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Development and application of a next-generation-sequencing (NGS) approach to detect known and novel gene defects underlying retinal diseases

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Abstract

Background: Inherited retinal disorders are clinically and genetically heterogeneous with more than 150 gene defects accounting for the diversity of disease phenotypes. So far, mutation detection was mainly performed by APEX technology and direct Sanger sequencing of known genes. However, these methods are time consuming, expensive and unable to provide a result if the patient carries a new gene mutation. In addition, multiplicity of phenotypes associated with the same gene defect may be overlooked.

Methods: To overcome these challenges, we designed an exon sequencing array to target 254 known and candidate genes using Agilent capture. Subsequently, 20 DNA samples from 17 different families, including four patients with known mutations were sequenced using Illumina Genome Analyzer IIx next-generation-sequencing (NGS) platform. Different filtering approaches were applied to identify the genetic defect. The most likely disease causing variants were analyzed by Sanger sequencing. Co-segregation and sequencing analysis of control samples validated the pathogenicity of the observed variants.

Results: The phenotype of the patients included retinitis pigmentosa, congenital stationary night blindness, Best disease, early-onset cone dystrophy and Stargardt disease. In three of four control samples with known genotypes NGS detected the expected mutations. Three known and five novel mutations were identified in *NR2E3*, *PRPF3*, *EYS*, *PRPF8*, *CRB1*, *TRPM1* and *CACNA1F*. One of the control samples with a known genotype belongs to a family with two clinical phenotypes (Best and CSNB), where a novel mutation was identified for CSNB. In six families the disease associated mutations were not found, indicating that novel gene defects remain to be identified.

Conclusions: In summary, this unbiased and time-efficient NGS approach allowed mutation detection in 75% of control cases and in 57% of test cases. Furthermore, it has the possibility of associating known gene defects with novel phenotypes and mode of inheritance.

Keywords: NGS, retinal disorders, diagnostic tool.

Background

Inherited retinal disorders affect approximately 1 in 2000 individuals worldwide [1]. Symptoms and associated phenotypes are variable. In some groups the disease can be mild and stationary such as in congenital stationary night blindness (CSNB) or achromatopsia (ACHM), whereas other disorders are progressive leading to severe visual

impairment such as in rod-cone dystrophies, also known as retinitis pigmentosa (RP) or cone and cone-rod dystrophies. The heterogeneity of these diseases is reflected in the number of underlying gene defects. To date more than 150 genes have been implicated in different forms of retinal disorders <http://www.sph.uth.tmc.edu/Retnet/home.htm> and yet in a significant proportion of patients the disease causing mutation could not be identified, suggesting additional novel genes that remain to be discovered. Furthermore, recent studies have outlined that distinct phenotypes can

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be related to the dysfunction of the same gene [2-4]. Furthermore, there may be additional phenotype-genotype associations that are still not recognized. The state-of-the-art phenotypic characterization including precise family history and functional as well as structural assessment (i.e. routine ophthalmic examination, perimetry, color vision, full field and multifocal electroretinography (ERG), fundus autofluorescence (FAF) imaging and optical coherence tomography (OCT)) allows targeted mutation analysis for some disorders. However, in most cases of inherited retinal diseases, similar phenotypic features can be due to a large number of different gene defects.

Various methods can be used for the identification of the corresponding genetic defect. All these methods have advantages and disadvantages. Sanger sequencing is still the gold-standard in determining the gene defect, but due to the heterogeneity of the disorders it is time consuming and expensive to screen all known genes. Mutation detection by commercially available APEX genotyping microarrays (ASPER Ophthalmics, Estonia) [5,6] allows the detection of only known mutations. In addition, a separate microarray has been designed for each inheritance pattern, which tends to escalate the costs especially in simplex cases, for which inheritance pattern cannot be predetermined. Indirect methods with single nucleotide polymorphism (SNP) microarrays for linkage and homozygosity mapping are also powerful tools, which has proven its reliability in identifying novel and known gene defects [7-12]. However, in case of homozygosity mapping the method can only be applied to consanguineous families or inbred populations. To overcome these challenges, we designed a custom sequencing array in collaboration with a company (IntegraGen, Evry, France) to target all exons and part of flanking sequences for 254 known and candidate retinal genes. This array was subsequently applied through NGS to a cohort of 20 patients from 17 families with different inheritance pattern and clinical diagnosis including RP, CSNB, Best disease, early-onset cone dystrophy and Stargardt disease.

Methods

Clinical investigation

The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the local Ethics Committee (CPP, Ile de France V). Informed written consent was obtained from each study participant. Index patients underwent full ophthalmic examination as described before [13]. Whenever available, blood samples from affected and unaffected family members were collected for co-segregation analysis.

Previous molecular genetic analysis

Total genomic DNA was extracted from peripheral blood leucocytes according to manufacturer's recommendations

(Qiagen, Courtaboeuf, France). DNA samples from some patients with a diagnosis of RP were first analyzed and excluded for known mutations by applying commercially available microarray analysis (arRP and adRP ASPER Ophthalmics, Tartu, Estonia). In some cases, pathogenic variants in *EYS*, *C2orf71*, *RHO*, *PRPF31*, *PRPH2* and *RP1* were excluded by direct Sanger sequencing of the coding exonic and flanking intronic regions of the respective genes [13-17]. Conditions used to amplify *PRPH2* can be provided on request.

Molecular genetic analysis using NGS

A custom-made SureSelect oligonucleotide probe library was designed to capture the exons of 254 genes for different retinal disorders and candidate genes according to Agilent's recommendations (Table 1). These genes include 177 known genes underlying retinal dysfunction (<http://www.sph.uth.tmc.edu/retnet/sum-dis.htm>, October 2010, Table 1) and 77 candidate genes associated with existing animal models and expression data (Table 2). The eArray web-based probe design tool was used for this purpose <https://earray.chem.agilent.com/earray>. The following parameters were chosen for probe design: 120 bp length, 3× probe-tiling frequency, 20 bp overlap in restricted regions, which were identified by the implementation of eArray's RepeatMasker program. A total of 27,430 probes, covering 1177 Mb, were designed and synthesized by Agilent Technologies (Santa Clara, CA, USA). Sequence capture, enrichment, and elution were performed according to the manufacturer's instructions (SureSelect, Agilent). Briefly, three µg of each genomic DNA were fragmented by sonication and purified to yield fragments of 150-200 bps. Paired-end adaptor oligonucleotides from Illumina were ligated on repaired DNA fragments, which were then purified and enriched by six PCR cycles. 500 ng of the purified libraries were hybridized to the SureSelect oligo probe capture library for 24 h. After hybridization, washing, and elution, the eluted fraction underwent 14 cycles of PCR-amplification. This was followed by purification and quantification by qPCR to obtain sufficient DNA template for downstream applications. Each eluted-enriched DNA sample was then sequenced on an Illumina GAIIX as paired-end 75 bp reads. Image analysis and base calling was performed using Illumina Real Time Analysis (RTA) Pipeline version 1.10 with default parameters. Sequence reads were aligned to the reference human genome (UCSC hg19) using commercially available software (CASAVA1.7, Illumina) and the ELANDv2 alignment algorithm. Sequence variation annotation was performed using the IntegraGen in-house pipeline, which consisted of gene annotation (RefSeq), detection of known polymorphisms (dbSNP 131, 1000 Genome) followed by mutation characterization (exonic, intronic,

Table 1 Known retinal disease genes

<i>Number</i>	<i>Gene name</i>
1	<i>ABCA4</i>
2	<i>ABCC6</i>
3	<i>ADAM9</i>
4	<i>AHI1</i>
5	<i>AIPL1</i>
6	<i>ALMS1</i>
7	<i>ARL6</i>
8	<i>ARMS2</i>
9	<i>ATXN7</i>
10	<i>BBS10</i>
11	<i>BBS12</i>
12	<i>BBS2</i>
13	<i>BBS4</i>
14	<i>BBS5</i>
15	<i>BBS7</i>
16	<i>BBS9</i>
17	<i>BEST1</i>
18	<i>C1QTNF5</i>
19	<i>C2</i>
20	<i>C2orf71</i>
21	<i>C3</i>
22	<i>CA4</i>
23	<i>CABP4</i>
24	<i>CACNA1F</i>
25	<i>CACNA2D4</i>
26	<i>CC2D2A</i>
27	<i>CDH23</i>
28	<i>CDH3</i>
29	<i>CEP290</i>
30	<i>CERKL</i>
31	<i>CFB</i>
32	<i>CFH</i>
33	<i>CHM</i>
34	<i>CLN3</i>
35	<i>CLRN1</i>
36	<i>CNGA1</i>
37	<i>CNGA3</i>
38	<i>CNGB1</i>
39	<i>CNGB3</i>
40	<i>CNNM4</i>
41	<i>COL11A1</i>
42	<i>COL2A1</i>
43	<i>COL9A1</i>
44	<i>CRB1</i>
45	<i>CRX</i>
46	<i>CYP4V2</i>
47	<i>DFNB31</i>

Table 1 Known retinal disease genes (Continued)

48	<i>DMD</i>
49	<i>DPP3</i>
50	<i>EFEMP1</i>
51	<i>ELOVL4</i>
52	<i>ERCC6</i>
53	<i>EYS</i>
54	<i>FAM161A</i>
55	<i>FBLN5</i>
56	<i>FSCN2</i>
57	<i>FZD4</i>
58	<i>GNAT1</i>
59	<i>GNAT2</i>
60	<i>GPR98</i>
61	<i>GRK1</i>
62	<i>GRM6</i>
63	<i>GUCA1A</i>
64	<i>GUCA1B</i>
65	<i>GUCY2D</i>
66	<i>HMCN1</i>
67	<i>HTRA1</i>
68	<i>IDH3B</i>
69	<i>IMPDH1</i>
70	<i>IMPG2</i>
71	<i>INPP5E</i>
72	<i>INVS</i>
73	<i>IQCB1</i>
74	<i>JAG1</i>
75	<i>KCNJ13</i>
76	<i>KCNV2</i>
77	<i>KLHL7</i>
78	<i>LCA5</i>
79	<i>LRAT</i>
80	<i>LRP5</i>
81	<i>MERTK</i>
82	<i>MFRP</i>
83	<i>MKKS</i>
84	<i>MKS1</i>
85	<i>MTND1</i>
86	<i>MTND6</i>
87	<i>MT-AP6</i>
88	<i>MTND2</i>
89	<i>MTND5</i>
90	<i>MTND4</i>
91	<i>MYO7A</i>
92	<i>NDP</i>
93	<i>NPHP1</i>
94	<i>NPHP3</i>
95	<i>NPHP4</i>

