The Transcription Factor Foxf1 Binds to Serum Response Factor and Myocardin to Regulate Gene Transcription in Visceral Smooth Muscle Cells*

April M. Hoggatt[‡], Ju-Ryoung Kim[‡], Vladimir Ustiyan[§], Xiaomeng Ren[§], Tanya V. Kalin[§], Vladimir V. Kalinichenko[§], and B. Paul Herring[‡1]

From the [‡]Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202 and the [§]Division of Pulmonary Biology, Perinatal Institute of Cincinnati Children's Research Foundation, Cincinnati, Ohio 45229

Background: The role of Foxf1 in smooth muscle development is unknown.
Results: Foxf1 binds to serum response factor and myocardin to regulate transcription and affect contractility of visceral smooth muscle cells.
Conclusion: Foxf1 is required for normal development of gastrointestinal smooth muscle.
Significance: Forkhead proteins interact with the SRF/myocardin axis to control the phenotype of smooth muscle cells.

Smooth muscle cells (SMCs) modulate their phenotype from a quiescent contractile state to a dedifferentiated, proliferative and migratory state during the pathogenesis of many diseases, including intestinal pseudoobstruction. Understanding how smooth muscle gene expression is regulated in these different phenotypic states is critical for unraveling the pathogenesis of these diseases. In the current study we examined the specific roles of Foxf1 in visceral SMC differentiation. Data show that Foxf1 is specifically required for expression of several contractile and regulatory proteins such as telokin, smooth muscle γ-actin, and Cav1.2b in visceral SMCs. Mechanistically, Foxf1 directly binds to and activates the telokin promoter. Foxf1 also directly binds to serum response factor (SRF) and myocardin-related transcription factors (MRTFs). Unlike Foxo4 and Foxq1, which bind to MRTFs and block their interaction with SRF, Foxf1 acts synergistically with these proteins to regulate telokin expression. Knock-out of Foxf1 specifically in SMCs results in neonatal lethality, with mice exhibiting GI tract abnormalities. Mice heterozygous for Foxf1 in SMC exhibited impaired colonic contractility and decreased expression of contractile proteins. These studies together with previous studies, suggest that different forkhead proteins can regulate gene expression in SMCs through modulating the activity of the SRF-myocardin axis to either promote or inhibit differentiation and proliferation thereby altering gastrointestinal contractility and development.

Smooth muscle cells (SMCs)[2] are the primary contractile components of cardiovascular, respiratory, genitourinary, and digestive systems. SMCs modulate their phenotype in response to extracellular cues during the development and progression of a variety of diseases including chronic intestinal pseudo-obstruction, atherosclerosis, hypertension, and asthma. These diseases are associated with decreased expression of proteins required for the normal contractile function of smooth muscle cells (1). Understanding the mechanisms that control expression of contractile and regulatory proteins in SMCs is, therefore, an essential step toward determining how these processes are altered in pathological conditions. Toward this goal numerous studies have characterized the transcription factors that control the expression of contractile proteins in SMCs (1–3). Of the factors currently identified, serum response factor (SRF) and SRF-associated factors, such as myocardin and myocardin-related transcription factor A (MRTFA), play a central role in the expression of many different smooth muscle-specific genes (4–6). Knock-out of SRF in gastrointestinal (GI) SMCs of adult mice attenuates expression of smooth muscle-specific genes and results in a chronic intestinal pseudo-obstruction (7, 8). Alteration of the SRF-myocardin axis occurs during, and is likely a contributing cause to, pathological smooth muscle remodeling in many diseases. For example, following partial obstruction of the intestine in mice, the intestine undergoes a two-phase adaptive response resulting in hyperplasia followed by hypertrophy of intestinal smooth muscle (9). These adaptive responses are associated with decreased expression of myocardin and SRF during the proliferative phase when SRF binding to the promoters of smooth muscle contractile protein genes is decreased. In contrast, during the hypertrophic phase myocardin expression is increased and more SRF is found at the promoters of contractile protein genes (9).

In vascular smooth muscle, the Foxo family of forkhead (Fox) transcription factors have been shown to play important roles in regulating the differentiation and function of smooth muscle cells (10–13). Foxo4 has been shown to inhibit vascular smooth muscle differentiation through its ability to bind and inhibit the activity of myocardin (13). Conversely, Foxo4 has also been shown to activate myocardin transcription in a heterologous system (12). Foxo4 can promote SMC migration through stim-

[1] To whom correspondence should be addressed: Department of Cellular and Integrative Physiology, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis IN, 46202. Tel.: 317-278-1785; Fax: 317-274-3318; E-mail: pherring@iupui.edu.

[2] The abbreviations used are: SMC, smooth muscle cells; SRF, serum response factor; MRTF, myocardin-related transcription factors.

ulating expression of MMP9 and Foxo4 knock-out mice have decreased neointima formation following vascular injury (14). In contrast, expression of a constitutively active Foxo3 inhibited neointima formation through its ability to inhibit SMC proliferation and promote apoptosis via activation of p27Kip1, proapoptotic genes, and inhibition of Cyr61 (10, 11, 15, 16). Similarly, Foxo1 has also been shown to increase expression of p27Kip1 and caspase 3, promoting apoptosis of vascular SMCs (17). Importantly the activity of the Foxo transcription factors is controlled by Akt-mediated phosphorylation, which results in their binding to 14-3-3 proteins and nuclear exclusion that attenuates their transcriptional activity. Signaling from the IGFI-R, PDGF-R, and TNFα-R through Akt to Foxo is thus important during the development of vascular diseases (11, 13, 17–19).

Other Fox family members have also been shown to impact smooth muscle proliferation and differentiation in the GI tract. For example, Foxm1 is critical for SMC proliferation during development such that mice harboring a smooth muscle-specific deletion of Foxm1 have decreased smooth muscle in the walls of their arteries and esophagus (20). Foxf1 heterozygous mice display abnormalities in lung and gallbladder development (21–24). The gallbladders of Foxf1$^{+/-}$ heterozygous mice are significantly smaller than normal and their external smooth muscle layer is absent (21), indicating that wild type levels of Foxf1 are required for the correct differentiation of smooth muscle in the gallbladder. Heterozygous deletion and point mutations in the *FOXF1* gene locus were recently found in 40% of patients with Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins (ACD/MPV), a pediatric congenital disorder, which is characterized by severe abnormalities in development of the gallbladder, lung, and GI tract (25). A second Foxf protein, Foxf2 also plays an important role in intestinal development (26). Numerous alterations in the intestinal epithelia occurred in Foxf1/f2 mutant mice as a result of the defective signaling from mesenchymal cells (26).

In the current study we examined the specific roles of Foxf1 in visceral SMC differentiation. Data show that Foxf1 is specifically required for expression of telokin, Cav1.2b and other smooth muscle contractile proteins in visceral SMCs. Mechanistically, Foxf1 directly binds to and activates the telokin promoter. Foxf1 also directly binds to SRF and myocardin-related transcription factors. Unlike Foxq1 which blocks the interaction of MRTFs and SRF, Foxf1 acts synergistically with these proteins to regulate telokin expression. Knock-out of Foxf1 specifically in SMCs *in vivo* in mice results in GI tract defects and impaired GI contractility.

EXPERIMENTAL PROCEDURES

Constructs—A Foxf1 mammalian expression construct was amplified by PCR from an HFH8 clone obtained from Robert Costa (27). This resulted in expression of a Foxf1 protein of 353 amino acids identical to that encoded by NM_010426.1 This clone was also used as a template for PCR to generate bacterial expression constructs in pGEX4T1 and pET28 as detailed in figure legends. Foxf2 was amplified by RT-PCR from mRNA isolated from mouse intestine. The encoded protein of 446 amino-acids is identical to that encoded by NM_010225.2. The

Foxq1 expression plasmid was described previously (28). SRF pBind and myocardin pACT mammalian two-hybrid plasmids, GST-SRF and GST-myocardin bacterial expression plasmids were described previously (29–32). GST-NT MRTFA (encoding amino acids 1–628), and GST-CT MRTFA (encoding amino acids 618–929) were generated by PCR amplification of MRTFA fragments from the MRTFA mammalian expression vector described previously (29). All expression constructs were confirmed by DNA sequencing. Luciferase reporter genes used were as described previously (33).

