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The ERN1-Dependent Impact of Glucose Deprivation on TOB1, E2F1, CCNH, TWIST1, CPT2, and HBEGF Gene Expressions in U87MG Glioblastoma Cells

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Abstract

We studied the impact of glucose deprivation on E2F1, TOB1, CCNH, TWIST1, CPT2, and HBEGF gene expressions in U87MG glioblastoma cells in response to the inhibition of ERN1 enzymatic activities. It was shown that the expression level of TOB1, CPT2, and E2F1 genes was down-regulated when control glioblastoma cells were treated by glucose deprivation. At the same time, the ERN1 knockdown modified the sensitivity of all these genes to decreased glucose supply. At the same time, the expression of HBEGF, TWIST1, and CCNH genes in control glioblastoma cells was resistant to glucose deprivation conditions. However, inhibition of enzymatic activities of ERN1 signaling protein strongly increased the impact of glucose deprivation on HBEGF and CCNH gene expressions but down-regulated the expression of the TWIST1 gene. These results demonstrated that enzymatic activities of signaling protein ERN1 control the sensitivity of all studied genes to glucose deprivation in U87MG glioblastoma cells in a gene-specific manner. This is important for elucidating the endoplasmic reticulum stress-mediated sensitivity of key regulatory gene expression in glioblastoma cells to glucose supply.

Keywords: glucose deprivation; ERN1 knockdown; HBEGF; TOB1; mRNA expression; U87MG glioblastoma cells

Introduction

Glioblastoma, like other malignant tumors, uses the endoplasmic reticulum stress response to adapt and enhance cell proliferation under stressful environmental conditions, including low glucose and other nutrients (Colombo et al. 2011; Bravo et al. 2013; Guo et al. 2016; Almanza et al. 2019). It is well known that activation of the ERN1 branch of the endoplasmic reticulum stress response is tightly linked to cell proliferation, apoptosis, and cell death, and that inhibition of its function has been demonstrated to result in a significant anti-proliferative effect in glioblastoma growth (Auf et al. 2010, 2013; Gu et al. 2012; Logue et al. 2018; Hetz et al. 2019; Minchenko et al. 2021b, 2024a). Glucose is the most abundant amino acid in tissues and represents an essential substrate for tumor cell metabolism as a substrate for glycolysis. Reliance on glucose supply has been considered a hallmark of metabolism in many cancer cells, but the requirements for glucose in different cancers are heterogeneous (Gatenby et al. 2007; Denko et al. 2008; Colombo et al. 2011; Gu et al. 2012; Cluntun et al. 2017; Ediriweera and Jayasena 2023). Glucose and glutamine supply is important for glioblastoma development and a more aggressive behavior (Guo et al. 2016; Kodama et al. 2020; Yoo et al. 2020; Minchenko et al. 2021a, 2023; Bhowmick et al. 2023; Ediriweera and Jayasena 2023; Fasoulakis et al. 2023; Li et al. 2023). There is mounting evidence that there is a link between glucose metabolism and the proliferation of cancer cells, demonstrating that glucose metabolism is a vital mechanism for all cellular processes, including the development of cancer (Minchenko et al. 2002; Denko et al. 2008; Buono and Longo 2018). It is interesting to note that glucose deprivation as well as endoplasmic reticulum stress are significant and complementary factors for tumor growth and that ERN1/IRE1 (endoplasmic reticulum to nucleus signaling 1/inositol requiring enzyme 1) mediated stress signaling can significantly modify the effects of glucose deprivation on gene expressions (Minchenko et al. 2013, 2020, 2023, 2024b). Furthermore, ERN1 knockdown also modifies the impact of glucose deprivation on the expression of numerous factors related to insulin and glucocorticoid receptors (Minchenko et al. 2013, 2020, 2021a; Riabovol et al. 2019; Tsymbal et al. 2020).

