Retinal degeneration associated with **RDH12** mutations results from decreased 11-cis retinal synthesis due to disruption of the visual cycle

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Retinoid dehydrogenases/reductases catalyze key oxidation–reduction reactions in the visual cycle that converts vitamin A to 11-cis retinal, the chromophore of the rod and cone photoreceptors. It has recently been shown that mutations in **RDH12**, encoding a retinol dehydrogenase, result in severe and early-onset autosomal recessive retinal dystrophy (arRD). In a cohort of 1011 individuals diagnosed with arRD, we have now identified 20 different disease-associated **RDH12** mutations, of which 16 are novel, in a total of 22 individuals (2.2%). Haplotype analysis suggested a founder mutation for each of the three common mutations: p.L99I, p.T155I and c.806_810delCCCTG. Patients typically presented with early disease that affected the function of both rods and cones and progressed to legal blindness in early adulthood. Eleven of the missense variants identified in our study exhibited profound loss of catalytic activity when expressed in transiently transfected COS-7 cells and assayed for ability to convert all-trans retinal to all-trans retinol. Loss-of-function appeared to result from decreased protein stability, as expression levels were significantly reduced. For the p.T49M variant, differing activity profiles were associated with each of the alleles of the common p.R161Q **RDH12** polymorphism, suggesting that genetic background may act as a modifier of mutation effect. A locus (**LCA3**) for Leber congenital amaurosis, a severe, early-onset form of arRD, maps close to **RDH12** on chromosome 14q24. Haplotype analysis in the family in which **LCA3** was mapped excluded **RDH12** as the **LCA3** gene and thus suggests the presence of a novel arRD gene in this region.

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INTRODUCTION

The visual cycle consists of a series of enzymatic reactions and transport mechanisms involved in the metabolism of vitamin A needed to sustain the light response of the vertebrate retina (Fig. 1). In the dark, the vitamin A analog 11-cis retinal forms a covalent bond with rod and cone opsins to generate the photoreceptor visual pigments (reviewed in 1). Upon illumination, 11-cis retinal is isomerized to all-trans retinal needed to stabilize the active signaling conformation of these proteins. Following decay of the active state, all-trans retinal is released from the apoprotein, reduced to all-trans retinol (vitamin A), transported to the retinal pigment epithelium (RPE) and esterified to phospholipids. The resulting retinyl esters serve as substrates for a concerted isomerization/hydrolysis reaction that regenerates 11-cis retinal, which is subsequently oxidized to reform 11-cis retinal and returned to the photoreceptor cells.

Retinoid dehydrogenases/reductases (or oxidoreductases) located in the photoreceptor cells and the RPE catalyze key oxidation-reduction reactions in the visual cycle. *RDH12* (14q23.3–q24.1) (MIM no. 608830) encodes a dual specificity enzyme expressed in the retina that acts on both trans and cis retinoid substrates (2,3). Mutations in human *RDH12* were recently shown to be associated with certain forms of severe, childhood-onset autosomal recessive retinal dystrophy (arRD) (4), establishing a unique requirement for this isoform in visual cycle function. In an initial report of disease-associated *RDH12* mutations, we detected a homozygous p.Y226C change in 13 patients of a large consanguineous Austrian family that resulted in loss-of-function in *in vitro* assays, and four other most likely pathogenic changes (p.T49M, p.R62X, p.Q189X and c.806_810delCCCTG) (4). Additional disease-associated mutations were reported, including six missense mutations whose functional significance has not yet been established (5).

Here, we report the identification of 20 novel *RDH12* coding sequence variants, and the results of functional analysis of a total of 14 missense mutations with respect to catalytic activity and protein expression. Our studies define the range of *RDH12* mutations in patients with childhood-onset severe arRD and suggest that *RDH12* loss-of-function disrupts the cycle of synthesis of the visual pigment chromophore, 11-cis retinal, resulting in early-onset and progressive disease. The central role of *RDH12* in the visual cycle establishes it as an important retinal dystrophy gene that may be an important future therapeutic target.

