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Endoplasmic reticulum stress-dependent regulation of the expression of serine hydroxymethyltransferase 2 in glioblastoma cells

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Objective. Serine hydroxymethyltransferase (SHMT2) plays a multifunctional role in mitochondria (folate-dependent tRNA methylation, translation, and thymidylate synthesis). The endoplasmic reticulum stress, hypoxia, and glucose and glutamine supply are significant factors of malignant tumor growth including glioblastoma. Previous studies have shown that the knockdown of the endoplasmic reticulum to nucleus signaling 1 (ERN1) pathway of endoplasmic reticulum stress strongly suppressed glioblastoma cell proliferation and modified the sensitivity of these cells to hypoxia and glucose or glutamine deprivations. The present study aimed to investigate the regulation of the *SHMT2* gene in U87MG glioblastoma cells by ERN1 knockdown, hypoxia, and glucose or glutamine deprivations with the intent to reveal the role of ERN1 signaling in sensitivity of this gene expression to hypoxia and nutrient supply.

Methods. The control U87MG glioblastoma cells (transfected by an empty vector) and ERN1 knockdown cells with inhibited ERN1 endoribonuclease and protein kinase (dnERN1) or only ERN1 endoribonuclease (dnrERN1) were used. Hypoxia was introduced by dimethyloxalylglycine (500 ng/ml for 4 h). For glucose and glutamine deprivations, cells were exposed in DMEM without glucose and glutamine, respectively for 16 h. RNA was extracted from cells and reverse transcribed. The expression level of the *SHMT2* gene was studied by real-time qPCR and normalized to ACTB.

Results. It was found that inhibition of ERN1 endoribonuclease and protein kinase in glioblastoma cells led to a down-regulation of *SHMT2* gene expression in U87MG cells. At the same time, the expression of this gene did not significantly change in cells with inhibited ERN1 endoribonuclease, but tunicamycin strongly increased its expression. Moreover, the expression of the *SHMT2* gene was not affected in U87MG cells after silencing of XBP1. Hypoxia up-regulated the expression level of the *SHMT2* gene in both control and ERN1 knockdown U87MG cells. The expression of this gene was significantly up-regulated in glioblastoma cells under glucose and glutamine deprivations and ERN1 knockdown significantly increased the sensitivity of the *SHMT2* gene to these nutrient deprivation conditions.

Conclusion. The results of the present study demonstrate that the expression of the *SHMT2* gene responsible for serine metabolism and formation of folate one-carbon is controlled by ERN1 protein kinase and induced by hypoxia as well as glutamine and glucose deprivation conditions in glioblastoma cells and reflects the ERN1-mediated reprogramming of sensitivity this gene expression to nutrient deprivation.

Key words: ERN1 knockdown, hypoxia, nutrient deprivation, SHMT2, gene expression, U87MG cells

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The serine hydroxymethyltransferase (SHMT2) is one of the key enzymes associated with serine and folate metabolism and represents the mitochondrial form of a pyridoxal phosphate-dependent enzyme that catalyzes the reversible reaction of serine and tetrahydrofolate to glycine and 5,10-methylene tetrahydrofolate, an essential intermediate for the biosynthesis of purines, thymidine and methionine (Morscher et al. 2018). The mitochondrial SHMT2 and folate enzymes are strongly up-regulated in numerous human cancers, but antifolates, as well as SHMT2 knockdown, have anticancer effects (Tedeschi et al. 2013; Ducker et al. 2017; Morscher et al. 2018; Nguyen et al. 2021; Xie et al. 2022; Qiao et al. 2023; Wang et al. 2023; Zhang et al. 2023; Shan et al. 2024). Furthermore, disruption of whole-cell folate metabolism, by either folate deficiency or antifolate treatment, impairs the respiratory chain (Morscher et al. 2018). This enzyme requires for mitochondrial translation providing by methyl donors to produce the taurinomethyluridine base at the wobble position of some mitochondrial tRNAs and contributes to the *de novo* mitochondrial biosynthesis of thymidylate, which is required to prevent uracil accumulation in mitochondrial DNA (Anderson et al. 2011; Minton et al. 2018; Morscher et al. 2018). He et al. (2023) indicated that serine metabolism maintains metabolic homeostasis and treats human diseases. Thus, the SHMT2 enzyme plays a multifunctional role in mitochondria. The cytosolic isoform (SHMT1) and the mitochondrial isoform (SHMT2) have distinct cellular roles, but comparable catalytic properties, while SHMT2 undergoes a dimer-to-tetramer transition upon pyridoxal 5'-phosphate binding (Giardina et al. 2015).

