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DAPL1 deficiency in mice impairs antioxidant defenses in the RPE and leads to retinal degeneration with AMD-like features

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ABSTRACT

The decreased antioxidant capacity in the retinal pigment epithelium (RPE) is the hallmark of retinal degenerative diseases including age-related macular degeneration (AMD). Nevertheless, the exact regulatory mechanisms underlying the pathogenesis of retinal degenerations remain largely unknown. Here we show in mice that deficiencies in *Dapl1*, a susceptibility gene for human AMD, impair the antioxidant capacity of the RPE and lead to age-related retinal degeneration in the 18-month-old mice homozygous for a partial deletion of *Dapl1*. *Dapl1*-deficiency is associated with a reduction of the RPE's antioxidant capacity, and experimental re-expression of *Dapl1* reverses this reduction and protects the retina from oxidative damage. Mechanistically, DAPL1 directly binds the transcription factor E2F4 and inhibits the expression of MYC, leading to upregulation of the transcription factor MITF and its targets NRF2 and PGC1α, both of which regulate the RPE's antioxidant function. When MITF is experimentally overexpressed in the RPE of DAPL1 deficient mice, antioxidation is restored and retinas are protected from degeneration. These findings suggest that the DAPL1-MITF axis functions as a novel regulator of the antioxidant defense system of the RPE and may play a critical role in the pathogenesis of age-related retinal degenerative diseases.

1. Introduction

Oxidative stress is a common risk factor in aging and many neurodegenerative diseases. Retinal oxidative damage has been identified as one of the major causative factors in retinal degenerative diseases, including age-related macular degeneration (AMD), a major cause of irreversible blindness and a considerable health burden during aging [1,2]. In fact, about 80% of AMD patients exhibiting the so-called dry form of AMD (also named geographic atrophy), and in them degeneration or dysfunction of the retinal pigment epithelium (RPE) seems to be the primary event followed by secondary damage of retinal photoreceptors. Nevertheless, the underlying pathogenetic mechanisms have not been fully elucidated and effective therapeutic or preventive strategies for the diseases have not so far become available [3].

The RPE consists of a single layer of pigmented epithelial cells wedged between the neural retina and the choroid. It plays various roles in supporting retinal structure and homeostasis, including secreting trophic factors, maintaining the blood-retina barrier, removing retinal waste and clearing reactive oxygen species (ROS) [4–6]. Mutations in genes important for these functions can induce retinal degeneration and visual damage [7–9], and RPE senescence may trigger the development of AMD [10,11].

Age-related diseases are frequently associated with oxidative stress and organelle damage. Increased ROS can cause unfolded or misfolded

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proteins, rendering them prone to protein-protein or lipid/protein aggregation [12]. Hence, a major challenge for the field of retinal degenerative diseases is to protect retinas from oxidative damage, whereby the RPE with its antioxidant capacity may represent a prime target to achieve this goal [3,13]. It is known, for instance, loss of NRF2 (nuclear factor erythroid 2) and PGC-1α (peroxisome proliferator activated receptor gamma coactivator 1 PPARGC1) leads to RPE cell damage resembling dry AMD like phenotypes in mice [14]. AMD is a common polygenic disease and is caused by complex interactions between genetic and environmental factors including age [3,15,16]. Recently, a human candidate gene approach identified single nucleotide polymorphisms (SNPs) in the death-associated protein like-1 gene (*DAPL1)* to be associated with a reduction in the expression of some isoforms of DAPL1 and with AMD [17]. DAPL1 is expressed in corneal epithelial cells, suprabasal cells of the epidermis, the esophageal and tongue epithelium, and suprabasal precortical cells of anagen hair follicles. It has been speculated, therefore, that it plays roles in regulating epithelial cell differentiation [18]. We have previously shown that DAPL1 is highly expressed in mature RPE cells and acts as a suppressor of RPE cell proliferation [19]. Nevertheless, the functions of DAPL1 in RPE cells and its potential mechanisms in the pathogenesis of age-related retinal degenerative diseases remain elusive.

The microphthalmia-associated transcription factor, MITF, is a member of the MITF-TFE family of basic-helix-loop-helix-leucine zipper (bHLHZip) proteins that is expressed in diverse cell types, while it is specifically expressed in the RPE cells in the retina [8]. In humans,

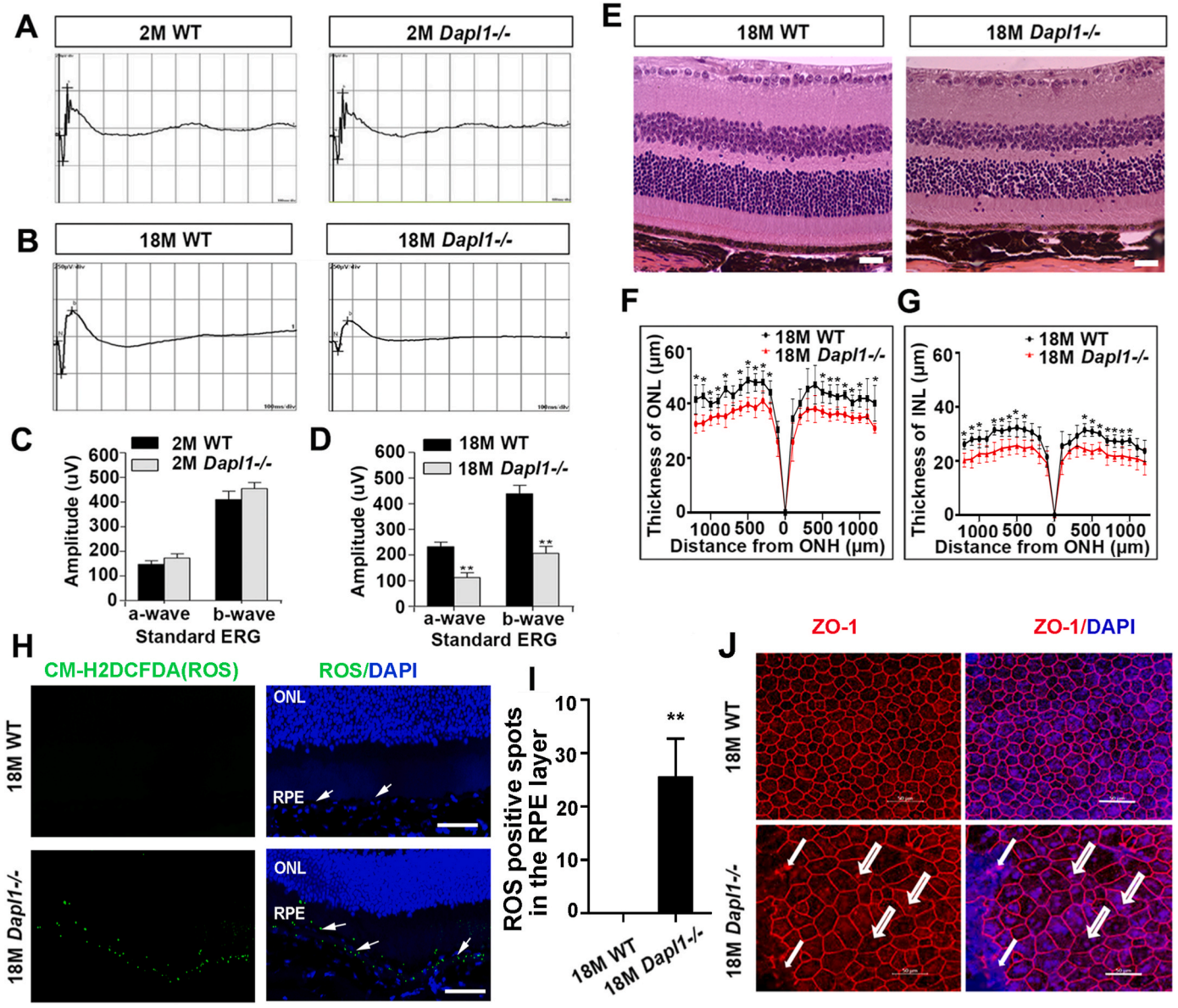

Fig. 1. ***Dapl1*−/− mice develop age-related retinal degeneration-like phenotypic features and increase oxidative stress in RPE cell.** (A–D) ERG traces of 2-month-old and 18-month-old WT and *Dapl1*−/− mice under the scotopic condition and quantification. Note that there was no significant difference between 2-month-old WT and *Dapl1*−/− mice, while the a- and b waves of 18-month-old *Dapl1*−/− mice were more severely impaired than those in WT mice. (E) Representative histological sections of 18-month-old WT and *Dapl1*−/− retinas. Sections represent an area around 400 μm from the ONH. (F, G) Quantification shows the mean number of the thickness of the ONL and INL in the mice retinal regions as indicated. (H) Green fluorescent probe CM-H2DCFDA detected ROS production in 18-month-old WT and *Dapl1*−/− retinas. (I) Quantification shows the mean number of ROS positive spots in the RPE layer based on the result (H). (J) ZO-1 immunostaining of flat-mounted RPE of 18-month-old *Dapl1*−/− and WT mice, showing multi-nuclear (empty arrows) RPE cells and damaged cell junctions of 18-month-old *Dapl1*−/− (solid arrows) but not WT mice. ERG, electroretinogram; ONH, optic nerve head; ONL, outer nuclear layer. N = 6, Scale bar: 50 μm, ** or * indicates $P < 0.01$ or $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

germline *MITF* mutations are associated with pigmentary/deafness syndromes, including Waardenburg Syndrome (WS) type IIa, Tietz syndrome and COMMAD syndrome, and an increased susceptibility to develop melanomas [20–25]. Mice homozygous for severe *Mitf* mutations exhibit microphthalmia, RPE abnormalities, retinal degeneration, depigmentation, and deafness [26–28]. Nevertheless, how RPE-expressed MITF contributes to retinal degenerative diseases is still not fully understood. We have recently shown that MITF regulates a number of factors in the RPE that are of importance in retinal physiology. They include PEDF (pigment epithelium derived factor), RDH5 (retinol dehydrogenase-5) and RLBP1 (retinaldehyde binding protein-1) [29–31], and NRF2 and PGC1α, both of which involved in regulating the antioxidant defense system of the RPE [32,33]. Interestingly, deliberate overexpression of MITF in the RPE can protect the retina against oxidative damage-induced retinal degeneration [33]. Here, we show in mice that MITF itself is regulated by DAPL1 and that DAPL1 influences the antioxidant defense system in RPE cells. We find that deficiencies in *Dapl1* lead to age-related retinal degeneration-like phenotypic features in aged mice and that re-expression of *Dapl1* in *Dapl1*−/− RPE increases its antioxidant capacity both in vitro and in vivo. We further find that DAPL1 directly binds E2F4 (E2F transcription factor 4) and so inhibits the expression of MYC (MYC proto-oncogene, a bHLH transcription factor), leading to an increase in the expression of MITF and in turn its targets NRF2 and PGC1α. The results suggest that the DAPL1/E2F4-MYC-MITF-NRF2/PGC1α axis provides an important link between the RPE, its antioxidant capacity, and retinal health.

