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Retina

# Sirt3 Protects Retinal Pigment Epithelial Cells From High Glucose-Induced Injury by Promoting Mitophagy Through the AMPK/mTOR/ULK1 Pathway

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**Purpose:** The regulation of mitophagy by Sirt3 has rarely been studied in ocular diseases. In the present study, we determined the effects of Sirt3 on AMPK/mTOR/ULK1 signaling pathway-mediated mitophagy in retinal pigment epithelial (RPE) cells in a high glucose environment.

**Methods:** The mRNA expression levels of Sirt3, AMPK, mTOR, ULK1, and LC3B in RPE cells under varying glucose conditions were measured by real-time polymerase chain reaction (RT-PCR). The expressions of Sirt3, mitophagy protein, and AMPK/mTOR/ULK1 signaling pathway-related proteins were detected by Western blotting. Lentivirus (LV) transfection mediated the stable overexpression of Sirt3 in cell lines. The experimental groups were NG (5.5 mM glucose), hypertonic, HG (30 mM glucose), HG + LV-GFP, and HG + LV-Sirt3. Western blotting was performed to detect the expressions of mitophagy proteins and AMPK/mTOR/ULK1-related proteins in a high glucose environment during the overexpression of Sirt3. Reactive oxygen species (ROS) production in a high glucose environment was measured by DCFH-DA staining. Mitophagy was detected by labeling mitochondria and lysosomes with MitoTracker and LysoTracker probes, respectively. Apoptosis was detected by flow cytometry.

**Results:** Sirt3 expression was reduced in the high glucose group, inhibiting the AMPK/mTOR/ULK1 pathway, with diminished mitophagy and increased intracellular ROS production. The overexpression of Sirt3, increased expression of p-AMPK/AMPK and p-ULK1/ULK1, and decreased expression of p-mTOR/mTOR inhibited cell apoptosis and enhanced mitophagy.

**Conclusions:** Sirt3 protected RPE cells from high glucose-induced injury by activating the AMPK/mTOR/ULK1 signaling pathway.

**Translational Relevance:** By identifying new targets of action, we aimed to establish effective therapeutic targets for diabetic retinopathy treatment.

## Introduction

Diabetes mellitus (DM) is one of the most common chronic diseases. Diabetes can lead to higher mortality and serious complications.[1,2] Diabetic retinopathy (DR) is the most common ocular complication of diabetes, occurring in approximately 35% of patients with diabetes , and it is a leading cause of acquired vision loss in the working population.[3,4] Hyperglycemia increases the inflammatory response, apoptosis, and oxidative stress disorders in

the retina and capillaries.[5,6] The retinal pigment epithelium (RPE) acts as a permeable barrier between the blood and the retina, maintaining the retina's internal homeostasis,[7–9] and it plays a pivotal role in maintaining visual acuity and retinal function.[10] Recent studies have reported that hyperglycemia can lead to abnormalities in retinal metabolic pathways, RPE cell dysfunctions, and ultimate apoptosis of retinal cells.[11–13] However, the mechanism and role in DR are unclear.

Mitophagy maintains a balance between mitochondrial number and mass, and normal cellular physiological functions in the face of malnutrition or external stimuli.[14] Mitophagy disorders are associated with a variety of ophthalmologic diseases, for example, DR.[15] In DR treatment, berberine is a type of isoquinoline alkaloid, which enhances autophagy and attenuates apoptosis in rat retinal Müller cells by regulating the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling.[6] AMPK has been reported to enhance autophagy by either directly activating Unc-51-like (ULK1) protein or indirectly inhibiting ULK1 phosphorylation by mTOR. Therefore, it has been suggested that AMPK/mTOR/ULK1-mediated mitophagy is linked to the development and progression of DR.[16]

Mitophagy is an intracellular physiological phenomenon that facilitates the timely removal of damaged mitochondria. Mitochondrial acetylation is one of the important regulatory mechanisms used to activate mitochondrial enzymes.[17,18] Mitophagy is also a major source of reactive oxygen species (ROS).[19] Sirt3, a major mitochondrial nicotinamide adenine dinucleotide ($NAD^+$)-dependent deacetylase, is required for the metabolic adaptation of mitochondria to environmental conditions.[20,21] Sirt3 is involved in maintaining mitochondrial functions, including adenosine triphosphate (ATP) production, nutrient oxidation, ROS production, and cell death.[22,23] During retinopathy, overexpression of Sirt3 prevents DR, and Sirt3 deficiency causes internal retinal dysfunctions. Furthermore, it has been reported that Sirt3 overexpression ameliorated the morphological changes of retinal tissues in models of DR, promoted autophagy-related protein expression, and prevented VEGF expression in a DR rat model.[24–26]

Previous reports found that Sirt3 regulated mitophagy to protect RPE cells, and AMPK/mTOR/ULK1 was an important signaling pathway for mitophagy; but it is unknown whether Sirt3 acts on RPE cells through the AMPK/mTOR/ULL1 signaling pathway. In the present study, we therefore determined the effects of Sirt3 on RPE cells involving the AMPK/mTOR/ULK1 pathway in a high glucose environment, to identify the mechanism of Sirt3 during DR.

