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Novel homozygous mutations in the transcription factor *NRL* cause non-syndromic retinitis pigmentosa

Mohammed E. El-Asrag,[1,2] Marta Corton,[3] Martin McKibbin,[1,4] Almudena Avila-Fernandez,[3] Moin D. Mohamed,[5] Fiona Blanco-Kelly,[3] Carmel Toomes,[1] Chris F. Inglehearn,[1] Carmen Ayuso,[3] Manir Ali[1]

(The first two authors contributed equally to this work.)

[1]Division of Molecular Medicine, Leeds Institute of Medical Research, St. James's University Hospital, University of Leeds, Leeds, UK; [2]Department of Zoology, Faculty of Science, Benha University, Benha, Egypt; [3]Department of Genetics & Genomics, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz University Hospital- Universidad Autónoma de Madrid (IIS-FJD, UAM), Centre for Biomedical Network Research on Rare Diseases (CIBERER), Madrid, Spain; [4]The Eye Department, St. James's University Hospital, Leeds, UK; [5]Ophthalmology Department, St Thomas' Hospital, London, UK

Purpose: To describe the clinical phenotype and genetic basis of non-syndromic retinitis pigmentosa (RP) in one family and two sporadic cases with biallelic mutations in the transcription factor neural retina leucine zipper (*NRL).*
Methods: Exome sequencing was performed in one affected family member. Microsatellite genotyping was used for haplotype analysis. PCR and Sanger sequencing were used to confirm mutations in and screen other family members where they were available. The SMART tool for domain prediction helped us build the protein schematic diagram.
Results: For family MM1 of Pakistani origin, whole-exome sequencing and microsatellite genotyping revealed homozygosity on chromosome 14 and identified a homozygous stop-loss mutation in *NRL*, NM_006177.5: c.713G>T, p.*238Lext57, which is predicted to add an extra 57 amino acids to the normal protein chain. The variant segregated with disease symptoms in the family. For case RP-3051 of Spanish ancestry, clinical exome sequencing focusing on the morbid genome highlighted a homozygous nonsense mutation in *NRL*, c.238C>T, p.Gln80*, as the most likely disease candidate. For case RP-1553 of Romanian ethnicity, targeted-exome sequencing of 73 RP/LCA genes identified a homozygous nonsense mutation in *NRL*, c.544C>T, p.Gln182*. The variants were either rare or absent in the gnomAD database.
Conclusions: *NRL* mutations predominantly cause dominant retinal disease, but there have been five published reports of mutations causing recessive disease. Here, we present three further examples of recessive RP due to *NRL* mutations. The phenotypes observed are consistent with those in the previous reports, and the observed mutation types and distribution further confirm distinct patterns for variants in *NRL* causing recessive and dominant diseases.

Retinitis pigmentosa (RP) is a group of inherited disorders of the retina that are characterized initially by the loss of rod photoreceptor function followed by cone photoreceptors. The age of onset and severity vary among affected individuals. Night blindness is often the earliest symptom, and clinical hallmarks include attenuated retinal vessels in the peripheral retina, bone spicule pigments throughout the fundus and absent α- and β-waves following electroretinography [1,2]. RP is the most common cause of hereditary blindness, with an incidence of approximately 1 in 4,000 in the population [3]. The majority of cases are non-syndromic, though about a quarter are syndromic, associated with non-ocular conditions such as obesity, polydactyly, renal malformation, hearing loss and intellectual disability [4]. RP is genetically heterogeneous, with autosomal dominant, autosomal recessive and X-linked patterns of inheritance. To date, mutations in 93 genes have been shown to cause the condition (RetNet). These genes encode proteins that are required for the development and maintenance of photoreceptor structure and its matrix membranes, visual transduction, ciliary trafficking and photoreceptor outer segment shedding. Recent advances in technology have helped to identify the genetic causes of up to 70% of RP patients, with the remaining group still of unknown etiology [5,6].

