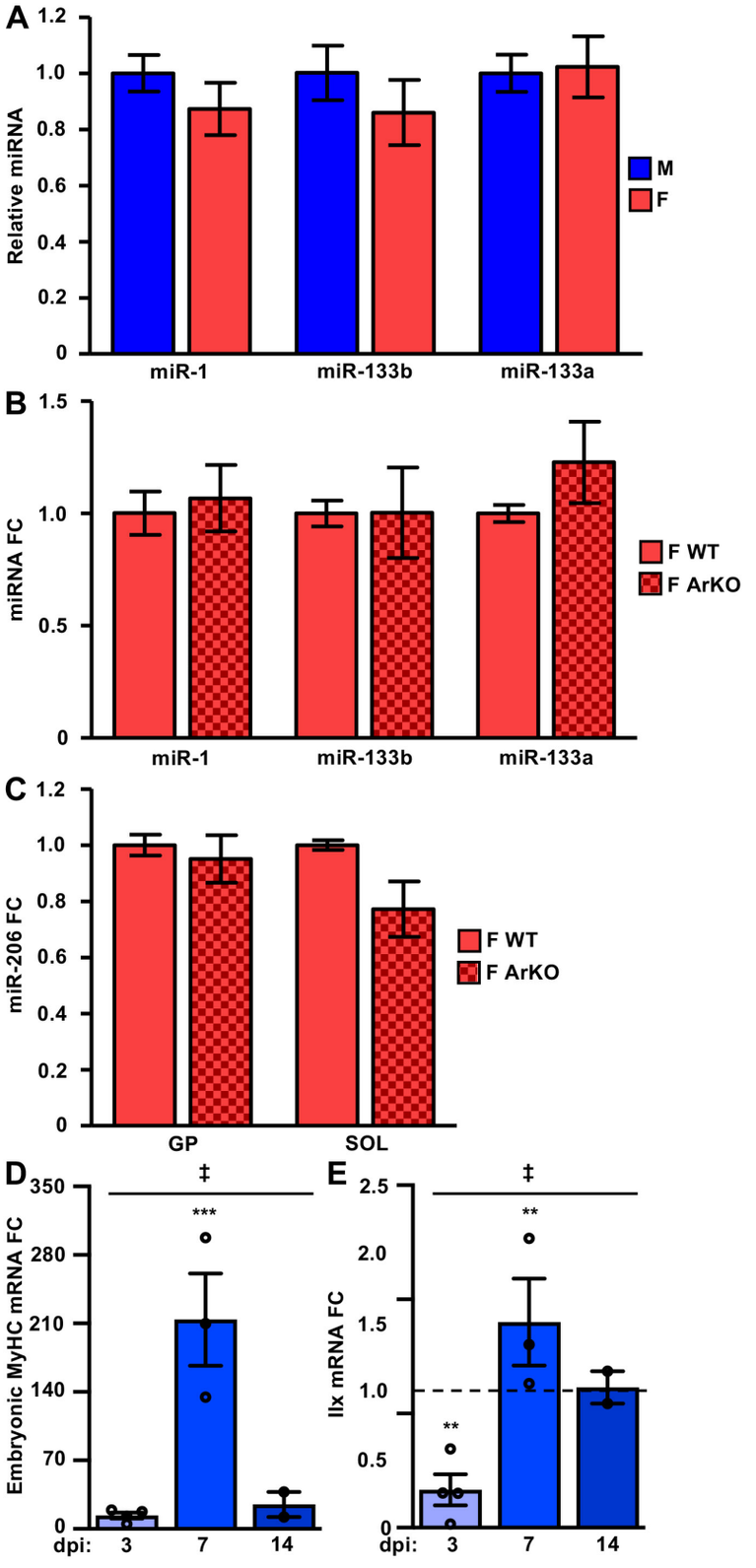
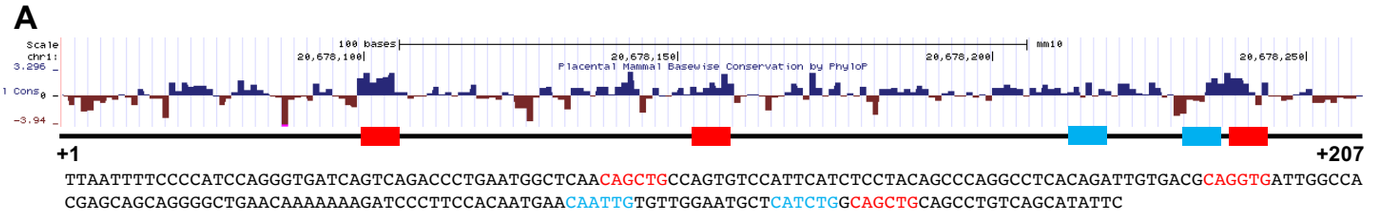
