A myocardin-adjacent lncRNA balances SRF-dependent gene transcription in the heart

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Myocardin, a potent coactivator of serum response factor (SRF), competes with ternary complex factor (TCF) proteins for SRF binding to balance opposing mitogenic and myogenic gene programs in cardiac and smooth muscle. Here we identify a cardiac lncRNA transcribed adjacent to *myocardin*, named CARDINAL, which antagonizes SRF-dependent mitogenic gene transcription in the heart. *CARDINAL*-deficient mice show ectopic TCF/SRF-dependent mitogenic gene expression and decreased cardiac contractility in response to age and ischemic stress. CARDINAL forms a nuclear complex with SRF and inhibits TCF-mediated transactivation of the promitogenic gene c-fos, suggesting CARDINAL functions as an RNA cofactor for SRF in the heart.

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Heart development and pathological remodeling are regulated by a network of transcription factors and noncoding RNAs that coordinate expression of genes involved in cardiomyocyte proliferation, differentiation, and contractility (Olson 2006; Miano 2010; Small and Olson 2011). SRF is a widely expressed transcription factor that binds the CArG-box DNA motif CC(A/T)$_6$GG found in many muscle-specific and growth factor-inducible promoters (Shore and Sharrocks 1995; Miano 2010). In response to extracellular cues, SRF associates with diverse transcriptional coactivators to switch between opposing mitogenic and myogenic gene programs (Wang et al. 2004; Gualdrini et al. 2016). Contractile and cytoskeletal genes are activated when SRF associates with the cardiac- and smooth muscle-specific coactivator myocardin (*Myocd*), or myocardin-related transcription factor-A (MRTF-A; MKL1) and myocardin-related transcription factor-B (MRTF-B; MKL2) in other tissues such as mammary myoepithelium and skeletal muscle (Wang et al. 2001, 2002a,b, 2003; Li et al. 2003; Wang and Olson 2004; Pipes et al. 2006). Alternatively, the control of mitogen-activated cell signaling is dependent upon the association of SRF with members of the TCF family, including ELK-1, ELK-3, and ELK-4 (Shaw et al. 1989; Janknecht et al. 1993). TCFs suppress myogenic gene expression by displacing Myocd/MRTFs from a common docking site on SRF; however, the mechanisms by which mitogenic genes are suppressed remain poorly understood. The coexpression of TCFs and Myocd/MRTFs in cardiomyocytes suggest that additional cofactors are required to counterbalance SRF-dependent gene programs in the heart.

Noncoding RNAs are an emerging class of transcriptional regulators of gene expression, many of which are expressed in tissue-specific patterns. Nucleotide polymorphisms in noncoding regions containing lncRNAs have long been associated with an increased risk for developing cardiovascular disease (Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007). However, only recent in-depth functional studies have identified lncRNAs as important regulators of heart development (Grote et al. 2013; Klattenhoff et al. 2013; Anderson et al. 2016) and disease progression (Han et al. 2014; Wang et al. 2016). LncRNAs play a variety of biological roles fundamental to regulating gene expression, including chromatin modification, protein synthesis, RNA processing, and gene silencing (Kaikkonen et al. 2011; Vance and Ponting 2014). Given the vast number of noncoding RNAs that have been recently discovered in mammalian genomes, of which more than half are associated with chromatin (Werner and Ruthenburg 2015), lncRNAs represent an untapped reservoir of transcriptional cofactors for fine-tuning essential gene networks.

Here we characterize a cardiac lncRNA gene upstream of the *Myocd* locus that we named the myocardin-adjacent long noncoding RNA (CARDINAL). The *CARDINAL* promoter, conserved in both mouse and human genomes, is robustly activated by the cardiac transcription factors MEF2 and Myocd/MRTFs. Genetic disruption of *CARDINAL* in mice did not affect *Myocd* expression but resulted in ectopic expression of SRF-regulated mitogenic genes and decreased heart function with age and in response to ischemic stress. We show that CARDINAL localizes to chromatin in cardiomyocytes, forms a complex with SRF, and is sufficient to inhibit TCF-mediated transactivation of the SRF-target gene *c-Fos*. Furthermore, CARDINAL is significantly up-regulated with *Myocd* during heart failure in humans and mice, suggesting it plays a role in controlling the SRF-dependent cardiac gene networks required to maintain heart function and ventricular remodeling in response to injury or stress.

