Generation and Characterization of Liver-Specific CD36 Knockout Mice (Postprint)

Authors: Su Chunxiao, Zhang Xiaoyu, Zeng Han, Chen Yaxi, Ruan Xiongzhong, Yang Ping

Date: 2018-05-23T00:00:00+00:00

Abstract

Objective: To construct and validate liver-specific CD36 knockout mice using the Cre/Loxp recombinase system, laying a foundation for studying the biological functions of CD36. Methods: Construct a CD36 targeting vector, electroporate and transfect embryonic stem cells, screen for positive clones with correct homologous recombination via long-chain PCR. After expansion, inject positive embryonic stem cell clones into C57BL/6J mouse blastocysts to obtain chimeric mice, then cross with Flp mice to screen for Flox heterozygous mice. These mice were crossed with imported Alb-Cre mice to obtain CD36fl/fl:Alb-Cre+ genotype mice in the F3 generation, which are liver-specific CD36 knockout mice. PCR was used to identify mouse genotypes, PCR, real-time quantitative PCR and Western blot were used to verify CD36 knockout efficiency in mouse liver, Western blot was used to detect CD36 expression in mouse kidney, adipose and cardiac tissues, and HE staining was used to observe morphological changes in mouse liver. Results: Flox heterozygous mice for the CD36 gene were established. After crossing with Alb-Cre mice, CD36fl/fl:Alb-Cre- and CD36fl/fl:Alb-Cre+ genotype mice were screened in the F3 generation. DNA-level analysis confirmed that the liver CD36 gene in CD36fl/fl:Alb-Cre+ genotype mice was knocked out via the Cre/Loxp recombinase system. Compared with CD36fl/fl:Alb-Cre- genotype mice, CD36fl/fl:Alb-Cre+ genotype mice showed significantly reduced liver CD36 mRNA and protein expression levels, no difference in CD36 protein expression in kidney, adipose and cardiac tissues, and no significant difference in liver morphological characteristics. Conclusion: Liver-specific CD36 gene knockout mice were successfully constructed through the Cre/Loxp recombinase system, providing an animal model for studying the function of CD36 in liver metabolism and liver diseases.

Full Text

Preamble

Establishment and Identification of Liver-Specific CD36 Knockout Mice

Su Chunxiao, Zhang Xiaoyu, Zeng Han, Chen Yaxi, Ruan Xiongzhong, Yang Ping**

(Centre for Lipid Research, Chongqing Key Laboratory of Lipid and Glucose Metabolism, Chongqing Medical University, Chongqing 400016, China)

Abstract

Objective: To generate and validate liver-specific CD36 knockout mice using the Cre/LoxP recombinase system, thereby establishing a foundation for investigating the biological functions of CD36. **Methods:** A CD36 targeting vector was constructed and electroporated into embryonic stem cells. Positive clones with correct homologous recombination were screened by long-chain PCR. Expanded positive embryonic stem cell clones were microinjected into C57BL/6J mouse blastocysts to generate chimeric mice, which were then crossed with Flp mice to obtain Flox heterozygous mice. These Flox mice were subsequently mated with Alb-Cre mice, yielding CD36fl/fl:Alb-Cre+ genotype mice in the F3 generation, which represent the liver-specific CD36 knockout mice. Mouse genotypes were identified by PCR. The knockout efficiency in liver was verified by PCR, real-time quantitative PCR, and Western blot. CD36 expression in kidney, adipose, and cardiac tissues was detected by Western blot, and hepatic morphological changes were observed by HE staining. **Results:** Flox heterozygous mice carrying the CD36 gene were successfully established. After mating with Alb-Cre mice, CD36fl/fl:Alb-Cre- and CD36fl/fl:Alb-Cre+ genotype mice were obtained in the F3 generation. DNA-level analysis confirmed that the CD36 gene was knocked out in the liver of CD36fl/fl:Alb-Cre+ mice via the Cre/LoxP recombinase system. Compared with CD36fl/fl:Alb-Cre- mice, CD36fl/fl:Alb-Cre+ mice exhibited significantly reduced CD36 mRNA and protein expression in liver, while CD36 protein expression in kidney, adipose, and cardiac tissues showed no difference. No significant morphological differences were observed in liver histology. **Conclusion:** Liver-specific CD36 knockout mice were successfully generated using the Cre/LoxP recombinase system, providing an animal model for studying CD36 function in hepatic metabolism and liver diseases.

