Inhibition of BUB3 shunts glucose to glycolytic pathway by inducing PFKFB3 accumulation

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Research article

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Abstract

Purpose: Metabolic reprogramming as a hallmark of cancer has countless connections with other biological behavior of tumor such as rapid mitosis. Mitotic checkpoint protein BUB3 as a key protein involved in the regulation of mitosis is modulated by PKM2, an important glycolytic enzyme. However the role of BUB3 in glucose metabolism remains unknown.

Methods: We analyzed the TCGA data to evaluate BUB3 expression in certain tumors. The uptake of glucose and CO2 incorporation was tested by isotopic tracer methods. The lactate, NADPH, NADP and metabolic enzyme activities were tested by assay kits accordingly.

Results: We show here that BUB3 is over expressed in cervical cancer and hepatocellular carcinoma. Interference of BUB3 increase the uptake of glucose and shunts the metabolic flux from pentose phosphate pathway to glycolytic pathway. The glycolysis metabolites lactate is increased by BUB3 interference whereas NADPH/NADP ratio is reduced. With regard to metabolic enzymes, interference of BUB3 increase PFKFB3 on protein level and enzyme activity, but not mRNA level. Moreover, the increasing of protein level is diminished when proteasome degradation pathway is blocked by MG132.

Conclusions: BUB3 is a potential tumor promoter and plays certain roles in cancer cellular metabolic reprogramming.

Background

Reprogramming of cellular metabolism has been reckoned as one of the hallmarks of cancer[1]. Cancer cells often use glucose as a carbon source for glycolysis, even in oxygenated environments, which is known as the Warburg effect. By this type of glucose metabolism, cancer cells produced sufficient nucleotides, proteins, and lipids, which are necessary for the rapid growth and division[2]. And one of the key enzymes in this highly glycolytic metabolic type is 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3).

PFKFB3 has been found to be up-regulated in numerous cancers[3]. It converts fructose-6-phosphate to fructose-2,6-bisP (F2,6BP). F2,6BP is an allosteric activator of 6-phosphofructokinase-1, stimulating glycolysis. It was more recently discovered that PFKFB3 degradation through the proteasome pathway, thus shunting glucose usage from glycolysis to the pentose phosphate pathway for NADPH generation [4]. These findings established a significant role of PFKFB3 in cancer glucose metabolism as a switcher between glycolysis and pentose phosphate pathway.

BUB3 mitotic checkpoint protein, also known as BUB3 or hBUB3, is involved with the regulation of the Spindle Assembly Checkpoint (SAC). BUB3 delays the start of anaphase by directing the localization of kinetochore during prometaphase[5]. SAC is activated during the process of cell division, preventing separation of duplicated chromosomes until each chromosome is properly attached to the spindle apparatus. When SAC is not active, BUB3 facilitates binding of APC/C and Cdc20 for substrate ubiquitination[6]. Tumor-specific pyruvate kinase M2 (PKM2), a key enzyme in aerobic glycolysis, binds to Bub3 during mitosis and phosphorylates Bub3 at Y207.

Thus facilitates BUB3 recruitment to kinetochores[7]. However, whether BUB3 plays a role in glucose metabolism remains unknown.

In the present study, we show that interference of BUB3 by siRNA shunts glucose from (pentose phosphate pathway) PPP to glycolytic pathway in cancer cells. It is possibly because inhibition of BUB3 stabilize PFKFB3 in protein level. Thus the relationship between BUB3 and PFKFB3, as well as the influence of BUB3 on glucose metabolism in cancer cells were revealed for the first time.

Methods

2.1 Materials

Cell culture reagents (DMEM and fetal bovine serum) were from Invitrogen/Gibco. [1- 14 C]-glucose and [6- 14 C]-glucose were from Shenzhen Zhonghe Headway Bio-Sci & Tech Co. MG132 were obtained from Sigma-Aldrich.

2.2 Cell culture

The human cervical cancer cell line Hela and hepatocellular carcinoma HepG2 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were maintained in DMEM medium. The media were supplemented with 10% FBS and 100 units/mL penicillin/streptomycin. Cell cultures were maintained in 5% CO_2 and air in a humidified 37°C incubator.

2.3 TCGA data analysis

The Cancer Genome Atlas (TCGA) online gene profile data for cervical cancer and hepatocellular carcinoma (http://cancergenome.nih.gov/) was downloaded. The relative mRNA expression levels of BUB3 were analyzed from a dataset of 304 cases of cervical cancer and 3 cases of normal cervical tissue, as well as a dataset of 371 cases of hepatocellular carcinoma and 50 cases of normal liver tissue.

2.4 Transfection of siRNA

Cells were transfected with oligo siRNAs using Lipofectamine 2000. The sequences of siRNA oligos used in this study are as follows: Non-specific, UUCUCCGAACGUGUCACGUTT, ACGUGACACGUUCGGAGAATT; BUB3, CAAGCAGGGUUAUGUAUUATT, UAAUACAUAACCCUGCUUGTT.

2.5 Measurement of cell viability

Cells were seeded in 96-well plates at 5x10 3 cells/well. After the indicated time, cell viability was determined using the CCK-8 assay (Dojindo) according to the manufacturer's instructions.

