Rapamycin Protects Mouse Skin from Ultraviolet B-Induced Photodamage by Modulating Hspb2-Mediated Autophagy and Apoptosis

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Abstract

Continuous exposure to UVB is the main extrinsic cause of skin photodamage, which is associated with oxidative stress, DNA damage, apoptosis and degradation of collagen. Rapamycin, a mechanistic target inhibitor of rapamycin complex 1 (mTORC1), has been shown to play a crucial role anti-tumor and aging retardation, but its mechanism of action in UVB-induced photodamage still remains unknown. In this study, we investigated the role of rapamycin and Hspb2(also known as Hsp27)in UVB-induced photodamage in mice. Histological results showed that knockout of the *hspb2* exacerbated the skin damage, as evidenced by thickening of the epidermis, breakage and disruption of collagen fibers and reduction in their number, which is reversed by rapamycin treatment. In addition, *hspb2* knockout promoted UVB-induced apoptosis and reduced autophagy levels, with a significant increase in p53 levels and Bax/Bcl-2 ratio, a reduction in LC3II/I ratio and an increase in p62 levels in the KO mice compared to those in WT mice after the same dose of UVB irradiation. Rapamycin was also found to inhibit collagen degradation induced by *hspb2* knockdown through activation of the TGF-β/Smad signaling pathway. There is some evidence that rapamycin may alleviate UVB-induced photodamage.

INTRODUCTION

As the outermost layer of our body, skin is the first barrier against external pathogens and environmental damages(1). UV light is widely present in our lives and affects our skin like a double-edged sword. On the one hand, artificial lamps emitting ultraviolet radiation can be used to treat many skin diseases such as atopic dermatitis, psoriasis and vitiligo(2, 3). On the other hand, overexposure of skin to UV radiation can induce genotoxicity, inflammation and oxidative stress, leading to acute photodamage, photoaging and even skin cancer(4, 5). Studies have shown that following UVB exposure, normal human skin fibroblasts activate activator protein 1(AP-1), which causes increased activity of matrix metalloproteinase MMPs and inhibits collagen synthesis by disrupting the transforming growth factor beta 1 (TGF-β1) pathway (6).

When the skin is exposed to UVB rays, DNA damage can be triggered and can be regulated by autophagy through nucleotide excision repair(7). Autophagy is a process of cellular self-digestion that degrades misfolded or unfolded proteins and damages the organelles within the cell to maintain cellular homeostasis, as well as an adaptive process that provides nutrients and energy to the organism during stressful conditions (8). UVB irradiation can also induce apoptosis by activating the tumor suppressor p53 or by mitochondrial damage and cytochrome C release (9). Apoptosis is an evolutionarily conserved form of procedural cell death and plays an essential role in the progression of many physiological and pathological processes. It has been shown that activation of autophagy can inhibit apoptosis, acting as a self-repair mechanism for halting cell damage (10).

Previous studies have demonstrated that Hspb2 is an important anti-apoptotic protein (11). Small heat shock protein 27 (Hspb2), a new member of the heat shock protein family, is highly represented in heart and skeletal muscle (11). Previous studies by our group have also shown that Hsp27 can protect the skin from UVB-induced photodamage by mediating autophagy and the production of reactive oxygen species

(12). Rapamycin, a specific inhibitor of mTOR, has been shown in many studies, both in vivo and in vitro, to have an important role in delaying aging and treating age-related diseases (13–16). In addition, it is often used as an immunosuppressant for acute rejection after organ transplantation and as an anti-tumor therapy for various cancers, such as advanced renal cell carcinoma, advanced breast cancer, progressive pancreatic neuroendocrine tumors (17). However, studies on the mechanism of rapamycin in UVB-induced photodamage, especially in vivo studies, are relatively scarce. In this research we investigate the role of Hspb2 in a UVB-induced skin photodamage model in mice.

MATERIALS AND METHODS

Establishment and transmission of hspb2 KO mice. *Hspb2* knockout mice were produced by Cas9/CRISPR-mediated genome editing (Cyagen Biosciences Inc., Guangzhou, China). The animal background chosen was C57BL/6J. The gDNA vector was first designed (Fig. 1A). The *hspb2* gene (NCBI reference sequence: NM_024441; collection: ENSMUSG00000038086) is situated in chromosome 9 of mice, and two exons were identified, with the ATG starting codon in exon 1 and the TGA terminating codon in exon 2 (transcript: ENSMUST00000042790). Exon 2 was chosen as the target site. The gRNA vector and Cas9 vector were then transcribed in vitro and co-injected them into the fertilized eggs, and the mice were identified by PCR and sequencing after birth to obtain positive F0 mice. The F1 heterozygous mice were mated and the toes of the newborn mice were taken for PCR identification. For amplification, the primer sequences are as follows: Forward primer (F1): 5'-GTACTTGGGATCA GGGCTACTAG-3', Reverse primer (R1): 5’-GAATGGGAGATGGTTCACCTCAG-3’ (Targeted allele: 222 bp; Wildtype allele: 253 bp), resulting in KO mice. WT mice show one band at 253bp, while KO mice show one band at 222bp and heterozygous mice show two bands, as shown in Fig. 1B, with mice 3/7 being the *hspb2* KO mice.