Keywords: CD36 gene; gene knockout; Cre/LoxP

Introduction

CD36, also known as fatty acid translocase (FAT/CD36), belongs to the class B scavenger receptor family. Human CD36 is located at chromosome 7q11.2, contains 15 exons, spans 32 kb, and has a molecular weight of approximately 88 kDa [1]. This transmembrane glycoprotein comprises two transmembrane domains,

two very short intracellular domains, and a heavily glycosylated extracellular domain. The extracellular domain near the carboxyl terminus contains three disulfide bonds, and this extensive glycosylation is crucial for CD36 anchoring in the membrane [2]. The amino-terminal region contains binding domains for fatty acids, oxidized low-density lipoproteins, phospholipids, thrombospondin, and Plasmodium falciparum-infected erythrocytes [3]. CD36 is widely expressed in various cell types including adipocytes, macrophages, cardiomyocytes, and hepatocytes. Through interactions with different ligands, CD36 regulates multiple physiological and pathological processes, including scavenger receptor function, fatty acid transport, lipid metabolism, and angiogenesis modulation [4].

The liver is a vital metabolic organ, and lipid metabolism disorders are closely associated with the development and progression of liver diseases. CD36 plays an important role in maintaining lipid homeostasis. Studies have shown that under pathological conditions such as diet-induced obesity and diabetes, elevated CD36 expression promotes hepatic uptake of circulating fatty acids [5]. CD36 has also been implicated in lipid metabolism disorders in nonalcoholic fatty liver disease (NAFLD) [6]. Recent research indicates that CD36 contributes to hepatocellular carcinoma, as the progression of epithelial-mesenchymal transition in primary liver cancer correlates closely with CD36 expression and free fatty acid levels [7]. While most current CD36 research relies on in vitro experiments, in vivo studies have utilized CD36 knockout mice, though these lack tissue specificity. To further investigate hepatic CD36 function, our research group imported Alb-Cre tool mice and constructed Flox heterozygous mice, successfully generating liver-specific CD36 knockout mice using Cre/LoxP recombinase-mediated gene knockout technology and validating the knockout efficiency.

Materials and Methods

1.1 Materials

PCR primers were synthesized by Tsingke Biotechnology (Beijing). 2×Taq Master Mix (with ddH2O) was purchased from Vazyme Biotech (Nanjing). PrimeSTAR® GXL DNA Polymerase, SYBR Green, reverse transcription kits, and DL500/DL2000 DNA Markers were obtained from TaKaRa Bio (Dalian). Agarose and BCA protein quantification kits were purchased from Beijing Dingguo Changsheng Biotechnology. 50×TAE buffer was from Sangon Biotech (Shanghai). CD36 antibody was from Novus Biologicals (USA), and ECL chemiluminescence substrate was from Bio-Rad (USA). Embryonic stem cells and animals for Flox heterozygous mouse construction and Alb-Cre mice were purchased from Shanghai Model Organisms Center, and C57BL/6 mice were obtained from Chongqing Medical University.

1.2.1 Generation of CD36fl/+ Genotype Mice

Targeting exon 5 of CD36, we constructed a conditional CD36 knockout (CD36-cKO) targeting vector containing a 2.7 kb 5' homology arm,

a 2.6 kb Flox region with PGK-Neo-polyA, a 3.4 kb 3' homology arm, and MC1-TK-polyA negative selection marker [Figure 1: see original paper]. After linearization, the vector was electroporated into embryonic stem (ES) cells. Positive clones with correct homologous recombination were identified by long-fragment PCR. For ES cell screening, the 5' arm primers were: forward 5' -CAAAACAGCCATACCATCCATACCAT-3' and reverse 5' -GGCCTACCCGCTTCCATTGCTC-3' . The 3' arm primers were: forward 5' -CCGTGCCTTCCTTGACCCTGG-3' and reverse 5' -CAACCTAACAAATCTGAAACAACCCAAG-3' . PCR conditions were: 94°C for 2 min (1 cycle); 98°C for 20 s, 66°C for 20 s, 68°C for 2 min 30 s (32 cycles); 68°C for 5 min (1 cycle). Positive ES cell clones were expanded and microinjected into C57BL/6J blastocysts to generate chimeric mice (detailed procedures in references [8-11]). High-percentage chimeric males were crossed with Flp mice to obtain Flox heterozygous mice, whose genotypes were confirmed by tail DNA extraction as described in section 1.2.3.

