

Regulation of 130kDa smooth muscle myosin light chain kinase expression by an intronic CARG element

Meng Chen, Wenwu Zhang, Xiao Lu, April M. Hoggatt, Susan J. Gunst, Ghassan S. Kassab, Johnathan D. Tune and B. Paul Herring\*

Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202

*Short title:* smMLCK regulates intestinal contractility and proliferation

\* To whom correspondence should be addressed: Paul Herring, Department of Cellular and Integrative Physiology, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis IN, 46202 Phone: (317) 278-1785 FAX: (317) 274-3318 Email: [pherring@iupui.edu](mailto:pherring@iupui.edu)

**Background:** Mechanisms regulating transcription of MLCK are poorly defined.

**Results:** Deleting a CARG element from the *mylk1* gene specifically decreased expression of the 130kDa smMLCK isoform, resulting in decreased intestinal contractility and proliferation.

**Conclusion:** The 130kDa smMLCK isoform has functions that cannot be compensated for by the 220kDa MLCK.

**Significance:** Floxed *mylk1* mice permit specific functions of the 130kDa smMLCK to be determined.

#### SUMMARY

The *mylk1* gene encodes a 220kDa nonmuscle myosin light chain kinase (MLCK), a 130kDa 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 intron15 of *mylk1*. As this CARG element is near to the promoter that drives transcription of the 130kDa smMLCK, we examined its role in regulating expression of this transcript. Results show that deletion of the intronic CARG region from a  $\beta$ -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 130kDa smMLCK by 40% without affecting expression of the 220kDa MLCK or telokin. This reduction in 130kDa 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 130kDa smMLCK and that the 130kDa 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 ~250kb, comprising 31 exons (1). *Mylk1* encodes at least three protein products: a 220kDa MLCK, a 130kDa 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 220kDa MLCK is also referred to as nonmuscle MLCK or endothelial MLCK, as it was first characterized in chick embryo fibroblasts and endothelial cells (2,3). The 130kDa MLCK is also called the smooth muscle MLCK (smMLCK), since 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

This is the author's manuscript of the article published in final edited form as:

Chen, M., Zhang, W., Lu, X., Hoggatt, A. M., Gunst, S. J., Kassab, G. S., ... & Herring, B. P. (2013). Regulation of 130-kDa smooth muscle myosin light chain kinase expression by an intronic CARG element. *Journal of Biological Chemistry*, 288(48), 34647-34657. <http://dx.doi.org/10.1074/jbc.M113.510362>

that is expressed at very high levels in intestinal, urinary, and reproductive tract smooth muscle, at low levels in vascular smooth muscle cells and undetectable levels in other tissues (6).

In the presence of  $\text{Ca}^{2+}$  and calmodulin, both the 220kDa and 130kDa smMLCK can phosphorylate serine 19 of the 20kDa myosin regulatory light chain (RLC) 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 RLC 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 knockout of the 220kDa 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 130kDa 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 220kDa and 130kDa smMLCK specifically from endothelial cells had very little effect on vascular permeability, bringing into question the importance of endothelial cell expressed MLCK in regulating endothelial barrier function (28). Because of the overlapping structure of the 220kDa and 130kDa smMLCK it is difficult to examine the function of the 130kDa smMLCK, without also affecting expression of the 220kDa isoform. To address this issue, we examined regulatory elements that

specifically regulate expression of the 130kDa smMLCK with the hypothesis that deletion of these elements may attenuate expression of the 130kDa 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 130kDa 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 130kDa MLCK (29).

The CArG element,  $\text{CC(A/T)}_6\text{GG}$ , is the cis-regulatory element that binds serum response factor (SRF), an evolutionarily conserved MADS (MCM1, agamous, deficiens, 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 130kDa smMLCK (33). SRF and myocardin enhanced the activity of the 130kDa smMLCK promoter reporter genes and induced expression of the 130kDa smMLCK in 10T1/2 fibroblast cells, while 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 130kDa smMLCK (intron 15 of *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 130kDa smMLCK *in vivo*. Here, we found that the intronic CARG element is important for regulating expression of transgenes driven by the 130kDa smMLCK promoter *in vivo* and for driving expression of endogenous 130kDa smMLCK in mice. Moreover, we show that targeting this element is an effective means to specifically decrease expression of the endogenous 130kDa smMLCK without affecting expression of the 220kDa 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 7.56kb fragment containing about 5.1kb extending 5' and 2.16kb extending 3' to the intronic CARG region, was subcloned from a C57BL/6 BAC clone (RP23: 5501, Source BioScience, UK) into pSP72 vector (Promega). To construct the targeting vector for homologous recombination, a loxP/FRT flanked Neo cassette was inserted 54bp 3' of the intronic CARG element, and a single loxP site containing engineered Afl II and BamH I sites for southern blot analysis was inserted 184bp 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 Fse I and Pml I to yield a 2.3kb fragment that included the intronic CARG element and surrounding loxP sites. The 130kDa smMLCK promoter, exon 1, intron 1 and portion of exon 2 were cut from the pGL2B construct described previously (29) and ligated into the pWhere lacZ reporter vector (InvivoGen). The smMLCK<sup>-389~+8427</sup> pWhere vector was then cut by Fse I and Pml I. The

resulting 2kb fragment that included the intronic CARG element was replaced with the corresponding 2.3kb fragment isolated from the targeting vector to generate the I<sub>CARG</sub>-smMLCK<sup>-389~+8427</sup> 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<sub>CARG</sub>-smMLCK<sup>-389~+8427</sup> pWhere plasmid it was introduced into bacteria expressing Cre recombinase to generate plasmid  $\Delta$ I<sub>CARG</sub>-smMLCK<sup>-389~+8427</sup> pWhere. Correct excision of the CARG element was confirmed by DNA sequencing. I<sub>CARG</sub>-smMLCK<sup>-389~+8427</sup> pWhere and  $\Delta$ I<sub>CARG</sub>-smMLCK<sup>-389~+8427</sup> 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 one month old by  $\beta$ -galactosidase staining as described previously (34). All animal experiments were conducted under the approval of Indiana University School of Medicine Institutional Animal Care and Use Committee.

### *Generation of knockout mice*

Knockout mice were generated by inGenious Targeting Laboratory (Stony Brook, NY). 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 Flpe mice to remove the Neo cassette. The deletion of Neo cassette was screened and confirmed by PCR and DNA sequencing. Germline floxed mice were mated to smMHC-Cre mice (from Michael Kotlikoff at Cornell)(also on a C57BL/6 background) to delete the intronic CARG region specifically in smooth muscle tissues. Genotyping primers are: 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 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  $\mu$ M 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 PSS-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 used to compensate for water transport across the vessel wall. The vascular contraction during endothelin 1 stimulation was measured as changes in intra-luminal 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 weeks old control and smooth muscle-specific 130kDa MLCK knockout mice. The mRNA expression levels were quantitated by reverse transcription-qPCR as described previously (37). Because the 130kDa smMLCK transcript has a unique 5' UTR region not present in the 220kDa MLCK transcript, we were able to design primers to specifically detect the 130kDa smMLCK mRNA. The primers used are: 220kDa MLCK F: GAA CCT CTG CAT CAA AGA AGG AG, 220 MLCK R: GAT GGC TTG CCC TTT TCT GTG CCA TG, 130kDa 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 previous (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).

