RESEARCH ARTICLE

Figures and figure supplements

Amelioration of non-alcoholic fatty liver disease by targeting adhesion G protein-coupled receptor F1 (*Adgrf1*)

Mengyao Wu *et al*.

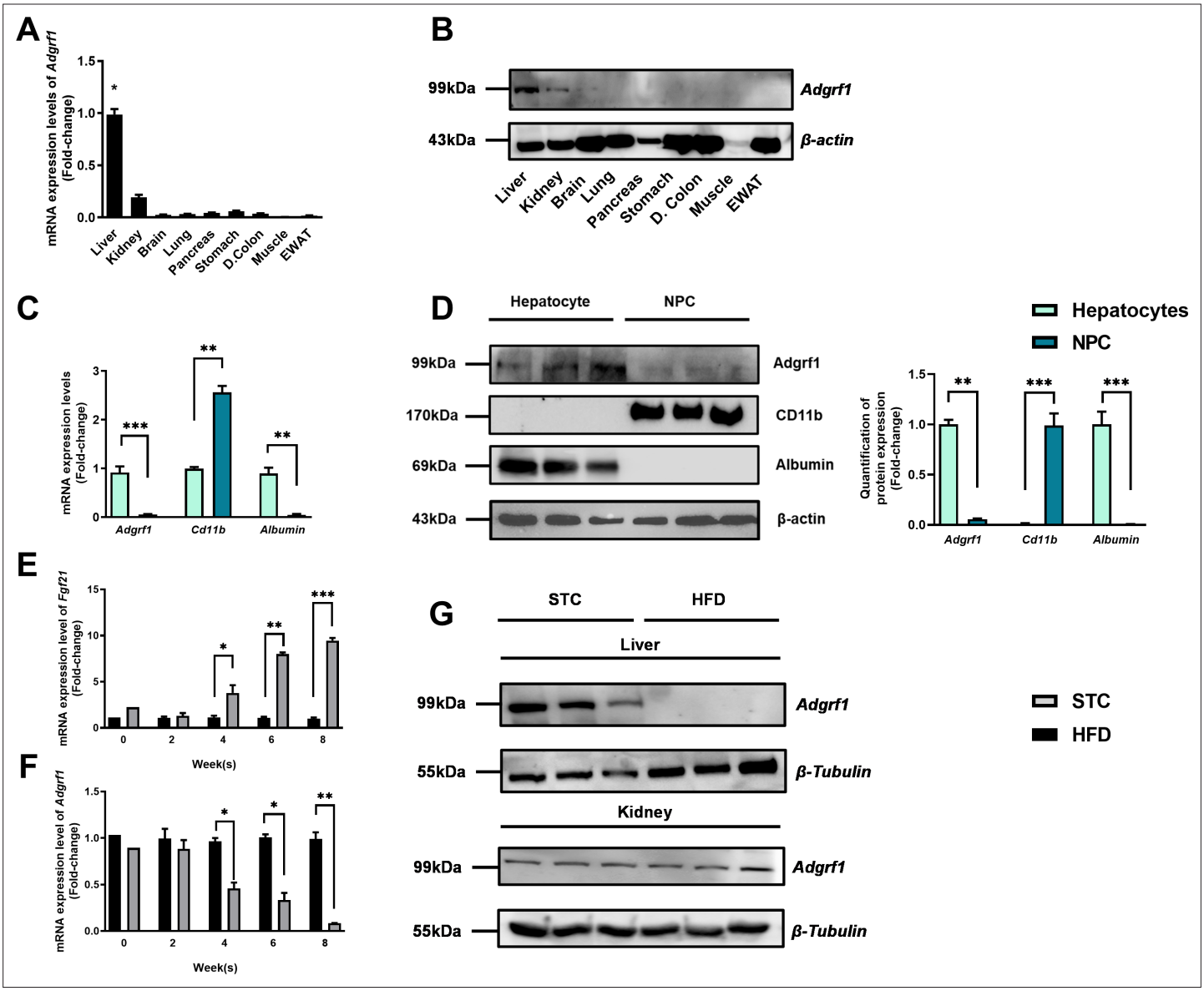

Figure 1. *Adgrf1* is mainly expressed in the liver and its expression is downregulated after HFD treatment. Eight-week-old male C57BL/6J mice were fed with either STC or HFD for 8 wk. (**A**) mRNA expression levels of *Adgrf1* in different organs as determined by RT-qPCR analysis (n = 5). (**B**) Representative immunoblotting analyses of Adgrf1 expression in different tissues of C57BL/J mice after STC for 8 wk (n = 3). (**C**) mRNA expression levels of *Adgrf1*, *Cd11b*, and albumin in factions of hepatocyte or NPCs isolated from STC-fed mice livers as determined by RT-qPCR. (**D**) Left panel: representative immunoblotting analyses of Adgrf1, Cd11b, and albumin in fractions of hepatocytes or NPC isolated from mice livers fed with STC, each lane is a sample from different individual; right panel: quantification of protein expression levels of Adgrf1, Cd11b, and albumin. Protein expression levels were normalized to the expression of *β-actin*. The fraction of hepatocytes was set as 1 for fold-change calculation. (**E**) mRNA expression levels of FGF21 in mice liver fed with 0, 2, 4, 6, and 8 wk of HFD as determined by RT-qPCR. (**F**) mRNA expression levels of Adgrf1 in mice liver fed with 0, 2, 4, 6, and 8 wk of HFD as determined by RT-qPCR. (**G**) Left panel: representative immunoblotting analyses of Adgrf1 in mice fed with either STC or HFD for 8 wk; right panel: quantification of protein expression levels of Adgrf1. Protein expression levels were normalized to the expression of β-tubulin. The sample from STC mice was set as 1 for fold-change calculation. Each lane is a sample from different individual. *Adgrf1*, G-protein-coupled receptor 110; STC, standard chow diet; HFD, high-fat diet; NPC, non-parenchymal cell. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

