mTORC2 links growth factor signaling with epigenetic regulation of iron metabolism in glioblastoma

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Abstract

In cancer, aberrant growth factor receptor signaling reprograms cellular metabolism and global gene transcription to drive aggressive growth, but the underlying mechanisms are not well understood. Here we show that in the highly lethal brain tumor glioblastoma (GBM), mechanistic target of rapamycin complex 2 (mTORC2), a critical core component of the growth factor signaling system, couples acetyl-CoA production with nuclear translocation of histone-modifying enzymes including pyruvate dehydrogenase (PDH) and class IIa histone deacetylases (HDACs) to globally alter histone acetylation. Integrated analyses in orthotopic mouse models and in clinical GBM samples reveal that mTORC2 controls iron metabolisms via histone H3 acetylation of the iron-related gene promoter, promoting tumor cell survival. These results nominate mTORC2 as a critical epigenetic regulator of iron metabolism in cancer.

Introduction

Metabolic reprogramming is a core hallmark of cancer (1, 2), enabling cancer cells to meet the coordinately elevated anabolic and energetic demands imposed by rapid tumor growth (3). Aberrant growth factor receptor signaling, frequently as a consequence of receptor tyrosine kinase (RTK) amplification and/or gain of function mutations, promotes tumor growth by coupling proliferative signaling with enhanced nutrient uptake and utilization. This process also alters gene expression, including via epigenetic regulation (4), although the molecular mechanisms are still incompletely understood. Persistent growth factor receptor signaling can enhance the cellular pool of acetyl-CoA, which provides the acetyl groups needed for histone acetylation (5, 6). At present, the components of the growth factor signaling system that link oncogenic mutations with epigenetic regulation, and the specific genes that may be regulated by this process, mandate further study.

We focus on mechanistic target of rapamycin complex 2 (mTORC2), a core component of the growth factor signaling in glioblastoma (GBM), the highly lethal form of brain tumor. Epidermal growth factor receptor (EGFR) is amplified in over 50% of GBMs (7), commonly in association with the gain of function mutation EGFRvIII. We have previously shown that EGFRvIII potently activates mTORC2, which is required for GBM pathogenesis via its effect on glucose, amino acid, and lipid metabolism (8-11). We have also shown that mTORC2 integrates aberrant growth factor signaling with glucose and amino acid availability, ensuring that aberrant growth factor receptor signaling is coupled with
sufficient environmental nutrients to support rapid tumor growth (9, 11, 12). We hypothesized that mTORC2 could also play a role in gene regulation, by controlling histone acetylation. Here, we report a previously unanticipated role for mTORC2 in driving GBM growth by promoter histone acetylation of iron metabolism genes.

Results

To examine the histone acetylation status in human GBM, we performed quantitative immunohistochemical analyses of EGFR/mTORC2 signaling, glycolytic enzyme lactate dehydrogenase A (LDHA) expression, and acetylation of histone H3 tails including lysines 9, 18 and 27 (H3K9ac, K18ac and K27ac) in surgical and autopsied cases of human GBM, revealing increased H3K9ac, K18ac and K27ac in EGFR/mTORC2-activated GBM clinical samples (Fig. 1, A-C and Fig. S1, B and D). Notably, GBM and breast cancer specimens display that EGFR-highly expressing tumor cells increase histone acetylation within tumor (Fig. S1C), suggesting that genetic and epigenetic interaction may be a phenomenon observed in a broader range of cancer types. Similar to persistent EGFR signaling, constitutive platelet-derived growth factor (PDGF)-mediated activation in vivo, using a retrovirus-mediated PDGF ligand overexpression to activate PDGF receptor (PDGFR) signaling induced GBM tumors including cerebrum as well as brainstem, with tumor cells that similarly displayed high levels of acetylated histone H3 in comparison with the control non-neoplastic brain regions (Fig. 2B and Fig. S2A). The expression of H3 acetylation was correlated with a proliferative marker Ki-67 (Fig. 2C). Interestingly, even in the control rat brain, H3 acetylation was observed in specific regions such as in the white matter and the subventricular zone (Fig. S2B), and these might reflect the glioma-initiating cells (13, 14). We next interrogated signaling pathways downstream of PDGF to identify those responsible for promoting histone acetylation. The level of mTORC2 signaling markers was compatible with the histone acetylation level in the tumor (Fig. 2, A and D and Fig. S2A). These data indicate that RTK-mTORC2-dependent histone acetylation is prevalent among GBM driven either by EGFR or PDGFR signaling.

We next examined whether mTORC2 directly regulates histone acetylation. Lentivirus-mediated genetic knockdown of Rictor (a core component of mTORC2) by shRNA decreases nuclear expression of histone acetylation in GBM cells (Fig. 3A). Of note, siRNA-mediated knockdown studies on signaling components downstream of EGFR
demonstrated that mTORC2 inhibition potently decreases histone acetylation whereas Akt, one of the effectors downstream of mTORC2, has the mild effect on it (Fig. 3B). We further investigated the effector to regulate histone acetylation downstream of mTORC2 and found that both Akt and SGK1 are the potential regulator of histone acetylation downstream of mTORC2 (Fig. 3C). We and others speculate that the level of intermediary metabolites derived from metabolic reactions could have a profound effect on epigenetic regulation (5, 6). Indeed, metabolomic analyses demonstrated the drastic change in intermediary metabolites by mTORC2 inhibition (Fig. S3, A and B).

