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RESEARCH REPORT

Cooperative activation of cardiac transcription through myocardin bridging of paired MEF2 sites

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ABSTRACT

Enhancers frequently contain multiple binding sites for the same transcription factor. These homotypic binding sites often exhibit synergy, whereby the transcriptional output from two or more binding sites is greater than the sum of the contributions of the individual binding sites alone. Although this phenomenon is frequently observed, the mechanistic basis for homotypic binding site synergy is poorly understood. Here, we identify a bona fide cardiac-specific *Prkaa2* enhancer that is synergistically activated by homotypic MEF2 binding sites. We show that two MEF2 sites in the enhancer function cooperatively due to bridging of the MEF2C-bound sites by the SAP domain-containing co-activator protein myocardin, and we show that paired sites buffer the enhancer from integration site-dependent effects on transcription *in vivo*. Paired MEF2 sites are prevalent in cardiac enhancers, suggesting that this might be a common mechanism underlying synergy in the control of cardiac gene expression *in vivo*.

KEY WORDS: AMPK, MEF2, *Prkaa2*, Mouse, Myocardin, Transcription

INTRODUCTION

Myocyte enhancer factor 2 (MEF2) transcription factors are crucial regulators of cardiac gene expression (Black and Cripps, 2010; Potthoff and Olson, 2007). MEF2 proteins have highly conserved N-terminal MADS and MEF2 domains, which facilitate binding to an AT-rich sequence found in the promoters and enhancers of numerous cardiac genes (Black and Cripps, 2010; Black and Olson, 1998). In mice, *Mef2c* is the earliest *Mef2* gene to be expressed in the heart, and mice lacking *Mef2c* die at embryonic day (E) 9.5 due to profound cardiac defects (Lin et al., 1997). Although MEF2C is widely appreciated as a regulator of cardiac gene expression during development and in adulthood (Black and Cripps, 2010; Potthoff and Olson, 2007), the mechanisms regulating MEF2C-dependent gene expression remain incompletely resolved.

The SAP domain protein myocardin is a positive-acting transcriptional co-activator that has been extensively studied as a co-factor for serum response factor (SRF) (Miano, 2015; Parmacek, 2007; Pipes et al., 2006; Wang and Olson, 2004). Alternative splicing of myocardin produces two isoforms: a short form, myocardin-856, which is expressed in smooth muscle and interacts with SRF; and a long form, myocardin-935, which is expressed in cardiac muscle and can interact with either MEF2 or SRF (Creemers et al., 2006). Interaction of myocardin-935 with MEF2 strongly potentiates the transcriptional activity of MEF2 (Creemers et al., 2006). However, the functional interaction of myocardin with MEF2 remains largely unexplored, and the role of this complex *in vivo* is not known.

Here, we identified a conserved, cardiac-specific enhancer of *Prkaa2*, the gene encoding the catalytic α2 subunit of AMP-activated protein kinase (AMPK). The *Prkaa2* enhancer is dependent on MEF2C for enhancer activity *in vivo*, and it is cooperatively activated by MEF2C and myocardin-935. Mechanistically, we found that the *Prkaa2* enhancer is cooperatively activated by the bridging of two conserved, essential MEF2 sites via myocardin dimerization and concomitant interaction with MEF2C. Moreover, the presence of paired MEF2 sites confers robustness to the *Prkaa2* enhancer *in vivo*, buffering it from integration site-dependent effects on transcriptional output.

RESULTS AND DISCUSSION

Identification of a myocardial-specific *Prkaa2* enhancer

Because of its central role in the heart as a master regulator of energy balance and homeostasis and due to its regulatory changes during heart failure, AMPK has been extensively studied, but, remarkably, the *in vivo* transcriptional regulation of the genes encoding AMPK subunits has not previously been investigated. The first intron of the *Prkaa2* gene contains a ∼1 kb evolutionarily conserved element marked by activating histone marks in an *in vitro* model of cardiomyocyte differentiation (Wamstad et al., 2012) (Fig. 1A). We tested this element for enhancer activity in transgenic mouse embryos and found that it functions as a cardiac enhancer from the cardiac crescent stage, throughout embryonic development, and in adulthood in a pattern that appeared essentially identical to the endogenous pattern of *Prkaa2* mRNA expression (Fig. 1B-P). Transverse sectioning of X-gal-stained embryos showed that staining was only present in cardiac progenitors at E7.75 and thereafter only in the myocardial layer of the heart at E9.5 and E11.5 (Fig. 1D,G,J,M). The *Prkaa2* enhancer did not appear to be active outside of the myocardium, although we cannot rule out activity at later developmental or adult stages in other non-myocardial cell types within the heart.

The location of the enhancer in the first intron of *Prkaa2* and the concordance of enhancer activity with endogenous *Prkaa2* expression (Fig. 1) strongly suggest that this enhancer regulates *Prkaa2* expression. As an explicit test of this notion, we used CRISPR/Cas9 to delete the enhancer from the mouse genome and

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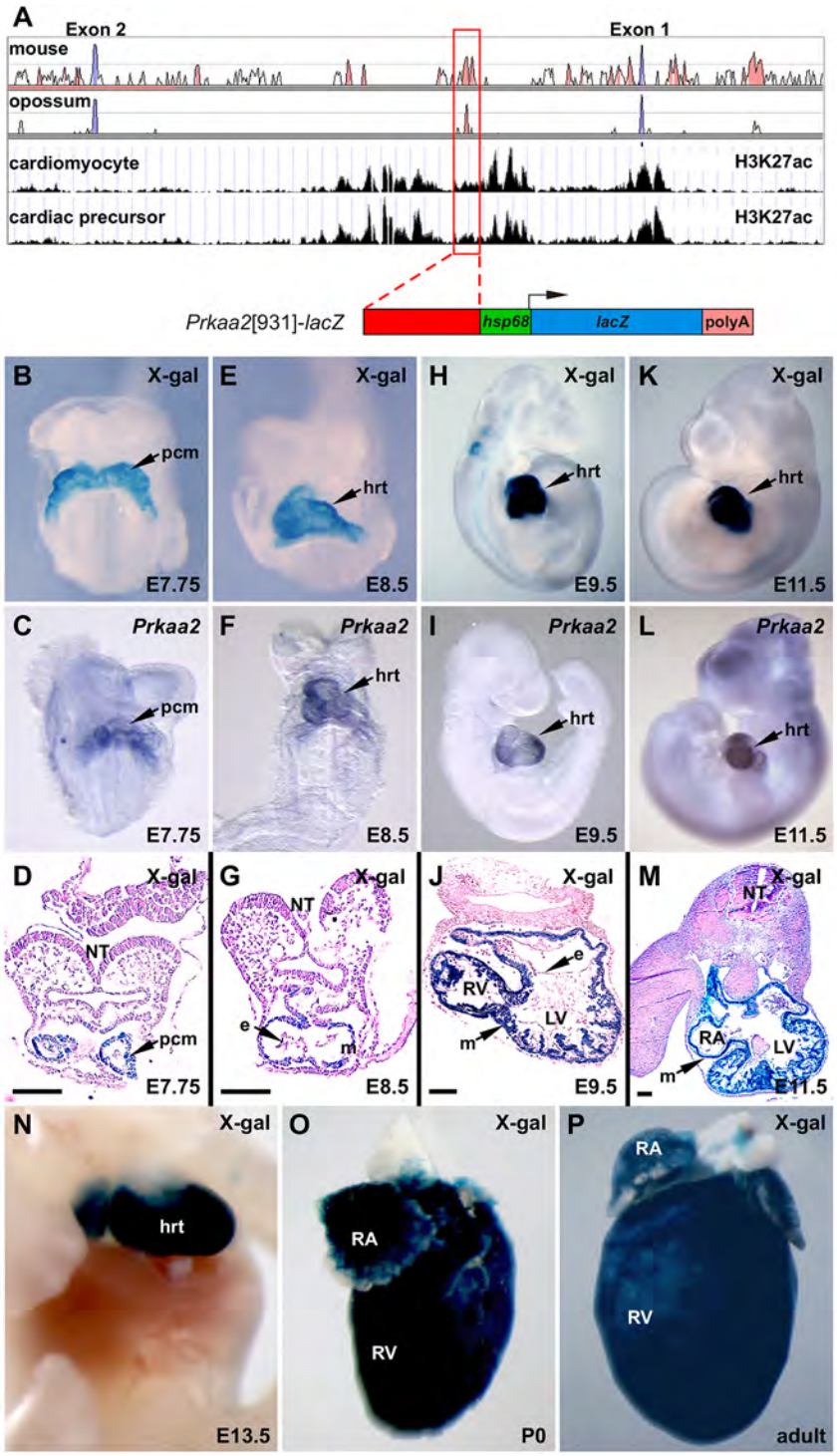

