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Original Contribution

Thioredoxin-like 6 protects retinal cell line from photooxidative damage by upregulating NF-κB activity

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ABSTRACT

Apoptosis is the common pathway to photoreceptor cell death in many eye diseases including age-related macular degeneration which affects more than 8 million individuals in the United States alone. RdCVF, a truncated mouse thioredoxin is specifically expressed by rod photoreceptor cells and prevents the apoptosis of cone cells. However the protective mechanism of RdCVF and the implications of its human homologue, thioredoxin-like 6 (TXNL6), on the apoptosis of retinal cells remain unknown. In this study, we examined the function of TXNL6 and investigated its mechanism of protection using a cone photoreceptor cell line, 661W. We found that the photooxidative stress-induced degradation of NF-κB proteins is rescued by overexpression of TXNL6, which enabled the NF-κB transactivation activity. Furthermore, the overexpression of TXNL6 rescued the photooxidative stress-induced apoptosis of 661W cells. Interestingly, this protective effect was significantly blocked by NF-κB specific inhibitors demonstrating that TXNL6 exerts its protective effect against apoptosis via NF-κB. Taken together, our study shows that the TXNL6 probably protects retinal cells from photooxidative damage-induced apoptosis via upregulation of NF-κB activity. The identification of TXNL6 and the demonstration of its protective mechanism offer new insights into treatment possibilities for photoreceptor cell degradation.

Introduction

Photoreceptor cell death via apoptosis is responsible for many eye diseases including age-related macular degeneration which is estimated to affect more than 8 million individuals in the United States alone [1–4]. It has largely been accepted that reactive oxidative species (ROS) and a series of caspase and calpain enzymes plays a key role in both the initiation and execution of apoptosis [2,5,6]. Therefore, the regulation of the cellular redox state and apoptotic process may serve as potential therapeutic strategies applicable to retinitis pigmentosa and other human retinal diseases [6,7].

The nuclear factor kappa B (NF-κB) is a family of transcription factors involved in regulating the expression of numerous genes participating in immune response and inflammation [8,9]. NF-κB is also an important regulator of apoptosis and it was suggested that reactive oxygen species (ROS) may be involved [10–12]. It has been shown that nerve growth factor-dependent activation of NF-κB contributes to the survival of sympathetic neurons [13] and the activation of NF-κB is necessary for the survival of S-type neuroblastoma [14]. Furthermore, Crawford *et al* [15] reported that the preservation of NF-κB binding activity in the nucleus may be essential for mouse cone photoreceptor cells, 661W, to survive photooxidative damage-induced apoptosis.

Thioredoxin (TRX) is a small, ubiquitous protein of 12 kDa which is conserved in all organisms, from *E. coli* to human [16]. It functions as a general protein-disulfide reductase, as it has two-redox-active half-cysteine residues in its catalytic active site. This active site is conserved, with the consensus amino acid sequence of –Cys-Gly-Pro-Cys- [17]. In the cytoplasm, TRX exists either in a reduced form with a dithiol, or in an oxidized form in which the Cys residues at the active site form an intramolecular disulfide bridge [18]. The protein participates in redox reactions via reversible oxidation of its active dithiol to a disulfide. TRX catalyzes dithiol-disulfide exchange reactions involving many thiol-dependent cellular processes, including the regulation of transcription factor DNA binding activity, anti-oxidant defense, modulation of apoptosis and the immune response [19,20].

Abbreviations: DCFH-DA, dichlorofluorescein diacetate; DLR, dual-luciferase reporter; DM, double mutant; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic gel mobility shift assay; GST, glutathione-S-transferase; JSH-23, NF-κB activation inhibitor II; PCR, polymerase chain reaction; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; RdCVF, Rod-derived cone viability factor; TNF-α, tumor necrosis factor; TXNL6, thioredoxin-like 6; TUNEL, terminal transferase dUTP nick end labeling.

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The human thioredoxin-like 6, previously known as TRX6, is now referred to as TXNL6. Recently, Leveillard *et al.* [21] showed that a mouse homologue of TXNL6, named as rod-derived cone viability factor (RdCVF), could slow down cone degeneration in both the chick and mouse models. However, the protective mechanism of RdCVF against apoptosis of retinal cells is still unknown. In our earlier study [22], we found that the human TXNL6 could enhance NF-κB DNA-binding activity. Therefore, we postulate that TXNL6 is a novel regulator of NF-κB and protects photoreceptor cells from apoptosis via the activation of NF-κB signaling pathway. Our main objective here is therefore to clarify whether the human TXNL6 regulates NF-κB activity and if so, how does it protect the photoreceptor cells from light-induced apoptosis? To address this question, we investigated the role of TXNL6 in HeLa and 661W photoreceptor cell lines, the latter of which is essential for investigating photoreceptor apoptosis [23]. The rationale for our choice of HeLa cells at the start of this study was that the human TRX1 has been reported to regulate NF-κB activity in HeLa cells [24]. We demonstrated that the TXNL6 upregulates NF-κB activity, thereby protecting retinal cells from apoptosis induced by photo-oxidative damage.

Materials and methods

Plasmid Construction

Wild type TXNL6 (GenBank Accession number: BC014127) was cloned with a C-terminal FLAG tag into the *XhoI-SalI* sites of the pCI-neo (Stratagene, CA, USA) and pEGFP-N3 (BD Biosciences, CA, USA) mammalian expression vectors. For the pEGFP-TXNL6 construct, we had incorporated a stop codon after the FLAG tag (Supplementary Fig. 2A). This was to ensure that the expression of EGFP does not affect the function of TXNL6 since EGFP (26 kDa) is even larger than TXNL6 (24 kDa). The two Cys residues in the active motif are known to be responsible for the oxidoreductase function of the TRX superfamily (Supplementary Fig. 1) [24]. The importance of the two Cys residues to the function of TXNL6 was investigated by mutating them into Ala residues using QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene). For bacterial expression, the ORF encoding the human TXNL6 was cloned into the *BamHI-XhoI* sites of the pGEX-4T-1 expression vector (GE Healthcare) and transformed into *E. coli* BL21 (DE3).

Bacterial Expression and Purification of human TXNL6

The induction of the fusion protein was performed as previously described [25]. Overexpressing bacteria were harvested and disrupted by sonication, and the supernatant was cleared by centrifugation at 15,000 ×*g* for 30 min and loaded onto a glutathione-Sepharose 4B column (GE Healthcare). Binding to the matrix was allowed to occur overnight at 4 °C. Protein concentration was determined by Bradford assay.

In vitro Interaction between TXNL6 and NF-κB p50

Based on a previous report [24], 100 ng of recombinant NF-κB p50 subunit (Promega, WI, USA) was incubated with 50 μl of 50% slurry of Sepharose-GST-TXNL6. The mixture was rotated end-over-end at 4 °C for 30 min with 10 μl of 1 mM dithiothreitol; then 10 μl of 10 mM diamide was added and the reaction was continued overnight at 4 °C, with 0.05% NP-40. The suspension was centrifuged and the resin was washed to reduce any unspecific binding, before the mixture was denatured by boiling. The protein was analyzed on 10% SDS-PAGE, and the presence of NF-κB p50 and TXNL6 was detected by Western blot using anti-p50 antibody (eBioscience, CA, USA) and anti-FLAG M2 (Sigma), respectively.

Cell Culture and Transfection

HeLa cells were maintained at 37 °C in Dulbecco's modified Eagle's medium, DMEM (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). At 12 h prior to transfection, cells were seeded in a 6-well plate at 0.8×10^6 cells/well. Transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. After incubation for 30 h, the cells were treated with indicated amounts of TNF-α or PMA for 6 h before harvesting. The 661 W cell line was originally isolated from mouse retinal tumors and has been shown to be of cone photoreceptor cell lineage [23]. 661W cells were routinely grown in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, 0.3 g/L L-glutamine and 40 μl/L 2-mercaptoethanol, at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. To perform gene reporter assays, dual luciferase activities were measured using the Dual-Luciferase Reporter (DLR) Assay System (Promega) at 36 h after transfection. The κB reporter was p5×NF-κB-luciferase (Stratagene). This reporter activity correlates with the transactivation ability of NF-κB transcription factor. pRL-CMV (Promega) was used as an internal control.