# Materials and Methods

## Materials

Materials and reagents were as follows: DEME/F12 medium (Hyclone, USA); fetal bovine serum (BI, Israel); RNA extraction kits (Biotec Biotechnology, China); reverse transcription kits (SYBR Green Raltime PCR Master Mix; Toyobo, Japan); primers (Shanghai Biotechnology, China); reactive oxygen species assay kits (Solarbio, China); Annexin V-PE/7AAD kit (Solarbio, China); mitochondrial red fluorescent probe (Solarbio, China); lysosomal green fluorescent probe (Beyotime, China); SDS-PAGE gel preparation kits (Aspen, America); RIPA total protein lysate kits (Aspen, America); BCA protein concentration assay kits (ECL Chemiluminescence Assay Kit; ECL, USA); protein markers (Thermo Fisher Scientific, USA); AMPK, mTOR, ULK1, p-AMPK, and p-ULK1 (CST, USA); and anti-p-mTOR (Abcam, UK); anti-$\beta$-actin (Tiande Yue, China), and Sirt3 and anti-LC3B (Wuhan Sanying, China).

## Cell Culture and Drug Treatment

Human RPE cells (ARPE-19, Lot number, 202202 17-02) were maintained in a 5% $CO_2$, 37°C cell culture incubator using DMEM/F12 (Hyclone, USA) medium containing 10% fetal bovine serum (BI, Israel) and 1% penicillin solution (Supplementary Materials 1 and 2). The medium was replaced every 2 days. To develop the hyperglycemic and hyperosmotic model groups of cells, RPE cells were maintained in a medium containing 5.5 mM D-glucose (normal group [NG]), 30 mM D-glucose (hyperglycemic group [HG]), and 5.5 mM D-glucose + 24.5 mM D-mannitol (hypertonic group) for 72 hours.[27,28]

## Lentiviral Infection of RPE Cells

RPE cells were inoculated in 6-well plates at $2 \times 10^5$ cells per well to achieve 30% cell confluency, the next day. For lentiviral infection, we added 1 mL of fresh medium to each well, adding polybrene (6 µg/mL) and the appropriate volume of the virus at a multiplicity of infection of 10, then we added 1 mL of fresh medium to each well after 4 hours of culture. After 24 hours of infection, we first aspirated the culture medium containing the virus from each well and replaced it with fresh complete culture medium to continue the

**Table.** The Primers Used in this Study Were Designed and Synthesized by Shanghai Biotechnology and are Listed in the Table

| Gene Name | Forward Sequences | Reverse Sequences |
|---|---|---|
| Sirt3 | CCCAGTGGCATTCCAGACTT | AAGCAGCCGGAGAAAGTAGTG |
| AMPK | TTTAGACCACAACCGCTCTAGTC | ATTATATGAGGATGCCTGAAAAGC |
| m-TOR | TCAGCCTGTCAGAATCCAAGTC | TTGAAGATGAAGGTGATGGCC |
| ULK1 | CTCTGCCTGTCGTCCACTGT | CTCTGCCTGTCGTCCACTGT |
| LC3B | TTCCGACTTATTCGAGAGCAGC | AGCATTGAGCTGTAAGCGCC |
| $\beta$-actin | GTCCACCGCAAATGCTTCTA | TGCTGTCACCTTCACCGTTC |

culture. Expression efficiency of the green fluorescent protein (GFP) was determined using a fluorescence microscope, 72 hours after infection. We then passaged the cells according to the percentage of cell confluency, then added puromycin (2 µg/mL) to the cells in subsequent experiments to screen-out puromycin-resistant cells for further experiments. The experimental groups were the following: NG (5.5 mM), hypertonic (30 mM), HG + LV-GFP, and HG + LV-Sirt3.

## Western Blot Analysis

Samples from each group of cells were collected and the total protein of cells was extracted using RIPA buffer (Aspen, USA) containing phosphatase inhibitor, then the concentration of total protein was determined using a BCA kit. Equivalent proteins were transferred to PVDF membranes after resolving them using SDS-PAGE (Aspen), then the membranes were incubated with primary antibodies overnight at 4°C. The membrane was then incubated with a secondary antibody (1:5,000) for 30 minutes at room temperature, and specific bands were detected using enhanced chemiluminescence reagents (ECL; Aspen, USA). The $\beta$-actin was used as an endogenous reference. Relative protein expression was calculated using ImageJ analyses (National Institutes of Health, Bethesda, MD, USA) of the band grayscales.[27,29]

## Real-Time PCR Analysis

After each group of cells was treated with the appropriate conditions, total RNA was extracted using TRIzol reagent (Bioteke, China), then measured using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). One µg of RNA was reverse transcribed to synthesize cDNA (cDNA Synthesis Kit; Applied Biosystems, USA) and RT-PCR (Applied Biosystems) StepOnePlus using a SYBR Green PCR kit (Toyobo). Each sample was tested three times. The *GAPDH* gene was used as a control. The primers used in this study were designed and synthesized by Shanghai Biotechnology (Shanghai, China) and are listed in the Table.

## Co-Localization Assays

After the treatment of each group of cells, MitoTracker Red (Solarbio Life Sciences, China) at a concentration of 25 nM and LysoTracker Green (Beyotime Biotechnology, China) at a concentration of 100 nM was added to 6-well plates and incubated with cells at 37°C for 60 minutes to label mitochondria and lysosomes, respectively. After staining, the staining solution was replaced with fresh medium, and the fluorescence was observed using a fluorescence microscope (IX73; Olympus, Japan) and photographed (cellSens Standard 1.17; Olympus). Pearson's correlation was calculated using ImageJ for the fluorescence co-localization analyses.

