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Posttranscriptional Regulation of PER1 Underlies the Oncogenic Function of IREα

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Abstract

Growing evidence supports a role for the unfolded protein response (UPR) in carcinogenesis; however, the precise molecular mechanisms underlying this phenomenon remain elusive. Herein, we identified the circadian clock PER1 mRNA as a novel substrate of the endoribonuclease activity of the UPR sensor IRE1α. Analysis of the mechanism shows that IRE1α endoribonuclease activity decreased PER1 mRNA in tumor cells without affecting PER1 gene transcription. Inhibition of IRE1α signaling using either siRNA-mediated silencing or a dominant-negative strategy prevented PER1 mRNA decay, reduced tumorigenesis, and increased survival, features that were reversed upon PER1 silencing. Clinically, patients showing reduced survival have lower levels of PER1 mRNA expression and increased splicing of XBP1, a known IRE-α substrate, thereby pointing toward an increased IRE1α activity in these patients. Hence, we describe a novel mechanism connecting the UPR and circadian clock components in tumor cells, thereby highlighting the importance of this interplay in tumor development.

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Introduction

The tumor microenvironment, and in particular, hypoxia and nutrient limitation, can lead to perturbations of endoplasmic reticulum functions, thereby resulting in the activation of an adaptive response named the unfolded protein response (UPR; refs. 1, 2). The UPR primarily provides tumor cells with the ability to cope with stress and to adapt for survival. In addition to its role in cellular adaptation, the UPR, and in particular IRE1α signaling, have been proposed to play significant roles during tumor development. This was supported by the identification of somatic mutations in the IRE1 gene (3) or the dysregulation of endoplasmic reticulum stress targets in various cancers (4–6). Moreover, the RNAse activity of IRE1α and the XBP1 transcription factor, whose mRNA is spliced by the combined action of IRE1α RNAse activity and a yet unknown ligase, have also been found to be necessary for tumor formation and growth in multiple myeloma, glioblastoma, and transformed embryonic fibroblast (7–9). Although our data have pointed toward a role for IRE1α signaling in tumor biology, IRE1α-dependent signaling pathways involved in such process still remain unclear.

In the present study, using glioblastoma as a model, we show that IRE1α endoribonuclease unexpectedly cleaves the mRNA encoded by the core circadian clock gene, PER1, thereby leading to its degradation. As PER1 is not a secretory protein but rather localizes to the cytosol/nucleus, this could therefore contribute to the regulation of a central signaling pathway and to an endoplasmic reticulum-dependent control of tumor growth. Collectively, we define a novel interplay between IRE1α and PER1 regulating tumor growth and angiogenesis, an observation consistent with the emerging role of PER1 in cancer (10, 11). Moreover, the analysis of clinical samples revealed that low PER1 mRNA expression and high XBP1 mRNA splicing correlated with poorer prognoses. These results identify IRE1α as a master regulator of cellular homeostasis in tumors, and provide the rationale for the development of IRE1α-targeted therapies in cancer cells.

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Materials and Methods

Recombinant protein expression

IRE1-cyt0 cDNA (AA 470–977) was cloned from human liver cDNAs using the Gateway technology (Invitrogen Corp.) into either pGEX-2TK or pDEST17. IRE1-cyt0 cDNA devoid of ATG, was amplified by PCR using the Platinum Taq DNA Polymerase High Fidelity (Invitrogen Corp.) and the following amplifica-
tion scheme: denaturation at 94 °C for at 40 seconds, annealing at 60 °C for 40 seconds, elongation at 68 °C for 2 minutes, 35 cycles. The PCR products were precipitated using PEG8000 and recombined into pDONR201 using the Gateway BP clonase (Invitrogen Corp.). The plasmids were then transformed into competent DH5α cells and positive clones selected and sequenced. Positive clones were recombined into destination vectors using LR clonase (Invitrogen Corp.). Five individual colonies were selected and pooled and plasmid DNA was ampli-
cified and subsequently transformed into competent BL21 bacterial cells. Recombinant protein expression in BL21 cells was induced using 1 mmol/L IPTG for 3 hours. Bacteria were then collected by centrifugation, lysed, and recombinant pro-
teins purified as recommended by the manufacturer (Gibco BRL). The resulting purified proteins were concentrated and diazoyed using Amicon ultra centrifugal filters (cutoff = 20,000 Da; Millipore Corp.), followed by functional testing as previously described (12, 13).

