THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of UVB-induced cellular mechanisms in human keratinocytes using a novel approach of delivering *in vitro* synthesized mRNA encoding cyclobutane pyrimidine dimer-specific photolyase

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Contents

Abbreviations

6-4PP	6-4 photoproduct or pyrimidine(6-4)pyrimidone
AKT	Protein kinase B
ATF3	Activating transcription factor 3
CCNE1	Cyclin E1
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15)
c-Myc	Avian Myelocytomatosis Viral Oncogene Homolog
CPD	Cyclobutane pyrimidine dimer
CPD-PL Ψ-mRNA	Pseudouridine-modified mRNA encoding CPD-photolyase
CPD-PL	CPD-specific photolyase
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
eGFP Ψ-mRNA	Pseudouridine-modified mRNA encoding eGFP
eGFP	Enhanced green fluorescent protein
EGR1	Early growth response 1
ELISA	Enzyme-linked immunosorbent assay
ERCC1	Excision repair cross-complementation group 1
FAD	Flavin adenine dinucleotide
FADH	Reduced forms of FAD
HPLC	High-performance liquid chromatography
HR23B	RAD23 homolog B
ID2	Inhibitor of DNA binding 2
IL-6	Interleukin 6
IVT mRNA	*In vitro* transcribed messenger RNA
JNK	c-Jun N-terminal kinase

MAPK	Mitogen-activated protein kinases
NER	Nucleotide excision repair
Ψ	Pseudouridine
PTGS2	Prostaglandin-endoperoxide synthase 2
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
RUNX1	Runt-related transcription factor 1
SMAD4	Mothers against decapentaplegic homolog, family member 4
SNAI1	Snail family zinc finger 1
SNAI2	Snail family zinc finger 2
TP53	Tumor protein p53
UV	Ultraviolet radiation
XPA	Xeroderma pigmentosum, Complementation group A
XPB	Xeroderma pigmentosum, Complementation group B
XPC	Xeroderma pigmentosum, Complementation group C
XPD	Xeroderma pigmentosum, Complementation group D
XPF	Xeroderma pigmentosum, Complementation group F
XPG	Xeroderma pigmentosum, Complementation group G

1. Introduction

Ultraviolet B (UVB) (280-315 nm) radiation is the main environmental risk factor for sunburn, skin carcinogenesis and premature skin aging. Major biological effects of UVB radiation are attributed to cyclobutane pyrimidine dimers (CPDs), which are the most frequently formed deleterious DNA photolesions. CPDs have been shown to induce inflammation, immune suppression and mutations that may lead to melanoma and non-melanoma skin cancers. However, so far, it has been unclear how CPDs change gene expression and cell activities. Until now, there was no suitable experimental platform to identify directly CPD-responsive genes in human cells, thus distinguish CPD-regulated cellular mechanisms from those mediated by other UVB-induced derivatives, including diverse photoproducts, reactive oxygen species, cross-linked protein-DNA and other damaged macromolecules.

The integrity of DNA can be quickly restored by a light-activated photolyase, which splits the CPDs using the energy of visible light in a process called „photoreactivation". However, this DNA repair enzyme is absent in placental mammals, including humans, that must rely on the slower and less accurate nucleotide excision repair (NER) system to repair UV-induced DNA-lesions. Our scientific idea was to transiently express functional CPD-specific photolyase in cultured human keratinocytes using a novel i*n vitro* transcribed (IVT) mRNA-based gene therapy method, as a platform to distinguish CPD-dependent and -independent events of UVB-induced cellular responses.

1.1 Biological effects of ultraviolet radiation on skin cells

UV radiation is divided into three wavelength ranges, UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). The UVC spectrum of solar radiation has no clinical relevance, because it is completely absorbed by the ozone layer. Human skin is exposed to UVA (95%) and UVB (5%). UVB radiation is mainly absorbed in the epidermis, while UVA also reaches the dermis and hypodermis (**Figure 1**) [1].

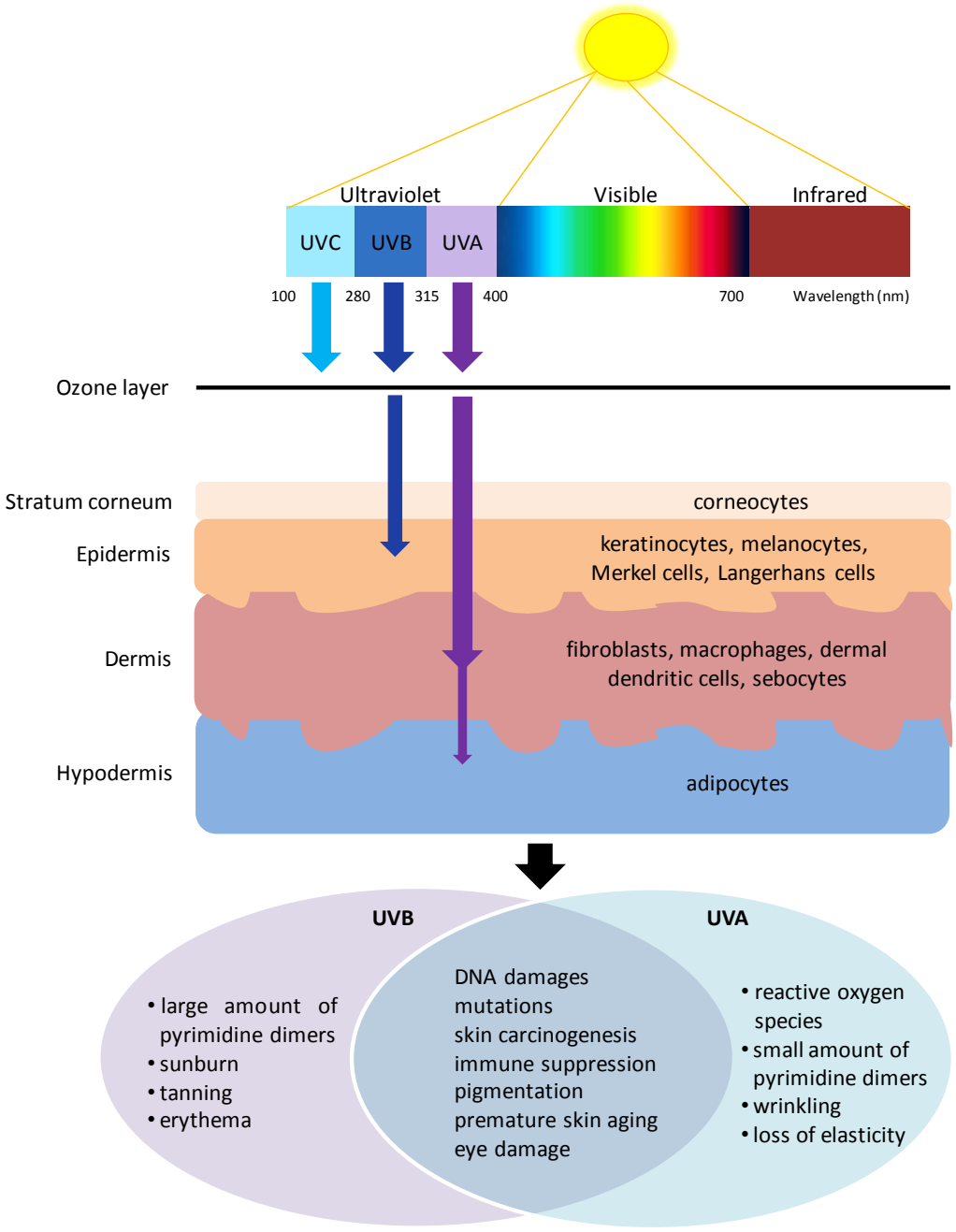


Figure 1. The spectrum of solar radiation and penetration of UV rays into the skin.

Solar radiation has many beneficial effects (e.g., vitamin D synthesis, improvement of skin diseases, antidepressant effect), but it is also responsible for pathological changes in the skin, including sunburn, photoaging and skin cancer [2]. UV radiation induces harmful photochemical reactions causing damage in nucleic acids, lipids, proteins and altering the cell structures [1]. The absorption of UVB photons by genomic DNA is one of the most important cell biological events upon solar radiation, because DNA absorbs UV light very effectively, it carries the genetic information for a cell and it is present the fewest copies in a cell [3]. A variety of DNA photolesions, including cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs) are induced by UVB [4]. The consequence is substantial change in the pattern of actively transcribed genes, including those involved in DNA repair, cell cycle regulation, cell growth, apoptosis and immune responses [5]. UVB-induced DNA damage can also result in mutations thereby playing a critical role in skin tumorigenesis [6].

1.2 The major UVB-induced DNA damages

1.2.1 Cyclobutane pyrimidine dimers

Cyclobutane pyrimidine dimers discovered by *R. B. Setlow et al.* [7] are the most frequently formed cytotoxic DNA photolesions. These photoproducts cause mild distortion of DNA structure leading to the interruption of DNA replication that can result in chromosomal imbalances and blockage of transciptional program [8, 9]. Available evidence seems to support that CPDs are mainly responsible for the cytotoxic and mutagenic effects of UV radiation in mammalian cells [10, 11]. It forms in double-stranded DNA when two adjacent pyrimidine bases in one strand fuse together (**Figure 2**) [12]. The formation of CPDs at different dipyrimidine sites depends on the wavelength of UV light. UVA radiation has a poor efficiency in producing CPDs while UVB and UVC readily and effectively induce these photolesions, predominantly at thymine dimers (TTs) followed by formation at TC, CT and CC sequences [13, 14]. The removal of CPDs from the mammalian genome is slow and

incomplete [15, 16]. Accumulated CPDs induce cell cycle arrest in order to prevent the multiplication of mutated chromosomes, but may lead to induction of apoptosis, genomic instability or skin carcinogenesis [17-20]. It has been known for many years that CPDs containing cytosine (CCs and CTs) have the most powerful mutagenic activity. This is due to deamination of cytosine or 5-methylcytosine resulted in deaminated CPDs, which are bypassed by DNA polymerase ε during a DNA damage tolerance process called translesion synthesis [21-23]. It has been shown in mammalian cells irradiated by UVB light that the most common mutations at dipyrimidine sequences are C → T transitions, including CC → TT double-base mutation events, later named "UV fingerprint" mutations [11, 24]. Indeed, strong correlation has been found between the occurrence of these "UV fingerpint" mutations in p53 gene and the development of non-melanoma skin cancer [25-27].

1.2.2 Pyrimidine (6-4) pyrimidone photoproducts

Another type of UV-induced photolesions formed in DNA is the pyrimidine (6-4) pyrimidone photoproduct, briefly called (6-4) photoproduct [28]. The first studies on DNA damages demonstrated that 6-4PPs primarily cause T → C transition with high replicating error frequency, followed by formation at CC and TT sites (**Figure 2**) [10].

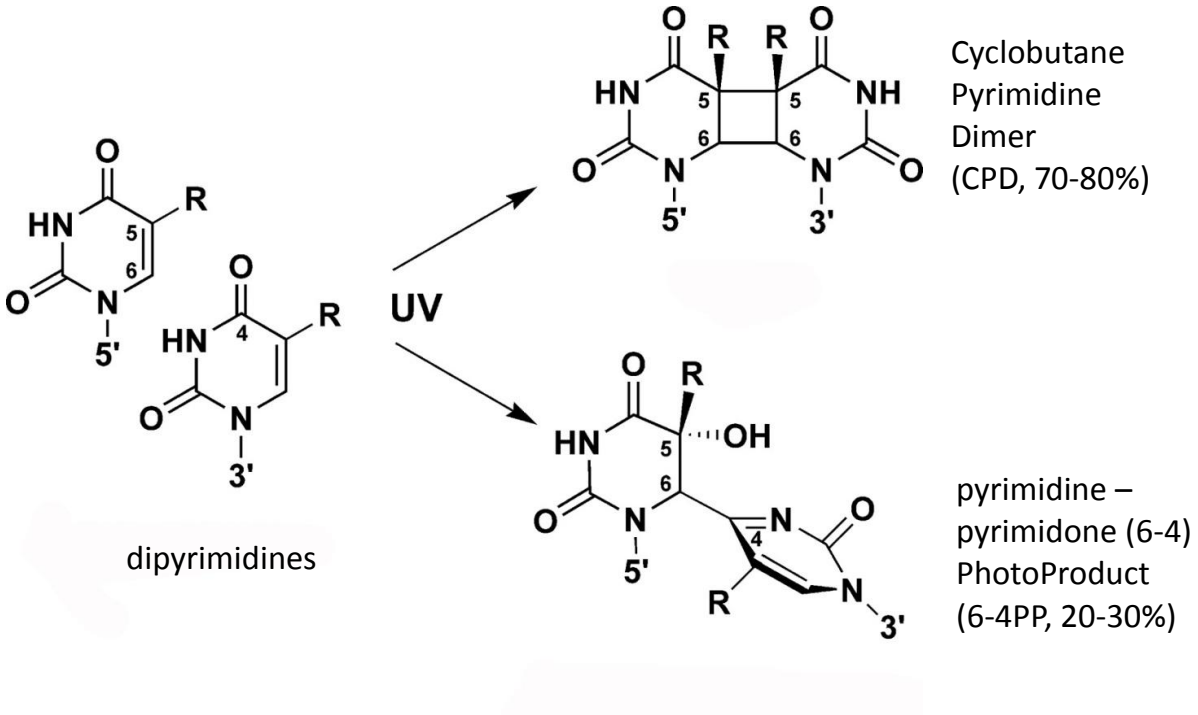


Figure 2. Structure of UV-induced DNA photoproducts. *Formation of cyclobutane pyrimidine dimer and (6-4) photoproduct at TpT site induced by UV irradiation. (Taken from Jiang Li et al., doi: 10.1074/jbc.M604483200).*

These photoproducts produce greater distortion in the structure of DNA double-helix and are less frequently induced by UV irradiation than CPDs [29]. However, the frequency of their formation significantly depend on the ratio of DNA bases, chromatin structure and the wavelength of UV light [30]. Despite the fact that 6-4PPs represent only 10-20% of all UV-induced DNA damages, they play a significant role in the toxic and premutagenic effects of UV radiation [30]. Nevertheless, there is noticeable evidence that the removal of 6-4PPs from the mammalian genome is provided at much higher rate compared to the repair rate of CPDs [15, 16]. The reason for this is not completely clear. The more pronounced DNA double-helix distorting effect of 6-4PPs might affect the efficiency of the DNA repair processes. There is considerable evidence that if a 6-4PP absorbs an additional UV photon at 320 nm, it is rearranged to another product, namely Dewar isomer [13]. These products show low mutagenic activity [31] and are repaired as efficiently as 6-4PPs in human cells [16].

Timely repair of damaged DNA is critical to prevent the adverse effects of UV radiation. All living organisms have DNA repair mechanisms to eliminate UV-induced DNA photolesions.

1.3 Repair of UVB-induced DNA photolesions

1.3.1 Nucleotide excision repair system

Naturally, UV-induced DNA photoproducts from the human genome are eliminated by the versatile nucleotide excision repair (NER) system [32]. Many researcher teams managed to demonstrate the existence of this repair process in bacteria, mammalian and human cells in the 1960s [33]. NER is involved in the repair of numerous structurally different DNA damages indicating its versatility [34, 35]. The main substrates of this complex mechanism are DNA photoproducts caused by UV radiation, however, NER repairs DNA damage induced by chemical substances including chemotherapeutics that may cause inter– or intrastrand crosslinks of DNA, alkaloids produced by fungi and polycyclic aromatic hydrocarbons. NER plays an important role in removal of UV-induced photolesions and works closely with other proteins involved in DNA replication, transcription, cell cycle progression and alternative repair pathways such as base excision repair.

NER is a multistep repair mechanism and more than 30 proteins are involved in the pathway [36]. An *in vivo* study provided evidence that the NER components are recruited sequentially [37]. There are two NER subpathways. Global genome NER (GG-NER) and transcription-coupled NER (TC-NER) basically differ in the initial steps of DNA damage recognition and recruitment of proteins to the lesions [36, 38]. This repair process consists of six main interconnected steps: 1) recognition of DNA damage, 2) verification of DNA lesion, 3) DNA unwinding, 4) dual-strand incision, 5) excision of DNA fragment containing lesion and finally 6) DNA resynthesis and rejoining (**Figure 3**).