Exon 5 was selected as the target, with LoxP sites inserted on both sides. When crossed with Alb-Cre mice expressing Cre recombinase specifically in liver, the Cre enzyme recognizes LoxP sequences and mediates homologous recombination to achieve liver-specific CD36 knockout. The breeding strategy was: first, CD36fl/+ Flox heterozygous mice and imported Alb-Cre+ mice were separately crossed with C57BL/6 mice at 1:1 ratio to expand the colonies; CD36fl/+ mice were then intercrossed to obtain CD36fl/fl mice, while CD36fl/+ mice were crossed with Alb-Cre+ mice to obtain CD36fl/+:Alb-Cre+ mice; finally, CD36fl/fl mice were crossed with CD36fl/+:Alb-Cre+ mice to generate CD36fl/fl:Alb-Cre+ mice (liver-specific CD36 knockout mice), with CD36fl/fl:Alb-Cre- mice serving as controls [Figure 2: see original paper].

1.2.3 Genotyping of Liver-Specific CD36 Knockout Mice

Three-week-old mice were weaned, toe-clipped for identification, and approximately 5 mm tail tips were collected in sterile EP tubes with 50 l tissue lysis buffer (25 mM NaOH and 0.2 mM EDTA). Tails were minced and fully digested, then heated at 99°C, 800W for 1 hour in a microwave oven. After cooling, 50 l tissue lysis stop buffer (40 mM Tris-HCl) was added, mixed, and centrifuged at 12,000g for 3 minutes at room temperature. The supernatant was used for PCR genotyping. Primer sequences for LoxP and Cre are listed in Table 1 . The 24 l reaction contained: 2×Taq Master Mix 12 l, forward and reverse primers (10 M) 0.4 l each, ddH2O 9.2 l, and template DNA 2 l. LoxP PCR conditions: 94°C for 5 min (1 cycle); 94°C for 30 s, 62°C for 30 s, 72°C for 1 min (35 cycles); 72°C for 5 min (1 cycle). Cre PCR conditions: 94°C for 2 min (1 cycle); 94°C for 30 s, 60°C for 30 s, 72°C for 30 s (33 cycles); 72°C for 5 min (1 cycle). After PCR, 7 l products were separated by 1.5% agarose gel electrophoresis and analyzed using a gel imaging system.

1.2.4 DNA-Level Verification of Cre Recombinase Activity

Three mice each of CD36+/+:Alb-Cre-, CD36fl/fl:Alb-Cre-, and CD36fl/fl:Alb-Cre+ genotypes were dissected. After liver perfusion, approximately 30 mg tissue was processed for genomic DNA extraction using a mouse genomic DNA extraction kit. PCR amplification used primers: forward 5' -TCCTCTTCCTCTTGAACACTCT-3'and reverse 5'-TACTTGCTGTACTGGTTGATGG-3' . The 20 l reaction contained: ddH2O 11.2 l, 5× PrimeStar GXL PCR Buffer 4 l, 2.5 mM dNTPs 2 l, CD36-P1 (10 pmol/ l) 0.5 l, CD36-P2 (10 pmol/ l) 0.5 l, PrimeStar GXL DNA Polymerase 0.8 l, and genomic DNA 1 l. Conditions: 94°C for 2 min (1 cycle); 98°C for 20 s, 63°C for 20 s, 68°C for 1.5 min (32 cycles); 68°C for 5 min (1 cycle). Products (7 l) were analyzed by 1% agarose gel electrophoresis to determine whether liver-expressed Cre recombinase was functional.

