# Loss of Tubedown Expression as a Contributing Factor in the Development of Age-Related Retinopathy

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**Purpose.** Tubedown (Tbdn), a cortactin-binding acetyltransferase subunit, regulates retinal vascular permeability and homeostasis in adulthood. Here the authors explore whether Tbdn loss during aging might contribute to the mechanisms underlying age-related neovascular retinopathy.

**Methods.** A conditional endothelial-specific transgenic model of Tbdn loss was compared with aged mouse and human specimens from 5- to 93-year-old individuals. Specimens were analyzed by morphometric measurements and for functional markers using immunohistochemistry and Western blot analysis.

**Results.** An age-dependent decrease in Tbdn expression in endothelial cells of the posterior pole of the eye correlated with pathologic changes in choroidal and retinal tissues of aged mice. In humans, aged specimens without eye disease exhibited a moderate decrease in retinal and choroidal endothelial Tbdn expression compared with younger persons, whereas a greater decrease in choroid vascular Tbdn expression was observed in patients with age-related macular degeneration. In mice, Tbdn loss resulting from old age or conditional Tbdn knockdown was associated with retinal lesions showing significant extravascularly localized albumin and correlated with increased activity of senescence-associated β-galactosidase in the retinal pigment epithelium. A range of abnormalities in RPE, Bruch's membrane, and choriocapillaris observable at the ultrastructural level in Tbdn-knockdown eyes recapitulate those present in human AMD.

**Conclusions.** This work provides evidence that the marked decrease in the level of expression of Tbdn in the retinal and choroidal vasculature during aging contributes to the multifactorial process that leads to the development of age-related retinopathy and choroidopathy. (*Invest Ophthalmol Vis Sci.* 2010;51:5267–5277) DOI:10.1167/iovs.09-4527

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During aging, vision loss greatly affects quality of life and functional autonomy. Age-related macular degeneration (AMD) is one of the leading causes of blindness in people older than 60.[1,2] The prevalence of AMD increases dramatically with age, one important risk factor. Because of aging demographics, by the year 2020, the number of people affected with blindness or low vision is projected to increase substantially.[3] AMD is a degenerative disease (see Refs. 1,4,5 for reviews) initiated by thickening of the Bruch's membrane (BM), which serves as the extracellular matrix layer separating the choroid from the retinal layers. Degeneration is complex but could involve the accumulation of deposits, changes in the retinal pigment epithelium (RPE) and retinal pigment epithelial cell loss leading to hypopigmented areas, and the development of hyperpigmented areas. This stage can progress to a proliferative neovascular (wet or exudative) form characterized by the growth of choroidal vessels (choroidal neovascularization) or to a geographic atrophy form characterized by atrophy of the RPE and specific geographic areas of the neural retina. In contrast to the geographic atrophy form, the neovascular form progresses rapidly. Proliferative choroidal blood vessels may break through the BM and enter the subretinal space and photoreceptor layers. Leakage of blood, plasma, and lipid stimulates fibroglial reorganization and promotes scarring and detachment of the RPE or retina. Neovascular AMD is associated with severe vision loss, but the mechanisms that cause the progression of AMD to neovascular AMD are still incompletely understood.[1,4,6]

During AMD the normal aging process itself poses potential risk and causative factors in the severity of age-related retinal disease. The aging process predisposes the eye to the development of physical changes that could play a role in promoting retinal and choroidal neovascularization.[1,4] Elucidating how the normal aging process affects the cellular and molecular mechanisms of proliferative retinal disease and designing therapies to prevent age-related molecular abnormalities of the retinal and choroidal vasculature may offer a preemptive means to replace current treatments and to prevent the vision loss associated with retinal disease. The molecular events leading to retinal neovascularization in diseases such as neovascular AMD are thought to involve increased expression of proangiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and insulin-like growth factor.[7] Changes in integrins and extracellular matrix component expression and glial cells have also been implicated in neovascular AMD.[1,7] VEGF has been recognized as a


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critical component of the pathologic neovascularization that develops during blinding eye diseases and mediates this process through the regulation of endothelial cell proliferation and permeability.[8] However, a range of factors is likely involved in the microenvironment, promoting retinal and choroidal blood vessel pathobiology. Therefore, the targeting of a single factor for antiangiogenic therapy may not be completely effective. The characterization of the common regulators acting in diverse proangiogenic pathways will be key to identifying targets that could have a more global effect on controlling retinal and choroidal vasculopathy.

Work from our laboratories has defined Tubedown (Tbdn, also now referred to as Narg1, mNat1, NATH) as a novel homeostatic factor necessary for the maintenance of a healthy retina by preventing blood vessel overgrowth and leakiness.[9–12] Tbdn is a subunit of the NatA acetyltransferase through its association with the catalytic subunit Ard1.[13,14] Tbdn expression during embryogenesis is regulated in developing blood vessels and neuronal tissues; in adults, high levels become restricted to the endothelial cells of only a few vascular beds, including the eye and the ovary.[9,10,13,15–17] Both yeast and mammalian NatA complexes associate with ribosomes and have been involved in the regulation of a wide range of processes from cell growth to cell differentiation.[13–25] Previous studies have shown that NatA mediates cotranslational acetylation of nascent polypeptides at specific residues in the second position of the N terminus on cleavage of the initial methionine by methionine aminopeptidases.[19,21,26] With N-terminal acetylation one of the most abundant protein modifications, a wide variety of potential substrates for NatA have been reported.[20–22,26,27] There is also evidence that Ard1 can acetylate lysine residues.[23,25] Homologues for Tbdn (mNat2, 70% identity) and Ard1 (Ard2, 81% identity) have also been described in mammalian cells.[13,28] More recently, we reported that Tbdn also localized at the cell cortex and along the actin cytoskeleton while forming a complex with the actin-binding protein cortactin.[12] Tbdn was found to play a role in regulating endothelial cell permeability, possibly through the regulation of endocytosis.[12,24] In adults, several lines of evidence support a critical role for Tbdn in the ocular blood vessels to maintain the homeostasis of the retina. The knockdown of Tbdn in retinal endothelial cells in vitro has been associated with increased angiogenesis and increased permeability to albumin.[12,15] Normal expression of Tbdn in retinal endothelial cells is specifically suppressed in the blood vessels of retinal lesions in patients with proliferative diabetic retinopathy (PDR) and retinopathy of prematurity and in some animal models of neovascular retinopathy.[10,15,29] Conditional endothelial knockdown of Tbdn in a bitransgenic mouse model (*TIE2/rtTA/Enh-TRE/ASTBDN*) results in neovascularization, specifically in the retina and the choroid.[11] The retinal pathology in this mouse model increases in severity with prolonged knockdown of Tbdn expression in endothelium, with thickening of the retinal and choroid tissues and the formation of a preretinal membrane harboring an expanding mass of fibrovascular elements reminiscent of retinal thickening secondary to the formation of neovascular membranes occurring in macular edema in humans during PDR.[11,30]

The pathology in the choroid layers in endothelial Tbdn knockdown mice[11] has suggested that the Tbdn knockdown may be useful for modeling the choroidal neovascularization in human AMD. Given that neovascular AMD is an age-related disease, the purpose of the present study was to determine whether the expression of Tbdn in retinal and choroidal vasculature is affected by normal aging and, if so, whether the age-related loss of Tbdn expression is a contributing factor in the pathologic changes occurring in patients with age-related retinal and choroidal neovascularization.

