Inhibition of kinase and endoribonuclease activity of ERN1/IRE1α affects expression of proliferation-related genes in U87 glioma cells

Abstract: Inhibition of ERN1/IRE1α (endoplasmic reticulum to nucleus signaling 1/inositol requiring enzyme-1α), the major signaling pathway of endoplasmic reticulum stress, significantly decreases tumor growth. We have studied the expression of transcription factors such as E2F8 (E2F transcription factor 8), EPAS1 (endothelial PAS domain protein 1), TBX3 (T-box 3), ATF3 (activating transcription factor 3), FOXF1 (forkhead box F1), and HOXC6 (homeobox C6) in U87 glioma cells overexpressing dominant-negative ERN1/IRE1α defective in endoribonuclease (dnr-ERN1) as well as defective in both kinase and endonuclease (dn-ERN1) activity of ERN1/IRE1α. We have demonstrated that the expression of all studied genes is decreased at the mRNA level in cells with modified ERN1/IRE1α; TBX3, however, is increased in these cells as compared to control glioma cells. Changes in protein levels of E2F8, HOXC6, ATF3, and TBX3 corresponded to changes in mRNAs levels. We also found that two mutated ERN1/IRE1α have differential effects on the expression of studied transcripts. The presence of kinase and endonuclease deficient ERN1/IRE1α in glioma cells had a less profound effect on the expression of E2F8, HOXC6, and TBX3 genes than the blockade of the endoribonuclease activity of ERN1/IRE1α alone. Kinase and endonuclease deficient ERN1/IRE1α suppresses ATF3 and FOXF1 gene expressions, while inhibition of only endoribonuclease of ERN1/IRE1α leads to the up-regulation of these gene transcripts. The present study demonstrates that fine-tuning of the expression of proliferation related genes is regulated by ERN1/IRE1α an effector of endoplasmic reticulum stress. Inhibition of ERN1/IRE1α, especially its endoribonuclease activity, correlates with deregulation of proliferation related genes and thus slower tumor growth.

Keywords: endoplasmic reticulum stress, ERN1/IRE1α, E2F8, EPAS1, HOXC6, ATF3, TBX3, FOXF1, U87 glioma cells, NHA/TS astrocyte cells

1 Introduction

The endoplasmic reticulum (ER) is the primary organelle able to activate a distinct cellular stress response, termed the Unfolded Protein Response (UPR) in which a moiety of factors (typically aggregates of misfolded proteins) triggers activation of a complex set of signaling pathways to execute a resolution to the causative stress. Malignant tumors utilize the endoplasmic reticulum stress response to adapt to stressful, environmental conditions [1-3]. The rapid growth of solid tumors generates micro-environmental changes in association to hypoxia, nutrient deprivation and acidosis, which induce new blood vessels formation and cell proliferation. Those processes rely on the activation of endoplasmic reticulum stress signalling pathways [2,3]. UPR is mediated by three interconnected, endoplasmic reticulum-resident sensors. ERN1/IRE1α (endoplasmic reticulum to nucleus signaling 1/inositol requiring enzyme-1α).
requiring enzyme-1alpha) is the most evolutionary conserved sensor that responds to protein misfolding with a highly tuned program aimed to either resolve the stress or direct the cell towards apoptosis in the case rectification is not viable; thus making it a key regulator of life and death processes[1,4-7].

The ERN1/IRE1α enzyme contains two distinct catalytic domains: serine/threonine kinase and endoribonuclease. Endoribonuclease activity is involved in the degradation of a specific subset of mRNA targeted to the ER to lessen the load of protein synthesis on the already stressed ER. Endonuclease activity also initiates the cytosolic splicing of the pre-XBP1 (X-box binding protein 1) mRNA, whose mature transcript encodes for a transcription factor that stimulates the expression of numerous UPR-specific genes, namely other key transcription factors [8-11]. Moreover, activation of the ERN1/IRE1α branch of the endoplasmic reticulum stress response is intimately linked to apoptosis. Ablation of this sensor’s function by a dominant-negative construct of ERN1/IRE1α (dn-ERN1) has been shown to result in a significant anti-proliferative effect in glioma growth [2,12]. This is due to down-regulation of prevalent pro-angiogenic factors and up-regulation of anti-angiogenic genes, both in vitro and in the CAM (chorio-allantoic membrane) model, as well as in mice engrafted intracerebrally with U87 glioma cell clones [13-15]. The executive mechanism of the exhibited anti-proliferative effects is not yet known. We propose that anti-proliferative effect is realized through mediation by transcription factors, which are integrated into the UPR signaling pathways to regulate cell cycle, apoptosis and senescence [16-21]. Possible involvement of such transcription factors such as E2F, HIF, TBX, ATF, FOX, and HOX families was made evidently pertinent through transcriptomic analysis of U87 glioma cells expressing the dominant-negative mutant of ERN1/IRE1α [14].

