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Endoplasmic reticulum stress-dependent regulation of carboxypeptidase E expression in glioblastoma cells

Oleksandr H. Minchenko, Anastasiia I. Abramchuk, Olena O. Khita, Myroslava Y. Sliusar, Yuliia M. Viletska, Dmytro O. Minchenko

Department of Molecular Biology, Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv, Ukraine
E-mail: ominchenko@yahoo.com

Objective. Carboxypeptidase E (CPE) plays an important role in the biosynthesis of neurotransmitters and peptide hormones including insulin. It also promotes cell proliferation, survival, and invasion of tumor cells. The endoplasmic reticulum stress, hypoxia, and nutrient supply are significant factors of malignant tumor growth including glioblastoma. There are data indicating that the knockdown of the endoplasmic reticulum to nucleus signaling 1 (ERN1) suppressed glioblastoma cell proliferation and increased invasiveness of these cells. The present study aims to investigate the regulation of the *CPE* 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 the regulation of this gene expression and function in tumorigenesis.

Methods. Human glioblastoma cells U87MG (transfected by an empty vector; control) and ERN1 knockdown cells with inhibited ERN1 endoribonuclease and protein kinase (dnERN1) or only ERN1 endoribonuclease (dnrERN1) were used. Hypoxia was introduced by dimethyloxalylglycine; for glucose and glutamine deprivations, the cells were cultured in DMEM medium without glucose or glutamine for 16 h, respectively. The expression level of the *CPE* gene was studied by quantitative RT-PCR and normalized to ACTB.

Results. It was found that inhibition of endoribonuclease and protein kinase activities of ERN1 led to a strong up-regulation of *CPE* gene expression in glioblastoma cells. The expression of this gene also increased in glioblastoma cells after silencing ERN1. At the same time, the expression of this gene did not significantly change in cells with inhibited ERN1 endoribonuclease only. The expression of the *CPE* gene was resistant to hypoxia in control U87MG cells, but increased in cells with ERN1 knockdown. The expression of this gene was up-regulated under glutamine deprivation in control glioblastoma cells, but decreased upon ERN1 knockdown. However, glucose deprivation decreased the expression of *CPE* gene in both types of used cells, but ERN1 inhibition enhanced this effect.

Conclusion. The results of the present study demonstrate that inhibition of ERN1 strongly up-regulated the expression of pro-oncogenic *CPE* gene through protein kinase activity of ERN1 and that increased *CPE* gene expression possibly participates in ERN1 knockdown-mediated invasiveness of glioblastoma cells.

Keywords: carboxypeptidase E, gene expression, ERN1 knockdown, ERN1 protein kinase, hypoxia, nutrient deprivation, glioblastoma cells

Corresponding author: Oleksandr H. Minchenko, Prof., Department of Molecular Biology, Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine; phone: +380442356151; e-mail: ominchenko@yahoo.com.

Carboxypeptidase E (CPE) plays an important role in the biosynthesis of neurotransmitters and peptide hormones, including insulin (Fricker 1988, 2018; Cawley et al. 2012; Chen et al. 2023). Mutations in CPE lead to obesity and type 2 diabetes mellitus in humans, and whole-body CPE knockout or mutant mice are obese and hyperglycemic and fail to convert proinsulin to insulin (Chen et al. 2023). However, CRE is a multifunctional protein that assists in many essential nonenzymatic roles in the endocrine and nervous systems and may participate in controlling cell proliferation, survival, epithelial-mesenchymal transition, and invasion to promote tumorigenesis (Cawley et al. 2012; Fan et al. 2019; Hareendran et al. 2022; Kuo et al. 2022). Silencing of CPE mRNA has anticancer effects: inhibits proliferation and invasion of pancreatic cancer cells (Lou and Loh 2021). Mechanisms driving tumor growth and metastasis are complex and involve the recruitment of many genes working in concert with each other. CPE is an important factor in driving growth, survival, and metastasis in many cancer types (Fan et al. 2019; Hareendran et al. 2022).