2.6 mRNA expression analysis

Total RNA was isolated using a Trizol kit (Omega, Norcross, GA, USA) and transcribed to cDNA with a cDNA synthesis kit (Takara, Otsu, Japan). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Takara) and the transcript levels of genes were detected by using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used for detection of specific genes are listed below.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
BUB3	GGTTCTAACGAGTTCAAGCTGA	GGCACATCGTAGAGACGCAC
PFKFB3	GGGCCATAATCGTCCTCACC	TTGCACAGCACAGGGAAGAT
HK2	GAGCCACCACTCACCCTACT	CCAGGCATTCGGCAATGTG
GLUT1	GCCAGAAGGAGTCAGGTTCAA	TCCTCGGAAAGGAGTTAGATCC
PKM2	GGGCCATAATCGTCCTCACC	TTGCACAGCACAGGGAAGAT
LDHA	AGGAGAAACACGCCTTGATTTAG	ACGAGCAGAGTCCAGATTACAA

2.7 Immunoblotting

Cells were lysed into the RIPA buffer containing protease inhibitors by incubating on ice for 30 min. Followed by centrifugation at 10 000 g for 15 min. The extracted proteins were subjected to electrophoresis on SDS-polyacrylamide gels and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK), which were blocked and probed with specific primary antibodies with appropriate dilution at 4°C overnight. The membranes were then incubated with the horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature, followed by three washes with 1 × TBST. The immunoreactive bands were visualized by ECL Plus system (Tanon, Shanghai, China).

2.8 [18F]-Fluorodeoxyglucose ([18F]-FDG) incorporation

FDG uptake assays were performed essentially as described[8]. The cells in 12 well-plates were washed with PBS and placed in a glucose and serum free DMEM medium, to assay FDG uptake of the cells. Then 74 kBq [18F] fluorodeoxyglucose (FDG) was added to the cultures and the cells were incubated at 37°C for 1h. The cells were washed 3 times with ice-cold PBS to eliminate free FDG, and then lysed by NaOH. The lysate and a standard solution were counted in a γ counter (Beckman LS6500). Cells in parallel wells were counted. The FDG incorporation was determined as the percentage of the original concentration and normalized with the number of cells.

2.9 Glucose oxidation

The incorporation of [1-14C] or [6-14C] glucose into $^{14}CO_2$ was determined as previously reported[9]. Briefly, cells were cultured in 10cm dishes, and the cells were exposed to DMEM supplemented with [1- 14 C] glucose (0.1 μCi/ml) or [6- 14 C] glucose(0.1 μCi/ml). The dish was placed in a container to collect CO_2 produced. Rates of glucose consumption were measured by incubating cells for 120 min at 37°C. Fresh air was pumped into the container by a ventilator. The $^{14}CO_2$ was driven into a vial and trapped by Hyamine hydroxide. No-cell controls were included to correct for unspecific CO_2 trapping.

2.10 Mass spectrometry

The metabolites extraction was carried out as previously reported [4]. Glucose metabolites in the cell lysate were tested by liquid chromatography-mass spectrometer (LC-MS) (Agilent, CA, USA). The quantity of metabolites was normalized to the total protein levels.

2.11 Metabolic enzymes assays

Enzymes of glucose metabolism were assayed using an assay kit (Comin Biotechnology Co. Ltd, Suzhou, China) according to the manufacturer's recommended protocol.

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Student's t-test were used to analyze the statistical significance, in which $p < 0.05$ or less were considered as significant differences.

Results

3.1 BUB3 is overexpressed in cervical cancer.

It has been proposed that BUB3 increase colorectal cancer risk by high-throughput sequencing analysis [10]. However the clinical significance of BUB3 on other cancer types remains unclear. We extracted TCGA (The Cancer Genome Atlas) online gene profile data and performed a comprehensive evaluation. Our analysis revealed that BUB3 mRNA was overexpressed significantly in primary solid tumors (cervical cancer and hepatocellular carcinoma) compared with normal solid tissue (figure 1A and 1B). Interference of BUB3 with siRNA in Hela cells showed relatively slower proliferation rate compared with non-specific control (figure1c).

3.2 BUB3 modulate glucose metabolism.

To test if BUB3 have regulating effect on glucose metabolism in cancer cells. We interference the expression of BUB3 with siRNA. Interference efficiency was evaluated with western blot. Firstly, we tested the effect of interference of BUB3 on glucose uptake with 18F-FDG (Figure 3A). 18F-FDG is a glucose analog, with the positron-emitting radionuclide fluorine-18 substituted for the normal hydroxyl group at the C-2 position in the glucose molecule. The uptake of 18F-FDG by tissues is a marker for the cell uptake of glucose, which in turn is closely correlated with certain types of cell metabolism. As is shown in figure 2A, the uptake of 18F-FDG of Hela cells was elevated by the interference of BUB3, which indicate inhibition of BUB3 could promote glucose uptake in certain cells.