Fig. 1. Identification of a cardiac-restricted *Prkaa2* enhancer. (A) Human:mouse conservation (top box), human:opossum conservation (second box), H3K27 acetylation (Wamstad et al., 2012) in cardiomyocytes (third box), and H3K27 acetylation (Wamstad et al., 2012) in cardiac precursors (fourth box) in the *Prkaa2* locus. The red-boxed peak highlights the 931 bp *Prkaa2* enhancer. Red-filled peaks, noncoding sequences conserved between 75% and 100%; blue-filled peaks, coding sequences conserved between 75% and 100%; white peaks, conservation between 50% and 75%. (B-P) Whole-mount (B,E,H,K,N-P) and sections (D,G,J,M) of X-gal-stained *Prkaa2*[931]-*lacZ* transgenic embryos and postnatal hearts. *Prkaa2*[931] enhancer activity recapitulates the expression pattern of endogenous *Prkaa2* detected by whole-mount *in situ* hybridization (C,F,I,L) from E7.75 through E11.5. e, endocardium; hrt, heart; LV, left ventricle; m, myocardium; NT, neural tube; pcm, precardiac mesoderm; RA, right atrium, RV, right ventricle. Scale bars: 100 µm.

compared *Prkaa2* expression in the presence and absence of this enhancer (Fig. S1). Mice of all genotypes (*Prkaa2*$^{+/+}$, *Prkaa2*$^{+/enh\Delta}$, *Prkaa2*$^{enh\Delta/enh\Delta}$) occurred at predicted Mendelian frequency, and no overt phenotypes were observed (data not shown). However, *Prkaa2*$^{enh\Delta/enh\Delta}$ mice had a 64.4% reduction in *Prkaa2* expression in the heart at E9.5 compared with *Prkaa2*$^{+/+}$ mice (Fig. S1B). This establishes that this intronic element is a bona fide *Prkaa2* transcriptional enhancer. These data also suggest that additional cardiac enhancers for *Prkaa2* must exist to account for the remaining 36% of cardiac gene expression in *Prkaa2*$^{enh\Delta/enh\Delta}$ embryos.

The *Prkaa2* enhancer is a transcriptional target of MEF2C

We next generated a small series of deletion fragments within the 931 bp *Prkaa2* enhancer and found that a 200 bp fragment from nucleotides 429-628 of the 931 bp intronic enhancer was necessary and sufficient to direct expression exclusively to the myocardium at E11.5 (Fig. S2). This 200 bp fragment contains two perfectly conserved MEF2 consensus sites (Fig. S2F), suggesting that this *Prkaa2* enhancer might be regulated by MEF2. Indeed, activity of the enhancer was completely abolished when the *Prkaa2*[931]-*lacZ* transgene was crossed

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onto a *Mef2c*-null background (Fig. 2A,A′). Moreover, endogenous *Prkaa2* expression was also significantly reduced by 77% in the hearts of *Mef2c*-null mice (Fig. S3). Notably, expression of endogenous *Prkaa2* was not completely abolished in the absence of MEF2C function, further supporting the likely existence of additional, MEF2C-independent *Prkaa2* cardiac enhancers. In electrophoretic mobility shift assays (EMSAs), MEF2C bound specifically to each of the *Prkaa2* MEF2 sites (Fig. 2B).

MEF2C significantly activated the *Prkaa2* cardiac enhancer in P19CL6, a cardiac progenitor-like cell line, and this activation was dependent on the presence of intact MEF2 sites (Fig. 2C). We also examined the ability of MEF2C-VP16, a fusion of MEF2C with a potent transactivation domain from herpes simplex virus, to activate the *Prkaa2* enhancer (Fig. 2D). Similar to wild-type MEF2C, MEF2C-VP16 activated the *Prkaa2* reporter in a MEF2 site-dependent fashion (Fig. 2D), but activation was much more robust than by wild-type MEF2C (~5-fold for MEF2C compared with >2000-fold for MEF2C-VP16).

Cooperative activation of the *Prkaa2* enhancer by MEF2C and myocardin

One possible explanation for the dramatic difference in transactivation of the *Prkaa2* enhancer by MEF2C-VP16 compared with MEF2C is that P19CL6 cells might be missing one or more MEF2C co-activator proteins required for robust activation, and fusion of the VP16 activation domain compensated for the missing co-factor. The long form of myocardin is restricted to the heart and has been shown to coactivate MEF2 (Creemers et al., 2006). Therefore, to determine if myocardin might be involved in *Prkaa2* regulation *in vivo*, we crossed the *Prkaa2*[931]-*lacZ* transgene onto a myocardin-null ($Myocd^{-/-}$) background and examined β-galactosidase activity at E9.5 (Fig. 3A). X-gal staining of $Myocd^{-/-}$ embryos was noticeably weaker than staining of wild-type embryos (Fig. 3A,A′). Using a quantitative luminescence assay, we found ~80% reduction in β-galactosidase activity in *Myocd*-null hearts compared with wild type or *Myocd* heterozygotes (Fig. 3A″). These data indicate that myocardin regulates the *Prkaa2* enhancer *in vivo*.

