Supplemental Materials and Methods

Generation of 3cCRISPR Targeted Mice

3cCRISPR comprises Cas9 mRNA (purchased directly from TriLink Biotechnologies), a single strand oligonucleotide (synthesized Ultrapure by Integrated DNA Technologies), and a gRNA comprising user-defined crRNA and the invariant tracrRNA within the px330 plasmid (Addgene). Substitutions within the HDR template are most efficiently integrated into the genome when they are relatively close to the Cas9-mediated DSB [1]. Thus, we selected a crRNA sequence that included the intronic CArG element up to but excluding the terminal 3 nucleotides comprising the PAM sequence (Figure 1A). Complementary oligonucleotides corresponding to the crRNA underlined in Figure 1A were annealed for cloning into the *BbsI* site of the px330 plasmid. PCR of the gRNA was done using a universal reverse primer to the tracrRNA and a forward primer containing the T7 promoter followed by 20 nucleotides of the crRNA sequence (Supplemental Table II). The PCR product was column purified and then used as a template for in vitro transcription according to the manufacturer's specifications (MEGAshortscript T7 kit, Life Technologies). The ~90 nucleotide gRNA was then purified using the MEGAclear kit (Life Technologies) and eluted in RNase-free water for analysis with a Bioanalyzer. Fertilized eggs from C57BL-6J/SJL hybrid mice were injected with Cas9 mRNA (100 ng/μl), gRNA (50 ng/μl), and a single strand oligonucleotide containing three base pair substitutions of an intronic CArG box (100 ng/ml) (Figure 1A, Supplemental Table II). Viable two-cell stage embryos were transferred to pseudo-pregnant ICR females and founder mice were tail-snipped for

extensive genotyping and sequencing. The three founder mice were each successfully bred to the F_1 generation using C57BL/6J mice for further analysis. All mouse procedures were performed according to NIH guidelines and approved by the Institutional Animal Care and Use Committee of the University of Rochester.

RFLP Analysis

Tail DNA was prepared using standard methods. PCR primers (Supplemental Table II) flanking the intronic CArG box region were used to amplify genomic DNA which was then gel and column purified for restriction digestion using *SalI* (New England Biolabs). Digested DNA was then run out on a 1% agarose gel and stained with ethidium bromide for UV illuminated image capture.

Multiplex PCR Assay

We developed a novel PCR approach to unambiguously genotype litters of mice derived from heterozygous interbreeding. In this assay, a common reverse primer is used with one of two forward primers that differ in length and 3' end sequence in order to discriminate between wildtype and correctly edited mutant mice (see Supplemental Figure I). Plasmids containing 523 nucleotides of wildtype or sequence-edited CArG box DNA (sequence-edit generated using QuikChange Mutagenesis kit, Stratagene) were used as templates to test the different primers shown in Supplemental Figure I and used as positive controls for genotyping litters of mice from the F_1 intercross (Figure 1C). The two forward primers were combined with the common reverse primer to empirically determine optimal PCR parameters. 2% agarose gels were run to resolve

the 30 nucleotide difference in product size between wildtype and mutant alleles (Supplemental Figure I and Figure 1C).

Analysis of Off-Target Events and Sequencing

Potential off-targets were predicted by searching the mouse genome (mm9) for matches to the 20-nt *Cnn* crRNA sequence allowing for up to four mismatches followed by NGG or NAG PAM sequences. Matches were ranked by ascending number of mismatches. The top 10 potential off-target sites were PCR amplified for Sanger sequencing using primers shown in Supplemental Table II. We focused on those off target sites with no more than two mismatches in the seed sequence. A list of the target sites and results of Sanger sequencing are found in Supplemental Table I. All sequencing was done through the Genomics Facility of the Cornell Biotechnology Resource Center (http://www.biotech.cornell.edu/brc/genomics). A complete listing of all predicted off-target sites as well as the sequences of those sites interrogated for indels is available upon request.

