Published in final edited form as:

Gastroenterology. 2010 January ; 138(1): 241. doi:10.1053/j.gastro.2009.08.064.

The hormone receptor GUCY2C suppresses intestinal tumor formation by inhibiting AKT signaling

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Abstract

Background & Aims—Gucy2c is the intestinal cell receptor for the paracrine hormones guanylin and uroguanylin that converts GTP to cyclic (c)GMP. It functions as a tumor suppressor; its loss

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Disclosure

SAW is a paid consultant to Merck, and the Chair (uncompensated) of the Scientific Advisory Board of Targeted Diagnostics and Therapeutics, Inc., which provided research funding that, in part, supported this work and which has a license to commercialize inventions related to this work. GMP receives research salary support from Merck. He is an inventor on patents which include information contained in this manuscript. All the other authors have no conflict of interest to disclose.

disrupts intestinal homeostasis and promotes tumorigenesis. We investigated the effects of *Gucy2c* loss on intestinal cell proliferation, metabolism, signaling, and tumorigenesis in mice.

Methods—Intestinal cell proliferation and metabolism were examined in *Gucy2c*^{−/−} and wild-type mice and human colon cancer cells by microscopy, immunoblot, and functional analyses. AKT regulation and signaling were examined and the role of AKT in *Gucy2c*-dependent tumorigenesis was defined in *Gucy2c*^{−/−}*Akt1*^{−/−} mice. Microarray analyses compared gene expression profiles of intestine of *Gucy2c*^{−/−} and wild-type mice.

Results—The size and number of intestinal crypts increased in *Gucy2c*^{−/−} mice; the associated epithelial cells exhibited accelerated proliferation, increased glycolysis, and reduced oxidative phosphorylation, which was reversed by oral administration of cGMP. Conversely, activating GUCY2C in human colon cancer cells delayed cell cycle progression (inhibiting DNA synthesis and colony formation), reduced glycolysis, and increased mitochondrial ATP production. AKT signaling pathways were activated in intestines of *Gucy2c*^{−/−} mice, associated with increased AKT phosphorylation. Disruption of AKT activity, pharmacologically or genetically, reduced DNA synthesis, proliferation, and glycolysis and increased mitochondrial biogenesis. Intestinal tumorigenesis increased following administration of azoxymethane to *Gucy2c*^{−/−} mice, compared with wild-type mice, but was eliminated in *Gucy2c*^{−/−}*Akt1*^{−/−} mice.

Conclusions—*Gucy2c* is a tumor suppressor that controls proliferation and survival of intestinal epithelial cells by inactivating AKT signaling. This receptor and its ligands, which are paracrine hormones, might be novel candidates for anti-colorectal cancer therapy.

INTRODUCTION

Disruption of developmental processes maintaining cellular homeostasis, including proliferation and metabolism, conveys an evolutionary advantage to cancer cells.^{1–4} Indeed, dysregulation of cell cycle progression and differentiation through targeted inactivation of p21 promotes APC mutation-dependent tumor formation by increasing proliferation and decreasing differentiation in intestine.⁵ Further, crypt hyperplasia, associated with disrupted differentiation, promotes intestinal tumorigenesis in mice with increased IGF2 expression⁶. Moreover, metabolic remodeling permits tumor cells to undergo energy-independent autonomous replication through intermediate metabolism, confers survival advantages under hypoxic conditions, and promotes further accumulation of mutations.⁷

GUCY2C is an intestine-specific receptor that catalyzes the conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) upon activation by the endogenous paracrine hormones guanylin and uroguanylin or the exogenous ligands, the diarrheagenic bacterial heat-stable enterotoxins (STs).^{8–13} Guanylin and uroguanylin exhibit a pattern of expression along the crypt-villus axis that is associated with the transition from proliferation to differentiation, absent in the crypt but present in the differentiated compartment.¹⁴ Additionally, these paracrine hormones are among the most commonly lost gene products in colorectal cancer, and their loss occurs early along the continuum of transformation.^{15–17} Further, elimination of GUCY2C signaling disrupts intestinal homeostasis and tumor susceptibility.^{18, 19} In order to define the functional relevance of GUCY2C to mechanisms underlying epithelial homeostasis and intestinal tumorigenesis, we performed analyses to identify signaling targets of GUCY2C in processes supporting transformation.

