New mouse models for recessive retinitis pigmentosa caused by mutations in the Pde6a gene

Kenji Sakamoto¹,†, Michael McCluskey¹, Theodore G. Wensel²,3,4, Jürgen K. Naggert¹ and Patsy M. Nishina¹,*

¹The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA, ²Department of Biochemistry and Molecular Biology, ³Department of Neuroscience and ⁴Department of Ophthalmology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

Received August 29, 2008; Revised and Accepted October 9, 2008

The heterotetrameric phosphodiesterase (PDE) 6 complex, made up of α, β and two γ subunits, regulates intracellular cGMP levels by hydrolyzing cGMP in response to light activation of G protein coupled receptors in cones and rods, making it an essential component of the visual phototransduction cascade [Zhang, X. and Cote, R.H. (2005) cGMP signaling in vertebrate retinal photoreceptor cells. Front. Biosci., 10, 1191–1204.]. Using a genetic positional candidate cloning strategy, we have identified missense mutations within the catalytic domain of the Pde6a gene in two mouse models from an ethyl nitrosourea chemical mutagenesis screen. In these first small rodent models of PDE6A, significantly different biochemical outcomes and rates of degeneration of murine photoreceptor cells were observed, indicating allelic variation and previously unrecognized structure–function relationships. In addition, these new models reveal that the mutations not only affect the function of the PDE6A protein itself, but also the level of PDE6B within the retina. Finally, we show that the variation of the disease phenotype by background modifier genes may be dependent upon the particular disease allele present.

INTRODUCTION

Retinitis pigmentosa (RP), characterized by progressive photoreceptor degeneration, vision impairment and often blindness, affects approximately 1 in 4000 individuals worldwide (1). According to RetNet (http://www.sph.uth.tmc.edu/retnet/home.htm), a database that summarizes heritable retinal disease loci reported to date, 21 autosomal recessive RP genes have been identified, many of which encode proteins important in the rod photoreceptor visual transduction cascade. Central among these is the heterotetrameric phosphodiesterase (PDE) 6 complex, consisting of α, β and two γ subunits. Each subunit is essential for photoreceptor function and maintenance, as mutations within PDE6A and PDE6B have been reported to cause recessive RP in humans (2–4), and mutations within Pde6g have been reported to cause retinal degeneration in mice (5,6). Several mouse models with mutations in Pde6b, such as rd1 (7), rd10 (8), rd11 (9) and those from the Medical Research Council, Human Genetics Unit, Western General Hospital (Edinburgh, Scotland, UK) (9) are available. The Cardigan Welsh corgi dog, however, is the only animal model reported to date with a PDE6A mutation (10,11). With the exception of a brief clinical report (2,4) and a report describing the mutation in PDE6A (10,11), studies to examine the biochemical consequences of a mutation in PDE6A have yet to be carried out.

Here, we report two new mouse models with Pde6a mutations identified in a G3 ethyl nitrosourea (ENU) mutagenesis screen. The original residues mutated in these Pde6a models encode for amino acids that are highly conserved across species both at the nucleotide and amino acid level. Despite the fact that both mutations are located within the catalytic domain of the PDE6A protein, the biochemical consequences of the mutations differ. cGMP levels are elevated in Pde6a(nmf137) (8) and Pde6anmf282/nmf282 mice when compared with their respective controls.
Photoreceptor degeneration also differs between the mutants. Photoreceptor degeneration occurs at a faster rate in Pde6a<sup>nmf282/nmf282</sup> mice. Our data suggest that the valine 685 residue is more critical to PDE6A function than aspartic acid 670. We also show that genetic background modifier(s) are able to delay photoreceptor cell death in dark-reared Pde6a<sup>nmf363/nmf365</sup> but not in Pde6a<sup>nmf282/nmf282</sup> mutants. The availability of this allelic series of Pde6a mutations will allow for detailed in vivo studies of the structure and function of PDE6A and provides a means to examine genetic modifying influences on disease progression.

