Identity-by-descent-guided mutation analysis and exome sequencing in consanguineous families reveals unusual clinical and molecular findings in retinal dystrophy

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Purpose: Autosomal recessive retinal dystrophies are clinically and genetically heterogeneous, which hampers molecular diagnosis. We evaluated identity-by-descent-guided Sanger sequencing or whole-exome sequencing in 26 families with nonsyndromic (19) or syndromic (7) autosomal recessive retinal dystrophies to identify disease-causing mutations.

Methods: Patients underwent genome-wide identity-by-descent mapping followed by Sanger sequencing (16) or whole-exome sequencing (10). Whole-exome sequencing data were filtered against identity-by-descent regions and known retinal dystrophy genes. The medical history was reviewed in mutation-positive families.

Results: We identified mutations in 14 known retinal dystrophy genes in 20/26 (77%) families: *ABCA4*, *CERKL*, *CLN3*, *CNNM4*, *C2orf71*, *IQCB1*, *LRAT*, *MERTK*, *NMNAT1*, *PCDH15*, *PDE6B*, *RDH12*, *RPGRIP1*, and *USH2A*. Whole-exome sequencing in single individuals revealed mutations in either the largest or smaller

identity-by-descent regions, and a compound heterozygous genotype in *NMNAT1*. Moreover, a novel deletion was found in *PCDH15*. In addition, we identified mutations in *CLN3*, *CNNM4*, and *IQCB1* in patients initially diagnosed with nonsyndromic retinal dystrophies.

Conclusion: Our study emphasized that identity-by-descent-guided mutation analysis and/or whole-exome sequencing are powerful tools for the molecular diagnosis of retinal dystrophy. Our approach uncovered unusual molecular findings and unmasked syndromic retinal dystrophies, guiding future medical management. Finally, elucidating *ABCA4*, *LRAT*, and *MERTK* mutations offers potential gene-specific therapeutic perspectives.

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Key Words: copy-number variation analysis; identity-by-descent mapping; retinal dystrophy; variant filtering; whole-exome sequencing

INTRODUCTION

Retinal dystrophies (RDs) have a profound impact on an individual's life. Patients develop significant visual impairment, often leading to irreversible blindness, with an age of onset ranging from birth to late adulthood. RDs are inherited, with autosomal recessive (AR), autosomal dominant, and X-linked inheritance patterns described.¹ RDs generally display a tremendous genetic heterogeneity: more than 240 disease loci (>200 genes) have been identified so far, each accounting for only a fraction of patients. Moreover, up to 50% of disease genes are still unidentified.² In addition, retinal phenotypes often overlap and might be associated with systemic features developing in a later disease stage.³ Overall, this profound genetic and phenotypic complexity severely hampers the establishment of a molecular diagnosis, which is essential in view of not only genetic counseling but also gene therapy.⁴

Molecular diagnostics for RDs recently shifted from commercial microarray-based testing for known mutations and Sanger sequencing of limited gene sets^{5,6} to massively parallel sequencing (MPS), allowing fast and cost-effective sequencing of comprehensive RD gene panels.⁷⁻⁹ One step further is the implementation of whole-exome sequencing (WES), in which all (coding) exons are enriched and sequenced, enabling the identification of mutations in known as well as novel RD genes. However, the enormous number of variants thus identified is extremely challenging to interpret.

Here, homozygosity or identity-by-descent (IBD) mapping offers an excellent filtering strategy because this technique pinpoints the location of the causal gene in inbred families in which an AR disease is segregating. IBD mapping is based on the concept that consanguineous individuals have a high risk of carrying two identical disease alleles originating from a common ancestor. The region encompassing the disease gene is

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Table 1 Overview of mutations identified in this study

Family no.	Initial diagnosis	Origin	Par cons	Prescreening	Chr	Homozygous region			Mutation
						Start (hg19) (bp)	Stop (hg19) (bp)	No. of SNPs	
F1	LCA	Sahrawi	FC	LCA chip 6 LCA genes ¹⁴	14	36,398,888	89,759,896	5,225	RDH12 c.912G>A
F2	EORD	Turkey	See pedigree	16 LCA genes ⁷ LCA chip <i>CRB1</i>	4	139,794,516	161,515,788	2,020	p.Trp304* <i>LRAT</i> c.326G>T p.Arg109Leu
F3	ARRP	Turkey	See pedigree	ARRP chip	14	63,582,469	91,816,089	2,634	RDH12 c.464C>T p.Thr155Ile¹⁷
F4	ARRP	Morocco	FC	/	14	50,519,022	73,048,944	2,143	RDH12 c.464C>T p.Thr155Ile¹⁷
F5	ARRP	Belgium	TC	ARRP chip	14	68,010,224	94,907,644	2,539	RDH12 c.803G>T p.Cys268Phe
F6	ARRP	Belgium	FC once removed	ARRP chip	1	208,130,418	230,905,723	2,281	USH2A c.12574C>T p.Arg4192Cys
F7	ARCRD	Algeria	See pedigree	ARRP chip	2	84,056,323	98,708,410	343	CNNM4 c.189del p.Asp63fs
F8	ARCRD	Turkey	NA	<i>ABCA4</i> chip	1	85,954,113	101,239,376	1,477	ABCA4 c.3819dup p.Leu1274fs
F9	SLS	Turkey	NA	LCA chip 6 LCA genes ¹⁴	3	109,503,273	137,519,670	2,422	IQCB1 c.1381C>T p.Arg461*²¹
F10	SLS	Belgium	FC	<i>CEP290</i>	3	117,023,557	147,755,330	2,598	IQCB1 c.424_425del p.Phe142fs²¹
F11	USH1	NA	NA	/	10	55,237,139	68,437,615	1,689	PCDH15 Exon 1 and 2 deletion

