

ORIGINAL RESEARCH ARTICLE

ERN1 signaling in the unfolded protein response regulates Ras homolog family member B expression in glioblastoma cells: Effect of hypoxia

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Abstract

Endoplasmic reticulum (ER) stress and hypoxia are essential factors for the effective growth of malignant tumors, including glioblastomas—the most malignant brain tumors, which are difficult to treat. Inhibition of the signaling protein ER to nucleus signaling 1 (ERN1) reduces the proliferation rate of glioblastoma cells by altering the expression of numerous genes. The present study aims to investigate the regulation of tumor suppressor Ras homolog family member B (RHOB) expression by *ERN1* knockdown and hypoxia using genetically modified glioblastoma cells (dnERN1 and dnrERN1) and mRNA silencing. The expression level of the *RHOB* gene was assessed using quantitative polymerase chain reaction. It was found that inhibition of the endoribonuclease activity of *ERN1* increased *RHOB* expression levels. However, a greater induction of *RHOB* expression was observed in dnERN1 cells, with inhibited ERN1 endoribonuclease and protein kinase activities, indicating their involvement in controlling *RHOB* expression. *ERN1* and X-box binding protein 1 mRNA silencing also increased *RHOB* expression levels. The expression of microRNA miR-21-5p and miR-223-3p, which have binding sites in *RHOB* mRNA, was decreased in dnERN1 glioblastoma cells. *RHOB* expression was also increased under hypoxia; however, in dnERN1 glioblastoma cells, the effect of hypoxia was reduced, indicating that ERN1 is involved in this regulation. Furthermore, this gene expression was unchanged by glucose deprivation in control cells; however, it was increased in dnERN1 glioblastoma cells. These findings highlight the significant role of ERN1 protein kinase and endoribonuclease activities in the transcriptional and possibly post-transcriptional regulation of *RHOB* expression.

Keywords: Ras homolog family member B; Gene expression; Endoplasmic reticulum to nucleus signaling 1 knockdown; Endoplasmic reticulum to nucleus signaling 1 protein kinase; Hypoxia; Glucose deprivation; Glioblastoma cells

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1. Introduction

Glioblastoma growth requires the unfolded protein response induced by endoplasmic reticulum (ER) stress and hypoxia. Glucose and glutamine supply are also important factors for tumor growth.¹⁻⁵ Metabolic reprogramming of tumor cells is a fundamental factor that promotes their progression and chemoresistance, which is mediated primarily by ER stress.⁶⁻⁹ Knockdown of ER to nucleus signaling 1 (ERN1), a key signaling protein of the unfolded protein response, significantly inhibited glioblastoma cell proliferation and tumor growth *in vivo*; however, it resulted in enhanced invasiveness of glioma cells.^{10,11}

The signaling protein ERN1 is localized in the ER membrane and has two enzymatic activities in its cytoplasmic domain: protein kinase and endoribonuclease.^{1,3,12} The endoribonuclease activity of *ERN1* initiates the splicing of the pre-mRNA of the transcription factor X-box binding protein 1 (*XBPI*) to form the active transcription factor *XBPI*, which is responsible for the synthesis of hundreds of proteins required for the stress response.^{4,12-16}

The activity of ERN1 protein kinase also participates in ERN1-mediated control of gene expression.¹⁵⁻¹⁹ It is important to note that inhibition of ERN1 protein kinase activity can both repress and activate gene expression.¹⁵⁻²⁰ This activity is responsible for the upregulation of homeobox transcription factors that control both invasion and proliferation. Notably, inhibition of *ERN1* upregulates several pro-oncogenic genes with pro-invasive properties, despite inhibiting the proliferation of glioblastoma cells.¹⁵ Thus, inhibition of the endoribonuclease and protein kinase activities of ERN1 in glioblastoma cells reduces their proliferative activity by affecting the expression of numerous genes; however, some pro-invasive genes are activated, which leads to an increase in the invasiveness of these cells.^{10,15-20} Similar results were obtained when *ERN1* was suppressed by silencing.^{15-17,20} *XBPI*-mediated effects of *ERN1* on gene expression were also investigated by silencing *XBPI* mRNA. The changes observed were similar to those observed upon inhibiting the endoribonuclease activity of *ERN1*.¹⁵⁻²⁰ There is evidence of pharmacological inhibition of ERN1 activity, achieved by targeting either the protein kinase domain or the endoribonuclease domain, resulting in the suppression of glioblastoma growth and an enhancement of the response to chemotherapy.^{8,21-23}

Ras homolog family member B (RHOB) is a Rho-related guanosine triphosphate-binding protein that plays a negative role in tumorigenesis by affecting growth factor signaling in transformed cells.²⁴⁻²⁶ Its expression is downregulated in clinical tumor samples and colon adenocarcinoma cell lines, as well as in other cancer cell lines, including

glioblastoma cells.²⁶⁻²⁹ Thus, decreased expression of the *RHOB* gene is a feature of lung cancer progression and is associated with increased epithelial-to-mesenchymal transition and cell invasiveness.³⁰ Furthermore, it was also found that *RHOB* mediates control of prostate cancer cell proliferation and chemoresistance via the phosphatidylinositol 3-kinase/protein kinase B pathway.³¹ It has been demonstrated that microRNAs (miRNAs) and other factors regulate *RHOB* gene expression in various malignant tumors.^{25-29,32} Thus, miR-19a is overexpressed in human glioblastoma cells, promoting their proliferation and invasion, but suppression of this miRNA expression inhibits both processes.²⁹ In contrast, *RHOB* knockdown decreases the cell proliferation and invasion induced by miR-19a.²⁹ Moreover, miR-223 is significantly increased in colon adenocarcinoma and controls cell proliferation by suppressing *RHOB* gene expression.²⁷ However, inhibition of this miRNA increases the expression level of the tumor suppressor *RHOB* and decreases the proliferation of adenocarcinoma cells.²⁷

Additionally, *RHOB* may play a dualistic role in the context of cancer development and progression, acting as both a tumor suppressor and, in some cancers, as an oncogene.³³ This may be because cell-specific post-translational modifications occur in the C-terminal region of *RHOB*, potentially contributing to the opposing functions of this protein in the tumor microenvironment.³³ Recently, Wang *et al.*³⁴ demonstrated that *RHOB* downregulation suppresses the progression of cervical cancer and enhances the sensitivity of these cancer cells to cisplatin. This was accompanied by an increase in the epithelial marker E-cadherin, while the level of vimentin decreased.

