The American Journal of Pathology, Vol. 169, No. 6, December 2006
Copyright © American Society for Investigative Pathology
DOI: 10.2353/ajpath.2006.060400

Cell Injury, Repair, Aging and Apoptosis

Studies of Optineurin, a Glaucoma Gene

Golgi Fragmentation and Cell Death from Overexpression of Wild-Type and Mutant Optineurin in Two Ocular Cell Types

Bum-Chan Park, Xiang Shen, Mishan Samaraweera, and Beatrice Y.J.T. Yue

From the Department of Ophthalmology and Visual Sciences, College of Medicine, University of Illinois, Chicago, Illinois

Optineurin (OPTN) has recently been linked to glaucoma, a major cause of blindness worldwide. Mutations in OPTN such as Glu[50]→Lys (E50K) have been reported in patients, particularly those with normal pressure glaucoma. Here, we show that the endogenous OPTN was not secreted in two ocular cell types, human trabecular meshwork and retinal pigment epithelial cells. It localized instead in the cytoplasm in a diffuse pattern without a distinct association with the Golgi apparatus. When overexpressed, however, wild-type OPTN-green fluorescent protein (GFP) formed foci especially around the Golgi, colocalizing partially with the common endocytic pathway marker transferrin receptor in both cell types. Fragmentation of the Golgi was also observed. On nocodazole treatment, the OPTN foci were dispersed into the cytoplasm. Overexpression of mutant $OPTN_{E50K}$-GFP resulted in a greater number ($P < 0.0055$) and size of the foci, compared with the wild type, and the Golgi alteration was potentiated. Cell loss observed in OPTN-expressing cultures was also more pronounced in $OPTN_{E50K}$-GFP compared with that of wild-type OPTN-GFP counterparts ($P < 0.01$). This study highlights a possible role of OPTN in vesicle trafficking and Golgi integrity. It also provides insights into the possible mechanisms why E50K would exhibit a propensity toward the development of glaucoma. ***(Am J Pathol 2006, 169:1976–1989; DOI: 10.2353/ajpath.2006.060400)***

Glaucoma, a major cause of blindness worldwide, is a group of diseases characterized by a progressive loss of retinal ganglion cells and their axons. Primary open angle glaucoma (POAG), the most common form of the disease, is age-related and is frequently associated with elevated intraocular pressure (IOP). The IOP is controlled by a balance between the production and outflow of the aqueous humor in the anterior chamber. The trabecular meshwork (TM), a specialized eye tissue neighboring the cornea, is the major site for regulation of the aqueous humor outflow.[1]

A subset of POAG that accounts for approximately 30% of the POAG cases[2] is called normal tension or normal pressure glaucoma (NPG). In this condition, the glaucomatous damage occurs with IOPs within the normal limits, although the progression of the damage is still believed to be IOP dependent.[3]

Genetic studies have established that POAG is genetically heterogeneous and is caused by several susceptibility genes[4,5] and perhaps also environmental factors.[6] To date, a total of 12 chromosomal loci, designated as GLC1A to GLC1L, have been mapped for POAG.[4,5] Three genes, *myocilin*, *optineurin* (*OPTN*), and *WDR36*, have been identified, respectively, as the *GLC1A* gene on 1q23-q25,[7,8] *GLC1E* gene on 10p15-p14,[9,10] and *GLC1G* gene on 5q22.1.[11] Among them, *OPTN* is linked particularly to NPG cases.[10,12] Mutations, including Glu[50]→Lys (E50K), Met[98]→Lys (M98K), and Arg[45]→Gln (R545Q), in OPTN have been found in 16.7% of families with hereditary POAG. Approximately 80% of those families had the most prevalent E50K mutation. Of the E50K-affected subjects, 18% had high and the remaining had normal IOP values.[10,12]

The human *OPTN* gene contains three noncoding exons in the 5′-untranslated region and 13 exons that code for a 577-amino acid protein.[10] Alternative splicing at the 5′-untranslated region generates at least three different isoforms, but all have the same open reading frame.[10] Sequence analysis indicates that OPTN is a protein containing multiple coiled coil domains, at least one leucine zipper (amino acids 143 to 164), and a carboxyl-terminal zinc

Supported by National Eye Institute grants EY 05628 and EY 03890 (to B.Y.J.T.Y.) and core EY 01792.

Accepted for publication August 15, 2006.

Address reprint requests to Beatrice Yue, Ph.D., Department of Ophthalmology and Visual Sciences, College of Medicine, University of Illinois, 1855 W. Taylor Street, Chicago, IL 60612. E-mail: beatyue@uic.edu.

finger.[13–15] OPTN was also identified previously as FIP-2 (14.7-interacting protein-2) and NRP (nuclear factor-κB essential modulator-related protein).[13,15] It was suggested that OPTN might have a role in TNF-α-induced apoptosis,[13] although this has not been well documented.

OPTN is expressed in many tissues, such as the heart, brain, liver, skeletal muscle, kidney, pancreas, and the eye.[10,13] Previous studies suggested that OPTN might be associated with the Golgi apparatus.[10,15–17] OPTN has been shown to interact with Rab8, huntingtin, and myosin VI.[14,17] Rab8 regulates membrane trafficking and is known to promote changes in cell shape by reorganizing actin and microtubules.[18,19] Huntingtin has been localized to endocytic and secretory membrane organelles.[20] Myosin VI is a multifunctional motor protein that moves toward the minus end of actin filaments and is found in a number of intracellular compartments, including endocytic vesicles, the Golgi, and secretory vesicles.[21]

Cellular and molecular biological studies of OPTN on eye tissues or cells have been limited. Immunolabeling for OPTN did locate this protein in the TM, cornea, nonpigmented ciliary epithelium, iris, and retina, especially the retinal pigment epithelium (RPE).[22,23] OPTN was also reported to be in the aqueous humor, suggesting that it may be a secretory protein.[10] Nevertheless, the exact properties and the role of OPTN in ocular cells and the pathogenic mechanisms of mutations such as E50K remain undefined.

In the present study, we examined the localization of OPTN in two ocular cell types, namely, normal human TM and RPE cells. We also investigated the consequences from overexpression of wild-type OPTN and OPTN containing mutation E50K. Our study implicated a role of OPTN in vesicle trafficking and Golgi integrity in TM and RPE cells. It also unveiled possible mechanisms why E50K mutation may lead to pathology. These findings are of clinical relevance in understanding of the development of glaucoma.

Materials and Methods

Cell Cultures

Normal human TM tissues excised from donor eyes (Illinois Eye Bank, Chicago, IL) were cultured on Falcon Primaria flasks in complete medium containing Eagle's minimum essential medium (Sigma, St. Louis, MO), 10% fetal bovine serum, 5% calf serum, essential and nonessential amino acids, and antibiotics. Donors were 14, 19, 21, 24, 26, 44, and 49 years old. When TM cells reached confluence, they were trypsinized and subcultured. Second- to fourth-passage cells were used in this study. RPE (ARPE-19) cells obtained from American Type Culture Collection (Manassas, VA) were grown and maintained in complete medium.

Secretion Analysis

Freshly confluent TM cells were equilibrated in basal medium (Eagle's minimum essential medium with 2 mg/ml bovine serum albumin, 0.1 mg/ml lima bean trypsin inhibitor, 1 μg/ml insulin, and 0.1 μg/ml transferrin) for 1 hour. Cells were then incubated with fresh basal medium, and the medium was collected for two sequential 30-minute periods. The medium was replaced with fresh basal medium containing 1 mmol/L $BaCl_2$ for another two sequential 30-minute periods.[24] The media collected were centrifuged to remove nonadherent cells. Protease inhibitor cocktail (Roche, Indianapolis, IN) was added, and the media were concentrated by Centricon YM-10 (Millipore, Bedford, MA). Cells were harvested in CelLytic-M cell lysis reagent (Sigma) containing protease inhibitor cocktail. Aliquots of each media sample and total cell lysate were analyzed by Western blotting. In brief, proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electroblotted for 1 hour onto Protran BA83 nitrocellulose membrane (Whatman, Sanford, ME). After blocking for 1 hour with 5% nonfat dry milk in 20 mmol/L Tris buffer (pH 7.4) containing 150 mmol/L NaCl and 0.1% Tween 20, the membrane was incubated for 1 hour with either anti-OPTN (1:2000; Cayman Chemical, Ann Arbor, MI) or anti-myocilin[25] (1:5000; a gift from Dr. Daniel Stamer, University of Arizona, Tucson, AZ). According to the manufacturer's specification, the anti-OPTN antibody, raised in rabbits against a peptide corresponding to amino acid 115 to 130 sequence, recognizes human OPTN with a molecular size of 74 kd.

The blot was further incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA), and protein bands were detected using SuperSignal Substrate (Pierce, Rockford, IL). For repeated probing, the blot was stripped for 1 hour at room temperature with ImmunoPure IgG Elution buffer (Pierce).

Immunofluorescence Staining

TM and RPE cells (5000 cells/well) plated onto Lab-Tek eight-well CC2 glass chamber slides (Nalge Nunc, Rochester, NY) were fixed in 4% paraformaldehyde for 15 minutes, washed with 100 mmol/L glycine in phosphate-buffered saline, and permeabilized in 0.2% Triton X-100 for 4 minutes. After 30 minutes of blocking with 3% bovine serum albumin, the cells were incubated for 1 hour at room temperature with anti-OPTN (1:200), anti-GM130 (1:100; BD Biosciences, San Jose, CA), or anti-golgin97 (1:200; Invitrogen, Carlsbad, CA). They were further incubated for 45 minutes with fluorescein isothiocyanate- or Cy3-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories) and mounted in Vectashield with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Photography was performed using a 63× oil objective on an Axioscope (Carl Zeiss MicroImaging, Thornwood, NY) with the aid of Metamorph software (Molecular Devices, Downingtown, PA). In some experiments, confocal microscopic analysis was performed on a Leica SP2 confocal system (Leica Microsystems, Bannockburn, IL) with a 63× oil objective using sequential scanning to minimize the bleed through.[26] The extent of colocalization was quantified using the ImageJ software by generating contour maps of each image by overlaying the green [wild-type OPTN ($OPTN_{WT}$)-green fluorescent protein (GFP)] and red (Golgi markers) channels. Total number of green foci and that overlapped with the red staining around the Golgi areas were counted.[27]

Subcellular Fractionation

TM and RPE (5×10^6) cells were harvested in homogenization buffer (0.25 mol/L sucrose, 10 mmol/L HEPES, pH 7.4, and 1 mmol/L ethylenediamine tetraacetic acid). The cells were broken by repeated strokes in a Dounce homogenizer. Cell debris and nuclei were pelleted by centrifugation at $1000 \times g$ for 10 minutes. The supernatant was overlaid onto the top of the discontinuous gradient, which consisted of 30, 25, 20, 15, and 10% iodixanol solution (OptiPrep; Accurate Chemical & Scientific Corp., Westbury, NY). After centrifugation at 4°C in a Beckman SW rotor at $100,000 \times g$ for 3 hours, 17 fractions were collected from top to bottom. One-sixth of each fraction was loaded on SDS-PAGE gels and immunoblotted with anti-optineurin, anti-GM130 (1:500), and anti-golgin97 (1:2000).