## Detection of ROS

After the cells were treated, 2 mL DCFH-DA stain (Solarbio) was added to 6-well plates, and then the cells were washed 3 times with serum-free cell medium at 37°C for 20 minutes, to fully remove the DCFH-DA not entering the cells. Fluorescence microscopy (IX73; Olympus, Japan) was used to detect ROS, followed by capturing the images using cellSens Standard 1.17 (Olympus). Fluorescence intensity was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

## Flow Cytometry

RPE cells were inoculated in six-well plates and cultured with the appropriate treatments. We collected the cell precipitates by centrifugation after EDTA-free trypsin digestion, washed with pre-cooled phosphate-buffered saline (PBS), then added 100 µL 1 × binding buffer to resuspend the cells. A total of 5 µL Annexin V/PE (Solarbio) was then added and incubated for

5 minutes at room temperature in the dark, followed by 10 µL 7-Aminoactinomycin D (7AAD) and finally 400 µL PBS. Cells were then detected using a flow cytometer (FACSAria; BD Biosciences, USA) within 1 hour.

## Statistical Analysis

All data are expressed as the mean ± standard deviation. Prism 8.0 software (GraphPad, USA) was used for statistical analysis. The analysis was performed using a homogeneity test of variance, an unpaired *t*-test for homogeneity of variance, and a nonparametric test for heterogeneity of variance. The $P < 0.05$ were considered statistically significant.

## Results

### Effect of High Glucose Environment on Sirt3, AMPK, mTOR, and ULK1 of ARPE-19

In the present study, the levels of mRNA of genes related to the autophagic pathway and the expression levels of key proteins in the AMPK/mTOR/ULK1 pathway were determined. The results showed that the mRNA expressions of Sirt3, AMPK, and ULK1 were significantly decreased in RPE cells in the high glucose group (Figs. 1a–c), whereas the expression of mTOR was significantly increased (Fig. 1d). Furthermore, the ratios of key proteins (Sirt3, p-AMPK/AMPK, and p-ULK1/ULK1) were significantly increased (Figs. 1e–g), and p-mTOR/mTOR was significantly decreased (Fig. 1h). Together, the results indicated that high glucose inhibited the expressions of the Sirt3 and AMPK/mTOR/ULK1 signaling pathways.

### Effect of High Glucose on Mitophagy Levels of ARPE-19 Cells

LC3B is a widely used marker for monitoring autophagy. LC3 I binds to phosphatidyl ethanol to form LC3 II and LC3 II, so the subunit expression can be used to identify the level of mitophagy. In the present study, we used MitoTracker (red) and LysoTracker (green) to label mitochondria and lysosomes, respectively, for co-localization analysis

