Angiopoietin receptor TEK mutations underlie primary congenital glaucoma with variable expressivity

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The molecular etiology of PCG is only partially understood, as only a few genes responsible for PCG have been identified (6, 10–14). Mutations in CYP1B1, which encodes a cytochrome P450 enzyme, is the most common cause of autosomal recessive PCG worldwide, accounting for up to 87% of familial cases in some inbred populations but only 25%–27% in populations with heterogeneous ethnicity (5, 9, 15–18). With CYP1B1 mutations account-

Primary congenital glaucoma (PCG) is a devastating eye disease and an important cause of childhood blindness worldwide. In PCG, defects in the anterior chamber aqueous humor outflow structures of the eye result in elevated intraocular pressure (IOP); however, the genes and molecular mechanisms involved in the etiology of these defects have not been fully characterized. Previously, we observed PCG-like phenotypes in transgenic mice that lack functional angiopoietin-TEK signaling. Herein, we identified rare TEK variants in 10 of 189 unrelated PCG families and demonstrated that each mutation results in haploinsufficiency due to protein loss of function. Multiple cellular mechanisms were responsible for the loss of protein function resulting from individual TEK variants, including an absence of normal protein production, protein aggregate formation, enhanced proteasomal degradation, altered subcellular localization, and reduced responsiveness to ligand stimulation. Further, in mice, hemizygosity for Tek led to the formation of severely hypomorphic Schlemm’s canal and trabecular meshwork, as well as elevated IOP, demonstrating that anterior chamber vascular development is sensitive to Tek gene dosage and the resulting decrease in angiopoietin-TEK signaling. Collectively, these results identify TEK mutations in patients with PCG that likely underlie disease and are transmitted in an autosomal dominant pattern with variable expressivity.

Introduction
Glaucoma is a group of heterogeneous diseases that is characterized by a chronic degenerative optic neuropathy, affecting more than 60 million people worldwide (1–3). Primary congenital glaucoma (PCG) (OMIM 231300) is a severe form of the disease with unclear etiology and is characterized by infant/early-childhood ocular hypertension, enlarged eye globes (buphthalmos), and optic neuropathy, which can result in vision loss and blindness—often
despite treatment (4–6). Indeed, PCG accounts for 18% of children enrolled in institutions for the blind worldwide (7). PCG occurs in all ethnic groups, but the disease incidence varies according to ethnic background, ranging from 1:1,250 in inbred populations to 1:30,000 in populations with heterogeneous ethnicity (6, 8–12). Families can exhibit autosomal recessive or dominant inheritance, although the majority of cases appear to be sporadic (5, 6, 10–14).

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It is well recognized that elevated intraocular pressure (IOP) is the key risk factor contributing to glaucoma, including PCG (1–3, 6, 19, 20). The elevated pressure in PCG is believed to result from defects in the aqueous humor outflow (AHO) pathway, rather than for PCG in less than a third of patients with heterogeneous ethnicity, alternate mechanisms must underlie the majority of these other cases. Furthermore, the mechanism of PCG due to mutations in CYP1B1 is not clearly understood.

Figure 1. Pedigree structures and TEK mutations identified in 10 families. (A) Pedigrees of the 10 families with TEK mutations. Specific mutations in TEK are listed below the different pedigrees, with those family members annotated as +/-M. Affected individuals are indicated by solid black/grey symbols (black for early-onset disease and grey for late-onset disease; see Table 1 for details). Note: White symbols do not exclude an undiagnosed late-onset disease. Unknown genotypes are marked as ??/?. (B) In family 2, a TEK SNP was inconsistent with father-to-son transmission (upper panel). Copy number variation analysis identified a 56-kb deletion that was passed from the unaffected father (annotated as 2; lower panel) to the unilaterally affected son (annotated as 3). The physical location of the TEK gene is shown, with arrows indicating the deletion breakpoints within introns 1 and 4. CN, copy number. (C) A schematic illustration of the TEK receptor with the locations of identified variants. Eight of 10 variants were identified in the ectodomain of the receptor. Tyr1102/Tyr1108 are the primary tyrosine phosphorylation sites in the C-terminal tail of the TEK receptor that initiate downstream signaling events (35, 36).
an increase in aqueous humor (AH) production (5, 6, 14). Ocular anterior chamber fluid (AH) is produced by the ciliary body and drained mainly through Schlemm’s canal (SC) and uveoscleral pathways (21, 22). An imbalance in fluid homeostasis results in elevated IOP. In humans, 80% of fluid drainage is conducted through the conventional, or trabecular, AHO pathway (23). AH first passes through the trabecular meshwork (TM) before entering the lymphatic-like SC for drainage (22, 24, 25). The remaining 20% of fluid egresses via the unconventional uveoscleral tract pathway, which includes the extracellular spaces within the iris, ciliary muscle, and sclera (21).

The angiopoietin receptor TEK (tunica interna endothelial cell kinase, also known as Tie2) is a receptor tyrosine kinase and regulates vascular homeostasis through its auto- and transphosphorylation (26–29). The primary ligand, angiopoietin-1 (ANGPT1), is expressed by pericytes and other vascular supporting cells, and exerts its proangiogenic and vascular stabilizing effects through activating TEK. A second ligand, ANGPT2, is secreted by the endothelium and acts as a context-dependent partial agonist of TEK (26, 27, 29, 30).