Animal experiments, intracranial injections, tumor size, and blood capillary measurements

The protocol used was as previously described (14, 15) and was approved by the local animal committee. Cell implantations (2 × 10⁶ cells) in Nude mice were at 2 mm lateral to the bregma and 3 mm in depth using empty vector and IRE1_DN cells stably expressing pGIPZ-GFP-shPer1 or pGIPZ-GFP alone. Twenty-eight days postinjection, brain sections were observed for GFP fluorescence and stained using hematoxy-
ylin and eosin for visualization of tumor masses. Tumor volume was then estimated by measuring the length (L) and width (W) of each tumor and was calculated using the following formula (L × W² × 0.5). CD31-positive cells and Ki-67–positive cells were enumerated from histo-
logic staining using rat antibodies against CD31 (Phar-
genie), mouse antibody against Ki-67 (Clone MIB1, Dako), and secondary antibodies coupled to HRP (Dako). Imaging was carried out using a Nikon E600 microscope equipped with a digital camera DMX1200.

Microarray analysis

Microarray assay and preprocessing analysis were conducted in the microarray core facility of the Research Institute for Biotherapy at Montpellier using the standard Affymetrix protocol. Total RNA was extracted using TRIzol reagent (Invitrogen). RNA integrity was verified on an Agi-
ent 2100 Bioanalyzer. For each of the samples, total RNA was reverse transcribed into cDNA, followed by in vitro transcription and biotin labeling to generate cRNA (Enzo Biochem). The fragmented, biotin-labeled cRNA was hybridized to the Human Genome U133 2.0 oligonucleotide arrays (Affymetrix) containing approximately 22,000 probes. Micro-
arrays were stained with streptavidin antibodies and strep-
tavidi-phycoerythrin in an Affymetrix Fluidics station. Arrays were scanned using a 3000 7G scanner. Raw data were processed into R/Bioconductor by using the Limma package (16). To determine genes whose expression increased when IRE1α is inactivated, probe set intensities were obtained by means of Gene Chip Robust Multiarray Averaging and were selected by using a corrected P value threshold of 0.05 and fold change threshold of |log2(fc)| ≥2.5 as previously described in ref. 15. The regulated genes are listed in Supplementary Table S1. Data are accessible on the NCBI Geo portal with the reference number GSE27306.

RNA isolation, reverse transcription PCR, and quantitative PCR analyses

Total RNA was prepared using the TRizol reagent (Invitro-
gen Corp.). Semiquantitative analyses were carried out as previously described (17). The primers used were designed using Primer depot software (18) and are listed in Supplementary Table S4. The PCR products were resolved on 1% to 3% agarose gels. For real-time quantitative PCR (qPCR), RNA was reverse transcribed with Superscript II (Promega). All PCR reactions were carried out with a Stratagene ×4000 thermo-
cycler (Stratagene) and the SYBR Green PCR Core Reagents Kit (Bio-Rad). Experiments were conducted in triplicate for each data point. Each sample was normalized toward the expression of the Rplp0 gene.