Primary Cell Culture—The colon and esophagus of four to 6 week old wild type, Foxf1$^{f/f}$, or Foxf2$^{f/f}$ mice were excised, cleaned of fatty tissue and external vasculature. Each organ was opened, the epithelial layer removed and the resulting smooth muscle layers minced and digested for ~30 min with 0.6 units of Liberase TM (Roche)/ml of HBSS for each organ. The digested mixture was strained through a 100 μM filter washed with 10% FCS-DMEM, and the cells plated in 6-well plates in 10% FCS-DMEM.

Adenoviral Transduction and Quantitative Real-time RT-PCR—For adenoviral transduction primary cultures of SMCs were trypsinized and replated into 12-well plates at 5×10^4 cells/well. The following day cells were transduced with adenovirus encoding Cre recombinase or YFP. 48 or 72 h later cells were lysed in Trizol and total mRNA prepared according to the manufacturer's directions. 0.5 μg of RNA was reverse transcribed to cDNA using random hexamer primers and MLV RT enzyme. Primers used for quantitative PCR were described previously (34, 35) together with primers for Foxf1: F-CAT ACC TTC ACC AAA ACA GTC ACA A, R-AAA CTC TCT GTC ACA CAT GCT, Foxf2: F-CCT ACT CGT TGG AGC AGA GCT ACT, R-GCA GTC CGA CTG AGA GAT CCT, BMP4: F-GCC AGC CGA CGA ACA, R-CTC ACT GGT CCC TGG GAT GT, Integrin β3: F-GTG GGA GGG CAG TCC TCT A, R-CAG GAT ATC AGG ACC CTT GG.

Cell Transfections—10T1/2 mouse fibroblast-like cells were cultured in 10%FCS-DMEM. Cells were transfected 16–18 h post plating at a 70–90% density in 12-well plates. 1 μg of high quality purified plasmid DNA was transfected with 2 μl of Fugene 6 (Roche) per well. Each well contained equal quantities of a mixture of minimal TK promoter *Renilla* reporter gene, the indicated firefly reporter plasmids and mammalian expression plasmids. 24–30 h post transfection, cells were lysed in 100 μl of Promega passive lysis buffer, 10 μl were assayed using a Promega dual luciferase kit.

Electrophoretic mobility shift assays, protein co-immunoprecipitation, GST pull-down assays and *in vitro* contractility of colonic rings were performed as described previously (28–30, 34).

Mouse Strains—Mice harboring either Foxf1 or Foxf2 floxed alleles were generated at Cincinnati Children's Hospital Medical Center using a standard gene targeting technique described previously (36). LoxP sites surrounded the first exons, which contain the DNA binding domains of the Foxf1 and Foxf2 genes. Foxf1$^{f/f}$ female mice were bred with smMHC-Cre-GFP$^{tg/-}$ male mice (obtained from Dr. Kotlikoff, Cornell University (37)) to generate smMHC-Cre-GFP$^{tg/-}$ Foxf1$^{f/f}$ double transgenic mice (smooth muscle-specific *Foxf1* knock-out mice
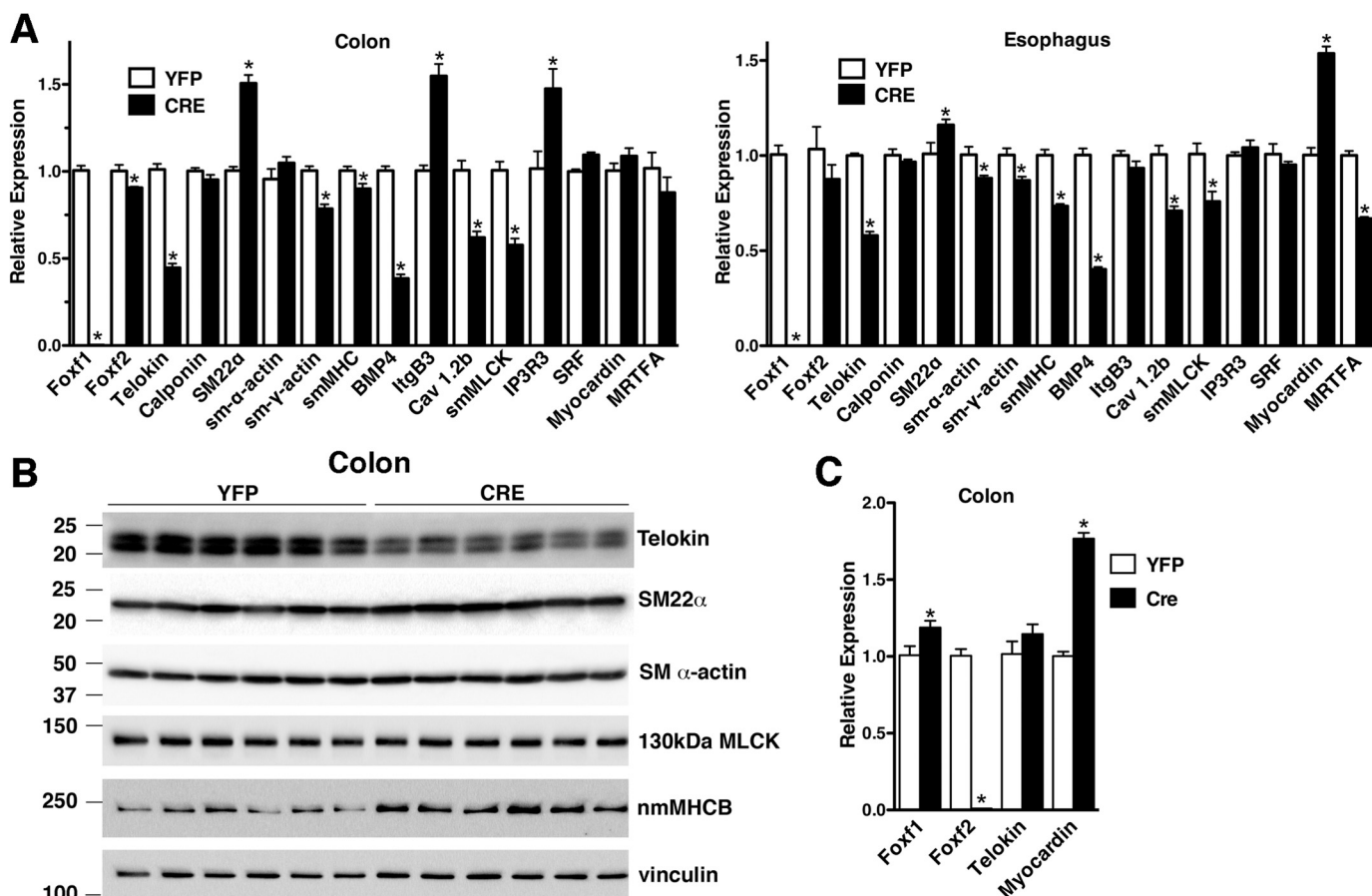
FIGURE 1. **Foxf1 knockdown decreases telokin expression.** *A* and *B*, primary smooth muscle cells from colon and esophagus of Foxf1$^{f/f}$ mice were transduced with either Cre-expressing adenovirus or YFP-expressing control virus. RNA and protein lysates were harvested 48 h post-transduction. *A*, qRT-PCR was performed to detect endogenous mRNAs in cells expressing Cre (*black bars*) or YFP (*white bars*). Transcript levels were first normalized to an hprt internal loading control and then samples from Cre-transduced cells were expressed relative to samples obtained from control YFP-transduced cells. Relative expression $= 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{Cre} - Ct_{hprt}) - (Ct_{yfp} - Ct_{hprt})$. Each column represents the mean $\pm$ S.E. of 6–22 samples obtained from 1–3 different cell preparations (*, $p < 0.05$). Foxf1 mRNA expression in Cre-transduced cells was undetectable in the primary SMCs demonstrating efficient knock-out of Foxf1. *B*, Western blot analysis of protein lysates of parallel plates of transduced SMC from colon. *C*, primary smooth muscle cells from colon of Foxf2$^{f/f}$ mice were transduced with either Cre virus or YFP control virus and mRNA expression analyzed as described in *A*.
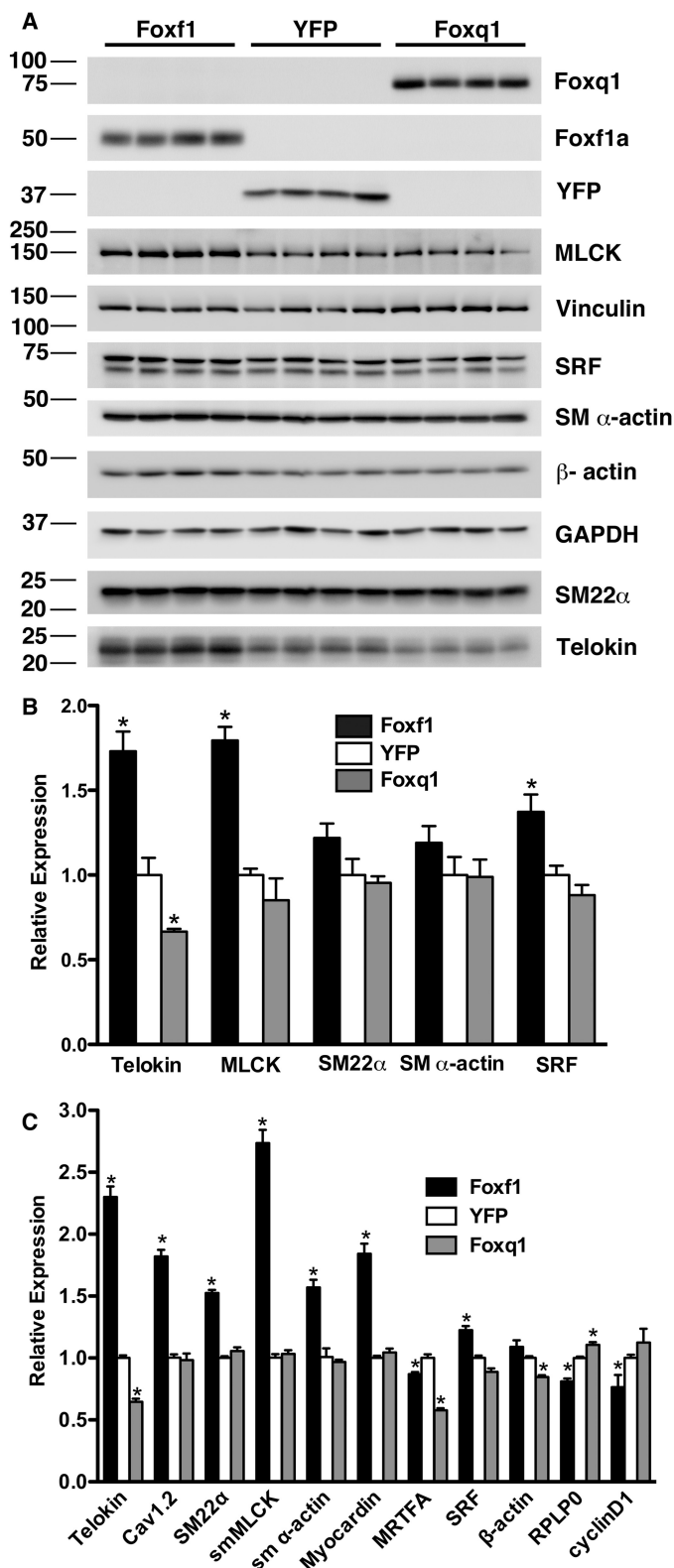
or smFoxf1$^{-/-}$) and smMHC-Cre-GFP$^{tg/-}$ Foxf1$^{f/+}$ (<u>s</u>mooth <u>m</u>uscle-specific Foxf1 heterozygous mice or smFoxf1$^{+/-}$). Animal studies were reviewed and approved by the Animal Care and Use Committee of Cincinnati Children's Hospital Research Foundation.