In the eye, the TEK receptor is highly expressed in the SC endothelium (24, 25). Recently, we discovered that angiopoietin-in-TEK signaling is required for SC development in mice (31). Deletion of TEK or both major angiopoietin ligands after embryonic day 16.5 circumvents embryonic lethality and results in developmental loss of SC, extremely elevated IOP, rapid and complete retinal ganglion loss, and glaucoma (31). Despite the critical role of this signaling pathway for SC development in mice, the relevance of this pathway in human disease has not, to our knowledge, been previously reported.

Here, we describe human mutations in TEK identified in a PCG cohort of 189 families that do not carry mutations in the known disease-causing genes CYP1B1 (16–18), LTPB2 (32), FOXCI (33), and MYOC (13). We identified 10 heterozygous novel/rare TEK variants as disease-causing mutations and assessed their effects on TEK function. We also determined in mice whether haploinsufficiency for Tek is sufficient to result in abnormal SC development and elevation of IOP. In addition to the identification of a pathway for human glaucoma, we assign key functional roles to specific domains of the TEK receptor.

### Results

#### TEK mutations are identified in PCG families with unknown etiology.

To identify novel PCG causative genes, we applied whole exome sequencing to a multiethnic cohort of PCG families with unknown molecular etiology. We performed an initial exome sequencing analysis on 21 affected individuals from 20 families including 2 affected individuals in family 1 (Figure 1A) and 19 sporadic cases. Under a monogenic recessive inheritance model, filtering for novel nonsynonymous, homozygous, or compound heterozygous variants in a single gene identified no candidate genes. An analysis of 2 complete family trios also excluded the possibility of de novo mutations being causative for the phenotype in these 2 families. We initially focused our analysis on a family demonstrating direct parent-to-child transmission for a known SNP (see Sanger sequencing traces in Figure 1B). A TEK variant identified in this family was confirmed in 20 additional families of PCG families (Figure 1A).

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We reasoned that loss-of-function (LoF) mutations in TEK could be involved in the etiology of PCG, given our findings of a PCG-like phenotype in Angpt1/Angpt2 and Tek cKO mice (31). Therefore, we sought additional novel/rare TEK variants in a collaborative multi-ethnic cohort totaling 209 individuals in 189 families. In family 2 (Figure 1A), we screened for TEK variants by Sanger sequencing and identified an inconsistency in father-to-son transmission for a known SNP (see Sanger sequencing traces in Figure 1B) that suggested the presence of a deletion. Copy number variation analysis was performed on the family trio using Affy-

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### Table 1. TEK variants identified in 10 PCG families

<table>
<thead>
<tr>
<th>ID</th>
<th>Ethnicity</th>
<th>Sex</th>
<th>Age at onset</th>
<th>Age of unaffected carrier(s)</th>
<th>Chromosome position</th>
<th>Exon(s)</th>
<th>Coding DNA mutation</th>
<th>Protein alteration</th>
<th>ExAC variant count</th>
<th>ExAC total alleles</th>
<th>ExAC ethnically matched alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lat</td>
<td>M &amp; F</td>
<td>Infant &amp; 4 months</td>
<td>–</td>
<td>27180257</td>
<td>7</td>
<td>c.921C&gt;A</td>
<td>NMD or p.Y307*</td>
<td>Novel</td>
<td>121,226</td>
<td>11,524</td>
</tr>
<tr>
<td>2</td>
<td>Rom</td>
<td>M</td>
<td>n/a</td>
<td>n/a</td>
<td>g.(27116823_27118707) (27170308_27172125)del</td>
<td>2–4</td>
<td>c.5362del</td>
<td>p.T19_R210del</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Eur (Am)</td>
<td>M &amp; F</td>
<td>2 yr &amp; n/a</td>
<td>30 yr &amp; 3 yr</td>
<td>27197520</td>
<td>12</td>
<td>c.1832A&gt;G</td>
<td>p.Y611C</td>
<td>Novel</td>
<td>121,268</td>
<td>66,710</td>
</tr>
<tr>
<td>6</td>
<td>Eur (I)</td>
<td>M</td>
<td>5 mo</td>
<td>39 yr</td>
<td>27215554</td>
<td>18</td>
<td>c.950G&gt;T</td>
<td>NMD or p.G984*</td>
<td>Novel</td>
<td>121,294</td>
<td>66,658</td>
</tr>
<tr>
<td>7</td>
<td>Eur (Au)</td>
<td>M</td>
<td>Birth</td>
<td>54 yr</td>
<td>27172478</td>
<td>5</td>
<td>c.760+2T&gt;C</td>
<td>NMD or p.A254Gfs*3</td>
<td>Novel</td>
<td>119,718</td>
<td>65,966</td>
</tr>
<tr>
<td>8</td>
<td>Eur (Au)</td>
<td>F</td>
<td>Birth</td>
<td>38 yr</td>
<td>27228305</td>
<td>22</td>
<td>c.3300+2del</td>
<td>p.Y1068Rfs*3</td>
<td>Novel</td>
<td>120,580</td>
<td>66,356</td>
</tr>
<tr>
<td>9</td>
<td>Eur (Am)</td>
<td>M</td>
<td>Birth</td>
<td>n/a</td>
<td>27204931</td>
<td>14</td>
<td>c.2232dupC</td>
<td>NMD or p.K745fs*76</td>
<td>Novel</td>
<td>121,392</td>
<td>66,726</td>
</tr>
<tr>
<td>10</td>
<td>Eur (Am)</td>
<td>F &amp; M</td>
<td>2 mo &amp; birth</td>
<td>25 yr</td>
<td>27168576</td>
<td>3</td>
<td>c.448G&gt;T</td>
<td>NMD or p.E150*</td>
<td>Novel</td>
<td>115,554</td>
<td>63,256</td>
</tr>
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</table>

