Ultraviolet Light Inhibits Translation through Activation of the Unfolded Protein Response Kinase PERK in the Lumen of the Endoplasmic Reticulum*

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Exposure to ultraviolet light can cause inflammation, premature skin aging, and cancer. UV irradiation alters the expression of multiple genes that encode functions to repair DNA damage, arrest cell growth, and induce apoptosis. In addition, UV irradiation inhibits protein synthesis, although the mechanism is not known. In this report, we show that UV irradiation induces phosphorylation of eukaryotic translation initiation factor 2 on the α-subunit (eIF2α) and inhibits protein synthesis in a dosage- and time-dependent manner. The UV-induced phosphorylation of eIF2α was prevented by the overexpression of a non-phosphorylatable mutant of eIF2α (S51A). PERK is an eIF2α protein kinase localized to the endoplasmic reticulum that is activated by the accumulation of unfolded proteins in the endoplasmic reticulum. Expression of trans-dominant-negative mutants of PERK also prevented eIF2α phosphorylation upon UV treatment and protected from the associated translation attenuation. The luminal domain of dominant-negative mutant PERK formed heterodimers with endogenous PERK to inhibit the PERK signaling pathway. In contrast, eIF2α phosphorylation was not inhibited by overexpression of a trans-dominant-negative mutant kinase, PKR, supporting the theory that UV-induced eIF2α phosphorylation is specifically mediated by PERK. These results support a novel mechanism by which UV irradiation regulates translation via an endoplasmic reticulum-stress signaling pathway.

UV irradiation causes the increased or reduced expression of multiple genes that encode functions to repair DNA damage (1), arrest cell growth (2, 3), and induce apoptosis (4). The transcriptional regulation upon UV irradiation has been extensively studied (5, 6). However, translational regulation upon UV irradiation has not been well investigated. In this study we elucidate a novel UV-activated signaling pathway that emanates from the endoplasmic reticulum (ER) and inhibits protein synthesis by phosphorylation of the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α).

Accumulation of unfolded proteins in the ER induces eIF2α phosphorylation through activation of the eIF2α kinase, PERK (7–10). PERK contains a signal peptide (amino acids 1–31) and a domain (amino acids 32–521) that resides in the ER lumen and shares 20% identity with the ER-stress-sensing luminal domain of mammalian IRE1 (an ER resident transmembrane kinase). The Ser7Thr protein kinase domain (amino acids 589–1114) is 40% identical to the kinase domain of the double-stranded RNA-activated eIF2α protein kinase PKR. PERK specifically phosphorylates the serine 51 residue of eIF2α in vitro and in vivo and thereby inhibits translation initiation. Phosphorylation of eIF2α limits the frequency of polypeptide chain initiation in general, although it can permit the preferential translation of certain mRNAs, possibly those that have open reading frames within the 5′-untranslated regions or those that have internal ribosomal entry sites (11–14). One mRNA selectively translated under these conditions encodes the basic leucine zipper-containing activating transcription factor ATF4 that likely contributes to ER-stress-induced transcription as a component of the unfolded protein response. Expression of a catalytically inactive PERK kinase mutant (K618A) acts in a trans-dominant-negative manner to protect eIF2α from phosphorylation (8). Overexpression of PERK results in spontaneous activation to inhibit translation of its own mRNA (8). Overexpression of the isolated luminal domain of PERK (PERKLAC) inhibits ER-stress-induced expression of a subset of unfolded protein-responsive genes such as BiP and CHOP10 (GADD153) (8, 15).

It was reported that UV irradiation inhibits protein synthesis in rat fibroblasts (16). However, the mechanism for UV-induced protein synthesis inhibition is not clear. It was proposed that UV-induced translational inhibition undergoes a ribotoxic stress signaling pathway, which is based on the fact that the UV-induced translational inhibition is correlated with rRNA damage and JNK activation (16). In this article we provide evidence that UV irradiation induces phosphorylation of eIF2α and inhibits protein synthesis in intact cells. We also provide evidence that the UV-induced eIF2α phosphorylation and translational inhibition are mediated by PERK through an ER-stress signaling pathway.