The neural retina leucine zipper gene (*NRL*, OMIM 162080) maps to human chromosome 14q11.2 [7] and encodes a 237 amino acid protein that belongs to the Maf subfamily of transcription factors, which are required for cell differentiation [8]. The protein has a Maf transactivation and a bZIP (basic leucine zipper) DNA-binding and dimerization domain. During mammalian retinal development, NRL is

Correspondence to: Manir Ali, Division of Molecular Medicine, Leeds Institute of Medical Research, St. James's University Hospital, University of Leeds, UK; Phone: 44 113 343 8420; FAX: 44 113 343 8603; email: m.ali@leeds.ac.uk

required for rod photoreceptor differentiation and synergistically interacts with CRX, NR2E3 and other transcription factors to regulate the activity of photoreceptor-specific genes such as rhodopsin [9-11]. The transgenic knockout mouse (*Nrl*$^{-/-}$) has an unusual retinal photoreceptor layer with shorter outer and inner segments, a functionally rod-less retina and super-normal cone function mediated by S-cones [12]. The transgenic overexpression of *Nrl* in mouse retina induces postmitotic photoreceptor precursors to drive differentiation toward rods instead of cones [13]. This function of *Nrl* to induce rod development while repressing the S-cone pathway acts through direct activation of the transcription factor *Nr2e3* [14]. Mutations in *NRL* have been associated with both dominant and recessively inherited retinal disease in humans [15,16]. The study reported herein describes novel recessive mutations in *NRL* causing non-syndromic RP in one family and two sporadic cases.

METHODS

Patient recruitment and sampling: Ethical approval for the study was obtained from the committees of the Leeds Teaching Hospitals National Health Service Trust ([17]/YH/0032) and the Fundación Jiménez Díaz University Hospital in Madrid (reference number PIC 134–2016_FJD). Consent was obtained from the individuals and their guardians where appropriate. The patients were diagnosed with non-syndromic RP following detailed clinical examination by an experienced ophthalmologist and after taking a clinical history. Blood samples were collected and stored in BD vacutainer® EDTA blood collection tubes (BD Biosciences, Oxford, U.K.). Genomic DNA was extracted from peripheral blood cells according to standard protocols using a QIAamp DNA blood midi kit (Qiagen Limited, Manchester, U.K.).

Exome sequencing: Targeted enriched libraries were prepared using either SureSelect Human Whole Exome reagent v.5 (Agilent Technologies, Santa Clara, CA), Clinical Exome solution (Sophia Genetics, Boston, MA) covering the exons of 4,490 known disease-related genes or a customized Haloplex reagent (Agilent) of the exons of 73 genes associated with RP/LCA as previously described [17]. Libraries were sequenced using a paired-end protocol on the HiSeq2500 or NextSeq500 platforms (Illumina, Little Chesterford, U.K.).

The quality of the output sequencing data was evaluated using FASTQ tools on the Galaxy platform [18]. After quality control filtering, the sequencing data was aligned against the reference human genome (hg19/GRCh37) using Bowtie2 and processed in SAM/BAM format using SAMtools and the Genome Analysis Toolkit (GATK). Potential PCR duplicates were removed using Picard, and the mean depth of reads per base was noted. Insertions, deletions and single nucleotide variants were noted in VCF format using the Unified Genotyper function of GATK.

The variant list was annotated with ANNOVAR software and filtered to include only non-synonymous coding variants, insertions and deletions affecting the coding sequence and any variants within 5 bp of splice donor and acceptor sites, with a minimum read depth of 10 and a minor allele frequency ≤1% in the Exome Variant Server, as well as the 1000 Genomes and Genome Aggregation databases. The pathogenicity of missense variants was assessed using the following prediction software: PolyPhen2, SIFT and CADD. For the analysis of whole exome and clinical exome data, the variant list was compared against the known retinal dystrophy genes in RetNet (accessed December 2021).

To detect homozygous regions from exome sequencing data, the AgileGenotyper software was used [19]. The exported variant list was analyzed using AutoSNPa, and the homozygous regions were visualized against a circular ideogram of Chromosomes 1 to 22 using AgileMultiIdeogram.

