Transgenic TBK1 mice have features of normal tension glaucoma

John H. Fingert1,2,*, Kathy Miller1,2, Adam Hedberg-Buenz1,2,3, Ben R. Roos1,2, Carly J. Lewis1,2,3, Robert F. Mullins1,2 and Michael G. Anderson1,2,3,4

1Department of Ophthalmology and Visual Sciences, Carver College of Medicine, University of Iowa, Iowa City, IA, USA, 2Stephen A. Wynn Institute for Vision Research, University of Iowa, Iowa City, IA, USA, 3Department of Molecular Physiology and Biophysics, Carver College of Medicine, University of Iowa, Iowa City, IA, USA and 4VA Center for the Prevention and Treatment of Visual Loss, Iowa City VA Health Care System, Iowa City, IA, USA

*To whom correspondence should be addressed at: John H. Fingert, University of Iowa, 3111B MERF, 375 Newton Road, Iowa City, IA 52242, USA. Tel: 319-335-7508; Fax: 877-434-9041; Email: john-fingert@uiowa.edu

Abstract

Duplication of the TBK1 gene is associated with 1–2% of normal tension glaucoma, a common cause of vision loss and blindness that occurs without grossly abnormal intraocular pressure. We generated a transgenic mouse that has one copy of the human TBK1 gene (native promoter and gene structure) incorporated into the mouse genome (Tg-TBK1). Expression of the TBK1 transgene in the retinae of these mice was demonstrated by real-time PCR. Using immunohistochemistry TBK1 protein was predominantly localized to the ganglion cell layer of the retina, the cell type most affected by glaucoma. More intense TBK1 labelling was detected in the retinal ganglion cells (RGCs) of Tg-TBK1 mice than in wild-type littermates. Tg-TBK1 mice exhibit the cardinal sign of glaucoma, a progressive loss of RGCs. Hemizygous Tg-TBK1 mice (with one TBK1 transgene per genome) had a 13% loss of RGCs by 18 months of age (P = 1.5 × 10⁻⁸). Homozygous Tg-TBK1 mice had 7.6% fewer RGCs than hemizygous Tg-TBK1 mice and 20% fewer RGCs than wild-type mice (P = 1.9 × 10⁻⁵) at 6 months of age. No difference in intraocular pressures was detected between Tg-TBK1 mice and wild-type littermates as they aged (P > 0.05). Tg-TBK1 mice with extra doses of the TBK1 gene recapitulate the phenotype of normal tension glaucoma in human patients with a TBK1 gene duplication. Together, these studies confirm the pathogenicity of the TBK1 gene duplication in human glaucoma and suggest that excess production of TBK1 kinase may have a role in the pathology of glaucoma.

Introduction

Primary open angle glaucoma (POAG) is the most common form of glaucoma worldwide and is a common cause of blindness and visual disability (1). The chief features of POAG are a characteristic pattern of optic nerve damage (cupping of the optic disc) and a corresponding, characteristic pattern of visual field loss. Elevated intraocular pressure (IOP) is a risk factor for glaucoma, however, the disease can occur at any IOP. When POAG occurs in the absence of elevated IOP it is frequently termed normal tension glaucoma (NTG). The overall prevalence of POAG ranges from 0.5 to 8.8% depending on age, ethnicity and diagnostic criteria employed and 30–92% of these POAG patients may be classified as NTG (2). NTG accounts for a significant proportion of glaucoma worldwide that is higher in Asian populations (76%) than in white populations (34%) (3).

Genetic variation is important in the pathogenesis of glaucoma. Some cases of POAG have a complex genetic basis and are caused by the combined actions of many genetic risk factors and environmental factors (4). Genome-wide association studies have identified many of these genetic risk factors for POAG including the following genes and loci: CAV1/CAV2 (5),...
of NTG by engineering and characterizing a transgenic mouse with the same genetic defect carried by humans with TBK1-associated glaucoma. We generated a transgenic strain of mice that has one wild-type copy of the human TBK1 gene and its native promoter incorporated into its genome (Tg-TBK1). The Tg-TBK1 mice have a total of three of these kinase genes in their genome (one human TBK1 transgene and two native Tbk1 genes). Here we investigate the Tg-TBK1 mice for a recapitulation of the glaucoma phenotype observed in human NTG patients, which would provide the strongest evidence that TBK1 gene duplications do in fact cause NTG.

