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Absence of the SRC-2 Coactivator Results in a Glycogenopathy Resembling Von Gierke's Disease

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Abstract

Hepatic glucose production is critical for basal brain function and survival when dietary glucose is unavailable. Glucose-6-phosphatase (G6Pase) is an essential, rate-limiting enzyme that serves as a terminal gatekeeper for hepatic glucose release into the plasma. Mutations in *G6Pase* result in Von Gierke's disease (glycogen storage disease–1a), a potentially fatal genetic disorder. We have identified the transcriptional coactivator SRC-2 as a regulator of fasting hepatic glucose release, a function that SRC-2 performs by controlling the expression of hepatic *G6Pase*. SRC-2 modulates *G6Pase* expression directly by acting as a coactivator with the orphan nuclear receptor RORα. In addition, SRC-2 ablation, in both a whole-body and liver-specific manner, resulted in a Von Gierke's disease phenotype in mice. Our results position SRC-2 as a critical regulator of mammalian glucose production.

In mammals, when exogenous glucose is not available, between meals and during fasting, the liver produces glucose from substrates such as glycogen, lactate, pyruvate, glycerol, and amino acids (1). During fasting, the liver contributes about 85 to 90% of the glucose load, and the remainder is contributed by the kidney (1,2). This function of the liver is vital for basal brain function, and thus for the survival of the fasting organism (1,3).

Glucose-6-phosphatase (G6Pase) is an essential, rate-limiting enzyme that catalyzes the terminal reaction that produces glucose during fasting (2,4,5). This reaction is common to glycogenolysis and gluconeogenesis, the two processes that provide the body with glucose when the organism is subjected to a fast. This metabolic location, at the crossroad between glycogenolysis and gluconeogenesis, allows G6Pase to control both the short-term and longer-

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Methods
Figs. S1 to S11
Table S1
References

term responses to fasting (2,4,5). G6Pase is present at the membrane of the endoplasmic reticulum in the liver and kidney, where it dephosphorylates glucose-6-phosphate to produce free glucose (2,4,5). Without dephosphorylation, glucose remains trapped in the liver, because the glucose transporters that shuttle it into the plasma cannot transport the phosphorylated form (2,4,5). Loss-of-function mutations in *G6Pase* result in Von Gierke's disease (glycogen storage disorder–1a), an autosomal recessive disorder with an incidence of 1 in 100,000 live births (6). Von Gierke's disease is clinically characterized by impaired growth, fasting hypoglycemia, and an increase in concentrations of triglycerides, cholesterol, free fatty acids, ketone bodies, uric acid, and lactic acid in the plasma of fasting animals. Increased liver glycogen stores and hepatic steatosis also occur (6).

Steroid receptor coactivator 2 (SRC-2), a member of the p160 family of transcriptional co-activators, has been implicated in a number of physiological processes, from reproduction, mammary morphogenesis, and uterine function to energy metabolism in adipose tissue via regulation of adaptive thermogenesis (7-10). Its role in liver-mediated glucose homeostasis, however, is unknown. Because fasting is a powerful inducer of the glycogenolytic and gluconeogenic response through increased transcription of the *G6Pase* gene, we tested the role of SRC-2 in fasting modulation of *G6Pase* expression. In the absence of SRC-2 in mice, there was a deficit in fasting *G6Pase* expression in both the liver and the kidney (Fig. 1A). This deficit in expression was accompanied by a deficiency in the catalytic activity of hepatic G6Pase.

Because *G6Pase* expression is enhanced by fasting and suppressed by feeding, we tested the effect of a lack of SRC-2 in those settings. SRC-2 was critical for both basal as well as induced expression of *G6Pase* (Fig. 1B). The fasting expression of other genes involved in the control of gluconeogenic activity such as *phosphoenolpyruvate carboxykinase* (*PEPCK*), *fructose-1,6-bisphosphatase 1* (*FBP1*), *peroxi-some proliferator-activated receptor gamma, coactivator 1 alpha* (*PGC-1a*), and *Glucose-6-phosphate translocase* (*G6P translocase*) was essentially unaffected by the absence of SRC-2 (Fig. 1C). Of 21 other genes that are important for fasting glucose homeostasis, in the glycogenolysis, glycogenesis, β-oxidation, ketogenesis, and hexose monophosphate shunt pathways, expression of none of these genes was significantly decreased in mice lacking SRC-2 (table S1). To determine whether other p160 coactivator family members (SRC-1 and SRC-3) function in *G6Pase* regulation during fasting, we determined *G6Pase* expression in the livers of SRC-1 and SRC-3 null animals that were fasted for 24 hours. The absence of SRC-1 and SRC-3 did not affect the expression of *G6Pase*, suggesting an SRC-2–specific effect on the regulation of fasting hepatic *G6Pase* expression (fig. S10).