RESULTS

nmf282 and nmf363 are caused by missense mutations in Pde6a

Mice with an abnormal fundus pigmentation were observed by indirect ophthalmoscopy in a colony of ENU mutagenized G3 A.B6-Tyr<sup>+/+</sup>/J mice in the Neuroscience Mutagenesis Facility (NMF) at The Jackson Laboratory. At 3 months of age, three out of 11 G3 offspring from a sibship were affected, suggesting a recessive inheritance of the disorder. Outcrosses of an affected mutant, named nmf282, to wild-type strain A.B6-Tyr<sup>+/+</sup>/J and an intercross of the resulting F1 mice confirmed the recessive inheritance pattern as no F1 mice were affected but 25% of F2 mice had a grainy fundus appearance upon examination. To map the disease-causing mutation, affected mice were outcrossed to strain DBA/2J and the resultant F1 mice were intercrossed. DNA samples from the segregating F2 intercross were used in a genome wide scan in which a DNA pooling strategy was employed, followed by genotyping of individual DNA samples with simple sequence length polymorphic (SSLP) markers to confirm the linkage. The nmf282 disease locus was mapped to Chromosome 18 between markers D18Mit51 and D18Mit81. Subsequently, all F2 mice were phenotyped for the grainy retinal appearance by indirect ophthalmoscopy and individually genotyped for the flanking markers, D18Mit51 and D18Mit81, to construct a fine structure map for the region containing nmf282. All unaffected mice carrying a recombination between the flanking markers were crossed to homozygous nmf282/nmf282 animals to determine whether the recombinants carried the nmf282 allele. The region encompassing nmf282 was narrowed to 1.26 Mb in size between markers D18Mit51 and SNP rs237402 (Fig. 1A, Supplementary Material, Fig. S1 and Table S1).

The critical nmf282 region identified by the fine structure map contained 12 genes, according to the Ensembl database (http://www.ensembl.org), including Pde6a, an excellent candidate gene. Sequencing of both Pde6a cDNA and genomic DNA from A.B6-Tyr<sup>+/+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> mice revealed a single nucleotide G to A missense mutation in exon 16 (Fig. 1B), predicted to cause an amino acid change from aspartic acid to glycine (D670G) in PDE6A (Fig. 1D). This Pde6a<sup>nmf282</sup> mutation, located in the catalytic domain of PDE6A, is 15 amino acids from the Pde6a<sup>nmf282</sup> mutation. The aspartic acid residue at position 670 is also highly conserved among zebrafish, mouse, dog, human and bovine (Fig. 1C).

Mutations within the Pde6a subunit lead to rapid photoreceptor degeneration

By histological analysis of retinal sections from A.B6-Tyr<sup>+/+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> mice and age-matched controls stained with hematoxylin-eosin, photoreceptor degeneration was clearly observable at P12 with an ~20% reduction in the outer nuclear layer (ONL) thickness and an ~50% reduction in the outer segment (OS) thickness (Fig. 2A and B). At P14, the thickness of the ONL and IS decreased to ~50% of that observed in heterozygote mice and all OS were degenerated. Nearly all photoreceptors were gone by P21. In contrast, at P12, retinal sections of C57BL/6J-Pde6a<sup>nmf363</sup> mice and age-matched controls until P16. By P14, an ~30% reduction in ONL thickness is observed in mutant mice. The thickness of the IS and OS is almost comparable to that of C57BL/6J-Pde6a<sup>nmf365</sup> mutants controls until P16. Degeneration of OS is clearly seen in C57BL/6J-Pde6a<sup>nmf363</sup> mutants at P19. However, even at P36, a single layer of cell bodies still remained in the ONL of the mutant mice. Therefore, the photoreceptor degeneration while rapid has a later onset and is prolonged in C57BL/6J-Pde6a<sup>nmf363</sup> mutants compared to A.B6-Tyr<sup>+/+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> mutant mice. Functional analyses by ERG are consistent with these histological observations (Fig. 2E). While the ERG response is extinguished in A.B6-Tyr<sup>+/+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> mutants at P14, some residual activity is still observed in C57BL/6J-Pde6a<sup>nmf363</sup> mutants at P21.

Retinas of A.B6-Tyr<sup>+/+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> and C57BL/6J-Pde6a<sup>nmf363/nmf365</sup> mutant mice have reduced PDE activity and subunit levels but accumulate different levels of cGMP

To determine the consequences of the missense mutations in Pde6a, the cGMP-specific PDE activities in A.B6-Tyr<sup>+/+</sup>
J-Pde6anmf282/nmf282 mutant retinal fractions were measured at a substrate concentration of 0.4 mM (Fig. 3A). As anticipated, the activity of cGMP-specific PDE in the Pde6anmf282/nmf282 mutant retinas was significantly reduced in comparison to heterozygous controls at P7, P10 and P12.

