Supporting Information

Vo et al. 10.1073/pnas.1013162107

Supporting Methods.

Cell Culture. C_2C_{12} cells were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin and were differentiated by serum starvation in media containing DMEM, 2% horse serum, and 1% penicillin/streptomycin. Stably transfected HEK293 were maintained in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, 200 μg/mL G418. Primary rat neonatal cardiomyocytes were harvested and cultured from P0-P1 rat pups (Sprague-Dawley) using standard protocols. Cardiomyocytes were dissociated with Ads buffer (116 mM NaCl, 20 mM Hepes, 1 mM NaH_2PO_4, 5.5 mM dextrose, 5.4 mM KCl, 0.8 mM $MgSO_4$, pH 7.35) containing trypsin (Sigma) and DNaseI (Roche). Noncardiomyocytes were removed by preplating the dissociated cells. Following the preplating step, cardiomyocytes were seeded in plating medium (67% DMEM, 17% M199, 10% horse serum, 5% newborn calf serum, and 1% penicillin/streptomycin) at a density of $\sim 3 \times 10^6$ cells per 10-cm dish and 275,000 cells/well for 12 well plates. Tissue culture vessels were precoated with 0.1% gelatin and 1 μg/mL fibronectin. Cardiomyocytes were then cultured in maintenance medium (78.5% DMEM, 20.5% M199, 1% penicillin/streptomycin.)

Expression Vectors. ***Plasmids.*** The MBP-MS2 expression construct was a gift from Melissa Moore (University of Massachusetts, Worcester). The MS2 RNA sequence containing three hairpins was derived from ref. 1: F: cgggatccgatatccgtacaccatcagggtacgagctagcccatggcgtacaccatcagggtacga, R: gctctagagaattccgtaccctgatggtgtacgagatctactagtcgtaccctgatggtgtacgc).

PCR primers. Murine Hand2 3′UTR (F: cacctctagatgaataagaagaggagagcagtgagccgggggc, R: cgggatcctgagaaccactgactc)

RT-PCR primers. GFPa [(lentivirus) F: gatgccacctacggcaagctacc, R: gaagtcgtgctgcttcatgtggtc; GFPb (pDEST53) F: tcgacacaatctgccctttcg, R: gtaatcccagcagcagttacaaa]; Hand2 (F tatgaagaggacactcccgtgtggtaag, R ctacacagccaagagggaacattcaca); B2M (F: cacacccaccgagaccgatgtat, R: tgggctccttcagagtgacgtgt); biotinylated miR1 (antisense: 5′-phosphorylated-uggaauguaaagaaguauguauuu-3′-biotin; sense: 5′-phosphorylated-auacauacuucuuuacauucuaag-3′).

Mutagenesis primers. Lowercase letters highlight mutated sequences. miR133a site A (F: GAAGAAGAGGAGAGCAGTGgaggggaccagccgGCGCCAGATGCAGAC, R: GTCTGCATCTGGCGCcggctggtcccctcCACTGCTCTCCTCTTCTTC); miR133a site B (F: CTCGGCCTTCGGATATAAATATgttgttaatggccgAAGTTTTATTTCAAAG, R: CTTTGAAATAAAACTTcggccattaacaacATATTTATATCCGAAGGCCGAG).

MS2 RNA oligonucleotides containing BamHI and XbaI restriction sites (1) were annealed and cloned into the pENTR-D-TOPO gateway vector (Invitrogen). The murine Hand2 3′UTR was also cloned by PCR into same vector. PCR primers for Hand2 contained XbaI (forward) and BamHI (reverse) restriction sites. Transcription stop sites were also included within the forward primer. The Hand2 3′UTR pENTR-D-TOPO construct was then cloned into the NotI and BamHI sites of the MS2 pENTR-D-TOPO vector. This generated a construct expressing the murine Hand2 3′UTR with an MS2 tag in the pENTR-D-TOPO vector (designated Hand2-MS2-pENTR). To create a GFP construct regulated by an MS2-tagged Hand2 3′UTR expression construct, the Hand2-MS2-pENTR construct was recombined using LR Clonase II (Invitrogen) into the pDEST53 gateway destination vector (Invitrogen) creating GFP- Hand2-MS2-pDEST53, which was used for transfection into HEK293 cells (for stable cell lines) and C_2C_{12} cells (for transient transfections). To create an MS2-tagged lentivirus construct expressing GFP regulated by the Hand2 3′UTR, the pENTR-D-TOPO construct was digested with BsrGI (which flanks the Hand2 3′UTR-MS2 sequence) and cloned into the BsrGI site within the FuGW lentivirus vector (2). Control constructs were generated similarly.

Validation of microRNA Regulation. Primary rat cardiomyocytes were transfected 100-nM microRNA inhibitors (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) or electroporation (Lonza). Cells were harvested and lysed 48 h after transfection in modified RIPA (Radioimmunoprecipitation assay) buffer and assayed for levels of Hand2 (Santa Cruz) and tubulin (Sigma) protein. RNA was also purified from these lysates for RT-PCR analysis of Hand2 mRNA levels.

C_2C_{12} cells were transfected using Lipofectamine (Invitrogen) with an MS2-tagged GFP transcript. The following day, cells were differentiated for 24 h and harvested with modified RIPA buffer. Western analysis was performed for GFP (BD Biosciences) and tubulin (Sigma). Quantification was performed using the BioRad ChemiDoc XRS imaging system.

Luciferase Assays in Primary Cardiomyocytes and HEK293 Cells. Primary rat neonatal cardiomyocytes or HEK293 cells were transfected with the pGL3 control firefly luciferase plasmid expressing the Hand2 3′UTR (Promega) as well as an SV40-Renilla luciferase plasmid (Promega) as an internal control. For cardiomyocytes, miR-133a or miR-143 inhibitors (100 nM, Dharmacon) were cotransfected. For HEK293 cells, microRNA mimics (5 nM, Dharmacon) were cotransfected. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega).

1. Zhou Z, Licklider LJ, Gygi SP, Reed R (2002) Comprehensive proteomic analysis of the human spliceosome. *Nature* 419:182–185.
2. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D (2002) Germline transmission and tissue specific expression of transgenes delivered by lentiviral vectors. *Science* 295:868–872.

Fig. S1. Abundant microRNAs in cardiomyocytes. Abundant microRNAs were detected in primary cardiomyocytes using the multiplex Taqman microRNA array. Data represent four independent experiments. (Mean ± SE are shown.)

Fig. S2. Combinatorial regulation of the Hand2 3′UTR by miR-1 and miR-133a. Coexpression of miR-133a and miR-1 mimics in HEK293S cells increases repression of the Hand2 3′UTR as compared to miR-133a and miR-1 alone. Neither microRNA alone reduced luciferase activity. (Luciferase activity is normalized to an SV40-Renilla internal control and to miR-143, a negative control microRNA.)

Fig. S3. Blocking miR-133a elevates luciferase reporter activity. Inhibition of miR-133a, but not miR-143, relieves repression of luciferase activity in primary cardiomyocytes.

Fig. S4. miR-146b associates with the Hand2 3′UTR in cardiomyocytes but not HEK293 cells. Multiplex Taqman assay show (*A*) association of miR-146b with the Hand2 3′UTR and (*B*) total miR-146b levels in cardiomyocytes and HEK293 cells. (Mean +/− S.E. are shown.) (*C*) Mutation of a putative miR-146b MRE within the full-length Hand2 3′UTR decreases miR-146b binding in primary cardiomyocytes.