Phenotype Driven Analysis of Whole Genome Sequencing Identifies Deep Intronic Variants that Cause Retinal Dystrophies by Aberrant Exonization

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Purpose. To demonstrate the effectiveness of combining retinal phenotyping and focused variant filtering from genome sequencing (GS) in identifying deep intronic disease causing variants in inherited retinal dystrophies.

Methods. Affected members from three pedigrees with classical enhanced S-cone syndrome (ESCS; Pedigree 1), congenital stationary night blindness (CSNB; Pedigree 2), and achromatopsia (ACHM; Pedigree 3), respectively, underwent detailed ophthalmologic evaluation, optical coherence tomography, and electroretinography. The probands underwent panel-based genetic testing followed by GS analysis. Mini-gene constructs (NR2E3, GPR179 and CNGB3) and patient-derived cDNA experiments (NR2E3 and GPR179) were performed to assess the functional effect of the deep intronic variants.

Results. The electrophysiological findings confirmed the clinical diagnosis of ESCS, CSNB, and ACHM in the respective pedigrees. Panel-based testing revealed heterozygous pathogenic variants in NR2E3 (NM_014249.3; c.119-2A>C; Pedigree 1) and CNGB3 (NM_019098.4; c.1148delC/p.Thr383Ilefs*13; Pedigree 3). The GS revealed heterozygous deep intrinsic variants in Pedigrees 1 (NR2E3; c.1100+1124G>A) and 3 (CNGB3; c.852+4751A>T), and a homozygous GPR179 variant in Pedigree 2 (NM_001004334.3; c.903+343G>A). The identified variants segregated with the phenotype in all pedigrees. All deep intrinsic variants were predicted to generate a splice acceptor gain causing aberrant exonization in NR2E3 [89 base pairs (bp)], GPR179 (197 bp), and CNGB3 (73 bp); splicing defects were validated through patient-derived cDNA experiments and/or minigene constructs and rescued by antisense oligonucleotide treatment.

Conclusions. Deep intrinsic mutations contribute to missing heritability in retinal dystrophies. Combining results from phenotype-directed gene panel testing, GS, and in silico splice prediction tools can help identify these difficult-to-detect pathogenic deep intrinsic variants.

Keywords: NR2E3, GPR179, CNGB3, RNA splice sites, introns, deep intrinsic variant, antisense oligonucleotide, enhanced S-cone syndrome, congenital stationary night blindness, color vision defects, achromatopsia, retinal dystrophies

Inherited retinal dystrophies (IRDs) are a leading cause of childhood blindness for which over 250 genes have been identified (RetNet, https://sph.uth.edu/retnet/). As we have entered the era of gene-specific patient management, there is increased incentive to clarify the genetic diagnosis of patients. Panel-based next generation sequencing (NGS) techniques primarily focus on detecting exonic and canonical splicing variants with a mutation detection rate of 60% to 70%. The mutation detection rate is different among various clinical classes of IRDs; it exceeds 90% in single-gene disorders (e.g., RS1 and CHM) and some well-defined forms of IRDs (e.g., achromatopsia [ACHM] and congenital stationary night blindness [CSNB]), but it is much lower in sporadic retinitis pigmentosa (<60%) and central...
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areolar choroidal dystrophy (35%). In a growing number of unsolved IRD cases it is becoming clear that the missing heritability is found in the intronic regions of known genes, such as ABCA4, CEP290, USH2A, CLRN1, CNGB3 and CAGNA1F. These in instances, a distinct phenotype or the presence of a heterozygous disease-causing variant in a known autosomal recessive (AR) gene initiated a comprehensive analysis of the noncoding regions of the gene of interest to uncover novel deep-intronic disease causing variants.

Intronic variants have the potential to result in mis-splicing pre-mRNA and generate an aberrant transcript that could be disease causing in some IRD cases. This can occur where cis-splicing motif sequences are gained or cryptic splice sites in an intron are activated. Common splice sites include 5′ donor sites, 3′ acceptor sites, branch point and polypyrimidine tract sequences, and splicing silencer or enhancer regions. Although most of these intronic variants are well tolerated, some of them have the potential to alter correct splicing through the creation or disruption of motifs read by the spliceosome. This can create a frameshift or a premature termination codon that can lead to nonsense mediated decay.

In this study, we combined phenotyping with NGS-based analysis of the noncoding intronic regions of genes of interest to uncover pathogenic deep intronic variants in three AR IRDs, enhanced S-cone syndrome ([ESCS; NR2E3] (OMIM: 604485)), complete CSNB ([cCSNB; GPR179] [OMIM: 614515]), and ACHM ([CNGB3] [OMIM: 605080]). Functional validation of the intronic variants were performed using patient-derived cDNA analysis and/or minigene constructs incorporating the deep intronic variants and antisense oligonucleotide rescue treatments.

