Glucose-dependent acetylation of Rictor promotes targeted cancer therapy resistance

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Cancer cells adapt their signaling in response to nutrient availability. To uncover the mechanisms regulating this process and its functional consequences, we interrogated cell lines, mouse tumor models, and clinical samples of glioblastoma (GBM), the highly lethal brain cancer. We discovered that glucose or acetate is required for epidermal growth factor receptor vIII (EGFRvIII), the most common growth factor receptor mutation in GBM, to activate mechanistic target of rapamycin complex 2 (mTORC2) and promote tumor growth. Glucose or acetate promoted growth factor receptor signaling through acetyl-CoA–dependent acetylation of Rictor, a core component of the mTORC2 signaling complex. Remarkably, in the presence of elevated glucose levels, Rictor acetylation is maintained to form an autoactivation loop of mTORC2 even when the upstream components of the growth factor receptor signaling pathway are no longer active, thus rendering GBMs resistant to EGFR-, PI3K (phosphoinositide 3-kinase)-, or AKT (v-akt murine thymoma viral oncogene homolog)-targeted therapies. These results demonstrate that elevated nutrient levels can drive resistance to targeted cancer treatments and nominate mTORC2 as a central node for integrating growth factor signaling with nutrient availability in GBM.

mTORC2 | Rictor acetylation | metabolic reprogramming | targeted therapy resistance | glioblastoma

Cancer cells reprogram their metabolism, converting the majority of glucose they take up into lactate, even in the presence of sufficient oxygen to support oxidative phosphorylation (1, 2). This biochemical adaptation, known as the Warburg effect, enables cancer cells to meet the demands imposed by their rapid growth through the provision of glycolytic intermediates as carbon-containing precursors for macromolecular biosynthesis (3, 4). The enhanced flux of glucose-derived carbons into metabolic intermediates enables tumor cells to meet their energetic and anabolic demands and may also globally alter gene transcription and the epigenetic landscape through generation of acetyl CoA (acetyl-CoA) (5–7). Cancer cells, including glioblastoma (GBM) cells, also avidly take up acetate, using it as a bioenergetic substrate and for macromolecular biosynthesis and histone modification (8–10). Understanding how cancer cells harness cellular metabolism and its metabolites for their survival may yield insights into cancer pathogenesis and the mechanisms that tumor cells use to resist targeted therapies.

Cancer metabolic reprogramming is a consequence of upstream mutations in the growth factor receptor–phosphoinositide 3-kinase (PI3K)–v-akt murine thymoma viral oncogene homolog (AKT)–mechanistic target of rapamycin (mTOR) signaling network (1, 4, 11, 12), and resistance to small molecule inhibitors that target this signaling network is associated with sustained PI3K-AKT-mTOR signaling, or its key components (13, 14). However, it is unclear whether elevated levels of glucose and/or other nutrients may promote resistance to targeted therapies and, if so, whether nutrient metabolism may be implicated in maintaining signal flux through mTOR to drive resistance. Drugs that target PI3K-AKT-mTOR signaling commonly lead to hyperglycemia (15), and associated treatments like steroids also elevate blood glucose levels (16), conferring considerable importance on this question.

Here, we report the surprising discovery that glucose-dependent acetylation of Rictor, a central component of mechanistic target of rapamycin complex 2 (mTORC2), promotes tumor growth and resistance to epidermal growth factor receptor (EGFR)-, PI3K-, and AKT-targeted therapies, and we identify the signaling mechanism underpinning this previously unidentified form of cancer drug resistance.

Results

mTORC2 Signaling Is Regulated by Glucose and Acetate Levels Through Acetyl-CoA. To examine how tumor cells use cellular metabolism for their survival, we analyzed the effect of intermediary metabolites on mTORC2 activity, which is essential in a variety of cellular functions in cancer (17–19). We added exogenous glucose and acetate to U87 GBM cells and measured mTORC2 activation [p-AKT S473 and p-NDRG1 (*N*-Myc downstream-regulated gene 1) T346] (Fig. 1 *A* and *C*). The effect of glucose on mTORC2 kinase activity was also confirmed directly by in vitro kinase assay (Fig. 1*B*). Similar results were seen with U87 cells expressing

Significance

Cancer cells reprogram their metabolism in response to growth factor receptor mutations. However, the effect of altered nutrient levels on oncogenic signaling and therapeutic response is not well understood. We demonstrate that glucose or acetate, two abundant "fuel" sources in the brain, promote epidermal growth factor receptor vIII (EGFRvIII)-dependent signaling through activation of mechanistic target of rapamycin complex 2 (mTORC2) by acetylation of its core component Rictor. This activity is mediated through elevated levels of acetyl-CoA. A surprising implication of this study is that glucose or acetate can contribute to targeted therapy resistance by maintaining signaling through downstream components of the growth factor receptor signaling cascade.