PERK (residues 1–536); eIF2α, α-subunit of eukaryotic initiation factor 2; GFP, green fluorescent protein; EGFP, enhanced GFP; JNK, c-Jun NH2-terminal kinase; OA, okadaic acid; HIT, hamster insulinoma tumor.
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**Fig. 1. UV inhibits nascent protein synthesis in a dosage- and time-dependent manner.** Total cell extracts (30 µg) from UV-irradiated and [35S]Met/Cys pulse-labeled MCF-7 cells were resolved by 12% SDS-PAGE, stained with Coomassie Blue R-250 (right panel), treated with En3Hance (PerkinElmer Life Sciences), and dried. The nascent protein synthesis was determined by autoradiography (left panel). A, dose-dependent inhibition of translation after 30 J/m² UVC.

**Fig. 2. UV induces endogenous eIF2α phosphorylation.** Total cell extracts prepared from UV-irradiated HIT cells or eIF2α transfected COS-1 cells were resolved by 12% SDS-PAGE and electroblotted to nitrocellulose membranes. The amount of total eIF2α and phosphorylated eIF2α were measured by Western blot analysis. The levels of eIF2α and phosphorylated eIF2α were determined using NIH Image (Version 1.62). A, endogenous eIF2α phosphorylation in HIT cells; B, dose-dependent phosphorylation of eIF2α at 4 h post-irradiation; C, time-dependent phosphorylation of eIF2α after 50 J/m² UV irradiation.

**MATERIALS AND METHODS**

**Construction of Mammalian Expression Vectors for PERK, Mutant PERK (K618A), the Luminal Domain of PERK (PERKΔC), and EGF-F**

The myc-tagged PERK and PERK (K618A) expression vectors (in pcDNA1, Invitrogen, San Diego, CA) were kindly provided by Dr. David Ron (New York University School of Medicine). Using restriction enzyme digestion and ligation we subcloned the genes into the mammalian expression vector pETFVA, which is identical to the other expression vectors (PKR mutants, eIF2α, and eIF2α-51A) used in our experiments. The luminal domain of PERK (PERKΔC, amino acids 1–536) was amplified from pcDNA1-PERK using the PCR method. A high fidelity DNA polymerase (Pwo, Roche Molecular Biochemicals) was used for the PCR. A T7 tag (MAMTGTTGQMG) was added to the C terminus of PERKΔC. The PCR products were subcloned into the mammalian expression vector pETFVA.

To construct the pBIP-EGFP vector the BiP promoter was excised from pGL3-BiP using restriction enzyme digestion and was subcloned into p2EGFP-1 (Stratagene, La Jolla, CA), which encodes a short-lived form of EGFP.

**Cell Culture and Derivation of the PERKΔC Stably Transfected MCF-7 Cell Line—**COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and penicillin/streptomycin (100 units/ml). All of the above reagents were purchased from Invitrogen.

For construction of an MCF-7 cell line that expresses PERKΔC the PERKΔC was subcloned from pETFVA-PERKΔC into the pZ vector to form pZ-PERKΔC. MCF-7 cells were transfected with pZ-PERKΔC using Fugene6 (Roche Molecular Biochemicals) and selected with G418 (0.5 mg/ml, Invitrogen). Expression of PERKΔC was detected by Western blot analysis using anti-PERK antibody. The PERKΔC stably transfected MCF-7-PERKΔC cells were identified and cultured in medium containing 0.5 mg/ml G418.

**Detection of BiP Promoter Activation—**MCF-7 cells were plated in a 6-well plate at a density of 5 × 10⁵ cells per well at 18 h pretreatment. The cells were transfected with p2EGFP-BiP (0.8 µg/well) using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s protocol. At 18 h post-transfection, the cells were irradiated with 30 J/m² UVC. The images of EGFP expression were then captured by an Olympus IX70 fluorescence microscope (Olympus America Inc., Melville, NY) using a Kodak M280 camera (Eastman Kodak Co.) with an exposure time of 0.25 s.

The expression of GFP was quantified by Imagepro Plus 4.1 (Media Cybernetics, Inc., Silver Spring, MD) for each cell count integrated optical density of the green channel. Merged cells were manually separated, and artifacts (items or noise too small to be cells) were removed before the quantitation. The average integrated optical density per cell was generated by Microsoft Excel (Microsoft Co., Seattle, WA).

