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Missense Mutations in *TCF8* Cause Late-Onset Fuchs Corneal Dystrophy and Interact with *FCD4* on Chromosome 9p

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Fuchs corneal dystrophy (FCD) is a degenerative genetic disorder of the corneal endothelium that represents one of the most common causes of corneal transplantation in the United States. Despite its high prevalence (4% over the age of 40), the underlying genetic basis of FCD is largely unknown. Here we report missense mutations in *TCF8*, a transcription factor whose haploinsufficiency causes posterior polymorphous corneal dystrophy (PPCD), in a cohort of late-onset FCD patients. In contrast to PPCD-causing mutations, all of which are null, FCD-associated mutations encode rare missense changes suggested to cause loss of function by an in vivo complementation assay. Importantly, segregation of a recurring p.Q840P mutation in a large, multigenerational FCD pedigree showed this allele to be sufficient but not necessary for pathogenesis. Execution of a genome-wide scan conditioned for the presence of the 840P allele identified an additional late-onset FCD locus on chromosome 9p, whereas haplotype analysis indicated that the presence of the *TCF8* allele and the disease haplotype on 9p leads to a severe FCD manifestation with poor prognosis. Our data suggest that PPCD and FCD are allelic variants of the same disease continuum and that genetic interaction between genes that cause corneal dystrophies can modulate the expressivity of the phenotype.

Introduction

FCD represents the most common form of genetic disorders of the corneal endothelium.[1,2] The disorder affects as much as 4% of the population over the age of 40 and accounts for a significant fraction of the corneal transplantation performed in the United States every year.[2–4] Clinically, FCD is marked by the development of guttae, excrescences of Descemet membrane that appear in the fourth or fifth decade and increase in number over time.[5,6] As the disease progresses, visual acuity decreases secondary to corneal edema and endothelial cell loss, with end-stage disease evidenced by the formation of painful epithelial bullae.[7]

FCD is genetically heterogeneous, exhibiting an autosomal-dominant mode of inheritance with variable penetrance and expressivity. The rare form of early-onset FCD is causally associated with mutations in *COL8A2* (MIM 120252), whereas the more common late-onset FCD has been localized to three loci: *FCD1, FCD2,* and *FCD3* on chromosomes 13, 18, and 5, respectively.[8–10] Although clinically distinct, corneal endothelial dystrophies share clinical features suggesting that genes implicated in one corneal dystrophy may also harbor mutations liable for other dystrophies. This premise was strengthened when pathogenic mutations in *SLC4A11* (MIM 610206), a borate transporter in which loss-of-function mutations cause autosomal-recessive congenital hereditary endothelial

dystrophy (CHED2 [MIM 217700]), were identified in sporadic late-onset FCD patients.[11] Therefore, we hypothesized that *TCF8* (MIM 189909), a transcription factor shown to be causally associated with another corneal dystrophy, PPCD (MIM 609141),[12,13] may contribute to the genetic load of late-onset FCD.

Here, we report five missense mutations in *TCF8* associated with late-onset FCD, which appear to be causal because of a loss of protein function based on an in vivo complementation assay. One of these mutations, encoding a p.Q840P change, was present in both a sporadic FCD patient and in a large family and was transmitted in a manner consistent with an autosomal-dominant trait. However, the 840P allele could not explain FCD in all patients, suggesting the presence of a second pathogenic allele in this pedigree. A genome-wide scan conditioned to the presence/absence of the *TCF8* mutant allele identified a new locus for late-onset FCD, *FCD4* on chromosome 9p subsequent genetic and clinical analyses suggested that although mutations in each of *TCF8* and *FCD4* are sufficient for pathogenesis, genetic interaction between these two loci can lead to a more severe form of the disease. Our data represent the first familial evidence for mutations that cause late-onset FCD, supporting the hypothesis that several corneal dystrophies, although clinically distinct, share the same molecular etiology. Additionally, our data suggest that the quality and quantity of mutations in FCD-associated loci can have a profound impact on the

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clinical presentation of the phenotype, which in turn will probably impact patient management.

Material and Methods

Recruitment

Family members were recruited through a proband with FCD presenting to our clinics at Johns Hopkins University or Duke University. Extended pedigrees were subsequently developed through interviews and patients were examined in locations proximal to their area of residence. Recruitment, examination, and procedures for DNA sample collection were approved by the Institutional Review Boards for Human Subjects Research at the Johns Hopkins University School of Medicine and Duke University Medical Center, respectively, according to the Declaration of Helsinki. Each study participant provided written consent.

Determination of Phenotype and Disease Severity

Individuals underwent detailed ophthalmic examination including slit-lamp biomicroscopy. Severity was graded on a modified scale according to Krachmer and colleagues with >12 central guttae in either eye (grade 1).[5] Progression of confluence was defined by three grades: horizontal width less than 2 mm (grade 2), from 2 to 5 mm (grade 3), and >5 mm (grade 4). The development of stromal and/or epithelial edema elevated this score to grade 5, and grade 6 is disease severe enough to require keratoplasty.

