OXFORD

Human Molecular Genetics, 2020, Vol. 29, No. 5 834–844

doi: 10.1093/hmg/ddaa007
Advance Access Publication Date: 21 January 2020
General Article One

GENERAL ARTICLE ONE

The signaling pathways implicated in impairment of hepatic autophagy in glycogen storage disease type Ia

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Abstract

Glucose-6-phosphatase-α (G6Pase-α or G6PC) deficiency in glycogen storage disease type-Ia (GSD-Ia) leads to impaired hepatic autophagy, a recycling process important for cellular metabolism and homeostasis. Autophagy can be regulated by several energy sensing pathways, including sirtuin 1 (SIRT1), forkhead box O (FoxO), AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor-α (PPAR-α), and mammalian target of rapamycin (mTOR). Using 10-day old global *G6pc*-deficient (*G6pc*−/−) mice, hepatic autophagy impairment was attributed to activation of mTOR and inhibition of AMPK signaling. In other studies, using adult liver-specific *G6pc*-deficient mice at both pre-tumor and tumor stages, hepatic autophagy impairment was attributed to downregulation of SIRT1 signaling and mTOR was not implicated. In this study, we provide a detailed analysis of the major autophagy pathways in young *G6pc*−/− mice over the first 4 weeks of life. We show that impaired SIRT1, FoxO3a, AMPK, and PPAR-α signaling are responsible for autophagy impairment but mTOR is involved minimally. Hepatic SIRT1 overexpression corrects defective autophagy, restores the expression of FoxO3a and liver kinase B1 but fails to normalize impaired PPAR-α expression or metabolic abnormalities associated with GSD-Ia. Importantly, restoration of hepatic G6Pase-α expression in *G6pc*−/− mice corrects defective autophagy, restores SIRT1/FoxO3a/AMPK/PPAR-α signaling and rectifies metabolic abnormalities. Taken together, these data show that hepatic autophagy impairment in GSD-Ia is mediated by downregulation of SIRT1/FoxO3a/AMPK/PPAR-α signaling.

Introduction

Glycogen storage disease type Ia (GSD-Ia, MIM232200) is caused by a deficiency in the enzyme activity of glucose-6-phosphatase-α (G6Pase-α or G6PC) that catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis in the liver and the kidney (1, 2). GSD-Ia patients manifest a phenotype of impaired glucose homeostasis, characterized by fasting hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, lactic acidemia, and growth retardation (1, 2). Untreated, GSD-Ia is juvenile lethal. Strict compliance with dietary therapies has enabled GSD-Ia patients to attain near normal growth and pubertal development (1, 2). However, the underlying pathological processes remain uncorrected and long-term complications including hepatocellular adenoma (HCA), which may undergo transformation to hepatocellular carcinoma (HCC)

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Received: August 22, 2019. **Revised:** December 19, 2019. **Accepted:** January 15, 2020

are common (1–4). Currently, a clinical gene therapy trial is exploring the efficacy of restoring hepatic G6Pase-α activity for human GSD-Ia (NCT03517085). However, it remains unclear whether HCA/HCC will respond to gene therapy in a clinical setting.

Autophagy is an evolutionary conserved, degradative process that facilitates the cellular clearance or turnover of misfolded proteins, protein aggregates, and damaged organelles (5). A deficiency in hepatic autophagy has been linked to many metabolic disorders, including hepatocarcinogenesis (6). Autophagy can be regulated by several signaling pathways, including sirtuin 1 (SIRT1) (7, 8), forkhead box O (FoxO) transcription factor family members (9, 10), AMP-activated protein kinase (AMPK) (11, 12), peroxisome proliferator-activated receptor-α (PPAR-α) (13, 14), and mammalian target of rapamycin (mTOR) (15). The FoxO transcription factors are critical regulators of autophagy that control the transcription of autophagy-related (*ATG*) genes (9, 10). SIRT1 is a NAD^+-dependent deacetylase whose activity can be increased by increased gene expression, interaction of the protein with activator molecules, or by increased cellular NAD^+ levels (7, 8). SIRT1 can regulate autophagy directly via deacetylation of key components of the autophagy network, including the ATG proteins, and indirectly via deacetylation and activation of FoxO transcription factors (7). AMPK is a key enzyme that regulates cellular energy state, growth, inflammation, and mitochondrial function (12). In the liver, AMPK suppresses *de novo* lipogenesis, increases fatty acid oxidation, promotes mitochondrial function-integrity, and activates autophagy (11, 12). PPAR-α is a nutrient sensing nuclear receptor that promotes fatty acid oxidation, serves as the master regulator of lipid metabolism, and promotes autophagy (16, 17). mTOR stimulates the synthesis of protein, lipid, and nucleotides and acts as a negative regulator of autophagy (15). Importantly, cross-talk can occur among these energy sensing pathways. SIRT1 and AMPK regulate each other and share many common targets, resulting in many of the convergent biological effects on energy metabolism (18). SIRT1 deacetylates the liver kinase B1 (LKB1), leading to LKB1 cytoplasmic localization and activation (19). The activated LKB1 in turn activates AMPK via activating phosphorylation of the AMPK-α subunit at residue T-172 (20). AMPK can enhance SIRT1 activity by increasing cellular NAD^+ levels, resulting in the deacetylation and modulation of the activity of many downstream SIRT1 targets (21). Studies have shown that liver specific deletion of SIRT1 impaired PPAR-α signaling, while increased hepatic SIRT1 levels stimulate PPAR-α activity (22). Using PPAR-α agonist, it was shown that PPAR-α can increase SIRT1 gene expression in fasting-induced anti-aging pathways (23).

To study the mechanisms underlying metabolic aberrations in GSD-Ia, we have generated two mouse models. One is a global *G6pc*-deficient (*G6pc*−/−) mouse line that mimics the phenotype of human GSD-Ia (24). However, even under intensive glucose therapy, less than 25% of *G6pc*−/− mice survive to weaning (21 days), making the follow-up study of metabolic aberrations difficult. The other is a liver-specific *G6pc*-deficient (L-*G6pc*−/−) mouse line, which readily survives to adulthood allowing the study of the adult onset HCA/HCC phenotype of GSD-Ia (25). Three recent studies have revealed that hepatic G6Pase-α deficiency leads to impaired autophagy in GSD-Ia (25–27). Using 10-day-old *G6pc*−/− mice, Farah *et al.* showed that impaired autophagy in GSD-Ia is caused by activation of mTOR and down-regulation of AMPK signaling (26). Using adult L-*G6pc*−/− mice at the pre-tumor stage, we showed that the underlying mechanism regulating hepatic autophagy impairment is downregulation of SIRT1 signaling (25). We further showed that treatment of L-*G6pc*−/− mice with rapamycin, a mTOR inhibitor failed to correct defective hepatic autophagy, demonstrating that mTOR signaling plays a minor role in regulating autophagy in GSD-Ia (25). Using adult L-*G6pc*−/− mice at the tumor stage, Gjorgjieva *et al.* showed that downregulation of SIRT1 signaling underlies impaired hepatic autophagy independent of the mTOR pathway (27). Since these studies used a different mouse model, the signaling pathways implicated in autophagy deficiency in young *G6pc*−/− mice remained unclear.