1.2.5 PCR Detection of Hepatic CD36 mRNA Expression

Liver tissue from CD36fl/fl:Alb-Cre- and CD36fl/fl:Alb-Cre+ mice was used for RNA extraction by Trizol method. After reverse transcription, qPCR was performed with primers: 18S internal control forward 5' -AGTCCCTGCCCTTTGTACACA-3', reverse 5'-CGATCCGAGGGCCTCACTA-3' ; CD36 forward 5' -GAGCCATCTTTGAGCCTTCA-3' , reverse 5' -TCAGATCCGAACACAGCGTA-3' . The 25 l reaction contained: ddH2O 10 l, forward and reverse primers 0.25 l each, SYBR 12.5 l, and cDNA 2 l. Two-step PCR was performed with annealing at 61°C. Data were quantified using the 2-ΔΔCT method to analyze hepatic CD36 mRNA expression levels.

1.2.6 Western Blot Analysis of CD36 Expression in Tissues

Total protein was extracted from liver, kidney, epididymal adipose, and cardiac tissues using RIPA lysis buffer, and protein concentration was determined by BCA assay. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, blocked with 3% BSA at 37°C for 1.5 hours, incubated with primary antibody overnight at 4°C, washed 3×15 minutes with TBST, incubated with secondary antibody at room temperature for 1 hour, washed again 3×15 minutes with TBST, and visualized by ECL chemiluminescence. Bands were quantified using ImageJ.

1.2.7 HE Staining for Liver Histology

Mouse liver paraffin sections were baked at 60°C for 20 minutes, deparaffinized in xylene I for 20 minutes and xylene II for 20 minutes, rehydrated through graded ethanol series (100%, 95%, 85%, 70%, 5 minutes each), rinsed in distilled water for 5 minutes, stained with hematoxylin for 5 minutes, washed in running water for 10 minutes, stained with eosin for 2 minutes, washed in running water for 2 minutes, dehydrated through 70%, 85%, 95%, and 100% ethanol (5 minutes

each), air-dried, cleared in xylene for 20 minutes, and mounted with neutral balsam for microscopic observation and imaging.

1.3 Statistical Analysis

All data were analyzed using GraphPad Prism 5 software and expressed as mean ± SD. Comparisons between two groups were performed using t-tests, with $P < 0.05$ considered statistically significant.

Results

2.1 Construction of Conditional CD36 Targeting Vector, ES Cell Screening, and Genotyping of Flox Heterozygous Mice

The constructed CD36-cKO targeting plasmid was linearized and digested with restriction enzymes, yielding 6500 bp and 8000 bp fragments [FIGURE:3(a)]. Genomic DNA from drug-resistant ES cell clones was extracted and screened by long-fragment PCR. Correct 5' arm recombination should produce a 3800 bp fragment (absent in negative clones), while correct 3' arm recombination should yield a 4300 bp fragment (absent in negative clones). Four positive ES cell clones (E1, E2, E3, E4) were obtained and confirmed by sequencing [FIGURE:3(b)]. Three high-percentage chimeric male mice were generated through embryo transfer. Crossing these with Flp mice produced Flox heterozygous mice, identified as C1-C5 in [FIGURE:3(c) and confirmed by sequencing (genotype CD36fl/+).

2.1 Generation and Genotyping of Liver-Specific CD36 Knockout Mice

F1 generation CD36fl/+ mice were crossed with Alb-Cre+ mice, and all F2 offspring were tagged and genotyped (mice 1-4 shown). F2 CD36fl/+:Alb-Cre+ mice were then crossed with CD36fl/fl mice, and F3 offspring were genotyped (mice 5-8 shown). For Flox identification: a single 379 bp band indicates wild-type CD36+/+; both 379 bp and 429 bp bands indicate heterozygous CD36fl/+; a single 429 bp band indicates homozygous CD36fl/fl. For Alb-Cre identification: a 450 bp band indicates Alb-Cre+, while absence of this band indicates Alb-Cre-. As shown in [Figure 4: see original paper], mouse #2 (CD36+/+:Alb-Cre-) is wild-type, mouse #6 (CD36fl/fl:Alb-Cre-) is a control mouse, and mouse #8 (CD36fl/fl:Alb-Cre+) is the liver-specific CD36 knockout mouse.

2.2.1 DNA-Level Verification of Hepatic CD36 Knockout

PCR amplification of the Flox region was used to qualitatively assess Cre activity. Based on primers designed for the Flox region, CD36fl/fl:Alb-Cre- genotype should amplify a 1.9 kb band, while after Cre recombination, CD36fl/fl:Alb-Cre+ genotype should amplify a 1.1 kb band. As shown in [Figure 5: see original paper], CD36fl/fl:Alb-Cre+ mice demonstrated functional Alb-Cre recombinase

activity that successfully deleted the CD36 Flox region.

