Deficiency of FLCN in Mouse Kidney Led to Development of Polycystic Kidneys and Renal Neoplasia

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Abstract

The Birt–Hogg–Dubé (BHD) disease is a genetic cancer syndrome. The responsible gene, BHD, has been identified by positional cloning and thought to be a novel tumor suppressor gene. BHD mutations cause many types of diseases including renal cell carcinomas, fibrofolliculomas, spontaneous pneumothorax, lung cysts, and colonic polyps/cancers. By combining Gateway Technology with the Ksp-Cre gene knockout system, we have developed a kidney-specific BHD knockout mouse model. BHDflox/flox/Ksp-Cre mice developed enlarged kidneys characterized by polycystic kidneys, hyperplasia, and cystic renal cell carcinoma. The affected BHDflox/+Ksp-Cre mice died of renal failure at approximate three weeks of age, having blood urea nitrogen levels over tenfold higher than those of BHDflox/+Ksp-Cre and wild-type littermate controls. We further demonstrated that these phenotypes were caused by inactivation of BHD and subsequent activation of the mTOR pathway. Application of rapamycin, which inhibits mTOR activity, to the affected mice led to extended survival and inhibited further progression of cystogenesis. These results provide a correlation of kidney-targeted gene inactivation with renal carcinoma, and they suggest that the BHD product FLCN, functioning as a cyst and tumor suppressor, like other hamartoma syndrome–related proteins such as PTEN, LKB1, and TSC1/2, is a component of the mTOR pathway, constituting a novel FLCN-mTOR signaling branch that regulates cell growth/proliferation.

Introduction

Birt–Hogg–Dubé (BHD) syndrome is an autosomal dominant genetic disease characterized by fibrofolliculomas (follicular hamartomas), renal cell carcinomas, spontaneous pneumothorax, and lung cysts[1]. Renal cysts were also observed in some patients[2,3]. The BHD gene (accession number, BC015687), located on chromosome 17p11.2, contains 14 exons spanning approximately 20 kb of genomic DNA and encodes a protein of 579 amino acids, folliculin (FLCN) that has no known functional domains [4,5,6]. Germ-line mutations, somatic alterations, and loss of BHD mRNA have been observed in patients with BHD, colorectal cancer, and in some cases of gastric cancer; thus, BHD may be viewed as a candidate tumor-suppressor gene[7,8,9,10]. Germ-line mutations of the counterpart BHD have also been identified in dogs and rats having renal cell carcinomas and renal multiple cysts [11,12,13].

As one of the hamartoma syndromes, BHD shares many clinical features (such as follicular hamartomas, mucosal fibromas, and internal malignancy) with Cowden syndrome (CD, affected gene PTEN), Peutz-Jeghers syndrome (PJS, affected gene LKB1), and tuberous sclerosis complex (affected genes TSC1/TSC2) [14,15,16]. Of these, Cowden syndrome shares the most clinical features with BHD. While PTEN, LKB1, and TSC1/2 are critical members of the mTOR pathway [17], the BHD protein FLCN has also been suggested to be involved [18,19]. These findings imply that FLCN, like PTEN, may also be a pivotal tumor suppressor gene and a potential player in mTOR pathway. Over the last few years, interest in FLCN has grown significantly. A few model organisms have been used to explore the physiological role of FLCN. However, these studies presented discrepant results, which leave the function of FLCN elusive. In Drosophila, the Bhd homologue was linked to JAK-STAT and Dpp pathway[20]. An in vitro experiment revealed that FLCN interacts with AMPK in mammalian cell lines, associating FLCN with the mTOR pathway[18], whereas in fission yeast, Bhd was reported to activate the mTOR counterpart Tor2, presenting an opposite role to Tsc1/2[19].
Since no in vitro experiments or nonmammalian model can replicate the complex processes of tumorigenesis in humans, the development of BHD-deficient animal models will shed light on the role of BHD in vivo and on the BHD-related biochemical pathways responsible for neoplasia, which eventually could lead to the development of therapeutic agents against BHD-related diseases. Although natural mutants could be used for experimental models, the possibilities of homozygous embryonic lethality and additional unknown genetic changes often impede further analysis of the phenotypes and the physiological role of the gene. The genetically engineered conditional knockout mouse model can bypass this barrier and provide a “cleaner” and more versatile system for functional studies of BHD gene protein FLCN. While it might be a suppressor of mouse cystogenesis demonstrated by a recent study[21], BHD is expected to be a potential tumor suppressor gene whose mutations have led to renal tumors and other diseases in BHD patients. Therefore, it is essential to further elucidate whether kidney-specific knockout of BHD in the mouse is also implicated in kidney tumorigenesis, and what mechanism is involved.

