Glucagon contributes to liver zonation

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Liver zonation characterizes the separation of metabolic pathways along the lobules and is required for optimal function. Wnt/β-catenin signaling controls metabolic zonation by activating genes in the perivenous hepatocytes, while suppressing genes in the periportal counterparts. We now demonstrate that glucagon opposes the actions of Wnt/β-catenin signaling on gene expression and metabolic zonation pattern. The effects were more pronounced in the periportal hepatocytes where 28% of all genes were activated by glucagon and inhibited by Wnt/β-catenin. The glucagon and Wnt/β-catenin receptors and their signaling pathways are uniformly distributed in periportal and perivenous hepatocytes and the expression is not regulated by the opposing signal. Collectively, our results show that glucagon controls gene expression and metabolic zonation in the liver through a counterplay with the Wnt/β-catenin signaling pathway.

The liver performs diverse functions essential for energy homeostasis, lipid and protein synthesis, biotransformation of xenobiotics and endogenous byproducts. The proper function of the liver depends on its structure, which consists of small units called lobules. Each lobule is composed of concentric layers of hepatocytes expanding from the central vein toward the periportal vein. Hepatocytes perform different metabolic functions along the periportal–central axis, a phenomenon referred to as metabolic zonation (1, 2). The hepatocytes closest to the branches of the portal vein are located in the periportal (PP) zone, whereas hepatocytes surrounding the central vein are in the perivenous (PV) zone (1, 2). Blood flows from the periportal to the central vein and consists of a mixture of nutrient-rich but poorly oxygenated blood from the portal vein and highly oxygenated blood from the hepatic artery (1). Due to the metabolic functions and signaling properties of the hepatocytes along the periportal–central axis, gradients of oxygen, nutrients, and hormones are created as the blood flows through the sinusoids. For these reasons, oxygen demanding pathways colocalize in the PP zone, which has the highest partial oxygen pressure. The functional division of hepatocytes also prevents futile cycling when fulfilling anabolic and catabolic requirements of opposing pathways and avoids competition for common substrates between pathways. Furthermore, efficient xenobiotic metabolism and biotransformation of endogenous byproducts of metabolism is accomplished by spatial separation of complementary pathways (1–4).

Zonation of liver functions is supported by pronounced heterogeneity in gene expression between hepatocytes along the sinusoid. RNA sequencing (RNA-seq) of single mouse hepatocytes revealed that half of the detected transcripts are non-randomly expressed (3,496 of 7,277 genes) (5). This suggests that liver zonation is a highly regulated process. Diffusible Wnt morphogens are secreted by the endothelial cells surrounding the central vein and regulate liver zonation by activation of the Wnt/β-catenin signaling pathway (6–9). Gene expression profiles of livers with hyperactivated [adenomatous polyposis coli (Apc) knockout mice] or inactivated (β-catenin–deficient mice) Wnt signaling revealed that 25% (884 of 3,496 genes) of all zonated genes are regulated by Wnt/β-catenin (5–7). Another 9% (298 genes) are regulated by hypoxia, Ras-dependent signaling pathways and pituitary hormones (5). Thus, the regulation of expression of two-thirds of the zonated liver genes remains to be established.

Glucagon is secreted from the α-cells of the endocrine pancreas and promotes hepatic glucose output to maintain normal blood glucose levels during fasting (10). In this study, we generated glucagon deficient (Gcg−/−) mice and demonstrate that glucagon is an important regulator of metabolic zonation in the liver. Unexpectedly, we found that glucagon modulates expression of a number of genes that are also regulated by the Wnt/β-catenin signaling pathway. Consistent with the drop in the blood glucagon concentration in the sinusoid along the periportal–central axis, we show that glucagon primarily affects gene expression and metabolic zonation in the periportal hepatocytes.

Results

Characterization of Gcg−/− Mice. Gcg−/− mice were generated using Velocigene technology (11). The targeting vector contained a specific deletion of the glucagon coding exon 3 leaving the glucagon-like peptide 1 (GLP-1) and 2 (GLP-2) exons (exons 4 and 5) to be transcribed and translated in-frame (Fig. 1A). To verify specific absence of glucagon coding exon 3 in pancreas from Gcg−/− mice, real-time quantitative RT-PCR (qRT-PCR) analysis was performed with three primer pairs, each recognizing a specific exon. Gcg exon 3 mRNA was not detected in Gcg−/− mice (Fig. 1B). Lack of glucagon was confirmed in plasma of Gcg−/− mice and by pancreas α-glucagon immunostaining (Fig. 1C and Fig. S1A). Disruption of glucagon signaling induces compensatory increase in proglucagon (Pgcg) gene transcription.