To examine the effect of metabolic reprogramming on histone acetylation, we analyzed the role of acetyl-CoA production on the acetylation of histone protein. Glucose and acetate are two major nutrients for acetyl-CoA production (15), and we found that glucose deprivation from the culture media or PP242 treatment (mTORC2 inhibition) significantly decreased global histone H3 acetylation, which was restored by the addition of exogenous acetate (Fig. S3, C and D). Further, Rictor overexpression augmented the histone H3K9 acetylation, and glucose withdrawal abrogated the effect of Rictor overexpression on histone acetylation, which was further rescued by the addition of acetate (Fig. S3E). Together, these findings indicate that mTORC2 facilitates histone acetylation via metabolic reprogramming and the production of a metabolite acetyl-CoA.

Acetyl-CoA-producing enzymes were reported to promote histone acetylation via metabolic reprogramming (16, 17). A recent report demonstrated that nuclear translocation of pyruvate dehydrogenase (PDH) complexes from mitochondria along with pyruvate kinase M2 (PKM2) isoform increase histone acetylation (20, 21). We found that mTORC2 activity significantly regulated the subcellular localization of PDH as well as mildly that of PKM2 (Fig. 4, A and B). Supporting our hypothesis, knockdown of Rictor decreased the total, cytoplasmic as well as nuclear level of acetyl-CoA (Fig. 4C), important in the acetylation of nuclear histone (16, 21). Consistent with this, an increase in global H3 histone acetylation by Rictor overexpression was mitigated by the concurrent knockdown of PDH (Fig. 4D). Another important source of acetyl-CoA was from glutamine via anaplerotic reactions (i.e. glutaminolysis) (22), and it may be related to mTORC2-dependent histone acetylation. However, the effect of glutaminolysis inhibitor (BPTES) on mTORC2-mediated histone acetylation was at least partly independent of the status of Rictor (Fig. S4), suggesting that PDH is the main enzyme to control acetyl-CoA and histone acetylation.
downstream of mTORC2. In addition to the acetyl-donor substrate (acetyl-CoA), histone tail acetylation necessitates the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) (18, 19). Considering the previous reports that mTORC2 mainly affects the status of class IIa HDACs among various HATs and HDACs (8), mTORC2 may promote the histone acetylation through the regulation of class IIa HDACs. We thus examined the additional effect of class IIa HDACs on mTORC2-mediated histone acetylation. Overexpression of class IIa HDACs in GBM cells with mTORC2 activation by EGFRvIII reduced histone acetylation, but had no effect on the methylation of histone H3 tails (Fig. 4E). Furthermore, reduction of histone acetylation by Rictor knockdown was partially rescued by the pharmacological inhibition of class IIa HDACs with tricostatin A (TSA) (Fig. 4F). Taken together, mTORC2 promotes histone acetylation both by increasing the acetyl-donor substrate via shifting the subcellular localization of acetyl-CoA producing enzymes PDH as well as by affecting the activity of histone modifying enzymes, class IIa HDACs.

Having shown that histone acetylation is dynamically controlled by mTORC2 through metabolic reprogramming, we next examined its epigenetic effect on the expression of tumor-promoting genes since histone acetylation is generally considered to relax the chromatin configuration and facilitate the gene transcription (23, 24). Notably, pathway analyses on RNA-sequencing-based transcriptome data demonstrated that mTORC2 inhibition affected the expression of genes related to not only RTK signaling, glioma and metabolism (Fig. S5A), but also reactive oxygen species (ROS) and mineral metabolism as well as glucose metabolism (Fig. 5A), suggesting that mTORC2 may regulate the genes related to iron metabolism in cancer cells through the epigenetic shift. Consistent with this idea, genes related to iron metabolism were upregulated in GBM cells treated with TSA and acetate which potentially increase the histone acetylation (Fig. S5B), further supporting the idea that iron-related genes could be epigenetically upregulated in cancer cells. On the contrary, RNA-seq data demonstrated that the expression of transcription factors to potentially regulate the ferritin gene such as SP1 (promotion) and ATF1 (repression) was not significantly different between control and Rictor-knockdown GBM cells (Fig. S5C) (25, 26). The histone mark of H3K9ac is a known modification of promoted transcription (27, 28), and we performed the ChIP-sequencing analyses to determine the effect of mTORC2 on the gene regulation through this histone modification. The twin peaks of H3K9ac
were observed within 1,000 bps around the transcription start site (TSS) (Fig. 5B), indicating its association with the gene promoter regions. Interestingly, mTORC2 suppression by Rictor knockdown reduced these dual peaks (Fig. 5B). We then sought after the specific groups of the genes regulated by this epigenetic shift, and unexpectedly, mTORC2 inhibition clearly reduced the H3K9ac peaks around TSS along with the expression of the genes relevant to the iron metabolism such as transferrin receptor (TFR), ferritin light chain (FTL) and heavy chain (FTH1) (Fig. 5C), which are essential in maintaining iron metabolism (29). Notably, mTORC2 activation by overexpression of Rictor promoted the H3K9 acetylation marks at the promoter region of FTL, which was abrogated by the concurrent withdrawal of glucose (Fig. 5D). Consistent with this, decreased expression of FTL transcripts by mTORC2 inhibition was recovered by the treatment with TSA (HDAC inhibition), acetate or 5-Aza-2'-deoxycytidine (DNA methylation inhibitor) (Fig. 5E and Fig. S5D), and knockdown of PDH and PKM2 reduced the level of FTL and FTH1 transcripts (Fig. S5E), suggesting the involvement of histone acetylation and potentially subsequent DNA demethylation on this process. These findings indicate that mTORC2 increases the expression of iron-related genes through the regulation of histone acetylation at their promoters.

