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ORIGINAL ARTICLE

Mutations in *POMGNT1* cause non-syndromic retinitis pigmentosa

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Abstract

A growing number of human diseases have been linked to defects in protein glycosylation that affects a wide range of organs. Among them, O-mannosylation is an unusual type of protein glycosylation that is largely restricted to the muscular and nerve system. Consistently, mutations in genes involved in the O-mannosylation pathway result in infantile-onset, severe developmental defects involving skeleton muscle, brain and eye, such as the muscle–eye–brain disease (MIM no. 253280). However, the functional importance of O-mannosylation in these tissues at later stages remains largely unknown. In our study, we have identified recessive mutations in *POMGNT1*, which encodes an essential component in O-mannosylation pathway, in three unrelated families with autosomal recessive retinitis pigmentosa (RP), but without extraocular involvement. Enzymatic assay of these mutant alleles demonstrate that they greatly reduce the POMGNT1 enzymatic activity and are likely to be hypomorphic. Immunohistochemistry shows that POMGNT1 is specifically expressed in photoreceptor basal body. Taken together, our work identifies a novel disease-causing gene for RP and indicates that proper protein O-mannosylation is not only essential for early organ development, but also important for maintaining survival and function of the highly specialized retinal cells at later stages.

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Introduction

Retinitis pigmentosa (RP) is a Mendelian disorder featured by night blindness, tunnel vision, progressive retinal degeneration and eventually complete blindness. Its prevalence worldwide is ~1 in 3000–7000 (1). The genetic etiology of RP is highly heterogeneous, with at least 79 disease-causing genes identified (RetNet, https://sph.uth.edu/retnet/). However, there are ~40% RP cases with unknown causative mutations, strongly suggesting novel genes involved in RP pathogenesis (2). RP can be linked to syndromic disorders since genes important for retina also play roles in other tissues (3). For example, defects of various lysosomal proteins in patients with neuronal ceroid lipofuscinosis (MIM no. 256730) have a wide range of neurological and behavioral abnormalities as well as retinal degeneration (4). Also, the loss of pantothenate kinase leads to HARP syndrome (MIM no. 607236) featured by central nervous system problems, hematological disorders and RP (5). Similar cases can be seen in other genetic disorders such as Wolfram syndrome (MIM no. 222300), hyper-IgD syndrome (MIM no. 260920) (6) and a series of ciliopathies (7).

With the advent of whole-exome sequencing (WES) era, large-scale WES in multiple cohorts have greatly improved our understanding about the molecular etiology of RP. Specifically, a number of novel genotype–phenotype correlations have been identified. In these studies, the phenotype spectrum of the mutations in syndrome-causing genes was expanded to non-syndromic RP. For instance, *MVK* mutations were known to cause hyper-IgD syndrome (8), which is a systemic inflammatory disorder. However, recent reports show that *MVK* defects also lead to non-syndromic RP (9). Another example is *CLRN1*, whose mutations were majorly associated with Usher syndrome (MIM no. 276902) (10), is also linked to non-syndromic RP (11). This phenomenon was observed in a long list of recent reports involving genes such as *WDR19* (12), *IFT172* (13), *IFT140* (14), *NEUROD1* (15) and *HGSNAT* (16). Although these reports were sporadic findings during the genetic screening of RP, they together account for a considerable proportion of RP cases. Furthermore, the identification of these genes will improve our ability of RP molecular diagnosis and our understanding of the basic biology of retina.

Glycosylation is a highly diverse category of protein post-translational modifications. It plays essential roles in regulating the protein subcellular localization, transport and quality control (17). Mutations in *DHDDS*, encoding an enzyme that plays a role in N-glycosylation, were reported to cause non-syndromic RP (18,19). Another specific type of glycosylation, protein O-mannosylation, was originally detected in the brains and muscles of mammals (20–22) and later found to be functionally important in these tissues (23,24). Correspondingly, disruption of enzymes in the O-mannosylation pathway will lead to a spectrum of genetic disorders majorly affecting human neuronal and muscular systems (25). One of the key member in the pathway is POMGNT1, a β-1,2-*N*-acetylglucosaminyltransferase enzyme that participates in O-mannosyl glycan synthesis (26). Recessive loss-of-function *POMGNT1* mutations can cause muscle–eye–brain disease (MEB, MIM no. 253280) (27), featured by extensive muscular, neurological and ocular abnormalities (28,29). Specifically, a series of ocular symptoms are seen in patients including retinal degeneration, optic nerve atrophy, impaired electroretinogram (ERG) signals, lens opacification, myopia and strabismus (30). The retinal abnormalities documented in MEB patients suggest the possibility that like other 'syndrome-causing genes with retinal involvement', *POMGNT1* mutations might also be associated with non-syndromic retinal disease.