Plasmid Construction

The open reading frame (ORF) without stop codon of *OPTN* was amplified by polymerase chain reaction (PCR) using pcDNA3-NRP (a gift from Dr. Robert Weil, Institute Pasteur, Paris, France) as template and primers 5′-GGCGAATTCCCACCATGTCCCATCAACCTCTCAGC-3′ and 5′-GGCGGATCCCGAATGATGCAATCCATCACGTG-3′. To generate the *Rab8* ORF, cDNAs synthesized from total RNA of human TM cells by Superscript II cDNA synthesis kit (Invitrogen) were used as template, and PCR was performed with primers 5′-GGCGAATTCTATGGCGAAGACCTACGATTA-3′ and 5′-GGCGGATCCTCACAGAAGAACACATCGGA-3′. The resulting PCR products of *OPTN* and *Rab8* were digested with *Eco*RI and *Bam*HI and cloned in frame into pEGFP-N1 and pDsRed-Monomer-C1 (BD Biosciences), respectively, at the same restriction sites to yield constructs containing wild-type OPTN and GFP, $pOPTN_{WT}$-GFP, and containing DsRedM and wild-type Rab8, pDsRedM-$Rab8_{WT}$, respectively. All mutant constructs were generated by QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The sense primer sequences used for mutagenesis were 5′-GAAAGAGCTCCTGACC<u>AAG</u>AACCACCAGCTGAAAG-3′ for $pOPTN_{E50K}$-GFP and 5′-GACACAGCCGGT<u>CTG</u>GAACGGTTTCGG-3′ for pDsRedM-$Rab8_{Q67L}$. The antisense primer sequences were the reverse. To construct untagged OPTN, PCR amplification of the *OPTN* ORF with stop codon was performed using pcDNA3-NRP as template and primers 5′-CCACCATGTCCCATCAACCTCTCAGC (sense) and 5′-TACTAAATGATGCAATCCATCACGTG-3′ (antisense). The antisense primer was also used to amplify FLAG-tagged OPTN along with a sense primer, 5′-CCACCATGGATTACAAGGATGACGACGATAAGATGTCCCATCAACCTCTCAGC-3′. The amplified PCR products were then inserted into pTarget (Promega, Madison, WI), yielding pTarget-OPTN and pTarget-FLAG-OPTN. Sequencing was followed to verify all of the constructs.

Transient Transfection

Transient transfection was performed using FuGENE6 reagent (Roche) per the manufacturer's instruction with minor modifications. In brief, cells were plated at 60 to 70% confluency overnight. Cells were washed with Dulbecco's modified Eagle's minimum essential medium (DMEM; Invitrogen) 1 hour before the transfection and transferred to DMEM containing 2.5% fetal bovine serum. FuGENE6 reagent in DMEM was mixed with plasmid DNA at a 3:1 ratio and added to the cells for indicated time periods. Two micrograms of plasmid DNA per milliliter of the final transfection mixture was used unless otherwise noted. The transfection efficiency was estimated from the number of GFP-expressing green cells versus total number of cells under a Zeiss fluorescence/phase contrast microscope.

Transgene Expression

TM and RPE cells in six-well plates were transfected with pEGFP-N1 (control) or $pOPTN_{WT}$-GFP for 16 hours. The media were collected and concentrated. The cells were lysed for 15 minutes in CelLytic-M cell lysis reagent containing protease inhibitor cocktail. Cell debris was removed, and the protein content was determined by bicinchoninic acid protein assay kit (Pierce). Protein extracts (10 or 20 μg) and concentrated media samples were resolved on SDS-PAGE and immunoblotted with anti-OPTN, anti-GFP (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000; Trevigen, Gaithersburg, MD).

Co-Distribution of OPTN Foci with Golgi Markers, Rab8, Myosin VI, and Transferrin Receptor

TM cells plated on eight-well glass chamber slides were transfected with $pOPTN_{WT}$-GFP, untagged pTarget-$OPTN_{WT}$, or pTarget-FLAG-$OPTN_{WT}$ for 16 hours. Cells were fixed for immunofluorescence staining with anti-OPTN, anti-GM130, anti-golgin97, anti-myosin VI (1:100; Sigma), anti-FLAG (1:500; Sigma), or anti-transferrin receptor (1:100; Invitrogen). For colocalization with Rab8, cells were co-transfected with $pOPTN_{WT}$-GFP and pDsRedM-$Rab8_{Q67L}$ (constitutively active Rab8).

Effects of Chemicals on OPTN Foci Distribution

The OPTN foci distribution was examined after treatment of transfected cells for 30 minutes with vehicle [dimethylsulfoxide (DMSO)], brefeldin A (BFA; 5 μg/ml), nocodazole (10 μg/ml), or cytochalasin D (CytoD; 1 μmol/L).

OPTN Foci Formation and the Golgi Integrity

TM and RPE cells were transfected with pEGFP-N1, $pOPTN_{WT}$-GFP, or $pOPTN_{E50K}$-GFP for 16 hours and stained with either anti-GM130 or anti-golgin97. Images were taken using a 63× oil objective as described above. The number of foci formed was counted in at least 60 transfected cells manually and through the use of Metamorph computer software. The integrity of the Golgi apparatus in the same set of transfectants was determined. The judgment of a broken Golgi complex was based on the presence of disconnected, small and round GM130-immunolabeled Golgi membranes dispersed in the cell.[28] Percentage of transfected cells that displayed broken or fragmented Golgi and the degree of Golgi fragmentation were analyzed manually and through the Metamorph software. Significance of the data was determined by Student's *t*-tests. Three sets of independent experiments were analyzed.

Quantitative Analysis of Cell Death

RPE cells on glass chamber slides were transiently transfected to express GFP alone, $OPTN_{WT}$-GFP, or $OPTN_{E50K}$-GFP. Five to six 10× fields, each containing at least 50 transfected cells, were selected. Images in the selected areas were captured after washing to remove nonsurviving cells at 24-hour intervals for 4 days. The number of remaining cells was counted using the Metamorph software. Three sets of independent experiments were performed. The significance of the data was determined by Student's *t*-tests.

Results

OPTN Is Not Secreted

OPTN was reported to be in the aqueous humor, suggesting that it might be secreted by cells in the anterior chamber such as TM and nonpigmented ciliary epithelial cells.[10] However, we did not detect any OPTN protein by Western blotting even on overloading or overexposure (data not shown) in culture media concentrated from either human TM or RPE cultures. To carefully address this issue, secretion assay was performed using $BaCl_2$, a secretagogue known to induce protein secretion.[24] TM cells were incubated in basal medium for two sequential 30-minute periods followed by the challenge of $BaCl_2$ for another two sequential 30-minute periods. The media collected along with total cell lysate were analyzed by immunoblotting (Figure 1). Myocilin, a protein well documented to be secreted in TM cells,[29] was used as a positive control. As expected, myocilin was found in the basal media, and the secretion was enhanced by $BaCl_2$. By contrast, OPTN was not discerned in any of the media samples (Figure 1, top, lanes 1 to 4) under the same conditions. Meanwhile, abundant OPTN was present in the total cell lysate (Figure 1, top, lane 5).

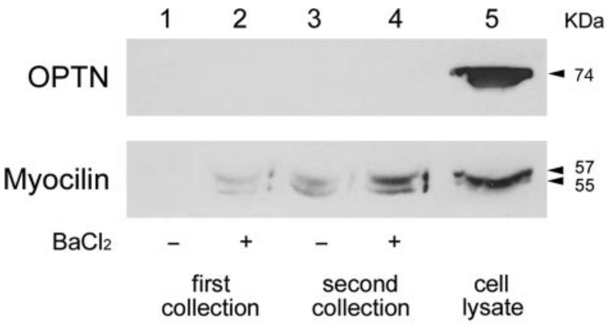

Figure 1. Secretion analysis of OPTN. TM cells were equilibrated in basal medium for 1 hour. Cells were transferred to fresh basal medium without and then with 1 mmol/L $BaCl_2$, each for two sequential 30-minute periods. The medium collected during each incubation period was concentrated, and equal aliquots were immunoblotted using anti-OPTN and anti-myocilin (positive control). The 55/57-kd (kDa) myocilin bands were found in the collected media, whereas the 74-kd OPTN band was only detected in the cell lysate.

Localization of Endogenous OPTN

OPTN has previously been suggested to localize to the Golgi apparatus in nonocular or transformed cell types.[10,15–17] We performed immunofluorescence (Figure 2) and noted that in human TM and RPE cells, the endogenous OPTN (Figure 2, A, a and d, and B, a and d), seen as punctuate staining products, was localized in the cytoplasm displaying a diffuse pattern. Double staining of OPTN with two Golgi markers, GM130 (Figure 2, Ab and Bb) for *cis*-compartment of the Golgi and golgin97 (Figure 2, Ae and Be) for the *trans*-Golgi network, indicated that there was no distinct association of OPTN with the Golgi in either TM or RPE cells (Figure 2, A, c and f, and B, c and f). The lack of major colocalization was confirmed by confocal microscopy after sequential scanning.[26]

To verify results further, subcellular fractionation was performed. TM and RPE cell extracts were fractionated on iodixanol density gradient and subjected to Western blotting. As shown in Figure 3, GM130 and golgin97 were detected in both cell types in fractions 4 to 13. GM130 was concentrated in slightly denser fractions (6 to 9, Figure 3A; 7 and 8, Figure 3B) than those of golgin97 (fractions 5 and 6, Figure 3, A and B) as expected. OPTN was retrieved in lighter fractions. It was distributed in TM and RPE cells, respectively, in fractions 1 to 5 (Figure 3A) and 2 to 4 (Figure 3B). There was only minor co-distribution with Golgi markers in fractions 4 and 5. These experiments confirmed that OPTN, to a large extent, is not associated with the Golgi.

