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A novel missense mutation in the gene for gap-junction protein α3 (*GJA3*) associated with autosomal dominant "nuclear punctate" cataracts linked to chromosome 13q

Thomas M. Bennett,[1] Donna S. Mackay,[1] Harry L. S. Knopf,[1] Alan Shiels[1,2]

Departments of [1]Ophthalmology and Visual Sciences and [2]Genetics, Washington University School of Medicine, St. Louis, MO

Purpose: Autosomal dominant cataracts are a clinically and genetically heterogeneous eye-lens disorder that usually present in childhood with symptoms of impaired vision. The purpose of this study was to map and identify the mutation underlying autosomal dominant nuclear punctate cataracts segregating in a six generation Caucasian pedigree.
Methods: Genomic DNA was prepared from blood leucocytes, genotyping was performed using microsatellite markers, and LOD scores were calculated using the LINKAGE programs. Mutation detection was performed using direct sequencing and restriction fragment length analysis.
Results: Significant evidence of linkage was obtained at marker D13S175 (LOD score [Z]=4.11, recombination fraction [θ]=0.0) and haplotyping indicated that the disease gene lay in the about 2 Mb physical interval between D13S1316 and D13S1236, which contained the gene for gap-junction protein α3 (*GJA3*) or connexin46. Sequencing of *GJA3* detected a C->T transition in exon 2 that resulted in the gain of an *Alu* 1 restriction site and was predicted to cause a conservative substitution of proline to leucine at codon 59 (P59L). Restriction analysis confirmed that the novel *Alu* 1 site co-segregated with cataracts in the family but was not detected in a control panel of 170 normal unrelated individuals.
Conclusions: The present study has identified a fifth mutation in *GJA3*, rendering this connexin gene one of the most common non-crystallin genes associated with autosomal dominant cataracts in humans.

Congenital and infantile forms of cataracts (lens opacities) present at birth and during the first year of life, respectively. Because these neonatal lens opacities can cause blurring of vision during the critical period of form-vision development, they are clinically important as a cause of deprivation amblyopia [1] and represent a significant cause of vision impairment, accounting for an estimated 10-20% of childhood blindness in developing countries [2] and about 4% of adult blindness in industrialized countries [3]. According to the US collaborative perinatal project [4], infantile cataracts present with a prevalence of 13.6 cases per 10,000 live births, with a similar prevalence of unilateral to bilateral cases, occurring either as an isolated, non-syndromic lens defect (about 43% of cases) or in association with other ocular and/or systemic disorders, including congenital rubella syndrome [4] and many diverse genetic syndromes (Online Mendelian Inheritance in Man). All three classical types of Mendelian inheritance have been described for non-syndromic cataracts. However, the majority of families reported display autosomal dominant transmission.

Currently, at least twenty loci for clinically diverse forms of non-syndromic Mendelian cataracts have been mapped on fourteen human chromosomes. No causative genes have been reported at six of the dominant loci on chromosomes 1p [5,6], 2p [7], 15q [8], 17p [9], 17q24 [10], and 20p [11] or at the two recessive loci on 3p [12] and 9q [13]. However, underlying mutations have been identified in several functionally diverse genes, including seven crystallin genes located on 2q (*CRYGC* and *CRYGD* [14-22]), 11q (*CRYAB* [23]), 17q (*CRYBA3/A1* [20,24,25]), 21q (*CRYAA* [26-28]), and 22q (*CRYBB1* and *CRYBB2* [29-32]), an aquaporin gene (*MIP/AQP*) on 12q [33], an intermediate filament-like gene (*BFSP2*) on 3q [34,35], a heat shock transcription factor gene (*HSP4*) on 16q [36], a tetraspan-like gene (*LIM2*) on 19q [37], two genes for gap-junction proteins α8 (*GJA8*) on 1q [38-41], and α3 (*GJA3*) on 13q [42-44].

