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Supplemental Information

ROCK inhibition enhanced hepatocyte liver engraftment by retaining membrane CD59 and attenuating complement activation

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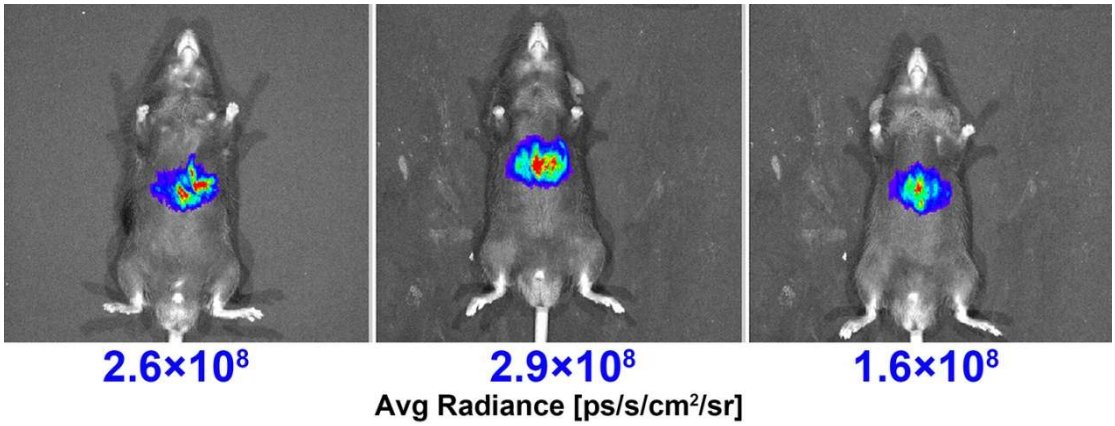

Figure S1. Representative bioluminescence images of mice transduced with replication-incompetent AAV2/8-CAG-EGFP-T2A-lucifrase.

