

## RESEARCH ARTICLE

# Mitochondrial functional impairment in *ARL3*-mutation related rod-cone dystrophy

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## Abstract

Mitochondria are vital for retinal cell function and survival, and there is growing evidence linking mitochondrial dysfunction to retinal degenerations. Although *ARL3* mutations have been linked to multiple forms of retinal degeneration, the relationship between *ARL3* and mitochondria remains unexplored. Herein, we investigated the effects of *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup>, and *ARL3*<sup>T31A/C118F</sup> mutations on mitochondrial function in fibroblasts obtained from patients with *ARL3*-related rod-cone dystrophy. Our findings revealed that these mutations led to a decrease in mitochondrial respiration, an increase in the accumulation mitochondrial reactive oxygen species (ROS), and induction of apoptosis in fibroblasts. Additionally, we conducted a comparative analysis of the effects of *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup>, and *ARL3*<sup>T31A/C118F</sup> proteins on mitochondria in ARPE-19 cells. Results showed that *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> not only affected mitochondrial function but also induced apoptosis in ARPE-19 cells. Conversely, *ARL3*<sup>C118F</sup> primarily influenced cell apoptosis with minimal effects on mitochondrial function in ARPE-19 cells. Transcriptome analysis further suggested the involvement of respiratory electron transport, response to ROS, and apoptotic signaling pathways in *ARL3*<sup>T31A/C118F</sup> cells. Our study demonstrated that *ARL3*-related mutations play a significant role in the diversity of mitochondrial function, providing novel insights into the functional analysis of *ARL3*-related mutations.

## KEYWORDS

apoptosis, *ARL3*, mitochondria, mutation, rod-cone dystrophy

## 1 | INTRODUCTION

Rod-cone dystrophy (RCD) is an inherited retinal dystrophy that affects approximately 1 in 40,000 individuals.<sup>1</sup> It is the most common form of retinitis pigmentosa, which is characterized by the progressive deterioration of both

rod and cone photoreceptor cells, leading to gradual vision loss. RCD is exceptionally heterogeneous with mutations in more than 65 genes being implicated,<sup>2,3</sup> characterized by their genetic etiology and mode of inheritance, including autosomal recessive, autosomal dominant, and X-linked patterns.<sup>4,5</sup> Nonetheless, roughly 40% of RCD cases

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remain unsolved emphasizing the urgency for disease mechanism to be discovered.<sup>6,7</sup>

ARL3, also known as ADP-ribosylation factor-like 3, is a small GTP-binding protein. It cycles between an inactive GDP-bound state and an active GTP-bound state.<sup>8–10</sup> Studies have shown that *ARL3* mutations have been associated with multiple forms of retinal degeneration. *ARL3*<sup>R99I</sup> variant causes autosomal recessive cone-rod dystrophy,<sup>11</sup> *ARL3*<sup>R149H</sup> or *ARL3*<sup>R149C</sup> variant causes recessive Joubert syndrome,<sup>12</sup> *ARL3*<sup>T31A/C118F</sup> compound heterozygote causes RCD,<sup>13</sup> *ARL3*<sup>Y90C</sup> causes autosomal retinitis pigmentosa, and *ARL3*<sup>D67V</sup> causes autosomal dominant retinal degeneration.<sup>14</sup> In a previous investigation, we identified a patient presenting with early-onset optic RCD,<sup>13</sup> who harbored compound heterozygous variants *ARL3* c.91A>G and c.353G>T (p.T31A and p.C118F). This patient displayed a severe clinical phenotype characterized by retinal thinning and atrophy, consistent with the clinical manifestation of retinal ciliopathy. Importantly, the patient's father carried an *ARL3* c.91A>G (p.T31A) mutation, which was associated with mild signs of retinal atrophy and a delayed onset of clinical manifestations. Furthermore, our recent studies have demonstrated that the *ARL3*<sup>T31A/C118F</sup> compound mutations lead to significantly elongated cilia and impaired retrograde transport, while the single *ARL3*<sup>T31A</sup> mutation does not.<sup>15</sup> These findings combined with clinical observations, suggest that distinct *ARL3* mutations may lead to varied clinical manifestations and cellular phenotypes. This variability could potentially be attributed to different functional alterations of *ARL3* resulting from various mutation sites.

*ARL3* is widely expressed in all eukaryotes and cell types and has been localized to the mitochondria, endosomes, lysosomes, proteasome, centrosome, cell projection and cilium.<sup>12,16</sup> In photoreceptors, *ARL3* play a crucial role in regulating the enrichment of lipidated proteins that are essential for eliciting the visual response within the outer segment, a modified primary cilium. Photoreceptors are abundantly rich in mitochondria, which are crucial for the survival and function of retinal cells and present a weak point in the antioxidant defense of photoreceptor cells.<sup>17</sup> Previous studies have shown that mitochondrial dysfunction is strongly associated with photoreceptor retinopathy.<sup>18</sup> It has been demonstrated that *ARL2*, the closest paralog of *ARL3*, plays a crucial role in controlling mitochondrial fusion and morphology,<sup>19–21</sup> suggesting that *ARL3* may also play a significant role in maintaining normal mitochondrial function in photoreceptors. In this study, we investigated the correlation between mitochondrial functional alterations and specific mutation sites in *ARL3* by utilizing fibroblast cells obtained from patients with *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> variants. Our findings

provide compelling evidence for the association between *ARL3* and mitochondrial function, thereby laying the groundwork for further investigations into *ARL3*-related diseases.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

Skin fibroblasts were cultured using a special primary skin fibroblast medium (Lab050-NP, Kuisai, China) with 10% serum and 100 units of penicillin and streptomycin. HEK293T cells were cultured in high glucose DMEM (SH30022.01, Hyclone, USA) with 10% FBS and 100 units of penicillin and streptomycin. ARPE-19 cells were cultured in DMEM/F-12 (10-092-CVRC, Corning, USA) with fetal bovine serum (10%v/v) (35-081-CV, Corning, USA) and 100 units of penicillin and streptomycin. All cells were cultured at 37°C in 5% CO<sub>2</sub> cell culture chamber.

