

## ERN1 inhibition modifies hypoxic regulation of EGLN1 expression in U87MG glioblastoma cells

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**Objective.** Glioblastomas are the most common and highly aggressive malignant brain tumors that are difficult to treat. Hypoxia is a significant factor in glioblastoma growth. Previous studies have shown that the inhibition of ERN1 (endoplasmic reticulum to nucleus signaling 1) significantly suppresses the glioblastoma cell proliferation and modifies the hypoxic regulation. The present study aimed to investigate the impact of hypoxia on the expression of endoplasmic reticulum stress-dependent genes including *EGLN1* in U87MG glioblastoma cells in relation to ERN1 inhibition to reveal a possible role of the ERN1 signaling pathway in the hypoxic regulation of these genes' expression.

**Methods.** The control U87MG glioblastoma cells (transfected by an empty vector) and ERN1 knockdown cells with inhibited ERN1 endoribonuclease and protein kinase (dnERN1) were used. Hypoxia was induced at normoxic conditions with dimethylxylglycine (0.5 mM for 4 h). RNA was extracted and reverse transcribed. The expression levels of *EGLN1*, *HSPA5*, *DDIT4*, *PA2G4*, *XIAP*, and *MTOR* genes were studied by real-time qPCR and normalized to *ACTB* mRNA.

**Results.** It was established that hypoxia increases the expression level of *EGLN1*, *HSPA5*, *DDIT4*, and *XIAP* in glioblastoma cells with native ERN1. However, the expression levels of two other stress-dependent genes (*PA2G4* and *MTOR*) were reduced in these glioblastoma cells under hypoxia. Furthermore, inhibition of ERN1 enzymatic activity attenuated the effects of hypoxia on the expression of most of the genes studied in glioblastoma cells, while *XIAP* gene expression was dramatically increased.

**Conclusion.** The results of this study showed that hypoxia differentially affects the expression of genes related to endoplasmic reticulum stress in glioblastoma cells and is dependent on ERN1 activity. This reflected the ERN1-mediated reprogramming of hypoxic regulation of gene expression.

**Keywords:** *EGLN1*, *DDIT4*, *HSPA5*, *mTOR*, gene expression, hypoxia, ERN1 inhibition, glioblastoma cells

One of the most well-known malignant brain tumors is glioblastoma. This solid tumor exhibits a high proliferation rate and invasiveness. Hypoxia and endoplasmic reticulum stress are key and mutually complementary factors in the metabolic reprogramming and growth of glioblastoma, as well as other malignant tumors (Fels and Koumenis

2006; Denko 2008; Chevet et al. 2015; Doultzinos et al. 2017; Almanza et al. 2019; Acosta-Alvear et al. 2025). ERN1/IRE1 (endoplasmic reticulum to nucleus signaling 1/inositol requiring enzyme 1) is a central mediator of the UPR (unfolded protein response) and plays a crucial role in the glioblastoma progression and resistance to chemotherapy (Logue

et al. 2018; Papaioannou and Chevet 2018; Hetz et al. 2020; Pelizzari-Raymundo et al. 2023). ERN1 represents a transmembrane protein and functions as a sensor of the UPR. It contains two distinct catalytic domains at the C-terminus, which display protein kinase and endoribonuclease activities (Almanza et al. 2019; Acosta-Alvear et al. 2025). Previous studies have shown that ERN1 knockdown significantly suppresses the glioblastoma cell proliferation and tumor growth *in vivo* in two experimental models: a mouse orthotopic brain model and the chicken chorio-allantoic membrane assay (Auf et al. 2010, 2013; Pelizzari-Raymundo et al. 2023; Minchenko et al. 2024b). Moreover, suppression of ERN1 endoribonuclease activity enhances the response to chemotherapy (Logue et al. 2018).

Hypoxia is a necessary factor for malignant tumor growth because it induces the expression of pro-oncogenic genes including *VEGF* and *PFKFB3*, which enhance angiogenesis and glycolysis. However, it is toxic to normal cells (Denko 2008; Minchenko et al. 1994, 2002, 2003). Tumor cells develop resistance to the toxic effects of hypoxia through genome reprogramming, which is mediated by endoplasmic reticulum stress signaling pathways (Minchenko et al. 2021). Hypoxic regulation of gene expression is facilitated by the hypoxia inducible factor (HIF) a heterodimeric transcription factor (Wang and Semenza 1993; Minchenko and Caro 2000; Wenger 2002; Minchenko et al. 2004; Knaup et al. 2014). At the same time, gene expression under hypoxia is gene-specific. Thus, the expression of diverse genes is changed differently upon hypoxia, even in the same cell line (Bobarykina et al. 2006; Minchenko et al. 2014; Sliusar et al. 2023; Krasnytska et al. 2024). It is also known that the level of expression of the pro-oncogenic gene *VEGF* changes differently in different organs of rats under *in vivo* hypoxia (Minchenko et al. 1994). Similar results were obtained when studying the effect of *in vivo* hypoxia on the expression of the *PFKFB1*, *PFKFB2*, and *PFKFB3* genes in different organs of mice (Minchenko et al. 2003).