## Methods

### Mouse Specimens

Wild-type or control single transgenic mice (*TIE2/rtTA/Enh* mice or *TRE/ASTBDN* mice)[11] were aged for periods of 2 to 20 months to assess choroidal and retinal Tbdn expression, albumin localization, RPE senescence–associated β-galactosidase activity, choroidal and retinal histology, and ultrastructure. Choroidal and retinal endothelial Tbdn expression was knocked down in *TIE2/rtTA/Enh-TRE/ASTBDN* bitransgenic[11] middle-aged (7 months) and aged (16 months) mice for a period of 6 or 10 weeks, as indicated. Conditional knockdown of Tbdn was facilitated by feeding the mice with commercially prepared mouse chow containing doxycycline (Dox) at 600 mg/kg (Bio-Serv, Frenchtown, NJ). Either control mice (middle-aged) were not treated with Dox, or wild-type and control single transgenic mice (*TIE2/rtTA/Enh* mice or *TRE/ASTBDN* mice) were fed with or without Dox for the same length of time. Mice were killed at appropriate time points and analyzed grossly and histologically by morphometric and immunohistochemical analysis of ocular tissues and blood vessels, as previously described,[11] to determine the extent and progression of the ocular pathology. For each eye specimen, sections were prepared, registered, and stored in serial or adjacent order. Every 20th paraffin section was stained with hematoxylin and eosin (H&E) to verify normal morphology in the controls and to map the retinal pathology in the experimental or aged mice. The care and use of animals in this study followed the guidelines set forward by the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of Memorial University.

### Human Eye Specimens

Expression of choroidal and retinal Tbdn was also evaluated by immunohistochemistry in normal specimens of young (≤12 years), adult (29–44 years), and aged (>72 years) persons. Human eye specimens were obtained from The National Disease Research Interchange (Philadelphia, PA) or the Georgia Eye Bank (Atlanta, GA). All research on human specimens followed the tenets of the Declaration of Helsinki and was performed under approval from the Human Investigation Committee of Memorial University. The young (group 1) specimens were from a 5-year-old boy and a 12-year-old girl who died in motor vehicle accidents. The adult (group 2) specimens were from 30- and 44-year-old men, both of whom died of cardiac arrest, and a 29-year-old man who died in a motor vehicle accident. The elderly (group 3) specimens were from a 73-year-old woman and an 81-year-old man who died of cardiac arrest, a 93-year-old man who died of myocardial infarction, and 91-year-old and 77-year-old women who died of respiratory failure. There were no pathologic abnormalities in the normal eye specimens. The eyes were grossly examined, processed routinely through increasing grades of alcohol, and embedded in paraffin. Sections of paraffin-embedded AMD specimens were from an 87-year-old woman with dry AMD who died of multiple organ failure and one additional human specimen displaying neovascular AMD. Immunocytochemistry was performed on the paraformaldehyde-fixed, paraffin-embedded sections of human eye tissues to detect Tbdn expression.

### Immunohistochemistry

Tbdn expression was analyzed by immunohistochemistry on paraffin-embedded sections of eye specimens using OE5 mouse monoclonal anti–Tbdn antibody.[17] For immunostaining, sections from paraffin-embedded tissues were deparaffinized, postfixed in 4% paraformaldehyde, and incubated overnight with primary antibody or negative control hybridoma supernatant supplemented with negative control isotype-matched IgG2a antibody (DakoCytomation, Glostrup, Den-

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mark) in 3% fat-free skim powdered milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST). Sections were developed using appropriate alkaline phosphatase-conjugated secondary antibodies (anti–mouse IgG or anti–mouse IgG2a) and substrate kit (Vector Red; Vector Laboratories, Burlingame, CA).

Paraffin sections from eyes from Dox-fed, endothelial-specific Tbdn knockdown mice, aged mice, and control mice killed at appropriate time points were analyzed by albumin immunohistochemistry to assess retinal albumin expression/localization. After deparaffinization, sections were treated for melanin bleaching by incubation with 0.25% $KMnO_4$ followed by 1% oxalic acid, as described.[12] Endogenous peroxidases were blocked in 0.3% $H_2O_2$, and sections were postfixed. Sections were stained with goat anti–albumin horseradish peroxidase (HRP)-conjugated antibody (GeneTex, San Antonio, TX) in 3% milk/TBST, as described.[12] Control, aged, and Tbdn knockdown sections were also stained with HRP-conjugated goat anti–rabbit IgG at the same concentration as the anti–albumin antibody as a negative control for the albumin staining. Adjacent sections were stained with H&E to enable assessment of tissue integrity and pathology.

## Western Blot Analysis

Mouse retinas were isolated from surrounding sclera, vitreous, and other ocular tissues. Western blot analysis was performed by standard procedures, as previously described,[12] using chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ) for measuring Tbdn with purified rabbit anti–Tbdn C755–766 antibody. Western blots were stripped and reprobed with anti–α-tubulin monoclonal antibody (DM1A; Sigma, St. Louis, MO) to determine loading equivalency. Densitometry analyses were conducted (Gel Logic Imaging System; Eastman Kodak, Rochester, NY), and intensities of the expressed bands were analyzed using molecular imaging software (version 4.0; Kodak).

## Choroid and Subretinal Ultrastructure

Transmission electron microscopy (TEM) was performed as previously described.[31] Briefly, eyes from both control mice (wild-type, *TIE2/rtTA/Enb* or *TRE/ASTBDN* mice) and *TIE2/rtTA/Enb-TRE/ASTBDN* bitransgenic mice, as described and of varying ages, were fixed with Karnovsky's fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.06 M sodium cacodylate buffer pH 7.4) and then postfixed with 1.0% osmium tetroxide. Next specimens were dehydrated and embedded in Epon 812 mixture. Semithin sections (0.5 μm) were prepared and stained with toluidine blue for preinspection by light microscopy. Ultrathin sections of areas of interest were generated and stained with electron-dense uranyl acetate and lead citrate, and choroid and retinal tissues were observed and photographed under TEM.

## Senescence-Associated β-Galactosidase Activity

Frozen sections were prepared from central retinal regions using a cryostat (CM1900; Leica, Bannockburn, IL) and were stained for senescence-associated β-galactosidase activity. Sections from 6-week Dox-fed, endothelial-specific Tbdn knockdown mice (middle aged), control mice (middle aged), and aged mice were fixed in 4% paraformaldehyde for 30 minutes and washed in phosphate-buffered saline (PBS). Sections were next incubated for 7 hours in the dark with senescence-associated β-galactosidase activity stain solution (1 mg/mL 5-bromo-4-chloro-3-indolyl P3-$^{D}$-galactoside [X-gal]; 40 mM citric acid/sodium phosphate [pH 6.0], freshly prepared 5 mM potassium ferrocyanide, freshly prepared 5 mM potassium ferricyanide 150 mM NaCl, and 2 mM $MgCl_2$).[32] After incubation, sections were washed with PBS and mounted in aqueous mounting medium (Gel/Mount; Sigma). Sections were photographed the following day for quantitative analysis of the staining intensity.

## Data and Statistical Analyses

Histochemical staining for Tbdn, albumin and senescence associated β-galactosidase activity were quantified by measuring staining intensities essentially, as previously described.[17,29] All sections were viewed and photographed using a microscope system (DMIRE2; Leica, Bannockburn, IL) equipped with a camera (RETIGA Exi; QImaging, Surrey BC, Canada) and software (Openlab, version 5; Improvision, Coventry, UK) for quantitation of the staining. Tbdn levels in retinal and choroidal blood vessels were expressed as the average staining levels of at least six separate mouse eye specimens or four separate human eye specimens (with the exception of AMD specimens, for which two specimens were available) from each category. Intensity of Tbdn staining in blood vessels and background staining (from neural retinal areas) was measured by determining red color intensity using a software tool (HIS Colorspy; Openlab). Background intensity measurements were subtracted from specific staining measurements. Relative intensities were expressed as the average staining levels ± SEM.