MATERIALS AND METHODS

For further details, please see Supplemental Experimental Procedures.

Animal models

Gucy2c^{-/-} and *Akt*^{-/-} C57BL/6 mice were bred, maintained, genotyped, and functionally characterized as described in accordance with the Thomas Jefferson University Animal Care and Use guidelines.¹⁹ Tumors were induced by azoxymethane, enumerated, and quantified as described (see supplemental procedures).¹⁹

Cell culture, siRNA transfection, and adenovirus infection

MC38 murine colon cancer cells were provided by J. Schlom (NCI, MD). A truncated mouse *Gucy2c*1-461 construct (*Gucy2c*-TM) containing extracellular ligand-binding and transmembrane domains, was generated as described.²⁰ In some experiments, cells were transfected with targeted or control siRNA (25 nM; Millipore) by Lipofectamine® RNAiMAX (Invitrogen). Adenovirus-expressing AKT1 and myr-AKT were provided by T. Chan (Thomas Jefferson University, PA). Adenovirus-expressing siAKT1 and GFP were from Millipore.

Human Tissue

Human tissues were obtained from patients undergoing surgery under a protocol approved by the IRB (control no. 01.0823).

Immunofluorescence

The anti-OxPhos complex IV subunit I antibody (Invitrogen) was used to stain mitochondria. The nuclear co-expression of pRb and cyclin D1 was quantified using anti-phospho-Rb and anti-cyclin D1 (Santa Cruz Biotechnology). Anti-hexokinase II antibody (Santa Cruz Biotechnology) was used to detect metabolic markers. DNA oxidized by ROS was quantified using anti-8-oxo-dG antibody (Trevigen). Stained tissues were quantified in 5 to 15 crypt-villus units/intestinal segment/mouse.

Immunoblot analyses

Protein was extracted from cells and tissues in M-PER reagent (Pierce) supplemented with protease and phosphatase inhibitors (Roche) and quantified by immunoblot analysis employing antibodies to: cyclin D1, β -catenin, GLUT1, PFK1, PFK2P, mtTFA, NRF1 (Santa Cruz Biotechnology); ATP5A1, COXI, Core II (Invitrogen); GLUT1, TSC1, TSC2 (Millipore); others were from Cell Signaling. Relative intensity reflects the mean of 5–15 individual animals per genotype, 12 paired human tumor and normal adjacent tissues, or ≥ 3 independent experiments with cultured cells.

Cell proliferation and crypt number

Ki-67 was used as a proliferation marker to quantify dividing cells by blinded-analysis from 5–15 crypt-villus units/segment/mouse.¹⁹ Results reflect the mean $\pm$ SEM of at least 9 animals in each group. DNA synthesis was quantified by [³H]thymidine incorporation.²¹ Crypt number was calculated from complete crypts in at least 5 transverse cross sections/intestinal segment/mouse.

Real-time PCR

Total RNA was subjected to one-step RT-PCR using TaqMan® EZ RT-PCR Core Reagents and specific primer/probes for TaqMan® Gene Expression Assays in an ABI 7000 (Applied Biosystems). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method and GAPDH or β -actin as reference.

Lactate production and glucose uptake

Lactate production and glucose uptake using ^3H -deoxyglucose (50 $\mu\text{Ci/mL}$), were quantified in isolated mouse intestinal mucosae. In cells, lactate production was quantified using a lactate measurement kit (Trinity Biotech) while glucose uptake was quantified employing 2-NBDG.

Mitochondrial function

Mitochondrial reductase activity was quantified by MTT analysis. Oxygen consumption was measured using the Oxygen Biosensor System (BD Bioscience). Mitochondria-specific ATP production was quantified using an ATP determination kit (Invitrogen). ROS production was quantified by Image-iT® LIVE Reactive Oxygen Species Kit (Invitrogen).

Microarray analyses

Microarray analyses, using the Affymetrix Mouse 430 2.0 3'-IVT platform, were performed on RNA extracted from intestine of *Gucy2c*^{+/+} and *Gucy2c*^{-/-} littermates (n=4 each).

Statistical analyses

Cell proliferation quantified by Ki-67 IHC was analyzed by linear mixed effects models while tumor incidence was analyzed by Pearson's chi-square test.¹⁸ Measurements at single time points were analyzed by ANOVA or, if appropriate, by t test unless otherwise specified. Unless otherwise indicated, results represent means $\pm$ SEM from at least 3 animals or 3 experiments performed in triplicate.