The organ-specific nature of gene expression changes may be due to the interaction of numerous proteins with HIF1A, which differentially alters its level and transcriptional activity (Minchenko and Caro 2000; Semenza 2017). Thus, more than 150 proteins have been identified that can interact with HIF1A altering its transcriptional activity and stability through different mechanisms including phosphorylation (Semenza 2017). We have previously shown that the hypoxic induction of the endothelin-1 gene expression in microvascular endothelial cells

is mediated by HIF-1 and inhibited by genistein, an inhibitor of tyrosine protein kinases (Minchenko and Caro 2000). It has also been found that inhibition of ERN1 modifies the hypoxic regulation of numerous gene expressions in glioblastoma cells (Minchenko et al. 2013, 2019, 2021, 2024c, 2025; Danilovsky et al. 2014; Sliusar et al. 2023; Viletska et al. 2026a, b). Inhibition of ERN1 may alter hypoxic regulation of gene expression by specific changes in proteins, which interact with HIF1A including XBPs and modulate its transcriptional activity (Chen et al. 2014; Semenza 2017).

For this study, we selected genes that regulate malignant tumor growth and whose expression is controlled by endoplasmic reticulum stress *EGLN1/PHD2*, (*HSPA5/BiP*, *DDIT4*, *PA2G4*, *XIAP*, and *MTOR*). Of particular interest is *EGLN1* (egl-9 family hypoxia inducible factor 1) known also as prolyl hydroxylase domain-containing protein 2 (*PHD2*). It is an oxygen sensor and controls the level of the HIF1alpha protein (Schofield and Ratcliffe 2004; Chan et al. 2016). The *EGLN1* gene is dysregulated in cancer and contributes to the epigenetic regulation of gene transcription and the reprogramming of cellular metabolism (Schodel et al. 2016; Losman et al. 2020; Jiang et al. 2023). *HSPA5/BiP* (heat shock protein family A, Hsp70, a member 5/immunoglobulin heavy chain-binding protein) known also as GRP78 (glucose-regulated protein 78 kDa) promotes folding and assembly of proteins and controls the quality of protein folding, is overexpressed in malignant tumors and acts also as a transcriptional regulator (Yin et al. 2017; Liu et al. 2023; Chen et al. 2024). *DDIT4* (DNA damage inducible transcript 4) also known as HIF-1 responsive protein RTP801 is induced in various cellular stress conditions, promotes malignancy, and regulates cell proliferation and survival by influencing the activity of the mechanistic target of rapamycin (*MTOR/mTOR*) (Lin et al. 2023; Tajik et al. 2023; Dai et al. 2024).

The *XIAP* (X-linked inhibitor of apoptosis) gene encodes a multifunctional protein that acts as an E3 ubiquitin-protein ligase and regulates apoptosis, inflammatory signaling, copper homeostasis, cell population proliferation, carcinoma growth, and invasion (Hiramatsu et al. 2014; Huang et al. 2019; Pei et al. 2024). The expression of *XIAP* gene is associated with malignancy and chemoresistance, but loss of *XIAP* leads to increased cell death (Pei et al. 2024). The expression level of *PA2G4* (proliferation-associated 2G4), also known as *EBP1* (erbB3 binding protein EBP1) is known to be increased in various tumors (He et al. 2023; Kumar et al. 2023).

*MTOR* (mechanistic target of rapamycin kinase) gene exhibits protein kinase activity and is a central regulator of cellular metabolism in response to stress signals particularly in various forms of cancer. It controls fundamental cellular functions, such as protein synthesis, metabolism, cell proliferation, growth, survival, and apoptosis (Mossmann et al. 2018; Murugan 2019; Marafie et al. 2024). In this regard, *MTOR*-mediated cell growth-promoting signaling is a target to fight various cancers (Murugan 2019; Marafie et al. 2024).

In this study, we investigate the impact of hypoxia on the expression of unfolded protein response (*HSPA5* and *DDIT4*) and hypoxia response (*EGLN1*, *XIAP*, *DDIT4*, and *PA2G4*) genes in ERN1 knockdown glioblastoma cells to reveal the role of the ERN1 signaling pathway in the hypoxic regulation of these gene expressions.

