Research Article
Experimental and Translational Hepatology

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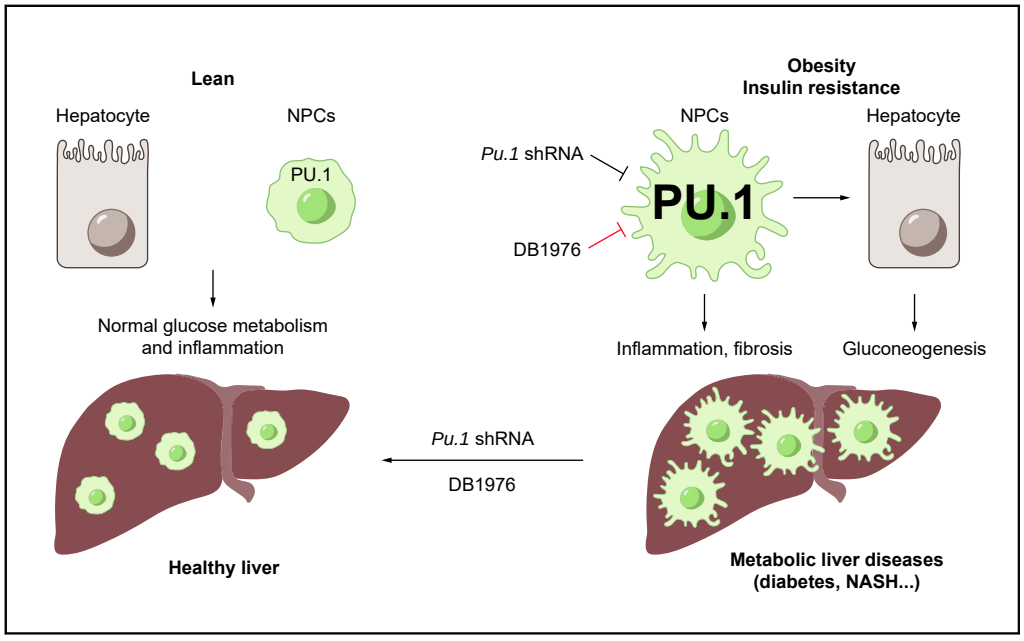
Graphical abstract



Highlights

- Liver, primarily macrophage, PU.1 expression is increased in obese mice and people.
- Targeting Pu.1 inhibits the inflammatory response in macrophages.
- Pharmacologic Pu.1 inhibition improves glucose homeostasis and alleviates liver inflammation, steatosis and fibrosis.
- Hepatocyte PU.1 does not play a major role in regulating its metabolic function.

Authors

Qiongming Liu, Junjie Yu, Liheng Wang, …, Utpal B. Pajvani, Jun Qin, Li Qiang

Correspondence

LQ2123@cumc.columbia.edu (L. Qiang), up2104@cumc.columbia.edu (U.B. Pajvani), jqin@bcm.edu (J. Qin).

Lay summary

Expression of the immune regulator PU.1 is increased in livers of obese mice and people. Blocking PU.1 improved glucose homeostasis, and reduced liver steatosis, inflammation and fibrosis in mouse models of non-alcoholic steatohepatitis. Inhibition of PU.1 is thus a potential therapeutic strategy for treating obesity-associated liver dysfunction and metabolic diseases.

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Inhibition of PU.1 ameliorates metabolic dysfunction and non-alcoholic steatohepatitis

Qiongming Liu[1,2,†], Junjie Yu[3,†], Liheng Wang[3,†], Yuliang Tang[4], Quan Zhou[2], Shuhui Ji[2], Yi Wang[5], Luis Santos[6], Rebecca A. Haeusler[1], Jianwen Que[7], Prashant Rajbhandari[6], Xiaoguang Lei[4], Luca Valenti[8], Utpal B. Pajvani[3,*], Jun Qin[2,5,*], Li Qiang[1,*]

[1]*Naomi Berrie Diabetes Center, Department of Pathology and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, 10032, USA;* [2]*State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Lifeomics, National Center for Protein Sciences (The PHOENIX Center at Beijing), Beijing 102206, China;* [3]*Naomi Berrie Diabetes Center, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York, 10032, USA;* [4]*Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, Department of Chemical Biology, College of Chemistry and Molecular Engineering, Synthetic and Functional Biomolecules Center, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China;* [5]*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, 77030, USA;* [6]*Diabetes, Obesity, and Metabolism Institute, Icahn School of Medicine at Mount Sinai, New York, United States;* [7]*Columbia Center for Human Development and Department of Medicine, Columbia University, New York, NY 10032;* [8]*Department of Pathophysiology and Transplantation, Università degli Studi Milano, and Internal Medicine and Metabolic Diseases, Fondazione IRCCS Ca' Granda Ospedale Policlinico, Milan, Italy*

Background & Aims: Obesity is a well-established risk factor for type 2 diabetes (T2D) and non-alcoholic steatohepatitis (NASH), but the underlying mechanisms remain incompletely understood. Herein, we aimed to identify novel pathogenic factors (and possible therapeutic targets) underlying metabolic dysfunction in the liver.

Methods: We applied a tandem quantitative proteomics strategy to enrich and identify transcription factors (TFs) induced in the obese liver. We used flow cytometry of liver cells to analyze the source of the induced TFs. We employed conditional knockout mice, shRNA, and small-molecule inhibitors to test the metabolic consequences of the induction of identified TFs. Finally, we validated mouse data in patient liver biopsies.

Results: We identified PU.1/SPI1, the master hematopoietic regulator, as one of the most upregulated TFs in livers from diet-induced obese (DIO) and genetically obese (*db/db*) mice. Targeting PU.1 in the whole liver, but not hepatocytes alone, significantly improved glucose homeostasis and suppressed liver inflammation. Consistently, treatment with the PU.1 inhibitor DB1976 markedly reduced inflammation and improved glucose homeostasis and dyslipidemia in DIO mice, and strongly suppressed glucose intolerance, liver steatosis, inflammation, and fibrosis in a dietary NASH mouse model. Furthermore, hepatic *PU.1* expression was positively correlated with insulin resistance and inflammation in liver biopsies from patients.

Conclusions: These data suggest that the elevated hematopoietic factor PU.1 promotes liver metabolic dysfunction, and may be a useful therapeutic target for obesity, insulin resistance/T2D, and NASH.

Lay summary: Expression of the immune regulator PU.1 is increased in livers of obese mice and people. Blocking PU.1 improved glucose homeostasis, and reduced liver steatosis, inflammation and fibrosis in mouse models of non-alcoholic steatohepatitis. Inhibition of PU.1 is thus a potential therapeutic strategy for treating obesity-associated liver dysfunction and metabolic diseases.

Introduction

Obesity and the associated metabolic syndrome are significant worldwide public health concerns and account for tremendous costs for the affected individuals, families, healthcare systems, and society. In particular, obesity is associated with the development of insulin resistance, type 2 diabetes (T2D), liver and cardiovascular diseases, and cancer. Insulin responsive tissues, including adipose tissue, skeletal muscle, and liver, are profoundly affected by obesity both at biomolecular and functional levels. Insulin sensitivity in the liver is pivotal in the regulation of glucose and lipid metabolism.[1] Insulin suppresses liver glucose production by inhibiting glycogenolysis and gluconeogenesis and stimulating glycogen synthesis, glycolysis and lipogenesis.[2] In insulin-resistant state, when insulin fails to adjust lipid and carbohydrate metabolism, hyperglycemia and dyslipidemia ensue, and exacerbate the incidence of non-alcoholic fatty liver disease (NAFLD).[3] Indeed, obesity and associated insulin resistance have been established as risk factors of liver fat accumulation, which promotes a liver disease spectrum ranging from NAFLD to non-alcoholic steatohepatitis (NASH), with possible progression towards cirrhosis and even hepatocellular carcinoma (HCC).[4–6] In parallel, liver diseases also contribute to the