For analysis of the impact of glucose deprivation on gene expression in U87MG glioblastoma cells in response to the inhibition of ERN1 enzymatic activities, we select genes encoding next important regulatory factors: E2F1, TOB1, CCNH, TWIST1, CPT2, and HBEGF. Heparin binding epidermal growth factor like growth factor (HBEGF) has multiple functions, enables growth factor activity, interacts with **EGFR (epidermal growth factor receptor),** promotes cell population proliferation, and participates in breast cancer metastasis to the brain (Zhou et al. 2014; Wu et al. 2021; Zhang et al. 2024). TOB1 (transducer of ERBB2, 1) has a relation to cancer growth and their radiosensitivity (Sun et al. 2013; Guan et al. 2017; Wang et al. 2020). Transcription factor E2F1 can mediate glioma cell proliferation, migration, invasion, and TP53-dependent apoptosis (Liu et al. 2023, 2024; Zhao et al. 2024). TWIST1 (Twist family bHLH transcription factor 1) gene is overexpressed in multiple human cancers, and the encoded transcription factor promotes tumor cell invasion, chemoresistance, and metastasis, interacting with p53 (Zhao et al. 2017; Peng et al. 2024; Xu et al. 2024). Carnitine palmitoyltransferase 2 (CPT2) is involved in the intramitochondrial synthesis of acylcarnitines from accumulated acyl-CoA metabolites and participates in fatty acid oxidation and tumorigenesis (Rouchen et al. 2023; Zeng et al. 2023). Its expression is consistently down-regulated in most cancer cell lines, which promotes proliferation and inhibits apoptosis through the p53 pathway (Zhang et al. 2021; Liu et al. 2022a,b; Mao et al. 2024). Cyclin H (CCNH) functions as a regulator of CDK kinases, is involved in cell cycle control, and participates in transcriptional regulation, and tumor growth (Mao et al. 2021; Liu et al. 2022c). Therefore, arctigenin suppresses numerous cancer cell growth by inhibiting cellular energy metabolism and induces tumor cell death under glucose deprivation possibly by suppressing the endoplasmic reticulum stress (Awale et al. 2006; Kim et al. 2010; Gu et al. 2012; He et al. 2018). Interestingly, low-glucose also protected primary glial cells but not six different glioblastoma and neuroblastoma cancer cell lines against the chemotherapy (Raffaghello et al. 2008). This study aimed to examine the expression of important regulatory genes in response to glucose deprivation in control and ERN1 knockdown glioblastoma cells to elucidate the role of ERN1 signaling in the sensitivity of glioblastoma cells to glucose deprivation.

Materials and Methods

Cell lines and culture conditions. The glioblastoma cell line U87MG was obtained from ATCC (USA) and grown in high glutamine (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 work we used two sublines of these cells described previously (Auf et al. 2013). One subline was obtained by selecting stable transfected clones with overexpression of "empty" vector pcDNA3.1, which was used for creation of genetic construct dnERN1. This untreated subline of glioblastoma cells (control glioblastoma cells) was used as control 1 in the study of the effect of glucose deprivation on the level of gene expressions. Second subline was obtained by selection of stable transfected clones with overexpression of ERN1 dominant/negative construct (dnERN1), having suppression of both the protein kinase and endoribonuclease activities of this signaling enzyme (Auf et al. 2013). It has been shown that cells with dnERN1 have a lower proliferation rate, do not express spliced XBP1, a key transcription factor in ERN1 signaling, and have not the phosphorylated isoform of ERN1 after induction of endoplasmic reticulum stress by tunicamycin (Auf et al. 2013). Both used in this study sublines of glioblastoma cells are grown in the presence of geneticin (G418) while these cells carrying empty vector pcDNA3.1 or dnERN1 construct. Glucose deprivation condition was created by changing the complete DMEM medium into culture plates on DMEM medium without glucose for 16h.

RNA isolation. Total RNA was extracted from glioblastoma cells using the Trizol reagent according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) as described previously (Auf et al. 2013). RNA concentration and spectral characteristic were measured using NanoDrop Spectrophotometer ND1000 (PEQLAB, Biotechnologie GmbH). Reverse transcription and quantitative PCR analysis. The expression levels of TOB1, HBEGF, TWIST1, E2F1, CPT2, and CCNH mRNAs as well as ACTB mRNA were measured in control glioblastoma cells and cells with a deficiency of ERN1, introduced by dnERN1, 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 (Minchenko et al. 2024a). Polymerase chain reaction was performed in triplicate. The expression of beta-actin mRNA was used as control of analyzed RNA quantity. The pair of primers specific for each studied gene was received from Sigma-Aldrich (St. Louis, MO, U.S.A.) and used for quantitative polymerase chain reaction (Table 1).