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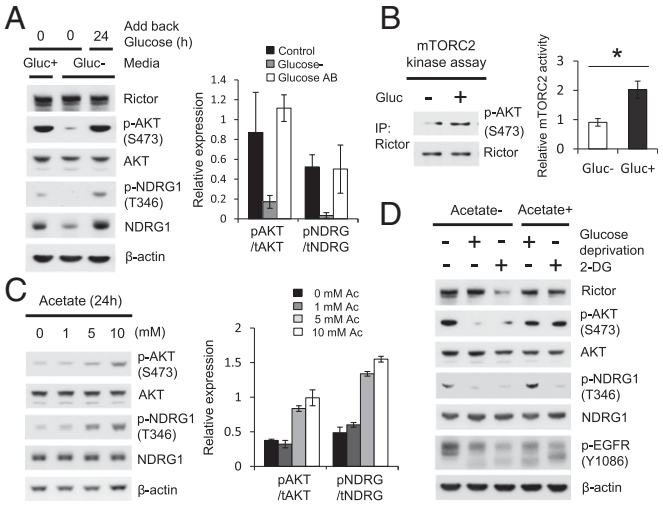

Fig. 1. Glucose or acetate promotes mTORC2 signaling. (*A*) Immunoblot analysis of mTORC2 activation (p-AKT S473, p-NDRG1) in U87 cells after 24 h of treatment with glucose deprivation (Gluc–), combined with an add-back (AB) of glucose for an additional 24 h; mean ± SEM of duplicates. tAKT, total AKT; tNDRG, total NDRG. (*B*) In vitro mTORC2 kinase assay to show the phosphorylation of the substrate protein AKT; mean ± SEM of duplicates. The lysates were prepared from U87-EGFRvIII cells that were cultured in the absence or presence of glucose (Gluc). (*C*) Quantified immunoblot analysis of mTORC2 activation in U87 cells with an indicated addition of acetate (Ac) for 24 h; mean ± SEM of duplicates. (*D*) Immunoblot analysis of mTORC2 activation in U87-EGFRvIII cells with 24 h of treatment with glucose deprivation or the glycolytic inhibitor 2-DG (10 mM) combined with an addition of exogenous acetate (50 mM).

EGFRvIII (constitutively active *EGFR* mutant) in which basal mTORC2 signaling is elevated, and glucose and acetate activated mTORC2 signaling in a dose- and time-dependent manner (Fig. S1*A*), suggesting that mTORC2 can respond to the availability of surrounding metabolites. Further, glucose deprivation or treatment with the glycolytic inhibitor 2-Deoxy-D-glucose (2-DG) reduced the mTORC2 activity, which was rescued by the addition of acetate (Fig. 1*D*), suggesting that mTORC2 can be activated by glycolytic intermediary metabolites produced from both glucose and acetate. Acetyl-CoA is one of the intermediate metabolites produced from both nutrients and is essential for cell growth and proliferation (20). We thus hypothesized that glucose and acetate can activate mTORC2 through acetyl-CoA production. We measured the level of acetyl-CoA in GBM cells exposed to various levels of glucose and acetate and found increases in the cellular production of acetyl-CoA (Fig. 2*A*). Consistent with this model, the production of acetyl-CoA depends on mTORC2 signaling (Fig. 2 *B* and *C*). Concurrent siRNA-mediated knockdown of pyruvate dehydrogenase (PDH), which is a critical step for glucose to be used for acetyl-CoA synthesis, and acyl-CoA synthetase short-chain family member 2 (ACSS2), the critical enzyme required by tumor cells for acetate utilization (8), suppressed AKT S473 phosphorylation, indicating that glucose and acetate may regulate mTORC2 activity through acetyl-CoA (Fig. 2*D*). Inhibition of both PDH and ACSS2 was required to lower the acetyl-CoA level and suppress AKT S473 phosphorylation (Fig. S1*B*), indicating that glucose and acetate converge on acetyl-CoA to regulate mTORC2 signaling. These results nominate mTORC2 as a critical integrator of nutrient status and growth factor receptor signaling and suggest that this activity is dependent upon levels of acetyl-CoA (Fig. 2*H*).

Rictor Acetylation Promotes mTORC2 Signaling. Lysine acetylation plays a critical role in regulating essential cellular functions (21, 22), including the activity of many metabolic enzymes (23, 24). Rictor contains several lysine residues that when acetylated increase mTORC2 activity, thus providing a critical link between nutrient-sensitive deacetylases and mTORC2 signaling (25). The acetyl-CoA produced from glucose and acetate could potentially provide the acetyl group (26) used for Rictor acetylation to regulate mTORC2 signaling (25). Therefore, we examined the possibility that glucose and acetate activate mTORC2 through acetyl-CoA–dependent

