LJ000328, a novel ALK2/3 kinase inhibitor, represses hepcidin and significantly improves the phenotype of IRIDA

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Supplementary methods

Kinase assays

The LJ000328 compound was synthesized with >99.9% purity in the form of HCL salt (Figure 1A). Full details of its synthesis and characterization will be detailed elsewhere. *In vitro* kinase reactions were carried out by Reaction Biology (Malvern, PA) using purified human enzymes[1].

Mouse studies

Wild-type and *Tmprss6*$^{-/-}$ mice (7-8 week-old) used in this study were all on a C57BL/6J background. They were given free access to tap water and standard laboratory mouse chow diet (180 mg iron/kg; SSNIFF, Soest, Germany). LJ000328 (20mg/kg; diluted in 2-Hydroxypropyl-β-cyclodextrin solution (45 % (w/v) in H_2O) was administered by intraperitoneal injections. Mock-injected mice received the same volume of 2-Hydroxypropyl-β-cyclodextrin solution.

8-week-old male wild-type mice on a C57BL/6J genetic background received, by oral gavage, 150 µl of LJ000328 (20mg/kg; diluted in 2-Hydroxypropyl-β-cyclodextrin solution (45 % (w/v) in H2O)) or 2-Hydroxypropyl-β-cyclodextrin solution (45 % (w/v) in H_2O) and were sacrificed after 4 hours.

Hematological parameters were assessed at sacrifice on a CELL-DYN Emerald system (Abbott, Lake Forest, IL). Mice were used in our studies under the guidelines and protocols approved by the Midi-Pyrénées Animal Ethics Committee.

Quantitative real-time PCR

Total RNA was isolated using UPzol lysis reagent (biotechrabbit, Hennigsdorf, Germany). First-strand cDNA synthesis was performed using MMLV-RT (Promega, Madison, WI). Quantitative real-time PCR were performed as previously described[2], with the primers listed in supplementary Table S1.

Western blot analysis

Protein extraction and western blot analysis were performed as previously described[2]. Briefly, livers were homogenized in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5mM

EDTA, pH 8, 0.1% NP-40). Equal amounts of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated overnight at 4°C with a rabbit anti-phospho-Smad5 antibody (Epitomics, Burlingame, CA). Membranes were then stripped and reprobed with a rabbit anti-Smad5 antibody (Epitomics).

Quantitative Iron Measurement

Transferrin saturation was deduced from serum iron and latent iron-binding capacity values (Biolabo, Maizy, France).

Serum sample analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatinine were determined using a COBAS-MIRA+ biochemical analyzer (Anexplo facility, Toulouse, France).

Serum erythropoietin levels were measured using the mouse EPO Quantikine set (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions

Histology

Organ samples were fixed in 4% buffered formalin, embedded in paraffin, and sliced into 5-µm sections, then deparaffinized, rehydrated, and stained with hematoxylin.

1. Hao J, Ho JN, Lewis JA, et al. In vivo structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. *ACS Chem Biol*. 2010;5(2):245-253.
2. Besson-Fournier C, Gineste A, Latour C, et al. Hepcidin upregulation by inflammation is independent of Smad1/5/8 signaling by activin B. *Blood*. 2017;129(4):533-536.

Supplementary Figure S1. LJ000328 treatment inhibits Bmp-Smad signaling and promotes iron absorption. WT C57BL/6 male mice injected with LJ000328 (blue boxes) (8-10/group) were sacrificed 2, 6, and 15 hours after injection and compared to mock-injected mice (grey boxes). Mice were analyzed for liver **(A)** *Id1* and **(B)** Smad7 mRNA expression, **(C)** serum iron content, **(D)** transferrin saturation Box-and-whisker plots are shown for *Id1* and *Smad7*, and means ± SEM are shown for iron parameters. Mean values were compared by ANOVA followed by Sidak's multiple comparison tests. ****P < .0001; ***P < .001; **P < .01; *P < .05

Supplementary Figure S2. LJ000328 does not modulate Bmp type I, type II and activin receptor mRNA expression. (A-F) WT C57BL/6 male mice injected with LJ000328 (blue boxes) (8-10/group) were sacrificed 2, 6, and 15 hours after injection and compared to mock-injected mice (grey boxes). Mice were analyzed for *Bmpr1a, Acvr1, Bmpr1b, Bmpr2, Acvr2a and Acvr2b* liver mRNA expression. **(G-L)** *Tmprss6*$^{-/-}$ mice were injected with LJ000328 (pink boxes) (n=5) for 7 weeks and compared with mock-injected mice (purple boxes) (n=5). Mice were characterized for liver *Bmpr1a, Acvr1, Bmpr1b, Bmpr2, Acvr2a and Acvr2b* liver mRNA expression. *ΔCts* are shown as box-and-whisker plots. Mean values were compared by ANOVA followed by Sidak's multiple comparison tests **(A-F)** or by Student's t-tests **(G-L).**

Supplementary Figure S3. LJ000328 injection suppresses Bmp-Smad signaling in *Tmprss6*$^{-/-}$ mice after 2hrs. *Tmprss6*$^{-/-}$ male mice injected with LJ000328 (blue boxes) (n=3) were sacrificed 2 hours after injection and compared to mock-injected mice (grey boxes) (n=3). Mice were analyzed for liver **(A)** *Id1* mRNA expression, and **(B)** *Smad7* mRNA expression. Box-and-whisker plots are shown for *Id1* and *Smad7* ΔCts. Mean ΔCt values were compared by Student's t-tests. *P < .05

Supplementary Figure S4. LJ000328 chronic treatment inhibits Bmp-Smad signaling in *Tmprss6*$^{-/-}$ mice. *Tmprss6*$^{-/-}$ mice were injected with LJ000328 (pink boxes) (n=5) for 7 weeks and compared with mock-injected mice (purple boxes) (n=5). Mice were characterized for **(A)** alopecia, and liver **(B)** pSmad-5 relative to total Smad5 protein expression, **(C)** *Id1* mRNA expression, **(D)** *Smad7* mRNA expression and **(E)** serum EPO level. For comparison, black boxes indicate levels measured in WT littermate mice. Box-and-whisker plots are shown for *Id1, and Smad7* ΔCts. For EPO measurement, results are expressed as mean ± SEM Mean. Mean values were compared by Student's t-tests. ***P < .001; **P < .01; *P < .05

Supplementary Figure S5. LJ000328 treatment does not induce toxicity in mice. *Tmprss6*$^{-/-}$ mice were injected with LJ000328 (pink boxes) (n=5) for 7 weeks and compared with mock-injected mice (purple boxes) (n=5). Mice were characterized for **(A)** weight, serum **(B)** alanine aminotransferase (ALT), **(C)** aspartate aminotransferase (AST), **(D)** creatinine, **(E)** lactate dehydrogenase (LDH), liver **(F)** *Crp* mRNA expression, **(G)** *Col1a1* mRNA expression and **(H)** *Col4a1* mRNA expression. Mean ± SEM are shown for weight, ALT, AST, LDH and creatinine and box-and-whisker plots are shown for *Crp, Col1a1,* and *Col4a1* ΔCts, and. Mean values were compared by Student's t-tests.

