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Regulation of 130-kDa Smooth Muscle Myosin Light Chain Kinase Expression by an Intronic CArG Element*

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Background: Mechanisms regulating transcription of MLCK are poorly defined.
Results: Deleting a CArG element from the *mylk1* gene specifically decreased expression of the 130-kDa smMLCK isoform, resulting in decreased intestinal contractility and proliferation.
Conclusion: The 130-kDa smMLCK isoform has functions that cannot be compensated for by the 220-kDa MLCK.
Significance: Floxed *mylk1* mice permit specific functions of the 130-kDa smMLCK to be determined.

The *mylk1* gene encodes a 220-kDa nonmuscle myosin light chain kinase (MLCK), a 130-kDa smooth muscle MLCK (smMLCK), as well as the non-catalytic product telokin. Together, these proteins play critical roles in regulating smooth muscle contractility. Changes in their expression are associated with many pathological conditions; thus, it is important to understand the mechanisms regulating expression of *mylk1* gene transcripts. Previously, we reported a highly conserved CArG box, which binds serum response factor, in intron 15 of *mylk1*. Because this CArG element is near the promoter that drives transcription of the 130-kDa smMLCK, we examined its role in regulating expression of this transcript. Results show that deletion of the intronic CArG region from a β-galactosidase reporter gene abolished transgene expression in mice *in vivo*. Deletion of the CArG region from the endogenous *mylk1* gene, specifically in smooth muscle cells, decreased expression of the 130-kDa smMLCK by 40% without affecting expression of the 220-kDa MLCK or telokin. This reduction in 130-kDa smMLCK expression resulted in decreased phosphorylation of myosin light chains, attenuated smooth muscle contractility, and a 24% decrease in small intestine length that was associated with a significant reduction of Ki67-positive smooth muscle cells. Overall, these data show that the CArG element in intron 15 of the *mylk1* gene is necessary for maximal expression of the 130-kDa smMLCK and that the 130-kDa smMLCK isoform is specifically required to regulate smooth muscle contractility and small intestine smooth muscle cell proliferation.

The *mylk1* gene is a large gene spanning ~250 kb, comprising 31 exons (1). *mylk1* encodes at least three protein products: a 220-kDa MLCK,[3] a 130-kDa MLCK, and a non-catalytic gene product, telokin. Each transcript from the *mylk1* gene is derived from a unique independent promoter within the gene (1). The 220-kDa MLCK is also referred to as nonmuscle MLCK or endothelial MLCK, because it was first characterized in chick embryo fibroblasts and endothelial cells (2, 3). The 130-kDa MLCK is also called the smooth muscle MLCK (smMLCK), because it is most abundant in smooth muscle tissues; however, it is also widely expressed in other tissues at lower levels (1, 4, 5). Telokin is a non-catalytic product of the gene that is expressed at very high levels in intestinal, urinary, and reproductive tract smooth muscle; at low levels in vascular smooth muscle cells; and at undetectable levels in other tissues (6).

In the presence of Ca^{2+} and calmodulin, both the 220- and 130-kDa smMLCK can phosphorylate serine 19 of the 20-kDa myosin regulatory light chain of smooth muscle and nonmuscle myosin II. In smooth muscle cells, phosphorylation of the myosin regulatory light chain is an obligatory step for the initiation of contraction. In many other cell types, phosphorylation of regulatory light chain induced by MLCK is important for regulating actomyosin-based cytoskeletal functions, such as focal adhesion and stress fiber formation, secretion, cytokinesis, neurite growth cone advancement, endothelial and epithelial barrier formation, and cell migration (7–13). Alterations in MLCK expression have been linked to a variety of pathologies, including colitis (14), inflammatory bowel disease (15), asthma (16, 17), inflammatory lung disease (18), familial aortic dissection (19), and hypertension (20, 21). The specific functions of the various MLCK isoforms in these processes, however, are not clear. Global knock-out of the 220-kDa MLCK in mice results in numerous defects in epithelial and endothelial barrier function, suggesting that this isoform has a specific role in regulating these processes (22–26). Through specific targeting of a portion of the catalytic domain shared by the 220- and 130-kDa MLCKs, it has been possible to determine the combined roles of these kinases in specific tissues and cell types (27). As anticipated, ablation of both MLCK isoforms in smooth muscle cells resulted in impaired contractility and decreased myosin light chain phosphorylation (20, 27). Surprisingly, deletion of both 220- and 130-kDa smMLCK specifically from endothelial cells had very little effect on vascular permeability, bringing into question the importance of endothelial cell-expressed MLCK

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[3] The abbreviations used are: MLCK, myosin light chain kinase; smMLCK, smooth muscle MLCK; MLC, myosin light chain; SUMO, small ubiquitin-like modifier; SRF, serum response factor; qRT-PCR, quantitative RT-PCR.

in regulating endothelial barrier function (28). Because of the overlapping structure of the 220- and 130-kDa smMLCK, it is difficult to examine the function of the 130-kDa smMLCK without also affecting expression of the 220-kDa isoform. To address this issue, we examined regulatory elements that specifically regulate expression of the 130-kDa smMLCK with the hypothesis that deletion of these elements may attenuate expression of the 130-kDa smMLCK without effecting expression of the other products of the *mylk1* gene. Toward this goal, we previously identified a promoter within intron 14 of the *mylk1* gene that specifically directs expression of the 130-kDa smMLCK (29). Within this promoter, there is a conserved CArG element that binds to serum response factor (SRF) and is required for myocardin-induced expression of the 130-kDa MLCK (29).

The CArG element, $CC(A/T)_6GG$, is the cis-regulatory element that binds SRF, an evolutionarily conserved MADS (MCM1, agamous, deficiens, and SRF) domain-containing transcription factor. SRF binding and crystal structure studies have shown that a functional CArG element can deviate by no more than 1 bp from the consensus sequence (30). Virtually all known CArG elements reside within 4 kb of the transcription start site of genes (30). Using computational algorithm prediction approaches with experimental validation, a genome-wide screen identified 60 target genes that are regulated by CArG elements. Among these, 26 of the validated SRF target genes encode for cytoskeletal/contractile or adhesion proteins (30, 31). When bound to a CArG element, SRF also provides a docking surface for interaction with numerous accessory co-factors to form ternary complexes, conferring tissue- or pathway-specific expression of target genes. For example, ternary complexes of SRF and Elk1 are important for growth factor regulation of immediate early genes such as c-*fos* (32). In smooth muscle cells, ternary complexes of SRF together with myocardin or myocardin-related transcription factors are very powerful activators of numerous smooth muscle-specific contractile and regulatory proteins, such as the 130-kDa smMLCK (33). SRF and myocardin enhanced the activity of the 130-kDa smMLCK promoter reporter genes and induced expression of the 130-kDa smMLCK in 10T1/2 fibroblast cells, whereas GATA-6 repressed promoter activity, possibly through disrupting SRF-myocardin complexes (29). Besides the CArG element in the promoter region, there is another highly conserved CArG element in the first intron of the 130-kDa smMLCK (intron 15 of the *mylk1* gene). Chromatin immunoprecipitation assays confirmed that this intronic CArG element also binds to SRF *in vivo* in smooth muscle cells (29). However, the previous studies did not determine if this intronic CArG element affects the expression of the 130-kDa smMLCK *in vivo*. Here, we found that the intronic CArG element is important for regulating expression of transgenes driven by the 130-kDa smMLCK promoter *in vivo* and for driving expression of endogenous 130-kDa smMLCK in mice. Moreover, we show that targeting this element is an effective means to specifically decrease expression of the endogenous 130-kDa smMLCK without affecting expression of the 220-kDa MLCK or telokin.

