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Forkhead box F2 Regulation of Platelet-Derived Growth Factor and myocardin/Serum Response Factor Signaling is Essential for Intestinal Development

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Running title: Foxf2 is Essential for Intestinal Development

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Keywords: Foxf2; gut development; smooth muscle hyperplasia; myocardin/SRF

Background: Transcriptional regulation of smooth muscle cells is an understudied component of intestinal development and physiology.

Results: Foxf2 deletion from smooth muscle causes intestinal malformations and colon remodeling.

Conclusion: Foxf2 regulation of PDGF and myocardin/SRF signaling is essential for intestinal development and homeostasis.

Significance: Better understanding transcriptional mechanisms regulating postnatal intestine development and homeostasis may provide therapeutic approaches for congenital and acquired gastrointestinal diseases.

ABSTRACT

Alterations in *Forkhead box F2* gene expression have been reported in numerous pathologies and *Foxf2*$^{-/-}$ mice are perinatal lethal with multiple malformations; however, molecular mechanisms pertaining to Foxf2 signaling are severely lacking. In the present study, Foxf2 requirements in murine smooth muscle cells were examined using a conditional knockout approach. We generated novel *Foxf2-floxed* mice, which we bred to *smMHC-Cre-eGFP* mice to generate a mouse line with *Foxf2* deleted specifically from smooth muscle. These mice exhibited growth retardation due to reduced intestinal length as well as inflammation and remodeling of the small intestine. Colons of Tg(*smMHC-Cre-eGFP*$^{+/-}$);*Foxf2*$^{-/-}$ mice had expansion of the myenteric nerve plexus and increased proliferation of smooth muscle cells leading to thickening of the longitudinal smooth muscle layer. Foxf2-deficiency in colonic smooth muscle was associated with increased expression of Foxf1, PDGFa, PDGFb, PDGFRα and myocardin. FOXF2 bound to promoter regions of these genes indicating direct transcriptional regulation. Foxf2 repressed *Foxf1* promoter activity in co-transfection experiments. We also show that knockdown of *Foxf2* in colonic smooth muscle cells *in vitro* and in transgenic mice increased myocardin/serum response factor signaling and increased expression of contractile proteins. Foxf2 attenuated myocardin/serum response factor signaling in smooth muscle cells through direct binding to the N-terminal region of myocardin. Our results indicate that Foxf2 signaling in smooth muscle cells is essential for intestinal development and serum response factor signaling.

The mammalian gut is derived from the endodermal and mesodermal germ layers, which give rise to gut epithelium, mesenchyme and smooth muscle (1). The enteric nervous system is derived from invading cells from the neural crest (1). Digestive diseases affect 60 to 70 million Americans resulting in 13.5 million hospitalizations annually (2). Digestive diseases run a gamut from viral infections to irritable bowel syndrome and hemorrhoids, and inflict all tissue layers (2). To this point, studies of gut development and diseases have primarily focused on the epithelial layer due to its obvious importance in digestion and nutrient absorption. However, smooth muscle is also of critical importance to gut anatomy and physiology, maintaining shape and allowing for motility of luminal content. Despite the great breadth of knowledge pertaining to vascular smooth muscle development and pathology, surprisingly little is known about molecular and transcriptional mechanisms critical for development of visceral smooth muscle lining the gut.

Development of visceral smooth muscle requires controlled cross-talk between epithelial, neuronal and mesenchymal cell layers (3-5). The muscularis of the gastrointestinal (GI) tract contains two distinct smooth muscle layers separated by the myenteric plexus; an inner circular layer and an outer longitudinal layer (1,6,7). The muscularis mucosa is also of mesenchymal origin and demonstrates features similar to smooth muscle (1). Numerous signaling pathways have been identified that play important roles in regulating visceral smooth muscle development during embryogenesis, including the TGF-β and bone morphogenetic protein (BMP) pathways (5,8,9), Wnt signaling (9,10), the Hedgehog pathway (3,4,9) and a number of growth factor-mediated pathways (i.e. PDGF, fibroblast growth factor (FGF) and insulin-like growth factor (IGF)) (5,7,11,12). However, knowledge pertaining to the regulation of postnatal visceral smooth muscle development remains scarce.

The forkhead box (Fox) family of transcription factors has been shown to mediate a wide variety of cellular activities including embryonic and postnatal cell growth, tissue repair after injury, cell migration and tumor formation (13). The Foxf subgroup contains two members, Foxf1 and Foxf2 (1,14). Lessons from knockout mice indicate that both Foxf members are critical for embryonic development as *Foxf1*$^{-/-}$ mice die *in utero* (15,16) and *Foxf2*$^{-/-}$ as well as compound heterozygotic (*Foxf1*$^{+/-}$/*Foxf2*$^{+/-}$) mice die shortly after birth (17). Several Fox proteins, including Foxo4, Foxq1 and Foxf1, have been shown to be critical for smooth muscle cell development via regulation of the myocardin/serum response factor (SRF) axis (18-20). Using *in situ* hybridization, embryonic *Foxf2* expression has been found in the mesenchyme of the oral cavity, limb buds, genitalia, CNS, eyes, lung, prostate, ear and placenta as well as the lamina propria region and smooth muscle of the developing GI tract (21,22). During embryonic development, Hedgehog signaling from the epithelium induces Foxf2 expression (17); however, postnatal Foxf2 expression and signaling remain poorly characterized. Studies have shown alterations in Foxf2 expression in prostate cancer (23) and that Foxf2 decreases the size and frequency of colonic polyps in colon adenoma (24). Foxf2 has been further shown to mediate cardiac metabolism (25) and a W174R amino acid substitution has been shown to cause anterior segment mesenchymal dysgenesis in the eye (*Foxf2*^{W174R}) (26). However, molecular mechanisms for Foxf2 signaling in smooth muscle cells have not been previously elucidated due to lack of mouse models allowing conditional inactivation of Foxf2.

In the present study, we generated a novel *Foxf2-floxed* (*Foxf2*^{fl}) mouse line to investigate the importance of Foxf2 in smooth muscle cells. By breeding these mice with *smooth muscle myosin heavy chain-Cre* mice (*smMHC-Cre-eGFP*) (27), we were able to efficiently delete Foxf2 from mature smooth muscle in the gut. *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice were viable and fertile. Juvenile mice showed no difference in size or morphology; however, adult *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice were significantly smaller in stature and had a significant decrease in the length of the GI tract (both large and small intestine). Adult mice had focal inflammatory regions in the small intestine and a significant increase in thickness of the smooth muscle and enteric neuron layers in the colon. We used colon smooth muscle from *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice and cultured *Foxf2$^{-/-}$* smooth muscle cells to demonstrate that Foxf2 regulates the PDGF pathway and physically interacts with myocardin to inhibit SRF signaling in visceral smooth muscle cells.

EXPERIMENTAL PROCEDURES

Generation of Foxf2-floxed mice and deletion of Foxf2 from smooth muscle myocytes. A LoxP site was inserted into the *Foxf2* promoter via Foxf2-targeting vector and PGK-gb2 LoxP/FRT-flanked Neomycin (neo) cassette was placed into the first intron (Figure 1D). Electroporation of mouse ES cells (C57Bl/6 x 129/SVEV) with the *Foxf2^{fl}*-targeting vector, following neo (G418) selection, was performed at the inGenious Targeting Laboratory (Stony Brook, NY). PCR analysis with multiple primer sets identified ES cells with the appropriate *Foxf2^{fl}*-targeted locus. *Foxf2^{fl}* ES cells were subsequently used to generate chimeric mice by injection into mouse blastocysts. Mice containing the *Foxf2^{fl}*-targeted allele were determined by PCR amplification with primers flanking the LoxP sequence located in the *Foxf2* promoter (P1 and P2) (Table 1) and primers located in the 3' region of the *Foxf2^{fl}* allele (P3 and P4). Chimeric mice were bred with C57Bl/6 mice in the animal facility of Cincinnati Children's Research Foundation to produce *Foxf2$^{fl/+}$* mice. The neo cassette was deleted by breeding *Foxf2$^{fl/+}$* mice with *ACT-FLP1* mice (Jackson Lab) (Figure 1D). The loss of neo in *Foxf2$^{fl/+}$* mice was confirmed by PCR using P5 and P6 primers (Table 1) and sequencing of the *Foxf2* locus using mouse tail DNA. *Foxf2$^{fl/+}$* mice were backcrossed to generate viable *Foxf2$^{fl/fl}$* mice that were bred into the C57Bl/6 background for ten generations. Deletion of the *Foxf2^{fl}* alleles from visceral smooth muscle was accomplished through breeding with *smooth muscle myosin heavy chain-Cre-eGFP* (*smMHC-Cre-eGFP*) transgenic mice (C57Bl/6) (27). Animal studies were approved by the Animal Care and Use Committee of Cincinnati Children's Research Foundation.