**Materials and Methods**

**Patient-related**

This project was approved by the Ethics Review Board at the Hospital for Sick Children, Toronto, and met the Tenets of the Declaration of Helsinki. Family members across three pedigrees were recruited at the Hospital for Sick Children after obtaining informed consent; this included three members from Pedigree 1 (ESCS; proband and unaffected parents; Fig. 1A), seven members from Pedigree 2 (cCSNB; proband, four unaffected siblings and unaffected parents; Fig. 2A) and five members from Pedigree 3 (ACHM; proband, two affected siblings and unaffected parents; Fig. 3A). The ocular phenotype was defined in all affected individuals by assessing various parameters of visual function and structure ([best corrected visual acuity (BCVA), color (Hardy Rand Rittler Charts) and contrast vision, fundus photography, spectral-domain optical coherence tomography (SD-OCT; Zeiss Cirrus (Pedigrees 1 and 2) or Heidelberg Spectralis (Pedigree 3)) and an International Society for Clinical Electrophysiology of Vision standard full-field electroretinogram (ERG)]). All unaffected members in Pedigree 2 (parents and siblings) underwent eye examinations including ERG. DNA was extracted from all participants for segregation analysis. All probands, as well as affected siblings in Pedigree 3, underwent candidate gene panel testing through the clinic (details in Supplementary Table S1). Fibroblast or lymphoblast cell lines were established from each proband for RNA extraction.

**Genome Sequencing and Variant Analysis**

Genome sequencing (GS) was performed in five subjects at The Center for Applied Genomics (TCAG) as previously published. Briefly, paired-end (2 × 150 base pairs [bp]) sequencing was performed on the three probands, one unaffected sibling in Pedigree 2 (II-3, Fig. 2A) and one affected sibling in Pedigree 3 (II-2, Fig. 3A) using Illumina Hi-Seq X platform and the reads were mapped to the GRCh37 reference sequence using bwa-mem v0.7.12. Duplicate reads were removed using MarkDuplicates Picard v2.5.0 ([https://broadinstitute.github.io/picard/]). Local read realignment and variant calling were accomplished using GATK v3.7.0, and calls were annotated using ANNOVAR.

Mobile transposable element (TE) insertion analysis was performed with Mobster v0.2.4.1. Copy number variants were called using ERDS v1.1 and CNVnator v0.3.2 using a window size of 500 bp.

From the pedigree information and clinical diagnoses, an AR mode of inheritance was assumed in each family. Variant analyses were performed using both candidate gene panel and genome wide approaches utilizing previously published customized pipelines, and included multiple in silico tools and control population databases. Prioritization of variants was established by variant frequency, gene relevance, pathogenicity scores, and segregation analysis. Supplementary Figures S1 to S3 details the candidate gene filtering approach, genome wide filtering approach, and variant prioritization in each pedigree. Segregation analysis was performed using the primer pairs 2, 9 and 13 for NR2E3, GPR179 and CNGB3, respectively (Supplementary Tables S2–S4).

**In Silico Splice Prediction of Intronic Variants**

The gain or loss of potential donor or acceptor sites in a 200 bp window around candidate variant sites were tested in silico using four splice prediction algorithms (Splice-SiteFinder, MaxEntScan, NNSPLICE, and GeneSplicer) implemented through Alamut Visual Software ([https://www.interactive-biosoftware.com/alamut-visual/]). Scores that met preset thresholds in three or more algorithms were flagged as significant (Table 1). SpliceAI (Illumina) prediction software was also used to assess aberrant splicing of rare intronic variants in candidate genes.

**Splicing Reporter Minigene Constructs, HEK293T Cell Transfection and RT-PCR**

NR2E3 and GPR179 minigene constructs were developed using similar techniques as previously described. The respective proband’s genomic DNA was amplified by polymerase chain reaction (PCR) using the primer pairs 1 (NR2E3) and 8 (GPR179) (Supplementary Tables S2 and S3). The primer pairs were located in the region complementary to introns 6 and 8 for NR2E3, and introns 1 and 4 for GPR179. The 5′ end of the forward and reverse primers were designed to include a HindIII site and Sall site, respectively, to facilitate plasmid cloning by restriction enzyme digest. Subsequent PCR amplification product was purified using QIAEX II Gel Extraction Kit. The purified DNA amplicons (3 µg) were double digested using HindIII-HF, Sall-HF and 10X CutSmart Buffer according to manufacturer’s instructions (New England Biolabs Cloner); the pcGFP-N1 plasmid vector was also double digested separately using identical protocol.
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FIGURE 1. Segregation of the NR2E3 variants and the functional validation of the deep intronic variant (c.1100+1124G>A) in Pedigree 1. (A) Pedigree structure and segregation results for NR2E3. (B) A schematic representation of NR2E3 and the location of the deep intronic variant (c.1100+1124G>A) in the top panel; bottom panel schema includes minigene construct, sequences around gained splice sites, the ASO sequence and its binding site. (C) RT-PCR of HEK293T cells transfected with minigenes: reference construct (NR2E3:EMG:REF; c.1100+1124:G) displayed product 1 [409 bp, reference transcript], the variant construct (NR2E3:EMG:MT; c.1100+1124:A) displayed a larger product 2 (501 bp, aberrant transcript), and after ASO treatment (NR2E3:EMG:MT+ASO_NR2E3), there was diminished amount of product 2. Green boxes denote reference transcripts and ASO-recovered reference transcripts. Red boxes denote aberrant transcripts. (D) RT-PCR results from control and patient-derived fibroblast cell lines with and without transfection of ASO_NR2E3. Control fibroblasts demonstrated product A (178 bp, reference transcript) and a nonspecific product F. Patient-derived fibroblasts demonstrated four distinct bands (products A, B, D and E); product A represents reference transcript as a result of leakage of the two splice mutations in the proband, products D (345 bp) and E (524 bp) are two aberrant splice products as a result of the deep intronic variant, and product B (219 bp) is likely an alternatively misspliced transcript. Treatment with ASO_NR2E3 in the patient fibroblasts reduced the relative levels of products D and E (aberrant splice products) and increased the level of normal transcript (product A); it is noted that a new aberrantly spliced product using the deep intronic splice acceptor gain site (product C, 268 bp) was observed. Green boxes denote reference transcripts. Red boxes denote aberrant transcripts. (E) Schematic representation of the cDNA products obtained in C and D. AS, acceptor site; BP, branch point sequence; CMV, cytomegalovirus promoter; DS, donor site; EGFP, enhanced green fluorescent protein; NeoR/KanR, neomycin/kanamycin resistance; PPT, polypyrimidine tract; PS/2′OMe, phosphorothioate/2′O Methyl.