It was possible that the expression deficit of *G6Pase* that we observed in SRC-2 null animals was due to an indirect systemic effect. To rule out this possibility we isolated primary hepatocytes from wild-type (WT) mice and depleted SRC-2 using RNA interference (RNAi). RNAi-mediated knockdown of SRC-2 resulted in a down-regulation of *G6Pase* expression, suggesting a cell-autonomous effect (Fig. 1D). *G6Pase* is positively regulated, at the transcriptional level, by fasting-associated hormones such as glucagon, catecholamines, and glucocorticoids. We exposed primary hepatocytes from WT and SRC-2 null animals to dexamethasone to stimulate the glucocorticoid receptor or forskolin to simulate hormonal stimulation of adenylyl cyclase. Ablation of SRC-2 decreased both control and hormone-mediated transcription of *G6Pase* (fig. S6, A and B).

The primary manifestations of Von Gierke's disease, which occur as a result of mutations in the *G6Pase* gene, are diminished growth; fasting hypoglycemia; increased concentrations of triglycerides, cholesterol, free fatty acids, ketone bodies, uric acid, and lactic acid in the plasma; increased liver glycogen stores; and hepatic steatosis (6). SRC-2 null mice showed fasting

hypoglycemia, although the animals maintained normoglycemia in the fed state (Fig. 2A). Considered along with the observation of fasting hypoinsulinemia (Fig. 2B), this suggests a defect in liver function. Further plasma analysis demonstrated increased concentrations of triglycerides, cholesterol, free fatty acids (FFAs), and ketone bodies in SRC-2$^{-/-}$ animals that were fasted for 24 hours (Fig. 2C). Histologically, liver morphology was distorted with a characteristic mosaic pattern like that of patients with Von Gierke's disease(Fig.2D)(11). Periodic acid–Schiff staining, Oil Red O staining, and electron microscopy (EM) revealed heavy accumulation of glycogen and triglycerides in the liver of fasted SRC-2 null animals (Fig. 2, E through G). Livers of 24-hour fasted SRC-2 null animals contained about twice as much glycogen and about three times as much triglycerides as did livers from WT animals (Fig. 2H). This was accompanied by increased concentrations of circulating glucagon and catecholamine in SRC-2 null animals, suggesting a compensatory attempt to mobilize liver glycogen (fig. S1). The growth deficit that accompanies the lack of SRC-2 has been described (12).

To unequivocally assess the effect of SRC-2 ablation on fasting (basal) liver glucose production, we measured hepatic glucose production using radiolabeled glucose in awake SRC-2 null animals and WT littermates that had been fasted overnight. Basal hepatic glucose production in SRC-2 null animals was depressed compared with that of WT littermates (fig. S3A). In vitro glucose production from gluconeogenic precursors lactate and pyruvate, by primary hepatocytes subjected to RNAi-mediated knockdown of SRC-2, was one-half that by control cells (fig. S3B).

Given that glucose homeostasis in fasting animals is primarily a function of the liver, we tested whether SRC-2 ablation, specifically in the liver, would reproduce the features of Von Gierke's disease. We infused adenovirus carrying the CRE recombinase gene into the tail veins of homozygous SRC-2 floxed (F/F) mice and exposed control SRC-2 F/F mice to empty adenovirus. This approach produces liver-selective transgenesis, because most of the adenoviral load is localized in the liver (13-15). Liver-selective depletion of SRC-2 resulted in decreased abundance of G6Pase mRNA in the liver of fasting animals (Fig. 3A). *G6Pase* expression in the kidney was increased, possibly as a compensatory response (Fig. 3A). By contrast, the results in fasted SRC-2 (whole-body) knockout mice showed decreased renal *G6Pase* expression, and thus exemplifies the critical nature of SRC-2's influence over *G6Pase* expression (Fig. 1A). Liver-selective depletion of SRC-2 in fasted animals resulted in hypoglycemia (Fig. 3B), along with increased concentrations of triglycerides, cholesterol, ketone bodies, and uric acid in the plasma (Fig. 3C). This plasma profile was accompanied by an accumulation of hepatic glycogen and triglycerides in fasted liver-selective SRC-2 null animals (Fig. 3D), thereby reproducing the phenotypic characteristics of Von Gierke's disease.

