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Identification of an *IMPDH1* mutation in autosomal dominant retinitis pigmentosa (RP10) revealed following comparative microarray analysis of transcripts derived from retinas of wild-type and Rho$^{-/-}$ mice

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Comparative analysis of the transcriptional profiles of approximately 6000 genes in the retinas of wild-type mice with those carrying a targeted disruption of the rhodopsin gene was undertaken by microarray analysis. This revealed a series of transcripts, of which some were derived from genes known to map at retinopathy loci, levels of which were reduced or elevated in the retinas of Rho$^{-/-}$ mice lacking functional photoreceptors. The human homologue of one of these genes, encoding inosine monophosphate dehydrogenase type 1 (*IMPDH1*), maps to the region of 7q to which an adRP gene (RP10) had previously been localized. Mutational screening of DNA from the Spanish adRP family, originally used to localize the RP10 gene, revealed an Arg224Pro substitution co-segregating with the disease phenotype. The amino acid at position 224 of the IMPDH1 protein is conserved among species and the substitution is not present in healthy, unrelated individuals of European origin. These data provide strong evidence that mutations within the *IMPDH1* gene cause adRP, and validate approaches to mutation detection involving comparative analysis of global transcription profiles in normal and degenerating retinal tissues. Other genes showing significant alterations in expression include some with anti-apoptotic functions and many encoding components of the extracellular matrix or cytoskeleton, a possible reflection of a response by Muller cells to preserve the remaining outer nuclear layer of the retina. We suggest that those genes identified are prime candidates for etiological involvement in degenerative retinal disease.

INTRODUCTION

The human retina consists of approximately 100 million photoreceptors linked via a series of secondary retinal neurones (horizontal, bipolar, amacrine and interplexiform cells) to approximately 3 million output neurones, or ganglion cells, the axons of which constitute the optic nerve (1). Hereditary neuroretinopathies involving death of photoreceptor cells (rod, cone, rod–cone or cone–rod dystrophies) and of ganglion cells (optic atrophies) cause visual handicap in young and middle aged people, probably affecting about 2 million people globally (2).

In retinopathies primarily involving degeneration of rod photoreceptors, including retinitis pigmentosa (RP), syndromes incorporating RP (Usher syndrome and Bardet–Biedl syndrome) and Leber congenital amaurosis (a congenital form of retinal degeneration), up to 30 genes have been implicated in disease pathology (http://www.sph.uth.tmc.edu/Retnet/). Such proteins include components of the visual transduction cycle, structural components of the rod and/or cone photoreceptor outer segment disc membranes, components of the retinoid cycle, transcription factors (including CRX and NRL), components of the cytoskeleton and extracellular matrices, splicing factors and, in one instance, a protein involved in circadian shedding of the outer segment discs of the photoreceptors into the pigmented epithelial layer (http://www.retina-international.com).

With respect to adRP specifically, the majority of mutations identified to date occur in genes encoding proteins expressed in photoreceptor cells. These include rhodopsin (3), peripherin/RDS

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(4,5), *ROM1* (6), cone–rod homeo box-containing (*CRX*) gene (7), *NRL* (8), *RP1* (9) and the retinal fascin gene (*FSCN2*) (10). In addition, mutations within two genes, *PRPC8* and *PRPF31*, encoding mRNA splicing factors have also been implicated in adRP (11,12). This is of particular significance in the context of the current report, in that *PRPC8* and *PRPF31* are expressed systemically, as opposed to other adRP genes, the expression of which is confined to photoreceptor cells.

Previous genetic linkage studies performed at this laboratory resulted in the mapping of an autosomal dominant RP (adRP) gene to the long arm of chromosome 7 (RP10) (13). The investigation involved a genome-wide search using DNA from 45 members of a large Spanish family, FA84, segregating classic adRP. RP has been traced through five generations of this kindred and the mean age of onset of the disease within the family is ~13 years with a range in onset from 5 to 28 years of age. Patients initially experience nyctalopia (night blindness) and subsequently suffer from a severe constriction of visual fields. All affected individuals show the classic clinical symptoms of RP, including optic disc pallor, bone spicule pattern pigmentary deposits and retinal vascular attenuation. They also exhibit either diminished or extinguished cone and rod electroretinograms (ERGs) (for a detailed clinical description of this family see 13).

The disease gene in an unrelated American adRP family, UTAD045, exhibiting a later onset and a slower progression of symptoms, was subsequently shown also to map to 7q31.3 (14). Data from both families have been combined in an attempt to map the disease locus with greater precision and critical recombinants in both families have placed the disease gene in an interval of 4.7 Mb between the proximal marker D7S2471 and the distal maker D7S530 on 7q31.3. A number of additional smaller RP10 families have now been identified, suggesting that this locus may be responsible for a significant proportion of all adRP (15,16).

The interval containing the RP10 gene is large and also relatively gene rich. It contains 23 known genes and a considerable number of sequences with predicted coding function (http://www.ensembl.org; http://genome.ucsc.edu). Methods enabling prioritization of disease candidates for this study, and other similar studies, were therefore of potential value.

Animal models of retinal degeneration provide useful systems in which to undertake such explorations. Mice carrying a targeted disruption of the rhodopsin gene lose their photoreceptors over a period of ~3 months, although the severity of degeneration is influenced by the nature of the genetic background upon which the mutation is expressed, retinas from Rho$^{-/-}$129Sv mice degenerating structurally and functionally more rapidly than those of Rho$^{-/-}$C57/BL6 mice (17–19). In the Rho$^{-/-}$129Sv strain all rod photoreceptors are lost at 4 months of age, and while residual cell bodies of cones persist, these cells are non-functional (19,20).