Tissue collection. Intestinal tracts were collected from *Foxf2$^{fl/fl}$* and *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice and placed in ice cold, calcium-free, sterile PBS. Colons and small intestines were separated at this time. Luminal contents were manually forced out by gentle manipulation with forceps. Tissues destined for immunohistochemistry were placed in 4% paraformaldehyde at this time. Tissues for whole organ RNA were placed in RNA-STAT-60. To enrich the smooth muscle layer of the colon, colons were split along their length and laid flat with the smooth muscle side down. A scalpel was then used to scrape away the epithelium and other layers leaving behind a smooth muscle enriched segment that was then placed in RNA-STAT-60 for RNA isolation or used for enzymatic digestion to prepare a single cell suspension as previously described (28).

Immunohistochemistry. To characterize FOXF2 expression throughout development of the GI tract, intestinal tissues were collected from wildtype mice at embryonic day 14.5 (E14.5), E17.5, postnatal day 1 (P1) as well as *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* and *Foxf2$^{fl/fl}$* mice at, E16.5, E18.5, P12 and in the adult (8-12 weeks). Intestines and abdomens were fixed in 4% paraformaldehyde overnight and embedded into paraffin blocks. Paraffin sections of 5 µm were immunostained with antibodies against FOXF2 (1:1000; Santa Cruz Biotech), CGRP (1:4000; Sigma), PDGFRα (1:200; Santa Cruz Biotech), α-smooth muscle actin (αSMA; 1:10,000; Sigma-Aldrich), γSMA (1:1000; 7 Hills Biotech), PECAM-1 (1:500; Pharminogen), FOXA2 (1:300; 7 Hills Biotech), FOXF1 (1:1000;(29)), pan-CYTOKERATIN (1:500; Sigma), Ki-67 (1:10,000; Dako) or Cyclin D1 (1:250; AbCam). Antibody-antigen complexes were detected using biotinylated secondary antibody followed by avidin-HRP complex and DAB substrate (Vector Labs, Burlingame, CA) as previously described (30-34). Sections were counterstained with nuclear fast red (Vector Labs). Intestinal sections were also stained with hematoxylin and eosin (H&E) to evaluate morphology, Masson's Trichrome to detect fibrosis or wheat-germ agglutinin (WGA; Sigma) to measure myocyte size. Size of myocytes was measured from WGA-stained slides as previously described (35,36). Slides were photographed using a Zeiss Axioplan2 microscope and Axiovision Rel 4.8 software.

To detect *Cre*-dependent reporter activity, *smMHC-Cre-eGFP* mice were crossed with the Cre-dependent reporter strain, *mT/mG (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J* (Jackson Lab). Tissues were harvested from adult double heterozygous mice, fixed in 4% paraformaldehyde and frozen in OCT tissue freezing media (Tissue-Tech). Frozen sections were washed in 100mM Tris pH7.6, 150mM NaCl, stained with Hoechst, mounted in Prolong Gold (Invitrogen) and visualized by confocal microscopy (Olympus Fluoview FV1000). Under these conditions cytoplasmic eGFP encoded by the *smMHC-Cre-eGFP* transgene is washed out of the tissue sections.

Quantitative real-time RT-PCR (qRT-PCR). Whole organ RNA was prepared from small intestine and P12 colon. Smooth muscle enriched RNA was prepared as described (28) from adult colon of individual *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ and control *Foxf2*$^{fl/fl}$ mice using RNA-STAT-60 (Tel-Test "B" Inc. Friendswood, TX). cDNA was generated using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Evaluation of expression levels of specific genes was performed by qRT-PCR using inventoried Taqman probes (Table 2) and the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) as previously described (37-42).

Chromatin immunoprecipitation (ChIP) assays. Stably transfected mouse rhabdomyosarcoma cells with a dual *His/Flag*-tagged *Foxf2* construct were generated and used for ChIP analysis. The pMIEG3 retroviral vector was used for dual-tagged protein expression in mammalian cells and has been described previously (43). In order to generate a double-tagged construct, *Foxf2* ORF was PCR-amplified using high-fidelity pfx Polymerase with N-terminal Flag and C-terminal *(His)6* affinity epitopes according to manufacturer's protocol (Invitrogen). Nuclear extracts from transfected mouse rhabdomyosarcoma cells were cross-linked by addition of formaldehyde, sonicated and used for immunoprecipitation with anti-6x HIS tag rabbit polyclonal antibodies (Abcam) as described previously (44-48). DNA fragments were approximately 500 base pairs as verified by agarose gel. Reverse cross-linked ChIP DNA samples were subjected to PCR, using oligonucleotides specific to promoter regions of mouse *PDGFa*, *PDGFb*, *PDGFRα*, *Foxf1* and *myocardin* genes (Table 3). Potential FOXF2 binding sites were identified using the MacVector program and the previously published FOXF2 consensus binding sequence TA(G)TTTA(G)T (44). DNA sites with the highest homology to the published FOXF2 consensus binding sequence were investigated via ChIP. DNA binding was normalized to control ChIP DNA samples, which were immunoprecipitated using control rabbit IgG.

Luciferase and mammalian two-hybrid assays. 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. A *Foxf1* mammalian expression construct was amplified by PCR from an HFH8 clone obtained from Robert Costa (44). This resulted in expression of a FOXF1 protein of 353 amino acids identical to that encoded by NM_010426.1. *Fox* expression plasmids were transfected together with SRF expression plasmid, telokin promoter luciferase reporter gene (-256 to +147) and TK-renilla luciferase internal control into 10T1/2 cells and luciferase assays performed as described previously (46). The Foxf1luciferase reporter (-5.3 kb Foxf1 + 3' RE) and Foxf2 luciferase reporter (6 repeats of Foxf2 binding sequence) genes were transfected into U2OS cells and luciferase assays performed as previously described (46,49). Mammalian two hybrid assays utilizing SRF fused to the GAL4 DNA binding domain and myocardin fused to the GAL4 activation domain were performed as described previously (50).

GST-pulldown assays. GST-SRF and GST-myocardin bacterial expression plasmids were described previously (50-53). 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 (52). All expression constructs were confirmed by DNA sequencing. Full length and fragments of *Foxf2* were PCR amplified and cloned into pET vectors for expression in bacteria. GST-pulldown assays were performed as described previously (53).

Statistical analysis. Student's T-test was used to determine statistical significance. P values <0.05 were considered significant. Values for all measurements were expressed as mean ± standard error of mean (SEM).

RESULTS

Generation of mice with Foxf2 deletion from smooth muscle cells. As Foxf2 expression has been poorly studied and the emergence of reliable commercial antibodies fairly recent, we performed immunohistochemistry on embryonic (E14.5 and E17.5), newborn, juvenile (P12) and adult (8-12 weeks) wildtype mice to investigate FOXF2 expression patterns in the GI tract during development. As early as E14.5 and at E17.5, FOXF2-positive nuclei were detected in the intestinal mesenchyme located adjacent to the epithelial layer (Figure 1A). In the newborn, P12 and adult, FOXF2 was detected in the lamina propria and inner circular smooth muscle layer of the muscularis externa in both the small intestine and colon (Figure 1B). FOXF2 was not detected in the outer longitudinal smooth muscle layer (Figure 1B). FOXF2 was not expressed in vascular smooth muscle cells (data not shown). qRT-PCR analysis indicated that Foxf2 expression increases along the cranial to caudal axis in the adult gut, with little expression in the duodenum and highest expression in the colon (Figure 1C).

To assess Foxf2 requirements in smooth muscle cells, we created a *PGK-Neo-Foxf2* targeting construct and used it for electroporation of ES cells (Figure 1D and Materials and Methods). A *Foxf2-floxed* (*Foxf2^{fl}*) mouse line was generated and confirmed by PCR amplification of tail genomic DNA (Figure 1F, G). The neo cassette was deleted by crossing the mice with ACT-FLP1 transgenic mice (Figure 1D). *Foxf2$^{fl/fl}$* mice contain LoxP sites flanking exon one of the *Foxf2* gene, which contains the DNA binding domain and one of two transcriptional activation domains of the FOXF2 protein. *Foxf2$^{fl/fl}$* mice were bred with *smMHC-Cre-eGFP* mice to delete *Foxf2* from intestinal smooth muscle. Analysis of Cre-dependent reporter mice demonstrated that the *smMHC-Cre-eGFP* transgene directs robust Cre activity in the smooth muscle layers as well as muscularis mucosa but not lamina propria of small intestine and colon (Figure 1E).