Microsatellite genotyping: Oligonucleotide primer pairs spanning polymorphic short tandem repeats were selected from the UCSC database. The PCR was performed with the primers on genomic DNA, and the products were genotyped on the ABI3130xl Genetic Analyzer (Applied Biosystems, Warrington, U.K.). Allele sizing was achieved using the GeneMapper v.4 software (Applied Biosystems).

PCR and Sanger sequencing: The PCR was typically performed on 30 ng of genomic DNA in 10 µl volumes according to standard protocols, which included 40 cycles of 94 °C for 30 s, 55–60 °C for 45 s and 72 °C for 45 s. An aliquot of the reaction product was visualized by agarose gel electrophoresis, and the remaining mixture was treated with ExoSAP-IT (GE Healthcare, Chalfont St. Giles, U.K.). The treated PCR product was cycle sequenced using the BigDye Terminator v.3.1 reaction mix (Applied Biosystems) and run on an ABI3130xl Genetic Analyzer (Applied Biosystems). The sequencing output was analyzed using the Sequence Analysis v.5.2 software package (Applied Biosystems) according to the manufacturer's instructions.

Bioinformatics: Genomic DNA sequence information was downloaded from the NCBI website. Oligonucleotide primer pairs were designed to amplify across the variant of interest using Primer3 software. The primers used in this study are listed in Appendix 1. The SMART tool was used to predict protein domains in the amino acid sequence.

RESULTS

One family of Pakistani origin (MM1) and two sporadic cases, one of Spanish ancestry (RP-3051) and the other of Romanian descent (RP-1553), were investigated in this study (Figure 1). All the affected subjects were diagnosed with early-onset retinitis pigmentosa (Table 1). None of the examined patients showed extraocular features. Where a family history of consanguinity was known, it suggested an autosomal recessive pattern of inheritance for disease transmission.

Clinical details and molecular analysis of family MM1: The proband (IV:2) in family MM1 is one of three affected individuals in a Pakistani family with consanguineous parents. She has had problems with her vision since early childhood and had surgery for strabismus when she was seven years old. At 19 years old, she was registered as visually impaired with grossly constricted visual fields and best corrected acuity of 6/24 and 6/36. Ophthalmic examination revealed typical clumped pigment deposits, and posterior subcapsular cataracts developed gradually in both eyes. At 43 years old, the