RESULTS

GUCY2C signaling coordinates proliferation and metabolism in intestinal epithelial cells

Elimination of GUCY2C signaling in mice (*Gucy2c*^{-/-}) expanded the size (Figure 1A) and number (Figure 1B) of crypts, associated with an increase in proteins promoting the cell cycle transition, including cyclin D1, cyclin-dependent kinase (CDK4), phosphorylated Rb (pRb), and β -catenin, while decreasing the cyclin-dependent kinase inhibitor p27 (Figures 1C). Expansion of the proliferating compartment was coupled with epithelial bioenergetic reprogramming, with increased glycolytic metabolism along the crypt-surface axis, resembling the tumor metabolic phenotype (Figures 2A).^{4, 22} Conversely, the mitochondrial genome and proteins of epithelial cells were decreased (Figures 2B). Further, staining of mitochondrial protein (Figure 2C) and direct visualization of mitochondria by electron microscopy (Figure 2D) revealed disruption of the crypt-surface gradient of mitochondria normally characterizing compartmentalization of proliferation and differentiation along that axis,²³ reflecting a decrease of mitochondria, which exhibited abnormal structure, in the differentiated villus compartment (Figure 2D).²⁴ Deregulation of the metabolic machinery increased glucose uptake and aerobic glycolysis reflected by increased lactate accumulation (Figure 2E). Further, mitochondrial oxygen consumption and dehydrogenase activity were reduced (Figure 2F). Proliferative and metabolic programming in the absence of GUCY2C signaling in mice recapitulated the phenotype in human colorectal tumors. Indeed, reduced guanylin in tumors (Figure S1A) was associated with increased cyclin D1 and decreased p27 regulating the G1/S transition (Figure S1B); increased GLUT1 and HKII regulating glycolysis (Figure S1C), and diminished electron transport complexes IV and V reflecting reduced mitochondrial oxidative phosphorylation (Figure S1D). Thus, beyond developmental programs controlling proliferation and differentiation important in spatiotemporal patterning, GUCY2C coordinates metabolic circuits along the crypt-surface axis.¹⁸ Moreover, disruption of GUCY2C expression, which mimics loss of guanylin and uroguanylin during transformation, induces proliferative and metabolic deregulation characterizing survival mechanisms in cancer.^{1, 2, 4, 19, 22}

Restoring GUCY2C signaling reverses neoplastic proliferative and metabolic phenotypes

Activation of GUCY2C signaling in human colon cancer cells by the heat-stable enterotoxin, ST, or its downstream effector, cGMP, reduced expression of cyclin D1 and pRb and increased p27 which, together, arrest the cell cycle (Figure 3A). Indeed, GUCY2C signaling delayed cell cycle progression (Figure 3B), and reduced DNA synthesis (Figure 3C) and colony formation (Figure 3D). Moreover, oral administration of the downstream effector of GUCY2C, cGMP, but not its inactive metabolite, GMP, restored crypt proliferative homeostasis in *Gucy2c*^{-/-} mice (Figure 3E), with a decrease in proliferating (Ki67-positive) cells in the crypt and absence of proliferating cells in the villus (Figure 3F), mimicking the effects of GUCY2C activation in human colon cancer cells.

Proliferative restriction was coupled with glycolytic reprogramming, reflected by decreased glucose uptake, lactate production (Figure 4A), and expression of rate-limiting enzymes mediating glycolysis that are typically over-expressed in tumors (Figure 4B).^{4, 22} ST effects were mediated by the canonical guanylyl cyclase catalytic domain, and GUCY2C constructs lacking that domain (TM) did not regulate proliferation or metabolism (Figure S2). The metabolic gap resulting from reduced glycolysis was bridged by increases in mitochondrial biogenesis. ST increased mtTFA, NRF-1, and PGC1 α in a GUCY2C-dependent fashion (Figure S3A), reflected by increases in mitochondrial content, including organelle-specific proteins (Figure S3B) and DNA (Figure S3C), and functional efficiency, including oxygen consumption, dehydrogenase activity, and ATP production (Figure 4C). Increased efficiency in coupling electron transport and ATP production was associated with reduced production of reactive oxygen species (ROS; Figure 4D). This might contribute to the increase in oxidative DNA damage (Figure 4E) and therefore an increase in tumor susceptibility¹⁹ due to loss of GUCY2C signaling. Similar to proliferative regulation, oral administration of cGMP reversed tumorigenic metabolism in *Gucy2c*^{-/-} mice (Figure 4F). Thus, survival pathways reflecting dysregulated GUCY2C and characterizing human colon cancer cells can be dynamically regulated in vitro and in vivo by reconstituting intestinal cGMP signaling.

