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PIK3CA mutant tumors depend on oxoglutarate dehydrogenase

Nina Illic-a,b, Kivanc Birsoy, Andrew J. Aguirre-a,b, Nora Kory-c,d,e,f,g, Michael E. Pacold-d,e,f,g,i, Shambhavi Singh-a,b, Susan E. Moody-a,b, Joseph D. DeAngelo-a,b, Nicole A. Spardy-a,b, Elizaveta Freinkman-d,2, Barbara A. Weinberg-b, Aviad Tsherniak-b, Glenn S. Cowley-b,3, David E. Root-b, John M. Asara-b, Francisca Vazquez-a,b, Hans R. Widlund-d, David M. Sabatini-d,e,f,g, and William C. Hahn-a,b,4

*Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215; 2Broad Institute of MIT and Harvard University, Cambridge, MA 02142; 3Laboratory of Metabolic Regulation and Genetics, The Rockefeller University, New York, NY 10065; 4Whitehead Institute for Biomedical Research, Cambridge, MA 02142; 5David H. Koch Institute for Integrative Cancer Research at MIT, Cambridge, MA 02139; 6Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139; 7Department of Medicine, Division of Signal Transduction, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115; and 8Department of Dermatology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115

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Oncogenic PIK3CA mutations are found in a significant fraction of human cancers, but therapeutic inhibition of PI3K has only shown limited success in clinical trials. To understand how mutant PIK3CA contributes to cancer cell proliferation, we used genome scale loss-of-function screening in a large number of genomically annotated cancer cell lines. As expected, we found that PIK3CA mutant cancer cells require PI3CA but also require the expression of the TCA cycle enzyme 2-oxoglutarate dehydrogenase (OGDH). To understand the relationship between oncogenic PIK3CA and OGDH function, we interrogated metabolic requirements and found an increased reliance on glucose metabolism to sustain PIK3CA mutant cell proliferation. Functional metabolic studies revealed that OGDH suppression increased levels of the metabolite 2-oxoglutarate (2OG). We found that this increase in 2OG levels, either by OGDH suppression or exogenous 2OG treatment, resulted in aspartate depletion that was specifically manifested as auxotrophy within PIK3CA mutant cells. Reduced levels of aspartate deregulated the malate–aspartate shuttle, which is important for cytoplasmic NAD+ regeneration that sustains rapid glucose breakdown through glycolysis. Consequently, because PIK3CA mutant cells exhibit a profound reliance on glucose metabolism, malate–aspartate shuttle deregulation leads to a specific proliferative block due to the inability to maintain NAD+/NADH homeostasis. Together these observations define a precise metabolic vulnerability imposed by a recurrently mutated oncogene.

PIK3CA | 2OG | OGDH | TCA cycle | glycolysis

Mutations in PI3K, particularly those involving the catalytic subunit PI3Kα, encoded by PIK3CA, are found in a number of cancer cell lineages, including breast, colorectal, endometrial, and lung (1). PI3K signaling is normally activated in response to membrane-bound receptor stimulation, which in turn activates several effector molecules ultimately promote cell proliferation, survival, and motility (2). Mutations in PI3Kα are clustered within the helical (E542K or E545K) or catalytic (H1047R) domain, each of which leads to constitutive kinase activation, hyperactive downstream signaling, and proliferative cues. Expression of mutant PIK3CA in cell or animal models induces tumorigenicity, confirming that these mutations are oncogenic (3).

Multiple PI3K inhibitors have been developed, and both pan-PI3K and PI3Kα-specific inhibitors are the subject of ongoing clinical trials (4). To date, these inhibitors have only shown limited clinical activity (5, 6). Because the mutant PI3Kα isoform appears to be the key driver of tumorigenic phenotypes in genetically engineered mouse models (2), development of mutation-specific PI3Kα inhibitors may lead to improved outcomes.

Although it is clear that oncogenic PI3Kα drives hyperactivity of normal downstream signaling cascades, accumulating evidence indicates that these mutant alleles also exhibit additional activities. Specifically, oncogenic PI3Kα is thought to promote glycolysis by enabling heightened glucose uptake through regulation of GLUT1/4 protein translation (7) and subsequent plasma membrane translocation (8), as well as regulating metabolite pathways (9, 10). However, enhanced glycolysis is also observed in rapidly proliferating cells, which requires increased glucose uptake (11). Consequently, it has been difficult to discern how individual oncogenes affect metabolism, because proliferation alone has broad impact on nutrient demand and utilization.

As an alternative to studies of candidate genes, genome-scale loss-of-function screens offer an unbiased means to discover novel and previously uncharted dependencies and functional relationships in cells. Project Achilles is an effort to identify and characterize cancer cell vulnerabilities by identifying gene dependencies at genome-scale in a large number of human cancer cell lines (12, 13). Using this dataset, we have focused on genes that drive cell transformation will therefore help identity strategies to target tumors harboring these mutations. Although targeting certain oncogenes has led to clinical responses in some cases, PIK3CA inhibition has been disappointing to date. Here, we show that cell proliferation and tumor growth of PIK3CA mutant cancers is inhibited by suppression 2-oxoglutarate dehydrogenase, which leads to increased metabolite 2-oxoglutarate (2OG) levels. Elevated 2OG affects the function of the malate–aspartate shuttle, which is important because of the glycolytic nature of these cancers. This work provides novel insights into how mutant PIK3CA drives tumor proliferation and identifies a metabolic dependency that can be exploited in these cancers.