Because SRC-2 is a “coactivator” and lacks specific DNA binding activity, it is presumably recruited to a gene promoter by a transcription factor in order to function. Although transcriptional coactivators often function at multiple genes, our results indicated that SRC-2 might regulate the expression of just one gene in the gluconeogenesis and glycogenolysis pathways—*G6Pase* (Fig.1,A and B, and table S1).We therefore searched the *G6Pase* promoter sequence for transcription factor response elements that might explain such selectivity. We found an evolution-arily conserved retinoid-related orphan receptor α (RORα) response element (RORE) close to the transcriptional start site (fig. S11). SRC-2 and RORα have been reported to bind and synergize in reporter assays (16). Consequently, we tested the human WT *G6Pase* promoter (−1227, +57) by coexpressing it with expression plasmids carrying SRC-2 and RORα. SRC-2 and RORα, when expressed together, synergized strongly to produce *G6Pase* promoter activation (Fig. 4A). This synergism (coactivation) was completely abolished upon specific mutation of the RORE (Fig. 4A). We also tested other transcription factors that bind SRC-2 and are important for regulation of fasting glucose homeostasis such

as the glucocorticoid receptor and orphan nuclear receptor Nurr77, and found that SRC-2 did not synergize with them in this context (fig. S7C).

To demonstrate the effect of RORα on trans-activation of *G6Pase* in the correct chromatin context, we transduced primary hepatocytes with adenoviral RORα. This resulted in increased expression of the endogenous *G6Pase* gene, without affecting the expression of other key genes involved in the gluconeogenic pathway such as *PEPCK*, *FBP1*, *PGC-1a* and *G6P translocase* (Fig. 4B). The RORα-mediated increase in *G6Pase* transactivation was abolished in a SRC-2 null background (fig. S4B). Furthermore, RNAi-mediated depletion of RORα in primary hepatocytes resulted in decreased expression of *G6Pase* (fig. S4). To assess whether RORα affects *G6Pase* regulation mediated by fasting hormones, we treated primary hepatocytes that had been exposed to RNAi-mediated depletion of RORα with dexamethasone or forskolin. Depletion of RORα diminished the effect of dexamethasone and forskolin stimulation of *G6Pase* gene expression (fig. S6C), although forskolin retained a strong activating effect. We also tested the effects of the promoter-proximal RORE on mediation of the signal from dexamethasone and forskolin (fig.S6D). Basal activity of the *G6Pase* promoter and that induced by a combination of dexamethasone and forskolin was inhibited upon mutation of the RORE, suggesting that this response element functions in the signaling from fasting hormones that results in *G6Pase* promoter activation. In vivo chromatin immunoprecipitation (ChIP) experiments showed that both SRC-2 and RORα proteins bound to the *G6Pase* promoter, selectively in the region of the RORE (Fig. 4C). To further substantiate that SRC-2 was recruited to the *G6Pase* promoter by RORα, we overexpressed RORα in primary hepatocytes. This resulted in increased promoter occupancy of RORα, which in turn increased the promoter occupancy of SRC-2, suggesting that RORα could indeed recruit SRC-2 to the *G6Pase* promoter (Fig. S5A). Conversely, SRC-2 promoter occupancy was abolished in cells depleted of RORα (Fig. S5B). These results suggest that RORα is necessary and sufficient to recruit SRC-2 to the *G6Pase* promoter.

The phenotype and the gene-expression profile of SRC-2 null animals suggests that the perturbation of glucose homeostasis is due to a deficiency in *G6Pase* expression. However, the possibility that SRC-2 might regulate another gene that might contribute to the phenotype cannot be ruled out. Therefore, we reintroduced G6Pase into SRC-2 null mice by infusion of a recombinant adenovirus containing *G6Pase* cDNA, or an adenovirus containing no cDNA insert. We measured blood glucose concentration in three independent feeding states—fed, fasted for 4 hours, and fasted for 18 hours. Fasting hypoglycemia caused by lack of SRC-2 was diminished in animals that received the G6Pase adenovirus compared to that in animals that were exposed to an empty adenovirus (Fig. 5). This result is consistent with the possibility that some of the glucose homeostasis deficit observed in fasted SRC-2 null animals results from a deficiency in *G6Pase* expression.