We lastly determined the functional role of mTORC2-dependent regulation of the components in iron metabolism. TCGA (The Cancer Genome Atlas) data sets (30, 31) demonstrated that the expression of iron metabolism-related genes such as TERC, FTL and FTH1 was upregulated in GBM in comparison with lower grade gliomas (LGG) which usually exhibit less activity of the mTOR signaling pathway (Fig. S6A). Human GBM (IDH-wildtype) tissue showed higher staining of ferritin molecules and Fe in comparison with normal brain (Fig. S6B). Further, expression of ferritin in surgical GBM specimens was concordant with the mTORC2 activation marker and the acetylated histone mark (Fig. 6A). Consistent with the clinical findings, GBM cells with Rictor knockdown markedly downregulated the expression of iron transporters such as TFR and divalent metal transporter 1 (DMT1) (Fig. 6B and Fig. S6C). More importantly, intracellular concentration of total iron was significantly reduced by Rictor knockdown (Fig. 6C and Fig. S6D). We then hypothesized that mTORC2 signaling may render GBM cells addicted to iron metabolism for their survival. Of note, promotion of GBM cell proliferation by Rictor overexpression was mitigated by the simultaneous knockdown of ferritin genes (FTL and FTH1) (Fig. 6D and Fig. S6E). Further, exogenous addition of iron promotes the proliferation of GBM cells in...
an mTORC2-dependent manner (Fig. 6E). Taken together, these results indicate that GBM cells with activated mTORC2 signaling facilitate cellular survival by enhancing iron metabolism through the regulation of acetylated histones in their promoters (Fig. 6F).

**Discussion**

Amplification and/or gain of function mutations in cancer are prevalent because they sit at the top of a cascade of interlacing events that integrate proliferative signaling and nutrient uptake and utilization with epigenetic regulation and gene transcription. Dissecting the critical nodes that integrate environmental nutrient levels with genetic alterations will be critical for better understanding cancer pathogenesis and for identifying potential therapeutic targets (32). Here, we have found that mTORC2 promotes GBM growth by altering iron metabolism, and we demonstrate that this activity is mediated by promoter histone modification. These results further nominate mTORC2 as an important regulator of oncogenic growth factor signaling in GBM, integrating the local tissue microenvironmental nutrient levels with tumor growth. Further studies will be needed to better understand its potential role in other cancer types.

Histone modifications are a dynamic chromatin mark with various important roles in gene regulation (33). In particular, histone H3 acetylation is tightly associated with the transcriptional start sites of genes, highly predictive of gene activity (27, 28). H3K9ac is one of the major marks reported to be found in actively transcribed promoters, and mTORC2 surprisingly increases H3K9ac at the promoter regions of the components in iron metabolism. Iron had been thought to be associated with cancer incidence, and a recent report demonstrated that GBM stem-like cells promoted iron trafficking, driving tumor growth (34). Although the previous comprehensive studies of lymphocytes with Rictor null mouse by Ye L et al did not demonstrate the significant transcriptome changes in iron metabolism pathways (GSE111536: Gene expression data from LCMV-specific Rictor-/- and WT follicular T helper cells), the present study demonstrates that mTORC2 affects the cancer cell survival at least partly through the epigenetic regulation of iron metabolism. The findings could be consistent with the idea that iron metabolism is regulated mainly according to the status of environmental amount of iron in normal cells whereas that may not be the case for cancer cells. Additionally, intriguing speculation would be that central carbon metabolism and mineral metabolism including iron might be
epigenetically interconnected in cancer cells and iron metabolism could be actively and autonomously regulated in cancer cells.

Intensive effort is currently aimed at targeting cancer cell metabolism and epigenetics. Unfortunately, however, therapeutics targeting the metabolism and epigenetics have not been a success thus far since a rapidly dividing normal constituents such as hematopoietic cells also harness the same kind of metabolic and epigenetic systems as cancer cells (2, 35). This study provides a strong evidence that mTORC2 could reside at the intersection of metabolism and epigenetics, which could be a therapeutic target against cancer metabolism as well as epigenetics. Our data suggest that GBM cells with mTORC2 activation are highly dependent on iron metabolism, suggesting that the autonomous iron metabolism in cancer cells may be therapeutically exploitable.

**Experimental procedures**

**Cell culture and human samples**

U87 and U87-EGFRvIII GBM cell lines were obtained as described previously (36). Cells were cultured in DMEM supplemented with 10% FBS (Omega Scientific, Tarzana, CA) in a humidified 5% CO2 incubator at 37°C. Autopsied cases of human GBM were a part of the collections from Tokyo Metropolitan Institute of Medical Science, and surgical tissues of glioma and breast cancer samples were obtained at Tokyo Women’s Medical University Hospital and the UCLA-affiliated hospitals. Physicians obtained informed consent from the patients. Gene expression analysis for TFRC, FTL and FTH1 in LGGs and GBMs were performed using TCGA data sets with eBioPortal for Cancer Genomics (http://www.cbioportal.org/). All methods and experimental protocols related to human subjects were approved by each institutional review board of Ethics Committee, and the procedures related to human subjects were carried out in accordance with each institutional review board-approved protocol and Declaration of Helsinki, 2013.