[*Keywords*: *LINC00670*; myocardin; SRF; long noncoding RNA; c-Fos; TCFs; ELK-1]

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Results and Discussion

Discovery of the cardiac lncRNA CARDINAL

In a bioinformatics screen for uncharacterized, cardiac-restricted RNAs, we discovered a lncRNA transcribed upstream of *myocardin* in human (*MYOCD*) and mouse (*Myocd*) genomes (Fig. 1A). We named this gene the *myocardin*-adjacent long noncoding RNA (*CARDINAL*). The human *CARDINAL* locus spans 87.4 kb and is comprised of four exons, terminating 28.8 kb upstream of the *MYOCD* transcriptional start site (Fig. 1A). *CARDINAL* is transcribed in tandem with *MYOCD* on human chromosome 17 and is 2.8 kb (also named *LINC00670*).

In mice, two partially overlapping transcripts are annotated upstream of the *Myocd* locus on chromosome 11 (ENSMUST00000130362 and ENSMUST00000128453). To determine the full-length CARDINAL sequence in mice, we performed rapid amplification of cDNA ends (RACE) using a primer specific to the ENSMUST 00000128453 transcript. We found that the two annotated transcripts in mice are transcribed as a single ~3.0-kb RNA, encoded by six exons (Fig. 1A). The full-length mouse *CARDINAL* locus spans 88.1 kb and terminates 7.6 kb upstream of the *Myocd* transcriptional start site (Fig. 1A). The previously identified *Myocd* cardiac enhancer lies within the first intron of the mouse *CARDINAL* gene, ~31 kb upstream of the *Myocd* transcriptional start site (Fig. 1A; Creemers et al. 2006). The genomic location of *CARDINAL* relative to *Myocd* was conserved in mice and humans; however, the exon organization was distinct, and only 57% nucleotide sequence homology was found between the mouse and human CARDINAL transcripts (Fig. 1A). Promoter-specific H3K4Me3 histone modifications demarcate a single promoter site for *CARDINAL* in the developing and adult mouse heart (Supplemental Fig. S1A; Shen et al. 2012). Northern blot analysis of multiple adult mouse tissues revealed a band corresponding to the predicted size of the full-length CARDINAL lncRNA in the heart (Supplemental Fig. S1B). We detected no conserved protein-coding open reading frames within the CARDINAL transcript, and transcriptome-wide ribosome profiling and mass spectrometry on human heart tissues have not detected peptides originating from CARDINAL (van Heesch et al. 2019).

