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Glucose metabolism induces mono-ubiquitination of histone H2B in mammalian cells

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ABSTRACT

Histone modifications play an important role in transcriptional regulation and are intimately involved in important biological and disease processes. Despite their functional significance, whether and how extracellular signals modulate histone modifications are not well defined. Using mono-ubiquitination of histone H2B as a model system, we have previously shown that mono-ubiquitination of histone H2B is induced by glucose through glycolysis in budding yeast *Sacchromyces cerevisiae* [1]. Because histones are well conserved proteins among eukaryotes and glycolysis is the most conserved metabolic pathway, we reasoned that the glucose–glycolysis–uH2B signal pathway originally discovered in yeast may be conserved in human cells. Using cultured human glioma cells as a model, we show in this report that extracellular media modulated global levels of mono-ubiquitination of histone H2B at K120 (uH2B). Nutrient deprivation removed the ubiquitin moiety of uH2B. Glucose-containing media induced uH2B in the cells while media lacking glucose had no effect on the induction of uH2B, suggesting that glucose was required for inducing uH2B in the cells. In contrast, non-metabolic glucose analogs were defective in inducing uH2B, suggesting that glucose metabolism was required for glucose-induced mono-ubiquitination in the cultured glioma cells. Moreover, shRNA knockdown of PKM2, an essential enzyme for glycolysis in malignant tumors, inhibited mono-ubiquitination of histone H2B in these cells. Taken together, our previous and current results demonstrate that the novel glucose–glycolysis–uH2B signal pathway is well conserved from yeast to mammalian cells, providing an evolutionarily-conserved regulatory mechanism of histone modifications.

1. Introduction

Histone modifications are intimately involved in fundamental processes such as transcription, DNA replication, recombination and DNA repair [2,3]. Despite their physiological significance, physiological signals of histone modifications remain poorly defined.

In budding yeast *Sacchromyces cerevisiae*, histone ubiquitination occurs at lysine residue 123 of histone H2B predominantly as a monomer [4]. Mono-ubiquitinated histone H2B (uH2B) is not thought for targeting H2B for degradation [3]. Although yeast Rad6 and Cdc34 ubiquitinate H2B *in vitro* without E3 [5–7], only Rad6 is required for inducing uH2B *in vivo* [4]. Bre1, a Rad6-associated RING finger protein, is E3 ligase involved in the specificity of mono-ubiquitination of H2B [8–10]. Additionally, factors in Paf1/RNA polymerase II complex are also required for inducing uH2B, which in turn directs methylation of histone H3 at lysine 4 and lysine 79 [10–19]. It appears that ubiquitin conjugation of H2B occurs through a co-transcriptional mechanism and is associated with the actively transcribed genes [3]. Mono-ubiquitination of histone H2B can also be reversed by Ubp8, a component of the transcriptional activator SAGA (Spt/Ada/Gcn5/Acetyltransferase) complex [20–22] and by Ubp10 [23,24].

Identification of Rad6 as an E2 for the mono-ubiquitination of H2B in yeast provides a genetic and biochemical basis for understanding the function of mono-ubiquitination of H2B [4]. A ubiquitin-conjugating mutant of Rad6 fails to complement the silencing defect, indicating that the ubiquitin-conjugating activity of Rad6 is critical for telomeric and HML silencing [8]. Consistent with the role of Rad6, uH2B acts in telomeric silencing [15,16]. In addition, uH2B may silence euchromatic genes. For instance, repression of *ARG*1 requires a functional Rad6 capable of H2B ubiquitination [25]. Both ubiquitination and deubiquitination of H2B are required for transcriptional activation of *GAL* genes [20,21,26]. The change in uH2B levels probably results from differential binding of Rad6 and Ubp8 to the *GAL*1 promoter [20,21], whereas the Ubp8-containing SAGA complex binds to the promoter during the entire

Abbreviations: uH2B, mono-ubiquitinated H2B; PBS, phosphate buffered saline; Glc, glucose; DMEM ($g^+g^+p^+$), DMEM media supplemented with 4.5 g/L glucose, 0.584 g/L glutamine and 110 mg/L pyruvate; DMEM ($g^-g^+p^-$), DMEM media lacking glucose and pyruvate but containing glutamine (0.584 g/L); DMEM ($g^-g^-p^-$), DMEM media lacking glucose, glutamine and pyruvate; *d*FBS, dialyzed fetal bovine serum.