## Results

### Generation of transgenic (Tg-TBK1) mice

We previously discovered that copy number variations of the TBK1 gene (i.e. duplications or triplications) are associated with NTG (27). Our subsequent studies showed that TBK1 mutations are responsible for approximately 1% of cases of NTG (28–31). The pathogenicity of TBK1 copy number variations is supported by their presence in patients with NTG, their absence in control subjects, and their absence in public databases (27–31). However, in order to provide stronger evidence that TBK1 gene duplications cause NTG, we generated a transgenic mouse with an extra copy of the TBK1 gene in an attempt to recapitulate human NTG in the mouse. A bacterial artificial chromosome (BAC) containing the entire human TBK1 gene (promoter, exons and introns) was obtained from BACPAC (RP11-6008, Oakland, CA) and used as a vector to produce transgenic mice. Mice carrying the transgene were identified and genotyped with a quantitative real-time PCR assay for the TBK1 gene (Fig. 1). One founder was identified that carried a single copy of the human TBK1 gene and was reiteratively backcrossed against C57BL/6J to produce the line B6.Tg(TBK1), which has one copy of the human TBK1 transgene in addition to the two copies of the mouse orthologue Tbk1 in its genome. These mice are hereafter referred to as ‘Tg-TBK1 mice’.

![Figure 1. Tg-TBK1 mice carry the human TBK1 transgene. Mice were genotyped using a quantitative real-time PCR assay to identify animals that carried the human TBK1 transgene. Approximately half the mice carried one copy of the transgene in their genome, while the half had no copies of the transgene. Genotyping offspring from an intercross of Tg-TBK1 mice with quantitative PCR identified mice that have two copies of the TBK1 transgene (homozygotes); mice that have one copy of the TBK1 transgene (hemizygotes); and wild-type mice with no copies of the transgene (above).](https://academic.oup.com/hmg/article/26/1/124/2595400)
Ocular production of TBK1

Quantitative real-time PCR with probes specific for the mouse Tbk1 mRNA was used to assess expression of the native mouse Tbk1 gene in retinal tissues from three Tg-TBK1 mice and from three age-matched, wild-type littermate mice. Mouse Tbk1 mRNA was detected at similar levels ($P = 0.83$) in the retinae from the Tg-TBK1 mice and their wild-type littersmates (Fig. 2A). The expression of the human TBK1 transgene in the retinae of the same mice was similarly assessed using quantitative real-time PCR with probes specific for human TBK1 mRNA. Robust expression of the human TBK1 transgene was detected in the retinae of the Tg-TBK1 mice and was absent from wild-type mice from the same litter (Fig. 2B), demonstrating that the transgene expresses human TBK1 mRNA and doesn’t influence expression of the endogenous ortholog in mouse retinae.

Tg-TBK1 mice, like human patients with TBK1-associated glaucoma, have more than the usual two copies of the Tbk1 gene per genome. We investigated the possibility that increased copies of the Tbk1 gene result in increased production of Tbk1 protein in Tg-TBK1 mice using immunohistochemistry in wild type and Tg-TBK1 retina. Tbk1 protein in wild-type mice is localized within the retinal ganglion cells (Fig. 3A and D), with weaker labelling in the inner and outer nuclear layers. More TBK1 labelling is detected within the retinal ganglion cells of transgenic TBK1 mice (Fig. 3B and E) than in wild-type littermates (Fig. 3A and D), with similar levels in the other retinal layers. No labelling was detected in a control experiment conducted without primary antibody (Fig. 3C). This pattern of increased TBK1 protein in the retinal ganglion cells of transgenic TBK1 mice was confirmed using two different antibodies and multiple mouse eyes ($n = 8$).

