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NFκB up-regulation of glucose transporter 3 is essential for hyperactive mammalian target of rapamycin-induced aerobic glycolysis and tumor growth

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ABSTRACT

Accumulating evidence indicates that mammalian target of rapamycin (mTOR) exerts a crucial role in aerobic glycolysis and tumorigenesis, but the underlying mechanisms remain largely obscure. Results from Tsc1- or Tsc2-null mouse embryonic fibroblasts (MEFs) and human cancer cell lines consistently indicate that the expression of glucose transporter 3 (Glut3) is dramatically up-regulated by mTOR. The rapamycin-sensitive mTOR complex 1 (mTORC1), but not the rapamycin-insensitive mTOR complex 2 (mTORC2), was involved in the regulation of Glut3 expression. Moreover, mTORC1 enhances Glut3 expression through the activation of the IKK/NFκB pathway. Depletion of Glut3 led to the suppression of aerobic glycolysis, the inhibition of cell proliferation and colony formation, and the attenuation of the tumorigenic potential of the cells with aberrantly hyper-activated mTORC1 signaling in nude mice. We conclude that Glut3 is a downstream target of mTORC1, and it is critical for oncogenic mTORC1-mediated aerobic glycolysis and tumorigenesis. Hence Glut3 may be a potential target for therapy against cancers caused by the aberrantly activated mTORC1 signaling.

Introduction

Unlike normal cells, tumor cells preferentially metabolize glucose through glycolysis even in the presence of sufficient oxygen, which is known as aerobic glycolysis or the Warburg effect [1,2]. Even though aerobic glycolysis is inefficient for energy production, it is believed to offer a selective advantage for the proliferation and survival of cancer cells. Currently, although the mechanism of aerobic glycolysis still remains largely obscure, the measurement of aerobic glycolysis in patients has been used clinically to diagnose cancers and monitor cancer progression in response to treatment [1,3]. Glycolytic inhibitors have also been tested for cancer treatment in clinical trials, such as 2-deoxyglucose (2-DG) and dichloroacetate (DCA) [4,5].

An extremely higher rate (up to 200-fold) of glycolysis is always detected in tumor cells, which is believed to compensate for the inefficiency on generating energy. This enhancement of glucose metabolism requires an accelerated glucose uptake into tumor cells. A family of glucose transporter proteins (Gluts) facilitates the glucose transport across the plasma membranes of mammalian cells in a tissue-specific manner. So far, 14 different Glut isoforms have been identified. These Gluts are divided into three subclasses (class I, class II, and class III) based on sequence similarities [6]. Class I Glut isoforms which have been well-characterized comprise Glut1-Glut4. Among this family, Glut1, Glut3, and Glut4 are high-affinity transporters, whereas Glut2 is a low-affinity transporter [7]. Under normal physiological conditions, Glut1 is ubiquitously expressed at low level, Glut4 is mainly expressed in adipose and muscle, and Glut3 is expressed primarily in neurons [8]. Most impressively, Glut3 has at least a 5-fold greater transporting capacity than Glut1 or Glut4

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[8]. Over-expression of Glut1 was observed in a variety of tumors, and its function in tumor development has been intensively investigated [4,7]. However, there is very limited research on Glut3. A recent study showed that Glut3 expression is correlated with poor survival in a broad range of tumor types [9], suggesting that many cancers may dysregulate Glut3 to drive tumor growth. However, the precise mechanisms leading to the up-regulation of Glut3 in tumor cells and the significance of Glut3 in tumor development are largely unknown.

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) (PI3K/AKT/mTOR) signaling pathway is frequently hyperactivated in human cancers, via gain-of-function mutations of proto-oncogenes such as EGFR, PI3K, and AKT, or loss-of-function mutations of tumor suppressors, including PTEN, LKB1, and the tuberous sclerosis complex 1 and 2 (TSC1/2) [10,11]. mTOR, a highly conserved serine/threonine protein kinase, is the key effector in this pathway via integrating multiple inputs, such as growth factor signaling and nutrient status to orchestrate a number of cellular processes, including cell growth, proliferation, differentiation, and survival [12,13]. As two upstream regulators of mTOR, TSC1 and TSC2 have distinct characteristics. These two proteins form a functional complex negatively regulating the activity of mTOR [14]. Aberrant activation of mTOR due to the loss of function of the TSC1/TSC2 complex is the major cause of tuberous sclerosis complex (TSC), a benign tumor syndrome that affects multiple organs [15,16]. mTOR can associate with different binding partners to form two functional complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is sensitive to rapamycin and activated by growth factor stimulation via PI3K/AKT pathway, whereas mTORC2 is rapamycin-insensitive, and its upstream regulatory signaling pathways remain poorly characterized [10]. Recently, the pivotal role of mTOR in glycolysis during tumorigenesis has been established, but the precise mechanisms still require further elucidation.

Our study demonstrates that mTORC1 positively regulates the expression of Glut3 through the activation of IKK/NFκB signaling. The knockdown of Glut3 results in reduction of aerobic glycolysis, inhibition of cell proliferation, suppression of colony formation, and delay of tumor growth. Since Glut3 is pivotal for hyperactive mTORC1-induced glycolysis and tumorigenesis, we suggest that Glut3 can serve as a potential target for the treatment of cancers associated with dysregulated mTOR signaling.

Materials and methods

Reagents, plasmids, and antibodies

Rapamycin and BAY 11–7082 were purchased from Sigma. WYE-354 was from Selleck Chemicals. Lipofectamine 2000 and 4–12% Bis-Tris Nu-PAGE gels were from Life Technologies. pNFκB-TA-Luc was from Clontech. pBabe Ha-RasV12 was from Addgene. Full-length mouse p65 cDNA and mouse Glut3 cDNA were obtained by PCR from mouse embryonic fibroblasts (MEFs) cDNA pools and cloned into a pLXIN retroviral vector. pLL3.7-GFP lentiviral vector and the pLXIN vector have been described previously [11]. Glut1 (ab652), Glut3 (ab41525), LaminB1 (ab133741) and Ki-67 (ab16667) antibodies were from Abcam. TSC2 (sc893) and β-actin (sc47778) antibodies were from Santa Cruz. TSC1 (#6935), S6 (#2217), p-S6 (Ser235/236) (#4857), AKT1 (#2967), p-AKT(ser473) (#4060), IKKα (#2682), IKKβ (#2370), p-IKKα/β (Ser176/180) (#2697), p65 (#8242), p-p65 (Ser536) (3033), mTOR (#2983), Raptor (#2280) and Rictor (#2114) antibodies were from Cell Signaling.

Cell cultures and treatments

All the mouse embryonic fibroblasts (MEFs) and rat uterine leiomyoma-derived Tsc2-null ELT3 cells used in this study have been described previously [11,17–19]. PT67 packaging cells were purchased from Clontech. HEK 293T and human glioblastoma cell line U87 cells were obtained from the ATCC. ELT3 cells were maintained and propagated in DMEM/F12 (1:1) with 10% FBS. The other cells were cultured in DMEM with 10% FBS. All cells were incubated at 37 °C in the presence of 5% CO_2. Production of retroviruses and subsequent generation of stable gene expression cell lines have been described previously [17]. Cells were harvested 24 h after final feeding at a confluence of approximately 80–90%, unless indicated differently. For drug treatment, cells were plated in 6-well plates at 30–40% density 24 h prior to treatment. The DMSO stocks of the agents used, including BAY 11–7082 and WYE-354 were diluted to appropriate concentrations with the cell culture medium. Rapamycin was diluted with ethanol.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol (Life Technologies) according to the protocol provided by the manufacturer. RNA was converted to cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa). qRT-PCR was performed using Power SYBR® Master Mix (Life Technologies) according to the manufacturer's protocol. The primer sequences are listed in Supplementary Table S1.