Trypsinization is reported to maximally activate cGMP-PDE activity (12) by cleavage of the PDE complex component, PDE6G, the inhibitory subunit of PDE6 and PDE6B (13). Therefore, the cGMP-specific PDE activities of trypsinized retinal cytosolic and membrane fractions of Pde6anmf282/nmf282 at P12 were measured (Fig. 3B). An activation of cGMP-PDE activity was not observed following trypsinization of the retinal fractions of the mutant mice, whereas trypsinization dramatically activated both cytosolic and membrane cGMP-PDE activity of the heterozygous controls. This suggests that the residue mutated in A.B6-Tyr+/J-Pde6anmf282/nmf282 mice is absolutely essential for the catalytic function of PDE6A.

The cGMP-specific PDE activities in C57BL/6J-Pde6anmf363/nmf363 were also significantly lower than those of heterozygous controls at P12, P16 and P19 (Fig. 3C). However, the activity after trypsinization in retina of the mutant mice remained significantly lower than that observed in the retinas of heterozygous controls.

To determine if the reduction in PDE activity was attributable to a reduction in PDE6A levels, western analysis was carried out. Immunoblots showed that PDE6A and PDE6B were abundant in the membrane fraction with significantly lower levels in the cytosolic fraction of homogenized retinas from heterozygous control mice. In contrast, the levels of both proteins were clearly reduced, and when corrected for loading, the cytosolic fraction appeared to contain more PDE6A and PDE6B than the membranous fraction in the retinas of A.B6-Tyr+/J-Pde6anmf282/nmf282 and C57BL/6J-Pde6anmf363/nmf363 mutant mice (Fig. 4). PDE6G was detected exclusively in the membrane fraction of the retinal extracts at equivalent levels in both heterozygous controls and mutant mice carrying either mutation.

It has been previously shown that cGMP content increases in mice carrying mutations affecting PDE6B activity (14,15). To determine if the observed reduction in cGMP-PDE activity also lead to the accumulation of cGMP in A.B6-Tyr+/J-Pde6anmf282/nmf282 and C57BL/6J-Pde6anmf363/nmf363 mutant mice, the retinal cGMP content of mutants and their respective controls were measured between postnatal day (P) 7 through P21 (Fig. 5A and B).
Figure 2. (A) Histological analysis of retinas from A.B6-Tyr+/+Pde6anmf282/+ and A.B6-Tyr+/+Pde6anmf282/+Pde6anmf282/+ mice at postnatal days (P) 11, P12, P14 and P21. All sections were stained with hematoxylin-eosin and photographed at the same magnification (200×). Scale bar represents 50 µm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments. (B) Temporal changes of retinal degeneration, presented as thickness of ONL (i), IS (ii) and OS (iii) normalized by INL thickness to correct for sectioning artifacts, in A.B6-Tyr+/+Pde6anmf282/+ and A.B6-Tyr+/+Pde6anmf282/+Pde6anmf282/+ mice at P11, P12 and P14. The earliest change is seen at P12 in the OSs of the mutant retina. Thicknesses of ONL and IS in the mutant mice at P14 are significantly decreased compared with those in the heterozygous control. OS are almost gone by P14 in mutant mice. *P < 0.05, A.B6-Tyr+/+Pde6anmf282/+ versus Pde6anmf282/+Pde6anmf282/+ by Student’s t-test. (C) Histological analysis of retinas from C57BL/6J-Pde6anmf363/+ and C57BL/6J-Pde6anmf363/+Pde6anmf363/+ at P12, P14, P19 and P36. All sections were stained with hematoxylin-eosin and photographed at the same magnification (200×). Scale bar represents 50 µm. (D) Temporal changes of retinal degeneration, presented as thickness of ONL (i), IS (ii) and OS (iii) normalized by INL thickness, in C57BL/6J-Pde6anmf363/+ and C57BL/6J-Pde6anmf363/+Pde6anmf363/+ at P12, P14, P19 and P36. All sections were stained with hematoxylin-eosin and photographed at the same magnification (200×). Scale bar represents 50 µm. (E) The a-wave (i) and b-wave (ii) amplitudes of dark-adapted electroretinograms from A.B6-Tyr+/+ (n=1) and A.B6-Tyr+/+Pde6anmf282/+Pde6anmf282/+ mice at P14 (n=3), and C57BL/6J (n=1) and C57BL/6J-Pde6anmf363/+Pde6anmf363/+ at P21 (n=4). While ERG response is extinguished in A.B6-Tyr+/+Pde6anmf282/+Pde6anmf282/+ mutants at P14, some residual activity is observed in C57BL/6J-Pde6anmf363/+Pde6anmf363/+ mutants at P21.
Figure 3. (A) Cyclic GMP-PDE activity in the retinal membrane fraction of A.B6-Tyr/J-Pde6a<sup>nmf282/+</sup> and A.B6-Tyr/J-Pde6a<sup>nmf282/nmf282</sup> mice at P7, P10 and P12. The PDE activity of mutant retinal membrane fractions was significantly lower than that in the heterozygous control. *P < 0.05, A.B6-Tyr/J-Pde6a<sup>nmf282/+</sup> versus Pde6a<sup>nmf282/nmf282</sup> by Student’s t-test. (B) Cyclic GMP-PDE activity in the retinal cytosol and membrane fractions of A.B6-Tyr/J-Pde6a<sup>nmf282/+</sup> and A.B6-Tyr/J-Pde6a<sup>nmf363/nmf363</sup> mice treated with trypsin at P10. Trypsinization-activated PDE activity in A.B6-Tyr/J-Pde6a<sup>nmf282/nmf282</sup> but not in A.B6-Tyr/J-Pde6a<sup>nmf363/nmf363</sup> retinal samples. (C) Cyclic GMP-PDE activity in the retinal membrane fractions of C57BL/6J/6-Pde6a<sup>nmf565/+</sup> and C57BL/63-Pde6a<sup>nmf565/nmf565</sup> mice at P12, P16 and P19. The PDE activity of mutant retinal membrane fractions was significantly lower in comparison to heterozygous controls. *P < 0.05, C57BL/63-Pde6a<sup>nmf565/nmf565</sup> versus C57BL/63-Pde6a<sup>nmf565/nmf565</sup> by Student’s t-test. (D) Cyclic GMP-PDE activity in the retinal cytosol and membrane fractions of C57BL/63-Pde6a<sup>nmf563/nmf563</sup> and C57BL/63-Pde6a<sup>nmf563/nmf563</sup> retinal samples.