Glycogenolysis is the primary process of endogenous glucose production in the first 4 hours after a meal and is followed by the transition to gluconeogenesis upon prolonged fasting (2, 4,5). During glycogenolysis, there is no requirement for the activation of PEPCK and FBP1, enzymes that are limited to the process of gluconeogenesis (1). We report the identification of two transcriptional entities, SRC-2 and RORα, that regulate *G6Pase* without regulating *PEPCK* and *FBP1*. Unlike systems in which transcriptional coactivators exert control over multiple downstream target genes to modulate physiology (17-19), SRC-2 appears to control glucose availability through the regulation of one key mediator (Fig. 1, A and B, and table S1). This study unmasks a biological entity, other than mutations in the *G6Pase* gene itself, that might contribute to cases of glycogen storage disorders and unexplained hypoglycemia. Accordingly, SRC-2 and RORα may represent new therapeutic targets against these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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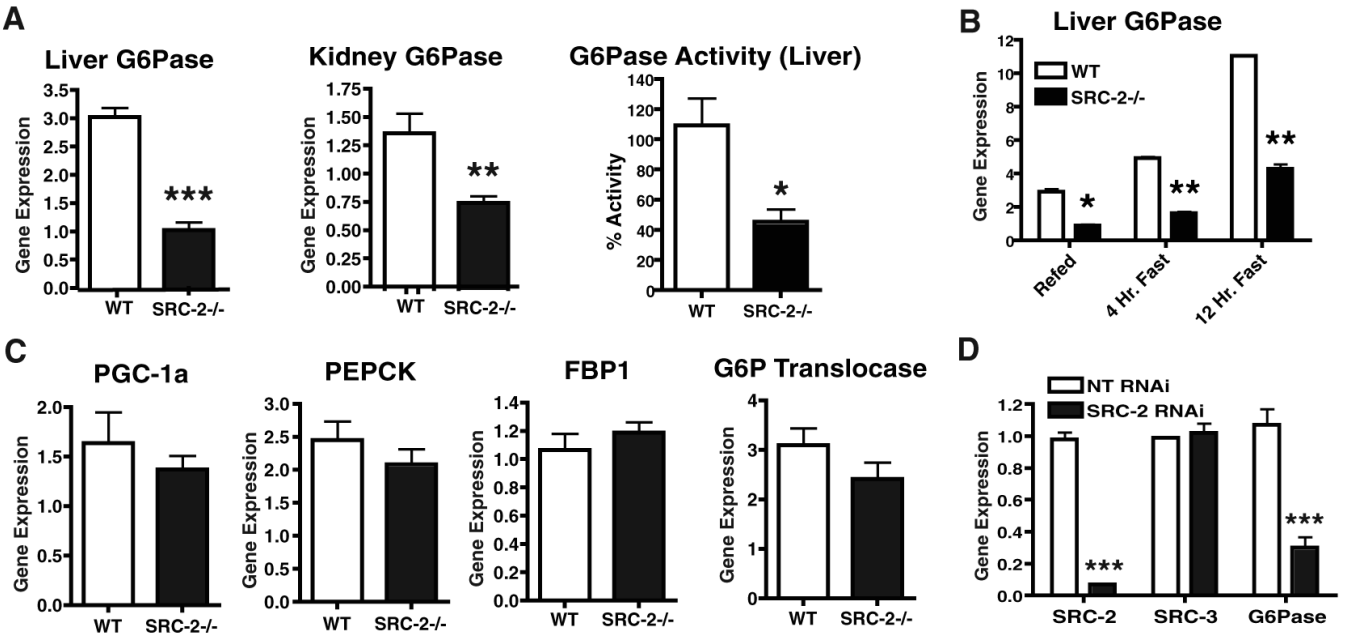

Fig. 1.