Selection of Stable Transformants

pEGFP-N3 vector which contains the neomycin resistant gene, was used for establishing stable cell lines. For transfection, cells were first seeded in a 6-well plate at a density of 5×10^5 cells/well. After transfection, the 661W cells were allowed to recover for 48 h and subjected to selection pressure at 900 μg/ml of G418 (BD Bioscience). Stable transfectants were maintained in 1000 μg/ml G418. The 661W cells were assessed for expression of EGFP-TXNL6 under an inverted phase contrast fluorescence microscope (Zeiss, Axiovert 25). As a negative control, an EGFP 661W stable cell line was selected in parallel.

Western Blot Analysis

HeLa and 661W cell lysates were prepared and immunoblot analysis was performed as described previously [26], with modifications. Mouse monoclonal antibody to FLAG (M2) and rabbit antibody to actin were obtained from Sigma. Polyclonal antibodies to NF-κB p50, p65 and IκBα were from eBiosciences (CA, USA). Rabbit polyclonal antibodies against β-tubulin and histone H1 were purchased from Santa Cruz (CA, USA).

Light treatment

To test the effects of daily-used light on cone cells, we employed a cool fluorescent lamp of 220 Volts and 27 Watts for our experiment. The intensity of the light was measured with a multi-range photometer (model S511, East Kilbride Instruments Ltd., Scotland, UK). 661W cells were exposed to 4.5 mW/cm^2 of light for the indicated times. The experiment was conducted within a biohazard cabinet located in a hepafiltered class 1000 air-conditioned clean-room with a constant ambient temperature maintained at 23 °C. To eliminate any bias such as light or temperature variations, dark control cells and light-stressed 661W cells were all prepared and treated in the same way.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of HeLa cells were prepared with the NE-PER nuclear extraction reagent (Pierce, IL, USA) according to the instruction manual. The mammalian consensus κB probe (5′-AGTTGAGGGGACTTTCC-CAGGC-3′) was synthesized and labeled with biotin using a Biotin 3′ End DNA Labeling Kit (Pierce). Twenty fmol of biotin end-labeled DNA was incubated with 2 μl of nuclear extract and 50 ng/μl Poly (dI•dC) for 30 min at room temperature. The reactions were electrophoresed at 100 V in a 4% polyacrylamide gel in Tris-Borate-EDTA for 1 h. The

resolved bands were transferred to the Hybond-N^+ nylon membrane (GE Healthcare). The biotin-labeled DNA was detected with LightShift chemiluminescent electrophoretic mobility shift assay (Pierce).

TUNEL Assay for Apoptosis

Apoptosis study was performed using In Situ Cell Death Detection Kit, TMR red (Roche). EGFP and EGFP-TXNL6 stable 661W cell lines were first plated onto glass coverslips at a density of 2.5×10^4 cells/coverslip and allowed to attach overnight. Cells were then exposed to 4.5 mW/cm^2 visible light for indicated times. The growth medium was removed and the coverslips were rinsed twice with PBS. The cells were then fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.4. The TUNEL reaction mixture was prepared immediately before use. Fifty μl TUNEL reaction mixtures were added onto the coverslip, and incubated in a humidified atmosphere for 60 min at 37 °C in the dark. Prolong® Gold (Molecular Probes) antifade reagent with DAPI was added onto the coverslip and the samples were viewed under fluorescence microscope (Olympus, BX60).

Cell Viability assay

Cell survival was measured with the CellTiter-Blue® Cell Viability Assay kit (Promega) according to the user manual. Briefly, the 661W EGFP-TXNL6 stable cells and control EGFP cells were seeded at 10^4 cells/well in 96-well plates. On the following day, the cells were subjected to light treatment with or without NF-κB inhibitors, over a time course. The NF-κB inhibitors were obtained from Calbiochem. Helenalin and JSH-23 were dissolved in dimethylsulfoxide, and PDTC was dissolved in water, all in 100× stock concentrations. After treatment, the CellTiter Blue reagent was added to each well and the fluorescence intensity was measured with a Perkin Elmer LS50B spectrofluorimeter using excitation and emission wavelengths of 560 and 590 nm, respectively. The fluorescence of the treated cells was compared to that of the untreated cells to obtain the percent cell viability.

Measurement of Reactive Oxygen Species

The generation of ROS in cells was assessed using dichlorofluorescein diacetate (DCFH-DA) from Molecular Probes (CA, USA) and further confirmed using Amplex® Red from Invitrogen. Within the cells, esterases cleave the acetate groups on DCFH-DA, thus trapping the reduced probe (DCFH) intracellularly and the generated ROS oxidizes DCFH, yielding the fluorescent product, DCF. Viable cells (10^4/well) were plated into 96-well plates (NUNC). Twelve hours after seeding, the original medium was removed and the cells in the plates were incubated with 50 μM DCFH-DA in the loading medium (DMEM with 10% FBS) at 37 °C for 30 min. After DCFH-DA was removed, the cells were washed and incubated with Krebs Ringer HEPES buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM HEPES, pH 7.4), and they were then subjected to light exposure over a time course, before the measurement of DCF fluorescence of the cells at 488/525 nm (as excitation/emission wavelengths). The H_2O_2 concentration was measured using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit according to the instruction manual.

Results

TXNL6 activates NF-κB in HeLa cell

Since the human classical 12 kDa TRX1 could regulate the NF-κB gene transactivation activity [24], it was pertinent to investigate whether this newly discovered human 24 kDa TXNL6 controls the activity of NF-κB. We manipulated cellular TXNL6 levels by means of transfection of TXNL6 expression plasmids and tested κB-dependent luciferase reporter gene expression under stress conditions. The level of luciferase expression correlates with the transactivation ability of NF-κB. HeLa cells were transfected with the TXNL6 expression plasmid containing the κB-responsive reporter, and cultured in the presence of TNF-α or PMA (phorbol 12-myristate 13-acetate). As reported previously, treatment with TNF-α resulted in a substantial increase in κB reporter activity. Interestingly, we showed that transient overexpression of wild type TXNL6 caused a further activation of TNF-α-induced κB-reporter expression (Fig. 1A). Similarly, addition of PMA caused an increase in the NF-κB activity, and the overexpression of TXNL6 in HeLa cells further augmented its activity by 2.5-fold (Fig. 1B). This suggests that indeed, TXNL6 could enhance the NF-κB transactivation activity during stress. Like the 12 kDa TRX1, the 24 kDa TXNL6 also contains two strategically located Cys residues in its potential active site (Supplementary Fig. 1). To investigate whether these two Cys residues are important in regulating NF-κB activity, we constructed the double mutant (DM-TXNL6) in which the 2 Cys residues were mutated to Ala. As shown in Fig. 1, the overexpression of DM-TXNL6 did not obviously affect the κB-reporter expression suggesting that the two Cys residues in the potential active motif of TXNL6 is essential to its NF-κB regulatory activity.