2. Results

2.1. Deficiency in Dapl1 leads to age-related retinal degeneration-like phenotypes and increases oxidative stress in RPE cell

To address whether DAPL1 plays any role in age-related retinopathy, we examined the eye phenotypes of previously generated *Dapl1*−/− mice [19]. As the data shown in Fig. 1, there was no significant difference in the electroretinograms (ERGs) between 2-month-old wild type (WT) and *Dapl1*−/− retinas (Fig. 1A, C), while the a- and b-wave amplitudes of standard combined ERGs were significantly decreased in 18-month-old *Dapl1*−/− retinas compared to age-matched WT retinas (Fig. 1B, D). To extend the characterization of the eye phenotypes of aged *Dapl1*−/− mice, we then focused on the retinas and the RPE directly. Consistent with the ERG results, in 18-month-old *Dapl1*−/− mice, the thickness both of outer nuclear layer (ONL) and inner nuclear layer (INL) were thinner compared to age-matched WT mice (Fig. 1E–G), while the retinas show no significant difference between the 2-month--old and 12-month-old WT and *Dapl1*−/− mice (Figs. S1 and S2). In addition, the ROS level was dramatically higher in the RPE layer of the 18-month-old *Dapl1*−/− mice compared to the age-matched WT mice (Fig. 1H and I), suggesting the increased oxidative stress in the aged *Dapl1*−/− mice RPE cells. Moreover, immunostaining for zonula occludens-1 (ZO-1, also named tight junction protein 1, TJP1) showed large, frequently multinucleated, and damaged RPE cells in aged *Dapl1*−/− mice, while age-matched WT mice showed smaller RPE cells with the typical hexagonal structure. These results suggest that deficiency in *Dapl1* impairs the antioxidant defense system in RPE cell and causes age-related retinal degeneration-like phenotypic features in mice.

2.2. Dapl1 deficiency decreases the antioxidative ability of RPE cell and exacerbates oxidative damage-induced retinal degeneration

The abovementioned results demonstrated that loss of *Dapl1* leads to RPE abnormalities and retinal pathologies in aged mice. To address whether *Dapl1* deficiency exacerbates oxidative damage-induced retinal degeneration in vivo, we tested whether deliberate application of an oxidizing reagent would cause more serious retinal and RPE damages in 2-month-old *Dapl1*−/− mice. To this end, we intraperitoneally injected mice of the respective genotypes with 50 mg/kg of *sodium iodate* ($NaIO_3$), which is a stable oxidizing agent that primarily targets the RPE [34,35]. Indeed, 12 h after $NaIO_3$ injection, immunostaining of ZO-1 results showed the structure of the *Dapl1*−/− mice RPE cell was dramatically damaged (Fig. 2A), while the ROS level was significantly higher in the RPE layer of the *Dapl1*−/− mice (Fig. 2B and C), suggesting that DAPL1 deficiency decreases the anti-oxidant ability of RPE cell. The antioxidant ability of *Dapl1*−/− RPE cell was also analyzed in the primary mouse RPE cells in vitro. Primary mouse RPE cells were isolated from the 2-month old WT and *Dapl1*−/− mice, and cultured in vitro for 10 days. $NaIO_3$ was added into the culture medium as the oxidizing agent, while cell activity was measured using CCK8 reaction and PI (Propidium Iodide) staining. Data showed that *Dapl1*−/− mice primary RPE cells have lower CCK8 activity and higher PI staining after the $NaIO_3$ treatment (Figs. S3A–C), suggesting that DAPL1 deficiency decreased the antioxidant ability of RPE cells. The neuronal protective roles of DAPL1 in oxidative damage-induced retinal degeneration in vivo was analyzed 4 days after the $NaIO_3$ injection. As the data shown, the thickness of the ONL was significantly reduced in *Dapl1*−/− mice compared to that in WT mice (Fig. 2D and E), the latter showing no significant differences to uninjected WT mice (Figs. S1A and B). Also, Rhodopsin and Opsin levels were lower in injected *Dapl1*−/− retinas compared to similarly injected WT retinas (Fig. 2F and G), the latter showing levels similar to those in uninjected controls (Figs. S1C and D). In addition, stronger TUNEL and GFAP-positive signals were observed in *Dapl1*−/− retinas (white arrow indicated) compared to those in WT retinas (Fig. 2H–J). Thus, after deliberate induction of oxidative stress, retinal degeneration was more severe in *Dapl1*−/− mice. These results suggest that *Dapl1* deficiency exacerbates oxidative damage-induced retinal degeneration in mice.

2.3. Overexpression of DAPL1 in the RPE increases its antioxidative capacity and protects the retina from oxidative damage

To confirm the above data suggesting DAPL1 deficiency to impair the antioxidant capacity of RPE cells, we overexpressed DAPL1 through AAV (adeno-associated virus)-mediated gene transfer using the RPE cell-specific *RPE65* (retinoid isomerohydrolase RPE65) promoter. As previously described, this promoter controls gene expression specifically in RPE cells [36]. Hence, we constructed the AAV9 expression vector AAV9-*p.RPE65-DAPL1-HA* allowing for expression of HA epitope-tagged DAPL1 and packaged it as described (hereafter called AAV9-DAPL1) (Fig. 3A) [33]. AAV9-DAPL1 was injected into the right eye, while an AAV9 empty vector virus was injected into the left eye of the subretinal space of 6-week-old *Dapl1*−/− mice. Two weeks after injection, western blotting results indicated that AAV9-DAPL1 injection led to increased levels of HA-tagged DAPL1 and its downstream target gene P21 (Fig. 3B and C). To determine whether AAV9-DAPL1-mediated gene transfer might alter the normal structure of the retina, injected *Dapl1*−/− and WT eyes were analyzed by H&E staining as well as Rhodopsin and Opsin immunostaining. No significant differences between the AAV9 and AAV9-DAPL1 injected *Dapl1*−/− (Figs. S4A–D) or WT retinas (Figs. S5A–D) were found.

To assess whether overexpression of DAPL1 in the RPE in 6-week-old *Dapl1*−/− mice might preserve its antioxidant capacity, the subretinal space of the right eye was injected with AAV9-DAPL1 and that of the left eye with AAV9 empty vector virus. Two weeks after injection, the mice were intraperitoneally injected with $NaIO_3$ (50 mg/kg) and the retinal structure was examined 4 days later. Compared to AAV9 injected eyes, AAV9-DAPL1 injected eyes showed a more intact RPE cell layer and the ONL was thicker (Fig. 3D and E). Consistent with these results, the expression levels of Rhodopsin and Opsin (Fig. 3F and G) were higher and TUNEL-positive signals lower in AAV9-DAPL1-injected eyes (Fig. 3H and I). But, we noticed that the Rhodopsin and Opsin were mislocalized to the inner retina of the AAV9-DAPL1-injected eyes, suggesting the normal retina structure was damaged. Interestingly, a