OPTN Forms Foci around the Golgi Complex on Overexpression and Causes Golgi Fragmentation

To evaluate the effects of wild-type OPTN ($OPTN_{WT}$) overexpression, TM and RPE cells were transiently transfected with constructs containing $OPTN_{WT}$ tagged with GFP ($pOPTN_{WT}$-GFP) and GFP alone (pEGFP-N1, mock control). Expression levels of the endogenous OPTN, $OPTN_{WT}$-GFP fusion protein, and GFP were subsequently

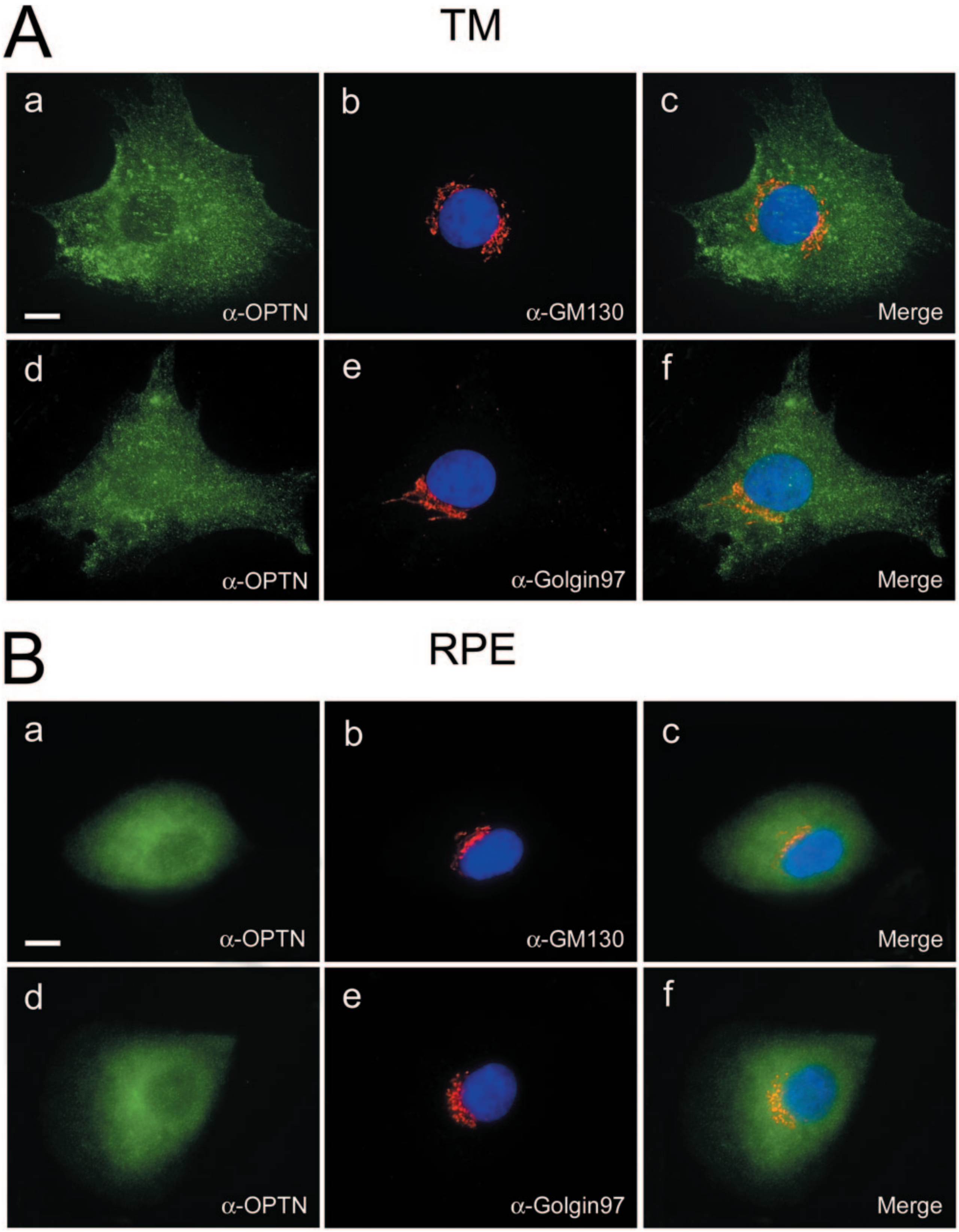

Figure 2. Localization of the endogenous OPTN in TM (**A**) and RPE (**B**) cells. Cells were fixed and double-stained with anti-OPTN antibody (α-OPTN) in green (**a** and **d**) and antibodies for Golgi markers α-GM130 (**b**) and α-Golgin97 (**e**) in red. 4,6-Diamidino-2-phenylindole staining for nuclei (blue) is shown with Golgi markers (**b** and **e**), and the merged images are shown in **c** and **f**. Images were taken using a 63× oil objective of an Axioscope and Metamorph software. Bar = 10 μm.

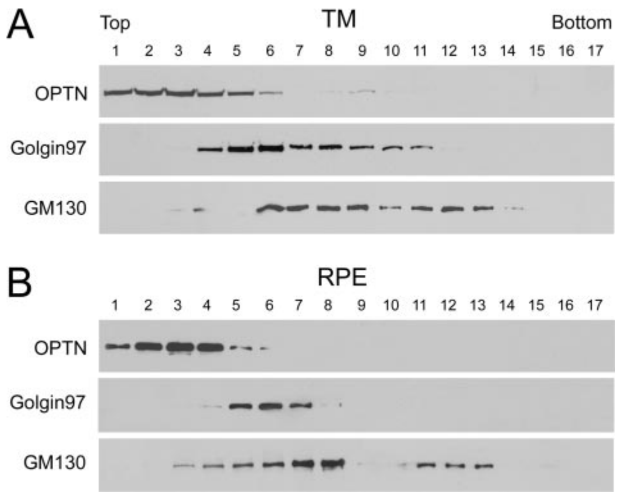

Figure 3. Distribution of OPTN and two Golgi markers after cellular fractionation. TM (**A**) and RPE (**B**) cell extracts were subjected to iodixanol density gradient flotation. Fractions were collected from the top (fraction 1) to the bottom (fraction 17) of the gradient. Aliquots of each fraction were analyzed by immunoblotting using anti-OPTN, anti-golgin97, or anti-GM130.

monitored by Western blotting (Figure 4, Aa and B, a and b). The GAPDH expression level was used as a protein loading control (Figure 4, Aa and Ba). In $OPTN_{WT}$-GFP-overexpressing cells (Figure 4, Aa and Ba; lane 2), the fusion protein with a molecular mass of 102 kd was detected as anticipated by OPTN and GFP antibodies. The endogenous OPTN was observed as a 74-kd band and GFP was observed as a 28-kd band in control samples (Figure 4, lane 1).

To determine whether OPTN is secreted when overexpressed, culture medium collected from RPE cultures 24 hours after $pOPTN_{WT}$-GFP transfection was concentrated. Immunoblotting with anti-OPTN demonstrated strong $OPTN_{WT}$-GFP and $OPTN_{WT}$ bands in the lysate of transfectants (Figure 4B, b, lane 3). In contrast, no protein bands were detected in the media (Figure 4B, b, lane 4), confirming the data in Figure 1 that OPTN was not a secreted protein.

By fluorescence microscopy, GFP in pEGFP-transfected controls was seen covering the entire cell area in the cytoplasm and in the nucleus (Figure 4B, f). $OPTN_{WT}$-GFP was found mainly in the cytoplasm of TM and RPE cells with a diffuse pattern (Figure 4, Ab and Bc) as was described for the endogenous OPTN in Figure 2. In addition, granular structures were observed to distribute in the cytoplasm including the cell periphery but most notably near the nucleus. They were variable in size and were referred to as the OPTN foci in this report. When $pOPTN_{WT}$-GFP-transfected cells were immunostained for OPTN, nearly all of the green fluorescent OPTN foci were decorated with anti-OPTN-immunoreactive products in red fluorescence (data not shown), verifying the specificity of the OPTN antibody and signifying that the foci formed from overexpression were made up of OPTN. The OPTN foci were also observed in cells expressing either untagged $OPTN_{WT}$ or FLAG-tagged $OPTN_{WT}$ after immunostaining with either anti-OPTN or anti-FLAG (data not shown). The distribution pattern of these foci was similar to that seen in $pOPTN_{WT}$-GFP-transfected cells, suggesting that the foci observed were not artifacts from the GFP tag.

The $pOPTN_{WT}$-GFP-transfected cells were immunostained for Golgi markers and imaged through sequential scanning using confocal microscopy. The foci, although in close proximity to the Golgi apparatus, were not overtly associated with the organelle. The extent of co-distribution or colocalization between the green OPTN foci and the red staining for golgin97 (Figure 4, Ac and Bd) around the Golgi areas was analyzed.[27] Results indicated that the overlap between the two was less than 6% ($5.3 \pm 2.3\%$, $n = 8$). The very minor, if any, co-distribution was likewise observed in cells expressing $OPTN_{WT}$-GFP co-immunostained with anti-GM130 (data not shown).

Whereas the GFP-expressing control cells exhibited robust Golgi staining and structure (Figure 4B, g), the Golgi complex in $OPTN_{WT}$-GFP-expressing cells appeared to be disconnected or fragmented (Figure 4, Ac and Bd). The percentage of transfectants that displayed fragmentated Golgi was subsequently determined. Almost 60% of the $OPTN_{WT}$-GFP-overexpressing cells, versus only 11% in the GFP control, showed Golgi alteration (Table 1).

OPTN Foci Co-Distribute with OPTN-Interacting Molecules and Transferrin Receptor

OPTN is known to interact with Rab8 and myosin VI.[14,17] To establish that the foci observed after transfection represent bona fide OPTN that possesses relevant properties, we examined the co-distribution of the OPTN foci with the constitutively active ($Rab8_{Q67L}$) form of Rab8 and myosin VI. Data depicted in Figure 5 revealed that there was colocalization between the OPTN foci and $Rab8_{Q67L}$ (Figure 5c) and myosin VI (Figure 5f). The colocalization was found in a majority, although not all, of the OPTN foci at the perinuclear region. Foci in the cell periphery largely did not co-distribute with either $Rab8_{Q67L}$ or myosin VI.

Apart from the Golgi apparatus, Rab8 and myosin VI have been associated with endocytic vesicles.[18,21] The lack of major Golgi colocalization and yet the connection with Rab8 and myosin VI suggested that the OPTN foci might be associated with endocytic compartments. This possibility was investigated by staining $pOPTN_{WT}$-GFP-transfected cells for transferrin receptor, a commonly used marker for endosomal vesicles. Results showed that the OPTN foci did co-distribute with a population of transferrin receptor molecules (Figure 5i).

