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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF THE CIRCADIAN OSCILLATION PATTERN OF UNFOLDED PROTEIN RESPONSE SIGNALING COMPONENTS IN HUMAN EMBRYONIC KIDNEY HEK293 CELLS

HEK293 İNSAN EMBRİYONİK BÖBREK HÜCRELERİNDE KATLANMAMIŞ PROTEİN YANITI SİNYALİ BİLEŞENLERİNİN SİRKADİYEN SALINIM MODELİNİN BELİRLENMESİ

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ABSTRACT

Objective: *The circadian rhythm is one of the primary regulatory systems with near 24-hour oscillations. It has a crucial role in regulating physiological conditions in the human body, including body temperature and the secretion of hormones. Numerous disorders, such as cancer and diabetes, have been linked to disruptions of the cellular circadian rhythm. Herein, we aimed to investigate the relationship between the circadian rhythm and unfolded protein response (UPR) signaling, which is one of the important physiological mechanisms in mammalian cells and has recently been associated with drug resistance, invasion and metastasis in cancer.*
Material and Method: *Human embryonic kidney cell line HEK293 was provided from the American Type Culture Collection and propagated in DMEM containing 10% FBS and growth ingredients. For in vitro circadian synchronization, cells were exposed to 50% and then the oscillation pattern of gene and protein expression of UPR-related target genes was analyzed by agarose gel electrophoresis and immunoblotting, respectively. The oscillation pattern was commented on through curve-fitting analysis.*
Result and Discussion: *Our findings demonstrated that UPR components, including IRE1α, XBP-1s, eIF2α, phospho(Ser51)-eIF2α, PERK, ATF4, GADD34 and ATF6, tightly exhibit oscillation patterns under a circadian rhythm on a 48-hour time scale like the PER1 gene that is a core component of the circadian rhythm. Moreover, endoplasmic reticulum (ER) stress genes, BiP/GRP78 and CHOP, were similar to UPR components under the circadian rhythm. Additionally, we found the activation of UPR signaling harmoniously modulated with the circadian rhythm. Present data indicated that the expression level of UPR components exhibited strict oscillation under the circadian rhythm. Our findings may guide experimental studies of new-generation UPR-targeted drugs to be developed to treat various pathologies in accordance with the circadian rhythm.*
Keywords: *Circadian clock, ER stress, unfolded protein response*

ÖZ

Amaç: *Sirkadiyen ritim, yaklaşık 24 saatlik salınımlara sahip temel düzenleyici sistemlerden biridir. Vücut ısısı ve hormon salgılanması da dahil olmak üzere insan vücudundaki fizyolojik koşulların düzenlenmesinde çok önemli bir role sahiptir. Kanser ve diyabet de dahil olmak üzere çok sayıda rahatsızlık hücresel sirkadiyen ritmin bozulmasıyla ilişkilendirilmiştir. Bu çalışmada memeli*

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hücrelerinde önemli fizyolojik mekanizmalardan biri olan ve son zamanlarda kanserde ilaç direnci, invazyon ve metastaz ile ilişkilendirilen katlanmamış protein yanıtı (UPR) sinyali ile sirkadiyen ritim arasındaki ilişkiyi araştırmayı amaçladık.

Gereç ve Yöntem: *İnsan embriyonik böbrek hücre hattı HEK293 Amerikan Tipi Kültür Koleksiyonundan sağlandı ve hücreler %10 FBS ve büyüme bileşenleri içeren DMEM besi ortamı içinde çoğaltıldı. İn vitro sirkadiyen senkronizasyon için hücreler %50 at serumuna maruz bırakıldı ve ardından UPR ile ilişkili hedef genlerin gen ifadesi ve protein düzeyindeki salınım modeli sırasıyla agaroz jel elektroforezi ve immünoblotlama ile analiz edildi. Salınım modeli eğri uydurma analizi yoluyla değerlendirildi.*

Sonuç ve Tartışma: *Bulgularımız, IRE1α, XBP-1s, eIF2α, fosfo(Ser51)-eIF2α, PERK, ATF4, GADD34 ve ATF6 dahil olmak üzere UPR bileşenlerinin, sirkadiyen ritmin çekirdek komponentlerinden PER1 geni gibi 48 saatlik bir zaman ölçeğinde sirkadiyen ritim altında sıkı bir şekilde salınım modelleri sergilediğini gösterdi. Ayrıca endoplazmik retikulum (ER) stres genleri, BiP/GRP78 ve CHOP da sirkadiyen ritim altında UPR bileşenlerine benzer şekilde davrandı. Ek olarak UPR sinyalinin aktivasyonunun sirkadiyen ritimle uyumlu bir şekilde modüle edildiğini bulduk. Mevcut veriler, UPR bileşenlerinin ekspresyon seviyesinin sirkadiyen ritim altında katı salınım sergilediğini gösterdi. Bulgularımız, çeşitli patolojileri sirkadiyen ritme göre tedavi etmek için geliştirilecek yeni nesil UPR hedefli ilaçların deneysel çalışmalarına yol gösterebilir.*

Anahtar Kelimeler: *ER stres, katlanmamış protein yanıtı, sirkadiyen saat*

INTRODUCTION

Circadian rhythms are identified as near-24-hour oscillations present in almost all physiological processes in the human body and brain [1]. The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the master circadian pacemaker. It is hierarchically organized and synchronized by light-dark cycles by neural and neuroendocrine pathways [2,3]. The circadian clock system also regulates many physiological conditions like body temperature, blood pressure, sleep/wake cycle, immune responses, and synthesis and secretion of hormones, such as cortisol and melatonin [4].

Cellular-level oscillations are originated by circadian clock genes such as clock circadian regulator (CLOCK), period circadian regulator 1-3 (Per 1-3), cryptochrome genes (CRY1 and CRY2) and brain and muscle ARNT-like1 (Bmal1) rhythmically regulating their transcription as well as the transcription of various clock-controlled genes [2,5]. The circadian timing system in mammals has long been a research subject due to its potential involvement in the pathogenesis of different diseases. While proper coordination of cellular- and tissue-level clocks is crucial for maintaining homeostasis, disruption of cellular circadian rhythm has been implicated in the pathogenesis of many diseases, including cardiovascular diseases, epilepsy, Alzheimer's disease, diabetes and cancer [6-8]. Therefore, it is extremely important to understand the relationship between circadian rhythm and physiologically important mechanisms in mammalian cells, such as endoplasmic reticulum-associated degradation (ERAD) and unfolded protein response (UPR) signaling and to characterize their oscillation patterns under a circadian rhythm [9]. In this context, it will be essential to determine oscillation pattern maps for the use of drugs that pharmacologically target these mechanisms to obtain more therapeutically effective results.