The endoplasmic reticulum (ER) stress, hypoxia, and nutrient (glucose and glutamine) supply are important factors of glioblastoma growth and its metabolic reprogramming as in many other malignant tumors (Denko 2008; Bravo et al. 2013; Chevet et al. 2015; Almanza et al. 2019; Minchenko et al. 2021). Previous studies show that the knockdown of ERN1 (endoplasmic reticulum to nucleus signaling 1) significantly suppressed glioblastoma cell proliferation, tumor growth *in vivo,* and response to chemotherapy through genome reprogramming but increased invasiveness (Auf et al. 2010, 2013; Logue et al. 2018; Minchenko et al. 2021). Metabolic reprogramming is a basic characteristic of tumor cells that 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).

The ERN1 is an ER transmembrane signaling protein with protein kinase and endoribonuclease activities in the cytoplasmic domain (Almanza et al. 2019). The endoribonuclease activity of ERN1 is responsible for alternative splicing of the XBP1 (X-box binding protein 1) pre-mRNA encoding splice variant of XBP1 (XBP1s), which regulates the expression of chaperons and enzymes for degradation of unfolded proteins and restoration of folding as well as many other proteins (Acosta-Alvear et al. 2007; Obacz et al. 2017; Doultsinos et al. 2017; Hetz et al. 2020; Pelizzari-Raymundo et al. 2024). The protein kinase activity of ERN1 also plays an important role in ERN1 signaling and controls the expression of many genes (Auf et al. 2013; Minchenko et al. 2015, 2019, 2020). Recently, it was shown that the protein kinase activity of ERN1 plays an important role in controlling the expression of homeobox genes associated with glioblastoma cell proliferation and invasion and that ERN1 knockdown increases their expression, although the proliferation of these cells is inhibited (Minchenko et al. 2024a).

There are 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, 2024b; Krasnytska et al. 2023; Sliusar et al. 2023). Hypoxic regulation of gene expression is realized preferentially through different mechanisms in a gene-specific manner (Minchenko and Caro 2000; Minchenko et al. 2002, 2004, 2021; Sun and Denko 2014; Semenza 2017). ER stress may modify hypoxic gene expression regulations by specific interaction of stress-signaling proteins with transcription factor HIF.

Thus, the ER stress and hypoxia as well as glucose and glutamine supply are 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 CPE, especially after suppression of glioblastoma cell proliferation by ERN1 knockdown. In this study, we are showing that the expression level of the *CPE* gene is strongly up-regulated by ERN1 knockdown of glioblastoma cells through protein kinase activity of ERN1 and possibly contributed to increased invasiveness of these cells.

Material and Methods

Cell lines and culture conditions. The human glioblastoma cells U87MG were grown 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 wild-type U87MG glioblastoma cells and three sublines of these cells described previously (Auf et al. 2013). One subline 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 a lower proliferation rate and did 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 did not have the phosphorylated isoform of ERN1 after induction of ER stress by tunicamycin (Auf et al. 2013). All three sublines of glioblastoma cells used in this study were grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium as described previously (Auf et al. 2013). All three sublines of U87MG glioblastoma cells were grown in the presence of geneticin (G418), while these cells carrying empty vector pcDNA3.1 or dominant/negative ERN1 constructs. Hypoxia was induced 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, the cells were cultured in DMEM without glucose and glutamine, respectively, for 16 h. The glioblastoma cells with inhibited endoribonuclease activity of ERN1 with tunicamycin (500 ng/ml) were used to clarify the role of other signaling pathways of ER stress all in the control of *CPE* gene expression.

Small interfering RNA knockdown experiments. The ERN1 mRNA in U87MG glioblastoma cells was silenced with a small interfering RNA (siRNA) as described previously (Auf et al. 2013). The ON-TARGETplus SMARTpool siRNA J-004951-(19-22) against human ERN1 (catalog: L-0049251-02-0005) and control siRNA (ON-TARGETplus Control Pool, Non-Targeting pool; catalog: D-001810-10-05) were 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.) according to 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. RNA concentration and spectral characteristic were measured using NanoDrop Spectrophotometer ND1000 (PEQLAB, Biotechnologie GmbH).

Reverse transcription and quantitative PCR analysis. The expression levels of CPE 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 previously (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 *CPE* and ERN1 genes was received from Sigma-Aldrich (St. Louis, MO, U.S.A.) and used for quantitative polymerase chain reaction: CPE forward 5'-atgggaatgaggctgttgga and CPE reverse 5'-attgcttcgacccacaaacc (NM_001873.4); ERN1 forward 5'-ccatgccgaagttcagatgg and ERN1 reverse 5'-gtgaggccgcatagtcaaag (NM_001433.5). Primers for ACTB have been described previously (Minchenko et al. 2024a).

