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Original Article

Genetic alterations in CREBRF influence prostate cancer survival and impact prostate tissue homeostasis in mice

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Abstract: Background: Risk factors for prostate cancer include age, environment, race and ethnicity. Genetic variants in cyclic-adenosine-monophosphate-response-element-binding protein 3 regulatory factor (CREBRF) gene are frequently observed in Pacific Islanders, a population with elevated prostate cancer incidence. CREBRF has been shown to play a role in other cancers, however its function in prostate homeostasis and tumorigenesis has not been previously explored. We determined the incidence of CREBRF alterations in publicly available databases and examined the impact of CREBRF deletion on the murine prostate in order to determine whether CREBRF impacts prostate physiology or pathophysiology. Methods: Alterations in CREBRF were identified in prostate cancer patients via in silico analysis of several publicly available datasets through cBioPortal. Male *Crebrf* knockout and wild-type littermate mice were generated and examined for prostate defects at 4 months of age. Immunohistochemical staining of murine prostate sections was used to determine the impact of *Crebrf* knockout on proliferation, apoptosis, inflammation and blood vessel density in the prostate. Serum adipokine levels were measured using a Luminex Multiplex Assay. Results: CREBRF alterations were identified in up to 4.05% of prostate tumors and the mutations identified were categorized as likely damaging. Median survival of prostate cancer patients with genetic alterations in CREBRF was 41.23 months, compared to 131 months for patients without these changes. In the murine model, the prostates of *Crebrf* knockout mice had reduced epithelial proliferation and increased TUNEL$^+$ apoptotic cells. Circulating adipokines PAI-1 and MCP-1 were also altered in *Crebrf* knockout mice compared to age-matched controls. Conclusions: Prostate cancer patients with genetic alterations in CREBRF had a significantly decreased overall survival suggesting that wild type CREBRF may play a role in limiting prostate tumorigenesis and progression. The murine knockout model demonstrated that CREBRF could modulate proliferation and apoptosis and macrophage density in the prostate. Serum levels of adipokines PAI-1 and MCP-1 were also altered and may contribute to the phenotypic changes observed in the prostates of *Crebrf* knockout mice. Future studies focused on populations susceptible to CREBRF mutations and mechanistic studies will be required to fully elucidate the potential role of CREBRF in prostate tumorigenesis.

Keywords: CREBRF, prostate cancer, pacific islanders, knockout mouse, prostate

Introduction

For US men, prostate cancer is the most frequently diagnosed cancer and has been the second leading cause of cancer death for the past several years [1]. Prostate cancer risk and mortality vary among racial and ethnic groups and these differences are likely driven by genetic, environmental, social and health care disparity factors [2, 3]. In the US, prostate cancer risk is elevated among Native Hawaiians and Samoans compared to non-Hispanic Whites [2, 4, 5]. Cancer is the leading cause of death for Native Hawaiians and other Pacific Islanders, including Samoans [6, 7]. These groups also have higher rates of diabetes, obesity, asthma and cardiovascular diseases [8]. These outcome studies highlight the impor-

tance of investigating the role of both external factors and genetic influences contributing to prostate cancer disparities in susceptible populations.

CREB3 regulatory factor (CREBRF) is a gene that is frequently altered in Pacific Islanders and Samoans [9]. For example, a missense CREBRF variant (Arg457Gln) is associated with higher body mass index in Pacific Islanders and has been identified in up to 15% of this population [10-12]. The CREBRF protein is evolutionarily conserved and widely expressed in human tissues, including the prostate [10, 13]. The functional role of CREBRF is not well understood, however it has been shown to facilitate recruitment of the CREB3 transcription factor to nuclear foci, resulting in repression of its transactivation activity and accelerated degradation [14]. A potential role for CREBRF in tumorigenesis has been reported for several cancers. For example, CREBRF acts as a tumor promoter in both gastric [15] and cervical cancer [16, 17] and its expression is high in these tumors. Conversely, CREBRF can act as a tumor suppressor in glioblastoma [18, 19], gallbladder cancer [20] and acute myeloid leukemia [21] and its expression is reduced compared to normal tissues in these cancers.

Although genetic variants of CREBRF are associated with other cancer types and common in a population that has a higher risk of prostate cancer than the general population, it is unclear whether CREBRF plays a role in prostate tumorigenesis. Since its potential impact on prostate cancer has yet to be explored, we queried several prostate cancer datasets available through the cBioPortal for Cancer Genomics [22, 23] to probe for any association between CREBRF gene variants and prostate cancer survival. We also assessed the functional consequences of *Crebrf* loss in the prostate of young mice to explore the potential functional role of CREBRF in prostate homeostasis and tumorigenesis.

Materials and methods

CREBRF alterations in human prostate tumors identified using cBioPortal

The cBioPortal for Cancer Genomics site (http://cbioportal.org) was utilized to identify genetic alternations in CREBRF alterations in prostate tumors [22, 23]. PolyPhen-2 (Polymorphism Phenotyping v2) was used to calculate the probability that genetic variants identified in prostate tumor specimens are damaging [24]. Probabilistic scores above 0.85 indicate that a variant is “probably damaging”, “possibly damaging” if its probabilistic score is above 0.15, and variants with a score ≤ 0.15 are classified as “benign” [25].

