CRISPR-Cas9 Genome Editing of a Single Regulatory Element Nearly Abolishes Target Gene Expression in Mice

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Abstract

Objective—To ascertain the importance of a single regulatory element in the control of *Cnn1* expression using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) genome editing.

Approach and Results—The CRISPR/Cas9 system was used to produce 3/18 founder mice carrying point mutations in an intronic CArG box of the smooth muscle cell (SMC)-restricted *Cnn1* gene. Each founder was bred for germ line transmission of the mutant CArG box and littermate interbreeding to generate homozygous mutant (*Cnn1* $^{CArG/\ CArG}$) mice. Quantitative RT-PCR, Western blotting, and confocal immunofluorescence microscopy showed dramatic reductions in *Cnn1* mRNA and CNN1 protein expression in *Cnn1* $^{CArG/\ CArG}$ mice with no change in other SMC-restricted genes and little evidence of off-target edits elsewhere in the genome. In vivo chromatin immunoprecipitation assay revealed a sharp decrease in binding of SRF to the mutant CArG box. Loss of CNN1 expression was coincident with an increase in Ki-67 positive cells in the normal vessel wall.

Conclusion—CRISPR/Cas9 genome editing of a single CArG box nearly abolishes *Cnn1* expression in vivo and evokes increases in SMC DNA synthesis. This facile genome editing system paves the way for a new generation of studies designed to test the importance of individual regulatory elements in living animals, including regulatory variants in conserved sequence blocks linked to human disease.

Keywords

CRISPR; SRF; CArG box; transgenic mouse; smooth muscle

Traditional approaches to studying the in vivo functionality of regulatory elements controlling gene expression involve pronuclear injection of promoter/enhancer sequences linked to a reporter gene (*e.g.*, *lacZ*). These experiments are limited by the out-of-genomic-

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context in which the sequences are studied and the necessity of generating multiple lines of mice to address issues surrounding copy number- and position-dependent effects on reporter gene activity as well as untoward consequences of foreign DNA sequences on the genomic landscape that could perturb endogenous gene expression[1]. The emergence of artificial chromosome-mediated transgenic mice represented an important step towards the study of gene expression in proper genomic context[2], but these large-capacity cloning vectors are difficult to work with and still require the generation of multiple independent lines of mice, which can take months to years to complete. Thus, newer facile methods of analyzing regulatory elements in their native genomic milieu are needed.

The CRISPR/Cas9 system, defined originally as a heritable, adaptive immune system in bacteria and archaea[3], has recently been harnessed as an RNA-guided nuclease method of genome editing in several animal species including zebrafish[4], pig[5], rat[6], mouse[7], rabbit[8], and monkey[9]. In what we shall refer to as three-component CRISPR (3cCRISPR), a guide RNA (gRNA) – comprising a 20 nucleotide CRISPR RNA (crRNA) plus the invariant transactivating RNA (tracrRNA) – is combined with Cas9 endonuclease mRNA and a single-strand oligonucleotide overlapping the gRNA targeted sequence for delivery to fertilized eggs. Precise genome editing occurs following sequential gRNA-Cas9-mediated DNA double-strand break at the genomic region of interest and homology-directed repair (HDR) of the double-strand break by a single-strand oligonucleotide harboring ~60 nucleotide homology arms flanking DNA substitutions (for mutating endogenous DNA sequences) or exogenous sequences (*e.g.*, restriction site, *loxP*) that are stably integrated at the site of repair[10, 11]. While this programmable endonuclease approach to genome editing has been deployed successfully to modify protein coding genes in vivo, there has yet to be a report on its use in altering regulatory elements controlling gene expression in a living animal[12]. In this context, there is an emerging need to elucidate the functionality of regulatory single nucleotide polymorphisms because most genomic variation associated with human disease occurs in noncoding sequence space where millions of regulatory elements reside[13]. Herein, we demonstrate CRISPR-Cas9-mediated mutation of a regulatory element that nearly abolishes expression of the endogenous *Cnn1* gene in mouse tissues.