RESULTS

Foxf1 Is Required for Contractile Protein Expression in Primary Cultures of Smooth Muscle Cells—To determine the importance of Foxf1 in regulating expression of genes in visceral SMCs, Foxf1 was knocked out in primary cultures of SMCs isolated from the colon and esophagus of Foxf1$^{f/f}$ mice by transduction with adenoviral expressed Cre recombinase (Fig. 1). Expression of a known Foxf1 target gene, BMP4, was attenuated in both SMC types. Knock-out of Foxf1 also attenuated expression of several markers of highly differentiated SMCs including telokin, the voltage-gated calcium channel α-subunit (Cav 1.2b), sm γ-actin, and smMHC (Fig. 1*A*). The attenuated telokin expression was also confirmed at the protein level in colon SMCs (Fig. 1*B*). In contrast, markers of more immature SMCs such as SM22α and SM α-actin were less affected by knock-out of Foxf1 with SM22α actually increasing a little (Fig.

1*A*). We also observed a small increase in myocardin expression and decrease in MRTFA expression in the knock-out esophagus cells but not colon cells (Fig. 1). There was no compensatory up-regulation of Foxf2 observed following Foxf1 knock-out suggesting that the observed changes were a specific consequence of loss of Foxf1. In further support of the specific requirement for Foxf1 there was no change in telokin expression in Foxf2 knock-out cells, although increased myocardin expression was seen following knockdown of Foxf2 in colon cells (Fig. 1*C*). Together these data suggest that Foxf1 is specifically required for expression of telokin and several other genes important for regulating contractility of GI SMCs. Similar to several other transcription factors, including SRF, overexpression of Foxf1 alone is not sufficient to induce telokin expression in 10T1/2 fibroblast cells that do not express endogenous telokin (data not shown). Overexpression of Foxf1 is, however, sufficient to increase endogenous telokin expression in telokin-expressing colon SMCs (Fig. 2). Overexpression of Foxf1 (about 30–40-fold overexpression) in colon SMCs also increased expression of smMLCK and to a lesser extent, Cav1.2, SM22α, sm α-actin, myocardin and SRF (Fig. 2). In contrast to these stimulatory effects of Foxf1, Foxq1, which

has been previously shown to repress telokin promoter activity (28), also repressed endogenous telokin expression without affecting the expression of most other smooth muscle contractile proteins (Fig. 2).

