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The ERN1 signaling pathway of unfolded protein controls the expression of EDEM1 and its hypoxic regulation in glioblastoma cells

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Objective. For the effective growth of malignant tumors, including glioblastoma, the necessary factors involve endoplasmic reticulum (ER) stress, hypoxia, and the availability of nutrients, particularly glucose. The ER degradation enhancing alpha-mannosidase like protein 1 (EDEM1) is involved in ER-associated degradation (ERAD) targeting misfolded glycoproteins for degradation in an N-glycan-independent manner. EDEM1 was also identified as a new modulator of insulin synthesis and secretion. The present study aims to investigate the regulation of the *EDEM1* gene expression in U87MG glioblastoma cells by hypoxia and glucose or glutamine deprivations depending on the knockdown of ERN1 (endoplasmic reticulum to nucleus signaling 1) with the intent to reveal the role of ERN1 signaling in the regulation of this gene expression and function in tumorigenesis.

Methods. The U87MG glioblastoma cells (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 (4 h). For glucose and glutamine deprivations, the cells were exposed to DMEM medium without glucose and glutamine, respectively, for 16 h. The expression level of the *EDEM1* gene was studied by quantitative RT-PCR and normalized to the ACTB mRNA.

Results. It was found that inhibition of endoribonuclease and protein kinase activities of ERN1 led to down-regulation of *EDEM1* gene expression in glioblastoma cells. Moreover, the expression of this gene was also decreased after silencing ERN1 in glioblastoma cells. At the same time, the expression of *EDEM1* gene did not significantly change in cells with inhibited ERN1 endoribonuclease only. The expression of the *EDEM1* gene was increased under hypoxia in control U87MG cells, but resistant to hypoxia in cells with ERN1 knockdown. Furthermore, the expression of this gene was up-regulated under glucose and glutamine deprivations in control glioblastoma cells. However, the ERN1 knockdown increased the sensitivity of *EDEM1* gene expression to glucose and decreased to glutamine deprivations.

Conclusion. The results of the present study demonstrate that inhibition of ERN1 down-regulated the expression of the *EDEM1* gene through protein kinase activity of ERN1 and that the regulation of this gene expression by hypoxia and nutrient supply, especially glucose, is differently controlled by ERN1 in glioblastoma cells.

Keywords: EDEM1, gene expression, ERN1 knockdown, ERN1 protein kinase, hypoxia, nutrient deprivation, glioblastoma cells

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Endoplasmic reticulum (ER) degradation enhancing alpha-mannosidase like protein 1 (EDEM1) is a quality control factor for folded glycoproteins and plays an important role in the degradation of misfolded glycoproteins in an N-glycan-independent manner in ubiquitin-dependent ER-associated degradation (ERAD) process (Oda et al. 2003; Olivari et al. 2005; Roth and Zuber 2017; Chiritoiu et al. 2020; Nowakowska-Golacka et al. 2021; Ghenea et al. 2022). The N-terminal disordered region of EDEM1 mediates the protein-protein interaction with misfolded proteins, whilst the absence of this domain significantly impairs their degradation (Chiritoiu et al. 2020). It has been shown that EDEM1 participates in the release of misfolded glycoproteins from the calnexin cycle (Molinari et al. 2003). Moreover, EDEM1 was recently identified as a new modulator of insulin synthesis and secretion in pancreatic β-cells (Flintoaca Alexandru et al. 2023). It also regulates systemic insulin signaling and metabolic homeostasis in Drosophila (Pathak and Varghese 2021). The silencing of EDEM1 increased the bioavailability of ER stress-induced ATF6 export to the Golgi complex through the stabilization of the natively unstable ATF6 protein (Papaioannou et al. 2018). Thus, the EDEM1 enhances ATF6 pro-survival signaling. There are data indicating that EDEM1 controls the effect of glucocorticoids on ER stress and ERAD (Das et al. 2013). It has also been shown that EDEM1 physically associates with epidermal growth factor receptor (EGFR) and enhances the EGFR degradation via ERAD (Miura et al. 2023). It is notable to note that EDEM1 as ERAD accelerating factor is turned over by the ERAD itself (Katsuki et al. 2024).

