Homozygosity Mapping and Whole-Genome Sequencing Links a Missense Mutation in *POMGNT1* to Autosomal Recessive Retinitis Pigmentosa

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Purpose. To identify the genetic cause in five families with autosomal recessive retinitis pigmentosa, a genetic disorder involving retinal degeneration and visual loss with high genetic heterogeneity.

Methods. We performed whole-genome single nucleotide polymorphism genotyping on 35 members from the five families to map the region of homozygosity shared by all patients. Whole-genome sequencing was then conducted on one of the patients and a novel variant was identified in *POMGNT1* from the homozygous region, which was confirmed by Sanger sequencing and sequenced in all family members. Mutant and wild-type POMGNT1 were expressed in heterologous cells to assess enzyme activity.

Results. A 1.8-Mb homozygous region was identified at 1p34-p33 shared by all 17 patients. Whole-genome sequencing revealed a novel missense mutation in *POMGNT1* (c.359A>C, p.Leu120Arg) from this homozygous region, which was shown to co-segregate with disease phenotype. The mutant protein carrying this missense mutation showed an approximately 80% decrease in POMGNT1 enzyme activity compared with the wild type.

Conclusions. We identified a novel mutation in *POMGNT1* that causes nonsyndromic autosomal recessive retinitis pigmentosa, adding to the genetic heterogeneity of this retinal disease. *POMGNT1* encodes a glycosyltransferase in O-mannosyl glycosylation and was previously found to be responsible for a group of congenital muscular dystrophies called dystroglycanopathies. Our discovery suggests the involvement of O-mannosyl glycosylation in retinitis pigmentosa and presents an instance of *POMGNT1* mutation that does not involve muscular dystrophy.

Keywords: retinitis pigmentosa, mutation screening, POMGNT1, homozygosity mapping, next-generation sequencing

Retinitis pigmentosa (RP; OMIM #268000) is an inherited disease involving retinal degeneration from the progressive loss of rod and cone photoreceptors. Consequentially, night vision is first lost, followed by peripheral and central vision, ultimately leading to total blindness in some cases.[1] Retinitis pigmentosa affects 1 in 4000 people worldwide and is both phenotypically and genetically heterogeneous, presenting with variable symptoms and different inheritance patterns.[2,3] Although more than 80 genes have been identified to cause the nonsyndromic hereditary form of RP (RetNet, http://www.sph.uth.tmc.edu/RetNet/),[4] these are collectively responsible for only approximately 60% of the entire patient population.[5]

Earlier studies identified RP genes such as rhodopsin (*RHO*) and those encoding phosphodiesterase enzymes in the phototransduction cascade (*PDE6A* and *PDE6B*) using a candidate gene approach, prioritizing genes in the mapped loci with relevant function and retina expression.[6–8] Subsequently, discoveries of RP genes through whole-genome linkage or homozygosity mapping without previous knowledge of the candidate genes successfully identified several genes without obvious retinal function, such as *EYS* and *SPATA7*, as well as genes with functions still unknown (e.g., *CERKL* and *PRCD*).[9,10]

Homozygosity mapping is a gene-mapping strategy that is commonly used to locate identical chromosomal regions in homologous chromosomes that are shared by patients in consanguineous families affected by autosomal recessive disease. Using high-density single-nucleotide polymorphism (SNP) arrays, it is a more cost-effective strategy compared with whole-genome sequencing on dozens of family members. The

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power of homozygosity mapping can extend beyond cases of consanguinity, as homozygous mutations have also been identified in nonconsanguineous families living in isolated communities.[11] More recently, next-generation sequencing has been successfully used to identify novel RP genes with the help of candidate region prioritization strategies, such as homozygosity mapping and cis-regulatory mapping.[12–14]

In the present study, we identified five families with inherited autosomal recessive RP (arRP). These families had no recent history of consanguineous marriages, but live on a closed and isolated small island off the southeastern coast of Taiwan and are presumed to share a gene pool from common ancestors. Using homozygosity mapping and whole-genome sequencing, we investigated the gene responsible for RP in these families.

Materials and Methods

Subject Assessment and Recruitment

The proband in each family was referred to ophthalmologists with a chief complaint of decreased night vision. All available family members were examined for their best-corrected visual acuity and visual field in addition to ophthalmoscopy and fundus photography for diagnosis. A total of 35 individuals were recruited, 17 of whom were diagnosed with RP. Blood was drawn for DNA extraction (Gentra Puregene DNA Isolation Kit; Qiagen, Hilden, Germany). Informed consent was obtained from all participants in accordance with approval from institutional review boards at National Yang Ming University in Taiwan.

Whole-Genome SNP Genotyping

Whole-genome SNP genotyping was performed with Affymetrix (Santa Clara, CA, USA) Genome-Wide Human SNP Array 6.0 according to the manufacturer's protocol. Genotypes were called using Genotype Console Software (Affymetrix) with default parameters suggested by the platform manufacturer. Sample call rates were all greater than 0.95, and the average call rate of the sample was 0.994 (SD 0.0036).