Supplementary Figure S6. LJ000328 treatment does not promote liver abnormality. *Tmprss6*$^{-/-}$ mice were injected with LJ000328 for 7 weeks and compared with mock-injected mice. Liver sections stained with H&E. Original magnification ×14

Supplementary Figure S7. LJ000328 treatment does not promote spleen abnormality. *Tmprss6*$^{-/-}$ mice were injected with LJ000328 for 7 weeks and compared with mock-injected mice. Spleen sections stained with H&E. Original magnification ×7

Supplementary Figure S8. LJ000328 treatment does not promote heart abnormality. *Tmprss6*$^{-/-}$ mice were injected with LJ000328 for 7 weeks and compared with mock-injected mice. Heart sections stained with H&E. Original magnification ×9

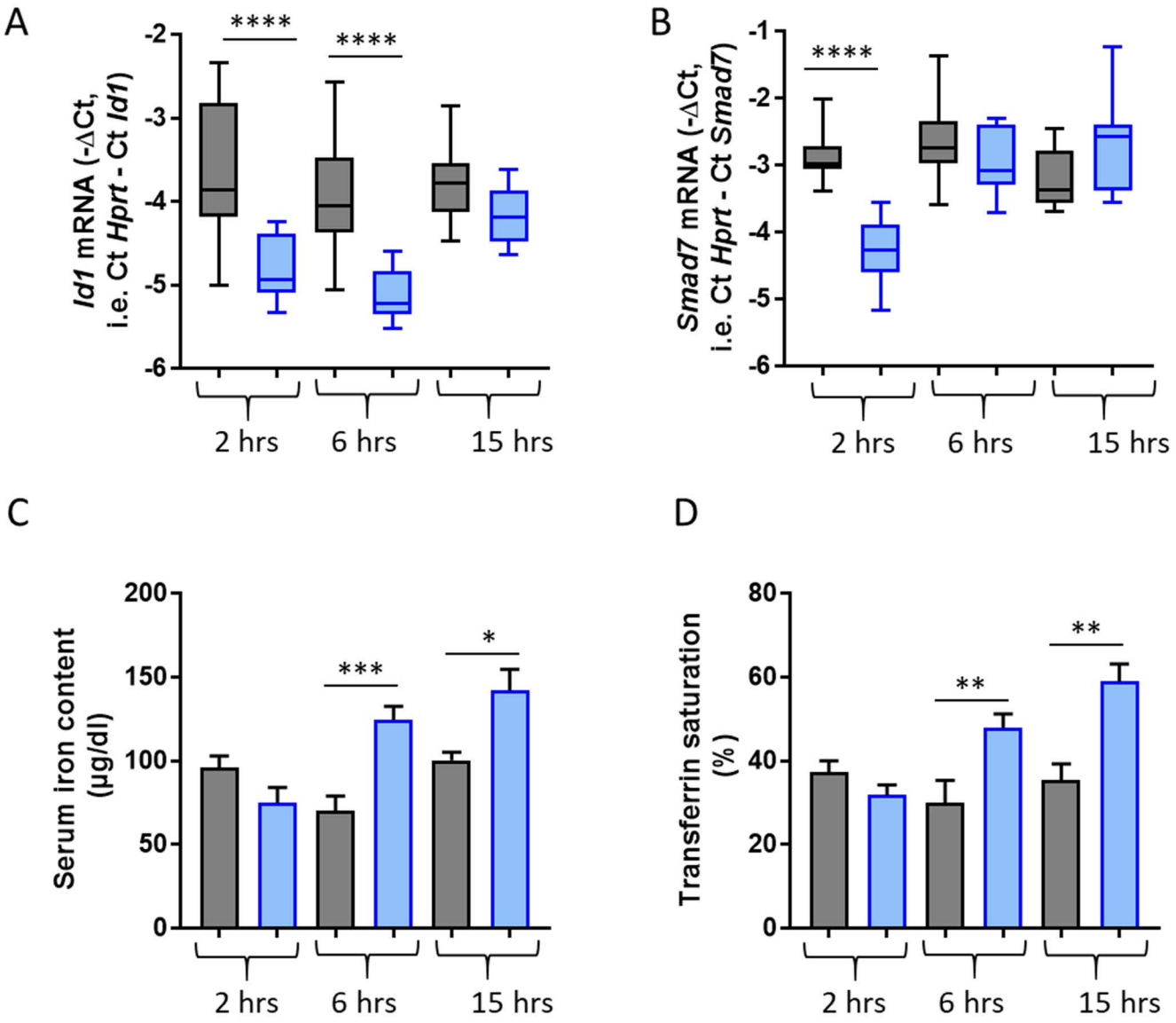
Supplementary Figure S9. LJ000328 treatment does not promote kidney abnormality. *Tmprss6*$^{-/-}$ mice were injected with LJ000328 for 7 weeks and compared with mock-injected mice. Kidney sections stained with H&E. Original magnification ×8

Supplementary Figure S10. LJ000328 reduces liver hepcidin expression after oral administration. WT C57BL/6 male received a single dose of LJ000328 (blue box; n=3) or 2-Hydroxypropyl-β-cyclodextrin solution (black box; n=3) by oral gavage and were sacrificed 4 hours later. Mice were analyzed for liver *Hamp* mRNA expression. Box-and-whisker plots are shown *Hamp* ΔCts. Mean values were compared by Student's t-test (F). ***P < .001