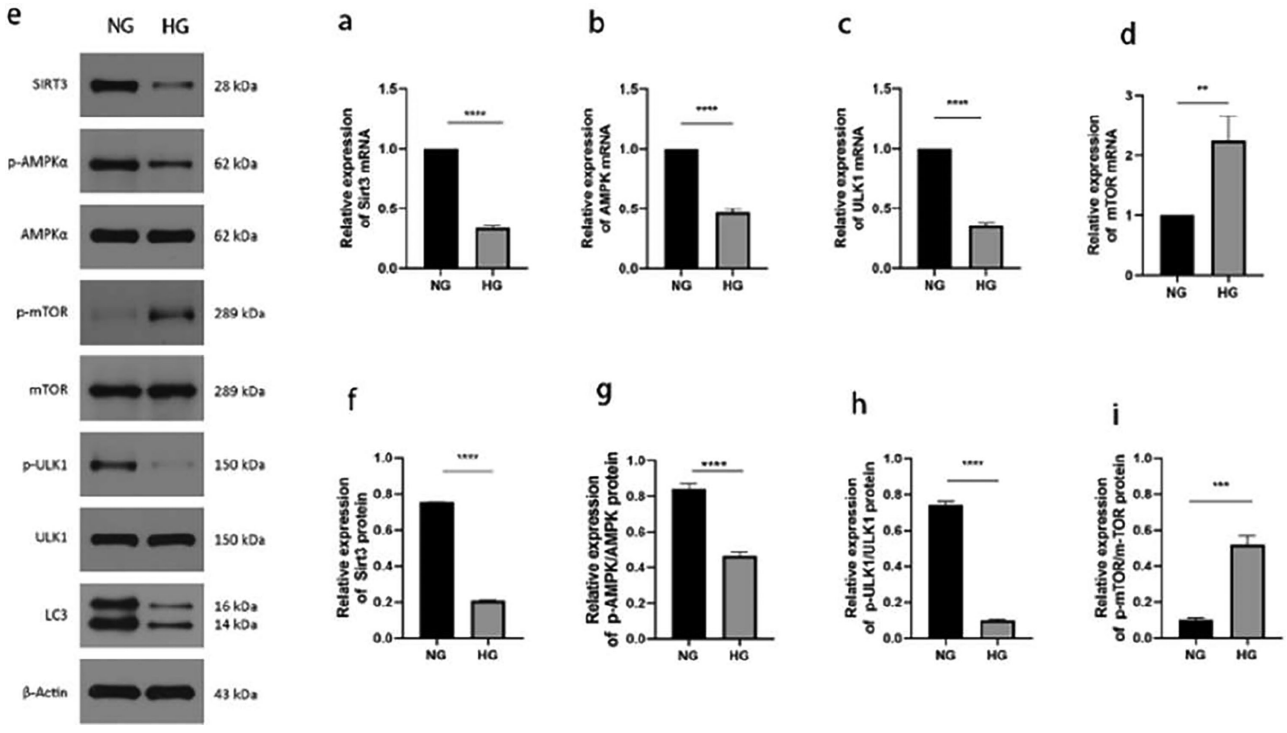


**Figure 1.** Inhibition of ARPE-19 mitosis under high glucose conditions and its effects on the Sirt3/AMPK/mTOR/ULK1 signaling pathway. (**a**–**d**) Real-time qPCR to detect the expression levels of Sirt3, AMPK, mTOR, and ULK1 mRNA in ARPE-19 cells in the high glucose and normal groups. (**e**) Western blotting to detect Sirt3, AMPK, mTOR, and ULK1 protein expression levels in ARPE-19 cells in the high glucose and normal groups. The bands represent protein expression levels. (**f**–**i**) Quantification of protein bands of Sirt3, AMPK, mTOR, and ULK1 using ImageJ software, normalized to levels of $\beta$-actin (data are expressed as the mean ± standard deviation (SD). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

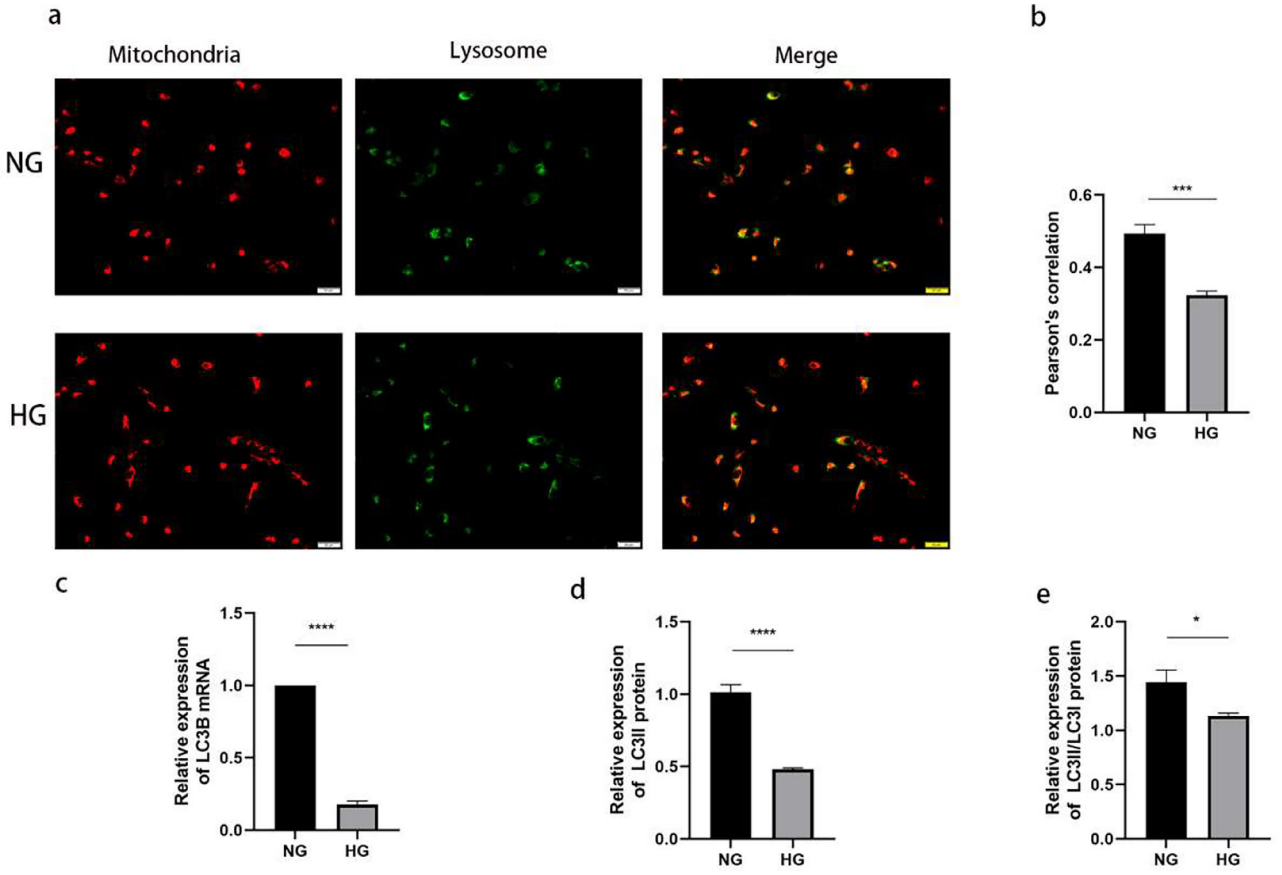


**Figure 2.** Mitophagy and fluorescence co-localizations in ARPE-19 cells induced by high glucose and normal glucose levels. (**a**) Representative images of Mito Tracker and LysoTracker staining co-localizations. Scale bar = 50 µm. (**b**) Pearson's correlation coefficients between mitochondria and lysosomes. (**c**–**e**) Protein bands of LC3 II and LC3 II /LC3 I were quantified using ImageJ software and normalized with $\beta$-actin. Data are expressed as the mean ± standard deviation (SD). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

after treatment of RPE with HG. The mitochondrial and lysosomal co-localization indicated positive mitophagy sites (Figs. 2a, 2b). After treating the cells in the same way, we examined the mRNA and protein expression levels of LC3B. The results showed that the relative mRNA expression of LC3B and LC3 II protein expression decreased during high glucose conditions (Figs. 2c–e). Overall, the results indicated that mitochondrial and lysosomal co-localization was downregulated during high glucose conditions.