The Rho$^{-/-}$ mouse provides a model that enables comparison of transcriptional profiles in mouse retinas with and without functional photoreceptors. Elevations or reductions in expression of transcripts from Rho$^{-/-}$ retinas are likely to be a reflection of the destruction of primary retinal neurones, and/or of a response by remaining retinal cells toward the degenerative processes taking place. Genes thus identified may rationally represent candidates for involvement in degenerative retinal disease processes. The studies reported here directly validate this hypothesis.

RESULTS

Microarray-based transcriptional analysis of wild-type and rho$^{-/-}$ retinas

Microarray studies were performed on RNA extracted from the retinas of wild-type 129Sv mice and Rho$^{-/-}$129Sv mice at 4 months of age. The ERG of Rho$^{-/-}$ mice on the original mixed genetic background and on pure C57BL/6J and 129Sv genetic backgrounds has previously been studied in detail, cone function being less well preserved on the latter background (19,20). At 4 months of age, cone ERG signals from Rho$^{-/-}$129Sv mice are virtually undetectable, indicative of essentially complete loss of cone function (in contrast, at this age in Rho$^{-/-}$C57s, cone function is still appreciably preserved indicating persistence of cone cell function on that particular background).

Four GeneChips® were used in total; two were hybridized with wild-type (Wt) retinal cRNA and two with Rho$^{-/-}$ cRNA. RNA from the retinas of 10 mice was pooled for each of the four RNAs used for analysis. Pooled samples were used to minimize the effects of variability that may occur between individual animals. Four different comparisons were made between the different cRNA samples; Wt1/Rho$^{-/-}$1, Wt2/Rho$^{-/-}$2, Wt1/Rho$^{-/-}$2 and Wt2/Rho$^{-/-}$1. 420 genes showed increased or decreased expression in at least two of four comparisons. While each comparison is not statistically independent of the others, this criterion allowed an initial selection of genes highly likely to show real differential expression between wild-type and Rho$^{-/-}$ mice.

Photoreceptors are essentially absent from the Rho$^{-/-}$ retina at 4 months and therefore there is less RNA present in a single mutant retina than in the wild-type. As mRNA levels were compared between the same concentrations of retinal RNA it was predicted that, as a result of compensation for the lack of photoreceptor RNA, non-photoreceptor layers would be over-represented in the Rho$^{-/-}$ sample. This prediction was borne out by the 107 genes showing a moderate increase in expression (1.7–2.5-fold) between wild-type and Rho$^{-/-}$ retinas, compared to only 38 with an equivalent decrease. The transcripts that showed an average increase or decrease of >1.5-fold in at least two of four comparisons were classified into categories of average fold change in expression levels between wild-type and Rho$^{-/-}$ retinas, illustrating the greater numbers of moderately increased genes and dramatically decreased genes (Fig. 1).

Of the 420 transcripts which showed increased or decreased expression in at least two of four comparisons, only those that had an average change of ≥3-fold in three or four comparisons were selected for further analysis. This selection process identified a total of 74 different candidates, 44 of which showed decreased expression in the Rho$^{-/-}$ retinas and 30 of which showed increased expression. By choosing these parameters, the large number of moderately increased genes are not included in the analysis. An average fold change was calculated for each gene with altered expression. The fact that the hybridization was performed in duplicate and that in cases where a gene was represented by more than one probe set, a very close correlation was observed between the expression

