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ARTICLE

Silibinin relieves UVB-induced apoptosis of human skin cells by inhibiting the YAP-p73 pathway

Wei-wei Liu[1], Fang Wang[1], Can Li[1], Xiao-yu Song[1], Wuxiyar Otkur[1,2], Yu-ying Zhu[1], Toshihiko Hayashi[1,3,4], Kazunori Mizuno[4], Shunji Hattori[4], Hitomi Fujisaki[4] and Takashi Ikejima[1,5]

Excessive exposure to UVB induces skin diseases. Silibinin, a flavonolignan used for treating liver diseases, is found to be effective against UVB-caused skin epidermal and dermal cell damage. In this study we investigated the molecular mechanisms underlying. Human nonmalignant immortalized keratinocyte HaCaT cells and neonatal human foreskin fibroblasts HFFs were exposed to UVB irradiation. We showed that pre-treatment with silibinin dose-dependently decreased UVB-induced apoptosis of HaCaT cells. Furthermore, we showed that silibinin treatment inhibited nuclear translocation of YAP after UVB irradiation. Molecular docking analysis and DARTS assay confirmed the direct interaction of silibinin with YAP. Silencing YAP by siRNA had no influence on the survival of HaCaT cells, whereas inhibiting classical YAP-TEAD signaling pathway by siRNA targeting TEAD1 or its pharmaceutical inhibitor verteporfin further augmented UVB-induced apoptosis, suggesting that YAP-TEAD pathway was prosurvival, which did not participate in the protective effect of silibinin. We then explored the pro-apoptotic YAP-p73 pathway. p73 was upregulated in UVB-irradiated cells, but reduced by silibinin cotreatment. The mRNA and protein levels of p73 target genes (*PML, p21* and *Bax*) were all increased by UVB but decreased by silibinin co-treatment. Inhibiting p73 by using siRNA reduced UVB-induced apoptosis, suggesting that downregulation of p73 was responsible for the cytoprotective effect of silibinin. In HFFs, the upregulated YAP-p73 pathway by UVB irradiation was also suppressed by silibinin. Collectively, YAP-p73 pathway is a major cause of the death of UVB-exposed epidermal HaCaT cells and dermal HFFs. Silibinin directly inhibits YAP-p73 pathway, exerting the protective action on UVB-irradiated skin cells.

Keywords: UVB; apoptosis; HaCaT cells; HFF primary fibroblasts; YAP; TEAD; p73; silibinin; verteporfin

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INTRODUCTION

Solar ultraviolet (UV) radiation, especially ultraviolet B (UVB, 280–320 nm) radiation, is important for humans [1]. Moderate exposure to UVB helps to maintain human health by promoting the conversion of cholesterol to vitamin D [2] and modulating mammalian immunity [3]. However, attention should be given to the risk of sun damage associated with being outdoors for long periods of time. Skin barrier disruption, especially severe apoptosis, frequently occurs after UVB exposure, which causes DNA damage to cells [4]. Here, we used human nonmalignant immortalized keratinocyte HaCaT cells and HFF primary fibroblasts to study epidermal and dermal cell apoptosis caused by UVB irradiation.

YAP, a conserved protein in higher eukaryotes [5], is an important transcriptional regulator with obvious biological roles in cell proliferation, differentiation and apoptosis [5–7]. Hippo signaling and extracellular mechanical signals, including the stiffness of the extracellular matrix and cell adhesive areas, are well-known pillars for YAP regulation; however, the upstream stimuli are far from fully revealed [8]. The main role of YAP is to regulate a series of target genes, including *CTGF*, *CYR61*, *ANKRD1* and others [7, 8]. Interestingly, YAP has no DNA binding domains, implying that this molecule binds to and cooperates with other transcription factors to exert its functions [8]. TEADs are the main YAP-interacting transcription factors that are typically involved in cell proliferation and differentiation [7, 8]. Other transcriptional factors interacting with the YAP molecule include MYC, p73, SMADs, PPAR-γ, FOXO1 and E2F. Among them, p73, which engages in a direct physical association with YAP in which the WW domain of YAP and the PPPPY motif of p73 are involved, is a classically proapoptotic transcription factor belonging to the p53 family [9, 10]. The proapoptotic function of YAP during DNA damage caused by cisplatin or doxorubicin is mediated by the p73 pathway [7, 8, 11]. In our study on UVB-induced cell injury in HaCaT and HFF cells, we found that the cell adhesive areas and cell shapes were considerably changed by UVB irradiation. Moreover, according to our experience in a UVB study, the digestion of UVB-irradiated cells by trypsin takes a longer time than that of nonirradiated cells and cells at a higher density seem more resistant to UVB irradiation. Considering that the YAP

[1]Wuya College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016, China; [2]CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China; [3]Department of Chemistry and Life science, School of Advanced Engineering, Kogakuin University, 2665-1, Nakanomachi, Hachioji, Tokyo 192-0015, Japan; [4]Nippi Research Institute of Biomatrix, Toride, Ibaraki 302-0017, Japan and [5]Key Laboratory of Computational Chemistry-Based Natural Antitumor Drug Research & Development, Shenyang 110016, China
Correspondence: Takashi Ikejima (ikejimat@vip.sina.com)

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pathway is closely related to cell shape, focal adhesion, cell density and other physical features [7, 8, 12, 13], we were curious about the modulation of the YAP pathway in cells under UVB stress. Interestingly, we found that UVB irradiation caused obvious YAP nuclear translocation in HaCaT cells and HFFs. Information regarding YAP signaling in UVB damage is quite limited; therefore, we studied the role of YAP in UVB-induced apoptosis, mainly focusing on the YAP-TEAD and YAP-p73 pathways.

Silibinin, isolated from the seeds of *Silybum marianum*, is the most studied flavonolignan to date and shows beneficial activities, including hepatoprotection and recently discovered anticancer, anti-inflammatory, neuroprotective and skin-protective activities [14–17]. Regarding the skin-protective role of silibinin, there are various possible mechanisms, including anti-inflammation, anti-oxidation, regulation of autophagy or p53 signaling, and some other mechanisms [17–19]. However, since silibinin is a drug with multiple cellular effects, until now, there has been no consolidated explanation for all the pharmacological effects. Although extensive studies on the YAP pathway have emerged in recent years, the modulation of the YAP pathway by silibinin has rarely been discussed. Interestingly, we found that silibinin inhibited the nuclear translocation of YAP in UVB-irradiated HaCaT cells and HFFs and investigated the role of YAP pathway in the current study.

MATERIALS AND METHODS

Cells and culture

Human nonmalignant immortalized keratinocyte HaCaT cells (CLS Cell Lines Service, 300,493) and HFF primary fibroblasts prepared by collagenase treatment of neonatal foreskin were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% (*v*/*v*) FBS (TBD Science, Tianjin, China), 100 μg/mL streptomycin and 100 U/mL penicillin. The cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere [20].

Reagents

Silibinin (Jurong Best Medicine Material, Zhenjiang, Jiangsu, China) with 99% purity determined by HPLC was used in our study. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and Hoechst 33342 were purchased from Sigma Chemical (St. Louis, MO, USA). Primary antibodies against caspase-3 (sc-7148), ICAD (inhibitor of caspase activated DNase, sc-9066), PARP (poly ADP-ribose polymerase, sc-7150), YAP (sc-101199), histone H3 (sc-517576) and β-actin (sc-47778) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against active caspase-3 (9664 S), p-YAP (Ser127, 13008 S), pan-TEAD (13295 S) and p73 (14620 S) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against Lamin B (12987-1-AP) were purchased from Proteintech (Rosemont, IL, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). SuperSignal® West Pico Chemiluminescent Substrates were purchased from Thermo Fisher Scientific (MA, USA).

