Pluquet et al., Supplemental information

Supplemental Tables

Table S1: **Genes up-regulated in IRE1_DN cells compared to EV cells.** To determine genes whose expression increased when IRE1α is inactivated, probe set intensities were obtained by means of GCRMA and were selected by using a corrected *P* value treshhold of 0.05 and fold change threshold of $|\log_2(fc)| \geq 2.5$. The gene set was analyzed by functional annotation using the web-based tools provided by the DAVID resource (http://david.abcc.ncifcrf.gov) among the non-secreted targets. The most significant biological process found was PANTHER BP00044: mRNA transcription regulation (pvalue< 0.01)

Gene	Name	Log2 (Fold Change)	Probesets
LIM domain binding 2	LDB2	4,68	206481_s_at
Forkhead box N3	FOXN3	3,96	218031_s_at
T-box 3	TBX3	3,85	219682_s_at
Kruppel-like factor 11	KLF11	3,84	218486_at
Transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	TLE4	2,90	204872_at
Retinoic acid receptor, beta	RARB	2,89	205080_at
Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	TLE1	2,84	203222_s_at
Period homolog 1 (Drosophila)	PER1	2,71	202861_at
Forkhead box O1A	FOXO1	2,68	202724_s_at
V-myb myeloblastosis viral oncogene homolog (avian)-like 1	MYBL1	2,58	213906_at
SRY (sex determining region Y)-box 9	SOX9	2,57	202935_s_at

Table S2: Identification of the potential IRE1α-mediated cleavage sites found on *Per1* mRNA. *This was carried out using the combination of sequence alignment and RNA structural analyses carried out with the M-fold program.*

Site	Potential 5' cleavage product (bases)	Potential 3' cleavage product (bases)
1	611	4106
2	1476	3241
3	1920	2797
4	3197	1520
5	3378	1339

Table S3: Characteristics of 20 glioblastoma samples towards XBP1s protein expression and localization. *The case # is indicated as well as the grade of the tumor, the type of staining observed using immunohistochemistry and revealed using diaminobenzidine and horseradish peroxidase. The age, sex and survival (days) of patients is also indicated.*

	Grade	XBP1s staining	Age	Sex	Survival (Days)
1	4	No staining (D)	49	F	530
2	4	Cytoplasmic (E)	71	M	207
3	4	Cytoplasmic (E)	51	F	261
4	4	No staining (D)	33	M	507
5	4	Cytoplasmic (E)	46	M	264
6	4	Nuclear (F)	57	F	144
7	4	No staining (D)	36	F	702
8	4	Cytoplasmic (E)	43	F	85
9	4	Cytoplasmic (E)	71	F	173
10	4	No staining (D)	74	M	13
11	4	Cytoplasmic (E)	53	F	261
12	4	Cytoplasmic (E)	41	M	305
13	4	No staining (E)	50	M	387
14	4	Cytoplasmic (E)	80	F	56
15	4	Cytoplasmic (E)	64	F	104
16	4	Cytoplasmic (E)	52	F	452
17	4	Cytoplasmic (E)	74	F	100
18	4	Cytoplasmic (E)	46	M	249
19	4	No staining (D)	44	M	469
20	4	Cytoplasmic (E)	57	M	64

Table S4: Primer Pairs used for PCR and qPCR studies.