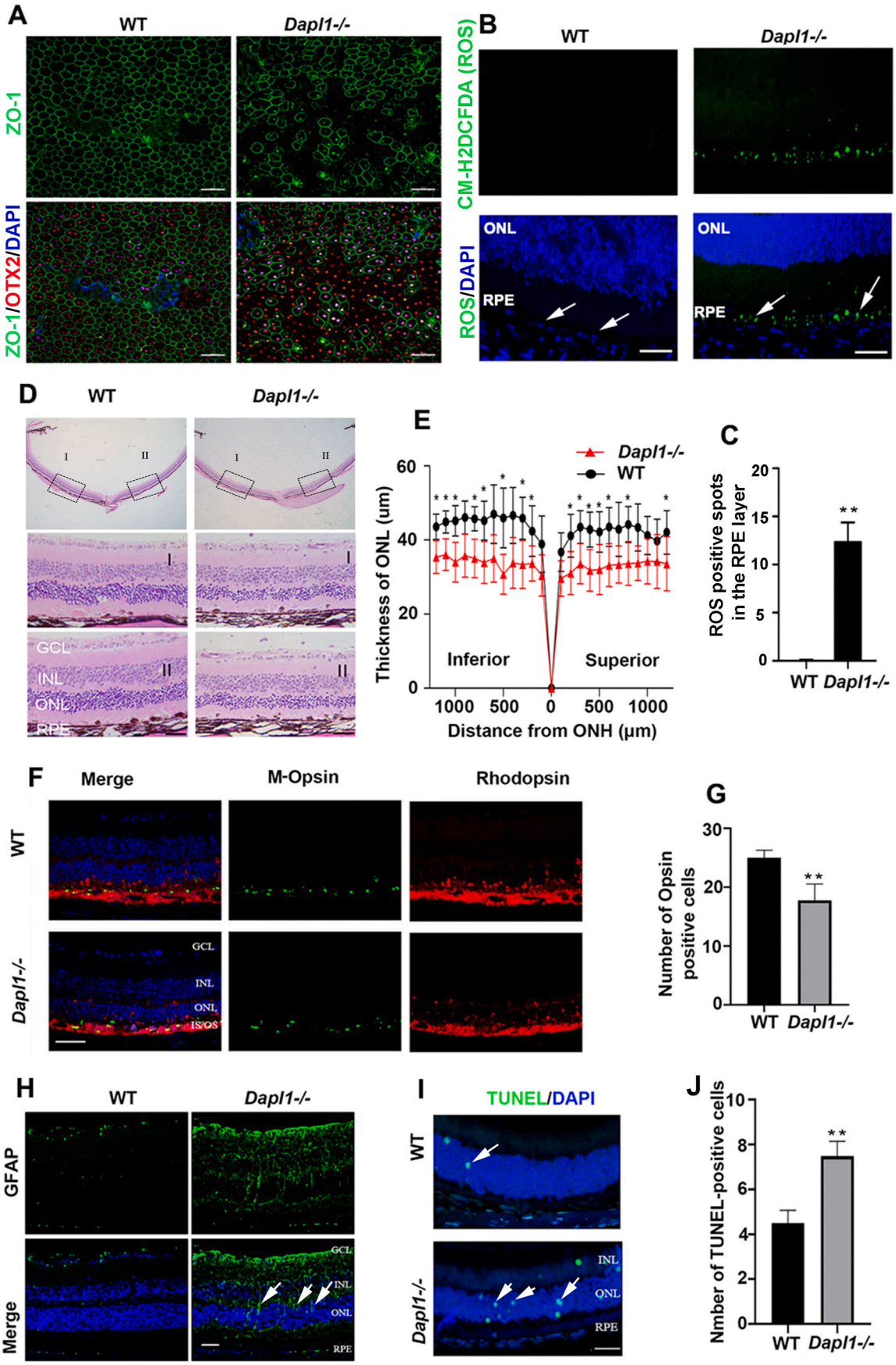

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Fig. 2. DAPL1 deficiency exacerbates oxidative damage-induced retinal degeneration. (A) 12 h after intraperitoneal injection of 50 mg/kg $NaIO_3$, immunostaining images of anti-ZO-1 and anti-OTX2 in RPE flat mounts of 2-month-old WT and *Dapl1*−/− mice. (B) 12 h after after intraperitoneal injection of 50 mg/kg $NaIO_3$, green fluorescent probe CM-H2DCFDA detected ROS production in 2-month-old WT and *Dapl1*−/− retinas. (C) Quantification shows the mean number of ROS positive spots in the RPE layer based on the result of (B). (D) Representative histological sections of 2-month-old WT and *Dapl1*−/− retinas 4 days after intraperitoneal injection of 50 mg/kg $NaIO_3$, I and II indicate the Inferior and Superior areas around 400 μm from the ONH. (E) Quantification showing the mean ONL thickness for the indicated retinal regions after the $NaIO_3$ injection in mice of the indicated genotypes. (F) Immunostaining for Rhodopsin and Opsin in retinas of 2-month-old *Dapl1*−/− and WT mice 4 days after $NaIO_3$ injection. (G) Quantification of the Opsin positive cells per section based on the results of (F). (H) GFAP immunostaining in the retinas of the indicated mice 4 days after injection of $NaIO_3$. (I, J) TUNEL staining in retinas of indicated mice 4 days after injection of $NaIO_3$ and quantification. ONH, optic nerve head; ONL, outer nuclear layer. Scale bar: 50 μm. for B, D, and G: N = 6; **or * indicates $P < 0.01$ or $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

protective role of DAPL1 overexpression was also observed in WT mice after injection of AAV9-DAPL1 followed by $NaIO_3$ injection (Figs. S6A–F). These data suggest that gene transfer-mediated overexpression of DAPL1 in the RPE can elevate the antioxidant capacity of the RPE and protect retinas from oxidative damage in both WT and *Dapl1*-deficient mice.

2.4. RPE-expressed DAPL1 acts at least in part by regulating MITF to promote antioxidation in vitro

MITF is a transcription factor with multiple and critical roles in both development and function of RPE cells, including antioxidation brought about by regulation of the expression of NRF2 and PGC1α [32,33]. This fact prompted us to analyze whether DAPL1 might regulate antioxidation through MITF. We found, firstly, that following infection with appropriate lentiviruses, DAPL1-overexpressing ARPE19 and D407 cells (another RPE cell line) expressed higher levels of MITF, NRF2 and PGC1α (Fig. 4A–D). Secondly, we found that after siRNA-mediated knock down of *MITF* in DAPL1-overexpressing cells (Fig. 4E), cell viability was decreased in response to GOX-exposure (Fig. 4F). Importantly, regardless of whether DAPL1-expressing cells were treated with control or MITF-specific si-RNAs, ROS levels were not changed, but they were specifically increased in si-MITF-treated DAPL1-expressing cells following GOX exposure (Fig. 4G–I). These results suggest that DAPL1 regulates the antioxidant capacity in RPE cells at least in part through MITF.

2.5. Dapl1 and Mitf interact genetically in vivo

To address the question of whether DAPL1 influences the levels of MITF in vivo, we first tested whether MITF levels were changed in *Dapl1*−/− RPE cells. Indeed, we found, that the protein levels of MITF, as well as those of NRF2 and PGC1α, were decreased in 2-month-old *Dapl1*−/− RPE cells compared to age-matched WT mice (Fig. 5A and B). We then tested whether a genetic reduction of MITF levels would promote oxidative damage in the retinas of 2-month-old *Dapl1*−/− mice, which normally is seen only in older *Dapl1*−/− mice (see above). Hence, we crossed *Dapl1*−/− mice with *Mitf* $^{mi\text{-}vga9}/^{mi\text{-}vga9}$ mice, the latter homozygous for a functional null mutation in *Mitf*. Further crosses generated *Dapl1*−/−;*Mitf* $^{mi\text{-}vga9}$/+ (hereafter called *Dapl1*−/−;*Mitf*+/−) mice with decreased MITF levels compared to those in *Dapl1*−/−;*Mitf*+/+ mice (Fig. 5C and D). *Dapl1*−/−;*Mitf*+/+ and *Dapl1*−/−;*Mitf*+/− mice showed no visible defects in either the structures of the RPE and neural retina nor in the expression of Rhodopsin and Opsin (Figs. S7A–D). In contrast, *Dapl1*−/−;*Mitf*+/− mice showed retinas that were more sensitive to oxidative damage induced by injection of $NaIO_3$, both with respect to the thickness of the ONL (Fig. 5E and F), the expression levels of Rhodopsin and Opsin (Fig. 5G and H) and TUNEL-positivity (Fig. 5I and J). These results suggest that *Dapl1*−/−;*Mitf*+/− mice are more susceptible to oxidative-induced retinal damage.

For confirmation, we also overexpressed MITF in *Dapl1*−/− mice. To this end, we used a *Dct-Mitf* transgenic mouse line, which has been shown to express high levels of MITF in the RPE [33]. Appropriate crosses finally yielded *Dapl1*−/−;*Mitf*+/+;*Dct-Mitf* mice, expressing high levels of MITF, and control *Dapl1*−/−;*Mitf*+/+ mice, expressing lower levels of MITF (Fig. 6A and B). Importantly, the extra levels of MITF in the former mice did not change the retinal structure or the expression of Rhodopsin and Opsin compared to MITF levels in the latter mice (Figs. S8A–D). Interestingly, 12 h after $NaIO_3$ injection, interestingly, immunostaining of ZO-1 results showed the RPE was less damaged in the *Dapl1*−/−; *Dct-Mitf* mice than the *Dapl1*−/− mice (Fig. 6C), meanwhile, the ROS level was significantly lower in the RPE layer of the *Dapl1*−/−; *Dct-Mitf* mice than the *Dapl1*−/− mice (Fig. 6D and E), suggesting that overexpression of MITF could reverse the antioxidant ability of *Dapl1*−/− mice RPE. Moreover, oxidative damage-induced retinal degeneration in *Dapl1*−/−; *Dct-Mitf* mice in vivo was analyzed 4 days after the $NaIO_3$ injection. As the data shown, the ONL thickness (Fig. 6F and G) and Rhodopsin and Opsin levels were higher in *Dapl1*−/−;*Mitf*+/+;*Dct-Mitf* mice compared to *Dapl1*−/−;*Mitf*+/+ mice (Fig. 6H and I), and TUNEL positivity was lower in the former (Fig. 6J and K). Further confirmation of these results came from studies using AAV9-mediated overexpression of MITF, using an AAV9-*p.RPE65-Mitf* virus, which has been shown to be RPE cell specifically expressed [33] (Fig. S9). Taken together, these data indicate that overexpression of MITF in the RPE indeed protects retinas against oxidative stress in *Dapl1*−/− mice.

2.6. DAPL1 up-regulates MITF by down-regulating MYC in RPE cells

Given that the cell cycle regulator MYC inhibits the expression of MITF [37], we hypothesized that DAPL1 may up-regulate MITF in the RPE at least in part by decreasing the levels of MYC. Hence, we first evaluated the protein levels of MYC in the RPE of 2-month-old *Dapl1*−/− mice and in ARPE-19 cells overexpressing DAPL1. As shown in Fig. 7A and B, MYC was increased at the protein level in *Dapl1*−/− RPE (which show reduced MITF levels, see above) as compared to WT RPE. In contrast, DAPL1 overexpression in vitro decreased MYC levels (Fig. 7C and D). As hypothesized, at least in vitro, MYC levels indeed inversely correlate with MITF levels: MYC overexpression, achieved by infection with an appropriate lentivirus, leads to lower MITF levels (Fig. 7E and F), and MYC knock down by siRNAs leads to higher MITF levels (Fig. 7G and H). To test whether DAPL1, MYC and MITF are linked in a pathway, we overexpressed MYC in ARPE-19-DAPL1 cells (in which, as shown above, MYC levels are normally decreased and MITF levels high). We found that indeed, this manipulation led to decreased MITF levels compared to appropriate controls (Fig. 7I and J), without, however, affecting the levels of DAPL1. These data indicate that DAPL1 regulates MITF at least in part by changing the levels of MYC.