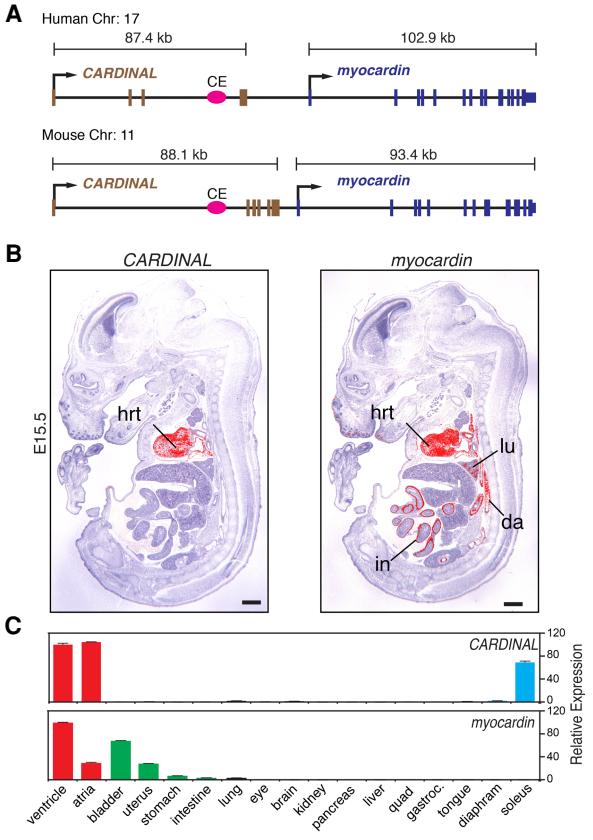

Figure 1. Discovery and expression of a *myocardin*-adjacent lncRNA (*CARDINAL*). (*A*) Diagram depicting the *CARDINAL* locus upstream of *myocardin* in mouse and human genomes. (CE) *Myocardin* cardiac enhancer. (*B*) Section in situ hybridization of mouse embryos at E15.5 using probes specific for *CARDINAL* and *myocardin*. Signal is pseudocolored red. (da) Diaphragm, (hrt) heart, (in) intestine, (lu) lung. Scale bars, 1 mm. (*C*) Quantitative real-time PCR analysis of CARDINAL and myocardin RNA expression across multiple adult mouse tissues. Values are expressed relative to liver.

To compare the expression patterns of *CARDINAL* and *Myocd*, we performed in situ hybridization on mouse embryo sections at embryonic day (E) 15.5. *CARDINAL* was detected exclusively in the heart, whereas *Myocd* was detected in the heart and the vascular and visceral smooth muscles of the dorsal aorta, lung, and intestines (Fig. 1B). In adult mice, qRT-PCR detected robust CARDINAL expression in the atria and ventricles of the heart, and in the soleus, a slow-type skeletal muscle in which *Myocd* is absent (Fig. 1C). In the developing heart, CARDINAL and *Myocd* were similarly enriched in cardiomyocytes and absent in cardiac fibroblasts (Supplemental Fig. S1C). Among different immortalized mouse cell lines, CARDINAL was detected in mouse HL-1 cardiomyocytes (Supplemental Fig. S1D). Subcellular fractionation of HL-1 cells showed enrichment of *CARDINAL* in the nuclear chromatin fraction, unlike two other RNAs, 18S and UpperHand (UPH), which were not associated with chromatin (Supplemental Fig. S1E). Thus, CARDINAL is a chromatin-associated lncRNA that shares an overlapping but distinct expression profile with the neighboring transcription factor Myocd.

Regulation of CARDINAL *by MEF2 and Myocd/MRTFs*

Previously, we reported that *myocardin* expression in heart and vascular smooth muscle is controlled by an upstream enhancer that requires binding of myocyte enhancer factor 2 (MEF2), a MADS-box transcription factor related to SRF (Creemers et al. 2006). Myocardin is capable of activating its own enhancer, but unlike most other *myocardin* target genes, this activation occurs through Mef2, independent of SRF. Interestingly, we noted that the *CARDINAL* promoter contains two highly conserved A/T-rich sequences, located 155 and 205 nucleotides upstream of the *CARDINAL* transcription initiation site, that resemble MEF2 binding sites (Fig. 2A,B). To determine whether MEF2 regulates *CARDINAL*, we generated a luciferase reporter using a 3.5-kb fragment of the *CARDINAL* promoter and first exon (3.5-kb *CARDINAL*-Luc). The 3.5-kb *CARDINAL*-Luc reporter was highly responsive to MEF2, which *trans*-activated the reporter ~60-fold (Fig. 2C). A 500-bp fragment of the *CARDINAL* promoter, retaining the two A/T-rich sequences, was