EXPERIMENTAL PROCEDURES

Generation of Targeting Vector for Homologous Recombination—An *mylk1* targeting vector was generated by inGenious Targeting Laboratory (Stony Brook, NY). A 7.56-kb fragment containing about 5.1 kb extending 5′ and 2.16 kb extending 3′ to the intronic CArG region, was subcloned from a C57BL/6 BAC clone (RP23: 55O1, Source BioScience) into pSP72 vector (Promega). To construct the targeting vector for homologous recombination, a *loxP*/FRT-flanked Neo cassette was inserted 54 bp 3′ of the intronic CArG element, and a single *loxP* site containing engineered AflII and BamHI sites for Southern blot analysis was inserted 184 bp 5′ of the intronic element.

Generation of Transgenic Reporter Mice—The neomycin resistance cassette was removed from the targeting vector described above by FLP recombinase-mediated recombination in bacteria. The vector was then digested by FseI and PmlI to yield a 2.3-kb fragment that included the intronic CArG element and surrounding *loxP* sites. The 130-kDa smMLCK promoter, exon 1, intron 1, and a portion of exon 2 were cut from the pGL2B construct described previously (29) and ligated into the pWhere *lacZ* reporter vector (InvivoGen). The smMLCK(−389/+8427) pWhere vector was then cut by FseI and PmlI. The resulting 2-kb fragment that included the intronic CArG element was replaced with the corresponding 2.3-kb fragment isolated from the targeting vector to generate the I_{CArG}-smMLCK(−389/+8427) pWhere plasmid. The integrity of the plasmid was confirmed by restriction enzyme digestion and DNA sequencing. In order to delete the CArG element from the I_{CArG}-smMLCK(−389/+8427) pWhere plasmid, it was introduced into bacteria expressing Cre recombinase to generate plasmid ΔI_{CArG}-smMLCK(−389/+8427) pWhere. Correct excision of the CArG element was confirmed by DNA sequencing. I_{CArG}-smMLCK(−389/+8427) pWhere and ΔI_{CArG}-smMLCK(−389/+8427) pWhere plasmids were linearized and microinjected into pronuclei of fertilized oocytes by standard procedures by the Indiana University School of Medicine transgenic mouse facility. Neonatal founder mice were genotyped for the presence of the transgene and were analyzed at 1 month old by β-galactosidase staining as described previously (34). All animal experiments were conducted under the approval of the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Generation of Knock-out Mice—Knock-out mice were generated by inGenious Targeting Laboratory. The targeting construct was linearized using NotI prior to electroporation into C57BL/6N embryonic stem cells. Positively selected ES cells were screened by PCR and then expanded for Southern blot confirmation of targeting. Correctly targeted ES cells were microinjected into BALB/c blastocysts. Resulting chimeras with a high percentage black coat color were mated to C57BL/6 FLP mice to remove the Neo cassette. The deletion of the Neo cassette was screened and confirmed by PCR and DNA sequencing. Germ line floxed mice were mated to smMHC-Cre mice (from Michael Kotlikoff (Cornell University, Ithaca, NY)) (also on a C57BL/6 background) to delete the intronic CArG region specifically in smooth muscle tissues. Genotyping primers were as follows: MLCK P1, GGC AAG CCA AAC CCT TAC

ACA; MLCK P2, GAC TGG AGA TAA CCT CCT CTC ACT; Cre F, CCA ATT TAC TGA CCG TAC ACC; Cre R, GTA CGT GAG ATA TCT TTA ACC CTG AT. For further analysis of the recombined flox allele, primer P2 was used in combination with MLCK P3: GGA TGT GAG CTG CGC TTC TGA G.

Contractility Measurement of Isolated Colon Rings—The proximal part of the colon (with intact epithelial layer) was cut into rings 0.5 cm in length, and contractility was measured as described previously (35). A 1.5-g preload amount was empirically determined to result in the optimal contraction. To induce colon smooth muscle contraction, colon rings were challenged with 60 mM KCl or 1 μM carbachol, a muscarinic agonist.

Contractility Measurement of Isolated Thoracic Aorta—Thoracic aortas (with intact endothelium) were dissected carefully, and their branches were ligated with thread before being used. The aortas were connected to a physiological saline solution-filled tube that was pressurized with a regulator to inflate the vessels to the desired pressure before chemical stimulation. A pressure transducer (SPR-524, Microtip catheter transducer, Millar) was used to monitor the intraluminal pressure, and a volume compensator was used to compensate for water transport across the vessel wall. The vascular contraction during endothelin 1 stimulation was measured as changes in intraluminal pressure (36).

Quantitative RT-PCR—Total RNAs were extracted from colon smooth muscle (the epithelial layer was removed by scraping), whole bladder, and thoracic aorta of 6-week-old control and smooth muscle-specific 130-kDa MLCK knock-out mice. The mRNA expression levels were quantitated by reverse transcription-quantitative PCR as described previously (37). Because the 130-kDa smMLCK transcript has a unique 5′-UTR not present in the 220-kDa MLCK transcript, we were able to design primers to specifically detect the 130-kDa smMLCK mRNA. The primers used were as follows: 220-kDa MLCK F, GAA CCT CTG CAT CAA AGA AGG AG; 220 MLCK R, GAT GGC TTG CCC TTT TCT GTG CCA TG; 130-kDa smMLCK F, CTC TTG CTA CTT TCT CTT TTT CCT TCA CTG; smMLCK R, CTG GTC TCC ACC CGT CTC TTC AAC AG; Telokin F, GAC ACC GCC TGA GTC CAA CCT CCG; Telokin R, GGC TTT TCC TCA GCA ACA GCC TCC.

Western Blot Analysis—Total protein lysates from colon smooth muscle (epithelial layer removed by scraping) and whole bladder were extracted and analyzed as described previously (37). Antibodies used for detecting MLCK were a polyclonal antibody raised against the common carboxyl terminus of MLCK and telokin (CT polyclonal) (38) and a polyclonal antibody raised against the full-length bovine smMLCK (FL polyclonal) (39). Vinculin was used as loading control (V4505, clone VIN-11-5, Sigma-Aldrich).

Myosin Light Chain (MLC) Phosphorylation—The proximal portion of the colon (epithelia intact) was cut into 0.5-cm-long circular rings, and the rings were hung in an organ bath, as described above for contractility measurements of colon. Tissues were flash-frozen in the basal non-contracted status or at the peak of contraction induced by 60 mM KCl. The phosphorylation levels of MLC were analyzed by Western blotting of proteins separated on urea/glycerol gels, as described previously (40).