Analysis and Mapping of the Homozygosity Region

Whole-genome SNP genotype data from each individual were analyzed for the presence of runs of homozygosity using the Runs of Homozygosity (ROH) tool from PLINK v1.06 (http://pngu.mgh.harvard.edu/purcell/plink).[15] Default settings of the "homozyg" command were used. Results from the ROH tool were depicted with an in house-developed graphical software for better visualization and determination of the common ROH shared by all patients.

Next-Generation Sequencing

Whole-genome sequencing was carried out on one of the recruited RP patients (F2–13 in Fig. 1) with HiSeq 2000 (paired-end of 100 bases) following the standard protocols provided by Illumina (San Diego, CA, USA). Primary analysis was carried out with Real Time Analysis version 1.7 set to default parameters. Alignment to the Human Genome reference (NCBI 36.3), calling of the SNPs, read depth, and detection of variants were performed with Illumina's software CASAVA 1.8. Further annotation and filtering of the detected genetic variations were carried out with VarioWatch (http://genepipe.ncgm.sinica.edu.tw/variowatch/main.do).[16]

Dideoxy Sequence Analysis of Candidate Genes and Mutation Confirmation

Primers for PCR amplification of exons, including the splicing junctions, were designed by PrimerZ (http://genepipe.ncgm.sinica.edu.tw/primerz/beginDesign.do).[17] Products from PCR were run on agarose gels to confirm single products. DNA sequencing was carried out using an ABI 3730 sequencer and BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) and the results were analyzed by Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA).

Cell Culture and Expression of FLAG-Tagged Wild-Type (WT) and Mutant *POMGNT1*

Full-length *POMGNT1* (NM_017739.3) cDNA was cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Plasmids were isolated with QIAprep Spin Miniprep kit (Qiagen). The sequence of the isolated plasmids was confirmed by Sanger sequencing. This sequence-verified *POMGNT1* plasmid then served as the template for site-mutagenesis PCR to produce the mutant *POMGNT1* cDNA containing the identified mutation c.359T>G. Wild-type and mutant *POMGNT1* were then subcloned into pCMV-Tag 2A vectors (Agilent Technologies, Santa Clara, CA, USA) with the FLAG tag attached before the gene, and extracted with Genopure Plasmid Midi Kit (Roche, Basel, Switzerland) or PureLink HiPure Plasmid Filter Purification Maxiprep Kits (Invitrogen, Carlsbad, CA, USA). Cells of HEK293T were transfected with TransIT-2020 transfection reagent (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions. After 24 hours of transfection, the cells were lysed with 1% Triton X-100 in Tris-buffered saline or mammalian protein extraction buffer (GE Healthcare Life Sciences, Marlborough, MA, USA) with complete EDTA-free protein inhibitor cocktail (Roche) for 10 minutes on ice, and then centrifuged at 16,000*g* at 4°C for 20 minutes. The supernatants were collected.

Western Blotting

Supernatants from the transfectant cell lysates were boiled with Laemmli sample buffer for 3 minutes before separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The anti-FLAG M2 antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as the primary antibody after blocking the membrane with 5% milk. Signals were visualized using ECL Western blotting detection reagents (Immobilon Western Chemiluminescent HRP Substrate; Millipore, Darmstadt, Germany).

Enzymatic Activity Assay

Total proteins obtained from HEK293T cells transfected with either vector only, or WT or mutant *POMGNT1* were purified by incubating with anti-FLAG M2 affinity gel (Sigma-Aldrich Corp.) to isolate the FLAG-tagged POMGNT1 proteins, according to the manufacturer's instructions for the immunoprecipitation of FLAG fusion proteins. The bound FLAG-tagged proteins were eluted with 3X FLAG peptide at a final concentration of 300 ng/μL. Eluted FLAG-tagged POMGNT1 proteins were concentrated on Savant SpeedVac SC110 (Thermo Fisher Scientific, Waltham, MA, USA) for 20 minutes and resuspended in 25 μL assay buffer (25 mM MES, 5 mM $MnCl_2$, 2.5 mM $CaCl_2$, 0.02% Brij-35, pH 6.5) for subsequent enzyme activity assays. The enzyme activity assay measures the ability of POMGNT1 to transfer N-acetyl-D-glucosamine from UDP-GlcNAc (Sigma-Aldrich Corp.) to methyl-α-D-mannopyronoside (Sigma-Aldrich Corp.), and was carried out with the Glycosyltransferase Activity Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's

