Nonsense Mutations in FAM161A Cause RP28-Associated Recessive Retinitis Pigmentosa


Retinitis pigmentosa (RP) is a degenerative disease of the retina leading to progressive loss of vision and, in many instances, to legal blindness at the end stage. The RP28 locus was assigned in 1999 to the short arm of chromosome 2 by homozygosity mapping in a large Indian family segregating autosomal-recessive RP (arRP). Following a combined approach of chromatin immunoprecipitation and parallel sequencing of genomic DNA, we identified a gene, FAM161A, which was shown to carry a homozygous nonsense mutation (p.Arg229X) in patients from the original RP28 pedigree. Another homozygous FAM161A stop mutation (p.Arg437X) was detected in three subjects from a cohort of 118 apparently unrelated German RP patients. Age at disease onset in these patients was in the second to third decade, with severe visual handicap in the fifth decade and legal blindness in the sixth to seventh decades. FAM161A is a phylogenetically conserved gene, expressed in the retina at relatively high levels and encoding a putative 76 kDa protein of unknown function. In the mouse retina, Fam161a mRNA is developmentally regulated and controlled by the transcription factor Crx, as demonstrated by chromatin immunoprecipitation and organotypic reporter assays on explanted retinas. Fam161a protein localizes to photoreceptor cells during development, and in adult animals it is present in the inner segment as well as the outer plexiform layer of the retina, the synaptic interface between photoreceptors and their efferent neurons. Taken together, our data indicate that null mutations in FAM161A are responsible for the RP28-associated arRP.

Retinitis pigmentosa (RP [MIM 268000]) is a hereditary blinding condition that affects approximately 1 million individuals worldwide. It is genetically heterogeneous, with mutations in more than 45 genes identified to date according to the RetNet database. Nevertheless, these mutations account for only about 50% of the existing disease alleles. Many of the known RP genes are expressed in rod photoreceptors, although during the disease process both rod and cones progressively degenerate as a result of mechanisms that are only partly known. The RP28 locus (MIM 606068) was mapped to chromosome 2p11-p15 in a large consanguineous family (PMK146) from India segregating autosomal-recessive RP (arRP), whereas the corresponding gene and the underlying genetic defect were not identified. Homozygosity mapping defined a 15 Mb interval between D2S1337 and D2S286 harboring 188 annotated sequences or putative genes. To search for the gene implicated in the RP28 phenotype, we implemented a synergistic strategy based on ultrahigh-throughput and chromatin immunoprecipitation coupled to UHTs (ChIP-Seq). Specifically, results from UHTs-based mutation screening of RP28 patient DNA were merged with data from genome-wide analyses of murine in vivo targets for the retinal transcription factor Crx for the identification of candidate genes localized within the RP28 interval. This approach was based on our observation that phylogenetically conserved Crx-bound regions (CBRs) detected by ChIP-Seq were present in 95% (61/64) of retina-enriched genes and in the majority of known RP-associated genes, suggesting that nearly all identified retinal disease genes with retina-enriched expression are direct Crx targets.

Our study involved human subjects and was carried out in accordance with the tenets of the Declaration of Helsinki and the ethical guidelines of our institutions. Written informed consent was obtained from all participants. Traces of DNA from patient V-4 of family PMK146 were amplified by whole-genome amplification (Qiagen, Venlo, The Netherlands). Fifteen micrograms of DNA were used in a sequence-capture experiment (SureSelect, Agilent, Santa Clara, CA, USA) with custom-designed probes targeting all of the 1643 exons (and their intronic vicinities) present in the RP28 interval. The captured DNA was then further processed for UHTs with a Genome Analyzer II (Illumina, San Diego, CA, USA) and mapped back to the human reference sequence.

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NC_000002.11. We obtained 14.8 million reads, corresponding to approximately 500 Mb of contiguous sequence, 62% of which could be aligned to exons from the candidate chromosomal region. The reads were assembled and evaluated as described previously and produced a 450-fold coverage per bp on average. In total, eight non-annotated homozygous variants were found with respect to the reference sequence (Table S1, available online).

