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The adaptor protein GIPC1 stabilizes the scavenger receptor SR-B1 and increases its cholesterol uptake

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The scavenger receptor class B type 1 (SR-B1), a high-density lipoprotein (HDL) receptor, is a membrane glycoprotein that mediates selective uptake of HDL-cholesterol and cholesterol ester (CE) into cells. SR-B1 is subject to posttranslational regulation; however, the underlying mechanisms still remain obscure. Here, we identified a novel SR-B1-interacting protein, GIPC1 (GAIP-interacting protein, *C* terminus 1) that interacts with SR-B1 and stabilizes SR-B1 by negative regulation of its proteasomal and lysosomal degradation pathways. The physiological interaction between SR-B1 and GIPC1 was supported by co-immunoprecipitation of wild-type and mutant GIPC1 constructs in SR-B1 ± GIPC1 overexpressing cells, in native liver cells, and in mouse liver tissues. Overexpression of GIPC1 increased endogenous SR-B1 protein levels, subsequently increasing selective HDL-cholesterol/CE uptake and cellular triglyceride (TG) and total cholesterol (TC) levels, whereas silencing of GIPC1 in the mouse liver was associated with blunted hepatic SR-B1 levels, elevated plasma TG and TC, and attenuated hepatic TG and TC content. A positive correlation was identified between GIPC1 and SR-B1 expression, and both expressions of GIPC1 and SR-B1 from human liver samples were inversely correlated with body mass index (BMI) from human subjects. We therefore conclude that GIPC1 plays a key role in the stability and function of SR-B1 and can also effectively regulate hepatic lipid and cholesterol metabolism. These findings expand our knowledge of the regulatory roles of GIPC1 and suggest that GIPC1 exerts a major effect on cell surface receptors such as SR-B1 and its associated hepatic lipid and cholesterol metabolic processes.

Scavenger receptor class B, type 1 (SR-B1) is an integral membrane glycoprotein that functions as a receptor for lipoproteins such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL) (1, 2). SR-B1 preferentially mediates selective uptake of HDL (and other lipoprotein)-derived cholesterol, cholesteryl esters (CEs), and unesterified cholesterol (UC), and it has also been shown that it facilitates free fatty acid (FFA) uptake in both primary adipocytes and model adipocyte cell lines (3). SR-B1 plays an important role in the reverse cholesterol transport (RCT), a process by which excess cholesterol from peripheral tissues is transferred to the liver for metabolism and excretion into the bile and provides cholesterol substrate to the steroidogenic cells of the adrenal gland, ovary, and testis for steroid hormone production (2, 4, 5). Given this, it is not surprising that SR-B1 is expressed at high levels in the liver and steroidogenic cells of the adrenal gland and ovary and testis (1, 2). Further studies demonstrated that SR-B1 participates in the uptake of several other molecules including vitamins, viruses (*e.g.*, hepatitis C virus, dengue virus, SARS-CoV-2 [COVID-19]), plasmodium parasite, phagocytosis of apoptotic cells and identified as a receptor for silica with a potential role in silica-induced pulmonary inflammation in macrophage (4, 6–9). SR-B1 is also implicated in tumor aggressiveness and in the prognosis of breast cancer (10, 11).

In both rodent and human steroidogenic tissues, the expression of steroidogenic SR-B1 is subject to transcriptional regulation by tissue-specific tropic hormones (*e.g.*, adrenal, ACTH, ovary, LH/FSH, and Leydig cells of testis, LH) *via* several transcription factors such as SF-1, LXRα and LXRβ, Sp1, PPARα, SREBP-1a, LRH-1, ERα and ERβ, CREB and NR0B1/DAX-1 (1, 12, 13). Our previous study demonstrated that DNA methylation status of SR-B1 promoter is also regulated by tropic hormone or its second messenger, cAMP, and participates in the regulation of SR-B1 expression in steroid-producing cells (14). Likewise, many dietary manipulations, hormones, and pharmacological agents transcriptionally regulate hepatic SR-B1 (4, 15, 16).

Both hepatic and steroidogenic SR-B1s are also subject to posttranscriptional and posttranslational regulation. The scaffold protein PDZK1/NHERF3 is a regulator of hepatic SR-B1; it interacts with and helps to maintain SR-B1's optimal expression, cell surface localization, and selective transport function in (17, 18). In contrast, PDZK1/NHERF3 is not detected in steroidogenic cells and thus, does not regulate steroidogenic SR-B1, but two other NHERFs family members,

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NHERF1 and NHERF2, interact with both hepatic and steroidogenic SR-B1s and negatively regulate their expression and function especially by promoting their degradation *via* ubiquitin/proteasome pathway (1, 13, 19). Moreover, previously, we provided evidence that two microRNAs, miR-125a and miR-455 inhibit HDL-supported steroid hormone production and downregulation of SR-B1 expression by directly binding to 3' UTR region of SR-B1 mRNA in steroidogenic cells (5). Interestingly, the expression of both these miRNAs in steroidogenic tissues/cells is suppressed by trophic hormones and its second messenger cAMP resulting in increased expression and function of SR-B1 (20). Three additional miRNAs, miR-185, miR-96, miRNA-24, and miR-223 also negatively regulate SR-B1 expression and function in the liver and macrophages (21–23). We further demonstrated that a salt-inducible kinase 1 (SIK1) positively regulates adrenal/gonadal steroidogenesis by stimulating the phosphorylation and activation of SR-B1 (24).

In an continuing effort to further enhance our understanding about the events connected with the post-transcriptional/posttranslational regulation of SR-B1 with a particular emphasis on the PDZ-domain containing proteins, we performed SR-B1 peptide pull-down/mass spectrometry (MS) assays, and identified that a novel PDZ protein, GIPC1, can physically interact with the intracellular tail of SR-B1 and elucidated the expression, function, and the structural organization of the GIPC1-SR-B1 complex in hepatocytes. GIPC1/GIPC (GAIP/RGS19-interacting protein C terminus), a single Postsynaptic density 95, Disks large, Zona occludens-1 (PDZ) domain adaptor protein, is a founding member of GIPC family, which also includes GIPC2 and GIPC3 (25).The central PDZ domain of GIPC1 binds type I C-terminal PDZ-binding motifs (PBMs) confirming to the consensus sequence (S/T)-X-**A**/V/L/I (26). GPIC1 also contains GIPC homology domains at their amino (GH1) and carboxyl (GH2) ends. The GH1 domain promotes self-dimerization, whereas the GH2 domain binds the globular domain of actin-based retrograde motor, MYOSIN6 (MYO6) driving endocytic vesicle internalization (26). GIPC1 is in one of the most versatile PDZ proteins known to date, with a large number of binding partners, most of which are transmembrane receptors, adhesion molecules, or proteins involved in endocytosis and trafficking of intracellular organelles (27–29). Our analysis indicates that GIPC1 interacts with hepatic SR-B1, upregulates its protein levels by promoting SR-B1 protein stability, and specifically controls the selective HDL-cholesterol function of SR-B1 in hepatocytes. Our data further reveal a critical role for GIPC1-SR-B1 cross talk in the regulation of hepatic lipid metabolism and cholesterol homoeostasis.

Results

Identification of GIPC1 as an SR-B1-binding/interacting protein

To explore the proteins that interact with SR-B1 and regulate SR-B1 function in hepatocyte, a biotin-labeled peptide containing *C*-terminal domain of mouse SR-B1 (Shanghai Bootech BioScience & Technology Co, Ltd) was incubated with mouse liver followed by incubation with streptavidin-agarose beads. Captured proteins were resolved by SDS-PAGE and separated bands were visualized by silver staining. Then the captured proteins were subjected to LC/MS assay (Fig. 1*A*). Analysis of the excised proteins by LC/MS identified GIPC1 as a binding partner of SR-B1. In addition, we detected the presence of PDZK1/NHERF3, a well-characterized hepatic SR-B1 interacting protein (see also Table S1) (18). SDS-PAGE/western blot analysis of pull-down samples probed with anti-GIPC1 demonstrated that Biotin-mCT45 interacts with endogenous GIPC1 present in liver extracts (Fig. 1*B*). Co-IP and western blotting experiments using liver extracts containing endogenous GIPC1 or cell extracts prepared from GIPC1 overexpressing cells further confirmed the specific interaction between SR-B1 and GIPC1 (Fig. 1, *C–F*). As noted above, GIPC family members including GIPC1 contain an amino-terminal GIPC homology 1 (GH1) domain, a central PDZ domain, and a carboxyl terminal GH2 domain (28). To identify which of these domains are required for GIPC1 targeting to SR-B1, we generated a series of domain-deletion constructs of GIPC1 and expressed them in HEK293, a human embryonic kidney cell line (Fig. 1*G*, *left*). Co-IP and western blotting results indicated that PDZ domain was essential for GIPC1-association with SR-B1 (Fig. 1*G*, *right*). In contrast, GIPC1 constructs lacking either *N*-terminal or *C*-terminal domain showed no effect on GIPC1 targeting to SR-B1. The different truncated SR-B1 was also constructed and cotransfected with GIPC1 into HEK293 cells for co-IP analysis (Fig. 1*H*, *left*). Interestingly, deletion of either *C*-terminal domain or *N*-terminal domain of SR-B1 had no effect on its ability to interact with GIPC1 (Fig. 1*H*, *right*).