Genotyping

An Affymetrix (Affymetrix, Santa Clara, CA) 5.0 SNP array was used for genome-wide linkage analyses. 500 ng of genomic DNA was used in a multiplexed SNP genotyping assay according to manufacturer's instructions. Arrays were then scanned in a Gene-Chip Scanner 3000 7G (Affymetrix). The output signal files were checked for quality control (QC) with the Affymetrix BRLMM analysis tool 2.0 (BAT 2.0). The default threshold score was set at 0.05 and was used as a cutoff for SNPs to be excluded. Cell files were processed further to generate genotypes with the BAT 2.0 Affymetrix genotyping console and locally written Perl scripts.

Linkage Analyses

SNP genotypes were incorporated into the family pedigree files to generate PED files with Plink software.[14] Pedigrees were analyzed assuming an autosomal-dominant mode of inheritance, 0.02 disease allele frequency, and 90% penetrance by MERLIN algorithms.[15] SNP allele frequencies from an ethnically matched control population were obtained from HapMap. The critical interval on 9p was delineated by genotyping STR markers; primers and conditions available upon request. Two point linkage analyses were performed with the FASTLINK version of MLINK from the LINKAGE Program Package.[16] Maximum LOD scores were calculated with ILINK under an autosomal-dominant model with a 0.02 disease allele frequency and 90% penetrance. The marker order and distances between the markers were obtained from the Marshfield database and the NCBI chromosome 9 sequence maps. Equal allele frequencies were assumed for the initial genome scan, while for fine mapping allele frequencies were derived from 96 unrelated and unaffected individuals.

Mutation Screening

Primer pairs for individual exons of TCF8 were designed with Primer3. The sequences and amplification conditions are available upon request. Each amplicon was purified and sequenced bidirectionally with Big Dye Terminator Ready Reaction Mix according to manufacturer's instructions (Applied Biosystems).

Construction of the TCF8 Vector

We cloned the TCF8 ORF into a pCS2+ plasmid as follows. The 3375 bp TCF8 ORF was amplified from HEK293T cDNA in two separate amplification reactions. In the first step, the 1875 bp fragment was amplified, digested with BamHI and BglII restriction endonucleases, and cloned into BamHI/BglII-digested pCS2+ plasmid. The orientation of the clone was detected by restriction digestion with BamHI, BglII, and XhoI. In the second step, a 1775 bp fragment was amplified, digested with BglII and XhoI restriction endonucleases, and cloned into BglII/XhoI digested pCS2+ plasmid harboring the N-terminal TCF8 fragment in the correct orientation. The sequence of TCF8 was confirmed by Big Dye Terminator Ready reaction mix according to manufacturer's instructions (Applied Biosystems).

Generation of Site-Directed Mutants

All five missense mutations identified in this study, as well as a previously identified mutation N696S,[17] were cloned in the TCF8-pCS2+ vector with QuikChange Site-Directed Mutagenesis Kit according to manufacturer's instructions (Stratagene, La Jolla, CA). The presence of each mutation was confirmed with bidirectional sequencing. Additionally, the entire TCF8 ORF for each clone was sequenced to rule out the presence of any mutations that may have been incorporated during the mutagenesis procedure.

In Vitro Transcription

The pCS2+ plasmids harboring the wild-type and mutant alleles of TCF8 were digested with NotI to linearize the plasmids. These linearized plasmids were used for in vitro transcription with mMessage mMachine transcription kit utilizing the SP6 promoter according to the manufacturer's instruction (Ambion Austin TX).

In Vivo Analyses

We used an antisense translation blocking morpholino (MO) to suppress translation of zebrafish tcf8. The MO was injected into one- to two-cell stage embryos. The exact concentration of MO necessary was determined by a dose-response curve. Embryos were raised to somite stages (approximately 14 hr) and scored for early developmental defects. Embryo phenotypes were rescued by coinjecting mRNA encoding full-length wild-type human TCF8 mRNA and the efficiency of rescue of the zebrafish tcf8MO was measured. Approximately 100 embryos were injected with either MO alone, MO and wild-type TCF8 mRNA, or MO and mRNA bearing one of each of the missense mutations and were scored blind to the injection cocktail.