In order to determine the actual conversion of glucose to the downstream pathways, [1-4C] glucose and [6-14C] glucose were incubated with Hela or HepG2 cells transfected with siBUB3 or siNC control. The CO_2 was collected and radiation activity of $^{14}CO_2$ was measured as mentioned in Methods. Since the CO_2 produced in PPP is from the C-1 position of the glucose molecule, [1-14C] glucose produced $^{14}CO_2$ was from both PPP and TCA cycle, whereas [6-14C] glucose produced $^{14}CO_2$ was only from the TCA cycle. When BUB3 was inhibited by siRNA, the [6-14C] glucose incorporated into $^{14}CO_2$ was increased (Figure 2A), but the [1-14C] glucose incorporated into $^{14}CO_2$ was decreased (Figure 2B). This result suggested that interference of BUB3 shifts glucose metabolic flux form PPP to the TCA cycle.

To further confirm that the pentose phosphate pathway was inhibited by siBUB3, cellular NADPH and NADP were determined. As is shown in figure 2C, the NADPH/NADP ratio was decreased when BUB3 was inhibited. Since the $^{14}CO_2$ decomposed from [6-14C] glucose in the TCA cycle has to through glycolytic pathway first. We asked if the glycolysis was up-regulated by BUB3 interference. As expected, lactate production was increased when BUB3 was inhibited (Figure 2D). These results suggest that BUB3 inhibition could promote both glycolysis and TCA cycle, but inhibit PPP. Or, in other words, interference of BUB3 shunts glucose to glycolytic pathway from the pentose phosphate pathway.

Differences in glucose biotransformation between Hela cells interference with BUB3 siRNA and non-specific control were examined by metabolome analysis based on liquid chromatography mass spectrometry (LC-MS). It was showing that the sum of metabolites belonging to glycolysis was increased, while those belonging to PPP were decreased significantly by BUB3 interference (Figure 2B-2G).

3.3 Interference of BUB3 up-regulate PFKFB3 enzyme activities.

To study how glycolytic pathway is regulated by BUB3, firstly we determined if the glycolytic enzymes are affected by interference of BUB3. As is well known, hexokinase, 6-phosphofructokinase, pyruvate kinase and lactate dehydrogenase have important roles in glycolysis. We determined the activity of these key enzymes with

enzyme activity assay kits accordingly. As shown in figure 4A and 4B, when the expression of BUB3 is inhibited, the activity of phosphofructokinase and hexokinase were increased, whereas the activity of pyruvate kinase and lactate dehydrogenase remained unchanged (Figure 4C and 4D). This result suggested that phosphofructokinase and hexokinase might be regulated by BUB3.

3.4 Interference of BUB3 induce PFKFB3 accumulation in protein level by inhibiting proteasome degeneration pathway.

To further confirm that HK2 and PFKFB3 are regulated by BUB3. We determined the protein levels of these enzymes in cells by western blot. In cancer cells, HK2, PFKFB3, PKM2 and LDHA are reckoned as pivotal enzymes in glycolysis. As is shown in figure 5A, the protein levels of HK2 and PFKFB3 were up-regulated by interference of BUB3, whereas PKM2 and LDHA did not have significant change in protein levels. To test whether the transcription of these enzyme genes were changed by interference of BUB3, quantitative PCR (qPCR) was applied to determine the mRNA levels of these enzymes. As is shown in figure 5B, all of the four enzymes did not have any change in mRNA levels when BUB3 was inhibited. These results suggest that HK2 and PFKFB3 were regulated by BUB3 in protein levels, but not mRNA levels.

It was reported that PFKFB3 protein is degenerated by APC/C regulated proteasome pathway [11]. Furthermore, BUB3 was reported that could promote activation of APC/C [12]. Thus, we asked that whether the proteasome pathway played any role in the accumulation of HK2 and PFKFB3 protein induced by interference of BUB3. As is shown in figure 5C, when the proteasome is blocked by MG132, a classic proteasome inhibitor, the accumulation effect induced by BUB3 inhibition was diminished. This result suggests that BUB3 inhibition induced PFKFB3 and HK2 protein accumulation are depending on proteasome pathway.

3.5 Shifts between glycolysis and PPP modulated by BUB3 are depended on PFKFB3.

PFKFB3 is a key switcher between glycolysis and PPP metabolic pathway[4]. In our previous study, the PFKFB3-binding protein was purified by immunoprecipitation and analyzed by LC-MS/MS[13]. BUB3 was revealed as potential binding partner of PFKFB3 by mass spectrometry. Next, we applied in vivo experiments to validate the protein–protein interaction between PFKFB3 and BUB3. Anti-BUB3 antibody was capable of immuno-precipitating PFKFB3 protein from Hela cell extracts (Figure 5A).

In our previous study, we knock-out PFKFB3 gene in Hela cells with CRISPR/Cas9[13].To verify whether BUB3 interference induced glucose biotransformation is depending on PFKFB3, [1-14C] and [6-14C] glucose oxidation were estimated in Hela cells with PFKFB3 knockout. As showed in Figure 5B and 5C, interference of BUB3 was not capable of shifting the metabolic flux between glycolysis and PPP in PFKFB3 knock-out Hela cells. This result indicates that the regulating effect of BUB3 on glucose biotransformation is, at least partially, rely on PFKFB3 expression.