For the quantitation of extravascularly localized albumin in the neural retina, albumin staining intensity in the inner retinal ganglion cell layer (which was elevated in Dox-fed Tbdn-knockdown and aged mice) and background staining (from the outer neural retina) were measured by determining brown color intensity (HIS Colorspy; Openlab). Background intensity measurements were subtracted from specific staining measurements. Relative intensities were expressed as the average staining levels of at least five separate mouse eye specimens from each group ± SEM.

Senescence-associated β-galactosidase activity staining in RPE areas of retinal tissues and background staining (from inner neural retinal layer areas) was measured by determining blue color intensity (HIS Colorspy tool; Openlab). Activity levels were expressed as the average staining of at least three individual mouse eye specimens from each group tested in at least two separate experiments. Background intensity measurements were subtracted from specific staining measurements. Relative staining intensity units were compared within micrographs of equivalent dimensions taken at equivalent magnifications and microscope and camera parameters. Values were expressed as the mean ± SEM percentage of the control.

Morphometric analysis of the retinal pathologic lesions in H&E-stained paraffin sections of mouse eyes was measured using Openlab software area outline tool. Retinal area measurements in all specimens were acquired within an invariable reference width encompassing the central retina of micrographs of equivalent magnification (areas with optic nerve were excluded). Relative retinal areas were expressed in arbitrary units and as the mean ± SEM percentage of the control.

Thicknesses of the RPE and the choroid in toluidine blue–stained semithin Epon sections of mouse eyes were measured using the Openlab software line measurement tool. Multiple measurements within the central retina (areas with optic nerve were excluded) of at least three separate specimens from each group were taken. Values were expressed as the mean ± SEM percentage of the control.

All quantitative analyses were compared using two-tailed Student's *t*-tests (Excel; Microsoft, Mississauga, ON, Canada). Data were considered statistically significant if $P \leq 0.05$.

# Results

## Choroidal and Retinal Endothelial Tbdn Expression during Aging and during AMD

We investigated whether normal aging influences the specific choroidal and retinal endothelial Tbdn expression in human and mouse eyes.[10] Tbdn expression was analyzed by immunohistochemistry of mouse retinal tissues using an

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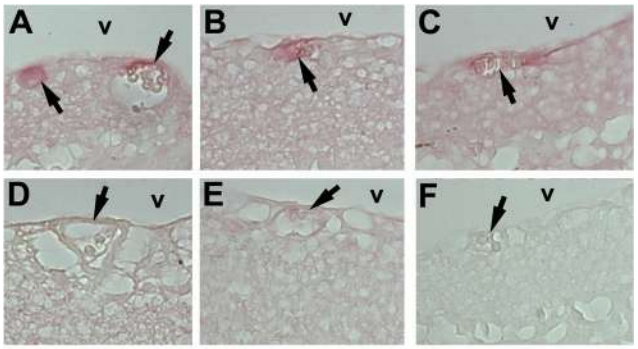


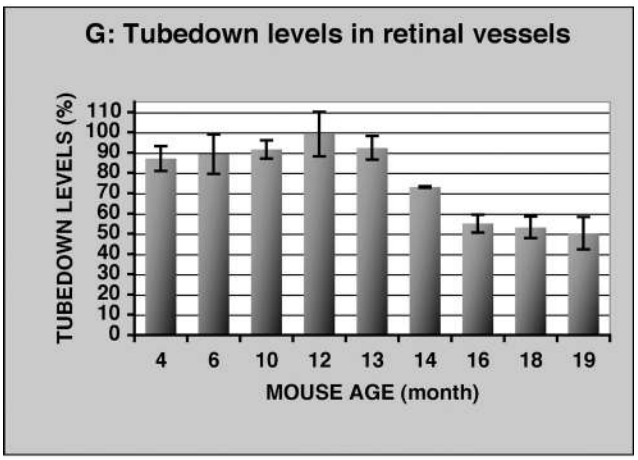


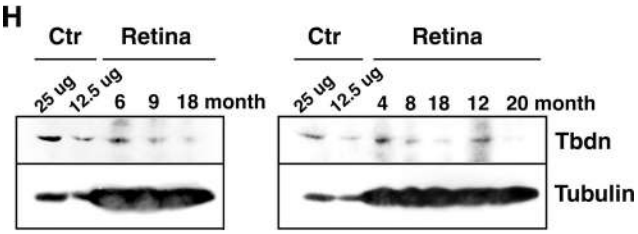


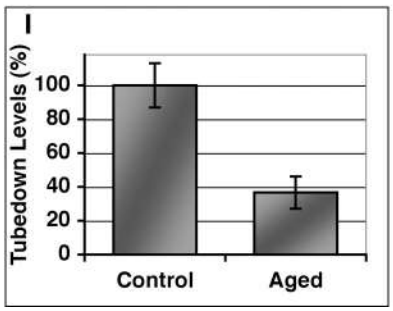


**FIGURE 1.** Suppression of Tbdn in retinal blood vessels of aged mice. Retinal blood vessels from young (**A**, 6 months old) and middle-aged (**B**, **C**, 12 months old) adult mice stained with anti–Tbdn mouse monoclonal antibody OE5 show intense endothelial staining for Tbdn expression (*red* staining reaction [*arrows*] blood vessel endothelial cells). In aged mice (**D**, 17.5 months old; **E**, 18 months old), blood vessel endothelial cells (*arrows*) show very low Tbdn expression. Sections from 12-month-old mouse (serial to section, **C**) incubated with an isotype-matched negative control antibody showed no staining of retinal blood vessels (**F**). Note that high Tbdn staining is restricted to retinal blood vessels. Sections were not counterstained to optimize clarity of Tbdn staining. (**A**–**F**) Original magnification, 400×All panels show close-up views of the inner retinal layer adjacent to the vitreous (v).(**G**) Average Tbdn levels in retinal blood vessels were quantitated by measuring staining intensities after immunohistochemical analyses of Tbdn protein expression for which a representative experiment is shown in **A** to **F**. Results are expressed as the mean ± SEM percentage of a 12-month-old reference specimen. (**H**) Representative Tbdn Western blot analyses (*top*) of retinal proteins (215 μg; retina) from young (4–6 months), middle-aged (8–12 months), and aged (18–20 months) mice, as indicated relative to control protein extracts from mouse IEM embryonic endothelial cells (Ctr; amounts indicated). Blots were reprobed and analyzed for α-tubulin expression as loading control (*bottom*). (**I**) Quantitative analysis of Tbdn Western blot analyses of retinal proteins from control (4–12 months) or aged (16–20 months) mice for which representative analyses are shown in **H**.

anti–Tbdn monoclonal antibody,[17] quantitation of immunohistochemical Tbdn staining in retinal endothelial cells, and Western blot analysis of mouse retinal protein extracts (Fig. 1). Immunohistologic analyses revealed that Tbdn protein expression levels in mouse retinal vessels are highest in young adults and middle-aged adults from 4 to 13 months. From 14 months to 16 months of age, Tbdn levels in mice gradually decrease to approximately 50% of levels observed in middle-aged mice. From 16 months of age (vs. 4–13 months; $P = 0.014$), Tbdn expression is maintained at this low level until at least 19 months of age (vs. 4–13 months; $P = 0.006$) (Fig. 1G). Western blot analysis confirmed immunohistochemical results of Tbdn downregulation during old age in the mouse retina (Figs. 1H, 1I). Western blot analysis revealed that the average levels of Tbdn in aged mouse retina (16–20 months) was only 37% of the average levels found in younger specimens (6–12 months) ($P = 0.014$) (Fig. 1I). Measurements of Tbdn expression in humans were also performed using histologic sections of eye specimens from a range of ages (5–93 years). Both retinal and choroidal blood vessels showed a significant decrease in levels of Tbdn expression as a function of age (Fig. 2). No significant differences ($P > 0.11$) were observed in human Tbdn levels between young (5–12 years) and early adult (29–44 years) normal specimens (Fig. 2F). Normal eye specimens from aged persons (older than 72 years of age) showed lower expression levels of Tbdn (approximately 50%) in both the retina and the choroid compared with younger persons ($P < 0.002$) (Figs. 2A–F). Moreover, the levels of expression of endothelial Tbdn in the choroidal blood vessels of AMD specimens (including both wet and dry AMD) were found to be sixfold decreased (17%; $P < 0.0002$) compared with the average levels in normal younger specimens (Fig. 2F), which represents an additional threefold decreased ($P < 0.001$) over the normal aged human specimens (older than 72 years of age).