Cell Proliferation—The intestines of littermate neonatal mice (day 9–10) were dissected, and the lowest portions of the ileum were incubated in 20% sucrose in PBS solution overnight at 4 °C. Tissue samples were frozen into Tissue-Tek O.C.T. compound (catalog no. 4583, Sakura), and 7-μm sections were cut. Sections were fixed with 3.7% formaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 10% FCS in 50 mM Tris, pH 7.6, 150 mM NaCl and then incubated with antibodies against Ki67 (catalog no. 15580, Abcam; 1:500) and smooth muscle α-actin (catalog no. A2547, Sigma; 1:500). Primary antibodies were visualized by incubation with rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:50) and FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch; 1:50) secondary antibodies.

Statistical Analysis—The χ^2 test was used to determine if the observed birth frequency of knock-out mice was lower than expected. For other statistical comparison, Student's t tests were performed (Prism, GraphPad Software). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Deletion of an Intronic CArG Element in the mylk1 Gene Abolished Transgene Expression Driven by the 130-kDa smMLCK Promoter—Previously, we reported a highly conserved CArG element located in intron 15 of the *mylk1* gene (first intron of the 130-kDa smMLCK) (29). To investigate the role of this intronic region in regulating 130-kDa smMLCK gene expression, we generated transgenic mice in which a *lacZ* reporter was driven by the 130-kDa smMLCK promoter, exon 1, intron 1, and a portion of exon 2 with (I_{CArG}-smMLCK(−389/+8427) pWhere) or without (ΔI_{CArG}-smMLCK(−389/+8427) pWhere) this intronic CArG region, as described under "Experimental Procedures." In two of the three independent founders harboring the wild type transgene, high levels of β-galactosidase staining were observed in visceral smooth muscle rich tissues, such as bladder, colon, small intestine, and ureters (Fig. 1). The third line had lower levels of expression but in a similar pattern (not shown). One of the two high expressing founders also exhibited staining in bronchi as well as in the lung and small vessels of skeletal muscle and liver (Fig. 1). In contrast to the wild type transgenes, no β-galactosidase expression could be detected in any of the seven founder mice harboring the CArG-deleted transgene (Fig. 1). Weak background staining seen in bladder, kidney, and colon is similar to that seen in non-transgenic mice (Fig. 1, *bottom right*). These data demonstrate that the intronic CArG region is critical for expression of a 130-kDa smMLCK-driven transgene.

Deletion of the Intronic CArG Region from the Endogenous mylk1 Gene Resulted in Decreased Expression of the 130-kDa smMLCK—To determine if deletion of the intronic CArG region would decrease expression of the endogenous 130-kDa smMLCK, we crossed mice harboring the floxed CArG region with mice expressing Cre recombinase under the control of the smooth muscle myosin heavy chain promoter (Fig. 2) (41). We have previously shown that this Cre transgene results in high levels of recombination specifically in smooth muscle tissues (35). PCR analysis of genomic DNA isolated from colon and aorta of knock-out and control mice further showed that there

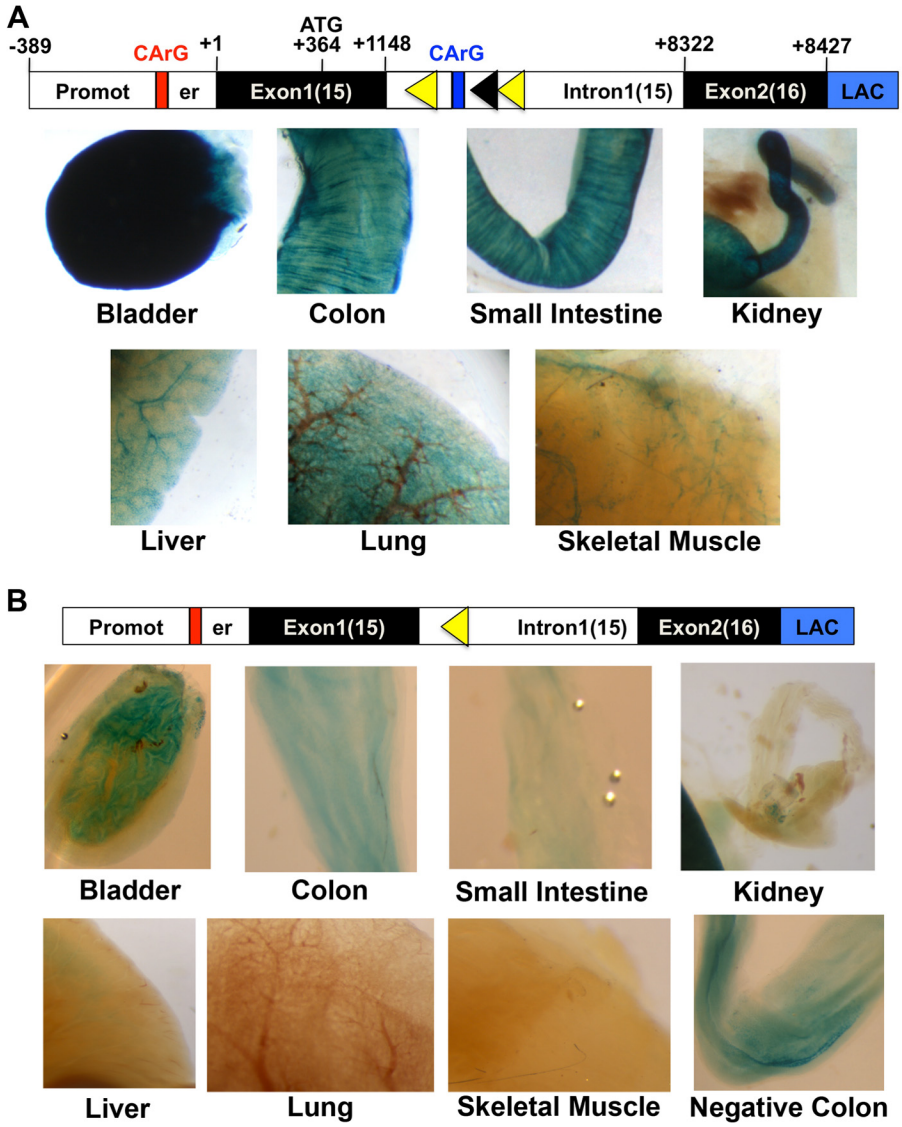
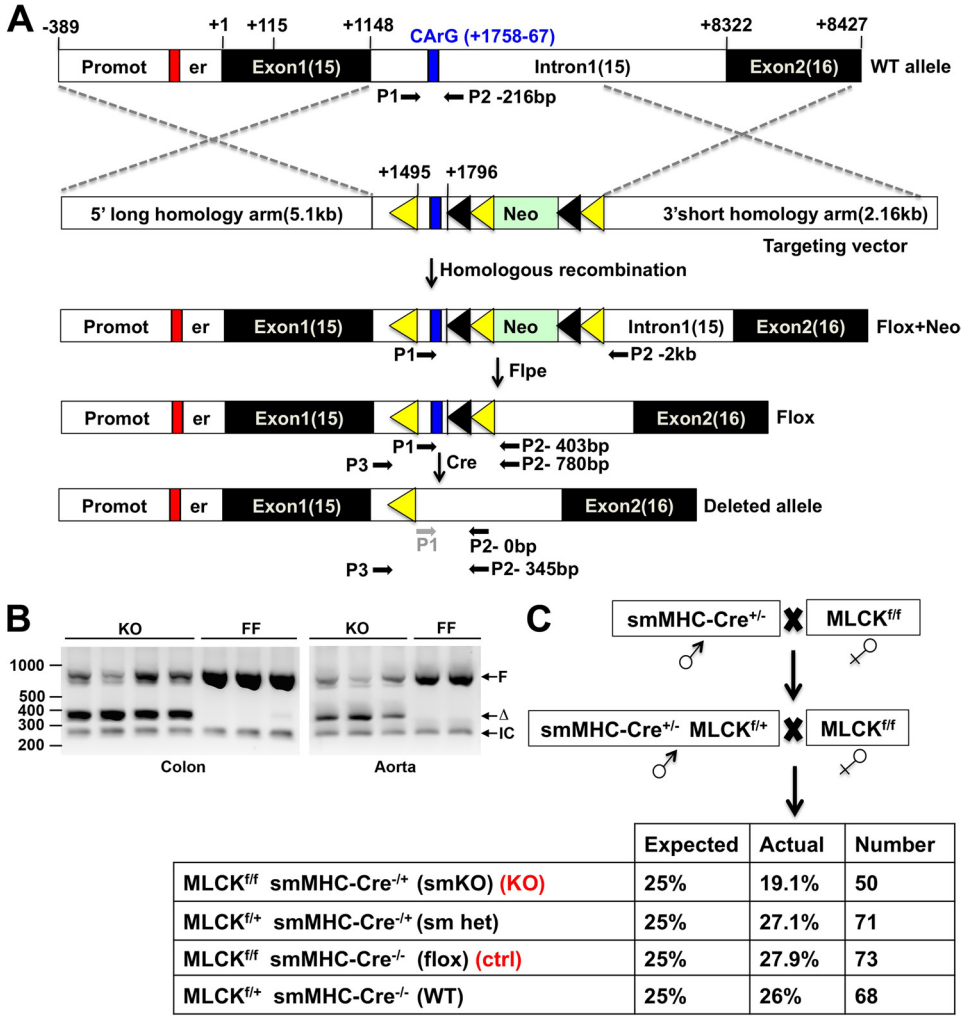