2.2.2 mRNA-Level Verification of Hepatic CD36 Knockout

Real-time quantitative PCR revealed that CD36fl/fl:Alb-Cre+ mice (liver-specific CD36 knockout) had significantly reduced hepatic CD36 expression compared with control CD36fl/fl:Alb-Cre- mice ($P < 0.05$), confirming successful construction of liver-specific CD36 knockout mice [Figure 6: see original paper].

2.2.3 Protein-Level Verification of Hepatic CD36 Knockout

To further validate the knockout efficiency, Western blot was performed to compare CD36 protein levels in CD36fl/fl:Alb-Cre- and CD36fl/fl:Alb-Cre+ mouse livers. As shown in [Figure 7: see original paper], CD36fl/fl:Alb-Cre+ mice exhibited significantly reduced hepatic CD36 protein expression compared with controls ($P < 0.05$). Analysis of other tissues showed no significant difference in CD36 expression in kidney, adipose, and cardiac tissues between genotypes ($P > 0.05$), further confirming the liver-specificity of the knockout.

2.3 Effects of Liver-Specific CD36 Knockout on Mouse Reproduction, Growth, and Liver Structure

Liver-specific CD36 knockout did not affect mouse reproduction or growth. At 6 weeks, CD36fl/fl:Alb-Cre- mice ($n = 6$) weighed 20.28 ± 0.90 g, while CD36fl/fl:Alb-Cre+ mice ($n = 6$) weighed 20.68 ± 0.45 g, with no significant difference between groups ($P > 0.05$). HE staining revealed intact hepatic lobular architecture in both genotypes, with hepatocytes radiating from central veins and showing normal cellular structure without significant lesions [Figure 8: see original paper], indicating that liver-specific CD36 knockout does not affect liver morphology.

Discussion

CD36, a fatty acid translocase, mediates fatty acid transport across membranes and plays crucial roles in intracellular lipid distribution and metabolism. CD36 facilitates fatty acid uptake in multiple cell types, though it is expressed at very low levels in normal hepatocytes. Its expression increases in hepatocytes of diet-induced obesity models, correlating with enhanced fatty acid uptake [12]. Studies have shown that hepatic CD36 is closely associated with NAFLD pathogenesis [13,14], and CD36-mediated fatty acid transport to the hepatocyte plasma membrane may contribute to hepatic steatosis in NAFLD patients [15]. Cancer cells frequently exhibit altered fatty acid metabolism to meet energy demands and sustain growth and proliferation [16], and CD36 has been shown to facilitate fatty acid uptake from the microenvironment to fuel tumor metastasis [17]. Recent studies also demonstrate that CD36 promotes sterile inflammation

by assembling Toll-like receptor 4 and 6 heterodimers [18], and may act as a pro-inflammatory factor in concanavalin A-induced liver injury by promoting hepatic inflammation and mediating the pro-apoptotic effects of chemokine CXCL10, suggesting a potential therapeutic target for immune-mediated hepatitis [19]. However, the precise roles and mechanisms of CD36 in liver diseases require further investigation.

Generating knockout mice lacking specific genes globally or in particular tissues represents a primary approach for studying gene function. The Cre/LoxP recombinase system enables tissue-specific gene knockout by placing Cre recombinase under the control of tissue- or cell-specific promoters, allowing Cre to recognize LoxP sequences and catalyze recombination between them to delete the floxed region [20,21].

To construct liver-specific CD36 knockout mice, we first generated Flox heterozygous mice with LoxP sites flanking exon 5 of CD36. Crossing these with Alb-Cre mice expressing Cre recombinase specifically in liver resulted in CD36fl/fl:Alb-Cre+ offspring in which Alb-Cre specifically recognized and deleted the floxed CD36 region in hepatocytes. We validated the knockout at DNA, mRNA, and protein levels, all confirming successful hepatic CD36 deletion. Analysis of other tissues demonstrated comparable CD36 expression in kidney, adipose, and cardiac tissues between CD36fl/fl:Alb-Cre+ and CD36fl/fl:Alb-Cre- mice. Notably, liver-specific CD36 knockout did not affect mouse growth, reproduction, or liver architecture as assessed by HE staining. Using the Cre/LoxP recombinase system, we have successfully established a liver-specific CD36 knockout mouse model for further functional studies of hepatic CD36.

