXB8* mRNA was a natural target of miR-196–directed cleavage. Having determined that miR-196 is expressed during mouse embryogenesis starting at or before day 7 (*10*) (fig. S1), total RNA from day 15 to day 17 mouse embryos was chosen for this analysis. Of the eight 5′-RACE clones that ended in the vicinity of the miR-196 complementary site [i.e., within the 150-nucleotide (nt) segment centering on the complementary site], seven terminated precisely at the position diagnostic of miR-196–directed cleavage (Fig. 2A, the eighth terminated 41 nt downstream).

By analyzing RNA isolated from the mouse, the *HOXB8* 5′-RACE results validated this miRNA-target interaction in the animal, whereas previous experimental support for predicted mammalian miRNA targets has come from reporter assays in cultured cells (*11*). Moreover, this experiment detected miRNA-mediated cleavage of the targeted message, whereas, in the previously examined metazoan examples, translational inhibition had been the mechanism of endogenous miRNA-mediated repression (*12*, *13*). Nevertheless, the *HOXB8* 5′-RACE results do not rule out the possibility that the predominant mode of silencing is translational inhibition, as illustrated for miR172 regulation of *Arabidopsis APETELA2* (*7*, *14*, *15*). To explore the mechanism of *HOXB8* repression, the miR-196 complementary site from *HOXB8* or control complementary sites were placed into the 3′ UTR of the firefly luciferase reporter gene, and the reporter plasmid was cotransfected into HeLa cells together with a transfection control and either cognate or noncognate miRNAs. As expected, miR-196a inhibited luciferase expression from the construct with the complementary site from *HOXB8* mRNA (Fig. 2B). Inhibition was essentially the same as that observed for a reporter with perfect antisense complementarity to the miRNA (miR-196a-as), indicating that the conserved G:U wobble involving U5 of miR-196 (Fig. 2A) does not substantially decrease miRNA-directed inhibition. Accompanying this inhibition was a substantial decrease in the amount of reporter mRNA, again similar to that seen with perfect antisense complementarity (Fig. 2C), indicating that a large fraction of the miR-196–directed repression occurred through mRNA degradation.

Genes from each of the other clusters also appear to be miR-196 targets (Fig. 1). The *HOXA7* 3′ UTR has multiple conserved matches to residues 2 to 8 of miR-196, called "seed matches," which previously identified it as a likely miR-196 target (Fig. 3A) (*11*). *HOXC8* and *HOXD8* UTRs have both seed matches and more extensive complementary sites (Fig. 3A), although none of these sites resemble the HOXB8 site in having perfect pairing at their center (fig. S2). Segments from all three UTRs imparted miR-196–dependent repression to the luciferase reporter without a substantial decrease in reporter mRNA, indicating predominantly translational inhibition (Fig. 3, B and C). To the extent that these experiments in cell culture reflect regulation in animals, miR-196 represses its targets by two posttranscriptional mechanisms. As reported for engineered targets (*8*, *9*, *16*, *17*), the choice of mechanism appears to depend on

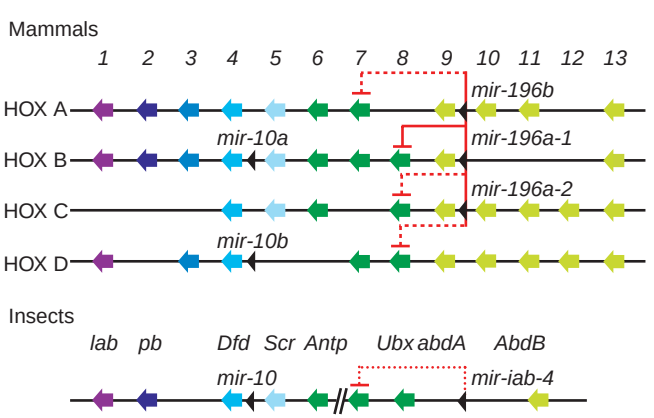

Fig. 1. Genomic organization of HOX clusters. Colored arrows indicate HOX genes representing 13 paralogous groups; black arrowheads depict miRNA genes. Repression supported by bioinformatic evidence only (dotted red line), cell-culture and bioinformatic evidence (dashed line), or in vivo, cell culture, and bioinformatic evidence (solid line) are indicated. The vertical red line indicates that miRNAs from any of the three loci could repress the targets.

