Online Supplementary Materials

Cathepsin K Activity Controls Cardiotoxin-Induced Skeletal Muscle Repair in Mice

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Table S1: Primer sequences for mice used for quantitative real-time PCR

Genes	Forward Primers	Reverse Primers
TLR-4	AGTGGGTCAAGGAACAGAAGCA	CTTTACCAGCTCATTTCTCACC
TLR-2	AAGAAGCTGGCATTCCGAGGC	CGTCTGACTCCGAGGGGTTGA
CatS	GTGGCCACTA AAGGGCCTG	ACCGCTTTTGTAGAAGAAGAAGGAG
CatK	AGCAGGCTGGAGGACTAAGGT	TTTGTGCATCTCAGTGGAAGACT
CatL	GGCAACCCGATGCGC	TGTGTGACTCCTGTGAAGAACCA
MMP-2	CCCCATGAAGCCTTGTTTACC	TTGTAGGAGGTGCCCTGGAA
MMP-9	CCAGACGCTCTTCGA GAACC	GTTATAGAAGTGGCGGTTGT
TIMP-1	GCCTACACCCCAGTCATGGA	GGCCCGTGATGAGAAACTCTT
TIMP-2	GTCCCATGATCCCTTGCTACA	TGCCCATTGATGCTCTTCTCT
MCP-1	GCCCCACTCACCTGCTGCTACT	CCTGCTGCTGGTGATCCTCTTGT
TNF-α	AGCCGATGGGTTGTACCTTG	ATAGCAAATCGGCTGACGGT
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCT

Abbreviations: TLR-2, toll-like receptor-2; CatS, cathepsin S; CatK, cathepsin K; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1; TIMP-2, tissue inhibitor of metalloproteinase-2; MCP-1, monocyte chemoattractant Protein-1; TNF-α, tumor necrosis factor-α; GAPDH, gluceradehyde-3-phosphate dehydrogenase.

Supplementary Figure Legends

Figure S1. Expressions of matrix metalloproteinase (MMP) and cathepsin family genes in the muscles of $CatK^{+/+}$ mice at the indicated time points after cardiotoxin (CTX) injection. **A:** Photos of both gastrocnemius mass and representative microscopy images of H&E staining of the non-injured and injured muscles of $CatK^{+/+}$ mice on days 3 and 14 after injury. **B–D:** Quantitative real-time PCR data show the levels of CatK, CatS, CatL, MMP-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, and TIMP-2 at days 0, 3, 7, and 14 after injury. Results are the mean±SD (n=6). **E:** A representative result of gelatin zymography shows the levels of MMP-2 and MMP-9 gelatinolytic activities of the non-injured and injured muscles of $CatK^{+/+}$ mice at day 3 after CTX injection. *p<0.05, **p<0.01 vs. the corresponding day 0 by one-way ANOVA followed by Tukey post hoc tests.

Figure S2. Effects of CatK inhibition on the levels of caspase-3 and cleaved caspase-3 in the muscles. **A-B:** Representative images of Western blots and the combined quantitative data show the ratio of cleaved caspase-3 to caspase-3 band intensities in the injured muscles of $CatK^{+/+}$ and $CatK^{-/-}$ mice. **C-D:** Representative images of Western blots and the combined quantitative data show the ratio of cleaved caspase-3 to caspase-3 band intensities in the injured muscles of CONT, LKI (low-dose CatK inhibitor) and HKI (high-dose CatK inhibitor) mice.

Figure S3. The gene expressions of cathepsin and MMP families in the skeletal muscles of mice of both genotypes at day 3 after injury. **A–C:** Quantitative real-time PCR data show the levels of CatS, CatL, MMP-2, MMP-9, TIMP-1, and TIMP-2 genes in the non-injured and injured gastrocnemius muscles of mice of both genotypes. Results are the mean±SD (n=5). **D,E:** Representative images of gelatin zymography and combined quantitative data show the gelatinolytic activities of MMP-2 and MMP-9 in the non-injured and injured gastrocnemius muscles of mice in both experimental groups. Results are the mean±SD (n=5). *p<0.05, **p<0.01 vs. the corresponding control groups by Student's *t*-test or one-way ANOVA followed by Tukey post hoc tests. NS: not significant.