Transgenic TBK1 mice have normal IOP

Humans with TBK1 copy number variations have NTG, glaucoma that occurs without elevated IOP (27–31). Consequently, we measured IOP in transgenic TBK1 and matched littersmates at 5 weeks, 3 months, 7 months, 12 months and 18 months of age (Fig. 4). Transgenic TBK1 mice have a mean IOP that is within the normal range for C57BL/6J mice (43,44) and indistinguishable from the IOP of matched wild-type littersmates ($P > 0.05$) at all ages. These data indicate that transgenic TBK1 mice have IOPs within the normal range as is expected for an animal model of human NTG.

Tg-TBK1 mice have progressive retinal ganglion cell loss

A central feature of glaucoma is damage to the retinal ganglion cells and their axons that make up the optic nerve. Animal models are typically assessed for such signs of glaucoma by using quantitative histological methods (i.e., counting retinal ganglion cells) (44–46). We evaluated Tg-TBK1 mice for a loss of retinal ganglion cells typical of glaucoma using a manual counting strategy. Flat mounts of retinae were prepared from 92 Tg-TBK1 mice and 96 matched wild-type littersmates at a range of ages and retinal ganglion cells were labelled with antibody directed against $\gamma$-synuclein.

At 5 weeks of age, there was no difference ($P = 0.82$) between the number of retinal ganglion cells between Tg-TBK1 mice ($n = 10$) and wild-type littersmates ($n = 10$). However, progressive retinal ganglion cell loss was detected as Tg-TBK1 mice aged (Fig. 5 and Supplementary Material, Fig. S1). A 9% decrease in retinal ganglion cells ($P = 0.00040$) in Tg-TBK1 mice ($n = 11$) was detected at 3 months of age when compared to wild-type littersmates ($n = 10$). Increasing losses of retinal ganglion cells were detected between transgenic mice and control mice aged to 7 months ($n = 50$) and in mice aged to 12 months ($n = 49$). At 18 months of age, Tg-TBK1 mice ($n = 23$) had a 13% loss of retinal ganglion cells when compared to littersmate controls ($n = 25$, $P = 1.5 \times 10^{-4}$).

An intercross of Tg-TBK1 mice that are hemizygous for the human TBK1 transgene produced F1 offspring with three genotypes: wild-type (no transgenes), hemizygous (one TBK1 transgene), or homozygous (two TBK1 transgenes). Twenty-three F1 mice were aged to 6 months and assessed for glaucoma by manually counting retinal ganglion cells (Fig. 6 and Supplementary Material, Fig. S2). Homozygous mice with two TBK1 transgenes had 7.6% fewer retinal ganglion cells than hemizygous mice with one TBK1 transgene and 20% fewer retinal ganglion cells than wild-type littersmates ($P = 1.9 \times 10^{-5}$). These experiments demonstrated a dose-response of retinal ganglion cell counts to the number of TBK1 transgenes. More TBK1 transgenes resulted in more retinal ganglion cell loss.

We also investigated the retinal ganglion cell counts in the same Tg-TBK1 mice and matched controls with a second methodology using hematoxylin and eosin (H&E) staining of retinal whole mounts and our previously reported automated image analysis procedure using hematoxylin and eosin (H&E) staining of retinal whole mounts and our previously reported automated image analysis procedure.

![Figure 2](https://example.com/figure2.png)

Figure 2. Expression of TBK1 mRNA in the retinae of Tg-TBK1 mice. Quantitative real-time PCR was used to measure expression of native mouse TBK1 gene (A) and the expression of the human TBK1 transgene (B) in both Tg-TBK1 mice and age-matched wild-type littersmates. Expression was normalized to GAPDH. Gene expression is depicted as a fraction of the highest level detected in a sample and the error bars represent standard error.
This approach identifies and counts the ganglion cells, amacrine cells and endothelial cells within the retinal ganglion cell layer based on recognition of features of cell nuclei that differentiate between these cell types. Flat mounts of retinae initially examined with \(\alpha\)-synuclein immuno-labelling were washed, relabelled with H&E and analysed to count retinal ganglion cells with RetFM-Class (Fig. 7 and Supplementary Material, Fig. S3). Retinae from 5-week-old wild-type mice (\(n = 3\)) and Tg-TBK1 mice (\(n = 2\)) had similar retinal ganglion cell counts. When retinae from mice aged to 18 months were analysed, a 11% reduction in ganglion cells (\(P = 0.043\)) was detected in Tg-TBK1 mice (\(n = 9\)) when compared to wild-type littermate mice (\(n = 6\)). The 11% loss of retinal ganglion cells detected in 18 month old Tg-TBK1 mice is similar to the 13% measured loss in the same 18 month old Tg-TBK1 mice using \(\alpha\)-synuclein labelling. Moreover, this analysis indicates that cell loss is largely confined to the retinal ganglion cells in Tg-TBK1 mice (Supplementary Material, Fig. S3).

