Glycogen metabolism is dispensable for tumour progression in clear cell renal cell carcinoma

Hong Xie1,2,7, Jun Song1,3,7, Jason Godfrey1,1,2, Romain Riscal1,1,2, Nicolas Skuli1,1,2, Itzhak Nissim1,4,5 and M. Celeste Simon1,2,6✉

Glycogen accumulation is a highly consistent, distinguishable characteristic of clear cell renal cell carcinoma (ccRCC). While elevated glycogen pools might be advantageous for ccRCC cells in nutrient-deprived microenvironments to sustain tumour viability, data supporting a biological role for glycogen in ccRCC are lacking. Here, we demonstrate that glycogen metabolism is not required for ccRCC proliferation in vitro nor xenograft tumour growth in vivo. Disruption of glycogen synthesis by CRISPR-mediated knockout of glycogen synthase 1 (GYS1) has no effect on proliferation in multiple cell lines, regardless of glucose concentrations or oxygen levels. Similarly, prevention of glycogen breakdown by deletion or pharmacological inhibition of glycogen phosphorylase B (PYGB) and L (PYGL) has no impact on cell viability under any condition tested. Lastly, in vivo xenograft experiments using the ccRCC cell line, UMRC2, reveal no substantial changes in tumour size or volume when glycogen metabolism is altered, largely mimicking the phenotype of our in vitro observations. Our findings suggest that glycogen build-up in established ccRCC tumour cells is likely to be a secondary, and apparently dispensable, consequence of constitutively active hypoxia-inducible factor 1-alpha (HIF-1α) signalling.

Kidney cancer ranks 8th in yearly incidence rates out of the nearly 200 distinct cancer classifications in the United States1. In fact, Surveillance, Epidemiology, and End Results Program data up to 2017 indicate that kidney cancer incidence had among the greatest percentage increase among males and females for all cancer types in this latest period analysed, highlighting a need for deeper understanding of the disease. ccRCC is the predominant disease subtype, accounting for more than 70% of patient cases, with most tumours harbouring loss-of-function mutations in the von Hippel–Lindau (VHL) tumour suppressor2. pVHL is an E3 ligase component that recognizes oxygen-dependent hydroxyl-lation sites on hypoxia-inducible factors (HIFs) to negatively regulate HIF protein levels through the ubiquitin–proteosome system. Only during acute hypoxia is pVHL unable to interact with HIFs; by extension, HIF proteins are stabilized to initiate an adaptive response to low oxygen. The vast majority of ccRCC tumours lack pVHL expression or function and uniquely exhibit chronic HIF-α stabilization, regardless of tumour oxygenation. As such, these tumours categorically display transcriptomic profiles driven by either HIF-1α and HIF-2α together (H11H2) or HIF-2α solely (H2). Recent pharmacological advances in the development of small-molecule inhibitors of HIF-2α, such as PT2385, have shown therapeutic promise in preclinical and clinical settings, supporting the oncogenic addiction to HIFs in ccRCC3,4. However, the scope of HIF-controlled downstream pathways that are clinically relevant for disease progression is incompletely defined.

Perhaps the most striking phenotypic aftermath of pVHL deficiency in ccRCC tumours is a histologically clear cytoplasm. This is due to the removal of large intracellular lipid droplets, harbouring cholesterol esters and triglycerides, and accumulated granules of glycogen during tissue processing for pathology4,5. We previously reported that HIF-2α promotes lipid storage by regulating the expression of an essential lipid droplet coat protein, perilipin-2 (PLIN2), in ccRCC. PLIN2 knockdown eradicates lipid droplets and strongly abrogates cell viability, illuminating the importance of lipid homeostasis in ccRCC6. However, regulation or biological relevance of glycogen metabolism in ccRCC has not been investigated in careful detail, despite emerging data that support a pro-tumorigenic role for glycogen in other cancers, such as glioblastoma (GBM) and non-small-cell lung cancer (NSCLC), under metabolic stress conditions6,7. Therefore, a thorough assessment of glycogen accretion in ccRCC was warranted.

The biochemical function of glycogen is to store glucose when ATP levels are high and release glucose, in the form of glucose-1-phosphate (G1P), when ATP levels decline8. Briefly, glycogen is constructed via a core protein unit, glycogenin, from which glucose molecules are covalently linked together through linear α(1–4) and branched α(1–6) bonds, and reciprocally degraded by hydrolysis to yield both monosaccharides (glucose) and polysaccharides (e.g., maltose) (Fig. 1a). This dynamic process is tightly regulated on multiple levels in mammals, both systemically by the insulin–glucagon axis and intracellularly through energy-sensing mechanisms, such as 5’-AMP-activated protein kinase and HIF-1α. In normal human physiology, the vast majority of glycogen resides in the gluconeogenic organs, namely the liver and kidney, and high-energy consuming organs, including muscle and brain9. However, increased glycogen abundance in cancer is a common event across a variety of originating tissues, including the kidney, suggesting that glycogen may confer some metabolic benefit within the tumour microenvironment10.

To address whether the glycogen pathway is consistently altered in ccRCC, elevated glycogen levels were confirmed in the tumours of patients compared to matched normal kidney tissue (Fig. 1b). Comparable quantities of glycogen were also detected in six cell...
line models of ccRCC: 786-O, UOK101, 769P, UMRC2, RCC4 and RCC10 (Extended Data Fig. 1a). To provide a more comprehensive and informative picture of glycogen production and degradation, we queried our previously generated metabolomics data in the same patient paired tumour and normal samples for glycogen-related metabolites. Many products of glycogen metabolism were relatively increased in ccRCC, including G1P and oligosaccharide branch intermediates, that is, maltose, maltotriose and maltotetraose (Fig. 1c). These findings are consistent with those from a separate metabolomics assessment of 138 matched ccRCC/normal tissue pairs we analysed further (Extended Data Fig. 1b). Lastly, The Cancer Genome Atlas (TCGA) RNA sequencing (RNA-Seq) analysis revealed that all genes involved in glycogen metabolism were differentially expressed, including overexpression of those encoding critical synthetic enzymes (PGM1, GYS1 and GBE1) and catabolic enzymes (PYGL and PYGM) (Fig. 1d,e). Some patients stratify into high or low levels of expression (relative to normal kidney), which may indicate that elevated glycogen abundance is a result of a variety of transcriptional inputs. This was also consistent with findings in our patient samples, where tumours sometimes showed higher or lower expression of a gene given related to glycogen metabolism compared to normal tissue (Extended Data Fig. 1c). Nevertheless, based on increased glycogen content, elevated glycogen-derived metabolites and differential expression of glycogen-modifying genes, we concluded that glycogen metabolism is deregulated in ccRCC tumours.