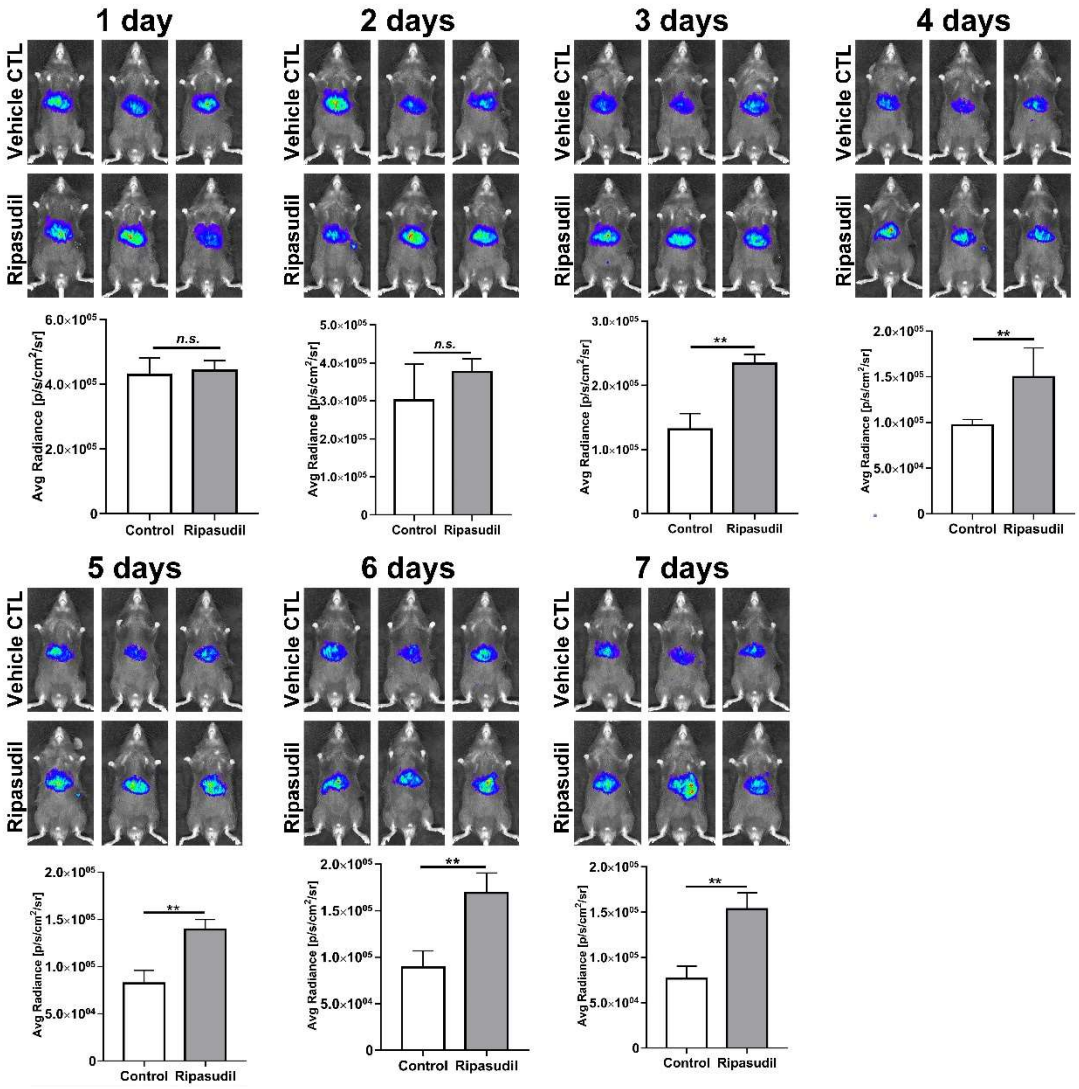

Figure S2. The daily time course of flux values of transplanted hepatocytes with ripasudil treatment for 7 days. Data are shown as the means ± SEMs (n=3). n.s, no significance. ** P<0.01.

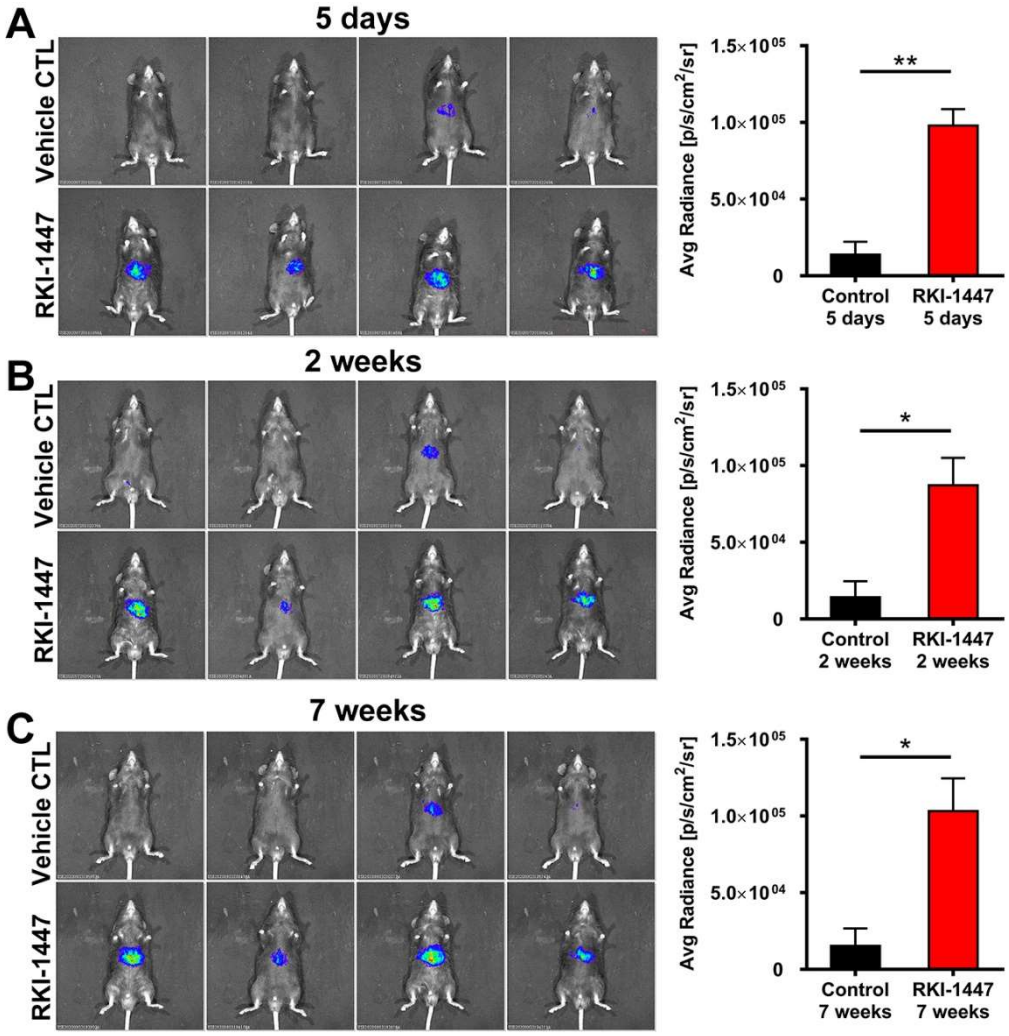

Figure S3. RKI-1447, another ROCK inhibitor with a different chemical scaffold from that of ripasudil, enhances hepatocyte liver engraftment. RKI-1447 (ROCK inhibitor, 80 mg/kg) was administered once intraperitoneally soon after cell transplantation. The mice were analyzed at 5 days, 2 weeks, and 7 weeks after transplantation. Data are shown as the means ± SEMs (n=4). *p < 0.05. ** P<0.01.

