### SUPPLEMENTARY INFORMATION

Title:

### SENP1 prevents steatohepatitis by suppressing RIPK1-driven apoptosis

and inflammation

### Author list:

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### Inventory of Supporting Information:

Supplementary Figures 1 to 9



# Supplementary Figure 1. Deletion of SENP1 in hepatocytes causes RIPK1-driven inflammation and liver damage.

a-d, Immunoblotting analysis of SENP1 protein levels in the livers of mice fed a HFD for indicated number of weeks (W) (n = 4) (a), and isolated hepatocytes, Kupffer cells and endothelial cells from livers of control mice and HFD-fed (24 weeks) mice (n = 2) (b). The purity of isolated each type of cells was determined by measuring the hepatocyte-specific gene Alb, Kupffer cell-specific gene *Clec4f*, and endothelia cell-specific gene Tek (n = 6) (b). c, d, Immunoblotting analysis of SENP1 expression in livers (c) and hepatocytes isolated from livers (d) of mice with indicated genotypes (n =3). e, The z scores of RIPK1, CYLD, BIRC2 and SENP1 in the genome-wide siRNA screen for regulators of RIPK1-dependent cell death. CYLD and BIRC2 are positive controls. f, Immunoblotting analysis of RIPK1 activation, apoptosis and necroptosis in hepatocytes isolated from livers of 8-m-o mice (n = 4 for Senp1<sup>t/t</sup>, n = 3 for Senp1<sup>t/t</sup>; Alb-Cre and Senp1<sup>t/t</sup>; Alb-Cre; Ripk1<sup>D138N/D138N</sup>). Lysate from MEFs treated with T(10 ng/ml)/CHX(C, 2 µg/ml)/zVAD(Z, 10 µM) for 4 hrs were used as a positive control for p-RIPK3(T231/S232) signal. \*: non-specific band. g, h, Representative images of histological analysis and IHC of CD45 of liver sections (g), and quantitative RT-PCR analysis of the mRNA expression of cytokines and chemokines in livers (h) from 3-m-o mice (n = 6). i-k, Heatmap (i) and GO analysis (**j**, **k**) of genes differentially expressed in livers from 3-m-o mice (n = 3). **I**, Quantitative RT-PCR analysis of the mRNA expression of Tnf and Cxcl1 from hepatocytes isolated from 8-m-o mice (n = 5). **m**, Oil Red O staining of lipids in livers from 3-m-o mice (n = 6). **n**, Relative intracellular TG content in hepatocytes isolated from 8-m-o mice (n = 5). **o**, Representative images of Masson's trichrome stained liver sections from 3-m-o mice. The quantification is shown at right (n = 5). Data are presented as mean ± s.e.m. (**b**, **h**, **l**, **n**, **o**). One-way ANOVA, post hoc Dunnett's test (**l**, n). Unpaired two-tailed t-test (h, o). Source data are provided in Source Data file.



## Supplementary Figure 2. The deSUMOylation activity of SENP1 is required for suppressing RIPK1 activation and liver damage.

**a**, Representative fluorescent microscopy images out of n = 3 livers two months after injections with pAAV-CBh-EGFP-P2A-SENP1-3xFLAG-tWPA or AAV-CBh-EGFP-P2A-SENP1(C599S)-3xFLAG-tWPA. The construct expresses EGFP, which allows for verification of the site and efficacy of infection. **b**, **c**, p-S166 RIPK1 (**b**) and CC3 (**c**) staining of liver sections from *Senp1<sup>t/t</sup>;Alb-Cre* control mice, *Senp1<sup>t/t</sup>;Alb-Cre;*AAV-*Senp1*<sup>WT</sup> (SENP1-WT) and *Senp1<sup>t/t</sup>;Alb-Cre;*AAV-*Senp1*<sup>C599S</sup> (SENP1-C599S) mice. Graph depicting numbers of p-S166 RIPK1<sup>+</sup> staining (**b**) and numbers of CC3<sup>+</sup> cells (**c**) on liver sections of indicated genotypes, respectively. Each dot represents an individual mouse. Mean ± s.e.m. of n = 5 mice for each genotype. One-way ANOVA, post hoc Dunnett's test. **d**, Quantitative RT-PCR analysis of the mRNA expression of cytokines and chemokines in livers of mice with indicated genotypes (n = 5 mice for each genotype). Each dot represents an individual mouse. Mean ± s.e.m. One-way ANOVA, post hoc Dunnett's test. **e**, Oil Red O staining of lipids in liver sections from mice of indicated genotypes. Representative images out of n = 5 mice for each genotype are represented. Graph depicting percentage of lipid content on liver sections of indicated genotypes. Each dot represents an individual mouse. Dunnett's test. Source data are provided in Source Data file.