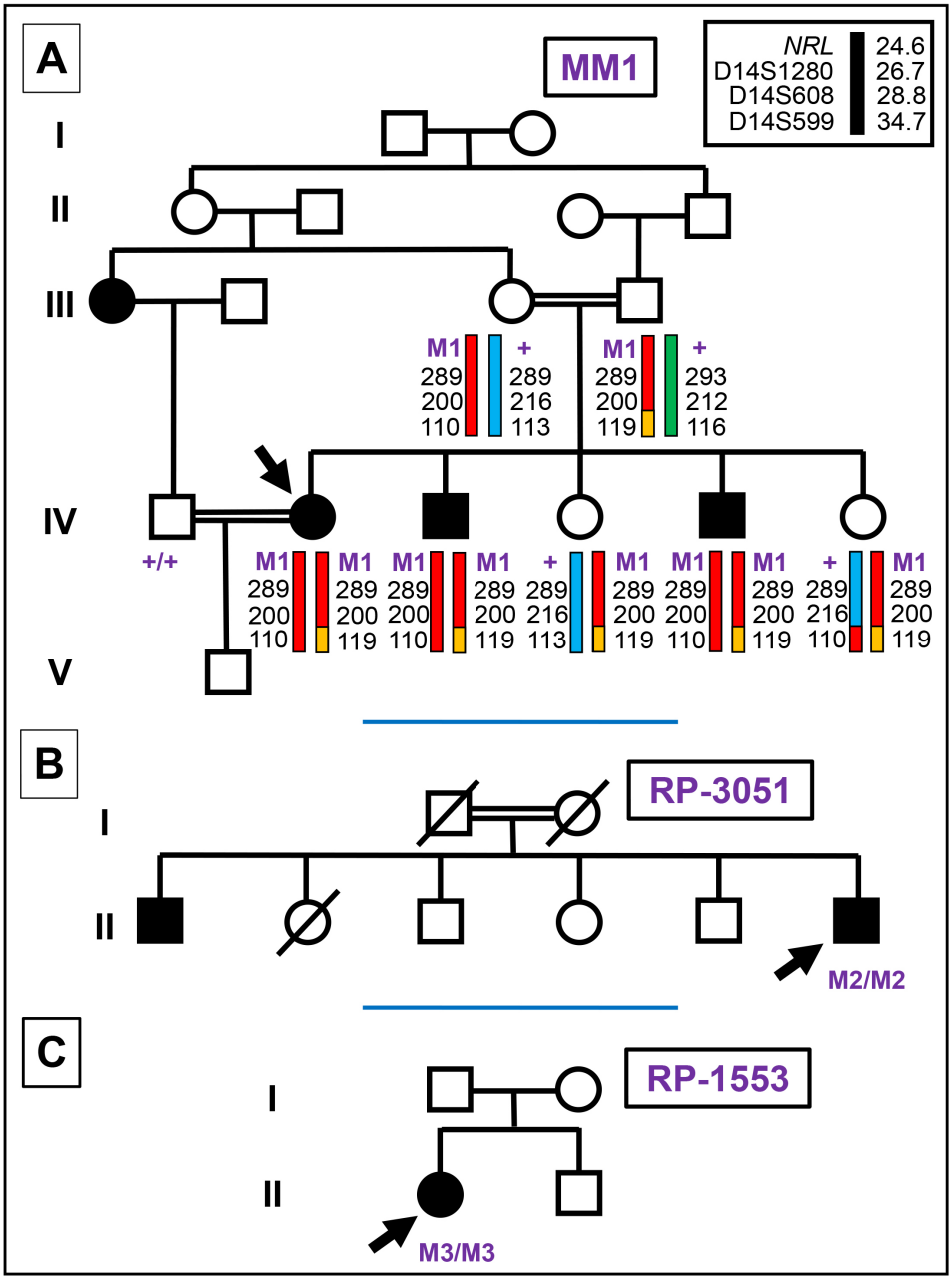

Figure 1. Families analyzed in this study. The structures of the pedigrees are shown for **A:** family MM1, **B:** case RP-3051 and **C:** case RP-1553. The proband in each pedigree is highlighted with an arrow. Microsatellite genotyping in family MM1 around the *NRL* locus is shown with allele sizes and haplotypes for three microsatellite markers, D14S1280, D14S608 and D14S599, located at 26.7, 28.8 and 34.7Mb from the top of chromosome 14 using the hg19/GRCh37 human reference genome. Haplotype analysis (red block) confirmed homozygosity in family MM1 around the *NRL* locus only in the affected cases. The *NRL* mutation (located at 24.6 Mb) is stated as M1, M2 and M3. Genomic DNA was only available from members to whom a genotype has been assigned. Note that only the affected individuals are homozygous for the mutation.

Table 1. Clinical details of retinal dystrophy patients examined in this study.

Family ID	Ethnicity	Subject	Gender	Age at examination (years)	Best corrected visual acuity (OD & OS)	Fundus examination	Diagnosis	Additional Findings
MM1	Pakistani	IV:2	F	19	6/24 & 6/36	Pigmentary retinopathy	Retinitis pigmentosa	Posterior subcapsular opacification
				43	6/36 & 6/60			
		IV:3	M	20	6/9 & 6/36	Pigmentary retinopathy	Retinitis pigmentosa	Amblyopia (OS)
				33	NA			Posterior subcapsular opacification
RP-3051	Spanish	II:6	M	57	NA	Pigmentary retinopathy	Retinitis pigmentosa	Photophobia, dyschromatopsia
RP-1553	Romanian	II:1	F	21	6/60 & 6/60	Pigmentary retinopathy	Retinitis pigmentosa	Nystagmus, complete scotoma