RNA cleavage assay

Total RNA (10 μg) from U87 or HepG2 was incubated with the cytoplasmic domain of human GST-IRE1α (5 μg) at 37 °C for the indicated amounts of time in a buffer containing 250 mmol/L Tris pH 7.5, 600 mmol/L NaCl, 5 mmol/L MgCl2, 5 mmol/L MnCl2, 25 mmol/L β-mercaptoethanol, supplemented with or without 10 mmol/L ATP as previously described (12). As control, we used heat-denatured GST-IRE1α. Reverse transcriptase (RT)-PCR was then conducted using Per1 pri-
mers and Gapdh as internal control. The pcDNA3.1-hPer1 expression vector was linearized by using Sspl and used as a template for in vitro transcription by using T7 polymerase (Promega) in the presence of dNTP and 32PdUTP. In vitro transcribed radiolabeled RNA was incubated in kinase buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L MgCl2, 1 mmol/L MnCl2, 5 mmol/L 2-mercaptoethanol, and 2 mmol/L ATP) with the cytoplasmic domain of human GST-
IRE1α at 37 °C for increasing amounts of time. Fragments resulting from the enzymatic reaction were resolved by Tris Borate EDTA-Urea electrophoresis and visualized by radioau-
tography on X-ray films. Secondary structure of Per1 mRNA was predicted using M-FOLD (19). For actinomycin D pulse-
chase experiments, actinomycin D was applied to 50% con-
fluent empty vector or IRE1_DN cells at a final concentration of 5 μg/mL for the indicated amounts of time. Total RNA was then extracted and reverse transcribed before qPCR analy-
sis using the following primers targeting Per1: forward 5′-
ctcagtggctgtctccttcc and reverse 5′-gagctgcttgccagaag (fragment 898–1016) or forward 5′-gatgtgcatctggtgaagct and reverse 5′-ccttgaagcttacgagga (fragment 1891–1991).
Lentiviral transduction and PER1 knockdown by shRNA
For PER1 knockdown experiments, we used the pGIPZ-GFP-lentiviral vectors expressing PER1 shRNA (shRNA) as previously described (Open Biosystems). Lentivirus-containing supernatant was collected 48 hours after transfection in LTA-HEK293T cells, 0.2 μm filtered, and snap frozen at −80°C. U87 cells were infected with lentivirus at low multiplicity according to the manufacturer’s instructions. Cells were selected in puromycin (2.5 μg/mL) and polyclonal populations were expanded and analyzed.

Luciferase reporter gene assay
The human PER1 promoter luciferase reporter gene construct was kindly provided by U. Albrecht (Freiburg, Switzerland). The hPER1-Luc plasmid was generated as previously described (20). Following transfection, cells were incubated for 24 hours and stimulated or not with drugs for an additional 16 hours. Luciferase activity was measured using the dual luciferase kit (Promega) according the manufacturer’s instructions. Light emission was measured in a luminometer (Lumistar).

RNA interference
siRNA were designed using Greg Hannon’s webtool and listed in Supplementary Table S5. Cells were transfected by using the siRNA Max Lipofectamine reagent (Invitrogen Corp.). Following incubation for 48 to 72 hours, total RNA was extracted and used for semi-quantitative RT-PCR.

Colony formation assay
Cells were plated at density of 104 per well in 12-well plates and cell proliferation rate was measured by cell counting (Beckman Coulter). For colony formation, 2,500 cells were seeded in 6-well plates. Twenty-four hours later, fresh medium was added and the cells were allowed to form colonies. After 2 weeks, the colonies were stained with 0.1% crystal violet and counted. The experiments were carried out at least twice in triplicate.

Antibody-based analyses
Total protein extracts and immunoblotting were conducted as previously described (14). Antibodies against PER1 and tubulin were purchased from Cogensics and Santa Cruz Biotechnology. Proteins were detected using secondary antibodies coupled to HRP (Dako) and immunoblots revealed using enhanced chemiluminescence and radioautography. For immunohistochemistry, analyses were carried out as previously described (15) using an antibody that was raised against the protein translated from Xbp1s mRNA (21).

Cell culture, transfections, and treatments
HepG2 and U87 cells were grown in Dulbecco’s Modified Eagle Medium, supplemented with 10% FBS, L-glutamine, and antibiotics. U87 and HepG2 cells were stably transfected with pcDNA3/IRE1-NCX-1, an expression vector encoding a cytoplasmic-defective IRE1α mutant (17). U87 cells were transiently transfected with pED-IRE1 WT or mutant K599A (22) expression vectors. Transfections were conducted using Lipofectamine (Invitrogen Corp.) according to the manufacturer’s recommendations. Actinomycin D were purchased from Sigma and used as indicated.

Statistical analyses
Data are presented as means ± SD. Statistical significance (P < 0.05 or lower) was determined using the Student t test 2-tailed distribution, assuming equal variance for the samples (GraphPad Prism). For in vivo studies, Kaplan–Meier curves and log-rank analysis were conducted using GraphPad 5.0.

Human samples
A total of 29 human glioblastoma samples were collected from The Bordeaux Tumor Bank and 31 + 20 samples from the Mayo Clinic. Twelve samples from normal or peritumoral brain tissues were also collected. Samples were collected according to the recommendations of the local ethics committees and informed consent was systematically obtained.