In this study, we sought to reconcile the reported differences and elucidate the various energy sensing signaling pathways regulating autophagy in *G6pc*−/− mice of 1–4 weeks of age. We show that consistent with the adult L-*G6pc*−/− mice, multiple signaling pathways, including SIRT1, FoxO3a, AMPK, and PPAR-α are downregulated in *G6pc*−/− mice but the activation status of mTOR is unaltered. We further show that, SIRT1 overexpression in *G6pc*−/− mice corrects hepatic autophagy and restores FoxO3a and LKB1 expression, indicating that SIRT1 is a key regulator of hepatic autophagy in GSD-Ia. SIRT1 overexpression, however, does not normalize PPAR-α expression or correct the hallmark metabolic abnormalities of GSD-Ia. Finally, we show that restoration of hepatic G6Pase-α expression via gene transfer normalizes defective autophagy, restores SIRT1, FoxO3a, AMPK, and PPAR-α signaling, and corrects metabolic abnormalities associated with G6Pase-α deficiency. We conclude that gene therapy offers a promising therapeutic strategy for GSD-Ia.

Results

Young *G6pc*−/− mice display impaired hepatic autophagy

Both young global G6Pase-α-knockout (*G6pc*−/−) (26) and adult liver-specific G6Pase-α-knockout (L-*G6pc*−/−) (25, 27) mice display impairment of hepatic autophagy but the signaling pathways regulating autophagy in GSD-Ia remain unclear. We first examined the expression of genes in components of the autophagy network in the livers of 1-, 2-, 3-, and 4-week-old *G6pc*−/− mice. Similar results were obtained and data for 2- and 4-week-old *G6pc*−/− mice are shown (Fig. 1). Impaired autophagy was evidenced by decreased expression of ATG101, Beclin-1, LC3B-I (microtubule-associated protein 1 light chain 3B-I) and LC3B-II in the livers of *G6pc*−/− mice at age 2 weeks (Fig. 1A) and 4 weeks (Fig. 1B). LC3B is a marker of autophagosome formation and the decreased expression is consistent with impaired formation (28). Impairment of autophagy is usually accompanied by the accumulation of p62, a selective substrate for autophagy (5). Compared to the controls, hepatic levels of p62 were increased in both 2- and 4-week-old *G6pc*−/− mice (Fig. 1). Intriguingly, the increase in p62 was more marked at age 2 weeks than that at age 4 weeks (compare Fig 1A and 1B).

Impaired hepatic SIRT1/FoxO3a/PPAR-α signaling in young *G6pc*−/− mice

Using L-*G6pc*−/− mice, we have shown downregulation of SIRT1/FoxO3a signaling underlies hepatic autophagy deficiency in adult GSD-Ia mice (25). In this study of young *G6pc*−/− mice, we show that hepatic protein levels of SIRT1 and FoxO3a were markedly decreased in both 2- and 4-week-old *G6pc*−/− mice, compared to the controls (Fig 2A). Consistent with the attenuated expression of SIRT1, the ratios of acetylated FoxO3a (inactive form) to total FoxO3a in nuclear extracts of 4-week-old *G6pc*−/− livers were higher than those of control livers

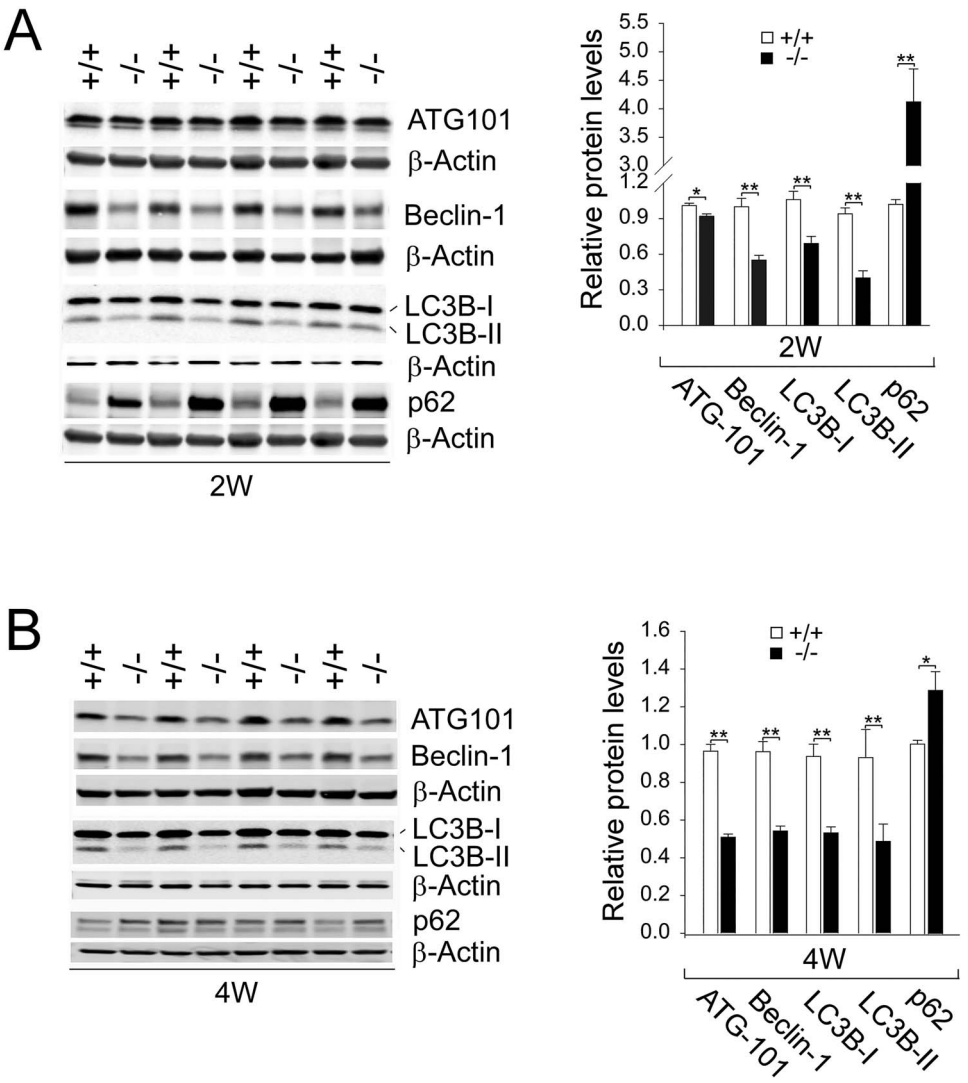

Figure 1. Impaired hepatic autophagy in young *G6pc*−/− mice.
Western-blot and densitometry analyses of hepatic ATG101, Beclin-1, LC3B, p62, and β-actin in 2-week-old (n = 8) (A) and 4-week-old (n = 8) (B) *G6pc*−/− mice. (+/+), WT mice; (−/−), *G6pc*−/− mice. Data represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$.