Animals. *Hspb2* KO mice (n = 10, 18–22 g) and age-matched WT mice (n = 10, 18–22 g, purchased from the Animal Experiment Center of Chongqing Medical University) were used in this study. They were housed at an ambient temperature of 23 ± 2°C, 60% relative humidity, and 12/12 h day/night alternation, with free access to feed and water. This study conformed to the ethical criteria set by the Experimental Animal Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Preparation of rapamycin mixture. 25 mg of rapamycin powder (cat.no. HY-10219, MCE, USA) was added into 1367 µl of dimethyl sulfoxide (DMSO) to configure a 20 mM master mix. 1.7 µl of the master mix was added 600 µl of pro-solvent (10% DMSO (Beyotime Biotechnology, China), 40% PEG300, 5% Tween-80 (MCE, USA) and 45% saline in that order). Each mouse was administered subcutaneously in the ear at 0.1 mg/kg, and the same volume of pro-solvent was given to the control group.

UVB radiation. Based on the study by Sajo et al.(18), we created a UVB-induced photodamage model of mouse ear skin. In the UVB irradiation group, the ear skin of each mouse was irradiated with a UVB lamp (TL20W/01, Philips, The Netherlands) emitting at 280–311 nm, at a total dose of 2700 mj/cm², once a day for two times.

Quantitative RT-PCR (qRT-PCR) analysis. After 24 h of the last illumination, the mice were executed and the skin tissue of the ears was taken. Total RNA was extracted by RNAiso Plus kit (TaKaRa, Japan) and reverse transcribed into cDNA by RT Master Mix for qPCR (MCE, USA). SYBR Green qPCR Master Mix (MCE, USA) was used to perform qRT-PCR, and GAPDH was amplified and used as an internal control. The primer sequences for the targeted genes are listed as follows:

p62, forward: 5'-ACTACCCCAGAAAGTTCCAGC-3',reverse: 5'-TTTTCCCGACT CCATCTGTTC-3'; *LC3B*, forward: 5'-GCTAACCAAGCCTTCTTCCTC-3',reverse: 5'-TGCTGTCCCGAATGTCTCC-3';*GAPDH*,forward:5'-GACATCAAGAAGGTGG TGAAGC-3',reverse:5'-GAAGGTGGAAGAGTGGGAGTT-3';*hspb2*,forward:5'-CG AGTACGAATTTGCCAACCC-3',reverse:5'-CAGAAACGCCTGGAACTTGC-3'; *ATG5*,forward:5'-GTTTTGGCTTTGGTTGAAGGAAGA-3',reverse: 5'-AATTCGTC CAAACCACACATCTC-3'; *CollagenI*,forward:5'-CCCGAGGTATGCTTGATCTGT AT-3',reverse:5'-TCCCTCGACTCCTACATCTTCTG-3';*Bax*,forward:5'-TTTTGCTA CAGGGTTTCATCCAGG-3',reverse:5'-TCATCCTCTGCAGCTCCATATTG-3';*Bcl-2*,forward:5'-GGATTGTGGCCTTCTTTGAGTTC-3',reverse:5'-CTTCAGAGAGAC AGCCAGGAGAAAT-3'; *P53*, forward: 5'-AGACCGCCGTACAGAAGAAGAAA-3',reverse:5'-CGGAACATCTCGAAGCGTTTAC-3';*MMP1*,forward:5'-GAGTGCCT GATGTGGGTGAATAC-3',reverse:5'-ATGTCAGCAGTGCCATCATAGAT-3'; *MMP3*,forward:5'-TGTGCAGCTCTACTTTGTTCTTTG-3',reverse:5'-CCTCGTATA GCCCAGAACTGATT-3';*MMP9*,forward:5'-CCATGTCACTTTCCCTTCACCTT-3', reverse:5'-TCTCACTAG GGCAGAAACCAAAT-3'

Western blot analysis. Proteins from mouse ear skin were extracted by T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher, USA), and the total protein concentration of each sample was determined using a BCA assay kit (Bimake, China). Protein samples were separated on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to nitrocellulose membranes (NC membranes), which were placed at room temperature in 7% skimmed milk for 2 h at room temperature and incubated overnight at 4°C in the following primary antibodies: GAPDH (Bimake, USA), P62 (Bimake, USA), LC3B (Sigma, USA), ATG5 (Bimake, USA), Bax (Proteintech, China), Bcl-2 (ZEN BIO, China), P53 (Proteintech, China), Hspb2 (Abclonal, China), and Collagen I (Abclonal, China). This was followed by incubation in secondary antibodies at room temperature for 1 h. Finally, the protein bands were imaged using the SuperSignal™ West Femto Maximum Sensitivity Substrate Kit (Thermo Fisher, USA).