The gender, age at examination, corrected visual acuity, ophthalmoscopy results, diagnosis and any additional findings are summarized. M=male, F=female, OD=right eye, OS=left eye, NA=not available.

best corrected acuity was 6/36 and 6/60. Her younger brother (IV:3), when examined at 20 years old, had amblyopia in his left eye and best corrected visual acuity of 6/9 and 6/36, with a fundus consistent with typical RP changes. At 33 years old, when he was last examined, he had no response to electroretinography and had developed posterior subcapsular cataracts. The other affected case (IV:5) in the family was not available for further examination.

Whole exome sequencing of the genomic DNA from subject IV:2 identified 60,603 total variants that differed from the reference genome sequence. After filtration, 119 variants remained, of which 25 were homozygous changes. After comparing the variant list against the known RetNet genes, a single candidate—a novel homozygous stop-loss mutation, NM_006177.5: c.713G>T, p.*238Lext57 (ClinVar accession SCV001478096) in the transcription factor gene *NRL*—was identified as the best candidate. AgileMultiIdeogram analysis of the WES data highlighted a 3.4 Mb homozygous region that contained the *NRL* gene (Appendix 2), and microsatellite genotyping with polymorphic markers confirmed this homozygosity (Figure 1A). The *NRL* variant was present once in 115,494 alleles in the gnomAD database, and Sanger sequencing (Figure 2A) confirmed that the mutation segregated with the disease phenotype in the family (Figure 1A) as expected for a recessive condition.

Clinical details and molecular analysis of case RP-3051: The index case (II:6) in the family RP-3051 is the sixth child of a consanguineous Spanish couple from a small village with a relatively high level of endogamy. He was referred for genetic testing at 57 years of age with a clinical diagnosis of RP. He had night blindness since childhood, loss of visual acuity since the third decade, and was now experiencing photophobia and slight dyschromatopsia. A second sibling manifested a similar presentation of RP, but clinical data were unavailable for that person.

Clinical exome sequencing of the index case II:6 followed by filtration analysis identified a novel homozygous nonsense mutation, c.238C>T, p.Gln80* (ClinVar accession SCV001478094) in the *NRL* gene, as the most likely cause of RP in the patient. This variant, which was absent in the gnomAD database, was verified by Sanger sequencing in patient genomic DNA (Figure 2B).

Clinical details and molecular analysis of case RP-1553: The patient (II:1) in the family RP-1553 is the only affected child of a Romanian couple who are not known to be related. She had visual impairment since two years old and was diagnosed with early-onset RP at five years of age. Her most recent examination at 21 years old showed nystagmus, complete loss of the visual field, best corrected visual acuity of 6/60 in both eyes, non-recordable electroretinography and typical RP presentation upon funduscopy.

Genomic DNA from patient II:1 underwent targeted exome sequencing for the 73 RP/LCA genes. Following variant prioritization, a novel homozygous mutation in *NRL*, c.544C>T p.Gln182* (ClinVar accession SCV001478095), was found as the most likely disease candidate. This variant was confirmed by Sanger sequencing the DNA of the patient (Figure 2C) and was absent from the gnomAD database.

DISCUSSION

Mutations in the *NRL* gene have been reported to cause either recessive or dominantly transmitted retinal disease in humans (see Figure 3). The type of mutation, null allele or missense, and the location of missense mutations in the protein