Growth retardation and diminished intestinal length in Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$ mice. *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice were born in normal Mendelian ratio and were viable and fertile. As early as E16.5, *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice had a noticeable decrease in FOXF2-staining in smooth muscle cells of the circular smooth muscle layer, without loss of FOXF2 from the lamina propria (Figure 2A), a finding consistent with GFP reporter studies (Figure 1E). Similar results were obtained from adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice. *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice exhibited loss of FOXF2 from the inner circular muscle layer (Figure 2A) and decreased Foxf2 mRNA in the colon and small intestine (Figure 2B). Although intestine structure and size was unchanged in juvenile *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice at P12, 8-12 week old adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice had a truncated GI tract with shorter small and large intestines (Figure 2 D, E). Adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice also had lower body weight than control littermates (19.8±1.2 g for *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice compared to 26.4±1.4 g for control mice) and less fecal production (1.3±0.3 g/day for *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice compared to 2±0.1 g/day for control mice), indicating diminished intestinal function. No intestinal abnormalities were observed in mice containing only the *smMHC-Cre-eGFP* transgene (39).

Focal inflammation in Foxf2 deficient small intestine. Decreased Foxf2 protein and mRNA were found in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* small intestines, a finding consistent with efficient deletion of *Foxf2* by the *smMHC-Cre-eGFP* transgene (Figure 2A, B). Adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* small intestines were mostly normal in morphology but exhibited ulcer-like patches with loss of villus structure and inflammatory cell infiltrates (Figure 3A, B). Morphological appearance of smooth muscle layers was normal (Figure 3A). Interestingly, the small intestine of juvenile (P12) and embryonic (E17.5) *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice were morphologically normal (Figure 3C and data not shown), indicating that Foxf2 is important for maintaining intestinal structure in adults.

Increased smooth muscle cell proliferation in Foxf2 deficient colons. The outer longitudinal smooth muscle layer was significantly thicker in adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons compared to control littermates (Figure 4A, B and 2C). Thicker muscle in the colon of *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice was not due to pronounced hypertrophy as there was no detectable difference in myocyte size between control and *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice (Figure 4C, D). This would suggest that increased muscle thickness was due to hyperplasia. Although no myocyte proliferation was observed in adult colons, neonatal P12 *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice exhibited increased Ki-67 and cyclin D$_1$ staining in myocytes from the outer longitudinal smooth muscle layer (Figure 4E). qRT-PCR analysis confirmed increased expression of the cell cycle regulators Cdc 25B, cyclin B$_1$ and cyclin D$_1$ in P12 *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice compared to age-matched controls (Figure 4F). Therefore, increased smooth muscle thickness in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons likely results from increased or prolonged myocyte proliferation during postnatal development and not from hypertrophy of individual myocytes.

Expansion of the myenteric plexus in Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$ colons. Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$ mice also had an expanded myenteric nerve plexus. Immunohistochemical staining using the calcitonin-related gene protein (CGRP), which marks serotonergic neurons, showed a clearly enlarged network of enteric neurons in adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice (Figure 4G). Greater CGRP staining of the myenteric plexus was also observed at P12, prior to thickening of the longitudinal muscle layer (Figure 4G inset). Expansion of the myenteric plexus in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice was confirmed by immunostaining with antibodies against the platelet-derived growth factor receptor alpha (PDGFRα) (Figure 4G) which is highly expressed in neurons (54-56) and weakly expressed in smooth muscle cells (57). Consistent with increased PDGFRα staining, qRT-PCR analysis showed increased expression of PDGFRα, as well as its ligands PDGFa and PDGFb, in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons (Figure 5A).

Altered expression of genes critical for gut morphogenesis in Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$ colons. To determine molecular mechanisms underlying increased proliferation in *Foxf2*-deficient colons, we examined expression of genes critical for the Hedgehog pathway, which has been shown to be important for proliferation of smooth muscle cells and their mesenchymal precursors (3,4,9). Indian Hedgehog (IHH) mRNA was increased in adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons, but was not changed at P12 (Figure 5B, data not shown). In agreement with increased Hedgehog signaling there was increased mRNA expression of Hedgehog target genes Foxf1, Gli1, Gli2 and the Hedgehog receptor Patched in smooth muscle-enriched tissue from *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice (Figure 5A). Considerably more FOXF1-positive myocytes were observed in the colon of *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* compared to control mice (Figure 5C), further indicating increased Hedgehog signaling. qRT-PCR analysis showed elevated mRNA levels of Wnt5a, BMP4 and myocardin (Figure 5A), factors critical for gut development. Despite significant alterations in smooth muscle regulatory genes in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice, muscle markers αSMA and γSMA were unaltered following Foxf2 deletion (Figure 5C), indicating that *Foxf2*-deficient smooth muscle cells are fully differentiated. No fibrosis was detected in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice as visualized by Masson's trichrome stain (Figure 5C). In addition, there was no observed difference in vascularization of the muscle layers of the colon as indicated by PECAM-1 immunostaining (Figure 5D). Furthermore, neither FOXA2 nor CYTOKERATIN immunostaining demonstrated any difference in the structure of colon epithelium (Figure 5D). Altogether, our data indicate that *Foxf2* deletion from smooth muscle cells alters expression of genes critical for intestinal morphogenesis.

Foxf2 binds to promoter regions of PDGFa, PDGFb, PDGFRα, myocardin and Foxf1 genes. Since expression of genes critical for the PDGF and Hedgehog signaling pathways was altered in smooth muscle of *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons (Figure 5A), we examined whether FOXF2 could directly bind to promoter regions of these genes. Potential FOXF2-binding sites (TGTTTAT, TATTTAT or TATTTGT (44)) were identified in the promoter regions of *PDGFa*, *PDGFb*, *PDGFRα* and *Foxf1* genes. Subsequently, a mouse myosarcoma cell line was created that stably expressed a *His*- and *Flag*-tagged *Foxf2* construct. Chromatin immunoprecipitation (ChIP) with anti-HIS antibodies demonstrated that HIS-FOXF2 bound to the promoter regions of the *PDGFa*, *PDGFb* and *PDGFRα* genes (Figure 6A). HIS-FOXF2 protein also bound to promoter regions of *myocardin*, a critical co-activator of SRF signaling, and *Foxf1* transcription factor, a downstream target of Hedgehog signaling (Figure 6B). In co-transfection experiments, FOXF2 repressed *Foxf1* promoter activity in a concentration dependent manner as shown by Luciferase assay (Figure 6C). Thus, Foxf1 is a direct transcriptional target of Foxf2.

Foxf2 directly interacts with myocardin and influences myocardin/SRF signaling. Gene expression studies in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice suggest that Foxf2 may regulate multiple signaling pathways during gut morphogenesis. Next, we focused our studies on the myocardin/SRF signaling pathway. SRF and myocardin are known to be essential for smooth muscle development and function (18). Having shown that FOXF2 was able to bind to the *myocardin* promoter, we investigated the effects of the FOXF proteins on myocardin/SRF signaling *in vitro*. Co-transfection experiments with an SRF-dependent luciferase reporter (telokin promoter) showed that both FOXF proteins, FOXF1 and FOXF2, can increase SRF dependent stimulation of the *telokin* reporter gene in the absence of myocardin, though FOXF2 to a lesser extent (Figure 7A). A mammalian two-hybrid assay, examining the binding of SRF to MYOCARDIN also showed that FOXF1 promotes the interaction of SRF with MYOCARDIN whereas FOXF2 does not (Figure 7B). Utilizing a glutathione pull-down assay we showed that FOXF2 protein has a direct, physical interaction with MYOCARDIN protein (Figure 7C). FOXF2 was found to bind strongly to the N-terminus of MYOCARDIN (Myo NT 1-585). FOXF2 weakly bound to SRF, the C-terminus of MYOCARDIN (Myo CT 585-935) and the N-terminus of the myocardin family member, MRTFA (Figure 7C). Further analysis of *myocardin* deletion mutants determined that FOXF2 binds to a region within the first 350 amino acids of MYOCARDIN (Figure 7D), which contains two RPEL motifs (58). Interestingly, this is distinct from FOXF1 which binds to the MYOCARDIN SAP domain (18).