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

Gene symbol	Gene name	Primer's sequence	Nucleotide number in sequence	GenBank accession number
CEBPB	CCAAT enhancer binding protein beta	F: 5'- caagaagaccgtggacaagc R: 5'- agctgctccaccttcttctg	993-1012 1163-1144	NM_005194.4
CEBPG	CCAAT enhancer binding protein gamma	F: 5'- aagcacaagacacactgcag R: 5'- ctactgtcctgcattgtcgc	540-559 736-717	NM_001806.4
PTEN	Phosphatase and tensin homolog	F: 5'- actattcccagtcagaggcg R: 5'- gaacttgtcttcccgtcgtg	1344-1363 1559-1540	NM_000314.8
CKS2	CDC28 protein kinase regulatory subunit 2	F: 5'- gtttcattttctgcagcgcg R: 5'- ccaagtctcctccactcctc	71-90 238-219	NM_001827.3
CCNH	Cyclin H	F: 5'- gttcggtgtttaagccagca R: 5'- tgccttctcctgtccaagag	339–358 553–534	NM_001239.4
ACTB	beta-actin	F: 5'- catccgcaaagacctgtacg R: 5'- cctgcttgctgatccacatc	948–967 1165–1146	NM_001101.5

Quantitative PCR analysis was performed using a special computer program "Differential expression calculator" and statistical analysis using GraphPad Prism 8.0.1 program as described previously (Minchenko et al. 2024a). Comparison of two means was performed by the use of two-tailed Student's *t*-test. $p<0.05$ was considered significant in all cases. The values of studied gene expressions were normalized to the expression of beta-actin mRNA and represent as percent of control (100%). All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments. The amplified DNA fragments were also analyzed on a 2.5 % agarose gel and that visualized by SYBR* Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, USA).

Results

To investigate a possible role of endoplasmic reticulum stress response in the control of *E2F1*, *TOB1*, *TWIST1*, *CPT2*, *HBEGF*, and *CCNH* gene expressions in U87MG glioblastoma cells under low glucose conditions we studied the impact of glucose deprivation on the expression of these genes in control glioblastoma cells (transfected by an empty vector) and cells with ERN1 knockdown. As shown in Figure 1, the expression of heparin binding EGF like growth factor (HBEGF) mRNA is resistant to glucose deprivation conditions in control glioblastoma cells in comparison with the cells growing in complete DMEM medium. Furthermore, inhibition of ERN1 signaling enzyme function by dnERN1 is significantly enhanced the sensitivity of *HBEGF* gene expression to this experimental condition. Thus, the level of this gene expression is strongly increased (+359 %) in glioblastoma cells without the enzymatic activities of ERN1 signaling protein as compared to control 2 (Figure 1).

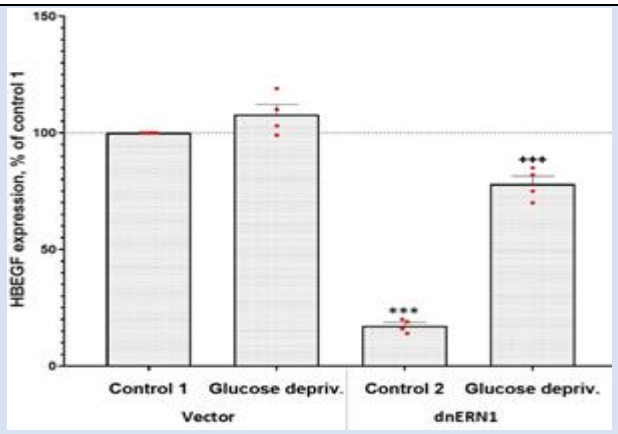

Figure 1: Effect of glucose deprivation on the expression level of heparin binding EGF like growth factor (HBEGF) mRNA in control U87 glioblastoma cells (Vector) and cells with a blockade of the ERN1 (dnERN1) measured by qPCR. Values of this mRNA expression were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4; ***- $p < 0.001$ vs control 1; $^{+++}$- $p < 0.001$ vs control 2.