## Materials and Methods

**Cell lines and culture conditions.** In this study, we used two sublines of U87MG glioblastoma cells previously described by Auf et al. (2013). A subline of glioblastoma cells with overexpression of vector pcDNA 3.1 was used as control glioblastoma cells. Another glioblastoma cell subline had suppressed ERN1 protein kinase and endoribonuclease activities due to overexpression of the ERN1 dominant/negative construct in pcDNA 3.1 (dnERN1) (Auf et al. 2013). These glioblastoma cells with ERN1 knockdown have reduced proliferation and do not express phosphorylated ERN1 and XBPs, a key transcription factor of ERN1 signaling in response to the induction of endoplasmic reticulum stress by tunicamycin (Auf et al. 2010, 2013; Minchenko et al. 2024b). The expression level of XBPs mRNA in these tunicamycin-treated glioblastoma cells was suppressed by 98% compared with control cells indicating high efficiency of ERN1 knockdown (Auf et al. 2013).

Both sublines of these 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<sub>2</sub> (Auf et al. 2013). Since these cells were carrying the empty vector pcDNA3.1 or dominant/negative ERN1 construct, geneticin (G418) was added to the medium during their growth (Auf et al. 2013). Hypoxia was formed using dimethyloxalylglycine (0.5 mM for 4 h) (Sigma-Aldrich, St. Louis,

MO, U.S.A.) as described previously (Minchenko et al. 2002, 2024a).

**RNA isolation.** RNA was obtained 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. RNA concentration and spectral characteristic were measured using a NanoDrop spectrophotometer.

**Reverse transcription and quantitative PCR analysis.** Thermo Scientific Verso cDNA Synthesis Kit (Germany) was used for reverse transcription as described (Rudnytska et al. 2021). The expression of *EGLN1*, *HSPA5*, *DDIT4*, *PA2G4*, *XIAP*, *MTOR*, and *ACTB* genes were measured in U87MG glioblastoma cells by quantitative polymerase chain reaction (qPCR) using The Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) and "QuantStudio 5 Real-Time PCR System" (Applied Biosystems, USA). Polymerase chain reaction was performed in triplicate. *ACTB* mRNA expression was used as a control for the amount of mRNA analyzed. The pair of primers specific for the genes under study was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) and used for qPCR: *EGLN1* forward 5'-ttgctgacattgaacccaaa and *EGLN1* reverse 5'-ttaccgaccgaatctgaagg; *HSPA5* forward 5'-aggacaagaaggaggacgtg and *HSPA5* reverse 5'-atcagacgttccccttcagg; *DDIT4* forward 5'-ctgttagctccgccaactc and *DDIT4* reverse 5'-ccaccccaaaagttcagtcg; *PA2G4* forward 5'-agaccagcagaagaaggacc and *PA2G4* reverse 5'-caaaacgccttcacactca; *XIAP* forward 5'-gccatctgagacacatgcag and *XIAP* reverse 5'-ctcttggggttaggtgagca; *MTOR* forward 5'-agtagcatgaaccctgcctt and *MTOR* reverse 5'-ctgggttggatcagggtct. Primers for *ACTB* were described previously (Minchenko et al. 2024b). The amplified DNA fragments were analyzed on a 2.5% agarose gel and then visualized by SYBR<sup>®</sup> Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, USA). The amplicons were represented by a single band of the expected size: *EGLN1* – 211 bp, *HSPA5* – 166 bp, *DDIT4* – 188 bp, *PA2G4* – 203 bp, *XIAP* – 155 bp, and *MTOR* – 223 bp.

**Statistical analysis.** The quantitative PCR analysis was performed using a special computer program, "Differential Expression Calculator", which represents an Excel sheet established by Livak and Schmittgen (2001). GraphPad Prism 8.0.1 was used for scientific data analysis and graphing. The values of hypoxic regulation of studied gene expression in glioblastoma cells with native ERN1 were normalized to the expression of beta-actin mRNA and expressed as a percentage of control 1

(cells without hypoxia; 100%). At the same time, the values of hypoxic regulation of the expression of all studied genes in ERN1 knockdown glioblastoma cells were compared with control 2 (ERN1 knockdown cells without hypoxia). All values were expressed as mean $\pm$ 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 observed for all analyzed data sets.