Gap-junctions are specialized arrays of cell-to-cell channels that facilitate the cytoplasmic exchange of ions, second messengers, and small (<1 kDa) metabolites (reviewed in [45]). Each gap-junction channel is composed of two hemi-channels, or connexons, which dock in the extracellular space between adjacent cells, and each connexon is comprised of six integral transmembrane protein subunits known as connexins. At least twenty genes for connexins of varying molecular mass (26-62 kDa) have been identified in the human genome and, in addition to *GJA3* and *GJA8*, at least six other connexins have been associated with human disease, including Charcot-Marie-Tooth neuropathy (*GJB1*) on Xq [46], oculodentodigital dysplasia (*GJA1*) on 6q [47], and various skin and/or hearing disorders (*GJB2*, *GJB3*, *GJB4*, and *GJB6*) mapping to 1p and 13q (reviewed in [48] and [49]). In this study we have mapped non-syndromic autosomal dominant cataracts to chromosome 13q and identified a novel mutation in GJA3 associated with "nuclear punctate" lens opacities.

Correspondence to: Alan Shiels, Ph.D., Department of Ophthalmology and Visual Sciences, Box 8096, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO, 63110; Phone: (314) 362-1637; FAX: (314) 362-3131; email: shiels@vision.wustl.edu

METHODS

Genotyping and linkage analysis: This study was approved by the institutional review board at Washington University School of Medicine and all participants provided informed consent prior to enrollment. Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA blood maxi kit (Qiagen, Valencia, CA). Microsatellite (CA)n repeat markers from the Généthon map [50] and the Marshfield genetic database were amplified using the polymerase chain reaction (PCR) and detected using a Li-Cor 4200 DNA analyzer

Table 1. PCR primers for mutation screening of *GJA3*

Location	Strand	Sequence (5'-3')
5' non-coding	Sense	TGCGGACCCGGCACTCAGC
Codons 223-229	Antisense	CTTCTTCCAGCCCAGGTGGTA
Codons 29-36	Sense	CTGTTCATCTTCCGCATTTTGG
Codons 96-103	Antisense	TCCATGCGCACGATGTGCAGCA
Codons 182-189	Sense	ACCGCTGGCCCTGCCCCAACAC
3' non-coding	Antisense	TCTTCTTCCAGCCCAGGTGGTA

Primer pairs used for amplification and sequencing of the coding exon for *GJA3* located on 13q.



Figure 1. Photograph of punctate nuclear cataract. Photograph of punctate nuclear lens opacities in individual V1:4 from the pedigree (Figure 2, arrow) prior to surgery at about 3 months of age. The opacities appear as dark specks in the retinal red reflex.