Discussion

BUB3 is a core component of the key mitotic surveillance mechanism spindle assembly checkpoint (SAC). It prevents anaphase onset until all chromosomes have attached to microtubules successfully and properly. It has been proposed that BUB3 increase colorectal cancer risk by high-throughput sequencing analysis[10]. However the clinical significance of BUB3 on other cancer types remains unclear. Here, we extracted TCGA (The Cancer Genome Atlas) online gene profile data and performed a deep analysis. We found that compared with normal tissue, BUB3 mRNA levels were significantly increased in cervical cancer and hepatocellular carcinoma.

BUB3 assists in the inhibition of the anaphase-promoting complex (APC/C) by modulating the phosphorylation of CDC20. By inhibiting APC/C ^{Cdc20} substrate ubiquitination, BUB3 delays cells in metaphase until all kinetochores properly attach to spindle microtubules. Which allow the spindle checkpoint additional time to correct errors in attachment. Although BUB3 has a role of delaying anaphase onset during spindle checkpoint activation, we found here that Hela cells interference BUB3 are slower in cell proliferation. This result was consistent when considering work from previous studies. Lacefield et al have proposed a model in which kinetochore localized BUB3 facilitates the binding of Cdc20 and the APC/C in metaphase. In the presence of unattached kinetochores, BUB3 prevent the onset of anaphase. But when all kinetochores are attached to microtubules properly, this role of BUB3 allows the normal activity of APC/C to ubiquitinate its substrates for timely progression into anaphase. Thus the presence of BUB does not procrastinate cell from devision, but ensures it goes properly.

It was reported that BUB3 is regulated by PKM2, a glycolysis enzyme which is instrumental in both aerobic glycolysis and gene transcription[7]. However, whether BUB3 plays a role in glucose metabolism remains unknown. In this study, we shown that glucose uptake was increased by interference of BUB3. BUB3 interference also accompanied with a shifting of metabolic flux from PPP to glycolysis. And in the metabolite level, NADPH/NADP ratio was diminished whereas lactate was increased after BUB3 interference. These results indicated that inhibition of BUB3 shunts glucose from PPP to glycolytic pathway.

6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) is a master regulator of glycolysis by its ability to synthesize fructose-2,6-bisphosphate, a potent allosteric activator of 6-phosphofructo-1-kinase. It was reported that PFKFB3 is a substrate of APC/C. APC/C plays a crucial role in brain metabolism by promoting PFKFB3 ubiquntination and thus protein degeneration[14]. In this study, we find that interference of BUB3 in Hela cells showed an increase in PFKFB3 and HK2 protein level, as well as their enzyme activities, but not mRNA level. When blocking the proteasome degradation pathway by MG132, the accumulation effect of BUB3 interference on PFKFB3 and HK2 protein level was minimized. On the other hand, in PFKFB3 knock-out Hela cells, BUB3 induced glucose biotransformation was partially blocked. These results support the idea that inhibiting BUB3 could stabilize PFKFB3 and HK2 through the proteasome degradation pathway.

Conclusions

Taken together, this study demonstrated elevated BUB3 expression accompanied by tumor generation and poor prognosis. Inhibition of BUB3 shunts glucose from the pentose phosphate pathway to glycolytic pathway by inducing PFKFB3 accumulation. However, there is still be limitation in this study. First, the effect of BUB3 on glucose metabolism was studied only in Hela cells in vitro. More types of cancer cell lines and in vivo experiments are still been needed to verify this conclusion. Moreover, the exact mechanisms of how BUB3 regulates the PFKFB3 and HK2 protein level still need to be further interpreted.

Declarations

Abbreviations

PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; BUB3: BUB3 mitotic checkpoint protein ; SAC: spindle assembly checkpoint ; PPP: pentose phosphate pathway.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Cell culture reagents (DMEM and fetal bovine serum) were from Invitrogen/Gibco. [1- 14 C]-glucose and [6- 14 C]-glucose were from Shenzhen Zhonghe Headway Bio-Sci & Tech Co. MG132 were obtained from Sigma-Aldrich.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JLi conceived the study. JLi and RZ ran the computational experiments. JLi analyzed the experimental results. GH and JLiu supervised the study. JLi wrote the manuscript. All authors read and approved the final manuscript.