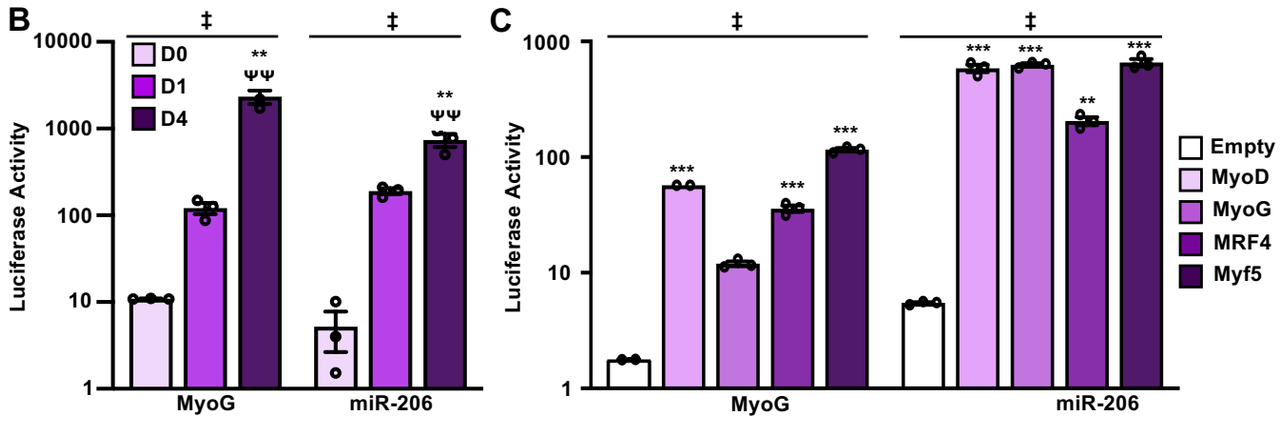
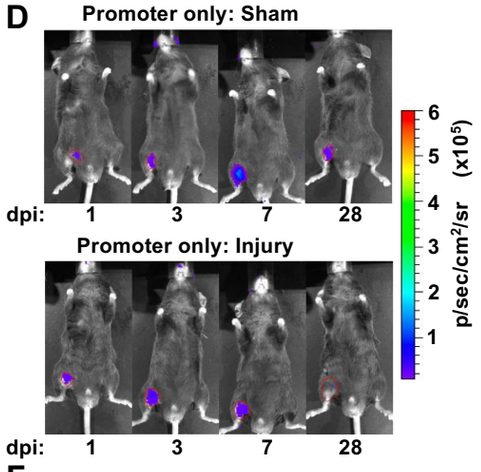