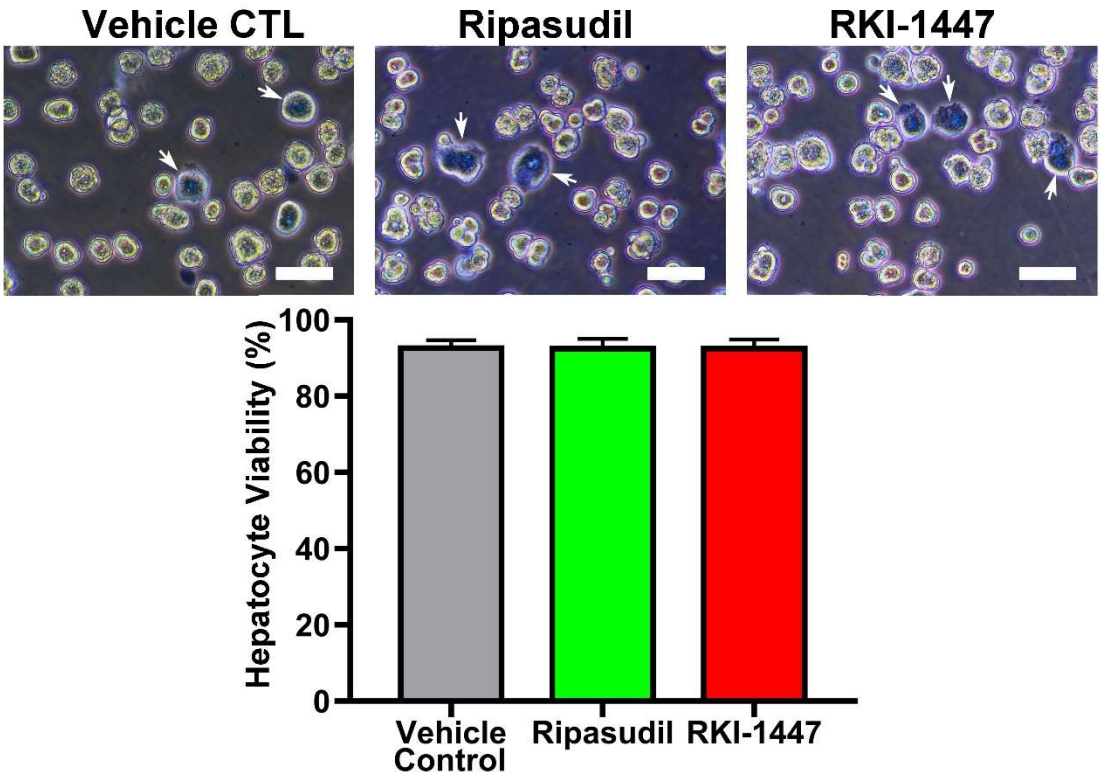

Figure S4. The viability of hepatocytes isolated with perfusion and digestion buffer supplemented with 4 μM ripasudil or RKI-1447. Arrow indicated the cells stained with trypan blue. The scale bars represent 50 μM. Data are expressed as the means ± SEMs of four independent hepatocyte isolation experiments.