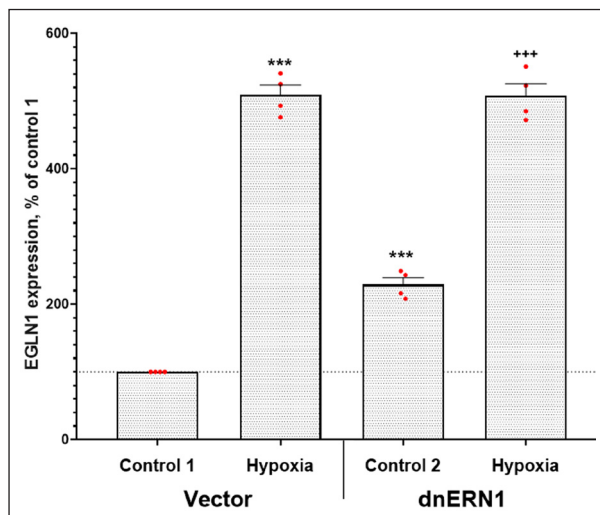
## Results

The possible role of endoplasmic reticulum stress signaling in hypoxic regulation of *EGLN1*, *HSPA5*, *DDIT4*, *XIAP*, *PA2G4*, and *MTOR* gene expressions was evaluated in control and ERN1-knockdown glioblastoma cells. As shown in Figure 1, the level of *EGLN1* gene expression strongly increased (+409%,  $p < 0.001$ ) in hypoxia-treated control glioblastoma cells (Vector) compared to control 1. Hypoxia also increased *EGLN1* gene expression in glioblastoma cells with suppressed activity of the ERN1 signaling

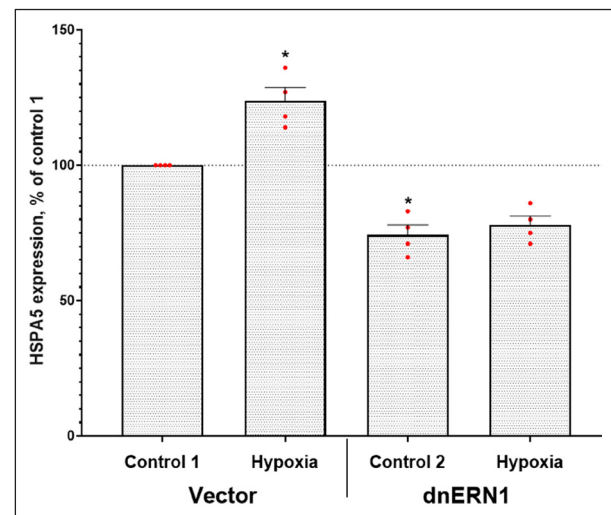
protein, but to a significantly lesser extent (+122%,  $p < 0.001$ ) (Figure 1). It is worth noting that the gene expression level of this 2-oxoglutarate-dependent dioxygenase was also increased (+129%,  $p < 0.001$ ) in glioblastoma cells with suppressed ERN1 signaling protein activity compared to control 1 (Figure 1).

Figure 2 demonstrates that *HSPA5* gene expression was increased (+24%;  $p < 0.05$ ) in hypoxia-treated control glioblastoma cells (Vector) compared to control 1 (cells without hypoxia treatment). At the same time, the sensitivity of *HSPA5* gene expression to hypoxia was reduced in glioblastoma cells with suppressed ERN1 activity compared to control 2 (dnERIN1 cells without hypoxia) (Figure 2). Additionally, it was demonstrated that inhibition of ERN1 reduces the expression level of this gene by 26% ( $p < 0.05$ ) in glioblastoma cells (Figure 2). Thus, ERN1 knockdown not only reduces the expression level of *HSPA5*, but also removes its hypoxic induction.

Larger changes were also detected in *DDIT4* gene expression in hypoxia-treated control U87MG glioblastoma cells (+297%,  $p < 0.001$ ; Figure 3). However, inhibition of ERN1 enzymatic activity decreases the sensitivity of this gene expression to hypoxia in glioblastoma cells compared to control 2. Thus,



**Figure 1.** The impact of hypoxia on the expression of egl-9 family hypoxia inducible factor 1/prolyl hydroxylase domain-containing protein 2 (*EGLN1*/PHD2) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERIN1) measured by qPCR. The values of *EGLN1* 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 (dnERIN1); mean $\pm$ SEM; \*\*\* $p < 0.001$  vs. control 1; \*\*\* $p < 0.001$  vs. control 2.



**Figure 2.** The impact of hypoxia on the expression of heat shock protein family A (Hsp70) member 5/immunoglobulin heavy chain-binding protein (*HSPA5*/BiP) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERIN1) measured by qPCR. The values of *HSPA5* 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 (dnERIN1); mean $\pm$ SEM; \* $p < 0.05$  vs. control 1.

