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Hypoxia controls the expression of genes responsible for serine synthesis in U87MG cells on ERN1-dependent manner

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Objective. Serine synthesis as well as endoplasmic reticulum stress and hypoxia are important factors of malignant tumor growth including glioblastoma. Previous studies have shown that the knockdown of ERN1 (endoplasmic reticulum to nucleus signaling) significantly suppressed the glioblastoma cell proliferation and modified the hypoxia regulation. The present study is aimed to investigate the impact of hypoxia on the expression of *PHGDH* (phosphoglycerate dehydrogenase), *PSAT1* (phosphoserine aminotransferase 1), *PSPH* (phosphoserine phosphatase), *ATF4* (activating transcription factor 4), and *SHMT1* (serine hydroxymethyltransferase 1) in U87MG glioblastoma cells in relation to knockdown of ERN1 with the intent to reveal the role of ERN1 signaling pathway on the endoplasmic reticulum stress-dependent regulation of expression of these genes.

Methods. The control U87MG glioblastoma cells (transfected by empty vector) and ERN1 knockdown cells (transfected by dominant-negative ERN1) were exposed to hypoxia introduced by dimethyloxalylglycine for 4 h. RNA was extracted from cells and reverse transcribed. The expression level of *PHGDH*, *PSAT1*, *PDPH*, *SHMT1*, and *ATF4* genes was studied by real-time qPCR and normalized to ACTB.

Results. It was found that hypoxia up-regulated the expression level of *PHGDH*, *PSAT1*, and *ATF4* genes in control U87MG cells, but *PSPH* and *SHMT1* genes expression was down-regulated. The expression of *PHGDH*, *PSAT1*, and *ATF4* genes in glioblastoma cells with knockdown of ERN1 signaling protein was more sensitive to hypoxia, especially *PSAT1* gene. At the same time, the expression of *PSPH* gene in ERN1 knockdown cells was resistant to hypoxia. The expression of *SHMT1* gene, encoding the enzyme responsible for conversion of serine to glycine, showed similar negative sensitivity to hypoxia in both control and ERN1 knockdown glioblastoma cells.

Conclusion. The results of the present study demonstrate that the expression of genes responsible for serine synthesis is sensitive to hypoxia in gene-specific manner and that ERN1 knockdown significantly modifies the impact of hypoxia on the expression of *PHGDH*, *PSAT1*, *PSPH*, and *ATF4* genes in glioblastoma cells and reflects the ERN1-mediated reprograming of hypoxic regulation at gene expression level.

Key words: ERN1 knockdown, hypoxia, PSAT1, PSPH, ATF4, gene expression, U87MG cells

The endoplasmic reticulum (ER) stress and hypoxia are important factors of tumor metabolic reprogramming and growth including glioblastoma (Denko 2008; Bravo et al. 2013; Chevet et al. 2015; Almanza et al. 2019; Minchenko et al. 2021). Serine synthesis is also essential for malignant tumor progression and therapeutic resistance (Vie et al. 2008; Mattaini et al. 2016; Yang and Vousden 2016; Engel et al. 2020;

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Li and Ye 2020; Hennequart et al. 2021). Previous studies have shown that the knockdown of ERN1 (endoplasmic reticulum to nucleus signaling 1) significantly suppresses the glioblastoma cell proliferation as well as tumor growth *in vivo* and the response to chemotherapy through genome reprogramming (Auf et al. 2010, 2013; Minchenko et al. 2015a, 2021; Logue et al. 2018). There are also data indicating that inhibition of ERN1 significantly modifies the hypoxic regulation of key regulatory genes expression in tumor cells (Minchenko et al. 2015b, 2019, 2020, 2021). Hypoxic regulation of gene expression is preferentially realized through transcription factor hypoxia inducible factor (HIF), but there are many other factors, which can modulate the expression of genes in gene-specific manner (Minchenko and Caro 2000; Sun and Denko 2014; Semenza 2017). It is possible that ER stress controls the hypoxic regulation of gene expression by specific changes in these additional factors, which can interact with HIF and modulate its activity.