## Supplementary Figure 3. Knockdown of SENP1 promotes RIPK1-dependent apoptosis in response to TNF $\alpha$ .

a, MEFs were transfected with siRNAs targeting SENP1, or control (NC), for 48 hrs. The cells were then pre-treated with 5z7 (100 nM) in the presence or absence of Nec-1s (10 µM) for 0.5 hr followed by TNFα (1 ng/ml) for 12 hrs. Cell survival was measured by CellTiter-Glo assay. One-way ANOVA, post hoc Dunnett's test. Knockdown efficiency is shown by immunoblotting with SENP1. b, MEFs stably expressing the indicated shRNA were pre-treated with 100 nM 5z7 for 0.5 hr followed by 1 ng/ml TNFα for indicated time. Cell death was measured as a function of time by SytoxGreen positivity assay. c, d, shNC (hereafter referred to Senp1-WT) and shSenp1-2 (hereafter referred to Senp1-KD) MEFs were treated with 100 nM 5z7 in the presence or absence of Nec-1s (10 µM) for 0.5 hr followed by 1 ng/ml TNF $\alpha$  for indicated time. Cell death was measured as a function of time by SytoxGreen positivity assay (c). The levels of p-S166 RIPK1 and cleaved caspase-3 (CC3) were determined by immunoblotting (d). e, MEFs stably expressing the indicated shRNA were stimulated with 10 ng/ml TNF $\alpha$  for indicated periods of time and the whole-cell lysates were immunoblotted as indicated. Quantification of relative p-IkBa and p-JNK levels that normalized to Tubulin levels was shown on the right. Mean  $\pm$  s.e.m. of n = 3 biologically independent experiments. f, MEFs stably expressing the indicated shRNA were treated with different concentrations of TNFα for 24 hrs. Cell death was measured by SytoxGreen positivity assay. g, MEFs stably expressing the indicated shRNA were treated with TNF $\alpha$  (10 ng/ml) in the presence or absence of Nec-1s (10  $\mu$ M) for 24 hrs. Representative images of SytoxGreen staining out of three independent experiments are represented. Graph depicting percentage of dead cells. Cell counting was performed manually using ImageJ. Data are presented as mean ± s.d. of three (a, f, g) and six (b, c) independent samples of one representative experiment out of n = 3 independent experiments. Source data are provided in Source Data file.



Supplementary Figure 4. Knockdown of SENP1 promotes RIPK1-dependent apoptosis and necroptosis.