Significance

Oncogenic lesions give rise to genotype-specific dependencies in tumors by altering cell physiology. Understanding how oncogenes drive cell transformation will therefore help identify strategies to target tumors harboring these mutations. Although targeting certain oncogenes has led to clinical responses in some cases, PIK3CA inhibition has been disappointing to date. Here, we show that cell proliferation and tumor growth of PIK3CA mutant cancers is inhibited by suppression 2-oxoglutarate dehydrogenase, which leads to increased metabolite 2-oxoglutarate (2OG) levels. Elevated 2OG affects the function of the malate–aspartate shuttle, which is important because of the glycolytic nature of these cancers. This work provides novel insights into how mutant PIK3CA drives tumor proliferation and identifies a metabolic dependency that can be exploited in these cancers.


Conflict of interest statement: W.C.H. is a consultant for Novartis.

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1Present address: Department of Radiation Oncology, New York University Langone Medical Center, New York, NY 10016.

2Present address: Metabolon, Inc., Research Triangle Park, NC 27709.

3Present address: Discovery Science, Janssen Research & Development (Johnson & Johnson), Spring House, PA 19477.

4To whom correspondence should be addressed. Email: william.hahn@dci.harvard.edu.

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that are specifically required for proliferation or survival of cancer cells that bear oncogenic PIK3CA mutations. This approach identified the tricarboxylic acid cycle (TCA) cycle enzyme 2-oxoglutarate dehydrogenase (OGDH) as an essential requirement to maintain PIK3CA mutant tumor cell proliferation or survival.

Results

Identification of OGDH as a Dependency Associated with PIK3CA Mutation. To identify genes and pathways that are required in cancer cells that harbor PIK3CA mutations, we used genome-scale shRNA data from Project Achilles (12, 13). Specifically, we used data derived from screening 17 PIK3CA mutant (MUT class) and 68 PIK3CA wild-type (WT class) cell lines, where individual covariant shRNA values (from a pool of ≥5 shRNAs per gene) were condensed to gene level dependencies using ATARiS (14). We then performed a two-class (MUT vs. WT) comparison among the two cell line classes by computing rescaled and normalized mutual information (RNMI) scores using the PARIS module in GenePattern (13) (Fig. 1A). To identify pathways or cell processes that represent specific vulnerabilities in PIK3CA MUT cells, we then performed Gene Set Enrichment Analysis (GSEA) (15) using the highest probability ranked genes, which revealed an enrichment for gene sets associated with the spliceosome, the TCA cycle, and lysine degradation (Fig. 1B). As expected, the gene with the highest association to the PIK3CA MUT class was PIK3CA (12) (Fig. 1C and Dataset S1). Among the 25 highest-ranked dependencies, we found all three components of the OGDH complex, including OGDH, dihydrolipoamide S-succinyltransferase (DLST), and dihydrolipoamide dehydrogenase (DLD) (Fig. 1C and D) (16). OGDH is a highly conserved TCA mitochondrial enzyme complex that catalyzes the irreversible conversion of 2-oxoglutarate (2OG) and CoA-SH to succinyl-CoA and CO₂, while reducing NAD⁺ to yield NADH (16).

To validate these observations, we individually assessed OGDH (Fig. 2 and Fig. S1A), DLST (Fig. S2A), DLD (Fig. S2B), and PIK3CA (Fig. 2 and Fig. S1A) as dependencies in multiple PIK3CA MUT and WT cell lines. Using at least two individual shRNAs per gene, we confirmed that these shRNAs suppressed the expression of each of these genes independent of mutation status (Fig. 2 and Figs. S1B and S2) and found that cell lines harboring PIK3CA MUT uniquely depended on each of the OGDH complex components for proliferation/survival.

OGDH Is Necessary to Maintain Mutant PIK3CA Tumor Growth in Vivo. Because the genes and pathways used by cells grown in culture may differ from genes required to sustain tumor growth in vivo, we investigated whether OGDH suppression affects tumor xenograft growth. We engineered PIK3CA MUT (RKO and HCT116) and WT (LS411N and HT55) cancer cell lines to express doxycycline (dox)-inducible shRNAs targeting OGDH and controls targeting GFP/RFP. Dox treatment induced shRNA suppression of OGDH (Fig. S3 A and B) and inhibited the proliferation of PIK3CA MUT cells in vitro (Fig. S3 C and D). To validate that these observations were due to on-target effects

Fig. 1. Genome-scale loss-of-function analyses identify the OGDH complex as a dependency in PIK3CA MUT cell lines. (A) Two-class comparative analysis of combined gene-level shRNA scores (ATARiS values) between PIK3CA MUT and WT cancer cell lines using PARIS module RNMI statistics for genes required for PIK3CA MUT cells (P < 0.01) used for (B) pathway identification by GSEA (P < 0.25, P < 0.01) and (C) gene rank list of top 25 PIK3CA mutation-associated dependencies. (D) Average ATARiS score sensitivity among PIK3CA WT (n = 68) and MUT (n = 17) cell lines for PIK3CA and OGDH (⁎⁎⁎P < 0.001; **⁎⁎⁎P < 0.0001 calculated using an unpaired two-sided t test).
of these shRNAs, we generated and introduced a variant OGDH cDNA allele (OGDH wobble) carrying silent mutations within the targeting motifs for each of the three shRNAs (Fig. S3E). Compared with controls, expression of this OGDH wobble allele rescued the effects of the OGDH-specific shRNAs (Fig. S3F).