**Antibodies and reagents**

Cell Signaling antibodies: EGF Receptor vIII (D6T2Q; Cat# 64952), Akt (Cat# 9272), p-Akt (S473; Cat# 4060), p-NDRG1 (T346; Cat# 5482), Raptor (Cat# 2280), Rictor (Cat# 2114), LDHA (Cat# 3582), H3K9ac (Cat# 9649), H3K18ac (Cat# 9675), H3K27ac (Cat# 4353), H3K4me2 (Cat# 9725), H3K9me2 (Cat# 4658), H3K27me2 (Cat# 9755), Histone H3 (Cat# 4499), GAPDH (Cat# 5174), PKM2 (Cat# 4053), Lamin A/C (Cat# 4777), Myc-tag (Cat# 2276) and β-actin (Cat# 3700), HRP-linked anti-rabbit IgG (Cat# 7074) and HRP-linked anti-mouse
IgG (Cat# 7076). Santa Cruz (Dallas, TX) antibodies: PDH (E1α; Cat# sc-377092).
Sigma antibodies: EGFR (Cat# SAB4300352), Flag-tag (Cat# F1804).
MBL International (Woburn, MA) antibodies: H3K9ac (Cat# MABI0305).
DAKO (Glostrup, Denmark) antibodies: Ki-67 (Cat# M7240) and ferritin (Cat# A113).
Thermo Fisher antibodies: transferrin receptor (H68.4) (Cat# 13-6890).
Reagents used are sodium acetate (Sigma-Aldrich, St. Louis, MO; Cat # S5636), PP242 (Cayman Chemical, Ann Arbor, MI; Cat# 13643), Trichostatin A (Sigma; Cat# T1952), 5-Aza-2'-deoxycytidine (WAKO, Osaka, Japan; Cat# 018-20941), ferric ammonium citrate (FAC; Sigma Cat# 5879), BPTES (Selleck, Houston, TX; Cat# S7753), Akti-1/2 (Calbiochem, La Jolla, CA; Cat# 124018), Bisindolylmaleimide I (Bis-I; Santa Cruz Cat# sc-24003) and GSK 650394 (Tocris Bioscience, Bristol, UK; Cat# 3572/10).

**DNA plasmid, siRNA and shRNA transfection**

GFP-NTD, Myc-Rictor and Flag-HDAC4-3A DNA plasmids were obtained from Addgene. Lentiviral shRNA vectors targeting human Rictor and scramble sequences were also obtained from Addgene. Transfections of DNA plasmids were performed using X-tremeGENE HP (Roche) in full serum, with medium change after 24 hours, and cells were typically harvested 48 hours post-transfection. Transfection of siRNA into cell lines was carried out using Lipofectamine RNAiMAX (Invitrogen) in full serum, with medium change after 24 hours. siRNAs were used at 10 nM, and cells were harvested 48 hours post-transfection. Lentivirus-mediated delivery of shRNA was performed as described previously (37). Cells were infected in the presence of 6 μg/ml protamine sulfate, selected for puromycin resistance, and analyzed on the seventh day after infection.

**Antibody validation, immunostaining and image analysis-based scoring**

Immunostaining was performed as previously described (38). Antibodies against Rictor (at a dilution of 1:50) and H3K27ac (at a dilution of 1:100) were validated for the immunostaining applicability with formalin-fixed, paraffin-embedded (FFPE) cell blocks from Rictor KD U87-EGFRvIII cells and TSA-treated U87 cells, respectively (Fig. S1A). Additionally, the antibody against ferritin (at a dilution of 1:500) was also immunohistochemically validated with the use of negative and positive staining controls in the study of human amyotrophic lateral sclerosis cases (Niida-Kawaguchi M et al. in submission). Prior to staining for Rictor and H3K27ac, sections were pre-treated with microwaving in citrate buffer, pH
6.0 for 10 min at 500 W for antigen retrieval. Slides were counterstained with hematoxylin or DAPI (Invitrogen, Carlsbad, CA) to visualize nuclei. Immunostained sections underwent immunohistochemical analysis in which the results were evaluated independently by two pathologists who were unaware of the findings of the molecular analyses. Immunofluorescent samples were analyzed with a fluorescent microscope (Olympus BX53 Digital Fluorescence Microscope). Quantitative image analysis was performed with Soft Imaging System software (Visiopharm, Hørsholm, Denmark) for cytoplasmic proteins. Images from each immunostained section were captured from representative regions of the tumor with sufficiently high tumor cell content based on H&E staining evaluation. Negative control staining was also performed for each section without primary antibodies to determine the threshold for immunopositivity. Nuclear scoring was performed by Image J-based scoring systems (ImageJ version 1.49, NIH). Quantification of the immunofluorescent staining for the cultured cells was performed with WinROOF V6.5 (MITANI Co., Tokyo, Japan) according to the manufacturer’s instructions.

Metabolome analysis
Metabolome analysis was conducted by C-SCOPE package of Human Metabolome Technologies (HMT, Yamagata, Japan) using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) for cation analysis and CE-tandem mass spectrometry (CE-MS/MS) for anion analysis based on the methods described previously (39, 40). Peaks were extracted using MasterHands, automatic integration software (Keio University, Tsuruoka, Yamagata, Japan) (41) and MassHunter Quantitative Analysis B.04.00 (Agilent Technologies) in order to obtain peak information including m/z, peak area, and migration time (MT). Signal peaks were annotated according to the HMT metabolite database based on their m/z values with the MTs. Concentrations of metabolites were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and by using standard curves with three-point calibrations. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed by HMT proprietary software, PeakStat and SampleStat, respectively. Detected metabolites were plotted on metabolic pathway maps using VANTED software (42).

