TLR Signaling Interacts with CREBH to Modulate HDL in Response to Bacterial Endotoxin

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Running title: TLR interacts with CREBH to modulate HDL

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Materials and Methods

Reagents - All the chemicals used for this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Lipofectamine™ 2000 transfection reagent was from Invitrogen (Grand Island, NE). Plasmids expressing CREBH and its truncated mutants were as previously described (1). Myc-tagged TRAF6 expression plasmid and its C70A mutant were as previously described (2). LPS was purchased from Sigma-Aldrich (St. Louis, MO). The antibodies against Flag, β-actin, and Tubulin were from Sigma-Aldrich (Santa Cruz, CA). The antibodies against c-Myc, HA , and TRAF6 were from Santa Cruz Biotech. The hepatocarcinoma cell line Huh-7 was kindly provided by Dr. Charles Rice at the Rockefeller University. The kits used to measure mouse serum cholesterol, aspartate transaminase (AST), and alanine transaminase (ALT) were from Bioassay Systems, In (Hayward, CA).

ChIP assays- Mouse liver chromatin was fragmented to an average size of 500 bp and cleared of debris by centrifugation at 20000 g for 30 min at 8°C. The supernatant was harvested and diluted 10-fold with ChIP Dilution Buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 167 mM NaCl; 16.7 mM Tris-HCl, pH8.0). CREBH-binding complexes were pulled down by using 2 μg/mL of a rabbit anti-CREBH antibody developed in our laboratory (3). As controls, the pre-cleared chromatin samples were pulled down using a rabbit anti-HA antibody. Immunoprecipitated chromatin fragments were reverse cross-linked, digested by proteinase K, and purified using QIAquick PCR Purification Kit (Qiagen, Germantown, MD). Presence of CREBH in gene promoters were quantified by semi-quantitative PCR or quantitative real-time PCR and expressed relative to the input genomic DNA as previously described (4). The sequences of the primers used for the ChIP-PCR assay are described in S-Table 2.

Isolation and culture of mouse hepatocytes - Mouse primary hepatocytes were prepared as described previously (5). Briefly, *in situ* liver perfusion was performed with 0.02% collagenase type IV (Sigma) in Hanks' balanced salt solution through a portal vein at a rate of 8 ml/min. After complete perfusion, liver cells were dispersed in DMEM medium. Cell suspensions were then filtered through 100 μm nylon cell strainer (BD Falcon) to remove tissue debris and cellular aggregates. The filtrates were centrifuged at 50 g for 2 min, and hepatocytes-enriched pellet and non-parenchymal cells (NPC)-enriched supernatant will be collected. Hepatocytes-enriched pellet were washed with PBS before they were seeded in culture flasks and maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Quantitative real-time PCR analysis - For quantitative real-time PCR analysis, the reaction mixture containing cDNA template, primers, and SYBR Green PCR Master Mix (Applied Bio systems) was run in a 7500 Fast Real-time PCR System (Applied Bio systems, Carlsbad, CA). Fold changes of mRNA levels were determined after normalization to internal control β-actin mRNA Levels. The sequences of real-time PCR primers used in this study are shown in Supplemental table 1.

Sirius-red staining of hepatic collagens – Sirius-red staining of collagen deposition in liver tissue section for fibrosis evaluation was as previously described (3). Briefly, paraffin-embedded mouse liver tissue sections (5μm) were pretreated with xylene, ethanol, and water according to the standard protocol. The liver tissue sections were stained for 1 hr with Sirius red solution (Sigma) and then washed with acidified water (0.5% acetic acid). The stained tissue sections were dehydrated with 100% ethanol and cleared in xylene before being mounted with mounting solution. The histological analysis of liver fibrosis were as described previously (6). Hepatic fibrosis were scored according to the modified Scheuer scoring system for fibrosis and cirrhosis (6, 7). The fibrosis stage scores were based on the 0-4 stage system: 0, none; 1,

zone 3 perisinusoidal fibrosis; 2, zone 3 perisinusoidal fibrosis plus portal fibrosis; 3, perisinusoidal fibrosis, portal fibrosis, plus bridging fibrosis; and 4, cirrhosis.

