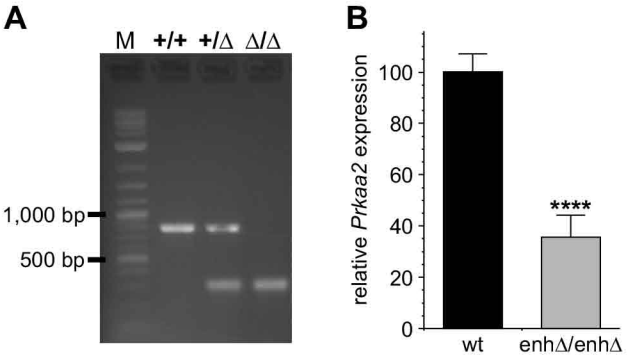
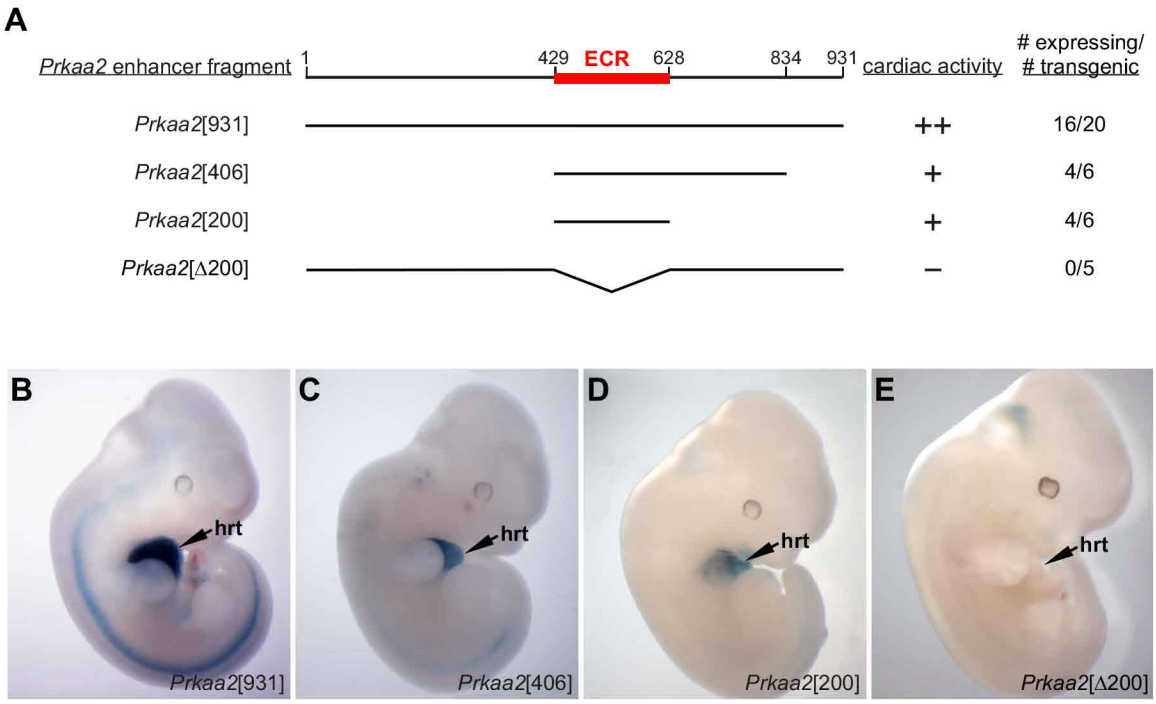


Figure S1. The *Prkaa2* enhancer is required for endogenous *Prkaa2* expression in the heart. (A) PCR detected the wild type and *Prkaa2* enhancer-Δ (1.1-kb deletion) alleles in wild type (+/+), heterozygous (+/Δ), and homozygous enhancer deletion mutants (Δ/Δ). The wild type allele results in the detection of an 820-bp PCR product; the enhancer deletion allele results in the detection of a 325-bp product. (B) Deletion of the *Prkaa2* cardiac enhancer (enhΔ/enhΔ) resulted in a 65% reduction in *Prkaa2* expression in the heart at E11.5 compared to wild type (wt) as determined by RT-qPCR. Results are reported as the mean plus SD; ****, $p<0.0001$ by two-tailed student's *t*-test; $n=5$ hearts for each group.