The ERN1 is an ER transmembrane signaling protein with protein kinase and endoribonuclease activities in cytoplasmic domain. The main function of ERN1 endoribonuclease is unconventional splicing of the XBP1 (X-box binding protein 1) pre-mRNA by excision of 26 bp fragment from the coding part. The resulting alternative splice variant of XBP1 mRNA encodes a bigger transcription factor with modified C-terminus, which regulates the expression of numerous genes that encode proteins for protein folding and degradation of unfolded proteins as well as affects broad aspects of cell fate and the metabolism of proteins, amino acids, and lipids (Acosta-Alvear et al. 2007; Manie et al. 2014; Doultsinos et al. 2017; Obacz et al. 2017; Almanza et al. 2019; Hetz et al. 2020).

The ERN1 endoribonuclease activity is also responsible for the degradation of specific mRNAs by special mechanism known as ERN1-dependent decay of mRNA (RIDD), which has revealed many unexpected features and can either preserve ER homeostasis or induce cell death (Maurel et al. 2014). ERN1 protein kinase plays also an important role in the implementation of ERN1 signaling and controls the expression of genes such as *EREG* (epiregulin), *EDN1* (endothelin 1), *ATF3* (activating transcription factor 3), and some others (Minchenko et al. 2013, 2015a, 2019, 2020). The transcription factor ATF3 promotes the expression of serine biosynthesis genes by binding to the promoters of *PHGDH* (phosphoglycerate dehydrogenase), *PSAT1* (phosphoserine aminotransferase 1), and *PSPH* (phosphoserine phosphatase) genes (Li et al. 2021; Luo et al. 2022).

The metabolic reprogramming is a basic characteristic of tumor cells and promotes their rapid growth and resistance to treatment preferentially through ER stress (Chevet et al. 2015; Avril et al. 2017; Logue et al. 2018; Papaioannou and Chevet 2018; Rathore et al. 2020). In tumor cells, *de novo* synthesis of serine is an important branch of glycolysis and is completed by means of a three-step enzymatic reaction, in which important metabolic enzymes PHGDH, PSAT1, and PSPH are involved (Possemato et al. 2011; Reid et al. 2018; Liao et al. 2019; Li et al. 2021; Itoyama et al. 2021; Tajan et al. 2021). The PHGDH is a rate-limiting enzyme for the *de novo* synthesis of serine, which catalyzes the first step of serine biosynthesis from 3-phosphoglycerate. It is highly expressed in a variety of cancers and is necessary for the rapid growth of tumor cells and resistance to chemotherapy (Rathore et al. 2020; Zhao et al. 2020; Itoyama et al. 2021).

Studies have shown that inhibitor of PHGDH can enhance the chemotherapeutic effect of temozolomide on brain tumors (Jin et al. 2022). It has also been shown that over-expressed PSAT1 stimulates the cell growth and increases the chemoresistance of cancer cells through modulating cell cycle and that microRNA-145-5p repressed cancer cell cycle progression and cell proliferation via targeting PSAT1 (Vie et al. 2008; Ding et al. 2022). PSAT1 is regulated by transcription factor ATF4 and enhances cancer cell proliferation through cyclin D1 (Gao et al. 2017). PSPH catalyzes the last irreversible step in the biosynthesis of L-serine and promotes tumor growth and metastasis (Rawat et al. 2021). However, this enzyme also promotes lung cancer progression by a noncanonical L-serine-independent pathway through the dephosphorylation of insulin receptor substrate 1 and MAPK signaling pathways (Liao et al. 2019; Park et al. 2019). Serine hydroxymethyltransferase 1 (SHMT1) also plays an important role in the control of serine level in the cell because it catalyzes the reversible conversion of serine to glycine (Pikman et al. 2022).

The ER stress and hypoxia are important factors of malignant tumor progression, metabolic reprogramming, and therapeutic resistance, but there are no available data concerning the interaction of these factors in the regulation of genes controlling the serine synthesis, especially after suppression of glioblastoma cell proliferation by inhibition of ERN1. In this study, we are showing that the expression level of genes responsible for serine synthesis was affected by hypoxia in U87MG cells on ERN1-dependent manner.

Materials and Methods

Cell lines and culture conditions. The U87MG glioblastoma cells were grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/ml, Gibco), and streptomycin (0.1 mg/ml, Gibco) at 37°C in incubator with 5% CO_2. In this study, we used two sublines of U87MG cells. One was obtained by selection of stably transfected clones with overexpression of vector pcDNA 3.1 and used as control (control glioblastoma cells). Second subline was obtained by selection of stably transfected clones with overexpression of ERN1 dominant/negative construct in pcDNA 3.1 (dnERN1) having suppression of both the ERN1 protein kinase and endoribonuclease activities (Auf et al. 2010, 2013).