Role of SRC-2 in *G6Pase* expression in the mouse liver and kidney. (**A**) Diminished expression and activity of G6Pase in the liver and kidney of SRC-$2^{-/-}$ mice. *G6Pase* expression was measured via relative quantitation by quantitative polymerase chain reaction (QPCR) in the liver and kidney of SRC-2 null mice and WT littermates upon 24 hours of fasting (n = 5 mice per group). For G6Pase activity analysis, the first value (WT#1) was fixed at 100, and the rest of the values were compared relative to that (n = 5 mice per group). (**B**) Role of SRC-2 in basal as well as induced hepatic *G6Pase* expression. *G6Pase* expression was measured via relative quantitation by QPCR in the liver of WT and SRC-2 null mice that were refed for 2 hours after a 4-hour fast, fasted for 4 hours, or fasted for 12 hours (n = 3 to 4 mice per group). (**C**) SRC-2 does not regulate the expression of other rate-limiting genes in the gluconeogenesis program. Expression of *G6P translocase*, *PEPCK*, *FBP1*, and *PGC-1a* was measured via relative quantitation by QPCR in the liver of SRC-2 null mice and WT littermates upon 24 hours of fasting (n =5 mice per group). (**D**) SRC-2 regulates *G6Pase* expression in a cell-autonomous manner. RNAi to SRC-2 results in down-regulation of G6Pase mRNA levels compared with nontargeting (NT) RNAi, as determined via relative quantitation by QPCR. SRC-3 expression level is used as a control for specificity of SRC-2 knockdown. Data are represented as the mean ± SEM. Unpaired student's t test was used for evaluation of statistical significance. *P < 0.05, **P < 0.01, and ***P < 0.001.

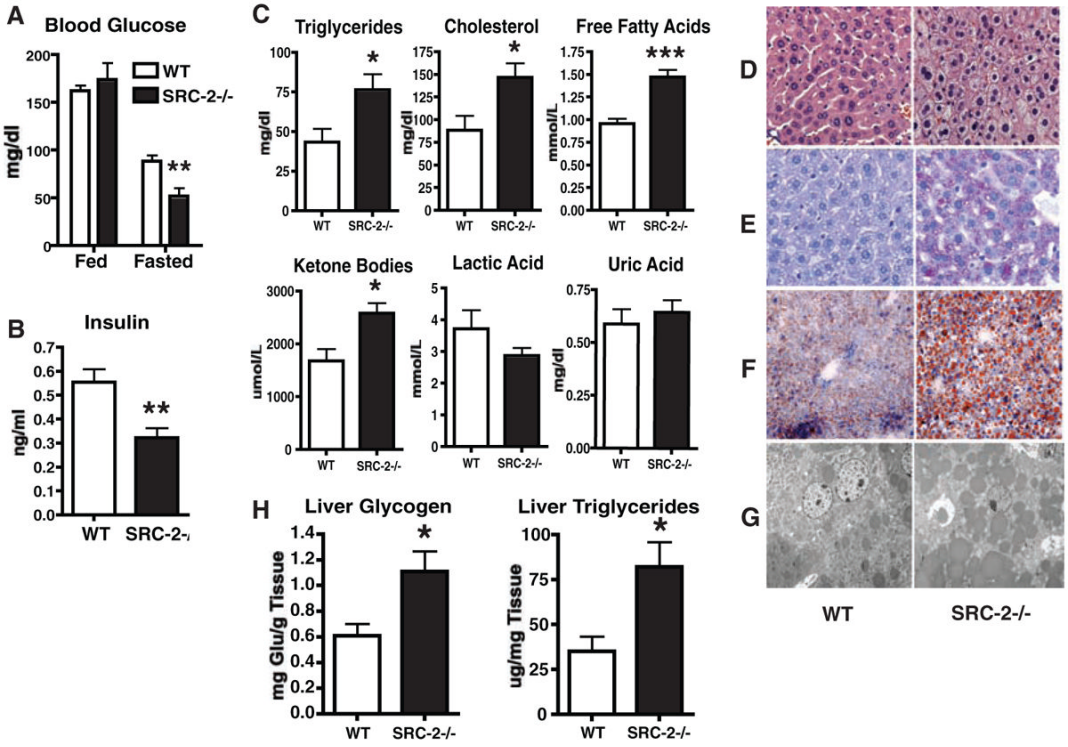

Fig. 2.

