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6 **Genetic background and light dependent progression of photoreceptor cell**
7 **degeneration in Prominin1 knockout mice**
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Running title (within 50 characters) Genetic and light-dependent disease progression

Precis Mutations in the transmembrane Prominin 1 cause photoreceptor degeneration. By using Prominin 1-deficient mice, we show that the degeneration is light dependent and is partially alleviated by fenretinide.

Abstract

PURPOSE: Mutations in *Prominin-1* (*Prom1*) gene are known to cause retinitis pigmentosa and Stargardt disease, both of which are associated with progressive photoreceptor cell death. There are no effective therapies for either disorder. The aim of this study was to investigate the mechanism of the retinal degeneration in *Prom1*-deficient mouse models.

METHODS: We constructed *Prom1* knockout mice with two distinct genetic backgrounds of C57BL/6 and C57BL/6xCBA/NSlc, and investigated the photoreceptor degeneration by means of histology and functional tests. In addition, we examined the effect of light on the *Prom1*^{-/-} retina by rearing the mice in the normal light/dark cycle and completely dark conditions. Finally, we investigated if the retinoic-acid derivative Fenretinide slowed the pace of retinal degeneration in these mouse models.

RESULTS: The *Prom1*^{-/-} knockout mice with both backgrounds developed photoreceptor degeneration after eye opening, but the C57BL/6 background mice developed photoreceptor cell degeneration much faster than the C57BL/6xCBA/NSlc mice, demonstrating genetic background dependency. Interestingly, our histological and functional examination showed that the photoreceptor cell degeneration of *Prom1* knockout mice was light dependent, and was almost completely inhibited when the mutant mice were kept in the dark. The *Prom1* knockout retina showed strong downregulation of expression of the visual cycle components, *Rdh12* and *Abca4*. Furthermore, administration of Fenretinide, which lowers the level of the toxic lipofuscin, slowed the degeneration of photoreceptor cells.

CONCLUSIONS: These findings improve our understanding of the mechanism of cell death in Prominin1 related disease and provide evidence that fenretinide may be worth studying in human disease.

Short title: Genetic background and light dependent photoreceptor degeneration

Key words: Prominin1, retinitis pigmentosa, Stargardt disease, Fenretinide, photoreceptor degeneration, light, modifier gene

1 Introduction

2
3 *Prominin-1 (Prom1)* (NCBI Gene ID 8842; also known as *RP41*, *AC133*, *CD133*, *MCDR2*, *STGD4*,
4 *PROML1*) encodes a 120 kDa pentaspan transmembrane glycoprotein [1], originally identified as a
5 surface antigen of human haematopoietic stem and progenitor cells [2]. Prom1 is expressed in a
6 number of tissues throughout embryogenesis and adulthood [1,3-6], and has been implicated in the
7 maintenance of stem cell characteristics and cell proliferation [6-14]. In addition to these functions,
8 Prom1 has been shown to be involved in a number of genetic disorders that cause photoreceptor
9 degeneration, including retinitis pigmentosa (RP; OMIM 268000; autosomal dominant and autosomal
10 recessive forms) and Stargardt's type 4 (STGD4; OMIM 603786) [15-17].

11
12 RP encompasses a genetically heterogeneous group of inherited disorders causing progressive
13 photoreceptor cell death [15,17] and affects about 1 in 4000 people. It usually starts with early rod
14 photoreceptor damage, manifesting as nyctalopia. Later there is loss of peripheral visual field, and in
15 advanced disease loss of central vision due to death of foveal cones. More than 60 causal genes have
16 been identified, including those encoding transcription factors, splicing regulators, membrane proteins
17 and proteins involved in visual cycle [17].

18
19 Stargardt's disease is one of the macular dystrophies, which predominantly affect the central retina.
20 The most common form of the dystrophy, Stargardt's type 1 (STGD1; OMIM 248200) is an autosomal
21 recessive disorder that is characterized by macular atrophy with or without flecks at the level of the
22 retinal pigment epithelium [16,18] and has a prevalence of at least 1 in 10,000 births; it is usually
23 diagnosed in the first or second decade of life, [19,20] and is recognized as the most common cause
24 of macular disease in children. STGD1 is caused by mutations in the *ATP-binding cassette, sub-family*
25 *A member 4 (ABCA4)* gene (NCBI Gene ID 24; also known as *RP19*, *ABCR*) [21]. There are four
26 types of Stargardt's disease known so far. Three other forms of macular dystrophy have some
27 phenotypic similarities to STGD1 but some of them show autosomal dominant inheritance; Stargardt's
28 type 2/3 (STGD2/3; OMIM 600110) caused by defects in the *ELOVL Fatty Acid Elongase 4 (ELOVL4)*
29 (NCBI Gene ID 6785) and Stargardt's type 4 caused by *Prominin-1 (Prom1)* genes [22-24],
30 respectively. Although there have been a number of different therapeutic approaches suggested [25],
31 there are as yet no effective treatments for this group of disorders.