a, b, SENP1 knockdown sensitized MEFs to T/C-induced cell death and converts RIA to RDA. Senp1-WT and Senp1-KD MEFs were treated with 2 µg/ml cycloheximide (CHX, C) in the presence or absence of Nec-1s (10 μM) for 0.5 hr followed by 10 ng/ml TNFα for indicated time. Cell death was measured as a function of time by SytoxGreen positivity assay, data are represented as mean ± s.d. of six independent samples of one representative experiment out of n = 3 independent experiments (a). The levels of p-S166 RIPK1 and CC3 were determined by immunoblotting, similar results were obtained from n = 3 independent experiments (b). c, d, SENP1 knockdown sensitized MEFs to T/C/Z-induced necroptosis. Senp1-WT and Senp1-KD MEFs were treated with CHX (2  $\mu$ g/ml) and zVAD.fmk (Z, 10  $\mu$ M) in the presence or absence of Nec-1s (10  $\mu$ M) for 0.5 hr followed by 1 ng/ml TNFα for indicated time. Cell death was measured as a function of time by SytoxGreen positivity assay, data are represented as mean ± s.d. of six independent samples of one representative experiment out of n = 3 independent experiments (c). The levels of p-S166 RIPK1, p-T231/S232 RIPK3 and p-S345 MLKL were determined by immunoblotting, similar results were obtained from n = 3 independent experiments (d). e, Senp1-WT and Senp1-KD MEFs were pretreated with CHX (C, 2 μg/ml) and zVAD.fmk (Z, 10 μM) for 0.5 hr followed by 1 ng/ml TNFα for indicated time. The necrosome was isolated by immunoprecipitation of RIPK3, and RIPK1 binding was revealed by immunoblotting. Similar results were obtained from n = 3 independent experiments. Source data are provided in Source Data file.



#### Supplementary Figure 5. SENP1 suppresses RIPK1 activation during development.

a, RIPK1 is hyperactivated in Senp1<sup>-/-</sup> fetal liver. Sections from E14.5 embryos of indicated genotype were immunostained for p-S166 RIPK1 and DAPI for nuclei. Representative images out of n = 8embryos (for each genotype) are represented. b-d, Heatmap (b) and GO analysis (c, d) of genes differentially expressed (fold change > 1.5, P-value < 0.05) in E14.5 fetal livers of mice of indicated genotypes. n = 3 embryos for each genotype. e. Venn diagram of genes upregulated in Senp1<sup>-/-</sup> fetal livers (vs Senp1<sup>+/+</sup>, fold change > 1.5, P-value < 0.05) and genes downregulated in Senp1<sup>-/-</sup> ;Ripk1<sup>D138N/D138N</sup> fetal livers (vs Senp1<sup>-/-</sup>, fold change > 1.5, P-value < 0.05). f, Numbers of offspring from intercrossing Senp1<sup>+/-</sup>; Ripk1<sup>D138N/D138N</sup> parents. **g**, **h**, RIPK1-D138N mutation does not alter the downregulation of genes that are involved in blood vessel development in Senp1<sup>-/-</sup> embryos. Heatmap (g) and GO analysis (h) of genes that are involved in blood vessel development, which are not rescued by RIPK1-D138N knockin mutation in Senp1<sup>-/-</sup> fetal livers. i, Immunohistochemistry and guantification of EPO in E14.5 fetal liver of indicated genotypes. Representative images out of n = 5embryos (for each genotype) are represented. Graph depicting percentage of EPO expression area on fetal liver sections of indicated genotypes. Each dot represents an individual mouse. Mean ± s.e.m. One-way ANOVA, post hoc Dunnett's test. j, Quantitative RT-PCR analysis of the mRNA expression of Epo and Epor (n = 3 embryos for each genotype). Each dot represents an individual mouse. Mean ± s.e.m. One-way ANOVA, post hoc Dunnett's test. Source data are provided in Source Data file.



