



# Regulation of Cholesterol Sulfotransferase SULT2B1b by Hepatocyte Nuclear Factor 4 $\alpha$ Constitutes a Negative Feedback Control of Hepatic Gluconeogenesis

Yuhan Bi,<sup>a</sup> Xiongjie Shi,<sup>a</sup> Junjie Zhu,<sup>a</sup> Xiudong Guan,<sup>b</sup> Wojciech G. Garbacz,<sup>a</sup> Yixian Huang,<sup>a</sup> Li Gao,<sup>a,c</sup> Jiong Yan,<sup>a</sup> Meishu Xu,<sup>a</sup> Songrong Ren,<sup>a</sup> Shunlin Ren,<sup>d</sup> Yulan Liu,<sup>c</sup> Xiaochao Ma,<sup>a</sup> Song Li,<sup>a</sup> Wen Xie<sup>a,e</sup>

<sup>a</sup>Center for Pharmacogenetics and Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

<sup>b</sup>Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing, China

<sup>c</sup>Department of Gastroenterology, Peking University People's Hospital, Beijing, China

<sup>d</sup>Department of Medicine, Virginia Commonwealth University, Veterans Affairs McGuire Medical Center, Richmond, Virginia, USA

<sup>e</sup>Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

**ABSTRACT** The cholesterol sulfotransferase SULT2B1b converts cholesterol to cholesterol sulfate (CS). We previously reported that SULT2B1b inhibits hepatic gluconeogenesis by antagonizing the gluconeogenic activity of hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ). In this study, we showed that the SULT2B1b gene is a transcriptional target of HNF4 $\alpha$ , which led to our hypothesis that the induction of SULT2B1b by HNF4 $\alpha$  represents a negative feedback to limit the gluconeogenic activity of HNF4 $\alpha$ . Indeed, downregulation of Sult2B1b enhanced the gluconeogenic activity of HNF4 $\alpha$ , which may have been accounted for by the increased acetylation of HNF4 $\alpha$  as a result of decreased expression of the HNF4 $\alpha$  deacetylase sirtuin 1 (Sirt1). The expression of Sult2B1b was also induced by HNF4 $\alpha$  upon fasting, and the Sult2B1b null (Sult2B1b<sup>-/-</sup>) mice showed increased gluconeogenic gene expression and an elevated fasting glucose level, suggesting that SULT2B1b also plays a restrictive role in HNF4 $\alpha$ -mediated fasting-responsive gluconeogenesis. We also developed thiocholesterol, a hydrolysis-resistant derivative of CS, which showed superior activity to that of the native CS in inhibiting gluconeogenesis and improving insulin sensitivity in high-fat-diet-induced diabetic mice. We conclude that the HNF4 $\alpha$ -SULT2B1b-CS axis represents a key endogenous mechanism to prevent uncontrolled gluconeogenesis. Thiocholesterol may be used as a therapeutic agent to manage hyperglycemia.

**KEYWORDS** sulfotransferase, HNF4 $\alpha$ , gluconeogenesis, cholesterol sulfate, thiocholesterol

**T**ype 2 diabetes (T2D) is characterized by an uncontrolled increase of blood glucose. Sustained hyperglycemia can lead to an array of diabetes-associated complications. As such, maintaining a normal blood glucose level is the primary goal in the clinical treatment of T2D, which can reduce the risk of developing diabetes-associated complications (1, 2). The whole-body glucose homeostasis is achieved through an intricate balance between glucose production, mostly by the liver, and glucose uptake by the peripheral tissues (3). Hepatic gluconeogenesis is a major component of hepatic glucose production, the rate of which is controlled by the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and the glucose 6-phosphatase (G6Pase) (4).

Hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) is a liver-enriched orphan nuclear receptor that plays a pivotal role in energy homeostasis by regulating the metabolism of glucose

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Address correspondence to Wen Xie, [wex6@pitt.edu](mailto:wex6@pitt.edu).

and lipids (5, 6). HNF4 $\alpha$  promotes gluconeogenesis through its positive regulation of PEPCK and G6Pase genes (7, 8). Although the HNF4 $\alpha$ -promoted gluconeogenesis is physiologically essential, uncontrolled gluconeogenesis is a major pathogenic event in the development of T2D (9–11). The expression and activity of HNF4 $\alpha$  are elevated in response to fasting, which is essential to maintain the fasting glucose level (9). Increased gluconeogenesis is also responsible for the increased whole-body glucose production in T2D patients after an overnight fasting (10, 11). The activity of HNF4 $\alpha$  can be regulated by posttranslational modifications, including acetylation (12). The acetylation homeostasis of HNF4 $\alpha$  is controlled by both deacetylases, such as sirtuin 1 (Sirt1) (13), and acetylases, such as CBP/p300 (14).

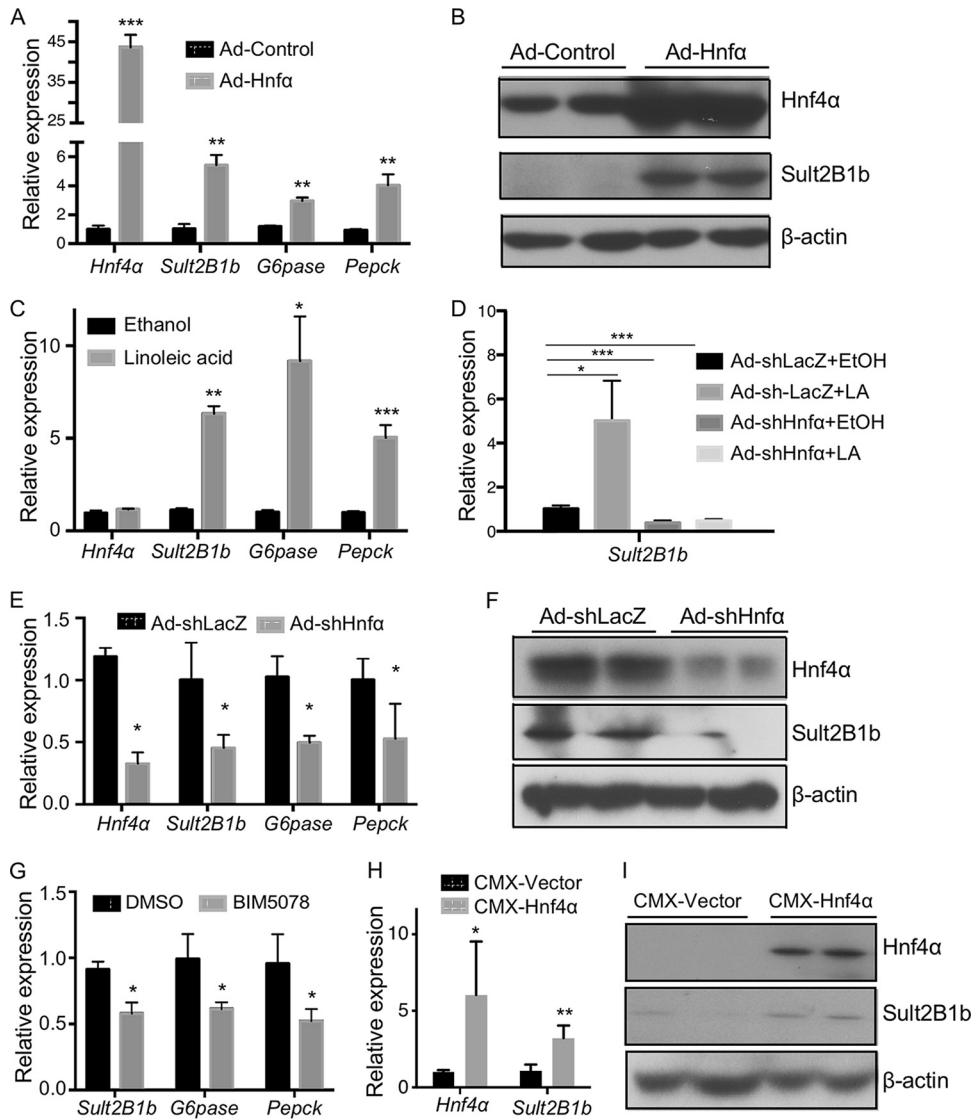
Sulfotransferases (SULTs) catalyze the transfer of a sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to an acceptor molecule (15). Sulfation plays an essential role in regulating the chemical and functional homeostasis of endogenous and exogenous molecules (16–18). Among SULTs, the cholesterol sulfotransferase SULT2B1b preferentially catalyzes the sulfoconjugation of cholesterol to synthesize cholesterol sulfate (CS) (19, 20). Previous studies have suggested a critical role of SULT2B1b in hepatic energy homeostasis. SULT2B1b can inhibit lipogenesis by sulfonating and deactivating oxysterols, the endogenous agonists for the lipogenic nuclear receptor liver X receptor (LXR) (21, 22). The expression and regulation of Sult2B1b were found to be important for the antilipogenic activity of the constitutive androstane receptor (23). We recently reported that SULT2B1b and its enzymatic by-product CS attenuate gluconeogenesis by inhibiting HNF4 $\alpha$  (6).

