Glucoma is an eye disease characterized by progressive degeneration of the optic nerve, eventually resulting in irreversible loss of vision. Glaucoma is a leading cause of blindness, and the number of individuals with glaucoma will increase to 79.6 million worldwide by 2020; of those, 11.1 million will be bilaterally blind.1 The most common form of this disease is POAG, which is associated with elevated IOP. Normal-tension glaucoma (NTG), a progressive optic neuropathy with irreversible loss of vision. Glaucoma is a leading cause of vision loss. Several mutations in the gene encoding optineurin (OPTN), the receptor for amyotrophic lateral sclerosis (ALS). ALS mutations in the ubiquitin-binding domain of OPTN impair Parkin-dependent mitophagy. However, the effects of glaucoma mutations in this region remain unknown. We examined the impact of glaucoma-associated OPTN mutations on Parkin-dependent mitophagy.

Methods. The mitochondria-localized, pH-sensitive fluorescent protein mito-Keima was used to monitor mitophagy. HeLa cells expressing Parkin were treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or oligomycin/antimycin A (O/A) to induce Parkin-dependent mitophagy. Two complementary mitophagy receptors, OPTN and NDP52, were deleted in HeLa cells expressing mito-Keima and Parkin (DKO_HeLa). The mutant OPTN genes were reintroduced into DKO_HeLa cells using retroviruses or through transfection. Mitophagy activity and OPTN localization were evaluated via microscopic analyses. OPTN binding to ubiquitin was examined using an immunoprecipitation assay.

Results. Parkin-dependent mitophagy was inhibited in DKO_HeLa cells. Introduction of two glaucoma mutations in the ubiquitin-interacting region of OPTN restored mitophagy in CCCP-treated DKO_HeLa cells, whereas the two ALS mutations failed to replicate this effect. Under treatment with CCCP, the two glaucoma-mutant OPTN proteins normally translocated to mitochondria and bound to ubiquitinated proteins. Furthermore, five additional glaucoma-mutant OPTN proteins restored CCCP-induced mitophagy. Moreover, treatment with O/A exhibited similar results.

Conclusions. Glaucoma-mutant OPTN proteins retain their normal properties as mitophagy receptors, suggesting that mutations in the OPTN gene cause glaucoma through a mechanism independent of mitophagy defects.

Keywords: glaucoma, mitophagy, mitochondria, optineurin, parkin
limited Impact of Glaucoma OPTN Mutations on Mitophagy

**METHODS**

**Antibodies**

The primary antibodies used in this study were anti-OPTN (ab23666; Abcam, Cambridge, UK), anti-NDP52 (#ab9036; Cell Signaling Technologies, Danvers, MA, USA), anti-hemagglutinin (anti-HA; M180-3; MBL, Nagoya, Japan), anti-Keima-Red (M126; BML), anti-green fluorescent protein (anti-GFP; #598; BML), anti-multipubiquitin (D058-3; BML), and anti-actin (sc-8432; SantaCruz, Dallas, TX, USA). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA).

**Plasmid Construction and Mutagenesis**

The wild-type (WT) OPTN coding sequence from a HeLa cell cDNA library was amplified through PCR and ligated into a pcDNA3.1-EGFP/Zeo vector to generate an OPTN expression plasmid. For the introduction of point mutations in the pcDNA3.1-EGFP_OPTN(WT) vector, the following primers were used: H26D; 5'-GGG AAT GGA CCC GAC CTG GCC-3' and H26E; 5'-GGA AAT GGA CCC GAC CTG GCC-3'. Mutations were introduced via PCR-based mutagenesis following the depolarization of mitochondria, PINK1 can accumulate on the mitochondrial outer membrane and recruit the ubiquitin ligase Parkin to the mitochondria. Subsequently, mitochondrial proteins are ubiquitinated by Parkin. Autophagy receptor proteins, including OPTN, NDP52, p62, NBR1, and TAX1BP1, are translocated to the damaged mitochondria via binding to ubiquitinated mitochondrial proteins and direct interactions with microtubule-associated protein 1 light chain 3 (LC3) to link damaged mitochondria with autophagosome formation. Mitochondria enveloped in autophagosomes are delivered to lysosomes (autolysosomes) and degraded. OPTN is not required for the execution of PINK1/Parkin-independent mitophagy.