(Fig 2B), demonstrating that hepatic levels of active FoxO3a were also reduced in *G6pc*−/− mice. Hepatic steatosis negatively regulates autophagy (29, 30) and reduces SIRT1 expression (25). The *G6pc*−/− mice displayed marked hepatic steatosis during postnatal development and hepatic levels of triglyceride (TG) in 1-, 2-, 3-, and 4-week-old *G6pc*−/− mice were 10.7-, 5.4-, 2.2-, and 4-fold higher, respectively, than that in control mice (Fig. 2C). Oil Red O staining confirmed that 4-week-old G6pc−/− mice exhibited a marked hepatic steatosis (Fig. 2D). Consistently, hepatic levels of PPAR-γ, a ligand-regulated nuclear receptor that governs adipocyte differentiation and a transcriptional inhibitor of SIRT1 (31) were significantly higher in *G6pc*−/− mice as compared to the control mice (Fig. 2E). The transcription of SIRT1 can also be stimulated by PPAR-α (23) that positively regulates hepatic autophagy (13, 14), suggesting that downregulation of PPAR-α in G6Pase-α-deficient liver (Fig. 2E) also contributes to defective autophagy. Taken together, downregulation of SIRT1/FoxO3a/PPAR-α signaling contributes to autophagy deficiency in GSD-Ia.

Impaired AMPK and unaltered mTOR signaling in the liver of young *G6pc*−/− mice

AMPK is a positive regulator of autophagy (11, 12). In the liver, LKB1 activates AMPK activity via phosphorylation of the AMPK α-subunit at T172 (20). In our study of 2- and 4-week-old *G6pc*−/− mice, hepatic levels of total AMPK were increased, but hepatic levels of the active p-AMPK-T172 were markedly decreased (Fig. 3A). Consistently, hepatic levels of LKB1 were markedly decreased in *G6pc*−/− mice (Fig. 3B), indicating that downregulation of AMPK signaling contributes to autophagy deficiency in GSD-Ia.

Studies have shown that AMPK activates autophagy via activating phosphorylation of unc-51-like kinase1 (ULK1), a mammalian protein kinase that plays a key role in the initial stages of autophagy (32, 33). We showed that while hepatic levels of total ULK1 was not statistically different between control and *G6pc*−/− mice, hepatic levels of p-ULK1-S555 and p-ULK1-S317, two major AMPK-dependent phosphorylation sites, and the ratios of p-ULK1-S555 to total ULK1and p-ULK1-S317 to total

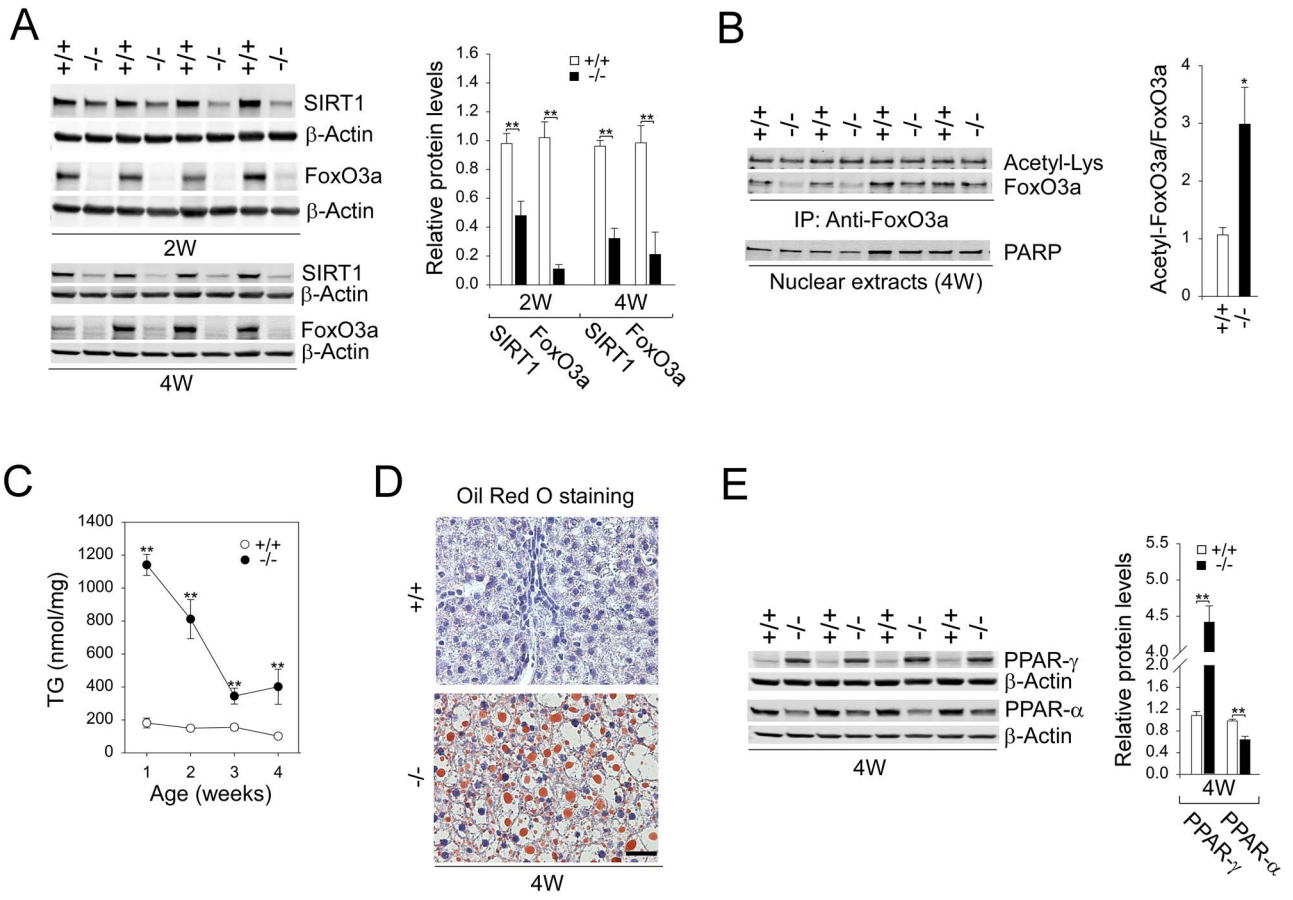

Figure 2. Impaired hepatic SIRT1, FoxO3a, and PPAR-α signaling in young *G6pc*−/− mice.
(A) Western-blot and densitometry analyses of hepatic SIRT1, FoxO3a, and β-actin in 2-week-old (n = 8) and 4-week-old (n = 8) *G6pc*−/− mice. (B) Western blots of acetylated and total FoxO3a after immunoprecipitation of nuclear extracts using anti-FoxO3a, and quantification of the acetylated FoxO3a/total FoxO3a (n = 8). (C) Hepaic triglyceride (TG) levels in 1-week-old (n = 8), 2-week-old (n = 8), 3-week-old (n = 8), and 4-week-old (n = 8) *G6pc*−/− mice. (D) Oil Red O staining of livers from 4-week-old control (+/+) and *G6pc*−/− (−/−) mice. Scale bar, 25 μm. (E) Western-blot and densitometry analyses of PPAR-γ, PPAR-α, and β-actin in 4-week-old (n = 8) *G6pc*−/− mice. (+/+), WT mice; (−/−), *G6pc*−/− mice. Data represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$.

ULK1 were decreased significantly in the *G6pc*−/− mice (Fig. 3B), suggesting that ULK1 is the kinase mediating the AMPK action in *G6pc*−/− mice.