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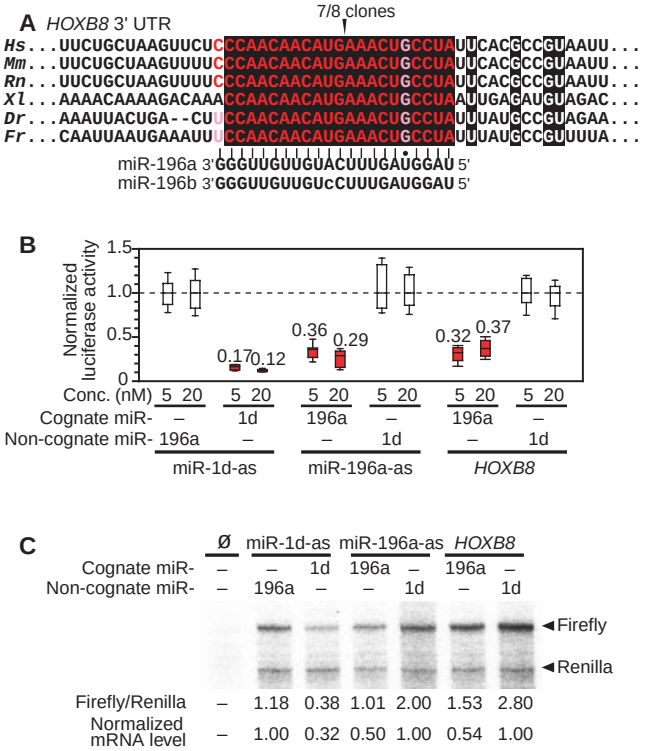

Fig. 2. MiR-196–directed cleavage of *HOXB8* mRNA. (**A**) Sequence alignment of the miR-196 complementary site in the 3′ UTRs of *HOXB8* genes (table S2). Absolutely conserved nucleotides are highlighted in black; those of the complementary site are either red (Watson-Crick pairing to miR-196a) or pink (G:U wobble). The vertical arrowhead indicates the 5′ end of cleavage products detected by 5′-RACE, with the frequency of clones noted. *Hs*, human; *Mm*, mouse; *Rn*, rat; *Xl*, frog; *Dr*, zebrafish; *Fr*, pufferfish. (**B**) Response to miR-196a in HeLa cells (*16*). Firefly luciferase reporters containing either the miR-196 complementary site from mammalian *HOXB8*, or the perfect antisense sequence of miR-196a or miR-1d (*HOXB8*, miR-196a-as, and miR-1d-as, respectively) were cotransfected with the indicated amount of cognate or noncognate miRNA (*10*). Firefly luciferase activity was normalized to that of the Renilla transfection control, then activity with cognate miRNA (solid) was normalized to the median activity with noncognate miRNA at the same concentration (open). Each box represents the distribution of activity measured for each reporter ($n = 9$; bars define 10th and 90th percentiles; box spans 25th and 75th percentiles; line and number indicate median normalized activity). In each case, the expression observed for the cognate miRNA significantly differed from that for the noncognate miRNA ($P < 0.001$, Mann-Whitney test). (**C**) Reporter mRNA levels monitored with an ribonuclease protection assay (*10*, *25*). Protected fragments of each probe are indicated (arrowheads), and were absent when cells did not receive reporter plasmid (∅). Intensity of firefly relative to Renilla bands (Firefly/Renilla) is shown and normalized to that of the noncognate miRNA. In three independent repetitions of this experiment, normalized mRNA levels were 0.35 ± 0.03, 0.53 ± 0.02, and 0.52 ± 0.03 for miR-1d-as, miR-196a-as, and *HOXB8*, respectively (±SD).

the nature of the complementary sites, with translational inhibition apparently requiring more complementarity sites but less pairing within each site.

For some HOX genes, posttranscriptional processes combine with complex transcriptional regulation to generate the pattern of HOX expression seen in early development (*18*, *19*). Our results indicate that miR-196 delimits or dampens the expression of *HOXB8* and probably also *HOXC8*, *HOXD8*, and *HOXA7*, thus pointing to additional posttranscriptional control of genes in the HOX clusters. The temporal and spatial order of HOX gene expression along the anterior-posterior axis is initially colinear with the physical position of the HOX genes on their chromosomes (*4*, *20*). If the same is true for *mir-196* genes, their locations suggest that they would initially be transcribed slightly later than their targets and could help define the posterior boundary of target gene expression. Close orthologs of miR-196 have not been found in invertebrates, although miR-196 is a distant homolog of the *let-7* miRNAs (*21*). Nonetheless, an unrelated miRNA, miR-iab-4, maps to the corresponding region in the Bithorax complex of insect HOX genes (*22*), and this miRNA is predicted to target *Ubx* (*23*), an insect counterpart of the HOX 6 to 8 paralogous groups, suggesting that analogous regulation might extend to insects (Fig. 1).