## Age-Dependent Retinal Pathology Associated with Tbdn Suppression

Given that endothelial Tbdn expression is suppressed in retinal tissues during AMD (Fig. 2), PDR,[10] and ROP[29] while endothelial Tbdn knockdown in mouse transgenics leads to fibrovascular growth, leaky retinal vessels, and thickening of the retinal tissues,[11,12] we investigated whether the suppression of Tbdn during aging in mice (Fig. 1) is also associated with retinal or choroidal pathology. To compare the effects of transgenically induced endothelial Tbdn suppression on choroid and retinal integrity with those observed during natural aging, Tbdn expression was knocked down in young to middle-aged *TIE2/rtTA/Enh-TRE/ASTBDN* bitransgenic adult mice by dietary supplementation with Dox for 6 to 10 weeks (Fig. 3).[11] As previously reported,[11,12] 6 to 12 weeks of endothelial Tbdn-knockdown in young to middle-aged (5–10 months) adult mice led to significant retinal pathology

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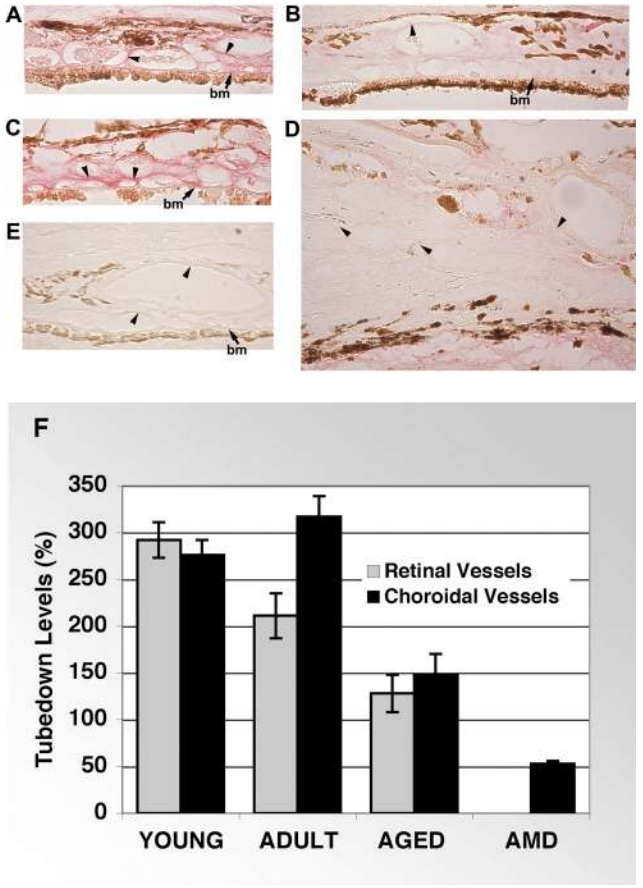


**FIGURE 2.** Tbdn loss in blood vessels in aged human persons and in patients with AMD. (**A**–**E**) Representative immunohistochemical analysis of Tbdn protein expression in human eye specimens using anti-Tbdn mouse monoclonal antibody OE5. The *bright red staining* (*arrowheads*) in the control adult specimens (**A**, **C**) indicates Tbdn expression in choroidal blood vessels. The normal melanin pigment granules present in the RPE cells and in choroidal melanocytes are *dark brown dots* seen in all panels. In the aged specimen (**B**, 91 years old), the choroid shows vascular dilatation and a decrease in levels of endothelial Tbdn expression in these regions (*arrowheads*, blood vessel endothelium). The neovascular AMD specimen (**D**) shows fibrovascular proliferation, and endothelial Tbdn expression is very low or undetectable (*arrowheads*, blood vessel endothelium). (We reported previously that Tbdn loss in blood vessels in neovascular retinopathy is not simply due to loss of endothelial cells.[13]) Sections were also incubated with negative control IgG2a (**E**) and showed no staining of blood vessels. BM is indicated by *arrows* in all panels except the AMD in **D** because BM is indiscernible amid the pathologic disorganization in this specimen. Not counterstained to emphasize Tbdn staining. The photoreceptor layer, which would be situated under BM at the bottom of all panels, has detached during processing in some of the specimens. Pictures were taken in the central retinal tissues. (**A**–**E**) Original magnification, 400×. (**F**) Average Tbdn levels in retinal and choroidal blood vessels of specimens from young (≤12 years old), adult (29–44 years old), and aged (>72 years old) healthy persons and those with AMD were quantified by measuring staining intensities after immunohistochemical analyses of Tbdn protein expression for which a representative experiment is shown in **A** to **E**. Results are expressed as the mean ± SEM percentage of an aged reference specimen.

characterized by abnormal retinal fibrovascular growth lesions and thickening of the retinal tissues (Figs. 3, 4). Ninety-one percent (10 of 11) of aged mice (16–19 months old) had retinal lesions. Among the aged specimens, only one was not suppressed for retinal endothelial Tbdn expression and did not have a retinal abnormality. No retinal lesions were detected in the young and middle-aged (5–10 months old) control mouse specimens with normal retinal endothelial Tbdn expression. Aged Tbdn knockdown specimens (17.5–19 months) had retinal abnormalities similar to those of middle-aged Tbdn knockdown mice (Fig. 3). Morphometric measurements of the retinal area sampled within a reference width revealed that aged specimens showed thickening of the retinal tissues similar to that of Tbdn knockdowns. Figure 3B shows an inverse correlation between retinal area measurements and retinal endothelial Tbdn expression for all specimens and the averages of the different groups of specimens. Retinal areas for all three Tbdn-suppressed groups were approximately 2.3-fold higher than for controls ($P < 0.0003$).