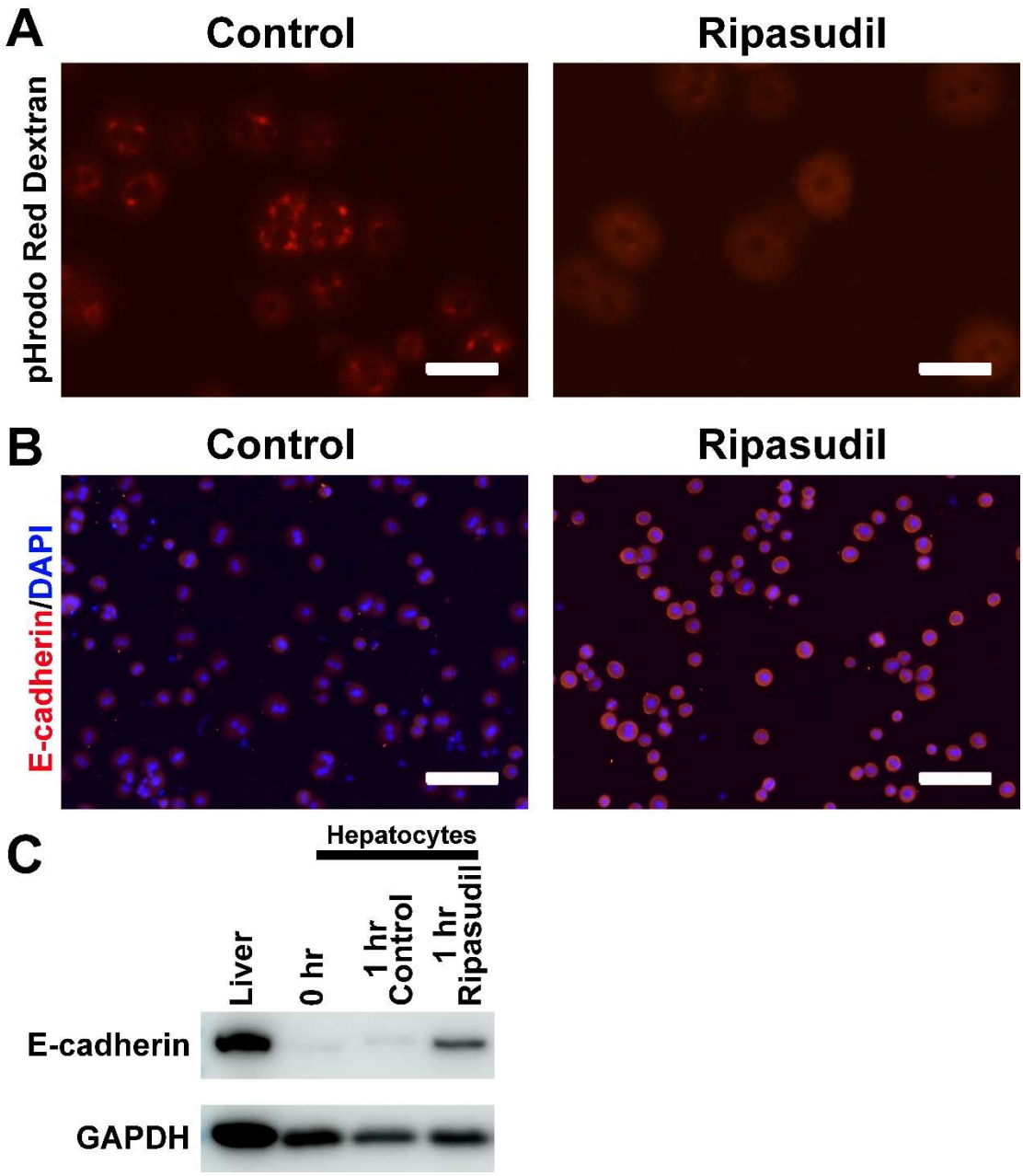

Figure S5. Ripasudil inhibited hepatocyte endocytosis and retained cell membrane E-cadherin. (A) Endocytosis of isolated hepatocytes was demonstrated by pHrodo Red dextran staining. (B) The expression of E-cadherin in isolated hepatocytes was analyzed by immunostaining after treatment with ripasudil or vehicle control. (C) The expression of E-cadherin in the liver and isolated hepatocytes was analyzed by western blotting. The scale bars represent 50 μM in Panel A and 100 μM in Panel B.