Distribution of OPTN Foci Is Microtubule Dependent

To assess whether the distribution of OPTN is microtubule- or actin network-dependent, we treated $pOPTN_{WT}$-GFP-transfected cells with vehicle (DMSO) alone, BFA, nocodazole, or CytoD. BFA is a fungal metabolite that causes Golgi dispersion into the cytoplasm by blocking the transport of proteins to the Golgi.[30] Nocodazole de-

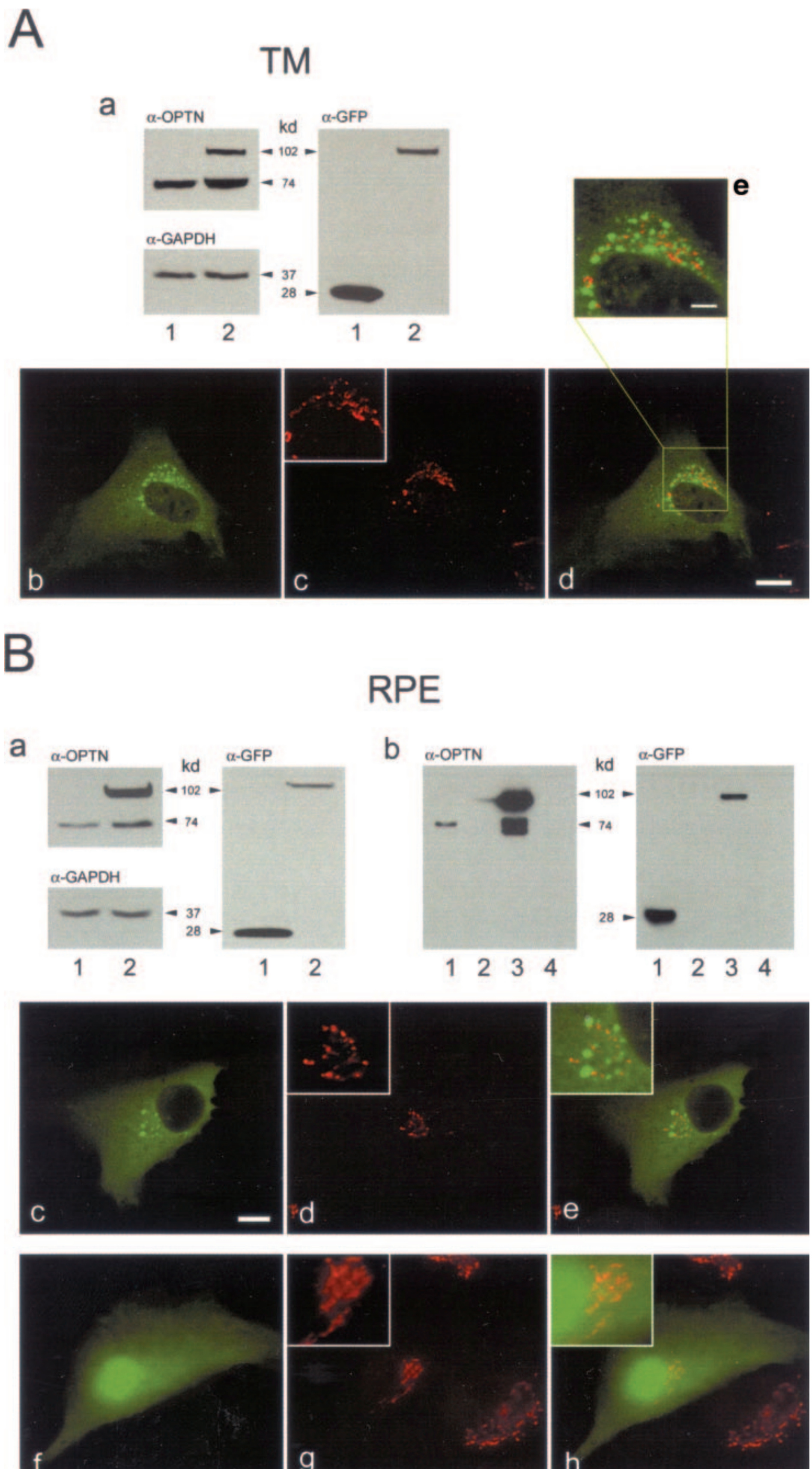
A
TM
a
α-OPTN
kd
α-GFP
102
74
α-GAPDH
37
28
1 2
1 2
e
b
c
d
B
RPE
a
α-OPTN
kd
α-GFP
102
74
α-GAPDH
37
28
1 2
1 2
b
α-OPTN
kd
α-GFP
102
74
28
1 2 3 4
1 2 3 4
c
d
e
f
g
h

Table 1. Number of OPTN Foci and Golgi Integrity in $OPTN_{WT}$- and $OPTN_{E50K}$-Overexpressing TM Cells

	Average no. of transfected cells examined per experiment	Average no. of foci per transfected cell	Golgi integrity	
			Percentage of cells with Golgi fragmentation	Degree of Golgi fragmentation per affected cell
GFP alone	70	0.4 ± 0.1	11.3 ± 2.4	1.2 ± 0.2
$OPTN_{WT}$-GFP	69	64.6 ± 2.1*	59.1 ± 2.3*	1.7 ± 0.1†
$OPTN_{E50K}$-GFP	69	152.9 ± 10.7*‡	87.6 ± 1.0*§	2.6 ± 0.1†‡

Transiently transfected TM cells (shown in Figure 7A) were stained with anti-GM130. The number of OPTN foci was counted as in Figure 7B, and numerical values are presented. The percentage of transfected cells that displayed broken or fragmented Golgi was determined. The degree of Golgi fragmentation in the same set of transfected cells was scored on a scale of 1 to 4, representing minor to increasingly severe alterations. Three independent experiments were analyzed, and data were averaged and presented as mean ± SD.

*$P < 0.004$ compared with the GFP control.

†$P < 0.035$ compared with the GFP control.

‡$P < 0.0055$ compared with the $OPTN_{WT}$-GFP.

§$P = 0.0036$ compared with $OPTN_{WT}$-GFP.

polymerizes microtubules.[31] CytoD is a fungal toxin that disrupts actin filaments and inhibits actin polymerization.[32] As shown in Figure 6, little effect was elicited by CytoD (Figure 6, j and l), whereas the OPTN foci were dispersed completely from the perinuclear/Golgi region into the cytoplasm by nocodazole (Figure 6, g and i). The dispersion was also seen with BFA (Figure 6, d and f) but only to a minor extent. Again, the OPTN foci did not co-distribute with the Golgi apparatus. These results suggested that the OPTN foci distribution was dependent on integrity of the microtubule, not the actin cytoskeleton.

Foci Formation and Golgi Integrity in Cells Overexpressing $OPTN_{E50K}$

Construct $pOPTN_{E50K}$-GFP was made by site-directed mutagenesis. TM and RPE cells were transiently transfected with pEGFP-N1, $pOPTN_{WT}$-GFP, or $pOPTN_{E50K}$-GFP. As was described for the $OPTN_{WT}$-GFP-expressing cells, foci were prominently observed in $OPTN_{E50K}$-GFP transfectants in the perinuclear region around the Golgi apparatus (Figure 7A). The foci formation was more striking in the latter than the former; the number and size of the foci were greater. We counted the foci in transfected cells and calculated the average number of foci per transfectant (Figure 7B; Table 1). The number of foci in $OPTN_{E50K}$-GFP-expressing cells was determined to be significantly ($P < 0.0055$) higher than that in $OPTN_{WT}$-GFP-transfectants. The foci number in the GFP control was, as expected, close to zero.

The transfected cells were further immunostained with either anti-GM130 or anti-golgin97. The Golgi structure in GFP-expressing control cells was indistinguishable from that in the neighboring nontransfected normal cells (Figure 8, a and d). The Golgi, as was depicted above (Figure 4), was fragmented in cells expressing $OPTN_{WT}$-GFP (Figure 8, b and e). The Golgi fragmentation was even more severe when cells were expressing $OPTN_{E50K}$-GFP (Figure 8, c and f).

Quantitative analyses were performed. The Golgi fragmentation was defined as the presence of disconnected, small and round Golgi fragments dispersed in the cell.[28] The percentage of TM cells with Golgi alteration (Table 1) was greater ($P = 0.0036$) in cells expressing $OPTN_{E50K}$-GFP (87.6 ± 1.0%) than in those expressing $OPTN_{WT}$-GFP (59.1 ± 2.3%). Both were significantly ($P < 0.004$) higher than the GFP-expressing controls (11.3 ± 2.4%). We also scored the Golgi alteration per affected cell on the scale of 1 to 4 in terms of its severity (Table 1). The more severe fragmentation was noted again in cells expressing $OPTN_{E50K}$-GFP. The Golgi alteration also appeared to be accompanying the foci formation. Generally, the more prominent the foci formation in number, the more austere was the Golgi fragmentation.

Overexpression of $OPTN_{WT}$ and $OPTN_{E50K}$ Results in Cell Death

During the course of the study, we noticed that the number of transfectants decreased with time after OPTN transfections. To provide quantitative assessments, the number of RPE cells expressing GFP alone or $OPTN_{WT}$- or $OPTN_{E50K}$-GFP was monitored after transfection at 24-hour intervals for 4 days. It was discovered that, at the 48-hour time point, the cell

Figure 4. Cellular localization of overexpressed OPTN. **A:** TM cells were transfected with pEGFP-N1 or $pOPTN_{WT}$-GFP for 16 hours. **a:** Twenty μg of protein extracts from pEGFP-N1-transfected (**lane 1**) and $pOPTN_{WT}$-GFP-transfected (**lane 2**) cells were subjected to Western blotting with anti-OPTN (α-OPTN), anti-GFP (α-GFP), or anti-GAPDH (α-GAPDH). **b–e:** TM cells were transfected with $pOPTN_{WT}$-GFP, fixed, and immunostained with anti-golgin97 (**c**; red). OPTN foci (green) are seen in **b**, **d**, and **e**. The Golgi apparatus is presented at a higher magnification in the **inset** in **c**. Merged image is shown in **d**. The boxed perinuclear/Golgi region in **d** is enlarged in **e**. OPTN foci were predominantly distributed in the perinuclear region around the Golgi with little colocalization observed with the Golgi marker golgin97. Scale bars: 10 μm (**b–d**); 4 μm (**e**). **B:** RPE cells were transfected with 0.4 μg (**a**) or 2 μg (**b**) of $pOPTN_{WT}$-GFP or pEGFP-N1 per milliliter of transfection solution. **a:** Twenty μg of protein extracts from pEGFP-N1- (**lane 1**) and $pOPTN_{WT}$-GFP-transfected cells (**lane 2**) were immunoblotted with α-OPTN, α-GFP, or α-GAPDH. **b:** Ten μg (1/20 of total lysate) of protein extracts (**lanes 1** and **3**) and one-sixth of concentrated media (**lanes 2** and **4**) from pEGFP-N1-transfected (**lanes 1** and **2**) and $pOPTN_{WT}$-GFP-transfected (**lanes 3** and **4**) cultures were probed with α-OPTN and α-GFP. In parallel experiments, the $pOPTN_{WT}$-GFP-transfected (**c–e**; GFP in green) and pEGFP-N1-transfected (**f–h**; GFP in green) cells were immunostained with anti-golgin97 (**d** and **g**; red). The Golgi apparatus is shown at a higher magnification in **insets** in **d** and **g**. Merged images are presented in **e** and **h**. Images were taken using a 63× oil objective. Bar = 10 μm.