Generation of Crebrf deletion mice

Mice with global deletion of the *Crebrf* gene were generated by crossing *Crebrf*$^{+/-}$ mice obtained under a material transfer agreement from the laboratory of Dr. Ray Lu (University of Guelph, Guelph, ON, Canada) [26]. Experimental cohorts were litter-matched virgin wild-type and *Crebrf*KO male animals on a C57BL/6J background. Mice were provided food (LabDiet 5P75, LabDiet, St. Louis, MO, USA) and water *ad libitum* and housed in a University of Pittsburgh vivarium. PCR genotyping of genomic DNA was performed using mouse tail or ear punch at age 19 days and muscle following euthanization at 4 months of age [27]. The Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh reviewed and approved all animal studies, which were conducted in strict accordance with the standards for humane animal care and use as set by the Animal Welfare Act and the National Institutes of Health guidelines for the use of laboratory animals under Animal Welfare Assurance number A3187-01.

A cohort of eight wild-type and ten *Crebrf*KO male mice were euthanized via CO_2 asphyxiation followed by decapitation at 4 months of age. Body mass and nose-to-rump length was measured for each mouse. Bladder size was measured *in situ* with a precision caliper and bladder volume was calculated using the formula for the volume of an ellipsoid [28]. Blood serum samples were taken via cardiac puncture after euthanasia. Urogenital tract necropsy was performed following cardiac puncture, and organs were fixed in 10% formalin. Fixed tissues were cleaned of excess fat and membrane with phosphate-buffered saline; mass of the seminal vesicles, prostate lobes and bladder was determined using a microbalance after blotting with filtration paper to remove excess liquid.

Serum adipokine analysis

Adipokines were simultaneously measured in duplicate in each serum sample using the

MILLIPLEX MAP Mouse Adipokine Magnetic Bead Panel-Endocrine Multiplex Assay|MADKMAG-71K (emdmillipore.com) Luminex xMAP® technology (multiplexed fluorescent bead-based immunoassay) (Luminex Corporation, Austin, TX, USA). Serum analyte quantitative analyses were performed with Luminex xPONENT 3.1 Software (Luminex Corporation, Austin, TX, USA) using a five-parameter logistic curve fitting.

Histopathologic analysis

Samples were fixed in 10% formalin for at least 24 hr, then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Prostate tissues were examined under light microscopy by a board-certified animal pathologist (LHR, V.M.D.) in a blinded fashion according to the criteria commonly used to score prostatic lesions in mice published by Shappell, et al. [29].

Immunohistochemistry and terminal transferase-mediated DNA end labeling (TUNEL) assay

Immunohistochemical staining was performed as described previously [30]. Primary antibodies were rabbit monoclonal anti-Ki-67 (D3B5, Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-CD68 (KP1, CM033, BioCare Medical), rat monoclonal anti-PECAM1/CD31 (550274, BD Biosciences, San Jose, CA, USA), and rabbit polyclonal anti-PAI-1 (ab66705, Abcam). Slides were then counterstained in hematoxylin.

TUNEL histochemical staining was performed using Apoptag TUNEL Stain (EMD Millipore, Burlington, MA, USA), and Masson's Trichrome histochemical staining was performed using HT15 (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's protocols. Stained sections were imaged with an Olympus BX51 (Olympus, Center Valley, PA, USA) equipped with an F-View II camera (Olympus) and DP-BSW software (Olympus).

Ki-67- and TUNEL-positive cell density were determined for luminal epithelial or stromal cells by analysis of sections from at least eight independent mice from each genotype and from all lobes of the prostate. The average number of positive cells in each prostate lobe for each mouse was determined from at least four nonoverlapping fields imaged at 20× magnification. The average number of CD68-positive macrophage cell and the CD31-positive microvessel density of each prostate lobe were determined similarly. PAI-1 expression was assessed as a function of immunostaining intensity and the percentage of positive cells. The intensity was based on a 4-point scale: none, faint/equivocal, moderate, and intense. An H-score was calculated by cell type using the formula: H-score = 0 (% no stain) + 1 (% faint/equivocal) + 2 (% moderate) + 3 (% intense).

Statistical analysis

Overall survival of prostate cancer patients was analyzed using the Kaplan-Meier method, and comparison between groups with and without alterations was calculated using log-rank and Gehan-Breslow-Wilcoxon tests. Comparisons between animal cohorts were calculated using the Student's t-test. A *P*-value of $P < 0.05$ was considered significant. GraphPad Prism version 9 was used for graphics (GraphPad Software, San Diego, CA, USA). Values are expressed as means ± S.D.

Results

CREBRF expression and genetic variants in prostate cancer

We queried several publicly available datasets through cBioPortal (cBioPortal.org) for the presence of genetic variants in CREBRF in prostate cancer patient specimens [22, 23]. Alteration data included single nucleotide changes, amplifications and deep deletions, and was available in 11 non-overlapping datasets, with alteration frequencies ranging from 0.42-4.05% (**Figure 1A**). Nine unique missense mutations, one splice variant and one truncating frameshift mutation in CREBRF were identified that collectively affected 0.2% (70/4135) of prostate cancer patients (**Figure 1B**; **Table 1**). Functional impact analysis predicted that seven of these mutations were either possibly or probably damaging to CREBRF function (**Table 1**). Overall survival was 41.23 months (23.32-NA) for patients with CREBRF genetic alterations compared to 131 (115.05-156.00) for patients without CREBRF alterations (*P*-value 0.07 log-rank test, $P < 0.001$ Gehan-Breslow-Wilcoxon test, p^1 and p^2) (**Figure 1C**; **Table 2**). These findings suggest that genetic variants in CREBRF