Results
IRE1α loss-of-function results in PER1 mRNA posttranscriptional upregulation
Using global expression profiles of U87 cells stably transfected with an empty vector or a well-established dominant-negative (DN)-IRE1α vector (IRE1_DN; ref. 17), we identified PER1 mRNA as a potential target of IRE1α signaling (Supplementary Table S1). PER1 mRNA expression was increased in both IRE1_DN cells and IRE1α-silenced cells, whereas XBP1 silencing had no effect (Fig. 1A). This indicates that PER1 mRNA expression regulation is dependent on IRE1α activity but not on XBP1. Similar results were obtained in additional empty vector or IRE1_DN (Supplementary Fig. S1A) HepG2 stable transduced cells (Supplementary Fig. S1B), thus ruling out any clonal or cell line-specific effects. Changes were specific of PER1, as PER2 mRNA levels were not altered in these conditions (Fig. 1A). To further investigate the relationship between IRE1α activity and PER1 mRNA expression, parental U87 cells were transiently transfected with increasing amounts of IRE1α WT or kinase dead mutant IRE1αK599A (22, 23). PER1 mRNA expression was reduced in cells overexpressing IRE1α WT in a dose-dependent manner (Fig. 1B; compare lanes 2 and 3 to lane 1). In contrast, PER1 mRNA accumulation in cells overexpressing IRE1αK599A (Fig. 1B; compare lanes 4 and 5 to lane 1). The impact of IRE1α activity inhibition on PER1 mRNA was also concomitant with an increase in PER1 protein levels in IRE1_DN cells (Fig. 1C).

PER1 mRNA expression was previously found to be under the control of UPR-regulated transcription factor ATTF6 (24). To determine whether the observed IRE1α-dependent regulation of PER1 mRNA occurred at the transcriptional level, empty vector and IRE1_DN cells were transfected with a PER1 promoter reporter construct containing the −1,500 bp upstream of the transcriptional start site. These experiments were carried out under control conditions (CTL) or upon overexpression of spliced XBP1 (XBP1) or the circadian clock regulator BMAL1 that is known to control PER1 expression (as a positive control). PER1 promoter activity remained unchanged in IRE1_DN or IRE1α-silenced cells, whereas it doubled in BMAL1-overexpressing cells and remained unchanged in cells
overexpressing XBP1s (Fig. 2B). These results indicate that the increase in PER1 mRNA in the absence of functional IRE1α may occur posttranscriptionally and independently of XBP1s. We then tested whether PER1 mRNA expression increase in IRE1_DN cells was associated with an increase in PER1 mRNA half-life. This was assessed using an actinomycin D pulse-chase experiment followed by qRT-PCR (Fig. 2C). Under these conditions, PER1 mRNA half-life was significantly prolonged going from 2.3 hours in empty vector cells to 3.5 hours in IRE1_DN cells. Together, these data identify PER1 mRNA as an IRE1α-regulated target in cancer cells and provide a novel role for IRE1α activity on PER1 mRNA stability.

PER1 mRNA is cleaved by IRE1α

Next, as IRE1α was shown to control mRNA levels through direct cleavage (25), we examine whether PER1 mRNA was a direct target of IRE1α endoribonuclease activity. We studied the effects of IRE1α activity on PER1 mRNA regulation through internal cleavage sites. PER1 mRNA potential cleavage fragments amounts were measured upon siRNA-mediated silencing of the ribonucleases XRN1/2 and SKI2, which respectively contribute to RNA degradation 5′-3′ and 3′-5′, as previously described (26). We confirmed that treatment with XRN1/2 or SKI2 siRNAs specifically reduced the expression of target mRNAs without affecting the expression of endogenous Irelα mRNA (Supplementary Fig. S2). Treatment with XRN1/2 or SKI2 siRNA did not affect PER1 mRNA sequences corresponding to 5′ (exons 4–8) and 3′ (exon 23) mRNA ends in IRE1_DN cells (Fig. 2D), showing that no PER1 mRNA cleavage fragments were present in these cells. In empty vector cells, SKI2 knock-down led to the accumulation of the 5′ PER1 mRNA sequence corresponding to the exons 4 to 8, and thus located upstream of potential IRE1α cleavage sites. In contrast, the fragment located downstream of these sites (exon 23) did not accumulate when compared with control irrelevant siRNA (GL2; Fig. 2D). Conversely, treatment with siRNA targeting the 5′ to 3′ exonucleases XRN1/2 only led to the increase of the fragment, downstream of this site (exon 23; Fig. 2D).

