

ARTICLE

Rare *FOXC1* variants in congenital glaucoma: identification of translation regulatory sequences

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Primary congenital glaucoma (PCG) is the cause of a significant proportion of inherited visual loss in children, but the underlying mechanism is poorly understood. In this study, we assessed the relationship between PCG and *FOXC1* variants by Sanger sequencing the proximal promoter and transcribed sequence of *FOXC1* from a cohort of 133 PCG families with no known *CYP1B1* or *MYOC* mutations. The pathogenicity of the identified variants was evaluated by functional analyses. Ten patients (7.5%) with no family history of glaucoma carried five different rare heterozygous *FOXC1* variants with both increased (rs77888940:C>G, c.-429C>G, rs730882054:c.1134_144del(CGCGCGCGCGG), p.(G380Rfs*144) and rs35717904:A>T, c.*734A>T) and decreased (rs185790394: C>T, c.-244C>T and rs79691946:C>T, p.(P297S)) transactivation, ranging from 50 to 180% of the wild-type activity. The five variants did not show monogenic segregation, and four of them were absent in a control group ($n = 233$). To the best of our knowledge, one of these variants (p.(G380Rfs*144)) has not previously been described. One of the *FOXC1* variant carriers (p.(P297S)) also coinherited a functionally altered rare *PITX2* heterozygous variant (rs6533526:C>T, c.*454C>T). Bioinformatics and functional analyses provided novel information on three of these variants. c.-429C>G potentially disrupts a consensus sequence for a terminal oligopyrimidine tract, whereas c.-244C>T may alter the RNA secondary structure in the 5'-untranslated region (UTR) that affects mRNA translation. In addition, p.(G380Rfs*144) led to increased protein stability. In summary, these data reveal the presence of translation regulatory sequences in the UTRs of *FOXC1* and provide evidence for a possible role of rare *FOXC1* variants as modifying factors of goniodysgenesis in PCG.

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INTRODUCTION

Primary congenital glaucoma (PCG; MIM# 231300) is the most common form of childhood glaucoma and represents a significant cause of visual loss and blindness in children. PCG results from developmental abnormalities of the anterior segment structures of the eye that are required for aqueous humor (AH) drainage. These abnormalities lead to an increase in AH outflow resistance, elevated intraocular pressure (IOP) and optic nerve degeneration. The PCG incidence varies between different populations, with an estimated overall occurrence of approximately 1 in 10 000 births. PCG is genetically heterogeneous. Four PCG loci have been mapped (*GLC3A* at 2p21, *GLC3B* at 1p36, *GLC3C* at 14q24.3 and *GLC3D* at 14q24.3), although only two glaucoma genes have been identified, *cytochrome P450, subfamily I, polypeptide 1* (*CYP1B1* MIM# 601771) and *latent transforming growth factor- β -binding protein 2* (*LTBP2* MIM# 602091), situated on the *GLC3A* and the *GLC3D* loci, respectively. In previous studies we showed that ~30% of Spanish PCG patients carry loss-of-function *CYP1B1* variants, with most of these variants resulting in null genotypes.¹ Interestingly, the existence of incomplete penetrance, variable expressivity and the fact that a significant proportion of patients carry nondominant heterozygous *CYP1B1* mutations¹ suggest that more than one gene can be involved in PCG inheritance. Mutations in *LTBP2*,² *MYOCILIN* (*MYOC*, MIM# 601652)^{3,4} and

forkhead box C1 (*FOXC1* MIM# 601090) have also been found in a small number of Indian PCG cases.⁵

FOXC1 mutations have been found to be involved in several dominant ocular defects, including Axenfeld-Rieger syndrome type 3 (ARS; MIM# 602482), a disease that is associated with increased glaucoma risk and variability in iris and extraocular abnormalities.⁶ The *FOXC1* gene encodes a transcription factor that regulates craniofacial, cardiovascular and ocular development.⁷ The *FOXC1* protein belongs to the FOX class of transcription factors and is characterized by a conserved 110 amino-acid DNA-binding domain, called the forkhead domain (FHD). *FOXC1* is present in mesoderm and neural crest-derived cells, including cells of the anterior segment of the eye, and the periorbital mesenchyme and mesenchymal cells that migrate into the eye.

In this study, we show the existence of regulatory signals in the untranslated regions (UTRs) of *FOXC1* that are involved in down-regulating its translation, and we provide evidence for the putative role of rare functionally altered *FOXC1* variants as modifying factors in PCG.

MATERIALS AND METHODS

Subjects

A total of 133 unrelated families affected by PCG were included in this study. The study and informed consent procedures were approved by the Ethics

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Committee for Human Research of the Hospital Clínico San Carlos, Madrid (Spain), and followed the tenets of the Declaration of Helsinki. Informed written consent was obtained from all of the participants and was recorded by staff involved in the study.

All subjects were clinically evaluated by glaucoma specialists. The ophthalmic examination included slit lamp biomicroscopy, gonioscopy, biometry, IOP measurement and ophthalmoscopy. The PCG clinical diagnosis was performed as previously described.¹ Patients over 3 years of age who were diagnosed with abnormal gonioscopy of the anterior segment (high iris insertion and absence of an angle recess), were considered to be late congenital glaucoma (LCG) cases. Patients with pediatric glaucoma that was either related to other ocular disorders or associated with systemic abnormalities were excluded. Ocular and nonocular features of ARS were ruled out in the patients. Control individuals were recruited from among those who attended the clinic for conditions other than glaucoma, including cataracts, floaters, refractive errors and itchy eyes. They also underwent full ocular exploration, including IOP measurement, determination of best visual acuity with optical correction, gonioscopy and eye fundus examination.

