Tristetraprolin-mediated hexokinase 2 expression regulation contributes to glycolysis in cancer cells

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ABSTRACT Hexokinase 2 (HK2) catalyzes the first step of glycolysis and is up-regulated in cancer cells. The mechanism has not been fully elucidated. Tristetraprolin (TTP) is an AU-rich element (ARE)-binding protein that inhibits the expression of ARE-containing genes by enhancing mRNA degradation. TTP expression is down-regulated in cancer cells. We demonstrated that TTP is critical for down-regulation of HK2 expression in cancer cells. HK2 mRNA contains an ARE within its 3′-UTR. TTP binds to HK2 3′-UTR and enhances degradation of HK2 mRNA. TTP overexpression decreased HK2 expression and suppressed the glycolytic capacity of cancer cells, measured as glucose uptake and production of glucose-6-phosphate, pyruvate, and lactate. TTP overexpression reduced both the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) of cancer cells. Ectopic expression of HK2 in cancer cells attenuated the reduction in glycolytic capacity, ECAR, and OCR from TTP. Taken together, these findings suggest that TTP acts as a negative regulator of HK2 expression and glucose metabolism in cancer cells.

INTRODUCTION Cancer cell metabolism is characterized by enhanced uptake and utilization of glucose, a phenomenon known as the Warburg effect. While normal cells produce energy mainly through oxidation of pyruvate in mitochondria, cancer cells predominantly produce energy via enhanced glycolysis in the cytosol, regardless of whether they are under normoxic or hypoxic conditions (Warburg, 1956; Koppenol et al., 2011). Cancer cells prefer to metabolize glucose by glycolysis to support proliferation and anabolic growth. They avoid oxidative phosphorylation even in the presence of sufficient amounts of oxygen, a property first observed by Otto Warburg (House et al., 1956). The altered metabolism of cancer cells is connected with elevated lactate production and proton accumulation, which causes a drop in extracellular pH that promotes cancer metastasis (Bellone et al., 2013). A correlation between glycolytic ATP production and tumor malignancy is reported (Simonnet et al., 2002).

Hexokinase catalyzes the first committed step of the glycolytic pathway, in which glucose is phosphorylated to glucose-6-phosphate with concomitant dephosphorylation of ATP. Mammalian cells have four major HK isoforms: HK1, HK2, HK3, and HK4 (Wilson, 2003). Whereas HK1 is the most highly expressed hexokinase isoform and has no documented function in human cancer, HK2 is involved in cancer progression (Peschiarioli et al., 2013). Up-regulation of HK2 is seen in multiple human cancers (Fang et al., 2012), and HK2 is the most pivotal HK, with a direct function in the Warburg effect. HK2 is also essential for cancer growth, survival, and metastasis (Pedersen, 2007).

HK2 expression is reported to be regulated at epigenetic, transcriptional, and posttranscriptional levels. At the transcriptional level, HK2 expression is up-regulated by hypoxia-inducible factor-1α (HIF-1α) in cancer cells. The HK2 promoter has a consensus motif...
for HIF-1 (Riddle et al., 2000; Mathupala et al., 2001) and expression is enhanced by hypoxia (Riddle et al., 2000; Mathupala et al., 2001; Gwak et al., 2005; Kim et al., 2007). Thus, HK2 is considered to be a HIF-1α target gene (Semenza, 2003). In addition, c-Myc is reported to be involved in up-regulation of HK2 in fibroblast growth factor-stimulated endothelial cells (Yu et al., 2017). HK2 expression might be epigenetically regulated, because DNA methylation of an HK2 promoter CpG island suppresses HK2 expression by inhibiting interaction between HIF-1α and a hypoxia response element in the HK2 promoter (Lee et al., 2016). At the posttranscriptional level, Roquin (An et al., 2017) and several microRNAs (miRs) such as miR-199a-5p (Guo et al., 2015), miR-4458 (Qin et al., 2016), and miR-143 (Fang et al., 2012; Pescharioli et al., 2013) are reported to target HK2 mRNA and down-regulate HK2 expression. Despite these findings, the regulation of HK2 expression in cancer cells remains elusive.