**UV Irradiation—**UVC was generated from a 15-watt UVC light source (UVP Inc., Upland, CA). The intensity of UVC was standardized before the quantitation. The average integrated optical density per cell was determined using a UV meter (UVP Inc.) and set at 3 watts/m². The medium was withdrawn during the UVC irradiation. After UV irradiation fresh medium was added to each plate.

**Analysis of Protein Synthesis—**The UV-irradiated cells were labeled with RediVue Pro Mix ([35S]Met/Cys (100 µCi/ml, 1,000 Ci/mmol; American Biosciences) for 20 min in methionine/cysteine-free minimal essential medium (Invitrogen). After washing with phosphate-buffered saline cell extracts were prepared by lysing the cells in Nonidet P-40 lysis buffer (2% Nonidet P-40, 80 mM NaCl, 100 mM Tris-HCl, 0.1% SDS). The protein concentration was measured using the Bio-Rad protein assay kit. Equal amounts of proteins were loaded to 12% SDS-PAGE and electroblotted to nitrocellulose membrane. The total amount of eIF2α and phosphorylated eIF2α were measured by Western blot analysis. The levels of eIF2α and phosphorylated eIF2α were determined using NIH Image (Version 1.62). Endogenous eIF2α phosphorylation in HIT cells; B, dose-dependent phosphorylation of eIF2α at 4 h post-irradiation; C, time-dependent phosphorylation of eIF2α after 50 J/m² UV irradiation.
siently transfected with eIF2α expression vector (pETFVA-2α) by the DEAE-dextran procedure as described previously (18). After 48 h the transfected cells were UV-irradiated and lysed in the Nonidet P-40 lysis buffer containing proteinase inhibitor mixture (Complete™, Roche Molecular Biochemicals) at the indicated postirradiation time. The expression levels of eIF2α in each sample were first determined by Western blot analysis using a mouse anti-eIF2α monoclonal antibody (19). After quantification of the expression levels of eIF2α equal amounts of eIF2α were subjected to SDS-PAGE followed by Western blot analysis. The levels of eIF2α expression and phosphorylation were determined using the same methods as described above.

**Dimerization Analysis of Intact PERK and the Luminal Domain of PERK**—COS-1 cells were transiently transfected with pETFVA-PERK (K618A)-myc and pETFVA-PERKAC-T7 using the DEAE-dextran procedure. After 48 h the cells were pulse-labeled using [35S]Met/Cys for 20 min in Met/Cys-free minimal essential medium. Cell extracts were prepared by lysis in the Nonidet P-40 lysis buffer. The dimerization of PERK (K618A)-myc and PERKAC-T7 was detected by co-immunoprecipitation using anti-T7 antibody or anti-myc antibody in radioimmune precipitation buffer. Antibody-antigen complexes were absorbed by protein A/G-agarose (Pierce) and resolved by 10% SDS-PAGE. The gels were fixed in 40% methanol, 10% acetic acid, prepared for fluorography by treatment with En3Hance (PerkinElmer Life Sciences), and dried. The dried gels were autoradiographed with Kodak BioMax-MR film.

**RESULTS AND DISCUSSIONS**

**UV Irradiation Inhibits Protein Synthesis in a Dosage- and Time-dependent Manner**—To further elucidate the mechanism of UV-induced translational inhibition we examined whether UV irradiation induces the phosphorylation of endogenous eIF2α. Because endogenous eIF2α is not detectable in MCF-7 cells we used hamster insulinoma tumor (HIT) cells, which express a higher level of eIF2α. The cells were irradiated with 50 J/m² UVC. To prevent the dephosphorylation and degradation of the phosphorylated proteins cells were cultured in medium containing OA and ALLN for 4 h after irradiation and lysed in Nonidet P-40 lysis buffer. The phosphorylation state of eIF2α was then examined. Phosphorylation of eIF2α increased when cells were treated with OA and ALLN alone (Fig. 2A) and was likely caused by the inhibition of phosphatase-1 and phosphatase-2A by OA as previously reported (20). When the cells were treated with UV the phosphorylation of eIF2α increased further by 70% (Fig. 2A). These results show that UV irradiation induces endogenous eIF2α phosphorylation. PERK activation was also examined in these cells. However, we were unable to detect a significant increase in PERK phosphorylation in the UV-irradiated cells (data not shown). This may be because UV-induced PERK activation is a prolonged process that does not produce a clear up-shifting band as is normally observed for phosphorylated PERK (21).