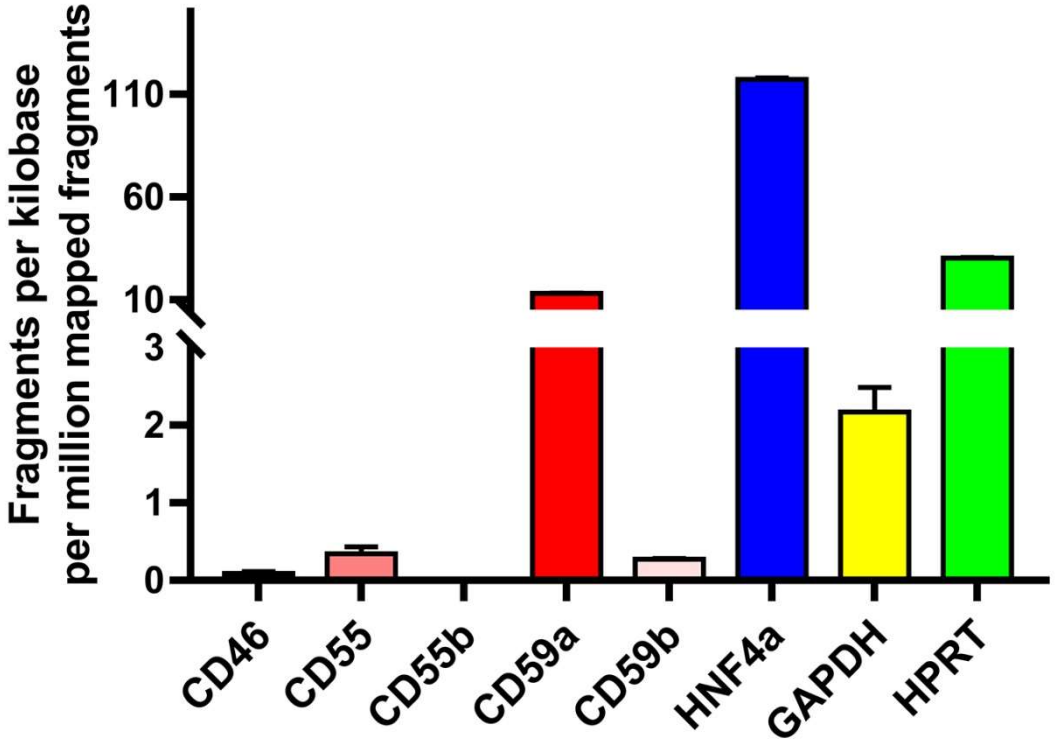

Figure S6. The RNA-seq data (GSE138158) showed that freshly isolated hepatocytes expressed the complement inhibitor CD59a. GSE138158 contains 2 biological repeats of RNA-seq data for freshly isolated hepatocytes.

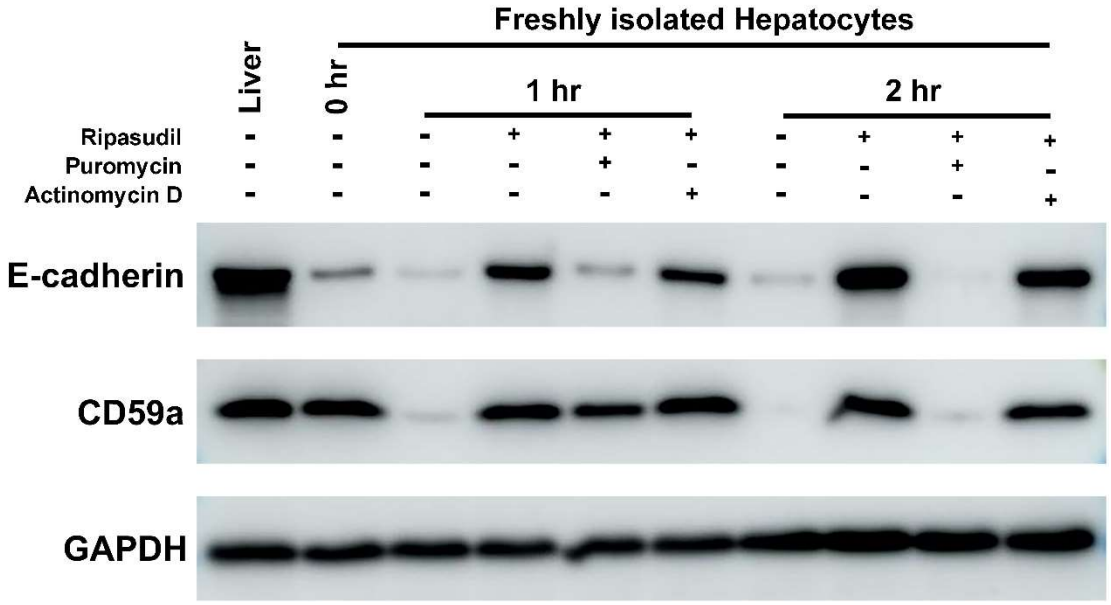

Figure S7. Ripasudil maintains hepatocyte membrane protein homeostasis dependent on protein neosynthesis. The expression of E-cadherin and CD59a in isolated hepatocytes was analyzed by western blotting after treatment with ripasudil, puromycin and actinomycin D.