TABLE 1. In Silico Splice Predictions

| Variant  | Acceptor Site Gain (gene; transcript; position) | SSF [0-100] | MaxEnt [0-16] | NNSPLICE [0-1] | GeneSplicer [0-15] | SpliceAI [0-1] | Donor Site Gain (SpliceAI) |
|----------|-----------------------------------------------|-------------|--------------|----------------|-------------------|---------------|---------------------------|
| NR2E3    | c.1100+1124G>A | Position: c.1100+1126 | 78.36 | 7.47 | 0.88 | 1.67 | 0.63 | Position: c.1100+1214 |
| GPR179   | NM_001004334.3; c.903+343G>A; g.15:36494957C>T | Position: c.903+345 | 90.61 | 12.24 | 0.97 | 3.07 | 0.86 | Position: c.903+542 |
| CNGB3    | Transcript NM_019098.4; c.852+4751A>T; g.8:87674402T-A | Position: c.852+4756 | 83.92 | 8.08 | - | 5.97 | 0.60 | Position: c.852+4829 |

In silico splice prediction scores using Alamut Visual Software included SSF, MaxEnt, NNSPLICE, and GeneSplicer flagged these deep intronic mutations as pathogenic acceptor gain mutations when greater than the default threshold values (SSF ≥ 70, MaxEnt ≥ 0, NNSPLICE ≥ 0.4, GeneSplicer ≥ 0, SpliceAI ≥ 0.5). SpliceAI (Illumina) also flagged these mutations as a pathogenic acceptor gain score. These scores also provided predicted aberrant exonization start and end positions. Genomic coordinates refer to the build GRCh37/hg19 for GPR179 and CNGB3, and GRCh38/hg38 for NR2E3.
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FIGURE 2. Segregation analysis in Pedigree 2 and the functional validation of the GPR179 deep intronic variant (c.903+343G>A). (A) Pedigree structure and segregation results for the GPR179 variant. (B) Top panel depicts a schematic representation of GPR179 and the location of the deep intronic variant (c.903+343G>A); bottom panel schema includes minigene construct, sequences around gained splice sites, and the ASO sequence and its binding site. (C) RT-PCR of HEK293T cells transfected with minigene reference construct (GPR179:EMG:REF; (c.903+343:G)) displayed two products (A and B, 108 and 196 bp, respectively, reference transcripts) and the variant construct (GPR179:EMG:MT; c.903+343:A) displayed two larger splice products that used the novel splice acceptor site (C and D, 305 and 393 bp, respectively, aberrant transcripts); following ASO treatment (GPR179:EMG:MT + ASOGPR179) there was relative reduction in the levels of product C, retained product D and rescue of product A. Green boxes denote reference transcripts. Red boxes denote aberrant transcripts. (D) RT-PCR results from control and patient-derived lymphoblast cell lines with and without transfection of ASOGPR179. Control lymphoblasts demonstrated product B (reference transcript); patient-derived lymphoblasts demonstrated product D (aberrant transcript) and on treatment with ASOGPR179 product D was still detected, but there was recovery of reference transcript (product B). Green boxes denote reference transcripts. Red boxes denote aberrant transcripts. (E) Schematic representation of the cDNA products obtained in C and D. A5, acceptor site; BP, branch point sequence; CMV, cytomegalovirus promoter; DS, donor site; EGFP, enhanced green fluorescent protein; NeoR/KanR, neomycin/kanamycin resistance; PPT, polypyrimidine tract; PS/2′OMe, phosphorothioate/2′O methyl.

T4 DNA Ligase was used to deliver the amplicon into the vector pEGFP-N1 backbone containing a cytomegalovirus promoter (Clontech, Takara, Figs. 1B, 2B) in a 3:1 molar ratio of insert to vector. The ligated product was transformed into One Shot TOP10 chemically competent Escherichia coli (ThermoFisher Scientific, Waltham, MA, USA). Kanamycin was used for clone selection; selected clones were grown in 5ml LB medium containing 250 μg of kanamycin and incubated at 37° C and 225 rpm for 24 hours. The plasmid DNA from each incubated colony was purified using the QIAprep Spin Miniprep kit. On Sanger sequencing, the minigene clones were confirmed to only include mutant constructs. The constructs were labeled NR2E3:EMG:MT and GPR179:EMG:MT, respectively.

