Nkx3.2 Inhibits Retinal Degeneration by Modulating Inflammation and Cell Death in RPE

Minsun Park,[1,2] Seung-Won Choi,[1,2] Da-Un Jeong,[1] Hae-Sol Shin,[3,4] Jiyeon Han,[5] Hye-Kyoung Oh,[2] Jun-Ho Jang,[2] Nari Ryu,[2] Hyunjin Jeong,[2] Young-Do Song,[1,2] Kyoung Yul Seo,[3,4] Sanghyuk Lee,[5] and Dae-Won Kim[1,2]

[1]Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea
[2]ICM Company Limited, Seoul, Republic of Korea
[3]Department of Ophthalmology, Severance Hospital, Institute of Vision Research, Yonsei University College of Medicine, Seoul, Republic of Korea
[4]Korea Mouse Sensory Phenotyping Center, Yonsei University College of Medicine, Seoul, Republic of Korea
[5]Department of Life Science, College of Science, Ewha Womans University, Seoul, Republic of Korea

Correspondence: Dae-Won Kim, Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Room 467, Building 102, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea; kimdw@yonsei.ac.kr.

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Purpose. To identify the expression of Nkx3.2 in retinal pigment epithelium (RPE) and evaluate its physiological role in association with retinal degeneration.

Methods. Nkx3.2 expression in RPE was examined by biochemical and histological analyses. Various in vitro and in vivo assays were employed to reveal the molecular mechanisms by which Nkx3.2 regulates inflammatory responses and cell survival in RPE. In addition, by investigating multiple animal models, the biological significance of Nkx3.2 in retinal degeneration was assessed.

Results. Nkx3.2 expression was verified in human cadaveric and mouse eye tissues and shown to be regulated by aging and oxidative stress. Mouse model analyses demonstrated retina protection activity of Nkx3.2 against aging, oxidative stress, vascular endothelial growth factor (VEGF) hyperactivation, and laser-induced damage. In vitro studies showed that Nkx3.2 downregulates pro-inflammatory cytokines and chemokines, but it upregulates anti-inflammatory factors. In addition, Nkx3.2 induced proteasomal degradation of receptor-interacting protein kinase 3 (RIP3), which, in turn, inhibited necroptosis. Consistent with these results, transcriptome analysis of mouse retina tissues indicated that Nkx3.2 can modulate gene expression profiles related to inflammatory responses, cell death, and visual function under oxidative stress.

Conclusions. Nkx3.2 can suppress inflammatory responses and necroptic cell death in RPE. By employing these mechanisms, Nkx3.2 may play a significant role in inhibiting retinal degeneration caused by aging and oxidative stress.

Keywords: Nkx3.2, RPE, retinal degeneration

The retina has a multilayered structure of distinct cell populations, and the outer nuclear layer is composed of two light-sensitive cell types (rods and cones) that convert light into electric signals and visual function.[1,2] Despite their functional importance, photoreceptors are unable to survive or function without necessary support from neighboring cells.[1,2] The structural integrity and function of the retinal pigment epithelium (RPE) is critical to maintaining normal visual function, as well as inhibiting retinal degeneration.[1,2] RPE dysfunctions can be caused by genetic mutation, external stress, and aging, which result in a broad range of degenerative retinal diseases including age-related macular degeneration and diabetic retinopathy, as well as various inherited retinal diseases.

As crucial factors causing retinal degeneration, oxidative stress, neuroinflammation, and cell death have been well documented.[3–6] Clinical and experimental data have shown that chronic oxidative stress is a persistent threat to the structural and functional integrity of RPE. Oxidative stress in retina activates inflammation and cell death pathways, which aggravate retinal degeneration by altering the microenvironment of RPE and photoreceptors.[3,6] Under these pathological conditions, microglia and macrophages are stimulated to secrete IL-1β, which further activates RPE cells to release pro-inflammatory factors and facilitates the invasion of inflammatory cells in the inflamed region.[5] Thus, it is important to control the inflammatory events occurring in the RPE to suppress the onset and progression of degenerative retinal diseases.

Programmed cell death pathways regulate normal cell physiology and human diseases. Recent studies have demonstrated that RPE cell death occurs in the form of necroptosis, and various factors causing RPE necroptosis can induce retina damage, suggesting that RPE necroptosis plays a significant role in retinal degeneration.[7–9] Necroptosis is defined by cell swelling and plasma membrane rupture,

resulting in the release of intracellular components into the extracellular space, causing an inflammatory response in the surrounding tissues. Receptor-interacting protein kinase-3 (RIP3) is a key regulator of necroptosis that phosphorylates mixed lineage kinase domain-like protein (MLKL) to promote MLKL oligomerization, leading to membrane disruption and cell death.[10,11]

Osteoarthritis (OA) is a degenerative disease characterized by cartilage loss and synovitis.[12–14] Oxidative stress is well known to cause cartilage damage and chronic inflammation, which promotes osteophyte maturation in conjunction with atypical angiogenesis and aberrant cartilage hypertrophy.[14,15] Nkx3.2 (also known as Bapx1) has been demonstrated to promote chondrocyte differentiation and survival, and it inhibits chondrocyte hypertrophy and angiogenesis in the context of skeletal development.[16–21] Furthermore, it has been shown that Nkx3.2 can suppress OA progression in both posttraumatic and age-driven OA models in mice.[22] Although the anatomical contexts differ, many degenerative diseases share common pathogenic mechanisms, including chronic inflammation, oxidative stress, and progressive cellular and structural deterioration. Given that OA and degenerative retinal diseases, such as age-related macular degeneration, are both age-related disorders that share these pathophysiological features, insights gained from one condition may offer valuable perspectives for understanding and managing the other. Considering the molecular functions of Nkx3.2 related to its disease-modifying activity for OA, it is feasible to hypothesize that Nkx3.2 may regulate retinal degeneration in association with oxidative stress, inflammation, and cell death.

In this work, we first identified Nkx3.2 expression in mouse and human RPE and showed Nkx3.2 can inhibit inflammatory responses and necroptosis in RPE. We further revealed that Nkx3.2 can alleviate retinal damage caused by aging and oxidative stress. These findings indicate that Nkx3.2 plays a role in suppressing retinal degeneration and maintaining RPE homeostasis.

Materials and Methods

Mice and Human RPE Tissue

C57BL6 mice were used to generate ciTg-Nkx3.2 and Nkx3.2 cKO mice, and the overall scheme and genotyping strategy are shown in the Supplementary Materials. Mouse genotyping was performed as previously described,[18] and the primer sequences are listed in Supplementary Table S1. All animal care procedures adhered to the guidelines set by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Yonsei Laboratory Animal Research Center (permit no. 2009-0033). Human donor eye tissues ($n = 10$) (see Supplementary Table S2) were obtained from the Center for Vision and Eye Banking Research, Eversight (Chicago, IL, USA, and Ann Arbor, MI, USA) within 12 hours after death.

Electroretinography, Optokinetic Nystagmus, and Optical Coherence Tomography

Electronretinogram (ERG) analysis was performed using MICRON Ganzfeld ERG (Phoenix-Micron, Bend, OR, USA) as described previously.[23] Spatial frequency thresholds (i.e., visual acuity) were assessed by optokinetic nystagmus (OKN) using a virtual optokinetic system (OptoMotry; Cerebral Mechanics, Medicine Hat, AB, Canada). The maximum spatial frequency was determined at which head tracking was achievable. Optical coherence tomography (OCT) scans were taken using the MICRON IV (Phoenix-Micron). Retinal thickness was measured using InSight-Animal OCT Segmentation Software (Phoenix-Micron).