It has been shown that cells with dnERN1 have a lower proliferation rate and do not express spliced variant of XBP1, a key transcription factor in ERN1 signaling, and do not have the phosphorylated isoform of ERN1 after induction of ER stress by tunicamycin (Auf et al. 2010, 2013; Minchenko et al. 2015a). Both sublines of glioblastoma cells used in this study grew in the presence of geneticin (G418), while carrying empty vector pcDNA3.1 or dnERN1 construct. Hypoxia was created by 0.5 mM dimethyloxalylglycine (Sigma-Aldrich, St. Louis, MO, U.S.A.) as described previously (Minchenko et al. 2002) and culture plates were exposed for 4 h.

RNA isolation. Total RNA was extracted from glioblastoma cells using the Trizol reagent according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification, all RNA samples were re-precipitated with 95% ethanol and re-dissolved again in nuclease-free water. RNA concentrations and spectral characteristics were measured using NanoDrop Spectrophotometer ND1000 (PEQLAB, Biotechnologie GmbH).

Reverse transcription and quantitative PCR analysis. The expression levels of PHGDH, PSAT1, PSPH, SHMT1, and ATF4 mRNAs as well as ACTB mRNA were measured in control U87MG cells and cells with a deficiency of ERN1 by quantitative polymerase chain reaction using SYBRGreen Mix (ABgene, Thermo Fisher Scientific, Epsom, Surrey, UK) and "QuantStudio 5 Real-Time PCR System" (Applied Biosystems, USA). Thermo Scientific Verso cDNA Synthesis Kit (Germany) was used for reverse transcription as described (Rudnytska et al. 2021). Polymerase chain reaction was performed in triplicate. The expression of beta-actin mRNA was used as a control of analyzed mRNA quantity. The pair of primers specific for each studied gene was received from Sigma-Aldrich (St. Louis, MO, U.S.A.) and used for quantitative polymerase chain reaction (Table 1).

The quantitative PCR analysis was performed using a special computer program "Differential expression calculator" and statistical analysis using GraphPad Prism8 program. The values of studied genes expression were normalized to the expression of beta-actin mRNA and expressed as a percentage of controls (100%). All values were expressed as mean±SEM from triplicate measurements performed in 3 independent experiments. A value of $p<0.05$ was considered significant in all cases. All experimental qPCR data were analyzed for the normality

Table 1
Characteristics of the primers used for quantitative real-time polymerase chain reaction

Gene symbol	Gene name	Primer's sequence	Nucleotide number in sequence	GeneBank accession number
PHGDH	Phosphoglycerate dehydrogenase	F: 5'- tcagttcgtggacatggtga R: 5'- tctttcaggaggccgacaat	992-1011 1231-1212	NM_006623.4
PSAT1	Phosphoserine aminotransferase 1	F: 5'- tgtcaagggagcagtactgg R: 5'- gcctgcaccttgtattccag	587-606 787-768	NM_021154.5
PSPH	Phosphoserine phosphatase	F: 5'- agcactggagaaacgaggaa R: 5'- agagcagctttgaaaggcac	566-585 783-764	NM_004577.4
SHMT1	Serine hydroxymethyltransferase 1	F: 5'- tctgccacgtccatcttctt R: 5'- cgggcatattccaggtttcg	679-698 836-817	NM_004169.5
ATF4	Activating transcription factor 4	F: 5'- gtccctccaacaacagcaag R: 5'- actttctgggagatggccaa	1093–1112 1328–1309	NM_005749.4
ACTB	beta-Actin	F: 5'- catccgcaaagacctgtacg R: 5'- cctgcttgctgatccacatc	948–967 1165–1146	NM_001101.5

of distribution using a graphical tool (normal probability plot) and a histogram as described previously (Rudnytska et al. 2021). A normal distribution was shown for all analyzed data sets. The amplified DNA fragments were analyzed on a 3% agarose gel and then visualized by SYBR* Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, USA).