The ER stress, hypoxia, and nutrients (glucose and glutamine) supply are important factors of glioblastoma growth and its metabolic reprogramming (Denko 2008; Sun and Denko 2014; Almanza et al. 2019; Minchenko et al. 2021; Pelizzari-Raymundo et al. 2024). Glioblastoma cell proliferation and tumor growth *in vivo* are significantly inhibited by the knockdown of ERN1 (ER to nucleus signaling 1) although the invasiveness of these cells is increased (Auf et al. 2010; Logue et al. 2018; Minchenko et al. 2021; Pelizzari-Raymundo et al. 2023; Minchenko et al. 2024b). 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; Minchenko et al. 2021). 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). This transcription factor regulates the expression of chaperons and enzymes for degradation of misfolded proteins and restoration of folding (Acosta-Alvear et al. 2007; Obacz et al. 2017; Doultsinos et al. 2017; Hetz et al. 2020; Pelizzari-Raymundo et al. 2024). It is worth that XBP1s participates in EDEM1-controlled insulin synthesis in pancreatic β-cells (Flintoaca Alexandru et al. 2023). The protein kinase activity of ERN1 also plays an important role in the ERN1 signaling and controls the expression of many genes (Auf et al. 2013; Minchenko et al. 2019, 2020, 2024b). Recently, it has been 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 the 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 preferentially realized through different mechanisms in a gene-specific manner (Minchenko and Caro 2000; Minchenko et al. 2002, 2004, 2021; Sun and Denko 2014; Semenza 2017; Batie and Rocha 2020). The ER stress may modify the hypoxic gene expression regulation by specific interaction of stress-signaling proteins with transcription factor HIF (Minchenko et al. 2021).

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 *EDEM1* gene expression, especially after suppression of glioblastoma cell proliferation by ERN1 knockdown. In this study, we are showing that the expression level of the *EDEM1* gene is down-regulated by ERN1 knockdown of glioblastoma cells through protein kinase activity of ERN1 abolishes the hypoxia-dependent *EDEM1* gene expression, enhances sensitivity to glucose deprivation, and possibly contributes to the proliferation reduction of these cells.

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 wild-type U87MG glioblastoma cells and three sublines of these 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 a lower proliferation rate and do not express a spliced variant of XBP1, a key transcription factor in the ERN1 signaling (Auf et al. 2013; Minchenko et al. 2024b). 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 were grown in the presence of geneticin (G418) as described previously (Auf et al. 2013; Minchenko et al. 2024b). Hypoxia was created by 0.5 mM dimethyloxalylglycine (Sigma-Aldrich, St. Louis, MO, USA) as described previously (Minchenko et al. 2002; Minchenko et al. 2024b) and culture plates were exposed for 4 h. For glucose and glutamine deprivations, cells were cultured in DMEM without glucose and glutamine, respectively, for 16 h. We also exposed the glioblastoma cells with inhibited endoribonuclease activity of ERN1 with tunicamycin (500 ng/ml) to clarify the role of other signaling pathways of ER stress all in the control of *EDEM1* gene expression.

Small interfering RNA knockdown experiments. The ERN1 mRNA in U87MG glioblastoma cells was silenced with small interfering RNA (siRNA) mainly 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) 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 ERN1 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 EDEM1 and ACTB mRNA were measured in control U87MG cells and cells with a deficiency of ERN1 by quantitative PCR 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 *EDEM1* and *ERN1* genes was received from Sigma-Aldrich (St. Louis, MO, USA) and used for quantitative PCR: EDEM1 forward 5'-tggaaacgatatggtgccct and reverse 5'-tctccatccggtcttctgtg (NM_014674.3); ERN1 forward 5'-ccatgccgaagttcagatgg and reverse 5'-gtgaggccgcatagtcaaag (NM_001433.5). Primers for ACTB have been described previously (Minchenko et al. 2024a).

The results of quantitative PCR were analyzed using "Differential Expression Calculator". Statistical analysis of the obtained results was performed 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. In all cases, a value of $p<0.05$ was considered significant. 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).