Ethnicity: Lat, Latino; Rom, Roman; Eur, European; Afr, African; I, Italian; Am, American; Au, Australian. Sex: M, Male; F, Female. Eyes affected: U, unilateral; B, bilateral. n/a, data not available. NMD, nonsense-mediated decay of mRNA transcript predicted (no protein product). Chromosome position in accordance with GRCh37/hg19 assembly. TEK mRNA reference sequence NM_000459.4. TEK protein reference sequence NP_000450.2.
metrix SNP 6.0 arrays, which identified a 56-kb deletion encompassing TEK exons 2–4 (p.T19_R210del) that was passed from the father (unknown affection status) to the unilaterally affected son (Figure 1B). In total in our cohort, whole exome and direct Sanger father (unknown affection status) to the unilaterally affected son passing metrix SNP 6.0 arrays, which identified a 56-kb deletion encompassing TEK exons 2–4 (p.T19_R210del) that was passed from the father (unknown affection status) to the unilaterally affected son.

Nine of these 10 mutations (p.Y307*, p.T19_R210del, p.C233Y, p.Y307*, p.K745fs, p.G984*, c.760+2>T>C, and c.3300+2>delT) were absent from publicly available exome databases (Exome Sequencing Project [NHLBI-ESP, http://evs.gs.washington.edu/EVS/], 6,500 exome sequences; Exome Aggregation Consortium [ExAC, http://exac.broadinstitute.org], 60,706 exome sequences) and 203 in-house control unrelated same-batch exome-sequenced samples. In all cases, no computational null alleles (Figure 2A). The highest allele frequency observed for the wild-type allele was extracted from the 7 PCG exomes and compared with the average read depth obtained from a number of unrelated same-batch exome-sequenced samples. In all cases, no evidence for additional exon-spanning duplications or deletions could be found (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI85830DS1).

Functional prediction for splicing donor variants were performed using Human Splicing Finder version 3.0 (HSF3.0). A score of more than 65 for position weight matrices (HSF) and more than 3 for maximum entropy (MaxEnt) is predictive for a functional splice site. Moreover, if the prediction score (between WT and mutant) is under −10% for HSF and −30% for MaxEnt, the tool considers that the mutation affects the splice site. See the predicted effects of the disruption of splice donor sites in Supplemental Figure 3.

### Table 2. Results of the TEK splicing donor variants analysis

<table>
<thead>
<tr>
<th>Splicing donor variant</th>
<th>Prediction algorithm</th>
<th>WT</th>
<th>Mutant</th>
<th>Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.760+2&gt;T&gt;C</td>
<td>HSF</td>
<td>94.19</td>
<td>67.35</td>
<td>−28.5, WT site broken</td>
</tr>
<tr>
<td></td>
<td>MaxEnt</td>
<td>10.45</td>
<td>2.7</td>
<td>−74.16</td>
</tr>
<tr>
<td>c.3300+2&gt;delT</td>
<td>HSF</td>
<td>99.05</td>
<td>49.22</td>
<td>−50.31, WT site broken</td>
</tr>
<tr>
<td></td>
<td>MaxEnt</td>
<td>11</td>
<td>−2.48</td>
<td>−122.55</td>
</tr>
</tbody>
</table>

### Table 3. Functional prediction results for missense TEK variants

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SIFT: TEK orthologs</td>
<td>64, 64, 63</td>
<td>Affect protein function, 0.01</td>
<td>Affect protein function, 0.02</td>
<td>Affect protein function, 0.0</td>
</tr>
<tr>
<td>SIFT: TEK family</td>
<td>141, 139, 140</td>
<td>Affect protein function, 0.0</td>
<td>Tolerated, 0.11</td>
<td>Affect protein function, 0.0</td>
</tr>
<tr>
<td>SIFT: BlinkB</td>
<td>102, 106, 172</td>
<td>Affect protein function, 0.0</td>
<td>Tolerated, 0.44</td>
<td>Affect protein function, 0.0</td>
</tr>
<tr>
<td>PolyPhen-2</td>
<td>n/a</td>
<td>Probably damaging, 1.0</td>
<td>Benign, 0.298</td>
<td>Probably damaging, 1.0</td>
</tr>
<tr>
<td>FoldX average ddG, Kcal/mol (SD)</td>
<td>n/a</td>
<td>7.52 (3.48)</td>
<td>0.93 (0.004)</td>
<td>2.02 (0.020)</td>
</tr>
</tbody>
</table>

*Sequences may not be diverse enough for confident prediction.