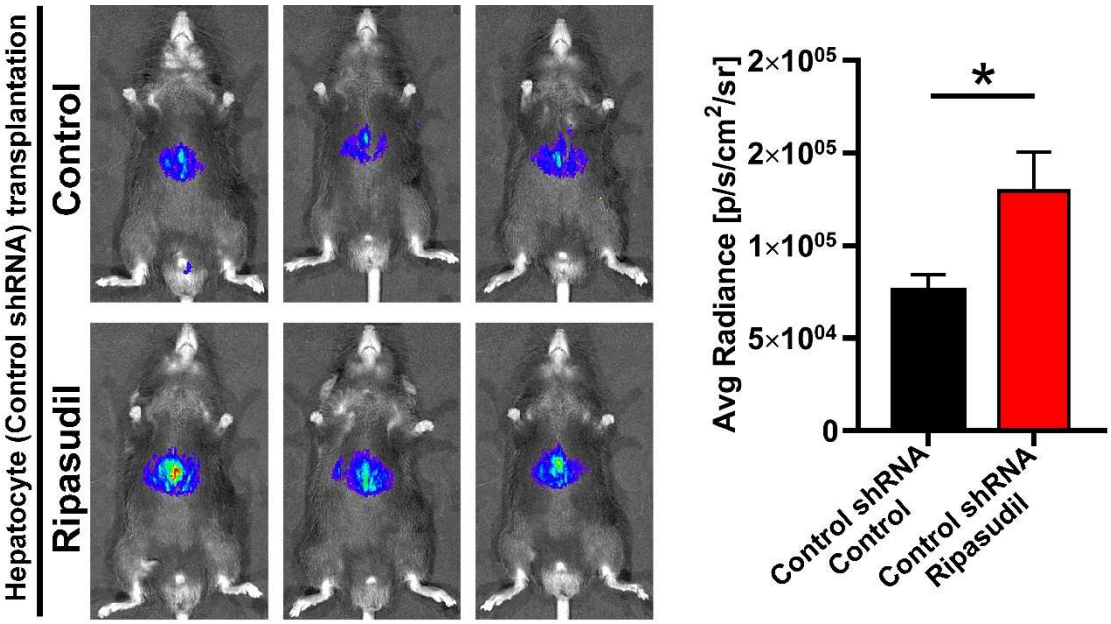

Figure S8. The liver engraftment of hepatocytes expressing control shRNA was analyzed by bioluminescence imaging 3 days after cell transplantation. Data are shown as the means ± SEMs of three independent experiments. *p < 0.05.

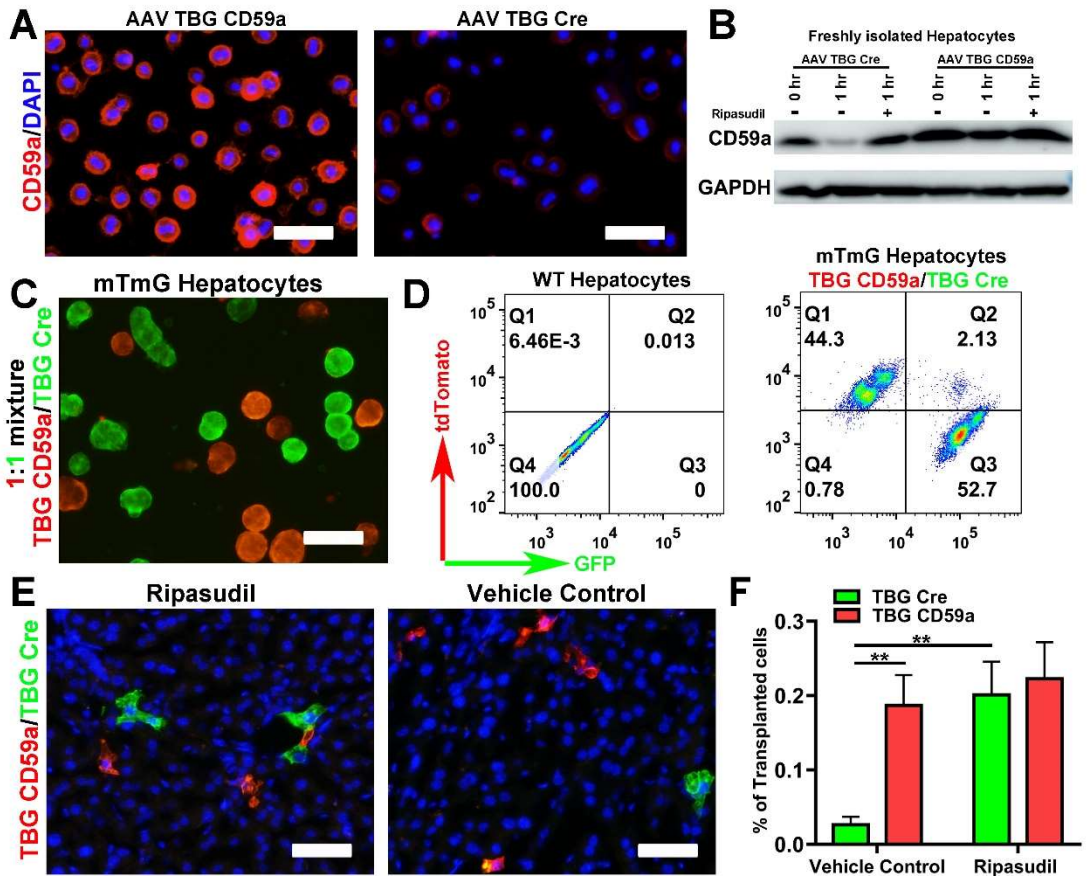

Figure S9. Overexpression of CD59a enhanced hepatocyte liver engraftment. (A) The expression of CD59a in isolated hepatocytes transduced with pAAV-TBG-Cd59a-WPRE or pAAV-TBG-Cre-WPRE was analyzed by immunostaining. (B) CD59a expression by hepatocytes transduced with pAAV-TBG-Cd59a-WPRE or pAAV-TBG-Cre-WPRE was analyzed by western blot. (C and D) Fluorescence images and flow cytometry analysis of 1:1 pooled mTmG hepatocytes transduced with pAAV-TBG-Cd59a-WPRE (red hepatocytes) and pAAV-TBG-Cre-WPRE (green hepatocytes). (E) Fluorescence images of liver sections transplanted with pooled mTmG hepatocytes transduced with pAAV-TBG-Cd59a-WPRE (red hepatocytes) and pAAV-TBG-Cre-WPRE (green hepatocytes). The mice were analyzed at 7 days after transplantation. (F) Quantitative analysis of Panel E. The scale bars represent 50 μM. Data are shown as the means ± SEMs of three

independent experiments. **p < 0.01.