GUCY2C regulates AKT signaling in intestinal cells

To identify the mechanism mediating preneoplastic transformation of intestinal epithelia in the absence of GUCY2C signaling, we performed microarray analysis to profile mRNA expression using both KEGG (Kyoto Encyclopedia of Genes and Genomes) and Biocarta pathway database annotations. AKT, a common signaling node regulating proliferation and metabolism, integrates cell survival circuits driving crypt developmental programs and its over-activation characterizes early colorectal tumorigenesis.^{25–30} Microarray profiling revealed that AKT pathways are the most significantly differentially activated in intestine from *Gucy2c*^{-/-}, compared to *Gucy2c*^{+/+} mice (KEGG database, $p=1.1 \times 10^{-6}$; Biocarta database, $p=8.5 \times 10^{-4}$; Figure 5A). Indeed, among 18 AKT-related pathways, 17 are significantly altered ($p<0.029$) by loss of GUCY2C (Figure 5A2). Eliminating GUCY2C resulted in increased AKT phosphorylation in intestinal cells (Figure 5B) at Thr308 and Ser473 (Figure 5C, pink), both of which are essential for AKT activity, recapitulating the over-activation of AKT in human colorectal cancer. Activation of canonical survival pathways by AKT1 induced by loss of GUCY2C was reflected by increased downstream TSC1/TSC2 degradation following TSC2 phosphorylation, which, in turn, increased mTOR signaling, a key integrator coordinating proliferative and metabolic signals required for cell growth and survival (Figure 5C, green).^{28, 31} Further, mTOR increased phosphorylation of S6K (Figure 5C, green), activating the translational machinery required for proliferation,³¹ and FOXO1, a key transcription factor promoting PGC1 α function and mitochondrial biogenesis,^{32, 33} resulting in its nuclear exclusion and degradation (Figure 5C, blue), coordinately rebalancing metabolism favoring glycolysis.²⁶ Conversely, restoring GUCY2C signaling by oral administration of cGMP specifically reduced AKT (Figure 5D, pink), but not MAP kinase (Figure 5D, brown), signaling

in *Gucy2c*^{-/-} mice. Moreover, in human colon cancer cells, GUCY2C signaling reversed oncogenic AKT activation and downstream effector mechanisms sustaining proliferation and metabolic reprogramming, including TSC2, mTOR, and FOXO1 (Figure 5E).

GUCY2C restricts oncogenic replicative and metabolic circuits through AKT inhibition

To further explore the causality between GUCY2C-mediated replicative and metabolic homeostasis and AKT regulation, we utilized pharmacological inhibition and adenovirus-mediated gene transfer to modulate AKT signaling. AKT can be silenced with SH6 (Figure 6A), which blocks membrane translocation and phosphorylation, or with AKT siRNA (siAKT; Figure 6B–E).^{25–27, 29} Eliminating AKT activity with SH6 (Figure 6A) or siAKT (Figure 6B) mimics GUCY2C signaling and blocks the effects of ST and cGMP, examined by proliferative (CyclinD), glycolytic (HKII) and mitochondrial (COXI) markers. The role of AKT in GUCY2C-mediated proliferative restriction was further confirmed by decreased DNA synthesis (Figure 6C), associated with metabolic rebalancing by increased mitochondrial content (Figure 6D) and a reciprocal reduction in glycolysis (Figure 6E). Conversely, myristoylation directly localizes AKT to membrane sites for phosphorylation, producing constitutive activation (myrAKT; Figure 6B–E).³² MyrAKT mimics elimination of GUCY2C signaling, and blocks the reverting effects of ST and cGMP, promoting expression of cell cycle mediators (Figure 6B, CyclinD), DNA synthesis (Figure 6C) and tumorigenic metabolism, reducing mitochondria protein (Figures 6B, COXI, and D) and enhancing glycolysis (Figure 6B, HKII, and E). Moreover, reducing AKT expression through gene disruption rescued the amplification of AOM-induced tumorigenesis produced by gene-targeted silencing of GUCY2C signaling in both small intestine and colon (Figure 7).