### 2.2 | Antibodies and reagents

Antibodies and reagents were purchased from: rabbit anti-flag (ab205606, Abcam, USA); rabbit anti-*ARL3* (10961-1-AP, Proteintech, USA); cycloheximide (CHX) (66-81-9, Selleck, USA); FCCP (HY-10041, MCE, USA); MG132 (HY-13259, MCE, USA).

### 2.3 | Plasmids and transfection

The empty vector (pcDNA3.1), pcDNA3.1-*ARL3*, pcDNA3.1-*ARL3*<sup>T31A</sup>, pcDNA3.1-*ARL3*<sup>C118F</sup>, pcDNA3.1-*ARL3*<sup>T31A/C118F</sup> containing a C-terminal Flag tag were synthesized cloned into the pcDNA3.1 vector, respectively. All constructed plasmids were the same as the research before.<sup>13</sup> Cells were transfected with EZ Trans (AC04L092, Life-iLab, China) and MG132 (M8699, MCE, USA) treatment for 4 h following the protocol. The pAAV-H1s-gRNA<sub>v2.0</sub>(*ARL3*)-tCMV-spCas9 was used to knock-down *ARL3* expression following manufacturer's protocol.

### 2.4 | Western blotting

Western blot was performed as reported elsewhere. Briefly, monocytes were lysed in RIPA buffer containing 1% protease inhibitor cocktail. and proteins were quantified using BCA protein assay kit (P0011, Beyotime, China) according to the instruction of the manufacturer, proteins from each sample were resuspended in SDS loading buffer

and denatured for 5 min at 100°C. Protein was separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system, and subsequently transferred on PVDF membrane (IPVH00010, Millipore, USA). After blocking in 5% skimmed milk for 2 h, the membranes incubated in the primary antibody at 4°C overnight. Blots were washed three times with TBS plus 0.1% Tween20 and incubated for 2 h at RT with second antibody-HRP conjugate. Then blots were visualized with Chemiluminescent detection reagent (WBKLS0500, Millipore, USA). Bands were analyzed using the ImageJ software (Version 1.52a, NIH).

## 2.5 | ROS measurement

Cells were incubated in six-well plates in cell culture chamber and cultured overnight. MitoSOX™ (M36008, Invitrogen, California, USA) was diluted in HBSS balanced salt solution at a ratio of 1:1000. Diluted MitoSOX™ was added to the wells and incubated in a 37°C incubator for 30 min. After washing three times with HBSS protected from light, fluorescence images were taken with a fluorescence microscope.

## 2.6 | Mitochondrial membrane potential (MMP) assay

MMP assay was performed using TMRM staining. Cells were incubated overnight. In each well, 1 µL of TMRM staining working solution and 1 mL of serum-free medium were added. Incubate for 30 min at 37°C in an incubator. After incubation, the cells were given three PBS buffer washes before the supernatant was removed. Finally, 1 mL of PBS was added and photographed with a fluorescence microscope.

## 2.7 | Measurement of oxygen consumption rate (OCR)

With an XFe analyzer (XFe96, Agilent Seahorse Technologies, USA) to detect cellular oxidative respiratory function, cells were spread on cell culture plates (102601–100, Agilent Seahorse Technologies). The probe plate (W21021, Agilent Seahorse Technologies) was placed with XF Calibrant (09021005, Agilent Seahorse Technologies) in a 37°C incubator for 1 h. Cell culture medium was replaced with XF medium (103334–100, Agilent Seahorse Technologies) containing 2 mM glutamine, 1 mM pyruvate and 10 mM glucose; and the cell culture plates were placed 37°C in a CO<sub>2</sub>-free incubator for 1 h. Adding 15 µM

oligomycin, 5 µM FCCP, and 5 µM rotenone/antimycin-A in probe plate for data detection. Protein concentration was measured for normalization. The software Wave 2.6.3 was used for results analysis.

## 2.8 | Apoptosis assay

The apoptosis assay was carried out in accordance with the directions of the Apoptosis Detection Kit (559,763, BD Biosciences, USA). The quantity of  $1 \times 10^6$  cells were gathered for detection. Using cold PBS, cells were washed and then resuspended in 100 µL 1× binding buffer, 5 µL PE-annexin V and 5 µL 7 AAD were incubated after samples were put to the tube at RT in the dark for 30 min after gently vortexing. Samples were introduced to 400 µL 1× binding buffer and then for flow cytometry measurement.

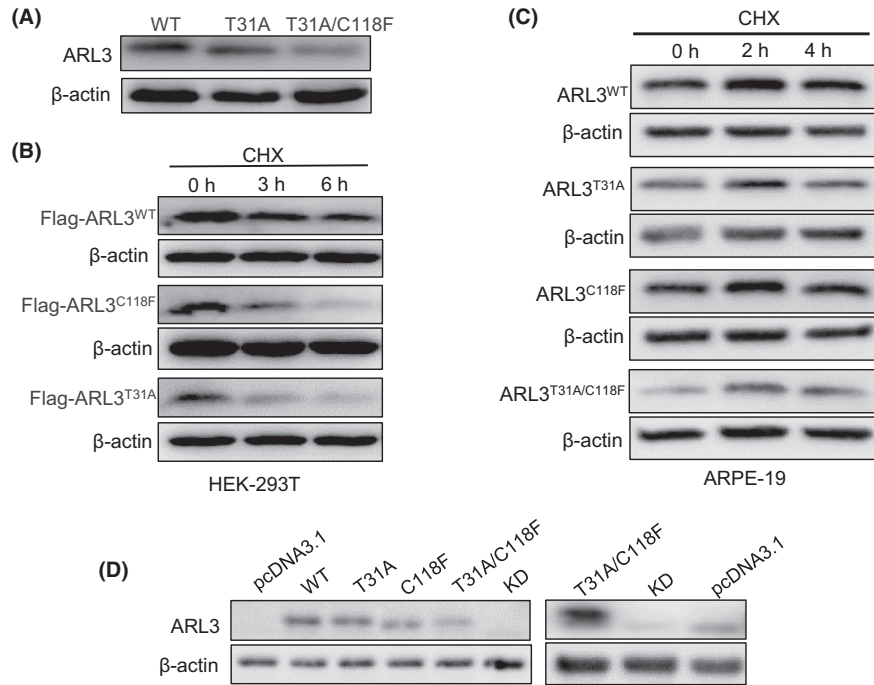
## 2.9 | Statistical analysis

GraphPad Prism 8 software (GraphPad, La Jolla, CA, USA) was used for statistical analyses. Student's *t*-test or one-way ANOVA followed by Bonferroni correction were applied to analysis the data. The *p* value not more than 0.05 was seen as significantly different.