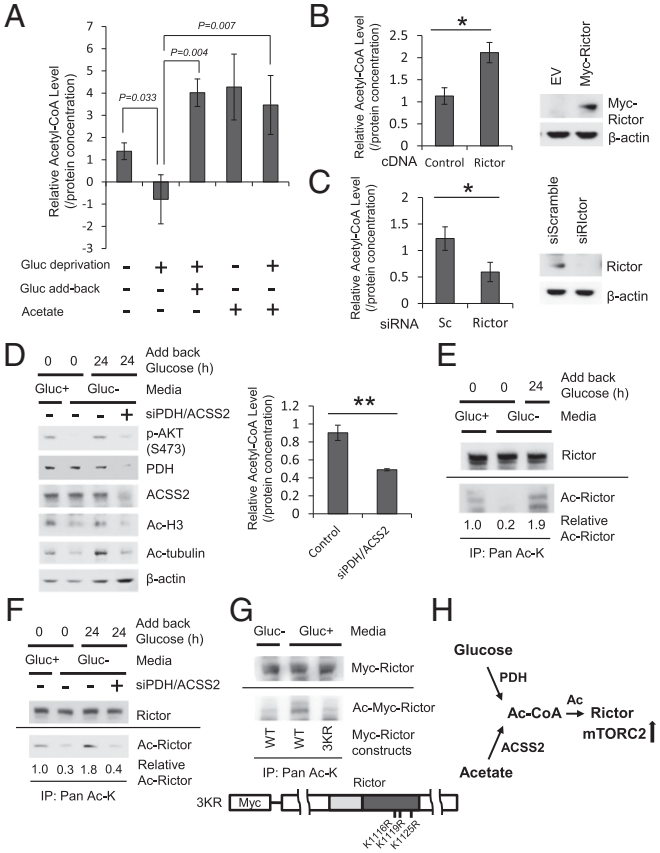

Fig. 2. Glucose or acetate promotes mTORC2 signaling via generation of acetyl-CoA and acetylation of Rictor. (*A*) Total cellular concentration of acetyl-CoA was measured in U87-EGFRvIII cells deprived of glucose, combined with an AB of glucose and an addition of exogenous acetate (50 mM) for 24 h; mean ± SEM of triplicates. (*B*) Total cellular concentration of acetyl-CoA was measured in U87 cells transfected with control (empty vector, EV) or Myc-Rictor plasmids; mean ± SEM of triplicates (*$P < 0.05$). (*C*) Total cellular concentration of acetyl-CoA in U87-EGFRvIII cells transfected with Scramble or Rictor siRNAs; mean ± SEM of triplicates (*$P < 0.05$). (*D*) Immunoblot analysis of mTORC2 activation in U87-EGFRvIII cells after 24 h of treatment with glucose deprivation (Gluc–), combined with an AB of glucose and siRNA-mediated knockdown of PDH and ACSS2 for an additional 24 h. Acetyl-histone 3 (Ac-H3) and acetyl-tubulin (Ac-tubulin) were used as controls to show the link between acetyl-CoA levels and protein acetylation. The bar graph shows total cellular concentration of acetyl-CoA in U87-EGFRvIII cells with control or PDH/ACSS2 siRNAs; mean ± SEM of triplicates (*$P < 0.05$, **$P < 0.01$). (*E*) IP analysis of Rictor acetylation in U87 cells from Fig. 1*A* after 24 h of treatment with glucose deprivation (Gluc–), combined with an AB of glucose for an additional 24 h. Ac-K, acetylated lysine; Ac-Rictor, acetylated Rictor. (*F*) Immunoblot analysis of Rictor acetylation in U87-EGFRvIII cells after 24 h of treatment with glucose deprivation (Gluc–), combined with an AB of glucose and siRNA-mediated knockdown of PDH and ACSS2 for an additional 24 h. (*G*) IP analysis of Myc-Rictor acetylation in U87 cells in the presence or absence of glucose, combined with an overexpression of wild-type (WT) or 3KR acetylation-resistant mutant of Rictor constructs. Schematic illustration represents Myc-tagged Rictor with lysine residues 1116, 1119, and 1125 replaced by R, which resists acetylation. (*H*) Glucose and acetate, which are the source for acetyl-CoA (Ac-CoA), facilitate the activation of mTORC2 via acetylation of Rictor.

Rictor acetylation. Consistent with the observed effects of glucose and acetate on mTORC2 activity (Fig. 1 *A* and *C*), either glucose or acetate was sufficient to increase Rictor acetylation in GBM cells including neurosphere cell lines (Fig. 2*E* and Fig. S2 *A* and *B*). Further, siRNA-mediated knockdown of both PDH and ACSS2, which reduced the intracellular acetyl-CoA level (Fig. 2*D*), suppressed the glucose-mediated acetylation of Rictor (Fig. 2*F*). This indicated that the effects of glucose and acetate on Rictor acetylation were mediated through acetyl-CoA. To better understand this phenomenon, we determined which lysine residues within Rictor were targets of glucose-mediated acetylation. Multiple putative acetylation sites were identified using a web server for KAT-specific Acetylation Site Prediction [ASEB (acetylation set enrichment-based) program] (27, 28) (Fig. S3*A*) and were located in the previously shown acetylation domains of Rictor (25). Of these, three lysine residues—K1116, K1119, and K1125—received high predictive scores (Fig. S3*A*) and are evolutionarily conserved from sea slugs to mammals (Fig. S3*B*). We then generated a series of Rictor deletion constructs in the predicted acetylation sites (Fig. S3*C*). Expression of the deletion construct of Rictor, Δ1110–1128, which eliminates lysine residues K1116, K1119, and K1125, resulted in a maximum loss in the activity of mTORC2 as demonstrated by phosphorylation of AKT and NDRG1 (Fig. S3*D*), indicating that these three lysines are the major acetylation sites of Rictor that are essential to promote mTORC2 activity. Additionally, to examine the importance of these lysine residues in glucose-mediated Rictor acetylation, we constructed a 3KR-mutant of Rictor that substitutes arginine (R) for lysine at K1116, K1119, and K1125 and confers resistance to acetylation. An immunoprecipitation (IP) analysis demonstrated that the presence of glucose significantly increased the acetylation of the wild-type Rictor construct but could not promote that of the 3KR mutant (Fig. 2*G*), indicating that K1116, K1119, and K1125 are essential in the glucose-dependent acetylation of Rictor. We further demonstrated that the substitution of the lysine residues by R (3KR) in Rictor indeed prevents glucose-driven augmentation of mTORC2 signaling (Fig. S4). These results demonstrate that glucose promotes Rictor acetylation to activate mTORC2 (Fig. 2*H*).

