TRIM69 inhibits cataractogenesis by negatively regulating p53

Xianfang Rong[a,b,c,d,e,1], Jun Rao[f,1], Dan Li[a,b,c,d,e], Qinghe Jing[a,b,c,d,e], Yi Lu[a,b,c,d,e], Yinghong Ji[a,b,c,d,e,*]

[a] *Department of Ophthalmology, Eye & ENT Hospital of Fudan University, No. 83 Fenyang Road, Shanghai, 200031, China*
[b] *Eye Institute, Eye & ENT Hospital of Fudan University, No. 83 Fenyang Road, Shanghai, 200031, China*
[c] *National Health Commission (NHC) Key Laboratory of Myopia (Fudan University), No. 83 Fenyang Road, Shanghai, 200031, China*
[d] *Laboratory of Myopia, Chinese Academy of Medical Sciences, No. 83 Fenyang Road, Shanghai, 200031, China*
[e] *Key Laboratory of Visual Impairment and Restoration of Shanghai, No. 83 Fenyang Road, Shanghai, 200031, China*
[f] *Jiangxi Provincial Key Laboratory of Translational Medicine and Oncology, Jiangxi Cancer Hospital, Jiangxi Cancer Center, Nanchang, 330029, China*

ARTICLE INFO

ABSTRACT

Ultraviolet B (UVB) irradiation can induce reactive oxygen species (ROS) production and apoptosis in human lens epithelial cells (HLECs), thus leading to the formation of cataracts. We studied the role of tripartite motif 69 (TRIM69) in cataract formation. The expression of TRIM69 protein was down-regulated in both human cataract capsule tissues and HLECs treated with UVB, whereas the expression of p53 protein exhibited an opposite trend. Ectopic expression of TRIM69 in HLECs significantly suppressed UVB-induced apoptosis and ROS production, whereas knockdown of TRIM69 promoted apoptosis and ROS production. TRIM69 can interact with p53 and induce its ubiquitination. The effects of TRIM69 overexpression in UVB-induced cell apoptosis and ROS production was clearly weakened by p53 overexpression, thus suggesting a role for p53 in TRIM69 functions. Furthermore, inhibition of ROS mitigated the effects of UVB irradiation on ROS production, cell apoptosis, forkhead box protein 3a (Foxo3a) phosphorylation, and TRIM69 expression. Additionally, Foxo3a overexpression significantly enhanced TRIM69 promoter activity, whereas Foxo3a knockdown had the opposite effect. In conclusion, we provide the first demonstration that Foxo3a is a potential transcription factor for TRIM69, and TRIM69 induces p53 ubiquitination. These results suggest that the Foxo3a/TRIM69/p53 regulatory network may be involved in cataract formation.

1. Introduction

A cataract is characterized by a loss of transparency of the ocular lens and is a frequently acquired cause of visual impairment in people > 40 years of age [1]. Cataracts affect approximately 20 million people and are the main cause of blindness worldwide [2]. Ultraviolet B (UVB [295–320 nm wavelength]) irradiation is one of the most important environmental risk factors for cataract development [3–5]. UVB irradiation may attack the oxidative pathways and induce the production of reactive oxygen species (ROS), which directly causes oxidative damage to DNA and proteins in the lens [6,7]. UVB-induced cataract formation begins with damage to human lens epithelial cells (HLECs), and apoptosis of these cells is an early event during cataract development [8]. Apoptosis is tightly regulated by B-cell lymphoma-2 (Bcl-2) family proteins, in which Bcl-2 inhibits cell apoptosis, whereas Bcl-2-associated X protein (Bax) reverses the anti-apoptotic effect of Bcl-2 [9,10]. We have previously reported increased expression of Bax and decreased expression of Bcl-2 during UVB-induced HLECs apoptosis, thus indicating that both proteins play key roles in cataract formation [11].

Cumulative evidence indicates that p53 plays a central role in response to diverse types of cellular stress, such as oxidative stress, deoxyribonucleic acid (DNA) damage, and nucleotide imbalance [12]. Under certain conditions, p53 can prevent cell cycle progression, direct damage repair, and induce cell apoptosis by regulating downstream genes [13]. Maltzman and Czyzyk (1984) have found that UV radiation stabilizes p53, and subsequent studies have suggested that p53 is actively involved in UV irradiation-provoked cell responses [14–17]. A recent study has reported that p53 expression is enhanced in the anterior lens capsules of age-related cataracts, and knockdown of p53 significantly inhibits UVB-induced cell apoptosis of HLECs [18]. These findings suggest that p53 may be involved in the pathogenesis of cataracts.

TRIM69 is a member of the tripartite motif (TRIM) family proteins, which have been reported to participate in various biological processes, such as cell growth, apoptosis, differentiation, innate immune responses, and cancer development [19,20]. The TRIM family proteins contain a RING finger domain, which may contribute to E3 ubiquitin ligase activity [21]. Several members of the TRIM family proteins (TRIM24, TRIM32, TRIM39, and TRIM59) have been reported to bind and ubiquitylate p53 [22–25]. TRIM69 is evolutionarily conserved in vertebrates. Knockdown of TRIM69 activates the p53 pathway and increases apoptosis during embryogenesis in zebrafish [26]. Whether TRIM69 plays a role in apoptosis of HLECs and the pathogenesis of cataracts is unclear.

In the present study we found that TRIM69 is down-regulated in cataract lens anterior capsular specimens, and TRIM69 overexpression attenuates UVB-induced cell apoptosis and ROS production by inducing p53 ubiquitination. Additionally, forkhead box protein 3a (Foxo3a), which is inactivated by UVB exposure, is a potential transcription factor for TRIM69. Our study suggests that the Foxo3a/TRIM69/p53 regulatory network may be involved in cataract formation.

2. Materials and methods

2.1. Human tissue samples

Anterior lens capsule specimens were collected from four cataract patients without other ocular diseases at the Department of Ophthalmology at the Eye & ENT Hospital of Fudan University (Shanghai, China). Four control anterior lens capsule specimens were obtained from the Shanghai Red Cross Eye Bank. All participants gave informed consent, and the study was approved by the Research Ethics Committee of Eye & ENT Hospital of Fudan University.

2.2. Western blot assays

The specimens and HLECs were lysed with RIPA lysis buffer at 4 °C. A bicinchoninic acid assay kit (Sigma Chemical Co., St. Louis, MO) was used for protein quantification. Proteins of each sample (25 μg per sample) were subjected to 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot assays were performed with primary antibodies to the following: TRIM69 (Ab111943; Abcam, Cambridge, MA, USA); p53 (Ab26; Abcam); Bax (Ab32503; Abcam); Bcl-2 (Ab32124; Abcam); Foxo3a (Ab53287; Abcam); ubiquitin (Ab7780; Abcam); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#5174; Cell Signaling Technology, Danvers, MA, USA). GAPDH protein was used as the loading control.