#### *MLC phosphorylation*

The proximal portion of the colon (epithelia intact) was cut into 0.5cm-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 myosin light chain (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 (Cat#4583, Sakura) and 7 $\mu$ m sections were cut. Sections were fixed with 3.7% formaldehyde, permeablized in 0.2% triton X-100 and blocked with 10% FCS in 50mMTris pH7.6, 150mM NaCl, then incubated with antibodies against Ki67 (Cat#15580, Abcam, 1:500) and smooth muscle  $\alpha$ -actin (Cat#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*

Chi-square test was used to determine if the observed birth frequency of knockout mice was lower than expected. For other statistical comparison, student's t-tests were performed (Prism, GraphPad Software, CA). 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 130kDa smMLCK promoter.* Previously we reported a highly conserved CARG element located in intron 15 of the *mylk1* gene (1<sup>st</sup> intron of the 130kDa smMLCK) (29). To investigate the role of this intronic region in regulating 130kDa smMLCK gene expression, we generated transgenic mice in which a lacZ reporter was driven by the 130kDa smMLCK promoter, exon1, intron1 and a portion of exon 2 with (I<sub>CARG</sub>-smMLCK<sup>-389~+8427</sup> pWhere) or without ( $\Delta$ I<sub>CARG</sub>-smMLCK<sup>-389~+8427</sup> pWhere) this intronic CARG region, as described in Methods. In 2 of the 3 independent founders harboring the wild type transgene, high levels of  $\beta$ -galactosidase staining were observed in visceral smooth muscle rich tissues, such as bladder, colon, small intestine and ureters (Figure 1). The third line had lower levels of expression but in a similar pattern (not shown). One of the 2 high expressing founders also exhibited staining in bronchi as well as in the lung and small vessels of skeletal muscle and liver (Figure 1). In contrast to the wild type transgenes, no  $\beta$ -galactosidase expression could be detected in any of 7 founder mice harboring the CARG deleted transgene (Figure 1). Weak background staining seen in bladder, kidney and colon is similar to that seen in non-transgenic mice (Figure 1, lower right panel). These data demonstrate that the intronic CARG region is critical for expression of a 130kDa smMLCK-driven transgene.

*Deletion of the intronic CARG region from the endogenous mylk1 gene resulted in decreased expression of the 130kDa smMLCK.* To determine if deletion of the intronic CARG region would decrease expression of the endogenous 130kDa 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 (Figure 2) (41). We have previously shown that this Cre transgene results in high levels of recombination specifically in smooth muscle tissues (42). PCR analysis of genomic DNA isolated from colon and aorta of knockout and control mice further showed that there is more efficient recombination of the floxed allele in colon smooth muscle as opposed to aortic smooth muscle (Figure 2B). Although the recombined allele was readily detectable in both tissues there also remained significant amounts of the non-recombined floxed allele. The latter likely 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<sup>f/f</sup> (global heterozygous) rather than the expected CARG<sup>f/f</sup>. Both control (Cre<sup>-/-</sup>CARG<sup>f/f</sup> and Cre<sup>-/-</sup>CARG<sup>f/-</sup>) and smooth muscle-specific CARG knockout (Cre<sup>-/+</sup>CARG<sup>f/f</sup> and Cre<sup>-/+</sup>CARG<sup>f/-</sup>) mice reached adulthood without any obvious growth and behavioral abnormalities. However, knockout mice were born with a slightly lower than expected frequency of 19% as compared to 25% (Chi-square test  $p = 0.033$ , degree of freedom=1, Figure 2C). 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 approximately 40% decrease in 130kDa smMLCK mRNA levels of CARG<sup>f/f</sup> Cre<sup>-/+</sup> knockout mice compared with control CARG<sup>f/f</sup> Cre<sup>-/-</sup> mice, while there was no significant alteration in 220kDa MLCK or telokin mRNA expression levels (Figure 3A). Similar results were seen when CARG<sup>f/-</sup> Cre<sup>-/+</sup> knockout mice were compared with CARG<sup>f/-</sup> Cre<sup>-/-</sup> control mice. As we did not see any significant differences in 130kDa smMLCK expression between the two

control strains (CArG<sup>fl/fl</sup> Cre<sup>-/-</sup> and CArG<sup>fl/-</sup> Cre<sup>-/-</sup>), and our standard genotyping does not distinguish between these strains, in all subsequent experiments control mice were a mixture of CArG<sup>fl/fl</sup> Cre<sup>-/-</sup> and CArG<sup>fl/-</sup> Cre<sup>-/-</sup>. Similarly, all smooth muscle-specific knockout mice were a mixture of CArG<sup>fl/fl</sup> Cre<sup>-/+</sup> and CArG<sup>fl/-</sup> Cre<sup>-/+</sup>.

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

*Deletion of the intronic region and subsequent decreases in 130kDa smMLCK expression attenuated smooth muscle contractility.* In order to investigate whether the decreased expression of the 130kDa 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 knockout mice was dramatically decreased compared with control mice (Figure 4A,C). Similarly carbachol induced contractions were also impaired in tissue from knockout mice (Figure 4B,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 knockout mice compared with controls (Figure 4D).

*Decreased contraction of smooth muscle tissues in knockout mice was associated with decreased myosin light chain phosphorylation.* The 130kDa smMLCK induces contraction of

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

*Decreased 130kDa smMLCK expression resulted in shortened small intestine.* Further analysis of both control and knockout mice showed that the length of small intestine was shorter by about 24% in the intronic CArG knockout mice, while the length of colon was not significantly different (Figure 5A, B). The shorter small intestine was not associated with any change in body weight (Figure 5C). We also did not detect any inflammation in knockout mice as determined by lack of changes in expression of inflammatory genes, IL1 $\beta$ , IL6, CCL2, Trem1 or CXCL10 (Figure 5D). 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 knockout mice (Figure 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 130kDa smMLCK. Deletion of this intronic CArG box attenuated expression of the 130kDa smMLCK without affecting expression of either the 220kDa MLCK or telokin. Moreover, decreased expression of only the 130kDa 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 130kDa smMLCK isoform is specifically required to

regulate not only smooth muscle contraction, but also intestinal smooth muscle cell proliferation.

Deletion of both the 220kDa and 130kDa MLCK from smooth muscle tissues has been previously shown to impair contractility, myosin light chain phosphorylation and gastrointestinal motility in mice (27). The current studies suggest that it is primarily the 130kDa smMLCK rather than the 220kDa 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 220kDa MLCK knockout mice (44). Results also suggest that the 130kDa smMLCK has a specific role in regulating the proliferation of small intestinal smooth muscle cells during early neonatal growth (Figure 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 130kDa smMLCK isoform in this process. This conclusion should, however, be viewed with caution as 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 knockout 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 130kDa smMLCK-lacZ reporter transgene in the visceral smooth muscle tissues, whereas deletion of this element from the endogenous gene only decreased endogenous 130kDa smMLCK expression by about 40%. The relatively small decrease in 130kDa 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 demonstrate variable levels of floxed alleles remaining in these tissues (Figure 2B). 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 130kDa

smMLCK. In support of this possibility, a notch responsive element has been identified at -3687 that plays an important role in activating 130kDa 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 to +8427. This may also explain why the reporter genes exhibited very low levels, or undetectable, lacZ expression in vascular smooth muscle tissues (Figure 1). Although the endogenous 130kDa 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). As 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 220kDa MLCK may modulate the chromatin structure of the gene to facilitate the activity of these internal elements. Although analysis of reporter genes suggest that the promoter and first intron of the 130kDa smMLCK are not sufficient to fully recapitulate expression of the endogenous 130kDa MLCK, deletion of the intronic CARG region from the endogenous gene decreases 130kDa 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 300bp of flanking sequence. The CARG element is located within a region of 63bp 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 130kDa 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, as 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 130kDa 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 (52,53). 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 130kDa smMLCK in SHR rats has been proposed to increase 130kDa 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 mouse) has also been shown to regulate 130kDa smMLCK expression and to be linked to inflammatory lung disease (54). As 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 130kDa smMLCK and telokin transcripts, it does so by binding to distinct CArG elements. These elements appear to be functionally separated from each other, as 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 200kDa or 130kDa smMLCKs (52). Similarly, deletion of the CArG element from intron 1 of the 130kDa smMLCK gene (in *mylk1* intron 15) decreased expression of the 130kDa smMLCK without affecting expression of the 220kDa MLCK or telokin (Figure 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 73kb from the 130kDa 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 knockout mouse model demonstrate that a CArG region within intron 15 of the *mylk1* gene plays an important role in specifically regulating expression of 130kDa smMLCK. Moreover, the I<sub>CArG</sub> flox mice provide a novel model system for further interrogating the specific functions of the 130kDa smMLCK isoform in different cell types *in vivo*.

