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Myocardin is a direct transcriptional target of Mef2, Tead and Foxo proteins during cardiovascular development

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Myocardin is a transcriptional co-activator of serum response factor (Srf), which is a key regulator of the expression of smooth and cardiac muscle genes. Consistent with its role in regulating cardiovascular development, myocardin is the earliest known marker specific to both the cardiac and smooth muscle lineages during embryogenesis. To understand how the expression of this early transcriptional regulator is initiated and maintained, we scanned 90 kb of genomic DNA encompassing the myocardin gene for cis-regulatory elements capable of directing myocardin transcription in cardiac and smooth muscle lineages in vivo. Here, we describe an enhancer that controls cardiovascular expression of the mouse myocardin gene during mouse embryogenesis and adulthood. Activity of this enhancer in the heart and vascular system requires the combined actions of the Mef2 and Foxo transcription factors. In addition, the Tead transcription factor is required specifically for enhancer activation in neural-crest-derived smooth muscle cells and dorsal aorta. Notably, myocardin also regulates its own enhancer, but in contrast to the majority of myocardin target genes, which are dependent on Srf, myocardin acts through Mef2 to control its enhancer. These findings reveal an Srf-independent mechanism for smooth and cardiac muscle-restricted transcription and provide insight into the regulatory mechanisms responsible for establishing the smooth and cardiac muscle phenotypes during development.

KEY WORDS: Mouse, Myocardin, Smooth muscle, Enhancer, Transcriptional regulation, Transgene

INTRODUCTION

Differentiation of cardiac, smooth and skeletal muscle cells is accompanied by the expression of overlapping but distinct sets of muscle-restricted genes. Nearly all smooth muscle genes and numerous cardiac and skeletal muscle genes are controlled by a *cis*-regulatory sequence known as a CArG box ($CC(A/T)_6GG$), which serves as the binding site for serum response factor (Srf) (Shore and Sharrocks, 1995). Srf is expressed in a wide range of cell types and confers cell type specificity and signal responsiveness to its target genes by recruiting specific transcriptional cofactors.

Myocardin and myocardin-related transcription factors (MRTFs) comprise a family of Srf coactivators that associate with the MADS (MCM1, agamous, deficiens, Srf)-box of Srf and contribute their powerful transcription activation domains to Srf target genes (Pipes et al., 2006). Myocardin is expressed specifically in cardiac and smooth muscle cells (SMCs) and activates muscle genes associated with the differentiation of these cell types (Wang et al., 2001; Wang et al., 2003). In transfected fibroblasts and stem cells, myocardin can activate a large array of smooth muscle genes (Chen et al., 2002; Du et al., 2003; Pipes et al., 2005; Wang et al., 2003; Yoshida et al., 2003), whereas ectopic expression of myocardin in *Xenopus* embryos results in activation of cardiac muscle genes even in spinal cord neurons (Small et al., 2005; Wang et al., 2003). Knockout mice lacking myocardin die during early embryogenesis with a lack of differentiated SMCs (Li et al., 2003). The myocardin-related transcription factors Mrtfa (Mal/Mkl1) and Mrtfb (Mkl2) (Cen et al., 2003; Du et al., 2004; Ma et al., 2001; Oh et al., 2005; Wang et al., 2002) are expressed in a broad range of cell types. Mrtfb has been shown to play an essential role in the development of a specific subset of vascular SMCs derived from the neural crest (Li et al., 2005; Oh et al., 2005). *Mrtfb* knockout mice die by embryonic day (E) 14.5 and display severe defects in the anatomy of branchial arch arteries, and cardiac outflow tract accompanied by a defect in smooth muscle differentiation. Mice lacking Mrtfa are viable and display a defect specific to lactating females in which myoepithelial cells, which provide contractility required for secretion of milk from the mammary gland, fail to differentiate and undergo apoptosis (Li et al., 2006; Sun et al., 2006).

During embryogenesis, the onset of myocardin expression coincides with specification of the cardiac lineage in the cardiac crescent, a specialized region of the developing mesoderm that adopts a cardiac fate in response to inductive cues from adjacent tissues (Fishman and Chien, 1997; McFadden and Olson, 2002). Expression of myocardin in early cardiac progenitors coincides with that of Nkx2-5, the earliest known marker of the cardiac lineage (Lints et al., 1993). Thereafter, myocardin is expressed throughout the atrial and ventricular chambers of the heart, as well as in a subset of SMCs in the dorsal aorta and outflow tract of the heart, the esophagus, gut, lung and a subset of head mesenchyme cells (Wang et al., 2001).

While the downstream target genes of myocardin have been described in detail, little is known of the mechanisms that regulate this early regulator of cardiovascular development and gene expression. Here we describe an enhancer upstream of the mouse myocardin gene that is sufficient to direct cardiovascular expression of myocardin during mouse embryogenesis. This enhancer serves as a direct transcriptional target of Mef2, Tead and Foxo transcription factors. In contrast to nearly every known smooth muscle gene, the myocardin enhancer is independent of Srf. Intriguingly, however, myocardin activates its own enhancer via Mef2. These findings reveal a unique transcriptional strategy for the activation of smooth and cardiac-muscle-specific transcription and suggest the existence of a positive feedback loop, through which myocardin regulates its own expression in collaboration with Mef2.

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MATERIALS AND METHODS

Transgenic mice

To search for *cis*-regulatory elements responsible for cardiac and smooth muscle expression of myocardin, we fused a series of 5′ genomic and intronic fragments of the mouse myocardin gene to the *hsp68* basal promoter upstream of a *lacZ* reporter gene and tested for expression in F0 transgenic mouse embryos. A total of 12 constructs, spanning a region of 90 kb of genomic sequence, were amplified by high fidelity PCR and the fragment boundaries were sequence-verified (Fig. 1). Regions of genomic sequences contained in the constructs used in Fig. 1 are summarized here (base pair numbering refers to the exact location of the construct relative to the translational start in exon 1 of the myocardin gene): construct 1: –68,232/–59,052 bp; construct 2: –59,086/–50,195 bp; construct 3: –50,312/–40,889 bp; construct 4: –41,562/–31,683 bp; construct 5: –32,521/–22,637 bp *(MyE1)*; construct 6: –25,043/–15,711 bp; construct 7: –17,345/–8,532 bp; construct 8: –10,692/–251 bp; construct 9: –5,158/+3,283 bp; construct 10: –1,501/+7,251 bp; construct 11: +5,885/+14,892 bp; construct 12: +14,336/+21,578 bp.

To identify discrete enhancer elements residing within MyE1 (Fig. 3B), we generated deletion constructs by PCR amplification and cloned them into the Hsp68*lacZ* vector for the production of transgenic mice.

For the generation of transgenic mice, constructs were digested with *Sal*I to remove vector sequences. DNA fragments were purified using a QiaQuick spin column (Qiagen, MD), injected into fertilized eggs from B6C3F1 female mice, and implanted into pseudopregnant ICR mice as previously described (Lien et al., 1999). Embryos were collected and stained for β-galactosidase activity. Sectioning, histology and Nuclear Fast Red staining were performed on the embryos as previously described (McFadden et al., 2000).

Primer sequences

MyE1, 5′-TTCCCACTAACATTGAGTACCAGGG-3′ (forward) and 5′-AGGCCTCTTGATTATGTTTAAAATTGG-3′ (reverse);
MyE2, 5′-TTCCCACTAACATTGAGTACCAGGG-3′ (forward) and 5′-AGATACAGTGTCCACAACATACAC-3′ (reverse);
MyE3, 5′-AGTTGGTTTCCATCAACAGGAG-3′ (forward) and 5′-AGGCCTCTTGATTATGTTTAAAATTGGG-3′ (reverse);

