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Supplementary Data

Generating a CRIPSR knock-out mouse through a strong premature termination codon: a cautionary tale

Qing Rex Lyu[1,2,3,✉], Peng Yao[1], Joseph M. Miano[1,2]

[1]*Aab Cardiovascular Research Institute, University of Rochester School of Medicine & Dentistry, Rochester, NY 14642, USA;*
[2]*Vascular Biology Center, Medical College of Georgia at Augusta University, Augusta, GA 30912, USA;*
[3]*Institute of Biomedicine and Health, Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, Chongqing 400714, China.*

Supplementary materials and methods

Synthesis and validation of sgRNAs

The sgRNAs were designed with CRISPOR program (http://crispor.tefor.net/) using DNA sequence from mouse genome database (mm9). Both sgRNAs and Cas9 protein were purchased from Synthego (http://www.synthego.com). The validation of sgRNA functioning was performed by incubating sgRNA, Cas9 2NLS nuclease and PCR-derived target DNA fragment for 1 hour at 37 °C. Agarose gel running was performed to detect the efficiency of sgRNA.

Generating of gene knock-out mice by CRISPR-Cas9 genome editing

For generating *Kank1*-PTC mouse, *Kank1*-sgRNA1 (***Supplementary Table 1***), Cas9 2NLS nuclease, and a single-strand homology-directed repair (HDR) template with insert and PAM mutation (5′-AAA AGAAAGACCCGTATTTTGTGGAAACCCCTTAC GGTTTTCAGCTAGACTTAGATTTCGTCAAATA ATAAACGTCGATGACATACAAAAGGGAAATA CTATCAAGAAACTGAACATCCAGAAGAGGCG AAAGCCATCTGTG-3′) were co-injected into strain C57BL6/J mouse zygotes. The founder mice with correct genotyping band after Sanger sequencing validation were subjected to breeding for germline transmission followed by heterozygous intercrossing to generate homozygous mutant mice.

Western blotting

Mouse tissues (aorta and bladder) were collected and homogenized in 200 μL of Cell Lysis Buffer (Cat. No. 9803, Cell Signaling Technology, USA) containing complete and EDTA-free protease inhibitor cocktail (Cat. No. 4693159001, Sigma-Aldrich, USA). The protein concentration was determined using DC Protein Assay Kit Ⅱ (Cat. No. 5000112, Bio-Rad, USA). Tissue lysate samples were boiled with 4× NuPAGE LDS sampling buffer (Cat. No. NP0008, Thermo Fisher, USA) and loaded into NuPAGE 4-12% Bis-Tris protein gel (Cat. No. NP0321BOX, Thermo Fisher) in 1× NuPAGE MES SDS Buffer (Cat. No. NP0060, Thermo Fisher). Electrophoresis was performed using 150 V for 40 minutes at room temperature. Membrane transfer was performed using Trans-Blot Turbo transfer system (Cat. No. 1703812, Bio-Rad) with Trans-Blot Turbo RTA Mini 0.2 μm PVDF transfer kit (Cat. No. 1704272, Bio-Rad). Membrane was then blocked with EveryBlot Blocking Buffer (Cat. No. 12010020, Bio-Rad) for 10 minutes and indicated antibodies (***Supplementary Table 2***) were applied. After a final wash with 1× PBS-T buffer, membranes were developed using SuperSignal West Pico PLUS chemiluminescent substrate (Cat. No. 34580, Thermo Fisher) in a Bio-Rad ChemiDoc

✉Corresponding author: Qing Rex Lyu, Institute of Biomedicine and Health, Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Sciences, 266 Fangzheng Avenue, Beibei District, Chongqing 400714, China. Tel: +86-18575532263, E-mail: lvqing@cigit.ac.cn.

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MP imaging system (Cat. No. 17001402, Bio-Rad).

Real-time qPCR

Mouse tissues (aorta and bladder) were homogenized and total RNA was extracted using RNeasy Mini kit (Cat. No. 74104, Qiagen, Germany). Total RNA concentration was determined, and cDNA was synthesized by using Bio-Rad iScript cDNA synthesis kit (Cat. No. 1708891, Bio-Rad). Real-time qPCR was performed using iTaq Universal SYBR Green SuperMix (Cat. No. 1725121, Bio-Rad) using primers shown in ***Supplementary Table 3***.