To determine whether glycogen serves a key biological role in ccRCC, the rate-limiting enzyme, glycogen synthase (GYS), was initially evaluated because it directly controls glycogen synthesis (Fig. 2a). In mammalian cells, GYS has two isoforms, GYS1 (expressed in skeletal muscle and other tissues) and GYS2 (expressed predominately in the liver)18. Analysis of the TCGA RNA-Seq database and primary samples revealed that GYS1 messenger RNA and protein levels were increased in tumour samples (Fig. 2b,c). Since constitutive activation of HIF-α proteins through pVHL loss of function is a major driver of ccRCC pathogenesis, the capability of HIFs to facilitate increased GYS1 transcription was assessed. Based on short hairpin RNA knockdown, GYS1 mRNA levels and glycogen deposition were selectively controlled by HIF-1α in HIF-1α-expressing cells (H1H2) (Extended Data Fig. 2a,b) and HIF-independent mechanisms in HIF-2α-only-expressing cells (H2) (Extended Data Fig. 2c). Based on previous transcriptomics analyses, the expression levels of GYS1, PYGB, PYGL and PYGM were not substantially different between H1H2 and H2 ccRCC tumours. Additionally, 786-O cells stably re-expressing pVHL did not show altered expression of glycogen metabolic enzymes (Extended Data Fig. 2c), confirming regulation by pathways other than the HIF-prolyl hydroxylation–pVHL system when HIF-1α is absent.

In addition to transcriptional regulation, post-translational control of GYS is achieved through phosphorylation, alternating between the phosphorylated inactive state and the dephosphorylated active state (Extended Data Fig. 2d)18. GYS dephosphorylation is catalysed via hydrolysis by protein phosphatase 1 (PP1), which is bound to glycogen targeting subunit proteins, that is, protein phosphatase 1 regulatory subunit 3 (PPP1R3) (ref. 19). At least seven distinct genes encode PPP1R3 (PPP1R3A–G) that are differentially expressed across tissues19. Previous studies demonstrated that PPP1R3C overexpression promotes glycogen accumulation in different tissues, suggesting its essential role in activating GYS and subsequent glycogen build-up20–23. In ccRCC tumours, PPP1R3B and PPP1R3C expression was elevated in all tumour stages (Extended Data Fig. 2e). Consistent with glycogen build-up, these data suggest that elevated GYS1 mRNA and protein levels in ccRCC is a result of HIF-1α stabilization, whereas enhanced GYS1 activity is due to greater expression of accessory proteins important for GYS1 dephosphorylation.

To assess the functional consequences of elevated GYS1 expression and glycogen accumulation in ccRCC, GYS1 protein was reduced using two independent single guide RNAs in multiple cell lines with variable baseline glycogen levels (UMRC2, 786-O, RCC4 and UOK101; Extended Data Fig. 1a). As shown in Fig. 2d and Extended Data Fig. 3a, both sgRNAs targeting GYS1 robustly decreased GYS1 protein levels, resulting in rapid glycogen depletion in these cells (Fig. 2e and Extended Data Fig. 3b). We hypothesized that cell proliferation would be inhibited if cells were unable to store glucose as glycogen for future use under acute conditions of nutrient deprivation. Surprisingly, GYS1 knockout did not affect growth in UMRC2 cells, regardless of culture conditions (25 mM versus 0 mM of glucose, 21 versus 1% O2, 1% FCS) (Fig. 2f). UMRC2 GYS1 wild-type (WT) and knockout cells were also embedded in Matrigel for spheroid growth to better mimic nutrient gradients and the tissue pressure observed within the tumour environment. Again, GYS1 knockout did not affect spheroid volume during the course of this assay (Fig. 2g). The same trend was observed in spheroid assays for 786-O cells and in two-dimensional proliferation assays for RCC4 and UOK101 cells (Extended Data Fig. 3c,d). Of note, both GYS1 WT and knockout UOK101 cells were particularly sensitive to growth in 25 mM of glucose at 1% O2 after 2 d due to lactic acid accumulation. These results suggest that although GYS1 mRNA and protein levels are overexpressed in the tumours of patients with ccRCC and cell lines and associated with a concomitant accumulation in glycogen, neither GYS1 nor glycogen production is required for two- or three-dimensional growth under normal and tumour-relevant stress conditions.

The data showing that glycogen depletion does not affect in vitro ccRCC cell proliferation were rather unexpected since GYS1 inhibition has been shown to reduce cell proliferation of other types of cancer, such as leukaemia24. An alternative hypothesis to explain this apparent lack of phenotype on GYS1 inhibition in ccRCC cells suggests that increased GYS1 expression and glycogen accumulation is a collateral effect of HIF-1α activation and other factors, simply making glycogen generation a by-product. Cells may need to simultaneously increase activity of the breakdown pathway to maintain glycogen homeostasis and avoid aberrant glucose storage.

**Fig. 1** | Glycogen synthesis and breakdown are hyperactive in ccRCC tumours. a, Schematic representation of the glycogen synthesis and breakdown pathway in the cytosol. Briefly, glucose conversion into glucose-1-phosphate (G1P) is added or subtracted from oligosaccharide chains scaffolded by the core protein, glycogenin. Green hexagon, singly added G1P; pink hexagon, polysaccharide molecules added; blue hexagon, units added as a new branch. b, Top: glycogen extracted from 20 pairs of fresh-frozen tumours of patients with ccRCC and adjacent normal kidneys and then quantified using the glycogen assay kit (Methods). Bottom: Summary of tumours analysed for glycogen levels and subdivided according to tumour stage; n = 20 biologically independent human ccRCC tumour/normal paired samples. Box plots (minimum to maximum all points): centre, median; boundaries, 25th and 75th percentiles; whiskers, minimum and maximum values. c, Abundance of glycogen metabolism-related metabolites in the same human ccRCC tumour/normal paired samples. Data are presented as the mean ± s.e.m. d, e, Normalized RNA-Seq reads of glycogen synthesis genes (PGM1, UGP2, GYS1 and GBE1) (d) and glycogen breakdown genes (PYGL, PYGB, PYGM and AGI) (e) in ccRCC (n = 428) and normal kidney (n = 66) samples; n denotes biologically independent human tissue samples. RNA-Seq data were obtained from the TCGA. Box plots (minimum to maximum all points): centre, median; boundaries, 25th and 75th percentiles; whiskers, 5th and 95th percentiles. P values were determined by two-tailed Student’s t-test. Red denotes an increase in gene expression.
into glycogen. Therefore, pVHL-deficient ccRCC cells may require enzymes involved in glycogen breakdown to balance enhanced GYS1 activity for metabolic homeostasis.