## REFERENCES

1. Herring, B. P., El-Mounayri, O., Gallagher, P. J., Yin, F., and Zhou, J. (2006) Regulation of myosin light chain kinase and telokin expression in smooth muscle tissues. *Am J Physiol Cell Physiol* **291**, C817-827
2. Shoemaker, M. O., Lau, W., Shattuck, R. L., Kwiatkowski, A. P., Matrisian, P. E., Guerra-Santos, L., Wilson, E., Lukas, T. J., Van Eldik, L. J., and Watterson, D. M. (1990) Use of DNA sequence and mutant analyses and antisense oligodeoxynucleotides to examine the molecular basis of nonmuscle myosin light chain kinase autoinhibition, calmodulin recognition, and activity. *J Cell Biol* **111**, 1107-1125
3. Garcia, J. G., Lazar, V., Gilbert-McClain, L. I., Gallagher, P. J., and Verin, A. D. (1997) Myosin light chain kinase in endothelium: molecular cloning and regulation. *Am J Respir Cell Mol Biol* **16**, 489-494



4. Herring, B. P., Dixon, S., and Gallagher, P. J. (2000) Smooth muscle myosin light chain kinase expression in cardiac and skeletal muscle. *Am J Physiol-Cell Ph* **279**, C1656-C1664
5. Blue, E. K., Goeckeler, Z. M., Jin, Y. J., Hou, L., Dixon, S. A., Herring, B. P., Wysolmerski, R. B., and Gallagher, P. J. (2002) 220- and 130-kDa MLCKs have distinct tissue distributions and intracellular localization patterns. *Am J Physiol-Cell Ph* **282**, C451-C460
6. Herring, B. P., Lyons, G. E., Hoggatt, A. M., and Gallagher, P. J. (2001) Telokin expression is restricted to smooth muscle tissues during mouse development. *Am J Physiol Cell Physiol* **280**, C12-21
7. Kamm, K. E., and Stull, J. T. (2001) Dedicated myosin light chain kinases with diverse cellular functions. *Journal of Biological Chemistry* **276**, 4527-4530
8. Totsukawa, G., Yamakita, Y., Yamashiro, S., Hartshorne, D. J., Sasaki, Y., and Matsumura, F. (2000) Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J Cell Biol* **150**, 797-806
9. Garcia, J. G. N., Davis, H. W., and Patterson, C. E. (1995) Regulation of Endothelial-Cell Gap Formation and Barrier Dysfunction - Role of Myosin Light-Chain Phosphorylation. *J Cell Physiol* **163**, 510-522
10. Iida, Y., Senda, T., Matsukawa, Y., Onoda, K., Miyazaki, J., Sakaguchi, H., Nimura, Y., Hidaka, H., and Niki, I. (1997) Myosin light-chain phosphorylation controls insulin secretion at a proximal step in the secretory cascade. *Am J Physiol-Endoc M* **273**, E782-E789
11. Matsumura, F. (2005) Regulation of myosin II during cytokinesis in higher eukaryotes. *Trends Cell Biol* **15**, 371-377
12. Schmidt, J. T., Morgan, P., Dowell, N., and Leu, B. H. (2002) Myosin light chain phosphorylation and growth cone motility. *J Neurobiol* **52**, 175-188
13. Shen, L., Black, E. D., Witkowski, E. D., Lencer, W. I., Guerriero, V., Schneeberger, E. E., and Turner, J. R. (2006) Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. *J Cell Sci* **119**, 2095-2106
14. Su, L. P., Shen, L., Clayburgh, D. R., Nalle, S. C., Sullivan, E. A., Meddings, J. B., Abraham, C., and Turner, J. R. (2009) Targeted Epithelial Tight Junction Dysfunction Causes Immune Activation and Contributes to Development of Experimental Colitis. *Gastroenterology* **136**, 551-563
15. Blair, S. A., Kane, S. V., Clayburgh, D. R., and Turner, J. R. (2006) Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease. *Lab Invest* **86**, 191-201
16. Leguilette, R., Laviolette, M., Bergeron, C., Zitouni, N., Kogut, P., Solway, J., Kachmar, L., Hamid, Q., and Lauzon, A. M. (2009) Myosin, Transgelin, and Myosin Light Chain Kinase Expression and Function in Asthma. *Am J Resp Crit Care* **179**, 194-204
17. Flores, C., Ma, S. F., Maresso, K., Ober, C., and Garcia, J. G. N. (2007) A variant of the myosin light chain kinase gene is associated with severe asthma in African Americans. *Genet Epidemiol* **31**, 296-305
18. Han, Y. J., Ma, S. F., Wade, M. S., Flores, C., and Garcia, J. G. N. (2012) An intronic MYLK variant associated with inflammatory lung disease regulates promoter activity of the smooth muscle myosin light chain kinase isoform. *J Mol Med* **90**, 299-308
19. Wang, L., Guo, D. C., Cao, J. M., Gong, L. M., Kamm, K. E., Regalado, E., Li, L., Shete, S., He, W. Q., Zhu, M. S., Offermanns, S., Gilchrist, D., Elefteriades, J., Stull, J. T., and Milewicz, D. M. (2010) Mutations in Myosin Light Chain Kinase Cause Familial Aortic Dissections. *Am J Hum Genet* **87**, 701-707
20. He, W. Q., Qiao, Y. N., Zhang, C. H., Peng, Y. J., Chen, C., Wang, P., Gao, Y. Q., Chen, X., Tao, T., Su, X. H., Li, C. J., Kamm, K. E., Stull, J. T., and Zhu, M. S. (2011) Role of myosin light chain kinase in regulation of basal blood pressure and maintenance of salt-induced hypertension. *Am J Physiol Heart Circ Physiol* **301**, H584-591