Results

Generation of BHD conditional knockout construct and mice

To generate a conditional knockout construct, we adopted the MultiSite Gateway® Three-Fragment Vector Construction system (Invitrogen, Carlsbad, CA) to inactivate the BHD gene by deleting exons 3 and 4 (Figure 1A). The construct was electroporated into 129/Sv strain embryonic stem (ES) cells. Correctly targeted ES cell clones were obtained after being selected with G418, screened by long-range PCR, and confirmed using PCR and Southern blot analysis (Figure 1B–E). For the generation of chimeras, ES cells heterozygous for the BHD-flox allele were injected into C57BL/6 blastocysts by standard procedures. Chimeras were bred to C57BL/6 mice to produce BHDflox/+ heterozygotes, and germ-line offspring were identified by PCR genotyping (Figure 1C).

BHD null mice are embryonic lethal

To determine whether ablation of BHD affected the viability of mice, we first generated a conventional BHD-deficient mouse model by intercrossing BHDflox/flox mice with CMV-Cre transgenic strains that express Cre recombinase in all tissues. While most heterozygous BHD+/−/CMV-Cre mice showed no obvious abnormalities at age of 18 months, the homozygous mutation was embryonic lethal and BHD−/− mutants died between 3.5 dpc and 8.5 dpc, underscoring the importance of BHD in development. Indeed, genes that are important in embryonic development are frequently found to be the culprits in human cancers.

Kidney-specific inactivation of bhd results in renal cysts and RCC

BHD patients have a strong predisposition to develop bilateral and multifocal renal tumors with a variety of histologies [22], implying an effect of BHD on kidney tumorigenesis. We thus generated a kidney-specific knockout by breeding BHDflox/flox mice with Ksp-Cre transgenic strains that express Cre recombinase under the control of the kidney-specific cadherin promoter [23]. While the BHD+/−/Ksp-Cre heterozygous mice showed a normal phenotype at the age of 18 months, the homozygous BHDflox/flox/Ksp-Cre mice developed bilateral polycystic kidneys that were on average twofold heavier than those of BHDflox/+Ksp-Cre and wild-type littermate controls (Figure 2A,B). The BHDflox/flox/Ksp-Cre mice died of kidney failure at the age of 3 weeks, having over 10 times higher levels of blood urea nitrogen (BUN) than normal littermate controls (Figure 2C). The considerably low levels of BHD mRNA detected by real time RT-PCR demonstrated inactivation of BHD in most of the kidney cells (Figure 2D). The appearance of the cysts here is similar to that found in polycystic kidney disease caused by mutated PKD genes (Figure 3A,B). Histopathological examination of the BHDflox/flox/Ksp-Cre kidneys revealed extremely dilated renal tubules that predominantly originated from collecting ducts due to high expression of Ksp-Cre recombinase. While some proximal tubules were highly or moderately dilated, most of the other proximal tubules remained relatively normal as a result of extremely low expression levels of Ksp-cre recombinase (Figure 3C–F). Atrophic, compressed glomeruli were also observed, and degeneration, necrosis, and haemorrhage were frequently observed in the late stages. These morphological changes suggest that homozygous BHD inactivation in the kidney may cause loss of growth control in tubular epithelial cells.

Kidney-specific inactivation of bhd produced renal cell carcinoma (RCC)