To investigate whether OGDH suppression induced a proliferative arrest or cell death in PIK3CA MUT cells, we used the fluorescent nucleic acid stain, SYTOX, which does not permeate live cells, thus staining only dead cells. By quantifying SYTOX-positive cells, we failed to detect cell death following OGDH suppression (Fig. S3G). We confirmed these observations using trypan blue staining of cells and quantifying cell numbers following shRNA induction (Fig. S3H). To assess whether OGDH suppression affects the cell cycle, we performed BrdU labeling experiments and found that PIK3CA MUT, but not WT, lines exhibit a decrease in BrdU incorporation following OGDH suppression (Fig. S3I), suggesting a block in the cell cycle, similar to the effects of pharmacological PI3K inhibition (17).

To examine the requirement for OGDH for PIK3CA mutant tumor growth, we implanted the dox-inducible cell lines into immunocompromised mice and induced the expression of the shRNAs either at time of implantation (Fig. 3A–C) or subsequent to tumor formation (Fig. 3D–F). Confirming our in vitro observations, PIK3CA MUT xenografts displayed a significant suppression of tumor growth upon OGDH shRNA induction ($P < 0.01$; Fig. 3B and E), whereas suppression of OGDH had no effect on PIK3CA WT xenograft tumors (Fig. 3C and F). Taken together, these data indicate that OGDH is a genuine dependence in PIK3CA mutant cancer cells supporting proliferation.

**PIK3CA Mutant Cell Lines Display an Enhanced Requirement for Glucose Metabolism.** We next examined how OGDH function affects PIK3CA mutant cancer cell proliferation. OGDH is an integral component of the TCA cycle, and the PI3K/mTOR pathway has been suggested to regulate both glucose (7, 8) and glutamine metabolism (18, 19). Hence, we examined the dependence of the PIK3CA MUT and WT lines on limiting availability of these two nutrients. Although we found that glutamine deprivation similarly affected proliferation of both PIK3CA MUT and WT cells (Fig. 4A), reducing glucose availability preferentially affected PIK3CA MUT cell line proliferation (Fig. 4B). These observations clearly suggested that PIK3CA MUT cell lines exhibit a higher...
glucose requirement to sustain proliferation, which is in agreement with previous studies (8, 20). To confirm these findings, we measured sensitivity to 2-deoxyglucose (2DG), a competitive inhibitor of the first step of glycolysis [conversion of glucose to glucose-6-phosphate (G6P)] (Fig. 4C). We found that the PIK3CA MUT cell lines exhibited higher sensitivity to 2DG compared with the WT cells, paralleling their requirement for higher glucose concentration (Fig. 4B). Elevated glycolytic demand could point toward reduced reliance on oxidative phosphorylation; therefore, we examined whether complex I inhibitors rotenone and phenformin (Fig. S4 A and B), complex V/ATP synthase inhibitor oligomycin A (Fig. S4C), and a mitochondrial proton gradient uncoupler 2,4-dinitrophenol (2,4-DNP; Fig. S4C) also affected proliferation. These inhibitors affected proliferation nearly uniformly across the cell lines, but independent of PIK3CA mutation status. In addition, we measured oxygen consumption rates among PIK3CA MUT and WT cell lines (Fig. S4 D and E) but failed to observe significant differences between the two groups. Hence, it is unlikely that the sensitivity to OGDH suppression in PIK3CA mutant cells is due to differences in mitochondrial bioenergetics but rather a consequence of their increased reliance on glucose metabolism.

To investigate the source of differential nutrient use between PIK3CA MUT and WT cell lines, we used [U-13C6]-glucose or [U-13C6]-glucose in media lacking the labeled nutrient. After allowing cells to proliferate for 6 or 7 h, respectively, we performed LC-MS to assess fractional labeling of metabolites. In glucose-labeled cells, we failed to find consistent differences in steady-state glutamine-to-TCA flux between PIK3CA MUT and WT cell lines (shOGDH-sensitive vs. resistant; Fig. S8 F and G). This finding suggested that utilization or activity of OGDH reaction did not explain the observed differential sensitivity to OGDH suppression. In glucose-labeled cells, however, we observed consistent increases in labeling along the three branches downstream of G6P—glycolysis (pyruvate), pentose phosphate pathway (adenosine and uridine), and nucleotide sugar metabolism (UDP-α-glucuronate; Fig. 4D and Fig. S5 A and B) across the PIK3CA mutant cancer cell lines. In addition, total levels of lactate were higher in MUT cells (with no significant difference in fractional labeling; Fig. 4D), suggesting altered glucose utilization and flux between pyruvate and lactate. We also found that secreted lactate levels were consistently higher in the MUT cell lines compared with WT measured over 72 h (Fig. 4E), as were their basal extracellular acidification rates (ECAR, Fig. 4F). When we propagated cells in the presence of the PIK3CA-specific catalytic inhibitor, BYL719 (21) at a dose that discriminates between PIK3CA MUT and WT cell lines (Fig. S5C), we found that total levels of glutamine-derived metabolites, glucose, and pentose phosphate pathway were generally reduced in both MUT and WT cells, with overall more potent effects in MUT cells (21) (Fig. SSD). Collectively, these data suggest that PIK3CA MUT cancer cells exhibit an enhanced requirement and utilization of glucose.