Next, we investigated the effect of glucose deprivation on the expression of gene encoding cyclin H (CCNH) relative to inhibition of ERN1 function. As shown in Figure 2, this gene expression does not significantly change in control glioblastoma cells exposure under glucose deprivation conditions, but strongly up-regulated (+60 %) in ERN1 knockdown glioblastoma cells in comparison with cells growing in complete medium (control 2).

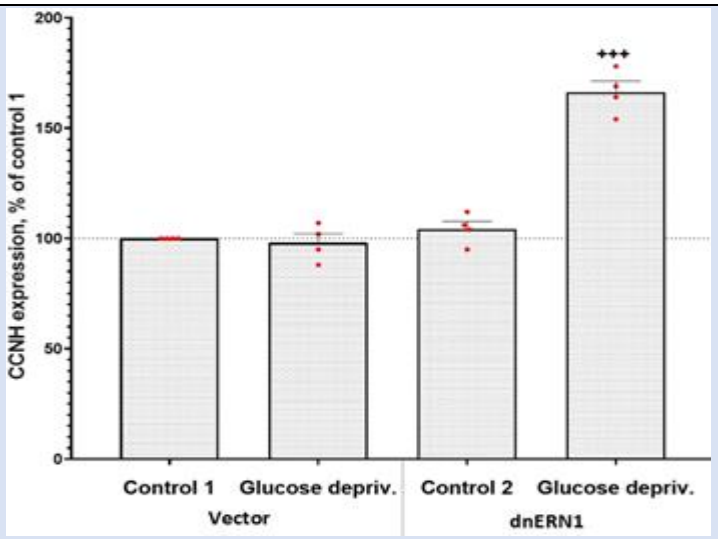

Figure 2: Effect of glucose deprivation on the expression level of cyclin H (CCNH) mRNA in control U87 glioblastoma cells (Vector) and cells with a blockade of the ERN1 (dnERN1) measured by qPCR. Values of this mRNA expression were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4; $^{+++}$- $p < 0.001$ vs control 2.

At the same time, the expression level of other studied genes (*TOB1*, *E2F1*, and *CPT2*) is down-regulated under glucose deprivation in control glioblastoma cells in comparison with cells growing in regular medium except *TWIST1*, which was resistant to this experimental condition (Figures 3-6). As shown in Figure 3, the expression of TOB1 mRNA is down-regulated in control glioblastoma cells treated with glucose deprivation (-57 %). However, the effect of glucose deprivation on this gene expression was significantly lesser in glioblastoma cells with ERN1 knockdown (-21 %) as compared to ERN1 knockdown cells growing with glucose (Figure 3). When studying the expression of the *TWIST1* gene was shown that its expression level does not significantly change in control glioblastoma cells treated by glucose deprivation (Figure 4). At the same time, inhibition of enzymatic activities of ERN1 in glioblastoma cells introduces stronger suppression of *TWIST1* gene expression (-57 %) as compared to corresponding cells growing in a complete medium (Figure 4).

