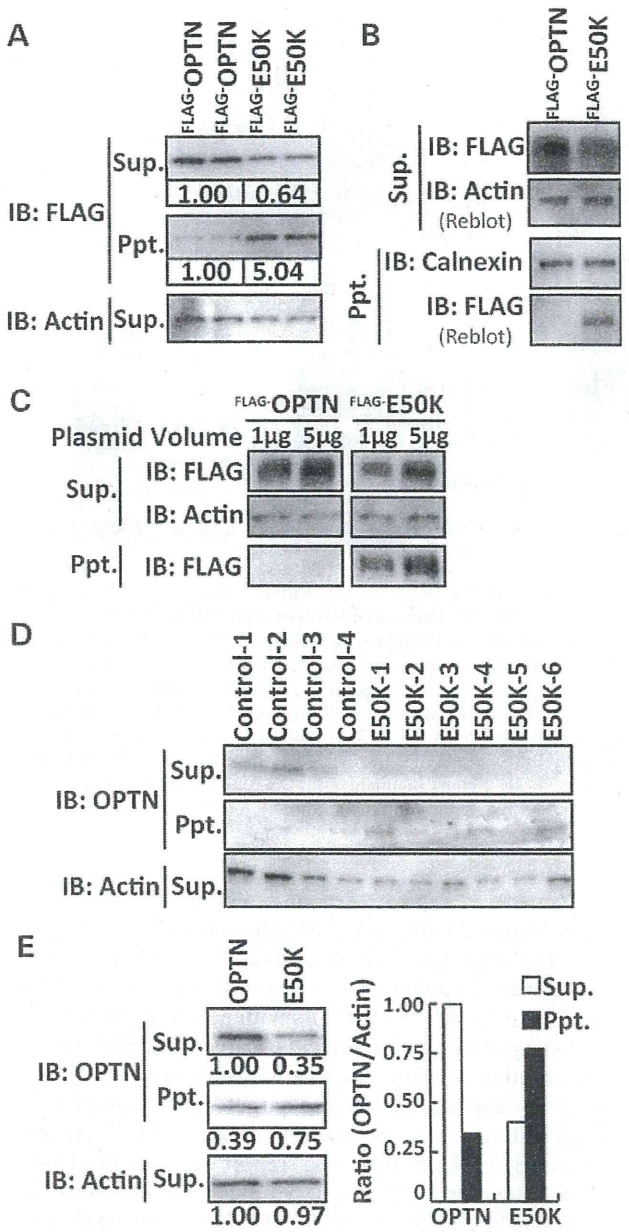

Figure 4. Distinct protein solubility of wild-type OPTN and the E50K mutant. (**A**) Wild-type OPTN and E50K expression under the same transfection condition. There were no differences in mRNA expressions under these transfection conditions (Supplementary Material, Fig. S4A). The 'Missing' E50K mutant protein was detected in the precipitated fraction (Ppt.), after supernatant (Sup.) collection. Semi-quantitative western blotting analysis was performed using Chemidoc (BioRad) with imaging software and the results are shown under each band. Approximately 2-fold reduction of E50K mutant protein in the Sup. fraction and 2- to 5-fold induction in the Ppt. fraction were observed. (**B**) Although calnexin, an ER membrane marker, is detected in both the Ppt. fraction of wild-type OPTN-expressing and E50K mutant-expressing cells, only the E50K mutant is detected in the Ppt. fraction. (**C**) The E50K mutant in the Ppt. fraction was increased in an E50K expression-dependent manner. (**D**) Endogenous expression and higher hydrophobicity of OPTN in iPSCs with the E50K mutation. Endogenous OPTN is also detected in the Ppt. fraction in iPSCs from E50K mutation-carrying NTG patients. (**E**) Abundant endogenous expression and higher hydrophobicity of OPTN in iPSC-derived neural cells 10 days after induction from E50K mutation-carrying NTG patients. Semi-quantitative western blotting analysis by Chemidoc with imaging software was performed and the results are shown under each band. The OPTN amounts in each fraction were normalized to the actin amount and then plotted. Sup., supernatant fraction; Ppt., precipitated fraction.