mTORC2 Forms an Autoactivation Loop Through Class IIa HDAC-Mediated Rictor Acetylation. Protein acetylation, including the acetylation of Rictor, can be controlled, at least in part, through the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities (25). We recently demonstrated that the lower levels of class IIa HDACs in *EGFR* mutant GBMs are achieved through inactivating phosphorylation by mTORC2 and possible subsequent degradation of HDAC4 and HDAC5 (19). Thus, if class IIa HDACs are the negative regulator of mTORC2 in response to extracellular glucose and acetate via deacetylation of Rictor, mTORC2 can establish a feed-forward autoactivation loop through inactivation of class IIa HDACs to keep Rictor in an acetylated state, maintaining downstream signaling.

First, we analyzed the downstream signaling of mTORC2, which regulates class IIa HDACs. Among the downstream components of mTORC2, including the AGC subfamily of kinases [AKT, serum and glucocorticoid-regulated kinase 1 (SGK1), and protein kinase Cα (PKCα)], genetic and pharmacologic analyses indicated PKCα phosphorylates and inactivates class IIa HDACs (Fig. S5 *A–D*). Although the residues in class IIa HDACs phosphorylated by PKCα are relatively well-conserved across the species (Fig. S5*E*), they are not perfectly matched to the consensus phosphorylation motif for PKC (29). We further showed that protein kinase D (PKD) may be involved in the PKCα-mediated phosphorylation of class IIa HDACs downstream of mTORC2 (Fig. S5*F*), as has been suggested previously (30).

Next, we investigated whether phosphorylation of HDAC4 and HDAC5 is involved in maintaining mTORC2 signaling by keeping Rictor in an acetylated state. Rictor physically associated with class IIa HDACs, and their interaction was enhanced by glucose and acetate (Fig. S6 *A* and *B*). Knockdown of class IIa HDACs significantly increased Rictor acetylation (Fig. S6*C*), whereas overexpression of class IIa HDACs reduced acetylation of Rictor, and this effect was more prominent when we used phosphorylation-resistant mutants of class IIa HDACs (Fig. S6*D*). Compatible with the status of Rictor acetylation, overexpression of class IIa HDACs reduced mTORC2 activity (Fig. S6*E*). Further, siRNA suppression of class IIa HDACs increased mTORC2 activity, and this was reversed by concurrent glucose deprivation (Fig. S6*F*), suggesting the involvement of acetyl-CoA. Finally, the deletion 1110–1128 construct (that removed lysines 1116, 1119, and 1125) was the most potent in suppressing class IIa HDAC-mediated Rictor acetylation (Fig. S6*G*), and reduction of mTORC2 activity by the overexpression of class IIa HDAC was rescued not by wild-type Rictor but by the 3KQ-mutant of Rictor, which has the substitution of glutamine (Q) for lysines K1116, K1119, and K1125 that mimics constitutive acetylation (Fig. S6*H*). Together, these results indicate that class IIa HDACs are regulated by mTORC2 through PKCα, forming an autoactivation loop of mTORC2 (Fig. S6*I*).

mTORC2–Class IIa HDAC–Rictor Acetylation Circuits in Vivo and in Patients. To explore the in vivo and clinical implications of the regulation of class IIa HDACs by mTORC2, and resultant Rictor acetylation, we analyzed GBM xenograft mouse models and primary human GBM samples. Immunoblot analysis of GBM xenografts revealed that Rictor knockdown reduced the activity of mTORC2 downstream signaling (p-AKT S473, p-NDRG1, and p-PKCα) (Fig. S7*A*). Cell-based analyses of immunostained GBM xenografts showed the association between class IIa HDAC phosphorylation and Rictor expression (Fig. S7*B*). Differences in HDAC phosphorylation may be alternatively explained by different rates of tumor progression in shScramble and shRictor tumor cells, as class IIa HDAC phosphorylation was recently demonstrated to be involved in mitogenic signaling (31). Immunohistochemical analysis of primary human brain and GBM samples (26 normal brain and 60 GBM) revealed that Rictor expression and class IIa HDAC phosphorylation were both elevated in GBMs relative to the normal brain (Rictor, $P = 0.0209$; phosphorylated class IIa HDACs, $P = 0.0005$) (Fig. S8*A*). Rictor expression and class IIa HDAC phosphorylation were significantly intercorrelated with each other ($P = 0.002$) (Fig. S8*B*). Compatible with the histological findings, IP analysis of GBM autopsy samples confirmed coordinate increases in the acetylation of Rictor as well as the phosphorylation of class IIa HDACs in tumor tissue relative to the contralateral normal brain (Fig. S8*C*). Consistent with our model that Rictor is regulated primarily by acetylation, and not by elevation of its transcript level, Rictor mRNA was not overexpressed relative to the normal brain in The Cancer Genome Atlas database of GBMs (Fig. S9 *A* and *B*). These results indicate that mTORC2 signaling, phosphorylation of class IIa HDACs, and Rictor acetylation are coordinately up-regulated in human GBM patients and may be involved in the GBM pathogenesis in the clinic.

