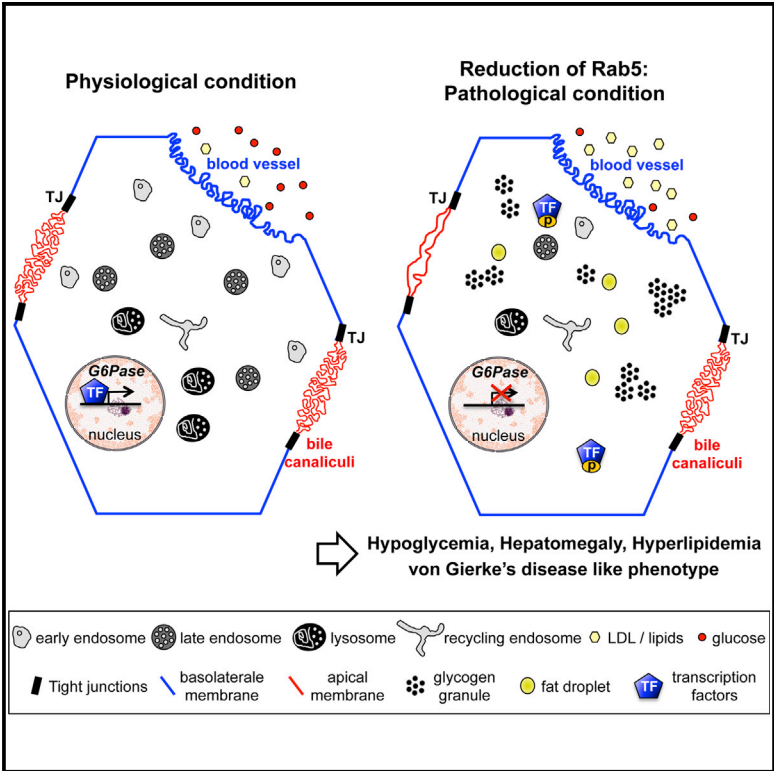


Regulation of Liver Metabolism by the Endosomal GTPase Rab5

Graphical Abstract



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In Brief

Zeigerer et al. uncover a requirement of Rab5 and the endosomal system for the regulation of hepatic metabolism that goes beyond the classical view of controlling surface expression of nutrient transporters. Rab5 depletion leads to reduced gluconeogenesis, similar to von Gierke's disease, suggesting important implications for metabolic diseases.

Highlights

- Knockdown of Rab5 in the liver leads to hypoglycemia and hepatomegaly
- Rab5 and the endosomal system are required for G6Pase and Pepck gene expression
- Loss of Rab5 inhibits the activity of transcription factors for gluconeogenic genes

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Regulation of Liver Metabolism by the Endosomal GTPase Rab5

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SUMMARY

The liver maintains glucose and lipid homeostasis by adapting its metabolic activity to the energy needs of the organism. Communication between hepatocytes and extracellular environment via endocytosis is key to such homeostasis. Here, we addressed the question of whether endosomes are required for gluconeogenic gene expression. We took advantage of the loss of endosomes in the mouse liver upon Rab5 silencing. Strikingly, we found hepatomegaly and severe metabolic defects such as hypoglycemia, hypercholesterolemia, hyperlipidemia, and glycogen accumulation that phenocopied those found in von Gierke's disease, a glucose-6-phosphatase (G6Pase) deficiency. G6Pase deficiency alone can account for the reduction in hepatic glucose output and glycogen accumulation as determined by mathematical modeling. Interestingly, we uncovered functional alterations in the transcription factors, which regulate G6Pase expression. Our data highlight a requirement of Rab5 and the endosomal system for the regulation of gluconeogenic gene expression that has important implications for metabolic diseases.

INTRODUCTION

The liver plays an essential role in the metabolic adaptation to nutrients through processes such as gluconeogenesis, glycogenolysis, lipogenesis, and fatty acid oxidation that are under the control of glucagon and insulin. In the absorptive state, when high glucose levels induce insulin secretion, anabolic pathways, such

as glycogen production and lipogenesis are activated, whereas catabolic processes are inhibited. In the fasted state, when glucagon is elevated relative to insulin, fatty acid oxidation, glycogenolysis, and gluconeogenesis are induced to maintain blood glucose homeostasis (Lin and Accili, 2011). Dysfunction of glucose production leads to severe metabolic diseases. The most common pathology is associated with insulin resistance, which contributes to type 2 diabetes and the metabolic syndrome (Lin and Accili, 2011). In this disease, insulin fails to exert an inhibitory role on gluconeogenesis. Mutations in genes involved in gluconeogenesis can cause glycogen storage diseases with marked alterations in glucose homeostasis. For example, von Gierke's disease, the glycogen storage disease type Ia (GSD-Ia), is an autosomal recessive disorder caused by a deficiency of glucose-6-phosphatase (G6Pase) and characterized by hypoglycemia, abnormal glycogen accumulations (glycogenesis), hepatomegaly, and hyperlipidemia, among others (Chou et al., 2010).

Glucose homeostasis is regulated by endocytosis. Endocytosis is an essential mechanism for the uptake and signal transduction of growth factors and hormones (Sorkin and von Zastrow, 2009) but also for regulating metabolic activities, by changing the expression of hormone receptors and nutrient transporters on the cell surface. For example, in fat and muscle cells, the amount of internalized glucose can be modulated by varying the rates of endo- and exocytosis of the insulin-responsive glucose transporter, GLUT4 (Antonescu et al., 2014). Endocytosis governs the clearance of low-density-lipoproteins (LDLs) from the circulation especially in the liver, where mutations in this process cause hypercholesterolemia (Koivisto et al., 2001). New evidence suggests that the endo-lysosomal system is also involved in the regulation of metabolic gene transcription. For example, the transcription factor EB (TFEB) translocates from lysosomes to the nucleus in response to starvation and activates genes involved in lysosomal biogenesis, autophagy, and lipid metabolism

(Settembre et al., 2013). Conversely, metabolic activities also regulate endocytosis, since knockdown of metabolic genes in a genome-wide RNAi screen had dramatic effects on endocytic trafficking (Collinet et al., 2010). Together, these findings suggest that endocytosis and metabolism are functionally coupled.