The endoplasmic reticulum (ER) stress and hypoxia as well as glucose and glutamine supply 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). Furthermore, serine synthesis and metabolism are also essential for malignant tumor progression and therapeutic resistance (Maddocks et al. 2013; Mattaini et al. 2016; Yang and Vousden 2016; Engel et al. 2020; Li and Ye 2020; Hennequart et al. 2021; Tajan et al. 2021). Previous studies have shown that the knockdown of ERN1 (endoplasmic reticulum to nucleus signaling 1) significantly suppressed the glioblastoma cell proliferation as well as tumor growth *in vivo* and response to chemotherapy through genome reprogramming (Auf et al. 2010, 2013; Logue et al. 2018; Minchenko et al. 2021).

The ERN1 is an ER transmembrane signaling protein with protein kinase and endoribonuclease activities in the cytoplasmic domain. The main function of ERN1 endoribonuclease is unconventional splicing of the XBP1 (X-box binding protein 1) pre-mRNA. The resulting alternative splice variant of XBP1 mRNA (XBP1s) regulates the expression of numerous genes that encode proteins for protein folding and degradation of unfolded proteins (Acosta-Alvear et al. 2007; Doultsinos et al. 2017; Obacz et al. 2017; Almanza et al. 2019; Hetz et al. 2020). Furthermore, ERN1 protein kinase also plays an important role in the implementation of ERN1 signaling and controls the expression of genes such as *EREG* (epiregulin), *EDN1* (endothelin 1) and some others (Auf et al. 2013; Minchenko et al. 2015, 2019, 2020). 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).

There is also data indicating that inhibition of ERN1 significantly modifies hypoxic regulation of key regulatory gene expressions as well as their sensitivity to glucose and glutamine deprivations in glioblastoma cells (Minchenko et al. 2019, 2020, 2021, 2024; Krasnytska et al. 2023; Sliusar et al. 2023). Hypoxic regulation of gene expression is realized preferentially through transcription factor HIF (hypoxia-inducible factor), but there are many other factors, which can modulate the expression of genes in a gene-specific manner (Minchenko and Caro 2000; Minchenko et al. 2002, 2004; Sun and Denko 2014; Semenza 2017). It is possible that ER stress controls hypoxic regulations of gene expression by specific changes in these additional factors, which can interact with transcription factor HIF and modify its activity. It is important to note that the mitochondrial SHMT2 is required for robust mitochondrial oxygen consumption and proliferation at low glucose supply (Minton et al. 2018).

The ER stress and hypoxia as well as glucose and glutamine supply are the important factors of malignant tumor progression, metabolic reprogramming and therapeutic resistance. However, there are still not available data concerning the interaction of these factors in the regulation of gene controlling the serine metabolism, especially after suppression of glioblastoma cell proliferation by inhibition of ERN1. In this study, we are showing that the expression level of the *SHMT2* gene is affected by ERN1 knockdown and is very sensitive to glucose and glutamine deprivation in U87MG glioblastoma cells in an ERN1-dependent manner.

Material 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 three sublines of U87MG cells described previously (Auf et al. 2013). One was obtained by selection of stably transfected clones with overexpression of vector pcDNA 3.1 and used as control (control glioblastoma cells). The 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. The third subline has inhibited ERN1 endoribonuclease only by dnrERN1 constructs (Auf et al. 2013).
The cells with dnERN1 and dnrERN1 have lower proliferation rate and do not express a spliced variant of XBP1, a key transcription factor in ERN1 signaling (Auf et al. 2010, 2013; Minchenko et al. 2015). Moreover, the cells with dnERN1 do not have the phosphorylated isoform of ERN1 after induction of ER stress by tunicamycin (Auf et al. 2013). All sublines of glioblastoma cells used in this study grew in the presence of geneticin (G418), while carrying empty vector pcDNA3.1 or dominant/negative ERN1 constructs. 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. For glucose and glutamine deprivations, cells were exposed in DMEM without glucose and glutamine, respectively for 16 h. We also treated the glioblastoma cells with inhibited ERN1 endoribonuclease by tunicamycin (500 ng/ml) to clarify the role of other signaling pathways of ER stress al in the control of *SHMT2* gene expression.