Fig. S1. myomiR expression in male, female, and female ArKO muscles and MyHC expression in regenerating TA. (A) Expression of miR-1, miR-133b, and miR-133a is not sexually dimorphic in the TA. We measured miRNA levels in male (M) and female (F) TAs. Ns are 7-8 per group. **(B)** Expression of miR-1, miR-133b, and miR-133a is not different between WT and ArKO female mice in the TA. Ns are 5-6 per group. **(C)** miR-206 expression is not different between WT and aromatase KO (ArKO) females in the gastrocnemius and plantaris (GP) and the soleus (SOL). Ns are 5-6 per group. **(D)** Embryonic MyHC and **(E)** IIx MyHC expression in regenerating male TA at the indicated days post-$BaCl_2$ injury (dpi). Expression levels relative to the uninjured (PBS-injected) contralateral control TA. Control levels are represented as a dashed line at y = 1. Ns are: 4 3dpi, 3 7dpi, and 2 14dpi. ANOVA indicated a significant effect from injury (line with ‡; $p \leq 0.0001$). Asterisks indicate post-test results. ** = $p \leq 0.01$, *** = $p \leq 0.001$ vs. control.







E

Muscle Consensus: (C/G)N(G/A)(G/A)CA(C/G)(C/G)TG(C/T)(C/T)N(C/G)

E-box #	Mouse	Score	Human	Score
1	TCAACAGCTGCCAG	= 7.5	TCAACAGCTGCCAA	= 7.0
2	GACGCAGGCGATTG	= 7.0	GGGGCAGGTGATGG	= 7.5
3	TGAACAATTGTGTT	= 5.5	Ø	
4	TGCTCATCTGGCAG	= 5.5	Ø	
5	CTGGCAGCTGCAGC	= 7.5	TGGGCAGCTGCTGC	= 7.5

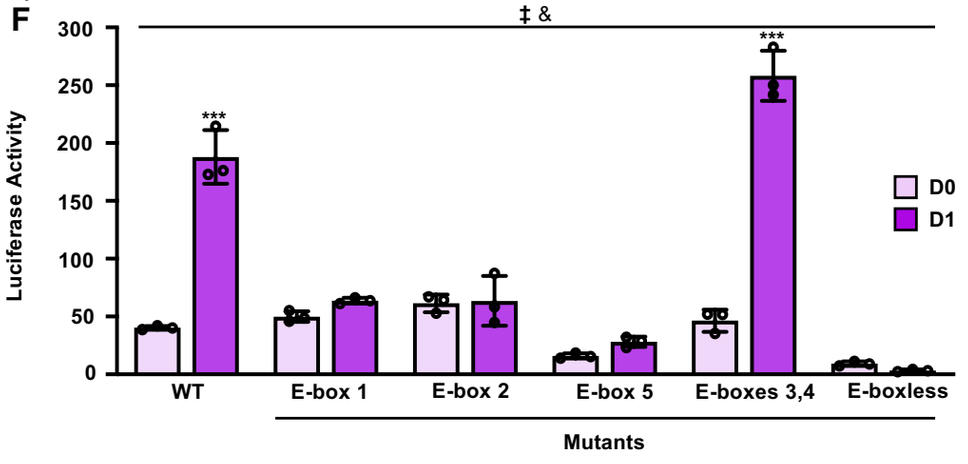


Fig. S2. The miR-206 transcriptional enhancer is dependent on conserved, muscle-specific E-boxes. (A) A 207-bp region approximately 1 kb upstream of the miR-206 locus contains spikes in conservation corresponding to E-box consensus sites. The line drawing below the image (image from Genome Browser (genome.ucsc.edu); blue histogram illustrates conservation across placental mammals) indicates the location of the E-boxes conforming to the consensus sequence CANNTG, with E-boxes conserved in human, mouse, and rat shown in red and those not conserved in human shown in cyan. The complete mouse sequence cloned for reporter gene analysis is also shown with E-boxes similarly color-coded. **(B)** Activity of a firefly luciferase reporter gene driven by the 200-bp genomic region increases during differentiation of C2C12 cells. We transfected C2C12 cells with the constructs indicated on the x-axis. We induced differentiation 24 hours post-transfection and measured reporter activity at Day 0 (proliferating myoblasts), Day 1, and Day 4 after differentiation initiation. MyoG serves as a positive control and has the myogenin enhancer driving firefly luciferase. 1-way ANOVA indicated a significant effect from day of differentiation (line with ‡; $p \leq 0.01$). Asterisks indicate post-test results. ** = $p \leq 0.01$ vs. Day 0, ψψ = $p \leq 0.01$ vs. Day 1. **(C)** The miR-206 200-bp reporter responds to expression of muscle regulatory factors (MRFs) in mouse 10T1/2 fibroblasts. We transfected 10T1/2 cells with the reporters indicated on the x-axis and either empty vector control or overexpression constructs for Flag-tagged MRFs. MyoG and miR-206 200-bp both respond to all 4 MRFs, but the strength of activation in the presence of the various MRFs differs between the reporters. 1-way ANOVA indicated a significant effect from MRF expression (line with ‡; $p \leq 0.0001$). Asterisks indicate post-test results vs Empty Vector. ** = $p \leq 0.01$; *** = $p \leq 0.001$. **(D)** *In vivo* imaging of basal promoter-driven reporter gene expression. Reporter gene activity imaging of minTATA negative control reporter as described and quantified in Fig. 2E. Signal intensity was false colored according to the color bar below. **(E)** The miR-206 transcriptional enhancer contains 3 muscle-specific conserved E-boxes. We analyzed E-boxes from the mouse 200-bp region (numbered from the 5’ to the 3’ end of the cloned region) and the orthologous human region for conformation to the longer muscle-specific E-box consensus sequence described by Buskin and Hauschka (Buskin and Hauschka, 1989). The consensus sequence is shown above the table with the core E-box in red text. The scoring rubric is 0 points for divergence, 0.5 points for a degenerate sequence match, and 1 point for an invariant sequence match (8 = highest possible score). Asterisks indicate divergence from the muscle