RESULTS

**RDH12 mutations in individuals with arRD**

We analyzed samples from 1011 individuals with retinal dystrophy originating from Germany, Spain, Austria, The Netherlands, Canada and the United States and identified 20 different disease-associated *RDH12* mutations, 16 of which represent novel mutations, present in homozygous or compound heterozygous form in a total of 22 probands (Table 1). The data from our cohort suggest that *RDH12* mutations account for 2.2% of cases of arRD. The majority (12/20, 60%) of sequence changes were missense mutations. Other mutations predicted premature protein termination, including nonsense mutations and rearrangements affecting a few nucleotides. Loss of the methionine initiation codon, c.2T>C (p.M1?) was also observed. Mutations for which a premature termination of translation was predicted or which resulted in loss of enzyme activity in *in vitro* assays, and four other most likely pathogenic changes (p.T49M, p.R62X, p.Q189X and c.806_810delCCCTG) (4). Additional disease-associated mutations were reported, including six missense mutations whose functional significance has not yet been established (5).

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Family 17 as the younger affected sister does not carry this particular change (data not shown). Direct sequencing of all seven coding RDH12 exons did not reveal a second disease-causing change in the three patients carrying c.577C > T (p.R193C), c.617C > T (p.A206V) or c.689C > T (p.P230L) in heterozygous form. Although these variants were not detected in 10 unaffected individuals, and segregated with the disease phenotype in the respective (small) families (data not shown), their role in the pathogenesis of the patients’ disease remains to be determined by future studies including functional assays that were not part of this study. Variant c.301G > A (p.D101N; Table 2) was detected in heterozygous state in a patient who was not part of the original cohort. The proband was from a three-generation family in which a most likely autosomal dominant form of RD is segregating. However, the mutation was transmitted to the patient by his unaffected mother, making it unlikely to be pathogenic. A number of rare sequence variants were also identified, both in patients and unaffected individuals, which were not likely to be pathogenic, including c.-123C > T (5′-UTR), c.188-31A > T (intron 2), c.188-14insT (intron 2), c.188-14insTTT (intron 2), c.333C > A (p.G111), c.343 + 101C > T (intron 3), as well as the previously reported variants c.-152A > G (5′-UTR), c.187 + 54A > T (intron 2), c.187 + 60G > A (intron 2) and c.195A > C (p.R65Q) (4). A previously identified commonly occurring polymorphism, c.482G > A (p.R161Q), was also detected in the present study, for which the minor allele c.482A (p.Q161) is present at a frequency of 0.2 in the general population (4).

A summary of all RDH12 disease-associated mutations identified to date is shown on a schematic of the gene structure in Figure 2.

### Table 1. Disease-associated RDH12 mutations identified in the present study in patients carrying the changes in homozygous or compound heterozygous form