We further examined whether BHDflox/flox/Ksp-cre mice develop renal carcinomas along with the cysts. We observed that kidneys from mice less than two weeks old predominately presented dilated tubules and cysts, whereas mice more than 18 days old also developed hyperplasia and renal cell carcinoma in their polycystic kidneys (Figure 3G–J). Hyperplastic areas frequently exhibited as multiple layers of epithelial cells along the inner surface of the tubules (Figure 3G, H). Renal cell carcinoma, which presents as cystic RCC, was frequently observed in the extremely enlarged kidneys. Cystic RCC was first described in 1986 and more cases have been reported since then [24,25,26,27,28,29,30,31,32]. Images of human cystic RCC are also available in the webpathology website (http://www.webpathology.com/image.asp?case=66&n=8; http://www.webpathology.com/image.asp?case=66&n=9). The occurrence of cystic RCC in the general population is 4 to 10%, or 1 to 2% of all renal tumors. The cystic RCC does not present as a solid mass, but rather as a unicellular or multilocular cystic mass that is composed of cancer cells growing in the form of cysts that are distinct from regular cysts (Figures 3I,J and S1). While some of the tumor cells lined the septa, the others protruded into the cystic lumen. Most of the tumor cells were larger than the regular cystic cells. Biradial cystic RCC cells were also observed. Many cystic spaces are filled with hemorrhage or proteinaceous fluid. No solid tumors were observed in any of the affected mice, which may be attributed to their short lifespan; three weeks might not be sufficient for solid tumor development.

Deficiency of FLCN and subsequent activation of mTOR contributed to renal cysts and RCCs

To elucidate the biochemical mechanisms of the cystogenesis and carcinogenesis related to inactivation of the BHD gene, we investigated the possible relevance of BHD to the mTOR signaling pathway for the following reasons: 1) our microarray analysis revealed that ectopic expression of the BHD gene product, FLCN, led to down-regulation of the AKT-related mTOR pathway signature (Figure S2); 2) BHD, PTEN, LKB1, and TSC1/2 are all hamartoma syndrome–related genes, and the roles of PTEN, LKB1, and TSC1/2 in the mTOR pathway have been well-established; and 3) in vitro experiments indicated that FLCN interacted with AMPK, a member of the mTOR pathway [18]. All these clues implied that BHD gene may play an important role in suppression of cystogenesis and tumorigenesis and that its inactivation could lead to the formation of renal cysts and RCC through the mTOR pathway.

Before investigating the correlation of FLCN with mTOR pathway, we first examined the distribution of FLCN in normal...
mouse kidney and polycystic kidney. To do this, we designed and developed a human BHD monoclonal antibody that is compatible with immunohistochemical analysis in the mouse. While FLCN was predominantly expressed in the normal proximal tubules and collecting ducts in the cortex, obvious expression was rarely observed in the kidney distal tubules of mice at age of 3 weeks (Figure 3K–N). In the polycystic kidney, FLCN was only detected in relatively normal tubules (Figure 3C–F), which are mainly proximal tubules. A small number of proximal tubules were also enlarged due to moderate expression of Ksp-Cre recombinase (Figure 3F, arrow), which is different from the previous report where the proximal tubules are not involved. All the enlarged tubules were FLCN-negative (Figure 3C,D), indicating a correlation of the formation of cysts with inactivation of the BHD gene.

We then explored whether the inactivation of BHD resulted in the activation of mTOR in affected cysts and RCCs. Immunohistochemical analysis showed that mTOR was activated through phosphorylation in cysts and cystic RCCs (Figure 4A–C), which stained FLCN-negative (Figure 4B). We further examined the phosphorylation status of the downstream target S6 (Figure 4D). Phosphorylated S6 has been observed in some cysts and in cystic RCC. Although FLCN was reported to be a possible downstream
effector of mTOR in an in vitro experiment[18], our data revealed that deficiency of FLCN activated mTOR pathway in vivo, suggesting mTOR might a downstream target of FLCN. To further elucidate the correlation of FLCN and mTOR, we applied the mTOR inhibitor rapamycin to affected mice to see whether we could inhibit or reverse the development of cysts. Rapamycin treatment significantly extended the survival period of BHDflox/flox/Ksp-Cre mice and inhibited the development of cysts relative to control mice; some mice survived more than 50 days. However, once the rapamycin treatment was stopped, cysts redeveloped rapidly and the mice died within 10 days. This result indicated that rapamycin can inhibit cystic cell growth, but cannot reverse the cystic kidney phenotype. We also tested a few other members of the mTOR pathway (e.g. AKT) through IHC; no significant changes were observed or inconsistent results were obtained following inactivation of BHD, implying a novel FLCN-mTOR pathway branch may exists. In addition, FLCN might be related to other signaling pathways. Obviously, the precise in vivo mode of action of FLCN merits more investigation.