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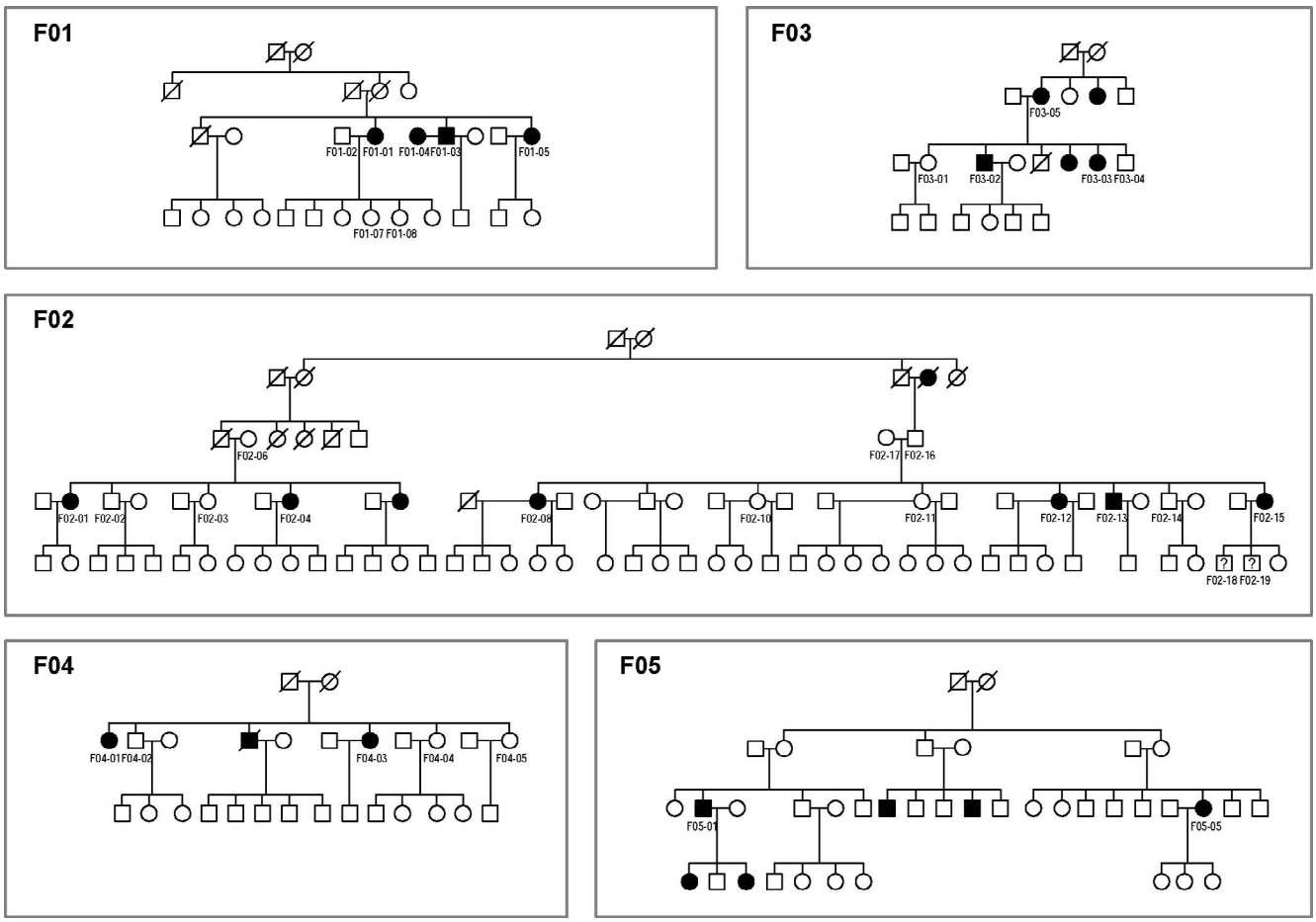

Figure 1. Pedigrees of five families with autosomal recessive RP. A total of 35 individuals from five different families were recruited and are numbered in the pedigrees. Among them, 17 were diagnosed with RP, shown as the *filled box*. Patients recruited below the age of 30 may not have yet reached the onset age and were assigned with a question mark denoting unknown status.

instructions.[18] Specifically, a phosphate standard curve was established to build a linear relationship between phosphate content and absorbance at 620 nm. Then, equal parts of eluted POMGNT1 proteins, along with different dilutions of rhPOMGNT1 (R&D Systems) containing 4 ng, 2 ng, 1 ng, 0.5 ng, and 0.25 ng of the protein were added to a 25-μL reaction mixture containing equal parts of coupling phosphatase (24 μg/mL), UDP-GlcNAc (6 mM), and 1 M methyl-alpha-D-mannopyranoside. The reaction was incubated at 37°C for 20 minutes, and 30 μL Malachite Green Reagent A and 100 μL deionized water were added and mixed. Finally, 30 μL Malachite Green Reagent B was added and incubated for 20 minutes at room temperature for color development, and the absorbance was read on a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 620 nm. The final optical density readings of the proteins from the vector-only transfectants were subtracted from those of the mutant and WT proteins, and the mutant enzyme activity was normalized to that of WT POMGNT1 to determine relative enzyme activity.

