

Myogenic factors that regulate expression of muscle-specific microRNAs

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Contributed by Harvey F. Lodish, April 6, 2006

Since their discovery as key regulators of early animal development, microRNAs now are recognized as widespread regulators of gene expression. Despite their abundance, little is known regarding the regulation of microRNA biogenesis. We show that three highly conserved muscle-specific microRNAs, miR-1, miR-133 and miR-206, are robustly induced during the myoblast-myotube transition, both in primary human myoblasts and in the mouse mesenchymal C₂C₁₂ stem cell line. These microRNAs were not induced during osteogenic conversion of C₂C₁₂ cells. Moreover, both loci encoding miR-1, miR-1-1, and miR-1-2, and two of the three encoding miR-133, miR-133a-1 and miR-133a-2, are strongly induced during myogenesis. Some of the induced microRNAs are in intergenic regions, whereas two are transcribed in the opposite direction to the nonmuscle-specific gene in which they are embedded. By using CHIP analysis, we demonstrate that the myogenic factors Myogenin and MyoD bind to regions upstream of these microRNAs and, therefore, are likely to regulate their expression. Because miR-1 and miR-206 are predicted to repress similar mRNA targets, our work suggests that induction of these microRNAs is important in regulating the expression of muscle-specific proteins.

development | myogenesis | transcription

Small ribonucleotide-based regulators of gene expression known as microRNAs play important roles in regulatory gene expression (see refs. 1 and 2 for reviews). Although initially identified through their role in the development of *Caenorhabditis elegans* larvae (3, 4), microRNA abundance (5–7) and conservation is suggestive of a much broader role within both the animal and plant kingdoms. Studies in vertebrates and invertebrates have confirmed this notion: microRNAs play a fundamental role in diverse biological and pathological processes including apoptosis (8), cell fate determination (9–11), metabolism (12, 13) and tumorigenesis (14–16). microRNAs are synthesized as longer primary microRNAs (17, 18) that are sequentially processed in the nucleus and cytoplasm by distinct multiprotein complexes containing Drosha/DGCR8 (19–23) and Dicer/TRBP (24–26), respectively. The resulting duplex is unwound by a helicase activity, and the mature single-stranded RNA is loaded onto the RNA-induced silencing complex, wherein interactions between microRNAs and mRNAs occur. microRNAs repress gene expression by cleaving target mRNAs or by inhibiting the ability of the target mRNA to be translated into protein. Computational approaches have yielded hundreds of putative mRNA targets for individual microRNAs (27–31), but the number of verified targets with biological relevance is still very small.

We are interested in studying the ability of microRNAs to regulate cell differentiation. Our work focuses on three microRNAs specifically and abundantly expressed in muscle tissue, miR-1, miR-133, and miR-206 (32–34). The overrepresentation of these and other microRNAs in various differentiated tissues implicates microRNAs in the determination or maintenance of the differentiated state (35). Studies aimed at the perturbation of miR-1 expression in mice and flies have suggested both types of function for this microRNA. Using a

gain-of-function approach, a role for miR-1 in mice has been postulated in the regulation of cardiomyocyte proliferation, thereby implicating miR-1 in the determination of the differentiated state (36). Using the complementary loss-of-function approach in flies, Sokol and Ambros (37) have determined that miR-1 is dispensable for formation of the musculature and suggest that miR-1 may play a role in the maintenance of the differentiated state. Authors of a more recent study (38) have proposed a model in which miR-1 and miR-133 regulate myogenesis by controlling distinct aspects of the differentiation process.

Here we examine the myogenic regulation of microRNAs that belong to distinct families, the miR-1 family (miR-1-1, miR-1-2, and miR-206) and the miR-133 family (miR-133a-1, miR-133a-2, and miR-133b). We show that five of six microRNAs that belong to these families are specifically induced during myogenesis, suggesting these microRNAs play crucial roles during the process of skeletal muscle formation. The myogenic transcription factors myogenin and myogenic differentiation 1 (MyoD) bind to regions upstream of the miR-1 and miR-133 stem loop, thereby providing a molecular explanation for the observed induction during myogenesis. These results, along with similar studies by Chen *et al.* (38), lay the groundwork for analyzing putative contributions of microRNAs to the process of myogenesis.