2.3. Cell culture and UVB exposure

A HLEC cell line was obtained from the Cell Bank of Academia Sinica (Shanghai, China), grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum, and passaged as previously described [11]. Before UVB exposure, the cells were grown to 80% confluence and washed twice with phosphate buffered saline (PBS [pH 7.4]). Cells in PBS were irradiated for 10 min with a UVB radiometer (Photoelectric Instrument Factory, Beijing Normal University, Beijing, China) at a wavelength of 297 nm. After UV irradiation, PBS was replaced with fresh RPMI-1640 medium, and the cells were cultured for 24 h at 37 °C in 5% CO_2, and collected for experiments.

2.4. Overexpression of TRIM69, p53, and Foxo3a

To overexpress TRIM69, p53, and Foxo3a, we amplified the complete coding sequences with the following primers and cloned them into pLVX-puro (Clontech, Palo Alto, CA, USA): TRIM69 (NM_182985.4) forward, 5′- GCGAATTCATGGAGG TATCCACCAACC -3′ and reverse, 5′-CGGGATCCTTACTGTGGATGTAAGATGTGC -3'; p53 (NM_000546.5) forward, 5′- GCGAATTCATGGAGGAAGACCAGGCTCTGG -3′ and reverse, 5′- CGGGATCCCTAGGGTACAAATGACCTCAGCGT -3'; and Foxo3a (NM_001455.3) forward, 5′- GCGAATTCATGGCAGAGGCACCGGCTTC -3′ and reverse, 5′- CGGGATCCTCAGCCTGGCACCCAGCTCT -3'. Lentivirus packaging was performed as described above.

2.5. Experimental groups

To study the effects of UVB irradiation on TRIM69 expression, HLECs were exposed to different UVB intensities (0.5, 1, 2, or 4 W/m^2) for 10 min, and the expression of TRIM69 protein was detected with a western blot assay after 24 h of culture. Control cells were treated similarly but were not exposed to UVB. To investigate the role of the TRIM69 gene in UVB-induced apoptosis, HLECs were divided into the following four groups: control, cells without UVB exposure; UVB, cells subjected to 2 W/m^2 of UVB for 10 min; vector + UVB, cells transduced with vector virus for 24 h, then subjected to 2 W/m^2 of UVB for 10 min; and TRIM69 + UVB, cells transduced with TRIM69 OE virus for 24 h, then subjected to 2 W/m^2 of UVB for 10 min.

To determine the function of p53 in UVB-induced cell apoptosis, HLECs were divided into the following five groups: control; Pifithrin-α (PFTα); vector; TRIM69 Overexpression (OE); and TRIM69 OE + p53 OE. All the groups were subjected to 2 W/m^2 of UVB for 10 min. The PFTα group was treated with 10 μM PFTα (Selleck Chemicals, Houston, TX, USA) 1 h before UVB irritation. The cells in the vector, TRIM69 OE, and TRIM69 OE + p53 OE groups were transduced with vector, TRIM69 OE, and TREIM69 OE plus p53 OE 24 h before UVB irritation. The control group received only UVB exposure.

To explore the role of ROS, we divided HLECs into three groups: control, cells without UVB exposure; UVB, cells subjected to 2 W/m^2 of UVB for 10 min; and UVB + *N*-acetyl-ʟ-cysteine (NAC), cells treated with 10 mM NAC (Selleck Chemicals, Houston, TX, USA) for 1 h, then exposed to 2 W/m^2 of UVB for 10 min.

2.6. Inhibition of TRIM69 and Foxo3a expression

Short hairpin RNA (shRNA) oligonucleotides targeting TRIM69 (TRIM69#1, CCGGTCCAGGCGACTATGTTGAAACTCGAGTTTCAACAT AGTCGCCTGGTTTTTG; TRIM69 #2, CCGGTGCATGGAGAGAACCTGA AACTCGAGTTTCAGGTTCTCTCCATGC TTTTTG; TRIM69#3, CCGGTC CTGAAACTGTTCAGTAAACTCGAGTTTACTGAACAGTTTCAGGTTT TTG; and TRIM69#4, CCGGTCCTATCCAGTACATGGTATCTCGAGATA CCATGTACTGGATAGGTTTTTG) and Foxo3a (Foxo3a#1, CCGGTCGA GGAGGAGGACGATGAACTCGAGTTCATCGTCCTCCTCCTCG TTTTTG; Foxo3a#2, CCGGTGCTGCTGCAGCCTCAGCAACTCGAGTTGCTGAGGC TGCAGCAGC TTTTTG; and Foxo3a#3, CCGGTCCATGGACAATAGCAA CAACTCGAGTTGTTGCTATTGTCCATGG TTTTTG) were cloned into *Age*I- and *Eco*RI-digested pLKO.1 (Addgene, Cambridge, MA, USA). The shRNA constructs were transfected into 293T cells along with packaging plasmids (psPAX2 and pMD2.G; Addgene, Watertown, MA, USA) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. At 48–72 h post-transfection, lentiviruses

were collected to transduce HLECs.

2.7. Flow cytometry analysis of apoptosis and ROS production

After the indicated treatments, the HLECs were collected. For analysis of apoptosis, the HLECs were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Beyotime, Shanghai, China) for 15 min. For detection of ROS, the HLECs were incubated with RPMI-1640 medium containing 10 μM 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime) and dihydroethidium (DHE) at 37 °C for 20 min as previously described [27–30]. After incubation, all samples were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The cells stained positive with Annexin V and negative with PI were undergoing apoptosis.

2.8. Immunoprecipitation

Cell lysates were incubated with anti-TRIM69 (Ab111943; Abcam), anti-p53 (Ab26; Abcam), or IgG at 4 °C for 2 h, then incubated with protein G agarose beads (Roche, Indianapolis, IN, USA) at 4 °C for 1 h. Subsequently, immunoprecipitates were washed three times with Radio Immunoprecipitation Assay (RIPA) lysis buffer, collected by centrifugation, resuspended in SDS-PAGE sample buffer, and analyzed with western blot assays.

2.9. Dual luciferase reporter assays

HLECs were seeded in six-well plates and cultured overnight. The cells were transfected with pGL3-basic plasmid (Promega, Madison, WI, USA) containing TRIM69 and pRL-TK promoters (Promega) with Lipofectamine 2000, then transduced with Foxo3a OE/Vector or shNC/shFoxo3a#2. At 48 h after treatment, luciferase activity (Firefly and *Renilla*) was determined with the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol.