Table 1 Known retinal disease genes (Continued)

96	<i>NR2E3</i>
97	<i>NRL</i>
98	<i>NYX</i>
99	<i>OAT</i>
100	<i>OFD1</i>
101	<i>OPA1</i>
102	<i>OPA3</i>
103	<i>OPN1LW</i>
104	<i>OPN1MW</i>
105	<i>OPN1Sw</i>
106	<i>OTX2</i>
107	<i>PANK2</i>
108	<i>PAX2</i>
109	<i>PCDH15</i>
110	<i>PCDH21</i>
111	<i>PDE6A</i>
112	<i>PDE6B</i>
113	<i>PDE6C</i>
114	<i>PDE6G</i>
115	<i>PDZD7</i>
116	<i>PEX1</i>
117	<i>PEX2</i>
118	<i>PEX7</i>
119	<i>PGK1</i>
120	<i>PHYH</i>
121	<i>PITPNM3</i>
122	<i>PRCD</i>
123	<i>PROM1</i>
124	<i>PRPF3</i>
125	<i>PRPF31</i>
126	<i>PRPF8</i>
127	<i>PRPH2</i>
128	<i>RAX2</i>
129	<i>RB1</i>
130	<i>RBP3</i>
131	<i>RBP4</i>
132	<i>RD3</i>
133	<i>RDH12</i>
134	<i>RDH5</i>
135	<i>RGR</i>
136	<i>RGS9</i>
137	<i>RGS9BP</i>
138	<i>RHO</i>
139	<i>RIMS1</i>
140	<i>RLBP1</i>
141	<i>ROM1</i>
142	<i>RP1</i>

Table 1 Known retinal disease genes (Continued)

143	<i>RP1L1</i>
144	<i>RP2</i>
145	<i>RP9</i>
146	<i>RPE65</i>
147	<i>RPGR</i>
148	<i>RPGRIP1</i>
149	<i>RPGRIP1L</i>
150	<i>RS1</i>
151	<i>SAG</i>
152	<i>SDCCAG8</i>
153	<i>SEMA4A</i>
154	<i>SLC24A1</i>
155	<i>SNRNP200</i>
156	<i>SPATA7</i>
157	<i>TEAD1</i>
158	<i>TIMM8A</i>
159	<i>TIMP3</i>
160	<i>TLR3</i>
161	<i>TLR4</i>
162	<i>TMEM126A</i>
163	<i>TOPORS</i>
164	<i>TREX1</i>
165	<i>TRIM32</i>
166	<i>TRPM1</i>
167	<i>TSPAN12</i>
168	<i>TTC8</i>
169	<i>TTPA</i>
170	<i>TULP1</i>
171	<i>UNC119</i>
172	<i>USH1C</i>
173	<i>USH1G</i>
174	<i>USH2A</i>
175	<i>VCAN</i>
176	<i>WFS1</i>
177	<i>ZNF513</i>

silent, nonsense etc.). For each position, the exomic frequencies (homozygous and heterozygous) were determined from all the exomes already sequenced by IntegraGen and the exome results provided by HapMap project.

Investigation of annotated sequencing data

We received the annotated sequencing data in the form of excel tables. On average 946 SNPs and 83 insertions and deletions were identified for each sample (Figure 1). By using the filtering system, we first investigated variants (nonsense and missense mutations, intronic

Table 2 Candidate genes for retinal disorders

Number	Gene name	Reason	References
1	ADCY1	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
2	ANKRD33	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
3	ANXA2	Promotion of choroidal neovascularization	[36]
4	ARL13B	Cilia protein, mutations lead to Joubert Syndrome	[37]
5	BMP7	Regulation of Pax 2 in mouse retina	[38]
6	BSG	-	Thierry Leveillard personal communication
7	CAMK2D	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
8	CCDC28B	Modifier for BBS	[39,40]
9	CLCN7	Cln7 ^{-/-} mice severe osteopetrosis and retinal degeneration	[41]
10	COL4A3	Alport syndrome, with eye abnormalities	[42,43]
11	COL4A4	Alport syndrome, with eye abnormalities	[42,44]
12	COL4A5	Alport syndrome, with eye abnormalities	[42,45]
13	CUBN	-	Personal communication Renata Kozyraki
14	CYP1B1	glaucoma	[46]
15	DOHH	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
16	DSCAML1	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
17	ESRRB	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
18	FIZ1	Interactor of <i>NRL</i>	[47]
19	GJA9	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
20	GNAZ	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
21	GNGT1	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
22	GPR152	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
23	HCN1	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
24	HEATR5A	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
25	HIST1H1C	Expressed in retina	Expression databases
26	IMPG1	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
27	INSL5	diff. Expression rd1 mouse	Chalmel et al., manuscript in preparation
28	KCNB1	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation
29	KCTD7	Expressed in retina	Expression databases
30	LASS4	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation
31	LRIT2	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation Rd1 mouse
32	LRP2	-	Personal communication Renata Kozyraki
33	MAB21L1	diff. expression Rd1 mouse	Chalmel et al., manuscript in preparation
34	MAP2	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation
35	MAS1	Degeneration of cones due to expression of <i>Mas1</i>	[48]
36	MAST2	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation
37	MPP4	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation
38	MYOC	glaucoma	[49]
39	NDUFA12	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation
40	NEUROD1	BETA2/NeuroD1 ^{-/-} mouse: photoreceptor degeneration	[50]
41	NOS2	glaucoma	[51]
42	NXNL1	Rod-derived cone viability factor	[52]
43	NXNL2	Rod-derived cone viability factor 2	[53]
44	OPN1MW2	Cone opsin, medium-wave-sensitive2	[54]
45	OPTN	glaucoma	[55]
46	PFKFB2	diff. expression rd1 mouse	Chalmel et al., manuscript in preparation
47	PIAS3	Rod photoreceptor development	[56]

Table 2 Candidate genes for retinal disorders (Continued)

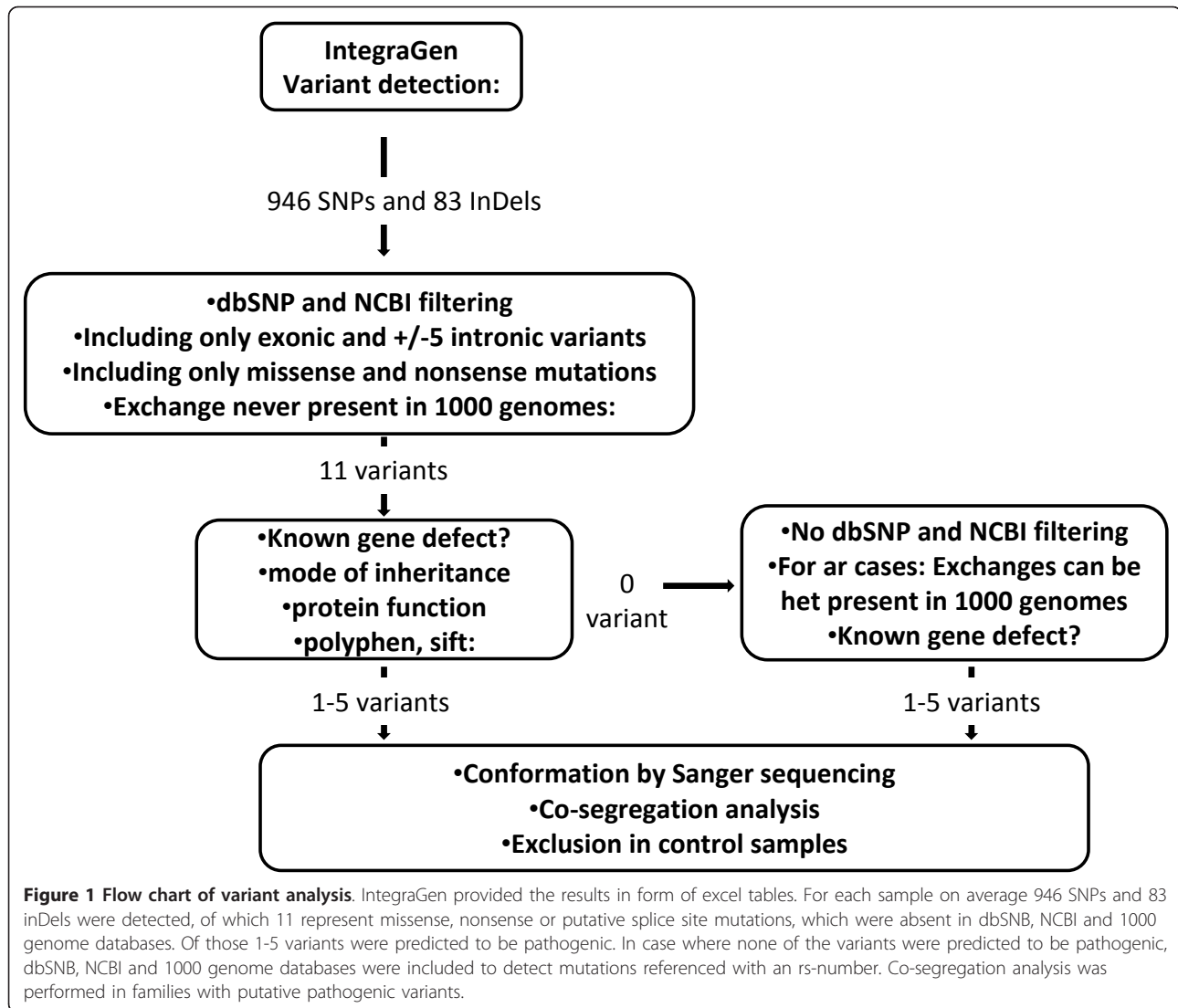
48	PKD2L1	Diff. expression in human retinal detachment	Delyfer et al. 2011 submitted
49	PLEKHA1	Age-related macular degeneratiom	[57]
50	PPEF2	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
51	RAB8A	Interacts with RPGR, role in cilia biogenesis and maintenance	[58]
52	RABGEF1	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
53	RCVRN	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
54	RGS20	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
55	RNF144B	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
56	RORB	Rod photoreceptor development in mice	[59]
57	RXRG	Retinoic acid receptor, highly expressed in the eye	Expression databases
58	SGIP1	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
59	SLC16A8	Altered visual function in ko-mice	[60]
60	SLC17A7	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
61	STAM2	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
62	STK35	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
63	STX3	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
64	SV2B	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
65	TBC1D24	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
66	THRB	Essential for M-cone development in rodents	[61]
67	TMEM216	Cilia protein, mutations lead to Joubert and Meckel syndrome	[62]
68	TMEM67	Cilia protein, mutations lead to Joubert	[63]
69	TRPC1	diff. expression rd1 mouse	diff. expression Rd1 mouse
70	UHMK1	diff. expression rd1 mouse	diff. expression Rd1 mouse
71	VSX1	Stimulator for promoter NXNL1	[64]
72	VSX2	Stimulator for promoter NXNL1	[64]
73	WDR17	diff. expression rd1 mouse	diff. expression Rd1 mouse
74	WDR31	diff. expression Nxn1 ^{-/-} mouse	[65]
75	WISP1	diff. expression rd1 mouse	Chalmel et al., manuscript in preparatiom
76	XIAP	Protects photoreceptors in animal models of RP	[66]
77	ZDHHC2	diff. expression Rd1 mouse	Chalmel et al., manuscript in preparatiom