In A.B6-Tyr<sup>+</sup>/J-Pde6a<sup>nmf282/+</sup> (Fig. 5A), the retinal cGMP level was 1.5 ± 0.2 pmol/mg protein at P7, but gradually increased as photoreceptor OSs matured. In the retina of mutant mice, cGMP levels were slightly but significantly higher than that observed in controls at P7, similar to controls at P8, and lower than controls at P10. After P11, mutant cGMP levels rose significantly, reaching 28.8 ± 3.2 pmol/mg protein at P12, which is approximately 2-fold higher than that observed in control retinas. The cGMP content in retinas of C57BL/63-Pde6a<sup>nmf363/+</sup> control and C57BL/63-Pde6a<sup>nmf363/nmf363</sup> mutant mice were measured between P12 through P21 (Fig. 5B). The cGMP level in the heterozygous control retina was 18.9 ± 1.7 pmol/mg protein at P12, and gradually increased as photoreceptor OSs matured. However, unlike A.B6-Tyr<sup>+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> mutants, cGMP levels of C57BL/63-Pde6a<sup>nmf363/nmf363</sup> mutant mice were similar to the control at P12, but declined to significantly lower levels than controls between P14 through P21. To determine if the lower cGMP content was due to a reduction in the generation of cGMP, the activity of the key enzyme necessary for its generation, guanylate cyclase (GC), was assessed. The basal activity of GC in the membranous fraction of retinal extracts in the presence of 30 mM EDTA and absence of exogenous guanylate cyclase activating proteins (GCAPs) was not different in heterozygote controls and mutants at P10, but GC activity was significantly reduced in Pde6a<sup>nmf282/nmf282</sup> mutants compared to heterozygous controls at P12 and P14 (Fig. 6A). In C57BL/63-Pde6a<sup>nmf363/nmf363</sup> mutants, GC activity did not differ from heterozygous control until P13, but at P16, GC activity was significantly lower than that of heterozygous controls (Fig. 6D). The reduction in activity was reflected in the levels of GC protein which was reduced in both Pde6a<sup>nmf282/nmf282</sup> retinas at P14 (Fig. 6B and C) and C57BL/63-Pde6a<sup>nmf563/nmf563</sup> retinas at P16 (Fig. 6E and F).