Understanding the role of the endocytic system in the regulation of metabolism would require molecular perturbations capable of ablating the function of endosomes. The small GTPase Rab5 regulates the membrane assembly of a multi-protein machinery conferring early endosome identity and function, such as endosome tethering, fusion, and motility (Wandinger-Ness and Zerial, 2014). Remarkably, liver-specific knockdown of Rab5 by RNAi in mice causes a massive depletion of endosomes and lysosomes, indicating that it is necessary for endosome biogenesis (Zeigerer et al., 2012). Interestingly, ultrastructural analysis of liver sections also revealed a strong accumulation of glycogen granules and lipid droplets in the liver, raising the question of why depletion of Rab5 and, consequently, of the endosomal system causes severe alterations in hepatocellular glucose and lipid metabolism. In the present study, we addressed this question through a systems biology approach.

RESULTS

Rab5 Is Essential for Lipid and Glucose Metabolism in the Liver

The three Rab5 isoforms were silenced in mouse liver by RNAi through tail vein injection of lipid nanoparticles (LNPs) containing small interfering RNAs (siRNAs) (against Rab5a,b,c or luciferase), as previously described (Zeigerer et al., 2012). Five days after injection, when the reduction in endosome number in Rab5 knockdown (Rab5KD) livers is maximal (Zeigerer et al., 2012), mice were starved for 6 hr, their liver extracted and analyzed by transcriptomics, proteomics, and lipidomics. The transcriptomics analysis was performed by microarray and RNA sequencing (RNA-seq). Comparison of the hits from the differentially expressed genes (DEG) in both data sets revealed 451 common DEG, resulting in 77% overlap (RNA-seq to microarray), showing a high Spearman correlation (Figures S1A and S1B; Table S1). The data set was split into 328 up- and 123 downregulated genes, which were considered separately for pathway enrichment analysis (Table S1). Strikingly, we observed severe alterations in genes involved in metabolic pathways. Among the upregulated genes, there was a significant enrichment for genes associated with cell-cycle and steroid biosynthesis, suggesting that loss of Rab5 affects cell proliferation and lipid metabolism (Figure S1C). The upregulation of steroid biosynthesis can be explained by a compensatory increase consequent to the block of LDL internalization (Brown and Goldstein, 2009; Zeigerer et al., 2012). For the downregulated genes, metabolism of xenobiotics by cytochrome p450, fatty acid, amino acid metabolism, and glucose metabolism were significantly affected (Figure S1D). These data show that Rab5 depletion induces alterations in gene expression for specific metabolic pathways in the liver.

For quantitative proteomics, control and Rab5KD liver tissues were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS), and a combined analysis of the spectra from all samples resulted in the identification of ~8,000 proteins

at a false discovery rate (FDR) of 1%. Using the label-free quantification algorithm in MaxQuant, we measured the abundance of 5,800 proteins in at least one pairwise comparison (Table S2). We detected 980 differentially expressed proteins (DEPs), of which 553 were up- and 427 downregulated compared to control t test ($p < 0.05$) (Table S2). Consistent with the transcriptomics data, most proteins were again involved in cellular metabolism (Figure 1).

Next, we subjected the DEP to pathway enrichment analysis using KEGG (Figures 1A and 1B; Table S2) and a topological model of central metabolism (Figure S2A). In agreement with the transcriptomics, proteins involved in steroid biosynthesis were upregulated. Strikingly, most metabolic pathways, such as β -oxidation, fatty acid, and glucose metabolism, etc., were downregulated (Figure 1; Table S2). Comparison of the proteomics with the transcriptomics data sets revealed 88 common genes/proteins (Figure S1E; Table S3). Most hits were well correlated (Figure S1F), suggesting a regulation at the mRNA level. These candidates were again enriched in metabolic pathways with an upregulation in steroid and terpenoid backbone biosynthesis and a downregulation of fatty acid and amino acid metabolism (Figures S1G and S1H). Taken together, these data indicate that the liver responds to Rab5KD by boosting the pathways for steroid biosynthesis while reducing glucose and energy metabolism.

The levels of lipids in the liver and serum were determined by shotgun lipidomics. Consistent with transcriptomics and proteomics, we found a 2-fold enrichment of cholesterol and cholesterol ester (CE) in liver tissue of Rab5KD mice, with all CE species increased (Figure 1C). The excess of CE could explain in part the increase in lipid droplets (Miller and Bose, 2011) observed by EM upon Rab5KD (Zeigerer et al., 2012). In addition, we could not detect an induction of autophagosomes (data not shown), consistent with the loss of endosomes and lysosomes (Lamb et al., 2013). This implies that also lipophagy may be inhibited (Dong and Czaja, 2011), thus contributing to the accumulation of lipid droplets.

Interestingly, lipidomics analysis of sera from Rab5KD animals revealed a 2- to 5-fold increase in all major lipid classes (Figure 1D), leading to an induction of hyperlipidemia and hypercholesterolemia at 5 days after LNP injection. Importantly, this increase was reversible and serum lipids returned back to normal levels after recovery of the endosomal system at 10 days after injection (Figure 1D; Zeigerer et al., 2012). Altogether, Rab5 depletion causes severe changes in the expression of metabolic pathways associated with steroid biosynthesis, fatty acid, amino acid, and glucose metabolism.

Rab5 Depletion Causes Hepatomegaly

At 5 days post-injection, Rab5KD animals were similar in weight (Figure S3A) but exhibited an increase in liver size (Figure 2A), raising the liver/body weight ratio from 5% to 7% (Figure 2B). Interestingly, the induction of hepatomegaly coincided with the accumulation of glycogen (Zeigerer et al., 2012), suggesting that the two events may be linked. Hepatomegaly could either be caused by an increase in the size or the number of hepatocytes, or both. To distinguish between these possibilities, we performed a morphological analysis of liver sections and measured a 20%–30% increase in hepatocyte area in vivo (Figures S3B and S3C).