Variant screening

The genomic DNA was extracted from peripheral blood, using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). The DNA sequence variation analyses were carried out by automatic Sanger sequencing. A fragment of the promoter region (nucleotides -1 to -875), the coding region and the 5'- and 3'-UTRs of *FOXCI*, and the *PITX2* coding region and its 5'- and 3'-UTRs were amplified by PCR, using the primers described in Supplementary Table 1. The variants identified in each subject were confirmed by sequencing a new amplification product. The rs35717904:A>T, c.*734 A>T *FOXCI* variant was genotyped in controls by Taqman SNP genotyping assay (4351379, Applied Biosystems, Foster City, CA, USA). The entire *MYOC* and *CYP1B1* coding regions were checked in all patients for disease-causing variants by Sanger DNA sequencing as previously described.^{8,9}

Site-directed mutagenesis and cloning of the *FOXCI* and *PITX2* variants

Two *FOXCI* variants (rs79691946:C>T, p.(P297S) and rs35717904:A>T, c.*734 A>T), one *PITX2* nucleotide substitution (rs6533526:C>T, c.*454C>T) identified in this study and the control p.(I126M) *FOXCI* variant were obtained by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), with the primers and PCR conditions indicated in the Supplementary Materials and methods. Variants p.(G380Rfs*144), c.1359_1361dup (p.(G456dup)), rs185790394:C>T, c.-244C>T and rs77888940:C>G, c.-429C>G were also obtained as indicated in the Supplementary Materials and methods section. The recombinant *FOXCI* and *PITX2* versions were transiently expressed in human embryonic kidney 293 T (HEK-293 T) cells that were cultured as indicated in the Supplementary Materials and methods section. Transient plasmid transfections were carried out with 50–500 ng of plasmid DNA using the Superfect Transfection Reagent (Qiagen) according to the manufacturer's instructions.

Luciferase assays

The *FOXCI* transactivation assays were performed using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. HEK-293 T cells were transfected with 500 ng of the recombinant *FOXCI* expression vector (pcDNA3.1), along with 50 ng of the recombinant pGL3-basic-*CXCR4* luciferase reporter, and 50 ng of the pMirTarget vector as indicated in the Supplementary Materials and methods section. Finally, the cells were assayed for firefly luciferase activity.

To analyze the functional effect of the *PITX2* rs6533526:C>T, c.*454C>T variant, the mutant and wild-type 737 bp of the 3'-UTR sequence (NM_000325) *PITX2* were fused to the 3'-end of the firefly luciferase coding region (cloned into the pMirTarget vector; PS100062, OriGene, Technologies, Inc., Rockville, MD, USA). HEK-293 T cells were transfected with either the wild-type or the mutant 3'-UTR sequence, and 48 h later luciferase activity was measured.

Protein stability and half-life calculation

The protein stability was studied via western blot of transfected cells treated with cycloheximide (300 µg/ml) at different times as previously reported.⁹ The *FOXCI* protein levels were determined by densitometry and the relative amounts at different time points after cycloheximide treatment were expressed as a percentage of the levels at time 0 h. At least three independent assays for each variant were performed. The transfection efficiency was assessed by co-transfecting the recombinant cDNA constructs encoding the different mutants (500 ng) with the pMirTarget vector (50 ng) that encodes the red fluorescent protein (RFP). The RFP was detected via western blotting. The loaded samples were normalized for total protein content using the Bradford reagent (Pierce, Rockford, IL, USA). *FOXCI* decay followed first-order kinetics. The slope of the decay line was calculated using standard linear regression, and the protein half-life was determined as previously described.¹⁰

Western blotting and antibodies

For the western blot analyses, the nuclear extracts, prepared as indicated in the Supplementary Materials and methods, were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the Mini-PROTEAN III Gel Electrophoresis System (Bio-Rad, Hercules, CA, USA) as previously described.¹¹ A commercial mouse monoclonal anti-myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody, diluted at 1:1000. Horseradish peroxidase-conjugated antibodies against mouse IgG (Pierce) were diluted to 1:1000–1:4000. Chemiluminescence detection and the densitometry for protein band quantification was performed as previously described.¹¹ As an additional sample loading control, the endogenous lactate dehydrogenase (LDH) protein was detected in cell extracts using a goat anti-LDH antibody diluted to 1:5000 (AB1222, Chemicon, Temecula, CA, USA) and an anti-goat IgG horseradish peroxidase-conjugated antibody (sc-2033, Santa Cruz Biotechnology, diluted to 1:2000). RFP (transfection control) was detected using a rabbit anti-RFP antibody (AB233, Evrogen, Moscow, Russia), diluted to 1:5000, and an anti-rabbit IgG horseradish peroxidase-conjugated antibody (1858415, Pierce), diluted to 1:1000.

Bioinformatic analyses

The *in silico* analyses of *FOXCI* and *PITX2* sequences and their different variants were carried out using the programs described in Supplementary Materials and methods. Variants were named using directions from Mutalyzer (<https://humgenprojects.lumc.nl/trac/mutalyzer>), according to RefSeq NM_001453.2 and NM_00325.5 for *FOXCI* and *PITX2*, respectively. The *FOXCI* transcription start site and 5'-UTR sequence were defined as previously described.¹² The first nucleotide of the translation initiation site was numbered as nucleotide +1. All variants have been submitted to dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>).

Statistical analysis

The significance of the difference in sex and variant proportions between patients and control subjects was determined by either the χ^2 test or Fisher's exact test when frequencies were below five. The statistical comparisons between groups were performed using either the *t*-test or the one-way analysis of variance (ANOVA). A Bonferroni correction was applied to adjust tests for multiple comparisons. The data were statistically processed by the SigmaStat 2.0 software (SPSS Science Inc., Chicago, IL, USA).

RESULTS

Clinical phenotype of patients

To analyze the role of *FOXCI* variants in PCG, we studied 133 apparently unrelated PCG families. The main clinical features are presented in Table 1. The age of PCG diagnosis ranged from birth to 36 months, 12.0% of these cases were diagnosed at birth and 14.2% before the first month of life, and 73.8% were diagnosed between months 2 and 36 of life. Consanguinity was documented in only one family (PCG-148). The majority of cases were of Spanish ancestry, although six were immigrants from European or Arab countries. Three probands had mixed European origins of Spanish–German

(PCG-43 and PCG-132) or Spanish–Italian (PCG-112). None of the patients carried disease-causing variants in the coding regions of *MYOC* and *CYP1B1*.