Posttranscriptional regulation of gene expression can be mediated by AU-rich elements (AREs) located in the 3′-untranslated regions (3′-UTRs) of a variety of short-lived mRNAs such as those for cytokines and proto-oncogenes (Shaw and Kamen, 1986). The destabilizing function of AREs is regulated by ARE-binding proteins (Shyu and Wilkinson, 2000). One of the most well-characterized ARE-binding proteins is tristetraprolin (TTP), which promotes degradation of ARE-containing transcripts (Carballo et al., 1998; Chen et al., 2001; Lykke-Andersen and Wagner, 2005; Brooks and Blackshear, 2013). TTP inhibits cancer cell growth by inhibiting expression of cancer-related ARE-containing genes and enhancing degradation of proto-oncogene transcripts (Marderosian et al., 2006; Young et al., 2009; Lee et al., 2010a,b). TTP inhibits the EMT through down-regulation of Twist1 and Snail1 (Yoon et al., 2016). TTP expression is significantly decreased in various cancers (Brennan et al., 2009), which may lead to abnormalities that contribute to cancer processes.

Here, we showed that TTP acted as a negative regulator of glycolysis by posttranscriptionally down-regulating HK2 expression in cancer cells. Overexpression of TTP reduced glucose uptake, glycolysis, and growth of cancer cells. TTP did not affect the expression of HK1, but decreased HK2 expression by enhancing degradation of HK2 mRNA. Exogenous expression of HK2 restored glucose uptake, glycolysis, and growth of cancer cells. These novel findings suggested that TTP served as a negative regulator of HK2 in cancer cells. Considering the low levels of TTP in various cancers (Brennan et al., 2009), these results suggested a mechanism for up-regulation of HK2 observed in human cancers.

**RESULTS**

TTP expression decreases the glycolytic capacity of cancer cells

We previously reported that TTP overexpression decreases mitochondrial potential and ATP production in cancer cells (Vo et al., 2017). We investigated whether TTP expression affected the glycolytic capacity of cancer cells. MDAMB231 and H1299 cells were transfected with pcDNA6/V5-TPP (MDAMB231/TPP and H1299/TPP) or empty vector pcDNA6/V5 (MDAMB231/pcDNA and H1299/pcDNA) for 24 h. (A) TTP level was determined by semi-qRT-PCR (top) and Western blot (bottom). Cells were analyzed for (B) glucose uptake, (C) pyruvate production, and (D) lactate production. Data represent three experiments and are mean ± SD (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001).

![FIGURE 1: TTP overexpression decreases glycolytic capacity of cancer cells. MDAMB231 and H1299 cells were transiently transfected with 1 μg pcDNA6/V5-TPP (MDAMB231/TPP and H1299/TPP) or empty vector pcDNA6/V5 (MDAMB231/pcDNA and H1299/pcDNA) for 24 h.](image-url)

(A) TTP level was determined by semi-qRT-PCR (top) and Western blot (bottom). Cells were analyzed for (B) glucose uptake, (C) pyruvate production, and (D) lactate production. Data represent three experiments and are mean ± SD (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001).
overexpression decreased only HK2 in both MDAMB231 and H1299 cells (Figure 3A). The inhibition of TTP by siRNA increased expression of HK2 and LDHA among the five genes tested in both MCF-7 and A549 cells (Figure 3B). We determined the effect of TTP overexpression on mRNA degradation of the five genes. TTP overexpression did not affect the mRNA stability of GLUT1, HK1, PKM2, and LDHA (Figure 3C), but enhanced degradation of HK2 mRNA (Figure 3D) in both MDAMB23 and H1299 cells. These results indicated that TTP decreased HK2 expression by enhancing degradation of HK2 mRNA. Previously, it has been reported that HK2 mRNA stability is regulated by BAG3, Roquin, and Imp3 in cancer cells (An et al., 2017). We thus tested whether TTP enhances HK2 mRNA degradation by modulating the expression of these molecules. As shown in Figure 3E, overexpression or inhibition of TTP did not affect the expression levels of these molecules in either MDAMB231 or H1299 cells. These results suggest that these molecules are not involved in TTP-mediated degradation of HK2 mRNA in cancer cells.

**FIGURE 2:** Inhibition of TTP by siRNA increases glycolytic capacity in cancer cells. MCF-7 and A549 cells were transfected with 60 pmol of TTP-specific siRNA (MCF-7/TTP-siRNA and A549/TTP-siRNA) or scRNA (MCF-7/scRNA and A549/scRNA) for 24 h. (A) TTP level was determined by semi-qRT-PCR (top) and Western blot (bottom). Cells were analyzed for (B) glucose uptake, (C) pyruvate production, and (D) lactate production. Data represent three experiments and are mean ± SD (n = 3; *, p < 0.05; **, p < 0.01; ***, p < 0.001).