**UV Irradiation Induces eIF2α Phosphorylation in HIT Cells**—To further elucidate the mechanism of UV-induced translational inhibition we examined whether UV irradiation induces the phosphorylation of endogenous eIF2α. Because endogenous eIF2α is not detectable in MCF-7 cells we used hamster insulinoma tumor (HIT) cells, which express a higher level of eIF2α. The cells were irradiated with 50 J/m² UVC. To prevent the dephosphorylation and degradation of the phosphorylated proteins cells were cultured in medium containing OA and ALLN for 4 h after irradiation and lysed in Nonidet P-40 lysis buffer. The phosphorylation state of eIF2α was then examined. Phosphorylation of eIF2α increased when cells were treated with OA and ALLN alone (Fig. 2A) and was likely caused by the inhibition of phosphatase-1 and phosphatase-2A by OA as previously reported (20). When the cells were treated with UV the phosphorylation of eIF2α increased further by 70% (Fig. 2A). These results show that UV irradiation induces endogenous eIF2α phosphorylation. PERK activation was also examined in these cells. However, we were unable to detect a significant increase in PERK phosphorylation in the UV-irradiated cells (data not shown). This may be because UV-induced PERK activation is a prolonged process that does not produce a clear up-shifting band as is normally observed for phosphorylated PERK (21).

**UV Irradiation Induces eIF2α Phosphorylation in a Dosage- and Time-dependent Manner**—To further confirm that UV irradiation induces eIF2α phosphorylation we analyzed whether UV-irradiated eIF2α phosphorylation is dependent on dosage and time. COS-1 cells were used in this experiment because the cell line is conveniently and efficiently transiently transfected and expresses high levels of proteins from transfected genes. In addition, the endogenous eIF2α was undetectable in COS-1 cells by Western blot analysis (18, 19) the eIF2α was transiently transfected into COS-1 cells by Western blot analysis (18, 19) the eIF2α expression vector encoding eIF2α was transiently transfected into COS-1 cells, and phosphorylation of the expressed eIF2α was detected using an antibody specific for the phosphorylated form of eIF2α and compared with reactivity for an antibody that is specific for total eIF2α (17). Because the levels of total expressed eIF2α did not significantly vary at these doses and time points (Fig. 2, B and C), differential reactivity to the antibody specific for the phosphorylated form of eIF2α is indic-

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2 D. Ron, personal communication.
Western blot analysis using anti-eIF2α/H9251 untreated or treated with 15 J/m² UVC, 30 J/m² UVC, or 200 nM thapsigargin (Tg) as indicated at 18 h post-transfection. A, EGFP fluorescence emitted at 505 nm was captured at 0 and 6 h postirradiation; B, expression of EGFP was determined by ImagePro Plus 4.1 (Media Cybernetics) for each cell count integrated optical density of green channel.

**FIG. 4.** UV activates the BiP promoter. The pBiP-EGFP (1.3 μg/ml) transfected MCF-7 cells were either left untreated or treated with 15 J/m² UVC, 30 J/m² UVC, or 200 nM thapsigargin (Tg) as indicated (Fig. 2A). The pBiP promoter is sufficient to inhibit protein synthesis initiation (22) lower doses of irradiation (such as 30 J/m²) may also inhibit protein synthesis through eIF2α phosphorylation although it is difficult to measure the increase in eIF2α phosphorylation at these doses.