GIPC1 upregulates SR-B1 protein levels and SR-B1-mediated selective HDL-CE uptake function

We and others previously reported that PDZ proteins, PDZK1/NHERF3, NHERF1, and NHERF2, can interact with SR-B1 and regulate its protein levels and modulate SR-B1-mediated selective HDL-CE transport function in model hepatocyte cell lines (13, 17, 19). Given this and the fact that GIPC1 can regulate cellular protein levels by interacting with the target proteins and modulating their cellular trafficking or degradation (30, 31), we next elucidated whether GIPC1 interaction with SR-B1 modulates its expression and/or function. HEK293 cells were transfected with SR-B1 ± GIPC1 cDNA constructs. Western blotting of cellular extracts demonstrated that overexpression of GIPC1 was associated with enhanced expression of SR-B1 protein levels in cellular extracts from SR-B1 and GIPC1 cotransfected HEK293 cells (Fig. 2*A*). There is nearly no detected SR-B1 in HEK293 cell, and the concentration of overexpressed SR-B1 in HEK293 cell is about threefold to its physiological concentrations in Hepa 1 to 6 cell, whereas the overexpression of GIPC1 is about twofold to its physiological concentrations in HEK293 cells (data not shown). We next examined the effect of GPIC1 on the selective HDL-C transport function of SR-B1. Results

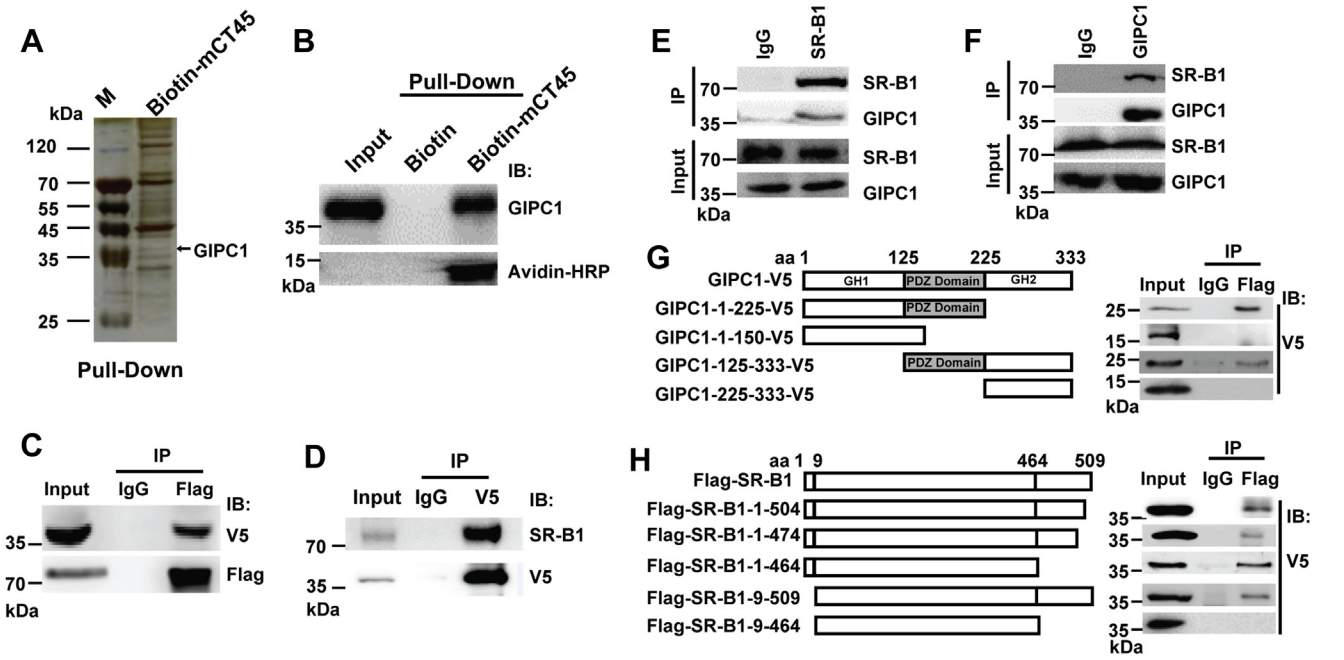

Figure 1. Identification of GPIC1 as a binding partner for SR-B1. Cellular proteins precipitated with biotin-labeled mSR-B1 c terminal domain (45 amino acids, mCT45)/Streptavidin-Agarose were isolated from adult mouse liver extracts. Interacting proteins were resolved by SDS-PAGE and detected by silver staining. Total cellular proteins representing the starting material (M) are shown in first lane (*A*). The resulting peptides were sequenced by LC/MS. *B*, liver lysates were incubated with the biotin mCT45/streptavidin-agarose as indicated and the resulting complexes were analyzed by immunoblotting with anti-GPIC1 or anti-SR-B1 antibody. *C* and *D*, western blots illustrating the interactions of overexpressed SR-B1 with the GIPC1. In (*C*) cellular extracts containing overexpressed Flag-SR-B1 and GIPC1-V5 proteins were incubated with anti-FLAG M2 magnetic beads. The western blots of bound proteins released from the washed beads were probed with anti-Flag and anti-V5 antibody to detect SR-B1-associated GPIC1. Likewise, in (*D*) cellular lysates were incubated with anti-V5 bound to protein A/G-agarose beads and western blots of bound proteins released from the washed beads were probed with rabbit anti-SR-B1 or anti-V5 antibody. *E* and *F*, interaction between endogenous SR-B1 and GPIC1. Anti-SR-B1 antibody bound to protein A/G-agarose beads (*E*) or anti-GIPC1 antibody (*F*) was incubated with mouse liver lysates as indicated. The western blots of bound proteins released from washed beads were probed with anti-SR-B1 and anti-GIPC1 antibody. *G*, *Left lane*, schematic diagram of three major domains of GIPC1 and positions of deleted sequences. *Right lane*, anti-Flag antibody bound to protein A-agarose beads was incubated with HEK293 cell extracts cooverexpressing Flag-SR-B1 and one of the truncated GIPC1 proteins. The western blots of bound proteins released from washed beads were probed with anti-Flag and anti-V5 antibodies. *H*, identification of SR-B1 domains that interact with GIPC1. *Left lane*, schematic diagram of SR-B1 truncated constructs. *Right lane*, anti-FLAG M2 magnetic beads were incubated with HEK293 cell extracts coexpressing GPIC1-V5 and different Flag-SR-B1 truncated constructs. The western blots of bound proteins released from washed beads were probed with anti-SR-B1.

presented in Figure 2*B* indicate that overexpression of GIPC1 leads to upregulation of SR-B1-mediated Dil-HDL-CE uptake (Fig. 2*B*). We next determined the impact of GIPC1 on the expression of various truncated forms of SR-B1 constructs. Overexpression of GIPC1 upregulated the expression of all SR-B1 constructs (*i.e.*, SR-B1-1-464, 1-474, 1-504, and 9-509) with the exception of SR-B1-9-464 (Fig. 2*C*). These results are in agreement with the co-IP data shown above in Figure 1*H*. In comparison to wild-type GPIC1, GPIC1 constructs lacking either PDZ domain or GH1 domain were severely impaired in their abilities to upregulate the expression of SR-B1 (Fig. 2*D*).

GIPC1 increases SR-B1 protein stability

Our previous studies demonstrated that the protein levels of SR-B1 were degraded in a time-dependent manner following cycloheximide (CHX) inhibition of cellular protein synthesis (13). Therefore, we investigated whether the GIPC1 regulates SR-B1 protein levels by modulating SR-B1 protein stability. HEK293 cells were transfected with SR-B1 ± GIPC1 constructs for 36 h and cells were then treated with CHX. Protein levels were measured at different time points by western blotting. These data demonstrated that overexpression of GIPC1 stabilizes cellular SR-B1 protein levels (Fig. 2, *E* and *F*). Next, we sought to determine protein degradation pathway whose function is inhibited by the GPIC1 and in turn leads to increased SR-B1 protein stability. Mouse hepatoma Hepa 1 to 6 cells and mouse primary hepatocyte cells were treated with leupeptin hemisulfate (Leu, lysosomal proteolysis inhibitor), MG-132 (proteasome inhibitor), or chloroquine diphosphate (autophagy inhibitor, block the fusion of the autophagosome with the lysosome), respectively. Both MG-132 treatment and Leu treatment upregulated SR-B1 protein levels in Hepa 1 to 6 cells, whereas chloroquine treatment had no effect on SR-B1 expression (Fig. 2*G*). Similar results were obtained using primary mouse hepatocytes (Fig. 2*G*). The coexpression of SR-B1 with GIPC1 in HEK293 cells or treatment of SR-B1 expressing HEK293 cells with MG-132 cells significantly enhanced the SR-B1 protein levels. However, MG-132 had no effect on the GIPC1-enhanced SR-B1 protein expression (Fig. 2*H*). Leu treatment also enhanced SR-B1 protein levels in SR-B1 overexpressing HEK293 cells and further upregulated the SR-B1 protein levels in HEK293 cells coexpressing SR-B1 and GPIC1 (Fig. 2*I*). These results indicate that GIPC1 upregulates SR-B1 protein levels by improving the protein stability *via* the negative regulation of proteasomal and lysosomal protein degradation pathways.