variants located +/- 5 apart from exon), which were absent in dbSNP and NCBI databases <http://ncbi.nlm.nih.gov/>. In the absence of known gene defects or putative pathogenic variants (see below) in the first step, we selected known genes, which were previously clinically associated including variants present in dbSNP and NCBI databases (Figure 1). Each predicted pathogenic variant was confirmed by Sanger sequencing.

Assessment of the pathogenicity of variants

Following criteria were applied to evaluate the pathogenic nature of novel variations identified by NGS: 1) stop/frameshift variants were considered as most likely to be disease causing; 2) co-segregation in the family; 3) absence in control samples; 4) for missense mutations amino acid conservation was studied in the UCSC Genome Browser <http://genome.ucsc.edu/> across species from all different evolutionary branches. If the amino

acid residue did not change it was considered as “highly conserved”. If a different change was seen in fewer than five species and not in the primates then it was considered as “moderately conserved” and if a change was present in 5-7, it was considered as “weakly conserved”, otherwise the amino acid residue was considered as “not conserved”, 5) pathogenicity predictions with bioinformatic tools (Polyphen: *Polymorphism Phenotyping*, <http://genetics.bwh.harvard.edu/pph/> and SIFT: *Sorting Intolerant From Tolerant*, <http://blocks.fhcrc.org/sift/SIFT.html>) if at least one of the program predicted the variant to be possibly damaging, it was considered to be pathogenic; 6) presence of the second mutant allele in the case of autosomal recessive inheritance. Mutations were described according to the HGVS website <http://www.hgvs.org/mutnomen>. In accordance with this nomenclature, nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG



translation initiation codon in the reference sequence. The initiation codon is codon 1. The correct nomenclature for mutation was checked applying Mutalyzer <http://www.lovd.nl/mutalyzer/>.

Results

The overall sequencing coverage of the captured regions was 98.4% and 90.4% for a 1x and a 10x coverage respectively. The overall sequencing depth was > 120x. The number of reference and variant sequences detected by NGS, reflected the correct zygosity state of the variant; on average if 50% of the sequences represented the variant, then a heterozygous state was called, while if 100% of the sequences represented the variant, then a homozygous or hemizygous state was annotated by IntegraGen.

Validation of the novel genetic testing tool for retinal disorders

To validate the novel genetic testing tool for retinal disorders, we used four DNA samples from families, in which we had previously identified different types of mutations by Sanger sequencing: one 1 bp duplication and one 1 bp deletion in *PRPF31* and missense mutations in *TRPM1* and *BEST1* (Table 3). Three of the four mutations were detectable by NGS, whereas the deletion in *PRPF31* was not identified. To validate if this was due to a technical problem of deletion detection in general or low coverage at this position, the sequencing depth was investigated in detail. Indeed the coverage at this position reflected by the mean depth was only ~1-6 for all samples. This indicates that although the coverage in

Table 3 Patients with known mutations used to validate the novel genetic approach for retinal disorders

Index	Phenotype	Gene	Mutation	Allele State	Read reference NGS	Read variant NGS	Mutation detected by NGS	Mean depth
CIC00034, F28	adRP	<i>PRPF31</i>	c.666dup p. I223YfsX56	het	11	13	yes	21.3-22.5
CIC00140, F108	adRP	<i>PRPF31</i>	c.997delG p. E333SfsX5	het	-	-	no	5.0-5.2
CIC00238, F165	arCSNB	<i>TRPM1</i>	c.1418G > C p.R473P	homo	0	38	yes	36.7
CIC00707, F470	Best and adCSNB see Table 5	<i>BEST1</i>	c.73C > T p.R25W	het	40	38	yes	99.4

general was very good, specific probes used here need to be redesigned to improve the capture for specific exons.

Detection of known and novel mutations

Some of the patients from the 14 families with no known gene defect were previously excluded for known mutations using microarray analysis and by Sanger sequencing in the known genes *EYS*, *C2orf71*, *RHO*, *PRPF31*, *PRPH2* and *RP1*. Other samples were never genetically investigated. In four DNA samples known mutations were detected (Table 4) from three different families with autosomal dominant (ad) or recessive (ar) RP. All mutations co-segregated with the phenotype (Figure 2). In seven samples, novel mutations in known genes were identified. These mutations co-segregated with the phenotype from five different families with adCSNB, x-linked incomplete CSNB, adRP, arRP and x-linked RP (Table 5, Figures 3 and 4). One of the cases from these five families was also used as a control for Best disease carrying a known *BEST1* mutation (Table 3). In addition to the Best phenotype, ERG-responses of this patient resembled those of complete CSNB, i.e. showing selective ON-bipolar pathway dysfunction. This phenotype was independent of the Best phenotype (Figure 3). The most likely disease causing mutation

detected by NGS was a novel heterozygous *TRPM1* mutation (Table 4, Figure 3).

Unsolved cases

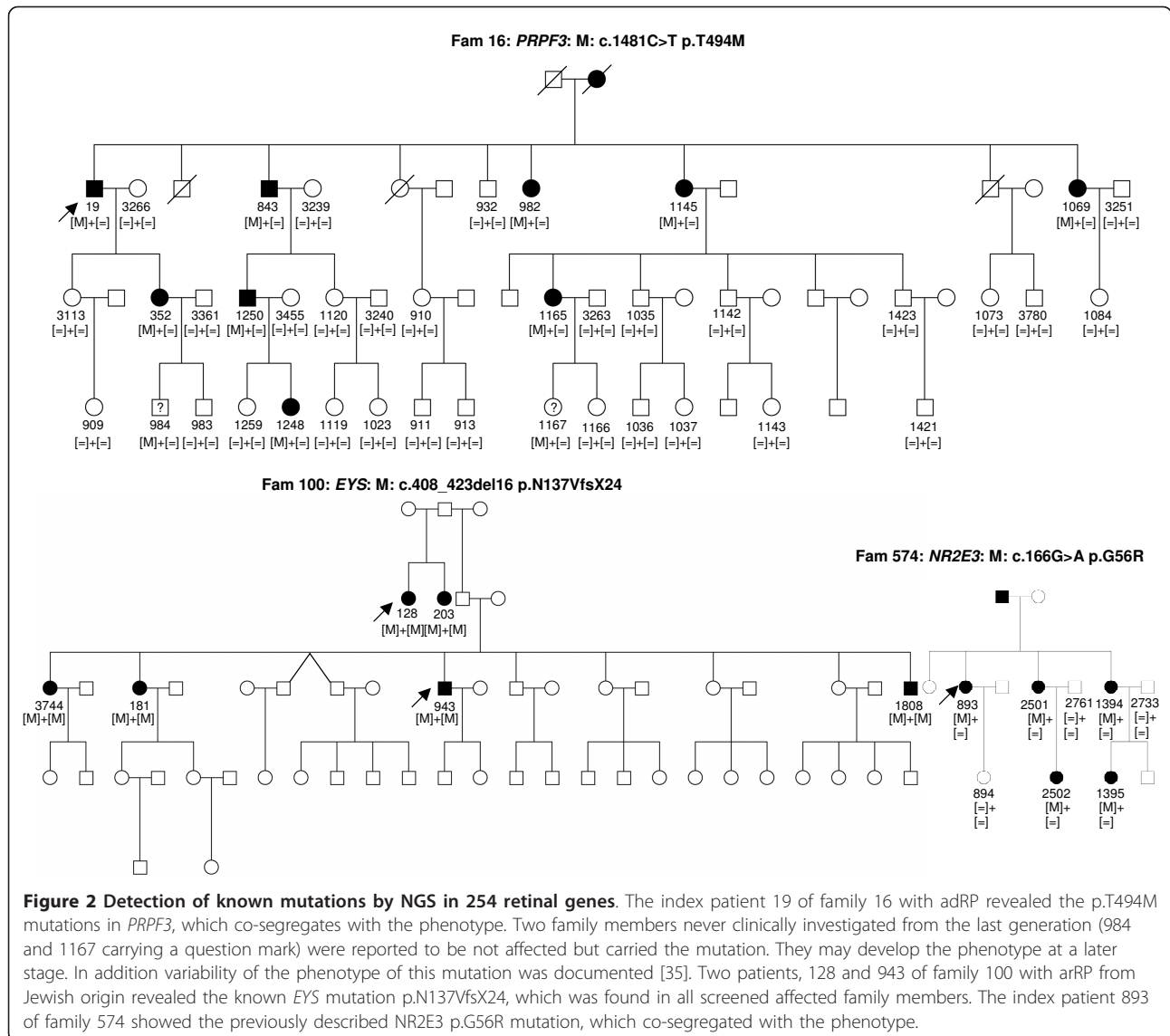
In six of the 14 families with Stargardt disease, adRP, adCD with postreceptoral defects, arRP, early onset arCD with macrocephaly and mental retardation described in affected sister and x-linked cCSNB, the disease associated mutations remain to be elucidated or validated (Table 6, Figure 5).