To investigate this further, glycogen catabolism (glycogenolysis) was functionally examined in ccRCC (Fig. 3a). Glycogen phosphorylase is the rate-limiting enzyme for glycogen degradation and...
Fig. 2 | Elevated levels of the glycogen synthesis enzyme GYS1 in ccRCC tumours does not affect proliferation in vitro. 

a, Schematic model of the simplified glycogen metabolism pathway and hypothetical effect of GYS1 blockade on glycogen and free glucose. 
b, Normalized RNA-seq reads of GYS1 in stage-stratified ccRCC (n = 428) and normal kidney (n = 66) samples; n denotes biologically independent human tissue samples. RNA-seq data were obtained from the TCGA. Box plots (minimum to maximum all points): centre, median; boundaries, 25th and 75th percentiles; whiskers, 5th and 95th percentiles; centre line, median. 
c, RT-qPCR (left) and immunoblots (right) of GYS1 in matched ccRCC and normal kidney samples; n = 20 biologically independent human ccRCC tumour/normal paired samples. For RT-qPCR, TBP and ACTB were utilized as endogenous control genes. Relative mRNA expression was determined by normalizing to expression in normal tissues. 
d, UMRC2 ccRCC cells transduced with two independent sgRNAs against GYS1 (sg1 and sg3) or a control sgRNA (sgC). Western blot analysis was performed 7 d after virus infection to assess GYS1 expression. 
e, Glycogen content measured in cells described in d on day 14 after virus infection; n = 3 technical replicates as an example of reproducible experiments. Data are presented as the mean ± s.d. Relative glycogen amount was determined by normalizing to glycogen level in sgC cells. 
f, Growth curves for the cells described in d cultured in medium containing 1% FCS combined with the indicated glucose and oxygen concentrations; n = 6 biologically independent cell populations. Data are presented as the mean ± s.e.m. Relative absorbance was determined by normalizing to values at day 0. 
g, Representative images acquired at ×40 magnification and relative volumes of spheroids formed by the cells described in d after 19 d of culture; n = 24 biologically independent spheroids. Data are presented as the mean ± s.e.m. Relative volume was determined by normalizing to that of the sgC spheroids. The numbers denote the average relative volumes. P values were determined by two-tailed Student’s t-test. 

Data are presented as mean values ± s.e.m.
comprises three isoforms in mammals: liver (PYGL); muscle (PYGM); and brain (PYGB). As shown in Fig. 1e, PYGL and PYGM mRNA abundance was elevated in ccRCC. Since PYGM expression at baseline was much lower than PYGL, PYGL was evaluated initially. Like those of GYS1, PYGL mRNA levels were amplified in all tumour stages (Fig. 3b). To genetically block glycogenolysis, two independent sgRNAs were pooled to target PYGL, producing a nearly complete loss of PYGL protein (Extended Data Fig. 4a,b). Because glycogen was depleted in vitro within 12h after glucose deprivation (Extended Data Fig. 4c), glycogen levels were measured at 6h in glucose-free medium to characterize the functional consequence of PYGL protein reduction. This time point allows cells to engage glycogenolysis without complete depletion before glycogen could be collected. PYGL knockout did not protect glycogen from degradation during this time frame (Extended Data Fig. 4d), suggesting that these cells either did not utilize PYGL to mobilize glycogen under glucose-deprived conditions or there is redundancy in this process. In agreement with our finding, Favaro et al. showed that in the GBM cell line U87, PYGL knockdown did not increase glycogen content under normoxia. However, they obtained the opposite result under hypoxia, where glycogen accumulated and proliferation decreased in shPYGL cells. To examine whether this could be recapitulated in ccRCC cells, PYGL WT/knockout cells were subjected to hypoxia treatment (0.5% O₂). In ccRCC cells, unlike U87 cells, PYGL depletion did not accumulate more glycogen under hypoxia (Extended Data Fig. 4e). In addition, there was no difference in cell growth between WT and PYGL knockout cells when cultured in any condition tested (replete, low-serum, low-glucose, low-oxygen) (Extended Data Fig. 4f). Taken together, these data suggest that although PYGL expression is upregulated in ccRCC, it is not necessary for glycogenolysis, nor is it required for in vitro cell growth.

Since PYGL was insufficient to control glycogen breakdown, other isoforms were analysed for functional redundancy. Although PYGB expression was downregulated in ccRCC tumours, it had comparable baseline mRNA levels as PYGL (Fig. 1e). Neither genetic knockout nor constitutive overexpression of PYGB protein disrupted proliferation of UMRC2 under metabolic stress conditions (Extended Data Fig. 4g,h). On reduction of PYGB protein, PYGL protein levels were increased, suggesting possible compensation (Extended Data Fig. 4g). Consistent with TCGA patient data, PYGM mRNA abundance was very low compared to the other two isoforms in UMRC2 cells (Extended Data Fig. 4i) and PYGM was not evaluated further. Therefore, PYGL and PYGB were simultaneously depleted with sgRNAs to avoid compensation from either enzyme (Fig. 3c and Extended Data Fig. 5a). Notably, PYGB/L double knockout completely prevented glycogenolysis under glucose deprivation, suggesting that both isoforms are active in ccRCC (Fig. 3d and Extended Data Fig. 5b). However, PYGB/L double knockout cells did not exhibit higher glycogen content than WT cells under hypoxia, despite a trend towards increased deposition of glycogen with low oxygen exposure (Extended Data Fig. 5c). This result suggests that ccRCC cells do not use any glycogen-derived glucose under hypoxic conditions, perhaps due to the increased uptake of extracellular glucose downstream of even greater hypoxia-mediated HIF stabilization.

In addition, PYGB/L double knockout did not affect cell proliferation under any conditions (Fig. 3e and Extended Data Fig. 5d). To verify this result independently, a selective glycogen phosphorylase inhibitor (GPI) was employed. GPI dose-dependently maintained glycogen content in glucose-free culture condition (Fig. 3f and Extended Data Fig. 5e); during long-term treatment, GPI blocked glycogen degradation when cells were cultured in low-glucose (2mM) or no glucose (0mM) conditions, regardless of oxygenation (Fig. 3g and Extended Data Fig. 5f). Consistent with PYGB/L double knockout, GPI treatment did not affect in vitro ccRCC cell growth (Fig. 3h and Extended Data Fig. 5g). These findings indicate that ccRCC tumour cells do not rely on glycogen breakdown for growth.

Despite no observable role in maintaining ccRCC viability, glycogen is rapidly broken down under low-glucose conditions (Extended Data Fig. 4c), suggesting that glycogen-derived glucose is metabolized in some way. Therefore, glycogen was labelled with [U-13C] glucose and then either allowed to or prevented from breakdown to specifically determine how glycogen-derived glucose was utilized (Fig. 3i and Extended Data Fig. 6a,b). As proof of principle, the percentage of intracellular labelled glucose relative to unlabelled glucose was greater in cells that can properly catabolize glycogen under low-glucose conditions (Extended Data Fig. 6c). The mass spectrometry results indicated that glycogen-derived glucose was diffusely processed into various metabolites (Extended Data Fig. 6d) but perhaps more concentrated in glycolytic intermediates and serine rather than the tricarboxylic acid cycle (Fig. 3i and Extended Data Fig. 6d).