TA cloning and Sanger sequencing

PCR for genotyping was performed with primers (***Supplementary Table 3***) using GoTaq Green Master mix (Cat. No. M7123, Promega, USA) and run in 1% to 2% agarose gel. Bands were cut and DNA was recovered using QIAquick Gel Extraction kit (Cat. No. 28704, QIAGEN). Purified PCR product was subjected to TA cloning using TOPO TA Cloning Kit (Cat. No. K457501, Thermo Fisher) and colonies were pick for mini plasmid DNA preparation (Cat. No. A1460, Promega). Plasmid DNA was quantified with Nanodrop and Sanger sequencing was performed by Genewiz (China).

Statistical analysis

All experiments were performed in at least three independent biological replicates, and paired *t*-test was applied for comparing gene knock-out and wild-type samples. All data analysis was done in GraphPad

Supplementary Table 1 **Single guide RNA sequence**

sgRNA name	Sequence (5′ to 3′)	Usage
Kank1-sgRNA1	CTTAGATTTCGTCAAATACG	For *Kank1*-PTC
Kank1-sgRNA2	CAGCCCGATTTAAGACTTGT	For *Kank1* exon 5 deletion (upstream)
Kank1-sgRNA3	ATAGACTGATGTCGCTATCC	For *Kank1* exon 5 deletion (downstream)

sgRNA: single guide RNA.