**Effects of Suppressing OGDH on Metabolic Pathways.** To examine the metabolic consequences of OGDH suppression, we performed LC-MS metabolite profiling following suppression of OGDH in PIK3CA MUT and WT cells (Fig. 5 A and B). In both PIK3CA MUT and WT cells, OGDH suppression resulted in a comparable increase in the levels of its substrate, 2OG (Fig. 5C), and an expected decrease in the pools of TCA cycle metabolites fumarate, malate, and aspartate (Fig. 5D). Furthermore, several metabolites involved in transamination reactions with 2OG (2-oxobutanoadipate, phenylpyruvate and 2-ketoisovalerate), or products from reactions that use 2OG (acetoacetate), were also affected. Expression of the OGDH wobble allele (Fig. S3 E and F) reversed these changes (Fig. S6 A–F).

In addition, we observed that the NAD+/NADH ratio was reduced following OGDH suppression in the sensitive PIK3CA MUT cells, indicative of an altered redox state that paralleled sensitivity to OGDH (Fig. 5E). NADH produced in the cytosol must be regenerated to NAD+ for glycolysis to proceed, which can be achieved through the activity of lactate dehydrogenase (22–24), glycerol-3-phosphate shuttle (25), or the malate–aspartate shuttle. The malate–aspartate shuttle activity regenerates NAD+ in the cytosol, which brings the reducing equivalents into the mitochondria (26, 27) (Fig. 5F). The direct relationship between the malate–aspartate shuttle and OGDH is through the substrate 2OG, which is consumed by transamination reactions on both sides of the mitochondrial membrane (Fig. S7A) (27).

To specifically examine the effects of increasing 2OG levels (Fig. 5C), we treated PIK3CA WT and MUT cells with a membrane-permeable 2OG-ester derivative, dimethyl-(dm)-2OG (28, 29) and assessed effects on cell proliferation. We confirmed an increase in intracellular 2OG levels, and also decreased levels of aspartate (Fig. 5G), both of which were comparable to what we observed when we suppressed OGDH (Fig. 5C and D). The levels of malate and fumarate remained unaffected (Fig. S6 G and H), consistent with cytosolic delivery of dm-2OG. In consonance with these observations, we found that dm-2OG treatment potently suppressed the proliferation of PIK3CA MUT cells, but had more
moderate effects on the WT cells (Fig. 5H). To confirm the specificity of proliferative effects observed by dm2OG treatment, we treated cells with two additional TCA cycle metabolite esters, dimethyl succinate and diethyl malate, and did not observe differential proliferative suppression among the MUT and WT group (Fig. S6 I and J).

Because an increase in 2OG levels, either by shOGDH or dm-2OG, preferentially affected PIK3CA MUT cell proliferation, we examined whether transamination reactions linked to the malate–aspartate shuttle were affected by these manipulations (Fig. 5F and Fig. S7A). Specifically, we treated cells with aminooxyacetic acid (AOA), a transaminase inhibitor that exhibits some preferential inhibition of aspartate transaminase (Fig. 5F) (27, 30) and subsequently assessed effects on cell proliferation. We found that AOA preferentially suppressed proliferation of the PIK3CA MUT cells (Fig. 5I). In addition, low-dose AOA sensitized PIK3CA WT cell lines to OGDH suppression (Fig. S6 K and L), indicating that further decrease in aspartate transaminase-mediated oxaloacetate-to-aspartate transamination affects OGDH sensitivity as observed in PIK3CA MUT cells. Together, these data show that OGDH suppression causes an expected increase in the levels of 2OG, accompanied by a decrease in the levels of downstream TCA cycle metabolites, as well as a concomitant reduction in NAD\(^+\)/NADH ratios. These changes are consistent with an altered redox state that can be explained as a consequence of malate–aspartate shuttle transamination reaction inhibition driven by increased 2OG levels (Fig. 5F).