Histological analysis. Mouse ear skin samples were obtained, fixed in 4% paraformaldehyde for 24 h, dehydrated in ethanol, and then embedded in paraffin and sectioned to a thickness of approximately 3–4 μm. The paraffin was then removed, and hematoxylin-eosin staining (HE staining) and Masson trichrome staining were performed for histological evaluation. Immunohistochemical staining was performed with TGF-β(1:100), Collagen I (1:100) for labelling, p62 (1:50), and LC3B (1:100) for immunofluorescence labelling, and all stained skin samples were observed using a section scanner (Pannoramic DESK, P-MIDI, P250, Hungary).

Statistical analysis. All experiments were repeated at least 3 times and statistical analyses were performed using Graphpad Prism 8.0.2 (GraphPad Software, Inc., USA). All the data are expressed as mean ± standard deviation. Student's t-test and one-way ANOVA were used to compare the differences

between 2 groups as well as between multiple groups, respectively, and p-values < 0.05 were considered statistically significant.

RESULTS

UVB radiation attenuates autophagy and promotes apoptosis in mouse skin

To assess the effects of UVB radiation on the levels of autophagy and apoptosis in mice, we created a UVB-induced mouse skin photodamage model. As shown in Fig. 1E and F, compared with the control group, the UVB irradiated group showed no significant change in the protein level of Hspb2, but a significant increase in the mRNA level and in the protein level of its phosphorylated form, p-HSP27, suggesting that Hspb2 may play a protective role in UVB-induced photodamage. Western blotting results showed that at the protein level, the expression of ATG5 was decreased, the expression of p62 was increased and the ratio of LC3II/ was decreased (Fig. 1E and I), and the same results were demonstrated in qRT-PCR (Fig. 1F). The results suggest that UVB radiation could lead to an attenuation of autophagy levels. Furthermore, after UVB irradiation, the levels of p53 and Bax were increased and Bcl-2 levels were decreased, which was observed at both protein and mRNA levels (Fig. 1E and H).

<Figure 1>

Rapamycin can alleviate UVB-induced skin damage

Studies have shown that UVB-induced skin damage is histologically characterized by thickening of the epidermis(19) and can damage the dermal connective tissue (20). In order to investigate the protective role of Hspb2 in UVB-induced skin photodamage, acute skin photodamage models were constructed in KO mice and WT mice. It was found that the epidermis of the WT group was thickened and blue collagen fibres were reduced, broken and disorganised, and rapamycin treatment reversed these changes (Fig. 2A, B). After UVB radiation, the skin accumulates large amounts of ROS intracellularly, leading to an increase in matrix metalloproteinase MMPs in the skin (21). The results of western blotting, qRT-PCR, and IHC (Fig. 3C ~ F) showed that deletion of the *hspb2* gene led to further accumulation of MMP1, 3, and 9 and a decrease in collagen fibril I levels; while rapamycin reduced the levels of matrix metalloproteinases, thereby slowing the degradation of collagen fibrils and acting as a photoprotective agent.

<Figure 2>

Rapamycin reverses reduced autophagy levels in hspb2 KO mice

After UVB radiation, the protein and mRNA expression levels of p62, ATG5 and LC3 in the skin tissues were examined. It was found that compared to wild-type mice, the *hspb2* gene knockout mice had significantly higher levels of p62 and lower LC3II/I ratios, while no significant change of the levels of

ATG5 in protein or mRNA levels (Fig. 3A, B, C). This suggests that Hspb2 may promote autophagy in UVB-induced skin damage and thus play a protective role. Furthermore, rapamycin treatment elevated autophagy levels. Within fixed, paraffin-embedded and sectioned skin tissues in parallel with immunofluorescence staining, the results again corroborated this contention (Fig. 3D, E, F).

<Figure 3>

Rapamycin inhibits the elevated apoptosis induced by hspb2 knockout

As a repressor of transcription, p53 can be highly induced by a variety of stress signals such as DNA damage. Activation of p53 can induce cell cycle arrest as well as apoptosis (22). In previous studies by our group, it has been shown that knockdown of Hspb2 accelerates apoptosis in photodamaged Hacat cells by activating the p53/Bax/Bcl-2-dependent mitochondrial apoptosis pathway (23). To determine the effect of Hspb2 as well as rapamycin on the level of apoptosis in photodamaged skin of mice, we examined the differences in protein and mRNA expression levels of p53, Bax and Bcl-2. As shown in Fig. 4A ~ C, knockdown of *hspb2* in mouse skin further increased the level of p53 as well as the Bax/Bcl-2 ratio, further accelerating apoptosis. Rapamycin treatment, on the other hand, inhibited the progression of apoptosis.