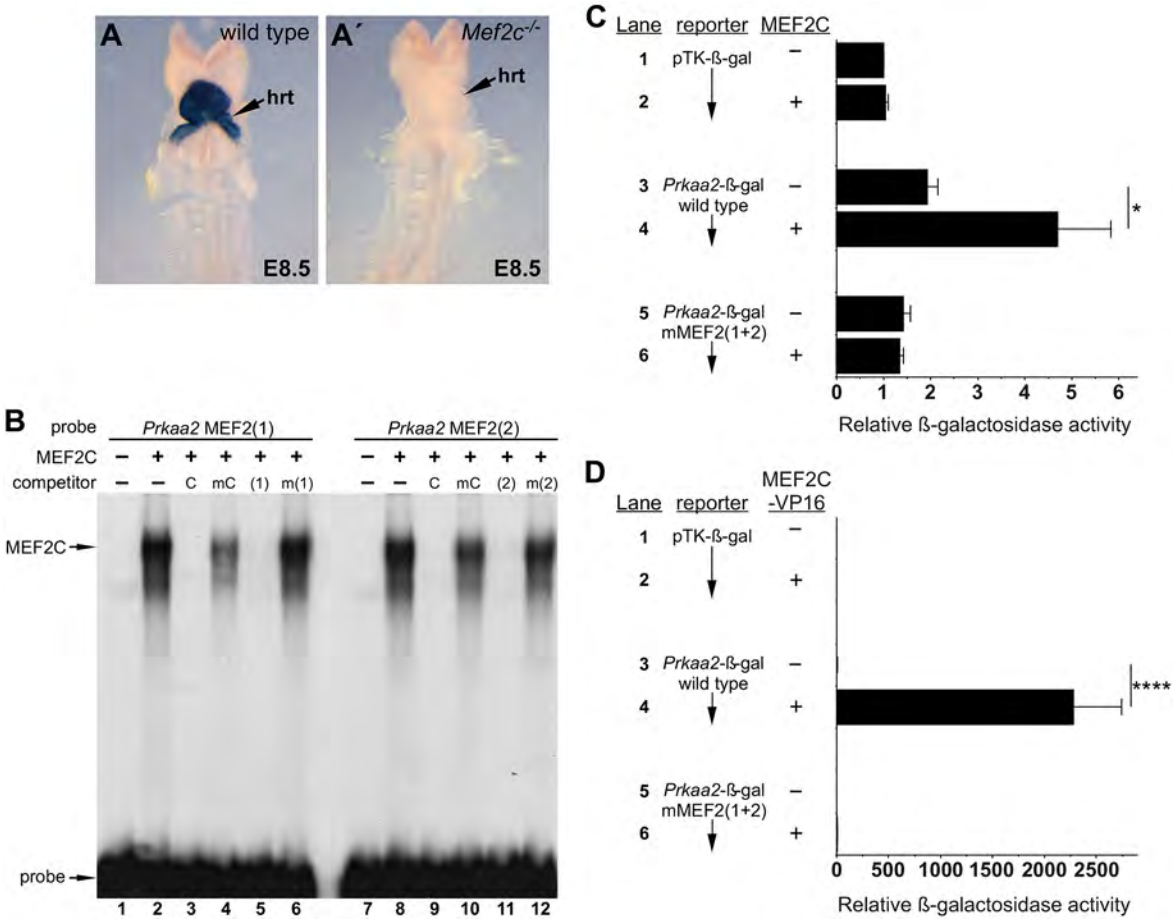

Fig. 2. The *Prkaa2* cardiac enhancer requires MEF2C for activity. (A,A′) *Prkaa2*[931]-*lacZ* transgenic mice were crossed into $Mef2c^{+/+}$ (wild type, A) and $Mef2c^{-/-}$ (A′) backgrounds, and enhancer activity was examined by X-gal staining at E8.5; hrt, heart. (B) *Prkaa2* MEF2 site 1 (lanes 1-6) or *Prkaa2* MEF2 site 2 (lanes 7-12) was used in EMSA with reticulocyte lysate (–, lanes 1 and 7) or with recombinant MEF2C (+, lanes 2-6 and 8-12). MEF2C efficiently bound to both of the *Prkaa2* MEF2 sites (lanes 2 and 8). Binding to each site was competed by an excess of unlabeled control MEF2 site from the myogenin gene (C) or by unlabeled self probe (1) or (2), respectively, but not by mutant versions of the unlabeled competitors [mC, m(1), m(2)]. (C,D) P19CL6 cells were co-transfected with parental pTK-β-gal reporter (lanes 1, 2), wild-type *Prkaa2*-β-gal (lanes 3, 4), or a mutant version of the *Prkaa2*-β-gal reporter with both MEF2 sites disrupted (lanes 5, 6). Co-transfection of an expression plasmid for MEF2C (C) or MEF2C-VP16 (D) is indicated with a plus symbol; a minus symbol indicates that an equivalent amount of the parental expression plasmid was added. Results are reported as mean+s.e.m.; n=14 (C) or n=8 (D) independent biological replicates. Note the difference in the values on the x-axes for transactivation by MEF2C (C) versus MEF2C-VP16 (D). *P<0.05, ****P<0.0001, two-way ANOVA with Bonferroni's post-hoc test.