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course of transcriptional activation [27–29]. Therefore, upon dissociation of Rad6 from the promoter, uH2B levels decrease presumably caused by removal of ubiquitin from uH2B by Ubp8 [20,21].

The role of uH2B has emerged recently in human tumor cells. hRNF20/Bre1, human histone H2B (K120) ubiquitin ligase and a homolog of yeast Bre1, physically interacts with the tumor suppressor protein p53, functioning as a transcriptional co-activator of p53 [30]. hRNF20/Bre1 is also required for p53 expression. hRNF20 depletion enhances transcriptional effects of epidermal growth factor, increases cell migration, and elicits transformation and tumorigenesis, suggesting that hRNF20/Bre1 may be a putative tumor suppressor [31]. Usp22, a human homolog of yeast ubp8, is thought to be a cancer stem cell marker [32] that is required for Myc-mediated cell transformation [33].

The evolutionary conservation of modifying enzymes of uH2B in yeast and human suggest the importance of mono-ubiquitinated histone at H2B K123 throughout the eukaryotic evolution. To dissect the upstream signaling events of uH2B, we have previously used uH2B in budding yeast as a model system. We show that glucose induces uH2B through glycolysis, revealing a novel paradigm of chromatin regulation [1]. Since glycolysis is the most conserved metabolic pathway and histones are among the most conserved proteins, we reasoned that the glucose–glycolysis–uH2B pathway may be evolutionarily conserved from yeast to man. In this report, we show that extracellular media regulated global levels of mono-ubiquitinated histone H2B at K120 (uH2B) in human glioma cells. Nutrient deprivation of glioma cells removed the ubiquitin moiety of uH2B. Like in yeast, glucose-containing media induced uH2B in glioma cells while media lacking glucose was defective in inducing uH2B. Non-metabolic glucose analogs were also defective in inducing uH2B, suggesting that glycolysis was required for glucose-induced uH2B. Furthermore, depletion of PKM2, a critical enzyme for glycolysis in malignant tumors, inhibited mono-ubiquitination of histone H2B. Taken together, our previous and current findings demonstrate that the novel glucose–glycolysis–uH2B signal pathway is well conserved from yeast to man, providing an evolutionarily-conserved regulatory mechanism of histone modifications.

2. Materials and methods

2.1. Cell lines, chemicals, antibodies and culture media

The U87 MG and U118 MG (hereafter abbreviated as U87 and U118, respectively) human glioblastoma (grade IV glioma) cells were kindly provided by Drs. Hui Zhang and Hong Sun. U118 and U87 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (4.5 g/L glucose, 0.584 g/L glutamine and 110 mg/L pyruvate, denoted DMEM$g^+g^+p^+$ in this report, Cat# 11995, Gibco, Carlsbad, CA, supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Dialyzed fetal bovine serum (FBS) was done with dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) in PBS (pH 7.4) (6×1 l) during the 48-h period at 4 °C. The dialyzed FBS (*d*FBS) was filtered through a 22 μm filter unit (Millipore, Billerica, MA) and stored at 4 °C before use. L-Glucose, 2-deoxyglucose, and 3-*O*-methyl-D-glucopyranose) were purchased from Sigma–Aldrich (St. Louis, MO). Antibody to H2B was from Millipore (Billerica, MA), the antibody raised against the branched ubiquitinated H2B at K120 was from MediMabs (Cat# MM-0029, Montreal, Canada) [31], and antibody to PKM2 (ab55602) is from Abcam (Cambridge, MA). Immunoreactive bands were detected using SuperSignal West Dura Extended Duration Substrate kit (Thermo Scientific, Waltham, MA). Glucose-minus DMEM was purchased from Gibco (Cat# 11966) and contained 584 mg/L glutamine but no glucose and pyruvate (denoted as DMEM$g^-g^+p^-$). DMEM with no glucose, no glutamine, no sodium pyruvate (denoted as DMEM$g^-g^-p^-$) was purchased from Hyclone (Cat# RR11983.01, Logan, UT).