Sequence analysis revealed that five IRE1α consensus cleavage sites were present on human PER1 mRNA (Fig. 3A). Moreover, these cleavage sites were associated with P-loops structures, thereby creating potential cleavage sites for IRE1α endoribonuclease (Supplementary Fig. S3A). We then tested whether IRE1α could directly cleave PER1 mRNA. Total RNA from U87 cells was subjected to an in vitro IRE1α-mediated cleavage assay (12). This reaction was followed by RT-PCR to monitor PER1 mRNA levels (Fig. 3B). A strong decrease in PER1 mRNA level was observed when total RNA was incubated with IRE1α and ATP, whereas mRNA levels of the housekeeping genes ORP150 or GAPDH were unchanged (Fig. 3B). A positive control for IRE1α endoribonuclease activity was obtained using XBP1 mRNA as a substrate (Supplementary Fig. S3B). These results show that IRE1α cleaves PER1 mRNA in vitro. Then, to identify the cleavage products resulting from PER1 mRNA, in vitro transcribed and radiolabeled PER1 mRNA was subjected to IRE1α cleavage as described above. These experiments showed a major radiolabeled fragment of approximately 4 kb corresponding to the mRNA transcribed from the PER1 cDNA. In addition, three bands corresponding to entities of, respectively, 2.7, 1.7, and 1 kb were also present in the original transcription reaction (Fig. 3C, lane 2). All the radiolabeled material was RNA as shown by RNase
A-mediated degradation (Fig. 3C, lane 1). In the presence of GST-IRE1\(\alpha\)-cyto, a band of approximately 2 kb (Fig. 3C, lanes 3–5) appeared across time and could correspond to the product generated following IRE1\(\alpha\)-mediated cleavage at nucleotide 1920 (Site 3, Supplementary Table S2), thus suggesting that IRE1\(\alpha\) cleaves PER1 mRNA at least at the cleavage site #3. To determine whether IRE1\(\alpha\) can also cleave PER1 mRNA at others sites, the five putative sites were mutated by insertion of a single mutation with the site CUGCAC where G was replaced by A. Mutated cDNA were in vitro transcribed and subjected to IRE1-mediated cleavage as above. Site-specific PCR amplification was then carried out for each reaction (Fig. 3D). This revealed that out of the 5 potential cleavage sites identified, only 3, namely 1920, 3197, and 3378 were cleaved by IRE1\(\alpha\) in vitro (Fig. 3D). Taken together, these data are consistent with an IRE1\(\alpha\)-dependent cleavage of PER1 mRNA.

IRE-dependent PER1 degradation modulates cancer cell survival and tumor progression in vivo