#### Supplementary Figure 6. SENP1 interacts with RIPK1 and suppresses its SUMOylation.

a, Schematic of mass spectrometry analysis for identification of SUMOylation targets in response to TNFα stimulation. Created with BioRender.com. b, HEK293T cells were transfected with Flag-RIPK1, and then immunoprecipitated using anti-Flag resin followed by incubation with recombinant catalytic domain (CD) of USP2 as indicated at 37 °C for 1 hr. The samples were analyzed by immunoblotting with RIPK1. c, d, HEK293T cells were co-transfected with expression vectors of Flag-SENP1 and Myc-RIPK1 (c), Flag-SENP1 and HA-RIPK1 (d) or Flag-SENP1 and HA-A20 (d) as indicated for 24 hrs. The interaction between SENP1 and RIPK1 or SENP1 and A20 was revealed by immunoprecipitation-immunoblotting assay as indicated. e, HEK293T cells were co-transfected with expression vectors of Flag-SENP1 and Myc-tagged full-length RIPK1, or truncation mutants of RIPK1 as indicated for 24 hrs. The cell lysates were immunoprecipitated with anti-Flag conjugated beads. The interaction of SENP1 and RIPK1 truncation mutants was revealed by immunoblotting. KD: Kinase domain; ID: Intermediate domain; DD: Death domain. f, HEK293T cells were cotransfected with expression vectors of Flag-RIPK1 and Myc-tagged full-length SENP1 or truncation mutants of SENP1 as indicated for 24 hrs. The cell lysates were immunoprecipitated with anti-Myc conjugated beads. The interaction of RIPK1 and SENP1 truncation mutants was revealed by immunoblotting. NTD: N-terminal domain; CD: catalytic domain. g, HEK293T cells were cotransfected with expression plasmids of Flag-RIPK1, SUMOylaiton system (His-SUMO3 + Ubc9) and wild-type SENP1 (SENP1-WT) or its catalytic inactive mutant (SENP1-C603S) as indicated for 24 hr. The whole-cell lysate was subjected to pull-down with Ni-NTA agaroses, and SUMOylated RIPK1 was revealed by immunoblotting with anti-RIPK1 antibody. h. Representative western blot analysis of RIPK1 SUMOylation in the livers of individuals with non-steatosis (n = 3) or NASH (n = 3). i, Representative western blot analysis of RIPK1 SUMOylation in the livers of mice fed a HFD for the indicated number of weeks (W). n = 3 mice per group. Similar results were obtained from n = 3independent experiments (b-g). Source data are provided in Source Data file.

Ubiquitin	RLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG76
SUMO1	RFLFEGQRIADNHTPKELGMEEEDVIEVYQEQTGG97
SUMO2	RFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG93
SUMO2	RFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG93
SUMO3	RFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGG92
SUMO3(T90R)	REREDGOPINETDIPAQLEMEDEDITDVFQQQIGG92

L		
-	-	

Lysine (K)	)	Intensity		Ratio (C/B)
sites	A	В	С	
550	0	331650000	78313000	0.24
132	0	212820000	20072000	0.09
185	0	66148000	10164000	0.15
226	0	38340000	15052000	0.39
167	0	16917000	1791100	0.11
205	0	12923000	4622600	0.36
11	0	4393700	0	0
619	0	3767500	1034300	0.27

f









b

а

#### Supplementary Figure 7. Lys550 is the main SUMOylation site of RIPK1.

**a**, Sequence alignment of the C-terminal amino acid sequences of ubiquitin, SUMO1, SUMO2, SUMO3 and SUMO3-T90R mutant with the artificial introduction of a tryptic cleavage site. **b**, Schematic of mass spectrometry analysis for identification of SUMOylation site(s) of RIPK1. Details are described in Methods. **c**, RIPK1 SUMOylation sites identified by mass spectrometry. **d**, K550A mutation reduces the SUMOylation levels of RIPK1. HEK293T cells were co-transfected with the SUMOylation system (His-SUMO3 and Ubc9) and wild-type RIPK1 (RIPK1-WT) or its mutants as indicated for 24 hrs. The whole-cell lysate was subjected to pull-down with Ni-NTA agaroses, SUMOylated RIPK1 was revealed by immunoblotting with anti-RIPK1 antibody. Similar results were obtained from n = 3 independent experiments. **e**, Immunoblot analysis of RIPK1 and SENP1 expression in reconstituted lines. Similar results were obtained from n = 3 independent experiments. **f**, Quantification of indicated protein in TNF-RSC from Fig. 6d, data are represented as mean  $\pm$  s.e.m of n = 3 independent experiments. Source data are provided in Source Data file.