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Not Applicable

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Figures

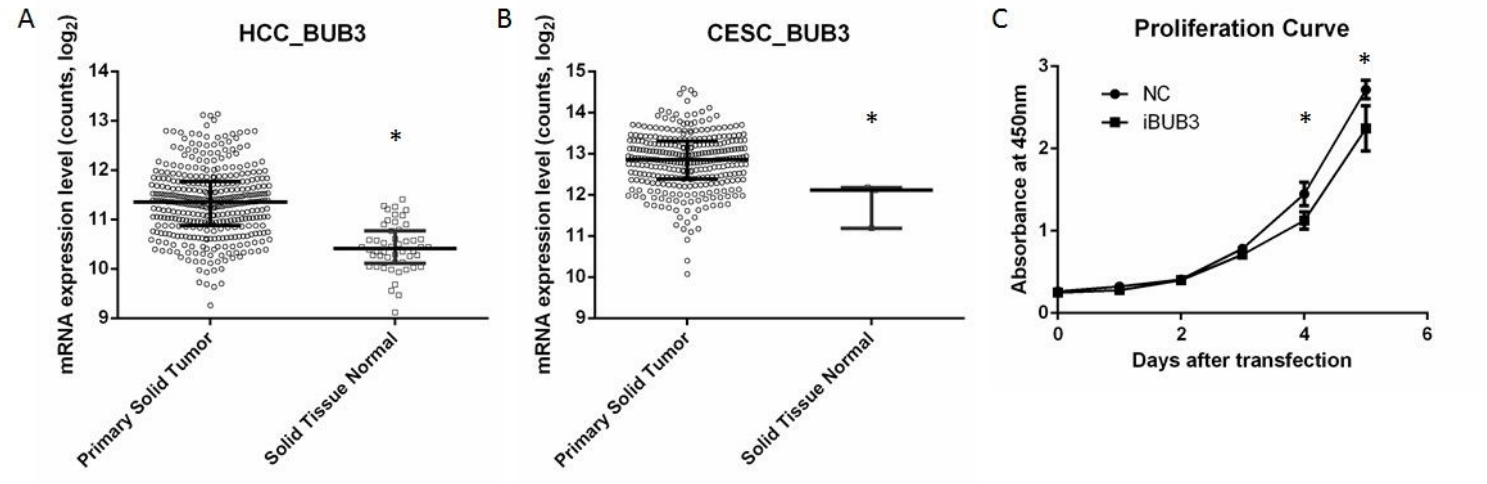

Figure 1

BUB3 is overexpressed in tumor. (A) BUB3 is overexpressed in hepatocellular carcinoma (HCC). (B) BUB3 is overexpressed in cervical cancer (CESC). (C) Interference of BUB3 decreases proliferation rate in Hela

cells.

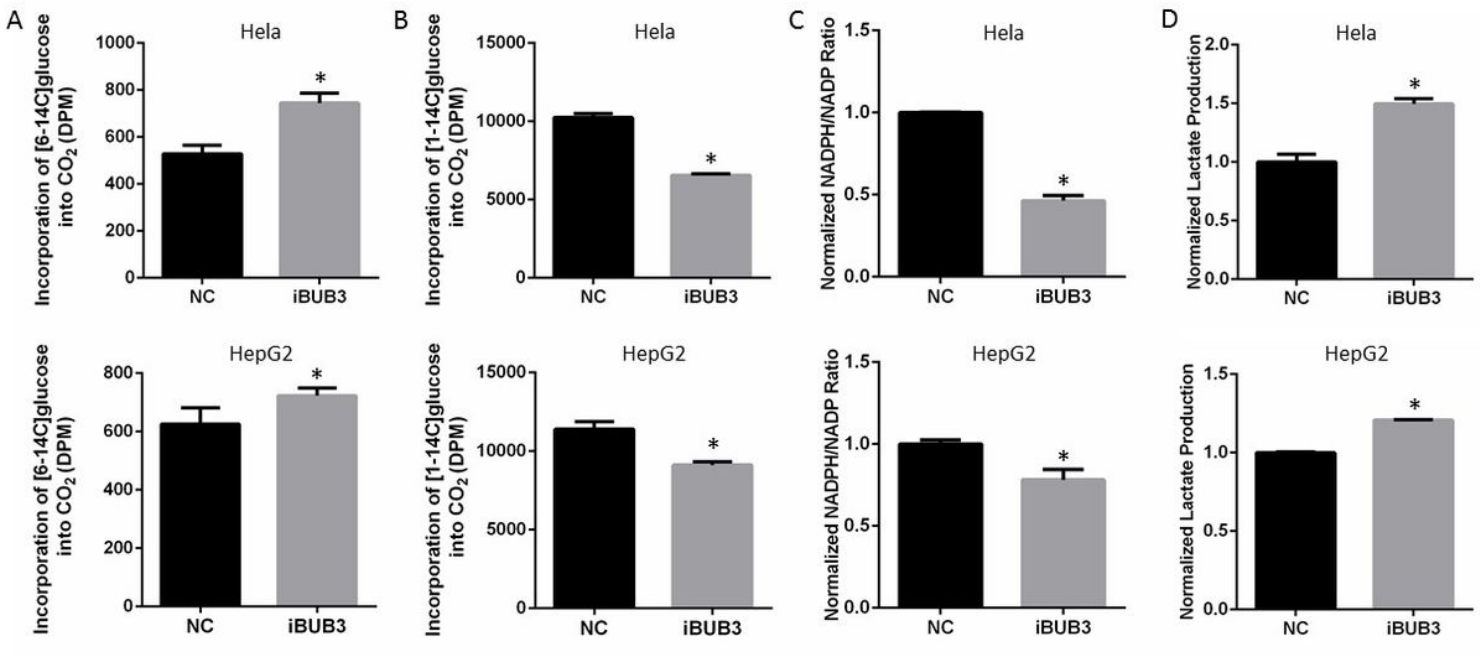

Figure 2

BUB3 modulates glucose metabolism. (A) Interference of BUB3 increases the incorporation of [1- 14C] glucose into CO2. (B) Interference of BUB3 decreases the incorporation of [6- 14C] glucose into CO2. (C) Interference of BUB3 decreases NADPH/NADP ratio. (D) Interference of BUB3 increases lactate production in Hela and HepG2 cells.