Results

Mutations in TCF8 Are Sufficient to Cause FCD

The association of SLC4A11 with late-onset FCD[11] raised the possibility that genes that cause other corneal endothelial dystrophies might also contribute to FCD. To investigate this hypothesis, we focused on TCF8, a transcription factor that has been causally linked to PPCD, another endothelial dystrophy with some phenotypic similarities to FCD.[12,13] We therefore sequenced the entire coding
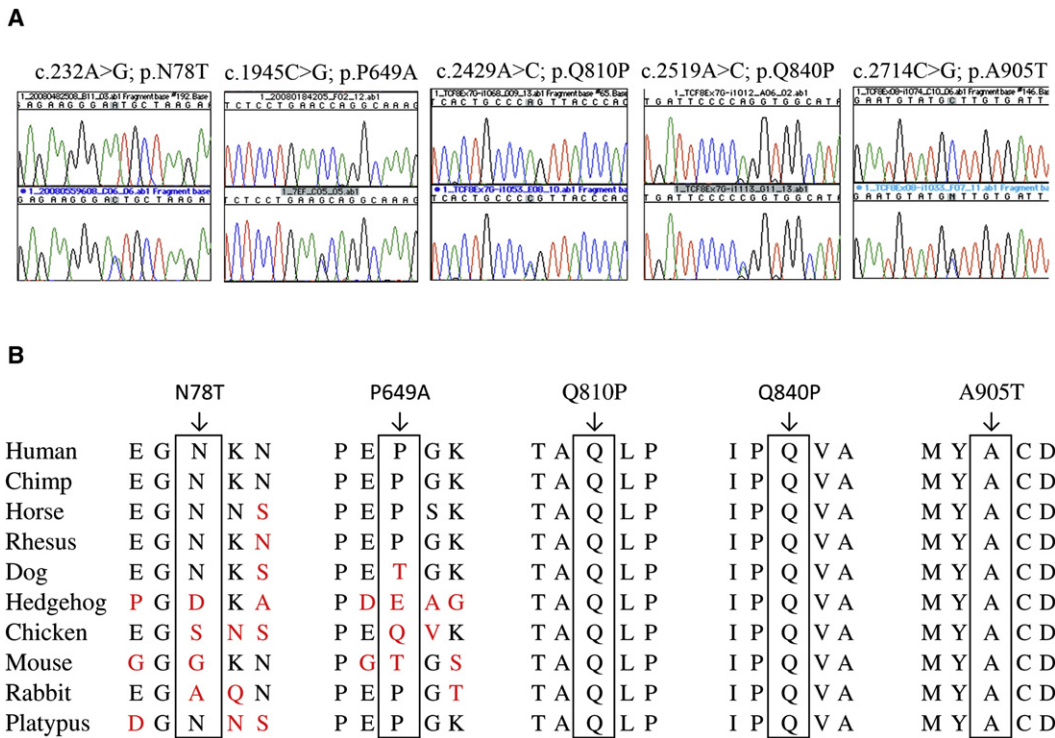
Figure 1. Sequence Analyses and Evolutionary Assessment of Late-Onset FCD Mutants
(A) Sequence chromatograms of the five *TCF8* mutations (c.232A>G, c.1945C>G, c.2429A>C, c.2519A>C, c.2714C>G) identified in late-onset FCD patients.
(B) Illustration of the evolutionary conservation of Asn78, Pro649, Gln810, Gln840, and Ala905 in other *TCF8* orthologs.

region of *TCF8* in 192 unrelated adult-onset FCD patients of northern European descent. In addition to a number of known SNPs, whose frequency in our cohort was indistinguishable from reported HapMap data, we also identified four unique alleles encoding the missense changes p.N78T, p.Q810P, p.Q840P, and p.A905G. Each of these variants affect evolutionary conserved residues (Figure 1) and was not found in 560 unrelated, ethnically matched control chromosomes or in HapMap samples of any ethnicity. Additionally, computational predictions with both SIFT[18] and PolyPhen[19] suggested that they were pathogenic, raising the strong possibility that missense alleles in *TCF8* can contribute to late-onset FCD. To confirm these data, we sequenced a second, independent cohort of 192 sporadic samples of Caucasian descent, ascertained under the same clinical criteria. We found three missense changes: a novel p.P649A that is also evolutionarily conserved and computationally predicted to be pathogenic (Figure 1) and p.N78T and p.Q840P, each of which was also found in the first cohort with no evidence for a shared ancestral haplotype. Six of the seven FCD patients were sporadic and as such, segregation analysis was not possible. The seventh patient bearing the recurring p.Q840P mutation, however, belongs to a large, multigenerational FCD family, acronymed TH. Targeted recruitment within TH (Figure 2) enabled us to further identify 12 individuals (seven females, five males) who fulfill the FCD phenotypic criteria (Table 1). A 13th

individual (Figure 2; individual IV:10) exhibited early signs of FCD; however, the patient did not have sufficient guttae for a positive diagnosis. In view of her younger age (38 years) and the number of guttae present bilaterally (<12), she was designated as a subclinical case.