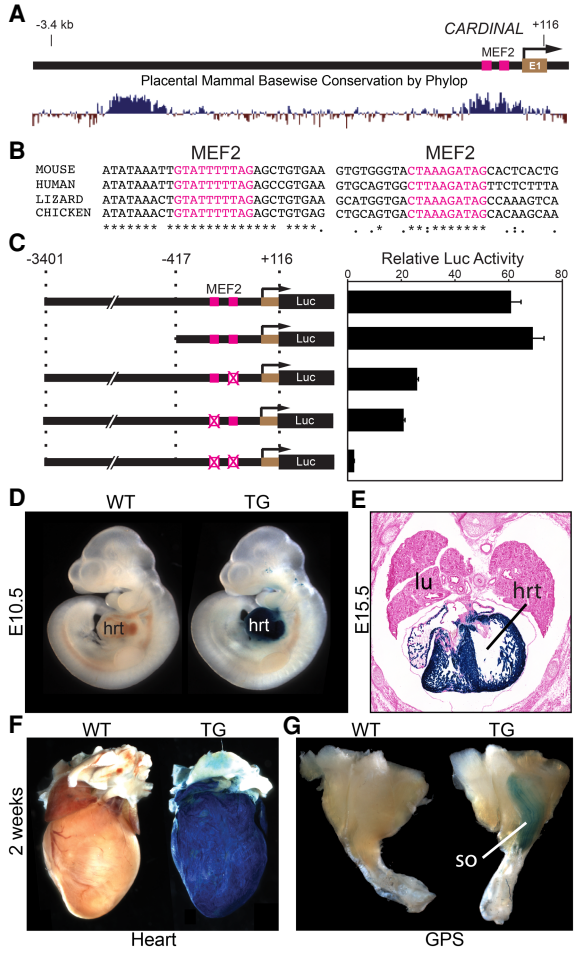

Figure 2. Regulation of *CARDINAL* by MEF2 in the heart. (*A*) Diagram showing conservation of the *CARDINAL* promoter and exon 1, with the two MEF2 binding sites (pink boxes). (*B*) Sequence alignment of the two MEF2 sites in the *CARDINAL* promoter, highlighted in pink. (*C*) Luciferase reporter assay showing activation of *CARDINAL* promoter constructs by addition of MEF2 in transfected COS-7 cells. (Pink X) MEF2 site mutations. Data are represented as mean ± SEM. (*D–G*) β-Galactosidase staining of CARDINAL-LacZ transgenic (TG) and wild-type (WT) E10.5 embryos (*D*), E15.5 fetus (*E*), and adult heart (*F*) and soleus (so) skeletal muscle (*G*). (hrt) Heart, (lu) lung, (GPS) gastrocnemius–plantaris–soleus.

equally responsive to MEF2 (Fig. 2C). Mutation of either MEF2 site in the 3.5-kb *CARDINAL*-Luc reporter reduced luciferase activity by about half, with mutation of both sites abolishing transactivation by MEF2 (Fig. 2C). The 3.5-kb *CARDINAL*-Luc promoter was also robustly transactivated by the cardiac- and smooth muscle-specific isoforms of *Myocd* and the *Myocd*-related transcription factors MRTF-A and MASTR (Supplemental Fig. S2A). These data suggest that *CARDINAL* is activated by promyogenic cardiac transcription factor gene programs.

To examine the regulation and expression of *CARDINAL* in vivo, we generated a transgenic reporter vector by fusing the 3.5-kb *CARDINAL* promoter sequence upstream of the β-galactosidase (LacZ) reporter gene (3.5-kb *CARDINAL*-LacZ). *CARDINAL*-LacZ transgenic reporter mice showed robust LacZ expression in the heart at embryonic, fetal, and adult time points (E8.5 to 2 wk) (Fig. 2D–F; Supplemental Fig. S2B–E). In addition to the heart, LacZ staining was observed in the soleus but was absent from fast-type skeletal muscle, consistent with the expression of endogenous *CARDINAL* RNA (Fig. 2G). LacZ staining was also detected in the developing mammary gland myoepithelium, a smooth muscle-like tissue whose development has been shown to be dependent upon MRTF-A expression (Supplemental Fig. S2F; Li et al. 2006). Consistent with endogenous *CARDINAL* expression, LacZ staining was absent from the developing vascular and visceral smooth muscles, which abundantly express *Myocd* (Wang et al. 2001). Simultaneous mutation of both MEF2 binding sites within the 3.5-kb *CARDINAL*-LacZ construct significantly attenuated LacZ expression in the heart, highlighting the importance of MEF2 as a requisite activator of the *CARDINAL* promoter in vivo (Supplemental Fig. S2G).