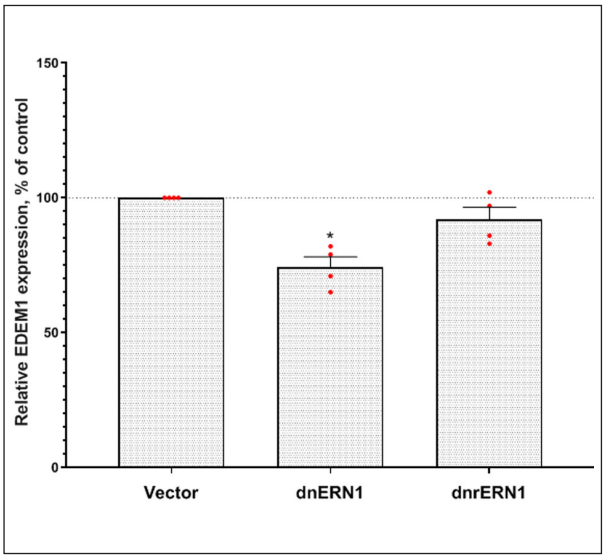

Figure 1. The expression of endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (EDEM1) in control U87MG glioblastoma cells (transfected by an empty vector; Vector), cells with suppressed endoribonuclease and protein kinase activities of ERN1 (endoplasmic reticulum to nucleus signaling 1) (dnERN1) or only endoribonuclease activity of ERN1 (dnrERN1) measured by qPCR. The values of this mRNA expression were normalized to ACTB mRNA and represented as a percent of the control (Vector, 100%); mean±SEM; *p<0.05.

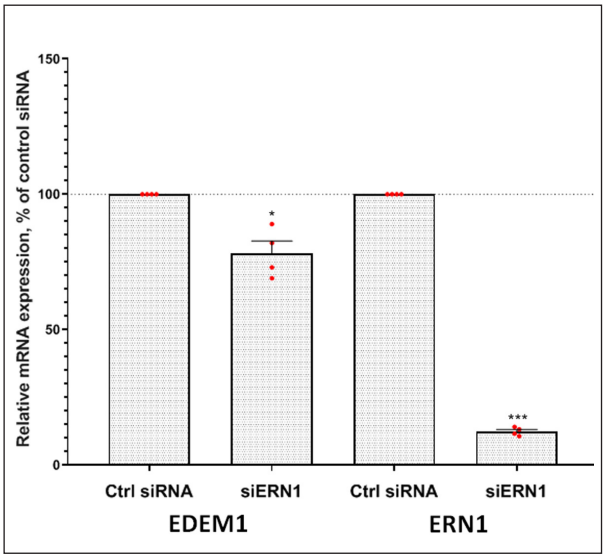

Figure 2. The impact of ERN1 (endoplasmic reticulum to nucleus signaling 1) mRNA silencing by specific for ERN1 siRNA (48 h) on the expression of endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (EDEM1) and ERN1 mRNAs in wild-type glioblastoma cells (qPCR analysis). The values of these mRNA expressions were normalized to beta-actin mRNA and represented as a percent of the control (Ctrl siRNA; 100%); mean±SEM; *p<0.05, ***p<0.001.

Results

First, we studied the impact of ERN1 knockdown on the expression of *EDEM1* gene expression in U87MG glioblastoma cells to evaluate the role of this signaling pathway in the regulation of *EDEM1* expression. The expression level of the *EDEM1* gene was down-regulated (–26%; p<0.05) in glioblastoma cells with suppressed protein kinase and endoribonuclease activities of ERN1 in comparison to transfected by empty vector control cells (Figure 1). At the same time, no significant changes were detected in the expression of the *EDEM1* gene in U87MG cells with inhibited endoribonuclease activity of ERN1 (Figure 1). Next, we studied the expression of the *EDEM1* gene in glioblastoma cells after silencing of ERN1 mRNA. As shown in Figure 2, the expression level of *EDEM1* gene was decreased (–22%; p<0.05) 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 *EDEM1* 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 *EDEM1* gene expression in cells without ERN1 endoribonuclease. We showed that tunicamycin strongly increased the expression of the *EDEM1* gene in these glioblastoma cells, indicating the involvement of other ER stress signaling pathways in the control of *EDEM1* gene expression by ER stress (Figure 3).