In an effort to normalize the data above with respect to the actual amount of OSs present, immunoblots of retinal lysates from the different mutants and controls were probed with anti-rhodopsin antibody in a longitudinal analysis. Interestingly despite significant shortening of OSs (Fig. 2B and D), rhodopsin levels did not differ across the time points described above for cGMP-PDE activity and cGMP level assays (data not shown). This led us to examine rhodopsin localization in the Pde6a mutants. Rhodopsin is localized not only in OS but also the perinuclear region of ONL in A.B6-Tyr<sup>+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> mice at P11 (data not shown). The same phenomenon was also observed in C3H-Pde6a<sup>rad1/rad1</sup> mice at P11 (data not shown).

Natural disease progression and biochemical differences between A.B6-Tyr<sup>+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> and C57BL/63-Pde6a<sup>nmf563/nmf563</sup> are due to allelic variance and not to the influence of genetic background

To determine whether the biochemical differences and rate of photoreceptor degeneration between A.B6-Tyr<sup>+</sup>/J-Pde6a<sup>nmf282/nmf282</sup> and C57BL/63-Pde6a<sup>nmf563/nmf563</sup> raised in standard vivarium conditions were attributable to allelic...
or genetic background influences, we intercrossed male and female F1 C57BL/6J;A.B6-Tyr\(^+\)/J-Pde6anmf282/nmf363 compound heterozygous mice to generate F2 mice homozygous for either allele and compound heterozygotes on a mixed C57BL/6J and A.B6-Tyr\(^+\)/J segregating background. The photoreceptor degeneration observed at P14 in mutants in a mixed genetic background was similar to that observed for the same alleles in their original fixed inbred backgrounds (Fig. 7A and B). The reduction of the thickness of ONL and OS in compound heterozygous mutants lay between the two homozygous parental alleles in the same segregating background. In addition, the pattern of retinal cGMP levels at P14 in mixed background animals of either variant was also similar to that observed in those obtained for the original inbred background (Fig. 7C). These data strongly indicate that the differences between the two Pde6a mutants are due to the specific mutations and not to genetic background influences.

No activation of calpain or caspase-3 is observed in Pde6a mutants

High intracellular cGMP concentrations have been shown to lead to an increased intracellular Ca\(^{2+}\) influx (16), presumably due to the opening of cGMP-gated channels. High intracellular Ca\(^{2+}\) concentrations are in turn thought to activate calpain, one of the calcium-activated neutral proteases, and/or caspase-3 (17,18). To test whether calpain and/or caspase-3 are activated in A.B6-Tyr\(^+\)/J-Pde6a\(^{nmf282/nmf282}\) mutants, in which positive ONL TUNEL staining was observed at P11 (Fig. 8A) or C57BL/6J-Pde6a\(^{nmf363/nmf363}\) at P14, we examined the cleavage of \(\alpha\)-fodrin, a substrate for both calpain and caspase-3. \(\alpha\)-fodrin, with a molecular weight of 240–280 kDa, is cleaved to 145 and 150 kDa forms by calpain and to 145 and 120 kDa by caspase-3 (16,18). As anticipated for the C57BL/6J-Pde6a\(^{nmf363/nmf363}\) mutant mice in which cGMP levels were not elevated, cleavage of fodrin and caspase-3 was not observed. Similarly, in A.B6-Tyr\(^+\)/J-Pde6a\(^{nmf282/nmf282}\) mutant retinas cleavage of fodrin and caspase-3 was also not observed, despite the increased cGMP levels in this mutant. To confirm the results, immunoblots probed with anti-cleaved caspase-3 antibody showed an absence of cleaved caspase-3 in both mutants (Fig. 8B and C).

Dark-rearing slows the progression of photoreceptor degeneration in C57BL/6J-Pde6a\(^{nmf363/nmf363}\) but not in A.B6-Tyr\(^+\)/J-Pde6a\(^{nmf282/nmf282}\) mutant mice and is due to genetic background influences

Progression of photoreceptor degeneration in some RP mouse models can be retarded by dark-rearing, suggesting that the exposure to light can precipitate or hasten the degenerative process (7). To determine if this was also the case with Pde6a mutants, both strains were bred and dark-reared until...
sacrifice at P14 (Pde6a<sup>nmf282/nmf282</sup>) or P19 (Pde6a<sup>nmf363/nmf363</sup>). At P14, the ONL thickness of dark-reared A.B6-Tyr<sup>+/J-Pde6a<sup>nmf282</sup>/-</sup> mice did not differ from that of mutants reared under standard lighting conditions (Fig. 9A and B). The ONL thickness at P19 of dark-reared C57BL/6J-Pde6a<sup>nmf363/nmf363</sup> mutant mice, however, was significantly greater than that observed for mutants reared under standard lighting conditions. The functional analyses by ERG were consistent with the histological observations. At P21 when the ERG a-wave is extinguished in C57BL/6-Pde6a<sup>nmf363/nmf363</sup> mutants reared under standard lighting condition, a clear a-wave, whose amplitude is smaller than normal C57BL/6J (Fig. 2E), is still observed in dark-reared mutants (Fig. 9C). The b-wave amplitude of dark-reared animals is also larger than that of the mutants reared under standard lighting conditions.