**OGDH Suppression Results in Malate and Aspartate Insufficiency.** To gain further insight into acute metabolic changes following OGDH suppression, we performed a 3-h pulse with [U-\(^{13}\)C\(_6\)]-glucose tracer in PIK3CA WT and MUT cells to assess effects on glucose-derived metabolites (Fig. S5A). We found that labeled glucose-derived lactate and glyceroldehyde-3-phosphate were reduced specifically in PIK3CA MUT cells following OGDH suppression (Fig. 5A). Venn diagrams depicting overlapping metabolite level changes following OGDH suppression in PIK3CA WT (HT55, C2BBe1) and MUT (RKO, HT29) cell lines. (B) Schematic of the TCA cycle with emphasis on overlapping metabolites whose levels are altered following OGDH suppression (blue, decreased; red, increased). (C–E) Relative levels of 2OG (C), malate, fumarate, and aspartate (D), or NAD\(^+\)/NADH ratio (E) following OGDH suppression. (F) Schematic representation of the cytoplasmic–mitochondrial malate–aspartate shuttle that regenerates cytoplasmic NAD\(^+\) to enable glycolysis to proceed. (G) MS analysis of metabolite changes in PIK3CA WT (C2BBe1, COV362, LS411N, HT55) or MUT (TOV21G, DLD1, HCT116, HT29) lines mock treated or propagated in 7 mM dm-2OG for 72 h; relative levels (normalized to mock treated) of 2OG and aspartate are shown; H. Proliferation effects in PIK3CA WT (C2BBe1, KM12, HT55, LS411N, COV362) and MUT (HT29, GP2D, TOV21G, RKO, HCT116, DLD1) lines following 96 h treatment with 7 mM dm-2OG. (I) Proliferation effects in PIK3CA WT (C2BBe1, KM12, HT55, LS411N, COV362) and MUT (AGS, HT52, GP2D, TOV21G, RKO, HCT116, DLD1) lines following 96-h treatment with 1.75 mM AOA. OA, oxaloacetate; Succ-CoA, succinyl-CoA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. P values were determined by an unpaired t test with Welch’s correction (C–E and G) or a one-way ANOVA with multiple comparisons (H and I). Error bars represent SD of the mean of three (C–E and G) or four replicates (H and I).
suppression (Fig. S6 M and N), suggestive of impaired glycolytic flux. As an alternative means to examine the effects on glutamine-derived metabolites, we also used a 7-h pulse with [U-\(^{13}\)C\(_5\)]-glutamine to label cells (Fig. 6B), accompanied by a 30–50% decrease in one-, two-, and three-carbon labeled (C1, C2, and C3) fractions of 2OG in MUT cells (Fig. 6C). Furthermore, we observed a shift toward reductive carboxylation of 2OG toward citrate, reflected in an increase in the C5-labeled fraction, in both of the MUT cell lines, but only one of the WT lines, LS411N (Fig. S6P) (31). Despite the threefold increase in C5-labeled citrate observed in MUT cells, this species constituted only ~1–6% of total citrate, suggesting that this alternate path of glutamine-derived carbons likely does not explain sensitivity to OGDH suppression. In agreement with our previous experiments (Fig. 5D), we confirmed the reduction in total malate levels in PIK3CA MUT cells upon OGDH suppression (Fig. S6Q), with more potent reduction in the C1 and C2 fractions in MUT cells (Fig. 6D). We also confirmed reduction in total aspartate levels (Fig. 5D and Fig. S6R), accompanied by more potent decrease in labeled species in MUT cells (Fig. 6E).

**OGDH Suppression Induces Aspartate Auxotrophy in PIK3CA Mutant Cells.** Given the profound and consistent decrease in aspartate levels caused both by OGDH suppression (Fig. 5D and Fig. S6R) and exogenous 2OG (Fig. 5G), we considered whether depletion of this amino acid following OGDH suppression drives the observed sensitivity in PIK3CA MUT cells. Aspartate is a nonessential

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**Fig. 6.** Aspartate supplementation reduces sensitivity to OGDH suppression or 2OG treatment. (A) Diagram of anaplerotic glutamine flux into the TCA cycle, depicting both oxidative and reductive pathways of aspartate generation from 2OG. Filled circles depict \(^{13}\)C atoms originating from [U-\(^{13}\)C\(_5\)]-L-glutamine. (B–E) MS analysis in PIK3CA MUT (HCT116 and RKO) and WT (HT55 and LS411N) cell lines cultured in the presence of dox for 72 h and with [U-\(^{13}\)C\(_5\)]-glutamine for 7 h; total pool sizes for 2OG (B) are shown separately from fractions of glutamine-derived labeled 2OG (C), malate (D), and aspartate (E). (F) Proliferation assay for PIK3CA MUT cell lines (HCT116 and RKO) expressing dox-inducible shRNAs, without or with ectopic SLC1A3, 96 h post dox induction of shRNAs and concurrent addition of L-aspartate. (G) Proliferation of PIK3CA MUT cell lines (RKO and HCT116) overexpressing SLC1A3, in the presence of 10 mM aspartate and mock treated or treated with 5 mM dm-2OG for 96 h. (H and I) MS analysis of PIK3CA MUT cell line RKO expressing dox-inducible shRNAs, without or with ectopic SLC1A3. Cells were cultured in the presence of dox with or without 5 mM L-aspartate or 1 mM sodium pyruvate for 72 h and with [U-\(^{13}\)C\(_5\)]-glutamine for 7 h. Total pool sizes for aspartate (H) are shown separately from fractions of glutamine-derived labeled aspartate (I). (J) MS analysis of NAD\(^+\)/NADH ratios in PIK3CA MUT cell line RKO. Cells were cultured in the presence of dox with or without 5 mM L-aspartate for 72 h. Each bar represents the mean \(\pm\) SD of three (B–E and H–J) or four (F and G) replicates. *\(P\) < 0.05, **\(P\) < 0.01, ***\(P\) < 0.001, ****\(P\) < 0.0001. P values were determined by unpaired two-tailed t-test statistics with Welch's correction for unequal variance (B–E), unpaired two-tailed t test (G), or a one-way ANOVA (F and H–J).
amino acid that is generated in the cell through 2OG-dependent transamination to and from oxaloacetate (Fig. S7A). Following OGDH suppression in PIK3CA MUT cells, reduced aspartate levels (Fig. 5D and Fig. S6F) could be caused by mitochondrial 2OG accumulation and matrix-side malate–aspartate shuttle deregulation (Fig. 5F) (32), and conversely exogenous 2OG addition would result in the same changes but initiated on the cytoplasmic side. Interestingly, in mitochondrial respiration deficient cells, or in the presence of complex I inhibitor phenformin, aspartate supplementation was recently shown to support cell proliferation and bypass oxidative phosphorylation inhibition as well as genetic defects in mitochondrial DNA (33, 34). To assess whether aspartate supplementation could reduce sensitivity to OGDH suppression, we used high-dose aspartate supplemented media and measured cell proliferation. We found that exogenous addition of aspartate could not restore proliferation (Fig. S7B). This result was, however, not surprising, given that most tissues outside the CNS do not normally express aspartate transporters at the plasma membrane (33). However, ectopic expression of the aspartate/glutamate transporter, SLC1A3 (33) (Fig. S7C), in combination with aspartate supplementation, caused a robust and dose-dependent sensitivity decrease to OGDH suppression (Fig. 6F). Furthermore, overexpression of SLC1A3 in the presence of exogenous aspartate reduced sensitivity to exogenous dm-2OG (Fig. 6G), suggesting that intracellular 2OG increases drive differential OGDH sensitivity. Notably, severe aspartate supplementation following OGDH suppression did not reduce sensitivity (Fig. S7D), likely because aspartate generated from oxaloacetate, which originates from malate or anaplerotically from pyruvate, requires a transamination that is imbalanced due to excess 2OG (Fig. S7A).