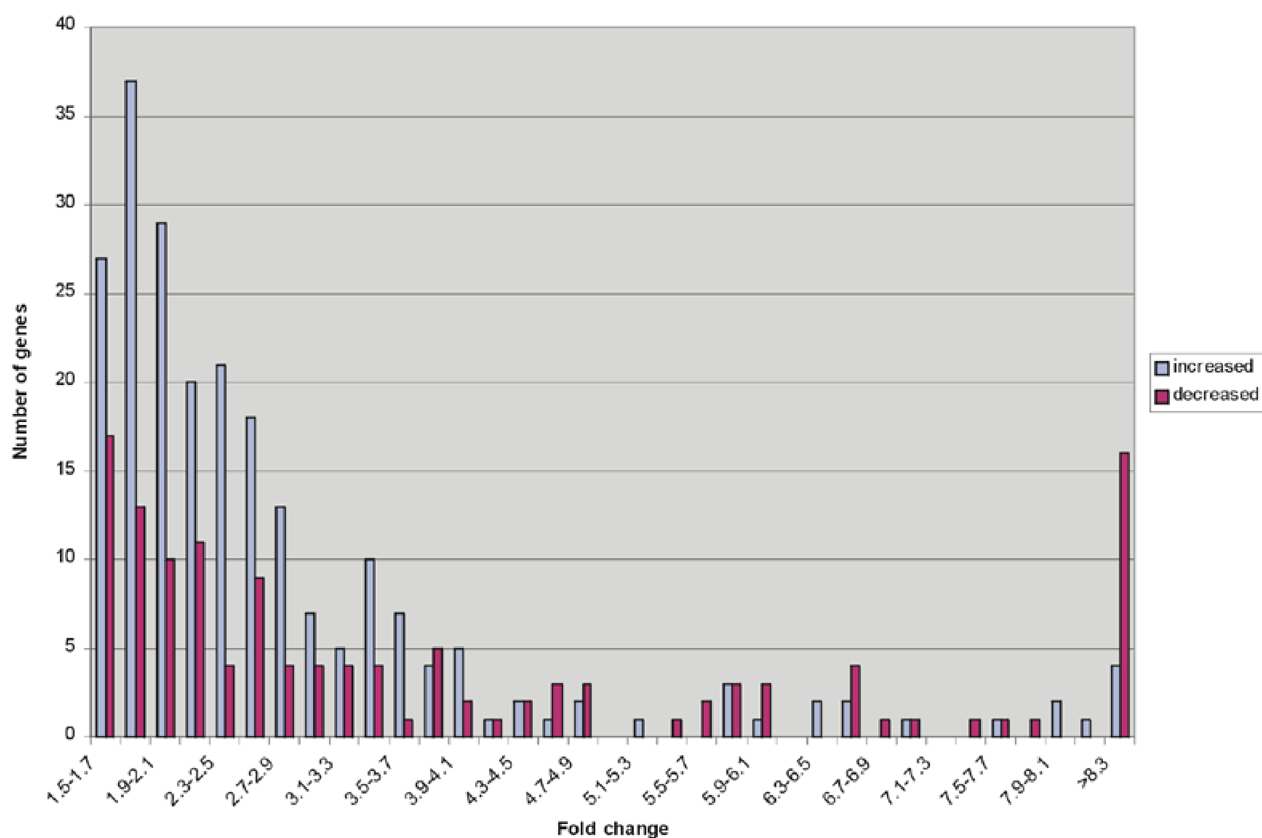

Figure 1. Comparison of the number and extent of genes up-regulated from wild-type to Rho$^{-/-}$ retina with those down-regulated. The 228 transcripts showing an average increase of >1.5-fold and 131 transcripts showing an average decrease of >1.5-fold in Rho$^{-/-}$ retinas are classed into categories of fold change. This illustrates the greater numbers of moderately increased genes and dramatically decreased genes.

levels reported, indicates a high level of reliability of data within these levels of change.

In order to validate the transcriptional variation observed in the microarray results, a number of genes showing either up- or down-regulation at the transcriptional level were analysed using relative quantitative RT–PCR on the RNA samples used for the chip experiments (it should be noted that the terms up- and down-regulation, as used in this script, refer to the relative transcript levels in equal amounts of RNA from wild-type and Rho$^{-/-}$ retinas). The genes chosen for further analysis were the β-subunit of rod cGMP phosphodiesterase (*PDE6B*), peripherin/RDS, hexokinase II, high mobility group 2 (*HMG2*), testican, cathepsin S (*CTSS*) and a gene homologous to myosin regulatory light chain 2 (*LC2*). The change in expression levels observed for these genes in the chip results spanned a range of fold change values, allowing the reliability of both large and moderate fold changes seen in the chip data to be tested. The fold changes calculated from the quantitative RT–PCR experiments, while not matching the array results in terms of precise calculations, showed agreement for the direction of the change in all cases (Table 1).

Table 1. Validation of fold changes observed for a number of genes in the microarray analysis using relative quantitative RT–PCR

Gene name	Average microarray fold change	Average RT–PCR fold change
Peripherin/RDS	–12	–58
PDE6B	–276	–123
Hexokinase II	–6	–4
HMG2	–6	–6
Testican	8	3
Cathepsin S precursor	4	4
Homologous to LC2	5	2

Fold changes presented are averaged for the four comparisons of the microarray data and for four RT–PCRs carried out for each gene

Genes showing significant changes in transcription level in Rho$^{-/-}$ retinas

Predictably, seven genes involved in the visual transduction cycle are among the 44 genes most significantly down-regulated in Rho$^{-/-}$ retinas in comparison with wild-type. These genes encode the photoreceptor-specific α and γ subunits of rod transducin, the α, β and γ subunits of rod cGMP phosphodiesterase, peripherin/rds and recoverin. It is of interest to note that five of these genes have previously been implicated in some form of retinal degeneration (http://www.sph.uth.tmc.edu/Retnet/). Just two other retinal degeneration genes, those

Table 2. Differentially expressed genes identified in the $Rho^{-/-}$ knockout mouse that map to known human retinal degeneration loci

Gene name	Average fold change	Human homologue chromosomal location	Retinal disease locus
Zinc finger protein A20	–12.5	6q24.1	Dominant retinal-cone dystrophy 1 6q25–q26; Deletion mapping, no marker data
Cerebellar degeneration-related protein 2	–12.3	16p12.2	Recessive RP (RP22) 16p12.1–p12.3; D16S287–D16S420
Hexokinase 2	–6	2p12	Recessive RP (RP28) 2p11–p16; D2S1337–D2S286
Myocyte specific enhancer factor 2C	–6.8	5q14.3	Usher syndrome, type 2 5q14.3–q21.2; D5S428–D5S433
High mobility group 2	–6	4q34.1	Recessive RP (RP29) 4q32–34; D4S621–D4S2417
Inosine monophosphate dehydrogenase type 1 (IMPDH1)	–5.8	7q31.3	Dominant RP (RP10) 7q31.3; D7S2471–D7S530
Ecto-5-prime nucleotidase (Cd73)	–3.7	6q14.3	Recessive Leber congenital amaurosis 6q12–6q16; D6S1551–D6S1694
CD44 antigen	+3	11p13	Dominant familial exudative vitreoretinopathy 11p13–p12; GATA34E08–D11S4102
Amyloid β (A4) precursor protein	+3	21q21.3	Usher syndrome, type 1 21q21; D21S1905–D21S1913

Locus information obtained from Retnet (http://www.sph.uth.tmc.edu/Retnet/)

encoding blue cone opsin and retinal-specific guanylate cyclase (*retGC*), were present on the microarrays. The blue cone opsin gene is among the 420 genes showing decreased expression in at least two of four comparisons. The *retGC* gene shows decreased expression in the $Rho^{-/-}$ retinas but the values obtained for it are not in the range considered to be significant. A recent report using a cDNA microarray with 960 genes to analyse expression in the $Crx^{-/-}$ mouse retina also showed down-regulation of a number of photoreceptor specific genes (21).