The endoplasmic reticulum (ER) has multiple roles in eukaryotic cells and is responsible for coordinating several functions, including protein synthesis, transport and folding, lipid and steroid biosynthesis, carbohydrate metabolism and Ca^{+2} storage [10-13]. UPR signaling organized in the ER plays a vital role in the re-adaptation of the ER against altered cellular physiological conditions and also mitigates stress responses through setting the ER capacity [14]. UPR signaling orchestrates the ER membrane resident three transmembrane proteins, inositol-requiring enzyme 1α (IRE1α), double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [15]. Accumulating misfolded or unfolded proteins in the ER lumen or specific stressful conditions activates the UPR signaling through autophosphorylation and dimerization or trimming of ER stress sensor proteins. Subsequently, downstream effectors coordinate the continuation of signal transmission and eventually, specialized transcription and translational programs are activated in the cells [15]. Excessive activation of the UPR signaling has been associated with emerging acquired drug

resistance, cancer cell invasion and metastasis [14,16]. Unusual alterations in the UPR signaling have been associated with numerous diseases, including neurodegenerative diseases, diabetes, inflammatory disease and cancer [17-20]. Therefore, determining the circadian oscillation pattern of UPR components is crucial in terms of organizing the use of UPR-targeted drugs according to the oscillatory pattern. However, the detailed behavior of the UPR signaling under circadian oscillation currently remains unclear.

In the present study, we investigated the daily physiological oscillation pattern of UPR signaling under circadian rhythmicity in human embryonic kidney HEK293 cells. For this aim, we evaluated the oscillation pattern of UPR signaling-related genes, IRE1α, XBP-1s, PERK, ATF4, GADD34, ATF6 and also ER stress genes, BiP/GRP78 and CHOP. Additionally, protein expression levels of some downstream effects of UPR signaling components, XBP-1s, eIF2α, phospho(Ser51)-eIF2α and BiP/GRP78 were tested by immunoblotting. Our results revealed a detailed physiological release pattern of UPR signaling. Present data indicated that the expression level of UPR components exhibited strict oscillation under circadian rhythm. Our findings may guide experimental studies of new-era UPR-targeted drugs in treating several pathologies.

MATERIAL AND METHOD

Material

Rabbit polyclonal antibodies anti-BiP/GRP78 (11587-1-AP)(1:10000), anti-Bmal1 (14268-1-AP)(1:2000) and anti-XBP-1s (24868-1-AP)(1:2500) were provided from Proteintech, anti-eIF2α (#9722)(1:2000) and anti-phospho-eIF2α (Ser51) (#9721)(1:2000) from Cell Signaling Technology. Mouse monoclonal β-actin antibody (A5316)(1:10000) was obtained from Sigma Aldrich. Horseradish peroxidase (HRP)-conjugated anti-mouse (#31430)(1:5000) or anti-rabbit (#31460)(1:5000) IgG (H+L) was purchased from Thermo Scientific.

Cell culture materials and reagents, including plastic ingredients, growth media, fetal bovine serum (FBS) and additional growth requirements were obtained from Sartorius. Horse serum was obtained from Biowest.

Cell Culture

Human embryonic kidney cell line HEK293 (CRL-1573TM) was obtained from the American Type Culture Collection (ATCC, USA). Cells were routinely propagated in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 5 mg ml^{-1} penicillin/streptomycin and 2 mM L-glutamine (Lonza). Cells were kept in a humidified atmosphere of 5% CO_2 and 95% air at a temperature of 37°C. The absence of mycoplasma contamination was periodically verified using MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza).

In vitro Circadian Synchronization of HEK293 Cells

Circadian synchronization was performed as previously described [9,21]. Cells were seeded on 10mm cell culture dishes. At confluence, the cells were propagated for 6 days and then cells were subjected to 50% horse serum (serum shock) or 10% FBS containing regular media for 2 hours for circadian synchronization. After serum shock synchronization, the shock medium was replaced with a serum-free medium. Cells were collected at 4-hour intervals for 48 hours and lysed for RNA extraction and protein isolation.

Total RNA Extraction and Complementary DNA Synthesis

Total RNA was extracted from cells by Monarch® Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's instructions. The concentration and purity of isolated RNAs were determined by a micro-spectrophotometer (Allsheng). Complementary DNA was synthesized using 1 μg RNA by iScript™ cDNA Synthesis kit (Bio-Rad). The reaction mix was incubated for 5 min at 25°C, 20 min at 46°C and 1 min at 95°C, respectively.

Reverse Transcription PCR (RT-PCR) and Agarose Electrophoresis

Specific primers were used for the PCR amplification of interested genes with Taq DNA polymerase (Thermo Scientific). The following PCR conditions were used: pre-denaturation at 95°C for 5 min, followed by 36 cycles of denaturation 30 s at 95°C, annealing 30 s at 60°C and extension 30 s 72°C and final extension 2 min at 72°C. After that, PCR products were electrophoretically separated in electrophoresis on 2% agarose gel containing ethidium bromide (Et-Br) and visualized by UV-transilluminator (Vilber Lourmat). The density of PCR amplicons was quantified with ImageJ software (National Institutes of Health, USA) (http://imagej.nih.gov/ij/). Primers were designed specifically for genes of interest, including PER1, IRE1α, XBP-1s, PERK, ATF4, GADD34, ATF6, BiP/GRP78, CHOP and RPLP0 and verified the optimized working. The primer sequences are available upon request. RPLP0 was used for the housekeeping gene. Each cDNA sample was analyzed in triplicates for each PCR.