Cell fractionation and western blot

Cytoplasmic and nuclear protein fractions were extracted using a NE-PER Reagent Kit (Pierce) according to the manufacturer's instructions. Western blot analysis of protein expression was performed as described previously [11].

RNA interference

Cells seeded in 12-well plates were transfected with siRNAs using Lipofectamine 2000 following the manufacturer's instructions. All siRNA oligonucleotides were synthesized by GenePharma (Shanghai, China). The siRNA target sequences are listed in Supplementary Table S2.

Reporter constructs and luciferase reporter assay

Mouse Glut3 promoter-intron 1 fragments (from –938 to +1132 bp) were generated by PCR amplification using mouse genomic DNA as template and then cloned into the *Nhe* I/*Hind* III sites of the luciferase reporter plasmid pGL3-basic (Promega). The primer sequences were as follows: forward, 5′-CGGCTAGCTCAGTTCAGTCCATCAGTCT-3′; reverse, 5′-GATGAAGCTTGGCCTGCTACAACCTCT-3′. The potential NFκB binding site on the first intronic region of mouse Glut3 gene was mutated using the Quick Change site-directed mutagenesis kit (Promega). The primer sequences were as follows: forward, 5′-GTATTTGTAAGGAAAACTCCATTTCCACGAAGATTAACTTG-3′; reverse, 5′-CAAGTTAATC TTCGTGGAAATGGAGTTTTCCTTACAAACAT-3′. Cells were cultured in triplicate to 80% confluence in 24-well plates and transfected the promoter constructs (200 ng) in combination with the plasmid pRL-TK (20 ng) as an internal control. Luciferase activity was detected with the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assay

Immunoprecipitation experiments with an anti-p65 antibody to detect protein–DNA interactions were performed using a SimpleChIP® Enzymatic Chromatin IP kit (Cell Signaling) according to the manufacturer's protocol. The immunoprecipitated DNA was purified and analyzed by qRT-PCR. The primer sequences used in the analysis of the target regions are as follows: the putative NFκB-binding sites region (PBR) of mouse Glut3 forward, 5′-GTCCTTACTATGGTTGTGAG-3′, reverse, 5′-CACGGTCTCTTAATTTACTT-3′; a nonspecific NFκB-binding region (NBR) of mouse Glut3 forward, 5′-GCAA GCATAATTTTCTCTGTTTC-3′, reverse, 5′-CAAAACACCCATAATGCATAAA-3′. The PBR of human Glut3 forward, 5′-CCGCTTCATCAGTTCTCTTTG-3′, reverse, 5′-CCTCAGT GGCATATGGTTACC-3′; the NBR of human Glut3 forward, 5′-CTAGGCCCTCAACCAAAACC-3′, reverse, 5′-TGCAACCCTTTCAGTACTGGA-3′.

Lentiviral vector production and transduction

pLL3.7-GFP plasmids were digested with *Xho* I and *Hpa* I enzymes, and then followed by insertion of annealed oligonucleotides. The sequences of the oligonucleotides are listed in Supplementary Table S3. HEK 293T cells were transfected with pLL3.7-GFP vector containing either of these sequences together with packaging vectors (pVSVG, pREV, and pMDL). Culture supernatants were collected after 48 h of transfection and then used to infect target cells.

Cell proliferation assay

Cell proliferation was measured using an MTT assay as described previously [11,15]. In brief, cell were seeded into 96-well plates in triplicate at a starting density of 1×10^3 per well and the proliferation was monitored for up to 4 days according to the manufacturer's specifications.

Measurements of glucose consumption and lactate production

Cells were seeded in culture dishes and cultured for 8 h. The culture medium was then changed and cells were incubated for an additional 16 h. Subsequently, the culture medium was collected for determination of glucose concentration and lactate levels using a Glucose assay kit and a Lactate assay kit (Eton Bioscience). Data were normalized to final cell counts. Glucose consumption was calculated as the difference in glucose concentration between fresh medium and supernatant.

Measurement of oxygen consumption

Oxygen consumption was measured using the Seahorse XF24 analyzer (Seahorse Bioscience). In brief, cells were plated in XF24 culture plates and grown for 24 h to form a confluent monolayer. One hour prior to the assay, the cells were rinsed and cultured in unbuffered DMEM (Sigma) according to the manufacturer's instructions. Oxygen consumption rate (OCR) was measured under basal conditions, in the presence of the ATP synthase inhibitors oligomycin (1 μM), or the mitochondiral complex III inhibitor antimycin A (0.5 μM), or in the presence of the mitochondrial uncoupler FCCP (0.5 μM) to assess maximal oxidative capacity.

Soft agar

A 0.6% (wt/vol) bottom layer of low melting point agarose in normal culture medium was prepared in 6-well plates. On top, a layer of 0.35% agarose containing 1×10^3 infected cells was placed. The number of colonies formed in the soft agar was counted after three weeks.

Induction of subcutaneous tumors in nude mice

Subcutaneous tumors were established in immunodeficient nude mice (BALB/c, 6 week old) as described previously [11,20]. Six male mice were used in each cohort. Tumor growth and mouse survival were assessed over 70-day periods following s.c. inoculation of 5×10^6 Tsc1-null cells expressing shGlut3-1 or shScramble in 0.2 ml DMEM into the right posterior back region. Mice were euthanized when tumor size was greater than 1000 mm^3, there was ulceration over the tumor or weight loss of more than 10% occurred. All animals were maintained and used in strict accordance with the guidelines of the Animal Center of Anhui Medical University.

Immunohistochemistry (IHC)

Immunohistochemical staining for Glut3 and Ki-67 was carried out using the Envision Plus/Horseradish Peroxidase System (Dako) according to the manufacturer's protocol.

Statistical analysis

Data were analyzed using a 2-tailed paired Student's t-test as previously indicated [15]. The Kaplan–Meier log-rank test was used for analysis of tumor development and survival data. Statistical significance was defined as $P < 0.05$.

Results

TSC1/TSC2 complex negatively regulates the expression of Glut3

Previous data from our laboratory and others have shown that the loss of TSC2 promotes aerobic glycolysis [11,20]. Since glucose uptake mediated by Gluts in mammalian cells is a rate-limiting step for glycolysis, we hypothesized that the expression of Gluts is also up-regulated to coordinate with the elevated consumption of glucose in Tsc2-deficient cells. To test this hypothesis, we compared the expression of Gluts between Tsc2+/+ and Tsc2–/– MEFs by analyzing our microarray database. Interestingly, among 12 Glut genes we examined, only Glut1 and Glut3 showed a substantial increase of their mRNA levels in Tsc2-null cells compared with the control cells (Supplementary Table S4). Also, Glut3 showed a significantly bigger increase than Glut1. Subsequently, qRT-PCR analysis confirmed that Glut3 mRNA level was dramatically increased in Tsc2-null MEFs, whereas the increase of Glut1 mRNA was relatively modest and the abundance of Glut2 and Glut4 was almost undetectable (Fig. 1A left panel). Consistent with the qRT-PCR data, the protein levels of Glut1 and Glut3 were also increased in Tsc2–/– MEFs (Fig. 1A right panel). To further verify the relationship between TSC2 and Gluts, a wild-type (WT) human TSC2 and a patient-derived GAP domain mutant TSC2 (N1651S) were introduced into the Tsc2–/– MEFs. As shown in Fig. 1B, ectopically expressed WT TSC2, but not the mutant TSC2, led to a significant down-regulation of Glut1 and Glut3. These results together indicate that TSC2 is a negative regulator of Glut1 and Glut3.