Statistics analysis - Experimental results are shown as mean ± SEM (for variation between animals or experiments). All in vitro experiments were repeated with biological triplicates at least three times independently. Mean values for biochemical data from the experimental groups were compared by paired, 2-tailed Student's *t* Tests. Multiple comparisons were performed with ANOVA and followed by ad hoc statistical test when necessary. Statistical tests with $P < 0.05$ were considered significant.

References

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Supplemental Figure Legends

S-Fig 1. (A) Semi-quantitative RT-PCR analysis of the expression levels of mRNAs encoding TLR2, TLR3, and TLR4 in mouse primary hepatocytes. Expression levels of *β-actin* mRNA were examined as loading controls. (**B**) Mouse primary hepatocytes were treated with LPS (100 ng/ml) or PBS. Western blot analysis was performed to detect endogenous CREBH cleavage. Levels of β-actin were determined as loading controls. **(C)** The ratios (%) of the activated form vs total CREBH protein signals in HEK293T cells expressing Flag-tagged full-length CREBH and different TRAF6 variants, including the full-length TRAF6 (TRAF6-WT), the truncated TRAF6 containing the trans-membrane domain (TRAF6-TF), the truncated TRAF6 containing the C-terminal MATH domain (TRAF6-C), and the truncated TRAF6 containing the N-terminal RING finger domain (TRAF6-N), as described in Fig 2A. The cleaved and total CREBH protein signals, determined by Western blot densitometry, were normalized to that of β-actin.

S-Fig 2. (**A**) Total RNAs were isolated from liver tissues of CREBH$^{-/-}$ and WT control mice after LPS (2μg/gm body weight) or PBS challenge for 18 hrs. qPCR analysis was performed to determine expression levels of the genes involved in apolipoproteins. Expression values were normalized to *β-actin* mRNA levels. Fold changes of mRNA levels are shown by comparing to one of the control mice treated with PBS. Each bar denotes the mean ± SEM (n=3). (**B**) Expression levels of the genes encoding inflammatory cytokines, chemokines, and acute-phase responsive proteins in the livers of CREBH$^{-/-}$ and WT control mice. Total RNAs were isolated from liver tissues of the CREBH$^{-/-}$ and WT as described in A. qPCR analysis was performed to determine expression levels of the genes involved in inflammation and hepatic acute-phase response. Expression values were normalized to *β-actin* mRNA levels. Fold changes of mRNA levels are shown by comparing to one of the control mouse treated with PBS. Each bar denotes the mean ± SEM (n=3). * $P<0.05$.

S-Fig 3. (**A-B**) Luciferase reporter analysis of the *ApoA4* gene promoter activation by CREBH and TRAF6. Huh7 cells were co-transfected with the plasmid vectors expressing full-length human CREBH, ApoA4 reporter, and TRAF6 or its mutant isoform C70A. The luciferase activities were examined at different time periods (as indicated) post the transfection. The experiment was done by triplicate samples. Each bar denotes the mean ± SEM (n=3). ** P<0.01. Rep, Reporter; CR, CREBH; T6, TRAF6.

S-Fig 4. Body temperature, body weight reduction and histological analysis of CREBH$^{-/-}$ and WT control mice under acute endotoxemia. CREBH$^{-/-}$ and WT control mice were IP injected with LPS of 20 μg/gram body weight. Health assessment based on body posture, body hygiene, skin dryness, eyes and head position, cage movement, food intake, body weight, and body temperature were conducted for 96 hours post injection. (**A-B**) Body temperature (A) and body weight reduction (B) of CREBH$^{-/-}$ and WT control mice under the different time points after the LPS injection for the survival experiment as described in Fig 4A. 12 mice per group (n=12) were culled for the experiment. Each point denotes the mean ± SEM. * P<0.05. (**C**) HE and Sirius-red staining of the kidney tissue sections of the CREBH$^{-/-}$ and WT control mice at 96 hours after the LPS injection. Magnification: 200 ×.

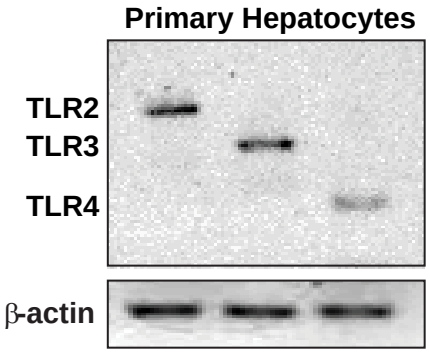
S-table 1. Sequences of quantitative real-time PCR primers used in this study.