F

MEF2(2)

```
mouse    ccacttaagcaaggagactgctcccttgaaagggcgaagaaagaaagtttctattattagcagagataaaaactgcctga
human    ccacttaagcaagaatactgttcccttgaaggggtgaagaag----gcttctattattagcagagat---aactgcctga
opossum  c-aattcagcaatgacactgttccctt--tagtgtgaggaag----gtcactattattagcagggataagaactgcctgg
         * * ** *****  * **** ******    * * ** ***     *   ************* ***   *********
```

MEF2(1)

```
mouse    aattccttcctcactagcaccatgctaaaaataaaatggtttaccacacatcagaataaaca--ttgctttgcatattca
human    aattctttccttaataacaccatactaaaaataaaaggttcatcatatttcaaaataaacacattgcttttcatacgca
opossum  aattctttcctaaataacaccattctaaaaataaagttgttca-catacttcaaaataaaaacactactctgaaaaccca
         ***** ***** * ** ****** **********   *** * ** *  *** ****** *   * ** *  * *  **
```

Figure S2. Deletion analyses of the *Prkaa2* cardiac enhancer identify a conserved 200-bp element that is necessary and sufficient for enhancer function *in vivo*. (A) Schematic diagram depicting the deletion constructs from the *Prkaa2* cardiac enhancer. Myocardial expression directed by each construct is depicted on the right. ++, strong myocardial expression; + myocardial expression; minus sign (-), not detectable in the heart. The column on the far right indicates the number of independent founder (F0) transgenic embryos that expressed β-galactosidase in the myocardium as a fraction of the total number of transgene-positive F0 embryos. ECR, evolutionarily-conserved region. (B-E) Representative transgene-positive E11.5 F0 embryos from each of the four fragments shown in (A) stained with X-gal to detect β-galactosidase activity. The 406-bp (*Prkaa2*[406]) and 200-bp (*Prkaa2*[200]) fragments directed specific expression to the myocardium (C,D) and recapitulated the expression pattern of the full-length 931-bp (*Prkaa2*[931]) enhancer (B). Deletion of the 200-bp ECR (*Prkaa2*[Δ200]) completely abolished *Prkaa2* enhancer activity (E). (F) Sequence comparison of the core region of the 200-bp ECR of the *Prkaa2* enhancer. The two perfect consensus MEF2 sites are indicated. Asterisks mark nucleotides conserved among the mouse, human, and opossum sequences.

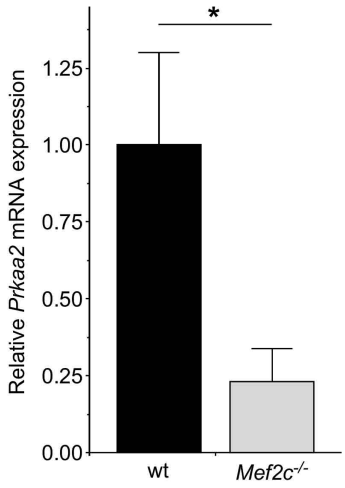


Figure S3. MEF2C regulates expression of *Prkaa2* in the heart *in vivo*. Wild type and *Mef2c*$^{-/-}$ embryos were isolated at E8.5 and RNA was isolated from mechanically dissected hearts and subjected to quantitative RT-PCR (qPCR) analysis of endogenous *Prkaa2* expression. *Prkaa2* expression was reduced by 77% in *Mef2c*$^{-/-}$ compared to wild type hearts. Results are reported as the mean plus SEM; n=7 wild type hearts; n=8 mutant hearts. *, $p<0.05$ by two-tailed student's t-test.