To investigate functional consequences of FOXF2 binding to MYOCARDIN on expression of SRF target genes, adenoviral Cre-mediated deletion of *Foxf2-floxed* alleles was performed in a primary culture of colon smooth muscle cells purified from *Foxf2$^{fl/fl}$* mice. Foxf2 mRNA was nearly completely ablated indicating efficient *Foxf2* deletion from primary smooth muscle cells (Figure 7E). *Foxf2* deletion significantly increased Foxf1 mRNA (Figure 7E), a finding consistent with increased Foxf1 expression in Foxf2-deficient colons (Figure 5A). *Foxf2* knockdown increased expression of SRF target genes smooth muscle 22α (sm22α), αSMA, γSMA, smMHC and myocardin *in vitro* (Figure 7E). Furthermore, increased mRNA levels of SRF target genes were observed in colon tissue obtained from adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice (Figure 7F). Thus, *Foxf2*-deficiency in colonic smooth muscle cells causes increased expression of genes regulated by the SRF signaling pathway. Altogether, our results indicate that FOXF2 directly binds to myocardin and inhibits myocardin/SRF signaling in smooth muscle cells.

DISCUSSION

To circumvent perinatal lethality in *Foxf2$^{-/-}$* mice, we generated a novel mouse model in which *Foxf2* is selectively deleted from smooth muscle cells beginning at mid-gestation and throughout the life of the mouse. Loss of *Foxf2* from smooth muscle decreased the length of the intestinal tract, truncating both the small intestine and colon. *Foxf2* deletion resulted in increased thickness of longitudinal smooth muscle and expansion of the myenteric nerve plexus. PDGFRα-positive nerves have been previously observed in the GI tract (54), and staining of control and *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ colons showed PDGFRα clearly localized to the myenteric plexus with increased staining in *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice. *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ colons also had elevated expression of PDGFa and PDGFb. Increased PDGF signaling can contribute to expansion of the myenteric plexus in *Foxf2*-deficient colons as PDGF signaling has been previously shown to induce neural mitosis and hyperplasia (55,59,60). Interestingly, *Foxf2*$^{-/-}$ mice have been reported to lack ganglia in the colon (17), which is in stark contrast to the increased myenteric plexus observed in our *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice. The reason for this discrepancy could be due to the contribution of Foxf2-regulated signals from the lamina propria, which is not targeted by *smMHC-Cre-eGFP* and thus not *Foxf2*-deficient in our mouse model. Foxf2 may regulate expression of genes essential for neural crest cell migration, proliferation or survival. Alternatively the difference may be due to the timing in which *Foxf2* is deleted, as *smMHC-Cre-eGFP* is not detected prior to E12.5 (27), which is after the initiation of neural crest infiltration of the colon (61).

In addition to expression in enteric neurons, PDGFRα and PDGF ligands are expressed in colon smooth muscle (1,57). PDGF has been shown to be a potent mitogenic agent in smooth muscle cells (62), and a cell autonomous role for PDGF signaling in smooth muscle proliferation has been previously demonstrated (63). Although we did not observe any smooth muscle cell proliferation in the colon of adult *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice, we did observe increased proliferation during early postnatal development. Unlike vascular smooth muscle, smooth muscle of the GI tract continues to proliferate for the first few days after birth. By 12 days after birth, proliferation of colon smooth muscle cells in control mice appears to have ceased. In contrast, in *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice there was still detectable proliferation in the circular muscle layer of the colon and marked proliferation in the longitudinal muscle layer. The increased expression of PDGF ligands and receptor in *Foxf2*-deficient colons could thus be stimulating proliferation of the neonatal colonic smooth muscle and/or delaying cell cycle exit. It has been previously shown that PDGF ligands originate from the circular muscle layer and myenteric plexus of the gut and PDGFRα is more highly expressed in the longitudinal muscle than the circular smooth muscle layer (57). This expression pattern could explain why the circular muscle layer is less affected by *Foxf2* deletion, because it lacks the receptor to respond to PDGF ligands. Increased expression of PDGF proteins, however, does not appear to prevent colonic smooth muscle cells from ultimately exiting the cell cycle and differentiating. Cell cycle exit coupled with the direct increase in myocardin expression seen following loss of *Foxf2* likely contributes to increased expression of contractile proteins in the adult colon.

The experiments performed in this study provide novel insight into the *in vivo* signaling of Foxf2 as well as regulatory pathways for postnatal visceral smooth muscle development, particularly in the intestines. It was previously shown that FOXF1 and FOXF2 bind to the same consensus cis-acting regulatory element sequence (64). This together with analysis of global knockout and compound *Foxf1/f2* heterozygous mice suggests that Foxf1 and Foxf2 may be partially redundant (17). In contrast, our studies have clearly shown that Foxf1 and Foxf2 have quite distinct roles in GI smooth muscle cells. We have previously shown that loss of *Foxf1* from smooth muscle cells results in decreased contractile protein expression (18), whereas in this manuscript we found that loss of *Foxf2* increases expression of these genes. Binding experiments show that FOXF1 and FOXF2 bind to different regions of MYOCARDIN ((18) and this study) and the effects of this binding are quite distinct. FOXF2 is a weak activator of SRF-regulated genes as compared to FOXF1 and binding of FOXF1 to MYOCARDIN promotes its interaction with SRF, whereas the binding of FOXF2 to MYOCARDIN does not. Our data indicate that Foxf2 may act as a functional decoy for Foxf1, having only minimal co-stimulatory activity, at least as pertains to myocardin/SRF signaling. In addition, FOXF2 inhibits *Foxf1* promoter activity, which can further exacerbate diminished myocardin/SRF signaling.

In addition to regulating the activity of MYOCARDIN/SRF complexes through protein-protein interactions, FOXF2 also directly affects the expression of *myocardin* through binding to a forkhead site in the *myocardin* promoter. ChIP analysis showed that FOXF2 can bind to the promoter region of *myocardin*, implicating Foxf2 in transcriptional regulation of the *myocardin* gene. Our data indicates that Foxf2 influences GI tract development via regulation of PDGF, SRF and Hedgehog pathways, all of which have been shown to be critical for GI tract development and physiology. Foxf2 may directly or indirectly regulate expression of genes critical for all three pathways and through this regulation mediate postnatal intestinal development and maintenance.

In summary, we generated a novel mouse line containing *Foxf2-floxed* alleles and used it to create smooth muscle-specific *Foxf2* knockout mice. Utilizing this model we demonstrated a distinct phenotype for smooth muscle *Foxf2* deletion compared to $Foxf2^{-/-}$ mice. We also showed that FOXF2 bound to the promoter region of the *myocardin* gene as well as several members of the PDGF and Hedgehog signaling pathways. FOXF2 directly binds to and inhibits *Foxf1* promoter activity and regulates SRF signaling through a protein-protein interaction with MYOCARDIN. Via its capacity to regulate PDGF, Hedgehog and SRF signaling, Foxf2 is a critical regulator of GI tract development and maintenance.

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FOOTNOTES

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[3] Abbreviations used: GI, gastrointestianal; Fox, Forkhead box; smMHC, smooth muscle myosin heavy chain; P, postnatal day; E, embryonic day; ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; CGRP, calcitonin gene-related peptide; PDGF, platelet-derived growth factor.

FIGURE LEGENDS

Figure 1. Deletion of *Foxf2* from GI smooth muscle. *Foxf2* is expressed in the mouse GI tract from embryonic time points through adulthood. (A-B) Immunostaining with anti-FOXF2 antibodies showed restriction of FOXF2 to mesenchymal layers of the developing GI tract at E14.5 and E17.5. Postnatally, Foxf2 was expressed in lamina propria [L] and circular muscle layer [C] but was absent from the longitudinal muscle layer [LM] and epithelium [E]. (C) qRT-PCR analysis of adult whole organ mRNA showed that Foxf2 is more highly expressed in the jejunum and ileum than the duodenum of the small intestine and more highly expressed in the colon than small intestine. Expression levels were normalized to β-actin (n=3). (D) Schematic shows *Foxf2-floxed* targeting construct. LoxP sites (solid triangles) were inserted to flank the first exon [E1] of the *Foxf2* gene. Locations of primers [P] and sizes of PCR products are indicated. Primers used are listed in Table 1. *Foxf2*$^{fl/fl}$ mice were bred with *ACT-FLP1* mice to remove *neo* and later with *smMHC-Cre-eGFP* mice to delete *Foxf2* from smooth muscle cells. (E) Visualization of immunofluorescence in frozen-fixed cross sections of adult ileum and colon from *smMHC-Cre-eGFP* mice crossed with the Cre-dependent *mT/mG* reporter strain. Membrane bound eGFP is detected in the smooth muscle layers as well as the muscularis mucosa, but not lamina propria. meGFP is expressed stronger in the muscularis mucosa of colon compared to ileum. Abbreviations: CM, circular muscle. LM, longitudinal muscle. MP, myenteric nerve plexus. MM, muscularis mucosa. SMF, submucosal fibroblasts. EP, epithelium. (F-G) PCR analysis of mouse tail DNA shows the presence of *neo* cassette, *Cre* transgene, as well as wildtype and *Foxf2-floxed* alleles. Scale bar = 10 μm.

