Rho-associated protein kinase (ROCK) 1 inhibition in hepatocytes attenuates nonalcoholic steatohepatitis

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**Supplemental Figure 1. Hepatocyte-specific ROCK1 deletion does not affect metabolic phenotype in FFC-fed mice**

(A) Hepatocytes were isolated from ROCK1^f/f^ (n=3) and ROCK1^Δhep^ (n=3) mice and western blot was performed for ROCK1 and GAPDH (a loading control) to confirm the efficiency of ROCK1 Cre-mediated deletion; (B) At 16 weeks of feeding, fasting glucose was measured from tail blood and fasting insulin was assessed by ELISA; (C) After 18 weeks of feeding, body composition was measured by Echo-MRI (D-E) and mice were placed into automatic Comprehensive Lab Monitoring System (CLAMS) to measure food intake, metabolic rate, physical activity; (F) Mice were weighed every other week to monitor weight changes; (G) Epidydimal white adipose tissue (eWAT) weight normalized to body weight; (H) Whole liver lysate from chow ROCK1^f/f^ (n=4), chow ROCK1^Δhep^ (n=5), FFC ROCK1^f/f^ (n=7), FFC ROCK1^Δhep^ (n=8) were isolated and western blot was performed for ROCK1 and HSP90 (as a loading control). ROCK1 levels were normalized to HSP90 for quantification. N of samples indicated in the graphs. ***p < 0.001, **p < 0.01, *p < 0.05, ns, non-significant.
Supplemental Figure 2. Changes in liver immune cell populations in FFC-fed hepatocyte-specific ROCK1 knockout mice.

Liver leukocytes were isolated from chow-fed ROCK1\textsuperscript{fl}, FFC-fed ROCK1\textsuperscript{fl}, and FFC-fed ROCK1\textsuperscript{hep} mice and subjected to mass cytometry, CyTOF. (A) Twenty-nine unique clusters were identified by a 30-marker panel using an R-phenograph clustering algorithm and visualized on a tSNE plot indicating relative abundance. Red indicates high frequency of cells; blue indicates low frequency of cells. (B) Cell frequencies and relative intensity of protein markers for each cluster of liver leukocytes. N of samples indicated in the graphs. ***p < 0.001, **p < 0.01, *p < 0.05, ns, non-significant.
Supplemental Figure 3. ROCK1 deletion prevents lipotoxicity-induced release of extracellular vesicles from hepatocytes.

Hepatocytes were isolated from \( \text{ROCK}^{1\text{ff}} \) (n=5) and \( \text{ROCK}^{1\text{Δhep}} \) (n=5) mice and treated with lipotoxic lipid lysophosphatidylcholine (LPC) or vehicle (Veh) for 4 hours. Extracellular vesicles (EVs) were isolated from cell culture supernatants by differential ultracentrifugation and subjected to the following analyses: (A) concentration quantification using nanoparticle tracking analysis (data are expressed as fold change over vehicle treatment of \( \text{ROCK}^{1\text{ff}} \) hepatocytes), and (B) Western blot for CXCL10 and TSG101 (established EV marker) using equal EV protein loading. \(*p < 0.01\)
Supplemental Figure 4. Hepatocyte-specific ROCK1 deletion attenuates inflammation and fibrosis during NASH

Whole liver RNA was isolated, and qPCR was performed for Collagen 6a1 and TGFβ. N of samples indicated in the graphs. **p < 0.01, *p < 0.05, ns, non-significant.
Supplemental Figure 5. Myeloid cell-specific ROCK1 deletion does not affect on metabolic phenotype and liver inflammation in NASH

(A) Bone marrow cells were isolated from ROCK1^{f/f} (n=3) and ROCK1^{Δmye} (n=3) and differentiated into macrophages (BMDM). Western blot was performed for ROCK1 and GAPDH was used as a loading control. ROCK1 levels were normalized to GAPDH; (B) At 16 weeks of feeding, fasting glucose and fasting insulin were...
measured; (C) At 18 weeks of feeding, body composition was assessed by Echo-MRI; (E) After 18 weeks of feeding, mice were placed into automatic Comprehensive Lab Monitoring System (CLAMS) to measure food intake, adjusted food intake, energy expenditure, metabolic rate, and physical activity; (F) Epididymal white adipose tissue (eWAT) weight normalized to body weight at the time of sacrifice; (G-J) Liver leukocytes were isolated from chow-fed ROCK1\textsuperscript{f/f}, FFC-fed ROCK1\textsuperscript{f/f} and FFC-fed ROCK1\textsuperscript{amy} mice and CyTOF was performed. (G) Twenty-seven unique clusters were identified by a 30-marker panel using an R-phenograph clustering algorithm and visualized on a tSNE plot; (H) The heatmap demonstrates the distribution and relative intensity of markers; (I) The heatmap shows the relative abundance of each cluster for each mouse; (J) Relative abundance of each cluster for each group of mice visualized by tSNE plot. Red indicates high frequency of cells; blue indicates low frequency of cells. N of samples indicated in the graphs. ***p < 0.001, *p < 0.05, ns, non-significant.
Supplemental Figure 6. ROCKi properties and effect on metabolic parameters in FFC-fed mice

(A) The chemical structure of ROCKi; (B) The pharmacokinetic properties of ROCKi. (C) A single oral dose of ROCKi (100 mg/kg of body weight, in 0.4% carboxymethyl cellulose) was administrated to male C57Bl/6J mice, after which pharmacokinetic parameters were determined; (D) Huh7 cells (hepatocyte cell line) were treated with lysophosphatidylcholine (LPC) in the presence or absence of 1 µM ROCKi. MYPT1, a substrate for ROCKs, and its phosphorylated form, pMYPT1, were assessed by western blot; (E-H) C57Bl/6J mice were fed chow or high fat, high fructose, and high cholesterol (FFC) diet for 24 weeks. Then they received ROCK inhibitor (ROCKi) or vehicle by daily oral gavage for additional 3 weeks while continuing the diets. (E) Daily body weight monitoring during ROCKi treatment; (F) After 12 days of treatment, fasting glucose and fasting insulin were measured; (G) Food intake during the ROCKi treatment. (H) Liver weight and epididymal white adipose tissue (eWAT) weight normalized to body weight at the time of sacrifice. (I) Transwell migration assay using mouse monocytes.
Monocytes were treated with ROCKi (1 µM or 10 µM), CCR2/5 antagonist cenicriviroc (1 µM) or vehicle (DMSO) for 30 minutes before allowing to migrate towards CCL2 (100 ng/mL) for 2 hours. N of samples indicated in the graphs. ****p < 0.001, **p < 0.01, *p < 0.05, ns, non-significant.