The locations of genes encoding transcripts significantly up- or down-regulated in degenerating $Rho^{-/-}$ retinas were compared with the known locations of retinopathy-causing genes. Nine of the genes identified in this study map at the locations of retinopathy loci and therefore may arguably represent disease candidates (Table 2). All genes showing elevations or reductions of >3-fold in expression levels in microarray comparisons of wild-type and $Rho^{-/-}$ retinas are listed in Table 3 and proteins encoded by such genes have been placed into functional groups in Table 4. It is of interest to note that a number of proteins with anti-apoptotic functions are shown to be down-regulated in the degenerating retinas of $Rho^{-/-}$ mice. Evidence accrued to date suggests that apoptosis may be a general mechanism of photoreceptor cell death in mutation-induced retinopathies. In addition, a surprisingly large number of those genes shown to be transcriptionally activated in degenerating retinas encode proteins either of the extracellular matrix (ECM) or the cytoskeleton. The significance of these findings is discussed below.

Mutation in *IMPDH1* in adRP

The gene encoding inosine monophosphate dehydrogenase type 1 (*IMPDH1*), which showed an average down-regulation of 5.8-fold in the $Rho^{-/-}$ retina, maps to the precise location of the RP10 locus. There were no other genes in the disease interval which showed a similar down-regulation. While this gene is known to be expressed in many tissues the result nevertheless suggests that it may, within the retina, be preferentially expressed in photoreceptor cells as opposed to secondary retinal neurones. Relative quantitative RT–PCR analysis confirmed down-regulation of the *IMPDH1* gene, with an average fold change of –4 in $Rho^{-/-}$ retinas. The *IMPDH1* gene was thus considered a strong candidate for the adRP gene mapping to chromosome 7q31.3.

DNA from members of FA84, in which linkage to the RP10 locus was first established, was subjected to direct sequence analysis using primers specific for amplification of the fourteen *IMPDH1* coding exons. This revealed a CGC→CCC change within exon 7 of the gene (Fig. 2). The mutation was shown to segregate in all affected members of the FA84. It was not present in an analysis of 200 chromosomes from non-related people of European origin and 50 Spanish chromosomes of unaffected and married members of FA84. The

mutation is predicted to bring about an arginine→proline substitution at codon 224 of the *IMPDH*1 gene. It is of note that arginine at codon 224 of the IMPDH1 protein is conserved in all four eukaryotic species for which sequence was available, human, mouse, *Drosophila* and chinese hamster, suggesting that this amino acid is important in retaining the functionality of the IMPDH1 protein. These data therefore provide strong evidence that the Arg224 mutation in the *IMPDH1* gene is the cause of the retinal degeneration in adRP family FA84.

DISCUSSION

Subsequent to the localization of RP10, initial efforts to identify the disease gene were hampered by the large size of the disease interval and the lack of obvious candidate genes. The global approach described here to analyse a large number of genes for differential expression in the $Rho^{-/-}$ mouse, lacking functional photoreceptors, has been of direct value in the identification of the RP10 gene—*IMPDH1*. Notably the same approach may aid in highlighting other mapped or unmapped retinopathy-causing genes.

The *IMPDH1* gene is comprised of 17 exons, the first three of which are non-coding. The 14 coding exons span ~18 kb of genomic sequence and encode a protein subunit of 514 amino acid residues, with the active IMPDH1 enzyme consisting of a homotetramer of these subunits (22) (GenBank accession no. J05272). The reaction carried out by the IMPDH1 protein is the rate-limiting step in guanine nucleotide biosynthesis. It catalyses the conversion of inosine monophosphate (IMP) to xanthosine monophosphate (XMP) with the concomitant reduction of NAD to NADH. XMP is subsequently converted to GMP which gives rise to one of the building blocks of DNA (dGTP) and also plays an essential role in intracellular signalling pathways. Guanine nucleotides are therefore essential for normal cell proliferation and function.

The identified *IMPDH1* mutation lies in the cystathionine-β-synthase 2 (CBS2) domain of the protein. Each *IMPDH1* monomer consists of two domains; the larger forming a barrel containing the active site loop and the smaller (residues 110–244) comprised of two tandem CBS dimer domains (23). CBS domains, originally identified in the CBS gene, are motifs which code for modules of unknown function. However, studies on these domains have revealed that they can attach to a wide range of other protein domains, suggesting that they may play a regulatory role (24).

IMPDH activity in human tissues involves a second isoenzyme, IMPDH type 2 (IMPDH2), which bears 84% amino acid homology to IMPDH1 and maps to chromosome 3p21 (25). Notably, the amino acid Arg224 appears to be highly conserved in both proteins. The regulation of expression of the two *IMPDH* genes differs considerably. *IMPDH2* appears to exhibit inducible expression, and its level of expression is increased in proliferating and malignant cells (26,27). The *IMPDH1* gene is widely expressed (http://www4.ncbi.nlm.nih.gov/UniGene/), but there is variability of expression in different tissues (28). Three major RNA transcripts for *IMPDH1*, which are differentially expressed from three alternative promoters, have been identified (29) suggesting that *IMPDH1* expression may be regulated in a complex cell type-specific manner (29,30). PCR analysis has indicated that the level of expression of *IMPDH1* in the wild-type mouse retina is relatively low in comparison to that of other known RP genes (unpublished data). An IMPDH2 knockout mouse model has been generated (31) and in consideration of the newly identified role of *IMPDH1* mutations in adRP it would be of interest to observe whether there is a retinal pathology in these animals.