Loss of CARDINAL *results in ectopic SRF/TCF gene activation and decreased cardiac contractility in response to age and ischemic injury*

To characterize the function of *CARDINAL* in vivo, we inserted a Cre-ERT2 reporter followed by two polyadenylation cassettes into the first exon of the mouse *CARDINAL* locus. This strategy preserves enhancer or promoter sequences that may be required for *CARDINAL* or *Myocd* transcription, while prematurely terminating the *CARDINAL* RNA to generate a knockout (KO) allele (Fig. 3A). The *CARDINAL* locus was modified using homologous recombination in murine embryonic stem cells. Blastocyst injection of targeted ES cells yielded chimeric founder mice, which were bred to C57BL/6 WT mice and genotyped using a PCR-based strategy (Supplemental Fig. S3A). *CARDINAL*-Cre-ERT2 heterozygous mice, when crossed to a ROSA26-LacZ reporter line, showed tamoxifen-inducible LacZ expression in the heart, indicating the faithful expression of the modified locus (Supplemental Fig. 3B).

Homozygous *CARDINAL* mice showed no major morphological abnormalities or functional impairment through young adulthood (Fig. 3B,C). However, echocardiography revealed a significant decline in left ventricular pumping efficiency in *CARDINAL* KO mice by 20 wk (Fig. 3C). Quantitative PCR revealed the absence of *CARDINAL* in the heart and soleus of *CARDINAL* KO mice (Fig. 3D). Surprisingly, the loss of *CARDINAL* and the termination of transcription through the *CARDINAL* locus had no significant impact on the expression of *Myocd* (Fig. 3E,F).

Chromatin-associated lncRNAs can function as regulators of gene transcription (Rinn and Chang 2012); therefore, we profiled gene expression changes using RNA sequencing of the heart and soleus of *CARDINAL* KO and WT littermates at 8 wk of age. Consistent with our quantitative PCR data, *CARDINAL* was significantly down-regulated in both tissues, while the expression of *Myocd* was unchanged. Similarly regulated genes were found in the heart and soleus muscle of *CARDINAL* KO mice (Supplemental Fig. S3C,D; Supplemental Table S1). The prototypical SRF-dependent immediate early (IE) genes *c-Fos* (*Fos*), *Atf3*, and *Nr4a1* were significantly up-regulated in *CARDINAL* KO soleus and heart, with additional IE genes up-regulated within each tissue type (Supplemental Table S1). The muscle-specific SRF targets, α-actin cytoskeletal genes *Actc1* and *Acta1* (Balza and Misra