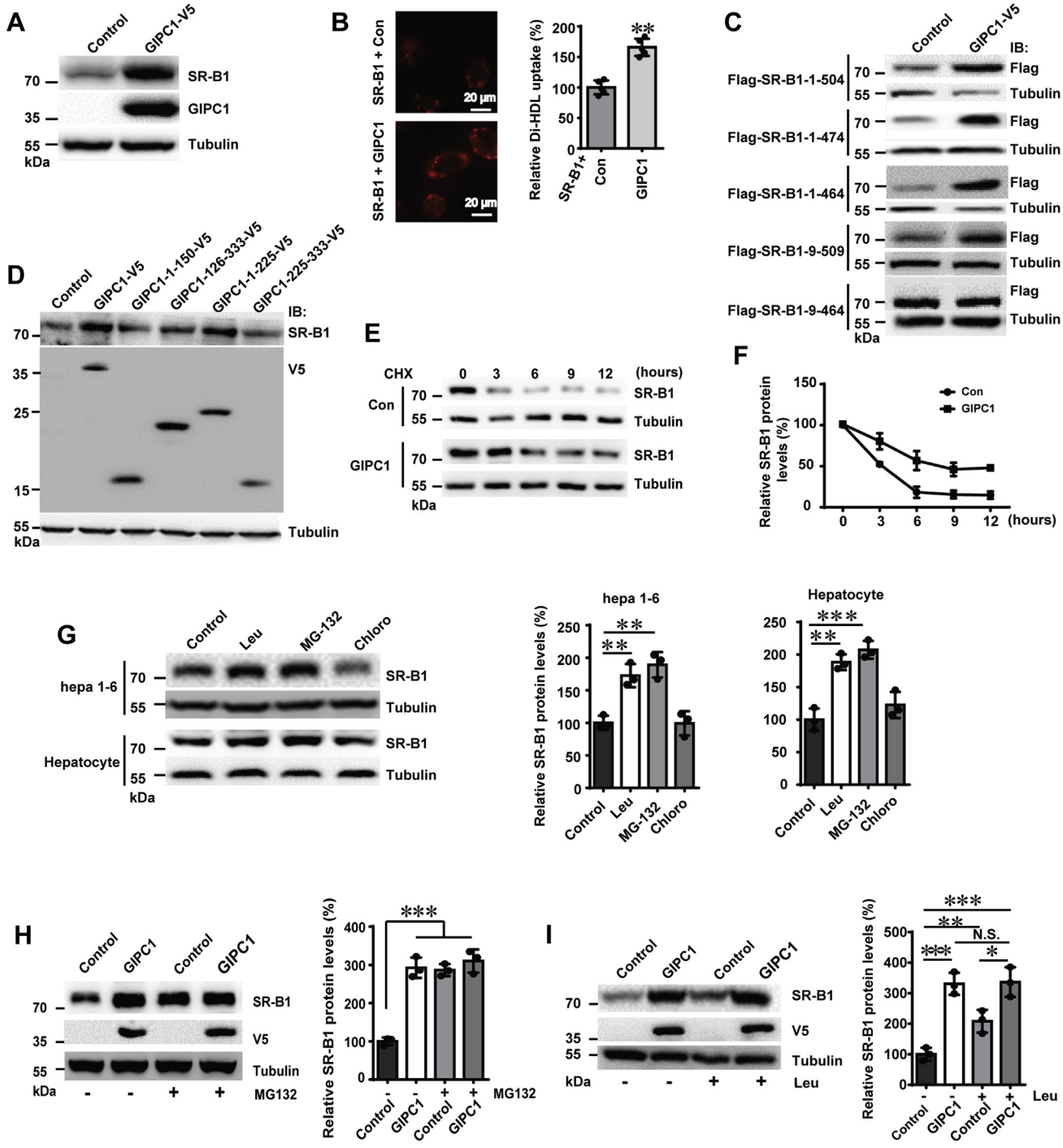

Figure 2. Overexpression of GIPC1 upregulates SR-B1 protein expression and function through modulating SR-B1 protein stability. *A*, HEK293 cells were cotransfected with SR-B1 and GIPC1 constructs, and 48 h after transfection, cell lysates were analyzed for SR-B1 and GPIC1 protein levels by western blotting. *B*, Dil-HDL uptake by HEK293 cells cotransfected with SR-B1 ± GIPC1 or control plasmid. *C*, HEK293 cells were cotransfected with GIPC1-V5 and different truncated versions of Flag-SR-B1 constructs, and 48 h after transfection, cell samples were analyzed for wild-type and truncated SR-B1 protein levels by western blotting using anti-Flag antibody. *D*, western blot analysis of the effects of variously truncated forms of GIPC1-V5 on Flag-SR-B1 protein expression in H239 cells. *E*, effect of overexpression of GIPC1 on time-dependent decay of the degradation of overexpressed Flag-SR-B1 following CHX treatment in the presence of CHX. *F*, a statistical quantification of panel *A*. HEK293 cells were transfected with Flag-SR-B1 ± GPIC1 for 36 h and subsequently cells treated with CHX. SR-B1 protein levels were determined at the indicated time points. *G*, Hepa 1 to 6 cells and primary mouse hepatocytes were treated with MG-132 (10 μM), leupeptin hemisulfate (Leu, 30 μM), or chloroquine diphosphate (10 μM) for 12 h, and cells were analyzed for SR-B1 protein levels by western blotting. *Right panel* shows quantitative values. *H*, HEK293 cells were cotransfected with Flag-SR-B1 and GIPC1-V5 construct or control plasmid, and 36 h after transfection, cell were treated with MG-132 for another 12 h, and subsequently, then cell lysates samples were analyzed for SR-B1 protein levels by western blotting. The statistical quantification quantitative values are shown in *right panel*. *I*, HEK293 cells were transfected under the identical as same as panel *D* and treated with Leu for another 12 h, and then cells were analyzed for SR-B1 protein levels by western blotting. The quantitative values are shown in *right panel*. The data presented are represents the mean ± SD from of three independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Effect of GIPC1 on the expression of SR-B1 expression and hepatic lipid metabolism in vitro

We next investigated whether GIPC1 is involved in the regulation of hepatic SR-B1 expression and lipid metabolism in a mouse hepatoma cell line (Hepa 1–6 cells) and primary mouse hepatocytes. Overexpression of GIPC1 enhanced SR-B1 protein levels without causing any significant changes in the SR-B1 mRNA levels (Fig. 3, *A–C*). We next measured the effect of increased expression of GIPC1 on SR-B1-mediated HDL-lipid uptake in cells using HDL-labeled with DiI lipophilic fluorescent dye probe (DiI-HDL) (Fig. 3*D*). In cells expressing GIPC1, the uptake of DiI-HDL was significantly increased as compared with control cells. We also examined the effect of increased expression of GIPC1, in this case measuring cellular lipid content using fluorescent neutral lipid dye, BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) (catalog # D3922, Thermo Fisher Scientific). As shown in Figure 3*E*, cells overexpressing GIPC1 showed increased accumulation of neutral lipids. In addition, cellular TG and TC levels were also increased in GIPC1 expressing Hepa 1 to 6 cells and mouse primary hepatocytes (Fig. 3, *F* and *G*). All the genes whose protein products participate in lipid and cholesterol metabolism that we quantified by qRT-PCR showed no changes in response to increased GIPC1 expression; only mRNA levels of *Scd1* were decreased modestly in GIPC1 expressing cells (Fig. 3*H*).