Four mutations (p.E150*, p.Y307*, p.K745fs, and p.G984*) are predicted to be null alleles likely to result in mRNA degradation via nonsense-mediated decay or truncated proteins (see predicted truncated proteins in Supplemental Figure 2A; EnzymeX [http://nucleoebtes.com/EnzymeX/] was used for predictions). Furthermore, the in silico predictive tool Human Splicing Finder (HSF 3.0, http://www.umd.be/HSF3/HSF.html) indicated that the two invariant splice donor mutations (c.760+2>T>C and c.3300+2>delT) affect splicing and result in functionally null proteins (Table 2 and Supplemental Figure 3). The strong enrichment for LoF mutations in our PCG cohort (6 of 189) compared with those observed in the ExAC database (2 of 60,706 individuals; \(P = 2.3 \times 10^{-14}\); odds ratio = 995.2 [95% confidence interval = 199.5–4,963.0]; see Methods for details), supports a role for TEK mutations in PCG pathogenesis. Additionally, according to analysis of evolutionary conservation and in silico predictive tools (Polymorphism Phenotyping v2 [PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/]; Sorting Intolerant from Tolerant [SIFT, http://sift.bii.a-star.edu.sg/]; and FoldX [http://foldx.crg.es/, http://foldxysara.switchlab.org/]), 2 of the 3 missense mutations are also expected to affect protein function (Table 3 and Supplemental Figures 4–6).

**TEK mutations identified in PCG patients exhibit LoF.** We experimentally tested the functional impact of PCG-associated TEK variants in cell-based assays to determine their effect on TEK protein production, phosphorylation, and trafficking within the cell. As mutations were identified sequentially, we performed analysis of the first 5 mutations discovered in our primary cohort. In our system, WT TEK protein underwent normal baseline autophosphorylation when expressed in HEK293 cells (Figure 2A). As predicted, the p.Y307* mutant cDNA (identified in family 1) did not produce a full-length intact protein with C-terminal FLAG tag, as the introduced stop codon is upstream of the tag (Figure 2A and Supplemental Figure 7). To test the possibility that the predicted p.Y307* mutant protein is produced and secreted into the medium, we generated a TEK306-Fc–expressing vector. TEK306-Fc protein, which contains the Ig1, Ig2, EGF1, and EGF2 domains, was detected in the medium (Supplemental Figure 2B and Supplemental Figure 7).

The p.T19_R210del (ΔT_R, family 2) and p.C233Y (family 3) mutations resulted in distinctly reduced protein levels accompanied by negligible autophosphorylation, making them functionally null alleles (Figure 2A). Interestingly, the p.K294N (family 2578 jci.org   Volume 126   Number 7   July 2016
investigate this possibility, we generated a p.C224S mutant and found that mutation of this paired cysteine led to a similar reduction in protein abundance (Figure 2B, right panels), confirming the critical importance of the disulfide bond for proper folding and subsequent expression.

Receptor tyrosine kinases mediate their specific signals through distinct tyrosine residues (28), and for TEK-mediated signaling the cytoplasmic C-terminal Tyr1102 and Tyr1108 residues play a central role (27, 35, 36). To investigate phosphorylation at individual tyrosine residues, we used mass spectrometry together with stable isotope labeling in cell culture (SILAC; Figure 2C and Supplemental Figure 8). While global phosphorylation of the p.K294N mutant was normal, SILAC revealed a 10-fold reduction in Tyr1102 phosphorylation (Figure 2C–E). This site is known to mediate signaling through p85-PI3K (35, 36), suggesting that the p.K294N mutation results in reduced p85-PI3K signaling activity. Based on the critical importance of this site for TEK function (35), we propose that p.K294N is a LoF allele. In contrast, no effect on basal phosphorylation (i.e., ligand-independent phosphorylation) was observed in the p.Y611C mutant (Figure 2C).

Deletion of exons 2–4 (p.T19_R210del, ΔT_R) results in loss of the terminal 4 aa of the signal peptide sequence (MDSLASLVLCGVSLLLSGGTVEG) and the entire Ig1 and Ig2 domains. To determine whether the loss of 4 aa from the signal peptide and/or loss of the Ig1 domain alone is sufficient to cause receptor degradation, we tested a natural splice variant (ENST00000519097) that lacks exon 2—encoding the terminal 5 aa of the signal peptide as well as the first Ig domain (Ig1). This splice variant (Ig1 del) was expressed and phosphorylated at normal levels (Figure 2B). Conversely, a form of TEK mutant carrying an in-frame somatic deletion of exon 3 and part of exon 4 (aa 122–165 of the Ig2 domain [Ig2 del]) (34) was poorly expressed and phosphorylated, indicating that the Ig2 domain is important for TEK stability and function (Figure 2B, left panels).

