factors) would be given as a boost in the event of documented smallpox. Further experiments to determine the longevity of protection, the consequences of delayed boosting and dosage effects are planned. □

## Methods

### Virus preparations

A vial of MVA passage 572 (2/22/1974), obtained from A. Mayr, was plaque purified, propagated in chick embryo fibroblasts, and purified by sedimentation through a sucrose cushion (to be described elsewhere). A vial of Dryvax (Wyeth Laboratories, Inc.), obtained from the US Centers for Disease Control and Prevention, was diluted as recommended and stored at 4 °C. Monkeypox virus strain Zaire 79 (V79-I-005), originally isolated from the scab of a fatally infected human in LLC-MK2 cells and passed twice in BSC40 cells, was obtained from J. Esposito and propagated in MA-104 cells. A clarified lysate of infected cells was used to make the challenge stock of virus.

### Animals

Cynomolgous monkeys (*Macaca fascicularis*) of Chinese origin were housed at Bioqual, Inc. for the immunological studies and transferred to the US Army Medical Research Institute of Infectious Diseases before the monkeypox virus challenge.

### Vaccinia virus neutralization assay[26]

Twofold serial dilutions of heat-inactivated sera were incubated with virus encoding the GFP for 1 h at 37 °C. HeLa S3 cells were added and incubated overnight. GFP-expressing cells were enumerated with a FACScaliber and analysed with FloJo software. $IC_{50}$ values were calculated using PRISM software (GraphPad Software, Inc.). All assays were performed in duplicate.

Received 26 November 2003; accepted 7 January 2004; doi:10.1038/nature02331.

1. Fulginiti, V. A., Papier, A., Lane, J. M., Neff, J. M. & Henderson, D. A. Smallpox vaccination: a review, part II. Adverse events. *Clin. Infect. Dis.* **37,** 251–271 (2003).
2. Mayr, A., Hochstein-Mintzel, V. & Stickl, H. Abstammung, eigenschaften und verwendung des attenuierten vaccinia-stammes MVA [Passage history, properties, and applicability of the attenuated vaccinia virus strain MVA]. *Infection* **3,** 6–14 (1975).
3. Meyer, H., Sutter, G. & Mayr, A. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* **72,** 1031–1038 (1991).
4. Antoine, G., Scheiflinger, F., Dorner, F. & Falkner, F. G. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology* **244,** 365–396 (1998).
5. Carroll, M. & Moss, B. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. *Virology* **238,** 198–211 (1997).
6. Drexler, I., Heller, K., Wahren, B., Erfle, V. & Sutter, G. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. *J. Gen. Virol.* **79,** 347–352 (1998).
7. Blanchard, T. J., Alcami, A., Andrea, P. & Smith, G. L. Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. *J. Gen. Virol.* **79,** 1159–1167 (1998).
8. Sutter, G. & Moss, B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc. Natl Acad. Sci. USA* **89,** 10847–10851 (1992).
9. Stittelaar, K. J. *et al.* Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques. *Vaccine* **19,** 3700–3709 (2001).
10. Hochstein-Mintzel, V., Huber, H.-C. & Stickl, H. Die orale und nasale immuniserung mit poxvirus vacciniae III. Mitteilung: tierexperimentelle untersuchungen [Oral and nasal immunization with poxvirus vaccine. Part III: experimental animal investigations]. *Zentralbl. Bakteriol. Hyg.* **156,** 30–96 (1972).
11. Hochstein-Mintzel, V., Hänichen, T., Huber, H. C. & Stickl, H. Vaccinia- und variolaprotektive wirkung des modifezierten vaccinia-stammes MVA bei intramuskulärer immunisierung [An attenuated strain of vaccinia virus (MVA). Successful intramuscular immunization against vaccinia and variola]. *Zentralbl. Bakteriol. Hyg.* **230,** 283–297 (1975).
12. Belyakov, I. M. *et al.* Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. *Proc. Natl Acad. Sci. USA* **100,** 9458–9463 (2003).
13. Stickl, H. *et al.* MVA-stufenimpfung gegen pocken. klinische erprobung des attenuierten pocken-lebendimpfstoffes, stamm MVA [MVA vaccination against smallpox: clinical trials of an attenuated live vaccinia virus strain (MVA)]. *Dtsch. Med. Wochenschr.* **99,** 2386–2392 (1974).
14. Mayr, A., Stickl, H., Müller, H. K., Danner, K. & Singer, H. Der pockenimpfstamm MVA: marker, genetische struktur, erfahrungen mit der parenteralen schutzimpfung und verhalten im abwehrgeschwächten organismus [The smallpox vaccination strain MVA: marker, genetic structure, experience gained with parenteral vaccination and behavior in organisms with debilitated defense mechanism]. *Zentralbl. Bakteriol. Hyg.* **167,** 375–390 (1978).
15. Mayr, A. History of variola, smallpox-eradication and MVA. *Berl. Munch. Tierarztl. Wochenschr.* **112,** 322–328 (1999).
16. Zaucha, G. M., Jahrling, P. B., Geisbert, T. W., Swearengen, J. R. & Hensley, L. The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (*Macaca fascicularis*). *Lab. Invest.* **81,** 1581–1600 (2001).
17. LeDuc, J. W., Damon, I., Relman, D. A., Huggins, J. & Jahrling, P. B. Smallpox research activities: U.S. interagency collaboration, 2001. *Emerg. Infect. Dis.* **8,** 743–745 (2002).
18. Arita, I., Jezek, Z., Khodakevich, L. & Ruti, K. Human monkeypox: a newly emerged orthopoxvirus zoonosis in the tropical rain forests of Africa. *Am. J. Trop. Med. Hyg.* **34,** 781–789 (1985).
19. Breman, J. G. & Henderson, D. A. Poxvirus dilemmas—monkeypox, smallpox, and biologic terrorism. *N. Engl. J. Med.* **339,** 556–559 (1998).
20. Smith, G. L., Vanderplasschen, A. & Law, M. The formation and function of extracellular enveloped vaccinia virus. *J. Gen. Virol.* **83,** 2915–2931 (2002).
21. Law, M. & Smith, G. L. Antibody neutralization of the extracellular enveloped form of vaccinia virus. *Virology* **280,** 132–142 (2001).
22. Hooper, J. W., Custer, D. M., Schmaljohn, C. S. & Schmaljohn, A. L. DNA vaccination with vaccinia virus L1R and A33R genes protects mice against a lethal poxvirus challenge. *Virology* **266,** 329–339 (2000).
23. Hooper, J. W., Custer, D. M. & Thompson, E. Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. *Virology* **306,** 181–195 (2003).
24. Galmiche, M. C., Goenaga, J., Wittek, R. & Rindisbacher, L. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. *Virology* **254,** 71–80 (1999).
25. Wolffe, E. J., Vijaya, S. & Moss, B. A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. *Virology* **211,** 53–63 (1995).
26. Earl, P. L., Americo, J. L. & Moss, B. Development and use of a vaccinia virus neutralization assay based on flow cytometric detection of green fluorescent protein. *J. Virol.* **77,** 10684–10688 (2003).
27. Appleyard, G., Hapel, A. J. & Boulter, E. A. An antigenic difference between intracellular and extracellular rabbitpox virus. *J. Gen. Virol.* **13,** 9–17 (1971).
28. Law, M., Hollinshead, R. & Smith, G. L. Antibody-sensitive and antibody-resistant cell-to-cell spread by vaccinia virus: role of the A33R protein in antibody-resistant spread. *J. Gen. Virol.* **83,** 209–222 (2002).
29. Breman, J. G. & Henderson, D. A. Diagnosis and management of smallpox. *N. Engl. J. Med.* **346,** 1300–1308 (2002).
30. Speller, S. A. & Warren, A. P. *Ex vivo* detection and enumeration of human antigen-specific $CD8^+$ T lymphocytes using antigen delivery by a recombinant vaccinia expression vector and intracellular cytokine staining. *J. Immunol. Methods* **262,** 167–180 (2002).