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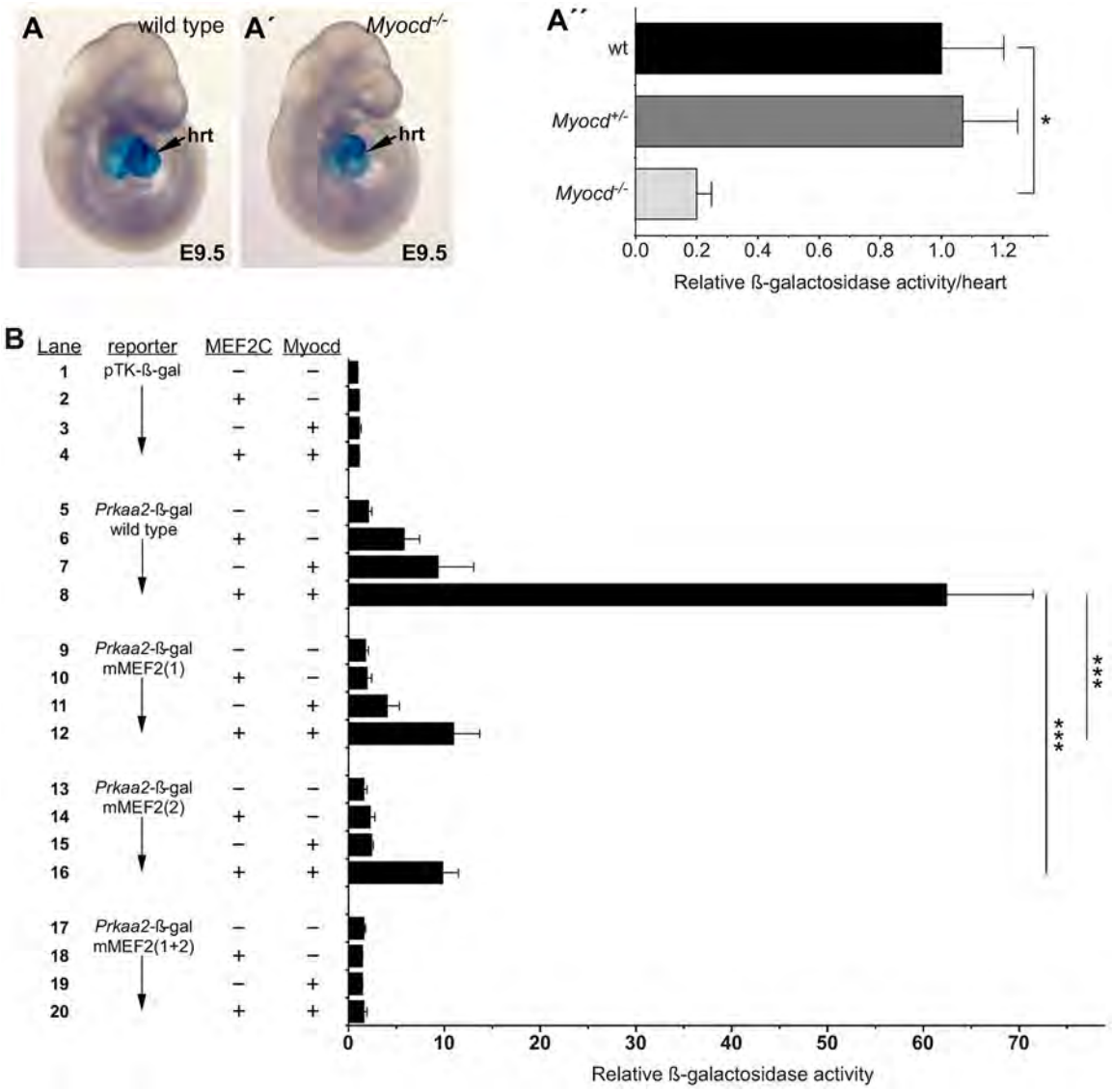

Fig. 3. Myocardin-935 regulates the *Prkaa2* cardiac enhancer. (A-A″) *Prkaa2*[931]-*lacZ* transgenic mice were crossed into *Myocd*$^{+/+}$ (wild type, A), *Myocd*$^{+/-}$ and *Myocd*$^{-/-}$ (A′) genetic backgrounds and enhancer activity was examined qualitatively by X-gal staining (A,A′) or quantitatively by chemiluminescence β-galactosidase assay (A″) at E9.5. hrt, heart. Data in A″ are expressed as the mean β-galactosidase activity (+s.e.m.) with the mean activity on the *Myocd*$^{+/+}$ background normalized to a value of 1. *n*=4 (*Myocd*$^{+/+}$), *n*=5 (*Myocd*$^{+/-}$) and *n*=3 (*Myocd*$^{-/-}$) independent biological replicates. **P*<0.05, one-way ANOVA with Bonferroni's post-hoc test. (B) P19CL6 cells were co-transfected with the parental pTK-β-gal reporter (lanes 1-4), wild-type *Prkaa2*-β-gal (lanes 5-8), or mutant versions of the *Prkaa2*-β-gal reporter containing disruptions in MEF2 site 1 (lanes 9-12), site 2 (lanes 13-16) or both MEF2 sites (lanes 17-20). Co-transfections with expression plasmids for MEF2C and myocardin-935 are indicated with a plus symbol; a minus symbol indicates that an equivalent amount of the parental expression plasmid was added. Results shown are the mean fold activation over the pTK-β-gal reporter in the presence of parental expression vectors+ s.e.m.; *n*=9 independent biological replicates. ****P*<0.001, two-way ANOVA with Bonferroni's post-hoc test.

We next examined cooperative activation of the *Prkaa2* enhancer by MEF2C and myocardin-935 in P19CL6 cells (Fig. 3B). MEF2C weakly, but significantly, activated the wild-type *Prkaa2*-β-gal reporter on its own (Fig. 3B, lanes 5 and 6; Fig. S4). Activation of the wild-type reporter was very potently augmented by cotransfection of a myocardin-935 expression plasmid (Fig. 3B, lanes 6 and 8; Fig. S4). The weak activation of the wild-type reporter by myocardin alone (Fig. 3B, lane 7) is likely to be due to low levels of MEF2 in P19CL6 cells, since mutation of the MEF2 sites in the *Prkaa2* enhancer abolished the myocardin-dependent activation of the reporter (Fig. 3B, lane 19). Mutation of either MEF2 site dramatically reduced, but did not abolish, cooperative activation by myocardin and MEF2C (Fig. 3B, lanes 9-16). Mutation of both MEF2 sites completely abolished activation of the reporter by MEF2C and myocardin (Fig. 3B, lanes 17-20). Importantly, no synergy was observed between MEF2C and the short form of myocardin (myocardin-856) on the *Prkaa2* enhancer under conditions in which myocardin-935 potently augmented MEF2C-dependent transactivation of the enhancer (Fig. S4).

Bridging of two MEF2 sites by myocardin dimerization

MEF2C and myocardin activated the *Prkaa2* reporters with a single intact MEF2 site by ∼10-fold but activated the wild-type reporter with two intact MEF2 sites by more than 60-fold (Fig. 3B), suggesting that the two sites function cooperatively. Previous work has shown that myocardin homodimerizes through a conserved leucine zipper (LZ) domain and that homodimerization facilitates stronger activation of SRF-dependent reporter genes

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containing two or more SRF binding sites (Wang et al., 2003). Based on these observations, we hypothesized that myocardin dimerization might facilitate interaction between the two *Prkaa2* MEF2 sites, and we tested this notion using an *in vitro* pull-down experiment (Fig. 4A,B). Addition of MEF2C or myocardin-935 alone resulted in minimal pull-down of MEF2 site 1 by MEF2 site 2 (Fig. 4B, lane 1). By contrast, inclusion of both MEF2C and myocardin-935 resulted in robust and highly significant pull-down of MEF2 site 1 by MEF2 site 2 (Fig. 4B, lane 4). Mutation of the myocardin LZ motif in a manner predicted to disrupt homodimerization (Wang et al., 2003) completely abolished the complex and resulted in only baseline pull-down of MEF2 site 1 (Fig. 4B, lane 5). These experiments demonstrate that myocardin-935 dimerization can facilitate interaction between two MEF2C-bound MEF2 sites.