E2F transcription factors, such as E2F8, are essential for orchestrating expression of genes required for cell cycle progression and proliferation, they promote angiogenesis through transcriptional activation of VEGFA in cooperation with HIF1 and are strongly up-regulated in human hepatocellular carcinoma [17,22,23]. The T-box transcription factor, TBX3 is a transcriptional repressor that plays multiple roles in both normal development and disease either by transcription repression or activation of target genes in a context-dependent manner. It controls the rate of cell proliferation as well as mediates cellular signaling pathways and the anti-proliferative role of TGF-β1 [16,24]. The transcription factor HOXC6 plays an important role in both proliferation and metastasis by regulating genes with both oncogenic and tumor suppressor activities and it may also contribute to the progression of gastric carcinogenesis [20,25,26]. The TP53 family, which we previously demonstrated to be modulated by ERN1/IRE1α, targets the forkhead box transcription factor, FOXF1. Ectopic expression of FOXF1 inhibits cancer cell invasion and migration, whereas the inactivation of FOXF1 stimulates both of these processes [21,27]. Consequently, FOXF1 overexpression is associated with epithelial-to-mesenchymal transition, the process mediated by chronic activation of UPR as well, in breast cancer, making it potentially a worthy player in the mechanistic progression of UPR mediated cancers [21].

Cyclic AMP-dependent activating transcription factor 3 (ATF3) is a cell-death regulator that is strongly induced during necrosis and can suppress the oncogenic function of mutant TP53, thereby contributing to tumor suppression. Although its suppressive function is speculated to be tumor specific, it has also demonstrated the promotion of other cancers [28-31]. The last transcription factor of interest, Endothelial PAS domain protein 1 (EPAS1), which is also known as hypoxia-inducible transcription factor-2alpha (HIF-2α), has been shown to correlate with tumor size, invasion and necrosis as well as with VEGF gene expression, which supports the correlation of EPAS1 up-regulation with tumor angiogenesis [32,33].

Therefore, based on the amalgamation of evidence listed above, the aim of this study was to investigate the possible roles of genes encoding for transcription factors: E2F8, EPAS1, HOXc6, ATF3, TBX3, and FOXF1 as they apply to the suppression of glioma cell proliferation via inhibition of the endoplasmic reticulum stress sensor ERN1/IRE1α with hopes of elucidating its mechanistic part in the development and progression of certain cancers and the contribution of UPR.

2 Material and Methods

Reagents. The glioma cell line U87 (HTB-14) was obtained from ATCC (USA) and grown in high glucose (4.5 g/l) Dulbecco’s modified Eagle’s minimum essential medium (DMEM; Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/ml; Gibco, USA) and streptomycin (0.1 mg/ml; Gibco) at 37°C in a 5% CO₂ incubator. The immortalized normal human astrocyte cells (line NHA/TS) were kindly provided by Drs. K. Sasai and S. Tanaka and were grown as reported [34].

Cell lines. In this work we used sublines of U87 glioma cells, which were described previously [13-15,35]. One subline was obtained by selection of stable transfected
clones overexpressing vector (pcDNA3.1), which was used for creation of dominant-negative constructs of ERN1/IRE1α (dn-ERN1 and dnr-ERN1). This untreated subline of glioma cells was used as a control (control glioma cells) in the study of the effects of inhibition of ERN1, in regards to the expression of the transcription factors of interest (Table 1). The second sub-line was obtained by the selection of stable transfected clones overexpressing dn-ERN1, having suppression of both the protein kinase and endoribonuclease activities of ERN1/IRE1α [14]. The third sub-line was obtained by the selection of stable transfected clones with the overexpression of dominant-negative ERN1/IRE1α endoribonuclease mutant (dnr-ERN1), which was obtained by truncation of the carboxy-terminal 78 amino acids of ERN1 [15]. It has recently been shown that these cells have a low rate of proliferation and do not express spliced XBP1, a key transcription factor in ERN1/IRE1α signaling, after induction of endoplasmic reticulum stress by tunicamycin [15]. For experiments with GRP78/HSPA5 we have also used U87 cells stable transfected by wild-type ERN1. The expression of the studied genes was compared with cells transfected with the previously mentioned, empty vector (control glioma cells, pcDNA3.1).

**Proliferation assay.** The rate of proliferation control glioma cells and ERN1 knockdown cells was measured via cell counter (Coultronics, Margency, France). Cell number was measured in triplicates after 3 days.

**Endoplasmic reticulum stress induction by tunicamycin.** Glioma cells with dnr-ERN1 were incubated with tunicamycin (0.01 mg/ml) for 2 hrs.

**RNA isolation.** Total RNA was extracted from both glioma and normal human astrocyte cells using Trizol reagent according to manufacturer protocols (Invitrogen, USA). The RNA pellets were washed with 75 % ethanol and dissolved in nuclease-free water. For additional purification RNA samples were re-precipitated with 95 % ethanol and re-dissolved again in nuclease-free water.