Evidence also indicates that inhibition of *ERN1* significantly modifies hypoxic regulation of key regulatory gene expression in glioblastoma cells.^{18,19,35-38} Hypoxic gene regulation is mediated primarily by the hypoxia-inducible factors (HIFs).³⁹⁻⁴² However, the effect of hypoxia on gene expression is modified by different mechanisms in a gene-specific manner.^{12,43-47} Furthermore, ER stress also modifies the effect of hypoxia on gene expression through crosstalk between ER-stress signaling and HIF-dependent transcription.^{12,35,37} It has been demonstrated that the expression level of numerous genes in glioma cells depends on glutamine and glucose availability, and the sensitivity and nature of the changes also mainly depend on the signaling protein ERN1.⁴⁸⁻⁵¹ It has recently been shown that both glucose and glutamine deficiency disrupt the expression of genes responsible for serine synthesis in a gene-specific manner in glioblastoma cells, with the effect of glutamine deficiency being more pronounced.⁴⁸ Inhibition of ERN1

enzymatic activity markedly enhances the dependence of *PHGDH*, *PSAT1*, *PSPH*, and *SHMT2* gene expression on both glucose and glutamine availability.⁴⁸

Therefore, the unfolded protein response and hypoxia are necessary for the efficient growth of gliomas, reprogramming of their metabolism, and establishment of chemoresistance. However, the interplay of these factors in regulating *RHOB* expression and the consequences of *ERN1* suppression remain unclear. Herein, we demonstrate that inhibition of *ERN1* via both its enzymatic activities upregulates the tumor suppressor *RHOB* in glioma cells, attenuates the effect of hypoxia on *RHOB* expression, and increases sensitivity to glucose deprivation.

2. Materials and methods

2.1. Cell culture

In this study, the U87MG glioblastoma cell line was used, including its genetically modified sublines, namely dn*ERN1* (suppressed for both enzymatic activities of *ERN1*) and dnr*ERN1* (inhibited *ERN1* endoribonuclease)—previously described and characterized in detail to confirm the loss of *ERN1* protein kinase and endoribonuclease activities.¹⁷ Glioblastoma cell sublines with dn*ERN1* and dnr*ERN1* do not express the spliced variant of *XBPI* mRNA, a key transcription factor in *ERN1* signaling, after induction of ER stress with tunicamycin.^{16,17}

Moreover, ER stress induced by tunicamycin leads to phosphorylation of *ERN1* in dnr*ERN1* and control cells, while in dn*ERN1* cells, the phosphorylated form of *ERN1* is not detected.¹⁷ The cells with dn*ERN1* and dnr*ERN1* have a lower proliferation rate, most pronounced in dnr*ERN1* cells.^{16,17} Hypoxia was induced by 0.5 mM dimethylxalylglycine for 4 h (Sigma-Aldrich, United States of America [USA]) as described previously.^{35,52} Effects of glutamine and glucose deprivation on *RHOB* expression were described previously.³⁸ ER stress was induced using tunicamycin (500 ng/mL).³⁸

2.2. Silencing of *ERN1* and *XBPI*

Conditions for silencing *ERN1* and *XBPI* mRNAs have been described in detail in our previous studies.^{15,17}

2.3. RNA extraction

RNA was isolated from cells as described previously.³⁸ After washing with ethanol, the RNA precipitates were dissolved in ribonuclease-free water and used for reverse transcription.

2.4. Reverse transcription and quantitative polymerase chain reaction analysis

The Thermo Scientific Verso cDNA Synthesis Kit (AB

1453A, Thermo Fisher Scientific, USA) was used for reverse transcription of RNA as described previously.¹⁷ The expression of *RHOB*, *ERN1*, *XBPI*, and *ACTB* mRNA was analyzed in glioblastoma cells by quantitative polymerase chain reaction (qPCR) using the Luna Universal qPCR Master Mix (New England Biolabs, USA) and QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). All reactions were performed in triplicate. The expression of *ACTB* mRNA was used as an internal control.

The primers specific for the *RHOB* gene were obtained from Sigma-Aldrich (USA) and were used for qPCR: *RHOB* forward 5'-CGACGTCATTCTCATGTGCT and reverse 5'-CGAGGTAGTCGTAGGCTTGG (NM_004040.4). Primers for *ERN1*, *XBPI*, and *ACTB* were described previously.¹⁶

The levels of miRNAs were determined using qPCR as described previously.¹⁶ For analysis of miRNA expression, a universal qPCR reverse primer and miRNA-specific forward primers were used: 5'-TAGCTTATCAGACTGATGTTGAA (for miR-21-5p) and 5'-TGTCAGTTTGTCAAATACCCCA (for miR-223-3p). miRNA expression levels were standardized to the U6 RNA level and represented as a percentage of control (100%). The qPCR results were evaluated using the differential expression calculator, which represents an Excel sheet established by Livak and Schmittgen.⁵³

2.5. Cell viability

Cell viability was assessed using the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (L3224, Abcam, UK.) according to the manufacturer's protocol. This kit allows the simultaneous identification of live and dead cells directly in culture plates by staining with green-fluorescent calcein-acetoxymethyl ester, indicating intracellular esterase activity, and red-fluorescent ethidium homodimer-1, indicating loss of plasma membrane integrity. Cells were imaged using NIS Elements imaging software (5.21.00 [Build 14583] 64 bit, Nikon, Japan) under a Nikon Eclipse Ti microscope (Nikon, Japan). Live and dead cells were quantified using ImageJ software (1.54p, Wayne Rasband and contributors National Institutes of Health, USA), following the method described by Schneider *et al.*⁵⁴

2.6. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.0.1 software (Dotmatics, USA). Gene expression values were standardized to *ACTB* mRNA expression and expressed as a percentage of control (100%). All data are presented as mean ± standard error of the mean (SEM) from triplicate measurements across four independent experiments. A *p*-value < 0.05 was considered statistically

significant. Normality of qPCR datasets was assessed as described previously.⁵⁵ All datasets showed a normal distribution.

3. Results

3.1. Effect of *ERN1* knockdown on *RHOB* expression in glioblastoma cells

As shown in Figure 1, *RHOB* expression was upregulated by 443% ($p < 0.001$) in dnERN1 cells compared to control cells. In dnrERN1 cells, *RHOB* expression was also increased but to a lesser extent (+236%; $p < 0.001$).