Identification of a Fourth FCD Locus
Segregation analysis showed that the p.Q840P allele is sufficient but not necessary for disease pathogenesis. None of the seven unaffected individuals carry the 840P allele, whereas the mutation is present in 7/12 affecteds (Figure 2), suggesting that a second, independent genetic event might account for the phenotype in the other five patients. To investigate this possibility, we performed a genome-wide screen by genotyping all family members with an Affymetrix 5.0 array. Approximately 481K SNPs passed quality control and were considered for linkage. However, because of the unattainable computational needs required to perform linkage with the entire marker set, we selected every 5th accepted SNP to simplify the data while maintaining uniform coverage across the genome and performed two-point linkage on ~96K SNPs. Initial analyses under various disease models failed to detect LOD scores even above 2.0 when considering the entire family. We therefore took into consideration the *TCF8* p.Q840P allele and recomputed linkage conditional to this mutation. This model predicated that (1) all affected individuals negative for the p.Q840P mutation must
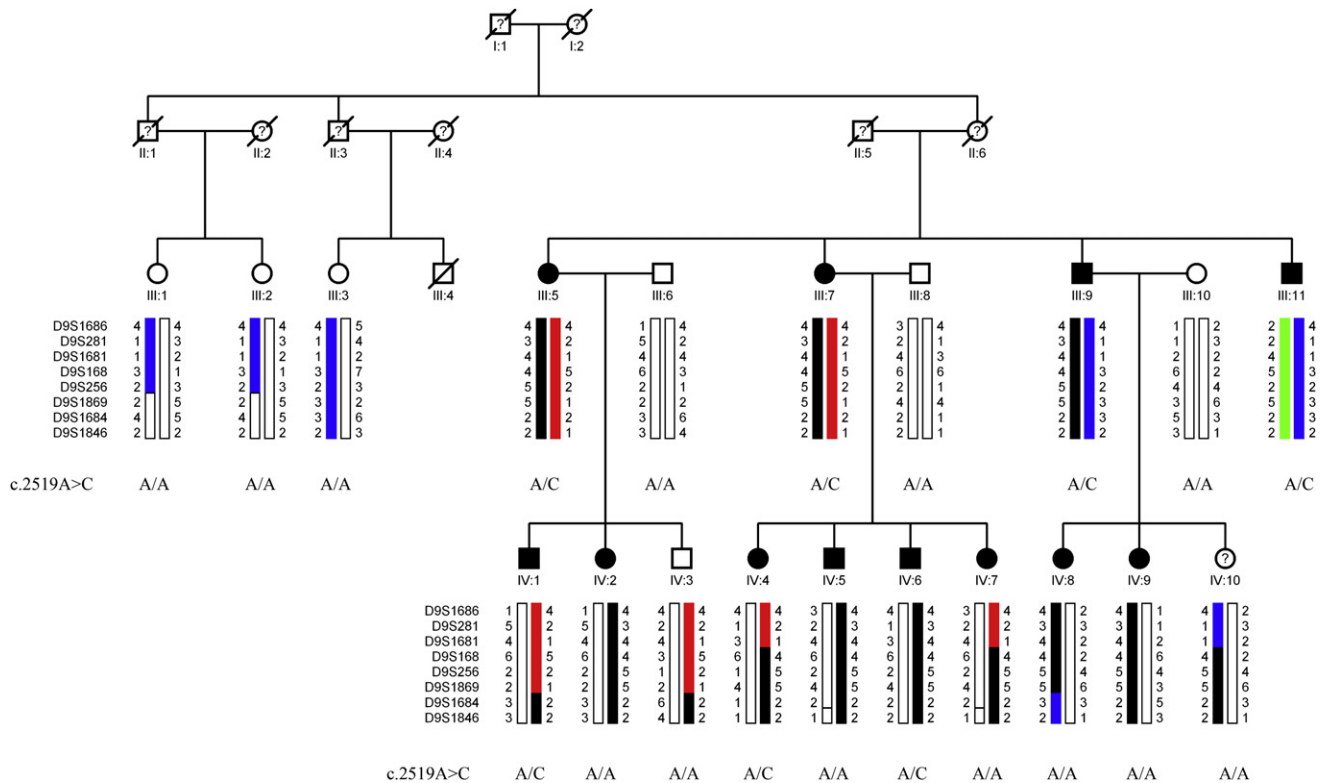
Figure 2. Pedigree of the Family TH Linked to the *FCD4* Locus
Symbols: filled symbol, affected; diagonal line through a symbol, deceased; question mark, affectation status known. Haplotypes of 12 adjacent chromosome 9p microsatellite markers are shown, with alleles forming the disease-bearing haplotype shaded black. The *TCF8* mutation; c.2519A>C identified in family TH is shown at the bottom.

harbor a pathogenic allele elsewhere in the genome; and (2) affected individuals heterozygous for the p.Q840P mutation may not necessarily harbor the pathogenic allele, with the exception of individuals with affected offspring who lack the p.Q840P mutation. Under this model, we found suggestive linkage to a single region in the genome on chromosome 9p (highest two-point LOD score of 2.43 with rs1410041). Importantly, a "reverse" genome scan was also consistent with a two-locus model. When we conditioned for the 9p risk allele (affected individuals or individuals with affected offspring negative for the 9p allele must harbor a second pathogenic allele elsewhere in the genome, whereas the rest of the affected individuals may not necessarily conceal this allele), we obtained the highest genome-wide two-point LOD score of 1.93 at *TCF8* (rs1148233), close to the theoretical maximum.