Western blot and immunoprecipitation (IP)
Immunoblotting was performed according to a modified version of the previously reported methods (8). Cell
lines or snap-frozen tissue samples were lysed and homogenized with radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)] from Boston BioProducts (Boston, MA). Protein concentration of each sample was determined using the BCA kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Equal amounts of protein extracts were separated by electrophoresis on 4-20% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Berkeley, CA), and then transferred to a nitrocellulose membrane with Trans-Blot Turbo Transfer System (Bio-Rad). The membrane was probed with the primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase. The immunoreactivity was detected with Super Signal West Pico Chemiluminescent Substrate or West Femto Trial kit (Thermo Fisher Scientific). Quantitative densitometry analysis was performed with an image analysis software (ImageJ version 1.49, NIH). For IP analyses, cells were lysed with the Pierce IP Lysis Buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% 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 3 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.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted by the use of RNeasy Plus Micro Kit (QIAGEN). Firststrand cDNA was synthesized by the use of iScript™ RT Supermix for RT-qPCR (Bio-Rad). Real-time RT-PCR was performed with the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Kyoto, Japan) on Thermal Cycler Dice Real Time System TP800 (Takara) following the manufacturer’s instructions. β-actin was used as an endogenous control. Primer sequences were available upon request.

RNA-sequencing and functional/canonical pathway analyses

U87-EGFRvIII cells were treated with siRNA against Scramble sequence or Rictor for 48 hours (n = 2 for each cell line). Total RNA was isolated by RNeasy Plus Mini Kit (QIAGEN, Venlo, The Netherlands) and submitted to Eurofins Genomics (Kanagawa, Japan) for library preparation and sequencing.
Gene expression data was analyzed by Chemicals Evaluation and Research Institute (CERI) (Tokyo, Japan), using Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, Redwood City, CA, USA). The data have been deposited in Gene Expression Omnibus (GEO: accession number GSE138475).

**Chromatin immunoprecipitation (ChIP)-sequencing and qPCR**

ChIP experiment was performed using SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer’s instruction. H3K9ac ChIP was performed from 5×10⁶ crosslinked U87-EGFRvIII cells treated with siRNA against Scramble sequence or Rictor for 48 hours (n = 2 for each cell line: ChIPed and input DNAs). ChIPed DNA was submitted to RIKEN GENESIS CO., LTD. (Tokyo, Japan) for library preparation and sequencing. Model-based analysis of ChIP-seq (MACS) normalizes the total tag counts between ChIPed and input DNAs. The data have been deposited in Gene Expression Omnibus (GEO: accession number GSE133117). For ChIP-qPCR analyses, H3K9ac ChIP was performed from 5×10⁶ crosslinked U87-EGFRvIII cells overexpressing Rictor in combination with glucose withdrawal for 24 hours. Immunoprecipitated chromatin was washed and de-crosslinked, and purified DNA was quantified by SYBR-Green real-time quantitative PCR. Recoveries were calculated as percent of input according to the previously reported methods (8).

**Global histone H3 detection assay**

Global histone H3 detection assay was performed using EpiQuik™ Global Histone H3 Acetylation Assay Kit (Epigentek, Farmingdale, NY) according to the manufacturer’s instructions. Histone extraction was performed with Histone Extraction Kit (Abcam) and 2 µg of each histone sample was applied to each well of the assay. The absorbance was read on a microplate reader (Multiskan GO Microplate Spectrophotometer; Thermo Fisher) at 450 nm.

**Subcellular fractionation and acetyl-CoA measurement**

Nuclear fractionation was prepared from sub-confluent 10 cm plates using a kit according to the manufacturer’s instructions (Active Motif, Carlsbad, CA). Intracellular acetyl-CoA level was calculated in a pmol range using PicoProbe Acetyl CoA Assay Kit (Abcam). Fluorescence was measured using Ex/Em = 535/589 nm with a microplate reader (Thermo Fisher). After correcting background from all readings, values for each sample were...
determined and normalized by protein concentration of each sample.

**Quantification of total intracellular iron by atomic absorption spectrometry**

U87-EGFRvIII cells (1.0 × 10⁶ cells per well) were seeded with siRNA against scramble (control) or Rictor for 2 days prior to use. The cells were collected and counted by trypsin treatment. The cell pellets were re-suspended in conc. HNO₃ (400 μL) and incubated for 6 hours. The lysates were diluted to 400 μL with distilled water. The concentrations of iron in the samples were measured by furnace atomic absorption spectroscopy with a Shimadzu AA-7000 atomic absorption spectrometer (43). The obtained values were normalized with the cell numbers.

**Quantitative measurement of soluble iron in the media**

U87-EGFRvIII with siRNA against Scramble sequence or Rictor were plated in 6-well plates and cultured with supplementation of 100 μM ferric ammonium citrate (FAC) for 48 hours (n = 3 for each cell lines). Medium was collected and the amount of soluble iron content of each sample was determined by the Ferrozine method using a Metalloassay FeII/FeIII kit (Metallogenics, Chiba, Japan) according to the manufacturer’s instructions. The iron concentration is determined by the formation of a chelate complex between Ferrozine and iron. Iron bound to transport proteins such as transferrin is dissociated from the protein by the action of weak acid, denaturant and reducing agent, and Fe³⁺ is reduced to Fe²⁺ to form Fe²⁺-Ferrozine complex. The iron concentration can be determined by measuring this complex at a wavelength of 560 nm. A value of soluble iron content on a microplate was normalized by protein concentration of each sample detected with a BCA protein assay.