32
33 Mutations in *Prom1* have been reported in both RP (autosomal dominant and autosomal recessive
34 forms) and STGD4 [15-17]. A homozygous frameshift mutation of *Prom1* gene, causing a premature
35 termination of the translation, was found in an Indian pedigree and resulted in inherited retinal
36 degeneration [26]. A homozygous mutation resulting in a truncated PROM1 protein was identified in a
37 family with severe retinitis pigmentosa (RP), where affected patients reported night blindness and
38 decreased visual acuity in early childhood with subsequent severe loss of central vision in adult life
39 [27]. More recent reports have identified recessive mutations in PROM1 in patients with cone-rod
40 dystrophy [28,29]. In contrast to these autosomal recessive mutations, another mutation,

Prom1/R373C (the mutation where the 373rd Arginine residue was substituted with Cysteine), is inherited as an autosomal dominant trait and causes STGD4 as well as bull's eye macular dystrophy (OMIM 608051) [24]. In this mutation, onset of Prom1 mediated STGD4 varies from 10 to 29 years old with a mean age of 19 years. Thus, Prom1 is associated with a wide range of human retinal dystrophy phenotypes; the disease severity can vary significantly even with individuals who harbor the same disease causing mutation(s).

Mouse models have been generated to recapitulate the retinal degeneration caused by PROM1. To date, a transgenic mouse expressing the human PROM1/R373C [24] and a *Prom1* knockout mouse [30] have been established and both show retinal degeneration. In both models, the photoreceptor layer (outer and inner segment layers) and the outer nuclear layer (ONL) start to degenerate at postnatal day 15 (P15), and are absent by 12 months. [24,30].

It is notable that the onset and progress of the diseases vary in patients, even within a single family [24,27,28,31,32]. Progression of the disease is rapid in some patients, but relatively slow in others. The molecular mechanisms underlying the variation in progression of Prom1-mediated retinal degeneration are however still largely unknown, but may reflect variation in environmental factors or modifier genes.

In this study, we have constructed new *Prom1* knockout mice to recapitulate the haploinsufficient-type of photoreceptor degenerative diseases [22,26]. We raised the mutant mice under distinct genetic backgrounds, and found that they showed evidence of photoreceptor cell degeneration with a different rate of progression. Using these models, we investigated the molecular mechanisms of the disease phenotype.

Methods

Animals and housing conditions

All animals in this study were subject to local and national ethical approval and guidance (University College London Ethical Committee and UK Home Office Regulations for Animal Use; RIKEN CDB Animal Care and Use Committee).

Two distinct genetic backgrounds (C57BL/6 and a hybrid of C57BL/6xCBA/NSIc) of *Prom1* knockout mice were analyzed. The generation of *Prom1*-deficient mice with C57BL/6 background (RIKEN strain) has been previously reported [10], and the information has been deposited in the database of RIKEN Center for Developmental Biology ([http://www.cdb.riken.jp/arg/mutant mice list.html](http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) (accession number: CDB0623K)). Due to breeding difficulties on this background, *in vitro* fertilization (IVF) using *Prom1* heterozygote sperm and eggs obtained from Wild Type CBA/NSIc mice was employed. The resulting heterozygotic sperm and eggs were then used for the second IVF to obtain *Prom1*^{-/-} mice. The mice with both backgrounds were used for histological analyses (Figure 1A-E, 3A-E), whereas all other experiments were performed only with the C57BL/6xCBA/NSIc background. *Prom1*^{-/-} and littermate controls were housed and maintained under standard laboratory conditions with a 12-hour day (160-lux)–night cycle and *ad libitum* access to food and water.

Effect of Light on *Prom1* retinal degeneration

For dark rearing (darkness luminescence <0.5 lx), female mice and their litters (P1 or P8) were transferred into a ventilated housing cabinet (Scantainer I-110, Scanbur, Denmark), which was further modified in house to reduce light exposure from the outside. Dark-housed animals were kept in the cabinet for approximately 4 weeks before analysis. The extent of photoreceptor degeneration was examined at P30. Electroretinograms (ERGs) were performed to assess retinal function. Mice were subsequently culled and the eyes enucleated under dim red light and processed for paraffin sectioning. Animal husbandry was performed under red light (emission spectrum above 600 nm, red lamp, #02580, British Electrical Lamps Limited, UK).

Histology

Eyes were enucleated and fixed in 4% buffered paraformaldehyde (PFA, Sigma-Aldrich, Gillingham, UK) in 1X PBS for 30 min at room temperature. To allow penetration of the fixative in adult eyes a circumferential cut was made around the posterior margin of the ciliary body and only the posterior eyecup was retained and fixed for another 30 min. Eyecups were processed with a series of ascending alcohols; cleared in trichloromethane; then embedded in paraffin. Retina paraffin blocks were cut sagittally at 5 µm thickness using a microtome. Sections for hematoxylin and eosin (HE) staining were first de-waxed in xylene, rehydrated, rinsed in water, and stained. The sections of the central part of the eye were analysed, and the images were taken at the top part.

Electroretinography (ERG)

Electroretinography (ERG) was performed using a ColorDome Ganzfeld ERG (Diagnosys LLC, Cambridge, UK). Briefly, mice were dark-adapted overnight for scotopic measurements and anesthetized under red light illumination by intraperitoneal injection of 50-75 mg/kg ketamine (Narketan, National Veterinary Services Ltd, Stoke-on-Trent, UK) and 10% medetomidine (Domitor, National Veterinary Services Ltd). Pupils were dilated using 10 mg/kg tropicamide and 0.5% phenylephrine hydrochloride. Animals were kept on a heating mat throughout the procedure to maintain the body temperature at 37°C. Subdermal needle electrodes (CareFusion, Middleton, WI, USA) were inserted as ground and reference electrodes near the hindquarter and between the eyes, respectively. Gold ring electrodes were placed on the corneal surfaces, which were lubricated using carbomer gel (Viscotears, Novartis Pharmaceuticals, UK). Following a further 10 minutes of dark adaptation the scotopic ERG was simultaneously recorded from both eyes with increasing stimulus strengths using mixed white light from 10^{-5} cd.s/m² to 10 cd.s/m² with every 10-fold interval. Four animals per genotype were analyzed in each set of experiments,

Immunohistochemistry

Following antigen retrieval (boiled in Tris-EDTA pH 9.0) sections were stained with either mouse monoclonal anti-Rhodopsin (Sigma) diluted 1:500 or rabbit polyclonal anti M-opsin (Millipore) diluted 1:500. Secondary antibodies included goat anti-mouse Alexa Fluor 488[®] or goat anti-rabbit Alexa Fluor 488[®]. Images were collected with LSM 710 confocal microscope (Zeiss).