The OPTN E478G mutation linked to sporadic ALS disrupts the ubiquitin-binding function of OPTN and affects Parkin-mediated mitophagy.27-29,48 However, it remains unclear whether glaucoma-associated OPTN mutations in the ubiquitin-interacting region affect Parkin-mediated mitophagy. In the present study, we hypothesized that mutant OPTN causes defects in the Parkin-dependent mitophagy pathway, eventually leading to POAG. We found that glaucoma-associated OPTN mutants rescued the mitophagy defect in mitophagy receptor-deficient HeLa cells (OPTN- and NDP52-deficient cells), whereas ALS-associated OPTN mutants (Q454E and E478G) did not.

**Cell Culture**

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; #013-30085; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; #10270; Life Technologies, Waltham, MA, USA), and maintained at 37°C under 5% carbon dioxide. For Parkin-dependent mitophagy, cells were cultured for 3 hours in DMEM/FBS containing 10 μM carbonyl cyanide 3-chlorophenylhydrazone.
Limited Impact of Glaucoma OPTN Mutations on Mitophagy

RNAs selected from OPTN (which encodes the ubiquitin receptor OPTN), were ligated into the pX330-U6-C000-0000-CAC CGA AAC CTG GAC -CAC CGC ATT TCA plasmids and transfected into HeLa cells. The samples were sonicated for a few seconds, incubated on ice for 30 minutes, and centrifuged at 14,000g at 4°C for 15 minutes. The supernatants were subjected to immunoprecipitation, and subsequently incubated with Dynabeads Protein G (#10005D; Invitrogen) conjugated with anti-GFP antibody (#598; MBL) for 30 minutes at room temperature. After washing in PBS containing 0.3% Tween20, the immunoprecipitated samples were eluted in 1X SDS sample buffer (50 mM Tris-HCl [pH8.8], 2% SDS, 6% β-mercaptoethanol, 10% glycerol) at 95°C for 5 minutes, and subjected to SDS-PAGE and Western blotting analysis. The blot was probed with anti-GFP antibody or anti-ubiquitin antibody, and visualized using EzWestLumi plus (#2332638; ATTO, Tokyo, Japan).

Statistics
Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Values are expressed as mean ± SEM. Statistical differences were assessed by Student’s t-test (Fig. 2B, shown in red), and visualized using EzWestLumi plus (#2332638; ATTO, Tokyo, Japan).}

RESULTS

Establishment of OPTN/NDP52 DKO HeLa Cells
We used the mitochondria-targeting pH-sensitive fluorescent protein mito-Keima to monitor mitophagy (Fig. 2A) We applied the CRISPR-Cas9 system to HeLa cell lines expressing the mito-Keima and HA-Parkin genes as previously described. We performed the mito-Keima assay to monitor mitophagy. Keima is a pH-sensitive fluorescent protein. When Keima is present in a neutral environment (i.e., mitochondrial matrix), this fluorescent protein is excited by 440 nm light (shown in green), but not by 590 nm light. However, when Keima is present in acidic conditions, (i.e., autolysosomes), this fluorescent protein is excited by 590 nm light (shown in red), but not by 440 nm light (Figs. 2A, 2B). Accordingly, the mitophagy activity was estimated by counting the number of autolysosomal punctate structures observed when excited by 590-nm wavelength using the MetaMorph 7 software (Molecular Devices, San Jose, CA, USA).