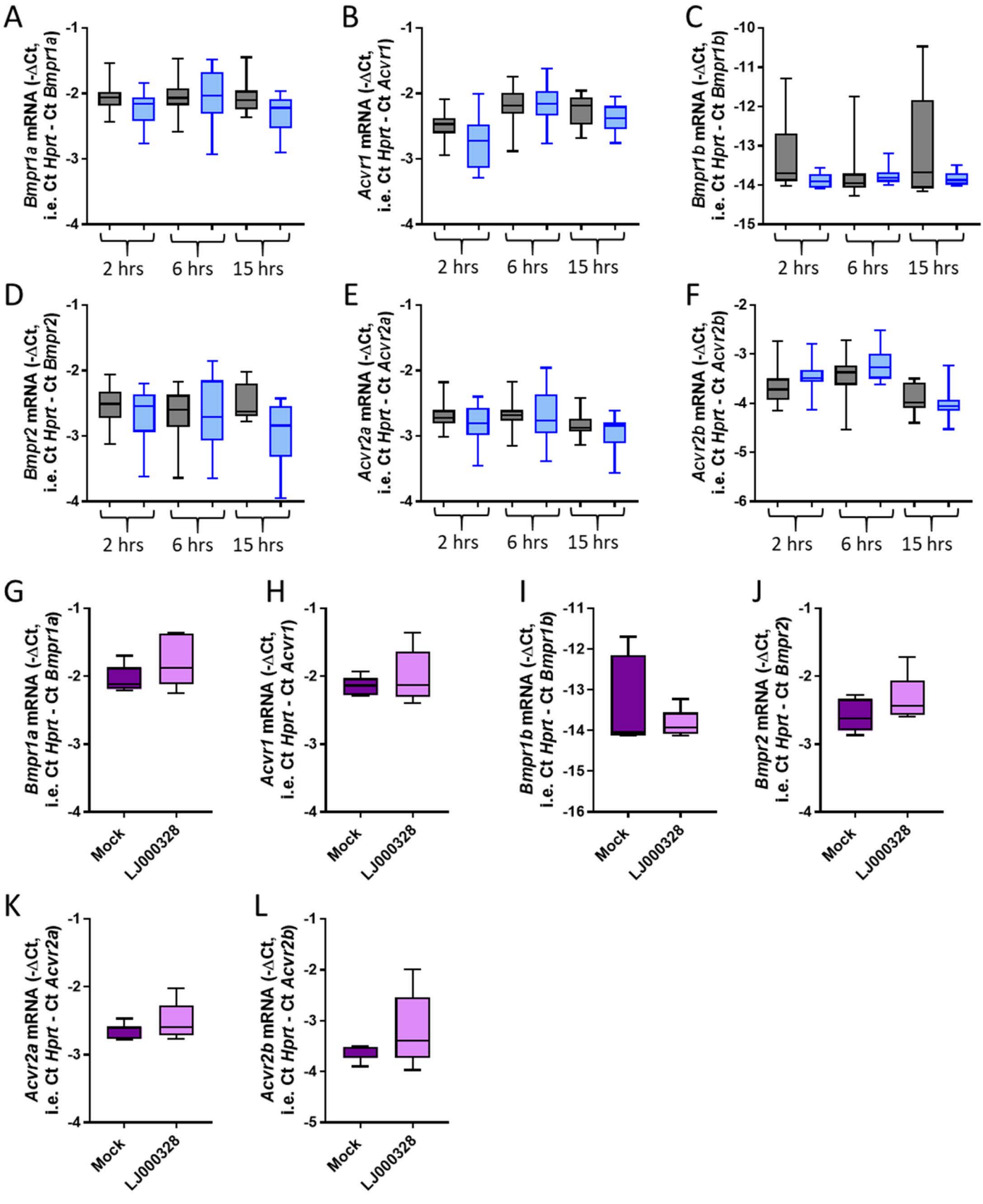
Supplementary Table 1. Primer sequences

Supplementary Table 2. Selectivity of LJ000328 and LDN-193189 for different kinases determined by *in vitro* kinase assays

Supplementary Figure S1

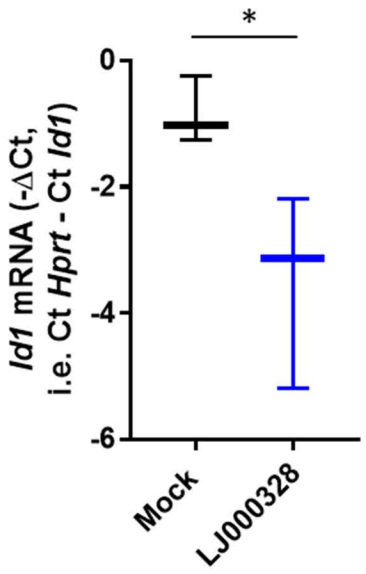


Supplementary Figure S2



Supplementary Figure S3

A



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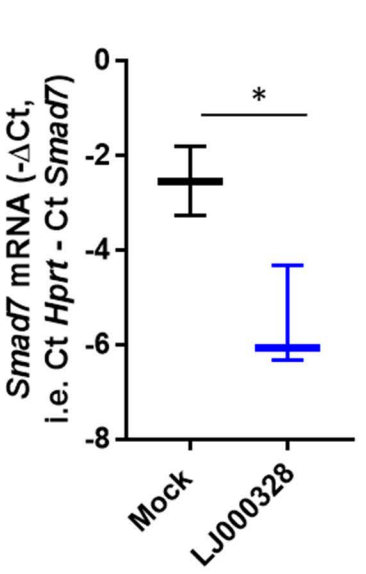