Laser-Induced Choroidal Neovascularization and Fundus Fluorescein Angiography Assessments

Both eyes of each mouse were subjected to laser photocoagulation (wavelength, 532 nm; diameter, 50 µm; duration, 80 mS; 240 mW), with four laser spots applied around the optic nerve head. Fundus fluorescein angiography (FFA) was performed with the MICRON IV retinal imaging microscope. Mice were anesthetized, and pupils were dilated and intraperitoneally injected with fluorescein AK-FLUOR (Long Grove Pharmaceuticals, Rosemont, IL, USA) at 5 µg/g body weight. The fluorescein leakages were represented by the corrected total fluorescence (CTF) calibrated with the ImageJ software (National Institutes of Health, Bethesda, MD, USA) using the below equation:

$$\text{CTF} = (\text{Integrated density}) - [(\text{Area of selected lesion}) \times (\text{Mean fluorescence of background readings})]$$

Lesions were excluded from the result analysis and statistics according to the exclusion criteria as previously suggested.[24]

Results

Identification of Nkx3.2 Expression in RPE and Its Regulation by Aging and Oxidative Stress

Nkx3.2 expression was analyzed in eye tissues from young and aged human cadaveric donors. Western blot (WB) analysis of RPE–choroidal tissues revealed that Nkx3.2 was clearly expressed in the young group, whereas its expression was markedly reduced in the aged group (Fig. 1A). In vitro RPE cell cultures also demonstrated evident Nkx3.2 expression in ARPE-19 and human retinal pigment epithelial cells (HRPEpiCs), which were further validated by RNA knockdown in ARPE-19; human artificial chromosome (HAC) and human embryonic kidney (HEK) 293T cells were included as positive and negative controls, respectively (Supplementary Fig. S1). Consistent with the findings in humans, Nkx3.2 was expressed in 2-month-old mouse RPE and was reduced in 29-month-old mouse RPE (Fig. 1B).

Next, Nkx3.2 expression was further analyzed in human posterior pole paraffin section by histological analyses. Prominent expression was observed in the RPE layer from the young subject, but its expression was significantly decreased in the aged subjects' RPE layer (Fig. 1C). Interestingly, hematoxylin and eosin (H&E) staining revealed age-related retinal changes showing an inverse correlation between Nkx3.2 expression levels and degeneration severity. Reduced expression of Nkx3.2 in the RPE was associated with outer nuclear layer (ONL) thinning, with subject I—who exhibited the lowest A expression—showing fewer than five ONL layers and the presence of

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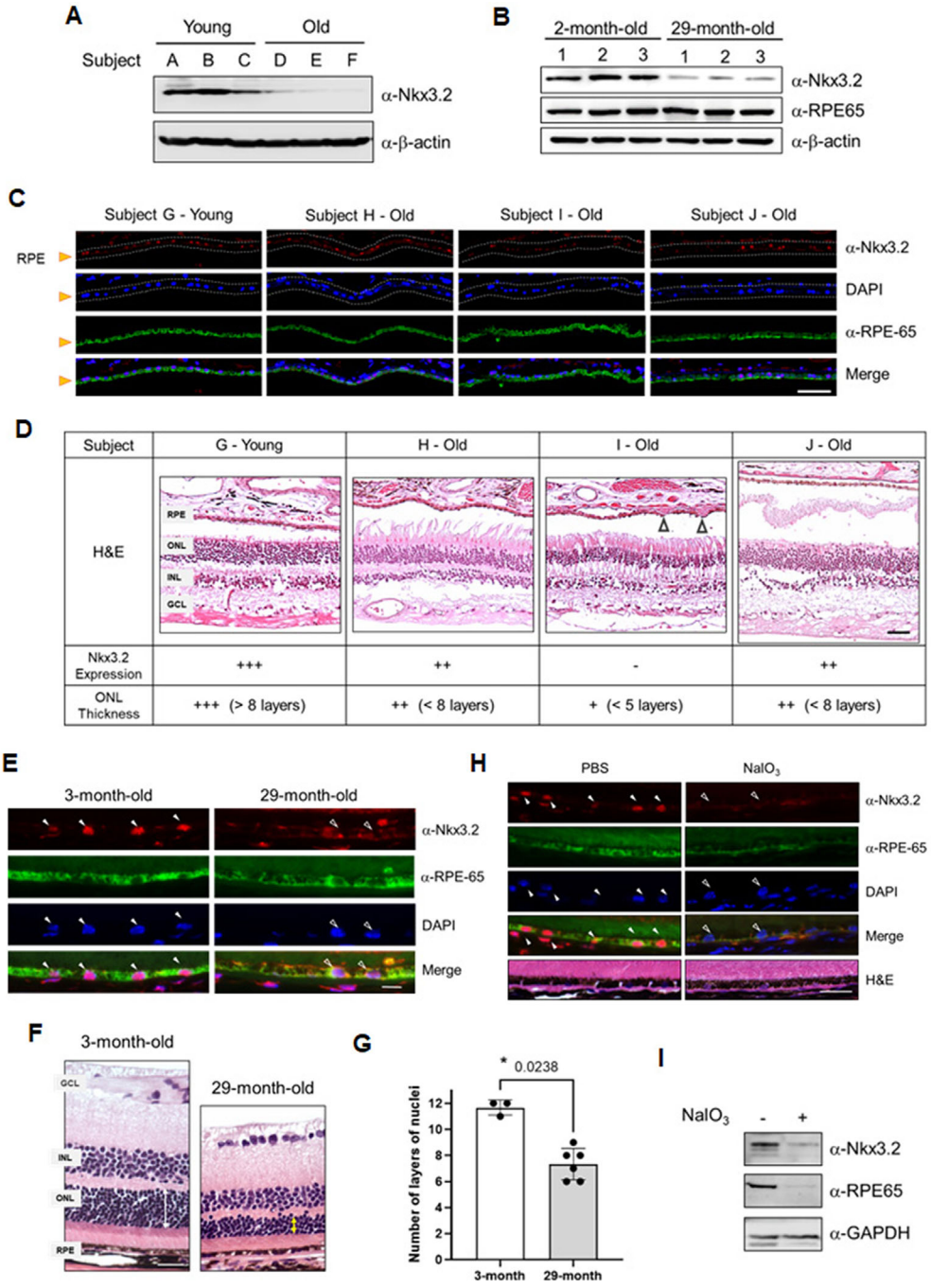

Figure 1. Nkx3.2 expression in RPE and its regulation by aging and oxidative stress. (**A**, **B**) Western blot analysis for endogenous Nkx3.2 in proteins extracted from RPE–choroid of human eye tissue (n = 3/group) (**A**) and total lysates from mouse posterior eyecup (n = 3/group) (**B**). (**C**) IF staining for endogenous Nkx3.2 and RPE65. (**D**) H&E staining with paraffin-embedded human retinal sections. *Scale bar*: 50 µm. The *arrowhead* indicates drusen-like deposits. (**E–G**) Representative image of IF staining (*scale bar*: 10 µm) (**E**), H&E staining (*scale bar*: 50 µm) (**F**), and quantification of number of layers of nuclei (**G**). Values are presented as mean ± SD. $^{**}P < 0.01$ by unpaired t-test. (**H**) Representative image of IF staining. (**I**) Western blot analysis in PBS or $NaIO_3$-treated (10 mg/kg for 3 days) mouse RPE. *Scale bar*: 20 µm.

drusen-like deposits (Fig. 1D). These findings were recapitulated in aged mice, where reduced Nkx3.2 expression in the RPE, as demonstrated by immunofluorescence (IF) staining (Fig. 1E) and RPE flatmounts (Supplementary Fig. S2), was accompanied by ONL thinning evident in H&E-stained sections (Fig. 1F). Statistical analysis of ONL layer thickness further confirmed a significant age-dependent decline, mirroring the human observations (Fig. 1G).