Results

microRNA Expression During Myogenesis in C₂C₁₂ Cells and Primary Human Myoblasts. Previous studies (32–35) as well as our own preliminary microRNA-array analysis (S.B. and P.K.R., unpublished data) had suggested that miR-1, miR-133, and miR-206 are highly expressed in heart and skeletal muscle tissue. To gain insights into microRNA function in skeletal muscle, we used C₂C₁₂ mesenchymal stem cells and examined expression patterns of these microRNAs as they mature into myotubes. C₂C₁₂ cells were differentiated along the myogenic pathway by placing them in 2% horse serum media for 4 days. Differentiation under these conditions is very efficient, and the steps preceding myotube formation, including Myogenin expression, cell cycle arrest, and cell cycle withdrawal, are well characterized (39). As shown in Fig. 1A Upper, miR-1 and miR-206 are induced during the C₂C₁₂ myogenesis. miR-1 is readily detectable by day 2, by which time the majority of the cells are expressing the cdk inhibitor p21 (39) and are mitotically inactive. By day 4, levels of sarcomeric proteins such as myosin are abundant; miR-1 levels are also high. The ≈60-base precursor to miR-1 also is induced in a manner similar to mature miR-1, implying that the regulation of miR-1 occurs primarily at the transcriptional level. miR-206 expression

Conflict of interest statement: No conflicts declared.

Abbreviations: CHIP, chromatin immunoprecipitation; DM, differentiation media; GM, growth media; MyoD, myogenic differentiation 1.

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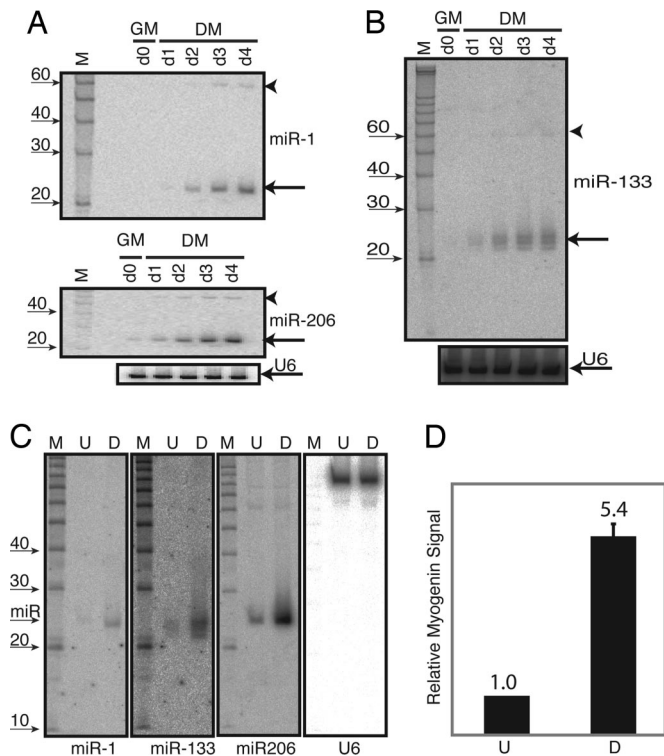


Fig. 1. Myogenic miRs. (A) Time course of miR-1 and miR-206 induction during C₂C₁₂ myogenesis. Total RNA from C₂C₁₂ cells in GM or DM for zero (d0), one (d1), two (d2), three (d3), or four (d4) days was subject to Northern blot analysis with a ³²P end-labeled miR-1, miR-206, or U6 probe. ³²P-labeled 10-bp RNA ladder (Ambion) is shown on the left. The miR-1 blot (*Top*) was stripped and reprobed sequentially for miR-206 (*Middle*) and U6 (*Bottom*). Mature and precursor microRNAs are labeled with an arrow and an arrowhead, respectively. (B) Same as in A except that a new set of samples was probed with a ³²P end-labeled miR-133 probe, stripped, and reprobed with a ³²P end-labeled U6 probe. (C) RNA was isolated from growing, undifferentiated primary human myoblasts in SkGM2 proliferation media (U) or from primary human myoblasts differentiated for 2 weeks in 2% horse serum-containing media (D). The position of the microRNA is indicated on the left. The membrane first was probed with miR-1, stripped, and subsequently probed for miR-133, miR-206 and U6 as indicated below the blots. ³²P-labeled 10-bp RNA ladder (Ambion) is shown on the left for the miR-1 blot. (D) Myogenin induction (by using quantitative RT-PCR) in the differentiated sample indicates efficient differentiation of the primary human myoblasts along the skeletal myogenic lineage. The same RNA samples was used for C and D.