2.7. DAPL1 binds E2F4 to regulate the expression of MYC

DAPL1 is not a transcription factor by itself, but conceivably controls gene expression by regulating bona fide transcription factors through protein-protein interactions. We first tested whether it was present in the nucleus. Both immunostaining and western blotting showed that DAPL1-HA is indeed located in the nucleus (Fig. S10) where it could potentially interact with E2F1 and E2F4, two transcription factors known to regulate the expression of MYC [38–40] and, interestingly, also PARP1 (poly (ADP-ribose) polymerase 1 and hence promote

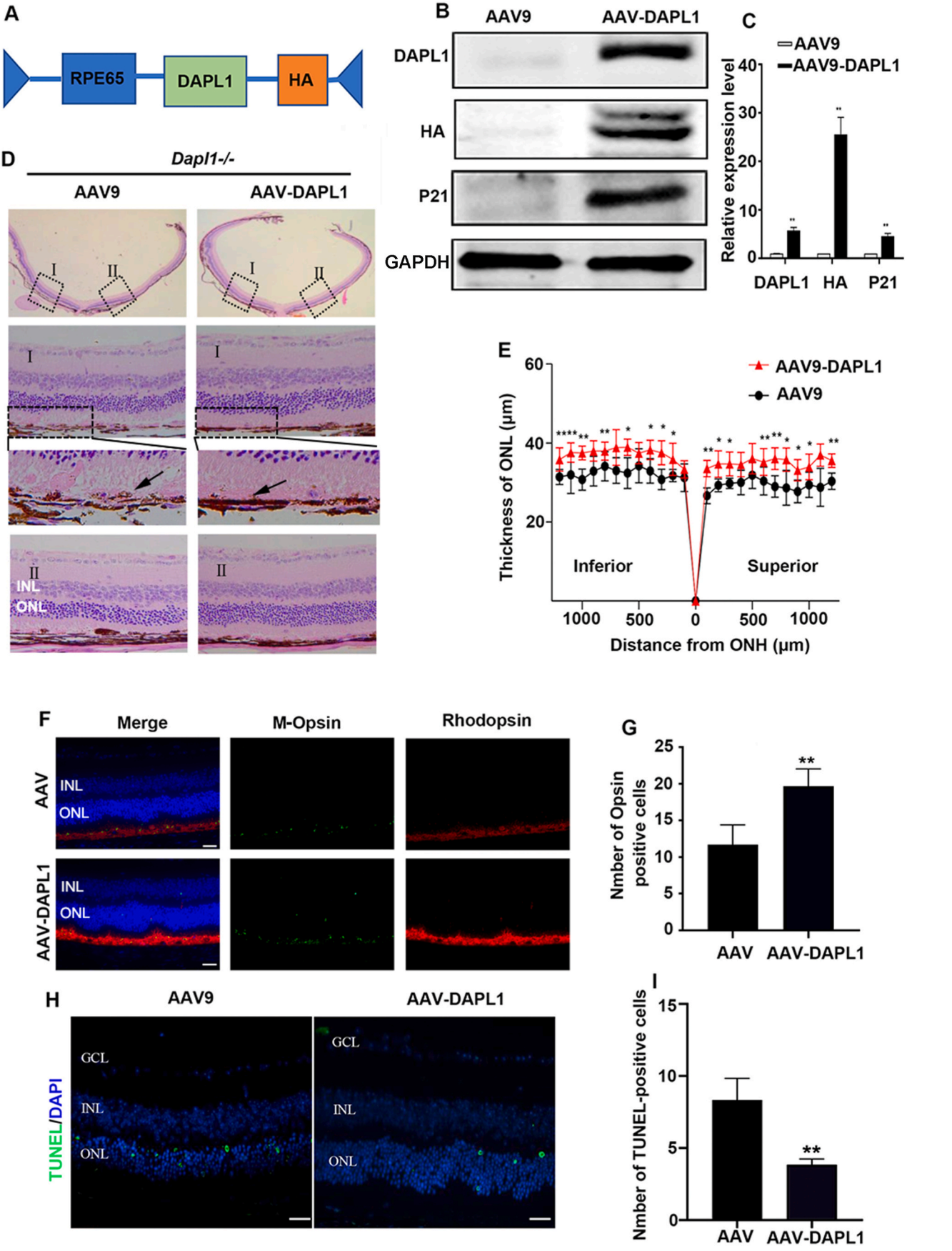

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Fig. 3. Specific overexpression of DAPL1 in RPE cells preserves retinal integrity in *Dapl1*−/− mice. (A) Schematic representation of the AAV9-*p.RPE65*-DAPL1 construct and subretinal injection scheme. (B, C) Western blots for the HA tag, DAPL1 and P21 in the RPE of *Dapl1*−/− mice 2 weeks after infection with AAV9 or AAV9-DAPL1-HA and quantification, N = 3. (D) H&E staining of 2-month-old *Dapl1*−/− retinal sections 2 weeks after AAV9 or AAV9-DAPL1-HA infection including 4 days period after $NaIO_3$ (50 mg/kg) injection, I and II indicate the Inferior and Superior areas around 400 μm from the ONH. (E) Quantification showing the mean ONL thickness of the indicated retinal regions of the indicated infected mice given $NaIO_3$ as indicated for D (N = 6). (F) Immunostaining for Rhodopsin and Opsin in *Dapl1*−/− retinas 2 weeks after infection with either AAV9 or AAV9-DAPL1-HA and injection of $NaIO_3$ as indicated in D. (G) Quantification of Opsin-positive cells per section based on the results in (F) (N = 6). (H, I) TUNEL staining in AAV9 or AAV9-DAPL1-HA infected *Dapl1*−/− eyes after injection of $NaIO_3$ and quantification (N = 6). ONH, optic nerve head; ONL, outer nuclear layer. Scale bar: 50 μm ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

oxidative stress [41]. We, therefore, used co-immunoprecipitation (pull down) assays of extracts of DAPL1-HA-overexpressing ARPE-19 cells to test for interactions with E2F1 and/or E2F4. As shown in Fig. 8A, DAPL1-HA pull down gave a signal for E2F4, and anti-E2F4 pull down a signal for DAPL1-HA. No signals were observed by using control IgG and anti-HA did not pull down E2F1. These results suggest that DAPL1 is at least capable of binding E2F4. To test whether this interaction leads to regulation of MYC, we manipulated the levels of E2F4 by overexpression and siRNA-mediated knock down in ARPE-19-DAPL1 cells. As shown in Fig. 8B–E, knock down of E2F4 led to a decrease and overexpression of E2F4 to an increase in the expression MYC.

Next, we analyzed through which protein domain DAPL1 might interact with E2F4. We have previously shown that a protein kinase C (PKC) phosphorylation domain of DAPL1 (aa24-aa28) is required to inhibit RPE cell proliferation [19]. We find that indeed, this domain is also needed for interaction with E2F4 as DAPL1 with a deletion of aa24-28 (DAPL1-Del-HA) is no longer capable of efficiently pulling down E2F4, nor is E2F4 capable of pulling down DAPL1-Del-HA (Fig. 8F and G). This DAPL1 domain is not only critical for interaction with E2F4 in pull down assays but is also functionally important to regulate MYC. As shown in Fig. 8H and I, after expression of DAPL1-Del-HA in ARPE-19 cells, MYC levels were increased and MITF levels were decreased as compared to cells in which full length DAPL1-HA was expressed. These results clearly show that the expression of MYC and MITF, and hence protection from oxidative stress, is controlled at least in part by DAPL1 binding to E2F4.

3. Discussion

The attention that *DAPL1* has so far received in functional and clinical studies is relatively limited. It has originally been speculated to be involved in an early stage of normal epithelial differentiation [18]. More recently, a candidate gene approach has identified *DAPL1* alleles to be associated with AMD [17]. How *DAPL1* and AMD may be linked mechanistically, however, has not so far been studied.

Here, we show in mice that absence of DAPL1 RNA or protein impairs the antioxidant defense system in RPE cells and induces an age-dependent manner a form of retinal degeneration. Our work clearly shows that DAPL1 acts as an antioxidant regulator in the RPE, the single cell layer that is juxtaposed to retinal photoreceptors and that plays an important role in retinal homeostasis. The pathway by which *Dapl1* controls the RPE's antioxidant function involves several transcription factors and a transcriptional co-activator. As schematically illustrated in Fig. 9, these factors seem to be arranged linearly in the following way: DAPL1 —| E2F4 → MYC —| MITF → NRF2 + PGC1α. The evidence for this is manifold: (1) *Dapl1* deficiency leads to age-related retinal degeneration-like phenotypic features and decreases the antioxidant capacity of RPE cell; (2) Overexpression of *Dapl1* in the RPE elevates the antioxidant capacity in both WT and *Dapl1*−/− mice and interferes with the development of retinal degeneration in *Dapl1*−/− mice; (3) DAPL1 binds E2F4 to inhibit the expression of MYC, leading to an increase in the expression of MITF in the RPE, which in turn increases antioxidation by regulating NRF2 and PGC1α; (4) deliberate overexpression of MITF in the RPE of *Dapl1*−/− mice rescues antioxidation and protects retinas from oxidative damage.

The link of DAPL1 to antioxidation is of particular interest because it is well established that antioxidant deficiency in the RPE is one of the main factors in the pathogenesis of retinal degenerative diseases including AMD [3,42]. Clearly, with increasing age, the amount of oxidized proteins, lipids, and damaged mitochondria accumulate, not the least also because of environmental factors such as smoking or excessive light exposure, and in addition they may be less efficiently removed [6,43,44]. Hence, future study will focus on the functional roles and precise regulatory mechanisms of DAPL1 in AMD. Besides RPE cells, DAPL1 might also play neuronal protective roles in the neural retina cells, which may also contribute the age-related retinal degeneration under the DAPL1 deficiency conditions.

It needs to be kept in mind, however, that even though the above pathway seems straightforward, the molecular mechanisms operating in aging RPE cells may be much more complex. For one, the pathway may include feedback loops that cannot be revealed if a given gene in a knockout mouse can no longer functionally respond to regulation. We have previously found in vitro, for instance, that at least one type of isoforms of MITF, so called (−) MITF, enhances DAPL1 expression in a "doubly" indirect pathway involving Musashi RNA binding protein-2 and a microRNA, miR-7 [45]. Combined with these results, it exists a positive feedback loop between the DAPL1 and MITF in RPE cells, which might work together to regulate functions in the RPE and maintain retinal homeostasis. Although this feedback loop is not expected to operate in our *Dapl1*−/− mice, it may well do so in humans, where it may be part of a regulatory circuit assuring RPE homeostasis.

Adding to the above complexity, it is also clear that each of the individual factors in the above pathway may have additional roles in the RPE or may influence antioxidation more indirectly. We here mention but a few of these additional roles. DAPL1, for instance, inhibits RPE cell proliferation by up-regulating the protein levels of P21, E2F1, P107 and P-RB [19], and *Dapl1* deficiencies in mice lead to RPE hyperproliferation [19]. Cell proliferation requires energy, provided by oxidative phosphorylation in mitochondria, which increases ROS levels and oxidative stress [41,46]. Nevertheless, that *Dapl1* deficiency leads to increased RPE cell proliferation need not necessarily confound the principle findings of the present study because the majority of the genetic and deliberate knockdown, overexpression and rescue experiments used here to demonstrate a link of DAPL1 to oxidative damage were performed in mice at an age at which the RPE hyperproliferation phenotypes are not yet manifested. Also, it is conceivable that inhibiting proliferation and at the same time maintaining antioxidation may be DAPL1's principle role in maintaining the RPE in a healthy state.