Immunoblotting Assay

Cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer (1xPBS, 1% nonidet P-40, 0.5% 7-DOC and 0.1% SDS, pH 8.0). After removing the insoluble phase by centrifugation at 14.000 rpm for 20 min at 4°C, total protein concentrations were determined by the bicinchoninic acid (BCA) (Thermo Scientific) assay. Typically, 25-40 μg of total cellular protein was used in immunoblotting studies. Protein samples were denatured in 4x Laemmli buffer at 70°C for 15 min and were separated on hand-cast polyacrylamide gels. Separated proteins were transferred to an Immobilon®-P polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween (PBS-Tween) for 1 h at room temperature and then incubated with the primary antibody prepared in PBS–Tween containing 5% skim milk for 1-2 h at room temperature or 4°C overnight. Secondary antibodies were applied for 1 h at room temperature. Protein bands were monitored using an enhanced chemiluminescence (ECL) solution (Thermo Scientific) in the Fusion Pulse system (Vilber Lourmat). The protein bands were quantitated by Image StudioTM Lite (LI-COR®) (https://www.licor.com/bio/image-studio-lite/).

Curve Fitting Analysis

Curve fitting analysis was carried out as previously described [9]. The circadian oscillation was conducted using a non-linear curve fitting algorithm using GraphPad Prism and MATLAB software, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the three replicates. Genes showing an R2 correlation greater than 0.8 in non-linear curve fitting analyses were kept.

Statistical Analysis

The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test with a minimum of 95% confidence interval by GraphPad Prism 7 (GraphPad Software, La Jolla CA, USA, www.graphpad.com). The significant level was set at 5% and probability values of $p<0.05$ were considered statistically significant for all tests.

RESULT AND DISCUSSION

Verifying the Circadian Synchronization of HEK293 Cells

To determine the success of the circadian synchronization of HEK293 cells, we applied the serum shock protocol as previously described and then investigated the expression level of the PER1 gene depending on the change over time [9,21]. PER1 gene is a characteristic circadian oscillator and is also rhythmically transcribed under circadian rhythms [22]. Therefore, PER1 is generally used to determine circadian synchrony in mammalian cells. The success of serum shock-mediated circadian synchronization of HEK293 cells was verified by examining the oscillation of PER1 expression. Our findings demonstrated that PER1 exhibited rhythmic oscillation, which peaked at 8-16 h and 32-40 h (Figures 1A, 1B). These results confirmed the achievement of circadian synchronization of HEK293 cells.

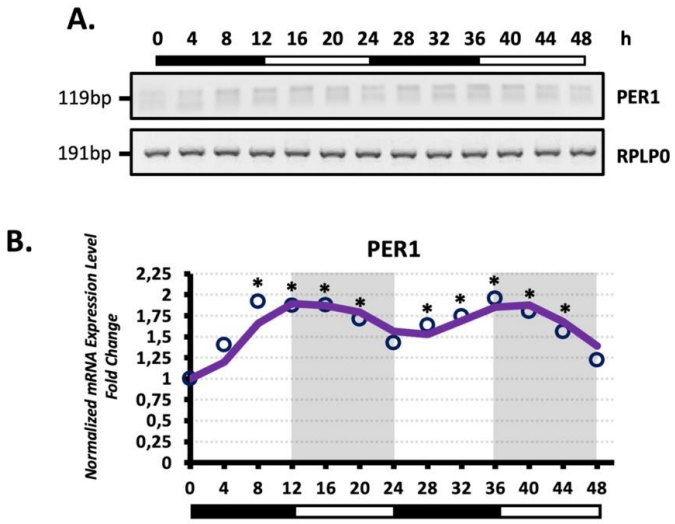

Figure 1. Verifying the circadian programming of HEK293 cells. **A,** Rhythmic mRNA expression levels of PER1 in the HEK293 cells across a 48 h circadian cycle. The total RNA was extracted from the HEK293 cells collected every 4 h during a 48 h circadian period and complementary DNA was synthesized and then target genes were amplified using specific primers. PCR amplicons were visualized on ethidium bromide-containing agarose gel. **B,** Relative gene expression quantities corresponding to three biological replicates were collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and the housekeeping gene RPLP0 was used as a loading control. Fold changes in mRNA expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled, via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the replicates. Genes showing an R^2 correlation greater than 0.8 in non-linear curve fitting analyses were kept. White-grey/black scale represents 4-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group. (*p<0.05) (n = 3)

Investigation of the Circadian Oscillation Pattern of UPR and ER Stress Genes

Expression levels of UPR signaling-related genes, including IRE1α, XBP-1s, PERK, ATF4, GADD34 and ATF6, were analyzed in serum shock-mediated synchronized HEK293 cells. Our results showed that all tested UPR genes tightly exhibited oscillation under the circadian rhythm similar to the PER1 expression pattern, which peaked at 8-16 h and 32-40 h and troughed at 24 h and 48 h (Figures 2A, 2B).

Next, we tested the ER stress-associated critical components, BiP/GRP78 and CHOP expression under the circadian rhythm in HEK293 cells. Consistent with the pattern of UPR genes, BiP/GRP78 and CHOP genes peaked at 8-16 h and 32-40 h and troughed at 24 h and 48 h (Figures. 3A, 3B).

Determination of the Protein Expression Levels of UPR Signaling-related Proteins under Circadian Oscillation

We investigated the protein expression level of some critical signal mediators of the UPR pathway, XBP-1s, a downstream effector of IREα signaling, and total-eIF2α, phospho(Ser51)-eIF2α for PERK signaling and also ER stress-related molecular chaperone BiP/GRP78 in serum shock mediated synchronized HEK293 cells by immunoblotting (Figures. 4A, 4B). Our results revealed that consistent with the mRNA data, XBP-1s, total-eIF2α, phospho (Ser51)-eIF2α and BiP/GRP78 proteins strongly exhibited robust circadian oscillation in synchronized HEK293 cells. The protein expression level of all tested downstream effectors of UPR and BiP/GRP78 peaked at 8-12 h and 34-32 h and troughed at 16-20 h and 36 h (Figures 4A, 4B).