21. Han, Y. J., Hu, W. Y., Chernaya, O., Antic, N., Gu, L., Gupta, M., Piano, M., and de Lanerolle, P. (2006) Increased myosin light chain kinase expression in hypertension: Regulation by serum response factor via an insertion mutation in the promoter. *Mol Biol Cell* **17**, 4039-4050
22. Usatyuk, P. V., Singleton, P. A., Pendyala, S., Kalari, S. K., He, D., Gorshkova, I. A., Camp, S. M., Moitra, J., Dudek, S. M., Garcia, J. G., and Natarajan, V. (2012) Novel role for non-muscle myosin light chain kinase (MLCK) in hyperoxia-induced recruitment of cytoskeletal proteins, NADPH oxidase activation, and reactive oxygen species generation in lung endothelium. *J Biol Chem* **287**, 9360-9375
23. Guo, M., Yuan, S. Y., Frederich, B. J., Sun, C., Shen, Q., McLean, D. L., and Wu, M. H. (2012) Role of non-muscle myosin light chain kinase in neutrophil-mediated intestinal barrier dysfunction during thermal injury. *Shock* **38**, 436-443
24. Sun, C., Wu, M. H., and Yuan, S. Y. (2011) Nonmuscle myosin light-chain kinase deficiency attenuates atherosclerosis in apolipoprotein E-deficient mice via reduced endothelial barrier dysfunction and monocyte migration. *Circulation* **124**, 48-57
25. Shen, Q., Rigor, R. R., Pivetti, C. D., Wu, M. H., and Yuan, S. Y. (2010) Myosin light chain kinase in microvascular endothelial barrier function. *Cardiovasc Res* **87**, 272-280
26. Clayburgh, D. R., Barrett, T. A., Tang, Y., Meddings, J. B., Van Eldik, L. J., Watterson, D. M., Clarke, L. L., Mrsny, R. J., and Turner, J. R. (2005) Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo. *J Clin Invest* **115**, 2702-2715
27. He, W. Q., Peng, Y. J., Zhang, W. C., Lv, N., Tang, J., Chen, C., Zhang, C. H., Gao, S., Chen, H. Q., Zhi, G., Feil, R., Kamm, K. E., Stull, J. T., Gao, X., and Zhu, M. S. (2008) Myosin light chain kinase is central to smooth muscle contraction and required for gastrointestinal motility in mice. *Gastroenterology* **135**, 610-620
28. Yu, Y., Lv, N., Lu, Z., Zheng, Y. Y., Zhang, W. C., Chen, C., Peng, Y. J., He, W. Q., Meng, F. Q., Zhu, M. S., and Chen, H. Q. (2012) Deletion of myosin light chain kinase in endothelial cells has a minor effect on the lipopolysaccharide-induced increase in microvascular endothelium permeability in mice. *Febs J* **279**, 1485-1494
29. Yin, F., Hoggatt, A. M., Zhou, J. L., and Herring, B. P. (2006) 130-kDa smooth muscle myosin light chain kinase is transcribed from a CArG-dependent, internal promoter within the mouse mylk gene. *Am J Physiol-Cell Ph* **290**, C1599-C1609
30. Miano, J. M., Sun, Q., Chen, G., Streb, J. W., Long, X. C., Yang, Y. M., and Stoeckert, C. J. (2006) Defining the mammalian CArGome. *Genome Research* **16**, 197-207
31. Miano, J. M., Long, X., and Fujiwara, K. (2007) Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. *Am J Physiol Cell Physiol* **292**, C70-81
32. Marais, R., Wynne, J., and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-393
33. Chen, J., Kitchen, C. M., Streb, J. W., and Miano, J. M. (2002) Myocardin: a component of a molecular switch for smooth muscle differentiation. *J Mol Cell Cardiol* **34**, 1345-1356
34. Touw, K., Hoggatt, A. M., Simon, G., and Herring, B. P. (2007) Hprt-targeted transgenes provide new insights into smooth muscle-restricted promoter activity. *Am J Physiol-Cell Ph* **292**, C1024-C1032
35. Zhang, M., Chen, M., Kim, J. R., Zhou, J., Jones, R. E., Tune, J. D., Kassab, G. S., Metzger, D., Ahlfeld, S., Conway, S. J., and Herring, B. P. (2011) SWI/SNF complexes containing Brahma or Brahma-related gene 1 play distinct roles in smooth muscle development. *Mol Cell Biol* **31**, 2618-2631
36. Lu, X. A., and Kassab, G. S. (2011) Assessment of endothelial function of large, medium, and small vessels: a unified myograph. *Am J Physiol-Heart C* **300**, H94-H100
37. Chen, M., and Herring, B. P. (2013) Regulation of microRNAs by Brahma-related gene 1 (Brg1) in smooth muscle cells. *J Biol Chem* **288**, 6397-6408

38. Gallagher, P. J., and Herring, B. P. (1991) The carboxyl terminus of the smooth muscle myosin light chain kinase is expressed as an independent protein, telokin. *J Biol Chem* **266**, 23945-23952
39. Kamm, K. E., Leachman, S. A., Michnoff, C. H., Nunnally, M. H., Persechini, A., Richardson, A. L., and Stull, J. T. (1987) Myosin light chain kinases and kinetics of myosin phosphorylation in smooth muscle cells. *Prog Clin Biol Res* **245**, 183-193
40. Touw, K., Chakraborty, S., Zhang, W., Obukhov, A. G., Tune, J. D., Gunst, S. J., and Herring, B. P. (2012) Altered calcium signaling in colonic smooth muscle of type 1 diabetic mice. *Am J Physiol Gastrointest Liver Physiol* **302**, G66-76
41. Xin, H. B., Deng, K. Y., Rishniw, M., Ji, G., and Kotlikoff, M. I. (2002) Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. *Physiol Genomics* **10**, 211-215
42. Zhang, M., Chen, M., Kim, J. R., Zhou, J., Jones, R. E., Tune, J. D., Kassab, G. S., Metzger, D., Ahlfeld, S., Conway, S. J., and Herring, B. P. (2011) SWI/SNF complexes containing Brahma or Brahma related gene 1 play distinct roles in smooth muscle development. *Molecular and Cellular Biology* **31**, 2618-2631
43. de Lange, W. J., Halabi, C. M., Beyer, A. M., and Sigmund, C. D. (2008) Germ line activation of the Tie2 and SMMHC promoters causes noncell-specific deletion of floxed alleles. *Physiological Genomics* **35**, 1-4
44. Wainwright, M. S., Rossi, J., Schavocky, J., Crawford, S., Steinhorn, D., Velentza, A. V., Zasadzki, M., Shirinsky, V., Jia, Y., Haiech, J., Van Eldik, L. J., and Watterson, D. M. (2003) Protein kinase involved in lung injury susceptibility: evidence from enzyme isoform genetic knockout and in vivo inhibitor treatment. *Proc Natl Acad Sci U S A* **100**, 6233-6238
45. Li, C., and Xu, Q. (2000) Mechanical stress-initiated signal transductions in vascular smooth muscle cells. *Cell Signal* **12**, 435-445
46. Maksym, G. N., Deng, L., Fairbank, N. J., Lall, C. A., and Connolly, S. C. (2005) Beneficial and harmful effects of oscillatory mechanical strain on airway smooth muscle. *Can J Physiol Pharmacol* **83**, 913-922
47. Tian, Y., Yue, X., Luo, D., Wazir, R., Wang, J., Wu, T., Chen, L., Liao, B., and Wang, K. (2013) Increased proliferation of human bladder smooth muscle cells is mediated by physiological cyclic stretch via the PI3KSGK1Kv1.3 pathway. *Mol Med Rep* **8**, 294-298
48. Basu, S., Srinivasan, D. K., Yang, K., Raina, H., Banerjee, S., Zhang, R., Fisher, S. A., and Proweller, A. (2013) Notch transcriptional control of vascular smooth muscle regulatory gene expression and function. *J Biol Chem* **288**, 11191-11202
49. Wang, J., Li, A., Wang, Z., Feng, X., Olson, E. N., and Schwartz, R. J. (2007) Myocardin sumoylation transactivates cardiogenic genes in pluripotent 10T1/2 fibroblasts. *Mol Cell Biol* **27**, 622-632
50. Matsuzaki, K., Minami, T., Tojo, M., Honda, Y., Uchimura, Y., Saitoh, H., Yasuda, H., Nagahiro, S., Saya, H., and Nakao, M. (2003) Serum response factor is modulated by the SUMO-1 conjugation system. *Biochem Biophys Res Commun* **306**, 32-38
51. Weger, S., Hammer, E., and Engstler, M. (2003) The DNA topoisomerase I binding protein topors as a novel cellular target for SUMO-1 modification: characterization of domains necessary for subcellular localization and sumolation. *Exp Cell Res* **290**, 13-27
52. Khromov, A. S., Wang, H., Choudhury, N., McDuffie, M., Herring, B. P., Nakamoto, R., Owens, G. K., Somlyo, A. P., and Somlyo, A. V. (2006) Smooth muscle of telokin-deficient mice exhibits increased sensitivity to Ca<sup>2+</sup> and decreased cGMP-induced relaxation. *Proc Natl Acad Sci U S A* **103**, 2440-2445
53. Touw, K., Hoggatt, A. M., Simon, G., and Herring, B. P. (2007) Hprt-targeted transgenes provide new insights into smooth muscle-restricted promoter activity. *Am J Physiol Cell Physiol* **292**, C1024-1032
54. Han, Y. J., Ma, S. F., Wade, M. S., Flores, C., and Garcia, J. G. (2012) An intronic MYLK variant associated with inflammatory lung disease regulates promoter activity of the smooth muscle myosin light chain kinase isoform. *Journal of molecular medicine* **90**, 299-308

## FOOTNOTES

This work was supported by a grant from the National Institute of Health HL085212 (to B.P.H.). Meng Chen was supported by a predoctoral fellowship from the American Heart Association (Midwest Affiliate). We would like to thank Dr. Adam Goodwill for helpful discussions and Dr. Simon Rhodes and Dr. Fred Pavalko for use of their equipment.