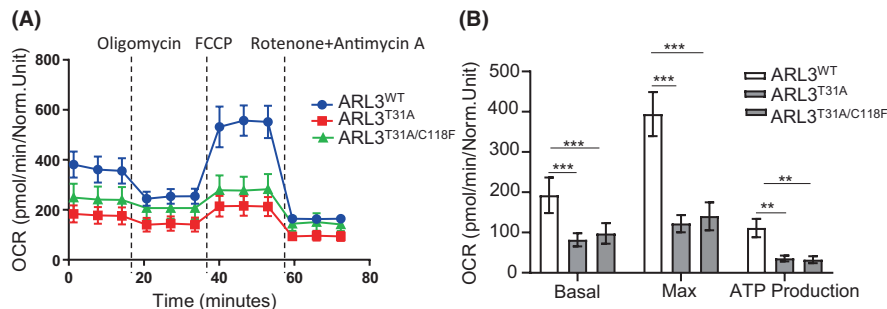
## 3 | RESULTS

### 3.1 | *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup> and *ARL3*<sup>T31A/C118F</sup> mutations decreased *ARL3* protein expression

Mutations within protein-coding genes have potential to disrupt protein expression and function. To assess the expression of *ARL3*, we constructed transformed immortal fibroblast (iFB) cell lines expressing *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup>, and subsequently examined the protein expression levels of *ARL3*<sup>WT</sup>, *ARL3*<sup>T31A</sup>, and *ARL3*<sup>T31A/C118F</sup> at low-passage number. Western-blot (WB) analysis revealed a decrease in protein expression of *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> compared to *ARL3*<sup>WT</sup> in fibroblast cells (Figure 1A). To evaluate protein stability, we treated HEK-293 T and ARPE-19 cells with CHX to inhibit new protein synthesis. Consistent with previous reports,<sup>13</sup> the stability of *ARL3*<sup>T31A</sup> and *ARL3*<sup>C118F</sup> is reduced in HEK-293 T cells (Figure 1B). However, *ARL3*-FLAG mutants degraded significantly slower in ARPE-19 cells (Figure 1C). To minimize stability-related effects, we used ARPE-19 cells for further molecular functional analysis and included the proteasomal inhibitor MG132 to prevent proteasome-dependent degradation (Figure 1D).



**FIGURE 1** *ARL3* Mutations induce alterations in protein expression in fibroblasts and ARPE-19 cells. (A) Decreased expression of *ARL3* protein in skin fibroblasts from patients ( $n=1$ ). (B) *ARL3*<sup>T31A</sup> and *ARL3*<sup>C118F</sup> mutations lead to reduced stability of *ARL3* protein in HEK293T cells. Following transfection with *ARL3*<sup>WT</sup>, *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup> plasmids for 24 h, HEK293T cells were subsequently exposed to 100  $\mu$ g/mL cycloheximide (CHX) for 0, 3, and 6 h, respectively. *ARL3* protein levels were detected using the flag antibody, while  $\beta$ -Actin levels served as an endogenous control ( $n=1$ ). (C) *ARL3* protein stability of *ARL3*<sup>T31A</sup> and *ARL3*<sup>C118F</sup> mutations degraded significantly slower in ARPE-19 cells. Following transfection with *ARL3*<sup>pcDNA3.1</sup>, *ARL3*<sup>WT</sup>, *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup>, *ARL3*<sup>T31A/C118F</sup> plasmids for 24 h, ARPE-19 cells were subsequently exposed to CHX for 0, 2, and 4 h, respectively. *ARL3* protein levels were detected using the flag antibody, while  $\beta$ -Actin levels served as an endogenous control ( $n=1$ ). (D) *ARL3* protein levels were detected in *ARL3*<sup>KD</sup> and *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup>, *ARL3*<sup>T31A/C118F</sup> transfected 48 h and MG132 treatment for 4 h ARPE-19 cells. KD, knockdown.



**FIGURE 2** The OCR in *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> fibroblasts. (A) The OCR were measured by adding oligomycin, FCCP and rotenone+antimycin sequentially with XFp seahorse respiration tester. (B) The statistics for basal respiration level, maximum respiration capacity, and ATP production. (\*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ). Values are means  $\pm$  SD ( $n=3-5$ ), (\*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ).

### 3.2 | *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> mutations result in reduced mitochondrial respiration in fibroblasts

Mitochondrial oxidative phosphorylation (OXPHOS) may be compromised when mitochondrial activity is compromised.<sup>22</sup> To assess the impact of *ARL3* mutations on OXPHOS, we utilized the Seahorse Cell Analyzer to

measure the OCR of *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> fibroblasts. The analysis of OCR revealed a significant decrease in mitochondrial respiration in *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> fibroblasts compared to *ARL3*<sup>WT</sup>. Specifically, *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> fibroblasts exhibited significantly decreased basal OCR, maximal OCR, and ATP-linked OCR in comparison to *ARL3*<sup>WT</sup> (Figure 2A,B). Notably, there were no significant differences observed between *ARL3*<sup>T31A</sup> and

ARL3<sup>T31A/C118F</sup> fibroblasts, thus confirming the inhibited respiration in both ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblasts.