Small interfering RNA knockdown experiments. The XBP1 mRNA in U87MG glioblastoma cells was silenced with small interfering RNA (siRNA) mainly as described previously (Auf et al. 2013). The ON-TARGETplus siRNA against human XBP1 (5'-GCUCUUUCCCUCAUGUAUAC) and control siRNA (ON-TARGET plus Control Pool, Non-Targeting pool; catalog: D-001810-10-05) was received from Dharmacon, a Horizon Discovery Group Company. Briefly, U87MG cells were seeded in 6-well plates and incubated until 50% confluency was reached. On the following day, the appropriate amount of siRNA against XBP1 and negative control siRNA were transfected into the cells using Lipofectamine RNAi/MAX reagent (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Transfection was performed for 48 h.

RNA isolation. Total RNA was extracted from glioblastoma cells using the Trizol reagent according to the 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 SHMT2 and 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 *SHMT2* and XBP1 genes was received from Sigma-Aldrich (St. Louis, MO, U.S.A.) and used for quantitative polymerase chain reaction: SHMT2 forward 5'-tggaagcctttgacctggat and SHMT2 reverse 5'-aagaagatggacgtggctga (NM_005412.6); XBP1 forward 5'-tgtcacccctccagaacatc and XBP1 reverse 5'-aagggaggctggtaaggaac (NM_005080.4). Primers for ACTB were described previously (Minchenko et al. 2024).

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 gene 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 4 independent experiments. A value of $p<0.05$ was considered significant in all cases. All experimental qPCR data were analyzed for the normality 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 regulation of *SHMT2* gene expression in glioblastoma cells was evaluated. As shown in Figure 1, the expression level of the *SHMT2* gene was down-regulated (-29%; $p<0.05$) in glioblastoma cells with suppressed both enzymatic activities of ERN1 (protein kinase and endoribonuclease) in comparison to control (transfected by empty vector) cells. At the same time, no significant changes were detected in the expression of the *SHMT2* gene in U87MG cells with inhibited ERN1 endoribonuclease only (Figure 1). Next, we studied the expression of the *SHMT2* gene in glioblastoma cells after silencing of XBP1 because the main function of ERN1 endoribonuclease is unconventional splicing of pre-mRNA of this transcription factor and splice variant of XBP1 controls of numerous gene expressions. As shown in Figure 2, the expression level of *SHMT2* gene did not significantly change in glioblastoma cells after 48 h of XBP1 silencing, while the level of XBP1 mRNA was effectively suppressed (-90%; $p<0.001$). We also studied the impact of tunicamycin on the expression of *SHMT2* gene in glioblastoma cells with inhibited ERN1 endoribonuclease only to clarify the role of other signaling pathways of ER stress in the control of *SHMT2* gene expression in cells without ERN1 endoribonuclease. As shown in Figure 3, tunicamycin strongly up-regulated the expression of this gene (+256%; $p<0.001$) indicating the participation of other signaling pathways of ER stress in the control of *SHMT2* gene expression by this stress.

We also studied the impact of hypoxia on the expression of the *SHMT2* gene in control glioblastoma cells and cells with suppressed both enzymatic activities of ERN1 (protein kinase and endoribonuclease) as compared to the corresponding control. As shown in Figure 4, the level of *SHMT2* gene expression is increased by hypoxia in both control glioblastoma cells (+22%; $p<0.05$) and cells with ERN1 knockdown (+18%; $p<0.05$) as compared to corresponding normoxic control cells.

We also studied the impact of glutamine deprivation on *SHMT2* gene expression in control cells with suppressed enzymatic activities of ERN1 protein kinase and endoribonuclease. As shown in Figure 5, the level of *SHMT2* gene expression is strongly increased in control glioblastoma cells exposure under glutamine deprivation conditions for 16 h (+95%; $p<0.001$) in comparison to control 1, but inhibition of ERN1 protein kinase and endoribonuclease significantly enhanced the sensitivity

