The Y227N mutation affects bestrophin-1 protein stability and impairs sperm function in a mouse model of Best vitelliform macular dystrophy

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ABSTRACT

Human bestrophin-1 (BEST1) is an integral membrane protein known to function as a Ca\(^{2+}\)-activated and volume-regulated chloride channel. The majority of disease-associated mutations in BEST1 constitute missense mutations and were shown in vitro to lead to a reduction in mutant protein half-life causing Best disease (BD), a rare autosomal dominant macular dystrophy. To further delineate BEST1-associated pathology in vivo and to provide an animal model useful to explore experimental treatment efficacies, we have generated a knock-in mouse line (Best1Y227N). Heterozygous and homozygous to explore experimental treatment efficacies, we have generated a knock-in mouse line carrying the autosomal dominant W93C BEST1 mutation (Best1W93C) displayed key features of the BD phenotype in the mouse model. This mouse model can be used to further delineate the autosomal dominant adult-onset vitelliform macular dystrophy (AVMD) (Krämer et al., 2000), the autosomal dominant vitreoretinal chorioretinal dystrophy (AVRCD) (Yardley et al., 2004) and the autosomal recessive bestrophinopathy (ARB) (Burgess et al., 2008). Key features of BEST1-related pathology include subretinal egg yolk-like (vitelliform) lesions (Mohler and Fine, 1981), fluid- and debris-filled retinal detachments (Mohler and Fine, 1981) and a reduction in the electro-oculogram (EOG) light peak (Cross and Bard, 1974).

Structural and functional analysis of BEST1 established the protein as a Ca\(^{2+}\)-activated and volume-regulated chloride channel (Sun et al., 2002; Hartzell et al., 2008; Xiao et al., 2008; Milenkovic et al., 2015) by forming a homo-pentameric protein complex (Kane Dickson et al., 2014; Yang et al., 2014). To further investigate disease pathology, several in vitro and in vivo disease models were generated. First, we and others showed that MDCKII BEST1-transfected cell culture models are well suited to demonstrate mis-localization (Johnson et al., 2014, 2013; Milenkovic et al., 2011) and reduced protein stability (Uggetti et al., 2016; Milenkovic et al., 2018) of autosomal dominant as well as autosomal recessive BEST1 mutations. These observations were further supported by studies in BD patient-derived human induced pluripotent stem cell (hiPSC) retinal pigment epithelium (RPE) (Milenkovic et al., 2015; Singh et al., 2015; Marmorstein et al., 2018). BD hiPSC-RPE cells displayed reduced protein expression (Milenkovic et al., 2015; Marmorstein et al., 2018) that was correlated with a diminished chloride conductance (Moshfegh et al., 2016) and delayed digestion of photoreceptor outer segments (POS) (Singh et al., 2015; Marmorstein et al., 2018). As such, MDCKII and hiPSC-RPE cells offer valuable in vitro tools to search for chemical compounds that modulate protein stability and possibly the degradative processes of mutant BEST1. Furthermore, spontaneous autosomal recessive mutations in the Best1 gene of dog breeds were reported providing a canine model of ARB (Guziewicz et al., 2007; Zangerl et al., 2010). Recently, for the canine ARB breed it was demonstrated that severity and progression can be attenuated by pharmacological intervention (Singh et al., 2015) or by adeno-associated virus 2 (AAV2)-mediated BEST1 gene augmentation (Guziewicz et al., 2018). A BD-associated Best1 knock-in mouse line carrying the autosomal dominant W93C BEST1 mutation (Best1W93C) displayed key features of the BD phenotype in the mouse eye (Zhang et al., 2010). While this phenotype was associated with potential abnormalities in calcium homeostasis, a direct link of these findings with the mutant Best1 protein and the ocular phenotype has not been established so far.

Here, we sought to establish a novel Best1 knock-in mouse model by introducing the recurrent human BD mutation Y227N into the mouse germline. This mouse model can be used to further delineate...
RESULTS

Generation of knock-in mice harboring the mBest1-Y227N mutation

Known disease-causing mutations in the BEST1 gene are clustered in the N-terminal half of the protein, with no striking preference for a particular codon or protein motif. The T to A transversion that encodes a tyrosine-to-asparagine change at codon 227 (Y227N) was identified in the affected members of an Iowa family of Dutch ancestry and also in a Canadian four-generation family (Milenkovic et al., 2015). The Y227N mutation was introduced into the mouse genome using homologous recombination (Fig. 1A). After removal of the neomycin cassette via Cre recombinase, PCR and Sanger sequencing was performed to confirm correct targeting into the mouse genome (Fig. 1B). Prior to phenotypic analysis, the mutant allele was crossed for more than ten generations onto a C57BL/6J (B6/J) and the outbred CD-1 mice characterized by high levels of mono-ubiquitinated Best1 protein. The findings in Best1Y227N mouse testis were directly associated with a decrease in sperm motility and the capability to fertilize eggs. This strong phenotype offers an excellent in vivo model to test drug efficacy of BEST1-associated pathology.