Discussion

In this study, we provide the first evidence that the BHD protein FLCN predominantly expresses in the proximal tubules and collecting ducts of the renal cortex (Figure 3K–N). By developing and subsequently analyzing the conditional BHD knockout mouse model, we demonstrate that the deletion of BHD in the mouse kidney leads to cystic renal cell carcinoma (cystic RCC) in addition to polycystic kidney and hyperplasia. The cystic RCC was only observed in the older affected mice (≥20 days old). This implies that most of the polycystic kidneys would only present regular cysts and various extents of hyperplasia if the affected mice are sacrificed earlier. Thus, although some kidney-specific knockout animal models of RCC-related genes failed to develop RCC[21,33,34], our data provide a connection between kidney-specific BHD gene inactivation and renal carcinogenesis. This finding suggests that BHD may act as a suppressor for both cystogenesis and tumorigenesis.

No solid kidney tumors were observed in any of the affected mice, which may be attributed to their short lifespan and mouse distinct genetic background. It is entirely possible that if the cysts had not caused kidney failure at age of three weeks, progression of these cystic RCC to solid tumors would have occurred. In addition, inactivation of BHD gene in the kidney causes a large proportion of tubules to form cysts. Once cystogenesis starts, fast-growing cysts become dominant and lead to highly cystic kidneys, kidney failure, and early death. Thus, lack of appropriate microenvironment might be another reason that the malignant/
pre-malignant cells failed to form solid renal tumors, which is a more complicated and slower process.

Our results further demonstrated that deficiency of BHD product FLCN led to activation of mTOR pathway in cystic cells, supporting the recent report and consolidating that FLCN is involved in mTOR pathway and mTOR may be downstream target of FLCN[21]. Interestingly, BHD is a member of the hamartoma syndrome family that includes Cowden syndrome (CD, affected gene PTEN), Peutz-Jeghers syndrome (PJS, affected gene LKB1), and tuberous sclerosis complex (affected genes TSC1/ TSC2) [14,15,16]. While PTEN, LKB1, and TSC1/2 have played pivotal roles in the mTOR pathway, our findings suggest that BHD protein FLCN, like other hamartoma syndrome–related proteins such as PTEN, LKB1, and TSC1/2, is an important component of the mTOR pathway, constituting a novel FLCN-mTOR signaling branch that regulates cell growth/proliferation, though FLCN may involve in other pathways.

Materials and Methods

Design and generation of BHD conditional knockout construct

The MultiSite Gateway® Three-Fragment Vector Construction system (Invitrogen, Carlsbad, CA) was modified for the purpose of fabricating recombination vectors [35]. Of the four vectors supplied in the system, the pDONR vectors, pDONR P4-P1R, and pDONR P2R-P3 were used to generate the 5' and 3' homology arm entry clones. Another vector, pENTR3C, was used to carry a targeted gene sequence of interest. To meet the gene targeting purpose, a 1.8-kb loxP-FRT-neo-FRT fragment excised from p-loxp-2FRTPGKneo (a gift of D. Gordon) was added to generate pENTR3CloxP-FRT-neo-FRT, which allowed later excision of BHD exons 3 and 4 and the neomycin-resistance gene by cre-mediated recombination in vivo. Synthetic oligonucleotides were used to insert an additional loxP site into the Drai site of the pENTR3G-loxPFRT- neo-FRT vector. Oligonucleotides loxF (5'-ATAACTTCGTATAGCATACATTATACGAAGTTATTT-3') and loxPR1 (5'-AAATAACTTCGTATAATGTATGCTATACGAAGTT-3') (IDT, Coralville, IA) were phosphorylated with T4 polynucleotide kinase (Invitrogen), annealed, and inserted into the Drai site of the pPGKneo/TK vector (a gift from T. Gridley).

Figure 3. Inactivation of FLCN in BHDfloxflox/Ksp-Cre mice led to polycystic disease, hyperplasia, cystic renal cell RCC. (A,B) Deficiency of FLCN resulted in polycystic disease. (C, D) No FLCN expression was detected in cysts (enlarged tubules). However, FLCN was still expressed in relatively normal tubules where BHD was not deleted or completely eliminated by Ksp-Cre due to no or low Ksp-Cre expression in some proximal tubules. (E, F) Most of the relative normal tubules were proximal tubules stained by proximal tubule-specific marker lotus tetragonolobus lectin (LTL). Many of the proximal tubules remained relatively normal, though some proximal tubules were also enlarged (indicated by arrow. (G, H) Hyperplasia (indicated by arrow) was frequently observed in the cysts. (I, J) Cystic renal cell carcinomas were also one of the important consequences of kidney-targeted BHD gene inactivation in BHDfloxflox/Ksp-Cre mice, which is morphologically distinct from regular cysts showed in A and B. (K, L) FLCN is predominately expressed in proximal tubules, which was demonstrated by the proximal-specific marker LTL (M). FLCN expression is quite weak in distal tubules, which was marked by the distal-tubule-specific marker, Na-Cl-cotransporter (TSC) (N). Scale bar = 50 μm.