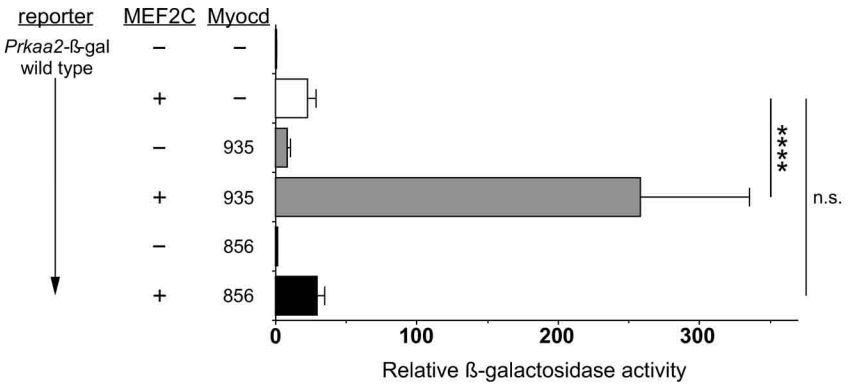


Figure S4. MEF2C and the short form of myocardin (myocardin-856) fail to cooperatively active the *Prkaa2* cardiac enhancer under conditions in which MEF2C and myocardin-935 cooperatively activate the enhancer. P19CL6 cells were co-transfected with the wild type *Prkaa2*-β-gal reporter and expression plasmids for MEF2C, myocardin-856, and myocardin-935. Co-transfected MEF2C is indicated with a plus (+) symbol; co-transfected myocardin-856 and -935 are indicated with the numbers "856" and "935", respectively. In each case, a minus (-) symbol indicates that an equivalent amount of the parental expression plasmid was transfected. Results are reported as the mean fold activation over the *Prkaa2*-β-gal reporter in the presence of parental expression vectors plus SEM; n=8 independent biological replicates for each transfection condition; n.s., not significant; ****, $p<0.0001$ by two-way ANOVA with Bonferroni's post-hoc test.

Table S1. Odds ratio and associated 95% confidence intervals (in parentheses) of detecting paired MEF2 sites in three genomic distance intervals in cardiac versus liver and cardiac versus embryonic stem (ES) cell enhancers.

Distance between paired MEF2 sites	Cardiac versus liver	Cardiac versus ES cell
Less than 10bp	**0.6 (0.5,0.7)**	**0.5 (0.4,0.6)**
Between 10bp and 200bp	**1.7 (1.5,1.9)**	**2.0 (1.7,2.3)**
Greater than 200bp	**0.7 (0.5,1.1)**	**1.6 (1.0,2.6)**

Table S2. List of cardiac enhancers from Wamstad et al (Cell 151, 206-220[2012]) with paired MEF2 sites. In each case site 1 was defined as having no mismatches from the consensus MEF2 site, YTAWWWWTAR. The second MEF2 site was allowed 0, 1, or 2, mismatches. Base locations for MEF2 site 1 and MEF2 site 2, relative to the start of the enhancer, are indicated. Nucleotide coordinates are from the Mouse July 2007 (NCBI37/mm9) Assembly.

Click here to Download Table S2

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids, cloning, and mutagenesis

Prkaa2[406] and *Prkaa2*[200] were generated by PCR from *Prkaa2*[931] by PCR using the following primers, 5′-agaggccagcaccacctaac-3′ and 5′-gggaaaatattttgcagaaa-3′ (*Prkaa2*[406]) and 5′-agaggccagcaccacctaac-3′ and 5′-gtctaagcaaatgagatgaa-3′ (*Prkaa2*[200]). *Prkaa2*[Δ200] was generated by PCR from *Prkaa2*[931] using the external primers Prkaa2-F, 5′-accctgtaaagagggaaaaccaaaac-3′ and Prkaa2-R, 5′-gccaaagcctcgtggttcctgccagc-3′ and the deletion generating primers 5′-aaagtggaaatccatcagaaaaggt-3′ and 5′-ggtttgtacctttctgatggattt-3′. The following mutant sequences were generated in the context of the 931-bp *Prkaa2* enhancer fragment for transgenic analyses and in the 200-bp fragment for transfection analyses: mMEF2(1), 5′-gcaccatgctaa**ccc**aaaatggtttac-3′ and mMEF2(2), 5′-gaaagtttctatt**ccc**agcagagataaa-3′. Plasmid pCDNA3-myocardin-935[mut LZ] was made using the same mutations as described for the short form of myocardin (Wang et al., 2003), using the following primers: mutLZ-F, 5′-gcagaaagtg**acc**aaccag**acc**acctggaag**acc**cggcaa-3′ and mutLZ-R, 5′-gctcttgccg**ggt**cttccaggt**ggt**ctggtt**ggt**cacttt-3′.