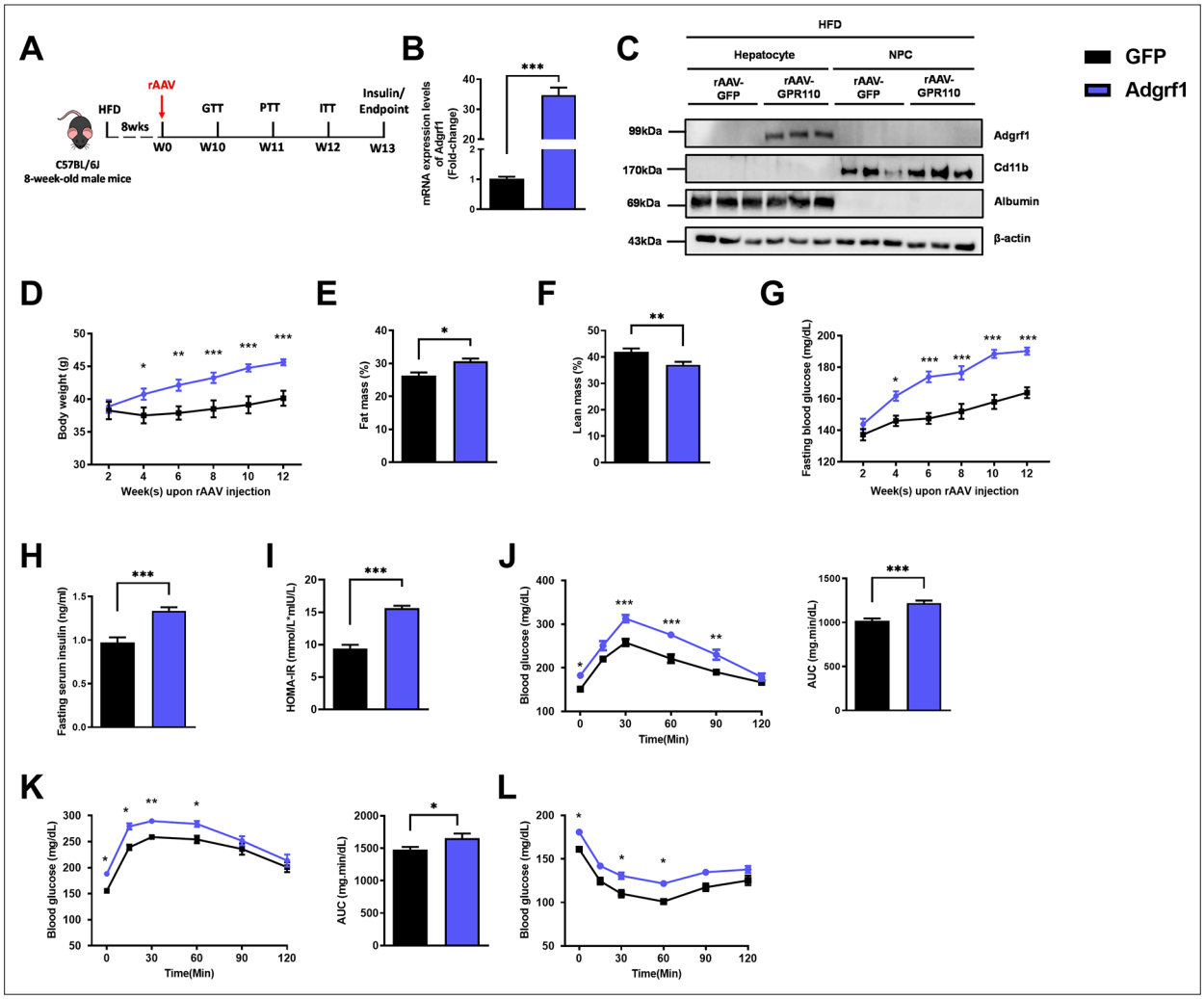

Figure 2. Overexpression of *Adgrf1* in hepatocytes exaggerates metabolic dysregulation by HFD treatment. Eight-week-old male C57BL/6J mice were infected with 3×10^{11} copies of rAAV encoding *Adgrf1* (rAAV-*Adgrf1*, i.v.) or control (rAAV-*GFP*, i.v.) and received HFD feeding, respectively. (**A**) Schematic illustration of viral treatments. (**B**) Hepatic mRNA expression levels of *Adgrf1* from rAAV-*Adgrf1* mice liver in fractions of hepatocyte or NPCs isolated from HFD-fed mice livers as determined by RT-qPCR. (**C**) Immunoblotting analyses of *Adgrf1*, *Cd11b*, and albumin from rAAV-*Adgrf1* mice liver in factions of hepatocyte or NPC isolated from HFD-fed mice livers. Each lane is a sample from a different individual. (**D**) Body weight, (**E**) the percentage of fat mass, and (**F**) lean mass were assessed in different groups. (**G**) Fasting blood glucose levels were measured biweekly upon rAAV injection. (**H**) The fasting serum insulin level and (**I**) HOMA-IR index were measured and calculated according to the formula [Fasting blood glucose (mmol/l) × Fasting blood insulin (mIU/l)]/22.5 for the HFD-fed rAAV-*Adgrf1* or rAAV-*GFP* mice at the end of the experiment. (**J**) GTT (1 g/kg BW, left) and area under curve (AUC, right) of serum glucose at week 10. (**K**) PTT (1 g/kg BW, left) and AUC (right) of serum glucose at week 11. (**L**) ITT (0.5 U/kg BW, left) and AUC (right) of serum glucose at week 12. mRNA expression levels of the target genes were normalized to the expression of mouse *Gapdh*. rAAV-NC group was set as 1 for fold-change calculation. n = 8 per group. *Adgrf1*, G-protein-coupled receptor 110; STC, standard chow diet; HFD, high-fat diet; NPC, non-parenchymal cell; BW, body weight; GTT, glucose tolerance test; PTT, pyruvate tolerance test; ITT, insulin tolerance test; AUC, area under curve; NC, negative control; HOMA-IR, homeostasis model assessment-estimated insulin resistance. Data represents as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

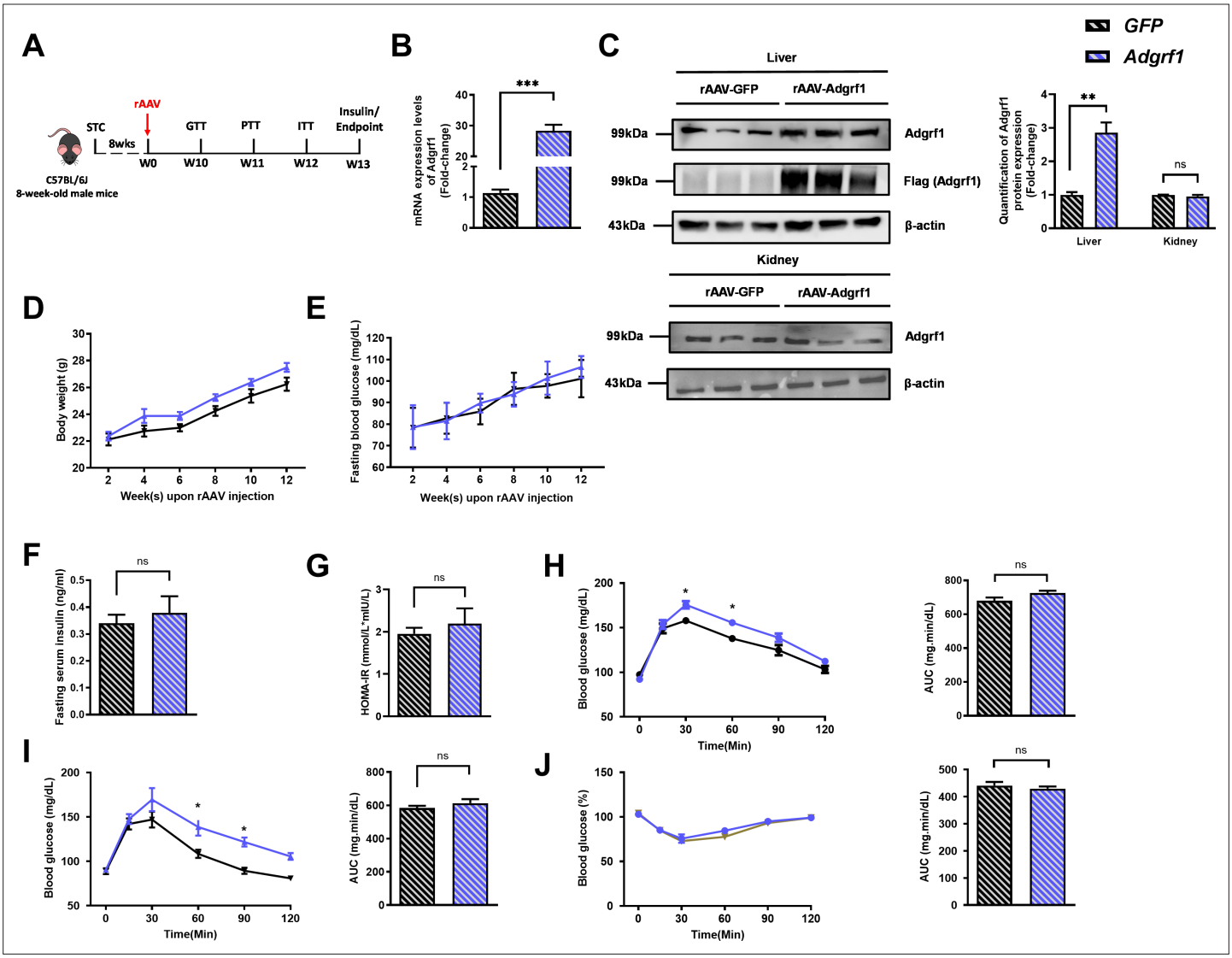

Figure 2—figure supplement 1. Hepatic overexpression of *Adgrf1* in STC-fed mice exhibits mild metabolic abnormalities. Eight-week-old male C57BL/6J mice were infected with 3×10^{11} copies of AAV encoding *Adgrf1* (rAAV-*Adgrf1*, i.v.) or control (rAAV-*GFP*, i.v.) and fed with STC diet. (**A**) Schematic illustration of viral treatments. (**B**) Hepatic mRNA expression levels of *Adgrf1* from STC-fed mice with liver-specific *Adgrf1* overexpression as determined by RT-qPCR analysis. (**C**) Left panel: immunoblotting analysis of hepatic protein expression level of Adgrf1 from STC-fed mice liver with Adgrf1 overexpression. Right panel: quantification of hepatic protein expression levels of Adgrf1. Each lane is a sample from a different individual; n = 3 per group. (**D**) Change of body weight and (**E**) fasting blood glucose at different weeks upon rAAV injection were measured. (**F**) Fasting blood insulin level and (**G**) HOMA-IR values were measured and calculated at the end the experiment. (**H**) GTT (1 g/kg BW, left) and area under curve (AUC, right) of serum glucose at the week of 10. (**I**) PTT (1 g/kg BW, left) and AUC (right) of serum glucose at week 11. (**J**) ITT (0.5 U/kg BW, left) and AUC (right) of serum glucose at week 12. mRNA expression levels of the target genes were normalized to the expression of mouse *Gapdh*. n = 8 per group. STC, standard chow diet; BW, body weight; i.v., intravenous injection; GTT, glucose tolerance test; PTT, pyruvate tolerance test; ITT, insulin tolerance test; AUC, area under curve; NC, negative control; HOMA-IR, homeostasis model assessment-estimated insulin resistance. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