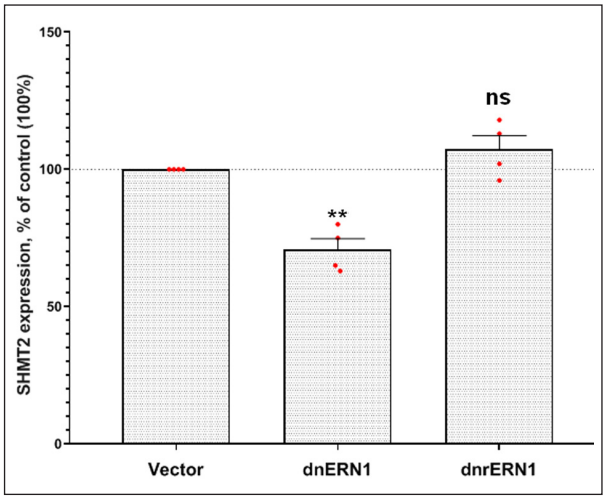

Figure 1. The expression of serine hydroxymethyltransferase 2 (SHMT2) in control U87MG glioblastoma cells (transfected by an empty vector; Vector), cells with suppressed ERN1 endoribonuclease and protein kinase (dnERN1) or only ERN1 endoribonuclease (dnrERN1) measured by qPCR. The values of this mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control (Vector, 100%); mean±SEM; **p<0.01; ns – not significant.

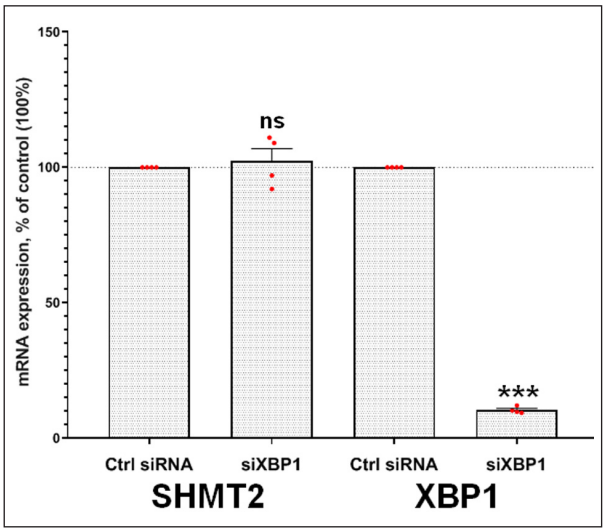

Figure 2. The impact of XBP1 mRNA silencing by specific for XBP1 siRNA (48 h) on the expression of SHMT2 and XBP1 mRNAs in control glioblastoma cells (qPCR analysis). The values of mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control (Ctrl siRNA; 100%); mean±SEM; ***p<0.001; ns – not significant.

of this gene expression to glutamine deprivation (+204%; p<0.001) as compared to control 2. Glucose deprivation conditions introduced similar changes in *SHMT2* gene expression in both control and ERN1 knockdown glioblastoma cells as shown in Figure 6. Thus, the exposure of control glioblastoma cells under glucose deprivation conditions for 16 h led to significant up-regulation of the expression of

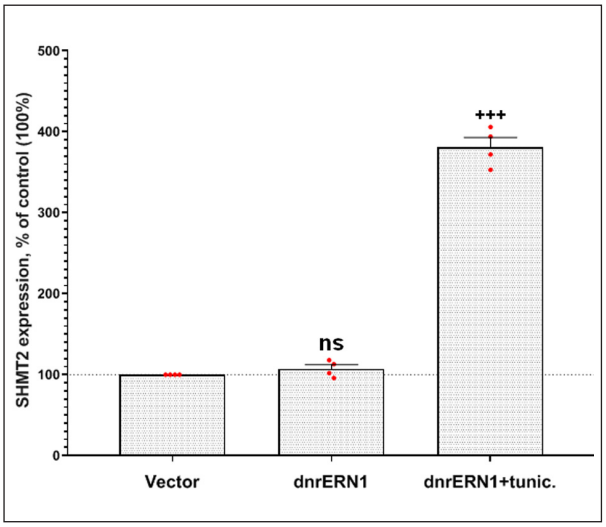

Figure 3. The impact of tunicamycin (500 ng/ml for 4 h) on the expression of SHMT2 mRNA in glioblastoma cells with a deficiency of ERN1 endoribonuclease only (dnrERN1) measured by qPCR. The values of this mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control (Vector; 100%); mean±SEM; $^{+++}$p<0.001; ns – not significant.