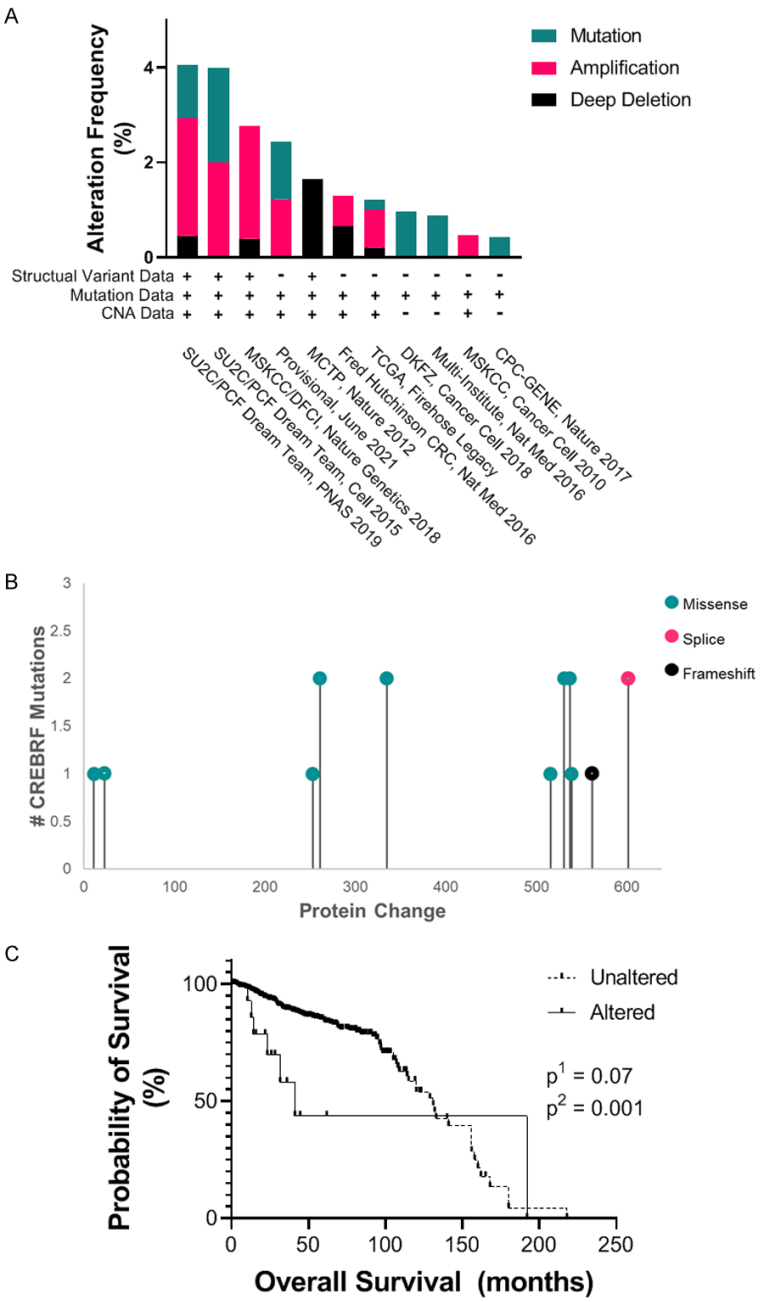

Figure 1. Identification of CREBRF alterations in prostate cancer specimens from the cBioPortal for cancer genomics [22]. A. Alteration frequency of types of CREBRF genetic alterations in prostate cancer patients, including mutation (teal), amplification (red), and deep deletion (black). B. Lollipop figure of CREBRF mutations detected in 70 patients (out of 4,135 total patients). Teal dots indicate missense mutations, red dots indicate splice variants, block dots indicate truncating frameshifts. C. Overall survival in patients with CREBRF alterations compared to patients with no alteration (unaltered). p^1, *P*-value acquired from the logrank test, p^2, *P*-value was acquired from the Gehan-Breslow-Wilcoxon test.

may be associated with poor prognosis in prostate cancer patients.

Lower urogenital tract phenotype of CrebrfKO mice

*Crebrf*KO mice have previously been shown to have reduced body weight at 6 months of age but no apparent gross abnormalities [26]. We found a similar non-significant decrease in the nose-to-rump length and body mass of *Crebrf*KO mice at 4 months of age (**Figure 2A**, **2B**). Gross examination of the lower urogenital tract of *Crebrf*KO mice revealed no morphological differences compared to wild-type controls and the prostates of *Crebrf*KO mice appeared to be histologically normal at 4 months of age (**Figure 2C**). However, the mass of seminal vesicles, the ventral-lateral and anterior prostate lobes was lower in *Crebrf*KO mice than in wild-type controls (**Figure 2D**, **2E**). There was no significant difference in the bladder mass or bladder volume (Supplementary Figure 1A, 1B).

The proliferative marker Ki-67 was used to detect dividing cells in the prostates of *Crebrf*KO and control mice. Overall, the number of Ki-67-positive epithelial cells was decreased in the KO mice vs control at 4 months of age. A statistically significant decrease in proliferation was identified in the ventral, dorsal and anterior prostate lobes (**Figure 3A**, **3C**). Stromal proliferation was also significantly decreased in the ventral and lateral prostate lobes of *Crebrf*KO mice (**Figure 3B**, **3C**). TUNEL immunostaining identified a statistically significant increase in the number of TUNEL positive epithelial cells of the ventral and lateral prostate, and a significant increase

Table 1. CREBRF mutations identified in prostate cancer patients (cBioPortal)

Protein Change	Mutation Type	Variant Type	Copy #	Functional Impact (PolyPhen-2)	Allele Frequency	Study of Origin
P11L	Missense	SNP	Diploid	1.00, probably damaging	0.04	[46]
S23L	Missense	SNP		0.09, benign		[47]
S253C	Missense	SNP	Diploid	0.79, possibly damaging	0.06	[48]
K261N	Missense	SNP	Diploid	0.01, benign	0.02	[49]
H335Q	Missense	SNP	Diploid	0.99, probably damaging	0.06	[49]
S516R	Missense	SNP		0.99, probably damaging		[47]
N531S	Missense	SNP		0.98		[50]
A537T	Missense	SNP	Gain	0.99, prob damaging	0.05	[49]
R539Q	Missense	SNP		0.98, probably damaging		[50]
X602_splice	Splice	SNP	Diploid		0.33	[49, 51]
N562Ifs*6	FS ins	INS	Diploid	0.99, probably damaging		[49]

SNP, single nucleotide polymorphism; FS ins, frame-shift insertion; INS, insertion. The results included here include the use of data from The Metastatic Prostate Cancer Project (https://mpcproject.org/), a project of Count Me In (https://joincountmein.org/).