Discussion

By using NGS in 254 known and candidate genes we were able to detect known and novel mutations in 57% of families tested. In order to achieve this goal, we applied a rigorous protocol (Figure 1). To our knowledge, this is the first report using NGS to investigate all inherited retinal disorders at once. In a study restricted to adRP, Bowne and co-workers used a similar approach including 46 known and candidate genes for adRP [18]. All their cases had previously been screened and excluded for most of the known genes underlying adRP. The authors were able to identify known or novel mutations in five out of 21 cases in genes not included in a pre-screening [18]. This added five patients to their

Table 4 Detection of known mutations by using the novel genetic approach for retinal disorders

Index	Phenotype	Pre-screening	Gene	Mutation	Allele State	Read reference NGS	Read variant NGS	Reference	Mutation verified by Sanger and co-segregation
CIC00019, F16	adRP	Linkage, <i>RHO</i> , <i>PRPF31</i> , <i>PRPH2</i> , <i>RP1</i>	<i>PRPF3</i>	c.1481C > T p.T494M	het	25	22	[67]	yes
CIC0000893, F574	adRP	<i>RHO</i> , <i>PRPF31</i> , <i>PRPH2</i> , <i>RP1</i>	<i>NR2E3</i>	c.166G > A p.G56R	het	5	3	[68]	yes
CIC000128, F100	arRP, consang.	-	<i>EYS</i>	c.408_423del p.N137VfsX24	homo	-	179	[13,69]	yes
CIC0000943, F100	arRP, consang	-	<i>EYS</i>	c.408_423del p.N137VfsX24	homo	0	193	[13,69]	yes



adRP cohort with known gene defects, indicating that 64% of their patients show known mutations with new genes still to be discovered in the remaining 36%. The current study provides a more exhaustive tool, since it incorporates screening of 254 genes implicated in various retinal disorders of different inheritance patterns and additional candidate genes for these phenotypes. With this approach a cohort of both pre-screened and unscreened samples, was investigated. The mutation detection rate of 57% is high and was never obtained before by high throughput screening methods. Furthermore, this approach is probably less time consuming and expensive than existing methods such as direct sequencing of all known genes or microarray analysis. Of note however is one of the variants detected with the NGS approach (i.e. p.V973L exchange in *GUCY2D*),

which was not confirmed by direct Sanger sequencing, suggesting the possibility of false positive using the high throughput screening. Verification by direct Sanger sequencing of most likely pathogenic variants is therefore essential to validate NGS data, although the false positive rate is assumed to be low (in our study 1/28 verified sequence variants represented a false positive).

Overall, the study of 20 subjects from 17 families by NGS showed that most of the targeted regions are well covered (more than 98%). However, some of the regions showed a lower coverage (GC-rich regions) or were not captured (repetitive regions). This was for instance the case for two genes underlying cCSNB, (i.e. *NYX* and *GRM6*) and the repetitive region of *ORF15* of *RPGR*. For GC-rich regions the capture design could be improved in the future by modifying NGS chemistry, as

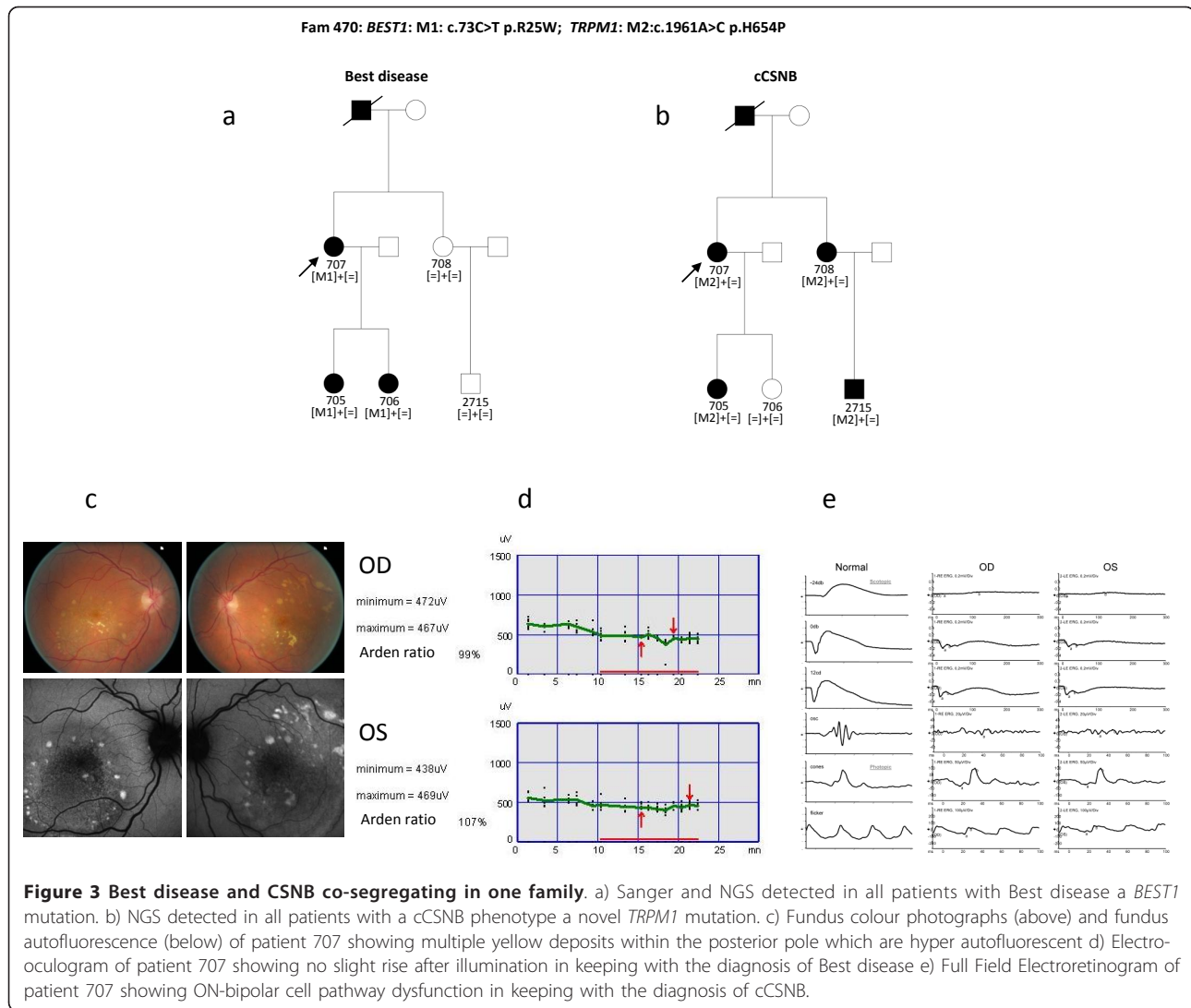
Table 5 Detection of novel mutations by using the novel genetic approach for retinal disorders

Index	Phenotype	Pre-screening	Gene	Mutation	Allele State	Read reference NGS	Read variant NGS	Mutation verified by Sanger and co-segregation	Conservation	Polyphen	Sift
CIC00707, F470	adCSNB and Best see Table 3	<i>RHO</i> , <i>PDE6B</i> , <i>GNAT1</i>	<i>TRPM1</i>	c.1961A > C p.H654P	het	39	38	yes	moderately conserved	possibly damaging	tolerated
CIC000348, F232	adRP, mild	<i>RHO</i> , <i>PRPF31</i> , <i>PRPH2</i> , <i>RP1</i> , adRP chip	<i>PRPF8</i>	c.6992A > G p.E2331G	het	13	10	yes	moderately conserved	possibly damaging	affect protein function
CIC000346, F232	adRP	-	<i>PRPF8</i>	c.6992A > G p.E2331G	het	5	9	yes	moderately conserved	possibly damaging	affect protein function
CIC000347, F232	as adRP	-	<i>PRPF8</i>	c.6992A > G p.E2331G	het	15	17	yes	moderately conserved	possibly damaging	affect protein function
CIC04240, F2025	arRP, consang., detailed clinic in [70]	<i>RS1</i>	<i>CRB1</i>	c.2219C > T p.S740F	homo	2	194	yes	highly conserved	probably damaging	affect protein function
CIC00199, F146	adRP or x-linked RP with affected carrier	<i>RHO</i> , <i>PRPF31</i> , <i>PRPH2</i> , <i>RP1</i> , adRP chip	<i>RPGR</i>	c.248-2A > G splice defect	hetero	30	22	yes	conserved splice site	n.a.	n.a.
CIC04094, F1915	icCSNB	-	<i>CACNA1F</i>	c.973C > T p.Q325X	hemi	0	28	yes	n.a.	n.a.	n.a.

it was successfully achieved for Sanger sequencing using different additives, which improved the amplification and subsequent sequencing. If repetitive regions like ORF15 of *RPGR* remain problematic for sequencing by NGS, direct Sanger sequencing of these targets might be the first screening of choice; in particular for disorders caused only by a few gene defects such as CSNB, and xl-RP.