Going downstream in the pathway, it has been previously demonstrated that E2F4 is required for cell cycle progression by binding tumor suppressor protein RB and its family members P107 and P130 [47,48]. Knockdown of *E2F4* decreases the expression of MYC and increases that of P21 [49]. *E2f4*−/− newborn mice display abnormalities in hematopoietic lineages as well as defects in the development of the gut epithelium [50], and the mice eventually die of an increased susceptibility to opportunistic infections [51]. Here we show in RPE cells that the levels of E2F4 directly correlate with those of MYC and that DAPL1 binding to a functionally important domain of E2F4 involving a PKC phosphorylation site reduces MYC expression, while compensatory overexpression of E2F4 in DAPL1-overexpressing cells increases MYC expression. MYC itself is a multipurpose oncogene with a wide array of functions. Mice heterozygous for a *Myc* deletion exhibit increased lifespan and resistance to several age-associated pathologies, including osteoporosis, cardiac fibrosis, and immunosenescence [52]. MYC

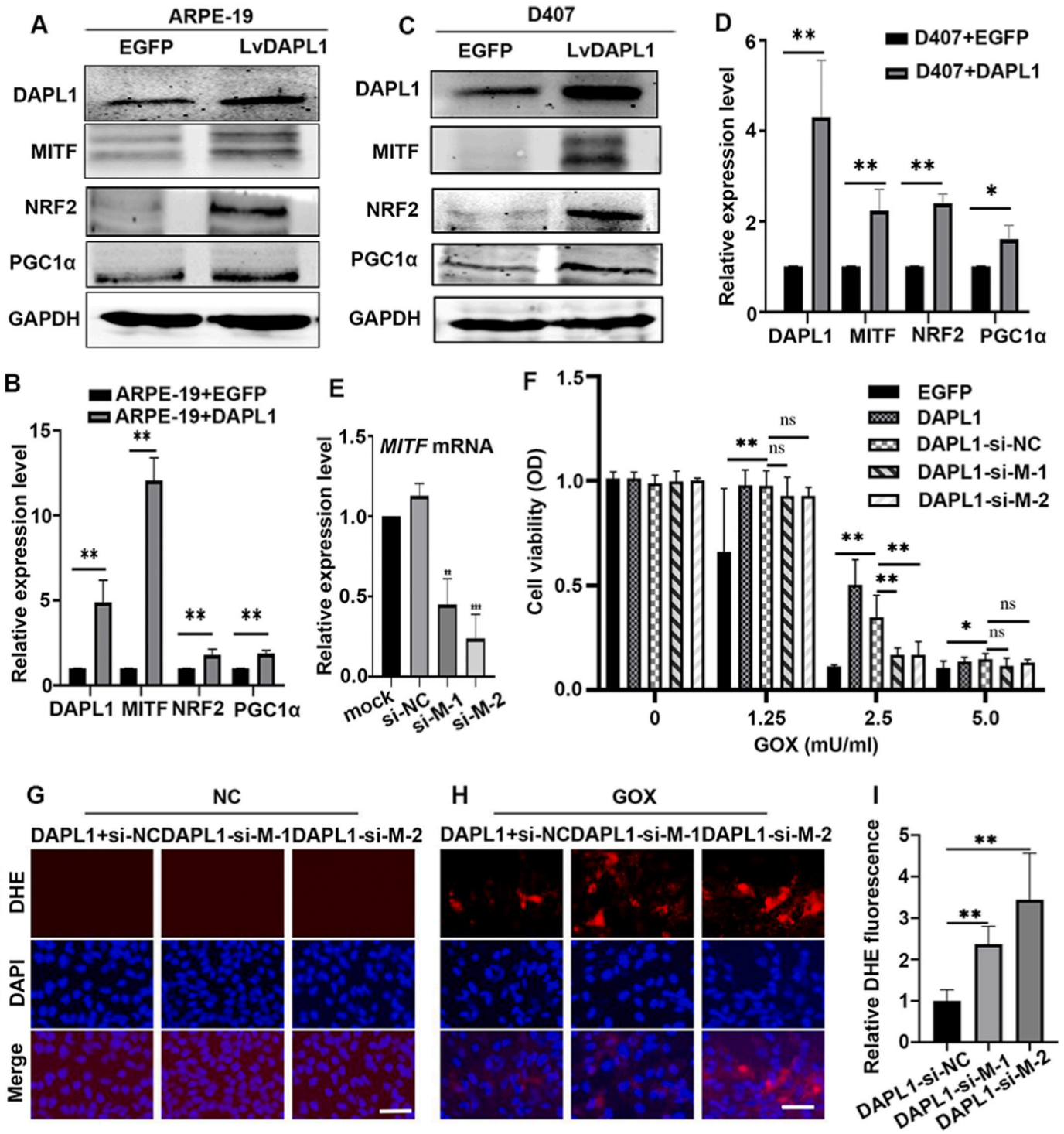

Fig. 4. DAPL1 regulates the antioxidant capacity in RPE cells by regulating MITF. (A–D) Western blots for DAPL1, MITF, NRF2, and PGC1α in DAPL1-overexpressing ARPE-19 and D407 cells and quantification. (E) *MITF* knockdown efficiency was measured by real-time PCR in ARPE-19+DAPL1 cells. Si-M-1 and si-M-2 indicate two distinct siRNAs against MITF. (F) Cell viability was measured by CCK8 after GOX treatment for 6 h in ARPE-19+DAPL1 cells with or without knockdown *MITF*. (G, H) Representative images and quantification (I) of DHE staining to detect the ROS levels in ARPE-19+DAPL1 cells after *MITF* knockdown with or without GOX treatment for 6 h. Scale bar: 50 μm. N = 3; $^{**}P < 0.01$, $^{*}P < 0.05$.

overexpression, on the other hand, causes oxidative stress and induces DNA damage [53]. Nevertheless, neither E2F4 nor MYC has so far been linked to the pathogenesis of retinal degenerative diseases in humans.

Going further downstream in the above pathway, MITF also regulates a variety of genes in the RPE. In fact, MITF is considered a master regulator of pigment cells and a major factor in their development and function. In the RPE, it regulates pigmentation genes but also *Pedf* and visual cycle genes such as *Rlbp1* and *Rdh5* [29–31]. Interestingly, in AMD patients, the expression of PEDF is lower in RPE cells, the RPE basal lamina, Bruch's membrane, and choroidal stroma, thereby potentially promoting choroidal neovascularization [54]. In mice, *Pedf* deficiency leads to an increase in susceptibility of retinal degeneration associated with the *rd10* mutation [55]. Hence, DAPL1 might play other functions through MITF in the RPE, which could also contribute to the