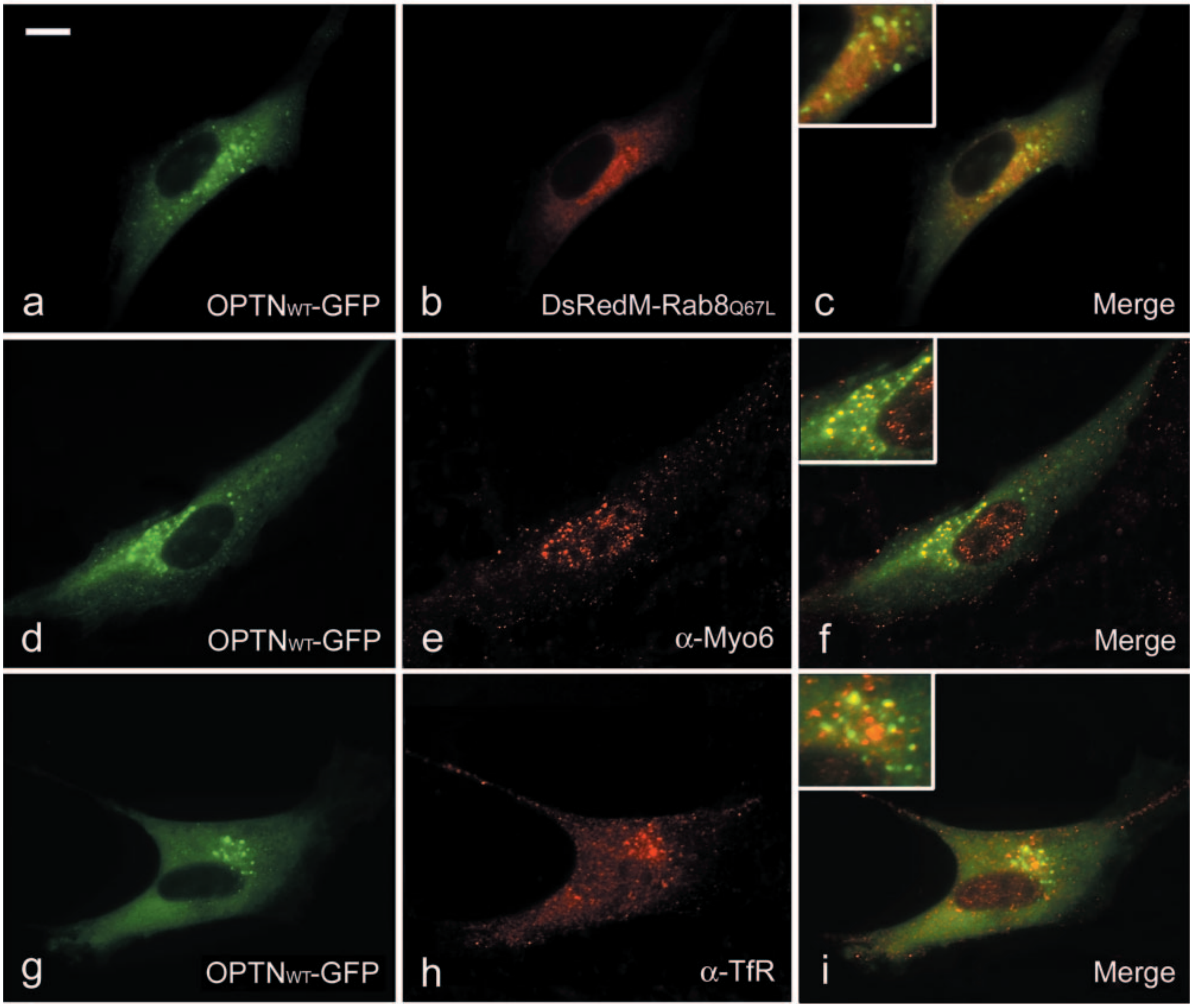

Figure 5. Colocalization of the OPTN foci with other proteins. For colocalization with Rab8, TM cells were co-transfected with pOPTN$_{WT}$-GFP (**a**; green) and pDsRedM-Rab8$_{Q67L}$ (**b**; red). For colocalization with myosin VI (**e**; red) and transferrin receptor (**h**; red), TM cells were immunostained with antibody to myosin VI (α-Myo6; **e**; red) and transferrin receptor (α-TfR; **h**; red) after transfection with pOPTN$_{WT}$-GFP (**d–i**; GFP in green). Colocalization is observed in merged images (**c**, **f**, and **i**). The perinuclear region is shown at a higher magnification in **insets**. Images were taken using a 63× oil objective. Bar = 10 μm.

number was increased for all cultures (Figure 9). At subsequent time points, the cell number remained roughly unchanged in the GFP control. On the contrary, a decrease in cell number, indicative of cell loss, was found in the wild-type and mutant OPTN-transfected cultures ($P < 0.001$ compared with the GFP control). The cell loss appeared to be more pronounced in the E50K cultures than in the wild type. The difference was statistically ($P < 0.01$) significant.

Discussion

OPTN protein, the product of the *GLC1E* gene, is ubiquitously expressed in many nonocular[13] and ocular[10,22,23] tissues. Mutations in the *OPTN* gene have only been found to date in patients with glaucomatous pathology in the eye, implying that the function, processing, or regulation of *OPTN* may be unique or specific in ocular cells. In the present study, we used two ocular cell types, human trabecular meshwork and RPE cells, for OPTN investigations.

The *OPTN* gene, although linked mostly to NPG, has also been linked to high-pressure POAG cases.[10,12] TM cells were chosen for the OPTN study because they are believed to be responsible for regulation of the aqueous humor outflow and control of the IOP, a major risk factor for POAG. RPE cells are not currently implicated in any forms of glaucoma. They were chosen for the study largely based on the finding that OPTN is prominently expressed in the RPE tissue in mouse and monkey eyes.[22,23] In addition, OPTN was found to be expressed at a reasonably high level in cultured TM and RPE cells, permitting systematic and extensive examinations. The basic information and protocols es-

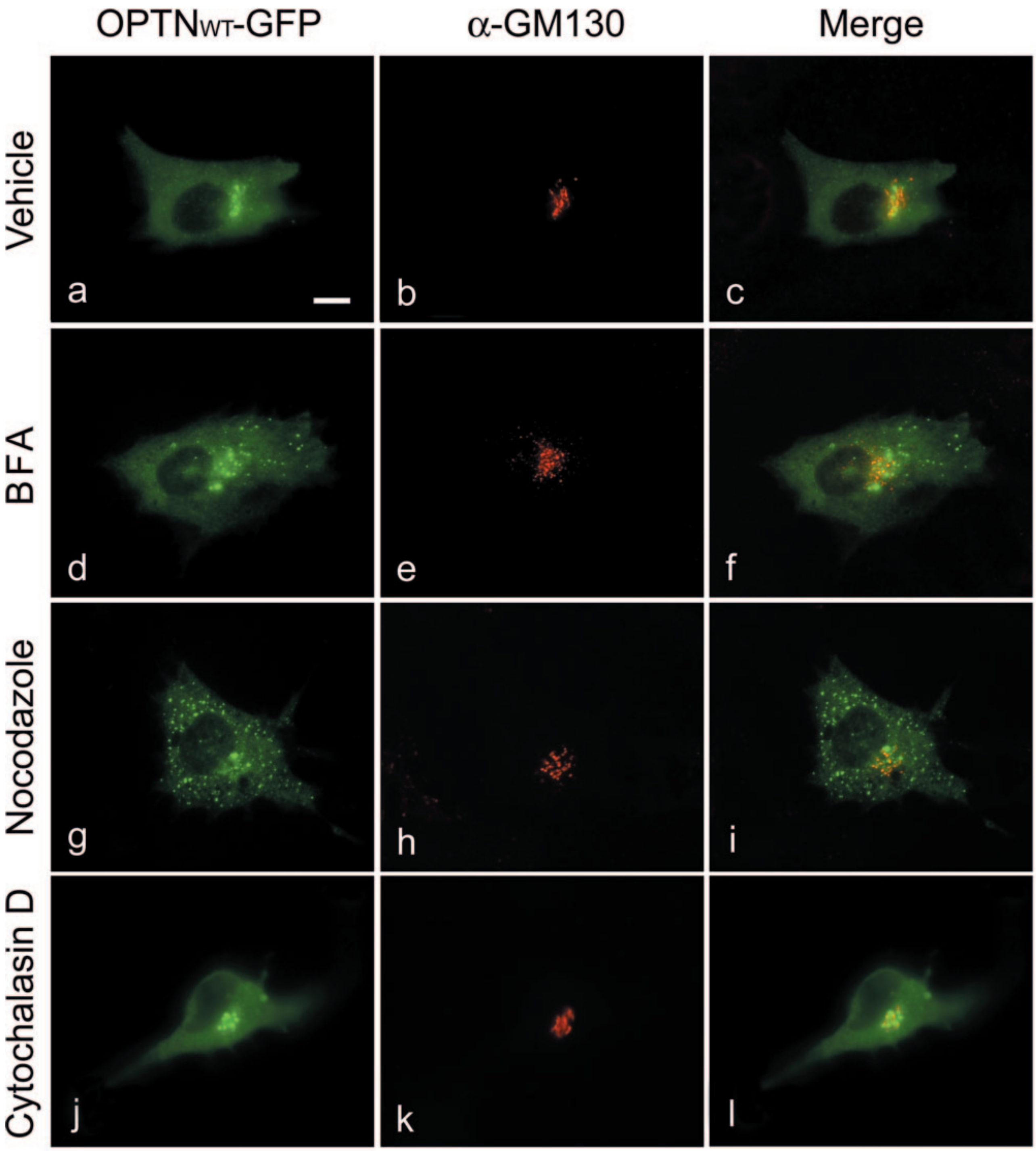

Figure 6. Effects of chemicals on the OPTN foci distribution. TM cells after transfection with pOPTN$_{WT}$-GFP were treated for 30 minutes with vehicle alone (DMSO; **a–c**), 5 μg/ml BFA (**d–f**), 10 μmol/L nocodazole (**g–i**), or 1 μmol/L cytochalasin D (**j–l**) for 30 minutes. Cells were fixed, permeabilized, and immunostained with anti-GM130 (α-GM130). Distribution of OPTN foci (**a**, **d**, **g**, and **j**) and the Golgi (**b**, **e**, **h**, and **k**) is shown in green and red, respectively. Merged images are presented in **c**, **f**, **i**, and **l**. Images were taken using a 63× oil objective. Bar = 10 μm.

tablished presently would facilitate future experimentation on cell types, including retinal ganglion cells and astrocytes, that are of direct relevance to NPG.