2.2. Lentiviral shRNA transduction particles

Lentiviral transduction particles for knockdown of human PKM2 gene were purchased from Sigma–Aldrich: clone TRCN 0000037611 contains the inserted sequence of CCGGCGGGTGAAC TTTGCCATGAATCTCGAGATTCATGGCAAAGTTCACCCGTTTTG; TRCN 00000195352 contains the inserted sequence of CCGGCTTTCCTG TGTGTACTCT GTCCTCGAGGACAGAGTACACACAGGAAAGTTTTTTG; TRCN 00000 195581 contains the inserted sequence of CCGGCAAC GCTTGTAGAACTCACTCCTCGAGGAGTGAGTTC TACAAGCGTTGTTTT TTG; TRCN 00000199494 contains the inserted sequences of CCGGGCCCGAGGCTTCTTCAAGAAGCTCGAGCTTCTTGAAGAAGCCTC GGGCTTTTTTG. The equal titer mixture of TRCN 00000-37611, -195352, -195581, -199494 clones is abbreviated as shPKM2 thereafter. Control lentiviral virus for knockdown Luciferase (SHC007, hereafter abbreviated as shLuciferase) was purchased from Sigma–Aldrich and contains the inserted sequence: CCGGCGCTG AGTACTTCGAAATGTCTCGAGGACATTTCGAAGTA CTCAGCGTTTTT. The transduction of lentiviral particles generally was according to the protocol provided by the manufacturer. Specifically, the U87 glioma cells were seeded at 0.5×10^5 cells per 60 mm dish while U118 glioma cells were seeded 1.0×10^5 cells per 60 mm dish [DMEM containing glucose, glutamine and pyruvate + 10% FBS + 1% antibiotics). Two days later, hexadimethrine bromide (Sigma–Aldrich) was added to cell culture medium (final concentration 8 μg/mL) to enhance transduction efficiency. shPKM2 or shLuciferase (multiplicity of infection (MOI) = 6 for U118 cell and MOI = 12 for U87 cells) were subsequently added and the plates were swirled gently to mix. The viral particle-containing medium was replaced with fresh medium Day 2. On Day 3, the medium was replaced by fresh medium containing 2 μg/mL puromycin (Sigma–Aldrich) for selecting transfected cells. The puromycin containing medium was then changed every 3 days.

2.3. Western-blotting analysis

Cell cultures (treated or non-treated) were harvested by scraping in their medium on ice, and then centrifuged. The cell pellets were re-suspended in $2 \times$ SDS protein sample buffer containing β-mercaptoethanol and heated at 100 °C for 5 min. Total protein extract was separated by 12% SDS polyacrylamide gel electrophoresis and used for Western blot analyses.

3. Results and discussion

3.1. Nutrient deprivation inhibited mono-ubiquitinated histone H2B (uH2B) in cultured glioma cells

Our previous studies show that nutrient (glucose) deprivation inhibits mono-ubiquitination of histone H2B in baker's yeast. To explore whether nutrient deprivation could also inhibit mono-ubiquitination of histone H2B in human cells, we first cultured human glioma cells to 40–60% (100 mm dish) in the standard cell culture medium—high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). We removed the DMEM medium/FBS from culture dishes and carefully rinsed the dishes twice with phosphate buffered saline (PBS) to remove residual amount of the DMEM/FBS media. We subsequently incubated the cells with PBS at 37 °C and collected cell samples after 1–6 h. During the PBS incubation, while some cells became de-attached, the rest were attached to the

Petri-dish plates. To collect both attached and de-attached cells, we scrapped attached cells in the PBS and collected the cell suspension by centrifugation at 300 relative centrifugal force (RCF) for 2 min. The collected cells were boiled in SDS–PAGE sample for Western analysis. Using the antibody that was raised specifically against ubiquitinated histone H2B at lysine 120 [31], we found that uH2B was present in the cells grown in the high-glucose-DMEM medium with FBS. Steady-state levels of uH2B significantly decreased over 6-h incubation period in PBS (Fig. 1A).

To examine whether uH2B levels could be recovered in the cells that had been incubated for 6 h in PBS, we added the high glucose DMEM with 10% FBS to the 6-h PBS-treated cells for at 37 °C. As shown in Fig. 1B, uH2B levels were largely recovered after 2 h of incubation with the high glucose media, indicating that signaling machineries of mono-ubiquitination and deubiquitination of histone H2B were largely functional during the 6-h incubation of PBS.