consensus sequence. Ø indicates that there is no orthologous human CANNTG sequence. **(F)** Only conserved E-boxes contribute to transcriptional activation from the miR-206 enhancer. We mutated E-boxes in the WT miR-206 200-bp construct from CANNTG to CANNTA. We mutated conserved E-boxes 1, 2, and 5 individually while we mutated unconserved E-boxes 3 and 4 together in one construct. Finally, we mutated all 5 E-boxes in the construct labeled E-boxless. We transfected C2C12 cells with each mutant construct as well as the WT and allowed them to differentiate for one day. We normalized firefly luciferase activity to control renilla luciferase activity. 2-way ANOVA indicated significant effects from reporter and day of differentiation (line with ‡ and &; $p \leq 0.0001$). Asterisks indicate post-test results vs D0. *** = $p \leq 0.001$.

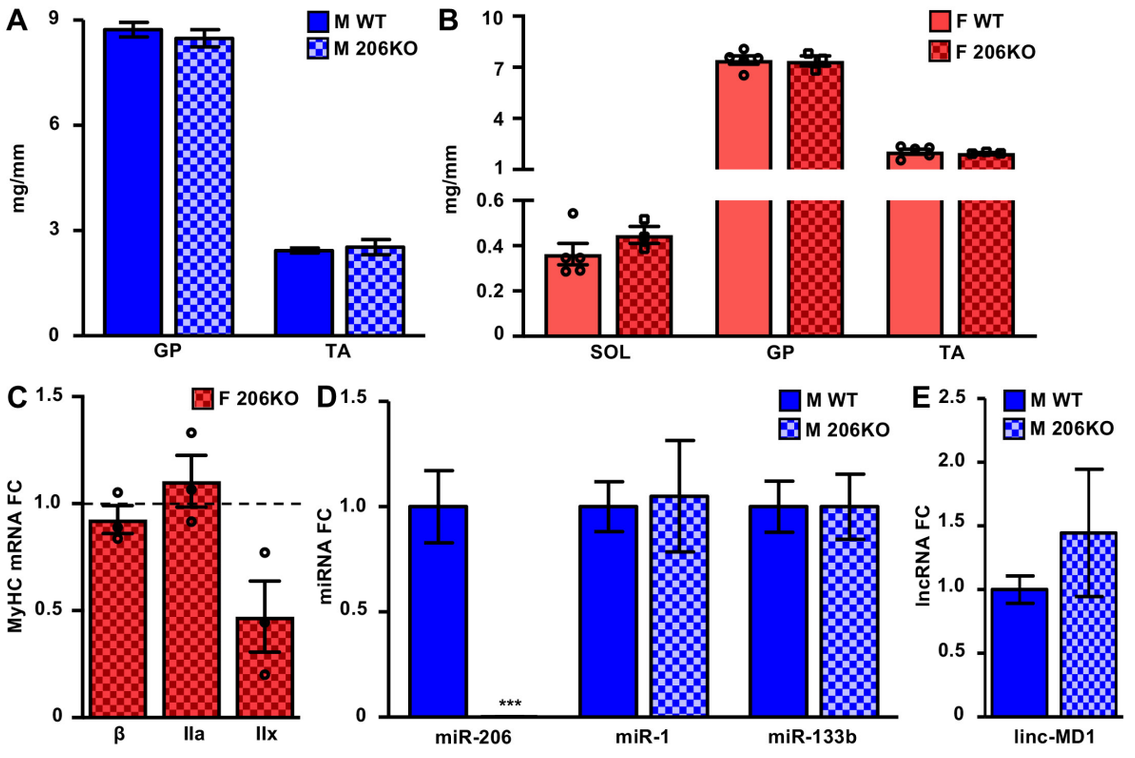


Figure S3. Muscle mass, myomiR, and linc-MD1 analysis in miR-206 KO vs WT mice. (A) Gastrocnemius and plantaris (GP) and tibialis anterior (TA) muscle masses are not different between 206KO and WT male mice. We normalized muscle masses to tibia length (TL) in male (M) mice. Ns are: 7 WT GP, 6 206KO GP, 7 WT TA, 5 206KO TA. **(B)** Soleus (SOL), gastrocnemius and plantaris (GP), and tibialis anterior (TA) muscle masses are not different between 206KO and WT female mice. We normalized muscle masses to tibia length (TL) in female (F) mice. Ns are 5 for WT, 3 for 206KO. **(C)** MyHC mRNA levels do not change in female 206KO SOL as measured by qPCR. WT levels are represented as a dashed line at y = 1. N was 5 for WT, 3 for 206KO. **(D)** miR-206 is undetectable in the 206KO male soleus and there is no compensatory change in miR-1 or miR-133b expression as measured by qPCR. Ns are 6 for both genotypes. *** = $p \leq 0.001$ vs. WT. **(E)** linc-MD1 expression is not different between male 206KO and WT soleus as measured by qPCR. Ns are 7 for WT, 6 for 206KO.