GUCY2C regulates AKT in a PTEN-dependent manner

Activation of AKT, in part, reflects the balance between oncogenic PI3K and the tumor suppressor PTEN, which together dynamically regulate phosphatidylinositol-3,4,5-triphosphate (PIP₃) available for directing membrane translocation and PDK-1 activation mediating AKT phosphorylation.^{25, 28} Elimination of GUCY2C in mice reduced PTEN expression and activity by increasing inhibitory phosphorylation (Figure 5C). Conversely, induction of cGMP signaling in human colon cancer cells increased PTEN expression and activity by reducing the ratio of phosphorylated (inactive) to total protein (Figure 8A). Moreover, silencing PTEN with siRNA (siPTEN) mimicked the effects of abolishing GUCY2C expression, and blocked the effects of cGMP on AKT activation and its downstream signaling (Figure 8B) and on key mediators of glycolysis (HKII), mitochondrial biogenesis (COXIV), and proliferation (CyclinD, Figure 8C). Taken together, these data indicate that GUCY2C regulates replicative and metabolic homeostasis in normal intestinal epithelium and tumors by modulating AKT activity, in part, in a PTEN-dependent manner.

DISCUSSION

There is a common process for transformation in all cells involving the evolution of autonomous growth and survival through deregulation of homeostatic processes underlying proliferation and metabolism.^{1, 22} This evolution is rooted in the genetic basis of cancer, in which the graded progression of the malignant phenotype reflects accumulation of sequential mutations.^{34, 35} In turn, this genetic instability reflects self-reinforcing cycles of proliferation uncoupled from normal DNA damage sensing and repair, promoted by metabolic remodeling and disabled apoptosis.^{1, 2, 4, 22, 34, 35} These amplifying mechanisms produce pervasive corruption of the genome and downstream homeostatic circuits, conferring phenotypic characteristics pathognomonic of the distal end of the transformation continuum including autonomous survival, invasion, metastasis, and resistance to therapy. Beyond this oncogenomic view of cancer, lineage-dependent tumorigenesis suggests that developmental

programs imprint tissue-specific mechanisms integrating proto-oncogenes and tumor suppressors in survival circuits, which coordinate fundamental homeostatic pathways.^{3, 35} Genomic or other pathophysiologic insults which corrupt these lineage-dependent programs, and the resultant deregulation of subordinate survival circuits, may represent the earliest events initiating or promoting the neoplastic continuum and the best opportunity for targeted prevention of cancer.

In that context, the present observations provide an adjunctive taxonomic perspective to colorectal tumorigenesis beyond a genetic disease reflecting sequential mutations in oncogenes and tumor suppressors.³⁴ Indeed, silencing of the intestinal homeostatic receptor GUCY2C, reflecting loss of paracrine hormone ligands,^{15, 16} promotes tumor initiation by inducing the proto-oncogene AKT which disrupts homeostatic replicative and bioenergetic programming, producing hyperproliferation and metabolic deregulation, both of which promote accumulation of mutations and tumor progression.^{1, 2, 4, 22} Hyperproliferation reflects the established role of GUCY2C and its ligands in organizing the crypt-surface axis, restricting the size of the crypt compartment, the number of cells proliferating, and the rate of the cell cycle through the G1-S transition.¹⁸ Metabolic deregulation extends the function of cGMP in programming mitochondrial biogenesis and oxidative metabolism with a previously unrecognized role in coordinately modulating aerobic glycolysis critical for energy independence and anabolic biosynthesis supporting hyperproliferation.^{4, 22, 36} Further, hyperproliferation, which expands the propagation of somatic mutations, and metabolic deregulation, which increases production of DNA-damaging ROS, cooperate to amplify genetic instability and promotes tumorigenesis.^{37, 38}