Results

Homozygosity Region Mapped to 1p34-p33 in Five Nonconsanguineous RP Families

Five families living on a small island off the southeastern coast of Taiwan with no record of recent consanguineous marriages were diagnosed with RP (Fig. 1). The island is home to an ethnic minority group who migrated to the island from the Batan Archipelago 800 years ago, who lived in a closed community with little interaction with outsiders until recently. Night blindness was noted in all RP patients and the onset was self-reported from the age of 12 to 40 years, although the actual onset may have been earlier and not detected by the subjects. In addition, peripheral field loss was observed. On ophthalmoscopy examination, all RP patients presented typical attenuated retinal arterioles, bone-spicule pigment deposits, and optic disc pallor (Fig. 2); rod responses were also severely reduced on electroretinography examination (not shown). Other than these ophthalmologic findings, the physical examinations were unremarkable; specifically, there was no mental retardation and no muscle weakness or atrophy observed. Compared with the generally accepted age of onset for typical RP of 10 to 30 years old, except for the two individuals who were assigned with unknown status (F02-18 and F02-19 in Fig. 1) since they were still in their adolescence and may develop RP later, all other recruited members were well beyond the onset age for RP. Autosomal recessive inheritance was obvious in F01, F02, and F04, in which all affected individuals were born to unaffected parents (Fig. 1). In F03 and F05, one of the affected individual's parents was also affected, suggesting different inheritance modes. However, because these families have been living on an isolated island with frequent intracommunity marriages, we hypothesize that the disease allele runs at a high

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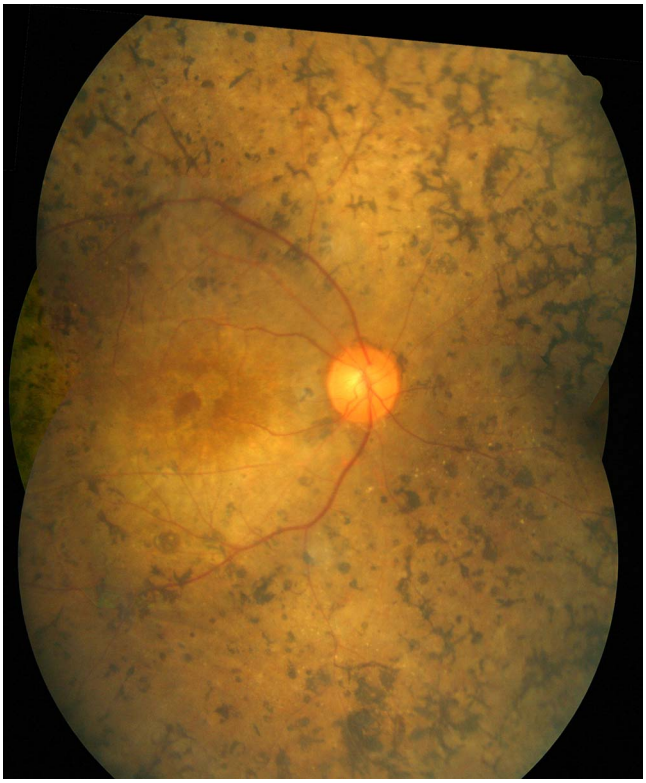

FIGURE 2. Fundus findings from an RP patient. Fundus photograph of individual F02-06 at age 59 showing typical RP-associated characteristics, including attenuation of the retina blood vessels, bone spicule-like deposits, and a waxy palor of the optic disc.

frequency in the local population with a substantial portion of heterozygote carriers; thereby, recessive inheritance is still possible in these two families (the affected parent carries a homozygous mutant, whereas the unaffected parent is a heterozygote carrier).

Whole-genome analysis of the homozygosity region was carried out on 35 individuals (numbered in Fig. 1) consisting of 17 RP patients and 18 unaffected family members. Homozygosity regions were identified for each individual and compared to reveal a shared homozygous region by all patients at 1p34-p33 (Fig. 3). The region is bounded by SNPs, rs2983715 and rs324423, and spans approximately 1.8 Mb from 44.9 Mb to 46.7 Mb on chromosome 1p (maximal logarithm of the odds score = 4.44). This homozygous region was also found in four unaffected individuals in our initial analysis (Fig. 3). After careful examination of the SNP genotypes from these unaffected individuals, we found that three of the unaffected individuals actually carried distinct haplotypes identified in the RP patients, although also homozygous. The homozygosity mapping program used identifies and defines stretches of homozygous regions but does not differentiate between genotypes (i.e., AA versus CC). However, one unaffected individual was found to carry the same homozygous haplotype as the patients. This individual did not show any RP symptoms but was still an adolescent and not far beyond the onset age; therefore, unknown status was assigned (F02-18 in Fig. 1). No other homozygous region was identified to be shared by all patients. This homozygous region at 1p34-p33 contains no known RP genes and does not overlap with any of the previously reported RP loci.