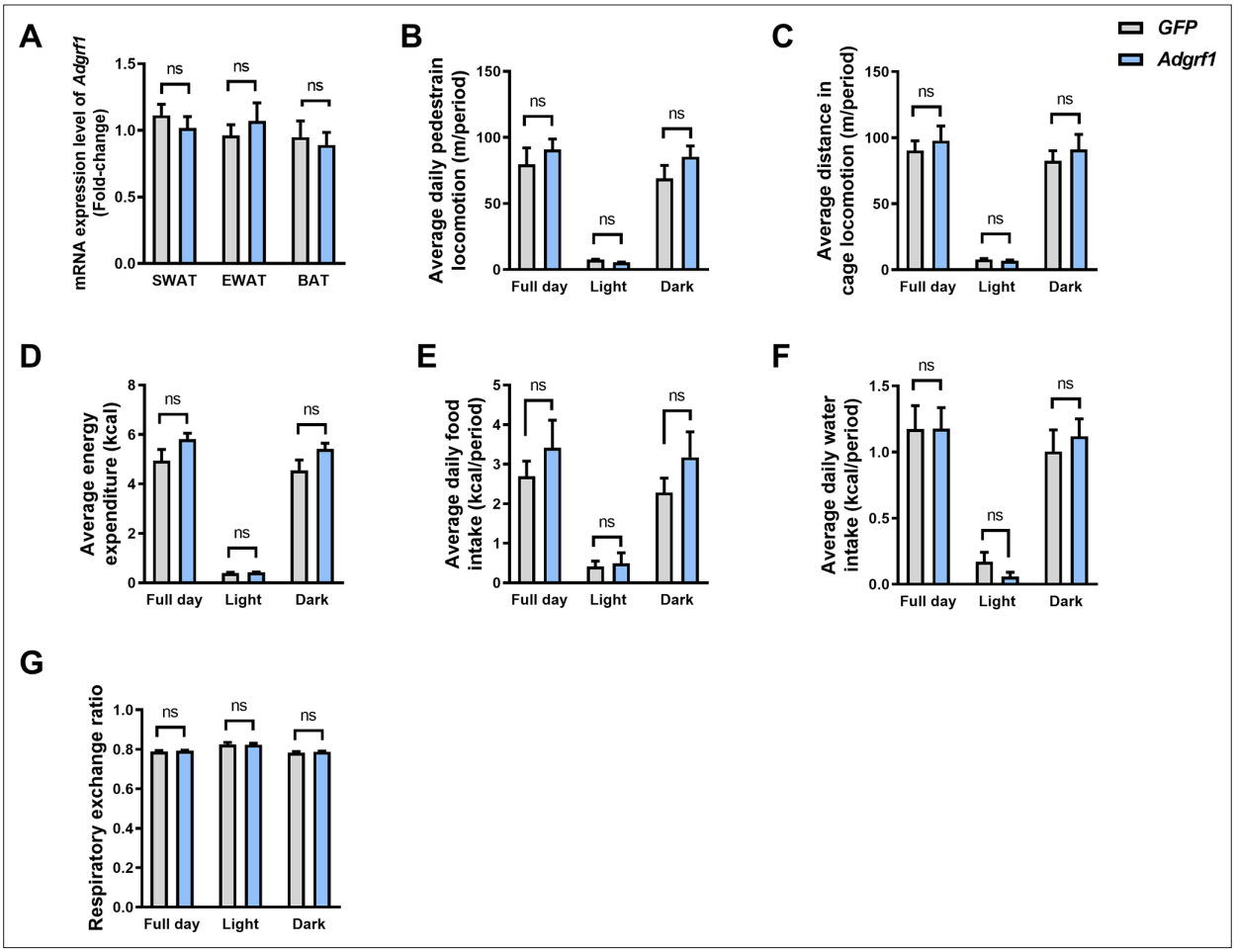

Figure 2—figure supplement 2. Overexpression of Adgrf1 did not interfere adipose tissues and other metabolic phenotypes. (**A**) mRNA expression levels of Adgrf1 in SWAT, EWAT, and BAT as determined by RT-qPCR analysis. (**B**) The average daily pedestrian locomotion, (**C**) average distance in cage locomotion, (**D**) average energy expenditure, (**E**) average daily food intake, (**F**) average daily water intake, and (**G**) respiratory exchange ratio was measured. n = 4 per group. SWAT, subcutaneous adipose tissue; EWAT, epididymal white adipose tissue; BAT, brown adipose tissue. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