The following study was focused on Glut3 since Glut3 showed the most significant change on expression among all the Gluts that we examined. It is reasonable to postulate that TSC1 was also involved in the regulation of Glut3, since TSC1 stabilizes TSC2 as a binding partner, via inhibiting the degradation of TSC2 mediated by ubiquitin/proteasomes [21]. As expected, loss of TSC1 led to down-regulation of TSC2 and a significant up-regulation of Glut3 at both protein and mRNA levels (Fig. 1C). Moreover, re-introduction of TSC2 blunted the expression of Glut3 in rat uterine leiomyoma-derived Tsc2-null ELT3 cells (Fig. 1D). Taken together, these results suggest that the TSC1/TSC2 complex negatively regulates Glut3 expression.

Loss of TSC1/TSC2 up-regulates Glut3 through the activation of mTORC1 signaling

Because the loss of TSC1 or TSC2 induces both mTOR hyperactivation and Glut3 over-expression, we speculated that mTOR is a positive regulator of Glut3 expression. mTOR exists in two multiprotein complexes, rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2. To explore which mTOR complex contributes to the elevated expression of Glut3, we first evaluated the effect of rapamycin, a specific inhibitor of mTORC1, on Glut3 expression. As depicted in the left panel of Fig. 2A, both Tsc1–/– and Tsc2–/– MEFs showed marked reduction in p-S6 (a marker of mTORC1 activity) and decreased Glut3 level in response to rapamycin treatment.

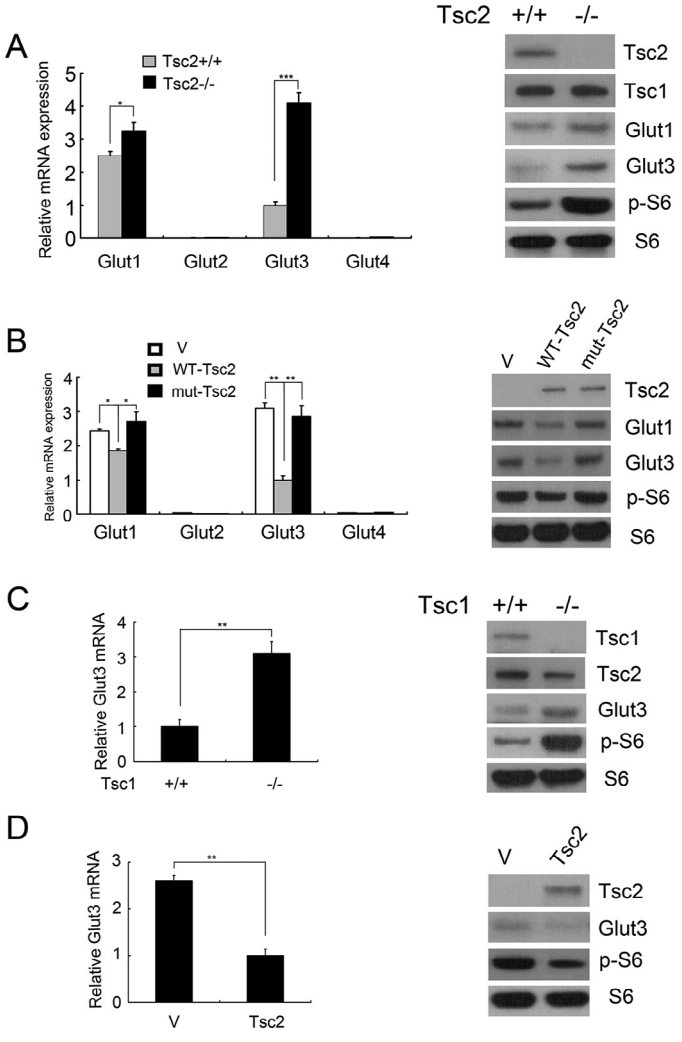

Fig. 1. TSC1/TSC2 complex negatively regulates Glut3 expression. (A) Tsc2+/+ and Tsc2–/– MEFs. (B) pLXIN (V), pLXIN-WT-TSC2, or pLXIN-mut-TSC2 retroviruses infected Tsc2–/– MEFs. (C) Tsc1+/+ and Tsc1–/– MEFs. (D) ELT3 cells transduced with the retroviruses for TSC2 in pLXIN or its control vector pLXIN (V). The levels of the indicated mRNAs were analyzed by qRT-PCR (left panel). Cell lysates were subjected to immunoblotting with the indicated antibodies (right panel). phospho-S6 (p-S6) is a downstream indicator of mTORC1 activity. S6 serves as a loading control. Error bars indicate mean ± SD of triplicate samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

