Supporting Information

Small et al. 10.1073/pnas.1000300107

SI Materials and Methods

RNA Isolation and miRNA Microarray Analysis. Forty-eight hours after adenovirus infection, total RNA was purified from cardiomyocytes (CMCs) using TRIzol reagent (Invitrogen). microRNA microarray analysis was performed using the mammalian microRNA probeset (LC Sciences). microRNA levels were validated using Northern blot and TaqMan microRNA real-time probes (Applied Biosystems).

Northern Blot. Ten micrograms of total RNA was loaded onto a denaturing 20% polyacrylamide gel and transferred to a Zetaprobe GT membrane (Bio-Rad), cross-linked by UV irradiation, and baked at 85 °C. Blots were hybridized overnight at 39 °C with ^{32}P-labeled antisense STARFIRE probes directed against the mature sequence of miR-486 (Integrated DNA Technologies). U6 RNA levels were detected as loading control.

In Situ Hybridization. Whole-mount and radioactive-section in situ hybridization was performed as described (1), using a probe designed against exon 39a of *sAnk1*.

lacZ Reporter Assay. Transgenic mice harboring various fragments of *miR-486* regulatory DNA fused to the hsp68-lacZ reporter construct were generated. Embryos and tissues were collected at indicated stages and stained for β-galactosidase activity as previously described (2).

Cell Culture and Luciferase Assay. COS cells were transfected using Fugene 6.0 (Roche) according to the manufacturer's protocol. For *miR-486* promoter analysis, various miR-486-luciferase reporter constructs were transfected with expression plasmids encoding MRTF-A and MyoD using the amount designated in Fig. 3. Total DNA was kept constant using empty pcDNA3.1. For 3′ UTR target assays, 20 ng of pMIR-Report luciferase vector was transfected with increasing amounts of CMV6-miR-486 expression vector. Empty CMV6 expression vector was used as the control to keep total plasmid DNA amounts constant.

Primary rat cardiomyocytes were prepared as described (3). Forty-eight hours after plating, cells were infected with adenovirus for 3 h in 10% FBS containing media at indicated multiplicity of infection (MOI) of MRTF-A, STARS, or combinations. βGal adenovirus was used as control. βGal–, STARS–, and Flag-MRTF-A–expressing adenoviruses have been previously described (4). Transfection of 100 pmol of 2′ O-Me–modified siRNA (IDT) directed against miR-486 was performed in a six-well plate using lipofectamine 2000 (Invitrogen). Modified siRNA against GFP was used as negative control.

Electrophoretic Mobility Shift Assay. Protein lysates were prepared from COS1 cells transfected with pcDNA3.1-flag or pcDNA-flag-serum response factor (SRF). In vitro binding analysis was performed as previously described (5), using double-stranded oligonucleotides that contain the CArG boxes (in boldface type) in the *sAnk1* intron 1 (Ank1 intron 39a): CArG1—5′-AGAAATGGCAA**GCATATAAGG**GCAGGGA and CArG2—5′-CTTCTCTTCTGC**TCTTATTAGG**AAAACC-3′. Oligonucleotides containing a mutated CArG were used as control (mutated nucleotides are in lowercase): mutCArG1—5′-AGAAATGGCAA**aCAcAcgtGG**GCAGGGA-3′ and mutCArG2—5′-CTTCTCTTCTGC**TCgTAcccGG**AAAACC-3′.

Plasmid Construction. A fragment of the miR-486 premiR was PCR amplified with LA Taq (TAKARA) and cloned into pCRII-TOPO using the following primers: For—5′-AATCAGCAGACATGCAGGAC-3′ and Rev—5′-GTGTGAGGGGTGTGCGTCAGG-3′. A MiR-486 premiR insert was subcloned into the SalI and NotI sites of the CMV6 expression vector.

For in situ hybridization, the alternative muscle-specific exon of the *sAnk1* gene was amplified using LA Taq and cloned into PCRII-TOPO using the following primers: For—5′-TTGGCAGGAAGGACATTTGGG-3′ and Rev—5′-TTCAGGAAGACCCGTCGGCG-3′. The plasmid was digested with BamHI and transcribed with T7 RNA polymerase for antisense RNA probe or with NotI and SP6 for sense probe. ^{35}S-UTP (PerkinElmer) or digoxigenin-labeled UTP (Roche) were incorporated into the transcript for radioactive-section or whole-mount in situ hybridization, respectively.