Highly metabolically active retina is extremely susceptible to oxidative stress, leading to RPE degeneration.[25] To examine the link between Nkx3.2 and oxidative stress, we employed a sodium iodate ($NaIO_3$)-induced retina degeneration (RD) model and found that intravenous (IV) injec-

tion of $NaIO_3$ caused a notable decrease of Nkx3.2 expression in RPE, as judged by IF (Fig. 1H) and WB (Fig. 1I). These findings suggest that Nk3.2 may play a protective role in maintaining retinal structure, and its downregulation with age could contribute to the progression of retinal degeneration.

Suppression of Retinal Damage and Preservation of Visual Function in ciTg-Nkx3.2 Mice

As Nkx3.2 expression in RPE was shown to be regulated by aging and oxidative stress, we next investigated the role of Nkx3.2 in RD by using RPE-specific Nkx3.2 transgenic mice (ciTg-Nkx3.2). To generate this transgenic mouse, we crossed ci-Nkx3.2 (a transgenic line with Cre-inducible HA-tagged mouse Nkx3.2 allele) mice[18] with BEST1-cre transgenic mice (Supplementary Fig. S3). Ectopic Nkx3.2 expression in RPE was confirmed by IF analysis (Supplementary Fig. S4, Supplementary Table S4).

Control (wild-type) and ciTg-Nkx3.2 mice were subjected to induced RD by IV injection of $NaIO_3$. Seven days after PBS or $NaIO_3$ administration, in vivo OCT and fundus images of the control and ciTg-Nkx3.2 mice were acquired (Supplementary Fig. S5). In control mice, $NaIO_3$ injection caused fundus abnormalities characterized by significant pigmentary changes and retina structural damage, while no obvious changes were observed in $NaIO_3$-injected ciTg-Nkx3.2 mouse (Figs. 2A, 2B). Consistent with these in-life imaging analyses, H&E staining and immunohistochemistry (IHC) for RPE65 indicated that oxidative stress-induced retina damage can be alleviated in the ciTg-Nkx3.2 mouse (Fig. 2C).

Next, visual function change by oxidative stress was compared between control and ciTg-Nkx3.2 mice by ERG. In control mice, ERG (scotopic and photopic) responses were almost abolished by $NaIO_3$ injections, but significant ERG responses were preserved in $NaIO_3$-injected ciTg-Nkx3.2 mice (Fig. 2D, Supplementary Table S5). Consistent with these findings, OKN analysis further indicates visual acuity can be maintained in ciTg-Nkx3.2 mice against oxidative stress, but not in control mice (Fig. 2E; Supplementary Table S6). In addition, we conducted laser-induced choroidal neovascularization (CNV) assays. Nine days after laser injury, the recovery from vascular leakage was significantly enhanced in ciTg-Nkx3.2 mice compared to control, as judged by FFA (Fig. 2F, Supplementary Fig. S6) and CTF area quantification (Fig. 2G, Supplementary Table S7).

Molecular Signatures Related to Retinal Protection in ciTg-Nkx3.2 Mice

To characterize the $NaIO_3$-induced retinal degeneration process at a molecular level, we performed RNA sequencing (RNA-seq) analysis with the posterior eye cup tissues isolated from mouse as a time series of 0, 3, and 7 days after $NaIO_3$ injection (Supplementary Fig. S7). The heatmap showed that most genesets related to inflammation and cell death were upregulated, but visual function–associated genesets were downregulated (Fig. 3A; Supplementary Tables S8, S9). These results were further supported by histological analyses; TUNEL-positive signal was detected in the RPE layer as well as ONLs at 24 hours after $NaIO_3$ injection, and this signal was further increased in the ONL and spread out to inner nuclear layer at 72 hours (Fig. 3B). In addition, aberrant melanin pigment cells were detected within the subretinal space near the RPE layer by H&E staining, and these cells were identified as markers for macrophage/monocyte monoclonal antibody (MOMA-2)–positive cells by IHC (Fig. 3C); the number of MOMA-2–positive cells in the subretinal space was counted (Fig. 3D).

Given these findings, we next tried to understand the molecular mechanisms by which Nkx3.2 protects retina against oxidative stress. To this end, we performed transcriptome analyses with wild-type and ciTg-Nkx3.2 mice in response to $NaIO_3$ treatment by using RNA-seq; the results are summarized in Figure 3E, and detailed methods are described in the Supplementary Materials. The gene set variation analysis (GSVA) score indicated that immune response, inflammation, and cell death signals were activated on day 3 and remained upregulated until day 7 in $NaIO_3$-injected wild-type mice. In contrast, in $NaIO_3$-injected ciTg-Nkx3.2 mice, the same set of genes was activated on day 3 but effectively suppressed down to the baseline by day 7. Additionally, genesets related to phototransduction and visual perception were suppressed on day 3 and stayed downregulated until day 7 in $NaIO_3$-injected wild-type mice. On the other hand, the same set of genes was suppressed on day 3 but restored back to the normal level by day 7 in $NaIO_3$-injected ciTg-Nkx3.2 mice. Taken together, these results indicate that ectopic expression of Nkx3.2 in RPE can alleviate structural and functional damage of retina under oxidative stress by inhibiting inflammation and cell death and preserving visual function.

Aggravation of Retinal Degeneration in Nkx3.2 cKO Mice

To reinforce the engagement of Nkx3.2 in retinal degeneration, we next investigated RPE-specific conditional knockout mice (Nkx3.2 cKO) mice. We generated Nkx3.2 cKO mice (Supplementary Fig. S8), and the loss of Nkx3.2 expression in Nkx3.2 cKO was verified by IF (Supplementary Fig. S9). It has been shown that fundus spots in the posterior retina can be generated during the aging process.[26] In control mice, there were no fundus spots at 12 months of age, and four out of eight eyes (50%) showed fundus spots at 18 months old. On the other hand, in Nkx3.2 cKO mice, four out of eight eyes (50%) had fundus spots at 12 months of age, and nine out of 11 eyes (82%) showed intense fundus spots at 18 months old; representative images are shown in Figure 4A, and collected individual images are shown in Supplementary Figure S10. OCT imaging demonstrated apparent potential deposits (yellow dotted circle) in aged Nkx3.2 cKO mice, but there was no obvious damage in the control aged mice (Fig. 4B). In addition, the measurements of total retinal thickness and ONL-RPE thickness in OCT images revealed that retinal tissue loss is significant in aged Nkx3.2 cKO mice, but not in control aged mice (Fig. 4C, Supplementary Table S11). Histological analyses also indicated that age-driven retinal degeneration is accelerated by Nkx3.2 deletion in RPE. Unlike control mice, Nkx3.2 cKO mice displayed distorted orientations of photoreceptor cells and aberrant MOMA-2–positive cells in subretinal spaces (Fig. 4D); the number of MOMA-2–positive cells in the subretinal space was counted (Fig. 4E, Supplementary Table S12). In addition, CNV assays demonstrated that the recovery from laser-induced lesions was significantly delayed in Nkx3.2 cKO mice compared to control mice, as judged by