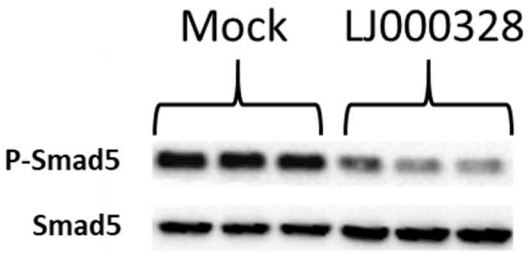
Supplementary Figure S4

A

No treatment

After treatment

B



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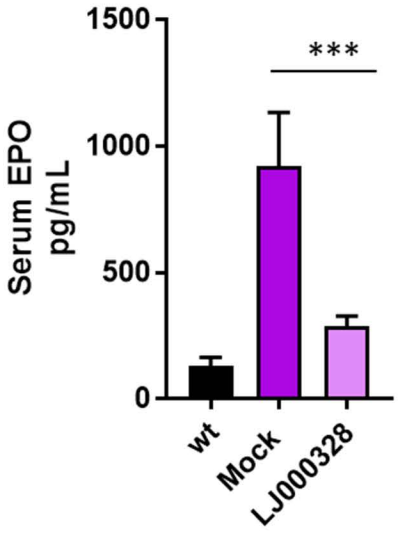
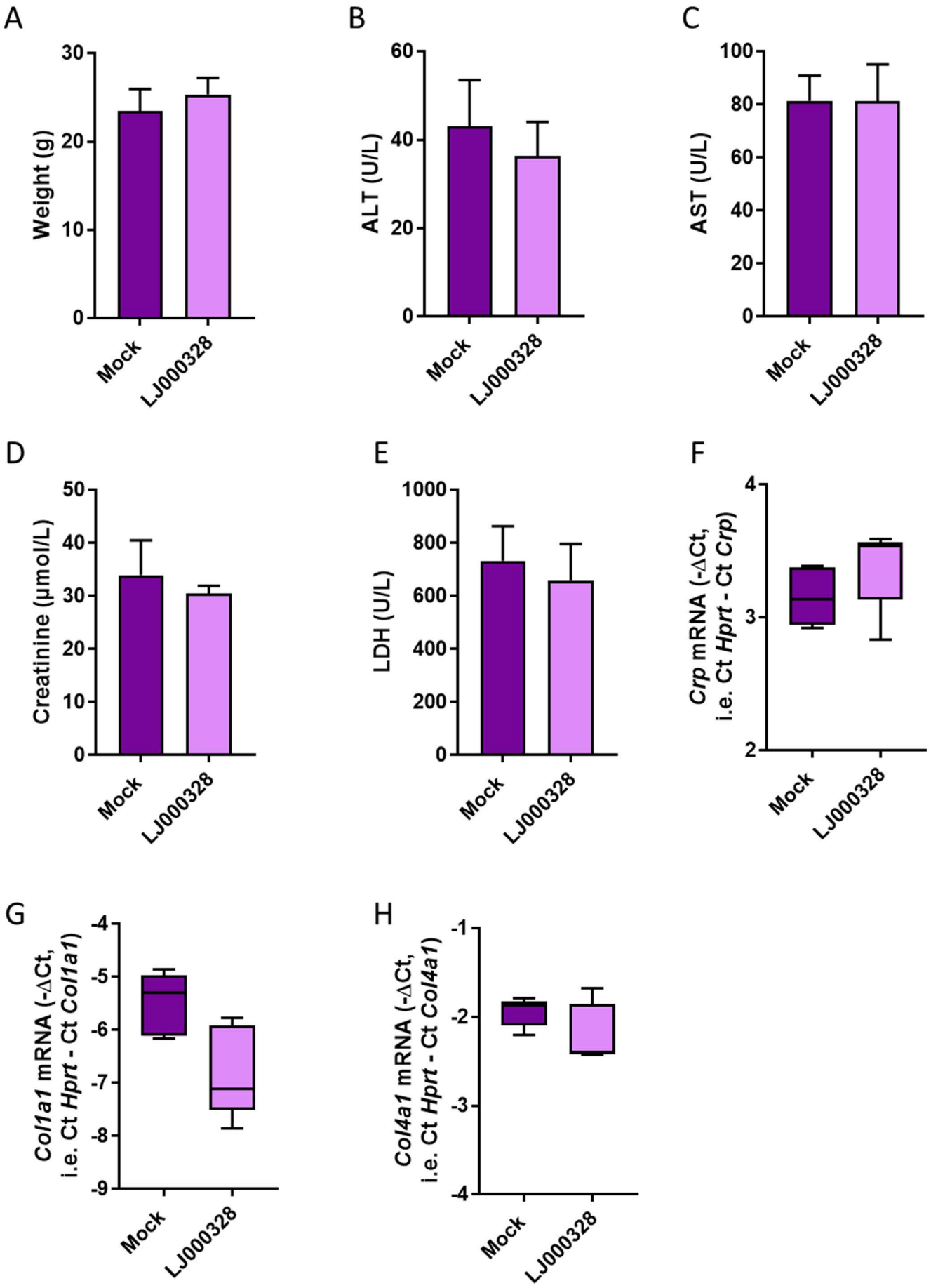
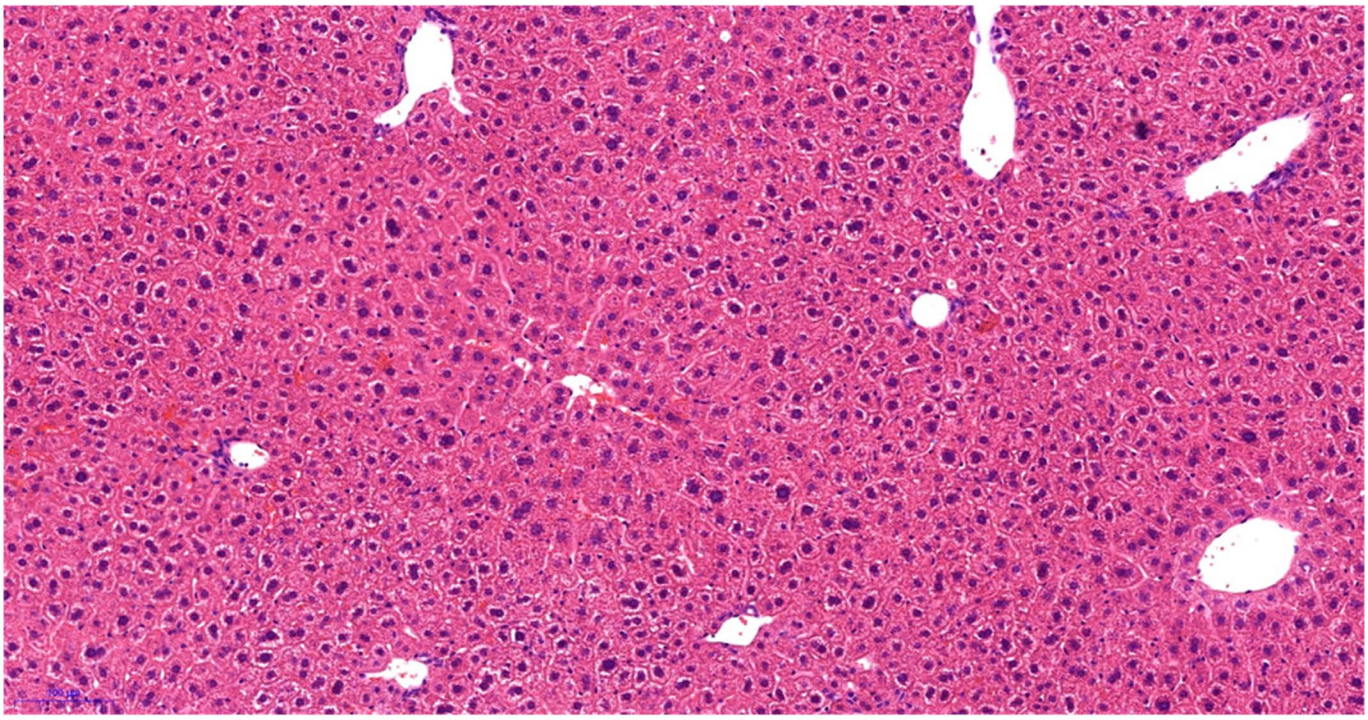