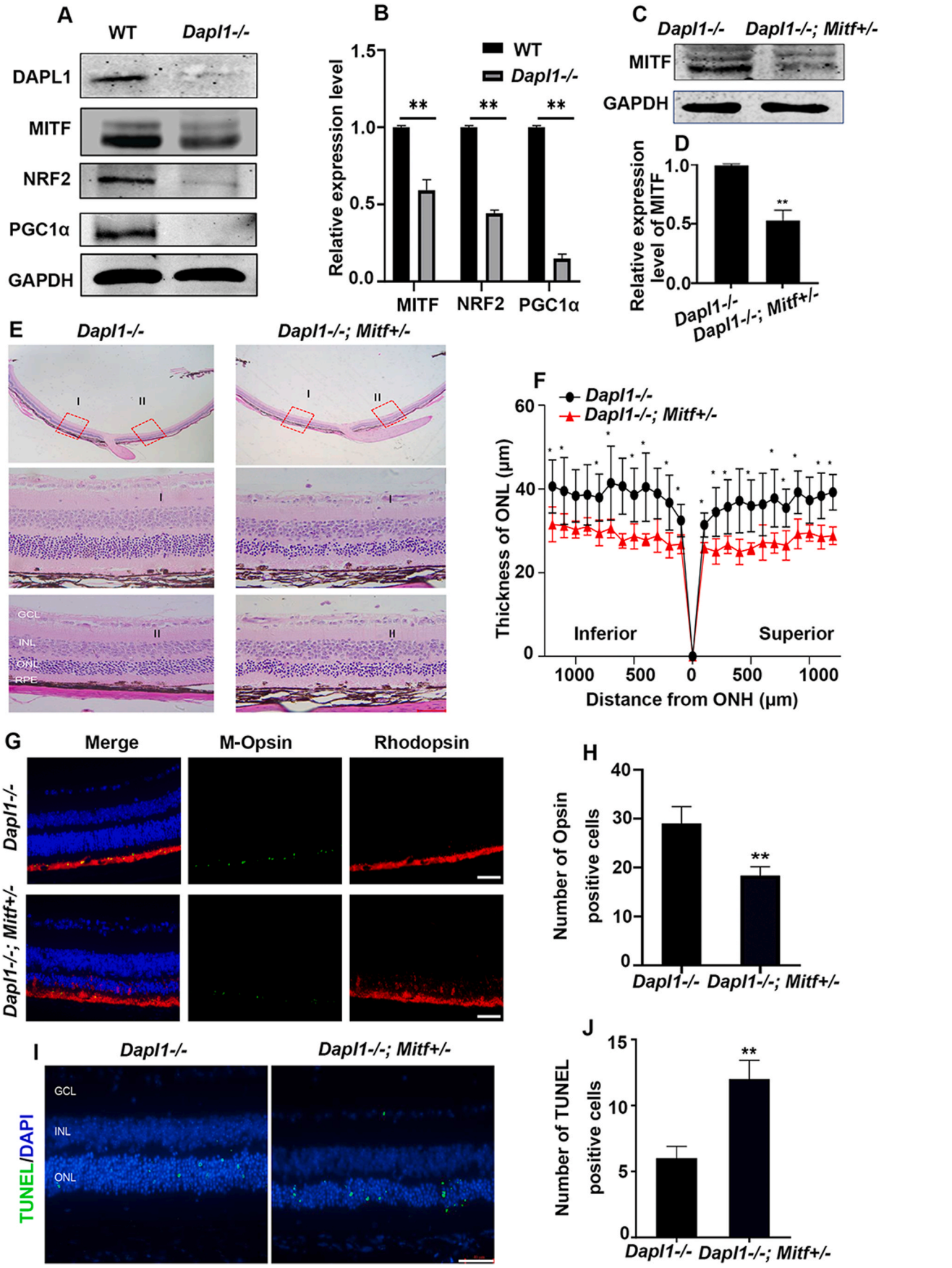

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Fig. 5. MITF haploinsufficiency exacerbates oxidative damage-induced retinal degeneration in *Dapl1*−/− mice. (A, B) Western blots for DAPL1, MITF, NRF2, and PGC1α in RPE cells in 2-month-old WT and *Dapl1*−/− mice and quantification (N = 3). (C) Western blots for MITF in RPE cells in 2-month-old *Dapl1*−/− and *Dapl1*−/−; *Mitf*+/− littermates and quantification (D) (N = 3). The 2-month-old *Dapl1*−/− and *Dapl1*−/−; *Mitf*+/− littermate mice were intraperitoneal injected with $NaIO_3$ (30 mg/kg), and the retinal structure analysis was carried out 4 days later. (E) H&E staining of the retinal sections of the indicated mice 4 days after intraperitoneal $NaIO_3$ injection, I and II indicate the Inferior and Superior areas around 400 μm from the ONH. (F) Quantification showing the mean thickness of the ONL in the indicated regions as based on the results in (E) (N = 6). (G) Immunostaining for Rhodopsin and Opsin in retinal sections of 2-month-old *Dapl1*−/− and *Dapl1*−/−; *Mitf*+/− littermates 4 days after $NaIO_3$ injection. (H) Quantification of the Opsin-positive cells per section based on the results in (G) (N = 6). (I) TUNEL staining of the retinas of 2-month-old *Dapl1*−/− and *Dapl1*−/−; *Mitf*+/− littermates 4 days after $NaIO_3$ injection and quantification (N = 6). ONH, optic nerve head; ONL, outer nuclear layer. Scale bar: 50 μm $^{**}P < 0.01$, $^{*}P < 0.05$.

occurrence of age-related retinal degeneration.

Finally, NRF2 has multiple roles in metabolism, mitochondrial physiology, autophagy and immune mechanisms [3,56–58] and PGC1α is a master regulator of mitochondrial biogenesis [59,60]. Both have also well-established roles in retinal physiology. *Nrf2/Pgc1α* double knockout mice, for instance, exhibit an AMD-like retinopathy [14], and overexpression of NRF2 protects retinas from oxidative damage [61]. Both genes are critical in regulating the cellular redox balance not only in the RPE but in other cell types as well. Moreover, we have previously found that MITF regulates the antioxidant defense system through the regulation of NRF2 and PGC1α [32,33], and that MITF overexpression in the RPE can protect retinas against premature degeneration [33].

In summary, then, our findings provide strong evidence that DAPL1 acts as an antioxidant regulator in RPE cells by binding E2F4 to regulate the MYC-MITF-NRF2/PGC1α axis. As *DAPL1* has been established as a susceptibility gene for AMD in humans, we believe that our findings not only provide novel insights into the regulatory mechanisms of the antioxidant defense system in the RPE, but also into the regulatory mechanisms operating during age-related retinal degeneration. It is hence important to elucidate whether the role of the *Dapl1* pathway in retinal degeneration in mice will be equally important in the pathogenesis of AMD and perhaps other forms of retinal degenerative diseases in humans and so potentially become a target for future therapeutic interventions.

4. Materials and methods

4.1. Animal studies and approval

Mitf $^{mi\text{-}vga9}$ mutant mice [26], here designated *Mitf*−/− mice were from Dr. Heinz Arnheiter. CRISPR/Cas9 mediated *Dapl1* knockout mice and *Dct-HA-Mitf* transgenic mice were generated and described in our previous work [19,33]. All mice were kept on the C57BL/6J background and the rd8 mutation of the *Crb1* gene was excluded. Protocols used for all animal studies were approved by the Wenzhou Medical University Animal Care and Use Committee and performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement on the use of Animals in Ophthalmic and Vision Research (WYDW2019-0353).

4.2. Cell culture

Primary mouse RPE cells were isolated from 2-month old WT or *Dapl1*−/−mice, and cultured in DMEM/F12 medium supplemented with 15% FBS. 10 days after the culture, the primary RPE cells were used for antioxidant analysis. The ARPE-19 and D407 cell lines were purchased from ATCC and cultured in DMEM/F12 and DMEM medium respectively (*Gibco*) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. The DAPL1-overexpressing ARPE-19 cells (ARPE-19+DAPL1) or EGFP-overexpressing cells (ARPE-19+EGFP) were produced as previously described [19]. The ARPE-19 and D407 cells to be used for experimental analysis were cultured for 2 weeks after plating.

4.3. $NaIO_3$-induced retinal oxidative damage

The $NaIO_3$-induced retinal oxidative damage mouse model has been described [33]. $NaIO_3$ (BBI Life Sciences) was dissolved in sterile normal saline and was given by intraperitoneal injection (30 mg/kg in *Dapl1*−/−; *Mitf*+/-mouse experiment or 50 mg/kg in other mouse experiments). The control group mice were injected with the same volume of normal saline. Four days after $NaIO_3$ injection, the eyeballs were harvested for the follow-up experimental analysis.

4.4. Scotopic electroretinogram (ERG) recordings

Mice were dark-adapted for 4 h and then kept under dim red light for preparation. After anaesthesia with ketamine (90 mg/kg) and xylazine (8 mg/kg), dilation of the pupils with 0.5% tropicamide and topical anaesthesia with 0.5% proxymetacaine HCl, standard ERG was recorded with corneal electrodes. Each stimulus (the optical power was 3 cds/m2, and the band pass was filtered from 1 to 300 Hz) was given to every responder once with a duration of 250 ms. Amplitudes were measured and recorded for both a- and b-waves by RETI2 scan vision electrophysiology detection (*Roland Consult, Brandenburg, Germany*). The average amplitudes of the a- and b-waves were calculated. The amplitude of the a-wave was measured from the baseline to the bottom of the a-wave, and the amplitude of the b-wave was measured from the bottom of the a-wave to the peak of the b-wave.

4.5. Retinal histological analysis and antibodies

After euthanasia of the experimental mice, both eye samples were obtained for histological analysis, immunofluorescence analysis and western blotting. For histopathological analysis, eye samples were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. For immunofluorescence analysis, eye samples were embedded in OCT (Optimal Cutting Temperature) compound at −80 °C. For immunofluorescence, retinal cuts were incubated in a blocking buffer containing 1% BSA for 1 h followed by 0.02% Triton X-100 for 30 min at room temperature. RPE flat mounts were incubated with anti-ZO-1 and anti-OXT2 primary antibodies overnight at 4 °C, followed by a 2-h incubation with the secondary antibody. Hematoxylin & Eosin (H&E) staining was carried out as previously described. The thickness of retinal ONL and INL layers were measured by ZEN 2.3 lite software of CarI Zeiss Microscopy at a distance between 100 μm and 1200 μm from the optic nerve head to the peripheral retina. The number of TUNEL or Opsin positive cells is represented as the average of the numbers counted separately in 6 different retinal sections. In addition, retinal tissues were stripped from other eye components for western blotting.

5. Western blotting

Mouse RPE cells or the RPE cell lines cultured in vitro were washed with PBS and lysed on ice for 30 min with RIPA Lysis Buffer (Biyuntian, China). The protein lysates were separated by 10% SDS-PAGE, then transferred to PVDF (Polyvinylidene fluoride) membranes, which were blocked with 5% skimmed milk at room temperature for 2 h, and incubated at 4 °C overnight with each primary anti-bodies. The antibodies used in this work and their dilution are listed in Table S2. The