In this study, we identified recessive *POMGNT1* mutations in three unrelated non-syndromic RP families by WES. Further biochemical studies showed that the mutations significantly impaired POMGNT1 enzymatic activity. Immunohistochemical analysis documented POMGNT1 expression in the photoreceptor layer. Our results greatly expand the phenotype spectrum of *POMGNT1* mutations and provide the first link between the protein O-mannosylation pathway and non-syndromic RP.

Results

Three non-syndromic RP families with *POMGNT1* mutations were identified

The index case we have investigated belongs to a medium-size autosomal recessive RP family of Italian origin diagnosed at the McGill University Health Centre (MUHC), Canada. The proband (MOGL2063) is a 69-year-old male. He was aware of visual problems at the age of 10 years old. He currently has a visual acuity of 20/100 and very restricted visual fields in both eyes (Goldmann visual field test shows 5°). His fundus images show bone spicules, narrow vessels, peripapillary atrophy and a tigroid appearance. The proband's sister is 78 years old (MOGL2064) and also affected with RP. She was aware of nyctalopia and vision problems when she was 12 years old. She currently has visual acuities of 20/80 OD and light perception OS, constricted visual fields (5°) (Fig. 1A and B) and peripapillary atrophy. Funduscopy featured bone spicules and attenuated retinal vessels. Optical coherence tomography (OCT) revealed chorioretinal atrophy, retinal thinning with absent inner segment/outer segment (IS/OS) junctions and cystoid macular edema in both eyes (Fig. 1C and D).

We first performed target capture sequencing on patient MOGL2064 to see if she has mutations in known RP-causing genes. The capture sequencing data did not identify any putative causative variants (data not shown). Then we performed WES on both affected individuals and particularly searched for biallelic variants shared by these two individuals. After data analysis, variants in *POMGNT1* were prioritized as top candidate due to its known link with a retinal phenotype. Both patients have a stop-gain variant (c.187C>T; p.R63*) and a missense variant (c.860T>G; p.I287S) in *POMGNT1*. The R63* variant is absent in the ExAC database (http://exac.broadinstitute.org) and the I287S variant has a frequency of 1 in 40 000, indicating they are rare in the population (Table 1). The stop-gain variant completely abolishes the catalytic domain of POMGNT1 and should confer no enzymatic activity according to previous structure–function studies (31). The I287S variant affects an amino acid (AA) site highly conserved in vertebrates (Fig. 2B) and it is considered damaging by all prediction algorithms (Table 2). The missense variant was not reported in MEB patients before. We performed Sanger sequencing and confirmed that the two variants are *in trans* and two unaffected siblings are wild-type (WT) or single heterozygous at these two loci (Fig. 2A, Supplementary Material, Fig. S1). We revisited the patients to see if they have muscular or neurological abnormalities as shown in MEB patients. Interestingly, both patients (now 69 and 78 years old) have no extraocular disease. These results strongly suggest that *POMGNT1* mutations are associated with non-syndromic RP.

To test our hypothesis that *POMGNT1* is a novel gene for non-syndromic RP, we then screened *POMGNT1* mutations in the WES data of 308 unsolved RP patients. Strikingly, we identified another two cases. Both patients were Han Chinese and diagnosed in Peking Union Medical College Hospital (PUMCH). The first patient is a 32-year-old female born in a consanguineous family (Fig. 2A).