Generation and analysis of transgenic and enhancer knockout mice

Generation and genotyping of transgenic mice was performed as described previously (De Val et al., 2004). *Mef2c* (MGI:1857491) and *Myocd* (MGI:2137495) knockout mice have been described previously (Li et al., 2003; Lin et al., 1997). The *Prkaa2*$^{enh\Delta}$ allele was generated by CRISPR-mediated genome editing, using previously described methods (Wang et al., 2013) and the following sgRNAs: sgRNA-1F, 5′-tacttgtgccccaaattccc**(tgg)**-3′ and sgRNA-2F, 5′-cacatgtacttcacatcaat**(ggg)**-3′ plus sgRNA-1R, 5′-gcacccttgtagtaattggg**(tgg)**-5′ and sgRNA-2R, 5′-agacagcaaacatgtactgc**(tgg)**-3′ (protospacer-adjacent motif [PAM] sequences are indicated in parentheses). Two guides on each side of the *Prkaa2* enhancer were

designed to delete an ~1.1 kb region encompassing the 931-bp intronic enhancer element. The *Prkaa2* gene is located on mouse chromosome 4 (Chr4:105029874-105109890). The sgRNAs used to delete the *Prkaa2* enhancer each have low predicted off-targeting (http://crispr.mit.edu; Ran et al., 2013). sgRNA-1F had no predicted off-targets on chromosome 4. sgRNA-2F has a low probability off-target sequence on chromosome 4 [Chr4: 64670185-64670207; tacagctatttcacatcaat(tgg)], containing 4 mismatches and located 40.3 Mb from *Prkaa2*. sgRNA-1R has a low probability off-target sequence on chromosome 4 [Chr4: 143030433-143030455; ggacccttgaagtaatttgg(gag), with 3 mismatches and an imperfect PAM site located 37.9 Mb from *Prkaa2*. sgRNA-2R has a low probability off-target sequence on chromosome 4 [Chr4: 30407325-30407347; atgcaagaaacatgtactgc(cag), with 4 mismatches and an imperfect PAM site located 74.6 Mb from *Prkaa2*. Importantly, none of the predicted off-targets were high confidence and none were in significant linkage disequilibrium with *Prkaa2*.

sgRNAs were transcribed *in vitro* using the MEGAshortscript T7 kit (Life Technologies, AM1354) and were then purified using the MEGAclear kit (Life Technologies, AM1908). Purified sgRNAs and *in vitro* transcribed Cas9 mRNA were co-injected into the cytoplasm of fertilized mouse oocytes using standard transgenic technology as described previously (De Val et al., 2004). Multiple F0 founders containing the predicted deletion were obtained. Founders were outcrossed to wild type mice to establish independent lines from F1 mice. The F1 offspring derived from distinct F0 founders were then intercrossed to generate F2 embryos; since the frequency of any individual off-targeting event is low (Ran et al., 2013), the likelihood of generating the same off-target mutation in two independently generated lines is statistically insignificant. F2 embryos were then analyzed for *Prkaa2* expression in the heart at E9.5 by real-time reverse transcriptase (RT)-quantitative real time PCR (RT-qPCR) using the SYBR green system (Applied Biosystems) and the following primers: 5′-gcggcggcgctcagagcccgcggc-3′ and 5′-cttaactgccactttatggcctg-3′. The presence of wild type and *Prkaa2* enhancer deletion alleles was detected by PCR using a standard 3-

primer genotyping protocol using the following primers: *Prkaa2* genotyping-forward, 5′-gcatcaagaatcatttaagccagt-3′; *Prkaa2* genotyping-reverse, 5′-ttctttacacagtgtgagaagtatgca-3′; *Prkaa2* genotyping-internal, 5′-tcagaggccagcaccacct-3′. The forward and reverse primers flank the deleted region. The binding site of internal primer is absent in the *Prkaa2* enhancer deletion ($Prkaa2^{enh\Delta}$) allele. The wild type allele results in the detection of an 820-bp product (genotyping internal + genotyping-reverse). The enhancer deletion allele results in the detection of a 325-bp product (genotyping forward + genotyping-reverse).

To detect *Prkaa2* mRNA expression in wild type and *Mef2c*-null hearts, embryos were collected at E8.5 and the entire trunk region at the level of the heart tube was mechanically dissected and frozen in liquid nitrogen. Yolk sacs were collected separately for PCR genotyping. RNA was extracted and subjected to RT-qPCR using the SYBR green system (Applied Biosystems) and the following primers: *Prkaa2*-F, 5′-acaggccataaagtggcagtta-3′; *Prkaa2* R: 5′-cgcccatgtttgcagatgta-3′; *Actb*-F: 5′-agtgtgacgttgacatccgt-3′; and *Actb*-R: 5′-tgctaggagccagagcagta-3′. Relative expression of *Prkaa2* to *Actb* (ΔCt) was determined for each sample and the difference between the means of wild type ($Mef2c^{+/+}$) and mutant ($Mef2c^{-/-}$) samples was calculated (ΔΔCt) and subjected to statistical analysis using unpaired, two-tailed student's t test.