FIGURE 1. **Comparison of transgene expression in reporter mice.** *A*, organs were harvested from 1-month-old reporter mice harboring the I_{CArG}-smMLCK(−389/+8427) pWhere transgene (shown schematically at the *top*), and *lacZ* expression was examined by X-Gal staining (*blue/green color*, $n = 3$). *Yellow triangle*, *loxP* site; *black triangle*, FRT site; *blue box*, intronic CArG element; *red box*, promoter CArG element. *B*, β-galactosidase expression in organs harvested from 1-month-old mice harboring the ΔI_{CArG}-smMLCK(−389/+8427) pWhere transgene (*schematic*, *top*) in which the intronic CArG element is deleted (images are representative of seven founder mice). *Bottom right*, β-galactosidase staining of colon from a nontransgenic mouse as a negative control (*Negative Colon*).

is more efficient recombination of the floxed allele in colon smooth muscle as opposed to aortic smooth muscle (Fig. 2*B*). Although the recombined allele was readily detectable in both tissues, there also remained significant amounts of the non-recombined floxed allele. The latter probably represents a combination of less than 100% efficiency of recombination, together with contamination from other cell types in the sample. Of note, we did not detect the recombined allele in the majority (about 75%) of our control flox/flox mice. This is in contrast to a previous report, which showed that transient expression of Cre recombinase driven by the smMHC promoter in sperm resulted in recombination of the floxed allele derived from the male Cre-positive parent in almost all mice (43). Recombination of the floxed allele in sperm results in progeny that are $CArG^{f/-}$ (global heterozygous) rather than the expected $CArG^{f/f}$. Both control ($Cre^{-/-}$ $CArG^{f/f}$ and $Cre^{-/-}$ $CArG^{f/-}$) and smooth muscle-specific CArG knock-out ($Cre^{-/+}$ $CArG^{f/f}$ and $Cre^{-/+}$ $CArG^{f/-}$) mice reached adulthood without any obvious growth and behavioral abnormalities. However, knock-out mice were born with a slightly lower than expected frequency of 19% as compared with 25% (χ^2 test $p = 0.033$, degree of freedom = 1; Fig. 2*C*). This suggests that deletion of the intronic CArG region from the native gene results in partial embryonic lethality or neonatal death. Quantitative real-time RT-PCR showed that there is an ~40% decrease in 130-kDa smMLCK mRNA levels of $CArG^{f/f}$ $Cre^{-/+}$ knock-out mice compared with control $CArG^{f/f}$ $Cre^{-/-}$ mice, whereas there was no significant alteration in 220-kDa MLCK or telokin mRNA expression levels (Fig. 3*A*). Similar results were seen when $CArG^{f/-}$ $Cre^{-/+}$ knock-out mice were compared with $CArG^{f/-}$ $Cre^{-/-}$ control mice. Because we did not see any significant differences in 130-kDa smMLCK expression between



	Expected	Actual	Number
MLCK$^{f/f}$ smMHC-Cre$^{-/+}$ (smKO) (KO)	25%	19.1%	50
MLCK$^{f/+}$ smMHC-Cre$^{-/+}$ (sm het)	25%	27.1%	71
MLCK$^{f/f}$ smMHC-Cre$^{-/-}$ (flox) (ctrl)	25%	27.9%	73
MLCK$^{f/+}$ smMHC-Cre$^{-/-}$ (WT)	25%	26%	68

FIGURE 2. **Generation of the intronic CArG knock-out mice.** *A*, schematic representation of the approach used to delete the intronic CArG region from the endogenous *mylk1* gene. The native *mylk1* gene is shown at the *top* with the targeting vector *below* it. The *numbers above* the native gene refer to nucleotide positions relative to the transcription start site of the 130-kDa smMLCK. The promoter and intronic CArG boxes are indicated (*red* and *blue boxes*, respectively). *Yellow triangles*, *loxP* sites; *black triangles*, FRT sites. *Below* the targeting vector are *schematic representations* of the correctly targeted allele (*Flox*+*Neo*), the targeted allele following FLP-mediated removal of the neomycin cassette (*Flox*), and the deleted allele generated following Cre-mediated recombination (*Deleted allele*). Positions of primers used for genotyping are indicated. *B*, ethidium bromide-stained agarose gel showing an example of PCR analysis of recombination of the intronic CArG element using primers P3 and P2 (*A*) and genomic DNA isolated from colon smooth muscle and aortic tissues. The bands corresponding to the floxed (*F*) and recombined or deleted (Δ) alleles and an internal loading control (*IC*) are indicated. *C*, breeding scheme used to generate knock-out mice together with the genotypes of the progeny, their expected frequency, observed frequency, and total numbers of pups analyzed.

the two control strains (CArG$^{f/f}$ Cre$^{-/-}$ and CArG$^{f/-}$ Cre$^{-/-}$), and our standard genotyping does not distinguish between these strains, in all subsequent experiments, control mice were a mixture of CArG$^{f/f}$ Cre$^{-/-}$ and CArG$^{f/-}$ Cre$^{-/-}$. Similarly, all smooth muscle-specific knock-out mice were a mixture of CArG$^{f/f}$ Cre$^{-/+}$ and CArG$^{f/-}$ Cre$^{-/+}$.