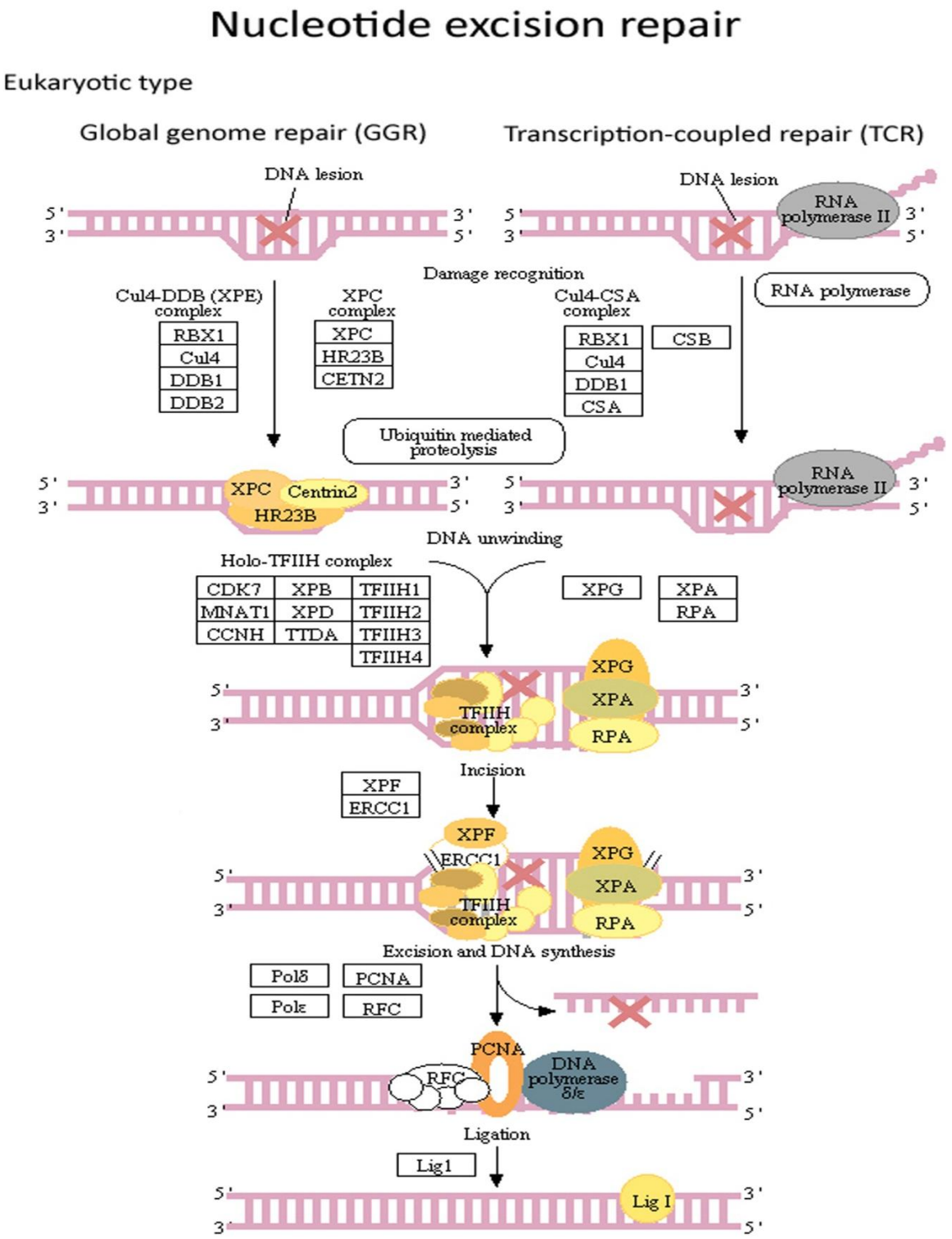


Figure 3. Schematic representation of both subpathways of the eukaryotic nucleotide excision repair system. *(Taken from Kanesiha Laboratories, KEGG pathway, http://www.genome.jp/kegg-bin/show_pathway?map=ko03420&show_description=show).*

In GG-NER, first activating enzyme complex, XPC/HR23B does not recognize the type of damage, but perceives distortion of the DNA double-helix [39]. Accordingly, this complex itself is unable or insufficiently able to recognize CPDs. 6-4PPs are recognized more efficiently due to the more pronounced DNA double-helix distorting effect of these photolesions. In this subpathway, DNA damage binding (DDB) protein activated by upregulation of a p53-responsive gene (p48) is essential for recognition of CPDs [40]. In TC-NER, the first signal of DNA damage recognition is the stalling of RNA polymerase II and inhibition of transcription elongation caused by UV-induced DNA photolesions (e.g. CPDs and 6-4PPs) on the template strand of active genes. This initial step in TC-NER requires the action of CSA and CSB (Cockayne syndrome protein A and B) proteins instead of the combined action of XPC/HR23B. The subsequent actions in NER are the same in both subpathways. The first important step after recruitment of the 10-subunit transcription repair factor TFIIH is that, two helicases (XPB and XPD) open the DNA double-helix surrounding the lesion. The second step is the verification of the DNA damage by XPA which is also a TFIIH subunit. After confirmation that the helix-distortion is not due to a specific DNA sequence, replication protein A (RPA) stabilizes these complexes and the unwound DNA structure becomes available for XPG and XPF/ERCC1 endonucleases. The former endonuclease is responsible for 3' incision, whereas the latter for 5' incision; at this point this dual cleavage process leads to an excised, single-strand DNA fragment of 24-32 nucleotides in length containing the lesion. Next, the gap created by removal of the damaged DNA portion is resynthesized by DNA polymerase δ/ε, with the help of additional factors such as proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). The repair process is completed by DNA ligase I that seals nick resulting in normal nucleotide sequence [41].

Inherited defects in NER genes result in photosensitive disorders, such as Xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy that are characterized by UV

sensitivity of patients with high incidence of skin cancers and/or accelerated aging [42].

1.3.2 Photoreactivation

Photoreactivation was first reported in 1949 [43]. This study demonstrated that *Streptomyces griseus* survived the lethal dose of UV, if the bacterial culture was also exposed to visible light immediately after UV treatment. Unlike NER system, photoreactivation is a light-dependent DNA repair mechanism which is carried out by photolyases [44]. These structure-specific DNA repair proteins bind specifically to pyrimidine dimers, either CPDs or 6-4PPs, and convert them back to the original state using the energy of visible light [45, 46]. Photolyases are monomeric proteins of 55-65 kDa that contain two chromophores (**Figure 4A**). The first one is a flavin adenine dinucleotide (FAD) which functions as electron donor and catalytic cofactor determining the enzymatic activity of photolyase. The second one is further classified into 2 groups, folate-type (5,10-methylenetetrahydrofolate; MTHF) and deazaflavin-type (8-hydroxy-5-deazaflavin; 8-HDF) chromophore, depending on the organism [47]. These cromophores act as a light-harvesting cofactor that increase more than 100-fold the visible light-absorbing capacity of photolyase [47]. All known photolyases rapidly remove UV-induced DNA lesions securing the genomic integrity in a light-dependent cyclic redox reaction with the help of the combined action of both chromophore cofactors (**Figure 4B**) [48].

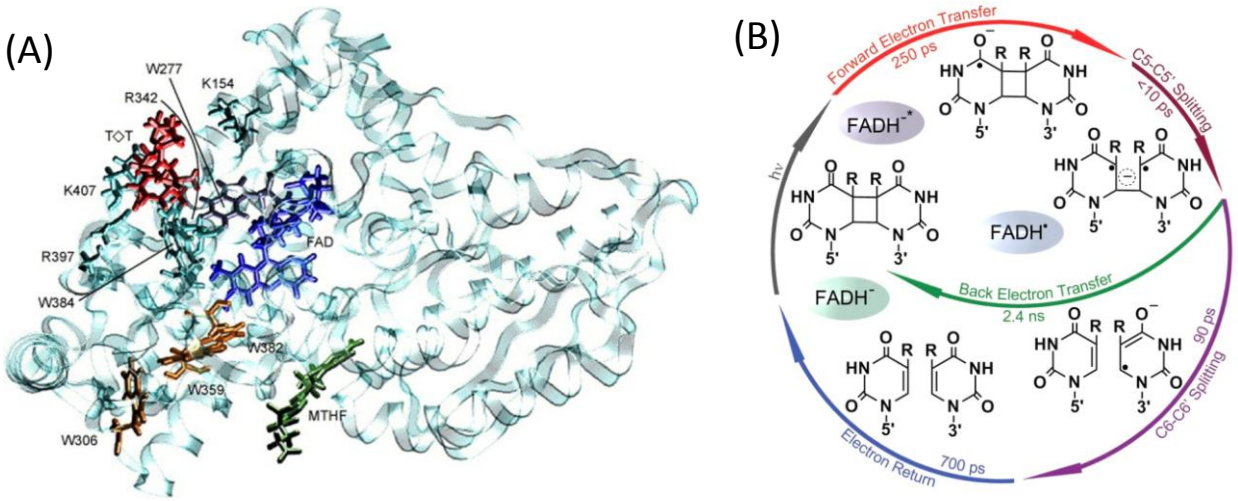


Figure 4. Schematic overview of a photolyase protein. ***(A)*** *The structure of Escherichia coli CPD-specific photolyase in a ribbon representation. The FAD cofactor and the second chromophore, MTHF, are shown in blue and green, respectively. A thymine dimer at a position that was predicted by molecular modeling calculations is shown in red. Selected amino acid residues that play important functional roles in substrate binding, photoreactivation and photoactivation are also depicted. (Taken from Stefan Weber, doi:10.1016/j.bbabio.2004.02.010).* ***(B)*** *Photocycle of UV-induced CPD repair by photolyase. As a first step, the enzyme specifically recognizes a CPD which enters the substrate binding pocket of photolyase where the catalytic cofactor (FADH-) is found. After substrate binding, the fully reduced FADH- absorbs visible light, directly or via energy transfer from the excited light-harvesting cofactor. Next, forward electron transfer occurs from the photoexcited FADH–* to the CPD; consequently splitting of two bonds of C5-C5' and C6-C6' in thymine dimer with subsequent electron return to the flavin cofactor in order to restore its catalytically active form (FADH-). (Taken from Zheyun Liu et al., doi: 10.1073/pnas.1110927108.)*

The enzyme has been described in diverse organisms from archaebacteria to marsupials, but seems to be absent in placental mammals like human [49]. Studies have been identified a protein, so called cryptochrome, which is present in mammals and shows high sequence homology to 6-4PP-specific photolyase, but it is unable to repair UV-induced DNA lesions [50]. Cryptochromes, functioning as blue-light receptors, regulate the circadian rhythms in many living organisms, including human [51].

1.4 Advantages and difficulties of synthetic mRNA-based gene therapy method

Gene therapy for the replacement of defective genes or for the expression of therapeutic proteins has made great progress in the last decade [52]. After exploring numerous viral and non-viral-based systems for expressing the encoded proteins, *in vitro* transcribed messenger RNA (IVT mRNA) seems to be the most suitable tool for transient protein expression [52]. The use of synthetic mRNA for developing vaccine formulations and delivery of genetic material into mammalian cells has grown into a novel and alternative tool to oust viral vector and DNA-based applications [53]. It has many features that make mRNA-mediated gene transfer especially valuable for functional characterization of encoded protein. The transfected mRNA is translated with high efficiency in any cell, including primary and non-dividing mammalian cells [54]. Importantly, when mRNA is delivered to the cell, only the encoded protein of interest is generated avoiding genomic integration, unlike other vectors, such as plasmids that contain sequences for additional proteins, or viral vectors that not only code for but also carry viral proteins into the cell. In addition, mRNA does not require nuclear localization or transcription to be functional, thus it can be safely used for gene transfer and expression [55]. In spite of these advantageous properties, *in vivo* application of IVT mRNA is limited owing to its labile nature and the fact that it stimulates innate immune system through RNA sensors, including Toll-like receptors (TLR3, TLR7, TLR8), retinoic acid-inducible gene 1 (RIG-I; also known as DDX58), melanoma differentiation-associated protein

5 (MDA5; also known as IFIH1) interferon-induced protein with tetratricopeptide repeats 1 and 5 (IFIT1 and IFIT5) and protein kinase RNA-activated (PKR; also known as EIF2AK2) [52]. The presence of modifying structure elements, such as chemically synthesized 5' cap analogues and long poly(A) tail, improved the stability of RNA and its translational efficiency opening new perspectives for therapeutic use [56]. However, unwanted side effects and the high sensitivity of IVT mRNA to exonucleases were still remained. In the last several years, mRNA-mediated transfection technology has improved tremendously [53]. Nucleoside modifications and HPLC purification were big step forward in the therapeutic applicability of mRNA. In addition, *in vitro* transcribed mRNA containing nucleoside modifications has been proved to be translated more efficiently than mRNA containing unmodified nucleosides [57]. It is now well documented that HPLC purification of RNA containing pseudouridine (Ψ), a naturally-occurring modified nucleoside that increase mRNA stability, completely abolishes intrinsic immunogenicity of the RNA and makes it highly translatable [58, 59]. A newly established preparative HPLC purification procedure was critical to obtain IVT mRNA that is free of aberrant transcription products including short RNAs and double stranded RNAs that activate RNA sensors even in the presence of pseudouridine or other nucleoside modification [59]. IVT mRNA containing nucleoside modifications are currently used in a wide variety of fields, including cancer immunotherapy, vaccines, protein replacement, gene editing and genetic reprogramming (**Figure 5**) [52].

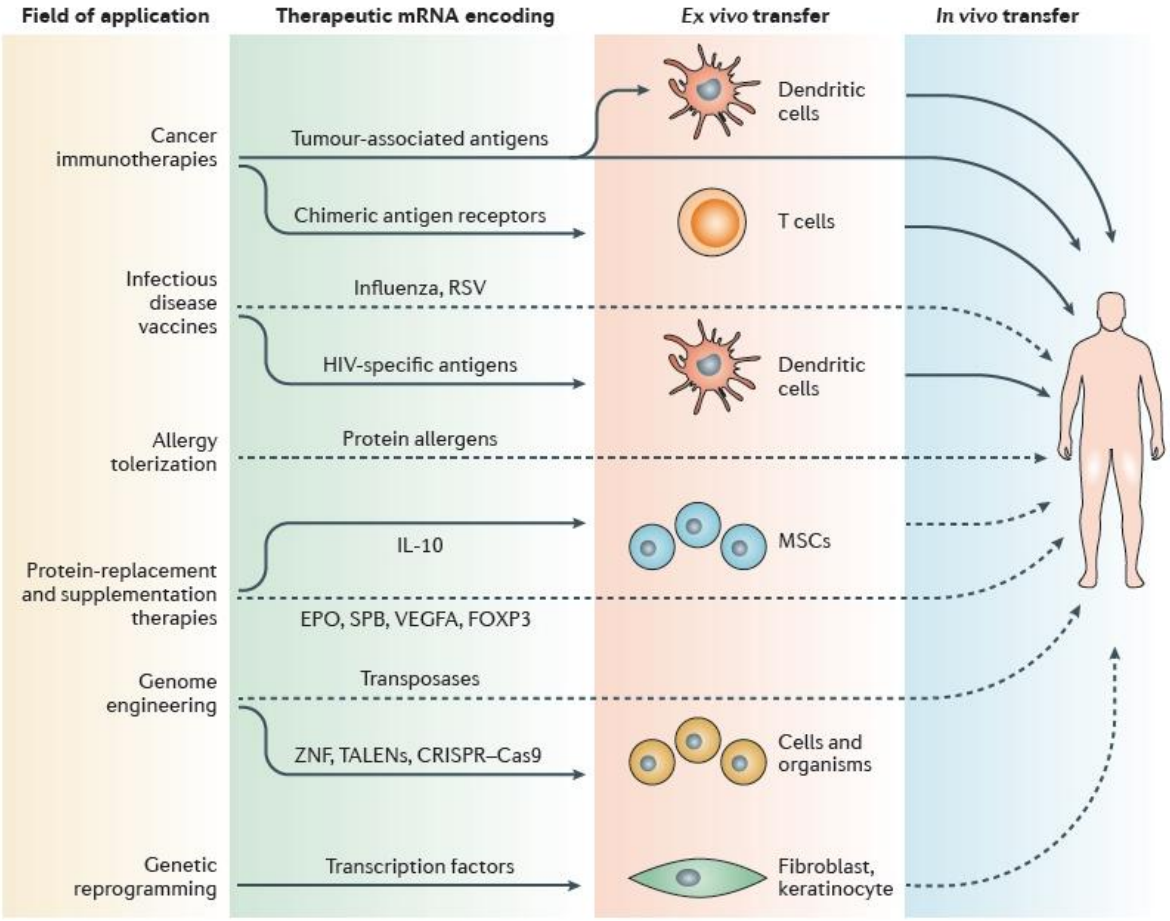


Figure 5. Major areas of potential therapeutic applications of in vitro transcribed mRNA. *The solid arrows pointing in the right hand column denote applications that are in the clinic, whereas stippled arrows refer to preclinical applications. (Taken from Ugur Sahin et al., doi: 10.1038/nrd4278).*

Nevertheless, *in vivo* delivery of IVT mRNA into living skin to express therapeutic proteins has not been resolved. The lipid-rich nature of the skin, the enhanced secretion of RNases on the skin surface and the *stratum corneum* are important limitations for IVT mRNA to development of *in vivo* skin therapeutic studies. Theoretically, CPD-photolyase mRNA applied topically on the skin might provide a new sun protection strategy with a potential to reduce acute sun damage and could serve as a prototype for expressing other specific proteins related to skin diseases.