## Effect of High Glucose Environment on ROS Production in ARPE-19 Cells

ROS levels were determined using a fluorescent probe, DCFH-DA, after 72 hours of treatment of RPE cells with HG. The results showed that high glucose significantly increased the level of intracellular ROS (Figs. 3a, 3b), and suggested that high glucose induced the production of cellular ROS.

## Overexpression of Sirt3 Regulates Mitophagy Through the AMPK/mTOR/ULK1 Signaling Pathway to Influence Cellular Regulation

We used lentiviral expression vectors encoding Sirt3 and GFP to induce Sirt3 overexpression (LV-Sirt3) and used a virus-negative control (LV-GFP) to detect the effects of Sirt3 on apoptosis via the AMPK/mTOR/ULK1 signaling pathway. The results showed that Sirt3 protein expression was increased in the HG + LV-Sirt3 group and decreased in the HG and HG + LV-GFP groups, with no significant difference in expressions between the hypertonic and NG groups (Fig. 4a). We found that the protein expressions of p-AMPK/AMPK, p-ULK1/ULK1, and LC3B in the HG + LV-Sirt3 group were significantly increased, when compared with the NG, HG, and HG + LV-GFP groups (Figs. 4b–e), whereas p-mTOR/mTOR protein expression was significantly decreased (Fig. 4f). The apoptosis percent of each group was measured by