Expression profiling of mBest1 in the mouse eye

Murine Best1 expression was found in a variety of tissues including the RPE/retina, although highest expression was eminent in the murine testis (Krämer et al., 2004; Milenkovic et al., 2015). To determine whether the introduction of the Y227N mutation affects mRNA or protein expression, we performed reverse transcription (RT)-PCR and western blot analysis from retina and RPE/choroid of mRNA or protein expression, we performed reverse transcription (RT)-PCR and western blot analysis from retina and RPE/choroid of mRNA or protein expression, we performed reverse transcription (RT)-PCR and western blot analysis from retina and RPE/choroid of mRNA or protein expression, we performed reverse transcription (RT)-PCR and western blot analysis from retina and RPE/choroid of mRNA or protein expression, we performed reverse transcription (RT)-PCR and western blot analysis from retina and RPE/choroid of mRNA or protein expression, we performed reverse transcription (RT)-PCR and western blot analysis from retina and RPE/choroid of mRNA or protein expression, we performed reverse transcription (RT)-PCR and western blot analysis from retina and RPE/choroid of mRNA or protein expression, 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Visual function in Best1<sup>Y227N</sup> mice

BD is characterized by a diminished EOG light peak with a normal electroretinogram (ERG) eventually leading to visual impairment (Blodi and Stone, 1990; Marmorstein et al., 2009). To assess visual function, we first analyzed ERG measurements in Best1<sup>Y227N</sup> mice. Single flash ERG responses of 6-month-old B6/J mice recorded under dark-adapted (Fig. 4A) and light-adapted (Fig. 4B) conditions using varying stimulus luminance intensities revealed no differences in retinal function among the three Best1 genotypes. Similarities in amplitude and waveform are highlighted in selected overlays of the three genotypes under scotopic and photopic conditions (Fig. 4C). To monitor the EOG light peak, we recorded the transepithelial standing potential across the RPE of B6/J +/+, +/N and N/N mice during light adaption using direct-current signal amplification of the ERG. For this purpose, the potential of fully dark-adapted 9-month-old B6/J animals was directly traced in response to a 10 min light stimulus at a fixed intensity (0.6 log cd/ m<sup>2</sup>), a stimulus luminance intensity similar to that used in human EOG recordings. Consistent with the characteristics of a light peak, the amplitude of the potential slowly increased during the period of light adaption although the final plateau was mostly lower than the pre-stimulus baseline (Fig. 4D). Amplitudes, waveform and stimulus for on and off responses in B6/J +/N and N/N revealed no significant difference from those of B6/J +/+ mice.

To further assess the impact of the Y227N mutation on visual function, we measured visual acuity of 3-month and 19–21-month-old B6/J mice using a virtual optomotor system as published earlier (Prusky et al., 2004). Mice were set on a platform surrounded by a rotating cylinder. By tracking the drifting gratings, reflexive head movements of the mouse were monitored and analyzed once a day for five consecutive days. Although we found an age-related decline in visual acuity when comparing the two age groups, visual acuity within the two groups was similar regardless of genotype (Fig. 4E).

RPE phagocytosis in Best1<sup>Y227N</sup> mice

Disk shedding is a most important function of the RPE to ensure continuous renewal of the light-sensitive rod and cone outer segments of photoreceptors (Young, 1967). This process follows a strict diurnal rhythm (LaVail, 1980). To examine the consequences of mutant Best1 on POS shedding and degradation of shed tips by phagocytosis, we first performed qRT-PCR of 13 phagocytosis-related genes. These genes are known to be involved in RPE phagocytosis during the so-called diurnal burst, shortly after light onset at 6:00am and are associated with the binding, internalization and degradation of POS in the RPE/choroid complex (Mazzoni et al., 2014). There was no statistical difference between the mRNA expression levels in B6/J +/+, +/N and N/N mice (Fig. 5A).

To further analyze engulfment and clearance of POS by the RPE in the diurnal course of retinal phagocytosis, we immunostained RPE flat mounts with antibody Rho-1D4 (1D4) specifically recognizing the C-terminus of rhodopsin. This facilitates the quantification of phagosomes containing 1D4-positive POS components at different time points relative to light onset. Consequently, fluorescence microscopy revealed a high number of 1D4-positive phagosomes at the apical RPE cell layer of B6/J and CD-1 +/+, penetration depth indicated by staining of the tight junction protein ZO-1, confirming regular POS internalization at 6:00am (Fig. 5B). The phagocytic burst is followed by a rapid decrease in the number of 1D4-positive phagosomes in a time-course of 1–12 h after light onset, representing efficient phagolyosomal digestion that is in line with previous reports (Damek-Poprawa et al., 2009). Quantification of phagosome numbers at defined time points in the RPE of B6/J and CD-1 +/+, +/N and N/N mice revealed comparable numbers of 1D4-positive phagosomes at all time points of the day (Fig. 5C). Of note, there was also no difference in the amount of immunostained POS between genotypes when using an antibody against the N-terminal epitope of mutant Best1 on POS shedding and degradation of shed tips by phagocytosis, we first performed qRT-PCR of 13 phagocytosis-related genes. These genes are known to be involved in RPE phagocytosis during the so-called diurnal burst, shortly after light onset at 6:00am and are associated with the binding, internalization and degradation of POS in the RPE/choroid complex (Mazzoni et al., 2014). There was no statistical difference between the mRNA expression levels in B6/J +/+, +/N and N/N mice (Fig. 5A).