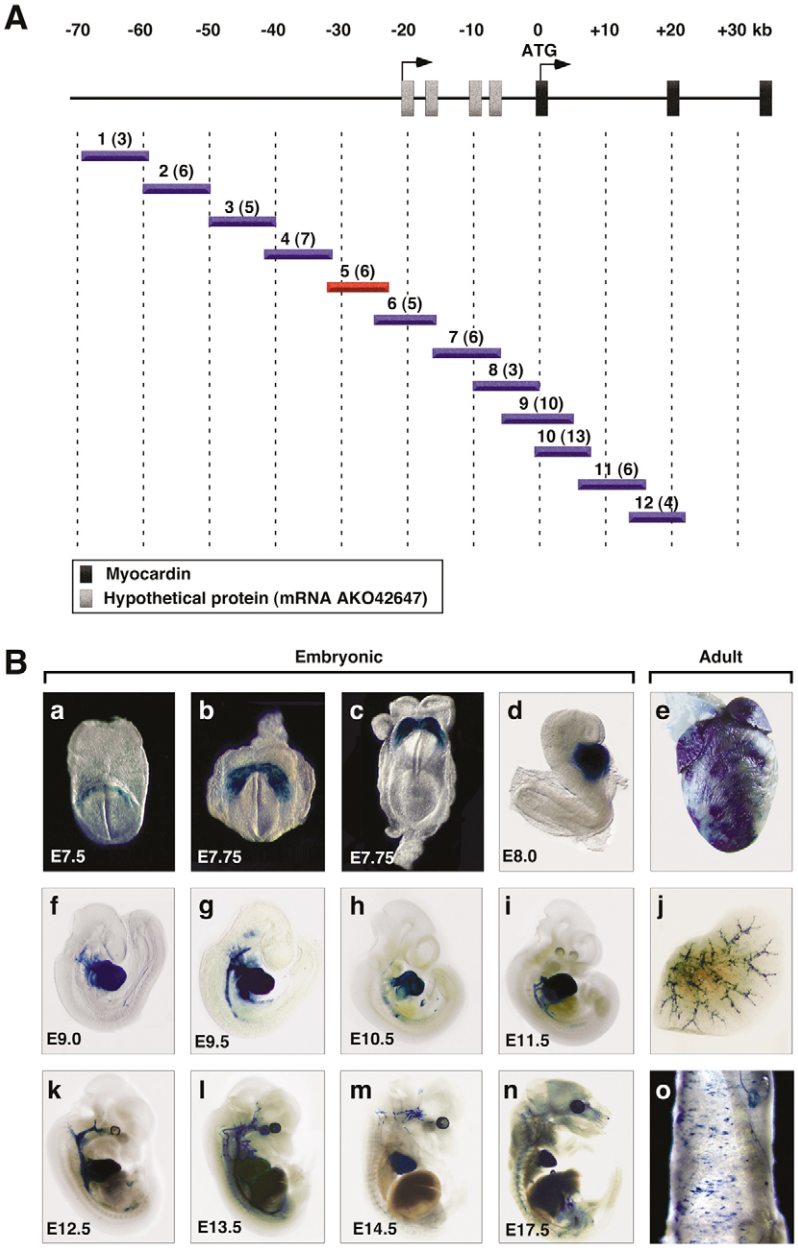

Fig. 1. Identification of *cis*-acting cardiovascular enhancer regions in the myocardin locus. (**A**) Schematic representation of the genomic organization of the myocardin locus and the constructs used for the creation of transgenic mice (see Materials and methods for construct details). The enhancer element that drives expression in heart and smooth muscle (fragment 5) is located 20-30 kb upstream of myocardin's translational start in exon 1, and is shown in red. The number of genotype-positive E12.5 embryos obtained from a total of three injections per construct are indicated between brackets. Shown at the top are the first three exons of myocardin (in black) and the exon organization of an upstream gene (mRNA AK042647) in gray. (**B**) Expresssion of the myocardin enhancer (MyE) throughout embryonic development was determined using a stable transgenic line bearing fragment 5, linked to Hsp68*lacZ*. Embryos harvested from staged matings demonstrated that the enhancer recapitulates the expression pattern of myocardin. Note strong *lacZ* expression in the cardiac crescent (a,b). As the linear heart tube forms (c) and loops (d,f), *lacZ* staining is detected at high levels throughout the entire heart. In the vasculature we detected *lacZ* expression in the developing dorsal aorta as early as E9.0-9.5 (f,g), in branchial arch arteries at E10.5 (h,i) and carotid arteries at E12.5 (k,l). In adult tissues, *lacZ* expression was detected in the heart (e) and in the vasculature of the lung (j) and aorta (o).

MyE4, 5′-TTCCCACTAACATTGAGTACC-3′ (forward) and 5′-TTTCTATGCATCATGGGTCCATGC-3′ (reverse);
MyE5, 5′-TCAATTTTTCGAAGAGTTCCAGTG-3′ (forward) and 5′-AGATACAGTGTCCACAACATAC-3′ (reverse);
MyE6, 5′-AGTTGGTTTCCATCAACAGG-3′ (forward) and 5′-TCCAAGCACCTCTATTTAAACC-3′ (reverse);
MyE7, 5′-TATCTACCATTCACACTGAGACC-3′ (forward) and 5′-AGGCCTCTTGATTATGTTTAAAATTGG-3′ (reverse); and
MyE8, 5′-TTCTGACTGGGTCCCTTACCACTGTCTTTGCA-3′ (forward) and 5′-TGCAGTAAAAAACAAATAGAACATTTGG-3′ (reverse).

Constructs, transfections and luciferase assays

Mef2c, Myocardin and Foxo4 murine expression constructs have been described (Liu et al., 2005; Martin et al., 1993; Wang et al., 2001). Expression vectors encoding rat Tead1 were kindly provided by Dr Ian Farrance (Baltimore, MD) (Zuzarte et al., 2000). The 350 bp, evolutionarily conserved, myocardin enhancer (MyE8) was cloned into the pGL2-E1b-luciferase reporter and the various deletion mutants were constructed through PCR-based mutagenesis and sequence-verified. Cells were transfected using Fugene 6 (Roche Molecular Biochemicals) with 250 ng of cDNA expression library together with 100 ng of MyE8-luciferase reporter and 30 ng of pCMV-*lacZ*. Forty-eight hours after transfection, cell extracts were assayed for luciferase expression using the luciferase assay kit (Promega). Relative promoter activities are expressed as luminescence relative units normalized for β-galactosidase expression in cell extracts. All transfection assays were performed at least three times.

cDNA expression screening

A mouse E10.5 cDNA expression library was purchased from Invitrogen. cDNA pools were prepared by using the PerfectPrep Plasmid 96 Vac Direct Bind kit (Eppendorf). Each cDNA pool used in the screening contained 50-100 single cDNA clones. In each well of a 24-well plate, 5×10^4 COS cells were cultured in DMEM with fetal bovine serum (10%), L-glutamine (2 mmol/l), and penicillin-streptomycin and transfected as described previously (Chang et al., 2005). For sib-selection, positive pools of cDNA expression plasmids were transformed into competent cells and plated on LB agar dishes. For each positive pool, 96 single clones were picked from a dish and grown in LB liquid media. Twelve single clones were combined as sub-pools, and plasmids were prepared and transfected for the reporter assays as described above. Single clones from the positive sub-pools were prepared and tested for their ability to activate MyE8-luciferase reporter.

Electrophoretic mobility shift assays

Oligonucleotides corresponding to the conserved Mef2, Foxo and Tead-binding and mutated binding sites were synthesized (Integrated DNA Technology) and used in gel mobility shift assay. Oligonucleotide sequences were as follows (the transcription factor binding sites in bold): Mef2 oligo, 5′-GGCCAAGATTGACAGCCA**CTATTTTAAG**AAGTGCTTTTAA-3′; Foxo oligo site 1, 5′-GGGCATTTCCA**TTAACA**TTTCTATTTCTGG-3′; Foxo oligo site 2, 5′-GGGCAAGTTCAAA**ATAACA**TTCCAGAAGGAGTCA-3′; Foxo oligo site 3, 5′-GGGAGTCACTAGA**AAAACA**TTCAAGGGAAGAG-3′; Foxo oligo site 4, 5′-GGAAGAGAAAAAAATTGTTTTCGTTTCGTAGCAGA-3′; Foxo oligo site 5, 5′-GGCAAATGTTCTATT**TGTTTT**TTACTGCAGAAAGCC-3′; Tead oligo, 5′-GGTGCAAGTTCAAAATAA**CATTCCA**GAAGGAGTCA 3′.

Annealed oligonucleotides were radiolabeled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase and purified using G50 spin columns (Roche). Proteins were translated in vitro in a coupled transcription-translation T7 reticulocyte lysate system (Promega). Reaction conditions of the gel mobility shift assays were previously described (McFadden et al., 2000). Mouse anti-TEF1 (BD biosciences Pharmingen) was used for Tead1 supershift, anti-AFX (goat polyclonal, Santa Cruz) for Foxo4 supershift and anti-Mef2c (rabbit polyclonal, Santa Cruz) for Mef2c supershift experiments. Unlabeled oligonucleotides used as competitors were annealed as described above and added to the reactions at the indicated concentrations. DNA-protein complexes were resolved on 5% polyacrylamide native gels and the gels were exposed to BioMax X-ray film (Kodak).