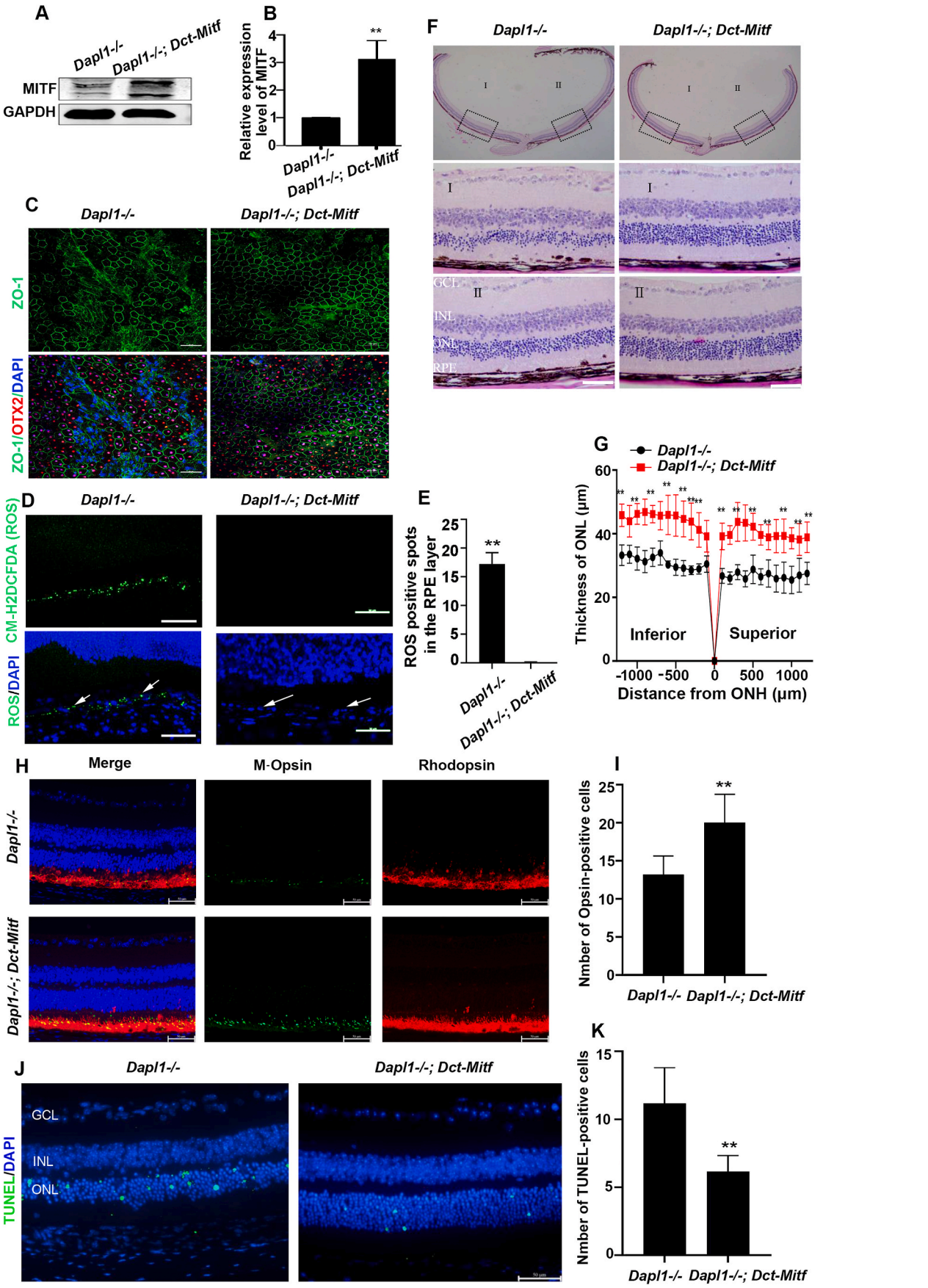

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Fig. 6. Transgenic overexpression of MITF in the *Dapl1−/−* RPE increases its antioxidant capacity. (A) Western blots for MITF in the RPE of 2-month-old *Dapl1−/−* and *Dapl1−/−; Dct-Mitf* littermates and quantification (B) (N = 3). For C-G, the mice were intraperitoneally injected with $NaIO_3$ (50 mg/kg), and the retinal structure analysis was carried out 4 days later. (C) 12 h after intraperitoneal injection of 50 mg/kg $NaIO_3$, immunostaining images of anti-ZO-1 and anti-OTX2 in RPE flat mounts of 2-month-old *Dapl1−/−* and *Dapl1−/−*; *Dct-Mitf* mice. (D) 12 h after after intraperitoneal injection of 50 mg/kg $NaIO_3$, green fluorescent probe CM-H2DCFDA detected ROS production in 2-month-old *Dapl1−/−* and *Dapl1−/−*; *Dct-Mitf* mice retinas. (E) Quantification shows the mean number of ROS positive spots in the RPE layer based on the result of (D). (F) H&E staining of retinal sections of the indicated mice 4 days after $NaIO_3$ injection, I and II indicate the Inferior and Superior areas around 400 μm from the ONH. (G) Quantification showing the mean thickness of the ONL in the indicated regions, based on (F). (H, I) Immunostaining for Rhodopsin and Opsin of retinal sections 4 days after injection of $NaIO_3$ and quantification (N = 6). (J, K) TUNEL staining in retinas 4 days after injection of $NaIO_3$ and quantification. N = 6. ONH, optic nerve head; ONL, outer nuclear layer. Scale bar: 50 μm $^{**}P < 0.01$, $^{*}P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

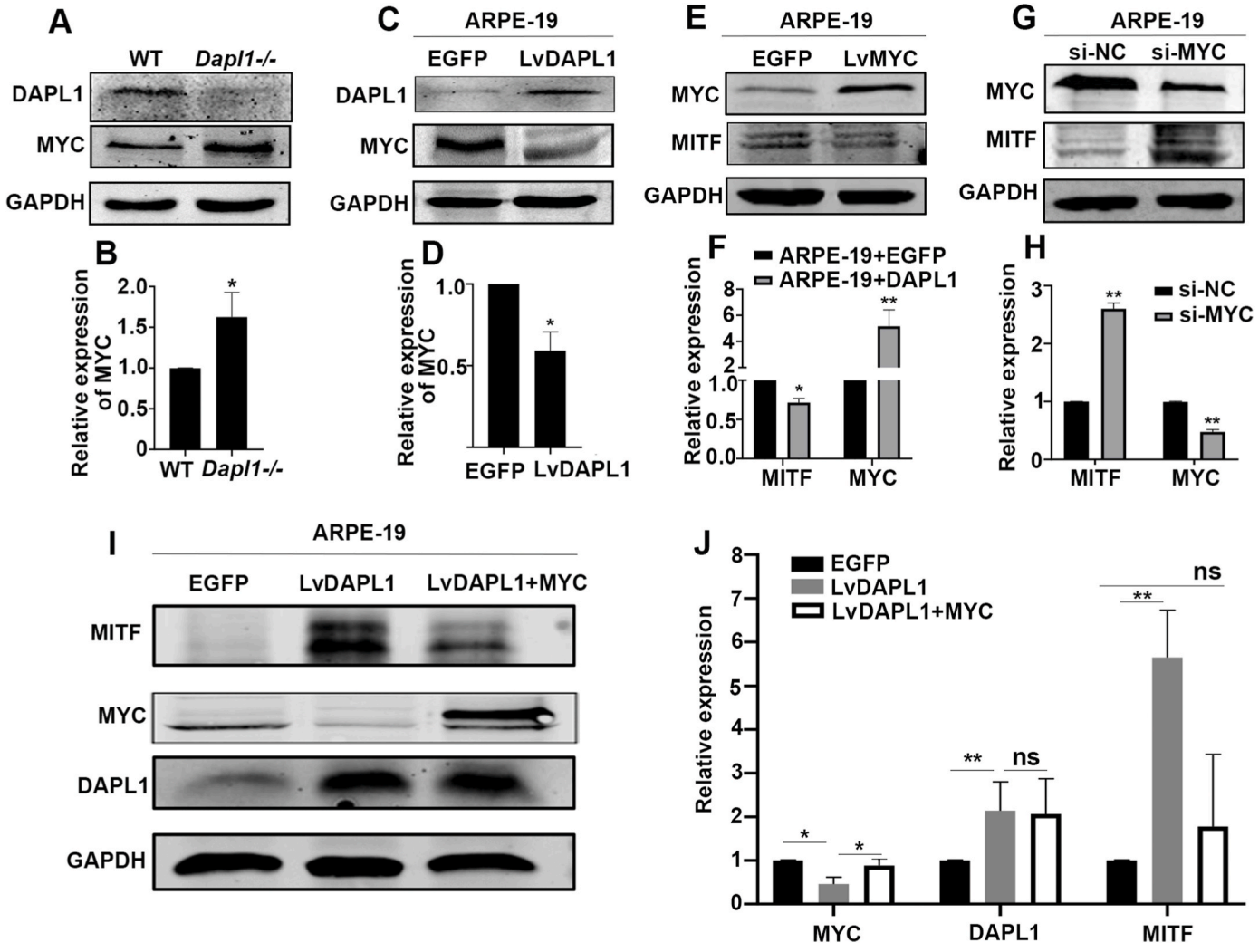

Fig. 7. DAPL1 upregulates MITF by down-regulating MYC in RPE cells. (A, B) Western blots and quantitative analysis for MYC and DAPL1 in the RPE of 2-month-old WT and *Dapl1−/−* mice. (C, D) Protein levels of MYC and DAPL1 in the DAPL1 overexpressing ARPE-19 cells (LvDAPL1) as seen by western blotting and its quantitative analysis. (E, F) Western blots and quantitative analysis for MYC and MITF in MYC-overexpressing ARPE-19 cells (LvMYC). (G, H) Western blots and quantitative analysis for MYC and MITF in si-MYC-treated ARPE-19 cells. (I) Western blots and quantitative analysis for MYC, MITF, and DAPL1 in cells overexpressing either DAPL1 or DAPL1 and MYC together. N = 3, $^{**}P < 0.01$, $^{*}P < 0.05$, ns, no significant difference.

membranes were then probed with fluorescein-conjugated secondary antibodies (1:5000, LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 2 h. Finally, the blots were analyzed using the Odyssey CLx system (LI-COR Biosciences), and quantitative densitometry of the bands were performed using ImageJ software.

6. ROS fluorescence assay

The method for determining ROS in the mouse retina section was described in our previous work [33]. Briefly, Green fluorescent probe CM-H2DCFDA (Genmed Scientifics Inc., Shanghai, China) was used to detect the ROS level in the prepare 10 μm thick unfixed retinal frozen sections according to the manufacturer's instructions. For each section, 200 μl of the preheated GENMED staining solution was added and incubated for 30 min in a light-protected moistened incubator at 37 °C. Immediate observation under a fluorescence microscope.