Figure S4. Evaluation of cathepsin and MMP gene expressions in the non-injured and injured gastrocnemius muscles of $CatK^{+/+}$ mice treated with vehicle (0.5%carboxymethylcellulose; CONT mice) or a low or high dose of the specific CatK inhibitor ONO-KK1-300-01 (3 mg/kg, LKI mice; 30 mg/kg, HKI mice) at day 3 after CTX injury. **A–D:** Quantitative real-time PCR data show the levels of CatS, CatK, CatL, MMP-2, MMP-9, TIMP-1, and TIMP-2 in the non-injured and injured muscles of the three experimental groups. Results are the mean±SD (n=6). **E,F:** Representative images of gelatin zymography and combined quantitative data show the gelatinolytic activities of MMP-2 and MMP-9 of the injured gastrocnemius in the three experimental groups (n=4). *p<0.05, **p<0.01 vs. the corresponding control groups by Student's t-test or one-way ANOVA followed by Tukey post hoc tests. NS: not significant.

Figure S5. CatK inhibition prevented CTX-induced muscle function. **A:** Grip strength was measured over 5 times in each mouse on 0, 3 and 14 days after CTX injury and averaged to obtain the grip strength for each time point. **B:** Workload in the vertical direction was calculated in consideration of the weight of the mouse (= weight × gravitational acceleration × mileage in the vertical direction). **C,D:** Running time and distance were calculated from the product of running time and speed. All items were expressed as the ratio of observed values to data on day 0 before CTX injection. Results are the mean±SD (n=3). *P<0.05, vs. the corresponding controls by 2-way repeated-measures ANOVA and Bonferroni post hoc tests.

Figure S6. Changes in the expression of CatK mRNA induced by CTX in a time- and dose-dependent manner. **A:** Microscopy images and quantitative real-time PCR data show the levels of CatK mRNA in the serum-free medium at the indicated time points. **B:** Representative images and quantitative real-time PCR data show changes in the CTX-induced CatK mRNA expression over time. **C:** Representative images and quantitative real-time PCR data show changes in the CTX-induced CatK mRNA expression with dose. Scale bars: A, 50 μm. *p<0.05, **p<0.01 vs. the corresponding control groups by Student's t-test or one-way ANOVA followed by Tukey post hoc tests. NS: not significant.