Statistical analysis. 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). 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 *CPE* gene expression in U87MG glioblastoma cells was evaluated. The expression level of the *CPE* gene was strongly up-regulated (+542%; $p<0.001$) in glioblastoma cells with suppressed both enzymatic activities of ERN1 (protein kinase and endoribonuclease) in comparison to control (transfected by empty vector)

cells (Figure 1). At the same time, no significant changes were detected in the expression of the *CPE* gene in U87MG cells with inhibited endoribonuclease activity of ERN1 (Figure 1). Next, we studied the expression of the *CPE* gene in glioblastoma cells after silencing of ERN1 mRNA. As shown in Figure 2, the expression level of *CPE* gene is strongly increased (+502%; $p<0.001$) in glioblastoma cells after 48 h of ERN1 silencing, while the level of ERN1 mRNA was effectively suppressed (-87%; $p<0.001$). We also studied the impact of tunicamycin on the expression of *CPE* gene in glioblastoma cells with inhibited only endoribonuclease activity of ERN1 to clarify the role of other signaling pathways of ER stress in the control of *CPE* gene expression in cells without ERN1 endoribonuclease. We showed that tunicamycin did not significantly alter *CPE* gene expression in these glioblastoma cells indicating the absence of possible involvement of other ER stress signaling pathways in the control of *CPE* gene expression by ER stress (Figure 3).

We evaluated the effect of hypoxia on the expression of the *CPE* 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 *CPE* gene expression is resistant to hypoxia in control glioblastoma cells but up-regulated in cells with ERN1 knockdown (+39%; $p<0.01$) as compared to control 2 (normoxic dnERN1 cells).

Furthermore, we also studied the impact of glucose deprivation on *CPE* gene expression in control cells and cells with suppressed both enzymatic activities of ERN1. As shown in Figure 5, the level of *CPE* gene expression is decreased in control glioblastoma cells exposure under glucose deprivation condition for 16 h (-22%; $p<0.05$) in comparison with control 1, but inhibition of both enzymatic activities of ERN1 increased the sensitivity of this gene expression to glucose deprivation (-37%; $p<0.001$) as compared to control 2. Glutamine deprivation condition led to up-regulation of *CPE* gene expression (+36%; $p<0.01$) in control glioblastoma cells and its down-regulation (-15%; $p<0.05$) in cells with ERN1 knockdown (Figure 6). The exposure of control glioblastoma cells under glutamine deprivation for 16 h introduced different changes in the expression of the *CPE* gene in control glioblastoma cells and in ERN1 knockdown cells indicating dependence of *CPE* gene expression under this deprivation condition from enzymatic activity of ERN1.

Discussion

The results of this study are summarized in Figure 7. The major finding reported here is that

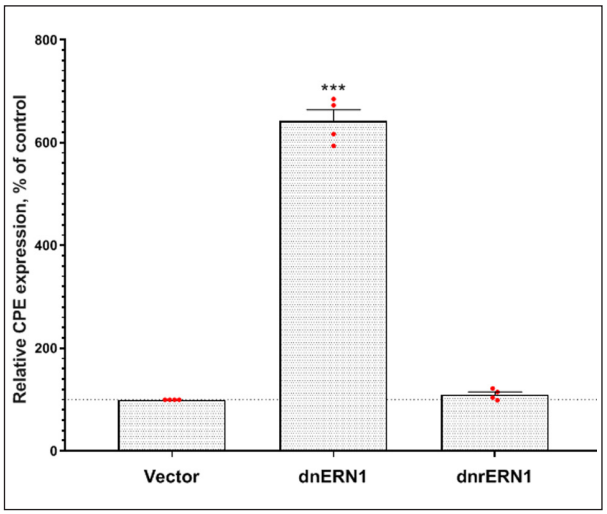

Figure 1. The expression of carboxypeptidase E (CPE) in control U87MG glioblastoma cells (transfected by an empty vector; Vector), cells with suppressed endoribonuclease and protein kinase activities of ERN1 (dnERN1) or only endoribonuclease activity of ERN1 (dnrERN1) measured by quantitative RT-PCR. The values of the mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control (Vector, 100%); mean±SEM; ***$p<0.001$ vs. controls (Vector).