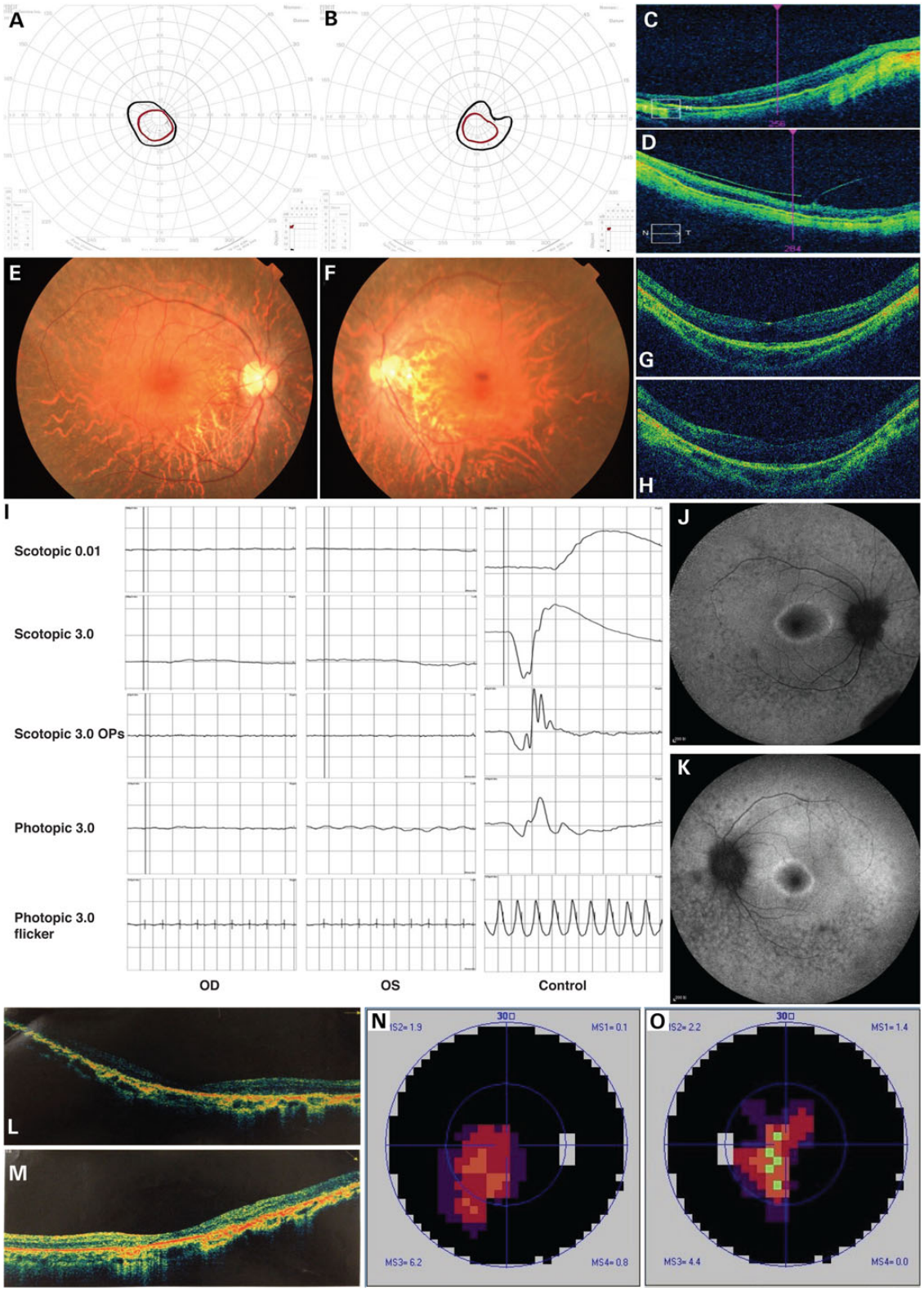

Figure 1. Clinical findings of non-syndromic RP patients in this study. Visual field test results of MOGL2064, right eye (**A**) and left eye (**B**), showing restricted visual field in both eyes. OCT images of MOGL2064, right eye (**C**) and left eye (**D**), featuring chorioretinal atrophy, retinal thinning with absent IS/OS junctions. Fundus images of SRF1105, right eye (**E**) and left eye (**F**), showing the tigroid appearance, thinning of mid-peripheral RPE and loss of choroid-capillaries. OCT images of SRF1105, right eye (**G**) and left eye (**H**), showing thinning of outer retinal layer with preserved foveal photoreceptors. (**I**) ERG results of SRF1105, showing no detectable ERG signals. Fluorescein angiography results of SRF1105, right eye (**J**) and left eye (**K**), showing oval shaped hypofluorescence with a hyperfluorescent ring in the macular and mottled hypofluorescence area in the peripheral retina. OCT images of SRF1630, right eye (**L**) and left eye (**M**), showing thinning of whole retina layers, a flat fovea and disappearance of IS/OS (right eye more severe than left eye). Visual field test results of patient SRF1630, right eye (**N**) and left eye (**O**), showing tunnel vision in both eyes.

Table 1. Clinical features and *POMGNT1* variants of RP patients in this study

ID/ethnicity	Dx	Sex	Age	MS	BR	Other	*POMGNT1* variants	O Freq	EM Freq
MOGL2063/Cau	RP	M	69	No	No	No	c.187C>T (p.R63*)	Absent	Absent
							c.860T>G (p.I287S)	3 in 121 400	1 in 66 738
MOGL2064/Cau	RP	F	78	No	No	No	c.187C>T (p.R63*)	Absent	Absent
							c.860T>G (p.I287S)	3 in 121 400	1 in 66 738
SRF1105/Chi	RP	F	32	No	No	No	c.466G>A (p.E156K) (homozygous)	Absent	Absent
SRF1630/Chi	RP	M	52	No	No	No	c.1895 + 1G>A (p.V633*)	Absent	Absent
							c.1505G>C (p.G502A)	Absent	Absent

Chi, Han Chinese; Cau, Caucasian; Dx, primary diagnosis; MS, if the patient has muscular abnormalities; BR, if the patient has brain abnormalities; Other, if the patient has other disorders. O Freq, overall frequency, occurrence in ExAC database (all population groups); EM Freq, ethnically matched frequency, occurrence in ExAC database within the ethnically matched (EM) population group. Specifically in the ExAC database, East Asian is the EM population group for Han Chinese patients. Non-Finnish European is the EM population group for Caucasian patients; cDNA and protein changes are based on reference cDNA sequence NM_017739.