Figure 3. Increased TBK1 protein in the Tg-TBK1 mouse retina. TBK1 protein is localized in the outer nuclear layer, inner nuclear layer and ganglion cell layer of the retina of Tg-TBK1 mice and wild-type littermates using immunohistochemistry. (A) qualitative increase in labelling of TBK1 protein is apparent in the retinal ganglion cells (indicated with an arrow) of the Tg-TBK1 mice (B) when compared with wild-type litter-mates. A no primary antibody control experiment shows minimal labelling of mouse retina (C). Sections of the retina of four Tg-TBK1 mice (D) and four wild-type litter-mates (E) also show increased TBK1 protein in the retinal ganglion cells of the transgenic mice (indicated with an arrow). The layers of the mouse retina are indicated with abbreviations: retinal ganglion cell layer (RGC), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and retinal pigment epithelium (RPE).

Figure 4. Tg-TBK1 mice do not have elevated IOP. Transgenic TBK1 mice have the same IOP as matched wild-type littermate mice at a range of ages (\(P > 0.05\)). Error bars indicate standard deviation.
The pathophysiology of retinal ganglion cell death in NTG is complex. Several potential disease processes have been explored including vascular mechanisms (49,50), autoimmune disease (51) and mechanisms involving abnormal intracranial pressure (52,53). The basic causes of NTG have been successfully investigated using a genetic approach. In 2002, Mansoor Sarfarazi discovered the first NTG gene, optineurin (OPTN), with linkage studies of a large glaucoma pedigree (22). Missense mutations in OPTN were subsequently shown to be responsible for 1–2% of NTG cases (23,24). More recently, we discovered that copy number variations of the TBK1 gene (duplications or triplications) are associated with ~1% of cases of NTG (27–31). Both OPTN and TBK1 encode proteins that have functions in autophagy and other biological processes including NF-kB signalling. These pedigree-based discoveries have identified a role for autophagy in the pathogenesis of NTG. Both known NTG-causing genes (TBK1 and OPTN) have important functions in autophagy. TBK1 encodes a kinase that phosphorylates and activates OPTN, while OPTN has been identified as an autophagy receptor that when activated promotes formation of the autophagosome and capture of proteins or structures targeted for degradation (54). These observations and studies suggest that dysregulation of autophagy in key tissues of the eye (i.e., retinal ganglion cells) are important in the development of NTG.

Numerous methods for counting retinal ganglion cells in mice have been employed using protein markers (γ-synuclein, Brn3A), mRNA markers (Sncg), histological staining patterns (Nissl and H&E) and retrograde labelling from axonal termini (47,48,55–59). Each has strengths and weaknesses with regards to sensitivity, specificity and technical limitations of the method (56,57,60). We used two methods to identify and count retinal ganglion cells. First, the cells labelled with γ-synuclein, antibody were manually counted. Second, retinal ganglion cells stained with H&E were automatically counted based on nuclear staining with H&E. Numerous methods for counting retinal ganglion cells in mice have been employed using protein markers (γ-synuclein, Brn3A), mRNA markers (Sncg), histological staining patterns (Nissl and H&E) and retrograde labelling from axonal termini (47,48,55–59). Each has strengths and weaknesses with regards to sensitivity, specificity and technical limitations of the method (56,57,60). We used two methods to identify and count retinal ganglion cells. First, the cells labelled with γ-synuclein antibody were manually counted. Second, retinal ganglion cells stained with H&E were automatically counted based on nuclear features using the RetFM-Class algorithm. The reduction in retinal ganglion cell density measured in Tg-TBK1 mice by both methods is strikingly similar.