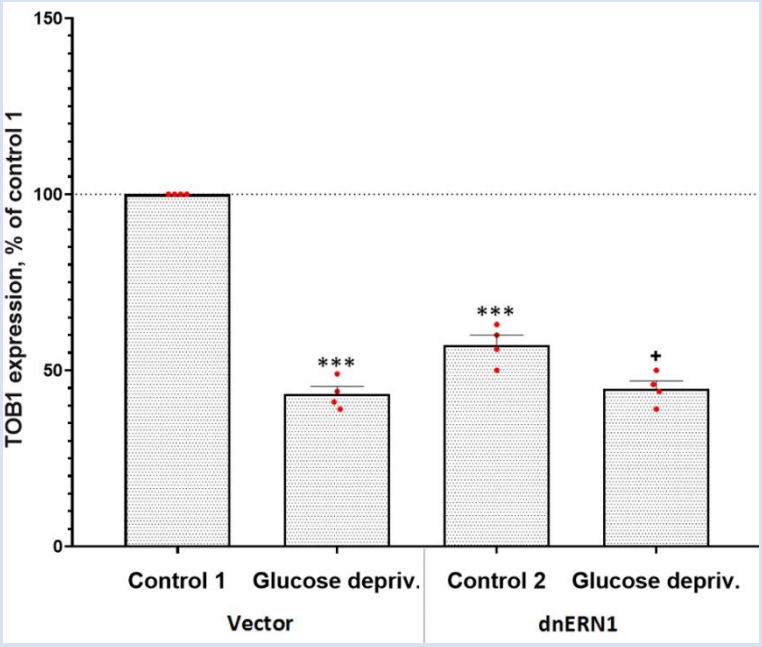

Figure 3: Effect of glucose deprivation on the expression level of transducer of ERBB2, 1 (TOB1) mRNA in control U87 glioblastoma cells (Vector) and cells with a blockade of the ERN1 (dnERN1) measured by qPCR. Values of this mRNA expression were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); *n*=4; ***- $p < 0.001$ vs control 1; $^{+}$- $p < 0.05$ vs control 2.

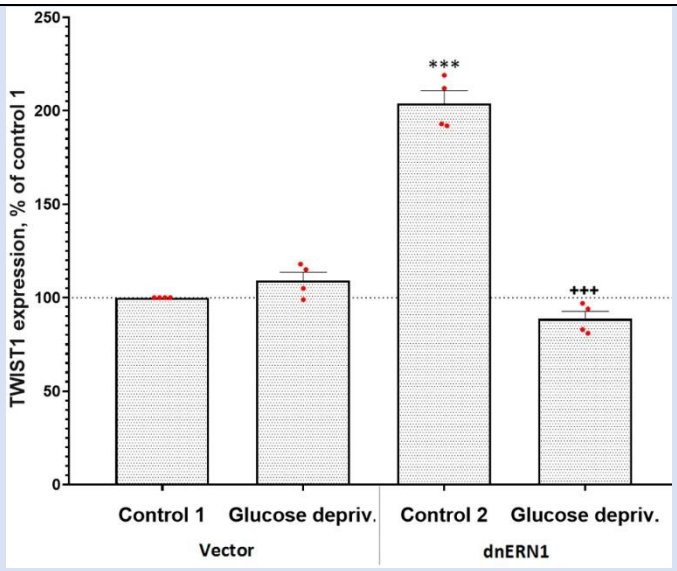

Figure 4: Effect of glucose deprivation on the expression level of TWIST family bHLH transcription factor 1 (TWIST1) mRNA in control U87 glioblastoma cells (Vector) and cells with a blockade of the ERN1 (dnERN1) measured by qPCR. Values of this mRNA expression were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); *n*=4; ***- $p < 0.001$ vs control 1; $^{+++}$- $p < 0.001$ vs control 2.

We also investigated the effect of glucose deprivation on the expression of the E2F1 gene encoding E2F transcription factor 1 in control and ERN1 knockdown U87MG glioblastoma cells. As shown in Figure 5, the expression level of E2F1 mRNA is also down-regulated in control glioblastoma cells (-16 %) and ERN1 knockdown cells (-13 %) treated with glucose deprivation in comparison to the corresponding controls. Thus, inhibition of enzymatic activities of ERN1 signaling protein does not significantly change the sensitivity of E2F1 mRNA expression to glucose deprivation (Figure 5). Furthermore, we have studied the effect of glucose deprivation on the expression of *CPT2* gene encoding carnitine palmitoyltransferase 2 in control glioblastoma cells and cells with inhibited enzymatic activities of ERN1. As shown in Figure 6, exposure of control glioblastoma cells under glucose deprivation conditions leads to down-regulation of the CPT2 mRNA expression (-32 %) in comparison with control

cells growing under condition with glucose. We also found that inhibition of ERN1 enhanced the sensitivity of this gene expression in glioblastoma cells to glucose deprivation (Figure 6).