To validate the aberrant splicing of the CNGB3 variant, a 962bp DNA fragment (gBlock) was designed and synthesized by GeneArt Strings (ThermoFisher Scientific, Waltham, MA, USA). This fragment contained exon 6 with flanking introns, connected to a unit of intron 6 containing the deep intronic variant with flanking intronic regions (169 bp and 131 bp, respectively), which connects to exon 7 with flanking introns (refer to Fig. 3B for details). A DNA fragment containing the normal reference nucleotide at the intronic variant site and another containing the mutant intronic variant were synthesized (Supplementary Table S5). The reference and variant design sequences were tested using
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FIGURE 3. Segregation analysis in Pedigree 3 and the functional validation of the CNGB3 deep intronic variant (c.852+4751A>T). (A) Pedigree structure and segregation analysis for the CNGB3 variants. (B) Top panel depicts a schematic representation of CNGB3 and the location of the deep intronic variant (c.852+4751A>T); bottom panel schema includes minigene construct, sequences around gained splice sites, ASO sequence and its binding site. (C) RT-PCR of HEK293T cells transfected with minigene reference construct (CNGB3:EMG:REF) displayed product A (182 bp, reference transcript), and the variant construct (CNGB3:EMG:MT) displayed products A (reference transcript due to leakage), B (409 bp, aberrant transcript), and C (684 bp, unspliced minigene); following ASO treatment (CNGB3:EMG:MT + ASOCNGB3), there was abrogation of the aberrant transcript (product B) with presence of reference transcript (product A). (D) Schematic representation of the cDNA products obtained in C. AmpR, ampicillin resistance; AS, acceptor site; PPT, polypyrrimidine tract; PS/2′OMe, phosphorothioate/2′O Methyl; T7, T7 promoter.

all of the splice prediction algorithms (described earlier) to ensure no new splice sites were created or destroyed. The DNA product was then blunt-end cloned using CloneJET PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA) and subsequently transformed into One Shot TOP10 chemically competent E. coli (Thermo Fisher Scientific). Ampicillin was used for clone selection; selected clones were grown in 5 mL LB medium containing 250 μg of ampicillin and incubated at 37°C and 225 rpm for 24 hours. Randomly selected colonies for the reference and mutant constructs underwent plasmid DNA purification using the QIAPrep Spin Miniprep Kit. DNA plasmid sequencing identity and fidelity was confirmed on Sanger sequencing. The reference and mutant minigene constructs (5695 bp long) were labeled CNGB3:EMG:REF and CNGB3:EMG:MT, respectively. Primers used for clone validation are listed in Supplementary Table S4. Similar modified minigene designs using shortened introns have been published.8,39

HEK293T cells were cultured in DMEM (Gibco, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS) added. For HEK293T cell transfection with clones of minigene constructs, 2.5 μg of the vector was added in a mixture of 250 μL Opti-MEM serum-free medium (Gibco, Gaithersburg, MD, USA) and 7.5 μL X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) and incubated for 25 minutes at room temperature to form a stable complex; this was then pipetted into a 6-well plate and incubated at 37°C for 72 hours. Similar techniques were used for all six minigene constructs (one reference and mutant construct per gene).

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The cDNA synthesis was conducted with Superscript IV One-Step RT-PCR System (Invitrogen) using equivalent RNA amounts (40ng each) in a 20μL reaction. Primer pairs 3, 10, and 16 were used for minigene splice detection for NR2E3, GPR179, and CNGB3, respectively (listed in Supplementary Tables S2–S4).

Patient-derived Cell Culture Experiments

Patient-derived fibroblasts (NR2E3) were cultured in AMEM (Wisent Bio Products) with 10% FBS added. Patient-derived
Table 2. Phenotype Summary of All the Cases

| Pedigree 1 (Proband) | Pedigree 2 (Proband) | Pedigree 3 (Proband) | Pedigree 3 (II-2) | Pedigree 3 (II-3) |
|----------------------|----------------------|----------------------|------------------|------------------|
| **Gene (Variants)**  | NR2E3: c.[119-2A>C]; [1100+124G>A] | GPR179: c.[903+343G>A]; [903+343G>A] | CNGB3: c.[11486delC]; [852+4751 A>T] | NA |
| **Symptoms**         | Nyctalopia at 3 years | Right exotropia – infancy | Nystagmus – infancy | Nystagmus – infancy |
|                      |                      |                      | Photophobia - infancy | Photophobia - infancy |
| **Age at Recent visit** | 17 years | 17 years | 23 years | 19 years |
| **Visual Acuity (Right Eye : Left Eye)** | 20/25: 20/25 | 20/70: 20/40 | 20/200: 20/200 | 20/160: 20/200 |
| **Contrast Sensitivity** | 1.50: 1.50 | 1.65:165 normal | 0.75:0.60 Strong RG & BY defect | 0.75:0.60 Strong RG & BY defect |
| **Color vision**     | Moderate RG defect | 0.75:0.60 Strong RG & BY defect | 0.75:0.60 Strong RG & BY defect | 0.75:0.60 Strong RG & moderate BY defect |
| **Refractive error** | +0.75+0.50 × 10: | −7.75+1.50 × 108: | −10.50/−4.00 × 105: | −11.00 /−11.00 |
| **(Right Eye : Left Eye)** | +1.50 white dots ST arcade | −7.75+1.50 × 70: | −10.50/−4.00 × 75: | −8.00/+1.00 × 70: |
| **Retinal exam (Both Eyes)** | Tilted disc; tessellated background; dull FR | Subtle foveal hyper AF | Subtle foveal hyper AF | Subtle foveal hyper AF |
| **Fundus Autofluorescence** | Hypo AF along ST arcade; hyper AF dots periphery | NA | Foveal hyper-AF | Foveolar hypo AF; parafoveal hyper AF |
| **Optical Coherence Tomography (Both Eyes)** | Normal CRT; ONL and OPL rosettes with thick and disorganized retina in mid-periphery | Normal CRT and lamination | Grade 1 FH; Mild disruption of OS in central sub-foveal region | Grade 1 FH; Mild disruption of OS in central sub-foveal region |
|                      |                      |                      | Grade 1 FH; Mild disruption of OS in central sub-foveal region | Grade 1 FH; Mild disruption of OS in central sub-foveal region |