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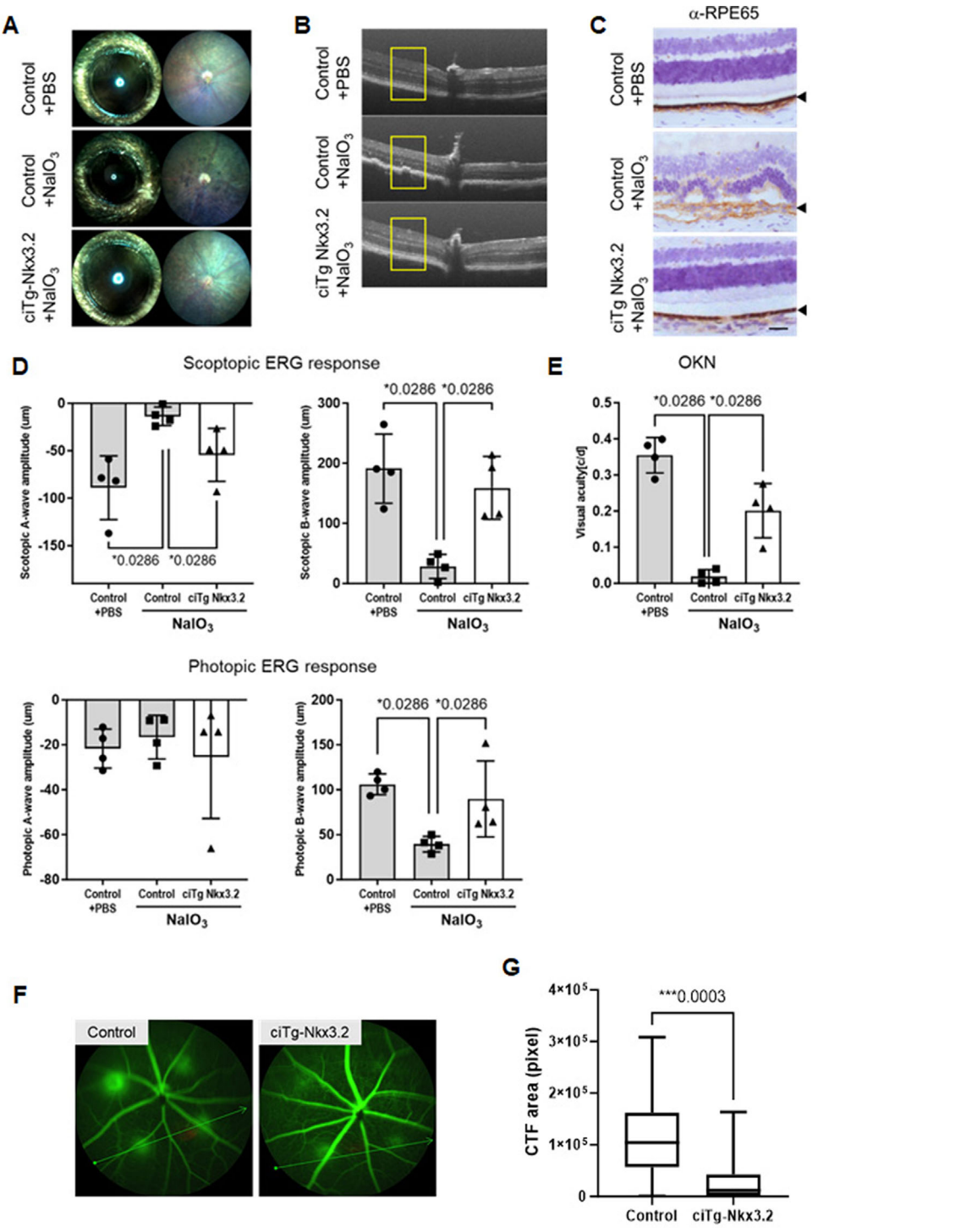

Figure 2. Suppression of retinal damage in ciTg-Nkx3.2 mice. PBS or $NaIO_3$ (20 mg/kg) were injected in control ($n = 6$) and ciTg-Nkx3.2 ($n = 4$) mice to induce retinal degeneration. (**A**) Representative image of fundus image. (**B**) Cross-sectional OCT images. (**C**) IHC staining of RPE65 (*arrowheads*). *Scale bar*: 20 µm. (**D**) Scotopic and photopic ERG a- and b-wave amplitudes. (**E**) OKN is shown in control mice with PBS ($n = 3$), control mice with $NaIO_3$ ($n = 3$), and ciTg-Nkx3.2 mice with $NaIO_3$ ($n = 4$). Values are mean ± SD. $^*P < 0.05$ by unpaired *t*-test; c/d, cycle per degree. (**F**, **G**) Observations of laser-induced CNV in control and ciTg-Nkx3.2 mice ($n = 3$/group) 9 days after induction. Shown are representative FFA images (**F**) and measurement results of the fluorescein leakage areas (CTF) (**G**). Data are shown as a minimum to maximum box plot. $^{***}P < 0.001$ by unpaired *t*-test.

FFA (Fig. 4F, Supplementary Fig. S11) and CTF change quantifications (Fig. 4G, Supplementary Table S13).

We next investigated whether the retinal degeneration in Nkx3.2 cKO mice can be associated with the alterations in retinal cell death. Because it has been indicated that oxidative stress contributes to degenerative retinal diseases by triggering RPE necroptosis,[6] we compared the levels of RIP3 expression in RPE between control and