Supplementary Figure S5

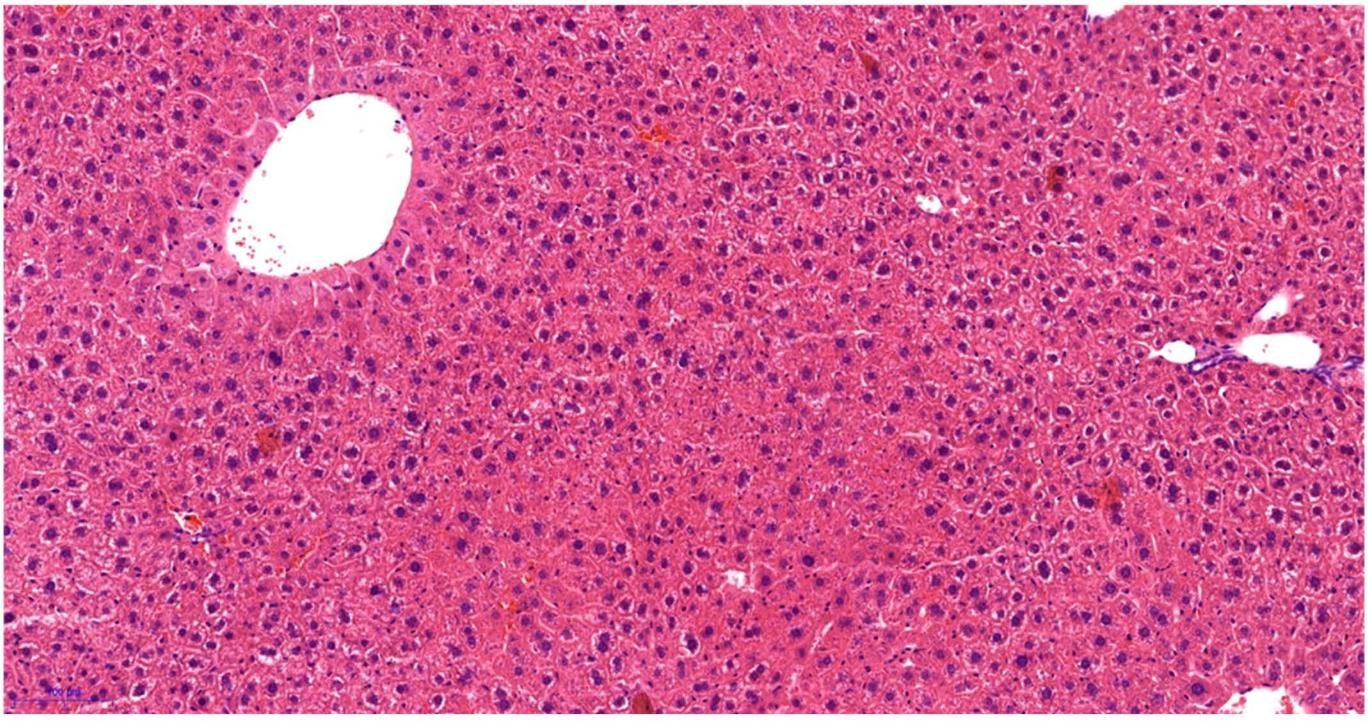


Supplementary Figure S6

Mock

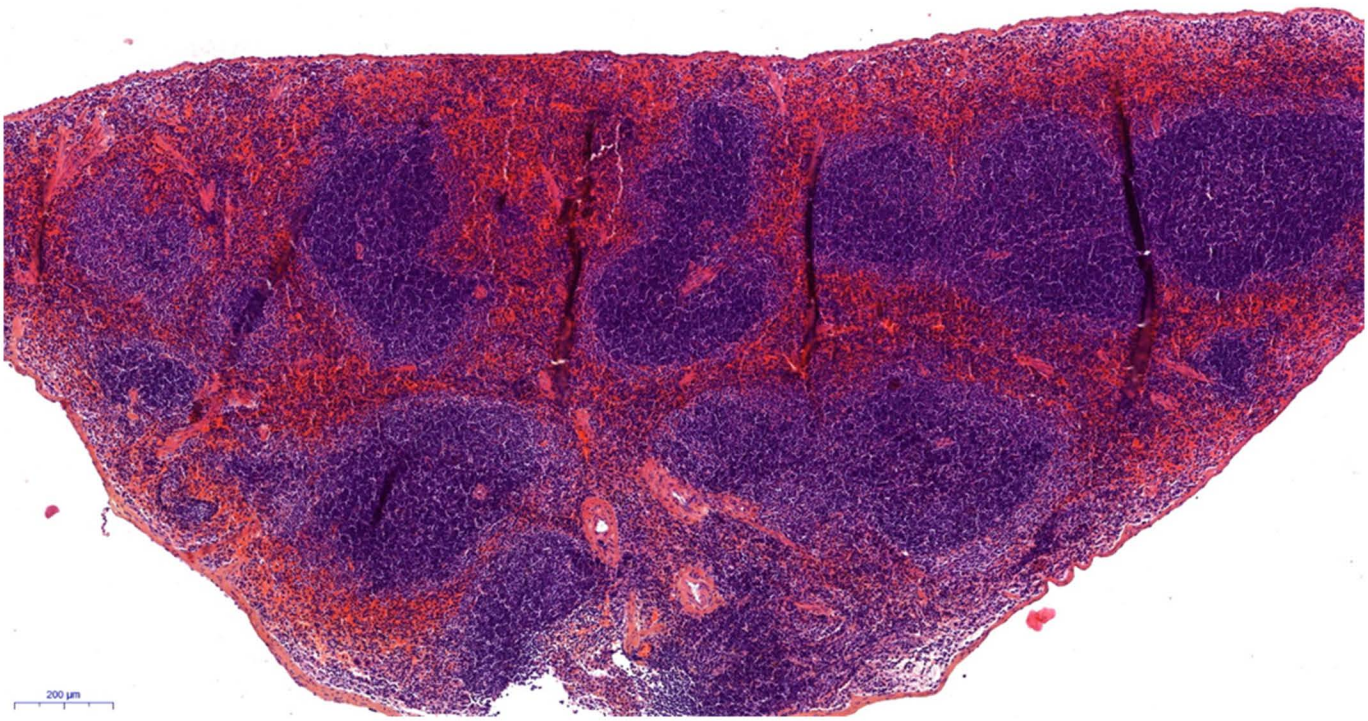


LJ000328

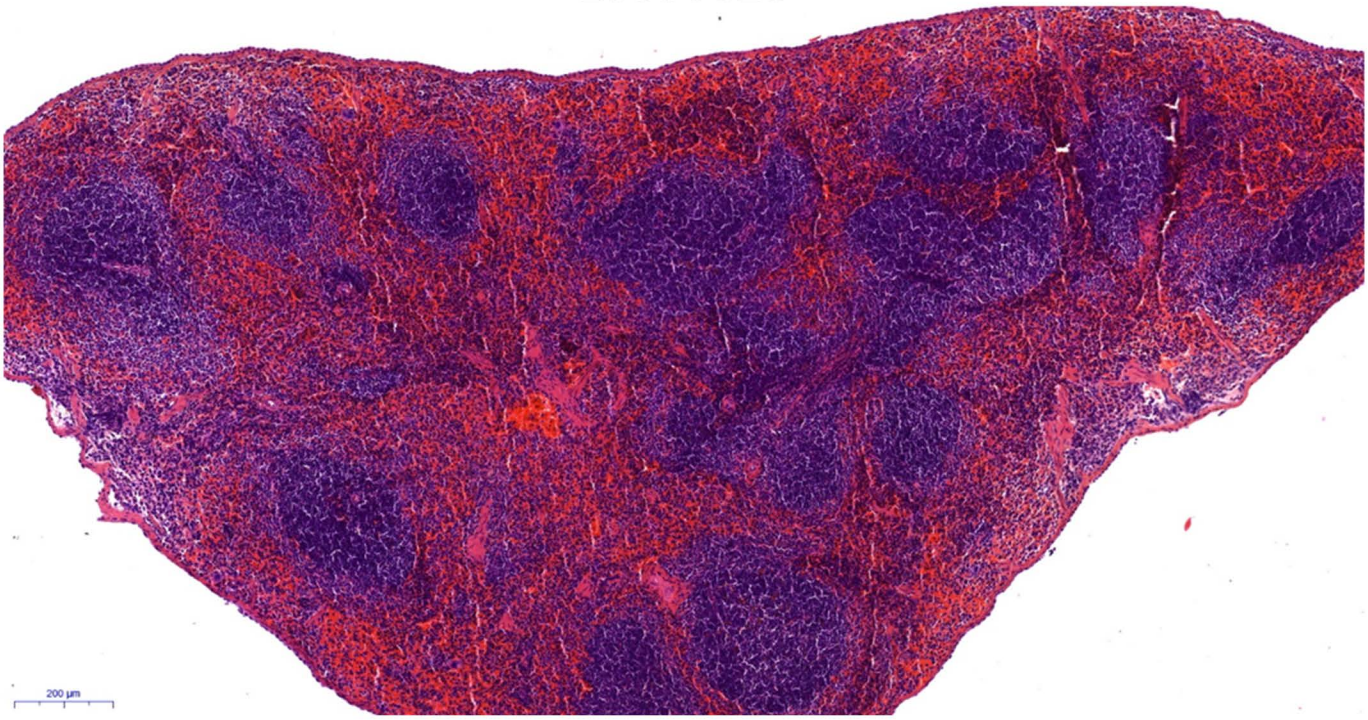


Supplementary Figure S7

Mock

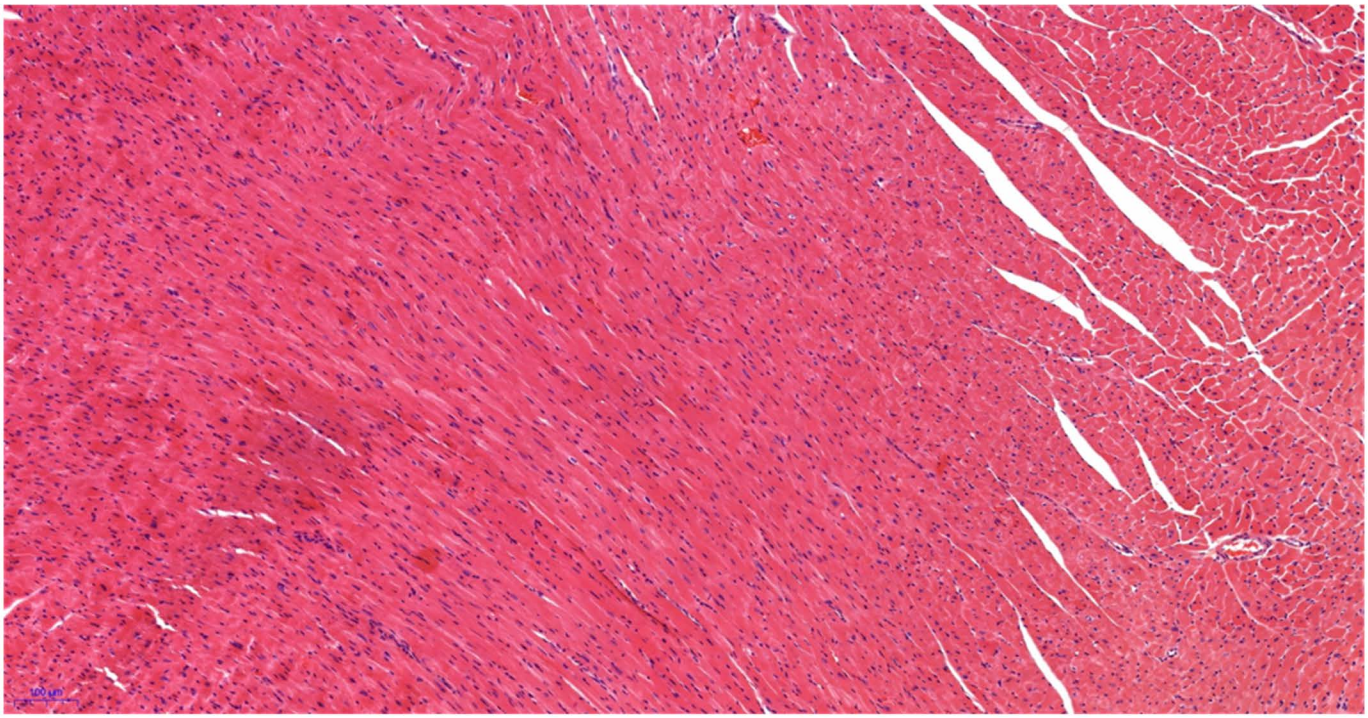


LJ000328

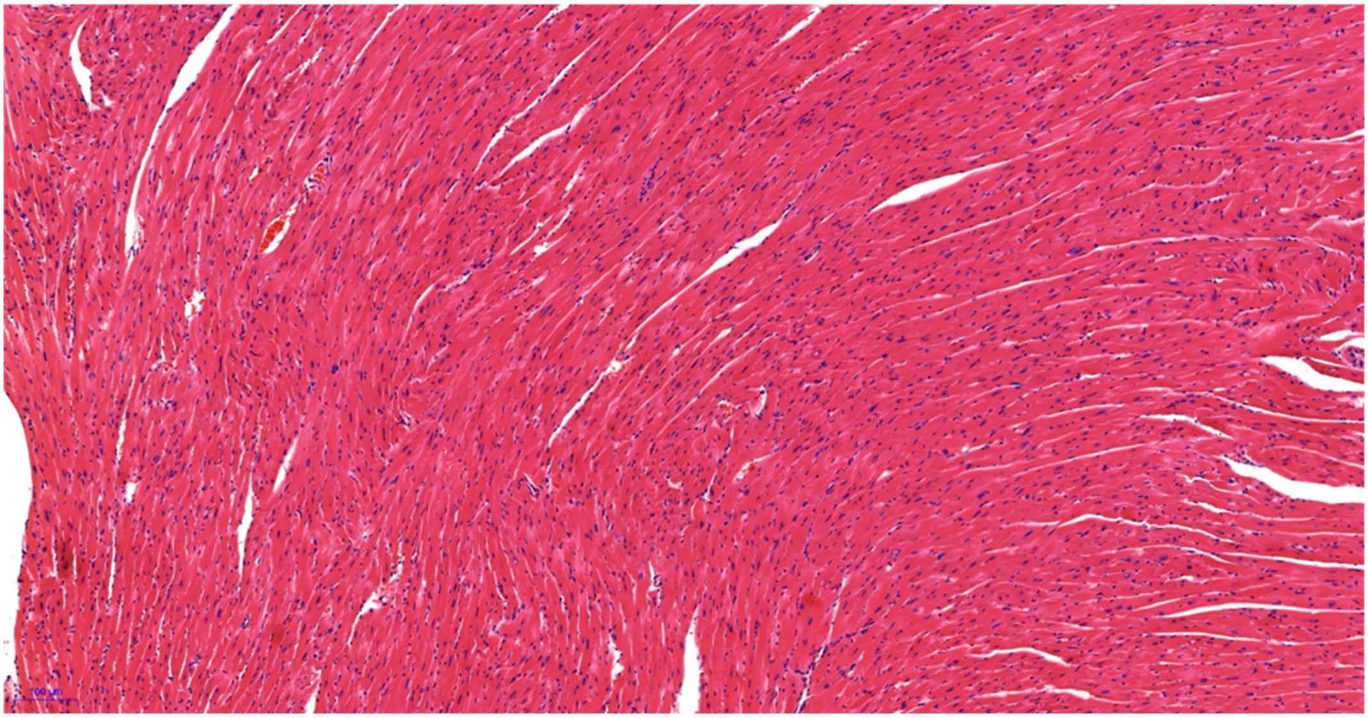


Supplementary Figure S8

Mock

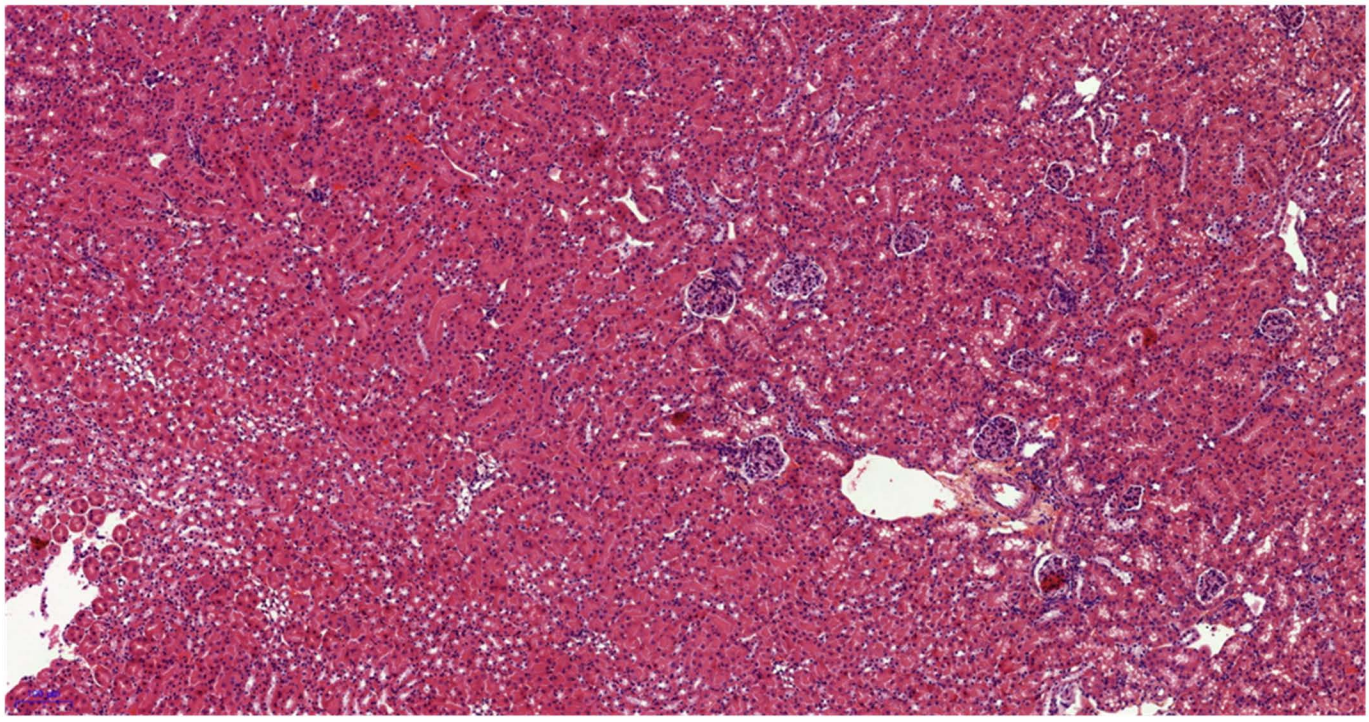


LJ000328

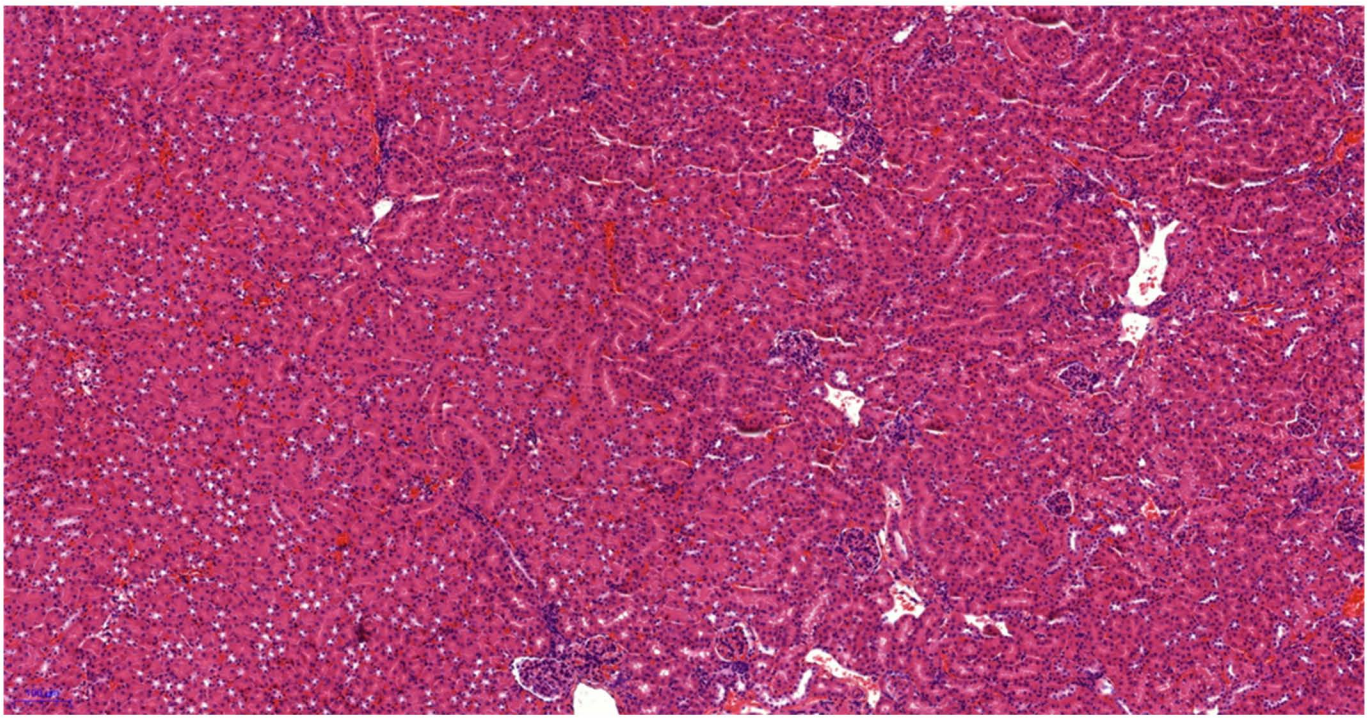