FOXC1 variants

Five different rare heterozygous sequence variants (Table 2) were identified in 10 of the 133 probands (7.5%). The rs185790394: C>T, c.-244C>T variant was identified in five probands in the heterozygous state, but additional familial samples for segregation analysis were only available for two of those families (PCG-12 and PCG-14, Figure 1a). The fathers of these two patients (PCG-12 and PCG-14) were heterozygous carriers of the c.-244C>T variant and manifested elevated IOP, but no other ocular or visual alterations were observed at the time of the study (PCG-12, I:1 and PCG-14, I:1; Figure 1a and Table 2). Two other index cases (PCG-96 and PCG-169) were heterozygous carriers for the rs77888940:C>G, c.-429C>G variant. The first patient showed bilateral glaucoma and inherited the nucleotide change from a healthy mother (Figure 1b), and the second carrier presented unilateral glaucoma (PCG-169, Table 2). The two variants present in these patients are listed in the Ensembl variation database, but no population frequency data are available. The

rs35717904:A>T, c.*734A>T variant was identified in a subject diagnosed with glaucoma in only one eye (Figure 1c). This nucleotide change has been described at low frequencies in Europeans (Ensembl variation database). rs730882054:c.1134_144delCGGCGGCGGG, p.(G380Rfs*144) was present in the PCG-54 proband (Figure 1d), who inherited it from his asymptomatic mother. This nucleotide deletion was considered to be novel because it was not present in the Ensembl variation database, the Human Gene Mutation Database¹³ (<http://www.hgmd.cf.ac.uk/ac/index.php>), GeneCards (www.genecards.org), Online Mendelian Inheritance in Man (OMIM, <http://omim.org/>) or PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>). In addition, c.1359_1361dup, p.(G456dup) (described as rs572346201:c.1337_1338insCGG, p.(G456_Q457insG) in dbSNP) was present in three of these patients (PCG-96, PCG-98 and PCG-54) in the heterozygous state. Pedigree analyses of the available family members did not support the involvement of all the identified variants in monogenic glaucoma transmission. All rare variants were genotyped in 233 Spanish control individuals (466 chromosomes) and only c.-244C>T was identified in two control subjects in heterozygosis (allelic frequency 0.4%).

Coinheritance of rare FOXC1 and PITX2 variants

Analyses of *PITX2* sequence in the carriers of rare *FOXC1* variants led to identification of the rs6533526:C>T, c.*454C>T nucleotide substitution in the heterozygous state in one patient (PCG-84) (Figure 1e). This subject was a double heterozygote for *FOXC1* and *PITX2* variants (rs79691946:C>T, p.(P297S)/+ and c.*454C>T/+, respectively) and was diagnosed with bilateral glaucoma at the age of 6 months. Among the three nonaffected siblings of the proband, subject II:3 harbored one copy of the *PITX2* variant, subject II:4 had the wild-type genotype for both nucleotide substitutions and subject II:5 had one copy of the *FOXC1* variant (Figure 1e). Finally, the proband's 2 year-old daughter (III:1) also inherited both the *FOXC1* and *PITX2* variants, and was asymptomatic at the time of the study (2 years, Table 2). p.(P297S) has previously been found in the heterozygous state in patients with microphthalmia and anterior

Table 1 Clinical and demographic features of the patients included in this study

Variable	PCG (n = 133)
Age (mean ± SD)	12.6 months ± 20.1
Female (%)	38.0
Male (%)	62.0
C/D/eye (mean ± SD)	0.53 ± 0.27
IOP (mm Hg)/eye (mean ± SD)	26.3 ± 9.9
Bilateral PCG (%)	73.9
Unilateral PCG (%)	26.1
Immigrants (%)	4.5

Abbreviations: C/D, cup/disc ratio of optic nerve at diagnosis; IOP, intraocular pressure.

Table 2 Rare FOXC1 and PITX2 gene variations identified in PCG and associated clinical features

Proband (family member)	Nucleotide change ^a	Amino-acid change	Phenotype/laterality	Age at diagnosis (months)/sex	IOP (mm Hg) at diagnosis (OD/OI)	C/D (OD/OS)	Treatment ^b
PCG-10	c.-244C>T	–	PCG/B	9/F	(30/30)	NA	Surgery
PCG-12	c.-244C>T	–	PCG/B	1.5/M	(22/24)	0.3/0.7	Surgery
PCG-12 (I:1)	c.-244C>T	–	OH/B	708/M	24/24	0.5/0.5	NA
PCG-14	c.-244C>T	–	PCG/B	1/M	(22/22)	0.3/0.5	Surgery
PCG-14 (I:1)	c.-244C>T	–	OH/B	612/M	22/22	0.2/0.2	NA
PCG-30	c.-244C>T	–	PCG/B	0/M	(23/28)	NA	Surgery
PCG-105	c.-244C>T	–	PCG/B	3/F	(30/31.5)	(0.6–0.7/0.7–0.8)	2+Surgery
PCG-96	c.-429C>G (rs77888940)	–	PCG/B	2/M	(32/36)	(0.2/0.2)	Surgery
PCG-169	c.-429C>G (rs77888940)	–	PCG/U (OD)	NA/F	NA	NA	2+Surgery
PCG-54	c.1134_144del	p.(G380Rfs*144)	PCG/B	4/M	(35/35)	(0.6/0.7)	Surgery
PCG-98	c.*734A>T (rs35717904)	–	LCG/U (OD)	60/F	(57/12)	(0.6/0.4)	1+Surgery
PCG-84 (II:2)	c.889C>T (<i>FOXC1</i>)	p.(P297S)	PCG/B	6/F	15/18	NA	Surgery
	c.*454C>T (rs6533526) (<i>PITX2</i>)	(<i>FOXC1</i>)					
PCG-84 (III:1)	c.889C>T (<i>FOXC1</i>)	p.(P297S)	Normal ^c	24 ^d	–	–	NA
	c.*454C>T (rs6533526) (<i>PITX2</i>)	(<i>FOXC1</i>)					

Abbreviations: LCG/PCG, late-onset primary congenital glaucoma/primary congenital glaucoma; B/U, bilateral/unilateral; C/D, cup/disk ratio; NA, not available; OH, ocular hypertension; OD/OS, right eye/left eye.

^aThe variants were present in the heterozygous state and were named according to RefSeq NM_001453.2 and NM_00325.5 for *FOXC1* and *PITX2*, respectively.

^bNumber of drugs and/or surgery.

^cAt the time of the study.

^dAge at the time of the study.