**Animal studies**

U87 and U87-EGFRvIII cell lines were implanted into immunodeficient SCID/Beige mice for subcutaneous xenograft studies as previously reported (8). Proneural GBM rat models were stereotactically induced by injecting pQ-PDGFB-HA-IRES-EGFP (PDGF-GFP) retroviruses into the subventricular zone of the lateral ventricle in the cerebrum or the tegmentum of the brainstem as described previously (44). The procedures related to animals were in accordance with the Guidelines for Animal Experiments of each institutional review board and the Law and Notification of the Japanese Government.

**Statistical analysis**

Statistical differences between the two groups were analyzed using Student’s two-tailed unpaired t-test, and
those among three or more groups using one-way ANOVA, followed by a Turkey test and Dunnett's test. Error bars represented standard deviation (SD) unless otherwise noted, and statistical significance was indicated as *p < 0.05, **p < 0.01, and ***p < 0.001.

Author contributions: K.M., W.K.C., P.S.M. and N.S. designed the experiments and wrote the manuscript. K.M. and M.H. conducted most of the experiments and data analysis. K.M. and S.I. performed image analysis-based scoring and data processing. H.Y. and H.O. helped with conducting the experiments and data analysis. W.H.Y., T.F.C., Y.M., T.K., N.A. and T.K. helped with collection of human materials and pathological data analysis. All authors discussed the results and helped revise the manuscript.

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Figure Legends

**Figure 1.** EGFR-mTORC2 signaling correlates with histone acetylation in human GBM. **A,** Histone H3K9, K18 and K27 acetylation immunostaining of GBM tissue (15 normal brain tissue and 17 GBM tissue). A bar graph showing the positive ratio of each histone mark [acetylated histone H3 (red) / total nuclei (red+blue)]. Scale bars, 50 µm (20 µm for insets). **B,** Quantitative immunhistochmical analysis of lactate dehydrogenase A (LDHA: a glycolytic enzyme) and histone H3 acetylation (K9ac, K18ac and K27ac), based on low and high expression regions in EGFR-mTORC2 signaling, obtained at autopsy from 7 patients with GBM or surgically resected 10 GBM tissue. The averaged mean saturation of Rictor for the mTORC2-low group is 5.39, and the mTORC2-high group is 8.83. Also shown are representative images of immunohistochemical quantification: cytoplasmic staining with Visiopharm and nuclear staining with Image J. AU, arbitrary unit. **C,** Representative images for differential expression in EGFR-mTOR signaling, glycolytic metabolism and histone acetylation in autopsied GBM samples. Scale bar, 40 µm.

**Figure 2.** mTORC2 signaling and histone acetylation are promoted in PDGF-induced GBM animal models. **A-C,** Cerebral and brainstem tissue with GBM tumors was harvested from rats infected with PDGFB-HA-IRES-EGFP retroviral vectors (n = 3 for each region). Immunohistochemistry was performed on paraffin-embedded tissue sections against an mTORC2 marker (p-Akt S473: **A**), a histone acetylation mark (H3K9ac: **B**) and a proliferation marker (Ki-67: **C**). mTORC2-high, -moderate and -low regions in GBM tissue was compared with contralateral non-neoplastic regions. Representative images are demonstrated with higher magnification of regions of interest. Scale bars, 40 µm (20 µm for insets). **D,** Quantification of histone H3 acetylation (K9ac, K18ac, K27ac) and Ki-67, based on low and high activity regions in mTORC2 signaling.

**Figure 3.** mTORC2 facilitates histone acetylation through its downstream effector Akt and SGK1. **A,** Immunofluorescent staining of H3K9ac, K18ac and K27ac (green) in U87-EGFRvIII cells with shRNA against Scramble sequence or Rictor. DAPI staining shows the counter nuclear staining (blue). Bar graphs demonstrate quantification of histone H3K9, K18 and K27 acetylation in U87-EGFRvIII cells with Scramble or Rictor shRNA (n = 3). Scale bar, 40 µm. **B,** Immunoblot detection and quantification of histone H3 acetylation (H3K9ac, H3K18ac, H3K27ac) in U87-EGFRvIII cells with indicated siRNAs targeting the effector molecules in EGFR-mTOR signaling including Akt, Raptor (mTORC1) and Rictor (mTORC2). Bar graphs showing the activation markers for Akt.
and mTORC2 pathways (p-Akt S473 and p-NDRG1 T346), and line graphs for H3 acetylation ratio. CBB, Coomassie Brilliant Blue staining. C, Immunoblot detection and quantification of histone H3K9 acetylation in U87-EGFRvIII cells treated with drugs targeting mTORC2 substrates including Akt (Akti-1/2: 2.5 µM), SGK1 (GSK650394: 2.0 µM) and PKC-α (Bis-I: 10 µM) for 48 hours. CBB, Coomassie Brilliant Blue staining; NS, not significant.

