

acid intermediate produces the aromatic hydroxylamine metabolite **C**, which, upon further reduction and fragmentation, produces **3** (fig. S5) (20).

To test the hypothesis that reduction of PA-824 by Ddn resulted in the release of reactive nitrogen species, we used the Griess reagent to directly detect nitrous acid. A PA-824-dependent production of HNO_2 by Ddn was consistently observed in the presence of F_{420}H_2 (Fig. 3A). NO could also be detected in PA-824-treated whole cells, under both aerobic (Fig. 3B) and hypoxic nonreplicating conditions (fig. S6), by preloading cells with diaminofluorescein (DAF-FM) diacetate, a probe that fluoresces specifically in the presence of NO (21, 22).

To correlate NO release with anaerobic killing, we selected a series of eight derivatives that had a broad range (from 2 to 125 μM , Table 1) of minimum anaerobicidal concentration (MAC) values (the aerobic potency of these compounds ranges from 0.04 to 6 μM). These derivatives revealed a strong correlation between the amount of the corresponding des-nitro product formed and anaerobic killing activity (Fig. 3C). Des-nitro formation did not correlate with aerobic activity, nor did any other metabolite correlate with either aerobic or anaerobic activity (fig. S7). The rate of NO release [an apparent V_{max}/K_m as RFU per hour divided by substrate affinity (the Michaelis constant) measured intracellularly] also correlated with anaerobic killing activity (Table 1). Finally, the effect of PA-824 on cells could be abrogated by a scavenger of NO, C-PTIO, which showed a dose-dependent decrease in NO production (Fig. 3D) and partially rescued hypoxic cells from killing by PA-824 (Fig. 3E). This effect was muted by both toxicity and poor cell penetrating ability of C-PTIO. Mtb mutants hypersensitive to NO, such as proteasome mutants, also display increased anaerobic sensitivity to PA-824 (fig. S8). The simplest interpretation of these data is that reactive nitrogen species are dominant in anaerobic activity but under aerobic conditions this is augmented by other effects.

Reactive nitrogen species play a major role in mammalian defense against mycobacterial infections, and mice deficient in the ability to produce NO are hypersusceptible to infection with Mtb (23). PA-824-resistant mutants in F_{420} biosynthesis have also been shown to be hypersensitive to killing by NO (24). Nitrite and NO both react with cytochromes and cytochrome c oxidase to interfere with electron flow and to cripple the coupling of respiration to reduction of oxygen. Thus, bicyclic nitroimidazoles may function as highly specific NO donors requiring activation by a powerful deazaflavin cofactor (F_{420}) that is unique to a small group of microorganisms and extremophiles, which makes cross-activation by mammalian enzymes highly unlikely. This unusual mechanism for nitroimidazole reduction avoids the single-electron-reduced nitrogen species typical of oxygen-sensitive, flavin-dependent enzymes and defines the chemistry of a class of F_{420} -dependent enzymatic nitroreductases.

NO-donating drugs have been shown to have potential for a wide variety of human diseases including cardiovascular disease, asthma, hypoxic-ischemic brain injury, glaucoma, and Alzheimer's disease (25). Indeed, it may be possible to more generally co-opt bacterial nitroreductases to reduce other nitro-containing heterocycles as an avenue for developing anti-infectives. It is noteworthy that the two candidate molecules in phase 2 trials (PA-824 and OPC-67683) were both optimized only for whole-cell aerobic activity and, as a result, have fairly modest anaerobic activity. Thus, these results have the potential to enable structure-based design of a drug specific for the treatment of latent tuberculosis.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S8
References

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

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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 regulates *G6Pase* expression directly by acting as a coactivator with the orphan nuclear receptor ROR α . 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-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 coactivators, 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)*, *peroxisome 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, gluconeogenesis, 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 stimulate hormonal stimulation of adenylyl cyclase. Ablation of SRC-2 decreased both control and hormone-mediated transcription of *G6Pase* (fig. S6, A and B).