Gene ID	Accession number	FWD	REV
PCR			
hPer1 e13-16	NM_002616	5'-GGGTCCTCCAGTGATAGCAA-3'	5'-GAGGAGGAGGCACATTTACG-3'
hGapdh	NM_002046	5'-ACCACCATGGAGAAGGCTGG-3'	5'-CTCAGTGTAGCCCAGGATGC-3'
hXbp1	NM_005080	5'-GGAACAGCAAGTGGTAGA-3'	5'-CTCAGTGTAGCCCAGGATGC-3'
hIre1α	NM_001433	5'-GGCCTGGTCACCACAATTAG-3'	5'-ATTCCCTCCTCTCCCTTCCT-3'
Ire1-Nck	----------	5'-TCATCACCTATCCCCTGAGC-3'	5'-AGATGCTTTCCGAGCACTGT-3'
hOrp150	NM_006389	5'-GAAGATGCAGAGCCCATTTC-3'	5'-TCTGCTCCAGGACCTCCTAA-3'
qPCR			
hPer1 e4-8	NM_002616	5'-CAAGGACTCAGAAGGAACTCATGACAG-3'	5'-GTACCGAGGCCCTGGATCCCGGTCAG-3'
hPer1 e23	NM_002616	5'-CAGGACTTGGCTATGGAGGA-3'	5'-AGGCTCAGCTGGAATATGGA-3'
hPer1	NM_002616	5'-AGGAAGGAGACAGCCACTGA-3'	5'-TATACCCTGGAGGAGCTGGA-3'
hPer2	NM_022817	5'-TACGCTGGCCACCTTGAAGTA-3'	5'-CACATCGTGAGGCGCCAGGA-3'
hXrn1	NM_019001	5'-GATGGATCTCAGAGCGGTATCC-3'	5'-CAGGTACAAGTTGTCAAATTCAGGAA-3'
hXrn2	NM_012255	5'-CGCAAGTACCCGTCCATCA-3'	5'-CTGGAATCTTTACACCATTGCATT-3'
hSki2	NM_006929	5'-ACTGGGAGCTGCTGAACTTG-3'	5'-CCATGGGGAAGGCTACTCTC-3'
Rplp0	NM_007475	5'-GGCGACCTGGAAGTCCAACT-3'	5'-CCATCAGCACCACAGCCTTC-3'
CXCL3	NM_002090	5'-TCCCCCATGGTTCAGAAAATC-3'	5'- GGTGCTCCCCTTGTTCAGTATCT-3'
Axin2	NM_004655	5'-TGCTCTGTTTTGTCTTAAAGGTCTTGA-3'	5'-ACAGATCATCCCATCCAACACA-3'
Cyclin D1	NM_053056	5'-AATGACCCCGCACGATTTCA-3'	5'-CTCCCCGCTGCCACCAT-3'
GPR49	NM_003667	5'-GAGGATCTGGTGAGCCTGAGAA-3'	5'-GATGCTGGAGCTGGTAAAGGT-3'
Cyclin B1	NM_031966	5'-CGGGAAGTCACTGGAAACAT-3'	5'-AAACATGGCAGTGACACCAA-3'
Cdc2	NM_001786	5'-CAGTCTTCAGGATGTGCTTATGC-3'	5'-GAGGTTTTAAGTCTCTGTGAAGAACTC-3'
Wee1	NM_003390	5'-ATTTCTCTGCGTGGGCAGAAG-3'	5'-CAAAAGGAGATCCTTCAACTCTGC-3'
Bip	NM_005347	5'-GCTTATGGCCTGGATAAGAGG-3'	5'-CCACAACTTCGAAGACACCAT-3'
Chop	NM_001195053	5'-AAGGCACTGAGCGTATCATGT-3'	5'-TGAAGATACACTTCCTTCTTGAAC-3'
Edem	NM_014674	5'-AGTCATCAACTCCAGCTCCAA-3'	5'-AACCATCTGGTCAATCTGTCG-3'
ERdj4	NM_012328	5'-TGGTGGTTCCAGTAGACAAAGG-3'	5'-CTTCGTTGAGTGACAGTCCTGC-3'
GRP94	NM_003299	5'-CCCCATGGCTTATATTCACTTTAC-3'	5'-TCTTGTTTTTCCTTCATTCTTTCC-3'
SEC61	NM_013336	5'-CTTAGTGTGCTGCCAGATTCC-3'	5'-CTCCATCAATGTGCCTCTGTT-3'
XBP1s	NM_001079539	5'-GGAACAGCAAGTGGTAGA-3'	5'-CTGGAGGGGTGACAAC-3'