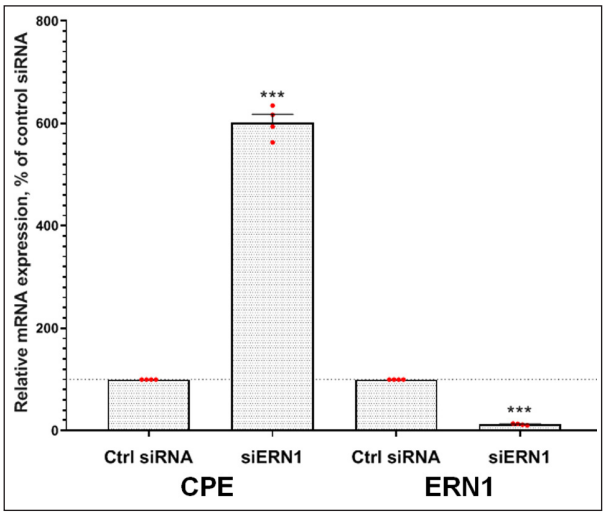

Figure 2. The impact of ERN1 (endoplasmic reticulum to nucleus signaling 1) mRNA silencing by specific for ERN1 siRNA (48 h) on the expression of carboxypeptidase E (CPE) and ERN1 mRNAs in wild-type glioblastoma cells (quantitative RT-PCR analysis). The values of mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control (Ctrl siRNA; 100%); mean±SEM; ***$p<0.001$ vs. controls (Ctrl siRNA).

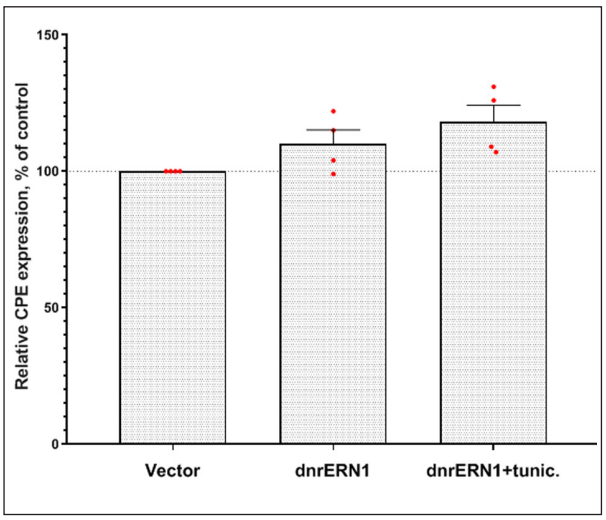

Figure 3. The impact of tunicamycin (500 ng/ml for 4 h) on the expression of carboxypeptidase E (CPE) mRNA in glioblastoma cells without endoribonuclease activity of ERN1 (dnrERN1) measured by quantitative RT-PCR. The values of CPE mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control (Vector; 100%); mean±SEM.

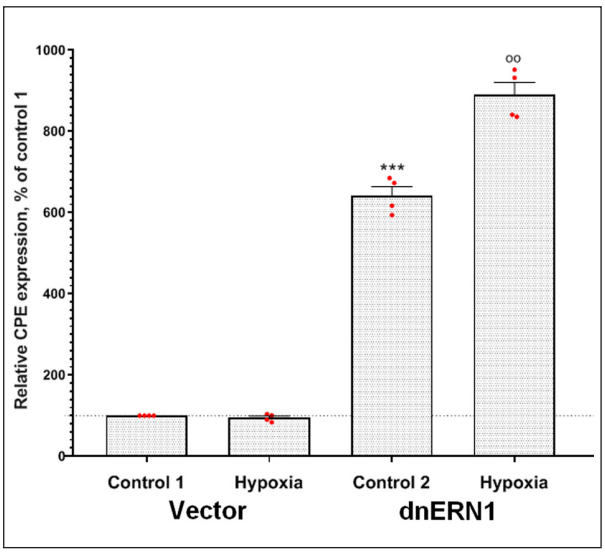

Figure 4. The impact of hypoxia on the expression of carboxypeptidase E (CPE) mRNA in control glioblastoma cells (Vector) and cells with ERN1 (endoplasmic reticulum to nucleus signaling 1) knockdown (dnERN1) measured by qPCR. The values of mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 (Vector; 100%). The impact of hypoxia in ERN1 knockdown cells was compared to control 2; mean±SEM; ***p<0.001 vs. control 1; ^{oo}p<0.01 vs. control 2.

the expression of the gene encoding the CPE does not change significantly in U87MG glioblastoma cells with inhibited ERN1 endoribonuclease, but is strongly up-regulated in cells with suppressed both enzymatic activities of ERN1 (protein kinase and endoribonuclease). These results indicate that ERN1 protein kinase plays an important role in the ERN1-dependent regulation of *CPE* gene expression and agree well with previously reported data concerning involvement of ERN1 protein kinase in the control of gene expression (Auf et al. 2013; Minchenko et al. 2015, 2019, 2020, 2024a).