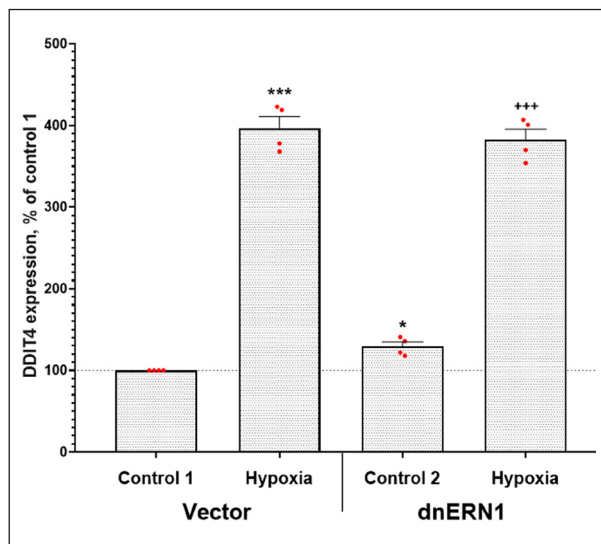
hypoxia increased *DDIT4* gene expression only by 197% ( $p<0.001$ ; Figure 3). The same figure shows data on the effect of ERN1 knockdown on *DDIT4* gene expression. It demonstrates a 29% increase ( $p<0.05$ ) in the expression level of this gene in glioblastoma cells with suppressed ERN1 activity (Figure 3).

Next, we demonstrated that X-linked inhibitor of apoptosis (*XIAP*) gene expression was also increased by hypoxia, however, the observed changes were relatively small (+15%,  $p<0.05$ ) compared to *EGLN1* and *DDIT4* genes (Figure 4). However, inhibition of ERN1 activity significantly suppressed *XIAP* gene expression (–70%,  $p<0.001$ ) in glioblastoma cells, but strongly increased the sensitivity of this gene expression to hypoxia. Thus, under hypoxia the level of *XIAP* gene expression is sharply increased (+120%,  $p<0.001$ ) in glioblastoma cells with suppressed ERN1 activity compared to control 2 (Figure 4).

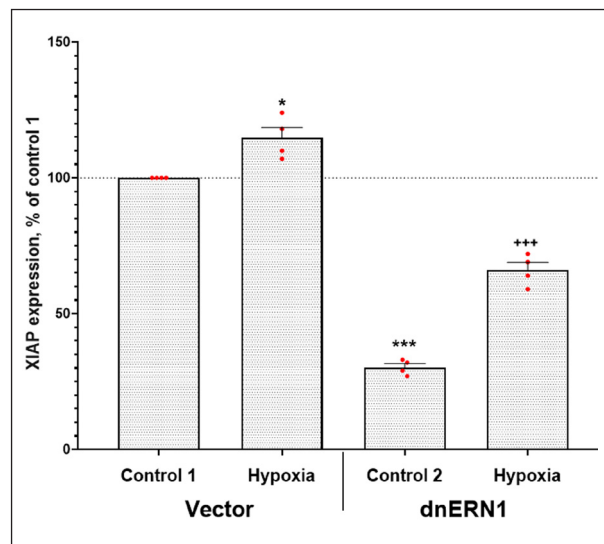
By studying the effect of hypoxia on the expression level of the *PA2G4* and *MTOR* genes, an opposite effect was found. As shown in Figure 5, the level of *PA2G4* gene expression was sharply reduced under hypoxia in glioblastoma cells with native ERN1 (–59%,  $p<0.001$ ). The suppressive effect of hypoxia on the expression level of this gene was also detected in hypoxia-treated glioblastoma cells with inhibited ERN1 activity, but it

was significantly smaller (–44%,  $p<0.01$ ; Figure 5). A similar negative effect of hypoxia was also shown for *MTOR* gene expression in glioblastoma cells. Thus, we showed that hypoxia strongly downregulates the expression of the *MTOR* gene (–62%,  $p<0.001$ ; Figure 6). However, the negative effect of hypoxia on this gene expression was significantly less pronounced in glioblastoma cells with suppressed ERN1 activity (–17%,  $p<0.05$ ; Figure 6). By analyzing the expression levels of *PA2G4* and *MTOR* genes, a suppressive effect of ERN1 knockdown on these gene expressions was also revealed. Thus, the level of *PA2G4* and *MTOR* gene expression was reduced under hypoxia (–48%,  $p<0.001$  for *PA2G4* and –12%,  $p<0.05$  for *MTOR*) in glioblastoma cells with inhibited activity of ERN1 compared to control dnERN1 cells without hypoxia (Figures 5 and 6).