In a complementary approach, alignment of mouse CBRs identified through ChIP-Seq and matched to the orthologous human RP28 interval yielded 15 presumptive Crx target genes (Table S2). The gene with the strongest Crx binding, as reflected by 205 ChIP-Seq reads at the corresponding mouse locus, was also the top candidate from the UHTs mutational screening. It corresponded to the uncharacterized gene FAM161A, bearing a homozygous c.685C>T (NM_032180.2) substitution in exon 3 that should result in the nonsense change p.Arg229X (Figure 1). As expected, this mutation was present in all four affected members of family PMK146 in a homozygous state, whereas unaffected relatives of the patients were either heterozygous or carried wild-type alleles. These data suggest FAM161A to be the RP28 gene previously mapped to 2p11-p15 in this family.

Further screening of FAM161A in 118 presumably unrelated patients from Germany with recessive or isolate forms of RP revealed the presence of another homozygous nonsense mutation (c.1309A>T [p.Arg437X]; Figure 1) in exon 3 of three patients (M09-0352, arRP173, and arRP323; Table 1) that cosegregated with the RP phenotype in the respective families. This mutation was absent in 400 ethnically matched control chromosomes (p = 5.4 × 10⁻³, Figure 1. Schematic Representation of the RP28 Interval and Mutations in FAM161A
(A) Linkage interval for RP28 on chromosome 2p is delimited by microsatellite markers D2S1337 and D2S286 and spans over 15 Mb of DNA. Black boxes represent the 188 genes screened by UHTs.
(B) Structure of the two protein-coding isoforms of FAM161A, which are transcribed in the reverse direction with respect to the indicated orientation of chromosome 2, and location of the nonsense mutations detected in patients with arRP. Arrows indicate the primers used in quantitative RT-PCR experiments for detection of FAM161A mRNA expression in human tissues. The forward primer for both RT-PCR reactions lies on exon 3, whereas the reverse primer is complementary to either the 3-4 or the 3-3a exon-exon junctions, depending on the isoform analyzed.
(C) Electropherograms of the c.685C>T (p.Arg229X) and c.1309A>T (p.Arg437X) mutations and the corresponding region in controls.
by chi-square). Both the c.685C>T and c.1309A>T changes represent likely functional null alleles with absence of the gene product, because the mRNA is predicted to undergo nonsense-mediated decay. The three German patients carrying the p.Arg437X mutation originated from different geographical regions, but they shared the same FAM161A haplotype composed of six intragenic SNPs (Table S3). The G allele of rs11125895:A>G was present in German controls (n = 178) at a frequency of 0.167. The finding of homozygosity in these apparently unrelated patients suggests that p.Arg437X represents a mutation originating from a common founder. Other DNA changes detected in FAM161A included rare missense or isocoding changes of unknown pathogenicity and SNPs (Tables S4 and S5). Three of the nonsynonymous sequence variants affect evolutionarily conserved residues and were not detected in 400 control chromosomes. However, all were found in a heterozygous state only. Thus, the significance of these rare variants in the pathogenesis of the retinal phenotype of the patients remains to be elucidated.