Results

Examining a possible role of the ER stress signaling mediated by ERN1 in the hypoxic regulation of the expression of *PHGDH*, *PSAT1*, *PSPH*, *SHMT1*, and *ATF4* genes, which are responsible for synthesis and metabolism of serine in control and ERN1 knockdown glioblastoma cells was evaluated. As shown in Figure 1A, the expression of *PHGDH* gene was up-regulated (+62%) by hypoxia in control glioblastoma cells in comparison with control (no treated) cells. Much smaller changes were detected in the expression of *PSAT1* gene in control U87MG cells treated by hypoxia (+23%) (Figure 1B). At the same time, hypoxia down-regulated the expression of *PSPH* gene in comparison with control (no treated) cells (-29%) (Figure 1C). As shown in Figures 2A and 2B, the expression of *ATF4* gene was significantly increased by hypoxia in control glioblastoma cells (+59%), but *SHMT1* gene expression was down-regulated in these cells upon hypoxia treatment (-69%) as compared to not treated control cells.

Investigating the impact of hypoxia on the expression of genes encoding PHGDH and PSAT1 proteins in relation to inhibition of ERN1 enzymatic functions, the exposure of ERN1 knockdown glioblastoma cells under hypoxia led to more pronounced changes in expression of these genes, especially *PSAT1* gene in comparison with cells growing without dimethyloxalylglycine (+101% for *PHGDH* gene and +226% for *PSAT1* gene (Figures 3A, B). At the same time, the exposure of ERN1 knockdown glioblastoma cells under hypoxia did not alter significantly the expression of *PSPH* gene in comparison with cells growing without dimethyloxalylglycine (Figure 3C). As shown in Figures 4A and 4B, the expression of *ATF4* gene is strongly increased by hypoxia in ERN1 knockdown glioblastoma cells (+121%), but *SHMT1*

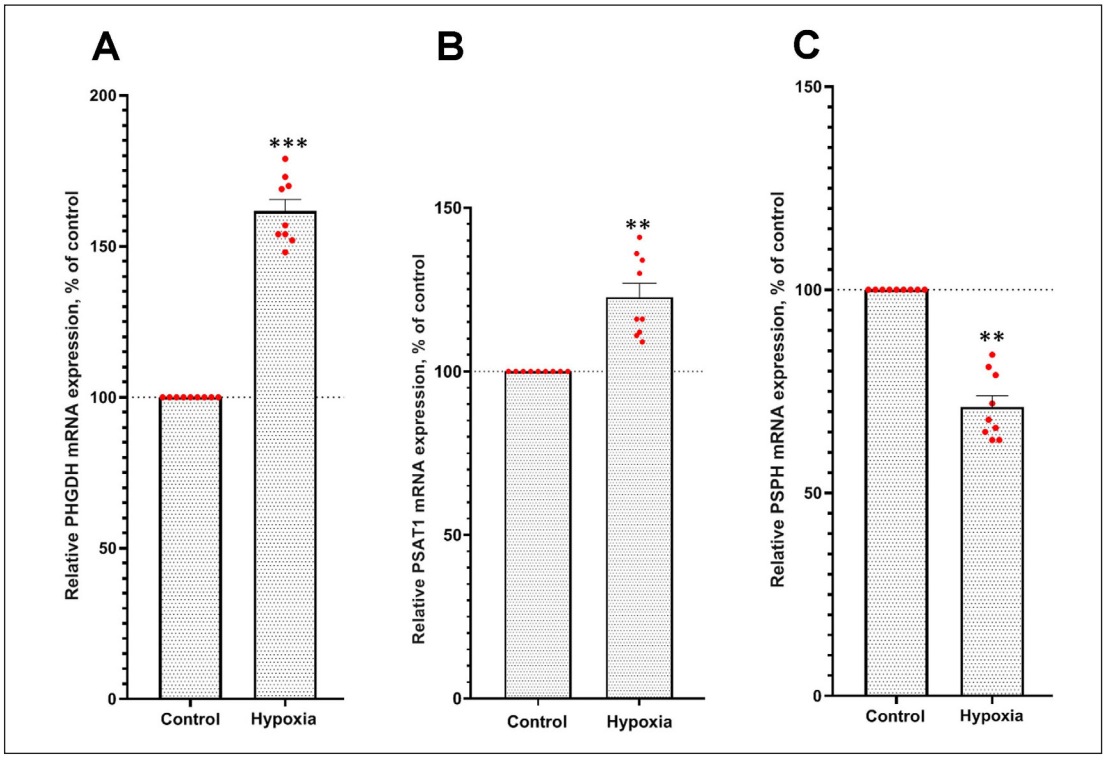

Figure 1. The impact of hypoxia on the expression levels of PHGDH (phosphoglycerate dehydrogenase) (**A**), PSAT1 (phosphoserine aminotransferase 1) (**B**), and PSPH (phosphoserine phosphatase) (**C**) in control U87MG glioblastoma cells (transfected by an empty vector) measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as percent of control (no treated cells, 100%). Data are presented as mean±SEM; **p<0.01; ***p<0.001 vs. control.