<Figure 4>

DISCUSSION

UV light is undeniably vital to our lives and has significant benefits for a number of systems, including the metabolism and cardiovascular systems (24). However, unlike other organs, the skin is the body's first barrier to the external environment and can cause sunburn, abnormal pigmentation and even malignant tumours after acute or prolonged exposure to UV light (25). In recent years, the increase in emissions of atmospheric pollutants has led to depletion of the ozone layer, resulting in a year-on-year increase in the amount of ultraviolet light on the earth's surface. In the face of the negative effects of ultraviolet light on people, prevention and treatment of photodamage to the skin caused by UV light has become one of the most important research topics(26). In our study, we established an acute photodamage model by UVB irradiation of mouse ear skin. Interestingly, similar to the manifestations of chronic photoaging, after acute UVB irradiation, a build-up of matrix metalloproteinases and a reduction in type I collagen fibres were observed.

Hspb2, as a molecular chaperone, ensures correct folding of proteins and promotes correct repetition of damaged proteins, and is thus essential for the maintenance of proper protein structure and function in cells (27). In previous studies by our group, we have demonstrated that Hsp27 plays an anti-apoptotic role in UVB-induced skin photoaging and photodamage of Hacat cells and rats' skin (28, 23). The experimental results in this study also confirmed this view. According to the previous findings of our group, the protein expression level of Hsp27 was not significantly altered in UVB-induced photodamage

models of epidermal keratin-forming cells and dermal fibroblasts, while its phosphorylated form, P-Hsp27, was significantly elevated and activated autophagy (12). Similar results were achieved in the experiments in this study. After UVB irradiation, the mouse ear skin showed no significant change in Hspb2 protein levels, but mainly elevated mRNA levels and significantly higher protein expression levels of P-HSP27 (Fig. 1E,F). Interestingly, unlike the cellular model, mouse skin showed a significant down-regulation of autophagy levels after photodamage. Furthermore, in the UVB-induced mouse skin photodamage model, the KO group showed increased p62 levels and decreased LC3II/I levels compared to the WT group, suggesting that Hspb2 may play a protective role in skin photodamage by activating autophagy.

It is well known that extensive exposure to UVB is a major risk factor for skin damage. This is because after UVB radiation, large amounts of reactive oxygen species are produced, leading to an inflammatory response, oxidative stress, DNA damage and apoptosis (29). In addition, excess reactive oxygen species activate the MAPK/AP-1 signaling pathway and NF-κB signaling pathway, promoting the transcriptional regulation of MMPs and ultimately leading to degradation of collagen and elastin(30), In our study, UVB-irradiated mouse skin showed diminished levels of autophagy, increased levels of apoptosis, increased MMP1/3/9, as well as decreased Col-1. Rapamycin, an inhibitor of the mTOR pathway, is also an immunosuppressant and antitumour agent that delays cell and organ ageing and extends life span (31). Rapamycin has been shown to protect mouse dermal fibroblasts from UVB-induced photoaging by inhibiting production of reactive oxygen species and maintaining normal levels of autophagy (32). This effect of rapamycin may be mediated by activation of the Nrf2-Keap1 signaling pathway, which promotes the expression of antioxidant proteins through induction of autophagy (33). In our previous study, rapamycin has been shown to reduce p53 expression to protect skin fibroblasts from UVA-induced photodamage (34). In the present study, rapamycin could exert its protective effect by reducing p53 and thus increase the Bax/Bcl-2 ratio to inhibit the progression of apoptosis.

Transforming growth factor beta (TGF-β) is involved in the regulation of cell growth and differentiation, apoptosis and extracellular matrix production, and its mediated activation of Smad2/3 molecules plays an important role in synthesis of Type I collagen fibrils (35, 36). In our study, KO mice reduced levels of TGF-β compared to WT mice. In contrast, rapamycin treatment activated the TGF-β/Smad signaling pathway, thereby promoting the expression of Type I collagen fibers and reducing the production of MMP1/3/9. These results suggest that Hspb2 and rapamycin play a synergistic role in regulation of the TGF-β pathway.

Although our study demonstrated that rapamycin can be a potential therapeutic agent for photodamage, we have not yet elucidated its association with Hspb2 and the mechanism by which they mediate the progression of autophagy and apoptosis, which will be the focus of more in-depth studies in our subsequent studies.

Declarations

ETHICAL APPROVAL The animal study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

COMPETING INTERESTS All the authors have no conflict of interest to declare.

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AUTHORS' CONTRIBUTIONS Conceptualization, C-AJ, PW ; methodology, AL and JX; software, AL and B-GL; validation, AL , PW and G-LB; formal analysis, G-LB and Wen-ZY; investigation, AL and JX; resources, C-AJ and PW; data curation, Wang-ZY, AL and J-YX; writing—original draft preparation, AL, Wen-ZY and PW; writing—review and editing, C-AJ and PW; supervision, PW; project dministration, C-AJ; funding acquisition, C-AJ and PW. All authors have read and agreed to the published version of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS The original contributions presented in the study are included in the article/Supplementary Ma-terial, further inquiries can be directed to the corresponding authors.