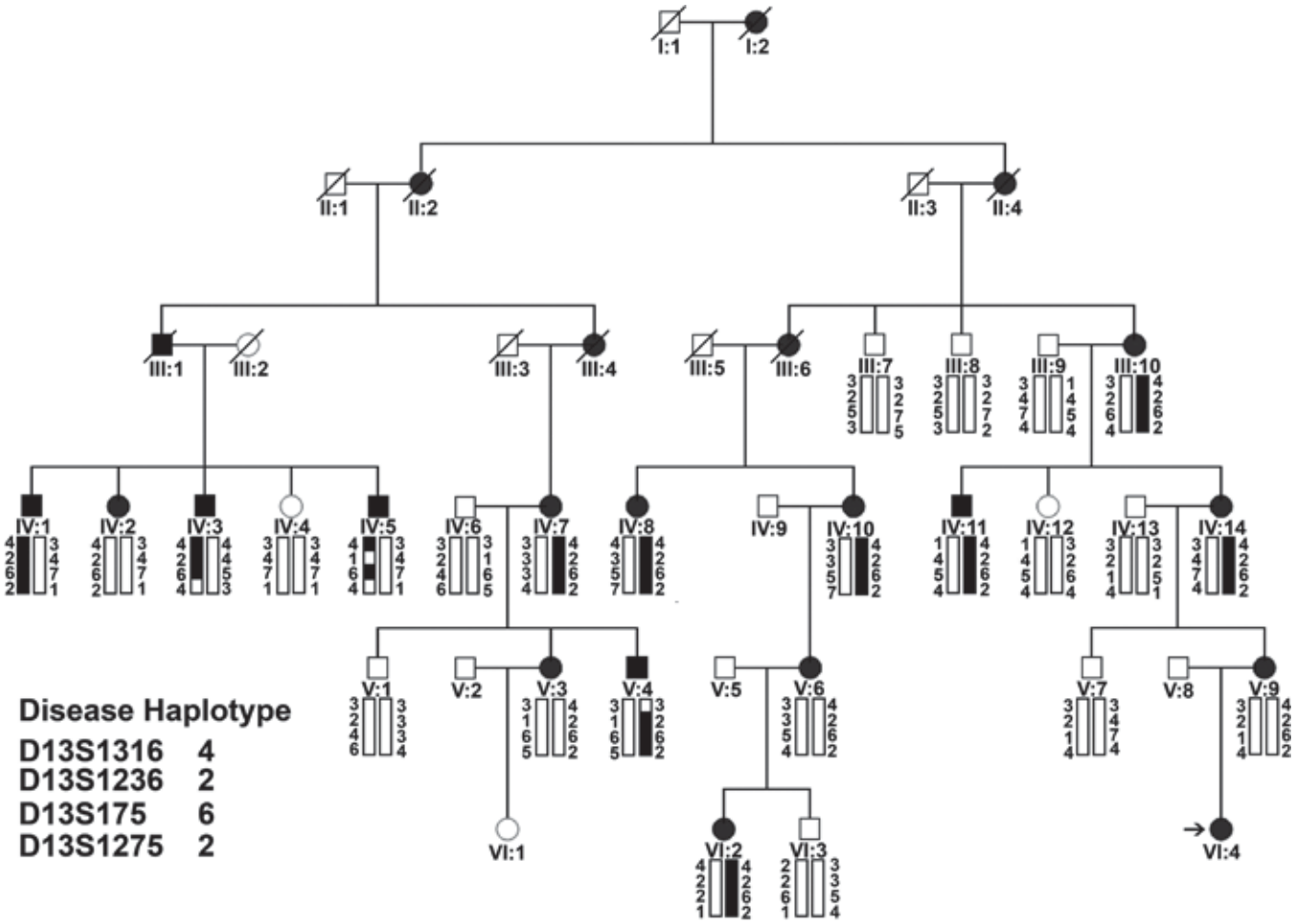

Figure 2. Cataract pedigree and haplotype analysis. Pedigree and haplotype analysis of the cataract family showing segregation of four microsatellite markers on chromosome 13q, listed in descending order from the centromere. Squares and circles symbolize males and females, respectively. Filled symbols denote affected status.

running Gene ImagIR software (Li-Cor, Lincoln, NE) as described previously [28]. Pedigree and haploptype data were managed using Cyrillic (version 2.1) software (FamilyGenetix Ltd., Reading, United Kingdom) and two-point LOD scores (Z) calculated using the MLINK sub-program from the LINKAGE (version 5.1) package of programs [51]. Microsatellite marker allele frequencies used for linkage analysis were those calculated by Généthon [50]. A gene frequency of 0.0001 and a penetrance of 100% were assumed for the disease locus.

Mutation analysis: Genomic sequence for *GJA3* was obtained from the Ensembl human genome browser and gene specific PCR primers were designed to anneal to coding regions and immediate 5' or 3' flanking, non-coding regions (Table 1). Genomic DNA (50-100 fmol) was PCR amplified with gene specific primers (25 pmol) for 35 standard cycles using a Peltier Thermal Cycler (PTC-200) DNA engine (MJ Research, Waltham, MA). PCR products were sized on 2% agarose gels containing 0.05% ethidium bromide (EtBr), visualized with a UV transilluminator, then purified using the QIAquick gel-extraction kit (Qiagen), and direct sequenced in both directions using the dye-terminator cycle-sequencing (DTCS) quick start kit on a CEQ8000 capillary-based genetic analysis system (Beckman-Coulter, Fullerton, CA). Restriction fragment length analysis was performed on gel-purified