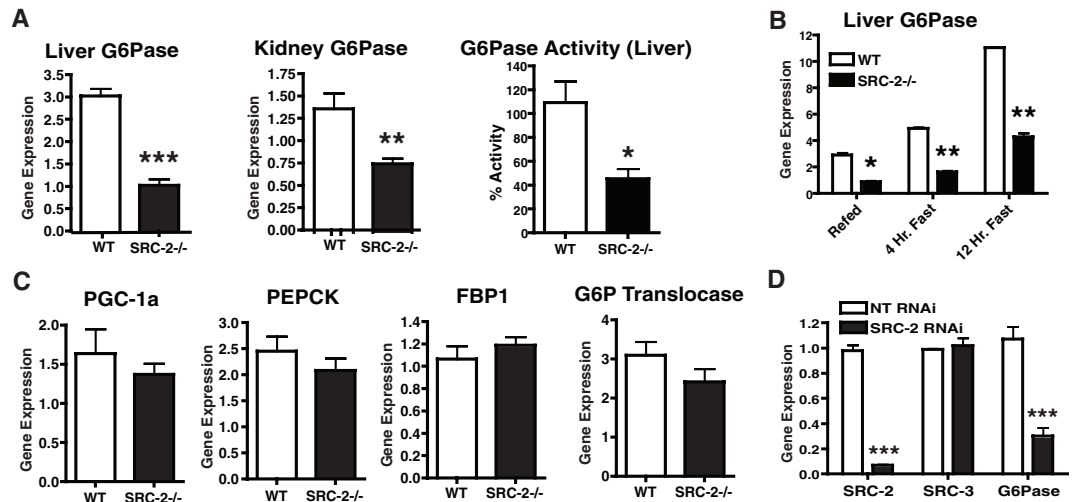
The primary manifestations of Von Gierke's disease, which occur as a result of mutations in the *G6Pase* gene, are diminished growth; fasting hypoglycemia; increased concentrations of triglycerides, cholesterol, free fatty acids, ketone bodies, uric acid, and lactic acid in the plasma; increased liver 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 char-

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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 regu-



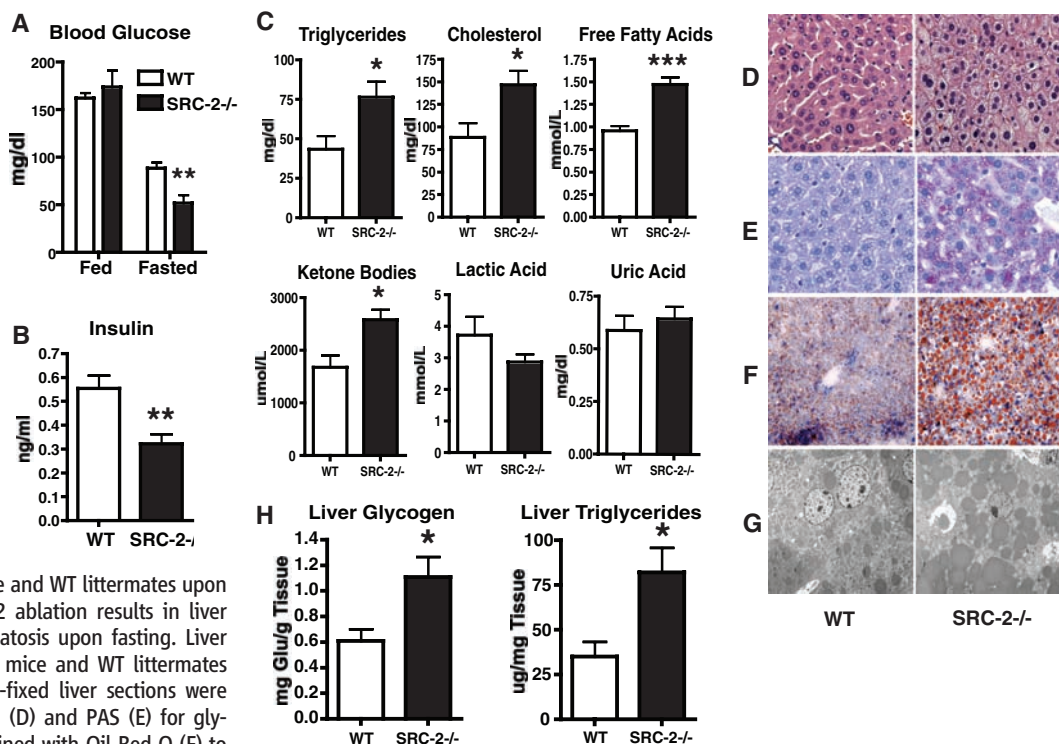
lates *G6Pase* expression in a cell-autonomous manner. RNAi to SRC-2 results in down-regulation of *G6Pase* mRNA levels compared with nontargeting (NT) RNAi, as determined via relative quantitation by QPCR. SRC-3 expression level is used as a control for specificity of SRC-2 knockdown. Data are represented as the mean \pm SEM. Unpaired student's *t* test was used for evaluation of statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