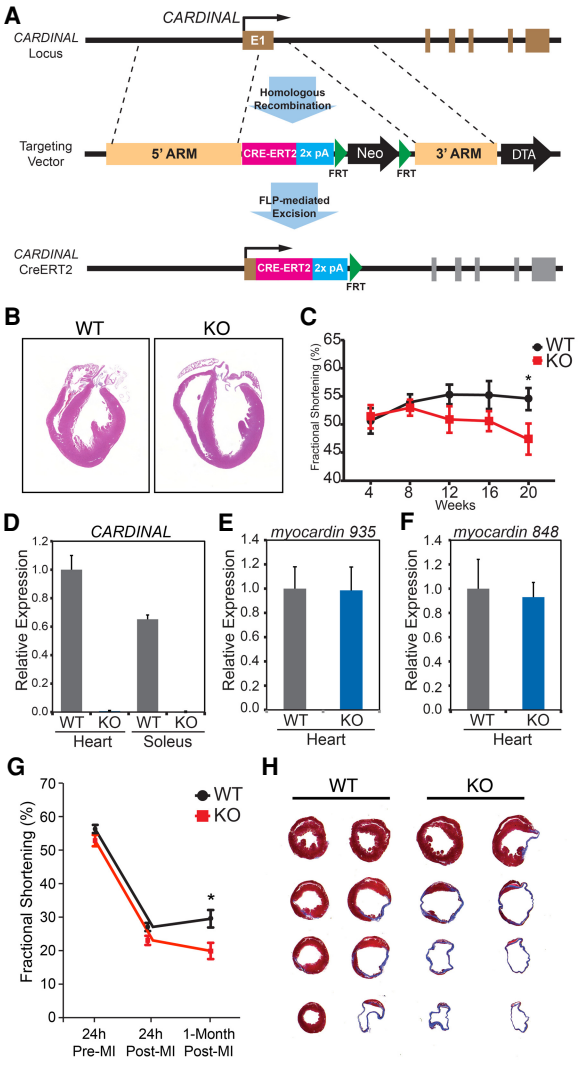

Figure 3. Disruption of *CARDINAL* in mice leads to contractile defects in response to age and ischemic injury. (*A*) Diagram of the *CARDINAL* KO targeting strategy. (*B*) Four-chamber histological section of hearts isolated from *CARDINAL* KO and WT littermates at 8 wk of age. (*C*) Percentage of fractional shortening of the left ventricle measured by echocardiography. (*D*,*E*) Real-time PCR using primers for *CARDINAL* (*D*), cardiac and smooth muscle *myocardin* isoforms (*E*,*F*) in the hearts of *CARDINAL* KO and WT littermates at 8 wk. (*G*) Measurement of cardiac function by fractional shortening using echocardiography in *CARDINAL* KO and WT littermates subjected to acute MI. $n = 8$ WT, $n = 7$ KO. (*H*) Transverse histological series stained with Masson's trichrome, evaluated at four levels below ligation, showing muscle and fibrotic scar from two independent WT and *CARDINAL* KO mice at 1 mo post-MI.

2006), were among the significantly down-regulated genes in both tissues (Supplemental Table S1). These findings reveal that SRF-dependent genes are altered in *CARDINAL* KO tissues, independent of changes in myocardin expression.

In response to cardiac stress, *Myocd* and *MRTF-A* are up-regulated and required for hypertrophic growth and ventricular remodeling (Xing et al. 2006; Liao et al. 2011; Trembley et al. 2018). Similarly, CARDINAL was significantly up-regulated in mice and patients with heart failure following acute myocardial infarction (MI) (Supplemental Fig. S3E,F; Molina-Navarro et al. 2013). We subjected 8-wk-old *CARDINAL* KO and WT littermates to MI and measured cardiac function 1 d and 1 mo post-MI. In sham-operated mice, no differences in heart function or morphology were observed (Supplemental Fig. S3G). However, while cardiac function was similar between *CARDINAL* KO and WT mice prior to and 1 d after MI, *CARDINAL* KO mice had significantly reduced fractional shortening, increased scar formation, and increased LV internal diameter 1 mo post-MI compared with WT littermates (Fig. 3G,H; Supplemental Fig. S3H). These findings support a role for *CARDINAL* as an RNA cofactor that is required for normal heart function.