**TTP decreases expression of luciferase mRNA containing the HK2 3′-UTR**

Analysis of the 2429–base pair human HK2 3′-UTR revealed three pentamer AUUUA ARE motifs (Figure 4A). To determine whether down-regulation of HK2 expression by TTP was mediated through the HK2 mRNA 3′-UTR, we used a luciferase reporter gene linked to the full HK2 3′-UTR or a fragment containing all three AREs in the plasmid psiCHECK2. When MDAMB231 cells were transfected with plasmid to overexpress TTP, luciferase activity from the full (Figure 4B) or fragment (Figure 4C) HK2 3′-UTR was inhibited. To determine the AREs within HK2 3′-UTR that responded to TTP, we generated a luciferase reporter gene linked to oligonucleotides containing the first and second (oligo-ARE-1,2) or third AUUUA motif (oligo-ARE-3) in the plasmid psiCHECK2. Oligo-ARE-1,2 did not respond to TTP (Figure 4D), but luciferase activity from oligo-ARE-3W was inhibited by TTP overexpression (Figure 4E). To determine the importance of the third AUUUA motif, we prepared a luciferase reporter gene with mutant oligonucleotides (oligo-ARE-3M, containing AUUUA sequences substituted with AGCA). Oligo-ARE-3W responded to TTP but oligo-ARE-3M did not (Figure 4E). Taken together, these results demonstrated that the third AUUUA motif in the HK2 3′-UTR was involved in TTP inhibition in MDAMB231 cells.

**TTP binds to the third ARE motif, ARE-3, in the HK2 mRNA 3′-UTR**

To determine whether TTP interacted with ARE-1,2 or ARE-3 of the HK2 3′-UTR, MDAMB231 cells were cotransfected with pcDNA6/V5-TTP and psiCHECK2-oligo-ARE-3W (oligo-ARE-3W), psiCHECK2-oligo-ARE-3M (oligo-ARE-3M), or psiCHECK2-oligo-ARE-1,2 (oligo-ARE-1,2). After immunoprecipitation with anti-V5 or control antibody (immunoglobulin G), the presence of TTP was determined by Western blots using anti-V5 (Figure 4F, bottom). Total RNA was extracted from immunoprecipitates, and the presence of luciferase mRNA was analyzed by RT-PCR using PCR primers specific to the luciferase gene. Amplified PCR product was observed in immunoprecipitates from cells cotransfected with oligo-ARE-3W and pcDNA6/V5-TTP (Figure 4F, top). No PCR products were detected in samples from cells cotransfected with pcDNA6/V5-TTP and oligo-ARE-3M or oligo-ARE-1,2 (Figure 4F, top). PCR product was also not detected in immunoprecipitates obtained using control antibody. We also determined the effect of TTP overexpression on mRNA degradation of the luciferase reporter genes linked to HK2 AREs in MDAMB231 cells. TTP overexpression enhanced the mRNA degradation of reporter genes linked to a fragment containing all three AREs or ARE-3W of HK2 but did not affect the mRNA stability of luciferase reporter genes linked to HK2 ARE-3M or ARE-1,2 (Figure 4G). These results demonstrated that TTP interacted specifically with HK2 ARE-3 and enhanced degradation of HK2 ARE-3-containing mRNA.

**TTP reduces hexokinase activity of cancer cells**

HK2 is a key enzyme in the phosphorylation of glucose in glycolysis in cancer cells (Pedersen, 2007). We determined the effects of TTP
expression on hexokinase activity. Expression of HK2 correlated negatively with TTP in cancer cells: expression of HK2 in MDA-MB231 and H1299 cells, which had low TTP, was higher than in MCF-7 and A549 cells, which had high TTP. Overexpression of HK2 in MDA-MB231 and H1299 cells (Figure 5A) reduced hexokinase activity (Figure 5C). Inhibition of TTP by siRNA (TTP-siRNA; Figure 5D) enhanced hexokinase activity in MCF-7 and A549 cells (Figure 5E). These results suggested that TTP expression reduced hexokinase activity in cancer cells.