2. Objectives

It is well known that CPDs are the principal mediators of the harmful effects of UVB radiation. CPD-specific photolyase can rapidly and efficiently repair these DNA photoproducts after photoreactivation, however, this enzyme is absent in humans. Transfection of human keratinocytes with *in vitro* transcribed nucleoside-modified mRNA encoding a marsupial CPD-specific photolyase seems to be an excellent model to study the cell biological effects of UVB with distinction between CPD-dependent and -independent cellular processes.

Our aims were:

1. To adjust the most appropriate experimental conditions for transfection of cultured human keratinocytes with IVT nucleoside-modified mRNA to achieve high yield translation of CPD-specific photolyase protein.
2. To investigate the effect of UVB pre-exposure on transfection efficiency.
3. To verify that the expressed CPD-specific photolyase is functionally active i.e. to demonstrate the rapid repair of UVB-induced CPDs in mRNA-transfected keratinocytes upon photoreactivation.
4. To determine UVB-induced CPD-dependent and -independent transcriptional responses in human keratinocytes combining the CPD-specific photolyase mRNA delivery with a genomics approach namely microarray and pathway analysis.
5. To investigate which signalling pathway plays a role in conversion of the cues generated by UVB-induced CPDs into a transcriptional response.

3. Materials and Methods

3.1 *In vitro* transcription of pseudouridine-modified mRNA encoding CPD-specific photolyase

Messenger RNAs encoding CPD-photolyase (CPD-PL Ψ-mRNA) and enhanced green fluorescent protein (eGFP Ψ-mRNA) (**Figure 6A**) generated *in vitro* transcription were gifted by the research group of Katalin Karikó (University of Pennsylvania, Department of Neurosurgery). A codon-optimized photolyase gene from *Potorous tridactylus* (rat kangaroo) containing GC-rich codons for high translational yields was synthesized by Entelechon (www.entelechon.com; Bad Abbach, Germany). The optimization increased the GC-content of the photolyase coding sequence (GenBank accession number: D26020) from 51.8% to 65.0%. The following plasmids were used as templates for *in vitro* mRNA transcription: pTEV-CPD-PL-A101 and pTEV-eGFP-A101. First, plasmids were linearized with NcoI and BamHI restriction enzymes (New England Biolabs, Beverly, MA, USA) to generate templates. Transcriptions were performed at 37°C for 3 hours using the Megascript T7 RNA polymerase kit (Ambion, Austin, TX, USA) in which UTP was replaced with pseudouridine triphosphate (TriLink, San Diego, CA, USA). Both mRNAs were synthesized with the 5' untranslated region (UTR) derived from the tobacco etch virus 5' leader RNA [60] and with the 3' UTR of *Xenopus* beta-globin mRNA [61]. Applying both UTRs ensures enhanced translation of the coding sequence [58]. To remove the template DNA, Turbo DNase (Ambion) was added to the reaction mix and further incubated for 15 minutes. Pseudouridine-modified mRNAs were HPLC-purified as described [59] and provided with cap1 generated by using the m7G capping enzyme and 2′-O-methyltransferase, according to the manufacturer's instructions (CellScript, Madison, WI, USA). The RNA was transcribed to contain an encoded 101-nt long 3' poly(A) tail, which was extended with ~ 300 nucleotides using poly(A) polymerase (USB, Cleveland OH, USA). Finally, lithium chloride (LiCl) was used to

precipitate the RNAs followed by washing steps with 75% ethanol. RNA samples were resuspended in nuclease-free water and analyzed by agarose gel electrophoresis for quality assurance. Small aliquots of RNA samples were stored in siliconized tubes at −20°C.

3.2 *In vitro* translation assay

In vitro protein synthesis from the mRNAs was determined by *Karikó Katalin et al.* using an *in vitro* translation system (Promega, Madison, WI, USA), according to the manufacturer's instructions. In briefly, aliquots of rabbit reticulocyte lysates were programmed with 0.5 μg RNA samples and incubated for 60 minutes at 30°C. The RNA-programmed rabbit reticulocyte lysates were also supplemented with ^{35}S-labeled methionine and cysteine. Aliquots of lysates were denatured and separated by 15% SDS-PAGE. Gel soaked in sodium salicylate is dried and to visualize the labeled samples, a fluorogram was generated by exposing the dry gel to BioMax MS film (Kodak, Rochester, NY, USA) (**Figure 6B**).

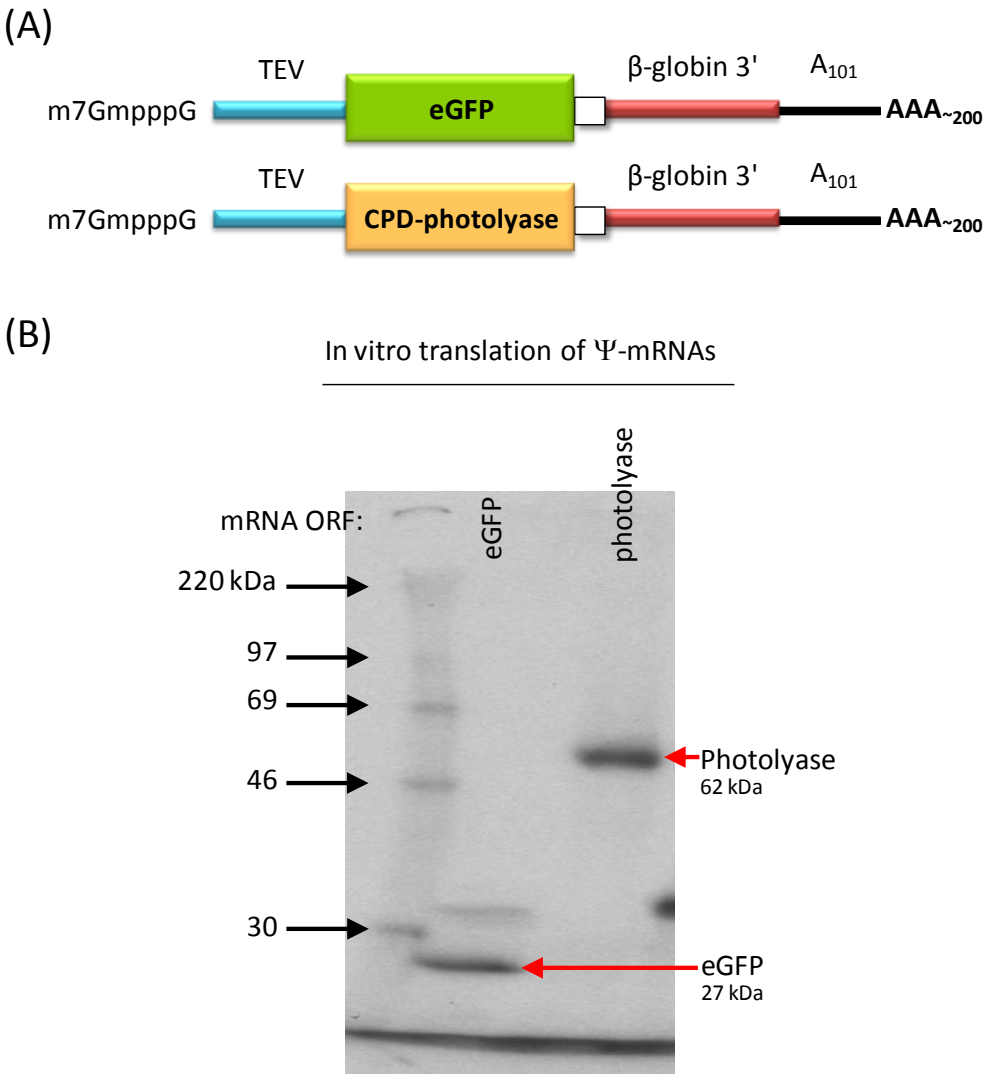


Figure 6. In vitro transcription and translation of pseudouridine-modified mRNAs. (A) *Schematic representation of all mRNAs used in this study. All in vitro transcribed mRNAs contain the same 7-methylguanylate cap, 5' UTR of TEV mRNA (144 nt), the coding sequence of eGFP (720 nt) or CPD-photolyase (1,599 nt), 3' UTR of Xenopus β-globin mRNA (162 nt), an extended poly(A) tail (300 nt).* ***(B)*** *To test whether selected transcripts encode the appropriate protein products in vitro translation (IVT) assay was performed. The arrows indicate the expected position of the 27 kDa eGFP and of the 62 kDa CPD-photolyase translated from in vitro synthesized mRNAs containing pseudouridine modifications.*

3.3 Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT established by *Boukamp et al* [62] was used at 75-85% confluency in each experiment. HaCaT cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) with 4.5 g/L glucose and 2 mM L-Glutamine and supplemented with 0.5% antibiotic / antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France) at 37°C in a 5% CO_2 atmosphere.

3.4 Transient transfection of human keratinocytes

HaCaT cells were seeded into 96-well plates at a density of 2×10^4 cells per well one day prior to transfection. Aliquots of RNA samples (0.25 µg) were complexed with 1.0 µl Lipofectamine LTX-PLUS (Life Technologies, Carlsbad, CA, USA) in a final volume of 100 µl of EpiLife and the complexed RNA was added to each well. The ratio of mRNA and Lipofectamine LTX-PLUS reagent that has been used in the first series of experiments was different (data not shown), but further optimization experiments demonstrated that the ratio described above results in higher transfection efficiency. The results presented in the dissertation are derived from experiments using the optimized transfection conditions. Following a two-hour transfection, the lipofectamine-RNA complex was replaced with 100 µl fresh culture medium.

3.5 UVB irradiation and photoreactivation

At 12 h post transfection, the cell monolayer in a well of a 96-well plate, was covered with 50 µl Dulbecco's phosphate buffer saline (DPBS) (Life Technologies) and subjected to 20 mJ/cm^2 UVB using two broadband TL-20W/12 tubes (Philips, Eindhoven, The Netherlands). Proper dosage of UVB was determined by a UVX Digital Radiometer (UVP Inc., San Gabriel, CA, USA). Immediately after UVB treatment, cells were either exposed to photoreactivating light (active CPD-photolyase) using two F18W/54-765/T8 white fluorescent

tubes (Havells-Sylvania, Erlangen, Germany) at a distance of 16 cm and using a 4 mm thick glass filter as a shield or kept in the dark (inactive CPD-photolyase) for one hour. Cells were further cultured in complete medium for the indicated times.

To investigate the effect of UVB pre-exposure on the transfection efficiency, HaCaT keratinocytes were first exposed to UVB (20 mJ/cm^2), then transfected with lipofectamine-complexed photolyase mRNA ninety minutes later. Immediately after mRNA transfection, cells were either exposed to photoreactivating light (active CPD-photolyase) or kept in the dark (inactive CPD-photolyase) for one hour. Cells were harvested at the indicated time point.

3.6 Fluorescence microscopy

HaCaT cells were plated on glass coverslips and subjected to 20 mJ/cm^2 UVB or left unirradiated prior to transfection of the encoding Ψ-modified mRNA. Cells were rinsed once in PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 20 min and permeabilized with 1% Triton X-100 (Amresco) for 10 min. Before immunolabeling, cells were blocked in 20% FBS and then probed with CPD-photolyase-specific polyclonal antibody (kindly gifted from Prof. Gijsbertus van der Horst; Erasmus University Medical Center, Rotterdam) diluted (1:500) in PBGTNa (PBS containing 0.5% bovine serum albumin (BSA) (Amresco, Solon, OH, USA), 0.05% gelatin (Sigma-Aldrich), 0.05% Tween-20 (Amresco) and 300 mM NaCl (VWR, San Diego, CA, USA) at 4°C overnight. After washing with PBS, goat anti-rabbit IgG Alexa Fluor 555 secondary antibody (1:2000; Life Technologies) was added at room temperature for 2 h. Nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA, USA) and cells were analyzed by fluorescence microscopy.

For the analysis of CPDs, before blocking, cellular DNA was denatured in 2 M HCl (VWR) at room temperature for 30 min. The samples were probed with a primary anti-CPD monoclonal antibody (TDM-2, Cosmo Bio, Tokyo, Japan) diluted (1:1500) in PBS containing 5% FBS for 30 min. Alexa Fluor 568-conjugated anti-mouse IgG was used as secondary antibody (1:2000;

Life Technologies). After washing with PBS, coverslips were mounted with Vectashield containing DAPI (Vector Laboratories) and cells were analyzed by fluorescence microscopy. Expression of eGFP in HaCaT cells transfected with eGFP Ψ-mRNA was documented using an epifluorescent Zeiss Axiovert 100 microscope mounted with a Zeiss AxioCam MRc 5 digital camera with AxioVision imaging system (version 4.8) and 0.4-second exposure time.

In post-UVB mRNA transfection experiment, translated CPD-photolyase protein was monitored at different time points after transfection by fluorescence confocal microscopy using an Olympus FV1000S confocal microscope (Olympus Co., Tokyo, Japan) equipped with 60×, oil immersion objective (NA: 1.3). For excitation, a laser of 543 nm was used. The average pixel time was 4 μs. The Z image series of 1 μm optical thickness were recorded in sequential scan mode. Confocal settings were identical for all scans and remained constant during imaging.

3.7 CPD-specific enzyme-linked immunosorbent assay (ELISA)

Genomic DNA was extracted by the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Flat-bottom 96-well plates were precoated with 0.003% protamine sulfate (50 μl/well, Sigma-Aldrich) and incubated with denatured DNA (15 ng/well) at 37°C overnight. The plates were washed with PBS containing 0.05% Tween-20 and incubated with 2% FBS at 37°C for 30 min to prevent non-specific binding of the antibody and washed again. Mouse anti-CPD monoclonal antibody (TDM2) diluted in PBS (1:1000) was added to each well and the plates were incubated at 37°C for 30 min. The plates were washed and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (1:3000, BioRad) at 37°C for 30 min. After washing, each sample were equilibrated with 150 μl of citrate-phosphate buffer (51.4 mM Na_2HPO_4, 24.3 mM citric acid monohydrate, pH 5.0), then substrate solution was added to each well (100 μl/well) containing 74 nM o-phenylenediamine (OPD) (Sigma-Aldrich) and 60 nM H_2O_2 in

citrate-phosphate buffer. Samples were incubated at 37°C for 15 min, and 2 M sulfuric acid (VWR) was added (50 µl/well) to stop the reaction and absorbance was measured at 492 nm using an Anthos 2020 microplate reader (Biochrom Ltd; Cambridge; UK).

3.8 Cell viability assay

The CPD-photolyase mRNA transfected cells were subjected to 20 and 60 mJ/cm^2 UVB, followed by exposure to one hour visible light or darkness. Cell viability was determined by EZ4U assay (Biomedica Gruppe, Vienna, Austria), according to the manufacturer's instructions.

3.9 Microarray analysis

Total RNA was isolated from HaCaT cells using guanidinium thiocyanate-phenol-chloroform extraction, according to *Chomczynski et al.* [63]. RNA samples were treated by DNase I (Fermentas, St. Leon-Rot, Germany) and their quality were analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Gene expression profiling of HaCaT cells transfected with CPD-photolyase mRNA was performed using a service provider (ChromoScience, Budapest, Hungary, http://www.chromoscience.hu). Briefly, cyanine 3-labeled cRNA was synthesized from 200 ng total RNA by the QuickAmp Labeling Kit (Agilent Technologies), according to the manufacturer's protocol. For each sample, 1650 ng of one-color labeled cRNA was hybridized to an Agilent 4x44 K Whole Human Genome Oligo (Agilent Technologies) microarray platform at 65°C for 17 h. All further steps were carried out according to the manufacturer's instructions (Agilent Technologies). The slides were scanned with the Agilent Microarray Scanner.

Data were normalized by the Feature Extraction software version 9.5 (Agilent Technologies) with default settings for one-color oligonucleotide microarrays and then transferred to GeneSpringGX program (Agilent Technologies) for further statistical evaluation. In GeneSpring, the normalization and data transformation steps recommended by Agilent

Technologies for one-color data were applied at each time point. Microarray data have been submitted to Gene Expression Omnibus (GEO) [64] database and can be retrieved at http://www.ncbi.nlm.nih.gov/geo/ under accession number GSE65034). Three replicates of the following 3 types of CPD-PL Ψ-mRNA transfected samples were studied by the microarray: non-irradiated, UVB-irradiated without photoreactivating light (inactive CPD-photolyase) and UVB-irradiated plus photoreactivated (active CPD-photolyase). Genes showing statistically significant 2-fold change were selected for further study by Ingenuity Pathway Analysis (IPA, Qiagen; http://www.ingenuity.com).