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* Corresponding authors. Addresses: Columbia University Medical Center, Russ Berrie Building Room 607A, 1150 St. Nicholas Ave, New York, NY 10032, USA. Tel.: 001-212-851-4929 (L. Qiang), or Columbia University Medical Center, Russ Berrie Building Room 121F, 1150 St. Nicholas Ave, New York, NY 10032, USA. Tel.: 001-212-851-4886 (U.B. Pajvani), or Verna and Marrs McLean Department of Biochemistry and Molecular Biology, One Baylor Plaza, Room 145E, Houston, TX 77030, USA. Tel.: 001-713-798-1507, fax: 001-713-796-9438 (J. Qin).
E-mail addresses: LQ2123@cumc.columbia.edu (L. Qiang), up2104@cumc.columbia.edu (U.B. Pajvani), jqin@bcm.edu (J. Qin).
† Contributed equally.
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development of diabetes.[7,8] However, the molecular mechanisms underlying this association remain insufficiently understood.[9]

With advances in mass spectrometry (MS)-based proteomics, substantial progress has been made in elucidating global protein changes involved in the development of T2D in β cells and adipocytes,[10,11] but has not been systematically applied to the liver. Aiming for quantitative measurement of transcription factors (TFs) on a proteomic scale, we developed a tandem proteomics strategy by combining TF response element (TFRE)-affinity enrichment with liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification of endogenous TFs.[12,13] Applying this approach to the liver may identify novel regulators of glucose homeostasis and lipid metabolism that contribute to the pathogenesis of liver dysfunctions in obesity and T2D.

In the present study, we applied TFRE-affinity enrichment and an LC-MS/MS approach to mouse liver to identify endogenous TFs that contribute to insulin resistance. This approach circumvents low endogenous expression of hepatic TFs, the discrepancy between protein and gene expression, and antibody reliability. Surprisingly, PU.1/SPI1 was identified and confirmed to be one of the most upregulated TFs in both diet-induced obese (DIO) and *db/db* mouse livers. PU.1 is predominantly expressed in hematopoietic and lymphatic cells[14] and is considered a "master" regulator responsible for macrophage terminal differentiation and maturation.[15–17] Compared to the immune and hematopoietic systems, less is known about the role of PU.1 in metabolic regulation. Our studies described herein reveal that PU.1 is a new therapeutic target for the treatment of liver dysfunction and metabolic diseases.

Materials and methods

Animals

Mice were maintained in accordance with Institutional Animal Care and Use Committee guidelines. Male C57BL/6J mice aged 6–8 weeks were purchased from HFK Biotech Co. (Beijing, China) or Jackson Laboratory (Bar Harbor, ME, USA). Diabetic *db/db* mice were obtained from the animal resource center of Nanjing University (Pengsheng Biotech, Nanjing, China). All mice were housed at 22–24°C with a 12 h/12 h light/dark cycle and provided water and standard rodent chow *ad libitum*. In some studies, male C57BL/6J mice were fed high-fat diet (HFD) (60% fat, 24% carbohydrates, and 16% protein based on caloric content, Research Diets D12492) or a NASH-provoking diet (Envigo, TD.160785) *ad libitum* for 4 months from 8 weeks of age,[18] with or without treatment with a PU.1 inhibitor (DB1976), synthesized in Dr. X. Lei's laboratory at Peking University.[19]

Pu.1$^{flox/flox}$ mice were previously described.[20,21] Hepatocyte *Pu.1* conditional knockout mice were generated by breeding *Pu.1*$^{flox/flox}$ mice with *Albumin-Cre* mice (Jackson Laboratory). Mice were maintained on C57BL/6J background and housed in a pathogen-free facility at Columbia University with a 12 h/12 h light/dark cycle and free access to water and standard irradiated rodent chow (5% energy from fat; Harlan Teklad). All animal experimental studies were approved and performed in accordance with animal license guidelines and regulations established by the Columbia University Animal Care Committee.

Metabolic characterization

Glucose tolerance test (GTT) was performed in overnight (16 h) fasted mice with an intraperitoneal bolus glucose injection of 1.0 or 2.0 g/kg of body weight. The insulin tolerance test was performed in 4 h fasted mice with an intraperitoneal injection of 0.5 U/kg of body weight. Blood glucose was determined from tail bleeds at the indicated times by glucose meter (Bayer). Liver triglyceride (TG) was extracted as previously described.[22] Plasma and liver TG were measured by the Infinity triglyceride kit (Thermo Fisher Scientific). Other plasma lipids were determined by total cholesterol E kit (Wako Diagnostics) and NEFA reagents (Wako Diagnostics).

Statistical analysis

Data are represented as mean ± SEM. $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001 correspond to p value according to the Student's t test. GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analyses.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Liver PU.1 is elevated in diet-induced obese mice

To systematically examine liver TFs that may contribute to obesity-induced metabolic dysfunction, we developed a quantitative proteomics strategy, as outlined in Fig. 1A, to survey functional TFs altered in obese mouse liver. Specifically, we concatenated our previously developed DNA-based affinity methods with a tandem array of the consensus TFRE or hormone response element (HRE) pull-down method named TFRE/HRE pull-down.[12,13] The endogenously expressed TFs from lean and obese mouse livers were enriched by TFRE/HRE pull-down and then characterized by LC-MS/MS. By this quantitative MS analysis, a list of TFs was identified with altered expression, either enriched (>3 fold) or decreased (<50%) in livers from DIO mice (Tables S2 and S3). Surprisingly, among the most upregulated TFs was PU.1/SPI1, a master regulator of myeloid cell activity (Fig. 1B and Fig. S1A). Consistently, the *Pu.1* mRNA level was increased ~5-fold in DIO mouse liver compared to chow diet-fed mice (Fig. 1C).

To determine the source of increased liver PU.1, we isolated parenchymal cells (PCs) and non-parenchymal cells (NPCs) from livers of C57BL/6J wild-type mice. As expected, Albumin was specifically expressed in PCs, whereas the macrophage marker F4/80 (*Adgre1*), stellate cell marker Desmin, and endothelial cell marker *Vwf* were only detected in fractionated NPCs (Fig. S2A). Though hepatocyte *Pu.1* expression was induced about 3.6-fold in DIO, *Pu.1* was primarily expressed in NPCs (Fig. 1D, E), predominately in macrophages and B cells (Fig. 1F, Fig. S2B). Intriguingly, although *Pu.1* expression did not change in these populations, macrophage and B cell number increased about 3-fold in DIO, though the latter remained a relatively minor contributor (Fig. 1G). Hepatic T cells and stellate cells (HSCs) did not substantially contribute to *Pu.1* expression in DIO liver (Fig. 1F, G). Overall, these data suggest that the induction of PU.1 in the obese liver is due to increased hepatocyte *Pu.1* expression and macrophage number.

Pu.1 knockdown in the liver improves glucose homeostasis in DIO mice

To explore the function of PU.1 in liver metabolism, we developed small hairpin RNAs (shRNAs) targeting *Pu.1* and administered the most efficient *Pu.1*-s6 to DIO mice using an adenovirus vector (Fig. 2A). *Pu.1* knockdown (KD) did not affect body weight