There are two possible mechanisms by which TXNL6 enhances NF-κB transactivation activity: (1) increasing NF-κB protein expression or nuclear translocation; (2) enhancing its DNA-binding activity. To understand how TXNL6 regulates NF-κB transactivation activity, we examined the protein synthesis and subcellular localization of NF-κB by Western blot analysis. With or without TXNL6 overexpression, the Western blot revealed equal expression of the NF-κB p50 protein suggesting that the TXNL6 did not affect the protein synthesis of the p50 subunit (Fig. 2A). Previously, overexpression of the 12 kDa TRX1

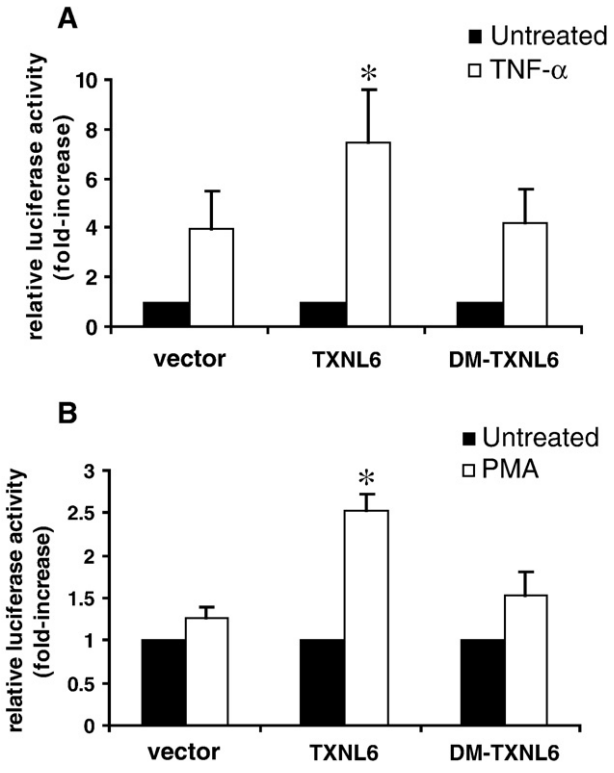

Fig. 1. TXNL6 augments NF-κB activity in HeLa cells. HeLa cells were transiently transfected with pNF-κB-Luc (experimental reporter) and pRL-CMV (control reporter), and 1.5 μg each of empty vector, TXNL6 or double mutant (DM) TXNL6. At 18 h post-transfection, the cells were treated for 6 h with *(A)* 10 ng/ml of TNF-α or *(B)* 10 ng/ml of PMA. Untreated: without TNF-α or PMA treatment. The luciferase activities were measured with DLR assay kit and were expressed as fold-increase relative to the internal control. Values represent the means ± S.E. from three independent experiments and presented as fold increases in luciferase activity over the untreated cells. * denotes statistical difference ($P<0.05$, T-Test) from the control group of HeLa cells transfected with empty vector.

has been shown to affect TNF-α-induced degradation of IκBα in HeLa cell [24,27]. Therefore, we examined the effects of TXNL6 on the IκBα degradation. Unlike TRX1, the transfection of cells with TXNL6 has no obvious effect on the TNF-α–induced degradation of IκBα (Fig. 2A). Then, we investigated whether the enhanced NF-κB activity is attributable to its nuclear translocation. As shown in Fig. 2B, the nuclear localization of NF-κB p50 and p65 was not significantly affected by the overexpression of TXNL6.

To examine whether the overexpression of TXNL6 would regulate the NF-κB DNA-binding activity, we performed EMSA. From Fig. 2C, it could be observed that without stress, the DNA-binding activity of NF-κB is minimal, and the transfectants expressing TXNL6 or DM-TXNL6 do not show a significant change. However, with stress induced by TNF-α, the overexpression of wild type TXNL6 markedly enhanced NF-κB DNA-binding activity. In contrast, the DM-TXNL6 has no significant effect on the NF-κB DNA-binding. This result is consistent with the κB-reporter assay in which the DM-TXNL6 has no obvious effect on the TNF-α-induced NF-κB activity (Fig. 1), further suggesting that the Cys residues within the putative active motif of this novel human TXNL6 are required to regulate the NF-κB activity. The binding of NF-κB was confirmed by supershift with anti-NF-κB p65 antibody (Fig. 2C, lane 8). Taken together, these results suggest that TXNL6 could activate NF-κB transactivation activity by enhancing its DNA-binding capability and that the Cys residues of TXNL6 are crucial for its function.

TXNL6 interacts with the p50 subunit of NF-κB

To examine whether TXNL6 directly associates with the NF-κB to regulate NF-κB DNA-binding activity, we investigated the interaction between the TXNL6 and the NF-κB proteins. It has been suggested that cross-linking reagents such as diamide, which converts free sulfhydryls to disulfides via Cys residue oxidation could be used to trap the transient physical association between TRX and NF-κB [24]. Thus, we

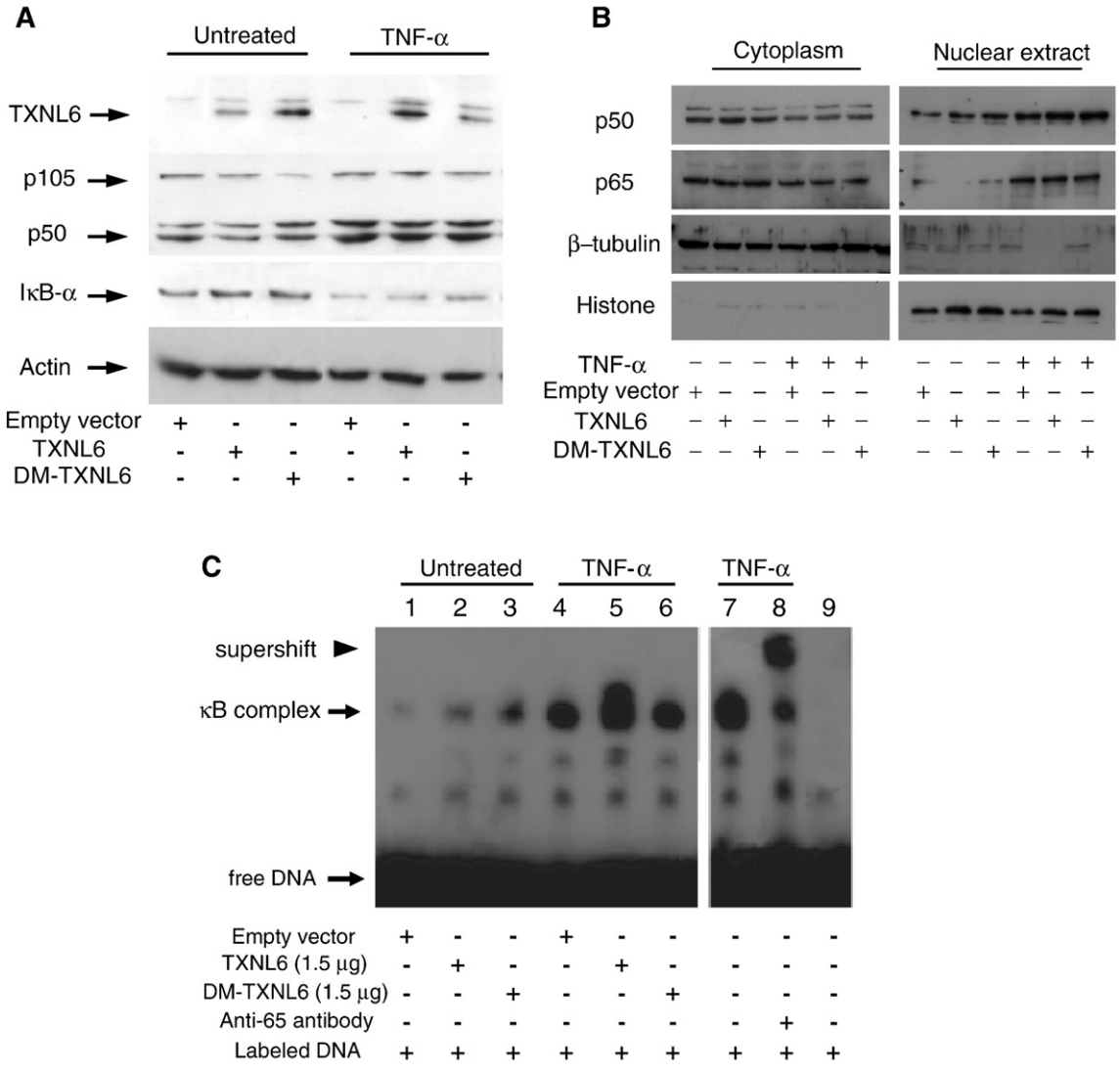

Fig. 2. TXNL6 did not affect the protein synthesis and subcellular localization of NF-κB but enhanced the DNA-binding activity of NF-κB. *(A)* Effects of TXNL6 overexpression on NF-κB p50 synthesis and IκBα degradation. HeLa cells were transfected with TXNL6 as mentioned in Fig. 1. Cell lysates were subjected to Western blot analysis as described in "Materials and Methods". The FLAG-tagged TXNL6 was detected with anti-FLAG antibody. *(B)* Effects of TXNL6 expression on the subcellular localization of NF-κB p50 and p65. HeLa cells were transfected with TXNL6 as described in Fig. 1. Nuclear and cytoplasmic extracts of HeLa cells were prepared with the NE-PER nuclear extraction reagent (Pierce). Nuclear and cytoplasmic fractions were subjected to Western blot analysis with p50 and p65 antibodies (eBiosciences). The histone H1 and β-tubulin were analyzed under the same conditions as the nuclear and cytoplasmic markers, respectively. *(C)* TXNL6 increases TNF-α-induced NF-κB DNA-binding activity. HeLa cells were transfected with indicated amounts of wild type TXNL6 and DM-TXNL6. Cells were harvested at 24-36 h after transfection, and nuclear extracts were prepared as described under "Materials and Methods". Two μl of nuclear extracts were analyzed by EMSA with the Pierce LightShift kit. The specificity of the binding of NF-κB was shown by supershift with NF-κB p65 antibody (lane 8). The p65 supershifted bands are marked by an arrowhead and the κB complex are indicated by an arrow.