Figure 2. Decreased Foxf2 and length of intestinal tract in *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice. (A-B) FOXF2 staining and Foxf2 mRNA were significantly decreased in *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ embryonic intestines at E16.5 as well as adult small intestine and colon smooth muscle. Expression levels were analyzed by qRT-PCR and normalized to β-actin (n=4). *p<0.05 versus control. (C) Representative images of H&E stained adult small intestine and colon from *Foxf2*$^{fl/fl}$ and *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ mice are shown. (D) Gross view of intestines from adult *Tg(smMHC-Cre-eGFP*$^{+/-}$*);Foxf2*$^{-/-}$ and control *Foxf2*$^{fl/fl}$ mice indicated shorter intestinal tract in *Foxf2*-deficient mice. (E)

Measurements of intestinal tract confirmed it was significantly shorter in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice due to decreased length of small and large intestines. (n=4). Scale bar = 10 μm in A, 100 μm in C.

Figure 3. Focal inflammation in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* small intestine. (A) Adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* small intestines had mostly normal morphology with focal regions of inflammatory infiltration leading to disruption of villus structure, though muscle morphology remained normal. (B) CYTOKERATIN (CK) staining shows disruption of villus epithelium in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* small intestines. (C) H&E staining of control and *Foxf2*-deficient small intestines showed normal morphology at P12. Scale bar = 10 μm.

Figure 4. Increased muscle thickness and expanded myenteric plexus in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons. (A) H&E staining indicated increased muscle thickness in adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons. Enteric neurons [EN] are shown with arrows, whereas longitudinal [LM] and circular [CM] smooth muscle layers are indicated by brackets. (B) Measurement of muscle thickness was performed in 12 random colon sections with n=4 mice per group. The longitudinal muscle layer was significantly thicker (*p<0.05 versus control) in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons. (C) Wheat germ agglutinin (WGA) staining and (D) measurement of cell size in adult mice eliminated hypertrophy as the cause of muscle thickening. (E) Immunostaining with Ki-67 and cyclin D_1 showed increased myocyte proliferation (arrows) in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons at P12. Longitudinal and circular smooth muscle layers are shown with brackets while epithelial layer is indicated as E (arrowheads show epithelial proliferation). (F) mRNA levels of Cdc 25B, cyclin B_1 and cyclin D_1 were increased but Foxm1 mRNA was unaltered in P12 *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons. Expression levels were determined by qRT-PCR and normalized to β-actin mRNA (n=4). (G) CGRP and PDGFRα immunostaining showed expansion of myenteric plexus (arrows) in adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons. Insets show expanded CGRP staining in P12 *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons. Scale bar = 10 μm.

Figure 5. Regulation of gene transcription by Foxf2. (A) Increased expression of Foxf1, Wnt, BMP, as well as members of the PDGF, Hedgehog and SRF pathways in smooth muscle isolated from adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons as determined by qRT-PCR. (B) Indian Hedgehog expression was increased in whole colon RNA from adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* mice. Epidermal growth factor receptor (EGFR) expression was unaltered. Expression levels were normalized to β-actin (n=4). *p<0.05 versus control. (C) FOXF1-positive myocytes were more abundant in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* smooth muscle than control myocytes as shown by immunostaining of the adult colon. Despite prolonged proliferation and altered gene expression, muscle markers αSMA and γSMA were observed in *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colon. Fibrosis was not observed in adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons as determined by Masson's Trichrome staining of intestinal paraffin sections. (D) Vasculature was unaltered in adult *Tg(smMHC-Cre-eGFP$^{+/-}$);Foxf2$^{-/-}$* colons as visualized by PECAM-1 staining. Epithelial staining for FOXA2 and CYTOKERATIN was normal in adult colons. Scale bar = 10 μm.

Figure 6. FOXF2 binds to *PDGF, myocardin* and *Foxf1* promoter regions. (A-B) FOXF2 binds to the promoter regions of *PDGFa*, *PDGFb*, *PDGFRα*, *myocardin* and *Foxf1* genes as determined by ChIP analysis. ChIP was performed using a rhabdomyosarcoma cell line which stably expressed His- and Flag-tagged FOXF2 protein. Anti-His antibodies or control IgG were used for ChIP. (n=3) Negative control for ChIP included a DNA region 1.7 kb 3' to the *Foxf1* regulatory element (Foxf1 3' Region). Anti-Flag antibody was used for ChIP of *Foxf1* to verify results obtained with anti-His antibody. (C) Luciferase reporter gene assays show that FOXF2 inhibits transcriptional activity of the *Foxf1* promoter (-5.3 kb

Foxf1 + 3’ RE) in a dose-dependent manner (left panel). Conversely FOXF2 activated a reporter gene driven by multiple copies of a FOXF2 binding site (right panel). (n=3)

Figure 7. FOXF2 regulates myocardin/SRF signaling in smooth muscle cells. (A) FOXF1 and, to a lesser extent, FOXF2 stimulate a SRF-dependent *telokin* reporter in the absence of myocardin. *SRF*, *Foxf1* and *Foxf2* expression plasmids were co-transfected with the SRF-dependent *telokin* reporter gene into 10TI1/2 cells. (B) Mammalian two-hybrid assay examining the binding between SRF and MYOCARDIN in the presence or absence of FOXF1 or FOXF2. In these assays *SRF* was fused to the GAL4 DNA binding domain [BD] and *myocardin* to the GAL4 activation domain [AD]. Interaction between SRF-BD and MYOCARDIN-AD results in activation of a GAL4-dependent reporter gene (SRF/Myo+vector compared to SRF/pACT+vector: pACT- empty GAL4 activation domain vector). FOXF1 promotes MYOCARDIN binding to SRF while FOXF2 has no effect on MYOCARDIN/SRF binding. (C) GST-pull down assays in which bacterial expressed FOXF2 was incubated with SRF, MYOCARDIN or MRTFA-GST fusion proteins. Following extensive washing, glutathione bound fusion proteins were analyzed by Western blot (upper panel). Input GST-fusion proteins visualized by Ponceau staining are shown in the lower panel. FOXF2 protein physically binds to SRF, the N-terminal half of MYOCARDIN (Myo NT 1-585) more weakly to the C-terminal half of MYOCARDIN (Myo CT 585-935) and weakly with the N-terminal half of MRTFA but not the C-terminal half of MRTFA. (D) Upper panel, schematic representation of MYOCARDIN structural domains. Middle panel, GST-pulldown assays using various fragments of MYOCARDIN fused to GST. The GST-fusion protein bound FOXF2 is shown in the middle panel and GST-fusion protein inputs in the lower panel. FOXF2 specifically bound to the first 220 amino acids of the MYOCARDIN protein, which contains RPEL motifs. Weak binding was also observed to amino acids 221-350 of MYOCARDIN. (E) Adenoviral Cre-mediated deletion of $Foxf2^{fl}$ alleles from primary colon smooth muscle cells increased mRNA levels of Foxf1 as well as expression of myocardin/SRF target genes SM22α, αSMA, γSMA, smMHC and myocardin. mRNA expression levels were quantitated by qRT-PCR and normalized to β-actin (n=6). *p<0.05 versus control. (F) mRNA levels of myocardin/SRF target genes SM22α, αSMA, γSMA and smMHC were increased in adult $Tg(smMHC\text{-}Cre\text{-}eGFP^{+/-});Foxf2^{-/-}$ mouse colons (n=4).