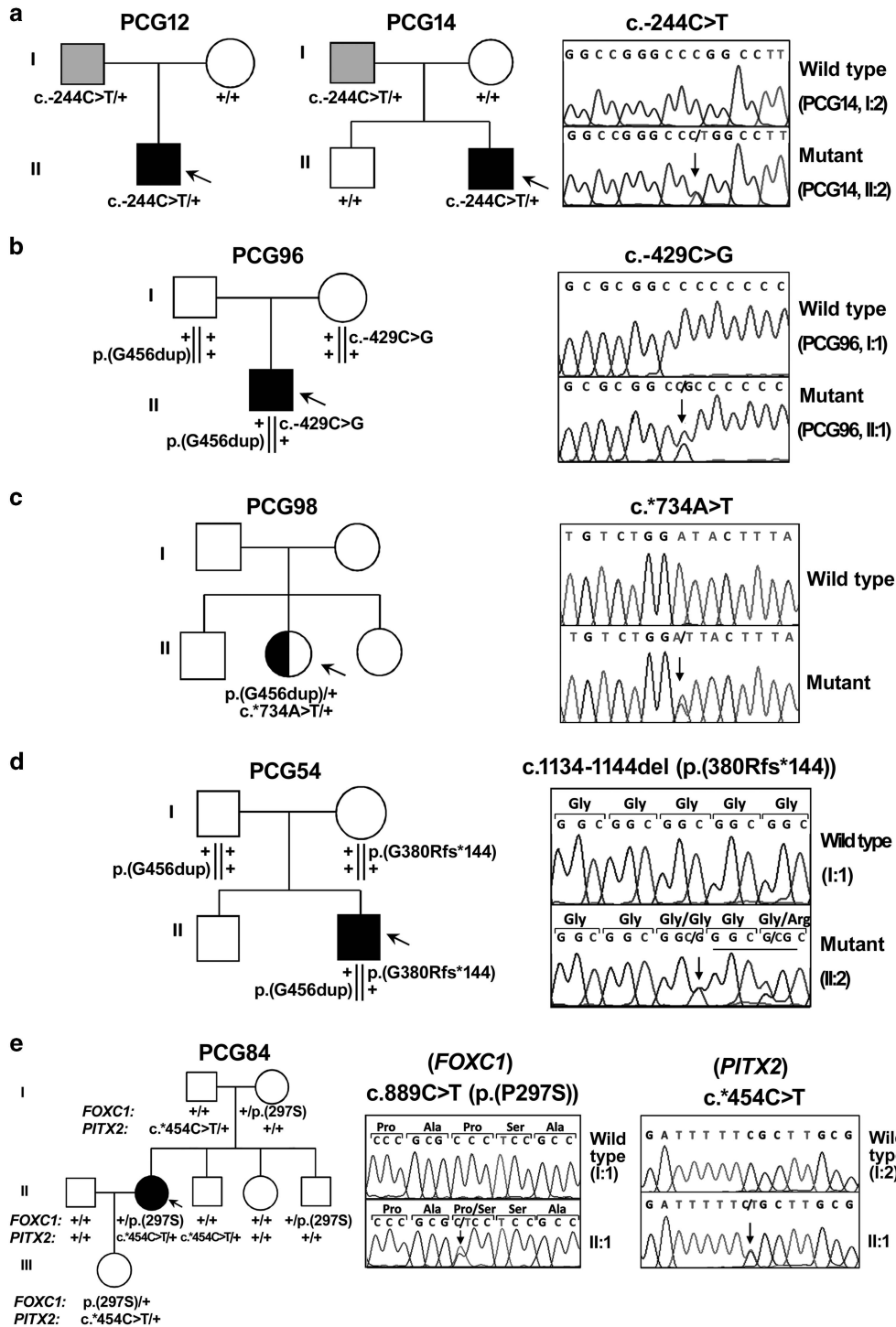


Figure 1 Segregation analysis of the *FOXC1* variants identified in PCG families. Pedigree of the families who carry the rs185790394: C>T, c.-244C>T (a), rs77888940:C>G, c.-429C>G (b), rs35717904:A>T, c.*734A>T (c), p.(G380Rfs*144) (d) and rs79691946:C>T, p.(P297S) (e) *FOXC1* variants. Segregation of the c.*454C>T *PITX2* variant is also shown in (e). The two vertical lines located below some of the pedigree symbols indicate haplotypes. Gray and black symbols indicate ocular hypertension and primary congenital glaucoma, respectively. The arrow in the pedigrees designates the index case. +: Wild-type allele. All of the variants were detected in the heterozygous state. The inset in each panel corresponds to representative electropherograms of the variants identified in each family. To facilitate comparison, the wild-type sequence is shown above the corresponding mutant sequence. Arrows in the insets indicate the location of variants.

segment dysgenesis (ASD).^{14,15} According to the Ensembl variation database, c.*454C>T is present at low frequencies in African and American subjects (5% and 2%, respectively), and is absent in European and Asian individuals. It was not present in 233 Spanish control individuals (466 chromosomes).

Bioinformatics analysis and evolutionary conservation of variants

Our analysis showed that the coding variants p.(P297S) and p.(G380Rfs*144) mapped at the inferred intrinsically disordered regions 2 and 3 (IDR2 and IDR3, respectively) of the inhibition domain (Figure 2a). The first of these variants affected an amino-acid residue situated in a Pro- and Ser-rich region (Figure 2a). The known variant p.(G456dup) localized at the Gly tract situated in the IDR4 (Figure 2a and c). The two noncoding variants (c.-429C>G and c.-244C>T), located in the 5'-UTR are predicted to alter a terminal oligopyrimidine tract (TOP) translation regulatory sequence and to increase the length of paired nucleotides in the mRNA secondary structure in this region, respectively (Figure 2b). Finally, the *FOXC1* c.*734A>T and *PITX2* c.*454C>T variants were localized in the predicted seed target sequences for hsa-miR-548l and hsa-miR-548p, respectively (Figure 2b and d, respectively).

Evolutionary conservation analyses revealed that the coding variants affected amino acids conserved among mammals (Figure 2c). Interestingly, among the noncoding variants, the c.-429C nucleotide was present exclusively in the humans (Figure 2c), whereas oligopyrimidine tracts, similar to that in which the human variant was embedded, were observed in macaque and rat DNA sequences (Figure 2c). The *PITX2* c.*454 cytosine was conserved in all analyzed mammal DNA sequences (Figure 2e).