The present observations underscore the evolving critical importance of the AKT signaling axis in tumorigenesis. Amplifications and mutations enhancing PI3K activity characterize tumors originating from a variety of tissues and drive transformation.^{39, 40} Indeed, *RAS* mutations producing constitutive activation support tumor initiation and maintenance through activation of AKT signaling.⁴¹ Moreover, *PTEN* is mutated, associated with loss of function, in a broad spectrum of tumors.⁴² Indeed, PTEN-PI3K/AKT signaling is one of the most frequently altered pathways in colorectal cancer.²⁷ This pathway constitutes a central node integrating mitogenic, pro-oncogenic, and tumor suppressing signals to coordinate programs, including the cell cycle, metabolism, DNA repair, and apoptosis, at the intersection of tissue homeostasis and tumorigenesis.^{26, 43} The present study suggests a model of colorectal preneoplasia in which pro-oncogenic signaling by the PTEN-PI3K/AKT axis is engaged, in the absence of component mutations, through a lineage-specific mechanism involving silencing of the intestinal gene product GUCY2C by loss of paracrine hormone expression. Targeting GUCY2C with receptor-specific ligands offers a unique approach to prevent or treat colorectal cancer, circumventing upstream oncogenomic alterations, including mutation or gene amplification, of RTKs, KRAS, or PI3K by suppressing AKT and dependent survival circuits critical to tumor cell autonomy, without off-target adverse effects in tissues devoid of GUCY2C expression.

It is worth noting that dysregulated GUCY2C alone does not induce spontaneous transformation, underscoring the significance of the integrity of compensatory apoptotic mechanisms in the context of hyperproliferation, metabolic reprogramming and loss of genomic integrity.^{18, 19} Rather, loss of GUCY2C signaling collaborates with mutations in key regulatory elements of the Wnt pathway, including APC and β -catenin, which, in part, uncouple apoptotic mechanisms to promote intestinal tumorigenesis.^{19, 44} Moreover, there may be a cause and effect relationship between silencing of GUCY2C and APC loss of heterozygosity early in transformation.¹⁹ These observations suggest a model of lineage-addicted tumorigenesis in colorectal cancer in which disruption of homeostatic cGMP signaling permits the universal tissue-specific inactivation of APC,^{3, 45} possibly through AKT-mediated

inhibition of Chk1 by phosphorylation,²⁶ or enhanced p53 degradation.⁴⁶ This paradigm in which GUCY2C silencing establishes the tissue-specific context for corruption of APC signaling suggests an appealing model for cancer prevention in which maintenance of cGMP homeostasis through paracrine hormone receptor activation preserves APC function, opposing neoplasia.⁴⁷

Loss of guanylin and uroguanylin at the earliest stages of premalignancy suggests a lineage-dependent model of intestinal tumorigenesis,^{3, 15–17} initiating as a disease of tissue-specific paracrine hormone insufficiency.⁴⁸ The resultant silencing of GUCY2C produces maladaptive spatiotemporal amplification of survival circuits and, subsequently, DNA damage and mutations contributing to the genetic basis of colorectal cancer. This unique model of intestinal transformation as a paracrine hormone deficiency has substantial implications for the prevention and treatment of colorectal cancer. Indeed, GUCY2C is over-expressed by intestinal tumors compared to normal adjacent tissues.^{49, 50} Early loss of hormones and compensatory over-expression of receptors offers a unique therapeutic window preceding the accumulation of mutations corrupting subordinate survival pathways to prevent maladaptive reprogramming and the evolution of cancer through oral GUCY2C hormone replacement therapy.⁴⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support

These studies were supported by grants from NIH (CA75123, CA95026) and Targeted Diagnostic and Therapeutics Inc. to SAW, and NIH (CA133950), the Pennsylvania Department of Health and the Prevent Cancer Foundation to GMP. The Pennsylvania Department of Health and NIH specifically disclaim responsibility for any analyses, interpretations or conclusions.

Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog
APC	adenomatous polyposis coli
cGMP	cyclic GMP
FOXO	the forkhead box O transcription factor
FZD	frizzled
GUCY2C	guanylyl cyclase C
GLUT1	glucose transporter I
HKII	hexokinase II
mTOR	mammalian target of rapamycin
PGC1 α	peroxisome proliferator-activated receptor gamma coactivator 1 α
PK	pyruvate kinase
pRb	phosphorylated retinoblastoma
Rheb	RAS homolog enriched in brain
ROS	reactive oxygen species
RTK	receptor tyrosine kinase

ST	diarrheagenic bacterial heat-stable enterotoxin
TSC	tuberous sclerosis complex

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Figure 1. GUCY2C regulates intestinal epithelial cell proliferation

(A) Immunohistochemical quantification of the proliferation marker Ki67. (B) Crypt number quantified in comparably-sized cross-sections. (C) Immunoblot analysis of cell cycle regulatory proteins. Each point in A and B represents the mean of results from a single mouse. Data in (C) represent means of $n \geq 9 \pm \text{SEM}$. * $p < 0.05$, *** $p < 0.001$.