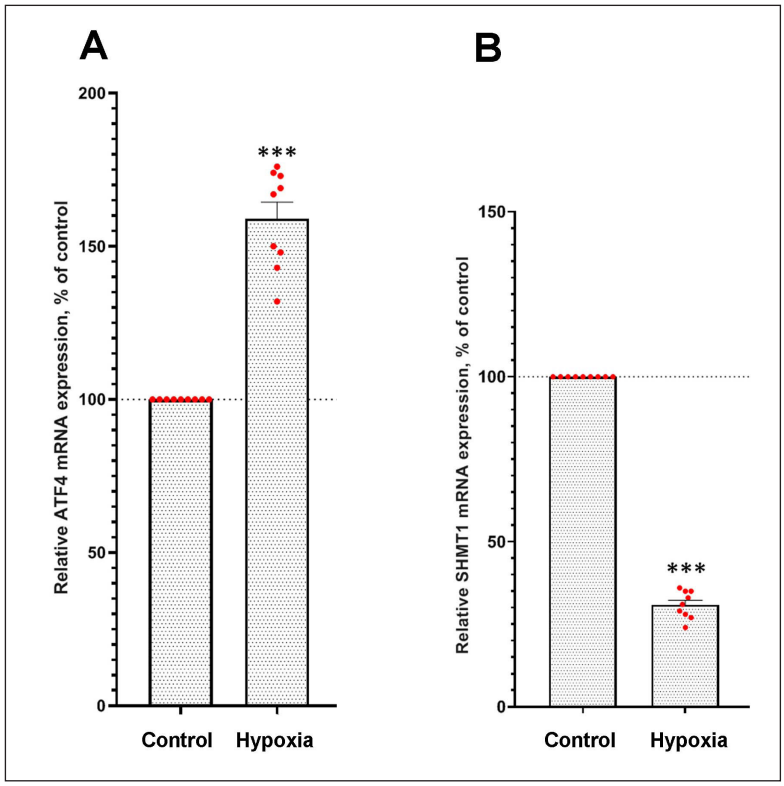

Figure 2. The impact of hypoxia on the expression level of SHMT1 (serine hydroxymethyltransferase 1) (**A**) and ATF4 (activating transcription factor 4) (**B**) in control U87MG glioblastoma cells measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as percent of control (100%). Data are presented as mean±SEM; ***p<0.001 vs. control.

gene expression is down-regulated by hypoxia treatment in these cells (-63%) as compared to corresponding control cells.

Discussion

The results of this study are summarized in Figure 5. The major finding reported here is that the expression of genes encoding the PHGDH and PSAT1 enzymes and transcription factor ATF4 are up-regulated under hypoxia in control and ERN1 knockdown U87MG glioblastoma cells. The results agree well with the prooncogenic role of these genes, which are responsible for serine biosynthesis and play an important role in tumor growth, metastasis, cell survival, and chemoresistance (Vie et al. 2008; Yang and Vousden 2016; Gao et al. 2017; Engel et al. 2020; Rathore et al. 2020; Zhao et al. 2020; Hennequart et al. 2021; Itoyama et al. 2021; Ding et al. 2022).

The knockdown of ERN1 signaling protein is associated with a much stronger up-regulation of *PHGDH*, *PSAT1*, and *ATF4* genes expression showing that hypoxic regulation of these genes expression in glioblastoma cells is ERN1-dependent. It is possible that strong hypoxic up-regulation of genes, which are responsible for serine biosynthesis, is connected with metabolic reprogramming, increased expression of ATF3 transcription factor and suppressed the proliferation of ERN1 knockdown glioblastoma cells (Auf et al. 2010, 2013; Minchenko et al. 2015b, 2021). At the same time, our results demonstrate down-regulation of *PSPH* gene expression by hypoxia in control glioblastoma cells. It catalyzes the last irreversible step in the biosynthesis of L-serine and promotes tumor growth and metastasis (Rawat et al. 2021). Although the hypoxia increases the expression of most genes involved in serine synthesis, it is not possible to talk about the hypoxic activation of serine synthesis under the conditions of a decrease in PSPH gene expression. It is more accurate to say that hypoxia may disrupt serine synthesis. It is interesting to note that PSPH has a non-canonical L-serine-independent pathway and promotes cancer progression through the