RESULTS

A 10 kb distal enhancer directs cardiovascular expression of myocardin in vivo

In an effort to identify the *cis*-acting regulatory sequences responsible for cardiac and smooth muscle expression of myocardin, we fused a series of intronic and 5′ flanking regions, spanning 90 kb of the mouse myocardin gene, to the *hsp68* basal promoter upstream of a *lacZ* reporter gene and systematically analyzed *lacZ* expression in F0 transgenic mouse embryos (Fig. 1A). Only one of the 12 transgenes, containing a DNA fragment located between –32.5 and –22.6 kb upstream of the first exon of the mouse myocardin gene, reproducibly directed *lacZ* in heart and smooth muscle of transgenic embryos at E12.5 (Fig. 1A). We refer to this 10 kb genomic fragment as MyE (myocardin enhancer). Of six independent F0 transgenic mice that carried the MyE-Hsp*lacZ* transgene, five showed similar patterns of *lacZ* expression in heart and smooth muscle. Notably, a previously uncharacterized gene (EST BB246147 or mRNA AK042647) is located between the transcription initiation site of the myocardin gene and the myocardin enhancer. Based on the mixed tissue distribution of these ESTs, this novel gene appears to be expressed ubiquitously. Thus, the myocardin enhancer must selectively recognize and activate the myocardin promoter despite being located closer to the promoter of mRNA AK042647.

The temporal and spatial expression pattern of the myocardin enhancer was determined using a stable transgenic line bearing MyE-Hsp*lacZ* (Fig. 1B). Enhancer activity marked cardiomyocytes in the cardiac crescent beginning at E7.5 (Fig. 1B, parts a,b). Transverse and sagittal sections through transgenic embryos showed intense staining of cells within the cardiogenic plate (Fig. 2A). By E7.75, the enhancer directed high levels of expression in the bilateral cardiogenic precursors and in no other regions of the embryo, an expression pattern very similar to markers of the primary heart field (atrial myosin light chain 2v and α-cardiac actin) at this stage (Cai et al., 2003; Kelly et al., 2001).

Recent studies have revealed a population of cardiac precursor cells, referred to as secondary heart field, which is derived from a region of the splanchnic mesoderm medial to and distinct from the primary heart field that makes up the cardiac crescent (Cai et al., 2003; Kelly et al., 2001; Kelly and Buckingham, 2002; Mjaatvedt et al., 2001; Waldo et al., 2001). Cells from the secondary heart field are added to the anterior region of the heart tube at the onset of looping and contribute to the formation of the outflow tract (OFT) and right ventricle (RV). The myocardin enhancer did not direct *lacZ* expression to cells in the splanchnic mesoderm (Fig. 2B,C), suggesting that myocardin is not expressed in the anterior heart field at these stages.

Between E8.0 and 9.5, high levels of *lacZ* expression were seen throughout the linear heart tube, and expression remained homogeneous throughout the heart during looping (Fig. 1B, parts d,f and Fig. 2B,C). *lacZ* continued to be expressed at high levels throughout the developing heart (with the exception of the cardiac cushions) until E13.5 (Fig. 2D,E). Thereafter, expression gradually diminished to lower but detectable levels and persisted in adult hearts (Fig. 1B, parts e,l-n and Fig. 2J). We conclude that the MyE is sufficient to direct the complete cardiac expression pattern of the endogenous myocardin gene during mouse embryogenesis.

Like the endogenous myocardin gene (Wang et al., 2001), the MyE-Hsp*lacZ* transgene also directed *lacZ* expression in SMCs of the developing dorsal aorta as early as E9.0 (Fig. 1B, parts f and g), which coincides with the time when SMCs populate and differentiate in large developing vessels (Owens, 1998). Smooth muscle markers, such as SM22α and SMα-actin, which are

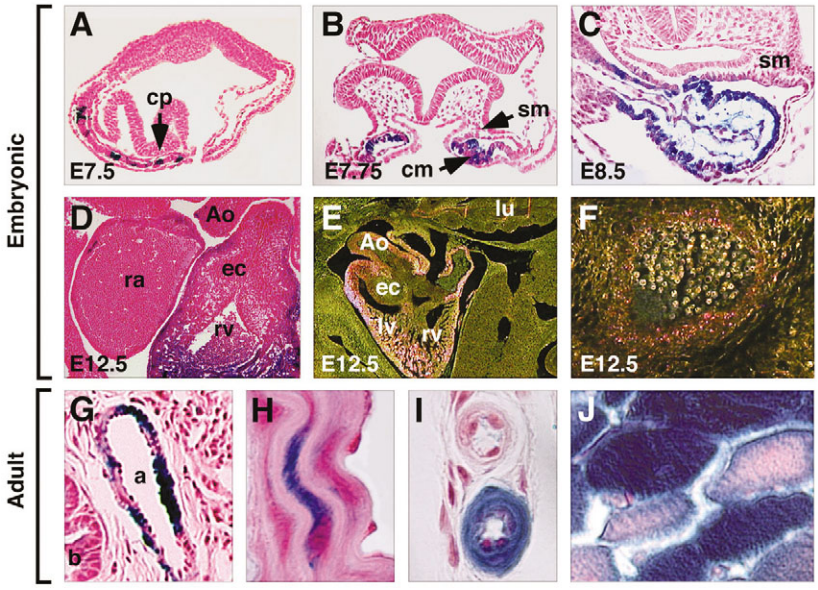

Fig. 2. Expression of the myocardin cardiac and smooth muscle enhancer. Embryos and adult tissues from the stable transgenic line harboring construct 5 (MyE1) were stained for β-galactosidase activity, sectioned and counterstained with Nuclear Fast Red. Note intense staining in the cardiogenic plate at E7.5 (**A**) and in the bilaterally symmetric cardiac mesoderm at E7.75 (**B**). At E8.5, expression is detected throughout the primitive myocardium, including the endocardial tissue lining the primitive heart (**C**). At E12.5 expression is seen in the ventricles and atria (**D**,**E**). Note that the endocardial cushion is devoid of *lacZ* expression. Using dark-field microscopy (E,**F**) *lacZ* expression could clearly be observed in the dorsal aorta at E12.5 (E,F). In adult vascular tissues (**G-J**), *lacZ* expression was detected in smooth muscle cells of the lung arteries (G) and aorta (H). A cross-section through the esophagus revealed strong expression in the vessel wall of small arteries but not veins (I). A high magnification of adult heart shows strong expression in cardiomyocytes (J). a, artery; ao, aorta; b, bronchus; cm, cardiac mesoderm; cp, cardiogenic plate; ec, endocardial cushion; ra, right atria; rv, right ventricle; sm, splanchnic mesoderm.

regulated by myocardin (Wang et al., 2001; Yoshida et al., 2003), become expressed at E9.5-10.5 (Owens, 1998), supporting previous findings that myocardin is required for the expression of these and other smooth muscle markers in vivo (Li et al., 2003). During the course of development, *lacZ* expression was apparent in developing large vessels such as the dorsal aorta and branchial arch arteries (Fig. 1B, parts g,h,k,l and Fig. 2F) and carotid arteries (Fig. 1B, part k). Expression in vessels in the head region, pulmonary outflow tract and intersomitic arteries became apparent after E13.5. In addition to its expression in the cardiovascular system, myocardin is expressed in SMCs within visceral organs, including lung, bladder, intestine and stomach (Wang et al., 2001). Transgene expression was not detectable in SMCs of the bladder and stomach, nor in the vascular plexus of the yolk sac or head mesenchyme, also sites where the endogenous gene is expressed (not shown). Thus the *lacZ* expression pattern recapitulates most, but not all of the expression pattern of the endogenous myocardin gene. In adult vascular tissues, *lacZ* expression was detected in SMCs of the aorta (Fig. 1B, part o and Fig. 2H, coronary arteries, lung arteries (Fig. 1B, part j and Fig. 2G) and other small arteries (Fig. 2I). Notably, in adult tissues, *lacZ* was expressed in a mosaic manner (Fig. 1B, parts e,o and Fig. 2G,H,J). It is not clear whether this reflects a heterogenous expression pattern of myocardin in adult heart and vascular SMCs or whether it is intrinsic to the usage of randomly integrated transgenes in which mosaic expression is not uncommon (Liu et al., 2006).

