DISCUSSION

We report the first NTG mouse model that was developed using the *OPTN* gene mutation found in NTG patients. Similar to human NTG patients, the *OPTN* E50K Tg mice show progressive RGC loss in the peripheral retina without elevation of IOP. OPTN Wt, 1st LZ deletion, 2nd LZ deletion, and H486R Tg mice did not show any sign of abnormality even after 16 month. Tg mice lines expressing OPTN E50K showed significant thinning of the NFL and INL (Fig. 2, 3, 4). Expression of transgene driven by ubiquitous chicken beta-actin promoter with CMV enhancer gave similar distribution of mutant OPTN as the endogenous OPTN (Fig. 1C). Considering the fact that mutant OPTN is expressed over the entire retina, it is noteworthy that the cell death occurred mainly in the RGCs and astrocytes. These cells may be more sensitive to deleterious effects of OPTN E50K expression than other retinal cells and this may be the reason for the NTG phenotype. The amount of RGCs was reduced by 20 % in *OPTN* E50K Tg mice as compared with Wt 16 month old animals. Slow progression of RGC death resembles that in human glaucomatous patients.

Retinal function was shown to be disrupted by *OPTN* E50K mutation. The amplitude of the photopic negative response (PhNR) by E50K Tg mice was marked as slow negative wave after the b-wave often being replaced by a prolonged positive phase above base line (Fig. 2D). The PhNR was probably primarily a reflection of the

spiking activity of RGCs although other spiking cells in the inner retina, such as amacrine cells, may also contribute to PhNR. These data agree with previous human ERG studies showing PhNR disturbances in glaucoma patients (Rangaswamy et al. 2007; Machida et al. 2008).

Surprisingly, apoptotic astrocytes were more abundant in the NFL of the peripheral retina and reduced around the optic nerve head (Fig. 5F, G). Previous studies have shown activated astrocytes in the glaucomatous retina and optic nerve lamina region (Wang et al., 2002; Howell at al., 2007). Glial cells are important structural and functional components of the nervous system, including the retinal astrocytes which originate embryologically in optic nerve and subsequently migrate to their final location in the whole retina (Watanabe and Raff, 1988). It is still unclear whether which cell type, an astrocytes or retinal ganglion cells, is affected before the other. Future study using this animal model may provide new information on why peripheral retinas are more affected in human glaucoma.

OPTN was discovered as the first gene shown to be associated with NTG (Rezaie et al., 2002). The molecular mechanism leading to the disease is still unknown. OPTN E50K mutation at the N-terminus of the protein is responsible for severe NTG while H486R mutation at the C-terminus is responsible for JOAG with elevation of IOP (Stone et al., 1997; Willoughby et al., 2004). Differences of phenotype observed for

mutations located in different parts of OPTN protein may originate in the modifications of different protein binding sites. OPTN was originally reported as the second protein (FIP-2) interacting with adenovirus protein E3-14.K (Li et al., 1998) and utilizing TNF-alpha or Fas-ligand pathways to mediate apoptosis, inflammation, and vasoconstriction (Sarfarazi et al., 2003). OPTN is known to interact with a number of proteins to perform multiple cellular functions: Rab8 for vesicle trafficking, Huntingtin for membrane trafficking, transcription factor IIIA and FOS for transcriptional activation (Rezaie et al., 2002; Hattula et al., 2000). Because OPTN is capable of interacting with multiple proteins, it is feasible that the E50K mutation can cause glaucoma by functionally affecting one or more protein interactions. Due to the location of the E50K mutation at the N-terminus of the OPTN protein, we speculated that interaction with Rab8 would be affected. Rab8 belongs to a family of small GTP-binding proteins which act as regulators of multiple cellular processes. Rab GTPases regulate all stages of membrane trafficking, including vesicle transport, cargo sorting, transport, tethering, and fusion (Zerial et al., 2001). Rab8 has been shown to be involved in polarized membrane transport and regulation of vesicular transport from the trans-golgi network (Huber et al., 1993). Docking and fusion of rhodopsin is impaired in photoreceptors by mutated Rab8 (Moritz et al., 2001), while treatment with antisense oligo to Rab8 inhibited membrane traffic in hippocampal neurons leading to