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Figures

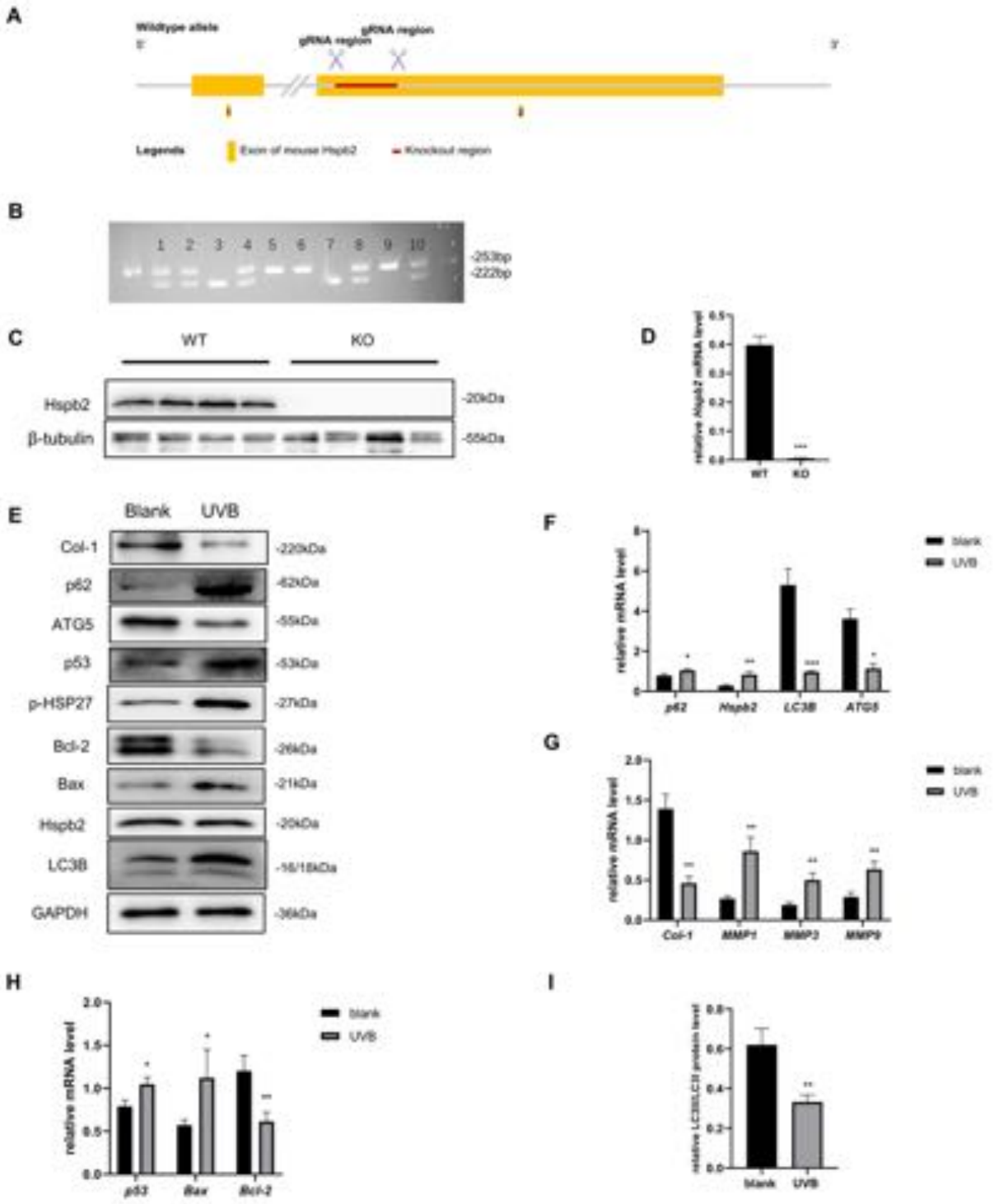

Figure 1

Establishment of *hspb2*knockout mouse model (C57BL/6J) and Effect of UVB irradiation on autophagy and apoptosis levels as well as collagen fiber expression in mouse skin. **(A)** Based on the genetic information, the knockout region was identified and the gDNA vector was designed. (**B)**The toes of the mice were cut at 7-10 days of age, DNA was extracted and used for PCR amplification, followed by agarose gel electrophoresis to detect their genotypes. (**C)** Differences in Hspb2 protein expression in the

ear skin tissues of WT and KO mice were detected by western blotting using β-tubulin as a reference. (**D)** Differences in *hspb2* at the mRNA level in the ear skin tissues of WT and KO mice were detected by qRT-PCR and quantified using GAPDH as a reference. **(E)** The expression of Hspb2, p-Hsp27, p62, ATG5, LC3B, p53, Bax, Bcl-2 and Collagen I proteins in mouse ear skin tissues was detected by Western blotting after UVB irradiation, using GAPDH as a reference. **(F–H)**The mRNA levels of *hspb2*, p62, ATG5, LC3B, p53, Bax, Bcl-2 and Collagen I, MMP1/3/9 in mouse ear skin tissues were measured by qRT-PCR after UVB irradiation, using GAPDH as reference. **(I)** LC3II/I protein expression after UVB irradiation. All results are expressed as mean ± SD, and experiments were repeated at least three times (*p<0.05, **p<0.01, ***p<0.005).