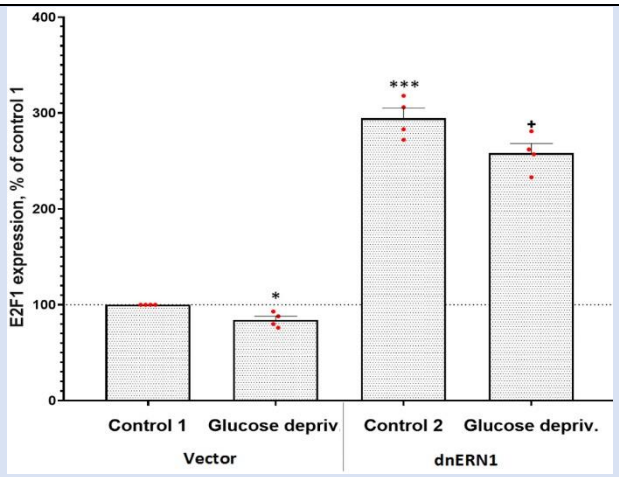

Figure 5: Effect of glucose deprivation on the expression level of E2F transcription factor 1 (E2F1) mRNA in control U87 glioblastoma cells (Vector) and cells with a blockade of the ERN1 (dnERN1) measured by qPCR. Values of this mRNA expression were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4; *- $p < 0.05$ and ***- $p < 0.001$ vs control 1; $^{+}$- $p < 0.05$ vs control 2.

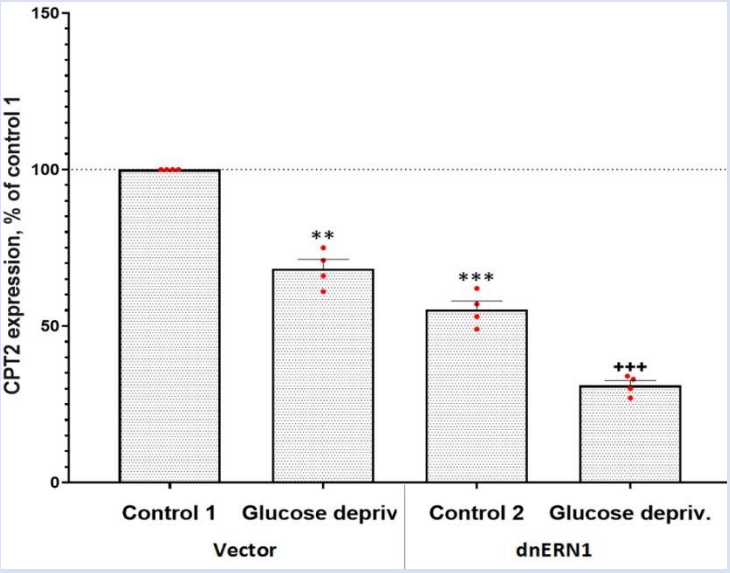

Figure 6: Effect of glucose deprivation on the expression level of carnitine palmitoyltransferase 2 (CPT2) mRNA in control U87 glioblastoma cells (Vector) and cells with a blockade of the ERN1 (dnERN1) measured by qPCR. Values of this mRNA expression were normalized to beta-actin mRNA level and represented as percent for control 1 (100%); n=4; ***- $p < 0.001$ vs control 1; $^{+++}$- $p < 0.001$ vs control 2.

Thus, glucose deprivation affects the expression of *E2F1*, *TOB1*, *TWIST1*, *CPT2*, *HBEGF*, and *CCNH* genes in a gene-specific manner, but the impact of glucose deprivation on most gene expressions depends on ERN1 signaling.

Discussion

In this work, we studied the effect of glucose deprivation on the expression of genes encoding important regulatory proteins in U87MG glioblastoma cells in relation to knockdown of ERN1, the major signaling pathway of the unfolded protein response. For this aim we used control glioblastoma cells, transfected by empty vector pcDNA3.1 and cells with full ERN1 deficiency introduced by dnERN1. This is important for the evaluation of possible significance of ERN1 signaling pathway-dependent control of glioblastoma cell proliferation because endoplasmic reticulum stress signaling mediated by ERN1 is involved in numerous metabolic pathways and knockdown of ERN1 has clear anti-tumor effects (Auf et al. 2010, 2013; Bravo et al. 2013; Logue et al.