Due to increased glyceraldehyde-3-phosphate (G3P), serine/glycine, and very slightly ribose-5-phosphate labelling, additional experiments were designed to test potential proliferation defects in cells that lacked glycogenolysis under nutrient conditions pertaining to these metabolites. Aside from the obvious role of G3P in glycolysis, G3P can also be catalysed to generate glycerol, which is necessary for lipid synthesis. Serine/glycine and the pentose phosphate pathway (PPP) both produce NAPDH, also needed for lipid synthesis. Therefore, UMRC2 control or PYGB/L double knockout cells were subjected to lipid starvation and assessed for relative cell numbers over 7d. Although UMRC2 cells typically grow poorly without an exogenous source of lipids, this was exacerbated somewhat in cells that cannot break down glycogen (Fig. 3i) (see below for further discussion).

To evaluate whether these metabolic perturbations could also affect other cellular functions aside from growth, such as migration, Boyden chamber assays were performed using control or PYGB/L

Fig. 3 | ccRCC tumour cells do not rely on glycogen breakdown for growth in vitro despite glycolytic entry of glycogen-derived glucose. a, Hypothetical effect of PYGL liver/muscle/brain blockade on glycogen breakdown. b, Normalized TCGA RNA-seq reads of PYGL in stage-stratified ccRCC (n=428 tumours) and normal kidney (n=66 tissue) samples. P values were determined by two-tailed Student’s t-test. c, Protein assessment comparing WT UMRC2 cells and PYGL knockout UMRC2 cells described in Extended Data Fig. 4b transduced with a control sgRNA against LacZ (sgC) or two combined sgRNAs targeting PYGB (sgPYGB/L), respectively. d, Glycogen quantification of the cells described in c cultured in 25mM or 0mM of glucose for 6h (normalized to sgC in 25mM of glucose). e, Growth curves for the cells described in c cultured in the indicated conditions; n=6. Normalized to day 0. f, Glycogen quantification of UMRC2 cells cultured in 25mM (blue) or 0mM (red) of glucose, treated with the indicated concentrations of DMSO or GPI for 6h. Normalized to 25mM of glucose. g, Glycogen quantification of UMRC2 cells cultured in 25mM, 2mM or 0mM of glucose and treated with DMSO (blue) or 5μM of GPI (red) in 21% O₂ for 48h. Normalized to 25mM of glucose condition plus DMSO. h, Growth curves for UMRC2 parental cells treated with DMSO, 2.5μM or 5μM of GPI and cultured in the indicated conditions; n=6 biologically independent cell populations. Normalized to day 0.1. Top: schematic of uniformly 13C-labelled ((13C)3) glucose release from glycogen. Bottom: fold change in APE of key glycolytic metabolites. Normalized to 0h; n=3. G3P = 3: glyceraldehyde-3-phosphate with three 13C carbons. LAC = 3: lactate with three 13C carbons. GPI concentration was 10μM. i, A two-way ANOVA with Sidak’s multiple comparison test was used to determine significance. For all glycogen measurements, data from n=3 technical replicates are presented as the mean ± s.d. for all growth curves, data from biologically independent cell populations are presented as the mean ± s.e.m.

LETTERS | NATURE METABOLISM | VOL 3 | MARCH 2021 | 327–336 | www.nature.com/natmetabolism
double knockout UMRC2 cells. Similarly to the lack of a proliferation phenotype, no observable change in migration was detected between cells that can or cannot engage in glycogenolysis (Extended Data Fig. 7a,b).
Lastly, to determine whether glycogen metabolism is important for ccRCC tumour progression in vivo, we systematically transplanted our UMRC2 models into both flanks of nude mice. For glycogen synthesis, no meaningful difference was evident between the growth of GYS1 knockout and WT tumours (Fig. 4a), despite robust glycogen and GYS1 protein depletion (Fig. 4b,c). Similarly,
for glycogen breakdown, no consistent, statistically significant effects on tumour volume or weight were observed when both PYGL and PYGB were effectively eliminated; glycogen levels were either maintained or slightly elevated in PYGB/L double knockout tumours (Fig. 4d–f). Collectively, these results indicate that neither glycogen accumulation nor homeostasis plays a major role in ccRCC tumour models.

In this study, we demonstrated that glycogen is largely unnecessary for ccRCC cell growth in a variety of models and stress conditions, clarifying an important clinical distinction for a classical biological feature of the disease. Only under low oxygen and low lipid conditions did inhibition of glycogen breakdown appear to modestly reduce cell growth. One explanation for this phenotype could be that ccRCC cells metabolize glycogen-derived glucose in pathways important for lipid synthesis, which is why no phenotype was observed in lipid-replete culture conditions. It is perplexing why 25 mM of exogenous glucose could not compensate for the lack of glycogen-derived glucose during lipid starvation. Nevertheless, since this phenotype was not observed in transplantable in vivo models, it is likely that overall deregulated glycogen metabolism in ccRCC is a side effect of constitutive HIF-1α signalling with minimal biological importance under conventional stress conditions (that is, low-glucose, low-oxygen). In other cancer types, such as GBM31 and NSCLC31, data exist supporting a pro-tumorigenic role of glycogen. Whether these tumour microenvironments have reduced fatty acid or triglyceride availability is unknown. Therefore, a key unanswered question is how cancer cells of distinct metabolic dependencies and microenvironments utilize glycogen. In our ccRCC models, we reproducibly observed glycogen degradation in low glucose and, conversely, glycogen retention in PYGB/L double knockout cells exposed to glucose deprivation. This indicates that ccRCC cells mobilize glycogen-derived glucose for some metabolic processes. Based on the glycogen labelling experiment, potential dependencies and microenvironments utilize glycogen. In our ccRCC models, we reproducibly observed glycogen degradation in low glucose and, conversely, glycogen retention in PYGB/L double knockout cells exposed to glucose deprivation. This indicates that ccRCC cells mobilize glycogen-derived glucose for some metabolic processes. Based on the glycogen labelling experiment, potential metabolic pathways include glycolysis, serine/glycine biosynthesis and the PPP. More in-depth metabolite analyses, such as antioxidant readouts or lipodomics, could clarify and connect a metabolic role of glycogen to a specific stress condition. In vivo models of PYGB/L double knockout tumour growth in either a low- or high-fat diet mimic changes in serum lipids, or with antiangiogenic treatment to intensify a hypoxic tumour microenvironment, could also be examined.

Future work on glycogen in ccRCC could identify distinct destinations of glycogen-derived glucose under specific culture conditions. This may reveal synthetic lethality approaches between GYS1 or PYGB/L inhibition, tailored to a patient’s unique tumour metabolism. For example, tumours addicted to glutamine, fatty acid or acetate availability may become vulnerable when glycogen cannot feed G1P into the central carbon pathway25–27. Additionally, it has recently been described that glycogen breakdown in the nucleus can regulate gene expression by altering histone acetylation in NSCLC31. Whether or not nuclear glycogen impacts the epigenetic landscape, or if the epigenetic profile itself can alleviate any proliferation defects from the loss of glycogen metabolism in ccRCC, has yet to be explored. Another avenue of synthetic lethality could be in serum/glycine biosynthesis (Extended Data Fig. 6d). Nevertheless, we suggest that elevated glycogen content in the tumours of patients with ccRCC should not be considered a therapeutic target on its own.