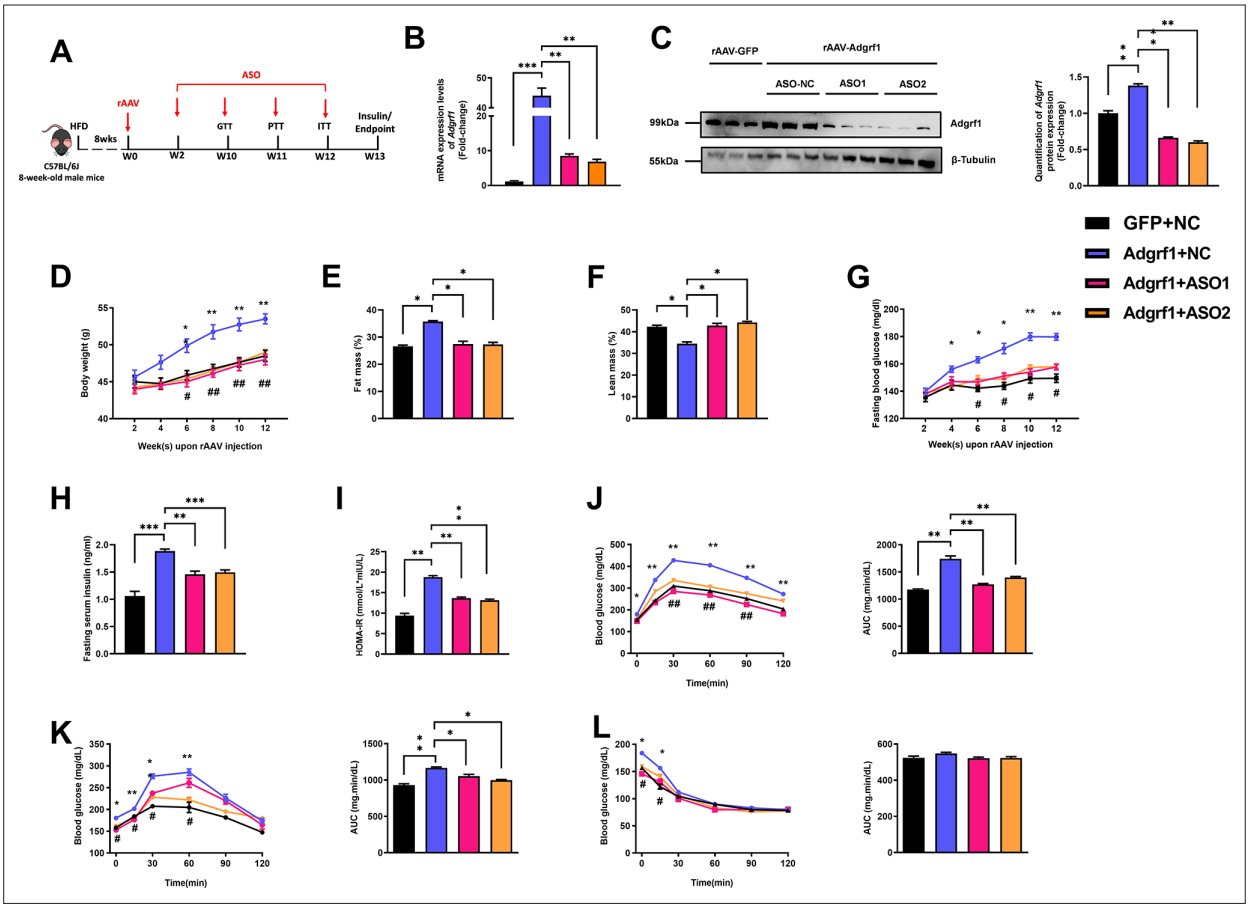

Figure 3. Deletion of hepatic *Adgrf1* protects against diet-induced glucose intolerance in *Adgrf1* overexpress mice. Eight-week-old male C57BL/6J mice were infected with either 3 × 10^{11} copies of rAAV encoding *Adgrf1* (rAAV-*Adgrf1*, i.v.) or control (rAAV-*GFP*, i.v.) and two different sequences of *Adgrf1* ASO (ASO1-*Adgrf1*, ASO2-*Adgrf1*, 5 mg/kg, one dose per week, s.c.) or scrambled control (ASO-NC, s.c.) received HFD feeding, respectively. (**A**) Schematic illustration of viral treatments. (**B**) Hepatic mRNA expression levels of *Adgrf1* from different groups of mice received either *GFP*-NC, *Adgrf1*-NC, *Adgrf1*-ASO1, or *Adgrf1*-ASO2 fed with HFD, respectively, as determined by RT-qPCR analysis. (**C**) Left panel: immunoblotting analyses of Adgrf1 and β-tubulin from livers of HFD-fed rAAV-GFP or rAAV-Adgrf1 mice treated with either ASO-NC or ASO-Adgrf1. Each lane is a sample from a different individual. Right panel: quantification of protein expression levels of Adgrf1 and β-tubulin. Protein expression levels were normalized to the expression of β-tubulin. (**D**) BW was measured biweekly upon rAAV and ASO injection. (**E**) The percentage of fat mass and (**F**) the percentage of lean mass were measured at the end of the experiment. (**G**) The fasting blood glucose level of different groups was measured upon rAAV and ASO injection. (**H**) Fasting serum insulin level and (**I**) HOMA-IR index were measured and calculated according to the formula [Fasting blood glucose (mmol/l)×Fasting blood insulin (mIU/l)]/22.5 for the HFD-fed rAAV-*Adgrf1* or rAAV-*GFP* mice at the end of the experiment. (**J**) GTT (1 g/kg BW, left) and AUC (right) of serum glucose at week 10. (**K**) PTT (1 g/kg BW, left) and AUC (right) of serum glucose at week 11. (**L**) ITT (0.5 U/kg BW, left) and AUC (right) of serum glucose at week 12. mRNA expression levels of the target genes were normalized to the expression of mouse *Gapdh*. rAAV-NC group was set as 1 for fold-change calculation. n = 8 per group. STC, standard chow diet; HFD, high-fat diet; ASO, antisense oligonucleotides; BW, body weight; GTT, glucose tolerance test; PTT, pyruvate tolerance test; ITT, insulin tolerance test; AUC, area under curve; NC, negative control; HOMA-IR, homeostasis model assessment-estimated insulin resistance. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

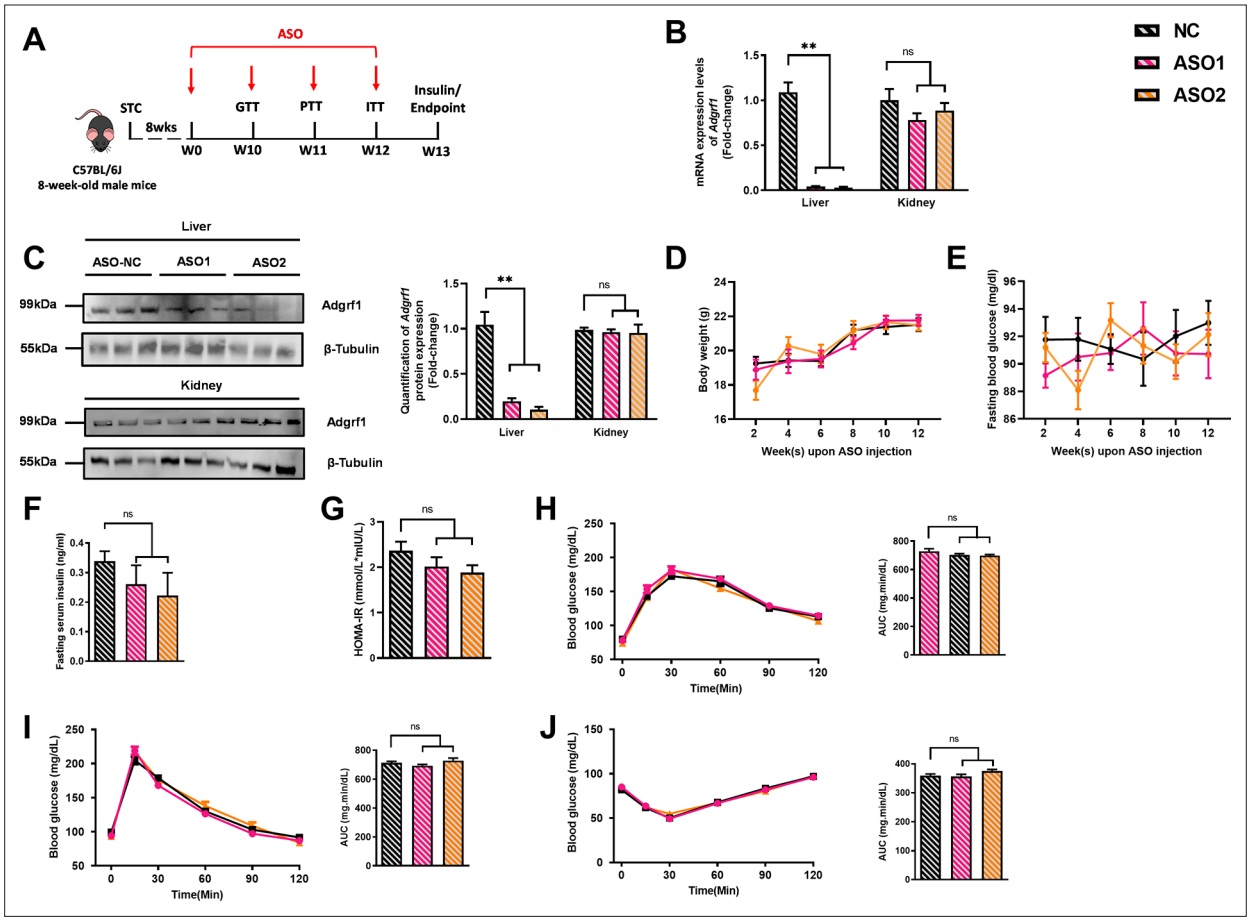

Figure 3—figure supplement 1. Hepatic knockdown of *Adgrf1* in STC-fed mice does not exhibit metabolic abnormalities. Eight-week-old male C57BL/6J mice were infected with two different sequences of *Adgrf1* antisense oligonucleotides (ASO1-*Adgrf1*, ASO2-*Adgrf1*, 5 mg/kg, one dose per week, s.c.) or scrambled control (ASO-NC, s.c.) received STC feeding, respectively. (**A**) Schematic illustration of viral treatments. (**B**) mRNA expression levels of *Adgrf1* in liver and kidney as determined by RT-qPCR analysis. mRNA expression levels of *Adgrf1* in different tissues were normalized to the expression of mouse *Gapdh*. (**C**) Left panel: immunoblotting analysis of hepatic protein expression level of Adgrf1 from STC-fed mice liver with Adgrf1 knockdown. Right panel: quantification of hepatic protein expression levels of Adgrf1. Each lane is a sample from a different individual. (**D**) Change of body weight and (**E**) fasting blood glucose at different weeks upon rAAV injection were measured. (**F**) Fasting blood insulin level and (**G**) HOMA-IR values were measured and calculated at the end the experiment. (**H**) GTT (1 g/kg BW, left) and area under curve (AUC, right) of serum glucose at the week of 10. (**I**) PTT (1 g/kg BW, left) and AUC (right) of serum glucose at week 11. (**J**) ITT (0.5 U/kg BW, left) and AUC (right) of serum glucose at week of 12. mRNA expression levels of the target genes were normalized to the expression of mouse *Gapdh*. STC, standard chow diet; s.c., subcutaneous injection; ASO, antisense oligonucleotides; BW, body weight; GTT, glucose tolerance test; PTT, pyruvate tolerance test; ITT, insulin tolerance test; AUC, area under curve; NC, negative control; HOMA-IR, homeostasis model assessment-estimated insulin resistance. Data represented as mean ± SEM; n = 8 mice per group; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*- test. *p<0.05, **p<0.01, ***p<0.001.