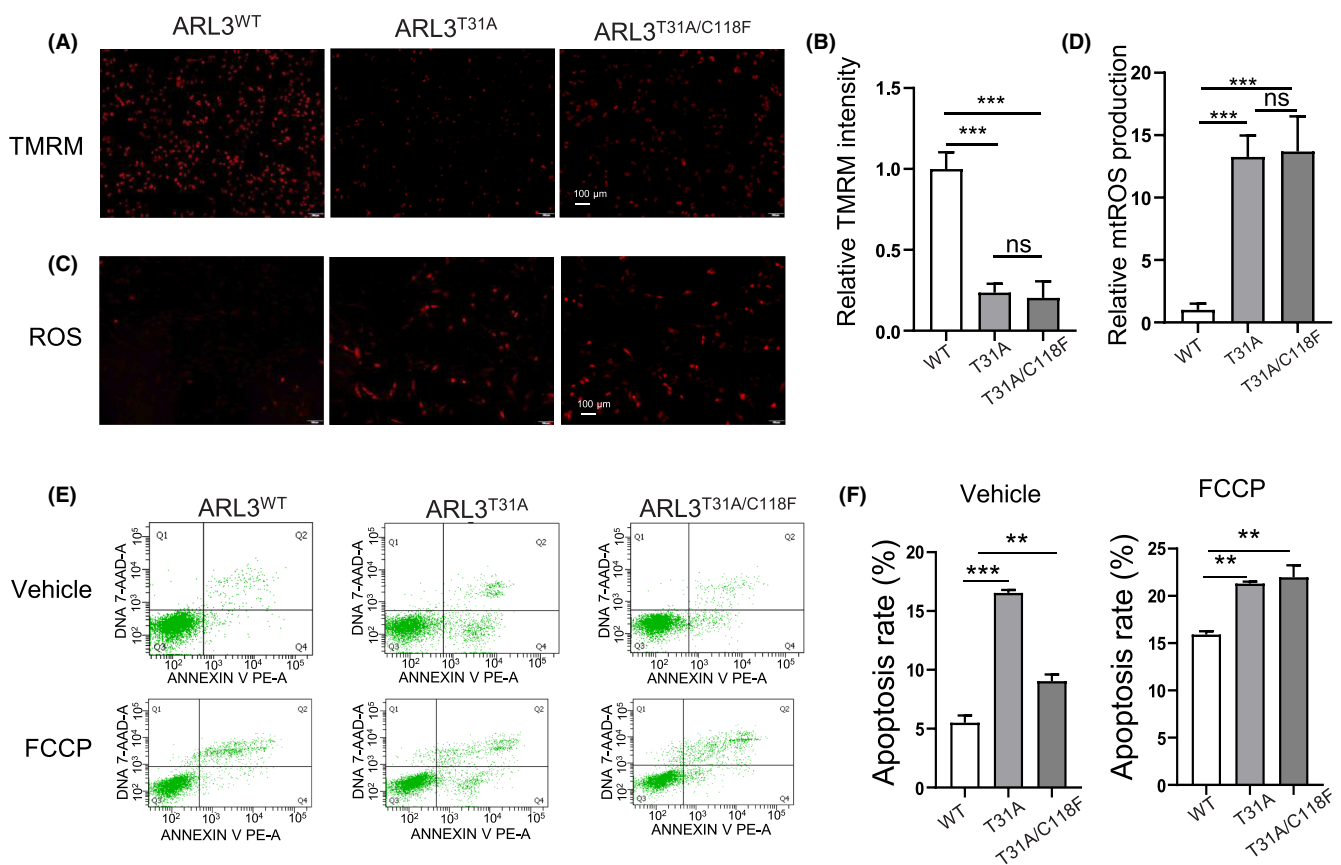
### 3.3 | ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> mutations induce mitochondrial ROS accumulation and MMP decrease in fibroblasts

MMP serves as an indicator of mitochondrial state. To explore the alternations of mitochondrial state in ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblasts, we assessed the MMP using tetramethylrhodamine methyl ester (TMRM). Our findings revealed a significant decrease in TMRM intensity in ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblasts compared to ARL3<sup>WT</sup> (Figure 3A,B). However, no significant difference was observed between ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblasts. Reactive oxygen species (ROS) are by-products of mitochondrial respiration and can disrupt MMP. To investigate the impact of ARL3 mutations on mitochondrial ROS, fibroblast cells were treated with MitoSOX, a dye specifically designed for detecting mitochondrial

ROS. Our results demonstrated a significant increase in mitochondrial ROS levels in ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblasts compared to ARL3<sup>WT</sup>. However, no significant differences were observed between ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblasts (Figure 3C,D). These findings suggest that both ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> mutations contribute to the accumulation of ROS within mitochondria and reduction of MMP.

### 3.4 | ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> mutations trigger apoptosis in fibroblasts

MMP reduction is the earliest event in the apoptotic cascade, and mitochondrial dysfunction or damage can induce stress responses, ultimately leading to apoptosis or cell death. To investigate the involvement of apoptosis in ARL3 mutant-related retinopathy, annexin V and PI double staining were used to assess the apoptosis rate. As shown in Figure 3E, the apoptosis rate of ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblast cells were higher compared to ARL3<sup>WT</sup>. Moreover, the induction of protonophore trifluorocarbonylcyanide



**FIGURE 3** The MMP, ROS level, and apoptosis rate in ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblasts. (A) Mitochondrial ROS levels were detected in ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblast cells. (B) The statistics for ROS level. (\*\*\*)  $p < 0.001$ , ns = not significant). Values are means  $\pm$  SD ( $n \geq 3$ ) (C) MMP were detected in ARL3<sup>T31A</sup> and ARL3<sup>T31A/C118F</sup> fibroblast cells. (D) The statistics for MMP. (\*\*\*)  $p < 0.001$ , ns = not significant). Values are means  $\pm$  SD ( $n \geq 3$ ) (E) Apoptosis rate measured by flow cytometry. Fibroblasts were treated before and after FCCP at concentrations of 40 μM for 4 h. (F) Statistical histogram of apoptosis rate in (E). (\*\*\*)  $p < 0.001$ , \*\*  $p < 0.01$ . Values are means  $\pm$  SD ( $n = 3$ ).

phenylhydrazine (FCCP) significantly augmented the apoptotic rate of  $ARL3^{T31A/C118F}$  fibroblast cells, surpassing 20% and exhibiting a substantial increase compared to  $ARL3^{WT}$  (Figure 3F). These findings suggest that both  $ARL3^{T31A}$  and  $ARL3^{T31A/C118F}$  mutations can trigger apoptosis in fibroblast cells, with the latter mutation leading to reduced cellular tolerance towards external stimuli.