Methods

Primary patient samples. De-identified fresh-frozen matched ccRCC tumour/normal samples were obtained from the Cooperative Human Tissue Network, which operates with the review and approval of local institutional review boards.

Cell culture and cell proliferation assays. Human ccRCC cell lines (UMRC2, 786-O, RCC4 and UOK101) were obtained from the ATCC and cultured in DMEM supplemented with 10% FCS and penicillin-streptomycin. All cells were routinely confirmed as Mycoplasma-negative (MycoAlert; tested every three months). For the culture conditions with various glucose concentrations, cells were maintained in glucose-free DMEM supplemented with 10% dialysed FCS (catalogue no. 100-108;Gemini Bio) and the indicated concentrations of glucose. Hypoxic conditions (0.5% and 1% O2) were achieved in a Baker Ruskinn InVivo2 work station by supplementing ambient air with balanced N2 and CO2. For the lipid depletion experiments, cells were cultured in charcoal-stripped FCS (catalogue no. 900-123; Gemini Bio). Cell proliferation assays were performed using the WST-1 reagent (catalogue no. 501594-9601; Sigma–Aldrich) cells were plated in 2-well plates at 800–1,500 cells per well and allowed to attach overnight. The medium was changed according to the indicated culture conditions on the following day, which was considered as day 0. Cells were subjected to the WST-1 assay according to the manufacturer’s protocol on each day. The cell proliferation rate was represented by relative absorbance, which was determined by normalizing to the absorbance number at day 0 of the assay. Additional proliferation assays were performed using Trypan Blue exclusion and labelled as total live cells on the y axis (Fig. 3j and Extended Data Fig. 4h, upper panel). Cells were plated in 6-well plates at 50,000–100,000 cells per well and allowed to adhere overnight. The next day, cells were given fresh medium and cultured under the experimental conditions. At each time point, cells were trypsinized and resuspended in FCS-containing medium.

The cell/Trypan Blue mixtures were counted by a Countess II (Thermo Fisher Scientific) and corrected for dilutions.

Boyd en chamber cell migration assay. A total of 50,000 UMR2C cells were seeded in 0.1 ml of 0% FCS, DMEM (0 mM of glucose) per transwell polycarbonate insert with 8-μm pores (catalogue no. 3422; Corning). Approximately 0% or 10% FCS DMEM (25 mM of glucose) was added to each bottom well. After overnight incubation, inserts were washed with PBS and then gently scraped with a cotton swab (topside only). Next, membrane inserts were incubated in approximately 0.6 ml of 0.5% Crystal Violet (catalogue no. C6158; Sigma–Aldrich) in 20% methanol for 10 min. Transwells were washed twice with PBS and allowed to dry overnight. Membrane inserts were cut out and mounted on a microscope slide for imaging.

Plasmids, lentivirus production and viral transduction. For shRNAs, the lentiviral vector pLKO.1 SCR (scrambled shRNA plasmid no. 17920) was obtained from Addgene. shRNAs targeting HIF1A (shHIF1A_52, shHIF1A_9) and EPAS1 (shEPAS1_6, shEPAS1_7) were previously described25. For genetic knockout using CRISPR–Cas9, the lentivector lentivector LentiCRISPR v2 (plasmid no. 52961) and LentiCRISPRv2 GFP (plasmid no. 82416) were obtained from Addgene.

Human sgRNAs targeting GYS1 no. 1 (GAAGAGCTGATGCTCCATCTTCTCGAGG) and no. 3 (CTCTACAGGTGTCGAGACGCACTGGA), and PYGL no. 1 (GAGCCAGCCTCTCGACATGTCTTCTC) and no. 3 (TAGCCAGCGGCCTGCTCCAGAC) and no. 4 (GAGGACCGGGAGAGATGTTCTCT) along with a control sgRNA targeting the mouse Rosas26 locus (AAGATGGGGGGATGGTCTT) were cloned into the LentiCRISPR v2 plasmid, while sgRNAs targeting PYGB no. 1 (CAACGTGGAAGACGACGAC) and no. 4 (CCACCCTGTGCGTGCTCACC) along with a control sgRNA targeting a mouse IgG control (TCTTGTTACGTAAGCTTCT) were cloned into the LentiCRISPR v2 GFP plasmid. To produce lentiviruses, 293T cells were cotransfected with srRNA or the CRISPR plasmid of interest along with the packaging plasmids pSPAX2 and pMD2.G using FuGENE 6 Transfection Reagent (catalogue no. E2691; Promega Corporation). Lentiviruses were collected 48 h after transfection. Viruses were used with 8 μg/ml of polybrene for infection.

For the LentiCRISPR v2 system, cells were selected with 4 μg/ml of puromycin for 5 d to establish stable cell lines; for the LentiCRISPR v2 GFP system, the top 50% green fluorescent protein-positive cells were sorted for future culture and analysis. For the PYGB overexpression analyses, 25 μl of the pre-made, constitutive expression lentiviral vector pLOC–PYGB (catalogue no. OH58899-202619959; Dharmacon), were added to 1.5 ml of UMR2C cells. Cells were selected with 5 μg/ml of blasticidin for 4 d before subsequent experimentation.

Western blot analysis. Cells and tumour tissue samples were lysed in radioimmunoprecipitation assay lysis and extraction buffer (catalogue no. 89900; Thermo Fisher Scientific) containing Roche Complete protease/phosphatase inhibitor (catalogue no. 0592791001). Protein concentration was quantified with the Pierce BCA Protein Assay Kit (catalogue no. 23225; Thermo Fisher Scientific). Isolated proteins were resolved by SDS–polyacrylamide gel electrophoresis and western blot analysis was performed. All primary antibodies were diluted 1:1,000 in 5% w/v non-fat milk. Blots were incubated with primary antibodies overnight at 4°C. GYS1 (catalogue no. ab40810) and PYGL (catalogue no. ab3280) antibodies were from Abcam; the HIF-1α antibody was obtained from Cayman (catalogue no. 10006421); the HIF-2α antibody was obtained from Novus Biologicals (catalogue no. NB100-122); the GAPDH antibody was obtained from Cell Signaling Technology (catalogue no. 2118); the PYGL antibody was obtained from Sigma–Aldrich (catalogue no. HPA000962); and the PYGB antibody was obtained from ProteinTech (catalogue no. 12075-1-AP). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (from Cell Signaling Technology catalogue no. 7074) followed by exposure to enhanced chemiluminescence substrate (catalogue no. NELE103001EA;
tumour tissues were homogenized with distilled H₂O on ice and then boiled for 5 min. Glycogen levels were measured using the Glycogen Quantification kit (Hs00958087_m1); (Hs00157863_m1); PYGB (Hs00176853_m1); EPAS1/HIF2A (Hs01026149_m1); CCND1 (Hs00765533_m1); PDK1 (Hs00176853_m1); GYS1 (Hs00157863_m1); PYGB (Hs00765686_m1); PYGL (Hs00958087_m1); PGM1 (Hs00989942_m1); and RNA18S/45S (Hs03929895_g1).