## FIGURE LEGENDS

**Figure 1. Comparison of transgene expression in reporter mice.** **A.** Organs were harvested from one month old reporter mice harboring the  $I_{\text{CArG-smMLCK}}^{-389\sim+8427}$  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.**  $\beta$ -galactosidase expression in organs harvested from 1 month old mice harboring the  $\Delta I_{\text{CArG-smMLCK}}^{-389\sim+8427}$  pWhere transgene (schematic, upper panel) in which the intronic CArG element is deleted (images are representative of 7 founder mice). Lower right panel shows  $\beta$ -galactosidase staining of colon from a nontransgenic mouse as a negative control (Negative colon).

**Figure 2. Generation of the intronic CArG knockout mice.** **A.** Schematic representation of 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. Numbers above the native gene refer to nucleotides positions relative to the transcription start site of the 130kDa 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 Flpe-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 (Panel A) and genomic DNA isolated from colon smooth muscle and aortic tissues. The bands corresponding to the Floxed (F) and recombined or deleted ( $\Delta$ ) alleles and an internal loading control (IC) are indicated. **C.** Breeding scheme used to generate knockout mice together with the genotypes of the progeny, their expected frequency, observed frequency and total numbers of pups analyzed.

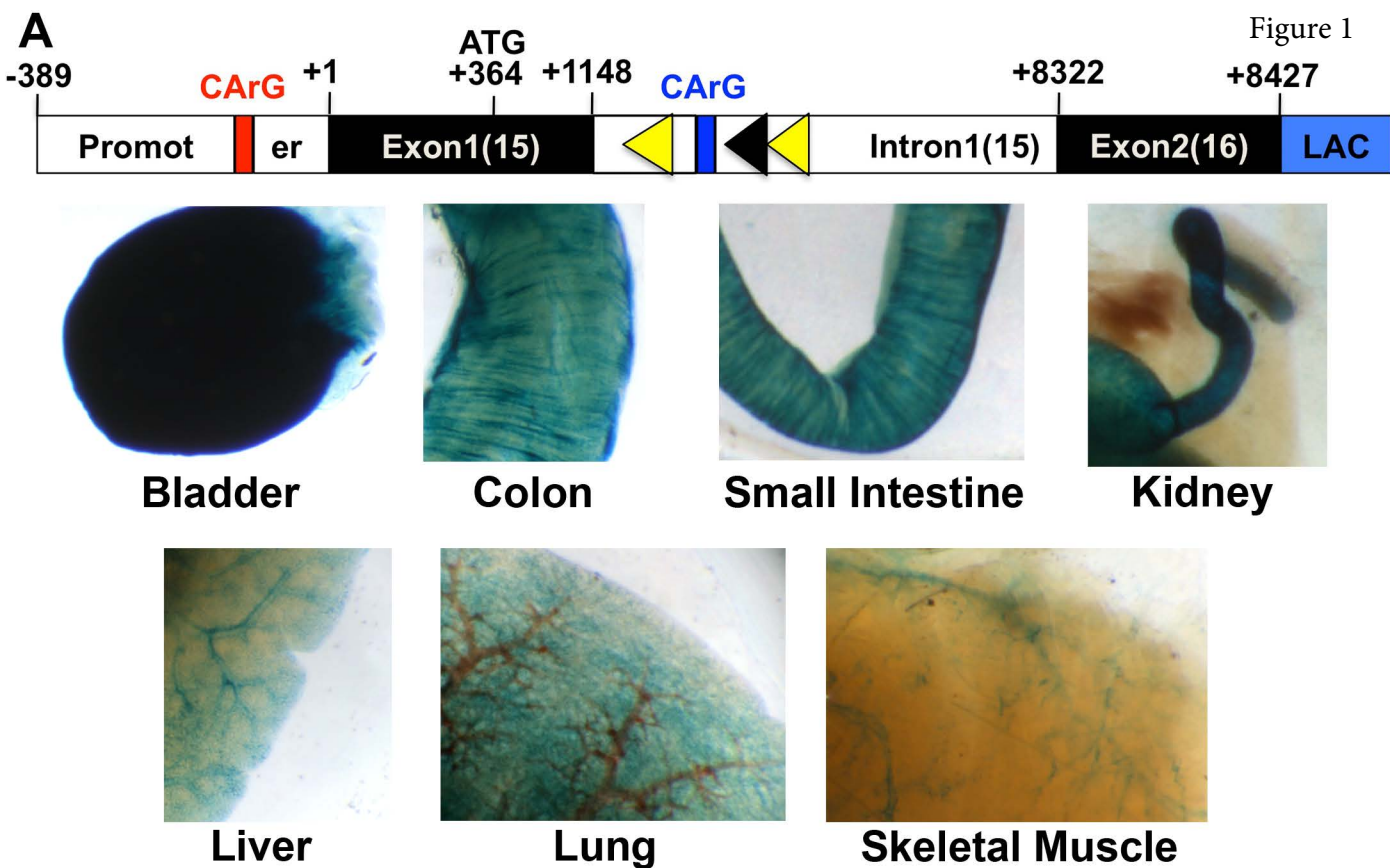
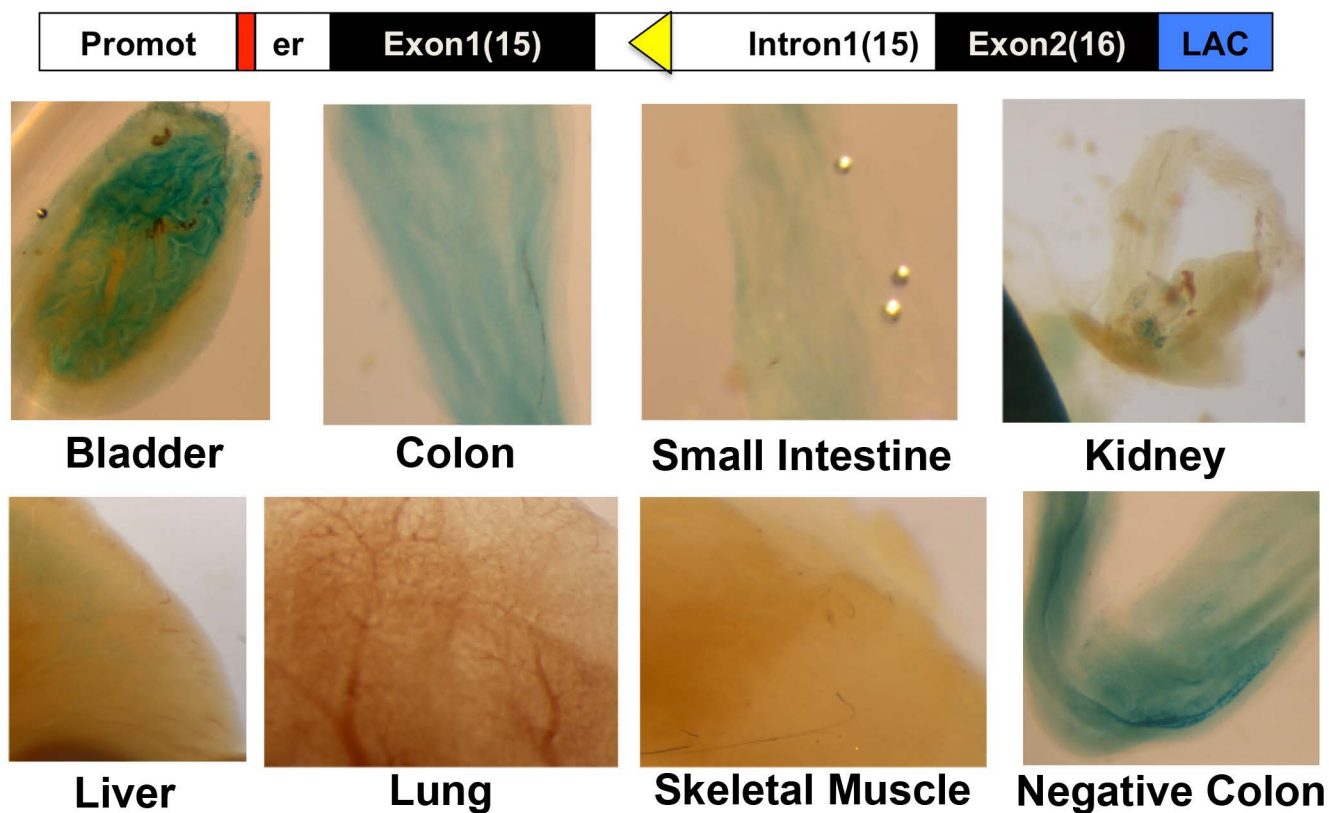
**Figure 3. The expression of the 130kDa smMLCK is decreased in CArG knockout mice.** **A.** Smooth muscle layers were isolated from the colon of control ( $\text{CArG}^{\text{flf}} \text{Cre}^{-/-}$ : FF), ( $\text{CArG}^{\text{flf}} \text{Cre}^{-/-}$ : F-) and knockout ( $\text{CArG}^{\text{flf}} \text{Cre}^{-/+}$ : FF KO) and ( $\text{CArG}^{\text{flf}} \text{Cre}^{-/+}$ : 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 =  $2^{-\Delta\text{Ct}}$ , where  $\Delta\text{Ct} = (\text{Ct}_{\text{expt}} - \text{Ct}_{\text{hprt}})$ . Each column represents the mean $\pm$ SEM of samples obtained from 5-14 mice. \*, p<0.05. **B.** mRNA levels in bladder and thoracic aorta from control ( $\text{CArG}^{\text{flf}} \text{Cre}^{-/-}$  +  $\text{CArG}^{\text{flf}} \text{Cre}^{-/-}$  combined: Ctrl) and knockout ( $\text{CArG}^{\text{flf}} \text{Cre}^{-/+}$  +  $\text{CArG}^{\text{flf}} \text{Cre}^{-/+}$  combined: KO) mice were measured by qRT-PCR. Transcript levels were quantitated as described in panel A. Each column represents the mean $\pm$ SEM of samples obtained from 11-13 mice. \*, p<0.05. **C.** Representative western blot of 130kDa smMLCK and vinculin in colon and bladder from two control (C) and two knockout (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 130kDa smMLCK protein expression in bladder tissue from wild type ( $\text{CArG}^{+/+} \text{Cre}^{-/-}$ : WT) and  $\text{CArG}^{\text{flf}} \text{Cre}^{-/-}$  (FF) mice. **G.** qRT-PCR analysis of 130kDa smMLCK mRNA expression in bladder and colon smooth muscle from