Significance

The lobules are the functional units of the liver. They consist of 15–25 layers of hepatocytes with specialized metabolic functions and gene expression patterns relative to their position along the lobule, a phenomenon referred to as metabolic zonation. The Wnt/β-catenin pathway regulates hepatocyte function but how the zonation is controlled to meet the metabolic demands of the liver is unclear. Glucagon regulates hepatic function. We now demonstrate that glucagon contributes to liver zonation by interacting and opposing the actions of the Wnt/β-catenin pathway. This open access article is distributed under a Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).
mice, since exon 4 and 5 mRNA expression in control mice pancreas. (Fig. S4C). Despite the interference with genes involved in amino acid metabolism and ammonia handling, we did not detect changes in plasma ammonia and urea levels in the Ggc<sup>−/−</sup> mice (Fig. S1 F and G). This is consistent with spare capacity of the urea cycle, which normally runs at 20–50% of capacity and most of the enzymes operate at or below their half-maximal capacity (19). Collectively, these data show that lack of glucagon reduces expression of PP genes, resulting in contraction of the PP zonation pattern.
Glucagon contributes to metabolic zonation. (A) Regulated PP genes in Gcg−/− mice liver. (B) Regulated PV genes in Gcg−/− mice liver. (C) The correlation of PP and PV gene expression between Gcg−/− and Pgcg−/− or Gcg−/− mice. (D) Gls2 RNA-ISH demonstrating down-regulation of Gls2 expression in Gcg−/− mice liver. (E) Quantification of Gls2 RNA-ISH positive area in liver. (F) Glul RNA-ISH, demonstrating extended Glul expression in Gcg−/− mice liver. (G) Quantification of Glul RNA-ISH positive area in liver. (H) GS immunohistochemistry (IHC) demonstrating extended GS expression in Gcg−/− mice liver. (I) Quantification of GS IHC positive area in liver. (J) Western blot demonstrating decreased expression of GLS and increased expression of GS in Gcg−/− mice liver. (K) Quantification of Western blot of GLS and GS in liver. All images are the same magnification. (Scale bar: 100 μm.) The insertions in D, F, and H were amplified with 4x magnification. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. RNA-seq comparison in A and B was between Gcg−/− and WT mouse liver samples.
Fig. 3. Gcg infusion restored Gcg−/− mice liver zonation pattern. (A) Heat map of 297 affected genes in Gcg−/− mice demonstrated dose-dependent effects of Gcg infusion. (B) Gene pathway analysis demonstrated dosage-dependent rescue effects of Gcg infusion on regulated PP gene signature. (C) Glu2 mRNA liver expression (RPKM) demonstrated Gcg induced dosage dependent up-regulation of Glu2 expression in Gcg−/− mice. (D) Glul mRNA liver expression with Gcg infusion (RPKM). (E) Representative Glu2 RNA-ISH images in liver demonstrated Gcg infusion restored Glu2 liver zonation pattern in Gcg−/− mice. (F) Quantification of RNA-ISH Glu2-positive area in liver with Gcg infusion. (G) Representative GS IHC images in liver demonstrated Gcg infusion restored GS liver zonation pattern in Gcg−/− mice and high dosage pushed GS toward the central vein further in WT mice. (H) Quantification of GS IHC-positive area in liver with Gcg infusion. (I) GLS and GS protein expression with Gcg infusion demonstrated Gcg inducing GLS expression but inhibiting GS expression. (J) Quantification of protein expression of GLS and GS in liver with Gcg infusion. All images are the same magnification. (Scale bar: 100 µm.) Data are mean ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001. In C and D, WT glucagon 0 µg/d group was compared with the other five groups. Significant genes were defined as fold change > 1.5 in either up or down direction and with P values < 0.01, and indicated by *.
pattern. On the contrary, glucagon deficiency expanded the PV zone as illustrated by the detection of Glul mRNA and GS protein in additional cell layers surrounding the central vein.