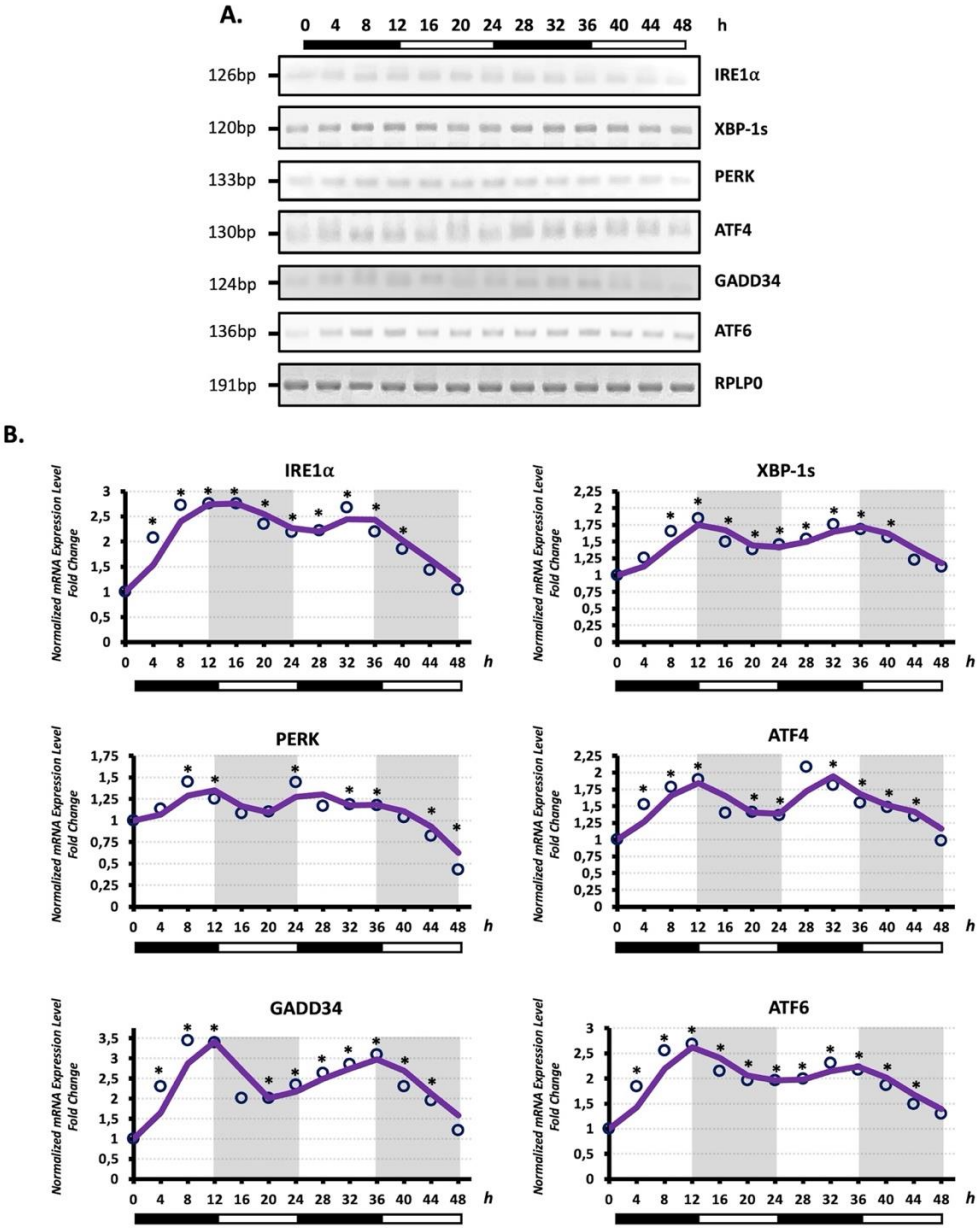

Figure 2. Evaluation of the circadian rhythmicity of the UPR components. **A,** Rhythmic mRNA expression levels of IRE1α, XBP-1s, PERK, ATF4, GADD34 and ATF6 in the HEK293 cells across a 48 h circadian cycle. The total RNA was extracted from the HEK293 cells collected every 4 h during a 48 h circadian period and complementary DNA was synthesized and then target genes were amplified using specific primers. PCR amplicons were visualized on ethidium bromide-containing agarose gel. **B,** Relative gene expression quantities corresponding to three biological replicates were collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and the housekeeping gene RPLP0 was used as a loading control. Fold changes in mRNA expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled, via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the replicates. Genes showing an R^2 correlation greater than 0.8 in non-linear curve fitting analyses were kept. White-grey/black scale represents 4-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group (*$p<0.05$) (n = 3)