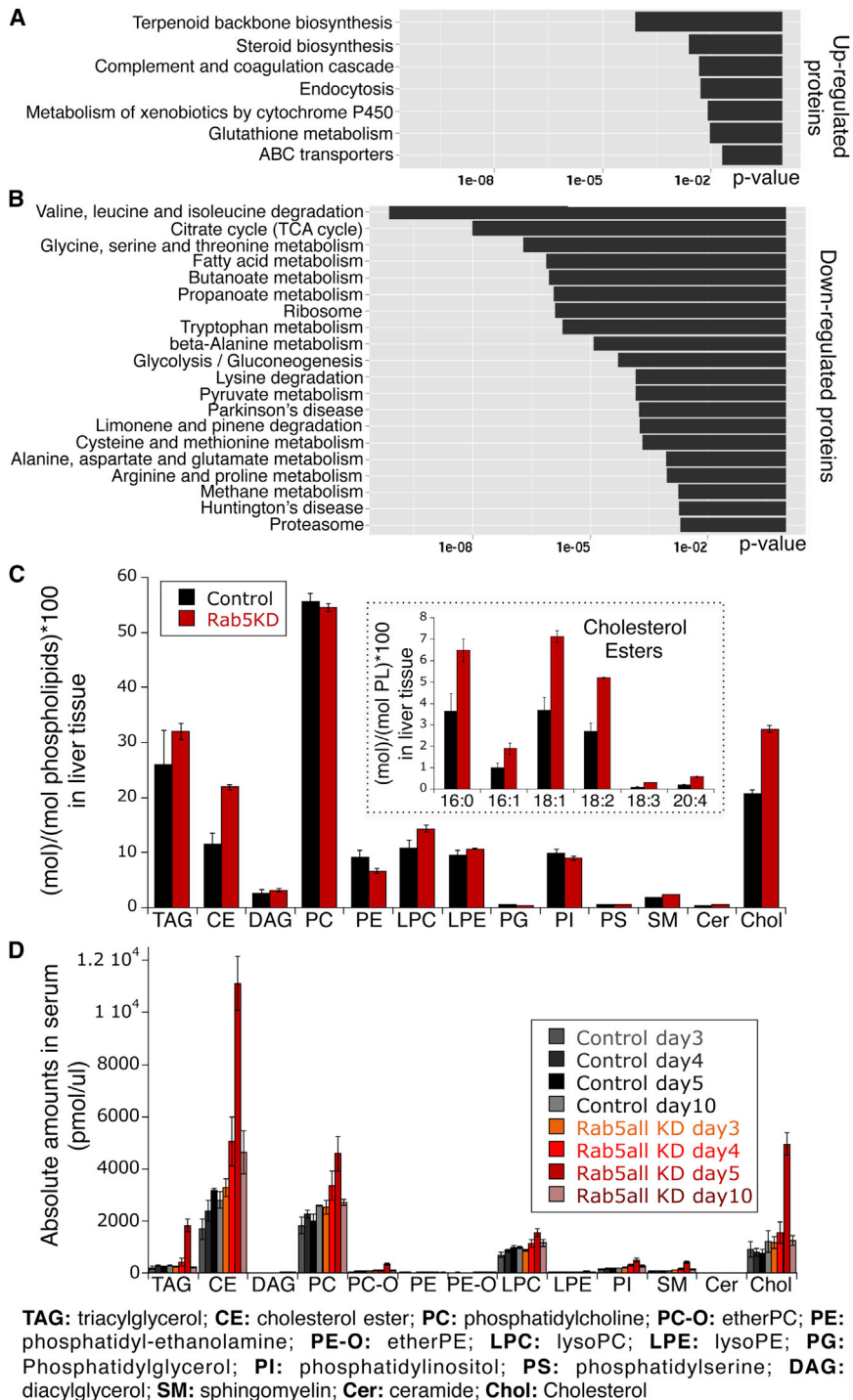


Figure 1. Proteomics and Lipidomics of Rab5KD Livers Reveal Alterations in Metabolism

(A–C) Significantly enriched KEGG pathways of up- (A) and downregulated (B) proteins. (C) Representative profiles of all lipid classes (normalized to phospholipids) ($p < 0.05$, $n = 4$ mice per condition). Inset: lipid species of cholesterol esters (mean \pm SEM). (D) Time course of lipid profiles in serum ($n = 4$ mice per condition, mean \pm SEM).

ure S3F) consistent with our transcriptomics analysis. Altogether, these data suggest that the hepatomegaly is accounted by an increase in hepatocytes volume and a mild increase in their number.

Rab5 Knockdown Induces Hypoglycemia

The alterations in glycolysis/gluconeogenesis together with the accumulation of glycogen granules suggest a connection between endosomes and the regulation of sugar metabolism. We therefore undertook a detailed analysis of the effects of Rab5 depletion on glucose homeostasis. We found a 30% reduction in fasting blood glucose levels compared to control at 5 days post-injection (Figure 2C). Surprisingly, no alterations in serum levels of insulin and glucagon were observed that could account for the decrease in blood glucose (Figure 2D), arguing for a more direct effect of Rab5 on glucose homeostasis in the liver. Interestingly, upon endosomal recovery at 10 days post-injection (Zeigerer et al., 2012), similar to liver size, blood glucose levels returned to normal (Figures 2B and 2C), suggesting that hypoglycemia and hepatomegaly correlate with the loss of Rab5 and endosomes.

Rab5KD Phenocopies the Loss of G6Pase in von Gierke's Disease

The accumulation of glycogen granules and lipid droplets, hypoglycemia, hepatomegaly, hypercholesterolemia, and hyperlipidemia observed upon Rab5KD

In addition, applying proteome ruler for the proteomics data set revealed a 1.4-fold increase in the total protein content per cell and cell volume (Figures S3D and S3E), in agreement with the image analysis. Such an expansion in cell volume agrees with the observation that glycogen accumulation causes swelling of hepatocytes (Froissart et al., 2011). However, H&E staining revealed also a mild increase in the proliferation index in Rab5KD livers (Fig-

strikingly resemble the alterations in von Gierke's disease (glycogen-storage disease type Ia) (Chou et al., 2010). von Gierke's disease is caused by a deficiency of G6Pase, the major enzyme controlling glucose release by the liver via glycogenolysis and gluconeogenesis. Therefore, we analyzed the G6Pase mRNA levels after 5 days post-injection. Strikingly, the expression of G6Pase was almost ablated (Figure 2E and Table S1).