Treatment with fenretinide and A1120

Administration of fenretinide and A1120 was performed as described previously [33,34], with slight modifications. Briefly, *Prom1*^{-/-} mice (P0) were injected intraperitoneally three times a week with 10 mg/kg of Fenretinide (N-[(4-hydroxyphenyl)amino]retinal; also known as 4-HPR; CAS number 65646-68-6) or the equivolume of DMSO (Dimethylsulfoxide) for 4 weeks. A1120 (2-(4-(2-(trifluoromethyl)phenyl)piperidine-1-carboxamido)benzoic acid; CAS number 1152782-19-8) [35,36], an RBP4 (NCBI gene ID 5950; Retinol Binding Protein 4) inhibitor, was tried at 30 mg/kg (see Discussion). Treated mice were subject to the analyses two days after the last injection.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from whole eye samples using RNeasy mini kit (Qiagen, Crawley, UK) following manufacturer's instructions. Reverse-transcription was performed using Superscript II Reverse Transcription Kit (LifeTechnologies). Real-time quantitative RT-PCR was performed with a thermal cycler (7900HT; Applied Biosciences). Reagents were obtained from Applied Biosystems and all primers were designed to span an intron to avoid amplifying genomic DNA. 18S was used as the internal control. The primer sequences are available in Supplementary Table 1. PCRs were conducted using at least three separate RNA preparations.

Electron microscopy

1 Eyes were enucleated and fixed in 3% glutaraldehyde and 1% paraformaldehyde in 0.08 M sodium
2 cacodylate-HCl (pH 7.4) for at least 30 h at 4 °C. The cornea and lens were removed and the eye
3 cups oriented and post-fixed in 1% aqueous osmium tetroxide for 2 h, dehydrated by an ascending
4 ethanol series (50%–100%) and propylene oxide, and infiltrated overnight with a 1:1 mixture of
5 propylene oxide and araldite resin (Agar Scientific Limited, Essex UK). After 8 h in full resin, the eyes
6 were embedded in fresh resin and incubated overnight at 60 °C. Semithin (0.7 µm) sections were cut
7 in the inferior–superior axis passing through the optic nerve head with a microtome (Ultracut S; Leica,
8 Wetzlar, Germany) Ultrathin sections were collected on copper grids (100 mesh, Agar Scientific),
9 contrast-stained with 1% uranyl acetate and lead citrate and analyzed using a JEOL 1010
10 transmission electron microscope at 80 kV. Retinal sections from three animals per genotype were
11 analysed.

12 **Statistics**

13 Electroretinogram (ERG) results were compared using two-way ANOVA on GraphPad Prism V4
14 (GraphPad software) with *p*-values of < 0.05 considered statistically significant.

Results

***Prom1* knockout mice develop photoreceptor cell degeneration in a genetic background dependent manner**

In order to examine the mechanism of *Prom1* mediated photoreceptor degeneration, we originally made a *Prom1* deficient mouse with pure C57BL/6 background [10], in which the wild type does not develop any degeneration or have retinal abnormalities (Figure 1A,B). At postnatal day 14 (P14), the layered structure, morphology of retinal neurons and glial cells, in particular morphology of the photoreceptor outer segment, and the numbers of retinal cells, showed that the retina was normal (Figure 1C,E). In contrast, at P20 the photoreceptor cell layer including the outer segment and cell body was completely missing (Figure 1D,E). Thus *Prom1*-knockout mouse developed photoreceptor cell degeneration (Figure 1C-E) as observed in the previously reported *Prom1* knockout mice [30].

Due to the fact that C57BL/6 background *Prom1*^{-/-} mice have difficulty in breeding, we generated the same *Prom1*-targeted mice with a hybrid background of C57BL/6xCBA/NSIc (see Material and Methods). This new knockout also started to exhibit extensive photoreceptor outer segment degeneration after P14 (Figure 1F,J,N), which coincided with eye opening. However, at P20, in contrast with the *Prom1*^{-/-} mice on the C57BL/6 background (Figure 1D,E), the outer plexiform layer (OPL), the outer nuclear layer (ONL) and outer/inner segments (OS/IS) were still preserved with thickness of 74%, 76% and 61%, respectively, compared to the wild-type mice (Figure 1G,K,N). This unexpected result prompted us to analyze the progression of the photoreceptor degeneration chronologically. The *Prom1*^{-/-} mice with C57BL/6xCBA/NSIc at P30 showed a substantial degree of retinal degeneration with approximately 22% of photoreceptor nuclei remaining (Figure 1H,L,N). By 4 months (P120) the entire ONL and OS/IS had been completely abolished (Figure 1I,M,N). On the other hand, the inner nuclear layer (INL) and inner plexiform layer (IPL) were largely unaffected (Figure 1I,M,N).