IMPDH1 is the first gene of its kind to be reported in the etiology of RP. It is therefore difficult to speculate at this stage as to how this mutation may bring about the degeneration of retinal photoreceptors. Inhibition of IMPDH causes a cessation of the cell cycle leading to the initiation of apoptosis. Mutation-induced alteration of the activity of IMPDH may be sufficient in photoreceptor cells to stimulate those processes leading to the initiation of apoptosis and hence degeneration of the retina (32,33).

However, the mutation identified in this study results in the synthesis of a protein that produces clinically recognizable disease only in retinal tissues. Photoreceptor cells are amongst the most physiologically active of any cell type in the human body. They require high levels of guanine nucleotides which are fundamentally important in signal transduction mechanisms, for example in the activation of transducin following light activation of the primary photoreactive component of rod cells, rhodopsin. Microarray analysis revealed that IMPDH1 is expressed at a higher level in photoreceptors than other retinal cell types in the mouse. This higher level of expression, together with the high GTP usage of photoreceptors, may go some way toward explaining the tissue-specific nature of the disease.

One possible explanation for the disease phenotype is that the mutation in the CBS2 domain hinders the interaction of an unknown photoreceptor-specific protein with IMPDH1. There is a precedence for such a scenario, with a similar situation being seen in the case of mutations in the RP GTPase regulator (*RPGR*) gene (34). As yet, the precise effect of the Arg224 mutation in RP10 remains unknown and detailed functional studies will be required to elucidate the mechanisms of disease pathology. It is of interest to note that there are many characterized pharmacological inhibitors of IMPDH (35) but the potential value of such inhibitors will only be established when further knowledge of the disease mechanism has been obtained. It should be noted that all other proteins in the guanine nucleotide synthesis pathway are now key candidates for retinal disease.

Presented here is a list of genes which show differential expression in the $Rho^{-/-}$ and wild-type mice. One of these, *IMPDH1*, has been shown to be implicated in the etiology of adRP, and therefore other members of this group may be regarded as strong disease candidates. While detailed consideration of the possible candidacies of all of these proteins is clearly beyond the scope of the current manuscript, it is nevertheless worth noting that several of those genes showing down-regulation in $Rho^{-/-}$ retinas encode proteins with established anti-apoptotic function, including the transcription factor A20 and cerebellar degeneration-related protein 2 (cdr2) (36,37). Data accrued largely from studies of animal models of RP indicate that a 'final common pathway' of photoreceptor cell death is apoptosis, although the molecular mechanisms linking the presence of primary mutations to the initiation of that process remain obscure at the present time. Down-regulation, within photoreceptor cells carrying primary mutations, or in other

Table 3. Genes showing down/up regulation of expression levels by ≥3-fold in a microarray comparison between wild-type and Rho$^{-/-}$ mouse retinas

GenBank accession number	Average fold change	Gene name
Genes showing an average fold change greater than or equal to +3× in all four comparisons		
X61800	+9.75	CCAAT/enhancer binding protein δ
X07411	+8.28	Adipocyte p27 protein/carbonyl reductase 2
X92864	+7.95	Testican
X58861	+6.65	Complement C1Q, α chain
W13739	+5.93	Homologous to pterin-4-α-carbinolamine dehydratase
W71543	+4.82	Homologous to myosin regulatory light chain 2
X06368	+4.35	c-fms proto-oncogene
U43541	+4.27	Laminin β 2
X00496	+4.05	Mouse la-associated invariant chain
AA146437	+3.98/+3.85	Cathepsin S
AA059700	+3.88/+3.1	β2-microglobulin
X70854	+3.73	Fibulin 1
Y10386	+3.65	Complement C1 inhibitor
X57337	+3.58	Procollagen C-proteinase enhancer protein
X62600	+3.52	CCAAT/enhancer binding protein β
W12941	+3.25	Homologous to interferon-inducible protein
V00835	+3.10	Metallothionein-1
X66081	+3.03	CD44 antigen precursor
AA067929	+3.00	Mouse amyloid β (A4) protein precursor
Genes showing an average fold change greater than or equal to +3× in three of four comparisons		
AA023669	+16.58/+3.40	Homologous to heat-shock 27 kDa protein
X15475	+8.43	Peripherin
X15789	+6.93	Cellular retinoic acid binding protein
AA044561	+5.75	Homologous to phosphoenolpyruvate carboxykinase
AA137902	+4.42	Na^+/K^+-transporting ATPase β-3 chain
AA003323	+4.05	Homologous to filamin A
X14194	+3.90	Entactin/nidogen 1
X99807	+3.75	Selenoprotein P
X58251	+3.63	Procollagen type 1α 2 chain
Y00305	+3.42	Voltage-gated potassium channel protein 1
X66449	+3.38	Calcium binding protein A6 (calcyclin)
Genes showing an average fold change greater than or equal to –3× in all four comparisons		
X55968	–276	Rod cGMP phosphodiesterase β-subunit
M25513	–214/–225	Rod transducin α-subunit
X60664	–39.15	Rod cGMP phosphodiesterase α-subunit
X66196	–32.97	Recoverin
Y00746	–19.75	Rod cGMP phosphodiesterase γ-subunit
AA087559	–18.45/–4.72	Alkaline phosphatase 2
M64228	–18.25	Mouse potassium channel protein (Shab)
AA051446	–18.02/–6.60	Cerebellar degeneration-related protein 2
X66983	–12.95/–6.60	Rck for protein kinase
X14770	–12.50	Retinal degeneration slow (rds)
U19463	–12.45	Zinc finger protein A20
U29055	–10.80	G protein (transducin) β-1
AA059557	–8.38	Similar to uridine kinase
Y11666	–7.40/4.72	Hexokinase II