**Reverse transcription and qPCR analysis.** QuantiTect Reverse Transcription Kit (QiAGEN, Germany) was used for cDNA synthesis according to manufacturer protocol. The expression level of E2F8, TBX3, EPAS1, ATF3, FOXF1, HOXC6, and ACTB mRNA were measured in U87 glioma cells and normal human astrocyte cells by real-time quantitative polymerase chain reaction using Mx 3000P QPCR (Stratagene, USA) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, Abgene House, UK). Polymerase chain reaction was performed in triplicate using specific primers, which were received from Sigma-Aldrich, USA (Table 1).

An analysis of quantitative PCR was performed using special computer program Differential Expression Calculator. The expression values of E2F8, TBX3, EPAS1, ATF3, FOXF1, HOXC6, and ACTB mRNA were normalized to beta-actin and represent a percent control (100 %).

**Western blot analysis.** E2F8, HOXC6, TBX3, and ATF3

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**Table 1: Characteristics of studied transcription factor genes and primers used for qPCR**

| Gene symbol | Gene name                         | Primer's sequence | Nucleotide numbers in sequence | GenBank accession number |
|-------------|-----------------------------------|-------------------|-------------------------------|--------------------------|
| TBX3        | T-box 3                           | F: 5’- acggaagctgctggaatggc | 1519–1540                     | NM_005996                |
|             |                                   | R: 5’- tgtctggtgacatctggcatc | 1519–1540                     |                          |
| EPAS1       | Endothelial PAS domain protein 1  | F: 5’- acggaagctgctggaatggc | 1519–1540                     | NM_005650                |
|             | (hypoxia-inducible factor 2 alpha)| R: 5’- tgtctggtgacatctggcatc | 1519–1540                     |                          |
|            |                                   | F: 5’- accaggctttgctttgcatctc | 788–807                      | NM_001430                |
|             |                                   | R: 5’- tgtctggtgacatctggcatc | 788–807                      |                          |
| E2F8        | E2F transcription factor 8        | F: 5’- accaggctttgctttgcatctc | 788–807                      | NM_001430                |
|             |                                   | R: 5’- tgtctggtgacatctggcatc | 788–807                      |                          |
|             |                                   | F: 5’- accaggctttgctttgcatctc | 788–807                      | NM_001430                |
|             |                                   | R: 5’- tgtctggtgacatctggcatc | 788–807                      |                          |
| ACTB        | beta-actin                        | F: 5’- accaggctttgctttgcatctc | 788–807                      | NM_001430                |
|             |                                   | R: 5’- tgtctggtgacatctggcatc | 788–807                      |                          |

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**Gene symbol**
- TBX3: T-box 3
- EPAS1: Endothelial PAS domain protein 1
- E2F8: E2F transcription factor 8
- ATF3: Activating transcription factor 3
- HOXC6: Homeobox C6
- FOXF1: Forkhead box F1
- ACTB: beta-actin
proteins were measured in glioma cells by Western blot analysis using mouse monoclonal anti-E2F8 antibody (H00079733-M01) from NOVUS Biologicals, mouse monoclonal anti-HOXC6 antibody (sc-376330), goat polyclonal anti-TBX3 antibody (sc-31657), rabbit polyclonal anti-ATF3 antibody (sc-188), and mouse monoclonal anti-ACTB (beta-actin; sc-47778) from Santa Cruz Biotechnology. ACTB was used as control of analyzed protein quantity. Western blot analysis was performed as described previously [36,37].

Statistical analysis was performed using OriginPro 7.5 software. All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments. Comparison of two means was performed by the use of two-tailed Student's t-test as described previously [38]. P < 0.05 was considered significant in all cases.

Ethical approval: The conducted research is not related to either human or animals use.

3 Results

3.1 ERN1/IRE1α modulates expression of E2F8, HOXC6, EPAS1, ATF3, FOXF1, and TBX3 transcription factor genes in glioma cells

The expression of these genes was studied by quantitative PCR and Western blot analysis. To test the effect of ERN1/IRE1α on expression levels of the transcription factor of interest in relation to the control of cell proliferation, we used U87 glioma cell sub-lines, constitutively expressing the dominant-negative mutant of ERN1/IRE1α, dn-ERN1 or dnr-ERN1, inhibiting endoribonuclease activity of endogenous ERN1/IRE1α or both endoribonuclease and kinase activities, respectively [14,15].