Foxf1 and SRF Synergistically Activate the Telokin Promoter— To verify that Foxf1 directly regulates transcription of the telokin gene, luciferase reporter assays were performed. A Foxf1 expression plasmid was transiently transfected into 10T1/2 cells with and without telokin, smMHC, smooth muscle α-actin, SM22α or thymidine kinase promoter-luciferase reporter plasmids as well as a *Renilla* internal control reporter gene. 10T1/2 fibroblast cells express no detectable endogenous Foxf1 mRNA (data not shown). Foxf1 overexpression increased telokin promoter reporter activity 2-fold and triggered a modest, but statistically significant decrease in smMHC promoter activity and a larger decrease in SM22α promoter activity (Fig. 3A). Foxf1 expression did not affect smooth muscle α-actin, or thymidine kinase promoter activity. These data are generally consistent with the knock-out studies that showed decreased telokin but increased SM22α expression following loss of Foxf1. A reporter gene harboring a mutation in the forkhead site of the telokin promoter was no longer activated by Foxf1 (Fig. 3B). As serum response factor and myocardin related transcription factors (MRTFs) are critical for regulating telokin promoter activity and previous studies have also shown that other Fox proteins can modulate SRF and myocardin activity we next sought to determine the relationship between Foxf1 and the SRF/MRTF axis. Using luciferase reporter assays we found that Foxf1 synergized with SRF to activate the telokin promoter (Fig. 3C). Analysis of SRF protein expression in parallel transfection replicates confirmed that Foxf1 did not significantly increase SRF expression (data not shown). Parallel experiments examining synergy between myocardin or MRTFA and Foxf1 were unsuccessful as we observed dramatic nonspecific effects of Foxf1 on exogenous myocardin and MRTFA expression. This complication together with the very low, to undetectable levels of myocardin and MRTFA required to avoid saturating the assay prevented us from unambiguously interpreting these experiments. To determine if Foxf1 affected the binding of myocardin to SRF we performed a mammalian two-hybrid assay in the presence or absence of Foxf1. Results of this assay demonstrated that Foxf1 promotes, rather than inhibits, the binding of myocardin to SRF. In contrast Foxq1 inhibited this interaction (Fig. 3D).

Foxf1 Binds to the Forkhead Binding Site in the Telokin Promoter—Using electrophoretic mobility shift assays we verified that Foxf1 can bind to the forkhead binding site in the telokin promoter and that the mutation characterized in the reporter assays abrogated binding (Fig. 4A, *mutant 10A-G*). Although Foxf1 and Foxq1 bind to the same region of the telokin promoter, they have opposing effects on promoter activity ((28), Fig. 3). To determine if this is due to specific binding differences, sequential point mutations were made in the probe used as an unlabeled competitor for electrophoretic
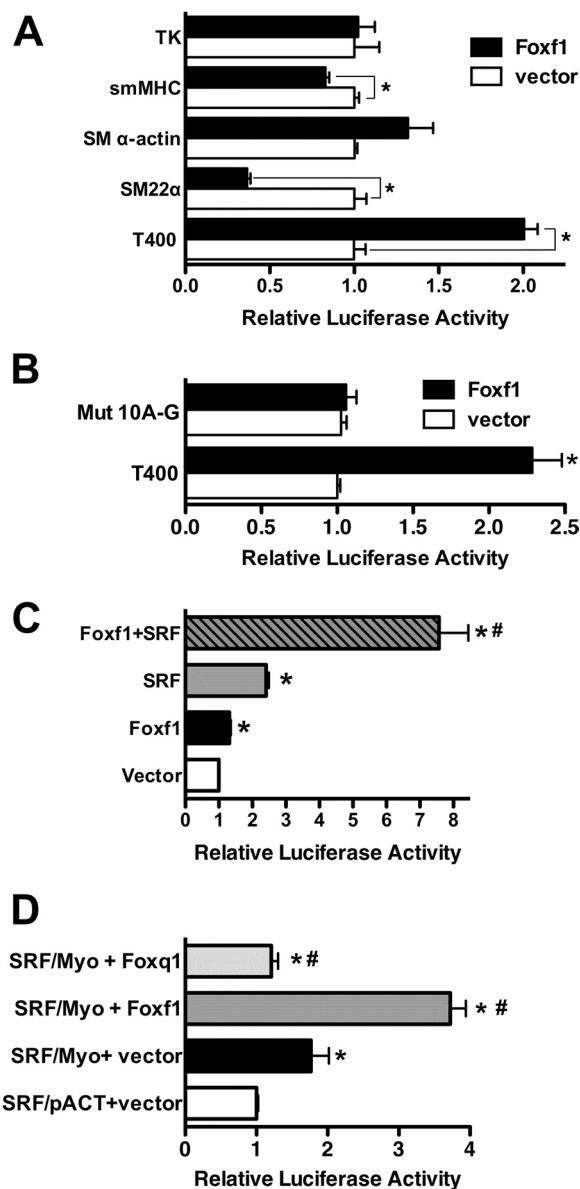
FIGURE 2. **Foxf1 increases and Foxq1 inhibits telokin protein expression in primary colon SMCs.** *A*, protein lysates from mouse colon SMCs transduced with Foxf1-HA, Foxq1-HA, or YFP-HA adenovirus were analyzed by Western blotting with the antibodies indicated. The blots shown are representative of three separate experiments using different SMC preparations, with each experiment including 4–6 independently transduced samples. *B*, Western blots were quantitated using Gene Tools (Syngene). Signals were normalized to GAPDH as an internal loading control. Data presented are the mean ± S.E. Statistical significance was evaluated using the nonpaired Stu-

dent's *t* test. * indicates $p < 0.05$. *C*, qRT-PCR analysis of parallel samples to those described in *panels A* and *B*. Data were normalized to an hprt internal control and are expressed relative to YFP control samples. Relative expression = $2^{-\Delta\Delta Ct}$. Data presented are the mean ± S.E. Statistical significance was evaluated from 4–8 samples using the nonpaired Student's *t* test. * indicates $p < 0.05$.

A

B

C

D

FIGURE 3. Foxf1 and SRF synergistically activate the telokin promoter and Foxf1 promotes SRF-myocardin binding. *A*, 10T1/2 cells were transiently transfected with a thymidine kinase (*TK*), smooth muscle myosin heavy chain (*smMHC*), smooth muscle α-actin (*SM-α actin*), SM22α or telokin (T400) promoter firefly luciferase reporter plasmids, and a minimal thymidine kinase *Renilla* luciferase control plasmid together with Foxf1 expression vector (0.5 μg) or empty expression vector (0.5 μg) control (vector). 24 h post-transfection cells were lysed and assayed for luciferase activity. Luciferase activity was first normalized to the internal control plasmid, then presented as fold change of mean luciferase activity $\pm$ S.E. in the presence of Foxf1 compared with activity with empty vector. $n = 6$, *, $p < 0.05$. *B*, 10T1/2 cells were transiently transfected with wild type telokin (T400) or Mut10A-G telokin promoter firefly luciferase reporter plasmids and a minimal thymidine kinase renilla luciferase control plasmid together with Foxf1 expression vector or empty expression vector control (vector). Luciferase activity was measured as described in *panel A*. Data shown are the mean $\pm$ S.E. of 16 samples, *, $p < 0.05$. *C*, 10T1/2 cells were transfected with a telokin promoter reporter gene (T400) and internal control TK-*Renilla* reporter gene together with expression plasmids encoding SRF (0.25 μg) and Foxf1(0.25 μg) as indicated. Empty expression vector was also added where appropriate to maintain a constant amount of total DNA. Luciferase activities were normalized to the internal control and expressed relative to the activity obtained from empty expression vector transfections. Data shown are the mean $\pm$ S.E. from 12 samples. *, $p < 0.05$ compared with vector control. #, $p < 0.05$ compared with SRF alone. *D*, mammalian two-hybrid assay using SRF fused to the GAL5 DNA binding domain and myocardin fused to the VP16 activation domain in the presence of Foxf1 or empty vector. Data shown

A

CGATCTGCAGTTGC$\overline{\text{CTTTATATAAACTATC}}$$\boxed{\text{CCTTTTACCC}}$C
1   14   CArG