Similar to colon, we observed ~40 and 30% reductions in 130-kDa smMLCK mRNA levels in the bladder and aorta of knock-out mice, respectively (Fig. 3*B*). Moreover, using an antibody to the common carboxyl terminus of MLCK and telokin, we found that deletion of the intronic CArG region reduced 130-kDa smMLCK protein expression by ~30 and 40% in colon and bladder, respectively (Fig. 3, *C* and *D*). Similar findings were observed using an MLCK antibody raised against the full-length bovine smMLCK (Fig. 3*D*). Additional experiments confirmed that the presence of the *loxP* sites in the control mice did not alter 130-kDa smMLCK expression compared with wild type mice (Fig. 3, *E*–*G*).

Deletion of the Intronic Region and Subsequent Decreases in 130-kDa smMLCK Expression Attenuated Smooth Muscle Contractility—In order to investigate whether the decreased expression of the 130-kDa smMLCK affects the contractility of both visceral and vascular smooth muscle, we analyzed the contractility of colon and aortic segments, *ex vivo*. Contraction elicited by high KCl-induced depolarization of colon from knock-out mice was dramatically decreased compared with control mice (Fig. 4, *A* and *C*). Similarly carbachol-induced contractions were also impaired in tissue from knock-out mice (Fig. 4, *B* and *C*). The L-type calcium channel inhibitor diltiazem blocked the contractile responses to high KCl in all mice (data not shown). ET1-mediated contraction of aortic segments was also decreased in knock-out mice compared with controls (Fig. 4*D*).

Decreased Contraction of Smooth Muscle Tissues in Knock-out Mice Was Associated with Decreased Myosin Light Chain Phosphorylation—The 130-kDa smMLCK induces contraction of smooth muscle by phosphorylating the regulatory myosin