We previously demonstrated that TBK1 is specifically localized to the retinal ganglion cells in the human retina (27,61). In this report, we identify native mouse Tbk1 in the inner and outer nuclear layer cells as well as in the retinal ganglion cells in the retinas of wild-type and Tg-TBK1 mice. However, increased labelling of this kinase protein is recognized in the retinal ganglion cells of Tg-TBK1 mice. These data suggest that human TBK1 protein produced by the TBK1 transgene may be somewhat confined to the retinal ganglion cells in Tg-TBK1 mice, mimicking the localization of native TBK1 protein in human retina. This expression pattern suggests that the Tg-TBK1 mouse is a good model of human NTG caused by CNVs of the

Figure 5. Tg-TBK1 mice have progressive loss of retinal ganglion cells. Flat mounts of retinas from Tg-TBK1 and normal littermates were labelled with γ-synuclein and retinal ganglion cell density was determined by manually counting labelled cells. Tg-TBK1 mice have the same number of retinal ganglion cells as their wild-type littermates when measured shortly after weaning (5 weeks of age). However, the Tg-TBK1 mice subsequently develop a progressive loss of retinal ganglion cells with 5% fewer cells at 3 months than littermates that increases to 13% fewer cells by 18 months. Data from each time-point was collected as a terminal measurement from different cohorts of mice (total n = 188). Error bars indicate standard deviation.

Figure 6. Dose response between the number of TBK1 transgenes and the loss of retinal ganglion cells. An intercross of Tg-TBK1 mice (hemizygous for the TBK1 transgene) produced mice homozygous for the TBK1 transgene (2 copies); mice hemizygous for the TBK1 transgene (1 copy); and wild-type mice with no copies of the TBK1 transgene. Mice were aged to 6 months, flat mounts of their retinas were labelled with γ-synuclein, and retinal ganglion cells were counted. Measurements were normalized to the retinal ganglion cell density determined for the wild-type mouse. The number of retinal ganglion cells counted was inversely proportional to the number of TBK1 transgenes per genome. Hemizygous mice had fewer cells than wild-type littermates and homozygous mice had the fewest cells. Error bars indicate standard deviation.

Figure 7. Automated retinal ganglion cell counts of Tg-TBK1 mice and wild-type littermates using RetFM-J. A subset of retinal whole mounts from mice analysed with γ-synuclein labelling were relabelled with H&E and the density of retinal ganglion cells in these whole mounts was calculated using RetFM-J as previously reported (43). Five-week old Tg-TBK1 and wild-type mice had similar numbers of retinal ganglion cells, while 18-month old mice Tg-TBK1 mice had significantly fewer retinal ganglion cells than wildtype littermates. The loss of retinal ganglion cells in 18 month old Tg-TBK1 mice detected by RetFM-J analysis (11%) is similar to the loss detected by γ-synuclein analysis (13%) depicted in Fig. 5. Error bars indicate standard deviation.
TBK1 gene. Moreover, analysis of aging Tg-TBK1 mice shows a significant loss of retinal ganglion cells over time.

Other transgenic models of NTG have also been created to model human disease. A missense mutation (E50K) in OPTN has been strongly associated with human glaucoma. In one transgenic mouse model, overexpression of the E50K OPTN mutant protein in mice resulted in features of NTG including thinning of all retinal layers and diffuse retinal apoptosis that occurred without elevated IOP. Diffuse apoptosis was detected in all cell types of the retina, which suggests that overexpression of OPTN mutants has a range of effects on retina (62). Another transgenic mouse made with an E50K optineurin transgene also showed signs of NTG. These mice had loss of retinal ganglion cells, loss of optic nerve axons and reduced visually evoked potentials (63). Transgenic OPTN mice along with transgenic Tg-TBK1 mice are powerful tools for investigating the pathogenesis of glaucoma. Recent studies of transgenic OPTN mice have suggested that autophagy involving mitochondria (mitophagy) may have an important role in glaucoma (64).