The enhanced affinity of TBK1 to the E50K mutant protein affects the proper oligomerization and solubility of OPTN

To elucidate the factors that affect the solubility of OPTN, we first examined the native state of wild-type OPTN and the E50K mutant. FLAG-tagged OPTN was expressed in cells and lysates were routinely prepared without detergent and separated by native-polyacrylamide gel electrophoresis (PAGE). Western blotting analysis after native-PAGE indicated more E50K-protein complexes compared with those formed by wild-type OPTN (Fig. 5A). The complexes were immunoprecipitated (IP) using an anti-FLAG antibody and then separated by SDS–PAGE, which revealed distinct binding partners of OPTN and E50K (Fig. 5B, OPTN, white arrowheads; E50K, black arrowheads). We identified each binding partner by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The OPTN partner was identified as OPTN itself, indicating tight oligomerization, while the E50K protein partner was identified as TBK1, which has been previously shown to interact with OPTN by a yeast two-hybrid screening (31). Each candidate interacting partner was further confirmed by IP and western blotting (Fig. 5C and D). Intriguingly, E50K exhibited enhanced affinity to TBK1, while its self-oligomerization was largely decreased (Fig. 5C, arrowhead). Oligomerized OPTN bands clearly seen in wild-type OPTN were restored by treatment with intracellular degradation inhibitors (Supplementary Material, Fig. S7A, left panel, Oligomer lanes), indicating the importance of OPTN oligomerization in intracellular traffic and intracellular degradation. In contrast, these intracellular inhibitors had no effect on the diminished oligomerization of the E50K mutant (Supplementary Material, Fig. S7A right panel, Oligomer lanes). Treatment with a specific inhibitor treatment for TBK1, BX975 (32), was used to examine the relevance of TBK1 binding and the abnormal insolubility of the E50K mutant. BX795 treatment had no effects on the trace amounts of either wild-type OPTN (Supplementary Material, Fig. S7B) or calnexin in the Ppt. fraction (Fig. 5E); on the other hand, the amount of the insolubilized E50K mutant in the Ppt. fraction was drastically decreased by treatment with BX795 in a concentration-dependent manner. Prolonged BX795 treatment was able to restore the E50K mutant protein to the Sup. fraction (Fig. 5F). These findings indicate that the enhanced affinity of E50K for TBK1 is one of the initial pathogenic events that trigger the intracellular insolubility of OPTN leading to improper OPTN transition from the ER to the Golgi body.

DISCUSSION

The OPTN E50K mutation is the only mutation currently affirmed as causative for NTG, and therefore, it is a clinically relevant mutation for elucidating the mechanism of disease onset at a molecular level (4). Although the E50K mutation is a rare event in familial POAG, the pathology is usually progressive, leading to full blindness even under strict IOP control (Supplementary Material, Fig. S1) (17). Previous reports on E50K mutant phenotypes were focused mainly on *in vitro* models using over-expression studies. Though our initial report on the phenotypic analyses of E50K^{-tg} mice was informative (19), there is a strong necessity for further establishment of the model for OPTN and its target molecules in the endogenous