### 3.5 | The $ARL3^{T31A}$ and $ARL3^{T31A/C118F}$ mutation demonstrate a greater degree of mitochondrial impairment in comparison to $ARL3^{C118F}$

To elucidate the relationship between mutation sites and mitochondrial functional alternations, we conducted a Seahorse assay in ARPE-19 cells transfected with various  $ARL3$  constructs ( $pcDNA3.1$ ,  $ARL3^{WT}$ ,  $ARL3^{T31A}$ ,  $ARL3^{C118F}$ ,  $ARL3^{T31A/C118F}$ ) followed by MG132 treatment 4 h and in  $ARL3$  knockdown ( $ARL3^{KD}$ ) cells. The results showed that  $ARL3^{KD}$  cells had significantly decreased basal and maximal OCR compared to  $pcDNA3.1$ . Overexpression of  $ARL3^{WT}$  did not significantly alter mitochondrial respiration, indicating that  $ARL3$  deficiency impairs mitochondrial function, while increased  $ARL3$  levels do not. Further analysis revealed that  $ARL3^{T31A}$  and  $ARL3^{T31A/C118F}$  overexpression resulted in decreased mitochondrial basal, maximal OCR, similar to  $ARL3^{KD}$  cells (Figure 4A,B). These findings, consistent with studies in iFB cells, suggested that  $ARL3^{T31A}$  and  $ARL3^{T31A/C118F}$  inhibits mitochondrial respiration. Moreover, we evaluated the ROS level after  $H_2O_2$  and MG132 treatment in ARPE-19 cells.  $ARL3^{T31A}$  and  $ARL3^{T31A/C118F}$  transfection

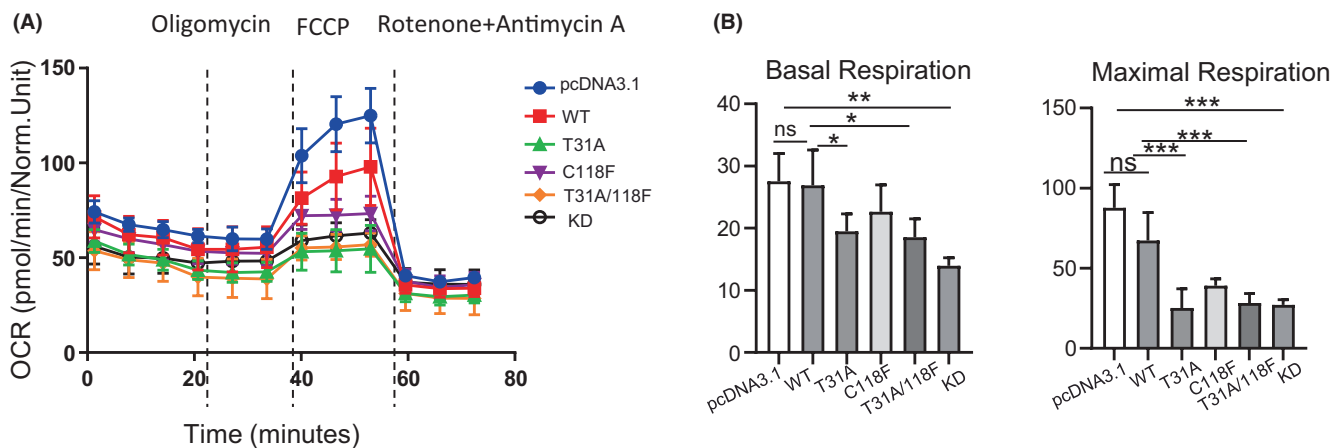
resulted in a notable increase in mitochondrial ROS levels compared to  $ARL3^{WT}$ , while no significant difference was observed in  $ARL3^{C118F}$ -transfected cells (Figure 5A,B). This indicates that  $ARL3^{T31A}$ ,  $ARL3^{T31A/C118F}$  may lead to more severe damage to the mitochondria in cells compared to  $ARL3^{C118F}$ .  $ARL3^{KD}$  lead to more severe damage to the mitochondrial respiration in cells compared to  $pcDNA3.1$ .