To ensure that the *hsp68* basal promoter had no influence on the timing or tissue specificity of myocardin regulatory sequences, we created a *lacZ* transgene in which the MyE enhancer was fused to the endogenous myocardin promoter (from –600 to –65 bp relative to the translational start). F0 embryos harboring this transgene showed β-galactosidase staining in a similar temporal and spatial pattern to that of MyE-Hsp*lacZ* transgene, although expression was weaker (data not shown).

Identification of cardiac and smooth muscle regulatory regions of the myocardin gene

To further delineate the regulatory elements within the 10 kb MyE fragment, we compared the mouse sequence with rat, human, dog and chicken sequences for evolutionary conservation (UCSC genome informatics: www.genome.ucsc.edu/). Based on the conservation pattern depicted in Fig. 3B, we tested a series of MyE deletion fragments for the ability to direct cardiac- and smooth-muscle-specific expression in transgenic embryos at E12.5. First, we divided the 10 kb MyE fragment into two equal, non-overlapping fragments of 5 kb (MyE2 and MyE3 in Fig. 3B). Interestingly, both fragments contained transcriptional enhancers sufficient to direct *lacZ* expression in the heart. Whereas MyE3 directed *lacZ* expression mainly to the heart, the MyE2 fragment showed strong expression in the myocardial layer of the heart *and* smooth muscle cells of the dorsal aorta and head vessels at E12.5. The MyE2 and MyE3 regions were further subdivided and all subfragments were tested with *hsp68-lacZ* (Fig. 3B).

Based on evolutionary conservation, a 350 bp subfragment (MyE8) of MyE2 was identified and found to be sufficient to direct *lacZ* expression in the heart and a distinct subset of SMCs (Fig. 3A,B). Deletion of the highly conserved 350 bp region in the context of the 3 kb MyE4 enhancer fragment (fragment MyE9) abolished *lacZ* expression completely, indicating that elements responsible for myocardin expression in differentiating SMCs and heart are present within the 350 bp sequence of MyE8. Fragment MyE3 was further delimited to a minimal enhancer of 2 kb (MyE6), which was sufficient to direct *lacZ* expression in the heart but not in vascular SMCs (Fig. 3A,B). As MyE8 contained *cis*-regulatory elements involved in the expression of both heart and smooth muscle, and could be narrowed down to a relatively small, highly conserved fragment, we chose to focus on this fragment for further studies. A summary of the expression patterns of all generated transgenic embryos is shown in Table S1 (in the supplementary material). Because the expression of active transgenes was lower in the vasculature than in the heart, animals with lower levels of transgene expression often did not show expression in the vasculature. Thus, the frequency of vascular versus cardiac expression was slightly reduced (see Table S1 in the supplementary material).

The myocardin enhancer is dependent on an essential Mef2 site

Inspection of the sequence of the MyE8 region revealed a highly conserved A + T-rich DNA sequence (CTATTTTAAG) (Fig. 3C) that resembled the binding site of Mef2 transcription factors, which bind the consensus sequence CTA(A/T)$_4$TAG (Black and Olson, 1998). In

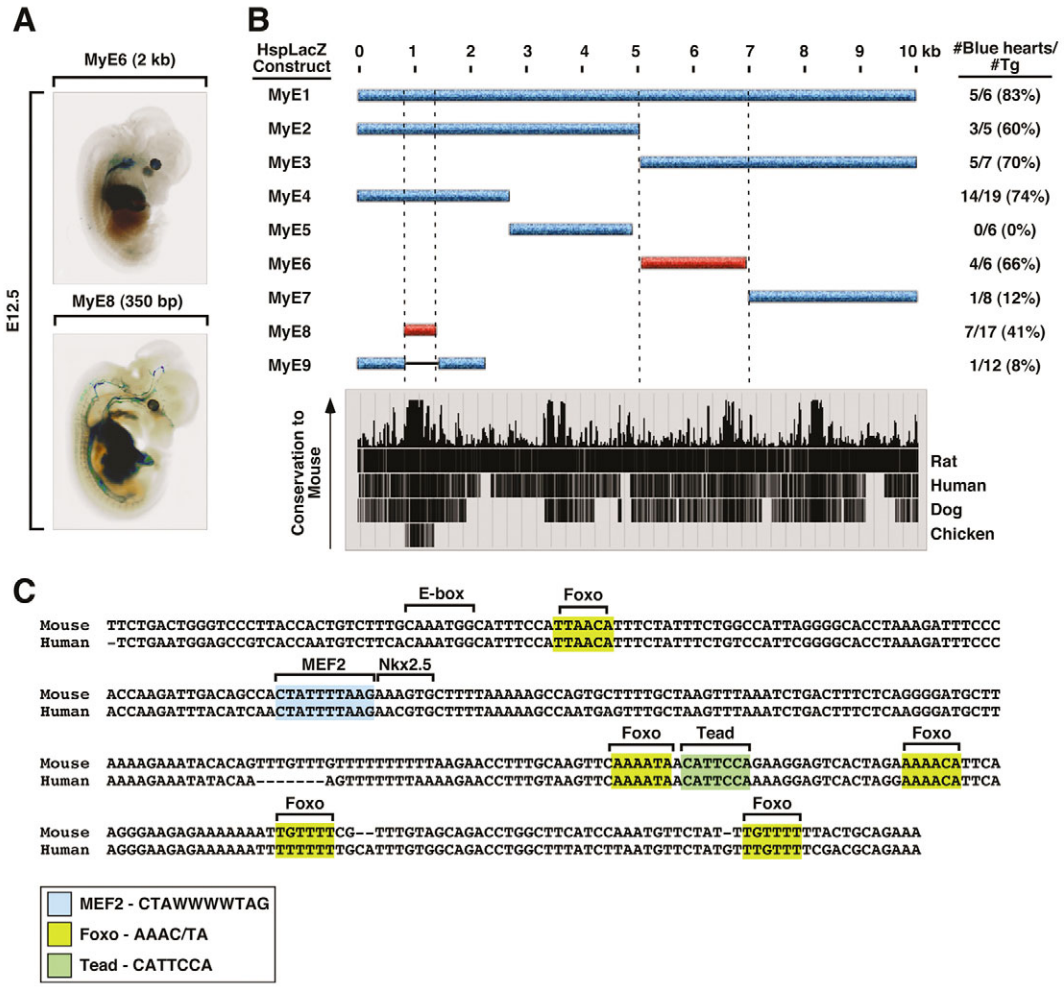

Fig. 3. Identification of an evolutionarily conserved minimal enhancer element sufficient to direct expression in heart and smooth muscle. (**A**) Representative expression pattern of F0 transgenic embryos with the two minimal transcriptional enhancers (MyE6 and MyE8). Whereas MyE6 directed *lacZ* expression to the heart, the 350 bp MyE8 fragment was sufficient to direct expression in heart *and* developing dorsal aorta and head vessels at E12.5. (**B**) Transgenic construct used to identify minimal elements of the myocardin enhancer (MyE). The construct numbers (MyE1-MyE9) are indicated on the left and fractions of F0 transgenic embryos showing cardiac expression at E12.5 are indicated in the right column. The 10 kb enhancer fragment MyE1 is identical to construct 5 in Fig. 1. An evolutionary conservation track shows the overall conservation score of MyE1 across all species, as well as pairwise alignment of rat, human, dog and chicken, each aligned to the mouse genome (Markov model, http://genome.ucsc.edu) (Kent et al., 2002). By transgenic analysis two evolutionarily conserved minimal enhancer elements, indicated in red, were identified (MyE6 and MyE8; 2 kb and 350 bp, respectively). Note that MyE8, but not MyE6, is conserved in the chicken. (**C**) Alignment of conserved mouse and human sequences in the myocardin enhancer MyE8. The conserved Mef2-binding site (blue shading), five candidate Foxo sites (yellow shading) and a conserved Tead-binding site (green shading) are noted.

light of the expression of Mef2c in the cardiac crescent, developing heart and vascular system (Black and Olson, 1998; Edmondson et al., 1994), and the essential role of Mef2c in cardiac and smooth muscle development (Lin et al., 1998; Lin et al., 1997), we tested whether Mef2c could bind this sequence in the myocardin enhancer.