The 3′ UTRs of the mouse PTEN and Foxo1a genes were amplified and cloned into the SacI/HindIII sites of the pMIR-Report luciferase vector using the following sequence-specific primers: PTEN, For—5′-GGCAATAGGACATTGTGTCAG-3′ and Rev—5′-TGACAAGAATGAGACTTTAAT-3′; Foxo1a, For—5′-GTTAGTGAGCAGGCTACATTT-3′ and Rev—5′-AAGACTTGGTGCTATGCGCTG-3′.

The seed region of the miR-486 target sites in the PTEN and Foxo1 3′ UTRs were mutated using the Quickchange II site-directed mutagenesis kit (Stratagene) and the following primers: Foxo1—5′-GTGCCAGCTTTGGATCGATCTTTTTCTATTG-3′; PTEN 1—5′-GTATATTTGTAGTGGGGGATCGAAATGAACCATCTAC-3′; and PTEN 2—5′-GGATACACAAATATGACGTGGATCGAATAATGCCTCATACC-3′.

Luciferase and lacZ reporters were constructed by ligating putative regulatory DNA into the KpnI and XhoI sites of the pGL2 e1b-minimal promoter-driven luciferase or pGH-lacZ plasminds, respectively. DNA fragments were amplified using LA Taq (TAKARA) and the following primers: upstream 1080, For—5′-CCCTTGTAACTCCCAGTGGCTG-3′ and Rev—5′-GATGAAGGTCCACATCCTCCT-3′; intron, For—5′-GTAACACCAGGCAAGCGGCAG-3′ and Rev—5′-CTGGAGTTAGAAGAAGGGAAA-3′; and truncated intron, Rev—5′-CTGGAGGTGTCTTGAGTTCTG-3′.

The Ebox or CArG boxes were mutated with the QuickchangeII site-directed mutagenesis kit (Stratagene) using the following primers (binding site in boldface type and mutant nucleotides in lowercase): 1080 mutEbox—5′-GGACAGCTCTAG**CcaCcG**GCTCGAGAGC-3′; intron mutCArG1—5′-GGAGAAATGGCAA**GCAcAgAAtG**CAGGGAGG-3′; and intron mutCArG2—5′-CCTTCTCTTCTG**CTCTgAggAGG**AAAACCCC-3′.

1. Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D (1999) HRT1, HRT2, and HRT3: A new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev Biol* 216:72–84.
2. Naya FJ, Wu C, Richardson JA, Overbeek P, Olson EN (1999) Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. *Development* 126:2045–2052.
3. Molkentin JD, et al. (1998) A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* 93:215–228.
4. Kuwahara K, Barrientos T, Pipes GC, Li S, Olson EN (2005) Muscle-specific signaling mechanism that links actin dynamics to serum response factor. *Mol Cell Biol* 25:3173–3181.
5. McFadden DG, et al. (2000) A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. *Development* 127:5331–5341.

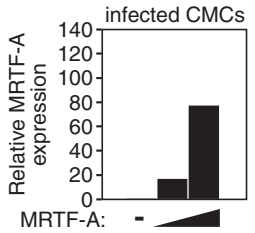

Fig. S1. Levels of MRTF-A expression by increasing MOI of adenovirus-infected CMCs is shown by real-time RT–PCR. Expression levels are normalized to GAPDH and relative to βGal control-infected CMCs.

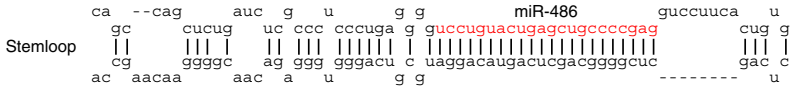

Fig. S2. The sequence of the stem-loop structure of miR-486.

Fig. S3. Sequence of the human and mouse CArG sequences from within the *sAnk1/miR-486* intronic enhancer.

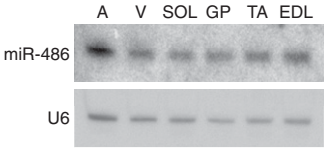

Fig. S4. Northern blot demonstrating the expression of miR-486 in adult tissues. U6 was detected as control for RNA loading. A, atrium; V, Sol, soleus; ventricle; GP, gastrocnemius plantaris; TA, tibialis anterior; EDL, extensor digitalis longus.