Table 2. Two-point LOD scores for linkage between the cataract locus and chromosome 13 markers

			Z(θ)							
Marker	cM	Mb	0.00	0.05	0.10	0.20	0.30	0.40	Z(max)	θ(max)
D13S1316	0	18.48	-∞	3.80	3.58	2.79	1.81	0.78	3.80	0.04
GJA3 (C->T)		18.51	7.41	6.81	6.19	4.83	3.32	1.65	7.41	0.00
D13S175	6.03	18.65	4.11	3.66	3.19	2.23	1.26	0.43	4.11	0.00
D13S1236	6.17	20.49	-∞	1.25	1.24	0.92	0.55	0.22	1.27	0.07
D13S1275	6.99	20.75	-∞	1.96	2.34	2.12	1.44	0.57	2.36	0.12

Two-point LOD scores (Z) for linkage between the cataract locus and four markers on 13q listed in genetic (sex-averaged) order Marshfield genetic database and physical order (UCSC Genome Bioinformatics) from p-tel, measured in centi-Morgans (cM) and megabases (Mb), respectively. The marker D13S1236 has been placed according to genetic location on the deCODE map [53], rather than the integrated Généthon-Marshfield map (2.77 cM).

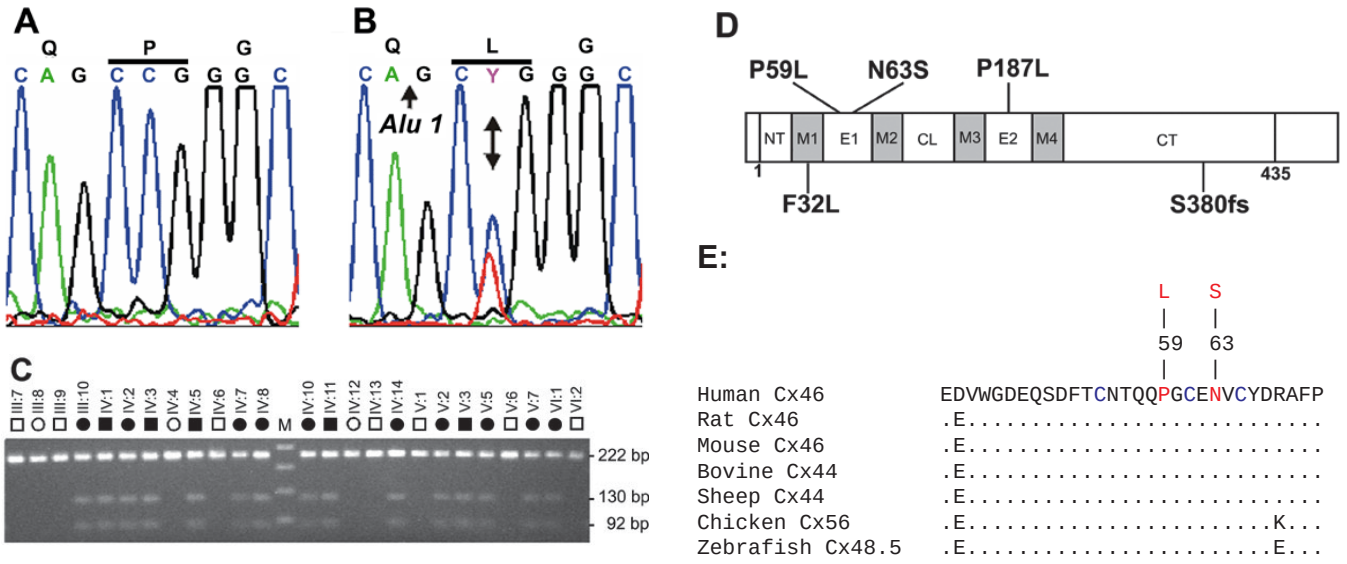

Figure 3. Mutation analysis of *GJA3.* Mutation analysis of *GJA3*. Sequence chromatograms of wild type allele (**A**) showing translation of proline (CCG) at codon 59 in exon 2 and mutant allele (**B**) showing a C->T transition at the second base of codon 59 that substituted leucine (CTG) for proline (P59L). **C**: Restriction fragment length analysis on agarose gels showing gain of an *Alu* 1 site (5'AG/CT) that co-segregated with affected individuals heterozygous for the mutant T-allele (130 bp and 92 bp) but not with unaffected individuals homozygous for the wild-type C-allele (222 bp). The letter "M" designates the 50 bp size ladder. **D**: Exon organization and mutation profile of *GJA3*. The entire coding region (435 amino acids) of connexin46 (Cx46) is located in a single exon. Based on hydrophobicity analysis [66], Cx46 has nine structural domains including a cytoplasmic amino-terminus (NT), 4 transmembrane domains (M1-M4), 2 extracellular loops (E1-E2), a cytoplasmic loop (CL), and a cytoplasmic carboxy-terminus (CT). The relative locations, with respect to the translation start codon, of the P59L mutation and three other mutations associated with dominant cataracts in humans are indicated. **E**: Amino acid sequence alignment of the E1 domain (codons 42-71) from human Cx46 and homologs from other species. Dots denote identical amino acids. Cysteine residues involved in connexon hemi-channel docking are in blue. The P59L and N63S substitutions are shown in red.