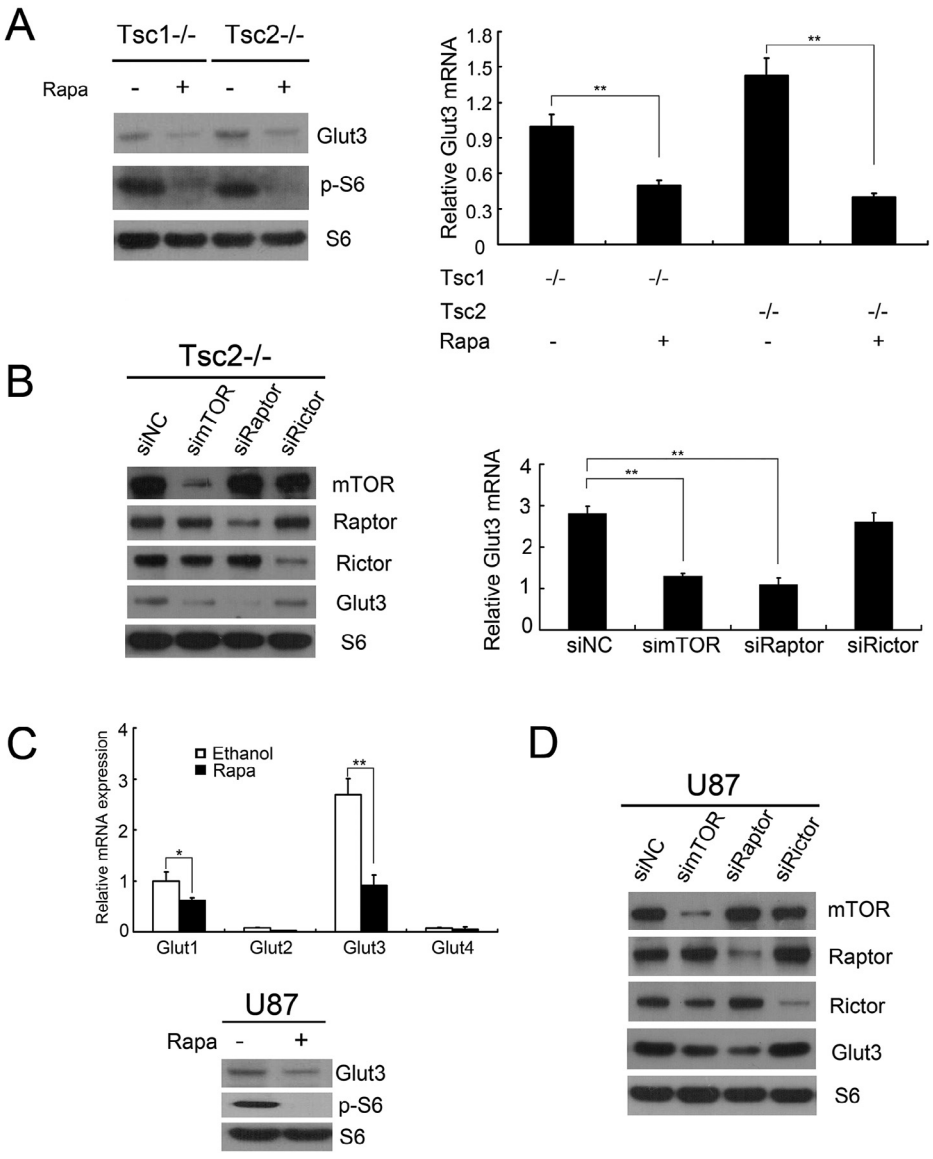

Fig. 2. mTORC1 positively regulates Glut3 expression. (A) Tsc1–/– and Tsc2–/– MEFs were treated with or without 20 nM rapamycin (Rapa) for 24 h. (B and D) Tsc2–/– MEFs or U87 cells were transfected with control siRNA (siNC) or siRNA for mTOR, Raptor, or Rictor for 48 h. (C) U87 cells were treated with or without 50 nM rapamycin (Rapa) for 24 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. The levels of the indicated mRNAs were detected by qRT-PCR. Error bars indicate mean ± SD of triplicate samples. $^{*}P < 0.05$; $^{**}P < 0.01$.

qRT-PCR analysis further showed that rapamycin down-regulated Glut3 expression occurs at transcriptional level (Fig. 2A right panel). To further confirm the role of mTORC1 in the regulation of Glut3, we investigated the expression of Glut3 in mTOR-, Raptor (a specific component of mTORC1)-, and Rictor (a specific component of mTORC2)-knockdown cells. Cells transfected with mTOR or Raptor siRNAs exhibited significantly decreased mRNA and protein levels of Glut3, whereas the cells transfected with Rictor siRNA showed similar Glut3 expression compared with those treated with control siRNA (Fig. 2B).

In addition to genetic loss of TSC1 or TSC2, mTORC1 can also be activated by up-regulation of the PI3K/AKT signaling pathway, such as the depletion of the PTEN tumor suppressor or the over-expression of the constitutively activated AKT1 [10]. Unsurprisingly, cells with either depleted PTEN or over-expression of constitutively activated AKT1 (AKT1-E17K) showed elevated Glut3 levels (Supplementary Fig. S1). Additionally, a human primary glioblastoma cell line U87 (PTEN-deficient) was employed to evaluate the correlation between mTORC1 activity and Glut3 expression in human cancer cells. As expected, inhibition of mTORC1 signaling in U87 cells by pharmacological or genetic strategies led to a similar result as in MEFs (Fig. 2C and D). Collectively, these data suggest that it is mTORC1, but not mTORC2, that positively regulates the expression of Glut3.

Both mTORC1 and mTORC2 activate IKKα/β-NFκB signaling

NFκB is a transcription factor which plays important roles in multiple physiological and pathological processes, including cell proliferation, apoptosis and tumorigenesis. It is a heterodimeric complex consisting of various subunits including p65, p50, cRel, and RelB. The heterodimer p65/p50 is the most frequent association in numerous cell types and referred as NFκB transcription factor. The NFκB dimmers are sequestered in the cytoplasm by inhibitory kappa B (IκB). Upon activation by the IκB kinase (IKK) complex, IκB is phosphorylated and degradated, allowing NFκB dimmers to translocate