These histological observations suggest that PROM1 is essential for the maintenance, rather than the development of retinal structure, and the phenotype with *Prom1* deficiency is dependent on the genetic background.

Expression of rhodopsin and M-opsin in the photoreceptor cells is downregulated and misplaced in *Prom1*-deficient mice.

We further analysed the ultrastructure of the *Prom1*^{-/-} in C57BL/6xCBA/NSIc retinal cells by using transmission electron microscopy (TEM). At P20, the photoreceptor outer segments of the retina were severely shortened in the *Prom1*^{-/-} mice compared to that of wild-type (Figure 2A,B), which is consistent with the histological findings (Figure 1K).

In the C57BL/6xCBA/NSIc mice, P20 is the beginning stage of the photoreceptor degeneration, and this enabled us to observe the intermediate stages of the degeneration. The tight junction structure at the outer limiting membrane seemed to be largely intact in the *Prom1*^{-/-} retina (Figure 2C,F; red

arrowheads) [37], and the mitochondria were formed normally in the inner segment (Figure 2D,G; red arrowheads). However, the structure of the outer segment was severely disorganized and misaligned (Figure 2E,H). This was consistent with the previous observation in the mice with the different genetic background [30].

We next examined the expression of photoreceptor specific proteins in the *Prom1* knockout by immunohistochemistry. At P20, WT retina showed a strong signal of the G-protein coupled receptor (GPCR) rhodopsin correctly localized to the outer segment in the rod photoreceptor (Figure 2I,I'). In contrast, in the *Prom1*^{-/-} retina, the signal was found in the inner segments and in the outer nuclear layer (Figure 2J,J') [30]. In addition, another GPCR M-opsin, normally localized to the outer segment of cone photoreceptors (Figure 2K,K') [38], was largely missing in *Prom1*^{-/-}, and was partially found in the outer nuclear layer, with the occasional cells showing positive signal (Figure 2L,L').

Taken together these observations demonstrate Prom1 is required for the maintenance of both rod- and cone- photoreceptor cells, and for the correct localization of opsin proteins that act in the outer segments.

Light-dependent impairment of photoreceptor cells in the *Prom1*-deficient mice

The photoreceptor degeneration in both mice strains of the pure C57BL/6 background and on the C57BL/6xCBA/NSlc background was initiated after P14, when they open their eyes. We thus hypothesized that Prom1 mediated photoreceptor degeneration was triggered by light exposure.

In order to examine the effect of light on the photoreceptor degeneration in *Prom1* knockout mice, the C57BL/6 mice were reared in complete darkness and compared with mice kept under normal light-dark cycle (see Material and Methods for details). At P30, the *Prom1*^{-/-} kept under normal light cycle indeed had no photoreceptor cells (OS,IS,ONL in Figure 3A,B). In contrast, the animals reared under the dark conditions still had some photoreceptor cells remaining, and their outer segments of comparable length to the wild type control (Figure 3C,D). Similar observations were made in the *Prom1*^{-/-} mice with the C57BL/6xCBA/NSlc background (Figure 3E-H); the *Prom1*^{-/-} mice reared under the normal light-dark cycle developed a severe photoreceptor degeneration (Figure 3E,F), while the same *Prom1*-depleted animals under the dark condition retained a significant proportion of the photoreceptor layer (Figure 3G,H). These observations suggest that the photoreceptor degeneration in *Prom1*-knockout mice developed in a light dependent manner both in C57BL/6 and C57BL/6xCBA/NSlc backgrounds.

We further analyzed the ultrastructure of the photoreceptor cells by transmission electron microscopy (TEM). The outer segment of the *Prom1*^{-/-} retina reared under dark conditions partially retained the structure of the cells (Figure 3I-L, compared to Figure 2B,F-H). In addition, as examined by immunohistochemistry in the *Prom1*^{-/-} mice, less mislocalized signal of rhodopsin and M-opsin staining was found in the inner segments compared to those under the light/dark condition (Figure 3M-O,

1 compared to Figure 2J,J',L,L'). Together these observations indicate that the *Prom1*^{-/-} photoreceptor cell
2 structure can be retained when the retina is protected from light.

3
4 We next investigated the function of photoreceptor cells in the C57BL/6xCBA/NSlc *Prom1*^{-/-} mice. To
5 this end, we examined the a and b waves of the electro-retinogram (ERG). ERG is conducted to
6 measure the electrical responses of the retinal cells upon external stimuli, and may be used to
7 evaluate progression of retinal diseases [39]. An ERG consists of two peaks; the initial peak (a-wave;
8 Figure 4A) reflects the physiological health of the rod or cone photoreceptors and the second (b-wave;
9 Figure 4A), the inner layers of retina, including bipolar, amacrine and Müller cells [40]. We performed
10 both scotopic (Figure 4A-F) and photopic (Figure 4G,H) ERGs to investigate the function of rod and
11 cone photoreceptors, respectively.

12
13 First we performed scotopic ERGs on the P30 mice (C57BL/6xCBA/NSlc) reared under the normal
14 light/dark condition. The *Prom1*^{-/-} mice showed marked impairment of a- and b-waves (Figure 4C and
15 red lines in Figure 4E,F).