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Predicted effect</th>
<th>Patient</th>
<th>Alleles a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>c.2T &gt; C</td>
<td>p.M1?</td>
<td>261 (m)</td>
<td>1</td>
</tr>
<tr>
<td>Exon 2</td>
<td>c.99_102dupAAAT</td>
<td>p.Val35LysfsX27</td>
<td>237 (m)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.139G &gt; A</td>
<td>p.A47T</td>
<td>3069 (p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.164C &gt; T</td>
<td>p.T55M</td>
<td>1047 (p)</td>
<td>1</td>
</tr>
<tr>
<td>Exon 3</td>
<td>c.193C &gt; T</td>
<td>p.R65X</td>
<td>915 (p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.295C &gt; A</td>
<td>p.L99I</td>
<td>237 (11)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>203 (m, p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>131 (m)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16 (m, p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>447 (m, p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3069 (m)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>02 (m, p)</td>
<td>1</td>
</tr>
<tr>
<td>Exon 4</td>
<td>c.375T &gt; A</td>
<td>p.N125K</td>
<td>0379 (p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.379G &gt; T</td>
<td>p.G127X</td>
<td>84 (m, p)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>c.429_432del4insGTT</td>
<td>p.His143GlnfsX19</td>
<td>679 (m, p)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>c.434G &gt; A</td>
<td>p.G145E</td>
<td>261 (p)</td>
<td>1</td>
</tr>
<tr>
<td>Exon 5</td>
<td>c.451C &gt; G</td>
<td>p.H151D</td>
<td>434 (p)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>c.464C &gt; T</td>
<td>p.T155I</td>
<td>19 (m, p)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>434 (m)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>217 (m, p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.582C &gt; A</td>
<td>p.Y194X</td>
<td>2975 (m)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.617C &gt; A</td>
<td>p.A206D</td>
<td>2975 (p)</td>
<td>1</td>
</tr>
<tr>
<td>Exon 6</td>
<td>c.701G &gt; A</td>
<td>p.R234H</td>
<td>3079 (m)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.715C &gt; G</td>
<td>p.R239W</td>
<td>1921 (m, p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>c.806_810delCCCTG</td>
<td>p.Ala269GlyfsX1</td>
<td>131 (m)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1921 (m, p)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>603 (m)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>915 (m)</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>93 (m, p)</td>
<td>2</td>
</tr>
<tr>
<td>Exon 7</td>
<td>c.821T &gt; C</td>
<td>p.L274P</td>
<td>74 (m, p)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>c.854G &gt; A</td>
<td>p.C285Y</td>
<td>496 (m, p)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>c.883C &gt; T</td>
<td>p.R295X</td>
<td>1047 (m)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

m, maternal allele; p, paternal allele; nd, parental origin unknown. Italics indicate change identified in this and our previous study (4).

aHow many alleles among the 22 individuals had each change.

### Table 2. RDH12 variants identified in the present study in patients carrying the changes in heterozygous form

<table>
<thead>
<tr>
<th>Mutation (heterozygous)</th>
<th>Predicted effect</th>
<th>Patient</th>
<th>Alleles a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 3</td>
<td>c.194G &gt; A</td>
<td>p.R65Q</td>
<td>0874 (p)</td>
</tr>
<tr>
<td></td>
<td>c.301G &gt; A</td>
<td>p.D101N</td>
<td>377 (m)</td>
</tr>
<tr>
<td>Exon 5</td>
<td>c.577C &gt; T</td>
<td>p.R193C</td>
<td>99 (p)</td>
</tr>
<tr>
<td></td>
<td>c.617C &gt; T</td>
<td>p.A206V</td>
<td>0613 (p)</td>
</tr>
<tr>
<td>Exon 6</td>
<td>c.689C &gt; T</td>
<td>p.P230L</td>
<td>242 (p)</td>
</tr>
<tr>
<td></td>
<td>c.805_810delCCCTG</td>
<td>p.Ala269GlyfsX1</td>
<td>98 (p)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

aHow many alleles among the 22 individuals had each change.
unglycosylated form of the protein (~35 kDa immunoreactive band) (4), with equivalent protein loading confirmed by analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoreactivity (Fig. 3, bottom panel). Immunohistochemical analysis of transfected COS-7 cells was consistent with localization of the 11 missense variants to the same intracellular membrane compartment(s) as the wild-type protein (Fig. 4). However, most likely because of higher expression levels, the wild-type protein appeared at higher intensity throughout the cells, often with high intensity focal build-up typical of overexpressed membrane proteins (data not shown).