Acetylated Rictor Makes GBM Cells Resistant to Loss of EGFR Signaling. Having shown that mTORC2 can establish an autoactivation feed-forward loop through Rictor acetylation by promoting acetyl-CoA production and inhibiting the activity of class IIa HDACs, we reasoned that mTORC2 activation would not depend on the upstream stimulation of EGFR after activation. To test this, we used GBM cell lines with tetracycline-regulated EGFRvIII and examined the effect of EGFR inhibition on mTORC2 activity. Consistent with our hypothesis, the expression of constitutively active EGFRvIII promoted Rictor acetylation (Fig. 3*A*), and once acetylated, the acetylation of Rictor persisted even after EGFR was turned off (Fig. 3*B*). In accordance with the status of Rictor

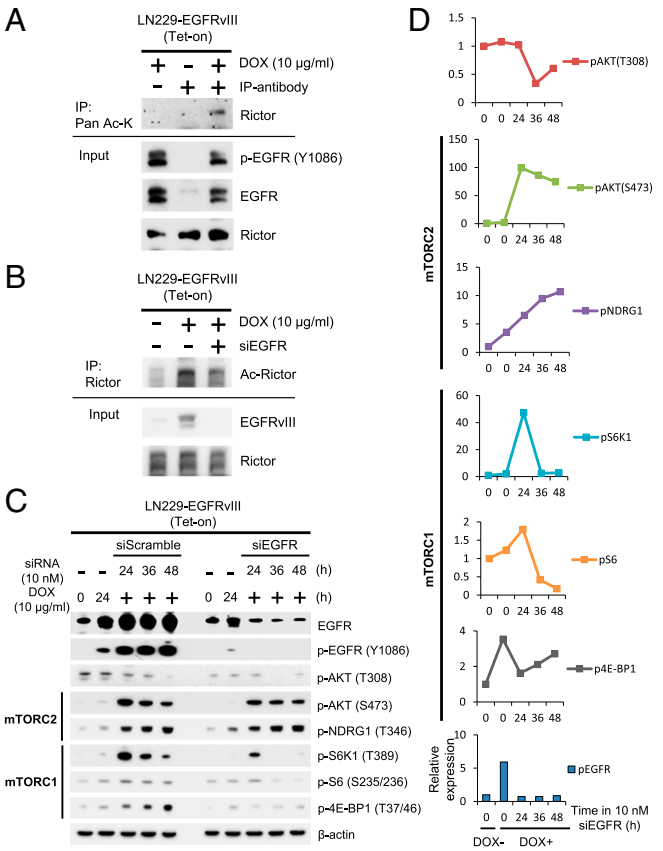

Fig. 3. Acetylated Rictor makes GBM cells resistant to loss of EGFR signaling. (*A*) IP assessment of the acetylation status of Rictor in LN229 GBM cells with doxycycline-inducible (Tet-on) EGFRvIII. Rictor acetylation corresponds to the activation of EGFR signaling. DOX, doxycycline. (*B*) IP assessment of the acetylation status of Rictor in LN229 GBM cells with doxycycline-inducible (Tet-on) EGFRvIII. Rictor acetylation is persistent after the loss of EGFR signaling by siRNA. (*C*) Immunoblot assessment of mTORC2 (p-AKT S473 and p-NDRG1 T346) and mTORC1 activation (p-S6K1 T389, p-S6 S235/236, and p-4E-BP1 T37/46) in LN229 cells with doxycycline-inducible (Tet-on) EGFRvIII, combined with time-course inhibition of EGFR by siRNAs. (*D*) Relative activation of mTORC2 and mTORC1 under EGFR inhibition, which was quantified using the immunoblot data in *C*. Note the relative conservation of mTORC2 signaling compared with mTORC1 signaling following the inhibition of EGFR signaling. Result is representative of two independent experiments.

acetylation, EGFR stimulation promoted both mTORC1 (p-S6K1, p-S6, and p-4E-BP1) and mTORC2 (p-AKT S473 and p-NDRG1) activities, and subsequent inhibition of EGFR suppressed mTORC1 signaling in a time-dependent manner. In contrast, mTORC2 signaling displayed resistance to EGFR loss and was persistently activated (Fig. 3 *C* and *D* and Fig. S10 *A* and *B*). Taken together, these results indicate that GBM cells maintain mTORC2 signaling even under the suppression of its upstream stimulator, EGFR, through an acetylation-mediated autoactivation loop.