To further compare the retinal lesions of aged mice with those of Tbdn-knockdown mice, immunostaining for plasma albumin was next examined as a measure of the integrity of retinal blood vessels and adjacent tissues (Fig. 4). Selective permeability of retinal endothelial cells usually limits plasma albumin to intravascular areas (blood vessel lumens) in normal disease-free retinas.[33,34] As shown in control mouse eye specimens, albumin immunostaining was restricted to the retinal blood vessel lumens (Fig. 4A). In contrast, albumin immunostaining of retinal lesions of both endothelial Tbdn knockdown[11,12] and aged (17.5 months and older) mouse

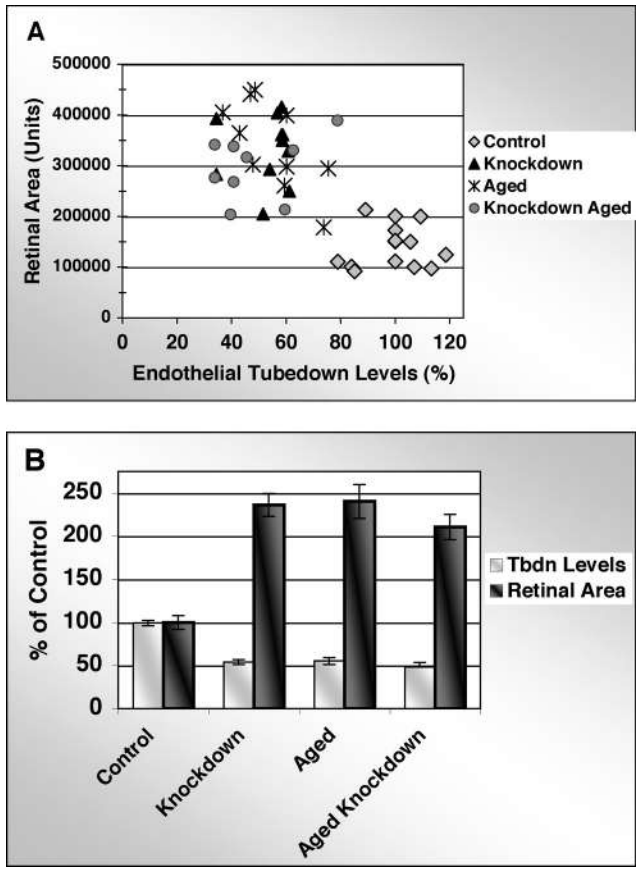


**FIGURE 3.** Retinal tissue pathology correlates with Tbdn expression loss in aged, Tbdn knockdown, and aged Tbdn knockdown mice. Retinal tissue area (cross-sectional area encompassed within a reference width of the central retina expressed as relative units) was inversely proportional to Tbdn levels in control, Tbdn-knockdown, aged, and aged Tbdn knockdown eyes. (**A**) Values from individual specimens. (**B**) Mean ± SEM values expressed in percentage of control.

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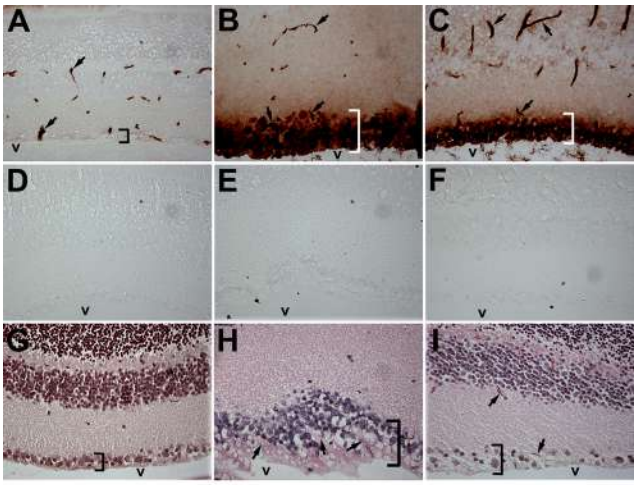


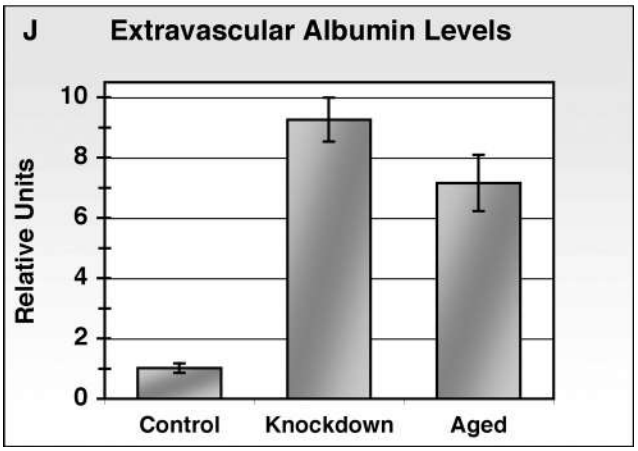


**FIGURE 4.** Retinal pathologic changes and extravascular localization of albumin in endothelial-specific Tbdn knockdown and aged mice. (**A–I**) Retinal tissue immunostaining for albumin (**A–C**, *brown stain*), negative control (**D–F**), or with H&E (**G–I**). Compared with control (middle age; **A**, **G**), endothelial-specific Tbdn-knockdown (middle age; **B**, **H**) and aged (**C**, **I**) mouse eyes showed extravascularly localized albumin (*brown stain*) in the neural retina, thickening of the retina, and abundant abnormal blood vessels. Brown albumin staining is confined mainly to blood vessel lumens in control retinas (**A**), whereas brown albumin staining is observed in extravascular locations in the neural retinal tissues in Tbdn-knockdown eyes (**B**) and aged eyes (**C**). Moreover, the area showing the highest levels of extravascularly localized albumin staining in Tbdn knockdown and aged specimens correlates with the larger capillary and vascular bed present in the ganglion cell layer of these specimens (bracketed areas in **B**, **H** and **C**, **I**). Control (**D**), Tbdn knockdown (**E**), and aged (**F**) eye sections stained with negative control IgG showed no staining. H&E staining in Tbdn knockdown and aged specimens did not reveal the presence of ruptured blood vessels (as evidenced by red blood cells in areas of the neural retina in which extravascularly localized albumin is detected). *Arrowheads*: blood vessels. *Brackets*: inner retinal layers (inner limiting membrane and ganglion cell layer). (**A**) to (**F**) are not counterstained to emphasize brown albumin staining in (**B**) and (**C**) and lack of staining in (**E**) and (**F**). (**A–I**) Original magnifications, 400×. (**J**) Quantitation of extravascularly localized albumin immunostaining in Tbdn knockdown and aged mouse eyes. Average levels of extravascularly localized albumin in the neural retinal tissues of control, Tbdn knockdown, and aged mouse specimens were quantified by measuring staining intensities after immunohistochemical analyses of albumin for which representative data are shown in (**A**) to (**I**). Results are expressed as mean ± SEM relative units in percentage.

eye specimens with suppressed retinal endothelial Tbdn expression revealed albumin immunoreactivity in extravascular locations of the neural retina (Figs. 4B, 4C). The extravascularly localized albumin in Tbdn knockdown and aged specimens was more prominent around inner retinal blood vessels situated within the ganglion cell layer, which we previously showed to contain fibrovascular abnormalities (Figs. 4B, 4C).[11] H&E analysis of the retinal pathology in Tbdn knockdown and aged specimens (Figs. 4H, 4I) did not display the presence of ruptured blood vessels (these can be revealed by red blood cells spilled into areas of the neural retina in which extravascularly localized albumin is present). Levels of extravascularly localized albumin were quantitated using digital colorimetric analysis of the albumin immunohistochemical staining signals. As shown in Figure 4J, both Tbdn knockdown and aged specimens showed significantly increased levels of extravascularly localized albumin immunoreactivity in the neural retina compared with control mice ($P < 6 \times 10^{-7}$).

## Age-Dependent Subretinal and Choroidal Abnormalities Associated with Tbdn Suppression

Analyses of the posterior pole of the eye using light microscopy and TEM and quantitative morphometric measurements were then performed on semithin toluidine blue–stained sections and on thin sections (Figs. 5, 6). Both endothelial Tbdn knockdown mouse eyes and aged (17.5–18 months old) mouse eyes had significantly thicker choroidal layers than did control mouse eyes ($P < 1 \times 10^{-6}$) (Fig. 5E).