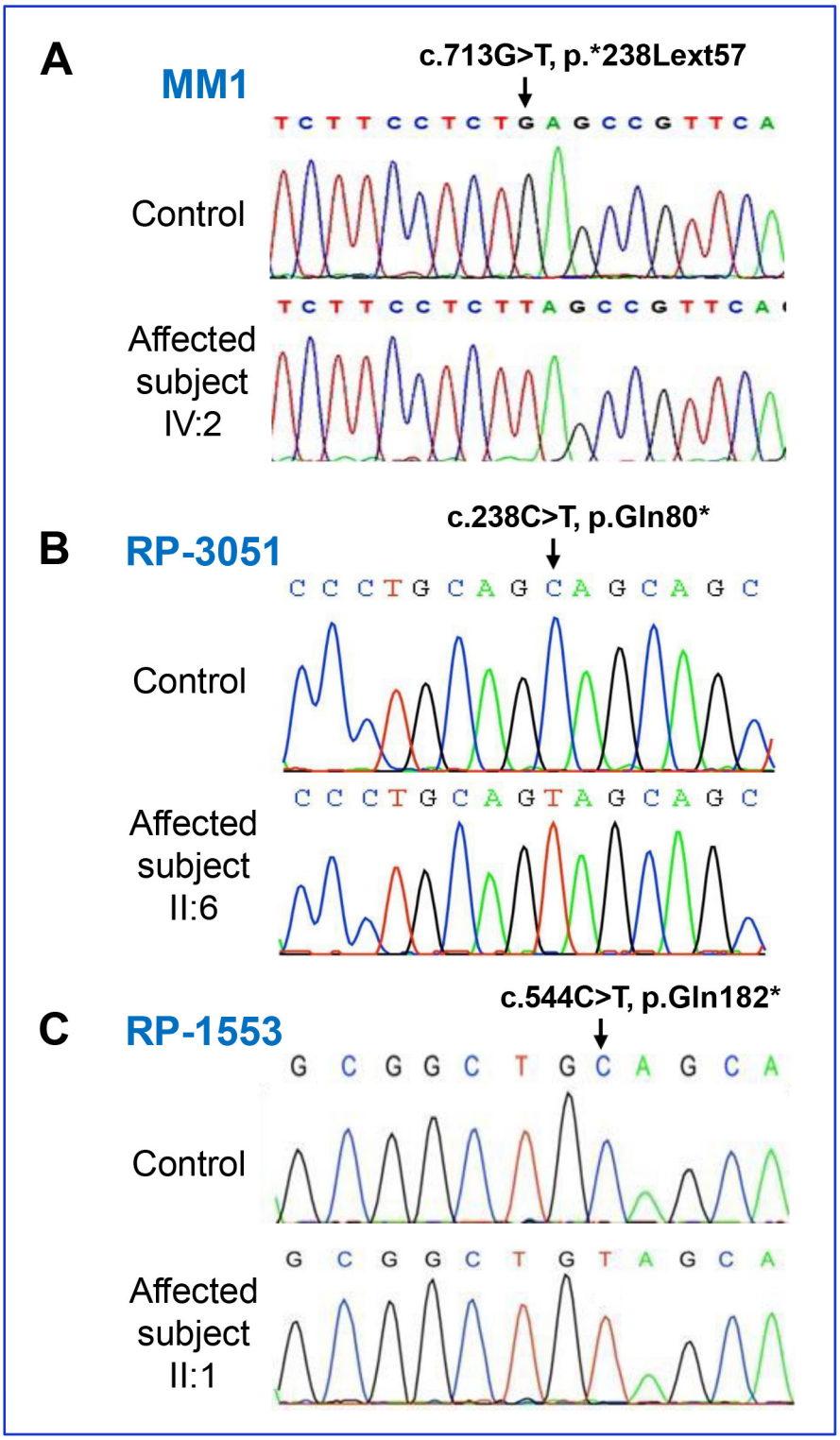

Figure 2. Confirmatory *NRL* sequence analysis. Chromatograms of the *NRL* sequence variants from a normal control individual and affected subjects, IV:2 from family MM1 **(A)**, II:6 from family RP-3051 **(B)** and II:1 from family RP-1553 **(C).** Note that homozygous mutations c.713G>T in family MM1, c.238C>T in family RP-3051 and c.544C>T in family RP-1553 are shown.