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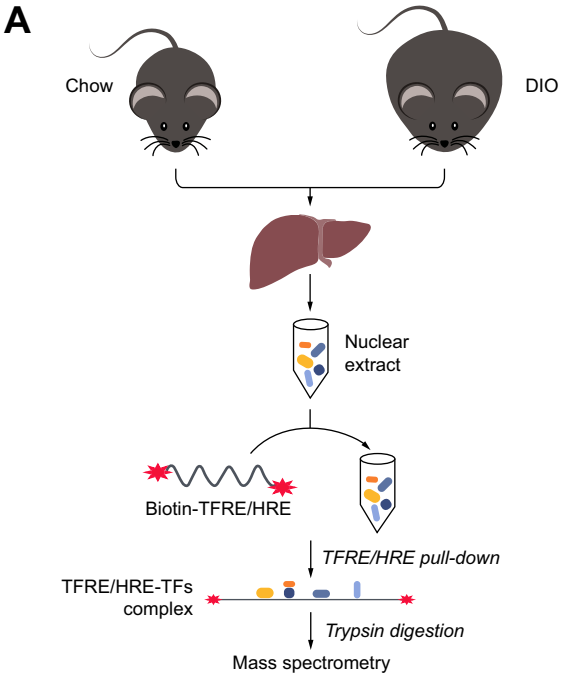
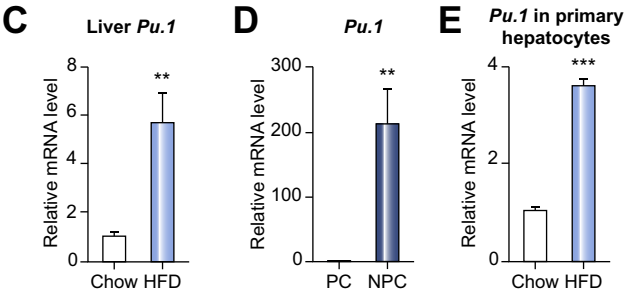
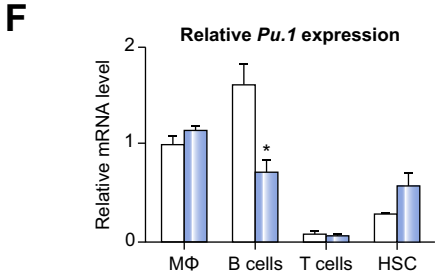


B

DNA element	Treatment	Peptide count	Unique peptide	iBAQ3
TFRE: consensus TF response element	Chow	0	0	0
	HFD	6	6	9.6
DR1: 12 copies of direct repeat 1 (DR1) of HRE	Chow	1	1	0.27
	HFD	3	3	10.1
IR3: 9 copies of invert repeat 3 (IR3) of HRE	Chow	4	4	2
	HFD	6	6	12.1
NRRE: combined direct repeats and invert repeats of HRE	Chow	3	0	0
	HFD	3	3	6.1





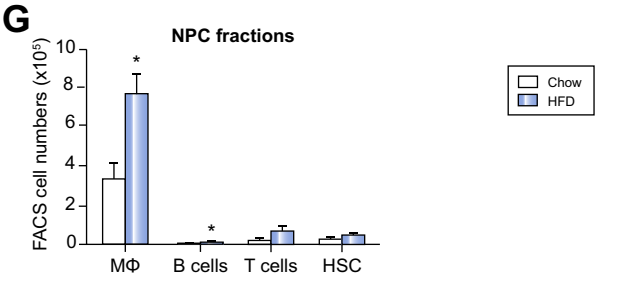


Fig. 1. Transcription factor PU.1 was upregulated in the DIO mouse liver. (A) Diagram of the DNA pull-down plus mass spectrometry strategy to quantitatively analyze DNA binding activities of TFs in chow diet-fed (Chow)/control and HFD-induced obesity (DIO) mouse liver. (B) Summary of the pulled-down PU.1 protein by different DNA elements in DIO mouse liver that were quantified by iBAQ-based quantitative mass spectrometry analysis. (C) Relative expression of *Pu.1* was analyzed by qPCR in chow- and HFD-fed mouse livers (n = 4). (D) mRNA expression of *Pu.1* in PCs and NPCs isolated from 8-week-old C57BL/6J mice after 16 h fasting and 4 h refeeding (n = 5, 5). (E) qPCR analysis of *Pu.1* expression in primary hepatocytes isolated from chow- and HFD-fed mice (n = 3, 3). (F) Relative *Pu.1* expression in FACS-sorted liver immune cells and hepatic stellate cells (HSCs) from chow- and HFD-fed mice (n = 3, 3). (G) Numbers of FACS-sorted immune cells and HSCs in NPCs from lean and DIO mice (n = 3, 3). All mice used in this study were males. Data are presented as mean ± SEM, **p* <0.05, ***p* <0.01, ****p* <0.001 by 2-tailed Student's *t* test. DIO, diet-induced obese; HFD, high-fat diet; HRE, hormone response element; HSCs, hepatic stellate cells; NPC, non-parenchymal cells; PCs, parenchymal cells; TF, transcription factors; TFRE, TF response element.

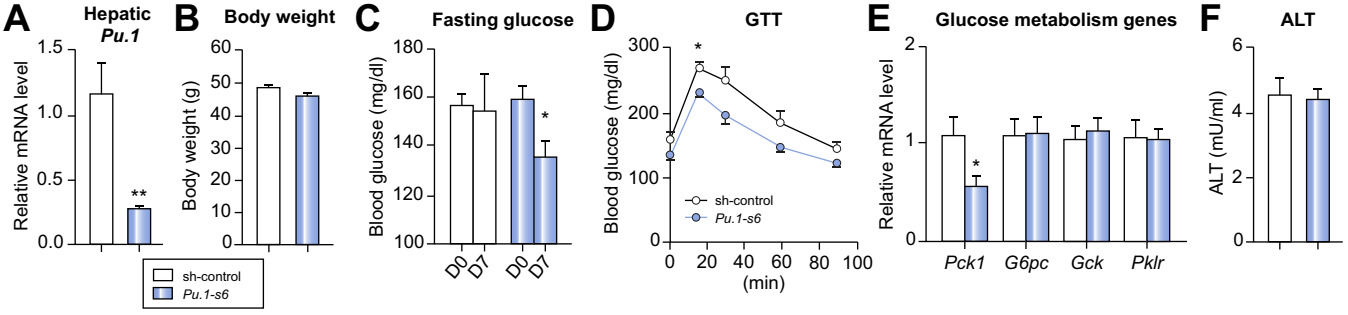


Fig. 2. *Pu.1* KD improves glucose homeostasis in DIO mice. Eight weeks' old male C57BL/6J mice were fed with HFD for 3 months and then treated with PU.1-s6 shRNA adenovirus for 7 days. (A) qPCR analysis of liver *Pu.1* expression. (B) Bodyweight. (C) Fasting blood glucose levels, solid bar for *Pu.1-s6*, and open bar for sh-control. (D) Intraperitoneal GTT. (E) mRNA expression of glucose metabolism genes. (F) Plasma ALT levels. n = 7/group. Data are presented as mean ± SEM. * *p* <0.05; ** *p* <0.01 *vs*. sh-control by 2-tailed Student's *t* test. ALT, alanine aminotransferase; DIO, diet-induced obese; GTT, glucose tolerance test; HFD, high-fat diet; KD, knockdown; shRNA, small hairpin RNA.

(Fig. 2B), but lowered fasting glucose levels (Fig. 2C), improved glucose tolerance (Fig. 2D), and repressed the expression of *Pck1*, a key regulator of gluconeogenesis (Fig. 2E). Livers of KD mice were slightly larger (Fig. S3A), but hepatic TG and cholesterol, plasma lipids and alanine aminotransferase (ALT) levels were not significantly affected (Fig. 2F and Fig. S3B–D). As expected, adenoviral KD of PU.1 in the liver had minimal effects on adipose tissue (Fig. S3E).

Pu.1 KD in liver improves glycemic control in diabetic *db/db* mice

Next, we asked whether hepatic PU.1 was also increased in a genetic model of obesity/diabetes, the leptin receptor-deficient *db/db* mouse. For better accuracy, we applied SWATH combined with LC-MS/MS to quantify the enrichment of Pu.1 in the liver from *db/db* mice. This approach excludes possible interference from non-specific detection by PU.1 antibody. We detected >2-fold enrichment of Pu.1 protein in *db/db* mouse liver at the λB motif of the Ig2–4 enhancer, a high-affinity PU.1 binding site,[23] in nuclear extracts of liver tissue from *db/db* mice compared to control littermates (Fig. 3A and Fig. S1B). We also confirmed the induction of PU.1 in *db/db* mice liver using alternative TFRE motif enrichment (Fig. 3B and Fig. S1C). These data indicated that PU.1 is similarly induced in the models of genetic and diet-induced obesity, and that targeting liver PU.1 would also show beneficial effects in *db/db* mice. Indeed, *Pu.1* KD (Fig. 3C), in the setting of a small, non-significant reduction in body weight (Fig. 3D), markedly reduced fasting glucose levels (Fig. 3E) without effects on plasma insulin (Fig. 3F). Similar to DIO mice, *Pu.1* KD improved glucose tolerance in *db/db* mice (Fig. 3G), and reduced *Pck1* expression (Fig. 3H), without changing plasma TG (Fig. 3I). Together, these data indicate that the induction of PU.1 in the liver contributes to the dysregulation of glucose homeostasis in *db/db* and DIO mice.