Supplementary Figure S9

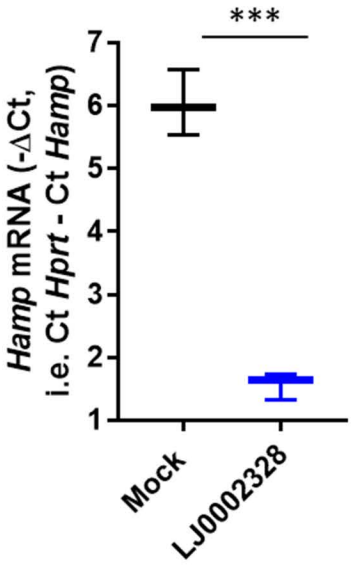
Mock



LJ000328



Supplementary Figure 10



Supplementary Table S1

	Forward	Reverse
Hprt	CTG-GTT-AAG-CAG-TAC-AGC-CCC-AA	CAG-GAG-GTC-CTT-TTC-ACC-AGC
Hamp	AAG-CAG-GGC-AGA-CAT-TGC-GAT	CAG-GAT-GTG-GCT-CTA-GGC-TAT-GT
Id1	ACC-CTG-AAC-GGC-GAG-ATC-A	TCG-TCG-GCT-GGA-ACA-CAT-G
Smad7	GCA-GGC-TGT-CCA-GAT-GCT-GT	GAT-CCC-CAG-GCT-CCA-GAA-GA