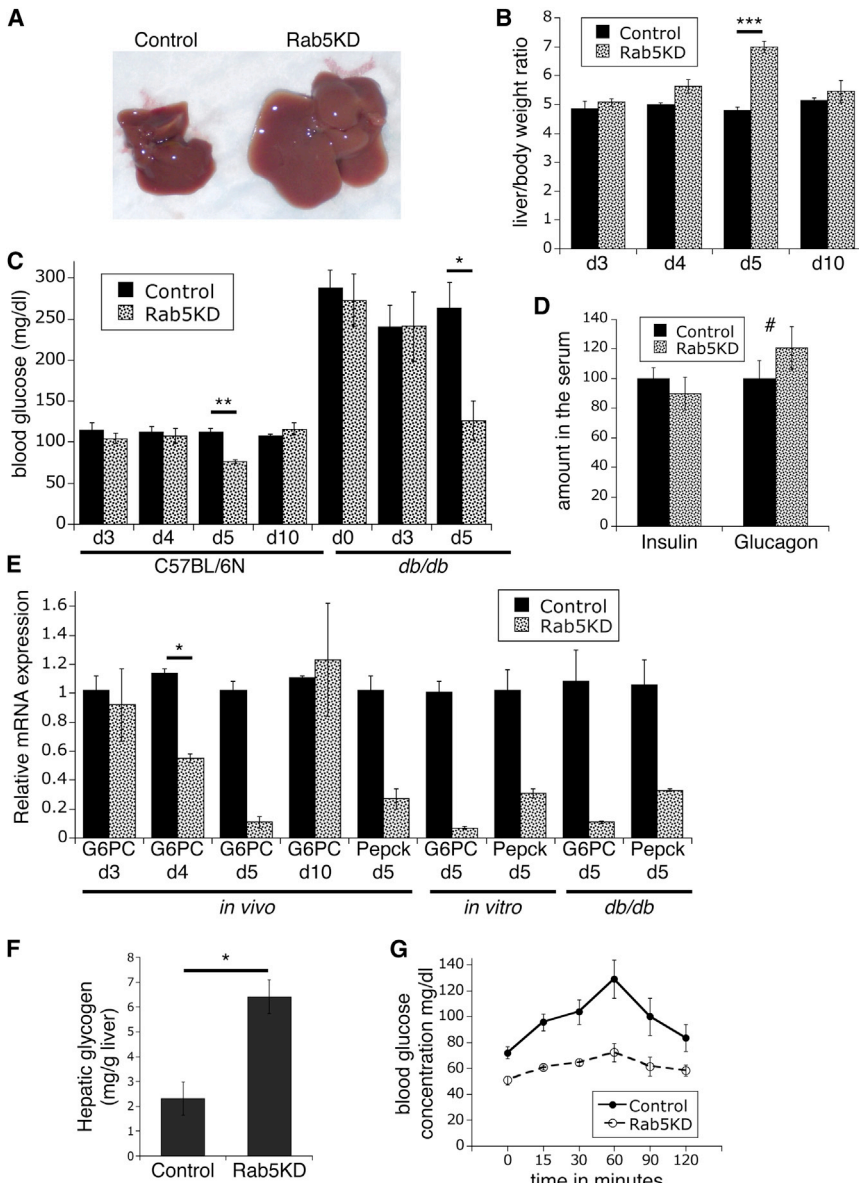


Figure 2. Metabolic Effects of Rab5KD
(A and B) Liver size (day 5) (A) and liver/body (B) weight ratio (time course) for 6-hr-fasted mice. (C) Time course of blood glucose levels for C57BL/6N and in *db/db* mice. (D) Insulin and glucagon levels of five independent experiments normalized to control. (E) Relative expression of *G6Pase* (time course) and *Pepck* in livers of C57BL/6N, *db/db* mice (day 5), and in primary hepatocytes. (F and G) (F) Liver glycogen content and (G) pyruvate tolerance test (2 mg/kg) at day 5. Representative experiments shown for (A)–(C) and (E)–(G); four mice per condition used for (B)–(G); mean \pm SEM; #*p* > 0.1, **p* < 0.05, ***p* < 0.001, ****p* < 0.0001.

von Gierke's disease (Cori and Cori, 1952), leading to the accumulation of G6P and activation of GS.

To directly test whether gluconeogenesis was inhibited, pyruvate tolerance tests were performed in control and Rab5KD mice. Rab5KD almost completely abolished glucose production by the liver (Figure 2G). This is consistent with transcriptomics and quantitative PCR (qPCR) analysis, which revealed a strong reduction in Phosphoenolpyruvate kinase (*Pepck1*), another rate-limiting enzyme for gluconeogenesis (Table S1 and Figure 2C). Importantly, after 10 days post-injection when the levels of Rab5 and the endosomal pathway recovered (Zeigerer et al., 2012), the expression of *G6Pase* was restored (Figure 2E), leading to normal glycemia and receding the metabolic phenotypes (Figures 2B, 2C, and 2E).

Although Rab5 was silenced specifically in the liver (Zeigerer et al., 2012), the reduction in *G6Pase* and *Pepck* expression could be triggered by endocrine signals from other organs. To rule out this possibility, we silenced Rab5 in isolated primary hepatocytes. Once again, we observed a strong decrease in *G6Pase* and *Pepck1* expression (Figure 2E), arguing that it is mediated by a hepatocyte-autonomous rather than a systemic effect. Taken together, these data suggest that the expression of *G6Pase* as well as other gluconeogenic genes is subjected to regulation by the endosomal system.

Since Rab5-depleted animals fail to mobilize their glycogen stores (Zeigerer et al., 2012), we quantified the glycogen in the liver and measured a 2-3-fold increase in glycogen levels in 6-hr-starved Rab5KD mice (Figure 2F). Glycogen levels are regulated by a balance of synthesis, via glycogen synthase (GS), and breakdown, through glycogen phosphorylase (GP), both regulated by phosphorylation. In addition, GS is also allosterically activated by glucose-6-phosphate (G6P) (von Wilamowitz-Moellendorff et al., 2013). We detected only a slight increase in GS ($p = 0.06$) but not GP phosphorylation in Rab5KD livers compared to control (Figures S4A and S4B). However, the G6P levels were increased over 2-fold (Figure S4C). Such increase is expected to activate GS, thus explaining the enhanced glycogen deposition. These data suggest that the glycogen storage phenotype is caused by the reduction in *G6Pase*, just as in

von Gierke's disease (Cori and Cori, 1952), leading to the accumulation of G6P and activation of GS.