By applying NGS sequencing to our retinal panel, known and novel mutations were detected in different patients. We believe that our diagnostic tool is particularly important for heterogeneous disorders like RP, for which many gene defects with different prevalence have been associated to one phenotype. It also allows the rapid detection of novel mutations in minor genes which are often not screened as a priority by direct Sanger sequencing. This was the case in our study for three individuals from one family with adRP in which NGS detected a novel *PRPF8* mutation in both affected and one unaffected family member (Table 4, Figure 4). In this family, the RP phenotype is mild and therefore it is possible that the unaffected member may develop symptoms later in life or alternatively it may be a case of incomplete penetrance as reported for another splicing

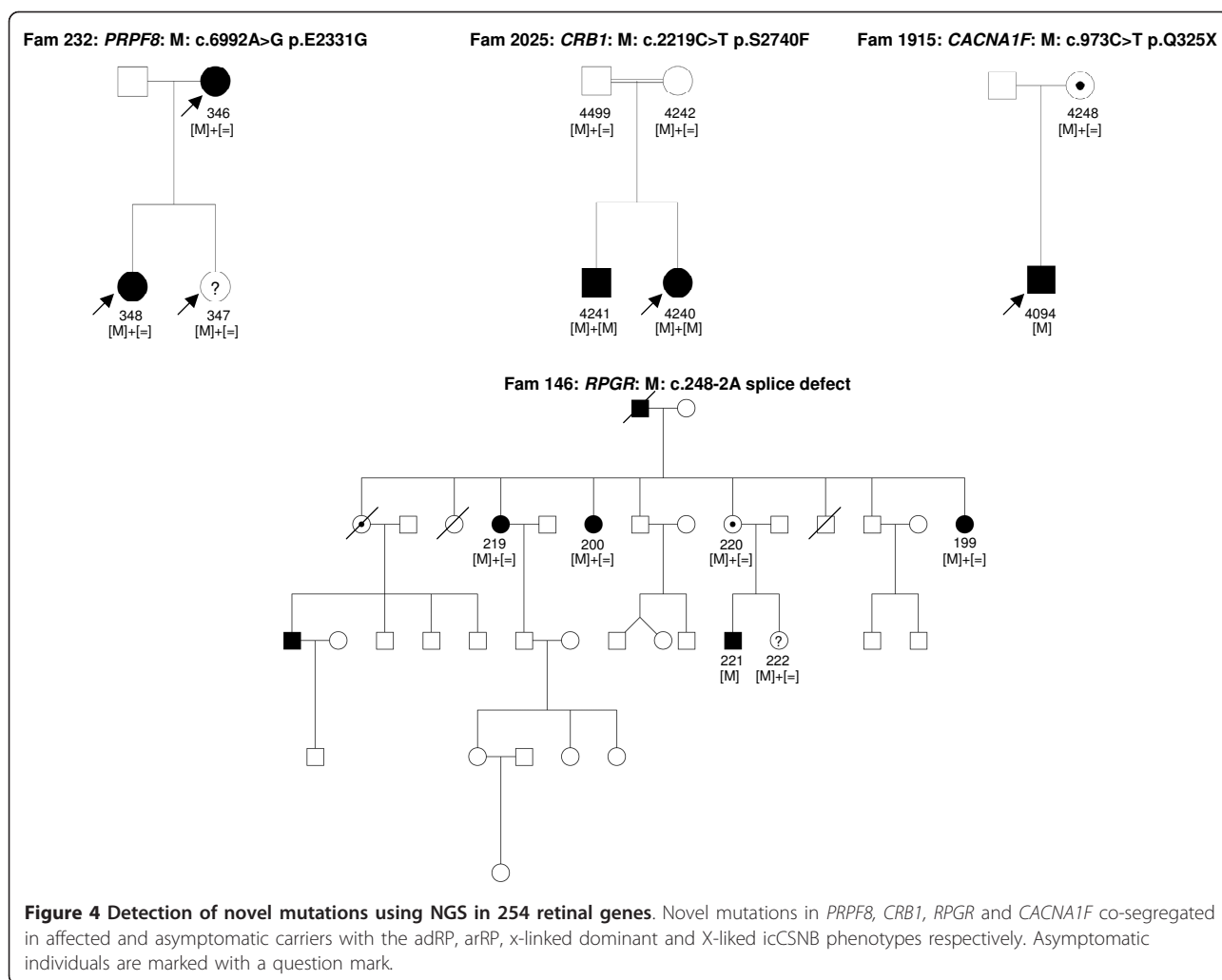
factor gene, *PRPF31* and recently for *PRPF8* as well [19-22]. Interestingly, a novel *TRPM1* mutation was identified in a patient with adCSNB, a gene previously only associated with arCSNB [23-26]. This is the first report of a *TRPM1* mutation co-segregating with ad Schubert-Bornschein type complete CSNB. Since the location of this mutation is not different compared to other mutations leading to arCSNB, it is not quite clear how *TRPM1* mutations might lead to either ad or arCSNB. Functional investigations are needed to validate the pathogenicity of this variant. Furthermore, this finding suggests that *TRPM1* heterozygous mutation carriers from arCSNB families should be investigated by electroretinography to determine whether they display similar retinal dysfunction as in affected members of the presented adCSNB family. Detection of a novel *RPGR* splice site mutation in family 146 presented a challenge. The actual disease causing change was concealed under a wrongly annotated rs62638633, which had previously been clinically associated to RP by a German group <http://www.ncbi.nlm.nih.gov/sites/varvu?gene=6103&rs=62638633>, (personal communication, Markus Preising). These observations indicate that the stringent filtering we applied initially can mask those



referenced disease causing variants. Bearing this in mind one can still first investigate unknown variants, but should then examine dbSNP for referenced variants either described to be disease causing, having a low minor allele frequency or present in interesting candidate genes. An accurate discrimination of non-pathogenic polymorphisms versus disease causing polymorphism in SNP databases is warranted to resolve this challenge.

In six families from the investigated cohort the disease causing mutations still remain to be identified. In the Stargardt patient with no pathogenic *ABCA4* mutations two variants in *CFH* were detected, one of which (rs1061170) had previously been reported to predispose to age related macular degeneration (AMD) [27-29]. The second *CFH* change is a novel variant, affecting a highly conserved residue, not found in NGS data from the other 19 samples and never associated with a disease. The

variants co-segregated in the only available family members, which were the patient's parents. Apart from the association with AMD, *CFH* mutations have been previously associated with renal diseases, the most common being membranoproliferative glomerulonephritis and hemolytic uremic syndrome, which can be also associated with an eye phenotype [30,31]. No renal dysfunction was present in our patient. To validate if the two variants identified in *CFH* are indeed disease causing, the DNA samples from other available family members for co-segregation analysis as well as characterization of functional consequences of the novel variant are needed. One patient with complete CSNB had an affected nephew and thus x-linked inheritance was assumed. However, neither Sanger nor NGS detected a mutation in the only known x-linked gene, *NYX*, causing cCSNB. To exclude recessive inheritance *TRPM1* and *GRM6* were investigated in detail. Indeed the patient carried a novel heterozygous



TRPM1 variant, which affects a highly conserved amino acid and was not identified in the other 19 samples investigated here (Table 6). However, direct Sanger sequencing of lower covered regions did not identify a second mutation in this gene. Similarly no mutations in *GRM6* were identified. These findings outline the need for additional family members to determine, through co-segregation, the pathogenicity of the numerous variants identified by NGS. This was also true for two other families with nonsense mutations in *CUBN* (Fam795) and *RP1L1* (Fam761) (Table 6). The nonsense mutation in *CUBN*, co-segregated with the phenotype in most of the family members (Figure 5). Had we not had access to additional family members, we might have retained this gene defect as the underlying cause for adCD and considered *CUBN* as a new gene involved in adCD. None of the other putatively pathogenic mutations identified in *CUBN*, *TRPM1* and *GUCY2D* co-segregated with the phenotype in this family (Table 6, Figure 5). *RP1L1* was already a candidate for adRP [32] but was previously

associated with occult macular dystrophy [33]. In our study, this variant did not co-segregate with the phenotype in other affected family members (data not shown).

This NGS study ended with six genetically unresolved families, which can be further investigated with whole exome sequencing. Although, no clear information about the actual percentage of missing gene defects underlying each group of inherited retinal disorders exists, previous studies have reported that in many cases the genetic cause still needs to be determined [18,34]. Whole exome sequencing approaches allow the detection of both, novel and known gene defects, but also generate numerous variants and therefore require the inclusion of more than one DNA sample for each family to rapidly exclude non-pathogenic variants. Due to the higher costs of exome sequencing for one sample compared to targeted sequencing, we propose to initially perform targeted sequencing in the index patient and proceed only after exclusion of a known gene defect to whole exome sequencing.

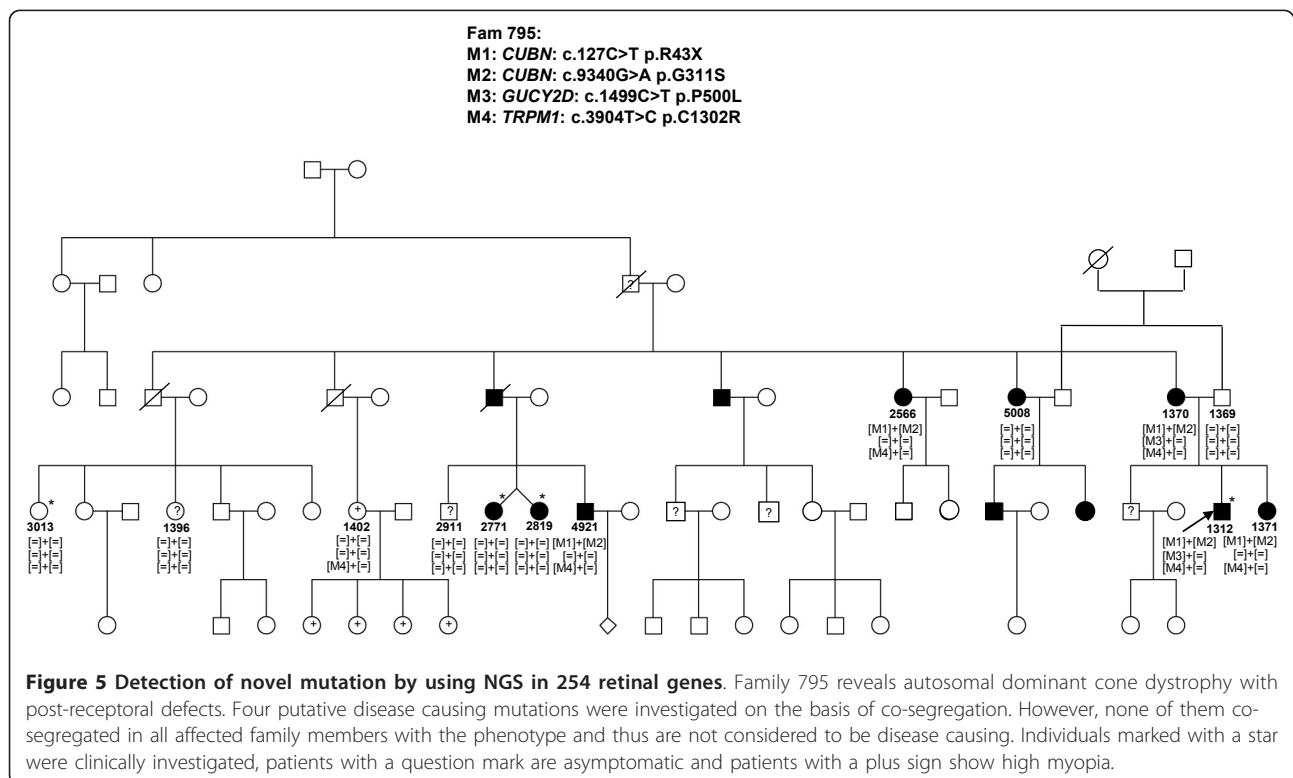
Table 6 Patients with unsolved genotype and unlikely disease causing mutations