Name	Type	Sequence
	Forward	GCTGCTGGAGGACGGTTACA
Fgf21	Reverse	CACAGGTCCCCAGGATGTTG
	Forward	AGCTTCCACGTTGTCTTAGGG
Apoa4	Reverse	TGTGACTCCACGTTGGAAGG
	Forward	CGTCTGGGCTCAAGATGAAGT
Apob	Reverse	CTGGACACCGCTGGAACTTT
	Forward	AGATGCGGCTAGTGGCAAAG
Bdh1	Reverse	CAGTTCCTTGACCCCAGCAT
ApoA5	Forward	TCCTCGCAGTGTTCGCAAG
	Reverse	GAAGCTGCCTTTCAGGTTCTC
Apoc2	Forward	CTCTGCTGGGCACGGTGCA
	Reverse	GCCGCCGAGCTTTTGCTGTAC
ApoA1	Forward	AGCTGAACCTGAATCTCCTG
	Reverse	CAGAGAGTCTACGTGTGT
Pck1	Forward	CTCAGCTGCATAACGGTCTG
	Reverse	CTTCAGCTTGCGGATGACAC
Cpt1a	Forward	AGAATCTCATTGGCCACCAG
	Reverse	CAG GGTCTCACTCTCCTTGC
Stat3	Forward	AACGTCAGCGACTCAAACTG
	Reverse	CCCGTACCTGAAGACCAAGTT
Saa	Forward	CGGGACATGGAGCAGAGG
	Reverse	TTGCCACTCCGGCCC
Sap	Forward	TGTCTGGGATTGAGATCTTACAACA
	Reverse	CTGCCGCCTTGACCTCTTAC
Tlr2	Forward	CCATTGAGGGTACAGTCGTCG
	Reverse	GGCATTAAGTCTCCGGAATTATC
Tlr 3	Forward	AGCCTTATACCATAAAAG
	Reverse	CAGTTCAGAAAGAACGG
Tlr 4	Forward	GGAAGGACTATGTGATGTGAC
	Reverse	GCTCTTCTAGACCCATGAAATTGG
Ccl2	Forward	CACTCACCTGCTGCTACTCA
.	Reverse	GCTTGGTGACAAAAACTACAGC
Ccl3	Forward	CCATATGGAGCTGACACCCC
	Reverse	GTCAGGAAAATGACACCTGGC
Rplpo	Forward	AGACAAGGTGGGAGCCAGCGA
	Reverse	GCGGACACCCTCCAGAAAGCG
Actin	Forward	GATCTGGCACCACACCTTCT
	Reverse	GGGGTGTTGAAGGTCTCAAA
Il6	Forward	CCCAATTTCCAATGCTCTCCT
	Reverse	TGAATTGGATGGTCTTGGTCC
Tnfα	Forward	CCA ACG CCC TCC TGG CCA AC
	Reverse	GAG CAC GTA GTC GGG GCA GC

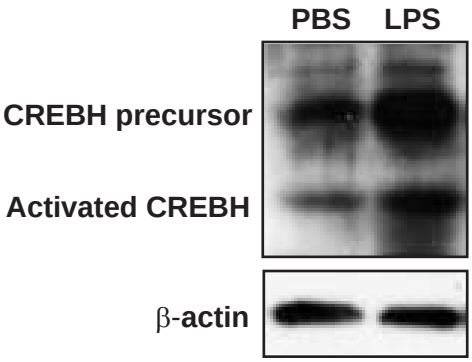
S-table 2. Sequences of ChIP-PCR primers used in this study.

Negative control primer	Forward	CATGGATGTATGCTCCCGACT
	Reverse	GGAGCTCAGTCTGTGTCCAG
Il6	Forward	GGAGAGGAGTGTGTGTCTT
	Reverse	GCGCATGACAGACGACACA
ApoA4	Forward	CAGGGTCCAGCCAACTCAAG
	Reverse	CTCCACGTTCGAAGGTGACA