Table S5: siRNA sequences

Gene ID	Sequence
GL2	5'-CGUACGCGGAAUACUUCGA-3'
Ire1α	5'-UUACUGGCUUCUGAUAGGA-3'
Xrn1	5'- GCCUUUGUCUCCUAACUGA-3'
	5'- UCCUCAAUGUGUAUAAUUA-3'
Xrn2	5'-UUCAGUAUUUCUUUGGAAA-3'
	5'-CAGUGGAUCUGAAUUUAUU-3'
Ski2	5'-CAGAGAAACGGCUAUGAGA-3'
	5'-CCGUUAUCCUGCUCUGCAA-3'
Xbp1	5'-CUCAUGGCCUUGUAGUUGA-3'

Supplemental Experimental Procedures

Assay for cell growth (SRB) - The SRB assay was performed as previously described (Vichai and Kirtikara, 2006). Briefly, cells were seeded into 96-well plates in 100 ml at a density of 5000 cells/well. After cell inoculation, the plates were incubated at 37°C for 24, to 96 h. Cell were then fixed in situ with trichloroacetic acid and stained with sulforhodamine B (Sigma, St Louis, MO, USA). Absorbance was measured at 510 nm.

Data analyses - All experiments were performed at least 3 times. Assays were analyzed using a paired or unpaired t-test or ANOVA following Newman-Keuls or Bonferroni test as appropriated, performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Antibodies- Antibodies against PDGFRB were purchased from Santa Cruz Biotechnology, against BAK, p53 and Bcl2 from Calbiochem, against p21 from Cell Signaling Technology, and against fibronectin from BD Biosciences. Proteins were detected using secondary antibodies coupled to HRP (Dako SA) and immunoblots revealed using enhanced chemiluminescence and radioautography.

Supplemental Figures

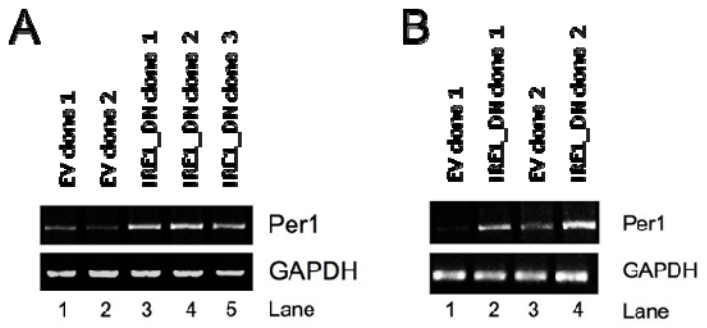

Figure S1: (**A**) Total RNA was isolated in different clones of U87 cells carrying either empty vector (EV) or IRE1_DN vector and assayed for *Per1* mRNA expression levels. (**B**) The same as in (A) in HepG2 cells.