Silencing of *ERN1* mRNA similarly upregulated *RHOB* expression in glioblastoma cells. As shown in Figure 2, *ERN1* mRNA was efficiently silenced (−89%; $p < 0.001$), and *RHOB* expression increased by 377% ($p < 0.001$) in these cells. *RHOB* expression was also elevated by 196% ($p < 0.001$) in glioblastoma cells with *XBPI* mRNA silencing (Figure 3), with *XBPI* silencing being high (−90%; $p < 0.001$).

To examine the contribution of other ER stress signaling pathways to *RHOB* regulation, we tested the

effect of tunicamycin in dnrERN1 cells. The findings revealed that tunicamycin did not significantly alter *RHOB* gene expression in these glioblastoma cells (Figure 4), indicating that other unfolded protein response pathways do not contribute to *RHOB* regulation.

3.2. Suppression of *ERN1* protein kinase and endoribonuclease activities modulates hypoxic regulation of *RHOB* expression

Two housekeeping genes (*ACTB* and *RPS16*) were used as references. As shown in Figure 5, hypoxia strongly upregulated *RHOB* expression in glioblastoma cells expressing native *ERN1* compared to control: +146% ($p < 0.001$) with *ACTB* as a reference (Figure 5A) and +141% ($p < 0.001$) with *RPS16* as a reference (Figure 5B).

In dnrERN1 cells, hypoxia increased *RHOB* expression to a lesser extent: +89% ($p < 0.001$) with *ACTB* (Figure 5A) and +94% ($p < 0.001$) with *RPS16* (Figure 5B) compared to the corresponding control (Control 2). These findings indicate that *ERN1* contributes to hypoxia-induced *RHOB* expression, and its inhibition reduces the effect of hypoxia on this gene.

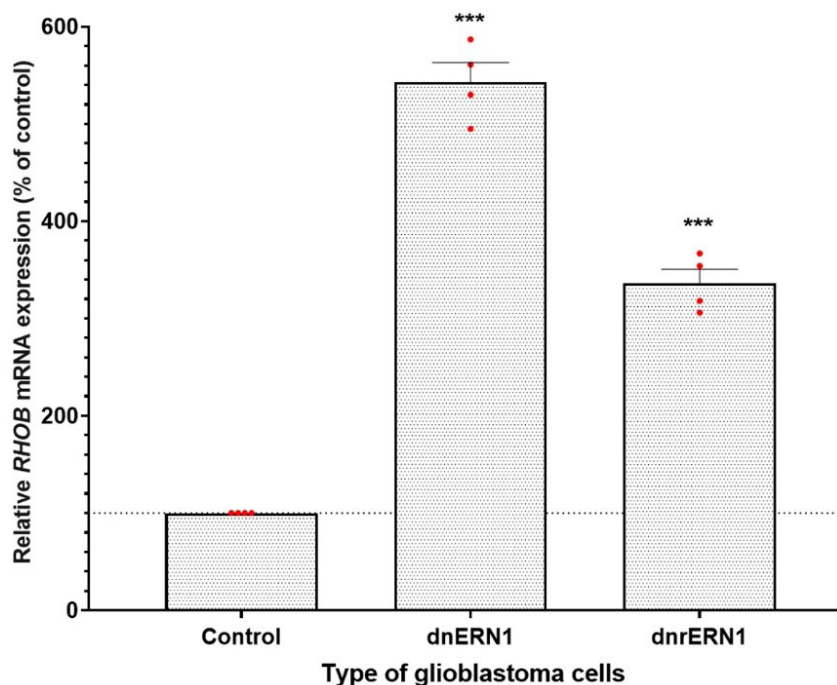


Figure 1. *RHOB* mRNA expression in U87MG glioblastoma cells with altered *ERN1* activity. *RHOB* mRNA levels were measured by quantitative polymerase chain reaction in control U87MG cells (empty vector), dnERN1 cells (with suppressed *ERN1* endoribonuclease and protein kinase activities), and dnrERN1 (with selective suppression of *ERN1* endoribonuclease activity). Expression levels were normalized to *ACTB* mRNA and expressed as a percentage of the control group (set at 100%). Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). *** $p < 0.001$ indicates a statistically significant difference when compared with control.

Abbreviations: *ERN1*: Endoplasmic reticulum to nucleus signaling 1; *RHOB*: Ras homolog family member B.

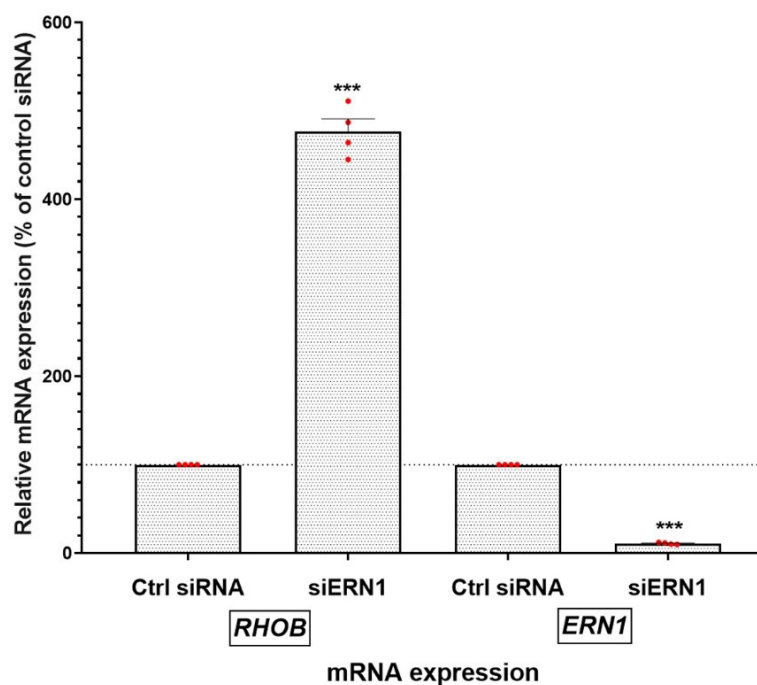


Figure 2. Effect of *ERN1* silencing on *RHOB* and *ERN1* mRNA expression in wild-type glioblastoma cells. *ERN1* mRNA was silenced using human-specific *ERN1* siRNA for 48 h, and *RHOB* and *ERN1* mRNA levels were analyzed by quantitative reverse transcription polymerase chain reaction. Expression levels were normalized to *ACTB* mRNA and expressed as a percentage of the control group (set at 100%). Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). *** $p < 0.001$ indicates a statistically significant difference when compared with Ctrl siRNA. Abbreviations: Ctrl: Control; *ERN1*: Endoplasmic reticulum to nucleus signaling 1; *RHOB*: Ras homolog family member B; siRNA: Small interfering RNA.