Figure S1

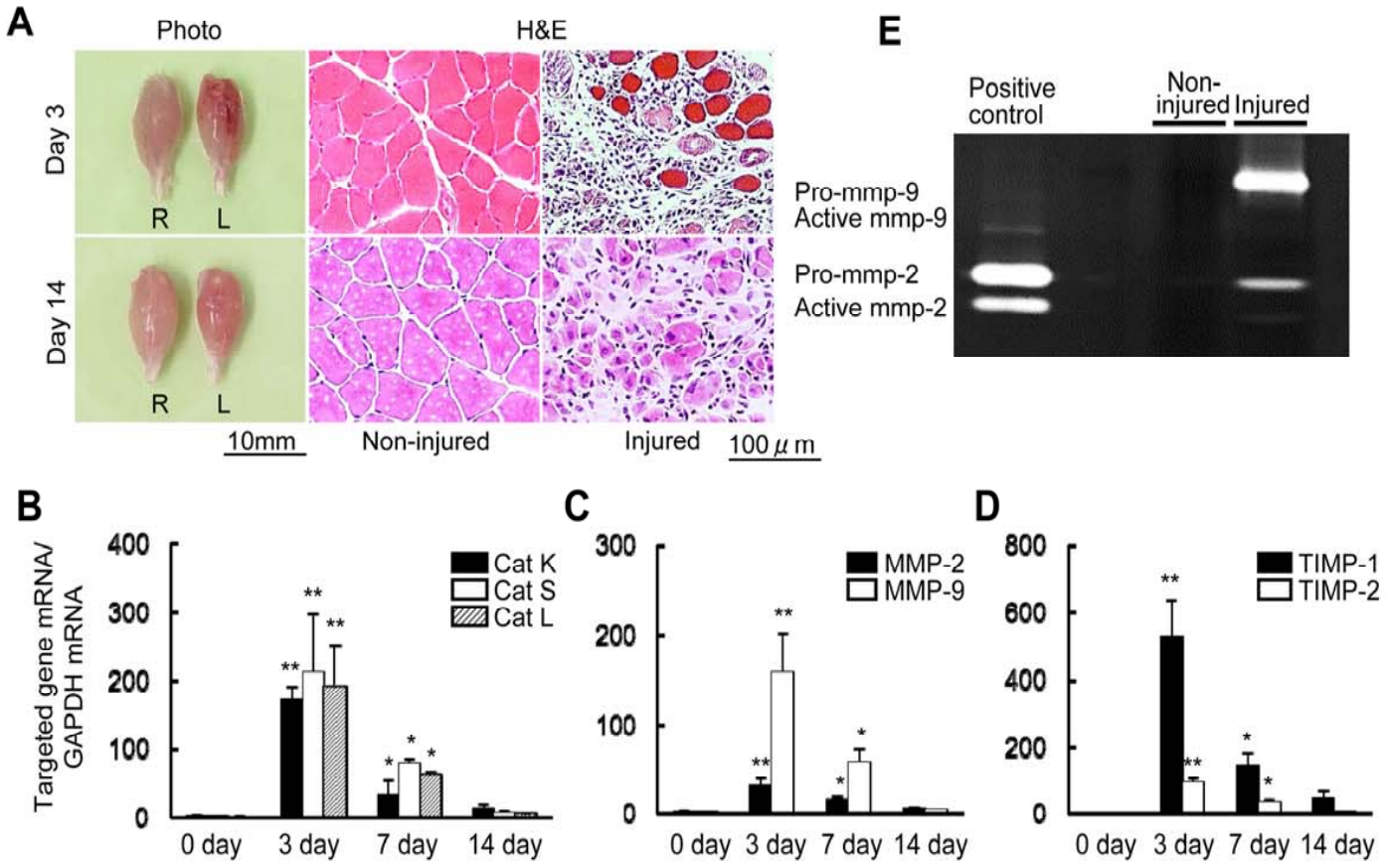