Hepatocyte-specific ablation of PU.1 has no effects on glucose metabolism

Since *Pu.1* was induced in hepatocytes in DIO mice (Fig. 1E), and hepatocytes are the exclusive glucose-producing cells in the liver, we asked whether the glucose-lowering effect of adenoviral *Pu.1* shRNA was due to reduced hepatocyte PU.1 action. To this end, we generated hepatocyte-specific *Pu.1* knockout mice by transducing lean $Pu.1^{flox/flox}$ mice with AAV8-TBG-Cre, to induce hepatocyte-specific recombination.[18] Intriguingly, these mice showed unchanged glucose tolerance (Fig. 4A). Even HFD-fed $Pu.1^{flox/flox}$ mice transduced with AAV8-TBG-Cre did not show altered glucose tolerance or insulin sensitivity compared to AAV8-TBG-GFP transduced mice (Fig. 4B, C). Finally, we crossed $Pu.1^{flox/flox}$ mice with *Alb-Cre* mice to conditionally delete PU.1 in hepatocytes (*Alb-Cre*:$Pu.1^{flox/flox}$) without affecting NPC *Pu.1* expression (Fig. 4D, E). After HFD feeding for 3 months, knockout mice showed similar body weight, glucose tolerance, and insulin sensitivity as control littermates (Fig. 4F–H). These negative results suggest that improved glucose homeostasis by adenovirus-mediated *Pu.1* KD is not due to loss of hepatocyte Pu.1.

KD of PU.1 in macrophages decreases liver inflammation in *db/db* mice

We next investigated a role of macrophage PU.1 in regulating liver metabolic function. There is accumulating evidence of the involvement of macrophages in the pathogenesis of insulin resistance and T2D,[24–26] and PU.1 has been shown to regulate the entire macrophage genomic landscape.[16,17,27] Consistent with the hypothesis that targeting PU.1 in liver macrophages induces metabolic improvements, *Pu.1*-s6 adenovirus transduces both hepatocytes and macrophages (Fig. S4A), without affecting *Pu.1* expression in B cells, T cells, or HSCs (Fig. S4B). With >80% down-regulation of macrophage *Pu.1* expression, *Pu.1* KD led to the repressed expression of proinflammatory genes tumor necrosis

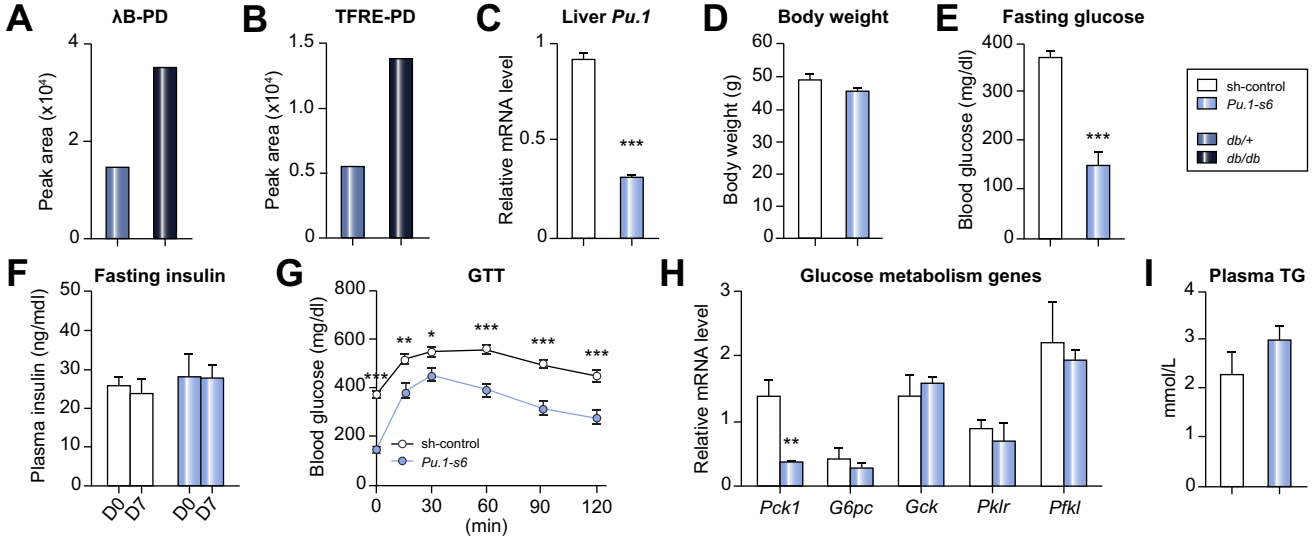


Fig. 3. *Pu.1* KD improves glucose metabolism in *db/db* mice. (A–B) PU.1 protein was enriched by λB-motif (A) or TFRE pull-down (B) from nuclear extracts of diabetic *db/db* mouse liver, and its level was quantified by SWATH analysis. (C–I) 12-week-old male *db/db* mice were injected with adenovirus control/*Pu.1*-shRNA (n = 7/group) for 7 days and their metabolic activities and hepatic gene expression were analyzed. (C) The KD efficiency of liver *Pu.1* was determined by qPCR. (D) Body weight. (E) Fasting blood glucose levels. (F) Fasting plasma insulin levels before and after virus administration. (G) GTT. (H) qPCR analysis of glucose metabolism genes in the liver. (I) Plasma TG levels. Data are presented as mean ± SEM. *p <0.05; **p <0.01; ***p <0.001, *Pu.1*-s6 *vs*. sh-control, by 2-tailed Student's *t* test. GTT, glucose tolerance test; KD, knockdown; shRNA, small hairpin RNA; TFRE, transcription factor response element; TG, triglyceride.