To determine the biologic significance of PER1 mRNA cleavage by IRE1\(\alpha\) on tumor cell growth, PER1 mRNA expression was attenuated using lentiviral-mediated delivery of GFP-shPER1 in empty vector and IRE1\(\alpha\)-DN cells (or GFP empty vector as control). We first confirmed that pGIPZ-GFP-shPER1 viral particles effectively reduced PER1 mRNA and protein expression in U87 cells using RT-PCR and immunoblotting (Fig. 4A and S4). Using these cells, the impact of PER1 regulation by IRE1\(\alpha\) anchorage-independent cell growth was investigated. After 2 weeks, the IRE1\(\alpha\)-DN cells showed a reduced ability to form colonies compared with empty vector cells (Fig. 4B). We then investigated the effects of PER1 silencing on tumor growth using our previously described in vivo orthotopic glioblastoma model (14). Fluorescence microscopy analysis of the tumors revealed GFP expression in tumor cells, thereby further confirming successful and stable lentiviral transduction in tumor cells up to 28 days postinjection (Fig. 4C). This also revealed that low PER1 expression in an IRE1\(\alpha\) wild-type background neither impacted on tumor volume (Fig. 4C and D), tumor shape (Fig. 4C and D), nor on the number of tumor proliferating cells (Fig. 3D). IRE1\(\alpha\)-DN cell-derived tumors were smaller (\(P < 0.001\)) with extensive tumor cell infiltration in surrounding parenchyma (Fig. 4D; \(P < 0.001\)). Interestingly, at 28 days postinjection, the size of IRE1\(\alpha\)-DNshPER1 cell-derived tumors was comparable with that of IRE1\(\alpha\)-DN cell-derived tumors (Fig. 4D), however, with reduced tumor cell infiltrates (Fig. 4D) and better-delimited perimeters as compared with empty vector-derived tumors. This phenotype was accompanied by a marked restoration of proliferation within the tumor (Fig. 4D). These results confirm a role of IRE1\(\alpha\) signaling in tumor growth in vivo and show the involvement of the IRE1\(\alpha\)/PER1 axis in this process. Moreover, tumor...
angiogenesis, which is abnormal in IRE1_DN-derived tumors (14, 15), was investigated in PER1-silenced cells using CD31 immunostaining (Fig. 4C). High vascular density was apparent in empty vector and EVshPER1 cell-derived tumors (Fig. 4C). Tumor vascularization was partially restored in IRE1_DNshPER1-derived tumors (Fig. 4C and D). These results establish that the loss of cancer cell proliferation and tumor vascularization due to impairment of IRE1 activity is in part mediated by increased PER1 expression and suggest a potential role for PER1 in tumor angiogenesis.

As the IRE1α/PER1 axis impacts on tumor growth capacity and angiogenesis, we then measured the consequences of its alteration on mouse survival following orthotopic injection (Fig. 5A). IRE1α signaling inhibition (IRE1_DN cell-derived tumors) increased the survival of tumor-bearing mice compared with those bearing empty vector cell-derived tumors. This survival advantage was lost in PER1 knocked-down tumors (Fig. 5A), thereby reinforcing the existence of functional interplay between IRE1α and PER1 underlying IRE1α.

Next, we sought to define mediating IRE1α/PER1 axis, to this end we identified the genes controlled by this axis and potentially involved in the control of tumor growth/angiogenesis. mRNA expression profiles in IRE1_DN and subjected or not to known endoplasmic reticulum stress inducers such as glucose or glutamine deprivation, hypoxia or Tn exposure were compared with those obtained in empty vector cells. These experimental conditions are also known to recapitulate microenvironmental stresses. We identified the top 50 genes up and downregulated all conditions included (Fig. 6A). As PER1 has been defined as a transcriptional repressor, we focused our attention on the genes downregulated in IRE1_DN cells, which were found to be enriched in both cytokine–cytokine receptor interaction and chemokine signaling pathways. To further explore the IRE1α/PER1–dependent cytokine and chemokine regulatory networks upon endoplasmic reticulum stress, validation of potential target genes was carried out using RT-qPCR. The proangiogenic chemokine CXCL3 was the most significant gene that showed restoration of its expression in IRE1_DNshPER1 cells upon glucose deprivation (Fig. 6B). We postulated
that this might be due to the coordinated regulation of CXCL3 expression by XBP1 that was already proposed by Gargalovic and colleagues (27) and the downregulation of PER1, which in this context would play a repressor role. As anticipated from our model, U87 cells transiently silenced for XBP1 and/or overexpressing PER1 and exposed or not to endoplasmic reticulum stress induced by Glc deprivation showed an attenuation of CXCL3 mRNA expression increase mediated by Glc deprivation (Fig. 6C). This shows that XBP1s increase and PER1 downregulation both contribute to the regulation of CXCL3 mRNA expression. Hence, integrated IRE1α signaling specifically controls chemokine expression upon stress.