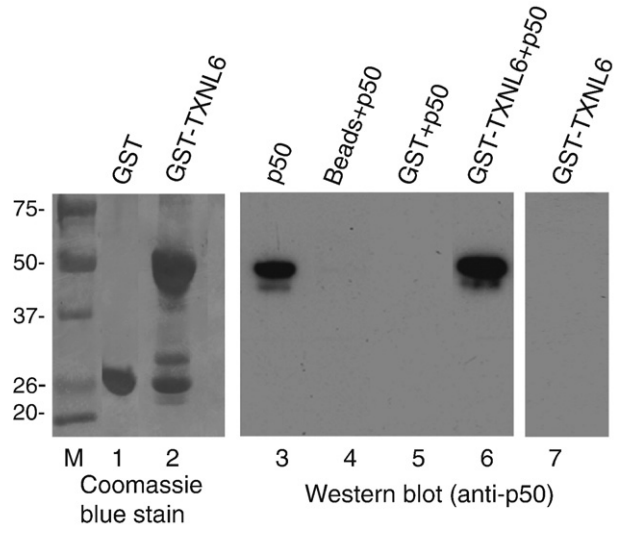

Fig. 3. *In vitro* interaction between TXNL6 and NF-κB p50 subunit. GST or GST-TXNL6 bound to glutathione Sepharose beads was incubated with human NF-κB p50 subunit. After washing, the bound protein was analyzed by Western blotting using anti-p50 antibody. Human recombinant p50 was loaded as positive control (lane 3). GST-TXNL6 interacted with the p50 protein specifically (lane 6). However, controls using Sepharose beads (lane 4) and GST (lane 5) did not bind the p50. Furthermore, the anti-p50 antibody did not cross-react with GST-TXNL6 (lane 7). After Western blot, the membrane was stained with Coomassie blue to show the GST (lane 1) and GST-TRX proteins (lane 2) used for pull-down assay. M: molecular weight marker.

incubated glutathione-S-transferase (GST) control protein and GST-TXNL6 fusion protein with NF-κB p50 in the presence of diamide. As shown in Fig. 3, indeed, the recombinant TXNL6 can interact specifically with NF-κB p50 in GST pull-down assay (lane 6). This interaction between TXNL6 and NF-κB p50 was specific as no interaction was observed between either control Sepharose beads or GST protein with NF-κB p50 (lanes 4-5). Furthermore, the p50 antibody did not cross-react with recombinant GST-TXNL6 (lane 7), suggesting that the Western blot specifically detected p50.

TXNL6 protects photoreceptor cells from photooxidative stress-induced apoptosis

Next, we investigated the role of TXNL6 in regulating the apoptosis of 661 W photoreceptor cells. The 661W cell line was selected as it is a homogenous cone photoreceptor cell culture [23]. It is known that RdCVF, the TXNL6 homologue in mice, is essential for cone viability [21]. To examine whether human TXNL6 could protect the 661W photoreceptor cells from light-induced apoptosis, we generated 661W stable cell lines expressing the EGFP-TXNL6 and EGFP (control) and maintained them under continuous selection pressure (Supplementary Figs. 2A & B). To investigate whether TXNL6 protects photoreceptor cells, the control EGFP 661W and EGFP-TXNL6 661W cell lines were subjected to various periods of light treatment. As shown in Fig. 4, without light exposure, the cells were generally healthy with no sign of apoptosis. Under light stress, apoptosis ensued and steadily increased with the duration of light exposure. Interestingly, after exposure to 4.5 mW/cm^2 of light for 40 min, apoptosis was substantially lower in TXNL6-stable cells compared to the control 661W cells. The morphological differences between the TXNL6 stable cells and control cells were also drastic. After 1 h light treatment, the control 661W cells had significantly shrunken and detached, with reduced filopodia extensions between them, suggesting apoptosis (data not shown). However in the TXNL6 stable cells, the extent of apoptosis is less severe.

We also sought to determine the effect of photooxidative stress on the metabolic activity of 661W photoreceptor cells and potential protection afforded by TXNL6. To measure viability, TXNL6 and control 661W cells that had been exposed to light for up to 1 h were evaluated for their ability to catalyze the reduction of resazurin to resorufin, which is proportional to the number of viable cells [28]. Similar to the TUNEL assay, the control EGFP 661W showed a striking decrease in survival after light treatment, while EGFP-TXNL6 stable 661W cells were effectively protected (Fig. 5A). Next, we tested if TXNL6 could

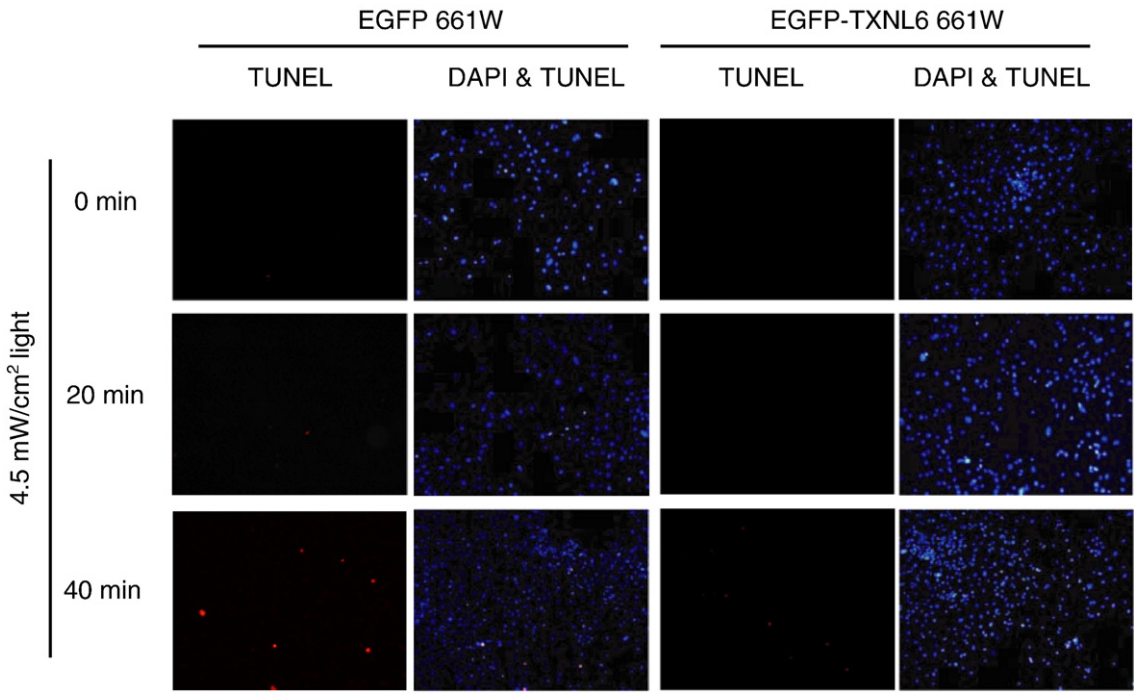

Fig. 4. TXNL6 protects 661W cells from apoptosis. TXNL6 protects 661W photoreceptor cells from light-induced apoptosis. EGFP and EGFP-TXNL6 stable 661W cells were exposed to 4.5 mW/cm^2 visible light for the indicated times, fixed with 4% paraformaldehyde, and processed for TUNEL labeling. There was a time-dependent increase in the number of cells labeled with fluoresceinated dUTP (red spots), suggestive of apoptosis with increasing duration of light exposure. Overexpression of TXNL6 protected the cells from apoptosis during light-stress.