Lack of SRC-2 results in features like those of Von Gierke's disease (glycogen storage disorder–1a). (**A**) SRC-2 ablation results in fasting hypoglycemia. Blood glucose concentrations were determined in mice upon ad libitum feeding and after 24 hours of fasting (n = 5 mice per group), by means of a hand-held glucometer. (**B**) Insulin concentrations were determined in plasma isolated from SRC-2 null mice and WT littermates upon 24 hours of fasting (n = 5 mice per group). (**C**) SRC-2 ablation results in increased concentrations of triglycerides, cholesterol, FFAs, and ketone bodies in the plasma upon fasting. Plasma concentrations of triglycerides, cholesterol, FFAs, ketone bodies, uric acid, and lactic acid were determined from plasma isolated from SRC-2 null mice and WT littermates upon 24 hours of fasting. (**D** to **G**) SRC-2 ablation results in liver glycogen accumulation and liver steatosis upon fasting. Liver tissue was isolated from SRC-2 null mice and WT littermates upon 24 hours of fasting. Formalin-fixed liver sections were stained with hematoxylin and eosin. (D) and PAS (E) for glycogen. Frozen liver sections were stained with Oil Red O (F) to demonstrate neutral lipids. Sections fixed in 2% gluteraldehyde and viewed by EM (G) demonstrated the abundance of lipid. (**H**) Measurement of hepatic glycogen and triglyceride content in mice fasted 24 hours. Data are represented as the mean ± SEM. Unpaired student's t test was used for evaluation of statistical significance. *P < 0.05, **P < 0.01, and ***P < 0.001.

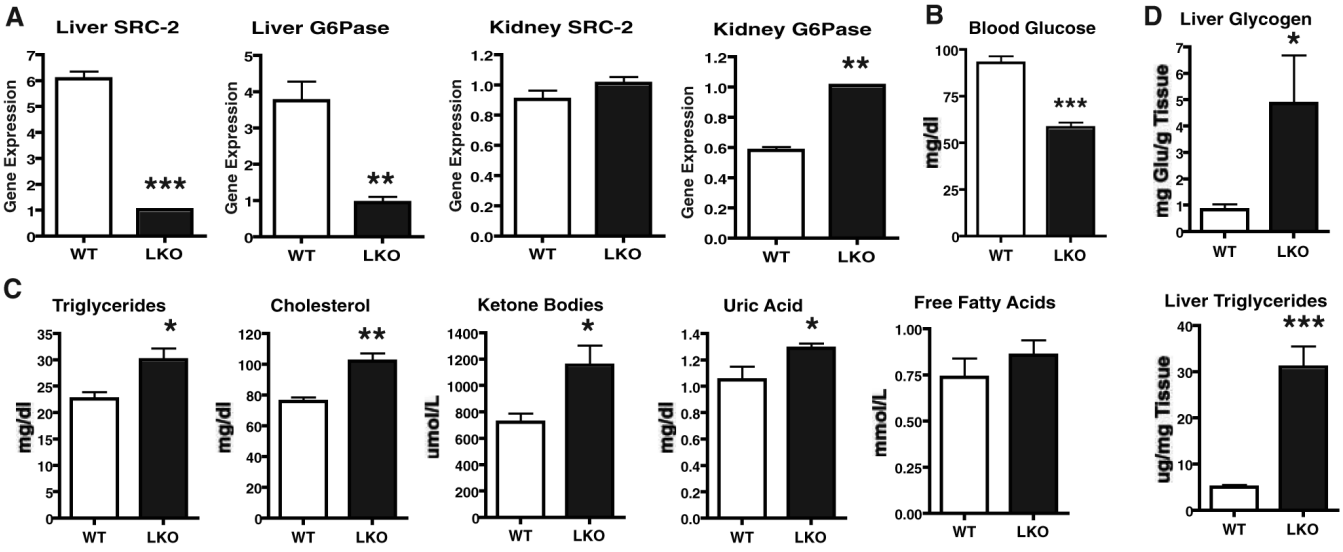

Fig. 3.