As shown in Fig. 4A, Mef2c translated in vitro bound avidly to the putative Mef2 site in the myocardin enhancer in gel mobility shift assays. The DNA-Mef2c complex could be supershifted using a Mef2c-specific antibody, and binding was competed by the cognate site, but not by a mutant site.

To further define the potential role of the Mef2 site for cardiac and smooth muscle activity of the myocardin enhancer, we mutated this site in the context of the 3 kb enhancer (fragment MyE4) and tested this mutant enhancer for its ability to direct *lacZ* expression in combination with the *hsp68* basal promoter in vivo. Examination of ten F0 transgenic embryos at E12.5 failed to show *lacZ* expression in heart and vessels with the mutant enhancer (Fig. 4B). In some embryos, ectopic expression of *lacZ* was observed, reflecting transcriptionally active sites of transgene integration. These results demonstrate that the Mef2 site in the myocardin enhancer is essential for expression in heart and smooth muscle in vivo.

Myocardin activates its own enhancer through an Srf-independent mechanism

In light of the recent discovery of a cardiac enriched isoform of myocardin containing a unique N-terminal peptide sequence that confers the ability to stimulate the transcriptional activity of Mef2 (Creemers et al., 2006), we tested whether myocardin could activate its own enhancer through the identified Mef2 site. Indeed, as shown in Fig. 4C, the cardiac isoform of myocardin (myocardin-956) and Mef2c synergistically activated the MyE8 enhancer linked to a luciferase reporter, whereas the enhancer in which the Mef2 site was

mutated was non-responsive. Responsiveness of the myocardin enhancer to myocardin and Mef2c required the N-terminal domain of myocardin that associates with Mef2, as the smooth muscle isoform of myocardin lacking this domain (myocardin-856) failed to activate the enhancer. Notably, the myocardin enhancer does not contain an Srf-binding site. These findings suggest that myocardin cooperates with Mef2 to activate its own enhancer through an Srf-independent mechanism.

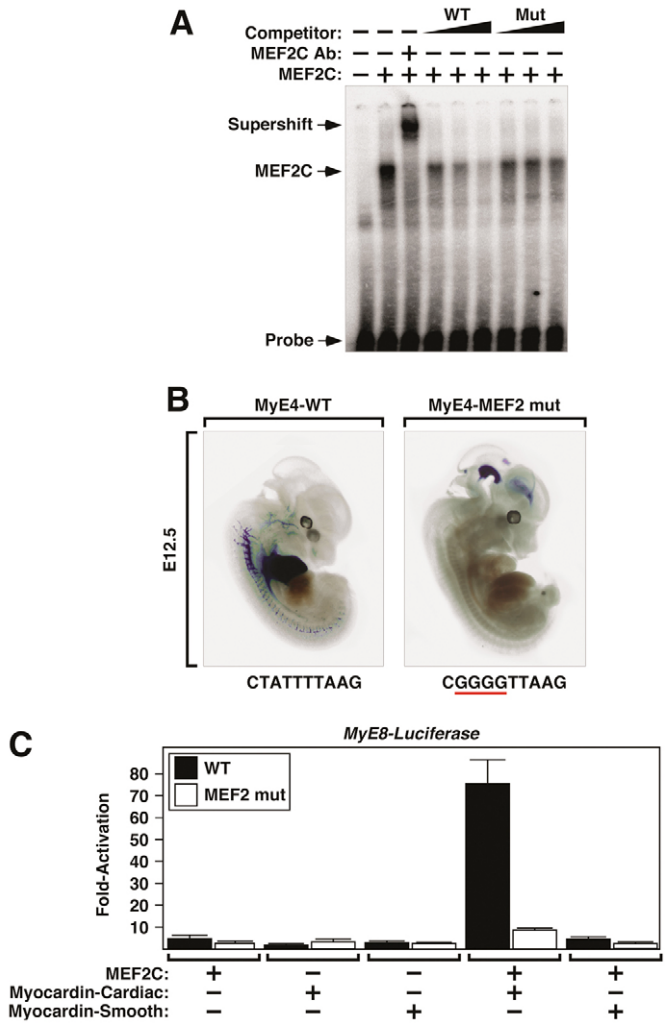

Fig. 4. Requirement of an Mef2-binding site for activity in heart and smooth muscle of the distal myocardin enhancer. (**A**) Binding of Mef2c to the Mef2-binding site in the minimal enhancer element MyE8. A ^{32}P-labeled oligonucleotide containing the conserved Mef2 site and Mef2c translated in reticulocyte lysate was used for electrophoretic mobility shift assays. DNA binding was seen only in reactions containing lysates with Mef2c. The DNA-Mef2c complex was supershifted using a Mef2c-specific antibody and unlabeled wild-type (WT) oligonucleotide efficiently competed for DNA binding, whereas unlabeled mutant (Mut) oligonucleotide did not. (**B**) Mutation of the Mef2-binding site in MyE4 (3 kb) enhancer completely abolishes transgenic expression at E12.5 in heart and smooth muscle. (**C**) Myocardin activates its own enhancer via Mef2. COS cells (24-well plates, 5×10^4 cells/well) were transfected with 100 ng of the indicated MyE8-luciferase reporters (wild-type and Mef2 mutant), 50 ng expression vectors encoding Mef2c and myocardin proteins and 30 ng of pCMV-*lacZ*. Activation of the MyE8 reporter by Mef2c was stimulated by the addition of cardiac isoform of myocardin and required the Mef2-binding site. The empty expression vector (pcDNA3.1) had no effect on the wild-type and mutated MyE8-luciferase reporter (not shown); therefore, the results are expressed as fold activation over empty pcDNA.

An expression screen reveals Tead and Foxo proteins as upstream regulators of myocardin expression

In an effort to further identify transcriptional regulators of the myocardin enhancer, we designed a eukaryotic cDNA expression screen for activators of the MyE8-dependent luciferase reporter. The reporter was transfected into COS cells along with pools of ~100 cDNA clones each from a mouse E10.5 expression library. We screened ~2000 cDNA pools, representing ~200,000 individual cDNA clones and identified a total of four positive pools. Sib-selections of the positive pools resulted in the identification of the transcription factors Tead2 and Foxo4 as activators of the MyE8 enhancer (Fig. 5A, Fig. 6A).

Four mammalian Foxo proteins have been described to date – Foxo1, Foxo3a, Foxo4 and Foxo6 (Van Der Heide et al., 2004) – with Foxo4 being most abundant in myocyte-containing tissues (Furuyama et al., 2000). Foxo proteins recognize and bind DNA sequences containing the core nucleotide sequence AAACA (Arden and Biggs, 3rd, 2002; Biggs, 3rd et al., 2001). We inspected the myocardin enhancer for evolutionarily conserved Foxo-binding sites and noted five potential sites in the MyE8 sequence (Fig. 3C). As shown in Fig. 5B, full-length Foxo4 potently activated the MyE8 reporter, but not the MyE8 reporter in which the Foxo sites were mutated. Gel mobility shift assays showed that Foxo4 bound to four of the five Foxo sites and could be supershifted using a Foxo4-specific antibody (Fig. 5C).

To test the function of the Foxo sites in vivo, we introduced mutations into each site in the context of the MyE4-*lacZ* transgene and determined the effect of those mutations on enhancer function at E10.5 (Fig. 5D; see Table S1 in the supplementary material). Mutation of all five Foxo sites resulted in a dramatic reduction in *lacZ* expression in heart and developing vessels, but the overall pattern of *lacZ* expression was unchanged, indicating that these sites have a quantitative effect on expression in vivo.

The Tead family of transcription factors consists of four members (Tead1-4), which share homology in a DNA-binding domain called a TEA domain. Tead genes are expressed widely in embryonic and adult tissues, and display distinct but overlapping expression patterns (Jacquemin et al., 1998; Kaneko et al., 1997). Tead1 mutant embryos die between E11 and 12 from heart defects (Chen et al., 1994). Analysis of the sequence of MyE8 revealed a consensus Tead-binding motif, CATTCCA (Davidson et al., 1988; Jacquemin et al., 1998; Xiao et al., 1991), within this enhancer (Fig. 3C).