AF, autofluorescence; BY, blue-yellow; CRT, central retinal thickness; FH, foveal hypoplasia; FR, foveal reflex; NA, not available; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments; RG, red-green; ST, superotemporal.

lymphoblasts (GPR179 and CNGB3) were cultured with RPM1 1640 medium (Gibco). For NR2E3, a nested PCR approach was used with 120ng of cDNA as the ectopic expression of this gene is low in fibroblasts (Primer pairs 6 and 7 in Supplementary Table S2; PCR conditions available on request). For GPR179, 200ng cDNA was used for reverse transcriptase (RT)-PCR in an AmpliTaq Gold 360 Master Mix (primer pair 10 in Supplementary Table S3; PCR conditions available on request).

Antisense Oligonucleotides Design and Rescue Experiments

Antisense oligonucleotides (ASO) were designed as a 20 bp sequence that would be complementary with a sequence that overlaps the deep intronic variant of interest in NR2E3, GPR179, and CNGB3, thereby acting as steric spliceosome inhibitors. A phosphorothioate (PS) backbone linkage was chosen so as to increase nuclease resistance and half-life. Each nucleotide ribose in the primer sequence was designed (Integrated DNA Technologies, Iowa) to contain a 2′-O-Me modification to prevent RNaseH pathway degradation of the transcript. ASOs used in this study were primers 5, 12, and 14 (Supplementary Tables S2–S4).

HEK293T cells were co-transfected with mutant minigene constructs (2.5 μg) and ASO (final culture concentration of 200nM). For transfection of ASOs in patient-derived fibroblasts or lymphoblasts, appropriate volume of ASO was added to reach a final concentration of 5 μmol/L. Transfection and RT-PCR were performed as previously described in earlier sections of the methods.

Electrophoresis and Imaging Techniques

The splicing assays for NR2E3 and CNGB3 (patient derived cDNA and/or minigene RT-PCR) were performed by agarose gel electrophoresis (1.5%) and imaged using ChemiDoc Imaging system (Bio-Rad, Hercules, CA, USA). The splicing assays for GPR179 were performed using high-resolution capillary electrophoresis where a digital image was generated (QIAxcel Advanced system; Qiagen).

RESULTS

Pedigree 1 (Enhanced S-Cone Syndrome)

Clinical Phenotype and Genetic Results. The proband, a male child of North European ancestry, had complaints of night blindness since three years of age. At 11 years, his BCVA was 20/25 in both eyes and had a strong red-green color deficit (Table 2). Retinal examination showed a few faint white dots along the superotemporal arcades but was otherwise unremarkable (Fig. 4A). The SD-OCT showed no evidence of foveal schisis, but there were retinal
rosettes in the outer nuclear and plexiform layers outside the macula; these regions showed retinal thickening and disorganization of middle and inner retinal layers (Fig. 4B). The ERG showed pathognomonic features of ESCS12 (nondetectable dim-light scotopic response; simplified, delayed and reduced scotopic response to a standard flash that was largely similar in morphology to the photopic standard flash response; and a markedly reduced photopic flicker response; Fig. 4D); control traces are shown in Figure 4E. At 13 years, wide-field autofluorescence (AF) revealed atrophy along the superotemporal arcades, and there were hyper-autofluorescent dots in the periphery (Fig. 4C). Panel-based testing identified a heterozygous previously reported NR2E3 mutation (c.119-2A>C) leading to skipping of exon 214,43; no variants were found in NRL. No copy number variations (CNV) were found when screening NR2E3.