PCR products, amplified with sense and antisense primers (Table 1) for codons 29-36 and 96-103, respectively, using *Alu* 1 at 37 °C for 1 h according to the manufacturer's instructions (Roche, Indianapolis, IN). Digestion products were analyzed on 2% agarose/0.05% EtBr gels. In order to distinguish the predicted mutation (with 95% confidence) from a polymorphism with 1% frequency we extended our *Alu* 1 restriction analysis to include genomic DNA samples from a panel of 170 unrelated control individuals as recommended previously [52].

RESULTS

13q linkage analysis: We studied a six generation Caucasian American family that segregated autosomal dominant cataracts in the absence of other ocular or systemic abnormalities. Ophthalmic records indicated that the cataracts were bilateral with coarse punctate opacities located in the central or nuclear region of the lens (Figure 1). The mean age at diagnosis was 4.7 years (range, birth-18 years), and the mean age at surgery was 8.6 years (range, 0-49 years).

Twenty-five members of the family (Figure 2), including fifteen affected individuals, seven unaffected individual, and three spouses were genotyped with microsatellite markers at eleven known loci for autosomal dominant cataracts on chromosomes 1q (*GJA8*), 2q (*CRYGC* and *CRYGD*), 3q (*BFSP2*), 11q (*CRYAB*), 12q (*MIP*), 13q (*GJA3*), 16q (*HSF4*), 17q (*CRYBA3/A1*), 19q (*LIM2*), 21q (*CRYAA*), and 22q (*CRYBB1* and *CRYBB2*). Following exclusion of ten of these loci ($Z<-1.0$, $\theta=0.0$-0.1), we obtained significant evidence of linkage (Table 2) for marker D13S175 ($Z=4.11$, $\theta=0$) on 13q11-q12.

Haplotyping of the pedigree (Figure 2) detected two affected males (IV:3, IV:5) who were obligate recombinants at D13S1275 and one affected male (V:4) who was recombinant at D13S1316. Apart from individual IV:5, no recombinant individuals were detected at two other intervening markers suggesting that the disease locus lay in the genetic interval, D13S1316-(2.77 cM)-D13S1236-(3.26 cM)-D13S175-(0.96 cM)-D13S1275 defined by the integrated Généthon-Marshfield maps. However, individual IV:5 was also recombinant at D13S1236 but not at D13S175 or D13S1316, suggesting that either a double recombination event had occurred or that marker order was inaccurate. Consistent with the latter, the deCODE genetic map [53] placed D13S1236 over 6 cM distal to D13S1316 and the chromosome 13 physical map (UCSC Genome Bioinformatics and Ensembl), placed D13S1236 between D13S175 and D13S1275 (Table 2), indicating that the disease locus lay in the physical interval, D13S1316-(0.17 Mb)-D13S175-(1.84 Mb)-D13S1236. Significantly, D13S1316 and D13S175 lie about 30 kb proximal and about 130 kb distal to *GJA3*, respectively, suggesting that the latter was a strong candidate gene for the cataract.