Figure 1A and Table 2 demonstrate that inhibition of ERN1/IRE1α gene function in U87 glioma cells by dn-ERN1 leads to the down-regulation of E2F8 mRNA (17fold). The inhibition of the endoribonuclease activity of ERN1/IRE1α alone by dnr-ERN1 has an even more robust effect on the expression of E2F8 (50 fold; Figure 1A and Table 2). The resultantly low level of E2F8 mRNA in cells expressing dnr-ERN1 is comparable to that of the normal human astrocyte cells line (NHA/TS) and not to immortalize U87 cells. Notably, normal human astrocyte cells are grown without the addition of geneticin (G418) while U87 cells carrying dn-ERN1 and dnr-ERN1 are grown in its presence. Therefore, the possibility exists that the differences in growing conditions could affect E2F8 expression; however, there were no significant differences found in the expression of E2F8 mRNA in glioma cells overexpressing the empty vector and wild-type U87 glioma cells. (Figure 1A). Therefore, inhibition of ERN1/IRE1α endoribonuclease affects the regulation of glioma cell growth by mediating E2F8 mRNA expression and lowering it approximately to a level normally observed in human astrocytes.

Thereafter, we tested how ERN1/IRE1α inhibition modulates the expression of transcription factors, HOXC6 and EPAS1. As shown in Figure 1B and Table 2, inhibition of ERN1/IRE1α endoribonuclease alone by dnr-ERN1 has a robust suppressive effect on HOXC6 expression (3.7 fold down-regulation); however, there is a slightly lesser effect on expression in the double mutant (dn-ERN1), which exhibits inhibition of both endonuclease and kinase activities. We found that the expression of HOXC6 mRNA in normal human astrocytes is very low (twelvefold less versus control glioma cells) and that in U87 glioma cells, inhibition of ERN1/IRE1α, especially its endoribonuclease activity, leads to more dramatized decrease in HOXC6 gene expression, making its expression akin to that of non-malignant, NHA/TS cells (Figure 1B).

mRNA expression of transcription factor EPAS1/HIF-2α (which mediates numerous hypoxia-induced processes including proliferation in cell-specific manner) is also affected by modulation of ERN1/IRE1α activity (Figure 1C, Table 2). Similar to the trend found with HOXC6

| Gene  | Tested condition | dn-ERN1- cells versus control cells | dnr-ERN1- cells versus control cells | Effect of tunicamycin in dnr-ERN1-cells |
|-------|-----------------|------------------------------------|-------------------------------------|----------------------------------------|
| E2F8  | Down 17fold     | Down 50fold                        | Down 8fold                          |
| TBX3  | Up 2.6fold      | Up 9.4fold                         | 34 % down                           |
| EPAS1 | Down 20fold     | Down 2.6fold                       | No changes                          |
| ATF3  | Down 7fold      | Down 21 %                          | Up 3fold                            |
| FOXF1 | Down 2fold      | Up 23fold                          | Down 2.4fold                        |
| HOXC6 | Down 1.9fold    | Down 3.7fold                       | Down 2fold                          |
Figure 1: Expression of E2F8 (E2F transcription factor 8; A), HOXC6 (homeobox C6 transcription factor; B), EPAS1 (endothelial PAS domain protein 1); HIF-2alpha (C), ATF3 (activating transcription factor 3; D), FOXF1 (forkhead box F1 transcription factor; E), TBX3 (T-box 3 transcriptional repressor; F), and HSPA5 (heat shock protein 5)/GRP78 (G) in glioma cell line U87 (Vector) and its sublines with a deficiency of both protein kinase and endoribonuclease of the signaling enzyme ERN1/IRE1α (dn-ERN1) and with a deficiency of ERN1/IRE1α endoribonuclease only (dnr-ERN1) as well as in normal human astrocytes line NHA/TS (Astroc.), wild-type U87 cells, and U87 glioma cells stable transfected by wild-type ERN1 (wtERN1) measured by qPCR. mRNA expressions values were normalized to beta-actin mRNA expression and represent as percent of control (vector, 100 %); mean ± SEM; n = 4.
expression, EPAS1 mRNA was found to be significantly lower in normal human astrocyte cells and in malignant cells harboring mutated ERN1/IRE1α than in control glioma cells. Thus, inhibition of ERN1/IRE1α modifies HOXC6 and EPAS1 expression in U87 glioma cells in an anti-proliferative manner. Specifically, inhibition of ERN1/IRE1α endoribonuclease alone is important for the suppression of HOXC6, while inhibition of both kinase and endonuclease activities is needed to block the expression of EPAS1.

We next tested whether ERN1 also participates in the regulation of ATF3 and FOXF1 genes. We found that expression of ATF3, the transcription factor that regulates transcription of numerous proliferative and apoptosis-related genes, is significantly decreased (sevenfold) in glioma cells stably transfected with dn-ERN1, (kinase and endonuclease deficient ERN1/IRE1α) (Figure 1D, Table 2), making its expression comparable to that of ATF3 mRNA in normal human astrocyte cells. Interestingly, inhibition of only the endoribonuclease activity of ERN1/IRE1α leads to a significantly lessened down-regulation of ATF3 gene expression in glioma cells (-21%; Figure 1D, Table 2). We concluded, therefore, that ERN1/IRE1α has a strong effect on ATF3 expression in glioma cells with its activation being needed for the up-regulation of ATF3 expression. Thus, the effect of ERN1/IRE1α on ATF3 strongly depends upon the type of ERN1/IRE1α inactivation.