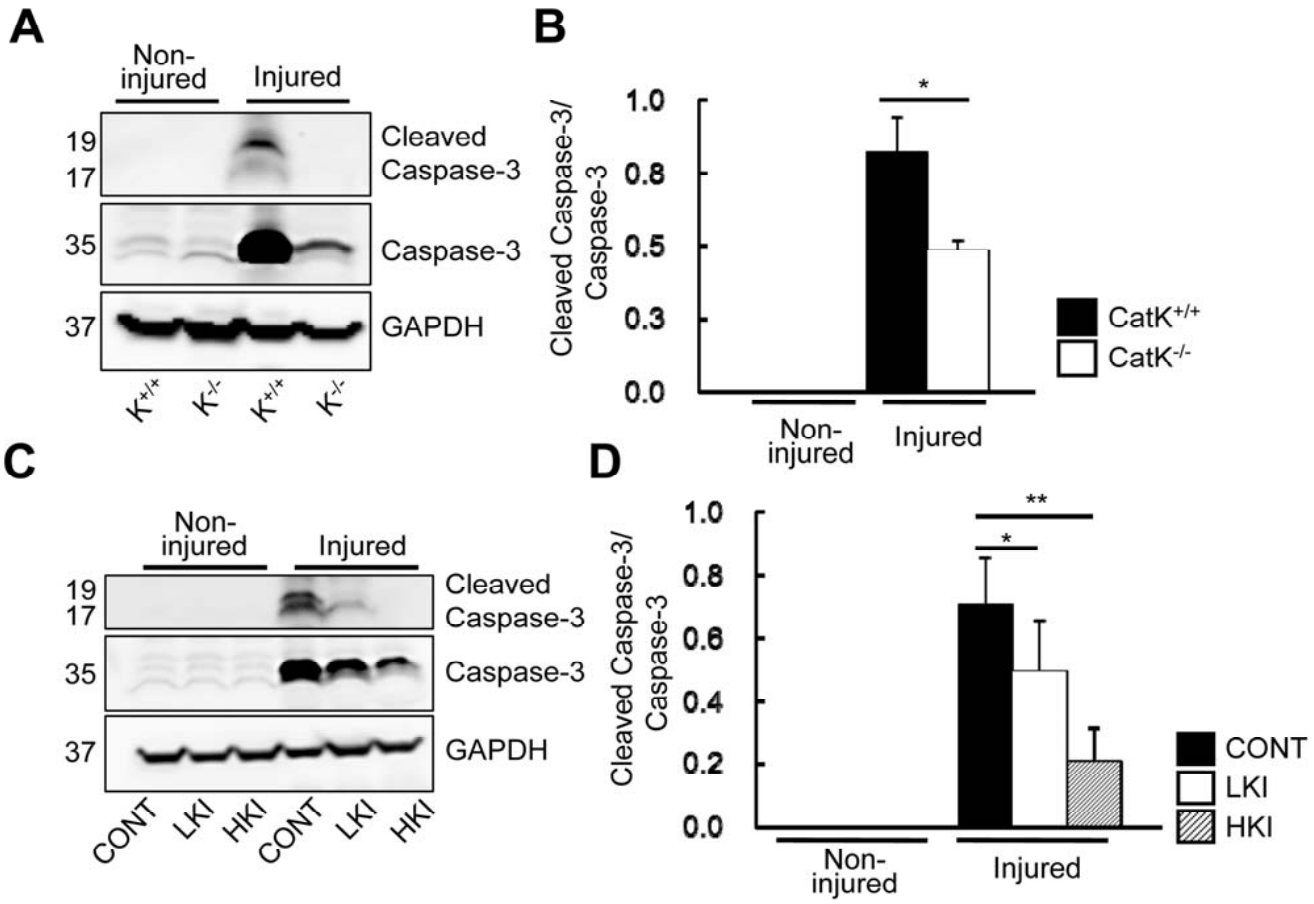