**Glucagon Infusion Restores Liver Zonation in Gcg<sup>−/−</sup> Mice.** To test whether glucagon application restores liver zonation in Gcg<sup>−/−</sup> mice, dosages of 30 and 120 μg/d glucagon were infused to Gcg<sup>−/−</sup> and WT mice for 7 d, resulting in a dose-dependent increase in blood glucagon and glucose levels (Fig. S5). Fig. 34 shows a dose-dependent reversal of expression of the 297 affected genes in Gcg<sup>−/−</sup> mice. Gene pathway analysis revealed a normalization of PP gene expression in Gcg<sup>−/−</sup> mice (Fig. 3B). Glucagon infusion normalized Gls2 expression and zonation pattern in Gcg<sup>−/−</sup> mice (Fig. 3 C, E, and F). Western blotting confirmed normalization of GLS protein in Gcg<sup>−/−</sup> mice (Fig. 3 I and J). Contrary to the effects in the PP zone, glucagon infusion decreased GS protein by 30% in both WT and Gcg<sup>−/−</sup> mice (Fig. 3 I and J), which was secondary to retraction of GS expression to the two to three cell layers closest to the central vein (Fig. 3 G and H). Glul RNA levels in the 120 μg/d dose trended lower without a significant difference (Fig. 3D). Collectively, these data show that glucagon restores Gcg<sup>−/−</sup> mouse liver PP gene expression and zonation pattern. Glucagon also reduced expression of the GS protein except in cells in the immediate vicinity to the central vein.

**Glucagon Receptors Are Expressed Throughout the Liver Lobules.** Next, we wanted to test whether the effects of glucagon on metabolic zonation in the liver is secondary to zonated expression of the glucagon receptor (Gcgr) and its downstream signaling components and whether the expression of the glucagon signaling pathway is compromised in Gcg<sup>−/−</sup> mice. Fig. 4A shows unperturbed hepatic expression of Gcgr mRNA in Gcg<sup>−/−</sup> mice. RNA-ISH detected Gcgr mRNA at comparable levels throughout the lobules (Fig. 4 B and C). The Gcgr RNA-ISH signal was slightly higher in Gcg<sup>−/−</sup> mice (Fig. 4C). Next, we extracted and plotted published expression data for Gcgr and 21 downstream signaling genes (5). The results confirm that expression of Gcgr and its signaling components are evenly distributed throughout the liver lobules (Fig. S6 A and B).

Insulin is the counterregulatory hormone of glucagon in the liver. Insulin receptor (Insr) RNA-ISH revealed even expression throughout the liver lobules and unchanged expression in Gcg<sup>−/−</sup> mice (Fig. 4 A, B, and D). Uniform distribution of Insr and its downstream 35 signaling genes was confirmed by reanalysis of published RNA expression data (5) (Fig. S6 A and C). Interestingly, Insr, but not Gcgr, is also expressed in the endothelial cells lining the perportal and central veins (Fig. 4E).

**Glucagon and Wnt/β-Catenin Regulated Genes Overlap in PP and PV Hepatocytes.** Our data show that glucagon contributes to liver zonation, although the glucagon receptor and its signaling genes do not show zonated expression. This raises the possibility that glucagon may interact with the Wnt/β-catenin signaling pathway to regulate gene expression and the metabolic zonation pattern. It has previously been reported that Wnt/β-catenin–inhibited genes (88 genes) are primarily expressed in PP hepatocytes,
whereas Wnt/β-catenin–activated genes (87 genes) are found in their PV counterparts (5). Supporting the hypothesis that the glucagon and Wnt/β-catenin signaling pathways interact, we found that 28% of the 88 PP expressed and Wnt/β-catenin–inhibited genes were regulated in the Gcg−/− mice (Fig. S4). Furthermore, expression of 9% of the PV and Wnt/β-catenin–activated genes were affected in the Gcg−/− mice (Fig. S5). These data suggest that many liver genes are regulated by both glucagon and Wnt/β-catenin, particularly in the PP hepatocytes.

**Wnt/β-Catenin Signaling Genes Are Not Zonated or Regulated by Glucagon.** Among 41 Wnt/β-catenin signaling genes in the mouse liver, only Fzd5 (−1.55) was slightly down-regulated in Gcg−/− mice (Fig. S5 C). Reanalysis of published data (5) revealed that 40 of these genes are express evenly throughout the lobules, whereas Ctnnb1 shows a trend toward higher expression in the PP zone (Fig. S6D).