Because SNPs are biallelic, they have reduced informativeness. Therefore, we genotyped all members of family TH with a series of 10 evenly spaced STR microsatellite markers (Figure 2). We observed LOD scores of 3.09 and 3.20 at $\theta = 0$ with markers *D9S168* and *D9S256*, respectively (Table 2), close to the theoretical maximum attainable with this family, thereby defining a fourth FCD locus, *FCD4*. Recombinants place the mutation in a 14 Mb interval between *D9S1681* proximally and *D9S1684* distally. Reconstruction of the disease haplotype was consistent with a two-locus model, in that all affected indi-

viduals negative for the p.Q840P *TCF8* mutation harbor the disease-transmitting 9p22.1-p24.1 haplotype (Figure 2). Notably, individual IV:10, with a borderline diagnosis, carries the disease haplotype as predicted by the presence of preclinical FCD findings. After considering her as affected, the LOD score is elevated to 3.48 (Table 2).

Genetic Interaction between *TCF8* and *FCD4*

Within family TH, five affected individuals are heterozygous for both the p.Q840P mutation and the disease haplotype on 9p22.1-p24.1 (Figure 2). We therefore wondered whether the presence of two pathogenic alleles influences the clinical phenotype and severity of the disease. To compare across the same age, we examined the clinical records of individuals III:5, III:7, III:9, and III:11 (all ages > 70). Individuals III:5, III:7, and III:9 harbor both p.Q840P mutation and the *FCD4* disease allele, whereas individual III:11 harbors only the p.Q840P mutation. The clinical records confirmed that all three individuals with both mutations have a severe FCD phenotype (modified Krachmer grading of 6 bilaterally) and have undergone corneal transplantations, bilaterally. In sharp contrast to his siblings, individual III:11 has a milder FCD phenotype (Krachmer grading of +2.5 bilaterally). Progression of disease for individuals in family TH with a single pathogenic allele (*TCF8* p.Q840P or disease haplotype on 9p) shows as a linear relationship between age and severity

Table 1. Clinical Characteristics of Affected Individuals of Family TH

Individual ID	Sex	Age at Time of the Examination (Years)	Age at Time of the Penetrating Keratoplasty (Years)	FCD Krachmer Grading	TCF8 Mutation	FCD4 Disease Haplotype
III:5	F	78	71	PK bilaterally	p.Q840P	yes
IV:1	M	55	N.A.	OD; +1.0/ OS; +1.5	p.Q840P	no
IV:2	F	57	N.A.	OD; +2.5/ OS; +2.5	wild-type	yes
III:7	F	76	70	PK bilaterally	p.Q840P	yes
IV:4	F	49	N.A.	OD; +1.0/ OS; +1.0	p.Q840P	yes
IV:5	M	53	N.A.	OD; +1.0/ OS; +1.5	wild-type	yes
IV:6	M	46	N.A.	OD; +1.0/ OS; +1.0	p.Q840P	yes
IV:7	F	42	N.A.	OD; +1.0/ OS; +1.0	wild-type	yes
III:9	M	72	68	PK bilaterally	p.Q840P	yes
IV:8	F	47	N.A.	OD; +1.0/ OS; +1.0	wild-type	yes
IV:9	F	45	N.A.	OD; +1.0/ OS; +1.0	wild-type	yes
IV:10	F	37	N.A.	trace bilaterally	wild-type	yes
III:11	F	70	N.A.	OD; +2.5/ OS; +2.5	p.Q840P	no

Individuals III:5, III:7, and III:9 (Figure 1) had bilateral corneal transplants. Penetrating keratoplasty was performed on each eye separately and only once the severity of disease had reached 6 on the modified scale proposed by Krachmer and colleagues.[5] Abbreviations: PK, penetrating keratoplasty; F, female; M, male; N.A., not applicable.

with an r^2 value of 0.84 (Figure 3). Notably, the severity of individuals III:5, III:7, and III:9, harboring pathogenic alleles in both TCF8 and FCD4, is not consistent with the progression pattern of family TH, and based on these observations, we predict that individuals IV:4 and IV:6 (both in their 40s at time of the examination) will develop a severe FCD phenotype compared with other affected siblings.

The fact that three affected individuals in family TH underwent a corneal transplant was surprising because the majority of the families in our cohort rarely have multiple affecteds that had corneal transplants. Therefore,