As Cys233 forms a disulfide bridge with Cys224 in the EGF1 domain (Supplemental Figures 4 and 5), we hypothesized that the p.C233Y variant may cause misfolding of the protein, due to the disruption of the disulfide bond, followed by degradation. To investigate this possibility, we generated a p.C224S mutant and found that mutation of this paired cysteine led to a similar reduction in protein abundance (Figure 2B, right panels), confirming the critical importance of the disulfide bond for proper folding and subsequent expression.

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Figure 2. TEK variants lead to reduced autophosphorylation. (A) Western blotting of WT and variant TEK expressed in HEK293 cells, following immunoprecipitation with Flag antibody. (B) The effect of deleting immunoglobulin-like domain (left), and the effect of breaking the disulfide bond formed between C233 and C224 in the EGF I domain (right). (C) Quantitative phosphorylation analysis of each tyrosine residue in the kinase domain (Tyr897, Tyr954, and Tyr976) and C-tail (Tyr1102 and Tyr1108) using SILAC. (D and E) Representative MS1 spectrum of a peptide (KTVNYTTYEKT) derived from either WT (red, light-labeled) or p.K294N mutant (blue, heavy-labeled). Tyrosine residues in this peptide correspond to Tyr1102 and Tyr1108. The spectrum of phosphorylated peptides (KTpYVNTTLpYEKT) is shown in D. The spectrum of nonphosphorylated peptides (KTYVNTTLYEKT) is shown in E. IgI del, natural variant lacking Ig1 domain; ΔT_R, PCG variant lacking Ig1 and Ig2 domain (p.T19_R210del); IgII del, disease-associated variant lacking Ig2 domain (34); KD, an artificial kinase-dead mutant (D982A substitution, disrupting DFG motif of kinase domain). Note that both the IgI del variant and the IgII del mutant were not identified in PCG cohorts. All blotting data are representative of more than 3 biological replicates.
As portions of the ectodomain are responsible for ligand binding (37, 38), subsequent multimerization (38), and subcellular localization (39–41), we predicted that the mutations in these regions could weaken or abolish ligand-mediated activation and signal transduction through the intracellular catalytic domain. Anticipating that TEK ectodomain mutations may affect ligand-mediated signaling, we expressed TEK variants in HUVECs and examined their subcellular localization after ligand stimulation. WT TEK was diffusely expressed on the membrane of resting cells (Figure 3A, control WT). When treated with ANGPT1, a major TEK agonist, WT TEK localized to cell-cell junctions and subsequently became internalized (Figure 3A and Supplemental Figure 9), as previously reported (39, 40). The p.K294N mutant also followed this dynamic receptor translocation. However, other mutants were unable to respond to ANGPT1 stimulus. p.T19_R210del formed intracellular aggregates, and p.C233Y was not found at the cell membrane (Figure 3A).

WT protein is typically detected within the soluble fraction of a cell lysate (Figure 3B), whereas mutant proteins can form aggregates inside cells and are found within the insoluble fraction. Consistent with subcellular localization studies, p.T19_R210del was found in the insoluble fraction (Figure 3B, ΔT_R). Our results clearly demonstrate that the deletion of Ig1 and Ig2 domains in ΔT_R reduces protein solubility, leading to protein aggregate formation, making this allele functionally null. The IgII deletion mutant was found in both fractions, and the p.C233Y mutant, although not insoluble, was less abundant, suggesting that solubility was not the cause of poor expression (Figure 3B). Other variants were largely found in the soluble fraction (Figure 3B).

To determine whether proteasomal degradation underlies the reduced levels of protein, we treated cells expressing the p.T19_R210del or p.C233Y mutant with the proteasomal inhibitor MG132. The inhibitor slightly increased WT protein expression in the soluble fraction. More prominently for WT and p.C233Y, there was a marked increase in insoluble protein following MG132 treatment (Figure 3C). These data suggest that TEK abundance is tightly regulated by proteasome-mediated degradation and that structural defects, such as p.C233Y, lead to enhanced protein degradation through this mechanism.

Interestingly, while the p.Y611C mutant is properly localized to the plasma membrane in resting cells, upon ANGPT1 stimulation it did not follow the normal course of dynamic clustering, junctional localization, and internalization, suggesting that this variant is unable to respond to ligand (Figure 3A, Y611C). In order to measure changes in ligand-mediated TEK phosphorylation, we cotransfected cells with protein tyrosine phosphatase recepto-
tor type B (PTPRB) to reduce baseline phosphorylation of coexpressed TEK (42). ANGPT1 stimulated the phosphorylation of WT TEK but not p.Y611C, further suggesting a defect in ligand-mediated activation (Supplemental Figure 10).

We also experimentally tested the functional impact of the two splice donor site mutations, c.760+2T>C (family 7) and c.3300+2delT (family 8), in cell-based exon trapping splicing assays to determine their effect on mRNA splicing (Figure 4).