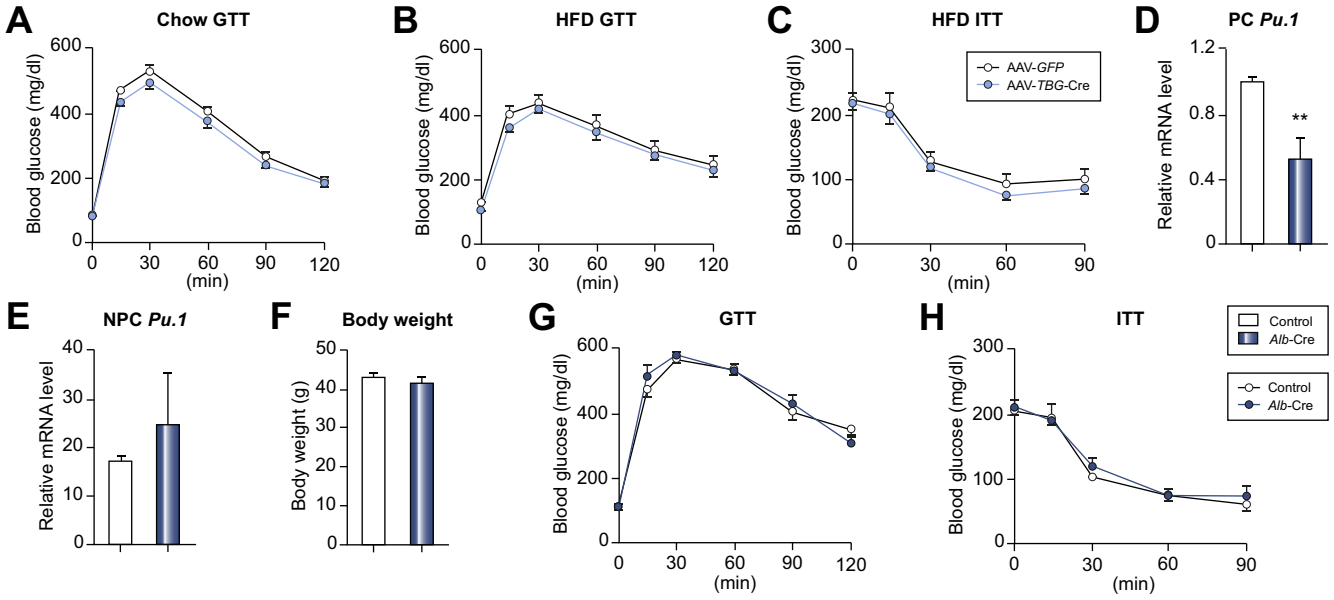


Fig. 4. Hepatocyte-specific knockout of PU.1 has no effect on metabolic activities. Metabolic characterizations of acute and congenital hepatocyte-specific *Pu.1* knockout mice. (A) GTT in chow-fed male *Pu.1*$^{flox/flox}$ mice 5 weeks post injection of AAV-TBG-Cre (n = 9) or AAV-TBG-GFP (as control, n = 8) virus. (B–C) In DIO (HFD-fed for 15 weeks) male *Pu.1*$^{flox/flox}$ mice, GTT, 10% glucose was used (B), and ITT (C) was performed after 4 weeks post AAV administration (n = 9, 10). (D–H) In DIO male *Pu.1*$^{flox/flox}$*:Alb-Cre* (n = 10) and control *Pu.1*$^{flox/flox}$ mice (n = 9), qPCR analysis of *Pu.1* expression in PCs (D) and NPCs (E), ** p <0.01 *vs.* control cells (n = 5, 2-tailed Student's *t* test); (F) body weight; (G) GTT, and (H) ITT. Data are presented as mean ± SEM. AAV, adeno-associated virus; DIO, diet-induced obesity; GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; NPC, non-parenchymal cells; PCs, parenchymal cells.

factor-α (*Tnfa*), interleukin *(Il)-6* and *Il-1b* in both Kupffer cells (KCs) and monocyte-derived macrophages (MoMFs) (Fig. 5A, B) and in the whole liver (Fig. 5C). Consistently, liver TNFα and IL-6 protein levels were both decreased (Fig. 5D).

PU.1 has been suggested to promote alternative macrophage polarization.[28] But anti-inflammatory macrophage markers *Arg1*, *Mrc1*, *Ym1*, and *Il-10* were all increased in isolated KCs and MoMFs by *Pu.1* KD (Fig. S4C, D). The alternative activation of macrophages involves peroxisome proliferator-activated receptor (PPAR)γ and PPARδ, members of the nuclear receptor superfamily.[29] The former regulates mitochondrial biogenesis and β-oxidation of fatty acids in alternatively activated murine macrophages[30]; the latter is absolutely required for the full expression of the effector phenotype of alternative activation of hepatic macrophages in response to IL-4, which ameliorates obesity-induced insulin resistance.[26] Furthermore, it is well known that the bindings of PPARγ and PU.1 on downstream target genes are mutually exclusive.[31] In support of this notion, both *Pparg* and *Ppard* were upregulated about 2-fold in KCs and MoMFs in *Pu.1* KD liver (Fig. S4C, D). To directly investigate the proinflammatory role of PU.1 in macrophages, we treated bone marrow-derived macrophages with lipopolysaccharide (LPS) to stimulate the inflammatory response and observed a 3.5-fold induction of PU.1 (Fig. 5E), comparable to its upregulation in DIO and *db/db* mouse livers. Importantly, concomitant treatment with DB1976, a highly specific heterocyclic diamidine inhibitor of PU.1,[32–35] inhibited its induction and activation of inflammatory markers *Tnfa* and *Il-6* (Fig. 5E). Together, these data indicate that PU.1 regulates macrophage inflammatory response, and KD of PU.1 mitigates liver inflammation and improves glucose homeostasis.

Inhibiting PU.1 by DB1976 improves metabolic dysfunctions in DIO mice

Since KD of PU.1 in liver macrophages improved glucose homeostasis and hepatic inflammation, we reasoned that targeting PU.1 may provide a novel therapy for metabolic diseases. Strikingly, treatment with the PU.1 inhibitor DB1976, reduced fasting glucose (Fig. 6A) and insulin levels (Fig. 6B), leading to normalized glucose tolerance in DIO mice (Fig. 6C). Consistently, hepatic gluconeogenic genes *G6pc* and *Pck1* were markedly repressed in DB1976-treated mice (Fig. 6D), and abnormally high basal Akt phosphorylation in DIO liver[36] and white adipose tissue (WAT) were corrected (Fig. S5A, B), in line with a reduction in fasting insulin levels (Fig. 6B). Improved glucose metabolism was not caused by hepatotoxicity, as serum ALT levels remained constant after DB1976 treatment (Fig. 6E). Instead, consistent with adenoviral KD, PU.1 inhibitor treatment repressed inflammatory genes (Fig. 6F) without observed alterations in liver macrophage infiltration (Fig. 6G). In parallel, macrophage alternative activation markers *Ym1*, and *Pparg* and *Ppard* were upregulated (Fig. S5C). T cell (*Cd3e*) and B cell markers (*Cd19* and *Cd138*) were downregulated (Fig. S5D), indicating reduced immune cell infiltration into the liver. DB1976-treated mice showed a lower body weight owing to reduced fat mass by MRI (Fig. 6H), with reduced visceral epididymal WAT (eWAT) and subcutaneous inguinal WAT (sWAT) deposit sizes (Fig. S5E), and downregulation of adipocyte genes (Fig. S5F). DB1976 treatment did not affect macrophage infiltration into eWAT (Fig. S5F, G), but as in the liver, increased macrophage alternative activation genes (Fig. S5F). Moreover, unlike acute *Pu.1* KD, chronic inhibition of PU.1 restored dyslipidemia associated with DIO (Fig. 6I) without affecting hepatic steatosis (Fig. 6J). Taken together, prolonged