UVB exposure procedure

HaCaT cells were seeded at a density of 1×10^5 cells/mL in 96-well plates (100 μL/well) or 3×10^5 cells/mL in six-well plates (2 mL/well), 60 mm dishes (4 mL/dish) or 100 mm dishes (10 mL/dish) and cultured for 24 h before UVB treatment. HFFs at passages 13–25 were seeded at a density of 2×10^4 cells/mL in 96-well plates or 4×10^4 cells/mL in six-well plates, 60-mm dishes or 100-mm dishes and cultured for 36 h before UVB treatment. Then, the cells were exposed to UVB lamps (Beijing Lighting Research Institute, Beijing, China) with an irradiation density of 20 μW/cm^2, as monitored by a UVB spectro radiometer (Photoelectric Instrument Factory of Beijing Normal University, Beijing, China), and a series of UVB doses were achieved by exposure for different time periods. To avoid possible UVB absorption by the proteins and other components in the medium, the culture medium was transiently replaced with 50 μL of PBS per well for 96-well plates, 250 μL of PBS for 24-well plates and 1 mL of PBS for six-well plates during UVB exposure [20]. Silibinin was added 1 h before UVB irradiation. Verteporfin, a YAP-TEAD binding inhibitor, is sensitive to light (300–700 nm wavelength) [21, 22] and was added immediately after UVB irradiation. HaCaT cells were further cultured for 12 h (or other indicated durations) while HFFs were further cultured for 36 h and then subjected to analysis.

MTT assay

An MTT assay was conducted as described previously [20, 23]. Cell viability was calculated using the formula below:

Relative cell numbers (%) $= 100 \times (A_{490,\ sample} - A_{490,\ blank})/(A_{490,\ control} - A_{490,\ blank})$

Viable cells after UVB (%) $= 100 \times (A_{490,\ UVB\text{-}irradiated\ sample} - A_{490,\ blank})/(A_{490,\ UVB\text{-}unirradiated\ sample} - A_{490,\ blank})$

Phase contrast microscopy

Morphological changes in the cells were observed with a phase contrast microscope (Leica, Nussloch, Germany).

Annexin V/PI staining and flow cytometry

An Annexin V-FITC/PI apoptosis detection kit was purchased from Bimake (Houston, TX, US) and used to stain cells. The cells were analyzed by using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The data were further analyzed by using FlowJo software.

Hoechst 33342 staining

Cells were incubated with Hoechst 33342 (2 μg/mL in medium) at 37 °C in the dark for 30 min and then observed with a fluorescence microscope (Olympus, Tokyo, Japan). Cells with fluorescence higher than the normal value were considered as Hoechst-positive cells. At least 6 fields for each treatment were selected for analysis.

Immunofluorescence confocal microscopy

Cells on coverslips were immunostained with corresponding antibodies following the procedure described previously [20]. Slides were visualized with a confocal microscope (Nikon C2 plus, Tokyo, Japan). The fluorescence density was quantified by ImageJ.

Western blotting

Both adherent and floating cells were collected and lysed with RIPA lysis buffer supplemented with PMSF to obtain whole cellular lysates. Alternatively, nuclear and cytoplasmic protein extraction kits purchased from Wanleibio (Shenyang, Liaoning, China) were used to obtain nuclear and cytoplasmic proteins. The cell lysates were then analyzed following Western blotting steps described previously [20]. ImageJ was used for the quantification of blots.

Quantitative RT–PCR

Cells were collected and lysed with RNAiso Plus (Takara Bio, Kusatsu, Shiga, Japan) to extract total RNA. Then, cDNA was synthesized by using PrimeScript™ RT Master Mix (Takara Bio, Kusatsu, Shiga, Japan). Finally, amplification of target genes was conducted by using TB Green® *Premix Ex Taq*™ II (Takara Bio, Kusatsu, Shiga, Japan) in a Stratagene Mx3000P® Multiplex Quantitative PCR (QPCR) System (Agilent Technologies, Santa Clara, CA, USA). The forward and reverse primers used were as follows: *YAP*, 5′-ATTTCCATAAGCCA GTTGCAG-3′, 5′-ACATTAACAGCAGCAATGGAC-3′; TAZ, 5′-AAGTGAGC CCTTTCTAACCTG-3′, 5′-TAAGGTCATGGCTACATCCAA-3′; *TEAD1*, 5′-AAAGTGCTGCCATAATAACGA-3′, 5′-GTTACTCATCCCGGTAAACCA-3′; *p73*, 5′-TCCAACGCACAAACATCCACT-3′, 5′-AGGGCGTCCATTAATAC TGCT-3′; *PML*, 5′-AGCTTCTCTTCACGCACTCCA-3′, 5′-GGACCCCAGCTT AGTTTCGAT-3′; *p21*, 5′-CACTTTGATTAGCAGCGGAAC-3′, 5′-TGTCCAT

AGCCTCTACTGCC-3′; *Bax*, 5′-CACTGCCTCTGGAATTGCTCA-3′, 5′-GC AACATAGCGAGACCCCTT-3′; and *β-actin*, 5′-ATGACTTAGTTGCGTTAC ACC-3′, 5′-AAACAAATAAAGCCATGCCAA-3′. The expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method, and *β-actin* was used for normalization.

Drug affinity responsive target stability (DARTS) assay

The DARTS assay, which takes advantage of a reduction in the protease susceptibility of a target protein upon drug binding is a universally applicable assay to identify drug-protein interactions [24, 25]. Pronase (Roche, Mannheim, Germany) was prepared as a 10 mg/mL stock solution in ultrapure water, and stored at −20 °C. Protein lysate was incubated with vehicle control (DMSO) or silibinin at 4 °C for 4 h. For proteolysis, each cellular lysate sample was subjected to treatment with pronase (1:50 or 1:100 ratios of pronase vs. cell lysate proteins) at room temperature (RT) or 0 °C for 2 min. Proteolysis was stopped by the addition of 0.5 M EDTA (pH 8.0) to each sample at a ratio of 1:10, and the mixtures were denatured by boiling with SDS loading buffer. The samples were subjected to SDS–PAGE for further analysis.

Molecular docking study

The structure of YAP (PDB code: 3KYS) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/) for a molecular docking study. The internal ligands of YAP (ID: PIL) were used to define the active pockets. Heteroatoms and water molecules were removed, and all hydrogen atoms were subsequently added to the protein using the Discovery Studio 3.4 program (Accelrys Inc., San Diego, USA). The structure was saved in PDB format for further docking study using Molegro Virtual Docker 4.4 (Molegro ApS, Aarhus, Denmark). All docking conformations were ranked based on the scores. The analysis was performed using the Discovery Studio 3.4 program.

siRNA transfection

The sense and antisense sequences of the siRNAs (GenePharma, Suzhou, China) used were as follows: si-YAP-1: 5′-GCAUCUUCGACAG UCUUCUTT-3′, 5′-AGAAGACUGUCGAAGAUGCTT-3′; si-YAP-2: 5′-GGU CAGAGAUACUUCUUAATT-3′, 5′-UUAAGAAGUAUCUCUGACCTT-3′; si-YAP-3: 5′-GGUGAUACUAUCAACCAAATT-3′, 5′-UUUGGUUGAUAG UAUCACCTT-3′; si-TAZ: 5′-AGGUACUUCCUCAAUCACATT-3′, 5′-UGUG AUUGAGGAAGUACCUTT-3′. si-TEAD1: 5′-GGAUCAGACUGCAAAGGA UTT-3′, 5′-AUCCUUUGCAGUCUGAUCCTT-3′; si-p73: 5′-CCCAAGGGU UACAGAGCAUTT-3′, 5′-AUGCUCUGUAACCCUUGGGTT-3′ and the negative control (si-NC): 5′-UUCUCCGAACGUGUCACGUTT-3′, 5′-ACG UGACACGUUCGGAGAATT-3′. Cells were transfected with 60 nM siRNA using Lipofectamine 2000 and incubated for 8 h; then, the transfection medium was replaced with fresh culture medium. The cells were subjected to subsequent experiments 72 h after transfection.

Statistical analysis

All experiments were replicated at least three times. The data are presented as the means ± SDs and were statistically analyzed by using GraphPad Prism 8.0 software. One-way or two-way ANOVA followed by Tukey's or Dunnett's multiple comparisons test was used to evaluate the statistical significance among groups. For comparisons between two groups, unpaired *t*-tests were used. A P value < 0.05 was considered to indicate statistical significance.

RESULTS

Silibinin inhibits UVB-induced HaCaT cell apoptosis

In epidermal HaCaT cells, UVB irradiation caused dose- and time-dependent cell loss; accordingly, in subsequent experiments, UVB irradiation of 200 J/m^2 was used, and cells were incubated for 12 h after irradiation to achieve loss of half of the cells (Fig. 1a). Next, the protective effect of silibinin against UVB-induced cell loss was found to increase in a concentration-dependent manner from 25 to 200 μM, reaching a maximum at 100 and 200 μM, but was decreased at 400 μM, which might be explained by the increased cytotoxicity of silibinin at higher doses (Fig. 1b). Therefore, 200 μM silibinin which showed the best protective effect and a low toxicity was used in this study.