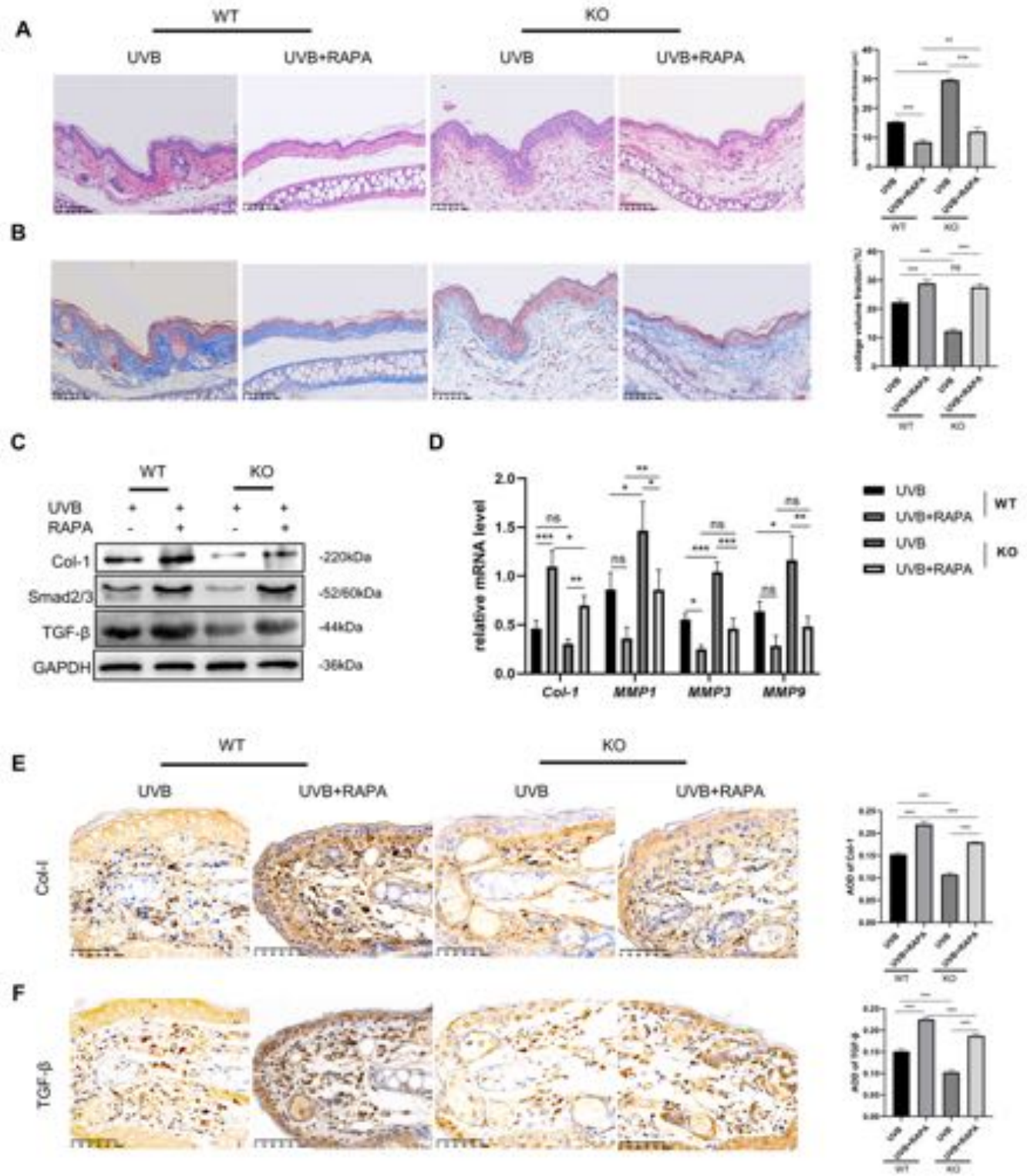

Figure 2

*Hspb2*knockout and the effect of rapamycin on UVB-induced skin damage in mice. **(A)** HE staining results and quantification of mean epidermal thickness. **(B)** Masson trichrome staining results and quantification of collagen fibre volume fraction. **(C)** The protein expression levels of TGF-β, Smad2/3 and Col-1 in skin tissues of WT and KO mice treated with rapamycin were measured by western blotting using GAPDH as a reference. (**D**) The mRNA expression levels of Col-1 and MMP1/3/9 in the skin tissues of WT

and KO mice treated with rapamycin were measured by qRT-PCR using GAPDH as the reference. **(E,F)** Results of immunohistochemical labeling of Collagen I and TGF-β in mouse skin tissues and quantification of the mean optical value (AOD). All results are expressed as mean ± SD and experiments were repeated at least three times (*p<0.05, **p<0.01, ***p<0.005).