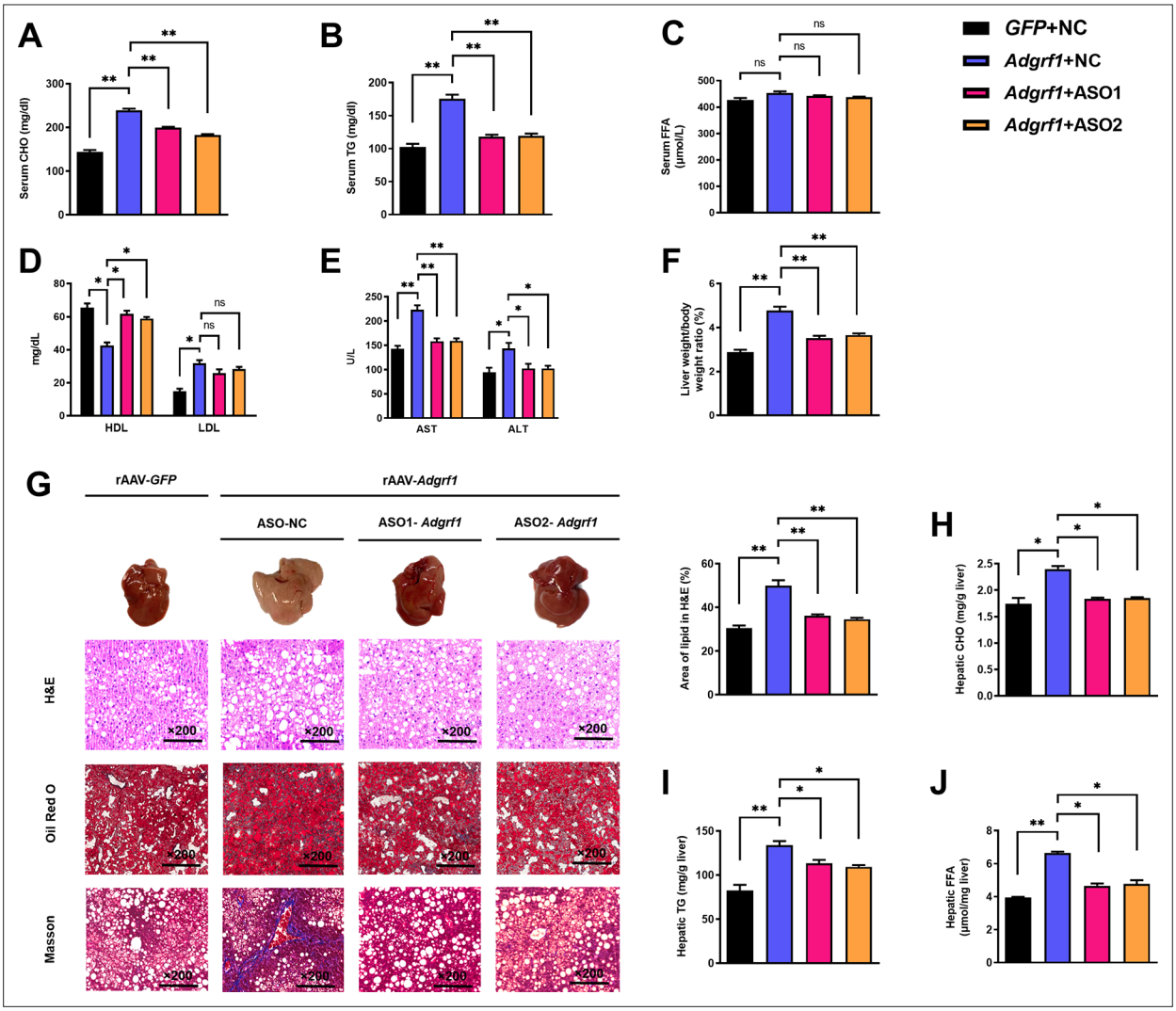

Figure 4. Upregulation of hepatic *Adgrf1* exaggerates liver steatosis in HFD-fed mice while downregulation of hepatic *Adgrf1* protects mice from diet-induced liver lipid accumulation. Eight-week-old male C57BL/6J mice were infected with either 3×10^{11} copies of rAAV encoding *Adgrf1* (rAAV-*Adgrf1*, i.v.) or control (rAAV-NC, i.v.) and two different sequences of *Adgrf1* antisense oligonucleotides (ASO1-*Adgrf1*, ASO2-*Adgrf1*, 5 mg/kg, one dose per week, s.c.) or scrambled control (ASO-NC, s.c.) received HFD feeding, respectively. (**A**) Serum CHO, (**B**) serum TG, and (**C**) serum FFA levels were measured at week 13. (**D**) Serum HDL and LDL. (**E**) The levels of serum AST ALT. (**F**) The ratio of the liver weight against body weight was calculated after sacrificing the mice from four different groups. (**G**) Representative gross pictures of liver tissues (upper panels), representative images of H&E (middle panels) and Oil Red O (lower panels) staining of liver sections (200 μm). The percentage of lipid area according to H&E staining (right panel). (**H**) Hepatic CHO, (**I**) hepatic TG, and (**J**) hepatic FFA were normalized by the weight of liver samples used for lipid extraction. n = 8 per group. i.v., intravenous injection; s.c., subcutaneous injection; STC, standard chow diet; HFD, high-fat diet; ASO, antisense oligonucleotides; BW, body weight; CHO, cholesterol; TG, triglyceride; FFA, free fatty acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; H&E, hematoxylin-eosin. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