**Supplementary Information** accompanies the paper on **www.nature.com/nature**.

**Acknowledgements** We are grateful to R. Elkins (NIAID) for animal acquisition and to R. Byrum (Bioqual, Inc.) for animal care and immunizations. The work was supported by the National Institute of Allergy and Infectious Diseases.

**Competing interests statement** The authors declare that they have no competing financial interests.

**Correspondence** and requests for materials should be addressed to B.M. (bmoss@nih.gov).

# Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression

**Zhigao Wang[1], Da-Zhi Wang[1,2], Dirk Hockemeyer[3], John McAnally[1], Alfred Nordheim[3] & Eric N. Olson[1]**

[1]*Department of Molecular Biology, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd, Dallas, Texas 75390-9148, USA*
[2]*Carolina Cardiovascular Biology Center, Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, North Carolina 27599-7126, USA*
[3]*Institute of Cell Biology, Department of Molecular Biology, Tuebingen University, D-72704, Tuebingen, Germany*

**Smooth muscle cells switch between differentiated and proliferative phenotypes in response to extracellular cues[1], but the transcriptional mechanisms that confer such phenotypic plasticity remain unclear. Serum response factor (SRF) activates genes involved in smooth muscle differentiation and proliferation by recruiting muscle-restricted cofactors, such as the transcriptional coactivator myocardin, and ternary complex factors (TCFs) of the ETS-domain family, respectively[2–9]. Here we show that growth signals repress smooth muscle genes by triggering the displacement of myocardin from SRF by Elk-1, a TCF that acts as a myogenic repressor. The opposing influences of myocardin and Elk-1 on smooth muscle gene expression are mediated by structurally related SRF-binding motifs that compete for a common docking site on SRF. A mutant smooth muscle**


©2004 Nature Publishing Group

**promoter, retaining responsiveness to myocardin and SRF but defective in TCF binding, directs ectopic transcription in the embryonic heart, demonstrating a role for TCFs in suppression of smooth muscle gene expression *in vivo*. We conclude that growth and developmental signals modulate smooth muscle gene expression by regulating the association of SRF with antagonistic cofactors.**