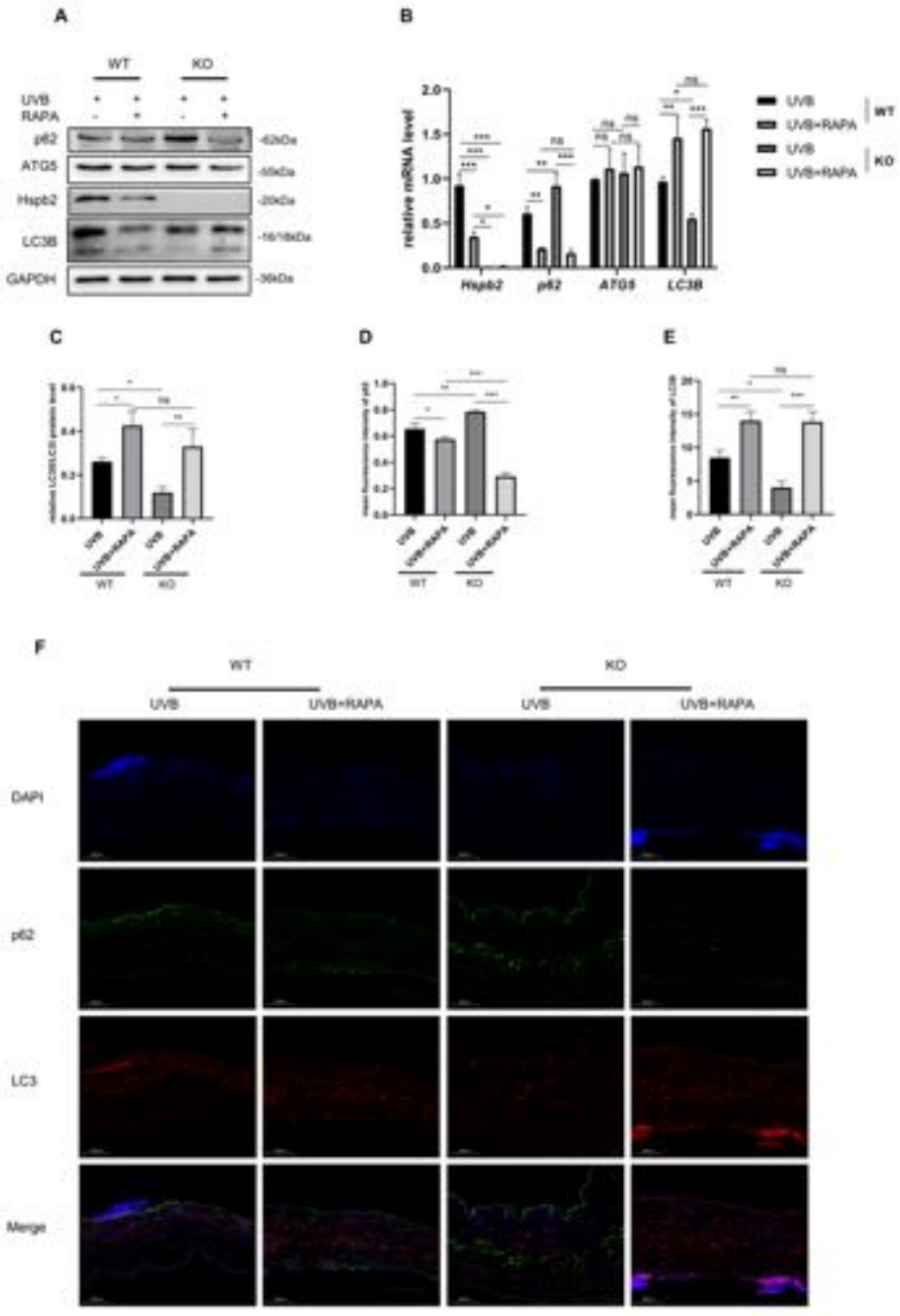

Figure 3

Effect of *hspb2*knockout and rapamycin treatment on skin autophagy levels in mice after UVB irradiation. **(A)** The protein expression levels of p62, ATG5, Hspb2 and LC3B in skin tissues of WT and KO mice receiving rapamycin treatment were detected by western blotting using GAPDH as a reference. **(B)** The mRNA expression levels of p62, ATG5, *hspb2* and LC3B in the skin tissues of WT and KO mice after receiving rapamycin treatment were measured by qRT-PCR using GAPDH as the reference. **(C)**Detection of changes in LC3II/I protein expression by *hspb2* knockout and rapamycin treatment. **(D,E,F)** Results of immunofluorescence labeling of mouse skin tissues and quantification of immunofluorescence intensity, DAPI is shown in blue, p62 in red and LC3 in green. All results are expressed as mean ± SD, and experiments were repeated at least three times (*p<0.05, **p<0.01, ***p<0.005).