3.10 Real-time quantitative RT-PCR analysis

To validate the microarray data, real-time quantitative reverse transcription (RT)-PCR was used. RT was performed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), according to the manufacturer's protocol. RT reactions contained 500-500 ng of RNA. To quantitate the expression of candidate genes, the following TaqMan Gene Expression assays (Life Technologies) were used: *ATF3* (Hs00231069_m1), *CCNE1* (Hs01026536_m1), *CDKN2B* (p15INK4b) (Hs00793225_m1), *EGR1* (Hs00152928_m1), *ID2* (Hs04187239_m1), *IL-6* (Hs00985639_m1), *PTGS2 (*also known as *COX-2)* (Hs00153133_m1), *RUNX1* (Hs00231079_m1), *SNAI2* (Hs00950344_m1), the sequences are proprietary and not released by the company. To determine mRNA expression of *SNAI1* the following custom-designed primers and probe set were used: forward primer: 5'-ACT ATG CCG CGC TCT TTC-3'; reverse primer: 5'-GCT GGA AGG TAA ACT CTG GAT-3'; and the probe sequence: 5'-[6-carboxyfluorescein (FAM)] AAT CGG AAG CCT AAC TAC AGC GAG C [tetramethylrhodamine (TAMRA)]-3'. Quantitative PCR was performed on the ABI 7900 HT Sequence Detection System (Life Technologies) using a reaction mixture containing 1× PCR buffer, 3 mM $MgCl_2$ and 0.1 mM dNTP mix (Fermentas), 1× TaqMan Gene Expression assay (Life Technologies), 1× ROX reference dye (Life Technologies),

0.0125 U Taq DNA polymerase (Fermentas) and 12.5 ng cDNA template in a total volume of 10 μl. Experiments were carried out in 384-well optical plates (Life Technologies) and the PCR program used for amplification was: 94°C 1 min, followed by 40 cycles of 94°C for 12 sec and 60°C for 45 sec. Relative RNA expression values were calculated using the $2^{-\Delta\Delta Ct}$ method [65] in which expression levels in samples containing active CPD-photolyase were compared to those containing inactive one. *SDHA* (Hs00188166_m1) and *PGK1* (Hs00943178_g1) mRNA levels that showed the smallest variation upon UVB exposure in keratinocytes [66] were used for normalization.

3.11 Western blot

Cells were lysed in RIPA buffer (R0278, Sigma-Aldrich) containing protease inhibitor cocktail (Sigma-Aldrich). The concentration of proteins was determined by the Pierce BCA protein assay kit (Life Technologies). Proteins (10 μg) were separated on 10% (CPD-photolyase) or 12% (CCNE1 and p15INK4b) polyacrylamide gels and transferred to nitrocellulose membrane (BioRad, Berkeley, CA). The membranes were blocked in 5% non-fat dry milk for 1 h and incubated with primary antibodies diluted in working solutions at 4°C overnight. The following primary antibodies were used: rabbit anti-CPD-photolyase polyclonal antibody (kindly gifted from Prof. Gijsbertus van der Horst) diluted (1:500) in PBGTNa, mouse anti-CCNE1, anti-β-actin (Cell Signaling Technology (Danvers, MA, USA) and anti-CDKN2B (Thermo Fisher Scientific (Rockford, IL, USA) antibodies diluted (1:750, 1:1000 and 1:750, respectively) in 5% non-fat dry milk. Goat anti-mouse or anti-rabbit IgG conjugated with HRP was used as a secondary antibody (1:3000, at room temperature, 1 h) (BioRad). The visualization of proteins was achieved with ECL Prime Western blotting detection system (GE Healthcare, Little Chalfont, UK) and densitometry was performed using ImageJ public software (version 1.45) (NIH, Bethesda, MD, USA).

3.12 Inhibition of protein kinase signaling pathways

Keratinocytes at 85% confluency were pretreated with specific inhibitors of JNK (SP600125), p38 MAPK (SB203580) or AKT (MK-2206 2HCl) at a final concentration of 40 μM, 10 μM and 10 μM, respectively for 1 hour before UVB irradiation. Samples were harvested at 6 and 24 h after UVB exposure. All chemical agents were purchased from SelleckChem (Selleck Chemicals, Houston, TX, USA) and were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Stock solutions were prepared according to the manufacturer's instructions and stored at –20°C.

3.13 Statistical analysis

Identification of genes differentially expressed in microarray experiments was carried out by the unpaired, Student's *t*-test followed by Benjamini-Hochberg correction. Statistical analysis of qRT-PCR data was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). The significance of differences in terms of mRNA expression comparing UVB-irradiated samples to non-irradiated ones, or UVB-irradiated plus photoreactivated (active CPD-photolyase) samples to non-photoreactivated (inactive CPD-photolyase) samples, respectively, was determined by the two-tailed, unpaired *t*-test. A *p* value of equal to or less than 0.05 was considered statistically significant. Each experiment was performed in triplicate obtained from three independently biological replicates.

4. Results

4.1 Synthesis of CPD-specific photolyase in human keratinocytes transfected with *in vitro* synthesized and pseudouridine-modified mRNA

To test whether pseudouridine-modified mRNA (Ψ-mRNA) is translated in human keratinocytes, eGFP-encoding Ψ-mRNA was complexed with lipofectamine LTX and delivered to the cells. The expression of eGFP was monitored by fluorescence microscopy at different time points after transfection. eGFP was detectable as early as 1 h, peaked between 6 and 12 h, and its level gradually declined, but remained detectable even at 48 h after mRNA transfection (**Figure 7**). This eGFP mRNA served as a control for photolyase experiments.

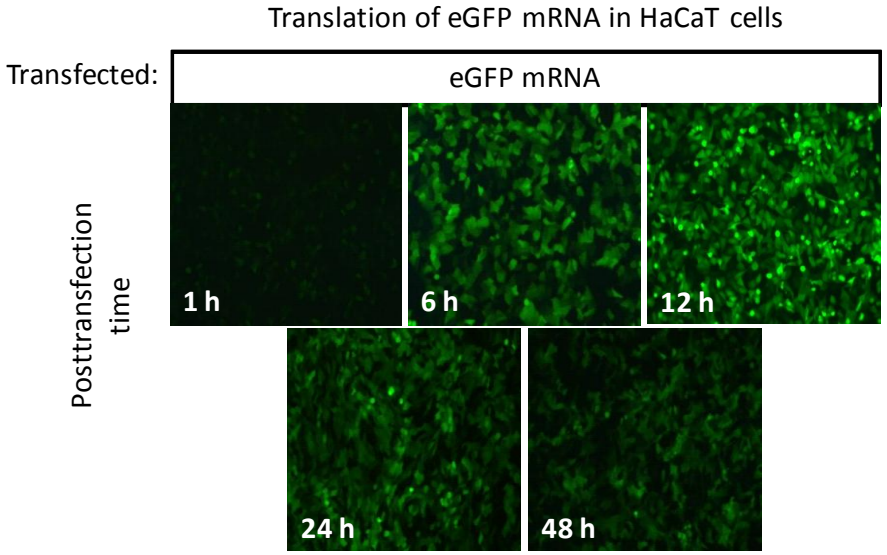


Figure 7. Translation of eGFP-encoding, Ψ-modified mRNA in keratinocytes. *HaCaT cells were transfected with Ψ-mRNA encoding eGFP. Translated eGFP was detected by fluorescence microscopy(original magnification ×200).*

Using western blotting, significant amount of CPD-photolyase synthesized from the transfected CPD-PL Ψ-mRNA could be detected not only in unirradiated HaCaT cells, but also in UVB-pretreated cells at already 1 h after delivery of encoding mRNA (**Figure 8A**). The photolyase migrated with the expected size of 62 kDa (**Figure 8A**) and localized in the

nuclei of keratinocytes, as demonstrated by immunofluorescence imaging (**Figure 8B**).

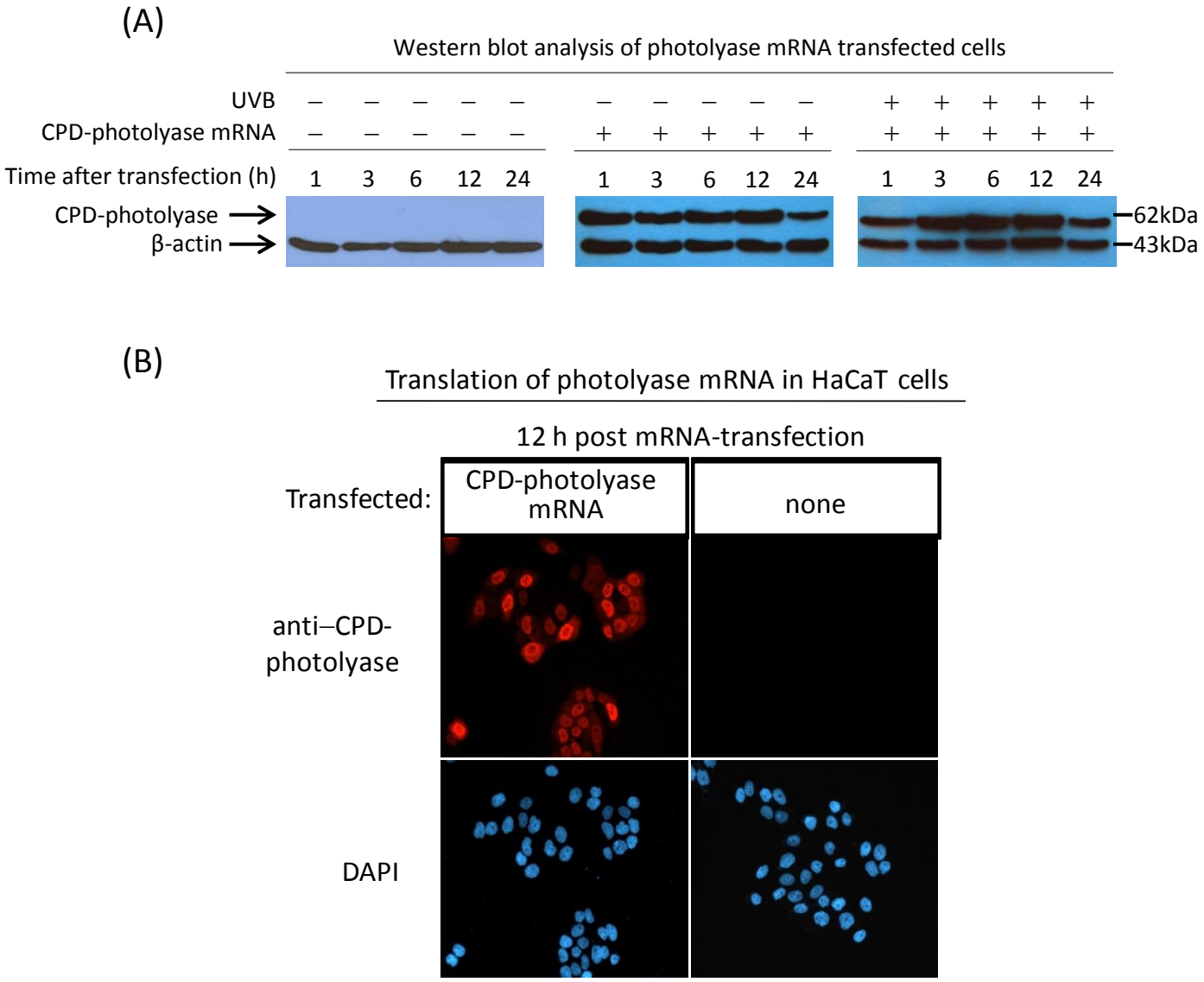


Figure 8. Translation of CPD-photolyase-encoding, Ψ-modified mRNA in keratinocytes. ***(A)*** *HaCaT cells preexposed to 20 mJ/cm^2 UVB (or not) were transfected with lipofectamine-complexed Ψ-mRNA encoding CPD-photolyase, or left untransfected. Cells were lysed at the indicated times post transfection and analyzed by western blot. Arrows mark positions of the 62 kDa CPD-photolyase and the loading reference, β-actin.* ***(B)*** *CPD-photolyase protein was observed in unirradiated HaCaT cells transfected with CPD-PL Ψ-mRNA using immunofluorescent detection. Nuclei were visualized by DAPI staining (original magnification ×400).*

Expression and subcellular localization of CPD-specific photolyase were monitored in UVB-exposed keratinocytes using fluorescence confocal microscopy (**Figure 9**). Strong red fluorescent signal was detected at first in the cytoplasm followed by fading of the cytoplasm and increasing fluorescence of the nuclei (**Figure 9**). Taken together, these results show that UVB irradiation of keratinocytes did neither reduce the efficacy of transfection nor influence the translation level of *in vitro* synthesized mRNA and, more importantly, confirm that CPD-photolyase accumulated in the nuclei where it can cleave cyclobutane pyrimidine dimers formed on the UV-damaged DNA.

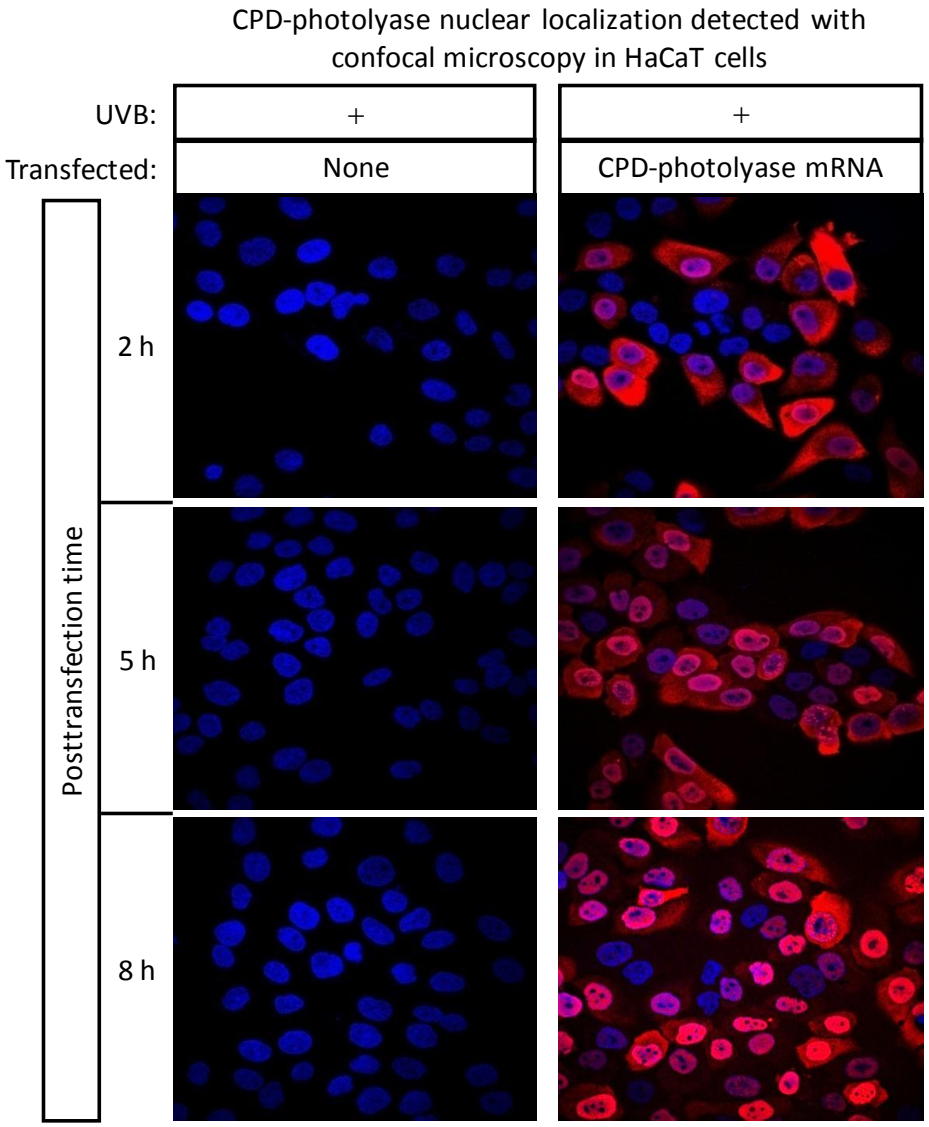


Figure 9. Synthesis and nuclear localization of CPD-specific photolyase in UVB-exposed human keratinocytes transfected with the encoding Ψ-mRNA. *HaCaT cells were subjected to 20 mJ/cm^2 UVB, then transfected with CPD-PL Ψ-mRNA 90 min later, or left untransfected (none). Using anti-CPD-specific photolyase polyclonal antibody and alexa fluor 555-conjugated secondary antibodies, subcellular localization of the encoded protein was monitored at the indicated times post-mRNA transfection by fluorescence confocal laser scanning microscopy. Nuclei were visualized by DAPI staining (original magnification ×600).*

4.2 Accelerated removal of CPD photolesions in keratinocytes transfected with CPD-photolyase mRNA

To investigate whether CPD-photolyase translated from the encoding Ψ-mRNA is functional and capable of cleaving CPDs, HaCaT cells were first transfected with lipofectamine-complexed CPD-PL Ψ-mRNA. Twelve hours later, the transfected cells were exposed to a physiological dose of UVB (20 mJ/cm^2). Immediately thereafter, cells were either exposed to photoreactivating light, or left in the dark for 1 hour. Using CPD-specific antibodies, efficient repair of these photolesions could be detected in CPD-PL Ψ-mRNA transfected cells that were subjected to photoreactivating light, while those deprived from this energy source or transfected with eGFP Ψ-mRNA contained high levels of CPDs, when it was analyzed immediately after the photoreactivation or incubation in the dark (**Figure 10A**).