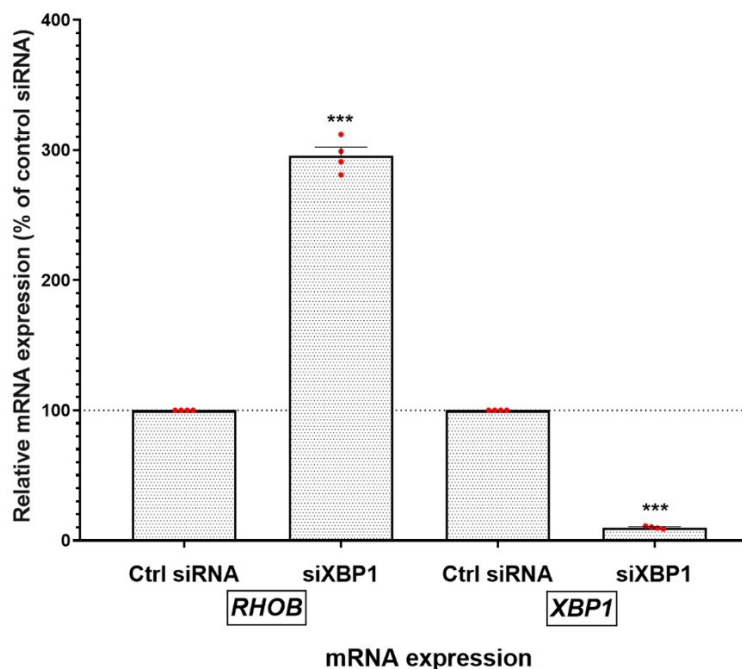


Figure 3. Effect of *XBP1* silencing on *RHOB* and *XBP1* mRNA expression in wild-type glioblastoma cells. *XBP1* mRNA was silenced using human-specific *XBP1* siRNA for 48 h, and *RHOB* and *XBP1* mRNA levels were analyzed by quantitative reverse transcription polymerase chain reaction. Expression levels were normalized to *ACTB* mRNA and expressed as a percentage of the control group (set at 100%). Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). *** $p < 0.001$ indicates a statistically significant difference when compared with Ctrl siRNA. Abbreviations: Ctrl: Control; *RHOB*: Ras homolog family member B; siRNA: Small interfering RNA; *XBP1*: X-box binding protein 1.

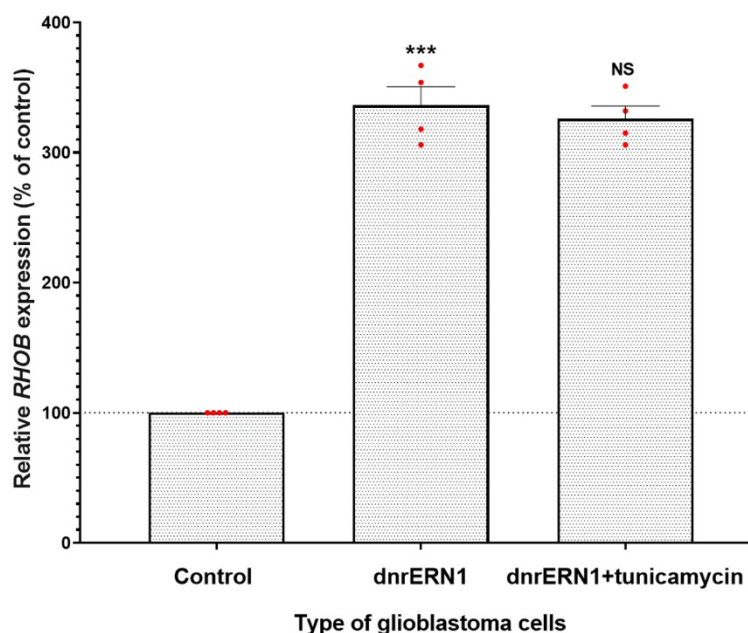


Figure 4. Effect of tunicamycin on *RHOB* mRNA expression in dnrERN1 glioblastoma cells. dnrERN1 cells (with selective suppression of ERN1 endoribonuclease activity) were treated with tunicamycin (500 ng/mL) for 4 h, and *RHOB* mRNA levels were measured by quantitative reverse transcription polymerase chain reaction. Expression levels were normalized to *ACTB* mRNA and expressed as a percentage of the control group (set at 100%). Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). *** $p < 0.001$ indicates a statistically significant difference when compared with control, whereas “NS” indicates no significant difference when compared with dnrERN1. Abbreviations: ERN1: Endoplasmic reticulum to nucleus signaling 1; RHOB: Ras homolog family member B.