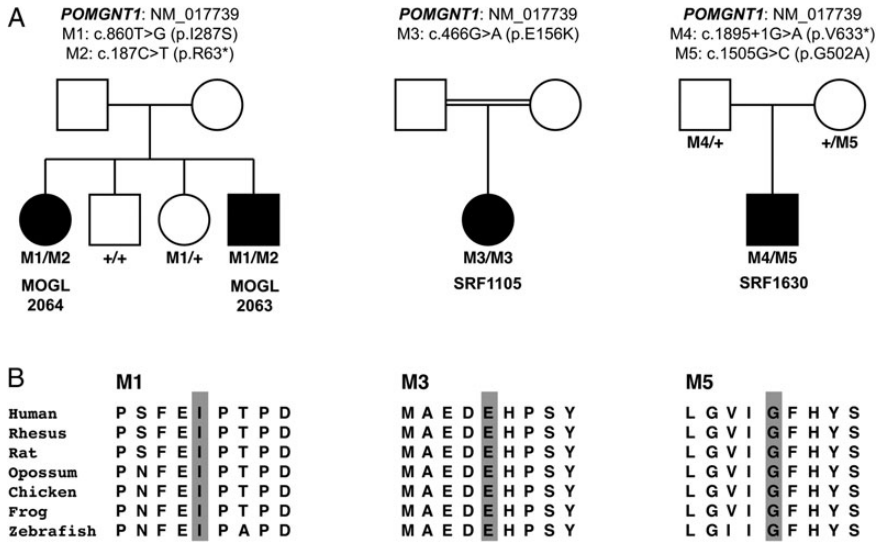

Figure 2. Genetic findings of non-syndromic RP patients in this study. (**A**) Four non-syndromic RP patients from three unrelated families with *POMGNT1* biallelic variants were identified. Genotypes (cDNA and protein changes based on reference cDNA sequence NM_017739) are labeled for each family. (**B**) POMGNT1 protein sequence alignment among selected vertebrates at the loci of *POMGNT1* missense variants identified in our study. All the disrupted amino acids are highly conserved in vertebrates.

Table 2. Predicted damaging effect of *POMGNT1* missense variants identified in this study.

POMGNT1 missense variants	SIFT	Polyphen2_HDIV	Polyphen2_HVAR	LRT	Mutation Taster	Mutation Assessor	FATHMM
c.860T>G (p.I287S)	D	PD	PD	De	Di	M	D
c.466G>A (p.E156K)	D	PD	PD	De	Di	M	D
c.1505G>C (p.G502A)	D	PD	PD	De	Di	M	D

SIFT, scale-invariant feature transform; Polyphen2, polymorphism phenotyping v2; LRT, likelihood-ratio test; FATHMM, functional analysis through hidden Markov models; D, damaging; PD, probably damaging; De, deleterious; Di, disease-causing; M, medium damaging.
cDNA and protein changes are based on reference cDNA sequence NM_017739.

She suffered night blindness since childhood. Her best corrected visual acuity is 20/25 OD and 20/50 OS. Her diopter is 6.00–2.75 × 169° OD and −9.75 to 3.00 × 6° OS. Her fundus showed a tigroid appearance, which possibly indicates thinning of mid-peripheral retinal pigment epithelium (RPE) and loss of choroid-capillaries (Fig. 1E and F). OCT revealed thinning of outer retinal layer with preserved foveal photoreceptor IS/OS junctions (Fig. 1G and H). Visual field testing demonstrated constricted visual field of both eyes. The ERG examination displayed non-detectable waves OU (Fig. 1I). Fundus auto-fluorescence result showed an oval shaped hypofluorescence with a hyperfluorescent ring in the macula and mottled hypofluorescence area in the peripheral retina (Fig. 1J and K). This patient was re-visited, but no syndromic abnormalities were identified. Her serum creatine kinase level is normal (60 U/l, reference: 24~170 U/l) and there is no evidence of periventricular white matter abnormality, ventriculomegaly, pontocerebellar hypoplasia or cerebellar cyst in magnetic resonance images. WES data show that she possesses a homozygous missense variant (c.466G>A; p.E156K) in *POMGNT1* (Fig. 2A). The missense variant is not found in any control database suggesting it is extremely rare (Table 1). It disrupts a highly conserved AA site (Fig. 2B) and considered damaging by all prediction algorithms

(Table 2). The variant is also not found in the list of known *POMGNT1* mutations identified in MEB patients.