is detectable when C₂C₁₂ cells are grown in proliferation media (d0/GM lane in Fig. 1*A Middle*; GM, growth medium), but its steady-state level increases manyfold as C₂C₁₂ cells differentiate. miR-133 (Fig. 1*B*) is undetectable at day 0 and abundant by day 4. Three distinct mature microRNA bands are seen in miR-133 blots and the exact identity of each band is not known, but many mature microRNAs are heterogeneous in size and vary from 17 to 24 nucleotides. Moreover, miR-133 is encoded by multiple loci (see below), and could also account for the size heterogeneity. Stripping and reprobing the blot with a U6 small nuclear RNA probe indicates equal loading of the lanes (Fig. 1*A Lower* and *B Lower*).

We repeated these analyses by using primary human myoblasts. These cells required a longer time to differentiate; after two weeks in culture, these myoblasts demonstrate a myotube-like morphology. Differentiation along the skeletal myogenic lineage was confirmed by detecting increased expression of myogenin (Fig. 1D). miR-1 expression is barely detectable in undifferentiated primary myoblastic cells but is induced during myotube differentiation. miR-133 also is induced upon differ-

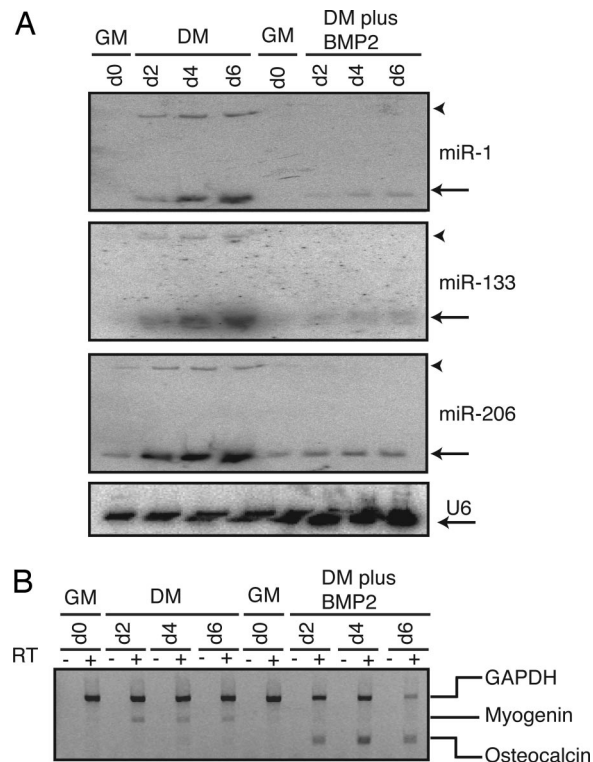


Fig. 2. miR-1, miR-133, and miR-206 induction is specific to myogenesis. (A) C₂C₁₂ cells were grown in GM or 2% horse serum (DM) in the presence or absence of 300 ng/ml BMP2. RNA from treated cells were harvested at the indicated times and probed for miR-1, miR-133, miR-206, and U6 small RNAs. Mature and precursor microRNAs are labeled with an arrow and arrowhead, respectively. (B) The same RNA samples used in A was DNase I treated and subject to RT-PCR for the detection of a myogenic marker (myogenin) or a osteoblastic marker (osteocalcin) and a housekeeping gene (GAPDH). Separate RT-PCR reactions were performed but were all loaded in the same well for simultaneous detection by using ethidium bromide staining. RT reactions performed without reverse transcriptase yielded no signal after PCR, and these samples are loaded in the “-” lanes.

entiation and displays the same heterogeneity in size as seen in C₂C₁₂ myotubes. Lastly, miR-206 is detectable in the myoblasts and is further up-regulated upon differentiation (Fig. 1C). The observation that miR-1, miR-133, and miR-206 are regulated similarly in primary human myoblasts and mouse mesenchymal stem cells implies that mechanisms underlying their expression have been conserved during mammalian evolution.