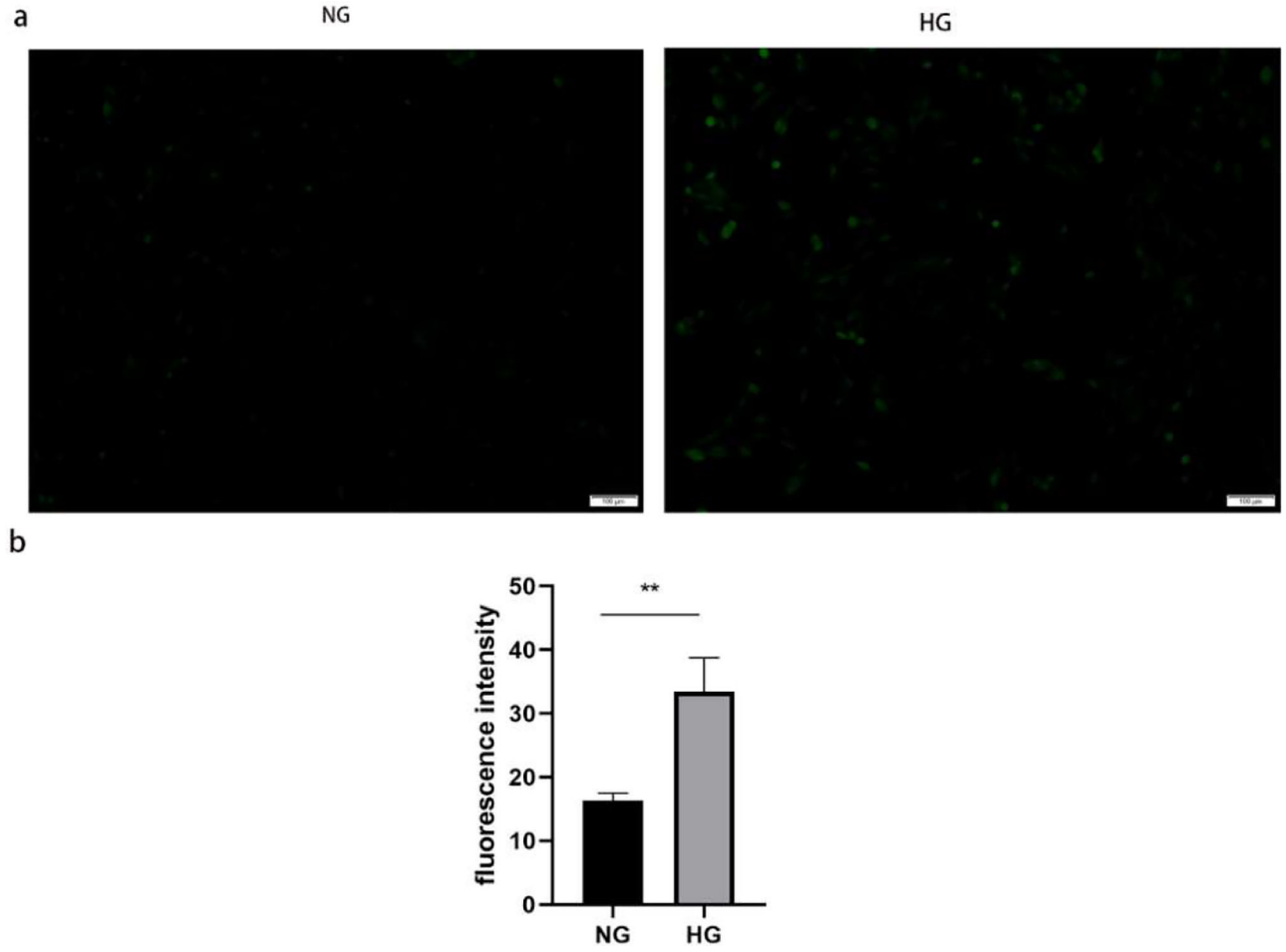


**Figure 3.** High glucose conditions induced a significant increase in reactive oxygen species (ROS) production in ARPE-19 cells, when compared to the normal group. (**a**) Representative images of ROS expression are shown using DCFH-DA. Scale bar = 100 µm. (**b**) Quantification of ROS fluorescence intensity using ImageJ software (data expressed as the mean ± standard deviation (SD), $^{*}P < 0.05$; $^{**}P < 0.01$).

flow cytometry, showing that high glucose promoted cell apoptosis, when compared with the normal group (Figs. 5a–e). Together, the results showed that high glucose decreased the expression of Sirt3 and inhibited activation of the AMPK/mTOR/ULK1 signaling pathway and mitophagy. However, overexpression of Sirt3 induced activation of the AMPK/mTOR/ULK1 pathway and increased the level of mitophagy, thereby inhibiting apoptosis.

## Discussion

In the present study, we showed that the expression of Sirt3 in RPE cells in a high glucose environment decreased, whereas it inhibited mitophagy, increased ROS levels, and promoted apoptosis in RPE cells. In contrast, when Sirt3 was overexpressed, it inhibited apoptosis by enhancing mitophagy through activation of the AMPK/mTOR/ULK1 pathway, which protected RPE cells from damage due to high glucose.

Excessive production of ROS causes genotoxicity and cellular damage, resulting in oxidative stress.[30] There is a strong correlation among mitochondrial oxidative stress, ROS production, and mitochondrial autophagy. It has been reported that increased oxidative stress, inflammatory responses, and increased ATP-driven oxygen consumption were associated with acute kidney injury, and that these conditions promoted mitochondrial damage, which triggered mitochondrial autophagy.[31] During myocardial ischemia-reperfusion injury, investigators have reported that mitochondrial autophagy can be used to remove damaged mitochondria and ROS, in a timely manner, which can effectively reduce reperfusion injury.[7] Similar results have also been reported on studies of diabetes. Lee et al.[32] found that induction