We provide herein experimental evidence that the endogenous OPTN is not a secreted protein. This protein was not found in the basal culture medium. Moreover, it was not secreted even under a condition in which secretion of proteins such as myocilin was promoted (Figure 1) or when OPTN is overexpressed (Figure 4B, b). In previous reports, OPTN was purported to be a secretory protein because it was detected in the aqueous humor and the culture medium.[10,12] Data, however, were not provided. Corroborating our results, Kroeber et al[33] in a recent study did not detect any OPTN in the aqueous

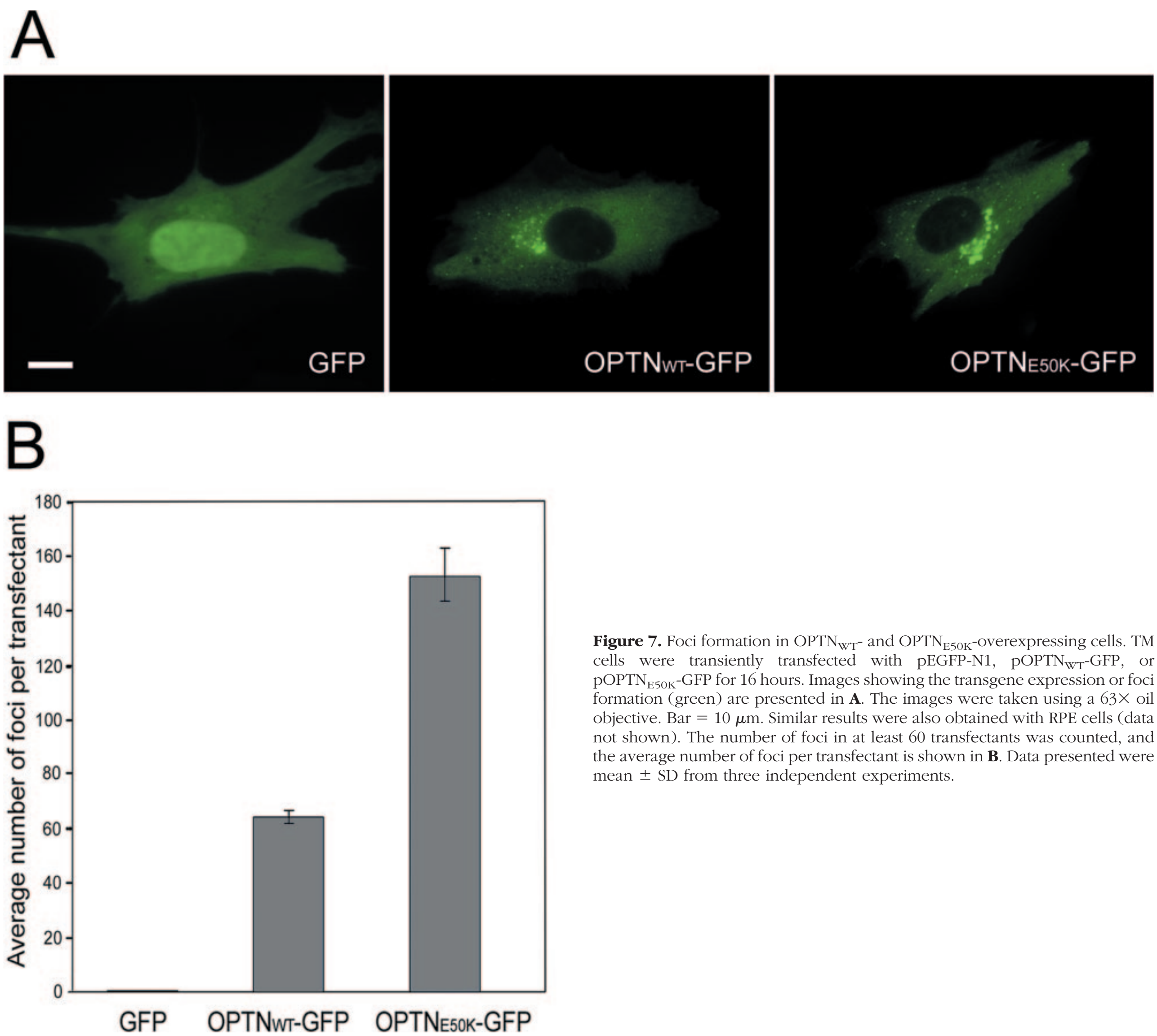

Figure 7. Foci formation in $OPTN_{WT}$- and $OPTN_{E50K}$-overexpressing cells. TM cells were transiently transfected with pEGFP-N1, $pOPTN_{WT}$-GFP, or $pOPTN_{E50K}$-GFP for 16 hours. Images showing the transgene expression or foci formation (green) are presented in **A**. The images were taken using a 63× oil objective. Bar = 10 μm. Similar results were also obtained with RPE cells (data not shown). The number of foci in at least 60 transfectants was counted, and the average number of foci per transfectant is shown in **B**. Data presented were mean ± SD from three independent experiments.

humor of transgenic mice overexpressing wild-type OPTN in the lens.

OPTN is demonstrated to distribute in the cytoplasm of human TM and RPE cells in a diffuse pattern (Figure 2). We also show that the punctate OPTN staining is not distinctly associated with the Golgi apparatus. This finding is disparate from previous observations that OPTN in cell types such as HeLa,[15,17] DU249 hepatoma,[16] dermal fibroblast,[10] and normal rat kidney[17] is localized to the Golgi. The disparity could reflect differences in cell types. However, it is noteworthy that the observations made previously were all through immunocytochemistry. We also performed immunofluorescence, but the lack of major colocalization was carefully scrutinized under a confocal microscope. Furthermore, biochemical analyses after subcellular fractionation indicated that the fractions segregating main portions of OPTN are different from those of the Golgi markers in TM and RPE cells (Figure 3). Most of the OPTN was found in the lighter cytosolic fractions, compatible with the immunofluorescence result. Although there may exist a slight overlap in distribution between OPTN and the Golgi (in fractions 4 and 5, for example; see Figure 3, A and B), it is evident that the endogenous OPTN is by and large not associated with the Golgi. Consistent with this conclusion, no more than marginal Golgi association was seen when TM and RPE cells were transfected to overexpress GFP-tagged wild-type OPTN. The granular structures or foci formed after the transfection, although seemingly residing in close proximity, did not appreciably colocalize with the Golgi complex after sequential confocal scanning (Figure 4).

The addition of C-terminal GFP tag to the wild-type OPTN may be a potential limitation in the transfection experiments. However, many proteins containing tags preserve properties comparable with those of the native or endogenous protein.[34] In the present case, the distribution pattern of $OPTN_{WT}$-GFP is similar to the endogenous protein. The foci are formed not only in cells overexpressing $OPTN_{WT}$-GFP but also in those expressing untagged or FLAG-tagged OPTN (data not shown), indi-

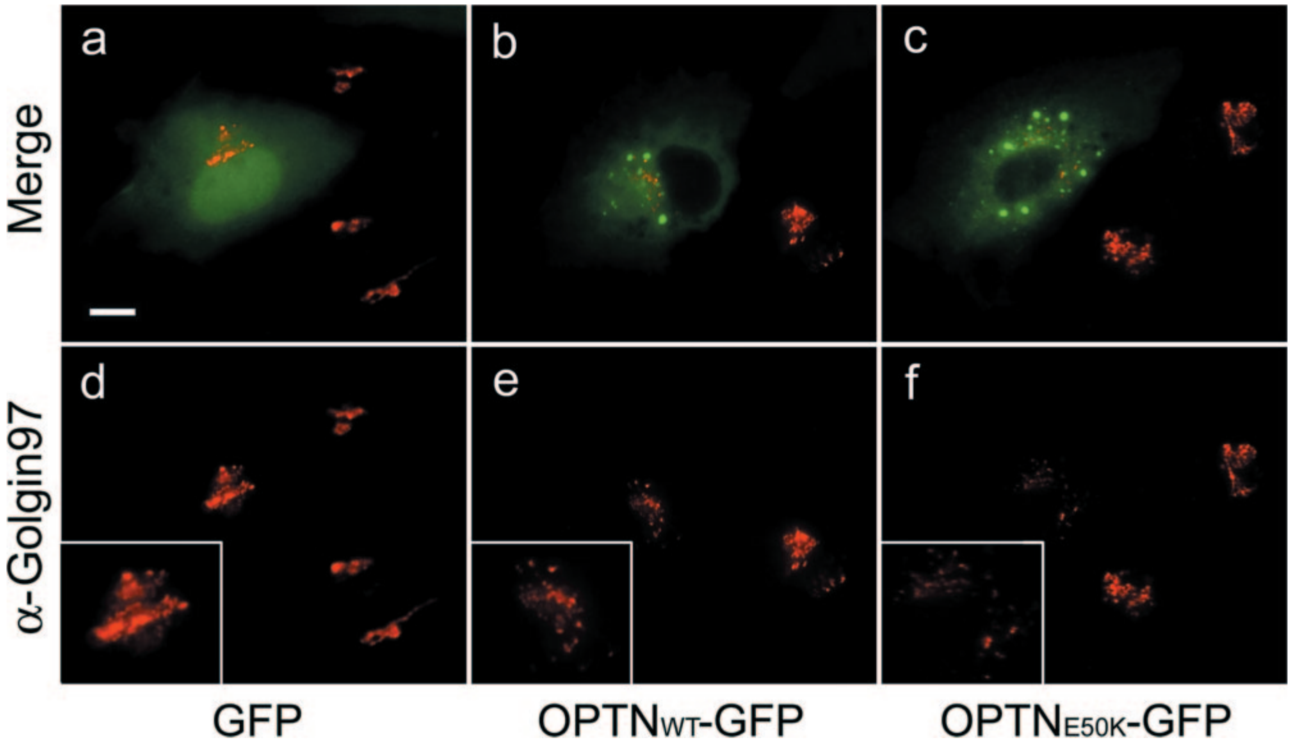

Figure 8. The Golgi integrity in $OPTN_{WT}$- and $OPTN_{E50K}$-overexpressing cells. RPE cells were transfected with pEGFP-N1 (**a** and **d**), $pOPTN_{WT}$-GFP (**b** and **e**), or $pOPTN_{E50K}$-GFP (**c** and **f**). Immunofluorescence staining using anti-golgin97 (α-Golgin97) was performed. The transfected cells are marked by the GFP green fluorescence, and the Golgi staining in the transfected and the neighboring nontransfected cells is seen in red. The merged images are shown in **a–c** and the images for Golgi staining alone are shown in **d–f**. The Golgi apparatus is presented at a higher magnification in **insets**. Images were taken using a 63× oil objective. Bar = 10 μm. Similar results were also obtained using TM cells (data not shown).