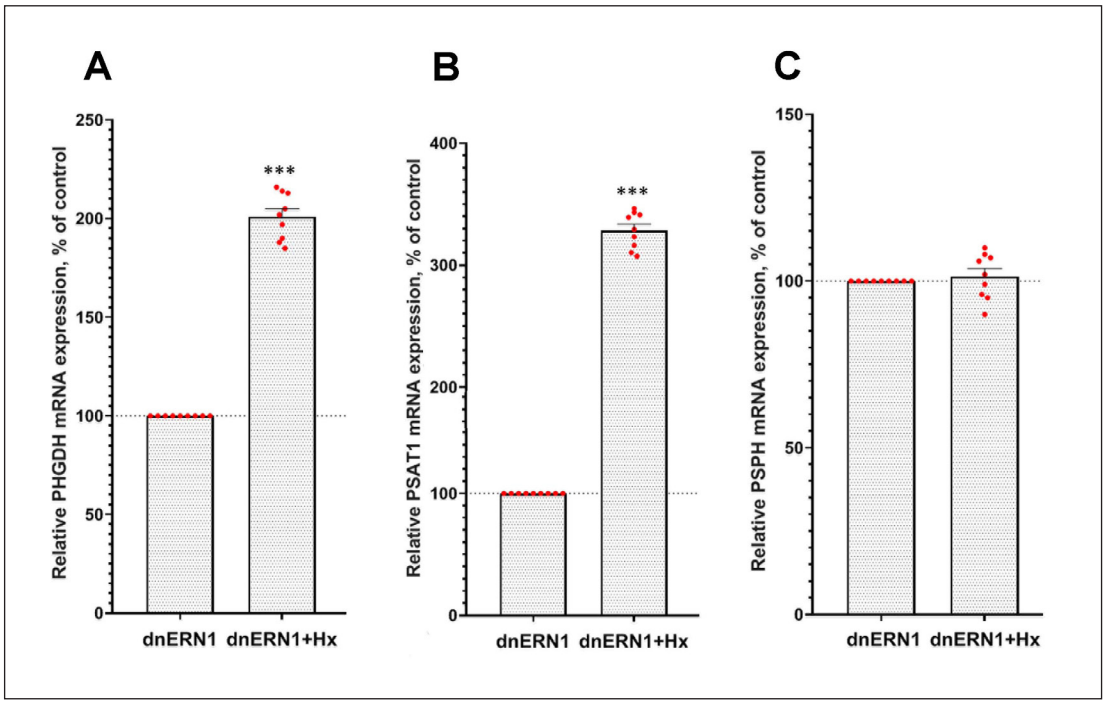

Figure 3. The impact of hypoxia on the expression levels of PHGDH (**A**), PSAT1 (**B**), and PSPH (**C**) in U87MG glioblastoma cells with a deficiency of both ERN1 protein kinase and endoribonuclease (dnERN1) measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as percent of control (dnERN1; 100%). Data are presented as mean±SEM; ***p<0.001 vs. control.

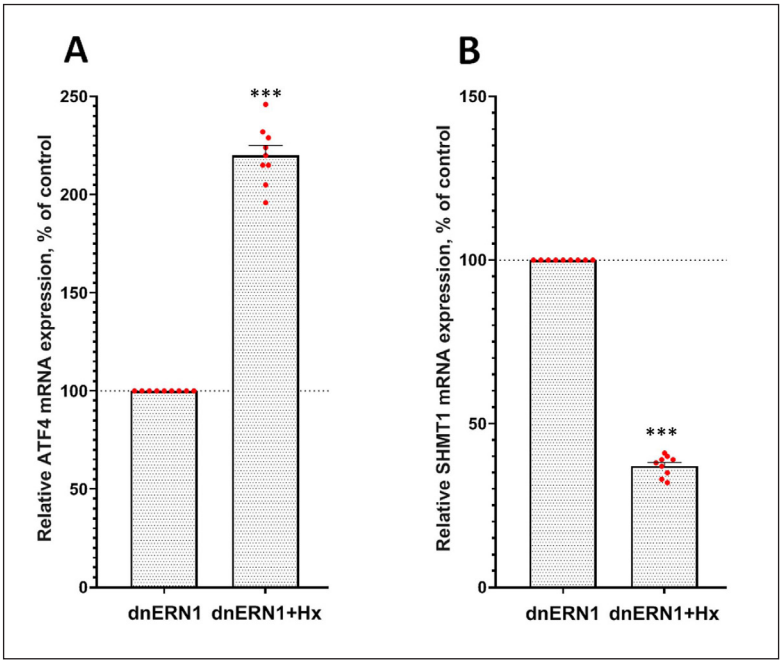

Figure 4. The impact of hypoxia on the expression level of ATF4 (**A**) and SHMT1 (**B**) in U87MG glioblastoma cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as percent of control (dnERN1; 100%). Data are presented as mean±SEM; ***p<0.001 vs. control.