Index	Phenotype	Pre-screening	Gene	Mutation	Allele State	Read reference NGS	Read variant NGS	Mutation verified by Sanger and co-segregation	Comment	
CIC03282, F1388	Stargardt	ABCA4 microarray	ABCA4	c.1268A > G p.H423R	het	77	61	yes	but reported as polymorphism [71]	
				c.6764G > T p.S2255I	het	2	7	yes	but reported as polymorphism [72]	
				no additional variants in lower covered exons						
				CFH	c.3482C > A p.P1161Q	het	77	52	yes	conserved, probably damaging
				c.1204C > T p.H402Y	het	94	87	yes	AMD	
CIC01269, F761	adRP	-	RP1L1	c.5959C > T p.Q1987X	het	145	150	yes, did not co-segregate	pass to whole exome sequencing	
CIC01312, F795	adCD with post-receptor defects	RHO, PDE6B, GNAT1 adRP chip	CUBN	c.127C > T p.R43X	het	139	102	yes, did not co-segregate	pass to whole exome sequencing	
			CUBN	c.9340G > A p.G3114S	het	61	44	yes, did not co-segregate		
			GUCY2D	c.1499C > T p.P500L	het	41	34	yes, did not co-segregate		
			TRPM1	c.3904T > C p.C1302R	het	102	99	yes, did not co-segregate		
CIC03225, F1362	arRP consang.	arRP chip	PROM1	c.314A > G p.Y105C	het	120	115	yes, but no additional mutation	no homo, no compound hets, pass to whole exome sequencing	
			GUCY2D	c.2917G > A p.V973L	het	6	2	false positive, not found by Sanger		
			DSCAML1	c.592C > T p.R198C	het	70	81	yes, but no additional mutation		
			TBC1D24	c.641G > A p.R214H	het	27	12	yes, but no additional mutation		
			TMEM67	c.1700A > G p.Y567C	het	80	58	yes, but no additional mutation		
CIC04757 F2364	Index and affected - sister early onset arCD, macrocephaly and mental retardation in affected sister consang.		IMPG2	c.3439C > T p.P1147S	homo	0	140	no	Polyphen and Sift benign, not conserved	
			PKD2L1	c.1027C > T p.R343C	het	63	68			
				c.1202T > G p.V401G	het	25	19		appeared also het in 11 of our samples appeared also het in affected sister but no other mutation in less covered exons	

Table 6 Patients with unsolved genotype and unlikely disease causing mutations (Continued)

			DFNB31	c.1943C > A p.S648Y	het	7	7	yes	affected sister also both variants but both come from father, no other variant in lower covered region.
				c.2644C > A p.R882S	het	27	14	yes	
			EYS	c.7597A > G p.K2533E	het	151	149	yes	Affected sister does not carry this variant
			RPGRIP1	c.2417C > T p.T806I	het	138	132	no	not conserved
CIC04152, F1955	male x-linked cCSNB, has affected nephew	<i>NYX</i>	TRPM1	c.470C > T p.S157F	het	118	130	yes, no other het mutation.	x-linked inheritance and phenotype verification

Index patients and respective gene defect are highlighted in bold. In some cases also family members were used for NGS.



Conclusions

In summary, our diagnostic tool is an unbiased time efficient method, which not only allows detecting known and novel mutations in known genes but also potentially associates known gene defects with novel phenotypes. This genetic testing tool can now be applied to large cohorts of inherited retinal disorders and should rapidly

deliver the prevalence of known genes and the percentage of cases with missing genetic defect for underlying forms of retinal disorders.

List of abbreviations

ad: autosomal dominant; ar: autosomal recessive; as: asymptomatic; het: heterozygous; homo: homozygous; hemi: hemizygous; - not noted; consang:

consanguinity was reported; n.a.: not applicable; CSNB: congenital stationary night blindness; RP: retinitis pigmentosa:

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Authors' contributions

IA was involved in the study design, participated in the choice of genes, interpreted the NGS data, clinically investigated patients, collected DNA samples, and has been involved in drafting the manuscript. KB participated in the choice of genes, interpreted the NGS data and has been involved in drafting the manuscript. TL was involved in the study design, participated in the choice of genes and has been involved in drafting the manuscript. SM-S clinically investigated patients and collected DNA samples. M-EL confirmed the NGS data by Sanger sequencing, performed control and co-segregation analysis. AG extracted DNA, confirmed the NGS data by Sanger sequencing, and performed control and co-segregation analysis. AA extracted DNA, confirmed the NGS data by Sanger sequencing, and performed control and co-segregation analysis. CM confirmed the NGS data by Sanger sequencing, and performed control and co-segregation analysis. J-PS performed NGS. ML performed the bioinformatic interpretation of NGS. J-AS clinically investigated patients and participated in the study design. SSB participated in the study design and has been involved in drafting the manuscript. CZ has made the study design, participated in the choice of genes, interpreted the NGS data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Sothocki MM, Daiger SP, Bowne SJ, Rodriguez JA, Northrup H, Heckenlively JR, Birch DG, Mintz-Hittner H, Ruiz RS, Lewis RA, Saperstein DA, Sullivan LS: **Prevalence of mutations causing retinitis pigmentosa and other inherited retinopathies.** *Hum Mutat* 2001, **17**:42-51.
2. Boon CJ, den Hollander AI, Hoyng CB, Cremers FP, Klevering BJ, Keunen JE: **The spectrum of retinal dystrophies caused by mutations in the peripherin/RDS gene.** *Prog Retin Eye Res* 2008, **27**:213-235.
3. Boon CJ, Klevering BJ, Leroy BP, Hoyng CB, Keunen JE, den Hollander AI: **The spectrum of ocular phenotypes caused by mutations in the BEST1 gene.** *Prog Retin Eye Res* 2009, **28**:187-205.
4. Schorderet DF, Escher P: **NR2E3 mutations in enhanced S-cone sensitivity syndrome (ESCS), Goldmann-Favre syndrome (GFS), clumped pigmentary retinal degeneration (CPRD), and retinitis pigmentosa (RP).** *Hum Mutat* 2009, **30**:1475-1485.
5. Jaakson K, Zernant J, Kulm M, Hutchinson A, Tonisson N, Glavac D, Ravnik-Glavac M, Hawlina M, Meltzer MR, Caruso RC, Testa F, Maugeri A, Hoyng CB, Gouras P, Simonelli F, Lewis RA, Lupski JR, Cremers FP, Allikmets R: **Genotyping microarray (gene chip) for the ABCR (ABCA4) gene.** *Hum Mutat* 2003, **22**:395-403.
6. Zeitz C, Labs S, Lorenz B, Forster U, Uksti J, Kroes HY, De Baere E, Leroy BP, Cremers FP, Wittmer M, van Genderen MM, Sahel JA, Audo I, Poloschek CM, Mohand-Said S, Fleischhauer JC, Huffmeier U, Moskova-Doumanova V, Levin AV, Hamel CP, Leifert D, Munier FL, Schorderet DF, Zrenner E, Friedburg C, Wissinger B, Kohl S, Berger W: **Genotyping microarray for CSNB-associated genes.** *Invest Ophthalmol Vis Sci* 2009, **50**:5919-5926.
7. Wang H, den Hollander AI, Moayed Y, Abulimiti A, Li Y, Collin RW, Hoyng CB, Lopez I, Bray M, Lewis RA, Lupski JR, Mardon G, Koeneke RK, Chen R: **Mutations in SPATA7 cause leber congenital amaurosis and juvenile retinitis pigmentosa.** *Am J Hum Genet* 2009, **84**:380-387.
8. den Hollander AI, McGee TL, Ziviello C, Banfi S, Dryja TP, Gonzalez-Fernandez F, Ghosh D, Berson EL: **A homozygous missense mutation in the IRBP gene (RBP3) associated with autosomal recessive retinitis pigmentosa.** *Invest Ophthalmol Vis Sci* 2009, **50**:1864-1872.
9. Collin RW, Littink KW, Klevering BJ, van den Born LI, Koeneke RK, Zonneveld MN, Blokland EA, Strom TM, Hoyng CB, den Hollander AI, Cremers FP: **Identification of a 2 Mb human ortholog of Drosophila eyes shut/spacemaker that is mutated in patients with retinitis pigmentosa.** *Am J Hum Genet* 2008, **83**:594-603.
10. Collin RW, Safieh C, Littink KW, Garzoni SA, Garzoni HJ, Rizel L, Abbasi AH, Cremers FP, den Hollander AI, Klevering BJ, Ben-Yosef T: **Mutations in C2ORF71 cause autosomal-recessive retinitis pigmentosa.** *Am J Hum Genet* 2010, **86**:783-788.
11. Bandah-Rozenfeld D, Collin RW, Banin E, van den Born LI, Coene KL, Siemiatkowska AM, Zelinger L, Khan MI, Lefeber DJ, Erdinest I, Testa F, Simonelli F, Voeselek K, Blokland EA, Strom TM, Klaver CC, Qamar R, Banfi S, Cremers FP, Sharon D, den Hollander AI: **Mutations in IMPG2, encoding interphotoreceptor matrix proteoglycan 2, cause autosomal-recessive retinitis pigmentosa.** *Am J Hum Genet* 2010, **87**:199-208.
12. Audo I, Bujakowska K, Mohand-Said S, Tronche S, Lancelot ME, Antonio A, Germain A, Lonjou C, Carpentier W, Sahel JA, Bhattacharya S, Zeitz C: **A novel DFNB31 mutation associated with Usher type 2 syndrome showing variable degrees of auditory loss in a consanguineous Portuguese family.** *Mol Vis* 2011, **17**:1598-1606.
13. Audo I, Sahel JA, Mohand-Said S, Lancelot ME, Antonio A, Moskova-Doumanova V, Nandrot EF, Doumanov J, Barragan I, Antinolo G, Bhattacharya SS, Zeitz C: **EYS is a major gene for rod-cone dystrophies in France.** *Hum Mutat* 2010, **31**:E1406-1435.
14. Audo I, Lancelot ME, Mohand-Said S, Antonio A, Germain A, Sahel JA, Bhattacharya SS, Zeitz C: **Novel C2orf71 mutations account for approximately 1% of cases in a large French arRP cohort.** *Hum Mutat* 2011, **32**:E2091-2103.
15. Audo I, Manes G, Mohand-Said S, Friedrich A, Lancelot ME, Antonio A, Moskova-Doumanova V, Poch O, Zanlonghi X, Hamel CP, Sahel JA, Bhattacharya SS, Zeitz C: **Spectrum of rhodopsin mutations in French autosomal dominant rod-cone dystrophy patients.** *Invest Ophthalmol Vis Sci* 2010, **51**:3687-3700.
16. Audo I, Bujakowska K, Mohand-Said S, Lancelot ME, Moskova-Doumanova V, Waseem NH, Antonio A, Sahel JA, Bhattacharya SS, Zeitz C: **Prevalence and novelty of PRPF31 mutations in French autosomal dominant rod-cone dystrophy patients and a review of published reports.** *BMC Med Genet* 2010, **11**:145.
17. Audo I, Mohand-Said S, Dhaenens CM, Germain A, Orhan E, Antonio A, Hamel C, Sahel JA, Bhattacharya SS, Zeitz C: **RP1 and autosomal dominant rod-cone dystrophy: Novel mutations, a review of published variants, and genotype-phenotype correlation.** *Hum Mutat* 2012, **33**:73-80.
18. Bowne SJ, Sullivan LS, Koboldt DC, Ding L, Fulton R, Abbott RM, Sodergren EJ, Birch DG, Wheaton DH, Heckenlively JR, Liu Q, Pierce EA, Weinstock GM, Daiger SP: **Identification of disease-causing mutations in autosomal dominant retinitis pigmentosa (adRP) using next-generation DNA sequencing.** *Invest Ophthalmol Vis Sci* 2011, **52**:494-503.
19. Vithana EN, Abu-Safieh L, Pelosini L, Winchester E, Horman D, Bird AC, Hunt DM, Bustin SA, Bhattacharya SS: **Expression of PRPF31 mRNA in patients with autosomal dominant retinitis pigmentosa: a molecular clue for incomplete penetrance?** *Invest Ophthalmol Vis Sci* 2003, **44**:4204-4209.
20. McGee TL, Devoto M, Ott J, Berson EL, Dryja TP: **Evidence that the penetrance of mutations at the RP11 locus causing dominant retinitis pigmentosa is influenced by a gene linked to the homologous RP11 allele.** *Am J Hum Genet* 1997, **61**:1059-1066.
21. Vithana EN, Abu-Safieh L, Allen MJ, Carey A, Papaioannou M, Chakarova C, Al-Magtheth M, Ebenezer ND, Willis C, Moore AT, Bird AC, Hunt DM,