2.10. Statistical analysis

Experiments were independently repeated three times, and the data are presented as the mean ± Standard Deviation (SD). One-way analysis of variance was performed in SPSS 17.0 software. A $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. TRIM69 and p53 are involved in cataract formation

We first evaluated the levels of TRIM69 and p53 proteins in cataracts and normal capsule tissues by using a western blot assay. The levels of TRIM69 protein were lower in cataract lens anterior capsules than in normal controls, whereas the p53 levels were higher (Fig. 1A). We also determined the levels of TRIM69 and p53 protein in HLECs treated with UVB (0.5, 1, 2, or 4 W/m^2) for 10 min. UVB exposed cells, compared with to non-irradiated HLECs (control), showed suppressed TRIM69 expression and enhanced p53 expression in a dose-dependent manner (Fig. 1B). These findings suggested that TRIM69 and p53 levels are strongly correlated with cataract formation.

3.2. Overexpression of TRIM69 decreased cell apoptosis, p53 expression, and ROS production after UVB irradiation

UVB irradiation induces HLEC apoptosis, an early event during the development of cataracts [8,11]. To determine the role of the TRIM69 gene in UVB-induced apoptosis of HLECs, we elevated TRIM69 expression through lentivirus transduction. HLECs were transduced with
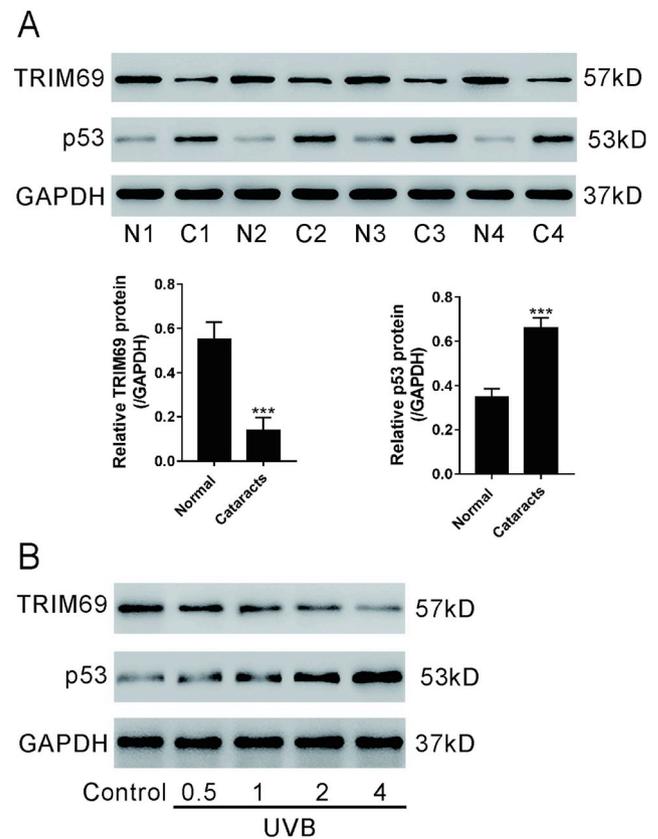
Fig. 1. TRIM69 and p53 involved in cataract formation. (A) Western blot results of the relative levels of TRIM69 and p53 protein in cataracts (C1–C4) and normal (N1–N4) capsule tissues. The band intensity was determined by Image J software (http://rsb.info.nih.gov/ij/, Bethesda, MD, USA) (lower panel). ***P < 0.001. (B) Western blot results of the relative levels of TRIM69 and p53 protein in HLECs exposed to UVB (0.5, 1, 2, or 4 W/m^2) for 10 min. HLECs without UVB irradiation served as the control.

TRIM69-overexpressing lentivirus (TRIM69 OE) or vector virus. Fig. 2A showed that the expression of TRIM69 protein in the TRIM69 OE group was higher than that in the vector group, whereas the level of TRIM69 protein in the vector group was similar to that in the control group (untreated cells).

Then, HLECs transduced with vector or TRIM69 OE were exposed to 2 W/m^2 of UVB for 10 min. Cell apoptosis was assessed with Annexin V-FITC/PI staining 24 h after UVB irradiation. UVB exposed cells, compared with control cells, showed significantly induced cell apoptosis. The percentage of apoptotic cells in the TRIM69 OE + UVB group was less than that in the vector + UVB group (Fig. 2B). p53 and apoptosis-regulated proteins (Bax and Bcl-2) were also analyzed with a western blot assay (Fig. 2C). p53 and the pro-apoptotic factor Bax were elevated by UVB exposure and suppressed by TRIM69 overexpression, whereas an opposite trend was observed in expression of the anti-apoptotic factor Bcl-2. These data suggest that elevated expression of TRIM69 attenuates HLEC apoptosis caused by UVB exposure.