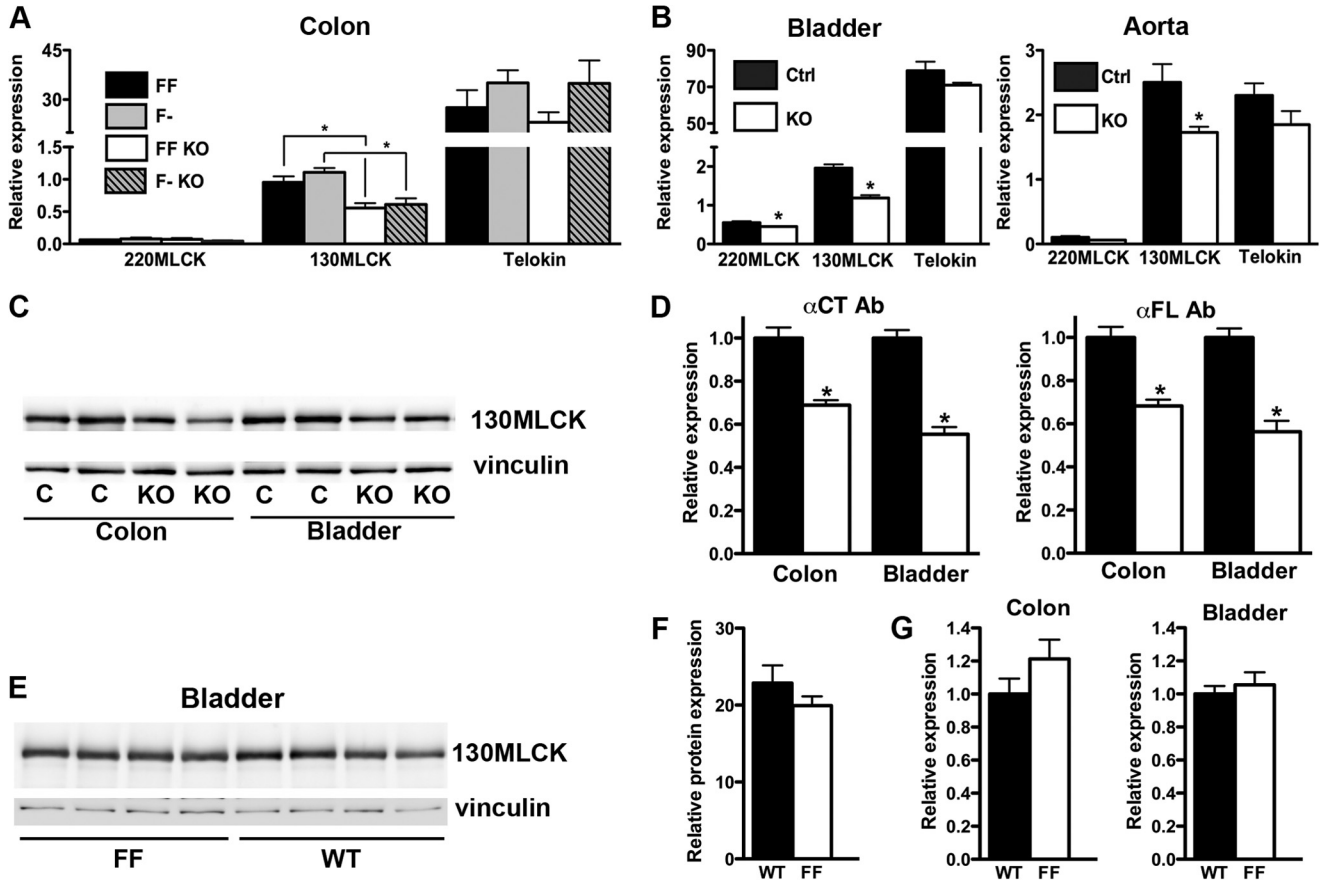


FIGURE 3. **The expression of the 130-kDa smMLCK is decreased in CArG knock-out mice.** *A*, smooth muscle layers were isolated from the colon of control ($CArG^{f/f}$ $Cre^{-/-}$ (*FF*) and $CArG^{f/-}$ $Cre^{-/-}$ (*F−*)) and knock-out ($CArG^{f/f}$ $Cre^{-/+}$ (*FF KO*) and ($CArG^{f/-}$ $Cre^{-/+}$ (*F− KO*)) mice, and total RNA was harvested. mRNA levels were measured by qRT-PCR. Transcript levels were normalized to *hprt* internal loading control, and relative expression levels (*RQ*) are shown. Relative expression $= 2^{-\Delta Ct}$, where $\Delta Ct = (Ct_{expt} - Ct_{hprt})$. Each *column* represents the mean ± S.E. (*error bars*) of samples obtained from 5–14 mice. *, $p < 0.05$. *B*, mRNA levels in bladder and thoracic aorta from control ($CArG^{f/f}$ $Cre^{-/-}$ + $CArG^{f/-}$ $Cre^{-/-}$ combined (*Ctrl*)) and knock-out ($CArG^{f/f}$ $Cre^{-/+}$ + $CArG^{f/-}$ $Cre^{-/+}$ combined (*KO*)) mice were measured by qRT-PCR. Transcript levels were quantitated as described in *A*. Each *column* represents the mean ± S.E. of samples obtained from 11–13 mice. *, $p < 0.05$. *C*, representative Western blot of 130-kDa smMLCK and vinculin in colon and bladder from two control (*C*) and two knock-out (*KO*) mice. *D*, quantitation of Western blots using different antibodies for smMLCK. Data were normalized to vinculin levels and are expressed relative to expression levels in control mice. *CT Ab*, a polyclonal antibody raised against the common carboxyl terminus of MLCK and telokin; *FL Ab*, a polyclonal antibody, raised against the full-length bovine smMLCK. $n = 11–13$. *, $p < 0.05$. *E*, Western blot analysis and quantitation (*F*) of 130-kDa smMLCK protein expression in bladder tissue from wild type ($CArG^{+/+}$ $Cre^{-/-}$; *WT*) and $CArG^{f/f}$ $Cre^{-/-}$ (*FF*) mice. *G*, qRT-PCR analysis of 130-kDa smMLCK mRNA expression in bladder and colon smooth muscle from wild type ($CArG^{+/+}$ $Cre^{-/-}$; *WT*) and flox/flox ($CArG^{f/f}$ $Cre^{-/-}$; *FF*) mice. No significant differences in 130-kDa smMLCK expression were observed. $n = 4$ (bladder). $n = 7$ (colon).

light chain. Thus, we sought to determine if the impaired contractile responses seen in the knock-out mice were associated with altered MLC phosphorylation. Under basal resting conditions, levels of MLC phosphorylation in both control and knock-out mice were very low and showed no significant difference (Fig. 4*E*). However, at the peak of contraction induced by high KCl, the level of phosphorylation of the MLC in knock-out mice was much less than that seen in control mice (18.3% as compared with 39.3%; Fig. 4*E*).

Decreased 130-kDa smMLCK Expression Resulted in Shortened Small Intestine—Further analysis of both control and knock-out mice showed that the length of small intestine was shorter by about 24% in the intronic CArG knock-out mice, whereas the length of colon was not significantly different (Fig. 5, *A* and *B*). The shorter small intestine was not associated with any change in body weight (Fig. 5*C*). We also did not detect any inflammation in knock-out mice, as determined by lack of changes in mRNA expression of inflammatory genes, IL1β, IL6, CCL2, Trem1, or CXCL10 (Fig. 5*D*). Immunofluorescence staining of cross-sections of ileum from neonatal mice for the cell proliferation marker Ki67 revealed that there were fewer positively stained smooth muscle cells in both circular and longitudinal smooth muscle layers of knock-out mice (Fig. 6).

DISCUSSION

Results of this study clearly show that a CArG box-containing region in intron 15 of the *mylk1* gene is required for expression of the 130-kDa smMLCK. Deletion of this intronic CArG box attenuated expression of the 130-kDa smMLCK without affecting expression of either the 220-kDa MLCK or telokin. Moreover, decreased expression of only the 130-kDa smMLCK in smooth muscle tissues attenuated smooth muscle contractility, which was associated with decreased myosin light chain phosphorylation and impaired small intestine smooth muscle cell proliferation. This demonstrates that the 130-kDa smMLCK isoform is specifically required to regulate not only smooth muscle contraction, but also intestinal smooth muscle cell proliferation.