Generation of OPTN/NDP52 Double Knockout (DKO) HeLa Cells
We applied the CRISPR-Cas9 system to HeLa cell lines expressing the mito-Keima and HA-Parkin genes as previously described to generate OPTN and NDP52 DKO cells. Guide RNAs selected from OPTN exon 4 (5′-CAC CGA AAC CTG GAC AGC TTT ACC C-3′) and NDP52 exon 2 (5′-CAC CGC ATT TCA TCC CTC GTC GAA-3′) were ligated into the pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (#42230; Addgene, Water- town, MA, USA) (OPTN-CRISPR1 and NDP52-CRISPR1 plasmids, respectively). HeLa cells stably expressing mito-Keima and HA-Parkin were transfected with OPTN-CRISPR1 and pcDNA3.1-hygro(-) plasmids using the FuGENE HD Transfection Reagent (E2511; Promega, Madison, WI, USA). The following day, the culture media were exchanged with DMEM/FBS containing 400 μg/ml hygromycin B for selection. After 3 days, the cells were cultured in DMEM/FBS without hygromycin B for 2 days, and subsequently re-plated for single colony selection. Each colony derived from a single cell was subjected to western blotting analysis to select OPTN-KO cells. Similarly, OPTN-KO mito-Keima/Parkin HeLa cells were transfected with the NDP52-CRISPR1 plasmid to generate OPTN/NDP52_DKO mito-Keima/Parkin HeLa cells.

Microscopic Analysis
Fluorescent microscopic analysis was performed without fixation using a microscope (IX73 Olympus, Tokyo, Japan) with an UPlanSApo ×60 oil objective lens (numerical aperture of 1.40).

Immunoprecipitation
OPTN/NDP52_DKO HeLa cells were transiently transfected with the described plasmids using FuGENE HD Transfection Reagent according to the manufacturer’s instructions. After 72 hours, the cells were treated with 10 μM CCCP for 3 hours and lysed in RIPA buffer (25 mM Tris-HCl [pH7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1X protease inhibitor cocktail (#04693123001; Roche, Basel, Switzerland). The samples were sonicated for a few seconds, incubated on ice for 30 minutes, and centrifuged at 14,000g at 4°C for 15 minutes. The supernatants were subjected to immunoprecipitation, and subsequently incubated with Dynabeads Protein G (#10005D; Invitrogen) conjugated with anti-GFP antibody (#598; MBL) for 30 minutes at room temperature. After washing in PBS containing 0.3% Tween20, the immunoprecipitated samples were eluted in 1X SDS sample buffer (50 mM Tris-HCl [pH6.8], 2% SDS, 6% β-mercaptoethanol, 10% glycerol) at 95°C for 5 minutes, and subjected to SDS-PAGE and Western blotting analysis. The blot was probed with anti-GFP antibody or anti-ubiquitin antibody, and visualized using EzWestLumi plus (#2332638; ATTO, Tokyo, Japan).

Statistics
Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Values are expressed as mean ± SEM. Statistical differences were assessed by Student’s t-test (Fig. 2B, shown in red), and visualized using EzWestLumi plus (#2332638; ATTO, Tokyo, Japan).
mitophagy (Figs. 3A, 3B). This suggests that WT OPTN is sufficient to restore mitophagy in DKO_Hela cells. The OPTN gene harboring a glaucoma or ALS mutation was expressed in DKO_Hela cells to investigate whether disease-associated mutations in this gene can restore Parkin-mediated mitophagy. We tested four disease-associated mutations in the ubiquitin-interacting region of OPTN, which are essential for Parkin-mediated mitophagy,14 including two glaucoma mutations5,8,9 (A377T and H486R) and two ALS mutations32,33 (Q454E and E478G) (Figs. 1A and 3). The E478G mutation of the OPTN gene was least effective in restoring the level of mitophagy among all mutations analyzed. This result is consistent with that reported in a previous publication, showing that ALS-associated E478G mutant OPTN was defective in ubiquitin-binding and autophagosome formation.27,29 Cells with another mutant OPTN harboring the ALS-associated Q454E also showed a significant, yet milder, reduction in the restored level of mitophagy relative to that observed with OPTN_WT. In contrast with ALS-associated mutants, the two glaucoma-associated OPTN mutations tested (A377T and H486R) did not alter mitophagy activity relative to OPTN_WT.