Next-Generation Sequencing Identified a Novel Mutation in *POMGNT1* From the Homozygous Region

Considering the heterogeneity of RP, we used next-generation sequencing on one patient (F2-13) for the initial screen of variation. A total of 108,750 Mb was obtained from the pair-end reads of 100 bases with an average 35-fold whole-genome coverage. Read 1 and read 2 mapped to 86.59% and 82.85% of the human reference genome (NCBI 36.3), respectively. After quality filtering, 4,907,422 variants were identified, and only two coding variants were located in the identified 1.8-Mb homozygous region on 1p: chr1:44650878G/A and chr1:46434332A/C. The first variant sits in exon 2 of *C1orf164* and is a synonymous change that does not affect the encoded amino acid. The second variant (NM_017739.3:c.359A>C, p.Leu120Arg, chr1:46434332A/C) is located in exon 5 of the *POMGNT1* gene and causes a missense mutation that has not been documented in the literature and was not recorded in any of the online databases.

Confirmation of the Novel *POMGNT1* Mutation in the RP Families

The exon containing the identified mutation in *POMGNT1* was sequenced in all recruited individuals. The mutation was found to co-segregate with the disease in all five families, with all 17 patients carrying the same homozygous mutation (c.359A>C, p.Leu120Arg). The individual (F02-18) with unknown status carrying the same homozygous stretch was confirmed to be a heterozygote carrier of this mutation. This mutation was not found in 470 randomly selected Han Chinese healthy controls.[19] This position, as well as the surrounding bases, is highly conserved among several different species (Fig. 4), and the mutation was predicted to be damaging by both PolyPhen and SIFT.[20,21]

Purified Mutant *POMGNT1* Proteins Showed Decreased Enzyme Activity

To confirm the pathogenicity of the identified mutation, WT and mutant POMGNT1 vectors were constructed and transiently expressed in HEK293T cells. Expressed POMGNT1 was purified from total proteins and subjected to a glycosyltransferase activity assay, which exhibited a linear relationship between the concentration of rhPOMGNT1 and absorbance, and could specifically measure the enzyme's ability to transfer N-acetyl-D-glucosamine from UDP-GlcNAc to methyl-α-D-mannopyronoside (Figs. 5a, 5b). The assay was repeated three times, and after adjusting for the absorbance of proteins from cells transfected with the empty vector, mutant POMGNT1 showed an average of only 21% of the enzyme activity of WT POMGNT1 (Fig. 5c). Western blot analysis showed comparable amounts of total and eluted proteins used for protein purification and the glycosyltransferase activity assay, with GAPDH as the control (Fig. 5d).

DISCUSSION

Autosomal recessive RP accounts for approximately 50% to 60% of all RP cases,[22,23] and 30% of arRP patients have unidentified genetic causes.[2] A gene mapping strategy involving a whole-genome approach followed by homozygosity mapping has been carried out to locate disease candidate regions and identify novel arRP genes,[24–27] and is particularly effective for populations with a tradition of consanguineous marriage, such as the Israeli and Palestinian populations, from