Table 3. *Continued.*

GenBank accession number	Average fold change	Gene name
U38495	–6.90	Rod transducin γ subunit
L13171	–6.80	Myocyte-specific enhancer factor 2
X84797	–6.68	Hematopoietic specific protein 1
J03482	–6.50	Histone H1, member 2
Z46757	–6.03	High mobility group 2 protein
AA061266	–5.88/–5.70	Oxysterol binding protein like 1
U00978	–5.77	Type I inosine monophosphate dehydrogenase
AA163305	–5.65/–5.47	Serum/glucocorticoid regulated kinase (Sgk)
L20343	–4.63	L-type calcium channel β 2a
U34277	–4.40	Platelet activating factor acetylhydrolase
L00919	–4.13	Protein 4.1
Y00051	–3.90	Neural cell adhesion molecule
W63974/U37353	–3.90/–3.00	Protein phosphatase 2A, reg. subunit B– β/γ isoform
X87817	–3.88	Ulip/dihydropyrimidinase-like 3
X14972	–3.88/–3.30	α-adaptin C
Y12650	–3.70	Hereditary haemochromatosis-like protein
L12059	–3.70	Ecto-5′-nucleotidase (Cd73)
W62420	–3.60	ADP-ribosylation factor 4-like
U28342	–3.32	Immunoglobulin heavy chain variable region
L02914	–3.08	Aquaporin 1
Genes showing an average fold change greater than or equal to –3× in three of four comparisons		
U19880	–8.52	D4 dopamine receptor gene
AA098332	–7.65	5′-AMP-activated protein kinase, β chain
U95167	–6.07	Transient receptor protein (trp)1
AA062247	–4.78	Homologous to transcription factor IIF, β subunit
X90778	–3.88	Histone H2B, testis
AA089097	–3.35	Homologous to phosphatidylcholine transfer
U07159	–3.25	Acetyl-coenzyme A dehydrogenase, medium chain
W77226	–3.13	ADP-ribosylation-like protein 3
AA103047	–3.13	Homologous to ADP-ribosylation factor-like protein 2

Genes are listed in order of decreasing fold change value. For genes that were present on the microarray twice, two values for fold change are presented.

retinal neurones, of transcripts encoding proteins which have been implicated in apoptotic mechanisms in other cell systems, may contribute to pathological disease mechanisms.

Approximately half of those transcripts showing elevations in levels of expression in $Rho^{-/-}$ retinas when compared to wild-type, encode proteins of the cytoskeleton or ECM and indeed some of these map to locations of known retinopathy-causing genes. Up-regulation of genes encoding such proteins may represent an attempt by retinal glial cells (Muller cells) to strengthen the interphotoreceptor matrix in an attempt to slow the degenerative process. Proteins showing elevated levels of transcript include laminin, fibulin, entactin, procollagen type 1 α 2 chain, procollagen C-proteinase enhancer protein, testican, cathepsin S, a protein with homology to myosin light chain 2, peripherin (distinct from peripherin/rds), a protein with homology to the actin binding protein filamin and CD44, a cell surface adhesion molecule specifically localized in the Muller cell microvilli that oppose the interphotoreceptor matrix.

It is worthy of note that a number of cytoskeletal/ECM proteins have already been implicated in hereditary neuroretinal degenerations, including the Usher syndrome type 1B protein, myosin 7A, which is thought to be involved with cell adhesion (38,39). A protein termed usherin, which has a domain involved in laminin network assembly, has been shown to be defective in Usher syndrome type IIA (40). Usherin has fibronectin type III motifs which are frequently observed in proteins comprising components of the ECM and basal lamina and in cell adhesion molecules (41). Mutations in the ECM gene *TIMP3* result in Sorsby’s fundus dystrophy (42) and, interestingly, the protein has been found to accumulate in retinas with RP not caused by *TIMP3* mutations (43). A cadherin protein, cdh23, has been implicated in Usher syndrome type 1D, and may be involved in intercellular

Table 4. Functional groups of genes showing differential expression in a microarray comparison between wild-type and $Rho^{-/-}$ mouse retinas