To confirm the results of the expression screen, in which we identified Tead2 as a potential activator of the myocardin enhancer, we expressed Tead2 in COS cells along with the MyE8-luciferase reporter. Tead2 activated the MyE8 reporter approximately eightfold over the empty reporter and a mutation in the Tead-binding site reduced this activation approximately twofold. As an antibody for supershift experiments was only available for Tead1, we tested for binding of Tead1 to the putative binding site in the MyE8 using Tead1 translated in vitro. As shown in Fig. 6C, Tead1 was able to bind the radiolabeled Tead site, and the DNA-Tead1 complex could be supershifted by the addition of Tead1-specific antibody. Specificity of the interaction was further demonstrated by competition with excess unlabeled probe but not with a mutated probe (Fig. 6C).

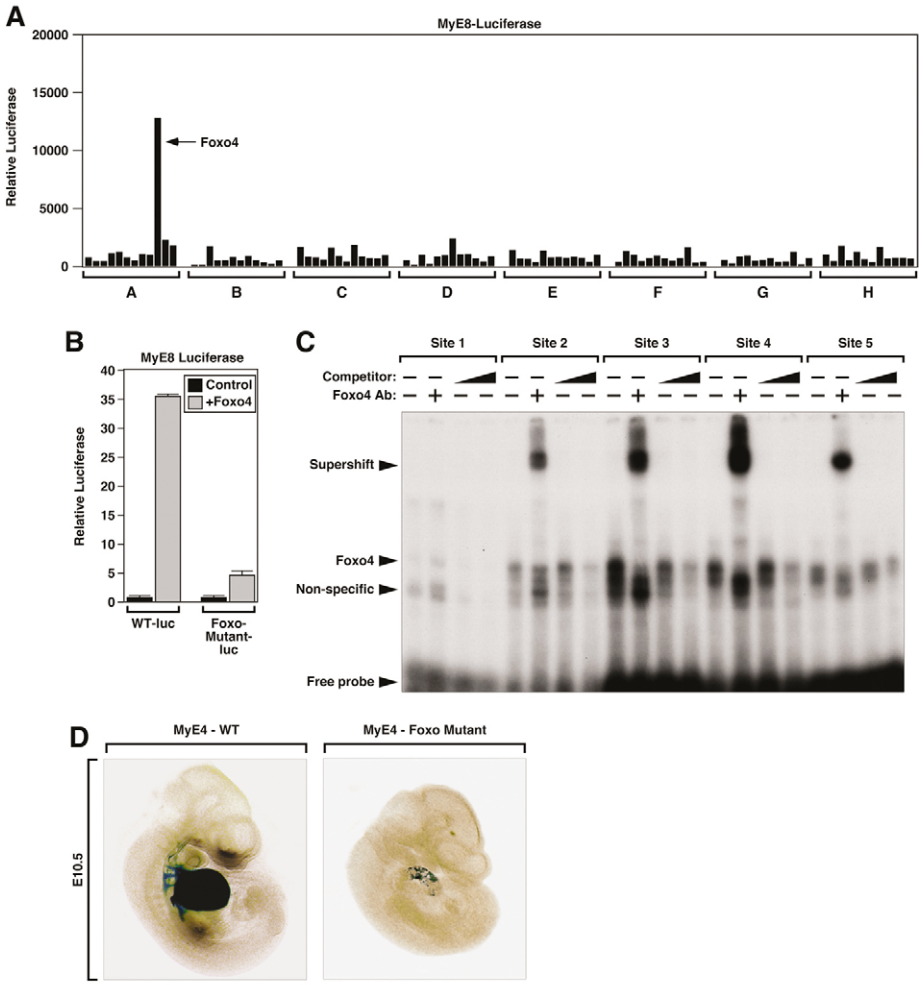

Fig. 5. Identification of Foxo proteins as mediators of myocardin expression. (**A**) Results from transfection assays in the 96-well plate that identified Foxo4 as an activator of the distal myocardin enhancer MyE8. In each well, we transfected the MyE8-luciferase reporter into COS cells along with pools of ~100 cDNA clones from a mouse E10.5 expression library. The MyE8-luciferase plasmid was specifically activated in well A10. Sibselection from this pool identified Foxo4 as the activating cDNA. (**B**) Transient transfections in COS cells show that Foxo4 activates the MyE8 reporter, which contains five predicted Foxo-binding sites, with the consensus sequence AAAC/TA (see Fig. 3C). Foxo4 was less effective in activating the MyE8 reporter in which all five Foxo-binding sites were mutated. (**C**) DNA binding of Foxo4 to the five predicted Foxo-binding sites (see Fig. 3C) in minimal enhancer element MyE8 is shown. Five ^{32}P-labeled oligonucleotides containing the predicted Foxo-binding sites and Foxo4 translated in reticulocyte lysate were used in electrophoretic mobility shift assays. The strongest binding was seen for Foxo sites 3 and 4, weaker binding for sites 2 and 5, whereas Foxo4 did not bind to the first site. The ^{32}P-DNA-Foxo4 complexes were supershifted using a Foxo4-specific antibody and unlabeled, wild-type oligonucleotides efficiently competed for DNA binding. (**D**) Requirement of Foxo sites for activity of the 3 kb myocardin enhancer in vivo. F0 transgenic embryos generated with construct MyE4, (see Fig. 3A) containing mutations of the five Foxo-binding sites abolishes transgenic expression at E10.5 in heart and smooth muscle.

As mutation of the Tead site in the MyE8 reporter did not abolish Tead activity completely (Fig. 6B), we searched for other possible Tead-binding sites within MyE8. We noticed a sequence ~20 bp downstream of the Tead-binding site that resembled, but did not completely fit, the consensus sequence for a Tead site (CATTCA instead of the CATTCCA) and gel shift analysis with probes for this site showed almost no Tead1 binding (not shown). Therefore, we do not consider this second site to be a Tead site, although we cannot rule out that it contributes to the residual luciferase activity seen in the MyE8-Tead mutant in Fig. 6B. Perhaps the residual activity of Tead2 on the mutated MyE8 reporter is indirect, via interaction with Mef2, or another factor. As Mef2 and Tead2 can physically interact (Maeda et al., 2002), we co-transfected Mef2c and Tead2 expression plasmids in COS cells, but saw no synergistic activation of the MyE8 reporter.

The potential role of the Tead site in the control of the myocardin enhancer in vivo was tested by mutating the site in the context of the 3 kb *lacZ* transgene (fragment MyE4). Analysis of seven independent F0 transgenic embryos at E10.5 showed that mutation of the Tead site did not affect *lacZ* expression in the heart (Fig. 6D-F). However, *lacZ* expression was consistently absent in the branchial arch arteries at this stage (Fig. 6D,F; see Table S1 in the supplementary material). In addition *lacZ* expression can be appreciated in the dorsal aorta of wild-type transgenic embryos but not in the Tead mutant (Fig. 6G). The requirement of the Tead-binding site for activation of the myocardin enhancer in neural-crest-derived SMCs is consistent with the recent identification of Tead2 as a neural-crest-specific regulator of *Pax3* transcription, one of the earliest markers of neural crest induction (Milewski et al., 2004). We conclude that Tead proteins act specifically to control myocardin expression and smooth muscle differentiation in branchial arch arteries and dorsal aorta.

In an attempt to identify potential molecular interactions in the regulation of the myocardin enhancer, we transfected all possible combinations of Mef2c, Foxo4 and Tead2 expression plasmids in COS cells along with the MyE8 reporter. Luciferase assays failed to show a combinatorial effect (synergistical activation or repression) of Mef2c, Foxo4 and Tead2 on the MyE8 reporter.

In addition to the Mef2, Tead and Foxo sites, we also tested candidate NKE- and E-box-binding sites to determine if these putative sites in the myocardin enhancer might be targets for Nkx2.5 and HAND proteins, respectively (Fig. 3C). Mutations introduced in those sites in the context of the MyE4-*lacZ* transgene demonstrated that these sites were dispensable for enhancer function in vivo (see Table S1 in the supplementary material). Together, the results of the in vivo and in vitro assays demonstrate that the binding sites for Mef2, Tead and Foxo proteins in the myocardin enhancer are required for complete enhancer function during cardiac and smooth muscle development in vivo.