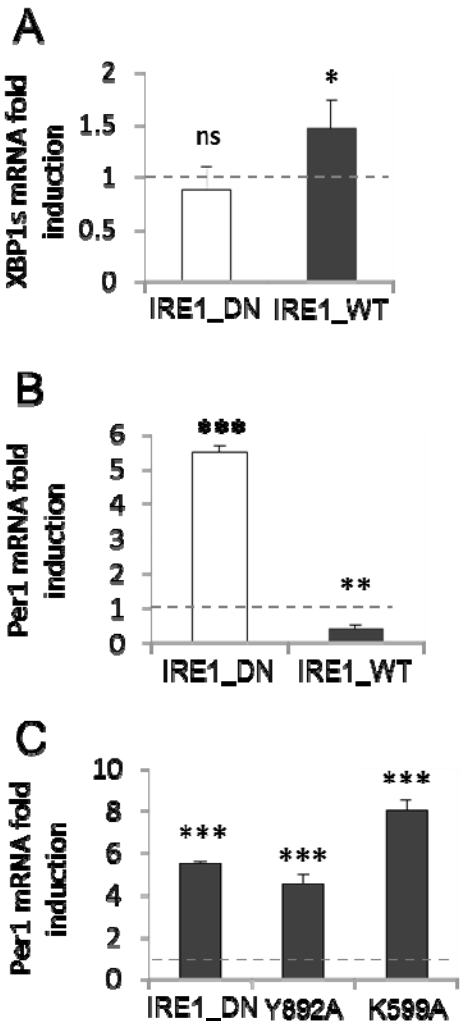

Figure S2: RT-qPCR of the spliced form of XBP1 (**A**) or *PER1* (**B**) and (**C**), performed on total RNA isolated from U87 transfected with IRE1α-WT or IRE1_DN and or RNase dead Y892A or kinase dead K599A IRE1α mutants. Data are representative of three independent experiments. Values were normalized to 18S rRNA and then to the control condition for each experiment. The dash line represents the control value (1). The experiment was carried out in triplicate and is presented as the mean ± SD. *, $p < 0.05$; **, $p < 0.01$, *** $p < 0.001$. As previously shown (Han et al., Cell, 2009), IRE1α overexpression resulted in XBP1 splicing (**A**). On the contrary, *PER1* mRNA levels increased in response to IRE1α mutant transfections (**B**) and (**C**).

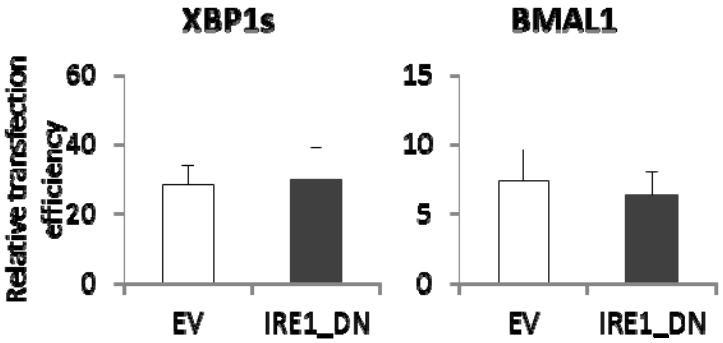

Figure S3: determination of the amount of plasmids (XBP1s or BMAL1) transfected in EV and IRE1_DN cells, as determined by PCR. Data are presented as the mean ± SD.

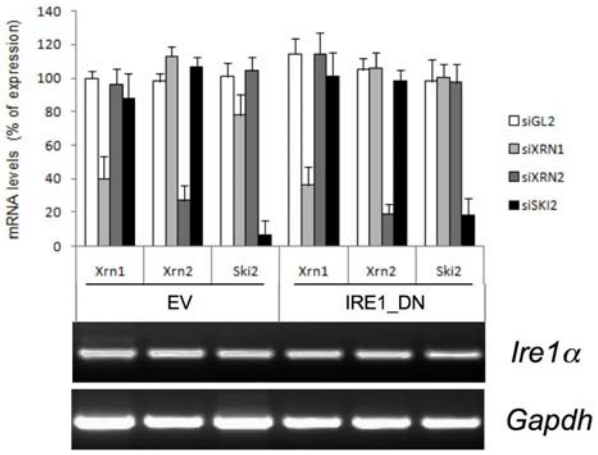

Figure S4: EV or IRE1_DN cells were transfected with siRNA against XRN1/2 or SKI2. RNA was isolated after 48 h and used to quantify XRN1/2 or SKI2 mRNA in control (siGL2) and RNAi-treated cells to determine the efficacy and specificity of RNA interference. Data are representative of at least three independent experiments. For each experimental condition, the expression level of *Ire1α* mRNA was determined by RT-PCR and reported to that of *Gapdh* (bottom panel).