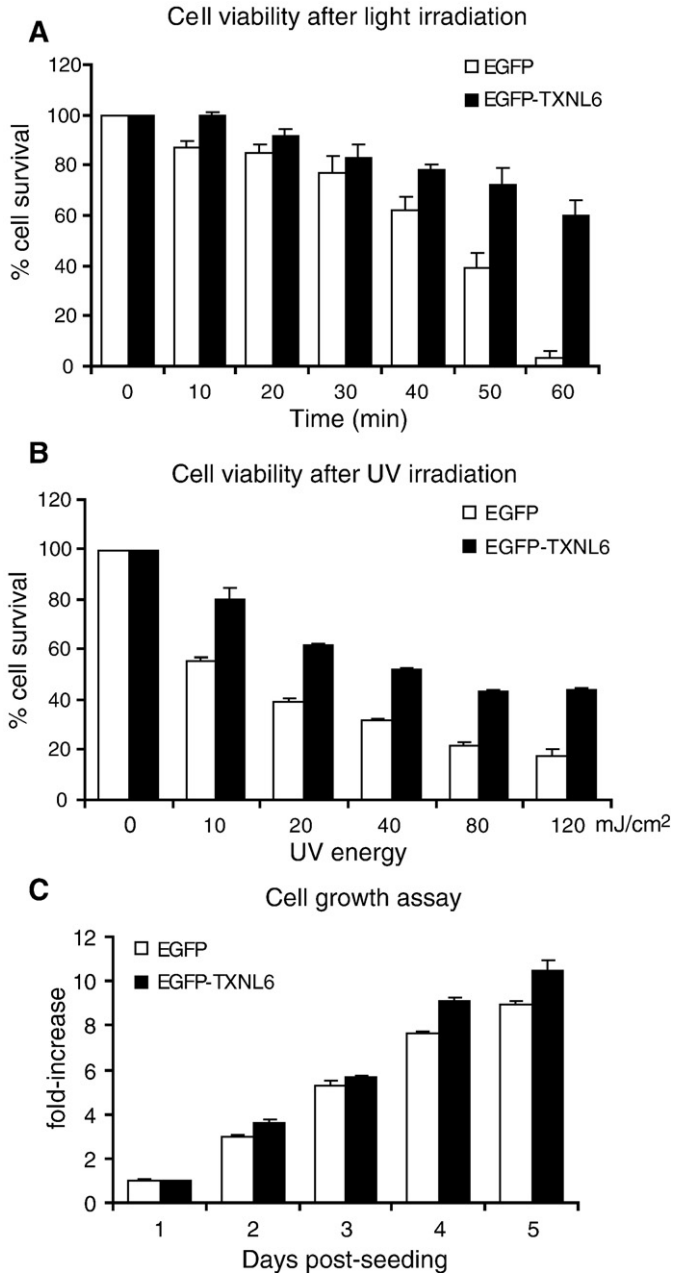

Fig. 5. Effect of TXNL6 overexpression on proliferation and viability of photoreceptor cells under stress. Control and TXNL6-overexpressing 661W cells were evaluated for viability changes induced by *(A)* light exposure and *(B)* UV irradiation by measuring the reduction of resazurin to resorufin. The percentage of cellular viability remaining in control and TXNL6-expressing cell lines was recorded. Results are representative of at least five independent experiments each carried out in triplicates and the data are presented as the means ± S.E. *(C)* Growth characteristics of 661W cells stably expressing the TXNL6-EGFP and EGFP control. Equal numbers of cells were seeded in triplicates in 96 well plates and counted over 5 days. The data showed no significant difference between the doubling times of control and TXNL6-expressing 661W cell lines. Results are representative of three independent experiments each carried out in triplicates each and data are presented as the means ± S.E.

protect UV-induced death of 661W cells. Indeed, as in the light exposure experiment, TXNL6 overexpression protected 661W photoreceptor cells from UV-induced cell death at 10 mJ/cm^2 to 120 mJ/cm^2 (Fig. 5B).

To examine whether the overexpression of TXNL6 will affect the growth rate of 661W cells, differences in growth characteristics between EGFP-661W and EGFP- TXNL6 661W stable cells were compared. There was no difference between the growth characteristics of 661W cell expressing EGFP, and 661W cells expressing EGFP and TXNL6 (Fig. 5C). Both cell types doubled between every 24-30 h. Although the EGFP-TXNL6 stable cells seemed to grow slightly more rapidly than the EGFP cells, these data indicate that the overexpression of TXNL6 did not obviously alter the inherent doubling time of the wild-type 661W photoreceptor cell.

The protective effect of TXNL6 on 661W cells was not due to ROS scavenging

To address the mechanism by which TXNL6 might protect cells from apoptosis, we examined whether TXNL6 could function as an ROS scavenger, since oxidative stress has been established to be a crucial mediator of photoreceptor cell death [29,30]. Two different dyes: dichlorofluorescein diacetate (DCFH-DA) and Amplex® Red were used to detect the generation of ROS. As shown in Supplementary Fig. 3, an increase in ROS level was observed in 661W cells upon light and UV treatment in a dose-dependent manner. However, there is no significant difference between the ROS level of TXNL6- and control-661W cells suggesting that the protective effect of TXNL6 on 661W cells is not due to the ROS scavenging (Supplementary Fig. 3). This is in contrast to the function of 12 kDa TRX1, which has been reported to scavenge ROS by itself or in association with other proteins [33].

TXNL6 rescues NF-κB activity in photoreceptor cells during light-induced stress

In our study, we found that TXNL6 could enhance NF-κB transactivation in HeLa cells. Therefore, we examined whether TXNL6 could regulate NF-κB activity and rescue the stress-induced apoptosis of 661W photoreceptor cells. Fig. 6A shows that exposure to 4.5 mW/cm^2 of visible light for 1 h caused a 10-fold decline in NF-κB activity in the cells. This is consistent with a previous report that exposure of photoreceptor cells to visible light down-regulates NF-κB activity [31]. Interestingly, light treatment of the TXNL6-expressing 661W cells only caused 5-fold decrease in NF-κB activity (Fig. 6A) suggesting that TXNL6 exerts a crucial role in a significant recovery of NF-κB activity when there is a change in the intracellular state of oxidation-reduction; similar to what was observed in the HeLa cells (Fig. 1).

In order to examine how TXNL6 regulates the NF-κB activity during photooxidative stress, we examined the levels of the NF-κB p50 and p65 proteins. Fig. 6B shows that exposure of the 661W cells to light of intensity 4.5 mW/cm^2 for 1 h caused a marked decline in the levels of p50 and p65 proteins. Interestingly, the decrease in the levels of p50 and p65 proteins was rapidly restored by the overexpression of TXNL6. Hence, we propose that light-induced oxidative stress targets the NF-κB pathway in the photoreceptor cells, resulting in the degradation of the NF-κB proteins, but, the introduction of TXNL6 prevented this degradation. However, at this juncture, the mechanism underlying the TXNL6-mediated protection of NF-κB proteins remains largely unknown.