In this study, we identified SULT2B1b as a novel transcript target of HNF4 $\alpha$ . We propose that the induction of SULT2B1b by HNF4 $\alpha$  constitutes a negative feedback to prevent the uncontrolled gluconeogenesis. Ablation of SULT2B1b increases the HNF4 $\alpha$ -mediated glucose production in hepatocytes and elevates the fasting blood glucose *in vivo*. We also developed the hydrolysis-resistant thiocholesterol (TC), which shows superior activity to that of CS in inhibiting gluconeogenesis and improving overall glucose homeostasis in high-fat diet (HFD)-induced diabetic mice.

## RESULTS

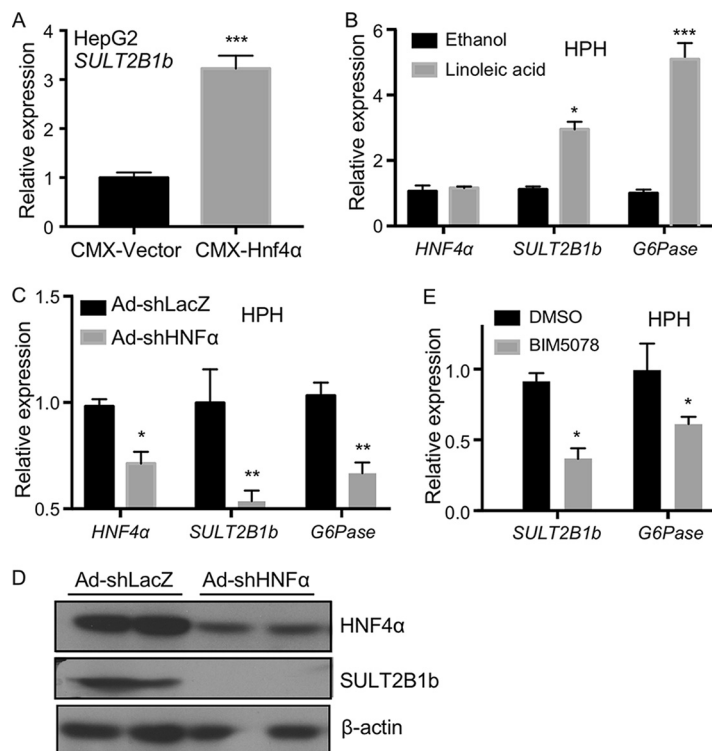
**HNF4 $\alpha$  positively regulates the expression of Sult2B1b in mouse primary hepatocytes and in mouse liver.** We have previously reported that SULT2B1b can negatively regulate the activity of HNF4 $\alpha$  (6). To our surprise, we found that the expression of SULT2B1b was positively regulated by HNF4 $\alpha$ . In the gain-of-function models, infection of primary mouse hepatocytes with adenovirus expressing Hnf4 $\alpha$  (Ad-Hnf4 $\alpha$ ) resulted in the mRNA induction of *Sult2B1b*, as well as the known HNF4 $\alpha$  target genes *G6pase* and *Pepck* (Fig. 1A). The overexpression of Hnf4 $\alpha$  and induction of Sult2B1b were verified at the protein level by Western blotting (Fig. 1B). In a pharmacological gain-of-function model, treatment of primary hepatocytes with linoleic acid, an endogenous Hnf4 activator (24), induced the expression of *Sult2B1b*, *G6pase*, and *Pepck* (Fig. 1C). The induction of *Sult2B1b* by linoleic acid was abolished when the expression of Hnf4 $\alpha$  was downregulated by Hnf4 $\alpha$ -targeting short hairpin RNA (shRNA) (Fig. 1D). Downregulation of Hnf4 $\alpha$  also decreased the basal expression of *Sult2B1b*, *G6pase*, and *Pepck* (Fig. 1D). The shRNA knockdown of Hnf4 $\alpha$  and downregulation of Sult2B1b were verified at the protein level by Western blotting (Fig. 1F). In a pharmacological loss-of-function model, treatment of primary mouse hepatocytes with BIM5078, an inhibitor of HNF4 $\alpha$  (25), downregulated the expression of *Sult2B1b*, *G6pase*, and *Pepck* (Fig. 1G). *In vivo*, hydrodynamic transfection of the mouse liver with the Hnf4 $\alpha$  expression vector increased the hepatic expression of Sult2B1b at both the mRNA (Fig. 1H) and the protein (Fig. 1I) levels.

**HNF4 $\alpha$  induces the expression of SULT2B1b in human liver cells.** The positive regulation of SULT2B1b by HNF4 $\alpha$  is conserved in human liver cells. In the human hepatoma HepG2 cells, transfection with the HNF4 $\alpha$  expression vector increased the expression of *SULT2B1b* (Fig. 2A). In human primary hepatocytes (HPH), treatment of cells with the HNF4 $\alpha$  activator linoleic acid induced the expression of both *SULT2B1b*



**FIG 1** HNF4 $\alpha$  positively regulates the expression of SULT2B1b in mouse primary hepatocytes and in mouse liver. (A to G) Experiments were conducted using primary hepatocytes isolated from 8-week-old WT male mice. (A) Relative mRNA expression of *Hnf4 $\alpha$* , *Sult2B1b*, *G6pase*, and *Pepck* in hepatocytes infected with adenovirus encoding Hnf4 $\alpha$  or the control virus as measured by real-time PCR. The expression of each gene was arbitrarily set as 1 in hepatocytes infected with the control virus. (B) Cells were the same as described for panel A. The protein expression of Hnf4 $\alpha$  and SULT2B1b was measured by Western blotting. (C) Relative mRNA expression of *Hnf4 $\alpha$* , *Sult2B1b*, *G6pase*, and *Pepck* in hepatocytes treated with the ethanol control or linoleic acid (25  $\mu$ M) for 24 h. The expression of each gene was arbitrarily set as 1 in cells treated with ethanol. (D) Hepatocytes were first infected with adenovirus encoding shHnf4 $\alpha$  or the control virus for 24 h before being treated with ethanol or linoleic acid (25  $\mu$ M) for another 24 h. The expression of *Sult2B1b* was measured by real-time PCR. (E and F) Hepatocytes were first infected with adenovirus encoding shHnf4 $\alpha$  or the control virus for 24 h, and the gene expression was evaluated by real-time PCR (E) and Western blotting (F), respectively. (G) Relative mRNA expression of *Sult2B1b*, *G6pase*, and *Pepck* in hepatocytes treated with vehicle (dimethyl sulfoxide [DMSO]) or the Hnf4 $\alpha$  inhibitor BIM5078 (20  $\mu$ M) for 24 h. (H and I) Eight-week-old WT male mouse livers were hydrodynamically transfected with the empty vector or the pCMX-Hnf4 $\alpha$  expression plasmid. The hepatic expressions of Hnf4 $\alpha$  and SULT2B1b mRNA (H) and protein (I) were measured by real-time PCR and Western blotting, respectively. Results are expressed as means  $\pm$  SD from three independent experiments (5 mice per group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

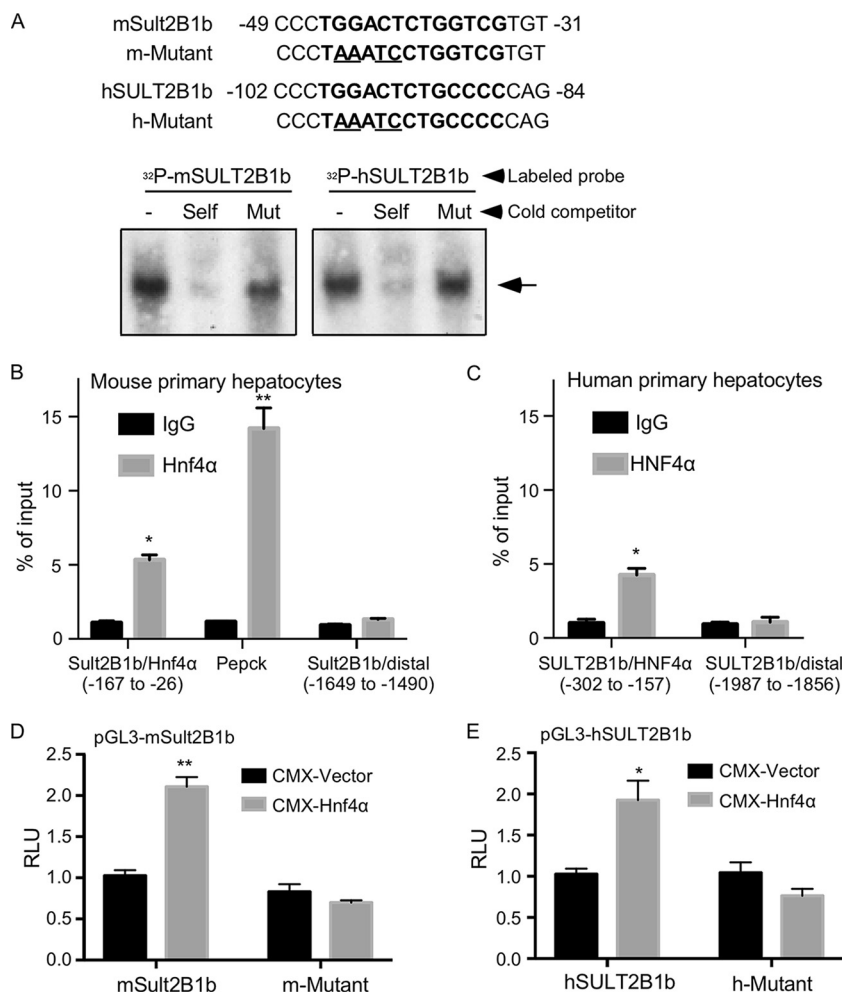
and *G6Pase* (Fig. 2B). In contrast, the basal mRNA expression of *SULT2B1b* and *G6Pase* was decreased when the expression of HNF4 $\alpha$  was knocked down by shRNA (Fig. 2C). The shRNA knockdown of HNF4 $\alpha$  and downregulation of SULT2B1b in human primary hepatocytes were verified at the protein level by Western blotting (Fig. 2D). The basal expression levels of *SULT2B1b* and *G6Pase* were decreased in human primary hepatocytes treated with the HNF4 $\alpha$  inhibitor BIM5078 (Fig. 2E).