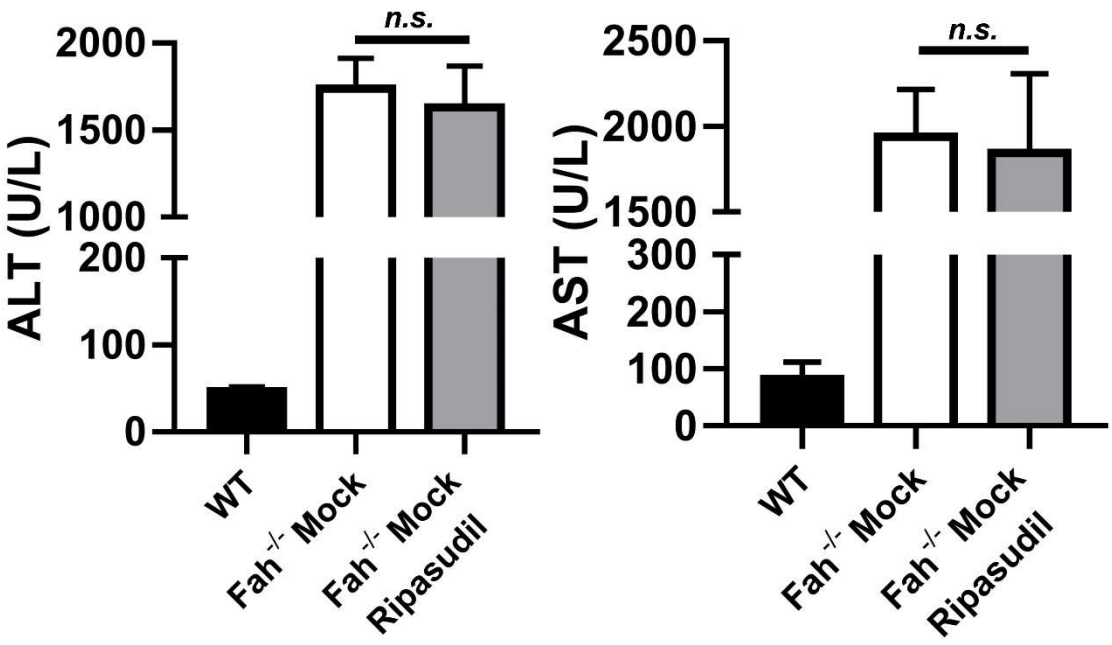

Figure S10. Ripasudil treatment has no impact on liver function in *Fah*$^{-/-}$ mice. Serum analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in *Fah*$^{-/-}$ mice at 4 weeks after mock transplantation under treatment with ripasudil or vehicle control.