References

[1] Gotoda T, Iizuka Y, Yamada N. Complex connection between CD36 and atherosclerosis, lipid metabolism, and insulin resistance syndromes. Curr Atheroscler Rep, 2000, 2(6): 453-454.
[2] Pepino MY, Kuda O, Samovski D, et al. Structure-function of CD36 and importance of fatty acid signal transduction in fat metabolism. Annu Rev Nutr, 2014; 34(1): 281-303.
[3] Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nat Rev Immunol, 2013, 13(9): 621-634.
[4] Febbraio M, Hajjar DP, Silverstein RL. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J Clin Invest, 2001, 108(6): 785-791.
[5] Wilson CG, Tran JL, Erion DM, et al. Hepatocyte-specific disruption of CD36 attenuates fatty liver and improves insulin sensitivity in HFD fed mice. Endocrinology, 2016, 157(2): 570-585.
[6] Pan X, Wang P, Luo J, et al. Adipogenic changes of hepatocytes in a high-fat diet-induced fatty liver mice model and non-alcoholic fatty liver disease patients. Endocrine, 2015, 48(3): 834-847.
[7] Nath A, Li I, Roberts LR, et al. Elevated free fatty acid uptake via CD36

promotes epithelial-mesenchymal transition in hepatocellular carcinoma. Sci Rep, 2015, 5: 14752.
[8] Liu P, Jenkins NA, Copeland NG. A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res, 2003, 13(3): 476-484.
[9] Chan W, Costantino N, Li R, et al. A recombineering based approach for high-throughput conditional knockout targeting vector construction. Nucleic Acids Res. 2007, 35(8): e64.
[10] Sun YP, Wang Y, Jin Z, et al. Establishment and phenotypic analysis of SHBG knockout mouse model. China Biotechnology, 2017, 37(8): 39-45.
[11] Chen JJ, Xing GC, Zhang LQ. Establishment of FBXL15 knockout mouse model based on LoxP/Cre system. China Biotechnology, 2015, 35(4): 74-79.
[12] Koonen DP, Jacobs RL, Febbraio M, et al. Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity. Diabetes, 2007, 56(12): 2863-2871.
[13] Luiken JJ, Arumugam Y, Dyck DJ, et al. Increased rates of fatty acid uptake and plasmalemmal fatty acid transporters in obese Zucker rats. J Biol Chem, 2001, 276(44): 40567-40573.
[14] Heebøll S, Poulsen MK, Ornstrup MJ, et al. Circulating sCD36 levels in patients with non-alcoholic fatty liver disease and controls. Int J Obes (Lond), 2017, 41(2): 262-267.
[15] Miquilena-Colina ME, Lima-Cabello E, Sánchez-Campos S, et al. Hepatic fatty acid translocase CD36 upregulation is associated with insulin resistance, hyperinsulinaemia and increased steatosis in non-alcoholic steatohepatitis and chronic hepatitis C. Gut, 2011, 60(10): 1394-1402.
[16] Currie E, Schulze A, Zechner R, et al. Cellular Fatty Acid Metabolism and Cancer. Cell Metabolism, 2013, 18(2): 153-161.
[17] Pascual G, Avgustinova A, Mejetta S, et al. Targeting metastasis-initiating cells through the fatty acid receptor CD36. Nature, 2017, 541(7635): 41-45.
[18] Stewart CR, Stuart LM, Wilkinson K, et al. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol, 2010, 11(2): 155-161.
[19] Gao H, Volat F, Sandhow L, et al. CD36 Is a Marker of Human Adipocyte Progenitors with Pronounced Adipogenic and Triglyceride Accumulation Potential. Stem Cells, 2017, 35(7), 1799-1814.
[20] Schmidt-Supprian M, Rajewsky K. Vagaries of conditional gene targeting. Nat Immunol, 2007, 8(7): 665-668.
[21] Baker LG, Lodge JK. Multiple gene deletion in Cryptococcus neoformans using the Cre-lox system. Methods Mol Biol, 2012, 845: 85-98.

Note: Figure translations are in progress. See original paper for figures.