In sum, the evidence linking TBK1 with NTG is growing. TBK1 copy number variations have been detected in NTG patients in multiple studies. Five additional studies of NTG patients (28–31) have replicated the discovery that TBK1 CNVs are associated with approximately 1 in 100 cases of NTG (27). Some preliminary studies have shown that duplication of the TBK1 gene has functional consequences including increased transcription of TBK1 mRNA (27) and activation of autophagy (37) in cell culture systems. However, in the current report, we provide the most compelling evidence that TBK1 copy number variations are pathogenic. We engineered Tg-TBK1 mice to have the same genetic defect (an extra dose of the TBK1 gene) as our human NTG patients with TBK1 gene duplications. Subsequently, we showed that Tg-TBK1 mice have a progressive loss of retinal ganglion cells that occurs without elevated IOP. Tg-TBK1 mice recapitulate human glaucoma and provide the strongest proof that CNVs involving the TBK1 gene cause glaucoma. Further study of these and other mice may provide new insights into the mechanisms by which autophagy genes lead to retinal ganglion cell damage and glaucoma. Moreover, this line of investigation has great potential for informing the development of novel therapies that are targeted to the specific causes of glaucoma.

Materials and Methods

Mouse husbandry

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental protocols were approved by the Animal Care and Use Committee of the University of Iowa. C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed and bred at the University of Iowa Research Animal Facility. Mice were maintained on a 4% fat NIH 31 diet provided ad libitum and were housed in cages containing dry bedding (Cellu-dri; Shepherd Specialty Papers, Kalamazoo, MI). The environment was kept at 21 °C with a 12-h light/12-h dark cycle.

Generation of Tg-TBK1 transgenic mice

A bacterial artificial chromosome (RP11-6008) containing the wild-type human TBK1 gene (natural promoter, exons and introns) was purchased from BACPAC (Oakland, CA) and was injected into C57BL/6J/SJL hybrid embryos in collaboration with the Transgenic Animal Facility at the University of Iowa. One resultant founder mouse was found to have a single human TBK1 transgene integrated into its genome. The founder and its offspring were backcrossed to C57BL/6J mice for seven generations by the onset of this study (>10 generations by the conclusion of the study). The integration of a single BAC (1 copy of the human TBK1 gene) was confirmed using a commercially available real-time PCR assay (TsqMan, TBK1 test probe Hs009808763, Thermo reference probe 4458370, Thermo Fisher) and a CFX96 real time PCR machine (BioRad Laboratories, Hercules, CA) following the manufacturer’s protocol. All mice were tested using this assay and those mice with the TBK1 transgene were termed ‘Tg-TBK1 mice’.

Gene expression studies

Retinas were harvested from Tg-TBK1 and age-matched wild-type mice of the same litter. Both retinas from each mouse were pooled together in a tube and flash frozen in liquid nitrogen for each animal. Total RNA was extracted using the RNaseasy Mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized using the BioRad iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative polymerase chain reaction was done using TaqMan Gene Expression probes (TBK1 test probe Hs00975477_m1, mouse Tbk1 test probe Mm00451150_m1, and Gapdh reference probe Mm9999915_g1, Thermo Fisher) following the manufacturer’s protocol. Each retinal cDNA library was amplified in triplicate in two separate experiments. Relative gene expression was calculated by the BioRad CFX Manager software. Gene expression levels were normalized using mGAPDH as the reference probe and reported as relative expression with the sample with the highest expression level set at 100%. The relative expression levels of mouse Tbk1 (quantitation cycle, Cq) were compared between Tg-TBK1 and wild-type littermate mice using a two-tailed paired t-test.