The transcription factor FOXF1 controls expression of some growth factors and can repress cell growth; however, tumor suppressor functions of FOXO transcription factors are lost in most cancer cells as a result of chromosomal translocation, deletion, miRNA-mediated repression, AKT-mediated cytoplasmic sequestration or ubiquitination-mediated proteasomal degradation [21,27,39]. FOXF1 mRNA, as expected, was found to be significantly higher in normal human astrocyte cells than in control glioma cells (Figure 1E, Table 2). The blocking of ERN1/IRE1α (kinase and endoribonuclease activities) by dn-ERN1 resulted in a down-regulation of FOXF1 mRNA, while inhibition of just the endoribonuclease activity of ERN1/IRE1α alone lead to a strong up-regulation of FOXF1 mRNA expression (in 23fold) (Figure 1E, Table 2), thus indicating the control of FOXF1 expression by ERN1/IRE1α. Interestingly, FOXF1 mRNA was found to be significantly higher in NHA/TS cells than in control glioma cells, being more similar to glioma cells harboring dnr-ERN1 (Figure 1E).

TBX3 is a transcription repressor that plays multiple roles in normal development and diseases of target genes in a context-dependent manner as well as controlling the rate of cell proliferation and mediating cellular signaling pathways [16,24]. It may mediate the antiproliferative role of TGF-β1, but is overexpressed in several cancers [24]. At the same time, normal human astrocyte cells have significantly higher amounts of mRNA for this transcription factor in comparison to control glioma cells. The expression of TBX3 mRNA is strongly induced in glioma cells harboring dnr-ERN1, indicating that activation of ERN1/IRE1α has a negative effect on TBX3 gene expression. Inhibition of endoribonuclease activity of ERN1/IRE1α leads to an even more robust induction of TBX3, most likely due to further transcriptional activation of this gene and/or stabilization of its mRNA (more than in ninefold) in glioma cells (Figure 1F, Table 2). Moreover, normal human astrocytes have a significantly higher level of TBX3 transcription repressor as compared to control and glioma cells overexpressing dnr-ERN1 (Figure 1F, Table 2). Thus, inhibition of ERN1/IRE1α endoribonuclease affects growth regulation, enhancing TBX3 gene expression and making its levels closer to that of non-malignant NHA/TS cells. Please note, different growing conditions were used for NHA/TS (-G418) and dnr-ERN1 glioma cells (+/-G418) as explained above. Therefore, these results may benefit from further validation; although, Figure 1A demonstrates that this difference in growing conditions does not affect E2F8 gene but we did not studied TBX3 gene.

Additionally, we studied the effect of ERN1/IRE1α inhibition on the expression of chaperone HSPA5/GRP78. As shown in Figure 1G, inhibition of ERN1/IRE1α by dn-ERN1 or dnr-ERN1 suppresses the expression of GRP78 gene in glioma cells, supporting the notion of “stippling” the endoplasmic reticulum stress response. At the same time, over-expression of wild-type ERN1 in glioma cells leads to up-regulation of this gene expression. We have also shown that the levels of GRP78/HSPA5 mRNA are similar in wild type glioma cells and in cells harboring the empty vector. Therefore, the presence of the empty vector or addition of G418 to the medium in glioma cells does not significantly change the expression level of GRP78 mRNA and consequently, the endoplasmic reticulum stress response.

In conclusion, we have demonstrated that the ERN1/IRE1α participates in the fine-tuning of mRNA levels of a subset of transcription factor genes important for control of proliferation. Tumor growth suppression in glioma cells with mutated ERN1/IRE1α maybe mediated by such transcription factors.
3.2 ERN1/IRE1α modulates protein levels of E2F8, HOXC6, ATF3, and TBX3 transcription factors in U87 glioma cells

To test whether changes in mRNA levels caused by inhibition of ERN1/IRE1α by dn-ERN1 or dnr-ERN1 correspond to the changes in the level of protein of E2F8, HOXC6, ATF3, and TBX3, we measured levels of these transcription factors by Western blot analysis. As shown in Figure 2A, the level of E2F8 strongly decreased in glioma cells harboring dn-ERN1 as well as dnr-ERN1, but inhibition of only the endoribonuclease activity of ERN1 leads to even more significant down-regulation of this transcription factor, and correlate to the changes in the mRNA level (Figure 1A). As shown in Figure 2B, the inhibition of only the endoribonuclease of ERN1/IRE1α by dnr-ERN1 in glioma cells has a strikingly suppressive effect on the level of HOXC6 protein, with a slightly lesser effect on this gene expression in double mutants of ERN1/IRE1α (dn-ERN1), which has both endonuclease and kinase activities affected. Thus, the changes in protein and mRNA levels are similar in glioma cells harboring dn-ERN1 and dnr-ERN1 as compared to control glioma cells (Figures 1B and 2B).