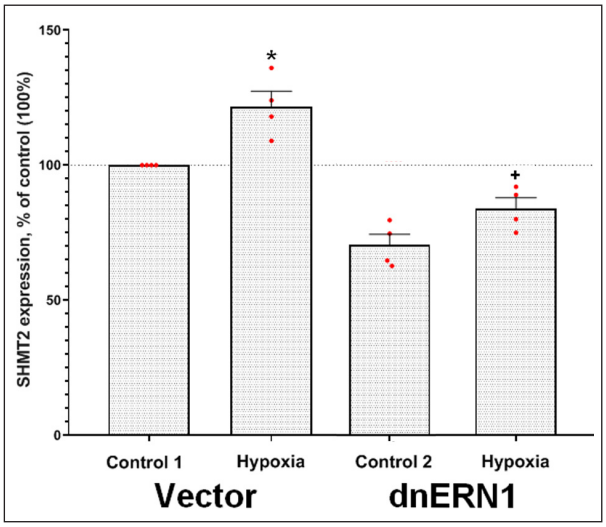

Figure 4. The impact of hypoxia on the expression of SHMT2 mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 for control glioblastoma cells (Vector; 100%) and control 2 for ERN1 knockdown cells (dnERN1; 100%); mean±SEM; *p<0.05 vs. control 1; $^{+}$p<0.05 vs. control 2.

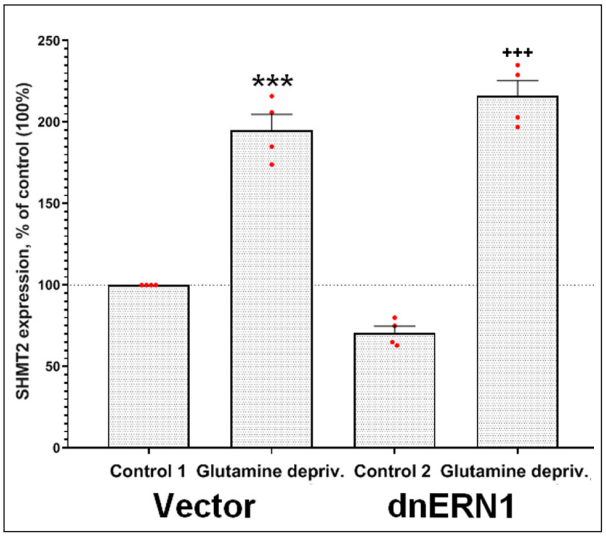

Figure 5. The impact of glutamine deprivation on the expression of SHMT2 mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 for control glioblastoma cells (Vector; 100%) and control 2 for ERN1 knockdown cells (dnERN1; 100%); mean±SEM; ***p<0.001 vs. control 1; $^{+++}$p<0.001 vs. control 2.

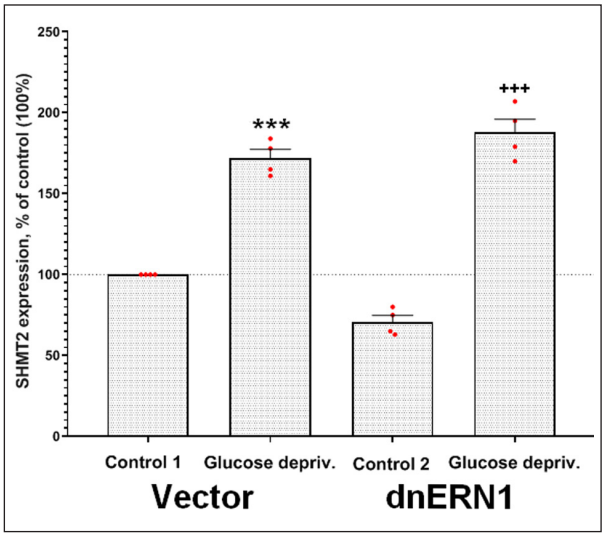

Figure 6. The impact of glucose deprivation on the expression of SHMT2 mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 for control glioblastoma cells (Vector; 100%) and control 2 for ERN1 knockdown cells (dnERN1; 100%); mean±SEM; ***p<0.001 vs. control 1; $^{+++}$p<0.001 vs. control 2.

the *SHMT2* gene (+72%; p<0.001) in comparison to control 1. The knockdown of ERN1 in glioblastoma cells also significantly increased the sensitivity of *SHMT2* gene expression to glucose deprivation (+165%; p<0.001) as compared to control 2.

Discussion

The results of this study are summarized in Figure 7. The major finding reported here is that the expression of the gene encoding the SHMT2 enzyme does not change significantly in U87MG glioblastoma cells with inhibited ERN1 endoribonuclease, but is down-regulated in cells with suppressed both ERN1 protein kinase and endoribonuclease. These results indicate that ERN1 protein kinase plays an important role in the ERN1-dependent regulation of *SHMT2* gene expression and agree well with previously reported data concerning the implementation of ERN1 protein kinase in the control of gene expression (Auf et al. 2013; Minchenko et al. 2015, 2019, 2020). The expression level of the *SHMT2* gene did not significantly change in glioblastoma cells after the effective silencing of XBP1, which is responsible for the control of numerous ERN1-dependent gene expressions. These results also support the ERN1 protein kinase-dependent mechanism of the regulation of *SHMT2* gene expression.