inhibition of neurite outgrowth. The Rab8 GTPase regulates apical protein localization in intestinal cells and cooperates with Bardet-Biedl syndrome proteins to promote ciliary membrane biogenesis (Sato et al., 2007; Nachury et al., 2007). Recently, OPTN was demonstrated to protect survival of NIH3T3 cells under oxidative stress by relocating to the nucleus in a Rab8-dependent manner, while OPTN E50K lost the ability to translocate itself to the nucleus (De Marco et al., 2006). OPTN was shown to be a link between Rab8 and myosin VI to Golgi complex. It plays a central role in Golgi ribbon formation and exocytosis (Sahlender et al., 2005). These data suggest that OPTN-Rab8 interaction is essential for Rab8 function and that disruption of Rab8-OPTN interaction may cause irregular transport within the cells. Gene mutations observed in NTG patients including E50K were located near the first zinc-finger domain which was predicted to be a binding site for Rab8 (Hattula et al., 2000). Additionally, glutamic acid at amino acid 50 is well conserved in 8 different species. Our data demonstrate that E50K mutation abolished interaction of OPNT with all forms of Rab8 on the Golgi complex.

Finally, we identified a three generation family with 4 NTG patients, all of whom carry the OPTN E50K mutation (Fig. 8). OCT analysis demonstrated morphological abnormality at the NFL, GCL and IPL in NTG patients as seen in OPTN E50K Tg mice (Fig. 8). These observation follow previous glaucoma reports showing thinning of the

NFL, GCL, IPL, and INL even before the detectable visual field changes occur (Tan et al., 2008; Guedes et al., 2003; Medeiros et al., 2005; Wassle et al., 1989).

In summary, we have proven that the E50K mutation in the *OPTN* gene can lead to NTG in mice. Although further studies are required to fully understand the detailed mechanism of disease progression, we obtained basic information about the affects of E50K mutation at molecular, cellular, and tissue levels. Due to the fact that OPTN interacts with multiple proteins involved in multiple levels of regulation, it is likely that different mutations in the *OPTN* gene may affect diverse cellular pathways. If Rab8 function is altered by *OPTN* mutation, it is likely that exocytosis of proteins regulated by the vesicle transport are affected in RGC or astrocytes. Following this path, the next question would be whether such alteration of protein secretion really exists, and what protein(s) would be involved. If these proteins can be identified, it may serve as potential therapeutic approach to treat patients with *OPTN* E50K mutation.

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FIGURE LEGENDS

Figure 1.

Development of transgenic mouse over expressing OPTN.

A, Schematic diagram of the OPTN constructs used in this study. The green region corresponds to the OPTN protein. Positions of mutations and deletions are shown in red. The HA tag is marked by yellow color. The CAGGS region corresponds to the

chicken beta-actin promoter. ***B,*** Fundus photographs of normal, Wt and E50K Tg mouse eyes at 16 month. Curvature of the retinal vessels indicates the excavation of the area including the optic disc in E50K Tg mouse eye. ***C,*** Total expression of endogenous and mutant OPTN (red) in the retina of normal and E50K Tg mice at 16 month. Anti-OPTN antibody and Anti-HA antibody were used to detect endogenous and mutant OPTN respectively. Scale bar, 50 μm.

Figure 2.

RGC loss and thinning of the retina thickness in the peripheral retina.

A, staining of retina sections of 16 month old normal and Tg mice. Scale bar, 200 μm (upper panel), 50 μm (lower panel). ***B,*** Quantification of the RGC number and retina thickness of 16 month old normal and Tg mice (n=6). Only 50K Tg mice at 16 months showed significant RGC loss and thinning of retina (**p<0.01).**C,** Quantification of the RGC number and retina thickness during development of E50K Tg mice(n=6). Tg mice showed statistically significant RGC loss and thinning of the retina starting from 12 months of age. **D,** Impaired ERG in E50K Tg mice. The amplitude of the PhNR by E50K Tg mice decreased and removed the negative wave to the transient b-wave (arrow), suggesting RGC loss and other abnormality.