Different results were obtained for two other missense variants identified in individuals diagnosed with arRD: p.R193C present in an individual in heterozygous form and p.R234H present in another individual in compound heterozygous form with p.N125K. In assays of recombinant protein activity in the presence of all-trans retinal and NADPH, decreased catalytic activity was observed ($P < 0.001$), however, p.C193 and p.H234 retained 79 and 44% the activity level of wild-type, respectively (Fig. 3, top panel). This compares with the activity of the p.Q161 polymorphic allele that exhibited 69% of the activity of the p.R161 variant (4). Co-transfection of the p.R62X variant had no effect on the activity of either p.T49 or p.M49 variants (data not shown). By segregation analysis, we found that the p.T49M change in the patient was present on the p.Q161 encoding maternal allele and the p.R62X change was present on the p.R161 encoding paternal allele, thus representing complex alleles. In order to perform a more detailed analysis of the altered enzymatic activity of the p.M49 variant, and possible effect of the genetic background, we generated expression constructs encoding all four possible combinations of p.T49, p.M49, p.R161 and p.Q161 variants. Western and immunohistochemical analyses of transfected COS-7 cells showed that all four proteins were expressed, with the levels of the p.T49/p.R161 recombinant protein (corresponding to the most common allele) consistently somewhat higher than that of the p.M49/p.R161, p.T49/p.Q161 and p.M49/p.Q161 proteins (Fig. 5C).

In assays of RDH12 retinoid reductase activity with all-trans retinal or 11-cis retinal as substrate, the change from p.T49 to p.M49 on the p.R161 background resulted in a significant stimulation of activity ($P < 0.05$) that was ~1.5–2-fold increased (Fig. 5A and B). The change from p.T49 to p.M49 produced a similar fold stimulation on the p.Q161 background. However, the p.T49/p.Q161 protein corresponding to the less frequent complex allele exhibited reduced retinoid reductase activity relative to the p.T49/p.R161 protein ($P < 0.05$). Thus, the p.T49 to p.M49 on p.Q161 change did not produce a final level of reductase activity significantly greater than that of the p.T49/p.R161 protein ($P < 0.05$).
In contrast, the change from p.T49 to p.M49 on the p.R161 background resulted in significant reduction of RDH12 retinoid dehydrogenase activity \((P < 0.05)\) that was \(\sim 0.5\)-fold decrease in assays with all-trans retinol or 11-cis retinol as substrate (Fig. 5A and B). The change from p.T49 to p.M49 also resulted in a similar fold reduction in activity on the p.Q161 background. However, as the dehydrogenase activity of the p.T49/p.Q161 protein was less than that of the p.T49/p.R161 protein \((P < 0.05)\), and only \(\sim 10\%\) that of the reductase activity, this situation resulted in very low levels of p.M49/p.Q161 dehydrogenase activity.

Our findings suggest that the p.T49M change shifts the catalytic efficiency of RDH12 to favor the conversion of retinal to retinol, with the final activity level influenced by the p.R161Q polymorphism. The significance of this shift will require further investigation.
establishing the direction and cellular location of the RDH12 reaction in vivo.

RDH12 mutations on a structural model

Although RDH12 is a member of a relatively large protein family, no structural data useful for evaluating RDH12 patient mutations in the context of the protein tertiary structure are currently available. Previously, a model of RDH5 tertiary structure was generated based on its homology (~24%) with 17β-hydroxysteroid dehydrogenase (6). In the case of RDH12, however, significantly lower homology (~12%) limits the usefulness of this strategy. Therefore, to generate a model of RDH12 tertiary structure, a low-resolution solution was predicted from the protein sequence using ab initio methods (7–9). The approach used here is capable of producing roughly correct models with complex topologies (10), although accuracy diminishes for large structures unassociated with a protein family (11,12).

The resulting structure with predicted coordinates was displayed and annotated to highlight the locations of the amino acid substitutions affected by RDH12 disease-associated missense mutations (Fig. 6). The protein has an apparently globular form with a core comprised alpha helical and beta sheet secondary structures. Sites of missense mutations are distributed widely across the predicted protein surface, although in most cases, they do not directly affect secondary structure elements that may participate in, for example, membrane association. The model provides no evidence that p.T49 and p.R161 are in close proximity, suggesting that activity effects resulting from substitutions at these sites are not due to simple steric interactions.
RDH12-associated phenotype