#### Supplementary Figure 8. PIAS1 promotes RIPK1 SUMOylation in SENP1-deficient cells.

a, HEK293T cells were co-transfected with expression vectors of Flag-RIPK1 and Myc-PIAS1 as indicated. The interaction between PIAS1 and RIPK1 was revealed by immunoprecipitationimmunoblotting assay. b, HEK293T cells were co-transfected with expression vectors of Myc-PIAS1 and Flag-tagged full-length RIPK1, or truncation mutants of RIPK1 as indicated for 24 hrs. The cell lysates were immunoprecipitated with anti-Flag conjugated beads. The interaction of PIAS1 and RIPK1 truncation mutants was revealed by immunoblotting. c, HEK293T cells were co-transfected with the indicated plasmids for 24 hrs. The whole-cell lysate was subjected to pull-down with Ni-NTA agaroses, RIPK1 SUMOvlation was revealed by immunoblotting with anti-RIPK1 antibody. d. Immunoblot analysis of SENP1 and PIAS1 expression in SENP1 and PIAS1 double knockout cells. e, MEFs were stimulated by Flag-TNFα (100 ng/ml) for indicated time. RIPK1 SUMOylation was revealed by tandem immunoprecipitation of TNF-RSC and immunoblotting with anti-RIPK1 antibody. f, MEFs were stimulated with Flag-TNF $\alpha$  (100 ng/ml) for the indicated time. The recruitment of PIAS1 into TNF-RSC was analyzed by immunoblotting. g, h, PIAS1-WT and PIAS1-KD (g) or SENP1-WT and SENP1-KD (h) MEFs were stimulated with Flag-TNF $\alpha$  (100 ng/ml) for the indicated times. The recruitment of PIAS1 and SENP1 in TNF-RSC was immunoprecipitated via α-Flag beads and analysed by western blots. i, j, MEFs were treated with 10 ng/mL TNFα in the presence or absence of Nec-1s (10 µM) for 24 hrs. Cell death was measured as a function of time by SytoxGreen positivity assay. Data are presented as mean ± s.d. of three independent samples of one representative experiment out of n = 3 independent experiments (i). The levels of p-S166 RIPK1 and CC3 were determined by immunoblotting (j). Similar results were obtained from n = 3 independent experiments (**a-h**, **j**). Source data are provided in Source Data file.



### Supplementary Figure 9. SENP1 deficiency exaggerates LPS-induced acute liver injury in a RIPK1-dependent manner.

**a**, Serum levels of ALT and AST of 2-m-o mice with indicated genotypes (n = 5 mice for each genotype). b, Plasma samples were harvested at 4 hrs after LPS (50 ng/g) injection for the measurement ALT and AST in 2-m-o mice with indicated genotypes (n=5 for each genotype). c, HE staining of liver sections of mice from **b**. Representative images out of n = 5 mice for each genotype are represented. d, TUNEL assay and p-S166 RIPK1 staining of liver sections from mice treated with LPS (n = 5 for each genotype). Graph depicting numbers of TUNEL<sup>+</sup> cells and percentage of p-S166 RIPK1<sup>+</sup> staining on liver sections of indicated genotypes, respectively. e. Quantitative RT-PCR analysis of the mRNA expression of cytokines and chemokines in livers of mice from b (n = 5 mice for each genotype). f, Representative western blot analysis of RIPK1 SUMOylation levels in primary hepatocytes derived from 2-m-o mice with indicated genotypes treated with 0.4 mM PA for 24 hrs. Similar results were obtained from n = 3 independent experiments. **g**, **h**, SENP1 deficiency sensitized primary hepatocytes to cell death induced by PA. Primary hepatocytes derived from 2-m-o mice with indicated genotypes were treated with 0.4 mM PA for 24 hrs. Cell death was measured by SytoxGreen positivity assay, data are represented as mean  $\pm$  s.d. of five independent samples of one representative experiment out of n = 3 independent experiments (g). The levels of p-S166 RIPK1 and CC3 were determined by immunoblotting, similar results were obtained from n = 3independent experiments (h). i, Liver lipid content, including TG, TC content per gram of liver from 6m-o mice with indicated genotypes fed with or without HFD for 4 months (n = 5 mice for each genotype). Data are presented as mean ± s.e.m. (a, b, d, e, i). One-way ANOVA, post hoc Dunnett's test (a, b, d, e, g, i). Source data are provided in Source Data file.