Table 2. Overall survival probability of patients with (altered) and without (unaltered) CREBRF alterations

CREBRF Status	Number of Cases	Number of Events	Median Months Overall (95% CI)
Altered	14	7	41.23 (23.32-NA)
Unaltered	967	135	131.00 (115.05-156.00)

in the number of TUNEL positive stromal cells in the lateral prostate of *Crebrf*KO mice (**Figure 4A-C**). These results suggest that reduced prostate size could be due in part to the reduced proliferation and increased apoptosis observed in *Crebrf*KO mice.

Serum adipokine levels

Serum adipokines were profiled in mice at 4 months of age. Adipokines IL-6, Insulin, Leptin, Resistin, PAI-1, MCP-1 and TNF-α were simultaneously measured in each serum sample. Compared to wild-type controls, PAI-1 was significantly increased and MCP-1 was significantly decreased in *Crebrf*KO mice (**Table 3**). PAI-1 immunostaining in prostate tissues revealed a statistically significant increase in staining intensity in the ventral and dorsal prostate lobes of *Crebrf*KO mice (**Figure 5A**, **5B**). No significant difference was detected in IL-6, Insulin, Leptin, Resistin or TNF-α (**Table 3**).

Macrophage and vessel density

The density of CD68-positive macrophages and CD31-positive vessels was determined using immunostaining of prostate tissues sections from *Crebrf*KO and control mice. Overall, the number of CD68-positive macrophages was significantly decreased in the ventral and dorsal lobes of *Crebrf*KO (**Figure 6A**). No detectable difference in prostate CD31-positive vessel density was identified between Control and *Crebrf*KO mice at 4 months of age (**Figure 6B**, **6C**).

Discussion

CREBRF variants are frequent in Samoans and Pacific Islanders [10, 11], populations that also have an increased risk for prostate cancer [2-4, 6-8]. However, the function of CREBRF in prostate has not been previously explored. By querying publicly available datasets, we identified CREBRF single nucleotide changes, amplifications and deep deletions in up to 4.05% of prostate cancer patients. These variants generated missense mutations, splice variants and insertions leading to frameshifts, of which the majority were scored as likely damaging. Furthermore, overall survival in patients with CREBRF alterations was significantly less than patients without CREBRF alterations. Interestingly, we did not identify the missense variant p.Arg457Gln frequently observed in Pacific Islanders that that has been correlated with elevated BMI in Samoans [10]. Thus, the different CREBRF variants found in Pacific Islanders may be distinct in their contributions to metabolic disorders