To test whether this protection is caused by genetic background influences, dark-reared F2 mice homozygous for either allele on a mixed segregating background were examined for protection from photoreceptor loss at P19. As anticipated, no protection from photoreceptor loss was observed in 9 F2 Pde6a<sup>nmf282/nmf282</sup> mutants. Interestingly, photoreceptor loss similar to the animal reared under standard lighting conditions was observed in all eight dark-reared F2 Pde6a<sup>nmf363/nmf363</sup> mutants examined (Fig. 10). These results suggest that the partial protection from photoreceptor loss seen in dark-reared C57BL/6-J-Pde6a<sup>nmf363/nmf363</sup> is probably a result of genetic background influences. Although a relatively small cohort, because none of the mice that were genotypically nmf363/nmf363 on the segregating background were protected, it suggests that the genetic modifiers act in a recessive manner or that multiple modifiers act in concert to contribute to the observed protection.

Figure 5. (A) Cyclic GMP content in retinas of A.B6-Tyr<sup>+/J-Pde6a<sup>nmf282</sup>/-</sup> mice between postnatal day (P) 7 through P14. The levels of cGMP in the mutant retina after P11 were higher than those in heterozygous controls. *P < 0.05, A.B6-Tyr<sup>+/J-Pde6a<sup>nmf282</sup>/-</sup> versus Pde6a<sup>nmf282/nmf282</sup> by Student’s t-test. (B) Cyclic GMP content in the retinas of C57BL/6-J-Pde6a<sup>nmf363</sup>/J- and C57BL/6-J-Pde6a<sup>nmf363</sup> mice between postnatal day (P) 12 through P21. The levels of cGMP in the mutant retina after P14 were lower than those in heterozygous controls. *P < 0.05, C57BL/6-J-Pde6a<sup>nmf363</sup>/J- versus C57BL/6-J-Pde6a<sup>nmf363/nmf363</sup> by Student’s t-test.

**DISCUSSION**

The PDE6 complex is an essential component of the visual phototransduction cascade. It regulates intracellular cGMP levels by hydrolysis of cGMP as a consequence of light activation of cone or rod G protein coupled receptors (20). Mutations within PDE6A and PDE6B have been identified in humans, accounting for ~3–4% of recessive RP in North America (4). A small rodent model for PDE6A mutations had, however, not yet been reported. The two new mouse models, generated by ENU chemical mutagenesis, C57BL/6-J-Pde6a<sup>nmf363/nmf363</sup> and A.B6-Tyr<sup>+/J-Pde6a<sup>nmf282/nmf282</sup></sup>, described here provide a unique resource to study both the biochemical and pathological consequences of different mutations within the PDE6A gene.