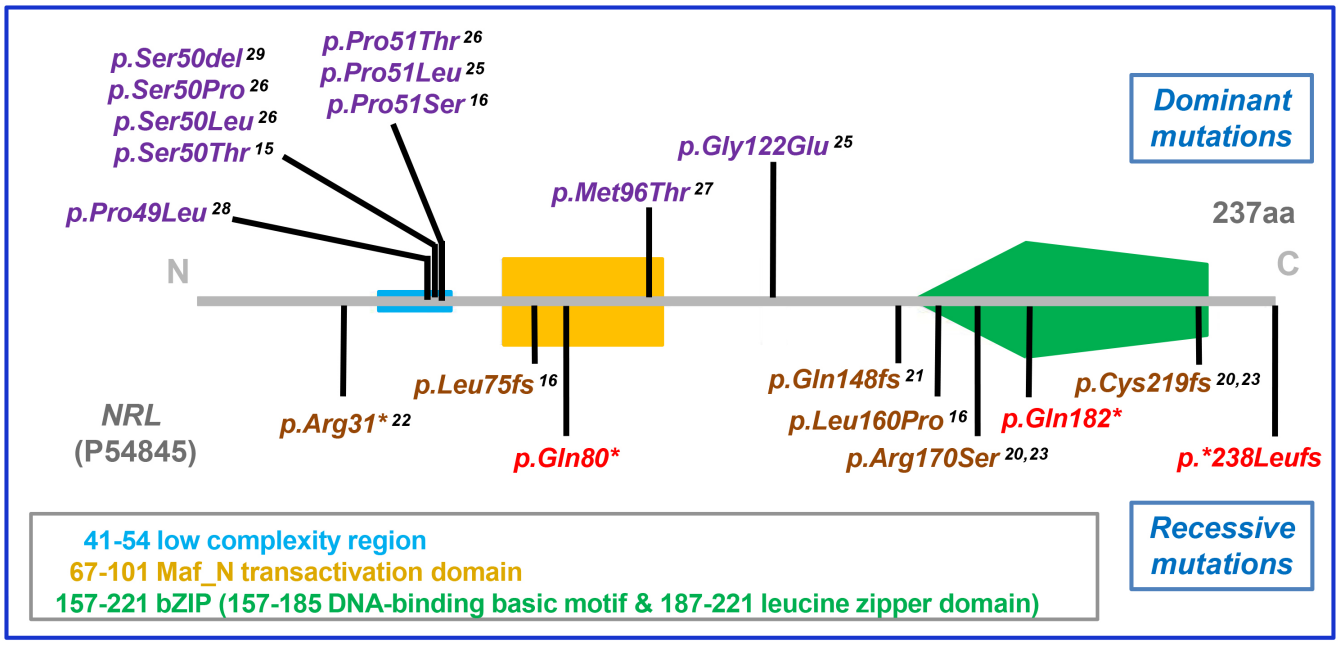

Figure 3. Mapping the *NRL* mutation spectrum onto the protein sequence. The domains in the 237 amino acid NRL protein are indicated. NRL mutations identified in dominant disease [15,16,25-29] are presented in purple text above the protein domain representation, and recessive disease [16,20-23] are shown in brown text below the illustration. The novel mutations described in this paper are highlighted in red in the diagram.

sequence determine the inheritance pattern of disease transmission. There are only five published reports of *NRL* mutations causing recessive disease [16,20-23]. These mutations are all either null alleles caused by nonsense or frameshift mutations or are missense mutations that map to the bZIP domain, which is required for DNA-binding, dimerization of NRL and interaction with the homeodomain of CRX [10]. Nonsense and frameshift mutations are likely to cause disease either by nonsense-mediated decay of the mutant transcript or, if a truncated protein is synthesized, due to complete or partial loss of the bZIP domain that would be detrimental to NRL function. However, missense mutations in the bZIP domain are also damaging by preventing DNA binding and reducing transcription activation [24]. These mutations therefore cause recessive disease by loss of normal protein function. Here, we report three further novel mutations causing recessive disease, consistent with this pattern. The p.Gln80* mutation removes the C-terminal end of the minimal transactivation domain, the DNA-binding basic domain and the leucine zipper dimerization domain, whereas the p.Gln182* removes only the leucine zipper domain. Despite the absence of segregation analysis of these mutations, the loss of key functional domains would certainly be consistent with loss of NRL function. Though the p.*238Lext57 stop-loss mutation, which segregated with disease in the family, contains all the functional domains of the protein, it is most likely that the extra amino acids at the C-terminus are problematic. Hypothetically, should the mutant transcript escape early decay, the extra amino acids would presumably interfere with NRL dimerization and protein interactions to impede normal function.