ARCRD, autosomal recessive cone-rod dystrophy; ARRP, autosomal recessive retinitis pigmentosa; EORD, early-onset RD; FC, first cousins; IBD, identity-by-descent; ID, intellectual disability; LCA, Leber congenital amaurosis; NA, not available; Par cons, parental consanguinity; RD, retinal dystrophy; SLS, Senior-Løken syndrome; SNP, single-nucleotide polymorphism; TC, third cousins; USH, Usher syndrome. Mutations in bold are new.

Table 1 Continued on next page

Table 1 Continued

Family no.	Initial diagnosis	Origin	Par cons	Prescreening	Homozygous region					Mutation
					Chr	Start (hg19) (bp)	Stop (hg19) (bp)	Length (Mb)	No. of SNPs	
F12	LCA	Turkey	NA	LCA chip 6 LCA genes ¹⁴	14	20,942,451	24,311,085	3.37	351	1 <i>RPGRIP1</i> c.2758C>T p.Gln920* <i>NMIVAT1</i> HeZ: c.205A>G p.Met69Val ²⁴ HeZ: c.769G>A p.Glu257Iys ²⁴ <i>CLN3</i> c.784A>T p.Lys262* <i>EYS</i> HeZ: c.9368dup p.Asn3123fs ²⁵ <i>MERTK</i> c.2180G>A p.Arg727Gln <i>C2orf71</i> c.1764del p.Glu589fs <i>PDE6B</i> c.121_125delins15 p.Pro41fs <i>USH2A</i> c.10721G>T p.Gly3574Val ⁹ <i>CERKL</i> c.847C>T p.Arg283* ²⁷ <i>IQCB1</i> c.1241_1242del p.Leu414fs
F13	LCA	Tunisia	NA	LCA chip 6 LCA genes ¹⁴ 16 LCA genes ⁷	/	/	/	/	/	/
F14	EORD	NA	FC	LCA chip	16	20,364,588	65,795,203	45.43	4,000	1
F15	EORD	NA	NA	LCA chip 6 LCA genes ¹⁴ 16 LCA genes ⁷	/	/	/	/	/	/
F16	ARRP	Turkey	FC	/	2	109,804,521	147,023,441	37.22	3,354	1
F17	ARRP	Belgium	FC	ARRP chip <i>RDH12</i>	2	16,205,386	48,592,202	32.39	3,221	1
F18	ARRP	Morocco	NA	ARRP chip	4	29,099	6,757,184	6.73	519	11
F19	ARRP	Turkey	NA	/	1	214,548,682	216,082,177	1.53	176	28
F20	ARCRD	Belgium	FC	/	2	182,024,031	182,884,959	0.86	90	38
F21	LCA + ID	Sicily	NA	LCA chip 6 LCA genes ¹⁴	3	121,324,957	122,649,522	1.32	135	23

ARCRD, autosomal recessive cone-rod dystrophy; ARRP, autosomal recessive retinitis pigmentosa; EORD, early-onset RD; FC, first cousins; IBD, identity-by-descent; ID, intellectual disability; LCA, Leber congenital amaurosis; NA, not available; Par cons, parental consanguinity; RD, retinal dystrophy; SLS, Senior-Loken syndrome; SNP, single-nucleotide polymorphism; TC, third cousins; USH, Usher syndrome. Mutations in bold are new.

homozygous and shared between different affected individuals of the same family.¹⁰ IBD mapping has played a major role in both RD gene discovery and routine screening, combined with both Sanger sequencing and MPS.^{5,11,12}

In this study, we performed IBD mapping and subsequent Sanger sequencing or WES in 26 consanguineous families with a nonsyndromic (19) or syndromic (7) AR RD. In addition to a high detection rate, our approach identified conventional subtle mutations as well as a large deletion. In addition, WES was instrumental in the identification of mutations located both within and outside IBD regions. Moreover, the molecular diagnosis revealed a complex clinical diagnosis in three families, directly influencing clinical follow-up and management. Finally, elucidating *ABCA4*, *LRAT*, and *MERTK* mutations offers gene-specific therapeutic perspectives.