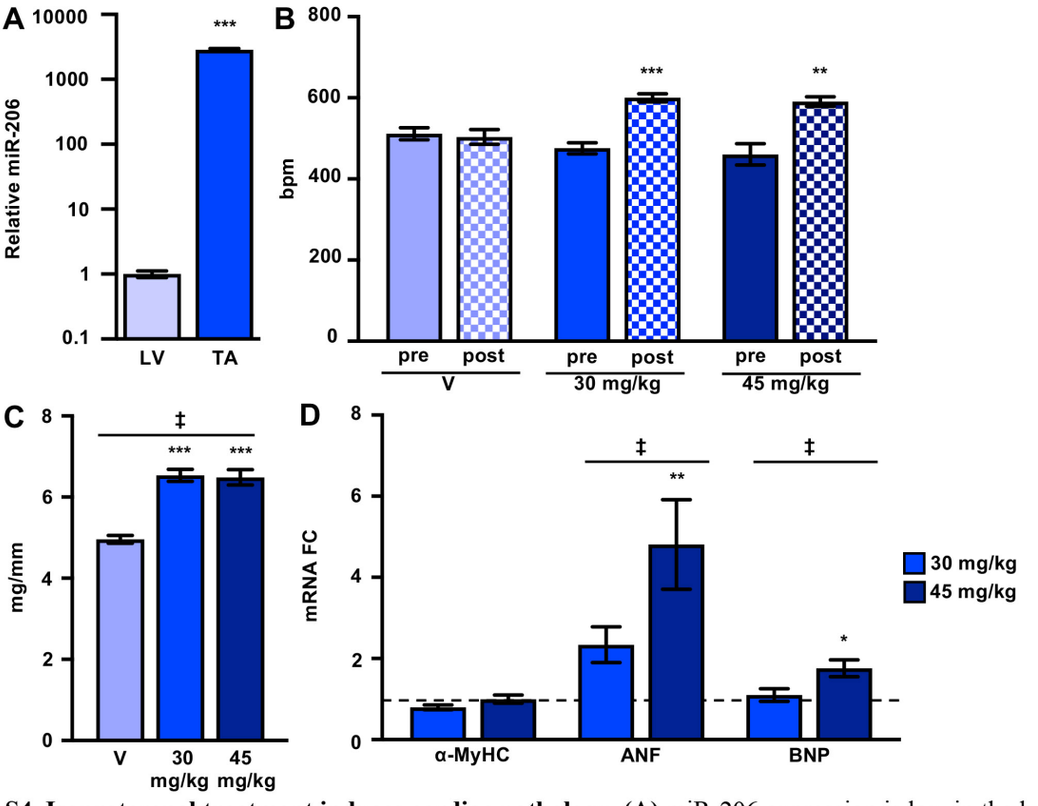


Figure S4. Isoproterenol treatment induces cardiac pathology. (A) miR-206 expression is low in the healthy LV. We measured miR-206 expression by qPCR in the male mouse LV and TA and found expression to be over 3 orders of magnitude higher in the TA. Ns are 6 for the LV and 8 for the TA. *** $p = 1.11 \times 10^{-9}$ vs. LV. **(B)** Heart rate significantly increased after 7 days treatment with both 30 mg kg^{-1} and 45 mg kg^{-1} isoproterenol. We measured heart rate in all animals before (solid bars) and after (patterned bars) treatment. ** = $p \leq 0.01$; *** = $p \leq 0.001$ vs. pre-treatment. **(C)** Left ventricle (LV) mass is higher in both groups of isoproterenol-treated animals compared to vehicle controls. We measured LV mass and normalized to tibia length (TL). ANOVA indicated a significant treatment effect (line with ‡). *** = $p \leq 0.001$ vs. vehicle. **(D)** Gene expression of β-MyHC, ANF, and BNP in the LVs of isoproterenol-treated mice as measured by qPCR. ANOVA indicated a significant treatment effect (line with ‡). Asterisks indicate post-test results. * = $p \leq 0.05$; ** = $p \leq 0.01$ vs. vehicle. Ns are 6 for vehicle control and 8 for both 30 mg kg^{-1} and 45 mg kg^{-1} for all measurements.