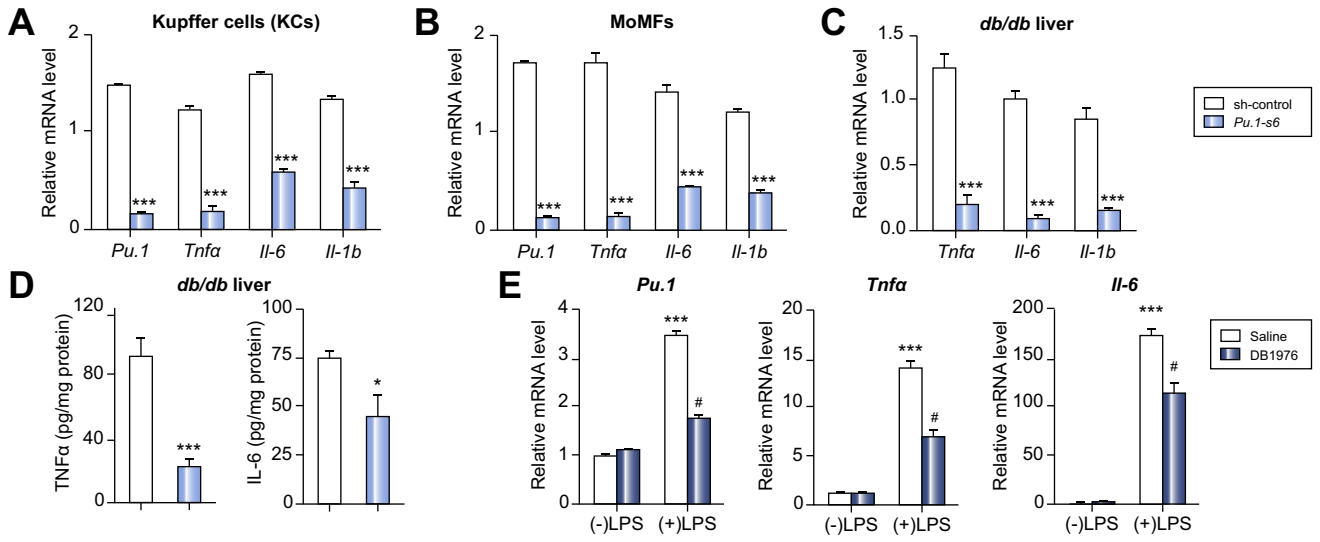


Fig. 5. Inhibiting PU.1 suppresses macrophage inflammatory response in the liver. (A–C) qPCR analyses of gene expression for *Pu.*1 and inflammation markers *Tnfα*, *Il-6*, and *Il-1b* in KCs (n = 4) (A), MoMFs (n = 4) (B), and whole liver (n = 7) (C). (D) The protein levels of inflammatory factors TNFα and IL-6 in the supernatants of homogenates of liver tissues were measured by ELISA (n = 7). $^{*}p$ <0.05; $^{***}p$ <0.001 for *Pu.1-s6 vs.* sh-control (2-tailed Student's *t* test). (E) Bone marrow-derive monocytes differentiated macrophages were stimulated with LPS (100 ng/ml) in the presence or absence of DB1976 (2.5 μM) for 24 hours. qPCR analyses of expression of *Pu.1*, *Tnfα*, and *Il-6*. $^{***}p$ <0.001 *vs.* (−) LPS Saline treatment, $^{\#}p$ <0.05 for DB1976 *vs.* (+) LPS Saline treatment, n = 5–6 (2-tailed Student's *t* test). Data are presented as mean ± SEM. KCs, Kupffer cells; LPS, lipopolysaccharide; MoMFs, monocyte-derived macrophages; sh, small hairpin.

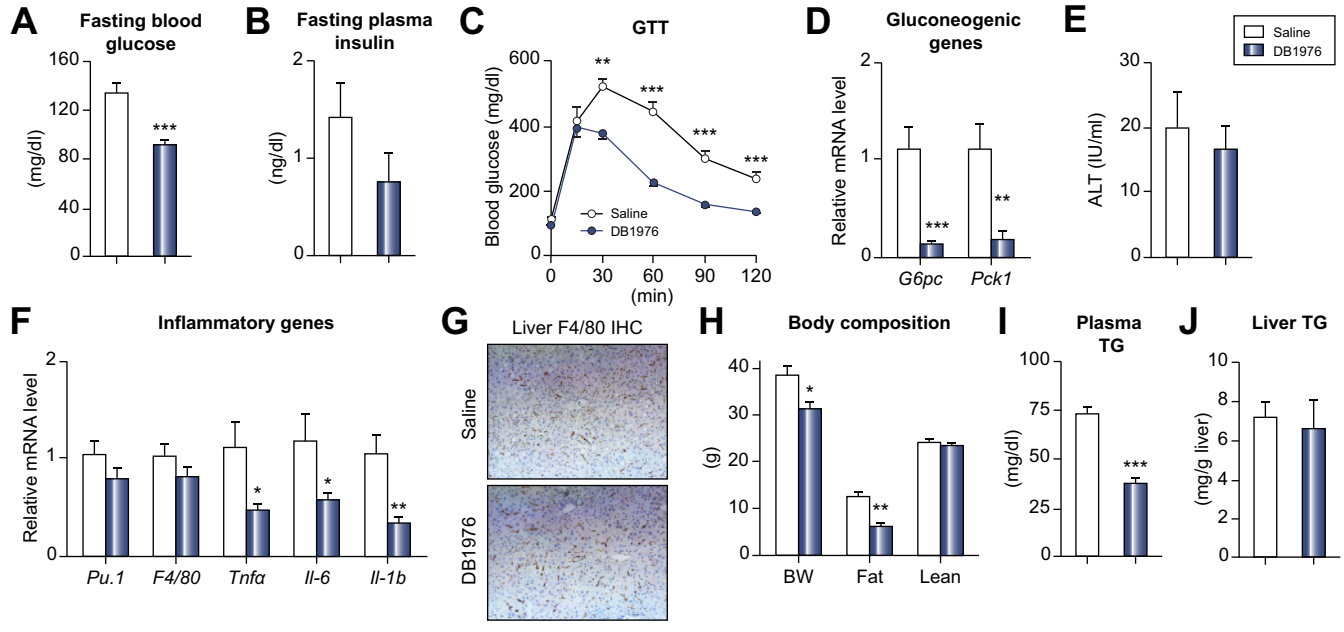


Fig. 6. PU.1 inhibitor DB1976 improves HFD-induced liver dysfunctions. Eight weeks' old male C57BL/6J mice were fed an HFD for 3 months, and then daily injected with either saline or DB1976 (2.5 mg/kg) for 5 weeks. The following parameters and experiments were measured or performed on both saline- and DB1976-treated mice (n = 6, 8). (A) Fasting blood glucose levels; (B) Fasting plasma insulin levels. (C) GTT. (D) qPCR analysis of liver gluconeogenic gene expression. (E) Plasma ALT levels. (F) mRNA expression of liver macrophage markers and inflammatory genes was determined by qPCR. (G) F4/80 immunohistochemical staining of liver sections. (H) Body weight and composition. (I) Plasma TG levels. (J) Liver TG content. Data were presented as mean ± SEM. $^{*}p$ <0.05; $^{**}p$ <0.01 $^{***}p$ <0.001 for DB1976- *vs.* saline-treated group by 2-tailed Student's *t* test. ALT, alanine aminotransferase; GTT, glucose tolerance test; HFD, high-fat diet; TG, triglyceride.

treatment with the PU.1 inhibitor DB1976 recapitulates the amelioration of hepatic inflammation, hyperglycemia, and glucose intolerance observed in *Pu.1* whole liver KD, and further demonstrates a pronounced improvement in adiposity and lipid metabolism.

PU.1 inhibition ameliorates NASH

The progression of NASH is closely related to insulin resistance and liver inflammation.[37,38] We hypothesized that PU.1 could be a drug target for NASH. To test this, we fed C57BL/6J mice a NASH-provoking diet for 10 weeks, then treated them with or

without DB1976 for another 6 weeks on the NASH diet. *Pu.1* was significantly induced in NASH diet-fed mouse liver (Fig. 7A), which was greatly attenuated by DB1976 treatment (Fig. 7B). DB1976 treatment improved glucose tolerance (Fig. 7C). As in DIO mice, inhibitor treatment decreased liver Akt phosphorylation (Fig. S6A). Hepatic steatosis was significantly reduced in DB1976-treated mice (Fig. 7D, E), as were plasma ALT levels (Fig. 7F). As expected, inflammatory genes *Tnfa*, *Il-6*, and *Il-1b* were markedly downregulated by PU.1 inhibition (Fig. 7G), together with reduced macrophage infiltration (Fig. 7H, I). The recently identified NASH-associated macrophage markers *Trem2* and *Cd9*[39] were also significantly downregulated (Fig. 7I). Consistent with the alleviated liver inflammation, the expression of T cell and B cell markers was also reduced (Fig. S6B). Importantly, DB1976 treatment significantly repressed fibrogenic gene expression (Fig. 7J) and decreased liver fibrosis (Fig. 7K). As for DIO mice, DB1976 treatment mildly reduced body weight and adiposity (Fig. S6C, D), associated with decreased macrophage infiltration and repressed adipocyte genes (Fig. S6E, F). These data indicate that inhibition of PU.1 by DB1976 improves multiple NASH-related pathologies.