In this system, the c.760+2T>C mutation eliminated the normal 5′ splice donor site of exon 5, which led to a cryptic splice site 21 bp into intron 5 (Figure 4, A and C). As the additional 21 bp sequence incorporated an in-frame termination codon into exon 5, it was expected to result in mRNA transcript destruction by the nonsense-mediated decay pathway or a severely truncated protein product (p.A254Gfs*3). In contrast, the c.3300+2delT splice donor site mutation resulted in complete skipping of exon 22, effectively removing 100 bp from the mRNA transcript and changing the codon reading frame by which the last exon would be translated (Figure 4, B and C). As a result, exon 23 would encode 2 different amino acid residues (Pro-Thr), followed by a termination codon instead of the normal 57 C-terminal residues (p.Y1068Pfs*3). Consequently, the mRNA would likely escape destruction by the nonsense-mediated decay pathway or encode proteins lacking at least a functional intracellular domain (See Supplemental Figure 2).

Collectively, our results have demonstrated that 7 of 10 TEK mutations identified in our PCG cohort lead to loss of functional protein by multiple cellular mechanisms — absence of intact protein production (p.Y307*, p.T19_R210del, c.760+2T>C [p.A254Gfs*3], and c.3300+2delT [p.Y1068Pfs*3]), enhanced proteasomal degradation (p.C233Y), impaired phosphorylation of key tyrosine residue (p.K249N), altered subcellular localization and reduced ligand responsiveness (p.Y611C), and protein aggregate formation (p.T19_R210del). We reason that the p.G984* (family 6), p.K745fs (family 9), and p.E150* (family 10) mutations are also likely LoF alleles, as their mRNA transcripts will either be destroyed by the nonsense-mediated decay pathway or encode proteins lacking at least a functional intracellular domain (See Supplemental Figure 2).

TEK haploinsufficiency results in abnormal AHO structure and elevated IOP. After proving that the heterozygous TEK mutations resulted in nonfunctional proteins, we hypothesized that TEK haploinsufficiency is sufficient to cause impaired SC development and ocular hypertension in mice. Using mice harboring a conditional by inversion allele (Te kCOIN mice) (43), we analyzed AHO drainage pathways in Te k-hemizygous and conditional knockout mice. Confocal microscopy revealed that Te k-haploinsufficient mice developed a severely hypomorphic canal with convolutions and focal narrowing, while SC was normal in control mice (Figures 5–7). SC
mmHg, Tek+/− 15.4 mmHg, P = 0.0046; Figure 5C and Supplemental Figure 11). The IOP levels were modestly correlated with the degree of SC hypomorphism (r = 0.3703, P < 0.01). These results demonstrate that reduced TEK signaling causes developmental defects of the AHO structure, a key feature of PCG, and correlates with elevated IOP.

Discussion

Ocular hypertension has been identified as the most important risk factor in human glaucoma, and current glaucoma therapy is focused on lowering IOP using pharmacological or surgical approaches (1–3, 20, 44, 45). While IOP is influenced by both production and drainage of the AH, defects in anterior chamber outflow have been identified as the source of ocular hypertension in glaucoma patients, including PCG patients (5, 46). Despite evidence that vascular growth factors are essential for SC development and AH outflow (25, 31, 47), links between these pathways and human glaucoma have not been identified. Herein, we describe 10 heterozygous novel/rare protein-altering mutations in TEK as a cause for PCG. Furthermore, our findings provide evidence that TEK gene dosage is critical for the proper development of AHO pathways, and ~50% reduced TEK signaling is sufficient to lead to defective SC development and compromised AH drainage in mice as well as in patients (Figure 8).

While PCG has clear genetic links, identification of causative alleles is complicated by variable disease penetrance and expressivity (5, 9, 12). Families identified in our patient cohort were no exception. TEK variants were heterozygous in all affected individuals, and the heterozygous disease allele was vertically transmitted from the affected parent to the affected child in two families (families 1 and 10), clearly indicating dominant transmission of the disease. Interestingly, the development of glaucoma in later decades in two mutation carriers in family 10 (one inferred) and the frequent unilateral involvement observed in mutation carriers (6 of 12 in our cohort with a TEK mutation versus 34 of 209 in our whole cohort) suggest that TEK-related disease can exhibit variable severity and age of onset (Figure 1 and Table 1). This highly variable expressivity may explain the high frequency of carriers without a typical early-onset PCG phenotype in family members. It was not possible to carefully examine many of the parents of the affected probands with PCG for evidence of glaucoma or raised IOP, and given the geographic and historical nature of some of the samples, it is not currently possible to gather this information. With the limited availability of human data, it is difficult to clearly distinguish variable expressivity from reduced penetrance. However, the biological plausibility is underscored by the mouse data clearly showing that haploinsufficiency for Tek is sufficient to lead to functional defects of the AHO pathway with variable expressivity. Therefore, we propose an autosomal dominant model with variable expressivity, consistent with other ocular disorders of developmental origin, such as those caused by mutations in FOXC1, MYOC, PAX6, and OPA1 (6, 10, 48–52). Variable expressivity may be explained by stochastic developmental events.
in SC formation (25) or oligo-/digenic inheritance. Although we have screened 19 “candidate genes,” including the signaling components of the ANGPT-TEK pathway (TEK, ANGPT1, ANGPT2, TIE1, ANGPT4, and PTPRB), genes expressed during SC development (PROXI, FLT4/VEGFR3, and VEGFC), and genes linked to various developmental glaucomas (FOXC2, CYPIBI, MYOC, FOXC1, OPTN, ASB10, TBK1, WDR36, OPA1, and NTF4), we did not identify possible pathogenic variants as a second allele for digenic inheritance. Our results also suggest that ascertaining future PCG subjects for genetic studies should incorporate thorough, longitudinal ophthalmic examinations of reportedly normal parents of affected probands, as they may have subclinical indicators of PCG/glaucoma.