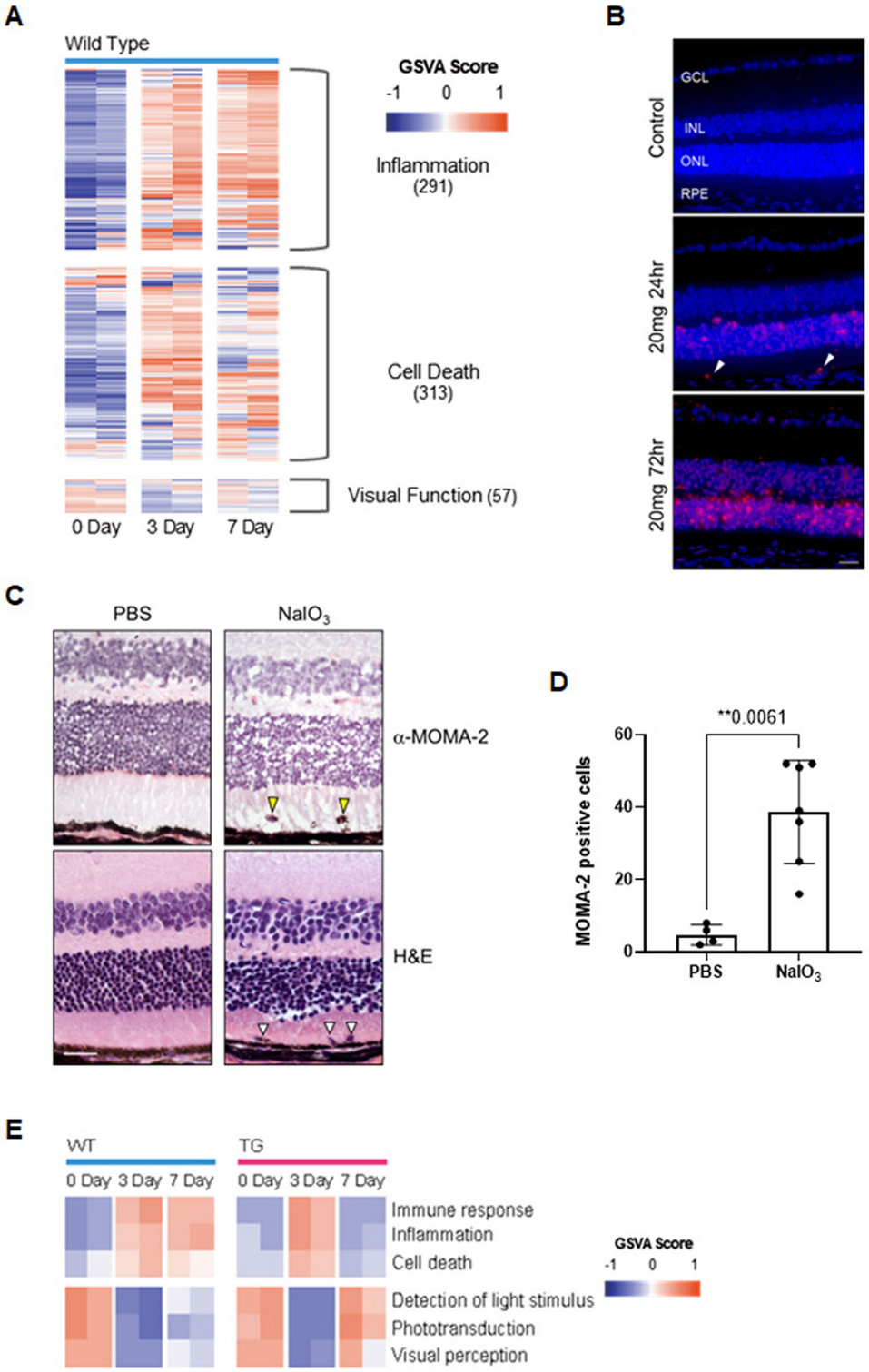

Figure 3. Molecular mechanisms of retina protection in ciTg-Nkx3.2. $NaIO_3$ (20 mg/kg) was injected into wild-type mice ($n = 3$/group at each time point) to induce retinal degeneration. (**A**) GSVA activity score of genesets related to inflammation, cell death, and visual function. Two columns for each day represent the replicate samples, and numbers in the parentheses indicate the number of genesets in each category. (**B**) Representative images after TUNEL reaction (*red*). *Scale bar*: 10 µm. (**C**) IHC and H&E staining for monocyte/macrophage (*arrowheads*) with paraffin-embedded retinal sections. *Scale bar*: 50 µm. (**D**) Quantification of MOMA-2–positive cells in subretinal space of $NaIO_3$-injected mice. Values are presented as mean ± SD. $^{**}P < 0.01$ by unpaired *t*-test. (**E**) Heatmap of geneset activities for representative genesets from GSVA in $NaIO_3$ (20 mg/kg)–injected wild-type and ciTg-Nkx3.2 mice ($n = 3$/group at each time point).

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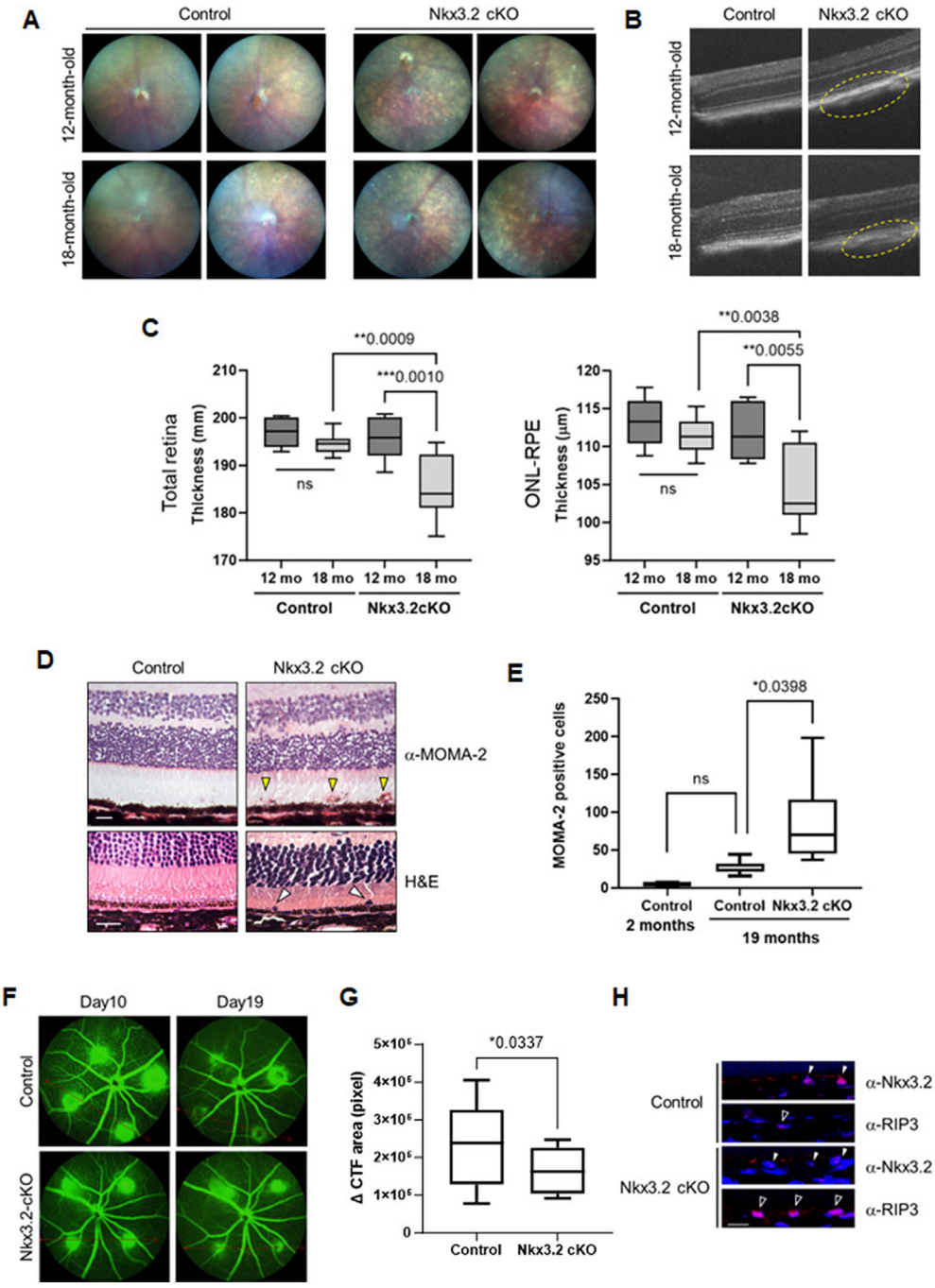