Qualitative assessment of TEM thin sections of posterior poles of control mice revealed normal structures of the RPE, BM, and choroid (Fig. 6). In these control specimens, the RPE showed numerous infoldings of the cell membrane along the basal borders of the cells and was separated from its underlying basement membrane (inner layer of BM) by a small distance. The cells contained nuclei, numerous melanosomes, melanin granules, Golgi complexes, lysosomes, rough and smooth endoplasmic reticulum, and mitochondria.[35,36] BM appeared similar to that observed in healthy humans[37,38] with the typical pentalaminar arrangement: choriocapillaris basal lamina, outer collagenous zone, middle elastic layer, inner collagenous zone, and inner retinal pigment epithelial basal lamina (Fig. 6A). As in normal specimens, the inner and outer collagenous zones presented an interlacing pattern and communicated with each other passing through the middle elastic layer. The choriocapillaris consisted of fenestrated endothelial cells enveloped by BM and presenting a single-layer cell membrane interfacing with the BM. Red blood cells were observed in the lumens of the choroidal capillaries. Melanocytic cells were also observed in the stroma. With the exception of some control mice showing limited irregularities of the basal infoldings of the RPE, in general control specimens (including those that were Dox induced) showed normal RPE, BM, and choroidal morphology, and no abnormal deposits were observed in any of these tissues. In contrast, TEM of the RPE-BM-choroid areas of Tbdn knockdown mice (Figs. 6B–E) revealed significant ultrastructural changes, including abnormal deposits of elastic and collagenous material in and around BM, complete loss of continuity of the elastic and collagen layers of BM, gaps in the membrane, and disorganization of the normally ordered nature of the five distinct pentalaminar layers of BM. Tbdn knockdown animals also appeared to have thicker BM. In some areas BM appeared thicker, whereas in other areas BM was disrupted and thinner. Tbdn knockdown eyes also showed invasion of the interior matrix layers of BM

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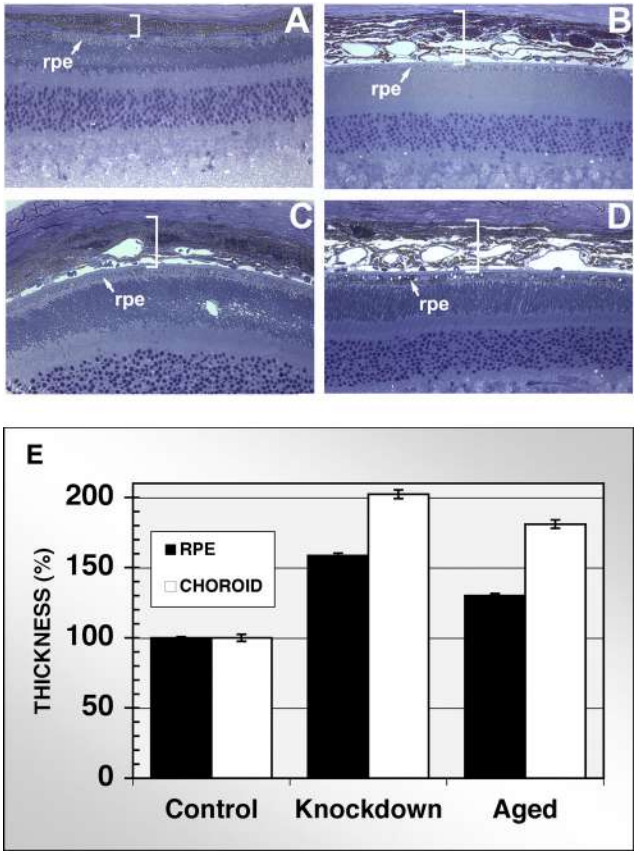


**FIGURE 5.** Morphologic changes in choroid-retinal tissues of endothelial-specific Tbdn knockdown and aged mice. (**A–D**) Compared with controls (**A**, middle age), Tbdn-knockdown mouse eyes (**B**, **C**; middle age) showed increases in RPE thickness (rpe, *arrows*), choroid (*bracketed areas*), choroidal neovascularization, deformation, and vasculopathy. An aged (18-month-old) mouse eye also shows choroidal thickening (**D**). Representative images are shown. Pictures were taken in the central retinal tissues. (**A–D**) Toluidine blue. Original magnification, 400×. (**E**) Increased thickness of the RPE and choroid of endothelial-specific Tbdn knockdown and aged mouse eyes. Tissue thickness of RPE and choroid of control mice (young adult and middle age), age-matched Tbdn knockdown mice (Knockdown), and aged mice was measured and quantitated by morphometric analysis of the central retinal tissues in histologic sections. Tbdn knockdown mice and aged mice showed similar patterns of increases in tissue thickness compared with controls. Results are expressed as the mean ± SEM percentage of the controls.

by cells, some of which were pigmented as evidenced by the presence of numerous cytoplasmic melanosomes (see Fig. 6B). In some cases, melanocytes, melanosomes, and cellular debris were present in the middle of BM (see Figs. 6B–E). These BM-invading materials displaced BM sufficiently to cause significant focal thickening of the membrane in and around the areas of invasion. TEM analyses of the choriocapillaries of Tbdn knockdown mice revealed multiple layers of endothelial cells per vessel, as evidenced by the presence of multiple layers of endothelial cell nuclei and of cytoplasms with organelles per capillary (data not shown). The choriocapillaries of control mice presented a single layer of endothelial cells. Ultrastructural analysis of the RPE cells of Tbdn knockdown eyes also revealed deposits, inclusion bodies, vacuoles, or membranous whorls located either in the cytoplasm or in a subepithelial space adjacent to the RPE cells (Figs. 6D, 6E). The RPE cells appeared to have more lysosomes in the basilar area and in those areas of the epithelium in transit through BM (Fig. 6E). Comparison of Tbdn knockdown mouse retinas with aged (17.5–18 months of age) mouse retinas revealed that the abnormal morphologic changes, such as thickening of the RPE, BM, and choroid in the ocular tissues of Tbdn knockdown mice were similar to morphologic changes occurring in much older mice (Fig. 6). Ultrastructural changes observed in aged specimens included greater variability in BM thickness, BM thick-