MATERIALS AND METHODS

Subject enrollment and clinical evaluation

Thirty-seven individuals from 26 consenting families were recruited from the Center for Medical Genetics Ghent and the Department of Ophthalmology, Ghent University Hospital, Ghent, Belgium; the Department of Ophthalmology, Queen Fabiola Children's University Hospital, Brussels, Belgium; and the Department of Ophthalmology and Center for Human Genetics, Leuven University Hospitals, Leuven, Belgium. Informed consent was obtained and the research protocols adhered to the tenets of the Declaration of Helsinki. Patients had histories and pedigree analysis taken and underwent ophthalmic examination, including best-corrected visual acuity testing, slit-lamp examination, Goldmann visual field testing, dilated fundus examination, and full-field flash electroretinography.¹³ Nineteen probands were initially diagnosed with one of the following nonsyndromic RDs: Leber congenital amaurosis (LCA) (4), early-onset RD (EORD) (3), AR retinitis pigmentosa (ARRP) (9), or AR cone-rod dystrophy (ARCRD) (3). In addition, seven probands were referred for a complex RD: Senior-Løken syndrome (2), LCA in combination with intellectual disability (ID) (2) or deafness (1), and Usher syndrome type I (1) or II (1).

Genetic prescreening, Sanger sequencing, and WES were performed on each proband (first child affected in the family). Following the identification of a causal mutation, medical records were reassessed.

Genetic prescreening

Genomic DNA was extracted from leukocytes (Puregene DNA isolation kit, Gentra, Qiagen, Venlo, The Netherlands). Depending on the RD, probands underwent the following prescreening prior to this study: LCA: LCA Arrayed Primer EXTension (APEX) chip (Asper Ophthalmics, Tartu, Estonia), followed by Sanger sequencing of *CEP290*, *CRB1*, *RPE65*, *AIPL1*, *GUCY2D*, and *CRX*;¹⁴ ARRP: ARRP APEX chip; and ARCRD: ARRP or *ABCA4* APEX chip. In addition, the probands of F1, F13, and F15 were included in MPS of 16 LCA genes.⁷ Table 1 contains information on patient-specific prior genetic screening.

IBD mapping

Genome-wide IBD mapping was performed in 37 individuals using Affymetrix GeneChip Human Mapping 250K *Nsp* arrays (DNAVision, Charleroi, Belgium). Single-nucleotide polymorphism (SNP) genotypes were analyzed with an in-house Perl script and with PLINK.¹⁵ Resulting homozygous regions were evaluated for their size and number of SNPs. Regions were excluded if unaffected siblings were homozygous for the same genotype as the affected individuals. Of note, this may result in incorrect exclusion of regions in the case that the affected sibling shows an overlapping identity-by-state interval.

Sequencing of candidate genes and WES

At the onset of this study, 16 families underwent IBD-guided Sanger sequencing. Genes were prioritized based on their location in homozygous regions and association with AR RDs.² Primers were designed using Primer3Plus¹⁶ to cover all coding exons and intron–exon boundaries. Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Life Technologies Europe, Ghent, Belgium) on a 3730xl DNA Analyzer (Applied Biosystems).

Following the introduction of MPS technology, IBD-guided WES was performed in the probands of 10 additional families. Exome enrichment and sequencing were performed by Aros AB (Aarhus, Denmark) using the Illumina TruSeq Exome Enrichment Kit (Illumina, San Diego, CA) (F13–F16, F18, F20, F21) and the Nextera Rapid Capture Expanded Exome kit (F12, F17, F19), followed by paired-end sequencing on a HiSeq 2000 (2 × 100 cycles). For the TruSeq kit, six indexed samples were pooled and sequenced across two flow cell lanes. For the Nextera kit, 12 indexed samples were pooled and sequenced across four flow cell lanes.

The CLC Genomics Workbench v6.0.4 (CLC bio, Cambridge, MA) was employed for read mapping against the human genome reference sequence (NCBI, GRCh37), postmapping duplicate read removal, coverage analysis, and quality-based variant calling. All mutations were confirmed with Sanger sequencing. Novel sequence variants were evaluated using the Alamut mutation interpretation software v2.2.1 (Interactive Biosoftware, Rouen, France), Alamut HT v1.1.5 software, literature search, and segregation analysis in all available individuals.