**FIG 2** HNF4 $\alpha$  induces the expression of SULT2B1b in human liver cells. (A) Relative mRNA expression of *SULT2B1b* in HepG2 cells transfected with the empty vector or the Hnf4 $\alpha$ -expressing plasmid. The expression of each gene was arbitrarily set as 1 in cells transfected with the empty vector. (B) Relative mRNA expression of *HNF4 $\alpha$* , *SULT2B1b*, and *G6Pase* in human primary hepatocytes (HPH) treated with ethanol or linoleic acid (25  $\mu$ M) for 24 h. The expression of each gene was arbitrarily set as 1 in cells treated with ethanol. (C) mRNA expression of *HNF4 $\alpha$* , *SULT2B1b*, and *G6Pase* in HPH infected with adenovirus encoding shHNF4 $\alpha$  or the control virus. The expression of each gene was arbitrarily set as 1 in cells infected with the control virus. (D) Cells were the same as described for panel C. The protein expression of HNF4 $\alpha$  and SULT2B1b was measured by Western blotting. (E) Relative mRNA expression of *SULT2B1b* and *G6Pase* in HPH treated with the vehicle (DMSO) or the HNF4 $\alpha$  inhibitor BIM5078 (20  $\mu$ M) for 24 h. The expression of each gene was arbitrarily set as 1 in cells treated with DMSO. Results are expressed as means  $\pm$  SD from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

### The mouse and human *SULT2B1b* genes are transcriptional targets of HNF4 $\alpha$ .

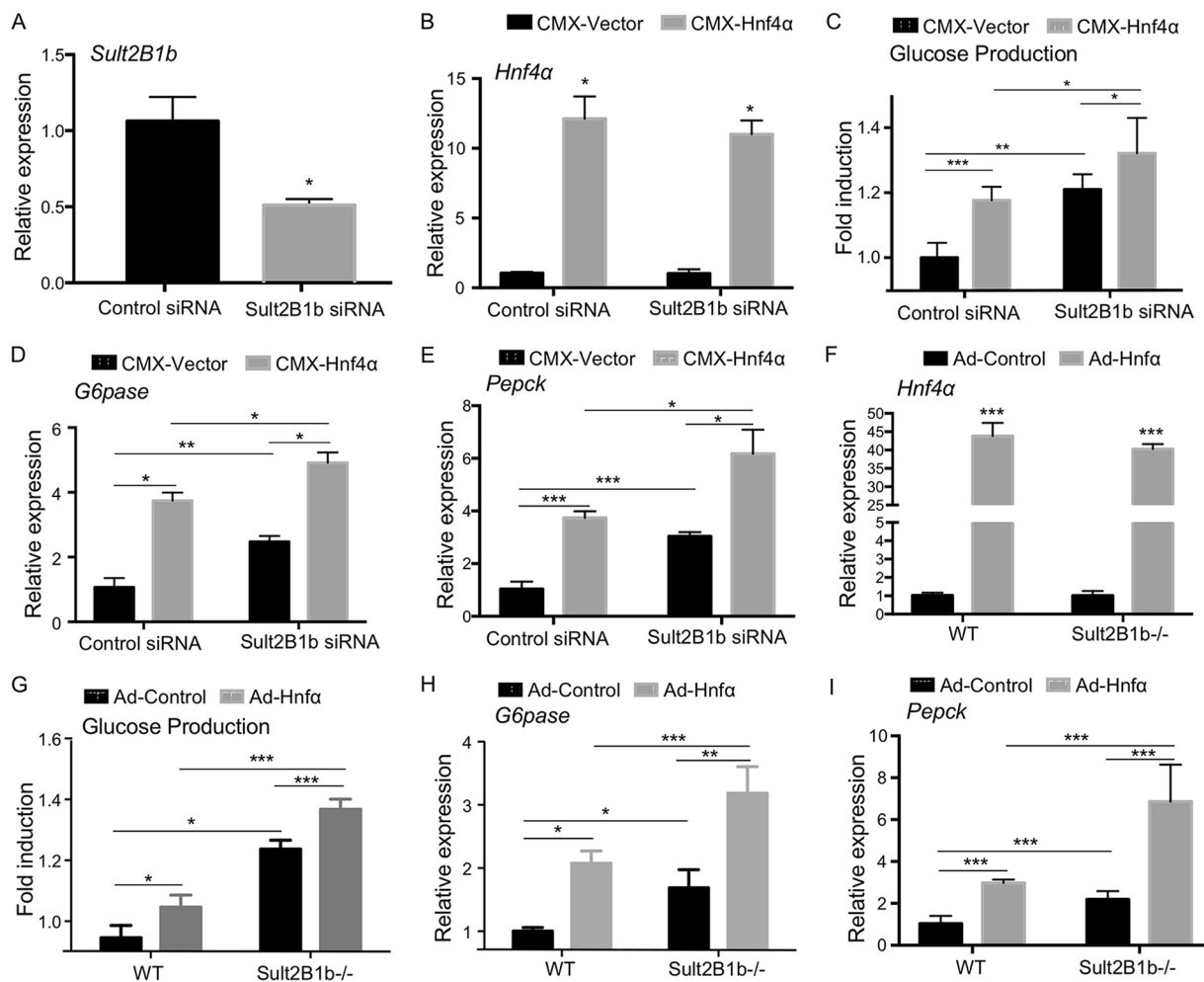
The regulation of mouse and human *SULT2B1b* by HNF4 $\alpha$  strongly suggested that the *SULT2B1b* gene is a direct transcriptional target of HNF4 $\alpha$ . As a transcriptional factor, HNF4 $\alpha$  often regulates gene expression by binding to the promoter regions of its target genes (26). Bioinformatic inspection of the mouse and human *SULT2B1b* gene promoters revealed a putative HNF4 $\alpha$  binding site on each of the promoter (Fig. 3A, top panel). The binding of HNF4 $\alpha$  to these two putative sites was confirmed by electrophoretic mobility shift assay (EMSA) (Fig. 3A, bottom panel). The binding was specific in that the binding was efficiently competed by unlabeled wild-type (WT) binding sites ("self") but not by their mutant variants. The recruitment of HNF4 $\alpha$  to the mouse *Sult2B1b* gene promoter was confirmed by chromatin immunoprecipitation (ChIP) assay on mouse primary hepatocytes (Fig. 3B), in which the recruitment of Hnf4 $\alpha$  to its known binding site in the *Pepck* gene promoter and a distal region in the *Sult2B1b* gene promoter were included as the positive and negative controls, respectively (Fig. 3B). The recruitment of HNF4 $\alpha$  to the human *SULT2B1b* gene promoter was confirmed by ChIP assay on human primary hepatocytes (Fig. 3C). To determine whether the mouse and human *SULT2B1b* gene promoters can be transactivated by HNF4 $\alpha$  and the functional relevance of the HNF4 $\alpha$  binding sites, we cloned the mouse and human *SULT2B1b* gene promoters, as well as their mutant variants in which the HNF4 $\alpha$  binding sites were mutated by site-directed mutagenesis. Both the mouse (Fig. 3D) and human (Fig. 3E) *SULT2B1b* gene promoter luciferase reporter genes were activated by the



**FIG 3** The mouse and human *SULT2B1b* genes are transcriptional targets of HNF4 $\alpha$ . (A) Sequences of the predicted HNF4 $\alpha$  binding sites in the mouse and human *SULT2B1b* gene promoters and their mutant variants (top). The bindings of HNF4 $\alpha$  to the putative HNF4 $\alpha$  binding sites were shown by electrophoretic mobility shift assay (EMSA) (bottom). (B and C) ChIP assay showing the recruitment of HNF4 $\alpha$  onto the mouse (C) and human (D) *SULT2B1b* gene promoters. (D and E) Activation of the mouse (D) and human (E) *SULT2B1b* promoter reporter genes by HNF4 $\alpha$ . Cells were cotransfected with the indicated WT or mutant reporter genes, and receptors were monitored by luciferase assay. Results are expressed as means  $\pm$  SD from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

cotransfection of HNF4 $\alpha$ , but the activation was abolished when the HNF4 $\alpha$  binding sites were mutated.