- Bhattacharya SS: A human homolog of yeast pre-mRNA splicing gene, PRP31, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (RP11). *Mol Cell* 2001, **8**:375-381.
22. Maubaret CG, Vaclavik V, Mukhopadhyay R, Waseem NH, Churchill A, Holder GE, Moore AT, Bhattacharya SS, Webster AR: **Autosomal Dominant Retinitis Pigmentosa with Intrafamilial Variability and Incomplete Penetrance in Two Families carrying Mutations in PRPF8.** *Invest Ophthalmol Vis Sci* 2011.
 23. Li Z, Sergouniotis PI, Michaelides M, Mackay DS, Wright GA, Devery S, Moore AT, Holder GE, Robson AG, Webster AR: **Recessive mutations of the gene TRPM1 abrogate ON bipolar cell function and cause complete congenital stationary night blindness in humans.** *Am J Hum Genet* 2009, **85**:711-719.
 24. van Genderen MM, Bijveld MM, Claassen YB, Florijn RJ, Pearing JN, Meire FM, McCall MA, Riemslag FC, Gregg RG, Bergen AA, Kamermans M: **Mutations in TRPM1 are a common cause of complete congenital stationary night blindness.** *Am J Hum Genet* 2009, **85**:730-736.
 25. Audo I, Kohl S, Leroy BP, Munier FL, Guillonneau X, Mohand-Said S, Bujakowska K, Nandrot EF, Lorenz B, Preising M, Kellner U, Renner AB, Bernd A, Antonio A, Moskova-Doumanova V, Lancelot ME, Poloschek CM, Drumare I, Defoort-Dhellemmes S, Wissinger B, Leveillard T, Hamel CP, Schorderet DF, De Baere E, Berger W, Jacobson SG, Zrenner E, Sahel JA, Bhattacharya SS, Zeitz C: **TRPM1 is mutated in patients with autosomal-recessive complete congenital stationary night blindness.** *Am J Hum Genet* 2009, **85**:720-729.
 26. Nakamura M, Sanuki R, Yasuna TR, Onishi A, Nishiguchi KM, Koike C, Kadowaki M, Kondo M, Miyake Y, Furukawa T: **TRPM1 mutations are associated with the complete form of congenital stationary night blindness.** *Mol Vis* 2010, **16**:425-437.
 27. Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J: **Complement factor H polymorphism in age-related macular degeneration.** *Science* 2005, **308**:385-389.
 28. Edwards AO, Ritter R, Abel KJ, Manning A, Panhuysen C, Farrer LA: **Complement factor H polymorphism and age-related macular degeneration.** *Science* 2005, **308**:421-424.
 29. Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, Gallins P, Spencer KL, Kwan SY, Noureddine M, Gilbert JR, Schnetz-Boutaud N, Agarwal A, Postel EA, Pericak-Vance MA: **Complement factor H variant increases the risk of age-related macular degeneration.** *Science* 2005, **308**:419-421.
 30. Ault BH: **Factor H and the pathogenesis of renal diseases.** *Pediatr Nephrol* 2000, **14**:1045-1053.
 31. Boon CJ, van de Kar NC, Klevering BJ, Keunen JE, Cremers FP, Klaver CC, Hoyng CB, Daha MR, den Hollander AI: **The spectrum of phenotypes caused by variants in the CFH gene.** *Mol Immunol* 2009, **46**:1573-1594.
 32. Bowne SJ, Daiger SP, Malone KA, Heckenlively JR, Kennan A, Humphries P, Hughbanks-Wheaton D, Birch DG, Liu Q, Pierce EA, Zuo J, Huang Q, Donovan DD, Sullivan LS: **Characterization of RP1L1, a highly polymorphic paralog of the retinitis pigmentosa 1 (RP1) gene.** *Mol Vis* 2003, **9**:129-137.
 33. Akahori M, Tsunoda K, Miyake Y, Fukuda Y, Ishiura H, Tsuji S, Usui T, Hatase T, Nakamura M, Ohde H, Itabashi T, Okamoto H, Takada Y, Iwata T: **Dominant mutations in RP1L1 are responsible for occult macular dystrophy.** *Am J Hum Genet* 2010, **87**:424-429.
 34. Berger W, Kloeckener-Gruissem B, Neidhardt J: **The molecular basis of human retinal and vitreoretinal diseases.** *Prog Retin Eye Res* 2010, **29**:335-375.
 35. Vaclavik V, Gaillard MC, Tiab L, Schorderet DF, Munier FL: **Variable phenotypic expressivity in a Swiss family with autosomal dominant retinitis pigmentosa due to a T494M mutation in the PRPF3 gene.** *Mol Vis* 2010, **16**:467-475.
 36. Zhao SH, Pan DY, Zhang Y, Wu JH, Liu X, Xu Y: **Annexin A2 promotes choroidal neovascularization by increasing vascular endothelial growth factor expression in a rat model of argon laser coagulation-induced choroidal neovascularization.** *Chin Med J (Engl)* 2010, **123**:713-721.
 37. Cantagrel V, Silhavy JL, Bielas SL, Swistun D, Marsh SE, Bertrand JY, Audollent S, Attie-Bitach T, Holden KR, Dobyns WB, Traver D, Al-Gazali L, Ali BR, Lindner TH, Caspary T, Otto EA, Hildebrandt F, Glass IA, Logan CV, Johnson CA, Bennett C, Brancati F, Valente EM, Woods CG, Gleeson JG: **Mutations in the cilia gene ARL13B lead to the classical form of Joubert syndrome.** *Am J Hum Genet* 2008, **83**:170-179.
 38. Sehgal R, Sheibani N, Rhodes SJ, Belecky Adams TL: **BMP7 and SHH regulate Pax2 in mouse retinal astrocytes by relieving TLX repression.** *Dev Biol* 2009, **332**:429-443.
 39. Beales PL, Badano JL, Ross AJ, Ansley SJ, Hoskins BE, Kirsten B, Mein CA, Froguel P, Scambler PJ, Lewis RA, Lupski JR, Katsanis N: **Genetic interaction of BBS1 mutations with alleles at other BBS loci can result in non-Mendelian Bardet-Biedl syndrome.** *Am J Hum Genet* 2003, **72**:1187-1199.
 40. Badano JL, Leitch CC, Ansley SJ, May-Simera L, Lawson S, Lewis RA, Beales PL, Dietz HC, Fisher S, Katsanis N: **Dissection of epistasis in oligogenic Bardet-Biedl syndrome.** *Nature* 2006, **439**:326-330.
 41. Kornak U, Kasper D, Bosl MR, Kaiser E, Schweizer M, Schulz A, Friedrich W, Delling G, Jentsch TJ: **Loss of the CIC-7 chloride channel leads to osteopetrosis in mice and man.** *Cell* 2001, **104**:205-215.
 42. Colville DJ, Savige J: **Alport syndrome. A review of the ocular manifestations.** *Ophthalmic Genet* 1997, **18**:161-173.
 43. Lemmink HH, Mochizuki T, van den Heuvel LP, Schroder CH, Barrientos A, Monnens LA, van Oost BA, Brunner HG, Reeders ST, Smeets HJ: **Mutations in the type IV collagen alpha 3 (COL4A3) gene in autosomal recessive Alport syndrome.** *Hum Mol Genet* 1994, **3**:1269-1273.
 44. Jefferson JA, Lemmink HH, Hughes AE, Hill CM, Smeets HJ, Doherty CC, Maxwell AP: **Autosomal dominant Alport syndrome linked to the type IV collagen alpha 3 and alpha 4 genes (COL4A3 and COL4A4).** *Nephrol Dial Transplant* 1997, **12**:1595-1599.
 45. Lemmink HH, Kluijtmans LA, Brunner HG, Schroder CH, Knebelmann B, Jelinkova E, van Oost BA, Monnens LA, Smeets HJ: **Aberrant splicing of the COL4A5 gene in patients with Alport syndrome.** *Hum Mol Genet* 1994, **3**:317-322.
 46. Stoilov I, Akarsu AN, Sarfarazi M: **Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (Buphthalmos) in families linked to the GLC3A locus on chromosome 2p21.** *Hum Mol Genet* 1997, **6**:641-647.
 47. Mitton KP, Swain PK, Khanna H, Dowd M, Apel IJ, Swaroop A: **Interaction of retinal bZIP transcription factor NRL with Flt3-interacting zinc-finger protein Fiz1: possible role of Fiz1 as a transcriptional repressor.** *Hum Mol Genet* 2003, **12**:365-373.
 48. Xu X, Quiambao AB, Roveri L, Pardue MT, Marx JL, Rohlich P, Peachey NS, Al-Ubaidi MR: **Degeneration of cone photoreceptors induced by expression of the Mas1 protooncogene.** *Exp Neurol* 2000, **163**:207-219.
 49. Kubota R, Kudoh J, Mashima Y, Asakawa S, Minoshima S, Hejtmancik JF, Oguchi Y, Shimizu N: **Genomic organization of the human myocilin gene (MYOC) responsible for primary open angle glaucoma (GLC1A).** *Biochem Biophys Res Commun* 1998, **242**:396-400.
 50. Pennesi ME, Cho JH, Yang Z, Wu SH, Zhang J, Wu SM, Tsai MJ: **BETA2/NeuroD1 null mice: a new model for transcription factor-dependent photoreceptor degeneration.** *J Neurosci* 2003, **23**:453-461.
 51. Liu B, Neufeld AH: **Expression of nitric oxide synthase-2 (NOS-2) in reactive astrocytes of the human glaucomatous optic nerve head.** *Glia* 2000, **30**:178-186.
 52. Leveillard T, Mohand-Said S, Lorentz O, Hicks D, Fintz AC, Clerin E, Simonutti M, Forster V, Cavusoglu N, Chalmel F, Dolle P, Poch O, Lambrou G, Sahel JA: **Identification and characterization of rod-derived cone viability factor.** *Nat Genet* 2004, **36**:755-759.
 53. Chalmel F, Leveillard T, Jaillard C, Lardenois A, Berdugo N, Morel E, Koehl P, Lambrou G, Holmgren A, Sahel JA, Poch O: **Rod-derived Cone Viability Factor-2 is a novel bifunctional-thioredoxin-like protein with therapeutic potential.** *BMC Mol Biol* 2007, **8**:74.
 54. Tsutsumi M, Ikeyama K, Denda S, Nakanishi J, Fuziwara S, Aoki H, Denda M: **Expressions of rod and cone photoreceptor-like proteins in human epidermis.** *Exp Dermatol* 2009, **18**:567-570.
 55. Rezaie T, Child A, Hitchings R, Brice G, Miller L, Coca-Prados M, Heon E, Krupin T, Ritch R, Kreutzer D, Crick RP, Sarfarazi M: **Adult-onset primary open-angle glaucoma caused by mutations in optineurin.** *Science* 2002, **295**:1077-1079.
 56. Onishi A, Peng GH, Hsu C, Alexis U, Chen S, Blackshaw S: **Pias3-dependent SUMOylation directs rod photoreceptor development.** *Neuron* 2009, **61**:234-246.
 57. Montezuma SR, Sobrin L, Seddon JM: **Review of genetics in age related macular degeneration.** *Semin Ophthalmol* 2007, **22**:229-240.
 58. Murga-Zamalloa CA, Atkins SJ, Peranen J, Swaroop A, Khanna H: **Interaction of retinitis pigmentosa GTPase regulator (RPGR) with RAB8A GTPase:**