UVB irradiation induces the generation of ROS, which may be associated with UVB irradiation induced-apoptosis [6,7]. ROS production was assessed with DCFH-DA and DHE staining and flow cytometry analysis. Both the fluorescence intensities were significantly increased by UVB irradiation, and were markedly decreased by TRIM69 overexpression (Fig. 2D and E). These findings indicated that UVB-induced ROS production in HLECs decreases after overexpression of TRIM69.
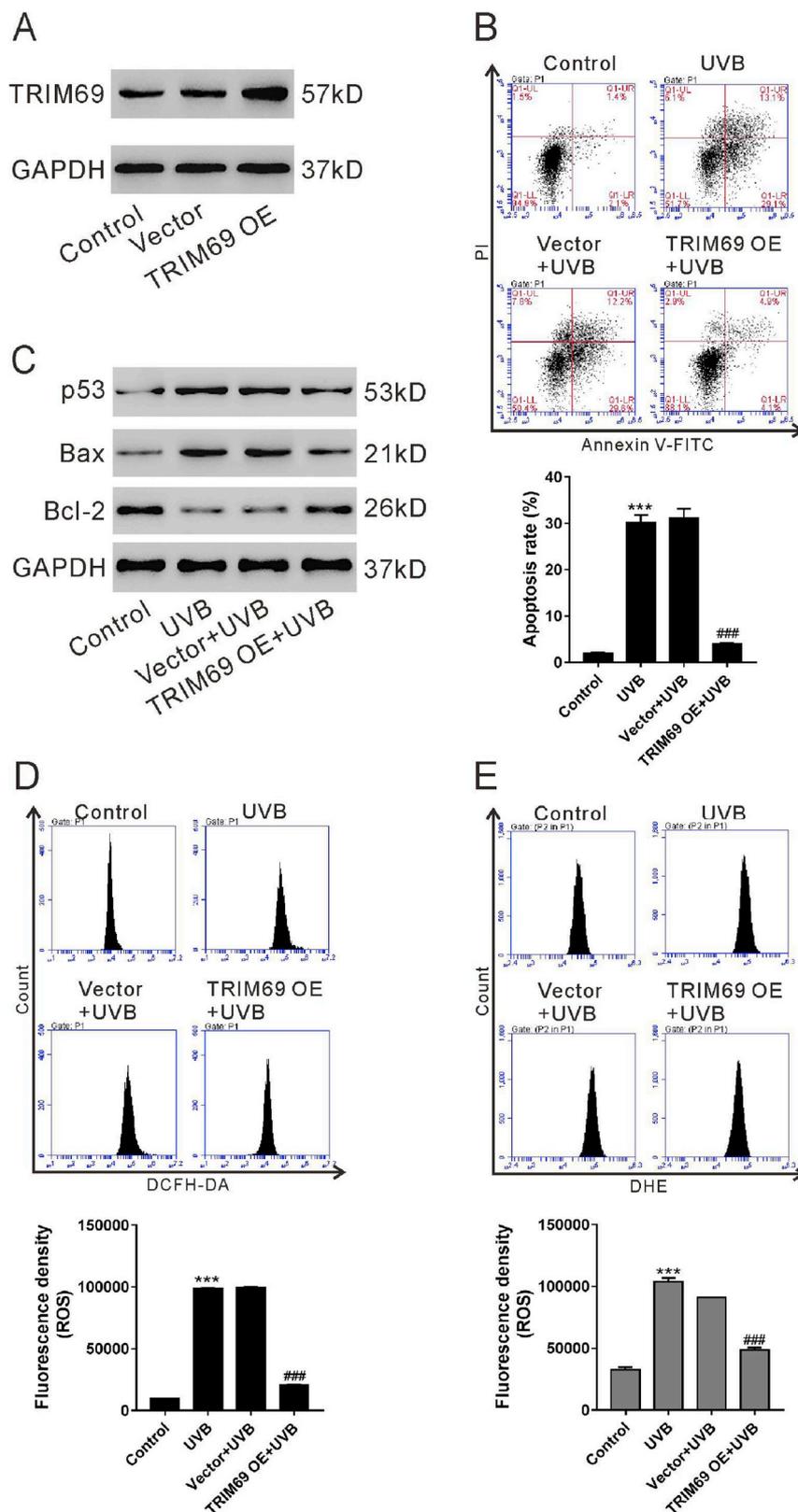
Fig. 2. Overexpression of TRIM69 decreased cell apoptosis, p53 expression, and ROS production caused by UVB irradiation. (A) HLECs were transduced with TRIM69-overexpression virus (TRIM69 OE) or control vector virus (vector). Expression of TRIM69 protein was determined with western blot assays at 48 h of culture. HLECs were transduced with TRIM69 OE or vector for 24 h, then subjected to UVB irradiation (2 W/m^2) for 10 min. (B) After 24 h of culture, Annexin V-FITC/PI staining was performed to analyze cell apoptosis. (C) Western blot assays were used to detect the expression of p53, Bax, and Bcl-2 protein. (D, E) DCFH-DA (D) and DHE (E) staining was performed to analyze ROS production. ***$P < 0.001$ vs. control; ###$P < 0.001$ vs. vector + UVB.

3.3. Inhibition of TRIM69 expression-induced apoptosis, p53 expression, and ROS production in HLECs

To further study the role of TRIM69 gene in HIEC apoptosis and ROS production under normal conditions, we knocked down TRIM69 expression through lentivirus transduction. As shown in Fig. 3A, all four