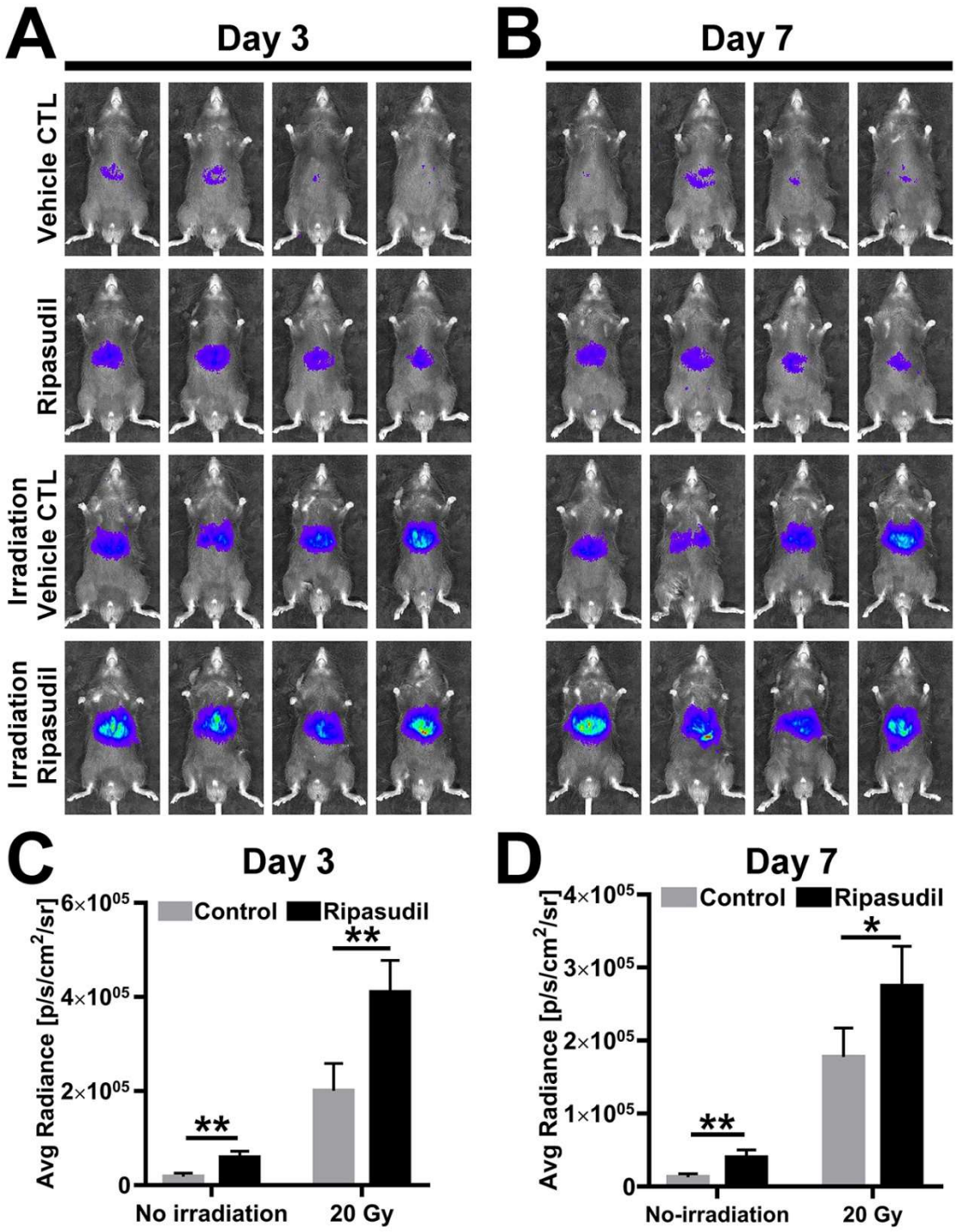

Figure S11. The engraftment of hepatocytes was analyzed by bioluminescence imaging in mice preconditioned with hepatic irradiation and treated with ripasudil. The mice were analyzed at 3 days and 7 days after transplantation. Data are shown as the means ± SEMs (n=4). *p < 0.05. ** P<0.01.

Table S1. shRNA sequences.

shRNA	Hairpin sequence (5'-3')
CD59a shRNA1	CACCGCGGTGGTTTCTTCATGCAATACTCGAGTATTGC ATGAAGAAACCACCGTTTTTT
CD59a shRNA2	CACCGTTGTCATGGTGAGATCATTATCTCGAGATAATGA TCTCACCATGACAATTTTTT
CD59a shRNA3	CACCGCCAGGATTCCTGTCTCTATTTCAAGAGAATAGAG ACAGGAATCCTGGTTTTTT
Scramble shRNA	CACCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTG ACACGTTCGGAGAATTTTTT

The target sequences are underlined.

Table S2. Antibodies used in immunocytochemistry, immunohistochemistry and western blot.

Antibodies	Source	Application	Dilution
Anti-E-Cadherin, rat monoclonal antibody	Santa Cruz, sc-59778	immunocytochemistry	200
Anti-DPP4, goat polyclonal antibody	R&D, AF954	immunohistochemistry	0.5 μg/ml
Anti-F4/80, rat monoclonal antibody	Santa Cruz, sc-52664	Immunocytochemistry/ immunohistochemistry	200
Anti-CD59a, rabbit monoclonal antibody	Sino Biological, 50724-R108	immunocytochemistry	200
Anti-C5b-9, mouse monoclonal antibody	Santa Cruz, sc-66190	Immunocytochemistry/ immunohistochemistry	200
Anti-E-Cadherin, rabbit monoclonal antibody	Cell Signaling Technology, 3195S	western blot	1000
Anti-CD59, rat monoclonal antibody	Santa Cruz, sc-59095	western blot	500
Anti-GAPDH, mouse monoclonal antibody	Proteintech, 60004-1-Ig	western blot	20000
Anti-β-Actin, HRP-conjugated monoclonal antibody	Proteintech, HRP-60008	western blot	2000

Table S3. PCR primers used in qRT–PCR experiments.

Genes	Forward (5’-3’)	Reverse (5’-3’)
Gapdh	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG
CD59a	TCAATCTGGCTGGGGATGTG	TGAGGCTAACAGCTGTGGAA