Figure 3.

Histopathology of retina and optic nerve of 16 month old Wt and E50K Tg mouse eyes.

A, Immunolabeling of the retina sections with calretinin, a specific marker for RGCs and amacrine cells. Synapse disruption was observed in the E50K Tg mouse retina (arrow). Scale bar, 20 μm. ***B,*** Hematoxylin-eosin staining and immunostaining with antibodies against tubulin ß III isoform in the optic nerve region. Significant thinning of the nerve fiber layer and the excavation of optic disc (arrow) was observed in E50K Tg mice. Scale bar, 100 μm.

Figure 4.

RGC degeneration in E50K Tg mice.

A, Immunostaining of normal, Wt, and E50K Tg mouse whole retinas with antibodies against SMI32, a specific marker of large type RGCs. Scale bar, 500 μm. White box indicate the location of photographs in lane B. **B,** Thinning of NFL, RGC loss. Scale bar, 50 μm. **C,** RGC axon abnormality (arrows) was also observed. Scale bar, 50 μm.

Figure 5.

Glia cells death in E50K Tg mice.

A, B, Flat mount retina of *E50K* Tg mice was double immunostained with SMI32 (red) and active caspase-3 (green) antibodies. Apoptotic cells were observed only in the whole mount retina of E50K Tg mice. Scale bar, 100 μm. **C-E,** Flat mount retina of E50K Tg mice was also double immunostained with active caspase-3 (green) and GFAP (red) antibodies showing apoptosis of astrocytes. Scale bar, 50 μm. **F,** Apoptotic astrocytes (green) in peripheral retina. **G,** Apoptotic astrocytes (green) in central retina. Scale bar, 100 μm.

Figure 6

IOP measurements for Wt and E50K Tg mice.

A, impact-rebound tonometer and ***B,*** optical interferometry tonometer. Both methods gave normal IOP of 15 +/-1 mmHg for Tg mice at all ages examined (n=6).

Figure 7

Disruption of OPTN-Rab8 interaction by E50K mutation.

A, A diagram of cDNA constructs used in experiments to study protein-protein interaction. ***B,*** The protein-protein interaction of OPTN Wt and E50K with Rab8 Wt, T22N inactive form, and Q67L active form as measured in RGC-5 cells. Interaction of OPTN Wt and Q67L active form of Rab8 increased two and five times over Rab8 Wt