Transactivation activity assay

The transactivation activity of the different *FOXC1* variants was assessed by transient co-transfection of the reporter luciferase gene coupled to the *CXCR4* promoter region that contains a FOX-binding element (FBE) (Figure 3a), with recombinant cDNA constructs encoding each *FOXC1* variant (Figure 3b and d). Three mutants (p.(G380Rfs*144), c.-429C>G and c.*734A>T) showed both increased protein levels and transactivation activity of ~130 to 180% (Figure 3b–d) compared with wild-type, and therefore these variants were classified as hypermorphic alleles. Conversely, the p.(P297S) and c.-244C>T variants are hypomorphic alleles because they reduced transactivation by ~50% (Figure 3b and c). The *FOXC1* protein levels associated with c.-244C>T were lower than those of the 3'-UTR wild-type sequence. The lowest transactivation activity, however, was associated with the control p.(I126M) mutation (previously identified in patients with ARS and glaucoma⁶) that also presented with decreased protein levels (Figure 3b). Finally, the polymorphic p.(G456dup) variant also increased transactivation activity to ~140% of the wild-type levels (Figure 3b). Western blot analyses of both RFP and LDH proteins showed no significant differences in either transfection efficiency or sample loading.

miRNA evaluation

The *PITX2* c.*454C>T variant was predicted to be embedded in a target sequence for hsa-miR-548p (Supplementary Figure 1A). The functional effect of this nucleotide substitution was evaluated by parallel expression in cells in cultures of mutant or wild-type *PITX2* 3'-UTRs, fused to the 3'-end of the luciferase coding sequence (Supplementary Figure 1B). Luciferase activity associated with the mutant sequence was ~40% lower than that of the wild-type (Supplementary Figure 1C), suggesting that the variant increases the

affinity of the target sequence for this endogenously expressed miRNA. Western blot analyses of both RFP and LDH proteins showed no significant differences in either transfection efficiency or sample loading.

Protein stability

The protein stability of the coding variants was assessed in transiently transfected cells treated with the protein synthesis inhibitor cycloheximide. Western blot analyses of both RFP and LDH proteins showed no significant differences in either transfection efficiency or sample loading within a time point (Figure 4a). The p.(P297S) variant was as stable as the wild-type protein (Figure 4b), with an estimated half-life of 12.37 h (Figure 4c). These data suggest that p.(P297S) alters transactivation activity independently of protein stability. In contrast, 8 h after cycloheximide treatment, the protein levels of the p.(G380Rfs*144) variant were almost 40% higher than those of the wild type (Figure 4a and b), with an estimated 2.6-fold increase in its half-life (12.95 h vs 34.14 h; Figure 4c). The lowest protein stability corresponded to the p.(I126M) control mutant (4.20 h; Figure 4).

DNA-binding ability and subcellular localization

The DNA-binding ability of the coding *FOXC1* variants was evaluated by electrophoretic mobility-shift assay (EMSA) analysis. These experiments showed the DNA-binding ability of the p.(P297S) and p.(G380Rfs*144) variants did not differ significantly from the wild type (Supplementary Figure 2A and B). In contrast, the intensity of the protein–DNA complexes formed by the p.(I126M) control mutation were significantly lower than those formed by the wild-type protein (Supplementary Figure 2A and B).

Fluorescence immunocytochemistry of recombinant wild-type *FOXC1* showed that the protein is localized exclusively in the nucleus (Supplementary Figure 3, wild type). The p.(I126M) control mutation and the other two coding variants showed normal subcellular nuclear localization (Supplementary Figure 3).

DISCUSSION

It is widely accepted that trabecular meshwork developmental abnormalities underlie the progression of PCG.^{16,17} Congenital glaucoma is frequently associated with severe functional alterations of *FOXC1* that lead to ARS phenotypes.⁶ Therefore, we hypothesized that moderate functional alterations of the *FOXC1* gene may contribute to relatively mild phenotypic alterations limited to congenital glaucoma. To test this hypothesis, we performed a comprehensive screening and a detailed functional analysis of *FOXC1* variants in, as far as we know, the largest European cohort of PCG patients that contained 133 Spanish probands with no *CYP11B1* or *MYOC* genetic alterations.

Presence of rare *FOXC1* variants in congenital glaucoma patients

In accordance with our working hypothesis, we identified rare heterozygous *FOXC1* variants that were associated with moderate alterations in transactivation activity in patients with no known familial congenital glaucoma history. Because these variants were either not found or underrepresented in the control group, it is unlikely that they would be present in the patients merely by chance, and therefore these data suggest that the variants that we identified may contribute to nonmonogenic PCG inheritance, although this hypothesis does require additional confirmation studies. In addition, one of the carriers of a *FOXC1* variant, diagnosed at the age of 6 months, PCG8-4 (Figure 1e), also coinherited a rare and hypomorphic *PITX2* variant in the heterozygous state, further