Immunohistochemistry

Mice were euthanized using CO2 asphyxiation followed by cervical dislocation. The eyes were placed in 4% paraformaldehyde for ~4 h at 4°C and then stored in PBS. The anterior segment and the lens were removed and eye cups were then infiltrated in sucrose solution and embedded in OCT compound. The eyes were then cut into 7 μm sections on a cryostat (MICROM GmbH, Walldorf, Germany). Sections were incubated in blocking solution (1:50 dilution of blocking serum from Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) for 30 min then in 1:500 anti-Tbk1 antibody (AbCam, Cambridge, MA) for 1 h. Sections were washed 3X with PBS and then incubated with biotinylated secondary antibody for 30 min (1:100 dilution from Vector Laboratories, Burlingame, CA). Following washes with PBS (3 × 5 min), sections were incubated in Vectastain ABC reagents (1:50 dilution reagent A and 1:50 dilution reagent B from Vectastain ABC Kit) for 30 min and then washed with PBS (3 × 5 min). Sections were then treated with avidin biotin horseradish peroxidase substrate (Vector VIP substrate kit, Vector Laboratories). Images were obtained using an Olympus BX41 microscope with a 20X objective. Sections of retinae from transgenic and wild-type littermate mice were developed in parallel under identical conditions, identical microscope settings and identical camera settings.
Measurement of IOP
IOP was measured using a rebound tonometer as previously described (65). Mice were acclimated to the procedure room, anaesthetized with 2.5% isoflurane+100% oxygen, and IOP was measured with a TonoLab tonometer (Colonial Medical Supply, Franconia, NH). All IOP cohorts included male and female mice.

Retinal ganglion cell counts
Retinal whole mounts of transgenic mice and littermates were prepared and retinal ganglion cells counted as previously described (66). Whole-mount preparations of retinas were incubated with 0.3% Triton X-100 for 4 h and were blocked with 0.1% filtered BSA solution with 0.1% Triton X-100 for 1 h. These retinas were then incubated with 1:300 anti-α-synuclein antibody (AbCam, Cambridge, MA) overnight, followed by incubation with secondary antibody Alexa Fluor 488 (Life Technologies, Madison, WI) for 30 min. For counting, four images of the mid-peripheral region were captured (one from each quadrant) at 100X magnification using an Olympus BX41 microscope. α-Synuclein-positive cells were counted in a 10,355 μm² area of each image using ImageJ software (NIH). Technicians were masked to the genotypes of the mice. Cell counts were compared between Tg-TBK1 mice and matched normal littersmates for each time point using a two-tailed t-test. Differences with P < 0.05 were considered significant.

Retinal whole mounts from some of the mice that were studied with immunohistochemical labelling were also reanalysed using a semi-automated image analysis software package, RetFM-Class, that classifies cells of the retinal ganglion cell layer as previously described (47,48). Briefly, whole mounts were washed in PBS and labelled with haematoxylin and eosin. Images of the retinal ganglion cell layer were obtained with a light microscope (BX52; Olympus, Tokyo, Japan) using a digital camera (DP72; Olympus, Tokyo, Japan). CellSens Standard imaging software (Olympus, Tokyo, Japan) was used for capturing colour images (RGB mode,.tif format), using fixed software settings for all images. Exposures were normalized using white balance and 1% spot option of the region tool before capturing images. The CellSens Extended Focal Imaging module was used as needed to correct for unevenness in the z-plane due to variable topography of whole-mounted retinas. Images were captured from each retinal whole mount in fixed topographic locations. Two images were captured from three regions (peripheral, mid-peripheral and central) of each of four retinal leaflets, for a total of 24 images per retina. Raw images from each retina were manually reviewed and regions containing artefacts (such as stain precipitate, tears and debris) were manually excluded from each image, and nuclei counts of each image obtained using RetFM-J. A minimum of two retinal leaflets per whole mounted retina were required for inclusion in the analysis. Many retinas that were successfully analysed with the α-synuclein were excluded from the RetFM-Class approach because they did not survive the re-mounting and re-staining process. Retinal areas were measured for each retina as previously described (47) and retinal ganglion cell densities were computed for each whole mount as mathematical averages of all images analysed per experiment (Fig. 7). Raw data from retinal ganglion cell layer analyses are plotted for each animal (Supplementary Material, Fig. S3).

Supplementary Material
Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

Funding
This work was supported by the National Institutes of Health [R01 EY023512]; American Glaucoma Society [Mid-Career Award]; the Glaucoma Research Foundation [Frank Stein and Paul S. May Grant for Innovative Glaucoma Research]; Research to Prevent Blindness [Physician-Scientist Award]; and Casey Mahon.

References