To measure the amount of CPDs removed from the genomic DNA of the CPD-PL Ψ-mRNA-transfected keratinocytes immediately and at 5 and 23 h after exposure to visible light, CPD-specific ELISA was used. In the first series of experiments we found that CPD-photolyase removed more than 60% of CPDs induced by UVB within the first hour of photoreactivation. Further optimization of the transfection experimental conditions assured quick removal of 90% of CPDs in CPD-PL Ψ-mRNA transfected cells subjected to photoreactivating light (photoreactivated), as compared to those kept in the dark (non-photoreactivated) or transfected with control eGFP Ψ-mRNA (**Figure 10B**). The results presented in the dissertation are derived from experiments using the optimized conditions. A reduced amount of CPD lesions was detected in all irradiated samples at 24 h vs. at immediate and 6 h time points due to slower endogenous NER-mediated DNA repair.

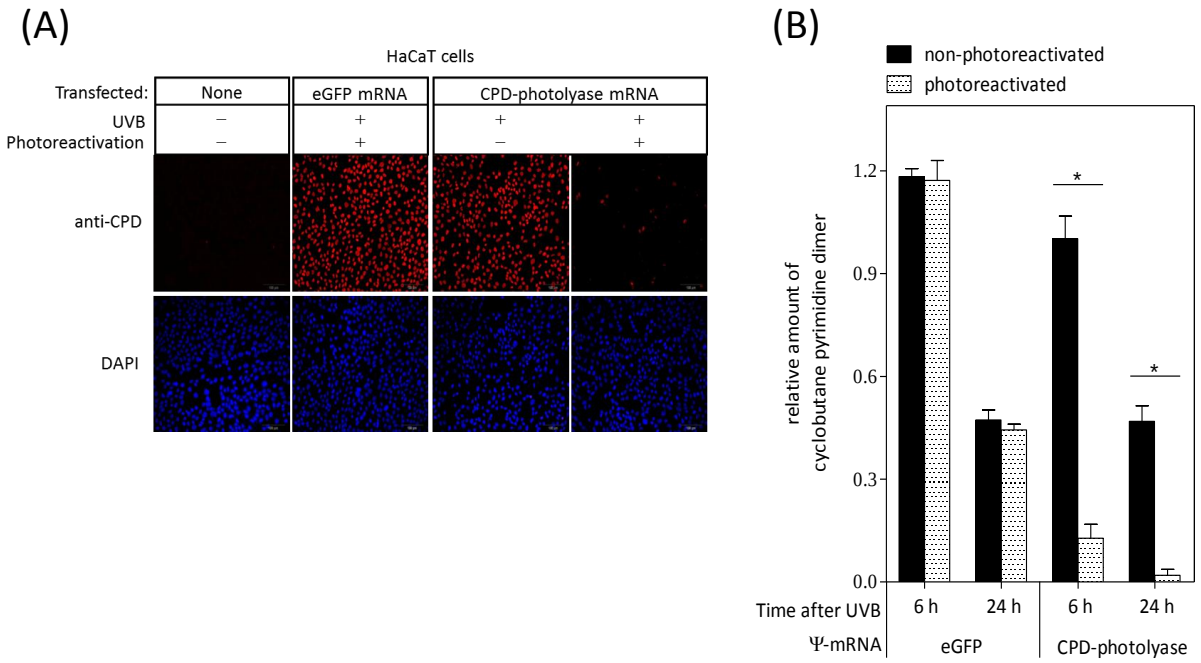


Figure 10. Enhanced repair of UVB-induced CPDs in keratinocytes is mediated by CPD-photolyase translated from the encoding Ψ-mRNA. (A-B) *HaCaT cells were transfected with lipofectamine-complexed Ψ-mRNA encoding CPD-photolyase or eGFP, or left untransfected (none). Twelve hours later, cells were subjected to 20 mJ/cm^2 UVB and immediately exposed to photoreactivating light or left in the dark for 1 h.* ***(A)*** *Immediately after photoreactivation or incubation in the dark, CPDs were detected by immunofluorescent labeling with CPD-specific antibody and Alexa 568-conjugated secondary antibody (red). Nuclei were visualized by DAPI staining. Images are representatives of three independently performed experiments (original magnification ×200).* ***(B)*** *Cells were harvested immediately and at 5 and 23 h after exposure to photoreactivating light (photoreactivated) or not (non-photoreactivated). Genomic DNA was isolated at the indicated times after UVB irradiation and the amount of CPDs was measured by ELISA. The values were calculated relative to those obtained with cells that were not UVB-irradiated. Significance was assessed by paired, two-sample t-test, $p<0.05$. Error bars represent the standard error of the mean from three experiments performed independently.*

For comparison, we also investigated the time kinetics of nucleotide excision repair mechanism in keratinocytes exposing them to 20 mJ/cm^2 UVB but not photoreactivating light. It took more than 48 hours for the physiologic NER system to reduce the amount of CPDs by 90% in UVB-irradiated HaCaT cells (**Figure 11**). Photoreactivation of UVB-irradiated, CPD-photolyase transfected HaCaT cells eliminated 90% of the CPDs within 6 hours after UVB exposure (**Figure 11**).

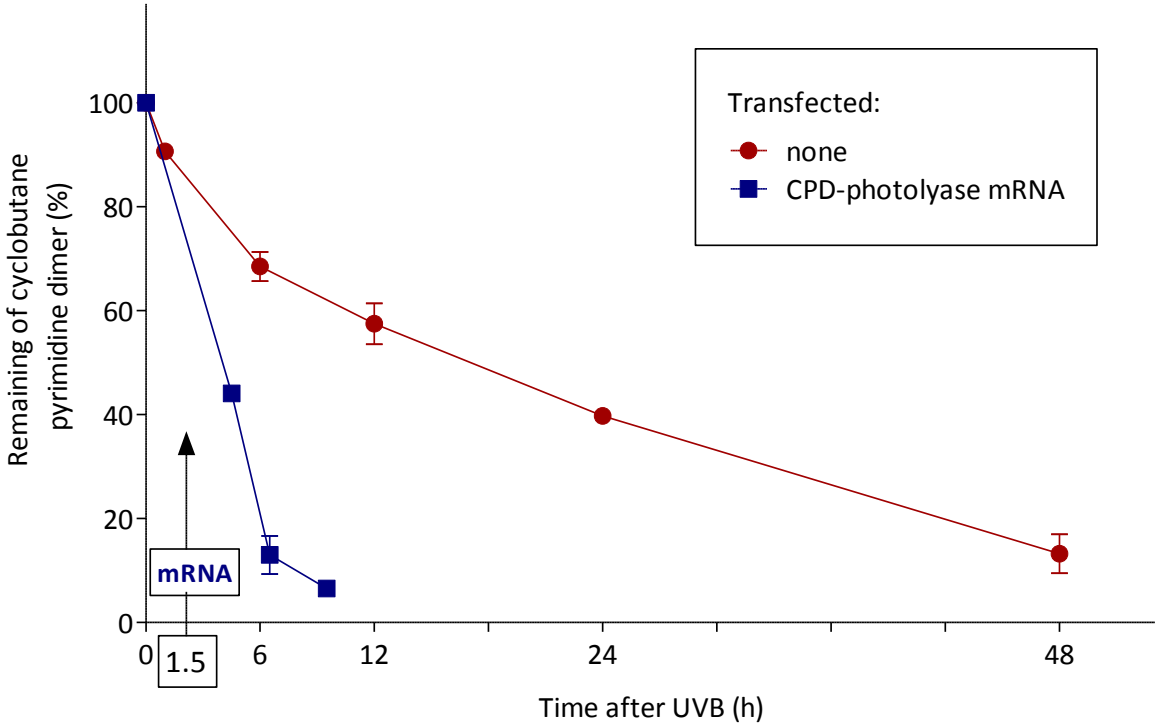


Figure 11. Time kinetics of repair of UVB-induced CPDs in human kerationcytes in the presence or absence of CPD-photolyase. *Keratinocytes preexposed to UVB radiation (20 mJ/cm^2) or kept in the dark were transfected with CPD-PL Ψ-mRNA and photoreactivated, or left untransfected (none). Cells were harvested immediately (0 h) and at the indicated times after UVB (or sham) irradiation. The amount of CPDs was measured by ELISA. CPD levels in cells exposed to UVB were calculated relative to those obtained with cells that were not UVB-irradiated, and shown as a percentage of the initial (0 h) value after UVB exposure. Error bars represent the standard error of the mean from three independent experiments.*

4.3 Rapid repair of CPDs in keratinocytes transfected with CPD-photolyase mRNA improves cell viability

HaCaT cells were irradiated with 20 and 60 mJ/cm^2 UVB 12 h after delivery of Ψ-mRNAs and immediately exposed to photoreactivating light (photoreactivated) or left in the dark (non-photoreactivated) for 1 h. Cell viability, determined 24 h after UVB irradiation, demonstrated that photoreactivation significantly increased cell survival of CPD-PL Ψ-mRNA transfected keratinocytes compared to those that were kept in the dark or transfected with eGFP Ψ-mRNA (**Figure 12**).

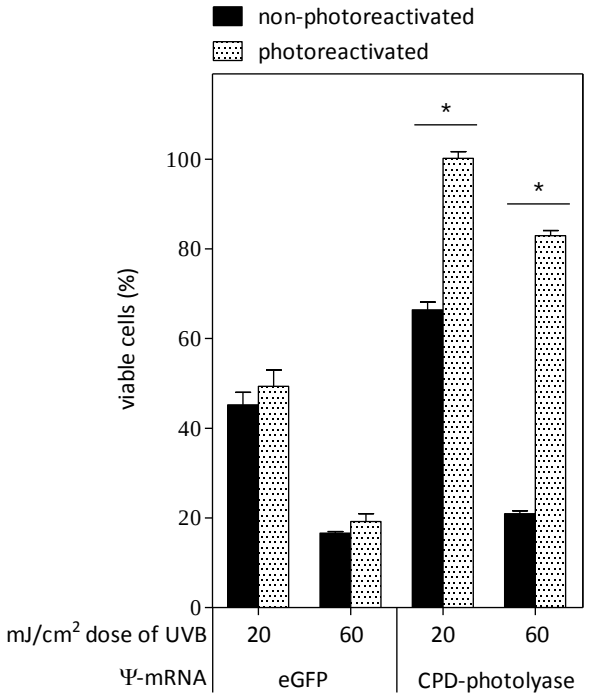


Figure 12. Photorepair of CPDs reduces the antiproliferative effect of UVB exposure on human keratinocytes. *To analyze cell viability in human keratinocytes transfected with Ψ-mRNAs EZ4U kit was used. Cell viability was determined after 20 and 60 mJ/cm^2 dose of UVB irradiation at 24 h, subsequently exposed to photoreactivating light (photoreactivated) or left in the dark (non-photoreactivated) for 1 h. Each experiment was performed in triplicate. Significance was assessed by paired, two-sample t-test, $p<0.001$. Error bars represent the standard error of the mean.*

4.4 CPD-dependent changes were distinguished within the UVB-induced alterations of gene expression in human keratinocytes

To determine the CPD-dependent gene expression profile of human keratinocytes exposed to UVB irradiation, an oligonucleotide microarray analysis was performed in which HaCaT cells transfected with CPD-specific photolyase mRNA were used. Total RNA was isolated from cells exposed to UVB subsequent photoreactivation or not and from non-irradiated cells at 6 and 24 h after UVB/sham irradiation. Gene expression values of UVB-irradiated plus photoreactivated samples (active CPD-photolyase) were compared to non-irradiated samples and to those that were UVB irradiated, but left without photoreactivation (inactive CPD-photolyase). UVB irradiated, but non-photoreactivated samples were also compared to non-irradiated samples. Cut-off values for changes in gene expression were set at ± 2-fold, and according to this criterion, expression level of 2,370 out of 41,000 genes were changed at 6 or 24 h after UVB exposure. Altered expression of 1,334 (56%) of UVB-regulated genes was restored in cells that contained active CPD-photolyase indicating that the changes in the expression of these genes were CPD-dependent (**Figure 13**). Importantly, more CPD-dependent gene expression changes (1,008 out of 1,334 genes) were observed at 6 h vs. 24 h, potentially due to the higher difference in CPD levels at 6 h comparing cells with active CPD-photolyase to those with inactive one (**Figure 13**). There are other reasons, such as transient expression of induced genes. On the other hand, CPD-dependent genes represented more than half of the UVB-regulated genes (1008 out of 1743 genes (58%) and 326 out of 627 genes (52%) at 6 and 24 h after UVB irradiation, respectively) at both time points after the exposure (**Figure 13**).

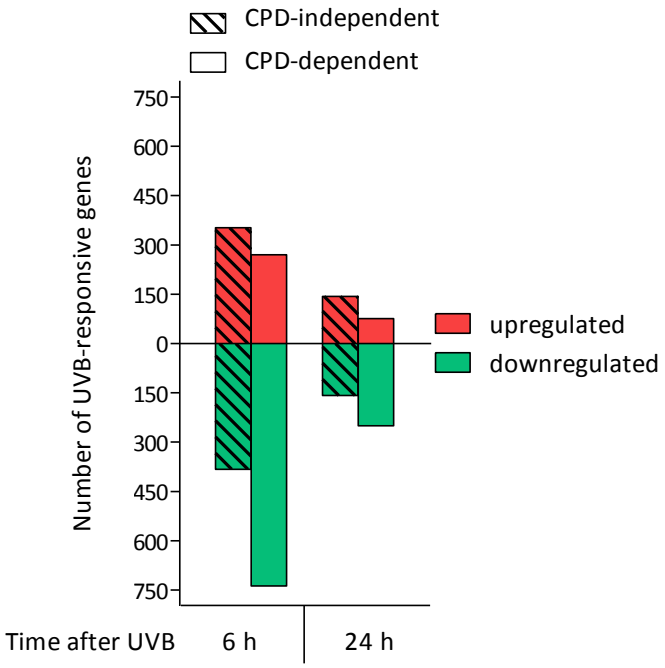


Figure 13. Divergence of UVB-mediated gene expression changes using CPD-specific photolyase encoded by in vitro transcribed mRNA. *Bar graphs represent the total number of UVB-responsive genes determined 6 and 24 h after the exposure. Bioset obtained from microarray experiment was divided into CPD-independent (the presence of active photolyase had no effect on the expression level of genes modified by UVB irradiation) and CPD-dependent (the presence of active photolyase has restored the expression level of genes modified by UVB irradiation) genes. Cut-off values for changes in gene expression were set at ± 2-fold.*

4.5 UVB-induced, CPD-dependent genes are associated with cellular stress responses

To determine possible network interactions and associated biological functions of CPD-regulated genes, datasets representing all the differentially expressed genes derived from microarray analyses were imported into Ingenuity Pathway Analysis (IPA) application.

Using IPA prediction analysis, we determined that a majority of CPD-dependent gene expression changes at 6 and 24 h after UVB exposure is linked to decreased cell viability and apoptosis induction (**Figure 14**). It is consistent with our observation that UVB irradiation significantly reduced the cell viability, while photoreactivation significantly increased cell survival of CPD-PL Ψ-mRNA transfected keratinocytes exposed to UVB (**Figure 12**). Interestingly, gene expression changes that were independent of CPDs seemed to influence the cell viability in the opposite direction at 24 h after UVB irradiation (**Figure 14**).