Figure 4. Aggravation of retinal degeneration in Nkx3.2 cKO mice. (**A**) Representative image of fundus image. (**B**) Cross-sectional OCT images. (**C**) Quantitative comparison of retinal thickness from OCT images of 12-month-old control mice or Nkx3.2 cKO mice ($n = 4$/group) and 18-month-old control mice ($n = 4$) or Nkx3.2 cKO mice ($n = 6$). Data are presented as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired *t*-test. (**D**) IHC and H&E staining for monocyte/macrophage (*arrowheads*) with paraffin-embedded retinal sections. *Scale bar*: 20 µm. (**E**) Quantification of MOMA-2–positive cells in subretinal space in 2-month-old mice ($n = 4$) or 19-month-old control mice ($n = 8$) and 19-month-old Nkx3.2 cKO mice ($n = 6$). Values are shown as a minimum to maximum box plot. There was a significant difference compared to 19-month-old control mice by one-way ANOVA ($^{*}P < 0.05$). (**F**) The FFA images of control mice and Nkx3.2 cKO mice ($n = 5$/group) on day 10 and day 19 after CNV induction. (**G**) Graphs were drawn based on values from changes in fluorescein leakage areas (CTF) from day 10 to day 19. Data shown as a minimum to maximum box plot. $^{*}P < 0.05$ by unpaired *t*-test. (**H**) IF for endogenous Nkx3.2 and RIP3 protein in control and Nkx3.2 cKO mice. *Scale bar*: 10 µm.

Nkx3.2 cKO mice. Whereas RIP3 expression in RPE was barely detectable in control mice, as expected, robust RIP3 expression was observed in Nkx3.2 cKO mice (Fig. 4H). These results suggest that Nkx3.2 may regulate RPE necroptosis.

Suppression of Inflammatory Responses by Nkx3.2

It has been well documented that inflammation plays a crucial role in a broad range of degenerative retinal

diseases.[5,27] Among various cytokines, IL-1β and TNFα have been considered as pivotal executors in association with RPE inflammation and retinal degeneration.[5] In this context, we hypothesized that Nkx3.2 may regulate inflammatory responses in RPE because Nkx3.2 has shown such activity in association with OA, a degenerative joint disease closely related to inflammation. Supporting this hypothesis, lentivirus-mediated Nkx3.2 overexpression in ARPE-19 cells inhibited the expression of pro-inflammatory cytokines and chemokines, induced by IL-1β or TNFα, as evidenced by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) (Fig. 5A, Supplementary Fig. S13) and ELISA (Fig. 5B, Supplementary Fig. S14). Conversely, anti-inflammatory *IL1RN* and IL1RAPL1 (Fig. 5C) and IL-1RA, encoded by the *IL1RN* gene, (Fig. 5D), were markedly increased by Nkx3.2 overexpression; lentivirus infection efficacy was shown (Supplementary Fig. S12). To further validate, we next examined whether Nkx3.2 knockdown can modulate the expression of inflammation-associated genes. Consistent with the findings from overexpression approaches, lentivirus-mediated Nkx3.2 knockdown potentiated IL-1β–mediated induction of pro-inflammatory cytokines and chemokines, as judged by RT-qPCR (Fig. 5E, Supplementary Fig. S16) and ELISA (Fig. 5F, Supplementary Fig. S17); lentivirus infection efficacy was shown (Supplementary Fig. S15). These in vitro findings were corroborated by observations in human ocular tissues, where IL-1Ra expression was notably higher in the young group and diminished in the aged group (Fig. 5G). These results support the physiological relevance of Nkx3.2 in suppressing multiple inflammatory pathways in the RPE that are known to play critical roles in retinal degeneration.

Nkx3.2-Mediated Inhibition of RPE Necroptosis Induced by Oxidative Stress

It has been shown that Nkx3.2 can regulate cell survival in chondrocytes.[22] Thus, it is feasible that Nkx3.2 may regulate cell death in RPE, as well. In accordance with this hypothesis, RPE cell death caused by oxidative stress (i.e., hydrogen peroxide [H_2O_2] or *tert*-butyl hydroperoxide [tBHP] treatment) was effectively inhibited by ectopic expression of Nkx3.2 in ARPE-19, as judged by TUNEL staining (Fig 6A). In addition, WST-1 cell viability assays and lactate dehydrogenase (LDH) release measurements further supported that oxidative stress-induced RPE cell death can be suppressed by Nkx3.2 overexpression (Figs. 6B, 6C) but further activated by Nkx3.2 knockdown (Supplementary Fig. S18).

RIP3 plays a crucial role during necroptic cell death by activating MLKL oligomerization and establishing necrosome complex.[11,28,29] Because we found that RIP3 expression in RPE can be elevated in Nkx3.2 cKO mice (Fig. 4H), we next explored the possibility that Nkx3.2 may regulate RIP3. In ARPE-19 cells, the levels of RIP3 were remarkably reduced by ectopic expression of Nkx3.2 in a dose-dependent manner (Fig. 6D) without affecting the levels of RIP1 and MLK1 (Supplementary Fig. S19). To further elucidate the molecular mechanisms by which Nkx3.2 suppresses RIP3, MG132, a proteasome-specific inhibitor, and chloroquine, a lysosome-specific inhibitor, were used. Nkx3.2-induced RIP3 protein degradation was inhibited by MG132 but not by chloroquine (Fig. 6E), indicating that Nkx3.2 promotes RIP3 protein degradation via proteasome.

Among ubiquitin E3 ligases, carboxyl terminus of Hsp70-interacting protein (CHIP) and pellino E3 ubiquitin protein ligase 1 (PELI1) have been implicated in RIP3 regulation,[30,31] and βTrCP1 has been shown to mediate Nkx3.2-induced IκBα degradation.[21] Thus, to identify the E3 ligase employed for RIP suppression by Nkx3.2, we examined CHIP, PELI1, and βTrCP1 and found that co-expression of βTrCP1-Δ inhibited Nkx3.2-induced RIP3 degradation, but CHIP-ΔU and PELI1-ΔR had no effects (Fig. 6F), suggesting that βTrCP1 is the E3 ligase employed for Nkx3.2-mediated proteasomal degradation of RIP3. Further, co-immunoprecipitation (co-IP) assays revealed that the interaction between RIP3 and βTrCP1 can be enhanced by Nkx3.2 co-expression (Supplementary Fig. S20) and reduced by Nkx3.2 knockdown (Supplementary Fig. S21). These results indicate that Nkx3.2 suppresses oxidative stress-induced necroptosis by enhancing RIP3–βTrCP1 complex formation, which, in turn, promotes RIP3 ubiquitination and subsequent proteasomal degradation.

Discussion

The retinal degeneration process is complicated and encompasses diverse interplay among aging, environmental risk factors, and genetic susceptibility.[4,32,33] Thus, despite extensive endeavors, the unmet medical needs related to degenerative retinal diseases remain substantial. It has been well documented that various factors causing RPE dysfunctions can lead to photoreceptor damage and loss of visual function.[5,34,35] In particular, oxidative stress and chronic inflammation have been considered to be the most common and significant factors promoting degenerative retinal diseases.[4,6,36] Current findings provide a significant novel insight to understand RPE regulation in conjunction with retinal degeneration.