Liver-selective ablation of SRC-2 results in development of the Von Gierke's disease phenotype. (**A**) Liver-selective SRC-2 ablation results in decreased expression of hepatic *G6Pase* and increased expression of renal *G6Pase*. SRC-2 F/F mice exposed to adenoviral CRE (liver knockout, LKO) were compared with SRC-2 F/F mice exposed to empty adenovirus (WT) by means of tail-vein infusion. Mice fasted for 24 hours were killed, and then plasma and various organs were isolated. Gene expression was measured via relative quantitation by QPCR. (**B**) Fasting hypoglycemia. Blood glucose concentrations were determined in mice fasted for 24 hours by means of a hand-held glucometer. (**C**) Concentrations of triglycerides, cholesterol, ketone bodies, and uric acid in plasma. Plasma concentrations of triglycerides, cholesterol, FFAs, ketone bodies and uric acid were determined from plasma isolated from mice fasted for 24 hours. (**D**) Hepatic steatosis and accumulation of liver glycogen. Measurement of hepatic glycogen and triglyceride content was done in mice fasted for 24 hours ($n = 7$ mice per group). Data are represented as the mean ± SEM. Unpaired student's *t* test was used for evaluation of statistical significance. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

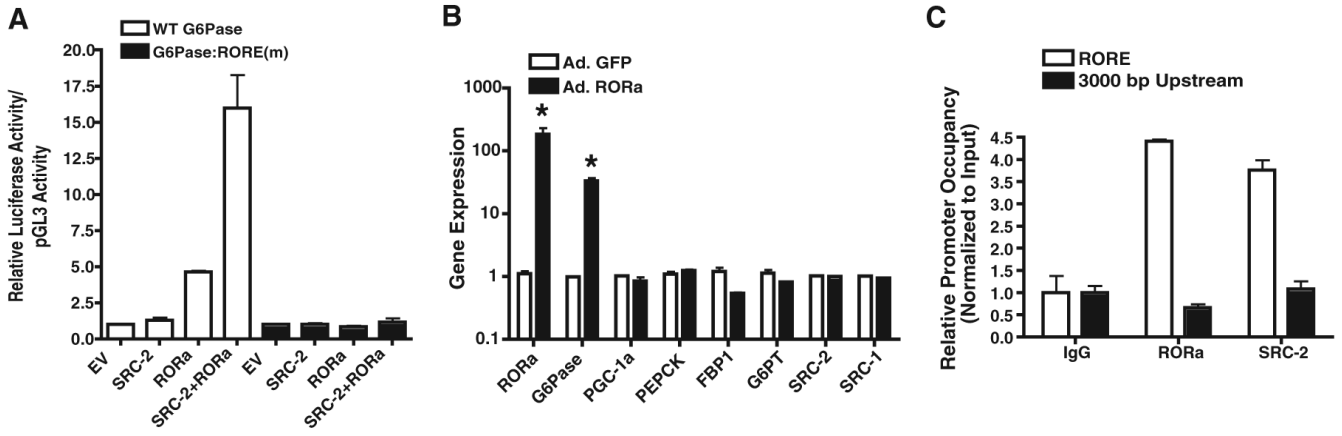

Fig. 4.

Cooperation of SRC-2 with orphan nuclear receptor RORα for transactivation of the *G6Pase* promoter. (**A**) Synergism of SRC-2 with RORα in producing trans-activation of the *G6Pase* promoter. HeLa cells were transfected with a reporter-gene plasmid driven by the WT human *G6Pase* promoter (−1227, +57) and the same promoter with the four central residues mutated in the hexameric RORE, together with expression plasmids for SRC-2 and RORα. Reporter-gene expression levels were determined 48 hours after transfection. The empty vector (EV) value was fixed at 1, and the rest of the values are compared relative to that. (**B**) RORα regulates *G6Pase* expression in a cell-autonomous manner. Adenoviral overexpression of RORα in mouse primary hepatocytes results in up-regulation of *G6Pase* expression as determined via relative quantitation by QPCR. (**C**) In vivo ChIP: In the mouse liver, SRC-2 and RORα bind the *G6Pase* promoter in the region containing the RORE. ChIP assays were performed with 150 to 200–base pair (bp) amplicons flanking the region containing the RORE and an irrelevant region 3000 bp upstream of the transcription start site. Sybr-Green QPCR (normalized to input) was used to assess SRC-2 and RORα occupancy of the *G6Pase* promoter upon ChIP, with SRC-2– and RORα-specific antibodies. Data are represented as the mean ± SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