Liver *PU.1* expression is associated with insulin resistance and inflammation in humans

To determine whether PU.1 is associated with insulin resistance in humans, we analyzed specimens from 38 outpatients undergoing percutaneous liver biopsy. Full demographics are presented in Fig. 8A. Notably, recruited individuals were predominantly male (97%), with BMI in the overweight range (25–30 kg/m^2). Although *PU.1* expression did not vary by age, BMI or abdominal circumference, plasma glucose or cholesterol levels, we found a significant positive correlation between *PU.1* expression and plasma insulin levels (Fig. 8B) or HOMA-IR (Fig. 8C), independent of potential confounding factors. At multivariate analysis, the association between *PU.1* expression and insulin resistance was independent of the demographic features of evaluated individuals, but the association with adiposity was not. Interestingly, in these individuals, we noted that *PU.1* expression was elevated in NASH but not steatosis livers (Fig. 8D) and showed a tight correlation with *TNFA* mRNA level (Fig. 8E), in line with our findings in mice. This study further suggests that PU.1 is involved in the pathogenesis of liver dysfunction in humans.

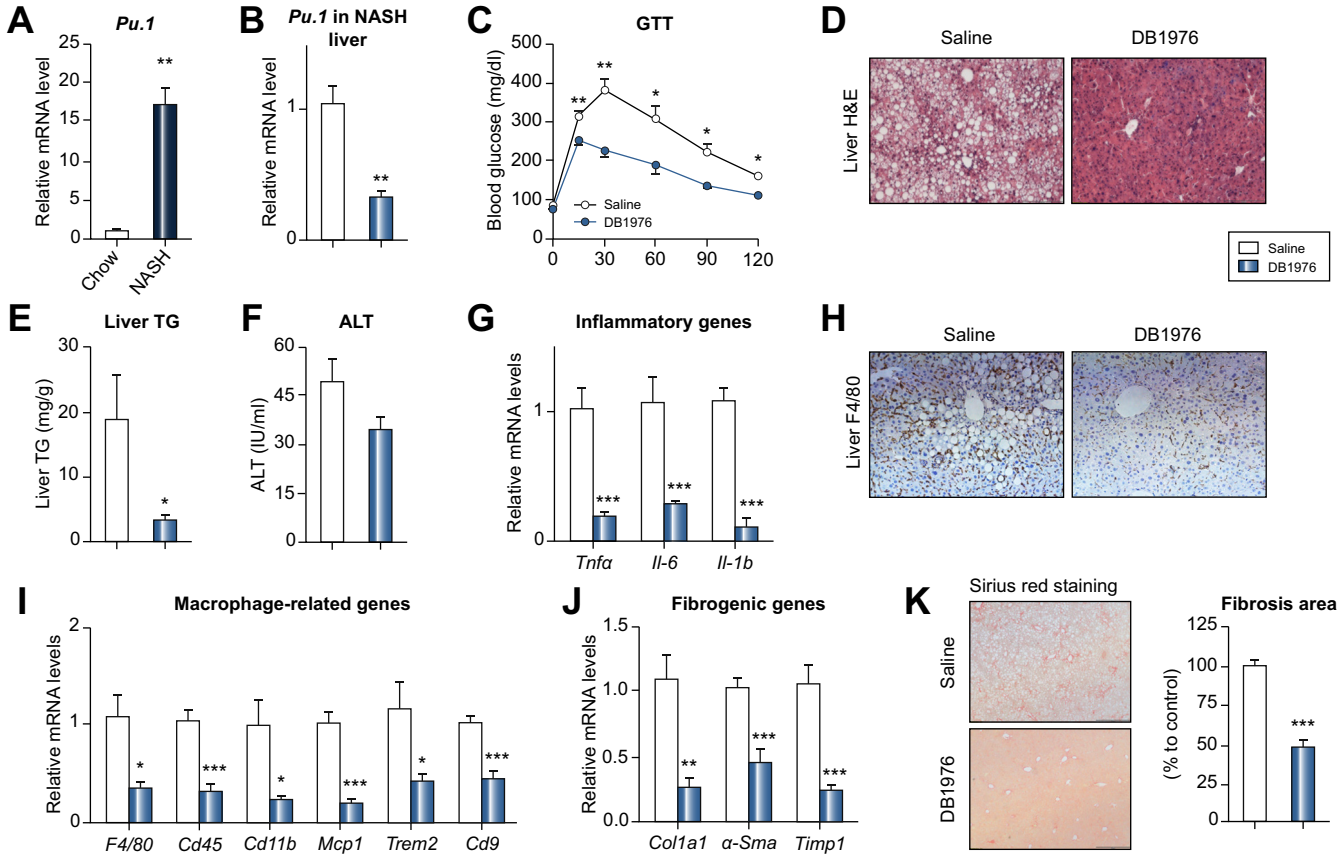


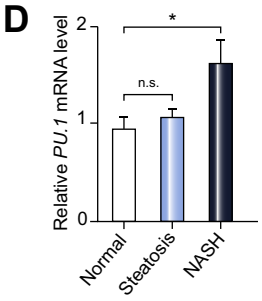
Fig. 7. PU.1 inhibitor ameliorates NASH in mouse model. (A) qPCR analysis of *Pu.1* expression in livers from Chow- or NASH diet-fed mice. 8 weeks' old male C57BL/6J mice were fed on Chow or NASH diet for 16 weeks. $^{**}p$ <0.01 (n = 4). (B–K) 8 weeks' old male C57BL/6J mice were fed on NASH diet for 10 weeks, and then daily injected with either saline (n = 7) or DB1976 (2.5 mg/kg, n = 7) for 6 weeks. (B) Liver *Pu.1* expression. (C) GTT. (D) H&E staining of liver sections. (E) Liver TG content. (F) Plasma ALT levels. (G) Liver inflammatory genes' expression. (H) F4/80 immunohistochemical staining of liver sections. (I) Expression of macrophage-related genes in the liver. (J) mRNA levels of fibrogenic genes. (K) Sirius red staining of liver sections (n = 4, 4), and fibrosis area quantification based on Sirius red staining. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 for DB1976 *vs.* Saline by 2-tailed Student's *t* test. Data are presented as mean ± SEM. ALT, alanine aminotransferase; GTT, glucose tolerance test; NASH, non-alcoholic steatohepatitis; TG, triglyceride.

A

Metabolic parameter	Average ± SD
Sex	31 M, 1 F
Age (yr)	49.5 ± 10.8
BMI (kg/m²)	26.9 ± 3.2
Abdominal circumference (cm)	99.5 ± 44.4
Glucose (mg/dl)	101 ± 24
Insulin (IU/ml)	13.8 ± 7.7
HOMA-IR	3.16 ± 1.87
Total cholesterol (mg/dl)	197 ± 41
Triglycerides (mg/dl)	112 ± 54
HDL (mg/dl)	50 ± 22
LDL (mg/dl)	125 ± 48

B

Metabolic parameter	Correlation	*p*
Glucose (mg/dl)	0.186	0.326
Insulin (IU/ml)	0.467	0.009
HOMA-IR	0.452	0.012
Total cholesterol (mg/dl)	-0.139	0.46
Triglycerides (mg/dl)	0.164	0.39
HDL (mg/dl)	-0.257	0.17
LDL (mg/dl)	-0.07	0.71



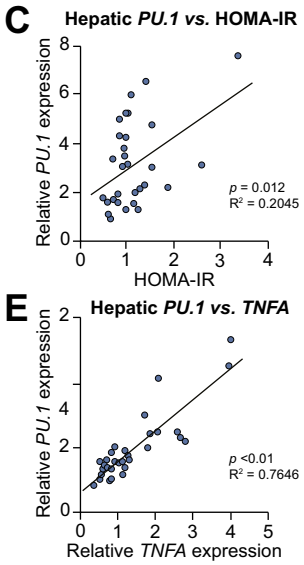


Fig. 8. Liver *PU.1* expression is associated with insulin resistance and inflammation in humans. (A) Demographics of patients for liver *PU.1* measurement. (B) Correlations of liver *PU.1* expression with metabolic parameters. (C) *PU.1* expression is positively correlated with insulin resistance indicator HOMA-IR in human liver biopsies. (D) Liver *PU.1* expression in grouped patients. (E) The positive correlation between *PU.1* expression and the inflammatory marker *TNFA* in human liver biopsies. **p* <0.05 by 2-tailed Student's *t* test, n.s., not significant. Data are presented as mean ± SEM. HOMA-IR, homeostatic model assessment of insulin resistance.