6.1. siRNA knock down

ARPE-19 cells were seeded in 6-well plates and allowed to grow to

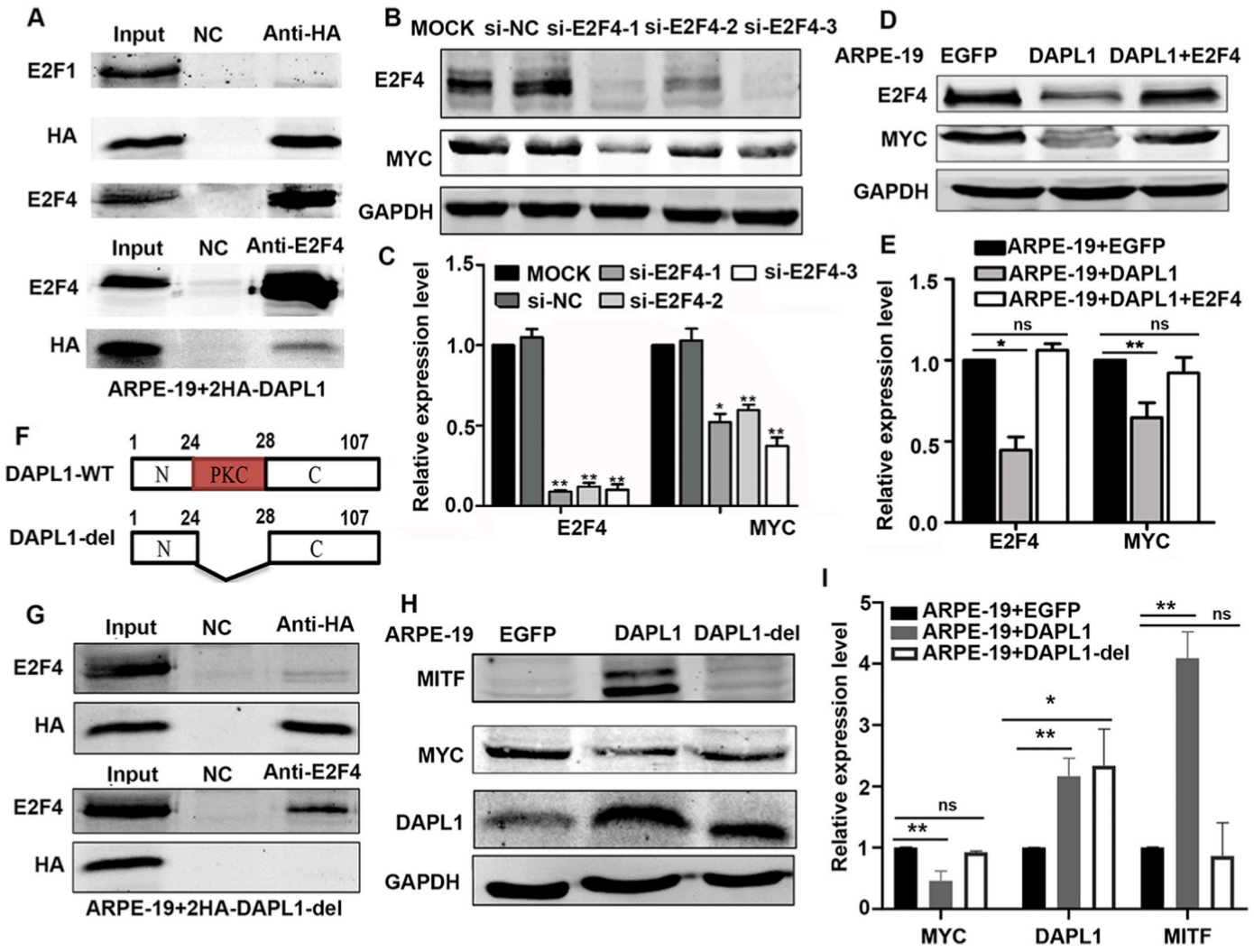

Fig. 8. DAPL1 binds E2F4 to regulate the expression of MYC and MITF. (A) Lentivirus-mediated DAPL1-HA expressing ARPE-19 cells were used for Co-IP analysis. In the anti-HA pull down samples, DAPL1-HA co-immunoprecipitates E2F4 but not E2F1. Also, Co-IP with anti-E2F4 pulls down DAPL1-HA as revealed by the HA signal. 2HA refers to constructs containing 2 HA tags. (B, C) Western blots for E2F4 and MYC in E2F4 knockdown ARPE-19 cells (si-E2F4) and quantification. (D, E) Western blots for E2F4 and MYC in cells overexpressing either DAPL1 alone or DAPL1 and E2F4 together (DAPL1+E2F4) and quantification. (F) Schematic diagram illustrating full-length DAPL1 and the PKC domain deletion (DAPL1-del). (G) Co-IP analysis of HA and E2F4 was carried out in ARPE-19+DAPL1-Del-HA cells. In anti-HA or anti-E2F4 to pull down samples, a weak E2F4 band was observed in the anti-HA pull down sample and no HA positive signal was observed in the anti-E2F4 pull down sample, indicating that E2F4 and DAPL1-del-HA protein show at best only weak interaction. (H, I) Western blot for DAPL1, MITF, and MYC in ARPE-19 cells overexpressing EGFP (ARPE-19+EGFP), DAPL1 (ARPE-19+DAPL1), or DAPL1-del (ARPE-19+DAPL1-del) and quantification. N = 3, $^{**}P < 0.01$, $^{*}P < 0.05$. ns, no significant difference.

70% confluence for 24 h. Transient transfection was performed using transfection reagent Lipofectamine™ 2000. Specific siRNA for human MITF, MYC and E2F4 isoforms and non-targeted control siRNAs were synthesized by Gene Pharma Co. (Shanghai, China) with the sequences listed in Table S1. 5 μL of siRNA and 5 μL of Lipofectamine™ 2000, respectively, were diluted with 95 μL of serum-free DMEM medium and mixed after a 5-min incubation. The mixture was then incubated for 20 min at room temperature and added drop-wise to each culture well containing 800 μL of serum-free DMEM medium. The medium was discarded 6 h thereafter and replaced with fresh complete culture medium with 10% FBS. Experiments such as western blotting were performed after culturing for 48 h.

6.2. AAV9 vector construction and virus injection

The AAV9-p.RPE65-MCS-SV40-PolyA vector was acquired from the Shanghai Genechem Co., LTD, China. Mouse HA-Mitf (NM_008601) was from Dr. Heinz Arnheiter, Human HA-DAPL1 (NM_001017920) was constructed as previously described [45]. Briefly, 0.5 μl of AAV9-MITF, AAV9-DAPL1 or the AAV9 empty vector virus supernatant (10^{12} genome copies/ml) was injected into the subretinal space of the mice, using a pulled angled glass pipette under direct observation aided by a dissecting microscope under dim light [33].

6.3. Lentivirus infection

DAPL1-HA was constructed into the vector of TG005, which was purchased from Bi'ang Biomedical Technology Co., Ltd (Shanghai, China). DAPL1-HA cDNA was amplified using the primers of DAPL1-HA-F and DAPL1-HA-R. DAPL1-Del-HA cDNA was amplified using the primers of DAPL1-Del-HA-F and DAPL1-Del-HA-R. The sequence of the primers are listed in Table S3. Lentiviral particles for E2F4 (LPP-Z0752-Lv105, NM_001950) and MYC (LPP-Z2845-Lv105, NM_002467) were purchased from Gene Copoeia Company. For infection, viruses were suspended in serum-free medium and added to ARPE-19 cells at a multiplicity of infection (MOI) of 100. After a 30-min incubation, fresh

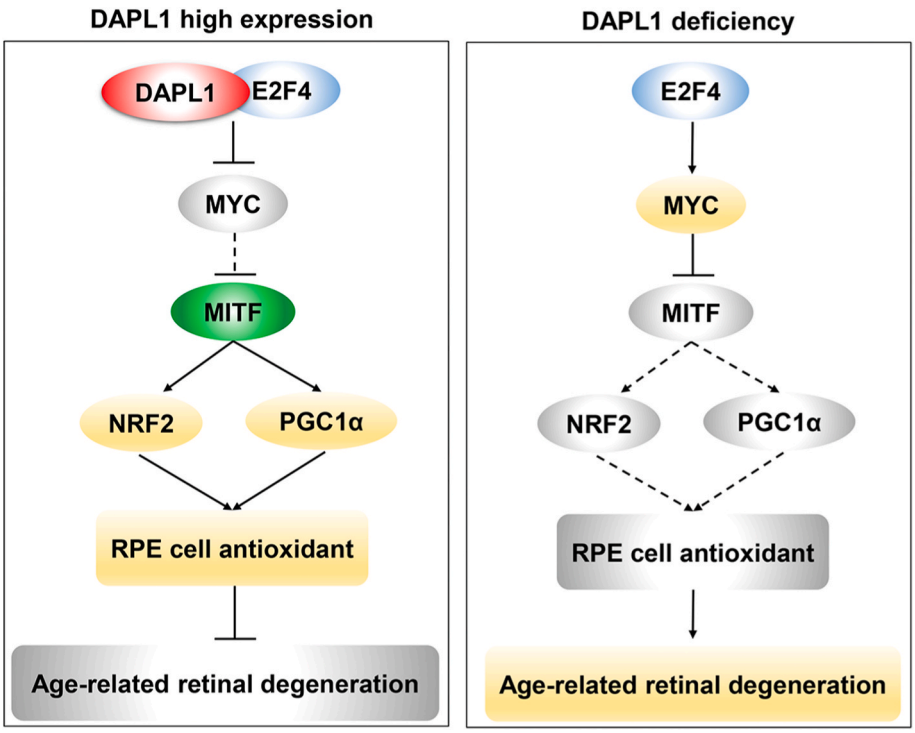

Fig. 9. Schematic summary of how DAPL1 acts as an antioxidant regulator in RPE cells. (Left) In normal RPE cells, DAPL1 is highly expressed and binding to E2F4 inhibits its activity. This leads to reduced expression of MYC, which in turn promotes the expression of MITF and hence activates the antioxidant defense system by increasing the expression of NRF2 and PGC1α. (Right) DAPL1 deficiency in RPE cells releases the inhibition of E2F4, leading to activation of MYC, which in turn decreases the expression of MITF and hence its targets NRF2 and PGC1α. This then exacerbates oxidative damage in the RPE and contributes to age-related retinal degeneration in mice with DAPL1 deficiency.

medium was added to the cells. Experiments such as western blots were performed after culturing for 48 h.

6.4. Immunoprecipitation

Using fresh samples of different cells, extracts (0.5 mg) were incubated for 4 h with nonspecific IgG, poly-clonal anti-HA, anti-E2F4 or anti-E2F1, followed by addition of 25 μl Protein A-sepharose beads (GE Life Sciences, 17-0780-01) overnight at 4 °C. Samples were washed five times with sterile PBS and denatured with SDS sample buffer. The immunocomplex was eluted in loading buffer by boiling at 95 °C for 5 min and then subjected to western blot analysis.

6.5. Statistical analysis

Each cellular experiment was repeated four times, while each mouse experiment was carried out using 6 mice (3 males, 3 females). All quantitative data were presented as the mean ± SEM. The statistical significance of differences between groups was obtained using one-way ANOVA in GraphPad (*San Diego, CA*). Differences were considered to be significant at $P < 0.05$.

Authors' contributions

Conceived and designed the experiments: L Hou, X Ma. Performed the experiments: X Ma, H Chen, S Jian, J He, Y Liu, S Han, X Hu, X Liu, L Chang, Z Liu. Analyzed the data: X Ma, H Chen, S Jian, J He, L Hou. Contributed reagents/materials/analysis tools: L Hou, X Ma, Y Chen. Wrote the manuscript: X Ma, L Hou, H Chen, S Jian, J He. Revised the manuscript: X Ma, L Hou.

Declaration of competing interest

The authors declared that they have no conflict of interest to this work.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.102675.

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