acteristic mosaic pattern like that of patients with Von Gierke's disease (Fig. 2D) (17). 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,

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% glutaraldehyde 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 \pm SEM. Unpaired student's t test was used for evaluation of statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.



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

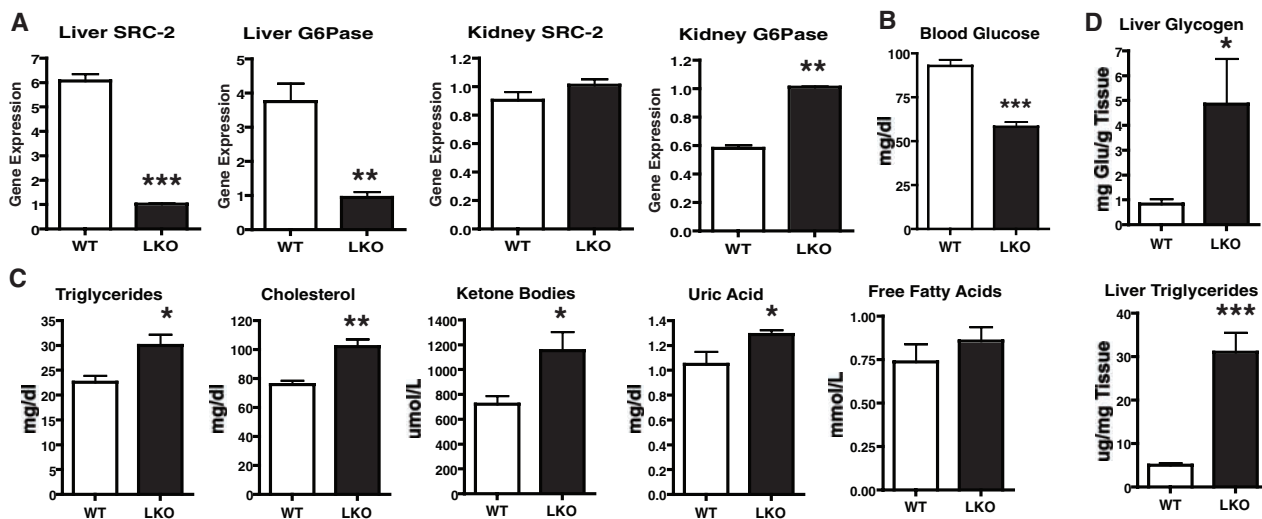


Fig. 3. Liver-selective ablation of SRC-2 results in development of the Von Gierke's disease phenotype. (A) Liver-selective SRC-2 ablation results in decreased expression of hepatic *G6Pase* and increased expression of renal *G6Pase*. SRC-2 F/F mice exposed to adenoviral CRE (liver knockout, LKO) were 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 \pm SEM. Unpaired student's t test was used for evaluation of statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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.