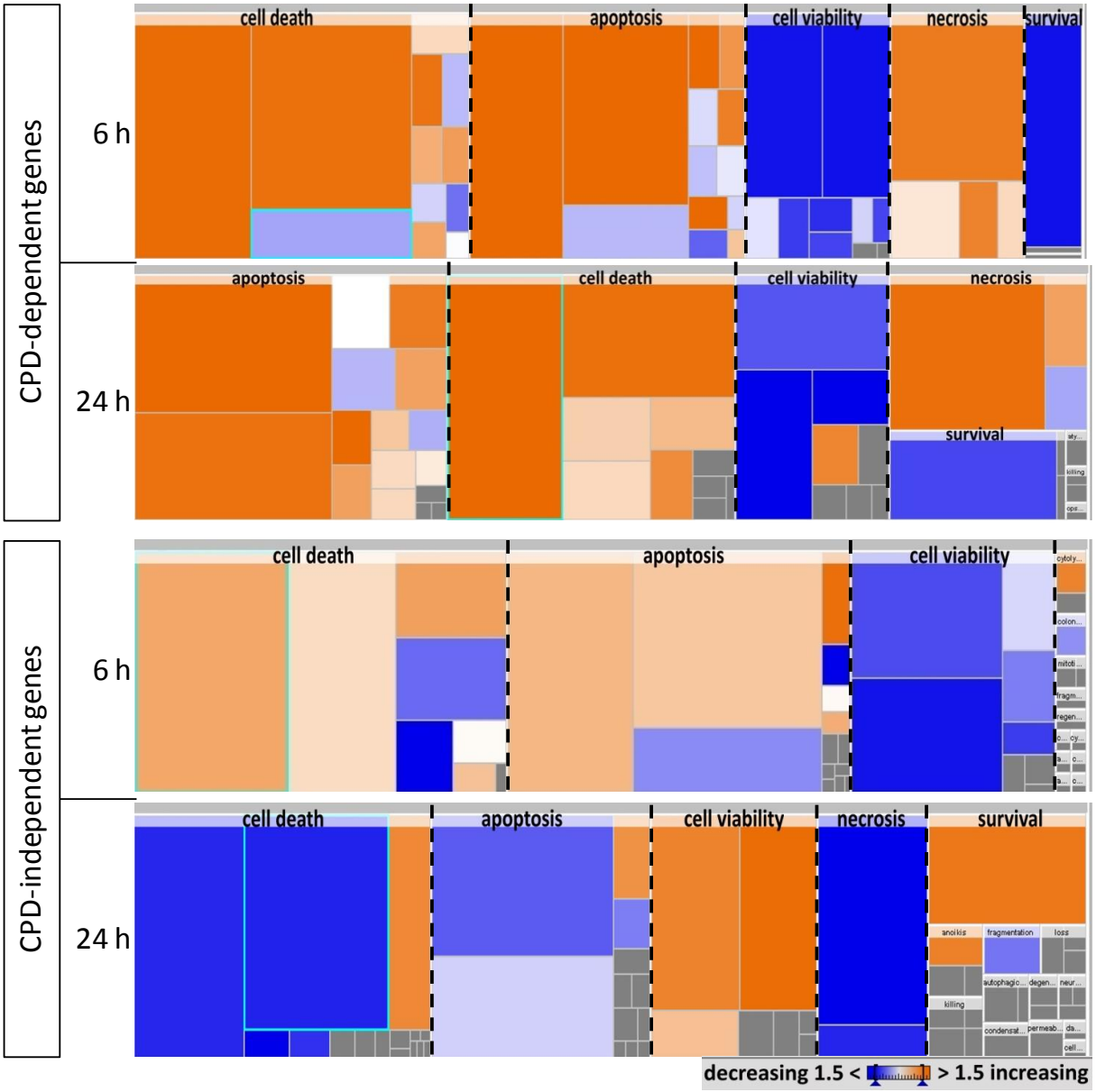


Figure 14. Effects of CPD-dependent and –independent gene expression changes on cell viability and cell death determined by IPA. *Squares in the heatmaps indicate genes related to cell survival and cell death signaling pathways, respectively. The color of squares reflects the effect of gene expression change on the biological process as predicted by IPA: promoting (orange squares), inhibitory (blue squares) or unknown (grey squares). The intensity of colors indicates the prediction strength. The overall state of cellular processes, whether inhibited or activated, is predicted based on Z-score algorithm.*

Analysing network interactions we found that a majority of CPD-dependent genes at 6 h after UVB exposure belongs to the regulatory network of *Gene Expression and Cell Cycle*, while those at the 24 h belong to the network of *Cell death and survival, Cellular Growth and Proliferation* (**Table 1**). 188 out of the 1008 CPD-regulated genes belonged to the two best-scored IPA networks at 6 h after UVB and 118 out of the 326 CPD-regulated genes at 24 h after the exposure (**Table 1**).

Top cellular functional categories among the total of 1334 CPD-regulated genes				
Time after UVB	**Associated Functions**	**CPD-regulated genes**	***p* value**	**Score**
6 h	Gene Expression, Cell Cycle, Cell Death and Survival	188	4.0E-23 – 1.9E-2	164
24 h	Cell Death and Survival, Cellular Growth and Proliferation	118	1.77E-6 – 3.3E-2	121

Table 1. Top functional categories among CPD-dependent genes determined at 6 and 24 h after UVB irradiation in CPD-PL Ψ-mRNA transfected keratinocytes. *The list of the top three networks and associated cellular functions of all CPD-related gene datasets are shown with their respective scores and p-values ($p < 0.05$) obtained from the Ingenuity Pathway Analysis (IPA) application. The score is derived from a p-value and indicates the likelihood of the focus genes in a network being found together due to random chance (defined as: - log10 (p-value)).*

The two top-rated networks of CPD-regulated genes at 6 and 24 h after UVB irradiation were centered around two transcriptional regulators and proto-oncogenes *c-Myc* and *c-Jun*. (**Figure 15 and 16**). In addition, two mediators of signal transduction *Smad4* and *TP53* were central nodes in the top network of CPD-dependent genes at 6 and 24 h after UVB exposure, respectively (**Figure 15 and 16**).

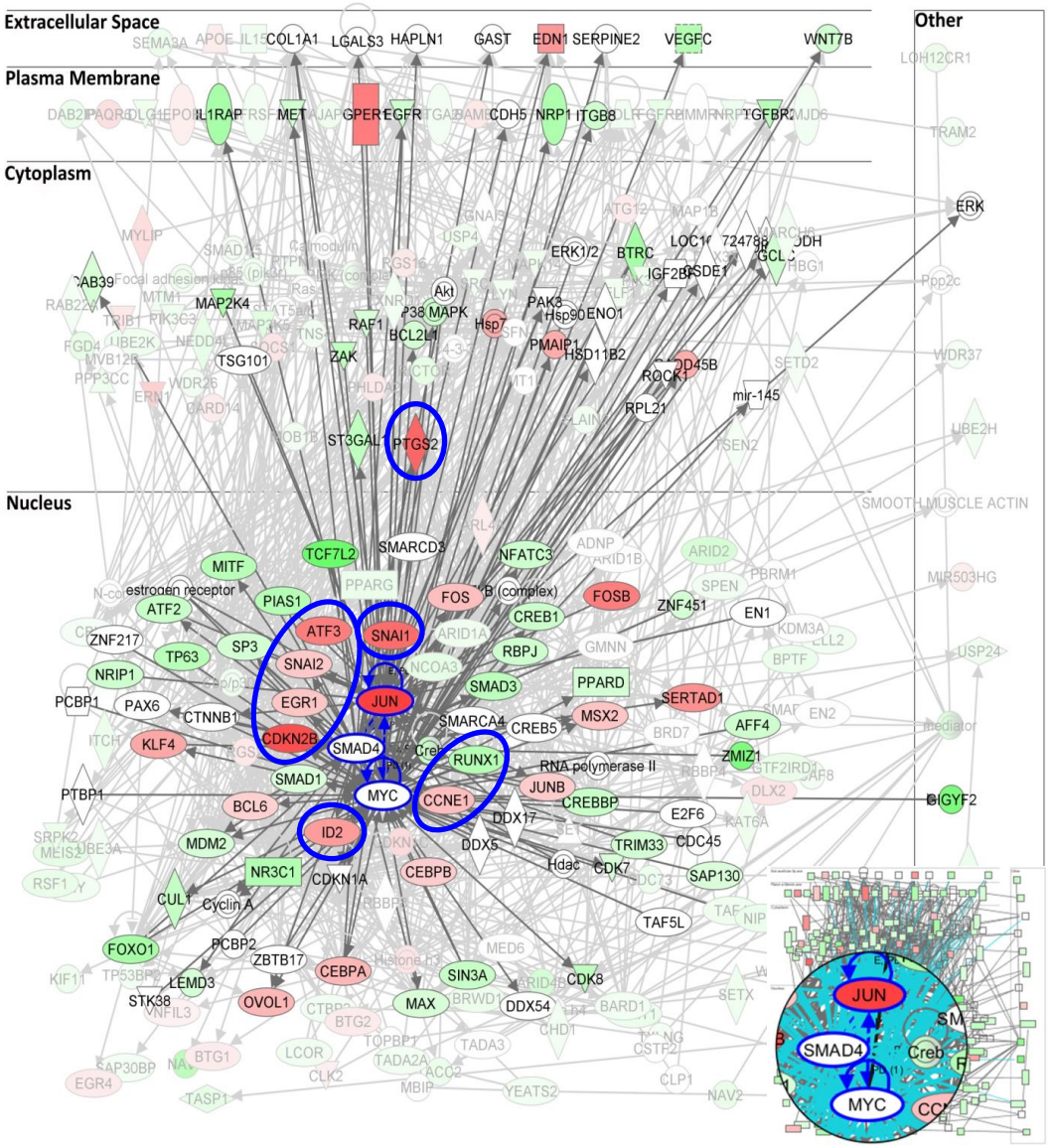


Figure 15. Top network interactions among CPD-dependent genes determined at 6 h after UVB irradiation in CPD-PL Ψ-mRNA transfected keratinocytes. *Direct interactions of genes derived from merging the two most highly rated networks, determined at 6 h after UVB irradiation, are illustrated in a view of subcellular compartments. Shaded nodes correspond to the significantly up- (red shaded) and downregulated (green shaded) genes modulated in a CPD-dependent manner. Genes in empty nodes (non-shaded) were not identified as differentially expressed in our experiment and were generated automatically by IPA Knowledge Base indicating a relevance to this network. The genes marked with blue circle have been validated by RT-qPCR.*

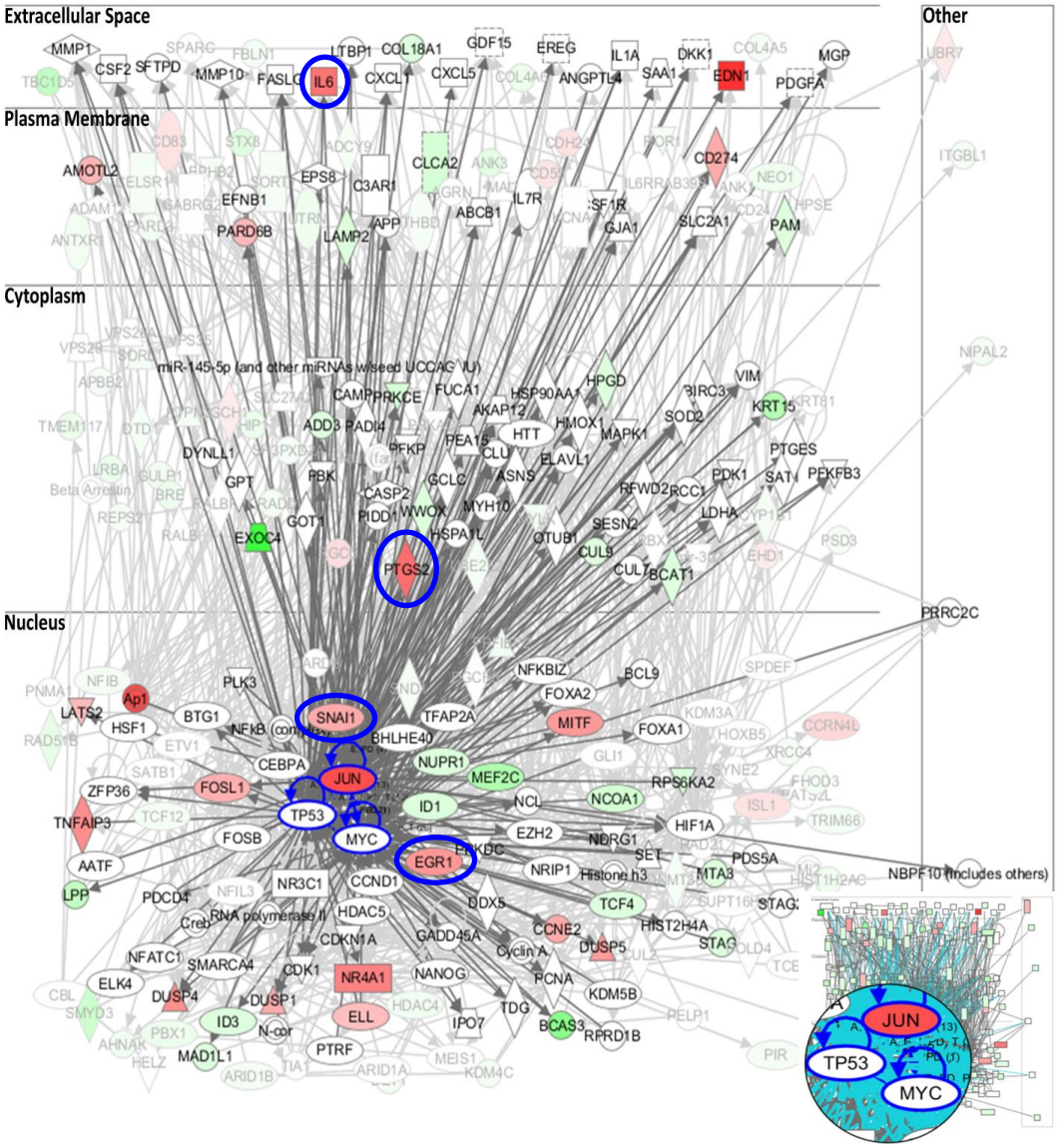


Figure 16. Top network interactions among CPD-dependent genes determined at 24 h after UVB irradiation in CPD-PL Ψ-mRNA transfected keratinocytes. *Direct interactions of genes derived from merging the two most highly rated networks, determined at 24 h after UVB irradiation, are illustrated in a view of subcellular compartments. Shaded nodes correspond to the significantly up- (red shaded) and downregulated (green shaded) genes modulated in a CPD-dependent manner. Genes in empty nodes (non-shaded) were not identified as differentially expressed in our experiment and were generated automatically by IPA Knowledge Base indicating a relevance to this network. The genes marked with blue circle have been validated by RT-qPCR.*

It is noteworthy that the expression of these key regulators, except *c-Jun*, was not changed at mRNA level after UVB exposure, while the expression of many of their target genes was changed, moreover, in a CPD-dependent manner. Ten target genes were selected for further investigation based on the microarray data and their involvement in cell cycle regulation *(CCNE1, CDKN2B)*, transcriptional regulation *(ATF3, EGR1, ID2, RUNX1)*, epithelial to mesenchymal transition *(SNAI1, SNAI2)* and inflammation *(IL-6, PTGS2)* (**Table 2**).

Table 2. Summarized fold change values by microarray results of the selected 10 CPD-dependent genes.

GenBank ID	Gene	Description	6 h after UVB irradiation		24 h after UVB irradiation	
			vs. non-irradiated	active PL vs. inactive PL	vs. non-irradiated	active PL vs. inactive PL
NM_001040619	ATF3	activating transcription factor 3	5.65	-3.30	1.85	-1.90
NM_001238	CCNE1	cyclin E1	2.87	-2.14	1.17	1.13
NM_004936	CDKN2B	cyclin-dependent kinase inhibitor 2B	7.76	-3.36	2.05	-2.06
NM_001964	EGR1	early growth response 1	2.51	-3.95	3.38	-2.39
NM_002166	ID2	inhibitor of DNA binding 2	4.35	-3.89	-2.09	1.45
NM_000600	IL-6	interleukin 6	2.93	-1.71	4.47	-2.41
NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2	6.75	-3.72	4.66	-3.04
NM_001001890	RUNX1	runt-related transcription factor 1	-4.45	4.36	1.05	1.09
NM_005985	SNAI1	snail family zinc finger 1	6.44	-3.44	2.76	-2.59
NM_003068	SNAI2	snail family zinc finger 2	2.39	-2.15	1.59	-1.24

active PL: samples containing active CPD-photolyase
inactive PL: samples containing inactive CPD-photolyase

4.6 CPD-dependency of expressional changes of 10 genes selected on the basis of microarray data were confirmed by qRT-PCR

To validate the microarray data, we employed real time quantitative RT-PCR analyses. Regarding *ATF3, CCNE1, EGR1, ID2, IL-6, PTGS2, SNAI1* and *SNAI2*, UV-induced

upregulation of these genes has been previously described [67-72], and our results were in agreement with the literature. We found significant upregulation of the expression of *CDKN2B* (p15INK4b), and downregulation of the expression of *RUNX1* in response to UVB (**Figure 17**). Furthermore, presence of active CPD-photolyase significantly prevented the changes in the expression of all tested genes (**Figure 17**), thereby confirming the results of the microarray analysis.