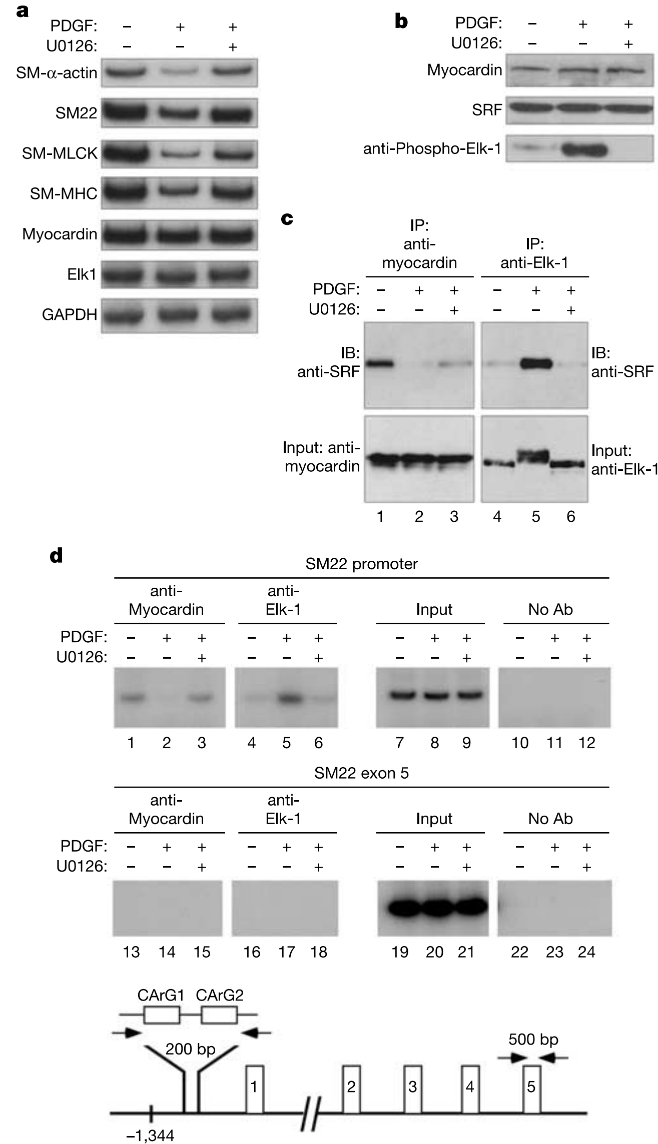


**Figure 1** Mutually exclusive interactions of myocardin and Elk-1 with SRF in SM cells. A7r5 cells were cultured in serum-free medium and then stimulated with or without PDGF-BB (20 ng ml$^{-1}$) for 24 h (**a**) or 30 min (**b**–**d**) in the presence or absence of U0126 (10 μM) as indicated. **a**, RNA was isolated and transcripts detected by RT–PCR, using SM-α-actin, SM22, SM-myosin light chain kinase (MLCK) and SM-myosin heavy chain (MHC) as markers of smooth muscle differentiation and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for RNA loading. **b**, Extracts were prepared from A7r5 cells and proteins were detected by western blot. **c**, Immunoprecipitations (IP) performed in A7r5 cell extracts with anti-myocardin or anti-Elk-1 antibody followed by immunoblot (IB) with mouse anti-SRF antibody. **d**, Chromatin was prepared from A7r5 cells and immunoprecipitated with anti-myocardin or anti-Elk-1, followed by PCR using primers from the *SM22* promoter or exon 5 of the *SM22* gene as a control. ChIP assays with primers flanking exon 5 of the coding region showed no response to PDGF.

Most smooth muscle contractile protein genes are controlled by SRF[10], which binds to a sequence known as a CArG box and recruits myocardin, a coactivator that is necessary and sufficient for smooth muscle gene expression[3–8]. In response to growth factor signalling, SRF also interacts with TCFs, a family of ETS-domain transcription factors that includes Elk-1, SAP-1a and NET/SAP-2/Erp (refs 11–13). Phosphorylation of these TCFs by MAP kinases facilitates their association with SRF and activation of growth-factor-inducible genes[14,15].

To investigate how extracellular signals enable SRF to select between muscle-specific and growth-regulated genes, which represent opposing transcriptional programmes, we examined the effect of platelet-derived growth factor (PDGF), an antagonist of smooth muscle differentiation[16], on myocardin/SRF target genes in the A7r5 smooth muscle (SM) cell line. PDGF specifically suppressed expression of contractile protein genes (Fig. 1a; Supplementary Fig. 1) and induced the phosphorylation of Elk-1, as detected by western blot with an anti-phospho-Elk-1 antibody or by an upshift of Elk-1 on SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1b, c). PDGF also increased the association of Elk-1 with SRF, which was paralleled by a decrease in association of myocardin with SRF, as detected by co-immunoprecipitation (Fig. 1c).

The association of TCFs with SRF is stabilized on SRF-binding sites that contain adjacent ETS-binding sequences (GGAA/T) (ref. 17), which are present in several smooth muscle gene promoters. Chromatin immunoprecipitation (ChIP) assays showed that suppression of the differentiated phenotype by PDGF was accompanied by an increase in the association of Elk-1 with SRF-binding sites in the promoters of the *SM22* and *SM-α-actin* genes, prototypical smooth muscle target genes of myocardin and SRF[18–20] (lanes 4 and 5 in Fig. 1d; Supplementary Fig. 2). The PDGF-dependent recruitment of Elk-1 to these SRF-binding sites was accompanied by the disappearance of myocardin from the same sites (lanes 1 and 2 in Fig. 1d; Supplementary Fig. 2).

Inhibition by U0126 of the MAP kinases MEK-1/2 blocked the phosphorylation of Elk-1 and the suppressive effects of PDGF on smooth muscle gene expression (Fig. 1a; Supplementary Fig. 1). U0126 also prevented the signal-dependent association of Elk-1 with SRF and the displacement of myocardin (Fig. 1b–d). Thus, modulation by PDGF of the differentiated SM cell phenotype was accompanied by the exchange of Elk-1 for myocardin on SRF in the control regions of native myocardin target genes; MEK-1/2, which phosphorylates ERK-1/2, which in turn phosphorylates Elk-1 (refs 13, 21), was a key effector in this process.