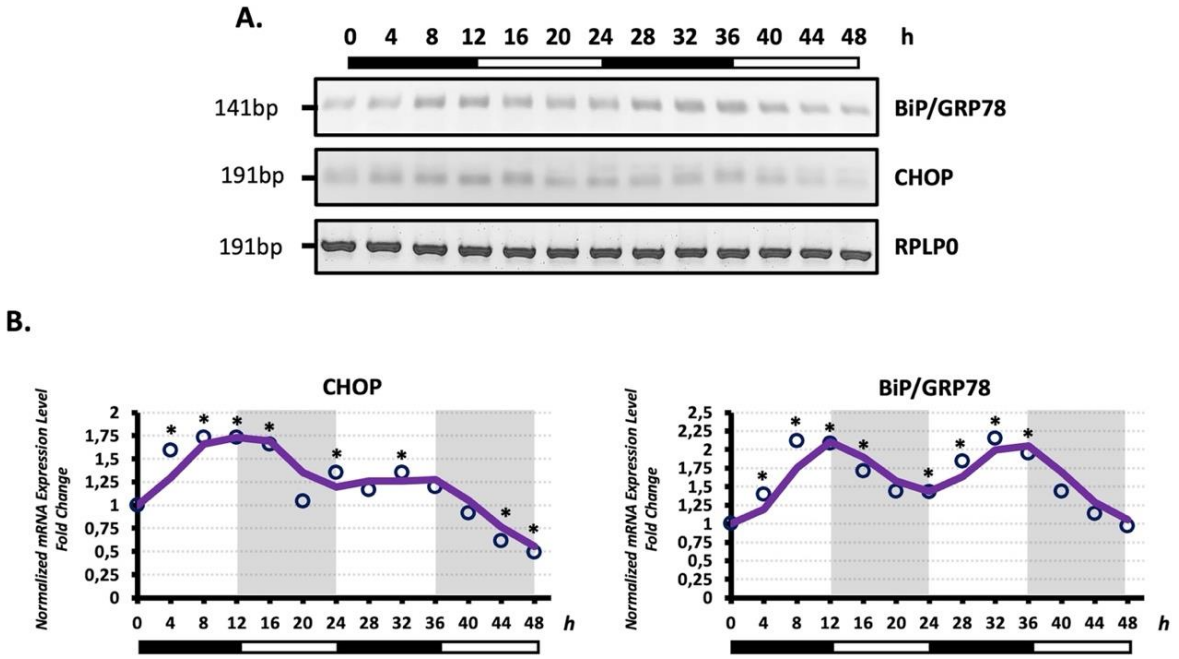

Figure 3. Evaluation of the circadian rhythmicity of BiP/GRP78 and CHOP**. A,** Rhythmic mRNA expression levels of BiP/GRP78 and CHOP in the HEK293 cells across a 48 h circadian cycle. The total RNA was extracted from the HEK293 cells collected every 4 h during a 48 h circadian period and complementary DNA was synthesized and then target genes were amplified using specific primers. PCR amplicons were visualized on ethidium bromide-containing agarose gel. **B,** Relative gene expression quantities corresponding to three biological replicates were collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and housekeeping gene RPLP0 was used as a loading control. Fold changes in mRNA expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled, via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data including the replicates. Genes showing an R^2 correlation greater than 0.8 in non-linear curve fitting analyses were kept. White-grey/black scale represents 4-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group. (*$p<0.05$) (n = 3)

The circadian system is a master regulatory system for nearly all physiological conditions and also consists of 24-hour. At the cellular level, molecular clocks originate circadian rhythms to promote coordination between internal time and the external world. This system mediates the regulation of the target genes by the circadian transcription factors, including CLOCK, BMAL1, PER1/2 and CRY1/2 [1]. The irregular circadian rhythmicity has been linked to increased susceptibility to digestive, immune, cardio-metabolic and neurological disorders, chronic conditions, such as diabetes, obesity, depression and bipolar disorder, as well as cancer [6,23-29]. Research studies have demonstrated that targeting the molecular and cellular mechanisms underlying circadian pathophysiology can be an alternative treatment way against diseases [6]. Therefore, understanding the circadian rhythmicity of physiologically important cellular mechanisms is very valuable as it has the potential to offer a new and promising treatment approach.

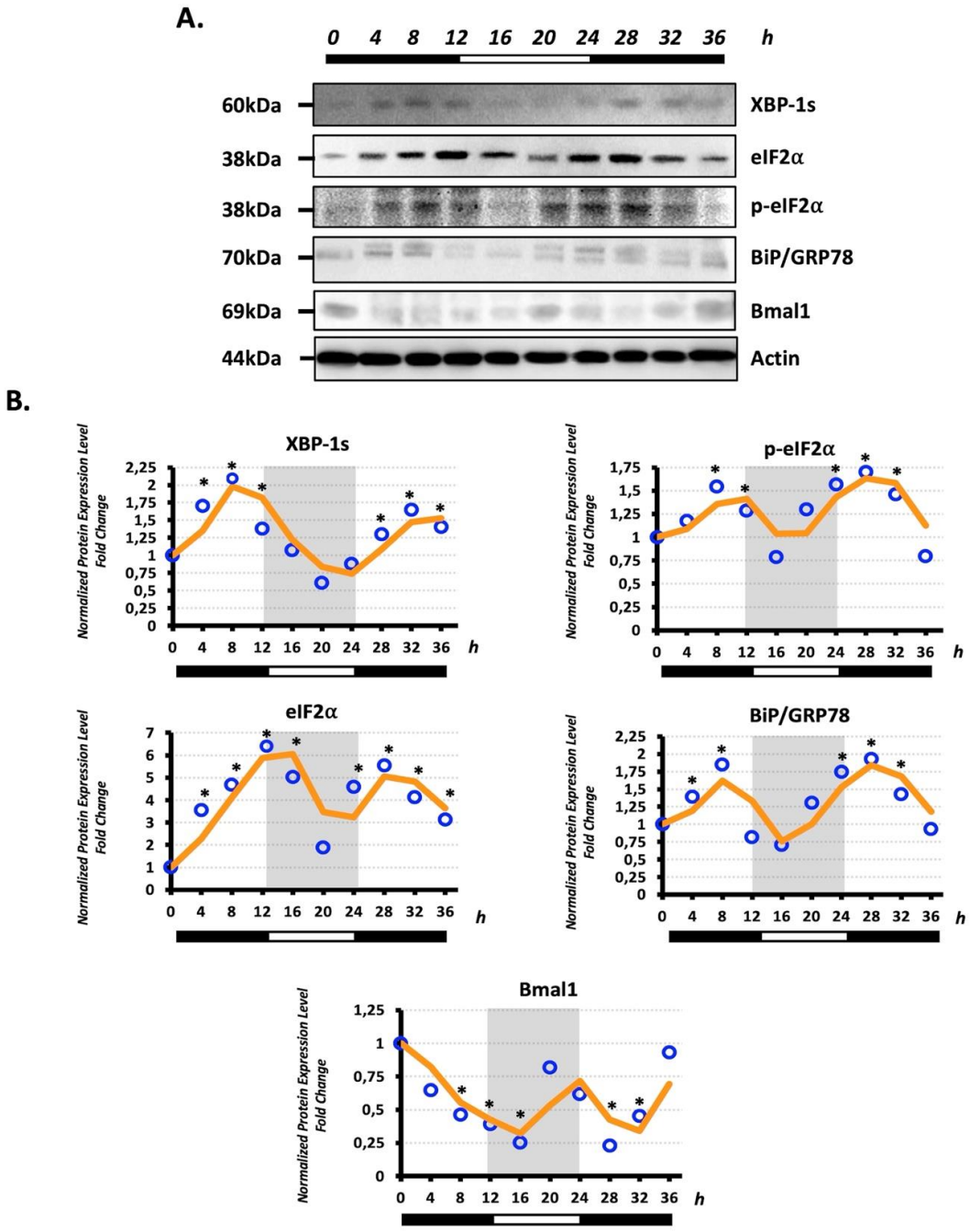

Figure 4. Expression level of UPR signaling-related proteins under circadian rhythmicity in HEK293 cells. Samples were collected every 4 h in a 36 h circadian period and then protein levels of XBP-1s, eIF2α, p-eIF2α, BiP/GRP78 and Bmal1 were analyzed by immunoblotting assay. Beta-actin was used as a loading control. **B.** Protein expression levels of XBP-1s, eIF2α, p-eIF2α, BiP/GRP78 and Bmal1 were quantified corresponding to three biological replicates collected every 4 h (navy blue circles). The band intensity was densitometrically analyzed and normalized using the beta-actin expression. Fold changes in protein expression levels were determined by comparison to the expression level at 0 h. Oscillation (represented as a continuous purple curve) was modeled via curve fitting analysis. A nonlinear curve fitting analysis was conducted, which fitted a sinusoid function *[A*sin(Bt + c)]* to the data, including the replicates. The white/black scale represents 12-hour periods. 0 h sample was set to 1. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test. '*' represents a comparison with the control group. (*$p<0.05$) (n = 3)