Low levels of PER1 gene correlates with poor survival in patients

To investigate whether the IRE1α/PER1 axis was of clinical relevance, human glioblastoma samples from 2 independent sources (CHU Bordeaux and Mayo Clinic) were analyzed for PER1 mRNA expression by qPCR. This revealed that both cohorts presented an expression of PER1 mRNA lower in tumors than that observed in normal or nontumoral tissues (red/Bordeaux; black/Mayo, Fig. 5B). To evaluate the impact of low PER1 expression on prognosis, postsurgery survival of 60 patients with glioblastoma was followed (Fig. 5C). These patients were classified into 2 groups in which PER1 expression...
was either lower or higher than average PER1 mRNA. The low PER1 group contained 31 patients and the high PER1 group contained 29 patients. Interestingly, high PER1 expression significantly correlated with increased survival with a median of 599 days compared with 411 days in the low PER1 group (P = 0.03; Fig. 5C). This result is in agreement with those obtained in Kaplan–Meier survival curves. Statistical difference between the 2 groups is indicated. Statistical difference between the 2 groups in indicated P = 0.004; log-rank test. D, Kaplan–Meier survival curves of patients displaying negative sXBP1 staining (6; XBP1−) or positive sXBP1 staining (14; XBP1+). P = 0.004; log-rank test.

Discussion

Our results identify PER1 mRNA as a novel and atypical substrate (coding for a cytosolic/nuclear protein) of RIDD contributing to cancer development. As PER1 is a core gene of the circadian clock, our observation could also be placed in the perspective of a previous report (29) that shows the significance of the UPR/circadian clock connection in the control of hepatic metabolism. In addition, another study showed that a connection between the eIF2α-dependent transcription factor ATF4 and the circadian clock transcription systems plays an important role in multidrug resistance in tumor cells (30). In this context, IRE1α-mediated PER1 mRNA decay could also represent another pathway in the well-described posttranscriptional regulation mechanisms of the circadian clock (31), whose relevance to cancer still remains to be investigated.
The findings included in this report show a direct clinical relevance of this newly identified IRE\(\alpha\)/Per1 axis as we have determined that PER1 mRNA may be a useful marker for predicting patient survival (Fig. 5). In addition to its role in the control of the circadian clock, PER1 has also directly been involved in cell stress response, through interactions with ATM and Chk2 to participate in \(\gamma\)-irradiation–induced apoptosis (11). Moreover, low PER1 mRNA expression was observed in a variety of cancers (10, 11, 32, 33), thereby suggesting its involvement in cancer development. Our data show that low PER1 together with high XBP1s expression are significantly associated with lower glioma patient survival. These observations point toward an instrumental role of IRE\(\alpha\) in glioma development. This was also supported by the suggested driver role of IRE\(\alpha\) mutations in cancer including glioma (3), however, the functional implication of these mutations remains to be shown.

IRE\(\alpha\) RNAse-dependent signaling in tumor could on the one hand regulate XBP1 mRNA splicing thereby leading to previously shown induction of proinflammatory cytokines as
reported in many instances (27, 34–36) and in parallel enhance this effect by repressing PER1 expression, which in turn could act as derepression of cytokine expression, as illustrated for other core circadian genes (37–39). Mutually exclusive phenotypic changes in glioma observed upon impairment of IRE1α signaling from massive/angiogenic to diffuse/avascular could either result from the IRE1α-dependent activation of a cell-autonomous proinflammatory/angiogenic phenotype or from the coordinated posttranscriptional stabilization of specific mRNA (RIDD substrates), thereby leading to major changes in tumor cell–microenvironment interactions.

As such, our study shows that CXCL3 mRNA is per se an IRE1α/XBP1s/PER1-dependent target in our model as determined in Fig. 6. These data are also consistent with the emerging role of CXCL3 as a key player in cancer development (27, 40) that also applies to glioblastoma (41, 42). Consequently, we identify here an IRE1α activity that coincidentally activates XBP1 mRNA splicing and PER1 mRNA decay. This provides a molecular link between IRE1α activation and tumor cell adaptation, and directly links IRE1α activity to proinflammatory/angiogenic phenotypes (Fig. 7).

Taken together, these data further support a specific and important role for IRE1α signaling in human glioblastoma and show that PER1 is a genuine signaling intermediate in glioblastoma progression. Moreover, these results suggest that IRE1α may constitute a suitable therapeutic target for patients with this disease. As a consequence, this suggests that controlling the interplay between UPR signaling and the circadian clock component might also be a suitable strategy to slow down cancer progression; our results may consequently define a model for novel therapeutic option for cancers.

Disclosure of Potential Conflicts of Interest
J.N. Sarkaria has a commercial research grant from Genentech, Basilea, Sanofi, and Merck. E. Chevet has a commercial research grant from Servier. No potential conflicts of interest were disclosed by the other authors.

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References

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