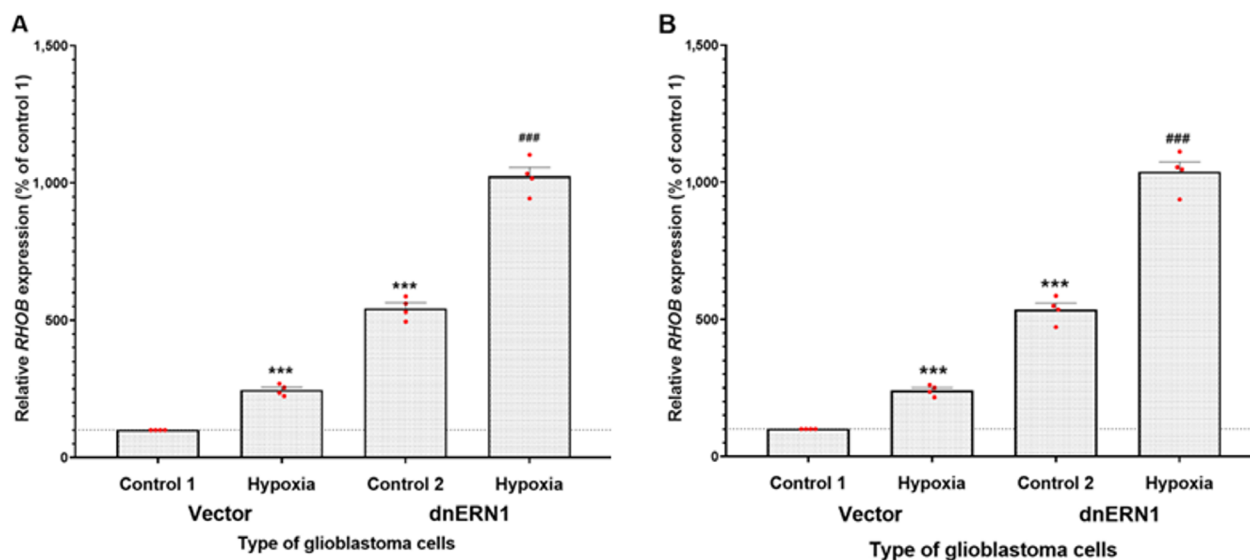


Figure 5. Effect of hypoxia on *RHOB* mRNA expression in control and *ERN1* knockdown glioblastoma cells. *RHOB* mRNA levels were measured by quantitative polymerase chain reaction in control cells (empty vector; Control 1) and *ERN1* knockdown cells (dnERN1; Control 2) under hypoxic conditions. Expression levels were normalized to *ACTB* and *RPS16* mRNAs and expressed as a percentage of the respective control group (set at 100%). The effect of hypoxia in dnERN1 cells was compared with Control 2, whereas hypoxia in vector-transfected cells was compared with Control 1. Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). *** $p < 0.001$ indicates a statistically significant difference when compared with Control 1, whereas ### $p < 0.001$ indicates a statistically significant difference when compared with Control 2. Abbreviations: ERN1: Endoplasmic reticulum to nucleus signaling 1; RHOB: Ras homolog family member B.

3.3. Suppression of ERN1 protein kinase and endoribonuclease activities reduces the sensitivity of *RHOB* expression to glutamine deprivation

Glutamine deprivation increased *RHOB* expression by 44% ($p < 0.01$) in glioblastoma cells expressing native *ERN1* compared to control (Figure 6). Suppression of ERN1 protein kinase and endoribonuclease activities dampened the response of *RHOB* expression to glutamine deprivation, resulting in a 25% increase ($p < 0.01$) in *ERN1* knockdown cells compared to the corresponding control (Control 2).

3.4. Suppression of both enzymatic activities of ERN1 upregulates *RHOB* expression under glucose deprivation

We investigated the effect of glucose deprivation on *RHOB* expression in dnERN1 and control cells using *ACTB* and *RPS16* as housekeeping genes. As shown in Figure 7A and 7B, *RHOB* expression was unchanged by glucose deprivation in control cells compared to Control 1, using either *ACTB* (Figure 7A) or *RPS16* (Figure 7B) as references. Inhibition of both enzymatic activities of *ERN1* removes this resistance, resulting in increased *RHOB* expression in

dnERN1 cells by 169% ($p < 0.001$) with *ACTB* (Figure 7A) and 166% ($p < 0.001$) with *RPS16* as references (Figure 7B) compared to the corresponding control.

3.5. Suppression of ERN1 protein kinase and endoribonuclease activities reduces microRNA expression targeting *RHOB* mRNA

We assessed the expression of miR-21-5p and miR-223-3p in control and dnERN1 cells, as analysis of the 3'-untranslated region of *RHOB* mRNA revealed binding sites for these miRNAs, as well as miR-19a-3p. As shown in Figure 8, inhibition of ERN1 protein kinase and endoribonuclease activities decreased miR-21-5p and miR-223-3p levels by 24% and 39%, respectively, compared to the control. These reductions likely contribute to post-transcriptional upregulation of *RHOB* expression.

3.6. Hypoxia and glucose deprivation do not affect glioblastoma cell viability

As shown in Table 1, the percentage of dead cells remained low, and hypoxia did not significantly affect cell viability. Glucose deprivation also had no impact on glioblastoma cell viability (Table 1). Furthermore, suppression of ERN1

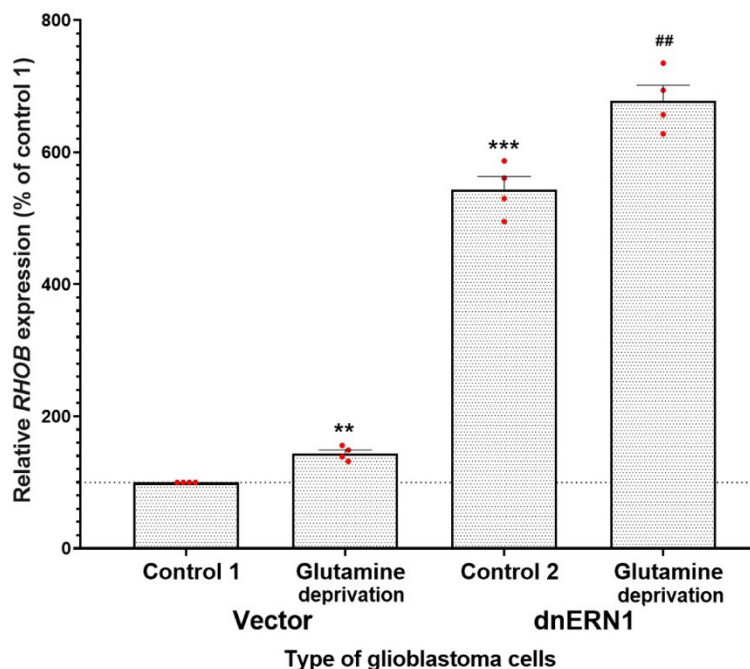


Figure 6. Effect of glutamine deprivation on *RHOB* mRNA expression in control glioblastoma cells (empty vector; Control 1) and dnERN1 cells (with inhibited ERN1 protein kinase and endoribonuclease activities; Control 2), measured by quantitative polymerase chain reaction. Expression levels were normalized to *ACTB* mRNA and expressed as a percentage of Control 1 group (100%). The effect of glutamine deprivation in dnERN1 cells was compared to Control 2. Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). ** $p < 0.01$ and *** $p < 0.001$ indicate a statistically significant difference when compared with Control 1, whereas ## $p < 0.01$ indicates a statistically significant difference when compared with Control 2.

Abbreviations: ERN1: Endoplasmic reticulum to nucleus signaling 1; RHOB: Ras homolog family member B.