We also investigated the impact of hypoxia introduced by dimethyloxalylglycine on the expression of the *EDEM1* 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 *EDEM1* gene expression is up-regulated by hypoxia (+28%; p<0.01) in control glioblastoma cells, but was resistant to hypoxia in cells with ERN1 knockdown as compared to control 2 (normoxic dnERN1 cells).

Finally, we studied the impact of glucose deprivation on *EDEM1* gene expression in control cells and cells with suppressed both enzymatic activities of ERN1. As shown in Figure 5, the level of *EDEM1* gene expression was increased in control glioblastoma cells cultured under glucose deprivation conditions for 16 h (+52%; p<0.001) in comparison to control 1, but inhibition of both enzymatic activities of ERN1 strongly increased the sensitivity of this gene expression to glucose deprivation (+117%; p<0.001)

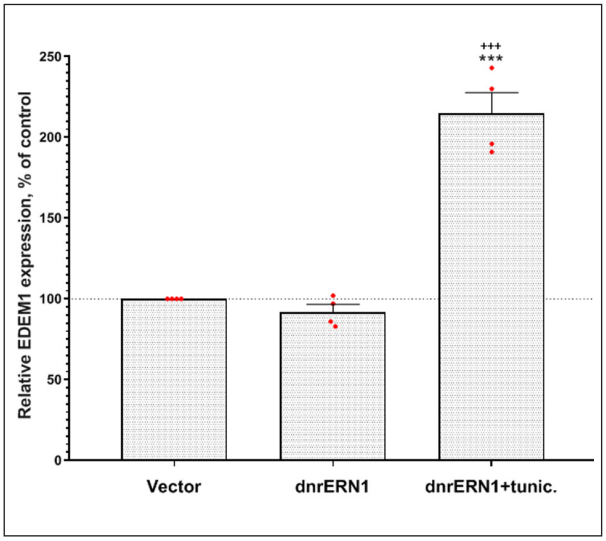

Figure 3. The impact of tunicamycin (500 ng/ml for 4 h) on the expression of endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (EDEM1) mRNA in glioblastoma cells without endoribonuclease activity of ERN1 (endoplasmic reticulum to nucleus signaling 1) (dnrERN1) measured by quantitative qPCR. The values of EDEM1 mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control (Vector; 100%); mean±SEM; ***p<0.001 vs. control (vector); $^{+++}$p<0.001 vs. dnrERN1.

as compared to control 2. Glutamine deprivation conditions lead to a strong up-regulation of *EDEM1* gene expression (+83%; p<0.001) in control glioblastoma cells and slightly lower increase (+55%; p<0.01) in cells with ERN1 knockdown (Figure 6). Thus, the exposure of control glioblastoma cells under glucose or glutamine deprivation for 16 h introduced different changes in the expression of the *EDEM1* gene in control glioblastoma cells and in ERN1 knockdown cells, indicating that *EDEM1* gene expression under these deprivation conditions depends on the enzymatic activity of ERN1.

Discussion

The major finding reported here is that the expression of the gene encoding the EDEM1 protein does not change significantly in U87MG glioblastoma cells with inhibited ERN1 endoribonuclease, but is down-regulated in cells with suppressed both enzymatic activities (protein kinase and endoribonuclease) of ERN1. The results of this study are summarized in Figure 7. These results indicate that ERN1 protein kinase plays an important role in the ERN1-dependent regulation of *EDEM1* gene