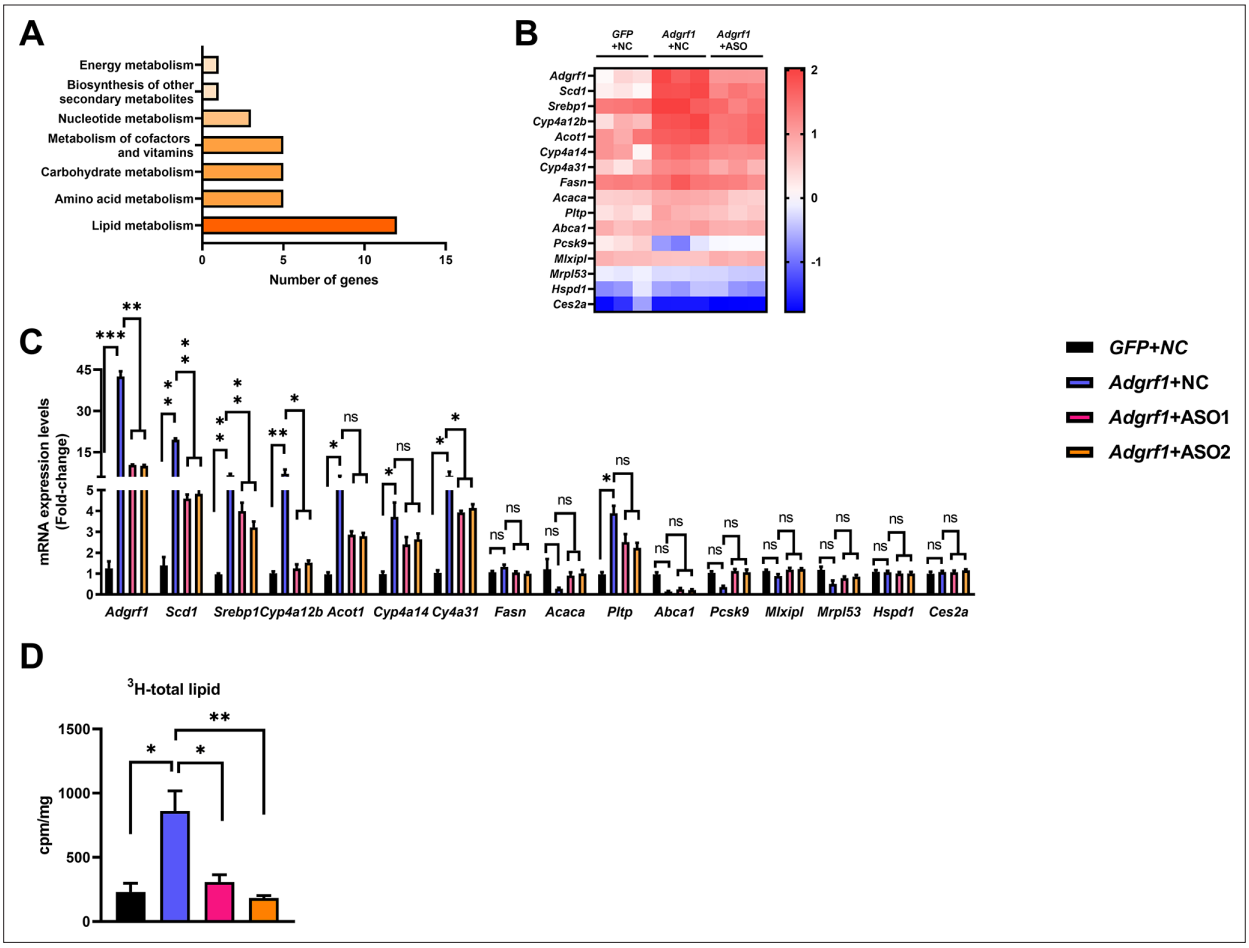

Figure 5. *Adgrf1* is a major regulator of hepatic lipid metabolism. Eight-week-old male C57BL/6J mice were infected with either 3×10^{11} copies of rAAV encoding *Adgrf1* (rAAV-*Adgrf1*, i.v.) or control (rAAV-NC, i.v.) and two *Adgrf1* antisense oligonucleotides (ASO1-*Adgrf1*, ASO2-*Adgrf1*, 5 mg/kg, one dose per week, s.c.) or scrambled control (ASO-NC, s.c.) and received HFD feeding, respectively. Mice were sacrificed and mRNA of liver from each group were extracted and RNA-seq analysis was conducted. (**A**) KEGG pathway assay of differential mRNA transcripts in rAAV and ASO groups identified by RNA-seq. (**B**) Heat map shows the $\log_2$ scale fold change in the expression levels of a set of genes involved in lipid metabolism from RNA seq data of livers in HFD-fed mice treated by rAAV-*Adgrf1* or rAAV-*Adgrf1* plus *Adgrf1*-ASO1. n = 3 per group. (**C**) mRNA expression levels of genes according to the heat map from different groups of mice received either *GFP*-NC, *Adgrf1*-NC, *Adgrf1*-ASO1, or *Adgrf1*-ASO2 fed with HFD, respectively, as determined by RT-qPCR analysis, n = 6 mice per group. (**D**) De novo lipogenic activity was measured the ^{3}H labeling of lipogenic Acetyl-CoA from 0.5 μCi ^{3}H-acetate. ASO, antisense oligonucleotides. STC, standard chow diet; HFD, high-fat diet; i.v., intravenous injection; s.c., subcutaneous injection; ASO, antisense oligonucleotides; KEGG, Kyoto Encyclopedia of Genes and Genomes; GEO, gene expression omnibus; NAFLD, non-alcoholic fatty liver disease. Data represented as mean ± SEM; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

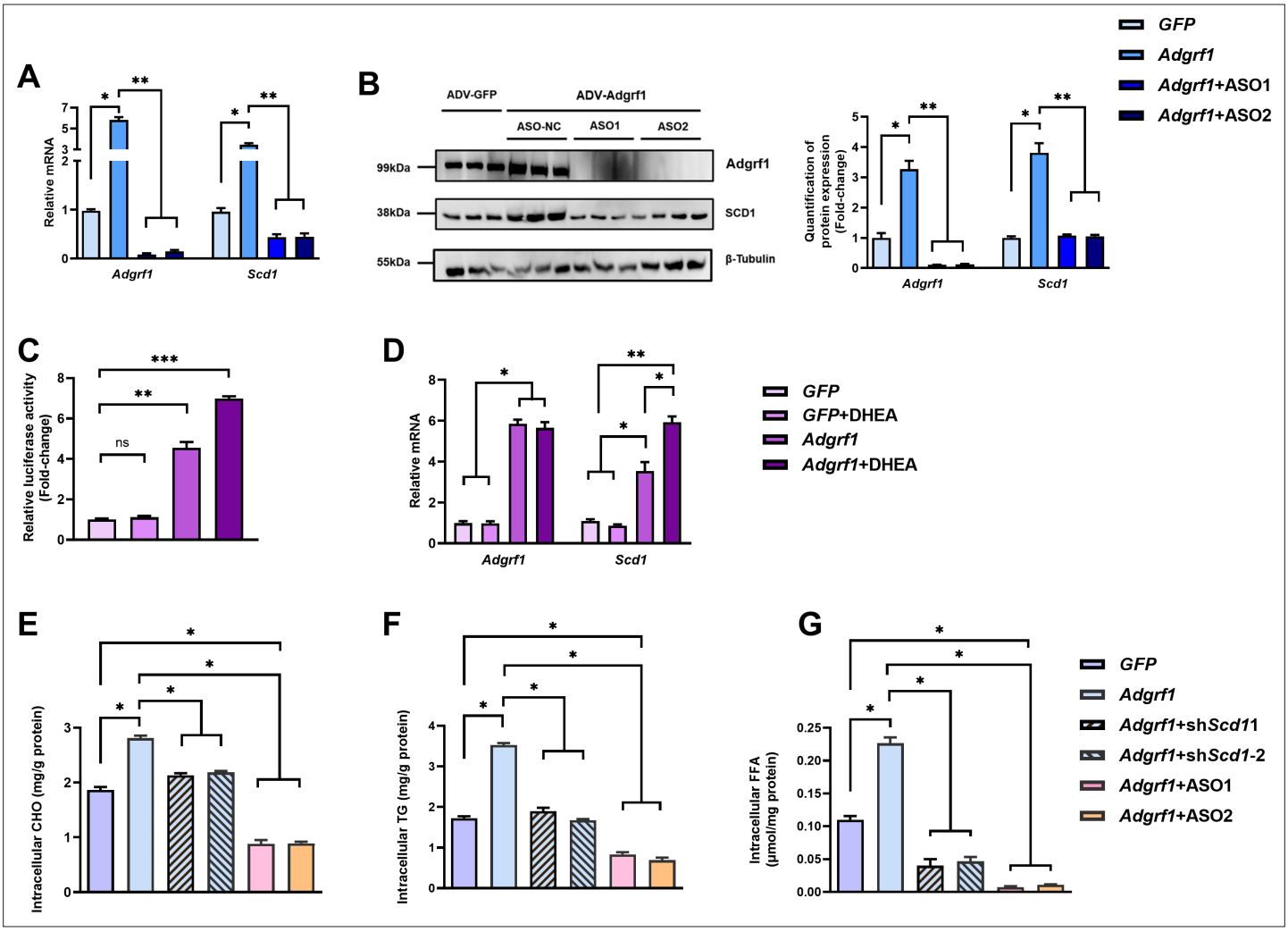

Figure 6. *Scd1* expression is regulated by *Adgrf1* in primary hepatocytes. Primary hepatocytes were isolated from 8-week-old male C57BL/6J mice with STC. (**A**) Primary hepatocytes were infected with either adenoviral vector expressing *Adgrf1* (ADV-*Adgrf1*) or control adenovirus expressing *GFP* (ADV-*GFP*) 24 hr after plating, followed by transfection with ASO1-*Adgrf1*, ASO2-*Adgrf1*, or ASO-NC for another 6 hr (n = 6). mRNA expression levels of *Adgrf1* and *Scd1* from different groups were assessed, as determined by RT-qPCR analysis. (**B**) Left panel: immunoblotting analysis for the expression level of Adgrf1 and Scd1 from different groups of primary hepatocytes. Right panel: quantification of protein expression levels of Adgrf1 and Scd1. Protein expression levels were normalized to the expression of β-tubulin. Each lane is a sample from a different plate. Right panel: quantification of protein expression levels of Adgrf1, Scd1, and β-tubulin. n = 3 per group. Protein expression levels were normalized to the expression of β-tubulin. The samples for GFP were set as 1 for fold-change calculation. (**C, D**) HEK293T cells were infected with pGL3-*Scd1* promoter-luciferase plasmid and adenoviral vector expressing *Adgrf1* (ADV-*Adgrf1*) or *GFP* (ADV-*GFP*) for 48 hr and DHEA was added to the transfected cells at the concentration of 100 μM for 24 hr. Cell lysates were used for (**C**) luciferase assay or (**D**) RT-qPCR analysis (n = 3). Lysates from the cell co-transfection with pGL3-*Scd1* promoter-luciferase plasmid and ADV-*GFP* without treatment of DHEA was set as 1 for fold-change calculation. (**E–G**) Primary hepatocytes were infected with either adenoviral vector expressing *Adgrf1* (ADV-*Adgrf1*) or control ADV-*GFP*, followed by transfecting with scramble or sh*Scd1*-1 or sh*Scd1*-2 plasmids for another 72 hr. Intracellular lipids were extracted and (**E**) CHO, (**F**) TG, and (**G**) FFA were assessed (n = 3). STC, standard chow diet; i.v., intravenous injection; s.c., subcutaneous injection; ASO, antisense oligonucleotides. CHO, cholesterol; TG, triglyceride; FFA, free fatty acid. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