### 3.6 | $ARL3^{T31A}$ , $ARL3^{C118F}$ , $ARL3^{T31A/C118F}$ variants induce higher cell apoptosis level

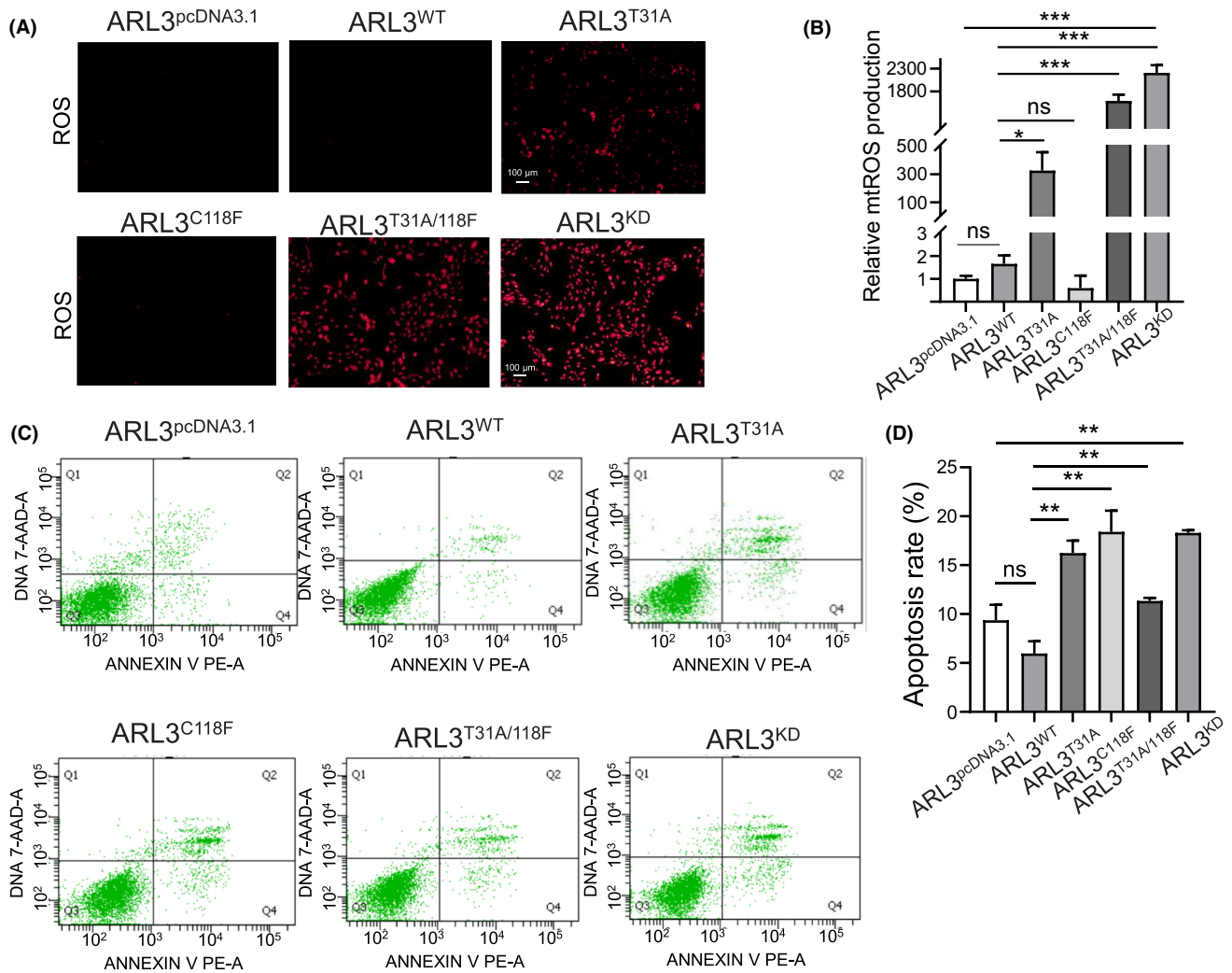
Mitochondria are sensors and responds to environmental stimuli. Stress reactions can result from mitochondrial damage and malfunction, leading to apoptosis. To investigate whether the  $ARL3^{T31A}$ ,  $ARL3^{C118F}$  and  $ARL3^{T31A/C118F}$  mutations induces apoptosis, we measured the apoptosis rate following MG132 treatment 4 h. We discovered that the apoptosis rate of the  $ARL3^{T31A}$ ,  $ARL3^{C118F}$  and  $ARL3^{T31A/C118F}$  transfected ARPE-19 cells was significantly higher than that of the  $ARL3^{WT}$  cells. What's more, the apoptosis rate was higher in  $ARL3^{KD}$  compared to  $ARL3^{pcDNA3.1}$  (Figure 5C,D). This finding demonstrates that the  $ARL3^{KD}$  and  $ARL3^{T31A}$ ,  $ARL3^{C118F}$ ,  $ARL3^{T31A/C118F}$  variants induce higher cell apoptosis level.

### 3.7 | Correlation between the transcriptomic data and proteomic data in $ARL3^{T31A}$ and $ARL3^{T31A/C118F}$ fibroblasts

To investigate the molecular mechanisms underlying mitochondrial dysfunction caused by  $ARL3$  mutations,



**FIGURE 4** The OCR in  $ARL3^{KD}$  and  $ARL3^{T31A}$ ,  $ARL3^{C118F}$ ,  $ARL3^{T31A/C118F}$  transfected ARPE-19 cells. (A) The OCR in transfected ARPE-19 cells with the  $ARL3^{pcDNA3.1}$ ,  $ARL3^{T31A}$ ,  $ARL3^{C118F}$ ,  $ARL3^{T31A/C118F}$  plasmid, followed by a 4-h treatment with MG132, and  $ARL3^{KD}$  cells were measured by adding oligomycin, FCCP and rotenone+antimycin sequentially with XFp seahorse respiration tester. (B) The statistics for basal respiration level, maximum respiration capacity, and ATP production are displayed in Graph B. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ns, not significant). Values are means  $\pm$  SD ( $n \geq 3$ ).