16
17 We next performed scotopic ERGs on the *Prom1*^{-/-} mice raised under complete darkness during the
18 period between P8 and P30. Strikingly, the a-wave and b-wave of the scotopic ERG in the dark-reared
19 *Prom1*^{-/-} mice were significantly improved compared to those reared in the normal light/dark condition,
20 and the b-wave reached almost the same level as that of the wild-type retina (Figure 4E,F; blue lines).
21 The amplitudes of a- and b-waves in the dark-reared wild-type mice appeared to be the same as those
22 in the wild-type mice reared in the normal light/dark cycle, suggesting that the light stimulation *per se*
23 did not influence the condition of the wild-type retina (Figure 4A,B,E,F; black and green lines). Taken
24 together, this set of analysis demonstrated that *Prom1*^{-/-} photoreceptor cells are functionally impaired
25 upon light exposure.

26
27 We additionally performed photopic ERGs. In agreement with the scotopic ERGs, the amplitudes of
28 both a-waves and b-waves in the photopic ERGs were reduced in the *Prom1*^{-/-} mice reared under the
29 normal light/dark cycle, but maintained if the knockout mice were protected from the light exposure
30 (Figure 4G,H).

31
32 Altogether these structural and functional examinations indicate that photoreceptor cells in the *Prom1*^{-/-}
33 mice are very vulnerable to light and damaged in a light dependent manner.

34 **Expression of genes involved in the visual cycle is affected in the *Prom1* knockout mice**

35 Light-dependent retinal degeneration is caused by a number of factors (see [41] and [42] for reviews).
36 Since some of these (e.g. *Abca4*, *Rdh8*, *Rpe65*, rhodopsin) are highly associated with the visual cycle,
37 we investigated the effect of the *Prom1* gene knockout on the visual cycle.
38
39

The visual cycle is a process which maintains the levels of the chromophore in photoreceptor outer segments [16]. Phototransduction starts with the conversion of 11-*cis*-retinal into all-*trans*-retinal on exposure of the photoreceptors to light. The all-*trans*-retinal is then recycled initially in the photoreceptors and later in the RPE (retinal pigmented epithelium) cells to reform 11-*cis*-retinal. All-*trans*-retinal is first reduced to all-*trans*-retinol (Vitamin A), a process which requires ABCA4 [19,43,44], RDH12 [44-47] and RDH8 [44]. All-*trans*-retinol is then transported with IRBP to the RPE cells, where it is converted, with the aid of LRAT to all-*trans*-retinyl-ester and further to all-*trans*-retinol with the help of the isomerase RPE65. This all-*trans*-retinol is oxidized into 11-*cis*-retinal by RDH5/11 and is transported back to the rod cells with IRBP, where it forms a complex with rhodopsin (reviewed in [16,48,49]).

In order to investigate how Prom1 is involved in this visual cycle, we examined the expression of visual cycle genes in PROM1-deficient mice. We isolated the eyes from the P14 wild-type or *Prom1*^{-/-} mice and performed qRT-PCR analysis. Expression of *Abca4* and *Rdh5/12/14* was significantly reduced in the *Prom1*^{-/-} retina. In contrast, the expression of *rhodopsin*, *IRBP*, *LRAT*, *S-opsin*, *Rdh11* and *Rdh13* were not significantly reduced compared to that in the wild-type tissue, whilst *M-opsin* was rather upregulated (Figure 5). This data suggests that PROM1 is required for the maintenance of the expression levels of *Abca4* and *Rdh12*, and is consistent with the idea that PROM1 is involved in the regulation of the visual cycle, especially at the reducing step of all-*trans*-retinal to all-*trans*-retinol.

Fenretinide can partially prevent the photoreceptor degeneration

Failure of the reduction of all-*trans*-retinal to all-*trans* retinol results in the formation of all-*trans* retinal dimer and A2E (a lipofuscin fluorophore; also known as N-retinylidene-N-retinylethanolamine) [48,50-53], both of which induce inflammation and subsequent photoreceptor cell dysfunction. The accumulation of these compounds has been observed in *Abca4*^{-/-}, *Rdh8*^{-/-} and *Rdh12*^{-/-} mice [33,44,53-55].

The synthetic retinoid derivative Fenretinide reduces the availability of retinoids to the eye by its interaction with RBP [33] (Retinol Binding Proteins; RBP1-4; NCBI gene IDs 5947-5950) [56,57] and thereby reduces accumulation of A2E in retinal pigment epithelium. As Fenretinide has been investigated in animal models of *Abca4*-mediated Stargardt diseases [33,34,58], we investigated whether Fenretinide may also reduce photoreceptor cell death in *Prom1*^{-/-} mice.

First, we examined the effect of Fenretinide on the *Prom1*^{-/-} mice. We injected 10 mg/kg of Fenretinide 3 times a week from P0 for four weeks. At P30, compared to the vehicle injected control, Fenretinide injected *Prom1*^{-/-} mice had thicker photoreceptor segments and ONL, and increased number of photoreceptor nuclei (Figure 6A-E). A similar result was obtained with a higher dosage (20 mg/kg) of Fenretinide (unpublished data). This finding suggests that Fenretinide protected, at least in part, the photoreceptor cells from *Prom1*-deficient mediated degeneration.