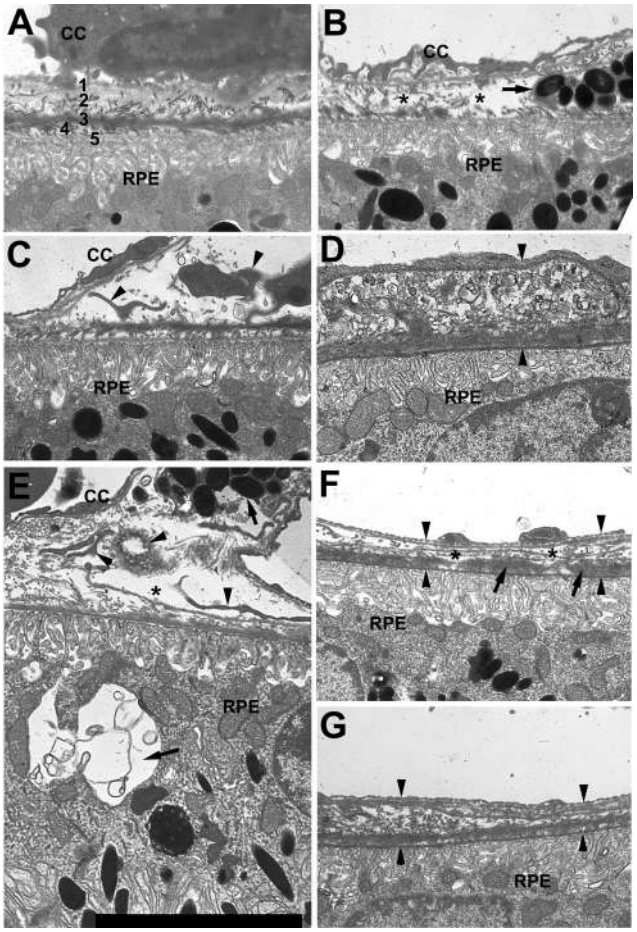


**FIGURE 6.** Ultrastructural changes of RPE, BM, and choroid in Tbdn knockdown and aged eyes. (**A**) Control mouse eye (middle age) showing the five normal layers of BM (5, RPE basal laminar membrane; 4, inner collagen layer; 3, elastic layer; 2, outer collagen layer; 1, choriocapillaris basal laminar membrane), which directly underlies the choriocapillaris endothelial cell layer (CC) and directly overlies the RPE. BM of age matched Tbdn-knockdown eyes (**B–E**) showed disruption of the normal five-layer structure, loss of definition, and continuity of elastic and collagen layers (**B**, **E**, *asterisks*), excess deposition and abnormal clumping of elastic and collagen materials (see thickened BM area delineated by *arrowheads* in **D**), invasion of BM by pigmented cells (**B**, **E**, *arrows*), and cellular material derived presumably from endothelial cells (**C**, **E**, *arrowheads*), which cause distortion and focal thickening of the invaded area of BM. Other changes apparent in the ultrastructure of Tbdn knockdown eyes were disorganization of the RPE, including the presence of vacuoles (**E**, *arrow*). BM of 18-month-old aged eyes (**F**, **G**) also showed some disruption of the normal five-layer structure, loss of definition, and continuity of the elastic and collagen layers (**F**, *asterisks*), presence of granular deposits within BM (**F**, *arrows*), and variations in BM thickness (areas delineated by *arrowheads* in **F**, **G**). Pictures were taken in the central retinal tissues. (**A–G**) Original magnification, 6000×. Representative images are shown. Scale bar, (**E**) 1 μm.

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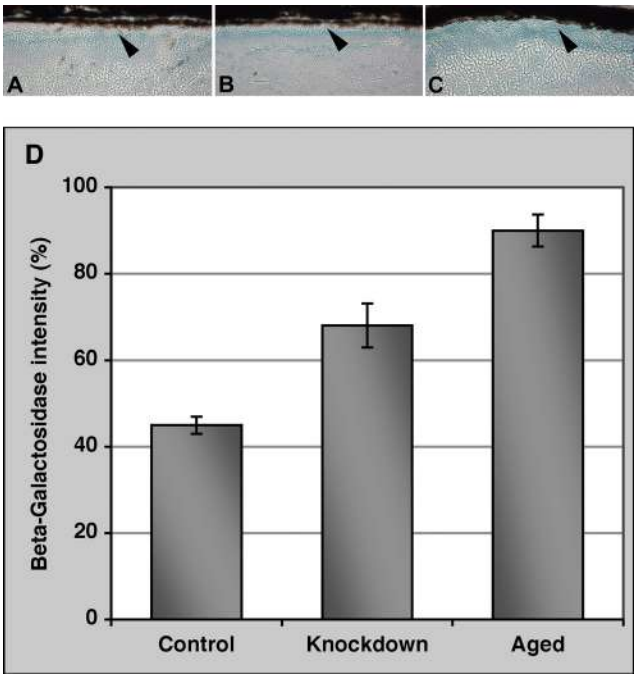


**FIGURE 7.** Senescence-associated β-galactosidase staining in aged and endothelial-specific Tbdn knockdown mice. (**A–C**) Representative senescence-associated β-galactosidase staining in eye specimens. Aged (**B**) and Tbdn knockdown mouse eye sections (**C**, middle age) showed significant senescence-associated β-galactosidase activity in RPE (*blue stain*, *arrow*) compared with control (**A**, middle aged). (**A–C**) Original magnification, 400×. (**D**) Average senescence-associated β-galactosidase activity in RPE of control mice (middle age), Tbdn knockdown mice (middle age), and aged mice was measured and quantitated by analysis of the central retinal tissues in histologic sections for which representative analysis is shown (**A–C**). Tbdn knockdown mice and aged mice showed similar patterns of increases in RPE senescence-associated β-galactosidase activity compared with controls. Results are expressed as the mean ± SEM percentage of the aged specimens.

ening, presence of granular deposits within BM, and disruption or deposition of the pentalaminar layer arrangement of BM (Figs. 6F, 6G).

Tissue morphometric analyses confirmed significant overall increases in thickness of the RPE layer and choroid of Tbdn-knockdown mice and aged (17.5 months and older) mice compared with control mice ($P < 1 \times 10^{-6}$) (Fig. 5E). The increased thicknesses measured in the RPE of Tbdn-knockdown and aged specimens were, respectively, 1.6-fold and 1.3-fold, whereas choroid measurements showed an approximate twofold increase for both types of specimens.

Senescence-associated β-galactosidase activity is a commonly used biomarker for evaluating replicative senescence in mammalian cells.[39] Because senescence-associated β-galactosidase activity increases in the primate RPE during aging,[40] this activity was assessed in eye sections of endothelial-specific Tbdn knockdown and control mice compared with aged mice. RPE of aged mice exhibited a level of senescence-associated β-galactosidase activity 2.1-fold higher than that in RPE of control mice ($P = 2 \times 10^{-12}$; Figs. 7B, 7D). RPE of Tbdn-knockdown mice exhibited a β-galactosidase activity 1.7-fold greater than that of RPE of control mice ($P = 9 \times 10^{-5}$; Figs. 7C, 7D). The β-galactosidase activity in the Tbdn-knockdown RPE was 73.8% that of aged control RPE ($P = 0.003$).

## DISCUSSION

This work reveals that Tbdn suppression in retinal blood vessels occurs during aging in mice. The knockdown of retinal vascular Tbdn expression during middle age to an extent similar to that in aged mice is sufficient to lead to the development of retinal fibrovascular lesions.[11] These results confirm that the highest levels of retinal Tbdn expression are required to maintain retinal homeostasis during middle age and during aging in the mouse. During aging in humans, decreases in endothelial Tbdn expression were observed in older persons compared with younger persons (Fig. 2G). Moderate (~50%) decreases in Tbdn expression were not associated with significant pathologic changes in humans and may not be sufficient to cause pathologic conditions in adulthood. However, in agreement with the mouse data, specimens from older persons with AMD displaying choroidal pathology were associated with a marked decrease (~83%) in choroidal endothelial Tbdn expression compared with specimens from younger persons. Our results in mouse and humans strongly suggest that a significant loss of choroid and retinal endothelial Tbdn expression in aged individuals is one of the contributing factors in the development of age-related choroidal-retinal pathology. These results are in agreement with the role of Tbdn in maintaining endothelial cell homeostasis.[9–12]

The pathologic changes in RPE, BM, and choroid of endothelial-specific Tbdn knockdown transgenic mice most closely resemble those occurring in humans and in other mouse models of neovascular AMD.[6] Well-described pathologic changes occurring in AMD include changes in the structural integrity of the elastic layer of the BM around the macula and increasing the formation of drusen consisting of basal laminar deposits and basal linear deposits.[1,41] The deposits of irregularly shaped acellular material observed in Tbdn knockdown BM are reminiscent of the drusen seen in aged human retinas and in AMD specimens.[1,4] Choroidal/subretinal amorphous deposits have also been described in other mouse models of AMD.[6] Interestingly, several of these mouse models require aging and/or change in diet or some kind of environmental influence. Moreover, the numbers, sizes, and severity of the choroidal deposits we observed in Tbdn knockdown eyes surpass those described at the BM-choroidal interface of several of these models in the absence of the added influence of aging or other manipulation.[6]