Using phase contrast microscopy, shrinkage and blebbing of cells in the UVB group were observed, which suggested the occurrence of apoptosis. After silibinin treatment, the apoptotic morphology was obviously eliminated (Fig. 1c). Annexin V/PI double staining showed that the ratio of apoptotic cells (Annexin V-positive cells) was increased in the UVB-irradiated group but reduced after silibinin treatment (Fig. 1d, e). Hoechst staining of the HaCaT cells clearly exhibited nuclear condensation and fragmentation in UVB-irradiated cells, confirming UVB-induced apoptosis, but in UVB- and silibinin-cotreated cells, the number of cells with apoptotic morphology was obviously reduced (Fig. 1f, g). In the UVB group, the apoptosis-executing protein caspase-3 was activated, after which the DNA repair enzyme PARP was cleaved and the apoptosis-inhibiting protein ICAD was degraded. However, after treatment with silibinin, the changes in these apoptosis-related proteins were all attenuated (Fig. 1h–k). The above results indicate that silibinin has an inhibitory effect on apoptosis induced by UVB.

Silibinin reduces UVB-induced YAP nuclear translocation in HaCaT cells

In addition to the apoptotic cell behaviors, we also observed some changes in the physical properties of UVB-treated HaCaT cells, including the flattening of cells (Fig. 1c). Moreover, in our UVB study, UVB irradiation increased the cell digestion time, and cells at high density always acquired resistance against UVB irradiation. YAP is a molecule well known for its integration of extracellular physical conditions including cell density, cell flattening and focal adhesion, with intracellular signal transduction, but the role of YAP in UVB injury has not been fully revealed, and the regulatory effect of silibinin on YAP is still unknown [5–8, 11, 12]. Therefore, we were interested in the role of YAP during UVB stress. The results from confocal microscopy showed that YAP was distributed evenly in the whole cell, but after exposure to UVB, YAP moved toward the nucleus in a time-dependent manner, and the nuclear localization reached a maximum at approximately 6 h after irradiation (Fig. 2a, b). Western blotting also showed that nuclear YAP levels increased time-dependently (Fig. 2c, d). The qRT–PCR results showed that the mRNA level of *YAP* was not obviously influenced by either UVB or silibinin (Fig. 2e). By measuring whole cellular proteins, we found that the YAP protein amount was not changed (Fig. 2f, g). Interestingly, the phosphorylation of YAP (at Ser127), which hinders YAP translocation into the nucleus [26, 27], was inhibited by UVB irradiation (Fig. 2f, g), accounting for the increased nuclear translocation of YAP during UVB stress. Silibinin restored the phosphorylation of YAP (Fig. 2f, g). The results of YAP immunostaining and Western blotting showed that the increase in YAP nuclear translocation was blocked by silibinin treatment (Fig. 2h–k). To verify whether there is a direct interaction between silibinin and the YAP protein, molecular docking studies and a DARTS assay were employed. The results from molecular docking indicated that silibinin forms complexes with the YAP domain (Fig. 3a–e) with favorable hydrogen bonds (Fig. 3b) and hydrophobic interactions (Fig. 3c). Silibinin even exhibits higher affinity for YAP than its ligand, as shown by the lower MolDock score (Fig. 3e). A DARTS assay was utilized as a simple, universally applicable target identification approach that analyzes direct drug binding to targets [24, 25]. The results of the DARTS assay showed that incubating the protein lysates with silibinin markedly enhanced the stability of YAP against pronase digestion (Fig. 3f, g), suggesting a direct interaction of silibinin with YAP. Since silibinin protected HaCaT cells against UVB-induced death and inhibited

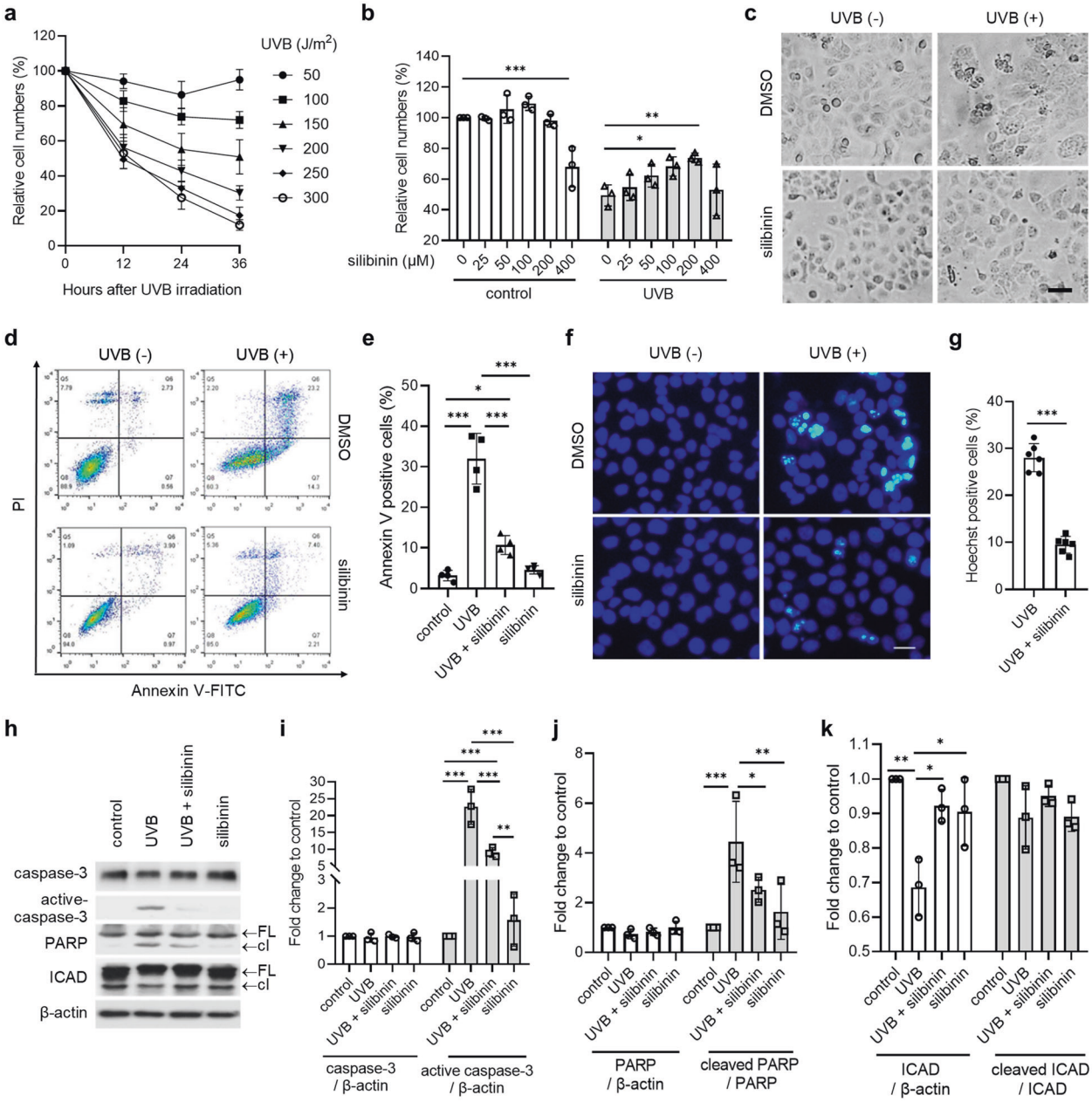

Fig. 1 Silibinin inhibits UVB-induced HaCaT cell apoptosis. a HaCaT cells were exposed to different doses of UVB irradiation, and the relative cell numbers were determined by MTT assay. **b** The relative cell numbers of HaCaT cells treated with different concentrations of silibinin were assessed with MTT assays 12 h after UVB exposure. **c** Morphology of cells. UVB, 200 J/m^2; silibinin, 200 μM. Bar, 20 μm. **d**–**e** HaCaT cells were stained with Annexin V-FITC/PI and analyzed by using flow cytometry. **f**–**g** The Hoechst-stained cells are shown. Bar, 10 μm. **h**–**k** The protein levels of apoptosis-related proteins were analyzed by Western blotting. FL = full length protein, cl = cleaved protein. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

YAP nuclear localization, we suspect that silibinin downregulates UVB damage to HaCaT cells by regulating the YAP pathway.