1 Next we evaluated the effect of Fenretinide on the function of *Prom1*^{-/-} retina by examining the
2 scotopic ERG responses. One caveat in this examination is that Fenretinide induces night-blindness
3 as a side-effect, because the function of rhodopsin is partially blocked and the visual cycle is slowed
4 down [34]. Consistently, the a-wave amplitude was decreased in the Fenretinide-treated retina (Figure
5 6F; gray line) compared to the non-treated retina (Figure 6F; black line), indicating that rod function is
6 affected by Fenretinide. In contrast, in the *Prom1*^{-/-} retina, which were almost insensitive to the
7 stimulus unless treated (Figure 6G, black line), the rod responses were improved when treated with
8 Fenretinide (Figure 6G, red line). This effect was more evident when the results (Figure 6F,G) were
9 replotted against the control (DMSO-treated) retina as the reference (Figure 6H). This observation
10 suggests that Fenretinide despite its inhibitory effect on normal rod cells function improves rod
11 function in *Prom1*^{-/-} mice possibly by reducing cell death. Likewise, the b-waves were decreased
12 (Figure 6I,K) when Fenretinide was treated in wild-type retina, but were improved in the *Prom1*^{-/-} retina
13 upon treatment with Fenretinide (Figure 6J,K).

14
15 Together the analyses indicate that the treatment with Fenretinide maintains the structure of
16 photoreceptor cells, which is accompanied by a partial recovery of function.
17

Discussion

Genetic background influences the progression of photoreceptor degeneration

This study demonstrates that variations in the genetic background significantly influence development of photoreceptor cell degeneration mediated by knockout of the *Prom1* gene. We examined the mutants with two genetic backgrounds; a pure C57BL/6 and a hybrid C57BL/6xCBA/NSIc. The *Prom1*^{-/-} mice of the pure C57BL/6 background exhibited complete photoreceptor degeneration within one week after eye opening (P20), while the retinal degeneration in the C57BL/6xCBA/NSIc mice developed much more slowly (Figure 1). The same mouse strain showed the same degeneration profile, suggesting that the variations in photoreceptor degeneration seem to be dependent on the genetic background, rather than the individual mice. Variations in disease severity are reported in patients with retinal disease caused by both autosomal-recessive (AR) [26,28,29] and autosomal-dominant (AD) *Prom1* mutations [32]. Mouse knockout models are good models for human disease caused by AR *Prom1* mutations but are not as useful as for studying the mechanism of AD disease. However it is possible that environmental or genetic modifiers may be common to both types of disorder.

A number of factors, both environmental and genetic, may account for this variation. In contrast to the studies in man, the environmental factors can be carefully controlled in experimental studies in mouse models; the mice used in our experiments were reared under carefully standardized conditions. Therefore, most of the variation in the progression of the photoreceptor degeneration is likely to be due to the genetic factors. The mechanisms underlying these strain differences are unknown and their identification will be challenging. The two types of the mice used in our experiments will be a good model system to identify such genetic factors for making this variation.

It should also be noted that there is diversity even in the same genetic background [30]. The previously reported *Prom1*^{-/-} mice with the same C57BL/6 background started the degeneration around P14, but even after 6 months the mice still had some remaining photoreceptor cells [10,30], which is much slower than our knockout mice with the same C57BL/6 background employed in our study (Figure 1A-E). This may be due to minor genomic variations raised by the difference of the mouse source [30]. More detailed study of these various mouse models will help identify these presumed genetic modifiers.

The divergence of the severity in the mutant has been defined as “expressivity” [59-61]. It has been suggested that expressivity is caused by modifier genes, which quantitatively influence the effect of the loss of the targeted gene. In the case of *Prom1*, *Abca4* and *Rdh12* may play such roles. This view is supported by the fact that the severity of light induced photoreceptor damage is dependent on the expression level [62] or genetic polymorphisms [63,64] of the *Rpe65* gene. Further work is needed to identify the modifier gene(s) for *Prom1*, including the investigations of genomic variation, for example SNP (Single-nucleotide polymorphism) and epigenetic status of the genomes in the different individuals or genetic backgrounds.

Light-dependent photoreceptor degeneration in *Prom1*-mutant mice and possible therapies for *Prom1*-causing diseases

It has been shown that STGD1 patients are sensitive to light, and undergo light-protective treatments [65]. This is consistent with the light-dependent photoreceptor degeneration found in the *Prom1*^{-/-} mice, raising the possibility that Prom1 is involved in the visual cycle function. Our qRT-PCR analysis revealed the reduction of *Abca4* and *Rdh12* expression at the onset of the photoreceptor degeneration, both of which are involved in the visual cycle (Figure 5). These two genes may therefore be downstream targets of Prom1. An alternative possibility is that *Prom1* mutant cells start to degenerate first, and *Abca4* and *Rdh12* expression is downregulated as a secondary effect of photoreceptor cell death. We would favor the former possibility, as the reduction of *Abca4* and *Rdh12* was highly selective – *Abca4* and *Rdh12* are downregulated whereas expressions of other genes were unchanged (Figure 5) – suggesting that the gene regulation by PROM1 is independent of the photoreceptor degeneration.

How does PROM1 control the expression of these genes? As PROM1 is a transmembrane protein, PROM1 may transduce the extracellular information or stimulation intracellularly. Future transcriptome studies will provide systematic information about downstream target genes and possible upstream regulatory pathways.

Accumulation of all-*trans*-retinal dimer and A2E has been reported in the STGD1 model mice with mutations of *Abca4* [51,66,67]. A2E is the major fluorophore of lipofuscin and high levels of lipofuscin are seen on fundus autofluorescence imaging in patients with Stargardt disease. As Prom1 deficiency in our mouse models results in downregulation of *Abca4* and *Rdh12* gene expression, it is likely that ensuing effects on the visual cycle will lead to accumulation of lipofuscin such as all-*trans*-retinal dimer and A2E in the *Prom1*^{-/-} mutants, although the direct measurement is yet to be performed.