Expression profiling of mutant Best1 in the mouse testis

RT-PCR, northern blot and western blot analysis was done in testis tissue from B6/J and CD-1 +/+, +/N and N/N mice. RT-PCR revealed no differences in mRNA expression in testis derived from the various genotypes (Fig. 6A). For confirmation, northern blot analysis was performed and showed a single transcript of ~2 kb in testis mRNA of all three genotypes confirming strong and
comparable Best1 expression of full-length Best1 mRNA in B6/J +/+, +/N and N/N mice (Fig. 6B). In line with previous data (Milenkovic et al., 2015), western blot analysis confirmed prominent Best1 protein expression in lysates from B6/J +/+ and CD-1 +/+ testis (Figs 6C and 2B). In contrast, Best1 protein expression was severely reduced in B6/J and CD-1 N/N (<18% of remaining Best1 protein) and to a lesser extent in B6/J +/N and CD-1 +/N mice (<80%) compared to B6/J +/+ and CD-1 +/+ controls (Fig. 6C and D), suggesting severe degradation of mutant Best1 protein. This was consistent with a reduced immunostaining of
Best1 protein in the B6/J N/N sperm head in contrast to B6/J +/+ animals where immunolabeling consistently stained the equatorial segment of the sperm head as reported earlier (Milenkovic et al., 2015) (Fig. 6E).

To examine whether reduced Best1 protein in mouse testis is caused by enhanced protein degradation, we examined ubiquitination in testis lysates from B6/J +/+ and B6/J N/N males as conjugation of a single ubiquitin to a protein can trigger degradation of the tagged protein (Terrell et al., 1998). Cell lysates were immunoprecipitated with α-ubiquitin and immunoblotted with α-Best1-C45 antibody solution. Western blot analysis identified a faint single immunoreactive Best1 molecular weight staining in +/+ testis, approximately ~8 kDa above the unmodified protein (Fig. 6F), consistent with Best1 being mono-ubiquitinated, and tagged for endo-lysosomal degradation (Haglund et al., 2003). In contrast to B6/J +/+ , testis lysates from B6/J N/N males showed increased amounts of mono-ubiquitinated Best1. Together, these findings indicate that reduced amounts of endogenous Best1 protein in the B6/J N/N mouse are likely resulting from decreased protein stability rather than reduced transcription.

Sperm motility and fertilization rates in Best1Y227N mice

Recently, we showed that complete loss of Best1 protein in the Best1 knockout mouse impairs sperm function and results in a severe sub-fertility phenotype (Milenkovic et al., 2015). To examine the functional consequences of reduced Best1 protein expression in the +/+ and N/N testis, we analyzed total motility from caudal testis, approximately 12 kDa above the unmodified protein (Fig. 6F), consistent with Best1 being mono-ubiquitinated, and tagged for endo-lysosomal degradation (Haglund et al., 2003). In contrast to B6/J +/+ , testis lysates from B6/J N/N males showed increased amounts of mono-ubiquitinated Best1. Together, these findings indicate that reduced amounts of endogenous Best1 protein in the B6/J N/N mouse are likely resulting from decreased protein stability rather than reduced transcription.

Sperm motility and fertilization rates in Best1Y227N mice

Fertilization rates when oocytes were incubated with sperm from CD-1 N/N (11% fertilized eggs) compared to CD-1 +/+ (36%) (Fig. 7B). In comparison, sperm from CD-1 Best1-deficient mice (+/-) revealed even lower fertilization rates with only 2% of fertilized eggs (Fig. 7B). This may explain why normal CD-1 females mated with CD-1 N/N males give birth to a normal number of pups (mean litter size 13±3.4 pups) (Fig. 7C) in contrast to normal females mated with CD-1 −/− males (0.5%) as reported earlier (Milenkovic et al., 2015) (Fig. 7D).

DISCUSSION

Mouse models of inherited retinal dystrophy have regularly been shown to be valuable tools for the analysis of molecular disease mechanisms but also for evaluating efficacy of novel therapeutic approaches (Chan et al., 2004; Isgrig et al., 2017). Here, we report the generation and characterization of a gene-modified mouse line carrying the human BD-associated mutation Y227N in the endogenous murine Best1 gene. Although this gene mutation in the human homologue is well established to cause autosomal dominant BD, we show that the mouse fails to reveal any of the typical features of human BEST1-associated disease.