Discussion

Obesity is an established risk factor for the development of T2D and NAFLD/NASH. Our method of TFRE/HRE pull-down and LC-MS/MS provides a powerful approach to identify novel TFs that contribute to these pathogenic changes. The emergence of PU.1 from our survey in the obese liver is surprising considering its traditional role in myeloid cell functions, as are its pronounced detrimental effects on hepatic insulin sensitivity, glucose metabolism, NAFLD and NASH. Our studies reveal PU.1 as a hitherto unknown mediator of the crosstalk between liver inflammation and metabolism, which thus emerges as a novel drug target for metabolic diseases.

The induction of PU.1 in the liver is due to increased hepatocyte *Pu.1* expression and increased macrophage number, although hepatocyte Pu.1's contribution to glucose homeostasis seems negligible, likely due to very low basal expression. In contrast, *Pu.1* is expressed at 200-fold higher amounts in NPCs, primarily macrophages and to a far lesser extent (~1%), B cells. HSCs could be another source of PU.1 as their moderate expression of *Pu.1* was induced in obesity. With respect to T cells, though *Pu.1* was expressed at less than 10% as in macrophages, they may contribute to the induction of PU.1 as their number was increased in obese livers. Last but not least, dendritic cells are a relatively sparse but highly heterogeneous population of antigen-presenting cells in the liver, and they play important roles in liver inflammation and fibrogenesis.[40,41] PU.1 is required for dendritic cell development and function[42]; and hence they could be among the PU.1-targeting cells to contribute to the metabolic improvements. *Pu.1* in B cells, T cells, and HSCs was not changed by *Pu.1* shRNA adenovirus and was thus unlikely to influence glucose homeostasis and inflammation after adenovirus treatment. But we cannot exclude the possibility that the small-molecule PU.1 inhibitor may affect all liver NPC populations, and given the versatility of PU.1 in immune cells as well as the local microenvironment-dependent functions of immune cells, the specific role of PU.1 in different NPCs deserves further study.

In the context of obesity and T2D, both KC activation and the number of MoMFs increase in the liver,[43–45] and they are considered as promising therapeutic targets for liver diseases.[46] Therefore, both MoMFs and KCs likely contribute to the induction of PU.1 in the obese liver. These data are in line with our analysis of a recent comprehensive single-cell RNA-sequencing analysis of NASH NPCs,[39] in which we found that *Pu.1* is predominantly expressed in macrophage clusters and a small subpopulation of dendritic cells (Fig. S7), and the Pu.1-expressing macrophages were markedly increased in NASH NPCs, similar to DIO model. Moreover, its expression largely overlaps with the NASH-associated macrophage marker *Trem2* (Fig. S7B and D), which was decreased by PU.1 inhibition (Fig. 7I). Macrophages display plasticity in their activation programs.[26,47] Accumulating evidence has established the detrimental role of proinflammatory macrophages and the beneficial role of anti-inflammatory macrophages in the progress of obesity-associated hepatic insulin resistance.[24–26] PU.1 appears necessary for the inflammatory activation of BMDM. Targeting PU.1 by shRNA or small-molecule inhibitors reduced the expression of proinflammatory genes (*i.e. Il-6*, *Il-1b*, and *Tnfa*) in the obese liver, but activated expression of anti-inflammatory macrophage markers, perhaps by an increase in PPARγ and PPARδ activity. PPAR activation can switch nutrient utilization to fatty acid oxidation,[48] and counteracts PU.1 to repress the inflammatory response in macrophages.[31] This could suggest that PPARγ (*i.e.*, pioglitazone)[49] and PPARδ (*i.e.*, elafibranor) agonists[50] may improve NASH phenotypes by inhibiting PU.1's activity in macrophages, a hypothesis worth exploring in patient-derived specimens. It would be further reasonable to speculate that PU.1 primes macrophages toward a proinflammatory polarization and the metabolic improvements

observed by targeting PU.1 involve the switch of macrophage polarization, but genetic tools to determine the specific function of PU.1 in KCs and MoMFs require greater characterization. The widely used LysM-Cre line also targets neutrophils and hematopoietic progenitors,[51] which are required for survival, and thus ablation of the essential PU.1 may cause lethality. An inducible Cre model may circumvent the neonatal lethality but remains a blunt tool, non-specifically targeting all macrophages.

The PU.1 inhibitor DB1976 showed similar but more pronounced benefits than *Pu.1* KD. The difference might be caused by the systemic inhibition of PU.1 beyond the liver. For example, during obesity, *Pu.1* is induced in adipocytes and contributes to insulin resistance and chronic inflammation,[52,53] while macrophage infiltration in adipose tissue is often associated with insulin resistance. In support of this notion, DB1976 treatment displayed anti-obesity effects in both DIO and NASH mice, accompanied by the downregulation of adipocyte genes and upregulation of macrophage alternative activated genes. Lean mice do not decrease body weight upon inhibitor treatment,[35] possibly owing to lower tissue macrophage abundance and raising the possibility that DB1976's functionality is condition-dependent.

PU.1 is vital for hematopoiesis and the development of myeloid and lymphatic cells.[14] Thus, the safety of potential PU.1 inhibitor for use in chronic T2D or NASH treatment needs clarification. For instance, although it has been shown that myeloid leukemia shows repressed PU.1, suggestive of a tumor-suppressive phenotype, leukemic cells are actually more vulnerable to PU.1 inhibition because of this partial PU.1 loss-of-function.[34] Thus, PU.1 inhibition by shRNA or small-molecule inhibitors may, in fact, be candidate treatments for leukemia.[19,34] DB1976 is an allosteric PU.1 inhibitor, functioning to disrupt binding of PU.1 to DNA rather than directly binding to PU.1. DB1976 is highly specific to PU.1 without affecting other E26 transformation-specific TFs owning to the unique AT-rich flanking motif in PU.1-binding sites. It has a relatively higher IC50 than other analogs (105 μM *vs.* <8 μM) and thus shows a better safety profile without affecting cell viability.[34] As a consequence, it shows little effect on normal hematopoiesis but rather specifically induces apoptosis and inhibits proliferation in leukemia cells,[34] without effects on hematopoietic stem cells and immune cells including B cells and T cells.[35] Similarly, we observed no side effects during our studies. Nevertheless, we should remain cognizant of these potential effects, and either optimize DB1976 dosing or pursue liver-specific delivery methods to maximize risk/benefit. These studies are important, as our work shows that PU.1 may be a promising therapeutic target for a broad spectrum of metabolic dysfunctions, including T2D and NAFLD/NASH.

Abbreviations

AAV, adeno-associated virus; ALT, alanine aminotransferase; DIO, diet-induced obese; eWAT, epididymal WAT; GTT, glucose tolerance test; HCC, hepatocellular carcinoma; HFD, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; HRE, hormone response element; HSCs, hepatic stellate cells; IL-, interleukin-; ITT, insulin tolerance test; KCs, Kupffer cells; KD, knockdown; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LPS, lipopolysaccharide; MoMFs, monocyte-derived macrophages; MS, mass spectrometry; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NPC, non-parenchymal cells; PCs, parenchymal cells; PPAR, peroxisome proliferator-activated receptor; shRNA, small hairpin RNA; T2D, type 2 diabetes; TF, transcription factors; TFRE, TF response element; TG, triglyceride; TNFα, tumor necrosis factor-α; WAT, white adipose tissue.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Q. Liu and L. Qiang conceived the study; Q. Liu, J. Yu, and Wang L. designed and performed experiments, analyzed the data and wrote the manuscript together with L. Qiang; Q. Zhou and S. Ji performed experiments; Y. Tang and X. Lei provided Pu.1 inhibitor DB1976; L. Santos and P. Rajbhandari analyzed NASH single-cell RNA-sequencing data; U. B. Pajvani, L. Valenti, Y. Wang, R. A. Haeusler, and J. Que edited the manuscript; L. Qiang, U.B. Pajvani, and L. Valenti obtained funding; L. Qiang and J. Qin supervised the study.

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Supplementary data

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Author names in bold designate shared co-first authorship

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