Affected study subjects found to have RDH12 mutations showed a progressive, severe and early-onset retinal dystrophy with visual impairment by the age of 2 years, in many cases with nystagmus, indicative of poor vision before the age of 6 months. In late-stage disease, visual acuity deteriorated to hand movement or light perception. Fundoscopic findings were waxy optic discs, severe attenuation of the arterioles, hyperpigmentation with bone spicule-like pigmentations in the mid-periphery and diffuse RPE/choroid atrophy. Visual fields were concentric restricted, often with eccentric small islands and multiple scotomas. Electroretinogram responses, both photopic and scotopic, were dramatically reduced at the time of first investigation in young patients (5–7 years old), with scotopic responses affected less than photopic ones. Electroretinogram responses were usually absent in older patients.

As mentioned earlier, the p.H234 allele retained 44% activity level of the wild-type enzyme. The proband carrying the p.R234H change (with the mutation p.N125K on the other allele) is 21-year-old and although his ophthalmologic parameters might indicate a somewhat milder overall disease course at this point, a conclusion in this regard would be premature.

LCA3 is not due to an RDH12 mutation

The LCA3 locus corresponds to a form of Leber congenital amaurosis, a severe autosomal recessive retinal degeneration, which maps to chromosome 14q24 within the 10 cM interval bounded by D14S284 and D14S68 (13). Thus, RDH12 is a candidate gene for LCA3. To test this hypothesis, several individuals from different branches of the large consanguineous family in which this locus was defined were studied for sequence changes in the RDH12 exons and adjacent intronic sequences. No disease-associated variants were identified. To test for larger deletions or rearrangements, PCR amplification primers were designed within each of the exons to span the intervening introns. All of these yielded the expected size products both in affected, heterozygote, and unaffected family members with the exception of the largest intron (intron 6; 4.4 kb), which could not be amplified from any family member. In addition, no disease-associated mutations were found in RDH11, which is also present in the critical genetic interval ~26 kb away from RDH12. Lastly, the genomic region containing these two genes was tested for co-segregation with the disease phenotype in the family by use of three SNPs within the introns of RDH12 and three microsatellite markers of which two flank the pair of genes and one is between them. This analysis showed lack of sharing of haplotypes both between the two affected siblings in generation VI and between the two parents who are first cousins (Fig. 7). On the basis of these results, it seems likely that a mutation in a novel retinal dystrophy disease gene is responsible for the phenotype of the family with the LCA3 locus.

DISCUSSION

Previous studies have shown that a number of different RDHs are present in the retina and RPE, including retSDR1, RDH8 (also named prRDH), RDH10, RDH11, RDH12, RDH13, RDH14, RDH15 and RDH16 (2,14–17). RDH5 is an RPE enzyme specific for 11-cis retinoid isomers and is involved in the oxidation of 11-cis retinol to 11-cis retinal (18). Mutations in human RDH5 cause fundus albipunctatus, a form of autosomal recessive, congenital stationary night blindness that usually does not progress to degenerative disease (19). These data in aggregate led to the general view that retinoid dehydrogenases/reductases serve in a redundant capacity in the visual cycle. However, the finding that RDH12 mutations are associated with childhood-onset severe arRD established that the encoded protein plays a unique role in retinal physiology. Patient mutations are divided between those predicting premature truncation (and likely absence of protein due to nonsense mediated decay) (20), and those encoding amino acid substitutions, with both classes of disease-associated variants likely resulting in altered visual cycle throughput.

Our studies of RDH12 function suggest that in the majority of cases, patient missense variants have significantly reduced expression and activity, and appear to represent disease-associated mutations that produce loss-of-function alleles due to disruption of the visual cycle. It is not yet clear whether loss-of-function can be attributed solely to decreased protein levels (most likely due to protein instability) or whether there are also effects on catalytic function, protein–protein interactions, etc. In a few instances, including one in which a second RDH12 change was not found, missense variants exhibited significant levels of activity and expression. However, it is possible that some of these variants have negative effects on in vivo function, alone or in combination,
which are not adequately measured by our in vitro assays of catalytic activity. Sites of missense mutations appear to localize on the protein surface in areas of unstructured sequence, suggesting that affected residues may disrupt functional domains involved in protein interactions, protein folding and catalytic activity, with decreased stability seen for the recombinant protein expressed in heterologous cells. Elucidating the relative contribution of these effects to loss-of-function awaits further studies of RDH12 specific activities and interactions, coupled with rigorous structural analysis.