The absence of functional defects in ocular tissue of Best1-modified mice, even in mutant mice up to 21 months of age, may be viewed in a more general debate of whether the nocturnal mouse is in fact a relevant model for macular degeneration in diurnal human. Of course, the mouse is known to be coneless with no anatomically comparable macular area. Nevertheless, Volland et al. demonstrated a greater density of photoreceptors in the mouse central retina than...
in the human macula suggesting that the phagocytic load is even higher in the mouse RPE (Volland et al., 2015). In line with these observations, a number of mouse models show remarkable overlaps with human retinal dystrophies, despite the fact that initial pathological damage is known to arise preferentially in the human macula (e.g. Weber et al., 2002a,b; Marmorstein et al., 2007). The absence of an ocular/retinal phenotype in the Best1Y227N mouse, even in two genetically divergent backgrounds, may simply be explained by findings in this study and by others that normal as well as mutant Best1 protein is below immunohistochemical detection in the murine RPE which is in striking contrast to the human situation (Mullins et al., 2005). This peculiarity in species-specific expression suggests that Best1 likely plays no crucial role in the murine eye. Nevertheless, Zhang et al. reported a knock-in mouse with a mutation at W93C, known to cause BD in human. Similar to our Y227N mutant mouse, the W93C mutation was also crossed onto the B6/J genetic background (Zhang et al., 2010). In contrast to the present study, these mice were reported to show some features of BD, including a deficit in the light peak, serous retinal detachment, accumulation of lipofuscin and abnormalities in calcium signaling in RPE cells. Interestingly, homozygous and heterozygous Best1-W93C mice (Zhang et al., 2010) but also Best1-deficient animals (Marmorstein et al., 2006) failed to reveal aberrant chloride currents as would be expected from earlier studies demonstrating that the introduction of mutation W93C diminishes human BEST1 (hBEST1) associated anion conductance (Sun et al., 2002; Lee et al., 2010; Marmorstein et al., 2015). Taken together, these inconsistencies suggest that the phenotypic abnormalities seen in the Best1W93C mouse may not be the result of impaired Best1 channel activity, although reasons to explain the different phenotypic retinal presentation of mutant mice harboring the Y227N or the W93C mutation are still unclear. It is possible though that the two mutations may have different effects on localization or stability of the mutant protein. While both, Y227N and W93C are known to mis-localize in Madin-Darby canine kidney (MDCKII) cells (Milenkovic et al., 2011, 2018), recent work from Johnson et al. (2013) demonstrated that the presence of properly localized wild-type Best1 can rescue the mis-localization caused by W93C. The Y227N mutation clearly reveals a dominant-negative effect (Mullins et al., 2005) as do most BEST mutations analyzed so far (Johnson et al., 2014). Unfortunately, the spurious Best1 expression in the murine RPE prevents a more thorough immunohistochemical examination of endogenous normal Best1 but also of mutant proteins Y227N and W93C.

In this study, we confirmed previous results demonstrating that Best1 protein is readily detectable in mouse testis (Milenkovic et al., 2015). Analysis of testis protein extracts from homozygous B6/J and homozygous CD-1 Best1-Y227N mice demonstrated a severe decrease in Best1 protein consistent with a number of in vitro studies (Uggetti et al., 2016; Marmorstein et al., 2018; Milenkovic et al., 2018). These findings suggest that a reduced protein stability and enhanced protein degradation of mutant protein play a key role in...
BD pathogenesis. In support of this, our in vivo data also demonstrate a reduced Best1 protein expression strongly arguing that this is due to increased protein degradation rather than to defective protein synthesis as Best1 mRNA expression is comparable in testis of +/+ , +/N and N/N mice. Also a covalent attachment of a single ubiquitin molecule, as found in this study, potentially targets mutant Best1 protein for lysosomal degradation (Haglund et al., 2003). Our recent in vitro study is in agreement with this conclusion where mis-localized and unstable BD-associated mutants are degraded via the endo-lysosomal degradation pathway (Milenkovic et al., 2018). Still, ultimate proof for an exclusion of a defective Best1 protein synthesis would require the inhibition of the underlying protein degradation pathway in cell lines of testicular somatic cells from BestY227N mice. Sperm cells, however, are not suited for appropriate cell culture experiments.

Phenotypic differences in mouse models due to their respective genetic background are well established (Eshraghi et al., 2016). To this end, we crossed the Best1Y227N mutation to an inbred as well as a genetically more diverse outbred mouse strain. For the latter, we decided on the CD-1 background where mis-localized and unstable BD-associated mutants are degraded via the endo-lysosomal degradation pathway (Milenkovic et al., 2018). Still, ultimate proof for an exclusion of a defective Best1 protein synthesis would require the inhibition of the underlying protein degradation pathway in cell lines of testicular somatic cells from Best1Y227N mice. Sperm cells, however, are not suited for appropriate cell culture experiments.

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In summary, here we report on a Best1-modified knock-in mouse line harboring the human Y227N mutation in the homologous murine gene. Mutant mice reveal no ocular/retinal phenotype but manifest a strong testicular effect on protein stability and sperm motility that is greatly modified by genetic background. Our data suggest that the observed phenotype in testis is due to increased protein degradation rather than defective protein synthesis. Due to this strong phenotype, our mouse strain offers an excellent in vivo model to analyze therapeutic approaches aiming at testing drug efficacies of compounds modifying protein stability and the

Fig. 6. Analysis of mutant Best1 protein (Y227N) in mouse testis. (A) RNA expression of Best1 in testis from CD-1 (left graph) and B6/J (right graph) +/+ , +/N and N/N mice by RT-PCR. Gusb served as control for RNA integrity. (B) Northern blot analysis of total RNA from B6/J +/+ , +/N and N/N mouse testis by hybridization to a probe targeting Best1 exon 7–10. Mouse smooth muscle actin served as control for equal loading and RNA integrity. (C) Representative western blot of whole testis lysates from CD-1 (left graph) and B6/J (right graph) +/+ , +/N and N/N mice using α-Best1-C45. Anti-beta actin served as loading control. (D) Quantification of Best1 protein expression relative to CD-1 and B6/J +/+ , respectively (n=4 mice of each genotype) obtained from C (data were extracted from two to three western blots of each triplet; one-way ANOVA: **P<0.01; ***P<0.001). (E) Immunofluorescence staining of sperm cells from B6/J +/+ and N/N males using α-Best1-C45. (F) Testis tissue from B6/J +/+ and N/N mice was immuno-precipitated with α-ubiquitin, transferred to nylon membranes and probed as indicated using α-Best1-C45 (n.b., fraction not bound). For each sample, the mean±s.d. is given.
degradative processes of mutant BEST1 protein. In a note of caution, it should be mentioned that pharmacological intervention in a tissue other than the one harboring the primary pathology (e.g. the RPE in BD) may be error prone as testicular protein degradation mechanisms may differ from those of the RPE. Further experiments will be needed to validate the utility of such an approach.