cating that the foci formation is not artifactual due to the tag. The size and the number of the foci are different in each cell, but their intracellular localization is consistently around perinuclear regions proximal to the Golgi. The foci also retain the OPTN properties, co-distributing with OPTN-interacting proteins Rab8 and myosin VI.

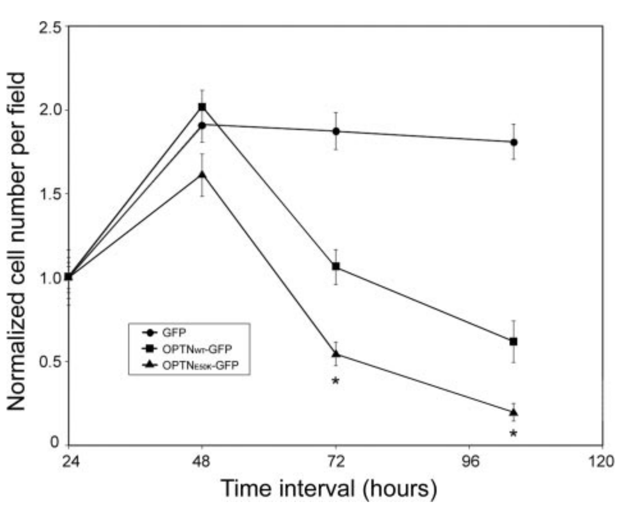

Figure 9. Quantification of cell death after transfection of $OPTN_{WT}$ and $OPTN_{E50K}$. RPE cells transiently expressing GFP alone (-●-), $OPTN_{WT}$-GFP (-■-), or $OPTN_{E50K}$-GFP (-▲-) were grown on glass chamber slides. Images in selected five to six 10× fields, each containing at least 50 transfected cells, were captured after washing at 24-hour intervals for 4 days. The number of transfected cells in the same fields was counted using Metamorph software. Results are expressed as number of transfectants per field (mean ± SEM, n = 6) after normalization to that at the 24-hour time point. Compared with the GFP control, cell death was noticed from 72 hours on in $OPTN_{WT}$- and $OPTN_{E50K}$-overexpressing cells (P < 0.001). *P < 0.01 compared with $OPTN_{WT}$-GFP.

OPTN foci in perinuclear areas in addition co-distributed with a population of transferrin receptor (Figure 5), a well-known marker frequently used in studies of endocytosis and recycling pathways. The transferrin receptor-transferrin complex is internalized through clathrin-coated pits,[35] transported to peripheral early-sorting endosomes, and then recycled back to cell surface either directly or via the pericentriolar recycling endosomes.[36] Transferrin receptor therefore can be found on the cell surface, early endosomes, pericentriolar recycling endosomes, and vesicles that traverse between these organelles. In our preliminary experiments (data not shown), the OPTN foci co-distributed neither with an early endosomal marker EEA1[37] nor with a late endosomal and lysosomal marker LAMP2,[38,39] suggesting that OPTN might be associated with recycling endosomes. Interestingly, the OPTN-interacting Rab8 has been reported to be localized on transferrin-positive recycling endosomes.[40] Myosin VI, a motor protein, has also been found on Rab8-positive vesicles perhaps through OPTN at the perinuclear region,[17] although its presence on recycling endosomes has yet to be verified. The transferrin receptor result thus points to a possibility that OPTN is associated with recycling endosomes, having a role in membrane trafficking. In accordance, the OPTN foci were dispersed on treatment with microtubule depolymerizing agent nocodazole (Figure 6), as was also observed previously in pericentriolar recycling endosomes.[41]

Overexpression of wild-type OPTN results in fragmentation of the Golgi (Figures 4 and 8). Generally, the more prominent the foci formation, the more severe is the Golgi phenotype. The question of why overexpressed OPTN causes an alteration of the Golgi is intriguing. This organelle has a central role in regulation of membrane traffic for secretion and sorting. It receives newly synthesized proteins and membrane from the endoplasmic reticulum and, after modification, repackages them for sorting to the plasma membrane, lysosomes, or secretory granules. The Golgi also receives recycled membrane through endocytosis and sends membrane components back to the endoplasmic reticulum for continued use. Perturbation during the processes for membranes into and out of the Golgi may affect its distribution and morphology.[42] For example, depletion of proteins that are involved in vesicle transport such as p115 and dynein causes fragmentation of the Golgi.[43,44] The fragmentation can also be induced by alteration in microtubule integrity, as was observed with nocodazole[31] or taxol[45] treatment or on overexpression of microtubule-associated proteins, including tau.[28] We speculate that the overexpressed OPTN might affect vesicle trafficking of membranes into the Golgi by overwhelming the critical limiting motor protein components and/or the microtubule network. A balanced level of OPTN in the cells may be vital for the Golgi steady-state organization and integrity.

In this study, we used the GFP technology to investigate the effects of an *OPTN* mutant, E50K, which is prevalent in patients with NPG.[10,12] We demonstrate that the foci formation and fragmentation of the Golgi are much more dramatic in cells expressing $OPTN_{E50K}$ (Figures 7 and 8; Table 1) than those overexpressing $OPTN_{WT}$, strongly implicating E50K as a dominant active mutation. Interestingly, NPG subjects who had E50K mutation were reported clinically to suffer glaucomatous defects more severe than those without this mutation.[46] It is also of note that PROSITE analysis[47] indicates that introduction of $Glu^{50}\rightarrow Lys$ mutation results in no particular changes either in the structure or the conformation of OPTN (data not shown). The E50K mutation may conceivably affect the protein activity or property by other mechanisms such as posttranslational phosphorylation.

Cell death was observed in $OPTN_{WT}$- and $OPTN_{E50K}$-overexpressing cells (Figure 9). As was seen with the foci formation and Golgi alteration, cell loss was also more pronounced in the $OPTN_{E50K}$ cultures. Cell death could be a consequence of fragmentation of the Golgi and/or defects in vesicle trafficking, as has been extensively described in the literature. For instance, Golgi disruption has been shown to be caused by ceramide before its other cellular effects, including detachment before apoptosis.[48] Expression of a caspase cleavage fragment of p115, a vesicle tethering protein, induces fragmentation of the Golgi apparatus and cell death.[49] Mutant Rab8 that impairs docking and fusion of rhodopsin-bearing post-Golgi membranes also causes cell death.[50]

Fragmentation of the Golgi in brain tissues has been reported in patients afflicted with many neurodegeneration diseases, including Alzheimer's disease,[51] amyotrophic lateral sclerosis,[52] Creutzfeldt-Jakob disease,[53] and multiple system atrophy.[54] Glaucoma has also been recognized or classified as a neurodegenerative disorder. In this condition, progressive cell loss in the TM and the retina is well documented.[55,56] We surmise therefore that chronic cell loss resulting from the Golgi fragmentation and/or defective trafficking may be the underlying basis of how the E50K mutation renders the patients predisposed for the glaucoma pathology. Of note, however, is that this hypothesis, derived from findings on TM and RPE cells, has to be tested using NPG-relevant cell types such as retinal ganglion cells.

In summary, the current study presents evidence that implicates a role of OPTN in vesicle trafficking and the Golgi organization. A known genetic mutation (E50K) is demonstrated to potentiate Golgi fragmentation and cause extensive cell death, a key occurrence in POAG. These findings may be of clinical relevance, providing an insight into the possible mechanisms why E50K would exhibit a propensity toward the development of glaucoma.

Acknowledgments

We thank Dr. Daniel Stamer for a generous gift of anti-myocilin antibody, Dr. Robert Weil for a plasmid pcDNA3-NRP, Drs. Paul Knepper and Takahisa Koga for critical review of the manuscript, Martin Tibudan for technical assistance, and Ms. Ruth Zelkha for assistance in expert imaging.