Glaucoma-Mutant OPTN Normally Localized to Mitochondria and Bound to Ubiquitinated Proteins

When Parkin-mediated mitophagy is induced, mitochondrial outer membrane proteins are ubiquitinated by Parkin and interact with OPTN via ubiquitin binding.30,41 Subsequently, OPTN interacts with LC3 to cause selective enveloping of mitochondria by the autophagosome (Fig. 1B).30,41 Thus, OPTN accumulates in mitochondria during Parkin-mediated

**FIGURE 2.** Generation of OPTN and NDP52 double KO HeLa cell line. (A) Schematic of the mito-Keima system. The fluorescent mito-Keima protein is localized to the mitochondrial matrix (“mitochondria”), and exhibits pH-dependent excitation. The excitation peak (“EX”) of the mito-Keima protein shifts from 440 to 590 nm when mitochondria are delivered to acidic lysosomes (“mitophagy”). (B) WT and OPTN/NDP52 DKO_Hela cells were treated with 10 μM CCCP for 3 hours, and analyzed through immunofluorescence microscopy. The mito-Keima signals transported to lysosomes were considered to represent “mitophagy” (red), and nondegraded mitochondria were designated as “mitochondria” (green). Scale bar: 10 μm. (C) WT and OPTN/NDP52 DKO_Hela cells were confirmed through western blotting with anti-OPTN, anti-NDP52, anti-Keima, anti-HA, and anti-actin antibodies. (D) Quantification of CCCP-induced mitophagy in WT and DKO_Hela cells. After treatment with CCCP for 3, 6, or 12 hours, mitophagy signals (shown in red in [B]) per cell were quantified. Values are expressed as mean ± SEM. White, WT; Black, DKO.
**Figure 3.** Glaucoma-mutant OPTN restored Parkin-dependent mitophagy in DKO_HeLa cells. (A) Representative pictures of mito-Keima signals observed in OPTN-transduced DKO_HeLa cells. DKO_HeLa cells were transduced with GFP alone or GFP-OPTN and treated with 10 µM CCCP for 3 hours. The mito-Keima signals transported to the lysosomes were considered to represent "mitophagy" (red), and nondegraded mitochondria were designated as "mitochondria" (green). Scale bars: 10 µm. (B) Quantification of mitophagy signals shown in (A). The levels of mitophagy were decreased in cells carrying ALS-mutant OPTN proteins (Q454E and E478G) relative to OPTN_WT; however, they were unaltered in cells carrying glaucoma-mutant OPTN proteins (A377T and H486R). Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01. White, treatment with DMSO; black, treatment with CCCP.
mitophagy. We examined whether a mutation in the UBD of OPTN alters the localization of OPTN. As expected, GFP-OPTN_WT efficiently co-localized with mitochondria after the induction of mitophagy by CCCP (Figs. 4A, 4B). The ALS-associated OPTN_Q454E and _E478G proteins localized differently during the induction of mitophagy. Mitochondria-localized GFP-positive signals were scarcely observed in DKO_Hela cells harboring OPTN_E478G; the majority of the GFP-positive signals were diffused in the cytoplasm. The OPTN_Q454E protein localized both to the mitochondria and the cytoplasm. Thus, the two ALS-associated mutations inhibited the translocation of OPTN to the mitochondria.

**Figure 4.** Glaucoma-mutant OPTN normally localized to mitochondria and bound to ubiquitinated proteins. (A) Representative pictures of GFP-OPTN signals observed in OPTN-transduced DKO_Hela cells. DKO_Hela cells were transduced with GFP alone or GFP-OPTN and treated with 10 μM CCCP for 3 hours. The subcellular localizations of exogenous OPTN were examined according to the GFP signals. Scale bars: 10 μm. (B) Quantification of GFP-OPTN localization shown in (A). The mitochondrial localization was diminished in cells carrying ALS-mutant OPTN proteins (Q454E and E478G) relative to OPTN_WT, but was normal in cells carrying glaucoma-mutant OPTN proteins (A377T and H486R). (C) Representative western blot of the immunoprecipitation assay. DKO_Hela cells were transfected with GFP-OPTN and treated with 10 μM CCCP for 3 hours. The immunoprecipitated samples from protein lysates with anti-GFP antibody were probed with anti-ubiquitin antibody. Glaucoma-mutant OPTN proteins (A377T and H486R) bound to ubiquitinated proteins (poly-Ub) as OPTN_WT, whereas ALS-mutant OPTN proteins (Q454E and E478G) did not.
Meanwhile, the glaucoma-associated OPTN_A377T and OPTN_H486R mutants localized normally to mitochondria after treatment with CCCP. In summary, glaucoma-associated mutant OPTN proteins can still translocate into mitochondria and induce mitophagy upon treatment with CCCP, whereas ALS-associated mutant OPTN proteins fail to translocate. These results suggest that glaucoma-associated mutations in OPTN have a markedly limited impact on CCCP-induced mitophagy compared with ALS-associated mutations.