Few studies have systematically addressed the functional consequences of complex alleles wherein two or more mutations occur on one chromosome. In such complex alleles, possibilities for functional interactions exist. For example, one constituent mutation may represent a neutral polymorphic variant with no apparent effect on function, each constituent mutation could contribute to the functional effect (21), or an apparent polymorphism may modify the functional consequences of a pathogenic mutation by virtue of being in cis (22). Our functional studies that pair the p.R161Q polymorphism with the p.M49 mutation as a complex allele clearly document the latter possibility. These studies show that the p.T49M change alters the catalytic efficiency of RDH12 to favor the conversion of retinal to retinol, with the level of total activity being influenced by the p.R161Q polymorphism. It remains to be determined how often coding SNPs may have functional consequences in the context of complex alleles.

There is growing evidence to support the notion that RDH12 functions as the key enzyme necessary for the reduction of all-trans retinal released from bleached photopigments in the recovery phase of the visual cycle. RDH12 can use either cis or trans retinoid isomers as substrates for dehydrogenase (retinol to retinal) and reductase (retinal to retinol).

Figure 7. Haplotype analysis in Family KKESH-019 in which the autosomal recessive LCA3 locus was mapped to chromosome 14q24 (13). The most informative portion of the family is shown with the original numbering of generations and individuals retained. Physical map and order of loci in the region the haplotypes span is D14S63 – 3.5 Mb – 5’-RDH11-3’ – 26 kb – 5’-RDH12 – dinucleotide repeat (DINUC) – 0.7 kb – RDH12-Intron 3 SNP – RDH12-Intron 5 SNP – RDH12-Intron 6 SNP – 3’-RDH12 – 9.0 kb – pentanucleotide repeat (PENTA). Note that in generation VI, the two affected offspring (1 and 4) from a consanguineous marriage carry different paternal haplotypes consistent with exclusion of both RDH11 and RDH12 as disease genes in this family.
activity with varying efficiency (2,3). RDH12 transcripts have been localized to the photoreceptor outer nuclear layer in human and mouse retinas using in situ hybridization (2). In addition, studies of knockout mice lacking prRDH1 (Rdh8), a different retinoid dehydrogenase expressed specifically in the photoreceptors, show that its loss has little effect on the murine phenotype, leading the authors to suggest that RDH12 is likely to be the key isoform necessary for the recovery phase (23). An important remaining question thus pertains to the roles played by other RDH isoforms present in the retina, including retSDR1, RDH13, RDH14 and RDH15. Are their activities in fact redundant or are there differences in, for example, the mechanisms of retinoid processing in rods versus cones? In this vein, RDH15 identified in cone-dominant Nrl-knockout mouse eyecups has been suggested to function in a proposed cone visual cycle (17). Alternatively, RDH12 may interact with other retinoid dehydrogenase/reductase isoforms to modulate function. Recent studies of Rdh5; Rdh11 double knockout mice suggest that such interactions are involved in regulating the activity of these two isoforms expressed in the RPE (24). Further insight into issues surrounding the function of RDH12 will require establishing whether RDH12 is expressed in rods or cones, or both, and defining the substrates and direction of the in vivo reaction(s). RDH12 mutations associated with arRD may provide important clues in this regard, in the form of differential effects on the oxidizing or reducing activity of the enzyme.