Mathematical Modeling and Simulations Suggest that the Block in Glucose Output and Accumulation of Glycogen Can Be Explained by Loss of *G6Pase* in Rab5KD Liver

How does *G6Pase* loss affect glucose output and glycogen production? We applied a mathematical kinetic model of hepatic

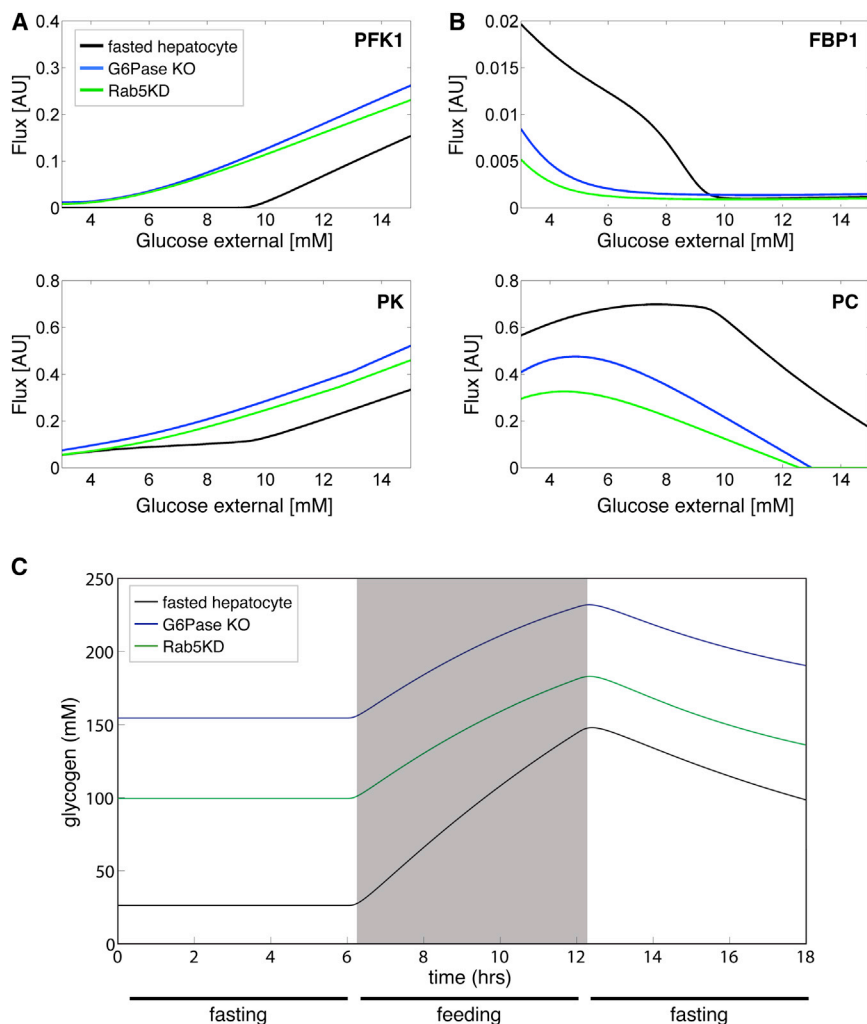


Figure 3. Mathematical Modeling of Glucose Fluxes and Glycogen Accumulation

(A and B) Model simulation of fluxes for glycolysis (PFK1, FBP1) (A) and gluconeogenesis (PK, PC) (B) for control (black), G6Pase knockout (blue), and the Rab5KD (green) livers.

(C) Simulation of glycogen dynamics in mouse livers during re-feeding and fasting.

limiting genes of gluconeogenesis (Table S1). The model simulations further support the suggestion that the reduction in G6Pase expression is sufficient to explain the decrease of glucose production upon Rab5KD.

To simulate glycogen dynamics associated with the fasting-refeeding-fasting cycle, we applied the same model to compare control, G6Pase KO, and Rab5KD (Figure 3C). Initial fasting and re-feeding (gray-shaded area) were simulated by blood glucose levels of 4 and 10 mM, respectively. Fluxes for glucokinase (GK), G6PPase (G6PP), glucose phosphate isomerase (GPI) (representing glycolytic/gluconeogenic flux), phosphoglucomutase (PGM) (representing glycogen synthesis/glycogen degradation), and glucose transporter (GlcT) are shown in Figure S2B. Glycogen is synthesized during the refeeding period and degraded in the fasting period in all conditions. Higher glycogen content leads to an increase in the flux through GP resulting in production of G6P. G6P can either be metabolized through glycolysis

glucose metabolism (Figure S2A) that takes into account metabolic regulation through substrates and allosteric effectors, reversible phosphorylation controlled by plasma insulin and glucagon, as well as changes in the amount of metabolic enzymes through variable gene expression. Model parameters (K_m and K_i values) were obtained from literature. V_{max} values for the reference state were calibrated to match physiological metabolite concentrations (S.B., H.-G.H., and N.B., unpublished data). Relative changes in protein levels of 24 proteins (Table S4) upon Rab5KD measured by proteomics were taken to scale the V_{max} values for the Rab5KD.

Glycolytic fluxes through PFK1 and PK and gluconeogenic fluxes through FBP1 and PC simulated with the model are depicted (Figures 3A and 3B). G6Pase knockout (KO) (blue) increases the glycolytic flux and decreases the gluconeogenic flux compared to control (fasted hepatocyte, black line). The proteome changes upon Rab5KD (green) decrease the futile fluxes between the opposing reactions (PFK1/FBP1 and PK/PC) in glycolysis and gluconeogenesis. Flux through gluconeogenesis was stronger affected by Rab5KD than by complete KO of G6Pase alone, consistent with the reduction in additional rate-

or converted to glucose via G6Pase. The loss of G6Pase in von Gierke's disease and upon Rab5KD leads to (1) a higher flux through glycolysis shown by GPI, (2) a rerouting back to glycogen production (through higher GS flux), and (3) to the accumulation of G6P and glycogen (Cori and Cori, 1952; Figures S4C and 2F). The simultaneous accumulation of G6P and glycogen lead to an increase in the flux through GS and GP without necessarily modifying their phosphorylation state. Interestingly, the proteome changes upon Rab5KD (Tables S2; Table S4) result only in a partial compensation causing higher G6P and glycogen content compared to control, as observed experimentally. Altogether, the model simulations support our conclusion that the reduction of G6Pase upon Rab5KD is sufficient to explain the accumulation of glycogen.