The expression level of the *CPE* gene is significantly increased in glioblastoma cells after the effective silencing of ERN1. These results also support the ERN1-dependent mechanism of *CPE* gene expression regulation. Recently, it was shown that the protein kinase activity of ERN1 plays an important role in controlling the expression of homeobox genes associated with glioblastoma cell proliferation and invasion and that ERN1 knockdown increases their expression although the proliferation of these cells is inhibited (Minchenko et al. 2024a). It is worth noting that inhibition of both enzymatic activities of ERN1 inhibits the proliferation of glioblastoma cells to a lesser extent than inhibition of one endoribonuclease (Minchenko et al. 2015). This could be explained by the induction of some pro-oncogenic genes under

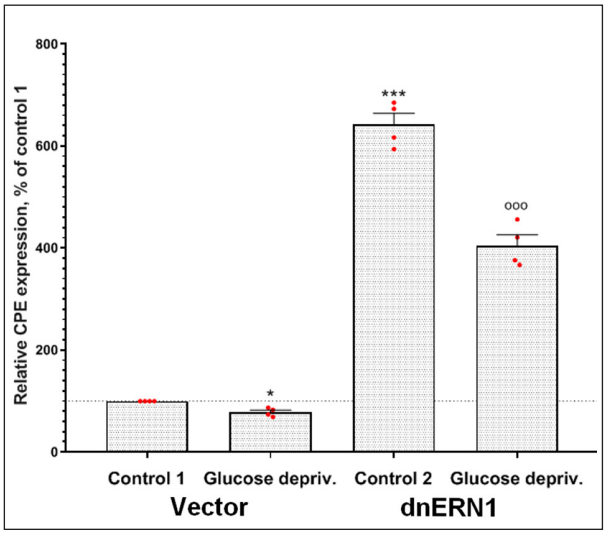

Figure 5. The impact of glucose deprivation on the expression of carboxypeptidase E (CPE) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (endoplasmic reticulum to nucleus signaling 1) (dnERN1) measured by qPCR. The values of CPE mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 (Vector; 100%). The impact of glucose deprivation in ERN1 knockdown cells was compared to control 2; mean±SEM; *p<0.05 and ***p<0.001 vs. control 1; ^{ooo}p<0.001 vs. control 2.

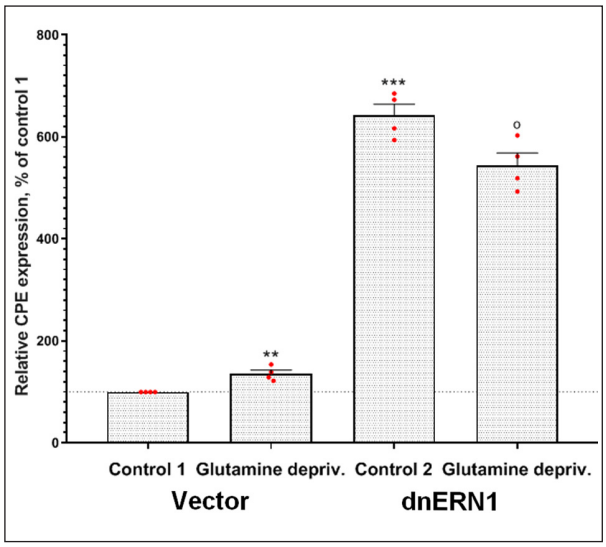

Figure 6. The impact of glutamine deprivation on the expression of carboxypeptidase E (CPE) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (endoplasmic reticulum to nucleus signaling 1) (dnERN1) measured by qPCR. The values of this mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 (Vector; 100%). The impact of glutamine deprivation in ERN1 knockdown cells was compared to control 2; mean±SEM; **p<0.01 and ***p<0.001 vs. control 1; °p<0.05 vs. control 2.

inhibition of ERN1 protein kinase (Minchenko et al. 2024a). However, inhibiting the protein kinase of ERN1 with a specific inhibitor suppresses glioblastoma growth (Pelizzari-Raymundo et al. 2023). It is possible that inhibition of ERN1 protein kinase suppresses the expression of proliferation-related genes, but increases some invasion-responsible pro-proliferative genes.