All experiments using animals complied with federal and institutional guidelines and were reviewed and approved by the UCSF Institutional Animal Care and Use Committee or the Animal Welfare and Research Committee at Lawrence Berkeley National Laboratory.

X-gal staining and *in situ* hybridization

X-gal staining to detect β-galactosidase activity was performed as described previously (Anderson et al., 2004). For sections, X-gal stained embryos were embedded in paraffin, and transverse and sagittal

sections were cut at a thickness of 7μm and counterstained with Nuclear Fast Red to visualize embryonic structures. To generate the *Prkaa2* antisense and sense probes, a region of the mRNA and 3′ UTR (899 to 1952) was cloned into pCR2.1-TOPO (Invitrogen) by PCR using the following primers: 5′-atttcctgaagacccctcctacgat-3′ and 5′-catgcaaacttgtcacaggcacagg-3′. Antisense probe was generated by linearizing the plasmid with BamHI and transcribing with T7 polymerase.

Cell culture, transfections, and luminescent β-galactosidase assays

P19CL6 cells were seeded at 1.25×10^4 cells/well in a 24-well plate. In each Fugene 6 transfection, 250ng of the indicated reporter plasmid was cotransfected with 250ng of each indicated transactivator. In samples where a cDNA expression plasmid was not transfected, an equal amount of the parental expression vector was transfected. For quantification of β-galactosidase activity in transgenic embryonic cardiac tissue, hearts were harvested at E11.5, quickly frozen in liquid nitrogen, and stored at -80°C. Cellular extracts from transgenic hearts were then prepared by resuspending the hearts in 0.1M $NaPO_4$ buffer and assaying for β-galactosidase activity by chemiluminescence as described previously (De Val et al., 2004).

Myocardin bridging assay

The myocardin bridging assay was conducted using *in vitro* translated MEF2C and myocardin proteins and oligonucleotides corresponding to *Prkaa2* MEF2 site 1 (plus primer extensions) and biotin-labeled *Prkaa2* MEF2 site 2 followed by qPCR using the SYBR green system to detect MEF2 site 1. 10 pmol *Prkaa2* MEF2 site 2 was conjugated to biotin (Integrated DNA Technologies) for subsequent precipitation by streptavidin-conjugated magnetic Dynabeads (Life Technologies, catalog # 65601), according to the manufacturer's recommendation. Streptavidin-conjugated Dynabeads bound to biotin-labeled MEF2 site 2 were then incubated for 4 h at 4°C with *in vitro* translated proteins and 2 pmol unlabeled *Prkaa2* MEF2

site 1 (plus primer extensions) in binding buffer (5mM Tris-HCl, pH 7.4, 0.5mM EDTA, 1M NaCl). Following incubation, immunoprecipitates were washed 5 times in binding buffer and then boiled for 5 min in 0.1% SDS to dissociate protein-DNA complexes. MEF2 site 1 with primer sequence extensions was then detected by qPCR using the SYBR green system (Applied Biosystems) and the following primers: 5′-ctaagcaaatgagatgaatatgca-3′ and 5′-ttccttcctcactagcaccatg-5′.

Bioinformatics and MEF2 site prediction

Cardiac, embryonic stem cell, and liver enhancers were defined as genome regions marked by H3K27ac (Creyghton et al., 2010; Wamstad et al., 2012). Cardiac enhancers included H3K27ac-marked regions in cardiac progenitors and/or cardiomyocytes (Wamstad et al., 2012). For each enhancer, the *matchPattern* function in the Biostrings package (Pages et al., 2014) within Bioconductor (Gentleman et al., 2004) and R (R Core Team, 2015) was used to identify MEF2 sites, defined by the consensus sequence YTAWWWWTAR. We compiled lists of paired MEF2 sites in enhancers with at least one site from each pair being a perfect match site. Other sites were allowed to have at most 2 mismatches. The A/T percentage in the genome in a 200-bp window around each perfect match site was computed. We estimated the odds of finding a paired MEF2 site within each of three distance intervals – less than 10 bp, between 10 and 200 bp, and greater than 200 bp – in a cardiac enhancer relative to the odds in either a liver or an embryonic stem cell enhancer using logistic regression to adjust for the A/T percentage (Table S1). The logistic regression was implemented using the *glm* function in R (R Core Team, 2015).

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