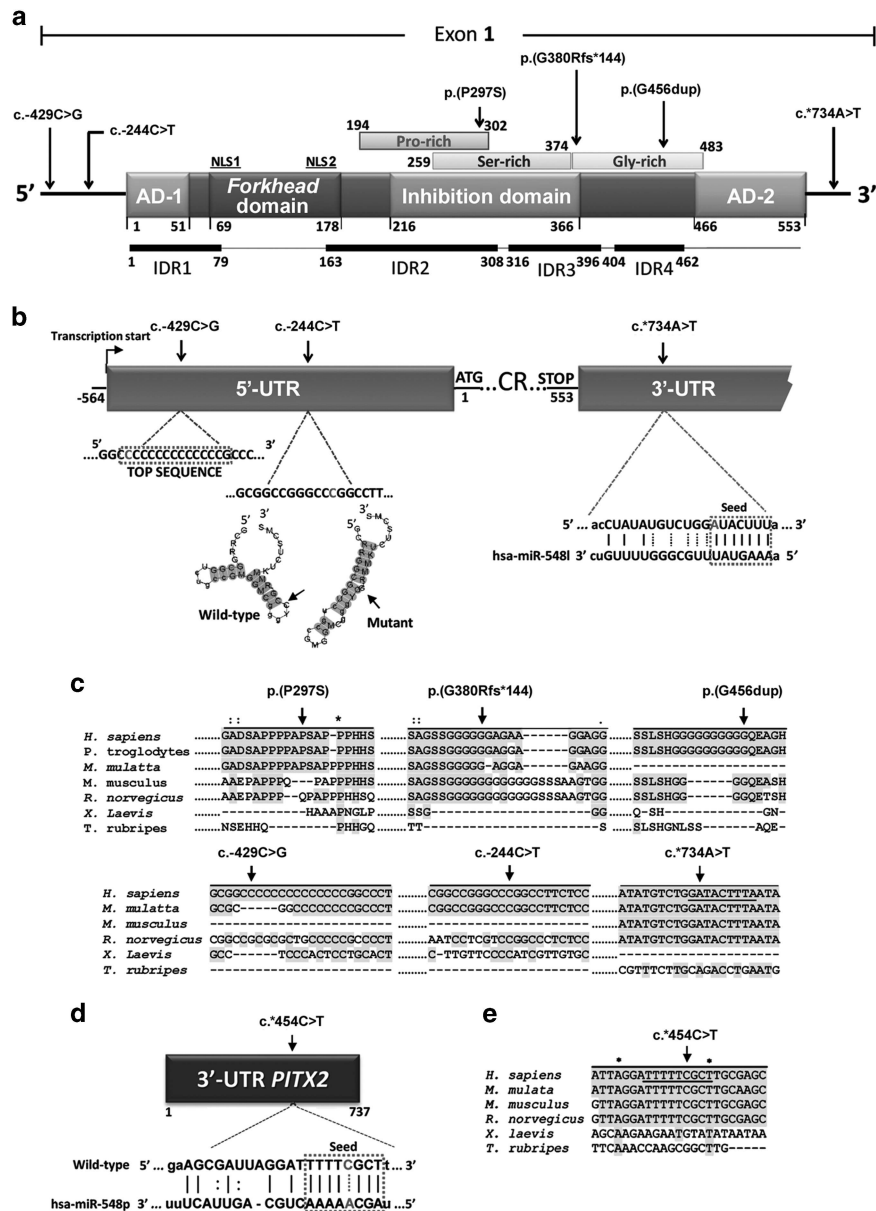


Figure 2 Localization of *FOXC1* and *PITX2* variants identified in this study. **(a)** Localization of the predicted and previously identified structural domains and motifs of *FOXC1* polypeptide chain. The predicted Pro-, Ser- and Gly-rich regions reported in the Prosite database are shown above the scheme. Four intrinsically disordered regions (IDRs) inferred with the DisEMBL²⁶ and Globplot²⁷ programs are shown below the protein. AD, activation domain; CR, coding region; NLS, nuclear localization signal. **(b)** Localization of the predicted *FOXC1* UTR regulatory sequences. The inference of functional RNA motifs in the 5'-UTR rsequence was carried out with RegRNA2 (<http://regrna2.mbc.nctu.edu.tw/>). The GeneBee-Molecular Biology Server (<http://www.genebee.msu.su/genebee.html>) was used for RNA secondary structure prediction. The hsa-miR-548l target sequence was inferred with the MicroSNIper software. The variable nucleotide is indicated in red. Vertical continuous and discontinuous lines in the miRNA target sequence indicate perfect Watson–Crick and G:U wobble pairings, respectively. Underlined nucleotides indicate the predicted seed target sequence for hsa-miR-548l. **(c)** Multiple alignments of variable amino-acid or nucleotide *FOXC1* sequences. Both nucleotide and amino-acid sequence alignments were carried out with Clustal W.²⁸ Arrows denote mutated positions. The asterisks indicate positions where all of the amino acids are identical, two vertical dots specify amino acids with similar chemical properties and one dot denotes amino-acid positions with weak chemical similarity. **(d)** Localization of the *PITX2* c.*454C>T variant in the predicted seed target sequence for hsa-miR-548p. The variable nucleotide is indicated in red. The target miRNA was predicted with the MicroSNIper software. The underlined nucleotides indicate the predicted seed target sequence for hsa-miR-548p. Vertical lines and dots in the predicted miRNA target sequence indicate perfect Watson–Crick and G:U wobble pairings, respectively. **(e)** Multiple alignments of the variable *PITX2* nucleotide sequence. Nucleotide sequence alignment was carried out as indicated in (c). The symbols are the same as in (c). A full color version of this figure is available at the *European Journal of Human Genetics* journal online.

suggesting the existence of nonmonogenic PCG inheritance and a possible oligogenic transmission pattern involving these two genes. The patient transmitted both variants to her daughter who was 2 years old and phenotypically normal at the time of the study. Although we

cannot rule out a delayed manifestation of the disease (variable expressivity), the current data suggest that the combination of only these two nucleotide changes is not enough to produce the disease, indicating a complex glaucoma inheritance. Follow-up of the infant is