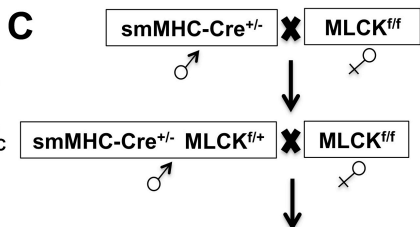
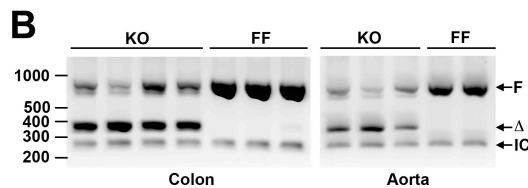
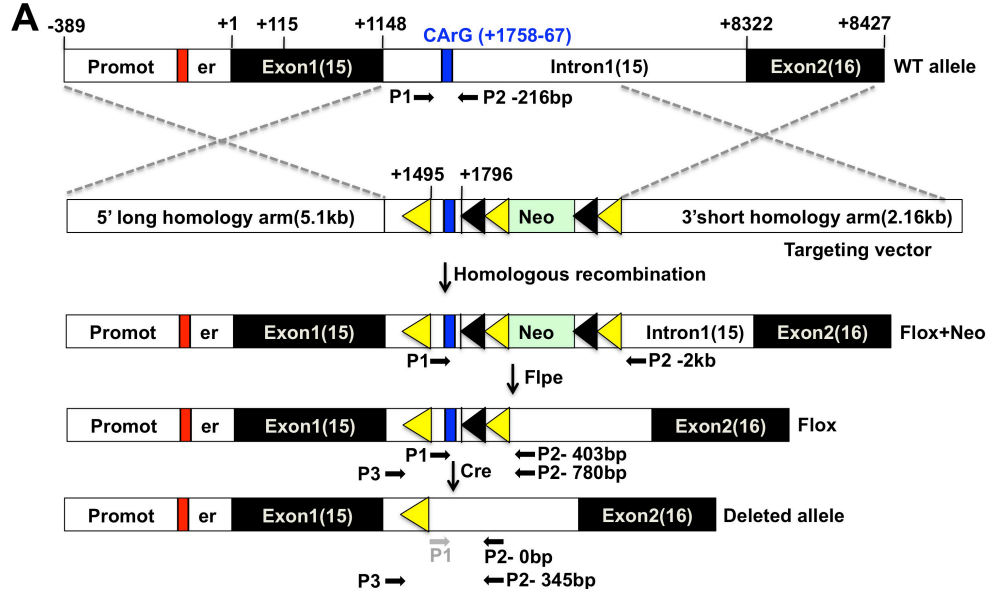
wild type (CArG<sup>+/+</sup> Cre<sup>-/-</sup>: WT) and flox/flox (CArG<sup>f/f</sup> Cre<sup>-/-</sup>: FF) mice. No significant differences in 130kDa smMLCK expression were observed. n=4(bladder). N=7(colon)

**Figure 4. Smooth muscle contractility and myosin light chain phosphorylation are decreased in knockout mice.** **A-B.** Colon rings were hung in an organ bath and stimulated to elicit contraction with 60mM KCl or 1 $\mu$ M Carbachol as described in methods. Data shown are the mean $\pm$ SEM changes in tension over time of 20 rings from control (CArG<sup>f/f</sup> Cre<sup>-/-</sup> + CArG<sup>f/-</sup> Cre<sup>-/-</sup>: Ctrl) mice and 12 from knockout (CArG<sup>f/f</sup> Cre<sup>-/+</sup> + CArG<sup>f/-</sup> Cre<sup>-/+</sup>: KO) mice. **C.** The average changes in peak contractile responses of colonic rings from control (Ctrl) and knockout (KO) mice. n=20 for control, n=12 for knockout. \*, 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 knockout. \*, p<0.05. **E.** Colon rings were either flash frozen under resting conditions or challenged by 60mM 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<sup>f/f</sup> Cre<sup>-/-</sup> and CArG<sup>f/-</sup> Cre<sup>-/-</sup> control; KO, CArG<sup>f/f</sup> Cre<sup>-/+</sup> and CArG<sup>f/-</sup> Cre<sup>-/+</sup>). 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 $\pm$ SEM are plotted in the right panel. \*, p<0.05.

**Figure 5. Knockout mice have shorter small intestines.** **A.** Representative pictures and **B,** quantitative data showing the lengths of colon and small intestine of adult control (CArG<sup>f/f</sup> Cre<sup>-/-</sup> + CArG<sup>f/-</sup> Cre<sup>-/-</sup>: Ctrl) and knockout (CArG<sup>f/f</sup> Cre<sup>-/+</sup> + CArG<sup>f/-</sup> Cre<sup>-/+</sup>: KO) mice. n=5. \*, p<0.05. **C.** Body weights of adult control and knockout mice. N=6. **D.** qRT-PCR analysis of inflammatory cell markers in smooth muscle from the small intestine of control or knockout mice. Transcript levels were quantitated as described in figure 3. n= 3-5 No significant differences in expression were observed in control and knockout mice.