In the present study, we identify two missense mutations within Pde6a that are predicted to alter highly conserved amino acids in the catalytic domain of the protein. Previous studies indicate that the aspartate residue at position 670, which is mutated in C57BL/6-J-Pde6a<sup>nmf363/nmf363</sup>, is conserved across the PDE superfamily. Although a crystal structure of PDE6A is currently not available, an X-ray crystallography determined structure of PDE5A. Because of the high similarity of the two proteins, by inference, the mutant residue in Pde6a<sup>nmf363/nmf363</sup> is predicted to be positioned very near the bound GMP in the crystal structure of the PDE5a catalytic domain (21) when GMP is bound (Fig. 12). This suggests that this residue may have functional importance across the PDE superfamily. In contrast, the valine
The activity was almost gone in the A.B6-Pde6a<sup>nmf282</sup> and A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup> mice between postnatal day (P) 10 through P14. The GC activity in the mutant retina after P12 was lower than that in heterozygous controls. <sup>*</sup> P < 0.05, A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup> versus Pde6a<sup>nmf282</sup>/Pde6a<sup>nmf282</sup> by Student’s t-test. (B) To determine the levels of GC, western analysis of retinal lysates isolated from A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup> and A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup> retinas at postnatal day 10, 12 and 14 was performed using anti-GC type E and anti-β-actin antibodies. (C) The band density of each PDE subunit was measured and normalized by the band density of β-actin. Each column shows the mean of four independent experiments. <sup>*</sup> P < 0.05, A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup> versus Pde6a<sup>nmf282</sup>/Pde6a<sup>nmf282</sup> by Student’s t-test. (D) GC basal activity in the retinas of C57BL/6J-Pde6a<sup>nmf363</sup> and C57BL/6J-Pde6a<sup>nmf363</sup> homozygotes was measured and normalized by the band density of β-actin antibodies. (E) To determine expression levels of GC, immunobLOTS were performed using anti-GC type E and anti-β-actin antibodies in retinal lysate isolated from C57BL/6J-Pde6a<sup>nmf363</sup> and C57BL/6J-Pde6a<sup>nmf363</sup> homozygous mice. (F) The band density of each PDE subunit was measured and normalized by the band density of β-actin. Each column shows the mean of four independent experiments. <sup>*</sup> P < 0.05, C57BL/6J-Pde6a<sup>nmf363</sup>/Pde6a<sup>nmf363</sup> and C57BL/6J-Pde6a<sup>nmf363</sup>/Pde6a<sup>nmf363</sup> by Student’s t-test.

As anticipated, both mutations lead to a decrease in cGMP-PDE activities. However, unexpectedly, the cGMP-PDE activity differed between the two mutants with C57BL/6J-Pde6a<sup>nmf363</sup> and Pde6a<sup>nmf282</sup> mutants having a milder reduction in activity than that observed in A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup> PDE activity. The total cGMP levels also differed. In A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup>, the retinal cGMP content from P11 through P14 was almost twice as high as that observed in the heterozygous controls. In contrast, the cGMP content in the C57BL/6J-Pde6a<sup>nmf363</sup> retina was significantly lower than that observed in the heterozygous controls between P14 and P21. The levels and activity of GC, the key enzyme necessary for the generation of cGMP, were reduced in both mutants. However, while the cGMP-PDE activity was almost gone in the A.B6-Tyr<sup>+/−</sup>/Pde6a<sup>nmf282</sup> retinas at P10, it was still partially active in the C57BL/6J-Pde6a<sup>nmf363</sup> retinas at P16. Therefore, it is possible that the residual activity of cGMP-PDE is involved in the lower cGMP content observed in C57BL/6J-Pde6a<sup>nmf363</sup> retinas. Alternatively, since ~90% of the total cGMP in OSs in WT animals is bound to the GAF domains of PDE6, the reduction of cGMP levels in Pde6a<sup>nmf363</sup> homozygotes may be due primarily to the decrease in PDE6 levels. The cytoplasmic cGMP levels may not be dramatically altered. In contrast, in Pde6a<sup>nmf282</sup> homozygous mice, a high level of cGMP in addition to greatly reduced PDE6 levels suggests that cytoplasmic levels of cGMP must be much higher than normal. This difference may partially account for the difference in severity and in effects of dark-rearing between the two alleles.

A reduction in retinal cGMP content in mice heterozygous for the Pde6<sup>a<sup>−/−</sup></sup> mutation (23,24) has been observed in association with mildly lower retinal cGMP-PDE activities compared to wild-type controls (19). Likewise, a similar difference in cGMP levels was observed between null and hypomorphic alleles of aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1), a protein essential for the biosynthesis of PDE6. While Aipl1 null mutants were reported to have an elevated retinal cGMP content (25), Aipl1-hypomorphic mice were shown to have a lower retinal cGMP content than controls (26). It is interesting to note that AIPL1 plays an essential role in processing of farnesylated proteins in retina (27). The carboxyterminus of PDE6A is farnesylated (28), and this modification is necessary for proper anchoring of the PDE6 complex to the OSs (29). Presumably, the observed changes in cGMP levels in Aipl1 mutants are in part due to its role in generating a fully functional PDE6 protein complex localized to the OSs. In both Pde6a mutants, the PDE6A content of the cytosolic fraction was greater than that for the membranous fraction suggesting that PDE6A is not bound to
the OS membranes as observed for the wild-type controls. Perhaps, the mutations play roles in the pathological consequences of the disease phenotype by a combination of indirect effects on defective binding to the membranous fraction and the reduction in PDE6B levels, and of the direct effects on catalytic activity of the PDE6A subunits.