**PERK Mediates UV-induced eIF2α Phosphorylation—**Both PKR and PERK were suggested to mediate stress-induced eIF2α phosphorylation (8, 23). Overexpression of a dominant-negative kinase mutant PKR (K296P) inhibits eIF2α phosphorylation catalyzed by endogenous PKR (19). In addition, a kinase-defective K618A mutant PERK acts in a trans-dominant-negative manner to inhibit eIF2α phosphorylation upon activation of endogenous PERK by ER-stress (8). Overexpression of the isolated luminal domain of PERK (PERKΔC) inhibits its ER-stress-induced transcriptional induction of CHOP10, a gene that acts as a marker of the unfolded protein response (8, 15). We examined whether overexpression of the dominant-negative mutant forms of PKR and/or PERK could also inhibit UV-induced phosphorylation of eIF2α. COS-1 cells were transiently co-transfected with eIF2α and the expression vectors indicated (Fig. 3). The transfected cells were irradiated with UV. At 4 h post-irradiation the cells were harvested, and the expression and phosphorylation of eIF2α were detected by Western blot analysis using anti-eIF2α and anti-eIF2α-P antibodies as described under “Materials and Methods.” This analysis demonstrated that the phosphorylation of overexpressed wild-type eIF2α increased with UV treatment. However, overexpression of a phosphorylation site mutant S51A eIF2α significantly prevented the phosphorylation of co-transfected eIF2α upon UV treatment (Fig. 3, lanes 4–6). These results support the theory that phosphorylation of eIF2α upon UV treatment occurs on residue Ser-51. Overexpression of wild-type PERK constitutively increased eIF2α phosphorylation, which was further increased after UV treatment (Fig. 3, lanes 10–12). Significantly, overexpression of either PERK (K618A) or PERKΔC inhibited UV-induced eIF2α phosphorylation (Fig. 3, lanes 13–18). Although a previous report suggested that UV irradiation (27 J/m², 45 min) does not induce autophosphorylation and activation of PERK (8) this analysis did not vary the time and dosage of UV treatment. We believe that the discrepancy is because of the time- and dosage-dependent activation of PERK. Our data show that increased eIF2α phosphorylation cannot be detected at 4 h after 30 J/m² irradiation (Fig. 2B). In contrast to mutant PERK overexpression of the dominant-negative mutant PKR (K296P) did not affect the UV-induced eIF2α phosphorylation (Fig. 3, lanes 7–9). These results support that UV-induced eIF2α phosphorylation is specifically mediated by PERK and not by PKR.

**UV Irradiation Activates BiP Promoter—**It was reported that UV irradiation induces the activation of CHOP10 (24), an ER-stress-inducible gene (25). ER-stress-induced CHOP10 activation may be mediated by ATF4, which is downstream of PERK activation and eIF2α phosphorylation (26). However, it is not known whether UV irradiation induces ER-stress to activate PERK. Because increasing levels of the ER luminal chaperone BiP inhibit PERK activation and eIF2α phosphorylation (21), we determined whether UV irradiation activates the BiP promoter. A BiP-regulated EGFP expression vector (pBiP-EGFP) was used as a reporter to measure BiP promoter activation after UV irradiation. UV irradiation induced GFP expression by 2.9-fold (15 J/m² UV) to 3.8-fold (30 J/m² UV) (Fig. 4B). In parallel, thapsigargin (200 nM) induced GFP expression by 3.4-fold (Fig. 4B). Our data indicate that UV induction of the BiP promoter is comparable with thapsigargin induction in MCF-7 cells (Fig. 4, A and B). Therefore, UV irradiation induces the ER-stress response, which results in the activation of the BiP promoter. These results suggest that UV-induced PERK activation with subsequent transcriptional induction of BiP would increase the level of BiP in the ER lumen and thereby prevent further PERK activation. Although UV treatment inhibits protein synthesis it is possible that the BiP mRNA is efficiently translated upon UV treatment because it has an internal ribosomal entry site element within the 5′ end of its mRNA. Indeed, recent studies demonstrated that internal
Ribosomal entry site-mediated translation is not inhibited upon eIF2\alpha phosphorylation (13, 14).

Expression of a Dominant-negative PERK (PERK\alpha/C) Reverses UV-induced Translational Inhibition—To confirm that PERK mediates UV-induced translational inhibition, protein synthesis after UV irradiation in MCF-7 and the MCF-7-PERK\alpha/C cell line was measured. Whereas protein synthesis was reduced to 18% in control MCF-7 cells at 24 h after 30 J/m² UVC, protein synthesis remained at 50% in MCF-7-PERK\alpha/C cells (Fig. 5B). The incomplete protection of UV-induced translational inhibition may be caused by the activation of endogenous PERK or other factors (such as a ribotoxic stress response) (16). Interestingly, when MCF-7-PERK\alpha/C cells were irradiated with a high dose (100 J/m²) of UVC, PERK\alpha/C failed to prevent UV-induced translational inhibition. Because expression of PERK\alpha/C in the MCF-7-PERK\alpha/C is not reduced after UV irradiation (Fig. 5C) the result suggests that another signaling pathway also may be involved in translational regulation after UV irradiation.
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UV irradiation. Our data show that UV-induced translational inhibition is at least partially mediated through PERK (Fig. 5, A and B).