Gene name	Gene function
Functional groups of genes in which expression is decreased between wild-type and $Rho^{-/-}$ retinas	
Intracellular signal transduction	
Cerebellar degeneration-related 2	Transcriptional co-repressor
Rck protein kinase	Ser/thr protein kinase involved in cell cycle regulation
Zinc finger protein A20	Inhibits NF-κB and TNF-induced apoptosis
Myocyte-specific enhancer factor 2	Transcription factor, neurogenesis
Hematopoietic specific protein 1	Transcription factor
Sgk	Ser/thr protein kinase, cell survival
Protein phosphatase 2A	Ser/thr phosphatase, cell growth/division
Ulip protein	May mediate signals involved in axonal outgrowth
ADP-ribosylation factor 4-like	Small GTP-binding protein (ARF family)
D4 dopamine receptor gene	Large G protein coupled receptor
5′-AMP-activated protein kinase	Metabolic sensor for AMP and ATP levels
ADP-ribosylation-like protein 3	Small GTP-binding protein (ARF family)
Homologous to ARF-like protein 2	Small GTP-binding protein (ARF family)
Ion channel/plasma membrane proteins	
Potassium channel protein (Shab)	Voltage-gated potassium ion channel
Transient receptor protein (trp) 1	Non voltage-sensitive store-operated Ca^{2+} channel
Calcium channel β 2a subunit	Voltage-gated calcium channel
Neural cell adhesion molecule	Cell–cell signalling, neurone adhesion
α-adaptin C	Found in coated vesicles of plasma membrane
Aquaporin 1	Molecular water channel protein
DNA processing and assembly	
Uridine kinase	Pyrimidine ribonucleoside kinase
Histone H1 gene	Compact DNA into nucleosomes
High mobility group 2	Increases transcription
Ecto-5′-nucleotidase (Cd73)	Catalyses cAMP into AMP
Transcription factor IIF, β subunit	Binds to RNA polymerase II
Histone H2B, testis	Compacts DNA into nucleosomes
Functional groups of genes in which expression is increased between wild-type and $Rho^{-/-}$ retinas	
ECM/cytoskeleton	
Testican	Proteoglycan–cell adhesion, migration, proliferation
Laminin β 2	Basement membrane constituent
Cathepsin S	Cleaves elastin, degradation of ECM
Fibulin 1	Glycoprotein incorporated into a fibrillar ECM
Procollagen C-proteinase enhancer	Cleavage of type I procollagen, ECM maintenance
Procollagen type I, α 2 chain	Fibrillar collagen family of ECM proteins
Entactin/nidogen	Binds type IV collagen and laminin
CD44 antigen	Glycoprotein with a role in matrix adhesion
β2-microglobulin	Cell surface amyloid protein
Amyloid β (A4) precursor	Membrane associated glycoprotein
Homologous to myosin regulatory light chain 2	Structural protein of muscle
Peripherin	Intermediate filament protein
Homologous to filamin A	Crosslinks actin filaments to membrane glycoproteins
Heat-shock protein 27 kDa	Inhibits actin polymerization, stabilizes protein structures
Immune response	
CCAAT/enhancer binding protein	Transcriptional activator of genes involved in immune responses
Complement C1Q subcomponent	Component of the serum complement system
Mouse la-associated invariant chain	Invariant polypeptide of MHC class II antigen-associated
Complement C1 inhibitor	Regulates complement activation

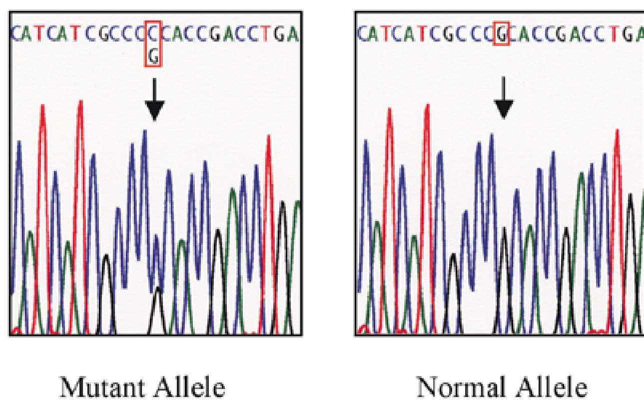

Figure 2. Partial sequence analysis of exon 7 of the *IMPDH1* gene showing the heterozygous C→G nucleotide substitution in an affected member of FA84.

adhesion (44). A mutation in the EGF-containing fibulin-like ECM protein 1 (*EFEMPI*) which, as the name suggests, is very closely related to fibulin, has also been linked to Doyne honeycomb retinal dystrophy (45). In the light of these observations the expression in the $Rho^{-/-}$ retina of three of these genes, *TIMP3*, *cdh23* and *EFEMP1*, was investigated by quantitative RT–PCR. All genes were found to be up-regulated: *TIMP3* by an average of 260%, *cdh23* by 300% and *EFEMPI* by 310%.

With respect to CD44, it has been found that the inherited retinal degeneration exhibited by the *rds* mouse, leads to an up-regulation of expression of CD44 in the retina (46). The current study now shows CD44 to be up-regulated in the rhodopsin knockout mouse. Transcripts derived from the genes encoding amyloid precursor protein (*APP*) and β-2-microglobulin (*B2M*) also show increased expression in $Rho^{-/-}$ retinas. Increased expression in these genes has been implicated in the death of neurones in Alzheimer's disease and amyloidosis, respectively, both of which are characterized by deposition of fibrillar amyloid proteins (47,48).

In conclusion, while immense progress has been made in the identification of genes involved in retinal degenerations, genes at a substantial number of retinopathy loci remain to be characterized in hereditary forms of disease and, with respect to age-related maculopathies, only one gene has been tentatively implicated in disease etiology to date (49). This paper demonstrates the value of studies of global transcriptional profiles of degenerating retinal tissues in the identification of genes which may, rationally, represent candidates for hereditary and multifactorial forms of retinal disease. It should be noted that five of the 35 genes that show a fold change of more than three in all four microarray comparisons, have previously been implicated in the etiology of retinal degenerations.