Fenretinide, originally recognized as an anti-cancer drug and later applied to retinal disease [68,69], has been investigated in mouse models of STGD1 disease [36]. The potential human use of Fenretinide will depend on the balance between the benefit (desired effect) to inhibit the accumulation of the toxic lipofuscin [56,57], and the systemic and ocular side effects. Fenretinide use in animal models (Figure 6F,H,I,K, Supplementary Figure S2A,C) [70], and in man [71] leads to rod dysfunction. On the other hand, the *Prom1*-mutant mice treated with Fenretinide showed improved retinal structure (Figure 6A-E) and improved scotopic ERG (Figure 6G,H,J,K) indicating that the positive effect on photoreceptor degeneration outweighed the inhibitory effect on rod function.

However, the recovery of the photoreceptor function from our fenretinide treatment experiments still needs to be improved. In particular, the photopic ERG responses were not significantly improved by fenretinide (Supplementary Figure S2). One of the possibilities for this limitation is the method of administration. Recent studies have shown fenretinide has very poor oral bioavailability due to low

1 solubility and low permeability [72-74]. Therefore, for ocular diseases, a controlled-release formulation,
2 which provides drug release over a longer period of time, may be advantageous [75]. We have tried
3 another RBP binding compound A1120, which has been used in STGD1 mouse models [35,36]. The
4 drug was however, less effective than Fenretinide, (MD and SO; unpublished data). As some primary
5 amines and GPCR/adenylate cyclase inhibitors have recently been introduced to improve the
6 photoreceptor survival in *Abca4*^{-/-} ; *Rdh8*^{-/-} mice [48,76], it would be interesting to explore their effects
7 in *Prom1*^{-/-} mice.

8
9 In addition to its effect on the visual cycle, it has also been shown that PROM1 interacts with
10 Protocadherin-21 (NCBI Gene ID; 92211; *PCDH21*, *CDHR1*) [24,77], which has also been implicated in
11 the photoreceptor degeneration [78]. The systematic identification of PROM1-interacting proteins will
12 help to better understand the direct function of PROM1.

13
14 In conclusion, we have provided evidence of light-dependent photoreceptor degeneration in the *Prom1*^{-/-}
15 mice, and have identified possible downstream signaling targets. Further investigation of the
16 downstream target genes and interacting proteins will allow a better understanding of the mechanisms
17 of photoreceptor cell death and more targeted therapeutic approaches in the future.

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28
29

Figure Legends

Figure 1 Outer cell retinal layers progressively degenerate in the *Prom1*-deficient mice in a genetic background dependent manner. (A-E) Photoreceptor and ONL areas are completely obliterated within 20 days after birth in *Prom1*^{-/-} mice with a C57BL/6 background (RIKEN strain). Hematoxylin and Eosin (HE) staining was performed on retinal sections of wild type (A,B) or *Prom1*^{-/-} (C,D) mice at 14 days (P14) (A,C) and 20 days (P20) (B,D) after birth. Scale bar in (A-D) = 25 μm. Relative size of each area in the *Prom1*^{-/-} to the wild-type is shown in (E). n=3 for each genotype. (F-N) HE staining of *Prom1*^{-/-} mice retina with a mixed background of C57BL/6xCBA/NSIc. Wild-type (F-I) and *Prom1*^{-/-} (J-M) mice retina with a mixed C57BL/6xCBA/NSIc background retinal sections at P14 (F,J), P20 (G,K), P30 (H,L) and P120 (I,M). Scale bar in (F-M) = 25 μm. (N) Relative size of each retinal cell layer in the *Prom1*^{-/-} mice compared to the wild-type mice. n=3 for each genotype. In (E) and (N), the data are represented with the mean value ± s.e.m. Asterisks; statistically significant (p-value less than 0.05). OS; outer segment, IS; inner segment, ONL; outer nuclear layer, OPL; outer plexiform layer, INL; inner nuclear layer, IPL; inner plexiform layer, **; degenerated area.

Figure 2 The outer segments of the photoreceptor cells are disordered and Opsin proteins are mislocalised in *Prom1*-deficient mice. (A-H) Outer segments are disorganized, while other parts of the photoreceptor cells are largely intact at P20. Wild-type (A,C-E) and *Prom1*^{-/-} mice retinae (B,F-H) were analyzed by transmission electron microscope. (A,B) Panorama images of wild-type (A) and *Prom1*^{-/-} (B) photoreceptor cells. Note that these are merged images from several individual shots. Original toluidine blue stained images are available in Supplementary Figure S1. RPE; Retinal Pigment Epithelium. (C-H) Detailed structure of outer segments in the wild-type (C-E) and in the *Prom1*^{-/-} (F-H) retina was analyzed at the levels labeled in (A) and (B). Tight-like junctions at the border of ONL and IS (red arrowheads in C,F), mitochondrial structure in IS (red arrowheads in D,G) and the OS structure (E,H) are shown. Scale bar in (A,B) = 2 μm and (C-H) = 500 nm. (I-L') Rhodopsin and M-opsin proteins are mislocalized in the *Prom1*^{-/-} retina. Wild-type (I,I',K,K') and *Prom1*^{-/-} retina (J,J',L,L') of P20 were analyzed by immunohistochemistry using rhodopsin (green in I,I',J,J') and M-opsin (green in K,K',L,L') antibodies. Ectopic localization is shown with brackets (I-J') and with arrowheads in (L,L'). DAPI is shown in blue (I',J',K',L'). Scale bar in (I) = 100 μm for (I-L'). PR; Photoreceptor Layer.