Mutation of Val 194 to Glu in the DNA-binding domain of SRF abolishes association with TCFs[22], and prevented ternary complex formation with myocardin (Fig. 2a), suggesting that these cofactors interact with the same region of SRF as has been observed for the myocardin-related transcription factor MAL[23]. Indeed, co-incubation of Elk-1 and SRF with increasing amounts of myocardin resulted in a loss of the SRF–Elk-1 complex on the SRF-binding site within the *SM22* promoter and the appearance of the SRF–myocardin complex (Fig. 2b, compare lanes 1–4 and 6–9). This competition required the basic region of myocardin (lane 10), which is essential for interaction with SRF (lane 5). Conversely, increasing the amount of Elk-1 in the DNA-binding assay resulted in the disappearance of the SRF–myocardin complex (Fig. 2b, compare lanes 11–14 and 16–19). Mutation of the TCF-binding site adjacent to the CArG box in the DNA probe did not affect the association of myocardin with SRF, but it impaired the ability of Elk-1 to compete with myocardin for interaction with SRF (Supplementary Fig. 3a).

TCFs interact with SRF through a B-box domain[17]. Replacement of Tyr 159 in the Elk-1 B-box with Ala abolishes interaction with SRF (lane 15 in Fig. 2b; ref. 24). This Elk-1 mutant (Y159A) was ineffective in displacing myocardin from SRF (lane 20 in Fig. 2b). Similar competition between myocardin and Elk-1 was observed


©2004 Nature Publishing Group

with a DNA probe corresponding to the serum response element from the *c-fos* promoter (Supplementary Fig. 3b, c).

There is no significant primary amino-acid homology between the SRF-binding region of myocardin and the B-box of Elk-1, but a stretch of amino acids in this region of myocardin resembles the α-helix-β-sheet-α-helix secondary structure of the B-box (Fig. 2c). Four hydrophobic amino acids, Tyr 153, Tyr 159, Phe 162 and Ile 164, in the Elk-1 B-box are critical for SRF binding[22,24] (Fig. 2c). In an effort to disrupt potential side-chain interactions between myocardin and SRF, while preserving the overall structure of the region, we replaced every hydrophobic residue in the above region of myocardin with Ala. Mutation of Leu 286 severely impaired the association of myocardin with SRF, as did Ala substitutions at positions 293 and 297 or a Pro substitution at position 291 (Fig. 2c, d). The ability of myocardin mutants to interact with SRF was roughly paralleled by their ability to activate the *SM22* promoter (Fig. 2e) and endogenous smooth muscle genes in transfected 10T1/2 cells (Supplementary Fig. 4).

A myocardin mutant in which the 21-amino-acid B-box-like region was replaced with the B-box of Elk-1 (Myo-Elk-B) formed a ternary complex with SRF (Fig. 2d) and activated smooth muscle gene expression (Fig. 2e; Supplementary Fig. 4). Replacement of the critical tyrosine with alanine (mutant Myo-Elk-Y159A) abolished myogenic activity of this mutant. These results suggest that myocardin, like Elk-1 (refs 24, 25), uses a short hydrophobic region to associate with a common docking site on SRF.

In contrast to myocardin, Elk-1 alone had no effect on the *SM22* promoter (Fig. 3a). However, Elk-1 could be converted to an activator of the *SM22* promoter by fusion to the transcription activation domain (TAD) of VP16 (Fig. 3a). The activity of this Elk-1–VP16 mutant was impaired by a mutation of the TCF site in the *SM22* promoter (Fig. 3a). Thus, Elk-1 associates with the TCF site in the *SM22* promoter, but it is an ineffective activator of this SRF target gene. In contrast, the activity of myocardin was enhanced on the promoter with a TCF-site mutation, consistent with the notion that endogenous TCFs interfere with myocardin activity (Fig. 3a; Supplementary Fig. 3d).

Myocardin was less effective in activating the *SM22* promoter when cells were stimulated by 20% serum than by 0.5% serum. The reduction in its transcriptional activity in response to high serum