Using 10-day-old *G6pc*−/− mice, Farah *et al.* showed that activation of mTOR signaling is responsible for autophagy deficiency in GSD-Ia (26). In contrast, mTOR signaling was shown to play a minimal role in autophagy impairment in adult L-*G6pc*−/− mice (25, 27). mTOR activity is activated by phosphorylation (34, 35). We therefore examined the activation status of mTOR in young *G6pc*−/− mice during weeks 1–4 of postnatal development. Similar results were obtained for all ages and data for 2- and 4-week-old *G6pc*−/− mice are shown. We showed that hepatic levels of total mTOR and the activated p-mTOR-S2448/p-mTOR-S2481 were similar between 2- and 4-week-old *G6pc*−/− and control mice (Fig. 3C), suggesting that mTOR signaling is not involved in regulating hepatic autophagy in GSD-Ia.

Studies have shown that mTOR inhibits autophagy via inhibiting phosphorylation of ULK1 at S757 (32, 36). We showed that hepatic levels of total ULK1, p-ULK1-S757, and the ratio of p-ULK1-S757 to total ULK1 were similar between *G6pc*−/− and control mice (Fig. 3B), again suggesting that mTOR plays a minimal role in regulating hepatic autophagy in GSD-Ia.

SIRT1 overexpression corrects hepatic autophagy impairment in young *G6pc*−/−mice

To demonstrate that down-regulation of SIRT1 signaling plays a key role in hepatic autophagy impairment in *G6pc*−/− mice, we examined the effects of adenovirus (Ad)-mediated SIRT1 overexpression on autophagy. An increase in hepatic SIRT1 expression in *G6pc*−/− mice corrected defective autophagy evident by normalization of hepatic levels of FoxO3a (Fig. 4A) and genes in the autophagy network, including ATG3, Beclin-1, LC3B-II, although hepatic levels of ATG101 and p62 were unaltered (Fig 4B). Studies have shown that the conjugation between ATG12 and ATG5 is essential for LC3 lipidation (37). We showed that an increase in hepatic SIRT1 expression in *G6pc*−/− mice also normalized hepatic levels of the ATG12-ATG5 conjugate (Fig. 4B).

SIRT1 deacetylation of LKB1 at residue K-48 (19) leads to activation of AMPK (20). We showed that SIRT1 overexpression normalized hepatic levels of total LKB1 and acetyl-LKB1 although the reduction in acetyl-LKB1 was not statistically significant (Fig. 5A). Interestingly, SIRT1 overexpression increased hepatic levels of total AMPK in both control and *G6pc*−/− mice (Fig. 5B). SIRT1 overexpression also increased hepatic levels of the activated p-AMPK-T172, although the increase was not statistically significant (Fig. 5B). Our results suggest that SIRT1 signaling may

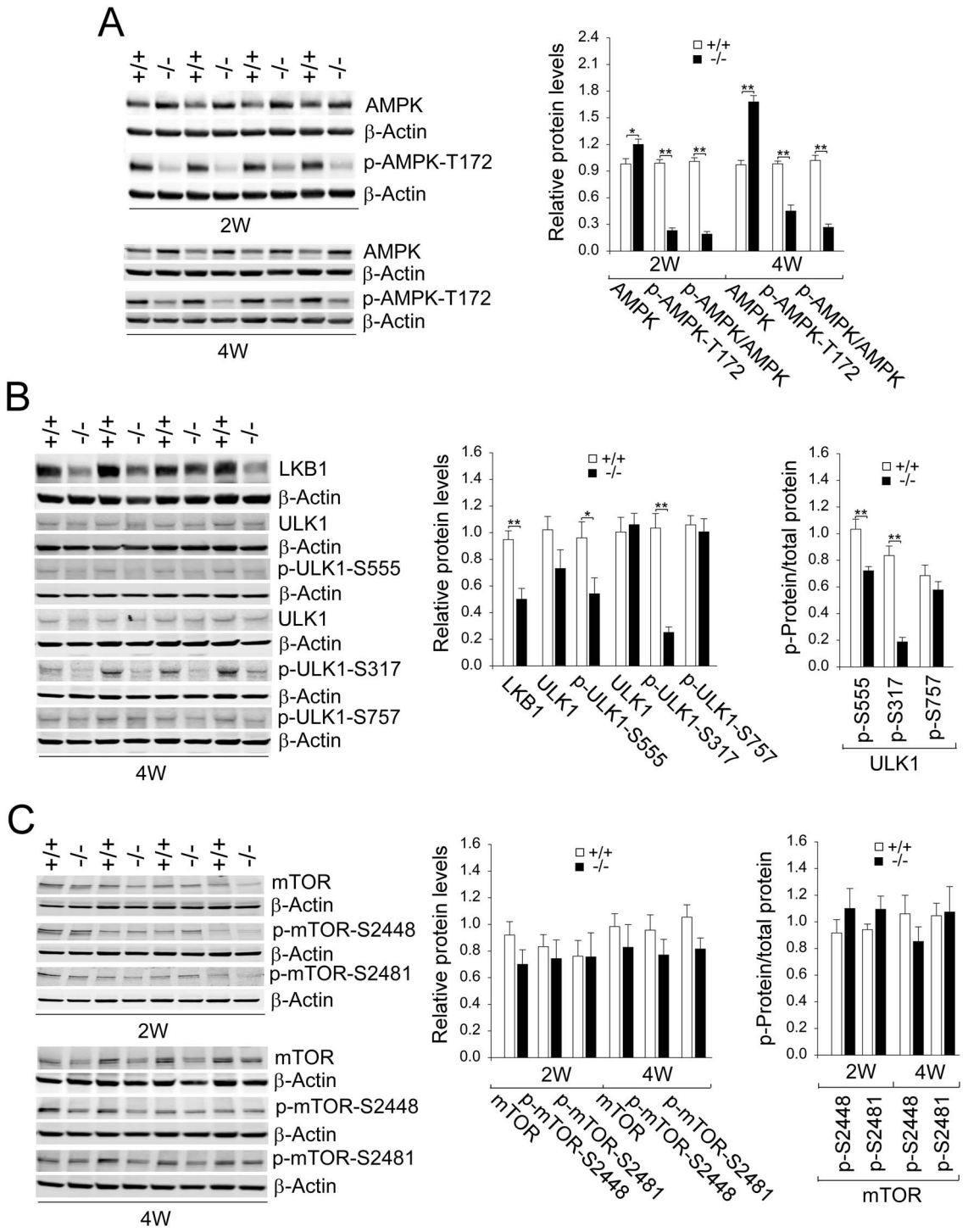

Figure 3. Impaired hepatic AMPK and unaltered mTOR signaling in young *G6pc*−/− mice.
(A) Western-blot and densitometry analyses of hepatic AMPK, p-AMPK-T172, and β-actin in 2-week-old (n = 8) and 4-week-old (n = 8) *G6pc*−/− mice. (B) Western-blot and densitometry analyses of hepatic LKB1, ULK1, p-ULK1-S555, p-ULK1-S317, p-ULK1-S757 and β-actin in 4-week-old (n = 8) *G6pc*−/− mice. (C) Western-blot and densitometry analyses of hepatic mTOR, p-mTOR-S2448, p-mTOR-S2481, and β-actin in 2-week-old (n = 8) and 4-week-old (n = 8) *G6pc*−/− mice. (+/+), WT mice; (−/−), *G6pc*−/− mice. Data represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$.

play a role in modulating AMPK activity in G6Pase-α-deficient livers.