2018; Almanza et al. 2019; Minchenko et al. 2021b). Furthermore, there are data that glucose deprivation can enhance the sensitivity of cancer cells to anti-cancer drugs, particularly arctigenin, which inhibits the growth of various cancer cells and induces tumor cell death under glucose deprivation condition possibly by blocking the unfolded protein response and inhibiting cellular energy metabolism (Awale et al. 2006; Kim et al. 2010; Gu et al. 2012; He et al. 2018). Results of our study clarify possible mechanisms of glucose deprivation on the proliferation/surviving of ERN1 knockdown glioblastoma cells through specific changes in the expression of genes encoding important regulatory proteins.

Results of this investigation are summarized in Figure 7, which clearly demonstrates the differential effect of glucose deprivation on the expression of studied genes and ERN1-dependent character of changes in the expression profile of all these genes in glioblastoma cells under glucose deprivation in a gene-specific manner. Therefore, removing of *HBEGF* and *CCNH* gene expression insensitivity to glucose deprivation in glioblastoma cells after ERN1 knockdown may reflect a decreased this tumor cell resistance and proliferation (Almanza et al. 2019; Mao et al. 2021; Minchenko et al. 2021b, 2024a).

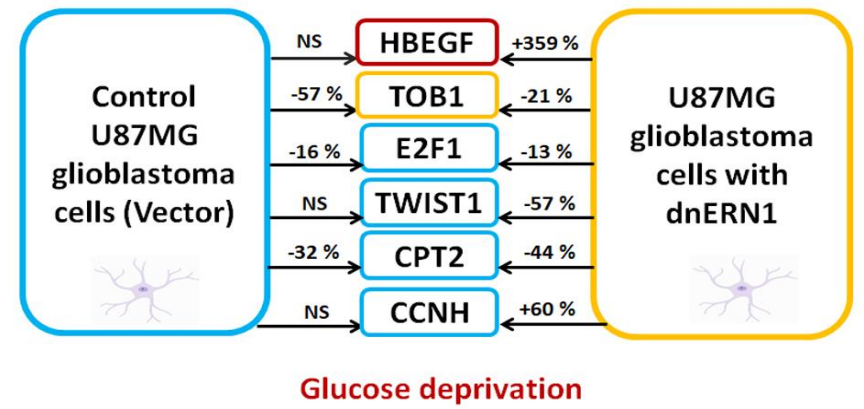

Figure 7: Schematic demonstration of changes in the expression profile of *HBEGF*, *TOB1*, *E2F1*, *TWIST1*, *CPT2*, and *CCNH* genes in the control and ERN1 knockdown (dnERN1) glioblastoma cells under glucose deprivation; NS – no significant changes.

Furthermore, we showed that the expression of transcription factor TWIST1, which has multiple biological functions, is also resistant to glucose deprivation in control glioblastoma cells which have multiple resistance, but strongly suppressed in cells with inhibited enzymatic activities of ERN1 (Figure 7). It agrees well with data Zhao et al. (2017), Peng et al. (2024), and Xu et al. (2024) that transcription factor TWIST1 promotes adenocarcinoma progression and chemoresistance in various cancers. More strong suppression of *CPT2* gene expression in glioblastoma cells with knockdown of ERN1 can also introduce decreased cell proliferation of these cells, which is consistent with the data Liu et al. (2022b) and Mao et al. (2024) about regulation of lactate signals and oxidative phosphorylation.

Thus, this study provides unique insights into the molecular mechanisms regulating the expression of genes encoding important regulatory proteins in glioblastoma cells in response to glucose deprivation and their correlation with inhibition of ERN1 activity and reduced cell proliferation in cells harboring dnERN1, attesting to the fact that endoplasmic reticulum stress as well as glucose supply is a necessary component of malignant tumor growth and cell survival. Furthermore, our results validate tight interaction of endoplasmic reticulum stress signaling pathway ERN1 with glucose supply in the regulation of the expression of genes encoding regulatory proteins, but the detailed molecular mechanisms of this regulation have not been yet clearly defined and requires further investigation.

Declarations

Acknowledgement

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Competing interest

All authors declare that they reviewed the manuscript and have no conflict of interest.

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