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, 2021). We have shown that hypoxia only increases the expression of the *CPE* gene in ERN1 knockdown cells. Thus, this gene expression is controlled by ERN1. There are data indicating that hypoxic regulation of numerous genes expression is significantly modified by ERN1 knockdown and many other factors by different mechanisms in a gene-specific manner (Minchenko and Caro 2000; Semenza 2017; Minchenko et al. 2004, 2019, 2020, 2021). 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

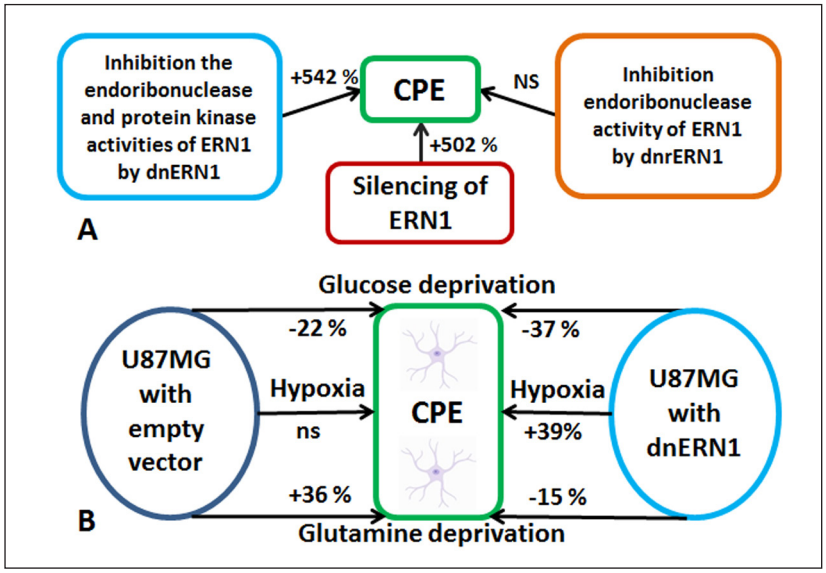

Figure 7. A) Schematic demonstration of the sensitivity of carboxypeptidase E (CPE) mRNA expression to inhibition of endoribonuclease and protein kinase activities of ERN1 (endoplasmic reticulum to nucleus signaling 1) by dnERN1 or only ERN1 endoribonuclease activity by dnrERN1 and by silencing of ERN1 mRNA (48 h). **B)** Schematic demonstration of the impact of hypoxia (Hx), glutamine, and glucose deprivations on the expression of CPE mRNA in control U87MG glioblastoma cells (with empty vector) and cells with a deficiency of both ERN1 protein kinase and endoribonuclease (with dnERN1).

its activity (Minchenko and Caro 2000; Semenza 2017).

The results of this study, regarding the dependence of *CPE* gene expression on ERN1 knockdown under glucose and glutamine deprivations, are in good agreement with our previous data on ERN1-dependent expression of numerous genes under similar nutrient deprivation conditions in a gene-specific manner (Minchenko et al. 2019, 2020, 2024b; Krasnytska et al. 2023). A decrease in the level of *CPE* gene expression under glucose deficiency may be a consequence of a decrease in the need for insulin, which is achieved by a decrease in the intensity of proinsulin processing, in which CPE is involved (Chen et al. 2023).

Conclusions. The data presented in this study identify the ERN1 protein kinase-dependent mechanism of the regulation of CPE expression in U87MG glioblastoma cells, which is a multi-functional protein that plays an important role in the biosynthesis of neurotransmitters and peptide hormones, promotes neuronal survival, and may participate in the cell proliferation control and invasion to promote tumorigenesis. Furthermore, the expression of the *CPE* gene is up-regulated in ERN1 knockdown glioblastoma cells and may contribute to the increased invasiveness of these cells. However, the detailed molecular mechanisms of the interaction of the ERN1-mediated signaling pathways with tumor cell proliferation and invasiveness are complex and warrant further investigation.

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

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