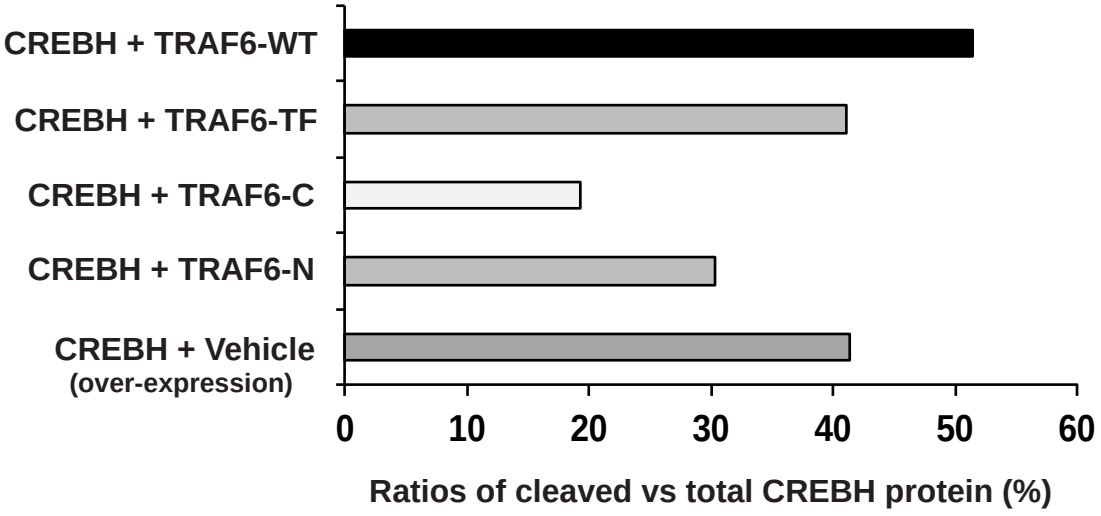
A


Primary Hepatocytes
TLR2
TLR3
TLR4
β-actin

B

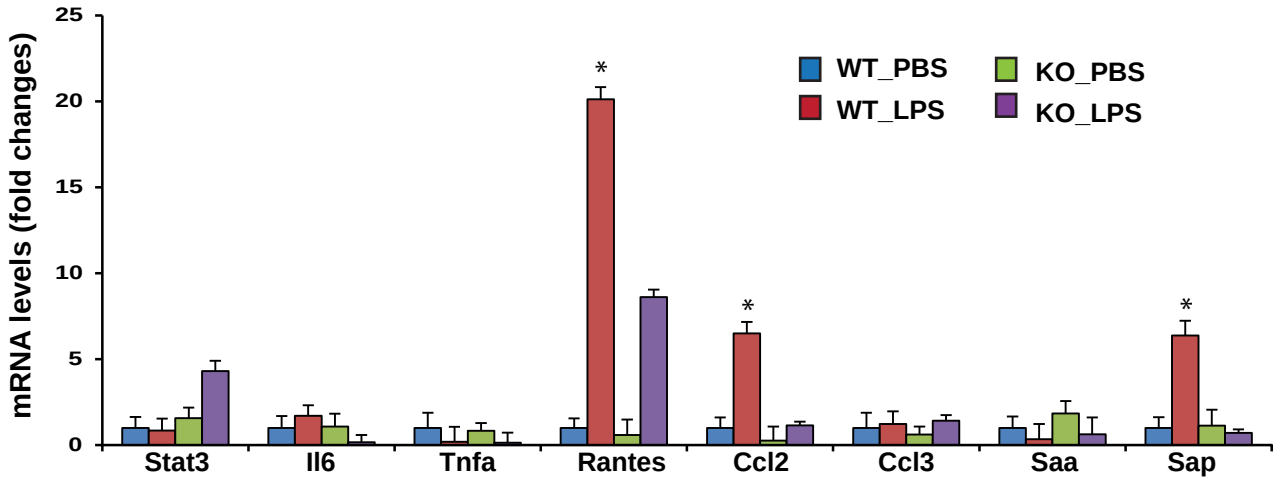

PBS
LPS
CREBH precursor
Activated CREBH
β-actin

C

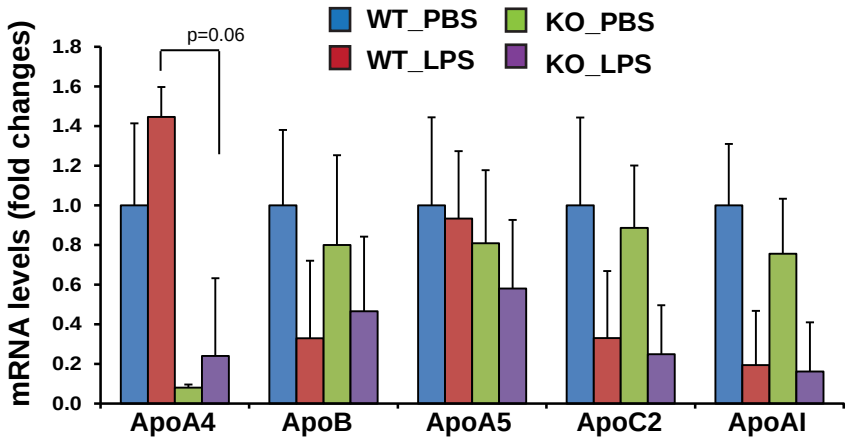

CREBH + TRAF6-WT
CREBH + TRAF6-TF
CREBH + TRAF6-C
CREBH + TRAF6-N
CREBH + Vehicle
(over-expression)
0
10
20
30
40
50
60
Ratios of cleaved vs total CREBH protein (%)