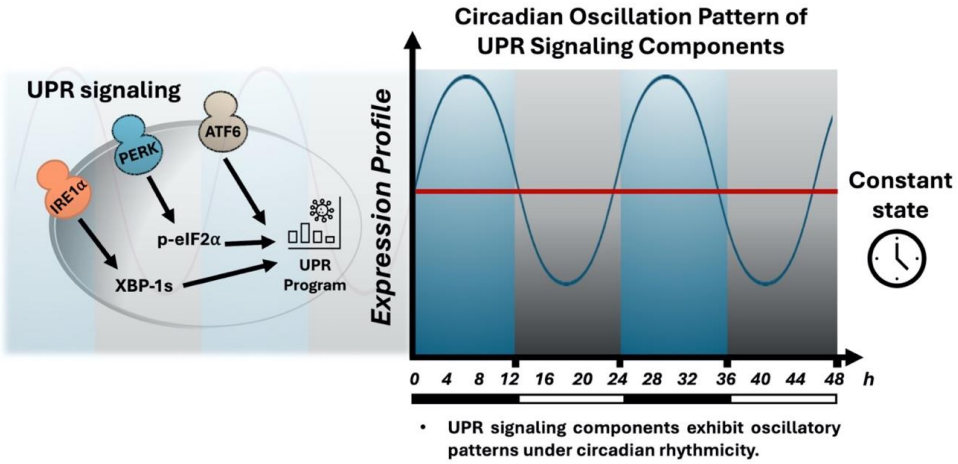

Figure 5. Representation of the expressional behavior of UPR signaling members under a circadian rhythm

Interest in the molecular biology of UPR signaling is exponentially growing based on the characterized roles of ER stress in numerous diverse pathologies, such as neurodegeneration, inflammation, diabetes, acquired drug resistance, invasiveness and metastasis capability-related roles in human cancer cells as well [30]. UPR activation can occur in various ways, such as elevated metabolic and oxidative stress, protein misfolding in the ER, impaired ER-associated degradation (ERAD) and altered ER Ca^{2+} stocks [31,32].

UPR signaling is coordinated by ER sensor transmembrane proteins, IRE1α, PERK and ATF6 and these proteins normally keep inactive form through interacting with BiP/GRP78. Upon ER stress, BiP/GRP78 is released from the sensor proteins. In this process, IRE1α and PERK proteins are activated by homodimerization and autophosphorylation and their downstream activators are warned. The activated PERK protein phosphorylates eIF2α at the serine 51 position, attenuating global translation in the cells. Phosphorylated eIF2α causes the selective increment in the translation of some of the group mRNAs, such as ATF4. GADD34, the phosphatase of phospho-eIF2α, negatively regulates the PERK signaling [19]. Additionally, PERK signaling controls the transcription of CHOP, a basic leucine zipper-containing transcription factor that promotes apoptosis in cells. Moreover, it increases the GADD34 expression [33,34]. IRE1α proteins function as a kinase and also as endoribonuclease enzyme. Activated IRE1α catalyzes the processing of the unspliced XBP1 (XBP-1u) to the spliced XBP1 (XBP1s). XBP-1s function as a transcription factor responsible for the transcription of UPR target genes [31,35]. Also, IRE1α can regulate of the subset of mRNAs and miRNAs through its IRE1α-dependent decay (RIDD) [35]. ATF6 activation allows the transition to the cis-Golgi compartment, which is cleaved by site-1 protease (S1P) and site-2 protease (S2P). The cleaved cytosolic N-terminal fragment translocates to the nucleus and coordinates the specialized transcriptional program of several genes, including ER chaperones and protein-folding enzymes [15,36]. Therefore, UPR signaling can lead to the adaptation or elimination of eukaryotic cells through reprogramming the cellular mechanism in response to cellular stresses and altered physiological conditions.

Herein, we clarified the circadian oscillation pattern of UPR signaling components in HEK293 cells at that transcriptional and translational levels. Our findings indicated that UPR components, including IRE1α, XBP-1s, PERK, ATF4, GADD34, and ATF6, robustly exhibit oscillation patterns under a circadian rhythm on a 48-hour time scale; likewise, the PER1 gene is the master component of the circadian rhythm (Figure 1a, b, 2a, b). Besides that, ER stress genes, BiP/GRP78 and CHOP were also manners similar to UPR components under the circadian rhythm (Figure 3a, b). To determine the relationship between UPR activation and circadian rhythmicity, we tested the downstream effectors of IRE1α and PERK branches of UPR signaling on a 36-hour time scale by immunoblotting. Similar to the oscillation pattern of mRNA expression of UPR components, protein levels of downstream effectors

of UPR signaling, XBP-1s, eIF2α, phospho(Ser51)-eIF2α and BiP/GRP78 peaked at 8-12 h and 28-32 h and troughed at 16-20 h and 36 h (Figure 4a, b). On the other hand, transcriptional activator circadian clock protein Bmal1 peaked at 20-24 h and 36 h and troughed at 12-16 and 28-32 h (Figure 4a, b). These results showed that UPR components and ER stress protein BiP/GRP78 exhibit oscillatory patterns not only at the transcriptional level but also consistently at the translational level.