GJA3 *mutation analysis:* According to the human genome browser, *GJA3* comprises one coding exon (Figure 3D). Sequence analysis of the entire coding region and immediate flanking regions in two affected individuals using *GJA3* specific primers (Table 1), detected a heterozygous C->T transition that was present in both of the affected individuals but not in either of the unaffected individuals (Figure 3A and Figure 3B). This single nucleotide change resulted in the gain of an *Alu* 1 restriction site (5'AG/CT) and restriction fragment length analysis confirmed the presence of the heterozygous C->T transition in all affected members of the pedigree and its absence in unaffected relatives and spouses (Figure 3C). Furthermore, when we tested the C->T transition as a bi-allelic marker, with a notional allelic frequency of 1%, in a two-point LOD score analysis of the cataract locus (Table 2) we obtained significant evidence of linkage ($Z=7.41$, $\theta=0$). Finally, we excluded the C->T transition as a single nucleotide polymorphism (SNP) in a panel of 170 normal unrelated individuals (data not shown).

At the level of protein translation, the C->T transition was predicted to result in a missense substitution of proline to leucine at codon 59 (P59L). This is considered a relatively conservative substitution of one non-polar hydrophobic residue for another, however, alignment of amino acid sequences for *GJA3* present in the Protein database using the BLAST algorithm [54] revealed that proline 59 is phylogenetically conserved from zebrafish to man (Figure 3E). Taken overall, the co-segregation of the C->T transition only with affected members of the pedigree and its absence in 340 normal chromosomes strongly suggested that the P59L substitution was a causative mutation rather than a benign SNP in linkage disequilibrium with the disease.

DISCUSSION

GJA3 encodes a lens abundant connexin of molecular mass about 46 kDa (Cx46) that functions in gap-junction communication between elongated fiber cells [55], which constitute the bulk of the lens mass and represent the target cells for cataract formation. Previous studies have identified three missense mutations (F32L, N63S, P187L) and an insertion mutation (1137insC), which resulted in a reading frame shift at codon 380 (S380fs), associated with autosomal dominant punctate cataracts segregating in extended pedigrees of English [42,56], Welsh [43,57], and Chinese [44] ancestry. Here we have identified a fifth mutation in *GJA3* linked with autosomal dominant nuclear punctate cataracts segregating in a six generation Caucasian American family.

Clinical descriptions of *GJA3* related cataracts share several genotype-phenotype similarities but also exhibit certain inter- and intra-familial differences with respect to the physical appearance and location of opacities within the juvenile lens. Thus, the F32L mutation [44] was associated with pulverulent (dust-like) or punctate opacities limited to the central (about 2 mm) zone or "embryonic nucleus" of the lens. Punctate opacities associated with the S380fs mutation [42] and the P59L mutation reported here, appeared coarse and granular located within the central zone (fetal nucleus) of the lens, with the former mutation also exhibiting a predominance fine dust-like opacities in the peripheral zone (juvenile cortex) of the lens. The N63S mutation [42,56] was also associated with fine dust-like opacities, which in some individuals formed a "zonular" or "lamellar" distribution with a clear peripheral cortex and minimal involvement of the central nucleus of the

lens. Others had more widely spread dust-like opacities extending into the cortex with no demarcation of the nucleus, and in very mild cases the dust-like opacities were clustered around the anterior and posterior "Y" shaped sutures of the lens fetal nucleus. Finally, the P187L opacities [43] were also described as central pulverulent affecting the embryonal, fetal, and infantile lens nuclei. However, they were surrounded by snowflake-like opacities in the anterior and posterior cortical region of the lens and also involved the posterior sub-capsular region.