CARDINAL *interacts with SRF and regulates SRF-dependent gene transcription*

To test whether *CARDINAL* regulates SRF-dependent gene transcription, we measured the effect of CARDINAL on the expression of SRF/CArG-dependent promoters of the muscle-specific gene *SM22* (*TAGLN*) and IE gene *c-FOS* in heterologous cell-based luciferase assays in COS-7 cells (Fig. 4A,B; Supplemental Fig S4A,B). Consistent with previous reports (Wang et al. 2004), robust activation of the *SM22* promoter by Myocd was repressed by coexpression of ELK-1 (Fig. 4A). In contrast, ELK-1 activation of the c-FOS promoter was not inhibited by coexpression

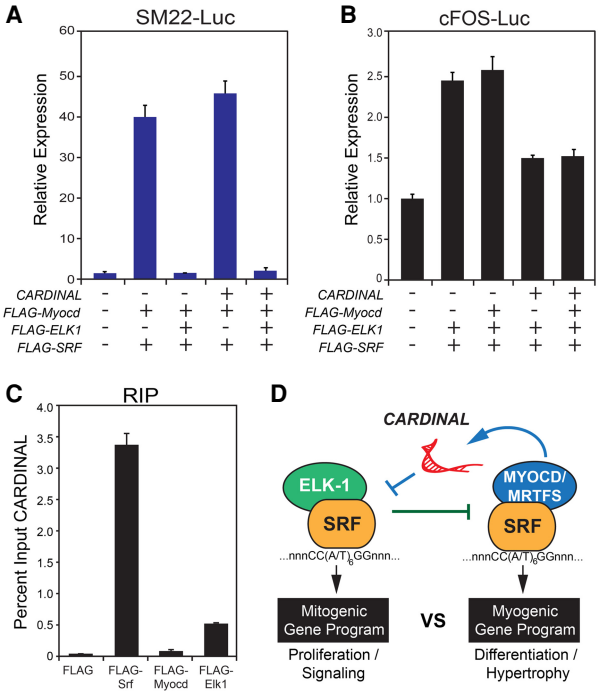

Figure 4. CARDINAL antagonizes SRF/TCF gene transcription. Regulation of the CArG-dependent promoters SM22 (*A*) and c-FOS (*B*) by SRF, the SRF cofactors ELK-1, *Myocd*, and *CARDINAL* in heterologous COS-7 cells transfected with SM22 or c-FOS promoters fused to a luciferase reporter cassette. (*C*) qRT-PCR detection of *CARDINAL* following RNA immunoprecipitation (RIP) of FLAG-epitope tagged SRF, ELK-1, or *Myocd* coexpressed with *CARDINAL* in heterologous COS-7 cells. (*D*) Model depicting a promyogenic role for CARDINAL as an SRF cofactor required to counter-balance SRF-dependent gene programs in the heart by antagonizing ELK-1/SRF mitogenic gene expression.

of *Myocd*, demonstrating the dominance of ELK1 on SRF-regulated promoters (Fig. 4B). While *CARDINAL* did not alter Myocd or ELK1-mediated changes to the SM22 promoter activation, coexpression of *CARDINAL* was sufficient to repress ELK1-mediated activation of the c-FOS promoter (Fig. 4A,B). These data demonstrate that unlike *Myocd*, *CARDINAL* can antagonize SRF/TCF-mediated gene expression.

To determine whether CARDINAL could mediate this repressive activity directly, we performed RNA immunoprecipitation (RIP) using FLAG-epitope tagged fusion proteins of SRF, *Myocd*, or ELK1 coexpressed with *CARDINAL* in COS-7 cells. *CARDINAL* formed a strong interaction with FLAG-SRF, weakly interacted with FLAG-ELK1, and did not interact with FLAG-*Myocd* (Fig. 4C). SRF lacks a typical RNA binding domain; therefore, the interaction with *CARDINAL* may be through a multiprotein complex. SRF interacts with >70 cofactors, many of which associate with RNA (Castello et al. 2012). As depicted in our model (Fig. 4D), we propose that *CARDINAL* promotes myogenic differentiation directed by *Myocd* and MRTFs in the heart by antagonizing TCF/SRF-mediated mitogenic gene transcription. Future studies on the biochemical nature of this interaction will shed light on the mechanistic role of CARDINAL as a novel RNA coregulator of cardiac gene transcription and contractility.