B

C
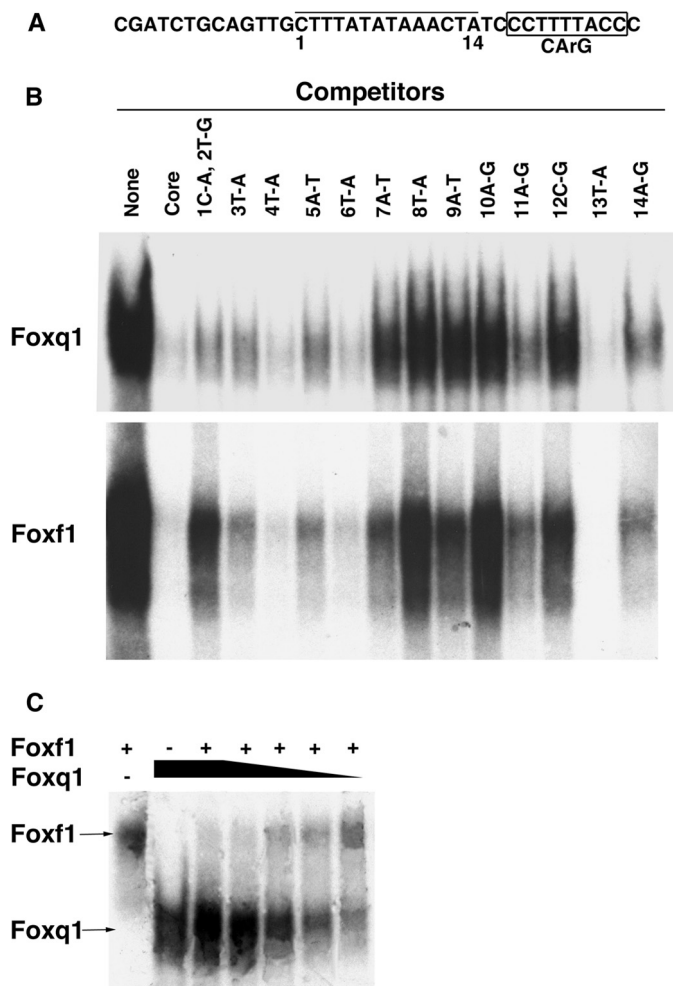
FIGURE 4. Foxf1 and Foxq1 require the same nucleotides within the AT-rich region of the telokin promoter for binding. *A*, sequence of the P^{32}-labeled telokin promoter probe used for gel mobility shift assays. *B*, labeled probe was incubated for 20 min with either Foxf1 or Foxq1 protein lysates followed by an additional incubation with a 200-fold excess of either no probe (*None*), an unlabeled oligo identical to the core (*Core*) or to unlabeled probes with the indicated mutations (numbering refers to the *overlined region* in panel A. *C*, P^{32}-labeled telokin core promoter probe was incubated for 20 min with a constant amount of Foxf1 extract and/or decreasing amounts of Foxq1 protein extracts as indicated. Samples were run on a 4% polyacrylamide gel and detected by autoradiography.

mobility shift assays. These results revealed that the nucleotide binding requirements for Foxf1 and Foxq1 are nearly identical within this region of the telokin promoter (Fig. 4*B*). Consistent with this observation, Foxf1 and Foxq1 competed with each other for binding to the telokin promoter (Fig. 4*C*).

Foxf1 Binds to SRF—The physical proximity between Foxf1 and SRF binding sites in the telokin promoter and the functional relationship between Foxf1 and SRF transcription factors suggest that Foxf1 and SRF may physically interact. In order to test this possibility, combinations of SRF and either Foxf1 or empty vector were overexpressed in A10 SMCs and coimmunoprecipitation assays were performed. Western blot analysis showed that Foxf1 specifically bound to SRF (Fig. 5*A*). A GST-

are the mean $\pm$ S.E. from 12–16 samples. *, $p < 0.05$ compared with SRF-pBind/empty pAct/vector control. #, $p < 0.05$ compared with SRFpBind/myocardin pAct/vector control.
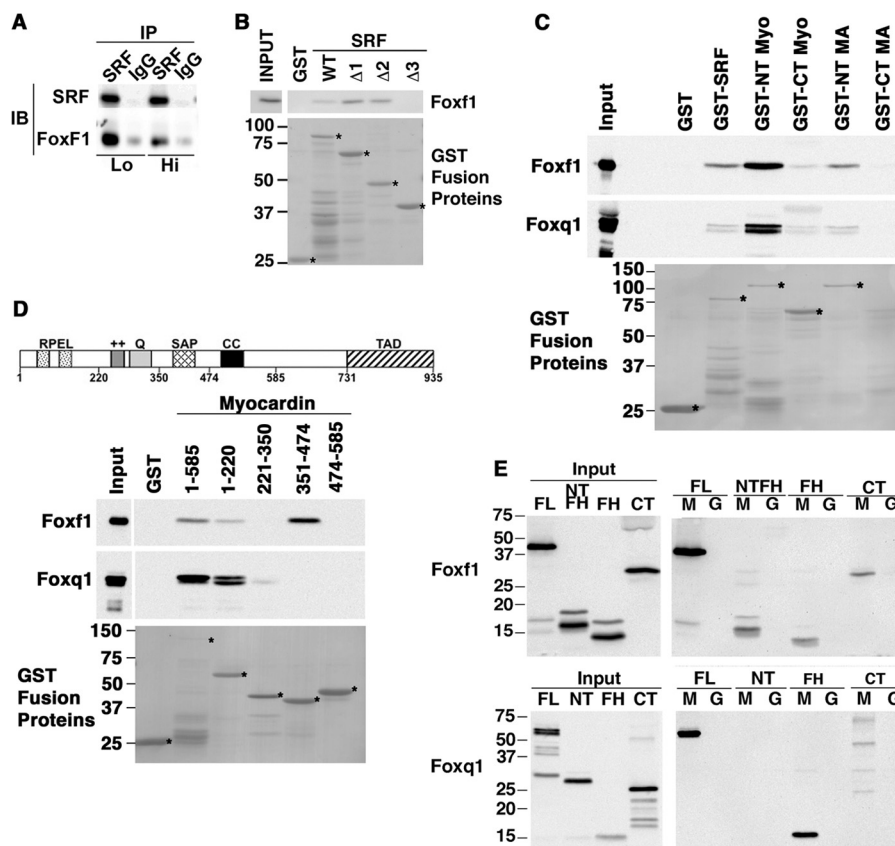
FIGURE 5. **Foxf1 binds to SRF and Foxf1 and Foxq1 directly bind to distinct regions of myocardin.** *A*, COS cells were transfected with SRF and Foxf1 expression plasmids. Nuclear proteins were extracted and immunoprecipitated with SRF or control IgG antibodies. Immunoprecipitated proteins were detected by Western blotting as indicated. *B–E*, GST-pulldown assays. Bacterial lysates containing empty GST vector (*GST*) or the indicated GST-SRF (*B*), GST-myocardin or GST-MRTFA (*C–E*) fusion proteins were mixed with bacterial expressed full-length Fox proteins as indicated. Glutathione-agarose-purified protein complexes were analyzed by Western blotting to identify the Fox proteins and Ponceau staining to visualize the GST fusion proteins. *B*, SRF WT (1–383), SRF Δ1 (1–338), Δ2 (1–222), Δ3 (1–132). *C*, NT-myocardin (1–585), CT-myocardin (585–935), NT-MRTF (1–628), CT-MRTFA (618–929). *D*, GST-Myocardin fusion proteins, or GST alone were incubated with bacterial expressed full-length Fox proteins as indicated. *E*, each of the Fox proteins was divided into the forkhead domain (*FH*), the region N-terminal of the forkhead domain (*NT*), and the region C-terminal of the forkhead domain (*CT*). As the N-terminal region of Foxf1 is very short, it was expressed together with the forkhead domain (NT FH). Foxf1 NTFH (1–118), FH (23–118), CT (119–353). Foxq1 NT (1–99), FH (100–215), CT (216–446). *Left panels* indicate the expression of the Fox protein fragments in the input material added to the pull-down assays. Fox protein fragments were incubated with GST-myocardin (*M*) or GST alone (*G*), and glutathione-agarose-purified protein complexes were analyzed by Western blotting to identify the Fox protein fragments that bound to myocardin.

pull down assay using proteins expressed in bacteria confirmed that this interaction was direct and that the SRF MADS domain was required for this interaction (Fig. 5B).