**MATERIALS AND METHODS**

**Antibodies**
Rabbit polyclonal antibodies included Best1-C45 (Milenkovic et al., 2015), diluted 1:5,000 for WB and 1:500 for ICC;ZO-1 (61-7300, Thermo Fisher Scientific, Waltham, USA), diluted 1:250; mouse monoclonal antibodies against β-actin (#5441, Sigma-Aldrich, Munich, Germany), diluted 1:1,000; Rpe65 (ab13826, Abcam, Cambridge, UK), diluted 1:5,000; Rs1, Rho-4D2 and Rho-1D4 (kindly provided by Dr Robert Molday, University of British Columbia, Vancouver, Canada), diluted 1:5,000, 1:2,500 and 1:10,000, respectively. Secondary antibody for immunofluorescence detection was goat Alexa 488 or 594-conjugated anti-rabbit and goat Alexa 594-conjugated anti-mouse (Thermo Fisher Scientific, dilution 1:500). Western blot experiments were performed with horsendish peroxidase-conjugated secondary antibodies (Calbiochem/Merck, Darmstadt, Germany; dilution 1:10,000).

**Immunofluorescence labeling**
Immunofluorescence was performed as previously described (Milenkovic et al., 2015). In brief, a 20 µl drop of sperm suspension was air-dried on microscope slides and stained with α-Best1-C45 at 4°C overnight (ON).

**Sample collection and preparation for RNA and protein expression**
Testis tissue was cut into small pieces and homogenized using a TissueLyzer (Thermo Fisher Scientific). For preparation of RPE/choroid and retinae, eyes were enucleated, bulbi were incised along the ora serrata and (Qiagen, Hilden, Germany). For preparation of RPE/choroid and retina samples, eyes were enucleated, bulbi were incised along the ora serrata and cornea, lens, iris and vitreous body were removed. After incubating the posterior eye cup in phosphate buffered saline (PBS, pH 7.4) at 37°C for 20 min the retina was removed. For western blot analysis and RT-PCR eye cups (sclera, RPE and choroid) and retinae were then homogenized using a TissueLyzer. For qRT-PCR, eye cups were treated enzymatically to isolate pure RPE cells. After removal of the retina, eye cups were incubated 30 min in PBS/1 mM EDTA, then transferred to a dissociation buffer of 3 mM L-cysteine (Sigma-Aldrich) in PBS/1 mM EDTA, 1 U/ml papain (Sigma-Aldrich), and 1 mg/ml BSA (Sigma-Aldrich) for 23 min. RPE cells were rinsed in DMEM culture medium (Thermo Fisher Scientific) supplemented with 2% FCS to stop the reaction (Thermo Fisher Scientific). After a washing step with PBS the isolated RPE cells were pelleted and subsequently stored at −80°C.

**Protein sample preparation, SDS page and quantitative western blot analysis**
Whole cell protein samples were prepared by homogenization in a modified RIPA buffer (20 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2% DOC, 0.25% NP 40, 0.025% SDS) supplemented with 1× Protease inhibitor cocktail (Roche, Mannheim, Germany). Protein extracts were sheared by passing through a 25-G needle several times on ice and subsequently with seven 1-s pulses at 32% amplitude using a Vibra-Cell (Sonics, Newtown, USA). Protein concentration was determined on a NanoDrop™ (Thermo Fisher Scientific) and equal amounts of protein were separated on SDS-Page and subsequently transferred onto Immobilon™-P PVDF membrane (Millipore, Bedford, USA). Incubation of primary and secondary antibodies was carried out at 4°C overnight. Protein labeling was visualized by chemiluminescence using the Odyssey™ FC Imaging System.

**RNA isolation, reverse transcription and RT-PCR**
Total RNA was extracted after DNase treatment (Roche) according to the manufacturer’s instructions using the RNaseasy Mini Kit (Qiagen). First strand cDNA synthesis from 1 µg of total RNA was performed with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific) and random hexamer oligonucleotide primers. For RT-PCR reactions 50 ng of cDNA was used as templates for PCR with Go Taq Polymerase (Promega, Mannheim, Germany). RT-PCR amplification of mBEST1 and mGus was performed using primer pairs mVM2d-2DNA-F (5′-CTG CAG GTG TCC CTG TGT T-3′)/mVM2d-2DNA-R (5′-TGT CTG AAG TGG AGT CTG T-3′) and mGus-ex11-F (5′-GAC CCG CCT CGC ATG TCC AG-3′)/mGus-ex12-R (5′-GCC CTG AAC GTG GAC CTC C-3′), respectively.
Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed with an ABI7900HT machine (Applied Biosystems, Darmstadt, Germany) using the 1× TaqMan Universal PCR Master Mix and dual-labeled probes (Roche ProbeLibrary, Roche Applied Science). For qRT-PCR reactions 25 ng of cDNA was used as templates for PCR. Measurements were performed in triplicates and results were analyzed with an ABI sequence detector software version 2.3 (Applied Biosystems) applying the ΔΔCt method for relative quantification. Primer sequences are listed in Table S1.