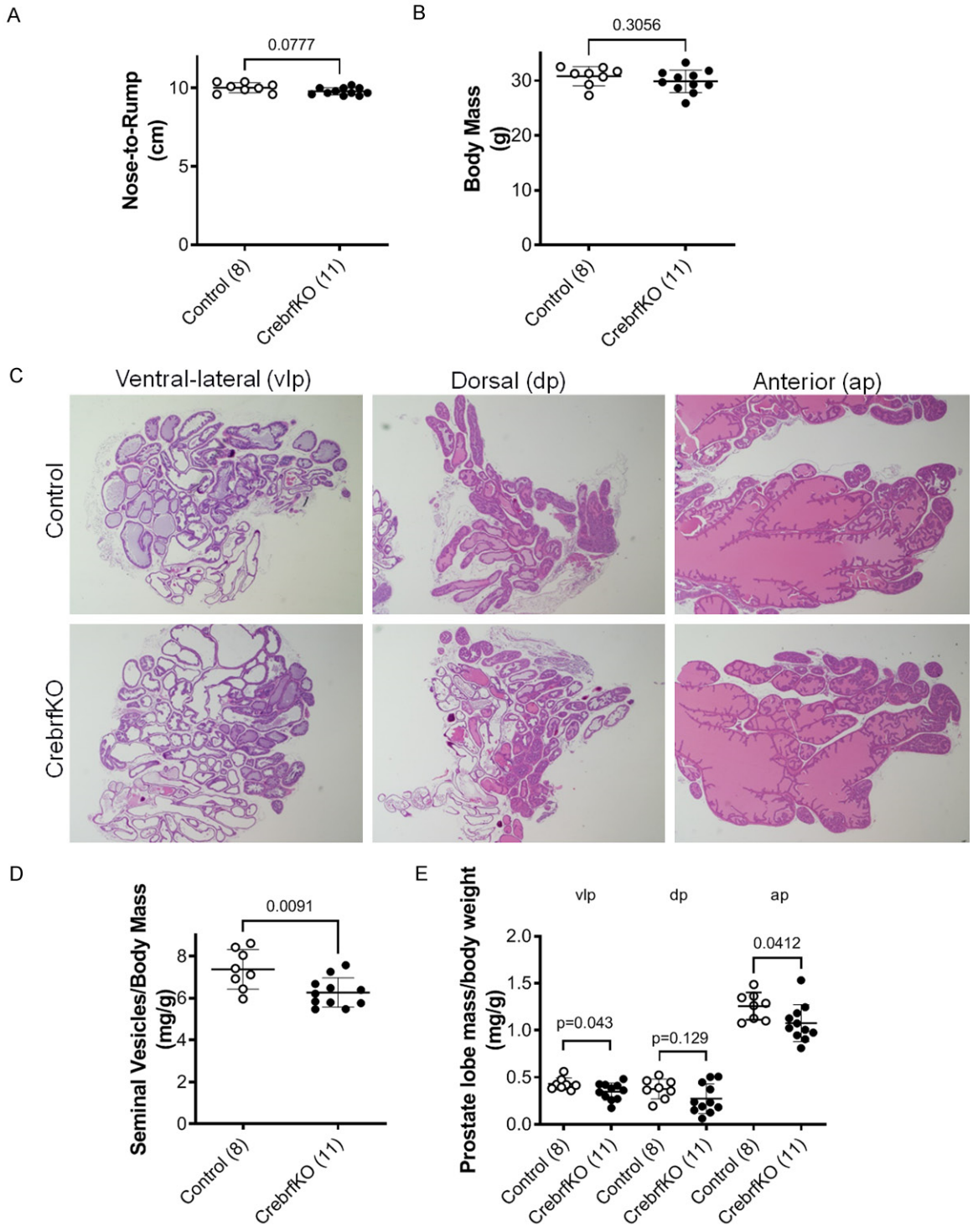

Figure 2. Impact of global *Crebrf* deletion in C57BL/6J mice at 4 months of age on the lower urogenital tract. A. Nose-to-rump length. B. Body mass. C. Hematoxylin and eosin staining of prostates. Original magnification, 2×. D. Mass:Seminal Vesicle ratio of seminal vesicles in combined mass of both seminal vesicles. E. Mass:Body mass ratio of prostate lobes. vlp, ventral-lateral prostate, dp, dorsal prostate, ap, anterior prostate. *P*-value acquired from the Student's t test, number of animals in each group indicated in parentheses.

and prostate cancer risk. Taken together, these findings suggest that genetic variants in CREBRF in prostate cancer patients are rare, but may be indicative of poor survival and thus