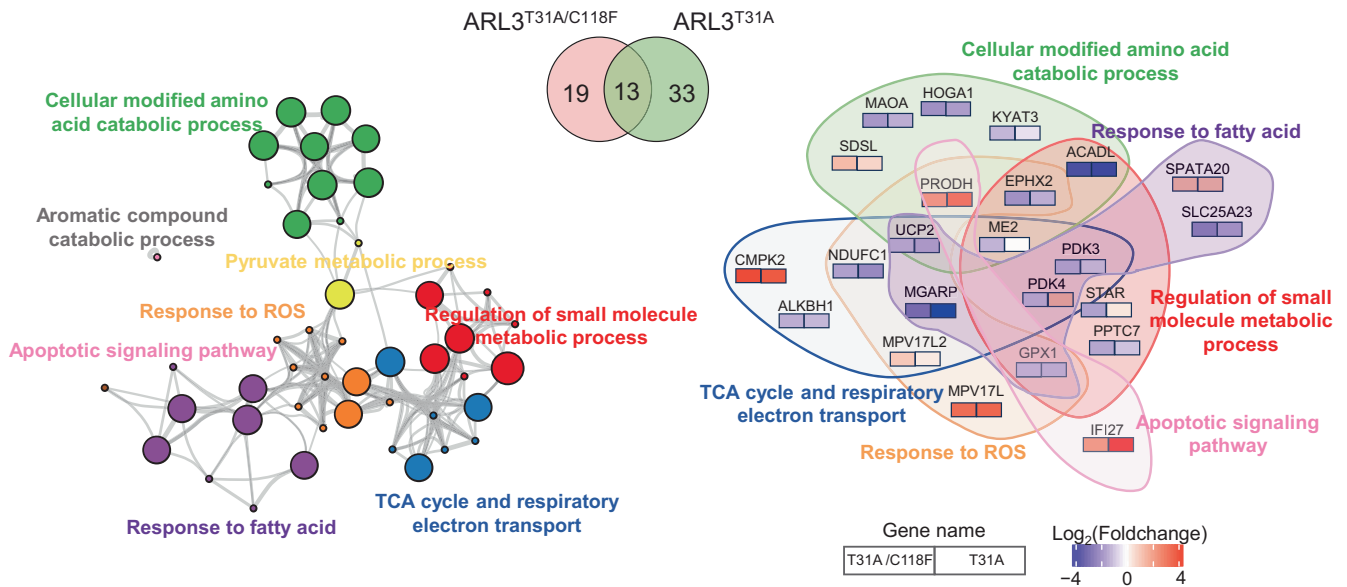


**FIGURE 5** Mitochondrial function measurement in ARL3<sup>KD</sup> and ARL3<sup>T31A</sup>, ARL3<sup>C118F</sup>, ARL3<sup>T31A/C118F</sup> transfected ARPE-19 cells. (A) Mitochondrial ROS level was detected in ARL3<sup>KD</sup> and ARL3<sup>pcDNA3.1</sup>, ARL3<sup>WT</sup>, ARL3<sup>T31A</sup>, ARL3<sup>C118F</sup>, ARL3<sup>T31A/C118F</sup> transfected ARPE-19 cells for 24 h, and MG132, H<sub>2</sub>O<sub>2</sub> (200 μM) treatment 4 h. (B) The statistics for ROS level. (\**p* < 0.05, \*\*\**p* < 0.001, ns = not significant). Values are means ± SD (*n* ≥ 3) (C) Apoptosis rate measured by flow cytometry in ARL3<sup>KD</sup> and ARL3<sup>pcDNA3.1</sup>, ARL3<sup>T31A</sup>, ARL3<sup>C118F</sup>, ARL3<sup>T31A/C118F</sup> transfected ARPE-19 cells for 24 h and MG132 treatment 4 h. (D) Statistical histogram of apoptosis rate in (C). Values are means ± SD (\*\**p* < 0.01). Values are means ± SD (*n* = 3).

and provide direction for further exploration into mitochondrial functional abnormalities. We conducted further analysis of our previously reported RNA-sequencing results of fibroblasts with ARL3<sup>T31A/C118F</sup> and ARL3<sup>T31A</sup> mutations. In this study, we integrated mitochondrial proteomic data (MitoCarta3.0) to enhance our understanding. Our analysis revealed 32 differentially expressed genes (DEGs) in the mitochondria of the ARL3<sup>T31A/C118F</sup> group, while 46 mitochondrial DEGs were identified in the ARL3<sup>T31A</sup> group, with 13 mitochondrial DEGs shared between the two groups (Figure 6).

Subsequent functional analysis using Metascape demonstrated that the DEGs in the ARL3<sup>T31A/C118F</sup> group were primarily associated with the regulation of small

molecule metabolic processes, the citric acid (TCA) cycle, respiratory electron transport, cellular modified amino acid catabolic processes, response to reactive oxygen species, and apoptotic signaling pathways, among others. These findings are consistent with our previous discovery that ARL3<sup>T31A/C118F</sup> mutations lead to reduced mitochondrial respiration, accumulation of mitochondrial ROS, and induction of apoptosis in fibroblasts. Additionally, we identified potential associations between mitochondrial respiratory dysfunction and alterations in the expression of PDK3, PDK4, ME2, UCP2, MGARP, NDUFC1, MPV17L2, CMPK2, and ALKBH1 proteins. Increased ROS levels may be correlated with changes in the expression of EPHX2, PRODH, UCP2, MGARP, NDUFC1, MPV17L2, GPX1, and



**FIGURE 6** DEGs in mitochondria. Venn diagram showing overlap of the mitochondrial genes and DEGs of *ARL3*<sup>T31A/C118F</sup> fibroblasts (left) and *ARL3*<sup>T31A</sup> in fibroblasts (right). Network of enriched items of the overlapped DEGs in *ARL3*<sup>T31A/C118F</sup> fibroblasts. Terms were colored according to the cluster. Heatmap showing DEGs of *ARL3*<sup>T31A/C118F</sup> and *ARL3*<sup>T31A</sup> in mitochondria. DEG, differentially expressed genes.

MPV14L proteins, while apoptosis may be associated with changes in the expression of PROD, GPX1, and IFI17 proteins.

## 4 | DISCUSSION

The *ARL3* Thr31 and Cys118 residues are highly conserved across species, and both missense mutations have been predicted to be pathogenic by various pathogenicity prediction algorithms.<sup>13</sup> In our previous study, the *ARL3*<sup>T31A/C118F</sup> was found to be associated with clinical phenotypes of optic RCD. Thr31 is located in the middle of *ARL3*'s p-loop domain and plays a role in the binding of GTP or GDP and Mg<sub>2</sub><sup>+</sup>. Another variant, *ARL3*<sup>T31N</sup>, is commonly used to construct *ARL3*-GDP conformational mimic.<sup>23</sup> T31A mutation may lead to dominant negative effects, hence possibly associated with the phenotype. Whether a serine (S) or threonine (T) mutated to an asparagine (N) or alanine (A) depends on the specific protein and the position within that region. This mutation could result in dominant negative effects. The *ARL3* T31 site is located in a critical region for GTP and GDP conversion, and mutation could impact the structure and function of the protein, potentially leading to dominant negative effects, which could be the underlying cause of the observed phenotype. And *ARL3*<sup>C118F</sup> was first reported in our previous study.<sup>13</sup> However, the functional alterations underlying the *ARL3*<sup>T31A</sup> and *ARL3*<sup>C118F</sup> mutations are not clear. A recent study by Giovanni Pagano

et al reported a significant influence of mitochondrial damage on retina degeneration.<sup>24</sup> Mari-Luz Moreno et al also demonstrated that the mitochondrial dysfunction may lead to retinitis pigmentosa.<sup>25</sup> These findings suggest a strong correlation between mitochondrial dysfunction and retina dysfunction. In this work, we investigated mitochondrial function of fibroblasts derived from skin tissues of patients with RCD carrying *ARL3*<sup>T31A/C118F</sup> and *ARL3*<sup>T31A</sup> mutations, and compared the effects of the two mutations.