Figure 2. GUCY2C coordinates metabolism in intestinal epithelial cells

(A) Immunoblot analysis of rate-limiting enzymes mediating glucose metabolism [glucose transporter 1 (GLUT1), hexokinase II (HKII), phosphofructokinases 1 and 2P (PFK1/2P), pyruvate kinase (PK), and lactate dehydrogenase (LDH)]. (B) Mitochondria quantified by PCR analysis of organelle-specific gene targets and immunoblot analysis of specific protein components [ATP synthase (ATP5A1), cytochrome oxidase I (COXI), complex III core protein II (Core II)]. (C) Mitochondrial protein (COXI immunofluorescence) and (D) organelle density (electron microscopy; arrow, mitochondria; scale bar = 500 nM). (E) Glycolysis quantified by glucose uptake and lactate production. (F) Mitochondrial function quantified by oxygen

consumption (respiration) and dehydrogenase activity (metabolic activity). Data represent means of $n \geq 3 \pm \text{SEM}$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. GUCY2C opposes proliferation in colon cancer cells and remodels the proliferative compartment in *GUCY2C*^{-/-} mice

(A) Immunoblot analysis of cell cycle regulatory proteins in T84 (*GUCY2C*-expressing) and HCT116 (*GUCY2C*-null) human colon cancer cells [ST, *GUCY2C* ligand, 8BrGMP (cGMP), downstream effector]. (B) Cell cycle analyzed by flow cytometry. (C) DNA synthesis quantified by ³H-thymidine incorporation. (D) Cell proliferation measured by colony formation. (E–F) *GUCY2C* signaling induced in *Gucy2c*^{+/+} and *Gucy2c*^{-/-} mice by oral supplementation with cell-permeable 8BrGMP (cGMP), the downstream mediator of *GUCY2C* [negative control, 8BrGMP (GMP), the cell-permeable metabolite of cGMP], for 7 d. (E) Immunoblot analysis of cyclin D. (F) Immunohistochemistry of Ki67. [in E and F, Blue

asterisk compared to *Gucy2c*^{+/+}; black compared to *Gucy2c*^{-/-} GMP]. Data represent means of n≥3 experiments ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Figure 4. GUCY2C signaling reverses the tumor metabolic phenotype in human colon cancer cells and *GUCY2C*^{-/-} mice

Glycolysis quantified by (A) glucose uptake and lactate production in human colon cancer cell lines [T84 (*GUCY2C*-expressing); HCT116, (*GUCY2C*-deficient); ST, *GUCY2C* ligand, 8BrGMP (cGMP), downstream effector]. (B) Immunoblot analysis of key metabolic enzymes specifically elevated in tumors, including GLUT1 and HKII, mediating glycolysis. (C) Mitochondrial activity quantified by oxygen consumption, dehydrogenase activity, and ATP production. (D) Reactive oxygen species quantified by 3'-(p-aminophenyl) fluorescein (APF). (E) DNA oxidative damage quantified by immunofluorescence [8-oxo-deoxyguanosine (8-oxo-dG)]. (F) Immunoblot analysis of glycolytic proteins (GLUT1, HKII), transcription factors

promoting mitochondrial biogenesis (PGC1), and mitochondrial protein (COXI) after oral supplementation with cell-permeable cGMP, the downstream mediator of GUCY2C, and the control analogue GMP, for 7 d in *Gucy2c*^{-/-} mice [in F, blue asterisk compared to *Gucy2c*^{+/+}; black compared to *Gucy2c*^{-/-} GMP]. Data represent means of n≥3 experiments ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Figure 5. GUCY2C regulates AKT signaling