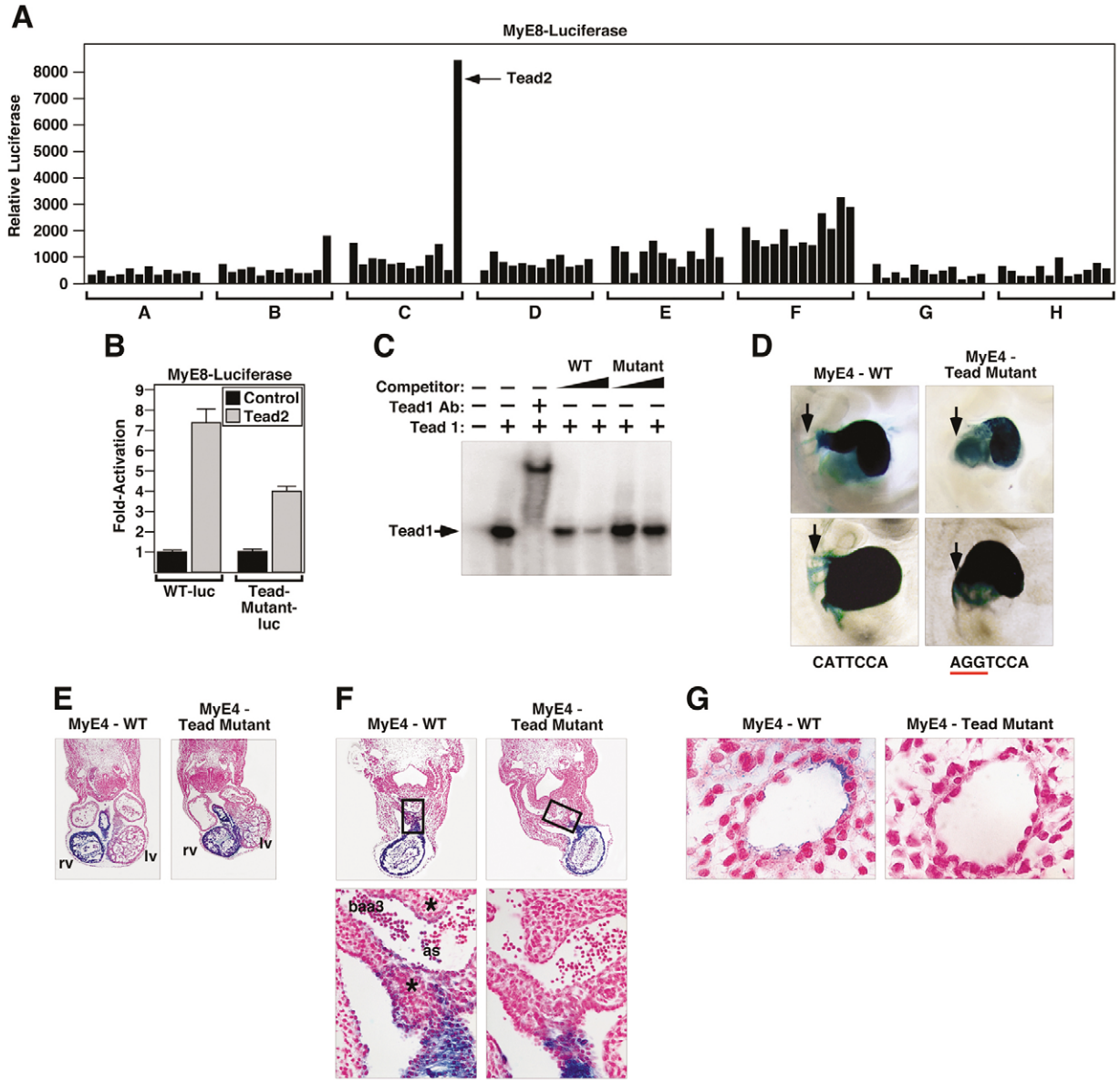

Fig. 6. Identification of Tead proteins as specific regulators of myocardin expression in branchial arch arteries and aorta. (**A**) A eukaryotic expression screen, in which we co-transfected MyE8 luciferase along with pools of ~100 cDNA clones from a mouse E10.5 expression library revealed Tead2 as an activating cDNA of the minimal myocardin enhancer (well C12). (**B**) Transient transfections in COS cells show that Tead2 activates the MyE8-luciferase reporter but was less effective in activating the MyE8 reporter in which the predicted Tead-binding site (see Fig. 3C) was mutated. (**C**) Binding of Tead1 to the Tead-binding site in minimal enhancer element MyE8. A ^{32}P-labeled oligonucleotide containing the conserved Tead site and Tead1 translated in reticulocyte lysate was used for electrophoretic mobility shift assays. DNA binding was seen only in reactions containing lysates with Tead1. The DNA-Tead complex was supershifted using a Tead1-specific antibody and unlabeled wild-type (WT) oligonucleotide efficiently competed for DNA binding, whereas unlabeled mutant (Mut) oligonucleotide did not. (**D**) Requirement of the Tead-binding site for activity of the myocardin enhancer (MyE4) specifically in branchial arch arteries at E10.5. Two independent F0 transgenic embryos generated with construct MyE4, and two independent F0 embryos with MyE4 in which the Tead-binding site was mutated, are shown. Note that *lacZ* expression is absent in the branchial arch arteries of the MyE4-Tead mutant embryos indicated by arrows. (**E**) Transverse cross-sections of E10.5 transgenic embryos show that cardiac *lacZ* expression, which is highest in the ventricles, is very similar in wild-type and Tead mutants. LV, left ventricle; RV, right ventricle. (**F**) A cross-section through the outflow tract region shows *lacZ* expression in cells (asterisk) surrounding the aortic sac (as) and third branchial arch artery (baa3) of wild-type but not Tead mutant embryos. (**G**) *lacZ* expression can be appreciated in the dorsal aorta of wild-type embryos but not in the Tead mutant embryos.

DISCUSSION

Myocardin is expressed specifically in cardiac and smooth muscle cells and is one of the earliest known markers of these myogenic lineages. Thus, elucidation of the mechanisms that activate myocardin transcription promises to provide insights into the initial steps involved in activation and maintenance of the cardiac and smooth muscle phenotypes. The results of this study reveal a distal upstream enhancer that recapitulates the expression of myocardin from the onset of cardiogenesis in the cardiac crescent and heart tube throughout the stages of looping and the formation of the four

chambered heart. This enhancer is also active in the developing dorsal aorta as early as E9.0-9.5, before the expression of smooth muscle differentiation markers (Owens, 1998), and in SMCs within large vessels such as the branchial arch arteries (E10.5-11.5) and carotid arteries (E12.5). The myocardin enhancer is a direct target of Mef2 and Foxo in cardiac and smooth muscle cells and is regulated by Tead factors in dorsal aorta and neural-crest-derived SMCs. Notably, myocardin can also activate its own enhancer, but in contrast to the majority of myocardin target genes, myocardin acts not through Srf, but through Mef2, to regulate its transcription. To our knowledge, the myocardin enhancer is the only *cis*-regulatory region described to date that relies on this combination of transcription factors for cardiovascular expression.

Nearly all smooth muscle genes are controlled by two or more CArG boxes (Owens et al., 2004; Sun et al., 2006). By contrast, our results show that expression of myocardin in smooth muscle does not require CArG boxes, reflecting its unique mode of regulation as an upstream regulator of subordinate CArG-independent genes. The histidine-rich calcium binding protein (*HRCBP*) gene is another rare example of a smooth muscle gene that is not controlled by CArG boxes (Anderson et al., 2004). Notably, Mef2 also serves as an essential regulator of *HRCBP* expression, but Tead and Foxo have not been shown to regulate the *HRCBP* enhancer.

Multiple, distant myocardin enhancers specify expression during development

Modular control of gene transcription, in which multiple independent regulatory elements combine to direct the complete expression pattern of a gene, has emerged as a common theme in the control of cardiac gene expression (Firulli and Olson, 1997). Through analysis of evolutionarily conserved DNA sequences and the testing of several deletion fragments in transgenic embryos, we were able to identify two independent enhancers (MyE6 and MyE8) that control myocardin expression. Both regions were sufficient to direct *lacZ* expression to the heart, but only one of those fragments (MyE8) was able to direct *lacZ* expression to SMCs. In addition to its expression in the cardiovascular system, myocardin is expressed in SMCs within visceral organs, including lung, bladder, intestine and stomach. The MyE8 enhancer is active in lung SMCs, but not in SMCs within these other organs, suggesting that expression in these tissues is controlled by additional enhancers, which either lie outside the 90 kb of genomic sequence we surveyed in transgenic mice or are influenced by negative regulatory elements that preclude their identification in the constructs we generated.