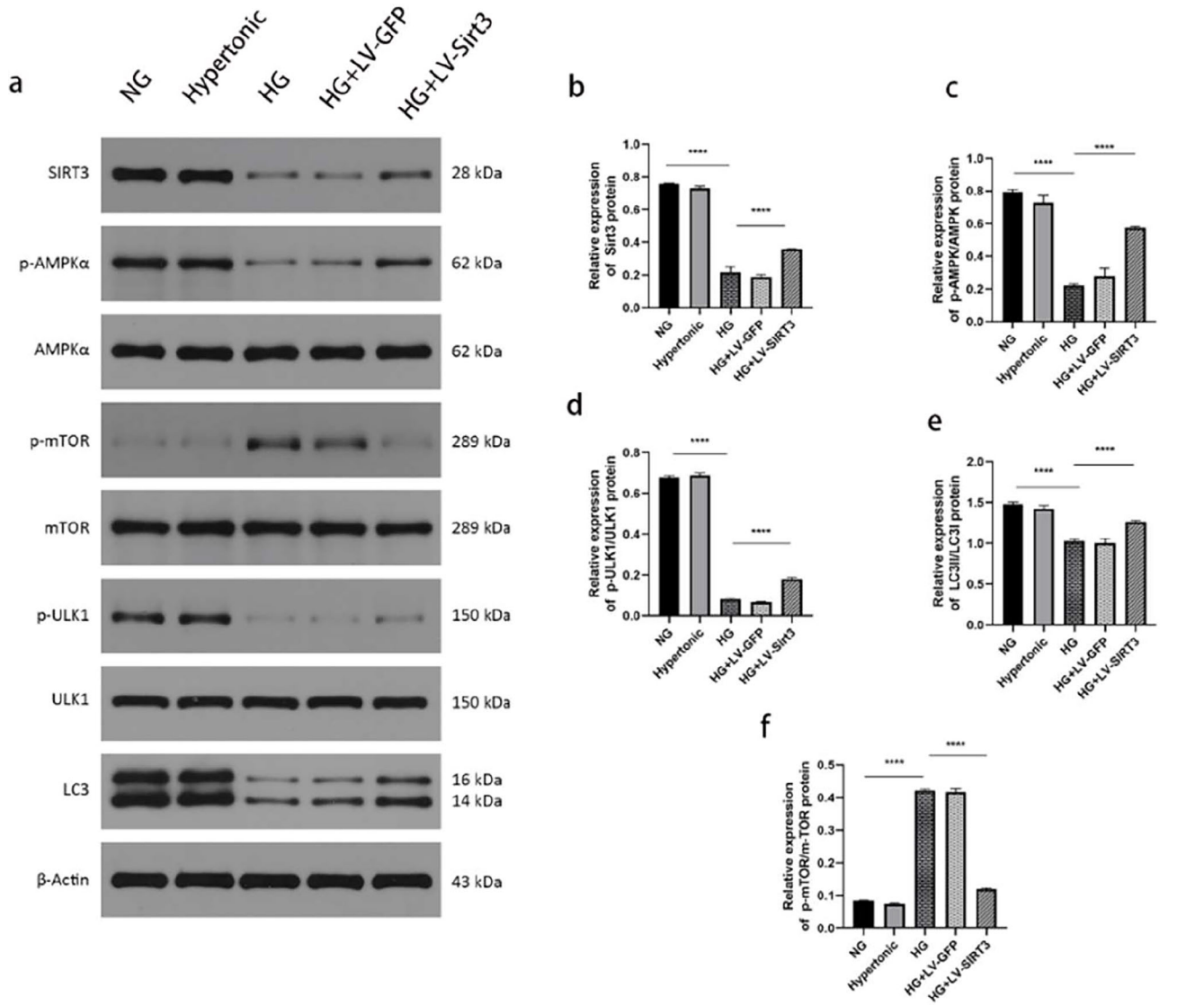


**Figure 4.** Effect of overexpression of Sirt3 during high glucose conditions on ARPE-19 cell mitophagy and the Sirt3/AMPK/mTOR/ULK1 signaling pathway. The experiments were divided into the NG, hypertonic, HG, HG + LV-GFP, and HG + LV-Sirt3 groups. (**a**) Western blotting to determine the expressions of Sirt3, AMPK, mTOR, ULK1, and LC3B proteins in ARPE-19 cells. (**b**–**f**) Protein bands of Sirt3, AMPK, mTOR, ULK1, and LC3II/LC3I were quantified using ImageJ software and *GAPDH* as a standard. Data are expressed as the mean ± standard deviation (SD). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

of mitophagy in platelets from patients with diabetes prevented severe oxidative stress. Seillirt et al.[33] and others have similarly reported that mitochondrial homeostasis can prevent metabolic syndrome through mechanisms that regulate oxidative stress. Thus, it can be argued that increased mitochondrial ROS production is central to the pathogenesis of diabetes, that excess ROS will stimulate mitochondrial damage, and that upregulation of mitophagy can remove damaged mitochondria in a timely manner. Our results also found that the relative mRNA expression of LC3B and protein expression of LC3 II in RPE cells were decreased in a high glucose environment, whereas mitophagy was inhibited, leading to intracellular ROS accumulation and causing cellular damage; so DR is closely associated with mitophagy. Furthermore, when we overexpressed Sirt3, we found that mitophagy was increased and apoptosis was decreased in RPE cells.

Sirtuins are nicotinamide adenine dinucleotide-dependent deacetylases that deacetylate target proteins, enhancing their activity and regulating a variety of biological processes. Seven Sirtuin proteins have been identified in mammals,[34] with Sirt3 predominating in the mitochondrial matrix.[35] Wei et al.[36] reported that inhibition of mitophagy exacerbated ROS production and impaired cardiovascular regen-