The other patient is a 52-year-old male. He presented with night blindness since his youth. Currently he has a visual acuity of 20/400 OD, 20/40 OS. OCT features thinning of the whole retina with a flat fovea and absent IS/OS junctions (Fig. 1L and M). Bone-spicule and salt-and-pepper pigment proliferation were scattered in the whole retina with macular involvement in both eyes. Visual field testing demonstrated tunnel vision in both eyes (Fig. 1N and O). He has a splicing (c.1895 + 1G>A; p.V633*) and a missense (c.1505G>C; p.G502A) *POMGNT1* variant inherited from one parent each. The splicing variant was previously reported in MEB patients and it causes intron retention and immediately generates a premature stop codon (32). The missense variant is absent in control databases, conserved among vertebrates and also predicated to be damaging by all algorithms (Fig. 2B, Tables 1 and 2).

In addition, we performed homozygosity mapping using SNP arrays and identified five index cases with homozygous regions that include *POMGNT1* (Supplementary Material, Table S1). However, mutation analysis of the whole-coding sequence using Sanger sequencing did not reveal any possible pathogenic mutation in these patients.

Functional analysis suggested the hypomorphic nature of the *POMGNT1* missense variants

We performed biochemical analyses to further test the pathogenicity of *POMGNT1* missense variants we identified. Specifically, we did site-directed mutagenesis on POMGNT1-expressing plasmids and transfected them into HEK293T cells. The culture supernatants were used for western blot analysis and POMGNT1 enzymatic activity measurement. Western blot results showed the successful expression of POMGNT1 proteins and their approximately equal molecular weights (Fig. 3A). During the enzymatic assay, we repeated the experiments five times. Compared with the WT POMGNT1 protein control, the two mutant POMGNT1 proteins we assayed (E156K and I287S) have significantly reduced ($P < 10^{-5}$) enzymatic activity, with only 30 and 10% of the WT level retained, respectively (Fig. 3B). This indicated that the *POMGNT1* variants identified in non-syndromic RP cases were hypomorphic mutations instead of complete loss-of-function ones.

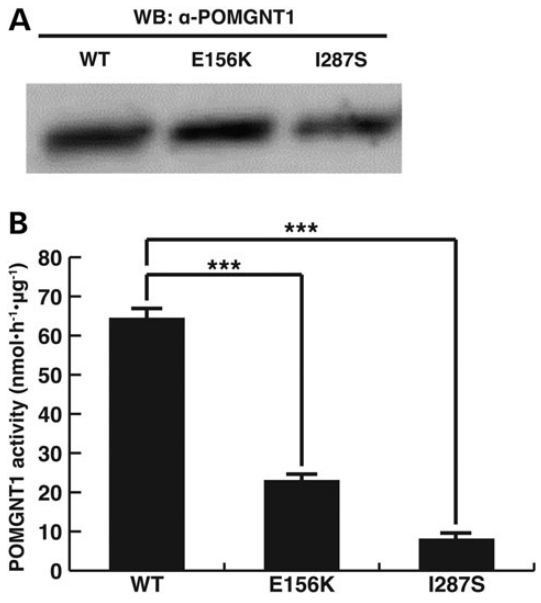

Figure 3. Biochemical studies to test the pathogenicity of two *POMGNT1* missense variants. (**A**) Western blot analysis of POMGNT1 WT and mutant proteins, showing their expression and approximately equal molecular weights. (**B**) Enzymatic activity of POMGNT1 WT and mutant proteins. Both mutants retained significantly lower activity compared with the WT level. Two-tailed Welch's *t*-test. ***$P < 10^{-5}$. Error bars indicate sample standard deviations.

POMGNT1 is expressed in photoreceptor cells

We further performed immunohistochemistry to explore the localization of POMGNT1 protein in the retina. In adult mouse retina cryo-sections, POMGNT1 shows no expression in nuclear layers or plexiform layers (Supplementary Material, Fig. S2), but clear expression in photoreceptor cells (Fig. 4). Specifically, we found that POMGNT1 is localized at the basal body and daughter centriole of photoreceptor cells, suggesting functional involvement at these sites (Fig. 4).

Discussion

POMGNT1 catalyzes the formation of GlcNAcβ1-2Man linkage of O-mannosyl glycans (27). O-mannosyl glycans play roles in the assembly and organization of the basal membranes in muscular and nervous systems, thus crucial to motor activity and neurodevelopment (33,34). As expected, *Pomgnt1*-null mice show typical MEB-like phenotype including muscular dystrophy, brain developmental defects and eye anomalies (35,36). Specifically, in the retina of *Pomgnt1*-null mice, the retinal layers are thinner than those in WT mice (36) and there is a reactive gliosis of astrocytes and Müller glial cells (35), which is also seen in other neurodegenerative disorders with retinal involvement (37). These mice also show abnormal retinal vasculature organization and reduced *a*- and *b*-wave ERG response levels (35,36), which are typical features of RP. The phenotypes in *Pomgnt1*-null mice indicated the essential role of POMGNT1 in the maintenance of retinal structures and functions. Previous studies have identified O-mannosylated dystroglycan and its binding partner Pikachurin are required for photoreceptor ribbon synapse formation (38,39), which is crucial for the neuronal connections between photoreceptors and second-order neurons. Here, we first showed that POMGNT1 is indeed expressed in photoreceptor cells, supporting its functional importance in this cell type. Interestingly, we found POMGNT1 is specifically localized near photoreceptor cilium basal body, a subcellular structure critical for protein transport from inner segments to outer segments, suggesting that POMGNT1 is not only critical for dystroglycan O-mannosylation, but also may modify outer segment proteins. Further proteomic studies would discover outer segment protein substrates of POMGNT1 and unravel the pathological mechanism of POMGNT1-associated retinal disease.