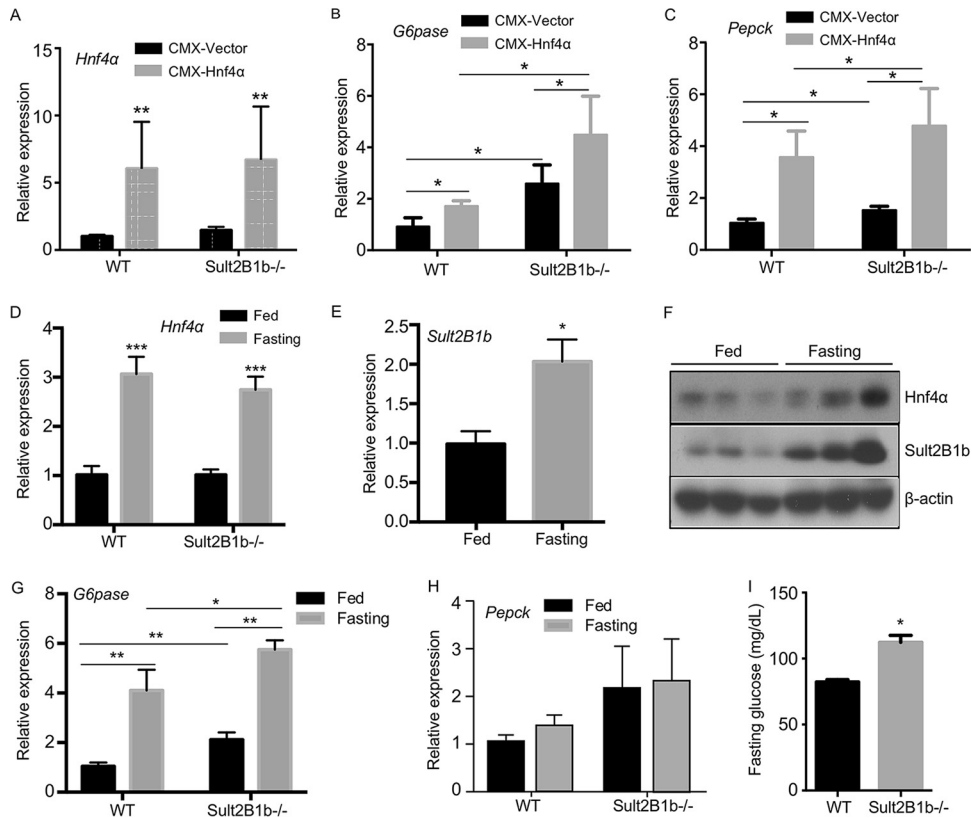
**Downregulation or ablation of Sult2B1b increases the gluconeogenic activity of Hnf4 $\alpha$  in mouse primary hepatocytes.** Having shown that *SULT2B1b* is an HNF4 $\alpha$  target gene and knowing that *SULT2B1b* can inhibit the gluconeogenic activity of HNF4 $\alpha$ , we hypothesized that the induction of *SULT2B1b* by HNF4 $\alpha$  may represent a negative feedback to limit the gluconeogenic activity of HNF4 $\alpha$ . Based on this hypothesis, we predicted that downregulation or ablation of *SULT2B1b* will increase the gluconeogenic activity of HNF4 $\alpha$  due to the lack of *SULT2B1b*-mediated inhibition. We first tested our hypothesis *in vitro* using mouse primary hepatocytes. In this experiment, hepatocytes were transiently transfected with the *Sult2B1b*-targeting small interfering RNA (siRNA) or the nontargeting control siRNA, in the presence or absence of the cotransfection of HNF4 $\alpha$ , followed by the measurement of forskolin (FSK)-responsive glucose production and the expression of gluconeogenic genes. The knockdown of *Sult2B1b* (Fig. 4A) and overexpression of *Hnf4 $\alpha$*  (Fig. 4B) were confirmed by real-time PCR (RT-PCR). As shown in Fig. 4C, knockdown of *Sult2B1b* increased the basal and



**FIG 4** Downregulation or ablation of Sult2B1b increases the gluconeogenic activity of Hnf4 $\alpha$  in mouse primary hepatocytes. (A) Hepatocytes isolated from 8-week-old WT male mice were transfected with scrambled or Sult2B1b-targeting siRNA for 48 h. The downregulation of *Sult2B1b* was verified by real-time PCR. The expression was arbitrarily set as 1 in cells transfected with scrambled siRNA. (B to E) Relative mRNA expression of *Hnf4 $\alpha$*  (B), *G6pase* (D), and *Pepck* (E) and glucose production (C) in hepatocytes transfected with scrambled or Sult2B1b-targeting siRNA for 24 h followed by transfection with the empty vector or the pCMX-Hnf4 $\alpha$ -expressing vector for another 24 h. The expression of each gene or glucose production was arbitrarily set as 1 in cells transfected with scrambled siRNA followed by transfection with the empty vector. (F to I) Relative mRNA expression of *Hnf4 $\alpha$*  (F), *G6pase* (H), and *Pepck* (I) and glucose production (G) in hepatocytes isolated from 8-week-old male WT or Sult2B1b<sup>-/-</sup> mice infected with adenovirus encoding Hnf4 $\alpha$  or the control virus. The expression of each gene or glucose production was arbitrarily set as 1 in cells isolated from WT mice infected with the control virus. Results are expressed as means  $\pm$  SD from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

Hnf4 $\alpha$ -responsive glucose production, which was accompanied by increased expression of *G6pase* (Fig. 4D) and *Pepck* (Fig. 4E). In another independent loss-of-function model, we infected hepatocytes isolated from the WT and Sult2B1b<sup>-/-</sup> mice with control adenovirus (Ad-Control) or Ad-Hnf4 $\alpha$ . The expression of transduced *Hnf4 $\alpha$*  was verified by real-time PCR (Fig. 4F). Again, increased Hnf4 $\alpha$ -responsive glucose production (Fig. 4G) and expression of *G6pase* (Fig. 4H) and *Pepck* (Fig. 4I) were observed in Sult2B1b<sup>-/-</sup> hepatocytes compared to what was seen in hepatocytes isolated from the WT mice.

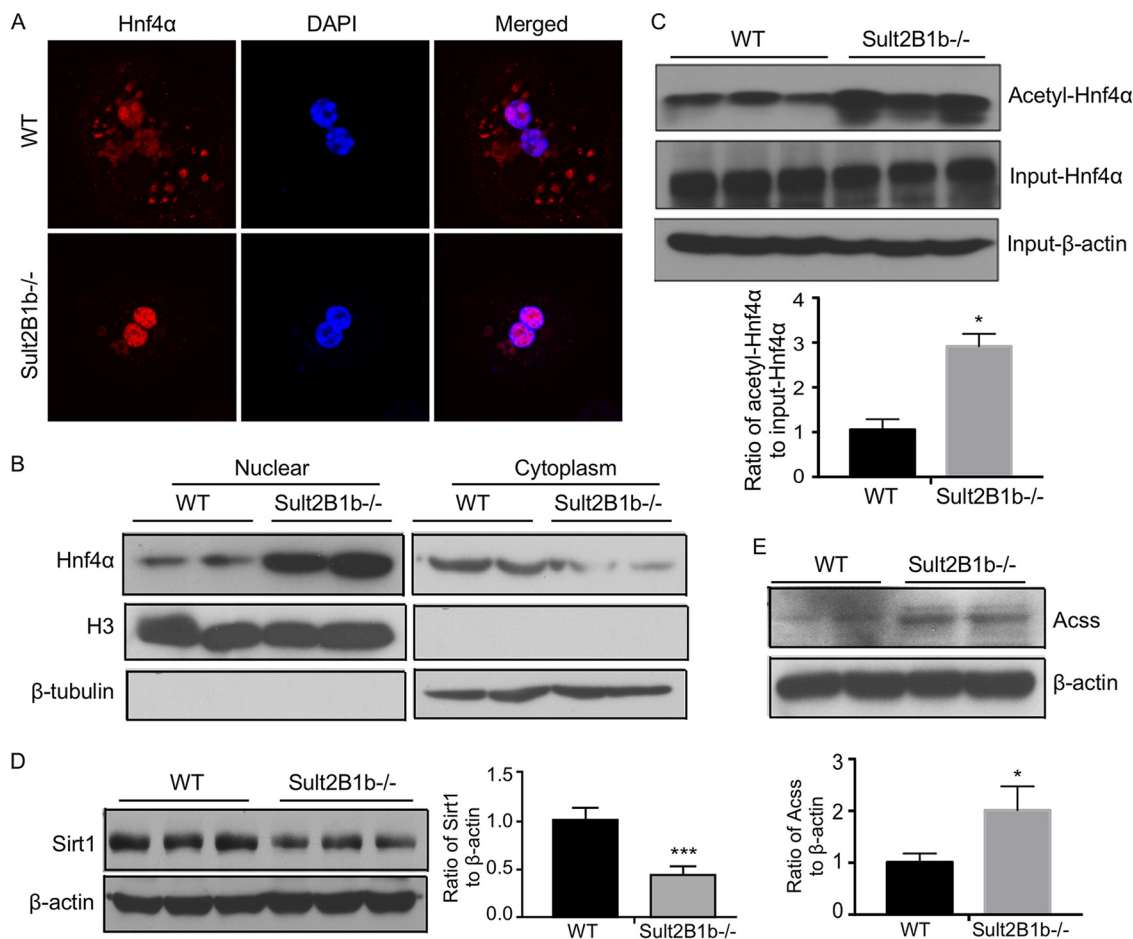
**Ablation of Sult2B1b increases the gluconeogenic activity of Hnf4 $\alpha$  in vivo, and Sult2B1b<sup>-/-</sup> mice exhibit elevated fasting blood glucose levels.** To determine the effect of Sult2B1b ablation on Hnf4 $\alpha$  activity *in vivo*, WT and Sult2B1b<sup>-/-</sup> mice were hydrodynamically transfected with the empty vector or the Hnf4 $\alpha$  expression vector. The overexpression of *Hnf4 $\alpha$*  in both genotypes was confirmed by real-time PCR (Fig. 5A). The Sult2B1b<sup>-/-</sup> mice showed increased basal and Hnf4 $\alpha$ -responsive expression of *G6pase* (Fig. 5B) and *Pepck* (Fig. 5C).