Western blot analysis of TBX3 protein demonstrates that it is induced in glioma cells harboring dn-ERN1 (Figure 2C), indicating that activation of ERN1/IRE1α also has a negative effect on TBX3 gene expression at proteinaceous level. Simultaneously, inhibition of the endoribonuclease activity of ERN1/IRE1α leads to a more heightened induction of TBX3 protein level in glioma cells (Figure 2C), making TBX3 protein levels comparable to the expression level of mRNA for same transcription factor in these experimental conditions. We also found that the protein level of transcription factor ATF3 is significantly lower in glioma cells stably transfected with dn-ERN1 (Figure 2D). These results are comparable to the expression of ATF3 mRNA in glioma cells. Interestingly, inhibition of only the endoribonuclease activity of ERN1/IRE1α leads to a significantly lower down-regulation of ATF3 protein level in glioma cells harboring dnr-ERN1 (Figure 2D), which also correlates to changes in ATF3 mRNA expression.

3.3 Induction of endoplasmic reticulum stress in U87 glioma cells, which constitutively expresses dnr-ERN1, modulates expression in most of transcription factor genes

To determine if endoplasmic reticulum stress regulates the genes tested above through the kinase activity of ERN1/IRE1α or other branches of ERSR, we investigated the effect of tunicamycin on the expression of E2F8, EPAS1, TBX3, ATF3, FOXF1 and HOXC6 genes. As shown in Figure 3A, induction of endoplasmic reticulum stress by tunicamycin in glioma cells containing dnr-ERN1 leads to strong suppression of E2F8 mRNA expression (more than sevenfold) and to a threefold up-regulation of ATF3 mRNA expression. We also found that expression of

![Figure 2](https://example.com/f2.png)
genes encoding for transcription factors FOXF1, HOXC6, and TBX3 are decreased in glioma cells lacking the endoribonuclease activity of ERN1 treated by tunicamycin (-57 %, -47 %, and -34 %, correspondingly) (Figure 3B, 3C). These results demonstrate that all of the studied genes are responsive to endoplasmic reticulum stress, but the mechanisms of its activation or deactivation are variable. Congruently, the regulation of FOXF1 and TBX3 mRNA expression in the circumstance of endoplasmic reticulum stress is realized through different signaling pathways and inhibition of ERN1/IRE1α endoribonuclease does not eliminate additional regulation of these gene expressions by tunicamycin (Figure 2B and 2C).

Contrary to the above, tunicamycin experiments have demonstrated that inhibition of endoribonuclease activity of ERN1/IRE1α by dnr-ERN1 (Figure 3C) leads to tunicamycin-resistance in the expression of EPAS1/HIF-2α mRNA. EPAS1/HIF-2α is an endoplasmic reticulum stress responsive gene, expression of which is increased in most malignant tumors [33], including U87 glioma cells (Figure 1C). Our results point out that induction of EPAS1/HIF-2α mRNA expression during endoplasmic reticulum stress is realized solely through ERN1/IRE1α and inhibition of its endoribonuclease leads to tunicamycin resistance.

In addition, Figure 3D demonstrates that treatment of glioma cells harboring dnr-ERN1 by 2 and 8 hours of tunicamycin significantly induces (2.6 and 2.4-fold, correspondingly) expression of GRP78/HSPA5 mRNA. Therefore, the presence of the empty vector or addition of G418 to the medium in glioma cells does not significantly change the expression level of GRP78 mRNA and consequently the endoplasmic reticulum stress response.

In conclusion, inhibition of the endoribonuclease activity of ERN1/IRE1α does not eliminate stress dependent regulation of all studied transcription factors, with the exception of EPAS1, by protein kinase of ERN1/IRE1α or by other branches of the endoplasmic reticulum stress response.

Figure 3: Effect of tunicamycin (0.01 mg/ml - 2 and 8h) on the expression levels of E2F8 and ATF3 (A), FOXF1 and HOXC6 (B), EPAS1 and TBX3 (C), and HSPA5/GRP78 (D) mRNAs (by qPCR) in glioma cells with a deficiency of ERN1/IRE1α endoribonuclease (dnr-ERN1). mRNA expression values were normalized to beta-actin mRNA expression and represent as percent of dnr-ERN1 non-treated cells as control, 100 %; mean ± SEM; n = 4.
3.4 Inhibition of endoribonuclease activity of ERN1/IRE1α strongly suppresses proliferation

We showed that inhibition of both kinase and endoribonuclease activities of ERN1/IRE1α results in changes in the expression of different genes which encode transcription factors related to control of proliferation and apoptosis. To see whether those changes affect cell proliferation, cells with and without ERN1/IRE1α were grown under normal conditions. Figure 4 demonstrates that the proliferation rate of glioma cells expressing dn-ERN1 is only twofold lower after 3 days in culture as compared to control U87 glioma cells; with a fourfold inhibition of proliferation in cells expressing dnr-ERN1.