Table 2. Two-Point LOD Scores Generated with 9p STR Markers

Marker	cM	Mb	0.00	0.01	0.05	0.10	0.20	0.30	Z_{max}	θ_{max}
D9S1686	12.50	4.63	0.672	0.658	0.598	0.567	0.520	0.357	0.672	0.00
			0.670	0.656	0.596	0.565	0.519	0.356	0.670	0.00
D9S1681	18.60	5.27	−0.484	−0.321	0.091	0.216	0.346	0.500	0.500	0.30
			−1.417	−1.225	−0.646	−0.448	−0.223	0.155	0.155	0.30
D9S281	15.33	6.71	−0.092	0.047	0.357	0.436	0.501	0.475	0.501	0.20
			−0.094	0.046	0.358	0.436	0.502	0.476	0.502	0.20
D9S168	20.40	10.57	3.094	3.038	2.807	2.689	2.507	1.862	3.094	0.00
			3.375	3.314	3.067	2.939	2.744	2.049	3.375	0.00
D9S256	22.80	11.01	3.201	3.145	2.916	2.798	2.617	1.972	3.201	0.00
			3.482	3.422	3.175	3.048	2.854	2.160	3.482	0.00
D9S1869	26.50	14.26	1.988	1.969	1.874	1.816	1.719	1.321	1.988	0.00
			2.268	2.246	2.133	2.066	1.955	1.508	2.268	0.00
D9S1684	34.42	19.60	−5.918	−2.870	−1.394	−0.839	−0.745	−0.232	−0.232	0.30
			−0.204	−0.185	−0.125	−0.102	−0.075	−0.022	−0.022	0.30
D9S1846	37.58	21.62	−3.221	−2.397	−1.403	−1.148	−0.873	−0.375	−0.375	0.30
			−4.299	−2.220	−1.169	−0.919	−0.658	−0.221	−0.221	0.30

LOD scores were calculated at different θ values for each marker with individual IV:10 as unaffected (top) and affected (bottom row).
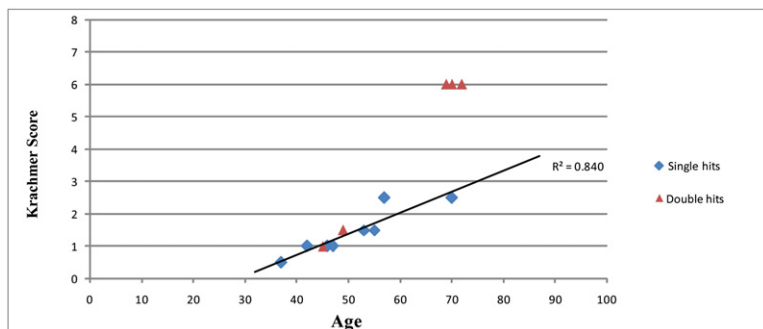
Figure 3. Progression of Late-Onset FCD with Age in Family TH
The diamonds represent the individuals with single pathogenic allele (*TCF8* p.Q840P or disease haplotype on 9p), whereas the triangles symbolize individuals harboring both pathogenic alleles. The age of each individual was recorded at the time of the examination or at the time of bilateral keratoplasty.

we wondered whether the presence of pathogenic alleles at two loci might have contributed to the higher incidence of corneal transplants in family TH. To examine this possibility, we interrogated our entire cohort setting the following two stringent criteria: (1) we restricted our analyses to families with three or more affecteds; and (2) we only considered affecteds with bilateral corneal transplants. We were able to examine 33 families and found a remarkably low frequency of bilateral transplants (9/246) which, despite the relatively modest sample size, was significantly different compared to 3/12 in family TH ($\chi^2 = 10.618$; p = 0.0006). Even under reduced stringency criteria, where all transplant events were considered in our cohort, the enrichment in family TH remained significant ($\chi^2 = 8.17$; p = 0.0042). As such, the most parsimonious explanation for these observations is that in family TH the p.Q840P allele interacts with the *FCD4* causal mutation to modulate the severity of the phenotype.

In Vivo Functional Assessment of *TCF8* Mutations
Taken together, our genetic data implicate missense mutations in *TCF8* in the development of late-onset FCD. All mutations affect conserved residues and have been found exclusively in patients (two of them recurrent). Importantly, phasing pedigree TH for the presence of the p.Q840P allele maps a new FCD locus supportive of a two-locus model. Although *TCF8* null mutations have been shown to cause PPCD, an earlier onset endothelial corneal dystrophy, we have determined that missense alleles in FCD are of potential mechanistic consequence. We considered two possibilities: (1) loss-of-function alleles lead to PPCD and dominant-negative or gain-of-function mutations cause FCD, or (2) both disorders can be induced by relative *TCF8* loss. To investigate these possibilities and to obtain further evidence for the functional relevance of the mutated residues, we took advantage of an in vivo complementation assay in zebrafish. Upon reciprocal BLAST, we identified the sole ortholog of *TCF8* in the zebrafish genome (81% similarity at protein level), also known as *kheper*. Previous studies have shown that expression of dominant-negative *tcf8* RNA results in severe defects during zebrafish gastrulation and neurulation.[20] Therefore, under the reasonable premise that morpholino (MO)-induced *tcf8* suppression should reproduce such

phenotypes, we initiated a series of in vivo complementation studies. Specifically, to interrogate the mechanisms by which mutations in *TCF8* cause FCD in humans, we sought to use these developmental defects as a functional readout and ask whether (1) suppression of endogenous *tcf8/kheper* produces similar phenotypes early in development; (2) whether wild-type (WT) human *TCF8* mRNA can rescue such phenotypes; (3) whether expression of human *TCF8* mRNA bearing each of the five point mutations can rescue the phenotype; and (4) whether expression of the same mutants might induce early developmental defects (indicative of a dominant-negative or gain-of-function effect).