We further examined the binding activity of mutant OPTN protein to ubiquitinated proteins in the context of CCCP-induced mitophagy through immunoprecipitation. We prepared protein lysates extracted from CCCP-treated GFP-OPTN transfected cells and immunoprecipitated GFP-OPTN from them with an anti-GFP antibody. The immunoprecipitated samples were probed with an anti-ubiquitin antibody. The two glaucoma-associated mutant OPTN proteins and the WT OPTN were associated with ubiquitinated proteins that appeared in bands more than 100 kDa. As expected, the two ALS-associated mutant OPTN proteins did not fully bind to ubiquitinated proteins (Fig. 4C).

**Five Additional Glaucoma-Mutant OPTN Proteins Restored Mitophagy and Localized to Mitochondria**

We extended our analysis to other glaucoma-associated mutations located outside the ubiquitin-interacting region of the OPTN gene. We selected five additional glaucoma-associated mutations throughout the gene (H26D, E50K, E105D, T202R, and A336G (Fig. 1A)). However, none of the glaucoma-associated mutant OPTN proteins impaired Parkin-mediated mitophagy, mitochondrial translocation, or ubiquitin-binding activity under treatment with CCCP (Figs. 5A–C). Surprisingly, the ubiquitin-binding activity of the OPTN_E50K protein was increased, despite normal mitophagy activity (Fig. 5C).

**Glaucoma-Mutant OPTN Also Restored the Mitophagy Induced by the Concomitant Treatment of Oligomycin and Antimycin A**

Finally, we examined the impact of mutant OPTN on oligomycin and antimycin A (O/A)-induced Parkin-dependent mitophagy, as the effect of CCCP is detrimental for cellular processes other than mitochondrial integrity. Treatment with O/A inhibits the mitochondrial respiratory complex III, leading to mitochondrial depolarization and increased levels of reactive oxygen species. The induction of Parkin-dependent mitophagy by treatment with O/A is generally considered more physiologically than that observed through treatment with CCCP.

We treated the DKO_HeLa cells with O/A (10 μM/4 μM) or DMSO to examine the impairment of mitophagy activity in these cells. Mitophagy was insufficiently induced in DKO_HeLa cells after treatment with O/A for 6 hours (Fig. 6A and Supplementary Fig. S5). In DKO_HeLa cells, exogenously expressed OPTN_WT proteins restored O/A-induced mitophagy and translocated normally to mitochondria (Fig. 6B and Supplementary Fig. S4B). This suggests that glaucoma-associated mutations in OPTN are not overtly compromised on the restoration of O/A-induced mitophagy, unlike ALS-associated mutations.

We further examined the binding activity of mutant OPTN proteins to ubiquitinated proteins in the context of O/A-induced mitophagy by immunoprecipitation. Seven glaucoma-associated mutant OPTN proteins and OPTN_WT were associated with ubiquitinated proteins that appeared in bands more than 100 kDa (Fig. 6D). Surprisingly, the OPTN_E50K mutant bound more to ubiquitinated proteins than the others. As expected, the two ALS-associated mutant OPTN proteins did not fully bind to ubiquitinated proteins (Fig. 6D).