Therefore, our results demonstrated that these nutrient starved cells would provide a simple cell-based assay platform for identifying extracellular signals that regulate mono-ubiquitination of histone H2B. The results also demonstrated that glucose-containing DMEM supplemented with 10% FBS contained signal molecule(s) for inducing uH2B in the cultured cells.

3.2. Glucose is an inducer of uH2B in cultured cells

We sought to identify which component(s) in the high glucose DMEM/FBS is responsible for inducing mono-ubiquitination of histone H2B in cultured glioma cells. Our previous research has already shown that glucose induces mono-ubiquitination of H2B in budding yeast (*S. cerevisiae*) [1]. To test if glucose-induced uH2B in the cultured human cells, we incubated the 6-h PBS-treated U87 and U118 cells with glucose-minus DMEM (Gibco, Cat# 11966, denoted DMEM ($g^-g^+p^-$)) with either regular or dialyzed FBS (Gibco). As shown in Fig. 2A, glucose-minus DMEM supplemented with undialyzed FBS could induce mono-ubiquitination of ubiquitination in H2B.

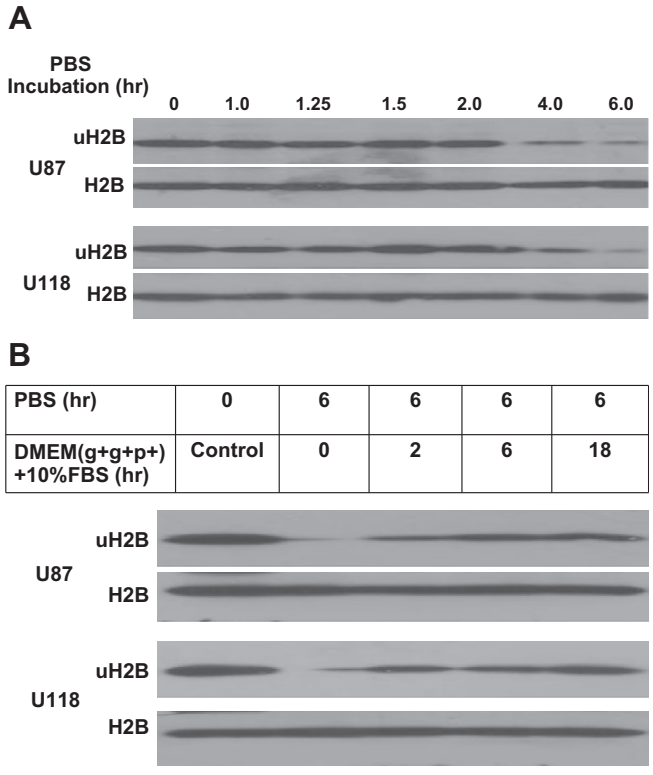

Fig. 1. Nutrient deprivation inhibited mono-ubiquitination histone H2B (uH2B) in cultured human glioma cells. (A) Human glioma cells (U87 and U118) were rinsed with PBS twice and incubated in PBS for indicated periods of time. Equal amounts of total proteins were separated by SDS–PAGE and analyzed for uH2B and H2B with their respective antibodies. (B) U87 and U118 were rinsed with PBS twice and incubated in PBS for 6 h. After centrifugation, cell pellets were re-suspended with DMEM ($g^+g^+p^+$) and 10% undialyzed fetal bovine serum (FBS) and incubated at 37 °C. The cells were then collected for Western analysis for uH2B and H2B. DMEM ($g^+g^+p^+$) denotes DMEM media supplemented with 4.5 g/L glucose, 0.584 g/L glutamine and 110 mg/L pyruvate.