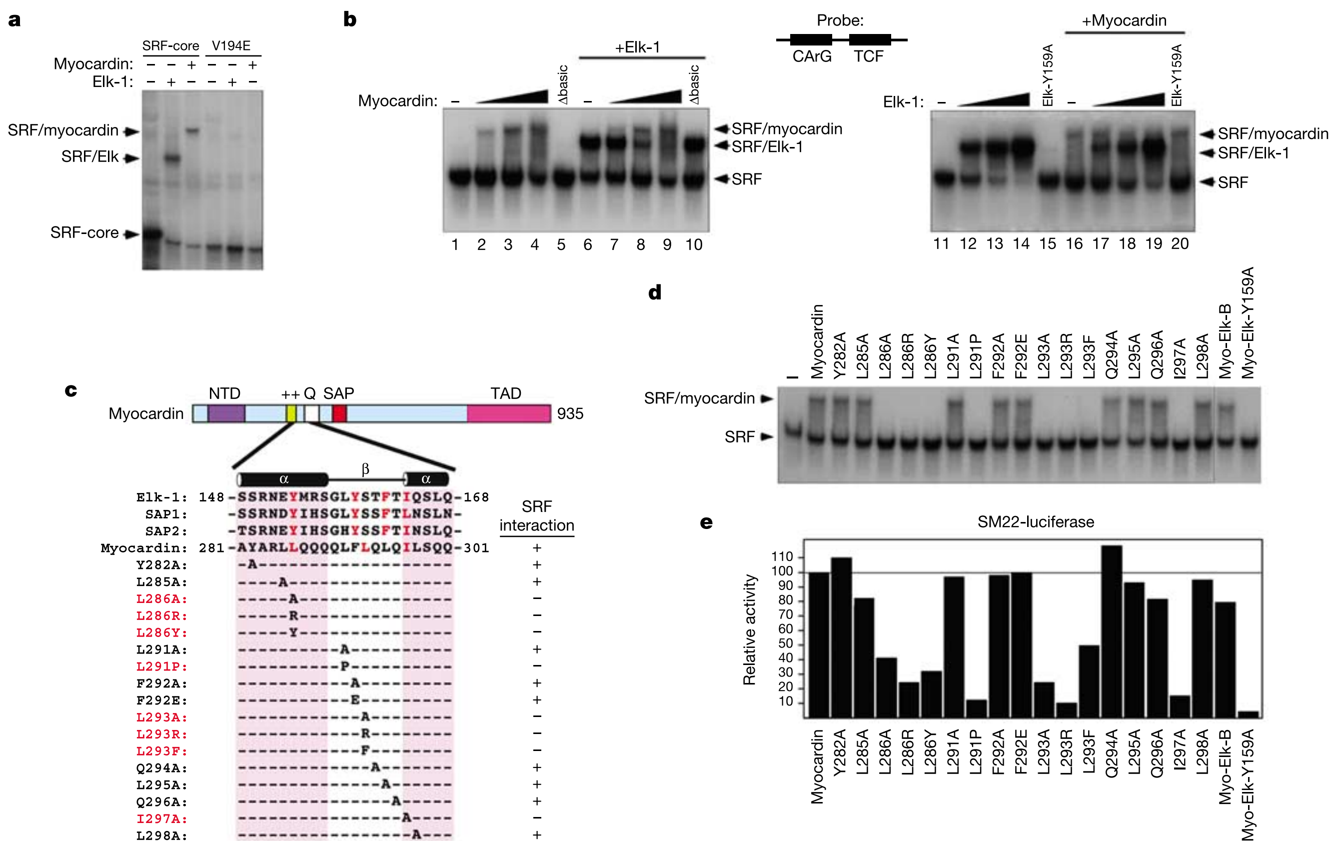


**Figure 2** Competition between Elk-1 and myocardin for interaction with SRF. **a**, Gel mobility shift assays were performed with a radiolabelled probe corresponding to the *c-fos* serum response element. SRF core region, the SRF core with a valine to glutamic acid mutation at position 194 (V194E), Elk-1 and myocardin were translated *in vitro*. Only the region of the gel containing the shifted probe is shown. **b**, Gel mobility shift assays were performed as in **a** using a probe corresponding to the distal CArG box from the *SM22* promoter. Left panel, increasing amounts of myocardin (0.5 μl, 2 μl or 5 μl) in the absence (lanes 2–5) or presence (lanes 7–10) of a constant amount of Elk-1 (0.3 μl) were used. Right panel, increasing amounts of Elk-1 (0.1 μl, 0.3 μl or 1 μl) in the absence (lanes 12–15) or presence (lanes 17–20) of a constant amount of myocardin (3 μl) were used. **c**, Schematic diagram of myocardin showing the SRF-interacting region. B-box regions of TCFs and the corresponding region of myocardin and myocardin mutants are shown in pink shading. Residues in red are required for SRF interaction. The amino-terminal domain (NTD), basic region (++) and the glutamine-rich domain (Q) are shown. **d**, Gel mobility shift assays were performed with SRF, myocardin, or the indicated myocardin mutants translated *in vitro* and a radiolabelled probe corresponding to the *c-fos* serum response element. The region of the gel containing shifted probe is shown. **e**, COS cells were transiently transfected with expression vectors for myocardin or the indicated myocardin mutants and an *SM22*-luciferase reporter. Values are expressed as the level of luciferase activity with each mutant relative to that with myocardin, which was assigned a value of 100.