SIRT1 overexpression, however, was unable to alter hepatic levels of PPAR-α and PPAR-γ (Fig. 5C). Moreover, SIRT1 overexpression also failed to correct metabolic alterations associated with GSD-Ia and hepatic levels of G6P, glycogen, and lactate remained elevated in Ad-SIRT1-treated *G6pc*−/− mice (Fig 5D). Taken together, down-regulation of SIRT1 signaling plays a key role in regulating hepatic autophagy in *G6pc*−/− mice but regulation of autophagy alone does not ameliorate the metabolic

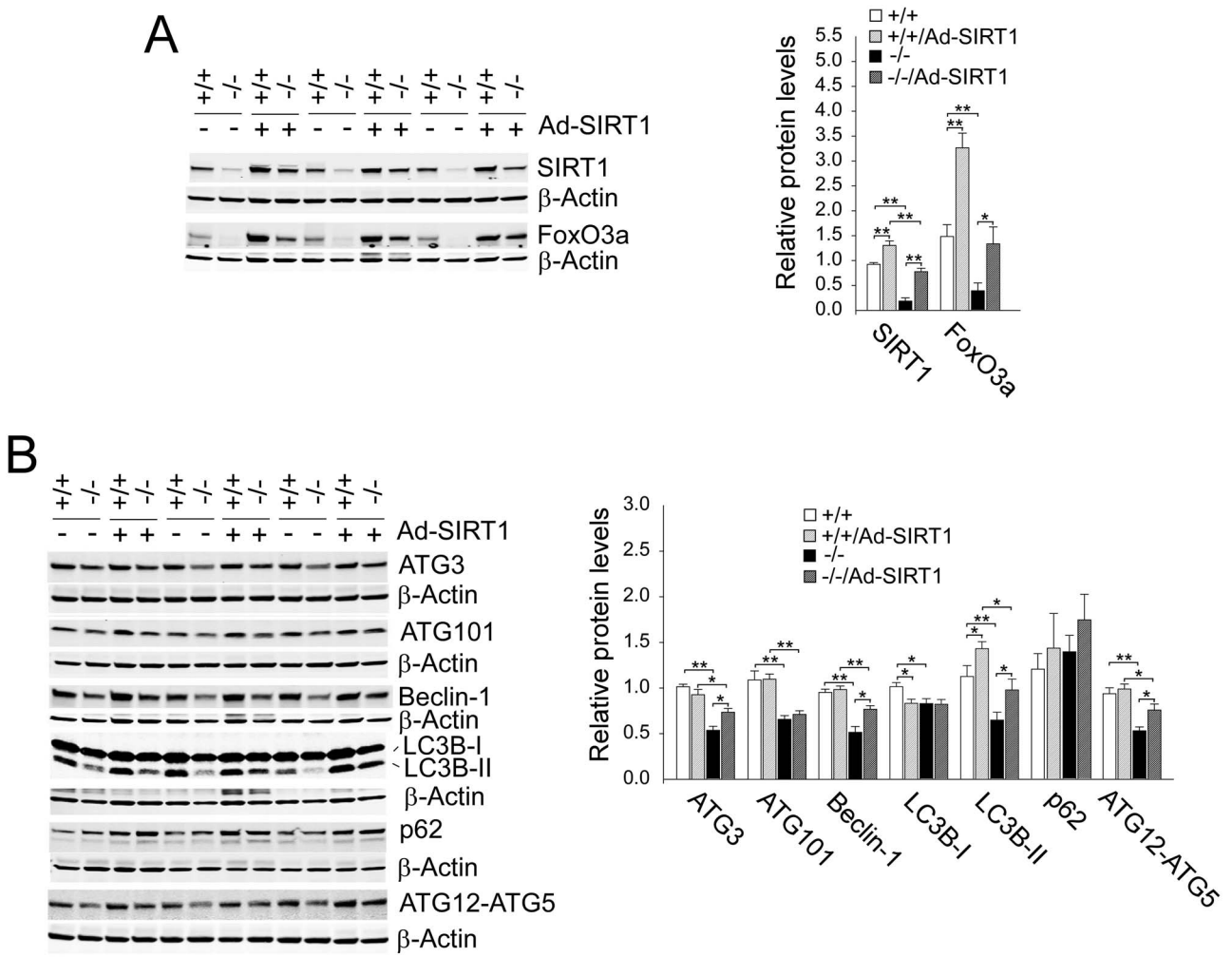

Figure 4. Effects of Ad-SIRT1 on hepatic autophagy and FoxO3a expresssion.
One-week-old control and *G6pc*−/− mice were treated with 1 x 10^8 pfu/mouse of Ad-SIRT1 and analyzed at age 3 weeks. (A) Western-blot and densitometry analyses of hepatic SIRT1, FoxO3a, and β-actin (n = 8). (B) Western-blot and densitometry analyses of hepatic ATG3, ATG101, Beclin-1, LC3B, p62, ATG12-ATG5 conjugate, and β-actin (n = 8). (+/+), WT mice; (−/−), *G6pc*−/− mice. Data represent the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.005$.

hallmark of GSD-Ia. Hepatic G6Pase-α plays additional role in maintaining liver homeostasis.

Restoration of hepatic G6Pase-α expression corrects autophagy impairment and restores liver homeostasis

We have developed two human G6Pase-α-expressing recombinant adeno-associated virus (rAAV) vectors, one (rAAV-WT) expressing wild-type G6Pase-α, the other (rAAV-S298C) expressing a novel G6Pase-α-S298C variant with increased enzymatic activity (38). We treated 2-week-old *G6pc*−/− mice with either 3 x 10^{12} vp/kg of rAAV-WT or rAAV-S298C. Hepatic G6Pase-α activity in 4-week-old control, rAAV-WT-treated, and rAAV-S298C-treated *G6pc*−/− mice averaged 272.8 ± 20.8, 24.8 ± 2.7, and 55.5 ± 3.2 nmol/min/mg, respectively. We have shown that rAAV-treated *G6pc*−/− mice expressing ≥3% of normal hepatic G6Pase-α activity maintain blood glucose homeostasis (39). As expected, the rAAV-WT- and rAAV-S298C-treated *G6pc*−/− mice expressing 9% and 19% normal hepatic G6Pase-α activity, respectively displayed a phenotype indistinguishable from that of the control mice, and were collectively named the rAAV mice.

The restoration of hepatic G6Pase-α expression in the *G6pc*−/− mice normalized hepatic levels of SIRT1, FoxO3a, ATG101, Beclin-1, LC3B-I, and p62, although the changes in LC3B-II were not statistically significant (Fig. 6A). While hepatic levels of AMPK remained elevated in untreated and rAAV-treated *G6pc*−/− mice, hepatic G6Pase-α restoration normalized hepatic levels of p-AMPK-T172 and LKB1 (Fig. 6B). Importantly, G6Pase-α restoration also normalized hepatic levels of PPAR-α and PPAR-γ (Fig. 6C), and hepatic levels of G6P, lactate, TG, and glycogen (Fig. 6D). Collectively, hepatic G6Pase-α restoration corrected defective autophagy, normalized SIRT1/FoxO3a/AMPK/PPAR-α signaling, and normalized metabolic abnormalities associated with GSD-Ia, demonstrating the multiple roles of G6Pase-α in liver homeostasis.