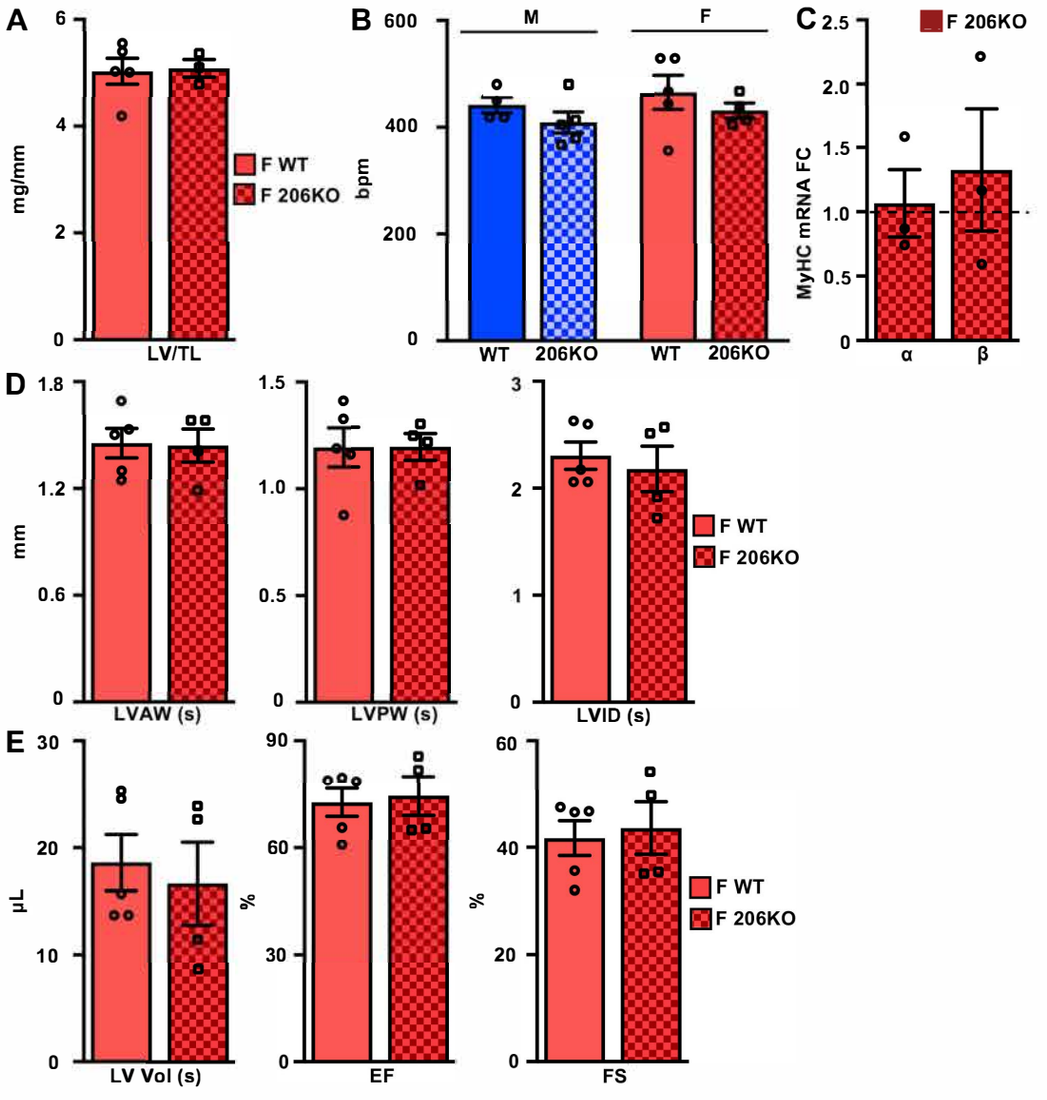


Figure S5. LV mass and M-mode echocardiography measurements. (A) Left ventricle (LV) mass is not different between 206KO females and WT. We measured LV mass and normalized to tibia length (TL) in female (F) mice. N was 5 for WT and 3 for 206KO. **(B)** Heart rate was the same between miR-206 KO and WT males and females. Ns were 4 for WT male (M), 5 for 206KO M, 5 for WT female (F), and 4 for 206KO F. **(C) (D)** M-mode echocardiographic measurements revealed no changes in LV dimensions in female (F) 206KO mice compared to WT. LV anterior wall at systole = LVAW (s), LV posterior wall at systole = LVPW (s), LV interior diameter at systole = LVID (s). Ns are 5 for WT and 4 for 206KO. **(E)** M-mode echocardiographic calculations revealed no changes in LV function in female (F) 206KO mice compared to WT. LV volume at systole = LV Vol (s), ejection fraction = EF, and fractional shortening = FS. Ns are 5 for WT and 4 for 206KO.