©2004 Nature Publishing Group

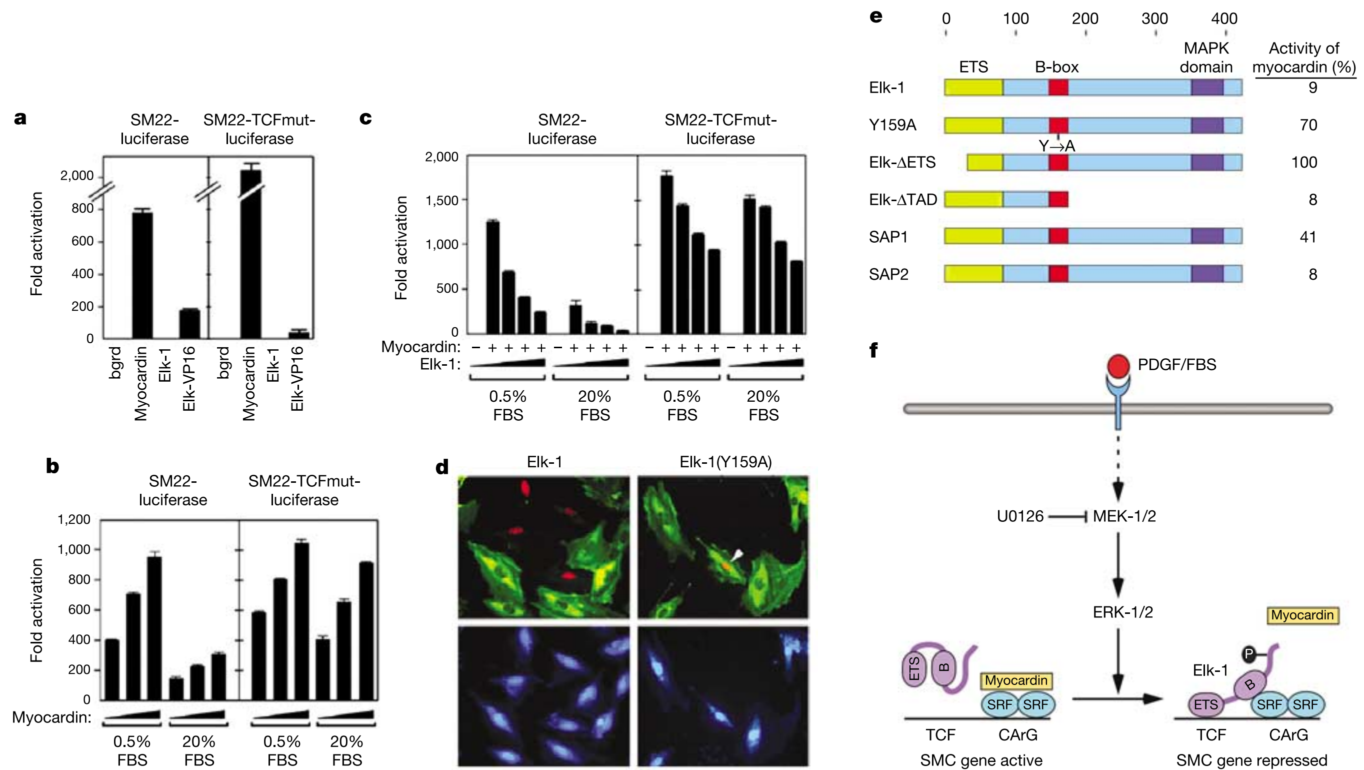


**Figure 3** Suppression of smooth muscle gene expression by Elk-1. **a**, COS cells were transiently transfected with expression vectors for myocardin, Elk-1 or Elk-1-VP16 and luciferase reporters linked to the wild-type *SM22* promoter or the promoter with a mutation in the TCF site. Values are expressed as fold activation above background. **b**, COS cells were transfected with myocardin expression vector and the indicated reporters and maintained in 0.5% FBS or stimulated with 20% FBS for 6 h. **c**, COS cells were transfected with expression vectors encoding myocardin and increasing amounts of Elk-1 and the indicated reporters and cultured under the indicated conditions. **d**, A7r5 cells were transfected with expression plasmids encoding Elk-1 or Elk-1(Y159A) with Myc-epitope tags. Upper panels, cultures were stained for SM-α-actin (green) and Myc (red). Note that cells expressing Elk-1 fail to express SM-α-actin (top left), whereas expression of Elk-1(Y159A) does not alter SM-α-actin expression (top right). Lower panels, Hoechst staining was used to detect nuclei. **e**, Mapping the regions of Elk-1 required for suppression of myocardin activity in transfected 10T1/2 cells. 10T1/2 cells were transfected with expression plasmids encoding myocardin and the indicated mutants of Elk-1. The relative number of SM-MHC-positive cells in each culture is expressed relative to the number in cultures transfected with myocardin alone, which was assigned a value of 100. Amino acids in Elk-1 are indicated along the top. **f**, A model to account for the modulation of smooth muscle genes by competition between myocardin and Elk-1 for SRF in response to growth signals.

was dependent on the TCF site (Fig. 3b) and was coupled to MEK-1/2-dependent phosphorylation of ERK-1/2, which stimulates the interaction of Elk-1 with SRF (Supplementary Fig. 5). Conversely, exogenous Elk-1 was a more effective repressor of the *SM22* promoter in the presence of 20% serum, and the TCF site enhanced its repressive activity (Fig. 3c). The Y159A mutant and an Elk-1 mutant lacking the ETS domain (Elk-1-ΔETS), which mediates binding to the TCF sequence, were ineffective inhibitors of myocardin (Supplementary Fig. 6a). Elk-1 showed similar repressive activity against a reporter linked to three tandem copies of a CArG box and adjacent TCF site (Supplementary Fig. 6b), confirming that its effects are dependent on SRF rather than another regulator.

Elk-1, as well as SAP-1 and -2, suppressed activation of smooth muscle genes by myocardin in 10T1/2 cells and in the A7r5 smooth muscle cell line, whereas the Y159A mutant was ineffective (Fig. 3d, e; Supplementary Fig. 6c–e). Mutants of Elk-1 lacking either the ETS domain (Elk-ΔETS) or a functional B-box were unable to interfere with myocardin activity (Fig. 3e). On the contrary, an Elk-1 mutant lacking the TAD was fully functional in suppressing myocardin activity (Fig. 3e, mutant Elk-ΔTAD).

To test whether TCFs modulate smooth muscle gene expression *in vivo*, we generated transgenic mouse embryos harbouring a *lacZ* transgene linked to the wild-type *SM22* promoter or the promoter with a mutation in the TCF site. Both promoters directed high expression of *lacZ* in the developing vasculature (Fig. 4a). The wild-type *SM22* promoter and the endogenous *SM22* gene[26], like many smooth muscle genes, are also expressed in the heart until embryonic day (E)13.5. In contrast, the mutant *SM22* promoter was still expressed at high levels in the heart at E15.5, at least two days after cardiac expression of the wild-type promoter had been extinguished (Fig. 4a). Immunostaining of cardiac sections with an anti-phospho-Elk-1 antibody showed the presence of phospho-Elk-1 in cardiomyocytes at this stage, where it could serve to repress transcription of SRF-dependent smooth muscle genes (Fig. 4b). Thus, the TCF site is required for the precise temporal control of this smooth muscle promoter in the developing heart at a time when phospho-Elk-1 is expressed there.