PERK Dimerizes with Intact PERK (K618A)—To examine the possibility that overexpressed PERK mutants form inactive dimers with endogenous PERK, a co-immunoprecipitation assay was established to measure dimerization between intact PERK and the N-terminal luminal domain of PERK in vivo. Because the wild-type PERK inhibits translation of its own mRNA and is expressed at a very low level, we used the kinase-inactive PERK mutant K618A in this analysis. COS-1 cells were transiently co-transfected with pETFVA′–PERK (K618A)–myc and pETFVA′–PERKΔC-T7. After pulse-labeling the transfected cells with [35S]Met/Cys, the interaction between intact PERK and PERKΔC was measured by co-immunoprecipitation with monoclonal anti-T7 antibody (Novagen Corp., Madison, WI) or anti-myc antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Total cell protein extracts and the immunoprecipitates were analyzed by SDS-PAGE. Immunoprecipitation of the T7-tagged PERKΔC yielded the expected polypeptide at 80 kDa as well as a 130-kDa polypeptide that represented the PERK (K618A) (Fig. 6, lane 6). Immunoprecipitation of the myc-tagged PERK (K618A) showed that expression levels of PERK (K618A) were slightly different in the transfected cells (67;100; Fig. 6, lane 8 versus lane 9). PERKΔC co-immunoprecipitated myc-tagged PERK (K618A) (Fig. 6, lane 9). The amount of dimerization was quantitated using NIH Image (Version 1.620). The intensity of PERK (K618A) was 24% of the associated PERKΔC (Fig. 6, lane 6). The intensity of the PERKΔC was 8.8% of the associated PERK (K618A). After correcting the numbers for the molecular weight of the peptides, the amount of heterodimerized peptides was 14.5 ± 0.2%. These results suggest that the overexpression of a mutated PERK may inhibit endogenous PERK by forming inactive heterodimers.

In summary, our findings demonstrate that UV irradiation induces an ER-stress-induced signaling pathway that activates PERK and inhibits protein synthesis in mammalian cells. These studies are a first step to understand the mechanistic details of how UV light activates an ER-stress response that may mediate downstream responses. We hypothesize that UV light disrupts protein folding in the ER resulting in the accumulation of unfolded proteins to promote PERK dimerization and activation. PERK-mediated phosphorylation of eIF2α subsequently results in protein synthesis inhibition. We also propose that upon PERK activation the translation of selective mRNAs (such as ATF4) (26, 27) increases. IRE1 is a protein kinase and site-specific endoribonuclease that signals transcriptional activation of a large set of genes upon activation of the unfolded protein response in the ER (28). Because PERK and the protein kinase/endoribonuclease IRE1 both are activated by identical degrees and types of ER-stress (29, 30), upon UV irradiation IRE1 may also be activated to signal the transcriptional activation of its target genes that include CHOP10, BiP, and GADD34 (21, 26, 31). It has been suggested that IRE1 can cleave ribosomal RNA (32) and that this may contribute to the ribotoxic effects associated with UV irradiation (16). Increased expression of the eIF2α phosphatase GADD34 may provide cell protection by reversing the transcriptional inhibition associated with PERK activation and eIF2α phosphorylation (31), which is consistent with the reversal of eIF2α phosphorylation and increased translation that we observed after longer periods of UV treatment. In addition, activated IRE1 can activate JNK to initiate an apoptotic program (33), a signaling pathway known to be activated by UV light (34, 35). Future studies should elucidate the factors that are required for PERK and/or IRE1 activation, and the studies also should clarify the mechanisms for downstream signaling in response to UV light. These studies should encourage the development of drugs to target PERK and/or IRE1 in order to prevent and treat UV-related development of skin cancers.

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