Myogenic Specificity in microRNA Expression Patterns. We next investigated whether miR-1, miR-133, and miR-206 are specifically induced during myogenesis or whether their expression was associated with differentiation in general. Accordingly, we induced osteogenic or myogenic differentiation of C₂C₁₂ cells by using low serum in the presence or absence of BMP2 (Bone Morphogenetic Protein 2), respectively. In this protocol, BMP2 overrides the myogenic signal induced by low serum and induces the cells along an osteoblastic lineage (40). As seen in Fig. 2A, neither miR-1, miR-133, nor miR-206 expression was up-regulated during C₂C₁₂ osteogenesis (“DM plus BMP2” lanes; DM, differentiation media). However, an increase in the levels of miR-1, miR-133, and miR-206 is seen during myogenesis (“DM” lanes). Parallel RT-PCR analyses (Fig. 2B) reveal appropriate induction of protein-encoding mRNA markers associated with myogenesis (myogenin) and osteogenesis (osteocalcin).

Identification of the Active Genomic Loci for Induced microRNAs. Of the three muscle-specific microRNAs, miR-1 and miR-133 are

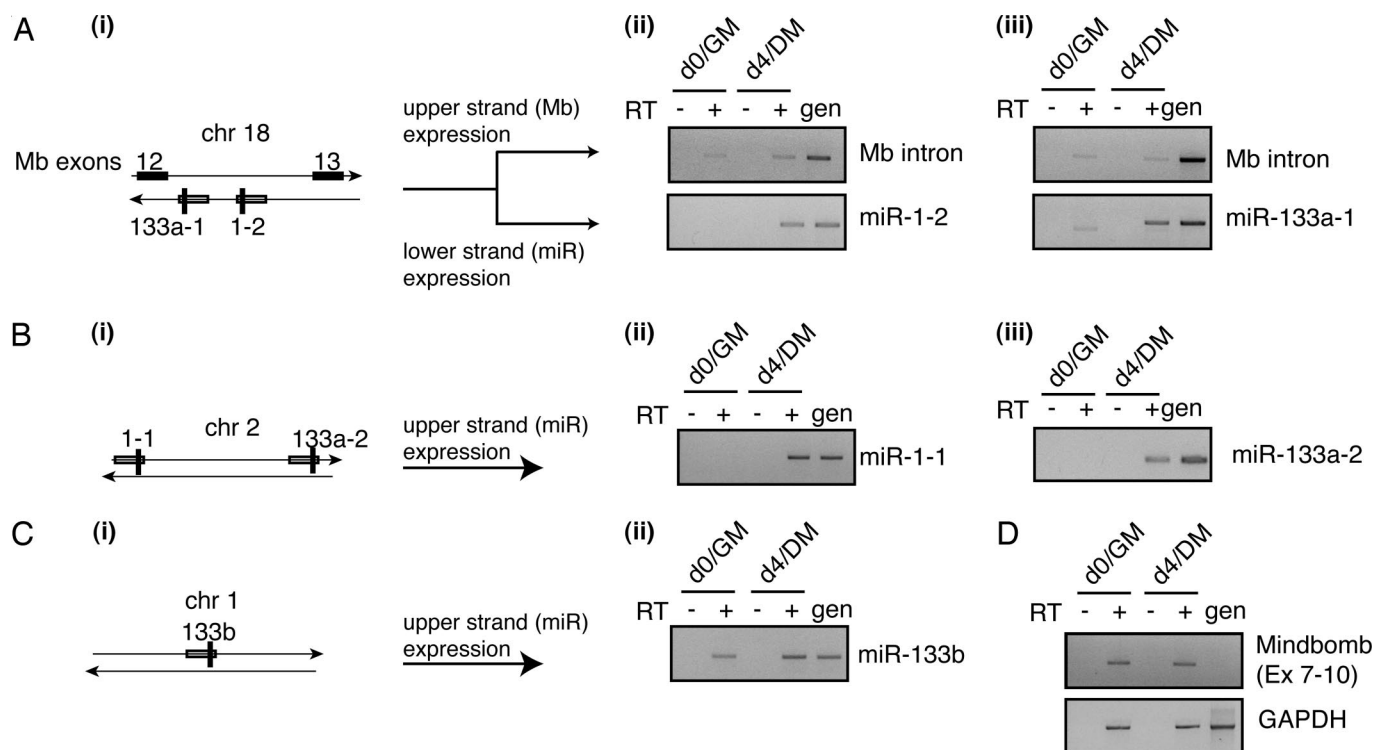


Fig. 3. Determination of the loci contributing to mature microRNAs. DNase I-treated RNA from either undifferentiated (day 0/GM) or differentiated (day 4/DM) C₂C₁₂ cells was used for RT-PCR. Genomic DNA (gen) was used as a positive control; lack of a signal from reactions without reverse transcriptase (– lanes) shows that there was no genomic DNA contamination. The RNA species being detected is indicated. (A) Intronic miRs. miR-1-2 and miR-133a-1 are located on chromosome 18 and are intronic (i); they are robustly induced during myogenesis (ii Lower and iii Lower). Mindbomb is not induced during myogenesis (ii Upper and iii Upper). (B and C) Intergenic microRNAs. (B) miR-1-1 and miR-133a-2 are located on chromosome 2 and are intergenic (i); they are also robustly induced during myogenesis (ii and iii). (C) miR-133b is located on chromosome 1 and is intergenic (i); it is not up-regulated as dramatically as the other miR-133 isoforms (ii). (D) Amplification of Mindbomb mRNA (Upper) confirms the lack of inducibility during myogenesis; GAPDH amplification (Lower) reveals equal loading of input RNA. Similar results were obtained in a duplicate experiment; results from one set are shown for consistency.

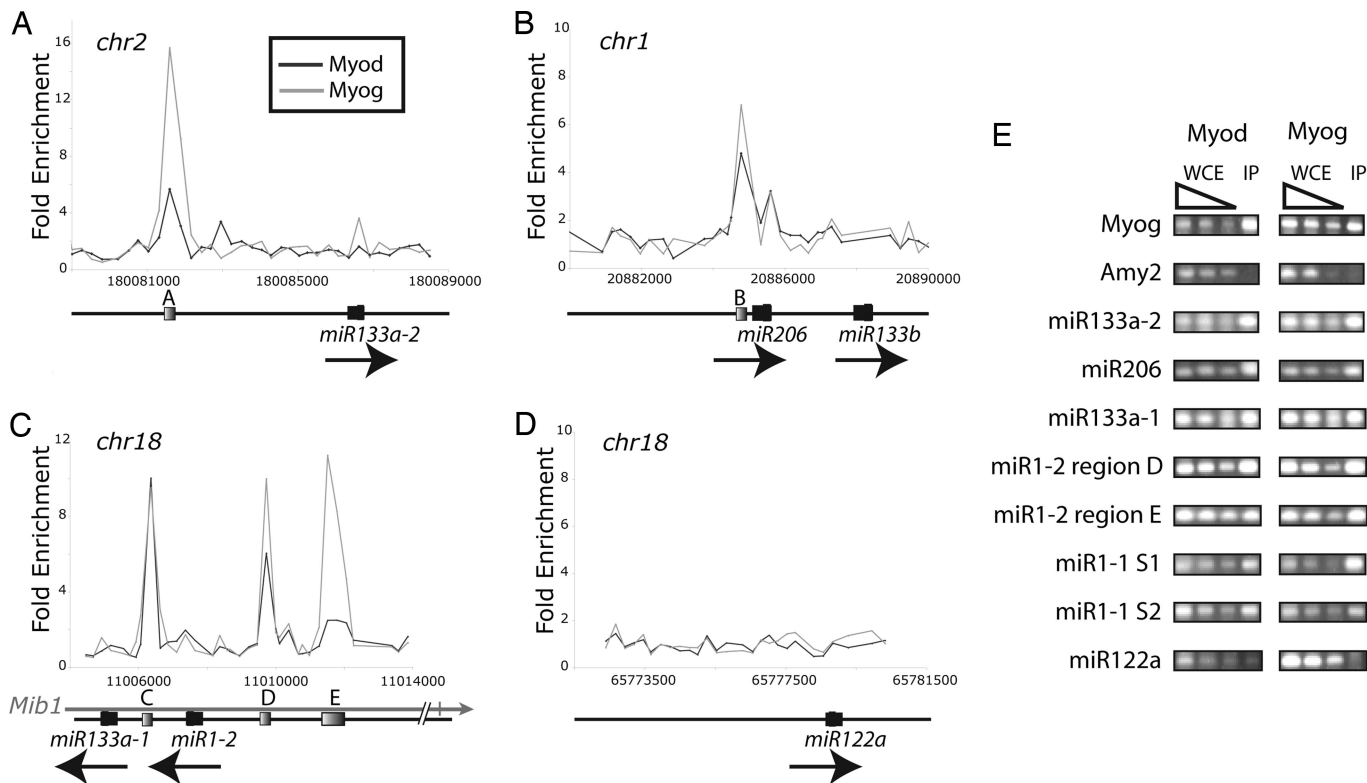
encoded by two and three loci, respectively. A schematic for these genomic loci is depicted in Fig. 3*Ai*, *Bi*, and *Ci*. In order to shed light on aspects of microRNA regulation that are still poorly understood, we set out to identify the specific miR-1 and miR-133 loci that are induced upon differentiation. To this end, we used RT-PCR transcribed sequences flanking the mature microRNAs, because these to amplify sequences differ in the multiple genes encoding the same mature microRNA.