The study not only highlights a range of potential candidate genes for retinal degeneration but also provides strong evidence that mutations in the *IMPDH1* gene play a role in adRP. Further studies will focus on elucidating the functional significance of *IMPDH1* mutants and on confirming the candidacy of those genes identified through microarray analysis.

MATERIALS AND METHODS

RNA extraction

Mice were initially generated carrying a targeted disruption of the rhodopsin gene using 129Sv-derived manipulated stem cells and C57BL/6J blastulas. Founders on the mixed 129/C57 background were backcrossed through a minimum of 12 successive generations onto essentially pure C57BL/6J and 129Sv genetic backgrounds. The latter were used in the current investigation. Purity of genetic background was confirmed using a series of genetic markers of both single nucleotide polymorphism (SNP) and microsatellite type. In all cases analysed, genotypes from $Rho^{-/-}$ animals backcrossed onto the 129Sv background were the same as 129Sv wild-type (unpublished data). Total RNA was isolated from pooled retinas of two sets of 10 4-month-old 129Sv wild-type and 129Sv $Rho^{-/-}$ mice by the method of Chomczynski and Sacchi (50).

cRNA preparation

Reverse transcription was performed on 12 µg of total RNA for 1 h at 42°C using a T7-oligo$(dT)_{24}$-primer and Superscript II RT (Gibco, Life Technologies, Rockville, MD). Second strand cDNA synthesis was done for 2 h at 16°C using *Escherichia coli* DNA Pol I, DNA ligase and RNAseH (Gibco, Life Technologies), followed by incubation in NaOH/EDTA for 10 min at 65°C in order to degrade rRNA and tRNA. After phenol-chloroform extraction transcription was performed for 6 h at 37°C using the Bioarray high-yield RNA transcript labelling kit with Bio-16-UTP and Bio-11-CTP nucleotides (Enzo Diagnostics, Farmingdale, NY). cRNA samples were purified with the RNeasy kit (Qiagen, Crawley, UK) followed by fragmentation for 35 min at 95°C.

CHIP analysis

Samples were analysed on GeneChips® (Affymetrix, Santa Clara, CA), where perfect match and mismatch are defined by an algorithm. To check the quality of each sample with regard to housekeeping genes GAPDH and β-actin, 15 µg of labelled cRNA was run on Test2-CHIPs (Affymetrix). Murine expression chips 'Mu11K subB' (Affymetrix) containing 6595 data sets were hybridized with 15 µg labelled cRNA for 16 h at 45°C under rotation. Mu11K subB-chips were stained in an Affymetrix Fluidics station using SAPE (Streptavidin/Phycoerythrin) followed by staining with an anti-streptavidin antibody and SAPE. Chips were scanned with a HP-Laserscanner and data were analysed with the Micrarray Suite Software (Affymetrix). Each microarray was scaled to '150' for normalization and then analysed as described previously in detail (51).

Data analysis and selection of candidate genes

Four comparison analyses were obtained by comparing expression data of each wild-type versus each $Rho^{-/-}$ sample using Affymetrix Software (Microarray Suite 4.0, Microdatabase 2.0 and Datamining Tool 2.0). After deletion of 55 Affymetrix spike-controls the remaining 6540 datasets derived from subB-Genechip analysis were sorted according to stringent criteria. 144 datasets were excluded from analysis because the absolute call was absent on all GeneChips® and 5497 datasets were excluded because the difference call was

not changed in all four comparisons. A ranking based on difference calls was done on the remaining 899 datasets. Genes were then excluded in cases where three of four comparisons were not changed or two of four comparisons were not changed, accompanied by one decreased and one increased comparison. The 420 remaining genes showing an increase or decrease in at least two of four comparisons were regarded as potential candidates. 275 genes were up-regulated and 145 were down-regulated in the $Rho^{-/-}$ mouse retinas. Seventy-four of these genes changed >3-fold in at least three comparisons and were regarded as the most interesting candidate genes (30 up- and 44 down-regulated and, of these, 19 genes were up- and 35 were down-regulated in all four comparisons).

Quantitative RT–PCR

Relative quantitative RT–PCR was used to verify the microarray data for a number of the differentially expressed genes. First-strand cDNA synthesis was carried out using 2 µg of total RNA from the pooled wild-type and knockout RNA samples with 4 U Omniscript RT (Qiagen, Crawley, UK) and 1 µM of random hexamers. Quantitative PCR was subsequently performed on a Roche LightCycler using the Quantitech PCR kit (Qiagen). Serial dilutions of cDNA were used to generate standard curves of crossing cycle number versus the logarithms of concentration for each gene of interest. A linear regression line calculated from the standard curves allowed relative transcript levels in both RNA samples to be determined. Values were normalized to the relative amounts of GAPDH present in the same cDNA preparations.

Mutational screening

For mutation analysis of the *IMPDH1* gene, coding exons were amplified from patient genomic DNA using primers located in flanking intron sequence. PCR reactions were carried out in 50 µl vols containing 10 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$, 25 pmol of each primer, 200 µM each dNTP, 500 ng patient DNA and 1.25 U HotStar DNA polymerase (Qiagen). The primers used for amplification of *IMPDH1* exon 7 were as follows: forward, ccctcctaaacatctcccaaa; reverse, ggtgaacctgggtcctcata. After an initial denaturation (94°C for 15 min), 30 cycles of PCR amplification were performed of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min with a final 10 min extension at 72°C. PCR products were purified using Qiaquick columns (Qiagen). Automated sequencing was carried out on an ABI 310 genetic analyser (Perkin Elmer, Shelton, CT) using Big Dye Terminator chemistries (Perkin Elmer).

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