S-Fig 1

A

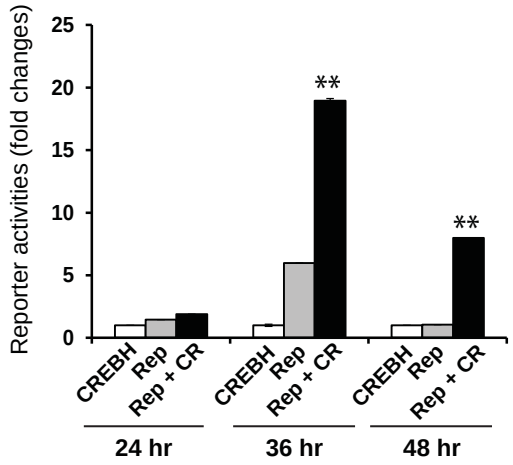

mRNA levels (fold changes)
25
20
15
10
5
0
WT_PBS
WT_LPS
KO_PBS
KO_LPS
*
*
*
Stat3
Il6
Tnfa
Rantes
Ccl2
Ccl3
Saa
Sap

B

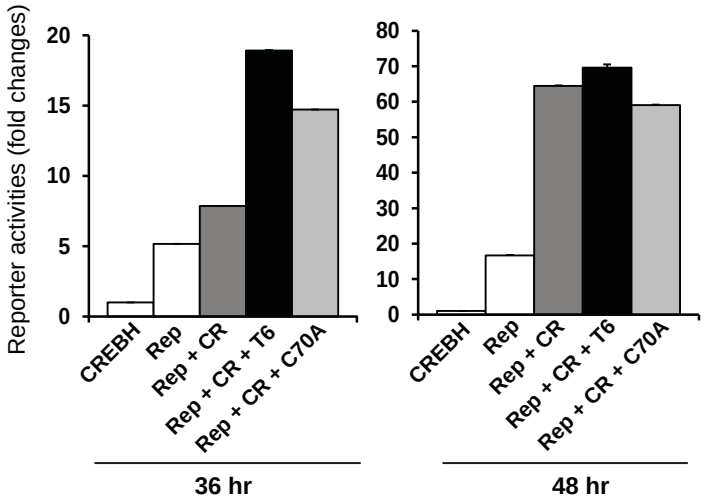

mRNA levels (fold changes)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p=0.06
WT_PBS
WT_LPS
KO_PBS
KO_LPS
ApoA4
ApoB
ApoA5
ApoC2
ApoAI