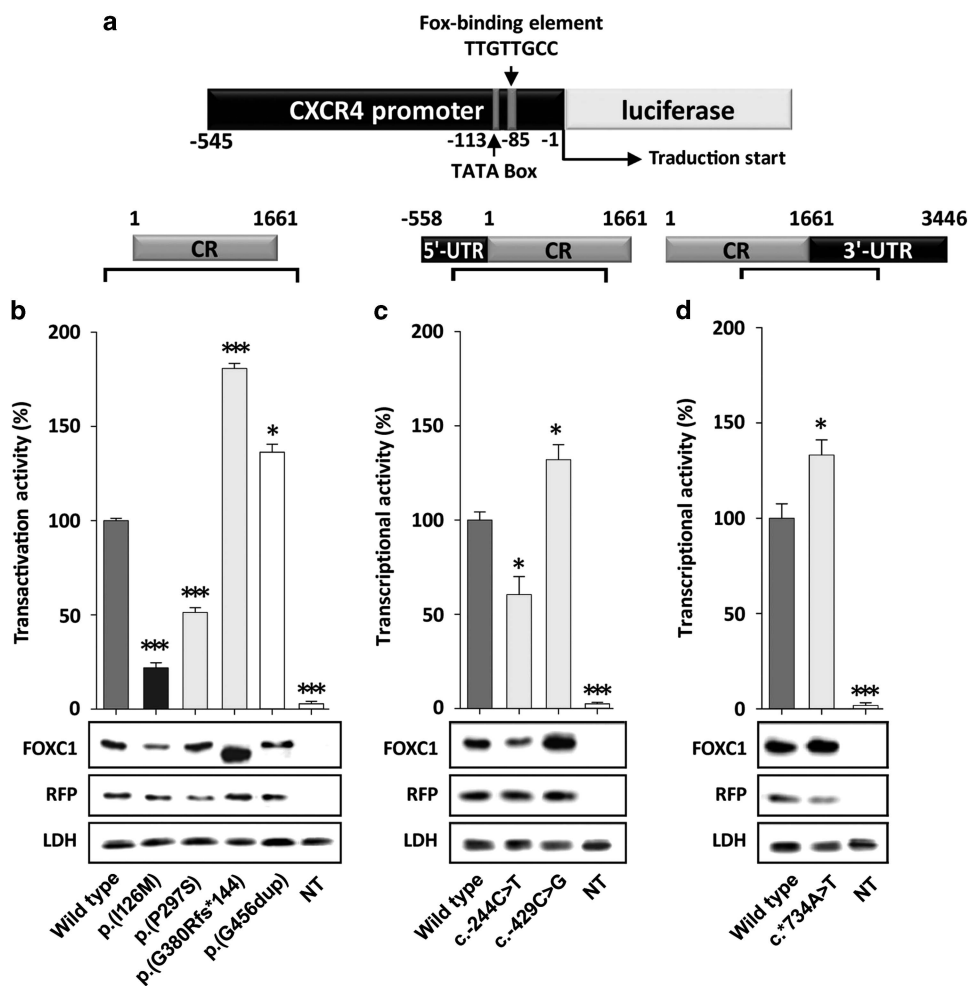


Figure 3 Altered transactivation activity of the *FOXC1* variants identified in PCG patients. (a) A scheme of the cDNA construct containing the *FOXC1*-binding element present in the *CXCR4* promoter, fused to luciferase and cloned into the PGL3 basic vector. This construct was used as a reporter of transactivation activity in co-transfection assays of the identified *FOXC1* variants and the empty pMirTarget vector that encodes RFP (transfection efficiency control). The numbers below the scheme correspond to nucleotide positions. The cartoons above (b–d) indicate the *FOXC1* mRNA regions incorporated into the different cDNA constructs that we used to assess the transcriptional activity of the different variants. CR, coding region. cDNA constructs encoding the indicated *FOXC1* variants corresponding only to the coding region (b), the coding region with the 5'-UTR (c) or the coding region fused to the 3'-UTR (d) were transiently coexpressed with the reporter cDNA construct in HEK-293 T cells. The transcription activity of variants, expressed as a percentage of the luciferase activity of the wild-type protein, was measured as indicated in the Materials and methods section. The protein levels of the different *FOXC1* versions present in HEK-293 T cells 48 h after transfection were determined by western immunoblot using a monoclonal anti-myc antibody (Santa Cruz Biotechnology). Each lane contained 15 μ g of the total protein obtained from the cell lysates. Transfection efficiency was assessed by western immunoblot using an anti-RFP antibody (Evrogen). The sample loading control, endogenous LDH, was also detected by immunoblot using an anti-LDH antibody (Chemicon). Error bars correspond to the SD of three independent experiments carried out in triplicate. Asterisks indicate statistically significant difference with respect to the control. The *P*-values were obtained using a one-way ANOVA followed by Tukey's multiple-comparison test: **P*<0.05; ****P*<0.001. NT, nontransfected.

required to clarify the inheritance pattern in this family. Nonmonogenic PCG transmission has long been suspected based on the existence of incomplete penetrance and variable expressivity. In addition, it has been found that a significant proportion (close to 20%) of PCG patients who carry *CYP11B1* mutations inherited them in the heterozygous state.¹ This finding led to the suggestion that PCG is not a simple Mendelian disease and that the action of modifier factors (genetic, stochastic and/or environmental) can significantly influence disease presentation.^{1,18} The possible existence of oligogenic glaucoma inheritance, caused by mutations in more than one gene, is not surprising because this disease is the result of abnormal development of the anterior eye segment, a complex phenomenon involving many intricate gene networks and molecular pathways. In accordance

with the idea of oligogenic glaucoma, inheritance involving both *CYP11B1* and *MYOC* has been reported in early-onset glaucoma¹⁹ and PCG.⁴ *FOXC1/PITX2* glaucoma transmission has also been described in ARS.²⁰

As noted above, our data also indicate a role for the moderately hypermorphic p.(G456dup) variant as a possible modifying factor. Interestingly, this variant was present in two patients who also had two other hypermorphic variants (c.-429C>G and p.(G380Rfs*144) in families PCG-96 and PCG-54, respectively) (Figure 1b and d), contributing to the overall increase in *FOXC1* activity associated with these genotypes. Moreover, we have reported that the p.(G456dup) variant coinherited in one patient along with another hypermorphic *FOXC1* variant (p.(Y47*)) seems to anticipate the glaucoma onset,²¹