Further analysis revealed that the distribution of the β-catenin (RNA, Ctnnb1; protein, β-catenin) protein was even throughout the liver lobules and the mRNA expression was similar in WT and Gcg−/− mouse livers (Fig. 5 C–E). RNA-ISH of Apc (RNA, Apc; protein, APC), a negative regulator of Wnt/β-catenin signaling was also evenly detected throughout the liver lobules and the expression was similar in WT and Gcg−/− mice (Fig. 5 C, D, and F). In summary, the expression of Wnt/β-catenin signaling genes was not altered in the Gcg−/− mouse liver. This suggests that glucagon and Wnt/β-catenin likely regulate gene expression by cross-talk at the transcriptional level.

**Wnt Signaling Inhibition Contracts the PV Zone in Gcg−/− Mice.** Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) binds to Rspandin (RSPO) to promote Wnt/β-catenin signaling (20–22). Therefore, to inhibit Wnt/β-catenin signaling, we delivered cDNA of the extracellular domain of Lgr5 fused with hFc by hydrodynamic DNA delivery (HDD) to livers of Gcg−/− and WT mice. Lgr5–hFc slightly increased Gls2 mRNA and GLS protein levels in Gcg−/− and WT mice (Fig. 6 A–E). Consistent with the primary action of Wnt/β-catenin in the PV zone, Lgr5–hFc reduced Glul mRNA and GS protein in Gcg−/− and WT mice (Fig. 6 A–C). Furthermore, the GS zonation pattern in Gcg−/− mice was retracted toward the central vein and was limited to one to two cell layers around the central vein in WT mice (Fig. 6 F and G). The finding that the GS zone was retracted but not missing is consistent with Lgr5–hFc binding to RSPO being a partial inhibitor of Wnt/β-catenin signaling (23, 24).

**Discussion**
Zonation of liver metabolic functions is supported by pronounced heterogeneity in gene expression between hepatocytes along the liver lobules. A recent single hepatocyte mRNA sequencing study revealed that half of the detected transcripts in mouse liver have zonated expression patterns, suggesting that liver zonation is a highly regulated process (5). Recent studies have highlighted that the Wnt/β-catenin signaling pathway is a key determinant of liver zonation (6–9). In fact, 25% of all zonated genes are regulated by Wnt/β-catenin (5). The regulation of only 9% of the other zonated genes is understood, leading to the question of which other hormones...
and mechanisms control liver zonation. Our data reveal an interaction between the glucagon and Wnt/β-catenin signaling pathways. Specifically, we demonstrate that these pathways counteract to regulate liver metabolic functions. Wnt/β-catenin signaling is more dominant in the PV zone regulating Wnt/β-catenin–activated gene expression, while glucagon action is more important in the PP zone.

Although the target gene expression of glucagon and Wnt/β-catenin showed restricted zonation patterns, the genes involved in their signaling were evenly distributed throughout the liver lobules. Previous studies have indicated that Apc IHC-positive staining was localized to the PP zone to inhibit Wnt/β-catenin signaling and that its counterplay maintains the homeostasis of liver zonation (6). Our RNA-ISH results show that Apc mRNA is evenly distributed throughout the liver lobules. Reanalysis of published single hepatocyte RNA sequencing data confirms that Apc mRNA is evenly distributed along the lobules (5).

Despite the established role of Wnt/β-catenin as the gatekeeper of liver metabolic zonation, the instructive mechanism controlling its spatiotemporal regulation is not known. With the even (nonzoned) distribution of the glucagon and Wnt/β-catenin signaling genes, the restricted expression patterns of their target genes could be explained by a combination of concentration gradients of the ligands between the PP and PV zones and by interactions between the signaling pathways at the transcriptional level. Diffusible Wnt morphogens are secreted by the endothelial cells surrounding the central vein and act upon nearby hepatocytes (25, 26). The efficiency of Wnt/β-catenin targeting depends on the local Wnt and Wnt regulator concentrations. The same accounts for glucagon but in the PP zone. Glucagon is released from the pancreatic α-cells and is distributed to the liver lobules with nutrient-rich blood from the portal vein where it flows through the PP and PV zones before reaching

![Image of graphs and diagrams](image-url)
the central vein. Importantly, 24% of glucagon is removed from the blood during the passage of the PP and PV zones (27). The concentration gradients of glucagon and Wnt ligands may only partially explain the zoned regulation of their target genes. The counterplay between them, as demonstrated in our study, also contributes to the regulation. Similar opposing transcriptional interactions have been described for Wnt/β-catenin and hepatocyte growth factor (HGF) (28). Thus, liver zonation may be influenced by the rate of glucagon secretion from the α-cells, a highly regulated process which involves neurohormonal factors and nutrients (10). Our understanding of the regulation of Wnt release from endothelial cells is quite limited. It has been hypothesized that injury-related chemokines may regulate release of Wnt ligands (29, 30).