The observation of endogenous miRNA-directed mRNA cleavage in animals raises the question of how many other metazoan miRNA targets might be down-regulated by cleavage. Few other human messages have such extensive complementarity to the known miRNAs. Nonetheless, analysis of off-target

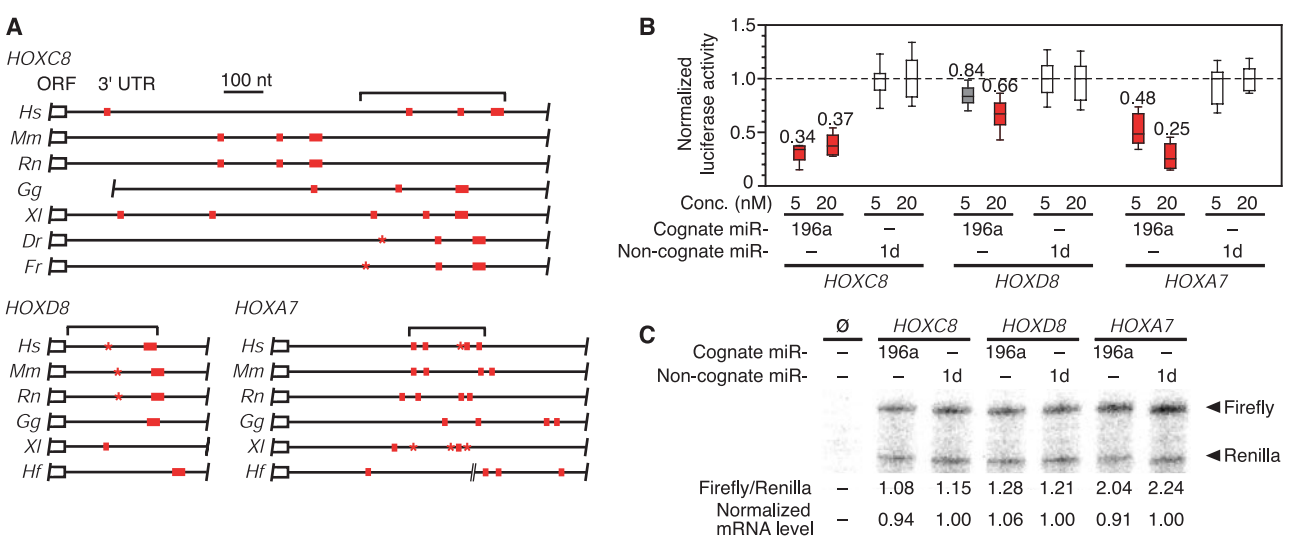

Fig. 3. Additional HOX genes predicted to be regulatory targets of miR-196. (**A**) miR-196 complementary sites in *HOXC8*, *HOXD8*, and *HOXA7* 3′ UTRs (table S2). Wide red bars indicate extensive complementarity, narrow bars indicate seed matches, and asterisks indicate slightly relaxed seed matches that permit a G:U wobble involving U8 of miR-196 (fig. S2). Abbreviations as in Fig. 1A, plus *Gg*, chicken, and *Hf*, hornshark. (**B**) Experimental support for predicted targets. Experiments were analogous to those of Fig. 2B, except the firefly reporters contained fragments of human *HOXC8*, *HOXD8*, and *HOXA7* UTRs bracketed in (A) (table S3). Red boxes denote statistically significant repression ($P < 0.001$, Mann-Whitney test). (**C**) Reporter mRNA levels monitored as in Fig. 2C. In three independent repetitions, normalized mRNA levels were 1.01 ± 0.08, 1.00 ± 0.09, and 0.95 ± 0.06 for *HOXC8*, *HOXD8*, and *HOXA7*, respectively (±SD).

siRNA-mediated regulation indicates that extensive complementarity is not always required (*24*), leaving open the possibility that a large fraction of metazoan miRNA regulation might be achieved through mRNA cleavage.