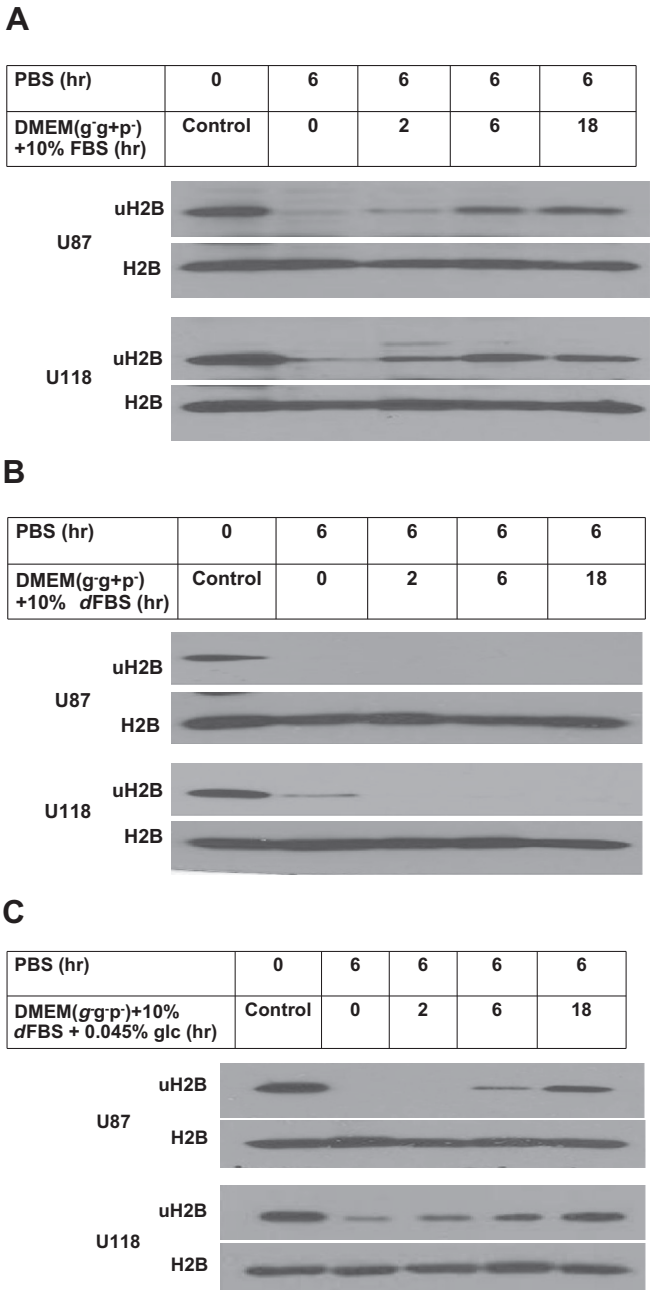

Fig. 2. Glucose is an inducer of uH2B in cultured human glioma cells. (A) U87 and U118 were rinsed with PBS twice and incubated in PBS for 6 h. After centrifugation, cell pellets were re-suspended with DMEM ($g^-g^+p^-$) with either 10% undialyzed FBS, dialyzed FBS (*d*FBS) or 0.045% glucose. Western analysis was done as described in Fig. 1. (A) uH2B levels in U87 and U118 treated with DMEM ($g^-g^+p^-$) with undialyzed FBS. (B) uH2B levels in U87 and U118 treated with DMEM ($g^-g^+p^-$) with dialyzed FBS (dFBS). (C) uH2B levels in U87 and U118 treated with DMEM ($g^-g^-p^-$) with dialyzed 10% FBS and 0.045% glucose (Glc). DMEM ($g^-g^+p^-$) denotes DMEM media lacking glucose but containing glutamine (0.584 g/L). DMEM ($g^-g^-p^-$) denotes DMEM media lacking glucose, glutamine and pyruvate.

In contrast, glucose-minus DMEM supplemented with dialyzed FBS supplementation of regular FBS was incapable of inducing uH2B (Fig. 2B). These data suggest that dialyzed FBS might have removed small molecules such as glucose and metabolites present in normal FBS. These data (Fig. 2B) also demonstrated that glutamine and other nutrients in the media were not required for inducing uH2B.

To determine if glucose was indeed responsible for inducing uH2B in the cultured glioma cells, we used glucose-minus DMEM medium (Hyclone) with dialyzed FBS supplemented with glucose to final concentration of 0.45 g/L. Specifically, we grew U87 and U118 cells in the regular high glucose DMEM media to 40–60% confluency and rinsed cells with PBS and subsequently incubated these cells in PBS for 6 h. After cells were collected as described earlier and replated with the glucose-minus media supplemented with dialyzed FBS and glucose (final concentration of 0.45 g/L). After 2-, 6-, and 18-h incubation, we collected the cells for Western analysis. As shown in Fig. 2C, uH2B was largely recovered following glucose incubation, demonstrating that glucose was required for inducing and mainstreaming uH2B.