Glycogen quantification. Glycogen levels were measured using the Glycogen Assay Kit II (Colorimetric) from Abcam (catalogue no. ab169558). Briefly, cells or tumour tissues were homogenized with distilled H₂O on ice and then boiled for 10 min. Homogenates were then spun at 13,000 r.p.m. for 10 min and supernatants were assayed for glycogen content. Results were normalized by protein content.

Matrigel-based spheroid growth assay. The Matrigel-based three-dimensional spheroid formation technique was described previously14. Briefly, 3,000 cells per well were plated in a 96-well ultra-low attachment plate (catalogue no. CLS3474; Sigma-Aldrich) along with DMEM plus 10% FCS and 2.5% Matrigel (catalogue no. 354230; Corning). Plates were centrifuged at 1,500 r.p.m. to promote spheroid formation and then imaged at the indicated time points using the EVOS FL Cell Imaging System. Final pictures were taken at ×40 magnification. Spheroid volume was calculated using a previously published ImageJ 1.53a (NIH) macro15.

Metabolites and glycogen tracing. Mass spectrometry-based metabolomics analysis of primary ccRCC was performed with Metabolon (Extended Data Fig. 1b), as described previously16. In addition, publicly available metabolomics data from Hakimi et al.17 were downloaded and values normalized to normal kidney tissue (Fig. 1c). For the glycogen tracing experiment, cells were seeded at 50–70% confluence in 15-cm dishes (day 0). The following day (day 1), the medium was changed to 1% dialysed FCS, 0 mM of glucose DMEM, to deplete unlabelled glycogen stores, and incubated at 1% O₂ overnight. The next day (day 2), 25 mM of uniformly labelled 13C ([U-13C]) glucose (catalogue no. CLM-1396-1; Cambridge Isotope Laboratories) was added to the cells to regenerate glycogen with labelled glucose and cultured overnight at 1% O₂. The following day (day 3), the medium was aspirated and cells were washed twice with 1× PBS to remove labelled glucose. Cells were then incubated at 1% O₂ overnight in fresh 1% dialysed FCS, 0 mM of glucose DMEM with 10 μM of GPI to prevent the breakdown of labelled glycogen. On the last day (day 4), the medium was changed to either dimethyl sulfoxide (DMSO) control or 10 μM of GPI in fresh 1% dialysed FCS, 0 mM of glucose DMEM and cultured for 0, 3 or 6 h. At these time points, cell metabolites were extracted by (1) aspirating the medium, (2) washing twice with cold PBS, (3) adding 90 μl of 4% perchloric acid and (4) transferring the semi-frozen supernatant to microtube flasks. All samples were submitted to the Metabolomics Core at the Children's Hospital of Philadelphia for liquid chromatography–mass spectrometry analysis. Data were normalized to internal metabolite controls and are presented as atom percentage excess (APE) to describe the percentage of a 13C-labelled metabolite relative to its unlabelled form.

Periodic acid–Schiff staining. Glycogen was detected in tumour sections after a standardized periodic acid–Schiff staining technique, which was performed by the Molecular Pathology & Imaging Core at the University of Pennsylvania.

Mice and xenograft experiments. The xenograft tumour experiments were approved by the Animal Care and Use Committee at the University of Pennsylvania. A total of 200 μl of 5 million UMRC2 control or knockout cells were injected subcutaneously into opposing flanks of the same 4–6-week-old female NIH/IL-6 mice (purchased from Charles River Laboratories), in a 1:1 mixture of DMEM and Matrigel. Once palpable tumours were established, tumour volume was monitored by calliper measurements. On completion of the experiment, the animals were killed by CO₂ inhalation, which was followed by cervical dislocation; xenograft tumours were dissected for downstream analyses.
18. Zois, C. E. & Harris, A. L. Glycogen metabolism has a key role in the cancer microenvironment and provides new targets for cancer therapy. J. Mol. Med. (Berl.) 94, 137–154 (2016).
19. Munro, S., Ceulemans, H., Bollen, M., DiPleseco, J. & Cohen, P. T. W. A novel glycogen-targeting subunit of protein phosphatase 1 that is regulated by insulin and shows differential tissue distribution in humans and rodents. FEBS J. 272, 1478–1489 (2005).
20. Shen, G.-M., Zhang, F.-L., Liu, X.-L. & Zhang, J.-W. Hypoxia-inducible factor 1-mediated regulation of PPP1R3C promotes glycogen accumulation in human MCF-7 cells under hypoxia. FEBS Lett. 584, 4366–4372 (2010).
21. Yang, R. et al. Loss of protein targeting to glycogen sensitizes human hepatocellular carcinoma cells towards glucose deprivation mediated oxidative stress and cell death. BioSci. Rep. 35, e00207 (2015).
22. Jurczak, M. J. et al. Transgenic overexpression of protein targeting to glycogen markedly increases adipocytic glycogen storage in mice. Am. J. Physiol. Endocrinol. Metab. 292, E952–E963 (2007).
23. Greenberg, C. C., Meredith, K. N., Yan, L. & Brady, M. J. Protein targeting to glycogen overexpression results in the specific enhancement of glycogen storage in 3T3-L1 adipocytes. J. Biol. Chem. 278, 30835–30842 (2003).
24. Bhanot, H. et al. Pathological glycogenesis through glycogen synthase 1 and suppression of excessive AMP kinase activity in myeloid leukemia cells. Leukemia 29, 1555–1563 (2015).
25. Varnier, M., Leese, G. P., Thompson, J. & Rennie, M. J. Stimulatory effect of glutamine on glycogen accumulation in human skeletal muscle. Am. J. Physiol. 269, E309–E315 (1995).
26. Lundsgaard, A.-M., Fritzen, A. M. & Kiens, B. Molecular regulation of fatty acid oxidation in skeletal muscle during aerobic exercise. Trends Endocrinol. Metab. 29, 18–30 (2018).
27. Hardin, C. D. & Roberts, T. M. Differential regulation of glucose and glycogen metabolism in vascular smooth muscle by exogenous substrates. J. Mol. Cell. Cardiol. 29, 1207–1216 (1997).
28. Vinci, M. et al. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. BMC Biol. 10, 29 (2012).
29. Ivanov, D. P. et al. Multiplexing spheroid volume, resazurin and acid phosphatase viability assays for high-throughput screening of tumour spheroids and stem cell neurospheres. PLoS ONE 9, e103817 (2014).

Acknowledgements
We thank the past and present members of the Simon laboratory for their helpful discussions on the project. We are grateful to Y. Daikin, O. Horyn and I. Nissim (Metabolomics Core Facility, Children’s Hospital of Philadelphia) for the glycogen tracing measurements. This work was supported by a National Institutes of Health National Research Service Award (no. F31CA239514-01 to J.G.) and National Cancer Institute grant nos. P01CA104838 and R35CA220483 to M.C.S.