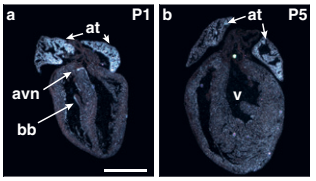

Fig. S5. In situ hybridization demonstrating early postnatal expression of sAnk1 in the heart. At postnatal day 1 (P1), (*A*) sAnk1 is expressed in the atria, AV node, and bundle branches of the ventricles. At P5 (*B*), cardiac expression of sAnk1 is enriched in the atria, AV node, and bundle branches and is detectable throughout the ventricular chambers. (Scale bar: 1 mm.) at, atrium; avn, atrioventricular node; bb, bundle branches; v, ventricle.

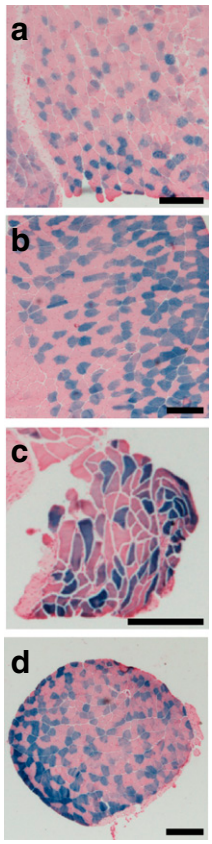

Fig. S6. *sAnk1* intronic enhancer directs adult skeletal muscle expression. lacZ staining of histological sections of adult skeletal muscle (*A*) Gastrocnemieus plantaris. (*B*) Tibialis anterior. (*C*) Soleus. (*D*) Extensor digitorum longus from transgenic mice harboring the truncated intron demonstrates muscle-fiber–specific β-gal activity. (Scale bar: 200 μm.)

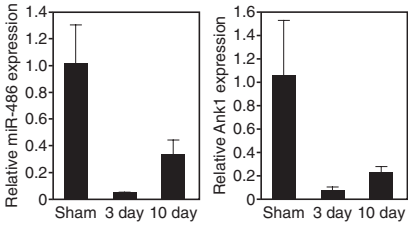

Fig. S7. miR-486 and sAnk1 expression detected by real-time RT–PCR and normalized to U6 and GAPDH expression, respectively, from RNA samples obtained from the tibialis anterior of a sham operated or denervated mouse 3 or 10 days after surgery.

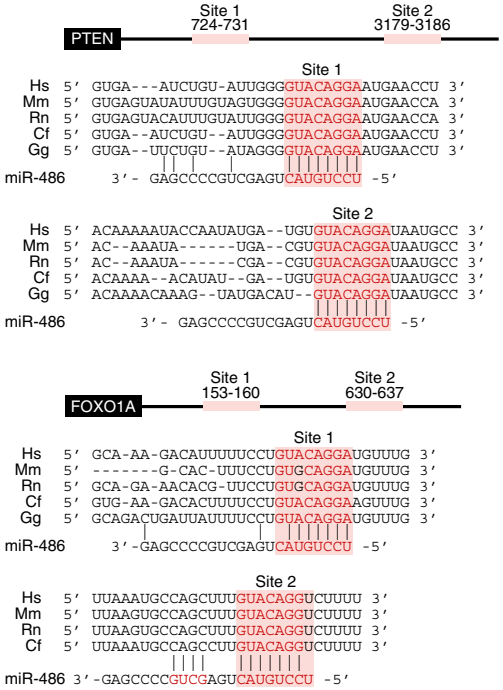

Fig. S8. Alignment of miR-486 seed sequence with target sites within the 3′ UTRs of PTEN and Foxo1a mRNAs demonstrates evolutionary conservation. Location of binding site within the UTR is indicated. The seed sequence of the miR-486 binding site is highlighted, and the species conservation within the seed region is indicated with red lettering.

Table S1. Predicted targets of miR-486 as determined by TargetScan 5.0 and miRanda algorithms

Gene Name	Sites	Gene Function
PTEN	2	Phosphatase; Tumor suppressor; Akt inhibitor
Foxo1a	2	Inhibited by Akt; Induces atrophy
IGF1	2	Insulin like growth factor 1
PI3KR1 (p85α)	1	Regulatory subunit of PI3K