Knocking down YAP protein does not alleviate UVB-induced cell death

To investigate the role of YAP in UVB-induced apoptosis, we used siRNA to silence the mRNA translation of YAP (Fig. 4a, b). Unexpectedly, the results showed that YAP knockdown had no effect on UVB-induced cell loss (Fig. 4c). Likewise, the Annexin V-positive ratio (Fig. 4d, e) and nuclear condensation, as shown by Hoechst staining (Fig. 4f, g), in UVB-treated cells were not changed by YAP silencing. Transcriptional coactivator with a PDZ-binding domain (TAZ) shares homology with YAP [28, 29]. Whether the ambiguous results of YAP silencing in UVB-irradiated cells were due to the compensatory effect of TAZ aroused our interest. We conducted TAZ silencing by transfecting siRNA (Fig. 4h), and the results showed that neither silencing TAZ nor simultaneously silencing YAZ and TAZ had an obvious influence on the cell viability of UVB-irradiated cells (Fig. 4i).

YAP is a protein that transcriptionally regulates many target genes to influence cell fate [8, 13]. However, YAP has no DNA binding domain; thus, it must interact with various cotranscription factors [7, 8]. The best-characterized transcription factors regulated by YAP are the TEAD family members, the prototype of which is TEAD1 (TEF-1) [30], which regulates the transcription of many prosurvival signals [30]. However, some other cofactors, especially p73, regulate the transcription of a series of proapoptotic proteins, leading to cell death

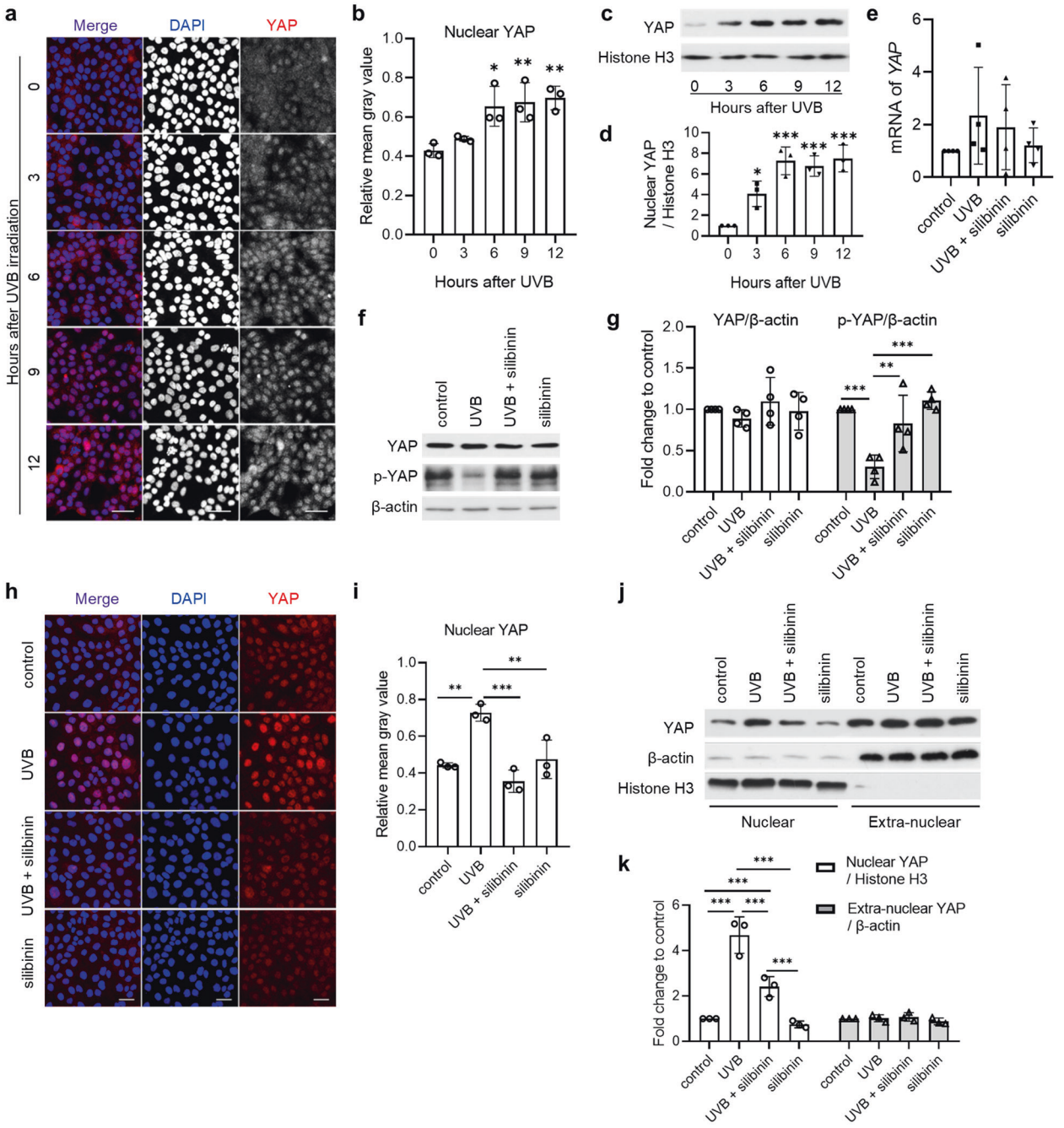

Fig. 2 YAP nuclear translocation enhancement by UVB irradiation is reversed by silibinin in HaCaT cells. a–**b** Immunostaining of YAP in HaCaT cells after UVB irradiation. Bar, 20 μm. HaCaT cells were fixed and immunostained with an antibody against YAP (red, TRITC), and the nuclei were counterstained with DAPI (blue). The nuclear YAP fluorescence quantified by Image J is presented in (**b**). *$P < 0.05$ vs. UVB 0 h group; **$P < 0.01$ vs. UVB 0 h group. **c**–**d** Nuclear YAP was analyzed by Western blotting. Histone H3 was used as the loading control for nuclear proteins. *$P < 0.05$ vs. UVB 0 h group; ***$P < 0.001$ vs. UVB 0 h group. **e** The mRNA amount of *YAP* was determined by qRT–PCR. **f**–**g** The protein levels of YAP and p-YAP were examined by Western blotting. **h**–**i** The YAP distribution in cells treated with UVB or silibinin was studied by confocal microscopy. Bar, 20 μm. **j**–**k** Nuclear and extranuclear YAP were analyzed by Western blotting. Histone H3 and β-actin were used as the loading controls for nuclear and extranuclear proteins, respectively. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[11, 31, 32]. The obscure results of YAP silencing were probably due to the complicated regulatory network lying downstream. Therefore, we studied the cofactors of YAP that have modulatory roles in apoptosis. First, we studied the role of the YAP-TEAD branch in UVB-treated cells.

TEAD1 downregulates apoptosis induced by UVB in HaCaT cells

The Western blotting results showed that nuclear TEAD was not influenced by UVB or silibinin treatment (Fig. 5a, b). Confocal microscopy confirmed the unchanged TEAD expression under UVB stress (Fig. 5c–d). The mRNA level of TEAD1 was also not altered (Fig. 5e). Verteporfin is a specific inhibitor of the YAP-TEAD interaction [33]. The results from the MTT assay showed that verteporfin treatment further aggravated the cell loss induced by UVB (Fig. 5f). Then, TEAD1 was knocked down by using siRNA transfection (Fig. 5g, h), which further reduced cell survival (Fig. 5i), augmented nuclear condensation (Fig. 5j, k) and increased the activation of caspase-3