**FIG 5** Ablation of *Sult2B1b* increases the gluconeogenic activity of *Hnf4 $\alpha$*  *in vivo*, and *Sult2B1b*<sup>-/-</sup> mice exhibit elevated fasting blood glucose levels. Eight-week-old WT and *Sult2B1b*<sup>-/-</sup> male mice were used. (A to C) Relative hepatic mRNA expression of *Hnf4 $\alpha$*  (A), *G6pase* (B), and *Pepck* (C) in the livers of WT and *Sult2B1b*<sup>-/-</sup> mice hydrodynamically transfected with the empty vector or the *Hnf4 $\alpha$* -expressing vector. The expression of each gene was arbitrarily set as 1 in mice transfected with the empty vector. (D to H) Relative hepatic expressions of *Hnf4 $\alpha$*  (D), *Sult2B1b* (E), *Hnf4 $\alpha$*  and *Sult2B1b* (F), *G6pase* (G), and *Pepck* (H) in fed and fasted WT and *Sult2B1b*<sup>-/-</sup> mice were measured by real-time PCR (D, E, G, and H) or Western blotting (F). The expression of each gene was arbitrarily set as 1 in fed WT mice. (I) Fasting glucose levels.  $n = 5$  mice per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

Fasting is known to induce the expression and activity of *Hnf4 $\alpha$*  and increase gluconeogenesis (9). To determine the effect of *Sult2B1b* ablation on fasting-responsive gluconeogenesis, WT and *Sult2B1b*<sup>-/-</sup> mice maintained on a chow diet were fasted for 24 h. As shown in Fig. 5D, fasting induced the expression of *Hnf4 $\alpha$*  in both genotypes. The induction of *Hnf4 $\alpha$*  in WT mice was accompanied by the induction of *Sult2B1b* (Fig. 5E), consistent with the notion that *Sult2B1b* is a transcriptional target of *Hnf4 $\alpha$* . The fasting-responsive induction of *Hnf4 $\alpha$*  and *Sult2B1b* protein expression was verified by Western blotting (Fig. 5F). The fasting-responsive expression of *G6pase* (Fig. 5G) but not of *Pepck* (Fig. 5H) was elevated in *Sult2B1b*<sup>-/-</sup> mice compared to their WT counterparts. Moreover, the *Sult2B1b*<sup>-/-</sup> mice exhibited higher levels of fasting glucose (Fig. 5I), which may have been contributed to by the increased gluconeogenesis.

**Ablation of *Sult2B1b* increases the acetylation of *Hnf4 $\alpha$*  by suppressing the *Hnf4 $\alpha$*  deacetylase *Sirt1*.** The transcriptional activity of HNF4 $\alpha$  can be regulated by acetylation. Acetylation of HNF4 $\alpha$  activates HNF4 $\alpha$  by promoting its nuclear translocation (6, 12). We first used immunofluorescence to determine whether *Sult2B1b* ablation impacted the subcellular localization of *Hnf4 $\alpha$*  in primary mouse hepatocytes. Indeed, compared to the WT hepatocytes, in which *Hnf4 $\alpha$*  was distributed in both the cytoplasm and nucleus, *Hnf4 $\alpha$*  was almost exclusively nuclear in hepatocytes isolated from the *Sult2B1b*<sup>-/-</sup> mice (Fig. 6A). The effect of *Sult2B1b* ablation on the subcellular distribution of *Hnf4 $\alpha$*  was further confirmed by nuclear-cytosolic fractionation of the liver and Western blotting (Fig. 6B). To examine the effect of *Sult2B1b* ablation on the acetylation of *Hnf4 $\alpha$* , hepatocyte lysates were immunoprecipitated with an anti-acetyl