Recessive *POMGNT1* mutations have been reported to cause MEB (27). The phenotype spectrum of MEB is highly variable, ranging from very severe Walker–Warburg syndrome-like phenotype with early lethality (40), to mild phenotype mimicking limb-girdle muscular dystrophy without mental retardation (41). In general, congenital or early-onset muscular dystrophy are the universal features presented, with most patients also developing neurological and ocular symptoms. Interestingly, the discoveries in our study marked a clearly different phenotype from the canonical phenotypes caused by *POMGNT1* mutations.

The non-syndromic feature of RP patients in our study probably resulted from two reasons. First, the missense *POMGNT1*

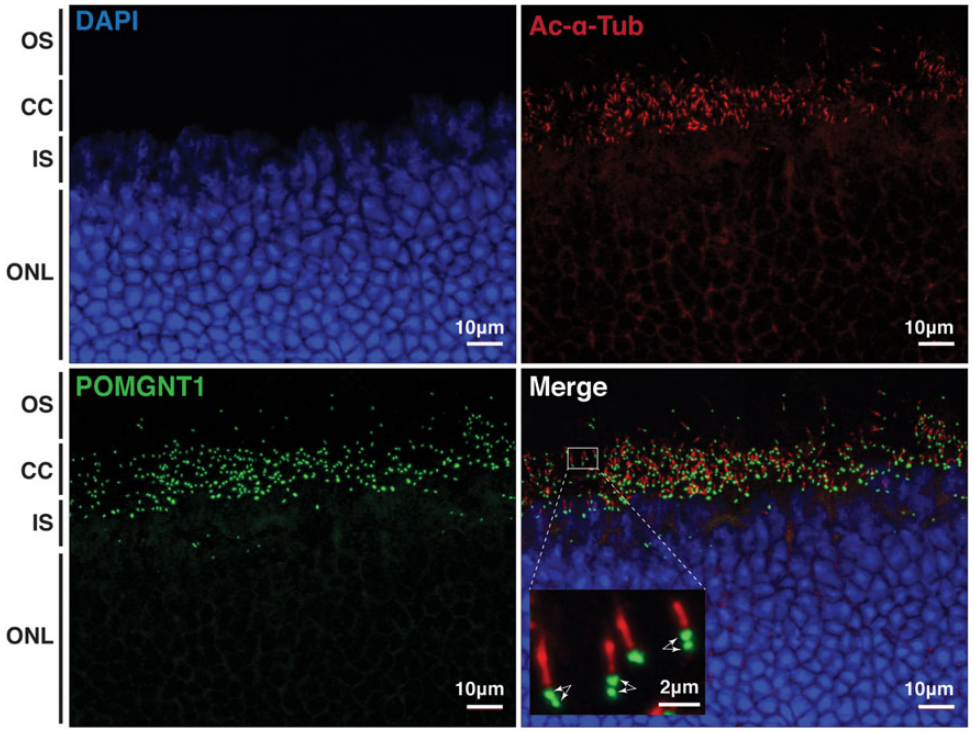

Figure 4. POMGNT1 is expressed in photoreceptor cells. For each individual photoreceptor, the POMGNT1 signal is next to the cilia marker (acetylated-α-tubulin) signal. Two-dot pattern (labeled by double arrows) indicates the localization of POMGNT1 at both the basal body and the daughter centriole of photoreceptor cells. Ac-α-Tub, acetylated-α-tubulin; OS, outer segments; CC, connecting cilium; IS, inner segments; ONL, outer nuclear layer.