Inhibition of NF-κB induces apoptosis of 661W photoreceptor cell

To further investigate the role of NF-κB in photoreceptor cell survival, 661W cells were treated with three chemical inhibitors of NF-κB, pyrrolidine dithiocarbamate (PDTC), helenalin and NF-κB Activation Inhibitor II (JSH-23). PDTC is an antioxidant that blocks NF-κB activity by suppressing the release of IκBα from NF-κB [32]. Helenalin is a specific NF-κB inhibitor which selectively alkylates the p65 subunit of NF-κB and inhibits its DNA-binding activity [33] and JSH-23 selectively blocks nuclear translocation of NF-κB p65 [34]. Here, we showed that after 5 h treatment, all three compounds dose-dependently killed 661W photoreceptor cells, with or without TXNL6 overexpression (Fig. 7), further supporting the notion that constitutive NF-κB activity is required for the survival of 661W photoreceptor cells. When visualized by phase contrast microscopy after treatment with

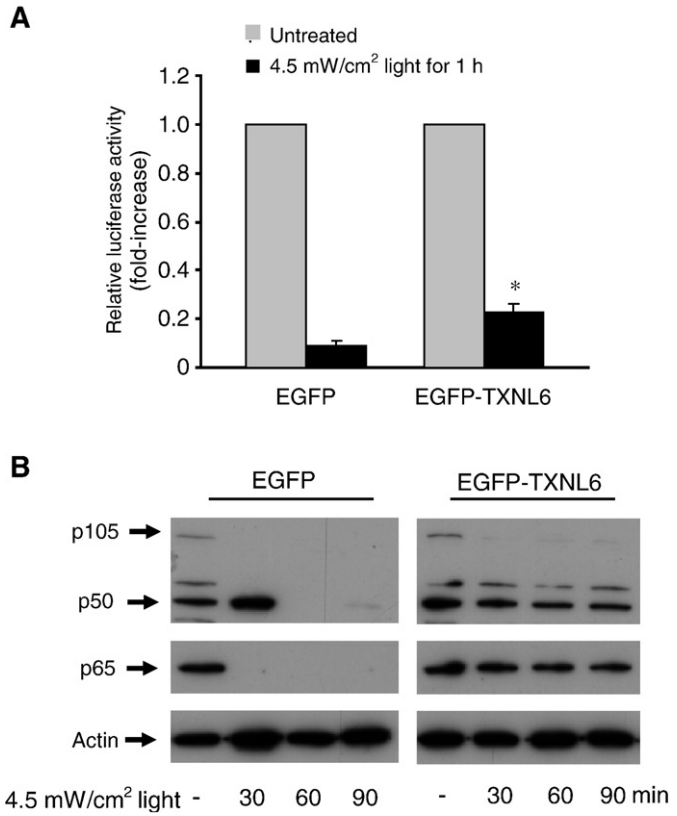

Fig. 6. TXNL6 protects NF-κB activity in 661W cells. *(A)* Overexpression of TXNL6 rescues light-induced decrease of NF-κB reporter activity. TXNL6-expressing and control 661W stable cells were transfected with pNF-κB-Luc and pRL-CMV (control) reporters and treated with 4.5 mW/cm^2 light for the indicated duration. The luciferase activities were measured with DLR kit and were expressed as percentages relative to the respective controls. Light treatment caused significant down-regulation of NF-κB transactivation activity. The overexpression of TXNL6 partially rescued the light-induced down-regulation of NF-κB activity. Values represent the means ± S.E. from three independent experiments. *(B)* TXNL6 overexpression prevents the degradation of NF-κB proteins. The EGFP or EGFP-TXNL6 stable cell lines were exposed to light for 15, 30 and 60 minutes and the levels of NF-κB subunit were studied by immunoblot analysis. There was a decrease in the levels of p50 and p65 subunits of NF-κB upon light exposure and the degradation of these NF-κB subunits was markedly rescued by the over-expression of TXNL6. Actin was used as a control for equal protein loading.

PDTC or Helenalin, 661W cells demonstrated an apoptotic appearance [14] including plasma membrane blebbing, nuclear condensation and detachment from the culture flask (Supplementary Fig. 4).

The TXNL6-induced NF-κB activity is essential for its neuroprotection against photooxidative stress-induced apoptosis

Since NF-κB activity is essential for the survival of 661W cells (Fig. 7), and TXNL6 can regulate NF-κB activity (Figs. 1 & 6) and protect light-induced apoptosis during light treatment (Fig. 5A), it was of interest to determine whether NF-κB activity is required for the protective effects of TXNL6 on photooxidative stress-induced apoptosis of 661W cell. We therefore tested the effect of NF-κB inhibitors on 661W cells with or without TXNL6, after light exposure. Our hypothesis is that, if TXNL6 protects retinal cell via the upregulation of NF-κB activity, the addition of a sub-lethal amount of NF-κB inhibitor (which only inhibits NF-κB but is not toxic to the cell) will abolish the protective effect of TXNL6. Furthermore, to show the effect of (i) the light treatment and (ii) the NF-κB inhibitors on the cell death of 661W cells with or without TXNL6, we had tested each of these controls (Fig. 5A and in Fig. 7, respectively). As shown in Fig. 8, the neuroprotective effect of TXNL6 at various periods of light exposure was markedly blocked by the addition of either helenalin (Fig. 8B) or PDTC (Fig. 8C). At several time points, the cell viability of TXNL6-expressing cells was even lower than the control cells. With the addition of NF-κB inhibitors, both EGFP and EGFP-TXNL6 stable 661W cell lines underwent apoptosis to the same extent. Compared with Fig. 8A, it is clear that NF-κB inhibitor abolished the neuroprotective effect of TXNL6. To further demonstrate that the NF-κB activity is essential, we tested another NF-κB-specific inhibitor, NF-κB Activation Inhibitor II (JSH-23), which is known to selectively block nuclear translocation of NF-κB p65 [34]. As shown in Fig. 8D, the protective effects of TXNL6 on photooxidative stress-induced apoptosis of 661W cell was abolished with the addition of JSH-23. These results strongly

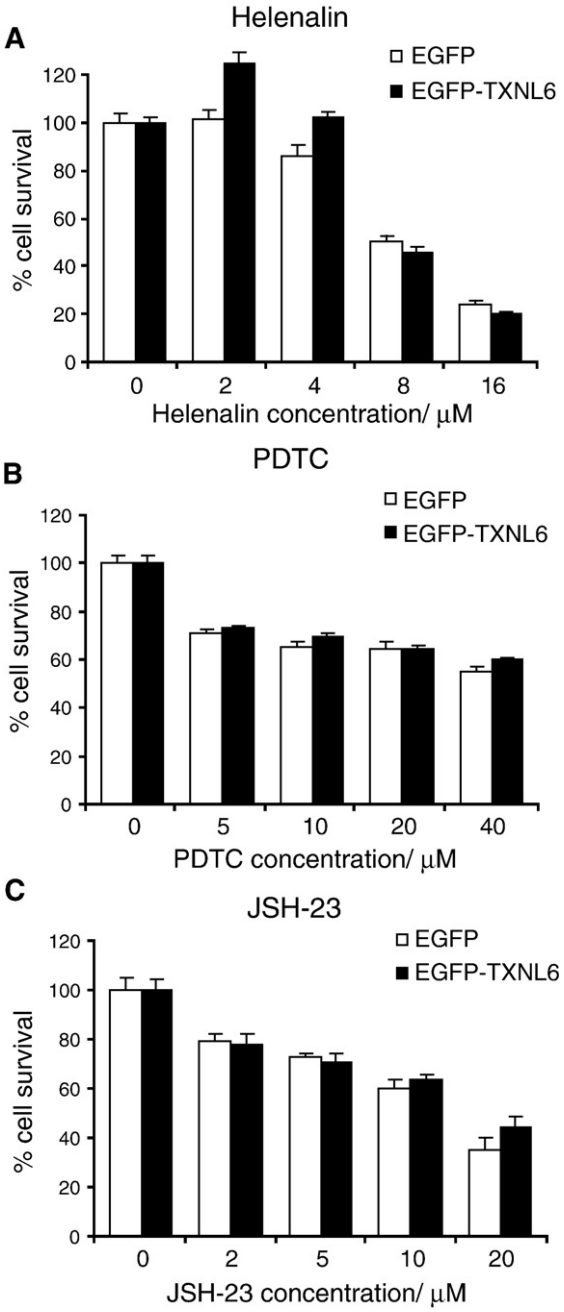

Fig. 7. NF-κB inhibitors, helenalin, PDTC and NF-κB Activation inhibitor II (JSH-23), kill 661W cells with or without TXNL6 overexpression. Cell viability was determined for 661W cells following treatment for 12 h with the indicated concentrations of *(A)* helenalin, *(B)* PDTC and *(C)* JSH-23. The 661W cells underwent apoptosis dose-dependently of helenalin, PDTC and JSH-23. The results are shown as a percentage of control cell (arbitrarily set at 100%), representating three independent experiments carried out in triplicate each and the data are presented as the means ± S.E.