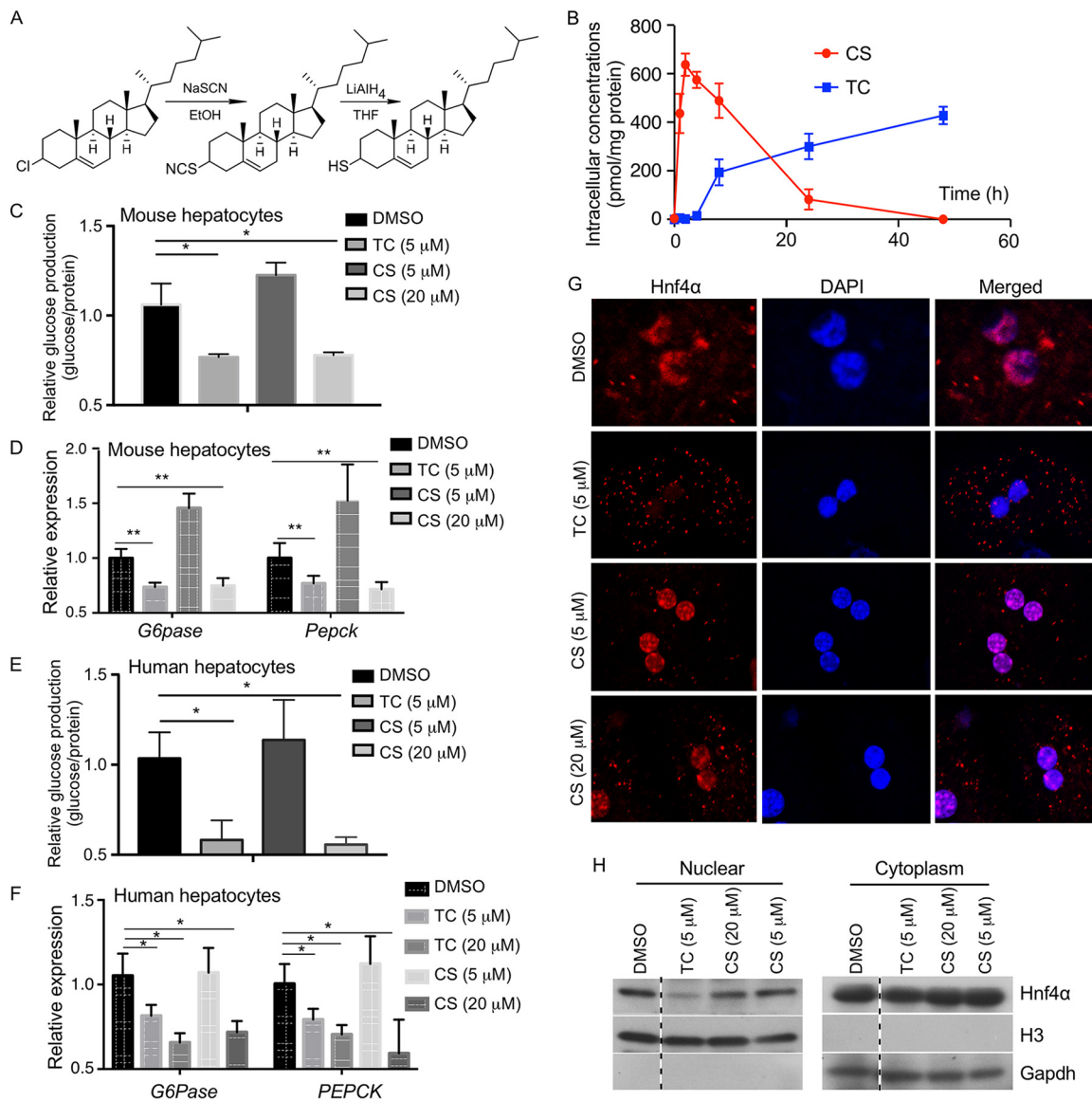


**FIG 6** Ablation of Sult2B1b increases the acetylation of Hnf4 $\alpha$  by suppressing the Hnf4 $\alpha$  deacetylase Sirt1. Eight-week-old WT and Sult2B1b<sup>-/-</sup> male mice were used. (A) The subcellular distribution of Hnf4 $\alpha$  was visualized by immunofluorescence using an anti-Hnf4 $\alpha$  antibody (red) in primary hepatocytes isolated from WT and Sult2B1b<sup>-/-</sup> mice. DAPI (4',6-diamidino-2-phenylindole; blue) was used for nuclear counterstaining. (B) Western blot analysis of the Hnf4 $\alpha$  protein levels in the nuclear and cytoplasmic fractions of WT and Sult2B1b<sup>-/-</sup> mouse liver. The purity of the nuclear and cytoplasmic fractions was confirmed by immunoblotting with histone H3 antibody (a nuclear marker) and  $\beta$ -tubulin antibody (a cytoplasmic marker), respectively. (C) Liver lysates of WT and Sult2B1b<sup>-/-</sup> mice were immunoprecipitated with an anti-acetyl lysine antibody before immunoblotting with an anti-Hnf4 $\alpha$  antibody. Shown below the blot is the densitometric quantification of the Western blotting results. (D and E) The hepatic expression levels of Sirt1 (D) and Acss (E) proteins were measured by Western blotting. Shown on the right of (D) or below (E) the blot is the densitometric quantification of the Western blotting results. Results are expressed as means  $\pm$  SD. \*,  $P < 0.05$ .

lysine antibody before immunoblotting with an anti-Hnf4 $\alpha$  antibody. As shown in Fig. 6C, the acetylation of Hnf4 $\alpha$  was increased in hepatocytes isolated from the Sult2B1b<sup>-/-</sup> mice. The increased acetylation of Hnf4 $\alpha$  in Sult2B1b<sup>-/-</sup> mice was associated with a decreased expression of Sirt1, a NAD<sup>+</sup>-dependent deacetylase that can deacetylate HNF4 $\alpha$  (13) (Fig. 6D), as well as an increased expression of acetyl coenzyme A (acetyl-CoA) synthetase (Acss) (Fig. 6E), the enzyme that catalyzes the formation of the acetyl donor acetyl-CoA (27).

**Thiocholesterol shows an improved intracellular stability and better efficacy in inhibiting gluconeogenesis in primary hepatocytes.** We previously reported that CS can inhibit Hnf4 $\alpha$ -mediated gluconeogenesis, which is an integral component of Hnf4 $\alpha$ - and Sult2B1b-mediated negative feedback regulation of gluconeogenesis. However, CS is readily hydrolyzed by the steroid sulfatase, limiting its utility as a therapeutic agent (28). We then synthesized thiocholesterol (TC), a structural analog of CS generated by replacing the sulfate with a thiol group that is predicted to be hydrolysis resistant (Fig. 7A). We hypothesized that TC may mimic the antigluconeogenic activity of CS but with a superior bioavailability due to its resistance to hydrolysis. To compare the intracellular stabilities of TC and CS, mouse primary hepatocytes were treated with





**FIG 7** Thiocholesterol (TC) shows an improved intracellular stability and better efficacy in inhibiting gluconeogenesis in primary hepatocytes. (A) Schematic depiction of the synthesis of TC from cholesteryl chloride. (B) Mouse primary hepatocytes isolated from 8-week-old male WT mice were treated with 5  $\mu$ M TC or CS for up to 48 h, and the intracellular concentrations of TC and CS were measured by using UPLC-mass spectrometry. (C and D) Glucose production (C) and expression of gluconeogenic genes (D) in mouse primary hepatocytes treated with the indicated concentrations of drugs for 24 h. Cells were treated with 10  $\mu$ M FSK for 2.5 h before the glucose production assay. The expression of each gene or glucose production was arbitrarily set as 1 in cells treated with DMSO. (E and F) The designs of the experiments were similar to those described for panels C and D, except that human primary hepatocytes were used. (G) The subcellular distribution of Hnf4 $\alpha$  was visualized by immunofluorescence using an anti-Hnf4 $\alpha$  antibody (red) in primary hepatocytes isolated from WT mice and treated with the indicated concentrations of drugs. DAPI (blue) was used for nuclear counterstaining. (H) The subcellular distribution of Hnf4 $\alpha$  was measured by nuclear-cytosolic fractionation of the hepatocytes and Western blotting. Histone H3 and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) are nuclear and cytoplasmic markers, respectively. All four lanes are from the same blot, with the dotted lines indicating noncontinuous lanes. Results are expressed as means  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

5  $\mu$ M TC or CS, and their intracellular concentrations were monitored over a 48-h period. As shown in Fig. 7B, in CS-treated cells, the intracellular concentration of CS peaked at 4 h but declined steadily thereafter, reaching a very low level after 24 h. In contrast, the intracellular concentration of TC continued rising until 48 h after the drug treatment. At the functional level, treatment of mouse primary hepatocytes with 5  $\mu$ M TC but not 5  $\mu$ M CS was effective in reducing FSK-stimulated glucose production (Fig. 7C) and inhibiting the expression of *G6pase* and *Pepck* (Fig. 7D). CS at 20  $\mu$ M could achieve an inhibitory effect similar to that obtained with 5  $\mu$ M TC (Fig. 7C and D). A

similar pattern of superior inhibitory effects of TC on glucose production (Fig. 7E) and gluconeogenic gene expression (Fig. 7F) was observed in human primary hepatocytes. Thiocholesterol was also more efficient than CS in inducing the nuclear exclusion of Hnf4 $\alpha$  in mouse primary hepatocytes as shown by immunofluorescence (Fig. 7G) and nuclear-cytosolic fractionation of the hepatocytes and Western blotting (Fig. 7H). Neither TC nor CS affected the mRNA expression of Hnf4 $\alpha$  in mouse primary hepatocytes as measured by real-time (RT) PCR (data not shown).