Figure 4. mTORC2 regulates histone acetylation through nuclear translocation of PDH/PKM2 and suppression of class IIa HDACs. A, Immunofluorescent staining of PDH and PKM2 in U87-EGFRvIII cells treated by PP242 (mTORC1/C2 inhibitors) for 24 hours. Arrowheads indicate each nucleus, and an arrow shows the mitochondrial distribution. Green, PDH staining; red, PKM2 staining; blue, DAPI staining. Scale bar, 10 µm. B, Subcellular fractionation of cytosolic and nuclear protein from U87 cells with or without Rictor cDNA overexpression, followed by immunoblotting against PDH (E1α) and PKM2. Lamin and GAPDH are loading controls for nuclear and cytoplasmic proteins, respectively. Bar graphs showing the nuclear/cytoplasmic ratio of each protein in control and Rictor-overexpressing U87 cells. C, Bar graphs showing the total, cytoplasmic and nuclear amount of acetyl-CoA from U87-EGFRvIII cells with Scramble or Rictor knockdown. D, ELISA-based detection of global histone H3 acetylation in U87 cells overexpressing Rictor, combined with siRNA-mediated knockdown of PDH or PKM2. E, Quantitative immunoblot analyses of histone modifications including methylation and acetylation in U87-EGFRvIII cells with the overexpression of class IIa HDAC (HDAC4-3A: phosphorylation-resistant mutant form). F, Immunoblot analysis demonstrates that H3K9ac reduction in Rictor-depleted U87-EGFRvIII cells was partially rescued by the concurrent inhibition of HDACs with TSA for 24 hours.

Figure 5. mTORC2-dependent histone acetylation promotes the expression of iron-related genes. A, Ingenuity canonical pathway annotations which were downregulated, but not upregulated on mTORC2 inhibition by Rictor knockdown. B, Average Peak densities of H3K9ac around the transcription start site (TSS) in U87-EGFRvIII cells with siRNA against Scramble sequences (red) or Rictor (blue). C, University of California, Santa Cruz, Browser image depicting H3K9ac peaks around the TSS of TER2 gene ( locus on chromosome 3), FTL gene (on chromosome 19) and FTH1 gene (on chromosome 11) in U87-EGFRvIII cells with Scramble or Rictor siRNAs. Red shades, potential promoter regions of each gene. D, ChIP-qPCR analysis on U87-EGFRvIII cells transfected with Control (GFP-NTD) or Rictor cDNA combined with glucose withdrawal for 24 hours,
assessing H3K9ac recovery on binding elements in FTL promoter regions. 

E, RT-qPCR analyses on FTL transcripts in U87-EGFRvIII cells transfected with shRNA, combined with supplementation of TSA (1.0 µM), acetate (10 mM) or 5-Aza-2'-deoxycytidine (100 nM) for 3 days.

**Figure 6.** mTORC2-dependent iron metabolism regulates GBM cell survival. 

A, Histological analyses for differential expression in mTORC2 signaling (p-NDRG1 T346), histone acetylation (H3K9ac, K18ac and K27ac) and ferritin expression in surgical GBM samples (n = 10). Scatter plots showing the association of each histone mark and ferritin expression with H3K9ac showing the strongest correlation with ferritin expression. Scale bar, 200 µm. 

B, Transcript and protein expression of transferrin receptor (TFR) in U87-EGFRvIII cells with siRNAs against Rictor.

C, Bar graph showing the difference in total intracellular concentration of iron between U87-EGFRvIII cells with siScramble and siRictor knockdown (n = 3), quantified by atomic absorption spectrometry. D, Bar graph showing the difference in cell proliferation (cell number) of U87 cells overexpressing Rictor with concurrent knockdown of FTL or FTH1 for 2 days (n = 3). E, Quantitative analyses of the proliferation in U87-EGFRvIII cells treated with exogenous addition of 100 µM ferric ammonium citrate (FAC), associated with siRNA-mediated knockdown of Rictor for 2 days (n = 3). Scale bar, 40 µm. 

F, RTK signaling activates mTORC2 which in turn promotes histone acetylation via the regulation of both the substrate and modifying enzymes, leading to the up-regulation of iron-related genes, a key downstream effector of cell survival in GBM.
A

H3K9ac  H3K18ac  H3K27ac

Normal

Tumor

Quantification

H3 acetylation ratio

Ac-H3K9  Ac-H3K18  Ac-H3K27

B

Cytoplasmic staining

Nuclear staining

LDHA

H3K9ac

H3K18ac

H3K27ac

C

Region #1 (EGFR-mTORC2-high)

Region #2 (EGFR-mTORC2-low)

H&E  EGFR  Rictor (mTORC2)  LDHA  H3K9ac  H3K18ac  H3K27ac

EGFR-mTOR signaling  Glycolysis  Histone modification

H&E

EGFR

Rictor

LDHA

H3K9ac

H3K18ac

H3K27ac

Quantification

Normal  Tumor

Positive ratio (%: acH3/total nuclei)

**  ***  *
A Genetic inhibition of mTORC2

| shScramble | shRictor |
|------------|----------|
| H3K9ac     |          |
| DAPI       |          |
| H3K18ac    |          |
| DAPI       |          |
| H3K27ac    |          |
| DAPI       |          |

H3 acetylation intensity

![Graph showing H3 acetylation intensity](image)

B Whole lysate

| siRNA          | H3K9ac | H3K18ac | H3K27ac |
|----------------|--------|---------|---------|
| Scramble       | 76     | 150     | 225     |
| siAkt          | 52     | 102     | 225     |
| siRaptor       | 76     | 52      | 38      |
| siRictor       | 38     | 38      | 38      |