TRIM69 shRNAs suppressed expression of TRIM69 protein in HLECs. Because TRIM69#1 and TRIM69#2 had better knockdown efficiency, we selected them for further experiments. Annexin V-FITC/propidium iodide staining (Fig. 3B), western blot analysis of apoptosis-regulated proteins (Fig. 3C), and DCFH-DA (Fig. 3D) and DHE staining (Fig. 3E) indicated that TRIM69#1 and TRIM69#2 induced HLEC apoptosis and
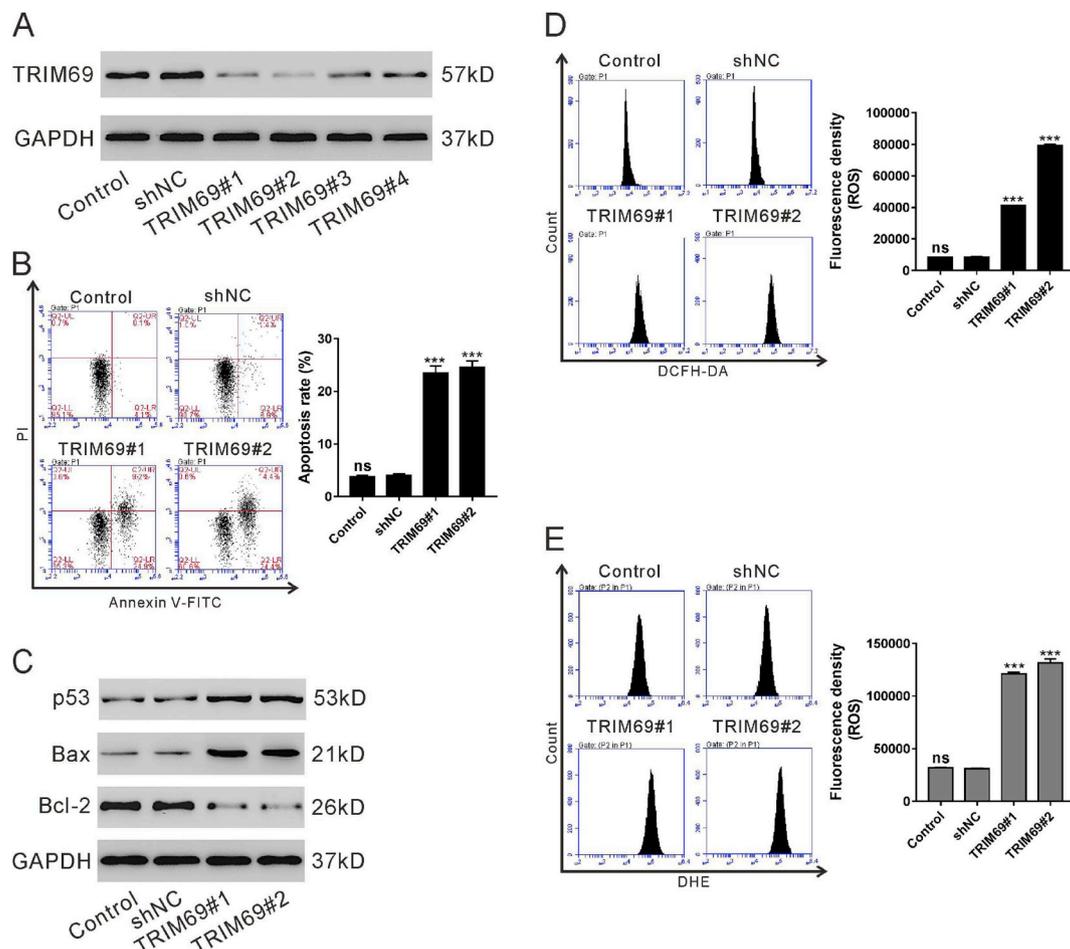
Fig. 3. Inhibition of TRIM69 expression induced apoptosis, p53 expression, and ROS production in HLECs. (A) HLECs were transduced with TRIM69 (TRIM69#1 ~ 4) or control shRNA virus (shNC). Expression of TRIM69 protein was determined with western blot assays at 48 h of culture. HLECs were transduced with TRIM69#1, TRIM69#2, or shNC for 48 h. (B) Annexin V-FITC/PI staining was performed to analyze cell apoptosis. (C) Western blot assays were used to detect the expression of p53, Bax, and Bcl-2 protein. (D, E) DCFH-DA (D) and DHE (E) staining was performed to analyze ROS production. ns: not significant; ***P < 0.001 vs. control.
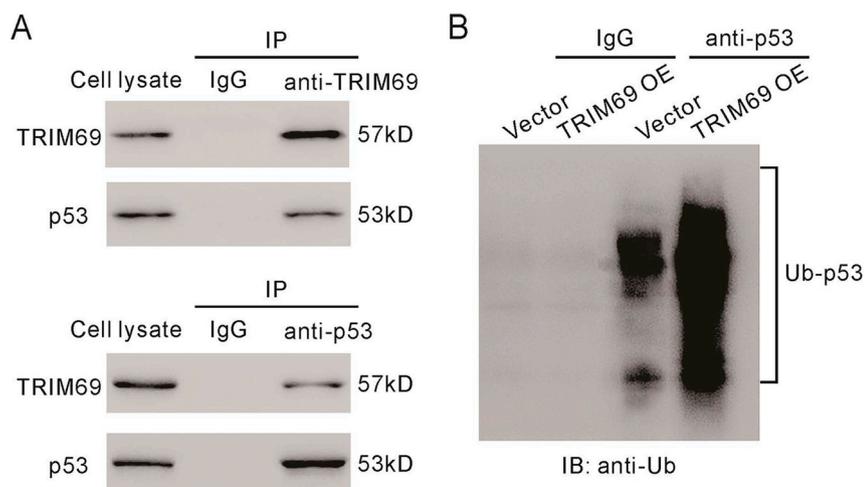
Fig. 4. TRIM69 interacted with p53 protein and induced ubiquitination. (A) HLEC lysates were immunoprecipitated (IP) with anti-TRIM69 or anti-p53, and western blot analysis was carried out as indicated. IgG was used as a negative control for IP. (B) HLECs were transduced with TRIM69 OE or vector for 48 h. HLEC lysates were subjected to IP with anti-p53, and western blot analysis was performed with anti-Ub. Half-open square bracket indicates the bands of ubiquitinated p53 (Ub-p53).