Mutation nomenclature uses numbering with the A of the initiation codon ATG as +1 (<http://www.hgvs.org/mutnomen>), based on the following RefSeqs: NM_000350.2 (*ABCA4*), NM_001030311.2 (*CERKL*), NM_001042432.1 (*CLN3*), NM_020184.3 (*CNNM4*), NM_201253.2 (*CRB1*), NM_001029883.1 (*C2orf71*), NM_001142800.1 (*EYS*), NM_001023570.2 (*IQCB1*), NM_004744.3 (*LRAT*), NM_006343.2 (*MERTK*), NM_022787.3 (*NMNAT1*), NM_000283.3 (*PDE6B*), NM_152443.2 (*RDH12*), NM_020366.3 (*RPGRIP1*), and NM_206933.2 (*USH2A*).

Copy-number variation screening and deletion delineation

Long-range amplicons were amplified with the iProof High-Fidelity DNA Polymerase (Bio-Rad, Nazareth Eke, Belgium)

and evaluated on the HT DNA 5K LabChip GX kit (Caliper, PerkinElmer, Zaventem, Belgium; HT DNA 5K).

Multiplex ligation-dependent probe amplification was performed using the SALSA MLPA P328 EYS probemix (MRC Holland, Amsterdam, The Netherlands) following the manufacturer's protocols.

RESULTS

The PLINK software was used for the detection of homozygous regions equal to or above 1 Mb in size. Following IBD mapping, known RD genes² were prioritized for Sanger sequencing in 16 families, whereas 10 families underwent WES (**Figure 1**). This revealed causal mutations in known RD genes in 20/26 families (**Table 1**). **Supplementary Table S1** online contains in silico predictions for all missense mutations, whereas **Supplementary Figure S1** online shows segregation analysis. **Figures 2** and **3** contain a selection of interesting molecular and clinical findings, respectively. In the following sections, results are discussed based on the applied approach (IBD + Sanger sequencing or IBD + WES).

Combined IBD mapping and Sanger sequencing

LCA and EORD. Using IBD-guided Sanger sequencing, one LCA and one EORD family were resolved out of three families (1 EORD, 2 LCA) (**Figure 1**, **Table 1**). In the first family (F1, LCA), a novel homozygous nonsense mutation was identified in *RDH12*: c.912G>A (p.Trp304*).⁷ This mutation is located in the last exon and thus not expected to induce nonsense-mediated decay. The proband was diagnosed with LCA at the age of 2 years and 3 months based on severe visual

deficiency from birth, congenital nystagmus, hyperopia, and night blindness. An electroretinography performed at the age of 2 years and 7 months showed absent rod-specific and only residual cone-specific responses. Subsequent segregation analysis demonstrated that the apparently unaffected, younger brother of the proband (IV:3) is also homozygous (see **Supplementary Figure S1** online) and will develop a similar phenotype. In the second family (F2, EORD), the result of Sanger sequencing of *IMPG2*, which is located in the largest IBD region, was negative, whereas Sanger sequencing of *LRAT*, which is located in the region with the second-largest number of consecutive SNPs, identified homozygosity for a novel missense mutation, c.326G>T (p.Arg109Leu) (see **Supplementary Table S1** online) (**Figure 3**).

Autosomal recessive retinitis pigmentosa. The molecular defect was identified in 4/5 ARRP families using combined IBD mapping and Sanger sequencing (**Figure 1**). The causal mutation was located in the largest (F3, F5, F6) or second-largest (F4) IBD region (**Table 1**).

In families F3, F4, and F5, mutations in *RDH12* are responsible for the phenotype. The causal mutation in F3 and F4 is a known missense mutation (c.464C>T, p.Thr155Ile; see **Supplementary Table S1** online), shown to reduce the conversion of all-*trans* retinal to all-*trans* retinol.¹⁷ No family members were available for segregation testing in F4. In F5, a novel missense mutation was identified, c.803G>T (p.Cys268Phe), which is predicted to affect protein function by a selection of prediction programs (**Supplementary Table S1** online).

Figure 1 Flowchart of the study. Unusual clinical findings and molecular causes are indicated in bold. ARCRD, autosomal recessive cone-rod dystrophy; ARRP, autosomal recessive retinitis pigmentosa; EORD, early-onset retinal dystrophy; IBD, identity-by-descent; ID, intellectual disability; LCA, Leber congenital amaurosis; RD, retinal dystrophy; SLS, Senior-Løken syndrome; USH, Usher syndrome.