Construction of the Best1 knock-in targeting vector

To introduce the Y227N mutation into exon six of the murine Best1 gene, a modified 5′-murine fragment, spanning exon 3 to exon 6, was generated by site-directed mutagenesis and co-ligated with an unmodified 5′-fragment into the pGADT7t vector. The 3′-fragment, spanning Best1 intron 6 to intron 9, was inserted into the ClaI site of the pGADT7t vector. A neomycin phosphotransferase (neo) gene flanked by loxP sites was integrated between the two arms. The final knock-in construct was linearized with restriction enzyme Xmal.

Homologous recombination in ES cells and generation of germ line chimeras

CJ7 ES cells were electroporated and ES cell clones were selected and screened for homologous recombination as previously described (Swaitek and Gridley, 1993). Recombinant ES cells were injected into B6/J blastocysts. Chimeric founders were bred to B6/J mice, and heterozygous mice were backcrossed into the inbred B6/J or outbred CD-1 (CD-1® IGS) genetic background (Schreve et al., 1994; Weber et al., 2002a) (Charles River Laboratories, Sulzfeld, Germany). Genotyping of animals was performed by PCR amplification using primer pair mVMD2-NdeI-F (5′-GTC TAG GGA GCT GCA TAT GG-3′)/mVMD2-R7 (5′-AAC AGC CAG TTG TAC CTG AC-3′). Prior to analysis, knock-in mice were backcrossed for at least ten generations into B6/J or CD-1 strains.

For analysis of testis and sperm cells, male mice were between 2–4 months of age. Retinal tissues were used at the indicated ages. All mice were maintained on a 12 h light/12 h dark cycle, housed under specific pathogen-free conditions and generally maintained under guidelines established by the institution for their use. Mice were euthanized by cervical dislocation after inhalation of carbon dioxide.

Histological analysis of retinal sections by light and electron microscopy

19–21-month-old +/+ and N/N mice and age matched littermate control animals were fixed by intracardiac perfusion with 1% paraformaldehyde (PFA) plus 1% glutaraldehyde containing 0.2 M cacodylate buffer. Eyes were enucleated and fixed overnight in Karnovsky’s buffer (2.5% glutaraldehyde, 2.0% paraformaldehyde) in 0.1 M cacodylate buffer, pH 7.2), washed with 0.2 M cacodylate buffer pH 7.4 and post fixed for 2 h in 1% osmium tetroxide and embedded in Epon (Serva, Heidelberg, Germany) after dehydration. Semi-thin sections (1 μm) from the central retina were cut along the vertical meridian of the eye at the optic nerve head (ONH) and counterstained with Methylene Blue and viewed on a Zeiss Axioskop-2 microscope using the AxioVision LE Rel. 4.5 software. Quantification of whole retinal thickness was assessed by dividing retinal areas into ten sections anterior and posterior of the ONH. Ultra-thin sections (50–80 nm) were contrasted with 4% uranyl acetate in 50% EtOH and 2% lead citrate in 1 M NaOH and viewed with an electron microscope (EM 902, Zeiss).

Analysis of retinal Docosahexaenoic Acid (DHA) concentration

DHA concentration was measured from retinae of CD-1 +/+ N/N and +/+ mice aged 10–12 months. Retinal protein concentrations were determined by a standard Bradford assay (Roti-quant®, Roth, Karlsruhe, Germany) after homogenization. DHA concentrations were analyzed by GC-mass spectrometry (MS). Briefly, 50 μg protein equivalents of each retinal homogenate were derivatized with acetyl chloride in methanol for 2 h at 80°C (Sparrow et al., 2012). An internal DHA standard mixture was added prior to methylation, followed by an extraction of methyl esters by hexane and analyzed using a Shimadzu QP-2010 GC-MS. DHA quantification was performed by external calibration and DHA levels are given as DHA [μg]/total retinal protein [μg].

Measurement of visual acuity

A Virtual Optomotor System (OptoMotry, CerebralMechanics, Lethbridge, Canada) was used to measure visual acuity as an index of visual function (Prusky et al., 2004). For that purpose, animals were exposed to moving and wave gratings of various spatial frequencies and reflexive head movements of mice were tracked. Acuity was assessed by starting with a low spatial frequency (0.1 cycle/degree) and incrementally increasing the spatial frequency of the grating until the animal failed to respond. The threshold was defined as the highest spatial frequency at 100% contrast. Each mouse was tested five times for acuity of the right and the left eye, respectively.

Diurnal RPE phagocytosis assay on RPE flat mounts

Eyes from 8–12-month-old +/+ and N/N and corresponding littermate control mice (+/+) were harvested at various time points after light onset (6:00am). After trimming the enucleated eyes, each eye was pierced once above the ora serrata (OS), prefixed by immersing in 2% PFA/BS for 4 min at room temperature followed by incising along the OS to remove lens and vitreous body. The retina was peeled away after an incubation step in PBS for 20 min at 37°C. After further 6 min fixation in 4% PFA/PBS immunofluorescence labeling was performed using primary antibody ZO-1 and Rho-1D4 or Rho-4D2. Incisions were made from the peripheral eye cup towards the optic nerve head to gain a flat mount preparation and mounted onto glass slides. Flat mounts were imaged on a Zeiss Axioskop-2 microscope using AxioVision LE Rel. 4.1 software and confocal microscope LSM 510 (Zeiss). The number of Rho-1D4-positive phagosomes per RPE cell, defined by ZO-1 staining, was assessed using ImageJ.