In conclusion, our data show that glucagon is required for the maintenance of liver metabolic zonation and that it counteracts the Wnt/β-catenin signaling pathway to regulate the expression of glucagon and Wnt/β-catenin–activated or inhibited genes.

Materials and Methods

Generation of Mouse Lines. Mice were generated using BAC-based homologous recombination (11). In Brief, we modified a BAC containing the Ggcg coding sequence by replacing the 1.86-kb region, consisting of introns flanking both ends of exon 3, with the neomycin cassette. After germline transmission was established, F1 Ggcg+/– mice were bred together to generate F2 Ggcg++ and Ggcg–/– mice. Also, F1 Ggcg–/– mice were backcrossed to C57BL6/J to generate N2 breeding heterozygote pairs that were used to generate N2F2 Ggcg++ and Ggcg–/– mice. The studies reported here were conducted on F2 or N2F2 littermates (8–12 wk of age) that were housed under 12 h of light per day in a temperature-controlled environment (22 ± 1 °C, 60–70% humidity). Mice deficient in the proglucagon gene (Ppgcg–/–) (87.5% C57BL6/N, 12.5% 129S6/SvEvTac) were generated by a 4.9-kb deletion of proglucagon gene exons 1–4 by homologous recombination using Regenener’s VelociClone technology (11). Mice deficient in the glucagon receptor (Gcgr−/–) (60% C57BL6/N, 40% 129S6/SvEvTac) were generated as described (16). All procedures were conducted in compliance with protocols approved by the Regenener Institutional Animal Care and Use Committee. Animals had free access to standard chow (LabDiet 5001).

Hydrodynamic DNA Delivery of Lgr5–hFc. Extracellular domain of Lgr5 was fused with hFc and cloned into expression vector pRGl97 construct. The construct was verified by DNA sequencing and secretion of the corresponding gene product was confirmed by ELISA analysis of culture media from tran- singly transfected HEK293 cells. To establish a baseline for serum chemistry parameters, serum samples were collected 7 d before HDD construct administration. Mice (n = 4–5 per group) were administered a single IV injection of Lgr5–hFc or control construct. One week after the injection, mice were killed and liver samples were collected for histology and Western blotting. LGR5–hFc expression was confirmed by plasma ELISA.

Glucagon Pump Infusion. Glucagon microosmotic pump implantation surgery was performed as described (31). Glucagon (Sigma) was dissolved in a cetrimide solution (Sigma) at a ratio of 6 mol cetrimide to 1 mol glucagon to maintain long-term solubility (31). Glucagon solutions were made to concentrations necessary to deliver 30 or 120 μg glucagon per day for 7-d microosmotic pumps (Alzet). Control littersmates were implanted with a pump containing the same concentration of cetrimide solution alone. Briefly, animals were anesthetized and a microosmotic pump (model 1002) was implanted s.c. in the back of the mice. Blood glucose was measured using a hand-held glucometer. One week later, blood was collected by cardiac puncture and liver was collected and processed for histology and RNA preparation.

RNA-Seq Preparation and Analysis. Total RNA was purified from all samples using MagMax-96 for Microarrays Total RNA Isolation Kit (Ambion by Life Technologies) according to manufacturer’s specifications. Genomic DNA was removed using MagMAXTurbo DNase Buffer and TURBO DNase from the MagMax Kit listed above (Ambion by Life Technologies). mRNA was purified from total RNA using Dynabeads mRNA Purification Kit (Invitrogen). Strand-specific RNA-seq libraries were prepared using KAPA mRNA-Seq Li- brary Preparation Kit (Kapa Biosystems). Twelve-cycle PCR was performed to amplify libraries. Sequencing was performed on Illumina HiSeq2000 by a multiplexed single-read run with 33 cycles. Raw sequence data (BCL files) were converted to FASTQ format via Illumina Casava 1.8.2. Reads were decoded based on their barcodes and read quality was evaluated with FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were mapped to the mouse transcriptome (NCBI GRCm38) using ArrayStudio software (OmicsSoft) allowing two mismatches. Reads mapped to the exons of a gene were summed at the gene level. Differentially expressed genes were identified by the DESeq2 package and significantly perturbed genes were defined with fold changes no less than 1.5 in either up or down di- rection and with P values of at least 0.01.