Aspartate Supplementation Restores a Truncated TCA Cycle. To assess which metabolic pathways were restored by aspartate supplementation, we performed [U-13C5]-glutamine tracer experiments (Fig. 6A and Fig. S4F). As expected, we confirmed that aspartate and malate levels were restored following aspartate, but not pyruvate, addition (Fig. 6 H and I and S7G), whereas 2OG and succinate levels remained elevated and depleted, respectively (Fig. S7 E and F). In addition, aspartate supplementation restored the NAD+/NADH ratio upon OGDH suppression to that of unperturbed control (Fig. 6I). These observations collectively indicate that the shOGDH-induced truncation of the TCA cycle causes 2OG to deplete malate and aspartate, both because of the effects on the TCA cycle but also due to affecting the operation of the malate–aspartate shuttle. Because of its important role in NAD+ regeneration for sustained glycolysis and maintained building-block synthesis, the malate–aspartate shuttle function is more critical for PIK3CA mutant cancer cell lines (Figs. 5F and 7 and Fig. S7H), leading to differential dependency on OGDH function to maintain intracellular 2OG balance.

Discussion

Mutations in metabolic genes, such as IDH1/2 (35), fumarate hydratase (36), succinate dehydrogenase (37, 38), and mitochondrial-encoded genes (39), although relatively rare, are actively pursued for therapy because of their causal involvement in the tumors that carry those lesions. In addition, hallmark oncogenes such as KRAS (40, 41) and MYC (42), as well as the mTORC1 pathway (18), provoke unique metabolic dependencies when hyperactivated. Because of their frequent involvement in promoting cancer development and growth, these dependencies are therefore being considered for improving cancer treatment.

From genome-scale vulnerability screens, we have identified the mitochondrial TCA cycle enzyme OGDH as a specific vulnerability in PIK3CA mutant cancer cells, required both for proliferation in culture, as well as in vivo tumor maintenance (Fig. S3 and Fig. 3). The PI3K pathway has previously been associated with multiple roles in metabolism, involving heightened glucose import through regulation of GLUT1 expression; GLUT4 translation and membrane localization (7, 8); stimulation of glutathione synthesis (43); and suppression of β-oxidation (9). In our analyses, and akin to previous reports (44), PIK3CA mutant cells do not exhibit altered reliance on mitochondrial metabolism (Fig. S4), suggesting an alternative requirement for OGDH, other than its role in the TCA cycle.

Using metabonomic and pharmacological approaches, we found that PIK3CA mutant cancer cells rely on OGDH through maintaining appropriate levels of the substrate 2OG. Following inhibition of OGDH, 2OG levels are increased (Figs. 5C and 6B), whereas the levels of malate and aspartate decrease (Fig. 5D and Fig. S6 Q and R), which in PIK3CA mutant cells reduces the NAD+/NADH ratio (Fig. 5E and Fig. S6F). In keeping with these results, it was recently reported that certain cancer cell lines depend on OGDH activity for aspartate and function of the malate–aspartate shuttle (45); however, without any association with tumor cell mutation status. The glycolytic nature of PIK3CA mutant cells, reflected in their reliance on glucose (Fig. 4B), sensitivity to inhibition of glycolysis (Fig. 4C), and elevated secretion of lactate (Fig. 4E and F), collectively imparts sensitivity to excess 2OG levels via deregulation of malate–aspartate shuttle activity, an important NAD+ regeneration pathway in the cytoplasm (Fig. 5F). Our observations suggest that dependence on OGDH activity is increased in cells with elevated glucose demand. Thus, we provide mechanistic insight into why a defined subset of cancer cell lines are more dependent on OGDH function through their heightened reliance on 2OG maintenance, which closely associates with the highlighted role of PIK3CA in promoting anabolic metabolism (7–10).