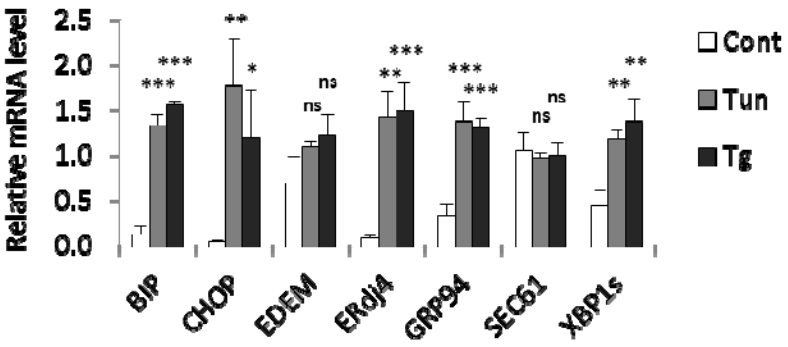

Figure S5: assessment of basal UPR in U87. Total RNA was isolated in U87 cells treated or not with ER stressors tunicamycin (Tun, 10 µg/ml, 6 h) or thapsigargine (Tg, 1 µM, 6 h) and assayed for UPR targets or control non-target (SEC61) mRNA expression levels. Data are presented as the mean ± SD. For each gene and experiment, values were normalized to the mean of all conditions. Statistical significance: *, $p < 0.05$; **, $p < 0.01$, *** $p < 0.001$. Excepted for EDEM and control SEC61, all UPR target gene expressions were statistically increased under stress.

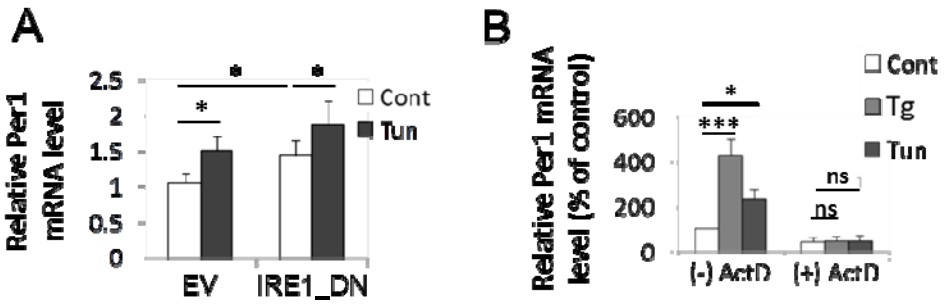

Figure S6: stress independent regulation of *PER1* by IRE1α. (**A**) Total RNA was isolated in EV or IRE1_DN cells treated or not with tunicamycin (Tun, 10 µg/ml, 6 h). (**B**) Total RNA was isolated in U87 cells treated or not with tunicamycin (Tun, 10 µg/ml, 6 h), thapsigargine (Tg, 1 µM, 6 h) and/or actinomycin D (ActD, 5 µg/ml, 6 h). *PER1* mRNA levels were measured by RT-qPCR. Data are presented as the mean ± SD. Statistical significance: *, $p < 0.05$; *** $p < 0.001$. In (**A**), Per1 mRNA levels increased under stress and in IRE1_DN compared to EV cells. In (**B**), following transcription blocking by ActD, the modulation of Per1 mRNA level by ER stressors was abrogated. This showed that *PER1* mRNA cleavage by IRE1α was not increased under ER stress.

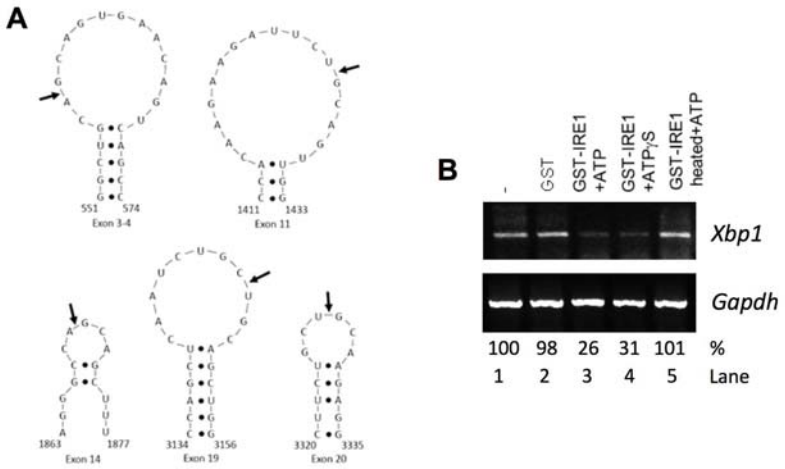

Figure S7: (**A**) Two-dimensional modeling of potential *Per1* mRNA cleavage sites using the M-Fold program. This reveals that most of the sites predicted from the alignment form P-Loops, structures necessary for IRE1α-mediated cleavage. (**B**) *In vitro* RNA cleavage assay. Total RNA extracted from U87 cells was incubated with GST or GST-IRE1α^{cyto} in the presence of ATP for 2h at 37°C. RT-PCR was then performed to determine *Xbp1* and *Gapdh* mRNA levels.