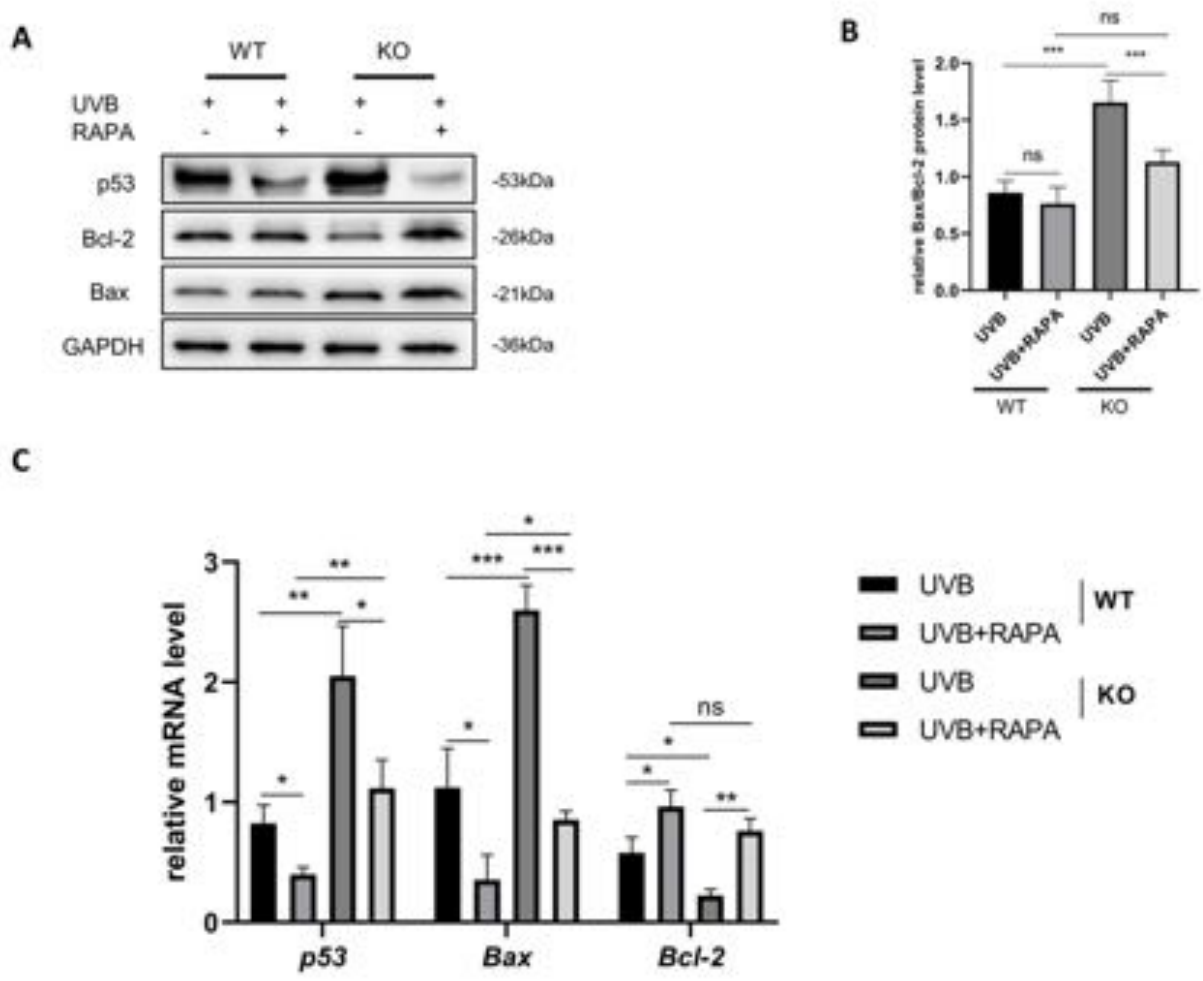

Figure 4

Effect of *hspb2*knockout as well as rapamycin treatment on the level of apoptosis in the skin of UVB irradiated mice. **(A)** The protein expression levels of p53, Bax and Bcl-2 in skin tissues of WT and KO groups of mice receiving rapamycin treatment were detected by western blotting using GAPDH as a reference. **(B)** Quantification of the ratio of Bax/Bcl-2 protein levels in skin tissues. **(C)** The mRNA

expression levels of p53, Bax and Bcl-2 in the skin tissues of WT and KO mice after receiving rapamycin treatment were detected by qRT-PCR using GAPDH as a reference. All results are expressed as mean ± SD and experiments were repeated at least three times (*p<0.05, **p<0.01, ***p<0.005).

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