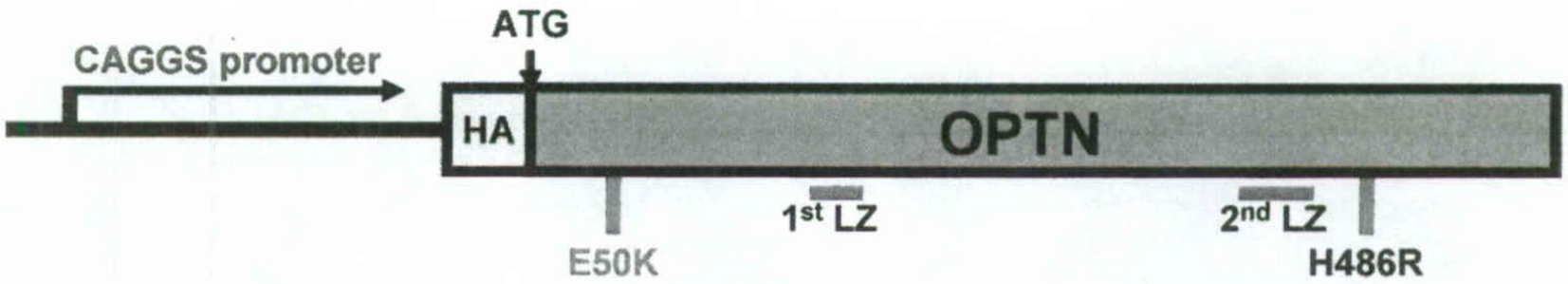
or T22N inactive form of Rab8 protein, respectively (**$p<0.01$). OPTN E50K did not show any interaction with any construct including the active form of Rab8 (n=6). ***C,*** Protein interaction of Wt or mutant OPTN E50K with Rab8 was measured by QCM technique. A sharp drop of QCM frequency was observed when control OPTN Wt was injected as guest sample, confirming the previous reports of OPTN-Rab8 interaction. Mutant OPTN E50K showed no interaction with Rab8. ***D,*** GST Pull-down assay to determine OPTN E50K-Rab8 interaction. The fusion protein GST-Rab8 was used for *in vitro* binding assay with purified OPTN Wt and OPTN E50K protein. For negative control OPTN Wt and OPTN E50K were reacted with GST alone (lane 1 & 2). *In vitro* translated OPTN Wt and OPTN E50K were analyzed by SDS-PAGE to show the protein size (lane 6 & 7). OPTN E50K showed significant loss of interaction with Rab8 compare with OPTN Wt (lane 3 & 4, graph). The illustration shows a diagram of the interaction experiment. ***E,*** Immunostaining the OPTN-Rab8 complex (green) with Golgi marker GM130 (red), indicate that these interactions take place adjacent to the Golgi network.

Figure 8.

NTG patient with OPTN E50K mutation.

A, A pedigree of a NTG family with OPTN E50K mutation. The patients were diagnosed as NTG with glaucomatous optic neuropathy and visual field loss. ***B,*** Optical coherence tomography (Cirrus HD-OCT, Carl Zeiss Meditec, Dublin , CA) and visual field test (Humphrey Field Analyzer, Carl Zeiss Medic, Dublin, CA) were shown on patient 2 and unrelated normal control. The retinal NFL thinning and glaucomatous visual field loss were observed in patients.

A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CAGGS promoter
HA
OPTN
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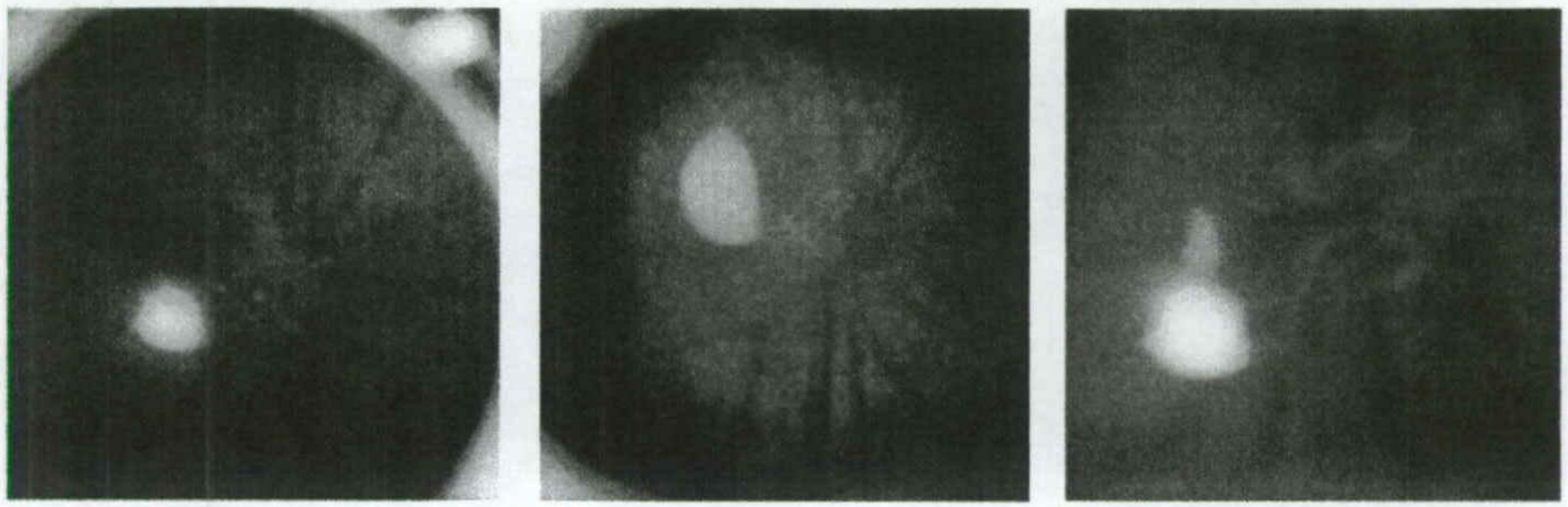
E50K
H486R