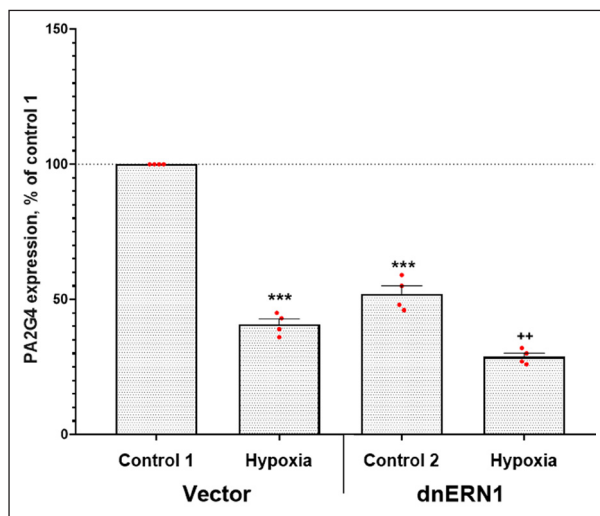
The hypoxia affects the expression of *EGLN1*, *HSPA5*, *DDIT4*, *XIAP*, *PA2G4*, and *MTOR* genes in glioblastoma cells in different ways. Moreover, the effect of hypoxia on the expression of the studied genes in glioblastoma cells is regulated by endoplasmic reticulum stress, particularly its signaling protein ERN1, since its inhibition modifies hypoxic regulation. Thus, the expression of all studied genes is dependent on ERN1.



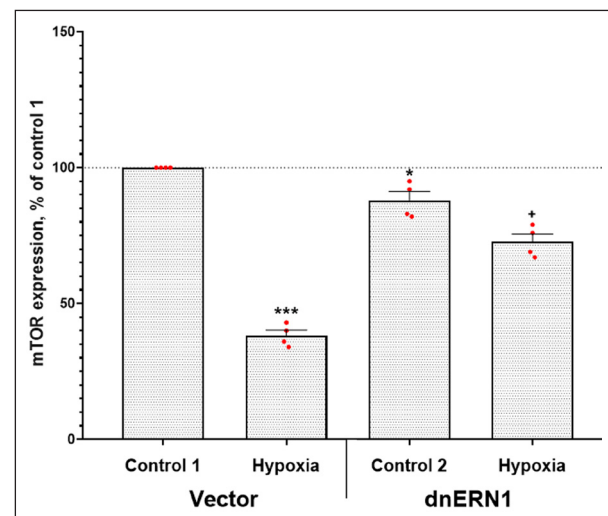
**Figure 3.** The impact of hypoxia on the expression of DNA damage inducible transcript 4 (*DDIT4*) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of this mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 for control glioblastoma cells (Vector; 100 %) and control 2 for ERN1 knockdown cells (dnERN1); mean±SEM; \* $p<0.05$  and \*\*\* $p<0.001$  vs. control 1; \*\*\* $p<0.001$  vs. control 2.



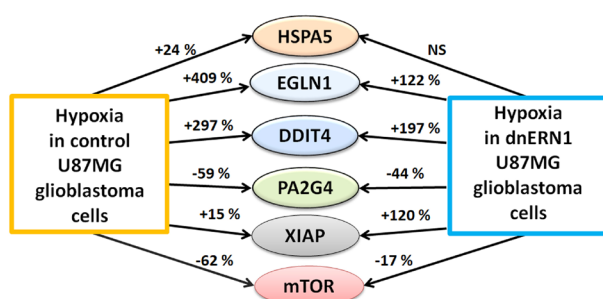
**Figure 4.** The impact of hypoxia on the expression of X-linked inhibitor of apoptosis (*XIAP*) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of *XIAP* mRNA expression were normalized to ACTB mRNA and represented as a percent of the control 1 for control glioblastoma cells (Vector; 100 %) and control 2 for ERN1 knockdown cells (dnERN1); mean±SEM; \* $p<0.05$  and \*\*\* $p<0.001$  vs. control 1; \*\*\* $p<0.001$  vs. control 2.



**Figure 5.** The impact of hypoxia on the expression of proliferation-associated 2G4/erbB3 binding protein EBP1 (PA2G4/EBP1) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of PA2G4 mRNA expression were normalized to ACTB mRNA and represented as a percent of the control 1 for control glioblastoma cells (Vector; 100 %) and control 2 for ERN1 knockdown cells (dnERN1); mean±SEM; \*\*\*p<0.001 vs control 1; \*\*p<0.01 vs. control 2.