**Ectopic expression of HK2 attenuates reduction of glycolysis by TTP**

On the basis of our results, we hypothesized that TTP controlled the glycolytic capacity of cancer cells by down-regulation of HK2. To...
test this, we cotransfected MDAMB231 and H1299 cells with pcDNA6/V5-TTP and FLHKII-pGFPN3, which did not contain HK2 3′-UTR. At 24 h after transfection, cells were analyzed for glycolytic capacity. Expression of HK2 (Figure 6A) significantly abrogated reduction by TTP of hexokinase activity (Figure 6B), glucose uptake (Figure 6C), and production of glucose-6-phosphate (G-6-P; Figure 6D), pyruvate (Figure 6E), and lactate (Figure 6F). These results indicated that TTP suppressed glycolytic capacity through down-regulation of HK2 in cancer cells.

TTP overexpression reduces extracellular acidification, oxygen consumption rates, ATP levels, and cell proliferation

Glycolysis is determined by measuring the extracellular acidification rate (ECAR) of media surrounding cells. Acidification is predominantly from excretion of lactic acid over time after conversion from pyruvate (Wu et al., 2007). To evaluate the glycolytic phenotype, control and TTP-overexpressing cells were subjected to Seahorse Extracellular Flux Analysis to assess cellular bioenergetic activity. Results from glucose stress tests indicated that TTP overexpression significantly decreased ECAR associated with glycolysis, glycolytic capacity, and glycolytic reserve in MDAMB231 and H1299 cells (Figure 7, A and B). TTP overexpression also altered mitochondrial respiration using Seahorse Mito stress tests. Oxygen consumption rates (OCRs) associated with basal and maximal mitochondrial respiration were significantly down-regulated in TTP-overexpressing MDAMB231 and H1299 cells (Figure 7, C and D). Ectopic expression of HK2 abrogated reduction of ECAR and OCR by TTP in both MDAMB231 and H1299 cells (Figure 7, A–D). We investigated ATP content and cell proliferation of TTP-overexpressing cells. Consistent with the decrease in ECAR and OCR, significant decreases in
ATP content and cell proliferation were observed in TTP-overexpressing MDAMB231 and H1299 cells (Figure 7, E and F). Ectopic expression of HK2 rescued the ATP content and cell proliferation in TTP-overexpressing MDAMB231 and H1299 cells (Figure 7, E and F). Taken together, these results demonstrated that overexpression of TTP reduced expression of HK2, followed by decreased glycolysis, mitochondrial respiration, ATP production, and cell proliferation in cancer cells.

**DISCUSSION**

HK2 is a key enzyme that catalyzes the rate-limiting first step of glycolysis and is highly up-regulated in multiple human tumors (Fang et al., 2012). Here, we demonstrated that TTP acts as a key regulator of HK2 expression and glycolysis in cancer cells. We provided evidence that HK2 was a target of TTP: the HK2 mRNA 3′-UTR contained an ARE, TTP decreased expression of a luciferase reporter gene linked to the HK2 mRNA 3′-UTR, TTP bound to the HK2 mRNA 3′-UTR, and overexpression of TTP enhanced degradation of HK2 mRNA and decreased expression of HK2 in cancer cells. In addition, we found that TTP overexpression decreased glycolytic capacity. Ectopic expression of HK2 abrogated reduction of the glycolytic capacity of cancer cells by TTP. Thus, our data indicated that TTP acted as a negative regulator of glycolysis through posttranscriptional down-regulation of HK2 in cancer cells.