The analysis of PCG mutations in TEK advances our understanding of structure-function relationships within the receptor. Interestingly, all the deletion and missense mutations identified in our PCG cohort were located in the ectodomain and are LoF. The location of PCG mutations is strikingly different from that of the gain-of-function (GoF) TEK mutations that have been linked with hereditary and sporadic venous malformations (VMs) (34, 53, 54). These VM mutations are located solely in the intracellular domain and result in enhanced kinase activity. Furthermore, our cell-based assays have identified new functional roles for various domains. The Ig2 domain, which functions as a ligand-binding domain (37, 38), is also critical for stable protein expression, as loss of this Ig domain in p.T19_R210del and IgII del leads to reduced protein levels. The mutation in the EGF1 domain highlights the critical role of cysteine residues, which are needed to disulfide cross-link the protein for stability (38, 55). Notably, the mutation in the membrane-proximal fibronectin type III (FN3) domain, which appears in ~40% of all receptor tyrosine kinases (ref. 28 and the Human Protein Reference Database [HPRD], http://www.hprd.org), sheds light on its novel role in ligand-mediated TEK receptor activation. The FN3 domain in the Eph family tyrosine kinase receptors directly stimulates intermolecular interactions to cluster the receptor into higher-order multimers at the plasma membrane (56). These Eph clusters act as efficient signaling centers to trigger cell responses and propagate phosphorylative signals. Although the TEK FN3 crystal structure is currently unavailable, the lack of membrane response following ANGPT1 stimulation as observed in HUVECs (Figure 3A) is likely due to impaired signaling activities.

GoF mutations in patients with VMs exhibit variable expressivity and led to the hypothesis that tight regulation of TEK activity is needed for vascular development (34, 54). Our findings that hemizygosity for TEK is sufficient to cause disease in mice and that heterozygosity for LoF mutations is associated with PCG of varying severity in patients suggest, for the first time to our knowledge, that there exists a dosage sensitivity for reduced activity of the receptor. It is reported that the conventional outflow path for AH drainage (i.e., SC) plays a less important role in mice than in humans, underscoring the significance of finding elevated IOP in the hemizygous mouse model and suggesting similar structural defects in SC would cause more severe disease in patients (57–59). Finally, our data indicate that TEK "dose" might be particularly important for proper development of the vasculature of the anterior chamber of the eye, suggesting that regulation of TEK signal strength may be a therapeutic option.

In summary, we have identified TEK mutations in patients with PCG that follow a dominant inheritance pattern with variable expressivity. Our experimental results further demonstrate that defects in the AHO pathway caused by reduced TEK signaling constitute a new mechanism of PCG disease in humans. It is likely
that investigating additional genes involved in the formation and maintenance of the AHO pathways will provide further insights to our understanding of this devastating eye disease and aid in the development of new therapeutic strategies for glaucoma.

Methods

Human study participants. PCG was defined by the following characteristics: (a) age at onset less than 3 years, (b) increased corneal diameter greater than 10 mm accompanied by corneal edema and/or Haab striae, and (c) IOP greater than 21 mmHg and/or optic nerve cupping greater than 0.4. Any patient with other ocular abnormalities or systemic conditions, other than iris stromal hypoplasia, was excluded from the study. Our studies adhered to the tenets of the Declaration of Helsinki and were compliant with the Health Insurance Portability and Accountability Act.

Whole exome sequencing. Exome sequencing of the initial PCG cohort of 20 families with at least one affected proband was performed using a Nimblegen EZ v3 capture kit (Roche) and 2 × 100 bp paired-end sequencing on a HiSeq2000 platform (Illumina). All exome variants were validated by Sanger sequencing. The TEK gene was Sanger sequencing screened in an additional 55 families in the primary cohort. An additional 114 families were also screened in a second international cohort (details are provided in the Supplemental Material). In total, 189 families containing 209 individuals affected by PCG participated in this study.

LoF gene-burden test. A LoF gene-burden test was performed by considering only the predicted LoF variants (i.e., stop gain, essential splice donor/acceptor, and frameshift insertion/deletion). While 7 of the 189 PCG probands carried heterozygous predicted LoF variants in TEK (p.E150*, p.Y307*, p.T19_R210del, p.K745fs, p.G984*, c.760+2T>C, and c.3300+2delT), only 2 heterozygous LoF TEK variants were present in 60,706 subjects in the ExAC cohort. Since large deletions (e.g., p.T19_R210del in family 2) would not have been called investigating additional genes involved in the formation and maintenance of the AHO pathways will provide further insights to our understanding of this devastating eye disease and aid in the development of new therapeutic strategies for glaucoma.

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by the VEP/LOFTEE pipeline used by ExAC (https://github.com/konradjk/loftee), we excluded this variant from our LoF gene-burden analysis. Thereby, we compared 6 of 189 PCG families with 2 of 60,706 individuals in ExAC.