Materials and methods

Study approval

Animal work described was approved and conducted under the oversight of the University of Texas Southwestern Institutional Animal Care and Use Committee and by the University of Rochester Medical Center's University Committee on Animal Resources.

Generation of CARDINAL KO mice

A targeting vector containing a CreERT2-2xpA-Frt-Neo-Frt expression cassette was targeted to exon 1 of the *CARDINAL* locus in mice. The targeting vector was linearized and electroporated into 129SvEv-derived embryonic stem cells. One clone with a correctly targeted *CARDINAL* locus was expanded and injected into 3.5-d C57BL/6 blastocysts. Chimeric male mice were crossed to C57BL/6 females to achieve germline transmission of the targeted allele and genotyped using PCR. Genotyping primer sequences are in Supplemental Table S2.

Mouse model of myocardial infarction

C57BL/6 (WT) and *CARDINAL* KO mice at 10–12 wk of age underwent permanent ligation of the left anterior descending artery, as previously described (van Rooij et al. 2008). Cardiac function and heart dimensions were evaluated by two-dimensional echocardiography on conscious mice using a VisualSonics Vevo 2100 system equipped with a 35-MHz transducer.

Generation of CARDINAL-LacZ transgenic mice

The 3.5-kb fragment of the upstream *CARDINAL* promoter and first exon (−3401 to +116) was cloned upstream of the β-galactosidase (LacZ) reporter gene in the vector BASIC-LacZ. Linearized LacZ reporter transgenes were injected into the pronuclei of fertilized oocytes using standard techniques.

Radiolabeled in situ hybridization

Radioisotopic in situ hybridization studies on sections were performed as previously described (Shelton et al. 2000). Antisense RNA probe templates for *CARDINAL* or *Myocd* were generated using mouse heart cDNA and subcloned into pCRII TOPO (Life Technologies). Primer sequences are listed in Supplemental Table S2.

RNA immunoprecipitation

RNA–protein complexes from formaldehyde cross-linked HL-1 cell lysates were immunoprecipitated using anti-FLAG M2 conjugated agarose beads (Sigma Aldrich). RNA purification and expression analysis was performed as previously described (Anderson et al. 2016).

Cellular and subcellular fractionations

Neonatal cardiomyocytes were isolated from ~50 postnatal day 1 (P3) C57BL/6 mice using the Neomyt kit (Cellutron nc-6031) and fractionated as previously described (Anderson et al. 2016).

Northern blot analysis

Northern blots were performed using a commercially prepared adult mouse multitissue RNA blot (Zyagen MN-MT-1) hybridized with a radiolabeled DNA probe specific to CARDINAL. Radiolabeled DNA probes for Northern and Southern blots were generated using a RadPrime kit (Life Technologies) (Supplemental Table S2).

Rapid amplification of cDNA ends (RACE)

The 5′ end of the mouse CARDINAL transcript in the heart was determined using Marathon RACE-ready adult mouse cDNA (Clontech). RACE-PCR was performed according to manufacturer's recommended protocol, using primers specific to the 3′ CARDINAL fragment and the Marathon adapter primer 1 (AP1). The gene-specific primer used to amplify the 5′ sequence of CARDINAL is listed in Supplemental Table S2.

Statistical analysis

Results are expressed as mean ± SEM. Unpaired two-tailed Student *t*-test with Welch correction was performed to determine statistical significance. *P*-values of <0.05 were considered significant.

Competing interest statement

The authors declare no competing interests.

Acknowledgments

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Author contributions: D.M.A. and K.M.A. designed and performed experiments, interpreted data, and wrote the manuscript. B.R.N. analyzed the RNA sequencing data. J.R.M. generated CARDINAL mice from plasmids designed and generated by D.M.A. and K.M.A. S.B. performed the Northern blot. J.M.S. performed histological analyses. R.B.-D. wrote the animal protocol and edited the manuscript. E.N.O. interpreted results and edited the manuscript.

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