Four isoforms, HK1–HK4, are found in mammalian HKs (Wilson, 2003). Among these, HK2 is the predominantly overexpressed form in multiple human tumors (Fang et al., 2012) and is essential for cancer growth, survival, and metastasis (Pedersen, 2007). We investigated the underlying mechanisms of increased expression of HK2 in tumors. Tumors use a multitude of genetic, epigenetic, transcriptional, and posttranslational strategies to enhance HK2 expression (Mathupala et al., 2006). During tumorigenesis, the HK2 gene is switched on by demethylation of the HK2 gene promoter (Goel et al., 2003). The HK2 promoter contains well-defined cis-elements for transcription initiation (TATA and CAAT) and for activation by protein kinase-A and protein kinase-C pathways (Mathupala et al., 1995; Rempel et al., 1996; Lee and Pedersen, 2003). In addition, functional response elements for hypoxia via HIF-1 and p53 are located in the HK2 promoter (Mathupala et al., 1997, 2001). Response elements in the proximal region of the HK2 promoter required for its activity are either absent or poorly conserved in the promoters of HK1, HK3, and HK4 (Lee and Pedersen, 2003). Roquin (An et al., 2017) and several miRs such as miR-199a-5p (Guo et al., 2015), miR-4458 (Qin et al., 2016), and miR-143 (Fang et al., 2012;...
 Peschiaroli et al., 2013) are reported to target HK2 mRNA and down-regulate HK2 expression. Although these findings help explain the selective expression of HK2 in cancer cells, they are not sufficient to explain the enhanced expression of HK2 in cancer cells, because these regulations may not occur only in tumor cells. In this study, we demonstrated that HK2, but not HK1, contains an ARE within its mRNA 3′-UTR. Thus, TTP reduced the expression of HK2 but not HK1 in human cancer cells. It has been reported that TTP expression is substantially decreased in 65% of human tumors derived from 19 different tissues and with particularly high frequency in tumors of the thyroid, lung, ovary, uterus, and breast (Brennan et al., 2009). Considering that TTP acts as a negative regulator of HK2 expression in cancer cells, down-regulation of TTP in human tumors would critically contribute to up-regulation of HK2 and the Warburg effect in cancer cells.

HK2 binds to voltage-dependent anion channels (VDACs) on the outer mitochondrial membrane (Rose and Warms, 1967; Wilson, 1995) and preferentially accesses and uses mitochondrially generated ATP to phosphorylate glucose to glucose-6-phosphate (Arora and Pedersen, 1988). HK binding to VDACs is proposed to

FIGURE 6: Overexpression of HK2 attenuates reduction of glycolytic capacity of cancer cells by TTP. MDAMB231 and H1299 cells were transiently transfected with empty vector pcDNA6/V5 or pcDNA6/V5-TTP and FLHKII-pGFPN3 for 48 h. (A) TTP and HK2 levels were determined by RT-PCR (top) and Western blot (bottom). Cells were analyzed for (B) hexokinase activity, (C) glucose uptake, (D) G6P, (E) pyruvate production, and (F) lactate production. Data are mean ± SD (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001).
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suppress mitochondrial function while stimulating glycolysis (Lemasters and Holmuhamedov, 2006). This proposal was in a report suggesting that inhibition of HK2 activity leads to a switch in bioenergetics from aerobic glycolysis to mitochondrial oxidative phosphorylation (Lu et al., 2015). However, whether HK2 suppresses mitochondrial oxidative phosphorylation is controversial. Dissociation of HK2 from mitochondria is reported to inhibit glycolysis and mitochondrial respiration and deplete intracellular ATP levels (Woldetsadik et al., 2017). Consistent with these findings, we found that down-regulation of HK2 by TTP decreased both the oxygen consumption rate, mediated by mitochondria, and the glycolytic capacity of cancer cells. In addition, TTP overexpression decreased intracellular ATP in cancer cells. We previously reported that TTP induces mitochondrial dysfunction through down-regulation of α-synuclein (Vo et al., 2017). Thus, we hypothesize that even though we did not determine the effect of TTP overexpression on α-synuclein level, TTP overexpression might inhibit mitochondrial oxidative phosphorylation through down-regulation of α-synuclein.

We found that ectopic expression of HK2 rescued OCR and ECAR in TTP-overexpressed cancer cells, indicating that inhibition of mitochondrial oxidative phosphorylation and glycolysis by TTP was mediated by HK2 down-regulation. Collectively, our data suggested

FIGURE 7: TTP overexpression decreases extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) and ectopic expression of HK2 attenuates TTP reduction of OCR and ECAR in cancer cells. MDAMB231 and H1299 cells were transiently transfected with empty vector pcDNA6/V5 or pcDNA6/V5-TTP and FLHKII-pGFPN3 for 48 h. (A–D) TTP overexpression decreases both ECAR and OCR in cancer cells. Mean ECAR in transiently transfected MDAMB231 cells (A) and H1299 cells (B) was measured before and after sequential addition of glucose (1 μM), oligomycin (1 μM), and 2-DG (1 μM). Each data point represents an ECAR measurement. Graphs show glycolysis, glycolytic capacity, and glycolytic reserve. Data are mean ± SD (n = 3; *p < 0.05; **p < 0.01). Mean OCR of transfected MDAMB231 cells (C) and H1299 cells (D) before and after addition of oligomycin (1 μM), FCCP (1 μM), or antimycin A (1 μM). Each data point represents an OCR measurement. Graphs are basal respiration, proton leak, and ATP production. Data are mean ± SD (n = 3; *p < 0.05; **p < 0.01). (E) TTP overexpression decreases cellular ATP levels in cancer cells. Cellular ATP from transfected MDAMB231 and H1299 cells, measured using the luminescent cell viability assays. Data are mean ± SD (n = 3; *p < 0.05; **p < 0.01; ***p < 0.001). (F) TTP overexpression inhibits proliferation of cancer cells. Proliferation of transfected MDAMB231 and H1299 cells was assessed as absorbance at 490 nm using MTS cell proliferation assays. Graphs show relative cell proliferation. Data are mean ± SD (n = 3; *p < 0.05; **p < 0.01).
that TTP reduced both mitochondrial oxidative phosphorylation and glycolysis through down-regulation of HK2 in cancer cells.