Currently, no dominant spontaneous or mutagen induced cataracts have been associated with the murine gene for GJA3 (*Gja3*), and *Gja3* appears to map about 6 cM proximal to two potentially allelic recessive mutations, rupture of lens cataract (*rlc*) and lens rupture 2 (*lr2*), which have been mapped to the mid-region of murine chromosome 14 [58,59]. However, mice homozygous for targeted disruption of *Gja3* have been shown to develop severe nuclear cataracts associated with γ-crystallin proteolysis [60]. The severity of the murine cataracts on a 129SvJ genetic background was found to be significantly reduced on a C57BL/6J background [61], suggesting the phenotype was influenced by genetic modifiers, which may also contribute to the inter- and intra-familial variation observed in human *GJA3* related cataracts. Electrophysiological studies of lenses from *Gja3* null mice have revealed that the mature terminally differentiated fibers within the opaque nucleus are not only uncoupled from each other but also from the surrounding peripheral fibers undergoing terminal differentiation within the clear outer cortex [62]. Moreover, genetic replacement of the coding region for gap-junction protein α8 (*Gja8*), or connexin50 (Cx50, the other abundant lens fiber connexin), with the Cx46 coding region by targeted knock-in prevented the lens opacities but not the lens growth defect characteristic of *Gja8* null mice [63]. These observations suggest that Cx46 plays a critical role in maintaining lens transparency, particularly within the nucleus.

Functional expression studies have demonstrated that wild type human Cx46 can form gap-junction channels in paired *Xenopus* oocytes and hemi gap-junction channels (connexons) in single oocytes [64]. Beyond gap-junction formation the physiological function of Cx46 hemi-channels in the lens is unclear. However in oocytes, they have been shown to open in response to membrane depolarization (voltage-sensitive) and reduced extracellular calcium concentrations in a manner similar to orthodox ion channels (reviewed in [65]). Based on the hydrophobicity profile [66] of Cx46, the P59L substitution lies close to the N63S substitution [42] in the first extracellular loop (E1) domain, within a phylogenetically conserved motif of twelve amino acids containing three cysteine residues (54-CNTQQPGCENVC-65). Similarly, the P187L substitution in Cx46 [43] is located in the second extracellular loop (E2) domain within another phylogenetically conserved motif containing three cysteines (181-CDRWPCPNTVDC-192). Both the E1 and E2 domains are believed to function in docking of connexon hemi-channels (connexin hexamers) via cysteine-cysteine disulfide bridges in the intercellular space [67], to form gap-junction channels (connexin dodecamers) and, conceivably, mutations in these extracellular domains may impair Cx46 mediated coupling of lens fiber cells triggering lens opacities. Consistent with a loss of hemi-channel docking ability, the N63S mutant failed to form gap-junction channels when expressed in paired *Xenopus* oocytes [64]. Moreover, the N63S mutant did not exert strong dominant negative inhibitory effects when co-expressed with its wild type counterpart in oocytes, consistent with a loss-of-function mechanism and suggesting that a lower threshold level of Cx46 function is required for maintaining lens transparency [64]. However, heterozygous loss of Cx46 was not sufficient to elicit lens opacities in mice [60] indicating that, in addition to loss of function effects detected in oocytes, there may be deleterious gain-of-function mechanisms associated with expression of Cx46 mutants in the lens. Interestingly, the N63S mutant also exhibited impaired ability to form hemi gap-junction channels in single oocytes [64]. These observations raise the possibility that the primary genetic defect operated at the connexin (monomer) level prior to connexon (hexamer) formation, perhaps as a result of impaired targeting to the cell surface, accelerated degradation, or both. Whether or not the P59L mutant reported here malfunctions in a manner similar to that of the N63S mutant remains to be established.

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