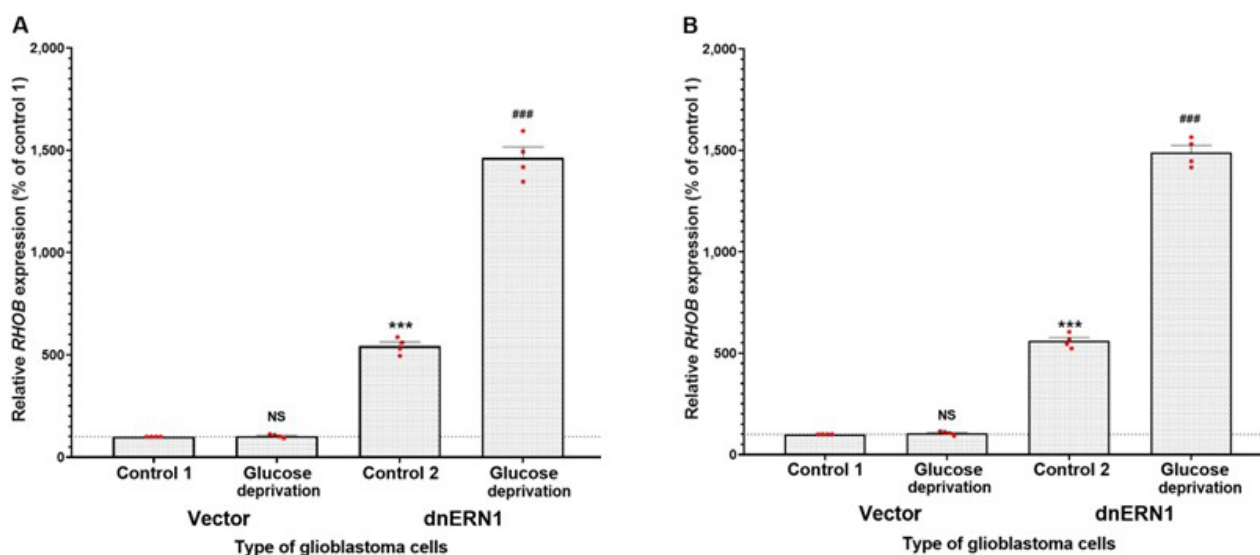


Figure 7. Effect of glucose deprivation on *RHOB* mRNA expression in control glioblastoma cells (empty vector; Control 1) and dnERN1 cells (with inhibited ERN1 protein kinase and endoribonuclease activities; Control 2), measured by quantitative polymerase chain reaction. Expression levels were normalized to *ACTB* or *RPS16* mRNAs and expressed as a percentage of Control 1 group (100%). The effect of glucose deprivation in dnERN1 cells was compared to Control 2. Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). *** $p < 0.001$ indicates a statistically significant difference when compared with Control 1, whereas *** $p < 0.001$ indicates a statistically significant difference when compared with Control 2. “NS” indicates no significant difference when compared to Control 1.

Abbreviations: ERN1: Endoplasmic reticulum to nucleus signaling 1; RHOB: Ras homolog family member B.

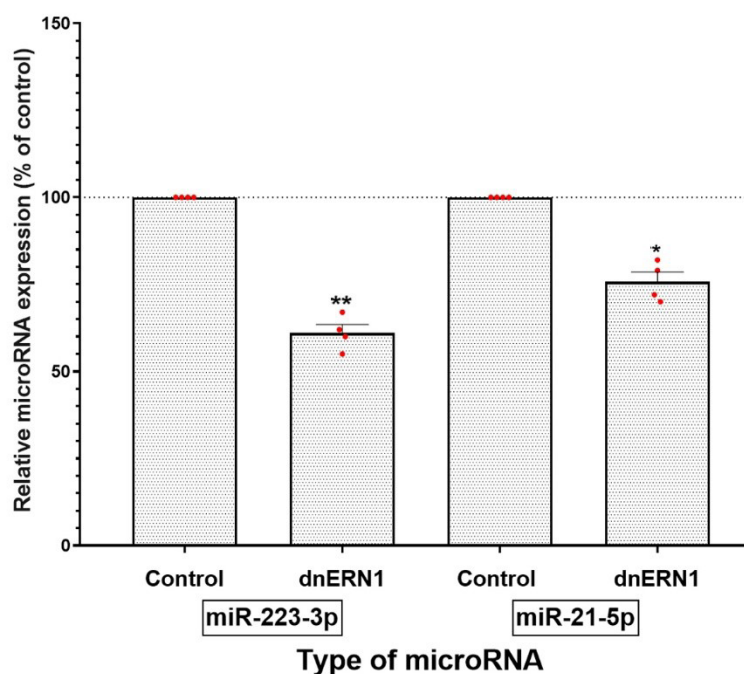


Figure 8. Inhibition of ERN1 protein kinase and endoribonuclease activities in dnERN1 cells decreases the expression of microRNAs miR-223-3p and miR-21-5p in glioblastoma cells. Expression levels were normalized to U6 RNA and expressed as a percentage of the control group (100%). Data are shown as mean \pm standard error of the mean ($n = 4$ independent experiments performed in triplicate). * $p < 0.05$ and ** $p < 0.01$ indicate a statistically significant difference when compared with control.

Abbreviation: ERN1: Endoplasmic reticulum to nucleus signaling 1.

protein kinase and endoribonuclease activities in dnERN1 cells did not alter cell viability under hypoxia or glucose deprivation.

4. Discussion

The findings of this study reveal robust upregulation of the tumor suppressor RHOB in *ERN1* knockdown glioblastoma cells. Inhibition of both enzymatic activities of *ERN1* induced significantly greater *RHOB* mRNA expression compared to inhibition of the endoribonuclease activity alone. Similar increases were observed after *ERN1* silencing, consistent with previous studies comparing different approaches to *ERN1* suppression on gene expression.^{15-17,20,37,38,49} These results indicate that *RHOB* expression is mediated through both enzymatic activities of *ERN1*.

In experiments with *XBPI* mRNA silencing, *RHOB* mRNA expression increased to nearly the same extent as in dnERN1 cells, suggesting that the effect of inhibiting ERN1 endoribonuclease activity is primarily mediated through XBPI. This observation effectively eliminates post-transcriptional regulation of *RHOB* via regulated Ire1-dependent decay.^{1,13,15-17} Figure 9 summarizes the main results of this study.