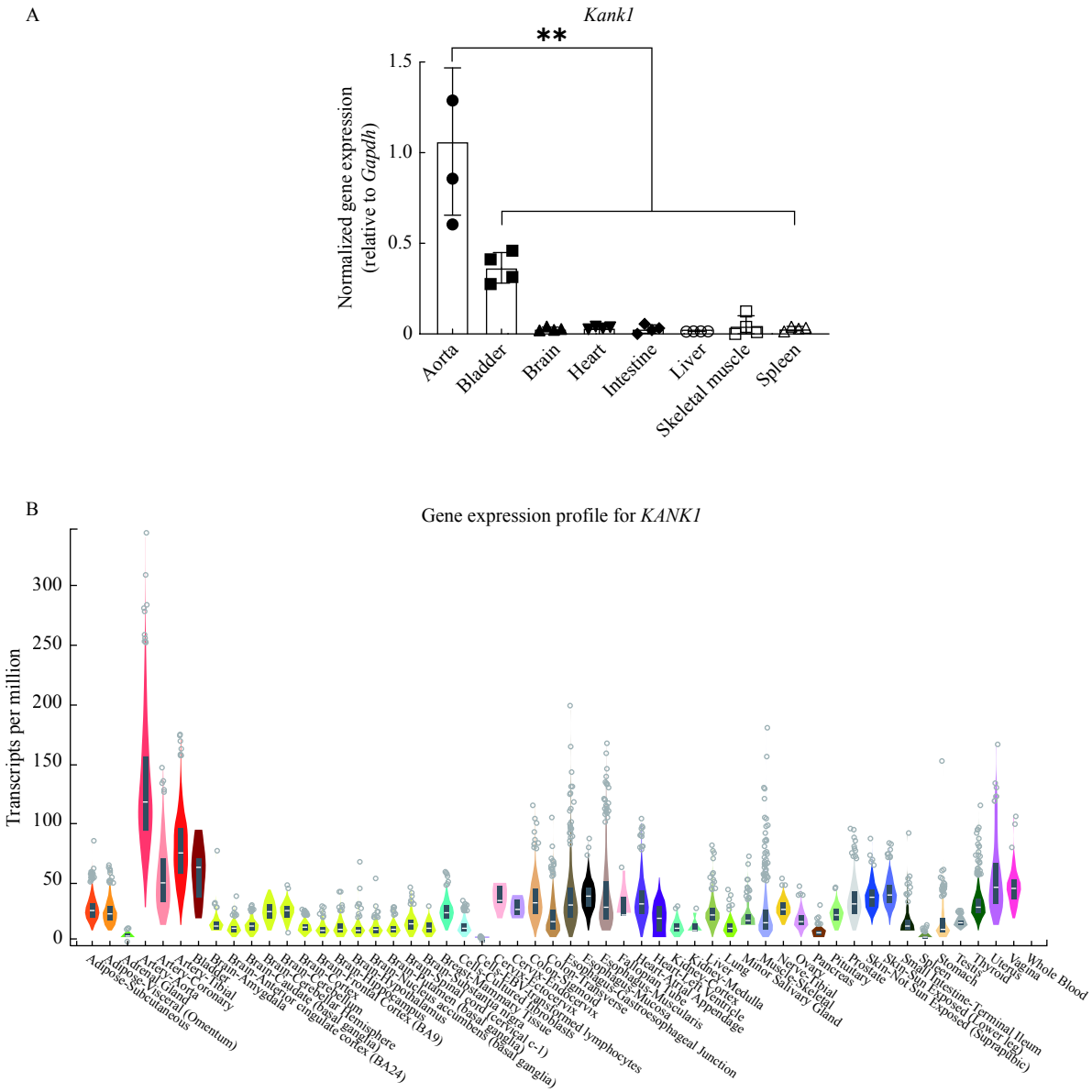
Supplementary Table 2 **Antibody list**

Antibody name	Vendor	Cat. No.	Dilution
Anti-Kank1 antibody	Bethyl Laboratories	A301-882A	1:1000
Anti-GAPDH antibody	Millipore	MAB374	1:5000
Anti-Tubulin antibody	Proteintech	10094-1-AP	1:2000

Supplementary Table 3 **Primer sequence**

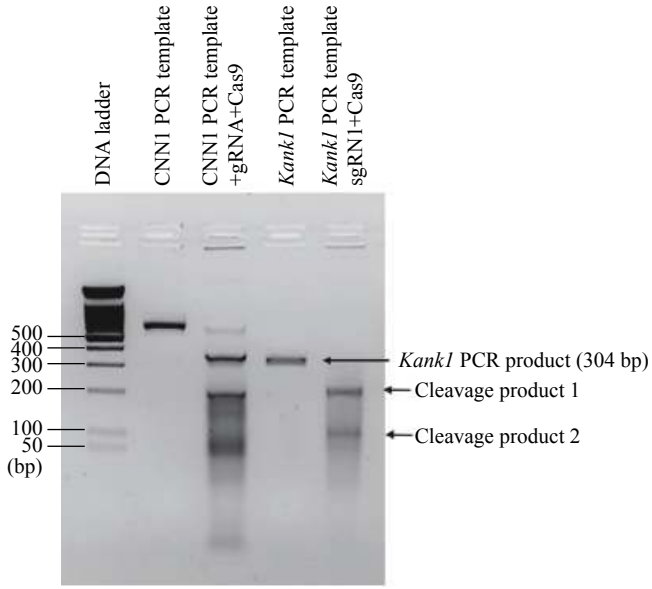
Primer name	Sequence (5′ to 3′)	Usage
sgRNA-target-F	AGCAGACGGAGTTCTGAATG	Test sgRNA by cleavage assay
sgRNA-target-R	TGAAGTAACGTGGCTTCTGG	
mKank1-F	ACCCTGTCGTCCATCAATTC	Detect *Kank1* with real-time qPCR
mKank1-R	CCTCCACACTTGCAGGTATATT	
mKank1-Mut-F1	GCTAGACTTAGATTTCGTCAAATAATAAACGTCG	*Kank1*-PTC mice genotyping, use F1-R pair for mutant, F2-R pair for wild-type
mKank1-Wt-F2	CGCTCGAGCTGCAGGAATTCGATATCAAGCTTATCGATACGTCAAATACGTGGATGAC	
mKank1-gt-R	TGAAGTAACGTGGCTTCTGG	
mKank1-e1-F1	GAACATAGAATCCTCCTGCCTTCC	*Kank1* exon 5 deletion genotyping (upstream)
mKank1-e1-R1	AAGAAGACATGGCAACACATGC	
mKank1-e1-F2	TGGTGAGATGGAGCAAGAATG	*Kank1* exon 5 deletion genotyping (downstream)
mKank1-e1-R2	AAAGGAGCTAGGGAGACAGTAG	

sgRNA: single guide RNA.



Supplementary Fig. 1 **Expression profiles of *Kank1* in mouse tissues and human *KANK1* mRNAexpression in GTEx portal.** A: Real-time qPCR results for *Kank1* mRNA expression level in mouse tissues. B: Gene expression profile for *KANK1* in GTEx portal. GTEx: genotype-tissue expression.

Prism 8. Results are expressed as mean±SD. *P* value less than 0.05 was considered significant.



Supplementary Fig. 2 **sgRNA cleavage assay.** The agarose gel image for cleavage assay to validate *Kank1* sgRNA1. Previously validated CNN1 sgRNA was used as positive control (lane 2 and 3). sgRNA: single guide RNA.

Supplementary Fig. 3 **The schematic for PTC insertion strategy.** The schematic image for the *Kank1* gene structure, gRNA target, PAM sequence and start codon. The second in-frame AUG start codon localizes 329 nucleotides downstream of PTC insertion site. PTC: premature termination codon.