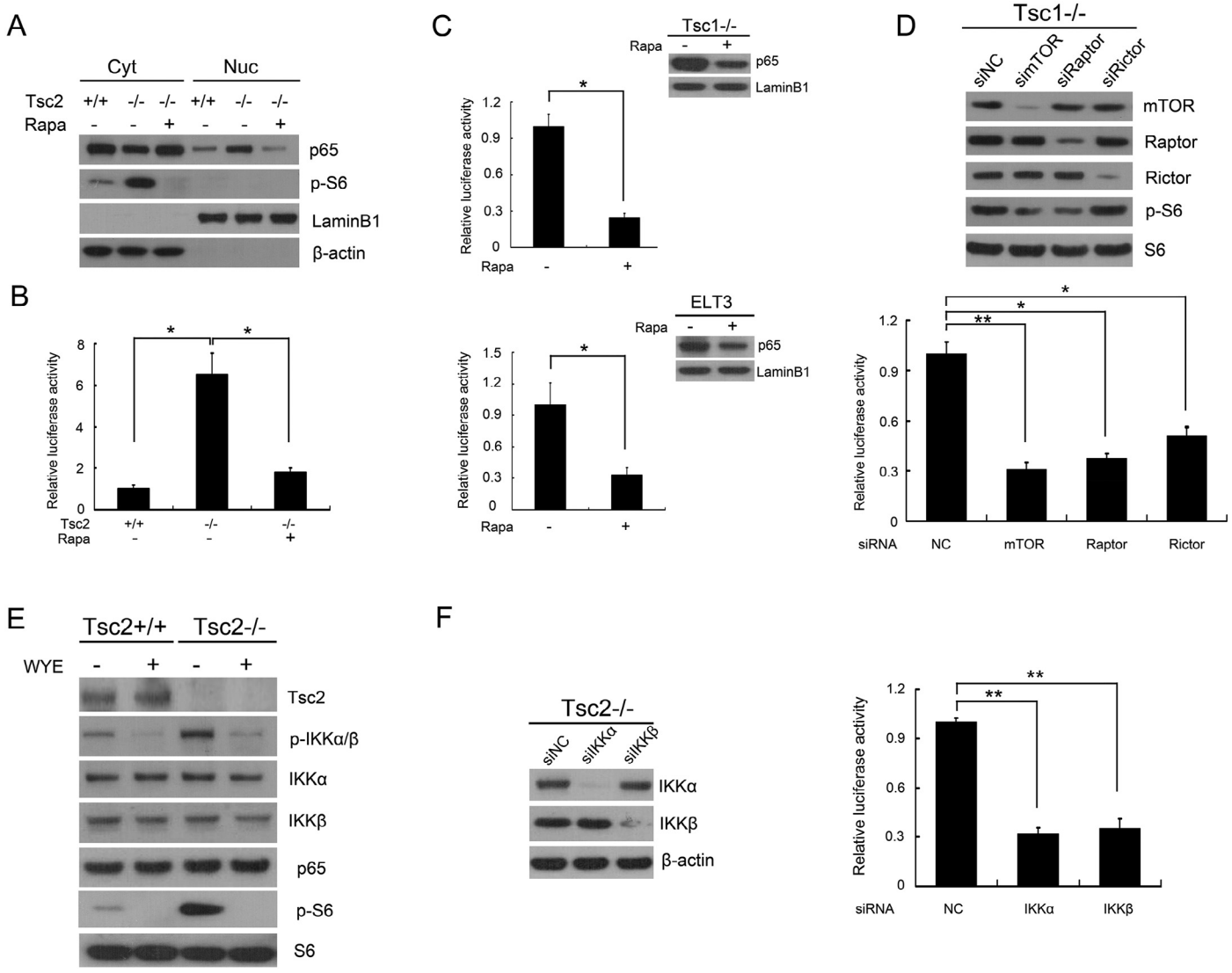

Fig. 3. Both mTORC1 and mTORC2 activate IKK/NFκB signaling. (A) Western blot analysis with the indicated antibodies of the cytoplasm (Cyt) and nuclear (Nuc) proteins from Tsc2+/+, Tsc2−/−, or rapamycin treated (20 nM, 24 h) Tsc2−/− MEFs. (B) Tsc2+/+, Tsc2−/−, or rapamycin-treated (20 nM for 24 h) Tsc2−/− MEFs were co-transfected with pNFκB-TA-Luc (200 ng) together with the internal control plasmid pRL-TK (20 ng). After 24 h of transfection, the relative luciferase activity was measured. (C) Tsc1−/− MEFs or ELT3 cells were treated with or without 20 nM rapamycin (Rapa) for 24 h. The nuclear proteins were analyzed by western blot with the indicated antibodies. The relative luciferase activity was measured as B. (D) Tsc1−/− MEFs were transfected with control siRNA (siNC), or siRNA for mTOR, Raptor, or Rictor, as indicated. Cell lysates were subjected to immunoblotting with the indicated antibodies. The relative luciferase activity was measured as B. (E) Tsc2+/+ and Tsc2−/− MEFs were treated with or without 5 μM WYE-354 (WYE), an ATP-competitive mTOR kinase inhibitor, for 24 h. The proteins were detected by immunoblotting with the indicated antibodies. (F) Tsc2−/− MEFs were transfected with control siRNA (siNC), or siRNA for IKKα or IKKβ, as indicated. Cell lysates were subjected to immunoblotting with the indicated antibodies. The relative luciferase activity was measured as B. Error bars indicate mean ± SD of triplicate samples. $^{*}P < 0.05$; $^{**}P < 0.01$.

into the nucleus and then promote the transcription of target genes [22,23].

We next determined whether NFκB signaling is stimulated by mTOR activation. As shown in Fig. 3A, nuclear p65 was dramatically elevated in Tsc2−/− MEFs compared to the control cells. Its level was markedly reduced by rapamycin treatment, indicating that the activation of NFκB signaling is mTORC1 dependent (Fig. 3A). Luciferase reporter assay further showed that the transcriptional and DNA-binding activities of NFκB were enhanced in Tsc2-null cells, and which were normalized by treatment with rapamycin (Fig. 3B). Furthermore, a consistent result was obtained in Tsc1−/− MEFs and ELT3 cells in response to rapamycin treatment (Fig. 3C). Moreover, ectopic expression of WT TSC2, but not the mutant TSC2, reversed the transcriptional and DNA-binding activities of NFκB in Tsc2-null MEFs (Supplementary Fig. S2). To further determine whether endogenous NFκB activity is controlled by mTOR, siRNAs against mTOR, Raptor, or Rictor were transfected into Tsc1−/− MEFs in parallel with a control siRNA. Interestingly, the knockdown of mTOR, Raptor, or Rictor all strikingly suppressed the NFκB-dependent reporter activity (Fig. 3D), which suggests that both mTORC1 and mTORC2 stimulate NFκB activity. As the activation of NFκB is achieved through the action of IKKα/β, it was considered that IKKα/β activity was elevated in mTOR-activated cells. As expected, phosphorylation of the active sites in IKKα/β were significantly increased in Tsc2-deficient cells compared to Tsc2+/+ cells (Fig. 3E). The enhanced phosphorylation on IKKα/β was abolished by treatment with WYE-354, an ATP-competitive mTOR kinase inhibitor that could suppress both mTORC1 and mTORC2 activities (Fig. 3E). Additionally, knockdown of IKKα or IKKβ blocked NFκB-dependent reporter activity in Tsc2−/− MEFs (Fig. 3F). Thus, we conclude that IKKα/β/NFκB signaling is activated by both mTORC1 and mTORC2.

mTORC1 up-regulates Glut3 through the activation of NFκB signaling

To examine whether mTORC1 up-regulates Glut3 via NFκB signaling, we treated Tsc1−/− and Tsc2−/− MEFs with BAY 11–7082, a specific NFκB inhibitor. As shown in Fig. 4A, both protein and mRNA levels of Glut3 were markedly decreased in the presence of BAY 11–7082. To further establish the relationship between NFκB signaling and Glut3 in the cells with hyper-activated mTORC1 signaling, siRNAs for p65 were transfected into Tsc1−/− and Tsc2−/− MEFs. As depicted in Fig. 4B, treatment with p65 siRNA led to significantly reduced expression of p65 as well as Glut3 at both mRNA and protein levels. Moreover, knockdown of p65 also resulted in a marked reduction of Glut3 expression in U87 cells (Supplementary Fig. S3). Taken together, these data indicate that mTORC1 up-regulates Glut3 via the activation of NFκB signaling.

In terms of the mechanisms by which NFκB controls Glut3 expression, we hypothesized that p65 regulates Glut3 expression by directly binding to the promoter of Glut3 gene. The in silico analysis suggested that the region between +877 and +886 in the intron 1 of the Glut3 gene contains a putative consensus sequence (GGGGGTCACC), which potentially could be bound by p65 (Fig. 4C). To determine whether the potential binding of p65 on Glut3 gene