Materials and methods

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To generate a BHD gene targeting construct, a 3.5-kb 5' homology arm containing exon 2 and a 3.0-kb 3' arm carrying exons 5 and 6, PCR-amplified using Pfx polymerase (Invitrogen), were integrated into the pDONR P1-P4R and pDONR P2R-P through BP reaction (attB and attP sites) to generate the BHD-5' and BHD-3' homology entry clones, respectively. A 1.3-kb fragment of genomic DNA bearing exons 3 and 4 of the BHD gene was inserted into the modified pDONR vector pENTR3C-loxPMCS-loxP-FRT-neo-FRT between the SalI and NotI sites to generate a BHD-exon3-4-pENTR3C entry clone. Finally, the three entry clones, in combination with the modified destination vector, were incubated to create a BHD-pDESTR4R targeting construct through BP recombination reaction.

Identification of homologous recombinant ES cells and generation of kidney-specific knockout mice

The generated BHD-pDESTR4R targeting construct carries an ampicillin-resistant gene and a neomycin-resistant gene flanked by FRT sites. The construct was linearized with ScaI for electroporation into 129/sj strain ES cells. After selection with 500 µg/ml G418 (Invitrogen), 1,039 ES cell clones were isolated. The G418-positive ES clones were first screened by long-range PCR and then confirmed by Southern blot analysis. For the generation of chimeras, ES cells heterozygous for the BHDflox/+ allele were injected into C57BL/6 blastocysts by standard procedures. Chimeras were bred to C57BL/6 mice, and germline offspring were identified by PCR genotyping. To remove the neomycin gene flanked by two FTR sites, BHDflox/+ mice were crossed to FlpeR transgenic mice that express the site-specific recombinase FLP (FLPe). Then, BHDflox/+ heterozygous mice were intercrossed to give rise to mice homozygous for the BHDflox allele, i.e., BHDflox/flox mice.

To obtain mice with kidney-specific inactivation of BHD, BHDflox/+ mice were first bred to Ksp-Cre transgenic mice to generate BHD heterozygous mice (BHDflox/+ /Ksp-Cre in kidneys). BHDflox/+ /Ksp-Cre mice then were backcrossed to BHDflox/+ mice to generate BHD homozygous mice (BHDflox/flox /Ksp-Cre in kidneys).

All mice were manipulated and housed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Van Andel Institute and conducted in an ethical, humane, and scientifically justified manner, and in full compliance with applicable regulations.

Genotyping, RNA and protein analysis

ES cell DNA and tail DNA was extracted by using automated DNA isolation system (Kurabo Industries) and subjected to regular PCR and long-range PCR genotyping analysis (see supplementary Table S1 for PCR primers). For genotyping by Southern blot analysis, DNA from ES cells or tissues was extracted using standard DNA extraction procedure. Purified DNA was digested by XmnI or HindIII, isolated by 0.8% agarose gel, and transferred onto nylon membrane. UV-linked or dried membranes were subjected to DNA hybridization with 5' or 3' probes.

Total RNA was isolated from various mouse tissues and cystic cell lines with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Purified RNA was used for quantitative analysis (real-time RT-PCR) through ABI Prism 7700 Sequences Detector (Applied Biosystems).
For protein detection by Western blot, cultured cells and kidney whole-cell extracts prepared by homogenization were lysed in 1% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 15% glycerol, plus standard protease inhibitors (protease inhibitor cocktail tablets, Roche Diagnostics). Equal amounts of mutant and control kidney cell protein extracts were size-separated by 10% SDS-PAGE, and transferred to PVDF membranes (Invitrogen). FLCN was detected with a mouse monoclonal anti-FLCN antibody (developed by Laboratory of Antibody Technology) at a dilution of 1:750 using the enhanced chemiluminescence detection system.