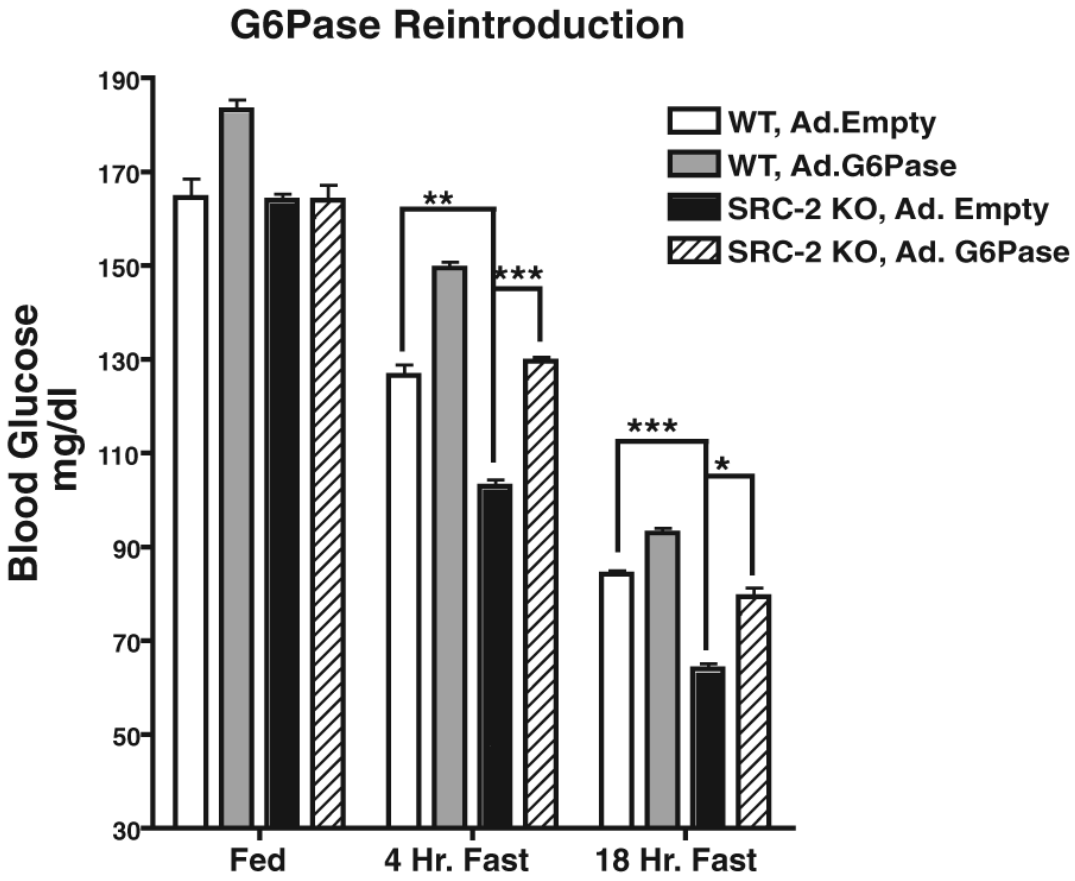

Fig. 5.

Correction of hypoglycemia in SRC-2$^{-/-}$ mice by overexpression of *G6Pase*. Adenoviral reintroduction of *G6Pase* in SRC-2 null mice and WT littermates. SRC-2 null mice (KO) exposed to canine helper-dependent adenoviral *G6Pase* (Ad. *G6Pase*) were compared with SRC-2 null (KO) and wild-type (WT) littermate mice exposed to empty helper-dependent adenovirus (Ad. Empty) by means of tail-vein infusion. Helper-dependent adenoviruses produce transgenesis for the duration of the mouse life-time and are associated with little inflammatory response (20). Thirty days after adenoviral infusion, mice were fasted for 24 hours before determination of blood glucose (KO + Ad. *G6Pase*, $n = 5$ mice; KO + Ad. Empty, $n = 6$ mice; WT + Ad. Empty, $n = 5$ mice; and WT + Ad. *G6Pase*, $n = 4$ mice). Data are represented as the mean ± SEM. Unpaired student's *t* test was used for evaluation of statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Statistical comparison between WT + Ad. Empty group and WT + Ad. *G6Pase* group achieved significance in the 4-hour fasted animals and in the 18-hour fasted animals. For clarity, this is not indicated in the figure.