Material and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

CArG boxes bind serum response factor (SRF) around a growing number of target genes, including most SMC-restricted genes[14]. We used 3cCRISPR to introduce nucleotide substitutions within a consensus intronic CArG box of the endogenous mouse *Cnn1* gene (Fig. 1A). This particular CArG box is suspected to play an important role in the endogenous expression of *Cnn1*[15, 16]. Initial pro-nuclear injections of plasmid DNA carrying Cas9 and the gRNA were unsuccessful in generating mutant mice. However, cytoplasmic injection of Cas9 mRNA, purified gRNA, and a single-strand oligonucleotide HDR template resulted in 3/18 pups with either one (n=2) or both (n=1) alleles correctly edited as indicated by a restriction fragment length polymorphism (RFLP) assay (Figure 1B) and a novel

multiplex PCR assay we have developed for the unambiguous genotyping of subtle mutations introduced into the genome (Figure 1C and Figure I in the online-only Data Supplement). Off-target mutations of related sequences in the genome were limited and, when present (2/10) in founder mice, affected only non-conserved intergenic sequences (Table I in the on-line Data Supplement). Importantly, repeat sequencing of these sites in F_1 mice revealed the absence of mutations indicating the off-target edits were successfully bred out of the genome. Quantitative RT-PCR analysis of the bi-allelic targeted founder mouse showed significant decreases in *Cnn1* mRNA expression in aorta and bladder (Figure II in the online-only Data Supplement). Each of the founders was successfully bred for germ line transmission of the CArG box mutation and F_1 littermate interbreeding. Levels of *Cnn1* mRNA in aorta and bladder were reduced ~50% in *Cnn1* $^{CArG/+}$ mice and virtually extinguished in *Cnn1* $^{CArG/\ CArG}$ mice (Figure 2A and Figure III in the online-only Data Supplement). Sanger sequencing verified precise genome editing (Figure 1D) with no mutations in sequences surrounding the CArG box, including the entire cDNA and 5' promoter region of *Cnn1* (data not shown), indicating reduced *Cnn1* mRNA expression was not the result of spurious mutations outside of the targeted CArG box. In agreement with mRNA expression analysis, Western blotting revealed low levels of CNN1 protein in the aorta of *Cnn1* $^{CArG/\ CArG}$ mice (Figure 2B and Figure III in the online-only Data Supplement). As expected from previous CArG mutagenesis experiments[15], in vivo chromatin immunoprecipitation assay showed essentially no binding of SRF to the mutant intronic CArG box (Figure 2C). Confocal immunofluorescence microscopy confirmed a clear reduction in expression of CNN1 protein in SMC of the carotid artery (Figure 2C) and other SMC-containing tissues (data not shown). There was no change in mRNA or protein expression of several SMC-restricted markers under similar CArG-SRF regulatory control (Figure III in the online-only Data Supplement). CNN1 has been reported to function as a tumor suppressor gene[17]. Consistent with this idea, we noted a significant increase in the number of Ki67 positive cells per longitudinal section of carotid artery in *Cnn1* $^{CArG/\ CArG}$ mice (21.7 ± 2.1) as compared to *Cnn1*$^{+/+}$ mice (7.7 ± 4.0) (mean Ki67+ cells $\pm$ std, n=3, p=0.0334, Figure 2E). Since *Cnn1* $^{CArG/\ CArG}$ mice are viable, they represent a valuable resource to further characterize the phenotypic changes associated with marked reductions in CNN1 under normal and pathological conditions.

Discussion

In this report, we used 3cCRISPR to mutate a regulatory element controlling gene expression in a living animal. We found that a three nucleotide substitution within an intronic SRF-binding CArG box, suggested previously to be of potential importance in the expression of the endogenous *Cnn1* gene[15, 16], nearly abrogates expression of *Cnn1* mRNA and CNN1 protein in SMC of the vessel wall, without altering expression of a number of other CArG-SRF-dependent genes. Detectable CNN1 protein in the vessel wall of mutant mice suggests there may be additional *cis* elements (such as downstream CArG boxes) directing low level expression of the gene. We also note an increase in the number of SMC undergoing DNA synthesis in the vessel wall of mice carrying the CArG box mutation. The fact that no other local mutations were present, and congruent results were found in independent founder mice, indicate the phenotypes observed are a consequence of a loss in

SRF binding to the mutant intronic CArG box. To our knowledge, this study provides the first genetic proof for a decisive role of a single regulatory element inducing a target gene's expression in vivo without the introduction of foreign DNA sequences or deletion of native sequences.