Although it has been demonstrated that Nkx3.2 plays a role in cartilage development as well as OA pathogenesis, little is known about its function in other cell types or tissues. In this work, we identified Nkx3.2 expression in RPE, which is shown to be regulated by aging and oxidative stress. It has been well documented that RPE and photoreceptors have an interdependent relationship, and maintenance of RPE homeostasis is crucial in preserving normal retinal functions. Although RPE degeneration can be a cause or result of photoreceptor damage, it is evident that both events eventually result in the immune privilege perturbation.[35,37,38] Current work indicates that Nkx3.2 can inhibit oxidative stress–induced inflammatory responses and necroptic cell death in RPE. In addition, RPE-specific overexpression of Nkx3.2 in mice protects the whole retina from the structural and functional damage caused by aging, oxidative stress, VEGF hyperactivation, and laser-induced photoablation. Furthermore, transcriptome profiling analyses revealed that Nkx3.2 expression in RPE can modulate a broad range of gene expression related to inflammatory responses, cell survival, and visual function. These findings align with previous reports showing that Nrf2 overexpression in the RPE promotes antioxidant defenses and slows degeneration.[39] While Nrf2 primarily targets oxidative stress pathways, Nkx3.2 appears to exert broader regulatory effects—simultaneously modulating inflammatory signaling and preserving retinal function. Therefore, these findings collectively highlight the significance of Nkx3.2 expression in RPE as a promising upstream regulator that contributes to maintaining retinal physiological homeostasis.

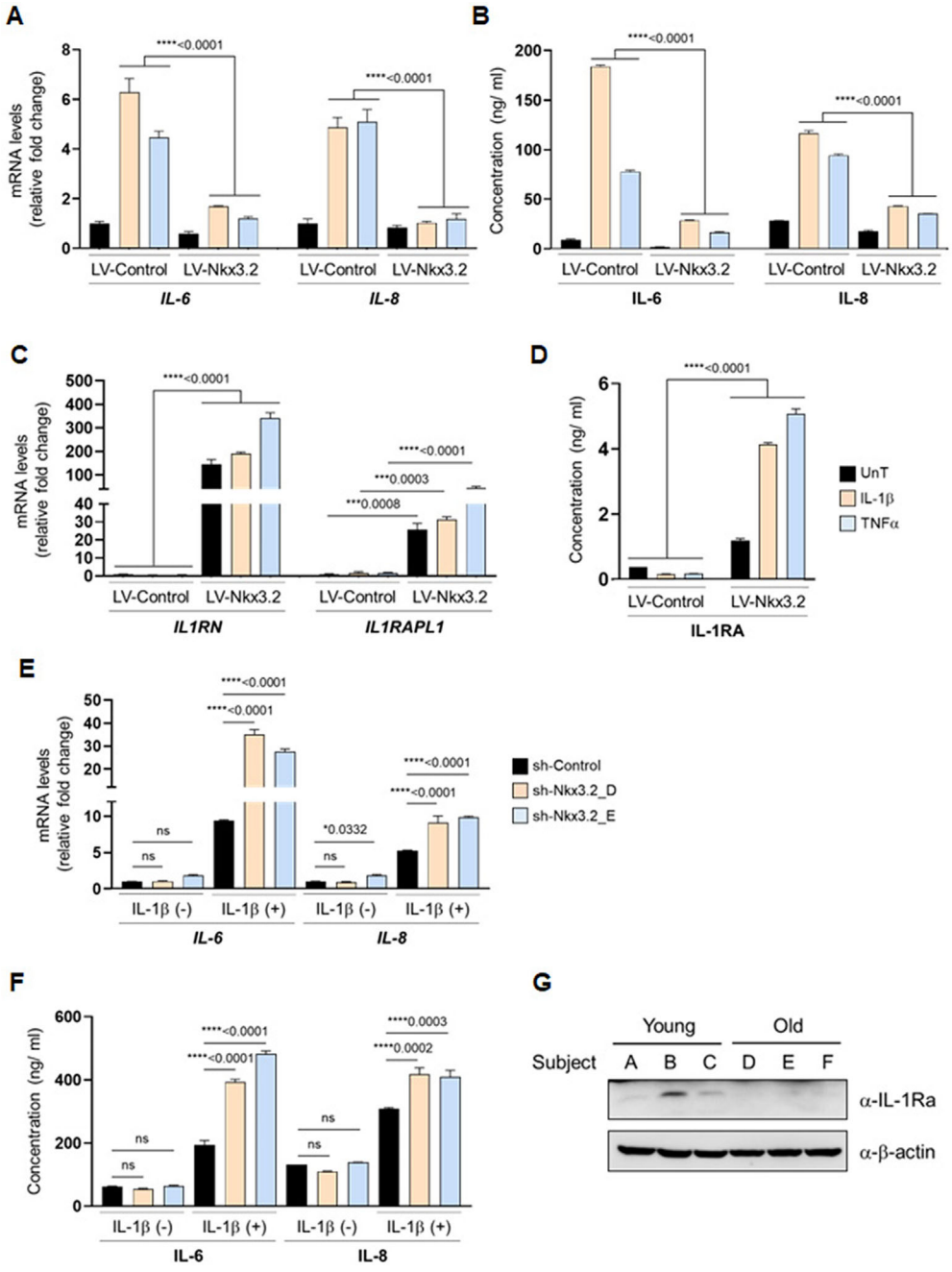

Figure 5. Suppression of inflammatory responses in RPE by Nkx3.2. (**A**–**D**) Control (LV-Control) or Nkx3.2 lentivirus (LV-Nkx3.2)–infected ARPE-19 cells were treated or not with IL-1β (10 ng/mL) and/or TNFα (10 ng/mL) for 24 hours. Shown are RT- qPCR analyses for genes associated with proinflammatory cytokines (**A**) and anti-inflammatory factors (**C**). Each value was normalized with GAPDH and compared fold changes relative to control. Secreted IL-6 and IL-8 (**B**) and IL-1RA (**D**) protein levels were quantified by ELISA. Values are mean ± SD. Statistical analysis using two-way ANOVA. ****$P < 0.0001$; ns, not significant. (**E**, **F**) Control or Nkx3.2 shRNA lentivirus–infected ARPE-19 cells were treated or not with IL-1β (10 ng/mL) and/or TNFα (10 ng/mL) for 24 hours. The mRNA levels of proinflammatory cytokines (**E**) were determined by RT-qPCR. Each value was normalized to that of GAPDH, and fold changes were compared relative to the untreated sh-Control. ELISA was used to determine secreted IL-6 and IL-8 (**F**). Values are mean ± SD. Statistical differences among groups were analyzed by one-way ANOVA. ****$P < 0.0001$; ns, not significant. (**G**) Western blot analysis for IL-1Ra expression in proteins extracted from the RPE–choroid of human eye tissue ($n = 3$/group).