GS analysis of the candidate genes revealed two variants in NR2E3; the previously identified pathogenic splice variant (c.119-2A>C) and a novel deep intronic variant in intron 7 (chr15: 71815241:G>A; c.1100+1124G>A;
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NM_014249.3; GRC chimpanzee/hg38). The deep intronic variant was not observed in any GS control databases (gnomAD (https://gnomad.broadinstitute.org/); BRAVO (https://bravo.sph.umich.edu/freez5/hg38/)). All in silico tools (Table 1) strongly predicted the gain of a splice acceptor site 2 bp downstream (c.1100+1124G); Sanger sequencing confirmed product 2 to have incorporated the aberrant exon of 89 bp (Figs. 1C, 1E), which is strongly predicted to incorporate a novel aberrant 41 bp exon 581 bp downstream (c.1100+1124A), and SpliceAI predicted an aberrant exon of 89 bp with a specific start and stop site. Genotyping showed that the variants segregated with the disease phenotype (Fig. 1A). A detailed analysis of GS did not reveal any other candidate gene with biallelic exonic or canonical splice variants (Supplementary Fig. S1).

**Splicing Reporter Minigene RT-PCR and ASO Rescue.** HEK293T cells were transfected with the NR2E3:EMG:REF (reference sequence, c.1100+1124G), NR2E3:EMG:MT (variant sequence, c.1100+1124A), and NR2E3:EMG:MT + ASONR2E3. After RT-PCR, the reference clone (c.1100+1124G) displayed a distinct favored band (product 1; 409 bp) whereas the variant clone (c.1100+1124A) displayed a larger band (product 2; 501 bp); Sanger sequencing confirmed product 2 to have incorporated the aberrant exon of 89 bp (Figs. 1C, 1E). The aberrant exon size and the start sites were as predicted by SpliceAI (Table 1; Fig. 1E). The variant clone cotransfected with ASO NR2E3 resulted in diminished amounts of mutant mRNA (product 2), although no increase in reference transcript (product 1) was visible (Fig. 1C). HEK293T cells with no transfection displayed no bands because they do not express NR2E3. It is noted that both reference and variant constructs used a different donor site at exon 7 boundary than predicted (Fig. 1E).

** Fibroblast RT-PCR and ASO Rescue.** In control fibroblasts and ASO NR2E3 transfected control fibroblasts product A (178 bp; Fig. 1D) was detected; Sanger sequencing confirmed normal splicing of exon 7 and exon 8. In patient fibroblasts compound heterozygous for c.1100+1124G>A and c.119-2A>C variants, four different transcripts [products A (178 bp), B (219 bp), D (345 bp) and E (524 bp)] were identified (Fig. 1D). Sanger sequencing confirmed product A to be reference transcript, and products D and E to contain the 89 bp aberrant exon as the intronic variant acted as an acceptor site. On translation, a stop codon is introduced after six amino acids into the aberrant exon. The product A present in patient fibroblasts reflects limited leakage of reference transcripts as a result of the two splice variants (c.119-2A>C and c.1100+1124G>A). Product B was determined to incorporate a novel aberrant 41 bp exon 581 bp downstream of the deep intronic variant (Figs. 1D, 1E), which is likely an alternate mis-spliced transcript. The ASO NR2E3 transfected patient fibroblasts displayed an absence or decrease of two of the aberrantly exonized bands (loss of product E, decrease of product D) and a relative increase in reference band signal (product A) compared with the nontransfection condition (Fig. 1D). It is noted that ASO NR2E3 transfected patient fibroblasts additionally displayed product C (268 bp), which was absent under nontransfection condition, and on Sanger sequencing confirmed an aberrant exonization.

**Pedigree 2 (Complete Congenital Stationary Night Blindness)**

**Clinical Phenotype and Genetic Results.** The proband is part of a sibship of five, born to consanguineous parents of Sudanese ancestry. The child had right exotropia since infancy, surgically corrected at 10 years of age. Night blindness was first noted at 11 years of age, and his BCVA was 20/60 and 20/25 in the right and left eyes, respectively. He had high myopia and related fundus changes (Table 2; Figs. 5A, 5B). The SD-OCT was normal (Figs. 5C, 5D), ERG was consistent with selective ON-bipolar dysfunction (Fig. 5E; control traces are shown in Fig. 5F), and the clinical phenotype was consistent with the diagnosis of cCSNB. Both parents and all unaffected siblings had a normal eye exam including an ERG. Panel-based testing for cSNB (14 genes) in the proband did not identify any pathogenic variants.

**GS Analysis of the six candidate genes known to cause cCSNB identified a homozygous intron 2 variant in GPR179 (chr17:36494957:C>T; c.903+343G>A; NM_001004334.3; GRCh37/hg19), flagged by all in silico tools as a gain of function splice acceptor site 2 bp downstream (Table 1). SpliceAI annotation predicted an aberrant exon of 197 bp size with a start site at c.903+345 and a stop site at c.903+542 (Table 1; Figs. 2B, 2E).** In gnomAD, this variant was recorded once in a heterozygous state (allele frequency of 3.187 × 10⁻⁵) and never in a homozygous state. The variant segregated with the disease phenotype in the family (Fig. 2A). Supplementary Figure S2 lists two genes that carried homozygous coding variants that segregated with the disease but had no known human disease association and, hence, were not considered for further investigation.