Genetically inactivating genes, whether protein-coding or otherwise, is common practice in the laboratory. For example, a CArG box in the proximal promoter of the *Telokin* gene was deleted with some flanking sequences using Cre-mediated excision, and this deletion resulted in the loss of *Telokin* expression with consequent changes in the tone of intestinal smooth muscle[18]. Such experimentation is crucial to study the functional consequences associated with loss of gene products and to gain insight into the potential role of regulatory elements such as CArG boxes in the setting of a living animal. Traditional methods of deleting a gene in this manner require complex design and validation of a targeting vector followed by the time-consuming steps of electroporating the targeting vector into embryonic stem cells, enrichment and screening of embryonic stem cells for correct targeting, transference of the embryonic stem cell clone into the blastocyst, and breeding of chimeric mice for germ line transmission of the targeted allele[19, 20]. Due to the protracted time in generating genetically altered mice with this traditional approach, coupled with the complexity and redundancy of gene regulation, there have been far fewer regulatory knockouts as compared to genic knockouts. Further, genic control regions, such as enhancers, have almost always been deleted via Cre recombinase-mediated excision of 100s-1000s of nucleotides, effectively leaving a genomic cavity[21–23]. While these studies certainly provide some insight into the role of enhancers in target gene expression and animal pathophysiology, they lack resolution in narrowing down specific elements within the enhancer that mediate gene expression. Moreover, foreign DNA sequences such as *loxP* sites or the neomycin selectable marker can alter expression of local and/or distal genes, thus confounding accurate interpretation of knockout phenotypes[24]. In contrast, application of 3cCRISPR, as implemented here, provides exquisite genomic editing such that subtle nucleotide substitutions are introduced without deleting sequences or introducing any foreign DNA sequences. This revolutionary technology, therefore, offers an unprecedented experimental approach to precisely model regulatory variants, including previously identified human CArG-SNPs[25], that may contribute to human disease. We predict 3cCRISPR will find widespread utility in the analysis of regulatory elements and will likely supplant traditional approaches to their functional study in transgenic animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

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3cCRISPR	Three-component CRISPR
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
crRNA	CRISPR RNA
gRNA	Guide RNA
HDR	Homology directed repair
PAM	Protospacer adjacent motif
RFLP	Restriction fragment length polymorphism
SMC	Smooth muscle cell(s)
SRF	Serum response factor
tracrRNA	Transactivating RNA