Using this assay for miR-1-1, miR-133a-2, and miR-133b is straightforward because these microRNAs are located in intergenic regions of the chromosome. However, both miR-1-2 and miR-133a-1 are located in an intron between exons 12 and 13 of Mindbomb (*Mb*), a protein-encoding gene. Moreover, miR-1-2 and miR-133a-1 are expressed from the same DNA strand, whereas *Mb* is expressed from the opposite strand (Fig. 3*Ai*). To distinguish expression from the *Mb* strand versus the miR-1-2/miR-133a-1 strand, we used strand-specific reverse transcription, followed by PCR to detect expression from either strand. Fig. 3 reveals the expression patterns of the miR-1 and miR-133 isoforms before (lanes labeled d0/GM) and after (lanes labeled d4/DM) differentiation of C₂C₁₂ cells along the myogenic lineage. miR-1-2 and miR-133a-1 are robustly induced during myogenesis (Fig. 3*Aii* Lower and *iii* Lower). Parallel RT-PCR reactions designed to detect expression of the *Mb* intron reveals constitutive expression (at low levels) from the *Mb* strand (Fig. 3*Aii* Upper and *iii* Upper). Expression from the other miR-1 and miR-133 loci is shown in Fig. 3*B* and *C*. miR-1-1 and miR-133a-2 are robustly induced. In contrast, miR-133b is not; we detect some miR-133b expression in myoblasts, but the level is not significantly enhanced after myogenesis. We confirmed that *Mb*

expression was indeed constitutive with another set of primers that amplified a fragment of mRNA encompassing exons 7–10 of *Mb* (Fig. 3*D* Upper). GAPDH mRNA was also amplified to demonstrate equal input of RNA in the various samples (Fig. 3*D* Lower). The relative physical orientation of *Mb* and miR-1-2/miR-133a-1, coupled with our expression data, collectively implies that distinct regulatory elements control the expression of miR-1-2/miR-133a-1 and *Mb*.

Binding of Myogenic Regulators to Induced microRNA Loci. The observation that miR-1, miR-133, and miR-206 are all specifically up-regulated during myogenesis suggests that regulators of muscle differentiation might be responsible for their activation. MyoD and myogenin both are myogenic transcriptional regulatory factors that activate a number of muscle-specific structural genes and transcription factors to drive myogenesis (41). MyoD is expressed in proliferating C₂C₁₂ myoblasts, and upon induction of differentiation, it activates expression of myogenin and other muscle-specific genes. MyoD and myogenin share a number of common targets and participate in a feed-forward circuit that helps to temporally pattern gene expression during myogenesis (42, 43). We used chromatin immunoprecipitation (ChIP) coupled with tiled DNA arrays or promoter-specific PCR to examine the occupancy of these myogenic factors at genomic regions upstream of the induced microRNAs in differentiated C₂C₁₂ cells.