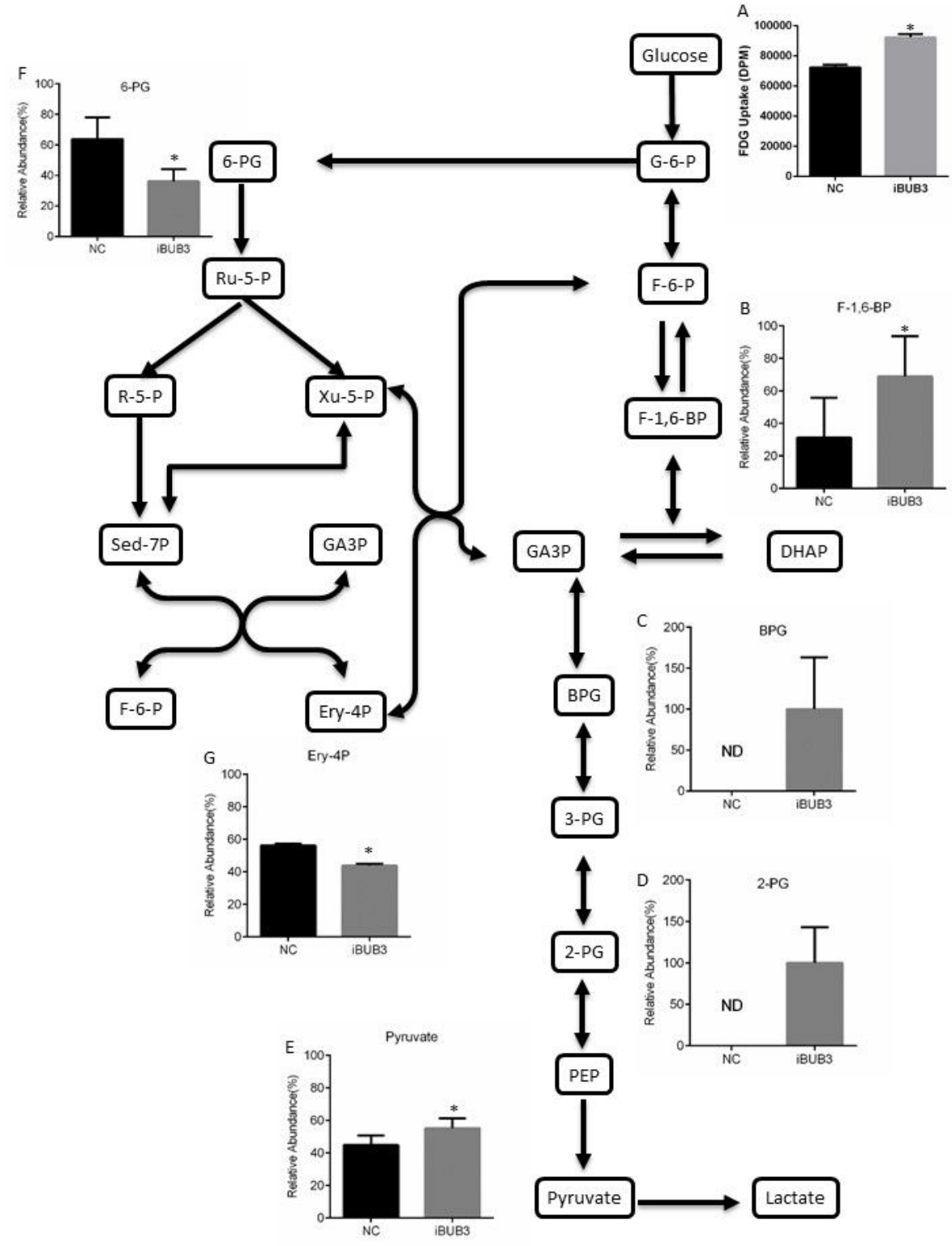

Figure 3

Inhibition of BUB3 shifts glucose metabolism towards glycolysis. (A) Interference of BUB3 promotes 18F-FDG uptake in Hela cells. (B-G)The relative abundance of metabolites in glycolysis and PPP between the control and BUB3 interference Hela cells. G-6-P, glucose 6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose 1,6-bisphosphate; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; BPG, bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; 6-

PG, 6-phosphogluconate; Ru-5-P, ribulose-5-phosphate; R-5-P, ribose-5-phosphate; Xu-5-P, xylulose-5-phosphate; Sed-7P, sedoheptulose-7-phosphate; Ery-4P, erythrose-4-phosphate.