Our results demonstrate that myocardin and Elk-1 compete for interaction with a common docking site on SRF and that Elk-1 acts as a signal-responsive repressor of smooth muscle gene expression by displacing myocardin from SRF within the context of native chromatin (Fig. 3f). The repressive influence of Elk-1 on smooth muscle genes is unexpected in the light of its role as an activator of growth-factor-inducible genes[9]. We propose that Elk-1, which is a weak activator compared with myocardin[27], is sufficient to suppress smooth muscle gene expression when bound to even a single SRF-binding site, owing to the dependence of myocardin on


©2004 Nature Publishing Group

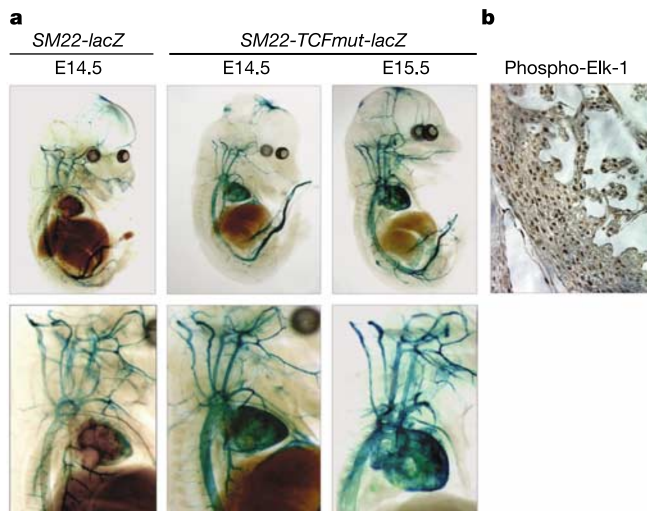


**Figure 4** Ectopic expression of a *lacZ* reporter controlled by an *SM22* promoter lacking the TCF site. **a**, Transgenic mouse embryos of the indicated ages were stained for *LacZ* expression. The transgene with the mutation in the TCF site continues to be expressed in the heart after the wild-type promoter has been downregulated. **b**, Staining of a histological section of an E15.5 mouse heart with anti-phospho-Elk-1 antibody reveals the presence of phospho-Elk-1 in cardiomyocytes.

binding to multiple sites for maximal transcriptional activation[3,28]. The mutually exclusive association of SRF with myocardin and Elk-1 (or other TCFs) provides a mechanism for modulating smooth muscle gene expression during development and in response to growth signalling. □

## Methods

### Cell culture and transfection

A7r5 cells were maintained as described previously[16]. Cells were treated with 20 ng $ml^{-1}$ PDGF-BB (Sigma) for 24 h and, when indicated, were pretreated with 10 μM U0126 (Cell Signalling) for 2 h before PDGF-BB treatment. Transfections and luciferase assays were performed as described[3,28]. Myocardin mutants were generated through polymerase chain reaction (PCR)-based mutagenesis using the QuickChange kit from Stratagene. The *SM22*-luciferase reporter contained the 1,434 base pair promoter[18] and the mutant promoter contained a mutation in the TCF site.

### ChIP and IP assays

ChIP assays were performed using the ChIP Assay Kit from Upstate Biotechnology as described in ref. 29. Immunoprecipitation assays were performed essentially as ChIP assays, except the immunoprecipitation products were separated on SDS–PAGE and analysed by western blot with anti-SRF antibody. Affinity-purified rabbit anti-myocardin antibody was generated against amino acids 141–159. Rabbit anti-Elk-1 antibodies were from Santa Cruz and Cell Signalling. Further details are in Supplementary Information.

### Analysis of SM and 10T1/2 cells

Immunocytochemistry, myogenic assays, western blots, and reverse transcription (RT)–PCR were performed as described[7].

### Gel mobility shift assays

SRF, myocardin, and Elk-1 were translated *in vitro* with a TNT T7-coupled reticulocyte lysate system (Promega). Gel mobility shift assays were performed using double-stranded probes as described (ref. 3; Supplementary Information). The sequences of the distal *SM22*-CArG box probes are shown below. The CArG box is in bold and the TCF site is italicized. Wild-type: AGCTGTTTCAGGGTCCTGC**CCATAAAAGG**TTT***TTCC***CGG CCGCC; the TCF mutant: AGCTGTTTCAGGGTCCTGC**CCATAAAAGG**TTT***TTAA*** CGGCCGCC.

### Histology

Generation of transgenic mice and LacZ staining were performed as described[18]. Paraffin sections of E15.5 mouse hearts were stained with anti-phospho-Elk-1 antibody according to manufacturer's instructions (Cell Signalling).

Received 17 December 2003; accepted 26 January 2004; doi:10.1038/nature02382.