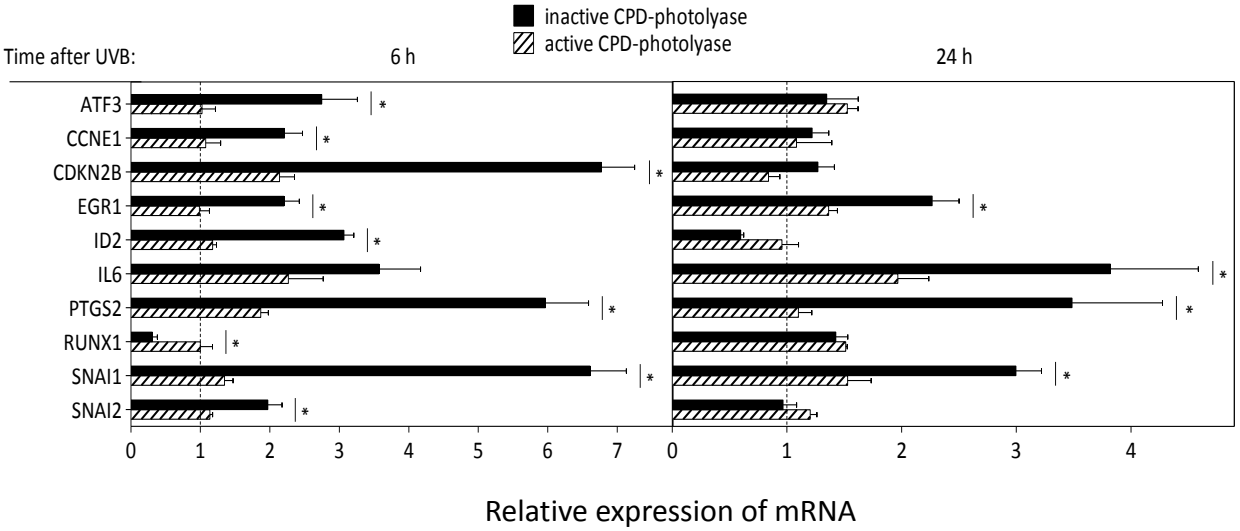
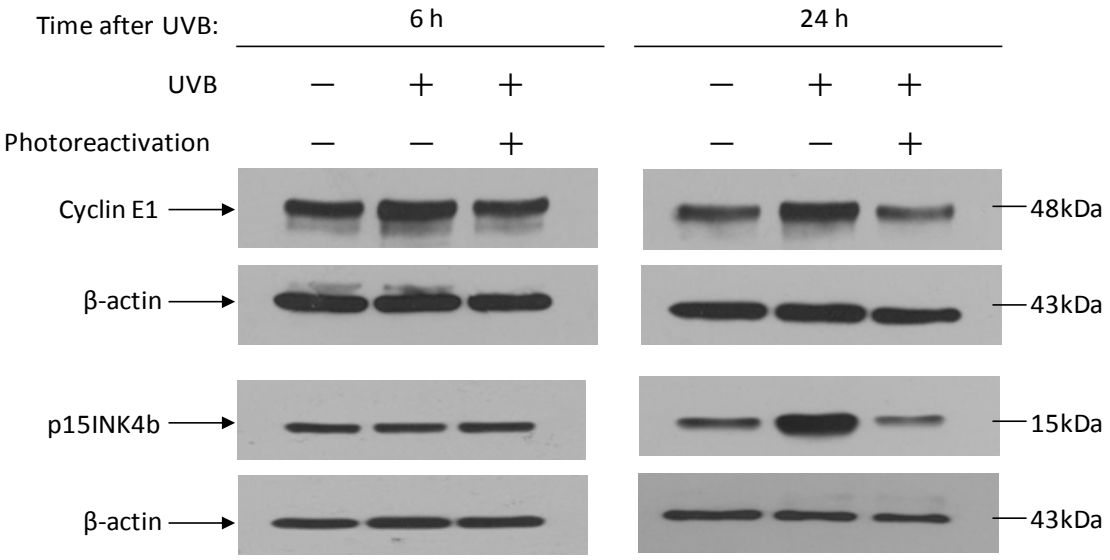


Figure 17. Experimental verification of microarray results for 10 selected genes. *HaCaT cells were exposed to 20 mJ/cm^2 UVB 12 h after delivery of CPD-PL Ψ-mRNA. Immediately thereafter, cells were either subjected to photoreactivating light (active CPD-photolyase) or left in the dark (inactive CPD-photolyase) for 1 h. Following incubation total RNA was extracted at 5 and 23 h, then real-time RT-qPCR was performed to validate the CPD-dependent expression of indicated genes. Values measured in UVB irradiated cells with or without photoreactivation were related to those measured in non-UVB irradiated cells that were transfected control Ψ-mRNA (pecked lines). Asterisks indicate significant differences (two-tailed, unpaired t-test; $p<0.05$) between photoreactivated (active CPD-photolyase) and non-photoreactivated (inactive CPD-photolyase) samples. The results of RT-qPCR are means ± SEM from three independent experiments in triplicate.*

4.7 Overexpression of cyclin E1 (CCNE1) and p15INK4b (CDKN2B) proteins in response to UVB is dependent on the generation of CPD and activation of JNK pathway

For further investigation, we selected two cell cycle-regulatory genes that were previously less characterized, related to their involvement in UVB-induced stress response. We have found that the expression of cyclin E1 and p15INK4b proteins were significantly increased at 24 h after UVB irradiation, but this increase was prevented by active CPD-photolyase (**Figure 18A, 18B**).

(A)



(B)

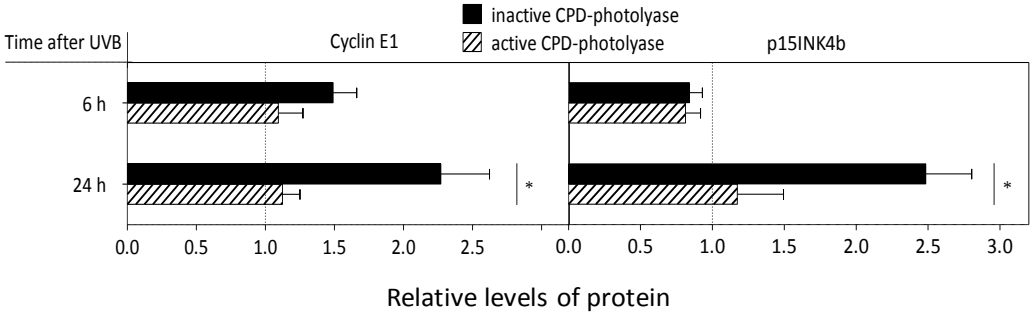


Figure 18. Photorepair of CPDs prevents altered expression of cyclin E1 and p15INK4b protein in UVB irradiated HaCaT cells. (A-B) *Cells were transfected with lipofectamine-complexed CPD-PL Ψ-mRNA, 12 h later irradiated with 20 mJ/cm^2 UVB and immediately exposed to photoreactivating light (active CPD-photolyase) or kept in the dark (inactive CPD-photolyase) for 1 h. Subsequently, cells were cultured at 37°C until harvested at the indicated time after UVB irradiation.* ***(A)*** *The expression of cyclin E1 and p15INK4b were analyzed by Western blot.* ***(B)*** *Quantitation of western blots displays relative changes in protein expression normalized to β-actin. Pixel densities were calculated relative to those obtained with cells that were not UVB irradiated (pecked lines). Significance was assessed by two-tailed, unpaired t-test (asterisk, $p<0.05$) showing differences between photoreactivated and non-photoreactivated samples. Error bars represent the standard error of the mean. The results are means of three independent experiments.*

UVB can activate various protein kinases to regulate cell proliferation and survival pathways in human keratinocytes [73]. To investigate the signalling pathways involved in modulation of cyclin E1 and p15INK4b expression upon UVB exposure, HaCaT cells were first exposed to specific inhibitors of JNK (SP600125), p38 MAPK (SB203580) or AKT (MK-2206 2HCl) for 1 h, then immediately subjected to 20 mJ/cm^2 of UVB. Subsequently, cells were cultured in keratinocyte medium containing inhibitor until harvested at 6 and 24 h. We determined the expression of cyclin E1 and p15INK4b proteins by western blot analysis. While suppression of p38 MAPK or AKT had no effect on UVB-induced increase in the levels of these proteins (**Figure 19**), we found that the treatment of cells with the JNK inhibitor SP600125 abolished the induction of cyclin E1 and p15INK4b protein expression at 24 h after UVB (**Figure 19**).

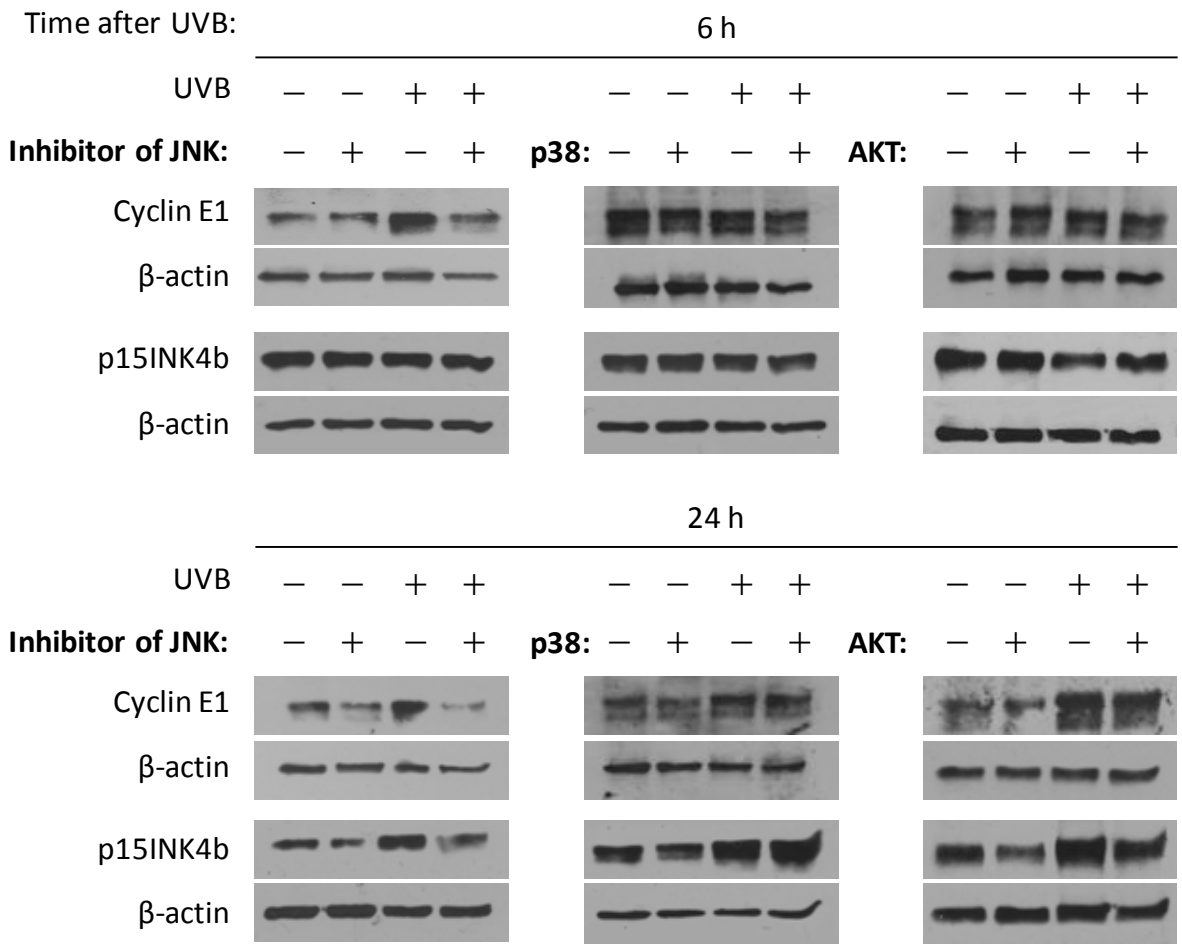


Figure 19. Induction of cyclin E1 and p15INK4b protein expression upon UVB exposure is regulated through the JNK signalling pathway in HaCaT cells. *Keratinocytes were incubated in serum-free medium supplemented with p38 MAPK (SB203580), AKT (MK-2206 2HCl) or JNK inhibitor (SP600125) for 1 h, then irradiated with a physiological dose of UVB or left untreated. The cells were cultured further in serum-free medium supplemented with the inhibitor. Cells were harvested for western blot assay at the indicated time. Protein levels of cyclin E1, p15INK4b, and β-actin are noted. The figure shows representative results from three independent experiments.*

5. Discussion

Intracellular delivery of therapeutic proteins has been performed using many different viral and non-viral techniques. Among them, the most promising in clinical point of view is the mRNA-based gene transfection. It is well documented that *in vitro* synthesized mRNA has great therapeutic potential to transiently express physiologically important proteins [52]. Here, we applied this novel mRNA-based platform to achieve functional non-human photolyase production in cultured human keratinocytes, thereby gaining insight into the UVB-induced cellular mechanisms. The CPD-specific photolyase of *Potorous tridactylus* encoded by the mRNA was correctly located to the nucleus and resulted in rapid and efficient repair of UVB-induced CPDs upon photoreactivation regardless of mRNA transfection was applied before or after UVB exposure. This experimental platform enabled us to distinguish between CPD-dependent and -independent cellular mechanisms induced by UVB irradiation. Transfection of HaCaT cells with photolyase mRNA led to a significant survival advantage indicating that CPDs are principal contributors to the UVB-induced decrease in cell viability. Furthermore, we confirmed that CPDs are the primary cause of transcriptional changes in response to UVB radiation.

This study demonstrated simple and highly efficient and effective synthesis of CPD-specific photolyase in human cells from transfected mRNA. Several studies have reported successful protection from UVB-induced damage using topical sunscreen lotions containing liposomal-encapsulated bacterial photolyase or CPD-specific endonuclease [72, 74-76]. However, direct evidence for nuclear localization of photolyase derived from the cyanobacterium *Anacystis nidulans* has not been provided, and sequence analysis of the 54.5 kDa protein identified neither a classical nor a non-classical nuclear localization signal. Using nucleoside-modified mRNA encoding marsupial CPD-photolyase, we could verify that the encoded protein correctly located to the nucleus of human keratinocytes and was functionally active as it

repaired 90% of CPD lesions within 6 hours after UVB irradiation in response to photoreactivating light exposure. In placental mammals, removal of UVB-induced CPD lesions is entirely dependent on the highly conserved repair system NER containing 9 major proteins and over 20 associated genes. In our experiments, more than 48 hours were required to reduce the amount of CPDs by 90% by the NER in UVB-irradiated HaCaT keratinocytes (**Figure 11**).

Accumulation of CPDs in cells with high proliferation rate is highly cytotoxic and mutagenic thereby explicitly contributing to UVB-induced carcinogenesis [29, 77]. Genetic defects in NER result in photosensitivity and skin cancer proneness [78]. Identification of the genes regulated by CPD photolesions might have clinical relevance, as it will facilitate defining novel targets for diagnosis or treatment of UVB-mediated skin diseases. To characterize the CPD-dependent and -independent cellular transcriptional responses to UVB irradiation microarray analysis was performed. Transcriptome analysis identified 1334 CPD-responsive genes, which represent more than 50% of the genes regulated by UVB at 6 and 24 h after the exposure (**Figure 13**), indicating that CPDs are major source of altered gene expression induced by UVB irradiation. A significant number of these genes (738 and 250 genes at 6 h and 24 h after the exposure, respectively) were downregulated, most likely due to the stalling of RNA polymerase II and inhibition of transcription elongation caused by UVB-induced DNA damage [79]. Furthermore, we found a markedly higher number of CPD-dependent genes to show altered expression levels at 6 h, as compared to 24 h owing to the slower naturally-occurring DNA repair mechanism.

It is well established that UVB irradiation promotes cell cycle arrest or induction of apoptotic machinery and inhibits cell proliferation [18, 80, 81]. Comparative analysis of downstream effects of gene expression changes observed in CPD-dependent and -independent genes revealed that CPDs are key determinants of cell death and survival upon UVB exposure.