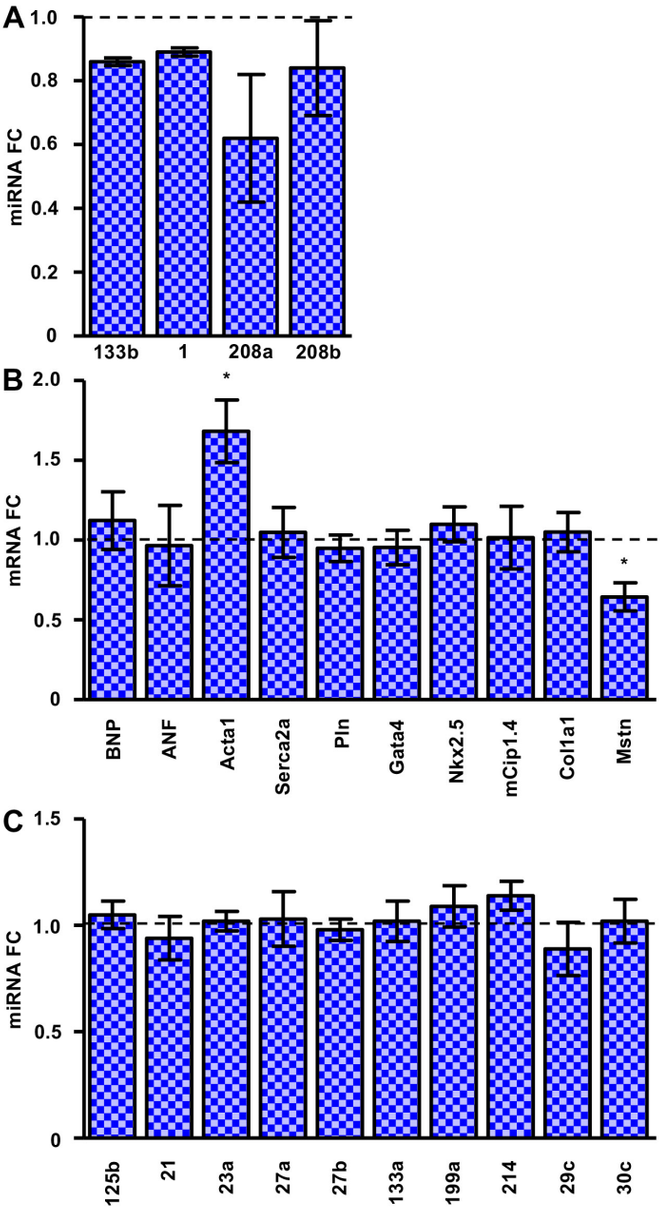


Figure S6. RNA analysis in left ventricles from male miR-206 KO vs WT mice. (A) myomiR expression as measured by qPCR. There is no difference in expression of miRs -133b, -1, -208a, or -208b. Ns are 4-6 per group. **(B)** Expression of mRNA corresponding to cardiac stress markers. Acta1 was significantly up-regulated while Mstn was significantly down-regulated. WT levels are represented as a dashed line at y = 1. Ns are 5-7 per group. * = $p \leq 0.05$ 206KO vs WT. **(C)** Expression of miRNA cardiac stress markers. WT levels are represented as a dashed line at y = 1. Ns are 7 for WT and 6 for 206KO.

Luciferase constructs

miR-206 enhancer SP	GGGGTACCTTAATTTTCCCCATCCAGGGTG
miR-206 enhancer ASP	CCAATGCATTGCAGCTGCCAGATGAGCAT

Mutagenic primers

E-box 1 SP	CTGAATGGCTCAACAGCTACCAGTGTCCATTCATC
E-box 1 ASP	GATGAATGGACACTGGTAGCTGTTGAGCCATTCAG
E-box 2 SP	GATTGTGACGCAGGTAATTGGCCACGAGCAGC
E-box 2 ASP	GCTGCTCGTGGCCAATTACCTGCGTCACAATC
E-box 5 SP	GGAATGCTCATCTGGCAGCTACAATGCAGAGGG
E-box 5 ASP	CCCTCTGCATTGTAGCTGCCAGATGAGCATTCC
E-boxes 3 4 SP	CCACAATGAACAATTATGTTGGAATGCTCATCTAGCAGCTGC
E-boxes 3 4 ASP	GCAGCTGCTAGATGAGCATTCCAACATAATTGTTCATTGTGG