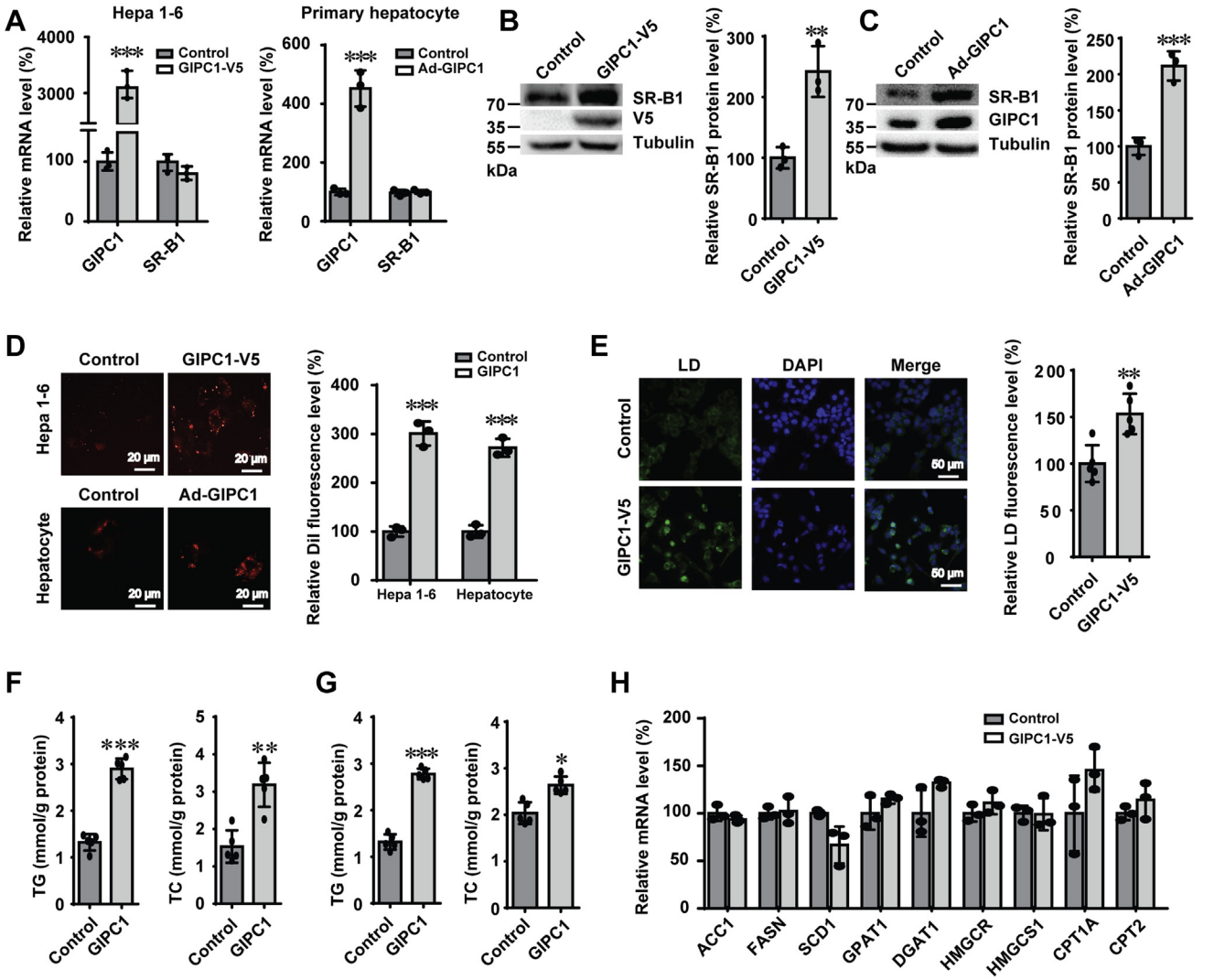

Figure 3. Increased expression of GIPC1 regulates SR-B1 expression, HDL uptake, and lipid metabolism *in vitro*. Hepa 1 to 6 cells were transfected with ± pcDNA6-GIPC1-V5 construct and mouse primary hepatocyte infected with ± adenoviruses encoding the full-length of GIPC1 cDNA, and 48 h after transfection or infection, cells were analyzed for mRNA and protein levels of SR-B1 and GIPC1, DiI-HDL lipid uptake, BODIPY lipid accumulation, triglyceride (TG), and cholesterol levels, or mRNA levels of *Acc1*, *Fasn*, *Scd1*, *Dgat1*, *Hmgcr*, *Hmgc1*, *Cpt1a* and *Cpt2*. *A*, mRNA levels of *Gipc1* and *Scarb1* measured by qRT-PCR. *B*, protein levels of SR-B1 and GIPC1 in Hepa 1 to 6 cells. Densitometric analysis data are shown in the *right panel*. *C*, protein levels of SR-B1 and GIPC1 in mouse primary A. Densitometric analysis data are shown in *right panel*. *D*, DiL-HDL uptake by Hepa 1 to 6 cells and hepatocytes. Quantitative data are shown in the *right panel*. *E*, accumulation of BODIPY-labeled neutral lipids in Hepa 1 to 6 cells. Quantitative data are shown in *right panel*. *F*, cellular TG and TC levels of Hepa 1 to 6 cells. *G*, cellular TG and TC levels primary hepatocytes. *H*, relative mRNA levels of *Acc1*, *Fasn*, *Scd1*, *Gpat1*, *Dgat1*, *Hmgcr*, *Hmgcs1*, *Cpt1a*, and *Cpt2* of Hepa 1 to 6 cells. The results presented are the mean ± SD of three independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Reduction of GIPC1 levels decreases SR-B1 expression and attenuates cellular lipid uptake and accumulation in a hepatic cell line in vitro

To further confirm the stimulatory effect of GIPC1 on SR-B1 expression and lipid metabolism, we knocked down GIPC1 expression in Hepa 1 to 6 cells using two different GIPC1 short hairpin RNA 1 and 2 (shRNA1 and shRNA2) and then measured various lipid-related metabolic parameters (Fig. 4, *A*–*F*). The SR-B1 protein reduced to 29.5% and 33.2% of control level for the cells treated with GIPC1 shRNA1 or shRNA2, respectively. However, we did not detect a reduction in the mRNA levels of SR-B1 in cells treated with GIPC1 shRNAs (Fig. 4, *A* and *B*). Knockdown of GIPC1 in Hepa 1 to 6 cells also diminished both Dil-HDL uptake and neutral lipid