Discussion

GSD-Ia is caused by a deficiency of the metabolic enzyme G6Pase-α that catalyzes the hydrolysis of G6P to glucose and phosphate and is a key enzyme in maintaining blood glucose homeostasis (1, 2). Recent studies have shown that one consequence of hepatic G6Pase-α deficiency is impairment of

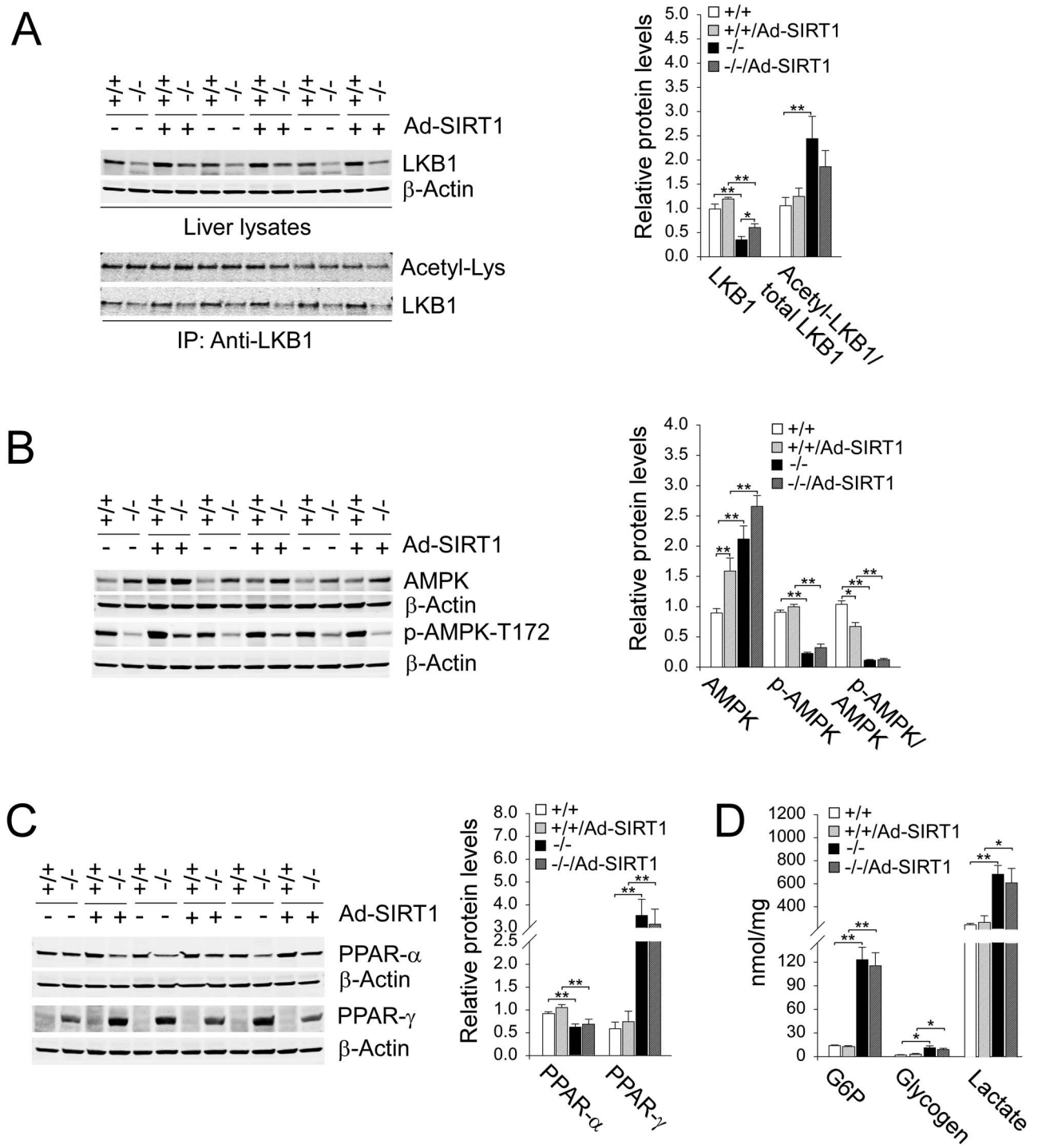

Figure 5. Effects of Ad-SIRT1 on the associated autophagy signaling pathways.
One-week-old control and *G6pc*−/− mice were treated with 1 x 10^8 pfu/mouse of Ad-SIRT1 and analyzed at age 3 weeks. (A) Western blots of total LKB1 as well as total and acetylated LKB1 after immunoprecipitation of liver extracts using anti-LKB1, and quantification of the acetylated LKB1/total LKB1 (n = 8). (B) Western-blot and densitometry analyses of hepatic AMPK, p-AMPK-T172, and β-actin (n = 8). (C) Western-blot and densitometry analyses of hepatic PPAR-α, PPAR-γ, and β-actin (n = 8). (D) Hepatic levels of G6P, glycogen and lactate (n = 4). (+/+), WT mice; (−/−), *G6pc*−/− mice. Data represent the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.005$.

autophagy but the signaling pathways that regulate autophagy in GSD-Ia are unclear. While disturbed AMPK/mTOR signaling was shown to mediate hepatic autophagy impairment in 10-day-old *G6pc*−/− mice (26), downregulation of SIRT1 signaling independent of mTOR was shown to underlie hepatic autophagy impairment in adult L-*G6pc*−/− mice (25, 27). To further elaborate the signaling pathways implicated in autophagy deficiency in the early stages of GSD-Ia, we now carried out a detailed study examining the signaling pathways regulating autophagy in *G6pc*−/− mice during weeks 1–4 of postnatal development.