*ARL3* is implicated in the development of optic retinitis pigmentosa, a disease with varying clinical presentations. We have reported that patients carrying a single mutation, *ARL3*<sup>T31A</sup>, who exhibited a milder clinical phenotype with a late onset of CRD phenotype, primarily presenting with visual loss. On the other hand, the variant *ARL3*<sup>C118F</sup> did not manifest any disease symptoms.<sup>13</sup> Therefore, different mutations in *ARL3* can lead to different clinical phenotypes. Protein quality control ensures the orderly performance of both protein expression and function.<sup>26</sup> In this study, we observed reduced *ARL3* protein expression in fibroblasts carrying *ARL3*<sup>T31A</sup> and *ARL3*<sup>T31A/C118F</sup> mutations, as well as in ARPE-19 cells transfected with plasmids containing *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup> and *ARL3*<sup>T31A/C118F</sup> mutations. This suggests that the *ARL3*<sup>T31A</sup>, *ARL3*<sup>C118F</sup> and *ARL3*<sup>T31A/C118F</sup> mutations decrease the expression of *ARL3* protein, thereby impacting its function and cellular phenotype. *ARL3*<sup>KD</sup> cells also showed decreased *ARL3* expression, which means that *ARL3* expression



did impact the function. Furthermore,  $ARL3^{KD}$ ,  $ARL3^{T31A}$  and  $ARL3^{T31A/C118F}$  mutation exhibited impaired mitochondrial function, characterized by decreased mitochondrial OCR, increased mitochondrial ROS, and decreased MMP. However, the  $ARL3^{C118F}$  mutation did not have the same effect.  $ARL3$  overexpression does not significantly impact OCR suggests that  $ARL3$ , in excess, does not alter basic mitochondrial functions or metabolic states. However, the significant reduction in basal and maximal OCR following  $ARL3$  knockdown indicates that  $ARL3$  is essential for maintaining mitochondrial function and energy metabolism at normal levels. Possible explanations include that  $ARL3$  might be involved in regulating the transport of proteins or lipids across the mitochondrial membrane, which are crucial for mitochondrial function. A decrease in  $ARL3$  could lead to a shortage of these important components, thereby affecting OCR. And  $ARL3$  may play a role in maintaining mitochondrial morphology and dynamic balance. Knockdown of  $ARL3$  could disrupt mitochondrial fusion or fission, affecting overall mitochondrial function. Moreover,  $ARL3$  might regulate key metabolic pathways, such as the assembly or function of respiratory chain complexes, impacting OXPHOS.  $ARL3$  knockdown might impair these metabolic pathways, reducing OCR. These findings indicate that  $ARL3$  plays a role in mitochondrial respiration during oxidative stress. Moreover, different mutations in  $ARL3$  lead to distinct cellular phenotypic heterogeneity, consistent with their clinical phenotypes.

Apoptosis is a form of programmed cell death, which plays an important role in the clearance of abnormal cells. Previous studies have demonstrated that the retinas of  $ARL3$  knockout mice often exhibit accompanying apoptosis.<sup>27,28</sup> Our findings indicate that  $ARL3^{T31A/C118F}$  and  $ARL3^{T31A}$  fibroblasts are more susceptible to apoptosis compared to control cells. Photoreceptor cells are particularly sensitive to adverse stimuli. Similar to fibroblasts, photoreceptor cells in individuals with  $ARL3^{T31A/C118F}$  and  $ARL3^{C118F}$  are more prone to apoptosis, leading to retinal degeneration. Our results suggest that the compound mutation of  $ARL3$  may result in mitochondrial functional impairment, subsequently leading to retinal cell damage, apoptosis, and retinal thinning, thereby contributing to a more severe clinical phenotype. However, multiple mechanisms contribute to this progression, encompassing the process from higher ROS, mitochondrial damage to apoptosis.<sup>29</sup> Further experimental validation is necessary to ascertain the precise pathway through which apoptosis is induced.

Additionally, mitochondria not only play a role in energy metabolism but also influence ciliary function

by regulating cellular signaling pathways and ion balance.<sup>30</sup> Thus, there might exist a close interaction and dependency between mitochondrial function and cilia. Mitochondria affect cell function and structure through energy supply, cellular signaling, and other mechanisms.<sup>31</sup> While impaired  $ARL3$  mitochondrial function may affect ciliary formation and transport. In our previous research, we found that the  $ARL3^{T31A/C118F}$  mutation resulted in decreased ciliogenesis, longer cilia length and disruptions in retrograde ciliary transport.<sup>15</sup> These changes could potentially be associated with alterations in mitochondrial function, and further experimental validation is needed to elucidate its specific mechanisms.

In summary, our study unveiled that different variants of  $ARL3$  can lead to various cellular phenotypes, including mitochondrial functional impairments and apoptosis. Moreover, we propose, for the first time, a correlation between  $ARL3$  mutations and mitochondrial function. These discoveries present fresh avenues for investigating  $ARL3$ -associated mutations, thereby broadening the horizons of future research endeavors.

#### AUTHOR CONTRIBUTIONS

X.L. Zhang, B. Lei, and X.X. Jin conceived and designed the research. Experiments were processed by X.L. Zhang, S. Yao, L.J. Zhang, B.S. Zhang, M.Z. Yang, Q.G. Guo and J. Xu. The original manuscript was written by X.L. Zhang and X.X. Jin. Z.F. Wang, B. Lei, and X.X. Jin reviewed and revised the article. All authors have read and approved the final version of this article.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The authors will give the original data that underpins the results of this research. The original data will be made available by the writers without any hesitation.

## ETHICS STATEMENT

In conducting this study, the Helsinki Declaration was followed. All subjects provided written, informed consent, and the study received ethical approval from the Henan Eye Hospital Ethics Committee [IRB approval number: HNEECKY-2019<sup>12</sup>].

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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