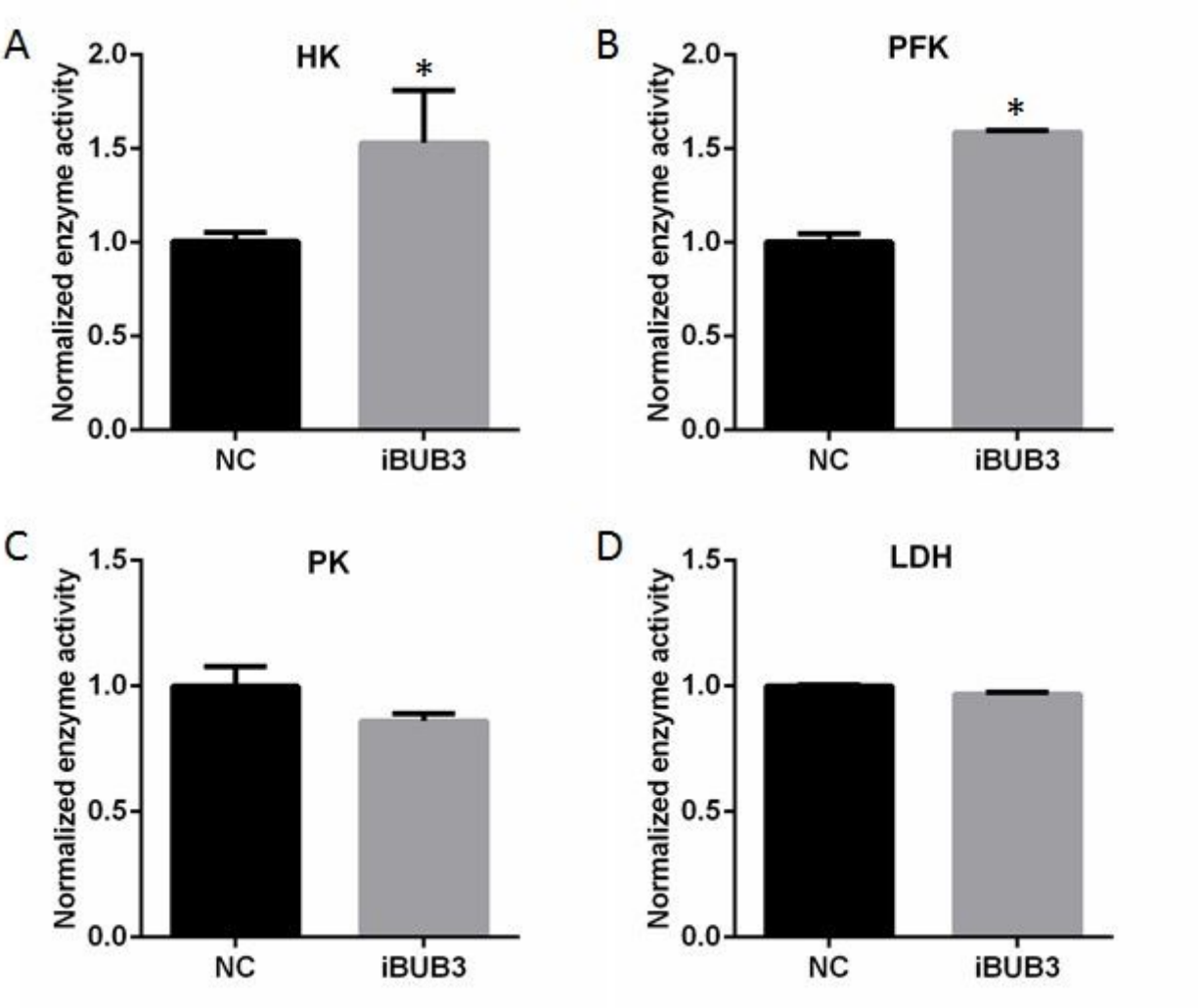

Figure 4

BUB3 regulates glucose metabolic enzymes. Interference of BUB3 increases hexokinase, HK (A) and phosphofructokinase, PFK (B)activities in Hela cells, whereas pyruvate kinase, PK (C) and lactic dehydrogenase, LDH (D) remains not any significant difference.