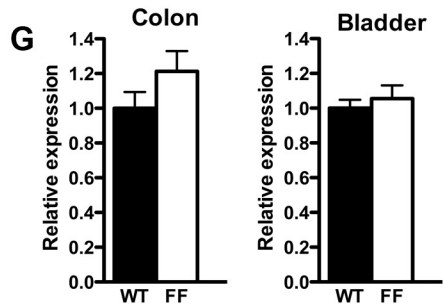
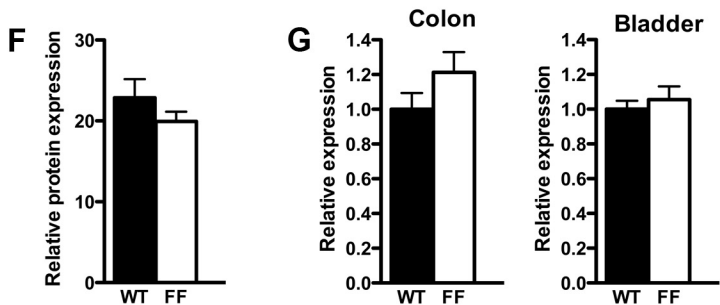
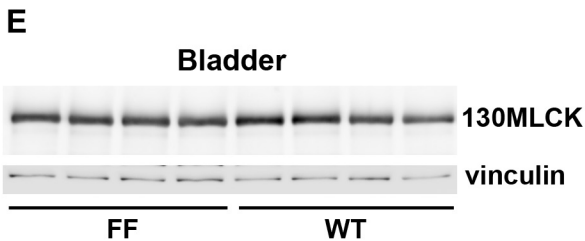
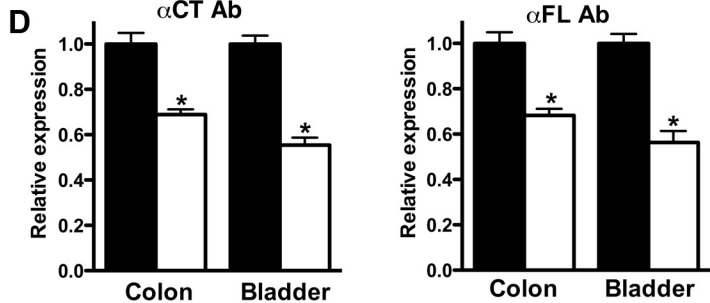
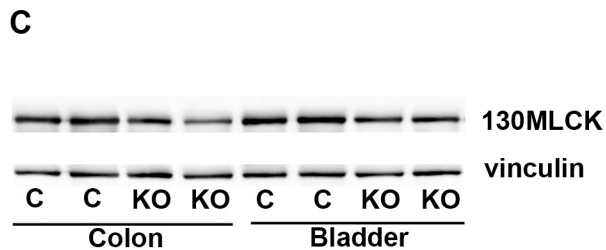
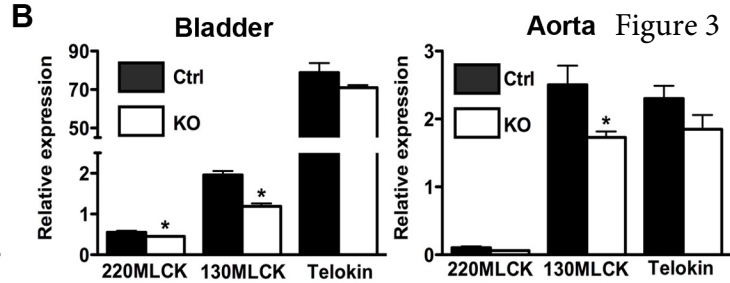
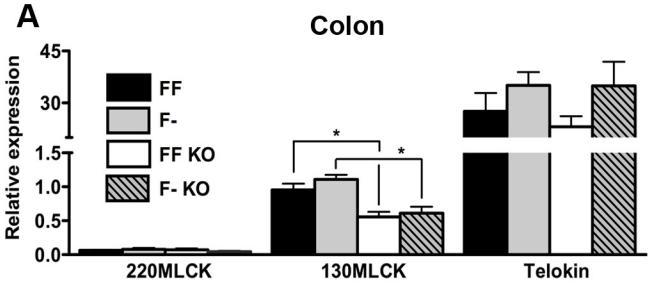
**Figure 6. Knockout mice have decreased intestinal smooth muscle cell proliferation.** **A.** Ki67 (red) and smooth muscle  $\alpha$ -actin (green) staining of cross sections of the lower portion of small intestines from neonatal control (CArG<sup>f/f</sup> Cre<sup>-/-</sup> + CArG<sup>f/-</sup> Cre<sup>-/-</sup>: Ctrl) and knockout (CArG<sup>f/f</sup> Cre<sup>-/+</sup> + CArG<sup>f/-</sup> Cre<sup>-/+</sup>: KO) mice. White arrows and white arrow heads 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 40x magnification in the circular layer, longitudinal layer and both smooth muscle layers of the small intestine. n=6-7 mice. \*, p<0.05.

**B**



	Expected	Actual	Number
MLCK <sup>fl/fl</sup> smMHC-Cre <sup>+/-</sup> (smKO) (KO)	25%	19.1%	50
MLCK <sup>fl/+</sup> smMHC-Cre <sup>+/-</sup> (sm het)	25%	27.1%	71
MLCK <sup>fl/fl</sup> smMHC-Cre <sup>-/-</sup> (flox) (ctrl)	25%	27.9%	73
MLCK <sup>fl/+</sup> smMHC-Cre <sup>-/-</sup> (WT)	25%	26%	68