Table1. ***Foxf2-floxed* allele mouse primers**

Primer name	**Sequence**
P1	TGGAGGAGTGTTCTCGAATGGAG
P2	CACTGGACGCCCTTGAGCTG
P3	CCAGAGGCCACTTGTGTAGC
P4	AAAGCTTGTGTCTAGCGGTTGTCCAC
P5	AGCATGTCTTCCTACTCGTTGGAG
P6	GCTCCTGCCGAGAAAGTATCC

Primer sets in table were used for genotyping from mouse tail DNA.

Table 2. Taqman Probes

Gene name	Assay number
acta2	Mm00725412_s1
actb	Mm00607939_s1
actg2	Mm00656102_m1
bmp4	Mm01321704_m1
ccnb1	Mm00838401_g1
ccnd1	Mm00432359_m1
cdc25b	Mm00499136_m1
Foxf1	Mm00487497_m1
Foxf2	Mm00515793_m1
Foxm1	Mm00514924_m1
gli1	Mm01160468_g1
gli2	Mm01293116_m1
myh11	Mm00443013_m1
myocd	Mm00455051_m1
pdgfa	Mm01205760_m1
pdgfb	Mm01298578_m1
pdgfra	Mm01211694_m1
ptch1	Mm00436026_m1
sox9	Mm00448840_m1
srf	Mm00491032_m1
tagln	Mm00441661_g1
wnt5a	Mm00437347_m1

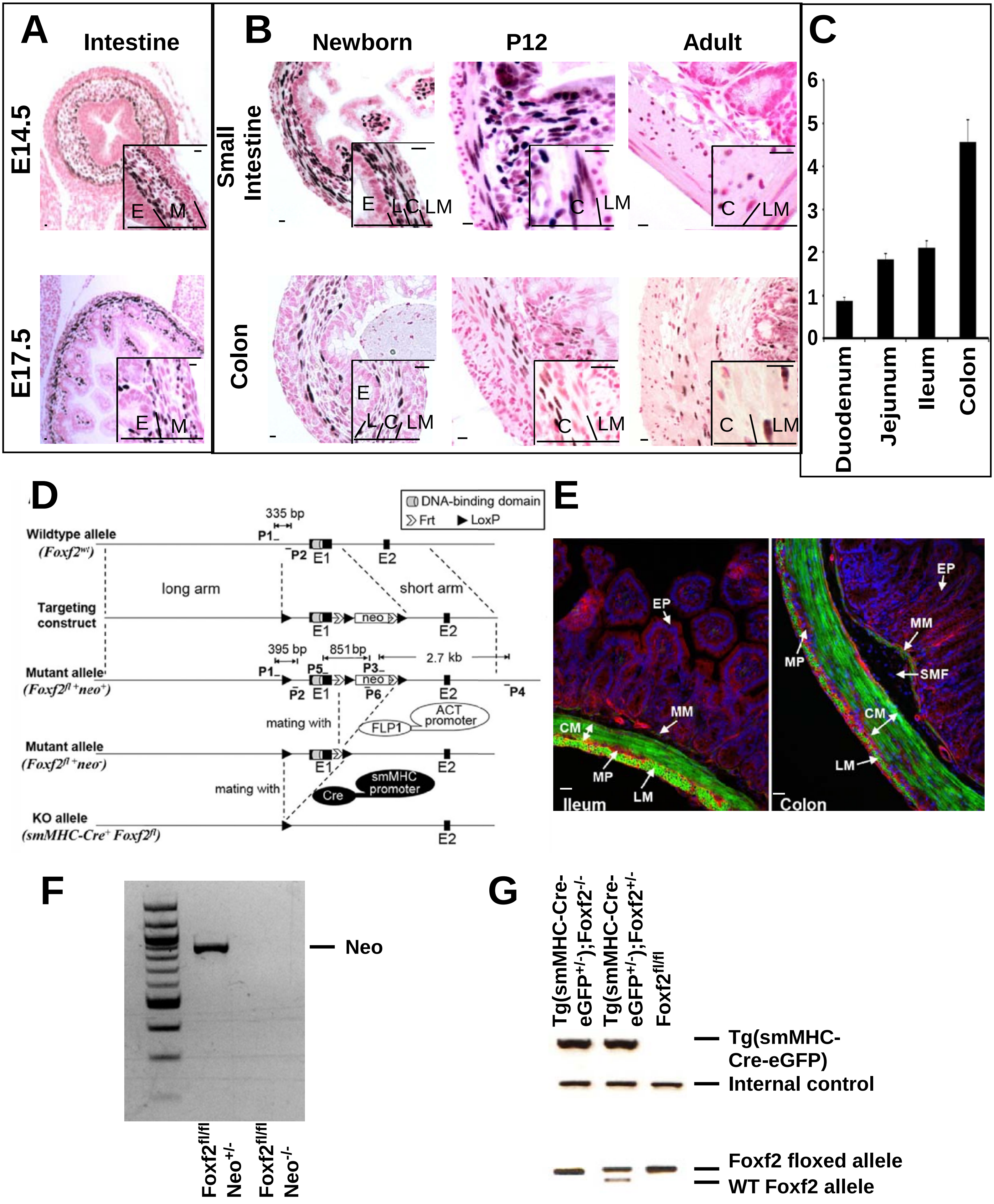
Table lists inventoried Taqman probes used for qRT-PCR analysis of gene expression.

Table 3. ChIP Primers

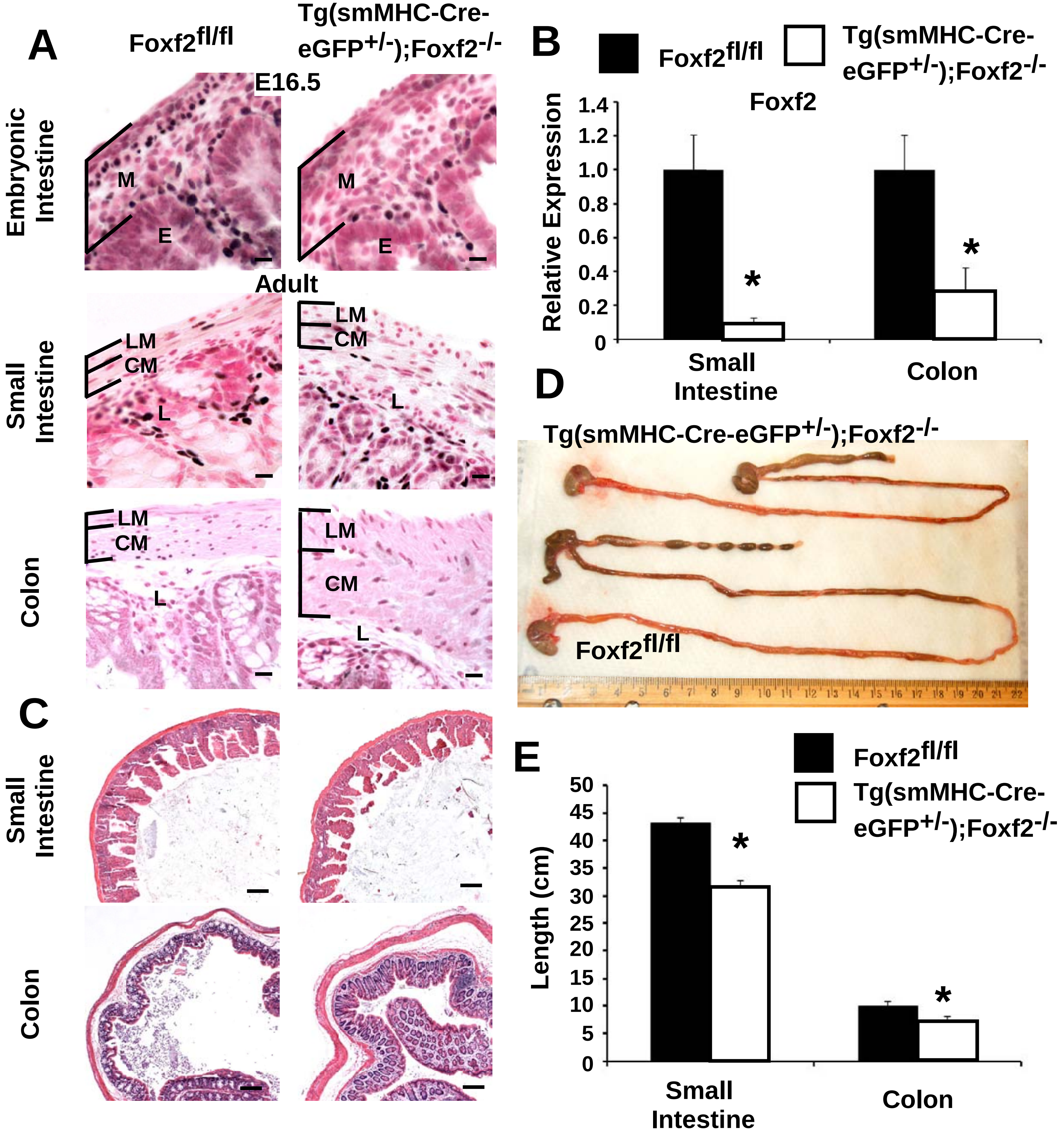
Gene (Promoter region)	Forward primer (5’-3’)	Reverse primer (5’-3’)
Foxf1 + Enhancer (248/255)	CCCCACCCCTAACGGATTATTTG	TCCCCTGCTTCTAAAAACTGTCCC
*Foxf1*3’Region (Negative Control)	ACCACCATCTCCATCACCAGCC	CCTCCCGTCCAGAATGTCAAGTC
Myocardin (-4532/-4525 & -4523/-4516)	CCTGGGACCACACAAACAAC	AGGCTCCGTCTCAGCGTG
PDGFa (-6421/-6414)	CCCATCATCCTCTCCTCTTTGC	GGTAGCCTTTAGAAATGTGTGCCAG
PDGFb (-1435/-1428)	TAGATGAGTTCTGGGACTGGACT	AGACATAACCGGAGGAGAAGAAG
PDGFRα (-531/-524)	TGTGCCTCCCAATGGATGAAATC	CCTCTGTGAGACGGAAGCCTGC