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Significance

The CRISPR/Cas9 system is a revolutionary approach to rapidly generate precision-guided mutations in any genome. There have been a number of reports detailing CRISPR/Cas9-mediated edits in protein coding and noncoding genes in vivo. However, no study has yet to report on the use of CRISPR/Cas9 to effectuate a mutation in a regulatory element controlling gene expression in an animal model system. This type of genome editing is highly significant given the increasing recognition of disease-associated variants linked to regulatory elements that determine the extent and duration of a target gene's expression and the need to model such mutations in a living organism. Here, we provide the first case study of CRISPR/Cas9 directing a subtle mutation within a key regulatory element controlling gene expression in vivo. This study provides a foundation for future experiments that will swiftly interrogate regulatory elements and their variants in animal models.
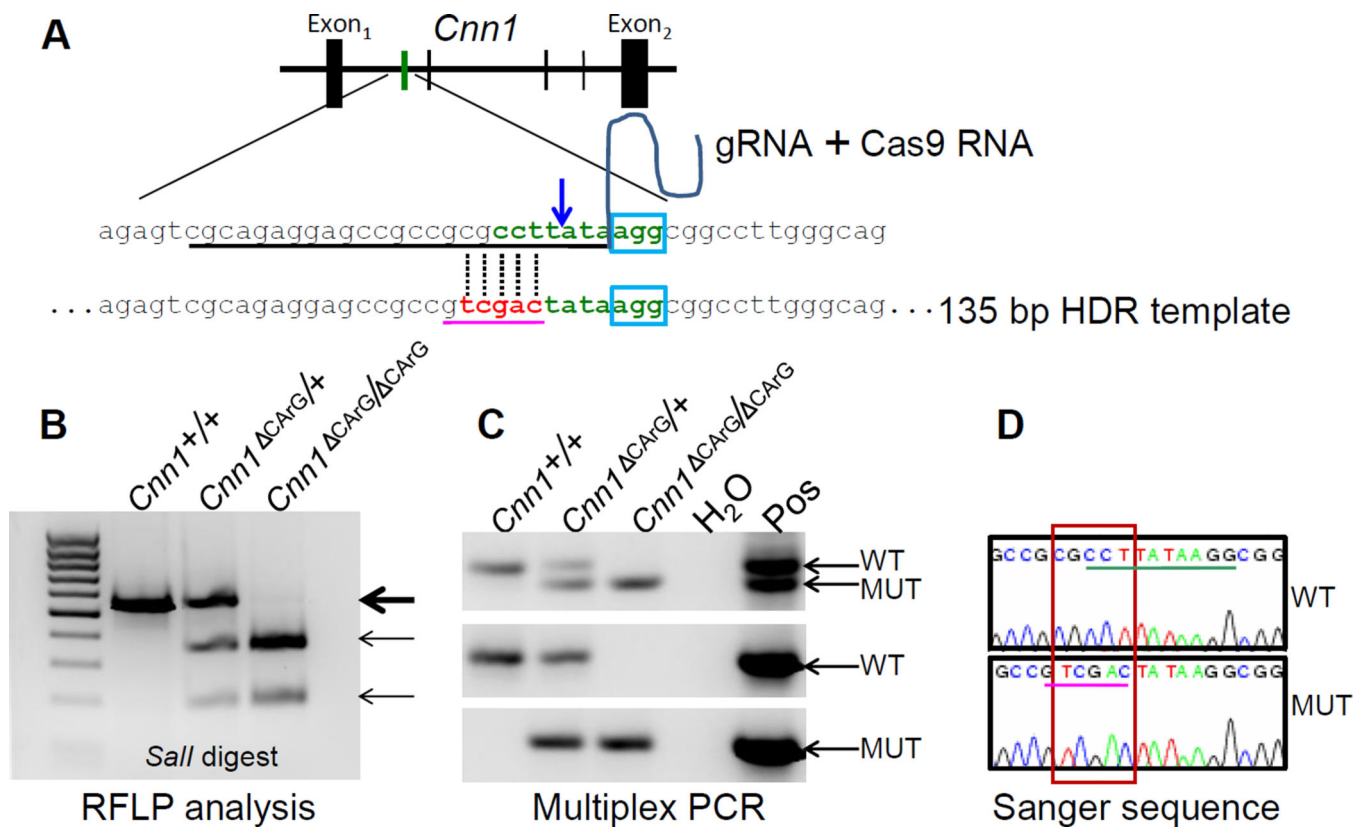
Figure 1. 3cCRISPR genome editing of a CArG box