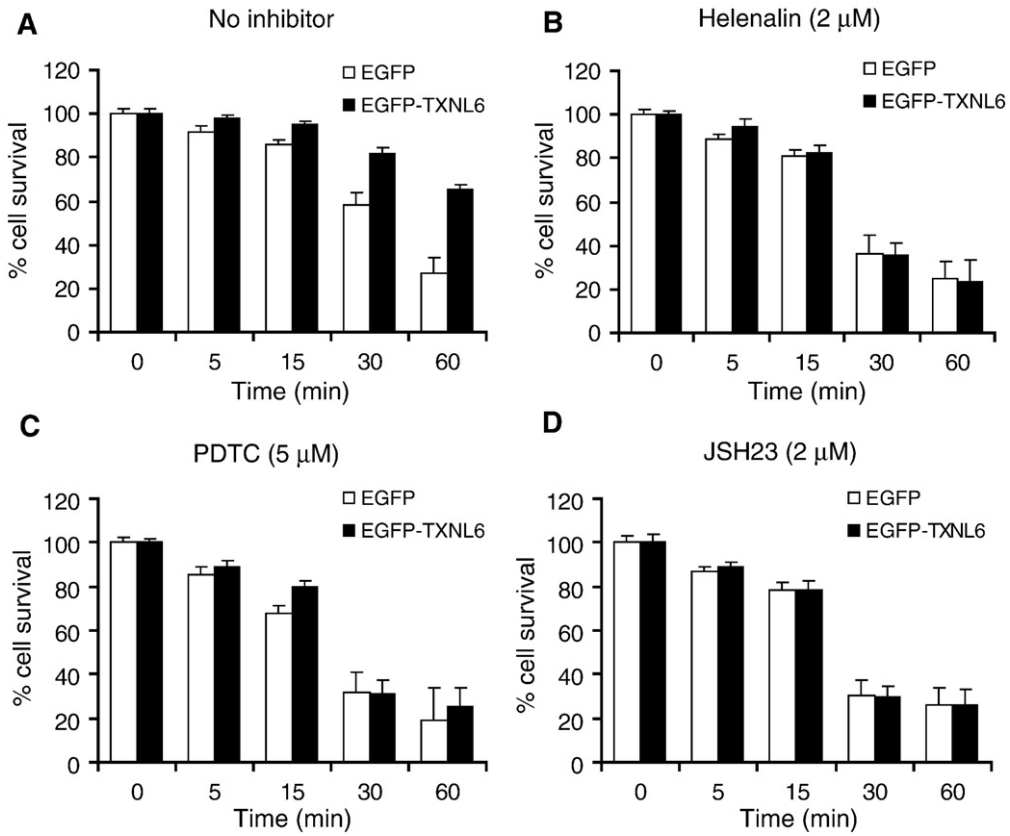

Fig. 8. NF-κB inhibitors abolish the protective effect of TXNL6 on light-induced apoptosis of 661W cells. Cell death was assessed by CellTiter Blue assay with or without NF-κB inhibitors after exposure to light at 4.5 mW/cm^2 for the indicated times. With the addition of NF-κB inhibitors: *(B)* helenalin, *(C)* PDTC and *(D)* JSH-23, the protective effect of TXNL6 on 661W cells *(A)* was completely abolished and light-exposure induced apoptosis of both cell lines occurred at the same rate. The results are shown as percentage of cells without light treatment (arbitrarily set at 100%). Results are representative of three independent experiments carried out in triplicates each and data are presented as the means±S.E.

suggest that TXNL6-induced NF-κB activity is essential for its neuroprotective effects against photooxidative stress-induced apoptosis of 661W photoreceptor cell.

Discussion

We have demonstrated the possible mechanism by which TXNL6 protects 661W photoreceptor cells against photooxidation-induced apoptosis. The protective effect of TXNL6 is not attributed to its ROS-scavenging capability but, it operates by enhancing NF-κB transactivation in the 661W cell. We also show that the constitutive activity of the NF-κB is crucial for the survival of this photoreceptor cell and importantly, the protective effects of TXNL6 against apoptosis were significantly blocked by the addition of NF-κB-specific inhibitors, demonstrating that TXNL6-induced NF-κB activity is essential for its neuroprotective effects against light-induced apoptosis of 661W photoreceptor cell.

In the cell, the NF-κB dimers are held in an inactive cytoplasmic complex with a family of inhibitory proteins, the IκBs. As an immediate response to foreign stimuli such as lipopolysaccharides, cytokines and oxidative stress, IκBs are phosphorylated and degraded by the proteasome resulting in the release of the active NF-κB which initiates the transcription of various κB-responsive genes [9]. However in the 661W photoreceptor cell, the NF-κB protein does not seem to follow such a consensus pathway of its activation by stressors such as visible and UV light. Our results evidently showed that after light treatment, the NF-κB protein was rapidly degraded, resulting in the significant downregulation of the NF-κB transactivation ability (Fig. 6). The light-induced cell death appears to result from the degradation of NF-κB, because the NF-κB-specific inhibitor alone is sufficient to cause apoptosis of the 661W cell (Fig. 7). All of these observations underscore the importance of NF-κB against photooxidative stress-induced apoptosis in 661W cells.

Our studies showed that the 24 kDa TXNL6 could positively regulate the TNF-α-induced NF-κB activation in HeLa cells. This control of NF-κB activity could be due to the redox regulation by TXNL6, as observed from the significant loss of the augmentation of NF-κB activity when the cells were transfected with the Cys-double mutant, DM-TXNL6 (Fig. 1). This suggests that the two strategically located Cys residues at the active site of TXNL6 could be crucial for its control and regulation of the NF-κB activity. With the overexpression of TXNL6, the formation of κB-DNA complex was more intense, in contrast to that of the mutant TXNL6 (Fig. 2C). Therefore, it is reasonable to postulate that the TXNL6 reduces the Cys residue in the DNA-binding motif of NF-κB to enhance its binding to the κB promoter site.

Since overexpression of TXNL6 did not appear to affect the accumulation of ROS (Supplementary Fig. 3) in the photoreceptor cells, we postulate that TXNL6 prevents photooxidative damage via mechanisms other than ROS scavenging. This is in contrast to the universally expressed human TRX1, which prevents photoreceptor cell damage by scavenging singlet oxygen and hydroxyl radicals [20]. We also found that the recombinant full length human TXNL6 did not have detectable oxidoreductase activity in insulin reduction assay and TRX reductase assay (data not shown). This lack of oxidoreductase activity is probably due to the marked change in the active site motif of TXNL6 in which the most conserved Trp residue that juxtaposes the first Cys has been substituted to Ala (Supplementary Fig. 1) [22]. This subtle difference in the amino acid residue in the catalytic site might confer diverse enzymatic activity and substrate specificity.