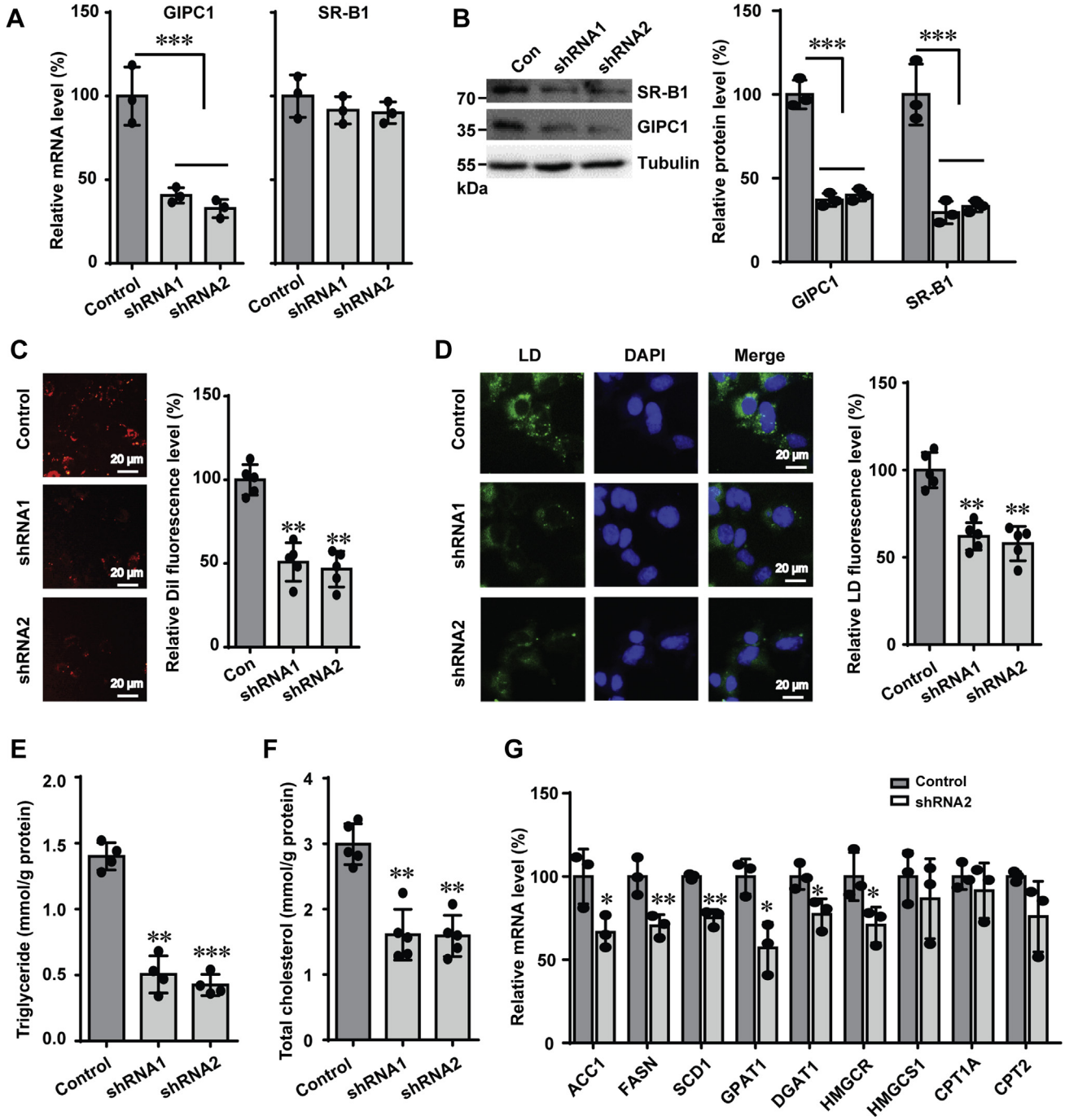

Figure 4. SR-B1 protein levels, Dil-HDL uptake, neutral lipid accumulation, and TG/TC levels are diminished in cells with decreased GIPC1 levels. Hepa 1 to 6 cells were transfected control shRNA, GIPC1 shRNA1, or GIPC1 shRNA2 and after 48 h, cells were subjected to various measurements. *A*, mRNA levels of *Gipc1* and *Scarb1*; *B*, protein levels of GIPC1 and SR-B1. Densitometric analysis data are shown in the *right panel*. *C*, Dil-HDL uptake. Quantitative data are shown in *right panel*. *D*, BODIPY-labeled lipid accumulation quantitative data are shown in *right panel*. *E* and *F*, TG levels and TC levels in Hepa 1 to 6 cells. *G*, relative mRNA levels of *Acc1*, *Fasn*, *Scd1*, *Gpat1*, *Dgat1*, *Hmgcr*, *Hmgcs1*, *Cpt1a*, and *Cpt2* with decreased GIPC1 levels. The data represent the mean ± SD of three independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

accumulation (Fig. 4, *C* and *D*). Analysis of cellular lipid content revealed that in comparison to cells treated with the control shRNA, cells demonstrated reduced DiI-HDL uptake, lipid accumulation, and TG and cholesterol levels, when GIPC1 levels were reduced by shRNAs treatment (Fig. 4, *C–F*). Several enzyme genes whose protein products catalyze fatty acid, cholesterol, and triglyceride synthesis, including *Acc1*, *Fasn*, *Scd1*, *Gpat*1, *Dgat1*, and *Hmgc*r were significantly decreased by knocking down GIPC1 expression, whereas two genes that participate in fatty acid β-oxidation, *Cpt1a* and *Cpt2*, exhibited no significant changes (Fig. 4*G*). Collectively, these data demonstrate that GIPC1 both enhances the SR-B1 protein levels and potentiates cellular lipid uptake and accumulation.

GIPC1 suppression inhibits SR-B1 expression and alters cholesterol and lipid metabolism in vivo

We assessed the effects of GIPC1 silencing (by administration of Ad-GIPC1 shRNA to mice vial tail vein) on SR-B1 expression and lipid metabolism. The qRT-PCR measurements indicated that administration of Ad-GIPC1 shRNA *via* tail-vein injection to mice is highly effective in silencing the expression of endogenous hepatic GIPC1 (Fig. 5*A*). Next, we used western blotting to evaluate hepatic SR-B1 protein expression. GIPC1 silencing induced a loss of SR-B1 protein expression (Fig. 5*B*). Likewise, hepatic TG and TC content and hepatic high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were downregulated with GIPC1 suppression (Fig. 5, *C–F*). In contrast, plasma levels of TG, TC, HDL-C, and LDL-C were increased in Ad-GIPC1 shRNA-treated mice (Fig. 5, *G–J*). Interestingly, the mRNA levels of SR-B1 decreased in the liver samples from mice treated with Ad-GIPC1 (Fig. 5*H*). The mRNA levels of *Scd1*, *Dgat1*, *Hmgcr*, *Hmgcs1*, and *Cpt1a* were downregulated in liver samples of GIPC1 knockdown mice as compared with data obtained using control mice. *Acc1*, *Fasn*, *Gpat1*, and *Cpt2* mRNA levels, however, exhibited no significant changes. These findings demonstrate that GIPC1 regulation of hepatic SR-B1 and lipid metabolism is quite different and complex between *in vitro versus in vivo* conditions.

Impact of human and rodent obesity on the functional expression of mRNAs and protein levels of GIPC1 and SR-B1

To further evaluate the functional interaction between GIPC1 and SR-B1, we carried out translational studies using liver samples collected from 32 obese and nonobese subjects at the time of surgery to remove the liver hemangioma (Table S2). As shown in Figure 6*A*, a negative correlation was observed between *Gipc1* mRNA levels and the BMI of obese subjects ($R = -0.6014$ and $p = 0.0003$, Fig. 6*A*). Similarly, the expression of *Scarb1* mRNA was also negatively correlated with the BMI of obese subjects ($R = -0.5333$ and $p = 0.0017$, Fig. 6*B*). In addition, *GIPC1* and *Scarb1* mRNA levels showed a positive association ($R = 0.7954$, $p < 0.0001$, Fig. 6*C*). Based on these findings, we performed similar studies using two mouse models of obesity: the high-fat diet (HFD)-fed mice and *ob/ob* mice. As shown in Figure 6*D*, feeding an HFD to mice suppressed the protein levels of both GIPC1 and SR-B1. Likewise, protein levels of both GIPC1 and SR-B1 were significantly attenuated in the liver samples of *ob/ob* mice as compared with wild-type control mice (Fig. 6*E*).

Discussion

We demonstrate here that the PDZ protein, GIPC1 interacts with SR-B1 and is mediated through the PDZ domain of GIPC. Changes in GIPC1 expression by overexpression of GIPC1 or reduction by GIPC-shRNA significantly affect SR-B1 expression, SR-B1-mediated selective HDL-cholesterol transport function, and SR-B1-linked hepatic lipid metabolism. Furthermore, we found that hepatic mRNA levels of both *Scarb1* and *Gipc1* are negatively correlated with the BMI of obese subjects. Likewise, hepatic protein levels of both GIPC1 and SR-B1 are suppressed in HFD-fed mice and *ob/ob* mice. Taken together, our results provide evidence that another PDZ protein, GIPC1 in addition to NHERF1, NHERF2, and PDZK1/NHERF3 plays a major regulatory role in the expression and function of hepatic SR-B1.

SR-B1, an HDL receptor, is the most abundant lipoprotein receptor in high cholesterol requiring tissues, such as the liver, adrenal glands, and gonads (1, 2). SR-B1 mediates the selective delivery of cholesterol esters and cholesterol from circulating HDL particles to the steroidogenic cells of the adrenal gland and gonads for steroid hormone production (1, 32); it also participates in reverse cholesterol transport (RCT) and facilitates cellular fatty acid uptake (3, 33). The SR-B1 expression is subject to transcriptional, posttranscriptional, and post-translational regulation (13). PDZ protein, PDZK1/NHERF3, is a major posttranslational regulator of hepatic SR-B1 but not steroidogenic SR-B1; it is required for the optimal expression of hepatic SR-B1 its cell surface localization and consequently, selective HDL-cholesterol transport function of SR-B1 (17). In addition, our previous studies identified two other PDZ family member of proteins, NHERF1 and NHERF2, that negatively regulate expression and function of both steroidogenic and hepatic SR-B1s *via* inhibition of their *de novo* synthesis and promote their increased ubiquitination/proteasomal degradation (13, 19). All these three PDZ proteins interact with hepatic SR-B1 and exhibit posttranslational modulation of SR-B1 at the protein level (only NHERF1 and NHERF2 also carry out these negative functions against steroidogenic SR-B1).