Figure 2





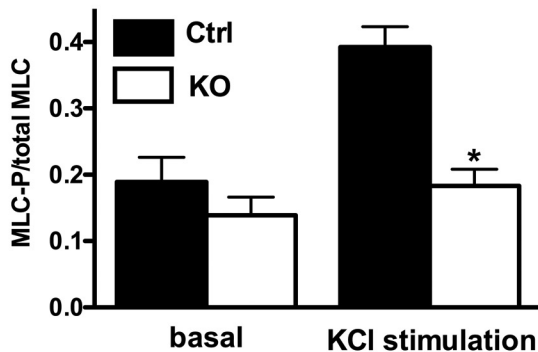
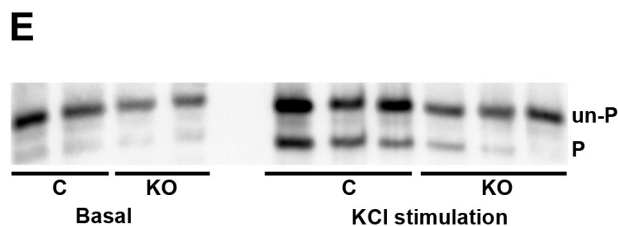
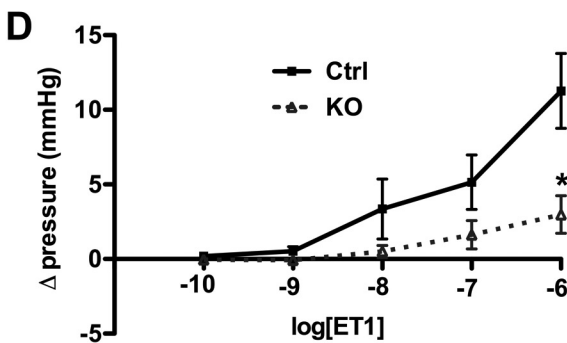
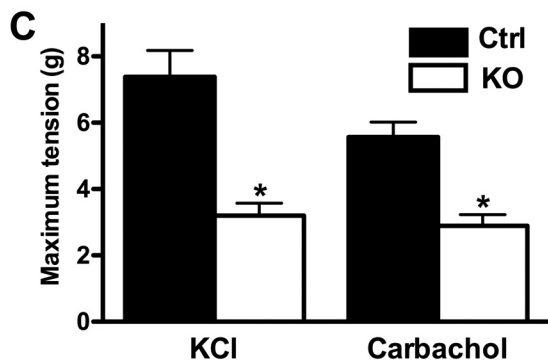
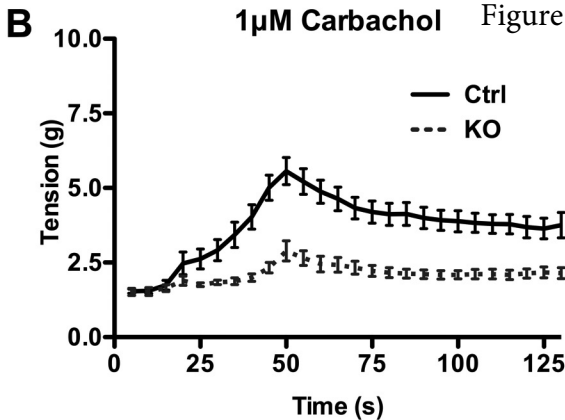
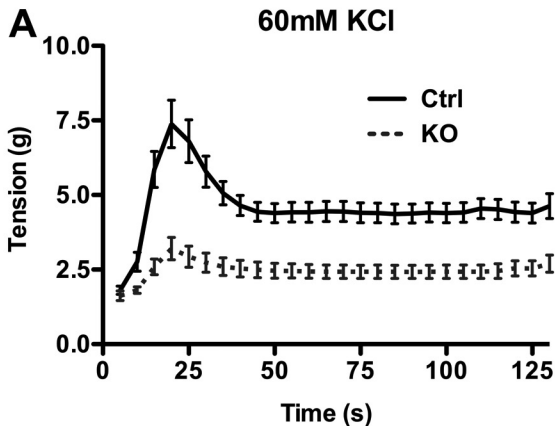
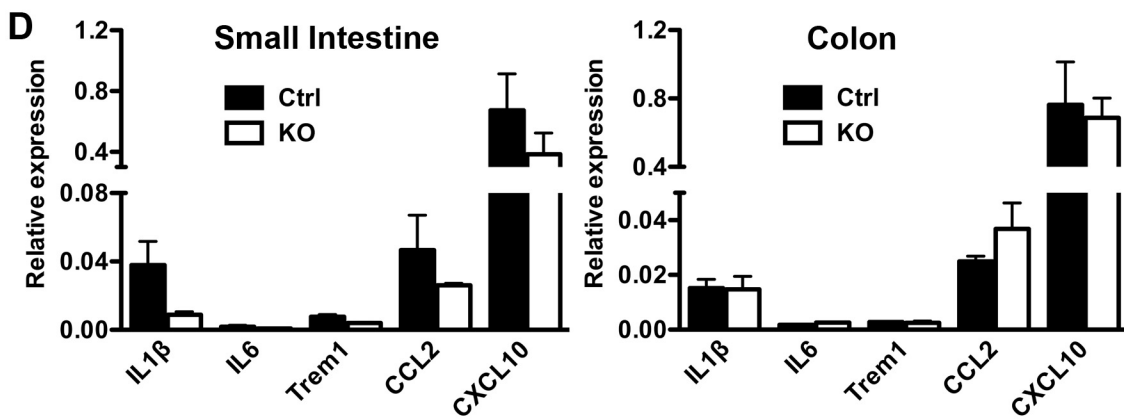
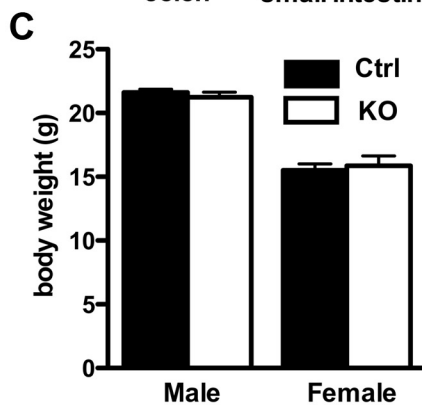
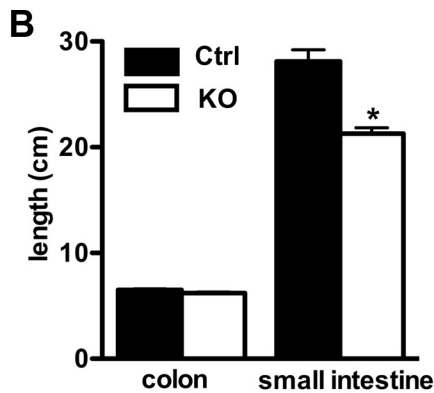


Figure 5



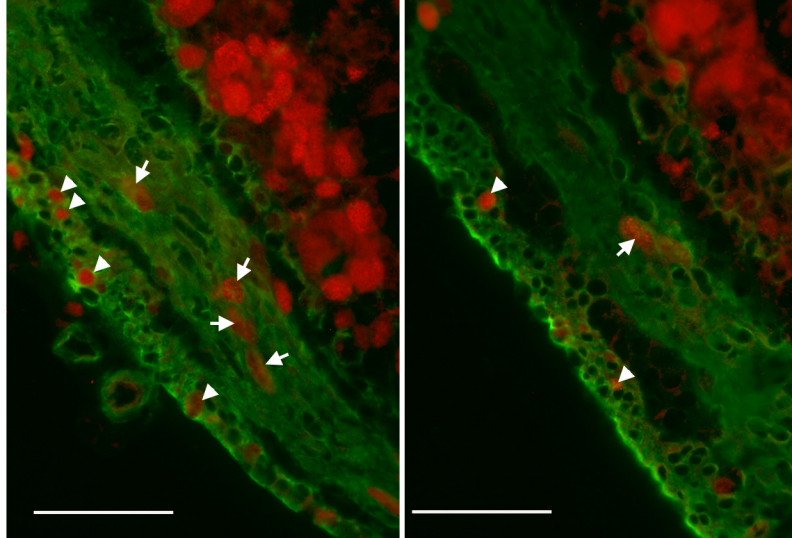
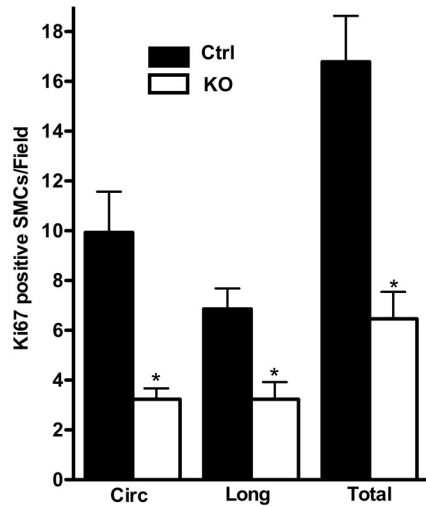
**A****Ctrl****KO****B**

Figure 6



**Gene Regulation:**

**Regulation of 130kDa smooth muscle  
myosin light chain kinase expression by an  
intronic CARG element**

Meng Chen, Wenwu Zhang, Xiao Lu, April  
M. Hoggatt, Susan J. Gunst, Ghassan S.  
Kassab, Johnathan D. Tune and B. Paul  
Herring

*J. Biol. Chem.* published online October 22, 2013

GENE REGULATION

CELL BIOLOGY

---

Access the most updated version of this article at doi: [10.1074/jbc.M113.510362](https://doi.org/10.1074/jbc.M113.510362)

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at  
<http://www.jbc.org/content/early/2013/10/22/jbc.M113.510362.full.html#ref-list-1>