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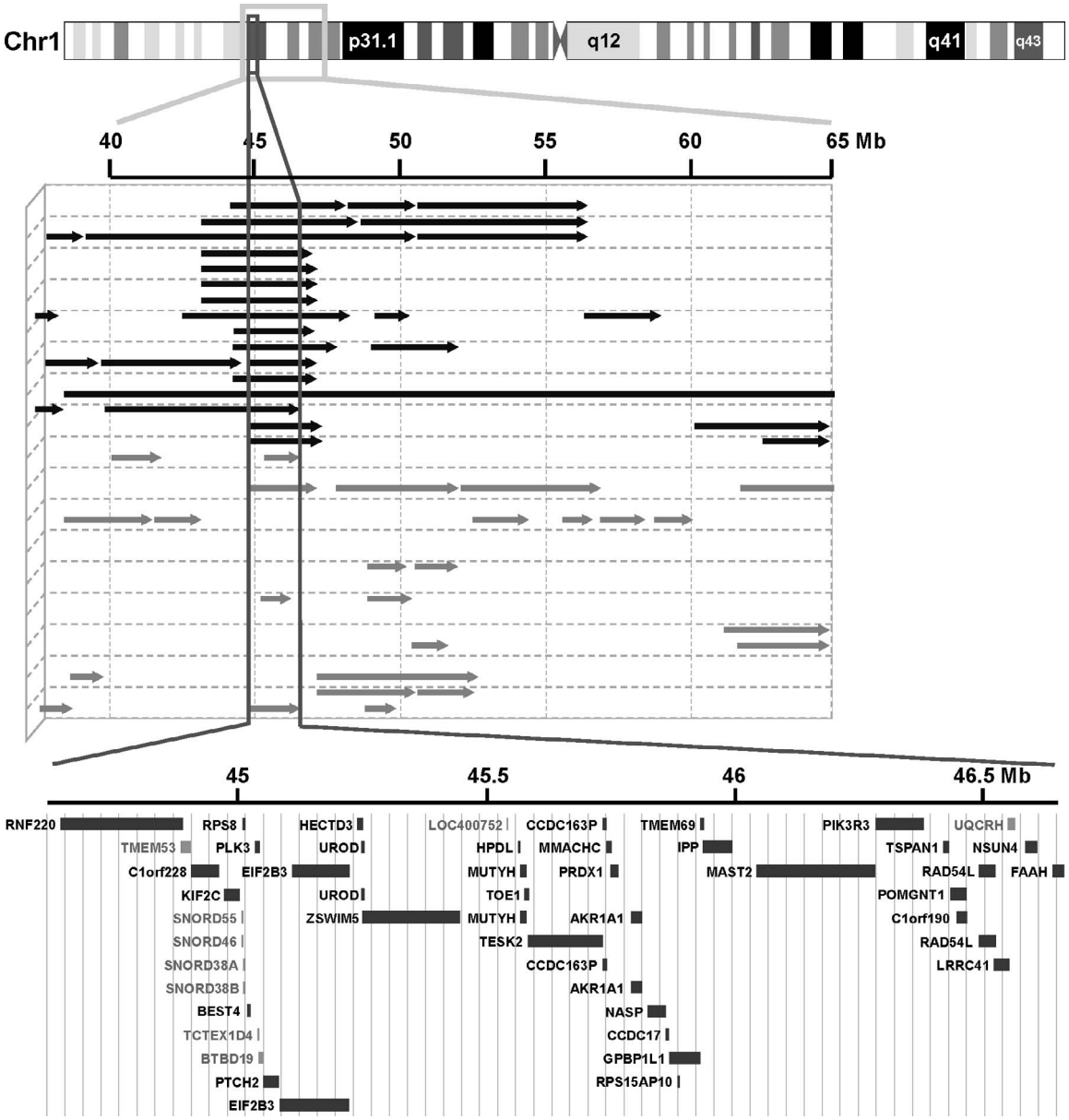

Figure 3. A common homozygous region mapped to 1p34-p33. Each *arrowhead stretch* represents the homozygosity segment identified by analysis of whole-genome SNP genotypes from each individual. *Darker stretches* on the *upper half* of the grid represent individuals affected with RP, and *lighter gray stretches* in the *lower half* represent nonaffected individuals. A 1.8-Mb region was shared by all RP patients on 1p34-p33, although four nonaffected individuals also carried homozygous stretches in the region. This is the only homozygous region shared by all affected members after whole-genome analysis. A total of 56 putative genes were found in this region.

whom discoveries of several arRP genes have been made.[28–30] Homozygosity mapping can be equally powerful in identifying novel arRP genes from nonconsanguineous families residing in areas with little migration or living in a closed community, as shown by Collin et al.,[31] who identified 21 mutations from 186 mainly nonconsanguineous arRP families living in the Netherlands. In the present study, we confirmed the power of combining homozygosity mapping and next-generation sequencing for identifying a novel mutation as the cause of RP in patients from five nonconsanguineous families residing on an isolated island with little migration.

POMGNT1 encodes an enzyme that catalyzes the transfer of N-acetylglucosamine to O-linked mannoses of glycoproteins. This enzyme was first identified as the cause of muscle-eye-brain (MEB) disease (OMIM 253280), an autosomal recessive disorder characterized by congenital muscular dystrophy, structural eye abnormalities, and cobblestone lissencephaly due to loss of the enzymatic activity in O-mannosyl glycosylation.[32,33] Mutations in *POMGNT1* were later linked to an extended group of congenital muscular dystrophies, including congenital with mental retardation (OMIM 613151),[34,35] and a milder limb-girdle form with normal intellect (OMIM 613157).[36] Although the cerebral and ophthalmologic findings in patients carrying *POMGNT1* mutations vary extensively, they always present a certain degree of muscular dystrophy. Our finding in RP patients without muscle involvement coincides with a recent report on *POMGNT1* mutations leading to nonsyndromic RP, and provides an independent