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Supporting Online Material

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Materials and Methods
Figs. S1 and S2
Tables S1 to S3

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Regeneration of Peroxiredoxins by p53-Regulated Sestrins, Homologs of Bacterial AhpD

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Acting as a signal, hydrogen peroxide circumvents antioxidant defense by overoxidizing peroxiredoxins (Prxs), the enzymes that metabolize peroxides. We show that sestrins, a family of proteins whose expression is modulated by p53, are required for regeneration of Prxs containing Cys-SO_2H, thus reestablishing the antioxidant firewall. Sestrins contain a predicted redox-active domain homologous to AhpD, the enzyme catalyzing the reduction of a bacterial Prx, AhpC. Purified Hi95 (sestrin 2) protein supports adenosine triphosphate–dependent reduction of overoxidized PrxI in vitro, indicating that unlike AhpD, which is a disulfide reductase, sestrins are cysteine sulfinyl reductases. As modulators of peroxide signaling and antioxidant defense, sestrins constitute potential therapeutic targets.

Reactive oxygen and nitrogen species (ROS and RNS, respectively) are by-products of metabolism that are destroyed by antioxidant systems. However, the same compounds function as important signal-transducing messengers. Hydrogen peroxide bursts are elicited by ligand-receptor interaction in major pathways, which include mitogen-activated protein kinase and nuclear factor κB pathways (*1*), affecting cell proliferation and cell death. Antioxidant mechanisms must be thus tightly regulated to enable signaling but prevent oxidative damage.

Peroxiredoxins (Prxs), a family of thiol-containing peroxidases conserved from bacteria to mammals (*2*), are major reductants of endogenously produced peroxides in eukaryotes (*3*). In addition, Prxs catalyze decomposition of RNS (*4*–*7*). How do hydrogen peroxide signals evade this antioxidant defense? The answer seems to lie in the structure of eukaryotic 2-Cys Prxs (*8*) which,

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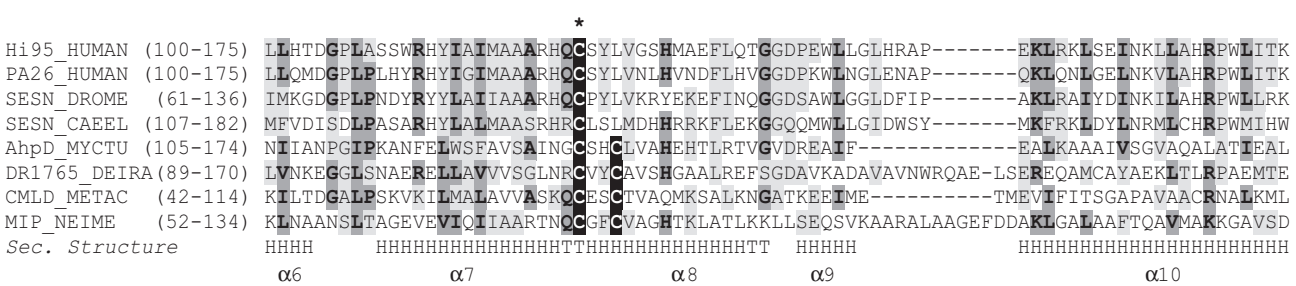

Fig. 1. Multiple alignment of sestrins and selected AhpD-family proteins (*31*). The alignment was constructed with the use of the MACAW program (*32*). The secondary structure assignments are from the AhpD structure (Protein Data Bank code 1KNC); H indicates α-helix [numbered as in (*23*)], and T indicates a hydrogen-bonded turn. Amino acid residues conserved (identical or similar) in aligned sequences are shown by shading; darker shading indicates identical or closely similar residues. The two cysteines of the AhpD family proteins that form a transient disulfide bridge are shown by reverse shading, and the cysteine that is conserved between the sestrins and the AhpD family is denoted by an asterisk. The positions of the aligned regions in the respective protein sequences are indicated in parentheses. CAEEL indicates *Caenorhabditis elegans*; DROME, *Drosophila melanogaster*; MYCTU, *M. tuberculosis*; DEIRA, *Deinococcus radiodurans*; METAC, *Methanosarcina acetivorans*; NEIME, *Neisseria meningitides*; SESN, sestrin; and CMLD, carboxymuconolactone decarboxylase. The GenBank accession number for Hi95_HUMAN is NP_113647; PA26_HUMAN, NP_055269; SESN_DROME, Q9W1K5; SESN_CAEEL, NP_490664; AhpD_MYCTU, NP_216945; DR1765_DEIRA, NP_295488; CMLD_METAC, NP_618609; and NMA1203_NEIME, NP_283969.