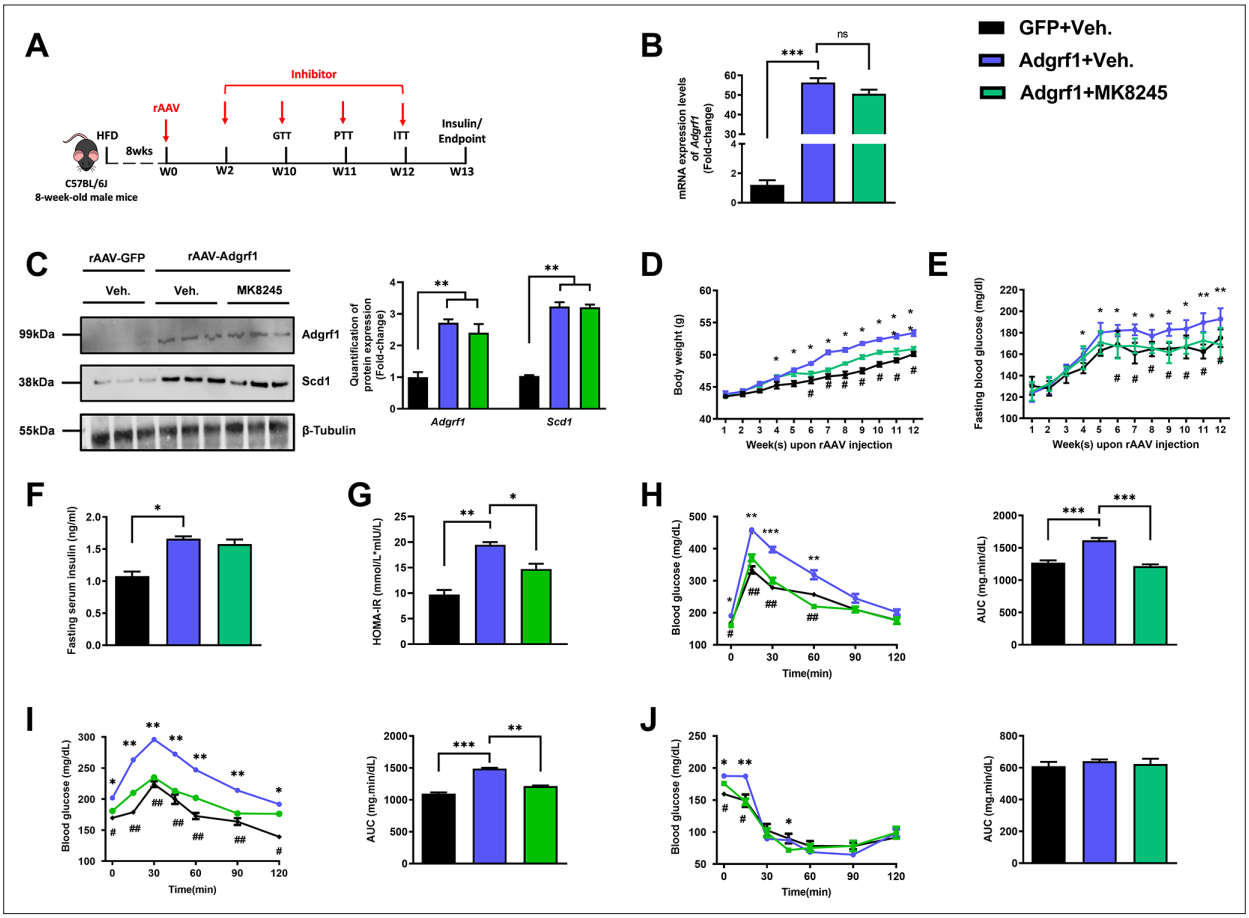

Figure 7. Inhibition of *Scd1* alleviates the glucose impairment in mice with hepatic *Adgrf1* overexpression. Eight-week-old male C57BL/6J mice were infected with either 3×10^{11} copies of rAAV encoding *Adgrf1* (rAAV-*Adgrf1*, i.v.) or control (rAAV-*GFP*, i.v.) and *Scd1* inhibitor (MK8245, 10 mg/kg/week, p.o.) or inhibitor vehicle (inhibitor-Veh., p.o.) received HFD feeding. (**A**) Schematic illustration of viral treatments. (**B**) Hepatic mRNA expression levels of *Adgrf1* from different groups of mice received rAAV and inhibitor fed with HFD, respectively, as determined by RT-qPCR analysis. (**C**) Left panel: immunoblotting analysis for the hepatic protein expression level of Adgrf1 and Scd1 from different groups of mice fed with HFD. Right panel: quantification of protein expression levels of Adgrf1 and Scd1. Protein expression levels were normalized to the expression of β-tubulin. Each lane is a sample from a different individual. (**D**) BW and (**E**) fasting blood glucose level were measured at different weeks upon rAAV and inhibitor injection. (**F**) The fasting blood insulin level and (**G**) HOMA-IR index were measured and calculated according to the formula [Fasting blood glucose (mmol/l)×Fasting blood insulin (mIU/l)]/22.5 for the HFD-fed rAAV-*Adgrf1* or rAAV-*GFP* mice at the end of the experiment. (**H**) GTT (1 g/kg BW, left) and AUC (right) of serum glucose at the week of 10. (**I**) PTT (1 g/kg BW, left) and AUC (right) of serum glucose at week 11. (**J**) ITT (0.5 U/kg BW, left) and AUC (right) of serum glucose at week of 12. mRNA expression levels of the target genes were normalized to the expression of mouse *Gapdh*. rAAV-NC group was set as 1 for fold-change calculation. n = 8 per group. HFD, high-fat diet; i.v., intravenous injection; p.o., oral administration; ASO, antisense oligonucleotides; BW, body weight; GTT, glucose tolerance test; PTT, pyruvate tolerance test; ITT, insulin tolerance test; AUC, area under curve; NC, negative control; HOMA-IR, homeostasis model assessment-estimated insulin resistance. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