RDH12 is one of several retinal dystrophy genes that disrupt the cycling of vitamin A analogs between the photoreceptor cells and the adjacent RPE (reviewed in 25). These include RPE65 that has been recently shown to encode the retinoid isomerase (26–29), RLBP1 that encodes the RPE cellular retinal binding protein CRALBP (30,31), RGR that encodes the retinal G protein-coupled receptor that enhances isomerase activity (32–34), and LRAT that encodes the retinyl ester synthase, lecithin retinol acyl transferase (35,36). In each case, the autosomal recessive mode of inheritance suggests that pathogenicity of disease-associated mutations in these genes results from loss-of-function. Considerable effort is now focused on developing strategies for therapeutic intervention in patients with RPE65 defects, involving both gene and retinoid replacement approaches (37–39). Because the diseases associated with RPE65 and RDH12 mutations share similarities in phenotype, and potentially in molecular pathology (chromophore loss), replacement strategies similar to those under development for RPE65 may be appropriate for RDH12, especially gene replacement therapy. However, strategies for retinoid replacement therapy in the case of RDH12 may be less straightforward in that it is not yet known whether the associated pathology results from deficits in the production of chromophore, or in the build-up of toxic intermediates (e.g. free aldehydes), or both. In the latter case, addition of exogenous chromophore (11-cis retinal) that would continue to push the visual cycle forward could, in the long term, exacerbate rather than ameliorate associated pathology and functional loss. Therefore, definition of the specific role of RDH12 in the visual cycle will be an essential prerequisite for moving toward therapeutic intervention. The hope is that targeted strategies can be developed, that will benefit the majority of patients with mutations in visual cycle genes.

**MATERIALS AND METHODS**

**Patients and families**

One hundred and eleven individuals with a clinical diagnosis of (juvenile) retinitis pigmentosa, Leber congenital amaurosis or unclassified retinal dystrophy were enrolled in this study, including simplex cases and cases with evidence for autosomal recessive inheritance. Informed consent was obtained from all participants, and protocols were approved by the institutional review boards of University of Michigan, Universität Innsbruck, Universitätsklinikum Hamburg-Eppendorf, Universität Tübingen, University of Pennsylvania, Columbia University, Radboud University Nijmegen Medical Center, Baylor College of Medicine, Hospitales Universitarios Virgen del Rocio, La Fundación Jiménez Díaz, Hospital San Pau, and McGill University Health Center. DNA was purified from venous blood or buccal smears. Patients underwent ophthalmoscopy and electroretinography.

**Mutation screening and genotyping**

The seven exons of RDH12 were amplified using standard PCR conditions and oligonucleotide primers positioned in the intronic regions. Mutation screening was performed using denaturing HPLC on a WAVE DNA Fragment Analysis System (Transgenomic, Crewe, UK) or by single-strand conformation analysis with 8% glycerol. Fragments exhibiting an abnormal retention/electrophoresis pattern were sequenced on an ABI 310 DNA sequencer using BigDye terminator mix (Applied Biosystems). Patient haplotypes were determined by analysis of intragenic SNPs for chromosomes with the three most prevalent RDH12 mutations, p.L99I (c.187/ IVS2 + 54A–c.187/IVS2 + 60G–c.482A/p.161Q), p.T155I (c.187/IVS2 + 54A–c.187/IVS2 + 60A–c.482G/p.161R) and c.806_810delCCCTG (c.187/IVS2 + 54A–c.187/ IVS2 + 60A–c.482G/p.161R). Primer sequences and screening conditions are available upon request.

**In vitro mutagenesis and expression analysis**

RDH12 cDNA (c.482G allele (p.R161) including 10 bp 5'- and 32 bp 5'-untranslated sequence) was cloned into pcDNA3.1/HIS which expresses the recombinant protein as a fusion with Xpress epitope (Invitrogen). RDH12 sequence variants identified in individuals with arRD were introduced using the QuikChange Kit (Stratagene) and constructs verified by DNA sequencing. COS-7 cells in six-well plates or on chamber slides were transiently transfected with mutant or wild-type RDH12 constructs, or empty vector, using FuGene6 (1 μg DNA/3 μl reagent) according to the manufacturer’s instructions (Roche), and were harvested at 44 h post-transfection. A β-galactosidase encoding expression construct (pCMV-βgal) was included as 5% of total DNA and assayed using o-nitrophenyl-β-galacto-pyranoside as substrate (A420 nm) and showed that all constructs were transfected with approximately equal efficiency.