In silico functional studies. PolyPhen-2 (60, 61) and SIFT (version 5.2.2) (62) were used to predict any functional effects that TEK missense variants may have on protein function. To analyze the effect of missense variants on protein structure, FoldX (63) analyses were performed using known crystal structures when available. For the two splice donor variants (c.760+2T>C, c.3300+2delT), we used HSF version 3.0 (64) and Automatic Analysis of SNP sites (AASites) (65) to analyze whether the variants can cause changes in splice sites. Detailed protocols are available in the Supplemental Material.

Tissue culture and proteomics. TEK expression constructs were created by cloning full-length TEK cDNA and incorporating a 3′ Flag-tag sequence into the pcDNA3.1 vector. Site-directed mutagenesis and synthesized DNAs were used to generate variant TEK-Flag expression vectors. Total TEK phosphorylation was determined by Western blotting following immunoprecipitation. TEK solubility was analyzed by separating cell lysates into soluble and insoluble fractions. Protein localization studies were performed by transfecting HUVECs with the TEK-Flag vectors and immunostaining using standard protocols. For SILAC analysis, WT TEK-Flag was transfected into light isotype-labeled cells and PCG variants into heavy isotope-labeled cells. The heavy/light ratio of phosphorylated peptides identified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was calculated by quantification node, and the ratio was normalized by protein abundance. Detailed protocols are available in the Supplemental Material.

Exon trapping splicing assay. Genomic DNA fragments containing TEK exons 5–6 and exon 22 from affected individuals and a control subject were cloned into the exon trapping vector pSPL3. Total RNA from exon trapping construct–transfected Cos-7 cells were reverse-transcribed and PCR-amplified. The resulting RT-PCR products from WT and mutant minigenes were visualized by gel electrophoresis, and their sequence composition was determined by Sanger sequencing. Detailed protocols are provided in the Supplemental Material.

Mice and breeding. Tek WT (Tek+/-), hemizygous (Tek-/-), and homozygous knockout (Tek cKO) mice were generated by crossing Tek+/- mice with ROSA-rtTA/Cre mice (Supplemental Figure 12). These mouse lines were described previously (30, 31). Doxycycline was added to the drinking water of pregnant dams from embryonic day 17.5 to induce Tek deletion. IOP was measured using a TONOLAB rebound tonometer (gCare). TEK expression in lung lysates was analyzed by standard blotting procedures. SC imaging was performed as previously described (31). Mice were maintained on a mixed background, and littermate controls were used for all analyses. Detailed protocols are available in the Supplemental Material.

Statistics. Comparisons of IOP between Tek+/- and WT mice were analyzed by unpaired 2-tailed Student’s t test using GraphPad Prism software. The LoF gene-burden test was performed using JMP Pro 11 software, and a two-tailed Fisher’s exact test was used for testing the statistical significance. A P value less than 0.05 was considered statistically significant.

Study approval. Subjects and their families were recruited from multiple international centers, each of which had received study approval from their respective institutional review boards: University of Wisconsin-Madison Health Sciences Institutional Review Board; Duke University Health System Institutional Review Board for Clinical Investigations; Children’s Hospital of Philadelphia Institutional Review Board; Massachusetts Eye and Ear Infirmary Human Studies Committee; Human Research Ethics Committee of the University of Western Australia; Southern Adelaide Clinical Human Research Ethics Committee; Flinders Medical Centre, Bedford Park, South Australia, Australia; Tasmanian Health and Medical Human Research Ethics Committee, Hobart, Tasmania, Australia; Human Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital; Ethical Review Board of the Medical Faculty of the Friedrich-Alexander University Erlangen-Nürnberg; Ethical Review Board of the Medical Faculty of Niguarda Ca’Granda Hospital; Commission of Ethics for Analysis of Research Projects—Clinical Board of Hospital Clinics and the Faculty of Medicine, University of São Paulo. Written informed consent for study participation was obtained from the subjects or subjects’ parents. All animal experiments were approved by the Animal Care Committee of Northwestern University.

Author contributions
SEQ and TLY conceived, designed, and supervised the study, and wrote the manuscript. TS designed and performed proteomic, cell biology, and comparative biology experiments, analyzed data, and wrote the manuscript. SWT performed and coordinated the human genetic analyses, designed and performed exon trapping experiments, analyzed data, and wrote the manuscript. JJ designed and supervised the proteomics and cell biology study, and wrote the manuscript. JLI supplied patient material, performed human genetic analyses, and wrote the manuscript. FP and JEC supplied patient material and performed human genetic analyses. BRT designed and performed comparative biology experiments, analyzed data, and wrote the manuscript. OMS performed human genetic analyses and wrote the manuscript. VM and SMS performed in silico analyses and wrote a portion of the manuscript. KNW analyzed genetic data and performed experiments. KK and SJ performed experiments and wrote the manuscript. SY, LF, BK, KNTV, and XL performed experiments. LK, DNA, SF, LM, ES, TZ, AWH, KBP, DAM, KFA, JBR, ShL, and SR contributed human subjects, performed genetic analyses, and contributed to writing the manuscript. All authors contributed to the review and approval of the manuscript.

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