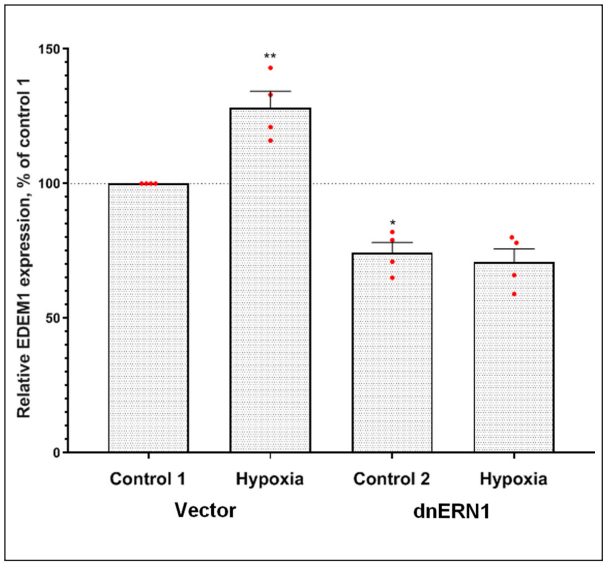

Figure 4. The impact of hypoxia on the expression of endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (EDEM1) mRNA in control glioblastoma cells (Vector) and cells with ERN1 (endoplasmic reticulum to nucleus signaling 1) knockdown (dnERN1) measured by qPCR. The values of this mRNA expression were normalized to beta-actin mRNA and represented as a percent of the control 1 (Vector; 100%). The impact of hypoxia in ERN1 knockdown cells was compared to control 2; mean±SEM; *p<0.05 and **p<0.01 vs. control 1.

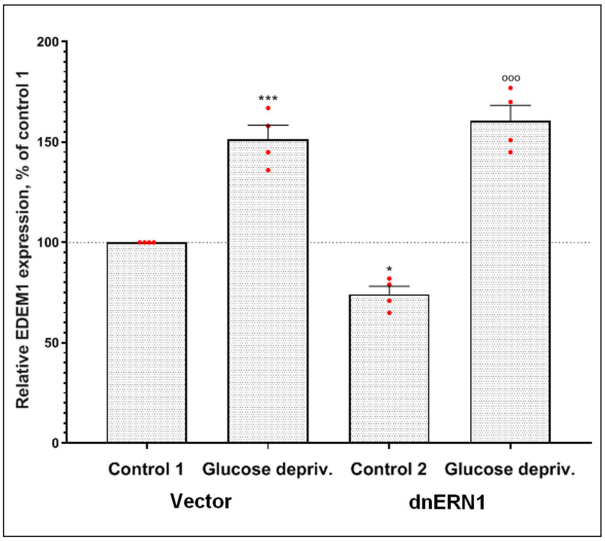

Figure 5. The impact of glucose deprivation on the expression of endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (EDEM1) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (endoplasmic reticulum to nucleus signaling 1) (dnERN1) measured by qPCR. The values of EDEM1 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.

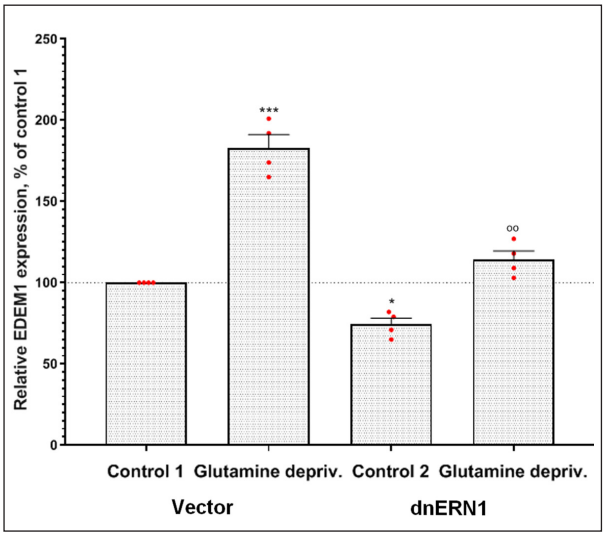

Figure 6. The impact of glutamine deprivation on the expression of endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (EDEM1) mRNA in control glioblastoma cells and cells with a deficiency of both enzymatic activities of ERN1 (endoplasmic reticulum to nucleus signaling 1) (dnERN1) 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<0.05 and ***p<0.001 vs. control 1; ^{oo}p<0.01 vs. control 2.