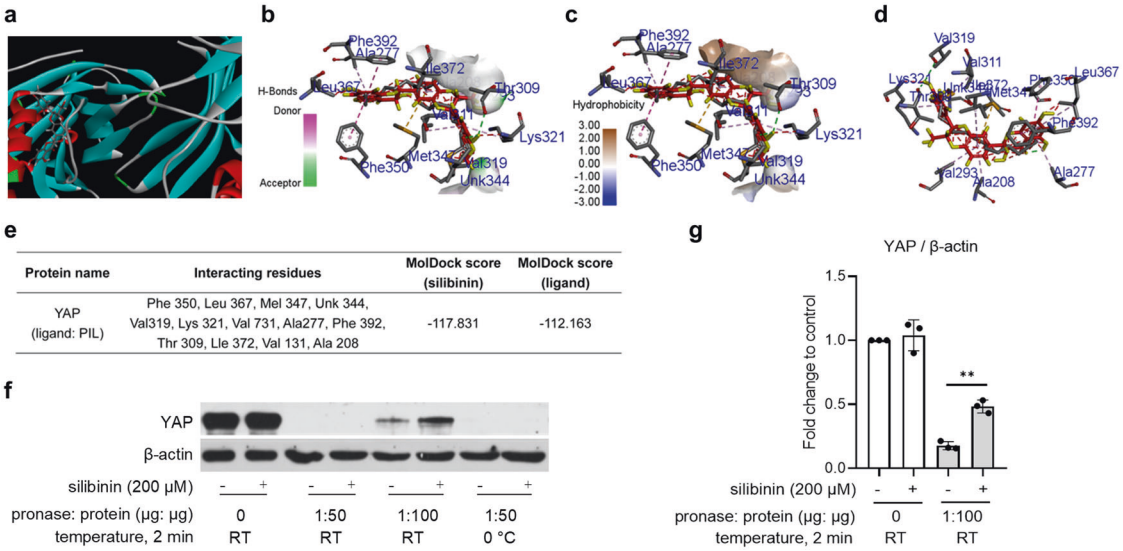

Fig. 3 Interaction of the silibinin molecule with the YAP protein. a Surface diagram of the YAP protein with the silibinin molecule from induced fit docking. **b** Hydrogen bonds formed between silibinin and the residues at the YAP binding pockets. **c** Hydrophobic bonds formed between silibinin and YAP. **d** Close-up view of the detailed interaction between YAP and silibinin. **e** The interacting residues and the molecular docking scores are listed. **f**–**g** The direct interaction between YAP and silibinin was studied by using a DARTS assay. Pronase was applied to the cell lysates (which had been incubated with silibinin or DMSO for 4 h at 4 °C) at a ratio of 1:50 or 1:100 (pronase (μg): protein (μg)) for 2 min of digestion at room temperature (RT) or 0 °C, and the residual YAP protein level was determined by Western blotting to study the interference of silibinin with YAP digestion. $^{**}P < 0.01$.

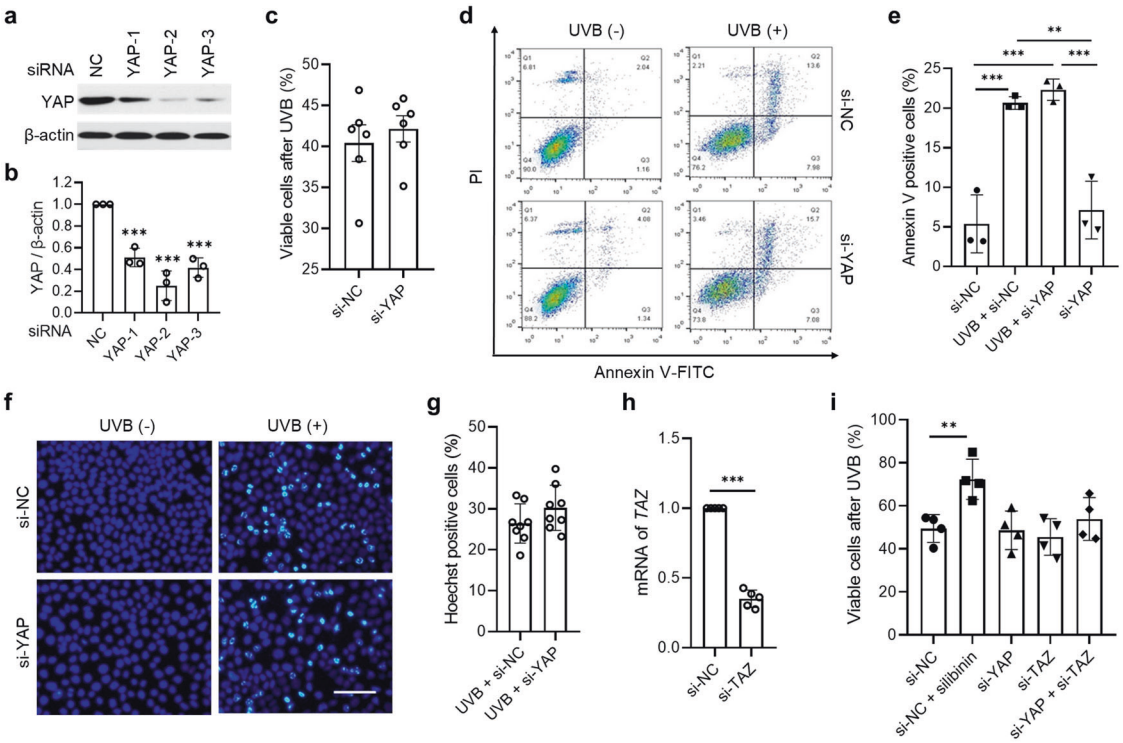

Fig. 4 Knocking out YAP does not alleviate UVB-induced cell death. a–**b** HaCaT cells were transfected with siRNAs with three sequences to silence YAP. Si-YAP-2 showed the best silencing efficiency and was selected for use in the following study. $^{***}P < 0.001$ vs. si-NC group. **c** Silencing YAP did not alleviate cell loss caused by UVB. **d**–**e** Annexin V-positive cells were analyzed after YAP was silenced. **f**–**g** Fluorescence microscopic images of HaCaT cells transfected with siRNA with Hoechst 33258 staining. Bar, 50 μm. **h** TAZ was silenced by using siRNA. **i** The viability of UVB-irradiated cells with YAP and TAZ silencing. $^{**}P < 0.01$; $^{***}P < 0.001$.

(Fig. 5l, m) in UVB-irradiated HaCaT cells, indicating that the YAP-TEAD branch had a protective effect against UVB-induced HaCaT cell apoptosis. This branch did not account for the protective effect of silibinin against UVB damage in HaCaT cells.

YAP-p73 pathway activation by UVB is inhibited by silibinin

Numerous studies on DNA damage caused by chemicals have shown that the YAP-p73 pathway is activated to initiate apoptosis. In this process, the transcriptional function of p73 is enhanced via