For western analysis of RDH12 fusion-protein expression, transfected cells were harvested in 10 mM Tris–HCl, pH 7.4, 250 mM sucrose, and protein concentrations determined by Lowry assay. Samples in SDS sample buffer
were electrophoresed on NuPage® Novex Bis–Tris acrylamide gels (4–12%) (Invitrogen) alongside SeeBlue® Plus2 standards (Invitrogen), transferred to nitrocellulose, incubated with Xpress antibody (Invitrogen), and then with alkaline phosphatase-conjugated anti-mouse IgG (Molecular Probes). Equivalent protein loading was verified by Coomassie blue staining and analysis of GAPDH immunoreactivity (Ambion).

For immunocytochemical analysis of protein localization, transfected cells were fixed using 4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.2 for 10 min at room temperature, incubated with the Anti-Xpress antibody (1:250) (Invitrogen) for 2 h at room temperature, washed, then incubated with Alexa-Fluor 555 anti-mouse IgG secondary antibody (1:500) (Molecular Probes) for 1 h at room temperature. Negative control was done for each transfection, excluding the primary antibody incubation step (data not shown). Slides were viewed under oil immersion (100×) and photographed on a Nikon Eclipse E800 microscope with fluorescence filter cube using a Nikon DMX1200 digital camera with the manufacturer’s data acquisition software.

Enzyme assay

In vitro analysis of recombinant RDH12 activity in COS-7 cell extracts was performed as described previously (4), using at least two independent clones of each mutation, assayed in triplicate. Briefly, cells were harvested, disrupted by sonication, and enzyme activity assayed in reactions containing 6 µg protein, 200 µM NADPH, 100 µM all-trans retinal or 11-cis retinal (for reductase activity) or 6 µg protein, 400 µM NADP⁺, 200 µM all-trans retinol or 11-cis retinol (for dehydrogenase activity) according to published methods (2), with retinoid concentrations calculated using extinction coefficients in Garwin and Saari (40). Retinoids were extracted into organic solvents and identified and quantitated by normal phase HPLC analysis using a Waters 2695 Alliance Separation Module and 2996 Photodiode Array Detector with a Supelcosil LC-31 column (25 × 4.6 mm², 3 µm) developed with 5% 1,4-dioxane in hexane. Peak identification was by comparison to retention times of retinoid standards and evaluation of wavelength maxima. Quantitative analysis was by comparison of peak areas at 325 nm for all-trans retinol, 318 nm for 11-cis retinol, 368 nm for all-trans retinal and 362.5 nm for 11-cis retinal. Data were analyzed using ANOVA (α = 0.05; Excel software), with significant statistical difference determined as P < 0.05. Assay conditions were optimized such that retinoid concentrations were not rate limiting, and product formation by RDH12 wild-type protein was linear during the reaction period (10–20-fold above background in 30 min), as well as with respect to amount of added protein.

RDH12 tertiary structure prediction

A low-resolution model of the tertiary structure of the RDH12 protein was derived, ab initio, by submitting the human RDH12 amino acid sequence using HMMSTR, a hidden Markov model based on protein structures in the invariant or initiation folding sites (I-sites) library of non-redundant short sequence motifs (supersecondary structures) that correlate with local structures (8), coupled with the Rosetta program to build structures from protein fragments using a Monte Carlo simulated annealing algorithm (9). The resulting tertiary structure with predicted coordinates was visualized and displayed with Discover Studio ViewerPro 5.0 (Accelrys, San Diego, CA, USA).

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Conflict of Interest statement. None declared.

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