GIPC1 is one of most versatile PDZ proteins known to date, with a large number of binding partners with a range of metabolic and functional implications including regulating cell surface receptor expression, intracellular trafficking of proteins and receptors, anchoring proteins at specific subcellular compartments, and signaling transduction (27–29). GIPC has also been shown to interact with other proteins including MYO6, integrin α5 subunit, and α-actinin-1. GIPC1 is involved in receptor trafficking and stabilization by protein–protein interaction with a variety of PDZ ligands, such as IGF1R, GLUT1, and NRP1 (28). The N-terminal portion of GIPC1 mediates its oligomerization, whereas its C-terminal region

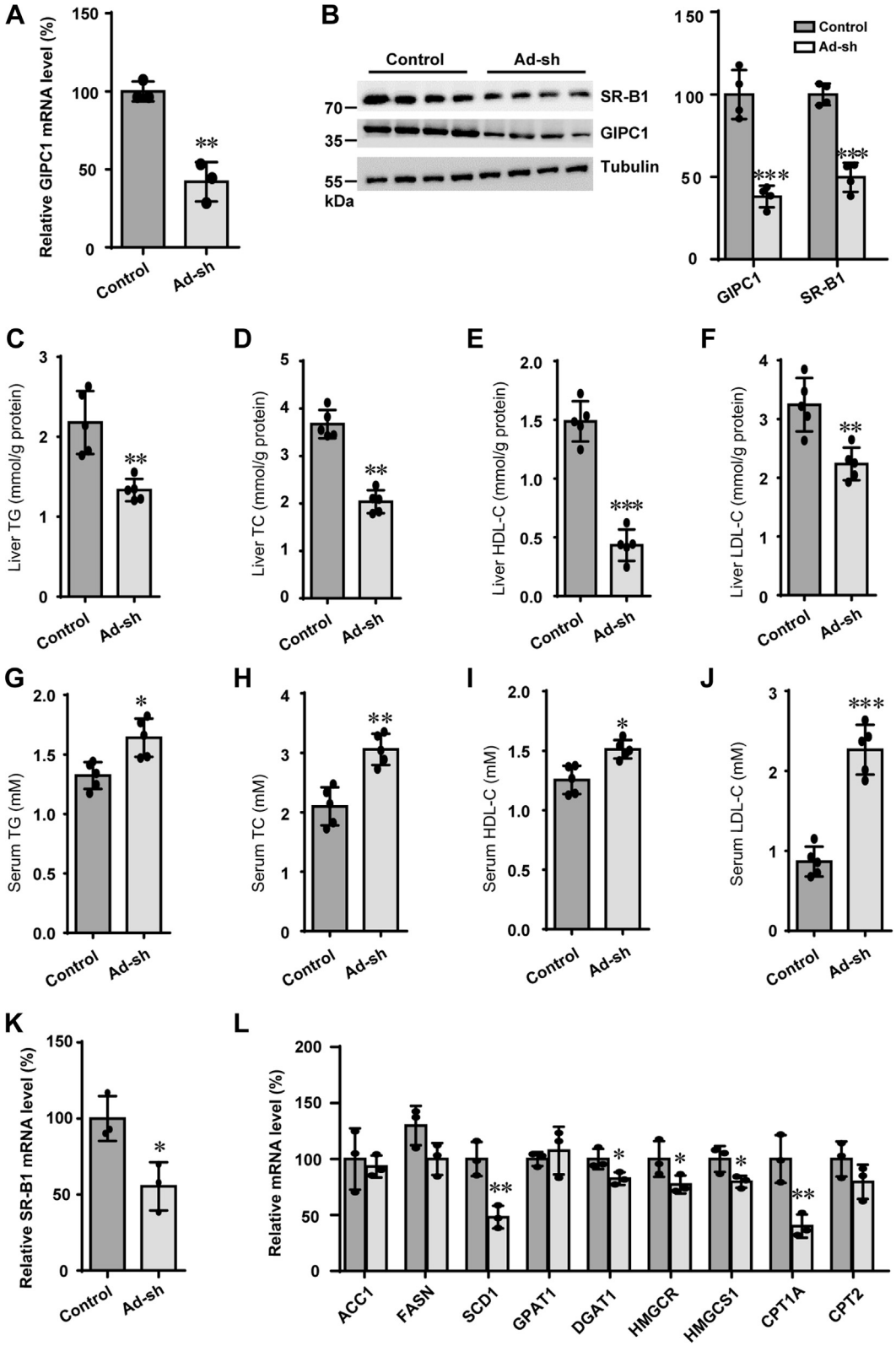

Figure 5. The effects of GIPC1 silencing *in vivo* on SR-B1 expression and cholesterol and lipid metabolism. *A*, the mRNA levels of *Gipc1* in the liver samples of mice with administration of adenoviruses (Ad)-GIPC1 shRNA (Ad-sh) (n = 5) or Ad-GFP control (n = 5). *B*, protein levels of SR-B1 and GIPC1 in the liver samples of mice with administration of Ad-sh or Ad-GFIP1 control. Densitometric analysis data are shown in *right panel*. *C–F*, serum TG, TC, HDL-C, and LDL-C levels in mice in response to administration of Ad-sh or Ad-GFP control. *G–J*, TG, TC, HDL-C, and LDL-C levels content in the liver samples of mice with administration of Ad-sh or Ad-GFP control. *K*, mRNA levels of *Scarb1* in the liver samples of mice treated with administration of Ad-sh or control Ad-GFP. *L*, relative mRNA levels of *Acc1*, *Fasn*, *Scd1*, *Gpat1*, *Dgat1*, *Hmgcr*, *Hmgcs1*, *Cpt1a*, and *Cpt2* in the liver samples of mice administered with Ad-sh or control Ad-GFP. The data represents the mean ± SD by Student's *t*-test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

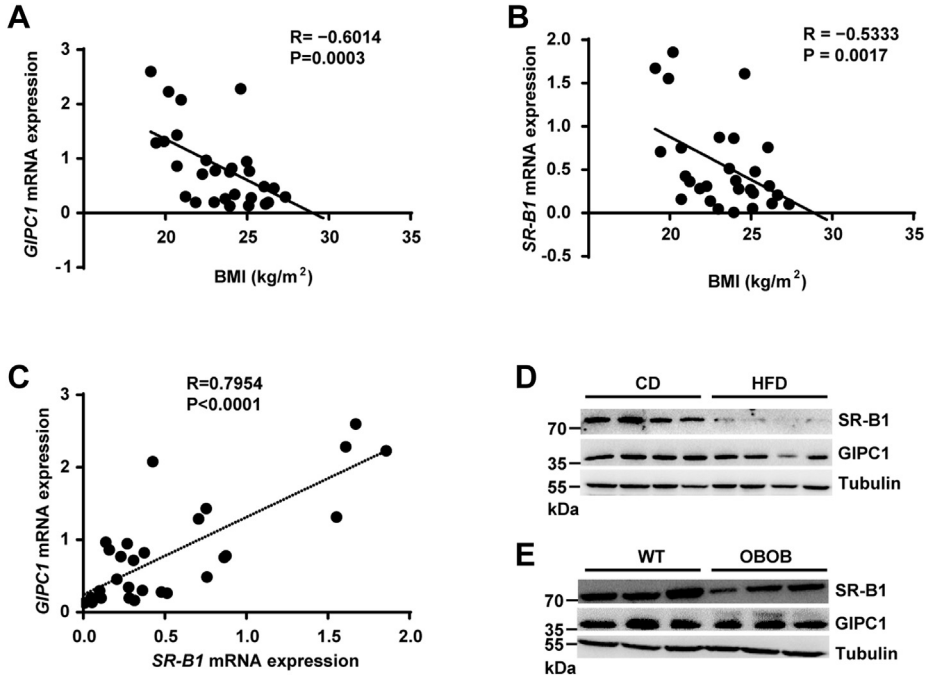

Figure 6. The expression of GIPC1 and SR-B1 in human clinical samples and liver of obese mice. Impact of human and rodent obesity on the functional expression of mRNA and protein levels of GIPC1 and SR-B1. *A*, negative correlation of liver *Gipc1* mRNA levels with BMI of obese and nonobese subjects. *B*, negative correlation of liver *Scarb1* mRNA levels with BMI of obese and nonobese subjects. *C*, positive correlation between hepatic *GIPC1* and *Scarb1* mRNA levels of liver samples derived from obese and nonobese subjects. The correlation coefficients (R values) and *p* values were calculated by Pearson analysis. *D*, western blots showing GIPC1 and SR-B1 protein levels in liver samples from chow diet (CD) and HFD-fed mice (in the livers of HFD-induced obese mice, n = 4). *E*, western blots showing protein levels of GIPC1 and SR-B1 in the liver samples from wild-type (C57BL/6J) and ob/ob mice (n = 3).

binds motor protein like MYO6. The central PDZ domain of GIPC1 can bind the C-terminal consensus (S/T)-X-**A**/V/L/I sequence of its regulating proteins (28). Our studies suggest that GIPC1 construct lacking PDZ domain loses its ability to bind to SR-B1. We also identified several motor proteins including MYO6 as the putative SR-B1-interacting proteins by pull-down/MS assays (Table S1). The related studies demonstrated that overexpression of GIPC1 upregulated both endogenous and ectopic expression of SR-B1 protein levels. These results are consistent with observations that GIPC1 is necessary for recycling and cell-surface expression of transmembrane receptors, such as IGF1R and TGFβR3 (28, 31, 34).