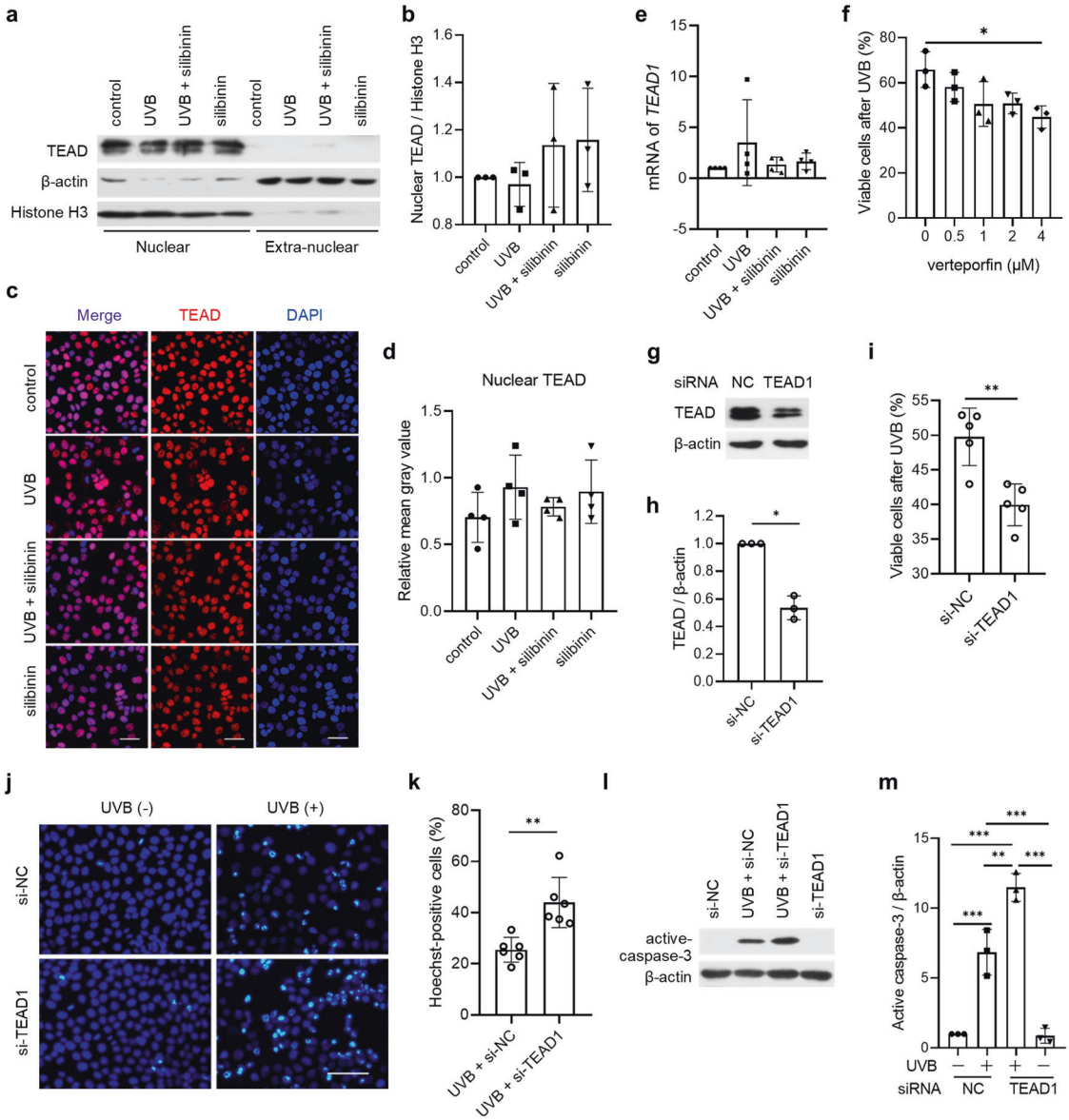

Fig. 5 The YAP-TEAD pathway promotes survival in UVB-treated HaCaT cells. a–b Nuclear and extranuclear TEAD (pan-TEAD) was analyzed by Western blotting. Histone H3 and β-actin were used as the loading controls for nuclear and extranuclear proteins, respectively. **c–d** TEAD (red, TRITC) was immunostained and observed under confocal microscopy. Bar, 20 μm. **e** The mRNA level of *TEAD1* was determined by qRT–PCR. **f** The immediate addition of the YAP inhibitor verteporfin after UVB irradiation further promoted HaCaT cell death. **g–h** HaCaT cells were treated with siRNA to silence TEAD1. **i** The silencing of TEAD1 further reduced cell viability in UVB-treated cells. **j–k** HaCaT cells with TEAD1 silencing were stained with Hoechst 33258. Bar, 50 μm. **l–m** The activation of caspase-3 was assessed by Western blotting. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

binding with YAP [10, 31, 32]. We next investigated the role of p73 in UVB-induced HaCaT cell apoptosis. Western blotting showed that nuclear p73 expression was upregulated in UVB-treated cells but reduced by cotreatment with silibinin (Fig. 6a, b). The confocal microscopy results showed that the fluorescence of nuclear p73 was markedly increased in UVB-treated cells but decreased with silibinin cotreatment (Fig. 6c, d). The mRNA levels of p73 were upregulated by UVB irradiation but downregulated by cotreatment with silibinin (Fig. 6e). The transcription of target genes in the YAP-p73 pathway, including *PML*, *p21* and *Bax* [11, 31, 34], was also stimulated by UVB but repressed by silibinin cotreatment (Fig. 6f–h), confirming the participation of the YAP-p73 pathway in this apoptotic process. The protein level of PML, as shown by confocal microscopy (Fig. 6i, j), and the protein levels of p21 and Bax, as shown by Western blotting (Fig. 6k, l), were all increased in UVB-treated cells, but decreased with silibinin cotreatment. Depletion of p73 by siRNA transfection (Fig. 6m, n) reduced the mRNA levels of *PML*, *p21* and *Bax*, and silencing YAP also resulted in a similar trend, confirming the regulation of these genes by the YAP-p73 pathway in UVB-irradiated HaCaT cells (Fig. 6o–q). The repression of these proapoptotic proteins in the p73 branch might be responsible for the antiapoptotic role of silibinin.

The YAP-p73 pathway accounts for the apoptosis caused by UVB irradiation

Silencing of p73 increased cell survival after UVB exposure (Fig. 7a). Moreover, after p73 was eliminated, the increase in Annexin V-positive cells (Fig. 7b, c), nuclear condensation and fragmentation (Fig. 7d, e), and activation of caspase-3 (Fig. 7f, g) in UVB-irradiated HaCaT cells were all attenuated. These results

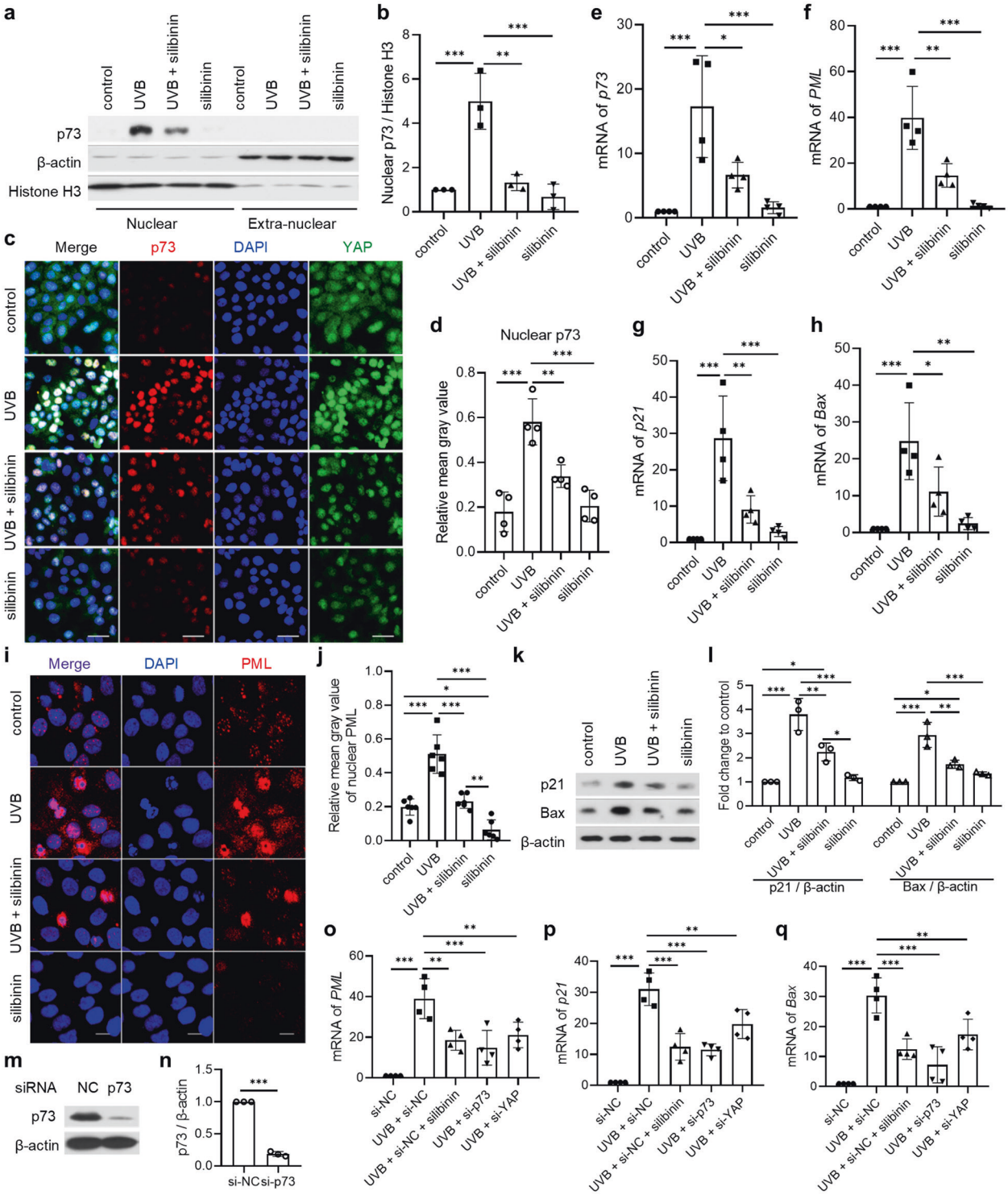

Fig. 6 The YAP-associated nuclear transcription factor p73 is regulated by UVB and silibinin treatment. a–b Nuclear and extranuclear p73 was examined by Western blotting. Histone H3 and β-actin were used as the loading controls for nuclear and extranuclear proteins, respectively. **c–d** p73 (red, TRITC) and YAP (green, FITC) were immunostained and observed by confocal microscopy. Bar, 20 μm. **e** mRNA of p73. **f–h** The mRNA levels of the p73 target genes *PML*, *p21* and *Bax* were quantified by qRT–PCR. **i–j** PML (red, TRITC) was immunostained and observed by confocal microscopy. Bar, 10 μm. **k–l** The protein levels of the p73 target genes p21 and Bax were examined by Western blotting. **m–n** The p73 protein was knocked down by transfection of siRNA. **o–q** mRNA levels of the p73 target genes *PML*, *p21* and *Bax* in cells transfected with siRNA targeting p73. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