The two MEF2 sites in the *Prkaa2* cardiac enhancer exhibit cooperative activity *in vivo*

To determine whether the *Prkaa2* MEF2 sites are required for enhancer activity *in vivo*, we generated transgenic mouse embryos with the MEF2 sites mutated singly and in combination (Fig. 4C-H). The wild-type (wt) *Prkaa2* enhancer directed robust expression in E11.5 and adult hearts (Fig. 4C,G). Mutation of both MEF2 sites resulted in complete loss of detectable X-gal staining in every transgenic founder examined at E11.5 (Fig. 4F) and in adult hearts (Fig. 4H). By contrast, mutation of either of the MEF2 sites alone

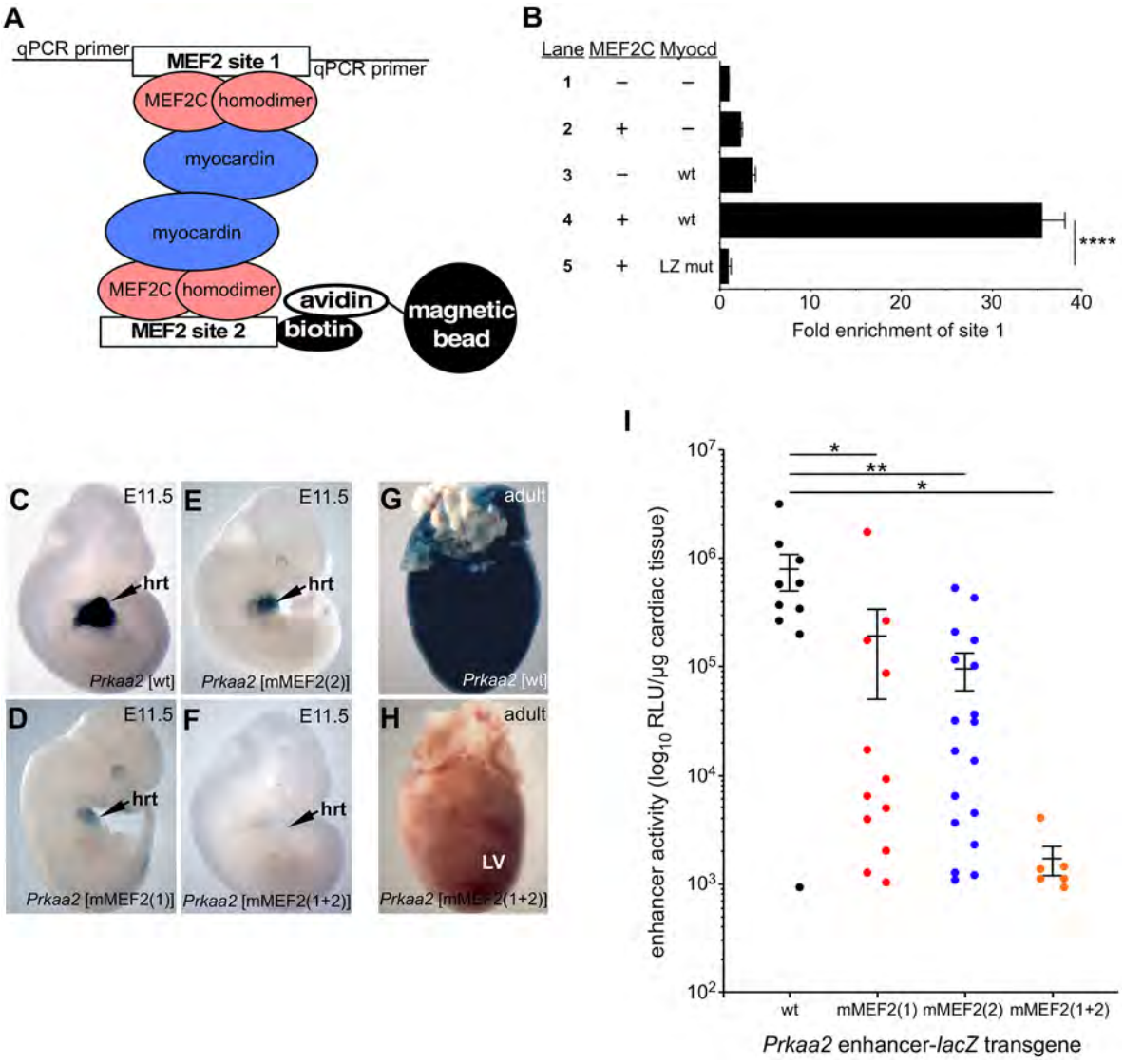

Fig. 4. Bridging of MEF2C-bound MEF2 sites by myocardin-935. (A) Schematic of the *in vitro* pull-down assay. (B) qPCR detection of co-precipitated MEF2 site 1 after incubation with biotinylated MEF2 site 2 in the presence of reticulocyte lysate control (lane 1), MEF2C alone (lane 2), myocardin-935 (wt) alone (lane 3), MEF2C plus myocardin-935 (lane 4), or MEF2C plus a leucine zipper mutant form of myocardin-935 (LZ mut) (lane 5). Results are presented as mean fold enrichment over the reticulocyte lysate control+s.d. ****P<0.0001, two-way ANOVA with Bonferroni's post-hoc test. (C-H) The MEF2 sites in the *Prkaa2* cardiac enhancer act synergistically *in vivo*. The wild-type *Prkaa2*[931]-*lacZ* transgenic reporter (C) and versions containing mutations in MEF2 site 1 (D), site 2 (E) or both MEF2 sites (F) were used to generate multiple independent transgenic lines or F0 embryos, and representative E11.5 embryos are shown. hrt, heart; LV, left ventricle. (G,H) The presence of both MEF2 sites is required for *Prkaa2* enhancer activity in the adult heart. (I) Quantitation of β-galactosidase activity in E11.5 hearts from each of the *Prkaa2*[931]-*lacZ* transgenic reporter constructs shown in C-F. Each point on the graph represents a single embryonic heart from an independently generated transgenic embryo. Data are expressed as mean RLU/µg of excised heart tissue+s.e.m. Note the log scale on the *y*-axis. *P<0.05, **P<0.01, two-way ANOVA with Bonferroni's post-hoc test.

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reduced (but did not completely abolish) enhancer activity (Fig. 4D,E). These observations are consistent with the transactivation data (Fig. 3B), where we observed that mutation of a single site profoundly reduced, but did not completely abolish, transactivation whereas mutation of both MEF2 sites completely abolished transactivation by MEF2C and myocardin-935.