Figure 2 Unusual molecular and clinical findings revealed in this study. (a) Sanger sequencing of the *CNNM4* gene, located in the largest IBD region of F7, revealed a novel frameshift mutation. Based on these molecular data, dental clinical records were revisited, showing features typical for dentinogenesis imperfecta: yellowish discoloration and quality loss of enamel, especially at tips of teeth, as well as fang-like malformation of canines (VI:10, age 3 years 10 months). Fundus picture of the left eye of patient VI:2 age 15 years 10 months: note yellowish discoloration of macula, and outer retinal atrophy with intraretinal pigmentation in (mid) periphery; macula around area of central atrophy has better preserved retinal quality. (b) Delineation of the homozygous *PCDH15* deletion in the proband of F11. Amplicons for which PCR amplification failed are indicated in green. The deletion, containing exons 1 and 2 of the *PCDH15* gene, was refined by two subsequent rounds of PCR walking, resulting in an estimated deleted region of 163.5 to 165.7 kb. This region was amplified by long-range PCR (purple bar) and sequenced, delineating the 164kb deletion at the nucleotide level (green bar). (c) Combined IBD mapping and WES identified a novel nonsense mutation in the *CLN3* gene in the proband of F14 (IV:4), initially diagnosed with EORD. Revisiting of the clinical record revealed psychiatric symptoms compatible with Batten disease. Predictive testing of the youngest sister (IV:6) uncovered her disease status. Individuals who underwent IBD mapping are indicated with an asterisk. ARCRD, autosomal recessive cone-rod dystrophy; EORD, early-onset retinal dystrophy; IBD, identity-by-descent; PCR, polymerase chain reaction; USH, Usher syndrome; WES, whole-exome sequencing.

Figure 3 Clinical phenotype of five retinal dystrophy patients with an established molecular diagnosis. Proband of F2, VI:2 (*LRAT*), left eye: fundus picture of left eye of proband of F2 with EORD due to biallelic mutations in *LRAT*. Typical grayish patches of outer retinal atrophy in peripheral macula and retinal periphery with much better preserved macula with some degree of preretinal fibrosis but otherwise without intraretinal pigment migration, at age 17 years. Picture typical for phenotype of *LRAT*-related EORD. Proband of F9, II:1 (*IQCB1*), right eye: fundus picture of right eye of proband of F9 with SLS due to biallelic mutations in *IQCB1*. Peripheral grayish patches of outer retinal atrophy, with some minor intraretinal pigmentation, with relatively slightly better preserved macula, at age 17 years. Proband of F10, II:1 (*IQCB1*), right eye: fundus picture of right eye of proband of F10 with SLS due to biallelic mutations in *IQCB1*. Peripheral grayish patches of outer retinal atrophy, with spicular intraretinal pigmentation and narrowing of the retinal vasculature, with relatively much better preserved macula, at age 20 years. Proband of F10, II:1 (*IQCB1*), right eye: blue-light autofluorescence image of proband of F10 showing mild hyperautofluorescent central perimacular ring. Two affected brothers of F20, IV:3 and IV:6 (*CERKL*), right macula: intrafamilial phenotypical variability can be seen in pictures of right macula of proband of F20 (IV:3), with ARCRD due to biallelic *CERKL* mutations, showing relatively mild atrophy in the central macula, whereas the right macula of his older brother (IV:6) is completely atrophic, but for one minute central foveal island. ARCRD, autosomal recessive cone-rod dystrophy; EORD, early-onset retinal dystrophy; SLS, Senior-Loken syndrome.

Finally, a homozygous missense mutation in *USH2A* (c.12574C>T, p.Arg4192Cys; see **Supplementary Table S1** online) was detected in the proband of F6 following candidate gene screening of *RD3* and *USH2A*—both located in the largest IBD region. A different missense variant (c.12575G>A, p.Arg4192His) has previously been described at the same

amino acid position.¹⁸ However, the latter variant is predicted to be benign (data not shown).

Autosomal recessive cone-rod dystrophy. This study included three families with ARCRD (**Figure 1**). Combined IBD mapping and Sanger sequencing revealed disease-causing mutations in two families (F7 and F8).

In F7, sequencing of *MERTK* and *CNNM4*, located in the two largest IBD regions, identified a novel homozygous frameshift mutation in *CNNM4*, c.189del (p.Asp63Glufs*12). Because *CNNM4* mutations are associated with Jalili syndrome (OMIM 217080), characterized by CRD and amelogenesis imperfecta,^{19,20} dental records were revisited, and these confirmed amelogenesis imperfecta in all affected family members (**Figure 2a**).

The second family (F8) contained one sporadic CRD patient, who tested negative for the *ABCA4* chip (**Table 1**). However, the *ABCA4* gene was located in the region with the largest number of consecutive homozygous SNPs. Subsequent *ABCA4* Sanger sequencing revealed a novel homozygous frameshift mutation (c.3819dup, p.Leu1274Serfs*8).

Syndromic RDs. In total, patients from seven families diagnosed with a syndromic RD were enrolled. Six of them were subjected to Sanger sequencing following IBD mapping (**Figure 1**).