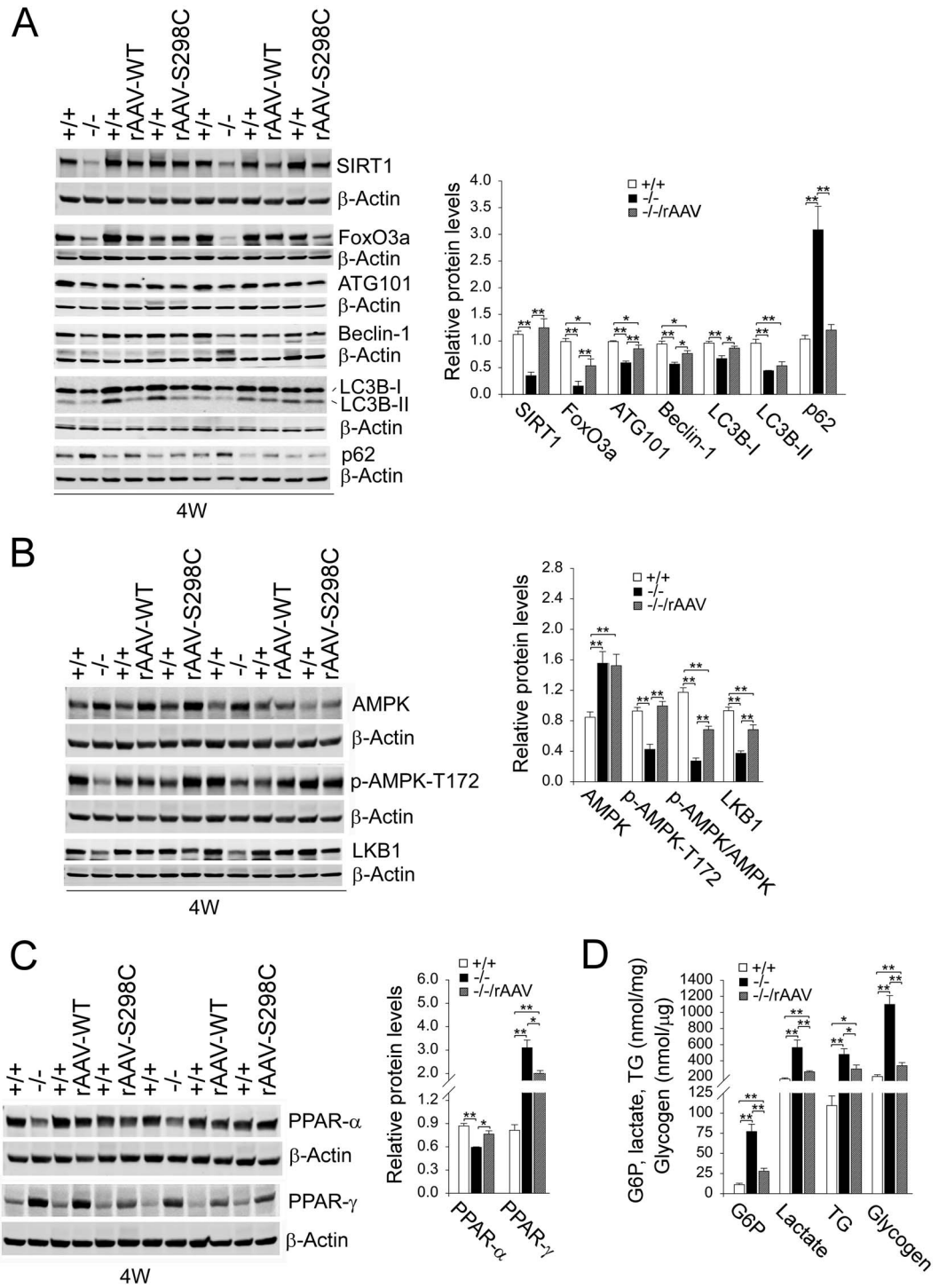

Figure 6. Restoration of hepatic G6Pase-α expression corrects autophagy and restores liver homeostasis.
Two-week-old control and *G6pc*−/− mice were treated with 3 x 10^{12} vp/kg of rAAV-WT or rAAV-S298C and analyzed at age 4 weeks. (A) Western-blot and densitometry analyses of hepatic SIRT1, FoxO3a, ATG101, Beclin-1, LC3B, p62, and β-actin (n = 12). (B) Western-blot and densitometry analyses of hepatic AMPK, p-AMPK, LKB1, and β-actin (n = 8). (C) Western-blot and densitometry analyses of hepatic PPAR-α, PPAR-γ, and β-actin (n = 8). (D) Hepatic levels of G6P, lactate, TG, and glycogen (n = 8). (+/+), WT mice; (−/−), *G6pc*−/− mice. Data represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$.

We showed that the impaired hepatic autophagy in *G6pc*−/− mice is mediated by downregulation of multiple energy sensing signaling pathways, including SIRT1, FoxO3a, AMPK, and PPAR-α (Fig. 7). As was shown in adult L-*G6pc*−/− mice (25, 27), mTOR signaling plays a minimal role in young *G6pc*−/− mice. We further showed that hepatic SIRT1 overexpression corrected autophagy impairment and normalized the expression of FoxO3a and LKB1, suggesting that SIRT1 may play a key role in regulating hepatic autophagy in GSD-Ia. Finally, we showed that hepatic G6Pase-α restoration corrected defective

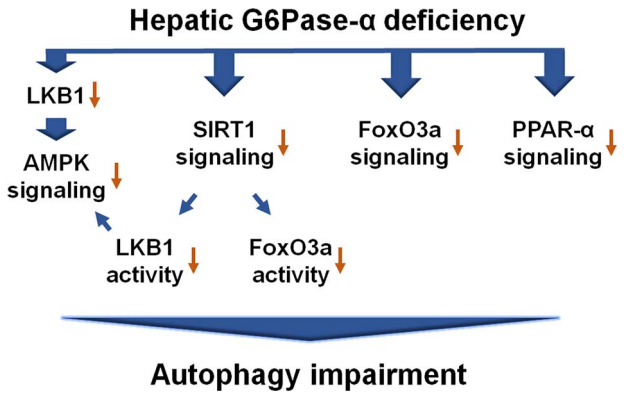

Figure 7.. The mechanism underlying hepatic autophagy impairment in young *G6pc*−/− mice.

Hepatic G6Pase-α-deficiency leads to autophagy impairment that is mediated by downregulation of multiple autophagy-activating signaling pathways, including AMPK, SIRT1, FoxO3a, and PPAR-α. Hepatic G6Pase-α-deficiency also results in reduced expression of LKB1 that can impair AMPK signaling. SIRT1 can regulate autophagy directly via deacetylation of key components of the autophagy network and indirectly via deacetylation and activation of FoxO3a. SIRT1 can also deacetylate and activate LKB1, leading to activating phosphorylation of the AMPK-α subunit.

autophagy, restored SIRT1/FoxO3a/AMPK/PPAR-α signaling, and normalized metabolic abnormalities associated with GSD-Ia.

G6Pase-α plays a vital role in maintaining liver homeostasis. Consequently, hepatic G6Pase-α deficiency leads to downregulation of many stress-related signaling pathways, such as SIRT1, FoxO3a, AMPK, and PPAR-α. Using 1- to 4-week-old *G6pc*−/− (this study) and adult L-*G6pc*−/− (25) mice, we showed that hepatic G6Pase-α deletion results in suppressed expression of PPAR-α that stimulates fatty acid oxidation, promotes autophagy, and enhances SIRT1 expression (13, 14, 23). Hepatic G6Pase-α deletion also leads to increased expression of PPAR-γ, a lipogenic factor capable of suppressing SIRT1 expression (31). The net outcome of PPAR-α suppression and PPAR-γ stimulation is a marked increase in hepatic steatosis that negatively regulates autophagy (29, 30) and reduces SIRT1 expression (25). Indeed, hepatomegaly, the hallmark of GSD-Ia is caused by a marked increase in neutral fat and glycogen storage (1, 2). The available evidence indicates that downregulation of SIRT1 signaling plays a key role in hepatic autophagy deficiency in GSD-Ia. SIRT1 can regulate autophagy directly via deacetylation of ATG proteins, and indirectly via deacetylation and activation of FoxO members (7) that transactivate *ATG* genes (9, 10). Indeed, the ratios of acetylated FoxO3a (inactive form) to total FoxO3a in 4-week-old *G6pc*−/− livers were higher than those of control livers. We further showed that hepatic SIRT1 overexpression in *G6pc*−/− mice corrected impaired autophagy and normalized hepatic levels of FoxO3a. SIRT1 can augment AMPK signaling via activation of LKB1 (19) that activates AMPK signaling via activating phosphorylation of the AMPK-α subunit (20). We showed that hepatic SIRT1 overexpression normalized hepatic levels of total LKB1, leading to a moderate increase in hepatic levels of p-AMPK.