**Figure 6.** The impact of hypoxia on the expression of mechanistic target of rapamycin (MTOR/mTOR) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (dnERN1) measured by qPCR. The values of this mRNA expression were normalized to ACTB mRNA and represented as a percent of the control 1 for control glioblastoma cells (Vector; 100 %) and control 2 for ERN1 knockdown cells (dnERN1); mean±SEM; \*p<0.05 and \*\*\*p<0.001 vs. control 1; \*p<0.05 vs. control 2.



**Figure 7.** Schematic demonstration of the impact of hypoxia on the expression of HSPA5, EGLN1, DDIT4, PA2G4, XIAP, and mTOR mRNAs in control U87MG glioblastoma cells (with native ERN1) and cells with a deficiency of ERN1 enzymatic activity (dnERN1).

## Discussion

Hypoxia is an important factor in tumor growth because endoplasmic reticulum stress induces resistance to the toxic effects of hypoxia through genome reprogramming. Hypoxic regulation of gene expression is mainly realized by transcription factor HIF (Wang and Semenza 1993; Wenger 2002; Schodel et al. 2016; Minchenko et al. 2002, 2004). It has previously been shown that HIF1A protein levels

are strongly increased upon hypoxia regardless of the type of inducers including dimethylxalylglycine (Minchenko et al. 2002, 2004, 2005; Bobarykina et al. 2006). At the same time, the changes in gene expression under hypoxia are gene-specific: different in the same cell line (Minchenko et al. 2013, 2019, 2024c, 2025; Danilovskiy et al. 2014; Sliusar et al. 2023; Krasnytska et al. 2024; Viletska et al. 2026a, b). It is known that many proteins that interact with HIF1A can differently alter hypoxic regulation of gene expression (Semenza 2017). The interaction of these proteins, which have different mechanisms of action with protein HIF1A, can either reduce or enhance its degradation and alter its transcriptional activity (Semenza 2017). Among the proteins interacting with HIF1A, co-activators and co-repressors have been identified, which can enhance and suppress the transcriptional activity of this transcription factor (Semenza 2017). This may be one explanation for HIF-dependent activation or repression of gene expression.

Protein kinases, which interact with HIF1A and can modify its activity, were also identified (Semenza 2017). This explains the inhibitory effect of the protein kinase inhibitor genistein on hypoxic induction of *EDN1* (endothelin-1) gene expression

in microvascular endothelial cells (Minchenko and Caro 2000). These results indicate the involvement of protein kinases in the HIF-mediated induction of *EDN1* gene expression under hypoxia. This is consistent with the data of Casillas et al. (2021) who have demonstrated that phosphorylation and stabilization of HIF1A by Pim-1 Proto-Oncogene, Serine/Threonine Kinase (PIM1) drive angiogenesis in solid tumors. Moreover, the multifaceted post-translational modification of HIF1A is important for its transcriptional activity and stability. Among the proteins interacting with HIF1A, some have been identified that regulate its post-translational modification, determining stability and activity of HIF1A in transcriptional regulation (Semenza 2017). These proteins can modify the hypoxic regulation of HIF-dependent gene expression.

Moreover, the effect of hypoxia on the expression of numerous genes depends on the endoplasmic reticulum stress, in particular its ERN1 signaling pathway, which is responsible for alternative splicing of the transcription factor XBP1. The transcription factor XBP1s regulates ERN1-dependent expression of numerous genes and is a co-activator of HIF1A (Semenza 2017). Therefore, knockdown of ERN1 alters hypoxic regulation of the expression of genes whose ERN1-dependent regulation is mediated by XBP1 (Minchenko et al. 2024b). This confirms that ERN1 is a co-activator of HIF1A.

The main results of this investigation are presented in Figure 7. The major finding reported here is that hypoxia affects the expression of *EGLN1*, *HSPA5*, *DDIT4*, *PA2G4*, *XIAP*, and *MTOR* in glioblastoma cells in a gene-specific, endoplasmic reticulum stress-dependent manner and that ERN1 knockdown modifies the effects of hypoxia. Likely, the effect of hypoxia on the expression of these genes in glioblastoma cells with native ERN1 may also be mediated by endoplasmic reticulum stress, particularly its ERN1 signaling pathway, since inhibition of ERN1 activity modifies the effect of hypoxia. This is in good agreement with the results of numerous investigations on the dependence of hypoxic regulation of various gene expressions on the activity of the ERN1 signaling protein (Minchenko et al. 2019, 2021; Sliusar et al. 2023; Krasnytska et al. 2024). Thus, in cells with suppressed ERN1 activity, the effects of hypoxia on most gene expressions are reduced due to the absence of ERN1-mediated enhancement of hypoxic regulation.