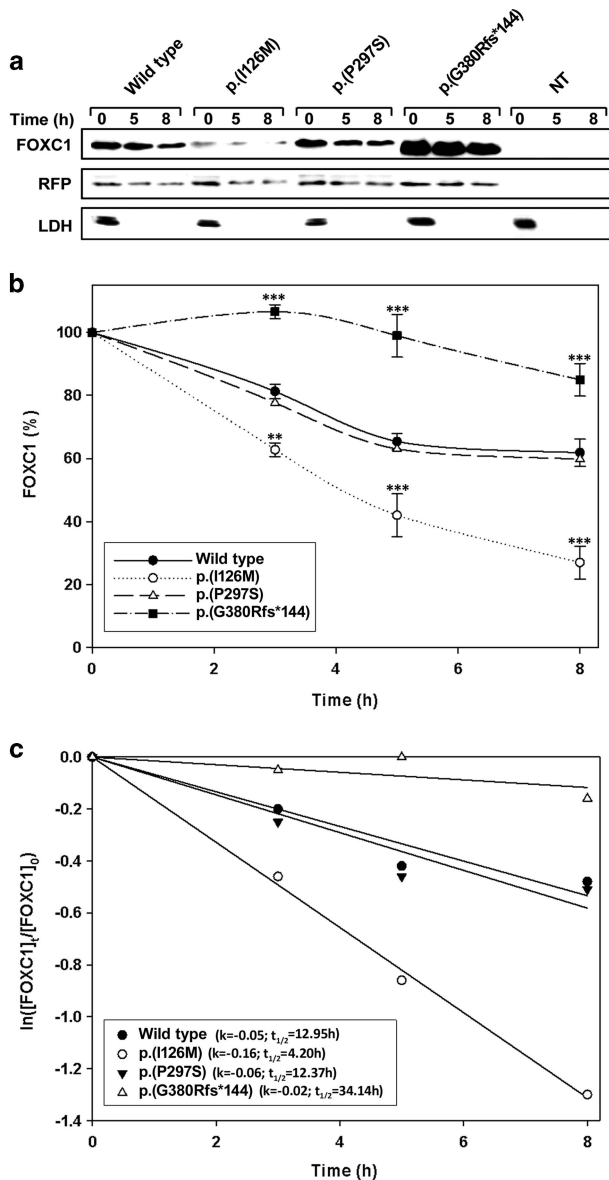


Figure 4 The *FOXC1* p.(G380Rfs*144) variant alters protein stability. (a) Time-course stability analyses of *FOXC1* mutant polypeptides found in PCG patients were carried out by transient expression of the corresponding cDNA constructs in HEK-293 T cells. The transfected cells were treated with the protein synthesis inhibitor cycloheximide and the different recombinant *FOXC1* variants were detected by western immunoblot using an anti-myc monoclonal antibody (Santa Cruz Biotechnology) at the indicated time points. Transfection efficiency was assessed by co-transfection with the nonrecombinant pMirTarget vector that encodes RFP. RFP was detected by western blot using an anti-RFP antibody (Evrogen). The sample loading control, endogenous LDH, was also detected by immunoblot using an anti-LDH antibody (Chemicon). (b) The amount of *FOXC1* at the indicated time points was determined by densitometry analysis of the signals detected via western blot. Relative amounts of *FOXC1* are expressed as a percentage of the protein levels at time 0 h. (c). The rate of decay (k) and half-lives ($t_{1/2}$) of the recombinant *FOXC1* variants at the indicated time points was determined using a linear regression analysis as described in the Materials and methods section. The error bars correspond to the SD of three independent experiments carried out in triplicate. The asterisks indicate statistically significant differences with respect to the control. The P -values were obtained using a one-way ANOVA followed by Tukey's multiple-comparison test: ** $P < 0.01$; *** $P < 0.001$.

further supporting its role as a modifier factor. We have observed that the p.(G456dup) variant moderately increases transactivation independent of protein stability and DNA-binding ability (unpublished data). As shown previously, p.(G456dup) is located in a Gly-rich region within an IDR of the protein that is important for molecular interactions. Therefore, the length of this region could affect protein-protein interactions and modulate the transactivation activity of *FOXC1*.

Functional evaluation of the rare variants and identification of regulatory sequences

Three of the five identified variants mapped at UTRs and two affected the coding region of *FOXC1*. The c.-429C>G purine/pyrimidine transversion was predicted to impair a putative TOP regulatory motif that is known to coordinate translation repression of vertebrate ribosomal proteins and translation elongation factors.²² This motif consists of 5–15 pyrimidines, beginning with a C residue and ending in a G nucleotide and, remarkably, it has been reported that the exchange of purines for pyrimidines in this motif results in unregulated translation.²² According to these data, the simultaneous increase in transactivation and *FOXC1* protein levels associated with the c.-429C>G variant supports the idea that the mutant has a diminished ability to repress mRNA translation. The second 5'-UTR variant that we found, c.-244C>T, was predicted to alter the mRNA secondary structure in this region. It is known that the 5'-UTR secondary structure influences mRNA translation,²³ and thus this variant has the potential to impair mRNA binding to regulators of protein synthesis. With regard to the c.*734A>T variant, we have observed that it disrupts the hsa-miR548l target sequence, leading to improved mRNA stability and thus to increased protein and transactivation levels.²⁴ Overall, our results support the hypothesis that these noncoding variants affect function. Moreover, the functional analyses of these variants provide the first evidence for the existence of translation negative *cis*-regulatory elements in both the 5'- and 3'-UTR *FOXC1* sequences and whose impairment likely participates in glaucoma. The p.(G380Rfs*144) variant generated a hypermorphic and truncated polypeptide lacking the C-terminal region that normally participates in *FOXC1* degradation via the ubiquitin 26S proteasome pathway.¹⁰ According to these data, p.(G380Rfs*144) showed remarkably increased protein stability that is likely the basis for its high transactivation activity.

Functional evaluation of the two gene variants identified in the patient with putative *FOXC1/PITX2* glaucoma inheritance showed that the reduced transactivation activity of *FOXC1* p.(P297S) does not depend on altered protein stability, DNA binding or nuclear localization. However, the fact that this variant maps to the predicted IDR3 opens the possibility that it interferes with the interactions of the transcription factor with other regulatory proteins, as discussed previously. In accordance with our results, it has been reported that p.(P297S) (1) shows reduced transactivation activity, (2) is associated with anterior segment digenesis and (3) is likely to disrupt *FOXC1* interactions with other unidentified factors involved in transactivation.¹⁴ In contrast to our results, however, a previous study has reported a moderately extended half-life for the p.(P297S) variant (45% longer than wild type).¹⁴ The second variant discussed, *PITX2* c.*454C>T, may increase the binding affinity of hsa-miR-548p to the mutant mRNA, leading to lower *PITX2* protein levels. Interestingly, this miRNA belongs to the same family as hsa-miR-548l that regulates *FOXC1* mRNA.²⁴ Thus, coinheritance of rare *FOXC1* and *PITX2* variants could contribute to glaucoma development by a simultaneous moderate alteration in translation of two related transcription factors.

Previous reports have suggested the existence of an upper and lower critical FOXC1 activity threshold associated with different phenotypes.²⁵ In accordance with these reports, our data suggest that moderate functional alterations of FOXC1, along with other genetic and/or environmental factors, may act as a modifier factor and contribute to the expression of relatively mild goniodysgenesis that results in congenital glaucoma.

In summary, this study supports the existence of novel *cis*-regulatory signals involved in FOXC1 translation and shows the existence of moderately altered rare variants of FOXC1 that may act as modifier factors in patients with PCG, suggesting the possible contribution of this gene to complex congenital glaucoma inheritance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)