Hypoxia is an important factor in tumor growth because ER stress induces resistance to the toxic effects of hypoxia through genome reprogramming including HIF-dependent mechanisms (Denko 2008; Sun and Denko 2014; Minchenko et al. 2019, 2020, 2024). We have shown that hypoxia increases the expression of the *SHMT2* gene in an ERN1-independent mechanism, while the expression of numerous genes is significantly modified by ERN1 knockdown by different mechanisms in a gene-specific manner (Minchenko and Caro 2000; Semenza 2017; Minchenko et al. 2019, 2020, 2024). ERN1 inhibition may modify 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 (Minchenko and Caro 2000; Semenza 2017). It is important to note that the mitochondrial SHMT2 is required for robust mitochondrial oxygen consumption and proliferation at low glucose supply (Minton et al. 2018). These data agree well with our result concerning strong up-regulation of the expression of the *SHMT2* gene in ERN1-dependent mechanism in cells exposure to glutamine and glucose deprivations. Previously, we have shown that ERN1 knockdown modified the sensitivity of numerous gene expressions to nutrient deprivations in a gene-specific manner (Minchenko et al. 2019, 2020, 2024; Krasnytska et al. 2023).

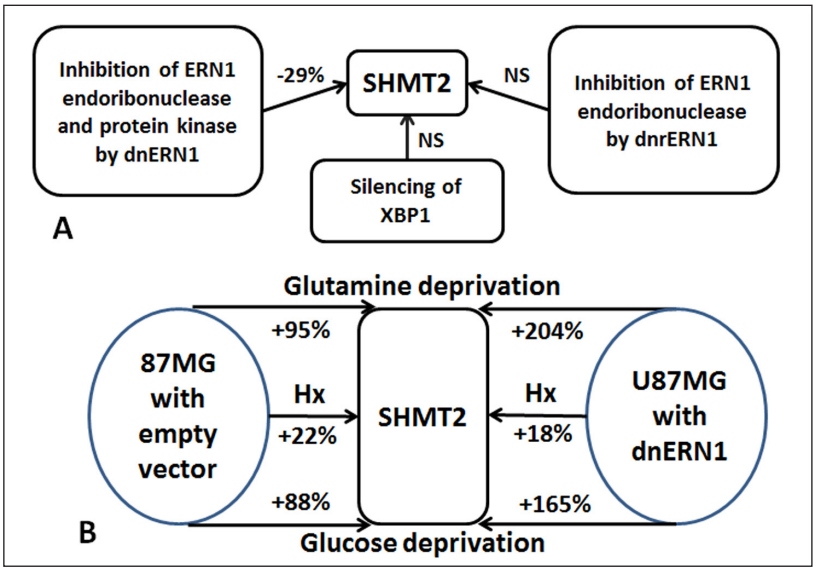

Figure 7. Schematic demonstration of the sensitivity of SHMT2 mRNA expression to inhibition of ERN1 endoribonuclease and protein kinase by dnERN1 or only ERN1 endoribonuclease only by dnrERN1 and after silencing of XBP1 mRNA (48 h) **(A)**. Schematic demonstration of the impact of hypoxia (Hx), glutamine and glucose deprivation on the expression of SHMT2 mRNA in control U87MG glioblastoma cells (with empty vector) and cells with a deficiency of both ERN1 protein kinase and endoribonuclease (with dnERN1) **(B)**.

Conclusion

The data presented in this study identify the ERN1 protein kinase-dependent mechanism of the regulation of *SHMT2* gene expression, which is responsible for serine metabolism and formation of folate one-carbon and induced by hypoxia as well as glutamine and glucose deprivation in U87MG glioblastoma cells under ERN1 knockdown. Furthermore, the expression of the *SHMT2* gene is up-regulated in glioblastoma cells and reflects the ERN1-mediated reprogramming of the sensitivity of this gene expression to nutrient deprivation. However, the detailed molecular mechanisms of the interaction of the ERN1-mediated signaling pathway with glutamine and glucose supply are complex yet and warrant further study.

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

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