Using a prediction model of IPA, we found that quick removal of CPDs by photoreactivation resulted in change of the gene expression pattern relating to cell death and survival at 24 h after UVB irradiation. A majority of CPD-dependent gene expression changes after UVB exposure could be linked to decreased cell viability and apoptosis induction, while gene expression changes that were independent of CPDs seemed to influence the cell viability in the opposite direction at 24 h after UVB irradiation (**Figure 14**). Accordingly, UVB irradiation significantly reduced the cell viability, while photoreactivation significantly increased cell survival of CPD-PL Ψ-mRNA transfected keratinocytes exposed to even high-dose UVB (**Figure 12**).

Top functional categories were determined among CPD-responsive genes, and we found that CPD-related gene expression changes were mainly associated with regulation of the transcriptional and cell cycle machineries in response to UVB irradiation in human keratinocytes. Molecular studies have provided evidence that recognition of DNA damage, including UVB-induced photolesions, can temporarily halt cell cycle progression, allowing time for cells to repair damages prior to replication [82, 83]. Unsuccessful repair of these photoproducts induces intracellular apoptotic signalling in order to prevent the multiplication of mutated chromosomes, but may lead to a permanent cell cycle block, genomic instability or skin carcinogenesis [17-19]. Network analysis of CPD-responsive genes revealed *c-Jun*, *c-Myc*, *Smad4* and *TP53* as the major regulating factors in CPD-induced cellular stress responses. The importance of these multifunctional transcription regulators in cell cycle progression, apoptosis and cellular transformation is supported by several studies [73, 84-87]. These data demonstrated that germline mutations or chemical inactivation of these key mediators, could result in perturbations of cell cycle checkpoint control leading to the transformation of cells and have been associated with a variety of tumours. Based on our microarray data, there was no detectable change in their expression at mRNA level upon UVB

exposure, except *c-Jun*, but the expression of many of their target genes has been changed in a CPD-dependent manner. These findings indicate that CPDs significantly contribute to UVB-induced cellular stress responses through regulation of target genes of these key regulatory molecules. We validated the microarray results for six transcriptional factors, *ATF3, EGR1, ID2, RUNX1, SNAI1* and *SNAI2*, and two apoptosis and –inflammation-related genes, *COX-2* and *IL-6*. Other studies have also suggested that upregulation of *ATF3*, *COX-2*, *EGR1*, *ID2*, *IL-6*, *SNAI1* and *SNAI2* has been implicated in UV-induced cellular stress responses [67-72]. Herein, we demonstrated that the expression of these genes is CPD-dependent, whereby they could serve as novel biomarkers for evaluation of the involvement of UVB in various photosensitive skin disorders or skin cancers. The most studied genes associated with UVB-mediated cellular processes are cyclooxygenase 2 *(COX-2)* and interleukin 6 (*IL-6*). Their upregulation is described as a diagnostic marker and predictive factor in many tumours [88-90] and other diseases related to chronic inflammation [91]. *In vivo* studies have demonstrated that the use of specific inhibitors of *COX-2* leads to accumulation of cells in G0/G1 phase of cell cycle and reduced proliferation rate of cancer cells [88].

Furthermore, we identified two CPD-dependent cell cycle-regulatory genes, *CDKN2B* and *CCNE1* that are involved in regulation of the G1 phase of cell cycle with less established roles in UVB-mediated cellular damage in keratinocyte biology. We showed that the CPD-photolyase mediated repair of UVB-induced CPDs prevented increased expression of *CDKN2B* (p15INK4b) and *CCNE1* mRNA and protein. It is well known that p15INK4b is a cyclin-dependent kinase inhibitor (CKI) that is related to cell cycle arrest in early to mid G1 phase of the cell cycle, whereas cyclin E1 promotes cell proliferation at the late G1 phase to S transition [92]. Loss of p15INK4b function and *CDKN2B* mutations has been described in several types of tumours [93-95] confirming that the inactivation of p15INK4b significantly contributes to the transformation of normal cells into cancer cells. A study using a mouse

model has shown that the cell cycle disturbance in epidermal keratinocytes subjected to an erythematogenic dose of UVB irradiation is at least in part caused by increased expression of cyclins, including cyclin E1 [96]. Their data suggests that UVB-induced hyperplasia and tumorigenesis is partly mediated by the upregulation of cyclin E1, it however has not been investigated in humans. Despite the apparently opposite function of *CDKN2B* and *CCNE1*, we found that UVB irradiation triggered overexpression of both genes in human keratinocytes. However, the effect of a cell cycle regulatory gene on cell cycle progression is highly dependent on cellular levels of other regulatory molecules and their posttranslational state. Taken together, our data strongly suggest the high relevance of CPDs in disruption of cell cycle progression.

The question of how CPD photolesions activate intracellular signalling and how they modulate the expression of genes in cellular mechanisms (e.g. cell cycle arrest) in response to UVB irradiation has not been answered. Using CPD-photolyase expressing transgenic mice, it has been shown previously that CPD photolesions are the major mediator of UVC-induced transcriptional responses and DNA strand-breaks [3]. UV radiation activates different signal transduction pathways in a wavelength- and dose-dependent manner [73]. It is well established that these signalling pathways are regulated by specific protein kinases, including mitogen-activated protein kinases (MAPK), protein kinase B (PKB or AKT) and protein kinase C (PKC) that play a crucial role in the response network to skin damage caused by UV exposure [73]. We examined how specific inhibitors of protein kinases, such as AKT, p38 kinase and JNK, affect gene expression changes caused by CPD photolesions. We found that only the JNK-specific inhibitor influenced UVB-induced overexpression of cyclin E1 and p15INK4b proteins. Reviewing the literature and comparing the data to our microarray findings, forty-two CPD-dependent genes (**Table 3**) could be identified that might be regulated through the activation of JNK.

Table 3. Candidate CPD-responsive genes regulated by JNK pathway		
Accession Number	**Gene symbol**	**Function[97]**
NM_001124	ADM	Metabolism, steroid
NM_000693	ALDH1A3	Energy/Detox
NM_001040619	ATF3	Transcription repressor
NM_003567	BCAR3	Cell cycle
NM_138931	BCL6	Transcription f. Zn finger
NM_006763	BTG2	Cell cycle inhibitor
NM_004233	CD83	Immunoglobulin
NM_000076	CDKN1C	Cell cycle inhibitor
NM_001008390	CGGBP1	Nucleoskeletal
NM_000104	CYP1B1	Cytochrome
NM_004405	DLX2	Transcription f. homeodom.
NM_004417	DUSP1	Phosphatase
NM_001394	DUSP4	Regulator, JNK, ERK, p38
NM_004419	DUSP5	Phosphatase
NM_003633	ENC1	Cytoskeletal, actin
NM_005252	FOS	Transcription factor
NM_000161	GCH1	Regulator, cAMP, cGMP
NM_003483	HMGA2	Transcription
NM_005114	HS3ST1	Golgi apparatus
NM_002166	ID2	Transcription repressor
NM_002203	ITGA2	Adhesion, integrin
NM_002228	JUN	Transcription factor
NM_000963	KLF4	Transcription factor
NM_003709	KLF7	Transcription factor
NM_002274	KRT13	Cytoskeletal, keratin
NM_033300	LRP8	Receptor
NM_002538	OCLN	Adhesion, junctional
NM_004235	PHLDA2	Apoptosis
NM_004235	PI3	Proteolysis inhibitor
NM_021127	PMAIP1	Oncogenesis
NM_177968	PPM1B	Phosphatase
NM_000963	PTGS2	Regulator, prostaglandin
NM_173174	PTK2B	Protein kinase
NM_016836	RBMS1	RNA metabolism
NM_002923	RGS2	G-regulated protein
NM_013272	SLCO3A1	Transporter
NM_022739	SMURF2	Proteolysis, ubiqutin
NM_003114	SPAG1	Tumor antigen
NM_006290	TNFAIP3	Apoptosis inhibitor
NM_012288	TRAM2	Membrane protein
NM_005429	VEGFC	Secreted
NM_004625	WNT7A	Regulator, WNT pathway

Table 3. List of 42 CPD-dependent targets defined as JNK-regulated genes in human keratinocytes. *Defining the functions of these genes derived from Gazel et al. [97].*

Our findings indicate an important role for JNK in the control of gene expression modulated by CPD photolesions. Activation of JNK is triggered by a variety of extracellular stimuli e.g., by UV radiation, and followed by nuclear translocation of the activated form. Multiple transcription factors including *c-Jun*, *c-Myc*, *Smad4* and *TP53* [73, 98] that were identified as central regulatory molecules in the top network of CPD-dependent genes after UVB exposure are known to be phosphorylated by active JNK. Genes regulated by JNK pathway are listed in **Table 3.** They are involved in a wide variety of cell processes including regulation of the cell cycle and transcriptional machineries, apoptosis, cellular growth and proliferation. It has been reported that active JNK is mainly associated with keratinocyte proliferation and differentiation [97, 99]. Some studies demonstrated enhanced JNK activity in psoriatic [100] and wound-healing epidermal cells [101], as well as in cylindromas and other hair follicle derived tumours [102]. Increased levels of the activated form of JNK have been shown in UV exposed keratinocytes [85].

In conclusion, this novel mRNA-based gene therapy method efficiently directs functional protein synthesis providing number of opportunities for future dermatological applications if the nucleic acid delivery to intact epidermis has been resolved. Concerning photolyase, thus it could be used in UV-sensitive diseases and in the prevention of skin cancer. Delivery of *in vitro* synthesized mRNA encoding CPD-specific photolyase into human keratinocytes described here is an excellent model to investigate UVB-induced cellular mechanisms. Our findings are important for those interested in studying the activities and effects of UV radiation and DNA photolesions in mammalian cells. This novel mRNA-based experimental platform opens up new horizons for understanding human physiology and pathophysiology.

6. Summary

We presented an original approach for studying the cellular mechanisms of UVB-induced DNA damage in cultured human keratinocytes using *in vitro* transcribed and nucleoside-modified mRNA encoding cyclobutane pyrimidine dimer (CPD)-specific photolyase.

Messenger RNA encoding CPD-photolyase of *Potorous tridactylus* was generated by *in vitro* transcription. Superior translation and non-immunogenic nature were achieved by codon-optimization, pseudouridine modification, long polyA-tail and cap1 structure incorporation, and HPLC purification.

It could be proved that the nucleoside-modified mRNA encoding non-human CPD-specific photolyase can be effectively delivered into human keratinocytes, the translated photolyase protein accumulated in the nuclei of cells, and it was functionally active. Rapid and highly efficient repair of UVB-induced CPDs could be demonstrated in transfected cells exposed to UVB and photoreactivating light. Photoreactivation significantly reduced the antiproliferative effect of UVB irradiation.

This experimental platform was suitable for investigating CPD-dependent and -independent cellular processes upon UVB exposure. We found that CPDs are the primary cause of transcriptional changes in response to UVB radiation. In addition, our findings suggested the involvement of JNK signalling pathway in CPD-dependent cellular stress responses upon UVB exposure.

In vitro transcribed mRNA can be a new platform for safe and effective delivery of therapeutic proteins opening wide perspectives for dermatological or other medical utilizations. Delivery of *in vitro* synthesized and pseudouridine-modified mRNA encoding CPD-photolyase into keratinocytes *in vivo* offers an innovative solution for skin cancer prevention.

7. Összefoglalás

In vitro szintetizált és nukleozid-módosított, ciklobután pirimidin dimer (CPD)-specifkus fotoliázt kódoló mRNS-alapú géntranszfekciós modellrendszer kialakításával újszerű megközelítésben vizsgáltuk az UVB sugárzás okozta DNS károsodások hatásait tenyésztett humán keratinocitákban.

A *Potorous tridactylus* CPD-fotoliáz génjét kódoló mRNS előállítása *in vitro* transzkripcióval történt. A szekvencia kodon-optimalizálásával, pszeudouridin módosítással, valamint hosszú poly(A) farok és cap1 struktúra mRNS-be történő beépítésével magas transzlációs hatékonyság és biológiai stabilitás volt kialakítható.

Bizonyítottuk, hogy CPD-fotoliáz fehérje hatékony szintézise érhető el humán keratinocitákban a nem-humán proteint kódoló nukleozid-módosított mRNS bevitelével. A CPD-specifikus fotoliáz a vártnak megfelelően a sejtek magjában lokalizálódott, és funkcionálisan aktív volt. Az UVB által indukált CPD-k gyors és hatékony kijavítódása volt kimutatható az UVB-irradiált, majd fotoreaktivált transzfektált sejtekben. A fotoreaktiváció szignifikánsan csökkentette az UVB antiproliferatív hatását.

Ez az *in vitro* modell alkalmas volt az UVB sugárzás által indukált CPD-függő és -független celluláris folyamatok elkülönítésére. Megállapítottuk, hogy az UVB-okozta transzkripcionális változások fő oka a CPD-képződés. Emellett eredményeink a JNK jelátviteli útvonal fontos szerepére mutattak rá az UVB-irradiációt követően kialakult, CPD-dependens stresszválaszokban.

Az *in vitro* szintetizált mRNS transzfekció a jövőben egy hatékony és biztonságos módját jelentheti a terápiás szempontból fontos fehérjék intracelluláris előállításának, bőrgyógyászati és más orvosi alkalmazások számára. CPD-fotoliázt kódoló, *in vitro* szintetizált mRNS keratinocitákba történő bejuttatása *in vivo* egy innovatív megoldást kínálhat a bőrdaganatok megelőzésére.

8. References

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Key words

in vitro synthesized mRNA, pseudouridine, ultraviolet B, cyclobutane pyrimidine dimer, photolyase, photoreactivation, DNA repair, human keratinocytes, cell death and survival, CPD-dependent genes

Kulcsszavak

in vitro szintetizált mRNS, pszeudouridin, UVB sugárzás, ciklobután pirimidin dimer, fotoliáz, fotoreaktiváció, DNS reparáció, humán keratinocita sejtek, sejthalál és sejttúlélés, CPD-függő gének

Publications related to dissertation

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List of publications related to the dissertation

1. **Boros, G.**, Mikó, E., Muramatsu, H., Weissman, D., Emri, E., Szegedi, A., Horkay, I., Emri, G., Karikó, K., Remenyik, É.: Identification of Cyclobutane Pyrimidine Dimer-Responsive Genes Using UVB-Irradiated Human Keratinocytes Transfected with In Vitro-Synthesized Photolyase mRNA.
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2. **Boros, G.**, Mikó, E., Muramatsu, H., Weissman, D., Emri, E., Rózsa, D., Nagy, G., Juhász, A., Juhász, I., van der Horst, G., Horkay, I., Remenyik, É., Karikó, K., Emri, G.: Transfection of pseudouridine-modified mRNA encoding CPD-photolyase leads to repair of DNA damage in human keratinocytes: A new approach with future therapeutic potential.
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List of other publications

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3. Emri, E., Mikó, E., Bai, P., **Boros, G.**, Nagy, G., Rózsa, D., Juhász, T., Hegedűs, C., Horkay, I., Remenyik, É., Emri, G.: Effects of non-toxic zinc exposure on human epidermal keratinocytes.
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Presentations

1. **23rd World Congress of Dermatology, Vancouver, Canada,** *Changes of mRNA expression during photoprotection accomplished by mRNA transfection – an in vitro study* (first author)
2. **44th Congress of European Society for Dermatological Research, Copenhagen, Denmark,** *Effect of cyclobutane pyrimidine dimer on gene expression is mediated by activation of c-Jun kinase in UVB irradiated human keratinocytes* (oral presentation)
3. **2nd Experimental Dermatological Conference, Szeged, Hungary,** *Cyclobutane pyrimidine dimer-dependent modulation of mRNA- and protein expression in human keratinocytes following UVB exposure* (oral presentation)
4. **16th Annual Meeting of American Society of Gene and Cell Therapy, Salt Lake City, Utah, USA,** *Transfection of pseudouridine-containing mRNA encoding CPD-photolyase into UVB-irradiated human keratinocytes results in rapid repair of DNA damage* (poster presentation)
5. **43rd Congress of European Society for Dermatological Research, Edinburgh, Scotland,** *Post-UV delivery of CPD-photolyase mRNA leads to repair of DNA damage in human keratinocytes* (poster presentation)
6. **42nd Congress of European Society for Dermatological Research, Venice, Italy,** *CPD-dependent gene expression changes in photolyase mRNA-transfected human keratinocytes irradiated by UVB* (poster walk)
7. **41st Congress of European Society for Dermatological Research, Barcelona, Spain,** *Enhanced repair of UVB-induced DNA lesions in photolyase mRNA-transfected keratinocytes* (poster presentation)

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