To date, the *NRL* mutations causing dominant retinal disease are all missense mutations apart from a single amino acid in-frame deletion [15,16,25-29]. In vitro assays have shown that these mutations exhibit reduced phosphorylation of NRL but enhanced transcriptional activation of the rhodopsin promoter, resulting in the gaining of function in excess of normal promoter activation [15,24,27,28]. Although other rod-specific promoters have not yet been directly investigated, this excessive activation of mutant NRL presumably affects several NRL target gene promoters that have been found to be important for maintaining rod photoreceptor function [30].

The clinical presentation and phenotype of the patients is different for recessive and dominant *NRL* retinopathy. To date, 25 patients have been studied with the dominant form of disease [15,16,25-29] and, including the patients described herein, 12 subjects have been described with recessive disease [16,20-23]. Night blindness from early childhood is a common symptom, followed by variable amounts of progressive visual

field constriction and reduced visual acuity. Other features co-existing in patients include nystagmus in 4/12 recessive [22,23], this paper] and 1/25 dominant cases [16], strabismus or amblyopia in 5/12 recessive [16,23], this paper] and 0/25 dominant subjects and posterior subcapsular cataracts in the more elderly, which was seen in 4/12 recessive [22,23], this paper] and 3/25 dominant patients [26]. For recessive *NRL* retinopathy, also called clumped pigment retinal degeneration, which is a subtype of autosomal recessive RP, these signs coincide with the early fundal appearance of clusters of clumped pigmented deposits in the periphery that is accompanied by chorioretinal atrophy and attenuated arterioles [16,22,23]. However, for dominant disease, fundal abnormalities tend to appear in the third decade with attenuated vessels and bone spicule–shaped pigment deposits that are more typically seen in patients diagnosed with RP [25-29]. Although patients with *NRL* retinopathy show markedly reduced rod and cone functions on electroretinography, patients with dominant disease also show loss of short wavelength (blue- or S-) cone function [16,28] whereas recessive disease patients show no detectable rod function and a relatively enhanced S-cone function [16,22,23]. This enhanced S-cone feature in recessive *NRL* retinopathy is similar to what is observed in *Nrl*-knockout mice [12] and patients with retinopathy due to *NR2E3* mutations [31]. It would be interesting to determine whether the patients described in this report share this enhanced S-cone phenotype, though limited patient access has precluded further study.

To summarize, we describe one family and two sporadic cases with different novel homozygous *NRL* mutations accounting for the disease phenotype. Previously, only five families and six mutations had been described with this form of RP, so the results contribute to the mutation spectrum for this condition. The phenotypes observed are consistent with those in previous reports, and the observed mutation types and distribution further confirm distinct patterns for variants causing recessive and dominant disease. Identifying the genetic cause of disease in these patients provides more accurate genetic counselling for the families and stratifies the patients into distinct groups as future treatments become available.

APPENDIX 1. OLIGONUCLEOTIDE PRIMER PAIRS FOR THE ANALYSIS OF THE *NRL* MUTATIONS.

To access the data, click or select the words "Appendix 1." The DNA sequences, PCR product size and annealing temperature of each primer pair are shown.

APPENDIX 2. HOMOZYGOSITY MAPPING IN FAMILY MM1 USING WES DATA.

To access the data, click or select the words "Appendix 2." MultiIdeogram of the WES data of patient IV:2 showing homozygous regions in blue. The *NRL* gene (arrowed) is located on chromosome 14 at ~24.6Mb (hg19) and maps within the homozygous region 21,860,360–25,288,227.

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