The studies presented in this article demonstrate the direct interaction of PDZ protein, GIPC1 with SR-B1, functions to upregulate the SR-B1 protein expression and SR-B1-mediated selective HDL-cholesterol transport in mouse liver cells. Gain and loss-of-function strategies further demonstrated that GIPC1 positively impacts cellular SR-B1 levels in both mouse model hepatic cell line (Hepa 1–6 cells) and primary hepatocytes. The TG and TC levels were attenuated by GIPC1-shRNA-mediated inhibition of GIPC1 expression, whereas overexpression of GIPC1 enhanced their cellular TG and TC levels. Several genes involved in hepatic lipid metabolism, including *Acc1* and *Fasn*, were altered in response to decreased expression of GIPC1. Silencing of GIPC1 in mouse liver *in vivo* was associated with increased serum levels of TG and TC, and decreased hepatic TG and TC content, and loss of SR-B1 in hepatocyte. Translational human studies indicated a negative correlation between hepatic GIPC1 expression and BMI of obese subjects. In addition, the expression of *Gipc1* was positively correlated with the *Scarb1* mRNA levels in human liver samples. These findings are also in close agreement with the data obtained using two mouse models of obesity: the HFD-fed mice and *ob/ob* mice. Feeding an HFD to mice inhibited the protein levels of both GIPC1 and SR-B1. Similarly, GIPC1 and SR-B1 protein levels were also attenuated in the livers of *ob/ob* mice compared with wild-type control mice. Overall these data suggest that GIPC1 modulation of SR-B1 expression and function may be another mechanism by which hepatic cells regulate their lipid metabolism including cholesterol metabolism.

As noted above, PDZK1 stabilizes SR-B1 through a protein–protein interaction, whereas NHERF1 and NHERF2 regulate its protein levels by promoting protein degradation *via* ubiquitination pathway (13, 17, 18, 35). Having established that GIPC1 upregulates the SR-B1 protein levels, we wished to determine whether GIPC1 does so by retarding the SR-B1 protein decay. To address this, HEK293 cells overexpressing SR-B1 and SR-B1 + GIPC1 were treated with CHX to arrest the cytosolic protein synthesis (translation), and subsequently, cell samples collected immediately and at different time points were analyzed for SR-B1 protein levels by western blotting. The half-life of SR-B1 was increased significantly in cells coexpressing SR-B1 and GIPC1 as compared with cells overexpressing SR-B1 alone. Furthermore, we found that treatment of hepatic cells with a proteasome inhibition, MG-132 or lysosome inhibitor, leupeptin hemisulfate enhanced SR-B1 protein levels in Hepa 1 to 6 cells, mouse primary hepatocytes, and HEK-293 cells overexpressing SR-B1 and

GIPC1 (Fig. 2*G*), suggesting that SR-B1 protein degradation is achieved by both proteasome and lysosomal pathways. Interestingly, expression of GIPC1 showed a slightly elevated levels of SR-B1 protein in cells treated with MG-132 or Leu, indicating that GIPC1 regulation of SR-B1 may also involve blocking SR-B1 degradation *via* proteasome and lysosomal pathways.

Conclusions

Taken together, these data strongly argue that SR-B1 interacting PDZ protein, GIPC1 is necessary for optimal expression and function of SR-B1 and that SR-B1 and GIPC1 work in concert to positively regulate hepatic cholesterol and lipid metabolism *in vitro* and *in vivo*. Interventions aimed at GIPC1-mediated increased expression of SR-B1 and/or SR-B1-linked regulation of hepatic cholesterol and lipid metabolism may prove effective in improving the health of the patients with dyslipidemia including hypercholesterolemia.

Experimental procedures

Reagents and antibodies

MG-132, leupeptin hemisulfate (Leu), chloroquine diphosphate, and protease inhibitor were purchased from MCE (MedChemExpress USA Co, Ltd). CHX was obtained from Glpbio Company. The PCR reagents were purchased from TAKARA Takara Biotechnology (Dalian) Co LTD Ltd. Most of the tissue culture supplies were procured from Thermo Fisher Scientific Inc through its Gibco Cell Culture Media Division. Antibodies against β-actin and ubiquitin were supplied by Santa Cruz Biotech. Anti-SR-B1 antibody (NB400-104) for co-immunoprecipitation was purchased from Novus Biologicals, LLC. The preparation of in-house generated polyclonal anti-SR-B1 antibody for western blotting has been described previously (23). The other following antibodies were also employed and obtained from the indication sources: anti-V5 tag from Invitrogen; anti-flag from Cell Signaling; anti-GIPC1 and anti-Tubulin from Abgent; secondary antibodies for western blotting, Goat-Anti-Rabbit and Goat-Anti-Mouse from Vazyme Biotech Co, Ltd.

Human samples

Liver specimens were collected from patients who underwent hepatic hemangioma surgery at the First Affiliated Hospital of Nanjing Medical University. This study was approved by the ethics committee at First Affiliated Hospital of Nanjing Medical University (Permit number 2018-SR-053), and abide by the Declaration of Helsinki principles. Patients provided informed consent at the time of surgery. Total RNA from liver tissue samples was isolated using a Trizol reagent (Invitrogen) and then was reversely transcribed into cDNA for qPCR.

Animals

All animal experiments were conducted in accordance with national and international guidelines on animal care and the procedures approved by the Laboratory Animal Care Committee at Nanjing Normal University (Permit 2090658, issued 20 April 2008). The 8-week-old male C57/BL6 mice and ob/ob mice were purchased from the Model Animal Research Center of Nanjing University and maintained on a 12-h light–dark cycle with free access to food and water. For long-term gene silencing, an oligonucleotide containing the siRNA sequence targeting GIPC1 was constructed into the adenoviral vector to endogenously express shRNA. The adenovirus (ad-GIPC1 shRNA) was packaged and purified by Vigene Biosciences. Adenovirus-expressing green fluorescent proteins (Ad-GFP) and scramble shRNA were used as controls. After a minimum 1-week acclimation period, animals were injected intravenously *via* the tail vein with adenoviruses at a dose of 1×10^9 plaque-forming units (PFU) per mouse. After 4 to 8 days, animals were euthanized by cervical dislocation, and serum and liver samples were collected for further analyses. The HFD-induced obesity was induced in C57BL/6J mice as described previously (23, 36).

Plasmids construction

The pCMV-flag-SR-B1 plasmid containing ORF of WT rat SR-B1 was constructed as previously described (13). A series of PCR products of rat SR-B1 with C-terminal or N-terminal deletion of amino acid residues 1 to 464 (flag-SR-B1-1-464), 1 to 474 (flag-SR-B1-1-474), 1 to 504 (flag-SR-B1-1-504), 9 to 509 (flag-SR-B1-9-509), and 9 to 464 (flag-SR-B1-9-464) were also generated and subcloned into pCMV-flag. Mouse full-length GIPC1 (amino acids [aa] 1–333; GIPC1) and truncated GIPC1 lacking putative GH2 (aa 1–225; GIPC1-1-225), PDZ and GH2 (aa 1–150; GIPC1-1-150), GH1 (aa 126–333; GIPC1-126-333), or GH1 and PDZ (aa 225–333; GIPC1-225-333) were amplified by PCR and subcloned into pcDNA6 with a V5 tag. The sequence of primers that were used are listed in Table S3. Nucleotide sequences of all the clones generated were confirmed by sequencing.

Cell culture and transfection

HEK293 and mouse hepatoma cells (Hepa 1–6) were purchased from the American Type Culture Collection (ATCC). HEK293 cells and Hepa 1 to 6 cells were cultured in Eagle's Minimum Essential Medium (EMEM) or Dulbecco's Modified Eagle's Medium (DMEM), respectively, supplemented with 10% fetal bovine serum and antibiotics (penicillin + streptomycin). All cell cultures were maintained at 37 °C in a humidified incubator in the presence of 5% CO_2/95% air. For transient transfection studies, cells were plated 12 h before transfection at a 50% confluence and then transfected with different plasmids using High Efficiency Transfection Reagent (Biomiga). Thirty-six to forty-eight hours after transfection, the cells were subjected to western blotting for detection and quantification of expressed protein or qRT-PCR for the quantification of mRNA levels. For inhibitor treatment studies, HEK293 cells were transfected with SR-B1 and control or GIPC1 plasmids for 30 h and then treated with vehicle (control) MG-132 (10 μM), Leu (30 μM), and chloroquine diphosphate (10 μM) for 12 h, and subsequently cells were

employed for western blotting. To perform SR-B1 protein decay experiments, HEK293 cells were transfected with control, SR-B1, or SR-B1 + GIPC1 plasmids for 36 h and then were treated with CHX (20 μg/ml) for different time points.

Primary hepatocytes were isolated from 8- to 10-week-old C57BL/6J mice by collagenase perfusion technique as described previously (37). Cells were cultured and maintained in 12-well or six-well plates containing DMEM/F-12 medium and subsequently, used for infection studies or studies involving treatment with inhibitors. For GIPC1 overexpression experiments, primary hepatocytes were infected with adenoviruses encoding the full-length of GIPC1 cDNAs (50 PFU) (Vigene Biosciences). The knockdown of endogenous GIPC1 expression, primary mouse hepatocytes were infected with adenoviruses (Vigene Biosciences) containing an shRNA targeting GIPC1.