We also demonstrated that loss of ERN1 abolishes XBP1s-mediated transcriptional enhancement of *HSPA5* expression in glioblastoma cells.

Furthermore, inhibition of ERN1 significantly reduces the effect of hypoxia on *EGLN1*, *DDIT4*, and *MTOR* gene expressions, however, enhances the hypoxic induction of *XIAP* gene expression. Inhibition of ERN1 downregulates the expression of multi-functional protein *XIAP*, which regulates not only caspases and apoptosis, but also modulates mitogenic kinase signaling, cell proliferation, and cell invasion (Hiramatsu et al. 2014; Pei et al. 2024). In response to such a decrease in *XIAP* expression, the induction of co-activator HIF1A may be initiated enhancing the hypoxic regulation of *XIAP* gene expression. Recently, it has been shown that ERN1 inhibition strongly upregulates *SHMT1*, which is also responsible for cell invasion (Minchenko et al. 2024b). A similar effect of ERN1 inhibition was also demonstrated for the *PBX3*, *PRRX1*, *PAX6*, and *EDN1* genes, which are pro-proliferative and pro-invasive (Minchenko et al. 2019, 2024a). It is important to note that ERN1-dependent expression of these genes is controlled by ERN1 protein kinase, but not its endoribonuclease activity.

However, for the *XIAP* gene a more pronounced effect of hypoxia was found precisely in cells with suppressed enzymatic activity of the ERN1 signaling protein. This may be due to ERN1-dependent resistance of this gene to hypoxia, since ERN1 inhibition leads to a sharp increase in the sensitivity of *XIAP* gene expression to hypoxia. These results are in good agreement with recent data from a study on hypoxic regulation of *PSAT1* expression in glioblastoma cells (Minchenko et al. 2024b). Furthermore, we have shown that hypoxia downregulates the expression of *PA2G4* and *MTOR* genes in glioblastoma cells. The effect of hypoxia, however, on the expression of these genes in glioblastoma cells with suppressed enzymatic activity of the ERN1 signaling protein was significantly less compared to control glioblastoma cells.

Thus, the different direction of changes in gene expression under hypoxia that we have identified, as well as the dependence of hypoxic regulation on ERN1 activity, indicates multifaceted mechanisms of hypoxic regulation of gene expression, which is consistent with the results of studies by other authors (Wenger 2002; Minchenko et al. 2004; Schodel et al. 2016). The expression of all studied genes (*HSPA5*, *DDIT4*, *EGLN1*, *PA2G4*, *XIAP*, and *MTOR*) is dependent on endoplasmic reticulum stress, and ERN1 inhibition leads to suppression of most of these gene expressions in U87MG glioblastoma cells. At the same time, *DDIT4* gene expression is increased in glioblastoma cells with ERN1 inhibition, although

increased *DDIT4* is associated with aggressive tumor behavior and invasion (Lin et al. 2002; Tajik et al. 2023). Moreover, ERN1 inhibition reduces hypoxic induction of *EGLN1* gene expression in glioblastoma cells, which may contribute to the inhibition of cell proliferation and survival (Minchenko et al. 2024b). Similar changes in sensitivity to hypoxia upon ERN1 inhibition were previously shown for the *SPAG4* gene (Krasnytska et al. 2024). These results are fully consistent with the reduced proliferative potential and increased invasiveness of ERN1 knockdown glioblastoma cells (Auf et al. 2010; Yin et al. 2017; Papaioannou and Chevet 2018; Minchenko et al. 2024a, b).

### Conclusion

Hypoxia affects the expression of *EGLN1*, *HSPA5*, *DDIT4*, *PA2G4*, *XIAP*, and *MTOR* genes in glioblastoma cells in dissimilar ways, both in terms of the extent of the changes and their direction.

Furthermore, the hypoxic regulation of the studied genes expression in glioblastoma cells is controlled by endoplasmic reticulum stress, particularly by its signaling protein ERN1, since its suppression modifies the effect of hypoxia in a gene-specific manner. This reflected the ERN1-mediated reprogramming of hypoxic regulation of gene expression possibly through changes in the activity of numerous HIF1A co-activators and co-repressors, as well as enzymes controlling its post-translational modification and ubiquitination. The analysis of ERN1-dependent hypoxic regulation of gene expression is required to optimize the efficiency of anti-glioblastoma treatment. However, the detailed molecular mechanisms of the interaction of hypoxia and the ERN1-mediated stress signaling pathway in the regulation of gene expression is a complex and requires further study.

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

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