The results also indicate that ERN1 protein kinase activity contributes to *RHOB* regulation, as inhibition of protein kinase activity enhances the effect of endoribonuclease inhibition on *RHOB* mRNA expression. Numerous studies have established that the unfolded protein response and *ERN1* signaling involve multiple mechanisms at both genomic and protein levels.^{1,3,56} Changes in gene expression are often complex and

multifaceted, resulting from enhancement or suppression of transcription, as well as modulation of mRNA stability via mechanisms including direct cleavage of mRNAs and miRNAs by ERN1 endoribonuclease (regulated Ire1-dependent decay mechanism).^{1,17,57-59}

It is generally accepted that *ERN1* regulates gene expression primarily at the transcriptional level, indirectly through *XBPI* and *ERN1* protein kinase activity, leading to genome reprogramming and activation of numerous transcription factors and their regulators.^{1,3,13,15-20,38,57}

The critical role of ERN1 protein kinase activity in regulating gene expression has been previously demonstrated for several genes.^{15-20,37,49} Upon *ERN1* knockdown, the decreased expression of *EREG*, *PHGDH*, *IDE*, *PITRM1*, and *TOBI* was shown to be mediated by its protein kinase activity.^{16,17,19,20} Signals from ERN1 protein kinase are transmitted through mitogen-activated protein kinase 8/c-Jun N-terminal kinase, which serves as an integration point for multiple regulatory signals and regulates transcription by targeting specific transcription factors, thereby influencing gene expression, particularly of the *EREG* gene. This is supported by the observation that the c-Jun N-terminal kinase inhibitor SP600125 tunicamycin-induced *EREG* expression in glioblastoma cells expressing native ERN1, but not in dnERN1 and dnERN1 cells.¹⁷

The protein kinase activity of ERN1 also mediates the upregulation of genes involved in proliferation and invasion (e.g., homeobox transcription factors, *EDN1*, and *SHMT1*).^{15,16,18} Interestingly, inhibition of both enzymatic activities of ERN1 reduces glioblastoma cell proliferation to a smaller extent than inhibition of endoribonuclease

Table 1. Percentage of dead glioblastoma cells under hypoxia and glucose deprivation with *ERN1* inhibition

Percentage of dead cells (%)	Vector		dnERN1		Vector		dnERN1	
	Control	Hypoxia	Control	Hypoxia	Control	Glucose deprivation	Control	Glucose deprivation
Mean	1.235	1.342	1.304	1.416	1.294	1.406	1.383	1.541
Standard error of the mean	0.203	0.327	0.278	0.321	0.255	0.362	0.246	0.316

Notes: Cell viability was assessed using the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells. Data are expressed as the percentage of dead cells (mean ± standard error of the mean; *n* = 4 independent experiments performed in triplicate). Inhibition of ERN1 protein kinase and endoribonuclease activities by dnERN1 did not significantly affect cell viability under hypoxic or glucose-deprived conditions.

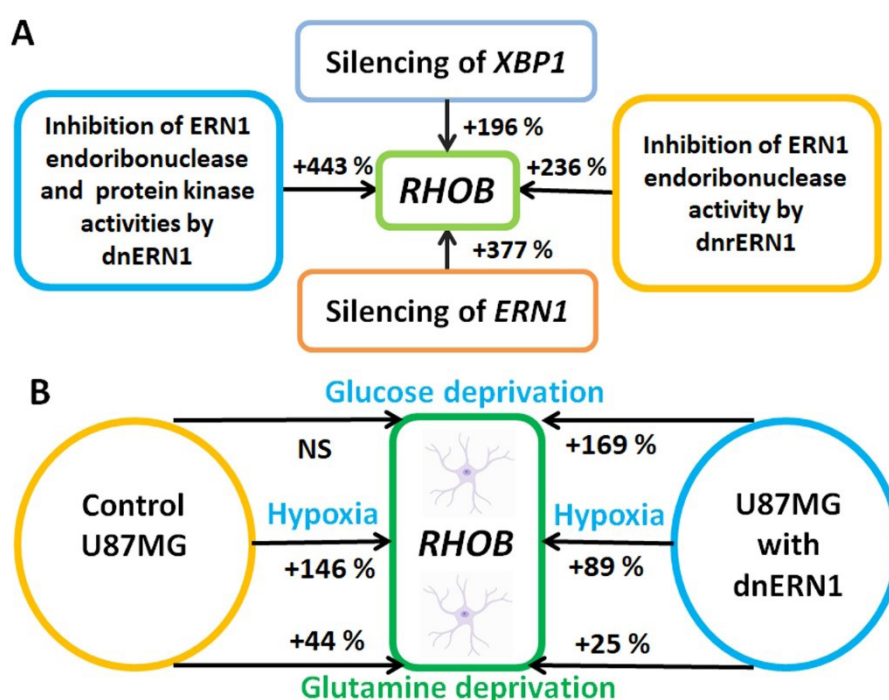


Figure 9. Schematic illustration of (A) the sensitivity of *RHOB* mRNA expression to inhibition of ERN1 protein kinase and endoribonuclease activities by dnERN1, selective inhibition of ERN1 endoribonuclease activity by dnrERN1, and silencing of ERN1 or XBP1 mRNAs (48 h), and (B) the effects of hypoxia, glucose deprivation, and glutamine deprivation on *RHOB* mRNA expression in control U87MG glioblastoma cells (empty vector) and cells lacking both ERN1 enzymatic activities (dnERN1). “NS” indicates no statistically significant difference when compared with control U87MG cells. Abbreviations: ERN1: Endoplasmic reticulum to nucleus signaling 1; RHOB: Ras homolog family member B; XBP1: X-box binding protein 1.

activity alone.¹⁶ This is explained by the induction of pro-oncogenic genes in *ERN1* knockdown glioblastoma cells via ERN1 protein kinase activity.^{15,16,18,20} Recently, a specific inhibitor of ERN1 protein kinase has been tested for its ability to inhibit glioblastoma progression.²³

The analysis of the 3'-untranslated region of *RHOB* mRNA revealed binding sites for miRNAs miR-21-5p, miR-223-3p, and miR-19a-3p. In this study, we demonstrated that expression levels of miR-21-5p and miR-223-3p were decreased in dnERN1 cells, possibly contributing to the regulation of *RHOB* mRNA expression. In these cells, miR-19a-3p expression was also reduced.¹⁸ These findings are consistent with previous studies showing the role of miRNAs in regulating *RHOB* mRNA expression in various cancer cells.^{25,27,29,32}

Additionally, *ERN1* regulates mRNA and miRNA expression post-transcriptionally.^{56,58} Moreover, some miRNAs are known to modulate ER-induced unfolded protein response pathways.^{58,60,61} This is consistent with studies by Cawley *et al.*,⁶² who demonstrated that loss of miRNA biogenesis increases resistance to stress-induced apoptosis, suggesting that miRNAs may act as modulators of the ER stress response. Therefore, it can be inferred that

changes in *RHOB* expression upon inhibition of ERN1 enzymatic activities are mediated by both transcriptional and post-transcriptional mechanisms.