The ~10 kb upstream of the induced microRNAs were tiled on DNA arrays, and immunoprecipitated DNA was labeled and hybridized to these arrays against a reference unenriched sample. Binding data from the arrays was confirmed by gene-specific



protocol and fluorescently labeled with Cy5-dUTP (Amersham Pharmacia). Labeled fragments were hybridized to DNA arrays in Agilent Technologies hybridization chambers at 40°C for ≈40 h against a Cy3-dUTP labeled reference sample of unenriched DNA. Arrays were washed and scanned by using an Agilent Technologies DNA microarray scanner BA as described in ref. 53. Unprocessed enrichment ratios were examined to look for evidence of MyoD or myogenin binding, and putative bound sequences were subjected to promoter-specific PCR to confirm enrichment. PCR reactions were performed as described in ref. 54. PCRs were performed on biological duplicate immunoprecipitations to confirm results. Primers used for miR-1-1 and

miR-122a ChIP-PCR were designed so as to flank conserved (between human and mouse) E boxes found in a 5,000-bp region upstream of the microRNA stem loop. The alignment and E box identification was performed by using RVISTA (<http://rvista.dcode.org>). All primer sequences used in Fig. 4E are listed in Table 2, which is published as supporting information on the PNAS web site.

We thank Drs. Dave Bartel, Richard Young, and I-Hung Shih for their intellectual support. This work was supported by Muscular Dystrophy Association Grant MDA-3882 (to P.K.R.), an American Cancer Society grant (to R.M.K.), National Institutes of Health Grant R01 DK068348 (to H.F.L.), and Richard A. Young Grants GM069400 and HG002668.