Studies have demonstrated that ER stress is regulated in a circadian manner as other physiological processes like the daily oscillation of metabolism and hormonal rhythm [37]. Today, the enhancing effect of the UPR signal on the levels of ERAD target genes is well understood, which is one of the significant protein quality control mechanisms in eukaryotic cells. It rigorously degrades the misfolded proteins and also selectively regulates the steady-state level of physiologically essential protein levels [38,39]. Recent research has suggested the regulatory links between certain components of the ER protein quality control mechanism and the circadian rhythm. Guo et al., (2020) showed that circadian control of Bmal1 through ubiquitination is facilitated by E3 ubiquitin ligase Hrd1, a key member of the ERAD pathway [40]. Kim et al. (2021) revealed the regulation of hepatic lipid and glucose homeostasis by the E3 ubiquitin ligase HRD1/Sel1L-controlled CREBH/PPARα transcriptional program in vitro and in vivo models [41]. Furthermore, Erzurumlu et al., (2023) reported that the mRNA and protein expression levels of ERQC components are extensively regulated under circadian rhythmicity. Moreover, the main E3 ligase enzymes associated with ERAD, Hrd1, and gp78, were suggested to regulate circadian oscillation through regulation of the stability of Bmal1, which is a master transcription factor known to regulate circadian rhythm [9]. 12 h period rhythmic activation of the IRE1α branch of UPR has been associated with the regulation of lipid metabolism in mouse liver [42]. Additionally, activation of UPR in cancer cells has been shown to lead to a 10-hour shift in circadian oscillation [43]. All these studies suggest that ER-mediated coordinated signaling mechanisms, which have physiological importance, are periodically regulated by circadian rhythmicity. Thus, a detailed characterization of the oscillation pattern of these mechanisms is essential for understanding the human pathologies associated with the circadian rhythm.

Our study revealed the detailed oscillation pattern of UPR components and also indicated the expression of UPR members is regulated at the transcriptional and translational levels in human embryonic kidney HEK293 cells with robust rhythmicity (Figure 5). Furthermore, IRE1α and PERK signaling activation also exhibited regular rhythmicity with circadian oscillation. In light of all this literature information, we hope that the present study will provide essential oscillatory map data for understanding the relationship between UPR and circadian rhythm in the molecular pathophysiology of many diseases, including cancer.

UPR is one of the most essential evolutionary conserved signaling mechanisms in mammalian cells. Today, we know that UPR, in addition to being a mechanism for maintaining cellular homeostasis and an adaptation mechanism against cellular stresses in healthy cells, is also a vital mechanism for cancer cells and is one of the essential mechanisms that limit the success of cancer treatments, such as the development of drug resistance. Moreover, the impairment in the UPR signaling has been associated with numerous pathophysiological diseases. The present *in vitro* study revealed the robust oscillation patterns of UPR signaling components under a circadian rhythm in healthy embryonic kidney cells. We hope that our research will guide experimental treatment protocols that will be designed according to the circadian rhythm map of the UPR in UPR-focused pharmacological interventions.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Reppert, S.M., Weaver, D.R. (2002). Coordination of circadian timing in mammals. Nature, 418(6901), 935-941. [CrossRef]
2. Rosenwasser, A.M., Turek, F.W. (2015). Neurobiology of circadian rhythm regulation. Sleep Medicine Clinics, 10(4), 403-412. [CrossRef]
3. Patke, A., Young, M.W., Axelrod, S. (2020). Molecular mechanisms and physiological importance of circadian rhythms. Nature Reviews Molecular Cell Biology, 21(2), 67-84. [CrossRef]
4. Hastings, M., O'Neill, J.S., Maywood, E.S. (2007). Circadian clocks: Regulators of endocrine and metabolic rhythms. Journal of Endocrinology, 195(2), 187-198. [CrossRef]
5. Trott, A.J., Menet, J.S. (2018). Regulation of circadian clock transcriptional output by CLOCK:BMAL1. PLoS Genetics, 14(1), e1007156. [CrossRef]
6. Lee, Y., Field, J.M., Sehgal, A. (2021). Circadian rhythms, disease and chronotheraphy. Journal of Biological Rhythms, 36(6), 503-531. [CrossRef]
7. Lee, Y. (2021). Roles of circadian clocks in cancer pathogenesis and treatment. Experimental & Molecular Medicine, 53(10), 1529-1538. [CrossRef]
8. Crnko, S., Du Pré, B.C., Sluijter, J.P.G., Van Laake, L.W. (2019). Circadian rhythms and the molecular clock in cardiovascular biology and disease. Nature Reviews Cardiology, 16(7), 437-47. [CrossRef]
9. Erzurumlu, Y., Catakli, D., Dogan, H.K. (2023). Circadian oscillation pattern of endoplasmic reticulum quality control (ERQC) components in human embryonic kidney HEK293 cells. Journal of Circadian Rhythms, 21, 1. [CrossRef]
10. Borgese, N., Francolini, M., Snapp, E. (2006). Endoplasmic reticulum architecture: Structures in flux. Current Opinion in Cell Biology, 18(4), 358-364. [CrossRef]
11. Braakman, I., Hebert, D.N. (2013). Protein folding in the endoplasmic reticulum. Cold Spring Harbor Perspectives in Biology, 5(5), a013201. [CrossRef]
12. Fagone, P., Jackowski, S. (2009). Membrane phospholipid synthesis and endoplasmic reticulum function. Journal of Lipid Research, 50, 311-316. [CrossRef]
13. Hebert, D.N., Garman, S.C., Molinari, M. (2005). The glycan code of the endoplasmic reticulum: Asparagine-linked carbohydrates as protein maturation and quality-control tags. Trends in Cell Biology, 15(7), 364-370. [CrossRef]
14. Adams, C.J., Kopp, M.C., Larburu, N., Nowak, P.R., Ali, M.M.U. (2019). Structure and molecular mechanism of er stress signaling by the unfolded protein response signal activator IRE1. Frontiers in Molecular Biosciences, 6, 11. [CrossRef]
15. Hetz, C., Zhang, K., Kaufman, R.J. (2020). Mechanisms, regulation and functions of the unfolded protein response. Nature Reviews Molecular Cell Biology, 21(8), 421-438. [CrossRef]
16. Madden, E., Logue, S.E., Healy, S.J., Manie, S., Samali, A. (2019). The role of the unfolded protein response in cancer progression: From oncogenesis to chemoresistance. Biology of the Cell, 111(1), 1-17. [CrossRef]
17. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., Kaufman, R.J. (2001). Translational control is required for the unfolded protein response and *in vivo* glucose homeostasis. Molecular Cell, 7(6), 1165-1176. [CrossRef]
18. Cheng, G., Feng, Z., He, B. (2005). Herpes simplex virus 1 infection activates the endoplasmic reticulum resident kinase PERK and mediates eIF-2alpha dephosphorylation by the gamma(1)34.5 protein. Journal of Virology, 79(3), 1379-1388. [CrossRef]