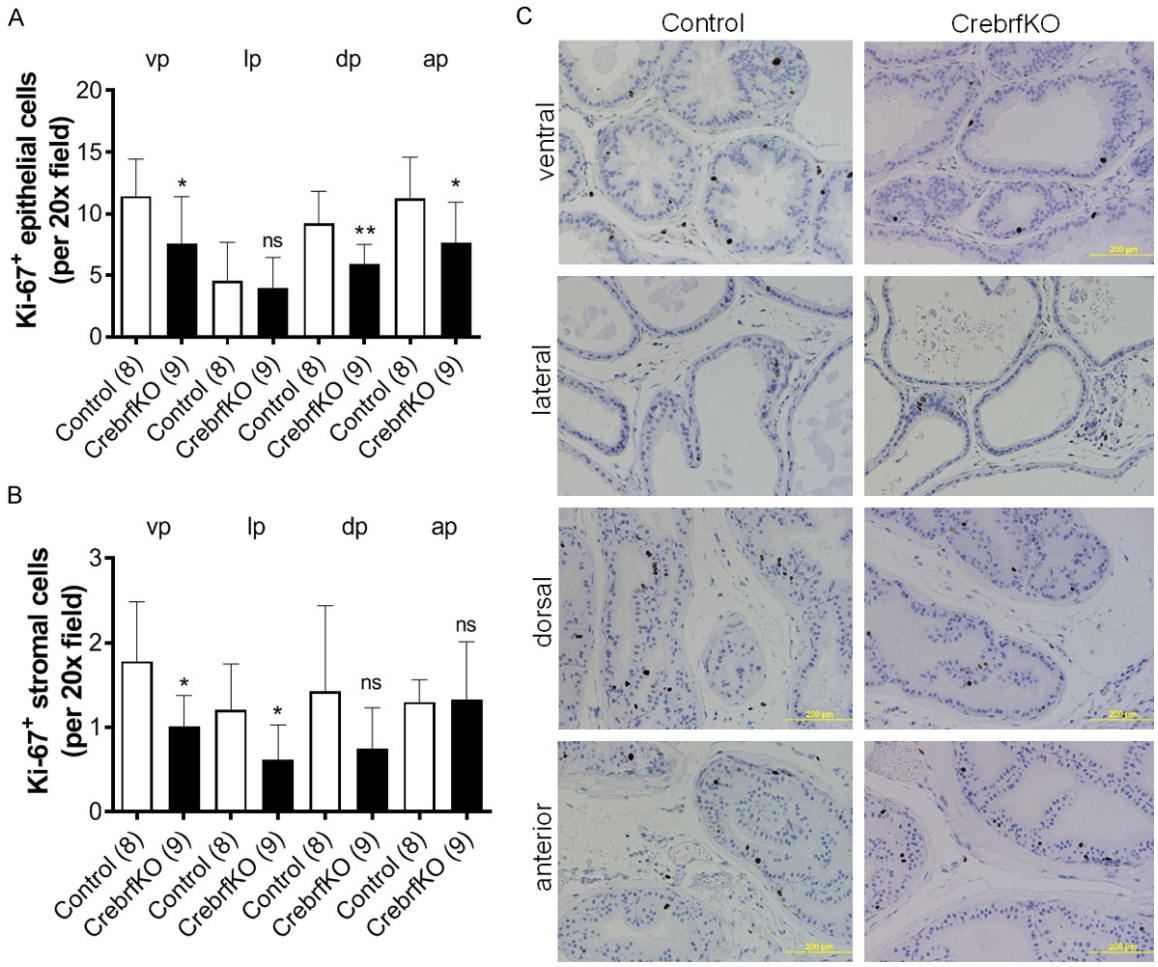

Figure 3. Impact of conventional *Crebrf* deletion on prostate epithelial and stromal proliferation in C57BL/6J mice at 4 months of age. A. Quantification of Ki-67$^+$ epithelial, and B. Stromal cells in the prostate lobes. Abbreviations: vp: ventral prostate; lp: lateral prostate; dp: dorsal prostate; ap: anterior prostate. C. Ki-67 immunostaining (brown) in the epithelial and stromal compartments of prostate ventral and lateral lobes. Original magnification, 20×. *, $P < 0.05$, **, $P < 0.01$, ns, non-significant. *P*-value acquired from the Student's t test, number of animals in each group indicated in parentheses.

may play a role in prostate cancer development or progression.

To further determine the potential functional role of CREBRF in the prostate, we examined a cohort of male mice with global loss of CREBRF [26, 27]. *Crebrf*KO mice have previously been shown to have reduced body weight at 6 months of age but no apparent gross abnormalities [26]. Here, we found a non-significant decrease in nose-to-rump length and body mass of *Crebrf*KO mice compared to age matched controls. The seminal vesicles and prostates were also decreased in mass. Since CREBRF knockdown was found to inhibit proliferation and enhanced apoptosis and autophagy in glioma cells [31], we quantified the number of Ki-67 proliferating and TUNEL positive apoptotic cells in the prostate. We found that epithelial proliferation was significantly decreased in the ventral, dorsal and anterior prostate lobes of *Crebrf*KO mice. Stromal proliferation was also decreased in the ventral and lateral lobes. Similarly, TUNEL positive apoptotic epithelial cells were increased in the ventral and lateral epithelial compartment and the lateral stromal compartment. These findings suggest that CREBRF is important for modulation of proliferation and apoptosis in the prostate.

Genetic variants in CREBRF have also been associated with obesity [10, 32], and prostate cancer risk is elevated in obese men [33-35]. The elevation of obesity-associated adipokines