The Rab5KD Liver Fails to Induce Gluconeogenic Gene Expression due to an Inhibitory Effect on CREB, ChREBP, and FoxO1 Function

The expression of G6Pase and *Pepck1* is under the opposite control of insulin and glucagon (Lin and Accili, 2011). As the levels of insulin and glucagon did not vary significantly

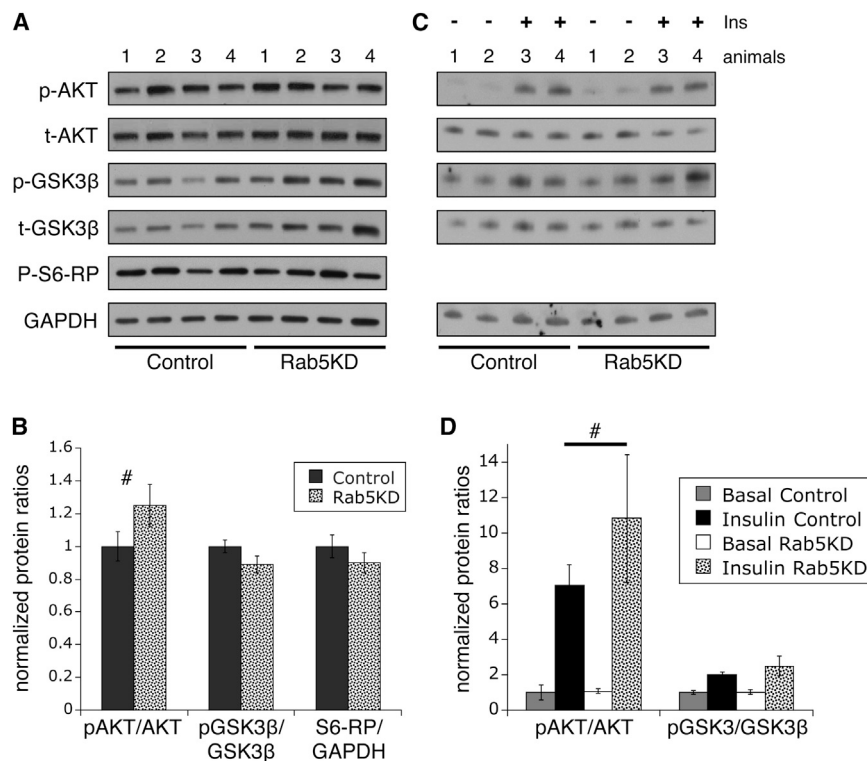


Figure 4. G6Pase Reduction Is Independent of Insulin Signaling

(A and C) Representative western blots for Akt, GSK3β (A and C), and p-S6-RP (A) in livers after 6-hr fast (A) and after insulin stimulation (0.75U/kg, 5 min) (C). (B) Quantification of five independent experiments from (A). (D) Quantification of representative experiment from (C) (mean ± SEM); #p > 0.1.

(Figure 2D), we inspected their signaling activity. First, we analyzed the activation of Akt and its downstream kinases. We observed a 20% increase in the p-Akt/Akt ratio in liver lysates from Rab5KD animals compared to control; however, this increase was not statistically significant (Figures 4A and 4B). Furthermore, the levels of the downstream kinase GSK3β were increased, but its phospho/total ratio and that of P-S6-RP were unchanged (Figures 4A and 4B). In addition, the response to insulin stimulation in vivo was not altered, as Rab5KD animals showed a similar activation of Akt and its downstream kinase GSK3β (Figures 4C and 4D). Occasionally, animals exhibited a stronger increase in p-Akt levels (up to 200%) in the basal state. However, since such an increase was not consistent between animals, it cannot be the sole mechanism underlying the loss of *G6Pase* and *Pepck1*.

We then verified that glucagon signaling was induced efficiently in response to hypoglycemia. Glucagon leads to an increase in cyclic AMP (cAMP) that activates protein kinase A (PKA), which, in turn, phosphorylates CREB, a key transcription factor controlling gluconeogenic gene expression (Altarejos and Montminy, 2011). The basal cAMP levels and PKA activity were increased in the liver of Rab5KD animals compared to control (Figures 5A and 5B). As expected, phospho-CREB was also increased (Figures 5C and 5D), suggesting that the liver responded normally to glucagon.

We next inspected the transcription factors responsible for *G6Pase* gene expression, CREB, *PGC1α*, *ChREBP*, and *FoxO1* using a combination of qPCR, western blot, and quantitative immunofluorescence microscopy analysis. Surprisingly, although the levels of *CREB* were increased, the expression of its target

PGC1α (Lin et al., 2005) was repressed (Figure 5E). In addition, *ChREBP* (Ma et al., 2006) was reduced by 50% (Figure 5E). *FoxO1* is mainly negatively regulated by phosphorylation (Matsumoto et al., 2007). *FoxO1* showed a modest (20%) decrease in gene expression but a 100% increase in phospho-*FoxO1*, suggesting a reduction in activity (Figures 5C–5E).

To verify whether the translocation of CREB, *FoxO1*, and phospho-*FoxO1* between the cytoplasm and nucleus occurred normally, we visualized their intracellular localization in primary hepatocytes and mouse liver upon Rab5KD.

CREB levels were unchanged and accumulated in the nucleus upon Rab5 depletion in vitro and in vivo (Figures S5A–S5D). However, total *FoxO1* remained largely cytoplasmic (Figures S5A and S5C) with a modest decrease in protein levels (Figure S5B). This is consistent with a strong increase in the levels of Akt-dependent phospho-Ser256-*FoxO1* (Figures 5F, 5G, and S5D) in primary hepatocytes and mouse liver. Therefore, although the reduction in *FoxO1* expression is modest, the increased phosphorylation and lack of nuclear translocation argue that its function is severely impaired. These data suggest that endosomes exert a regulatory role on the expression, activity, or nuclear translocation of the transcription factors for the gluconeogenic genes.