References

1. Bill A: The drainage of aqueous humor. Invest Ophthalmol Vis Sci 1975, 14:1–3
2. Hoyng PFJ, Kitazawa Y: Medical treatment of normal tension glaucoma. Surv Ophthalmol 2002, 47(Suppl 1):S116–S124
3. Anderson DR: Collaborative normal tension glaucoma study. Curr Opin Ophthalmol 2003, 14:86–90
4. Wiggs JL, Allingham RR, Hossain A, Kern J, Auguste J, DelBono EA, Broomer B, Graham FL, Hauser M, Pericak-Vance M, Haines JL: Genome-wide scan for adult onset primary open angle glaucoma. Hum Mol Genet 2000, 9:1109–1117
5. Fan BJ, Wang DY, Lam DS, Pang CP: Gene mapping for primary open angle glaucoma. Clin Biochem 2006, 39:249–258
6. Wang N, Chintala SK, Fini ME, Schuman JS: Activation of a tissue-specific stress response in the aqueous outflow pathway of the eye defines the glaucoma disease phenotype. Nat Med 2001, 7:304–309
7. Sheffield VC, Stone EM, Alward WL, Drack AV, Johnson AT, Streb LM, Nichols BE: Genetic linkage of familial open angle glaucoma to chromosome 1q21–q31. Nat Genet 1993, 4:47–50
8. Stone EM, Fingert JH, Alward WL, Nguyen TD, Polansky JR, Sunden LF, Nishimura D, Clark AF, Nystuen A, Nichols BE, Mackey DA, Ritch R, Kalenak JW, Craven ER, Sheffield VC: Identification of a gene that causes primary open angle glaucoma. Science 1997, 275:668–670
9. Sarfarazi M, Child A, Stoilova D, Brice G, Desai T, Trifan OC, Poinoosawmy D, Crick RP: Localization of the fourth locus (GLC1E) for adult-onset primary open-angle glaucoma to the 10p15–p14 region. Am J Hum Genet 1998, 62:641–652
10. Rezaie T, Child A, Hitchings R, Brice G, Miller L, Coca-Prados M, Heon E, Krupin T, Ritch R, Kreutzer D, Crick PP, Sarfarazi M: Adult-onset primary open-angle glaucoma caused by mutations in optineurin. Science 2002, 295:1077–1079
11. Monemi S, Spaeth G, DaSilva A, Popinchalk S, Ilitchev E, Liebmann J, Ritch R, Heon E, Crick RP, Child A, Sarfarazi M: Identification of a novel adult-onset primary open-angle glaucoma (POAG) gene on 5q22.1. Hum Mol Genet 2005, 14:725–733

12. Sarfarazi M, Rezaie T: Optineurin in primary open angle glaucoma. Ophthalmol Clin North Am 16:529–541, 2003
13. Li Y, Kang J, Horwitz MS: Interaction of an adenovirus E3 14.7-kilodalton protein with a novel tumor necrosis factor α-inducible cellular protein containing leucine zipper domains. Mol Cell Biol 1998, 18:1601–1610
14. Hattula K, Peranen J: FIP-2, a coiled-coil protein, links Huntington to Rab8 and modulates cellular morphogenesis. Curr Biol 2000, 10:1603–1606
15. Schwamborn K, Weil R, Courtois G, Whiteside ST, Israel A: Phorbol esters and cytokines regulate the expression of the NEMO-related-protein, a molecule involved in a NF-κB-independent pathway. J Biol Chem 2000, 275:22780–22789
16. Stroissnigg H, Repitz M, Miloloza A, Linhartova I, Beug H, Wiche G, Propst F: FIP-2, an IκB-kinase-γ-related protein, is associated with the Golgi apparatus and translocates to the marginal band during chicken erythroblast differentiation. Exp Cell Res 2002, 278:133–145
17. Sahlender DA, Roberts RC, Arden SD, Spudich G, Taylor MJ, Luzio JP, Kendrick-Jones J, Buss F: Optineurin links myosin VI to the Golgi complex and is involved in Golgi organization and exocytosis. J Cell Biol 2005, 169:285–295
18. Huber LA, Pimplikar S, Parton RG, Virta H, Zerial M, Simons K: Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. J Cell Biol 1993, 123:35–45
19. Peranen J, Auvinen P, Virta H, Wepf R, Simons K: Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. J Cell Biol 1996, 135:153–167
20. Velier J, Kim M, Schwarz C, Kim TW, Sapp E, Chase K, Aronin N, DiFiglia M: Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. Exp Neurol 1998, 152:34–40
21. Buss F, Spudich G, Kendrick-Jones J: Myosin VI: cellular functions and motor properties. Annu Rev Cell Dev Biol 2004, 20:649–676
22. Rezaie T, Sarfarazi M: Molecular cloning, genomic structure, and protein characterization of mouse optineurin. Genomics 2005, 85:131–138
23. Rezaie T, Waitzman DM, Seeman JL, Kaufman PL, Sarfarazi M: Molecular cloning and expression profiling of optineurin in the rhesus monkey. Invest Ophthalmol Vis Sci 2005, 46:2404–2410
24. El Meskini R, Mains RE, Eipper BA: Cell type-specific metabolism of peptidylglycine α-amidating monooxygenase in anterior pituitary. Endocrinology 2000, 141:3020–3034
25. Hardy KM, Hoffman EA, Gonzalez P, McKay BS, Stamer WD: Extracellular trafficking of myocilin in human trabecular meshwork cells. J Biol Chem 2005, 280:28917–28926
26. North AJ: Seeing is believing? A beginners' guide to practical pitfalls in imaging acquisition. J Cell Biol 2006, 172:9–18
27. Aivazian D, Serrano RL, Pfeffer S: TIP47 is a key effector for Rab9 localization. J Cell Biol 2006, 173:917–926
28. Liazoghli D, Perreault S, Micheva KD, Desjardins M, Leclerc N: Fragmentation of the Golgi apparatus induced by the overexpression of wild-type and mutant human tau forms in neurons. Am J Pathol 2005, 166:1499–1514
29. Jacobson N, Andrews M, Shepard AR, Nishimura D, Searby C, Fingert JH, Hageman G, Mullins R, Davidson BL, Kwon YH, Alward WL, Stone EM, Clark AF, Sheffield VC: Non-secretion of mutant proteins of the glaucoma gene myocilin in cultured trabecular meshwork cells and in aqueous humor. Hum Mol Genet 2001, 10:117–125
30. Lippincott-Schwartz J, Donaldson JG, Schweizer A, Berger EG, Hauri HP, Yuan LC, Klausner RD: Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell 1990, 60:821–836
31. Rogalski AA, Singer SI: Associations of elements of the Golgi apparatus with microtubules. J Cell Biol 1984, 99:1092–1100
32. Valderrama F, Babia T, Ayala I, Kok JW, Renau-Piqueras J, Egea G: Actin microfilaments are essential for the cytological positioning and morphology of the Golgi complex. Eur J Cell Biol 1986, 76:9–17
33. Kroeber M, Ohlmann A, Russell P, Tamm ER: Transgenic studies on the role of optineurin in the mouse eye. Exp Eye Res 2006, 82:1075–1085
34. Michaelson D, Philips M: The use of GFP to localize Rho GTPases in living cells. Methods Enzymol 2006, 406:296–315
35. Mellman I: Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 1996, 12:575–625
36. Sheff DR, Daro EA, Hull M, Mellman I: The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. J Cell Biol 1999, 145:123–139
37. Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A: EEA1 links PI3K function to Rab5 regulation of endosome fusion. Nature 1998, 394:494–498
38. Granger BL, Green SA, Gabel CA, Howe CL, Mellman I, Helenius A: Characterization and cloning of lgp110, a lysosomal membrane glycoprotein from mouse and rat cells. J Biol Chem 1990, 265:12036–12043
39. Uthayakumar S, Granger BL: Cell surface accumulation of overexpressed hamster lysosomal membrane glycoproteins. Cell Mol Biol Res 1995, 41:405–420
40. Ang AL, Fölsch H, Koivisto UM, Pypaert M, Mellman I: The Rab8 GTPase selectively regulates AP-1B-dependent basolateral transport in polarized Madin-Darby canine kidney cells. J Cell Biol 2003, 163:339–350
41. Daro E, van der Sluijs P, Galli T, Mellman I: Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. Proc Natl Acad Sci USA 1996, 93:9559–9564
42. Presley JF, Smith C, Hirschberg K, Miller C, Cole NB, Zaal KJ, Lippincott-Schwartz J: Golgi membrane dynamics. Mol Biol Cell 1998, 9:1617–1626
43. Puthenveedu MA, Linstedt AD: Gene replacement reveals that p115/SNARE interactions are essential for Golgi biogenesis. Proc Natl Acad Sci USA 2004, 101:1253–1256
44. Harada A, Takei Y, Kanai Y, Tanaka Y, Nonaka S, Hirokawa N: Golgi vesiculation and lysosome dispersion in cells lacking cytoplasmic dynein. J Cell Biol 1998, 141:51–59
45. Wehland J, Henkart M, Klausner R, Sandoval IV: Role of microtubules in the distribution of the Golgi apparatus: effect of taxol and microinjected anti-α-tubulin antibodies. Proc Natl Acad Sci USA 1983, 80:4286–4290
46. Aung T, Rezaie T, Okada K, Viswanathan AC, Child AH, Brice G, Bhattacharya SS, Lehmann OJ, Sarfarazi M, Hitchings RA: Clinical features and course of patients with glaucoma with the E50K mutation in the optineurin gene. Invest Ophthalmol Vis Sci 2005, 46:2816–2822
47. Bairoch A, Bucher P, Hofmann K: The PROSITE database, its status in 1997. Nucleic Acids Res 1997, 25:217–221
48. Hu W, Xu R, Zhang G, Jin J, Szulc ZM, Bielawski J, Hannun YA, Obeid LM, Mao C: Golgi fragmentation is associated with ceramide-induced cellular effects. Mol Biol Cell 2005, 16:1555–1567
49. Chiu R, Novikov L, Mukherjee S, Shields D: A caspase cleavage fragment of p115 induces fragmentation of the Golgi apparatus and apoptosis. J Cell Biol 2002, 159:637–648
50. Moritz OL, Tam BM, Hurd LL, Peranen J, Deretic D, Papermaster DS: Mutant Rab8 impairs docking and fusion of rhodopsin-bearing post-Golgi membranes and causes cell death of transgenic Xenopus rods. Mol Biol Cell 2001, 12:2341–2351
51. Gonatas NK, Gonatas JO, Stieber A: The involvement of the Golgi apparatus in the pathogenesis of amyotrophic lateral sclerosis, Alzheimer's disease, and ricin intoxication. Histochem Cell Biol 1998, 109:591–600
52. Stieber A, Gonatas JO, Moore JS, Bantly A, Yim HS, Yim MB, Gonatas NK: Disruption of the structure of the Golgi apparatus and the function of the secretory pathway by mutants G93A and G85R of Cu, Zn superoxide dismutase (SOD1) of familial amyotrophic lateral sclerosis. J Neurol Sci 2004, 219:45–53
53. Sakurai A, Okamoto K, Fujita Y, Nakazato Y, Wakabayashi K, Takahashi H, Gonatas NK: Fragmentation of the Golgi apparatus of the ballooned neurons in patients with corticobasal degeneration and Creutzfeldt-Jakob disease. Acta Neuropathol (Berl) 2001, 100:270–274
54. Sakurai A, Okamoto K, Yaguchi M, Fujita Y, Mizuno Y, Nakazato Y, Gonatas NK: Pathology of the inferior olivary nucleus in patients with multiple system atrophy. Acta Neuropathol (Berl) 2002, 103:550–554
55. Alvarado J, Murphy C, Juster R: Trabecular meshwork cellularity in primary open angle glaucoma and nonglaucomatous normals. Ophthalmology 1984, 91:564–579
56. Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ: Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. Invest Ophthalmol Vis Sci 1995, 36:774–786