Chronic oxidative stress and inflammatory conditions have been shown to promote necroptosis, which in turn exacerbates inflammatory responses.[6,10,40] IL-1β is the most critical cytokine that drives local and systemic inflammation, including retinal inflammation.[41–43] Because we found that Nkx3.2 can effectively inhibit the IL-1β pathway and its downstream targets, Nkx3.2-mediated suppression of the IL-1β signaling may play a central role in preserving RPE functions and preventing retinal degeneration.

The necroptic cell death signaling triggered by cytokines and oxidative stress typically involves the RIP1–RIP3–MLKL axis,[10,40] and Nkx3.2 has been shown to enhance chondro-

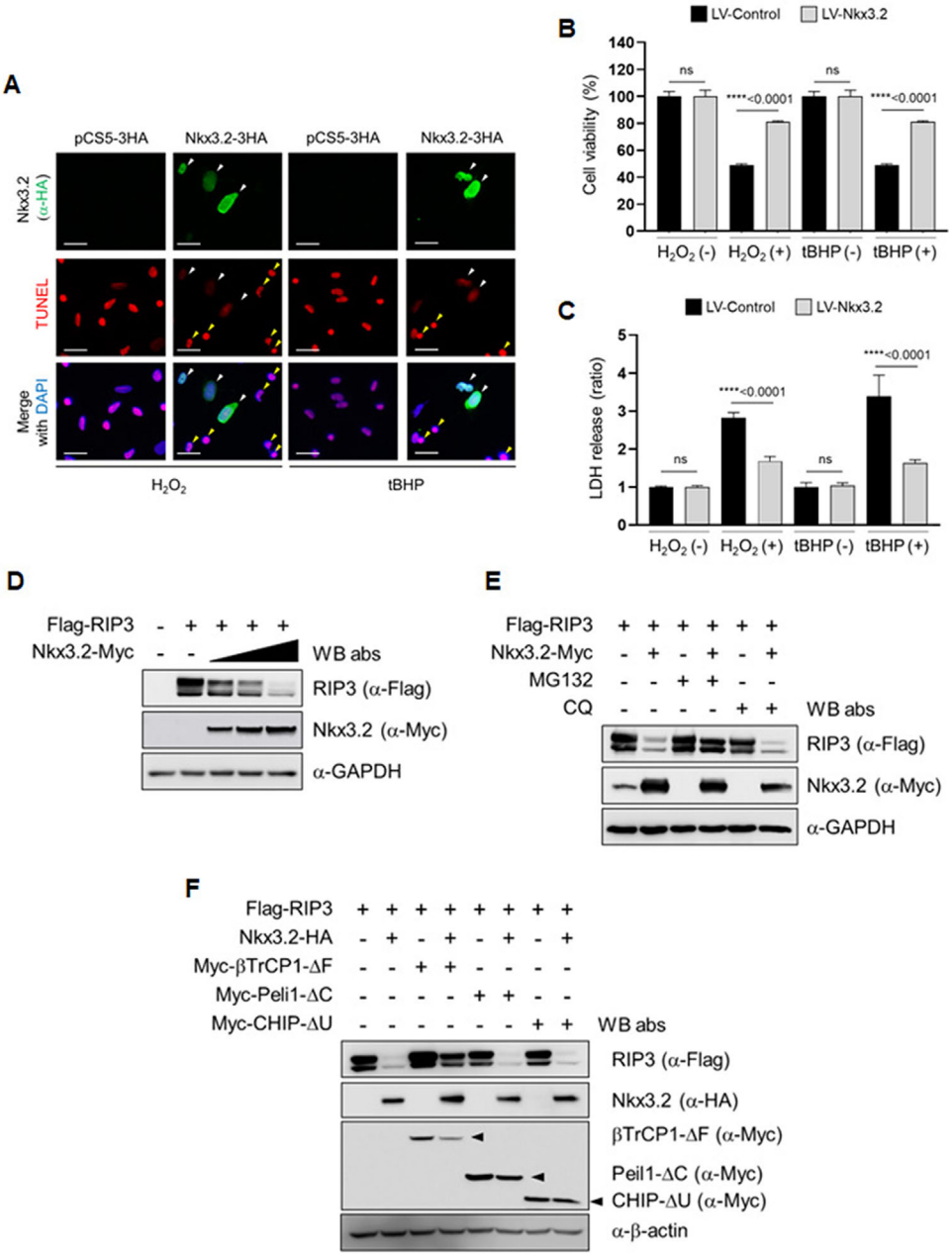

Figure 6. Inhibition of oxidative stress–induced RPE cell death by Nkx3.2. (**A**) ARPE-19 was transfected with HA-tagged empty vector or Nkx3.2-HA expression plasmid. Transfected cells were exposed to 500-mM H_2O_2 or 300-mM tBHP for the final 4 hours of incubation, and cell death was detected by TUNEL staining. The *yellow arrow* indicates untransfected cells, and the *white arrow* indicates Nkx3.2-HA-transfected cells. *Scale bar*: 20 mm. (**B**, **C**) ARPE-19 was infected with an empty vector or Nkx3.2 lentivirus. After 16 hours of 300-mM H_2O_2 or 100-mM tBHP incubation, cell viability was analyzed by the WST-1 assay (**B**) and LDH release assay (**C**). Data are shown as means ± SD. Statistical significance was determined by two-way ANOVA comparisons test. ****$P < 0.0001$; ns, not significant. (**D**–**F**) WB analysis results. ARPE-19 was transfected with Flag-RIP3 expression plasmid with different amounts of the Nkx3.2-Myc expression construct (0, 0.25, 0.5, 1 µg) (**D**). ARPE19 cells were transfected with Flag–RIP3 expression vehicle in the absence or presence of Nkx3.2–Myc expression plasmid. Transfected cells were exposed to 10-mM MG132 or 100-µM chloroquine diphosphate (CQ) for the final 8 hours of incubation (**E**). An expression vehicle for Flag–RIP3 was transfected into ARPE-19 cells with the Nkx3.2-HA expression construct in the absence or presence of Myc–βTrCP1–ΔF (F-box domain deletion mutant), Myc–Peli1–ΔR (RING-like domain deletion mutant), or Myc–CHIP–ΔU (U-box domain deletion mutant) for 48 hours (**F**). Total cell lysates were analyzed by western blotting.

cyte survival by activating RelA via βTrCP1-mediated proteasomal degradation of IκBα.[20,21] Our current results revealed that Nkx3.2 can inhibit RPE necroptosis under oxidative stress by selectively inducing proteasomal degradation of RIP3 by employing βTrCP1. Also, Nkx3.2 has been demonstrated to inhibit vascular invasion by triggering lysosomal degradation of hypoxia-inducible factor-1α (HIF-1α), a crucial regulator of VEGF expression, in the context of skeletal development.[17] Thus, it is intriguing to hypothesize that Nkx3.2 may also have a role in maintaining retinal structure integrity via Nkx3.2-mediated suppression of HIF-1α to inhibit aberrant vascular invasion.

The efforts on drug development for degenerative retinal diseases have been mainly focused on antioxidants, VEGF antagonists, and complement inhibitors, leaving significant unmet needs.[6,44,45] Thus, Nkx3.2 may serve as a promising target for the treatment of degenerative retinal diseases, considering its multifaceted activity regulating retinal inflammation, RPE necroptosis, and aberrant vascularization. We anticipate that a better understanding of Nkx3.2 function will provide valuable insights and contribute to the development of innovative therapeutic strategies for a broad spectrum of degenerative retinal diseases.

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