It seems that the FAM161A-type retinal dystrophy shows no unique clinical features. The course of the disease is rather slow, with severe visual handicap in the fifth decade and legal blindness in the sixth to seventh decades. Patient M09-0352 was a 19-year-old female who complained about progressive constriction of the visual field, disturbed night vision, slight hearing problems, and hyposmia at her first visit. She was the offspring of unaffected parents with no history of consanguinity. Best corrected visual acuity was 20/25 in both eyes. Funduscopy showed typical findings of RP, such as bone spicules in the midperiphery, narrowed vessels, and optic disc pallor. Fundus autofluorescence was mottled and reduced in the midperiphery and showed a fine, small ring of increased autofluorescence around the fovea. Goldmann perimetry demonstrated distinct constricted visual fields in both eyes. The outer borders were at 10° eccentricity. Full-field electroretinogram was not recordable. Patients arRP173 and arRP323 were 70 and 69 years old, respectively. These women were the only affected offspring of apparently unrelated parents. In both cases, age at onset was reported to be in the second to third decade. The diagnosis of RP was made at the ages of 30 (arRP323) and 42 (arRP173) years. Disease onset in the three affected siblings of the PMK146 family was reported to be around 15 years of age. At the ages of 42 and 47 years, respectively, patients V-2 and V-3 had a severe visual handicap but were not regarded to be legally blind. They presented with fundus changes typical for RP, including narrowing of the arteries, bone spicule pigments, and optic atrophy. The age at onset was considerably earlier (at the age of 5 years) in patient VI-2, with a more rapid disease progression: at the age of 15 years, she could count fingers close to face (OD) and had a visual acuity of 3/60 (OS). It therefore appears that FAM161A defects lead to a variable phenotype in terms of disease onset and progression. The differences could be due to genetic or environmental modifiers.

FAM161A is an uncharacterized sequence with multiple splicing variants, two of which are predicted to result in stable mRNA transcripts. The most abundant derives from six exons and encodes a predicted protein of 660 amino acid residues (76 kDa), whereas the second isoform contains a supplementary in-frame 168 bp exon.

Table 1. FAM161A Mutations in Patients with Retinitis Pigmentosa

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Figure 2. FAM161A mRNA Expression in Human and Mouse

(A and B) Quantitative RT-PCR in various human tissues of the FAM161A short isoform (A) and long isoform (B). Both graphs represent mRNA expression normalized to the housekeeping gene ACTB. Note that ranges of y axes are not the same.

(C) Fam161a expression in the mouse retina, as detected by quantitative RT-PCR with the use of the 18S rRNA as housekeeping gene at various developmental stages.

(D) Affymetrix GeneChip expression data from wild-type versus Crx−/− P14 retinas, as detected with the Fam161a-specific probe set 1443569_at. Error bars indicate standard errors of the mean (A and B) and standard deviations (C and D), calculated in all instances from three independent experiments.
between exons 3 and 4 defining additional 56 residues (Figure 1). Although in some species (including mouse) orthologous entries are incorrectly or only partly annotated, FAM161A is evolutionarily conserved in vertebrates (Figure S1) and has a paralog, named FAM161B. The putative protein products of FAM161A and FAM161B share approximately 30% identity over a 400 amino acid residue stretch. Data derived from in silico databases suggest that FAM161B is not expressed in the retina. The function of protein family 161 is currently unknown.

Quantitative RT-PCR of the major isoform of the FAM161A mRNA revealed elevated expression in the human retina and testis, whereas in other tissues FAM161A transcripts were present at lower abundance (Figure 2A). Considering that testis is an organ expressing almost 85% of all human genes at various levels,7 biologically relevant FAM161A expression in humans may be confined to the retina. Although present at different levels, the two protein-coding human isoforms of FAM161A showed a similar expression profile (Figure 2B), and none of them could be defined as retina-specific, as is the case for other RP genes presenting with multiple splicing isoforms.8–10

In the mouse, Fam161a mRNA was highly expressed in the developing and adult retina and its presence was critically dependent on Crx (Figures 2C and 2D). As shown in Figure 3A, four CBRs are present within the Fam161a region, two of which are evolutionarily conserved and reside in the promoter (CBR1) and the first intron of the gene (CBR2). To analyze whether these CBRs represent photoreceptor-specific cis-regulatory elements, we performed electroporations of CBR-reporter fusions into explanted living mouse postnatal day 0 (P0) retinas (Figure S2). Remarkably, although it is part of the Fam161a promoter region, CBR1 could not drive detectable expression in photoreceptors alone. In contrast, we found that CBR2 exerted strong photoreceptor-specific enhancer activity, as demonstrated by prominent DsRed fluorescence in the outer nuclear layer (ONL) (Figure 3B). These data establish Fam161a as a direct Crx target gene and highlight its retina-specific cis-regulatory regions.