Hypoxia is a critical factor in cancer progression, as the unfolded protein response reprograms cellular responses to hypoxia, thereby promoting resistance.^{2,12,18,38,44} In this study, we demonstrated that hypoxia upregulates *RHOB* expression in glioblastoma cells expressing native *ERN1* to a much greater extent than in dnERN1 cells (Figure 9). It is worth noting that the effect of hypoxia on *RHOB* expression was consistent when using either *ACTB* or *RPS16* as reference genes. These results indicate that *RHOB* mRNA expression is regulated by signaling protein ERN1.

A similar effect of *ERN1* knockdown on hypoxic regulation was observed for *EDN1* and *SPAG4* expression.^{18,36} Dimethylxalylglycine was used to mimic hypoxia. Previously, we demonstrated that dimethylxalylglycine-induced hypoxic conditions accurately recapitulate hypoxic gene regulation (e.g., via HIF-1α and HIF-2α) compared to low oxygen (1%).^{41,52,62-64} Specifically, HIF-1α protein levels in the PC-3 prostate cancer cell line were robustly induced by 1% oxygen hypoxia and to a similar extent by dimethylxalylglycine,

which mimics hypoxic effects at normal oxygen levels.⁴¹

In these cells, it was also demonstrated that both hypoxia (1% oxygen) and dimethyloxalylglycine significantly increased the expression level of HIF-dependent genes, including *PFKFB4*, *PFKFB3*, and *VEGFA*, as measured by ribonuclease protection assay.⁴¹ It is worth noting that the induction of these genes in PC-3 prostate cancer cells was similar under both hypoxic conditions, although slightly greater for *PFKFB3* and *VEGFA*.⁴¹ Moreover, analysis of *PFKFB4*, *PFKFB3*, and *GLUT1/SLC2A1* expression in other cancer cell lines (e.g., HeLa, HepG2, and Hep3B) revealed comparable changes under hypoxia induced by 1% oxygen or dimethyloxalylglycine.⁴¹

Similar results were observed when investigating the effects of hypoxia (1% oxygen) and dimethyloxalylglycine on *PFKFB4* and *PFKFB3* mRNA expression, as well as *PFKFB4* protein levels, in several pancreatic and gastric cancer cell lines (e.g., Panc1, PSN1, MIA PaCa-2, MKN45, and NUGC3).⁶² No significant differences were detected in the induction of these HIF-dependent genes under low oxygen or dimethyloxalylglycine treatment.^{62,63} Furthermore, *HIF1A* and *HIF2A* mRNA expression in MKN45 gastric cancer cells was similarly unaffected by either hypoxic condition.⁶² In PC-3 prostate adenocarcinoma cells, HIF-1 α protein levels increased significantly under both low oxygen and dimethyloxalylglycine treatment.⁶⁴

In contrast, *HIF1A* mRNA expression was suppressed, particularly under dimethyloxalylglycine,⁶⁴ while *HIF2A* mRNA expression remained unchanged by both hypoxic conditions.⁶⁵ Our data show that hypoxia differentially regulates gene expression, and its effects are strongly dependent on ERN1 signaling and additional regulatory factors.^{18,36,41,43-47} *ERN1* may modulate hypoxic gene regulation by altering genome-specific regulatory factors that interact with HIF and influence its activity.^{43,46,47}

In this study, we found that *RHOB* expression was unchanged by glucose deprivation in glioblastoma cells expressing native *ERN1*; however, *ERN1* inhibition rendered these cells sensitive to glucose availability. Two housekeeping genes (*ACTB* and *RPS16*) were used as reference controls. No significant differences were observed in the effects of glucose deprivation on *RHOB* expression in either control or dnERN1 cells when *ACTB* or *RPS16* mRNA served as reference genes. ERN1 may therefore contribute to the maintenance of *RHOB* expression under glucose deprivation. Notably, *RHOB* is generally suppressed in glioblastoma cells.^{28,29} Consistent with our findings, ERN1-dependent modulation of gene expression under glucose deficiency has been reported previously.^{19,37,38,48-51} Alterations in gene expression, including *RHOB*, under glucose/glutamine deprivation may reflect ERN1-mediated

stress adaptation mechanisms. Moreover, inhibition of ERN1 protein kinase and endoribonuclease activities by dnERN1 did not alter the viability of glioblastoma cells exposed under hypoxia or glucose deprivation.

The dependence of gene expression on glucose during ER stress underscores the close relationship between carbohydrate metabolism and the execution of genetic programs. This is consistent with the mechanism of ER stress induction, which involves disruption of post-translational protein modifications in the ER, particularly glycosylation, leading to genome-wide reprogramming and altered expression of numerous genes.^{1,13,14} These changes affect genes involved in glycosylation and other post-translational modifications, as well as post-transcriptional RNA regulation.^{19,20,36-38,66}

Glutamine also plays a critical role in glioblastoma growth by providing carbon and nitrogen to support cancer cell proliferation. However, glutamine can also modulate the tumor suppressor FBW7 through lysine glutamylation mediated by glutaminy-transfer RNA synthetase, thereby promoting cancer cell proliferation and survival.⁶⁷ Moreover, glutaminy-transfer RNA synthetase is regulated by MALAT1-associated small cytoplasmic RNA, a transfer RNA-like small non-coding RNA derived from the long non-coding RNA MALAT1, which enhances global protein translation.⁶⁸ Together, these findings highlight the multifaceted regulatory roles of glucose and glutamine in controlling metabolic and gene expression programs in both normal and malignant cells.

Ras homolog family member B is a tumor suppressor; however, studies using multicellular three-dimensional spheroid models have shown reduced migration and invasion following *RHOB* loss.⁶⁹ In contrast, *in vivo* mouse studies showed that neither downregulation nor upregulation of *RHOB* significantly affected tumor growth.⁶⁹ Currently, no data are available regarding the effects of *ERN1* knockdown on *RHOB* post-translational modifications, such as prenylation, which may influence its biological activity. This important question warrants further investigation.

5. Conclusion

In conclusion, these findings highlight the role of ERN1 protein kinase and endoribonuclease activities in regulating *RHOB* expression and modulating its responsiveness to hypoxia and glucose availability in glioma cells. Upregulation of the tumor suppressor *RHOB* in *ERN1* knockdown cells, particularly under glucose deprivation, may contribute to reduced glioblastoma cell proliferation.¹⁶ However, the molecular mechanisms underlying ERN1-dependent regulation of *RHOB* expression, as well as its

interaction with hypoxic signaling pathways, require further investigation.

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Conflict of interest

The authors declare that they have no competing interests.

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Not applicable.

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Not applicable.

Availability of data

Data is available from the corresponding author upon reasonable request.

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