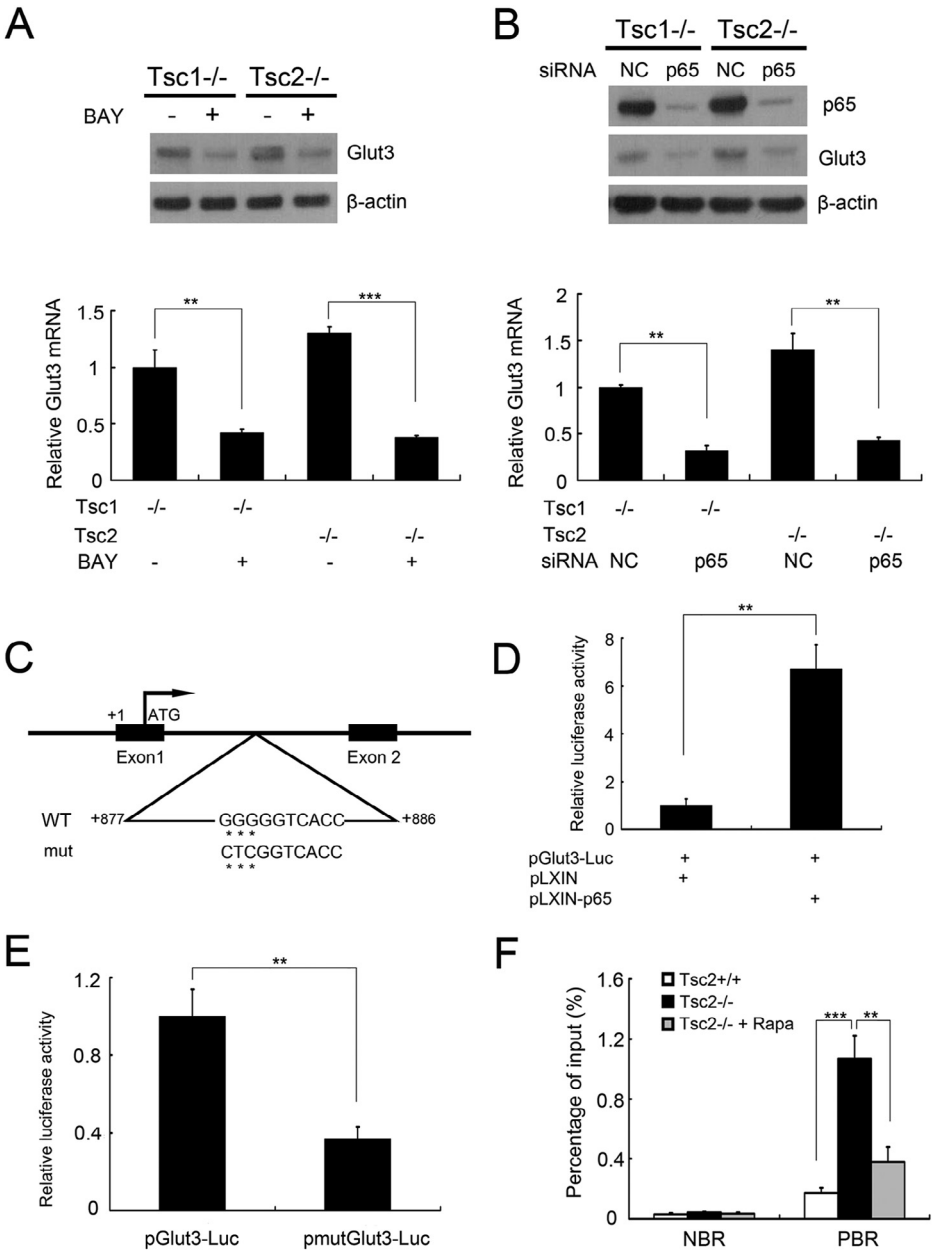

Fig. 4. mTORC1 up-regulates Glut3 expression through the activation of NFκB signaling. (A and B), Tsc1−/− and Tsc2−/− MEFs were treated with or without 10 μM BAY 11–7082 (BAY) for 24 h (A) or transfected with p65 siRNA or control siRNA (siNC) for 48 h (B). The proteins were detected by immunoblotting (top panel). qRT-PCR was performed to examine the expression of Glut3 (bottom panel). (C) Schematic representation of the putative wild-type (WT) and mutated (mut) NFκB-binding sites within the intron 1 region of mouse Glut3 gene. (D) HEK 293T cells were cotransfected with pGlut3-Luc reporter plasmid plus p65 expression vector or control vector and pRL-TK plasmid. (E) Tsc2−/− MEFs were cotransfected with p65 expression vector plus pGlut3-Luc or pmutGlut3-Luc reporter plasmid and pRL-TK plasmid. (D and E) Relative luciferase activity was evaluated 24 h after transfection. (F) Tsc2+/+, Tsc2−/−, or rapamycin (20 nM, 24 h) treated Tsc2−/− MEFs were subjected to ChIP assay using an anti-p65 antibody. Normal rabbit IgG antibody served as the negative control. qRT-PCR was conducted to amplify a region surrounding the putative NFκB-binding site (PBR) and a nonspecific NFκB-binding region (NBR). The data were plotted as the ratio of immunoprecipitated DNA subtracting nonspecific binding to IgG vs. total input DNA. Representative data from two independent experiments are shown. Error bars indicate mean ± SD of triplicate samples. $^{**}P < 0.01$; $^{***}P < 0.001$.

occurs *in vivo*, we inserted a 2070-bp fragment (from –938 to +1132 bp) of mouse Glut3 gene containing the potential binding sequence of p65, into the luciferase reporter plasmid to evaluate the transcriptional ability of p65 on this region. The recombinant reporter plasmid was then co-transfected into 293T cells together with p65 or empty control vector. Increased luciferase activity in p65-transduced cells indicated a significant transcriptional activation of Glut3 gene by p65 (Fig. 4D). Moreover, the transcriptional activity was markedly attenuated when the putative NFκB-binding site was mutated (Fig. 4E). To confirm the direct binding of p65 on this consensus sequence, we performed a ChIP assay. qRT-PCR analysis of ChIP DNA revealed that the direct binding of p65 to the putative NFκB-binding site of the Glut3 gene was significantly stronger in Tsc2–/– MEFs than in the control cells (Fig. 4F). Moreover, their interaction was disrupted by rapamycin treatment (Fig. 4F). In addition to MEFs, a putative NFκB-binding site was also to be found in the intron 1 region (from +462 to +472, GGAAAGTCACC) of human Glut3 gene (Supplementary Fig. S4A). Similar to MEFs, qRT-PCR analysis of ChIP DNA revealed that the binding of p65 to this region was disrupted by rapamycin treatment in U87 cells (Supplementary Fig. S4B). Taken together, p65 transduces mTORC1 signaling to Glut3 by directly up-regulating Glut3 gene transcription in mouse and human cells.

Glut3 is critical for aerobic glycolysis of the cells with active mTORC1 signaling

It has been demonstrated that mTORC1 promotes aerobic glycolysis [20], and our data here suggest that Glut3 is a downstream effector of mTORC1. Thus, we postulated that mTORC1 regulates aerobic glycolysis at least partially through Glut3. To test this hypothesis, two different shRNAs for Glut3 (shGlut3-1 and shGlut3-2) were used to knockdown Glut3 in Tsc1 or Tsc2-null cells, and then the glucose consumption and lactate production of these cells were measured. Western blot and qRT-PCR analysis revealed that both shGlut3-1 and shGlut3-2 dramatically decreased the expression of Glut3, and shGlut3-1 was more effective (Fig. 5A left and middle panel). Reduction of Glut3 expression led to a significant decrease in glucose consumption and lactate production (Fig. 5C and D), as well as an elevation in oxidative phosphorylation (Supplementary Fig. S5), suggesting that Glut3 exerts a critical role in aerobic glycolysis in mTORC1-activated cells. To further determine whether Glut3 is also involved in aerobic glycolysis of human cancer cells, we stably knocked down Glut3 in U87 cells. As shown in the right panel of Fig. 5A, both protein and mRNA levels of Glut3 were substantially reduced in the cells that were infected with shGlut3-1 or