To suppress expression of *tcf8* in embryos, we designed a translational blocking morpholino (tbMO). Injection of 2.5 ng of tbMO at the one- to two-cell stage resulted in defects in early development consistent with published observations in approximately 80% of embryos,[20] which could be categorized into two severity classes. Class I embryos survived gastrulation but had shortened body axis and pronounced detachment of cells along the dorsal axis at mid-somitic age, whereas class II embryos exhibited complete disruption of embryo morphology during gastrulation (Figure 4A). These phenotypes could be rescued efficiently by coinjection of human WT *TCF8* mRNA. Coinjection of 100 pg of human mRNA with 2.5 ng of tbMO resulted in a complete rescue of the morphant phenotypes (97.5% of embryos, n = 100–150 embryos scored blind) (Figure 4B). To determine whether mutations in *TCF8* result in disruption of protein function, we coinjected each mutant mRNA and assessed its ability to rescue morphant phenotypes. This assay further supported our human genetics evidence in that each of the five mutations reduced or abolished TCF8 function. Two mutations, p.N78T and p.Q810P, partially rescued the MO phenotype, indicating that these are probably hypomorphic, whereas rescue with human *TCF8* mRNA encoding either 649A, 840P, or 905G were indistinguishable from tbMO-injected embryos, suggesting that these alleles are severe (Figure 4B). The assay is not promiscuous; injection of tbMO with human mRNA encoding an N696S mutation reported previously in a Chinese FCD patient and reported to be benign[17] fully rescued the morphant phenotype (Figure 4B). Notably, though injection of wild-type mRNA
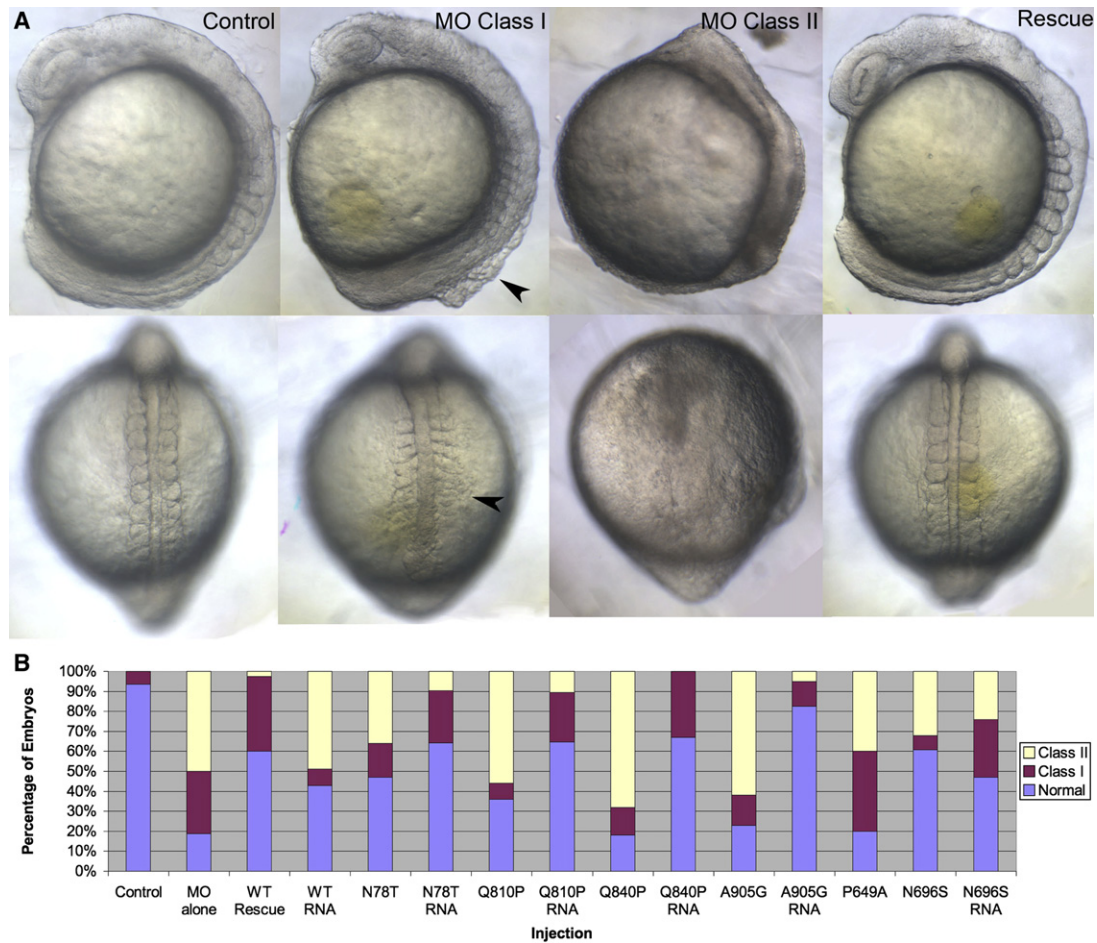
Figure 4. Suppression of *tcf8* Produces Specific Defects in Zebrafish Embryos
(A) Embryos injected with MO against *tcf8* have early developmental defects including short body axes and detachment of cells along the dorsal embryonic axis (arrowhead). These defects can be rescued by coinjection with full-length human mRNA.
(B) Proportions of unaffected embryos are rescued almost completely in coinjected embryos as compared to those injected with MO alone.

resulted in defects in early development, injection of each of the mutant mRNAs alone did not produce the same severity of defects, suggesting that these mutations are unlikely to exert a dominant-negative or gain-of-function effect (Figure 4B). As such, although caution must be exercised in translating the function of alleles in a developmental assay to late-onset corneal degeneration phenotypes, it is likely that a loss-of-function mechanism underlies the FCD-associated *TCF8* mutations.