In two cases, known null mutations in *IQCB1* were found to underlie Senior-Loken syndrome (F9: c.1381C>T (p.Arg461*)²¹; F10: c.424_425del (p.Phe142Profs*5); (**Figure 3**).²¹ Interestingly, in F9, only the youngest (II:2) of the affected brothers had been diagnosed with Senior-Loken syndrome and ID prior to his recent death, suggesting either the involvement of modifying factors or an independent AR entity.

The proband of F11 suffered from RP and congenital, bilateral, and profound sensorineural hearing loss, and did not develop speech. The fifth-largest IBD region contained the *PCDH15* gene, which is known to be associated with Usher syndrome type 1 (*USH1F*, OMIM 602083). Sanger sequencing of all exons revealed an inability to amplify the first two exons of *PCDH15*, indicative of a homozygous deletion. **Figure 2b** shows the delineation of this deletion. First, polymerase chain reaction walking with 13 amplicons delineated the deletion to a region ranging from 139.2 to 167.0kb. A second round of refinement using 20 additional amplicons reduced this interval to 163.5–165.7kb, allowing long-range polymerase chain reaction and Sanger sequencing of the junction product. This revealed a homozygous deletion of 164kb with a 2-bp region of microhomology (AT) at the junction (chr10:g.56408521-g.56572561). Of note, the IBD mapping data contained a region of no calls overlapping with this region.

Combined IBD mapping and WES

Following IBD mapping, WES was performed in the probands of 10 families: 2 families with LCA (F12, F13), 2 families with EORD (F14, F15), 4 families with ARRP (F16–F19), 1 family with ARCRD (F20), and 1 family with LCA and ID (F21) (**Figure 1**). For each sample, at least 73% of unique reads were aligned against the human genome. An average coverage of

60–98× was achieved for the regions enriched by both exome kits. Between 87 and 94% of these regions had a read depth of at least 10× (see **Supplementary Table S2** online).

Variants were called heterozygous or homozygous in case of a variant allele frequency of 20–70% and ≥70%, respectively. Variant filtering was performed in two steps. First, homozygous variants located in major IBD regions or known RD genes were selected (see **Supplementary Table S3** online). Subsequently, variants were selected based on their predicted translation effect and in silico predicted effect on protein function using Alamut HT (see **Supplementary Table S4** online).

Variant filtering based on IBD regions. Variant filtering based on IBD regions revealed the causal genetic defect in five families. In the proband of F12, a sporadic patient with LCA, several small homozygous regions were identified, suggesting a distant common ancestor of both parents (see **Supplementary Table S3** online). In the largest IBD region, a novel homozygous nonsense mutation was identified in the *RPGRIP1* gene, c.2758C>T (p.Gln920*).

In F14 (EORD), a novel nonsense mutation was identified in the *CLN3* gene (c.784A>T, p.Lys262*) (**Figure 2c**). Mutations in *CLN3* are associated with Batten disease or juvenile neuronal ceroid lipofuscinosis (OMIM 204200). Patients most often present with progressive visual loss between the ages of 5 and 10, followed by loss of motor coordination, mental decline, and seizures, starting at the age of 10–12 and leading to premature death. The majority of *CLN3* mutations produce prematurely truncated products and cause “classic” juvenile neuronal ceroid lipofuscinosis. However, some mutations cause a protracted course of juvenile neuronal ceroid lipofuscinosis.²² The proband of F14 was referred for genetic screening for EORD at the age of 10, when neurologic symptoms were not yet evident. Revisiting of the clinical records uncovered psychiatric manifestations from the age of 12 that are in keeping with a diagnosis of Batten disease. Segregation analysis showed that the youngest sister of the proband, aged 5, is also homozygous for the mutation, revealing a presymptomatic molecular diagnosis (**Figure 2c**).

In the third family, F16 (ARRP), filtering of the exome variants with the largest IBD region revealed only two novel or rare missense variants predicted to affect protein function, one of which was c.2180G>A (p.Arg727Gln) in the *MERTK* gene (see **Supplementary Table S1** online). The *MERTK* Arg727 residue is known to be involved in a hydrogen bond with the hydroxyl group of the ligand compound 52 (ref. 23).

In the fourth family, F17 (ARRP), a homozygous frameshift mutation was identified in *C2orf71*, c.1764del (p.Glu589fs). Segregation analysis showed that both parents and the older sibling are heterozygous carriers of the *C2orf71* mutation, whereas the younger sister is also homozygous (see **Supplementary Figure S1** online).

In the fifth family, F18 (ARRP), previous Sanger sequencing of four RD genes (*LRAT*, *RPI*, *ADAM9*, *CNGA1*) located in the five largest IBD regions did not identify any causal mutations. Because of this, a broader selection of 13 IBD regions was made for exome filtering (see **Supplementary Table S3** online). In

the 11th IBD region, a homozygous indel mutation was identified in *PDE6B*, c.121_125delins15 (p.Pro41Glufs*128; see **Supplementary Table S4** online). The CLC software initially reported this mutation as c.126_127insAG (p.Asp43fs). No family members were available for segregation analysis.