In this study, we found that the expression of Glut1 and LDHA was induced by TTP inhibition. It is not likely that these genes are direct targets of TTP since TTP overexpression did not affect their mRNA stability. Glycolytic genes including Glut1 and LDHA are induced by HIF-1α (Semenza et al., 1994), whose expression and activity are modulated by mitochondrial dysfunction (Chandel et al., 1998; Mansfield et al., 2005). Considering that TTP can induce mitochondrial dysfunction (Vo et al., 2017), it is possible that TTP may indirectly regulate the expression of Glut1 and LDHA by inducing mitochondrial dysfunction.

In conclusion, our data suggested that TTP reduced both glycolysis and mitochondrial energy generation of human cancer cells through destabilization of HK2 mRNA. We demonstrated that TTP enhanced degradation of HK2 mRNA through binding to an AUUUA motif in the HK2 mRNA 3′-UTR. HK1 mRNA did not contain an AUUUA motif within its 3′-UTR. Thus, its expression was not regulated by TTP. Since TTP expression is inhibited in a variety of human cancer cells (Brennan et al., 2009), HK2 up-regulation in cancer cells could be considered a consequence of low TTP levels in cancer. This study provided a molecular mechanism for the enhanced levels of HK2 in cancer cells. TTP-mediated enhancing of HK2 mRNA degradation expands our understanding of the regulation of HK2 expression in cancer cells.

**MATERIALS AND METHODS**

**Cell culture**

The human cancer cell lines MDAMB231, H1299, MCF-7, and A549 were from the Korean Cell Line Bank (KCLB-Seoul, Korea). MDAMB231 cells were cultured in DMEM. H1299, MCF-7, and A549 cells were cultured in RPMI media. All cell lines were supplemented with 10% FBS (heat-inactivated fetal bovine serum; Welgene, Korea) and were maintained at 37°C under a humidified atmosphere of 5% CO2.

**Plasmids, small interfering RNAs, transfections, and dual-luciferase assays**

The pcDNA6/V5-TTP construct was described previously (Lee et al., 2010a,b). The FLHKII-pGFPN3 construct (Hossein Ardehali, Northwestern University) was from Addgene. MDAMB231 and H1299 cells were transfected with pcDNA6/V5-TTP or FLHKII-pGFPN3 using the TurboFect in vitro transfection reagent (R0531; Thermo Scientific).

**Oligonucleotides**

| Oligo-ARE-1,2 | Sequences |
|---------------|-----------|
| F: 5′-TCGAGTGGAATCAGCTATCTTTCTATTATAATTTGAATAAAAATTATTCATCTGTATATGCTTCTTCGCCG-3′ | R: 5′-GGCCGCGAACAGCAGATCTCAAGAAGCTAAATAAAAATTATAATGAAATCGTCTTCGCCGAC-3′ |

| Oligo-ARE-3W | Sequences |
|---------------|-----------|
| F: 5′-TCGAGTGGAATCAGCTATCTTTCTATTATAATTTGAATAAAAATTATTCATCTGTATATGCTTCTTCGCCG-3′ | R: 5′-GGCCGCGAACAGCAGATCTCAAGAAGCTAAATAAAAATTATAATGAAATCGTCTTCGCCGAC-3′ |

| Oligo-ARE-3M | Sequences |
|---------------|-----------|
| F: 5′-TCGAGTGGAATCAGCTATCTTTCTATTATAATTTGAATAAAAATTATTCATCTGTATATGCTTCTTCGCCG-3′ | R: 5′-GGCCGCGAACAGCAGATCTCAAGAAGCTAAATAAAAATTATAATGAAATCGTCTTCGCCGAC-3′ |

Underlined sequences are restriction enzyme sites. Bold sequences are wild-type and mutant AU-rich elements.