Fox Proteins Directly Bind to Myocardin—As myocardin has been previously shown to bind to Foxo4 we also determined if myocardin could bind directly to other Fox proteins. GST-pull down assays revealed that both Foxf1 and Foxq1 can directly bind to the N-terminal half of myocardin (amino acids 1–585) and MRTFA (amino acids 1–628) (Fig. 5C). No or only very weak binding was observed to the C-terminal half of myocardin (amino acids 585–935) and MRTFA (amino acids 618–929). However, the Fox proteins each interacted with different regions of myocardin. Foxf1 primarily binds to the SAP domain, whereas Foxq1 binds mainly to the N-terminal RPEL repeat region (Fig. 5D). Similarly, the Fox proteins use different motifs for myocardin binding. Foxf1 requires the entire protein for high affinity binding, as all fragments of Foxf1 bound to myocardin with lower affinity than the full-length molecule (Fig. 5E). In contrast, Foxq1 binds to myocardin solely through its forkhead domain (Fig. 5E).

Deletion of Foxf1 from SMCs in Vivo Results in Neonatal Lethality and Impaired GI Contractility—Our *in vitro* studies demonstrated that Foxf1 regulates expression of genes such as telokin, Cav1.2b, smMHC, BMP4, and myocardin that modulate the contractility, proliferation, and differentiation of visceral smooth muscle (Fig. 1). To determine the importance of Foxf1 in SMCs *in vivo* Foxf1 flox mice were crossed with mice expressing Cre recombinase under the control of the smooth muscle myosin heavy chain promoter (37)(smFoxf1$^{-/-}$ mice). The majority of smFoxf1$^{-/-}$ mice died prior to or immediately after birth. They displayed GI tract abnormalities including a greatly distended esophagus (Fig. 6). This was associated with a thinning of the esophageal smooth muscle layer and decreased expression of sm γ-actin (Fig. 6). Analysis of Cre expression in E17.5 embryos revealed robust Cre expression in the smooth muscle layer of the esophagus and in the circular but not longitudinal smooth muscle of the intestine (Fig. 6C). This was associated with decreased expression of Foxf1 in these layers demonstrating efficient knock-out (Fig. 6C). The lack of Cre expression and Foxf1 knock-out in the intestine longitudinal
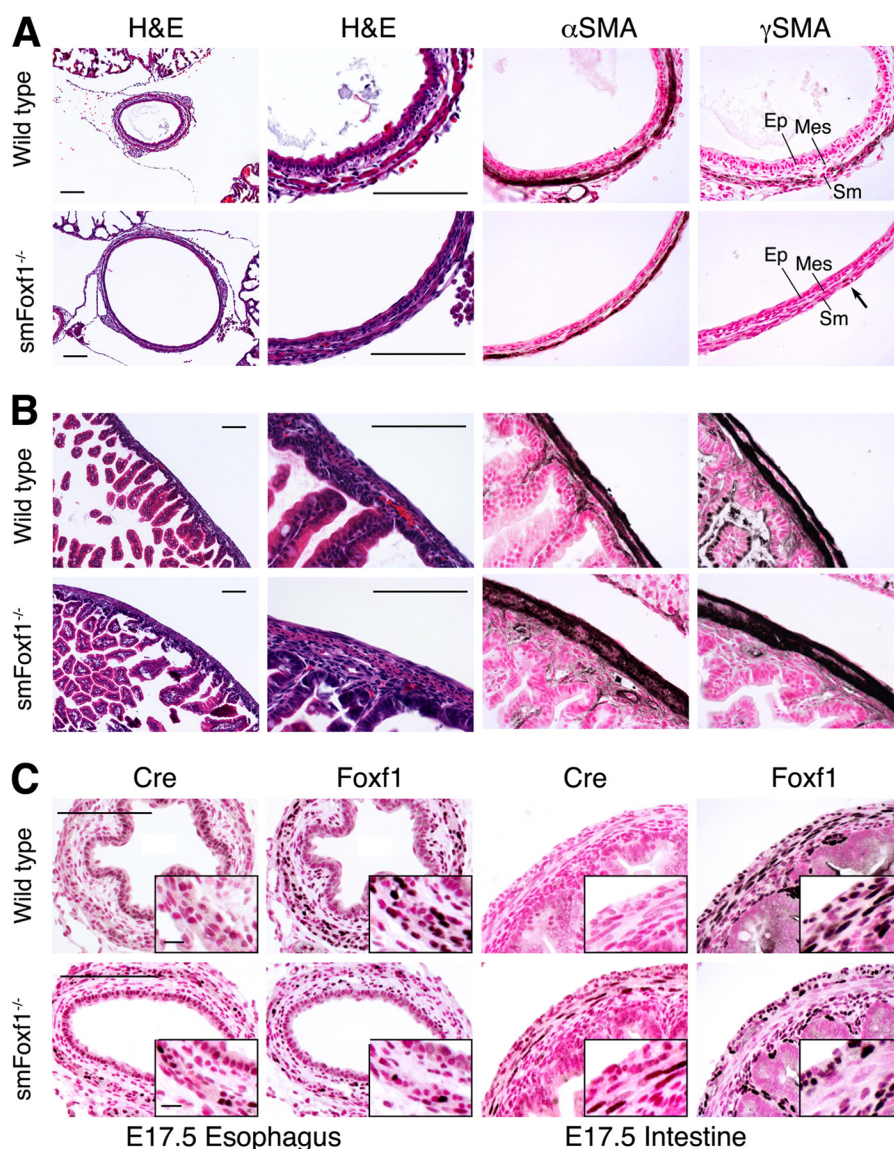
FIGURE 6. **Foxf1 deletion from smooth muscle cells causes esophageal defects in newborn mice.** Esophageal defects in Foxf1-deficient mice. *A* and *B*, paraffin sections from esophagus (*A*) and intestine (*B*) smFoxf1$^{-/-}$ and wild type Foxf1$^{f/f}$ newborn mice were stained with hematoxylin and eosin (H&E) or used for immunostaining with antibodies against smooth muscle α-actin (α*SMA*), smooth muscle γ-actin (γ*SMA*). Staining was visualized using biotinylated secondary antibody, avidin-HRP, and DAB substrate (*dark brown*), and then counterstained with nuclear fast red. Magnification: *left panels*, ×100; remaining panels, ×200. Scale bars represent 100 μm. *C*, immunostaining for Cre and Foxf1 in sections of the esophagus and intestine from E17.5 mice (Scale bars represent 100 μm). *Insets* show higher magnification images (scale bars represent 10 μm).

smooth muscle layer likely reflects the later differentiation of these cells, which occurs largely after birth. qRT-PCR analysis of whole esophagus and intestine isolated from embryonic day 18 smFoxf1$^{-/-}$ mice further demonstrated decreased expression of several contractile proteins (Fig. 7). Although smooth muscle-specific Foxf1 heterozygous mice (smFoxf1$^{-/+}$) are viable we noticed an increased mortality in breeding pairs. Moreover, these smFoxf1$^{-/+}$ heterozygous mice had impaired depolarization induced contractility of colonic rings (Fig. 8, *A* and *B*). The impaired contractility was still evident following treatment with 1 μM tetrodotoxin to block all neuronal input (Fig. 8*C*), indicating a true myogenic defect. Hematoxylin and eosin staining of cross sections of the colonic rings used for contractility studies revealed a slightly decreased thickness of the circular smooth muscle layer (Fig. 8*D*). The decreased thickness of the circular smooth muscle layer in the smFoxf1$^{-/+}$ mice is how-

ever, not likely to be sufficient to account for the decreased contractility observed. Further analysis of colonic smooth muscle (mucosa and submucosa removed) in these smFoxf1$^{-/+}$ mice revealed a marked decrease in expression of many contractile proteins in addition to decreased expression of myocardin and SRF (Fig. 8*E*).