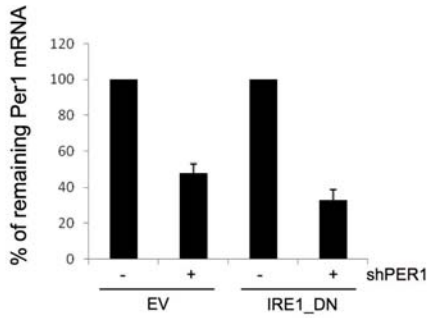

Figure S8: Expression of *Per1* mRNA in U87 cells upon lentiviral transduction. RT-PCR was used to measure *Per1* mRNA abundance. All values were normalized to *Gapdh* expression.

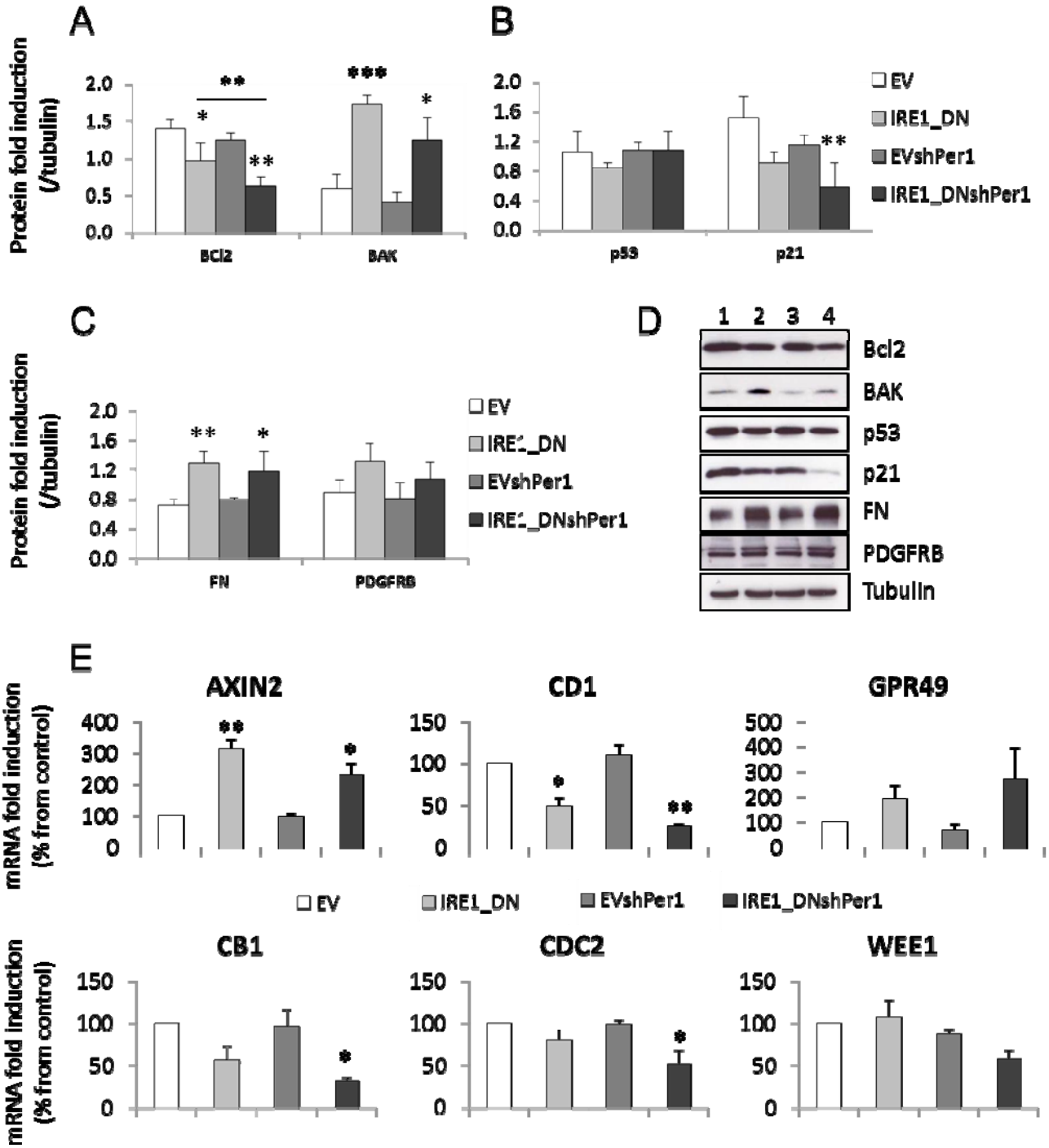

Figure S9: determination of protein (**A-D**) and mRNA (**E**) expression levels of potential PER1 targets in EV and IRE_DN cells transduced or not with shPER1. mRNA expression levels were determined using RT-qPCR. FN, fibronectin; CD1, cyclin D1; CB1, cyclin B1. Data are presented as the mean ± SD for protein and mean ± SEM for mRNA. Statistical significance: *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (**A-C**) show the quantification of immunoblots. (**D**) a representative immunoblot is presented for the corresponding proteins. Column 1, EV; 2, IRE1_DN; 3, EVshPER1; 4, IRE1_DNshPER1.