Rab5KD Rescues Hyperglycemia in Diabetic db/db Mice

Patients with type 2 diabetes fail to respond to insulin, a deficiency known as insulin resistance. Consequently, the liver enhances gluconeogenesis, contributing to hyperglycemia. If the regulation of gluconeogenic gene expression via endosomes were part of this mechanism, silencing Rab5 in the liver might rescue the hyperglycemia in diabetes. To test this possibility, we downregulated Rab5 in a mouse model of type 2 diabetes (*db/db*; *LepR KO*) and tested the effects on blood glucose and gluconeogenic gene expression in the liver of 10-week-old *db/db* mice. Strikingly, depletion of Rab5 5 days post-injection led to a 50% decrease in blood glucose, suggesting that loss of endosomes improved glucose homeostasis specifically in *db/db* mice (Figure 2C). Interestingly, the expression of *G6Pase* and *Pepck1* was strongly reduced upon Rab5 loss in livers of *db/db* mice (Figure 2E) supporting an involvement

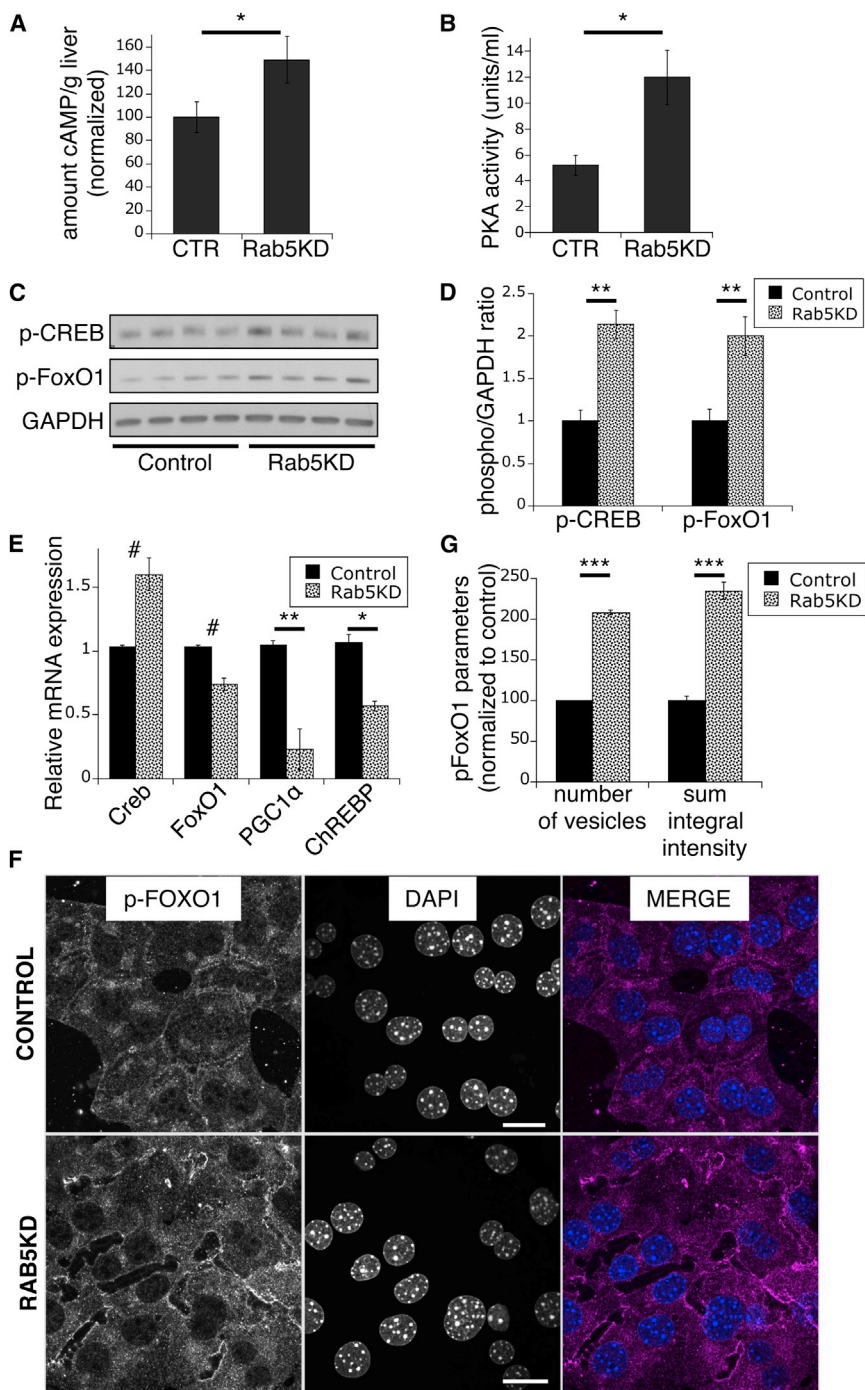


Figure 5. G6Pase Transcription Factor Alterations upon Rab5KD

(A–E) cAMP amount (A), PKA activity (B), western blots of p-CREB and p-FoxO1 (C), and quantification thereof (D), relative mRNA expression of CREB, FoxO1, PGC1 α , and ChREBP (E) from livers of 6-hr-starved control and Rab5KD mice (representative experiment shown; n = 4 mice per condition).

(F and G) (F) Representative confocal images (maximal projections of three merged middle sections) of primary hepatocytes stained with p-FoxO1 plus DAPI and (G) quantification thereof within cellular masks by QMPLA.

Scale bar, 20 μ m (mean \pm SEM) *p < 0.05, **p < 0.01, ***p < 0.0001.

gluconeogenesis. The induction of steroid biosynthesis is an expected consequence to the inhibition of LDL endocytosis (Brown and Goldstein, 2009; Zeigerer et al., 2012). Much more unexpected are the effects on glucose metabolism. The accumulation of glycogen granules, the increase in liver size, and the reduction in G6Pase gene expression all point at a striking similarity with the von Gierke's disease (Chou et al., 2010). The loss of G6Pase in von Gierke's disease appears to be sufficient to induce all the consequent metabolic abnormalities, e.g., accumulation of glycogen (Cori and Cori, 1952), hepatomegaly (Froissart et al., 2011), as well as hypercholesterolemia and hyperlipidemia (Bandsma et al., 2002). Mathematical kinetic modeling and simulation of hepatic glucose metabolism suggest that the reduction in G6Pase expression alone can explain the marked decrease in glucose production upon Rab5KD.

Rab5 may exert its function through its signaling effectors (Christoforidis et al., 1999; Schenck et al., 2008; Shin et al., 2005). However, we favor the interpretation that the metabolic alterations are the consequence of the loss of endosomes and lysosomes. First, the Rab5 signaling effectors are localized to endosomes (Schenck et al., 2008). Second,

of endosomes in the regulation of gluconeogenic gene expression.

DISCUSSION

In this study, we have uncovered a requirement of Rab5 and the endosomal system for the regulation of hepatic metabolism. Depletion of Rab5 boosts steroid biosynthesis while blocking

the metabolic alterations remarkably correlate with the depletion of endosomal compartments. Third, lysosomes play an important role in glycogenolysis (see below). Another indication of such a regulation is that Rab5 depletion was sufficient to overcome the insulin resistant phenotype of diabetic mice.