DISCUSSION

Results of the current study reveal important roles for Foxf1 in regulating gene expression in gastrointestinal SMCs. Foxf1 in smooth muscle cells is required for the development and normal physiological function of the gastrointestinal tract. Foxf1 regulates expression of many myocardin dependent smooth muscle-specific genes, although some genes are more sensitive to changes in Foxf1 than others. Consistently, telokin appears to be most sensitive to Foxf1 levels, being reduced following
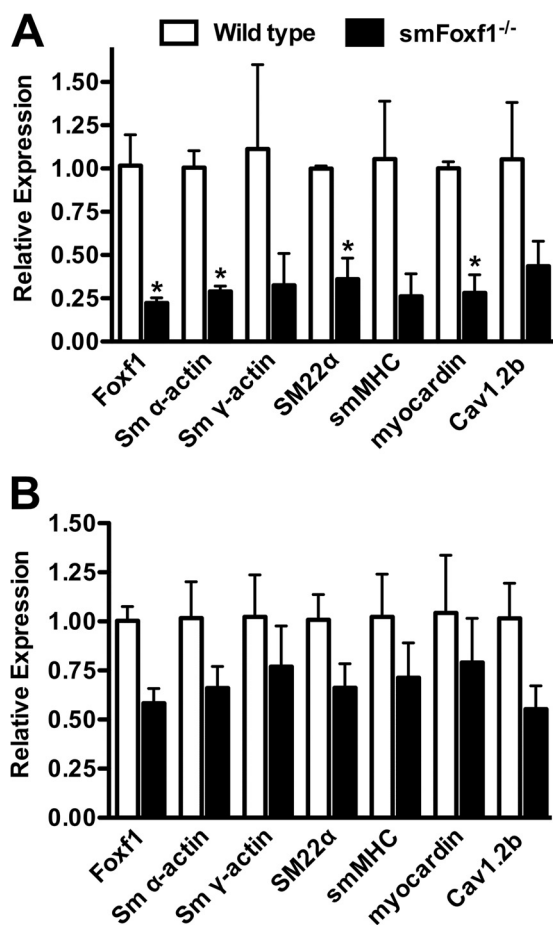
FIGURE 7. **Foxf1 deletion from SMCs results in deceased contractile protein expression.** qRT-PCR analysis of mRNA expression in the esophagus (*A*) and intestine (*B*) of E18.5 smFoxf1$^{-/-}$ and wild type Foxf1$^{f/f}$ mice. Data were normalized to a β-actin internal control and are expressed relative to Foxf1$^{f/f}$ wild type mice. Relative expression $= 2^{-\Delta\Delta Ct}$. Data presented are the mean $\pm$ S.E. of 2 pairs of mice. A statistically significant *p* value < 0.05 is shown with *asterisk*.

knock-out of Foxf1 in primary SMCs (Fig. 1), decreased in smFoxf1$^{-/+}$ tissues (Fig. 8) and increased following overexpression of Foxf1 (Fig. 2). Although expression of other SRF/myocardin dependent genes such as SM α-actin, SM22α and calponin were not significantly decreased following Foxf1 knock-out in SMCs *in vitro*, expression of these genes was increased following overexpression of Foxf1 and attenuated in tissues from heterozygous and knock-out mice (Figs. 1, 7, 8). The reason for these differences is not readily apparent although it may be related to differences in myocardin levels that are about 50-fold lower in cultures SMCs as compared with tissues. In cultured SMCs expression of genes such as SM22α and sm α-actin are less dependent on myocardin and thus less affected by changes in this pathway. The sensitivity of genes to Foxf1 regulation of the SRF/myocardin axis may reflect the sensitivity of specific genes to myocardin levels and/or the presence of Foxf1 binding sites adjacent to SRF/myocardin binding sites. For example, genes that are most sensitive to small decreases in myocardin levels may be more dependent on enhanced SRF-myocardin interactions facilitated by Foxf1. As the Foxf1 binding site in the telokin promoter was essential for Foxf1 stimulation of telokin promoter activity this would sug-

gest that direct binding of Foxf1 to the promoter may also help to stabilize binding of SRF-myocardin complexes to the promoter (Fig. 9*A*). Bioinformatic analysis of the mouse Cacna1c gene, that encodes Cav1.2b, revealed 6 potential Foxf1 binding sites within 4 kb of the translation start site of Cav1.2b, one of which is adjacent to a degenerate CArG box. This would suggest that a similar mechanism of Foxf1 and SRF synergy might regulate Cav1.2b expression. Foxf1-dependent regulation of Cav1.2b, the pore forming subunit of the voltage-gated calcium channel, is consistent with the decreased KCl-induced contraction observed in the smFoxf1$^{-/+}$ heterozygous mice (Fig. 8) as we have previously shown that KCl-induced contractions are primarily mediated through voltage gated calcium channels (34). Mechanistically, Foxf1 may also facilitate direct SRF-independent recruitment of additional myocardin molecules to enhance promoter activation (Fig. 9*A*). This would be consistent with the model in which myocardin dimerization or tetramerization is required for it to strongly activate gene expression (38). Most smooth muscle-specific genes have two or more CArG boxes, permitting recruitment of two or more SRF/myocardin dimers to facilitate strong promoter activation (Fig. 9*B*); whereas other growth factor responsive SRF-dependent genes that have only a single CArG box are poorly activated by myocardin (38). It has thus been a question why telokin, which has only a single CArG box, is strongly activated by myocardin (39). Previous studies have highlighted the importance of the AT-rich region that includes the Foxf1 binding site for efficient activation of the telokin promoter by myocardin (39). This observation together with our current findings suggest that perhaps Foxf1 binding to this AT-rich region may aid in the recruitment of a second myocardin dimer complex to the telokin promoter thereby facilitating strong myocardin-dependent gene activation (Fig. 9*A*).

The MADS domain of SRF provides a docking surface for the binding of numerous proteins, including Foxf1 (Fig. 5*B*), FOXK1, and myocardin (40, 41). In some cases, the binding of proteins to SRF is mutually exclusive, for example Elk1 and myocardin compete with each other for SRF binding (42). In others, such as we describe here with Foxf1 and myocardin, both proteins appear to be able to bind simultaneously. The findings that Foxf1 and Foxq bind directly to myocardin and MRTFA together with previous reports detailing the binding of Foxo4 to myocardin (13) suggests that many Fox proteins may be able to bind to and regulate the activity of myocardin family members. The outcome of the interaction of Fox proteins and myocardin is, however, dependent on the specific Fox protein involved. Foxq1 and Foxo4 inhibit myocardin binding to SRF while Foxf1 promotes this binding (Fig. 3*D* and Ref. 13). The differences in the effects of the Fox proteins can be explained, at least in part, by the distinct regions of myocardin to which they bind.