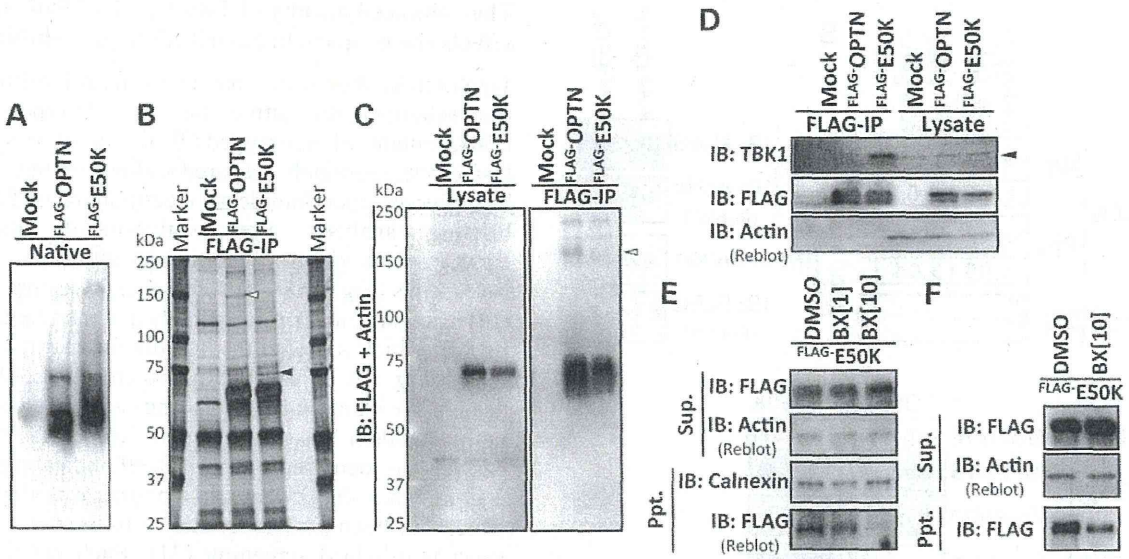

Figure 5. Constitutive interaction of the E50K mutant protein and TBK1 evokes the aberrant solubility of OPTN. (**A**) Native-PAGE of mock-transfected controls, wild-type OPTN-transfected cells and E50K-transfected cells revealed the distinct protein complex formation. (**B**) Silver-staining of immunoprecipitates of mock-transfected controls, wild-type OPTN-transfected cells and E50K-transfected cells using an antibody specific for the FLAG-tag. The relevant bands, wild-type OPTN specific binding molecule (white arrowhead) and E50K mutant-specific binding molecule (black arrowhead), were detected and further analyzed with LC–MS/MS. (**C**) Oligomerization of OPTN. The band indicated with white arrowhead in (B) turned out OPTN itself, i.e. wild-type OPTN is able to oligomerize, while E50K mutant protein largely lacks this oligomerization ability. (**D**) E50K mutant and TBK1 interaction. The band indicated with black arrowhead in (B) turned out TBK1 and E50K mutant protein exhibited higher affinity to TBK1 protein than wild-type OPTN. (**E**) The treatment with BX795, a TBK1 inhibitor, decreases the aberrant precipitation of E50K mutant protein in the Ppt. fraction in a concentration-dependent manner. Dimethylsulfoxide (DMSO) was used as the vehicle control and BX [1], BX [10] indicates the BX795 treatment concentrations of 1 μg/ml and 10 μg/ml, respectively, for 3 h. (**F**) Longer treatment with BX795 (6 h with 10 μg/ml) suppressed aberrant precipitation of E50K mutant protein in the Ppt. fraction and simultaneously restored E50K to the soluble fraction.

context to understand the exact molecular functions of OPTN and its mutations in glaucoma. In addition to the previously identified glaucomatous phenotypes, such as RGC loss, E50K^{-tg} mice also exhibit prominent retinal reactive gliosis with GFAP-positive Müller cells. It has been reported that GFAP-positive Müller cells can be experimentally induced in animal models mimicking glaucomatous phenotypes through various retinal insults, such as axonal damage, intravitreal injection and laser ablation (22–24). Thus, the persistent gliosis and inner layer cell death in E50K^{-tg} mice, without elevation of IOP, were of great interest, and this suggests that increased IOP is not the sole cause for POAG. The deposit-like E50K mutant protein seen in the INL of the retinas of E50K^{-tg} mice was encouraging, because similar abnormal protein inclusions are frequently found in clinical specimens of neurodegenerative tissues, including ALS (20, 33). Why E50K expression, which occurs throughout the body, only affects retinal homeostasis remains unknown. OPTN is also endogenously expressed in many other types of cells, like fibroblasts (7). In addition, most of the other cells expressing OPTN are proliferative and replaced usually within a few months, whereas the neural cells are usually non-proliferative and long-lived. We surmise that this is why the accumulation of E50K over time is critical in the pathogenesis of neurodegenerative diseases, including NTG. Though the E50K^{-tg} mice exhibit some representative neurodegenerative disease phenotypes, further investigation of the E50K accumulation in the endogenous context over time *in vivo* in the retina is needed, preferable in retinal specimens from E50K mutation-carrying NTG patient or from a mouse model, such as a site-specific knock-in mouse model.

Previous *in vitro* studies on E50K have shown large vesicle formation and Golgi fragmentation (10, 20), while there are no reports of endogenous E50K localization and behavior, especially in patient neuronal cells. In general, data pertaining to OPTN in clinical samples of patients with neurodegenerative diseases, including the retinal disease, is scarce. The iPSC technology is one solution to overcome this longstanding limitation by indirectly generating the desired target cells from iPSCs derived from patients with genetically driven neurodegenerative diseases (34). With this first report of the establishment of E50K-glaucoma iPSCs and their neuronal induction, molecular and cellular characterization of POAG onset can now be studied in the endogenous context. iPSC-derived neural cells from E50K mutation-carrying patients revealed for the first time that OPTN accumulated at the constricted Golgi body. In our current experiments, unlike the results of the E50K over-expression studies, Golgi was constricted but not fragmented. This discrepancy should be carefully examined to elucidate whether fragmentation of Golgi body is an endogenous phenotype or just an artifact induced by the over-expression. In any case, excess accumulation of E50K triggers Golgi body deformation and further deteriorates intracellular traffic, and eventually leads to cell death. It is well known that OPTN has a role in secretory vesicle transport and that E50K expression decreases the release of the neurotrophic factor NT3 (9, 35). Furthermore, prostaglandin E2 (PGE2) release via exocytosis is also decreased by E50K expression (Supplementary Material, Fig. S1B). These results indicate that due to the intracellular transport failure in cells expressing the E50K mutant, the paracrine activity for cellular protection and blood flow within the retina would also be attenuated.