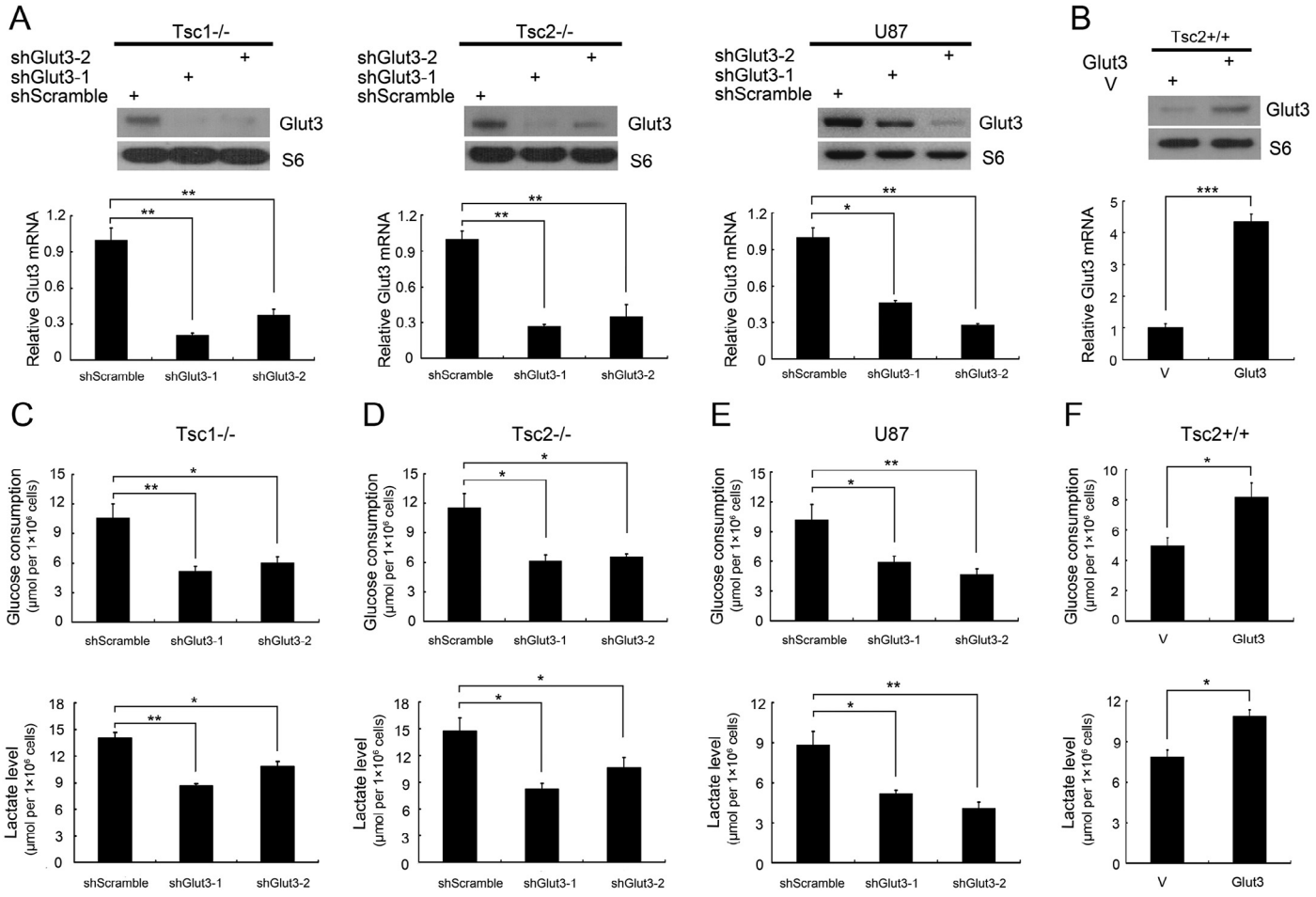

Fig. 5. Glut3 is critical for aerobic glycolysis of the cells with activated mTORC1 signaling. (A) Two independent shRNAs that silence Glut3 (shGlut3-1 and shGlut3-2) or a control shRNA (shScramble) were stably expressed in Tsc1–/– MEFs, Tsc2–/– MEFs, or U87 cells. (B) Tsc2+/+ MEFs infected with control vector (V) or Glut3 expressing-retrovirus. (A and B) The expression of Glut3 was checked by western blot (top panel) and qRT-PCR (bottom panel). (C–F) The glucose consumption (top panel) and lactate production (bottom panel) of the indicated cells were measured. Error bars indicate mean ± SD of triplicate samples. $^{*}P < 0.05$; $^{**}P < 0.01$.

shGlut3-2-expression viruses. Unsurprisingly, reduction in Glut3 expression decreased glucose consumption and lactate production in U87 cells (Fig. 5E). Moreover, to determine whether up-regulation of Glut3 is enough to induce metabolic changes in wild-type cells, we over-expressed Glut3 in Tsc2+/+ MEFs (Fig. 5B). As shown in Fig. 5F, over-expression of Glut3 led to a marked increase in glucose consumption and lactate production. Together, these observations indicate that Glut3 is pivotal for aerobic glycolysis of mouse and human cells with dysregulated mTORC1 signaling.

Depletion of Glut3 suppresses oncogenic mTORC1-mediated tumorigenesis

A shift from oxidative to glycolytic metabolism is considered to be beneficial for rapidly proliferating cancer cells that require fast building of macromolecules such as proteins, polysaccharides, lipids, and nucleic acids. Up-regulation of glycolytic metabolism has been shown to correlate with tumorigenesis and poor prognosis. Given that Glut3 contributes to increased aerobic glycolysis in mTORC1 activated cells, we considered that Glut3 is important for the proliferation and tumorigenesis of the cells with hyperactive mTORC1 signaling. Not unexpectedly, knockdown of Glut3 expression led to a marked reduction of cell proliferation and suppression of Ha-RasV12-induced anchorage-independent colony formation in Tsc1 or Tsc2-null MEFs (Fig. 6A and B). A consistent result was obtained in U87 cells when Glut3 expression was attenuated by shRNA (Fig. 6A and B). In contrast, over-expression of Glut3 promoted cell proliferation and increased colony formation (Fig. 6A and B). To further determine whether Glut3 depletion affects the ability of the cells with hyper-activated mTORC1 signaling to form tumors *in vivo*, we injected Tsc1-deficient cells expressing shGlut3-1 or shScramble subcutaneously into nude mice. Tsc1−/− MEFs expressing shGlut3-1 had markedly reduced tumorigenic capacity, in comparison to the control cells (Fig. 6C). Reduced Glut3 expression in the tumor tissues derived from Tsc1-null MEFs with shGlut3-1 was verified by immunoblotting (Fig. 6D). Moreover, IHC analysis of Ki-67 expression further confirmed that tumor cells with depleted Glut3 expression showed reduced proliferation properties *in vivo* (Fig. 6E). Taken together, these results demonstrate that Glut3 promotes the proliferation, colony formation, and tumor growth of the cells with aberrantly activated mTORC1 signaling.

Discussion

Aerobic glycolysis is a hallmark of cancer cells in which mTOR signaling is frequently hyperactivated. In the present study, we propose that the mTORC1 signaling plays a critical role in aerobic glycolysis and tumorigenesis, at least partially through the up-regulation of Glut3 expression. The transcription factor NFκB transcriptionally up-regulates Glut3 expression by acting as a downstream effector of mTORC1 signaling (Fig. 6F).

Growing evidence implicates that the mTORC1 signaling exerts a vital influence on glucose metabolism. Previous studies have shown that aberrantly activated mTORC1 promotes glycolysis, and glycolytic inhibitors suppress cell proliferation and tumor growth caused by oncogenic mTORC1 signaling [20]. Glut3, a high-affinity glucose transporter, is predominantly expressed in the brain under physiological conditions [6]. The abnormal over-expression of Glut3 has been documented in many types of cancers but the underlying mechanisms are less understood [24–26]. Here we demonstrated that Glut3 is a novel downstream effector of mTORC1, which plays a critical role in aerobic glycolysis and tumorigenesis. We propose that hyper-activated mTORC1 signaling is one of the main regulators responsible for the elevated Glut3 in cancers. Therefore, the mTORC1-specific inhibitor rapamycin could be used to treat the cancers with high Glut3 level.

The cells with aberrantly activated mTORC1 signaling are more susceptible than the control cells to glucose withdrawal [27,28]. Elevated Glut3 due to the activation of mTORC1 thus may contribute to maintaining transport of glucose at a high rate to ensure survival and proliferation of tumor cells. In fact, the key role of Glut3 in glucose metabolism and cell growth has been characterized recently in some human cancers. For example, a group reported that Glut3 up-regulation promotes the glucose uptake and the proliferation of human bladder cancer cells [29]. Another group showed that Glut3 over-expression led to the enhancement of glycolysis in human colon cancer cells [30]. In this study, we demonstrated that the increased Glut3 expression is responsible for accelerated glycolysis and tumor growth driven by aberrantly activated mTORC1 signaling. Our findings not only showed consistent results with previous studies that Glut3 is critical for glycolysis and cell proliferation, but also revealed a new molecular link between mTORC1 activation and tumorigenesis. Given that mTORC1 is frequently activated in bladder cancer and colon cancer due to mutations in the PI3K/AKT pathway, it is possible that it is dysregulated mTORC1 signaling in these cancers that promotes the expression of glut3 and subsequent acceleration of glycolysis and tumorigenesis. Thus, Glut3 may be a potential therapeutic target for the tumors with increased glycolysis resulting from hyperactive mTORC1 signaling.