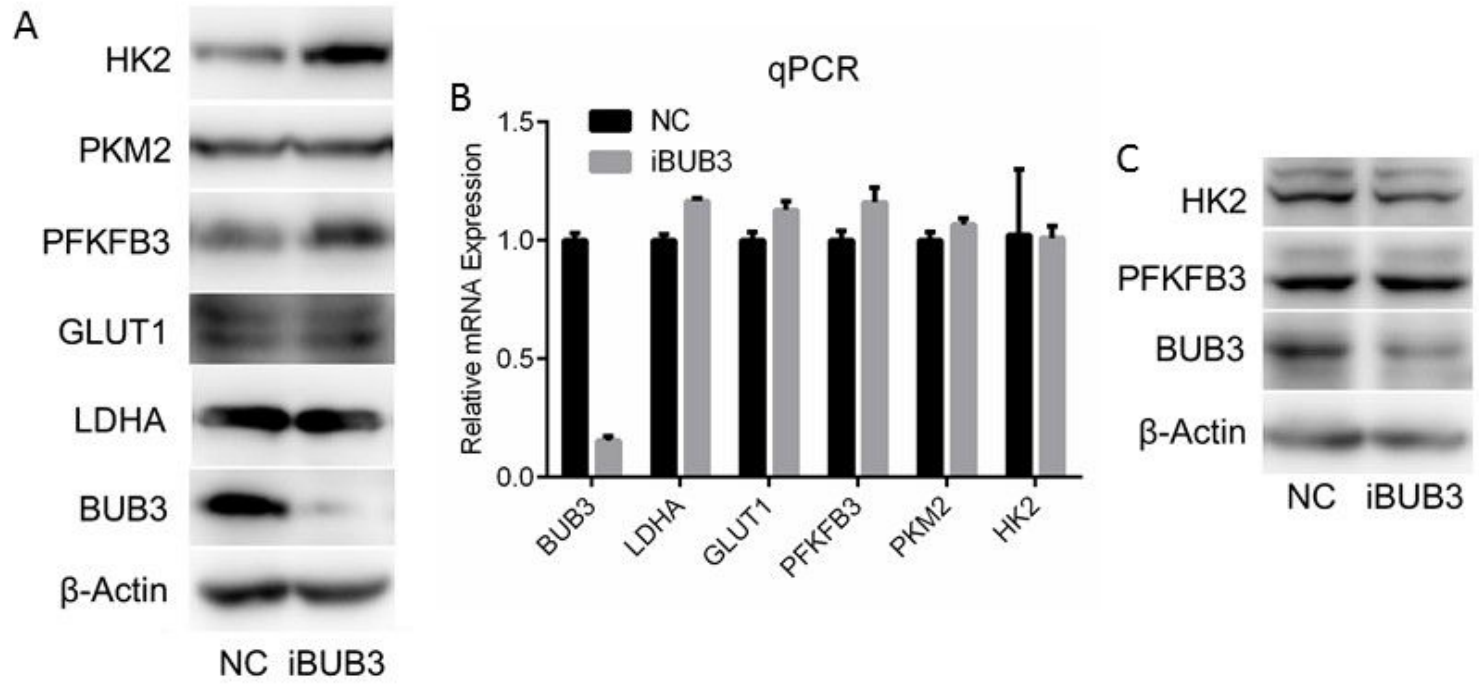

Figure 5

Interference of BUB3 promote PFKFB3 accumulation by inhibition of proteasome degeneration pathway. (A) HK2 and PFKFB3 protein are accumulated by interference of BUB3 in Hela cells. (B) Interference of BUB3 do not affect the mRNA expression of these enzymes accordingly. (C) MG132 treatment blocks the accumulation effects of HK2 and PFKFB3 induced by interference of BUB3.

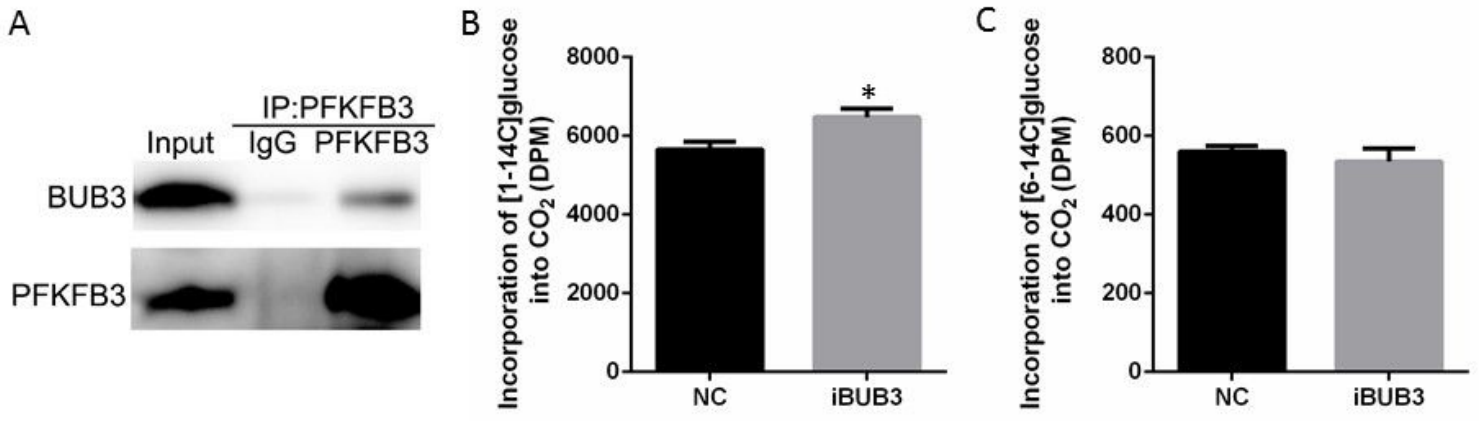

Figure 6

Modulation of BUB3 on glucose metabolic flux is depending on PFKFB3. (A) BUB3 is a binding partner of PFKFB3. In PFKFB3 knock-out Hela cells, (B) Interference of BUB3 slightly increases the incorporation of [1- 14C] glucose into CO2. (C). Interference of BUB3 has no significant effect on the incorporation of [6-14C] glucose into CO2.