Analysis of RPE lipofuscin pigments

RPE lipofuscin pigments were analyzed from 9- and 20-month-old BL/6J +/+ and N/N mice and their corresponding littermate controls as described (Kim et al., 2007; Wu et al., 2009). These measurements were performed specifically on the BL/6J background to ensure comparability to earlier studies (Zhang et al., 2010). Briefly, posterior murine eye cups including sclera, choroid, RPE and neural retina were homogenized in PBS using a glass tissue grinder and extracted in chloroform/methanol (2:1). Extracts were passed through a reverse phase cartridge with 0.1% trifluoroacetic acid (TFA) in methanol, dried under argon, re-dissolved in methanol and analyzed by reverse-phase HPLC (Waters Alliance system, Milford, MA, USA). A2E, atRAL-di-PE and A2-DHP-PE quantification was performed by external calibration. Data are given as molar quantity/eye cup.

Northern Blot

For northern blot analysis, total RNA (10 μg) was isolated from frozen testis, electrophoretically separated on a 1,2% agarose gel containing 1% formaldehyde and blotted onto nylon membrane (Amersham, Freiburg, Germany). Hybridization probes were generated by RT-PCR encompassing exon 9–10 of the mBest1 gene (NM_011913.2) using primer pair VMD2-Mmur-cDNA-F/R (5′-TGT CTG AAC TGG AGG GTG CT-3′)/VMD2-AGG GAG TAA TGG TTG GAA TGG G-3′). Mouse smooth muscle actin served as a control by using primer pair mSmActin_F/R (5′-AGG GAG TAA TGG TTG GAA TGG G-3′) and (5′-CAG ACG CAT GAT GGC ATG AGG-3′). The fragments were randomly labeled in the presence of [α-32P]dCTP (Redi Prime II DNA Labeling System, GE Healthcare, Munich, Germany). Removal of unincorporated nucleotides was achieved by Sephadex™ chromatography.

In vivo electroretinographic analysis

Electroretinograms (ERGs) were recorded binocularly according to previously described procedures (Tanimoto et al., 2012, 2015). The ERG equipment consisted of a Ganzfeld bowl, a direct current amplifier and a PC-based control and recording unit (Multiliner Vision; VIASYS Healthcare GmbH, Hoechberg, Germany). Mice were dark adapted overnight and
anaesthetized with ketamine (66.7 mg/kg body weight) and xylazine (11.7 mg/kg body weight). The pups were diluted and single flash ERG responses were obtained under dark-adapted and light-adapted conditions. Light adaptation was accomplished with a background illumination of 30 candela (cd) per square meter starting 10 min before recording. Single white-flash stimulus intensity ranged from $-4.1.5 \text{log cd}\text{s/m}^2$ under dark-adapted and from $-2.1.5 \text{log cd}\text{s/m}^2$ under light-adapted conditions, divided into ten and eight steps, respectively. Ten responses were averaged with an inter-stimulus interval of either 5 s or 17 s (for 0, 0.5, 1, and 1.5 log cd*s/m$^2$). Band-pass filter frequencies were 0.3 and 300 Hz.

In vivo standing potential analysis
There is a standing electrical potential across the eye, generated largely by the transscleral potential across the RPE which depends upon the state of ambient retinal illumination (Wimmers et al., 2007). As constant potentials are difficult to measure directly due to baseline drift and movement artefacts, EOG has been developed as an indirect method in patients that are able to move their eyes between two fixation sites (Constable et al., 2018). To obtain information about the standing potential changes with light exposure in mice that are not able to move their eyes (particularly when properly anesthetized), we performed a direct recording of the potential using the same recording equipment as for the ERG (see above). Briefly, gold wire ring electrodes (active electrodes) were moistened with methylcellulose and positioned on the surface of both murine corneae for binocular EOG recordings. Stainless steel needle electrodes were applied subcutaneously at the middle of the forehead region and the back near the tail as a reference and a ground electrode, respectively. A 10 min light pulse was used at a fixed intensity (0.6 log cd/m$^2$) in fully dark-adapted animals. Traces of 11 min duration (i.e. including 15 s before and 45 s after light exposure) were obtained using direct-current signal amplification (Band-pass filter frequencies were 0 and 300 Hz) (Tanimoto and Seeliger, 2018).

Sperm incubation media and sperm preparation
We used Toyoda Yokoyama Hoshi (TYH) media (Croquet et al., 2010) (138 mM NaCl, 4.8 mM KCl, 2 mM CaCl$\text{2}$, 1.2 mM KH$_2$PO$_4$, 1.0 mM MgSO$_4$, 5.6 mM Glucose, 0.5 mM Sodium Pyruvate, 10 mM L-Lactat, 10 mM Hepes) adjusted to pH 7.4 and equilibrated to an osmolality of 290 mosmol$^{-1}$. The cauda epididymis was removed, placed in one well of a four-well multi-dish (Thermo Fisher Scientific) and minced in 500 µl of TYH media. Sperm were allowed to swim out for 15 min. After removing epididymal tissue the sperm suspension was transferred into a 2 ml cup and kept in a 37°C incubator for further analysis.