Retinal vessel vulnerability in E50K^{-tg} mice is explained by these indirect extracellular E50K effects.

This study demonstrated that the E50K mutant is insoluble and is associated with the hydrophobic precipitate in lysates, compared with the wild-type OPTN, in iPSCs and iPSC-derived neural cells. Abnormal protein deposits, as shown in the retinas of the E50K^{-tg} mice, and protein hydrophobicity are frequently reported in neurodegenerative diseases (36–38). Recent reports in yeast models also supported the distinct hydrophobicities of wild-type OPTN and the E50K mutant (39). Although the prediction of isoelectric points (Compute pI/Mw, ExPASy) of wild-type OPTN and E50K do not differ (OPTN = 5.21, E50K = 5.26), their intracellular protein complex formation is considerably different. The amino acid characteristic of hydrophobic glutamate (E) against hydrophilic lysine (K) suggests that the E50K mutation is a possible charge swap mutation. E50K is located adjacent to the coiled-coil domain, which is a domain implicated in the interaction between OPTN and TBK1 (31, 15). The hydrophobicity of the E50K mutant was closely related with its enhanced interaction with TBK1, a well-known infection-responsive molecule. TBK1 induces macroautophagy by interacting with wild-type OPTN only under conditions of infection, and mediates crosstalk between innate immune response and autophagy (15). Additionally, the copy number variation of *TBK1* was associated with NTG onset (5, 6). The duplication of genes on chromosome 12q14 with familial POAG suggested that an extra copy of the *TBK1* gene and its copy number variation were responsible for NTG (40). More recently, NTG-related TBK1 mutations were also reported (41). Thus it is now well established that both *OPTN* and *TBK1* missense mutations are related with NTG onset. The abnormal physical protein interaction with TBK1 is responsible for the major cause of NTG in relation to the OPTN-E50K mutation. Together with the clinical facts, it has been reported that TBK1 has an important role in innate immunity pathways, and phosphorylated the ER-resident adaptor protein stimulator of IFN genes (STING) to enable IFN production (42, 43). Complexes of these molecules may be involved with the failure of the E50K OPTN protein to transition from ER to Golgi. Although TBK1 contributes to infection-related immunological responses, it also seems to contribute to the intracellular clearance of unnecessary components, such as by autophagy (15). Many other ophthalmic diseases, like macular diseases, are associated with abnormal protein metabolism (44); thus, the crosstalk of OPTN and TBK1 in the maintenance of intracellular clearance in retinal cells is likely to play a significant role in not only glaucomatous but also various other retinal diseases. Even though the exact function of TBK1 and the mechanism of the OPTN-TBK1 crosstalk in retinal homeostasis needs to be elucidated, compounds that abrogate the interaction between the E50K mutant and TBK1 are likely to be beneficial in the treatment of NTG patients.

Our current results pinpoint the molecular basis and concepts of NTG onset in E50K mutation-carrying patients and suggest that the RGC loss, the hallmark of glaucoma, is rather a terminal consequence of the sequential events, i.e. altered affinity of the E50K mutant inhibits self-oligomerization, leading to increased hydrophobicity, which affects downstream functions of OPTN, and eventually leads to cell death. Chronic and excessive accumulation of the E50K mutant protein recapitulated the partial neurodegenerative pathology, including reactive gliosis, vulnerability of retinal vessels and increased apoptotic cell death.

RGC loss is a hallmark of glaucoma; however, the results of this study showed that this phenomenon in E50K-NTG model is at the terminal stage of sequential abnormal events in the retina. In-depth characterization of the mutant protein in a physiologically relevant context and the proper choice/availability of a suitable animal model will help to elucidate and explore therapeutics for personalized treatment of glaucoma in the future.

MATERIALS AND METHODS

Antibodies and biochemical analysis

All the antibodies for biochemical studies were purchased from the following companies: anti-OPTN antibody (Cayman); anti-TBK1 antibody (Cell Signaling Technology); anti-FLAG (Sigma); anti-HA (Roche) and anti-Actin (Millipore). The TBK1 inhibitor, BX795, and cycloheximide were purchased from Calbiochem. Mini-PROTEAN TGX Gel and Transblot turbo system (BioRad) were used for native and SDS–PAGE western blotting according to the manufacturer's instructions. Quantitative western blotting was performed with ChemiDoc XRS+ with the Image lab software package (Biorad).