Therefore, inhibition of the endoribonuclease activity of ERN1 can result in a more substantial suppression of malignant cell proliferation, possibly though a stronger deregulation of key transcription factors responsible for the control of proliferation and apoptosis.

4 Discussion

This study has demonstrated that inhibition of the endoribonuclease activity alone or both endonuclease and kinase activities of ERN1/IRE1α together in U87 glioma cells, causes a strong decrease in the levels of E2F8 mRNA and protein (Figure 1A and 2A). Low amounts of E2F8 affect the composition and levels of its complexes with other members of E2F family of transcription factors and influence various cellular functions through the regulation of its target genes, of the cell cycle, apoptosis, and angiogenesis (including transcriptional activation of VEGFA in cooperation with HIF1) [17,22]. We have also demonstrated that ablation of only the endoribonuclease activity of ERN1/IRE1α in U87 glioma cells has a more pronounced suppressive effect on the expression of the E2F8 gene. Very low levels of E2F8 gene expression and protein observed in glioma cells with modulated ERN1/IRE1α are close to that in NHA/TS (Table 2, compare columns 3 and 2, and Figure 1A). Therefore, inhibition of ERN1/IRE1α endoribonuclease affects U87 glioma cell growth by regulating E2F8 mRNA expression and lowering it to the level observed in normal human astrocytes.

Similarly, but significantly less profound, were changes in HOXC6 gene expression (Table 2 and Figure 1B), which are consistent with its pro-proliferative role. Moreover, the expression of HOXC6 mRNA in glioma cells is significantly higher than compared to that of NHA/TS cells. Inhibition of ERN1/IRE1α, especially its endoribonuclease activity via dnr-ERN1, leads to a significant decrease in HOXC6 gene expression in U87 glioma cells, making its levels closer to non-malignant NHA/TS cells (Figure 1B). The HOXC6 gene encodes for a transcription factor that may contribute to the progression of gastric carcinogenesis as a pro-proliferative regulator because it plays an important role in proliferation, morphogenesis and metastasis. This is due to the fact that it regulates genes with both oncogenic and tumor suppressor activities [20,25,26]. Thus, down-regulation of HOXC6 mRNA in glioma cells harboring dnr-ERN1 (Figure 1B) conforms to the suppression of proliferation in these cells (Figure 4).

Figure 4: The rate of proliferation of control glioma U87 cells, transfected stably with vector (Vector) and its sublines with a deficiency of both protein kinase and endoribonuclease of the signaling enzyme ERN1 (dn-ERN1) and with a deficiency of only endoribonuclease of the ERN1/IRE1α enzyme (dnR-ERN1). Results represent as percent of control (100 %); n = 5.
Therefore, inhibition of the endoribonuclease activity of ERN1/IRE1α alone leads to a more robust suppression of malignant cell proliferation as compared to cells lacking both kinase and endoribonuclease activity (Figure 4). It is possible that the effect of ERN1/IRE1α inhibition on cell proliferation and gene expressions is mediated through its endoribonuclease activity and that a more robust effect of inhibition of the endoribonuclease activity of ERN1/IRE1α versus the inhibition of both its kinase and endoribonuclease activities is due to the additional role of ERN1/IRE1α kinase. We have also found that expression of E2F8 and HOXC6 mRNA in normal human astrocytes is very low compared to control glioma cells and that inhibition of ERN1/IRE1α, especially its endoribonuclease activity, changes the expression of both E2F8 and HOXC6 gene expressions in the direction of normalization (Table 3 and Figure 1A and B). Thus, down-regulation of E2F8 and HOXC6 gene expression nearing the levels seen in normal human astrocytes may contribute to suppression of glioma cell proliferation upon inhibition of ERN1/IRE1α endoribonuclease.

We have demonstrated that the expression of T-box transcription factor TBX3 is elevated in glioma cells when ERN1/IRE1α function is inhibited; with the effect being more pronounced when only the endoribonuclease activity of ERN1/IRE1α was suppressed (Table 2 and Figure 6). This increase may contribute to the suppression of cell proliferation and glioma growth because TBX3 is a transcriptional repressor that mediates cellular signaling pathways and controls the rate of cell proliferation [16,24]. This gene was found to be highly expressed in normal human astrocytes as compared to control glioma cells (Table 3 and Figure 1F). In tandem, the transcription factor TBX3 plays multiple roles in normal development and diseases by either repressing or activating transcription of its target genes in a context-dependent manner and it may mediate the anti-proliferative and pro-migratory role of TGF-β1 in breast epithelial and skin keratinocytes; however, its overexpression is associated with several cancers [24]. Thus, increased expression of TBX3 can mediate inhibition of cell growth as well as contribute to the suppression of glioma cell proliferation after inhibition of ERN1/IRE1α, since it has pleiotropic functions.