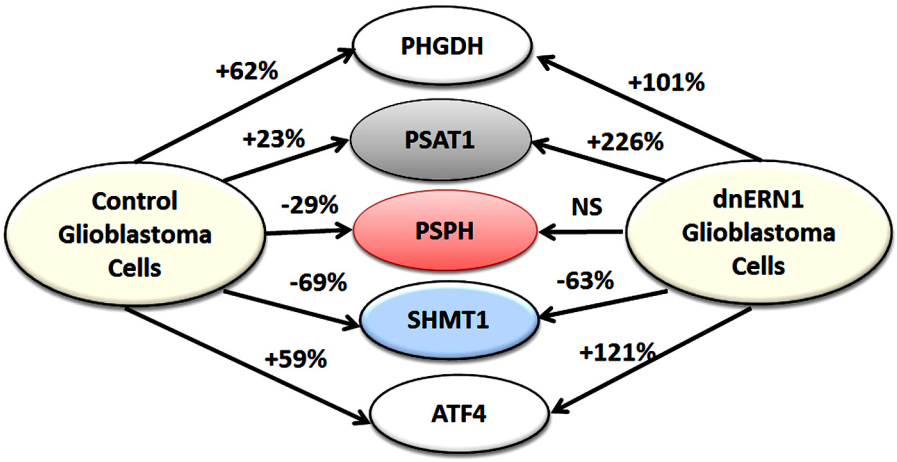

Figure 5. Schematic demonstration of the impact of hypoxia on the expression of PHGDH, PSAT1, PSPH, SHMT1, and ATF4 mRNAs in control U87MG glioblastoma cells and cells with a deficiency of both ERN1 protein kinase and endoribonuclease (dnERN1).

dephosphorylation of IRS1 and MAPK signaling (Liao et al. 2019; Park et al. 2019). It can be hypothesized that the reduced expression of PSPH under hypoxia is related to the importance for tumor cells of these additional, serine synthesis-independent functions of PSPH. We are also showing that hypoxia down-regulates the expression of *SHMT1* gene independently on the ERN1 signaling. It is possible that decreased expression of *SHMT1* gene can contribute to the serine level increase in glioblastoma cells upon hypoxia, because this enzyme is responsible for conversion of serine to glycine (Pikman et al. 2022).

Hypoxia is an important factor in tumor growth due to the fact that ER stress induces resistance to the toxic effects of hypoxia through genome reprogramming. Hypoxic regulation of gene expression is preferentially realized through transcription factor HIF (Denko 2008; Sun and Denko 2014). At the same time, the expression of genes under hypoxia are gene-specific, in the same cells differs (Minchenko et al. 2015a,b; 2019, 2020). It has been shown that there are many factors, which can modulate the impact of hypoxia on the expression of genes in gene-specific manner (Semenza 2017; Minchenko and Caro 2000). We have previously shown that hypoxic induction of the endothelin-1 gene was removed by a genistein, an inhibitor of protein kinases (Minchenko and Caro 2000). It is possible that ERN1 inhibition modifies the hypoxic regulation of gene expression by genome reprogramming through specific changes in the additional factors, which can interact with HIF and modulate its activity (Semenza 2017).

In conclusion, the data presented in this study identify hypoxic regulation of the expression of genes responsible for serine biosynthesis (*PHGDH*, *PSAT1*, *PSPH*, and *ATF4*) in U87MG glioblastoma cells on ERN1-dependent manner. The expression of *SHMT1* gene, which is responsible for serine metabolism, is down-regulated in these cells under hypoxia independently on ERN1 knockdown. However, the detailed molecular mechanisms of the interaction of hypoxia with ERN1-mediated stress signaling pathway are complex and need to be underwent to further studies.

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Conflict of interest: *The authors declare no conflicts of interest.*

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