Primer sets in table were used in ChIP analysis of Foxf2 binding to gene promoter regions (listed in parenthesis).

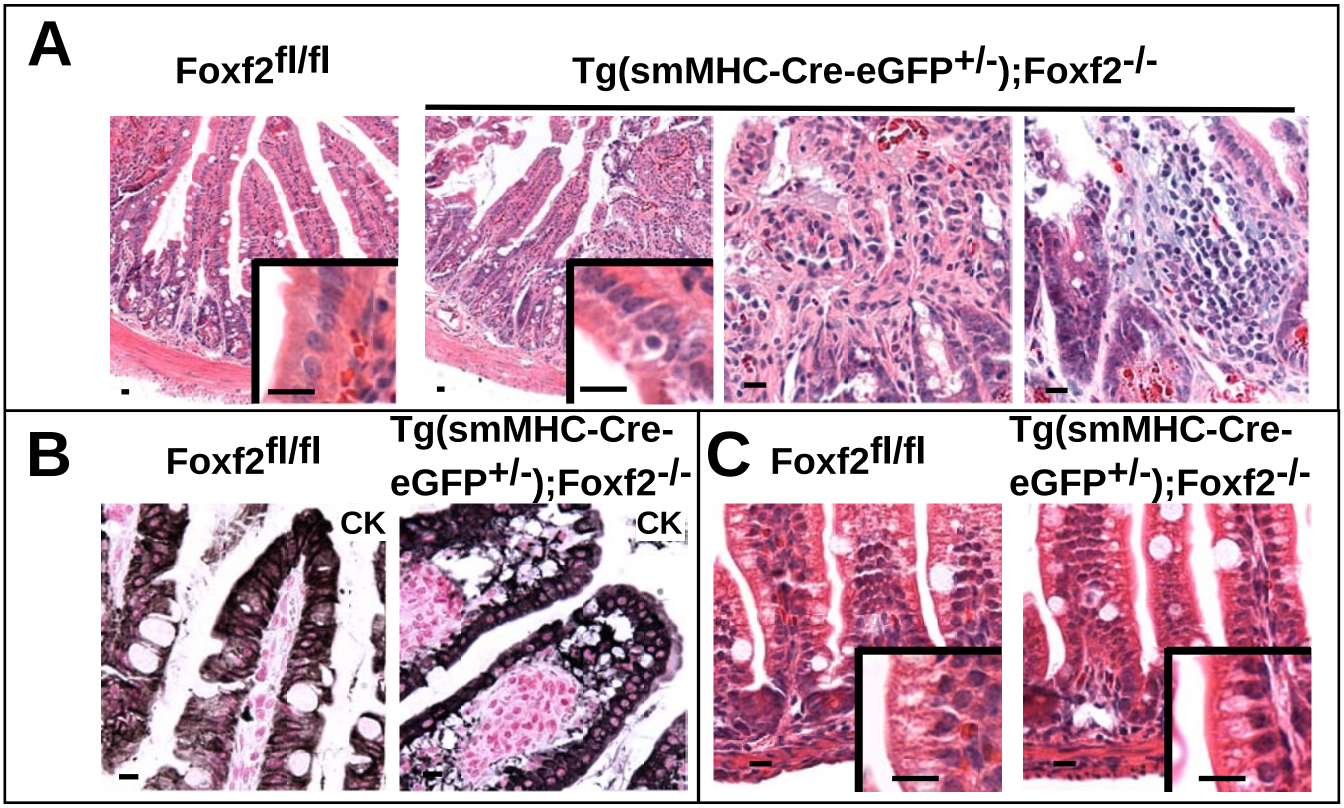
Bolte Figure 1



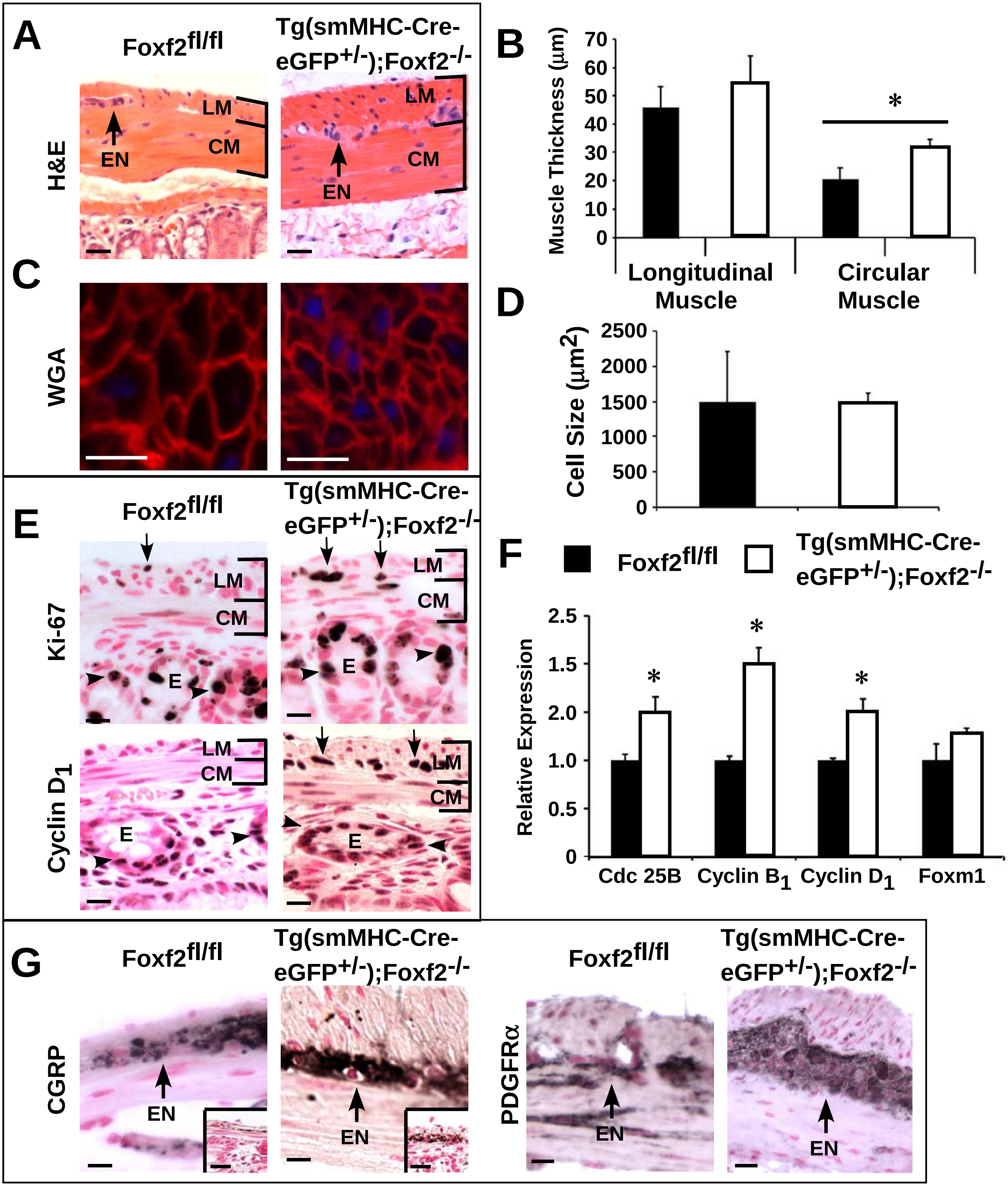