The characteristic of TXNL6 expression in 661W cell is interesting. We have shown that the TXNL6 mRNA was actively transcribed in the EGFP-TXNL6-stable cells (Supplementary Fig. 2C) but not in the EGFP-stable cells. However, Western blot analysis did not detect any TXNL6 protein in the actively transcribing EGFP-TXNL6-stable cells (Supplementary Fig. 2D). This result was surprising, since HeLa and HEK293 cells, which were transiently transfected with the same plasmid yielded readily detectable TXNL6 mRNA (data not show) as well as TXNL6 protein (Supplementary Fig. 2D), demonstrating both the transcriptional and translational competency of the TXNL6 plasmid. As suggested by our RT-PCR experiments with mRNA isolated from the EGFP-TXNL6-stable 661W cells, the TXNL6 mRNA might have undergone possible cleavages (data not shown), hence explaining the lack of TXNL6 protein in the 661W cells. Moreover, previous characterization of the RdCVF protein has shown that this protein might exist in two forms, and it was proposed that the truncated version slowed down cone cell degeneration [21]. This phenomenon might be applicable to the function of the TXNL6 protein in the 661W cells. It is possible that the expressed TXNL6 protein in the HeLa and HEK293 cells has remained intact and hence, detectable, since the TXNL6 is functionally irrelevant in these unnatural host cells. On the other hand, in the 661W photoreceptor cone cells, the expressed TXNL6 protein is directly in its milieu of physiological photoprotective action, and its existence might be tightly regulated. Therefore, although expressed in the 661W cells, this potential signaling protein could have undergone rapid degradation, hence escaping our detection methods. Nevertheless, the presence of TXNL6 mRNA in the EGFP-TXNL6-stable 661W cells proves it to be transcriptionally competent, and the visible expression of GFP supports the notion that the human TXNL6 protein was expressed in these 661W cells. Nevertheless further confirmation, possibly using anti-TXNL6 antibody, is necessary.

In summary, our study demonstrates that TXNL6 prevents the degradation of NF-κB proteins and significantly rescues the light- and UV-induced decrease in NF-κB transactivation activity. Overexpression of the human TXNL6 rescues the light- and UV-induced apoptosis of the photoreceptor cells, and the NF-κB activity is required for the neuroprotective effect of TXNL6. Our identification of this novel human TXNL6, and the demonstration of its protective mechanism offer new treatment possibilities for photoreceptor cell degradation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2008.04.028.

References

[1] Donovan, M.; Cotter, T. G. Caspase-independent photoreceptor apoptosis in vivo and differential expression of apoptotic protease activating factor-1 and caspase-3 during retinal development. *Cell Death Differ.* **9:**1220–1231; 2002.
[2] Kanan, Y.; Moiseyev, G.; Agarwal, N.; Ma, J. X.; Al-Ubaidi, M. R. Light induces programmed cell death by activating multiple independent proteases in a cone photoreceptor cell line. *Invest. Ophthalmol. Vis. Sci.* **48:**40–51; 2007.
[3] Chang, G. Q.; Hao, Y.; Wong, F. Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. *Neuron* **11:**595–605; 1993.
[4] Hartong, D. T.; Berson, E. L.; Dryja, T. P. Retinitis pigmentosa. *Lancet.* **368:**1795–1809; 2006.
[5] Gomez-Vicente, V.; Donovan, M.; Cotter, T. G. Multiple death pathways in retina-derived 661W cells following growth factor deprivation: crosstalk between caspases and calpains. *Cell Death Differ.* **12:**796–804; 2005.
[6] Carmody, R. J.; Cotter, T. G. Oxidative stress induces caspase-independent retinal apoptosis in vitro. *Cell Death Differ.* **7:**282–291; 2000.
[7] Sanvicens, N.; Gomez-Vicente, V.; Masip, I.; Messeguer, A.; Cotter, T. G. Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6. *J. Biol. Chem.* **279:**39268–39278; 2004.
[8] Bonizzi, G.; Karin, M. The two NF-kB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* **25:**280–288; 2004.
[9] Hayden, M. S.; Ghosh, S. Signaling to NF-kB. *Genes Dev.* **18:**2195–2224; 2004.
[10] Dutta, J.; Fan, Y.; Gupta, N.; Fan, G.; Gelinas, C. Current insights into the regulation of programmed cell death by NF-kappaB. *Oncogene* **25:**6800–6816; 2006.
[11] Gloire, G.; Legrand-Poels, S.; Piette, J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem. Pharmacol.* **72:**1493–1505; 2006.
[12] Burke, M. A.; Bothwell, M. p75 neurotrophin receptor mediates neurotrophin activation of NF-kappa B and induction of iNOS expression in P19 neurons. *J. Neurobiol.* **55:**191–203; 2003.
[13] Maggirwar, S. B.; Sarmiere, P. D.; Dewhurst, S.; Freeman, R. S. Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. *J. Neurosci.* **18:**10356–10365; 1998.
[14] Bian, X., et al. Constitutively active NFkappa B is required for the survival of S-type neuroblastoma. *J. Biol. Chem.* **277:**42144–42150; 2002.
[15] Crawford, M. J., et al. Bcl-2 overexpression protects photooxidative stress-induced apoptosis of photoreceptor cells via NF-kappaB preservation. *Biochem. Biophys. Res. Commun.* **281:**1304–1312; 2001.
[16] Powis, G.; Montfort, W. R. Properties and biological activities of thioredoxins. *Annu. Rev. Biophys. Biomol. Struct.* **30:**421–455; 2001.
[17] Holmgren, A. Thioredoxin. *Annu. Rev. Biochem.* **54:**237–271; 1985.
[18] Arner, E. S.; Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267:**6102–6109; 2000.
[19] Hirota, K.; Nakamura, H.; Masutani, H.; Yodoi, J. Thioredoxin superfamily and thioredoxin-inducing agents. *Ann. N. Y. Acad. Sci.* **957:**189–199; 2002.
[20] Tanito, M., et al. Cytoprotective effects of geranylgeranylacetone against retinal photooxidative damage. *J. Neurosci.* **25:**2396–2404; 2005.
[21] Leveillard, T., et al. Identification and characterization of rod-derived cone viability factor. *Nat. Genet.* **36:**755–759; 2004.
[22] Wang, X. W.; Liou, Y. C.; Ho, B.; Ding, J. L. An evolutionarily conserved 16-kDa thioredoxin-related protein is an antioxidant which regulates the NF-kappaB signaling pathway. *Free. Radic. Biol. Med.* **42:**247–259; 2007.
[23] Tan, E., et al. Expression of cone-photoreceptor-specific antigens in a cell line derived from retinal tumors in transgenic mice. *Invest. Ophthalmol. Vis. Sci.* **45:**764–768; 2004.
[24] Hirota, K., et al. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J. Biol. Chem.* **274:**27891–27897; 1999.
[25] Sadek, C. M., et al. Characterization of human thioredoxin-like 2. A novel microtubule-binding thioredoxin expressed predominantly in the cilia of lung airway epithelium and spermatid manchette and axoneme. *J. Biol. Chem.* **278:**13133–13142; 2003.
[26] Lee, K. K., et al. Purification, molecular cloning, and characterization of TRP32, a novel thioredoxin-related mammalian protein of 32 kDa. *J. Biol. Chem.* **273:**19160–19166; 1998.
[27] Das, K. C. c-Jun NH2-terminal kinase-mediated redox-dependent degradation of IkappaB: role of thioredoxin in NF-kappaB activation. *J. Biol. Chem.* **276:**4662–4670; 2001.
[28] Ahmed, S. A.; Gogal Jr., R. M.; Walsh, J. E. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J. Immunol. Methods* **170:**211–224; 1994.
[29] Carmody, R. J.; McGowan, A. J.; Cotter, T. G. Reactive oxygen species as mediators of photoreceptor apoptosis in vitro. *Exp. Cell. Res.* **248:**520–530; 1999.
[30] Donovan, M.; Carmody, R. J.; Cotter, T. G. Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent. *J. Biol. Chem.* **276:**23000–23008; 2001.
[31] Krishnamoorthy, R. R., et al. Photo-oxidative stress down-modulates the activity of nuclear factor-kappaB via involvement of caspase-1, leading to apoptosis of photoreceptor cells. *J. Biol. Chem.* **274:**3734–3743; 1999.
[32] Schreck, R.; Meier, B.; Mannel, D. N.; Droge, W.; Baeuerle, P. A. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J. Exp. Med.* **175:**1181–1194; 1992.
[33] Lyss, G.; Knorre, A.; Schmidt, T. J.; Pahl, H. L.; Merfort, I. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kB by directly targeting p65. *J. Biol. Chem.* **273:**33508–33516; 1998.
[34] Shin, H. M., et al. Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. *FEBS. Lett.* **571:**50–54; 2004.