ROS production. p53 expression was also induced by TRIM69#1 and TRIM69#2.

3.4. TRIM69 interacted with p53 protein and induced ubiquitination

The TRIM family proteins have been reported to possess E3 ligase activity for p53 [22–25]. The above results showed that TRIM69

overexpression decreased the levels of p53 protein in UVB-treated HLECs, whereas TRIM69 knockdown increased p53 protein expression. These findings prompted the question of whether TRIM69 might be an E3 ligase for p53 in HLECs. Immunoprecipitation (IP) experiments with anti-TRIM69 and anti-p53 (Fig. 4A) showed that TRIM69 and p53 forms a complex in HLECs. Additionally, TRIM69 overexpression clearly enhanced the ubiquitination of p53 (Fig. 4B), thus providing a preliminary
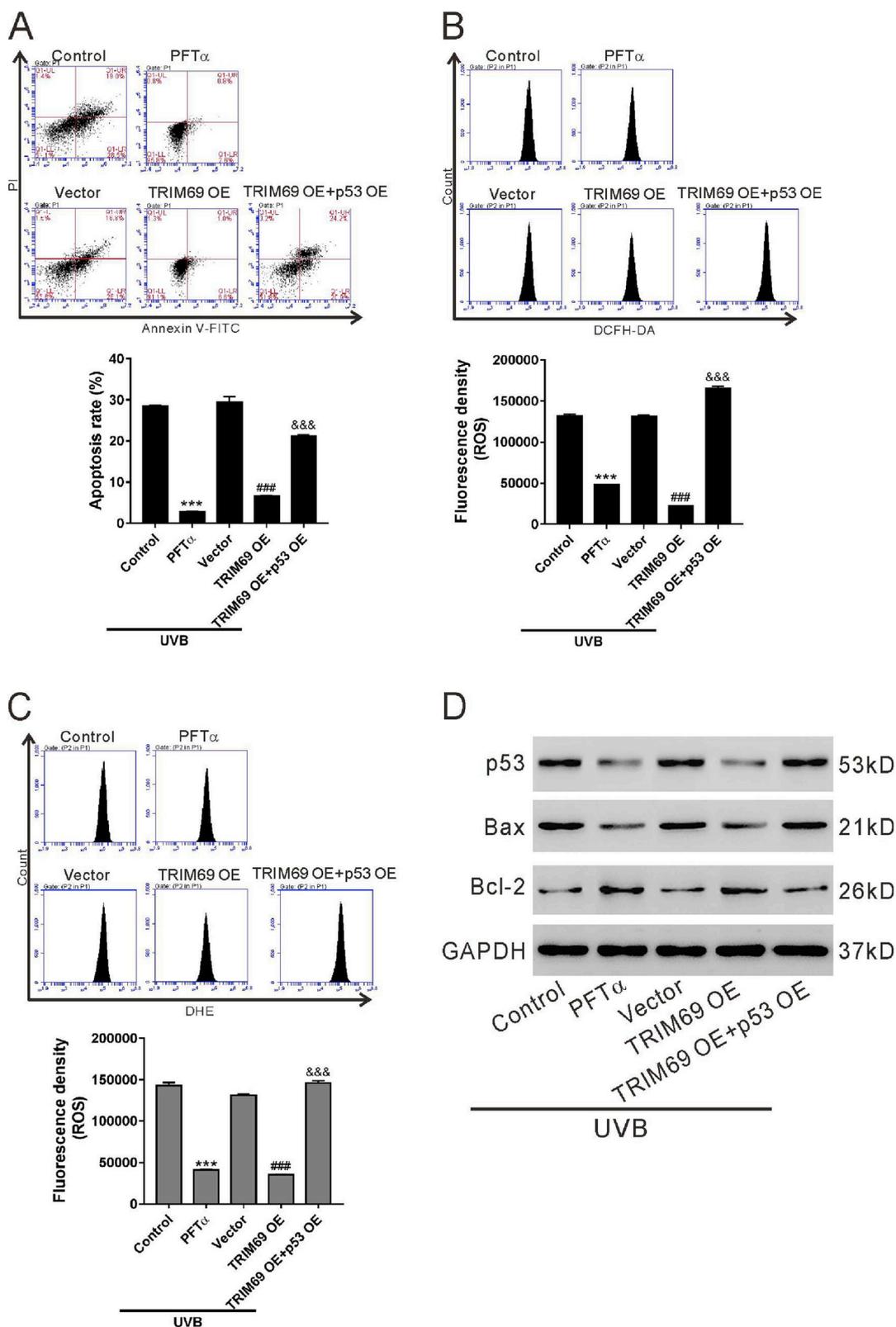
Fig. 5. p53 mediated the effects of TRIM69 in UVB-induced cell apoptosis and ROS production. HLECs were divided into the following five groups and treated as described in the Materials and Methods section before UVB irradiation: control; PFTα; vector; TRIM69 OE; and TRIM69 OE + p53 OE. (A) Cell apoptosis was detected with Annexin V-FITC/PI staining. (B, C) ROS production was assessed with DCFH-DA (B) and DHE (C). (D) Western blot assays were used to detect the expression of p53, Bax, and Bcl-2 protein. ***P < 0.001 vs. control, ###P < 0.001 vs. PFTα, &&&P < 0.001 vs. TRIM69 OE.
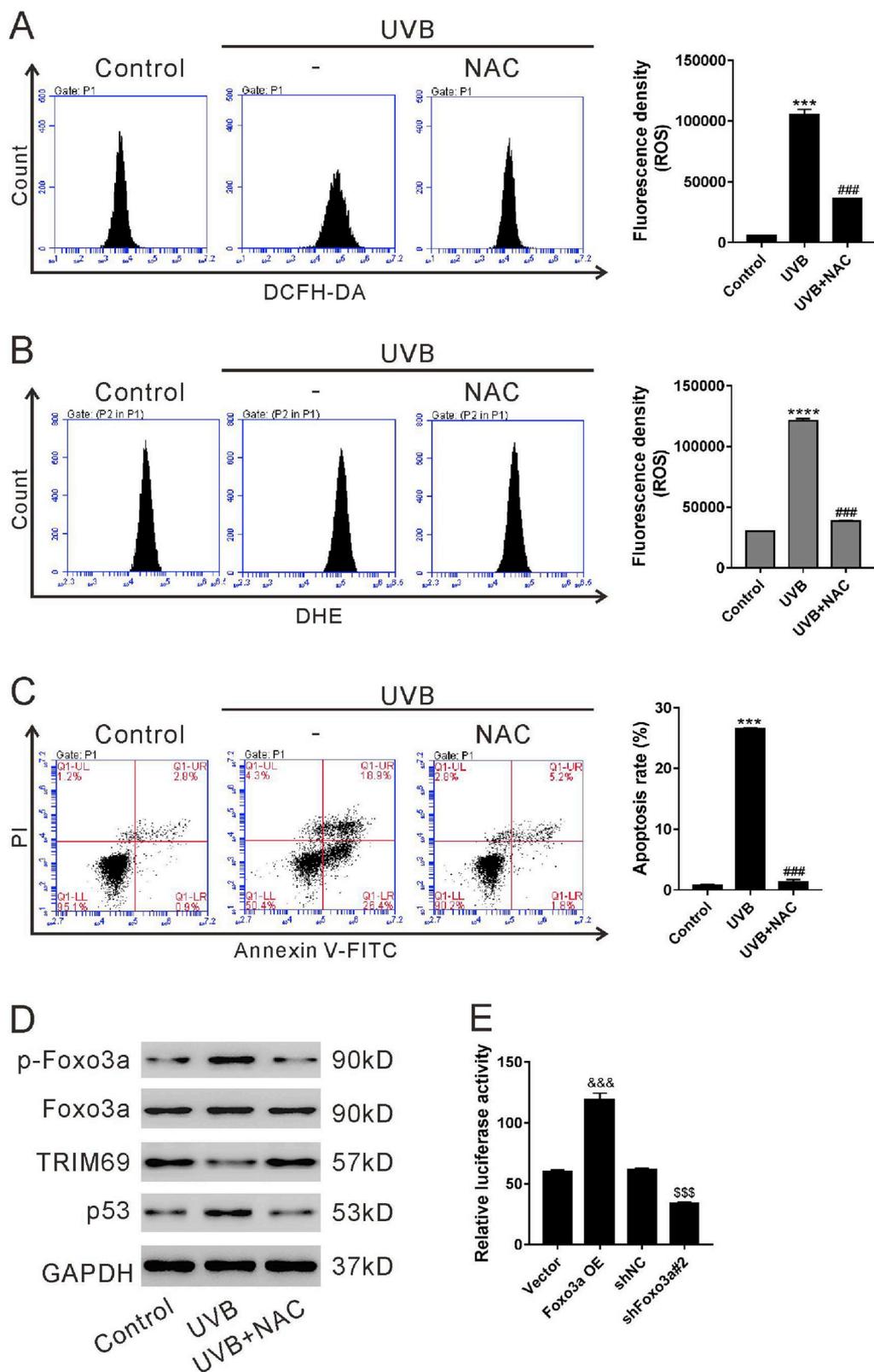
Fig. 6. Inhibition of ROS mitigated the effects of UVB irradiation on ROS production, cell apoptosis, and TRIM69 expression. HLECs were divided into the following three groups and treated as described in the Materials and Methods section: control; UVB; and UVB + NAC. (A, B) ROS production were assessed 24 h after UVB irradiation. (C) Cell apoptosis was assessed 24 h after UVB irradiation. (D) Protein expression was assessed 24 h after UVB irradiation. (E) HLECs were transduced with Fox3a overexpressing (Foxo3a OE)/vector or Fox3a shRNA (shFox3a#2)/control shRNA (shNC) and transfected with the TRIM69 promoter plasmid. The activity of the TRIM69 promoter was measured with dual luciferase reporter assays. ***$P < 0.001$ vs. control, ###$P < 0.001$ vs. UVB; &&& $P < 0.001$ vs. vector; $$$P < 0.001$ vs. shNC.

explanation for the mode of TRIM69's regulation of p53.

3.5. p53 mediated the effects of TRIM69 in UVB-induced cell apoptosis and ROS production

We then sought to assess the function of p53 in UVB-induced cell

apoptosis and ROS production. HLECs were pre-treated with a p53 inhibitor (PFTα), then subjected to UVB irradiation. PFTα significantly attenuated cell apoptosis (Fig. 5A), ROS production (Fig. 5B and C), and p53 expression (Fig. 5D) in response to UVB treatment. Furthermore, the effects of TRIM69 overexpression in UVB-induced cell apoptosis and ROS production were clearly weakened by p53 overexpression, thus

suggesting the involvement of p53 in TRIM69 functions.