qPCR

pri-miR-206	TCTGCGTGACAAGTGCCTCG
	TTCTCTGGGTGGAGCCATCG
β-MyHC	TTCCTTACTTGCTACCCTC
	CTTCTCAGACTTCCGCAG
MyHC IIA	ATTTCCCAGCTGCACCTTCT
	AAGACAGATGTTTTGGCATCAA
MyHC IIX	GGTCGAAGTTGCATCCCTAA
	GCTTGTTCTGAGCCTCGATT
α-MyHC	ACATTCTTCAGGATTCTCTG
	CTCCTTGTCATCAGGCAC
Embryonic MyHC	GGTCCCATACGTCCACAACT
	TGCAGCTATGCCAAACACTT
Mef2c	TCTGCCCTCAGTCAGTTGG
	CGTGGTGTGTTGTGGGTATC
Serca2a	GCTCAAGTTTGTGGCAATACTG
	TGCAGAGGGCTGGTAGATG
Pln	GTTGTGCCCTTTTTCTACAC
	AGAGAGAGCAGATTTGTGG
Mb	TGGCCATGGACAGGAAGT
	GGTCCTCTGAGCCCTTCATA
Tnni1	TCCACAACACCAGAGAGATCAAGG
	GCATGGCATCGGCTGAGACACG
Tnni2	GAGATGAGGAGAAGCGCAAC
	CGCTATCTGGAGCATCACAC
Six1	ACCGGAGGCAAAGAGACC
	GGAGAGAGTTGATTCTGCTTGTT
Eya1	GGGTCTTTAGACAGTTTCTCAGGT
	TGGGTATGATCTGTTGGAAGG
linc-MYH	GTGCAGCCAGAACAAGACAG

	CAAGATGGGAGGCTCTCAAA
BNP	AAGGTGCTGTCCCAGATG
	TTGGTCCTTCAAGAGCTGTC
ANF	CCAGGCCATATTGGAGCAAA
	GAAGCTGTTGCAGCCTAGTC
Acta1	CGACATCAGGAAGGACCTGTATGCC
	AGCCTCGTCGTACTCCTGCTTGG
Gapdh	AGGTCGGTGTGAACGGATTTG
	TGTAGACCATGTAGTTGAGGTCA
Gata4	CCCTACCCAGCCTACATGG
	ACATATCGAGATTGGGGTGTCT
Nkx2.5	CAAGTGCTCTCCTGCTTTCC
	CTTTGTCCAGCTCCACTGC
mCip1.4	AGCTCCCTGATTGCTTGTGT
	TGGAAGGTGGTGTCCTTGT
Col1a1	AATGGCACGGCTGTGTGCGA
	AACGGGTCCCCTTGGGCCTT
Mstn	GGAACTGATCGATCAGTACG
	CGCTTGCATTAGAAAGTCAG
18S	GCCGCTAGAGGTGAAATTCTTG
	CTTTCGCTCTGGTCCGTCTT
linc-MD1	GCAAGAAAACCACAGAGGAGG
	GTGAAGTCCTTGGAGTTTGAG

Genotyping primers

206KO Primer 1 WT SP	AAATCTCTAGTGGCCTTTGGGGGAGCC
206 KO Primer 3 KO SP	GCTCTATGGCTTCTGAGGCGGAAAGAACC
206KO Primer 2 Common ASP	GCATATTCTCTGGGTGGAGCCATCGG
ArKO Primer 1	CGTGGGCAGGTGATCAGTTTACCATGTCCTAATCTTCAC
ArKO Primer 2 WT	TCTTCTGAGGCCAAATAGCGCAAGATGTTC
ArKO Primer 3 KO	CTGCTAAAGCGCATGCTCCAGACTGCCTTG
mdx4cv WT SP	GATACGCTGCTTTAATGCCTTTAAGAACAGCTGCAGAACAGGAGAC
mdx4cv Mutant SP	CGGCCAGAACAGCTGCAGAACAGGAGAT
mdx4cv Common ASP	GCGCGGCTTGCCTCTGACCTGTCCTAT

Table S1. Primer sequences. Primers for cloning the miR-206 enhancer, mutagenizing the E-boxes in the miR-206 enhancer, qPCR analysis, and genotyping mice are presented 5' → 3'.

References:

Buskin, J. N. and Hauschka, S. D. (1989). Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. *Mol. Cell. Biol.* **9**, 2627–2640.