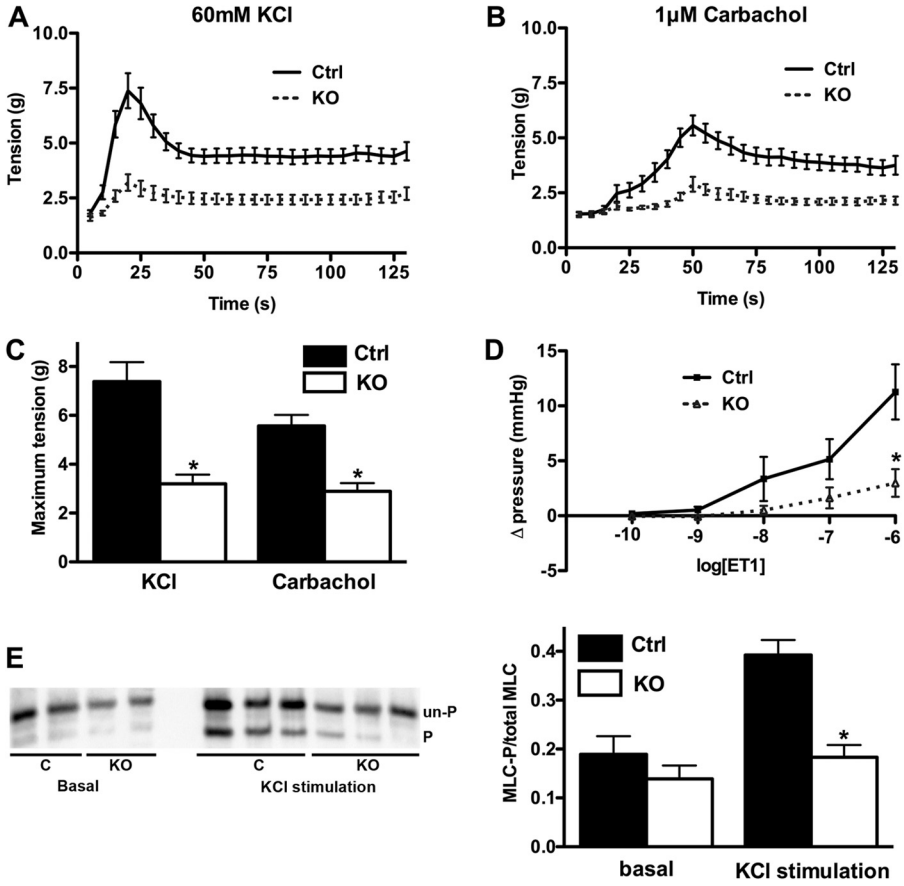


FIGURE 4. **Smooth muscle contractility and myosin light chain phosphorylation are decreased in knock-out mice.** *A* and *B*, colon rings were hung in an organ bath and stimulated to elicit contraction with 60 mM KCl or 1 μM carbachol as described under "Experimental Procedures." Data shown are the mean ± S.E. (*error bars*) changes in tension over time of 20 rings from control (CArG$^{f/f}$ Cre$^{-/-}$ + CArG$^{f/-}$ Cre$^{-/-}$; *Ctrl*) mice and 12 from knock-out (CArG$^{f/f}$ Cre$^{-/+}$ + CArG$^{f/-}$ Cre$^{-/+}$; *KO*) mice. *C*, the average changes in peak contractile responses of colonic rings from control (*Ctrl*) and knock-out (*KO*) mice. $n = 20$ for control; $n = 12$ for knock-out. *, $p < 0.05$. *D*, mean maximal contractile responses of thoracic aortic to increasing doses (*M*) of endothelin 1 (*ET1*). $n = 6$ for control, $n = 5$ for knock-out. *, $p < 0.05$. *E*, colon rings were either flash-frozen under resting conditions or challenged by 60 mM KCl and flash-frozen at the peak of contraction. Unphosphorylated and phosphorylated myosin light chains were separated by urea/glycerol gel electrophoresis and visualized by Western blotting. A representative Western blot is shown in the *left panel*. *un-P*, unphosphorylated MLC; *P*, phosphorylated MLC; *C*, CArG$^{f/f}$ Cre$^{-/-}$ and CArG$^{f/-}$ Cre$^{-/-}$ control; *KO*, CArG$^{f/f}$ Cre$^{-/+}$ and CArG$^{f/-}$ Cre$^{-/+}$. The ratios of MLC-P to total MLC under basal conditions ($n = 6$) and after KCl stimulation ($n = 10$) were calculated, and the mean values ± S.E. are plotted in the *right panel*. *, $p < 0.05$.

Deletion of both the 220- and 130-kDa MLCK from smooth muscle tissues has been previously shown to impair contractility, MLC phosphorylation, and gastrointestinal motility in mice (27). The current studies suggest that it is primarily the 130-kDa smMLCK rather than the 220-kDa MLCK that is responsible for regulating contraction in gastrointestinal smooth muscle. This is consistent with the lack of reported effects on smooth muscle contractility in the 220-kDa MLCK knock-out mice (44). Results also suggest that the 130-kDa smMLCK has a specific role in regulating the proliferation of small intestinal smooth muscle cells during early neonatal growth (Fig. 6). Although MLCK and myosin light chain phosphorylation are known to be important in cell division, these are the first data that suggest a specific role for the 130-kDa smMLCK isoform in this process. This conclusion should, however, be viewed with caution because it is possible that the alterations in proliferation are secondary to impaired contractility. In vascular, airway, and bladder smooth muscle, mechanical strain can induce smooth muscle cell proliferation (45–47). A decreased mechanical stimulus in CArG knock-out mice may thus also impair intestinal smooth muscle cell proliferation, resulting in attenuated intestinal elongation.

Deletion of the intronic CArG region completely abrogated expression of a 130-kDa smMLCK-*lacZ* reporter transgene in the visceral smooth muscle tissues, whereas deletion of this element from the endogenous gene only decreased endogenous 130-kDa smMLCK expression by about 40%. The relatively small decrease in 130-kDa smMLCK expression may be partially due to incomplete recombination of the floxed alleles by Cre recombinase. In support of this, PCR analysis of genomic DNA isolated from colon and aorta demonstrates variable levels of floxed alleles remaining in these tissues (Fig. 2*B*). Alternatively, it is also possible that the reporter transgene is missing additional positive cis-acting regulatory elements that play a role in regulating expression of the endogenous 130-kDa smMLCK. In support of this possibility, a Notch-responsive element has been identified at −3687 that plays an important role in activating 130-kDa smMLCK expression in vascular smooth muscle cells (48). This element is not present in the reporter genes described in our study, which extend from −389

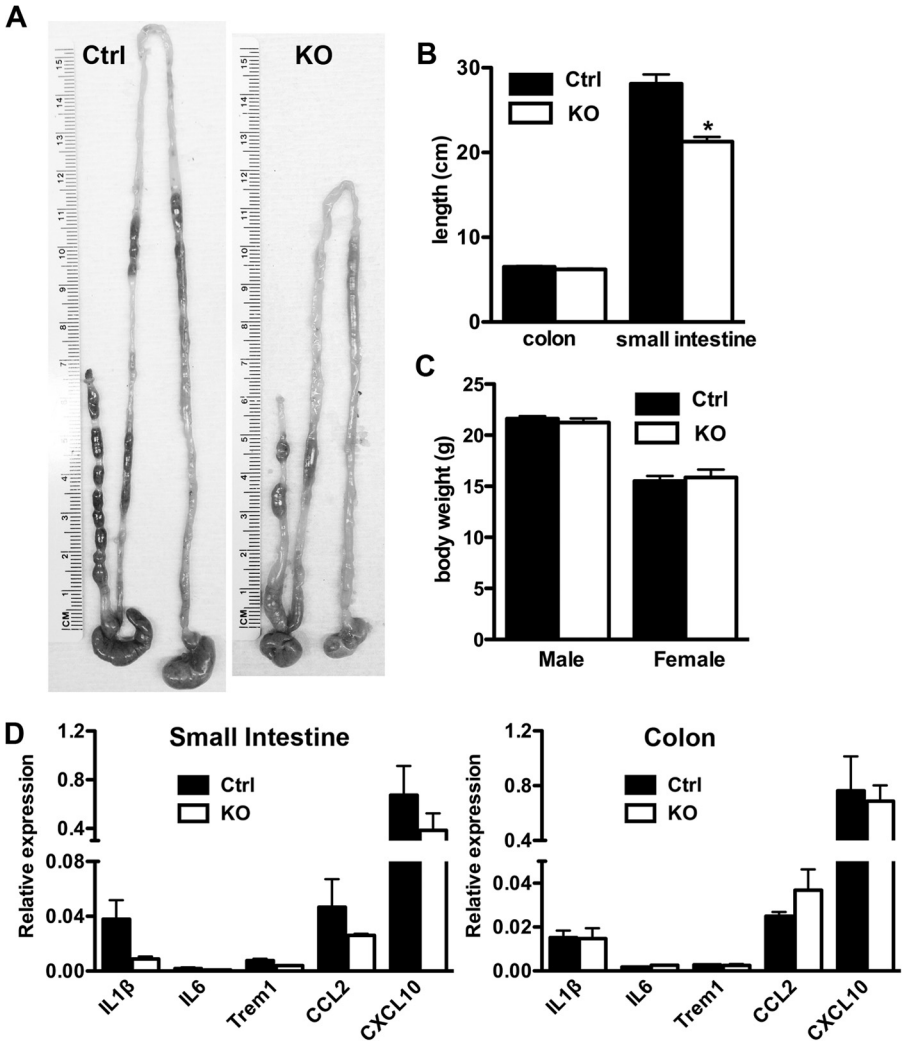


FIGURE 5. **Knock-out mice have shorter small intestines.** Representative pictures (*A*) and quantitative data (*B*) show the lengths of colon and small intestine of adult control ($CArG^{f/f}$ $Cre^{-/-}$ + $CArG^{f/-}$ $Cre^{-/-}$; *Ctrl*) and knock-out ($CArG^{f/f}$ $Cre^{-/+}$ + $CArG^{f/-}$ $Cre^{-/+}$; *KO*) mice. $n = 5$. *, $p < 0.05$. *C*, body weights of adult control and knock-out mice. $n = 6$. *D*, qRT-PCR analysis of inflammatory cell markers in smooth muscle from the small intestine of control or knock-out mice. Transcript levels were quantitated as described in the legend to Fig. 3. $n = 3$–5. No significant differences in expression were observed in control and knock-out mice. *Error bars*, S.E.