3.6. Inhibition of ROS mitigated the effects of UVB irradiation on ROS production, cell apoptosis, and TRIM69 expression

Oxidative stress has been reported to be associated with UVB irradiation induced-apoptosis [6,7]. HLECs were pre-treated with NAC, which directly scavenges ROS [31], before UVB exposure. NAC significantly decreased ROS production (Fig. 6A and B) and cell apoptosis (Fig. 6C) in response to UVB irradiation.

Foxo3a, a member of the forkhead transcription factor superfamily, has been reported to be associated with ROS [32]. Analysis based on PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) predicted that Foxo3a is a putative transcription factor for TRIM69. A western blot assay showed that Foxo3a phosphorylation increased in UVB-treated cells, and this effect was weakened by NAC pre-treatment (Fig. 6D). TRIM69 expression was higher in NAC- and UVB-treated cells than in UVB-treated cells, in which p53 expression was lower. We then elevated and knocked down Foxo3a expression through lentivirus transduction (Fig. S1). A Dual luciferase reporter assay (Fig. 6E) showed that the promoter activity of TRIM69 was inhibited by Fox3a knockdown but enhanced by Foxo3a overexpression. These data suggested the existence of a positive feedback loop among TRIM69, ROS, and Foxo3a that may be important in UVB-induced cell apoptosis in HLECs.

4. Discussion

TRIM69 belongs to the TRIM family, most members of which have E3 ubiquitin ligase activity [21]. The human TRIM69 gene was first cloned from a testis cDNA library and described to function as a ubiquitin E3 ligase via the RING finger domain [19]. The functions of human TRIM69 are poorly understood. Mouse Trim69 co-localizes with promyelocytic leukemia protein and induces HEK293 cell apoptosis [33]. Zebrafish TRIM69 regulates embryo development via down-regulation of the c-Jun and p53 pathways [26,34]. In the present study, we found that the expression of TRIM69 protein was down-regulated in cataract lens anterior capsules (Fig. 1A). In agreement with the above results, TRIM69 expression was lower in UVB-exposed HLECs than in control cells (Fig. 1B). In addition, p53 expression was up-regulated in cataract lens anterior capsules and UVB-treated HLECs, in agreement with results from a previous study [18]. These findings suggest a strong correlation among TRIM69, p53, and cataract formation.

UVB irradiation efficiently induces HLECs apoptosis, which is an early event during cataract development [8,11]. The Bcl-2 family proteins, the anti-apoptotic factor Bcl-2, and the pro-apoptotic factor Bax play key roles in cataract formation [11]. To validate the functions of TRIM69 *in vitro*, we constructed lentiviral vectors to elevate or knock down TIRM69 expression. As expected, overexpression of TRIM69 attenuated the effects of UVB irradiation on cell apoptosis, Bcl-2/Bax expression, and ROS production caused by UVB irradiation (Fig. 2), whereas knocking down TRIM69 expression promoted cell apoptosis and ROS production (Fig. 3). A significant elevation of Bax has been observed in zebrafish embryos with TRIM69 knockdown [26]. Increasing mouse Trim69 expression significantly increases the ratio of Bax-to-Bcl-2 in HEK293 cells [33]; however, our data on the expression of Bcl-2/Bax were inconsistent with those from the above studies, possibly because of species differences. Together, these data indicate that TRIM69 acts as an inhibitory factor during cataractogenesis. Novel pharmacological agents targeting TRIM69 may benefit for the treatment of UVB induced cataract formation.

p53 has been suggested to be closely involved in the pathogenesis of cataracts. Several TRIM family proteins possess E3 ligase activity for p53 [22–25]. Our data showed that TRIM69 overexpression decreased p53 protein expression in UVB-treated HLECs (Fig. 2C), whereas TRIM69 knockdown increased p53 protein expression (Fig. 3C).

TRIM69 formed a complex with p53 (Fig. 4A) and induced p53 ubiquitination in HLECs (Fig. 4B). Furthermore, the p53 inhibitor PFTα significantly attenuated cell apoptosis (Fig. 5A), p53 expression (Fig. 5D), and ROS production (Fig. 5B and C) in response to UVB treatment, results consistent with those from a previous study involving p53 small interfering RNA [18]. The effects of TRIM69 overexpression in UVB-exposed HLECs were clearly decreased by p53 overexpression, thus suggesting that p53 mediates the functions of TRIM69 in cataractogenesis.

UVB can efficiently induce ROS production [6,7]. The ROS inhibitor NAC decreased UVB-induced cell apoptosis (Fig. 6C), thus suggesting that ROS is associated with UVB induced-apoptosis. Foxo3a is an important transcription factor acting in response to ROS. Foxo3a becomes inactivated when it is phosphorylated at serine 253 [32]. Here, UVB irradiation increased p-Foxo3a, whereas ROS scavenging decreased p-Foxo3a in UVB-treated HLECs. It has been reported that ROS could activate AMP-activated protein kinase (AMPK), leading to Foxo3a inactivation [35,36]. Further study will be needed to determine whether AMPK signaling involves in the ROS-inactivated Foxo3a. Moreover, we found that Foxo3a transcriptionally regulated TRIM69 expression, according to dual luciferase reporter assays (Fig. 6E). Thus, UVB exposure inactivated Foxo3a by increasing ROS production. The decreased Foxo3a activity then caused down-regulation of TRIM69, thus leading to subsequent downstream cellular responses. Novel therapeutics based on targeting Foxo3a may also benefit for the treatment of UVB induced cataract formation.

Altogether, our results suggested that TRIM69 and p53 are closely involved in cataract formation. TRIM69 inhibited UVB-induced cell apoptosis and ROS production in HLECs by inducing p53 ubiquitination. Our data also revealed that Foxo3a, which is inactivated by ROS, is a potential transcription factor for TRIM69. These findings offer new insights into the molecular mechanisms associated with cataractogenesis and treatment strategies for cataracts.

Conflicts of interest

The authors declare that no competing interests exist.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101157.

References

[1] P.A. Asbell, I. Dualan, J. Mindel, D. Brocks, M. Ahmad, S. Epstein, Age-related cataract, Lancet 365 (2005) 599–609.

[2] D. Pascolini, S.P. Mariotti, Global estimates of visual impairment: 2010, Br. J. Ophthalmol. 96 (2012) 614–618.

[3] A. Midelfart, Ultraviolet radiation and cataract, Acta Ophthalmol. Scand. 83 (2005) 642–644.

[4] J.E. Roberts, Ultraviolet radiation as a risk factor for cataract and macular degeneration, Eye Contact Lens 37 (2011) 246–249.

[5] A. Shiels, J.F. Hejtmancik, Molecular genetics of cataract, Prog. Mol. Biol. Transl. Sci. 134 (2015) 203–218.

[6] J. Cejkova, Z. Lojda, Histochemical study on xanthine oxidase activity in the normal rabbit cornea and lens and after repeated irradiation of the eye with UVB rays, Acta Histochem. 98 (1996) 47–52.