Author contributions
H.X., J.S. and M.C.S. conceived the project and designed the experiments. H.X. and J.S. performed most of the experiments described. J.G., R.R. and N.S. helped with the final xenograft and revision work. I.N. performed the mass spectrometry analysis on the glycogen labelling experiment. H.X., J.G. and M.C.S. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s42255-021-00367-x.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42255-021-00367-x.

Correspondence and requests for materials should be addressed to M.C.S.

Peer review information Nature Metabolism thanks Adrian Harris, Scott Welford and the other, anonymous, reviewers for their contribution to the peer review of this work.

Primary Handling Editor: George Caputa.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2021
Extended Data Fig. 1 | Glycogen synthesis and breakdown are hyperactive in ccRCC tumors. Glycogen synthesis and breakdown are hyperactive in ccRCC tumors. a, Glycogen quantification of six ccRCC cell lines in replete conditions (10% FBS, 25 mM glucose RPMI) normalized to protein mass; n = 3 technical replicates. Data presented as mean ± SD. ‘H2’: cell lines exclusively expressing HIF-2α. ‘H1H2’: cell lines expressing both HIF-1α and HIF-2α. b, Abundance of glycogen metabolism-related metabolites (glucose-1-phosphate, maltose, maltotriose, and maltotetraose) in n = 138 biologically independent human ccRCC tumor/normal paired samples; data extracted from Hakimi AA, et al16. Data presented as mean ± SEM. c, qRT-PCR of GYS1, PYGB, PYGL, and PYGM in 20 matched ccRCC and adjacent normal kidney tissues; n = 3 technical replicates per tissue sample. Data presented as mean ± SD. Ribosomal subunit 45S RNA (45S) utilized as the endogenous control gene. P values determined by two-tailed Student’s t-test. ***, P < 0.001.
Extended Data Fig. 2 | Glycogen synthesis enzyme GYS1 is overrepresented in ccRCC and regulated by HIF-1α. Glycogen synthesis enzyme GYS1 is overrepresented in ccRCC and regulated by HIF-1α. a, UMRC2 and RCC4 (H1H2) ccRCC cells transduced with two independent shRNAs against HIF1A (shHIF1A_52 and shHIF1A_9), EPAS1 (shHIF2A_6 and shHIF2A_7), or a SCR (scrambled shRNA) control. qRT-PCR and Western blot for GYS1 shown. PDK1 and CCND1 included as positive controls for HIF-1α and HIF-2α suppression, respectively. For qRT-PCR, TBP and ACTB utilized as endogenous control genes, and relative mRNA expression determined by normalizing to expression in SCR samples; n = 3 technical replicates. Data are presented as mean ± SD. b, Glycogen quantification in UMRC2 cells transduced with indicated shRNAs after 4 days; n = 3 technical replicates. Data are presented as mean ± SD. Relative glycogen amount determined by normalizing to levels in SCR samples. c, EPAS1 (HIF-2A) depleted by two independent shRNAs (shHIF2A_6 and shHIF2A_7) in 786-O (H2) ccRCC cells, GYS1 expression shown by qRT-PCR; n = 3 technical replicates. Data are presented as mean ± SD. d, Schematic representation of GYS regulation by PP1 and PPP1R3 (see text for details). e, Normalized RNA-seq reads of PPP1R3B and PPP1R3C in stage-stratified ccRCC (n = 428) and normal kidney (n = 66) samples; n denotes biologically independent human tissue samples. RNA-seq data obtained from TCGA. Box plots (min. to max. all points): center = median, bounds = 25th and 75th percentiles, whiskers = 5th and 95th percentiles. P values determined by two-tailed Student’s t test. ***, P < 0.001.
Extended Data Fig. 3 | Glycogen is dispensable for ccRCC cell growth in vitro. Glycogen is dispensable for ccRCC cell growth in vitro. a, RCC4, UOK101, and 786-O ccRCC cells transduced with two independent sgRNAs against GYS1 (sg1 and sg3) or a control sgRNA (sgC). Western blot analysis performed 7 days after virus infection to assess GYS1 expression. b, Glycogen levels measured in cells described in a on day 7 after virus infection; n = 3 technical replicates. Data presented as mean ± SD. Relative glycogen amount was determined by normalizing to glycogen level in sgC cells. c, Representative images and relative volumes of spheroids formed by 786-O cells described in a after 19 days culture; n = 24 biologically independent spheroids. Data presented as mean ± SEM. Relative volume was determined by normalizing to that of sgC spheroids. Numbers denote average relative volumes. d, Growth curves for cells described in a cultured in medium containing 1% FBS combined with indicated glucose and oxygen concentrations; n = 6 biologically independent cell populations. Data presented as mean ± SEM. Relative absorbance was determined by normalizing to values at Day 0. P values determined by two-tailed Student’s t test. ***, P < 0.001.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | PYGL is not required for glycogen breakdown and in vitro ccRCC cell growth. PYGL is not required for glycogen breakdown and in vitro ccRCC cell growth. a, b, Protein assessment of 786-O and UMRC2 ccRCC cells transduced with three independent or two pooled sgRNAs against PYGL (sg1, sg3, sg4, or sg3 + 4) or a control sgRNA (sgC). Samples collected at 7 (a) or 6,8 (b) days after lentiviral infection. c, UMRC2 cells cultured in glucose-free medium for indicated time points, glycogen extracted and quantified. Relative glycogen amount determined by normalizing to glycogen level in cells at 0 hour. d, Cells described in b cultured in medium with 25 mM glucose or starved in glucose-free medium for 6 hours, glycogen extracted and quantified. Relative glycogen amount determined by normalizing to glycogen level in sgC cells cultured in medium with 25 mM glucose. e, Cells described in b cultured in 0.5% O2 for indicated time points, glycogen extracted and quantified. Relative glycogen amount determined by normalizing to glycogen level in sgC cells cultured in 21% O2. f, Growth curves for cells described in b cultured in indicated conditions; n = 6 biologically independent cell populations. Relative absorbance determined by normalizing to values at Day 0. g, Protein assessment of pooled sgRNAs 1 + 4 targeting PYGB (sgPYGB) or overexpression of PYGB (PYGB OE), upper and bottom panels respectively. sgC: control (guide targeting LacZ); sgPYGB/L: double knockout. h, Growth assays of UMRC2 under the indicated conditions. Live cell numbers were measured by Trypan Blue exclusion, and finalized values adjusted for dilution; n = 3 biologically independent cell populations. PYGB knockout and PYGB overexpression (upper and bottom panels respectively). Parental refers to uninfected UMRC2 cells. Sidak’s multiple comparison test was used to determine significance (ns, P > 0.05; *, 0.05 < P < 0.005; **, 0.005 < P < 0.0005; ***, P < 0.0005). i, qRT-PCR on UMRC2 cells for glycogen phosphorylase isoforms; n = 3 technical replicates. Data presented as mean ± SD. Ribosomal subunit 45S RNA (45S) utilized as the endogenous control gene. For all glycogen measurements, data from n = 3 technical replicates and presented as mean ± SD. For all growth curves, data are presented as mean ± SEM. All other P values determined by two-tailed Student’s t test. ***, P < 0.001.
Extended Data Fig. 5 | ccRCC tumor cells do not rely on glycogen breakdown for growth. ccRCC tumor cells do not rely on glycogen breakdown for growth. a, WT and PYGB KO 786-O cells transduced with a control sgRNA against LacZ (sgC) or combined two sgRNAs targeting PYGB (sgPYGB/L), respectively. Top 50% GFP positive cells sorted for culture. Western blot analysis performed 14 days after virus infection to assess PYGL and PYGB expression. SE, short exposure; LE, long exposure.