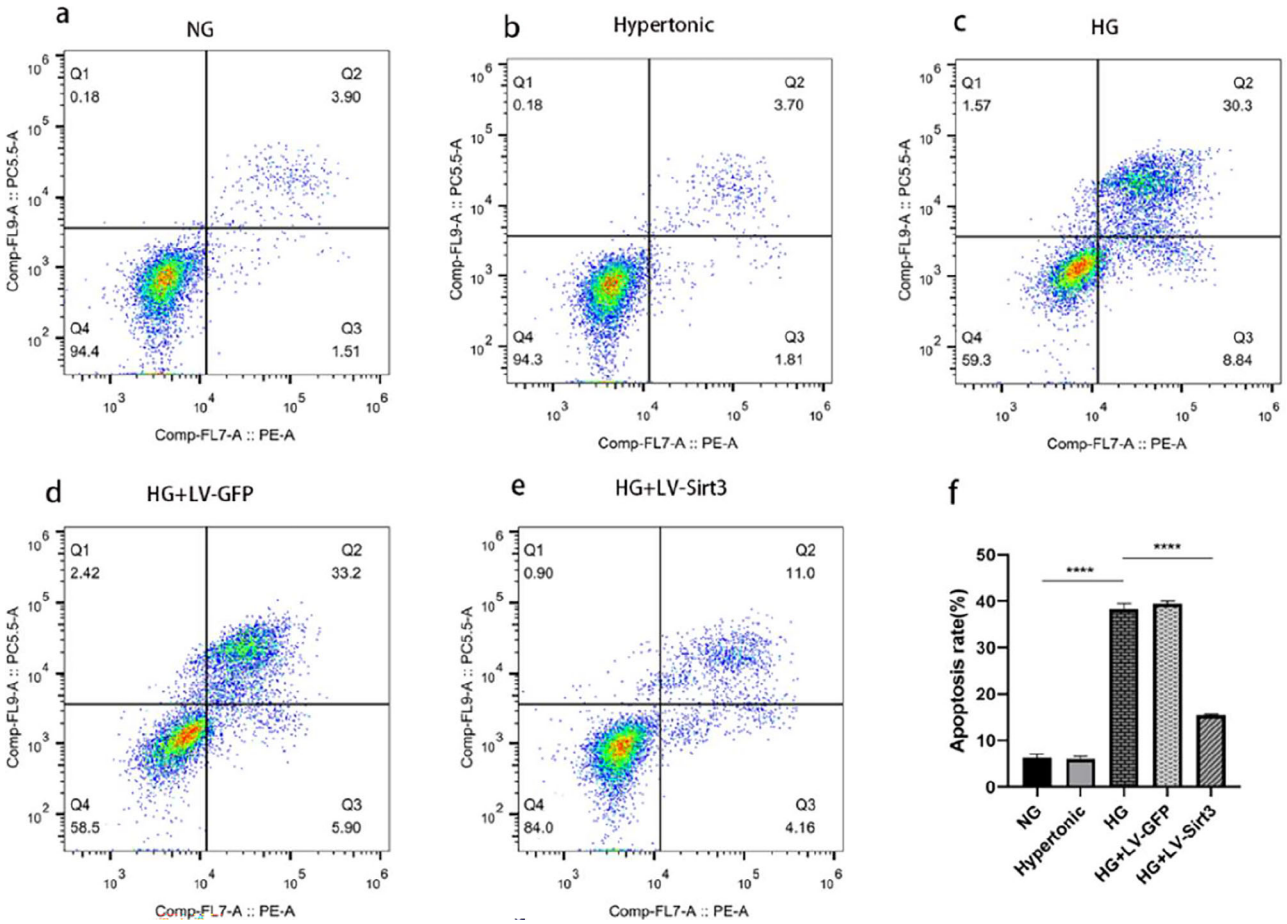


**Figure 5.** Detection of apoptosis in ARPE-19 cells under different treatments. (**a**–**e**) The effect of Sirt3 overexpression on the apoptosis of ARPE-19 cells was detected by flow cytometry. The experiment was divided into the NG, hyperosmotic, HG, HG + LV-GFP, and HG + LV-Sirt3 groups. (**f**) The percentages of apoptotic cells in each group. Data are expressed as the mean ± standard deviation (SD), $^{****}P < 0.0001$).

eration after knockdown of Sirt3, whereas specific overexpression reversed this phenomenon. In hypertensive heart disease, Sirt3 overexpression in cardiac vascular endothelial cells maintains angiogenesis and slows cardiac remodeling by activating mitophagy.[37] Moreover, knockdown of Sirt3 using siRNA in microglia further exacerbates the cytotoxicity mediated by microglial activation, including increased accumulation of ROS and enhanced apoptosis.[38] Based on these studies, we overexpressed Sirt3 by lentiviral transfection and we found that apoptosis was inhibited in the high glucose group. This is consistent with the results of other studies. However, we found that Sirt3 expression was increased in the malignant phenotype of some cancers, such as intestinal-type gastric and esophageal cancer cells.[39] This is contrary to our findings that the expression level of Sirt3 in RPE cells under high glucose conditions is lower than that in normal cells, because tumor cells, unlike RPE cells, are aberrantly proliferating cells that require more cellular energy than normal cells during their rapid growth and proliferation.[40]

The AMPK/mTOR/ULK1 pathway is an important signaling pathway for mitophagy,[41] but its role in RPE cells has not been reported. To the best of our knowledge, previous studies have focused on the effects between AMPK/mTOR and DR.[42,43] In osteoblasts, alginate enhances autophagy to inhibit apoptosis by upregulating the Sirt3-mediated AMPK/mTOR/ULK1 pathway.[44] In SH-SY5Y cells, Sirt3 can induce autophagy through the LKB1/AMPK/mTOR pathway to ameliorate cell damage.[45] Currently, there is a lack of knowledge on the role of Sirt3 during DR, and we have not seen any studies investigating the involvement of Sirt3 in DR, modulated by the AMPK/mTOR/ULK1 pathway. The experimental findings in the present study were intriguingly consistent, and found that Sirt3 may protect against high glucose-induced damage to RPE cells by regulating the AMPK/mTOR/ULK1 signaling

pathway that mediates mitophagy-induced RPE cell injury.

However, there was a limitation of the current study. We used an in vitro model of DR induced by high glucose. Furthermore, different microenvironments and various other factors may limit the reliability of the study or its clinical benefits. It is therefore our future goal to conduct in vivo experiments based on the findings from the in vitro studies.

## Conclusions

The present study showed that Sirt3 ameliorated high glucose-induced RPE cell injury, and inhibited apoptosis by inducing mitophagy. This process may involve the AMPK/mTOR/ULK1 pathway. Furthermore, this study suggested that Sirt3 could be a promising drug target for the treatment of DR, and therefore further research into the molecular mechanisms of Sirt3 action is needed in hopes of providing new therapeutic interventions for DR.

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**Author Contributions:** Wei Yang designed and conducted the research. Zhiru Zhang, Tianyu Yao, Wei Yang, and Li Huang collected and collated the data. Zhiru Zhang, Chen Qiu, Guihong Wu, and Xueqing Zhang prepared figures. Zhiru Zhang drafted the manuscript. Yue He reviewed the manuscript. All authors edited and revised the manuscript and approved the final version of the manuscript.

**Ethics Statements:** Ethical approval was not required for this study, which was reviewed and approved using the ethical standards of the Ethics Committee of Southwestern Medical University.

**Data Availability Statement:** All data generated or analyzed during the study are included in this article. Further questions can be directed to the corresponding author.

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