Persistent Rictor Acetylation in the Presence of Glucose Renders GBM Cells Resistant to EGFR-, PI3K-, and AKT-Targeted Therapies. If an acetylation-dependent autoactivation loop maintains mTORC2 signaling independent of upstream stimulation, then GBM cells with activated mTORC2 would be expected to be resistant to therapies that target its upstream elements including EGFR and PI3K (32). Consistent with this hypothesis, pharmacological inhibition of EGFR, PI3K, AKT, mTORC1, and mitogen-activated protein kinase (MAPK) failed to suppress phosphorylation of class IIa HDACs, Rictor acetylation, or AKT-independent mTORC2 signaling. Only PP242 (which inhibits both mTORC1 and mTORC2) reduced them (Fig. 4*A*). Additionally, an already activated "feed-forward autoactivation loop" through prior glucose stimulation can be efficiently stopped by mTOR inhibitor PP242 once it has been activated (Fig. S11). PI3K and AKT inhibitors showed some effect on Rictor acetylation without significantly impacting HDAC phosphorylation, suggesting that the PI3K/AKT pathway may affect Rictor acetylation independent of HDAC via an acetyl-CoA–producing enzyme such as ATP citrate lyase (33). Further, although PI3K inhibition alone could not suppress class IIa HDAC phosphorylation and Rictor acetylation (Fig. 4*A*), dual inhibition of PI3K and mTORC2 efficiently suppressed class IIa HDAC phosphorylation and Rictor acetylation in GBM cells including neurosphere cell lines (Fig. 4*B* and Fig. S12*A*), suggesting the potential effectiveness of a dual PI3K/mTOR inhibitor to overcome the resistance of GBM cells to monotargeted therapies. Finally, we evaluated the effect of class IIa HDAC and Rictor acetylation on cell proliferation in response to EGFR, PI3K, or AKT inhibitors. Introduction of class IIa HDAC construct HDAC4-3SA, which could not be phosphorylated and thus reduces Rictor acetylation more than the wild-type HDAC4 construct, sensitized GBM cells to EGFR-, PI3K-, or AKT-targeted inhibitors (Fig. S12*B*). In contrast, the GBM cells were more sensitive to the dual PI3K/mTOR inhibitor XL765, which blocked mTORC2 signaling (Fig. 4*B*), and the nonphosphorylatable HDAC4-3SA construct did not provide additional benefit (Fig. S12*B*). Further, knockdown of class IIa HDACs rendered GBM cells resistant to individual PI3K- and AKT-targeted therapies, but not when treated with this dual PI3K/mTOR inhibitor (Fig. S12*C*). Overexpression of the nonphosphorylatable HDAC4-3SA mutant made GBM cells vulnerable to EGFR, PI3K, and AKT inhibitors, which could be partially reversed by wild-type Rictor and much more so by the Rictor 3KQ mutant, which mimics constitutive acetylation (Fig. 4*C*). Compatible with these findings, glucose deprivation reduced Rictor acetylation and mTORC2 signaling and sensitized GBM cells to EGFR, PI3K, and AKT inhibitors (Fig. 4 *D* and *E*). Overexpression of a constitutively acetylated form (3KQ) of Rictor impacts cell proliferation and targeted therapy responses under glucose deprivation (Fig. 4*F*), indicating that Rictor acetylation is important in glucose-mediated targeted therapy resistance. Taken together, these results indicate that glucose-dependent acetylation of Rictor makes tumor cells resistant to therapies targeting key components of a growth factor receptor signaling pathway commonly hyperactivated in cancer by maintaining persistent downstream signaling (Fig. 4*G*).

Discussion

Dysregulation of cellular metabolism is a hallmark of cancer (2, 34). In addition to the proposed role of cancer metabolic reprogramming, which enables cancer cells to obtain a sufficient supply of macromolecular precursors required for rapid cellular proliferation while still meeting their energy requirements (3), it is important to decipher the mechanism(s) by which cancer cells use this metabolic shift to maximize their growth. In the present study, we have uncovered an acetylation-dependent mechanism of mTORC2 activation that could be promoted by cancer metabolic reprogramming, including the production of acetyl-CoA (Fig. 4*G*).

A recent report demonstrated that glucose availability can affect histone acetylation in response to growth factor stimulation, linking growth factor-induced increases in nutrient metabolism to the regulation of histone acetylation and gene expression (35, 36). Further, GBM cells avidly take up and use acetate as a fuel source and for macromolecular biosynthesis through its conversion into acetyl-CoA (8–10). At present, the role of nonnuclear protein acetylation and its role in cellular regulation and cancer are less well understood, but recent mass spectrometry-based proteomic analyses have identified a large number of potentially acetylated proteins, suggesting that reversible lysine acetylation might be a major regulatory mechanism in vital cellular functions (21–23). HDAC inhibitors, an emerging class of anticancer drugs,