Tbdn knockdown eyes also showed invasion of BM by cells, some of which may be choriocapillary endothelial cells. Normal choroidal capillaries extend cellular processes through their basal lamina that are thought to anchor and stabilize the choriocapillaris.[42] However, in the present cases of Tbdn knockdown eyes, the cellular structures invading the choriocapillaris basal lamina and BM were significantly more disruptive and appeared to be much larger and more abnormal than simple processes. This disruption of the BM is highly reminiscent of human choroidal neovascularization. Moreover, multiple layers of endothelial cells per choroidal vessel were observed in the Tbdn knockdown mice, suggesting that the endothelial cells are more migratory or more proliferative than control specimens. These results are in agreement with our previous study showing increased blood vessels in the choroid layers of Tbdn knockdown mouse eyes.[11] Other BM-invading cells observed in the present study could possibly be pigmented choroidally derived melanocytes containing cytoplasmic melanosomes. Early-phase AMD[1] is characterized by diffuse and focal thickening of the BM together with the development of hypopigmented and hyperpigmented areas, which could indicate derangements in the positions of pigmented and other cells. Other studies have pointed to the

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involvement of inflammatory responses in the pathogenesis of AMD, and macrophage-like cells invading the subretinal space in mouse models of AMD have been observed.[5,6] The increase in focal thickening of BM caused by cellular invasion or deposition of material was reflected in the higher degree of variability of measurements of the actual thickness of the BM in Tbdn knockdown eyes compared with control (data not shown). The loss and discontinuity of matrix material we observed in BM in Tbdn knockdown mice was also reminiscent of the defect in the deposition of the BM matrix layers reported in brachymorphic mice, which harbor deficiencies in the levels of the heparan sulfate proteoglycan (HSPG) decorin.[43] Interestingly, endothelial Tbdn knockdown in mice is also associated with a reduction in the expression of HSPG in the ocular vasculature.[11] Increased susceptibility to AMD has also recently been associated with a single nucleotide polymorphism of the *HTRA1* gene encoding a serine protease that regulates the degradation of extracellular matrix proteoglycans such as decorin.[5,44]

Retinal pathologic changes affecting RPE cells have been observed in aging eyes, during AMD, and in animal models of AMD.[1,4,6,40] We observed similar pathologic changes in the RPE of aged and endothelial Tbdn knockdown mice, including increased thickness, increased deposits in or around the RPE cells, and increased senescence-associated β-galactosidase activity. Increases in senescence-associated β-galactosidase can result from age-related replicative senescence or a stress-induced form of premature senescence that might involve oxidative damage.[39] Oxidative stress has been demonstrated to be a key component of a range of neovascular retinopathies, including AMD.[45] Given that endothelial Tbdn knockdown causes hyperpermeability and damage to the retinal and choroidal vasculature, it is possible that oxidative stress might occur and might lead to bystander effects of stress-induced premature senescence and increases in senescence-associated β-galactosidase activity in the RPE.[11,12,45,46] Recent morphometric analysis in human wet AMD suggests that choriocapillaris pathology precedes RPE damage.[47] Therefore, the fact that the vasculopathy associated with Tbdn suppression somehow causes RPE damage is consistent with the proposed sequence of events in wet AMD. Future studies will be needed to explore these possibilities.

Regulation of endothelial permeability is important for blood-retinal barrier and retinal homeostasis.[45,48] Retinal endothelial cell barrier function usually limits plasma albumin to intravascular areas (blood vessel lumens) in normal neural retinal tissues.[33,34] Extravasation of albumin from retinal blood vessels or extravascularly localized albumin increases with aging and has been described as a marker of retinal blood vessel damage in humans with PDR and in several rodent models of blood-retinal barrier breakdown.[49–51] This breakdown of blood-retinal barrier function has been shown to involve changes in permeability of retinal endothelial cells[51] and is associated with thickening of retinal tissues because of edema.[52] Recent studies using three-dimensional optical frequency domain imaging have also shown subretinal choroidal thickening and edema in neovascular AMD.[53] Our findings of retinal/choroidal thickening and albumin extravascular localization in the neural retina of Tbdn knockdown and aged mouse eyes are consistent with these studies and suggest that Tbdn loss is involved in the molecular mechanism, leading to retinal/choroidal leakage vasculopathy in aging. These results are also consistent with our recent data showing that the knockdown of Tbdn in retinal endothelial cells in vitro leads to increased permeability to FITC-albumin.[12] It is well established that hyperpermeability alone is an essential component of pathologic angiogenesis induced by the proangiogenic growth factor VEGF.[54,55] However, because endothelial Tbdn is a component of an intracellular acetyltransferase complex, the effect on endothelial hyperpermeability that we observed both in vitro and in vivo may be distinct from or parallel to the pathways of proangiogenic growth factors such as VEGF. At present, it remains to be confirmed whether endothelial Tbdn suppression impacts the production (from specific blood vessel wall cells) and biological function of proangiogenic growth factors or acts in a completely separate pathway.

Endothelial cell permeability and the transit of molecules across the endothelial cell barrier are mediated by either an intercellular junctional paracellular pathway or a transcellular pathway using a vesicular system.[48] Previous studies have shown that albumin makes use of the transcellular pathway in retinal endothelial cells in vivo.[49] Insights on how Tbdn might regulate endothelial cell permeability to albumin come from its interaction with cortactin,[12] a protein regulating the dynamics of the actin-cytoskeleton assembly, cell migration, endocytosis, and intracellular movement of vesicles.[48,56–59] Moreover, there is evidence that the Tbdn and Ard1 complex regulates endocytosis.[12,24] Actin and a number of other membrane-bound proteins, such as dynamin, caveolin-1, and gp60, are involved in a specific albumin transcytosis pathway in endothelial cells.[48] Whether Tbdn acetyltransferase complex with Ard1 can acetylate and regulate proteins involved in the albumin transcytosis pathway is unknown and will require further study. The mechanisms leading to changes in endothelial cell permeability during aging are poorly characterized. Both the change in distribution of claudin-5, a tight junction protein mediating blood-barrier function, and oxidative stress have been proposed as potential mechanisms.[60,61]

The mechanisms underlying the pathologic effects of vascular hyperpermeability include the stimulation of deposition of extracellular matrix around leaking vessels by leaked plasma proteins, which further drives the process of pathologic angiogenesis.[54] Interestingly, Tbdn was originally described as a protein that was downregulated by the exposure of endothelial cells to a material produced from and composed mainly of extracellular matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ).[9] Therefore, it is tempting to speculate that during old age, the dysregulation of extracellular matrix around hyperpermeable retinal blood vessels could further drive the suppression of Tbdn in a vicious circle of leakage, vascular matrix remodeling, and pathologic angiogenesis. Recently, a role for changes in the extracellular matrix protein ARMS2 in the development of AMD has been described.[62]

The retinas of middle-aged Tbdn knockdown mice displayed changes similar to those found in naturally aged mice, including increased variability in BM thickness, retinal and choroidal thickening, granular deposits within BM, disruption or deposition of material within BM, RPE senescence, and disruption of retinal blood vessels and neural retina integrity, as evidenced by extravascularly localized albumin. Our findings have implications for better understanding of the molecular and cellular mechanisms underlying AMD, which remains a leading cause of blindness worldwide. In light of the changes we observed in the retina, RPE cells, BM, and choroid in Tbdn knockdown eyes and similar changes in human eyes, Tbdn has emerged as an important protein, the presence of which protects retinal tissue from damaging effects of changes in extracellular matrix, plasma protein extravasation, and vasculopathy. Future therapies for neovascular eye diseases could target Tbdn with the hope of preventing, and not simply treating, these devastating causes of blindness.

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