mutations in our study are hypomorphic rather than the complete loss-of-function. The majority of the reported *POMGNT1* causal alleles in MEB patients were protein-truncating mutations (42,43), which will lead to the loss of protein activity since it was shown that the deletion of 19 AAs in the C-terminal is sufficient to abolish POMGNT1 enzymatic activity (31). Another two studies showed that nearly all missense *POMGNT1* MEB-causing variants also completely lose their activities (44,45). These results strongly argue that the total loss of POMGNT1 activity underlies typical MEB phenotype. While in our study, both missense variants we tested still maintains subnormal activity (30 and 10%), which seems plausible to correspond to the relatively milder non-syndromic phenotype. Interestingly, another report identified a homozygous hypomorphic *POMGNT1* variant in a patient diagnosed with limb-girdle muscular dystrophy, a phenotype milder than MEB (41), further supporting our contention that the POMGNT1 enzymatic activity level play an important role in determining the phenotype. In fact, studies in other genetic disease have also observed a correlation between disease severity and residual enzyme activity (46–48). The specialized structure, highly dynamic protein turnover and high-energy consumption in retina (49) probably confer higher threshold of POMGNT1 activity than other tissues for maintaining normal structure and functions. This model was also proposed recently in other similar studies identifying RP-related genotype–phenotype correlation (2,16,50). The unique biological profiles in the retina also explains why retinal defects often lie in the mild extremity of the spectra of syndromic phenotypes (51,52).

Secondly, genetic backgrounds or even non-genetic factors probably affect the phenotypes of individuals with *POMGNT1* variants. This has been proposed before (43,53,54) since remarkable phenotypic difference was observed in MEB patients, even with the same causative alleles in the same family. In addition, C57BL/6 *Pomgnt1*-null mice, which presumably possess the same genetic background, also show phenotypic variability in the eyes (35), further suggesting the notion that non-genetic factors have an impact on phenotype. Future studies on *Pomgnt1*-null mice in different genetic backgrounds or under various conditions might help to reveal the complicated mechanisms underlying phenotypic variability.

It should be noted that a number of MEB patients with *POMGNT1* mutations show no ocular symptoms or only non-retinal ocular symptoms including myopia, anterior chamber malformation, microphthalmia, nystagmus, strabismus and cataract (30,43,55). One reason may be that those MEB patients were too young to develop progressive retinal degeneration. MEB patients have a lifespan between 10 and 30 years (40) while the patients in our study are at older ages. In addition, early developmental defects in the eye would hinder the presentation and comprehensive clinical examination of late-onset retinal phenotype. As for the non-retinal ocular symptoms, since O-mannosyl glycan exist exclusively in neuronal and muscular tissues (20–22), they could probably be caused by secondary effects of neurodevelopmental defects or extraocular muscle anomalies as suggested by previous literature (30,56–60).

In summary, the present study identified *POMGNT1* as a novel disease-causing gene for non-syndromic RP. The significance of POMGNT1 specifically in the retina has been underappreciated before since muscular dystrophy and neurodevelopmental defects are the predominant symptoms and major cause for the short lifespan in MEB patients. Our results greatly expand the phenotype spectrum of *POMGNT1* mutations and highlighted the functional importance of POMGNT1 and O-mannosyl glycan in the retina. WES analysis in our cohort suggested *POMGNT1* mutations as a rare cause of non-syndromic RP. Nevertheless, future genetic screening of mutations in other genes involving

O-mannosylation pathway might improve the molecular diagnosis of non-syndromic RP cohorts and enhance our understanding of glycobiology in the retina.

Materials and Methods

Clinical diagnosis of patients and DNA sample collection

The patients were diagnosed by ophthalmic examinations including best corrected visual acuity testing, fundus examination, optical coherence topography (OCT, 3D OCT-2000 Spectral Domain; Topcon, Tokyo, Japan), visual field tests (Octopus, Interzeag, Schlieren, Switzerland), autofluorescence (Spectralis HRA + OCT; Heidelberg, Germany) and ERG (RetiPort ERG system, Roland Consult, Wiesbaden, Germany). RP was diagnosed according to medical and family history, typical fundus and OCT features, visual field defects and attenuated or abolished ERG responses. Blood samples were obtained from all patients and their family members if available. DNA was extracted using QIAamp DNA Blood Midi Kit as instructed by the manufacturer (QIAGEN, Hilden, Germany). This study adhered to the Declaration of Helsinki and was approved by the Institutional Review Board at the MUHC and PUMCH. We obtained informed written consent from all participating individuals for this study.

DNA library preparation and next generation sequencing

Approximately 1 μg of the genomic DNA sample was sheared into fragments of 200–500 bp in length. The sheared fragments were blunt-end repaired and a single-adenine base was added to the 3′ends using Klenow exonuclease. Illumina adapters were ligated to the repaired ends and DNA fragments were polymerase chain reaction (PCR) amplified for eight cycles after ligation. In each capture reaction, 50 pre-capture DNA libraries were pooled together. The targeted DNA was captured by customized retinal disease gene panel for retinal capture sequencing to screen for variants in known disease-causing genes. The detailed information of the retinal capture panel was described previously (2). If no plausible causative variants were identified, the DNA was then captured by NimbleGenSeqCap EZ Hybridization and Wash kit (NimblegenSeqCap EZ Human Exome Library v.2.0) following the manufacturer's protocols for WES. Captured libraries were sequenced on Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) as 100 bp paired-end reads according to the manufacturer's protocol.