Variant filtering based on known RD genes. Analysis of all variants located in the largest IBD regions identified in F13, F15, and F19–F20 did not reveal obvious causal mutations. In addition, despite presumed consanguinity, no large IBD regions were identified in the proband of F21. In these five families, variant filtering was performed against a list of all RetNet genes associated with both isolated as well as syndromic RDs.² Both homozygous and heterozygous variants were taken into account. Overall, this yielded on average 173 coding variants in the 209 genes per patient.

In the proband of F13 (LCA), 16 novel or rare missense changes were predicted to affect protein function by at least 2/4 in silico prediction programs (see **Supplementary Table S4** online). One of these was a known heterozygous missense mutation in *NMNAT1*, c.205A>G (p.Met69Val).²⁴ Subsequently, we evaluated *NMNAT1* variants in more detail and identified a second heterozygous missense mutation, c.769G>A (p.Glu257Lys), which is a known mutation not predicted to affect protein function (see **Supplementary Table S1** online).²⁴

In F15 (EORD), we identified a heterozygous known frameshift mutation in *EYS* (c.9368dup, p.Asn3123Lysfs*3).²⁵ Subsequent Sanger sequencing of exons with a coverage below 20× did not identify a second mutation. As several copy-number variations (CNV) have been described in *EYS*,²⁶ multiplex ligation-dependent probe amplification was performed. However, no CNV was detected.

The proband of F19 (ARRP) harbors several large IBD regions, suggesting close parental consanguinity (see **Supplementary Table S3** online). First, Sanger sequencing was performed for *MERTK*, *CEP290*, *FAM161A*, and *SNRNP200*, which are located in the two largest IBD regions. Because no mutations were found, the proband was subjected to WES. The application of the RetNet filter allowed the identification of the following homozygous missense mutation in the *USH2A* gene: c.10721G>T (p.Gly3574Val; see **Supplementary Table S1** online). This mutation, together with the known splice-site mutation c.3317-2A>G, has been described in a sporadic ARRP patient.⁹ Interestingly, it is located in a homozygous region of only 1.5 Mb, in which the *USH2A* gene is only partially located (see **Supplementary Figure S2** online).

In F20 (ARCRD), RetNet-based variant filtering revealed 170 variants resulting in an amino acid change. Of the six truncating variants, one was the known mutation c.847C>T (p.Arg283*)²⁷ in the *CERKL* gene, which is located in the 38th IBD region (**Figure 3**). Subsequent Sanger sequencing confirmed that the proband of F20 is homozygous for this *CERKL* mutation.

Variant filtering against the RetNet genes in the proband of F21 (LCA + ID) identified a novel homozygous frameshift mutation in *IQCB1* (c.1241_1242del, p.Leu414Hisfs*4). *IQCB1*

mutations have been associated with Senior-Loken syndrome²¹ as well as LCA.²⁸ When revisiting the patient's medical records, renal involvement with nephronophthisis was observed.

In conclusion, a variant-filtering strategy based on either IBD regions or RetNet genes allowed identification of causal mutations in 9/10 exomes.

DISCUSSION

The tremendous genetic and phenotypic heterogeneity of RDs poses a major challenge for establishing a molecular diagnosis. Here, we evaluated an integrated approach based on IBD-guided Sanger sequencing or WES to identify the causal gene in 26 consanguineous RD families.

The combination of IBD mapping and Sanger sequencing proved to be effective, as this strategy resulted in the identification of the causal mutation in 11/16 families (69%) (**Figure 1**). In 10/11 cases, the causal gene was located in the first- or second-largest homozygous region (**Table 1**). It has previously been observed that the causal gene resides in the largest homozygous region in about 17% of consanguineous families from inbred populations.²⁹ In the remaining group, however, gene-by-gene Sanger sequencing tends to be laborious and expensive.

In these patients, WES represents a more efficient way toward a molecular diagnosis. Indeed, using WES, we were able to identify causal genes located not only in high-ranked but also in markedly lower-ranked regions in four families (F18–F21, **Table 1**). Moreover, WES allowed the identification of a compound heterozygous genotype (F13), one of the pitfalls of IBD mapping. In total, WES revealed mutations in 9/10 families (**Figure 1**).