Pull-down for mass spectrometry

Biotin-labeled *C*-terminal 45 peptide of mouse SR-B1 (Biotin-mCT45) was synthesized by Shanghai Bootech BioScience &Technology Co, Ltd. Mouse liver tissue samples were excised, washed, and homogenized in a lysis buffer supplemented with protease inhibitors. The biotin-labeled mCT45 peptide was incubated with a suitable aliquot of liver lysate while rotating at 4 °C for 2 h and then incubated with Streptavidin-Agarose (Thermo Fisher Scientific Inc) for an additional 3 h. The samples were centrifuged and washed with cold PBS for three times. The proteins bound to mCT45 peptide Agarose beads were eluted with SDS-PAGE loading buffer.

Sample preparation for mass spectrometry

The IP samples were treated with 100 mM dithiothreitol at 100 °C for 5 min and then transferred into 10kd ultrafiltration centrifuge tube with 200 μl UA buffer (8 M Urea, 150 mM TrisHCl pH 8.0) after being cooled to room temperature. After centrifuged at 14,000*g* for 15 min, the precipitate were suspended with 100 μl IAA (50 mM IAA in UA), shaken at 600 rpm for 1 min, protected from light for 30 min at room temperature, and then centrifuged at 14,000*g* for 10 min. After washing with UA buffer and dissolution buffer, sample was digested in 40 μl Trypsin buffer (2 μg Trypsin in 40 μl Dissolution buffer), shaken at 600 rpm for 1 min, 37 °C for 16 to 18 h. The sample was collected into a new tube, centrifuged at 14,000*g* for 10 min, and applied to the filtrate to perform MS analysis.

Liquid chromatography and mass spectrometry identification

The sample was loaded to the Capillary High-Performance Liquid chromatographic column for separation. Solution A is 0.1% formic acid aqueous solution, and solution B is 0.1% formic acid acetonitrile aqueous solution (acetonitrile is 84%). Column, 0.15 mm*150 mm (RP-C18) (Column Technology Inc), was balanced with 95% solution A. The sample was loaded into Zorbax 300sb-c18 peptide traps (Agilent Technologies) by an automatic injector and then separated on a chromatographic column. The liquid gradient is as follows: 0 to 50 min, solution B linear gradient from 4% to 50%; 50 to 54 min, solution B linear gradient from 50% to 100%; 54 to 60 min, solution B maintained at 100%. After separated by capillary high-performance liquid chromatography, the sample was analyzed by Q Exactive mass spectrometer (Thermo Fisher). The detection method was positive ion. The mass charge ratio of polypeptide and polypeptide fragment was collected according to the following method: ten fragment maps (MS^2 scan) were collected after each full scan.

ESI mass spectrometry data analysis

For raw file, Mascot 2.2 software was used to search the corresponding database. Finally, the identified protein results were obtained. The relevant parameters are as follows: Enzyme = Trypsin, Missed cleavage = 2, Fixed modification: Carbamidomethyl (C), Variable modification: Oxidation (M). The Uniprot database (uniprot_mouse_74528_20140502.REVERSED.fasta, download date 2040502, 74,528 sequences) was used for peptides identification. Peptides tolerance: 20 ppm; MS/MS tolerance: 0.1 Da and Mascot result filter parameter: FDR ≤ 0.01. The LC-MS/MS and bioinformatics analysis was performed by Shanghai Hoogen Biotechnology Co, LTD.

Co-immunoprecipitation (co-IP)

The co-IP experiment was performed as descripted previously (19). For endogenous proteins, primary anti-SR-B1 antibody or anti-GIPC1 antibody was bound to mix protein A/G beads (ThermoFisher Scientific) and incubated with cell extracts in IP buffer for 3 h at 4 °C. To detect expressed FLAG or V5-tagged proteins, cell lysates were incubated anti-FLAG M2 magnetic beads (Sigma) or anti-V5 antibody (Invitrogen) to capture the relevant proteins. Proteins eluted from beads were subjected to SDS-PAGE and analyzed by immunoblotting using relevant antibodies.

Metabolite measurements

Blood samples were collected in nonheparinized tubes, incubated at 4 °C for 4 h, and centrifuged at 3000*g* for 10 min. Serum samples were then collected and stored at −80 °C until further analysis. Triglyceride (TG) and total cholesterol (TC) levels in Hepa 1 to 6 cells, mouse primary hepatocyte cells, and mouse serum and livers were measured using commercially supplied kits (Jiancheng Institute of Biotechnology).

Dil-HDL uptake assays

The Dil-HDL uptake assays were performed as previously described (23, 36). Aliquots of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil)-labeled HDL (Shanghai Jingke Chemical Technology Co), 5 μg/ml were incubated with transfected cells for 6 h at 37 °C. Then cells were then washed and analyzed for fluorescence using Nikon Eclipse Ti-S inverted fluorescence microscope (Nikon). The captured Dil cell images were analyzed using an Image J program (38).

Western blotting

Different cell or tissues samples were collected, homogenized in RIPA buffer with protease inhibitor. The extracted proteins were subjected to 12% SDS-PAGE. After electrophoresis the proteins were transferred to PVDF membranes (Millipore Corp) and the protein blots were incubated with appropriate antibodies overnight at 4 °C, then probed with HRP-conjugated anti-mouse or anti-rabbit secondary antibody (Vazyme) for 2 h at room temperature. The membrane was then washed and developed *via* Tanon-4500 luminescent imaging workstation (Tanon Science & Technology).

Real-time RT-PCR (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) of RNA samples was performed as previously described (36). For this, total RNA from cells or tissues was isolated using a Trizol reagent (Invitrogen) and RNA reverse transcribed to cDNA using a cDNA synthesis kit (Takara). Quantitative real-time PCR was performed employing an SYBR Green PCR Master Mix (Sigma), and the transcript levels were detected with a StepOnePlus Real-Time PCR System (Applied Biosystems). The 36B4 gene was used as an internal control. The sequence of primers used in qRT-PCR is shown in Table S4.

Statistical analysis

All data were tested for the normality by the Kolmogorov–Smirnov test and homogeneity of variance by Bartlett's test. The results were presented as the means ± SD and analyzed by unpaired Student's *t*-tests between the two groups. We used partial correlation analysis to examine correlations among the BMI, mRNA levels of *Gipc1* and *Scarb1* in hepatic samples from obese and nonobese human subjects. Statistical analyses were performed using the Graphpad prism 6. The significance level was set at 0.05.

Data availability

Mass spectrometry raw data and results are available in iProX: (https://www.iprox.org/page/HMV006.html; project ID: IPX0002856000). All other data are included in the article.

Supporting information—This article contains supporting information.

Author contributions—Z. H, and Z. G.: conceptualization; Z. Z., Q. Z., R. L., Y. -F. Q., and Z. H.: data curation; Z. Z., Q. Z., R. L., and Y. -F. Q.: formal analysis; Z. Z., Q. Z., and R. L.: investigation; Z. H, S. A., W. -J. S., Q. Z., and R. L.: methodology; Z. Z., Q. Z., and R. L.: visualization; Q. Z., Z. Z., and Z. H.: writing—original draft; Z. H., L. L., and Z. G.: project administration; W. -J. S., S. A., L. L., and Z. G.: writing—review and editing; Z. H.: supervision; Z. H., L. L., and Z. G.: resources; Z. H. and Z. G.: funding acquisition.

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Conflict of interest—All authors declare that they have no competing interest to report.

Abbreviations—The abbreviations used are: ACTH, adrenocorticotropic hormone; Ad-GFP, adenovirus-expressing green fluorescent proteins; Ad-sh, adenoviruses with GIPC1 shRNA; BMI, body mass index; CEs, cholesteryl esters; CHX, cycloheximide; CREB, cAMP response element binding protein; Dil, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; ERα, estrogen receptors α; ERβ, estrogen receptors β; FA, fatty acid; FSH, follicle-stimulating hormone; GH1, GIPC homology 1; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HFD, high-fat diet; IGF1R, insulin-like growth factor-1 receptor; LDL-C, LDL-cholesterol; Leu, leupeptin hemisulfate; LH, luteinizing hormone; LRH-1, liver receptor homolog 1; LXRα, liver X receptors α; LXRβ, liver X receptors β; MS, mass spectrometry; NR0B1 (DAX-1), nuclear receptor subfamily 0 group B member 1; NRP1, neuropilin 1; oxLDL, oxidized LDL; PFU, plaque-forming units; PPARα, peroxisome proliferator–activated receptor α; SF-1, steroidogenic factor 1; SIK1, salt inducible kinase 1; SR-B1, scavenger receptor class B type 1; SREBP-1a, sterol-regulatory element binding protein-1a; TC, total cholesterol; TG, Triglyceride; TGFβR3, transforming growth factorβ receptor type III; VLDL, very-low-density lipoprotein.

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