Crp	GGC-TTC-TTT-GAC-TCT-GCT-TCC-A	GCT-ACT-CTG-GTG-CCT-TCT-GAT-CA
Col1a1	CTG-ACG-CAT-GGC-CAA-GAA-GA	TAC-CTC-GGG-TTT-CCA-CGT-CT
Bmpr1a	GGC-TGC-AAA-TAC-TGG-TTG-CAC	TGC-AAG-GAT-TCA-CCG-AAA-GC
Acvr1	GCC-ATT-GCC-CAT-CGA-GAT-C	ATG-CAT-GAC-TGC-CAG-GCC
Bmpr1b	ATA-AGC-TTC-CCC-ATC-TGC-CTG	GCC-TTC-ATT-CCC-CAA-TCG-A
Bmpr2	GGC-CCA-ATT-CTC-TGG-ATC-TTT-C	CAC-CTG-ATC-CTG-ATT-TGC-CAT-C
Acvr2a	GCA-GGA-ATG-GCA-ATG-CTC-TGT	GAT-AAC-CTG-GCT-TCT-GCA-TCA-TGA
Acvr2b	CAG-ATT-CCG-CAG-TGC-CCT-A	TGG-TGT-GCA-TCA-CGA-AGG-A

Supplementary Table S2

	IC_{50} (nM)	
BMP type-I receptors	**LJ000328**	**LDN-193189**
ALK1	166.3	11.2
ALK2	10.9	3.3
ALK3	5.1	0.3
ALK6	29.4	3.5
TGFβ Type-I receptors	**LJ000328**	**LDN-193189**
ALK4	568.7	686.2
ALK5	1267	1 095
Type-II receptors	**LJ000328**	**LDN-193189**
BMPR2	> 50 000	3 845
TGFBR2	587.2	140
Other kinases	**LJ000328**	**LDN-193189**
AMPK	13 590	1 122
KDR	13 410	214.7
PDGFRb	7 450	n.d.