Bioinformatics analysis

Paired-end sequencing reads were obtained for each sample. Reads were mapped to human reference genome hg19 using Burrows–Wheeler aligner (61). Base-quality recalibration, local realignment and variant calling were performed as previously described (2). Since RP are rare Mendelian disorders, variants with a frequency >1/200 (for a recessive model) or 1/1000 (for a dominant model) in a series of public databases and internal control databases were filtered out. The list of databases can be found in previous literature (2). We also retrieved variant frequencies from the Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/) database. After frequency-based filtering, we filtered out synonymous variants, identified known retinal disease-causing variants and predicted the pathogenicity of variants using Scale-invariant feature transform (SIFT) (62), polymorphism phenotyping v2 (Polyphen2) (63), likelihood-ratio test (LRT) (64), MutationTaster (65), MutationAssessor (66) and functional analysis through hidden Markov models (FATHMM) (67) as previously described (2,68).

Sanger sequencing

For each suspected causative mutation, a 500 bp flanking sequence at both sides was obtained from the UCSC genome browser (hg19 assembly). RepeatMasker (http://www.repeatmasker.org/) was used to mask the repetitive sequences in human genome. Primer 3 (69) was used to design a pair of primers for generating a 400–600 bp PCR product to sequence the mutation site and at least 50 bp region surrounding it. After PCR amplification, the amplicons were sequenced on ABI 3730xl. Family members of patients were also Sanger-sequenced when available for confirming allele segregation. In addition, five cases with RP and a significant homozygous region covering *POMGNT1* were screened for mutations using primers (Supplementary Material, Table S2) covering all coding exons that were designed using the UCSC ExonPrimer tool (https://genome.ucsc.edu/index.html).

POMGNT1 enzymatic assay

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. The expression plasmids of soluble POMGNT1 were transfected into HEK293T cells, and the media were replaced with 10 ml fresh FBS-free Opti-MEM (Invitrogen) 2 days after transfection, and incubated for one more day. The FBS-free culture supernatants were subjected to western blot analysis and assay for POMGNT1 activity.

The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% gel) and transferred to a PVDF membrane. The membrane was blocked in PBS containing 5% skim milk and 0.5% Tween-20, incubated with anti-POMGNT1 C-terminus antibody (70), and subsequently treated with anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA). Blots were developed using an ECL kit (GE Healthcare). Purified sPOMGNT1 (70) was used as a mass standard to determine the amount of each protein. Optical density measurement of the bands (Image J software) produced a standard curve, which was used to calculate the concentration of each mutant protein.

POMGNT1 activity was obtained from the amount of [^{3}H]GlcNAc transferred to an O-mannosyl peptide (26). The enzyme reaction were carried out in 20 μl reaction buffer containing 140 mM MES (pH 7.0), 0.5 mM UDP-[^{3}H]GlcNAc (100 000 dpm/nmol), 0.5 mM O-mannosyl peptide [Ac-AAPT(Man)PVAAP-NH_2], 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM GlcNAc, 10% glycerol and 2 μl culture supernatant at 37°C for 2 h. After boiling for 3 min, the mixture was analyzed by reversed phase high-pressure (or high performance) liquid chromatography with a Wakopak 5C18–200 column (4.6 × 250 mM). Solvent A was 0.085% trifluoroacetic acid in distilled water and solvent B was 0.085% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 0–25% solvent B. The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter.

POMGNT1 immunohistochemistry

Immunohistochemistry was carried out as described in previous literature (71). Briefly, unfixed eyes of adult WT mice were

harvested and frozen in melting isopentane,16 μm cryo-sections were cut, post-fixed in 0.5% PFA for 10 min and treated with 0.1% Triton X-100 in PBS for 10 min and subsequently blocked in 10% normal goat serum with 0.1% Triton X-100 in PBS (blocking buffer) for 30 min to 1 h. Cryo-sections were then incubated for overnight at 4°C with primary antibodies (rabbit POMGNT1; 1:500; GeneTex) and anti-mouse acetylated α-tubulin (1:200; Santa-Cruz Biotechnology) diluted in blocking buffer. Slides were then washed in PBS, incubated with secondary antibody (Alexa 488 anti-rabbit; 1:500 dilution; Invitrogen) (Cy3 anti-mouse; 1:500; Jackson Immunochemicals) diluted in hybridization buffer at room temperature for 1 h, washed in PBS, DAPI (1:1000 dilution) was used for nuclear counter staining, slides were mounted with anti-fade medium (Prolong gold, Invitrogen) to reduce bleaching, and cover-slipped. Fluorescent images were captured with a Zeiss-Apotome.2 microscope, images were processed using ZEN and Adobe Photoshop CS4.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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