3.3. Non-metabolic glucose analogues were incapable of inducing uH2B in cultured cells

We further sought to determine if glucose metabolism was required for inducing uH2B. To test this, we used three non-metabolic analogs of glucose: L-glucose, 2-deoxyglucose, and 3-*O*-methyl-D-glucopyranose. L-Glucose cannot be transported into cells. 2-Deoxyglucose is transported and phosphorylated at the C-6 position but cannot be phosphorylated at the C-1 position, which is a critical step for glycolysis entry. 3-*O*-methyl-D-glucopyranose is transported; but cannot be phosphorylated at the C-6 position [1].

To test if non-metabolic glucose analogs had any effect on inducing uH2B, we incubated 6-h PBS-treated glioma cells in the glucose-minus DMEM medium (Hyclone) supplemented with dialyzed FBS and L-glucose, 2-deoxyglucose, or 3-*O*-methyl-D-glucopyranose to final 0.45 g/L concentration. We then collected cells after 2-, 6-, and 18-h incubation, prepared total cell extracts and analyzed uH2B. In contrast to glucose (Fig. 2C), none of the three non-metabolic glucose analogs was capable of inducing uH2B in the glioma cells (compared to Figs. 2C and 3). These results are remarkably consistent with our previous studies with baker's yeast. Taken together, these data suggest that glucose metabolism is required for inducing H2B in cultured glioma cells.

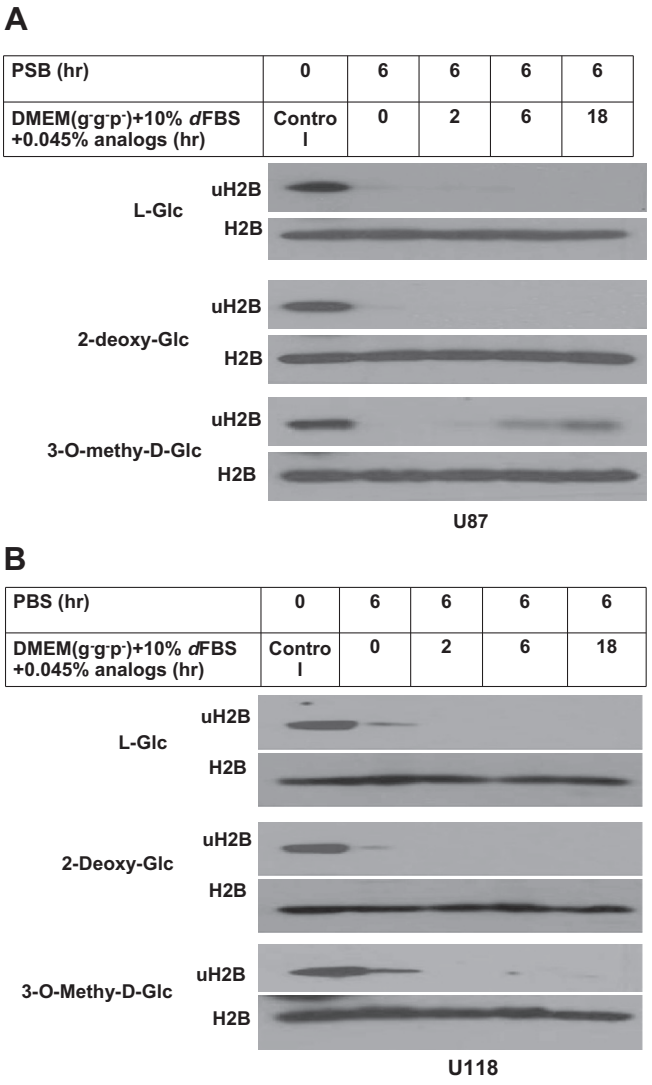

Fig. 3. Non-metabolic glucose analogues were incapable of inducing uH2B in cultured human glioma cells. U87 and U118 cells were treated as Fig. 3C except with non-metabolic glucose analogs, L-glucose, 2-deoxyglucose or 3-*O*-methyl-D-glucopyranose. (A) uH2B levels of U87 cells treated with the three non-metabolic glucose analogs. (B) uH2B levels of U118 cells treated with the three non-metabolic glucose analogs.