To determine if the *Prkaa2* MEF2 sites function cooperatively *in vivo*, we generated numerous independent F0 transgenic founder embryos with each of the transgene constructs, and quantified β-galactosidase activity in E11.5 hearts (Fig. 4I). The wild-type enhancer showed consistently strong activity (n=10 independent transgenic lines) with only a few outliers and a mean activity of 792,489 RLU/μg of cardiac tissue. Mutation (m) of either MEF2 site resulted in a profound and significant diminution of activity (mMEF2 site 1, n=12 independent transgenic lines, $\bar{x}$=194,591; mMEF2 site 2, n=18 independent transgenic lines, $\bar{x}$=96,627). Mutation of both MEF2 sites [mMEF2(1+2)] completely abolished enhancer activity in every transgenic founder examined (n=6 independent transgenic lines, $\bar{x}$=1700). These data demonstrate a cooperative (greater than additive) relationship between the two *Prkaa2* MEF2 sites *in vivo*. Interestingly, nearly all *Prkaa2-lacZ* transgenic lines with two intact MEF2 sites exhibited strong activity that ranged by less than a single order of magnitude, whereas transgenic embryos made from *Prkaa2-lacZ* constructs with only a single intact MEF2 site showed far greater variability ranging over nearly three orders of magnitude (Fig. 4I); this suggests that the presence of two functional MEF2 sites buffers the enhancer from silencing due to positional effects (Elgin and Reuter, 2013).

Paired MEF2 sites are prevalent in predicted cardiac enhancers

We analyzed predicted enhancers from mouse embryonic stem cells (ESCs), ESC-derived cardiac cells (cardiomyocytes and cardiac progenitors) and liver cells (Creyghton et al., 2010; Wamstad et al., 2012) and found that paired MEF2 sites occur in ∼30% of predicted cardiac enhancers. Interestingly, paired MEF2 sites occur 1.7 times more frequently in predicted cardiac enhancers than in liver enhancers (95% CI 1.5-1.9) and 2.0 times more frequently than in ESCs (95% CI 1.7-2.3) (Tables S1 and S2). The significant enrichment of paired MEF2 sites in predicted cardiac enhancers compared with liver and ESC enhancers suggests that natural selection has favored this arrangement in cardiac enhancers, possibly due to an advantage in gene expression conferred by myocardin bridging of the sites. Future studies will determine how the sequence and spacing of paired MEF2 sites in the *Prkaa2* enhancer and other *cis*-regulatory elements determines the timing and robustness of cardiac gene activation.

MATERIALS AND METHODS

Plasmids, cloning and mutagenesis

A 931 bp fragment of the mouse *Prkaa2* gene located between exons 1 and 2 was amplified by PCR using primers Prkaa2-F (5′-ACCCTGTAAA GAGGGAAAACCAAAAC-3′) and Prkaa2-R (5′-GCCAAAGCCTCG TGGTTCCTGCCAGC-3′) and then cloned into plasmid *hsp68-lacZ* (Kothary et al., 1989) to generate reporter plasmid *Prkaa2*[931]-*lacZ* for use in transgenic analyses and into plasmid pTK-β-gal (Robinson et al., 2014) to create plasmid *Prkaa2*-β-gal for use in transfection analyses. Plasmids pCDNA1-MEF2C, pCDNA1-MEF2C-VP16 and pCDNA3-Myocardin-935 have been described previously (Black et al., 1996; Black et al., 1995; Creemers et al., 2006). Details of deletion and site-specific mutations are provided in the supplementary Materials and Methods.

Generation and analysis of transgenic and enhancer knockout mice

Generation 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 (Wang et al., 2013). Additional details of CRISPR-mediated genome editing and mouse genotyping are provided in the supplementary Materials and Methods. Adult hearts were collected from female ICR mice at 12 weeks of age; embryos were collected from 6- to 52-week-old ICR mice. No inclusion or exclusion criteria were defined, and no animals were excluded from analyses. All experiments using animals complied with federal and institutional guidelines and were reviewed and approved by the UCSF IACUC.

X-gal staining and *in situ* hybridization

X-gal staining was performed as described previously (Anderson et al., 2004). Whole-mount and section *in situ* hybridization with digoxigenin-labeled antisense probes was performed as described (Rojas et al., 2005). Detailed protocol information is provided in the supplementary Materials and Methods.

Cell culture, transfections and luminescent β-galactosidase assays

P19CL6 cells (obtained from Richard Kitsis; Peng et al., 2002) were authenticated by differentiation assay and confirmed to be free of contamination during experiments, were maintained in Minimum Essential Medium (MEM) α supplemented with 10% fetal bovine serum, and were transfected using Fugene 6 (Roche) according to the manufacturer's recommendations. Cells were harvested 48 h post-transfection, and cellular extracts were prepared and assayed for β-galactosidase activity using the Luminescent β-galactosidase Detection Kit (Clontech) as previously described (Dodou et al., 2003). For details, see the supplementary Materials and Methods.

EMSA and myocardin bridging assay

EMSAs were performed as previously described (Dodou et al., 2003). The sense strand sequences of the *Prkaa2* oligonucleotides used for EMSA (with the MEF2 site underlined and mutant sequences indicated in bold) were: MEF2(1), 5′-GGGCA CCATGCTAAAAATAAAATGGTTT-3′; MEF2(2), 5′-GGGAAAG TTTCTATTATTAGCAGAGATA-3′; mMEF2 (1), 5′-GGGCACCATG CTAAA**CCC**AAAATGGTTT-3′; and mMEF2(2), 5′-GGGAAAGTTT CTATT**CCC**AGCAGAGATA-3′. Control and mutant control MEF2 sites from the myogenin promoter have been described (Yee and Rigby, 1993). Methods for the myocardin bridging assay are described in the supplementary Materials and Methods.

Bioinformatics and MEF2 site prediction

Cardiac enhancers (Wamstad et al., 2012) were analyzed using the matchPattern function in the Biostrings package (Pages et al., 2014) within Bioconductor (Gentleman et al., 2004) and R (R Core Team, 2015) to identify MEF2 sites, as defined by the consensus sequence YTAWWWTAR. For further details, see the supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.M.A., J.H., T.B.G., B.C., T.S. and A.B.H. performed experiments and analyzed data. S.-M.X., D.E.D. and L.A.P. generated transgenic and enhancer knockout mice. R.T. and K.S.P. performed bioinformatics analyses. B.G.B. helped identify the *Prkaa2* enhancer and made intellectual contributions. B.L.B. conceived and directed the project, analyzed data and wrote the paper. All authors discussed and commented on the manuscript.

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Supplementary information
Supplementary information available online at
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References

Anderson, J. P., Dodou, E., Heidt, A. B., De Val, S. J., Jaehnig, E. J., Greene, S. B., Olson, E. N. and Black, B. L. (2004). HRC is a direct transcriptional target of MEF2 during cardiac, skeletal, and arterial smooth muscle development in vivo. *Mol. Cell. Biol.* **24**, 3757-3768.

Black, B. L. and Cripps, R. M. (2010). Myocyte enhancer factor 2 transcription factors in heart development and disease. In *Heart Development and Regeneration* (ed. N. Rosenthal and R. P. Harvey), pp. 673-699. Oxford: Academic Press.

Black, B. L. and Olson, E. N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu. Rev. Cell Dev. Biol.* **14**, 167-196.