**TABLE 1:** Oligonucleotides used to analyze HK2 mRNA 3′-UTR.
and normalized by comparison with densities of internal control β-actin bands.

RNA kinetics, quantitative real-time PCR, and semiquantitative RT-PCR

For RNA kinetic analysis, we used actinomycin D (A9415; Sigma) and assessed HK2 mRNA expression using quantitative real-time PCR (qRT-PCR). DNase I–treated total RNA (2 µg) was reverse-transcribed using oligo-dT (79237; Qiagen) and MMLV reverse transcriptase (3201; Beamsbio) according to the manufacturer’s instructions. qRT-PCR was performed by monitoring increased fluorescence in real-time with the SYBR Green dye (MasterMix-R; Abm) using the StepOnePlus real-time PCR system (Applied Biosystems). Semiquantitative RT-PCR (semi-qRT-PCR) used Taq polymerase 2X premix (Solgent) and appropriate primers. PCR primer pairs were

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Semiquantitative RT-PCR (semi-qRT-PCR) used Taq polymerase 2X premix (Solgent) and appropriate primers. PCR primer pairs were used to quantitatively determine the expression of HK2 mRNA in HK2 cells. The expression of HK2 mRNA was calculated using the 2^ΔΔCT method. The relative expression of HK2 mRNA was normalized to the expression of the GAPDH housekeeping gene. The results were expressed as the mean ± SEM of at least three independent experiments.

Glucose-6-phosphate assays

Glucose-6-phosphate (G6P) was measured using glucose-6-phosphate assay kits (MAK014; Sigma) according to the manufacturer’s instructions: cells (1 × 10^6) were homogenized in two volumes of ice-cold PBS. After centrifugation at 13,000 × g for 10 min to remove insoluble materials, 50 µl of supernatant was added to duplicate wells of 96-well plates. After addition of 50 µl reaction mix containing G6P Assay Buffer, G6P Enzyme Mix, and G6P Substrate Mix, absorbance at 450 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Hexokinase assays

Hexokinase activity was measured using hexokinase colorimetric assay kits (MAK091; Sigma) according to the manufacturer’s instructions: cells (1 × 10^6) were homogenized in 200 µl of ice-cold HK Assay Buffer. After centrifugation at 13,000 × g for 10 min to remove insoluble materials, 50 µl supernatant was added to duplicate wells of 96-well plates. After addition of 50 µl reaction mix containing HK Assay Buffer, HK Enzyme mix, HK Developer, HK Coenzyme, and HK Substrate, absorbance at 450 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Pyruvate assays

Pyruvate production was measured using pyruvate assay kits (MAK071; Sigma) according to the manufacturer’s instructions: cells (1 × 10^6) were homogenized in four volumes Pyruvate Assay Buffer. After centrifugation at 13,000 × g for 10 min to remove insoluble materials, 50 µl supernatant was added to duplicate wells of 96-well plates. After addition of 50 µl reaction mix containing Pyruvate Assay Buffer, Pyruvate Probe Solution, and Pyruvate Enzyme Mix, absorbance at 570 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Lactate assays

Lactate production was measured using lactate assay kits II (MAK065; Sigma) according to the manufacturer’s instructions: cells (1 × 10^6) were homogenized in two volumes of Lactate Assay Buffer. After centrifugation at 13,000 × g for 10 min to remove insoluble materials, 50 µl of supernatant was added to duplicate wells of 96-well plates. After addition of 50 µl reaction mix containing Lactate Assay Buffer, Lactate Enzyme Mix, and Lactate Substrate Mix, absorbance at 450 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Glucose uptake assays

Glucose uptake was determined using Glucose Uptake-Glo assays (J1342; Promega) according to the manufacturer’s instructions: cells (1.5 × 10^6) were seeded in wells of 96-well plates. After being washed with 100 µl PBS, cells were incubated with 50 µl 1 mM 2-deoxyglucose (2DG) per well for 10 min at room temperature. Cells were treated with 25 µl Stop Buffer, 25 µl Neutralization Buffer, and 100 µl 2-deoxyglucose-6-phosphate (2DG6P) Detection Reagent for 30 min at room temperature. Luminescence intensity was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Ribonucleoprotein immunoprecipitation assays