Inherent stress from uncontrolled cancer cell growth provokes metabolic insufficiencies that must be satisfied by alternative nutrient uptake for TCA cycle catabolism, which involves β-oxidation of lipids, increased glutaminolysis, and breakdown of extracellular proteins. To this end, PIK3CA mutant colorectal cancer cell lines were recently shown to up-regulate the glutamate pyruvate transaminase 2 (GPT2) to fuel the TCA cycle with 2OG generated from pyruvate and glutamate (46). Notably, this role for GPT2 within PIK3CA mutant colon cancer cells was determined under glucose depletion; this represents an alternative pathway for pyruvate and glutamate to sustain the TCA cycle that would parallel the widely appreciated anaplerotic roles of pyruvate to generate oxaloacetate through pyruvate carboxylase and glutamate dehydrogenase to generate 2OG. In our experiments, conducted in complete media, OGDH suppression or exogenous 2OG addition, we failed to find deregulation of the TCA cycle attributable to PIK3CA mutation (Fig. 5G and Fig. S6 G and H). Importantly, we found that the effects of OGDH suppression or 2OG treatment were alleviated by increasing intracellular levels of aspartate using aspartate/glutamate transporter overexpression and exogenous aspartate supplementation.
Aspartate supplementation following OGDH suppression, however, did not restore the levels of the TCA cycle metabolites 2OG and succinate (Fig. S7 E and F), but restored the NAD+/NADH ratios (Fig. 6I) and cell proliferation (Fig. 6F–I and Fig. S7G and H). These observations suggest that it is the effects of elevated 2OG on depleting aspartate, rather than general suppression of the TCA cycle, that represents a unique vulnerability in \( \text{PIK3CA} \) mutant cells.

We speculate that pyruvate anaplerosis via oxaloacetate does not occur because aspartate is made from oxaloacetate, and this reaction is opposed by elevated 2OG (Fig. S74). Because \( \text{PIK3CA} \) mutant cell lines require NAD\(^+\) regeneration due to their inability to regulate glycolytic flux, our findings point toward 2OG imbalance as a critical metabolic vulnerability in this class of cancers. Although aspartate is integral to the operation of the malate-aspartate shuttle, we note that it is also a required metabolite for additional metabolic pathways, including the urea cycle (47); nucleotide synthesis (48–50); arginine (51) and asparagine synthesis (52); glutamate metabolism (53); and protein synthesis (54).

Hence, it is possible that additional pathways in the \( \text{PIK3CA} \) MUT cancers also require aspartate for sustained proliferation. 2OG mimetic tool compounds have been shown to have antiproliferative activity in a small set of cancer cell lines, albeit at high doses (mM) (55), making it difficult to extend these studies to animal models. Nevertheless, our observations suggest that further development of these or similar compounds may have potential therapeutic value if toxicity that may be caused by OGDH inhibition can be tolerated due to the reported association between its reduced activity and neurodegenerative disorders (56). As an alternative to OGDH inhibition, several recent studies have shown that aspartate becomes limiting for cancer cells bearing mutations in succinate dehydrogenase or under conditions of electron transport chain (ETC) suppression, suggesting inhibition of aspartate synthesis as a potential therapeutic strategy in those cancers (33, 34, 38). Furthermore, NAD\(^+\) depletion has been explored as a therapeutic option in certain subsets of cancer (57), including IDH1 mutant gliomas, which critically depend on NAD\(^+\) availability (58).

Collectively, our observations indicate that oncogenic \( \text{PIK3CA} \) confers a metabolic state that makes cells more sensitive to elevated levels of 2OG, revealing a heightened dependency on aspartate to maintain the malate–aspartate shuttle support for NAD\(^+\) regeneration. Further experimental exploration to assess aspartate synthesis, the operation of the malate–aspartate shuttle, or NAD\(^+\) regeneration may offer valuable prognostic or therapeutic insights for cancers bearing mutations in \( \text{PIK3CA} \). Importantly, these observations may extend to other tumors that do not bear mutations in \( \text{PIK3CA} \), but display a heightened glycolytic use and reliance on efficient NAD\(^+\) regeneration. In fact, when we analyzed data from Project Achilles (Fig. 1) we found that other cancer cell lines display sensitivity to OGDH suppression, and it remains to be determined which genetic features drive this dependency. It is plausible that these tumors generally display an increased glycolytic drive and would therefore exhibit comparable vulnerability to increases in 2OG levels. Alternatively, because of the dynamic plasticity of cancer cell metabolism, we could also envision that cell lines that initially exhibited sensitivity to 2OG fluctuation or OGDH suppression could acquire resistance to these insults. One way to achieve resistance would be by a metabolic shift toward increased oxidative metabolism, which would be more glucose efficient, and thus render reduced reliance on the malate–aspartate shuttle for NAD\(^+\) regeneration. Hence to prevent therapeutic resistance, cotargeting metabolic pathways involving mitochondrial metabolism may be advantageous, and would represent a confluence of pharmacological opportunities.