Quantitative RT-PCR

Total RNA was isolated from various mouse tissues of each genotype using the RNeasy kit (Qiagen). RNA integrity was assessed by spectrophotometry (NanoDrop). cDNA was synthesized from 1μg of total RNA using iScript (Bio-Rad) plus random decamers and oligodT primer. Quantitative RT-PCR was performed using IQ SYBR Green Supermix with a MyiQ single color real-time PCR detection system (BioRad). Experiments shown are representative of multiple independent experiments using

tissues from different lines of intronic CArG box mutant mice, performed by separate investigators to ensure quality control and accurate interpretation of observed changes in gene expression. Data are normalized to an internal control (*18 S*) by $2^{-\Delta\Delta Ct}$ and expressed graphically either relative to wildtype tissues (set to 1) or as absolute normalized numbers. Primers for quantitative RT-PCR are listed in the Supplemental Table II.

Western Blotting

Total protein was isolated from tissues harvested from different genotypes of mice using Lysis buffer (Cell signaling) plus protease inhibitor cocktail (Roche) and resolved in 15% acrylamide gels for Western blotting as previously described [2]. Antibodies and dilutions used were as follows: CNN1 (ProteinTech, 1:3000), LMOD1 (ProteinTech, 1:2000) [3], ACTA2 (Sigma, 1:1000), and alpha tubulin (Sigma, 1:4000).

Confocal Immunofluorescence Microscopy

All mouse tissues were fixed in methanol/H_2O/acetic acid (60:30:10, v/v) overnight at room temperature and then processed for paraffin sectioning.

Tissues were sectioned at 5 μm thickness, and slides were deparaffinized and rehydrated in PBS (pH, 7.4). Antigen retrieval was performed using a pressure cooker for 10 minutes on high pressure in pH, 6.0 Target Retrieval (Dako-S1699) and then blocked for 30 minutes (Dako-x0909). Primary antibodies were applied overnight at 4°C using 1:200 rabbit polyclonal anti-Calponin and 1:100 rat anti-Ki-67 (BioLegend) and then slides were washed in PBS. Fluorescently-conjugated secondary antibodies were

then applied for 30 minutes at room temperature. Slides were washed in PBS and mounted in Prolong Gold Antifade reagent supplemented with DAPI (Molecular Probes). Secondary antibodies were A11006 AlexaFluor 488 goat anti-Rat, 1:200 (for Ki-67) and A11012 AlexaFluor 594 goat anti-rabbit, 1:200 (for Calponin). Images were taken with a confocal microscope (Olympus FV 1000) with laser settings fixed to the wildtype genotype such that all other genotypes were imaged with the same parameters. Shown are z-stacks of 10-12 0.5 μm images processed through ImageJ software.

Chromatin Immunoprecipitation Assay

All experiments were carried out using Active Motif ChIP-IT High Sensitivity kit (#53040). Briefly, bladder tissue harvested from wildtype and *Cnn1*$^{\Delta CArG/\Delta CArG}$ mutant mice were cross-linked with fixation solution and then quenched with Stop Solution per kit instructions. Tissues were then lysed using a chilled dounce homogenizer. Chromatin was sonicated in the ChIP buffer to 300–600 bp with the Bioruptor UCD-200 sonication system (Diagenode) at high setting for 20 pulses of 30 seconds each in an ice water bath. Sonicated chromatin was incubated with 2μg anti-SRF (Santa Cruz sc-335) or 2μg IgG (Abcam, ab46540) overnight at 4°C, and then the immunoprecipitates were incubated with Protein G agarose beads for 3 hours at 4°C. The extraction and purification of DNA was done according to kit protocol. DNA was analyzed by end-point PCR and quantitative RT-PCR using IQ SYBR Green Supermix with a MyiQ single color real-time PCR detection system (BioRad). The primers were designed to amplify the intronic region spanning the consensus CArG box undergoing 3cCRISPR editing

(Supplemental Table II). Data are expressed as a percent of the amplified product obtained using input DNA. PCR product size was confirmed in a 2% agarose gel.

Statistical Analyses

Raw data were analyzed statistically using GraphPad Prism software. A one-way ANOVA with Tukey's post-hoc test for individual comparisons was carried out in Figure 2A. We only indicate the statistical differences between the homozygous mutant and wildtype mice. We used paired t-tests for data in Figures 2C and Supplemental Figure II.

References

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