To determine binding of TTP to HK2 ARE, ribonucleoprotein immunoprecipitation (RNP) assays were conducted as described previously (Lee et al., 2010b): MDAMB231 cells (1 × 10^6) were cotransfected with 10 µg pcDNA6/VS-TTP and psiCHECK2-HK2-ARE-3’W or psiCHECK2-HK2-ARE-3’M. At 24 h after transfection, cell suspensions were incubated in 1% formaldehyde for 20 min at room temperature. Reactions were stopped with 0.25 M glycine (pH 7.0), and cells were sonicated in modified radiolucent immunoprecipitation assay buffer containing protease inhibitors (Roche Applied Science). RNP complexes were immunoprecipitated using protein G-agarose beads preincubated with 1 µg anti-V5 Tag antibody (GWB-7DC53A, Genway Biotech) or 1 µg isotype control (Sigma). After cross-link reaction at 70°C for 45 min, RNA was isolated from immunoprecipitates and treated with DNase I (Qiagen). From the RNA, cDNA was synthesized, and the Renilla luciferase gene was amplified by PCR using Taq polymerase and Renilla luciferase-specific primers (Up, 5′-ACGTCGCTGACTCTCATC-3′; and Down, 5′-GACACTCTCA-GCTGACGAC-3′). HK1, HK2, GLUT1, and GAPDH, 5′-ACATCAAGAAGGTGGTGAAG-3′; HK2, 5′-GAGGTACAGATGACATG-3′; and HK1, 5′-TCTTACCGACGGTGACTG-3′. mRNA half-life was calculated by nonlinear regression of mRNA at 30, 60, 90, and 120-min time points following addition of actinomycin D using GraphPad Prism 5.00 software based on a one-phase exponential decay model.

Cell proliferation

Cells were transfected with a combination of pcDNA6/V5-TTP and FLHKII-pGFPN3 for 48 h. Cells were seeded in triplicate in 96-well culture plates at 5 × 10^3 cells/well and incubated for 24 h. Cells were measured with CellTiter 96 AQueous One Solution cell proliferation assays (Promega, 3580) according to the manufacturer’s instructions, and absorbance at 490 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

ATP assays

Cellular ATP levels were measured using CellTiter-Glo luminescent cell viability assay kits (G7570; Promega) according to the manufacturer’s instructions. MDAMB231 and H1299 cells were plated on 96-well white-walled plates with clear bottoms in 100 µl culture medium, and 100 µl CellTiter-Glo reagent was added to each well. Contents were mixed for 2 min on an orbital shaker to induce cellular lysis, followed by incubation at room temperature for 10 min to stabilize the signal. Luminescence was recorded immediately.
Seahorse extracellular flux analysis

Glycolysis and mitochondrial stress tests were performed using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacturer’s instructions. Cells were seeded at 1.5 × 10⁶ cells per well in XF96 cell culture microplates and incubated for 24 h to ensure attachment. To measure oxygen consumption, cells were equilibrated for 1 h in unbuffered XF assay medium supplemented with 25 mM glucose, 1 mM sodium pyruvate, 2 mM glutamax, 1× nonessential amino acids, and 1% FBS in a non-CO₂ incubator. Mitochondrial processes were examined through sequential injections of oligomycin (1 µM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 1 µM), and rotenone (1 µM)/antimycin A (1 µM). Indices of mitochondrial function were calculated as basal respiration rate (baseline OCR-rotenone/antimycin A OCR), ATP production (basal respiration rate-oligomycin OCR), and proton leak (oligomycin OCR-rotenone/antimycin A OCR). To measure glycolysis and glycolytic capacity, cells were cultured for 2 h in the absence of glucose. Three sequential injections of d-glucose (1 µM), oligomycin (1 µM), and 2-deoxyglucose (500 mM) provided ECAR associated with glycolysis, maximum glycolytic capacity, and nonglycolytic ECAR. Glycolysis was defined as ECAR following the addition of d-glucose and maximum glycolytic capacity was defined as ECAR following the addition of oligomycin. ECAR following treatment with 2-deoxyglucose was associated with nonglycolytic activity.

Statistical analysis

For statistical comparisons, p values were determined using Student’s t test or one-way analysis of variance. A p value of <0.05 was considered significant.

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