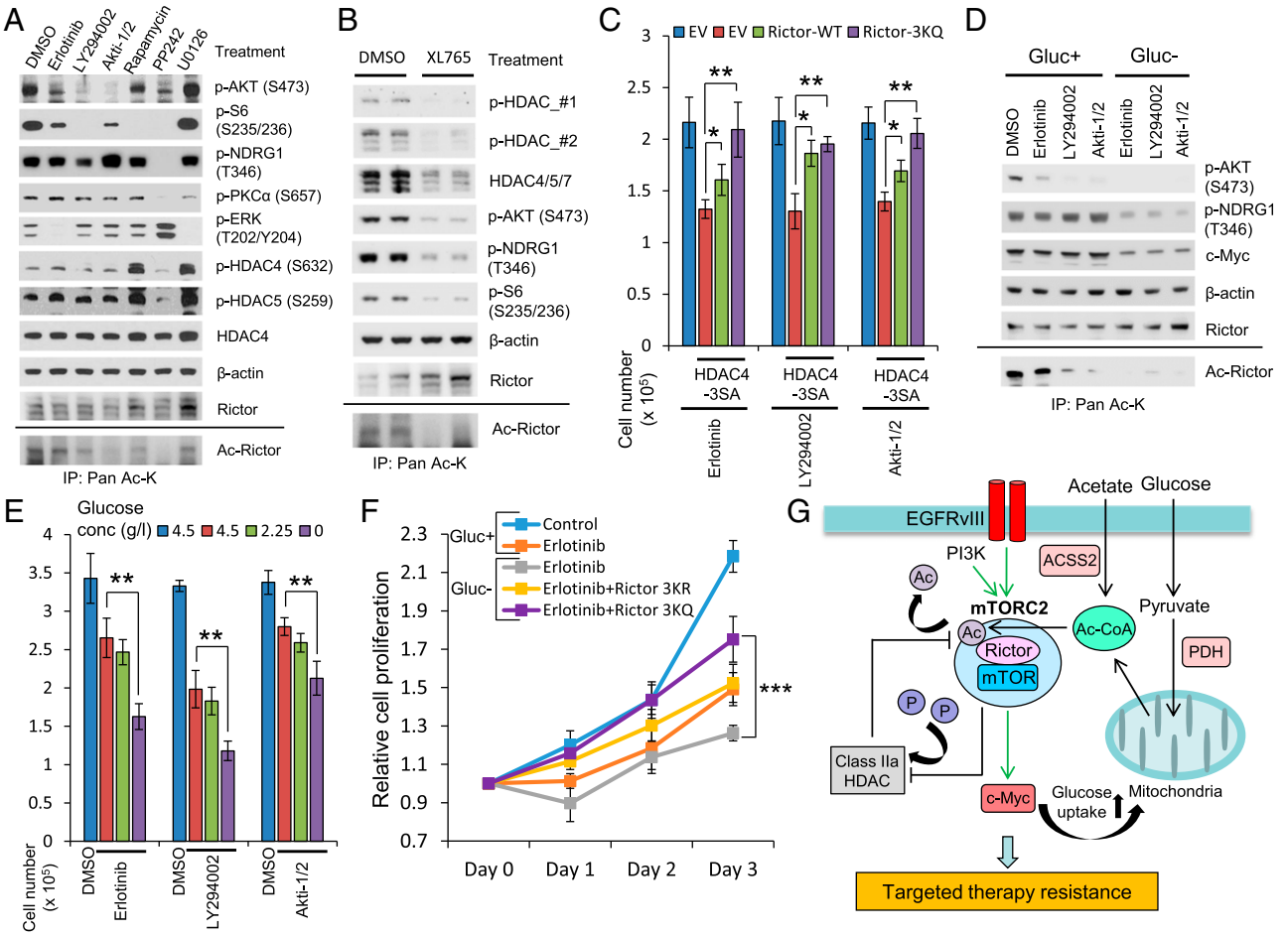

Fig. 4. Persistent Rictor acetylation renders GBM cells resistant to EGFR-, PI3K-, and AKT-targeted therapies. (*A*) Immunoblot analysis of the effect of the drugs on class IIa HDAC phosphorylation and Rictor acetylation in U87-EGFRvIII cells treated with various inhibitors against EGFR-PI3K/AKT-mTOR signaling. Akti-1/2, Akt inhibitor; DMSO, dimethyl sulfoxide; Erlotinib, EGFR inhibitor; LY294002, PI3K inhibitor; PP242, mTORC1/C2 inhibitor; Rapamycin, mTORC1 inhibitor; U0126, MAPK/ERK inhibitor. (*B*) Immunoblot analysis of the effect on class IIa HDAC phosphorylation and Rictor acetylation in U87-EGFRvIII cells treated with the dual PI3K/mTOR inhibitor XL765. p-HDAC4/5/7_#1, p-HDAC4 (Ser632)/HDAC5 (Ser498)/HDAC7 (Ser486); p-HDAC4/5/7_#2, p-HDAC4 (Ser246)/HDAC5 (Ser259)/HDAC7 (Ser155). (*C*) Decrease in cell proliferation of HDAC4-overexpressing U87-EGFRvIII cells treated by EGFR inhibitor (Erlotinib), pan PI3K inhibitor (LY294002), and AKT inhibitor (Akti-1/2) for 24 h was rescued mildly by wild-type Rictor and significantly by the 3KQ-Rictor mutant, which mimics constitutive acetylation; mean ± SD of triplicates (**$P < 0.01$). (*D*) Immunoblots demonstrated that glucose deprivation for 24 h augmented the effect of the EGFR inhibitor (Erlotinib), pan PI3K inhibitor (LY294002), and AKT inhibitor (Akti-1/2) in suppressing mTORC2 activity of U87-EGFRvIII cells including p-AKT S473, p-NDRG1, and c-Myc (19). (*E*) Glucose deprivation for 24 h augmented the effect of the EGFR inhibitor (Erlotinib), pan PI3K inhibitor (LY294002), and AKT inhibitor (Akti-1/2) in suppressing cell proliferation of U87-EGFRvIII cells; mean ± SD of triplicates (**$P < 0.01$). (*F*) Cell proliferation assay of U87-EGFRvIII cells treated by a combination of Erlotinib, glucose deprivation, and overexpression of Rictor-3KR or 3KQ mutants. $P < 0.01$ for comparison between cells treated with Erlotinib/Glucose- and 3KQ-overexpressing cells. Error bars, ±SD. (*G*) mTORC2 forms an autoactivation loop (*i*) by promoting glucose uptake and acetyl-CoA production through its downstream pathways of c-Myc (19) and (*ii*) by inactivating class IIa HDACs, which deacetylate Rictor and suppress mTORC2. By these mechanisms, GBM cells with activated mTORC2 are resistant to targeted therapies toward their upstream stimulators including EGFR and PI3K as well as their downstream effector AKT. The concentration of each drug is provided in *SI Materials and Methods*.