supported the proapoptotic effect of the YAP-p73 pathway in UVB-irradiated cells. We further treated the cells simultaneously with UVB, verteporfin and silibinin. Interestingly, silibinin treatment not only rescued the cells with UVB exposure but also rescued the cells cotreated with UVB and verteporfin, in which the YAP-TEAD branch was inhibited (Fig. 7h–j). These findings suggest that silibinin's inhibitory effect on the YAP-p73 pathway was protective in UVB-irradiated cells.

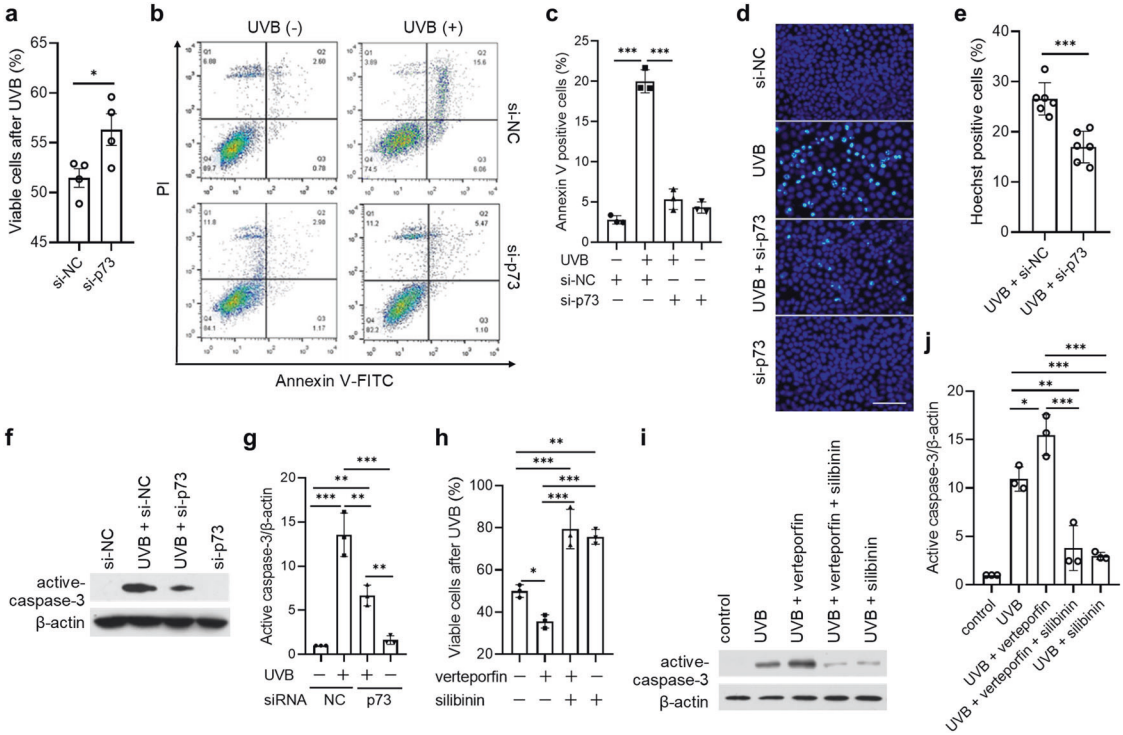

Fig. 7 Role of the YAP-p73 pathway in UVB-irradiated HaCaT cells. a Viability of UVB-treated cells after p73 was silenced. **b**–**c** The annexin V-positive cell ratios were analyzed after p73 was silenced. **d**–**e** HaCaT cells with p73 silencing were stained with Hoechst 33258. Bar, 50 μm. **f**–**g** Western blotting results of active caspase-3 in cells transfected with si-p73. **h** The viability of cells treated with verteporfin and silibinin was analyzed by MTT assay. Verteporfin, 4 μM. **i**–**j** Western blotting of active caspase-3 in UVB-treated cells treated with verteporfin and silibinin. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Inhibition of the YAP-p73 pathway plays a role in silibinin-mediated protection of HFFs against UVB irradiation

Our previous study found that silibinin protected dermal HFFs from UVB injury [20]. The role of the YAP-p73 pathway in HFFs was also studied here. Western blotting showed that the YAP protein was not changed by UVB irradiation or silibinin treatment but that p-YAP levels were increased by silibinin treatment in both UVB-irradiated and unirradiated cells, suggesting that silibinin has an inhibitory effect on YAP (Fig. 8a, b). Immunostaining of YAP showed that UVB irradiation increased the nuclear translocation of YAP, while silibinin treatment reduced YAP nuclear translocation (Fig. 8c, d). Western blotting showed that nuclear YAP levels were increased by UVB irradiation but decreased by silibinin cotreatment (Fig. 8e, f). The mRNA levels of *YAP* and *TEAD1* were found to be unaffected by either UVB or silibinin, while the levels of *p73* and its target genes, including *PML*, *p21* and *Bax*, were increased in UVB-treated cells but decreased in the cells cotreated with silibinin (Fig. 8g–l). Silencing p73 with siRNA increased the viability of UVB-treated HFFs (Fig. 8m). Taken together, these findings suggest that silibinin inhibits the YAP-p73 proapoptotic pathway in UVB-irradiated epidermal HaCaT cells and dermal HFFs, strongly suggesting that silibinin has the potential to be utilized in a protective strategy against skin injury caused by UVB irradiation.

DISCUSSION

The involvement of the YAP pathway in UVB-induced cell injury has not yet been extensively studied. Previous studies on UVC- or UV-induced YAP activation that involves the JNK and p38 pathways have suggested that the YAP-TEAD pathway has a protective effect [35, 36]. However, the role of p73 was not studied in their cell models. Our study strictly focused on UVB, and clearly distinguished the prosurvival TEAD branch and proapoptotic p73 branch of the YAP pathway in UVB-treated HaCaT cells, supplementing the gap between the UVB and YAP pathways.

However, the upstream mechanism by which YAP is regulated under UVB irradiation remains poorly understood. The possibility that the nuclear translocation of YAP is initiated by DNA damage-induced stress, oxidative stress, or inactivation of the Hippo pathway was suggested by the reductions in p-YAP levels in UVB-treated HaCaT cells [7, 8]. Under our assumptions, there is also another possibility: some other mechanical stimuli might be the main culprits. YAP nuclear translocation is reported to be influenced by the cell attachment area, substrate stiffness and shape of the nucleus [7, 8]. Cell and nuclear flattening caused by mechanical forces stretches nuclear pores, reduces their mechanical resistance to molecular transport, and increases YAP nuclear import [37]. Through morphological observations and routine experiments, we found that UVB-irradiated cells were more flattened in shape than control cells and were more resistant to trypsin digestion (the digestion time was prolonged 1.5- to 2-fold). These phenomena suggested that the interaction between cells and the extracellular matrix was changed, which may also explain why YAP cellular localization changes under UVB stress.