We raised a polyclonal rabbit antibody against a conserved part of FAM161A (peptide: N-NPITGARVAQYE-C, 100% identity between human and mouse) and performed immunohistochemistry by using this antibody at a final dilution of 1:40 and biotinylated goat anti-rabbit IgGs (1:200). Specificity of the antibody for the target protein was tested by competition experiments with the peptide used for immunization, as well as by siRNA-mediated silencing of FAM161A in cultured cells (not shown). Detection was achieved with the ABC-HRP Kit (Vector, Burlingame, CA, USA) and the use of 3,3′-diaminobenzidine (DAB) (Dako, Carpinteria, CA, USA). Staining revealed a developmentally regulated expression of Fam161a within the mouse retina. Specifically, we observed a light, diffuse labeling in the neural basal layer at P0 (Figure 4A). At
P10, a marked labeling was detected in all cell bodies of the outer nuclear layer (ONL), suggesting that Fam161a is expressed in both rods and cones. Note that the inner segments of the photoreceptors also stained positive for Fam161a (Figure 4B). In the adult retina (P30), Fam161a reaches a well-defined localization in the inner segment of photoreceptors, as well as in the outer plexiform layer. It is completely absent from the outer segment of photoreceptors. (D) Magnification of portions of (C), showing details of positive Fam161a staining. (E and F) Fam161a localization in retinas from the rd1 (rd) mouse at P12 and from the Rho−/− (rho) mouse at P60. Arrows indicate nuclear or perinuclear aggregates of Fam161a within photoreceptors. Magnification: A, B, and E, 200×; C and F, 400×; D, 800×. Abbreviations: NBL, neural basal layer; OS, outer segment of photoreceptors; IS, inner segment of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Figure 4. Fam161a Expression Pattern during Mouse Retinal Development and Retinal Degeneration
DAB immunostaining of Fam161a in murine retinas at various developmental stages, in wild-type animals (wt) and in models of retinal degeneration. (A and B) Fam161a is minimally expressed at P0 (A), whereas at P10 it shows a marked presence in the outer nuclear layer and in the inner segments of photoreceptors (B). Note that the outer plexiform layer is also positive. (C) In adult animals (P30), Fam161a reaches a well-defined localization in the inner segment of photoreceptors, as well as in the outer plexiform layer. It is completely absent from the outer segment of photoreceptors. (D) Magnification of portions of (C), showing details of positive Fam161a staining. (E and F) Fam161a localization in retinas from the rd1 (rd) mouse at P12 and from the Rho−/− (rho) mouse at P60. Arrows indicate nuclear or perinuclear aggregates of Fam161a within photoreceptors. Magnification: A, B, and E, 200×; C and F, 400×; D, 800×. Abbreviations: NBL, neural basal layer; OS, outer segment of photoreceptors; IS, inner segment of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Fam161a trafficking may be dependent on certain phototransduction proteins. However, this hypothesis is highly speculative and needs further experimental verification.

Analysis of a second consanguineous family from India has suggested refined boundaries of the putative RP28-chromosomal region and defined a smaller region of over-segregating in both families. Furthermore, linkage data do not support the assumption that FAM161A is implicated in the retinal dystrophy in the second family.

In this report, we show that mutations in FAM161A cause the RP28-associated form of arRP and that FAM161A mutations are present in the population of both India and Europe. The measured prevalence of FAM161A-associated RP in our cohort of German patients is in the range of 2%-3%, comparable to that of other arRP genes. Our data also indicate that these mutations are likely functional null alleles, making FAM161A an ideal target for constructing knockout animal models and for the option of gene replacement in the quest of seeking treatment for this disease.

Supplemental Data
Supplemental Data include two figures and five tables and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments
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Web Resources
The URLs for data presented herein are as follows:


Retinal Information Network (RetNet), http://www.sph.uth.tmc.edu/retnet/

References