Immunohistochemistry and tubular marker staining

Immunohistochemical analysis was performed following the manufacturer’s protocols. The antibodies used include anti-FLCN mAb, anti-Phospho-mTOR Rabbit mAb (Cell Signaling), anti-Phospho-S6 Ribosomal Protein (Cell Signaling). Proximal tubules were stained by biotinylated Lotus Tetragonolobus Lectin (LTL, Vector Laboratories), and distal tubules were detected by using rabbit anti-thiiazide-sensitive NaCl cotransporter affinity purified polyclonal antibody (TSC, Chemicon) Tubular markers. Marker biotinylated Peanut Agglutinin (PNA, Vector Laboratories) was used to stain collecting ducts.

Phenotyping and histopathology

Newborn mice were monitored daily. Sick mice were distinguished from healthy ones by enlarged abdomen at age of 10 days. Totally 73 BHD<sup>fox/fox</sup>/Ksp-Cre mice and 55 normal control littermates were collected for phenotyping and histopathological analysis. Mouse body weight, kidney weight were measured upon euthanasia by CO<sub>2</sub> inhalation. Tissues including kidneys, lung, liver, spleen, heart, stomach, intestine, brain, testes were collected and fixed in 4% paraformaldehyde for 24 hours. Kidneys, lung, liver, spleen, heart, stomach, intestine, brain, testes were sectioned at 3-μm thick and stained with hematoxylin and eosin (H&E). Stained slides were evaluated by a board-certified veterinary pathologist B. Sigler and pathologists X. Yang and J. Peng.

Blood biochemical analysis

Mouse blood was collected by cardiac puncture at age of 5 days, 10 days, and 15 days, and 20 days. Serum was collected after centrifugation and stored at −80°C for further biochemical analysis.

Rapamycin treatment of BHD<sup>fox/fox</sup>/Ksp-Cre and wild-type control littermates

Totally 29 mice from three litters were used for rapamycin treatment (n = 15) and control (n = 14). Mice from each litter at postnatal 7 days were randomly divided into two groups of rapamycin treatment and control. Rapamycin (LC Laboratories, Woburn, MA) was dissolved in ethanol at a concentration of 20 mg/mL and stored at −20°C. The rapamycin solution was freshly prepared by diluting the rapamycin stock to 250 μg/mL in buffer (1:1 10% PEG-400, 8% ethanol:10% Tween 80) and were injected by intraperitoneally daily at a dose of 2.5 mg/kg body weight for the duration of the treatment. Control animals received i.p. injection of equal amount of vehicle (5% PEG-400, 4% ethanol, and 5% Tween 80). Mice were treated for at least two weeks starting at postnatal day 7. Moribund mice were subjected to CO<sub>2</sub> euthanasia, dissection, and analysis in the duration of the treatment.

Supporting Information

Table S1 Primer used for BHD knockout genotyping

Found at: doi:10.1371/journal.pone.0003581.s001 (0.02 MB XLS)

Figure S1 Additional cystic RCC samples stained by hematoxylin and eosin. Cystic spaces are filled with proteinaceous fluid (A–J) or hemorrhage (K,L) in cystic RCC. The tumor cells have clear cytoplasm and hyperchromatic nuclei lining the septa or growing into the cystic lumina. Scale bar = 50 μm.

Found at: doi:10.1371/journal.pone.0003581.s002 (5.38 MB TIF)

Figure S2 Microarray analysis revealed that ectopic expression of FLCN led to down-regulation of the AKT- related mTOR pathway signature.

Found at: doi:10.1371/journal.pone.0003581.s003 (0.29 MB TIF)

Acknowledgments

We thank the following Van Andel Research Institute core facilities for their services: gene targeting core for generating the knockout; the vivarium for mouse husbandry; the sequencing core for nucleotide sequencing; the analytical, cellular, and molecular microscopy core for immunohistochemical analysis; and the flow cytometry core for blood analysis. In addition, we thank Dr. Bob Sigler for technical assistance in mouse pathology. We also thank David Nadziejka for technical editing and Sabrina Noyes for her administrative support.

Author Contributions

Conceived and designed the experiments: JC KF DP PW JK YL DH. Analyzed the data: JC JP CNQ SN. Contributed reagents/materials/analysis tools: SKK PZ BC. Wrote the paper: JC SKK BTT.

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