**Discussion**

The mechanism through which mutations in the OPTN gene lead to glaucoma or ALS has not been clarified, although there is evidence that OPTN plays crucial roles in Parkin-dependent mitophagy. In the present study, we investigated the impact of glaucoma-associated mutations in the OPTN gene in the context of Parkin-dependent mitophagy. Although these mutations had a limited effect on mitophagy regardless of the mutation site, two ALS-associated mutations in the ubiquitin-interacting region of OPTN exerted a more pronounced effect, as previously reported.

In the present study, we used HeLa cells to study the role of OPTN mutants in the context of Parkin-mediated mitophagy. This cell line has been widely used for the mechanistic study of mitophagy. The HeLa cell line is an immortalized cell line derived from cervical carcinoma tissue lacking endogenous expression of Parkin due to gene truncation. We used HeLa cells stably expressing Parkin to analyze Parkin-dependent mitophagy. Constitutive overexpression of Parkin may induce a larger magnitude of mitophagy even in the context of OPTN/NDP52 deficiency, which would obscure the impaired mitophagy of glaucoma-associated OPTN mutants. We confirmed the previously reported functional deficits of ALS-associated mutants in this cell line. The use of other cell types (e.g., neuronal cells) would be more suitable as the optic nerve is degenerated in patients with glaucoma. reported that acute overexpression of the OPTN_E50K mutant in rat primary retinal ganglion cells (RGCs) caused increased mitochondrial damage as well as activation of the apoptotic Bax pathway and increased oxidative stress in vitro. The results of our ubiquitin-binding assay using the OPTN_E50K mutant support these findings. Increased binding between OPTN and ubiquitinated proteins enhances mitophagy activity, despite the absence of alteration in restored mitophagy level demonstrated by our mitophagy assay. This discrepancy may be attributed to the cell type; RGCs are postmitotic neuronal cells, in which the introduced genes are undiluted, whereas HeLa cells are immortalized tumor cells, in which the introduced genes are diluted during cell division. Although this was beyond the scope of the present study, it would be interesting to analyze neuronal cells differentiated from pluripotent stem cells with specific OPTN mutations.

Duplication of the TBK1 gene was recently identified in patients with NTG. Both TBK1 and OPTN have functions in the autophagy machinery and nuclear factor-κB (NF-κB) signaling. Moreover, TBK1 phosphorylates and activates OPTN to promote autophagosome formation (Fig. 1B). Autophagy activity is enhanced in induced pluripotent stem cell–derived retinal cells from NTG patients harboring the TBK1 duplication.

Furthermore, TBK1-hemizygous transgenic mice have progressive loss of RGCs. Aged OPTN_E50K transgenic mice also have degeneration of RGCs and elevated mitophagy. Collectively, mild and persistent activation of mitophagy activity may govern the onset of POAG.
Mechanisms other than Parkin-mediated mitophagy may be involved in the pathogenesis of glaucoma. OPTN is a multifunctional adaptor protein that mediates a variety of cellular processes. One of the major targets for OPTN is NF-κB signaling, which regulates the expression of many genes involved in the immune response, apoptosis, and the cell cycle. OPTN is thought to negatively regulate TNFα-induced NF-κB signaling by competing with the NF-κB essential modulator (NEMO), an element of the inhibitor of the NF-κB kinase complex, for binding to ubiquitinated receptor-interacting kinase 1 (RIPK1) (Fig. 1B). OPTN also interacts with cylindromatosis (CYLD) (Fig. 1B), a deubiquitinating enzyme, to cleave polyubiquitin chains from the target proteins RIPK1 and NEMO, thus preventing the activation of NF-κB. ALS-associated E478G mutant OPTN abolished the inhibition of NF-κB signaling downstream of TNF-receptor activation, as well as the binding to polyubiquitinated proteins localized to damaged mitochondria during Parkin-mediated mitophagy. Moreover, the ALS-associated Q454E mutant OPTN exerts partial inhibitory effects on NF-κB signaling.