Figure 3 Retinal degeneration in *Prom1*-knockout mice is light dependent. Both C57BL/6 (A-D) and C57BL/6xCBA/NSIc (E-H) retinae were significantly protected from the photoreceptor degeneration when the animals were reared in dark conditions. Wild-type littermate controls (A,C,E,G) and *Prom1*^{-/-} (B,D,F,H) mice (C57BL/6xCBA/NSIc) were reared under normal light (A,B,E,F) or complete darkness (C,D,G,H) conditions from P8 until P30. Scale bar in (A-H) = 50 μm. (I-L) Ultrastructural analysis by transmission electron microscopy. The C57BL/6xCBA/NSIc animals were reared as in (H) and fixed at P30. Scale bar = 2 μm. Detailed structure at the levels denoted in (I) is shown in (J-L). Mitochondria (red arrowhead in K) and tight-like junctions (red arrowheads in L) are

shown. Scale bar = 500 nm. (M-N') The mislocalization of rhodopsin (green in M,M') and M-opsin (green in N,N') was reduced in the dark-reared mice. The *Prom1*^{-/-} animals were reared in the dark condition and analyzed by immunohistochemistry. DAPI is shown in blue in (M',N'). Scale bar in (M) = 100 μ m for (M-N'). (O) Quantification of the cells in which rhodopsin and M-opsin are mislocalized to ONL. Several squares of 100 μ m x 100 μ m were randomly chosen within the IS and ONL and the numbers of the positive cells for each protein were counted. The data of the wild-type and *Prom1*^{-/-} mice reared in the normal light-dark conditions were collected from Figure 2I-L'. The data are represented with the mean value $\pm$ s.e.m. Three retinal tissues for each were analysed. Asterisks; statistically significant (p-value less than 0.05).

Figure 4 *Prom1*^{-/-} mice show maintained their photoreceptor function when reared in dark. (A-D) Representative recordings of scotopic ERG responses (a-waves (light blue in A) and b-waves (orange in A)). C57BL/6xCBA/NSIc wild-type littermate (A,B) or *Prom1*^{-/-} mice (C,D) reared under the normal light/dark condition (A,C) or in the complete darkness from P8 until P30 (B,D) were subject to ERG using seven light amplitudes within the range of 10^{-5} and 10^1 (cd.s.m⁻²) under scotopic conditions. (E,F) Recordings of a-waves (E) and b-waves (F) from the wild-type under the normal light/dark (black lines and circle dots; n=5) or the dark (green lines and circle dots; n=6), *Prom1*^{-/-} under the normal light/dark (red lines and rectangular dots; n=8) or the dark (blue lines and triangular dots; n=3) conditions were summarized. Note that the horizontal axes are displayed in logarithmic scale. (G,H)

Figure 5 *Prom1* is essential for the expression of some of the visual cycle-related genes. The relative expression levels of the indicated genes were analyzed by qRT-PCR analysis. qRT-PCR was performed for C57BL/6xCBA/NSIc wild-type and P14 *Prom1*-deficient retina. The expression level of each gene in the wild type retina was defined as 1. Three retinal tissues were analysed. Primer sequences are indicated in Supplementary Table S1.

Figure 6 The visual cycle inhibitor Fenretinide delays PROM1 mediated retinal degeneration, and the retinal functions are partially preserved. C57BL/6xCBA/NSIc wild-type (A,B) or *Prom1*^{-/-} (C,D) mice treated with DMSO (vehicle) (A,C) or Fenretinide (B,D) were analysed at P30. Scale bar (A) = 50 μ m for (A-D). (E) Quantification of the photoreceptor nuclei in the ONL. (F-K) Fenretinide treatment shows an improvement of the ERG response. Responses of a-waves (F-H) and b-waves (I-K) were recorded under scotopic conditions in the wild-type retina treated with control DMSO (black lines and circle dots in F,I; n=5) or Fenretinide (FEN; gray lines and rectangle dots in F,I; n=3) and *Prom1*^{-/-} retina treated with control DMSO (black lines and circle dots in G,J; n=4) or Fenretinide (FEN; red lines and rectangle dots in G,J; n=3). (H,K) The relative ERG scores upon the Fenretinide treatment compared to those treated with the control DMSO were replotted from (F,G) and from (I,J) at 1 and 10 cd.s/m² (denoted as 0 and 1 in the horizontal axis; (I)) or at all data points (K). Note that the horizontal axes in (F-K) and the vertical axes in (I,K) are displayed in logarithmic scale. Asterisks; statistically significant (p-value less than 0.05). The data are represented with the mean value $\pm$ s.e.m.

Supplementary Figure S1 Toluidine blue staining of the samples for the EM images in Figure 2A-H. Semi-thin sections (1 μ m) of P20 mice stained with toluidine blue used for the analysis on a transmission electron microscopy. Scale bar = 25 μ m.

Supplementary Figure S2 Photopic ERGs in the retinas treated with Fenretinide. The a-waves (A,B) and b-waves (C,D) in C57BL/6xCBA/NSlc retinae were recorded under photopic conditions, as in Figure 6. The gray arrows in (C) indicate the points where scores were too low and are not visible.

Supplementary Table S1 Sequences for the qRT-PCR analysis

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