We note that Nkx2-5 has been reported to activate the proximal myocardin promoter in transfection assays via an Nkx-binding element (NKE), although the potential involvement of this DNA sequence in myocardin expression in vivo was not examined in that study (Ueyama et al., 2003). The distal upstream enhancer we identified is approximately 10 kb away from the region described by Ueyama et al. (Ueyama et al., 2003) and it does not appear to require Nkx2-5 for activity. Our results also indicate that the region they described is unable to direct expression in vivo.

Transcriptional regulation of myocardin by Mef2

The myocardin enhancer MyE8 contains a conserved Mef2 site, which is required for enhancer activity in vivo. Among the four members of the Mef2 family, Mef2c is the earliest marker of the cardiac lineage, and is required for morphogenesis and maturation of the heart and vascular system (Lin et al., 1998; Lin et al., 1997; Naya et al., 2002). Thus, Mef2c is likely to act through this enhancer element. As myocardin and Mef2 are co-expressed in the early cardiac crescent, we think Mef2c is most likely to participate in amplification and maintenance of myocardin expression rather than the initial activation of the gene.

Recently, we discovered that a cardiac isoform of myocardin, containing a unique N-terminal Mef2-binding motif, is able to interact with Mef2 and activate transcription through a subset of Mef2-dependent regulatory elements in the heart (Creemers et al., 2006). This cardiac isoform of myocardin was able to coactivate the myocardin enhancer with Mef2, and mutation of the Mef2 site abolished myocardin responsiveness of the enhancer. The dependence of the myocardin enhancer on Mef2 suggests the existence of a positive autoregulatory loop, through which myocardin may amplify and maintain its expression in the heart via this site. The predominant myocardin splice variant expressed in SMCs lacks the Mef2-binding peptide and is incapable of coactivating Mef2 (Creemers et al., 2006). Thus, although the Mef2 site in the myocardin enhancer is required for enhancer activity in cardiac and smooth muscle cells, this putative positive feedback loop would only be operative in the heart, suggesting that Mef2 cooperates with other cofactors to control myocardin transcription in SMCs. In this regard, Mef2 has been shown to cooperate with members of the Tead family (Maeda et al., 2002).

The existence of a possible autoregulatory loop to maintain myocardin expression specifically in cardiac muscle but not in SMCs may reflect one of the intrinsic differences between these two muscle cell types. In this regard, cardiomyocytes, like skeletal muscle cells, differentiate irreversibly. Positive autoregulation of the myocardin gene may serve to stabilize and maintain the cardiac phenotype in a manner similar to the autoregulation of the MyoD family in skeletal muscle (Thayer et al., 1989). By contrast, SMCs are highly plastic and modify their phenotype in response to developmental, physiological and pathological cues. The potential regulation of myocardin transcription by Mef2 and the resulting reinforcement of myocardin expression are schematized in Fig. 7.

Regulation of the myocardin enhancer by Foxo

The myocardin enhancer depends on multiple Foxo-binding sites for activity in vivo. The Foxo family of forkhead-type transcription factors regulates the transcription of genes involved in various cellular processes, including proliferation, differentiation, metabolism and survival (Accili and Arden, 2004). All four Foxo family members, Foxo1, Foxo3a, Foxo4 and Foxo6, are able to bind

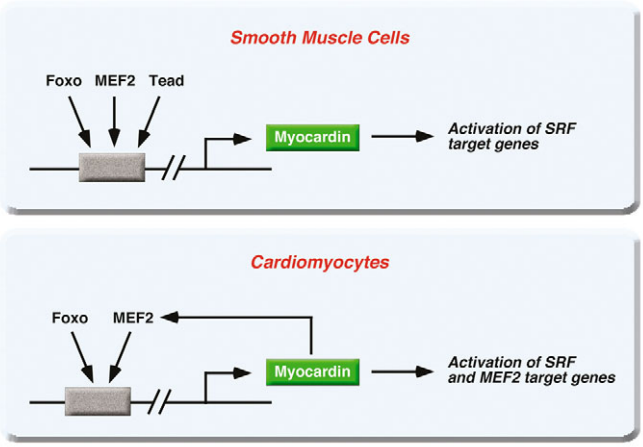

Fig. 7. Schematic diagram of the regulation of the myocardin enhancer.

the myocardin enhancer (data not shown), but we can only speculate as to which specific family members regulate the enhancer in vivo. In this regard, abundant expression of Foxo4 in myocyte-containing tissues (Furuyama et al., 2000) and recent evidence for the involvement of Foxo4 in phenotypic modulation of SMCs (Hayashi et al., 1998; Liu et al., 2005) support a possible role for Foxo4 as an upstream regulator of myocardin transcription. Interestingly, Foxo4 appears to trigger dedifferentiation of SMCs by interacting with and repressing the transcriptional activity of myocardin (Liu et al., 2005). This may counterbalance the actions of Foxo4 as an activator of myocardin transcription to finetune the potent transcriptional activity of myocardin.

Foxo1 expression overlaps that of myocardin in the heart, dorsal aorta and small vessels such as intersomitic and head vessels during early embryogenesis (Furuyama et al., 2004; Hosaka et al., 2004). Moreover, *Foxo1* null embryos die around E11 with defects in branchial arches and vascular remodeling (Hosaka et al., 2004). Whether these abnormalities reflect an essential role for Foxo1 solely in endothelial cells or whether there is an additional defect in SMC differentiation, possibly mediated by myocardin, is an interesting question for future investigation.

Regulation of the myocardin enhancer by Tead proteins

Through a eukaryotic cDNA expression screen we identified the transcription factor Tead2 as an activator of the myocardin enhancer. The Tead (TEA domain) family of proteins contains a highly conserved 72 amino acid DNA-binding domain, which is evolutionarily conserved among yeast, *Drosophila*, rat, chick, mouse and human (Jacquemin et al., 1996; Kaneko et al., 1997; Kaneko and DePamphilis, 1998). Tead proteins recognize the canonical M-CAT motif (CATTCCT/A) (Farrance and Ordahl, 1996; Larkin et al., 1996) found in promoters of muscle specific genes such as those encoding smooth muscle α-actin, skeletal α-actin, cardiac and skeletal troponin T and α- and β-MHC (Larkin et al., 1996; Swartz et al., 1998). Moreover, disruption of the *Tead1* gene disrupts heart development (Chen et al., 1994). Our results show that transcriptional activity of the myocardin enhancer crucially depends on a Tead-binding site, specifically in the branchial arch arteries and dorsal aorta. Previously reported expression data suggest that among members of the Tead family, Tead2 is most strongly expressed in regions of neural crest and branchial arches.

The requirement of a Tead-binding site for activation of the myocardin enhancer in neural-crest-derived smooth muscle cells is consistent with the recent identification of Tead2 as a neural-crest-specific regulator of the transcription of *Pax3,* which is one of the earliest markers of neural crest induction (Milewski et al., 2004). Interestingly, Pax3-expressing neural crest precursors become the SMCs of the aortic arch artery and major cranial vessels.

Implications

In addition to its important roles in the control of cardiac and smooth muscle development, myocardin has also been implicated in remodeling of the adult cardiovascular system during disease. In this regard, myocardin and CArG-dependent muscle genes are upregulated in the failing heart (Torrado et al., 2003; Xing et al., 2006). Overexpression of myocardin increases cardiomyocyte size and activates the expression of atrial natriuretic factor (Badorff et al., 2005; Xing et al., 2006), a sensitive Srf-dependent marker of cardiac hypertrophy. In addition, pathological remodeling of the vessel wall during atherosclerosis and restenosis involves a switch in SMC phenotype from a differentiated contractile to a proliferative 'synthetic' state (Owens et al., 2004). Signals that perturb myocardin activity in turn block transcription of SMC contractile genes and promote phenotypic switching (Liu et al., 2005).

Given the signal responsiveness of Mef2, Foxo and Tead factors (Accili and Arden, 2004; McKinsey et al., 2002; McLean et al., 2003), it is likely that different extracellular signals modulate myocardin expression via their effects on these activators. Thus, the discovery of the enhancer that controls myocardin expression in the cardiac and smooth muscle cell lineages provides insights, not only into the mechanisms governing the establishment of these cell types during embryogenesis, but also offers possibilities for manipulating myocardin expression in various therapeutic settings.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/21/4245/DC1

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