(A1) Expression profiling of signaling pathways suggest that genes altered in *Gucy2c*^{-/-} mice cluster in AKT-dependent processes. (A2) Fisher exact test for changes in AKT-dependent (18) and -independent (166) pathways quantified by relative microarray expression profiling of *Gucy2c*^{+/+}, compared to *Gucy2c*^{-/-}, mouse intestinal epithelia. Pathways, grouped by p values in rows and AKT dependence in columns, demonstrate that expression of genes altered in *Gucy2c*^{-/-} mice cluster in AKT-dependent processes (p=1.1×10⁻⁶). (B) Immunohistochemistry of pAKT. AKT is phosphorylated in both epithelial and stromal cells in *Gucy2c*^{-/-}, but only stromal cells in *Gucy2c*^{+/+}, mice. (C) AKT signaling quantified by immunoblot analysis of activated AKT, indicated by phosphorylation at T308 and S473 [pink],

and downstream targets. Activation of AKT induced phosphorylation of its immediate downstream target, TSC2, increasing the proteolytic degradation of the TSC1-TSC2 complex whose loss resulted in phosphorylation and activation of the mTOR kinase and subsequent phosphorylation of p70S6 kinase [green]. Also, AKT phosphorylated FOXO1, but not FOXO4, resulting in its nuclear exclusion and degradation [blue]. **(D)** Immunoblot analysis of active MAPK (pp38, pERK, pJNK, brown) and AKT signaling targets (pAKT, pp70S6K, pink) from *Gucy2c*^{-/-} mice following oral supplementation with cGMP for 1 d. **(E)** Evaluation of AKT signaling in human colon cancer cells (T84, GUCY2C-expressing) by immunoblot analysis of activated AKT (pAKT) and downstream targets. Reduced AKT phosphorylation, which stabilized the TSC1-TSC2 complex, reduced mTOR activation (pmTOR), and increased nuclear FOXO by decreasing FOXO phosphorylation. [ST, GUCY2C ligand; 8BrGMP (cGMP), GUCY2C downstream effector]. Data represent means of n≥4 experiments ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Figure 6. GUCY2C deprives cancer cells of replicative and metabolic survival advantages through AKT inhibition

(A) Immunoblot analysis of cell cycle regulatory protein (cyclin D), transcription factors mediating mitochondrial biogenesis (PGC1, mtTFA), mitochondrial protein (COXI), and rate-limiting proteins for glycolysis (GLUT1, HKII) in T84 cells (GUCY2C-expressing) following AKT inhibition by SH6 (AKT inhibitor). (B) Immunoblot analysis of activated AKT (pAKT), cell cycle regulatory protein (cyclin D), mitochondrial protein (COXI) and glycolytic protein (HKII), (C) DNA synthesis by thymidine incorporation, (D) mitochondrial content by fluorescence intensity of MitoTracker (FM), and (E) glucose uptake by fluorescence intensity of 2-NBDG in T84 cells expressing constitutively activated AKT (myrAKT), siRNA silencing AKT (siAKT), and wild-type AKT (AKT) [ST, GUCY2C ligand; 8BrGMP (cGMP), downstream effector]. Data represent means of $n \geq 3$ experiments $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7. GUCY2C regulates intestinal tumorigenesis through AKT
Tumorigenesis induced by AOM was quantified by (A) tumor enumeration, (B) tumor incidence and (C) tumor burden in *Gucy2c*^{-/-}, *Akt1*^{-/-}, and *Gucy2c*^{-/-}*Akt1*^{-/-} mice. In A and C, each point represents one mouse. * p<0.05, ** p<0.01, *** p<0.001, or n.s. p>0.1

Figure 8. GUCY2C regulates AKT in a PTEN dependent manner

Immunoblot analysis (**A1**) of PTEN expression (**A2**) and inactivation through phosphorylation (**A3**). Eliminating PTEN expression using siRNA (siPTEN) eliminated the effects of GUCY2C on (**B**) AKT signaling compared to a scrambled siRNA control (siCTR) and (**C**) proteins mediating oxidative phosphorylation (COXIV), glycolysis (HKII) and the cell cycle (Cyclin D). (**D**) Schematic model for GUCY2C signaling in tumorigenesis. Loss of GUCY2C supports pro-tumorigenic signaling through activation of AKT circuits, in part reflecting inhibition of PTEN, disrupting replicative and metabolic homeostasis and promoting tumorigenesis. Data represent means of $n \geq 3$ experiments $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.