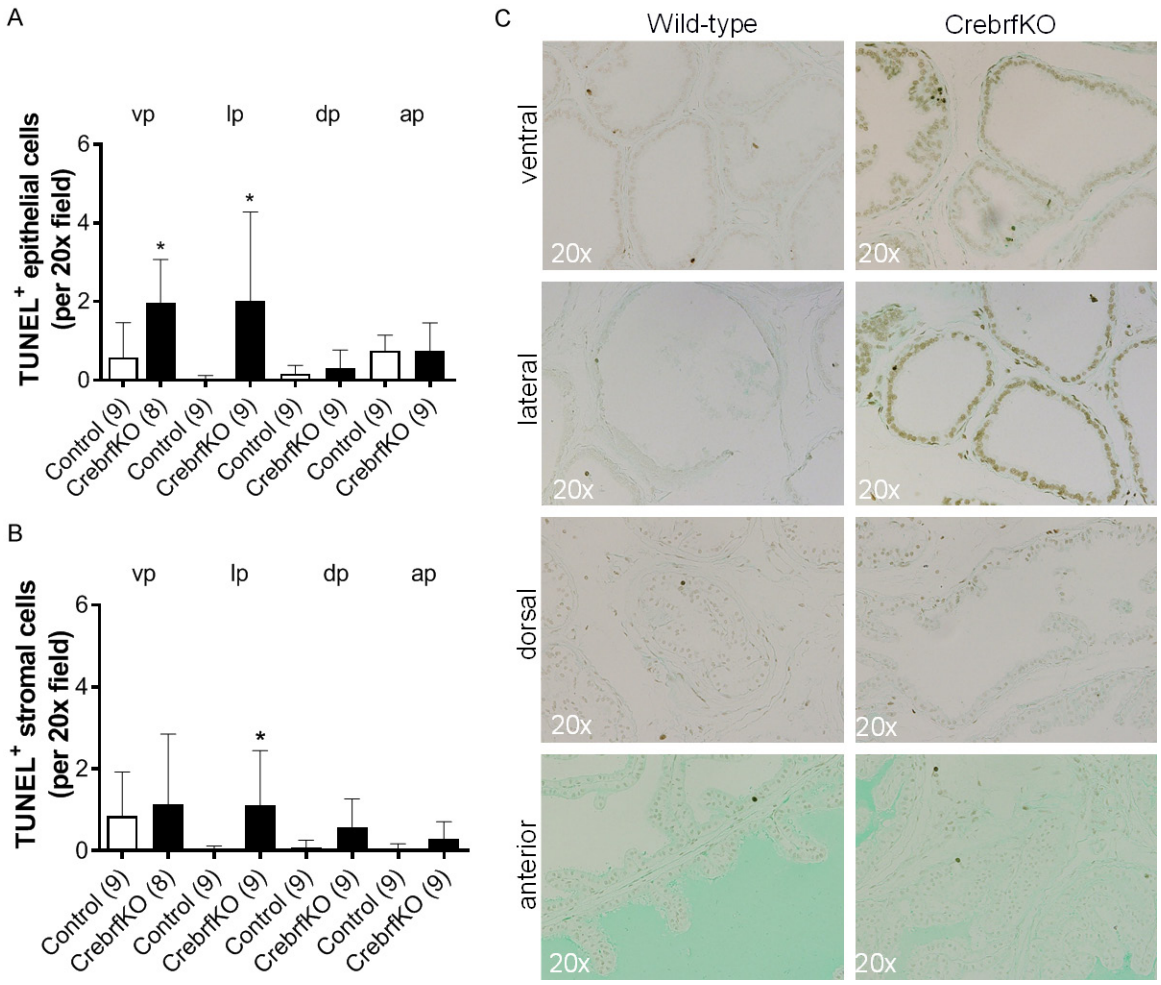

Figure 4. Impact of global *Crebrf* deletion on prostate epithelial and stromal apoptosis in C57BL/6J mice at 4 months of age. A. Quantification of TUNEL+ epithelial, and B. Stromal cells in the prostate lobes. Abbreviations: vp: ventral prostate; lp: lateral prostate; dp: dorsal prostate; ap: anterior prostate. C. TUNEL immunostaining (brown) in the epithelial and stromal compartments of prostate lobes. Original magnification, 20×. *, $P < 0.05$, ns, non-significant. *P*-value acquired from the Student's t test, number of animals in each group indicated in parentheses.

Table 3. Serum adipokines in CrebrfKO and wild-type control male mice at 4 months of age

Adipokine	Control (pg/ml)	*Crebrf*KO (pg/ml)	*P*-value
IL-6	6.14 (± 6.27)	9.026 (± 5.38)	0.18
Insulin	1516 (± 901)	1637 (± 1092)	0.78
Leptin	2361 (± 985)	3647 (± 3824)	0.31
PAI-1 (Total)	927 (± 454)	1512 (± 368)	0.003
MCP-1	21.8 (± 11.6)	13.0 (± 6.9)	0.0002
TNF-α	1.25 (± 1.49)	2.05 (± 1.87)	0.60

Data represent mean ± standard deviation.

has been reported in prostate cancer patients [36-39]. Thus we sought to identify alterations in serum adipokines in *Crebrf*KO mice. PAI-1 levels were increased almost 2-fold and MCP-1 levels were decreased almost 2-fold in *Crebrf*KO mice compared to wild-type. In agreement with the increased serum levels of PAI-1, PAI-1 immunostaining was significantly up-regulated in the prostate epithelial cells of *Crebrf*KO mice. PAI-1 is critical for controlling blood clotting, wound healing, and matrix remodeling and elevated PAI-1 can cause thrombosis, atherosclerosis and tissue fibrosis (Reviewed in [40]). PAI-1 is also a senescence marker in fibroblasts [41-43], and the significant reduction in epithelial and stromal proliferation in the *Crebrf*KO murine prostate may be due in part to increased expression of PAI-1. MCP-1 acts as a chemoattractant to recruit macrophages as well as to

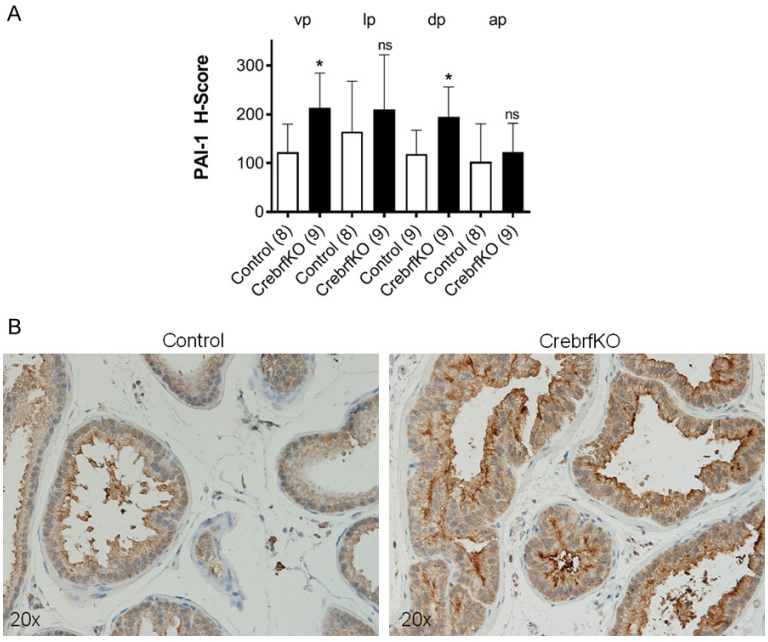

Figure 5. Impact of global *Crebrf* deletion on PAI-1 immunostaining in the prostate of C57BL/6J mice at 4 months of age. A. Quantification of PAI-1 immunostaining intensity and extent in the prostate lobes. Abbreviations: vp: ventral prostate; lp: lateral prostate; dp: dorsal prostate; ap: anterior prostate. B. PAI-1 immunostaining (brown) in the prostate ventral lobes. Original magnification, 20×. *, P < 0.05, ns, non-significant. *P*-value acquired from the Student's t test, number of animals in each group indicated in parentheses.