We have demonstrated that expression of E2F8, EPAS1, HOXC6, FOXF1, and ATF3 genes is decreased in glioma cells with inhibited ERN1/IRE1α via overexpressing dn-ERN1 (Table 2 and Figure 1). This decrease may also contribute to the suppression of cell proliferation (Figure 4) and tumor growth [14], because proteins encoded by these genes have predominantly pro-proliferative functions [18,19,28,29,30,33] and expression in normal human astrocytes are significantly less than in control glioma cells, with the exception of the FOXF1 gene.

Our results demonstrate that all of the genes studied are endoplasmic reticulum stress responsive, but the mechanisms of activation or suppression of expression, upon inhibition of ERN1/IRE1α differs. Tunicamycin experiments helped to clarify some aspects of these regulatory mechanisms. It is possible that in response to endoplasmic reticulum stress the up-regulation of E2F8 and HOXC6 mRNA is realized through the signaling pathway mediated by ERN1/IRE1α and inhibition of endoribonuclease does not lead to the up-regulation of E2F8 and HOXC6 by tunicamycin, which is significantly higher in U87 glioma cells compared to normal human astrocytes (Figures 1A and B). At the same time, the regulation of FOXF1 and TBX3 mRNA due to endoplasmic reticulum stress is likely mediated through different signaling pathways. Inhibition of ERN1/IRE1α endoribonuclease does not eliminate the down-regulation of these gene expressions by tunicamycin (Figure 2B and 2C), which is in agreement with the functional role of these transcription factors [16,24,39]. EPAS1/HIF-2α is an endoplasmic reticulum stress responsive gene, expression of which is increased in U87 glioma cells (Figure 1C) as well as in most malignant tumors [33]. EPAS1/HIF-2α expression in glioma cells harboring dnr-ERN1 is resistant to the induction of endoplasmic reticulum stress by tunicamycin (Figure 3C). It is possible that induction of EPAS1/HIF-2α expression, in the case of endoplasmic reticulum stress, is achieved through a ERN1/IRE1α mediated signaling pathway where inhibition of endoribonuclease leads to tunicamycin resistance.

Notably, endoplasmic reticulum stress modulates the function of various chaperones in the cell, including a central player in the unfolded protein response and a major endoplasmic reticulum chaperone, HSPA5/GRP78. HSPA5/GRP78 is overexpressed in many cancers, and implicated in cancer cell survival, since it has Ca(2+)-binding and anti-apoptotic properties and promotes tumor progression.

### Table 3: Summary of the expression level of transcription factor genes in glioma cells versus normal human astrocytes line NHA/TS

| Gene       | Tested condition | U87 glioma cells versus normal astrocyte cells |
|------------|------------------|-----------------------------------------------|
| E2F8       | 250 fold up      |                                               |
| TBX3       | 30 fold down     |                                               |
| EPAS1      | 2.2 fold up      |                                               |
| ATF3       | 1.4 fold down    |                                               |
| FOXF1      | 23 fold down     |                                               |
| HOXC6      | 12 fold up       |                                               |
proliferation, survival, metastasis, and resistance to a wide variety of therapies. Therefore, selective destruction of HSPA5/GRP78 could be potentially utilized as a novel anticancer strategy [40-42]. Our results demonstrating down-regulation of HSPA5/GRP78 expression in cells with inhibited ERN1/IRE1α (Figure 1G) and its correlation with a growth inhibition support this supposition. Moreover, sarcoplasmic reticulum Ca(2+) ATPase type 2 is down-regulated in some human cell carcinomas and its inhibition induces endoplasmic reticulum stress response, and exerts toxicity in glioma cells [43,44]. Endoplasmic reticulum stress also mediates both apoptosis and autophagy induced by cyclosporine A in malignant glioma cells via mTOR/p70S6K1 pathway [45].

In conclusion, inhibiting ERN1/IRE1α endoribonuclease affects tumor growth by lowering expression of transcription factors: E2F8, HOXC6, EPAS1, and ATF3, all of which have preferentially pro-proliferative properties and up-regulate expression of FOXF1 and transcriptional repressor TBX, helping to return the glioma cell to levels seen in normal human astrocytes (NHA/TS). Moreover, inhibition of the endoribonuclease activity of ERN1/IRE1α does not eliminate UPR-dependent regulation of transcription factors FOXF1, TBX3, E2F8, and HOXC6 by the kinase activity of ERN1/IRE1α or by other branches of endoplasmic reticulum stress response. Thus, the changes observed in the above studied transcription factors correlate well with slower cell proliferation in cells harboring dn-ERN1 or dnr-ERN1, attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant tumor growth and cell survival [2, 3, 6, 11].

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