S-Fig 2

A

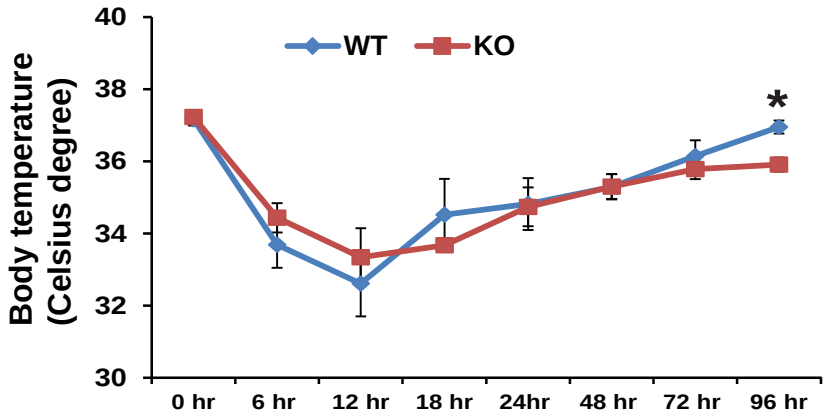

Reporter activities (fold changes)
25
20
15
10
5
0
**
**
CREBH
Rep
Rep + CR
CREBH
Rep
Rep + CR
CREBH
Rep
Rep + CR
24 hr
36 hr
48 hr

B

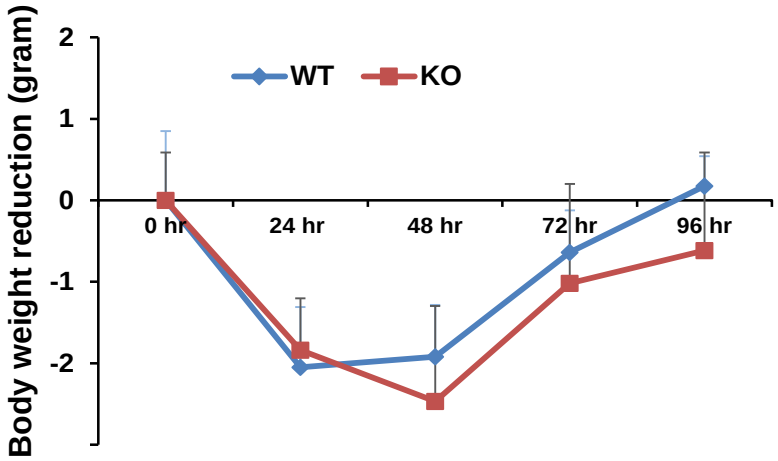

Reporter activities (fold changes)
20
15
10
5
0
80
70
60
50
40
30
20
10
0
CREBH
Rep
Rep + CR
Rep + CR + T6
Rep + CR + C70A
CREBH
Rep
Rep + CR
Rep + CR + T6
Rep + CR + C70A
36 hr
48 hr

S-Fig 3

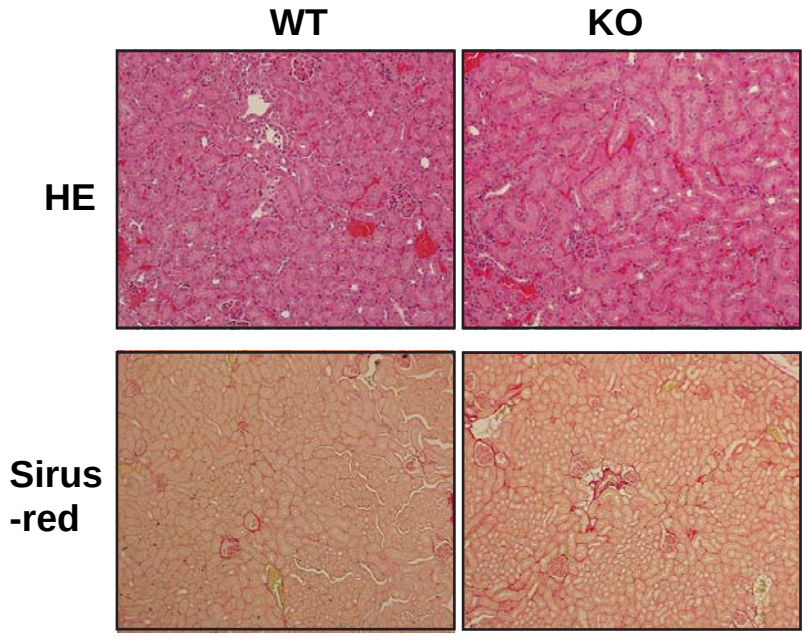
A


Body temperature (Celsius degree)
40
38
36
34
32
30
WT
KO
*
0 hr
6 hr
12 hr
18 hr
24hr
48 hr
72 hr
96 hr

B


Body weight reduction (gram)
2
1
0
-1
-2
WT
KO
0 hr
24 hr
48 hr
72 hr
96 hr

C


WT
KO
HE
Sirus
-red

S-Fig 4