B



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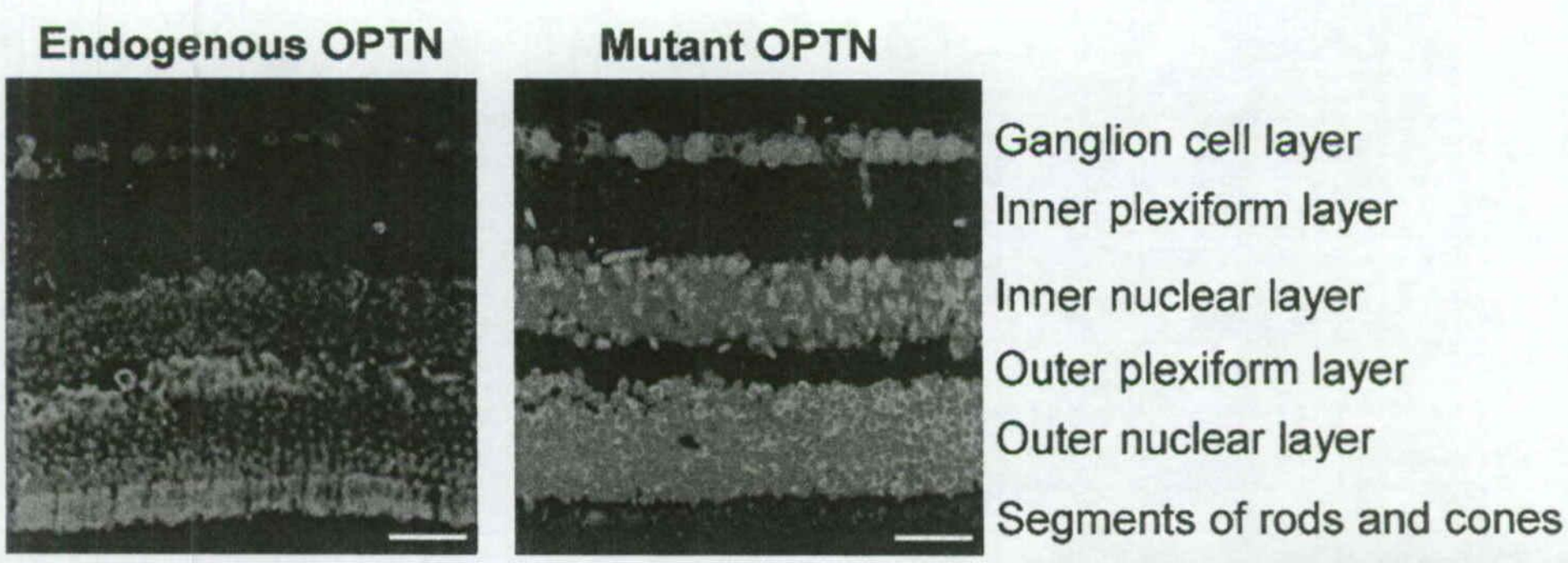
Endogenous OPTN
Mutant OPTN
Ganglion cell layer
Inner plexiform layer
Inner nuclear layer
Outer plexiform layer
Outer nuclear layer
Segments of rods and cones