**Figure 5.** Five additional glaucoma-mutant OPTN proteins restored mitophagy, localized to mitochondria, and bound to ubiquitinated proteins. (A) Quantification of mitophagy signals. The levels of mitophagy were unaltered in cells carrying five other glaucoma-mutant OPTN genes (H26D, E50K, E103D, T202R, and A336G). The three samples on the left (–, GFP, and WT) and the four samples on the right (A377T, Q454E, E478G, and H486R) are shown as the reference (see Fig. 3B), as all 12 samples were analyzed together in the original experiment. Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01. White, treatment with DMSO; Black, treatment with CCCP. (B) Quantification of GFP-OPTN localization. Mitochondrial localization was normal in cells carrying five other glaucoma-mutant OPTN genes. (C) Representative Western blot of the immunoprecipitation assay. Five additional glaucoma-mutant OPTN proteins bound to ubiquitinated proteins (poly-Ub) as OPTN_WT, whereas the ALS-mutant OPTN (E478G) did not.
Figure 6. Glaucoma-mutant OPTN proteins restored mitophagy and localized to mitochondria. (A) Quantification of O/A-induced mitophagy in DKO_Hela cells. After treatment with O/A for 3, 6, or 12 hours, mitophagy signals (shown in red in Supplementary Fig. S3) per cell were quantified. Values are expressed as mean ± SEM. White, WT; Black, DKO. (B) Quantification of mitophagy signals. The levels of mitophagy were unaltered in cells carrying seven glaucoma-mutant OPTN genes (H26D, E50K, E103D, T202R, A336G, A377T, and H486R). Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01. White, treatment with DMSO; Black, treatment with O/A. (C) Quantification of GFP-OPTN localization. Mitochondrial localization was normal in cells carrying seven glaucoma-mutant OPTN genes. (D) Representative Western blot of the immunoprecipitation assay. Seven glaucoma-mutant OPTN proteins bound to ubiquitinated proteins (poly-Ub) as OPTN_WT, whereas the ALS-mutant OPTN proteins (Q454E and E478G) did not.
Limited Impact of Glaucoma OPTN Mutations on Mitophagy

Our analyses revealed that the Q454E mutant OPTN showed partial impairment in Parkin-dependent mitophagy as well. Thus, ALS-associated mutations in the ubiquitin-interacting region of OPTN appear to cause dysregulations of both NF-kB signaling and mitophagy.

Similar to the ALS-associated mutant OPTN proteins, glaucoma-associated H486R mutant OPTN causes loss of interaction between OPTN and cytoskeleton, resulting in increased NF-kB signaling induced by inflammatory cytokines (i.e., TNFα, IL-1β, and lipopolysaccharide). In the TNFα-induced NF-kB signaling pathway, H486R mutant OPTN cannot bind sufficiently to ubiquitinated RIPK1, leading to the hyperactivation of NF-kB signaling. However, H486R mutant OPTN can interact with ubiquitinated mitochondrial outer membrane proteins in the context of the CCCP-induced Parkin-mediated mitophagy pathway. These discrepancies in the ubiquitin-binding capacity may be attributed to the context in which OPTN is recruited. It would be interesting to identify factors influencing the ubiquitin-binding activity of OPTN.

Furthermore, OPTN interacts with Rab8, myosin VI, and transferrin receptors, and is thought to play a role in intracellular trafficking (Fig. 1B). E50K mutant OPTN potentiates Golgi fragmentation and foci formation, and perturbs the interaction between OPTN and Rab8 (a critical regulator of trafficking), leading to neuronal degeneration. OPTN carrying an E50K mutation interacts more with transferrin receptors than OPTN_WT, and impairs transferrin uptake. M98K mutant OPTN enhances the interaction of OPTN with Rab12, a GTPase involved in the trafficking and lysosomal degradation of transferrin receptors. It is suggested that defective protein trafficking may be the cause of glaucoma in patients with OPTN mutations. Based on the present and previous data, we conclude that various mechanisms are involved in the development of glaucoma and ALS. Defects in Parkin-dependent mitophagy may not be the underlying mechanism of POAG.

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References
Limited Impact of Glaucoma OPTN Mutations on Mitophagy