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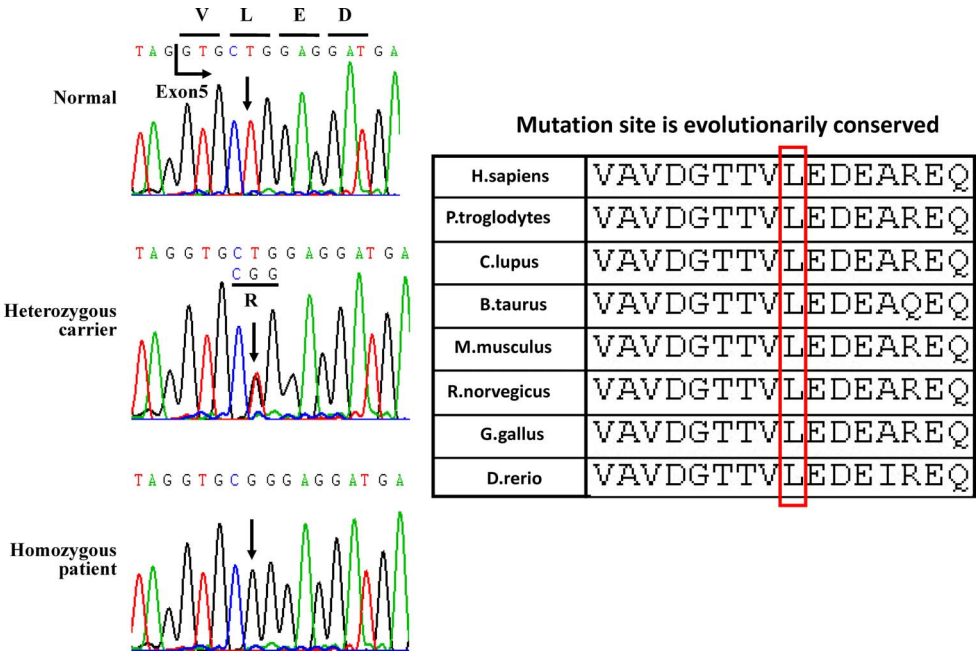

Figure 4. Novel mutation found in exon 5 of *POMGNT1* at a highly conserved region. Sequence profiles of the mutation site for healthy carriers and patients are shown. All patients carried the homozygous mutation, and carriers were heterozygotes. In addition to the mutation site itself, the surrounding bases are also highly conserved.

replication and confirmation of *POMGNT1* mutation leading to RP without causing muscular dystrophy.[37] Although the same gene was identified, we have here identified a novel mutation in this gene.

The novel missense *POMGNT1* mutation associated with RP in this study causes an amino acid change at residue 120, located in the putative stem region of the protein.[32,38] Other previously identified missense mutations in the stem region (E223K and C269Y) of POMGNT1 have been shown to result in the near complete loss of enzymatic activity when mutant constructs were transfected into HEK293T cells[33]; however, when these two mutations were inserted into the soluble form of POMGNT1 lacking 65 amino acids in the N-terminus, the enzymatic activity was restored to 30% and 10% of the soluble WT form, respectively.[39] In another study on the biochemical activity of mutant POMGNT1, missense mutations in the stem region (R265H and C269Y) resulted in residual enzymatic activity, compared with the total loss of activities from frameshift and nonsense mutations, or missense mutations in catalytic domains.[40] However, these stem region mutations were identified from patients carrying compound heterozygote mutations with the presence of one or more frameshift mutations, leading to truncated proteins, or other mutations in the catalytic domain that were shown to result in complete loss of enzyme activity. These patients showed typical MEB phenotypes with severity ranging from severe to moderate or mild.[41,42] The observed phenotypes result from different combinations of mutation effects, and therefore no one-to-one genotype-phenotype correlation can be deduced at present. The function of the POMGNT1 stem region is unclear, although only mutations found in this region have thus far shown residual enzymatic activities in vitro. This is consistent with our finding of the residual enzyme activity of mutant POMGNT1 carrying the L120R mutation, and the RP patients in this study differ from MEB patients in that both mutations are from the stem region of POMGNT1. This may explain why our patients exhibited a particularly mild phenotype compared with other MEB patients carrying at least one mutation that leads to total loss of enzymatic activity.

Our finding of a novel *POMGNT1* mutation in RP patients expands the heterogeneity of RP and suggests O-mannosylation as a possible pathway in retinal degeneration. POMGNT1 is a ubiquitous protein, and several recently identified RP genes (*IDH3B*[43] and splicing factors *PRPC8*, *HPRP3*[44,45]) show generalized expression and housekeeping-like functions; however, the reason for the retina-specific phenotype caused by these mutations remains unknown.

In 2011, two independent studies identified a mutation in *DHDDS* associated with arRP in Ashkenazi Jews.[13,29] *DHDDS* encodes a key enzyme that participates in the biosynthesis of glycoproteins in N-glycosylation, revealing the importance of this posttranslational modification of proteins for maintaining proper retinal function. Inhibition of the N-glycosylation pathway resulted in aberrant photoreceptor morphogenesis in vitro and retinal degeneration in living frogs.[46,47] In contrast to N-glycosylation, O-mannosylation is a less studied form of protein glycosylation, and our finding thus warrants further investigation of its role in RP pathogenesis.