Knock-out of Foxf1 specifically in smooth muscle cells resulted in embryonic/neonatal lethality clearly demonstrating a critical role of Foxf1 in smooth muscle development. Although we do not know exactly what is causing this lethality new born mice exhibited a dilated esophagus with a very thin smooth muscle layer (Fig. 6). The dilated esophagus was also associated with decreased expression of contractile proteins suggesting that it likely has impaired contractility and that new-
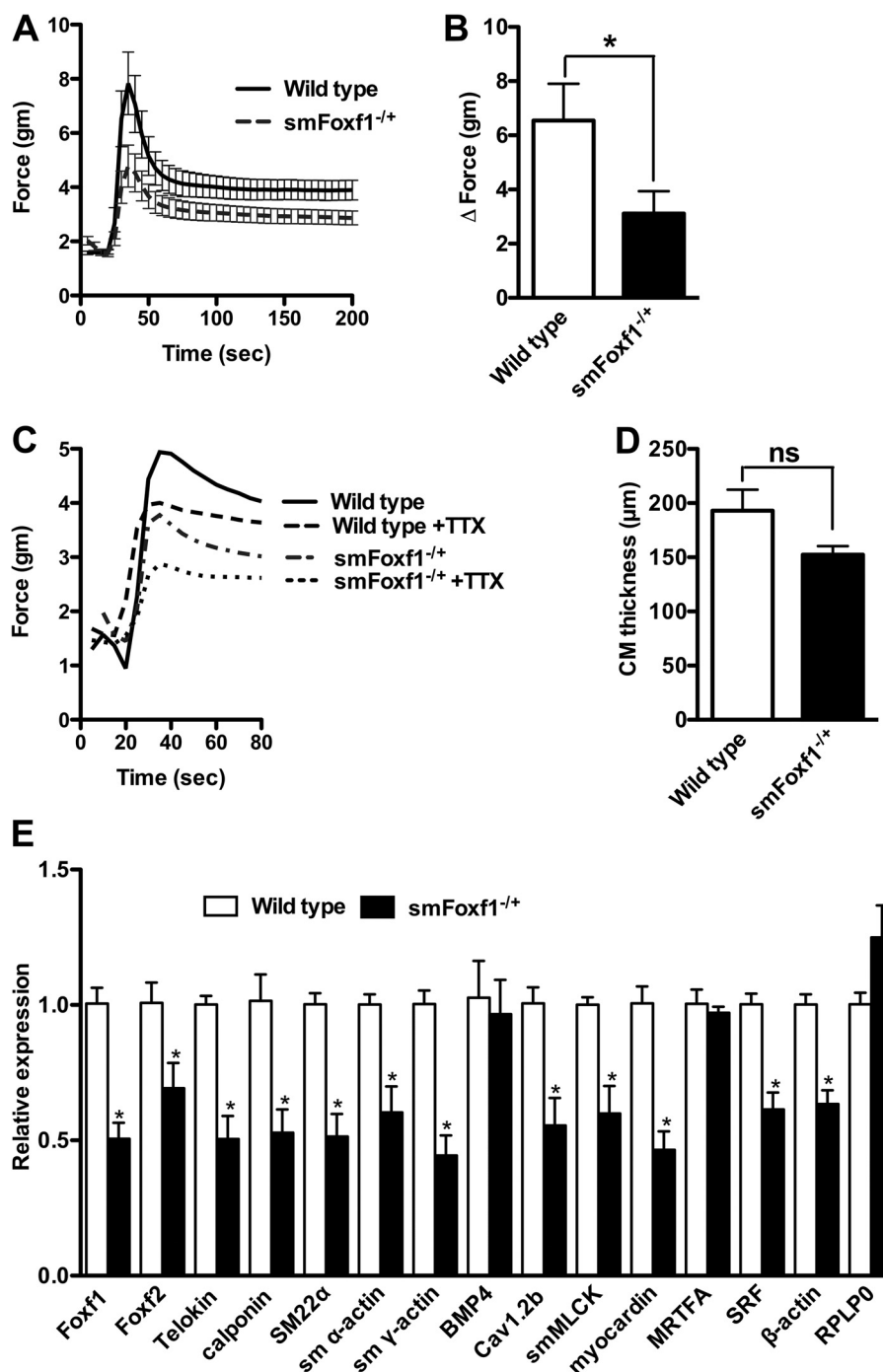
FIGURE 8. **Foxf1 deletion from SMCs results in impaired colonic contractility and decreased contractile protein expression.** Duplicate colonic rings were cut from the middle of the colon of smFoxf1$^{-/+}$ heterozygous mice or Foxf1$^{f/f}$ wild type mice. *A*, averaged tension recordings from colon rings contracted by 60 mM KCl stimulation. *B*, quantification of changes in peak force produced by colonic rings. Data shown are the mean ± S.D. of five different mice from each group. *, $p < 0.05$. *C*, average contractile responses of colonic rings from a wild type mouse and 3 smFoxf1$^{-/+}$ mice in response to 60 mM KCl prior to and following treatment with 1 μM tetrodotoxin (+*TTX*). *D*, circular smooth muscle thickness of colonic rings was measured from 4 different regions from at least 3 sections of each colonic ring (two rings from each of 3 smFoxf1$^{-/+}$ and 3 wild type mice). *D*, colonic smooth muscle was isolated from wild type and smFoxf1$^{-/+}$ mice and mRNA expression analyzed by qRT-PCR. Data were normalized to an hprt internal control and expressed relative to levels in wild type samples ($2^{-\Delta\Delta Ct}$). Data shown are the mean ± S.E. of 4 wild type and 6 smFoxf1$^{-/+}$ mice. A statistically significant *p* value < 0.05 is shown with *asterisk*.

born mice may be unable to feed. We also observed a slightly thinner circular smooth muscle layer in the colon of adult smFoxf1$^{-/+}$ heterozygous mice (Fig. 8*C*). Again this was associated with decreased expression of many contractile and regulatory proteins such as telokin and Cav1.2b. This decrease is unlikely to be attributed solely to fewer smooth muscle cells as

the samples analyzed were largely free of mucosa and thus composed primarily of smooth muscle cells together with some neuronal tissue. These data would suggest that Foxf1 is not only important for visceral smooth muscle development but also for maintaining expression of contractile proteins in adult GI smooth muscle tissues.
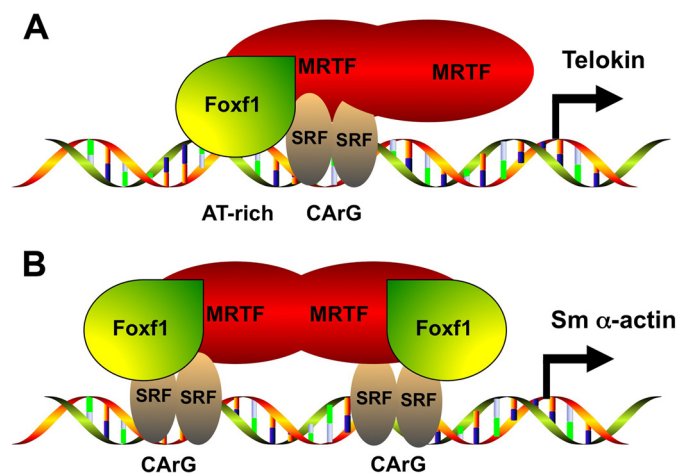
FIGURE 9. **Model depicting potential mechanisms by which Foxf1 regulates myocardin-dependent genes.** *A*, model single CArG box promoter, such the telokin promoter, in which Foxf1 directly binds to the promoter and recruits an additional MRTF. This Foxf1-bound MRTF then interacts with SRF-bound MRTF to activate transcription. *B*, model dual CArG box promoter, such as the sm α-actin promoter in which Foxf1 helps stabilize SRF-bound MRTF complexes to activate transcription.

In summary, our results demonstrate a critical role of Foxf1 in regulating gene expression in visceral SMCs *in vitro* and *in vivo* in mice. We propose a model in which Foxf1 interacts with the SRF/Myocardin axis to regulate gene transcription in visceral SMCs. The specific changes in gene expression, GI tract development and contractility, exhibited by the smooth muscle-specific Foxf1 knock-out mice, indicate that Foxf1 plays an important role in the regulation of GI smooth muscle that cannot be compensated for by Foxf2.

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