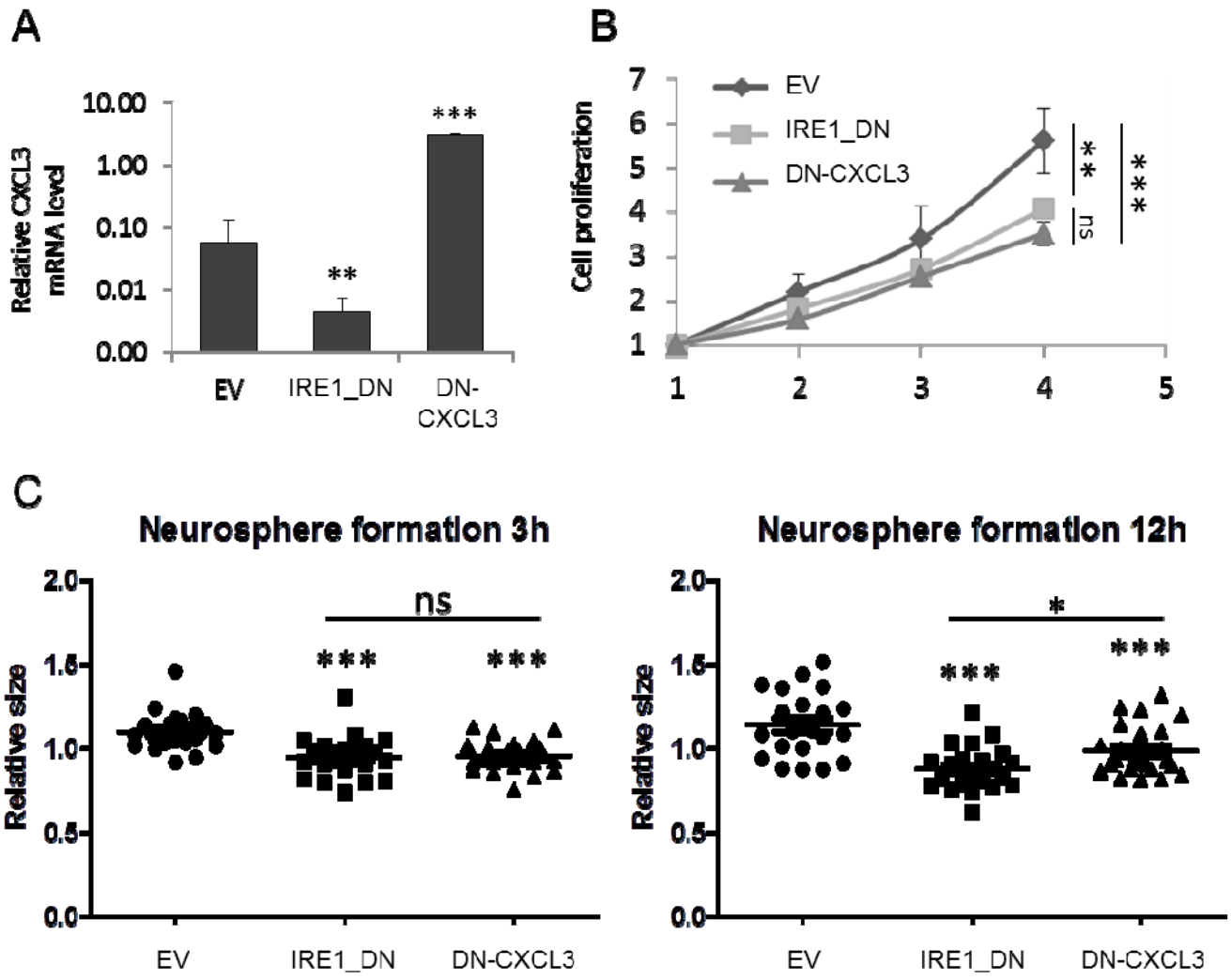

Figure S10: impact of CXCL3 re-expression on cell proliferation and neurosphere formation in IRE1_DN cells. (**A**) *CXCL3* mRNA level was measured by RT-qPCR in EV, IRE1_DN and IRE1_DN cells transduced with CXCL3 ORF (DN-CXCL3) (Fermentas, USA). (**B**) cell proliferation was assessed by sulforhodamine B assay for the corresponding time (days). (**C**) the ability of corresponding cells to form neurospheres was evaluated 3 and 12 