Although Glut3 has been shown to be involved in tumorigenesis, there is limited documentation on the transcriptional regulation of Glut3 expression. Only several transcription factors such as Sp1, HMGA1, and HIF1α have been indicated to be involved in the regulation of Glut3 [30–33]. Even though hyper-activation of mTOR led to enhanced HIF1α expression [20,34], knockdown of HIF1α has little effect on the expression of Glut3 (Supplementary Fig. S6), suggesting that mTORC1 up-regulated Glut3 expression is HIF1α independent. Here we demonstrate that NFκB, as a downstream target of mTORC1, up-regulates the expression of Glut3 at the transcriptional level. This result is consistent with a previous study which reported that NFκB signaling transcriptionally increases the expression of Glut3 upon loss of p53 [32]. However, in the current study, mTORC1/NFκB signaling pathway up-regulation of Glut3 was observed in both p53+/+ (Tsc1+/+ and Tsc1−/− MEFs) and p53−/− (Tsc2+/+ and Tsc2−/− MEFs) cells. Thus, mTORC1 induction of Glut3 expression is p53 independent. It is likely that mTORC1 activation stimulates the NFκB/Glut3 pathway in parallel with loss of function of p53.

The cross talk between mTORC1 and NFκB signaling has been extensively studied [17,35,36]. In addition to mTORC1, mTORC2 has also been shown to activate NFκB signaling recently. For example, a study showed that mTORC2-mediated activation of NFκB signaling promotes cancer cell proliferation in glioblastoma [37]. We have also reported that enhanced αB-crystallin by mTORC2/NFκB signaling has an essential role in TSC1/TSC2 complex deficiency-mediated tumorigenesis [38]. Consistent with these previous studies, we found that both mTORC1 and mTORC2 regulate the activity of NFκB signaling (Fig. 3). Furthermore, we suggest that mTOR activates NFκB signaling through promoting phosphorylation of the IKKα/β complex (Fig. 3E). Interestingly, although NFκB signaling is controlled by both mTORC1 and mTORC2 (Fig. 3), inhibition of mTORC1, but not mTORC2, decreased the expression of Glut3 (Fig. 2). It is possible that in addition to NFκB signaling, mTORC2 could manipulate other molecules, which are also involved in the regulation of Glut3 expression. It has been reported that GSK-3β is a positive regulator of Glut3 [39], and its activity is negatively regulated by mTORC2 [40]. Therefore, it is likely that increased GSK-3β activity may offset the effect of down-regulated NFκB signaling on the expression of Glut3 when mTORC2 activity is attenuated. Future works are required to investigate this possibility.

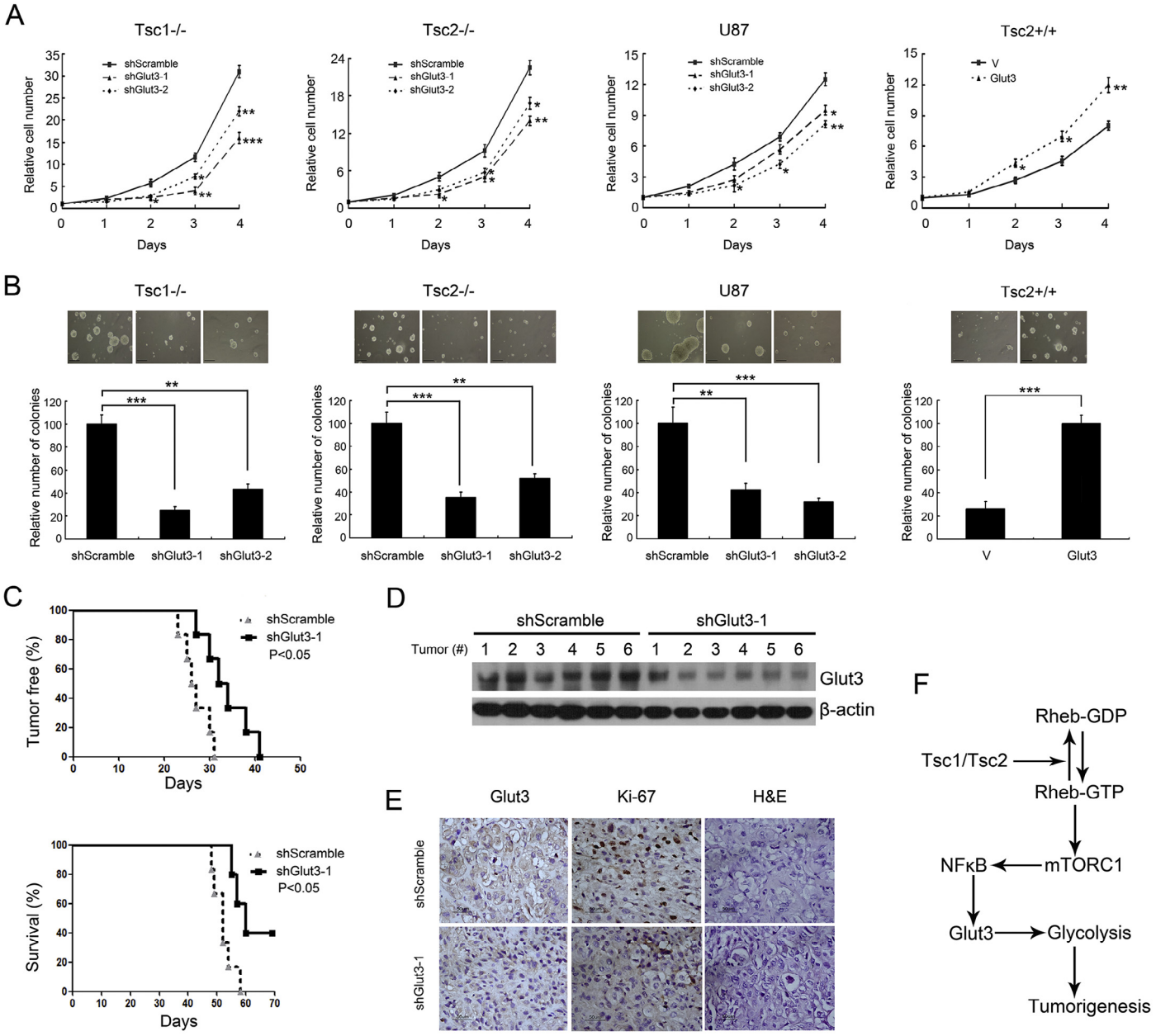

Fig. 6. Depletion of Glut3 suppresses cell proliferation, colony formation, and *in vivo* tumorigenicity. (A) The proliferation of the indicated cells was examined by MTT assay. (B) The number of colonies formed in soft agar by the indicated cells. Representative images were presented. Scale bar, 500 μm. Error bars indicate mean ± SD of triplicate samples. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. For soft agar assay, Tsc1–/–, Tsc2–/– or Tsc2+/+ MEFs were infected with the corresponding viruses together with a Ha-RasV12-expressing retrovirus. (C) Tsc1-null MEFs infected with shGlut3-1 or shScramble-expressing lentiviruses were inoculated subcutaneously into nude mice, and followed for tumor development (top panel) and survival (bottom panel). (D) Tumor lysates were analyzed for Glut3 expression by western blot. (E) The formalin-fixed and paraffin-embedded tumor sections were subjected to immunohistochemical staining. Representative images were presented. (F) Schematic illustration of the Tsc1/Tsc2/mTORC1 pathway regulated glycolysis and tumorigenesis through the NFκB/Glut3 network.

In conclusion, we demonstrated that hyperactive mTORC1 signaling contributes to accelerated glycolysis and carcinogenesis through the up-regulation of the NFκB/Glut3 signaling cascade. Our data may provide new insights into the regulation of Glut3, and lead to new diagnostic and therapeutic approaches for cancers with dysregulated mTORC1 signaling.

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Conflict of interest

None.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.01.001.

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