**Materials and Methods**

**Code Availability.**

\( \text{PARIS} \). The GenePattern module \( \text{PARIS} \) (13) (https://www.broadinstitute.org/achilles/resources) was used for RNNI statistics to generate a list of genes that are preferentially required for proliferation of \( \text{PIK3CA} \) MUT cell lines compared with WT cell lines (Dataset S1). The \( \text{ATARiS} \) (14) gene level dataset v2.0 was used as a data file (www.broadinstitute.org/achilles). The mutation annotation file used as classifier was generated using hybrid capture mutation data from the data from the Cancer Cell Line Encyclopedia (https://www.broadinstitute.org/cycle). A total of 85 cell lines (17 \( \text{PIK3CA} \) MUT) were used in the analysis, where cell lines without \( \text{PIK3CA} \) mutation data were removed.

\( \text{GSEA} \) (15) using KEGG 4.0 database was performed on the list of genes identified as required for the survival of \( \text{PIK3CA} \) MUT cell lines using \( \text{PARIS} \) two-class comparison analysis (13) with the significance cutoff of \( P < 0.01 \) among \( \text{PIK3CA} \) MUT-associated dependencies. \( \text{GSEA} \) was performed with 10,000 permutations and a threshold of at least three genes per gene set.

\( \text{PARIS}, \text{ATARiS}, \) and \( \text{GSEA} \) are publicly available as GenePattern modules (https://genepattern.broadinstitute.org).
Satisfy significance of $P < 0.05$ [unpaired two-sided test with Welch’s correction for unequal variance (for two groups) or a one-way ANOVA with multiple comparisons (for more than two groups)].

Glucose isoform tracing was conducted following the same protocol as for unlabelled metabolite profiling. Cells were incubated in regular medium for 24 h and then refed with media containing unlabelled 2.25 μl glucose (half of standard glucose concentration normally used) 24 h before harvesting metabolites. For baseline comparison among cell lines, the media was supplemented with 2.25 μl [U-13C]-glucose 6 h before metabolite isolation. For experiments with dox-inducible OGDH suppression, cells were incubated in the presence of dox for 48 h and refed with media containing dox and unlabelled 2.25 μl glucose for another 24 h. At 3 h before harvesting metabolites, the media was supplemented with 2.25 μl [U-13C]-glucose.

Glutamine Isoform Tracing. Glutamine isoform tracing was conducted on a QExactive benchtop Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe, coupled to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific). Cells were incubated in the presence of dox for 72 h and refed with glutamine-free DMEM supplemented with 1 mM [U-13C]-glutamine 7 h before harvesting polar metabolites with 80% methanol (with 10 ng/mL valve-d$_3$ as internal standard). Following 10 min vortex at 4 °C and a 10 min spin at 20,800 × g, samples were dried under nitrogen gas and resuspended in 100 μl water for analysis. Analysis was performed as previously described (33).

Bioenergetic Profiling. Oxygen consumption rate (OCR) and ECAR of intact cells were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). For OCR measurements, cells were seeded in four replicate wells on Seahorse XF24 cell culture plates in their standard growth media and assayed 24 h later. Two basal OCR measurements were taken in 9-min intervals, followed by sequential injections of 1. μl oligomycin, 4.μF CCP, and 1. μl antimycin A, taking two measurements at 9-min intervals following each treatment. Following analysis, the cells were trypsinized and counted for each well to normalize the OCR values. For basal ECAR measurements, cells were seeded in four replicate wells on Seahorse XF24 cell culture plates in their standard growth media, but lacking pyruvate. ECAR measurements were performed 24 h later after starving of glucose for 1 h and resaturation with 25 mM glucose for 35 min before the measurement. ECAR values were normalized to cell numbers.

SIXTOX Assay (Cell Death). Cell death assessment was performed using SYTOX staining (100 nM, SYTOX Green; Molecular Probes) followed by FACs analysis to measure relative SYTOX-positive cells as a function of total cells at 96 h post doxycycline induction of the indicated lentiviral shRNAs.

Cell Counting (with Trypan Blue Staining). Following 96 h of propagation in the absence or presence of doxycycline (to induce shRNA expression), cells were trypsinized and analyzed on the ViCell Analyzer for total cell numbers and percent of viable cells (Beckman). Trypan blue exclusion was used as an indicator of cell viability.

Metabolite Profiling and Analysis and Glucose Isoform Tracing. Metabolite extraction was performed as described previously (61). In brief, cells were cultured in low growth phase on 10-cm dishes and selected to transduced with non-puromycin or blasticidin the day after.

Cell Culture. The following human cancer cell lines were used: HT29, T47D, MDA-MB-453, KM12, HT53, C2BBe1, RKO, GP2D, DLD1, MCF7, SF767, AGS, SKCO1, LS411N, COV362, IGR39, Kuramochi, HCT116, TOV21G, and SU86.86. These cell lines were obtained from The Cancer Cell Line Encyclopedia (CCLE) - https://www.broadinstitute.org/ccle/home - and were originally acquired by CCLE from multiple commercial vendors. Before their use in Project Achilles and subsequent validation experiments, all cell lines were tested for mycoplasma contamination and SNP fingerprinted to confirm their identity (60).

All cell lines were cultured in DMEM containing 4.5 μg glucose (unless indicated otherwise), G L-glutamine, and sodium pyruvate (Life Technologies). The following cell lines were described previously (33).

(iii) FACS analysis

(iv) Glutamine Isotope Tracing.

(v) Glucose Isotope Tracing.

(vi) Bioenergetic Profiling.

(vii) Cell Counting (with Trypan Blue Staining).
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