**Splicing Reporter Minigene RT-PCR and ASO Rescue.** HEK293T cells were transfected with the following: GPR179:EMG:REF (reference sequence, c.903+343G), GPR179:EMG:MT (variant sequence, c.903+343A), and GPR179:EMG:MT + ASO GPR179. - RT-PCR of the reference clone (c.903+343G) displayed two bands [products B (196 bp) and A (108 bp)] which upon Sanger sequencing confirmed to contain spliced exon 2, 3, and 4 or exon 2 and 4, respectively (Fig. 2C). The variant clone (c.903+343A) displayed two distinct bands [product C (305 bp) and D (393 bp)]; Sanger sequencing confirmed both bands to incorporate the 197 bp aberrant exon as predicted by SpliceAI (Figs. 2C, 2E; Table 1). The variant clone cotransfected with ASO GPR179 showed an abrogation in one of the two aberrant transcripts (abrogated product C but retained product D) and a gain in the reference transcript (product A; Fig. 2C). HEK293T cells with no transfection displayed no bands because GPR179 expression levels are extremely low in non-neuronal tissue types.

**Lymphoblast RT-PCR and ASO Rescue.** In control lymphoblasts and ASO GPR179 transfected control lymphoblasts, normal splicing of exon 2, 3, and 4 was confirmed on Sanger sequencing [Product B (196 bp); Fig. 2D]. In patient’s lymphoblasts homozygous for c.903+343G>A, a different transcript (Product D [393 bp]; Fig. 2D) was identified; on Sanger sequencing this transcript was confirmed to have the 197 bp aberrant exon that introduced 44 amino acids and a premature stop. Hence, the deep intronic variant acted as an acceptor site for the existing exon 2 donor site. The reference transcript (product B) was not detected in the patient’s lymphoblasts but did appear upon ASO GPR179 transfection (Fig. 2D). A schematic for the observed transcripts is depicted in Figure 2E.

**Pedigree 3 (Complete Achromatopsia)**

**Clinical Phenotype and Genetic Results.** The proband, currently a 25-year-old male of Caucasian origin,
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FIGURE 5. Clinical phenotype of Pedigree 2 proband. (A, B) Fundus images of the right and left eyes, respectively. Both eyes show tilted discs and temporal crescents, and the background retina is tessellated. (C, D) SD-OCT horizontal line scan across the center of the macula showed relatively preserved retinal thickness and layering. (E) ERG showed non-detectable response to dark adapted (DA) 0.002 cd.s.m\(^{-2}\) stimulus; DA 0.01 ERG showed markedly reduced b-wave amplitude. The DA 3.0 and DA 10.0 responses showed borderline a-wave amplitudes and electronegative configuration consistent with generalized rod ON-bipolar dysfunction. The light adapted (LA) 3.0 2Hz ERG showed broadened trough of the a-wave suggesting selective involvement of cone ON-bipolar system and the 30-Hz flicker responses showed low-normal amplitudes. (F) ERG responses from a control subject.

had nystagmus and photophobia since infancy. His BCVA has remained stable over time and was 20/200 in each eye at the most recent visit. He had high myopia and a strong color vision defect (Table 2). Fundus showed macular atrophy and AF showed foveal hyper auto-fluorescence (Table 2; Figs. 6A, 6B). The SD-OCT showed bilateral grade 1 foveal hypoplasia and mild disruption of central macular photoreceptor outer segments (Table 2; Fig. 6C). The ERG results were consistent with complete ACHM (Fig. 6J); control traces are shown in Figure 6M.
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FIGURE 6.

Detailed clinical phenotype in Pedigree 3. (A, D, G) The fundus image from one eye in each affected. The fundus in the proband (A) showed macular atrophy, whereas, both siblings showed tilted disc, peri-papillary atrophy and foveal hypoplasia (D and G). (B, E, H) Fundus autofluorescence (AF) images from the corresponding eye in each affected. The proband (B) showed marked foveal hyper-AF; II-2 showed subtle para-foveal hyper-AF; II-3 also showed subtle foveal hyper-AF (H). (C, F, I) are corresponding SD-OCT line scans from the central macula in the three cases. All SD-OCTs showed disruption of photoreceptor outer segments in the central macula; all cases
Two younger siblings (II-2 & II-3) also had symptoms of nystagmus and photophobia since infancy. At their most recent visit, the BCVA was 20/160 and 20/200 in the right and left eyes respectively, in II-2 (18 years), and 20/200 and 20/250 in the right and left eyes respectively, in II-3 (13 years). Both siblings also had high myopia and associated peripheral retinal changes (Table 2; Figs. ID, 6G). The AF showed subtle increase in para-foveal (II-2) and foveal AF (II-3) (Table 2; Figs. 6F, 6H). Both siblings demonstrated bilateral grade 1 foveal hypoplasia, and mild central sub-foveal photoreceptor outer segment disruption on SD-OCT (Figs. 6F, 6I). The ERG was consistent with complete ACHM (Figs. 6K, 6L). Panel-based genetic testing identified a single heterozygous pathogenic variant in CNGB3 in all three siblings (c.1148delC/p.Thr383Ilefs*15).17

GS analysis of candidate genes for ACHM revealed two variants in CNGB3; the previously identified exonic variant c.1148delC (p.Thr383Ilefs*15) and a deep intronic variant (intron 6; chr8:87674402:T>A; c.852+4751A>T; NM_019098.4; GRCh37/hg19), flagged by four of the five in silico splice algorithms to result in a gain of splice acceptor site at c.852+4756 and an aberrant 73 bp long exon (Table 1). If translated, a stop codon will be introduced after 2 amino acids into this aberrant exon. The intronic variant was not observed in gnomAD and the two CNGB3 variants segregated with the disease phenotype in the pedigree (Fig. 3A). The c.1148delC is the most recurrent pathogenic variant reported in CNGB3.18 A comprehensive analysis of GS did not reveal any other candidate gene with biallelic coding or canonical splice variants (Supplementary Fig. S3).