Bolte Figure 2



Bolte Figure 3



Bolte Figure 4



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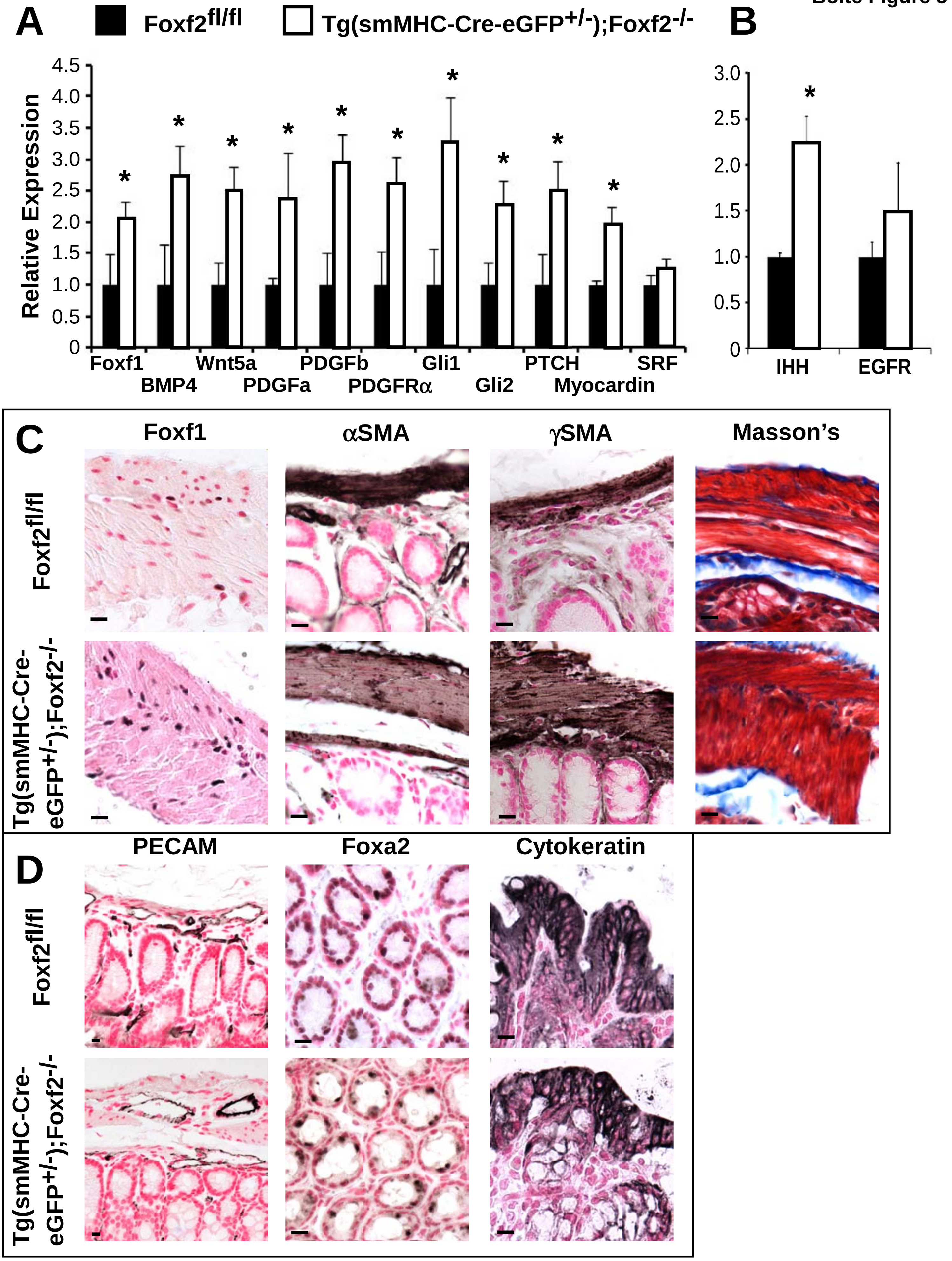
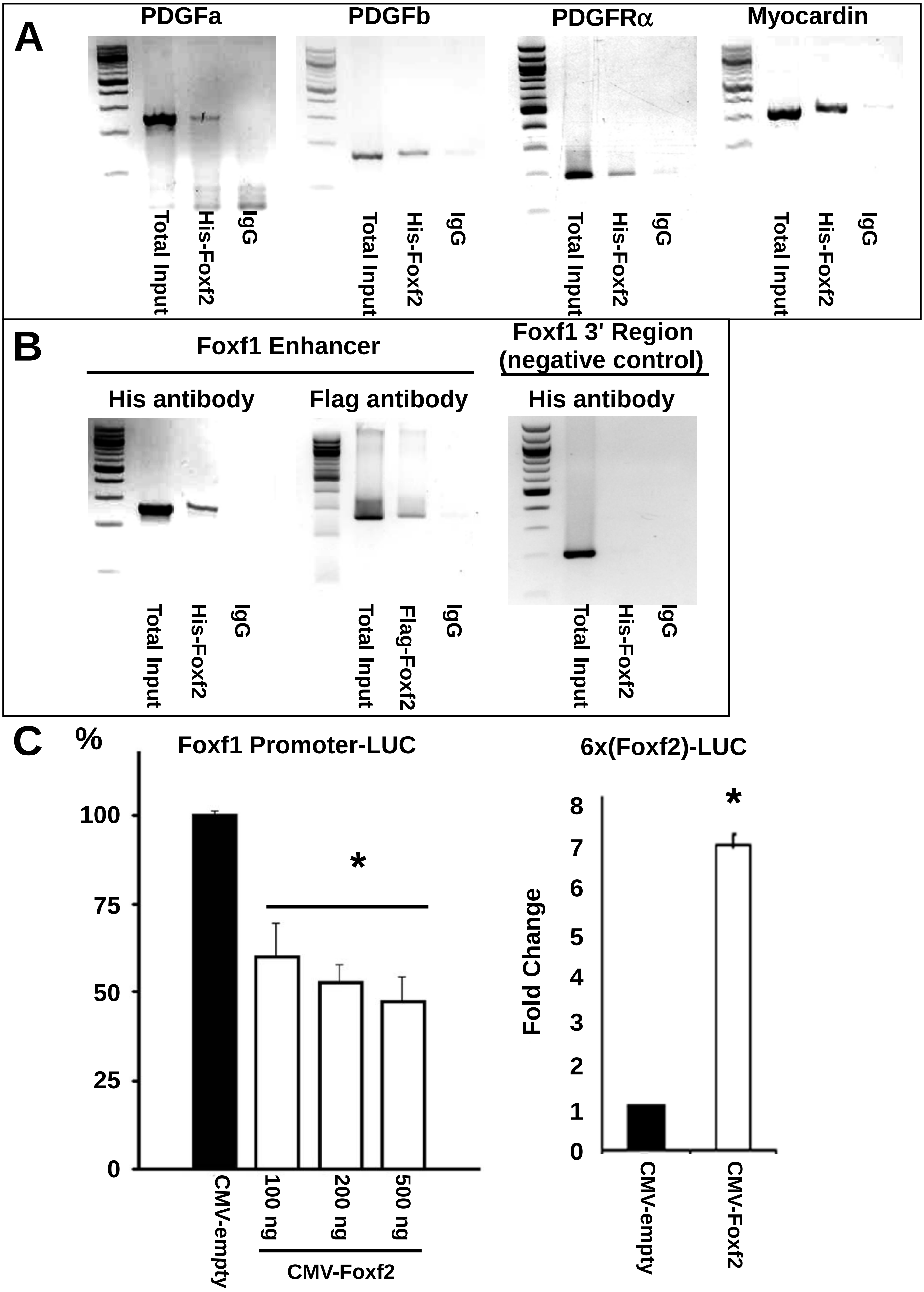


Bolte Figure 6



Bolte Figure 7