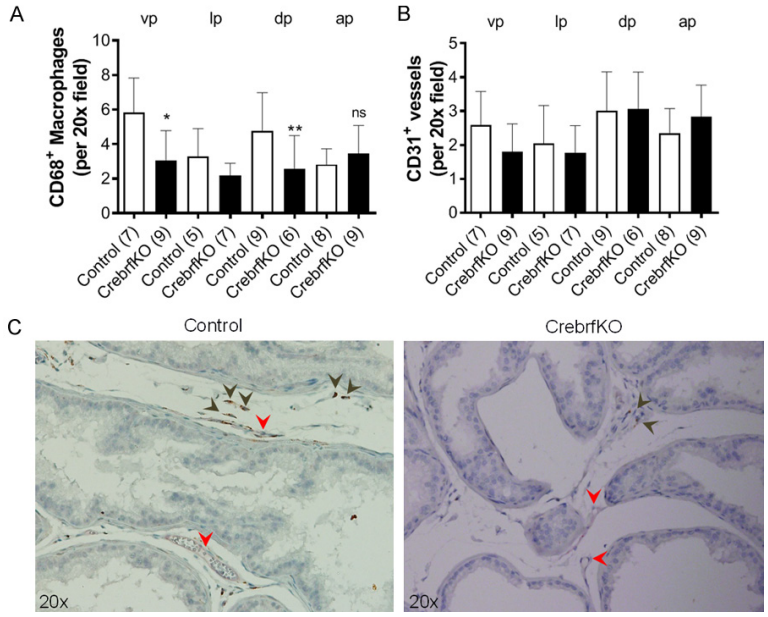

Figure 6. Impact of global *Crebrf* deletion on macrophage and vessel density in the prostate of C57BL/6J mice at 4 months of age. A. Quantification of CD68+ macrophages. B. CD31+ vessels in the prostate lobes. Abbreviations: vp: ventral prostate; lp: lateral prostate; dp: dorsal prostate; ap: anterior prostate. C. CD68 (brown, brown arrows) and CD31 (red, red arrows) immunostaining in the prostate ventral lobes. Original magnification, 20×. *, P < 0.05, ns, non-significant. *P*-value acquired from the Student's t test, number of animals in each group indicated in parentheses.

enhance epithelial proliferation in prostate cells [44]. Reduced serum levels of MCP-1 may contribute to the decreased number of macrophages observed in the ventral and dorsal lobes of *Crebrf*KO mice.

We also compared the number of CD31+ vessels in control and *Crebrf*KO mice and did not find any difference. In a recent study, CREBRF and TRIM2 expression was upregulated by high-density lipoprotein stimulation in response to angiogenic stimuli [45]. However, CREBRF did not appear to play a critical role in angiogenesis. Our findings that microvessel density was not altered in the prostates of *Crebrf*KO mice further suggest that CREBRF is not critical for angiogenesis.

In summary, genetic variants in CREBRF present in prostate cancer patients are associated with decreased overall survival. Furthermore, genetic ablation of CREBRF in mice induced a lobe-specific decrease in cellular proliferation and an increase in apoptosis, as well as a decrease in the density of macrophages in the prostate. Serum PAI-1 and MCP-1 levels were also altered in *Crebrf*KO mice. Our findings suggest that genetic variants affecting CREBRF function could alter prostate homeostasis and contribute to prostate tumorigenesis. More detailed mutation analyses will be of critical importance in examining the role of CREBRF in the development of prostate cancer, particularly in Native Hawaiian and Pacific Islanders. Racial and ethnic data are either not well characterized or predominantly composed of non-Hispanic White patients in current databases identifying prostate cancer risk alleles. Expansion of

these types of studies to include patients from more diverse populations is urgently needed. Prostate cancer is a disease strongly associated with aging, and it will be important to examine the long-term impact of CREBRF loss of function in the murine prostate in future studies. Since CREBRF has also been associated with elevated BMI and obesity is a risk factor for prostate cancer, murine studies could also explore potential mechanisms of the interaction between obesity and CREBRF loss in prostate tumorigenesis. In *Crebrf*KO mice, both epithelial and stromal changes were observed, suggesting that CREBRF is important for stromal-epithelial interaction. Thus, future studies on the potential impact of CREBRF mutations on prostate homeostasis are warranted.

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Disclosure of conflict of interest

None.

Abbreviations

CREBRF, Cyclic-adenosine-monophosphate-response-element-binding protein 3 regulatory factor; BMI, body mass index; DNA, Deoxyribonucleic acid; IHC, immunohistochemistry; IL-6, interleukin-6; MCP-1, Monocyte chemoattractant protein-1; PAI-1, Plasminogen activator inhibitor-1; PCR, polymerase chain reaction; TBS, Tris-buffered saline; TNF-α, Tumor necrosis factor-α; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling.

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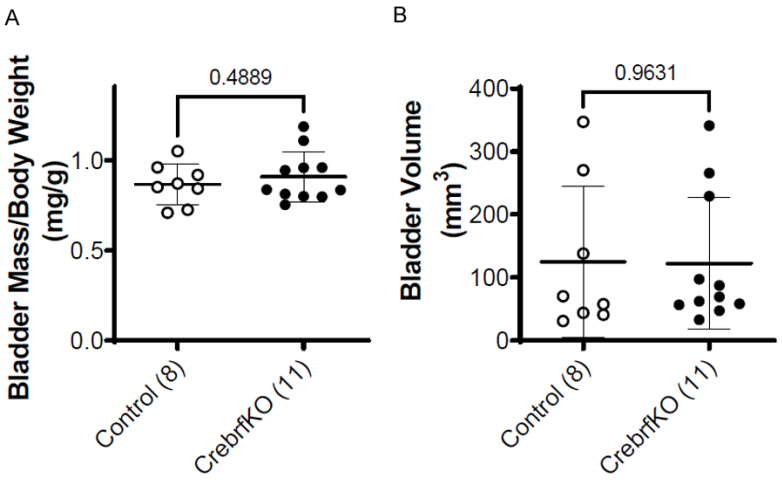

Supplementary Figure 1. Impact of global *Crebrf* deletion 2 in C57BL/6J mice at 4 months 3 of age on the bladder. A. Bladder Mass:Body Weight ratio of bladder in Control and *Crebrf*KO 4 mice at 4 months of age. B. Bladder volume in Control and *Crebrf*KO mice at 4 months of age.