Discussion

Here, we report five novel missense mutations in *TCF8* identified in two cohorts of patients diagnosed with late-onset FCD. These mutations affect evolutionary conserved alleles and were absent from ethnically matched control samples. Additionally, we identified a novel locus for late-onset FCD, *FCD4*, in a large family, with the critical interval mapped to chromosome 9p. Haplotype analyses refined the interval to a 14.3 Mb (15.8 cM) on 9p24.1-22.1 with

a maximum two-point parametric LOD score of 3.20. Although the maximum LOD score of 3.20 is only slightly higher than the traditional limiting value of 3.0, it represents the maximum theoretical obtainable value for this family. Our data reinforce the notion that there is extensive heterogeneity for late-onset FCD, contrary to early-onset that is causally associated with *COL8A2*, with *TCF8* contributing as much as 2% to the genetic load (7/384 unrelated patients), with no evidence for shared haplotypes among unrelated patients with the same mutation. Based on genetic and mutational data from all known *FCD* loci, *FCD2* remains the most common locus, with a predicted contribution of ~25%, followed by *SLC4A11*, in which we and others have found mutations in 3%–4% of sporadic late-onset FCD patients.[11] Each other mapped *FCD* locus represents, at present, a private mutation.

Mutations in *TCF8* have been associated previously with PPCD,[12,13] a dystrophy of the corneal endothelium. Interestingly, all mutations reported in PPCD either are nonsense mutations or result in premature termination of *TCF8*; in contrast, we found only missense mutations in

late-onset FCD patients. We have encountered this phenomenon before: *MKS1* (MIM 609883), one of the genes that causes Meckel-Gruber syndrome (MKS [604896]), a neonatal lethal condition, is always found to be null in Meckel patients. Under the hypothesis that hypomorphic mutations in that locus might cause a less severe phenotype, we were able to show that residual activity at this locus is causally related to Bardet-Biedl syndrome.[21] Given the fact the late-onset FCD represent a milder pathogenic phenotype compared with PPCD, the discovery of only missense mutations suggests that functionally deficient *TCF8* alleles are responsible for late-onset FCD phenotype, whereas nonsense mutations lead to PPCD, most probably because of haploinsufficiency.

The molecular mechanism of *TCF8* pathogenicity leading to two different corneal dystrophies remains unclear. *TCF8* is a transcription factor acting in some instances as an enhancer and in other instances as a repressor.[22] The two zinc finger domains present in the TCF8 define the DNA-binding specificity. Ikeda and colleagues investigated the DNA-binding properties of TCF8 protein and identified G/TT/GCACCTGT and C/TACCTG/TT as the optimal binding sites for the N- and C-terminal zinc finger motifs, respectively.[23] Interestingly, Krafchak and colleagues identified these consensus sequences present in the promoter regions of *COL4A*1 (MIM 120130), *COL4A2* (MIM 120090), *COL4A3* (MIM 120070), *COL4A*5 (MIM 303630), *COL4A6* (MIM 303631), and *COL8A*2.[12] Although *TCF8* transcripts have been detected in corneal samples,[12] it remains to be seen whether deficiency in the DNA-binding properties of TCF8 leads to late-onset FCD.

Our data provide the first familial evidence for mutations in adult-onset FCD and indicate that loss of function of *TCF8* can induce a range of corneal dystrophy phenotypes. Recently, it has been proposed that loss-of-function-mutations in *SLC4A11*, a gene mutated in CHED2, might also contribute to adult-onset FCD.[11] Taken together, these data suggest the presence of a continuum across several clinically distinct corneal dystrophies, where the nature of mutations might determine the severity and onset of the phenotype, a phenomenon we have encountered in other disorders.[21] As such, genes identified in CHED2, PPCD, and FCD represent bona fide candidates for all three clinical presentations. Moreover, our data also suggest that genetic interactions between FCD loci probably interact to modify the expressivity of the disorder; understanding the molecular basis of such interaction will be important both in terms of disease mechanism and also in the context of patient management and prognosis.

Acknowledgments

Web Resources

The URLs for data presented herein are as follows:

International HapMap Project, http://www.hapmap.org

Marshfield Clinic Research Foundation, http://www.marshfieldclinic.org/research/pages/index.aspx

National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov

Primer3, http://frodo.wi.mit.edu/primer3

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