h after seeding. Areas of the neurospheres were estimated by the Image J software. Data are presented as the mean ± SD (**A and B**) and mean ± SEM (**C**). Statistical significance: *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

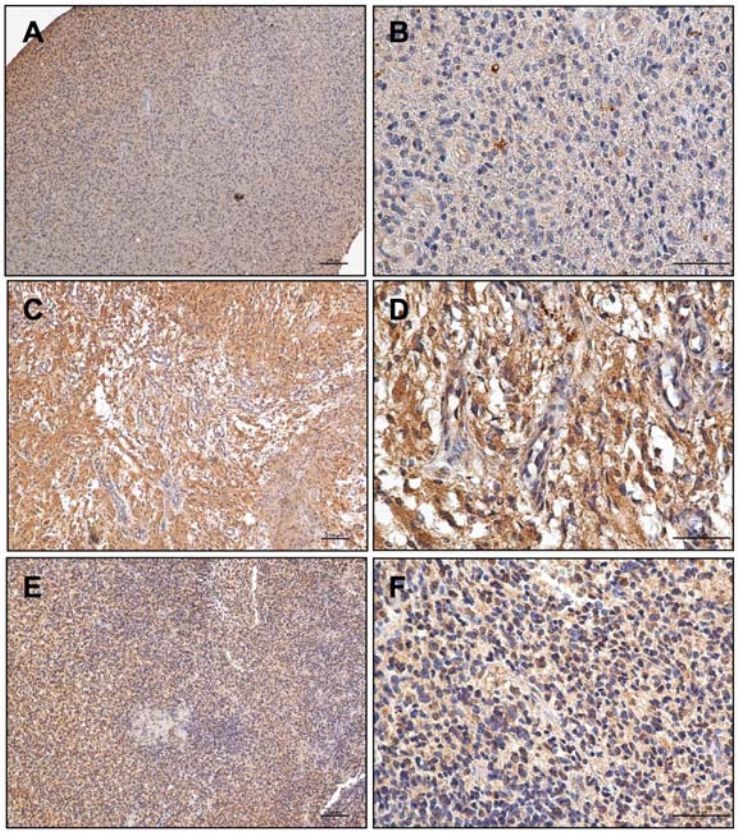

Figure S11: (**A-F**) Immunohistochemical analysis of 3 typical glioblastoma paraffin sections using anti sXBP1 antibodies. Higher magnification of A, C and E are shown in B, D and F respectively.