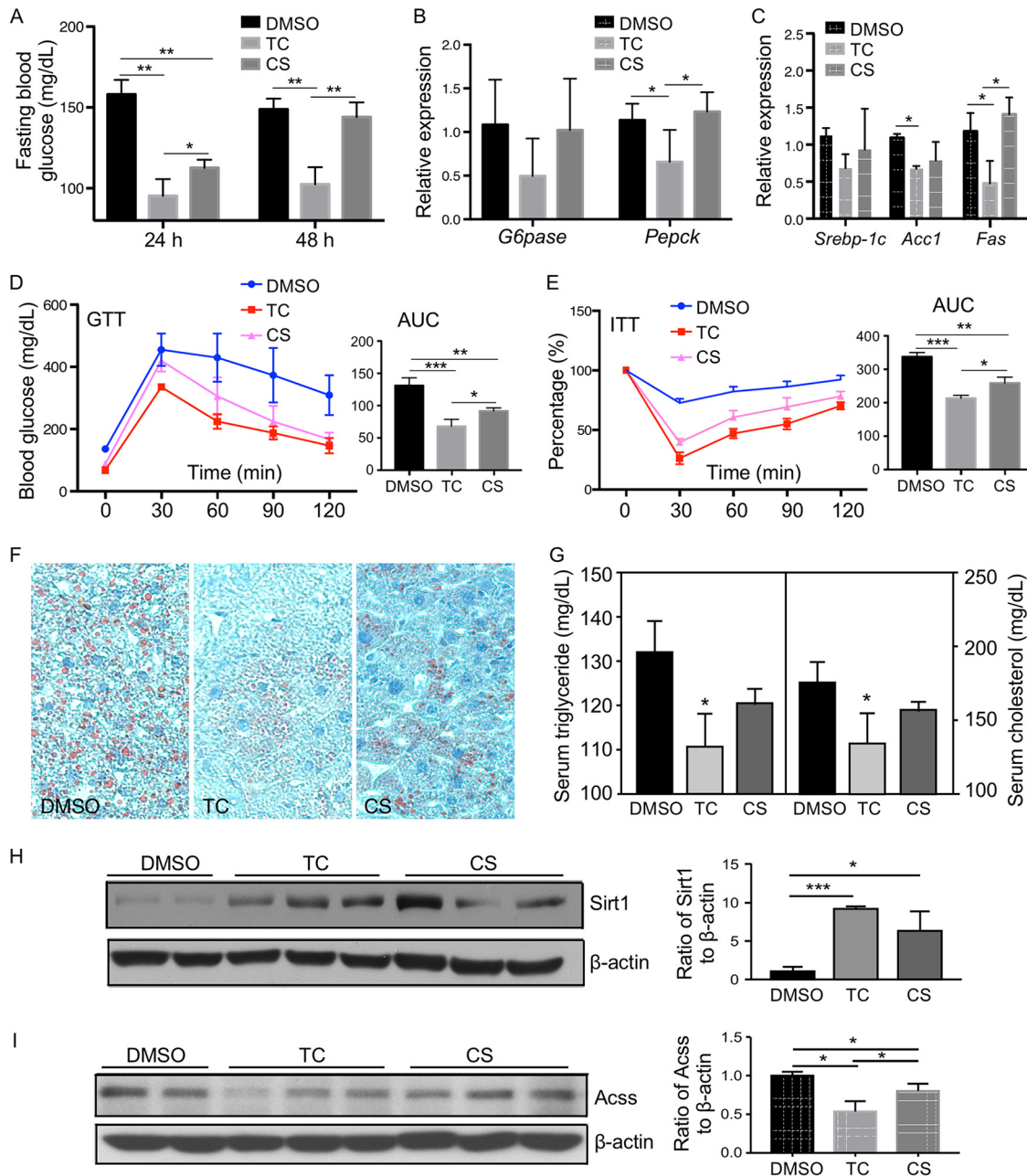
**Thiocholesterol exhibits a superior activity in reducing fasting blood glucose level and improving overall glucose homeostasis in HFD-fed mice.** We have previously reported that treatment of HFD-fed mice with CS can inhibit gluconeogenesis and improve insulin sensitivity (6). We then used the same HFD model to determine whether TC was more efficacious than CS in improving the overall glucose homeostasis. Mice were fed with HFD for 12 weeks before receiving intraperitoneal (i.p.) doses of TC or CS at 25 mg/kg of body weight 3 times per week for 2 weeks. At 24 h after the last dose of drug treatment, TC was more effective than CS in lowering the fasting blood glucose level (Fig. 8A). At 48 h after the last drug dose, TC remained effective in reducing the fasting blood glucose level, but CS was no longer effective (Fig. 8A). Thiocholesterol was also more effective than CS in reducing the gluconeogenic (Fig. 8B) and lipogenic (Fig. 8C) gene expression levels. Treatment with TC was more effective in improving the animal's performance in the glucose tolerance test (GTT) (Fig. 8D) and insulin tolerance test (ITT) (Fig. 8E), attenuating HFD-induced hepatic steatosis as shown by Oil red O staining (Fig. 8F), and lowering the serum triglyceride and cholesterol levels (Fig. 8G) without affecting the plasma insulin levels (data not shown). Treatment with TC was also effective in inducing the protein expression of Sirt1 (Fig. 8H) and suppressing the protein expression of Acss (Fig. 8I). This regimen of TC or CS was not toxic to the mice, because they did not elevate the activities of ALT and AST, nor did they affect the animal's food intake and body weight (data not shown). Treatment with TC or CS had little effect on the hepatic mRNA expression of Hnf4 $\alpha$  *in vivo* (data not shown).

## DISCUSSION

SULT2B1b catalyzes the sulfoconjugation of cholesterol to synthesize CS. We have previously shown that the expression of SULT2B1b in the liver was induced in diabetic obese mice and during the fasting-to-fed transition. We went on to show that SULT2B1b and CS inhibited gluconeogenesis by targeting HNF4 $\alpha$  in both cell cultures and transgenic mice (6). These results suggested that the induction of SULT2B1b may represent a protective response to metabolic stresses.

Knowing that SULT2B1b is a negative regulator of HNF4 $\alpha$ , it was interesting and intriguing to find that SULT2B1b itself is a transcriptional target of HNF4 $\alpha$ . HNF4 $\alpha$  is a transcriptional factor of diverse functions, including its gluconeogenic activity by regulating the key gluconeogenic enzymes. Although hepatic gluconeogenesis is an essential cellular function, uncontrolled gluconeogenesis can be pathogenic, including the development of T2D (7–11). Indeed, genetic variants of HNF4 $\alpha$  have been associated with an increased risk of T2D and metabolic syndrome in humans (29, 30). Therefore, it is necessary to have a cellular mechanism to limit gluconeogenesis, including that mediated by HNF4 $\alpha$ . Based on our results, we propose that the positive regulation of SULT2B1b by HNF4 $\alpha$  represents an effective cellular mechanism to keep the HNF4 $\alpha$ -mediated gluconeogenesis in check, including in the context of fasting response.

Consistent with our hypothesis, knockdown or knockout of Sult2B1b enhanced the gluconeogenic activity of HNF4 $\alpha$ , which was associated with increased acetylation and nuclear enrichment of HNF4 $\alpha$ . The increased acetylation of HNF4 $\alpha$  may have accounted for the nuclear enrichment of HNF4 $\alpha$ , because acetylation prevents the active export of HNF4 $\alpha$  to the cytoplasm and increases the DNA binding of HNF4 $\alpha$  (12, 14). The increased acetylation of HNF4 $\alpha$  in Sult2B1b<sup>-/-</sup> hepatocytes may be explained by the combined effects of a decreased expression of the HNF4 $\alpha$  deacetylase Sirt1 and an



**FIG 8** Thiocholesterol exhibits a superior activity in reducing fasting blood glucose level and improving overall glucose homeostasis in HFD-fed mice. Six-week-old WT C56BL/6 male mice were fed a high-fat diet (HFD) for 12 weeks followed by i.p. injections of vehicle (DMSO), TC (25 mg/kg), or CS (25 mg/kg) 3 times per week for 2 weeks while the mice remained on HFD. (A) Fasting blood glucose levels 24 h or 48 h after the last dose of drugs. Mice were fasted for 6 h before the blood glucose tests. (B and C) Hepatic expression of gluconeogenic genes (B) and lipogenic genes (C) 3 days after the last dose of drugs was measured by real-time PCR. The expression of each gene was arbitrarily set as 1 in mice treated with DMSO. (D and E) Shown on the right of each panel are the quantifications of GTT (D) and ITT (E) presented as areas under the curve (AUC). (F and G) Oil red O staining of the liver sections (F) and serum triglyceride and cholesterol levels (G). (H and I) Hepatic expression levels of the Sirt1 (H) and Acss (I) proteins was measured by Western blotting. Shown on the right are the densitometric quantifications of the Western blotting. Results are expressed as means  $\pm$  SD from three independent experiments (5 mice per group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

increased expression of the acetyl-CoA-generating enzyme Acss. The mechanisms by which Sirt 1 and Acss are regulated by Sult2B1b remain to be understood. CBP/p300 has been shown to acetylate HNF4 $\alpha$  (14), but the expression of CBP/p300 was not affected in the Sult2B1b<sup>-/-</sup> hepatocytes (data not shown).

In addition to inhibiting gluconeogenesis, SULT2B1b has been reported to have

many other functions in different cell types and tissues, ranging from inhibiting lipogenesis to promoting the liver cell proliferation (31), affecting prostate and colorectal cancer cells (32), and inhibiting the T cell receptor signaling (33). It remains to be determined whether HNF4 $\alpha$ , as a positive regulator of SULT2B1b, is also involved in these additional functions of SULT2B1b. Meanwhile, HNF4 $\alpha$  is a transcriptional factor that has numerous functions beyond promoting gluconeogenesis (34). It would be interesting to know whether and how the SULT2B1b gene, as an HNF4 $\alpha$  target gene, may have contributed to other cellular functions of HNF4 $\alpha$ .

Dysregulated gluconeogenesis is primarily responsible for the increased hepatic glucose production in T2D (11), suggesting that targeting the gluconeogenic pathway could attenuate hyperglycemia. Indeed, metformin, one of the most prescribed drugs for T2D, is believed to exert its therapeutic benefit by reducing hepatic gluconeogenesis (35). Based on our findings, it is tempting to explore CS as a potential therapeutic agent to manage metabolic disorders, because it is a key component in the HNF4 $\alpha$ -SULT2B1b negative feedback regulation of gluconeogenesis. However, the natural CS is chemically unstable, because it is readily hydrolyzed by the steroid sulfatase. In addressing the chemical instability of CS, we have developed and characterized TC, a hydrolysis-resistant derivative of CS. Our results showed that TC was superior to CS in inhibiting gluconeogenesis and improving overall glucose homeostasis in diabetic mice, which was attributed to the increased stability and bioavailability of TC. Certain cholesterol derivatives such as oxysterols have been shown to activate LXR, but neither TC nor CS affected the activity of LXR in reporter gene assays (data not shown).

In summary, we have identified the cholesterol sulfotransferase SULT2B1b as a novel transcript target of HNF4 $\alpha$ . The induction of SULT2B1b by HNF4 $\alpha$  constitutes a negative feedback mechanism to prevent uncontrolled gluconeogenesis in diabetes or fasting response. As a structural and functional analog of CS, the hydrolysis-resistant TC may be further explored as a therapeutic agent to inhibit gluconeogenesis and manage hyperglycemia.