Histone protein

|          | H3K9ac | H3K18ac | H3K27ac |
|----------|--------|---------|---------|
| 17       |        |         |         |
| 17       |        |         |         |
| 17       |        |         |         |

H3 acetylation ratio

![Graph showing H3 acetylation ratio](image)

C Whole lysate

| Drug treatment | p-Akt | p-NDRG1 | GAPDH |
|----------------|-------|---------|-------|
| DMSO           |       |         |       |
| Akti-1/2       |       |         |       |
| GSK650394      |       |         |       |
| Bis-1          |       |         |       |

Histone protein

|          | H3K9ac |
|----------|--------|
| 17       |        |
| 17       |        |
| 17       |        |

H3 acetylation ratio

![Graph showing H3 acetylation ratio](image)
**Figure 4**

**A**

Immunofluorescence images showing subcellular localization of PDH, PKM2, and DAPI in DMSO-treated and PP242 (10 µM)-treated U87-EFRvIII cells. Arrowheads indicate nuclear localization of PKM2.

**B**

**Cytosol** vs. **Nucleus** relative expression levels of PDH (E1α), PKM2, and GAPDH in U87-EFRvIII cells treated with Rictor cDNA or Control siRNA. 

**C**

Intracellular Acetyl-CoA levels in U87-EFRvIII cells treated with Rictor cDNA or Control siRNA, measured by Global H3ac ratio. 

**D**

Nuclear/Cytoplasmic ratio of PDH and PKM2 in U87-EFRvIII cells treated with Rictor cDNA or Control siRNA, with p value of 0.087. 

**E**

Western blot analysis of H3K4me2, H3K9me2, H3K27me2, H3K9ac, H3K27ac, and Total H3 in U87-EFRvIII and U87-vIII-HDAC4 cells treated with Control or shRictor. 

**F**

Western blot analysis of H3K9ac in U87-EFRvIII cells treated with Control, shRictor, and TSA (1.0 µM) Rictor siRNA, with Total H3 and Relative H3ac levels shown.
A

**Ingenuity Pathway: Down on mTORC2 inhibition**

| Metabolism: siRictor vs Scramble (U87-EGFRvIII) | Down | p value |
|-----------------------------------------------|------|---------|
| Free Radical Scavenging metabolism | metabolism of hydrogen peroxide | 4.10E-04 |
| Vitamin and Mineral Metabolism metabolism | metabolism of beta-estradiol | 9.38E-03 |
| Vitamin and Mineral Metabolism metabolism | metabolism of hydrocortisone | 1.23E-02 |
| Vitamin and Mineral Metabolism mobilization mobilization of Ca2+ | 1.14E-02 |
| Carbohydrate Metabolism uptake uptake of D-hexose | 4.07E-04 |
| Carbohydrate Metabolism uptake uptake of D-glucose | 1.01E-03 |
| Carbohydrate Metabolism absorption absorption of D-hexose | 5.60E-04 |
| Carbohydrate Metabolism absorption absorption of D-glucose | 9.38E-03 |
| Carbohydrate Metabolism distribution distribution of phosphatidylserine | 5.60E-04 |
| Carbohydrate Metabolism synthesis synthesis of inositol phosphate | 1.52E-03 |
| Carbohydrate Metabolism quantity quantity of fructose-1,6-diphosphate | 1.98E-03 |
| Carbohydrate Metabolism quantity quantity of fructose-6-phosphate | 3.88E-03 |
| Carbohydrate Metabolism accumulation accumulation of proteoglycan | 6.36E-03 |
| Carbohydrate Metabolism accumulation accumulation of inositol phosphate | 1.18E-02 |
| Carbohydrate Metabolism metabolism metabolism of fructose-2,6-diphosphate | 6.36E-03 |

B

**Average number of peaks per base**

Distance from TSS (bp)

C

**H3K9ac ChIP-seq**

TFR2 (Chr 7)

FTL (Chr 19)

FTH1 (Chr 11)

D

**FTL promoter**

| Rictor cDNA Glucose (4.5 g/L) | + | + | + | - |
|---|---|---|---|---|
| FTL transcripts | NS | NS | NS | NS |

E

**Relative expression**

shRictor | - | + | + | + |
|---|---|---|---|---|
| TSA | - | - | - | - |
| Acetate | - | - | - | - |
| 5-Aza | - | - | - | + |
A

p-NDRG1 (T346) high

low

H3K9ac

Ferritin

200 µm

B

TFR mRNA

Scramble Rictor RNAi

Rictor

Transferrin Receptor (84kDa)

GAPDH

C

Intracellular iron concentration

D

Cell number (x10^5)

Rictor cDNA

siFTL

siFTH1

E

No treatment

FAC (100 µM)

FAC (100 µM) + siRictor (10 nM)

40 µm

F

Cell proliferation

FAC siRictor

- + +

Cell number (x10^5)

- + +

RTK

mTORC2

Class IIa HDAC

Nuclear translocation

Metabolic reprogramming

PKM2

Ac CoA

Histone acetylation

Promoter of iron-metabolism genes
mTORC2 links growth factor signaling with epigenetic regulation of iron metabolism in glioblastoma

Kenta Masui, Mio Harachi, Shiro Ikegami, Huijun Yang, Hiromi Onizuka, William H. Yong, Timothy F. Cloughesy, Yoshihiro Muragaki, Takakazu Kawamata, Nobutaka Arai, Takashi Komori, Webster K. Cavenee, Paul S. Mischel and Noriyuki Shibata

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