Black, B. L., Martin, J. F. and Olson, E. N. (1995). The mouse MRF4 promoter is trans-activated directly and indirectly by muscle-specific transcription factors. *J. Biol. Chem.* **270**, 2889-2892.

Black, B. L., Ligon, K. L., Zhang, Y. and Olson, E. N. (1996). Cooperative transcriptional activation by the neurogenic basic helix-loop-helix protein MASH1 and members of the myocyte enhancer factor-2 (MEF2) family. *J. Biol. Chem.* **271**, 26659-26663.

Creemers, E. E., Sutherland, L. B., Oh, J., Barbosa, A. C. and Olson, E. N. (2006). Coactivation of MEF2 by the SAP domain proteins myocardin and MASTR. *Mol. Cell* **23**, 83-96.

Creyghton, M. P., Cheng, A. W., Welstead, G. G., Kooistra, T., Carey, B. W., Steine, E. J., Hanna, J., Lodato, M. A., Frampton, G. M., Sharp, P. A. et al. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc. Natl. Acad. Sci. USA* **107**, 21931-21936.

De Val, S., Anderson, J. P., Heidt, A. B., Khiem, D., Xu, S.-M. and Black, B. L. (2004). Mef2c is activated directly by Ets transcription factors through an evolutionarily conserved endothelial cell-specific enhancer. *Dev. Biol.* **275**, 424-434.

Dodou, E., Xu, S.-M. and Black, B. L. (2003). mef2c is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. *Mech. Dev.* **120**, 1021-1032.

Elgin, S. C. R. and Reuter, G. (2013). Position-effect variegation, heterochromatin formation, and gene silencing in Drosophila. *Cold Spring Harb. Perspect. Biol.* **5**, a017780.

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J. et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80.

Kothary, R., Clapoff, S., Darling, S., Perry, M. D., Moran, L. A. and Rossant, J. (1989). Inducible expression of an hsp68-lacZ hybrid gene in transgenic mice. *Development* **105**, 707-714.

Li, S., Wang, D.-Z., Wang, Z., Richardson, J. A. and Olson, E. N. (2003). The serum response factor coactivator myocardin is required for vascular smooth muscle development. *Proc. Natl. Acad. Sci. USA* **100**, 9366-9370.

Lin, Q., Schwarz, J., Bucana, C. and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-1407.

Miano, J. M. (2015). Myocardin in biology and disease. *J. Biomed. Res.* **29**, 3-19.

Pages, H., Aboyoun, P., Gentleman, R. and DebRoy, S. (2014). Biostrings: string objects representing biological sequences, and matching algorithms. R package version 2.36.4.

Parmacek, M. S. (2007). Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation. *Circ. Res.* **100**, 633-644.

Peng, C.-F., Wei, Y., Levsky, J. M., McDonald, T. V., Childs, G. and Kitsis, R. N. (2002). Microarray analysis of global changes in gene expression during cardiac myocyte differentiation. *Physiol. Genomics* **9**, 145-155.

Pipes, G. C. T., Creemers, E. E. and Olson, E. N. (2006). The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. *Genes Dev.* **20**, 1545-1556.

Potthoff, M. J. and Olson, E. N. (2007). MEF2: a central regulator of diverse developmental programs. *Development* **134**, 4131-4140.

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.

Robinson, A. S., Materna, S. C., Barnes, R. M., De Val, S., Xu, S. M. and Black, B. L. (2014). An arterial-specific enhancer of the human endothelin converting enzyme 1 (ECE1) gene is synergistically activated by Sox17, FoxC2, and Etv2. *Dev. Biol.* **395**, 379-389.

Rojas, A., De Val, S., Heidt, A. B., Xu, S. M., Bristow, J. and Black, B. L. (2005). Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. *Development* **132**, 3405-3417.

Wamstad, J. A., Alexander, J. M., Truty, R. M., Shrikumar, A., Li, F., Eilertson, K. E., Ding, H., Wylie, J. N., Pico, A. R., Capra, J. A. et al. (2012). Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage. *Cell* **151**, 206-220.

Wang, D.-Z. and Olson, E. N. (2004). Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Curr. Opin. Genet. Dev.* **14**, 558-566.

Wang, Z., Wang, D.-Z., Pipes, G. C. T. and Olson, E. N. (2003). Myocardin is a master regulator of smooth muscle gene expression. *Proc. Natl. Acad. Sci. USA* **100**, 7129-7134.

Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F. and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910-918.

Yee, S. P. and Rigby, P. W. (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.

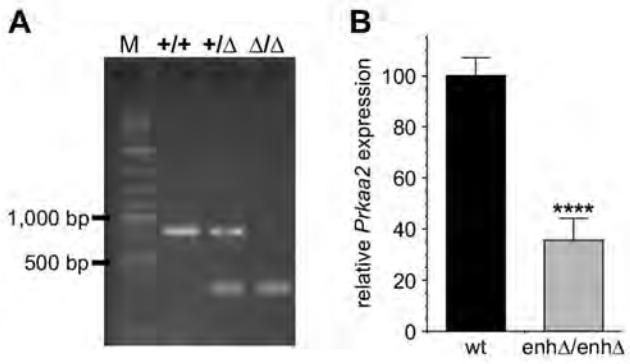
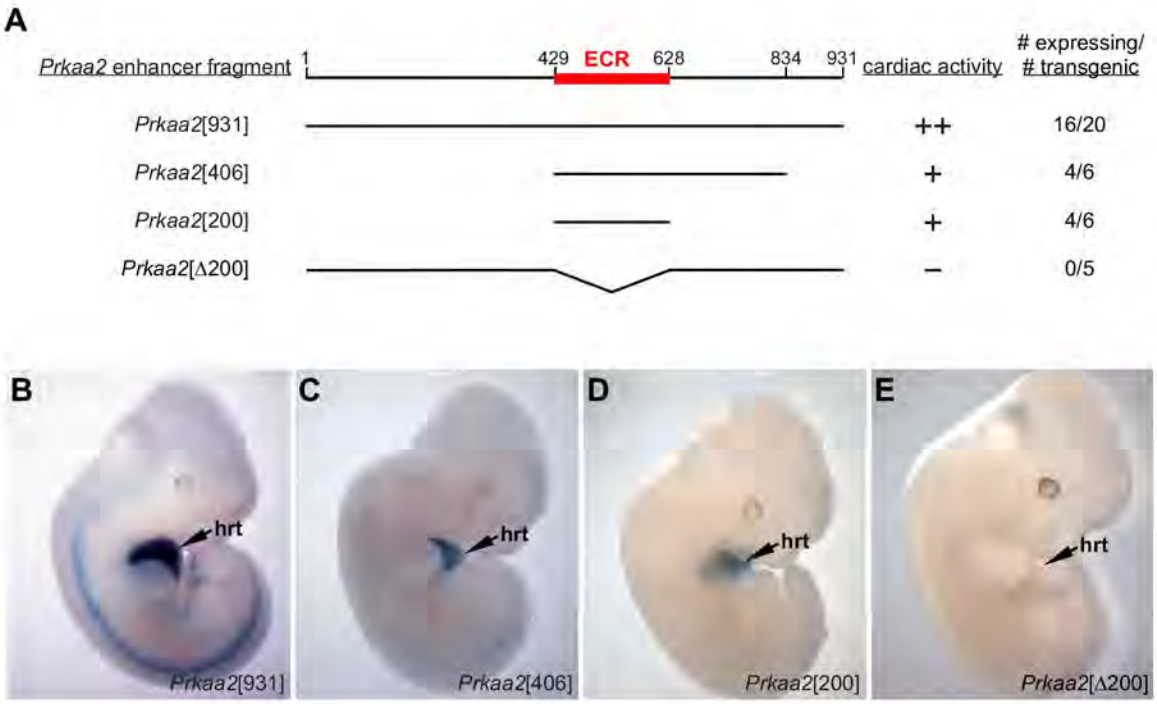