[7] U. Wölfle, P.R. Esser, B. Simon-Haarhaus, S.F. Martin, J. Lademann, C.M. Schempp, UVB-induced DNA damage, generation of reactive oxygen species, and inflammation are effectively attenuated by the flavonoid luteolin in vitro and in vivo, Free Radica. Bio. Med. 50 (2011) 1081–1093.

[8] W.C. Li, A. Spector, Lens epithelial cell apoptosis is an early event in the development of UVB-induced cataract, Free Radica. Bio. Med. 20 (1996) 301–311.

[9] J.M. Adams, S. Cory, The Bcl-2 protein family: arbiters of cell survival, Science 281 (1998) 1322–1326.

[10] J.C. Reed, Bcl-2 family proteins, Oncogene 17 (1998) 3225–3236.

[11] Y. Ji, L. Cai, T. Zheng, H. Ye, X. Rong, J. Rao, Y. Lu, The mechanism of UVB irradiation induced-apoptosis in cataract, Mol. Cell Biol. 401 (2015) 87–95.

[12] A.J. Levine, p53, the cellular gatekeeper for growth and division, Cell 88 (1997) 323–331.

[13] C.J. Brown, S. Lain, C.S. Verma, A.R. Fersht, D.P. Lane, Awakening guardian angels: drugging the p53 pathway, Nat. Rev. Cancer 9 (2009) 862–873.

[14] W. Maltzman, L. Czyzyk, UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells, Mol. Cell Biol. 4 (1984) 1689–1694.

[15] M. Ayala, H. Strid, U. Jacobsson, P.G. Söderberg, p53 expression and apoptosis in the lens after ultraviolet radiation exposure, Investig. Ophthalmol. Vis. Sci. 48 (2007) 4187–4191.

[16] C. Prives, P.A. Hall, The p53 pathway, J. Pathol. 187 (1999) 112–126.

[17] K.H. Vousden, X. Lu, Live or let die: the cell's response to p53, Nat. Rev. Cancer 2 (2002) 594–604.

[18] Y. Qin, J. Zhao, X. Min, M. Wang, W. Luo, D. Wu, Q. Yan, J. Li, X. Wu, J. Zhang, MicroRNA-125b inhibits lens epithelial cell apoptosis by targeting p53 in age-related cataract, Biochim. Biophys. Acta 1842 (2014) 2439–2447.

[19] Y. Han, R. Li, J. Gao, S. Miao, L. Wang, Characterisation of human RING finger protein TRIM69, a novel testis E3 ubiquitin ligase and its subcellular localisation, Biochem. Biophys. Res. Commun. 429 (2012) 6–11.

[20] S. Hatakeyama, TRIM proteins and cancer, Nat. Rev. Cancer 11 (2011) 792.

[21] E. Tai, S. Benchimol, TRIMming p53 for ubiquitination, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 11431–11432.

[22] K. Allton, A.K. Jain, H.M. Herz, W.W. Tsai, S.Y. Jung, J. Qin, A. Bergmann, R.L. Johnson, M.C. Barton, Trim24 targets endogenous p53 for degradation, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 11612–11616.

[23] J. Liu, C. Zhang, X.L. Wang, P. Ly, V. Belyi, Z.Y. Xu-Monette, K.H. Young, W. Hu, Z. Feng, E3 ubiquitin ligase TRIM32 negatively regulates tumor suppressor p53 to promote tumorigenesis, Cell Death Differ. 21 (2014) 1792–1804.

[24] L. Zhang, N.J. Huang, C. Chen, W. Tang, S. Kornbluth, Ubiquitylation of p53 by the APC/C inhibitor Trim39, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 20931–20936.

[25] Z. Zhou, Z. Ji, Y. Wang, J. Li, H. Cao, H.H. Zhu, W.Q. Gao, TRIM59 is up-regulated in gastric tumors, promoting ubiquitination and degradation of p53, Gastroenterology 147 (2014) 1043–1054.

[26] R. Han, Q. Zhao, S. Zong, S. Miao, L. Wang, A novel TRIM family member, Trim69, regulates zebrafish development through p53-mediated apoptosis, Mol. Reprod. Dev. 83 (2016) 442–454.

[27] C.P. LeBel, H. Ischiropoulos, S.C. Bondy, Evaluation of the probe 2', 7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress, Chem. Res. Toxicol. 5 (1992) 227–231.

[28] X. Cai, Y. Liu, Y. Hu, X. Liu, H. Jiang, S. Yang, Z. Shao, Y. Xia, L. Xiong, ROS-mediated lysosomal membrane permeabilization is involved in bupivacaine-induced death of rabbit intervertebral disc cells, Redox. Biol. 18 (2018) 65–76.

[29] S. Cui, X. Lv, W. Li, Z. Li, H. Liu, Y. Gao, G. Huang, Folic acid modulates VPO1 DNA methylation levels and alleviates oxidative stress-induced apoptosis in vivo and in vitro, Redox. Biol. 19 (2018) 81–91.

[30] M.S. Oliveira, M.I.F. Barbosa, T.B. de Souza, D.R.M. Moreira, F.T. Martins, W. Villarreal, R.P. Machado, A.C. Doriguetto, M.B.P. Soares, D.P. Bezerra, A novel platinum complex containing a piplartine derivative exhibits enhanced cytotoxicity, causes oxidative stress and triggers apoptotic cell death by erk/p38 pathway in human acute promyelocytic leukemia hl-60 cells, Redox. Biol. 20 (2019) 182–194.

[31] M. Halasi, M. Wang, T.S. Chavan, V. Gaponenko, N. Hay, A.L. Gartel, ROS inhibitor N-acetyl-L-cysteine antagonizes the activity of proteasome inhibitors, Biochem. J. 454 (2013) 201–208.

[32] J.W. Liu, D. Chandra, M.D. Rudd, A.P. Butler, V. Pallotta, D. Brown, P.J. Coffer, D.G. Tang, Induction of prosurvival molecules by apoptotic stimuli: involvement of FOXO3a and ROS, Oncogene 24 (2005) 2020–2031.

[33] H.W. Shyu, S.H. Hsu, H.M. Hsieh-Li, H. Li, Forced expression of RNF36 induces cell apoptosis, Exp. Cell Res. 287 (2003) 301–313.

[34] R. Han, R. Wang, Q. Zhao, Y. Han, S. Zong, S. Miao, W. Song, L. Wang, Trim69 regulates zebrafish brain development by ap-1 pathway, Sci. Rep. 6 (2016) 24034.

[35] F. Chiacchiera, C. Simone, The AMPK-FoxO3A axis as a target for cancer treatment, Cell Cycle 9 (2010) 1091–1096.

[36] D.G. Hardie, F.A. Ross, S.A. Hawley, AMPK: a nutrient and energy sensor that maintains energy homeostasis, Nat. Rev. Mol. Cell Biol. 13 (2012) 251.