Silibinin has multiple targets in cells, but modulation of the YAP pathway has not been frequently studied before. There has only been one report regarding the influence of silibinin on YAP signaling; the corresponding study found that silibinin suppresses YAP expression in glioblastoma cells [38]. In our study, we found that silibinin markedly reduced YAP nuclear translocation in UVB-irradiated epidermal HaCaT cells and dermal HFFs. Western blotting analyses showed that silibinin promotes the phosphorylation of YAP at Ser127, which promotes the cytoplasmic retention of YAP [27]. Moreover, the molecular docking and DARTS results indicate that silibinin has the potential to directly

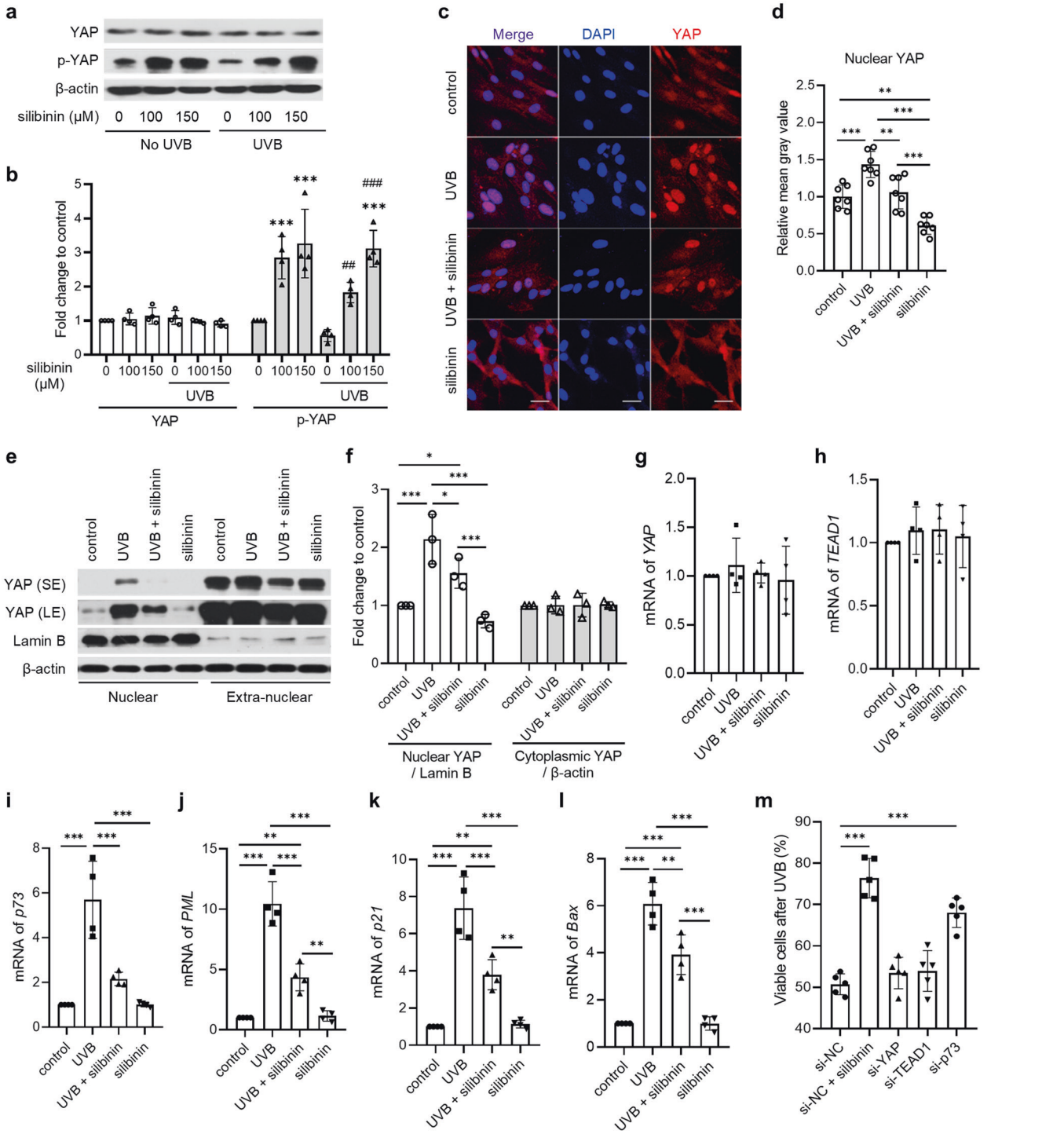

Fig. 8 The YAP-p73 pathway also plays a role in UVB-irradiated HFFs. a–**b** The protein levels of YAP and p-YAP in HFFs treated with UVB or silibinin. ***$P < 0.001$ vs. control group. ##$P < 0.01$ vs. UVB group; ###$P < 0.001$ vs. UVB group. **c**–**d** The nuclear distribution of YAP (red, TRITC) was studied by confocal microscopy. Bar, 20 μm. **e**–**f** Nuclear and extranuclear YAP were analyzed by Western blotting. Lamin B and β-actin were used as the loading controls for nuclear and extranuclear proteins, respectively. SE short exposure, LE long exposure. **g**–**l** mRNA levels of *YAP*, *TEAD1*, *p73*, *PML*, *p21* and *Bax* in HFFs treated with UVB or silibinin. **m** Cell viability was determined by MTT in UVB-irradiated HFFs with YAP, TEAD1 or p73 silencing. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

bind the YAP protein. A possible binding site in YAP, Val131, which is close to Ser127, may facilitate Ser127 phosphorylation. The inhibitory effect of silibinin on YAP signaling is possibly a common phenomenon across different cell types, since we have observed this phenomenon in HaCaT cells, human foreskin fibroblasts and MCF-7 human breast cancer cells (unpublished). This may account for the versatile or complex pharmacological effects of silibinin in different contexts. Silibinin has dual effects. For example, it shows cytoprotection during cellular stresses, including neuronal damage caused by overtraining [16], amyloid β [39] and LPS [40]; pancreatic β-cell injury induced by TNFα, IL-1β [41] and amylin [42]; and epidermal and dermal cell damage caused by

UVB exposure. However, silibinin also shows antiproliferative effects on cancer cells, including breast cancer cells [15], melanoma cells [43], fibrosarcoma cells [44] and cervical carcinoma cells [45]. It seems that silibinin has the potential to enhance cell function during various stresses, but it also restrains cell proliferation to some extent. Since the stresses induced by many different stimuli are all blocked by silibinin treatment, there must be some consolidated mechanisms. The YAP pathway also has a dual role in cell survival and death. This pathway employs transcriptional cofactors, including TEADs, to mediate cell survival signaling but relies on proapoptotic cofactors, including p73, to cause cell death [7, 8]. During unstressed cellular environments, the pro-apoptotic branch of YAP is not activated, and the pro-survival branch is dominant. Under these circumstances, inhibition of the YAP pathway by silibinin probably exhibits antiproliferative action. Under stressed conditions, the cytotoxic branch of YAP is activated. In these cases, the inhibitory effect of silibinin on the YAP pathway gives the cytoprotective results. Although it is difficult to fully reveal the modulatory mechanisms of silibinin in different directions, the regulation of YAP might provide some cues.

HaCaT, a spontaneously transformed aneuploid immortal keratinocyte cell line, closely approximates normal keratinocytes [46]. HaCaT maintains full epidermal differentiation capacity and expresses key markers of keratinocytes, thus offering a suitable model for human epidermal keratinocyte research [46–48]. However, HaCaT cells are still abnormal compared to primary epidermal keratinocytes, with hypotetraploid chromosomes and somewhat altered transcriptional profiles [46]. More studies using primary keratinocytes or in vivo studies should be performed to further confirm the role of the YAP-p73 pathway in UVB-irradiated skin cells and tissues.

Our study clarifies the role of the YAP pathway, especially the YAP-p73 branch, in apoptosis of UVB-irradiated epidermal HaCaT cells and dermal HFFs. Silibinin protects the irradiated cells from apoptosis by inhibiting the p73 pathway. However, the YAP-TEAD pathway is a prosurvival pathway in UVB-stressed cells. These findings emphasize the complexity of YAP pathways. We believe that maximal protection against UVB injury is achieved by inhibition of YAP-p73 together with promotion of YAP-TEAD.

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AUTHOR CONTRIBUTIONS

WWL and TI designed the research; WWL, FW, CL, WO and YYZ performed the research; WWL and FW analyzed the data; WWL wrote the paper; XYS performed the molecular docking study; SH and HF contributed to cell culture and contributed some reagents; and TI, TH and KM revised the paper.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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