Quantitative Real-Time PCR Analysis. qRT-PCR analysis was performed using TaqMan qRT-PCR chemistry and detection system (Applied Biosystems) with the primer pairs and labeled probes for Ggcg gene exons 3–5. Relative mRNA levels were calculated by the ΔΔct method, using a housekeeping gene (cytochrome A) for normalization and the mean value of WT mice as the reference value.

Liver Zonation Genes. PP- and PV-enriched genes were obtained from refs. 3 and 5. A total of 285 PS and 408 PV genes were identified. Gs-2 was manually added to the PP gene list. Wnt-activated (635 genes) or inhibited (249 genes) were extracted from liver gene signatures from Apc+/– and Apc−/− mice (28). Wnt-signaling genes were obtained from SABiosciences. Glucagon-signaling pathway genes were obtained according to the pathway diagram from https://rgd.mcw.edu/rgdweb/pathway/pathwayRecord.html?PW:0000676. Insulin-signaling pathway genes were obtained accord- ing to the pathway diagram from https://www.intechopen.com/book/hyperglycemia-metabolism-diabetes/mitochondrial-metabolism-and-insulin-action.

Annotation of Ggcg–/–, Ppgcg–/–, and Gcgr–/– Mouse Signature. Ggcg–/– mouse signature is annotated by the four compiled gene sets (PP, PV, Wnt-inhibited, and Wnt-activated) and Biological Process (BP) GO terms with P value < 0.1%. None of the glucagon and insulin signaling genes were regu- lated in the Ggcg–/– mice. Ppgcg–/– and Gcgr–/– mouse signature were generated using the same approach.

Tissue Morphology. Liver samples were collected, fixed in 4% paraformaldehyde for 48 h, equilibrated in 20% sucrose for 24 h, and then in 30% sucrose for 24 h at 4 °C before cryosectioning. The 10-μm sections were freshly prepared. IHC and RNAISH were done on separate sections. For RNAISH, sections were permeabilized and hybridized with mRNA probes to Glu2, Glut, Gcgr, Insr, or Apc (ACD Bio). Following probe hybridization and am- plification, mRNA was detected using RNAscope 2.5 HD Assay, brown kit (n = 3–4 mice from each genotype, n = 4–6 random pictures from each slide). Slides were scanned using a Zeiss Axio Scan Z1 slide scanner and the images were analyzed using Halo software (Indica Labs). For IHC, sections from each animal were stained with either α-GS or α-β-catenin antibodies (BD).

Blood Chemistry. Blood glucose was determined using ACCLU-CHEK Compact Plus (Roche Diagnostics). Plasma glucagon (Mercodia), active-GLP-1 (Meso research) and insulin (Merial) levels were determined using ELISA. Plasma total amino acid levels were measured using L-Amino Acid Quantification Kit (Sigma-Aldrich), which detects L-amino acids with exception of L-glycine. Concentrations of individual amino acids were measured by Metabolon with the use of gas chromatography–mass spectrometry. Ammonia and urea were assayed in a Siemens ADVIA Chemistry XPTB Clinical System.

Western Blotting. Liver samples were lysed with ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate dibasic and 1% Nonidet P-40) in the presence of protease and phosphatase inhibitor mixtures (Thermo Fisher Scientific), 1 mM DTT and 2 mM Na3VO4. Total sample lysates were mixed with 6× SDS loading buffer (Alfa-Aesar) and boiled for 5 min. Protein samples (10–100 μg) were loaded and separated on 4–20% gradient SDS/PAGE gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h with 5% BSA in 1x TBS supplemented with 0.1% Tween 20 (Bio-Rad) and incubated with the following antibodies: GS (610518, BD), GLS2 (150474A, Abcamab), and GAPDH (14C10, Cell Signaling). Bound antibodies were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:10,000; Jackson Immuno- research) and enhanced chemiluminescence reagent (Thermo Fisher Scientific). Band intensities were quantified in ImageJ software.

Data Analysis. All data are mean ± SEM. Statistical analyses were performed utilizing GraphPad software Prism 6.0. All parameters were analyzed by
Student’s t test, one-way ANOVA, or two-way ANOVA; a threshold of $P < 0.05$ was considered statistically significant.