Animal experiments, preparation of retinal flat-mounts for staining and immunohistochemistry

All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Vision Research and approved by the Tokyo Medical Center Experimental Animal Committee. The OPTN mutant E50K^{-tg} mouse used in this study has been described previously (19). Twenty-two to 24-month-old male E50K^{-tg} mice ($n = 4$) and their littermates ($n = 4$) were sacrificed for the assessment of retinal gliosis. Both eyes were dissected and immunostained in flat-mounts as previously described (19). Briefly, dissected eyes were fixed in 2% paraformaldehyde and permeabilized with 0.1% Triton-phosphate-buffered saline (PBS). Non-specific binding was prevented by blocking with DAKO's serum-free blocking buffer, and all specimens were incubated with Alex488-conjugated anti-GFAP antibody (Millipore) for 4°C, over two nights. After radial dissection, retinas were mounted in DAKO's fluorescent mounting medium. A total of 16 retinal specimens, with four micrographs per one retinal specimen, were imaged by LSM700 confocal fluorescence microscopy (Zeiss) using a blinded method. Image analysis was conducted using the ZEN software (Zeiss) and the GFAP-positive area per retinal area was scored. The anti-OPTN (Cayman) and anti-HA (COVANCE) antibodies were used under heated antigen-retrieval conditions. Endogenous peroxidase was quenched by 3% H_2O_2 in MeOH. After primary antibody reaction for 4°C overnight, simple rabbit IgG-horse radish peroxidase (HRP) stain and mouse IgG-HRP stain for mouse tissue (Nichirei) were used as secondary HRP-conjugated polymers. After developing with 3,3′-diaminobenzidine (DAB) substrate, specimens were counter-stained with Gill's hematoxylin.

Light microscopy was performed with an Eclipse 600 microscope (Nikon).

Cell culture, transfection and immunocytochemistry

HEK293T cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated FBS. The TransIT-PRO Transfection Kit (Mirus) was used according to the manufacturer's instructions. HEK293T cells were transfected with pAC-^{GFP}, pAC-^{GFP}-OPTN and pAC-^{GFP}-E50K to assess the intracellular localization of tagged OPTN. The ER-ID Red assay kit (Enzo) was used for endoplasmic reticulum staining. Anti-GM130 and Alexa633-conjugated anti-mouse IgG antibodies were used for Golgi immunostaining. The following constructs were used for over-expression studies: pEF-BOS-^{FLAG} (45), pEF-BOS-^{FLAG}-Optineurin and pEF-BOS-^{FLAG}-E50K.

Generation of iPSC and induction of differentiation to neural cells

Human E50K mutation-carrying iPSCs and the corresponding control iPSCs were established by Sendai-viral (DNAVEC) infection as previously reported (46) from circulating T-cells in the peripheral blood of human familial glaucoma patients with fully informed consent. All procedures were approved by the Ethics Committee of National Hospital Organization Tokyo Medical Center. For maintaining the pluripotency, iPSCs were cultured in bovine fibroblast growth factor (bFGF)-containing iPSC media on Matrigel-coated culture dishes. Oct3 and Nanog were used as pluripotency markers and Tuj1 was used as the neuronal marker. Neural cell induction was performed via embryoid body formation as described previously (27, 28), utilizing the Neuron differentiation Kit (R&D Systems) in accordance with the manufacturer's procedures.

Identification of E50K-binding proteins by LC–MS/MS

Samples for LC–MS/MS analysis were prepared by preparing lysates from HEK293T cells over-expressing FLAG-tagged OPTN from pEF-BOS-^{FLAG}, pEF-BOS-^{FLAG}-Optineurin or pEF-BOS-^{FLAG}-E50K. Each lysate sample was immunoprecipitated with M2-FLAG-Agarose (Sigma) for 2 h at 4°C. The immunoprecipitated beads were washed with lysis buffer five times and then eluted with 2 M urea. The eluates were electrophoresed on 7.5% SDS–PAGE gels and the gels were silver-stained with the Silver Quest Kit (Invitrogen). The band of interest was processed for in-gel digestion for further LC–MS/MS analysis. Samples were analyzed with LCQ-DECA XP (Thermo Scientific). The obtained binding candidates and their interaction with OPTN/E50K were confirmed by immunoprecipitation and western blotting.

AUTHORS' CONTRIBUTIONS

Y.M. and T.I designed the study; Y.M., D.I., H.K., Z.-L.C., H.K., performed the experiments; K.K., T.Y., T.S., S.Y., K.F. contributed new reagents/techniques; Y.M. and T.I. analyzed the data; Y.M. and T.I. wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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