Previous studies of *POMGNT1* and its glycosylation substrate have suggested its role in retina development, although a direct relation to RP is yet to be established. Pomgnt1 knockout mice exhibited a thinner retina with reduced density of retinal ganglion cells, and the inner limiting membrane showed frequent breaks, resulting in disruption of the membrane and subsequent reduction in retinal integrity.[48] Interestingly, dystroglycan (DG), the major substrate of POMGNT1, was shown to be important in eye morphogenesis and is required for proper retinal layering.[49] Further, the recently identified retina-specific α-DG ligand pikachurin was found to be necessary for the formation of photoreceptor ribbon synapse and is important for the physiological functions of visual perception.[50] Binding of pikachurin to α-DG was severely reduced in POMGNT1-deficient mice,[51] and overexpressing glycosyltransferase restored the interaction.[52] These

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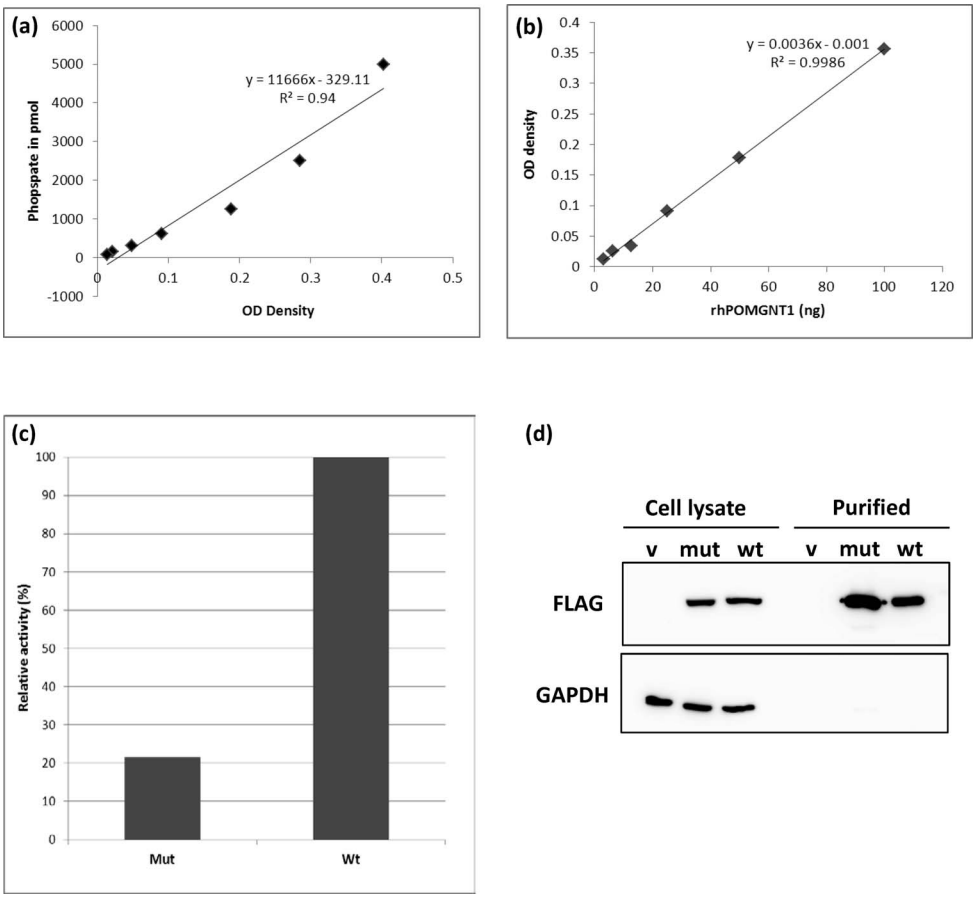

Figure 5. *POMGNT1* enzyme activity assay. (**a**) A standard curve was built with different dilutions of phosphate standards to show the linear relationship between absorbance and phosphate concentration. (**b**) Commercial rhPOMGNT1 was used and tested at different concentrations to show the linearity and effective range of this glycosyltransferase enzyme activity assay. (**c**) Purified proteins from cells expressing wild-type and mutant POMGNT1 were assayed for specific enzyme activity. Three independent experiments were performed and, on average, mutant proteins showed approximately 21.0% (range, 6.5%–36.0%) of the enzyme activity of wild-type proteins. (**d**) Western blot with anti-FLAG and anti-GAPDH antibodies showed that a comparable amount of proteins was used for the enzyme activity assay. v, vector only; mut, mutant POMGNT1; wt: wild-type POMGNT1.

findings suggest that the photoreceptor loss in the RP patients with *POMGNT1* mutation in this study may result from the absence of pikachurin binding as a consequence of the hypoglycosylation of α-DG. Further study is needed and a knockin mouse with c.359A>C would be valuable to elucidate the mechanism of O-mannosylation in retina development and function.

In summary, we here report a novel mutation in *POMGNT1* that causes RP, suggesting O-mannosylation defect in the retina as a possible mechanism for the disease.

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