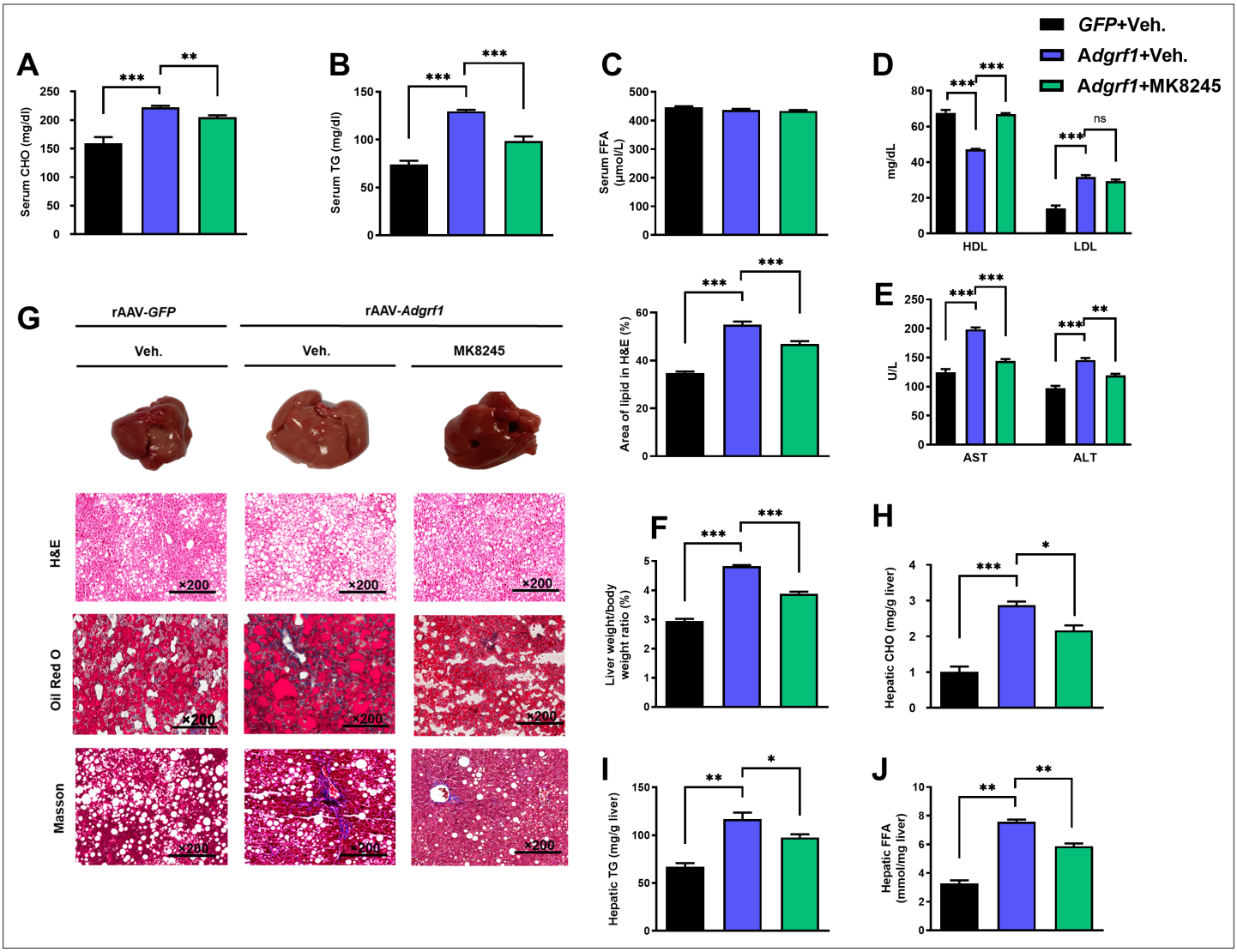

Figure 8. Inhibition of hepatic *Scd1* partially alleviates the severity of hepatic steatosis in *Adgrf1* overexpression mice. Eight-week-old male C57BL/6N mice were infected with either 3 × 10^{11} copies of rAAV encoding *Adgrf1* (rAAV-*Adgrf1*, i.v.) or control (rAAV-*GFP*, i.v.) and administered with *Scd1* inhibitor (MK8245, 10 mg/kg, p.o.) or inhibitor vehicle (inhibitor-Veh., p.o.) received HFD feeding. (**A**) Serum CHO, (**B**) serum TG, and (**C**) serum FFA levels were measured at the end of experiment. (**D**) Serum HDL and LDL, (**E**) AST and ALT level of each group of mice were measured at the end of the experiment. (**F**) The ratio of the liver weight against body weight was calculated after sacrificing the mice from four different groups. (**G**) Representative gross pictures of liver tissues (upper panels), representative images of H&E (middle panels) and Oil Red O (lower panels) staining of liver sections (200 μm). The percentage of lipid area according to H&E staining (right panel). (**H**) Hepatic CHO, (**I**) hepatic TG, and (**J**) hepatic FFA were normalized by the weight of liver samples used for lipid extraction. n = 8 per group. STC, standard chow diet; HFD, high-fat diet; i.v., intravenous injection; p.o., oral administration; CHO, cholesterol; TG, triglyceride; FFA, free fatty acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; H&E, hematoxylin-eosin. Data represented as mean ± SEM; repeated with three independent experiments; p-value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.

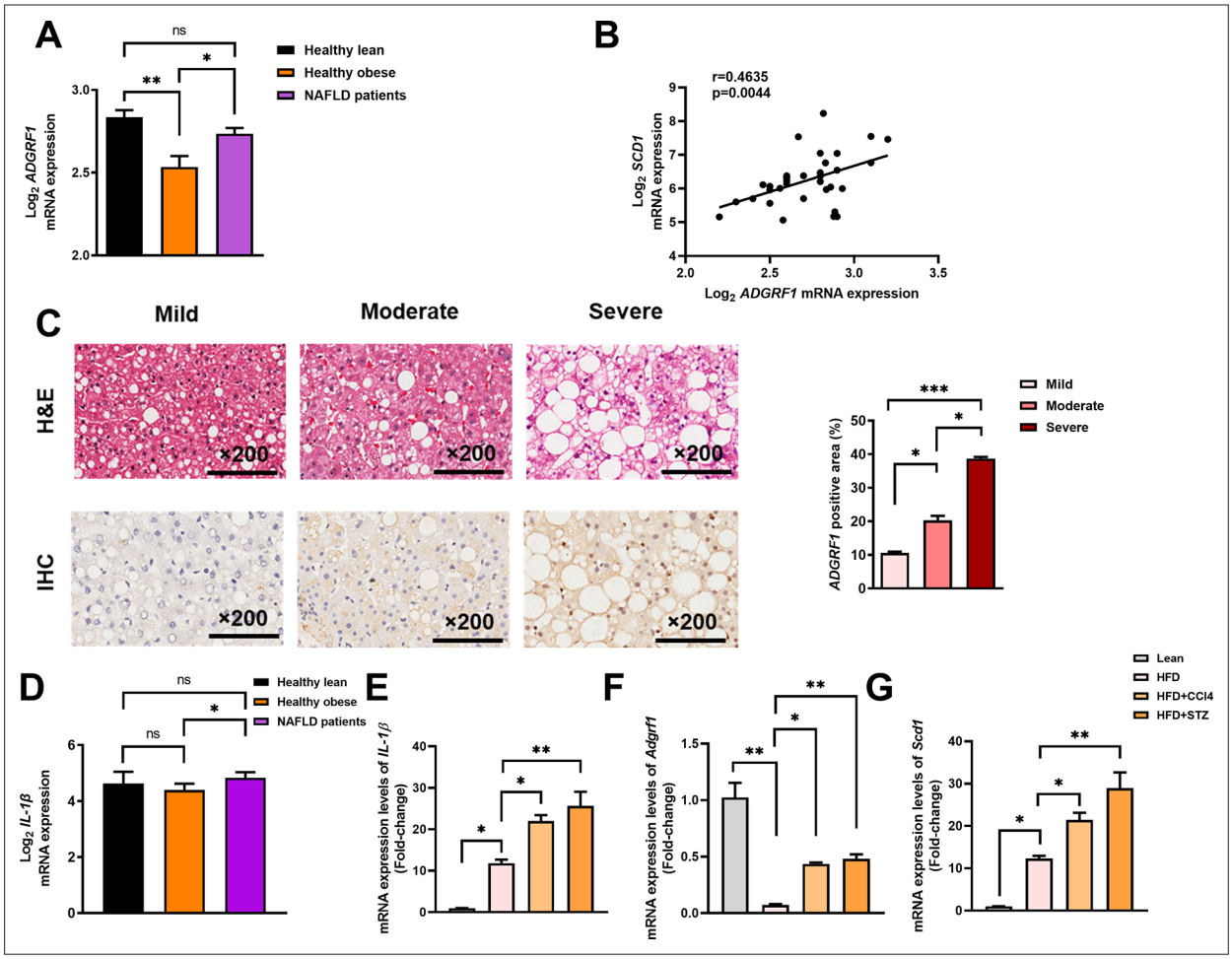

Figure 9. Hepatic expression of *ADGRF1* is upregulated in obese patients with hepatic steatosis when compared to those with normal liver morphology, which is positively associated with hepatic *SCD1* expression level. NAFLD patients have higher hepatic expression of *ADGRF1* accompanied with increased mRNA Scd1 expression. (**A**) Normalized Log_2 mRNA expression of *ADGRF1* in lean people without NAFLD (n = 12), obese people without NAFLD (n = 17), or obese patients with NAFLD (n = 8) according to the GEO database (GEO; Profile # GDS4881/8126820). (**B**) Correlation between *ADGRF1* and *SCD1* in liver of human subjects based on the GEO database. (**C**) Representative images of liver tissues with H&E staining (upper panels) and immunohistochemical staining (IHC) of *ADGRF1* (lower panels) from patients with different degree of NAFLD (200 μm). The percentage of *ADGRF1* positive area according to H&E staining (right panel). The percentage of *ADGRF1*-positive areas according to IHC staining (right panel); n = 3 per group. (**D**) Normalized Log2 mRNA expression of *IL-1β* in lean people without NAFLD (n = 12), obese people without NAFLD (n = 17), or obese patients with NAFLD (n = 8) according to the GEO database (GEO; Profile # GDS4881/8126820). (**E**) Hepatic mRNA expression levels of *IL-1β*, (**F**) *Adgrf1*, and (**G**) *Scd1* in either STC-fed mice or HFD-fed mice treated with CCl4 or STZ as determined by RT-qPCR. Data represented as mean ± SEM. p-Value analyzed by two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001.