1. Owens, G. K. Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75,** 487–517 (1995).
2. Chang, D. F. *et al.* Cysteine-rich LIM-only proteins CRP1 and CRP2 are potent smooth muscle differentiation cofactors. *Dev. Cell* **4,** 107–118 (2003).
3. Wang, D. *et al.* Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* **105,** 851–862 (2001).
4. Du, K. L. *et al.* Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. *Mol. Cell. Biol.* **23,** 2425–2437 (2003).
5. Chen, J., Kitchen, C. M., Streb, J. W. & Miano, J. M. Myocardin: a component of a molecular switch for smooth muscle differentiation. *J. Mol. Cell. Cardiol.* **34,** 1345–1356 (2002).
6. Yoshida, T. *et al.* Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. *Circ. Res.* **92,** 856–864 (2003).
7. Wang, Z., Wang, D. Z., Pipes, G. C. & Olson, E. N. Myocardin is a master regulator of smooth muscle gene expression. *Proc. Natl Acad. Sci. USA* **100,** 7129–7134 (2003).
8. Li, S., Wang, D. Z., Wang, Z., Richardson, J. A. & Olson, E. N. The serum response factor coactivator myocardin is required for vascular smooth muscle development. *Proc. Natl Acad. Sci. USA* **100,** 9366–9370 (2003).
9. Treisman, R. Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.* **4,** 96–101 (1994).
10. Miano, J. Serum response factor: toggling between disparate programs of gene expression. *J. Mol. Cell. Cardiol.* **35,** 577–593 (2003).
11. Shaw, P. E., Schroter, H. & Nordheim, A. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. *Cell* **56,** 563–572 (1989).
12. Giovane, A., Pintzas, A., Maira, S. M., Sobieszczuk, P. & Wasylyk, B. Net, a new ets transcription factor that is activated by Ras. *Genes Dev.* **8,** 1502–1513 (1994).
13. Sharrocks, A. D. Complexities in ETS-domain transcription factor function and regulation: lessons from the TCF (ternary complex factor) subfamily. The Colworth Medal Lecture. *Biochem. Soc. Trans.* **30,** 1–9 (2002).
14. Janknecht, R., Ernst, W. H., Pingoud, V. & Nordheim, A. Activation of ternary complex factor Elk-1 by MAP kinases. *EMBO J.* **12,** 5097–5104 (1993).
15. Marais, R., Wynne, J. & Treisman, R. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73,** 381–393 (1993).
16. Holycross, B. J., Blank, R. S., Thompson, M. M., Peach, M. J. & Owens, G. K. Platelet-derived growth factor-BB-induced suppression of smooth muscle cell differentiation. *Circ. Res.* **71,** 1525–1532 (1992).
17. Shore, P. *et al.* Determinants of DNA-binding specificity of ETS-domain transcription factors. *Mol. Cell. Biol.* **16,** 3338–3349 (1996).
18. Li, L., Liu, Z., Mercer, B., Overbeek, P. & Olson, E. N. Evidence for serum response factor-mediated regulatory networks governing SM22alpha transcription in smooth, skeletal, and cardiac muscle cells. *Dev. Biol.* **187,** 311–321 (1997).
19. Kim, S., Ip, H. S., Lu, M. M., Clendenin, C. & Parmacek, M. S. A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. *Mol. Cell. Biol.* **17,** 2266–2278 (1997).
20. Mack, C. P. & Owens, G. K. Regulation of smooth muscle alpha-actin expression *in vivo* is dependent on CArG elements within the 5′ and first intron promoter regions. *Circ. Res.* **84,** 852–861 (1999).
21. Heldin, C. H., Ostman, A. & Ronnstrand, L. Signal transduction via platelet-derived growth factor receptors. *Biochim. Biophys. Acta* **1378,** F79–113 (1998).
22. Ling, Y., West, A. G., Roberts, E. C., Lakey, J. H. & Sharrocks, A. D. Interaction of transcription factors with serum response factor. Identification of the Elk-1 binding surface. *J. Biol. Chem.* **273,** 10506–10514 (1998).
23. Miralles, F., Posern, G., Zaromytidou, A. I. & Treisman, R. Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113,** 329–342 (2003).
24. Ling, Y., Lakey, J. H., Roberts, C. E. & Sharrocks, A. D. Molecular characterization of the B-box protein-protein interaction motif of the ETS-domain transcription factor Elk-1. *EMBO J.* **16,** 2431–2440 (1997).
25. Hassler, M. & Richmond, T. J. The B-box dominates SAP-1-SRF interactions in the structure of the ternary complex. *EMBO J.* **20,** 3018–3028 (2001).
26. Li, L., Miano, J. M., Cserjesi, P. & Olson, E. N. SM22, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ. Res.* **78,** 188–195 (1996).
27. Cen, B. *et al.* Megakaryoblastic leukemia 1, a potent transcriptional activator for serum response factor (SRF), is required for serum induction of SRF target genes. *Mol. Cell. Biol.* **23,** 6597–6608 (2003).
28. Wang, D. Z. *et al.* Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc. Natl Acad. Sci. USA* **99,** 14855–14860 (2002).
29. Qiu, P. & Li, L. Histone acetylation and recruitment of serum responsive factor and CREB-binding protein onto SM22 promoter during SM22 gene expression. *Circ. Res.* **90,** 858–865 (2002).


**Supplementary Information** accompanies the paper on **www.nature.com/nature**.

**Acknowledgements** We thank A. Sharrocks, M. Parmacek and L. Schuger for reagents, A. Tizenor for graphics, J. Page for editorial assistance, L. Sutherland for technical assistance and R. Bassel-Duby, L. Li, M. Tallquist and H. Yanagisawa for discussions. This work was supported by grants from the NIH, the McGowan Foundation, the Donald W. Reynolds Foundation, and the Robert A. Welch Foundation to E.N.O., from the Muscular Dystrophy Association (MDA) and NIH to D.-Z.W., and by the DFG to A.N.

**Competing interests statement** The authors declare that they have no competing financial interests.

**Correspondence** and requests for materials should be addressed to E.N.O. (Eric.Olson@utsouthwestern.edu).


©2004 Nature Publishing Group