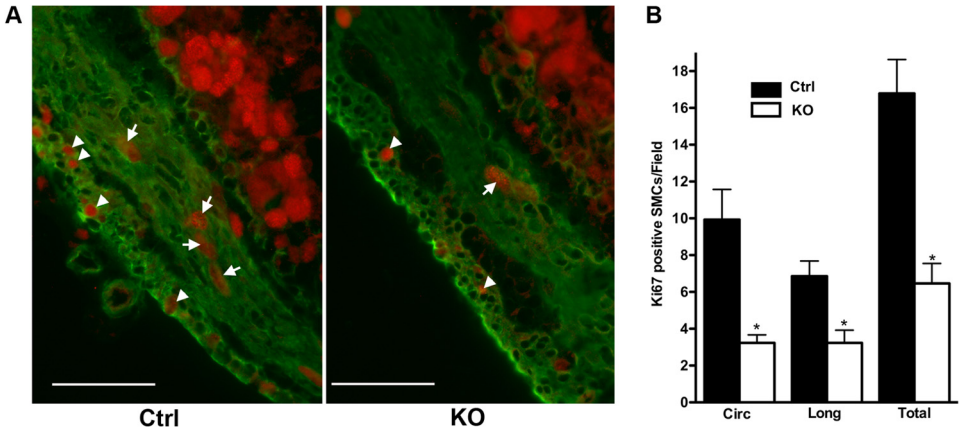


FIGURE 6. **Knock-out mice have decreased intestinal smooth muscle cell proliferation.** *A*, Ki67 (*red*) and smooth muscle α-actin (*green*) staining of cross-sections of the lower portion of small intestines from neonatal control ($CArG^{f/f}$ $Cre^{-/-}$ + $CArG^{f/-}$ $Cre^{-/-}$; *Ctrl*) and knock-out ($CArG^{f/f}$ $Cre^{-/+}$ + $CArG^{f/-}$ $Cre^{-/+}$; *KO*) mice. *White arrows* and *white arrowheads* point to examples of Ki67 positive smooth muscle cells in the circular and longitudinal smooth muscle layers, respectively. *B*, quantitation of the number of positive Ki67 smooth muscle cells per field at ×40 magnification in the circular layer, longitudinal layer, and both smooth muscle layers of the small intestine. $n = 6$–7 mice. *, $p < 0.05$. *Error bars*, S.E.

to +8427. This may also explain why the reporter genes exhibited very low level or undetectable *lacZ* expression in vascular smooth muscle tissues (Fig. 1). Although the endogenous 130-kDa smMLCK is expressed at lower levels in many nonmuscle tissues, we did not observe significant levels of *lacZ* transgene expression in many of these tissues. This may simply reflect the sensitivity of *lacZ* detection or the nonnative chromatin environment of reporter transgenes. Although the transgenic reporter mice were generated using a pWhere *lacZ* expression vector that is CpG-free and has H19 insulator elements flanking the transgene, we have previously shown that the telokin promoter also does not drive high levels of expression in many founder mice generated using this transgene vector (34). Because the promoter and regulatory elements analyzed are embedded within introns of the larger *mylk1* gene, it is possible that transcription from the promoters that drive expression of the 220-kDa MLCK may modulate the chromatin structure of the gene to facilitate the activity of these internal elements. Although analysis of reporter genes suggests that the promoter and first intron of the 130-kDa smMLCK are not sufficient to fully recapitulate expression of the endogenous 130-kDa MLCK, deletion of the intronic CArG region from the endogenous gene decreases 130-kDa smMLCK expression by 40%. This demonstrates that this element is required for full activation of the gene. The region deleted following Cre recombinase-mediated recombination of the *lacZ* reporter or the endogenous *mylk1* gene includes a conserved CArG box together with almost 300 bp of flanking sequence. The CArG element is located within a region of 63 bp that is highly conserved between species (29). Analysis using rVista identified conserved potential binding sites for the transcription factors SRF, Lun1, HoxA3, Oct, AP3, SRY, and DBP in this region. This raises the possibility that the decreased 130-kDa smMLCK seen following deletion of this region may be due to loss of not only SRF binding but also one or more of these other transcription factors. For example, Lun1, also named topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase (TOPORS), is particularly interesting in this regard, because this protein also has SUMO ligase activity, and both SRF and myocardin are known to be regulated by sumoylation (49–51). This raises the possibility that Lun1 may further regulate SRF and myocardin activity to control expression of the 130-kDa smMLCK.

We have previously shown that a CArG element within the telokin promoter is also critical for expression of telokin transcripts through analysis of transgenic reporter mice and targeting the endogenous telokin promoter (34, 42). Together with the current findings, these data show that SRF plays a key role in regulating expression of multiple transcripts from the *mylk1* gene. Although polymorphisms in these CArG elements have not yet been linked to diseases, an amplification of a CT repeat adjacent to the CArG element in the promoter of the 130-kDa smMLCK in SHR rats has been proposed to increase 130-kDa smMLCK expression and be the cause of the hypertension in these rats (21). A single nucleotide polymorphism (SNP) in intron 17 of the human *MYLK1* gene (equivalent to intron 15 in mice) has also been shown to regulate 130-kDa smMLCK expression and to be linked to inflammatory lung disease (18). Because this SNP is not in the conserved region deleted in the current study, these data suggest that there may be multiple important regulatory elements within this intron of the *mylk1* gene.

Although SRF is important for regulating expression of both 130-kDa smMLCK and telokin transcripts, it does so by binding to distinct CArG elements. These elements appear to be functionally separated from each other, because deletion of a single CArG element affects expression of one transcript but not the other. Deletion of the CArG element in the telokin promoter (in *mylk* intron 28) abolished telokin expression without affecting expression of transcripts encoding the 200- or 130-kDa smMLCKs (42). Similarly, deletion of the CArG element from intron 1 of the 130-kDa smMLCK gene (in *mylk1* intron 15) decreased expression of the 130-kDa smMLCK without affecting expression of the 220-kDa MLCK or telokin (Fig. 3). These data suggest that either the CArG elements are simply too far from the other promoters to affect their activity (*e.g.* the telokin CArG element is about 73 kb from the 130-kDa smMLCK promoter) or that there are perhaps insulator elements within the *mylk1* gene that restrict the activity of the elements to specific promoters. Additional studies are required to resolve these possibilities.

In summary, data from both transgenic reporter mice and a knock-out mouse model demonstrate that a CArG region within intron 15 of the *mylk1* gene plays an important role in specifically regulating expression of 130-kDa smMLCK. Moreover, the I_{CArG} flox mice provide a novel model system for further interrogating the specific functions of the 130-kDa smMLCK isoform in different cell types *in vivo*.

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