Splicing Reporter Minigene RT-PCR and ASO Rescue. HEK293T cells were transfected with the following: CNGB3:EMG:REF (reference sequence, c.852+4751:A), CNGB3:EMG:MT (variant sequence, c.852+4751:T), and CNGB3:EMG:MT + ASO-CNGB3. RT-PCR of the reference clone (c.852+4751:A) displayed a distinct band (product A [182 bp], Fig. 3C), which on Sanger sequencing contained the correctly spliced canonical exons 6 and 7. The variant clone (c.852+4751:T) displayed three distinct bands (products A, B, and C); on Sanger sequencing, product B (409 bp) incorporated an aberrant exon starting down 5 bp downstream of the variant at the SpliceAI predicted acceptor site (Figs. 3C, 3D; Table 1). The aberrantly spliced exon did not pick up the SpliceAI predicted donor site. The product C (684 bp) was confirmed to be the unspliced minigene on Sanger sequencing (Figs. 3C, 3D). The variant clone co-transfected with ASO-CNGB3 showed an abrogation of product B (aberrant transcript) and presence of only product A (reference transcript; Fig. 3C).

Lymphoblast RT-PCR. Repeated attempts to amplify the cDNA from control and patient lymphoblast cell line were unsuccessful due to the poor expression of CNGB3 outside the retina, and this challenge has been reported previously.8

Discussion
This study reports the solving of three distinct IRDs by following a similar principle of integrating knowledge from deep phenotyping and its application to GS analysis. Three novel deep intronic splice mutations leading to inclusion of an aberrant exon by way of a gain-of-function splice acceptor site were identified in three different cases of IRDs: ESCS, cCSNB, and complete ACHM. These are the first documented pathogenic deep intronic mutational events in NR2E3 and GPR179, genes that are always associated with specific phenotypes ESCS and cCSNB, respectively.15,16,42 Precise phenotyping guided the targeted assessment of GS, that led to mutational discovery in each instance. Functional assay using splice reporter minigene constructs and/or patient-derived cell lines confirmed mutation pathogenicity as predicted by in silico tools.

Enhanced S-cone syndrome (ESCS) is a condition where the electrophysiological phenotype is pathognomonic of mutations in NR2E3 or NRL.42–45 The proband's ERG (Pedigree 1) demonstrated these characteristic features that reflected the underlying anatomical defect of a rod-devoid retina with all detectable ERG responses being dominated by an excess of S-cones that cannot respond well to a fast flicker stimulus; the specific molecular mechanisms are poorly understood.46,47 It is noted that although the proband displayed night blindness, retinal findings were subtle and did not include the foveal schisis or nummular pigmentation often associated with ESCS.44,48 Furthermore, the proband's SD-OCT demonstrated characteristic retinal rosettes previously described in the rd7 mouse model mutant for Nr2e349 and human patients with NR2E3-related ESCS (Vincent, A, IOVS, 2013, 54, 15, ARVO E-Abstract). The identification of a known pathogenic variant in NR2E3 (c.119-2A>C)35 on panel-based sequencing and the precise phenotypic information guided the identification of the deep intronic variant (c.1100+1124G>A) by GS which was further validated by segregation and functional assays. The presence of some amount of reference NR2E3 transcript in the patient cDNA experiments due to leaky splicing of the two splice variants may account for the milder fundus findings in the proband; the c.119-2A>C variant assays (minigene) has been earlier reported to cause incomplete skipping of exon 2 of NR2E3 leading to some retention of the reference transcript.43

Complete CSNB (cCSNB), a usually non-progressive disorder, is genetically heterogeneous but is defined by characteristic features that include night blindness, high refractive error, largely normal retinal appearance, and selective retinal ON-bipolar cell dysfunction (rods and cones) on ERG. The proband in Pedigree-2 displayed these characteristic features.50 Panel-based gene analysis failed to identify any exonic variants in the known CSNB genes.50,51 The GS analysis prioritizing cCSNB genes identified a homozygous GPR179 variant (c.903+343G>A) that was validated using segregation analysis, splice reporter minigene assay, and patient-derived lymphoblasts.

Complete ACHM is a severe form of largely non-progressive cone dysfunction syndrome that encompasses clinical features of infantile onset nystagmus and photophobia, reduced vision, severe color vision deficiency, normal retinal appearance, and non-detectable photopic ERGs; the proband and affected siblings in Pedigree-3 demonstrated these phenotypic features.52 Panel-based clinical testing...
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In summary, the analytical and bioinformatics tools currently available for GS allow for improved analysis of noncoding regions of the genome and enhance our capability to detect deep intronic mutational events. Combining deep phenotyping with targeted GS analysis can accelerate detection of these deep intronic variants that account in part for missing heritability in IRDs.

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