can induce glioma cell death via induction of mitotic catastrophe (37), but we demonstrate that HDAC inhibition renders GBM cells resistant to targeted therapies due to elevated levels of Rictor acetylation. Acetylation of nuclear and cytoplasmic proteins may play a different role in cancer biology. The major acetyl group donor for protein acetylation is acetyl-CoA, which is a key metabolic intermediate produced and consumed by many metabolic reactions (7, 26). EGFR-mTOR signaling could elevate acetyl-CoA levels in GBM through glycolysis, glutaminolysis, and β-oxidation of fatty acids (6). Here we report a previously unidentified critical mechanism by which altered growth factor receptor signaling in cancer is "tuned" in response to glucose and acetate levels via protein acetylation, promoting the persistent growth of tumor cells in response to nutrients and rendering them insensitive to targeted treatments by maintaining key components of the downstream growth factor signaling cascade. We anticipate that mTORC2, like mTORC1, which plays a critical role in integrating cellular metabolism with signal transduction, will prove to be one among a number of key nodes that iteratively integrate oncogenic signaling and the nutrient environment to drive tumor growth in response to environmental constraints.

A surprising implication of this study is that GBM cells maintain mTORC2 signaling and cell survival through acetylation-dependent feed-forward activation of mTORC2 to maintain downstream signaling even after tumor cells are treated with inhibitors that target key upstream components of the growth factor receptor signaling system to which they are "addicted" (38). These observations make the previously unanticipated prediction that GBM cells may use nutrients to escape targeted therapies and at the same time provide a compelling rationale for the combined inhibition of EGFR/PI3K/AKT

and mTORC2, or interference with metabolic pathways by drugs such as a Myc inhibitor, to treat the deadly brain cancer GBM. AKT-independent mTORC2 signaling through NDRG1 has been shown to promote O-6-methylguanine-DNA methyltransferase (MGMT)-dependent resistance to alkylating chemotherapy in GBM, raising the possibility that glucose and acetate-dependent maintenance of mTORC2 signaling could contribute to temozolomide resistance (39). This also raises the possibility that targeted dietary interventions could potentially have an impact on the therapeutic response. Indeed, we demonstrate that glucose promotes Rictor acetylation and activates mTORC2 signaling, which was reduced by the concurrent suppression of acetyl-CoA production, indicating that the autoactivation loop cannot maintain itself irrespective of nutrition once activated. Thus, interference with nutritional condition or metabolism might sever the autoactivation loop of mTORC2. However, the ability of tumor cells to maintain Rictor acetylation in response to acetate when glucose is not available (Figs. 1*D* and 2 *A* and *D*) suggests that a ketogenic diet may not be sufficient to prevent nutrient-dependent maintenance of mTORC2 signaling. Dexamethasone, which is commonly given to GBM patients to limit brain swelling, is associated with hyperglycemia and shorter survival in patients (16). Future studies will be needed to examine the impact of diet and steroids on response to targeted therapy resistance.

Cancer is once again being recognized as a metabolic disease involving disturbances in energy production through respiration and fermentation (40). Cancer cells exploit metabolic adaptations such as the Warburg effect to meet their elevated anabolic and energetic demands (1), which may produce major global shifts in their epigenetic landscapes that favor tumor growth and survival (5, 7). The data presented here suggest that cancer metabolic adaptations also play a central role in resistance to targeted cancer therapies (Fig. 4*G*).

Materials and Methods

For IP analyses, cells were lysed with the Pierce IP Lysis Buffer [25 mM Tris•HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 5% (vol/vol) glycerol], supplemented with phosphatase and protease inhibitors (Thermo Scientific). Cell lysates were incubated overnight at 4 °C with 50 μL of the Dynabeads Protein A (Invitrogen) conjugated with 5 μL of each antibody. After washing three times with ice-cold PBS with Tween-20, the beads were boiled with denaturing elution buffer, and the eluted protein was analyzed by SDS/PAGE and immunoblotting. Details for cell culture, reagents, Western blot, mTORC2 kinase assay, acetyl-CoA measurement, Tet-regulatable EGFRvIII expression system, immunohistochemical analysis, protein acetylation site prediction, glucose and acetate addition cell analysis, site-directed mutagenesis, human and animal studies, and statistical analysis are provided in *SI Materials and Methods*. All in vivo experiments were conducted after approval by the Chancellor's Animal Research Committee of the University of California, Los Angeles (UCLA). GBM samples were acquired from the UCLA-affiliated hospitals. Physicians obtained informed consent from the patients. The procedures related to human subjects were performed according to an institutional review board-approved protocol by the Brain Tumor Translational Resource at UCLA.

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