(A) Strategy for targeting one of the 4 conserved intron 1 CArG boxes (vertical green and black lines) in mouse *Cnn1* gene. A synthetic crRNA (underlined sequence) was designed to include the first seven nucleotides of the CArG box (green) immediately 5' of a protospacer adjacent motif (PAM, blue box) comprising the remaining three nucleotides of the CArG box. The blue arrow indicates the predicted double strand break three nucleotides upstream of the PAM sequence. The crRNA was cloned upstream of the invariant tracrRNA (curved line) and this gRNA was then combined with Cas9 mRNA and the HDR template harboring three nucleotide substitutions of the CArG box following two nucleotide changes (red) that together create a novel *SalI* restriction site (pink underlined sequence), for cytoplasmic injection of fertilized mouse eggs. Genotyping of F_1 intercrossed mice was done using a *SalI* RFLP digest (B), a novel multiplex PCR assay (C) and Sanger sequencing (D). Thick arrow in panel B represents the wildtype PCR product and thin arrows represent the expected size products of one or both alleles with correct *SalI* site. Arrows in panel C represent the PCR products with multiplex PCR (top gel) and PCR using wildtype (middle) or mutant (bottom) forward primers (see Figure I in the online-only Data Supplement). The red box in panel D shows the confirmed sequence edit creating novel *SalI* site (pink underlined sequence) that changes the normal CArG box (green underlined sequence).
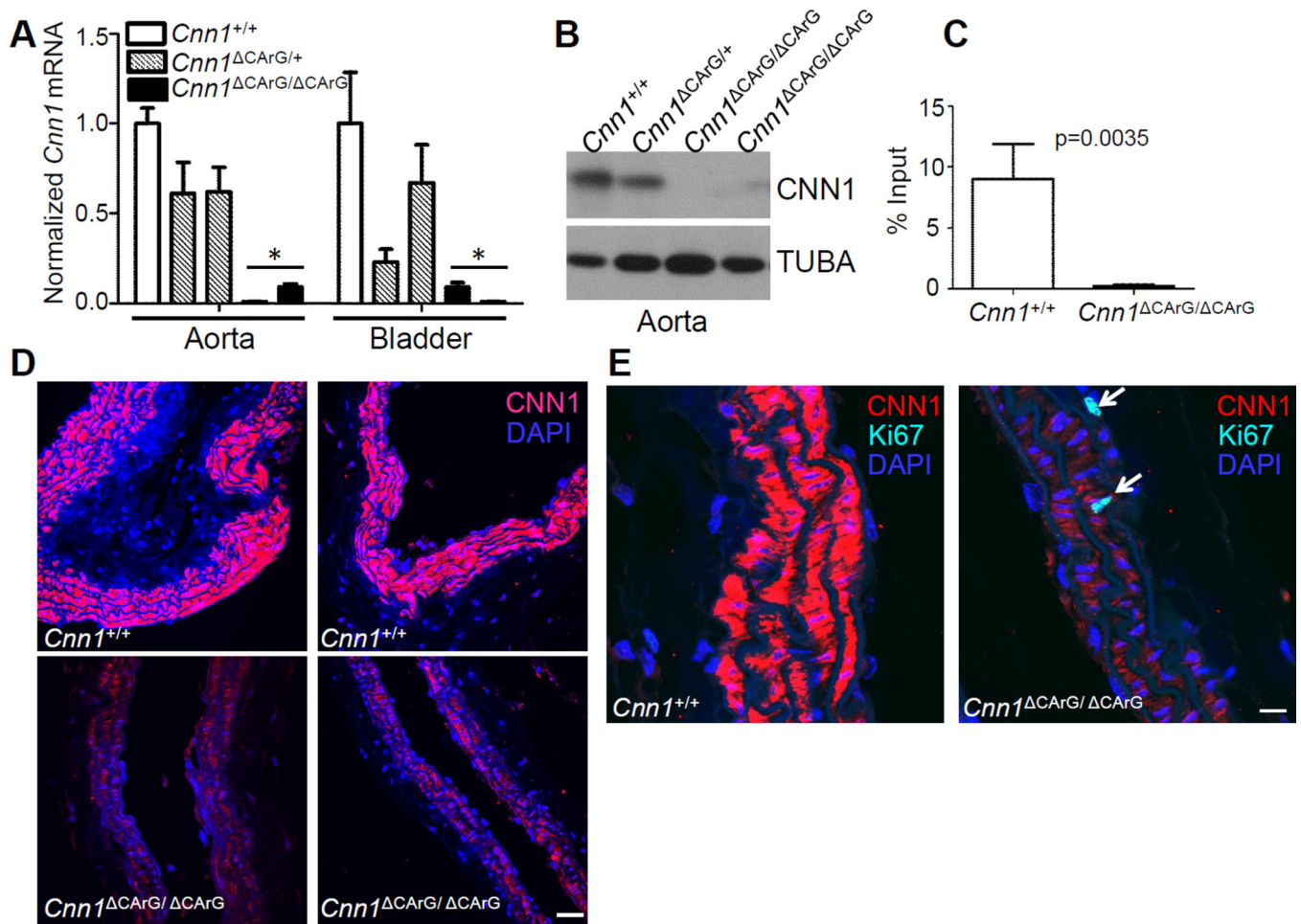
Figure 2. Expression of *Cnn1* mRNA and CNN1 protein in F$_1$ mice

(A) Mouse aorta or bladder analyzed for expression of *Cnn1* mRNA by quantitative RT-PCR with normalized *Cnn1*$^{+/+}$ set to 1.0; other genotypes expressed relative to *Cnn1*$^{+/+}$. (asterisks indicate p<0.01 compared to wildtype). See also Supplemental Figures II and III. (B) Expression of CNN1 protein in aorta by Western blotting. (C) In vivo chromatin immunoprecipitation assay of SRF binding to wildtype versus mutant intronic CArG box in bladder tissue. (D) Confocal immunofluorescence microscopy of CNN1 in wildtype versus mutant carotid arteries of two different founder lines. (E) SMC DNA synthesis (arrows) revealed with an antibody to Ki-67. Similar findings were seen in an independent founder line. Scale bars are 30 μm and 10 μm for panels D and E, respectively.