Analysis of sperm total motility
Sperm cell motility was analyzed using the computer-assisted sperm analysis (CASAII) system (HT CASA Ceros II, Hamilton Thorne, Beverly, USA) as per guidelines of the supplier. Briefly, sperm cell suspensions from CD-1 and B6J $+/+$ and N/N and the corresponding littermate control mice (+/+) were diluted three- to four-fold in TYH media before loading 3 µl on a counting chamber slide to obtain quantitative parameters of sperm motility. Three measurements were performed from each sperm suspension. Image collection and quantification of sperm function parameters were achieved with systems default settings. Total sperm cell motility was calculated as a percentage by subtracting the static sperm fraction from the total number of cells.

Immunoprecipitation
Testis tissue was homogenized in 750 µl lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton-X, 1 mM Na3VO4, 5 mM NaF, 1 mM PMSF, 1 Protease inhibitor cocktail). Protein extracts were sheared on ice with 15 1-s pulses at 30% amplitude using a Vibra-Cell sonicator (Sonics) and incubated for 30 min on a rotating wheel at 4°C. After centrifugation at 16,000 g for 15 min, the supernatant was incubated with 5 µl of anti-ubiquitin ON at 4°C. Immune complexes were precipitated with protein G-Sepharose (Pierce, Thermo Fisher Scientific) for 4 h at 4°C and the bead pellets were washed five times with ice-cold lysis buffer. 1 Lämml was added to the pellets and heated for 5 min at 92°C. After centrifugation the supernatant was subjected to SDS-page.

In vitro fertilization
The IVF protocol is based on methods developed by Naomi Nakagata’s laboratory and Infrafonter GmbH (Guan et al., 2014). Female CD-1 mice were superovulated by intraperitoneal injection of 5 IU PMSG (Intervet Deutschland GmbH) followed by 5 IU IUICG (Intervet Deutschland GmbH) 50 h later. Mice were euthanized by cervical dislocation 12 h post injection and cumulus-oocyte complexes (COCs) were collected from the oviducts. Cumulus cells were then removed by treating COCs with 300 µg/ml hyaluronidase (Sigma-Aldrich). Oocytes were washed in M2 media (Sigma-Aldrich), transferred to plates (~30 oocytes in each drop) containing 200 µl HTF media (Quinn et al., 1985) (102 mM NaCl, 4.7 mM KCl, 0.2 mM MgSO$_4$, 0.37 mM KH$_2$PO$_4$, 2.0 mM CaCl$_2$, 25 mM NaHCO$_3$, 2.8 mM Glucose, 0.33 mM Sodium pyruvate, 21.4 mM Sodium lactate, 0.075 g/l Penicillin G-potassium salt, 0.05 g/l Streptomycin sulfate, 4.0 g/l BSA), covered by mineral oil (Labotect GmbH, Rostock, Germany) and incubated until insemination. Sperm were incubated (37°C, 5% CO$_2$) in HTF media under mineral oil for 15–20 min to initiate capacitation. Next, 10 µl of sperm suspension was added to the insemination plates and incubated for 4–5 h at 37°C, 5% CO$_2$. After fertilization, oocytes were washed 5 times in HTF media and transferred for final development into KSOM$^{-}$ media (95 mM NaCl, 2.5 mM KCl, 0.35 mM KH$_2$PO$_4$, 0.2 mM MgSO$_4$, 0.2 mM Glucose, 0.06 g/l Penicillin G-potassium salt, 0.05 g/l Streptomycin sulfate, 10 mM Sodium lactate, 25 mM NaHCO$_3$, 0.001 g/l Phenol Red, 0.2 mM Sodium pyruvate, 1.7 mM CaCl$_2$, 0.01 mM Na$_2$EDTA, 1 mM L-Glutamine, 1.0 g/l BSA, add 0.5 ml of 100× MEM nonessential amino acid and 1 ml of 50× MEM essential amino acid/100 ml KSOM) at 37°C under 5% CO$_2$ ON (Ho et al., 1995). 24 h after initiation of insemination two-cell embryos and unfertilized oocytes were counted and imaged on a Leica M60 stereo microscope (Leica Mikrosystem, Wetzlar, Germany).

Mating and fertility
Thirty CD-1 females were mated with CD-1 $+/+$ males to assess the capacity to give birth. Reproductive capacity for Best1$^{Y227N}$ and Best1$^{-/-}$ males was assayed by breeding 7 CD-1 N/N and 8 $+/+$ males with two tested $+/+$ CD-1 females, respectively. After 19 days, females were isolated and kept in holding cages. The total number of litters and pups during the entire mating period was counted.

Statistical analysis
For comparison between two groups, statistical analysis was performed by two-tailed Student’s t-test. For comparisons among three or more groups, we used one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.H.F.W.; Methodology: N.T., M.W.S., J.R.S.; Validation: N.T., M.W.S., J.R.S.; Formal analysis: A.M., D.S., J.R.S.; Investigation: A.M., D.S., N.T., M.W.S., J.R.S.; Resources: D.S., B.H.F.W.; Data curation: B.H.F.W.; Writing - original draft: A.M.; Writing - review & editing: B.H.F.W.; Visualization: A.M., D.S., M.W.S., J.R.S.; Resources: D.S., B.H.F.W.; Data curation: B.H.F.W.; Funding acquisition: B.H.F.W.; Project administration: B.H.F.W., B.H.F.W.

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