SIRT1 and PPAR-α regulate each other. An increase in hepatic SIRT1 stimulates PPAR-α activity (22) and PPAR-α is a key molecule that increases SIRT1 gene expression (23). The inability of hepatic SIRT1 overexpression to normalize PPAR-α expression suggests that in *G6pc*−/− mice PPAR-α is a transcription factor that works upstream of SIRT1. We also showed that hepatic SIRT1 overexpression failed to normalize PPAR-γ expression, suggesting that PPAR-γ also acts upstream of SIRT1 in *G6pc*−/− mice. Autophagy can also be regulated by mTOR (15) which was implicated in inhibiting autophagy in 10-day-old *G6pc*−/− mice (26). We have previously shown that the activation status of mTOR was similar between control and L-*G6pc*−/− mice at the pre-tumor stage (25). We further showed that rapamycin treatment of L-*G6pc*−/− mice failed to normalize impaired hepatic autophagy, suggesting that mTOR plays a minimal role in autophagy deficiency in the liver-specific knockout model of GSD-Ia (25). Similarly, Gjorgjieva *et al.* showed that hepatic autophagy deficiency in L-*G6pc*−/− mice at the tumor stage is independent of the mTOR pathway (27). We confirmed this finding in the current study using 1- to 4-week-old *G6pc*−/− mice, in which we showed that SIRT1, FoxO3a, AMPK, and PPAR-α signaling were downregulated in G6Pase-α-deficient livers but the activation status of mTOR was unaltered. In summary, hepatic autophagy deficiency in GSD-Ia is regulated by multiple energy sensing signaling pathways including SIRT1, FoxO3a, AMPK, and PPARα.

One severe long-term complication associated with hepatic G6Pase-α deficiency is HCA/HCC. Recent studies begin to elucidate the molecular mechanisms regulating hepatocarcinogenesis in GSD-Ia using adult L-*G6pc*−/− mice. Firstly, hepatic G6Pase-α deficiency leads to SIRT1 signaling downregulation resulting in autophagy impairment (25, 27) and mitochondrial dysfunction (40) that can contribute to carcinogenesis. Secondly, hepatic G6Pase-α deficiency leads metabolic reprogramming (41) resulting in enhanced glycolysis and increased hexose monophosphate shunt and both can contribute to carcinogenesis. Therefore, multiple aberrations in G6Pase-α-deficient livers lead to the development of HCA/HCC in GSD-Ia. Importantly, restoration of hepatic G6Pase-α expression in L-*G6pc*−/− mice normalizes these aberrations (25, 40, 41) and prevents hepatic tumor initiation (42). We have shown that the rAAV-G6PC-treated *G6pc*−/− mice restoring ≥3% of normal hepatic G6Pase-α activity maintain glucose homeostasis and show no evidence of HCA/HCC (39). We now show that restoration of hepatic G6Pase-α expression in young *G6pc*−/− mice normalizes SIRT1, FoxO3a, AMPK and PPAR-α signaling, corrects autophagy impairment, and rectifies metabolic abnormalities. Taken together, our results suggest that gene therapy offers a promising therapeutic strategy to normalize glucose homeostasis and prevent HCA/HCC development in GSD-Ia.

Materials and Methods

Animals

All animal studies were conducted under an animal protocol approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Animal Care and Use Committee. To increase SIRT1 expression, 1-week-old *G6pc*−/− (24) mice were treated with 1 x 10^8 pfu/mice of Ad-SIRT1, a recombinant Ad vector expressing human SIRT1 (25) via retro-orbital sinus and liver samples were collected at age 3 weeks. To reconstitute hepatic G6Pase-α activity, 2-week-old *G6pc*−/− mice were treated with 3 x 10^{12} vp/kg of rAAV-WT or rAAV-S298C (38) via retro-orbital sinus and liver samples were collected at age 4 weeks. GSD-Ia is an autosomal recessive disorder and the phenotypes of *G6pc*+/+ and *G6pc*+/− were indistinguishable, therefore both mice were used as controls (referred collectively as WT or the control mice).

Metabolites determinations

Liver tissues were homogenized in 5% NP-40 and centrifuged to remove insoluble material.

The resulting supernatants were used to measure levels of G6P, lactate, glycogen, and TG using the respective assay kit from BioVision (Milpitas, CA).

Western-blot analysis

Western-blot images were detected with the use of the LI-COR Odyssey scanner and the Image studio 3.1 software (Li-Cor Biosciences, Lincoln, NE). The antibodies obtained from Cell Signaling Technology (Danvers, MA) were: Acetylated-Lysine (#9814), AMPK (#2532), ATG101 (#13492), ATG3 (#3415), ATG12 (#4180), Beclin-1 (#3738), FoxO3a (#12829), LKB1 (#3047), mTOR (#2983), p-mTOR-S2448 (#5536), p-mTOR-S2481 (#2974), PARP (#9542), ULK1 (# 8054S), p-ULK1-S757 (#14202S), and p-ULK1-S555 (#5869S). The antibodies obtained from Abcam (Cambridge, MA) were: p-AMPK (ab133448), LC3B (ab51520), and PPAR-α (ab24509). The antibodies obtained from Santa Cruz Biotechnology (Dallas, TX) were: β-actin (sc-47 778) and PPAR-γ (sc-7196). The antibody against SIRT1 (#07–131) was from Millipore (Burlington, MA), the antibody against p62 (NBP1–49956) was from Novus Biologicals (Centennial, CO), and the antibody against p-ULK1-S317 (#LS-C800719) was from Lifespan Bioscience Inc (Seattle, WA).

Immunoprecipitation

To detect the acetylated LKB1, liver tissues were homogenized in the cell lysis buffer (Cell Signaling # 9803) containing 1 X Halt Protease and Phosphatase Inhibitor Cocktails (Thermo Scientific, Waltham, MA), 1 mM okadaic acid (Sigma-Aldrich, St. Louis, MO), and 1 mM PMSF (Cell Signaling # 8553). After centrifugation, the resulting supernatants were subjected to immunoprecipitation with an antibody against LKB1 (Santa Cruz Biotechnology: sc32245) and the resulting precipitates were examined by Western-blot analysis using an antibody against either the acetylated lysine (Cell Signaling # 9814) or LKB1 (Cell Signaling #3047).

To detect the acetylated FoxO3a, liver nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) containing 1 X Halt Protease and Phosphatase Inhibitor Cocktails, 1 mM okadaic acid, and 1 mM PMSF. After centrifugation, the resulting supernatants were subjected to immunoprecipitation with an antibody against FoxO3a (Cell Signaling #12829) and the resulting precipitates were examined by Western-blot analysis using an antibody against either the acetylated lysine (Cell Signaling # 9814) or FoxO3a (Cell Signaling #12829).

Statistical analysis

The unpaired t test was performed using the GraphPad Prism Program, version 4 (GraphPad Software, San Diego, CA). Values were considered statistically significant at $p < 0.05$.

Conflict of Interest statement. None declared.

Funding

This research was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health.

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