1. Bartel, D. P. (2004) *Cell* **116**, 281–297.
2. Ambros, V. (2004) *Nature* **431**, 350–355.
3. Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993) *Cell* **75**, 843–854.
4. Wightman, B., Ha, I. & Ruvkun, G. (1993) *Cell* **75**, 855–862.
5. Lau, N. C., Lim, L. P., Weinstein, E. G. & Bartel, D. P. (2001) *Science* **294**, 858–862.
6. Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. (2001) *Science* **294**, 853–858.
7. Lee, R. C. & Ambros, V. (2001) *Science* **294**, 862–864.
8. Xu, P., Vernooys, S. Y., Guo, M. & Hay, B. A. (2003) *Curr. Biol.* **13**, 790–795.
9. Chang, S., Johnston, R. J., Jr., Frokjaer-Jensen, C., Lockery, S. & Hobert, O. (2004) *Nature* **430**, 785–789.
10. Chen, C. Z., Li, L., Lodish, H. F. & Bartel, D. P. (2004) *Science* **303**, 83–86.
11. Giraldez, A. J., Cinalli, R. M., Glasner, M. E., Enright, A. J., Thomson, J. M., Baskerville, S., Hammond, S. M., Bartel, D. P. & Schier, A. F. (2005) *Science* **308**, 833–838.
12. Poy, M. N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., Macdonald, P. E., Pfeffer, S., Tuschl, T., Rajewsky, N., Rorsman, P. & Stoffel, M. (2004) *Nature* **432**, 226–230.
13. Esau, C., Kang, X., Peralta, E., Hanson, E., Marcusson, E. G., Ravichandran, L. V., Sun, Y., Koo, S., Perera, R. J., Jain, R., et al. (2004) *J. Biol. Chem.* **279**, 52361–52365.
14. He, L., Thomson, J. M., Hemann, M. T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S. W., Hannon, G. J. & Hammond, S. M. (2005) *Nature* **435**, 828–833.
15. Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B. L., Mak, R. H., Ferrando, A. A., et al. (2005) *Nature* **435**, 834–838.
16. Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K. L., Brown, D. & Slack, F. J. (2005) *Cell* **120**, 635–647.
17. Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H. & Kim, V. N. (2004) *EMBO J.* **23**, 4051–4060.
18. Cai, X., Hagedorn, C. H. & Cullen, B. R. (2004) *RNA* **10**, 1957–1966.
19. Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. & Kim, V. N. (2003) *Nature* **425**, 415–419.
20. Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. & Shiekhattar, R. (2004) *Nature* **432**, 235–240.
21. Landthaler, M., Yalcin, A. & Tuschl, T. (2004) *Curr. Biol.* **14**, 2162–2167.
22. Han, J., Lee, Y., Yeom, K. H., Kim, Y. K., Jin, H. & Kim, V. N. (2004) *Genes Dev.* **18**, 3016–3027.
23. Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F. & Hannon, G. J. (2004) *Nature* **432**, 231–235.
24. Saito, K., Ishizuka, A., Siomi, H. & Siomi, M. C. (2005) *PLoS Biol.* **3**, e235.
25. Forstemann, K., Tomari, Y., Du, T., Vagin, V. V., Denli, A. M., Bratu, D. P., Klattenhoff, C., Theurkauf, W. E. & Zamore, P. D. (2005) *PLoS Biol.* **3**, e236.
26. Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K. & Shiekhattar, R. (2005) *Nature* **436**, 740–744.
27. Lewis, B. P., Burge, C. B. & Bartel, D. P. (2005) *Cell* **120**, 15–20.
28. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. & Burge, C. B. (2003) *Cell* **115**, 787–798.
29. Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C. & Marks, D. S. (2003) *Genome Biol.* **5**, R1.
30. Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P., da Piedade, I., Gunsalus, K. C., Stoffel, M. & Rajewsky, N. (2005) *Nat. Genet.* **37**, 495–500.
31. Kiriakidou, M., Nelson, P. T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z. & Hatzigeorgiou, A. (2004) *Genes Dev.* **18**, 1165–1178.
32. Sempere, L. F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E. & Ambros, V. (2004) *Genome Biol.* **5**, R13.
33. Baskerville, S. & Bartel, D. P. (2005) *RNA* **11**, 241–247.
34. Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W. & Tuschl, T. (2002) *Curr. Biol.* **12**, 735–739.
35. Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H. R., Kauppinen, S. & Plasterk, R. H. (2005) *Science* **309**, 310–311.
36. Zhao, Y., Samal, E. & Srivastava, D. (2005) *Nature* **436**, 214–220.
37. Sokol, N. S. & Ambros, V. (2005) *Genes Dev.* **19**, 2343–2354.
38. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L. & Wang, D. Z. (2006) *Nat. Genet.* **38**, 228–233.
39. Andres, V. & Walsh, K. (1996) *J. Cell Biol.* **132**, 657–666.
40. Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A. & Suda, T. (1994) *J. Cell Biol.* **127**, 1755–1766.
41. Tapscott, S. J. (2005) *Development (Cambridge, U.K.)* **132**, 2685–2695.
42. Blais, A., Tsikitis, M., Acosta-Alvear, D., Sharan, R., Kluger, Y. & Dynlacht, B. D. (2005) *Genes Dev.* **19**, 553–569.
43. Cao, Y., Kumar, R. M., Penn, B. H., Berkes, C. A., Kooperberg, C., Boyer, L. A., Young, R. A. & Tapscott, S. J. (2006) *EMBO J.* **25**, 502–511.
44. Velleca, M. A., Wallace, M. C. & Merlie, J. P. (1994) *Mol. Cell. Biol.* **14**, 7095–7104.
45. Eftimie, R., Brenner, H. R. & Buonanno, A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1349–1353.
46. Brennan, T. J., Edmondson, D. G. & Olson, E. N. (1990) *J. Cell Biol.* **110**, 929–937.
47. Kwon, C., Han, Z., Olson, E. N. & Srivastava, D. (2005) *Proc. Natl. Acad. Sci. USA* **102**, 18986–18991.
48. Farh, K. K., Grimson, A., Jan, C., Lewis, B. P., Johnston, W. K., Lim, L. P., Burge, C. B. & Bartel, D. P. (2005) *Science* **310**, 1817–1821.
49. Fazi, F., Rosa, A., Fatica, A., Gelmetti, V., De Marchis, M. L., Nervi, C. & Bozzoni, I. (2005) *Cell* **123**, 819–831.
50. O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V. & Mendell, J. T. (2005) *Nature* **435**, 839–843.
51. Sanes, J. R. & Lichtman, J. W. (1999) *Annu. Rev. Neurosci.* **22**, 389–442.
52. Nguyen, C. T., Parsadanian, A. S., Snider, W. D. & Lichtman, J. W. (1998) *Science* **279**, 1725–1729.
53. Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., et al. (2005) *Cell* **122**, 947–956.
54. Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., Chevalier, B., Johnstone, S. E., Cole, M. F., Isono, K., et al. (2006) *Cell* **125**, 301–313.