Interestingly, a functional genomics screen revealed that the endosomal system is modulated by a number of metabolic pathways, including glycolysis, gluconeogenesis, and steroid

biosynthesis (Collinet et al., 2010). This argues that the functional relationship between metabolism and endocytosis is bi-directional. Such a relationship goes beyond the classical view that endocytosis regulates the surface expression of nutrient sensors and transporters, e.g., GLUT4 (Antonescu et al., 2014). In our study, the decrease of gluconeogenic gene expression and occurrence of hypoglycemia upon Rab5KD cannot be explained solely by the 30% reduction of GLUT2 observed by proteomics. Insulin signaling was also not reproducibly affected.

Clearly, endocytosis is a key cellular process that, when perturbed, could have multiple direct or indirect effects on metabolism. Among the many possible interpretations, one is that endosomes could exert a regulatory role on the transcription factors responsible for gluconeogenic gene expression. We found that loss of *G6Pase* upon Rab5KD is due to impairment in the transcription factors regulating its expression. CREB was phosphorylated and translocated to the nucleus upon Rab5 depletion but failed to induce the expression of its downstream target *PGC1 α* . The levels of *ChREBP* were reduced, and the nuclear translocation of FoxO1 was inhibited, while its phosphorylation increased. These results suggest that endosomes are directly or indirectly necessary for the activity (CREB), expression (*ChREBP*), and phosphorylation (FoxO1) of the transcription factors for *G6Pase* expression. One possibility is that they could interact with Rab5 on the endosomal membrane, or through intermediary molecules. Endosomes could provide a scaffold for the transport toward the nucleus, as shown for CREB during the retrograde transport from the growth cone to the cell body in neurons (Cox et al., 2008), or for regulating their phosphorylation state, as they regulate signal transduction (Sorkin and von Zastrow, 2009). Akt phosphorylates FoxO proteins leading to their association with 14-3-3 and retention in the cytosol (Obsil and Obsilova, 2008). Addressing this question requires the development of new tools to detect the transcription factors intracellularly and follow how signals are transmitted from the endosomes to the kinases and phosphatases controlling their activity.

The functional connection between the endosomal system and metabolism is exemplified by the Target of rapamycin mTOR-TFEB pathway. The serine/threonine kinase mTOR is localized to lysosomes and responds to nutrients, growth factors, bioenergetic needs, and cellular stress (Laplante and Sabatini, 2012; Polak and Hall, 2009). When nutrients are available, mTORC1 phosphorylates the transcription factor TFEB and inhibits its activity, whereas under starvation TFEB translocates to the nucleus and induces the CLEAR transcriptional network required for lysosomal biogenesis, autophagy, and lipid catabolism (Settembre et al., 2013). Despite the severe loss of endosomes and lysosomes upon Rab5KD, we did not observe a significant induction of autophagy or TFEB activation. We found only 13 genes of the CLEAR network that are transcriptionally affected, 12 of them being upregulated upon Rab5KD. These results make it unlikely that the mechanism by which Rab5KD leads to the metabolic changes is TFEB dependent. Furthermore, we did not observe an induction of autophagosomes probably due to the loss of endosomes and lysosomes, which are necessary for their biogenesis (Lamb et al., 2013). The disturbed response to starvation could therefore add to the observed phenotypes. Clearance of intracellular lipids via

lipophagy is probably perturbed, contributing to the accumulation of lipid droplets. In addition, lysosomes have been implicated in the breakdown of glycogen granules (Dong and Czaja, 2011), further substantiating the lack of glycogenolysis.

It is likely that endosomal regulation of metabolic activities is not restricted to the liver but plays a more general role in different tissues. Dysfunctions of the endosomal system could play a regulatory role also in the development of metabolic diseases such as hepatic insulin resistance. On the other hand, pharmacological modulation of selective components of the endosomal system could be a possible strategy for therapeutic intervention. Our results showing that depletion of Rab5 reduces blood glucose levels in a mouse model of type 2 diabetes provide a proof of principle for such an approach.

EXPERIMENTAL PROCEDURES

Animals

Animal studies were conducted in accordance with German animal welfare legislation and in pathogen-free conditions in the animal facility of the MPI-CBG, Dresden, Germany. Protocols were approved by the Institutional Animal Welfare Officer (Tierschutzbeauftragter), and necessary licenses were obtained from the regional Ethical Commission for Animal Experimentation of Dresden, Germany (Tierversuchskommission, Landesdirektion Dresden).

Proteomics

Liver tissues were processed according to the FASP method and separated using strong anion exchange (Sharma et al., 2014). MS analysis of resulting peptides was performed by Q-Exactive mass spectrometer coupled on-line to a nanoflow UHPLC instrument (Thermo Fisher Scientific). Spectra were searched against the Uniprot FASTA database using MaxQuant (Cox and Mann, 2008), version 1.4.1.6. An FDR < 0.01 for proteins and peptides was required. Proteome quantification was performed using LFQ algorithm (Cox et al., 2014).

Shotgun Lipidomics

Lipids were extracted from mouse liver and plasma by a modified Folch protocol (Folch et al., 1957) and analyzed by shotgun mass spectrometry (Schuhmann et al., 2012). Molecular species of 15 lipid classes were identified and quantified by LipidXplorer software (Herzog et al., 2011).

Glucose Flux Modeling

The mathematical kinetic model comprises the pathways of glycolysis/gluconeogenesis and glycogen synthesis/glycogenolysis. Inputs are the external glucose and insulin and glucagon concentrations as well as protein abundances. Relative protein abundances are mapped as changes in V_{\max} values on the respective reactions. Simulations were performed for varying external glucose concentration between 3 and 15 mM. For the simulation of the Rab5KD condition, a total knockout of *G6Pase* was assumed.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO:GSE67227.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.04.018>.

AUTHOR CONTRIBUTIONS

M.Z., V.K., A.Z., and J.G. designed experiments. A.Z., R.L.B., K.S., S. Seifert, S. Sales, and R.C.J.D. conducted experiments. G.M., K.M., K.N., N.L., and

A.D. carried out experimental analysis. N.B. and S.B. performed mathematical modeling. M.M., A. Shevchenko, and H.-G.H. supervised experiments. M.Z. and A.Z. wrote the manuscript.

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