- implications for cilia dysfunction and photoreceptor degeneration. *Hum Mol Genet* 2010, **19**:3591-3598.
59. Jia L, Oh EC, Ng L, Srinivas M, Brooks M, Swaroop A, Forrest D: **Retinoid-related orphan nuclear receptor RORbeta is an early-acting factor in rod photoreceptor development.** *Proc Natl Acad Sci USA* 2009, **106**:17534-17539.
 60. Daniele LL, Sauer B, Gallagher SM, Pugh EN Jr, Philp NJ: **Altered visual function in monocarboxylate transporter 3 (Slc16a8) knockout mice.** *Am J Physiol Cell Physiol* 2008, **295**:C451-457.
 61. Ng L, Hurley JB, Dierks B, Srinivas M, Salto C, Vennstrom B, Reh TA, Forrest D: **A thyroid hormone receptor that is required for the development of green cone photoreceptors.** *Nat Genet* 2001, **27**:94-98.
 62. Valente EM, Logan CV, Mougou-Zerelli S, Lee JH, Silhavy JL, Brancati F, Iannicelli M, Travaglini L, Romani S, Illi B, Adams M, Szymanska K, Mazzotta A, Lee JE, Tolentino JC, Swistun D, Salpietro CD, Fede C, Gabriel S, Russ C, Cibulskis K, Sougnez C, Hildebrandt F, Otto EA, Held S, Diplas BH, Davis EE, Mikula M, Strom CM, Ben-Zeev B, et al: **Mutations in TMEM216 perturb ciliogenesis and cause Joubert, Meckel and related syndromes.** *Nat Genet* 2010, **42**:619-625.
 63. Baala L, Romano S, Khaddour R, Saunier S, Smith UM, Audollent S, Ozilou C, Faivre L, Laurent N, Foliguet B, Munnich A, Lyonnet S, Salomon R, Encha-Razavi F, Gubler MC, Boddaert N, de Lonlay P, Johnson CA, Vekemans M, Antignac C, Attie-Bitach T: **The Meckel-Gruber syndrome gene, MKS3, is mutated in Joubert syndrome.** *Am J Hum Genet* 2007, **80**:186-194.
 64. Reichman S, Kalathur RK, Lambard S, Ait-Ali N, Yang Y, Lardenois A, Ripp R, Poch O, Zack DJ, Sahel JA, Leveillard T: **The homeobox gene CHX10/VSX2 regulates RdCVF promoter activity in the inner retina.** *Hum Mol Genet* 2010, **19**:250-261.
 65. Cronin T, Raffelsberger W, Lee-Rivera I, Jaillard C, Niepon ML, Kinzel B, Clerin E, Petrosian A, Picaud S, Poch O, Sahel JA, Leveillard T: **The disruption of the rod-derived cone viability gene leads to photoreceptor dysfunction and susceptibility to oxidative stress.** *Cell Death Differ* 2010, **17**:1199-1210.
 66. Leonard KC, Petrin D, Coupland SG, Baker AN, Leonard BC, LaCasse EC, Hauswirth WW, Korneluk RG, Tsilfidis C: **XIAP protection of photoreceptors in animal models of retinitis pigmentosa.** *PLoS ONE* 2007, **2**:e314.
 67. Chakarova CF, Hims MM, Bolz H, Abu-Safieh L, Patel RJ, Papaioannou MG, Inglehearn CF, Keen TJ, Willis C, Moore AT, Rosenberg T, Webster AR, Bird AC, Gal A, Hunt D, Vithana EN, Bhattacharya SS: **Mutations in HPRP3, a third member of pre-mRNA splicing factor genes, implicated in autosomal dominant retinitis pigmentosa.** *Hum Mol Genet* 2002, **11**:87-92.
 68. Coppieters F, Leroy BP, Beysens D, Hellemans J, De Bosscher K, Haegeman G, Robberecht K, Wuyts W, Coucke PJ, De Baere E: **Recurrent mutation in the first zinc finger of the orphan nuclear receptor NR2E3 causes autosomal dominant retinitis pigmentosa.** *Am J Hum Genet* 2007, **81**:147-157.
 69. Bandah-Rozenfeld D, Littink KW, Ben-Yosef T, Strom TM, Chowers I, Collin RW, den Hollander AI, van den Born LI, Zonneveld MN, Merin S, Banin E, Cremers FP, Sharon D: **Novel null mutations in the EYS gene are a frequent cause of autosomal recessive retinitis pigmentosa in the Israeli population.** *Invest Ophthalmol Vis Sci* 2010, **51**:4387-4394.
 70. Bujakowska K, Audo I, Mohand-Said S, Lancelot ME, Antonio A, Germain A, Leveillard T, Letexier M, Saraiva JP, Lonjou C, Carpentier W, Sahel JA, Bhattacharya SS, Zeitz C: **CRB1 mutations in inherited retinal dystrophies.** *Hum Mutat* 2011, **33**: 306-315.
 71. Rivera A, White K, Stohr H, Steiner K, Hemmrich N, Grimm T, Jurklics B, Lorenz B, Scholl HP, Apfelstedt-Sylla E, Weber BH: **A comprehensive survey of sequence variation in the ABCA4 (ABCR) gene in Stargardt disease and age-related macular degeneration.** *Am J Hum Genet* 2000, **67**:800-813.
 72. Shroyer NF, Lewis RA, Lupski JR: **Analysis of the ABCR (ABCA4) gene in 4-aminquinoline retinopathy: is retinal toxicity by chloroquine and hydroxychloroquine related to Stargardt disease?** *Am J Ophthalmol* 2001, **131**:761-766.

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