3.4. Glycolytic machinery is required for inducing glucose-mediated mono-ubiquitination of histone H2B in cultured cells

In yeast, we have previously shown that glycolysis is required for inducing uH2B [1]. Genetic studies show that glycolysis is required for inducing uH2B in yeast [1] (Urasaki et al., in preparation). Like in yeast, human pyruvate kinase is the last irreversible and rate limiting step of glycolysis, catalyzing the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, generating ATP and pyruvate. The M2 subtype of pyruvate kinase (PKM2) is a pyruvate kinase subtype expressed in malignant human cells and is essential for glycolysis in malignant cells [34].

To further determine whether glucose metabolism is required for mono-ubiquitination of histone H2B, we knocked down pyruvate kinase (PKM2) in glioma cells using lentiviral PKM2 shRNA regular DMEM (containing glucose, glutamine and pyruvate) + 10% FBS + 1% antibiotics. We infected U118 cells with lentiviral shPKM2 (multiplicity of infection MOI = 6) or control lentivirus encoding shRNA that targets luciferase (shLuciferase) (MOI = 6). We selected transfected cells with puromycin and collected the cells immediately before lentivirus infection (Day 0 sample) as well as 3 and 6 days post lentiviral shRNA infection. Steady-state levels of uH2B were significantly reduced when PKM2 was depleted by shRNA. Whereas, mono-ubiquitinated levels of histone H2B remained unchanged in shLuciferase treatment (Fig. 4). Although knockdown of PKM2 in U87 required much higher titer of the lentivirus (MOI = 12), shPKM2 treatment also inhibited uH2B at Days 3 and 6 (data not shown). Taken together, our results demonstrate that glycolytic enzymes are required for inducing uH2B in cultured glioma cells.

The eukaryotic histone proteins undergo complex and dynamic modifications for regulating chromatin structure and gene transcription (e.g. acetylation, methylation, phosphorylation, and ubiquitination) [2,3,35]. Despite the importance of histone modifications, physiological signals to chromatin through histone modifications have not been well defined. We have previously shown the first example for linking glucose metabolism with histone modifications in baker's yeast, in which glucose induces mono-ubiquitination of histone H2B through glycolysis [1]. In this

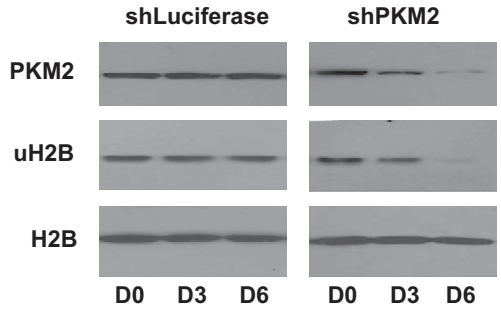

Fig. 4. PKM2 is required for inducing glucose-mediated mono-ubiquitination of histone H2B in cultured human glioma cells. PKM2 and uH2B levels of U118 infected with lentivirus encoding shRNA that targets PKM2 and control shRNA targeting luciferase at Days 0 (prior to viral infection), 3 and 6. Western analysis was done as described in Fig. 1.

study, we showed that the glycolytic regulation of uH2B was conserved in human cells. Specifically, we showed that glucose induced mono-ubiquitination of H2B at lysine 120 in human glioma cells. In contrast, non-metabolic glucose analogs were defective in inducing uH2B. Furthermore, shRNA depletion of PKM2, a pyruvate kinase of the last and rate-limiting step of glycolysis in malignant tumors, inhibited mono-ubiquitination of histone H2B. Taken together, we demonstrate that the novel glucose–glycolysis–uH2B pathway is conserved from yeast to cultured human cells.

The requirement of glycolysis for inducing uH2B is of particular interest. Metabolism in tumors is different from that in most quiescent tissues. Tumor cells take up glucose at higher rates than their adjacent normal tissues, a phenomenon known as aerobic glycolysis or the Warburg effect [36]. Pyruvate kinase isoform M2 (PKM2) is thought to critically regulate aerobic glycolysis for supplying metabolic intermediates for tumor cell proliferation [34,37,38]. Although histone H2B is also mono-ubiquitination at lysine 120 in cultured normal human dermal fibroblasts and normal human mammary epithelial cells (data not shown), the requirement of glycolysis for inducing uH2B warrants further studies to determine whether mono-ubiquitination of H2B at lysine 120 is different in tumor cells from that of adjacent normal cells in mouse models and cancer patients.

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