This study showed that combined IBD mapping and WES can lead to successful albeit challenging mutation discovery in a single individual of consanguineous origin, which is in agreement with a similar large-scale study in RD combining WES with IBD mapping¹² and two studies applying WES in RD.^{30,31} Given the large number of coding variants identified through WES, comprehensive variant filtering is of utmost importance. Variant filtering based on IBD regions or RetNet genes drastically reduced variant numbers (see **Supplementary Tables S4 and S5** online). Interestingly, our data demonstrated that RetNet gene-based filtering would have been superior to IBD filtering as a first-pass filter for several reasons. First, the RetNet filter generated markedly fewer variants to interpret, with an average of 173 heterozygous or homozygous coding variants per proband for the 209 RD genes assessed here—in contrast to up to 548 homozygous coding variants located in the IBD regions. Second, the RetNet filter allowed identification of a compound heterozygous genotype (F13) and mutations located in very small homozygous regions (F19–F21), which were not picked up by the IBD filter. Third, all mutations identified through the IBD filter (F12, F14, F16–F18) would have also been identified using the RetNet gene filter. These insights, together with efforts to apply IBD mapping directly to exome data,³² will eliminate the need for prior genome-wide SNP genotyping in future diagnostic WES applications in RD. However, for those families not having access to WES, IBD-guided targeted resequencing

of a selected number of genes using a small MPS benchtop sequencer will still be cost effective in consanguineous families.

One of the key findings of this study is the identification of mutations in syndromic RD genes that would not have been selected for routine first-pass screening based on the patient's initial diagnosis. Indeed, in syndromic RDs the retinal phenotype is often one of the first to arise, prior to other, later-onset, systemic manifestations. This is illustrated by the identification of a homozygous *CNNM4* frameshift mutation in a family initially referred to us with ARCD (F7, **Figure 2a**). It was only after review of clinical dental records that the presence of amelogenesis imperfecta was noticed, in agreement with Jalili syndrome.^{19,20} Second, the identification of a homozygous *CLN3* nonsense mutation in a proband initially diagnosed with EORD uncovered a link with emerging psychiatric and neurodegenerative manifestations with an important, unexpected impact on her clinical prognosis (F14, **Figure 2c**). Third, the identification of a truncating *IQCB1* mutation in a patient initially diagnosed with LCA and ID implies close renal follow-up. Of note, the detection of homozygous *RDH12*, *CLN3*, and *C2orf71* mutations in unaffected siblings of F1, F14, and F17, respectively, had important implications for clinical follow-up.

In addition to unexpected clinical and prognostic findings, our IBD-based approach revealed unusual molecular findings such as a large genomic rearrangement in *PCDH15* (**Figure 2b**). Besides a large IBD region encompassing this gene, the homozygous deletion appeared in the SNP data as a no call region. *PCDH15* large rearrangements are a known cause of Usher syndrome type 1. So far, six deletions with nonrecurrent breakpoints have been described, all removing at least one exon at the 5' end of the gene.^{33–35} It is presumed that the occurrence of distinct deletions in this region is related to the large size of *PCDH15*, in particular of the intronic regions between the first exons. This is in line with our findings, as bioinformatic analysis of the breakpoint regions suggests nonhomologous end joining or fork stalling and template switching as the most likely mechanisms for this novel deletion (data not shown). Although only a limited number of CNVs have been described in RDs, not surprisingly with low frequencies comparable to other single-base RD mutations,^{26,36} their occurrence warrants implementation of a CNV analysis algorithm in addition to IBD data analysis.

Overall, our approach revealed mutations in 20/26 (77%) families in the following 14 known RD genes: *ABCA4*, *CERKL*, *CLN3*, *CNNM4*, *C2orf71*, *IQCB1*, *LRAT*, *MERTK*, *NMNAT1*, *PCDH15*, *PDE6B*, *RDH12*, *RPGRIP1*, and *USH2A*. This overall mutation detection rate is in agreement with reported contributions of RD genes. In addition, the numbers of unique mutations and causal genes identified here illustrate the allelic and locus heterogeneity, respectively.

So far, the causal gene cannot be identified in six families. We anticipate these will likely be among “gene discovery” families, as known RD genes account for only 70–80% of cases. The combination of IBD mapping, WES, and recent variant filter tools specifically designed for RDs³⁷ provides a strong asset in gene identification in such families.¹² Of note, we cannot rule

out the presence of complex rearrangements and regulatory or deep-intronic mutations in known RD genes, taking into account the limitations of WES in detecting CNVs and enriching these regions, respectively. Whole-genome sequencing will offer a more suitable detection technique for these mutation mechanisms.³⁸

In conclusion, our study emphasized that IBD-guided mutation analysis and/or WES is a powerful tool for the molecular diagnosis of RD—even in a single individual. We unmasked unusual molecular and consequent clinical findings—the latter guiding future medical management and prognosis. Finally, elucidating *ABCA4*, *LRAT*, and *MERTK* mutations in three families offers potential gene-specific therapeutic perspectives.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

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DISCLOSURE

The authors declare no conflict of interest.

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