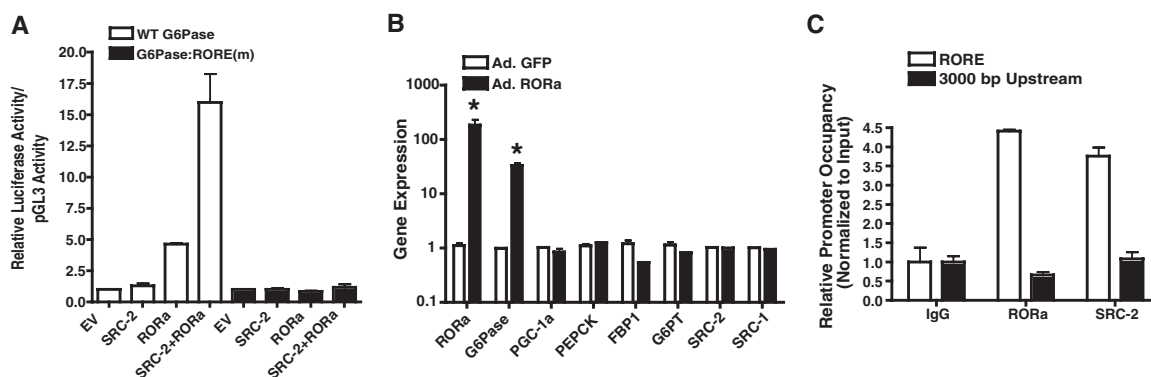
Because SRC-2 is a “coactivator” and lacks specific DNA binding activity, it is presumably recruited to a gene promoter by a transcription factor in order to function. Although transcriptional coactivators often function at multiple genes, our results indicated that SRC-2 might regulate the expression 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 evolutionarily conserved retinoid-related orphan receptor α (ROR α) response element (RORE) close to the transcriptional start site (fig. S11). SRC-2 and ROR α 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 ROR α . SRC-2 and ROR α , 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 ROR α on transactivation of *G6Pase* in the correct chromatin context, we transduced primary hepatocytes with adenoviral ROR α . 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-1 α* and *G6P translocase* (Fig. 4B). The ROR α -mediated increase in

G6Pase transactivation was abolished in a SRC-2 null background (fig. S4B). Furthermore, RNAi-mediated depletion of ROR α in primary hepatocytes resulted in decreased expression of *G6Pase* (fig. S4). To assess whether ROR α affects *G6Pase* regulation mediated by fasting hormones, we treated primary hepatocytes that had been exposed to RNAi-mediated depletion of ROR α with dexamethasone or forskolin. Depletion of ROR α 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 ROR α 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 ROR α , we overexpressed ROR α in primary hepatocytes. This resulted in increased promoter occupancy of ROR α , which in turn increased the promoter occupancy of SRC-2, suggesting that ROR α could indeed recruit SRC-2 to the *G6Pase* promoter (Fig. S5A). Conversely, SRC-2 promoter occupancy was abolished in cells depleted of ROR α (Fig. S5B). These results suggest that ROR α 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

Fig. 4. Cooperation of SRC-2 with orphan nuclear receptor ROR α for transactivation of the *G6Pase* promoter. (A) Synergism of SRC-2 with ROR α in producing transactivation 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 ROR α . 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) ROR α regulates *G6Pase* expression in a cell-autonomous manner. Adenoviral overexpression of ROR α 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 ROR α 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 ROR α occupancy of the *G6Pase* promoter upon ChIP, with SRC-2- and ROR α -specific antibodies. Data are represented as the mean \pm SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001.

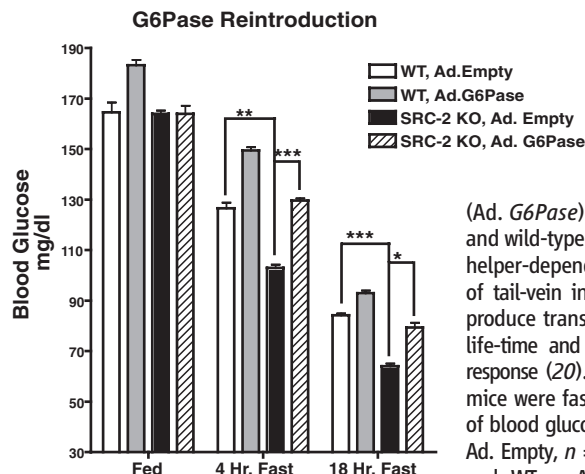


Fig. 5. Correction of hypoglycemia in SRC-2^{-/-} mice by overexpression of *G6Pase*. Adenoviral reintroduction of *G6Pase* in SRC-2 null mice and WT littermates. SRC-2 null mice (KO) exposed to canine helper-dependent adenoviral *G6Pase*

(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 \pm 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.

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 ROR α , 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 ROR α may represent new therapeutic targets against these diseases.

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Supporting Online Material

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Methods

Figs. S1 to S11

Table S1

References

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