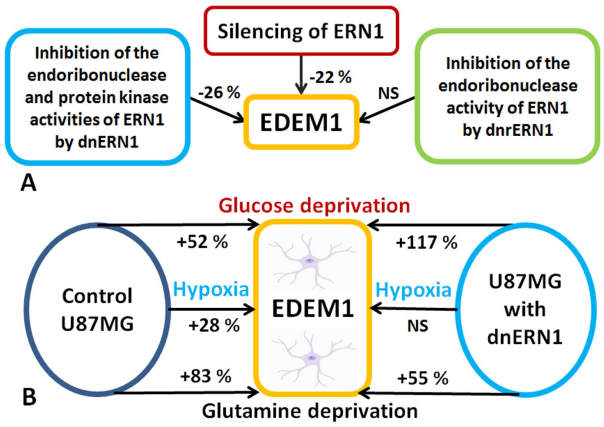

Figure 7. Schematic demonstration of the sensitivity of endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (EDEM1) mRNA expression to inhibition of endoribonuclease and protein kinase activities of ERN1 (endoplasmic reticulum to nucleus signaling 1) by dnERN1 or only ERN1 endoribonuclease activity by dnrERN1 and by silencing of ERN1 mRNA (48 h) **(A)**. Schematic demonstration of the impact of hypoxia (Hx), glucose, and glutamine deprivations on the expression of EDEM1 mRNA in control U87MG glioblastoma cells (with empty vector) and cells with a deficiency of both ERN1 protein kinase and endoribonuclease (with dnERN1) **(B)**.

expression and agree well with previously reported data concerning the involvement of ERN1 protein kinase in the control of gene expression (Auf et al. 2013; Minchenko et al. 2015, 2019, 2020, 2024a). Moreover, ERN1 protein kinase activity-mediated changes in gene expression were shown to be both positive and negative. The expression level of the *EDEM1* gene is decreased in glioblastoma cells after the effective silencing of ERN1. These results also support the ERN1-dependent mechanism of *EDEM1* gene expression regulation. Suppressive effect of ERN1 knockdown has been shown for *EREG*, *ATF3*, *IDE*, *PITRM1*, and *PHGDH* genes in glioblastoma cells (Auf et al. 2013; Minchenko et al. 2015, 2019, 2020, 2024b).

Recently, it has also been shown that the protein kinase activity of ERN1 is responsible for up-regulation of *EDN1*, *PBX3*, *PRRX1*, *PAX6*, *PBXIP1*, and *SHMT1* genes, which play an important role in controlling the cell proliferation and invasion (Minchenko et al. 2019, 2024a). It is worth to note 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, 2024b). This could be explained by the induction of some pro-oncogenic genes under inhibition of ERN1 protein kinase (Minchenko et al. 2024a). However, inhibiting the protein kinase of ERN1 with a specific inhibitor also suppresses the glioblastoma growth (Pelizzari-Raymundo et al. 2023). It is possible that inhibition of ERN1 protein kinase suppresses the expression of proliferation-related genes like *EREG*, *ATF3*, *PHGDH*, and *EDEM1*, 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 showed that hypoxia increases *EDEM1* gene expression only in control glioblastoma cells. Knockdown of ERN1 eliminates the hypoxic regulation of the expression of this gene. Thus, this gene expression is controlled by ERN1. Data have shown that hypoxic regulation of numerous gene expressions 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). The ERN1 may modify the impact of hypoxia on the expression of genes via genome specific changes in the additional regulatory

factors, which interact with HIF and modulate its activity (Minchenko and Caro 2000; Semenza 2017; Batie and Rocha 2020).

In this work, we showed that the expression of the EDEM1 is increased in glioblastoma cells under glucose and glutamine deficiency in ERN1-dependent manner. These results are in good agreement with our previous data on ERN1-dependent expression of numerous genes under glucose and glutamine deprivation conditions in a gene-specific manner (Minchenko et al. 2019, 2020, 2024b, 2024c; Krasnytska et al. 2023).

In conclusion, the data presented in this study identify the ERN1 protein kinase-dependent mechanism in the regulation of *EDEM1* expression in U87MG glioblastoma cells, which is a multifunctional protein that plays an important role in the quality control factor of folded glycoproteins and the degradation of misfolded glycoproteins, controls insulin synthesis, and the effect of glucocorticoids on ER stress and ERAD. However, the detailed molecular mechanism of the interaction of EDEM1 with ERN1-mediated signaling pathways warrants further study.

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

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