A
Photo
H&E
Day 3
Day 14
R
L
10mm
Non-injured
Injured
100 μm
E
Positive control
Non-injured
Injured
Pro-mmp-9
Active mmp-9
Pro-mmp-2
Active mmp-2
B
Targeted gene mRNA/ GAPDH mRNA
Cat K
Cat S
Cat L
0 day
3 day
7 day
14 day
C
MMP-2
MMP-9
D
TIMP-1
TIMP-2

Figure S2


A
Non-injured
Injured
19
17
Cleaved Caspase-3
35
Caspase-3
37
GAPDH
K+/+
K-/-
K+/+
K-/-
B
Cleaved Caspase-3/ Caspase-3
1.0
0.8
0.5
0.3
0.0
*
CatK+/+
CatK-/-
Non-injured
Injured
C
Non-injured
Injured
19
17
Cleaved Caspase-3
35
Caspase-3
37
GAPDH
CONT
LKI
HKI
CONT
LKI
HKI
D
Cleaved Caspase-3/ Caspase-3
1.0
0.8
0.6
0.4
0.2
0.0
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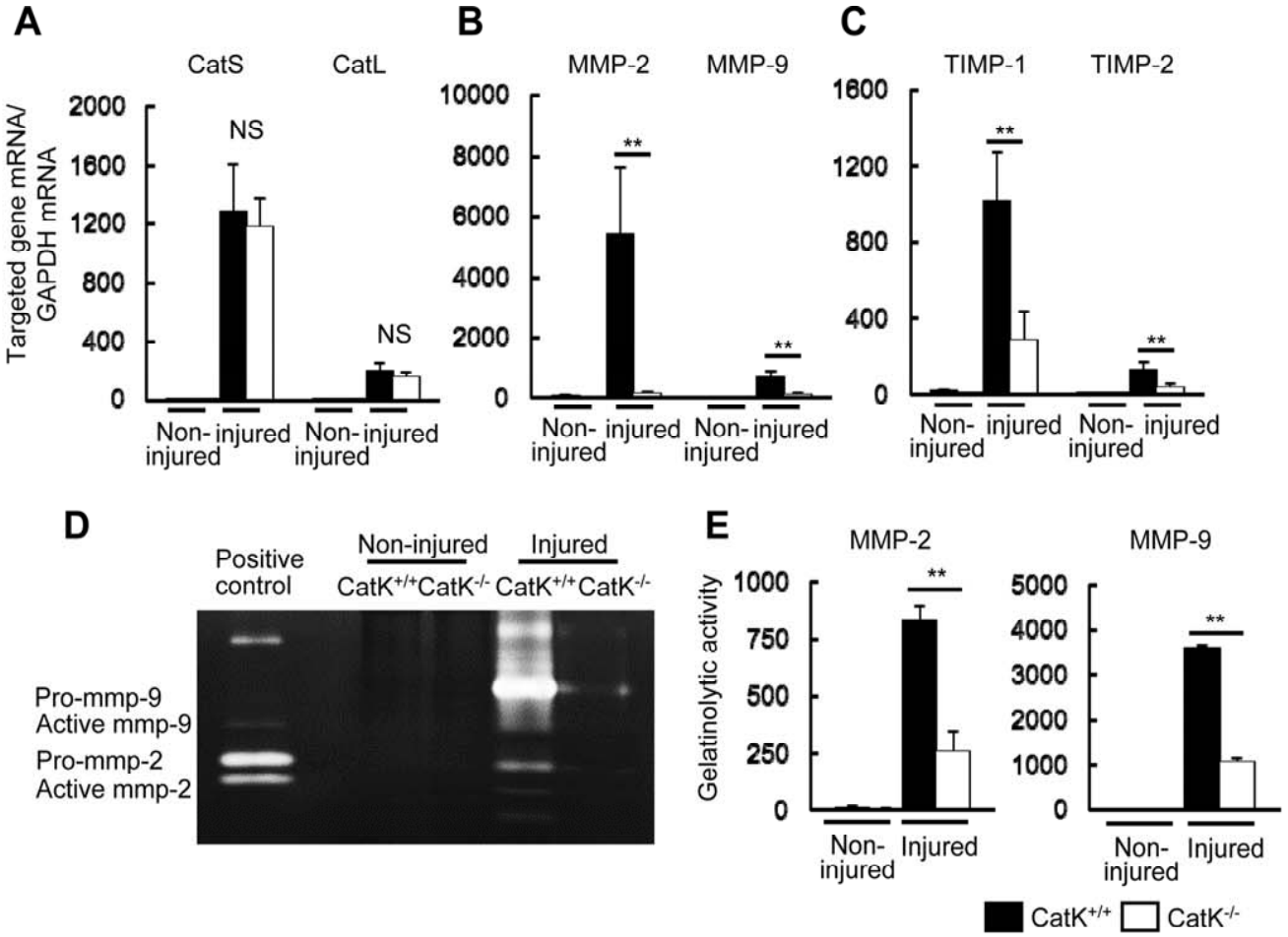
*
CONT
LKI
HKI
Non-injured
Injured

Figure S3


A
CatS
CatL
Targeted gene mRNA/ GAPDH mRNA
2000
1600
1200
800
400
0
NS
NS
Non-injured
injured
Non-injured
injured
B
MMP-2
MMP-9
10000
8000
6000
4000
2000
0
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Non-injured
injured
Non-injured
injured
C
TIMP-1
TIMP-2
1600
1200
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Non-injured
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D
Positive control
Non-injured
Injured
CatK+/+ CatK-/- CatK+/+ CatK-/-
Pro-mmp-9
Active mmp-9
Pro-mmp-2
Active mmp-2
E
MMP-2
MMP-9
Gelatinolytic activity
1000
750
500
250
0
5000
4000
3000
2000
1000
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**
**
Non-injured
Injured
Non-injured
Injured
CatK+/+
CatK-/-