## MATERIALS AND METHODS

**Mice, diet, and chemicals.** The Sult2B1b<sup>-/-</sup> mice (strain number 018773) were obtained from the Jackson Laboratory (Bar Harbor, ME). Standard chow from PMI Nutrition (St. Louis, MO) or HFD (S3282) from Bio-serv (Frenchtown, NJ) was used. All chemicals were purchased from Sigma (St. Louis, MO).

**Isolation of mouse and human primary hepatocytes.** Mouse primary hepatocytes were isolated by collagenase perfusion from 8-week-old male wild-type or Sult2B1b<sup>-/-</sup> mice as we have previously described (28). Human primary hepatocytes, also isolated by collagenase perfusion, were obtained through the NIH-sponsored Liver Tissue Procurement and Distribution System at the University of Pittsburgh.

**Histological and serum insulin analysis.** For Oil red O staining, snap-frozen liver tissues were sectioned into 8- $\mu$ m-thick samples and stained in 0.5% Oil red O in propylene glycerol. The serum insulin level was measured by an assay kit (catalog number 90080) from Crystal Chem (Downers Grove, IL).

**Immunoprecipitation, Western blot analysis, and ChIP assays.** Immunoprecipitation and Western blot analysis were performed as described previously (6). The primary antibody used for HNF4 $\alpha$  (catalog number MA1-199) was purchased from ThermoFisher Scientific (Pittsburgh, PA). Anti-acetylated lysine (catalog number 9441S), anti-Sirt1 (D60E1), and anti-FLAG (catalog number 2368) antibodies were purchased from Cell Signaling (Danvers, MA). The anti-Acss (sc-85258) and anti-SULT2B1b (sc-67103) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Western blotting images were quantified with the ImageJ software (<http://rsb.info.nih.gov/ij/>). The ChIP assay was performed as we have previously described (6).

**Glucose production assay.** Cells were washed three times with phosphate-buffered saline (PBS) to remove glucose. Cells were then incubated in glucose production buffer (glucose-free Dulbecco's modified Eagle medium [DMEM] [pH 7.4] containing 20 mM sodium lactate and 2 mM sodium pyruvate without phenol red) for 4 h, after which 500  $\mu$ l of medium was removed and centrifuged at 15,000  $\times$  g for 10 min. The glucose content in the supernatant was measured with a glucose assay kit from Sigma. Glucose concentrations were normalized to cellular protein concentrations.

**Immunofluorescence.** Cells were seeded on glass slides, fixed with 4% paraformaldehyde in PBS, and incubated overnight with the primary antibody at 4°C. Cells were then incubated with fluorescein-labeled goat anti-rabbit IgG and visualized by confocal microscopy.

**Gene expression analysis.** RNA was extracted by using the TRIzol reagent from Invitrogen (Carlsbad, CA). Reverse transcription into cDNA was performed by using reverse transcriptase Superscript II and oligoDT from Invitrogen. Quantitative RT-PCR was performed with an ABI Prism 7000 thermal cycler from Applied Biosystems by using the SYBR green detection reagent. The cyclophilin gene was used as the housekeeping control gene.

**GTT and ITT.** For the glucose tolerance test, mice received an intraperitoneal injection of D-glucose at 2 g/kg body weight after a 16-h fasting. For the insulin tolerance test, mice received an intraperitoneal injection of insulin at 0.5 unit/kg after a 6-h fasting.

**Adenoviral expression vectors.** Adenoviruses expressing Hnf4 $\alpha$  (Ad-Hnf4 $\alpha$ ), shRNA against Hnf4 $\alpha$  (Ad-shHnf4 $\alpha$ ), or  $\beta$ -galactosidase (Ad-shLacZ) were gifts from Yanqiao Zhang from the Northeast Ohio Medical University (36).

**Cloning of SULT2B1b gene promoters, transient-transfection and reporter gene assays, and EMSA.** The mouse Sult2B1b gene promoter (nucleotides [nt] -156 to +200) and the human SULT2B1b gene promoter (nt -197 to +154) were cloned by PCR using the following primers (5' to 3'): Sult2B1b Forward, CGACGCGTCGCGGCAAACCTTGCAGAGTAA; Sult2B1b Reverse, GAAGATCTTCTCAGACGAGCTCCACAGG; SULT2B1b Forward, CGACGCGTCGCCATTTCCCAATGAGCA; SULT2B1b Reverse, GAAGATCTTCTCAGAGGAGCA. The promoter sequences were then cloned into the pGL3-Luc reporter gene from Promega (Madison WI). Transient-transfection and luciferase reporter gene assays were performed as we have previously described, and the transfection efficiency was normalized against the  $\beta$ -galactosidase activities from a cotransfected CMX- $\beta$ -galactosidase vector (6). EMSA using [<sup>32</sup>P]dCTP-labeled oligonucleotides and *in vitro*-translated proteins was carried out as we have previously described (16).

**Stability assay of CS and TC in cells.** Primary hepatocytes were seeded onto 12-well plates and treated with vehicle, cholesterol sulfate (CS) (5  $\mu$ M), or thiocholesterol (TC) (5  $\mu$ M) for 0, 1, 2, 4, 8, 24, or 48 h. At the end of treatment, the medium was removed, 1 ml of methanol was added, and the cells were scraped and collected. The cells were sonicated for 15 s, and then 1 ml of dichloromethane was added. After vortexing for 1 min, the suspension was centrifuged at 13,600 rpm at 4°C for 20 min. The supernatant was transferred to a new tube and dried until analysis. For the detection of CS, the dried cellular extracts were reconstituted in 20  $\mu$ l of dichloromethane-methanol (1:1, vol/vol) and diluted with 180  $\mu$ l of water-isopropanol-acetonitrile (2:5:3, vol/vol/vol). After centrifugation, 5  $\mu$ l of the supernatant was injected into the Waters ultraperformance liquid chromatography and quadrupole time of flight mass spectrometry (UPLC-QTOF MS) system for analysis. For the detection of TC, the cellular extracts were reconstituted in 20  $\mu$ l of dichloromethane and diluted with 180  $\mu$ l of acetonitrile. After centrifugation, 50  $\mu$ l of the supernatant was transferred to a new tube and 150  $\mu$ l of 0.1 M borate buffer (pH 8.0) was added. One hundred microliters of 10 mM monobromobimane in acetonitrile was added for derivatization. The mixture was gently shaken for 2 h before taking 10  $\mu$ l for the UPLC-QTOF MS analysis. Chromatographic separation was performed using an Acquity UPLC BEH C<sub>18</sub> column (2.1 by 50 mm, 1.7  $\mu$ m; Waters Corporation, Milford, MA). For CS detection, the mobile phase A (MPA) was 10 mM ammonium acetate in water, and the mobile phase B (MPB) was 10 mM ammonium acetate in acetonitrile-water (95:5). The gradient began at 30% MPB and was held for 0.5 min, followed by 2.5 min of linear gradient to 95% MPB and holding for 3 min, and then decreased to 30% MPB for column equilibration. For TC detection, the MPA was 0.1% formic acid in water, and the MPB was 0.1% formic acid in acetonitrile. The gradient began at 30% MPB and was held for 0.5 min, followed by 2.5 min of linear gradient to 95% MPB and holding for 2.5 min, increased to 99% MPB in another 0.1 min, and held for 2.4 min and then decreased to 30% MPB for column equilibration. The flow rate of the mobile phase was 0.5 ml/min, and the column temperature was maintained at 50°C. The QTOF MS system was operated in a negative (for CS detection) or positive (for TC detection) high-resolution mode with electrospray ionization. The source and desolvation temperatures were set at 150 and 500°C, respectively. Nitrogen was applied as the cone gas (50 liters/h) and the desolvation gas (800 liters/h). Argon was applied as the collision gas. The capillary and cone voltages were set at 0.8 kV and 40 V, respectively. QTOF MS was calibrated with sodium formate and monitored by the intermittent injection of lock mass leucine encephalin ( $m/z = 556.2771$ ) in real time.

**Statistical analysis.** Data are expressed as the means  $\pm$  standard deviations (SD). One-way analysis of variance (ANOVA) Tukey's test or an unpaired Student *t* test was performed for statistical analysis using GraphPad Prism (San Diego, CA). *P* values of less than 0.05 were considered statistically significant.

**Study approval.** The Central Animal Facility of the University of Pittsburgh is fully accredited by the American Association for Laboratory Animal Care (AALAC). All procedures were performed in accordance with relevant federal guidelines and with the approval of the University of Pittsburgh ethical committee.

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Yuhan Bi and Wen Xie participated in research design. Yuhan Bi, Xiongjie Shi, Xiudong Guan, Junjie Zhu, Wojciech G. Garbacz, Yixian Huang, Li Gao, Meishu Xu, and Songrong Ren conducted the experiments. Yuhan Bi and Wen Xie performed data analysis. Yuhan Bi, Xiudong Guan, Jiong Yan, Shunlin Ren, Yulan Liu, Xiaochao Ma, Song

Li, and Wen Xie wrote or contributed to the writing of the manuscript. Wen Xie acquired funding.

We declare that no conflict of interest exists.

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