19. Fels, D.R., Koumenis, C. (2006). The PERK/eIF2alpha/ATF4 module of the UPR in hypoxia resistance and tumor growth. Cancer Biology & Therapy, 5(7), 723-728. [CrossRef]
20. Kakiuchi, C., Iwamoto, K., Ishiwata, M., Bundo, M., Kasahara, T., Kusumi, I., Tsujita, T., Okazaki, Y., Nanko, S., Kunugi, H., Sasaki, T., Kato, T. (2003). Impaired feedback regulation of XBP1 as a genetic risk factor for bipolar disorder. Nature Genetics, 35(2), 171-175. [CrossRef]
21. Balsalobre, A., Damiola, F., Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell, 93(6), 929-937. [CrossRef]
22. Lambert, C.M., Weaver, D.R. (2006). Peripheral gene expression rhythms in a diurnal rodent. Journal of Biological Rhythms, 21(1), 77-79. [CrossRef]
23. Segers, A., Depoortere, I. (2021). Circadian clocks in the digestive system. Nature Reviews Gastroenterology & Hepatology, 18(4), 239-251. [CrossRef]
24. Scheiermann, C., Gibbs, J., Ince, L., Loudon, A. (2018). Clocking in to immunity. Nature Reviews Immunology, 18(7), 423-437. [CrossRef]
25. Chellappa, S.L., Vujovic, N., Williams, J.S., Scheer, F.A.J.L. (2019). Impact of circadian disruption on cardiovascular function and disease. Trends In Endocrinology and Metabolism: TEM, 30(10), 767-779. [CrossRef]
26. Logan, R.W., McClung, C.A. (2019). Rhythms of life: Circadian disruption and brain disorders across the lifespan. Nature Reviews Neuroscience, 20(1), 49-65. [CrossRef]
27. Chakraborti, S. (2022). Handbook of Oxidative Stress in Cancer: Therapeutic Aspects. Springer Nature, p.4078.
28. Masri, S., Sassone-Corsi, P. (2018). The emerging link between cancer, metabolism, and circadian rhythms. Nature medicine, 24(12), 1795-1803. [CrossRef]
29. Reddy, S., Reddy, V. Sharma, S. (2023). Physiology, Circadian Rhythm. In StatPearls. Treasure Island (FL): StatPearls Publishing.
30. Schwarz, D.S., Blower, M.D. (2016). The endoplasmic reticulum: Structure, function and response to cellular signaling. Cellular And Molecular Life Sciences, 73(1), 79-94. [CrossRef]
31. Ron, D., Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews. Molecular Cell Biology, 8(7), 519-529. [CrossRef]
32. Benham A.M. (2019). Endoplasmic Reticulum redox pathways: In sickness and in health. The FEBS Journal, 286(2), 311-321. [CrossRef]
33. Novoa, I., Zeng, H., Harding, H.P., Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. The Journal of Cell Biology, 153(5), 1011-1022. [CrossRef]
34. Zhu, T., Jiang, X., Xin, H., Zheng, X., Xue, X., Chen, J.L., Qi, B. (2021). GADD34-mediated dephosphorylation of eIF2α facilitates pseudorabies virus replication by maintaining de novo protein synthesis. Veterinary Research, 52(1), 148. [CrossRef]
35. Clarke, R. (2019). The Unfolded Protein Response in Cancer. Springer. p.220. (Book).
36. Bashir, S., Banday, M., Qadri, O., Bashir, A., Hilal, N., Nida-I-Fatima, Rader, S., Fazili, K.M. (2021). The molecular mechanism and functional diversity of UPR signaling sensor IRE1. Life sciences, 265, 118740. [CrossRef]
37. Maillo, C., Martín, J., Sebastián, D., Hernández-Alvarez, M., García-Rocha, M., Reina, O., Zorzano, A., Fernandez, M., Méndez, R. (2017). Circadian- and UPR-dependent control of CPEB4 mediates a translational response to counteract hepatic steatosis under ER stress. Nature Cell Biology, 19(2), 94-105. [CrossRef]
38. Bhattarai, K.R., Riaz, T.A., Kim, H.R., Chae, H.J. (2021). The aftermath of the interplay between the endoplasmic reticulum stress response and redox signaling. Experimental & Molecular Medicine, 53(2), 151-167. [CrossRef]
39. Erzurumlu, Y., Dogan, H.K., Catakli, D., Aydogdu, E., Muhammed, M.T. (2023). Estrogens drive the endoplasmic reticulum-associated degradation and promote proto-oncogene c-Myc expression in prostate cancer cells by androgen receptor/estrogen receptor signaling. Journal of Cell Communication and Signaling, 17(3), 793-811. [CrossRef]
40. Guo, D., Zhu, Y., Wang, H., Wang, G., Wang, C., Ren, H. (2020). E3 ubiquitin ligase HRD1 modulates the circadian clock through regulation of BMAL1 stability. Experimental and Therapeutic Medicine, 20(3), 2639-2648. [CrossRef]
41. Kim, H., Wei, J., Song, Z., Mottillo, E., Samavati, L., Zhang, R., Li, L., Chen, X., Jena, B.P., Lin, J.D., Fang, D., Zhang, K. (2021). Regulation of hepatic circadian metabolism by the E3 ubiquitin ligase HRD1-controlled CREBH/PPARα transcriptional program. Molecular Metabolism, 49, 101192. [CrossRef]
42. Cretenet, G., Le Clech, M., Gachon, F. (2010). Circadian clock-coordinated 12 Hr period rhythmic

activation of the IRE1alpha pathway controls lipid metabolism in mouse liver. Cell Metabolism, 11(1), 47-57. [CrossRef]

43. Bu, Y., Yoshida, A., Chitnis, N., Altman, B.J., Tameire, F., Oran, A., Gennaro, V., Armeson, K.E., McMahon, S.B., Wertheim, G.B., Dang, C.V., Ruggero, D., Koumenis, C., Fuchs, S.Y., Diehl, J.A. (2018). A PERK-miR-211 axis suppresses circadian regulators and protein synthesis to promote cancer cell survival. Nature Cell Biology, 20(1), 104-115. [CrossRef]