b, c, d, e, f, g, UMRC2 and 786-O cells cultured in medium with 25 mM or 0 mM glucose, treated with indicated concentrations of DMSO or GPi for 6 hours. Glycogen extracted and quantified. Relative glycogen amount determined by normalizing to glycogen level in sgC cells cultured in medium with 25 mM glucose.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Glycogen-derived glucose broadly enters the central carbon pathway during glucose starvation. a, Glycogen labeling experimental design. [U-13C]: uniformly labeled heavy carbon (13C); GPI: glycogen phosphorylase inhibitor. Small blue or red circles denote free, unlabeled or labeled glucose, respectively. Large blue or red undefined shapes denote unlabeled or labeled glycogen, respectively. b, Sample collection scheme for metabolomic analysis following labeled glycogen breakdown or retention. c, Percentage of labeled glucose (Glucose M + 6) relative to unlabeled glucose in cells over 6 hours of glycogen breakdown or retention; n = 3 biologically independent cell populations. d, Fold change in APE relative to time 0 for indicated metabolites after 3 hours (upper panel) and 6 hours (bottom panel) of glycogen breakdown or retention; n = 3 biologically independent cell populations. For all metabolite measurements, data presented as mean ± SEM. CIT: citrate; SUCC: succinate; FUM: fumarate; MAL: malate; ASP: aspartate; GLU: glutamate; ALA: alanine; GLY: glycine; SER: serine; R5P: ribose-5-phosphate. (+1,2,3 denotes number of 13C carbons). Sidak’s multiple comparison test was used to determine significance (ns, P > 0.05; *, 0.05 < P < 0.005; **, 0.005 < P < 0.0005; ***, P < 0.0005).
Extended Data Fig. 7 | Glycogen availability does not alter cell migration. Glycogen availability does not alter cell migration. **a**, Representative field image of crystal violet-stained UMRC2 cells under the specified conditions and genetic alterations. Scale bar=200 μm. **b**, Quantification of cell migration calculated as average cell number per field; n=3 biologically independent cell populations (average count of 4 center-oriented fields per sample). Data presented as mean ± SEM. sgC: UMRC2 control sgLacZ; sgPYGB/L: UMRC2 PYGB/L double knockout. Sidak’s multiple comparison test was used to determine significance (ns, P > 0.05; *, 0.05 < P < 0.005; **, 0.005 < P < 0.0005; ***, P < 0.0005).
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
No software was used for data collection

Data analysis
Prism 7, 8, and 9 Graphpad, Adobe Photoshop and Illustrator 2020

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

(1) TCGA ccRCC RNAseq dataset: https://www.cabiportal.org. (2) Hakimi, et al. (2016) ccRCC metabolomics dataset: https://www.cell.com/cancer-cell/comments/S1535-6108%2815%2900458-7#secsectitle0145

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were chosen based on our previous work in ccRCC [Li, et al. 2014; Qiu, et al. 2015], where we found statistically significant differences in experiments performed |
| Data exclusions | None |
| Replication | Each cell and tumor proliferation experiment had at least 3 biological replicates. Each qPCR and glycogen quantification assays were performed with technical replicates. All these experiments were performed twice across multiple authors. Results were largely similar across all experiments, with some variation from experiment to experiment. Additionally, multiple human tumor samples and independent renal cancer cell lines were evaluated to ensure data reproducibility |
| Randomization | Mice were randomized according to control or genetic depletion of the genes of interest (GY51, PYG1/B). For in vitro experiments, cells were plated evenly across appropriate number of wells and then assigned conditions. Usually, wells at the top of the plate were assigned the vehicle or replete condition, followed by experimental conditions going down. Since cell populations should be fairly homogeneous, compared to tumors, this allocation seemed reasonable. |
| Blinding | Tumor measurements were performed in a blinded fashion. In vitro experiments were not blinded which we believe was not necessary due to the relative homogeneity of cells in established cell lines. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| □ | Antibodies |
| □ | Eukaryotic cell lines |
| ☒ | Palaeontology |
| □ | Animals and other organisms |
| □ | Human research participants |
| ☒ | Clinical data |

### Methods

| n/a | Involved in the study |
| --- | --- |
| ☒ | ChIP-seq |
| □ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

GY51 (AbCam, cat. ab40810), Beta-Actin (AbCam, cat. ab53280), HIF1-alpha (Cayman, cat. 10006421), HIF2-alpha (Novus Biologicals, cat. NB-100-122), GAPDH (Cell Signaling Technology, cat. 2118), PYG1 (Sigma, cat. HPA000962), PYG8 (ProteinTech, cat. 12075-1-AP), HRG-linked antibody (Cell Signaling Technology, cat. 7074). All antibodies were used at a 1:1000 dilution.

**Validation**

Validation was performed by the manufacturer. For GY51, validated by using a knockout line. For Beta-Actin, product discontinued and no validation was found. For HIF1-alpha, validated in CoCl2-treated cells. For HIF2-alpha, validated by biological, genetic, and orthogonal strategies (including culturing cells in hypoxia). For GAPDH and HRG-linked antibody, validated through strategies outlined in the Hallmarks of Antibody Validation. For PYG1, validated by independent and orthogonal RNAseq strategies. For PYG8, validated by immunoprecipitation of brain extracts.

### Eukaryotic cell lines

**Policy information about:** cell lines

**Cell line source(s)**

All cell lines were obtained from the American Type Culture Collection: UMRC2, RCC4, RCC10, 786-0, UOK101, 769P.

**Authentication**

ATCC cell lines are routinely authenticated by short tandem repeat analyses.

**Mycoplasma contamination**

All cell lines tested negative for mycoplasma.

**Commonly misidentified lines (See CTAC register)**

No commonly misidentified cell lines were used.
# Animals and other organisms

Policy information about [studies involving animals](https:// ARRIVE guidelines) recommended for reporting animal research.

| Category                  | Information                                                                 |
|---------------------------|-----------------------------------------------------------------------------|
| Laboratory animals        | NIH-III nude mice, 4-6 week old, female (strain code 201, Charles River)    |
| Wild animals              | No wild animals used in this study.                                         |
| Field-collected samples   | No samples collected from the field.                                        |
| Ethics oversight          | Mouse experiments approved by the Animal Care and Use Committee at the University of Pennsylvania |

Note that full information on the approval of the study protocol must also be provided in the manuscript.