Figure S1. The *Prkaa2* enhancer is required for endogenous *Prkaa2* expression in the heart. (A) PCR detected the wild type and *Prkaa2* enhancer-Δ (1.1-kb deletion) alleles in wild type (+/+), heterozygous (+/Δ), and homozygous enhancer deletion mutants (Δ/Δ). The wild type allele results in the detection 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.



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                                                                MEF2(2)
mouse     ccacttaagcaaggagactgctcccttgaaagggcgaagaaagaaagtttctattattagcagagataaaaactgcctga
human     ccacttaagcaagaatactgttcccttgaaggggtgaagaag----gcttctattattagcagagat---aactgcctga
opossum   c-aattcagcaatgacactgttccctt--tagtgtgaggaag----gtcactattattagcagggataagaactgcctgg
          * * ** *****  * **** ******    * * ** ***     *   ************ ***   *********

                                    MEF2(1)
mouse     aattccttcctcactagcaccatgctaaaaataaaatggtttaccacacatcagaataaaca--ttgctttgcatattca
human     aattctttccttaataacaccatactaaaaataaaaaggttcatcatatttcaaaataaacacattgcttttcatacgca
opossum   aattctttcctaaataacaccattctaaaaataaagttgttca-catacttcaaaataaaaacactactctgaaaaccca
          ***** ***** * ** ****** **********   *** * ** *  *** ****** *   * ** *  * *  **
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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.

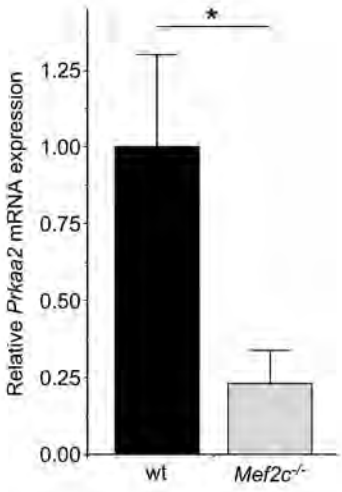

Figure S3. MEF2C regulates expression of *Prkaa2* in the heart *in vivo*. Wild type and *Mef2c*$^{-/-}$ embryos were isolated at E8.5 and RNA was isolated from mechanically dissected hearts and subjected to quantitative RT-PCR (qPCR) analysis of endogenous *Prkaa2* expression. *Prkaa2* expression was 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.

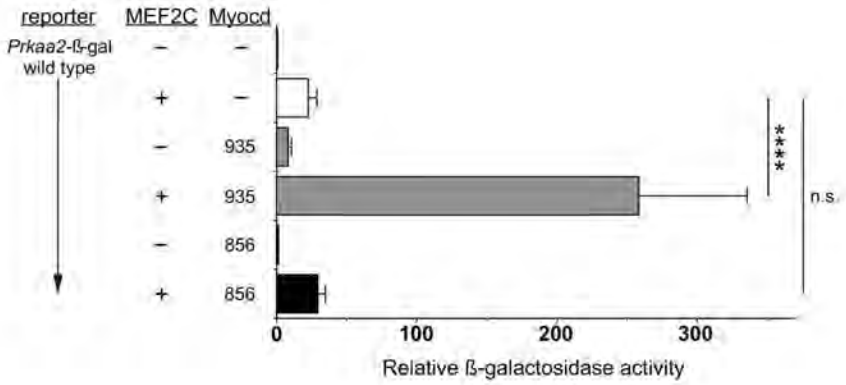

Figure S4. MEF2C and the short form of myocardin (myocardin-856) fail to cooperatively active the *Prkaa2* cardiac enhancer under conditions in which MEF2C and myocardin-935 cooperatively activate the enhancer. P19CL6 cells were co-transfected with the wild type *Prkaa2*-β-gal reporter and expression 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).

References for Supplementary Materials and Methods

Anderson, J. P., Dodou, E., Heidt, A. B., De Val, S. J., Jaehnig, E. J., Greene, S. B., Olson, E. N. and Black, B. L. (2004). HRC is a direct transcriptional target of MEF2 during cardiac, skeletal, and arterial smooth muscle development in vivo. *Mol Cell Biol* **24**, 3757-3768.

Creyghton, M. P., Cheng, A. W., Welstead, G. G., Kooistra, T., Carey, B. W., Steine, E. J., Hanna, J., Lodato, M. A., Frampton, G. M., Sharp, P. A., et al. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A* **107**, 21931-21936.

De Val, S., Anderson, J. P., Heidt, A. B., Khiem, D., Xu, S. M. and Black, B. L. (2004). Mef2c is activated directly by Ets transcription factors through an evolutionarily conserved endothelial cell-specific enhancer. *Dev Biol* **275**, 424-434.

Dodou, E., Xu, S. M. and Black, B. L. (2003). mef2c is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. *Mech Dev* **120**, 1021-1032.

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome biology* **5**, R80.

Li, S., Wang, D. Z., Wang, Z., Richardson, J. A. and Olson, E. N. (2003). The serum response factor coactivator myocardin is required for vascular smooth muscle development. *Proc Natl Acad Sci U S A* **100**, 9366-9370.

Lin, Q., Schwarz, J., Bucana, C. and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-1407.

Pages, H., Aboyoun, P., Gentleman, R. and DebRoy, S. (2014). Biostrings: string objects representing biological sequences, and matching algorithms. . *R Package Version 2.36.4.*

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. *https://www.R-project.org*.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature protocols* **8**, 2281-2308.

Wamstad, J. A., Alexander, J. M., Truty, R. M., Shrikumar, A., Li, F., Eilertson, K. E., Ding, H., Wylie, J. N., Pico, A. R., Capra, J. A., et al. (2012). Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage. *Cell* **151**, 206-220.

Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F. and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910-918.

Wang, Z., Wang, D. Z., Pipes, G. C. and Olson, E. N. (2003). Myocardin is a master regulator of smooth muscle gene expression. *Proc Natl Acad Sci U S A* **100**, 7129-7134.