Through genetic studies, we have shown that the differential reduction in retinal cGMP-PDE activity between the two mutants is due to allelic and not due to genetic background influences. Trypsinization which is reported to activate cGMP-PDE activity (12) was unable to increase cGMP-PDE activity in the retinal lysates of A.B6-Tyr+/J;C57BL/6-\textit{Pde6a} nmf282/nmf282 mice. This suggests that the \textit{nmf282} mutation renders the PDE6A protein non-functional, and that the valine at position 685 is important for the enzymatic activity of PDE6A. Alternatively, the mutation may affect both the PDE6A catalytic activity as well as the binding of PDE6G to PDE6A, rendering the trypsinization superfluous. However, the V685M amino acid change does not fall within the residues 751–763 of PDE6A which have been previously reported to be important for the enzymatic activity of PDE6A. The mutation that A.B6-Tyr+/J;C57BL/6-\textit{Pde6a} nmf282/nmf282 mice carry carries an accumulation of cGMP that could potentially lead to excessive calcium influx through cGMP-gated cation channels. Although intracellular calcium levels were not determined in the present study, intracellular cGMP (14,15) and calcium levels (16) have been reported to be elevated at P10 prior to the onset of apoptosis in \textit{Pde6brd1/rd1} mice. Calcium levels have also been shown to be elevated in light-induced photoreceptor damage models (30), suggesting that calcium may play a central role in retinal degenerations. It is still largely unknown how the sustained increase of intracellular calcium leads to photoreceptor degeneration. However, it has been suggested that calpain, one of the calcium-activated neutral proteases, and caspase-3 are activated and lead to the cleavage of \textit{\alpha}-fodrin, an essential cytoskeletal protein. Cleavage of \textit{\alpha}-fodrin is thought to contribute to the cellular collapse in apoptotic cells (18). This phenomenon has been reported in \textit{Pde6b} rd1/rd1 mutants (16) in which cGMP levels are elevated 4–5-fold and calpain activity increased significantly during retinal degeneration. Both changes are closely associated with the death of photoreceptor cells (31,32). In contrast, in neither of the \textit{Pde6a} mutants examined was cleavage of \textit{\alpha}-fodrin or caspase-3 observed. Therefore, a mechanism other than increased calcium influx may underlie the retinal degeneration observed in these particular mutants in which cGMP levels are either 2-fold elevated or reduced by 50% in comparison to wild-type controls.

Since dark-rearing of C57BL/6-\textit{Pde6a} nmf363/nmf363 mutants was able to slow the photoreceptor degeneration, oxidative stress may potentially be involved in the mechanism of cell death in this model. Alternatively, if the lower cGMP levels observed in C57BL/6-\textit{Pde6a} nmf363/nmf363 mice play an important role in the degeneration, dark-rearing may provide some protection by increasing the cGMP levels. Further investigation to clarify the mode through which apoptosis occurs is necessary.

Mutations in the \textit{PDE6A} gene have been described in families with autosomal recessive RP (2), and the ENU-induced mouse models described here are the first small rodent models of RP.
induced by mutations within \textit{Pde6a}. To date, the Cardigan Welsh corgi dog has been the only animal model available to study the disease pathology and progression as a result of a mutation within \textit{PDE6A} (9,10). The \textit{Pde6a} mouse models described here reveal complex changes, not only within the protein itself but in other molecules of the phototransduction cascade, and the allelic variation demonstrated by these models will yield further insight into the structure and function of the mutant residues. Additionally, the modification of the disease phenotype by dark-rearing will be valuable for identifying molecules that may be able to delay RP caused by \textit{PDE6A} mutations. Finally, these models will be useful for testing various treatment modalities such as the modification of environmental effects and the use of pharmaceutical agents or gene therapy that are expected to delay or ameliorate photoreceptor degeneration.

**MATERIALS AND METHODS**

**Animals, ENU mutagenesis and crosses**

Breeding and housing of the mice were conducted in the NMF or in the Research Animal Facility at The Jackson Laboratory under specific pathogen-free conditions. The mice were provided an autoclaved 6% fat chow diet (5K52, Lab Diet, PMI International, Brentwood, MO, USA) and acidified (pH 2.5; to retard bacterial growth) water in a vivarium with a 12:12 h dark–light cycle. To generate chemically mutanized mice, male A.B6-\textit{Tyr}\textsuperscript{+/J} or C57BL/6J mice were treated with three doses of ENU, and immediately mated with untreated females of the same background strain to produce G1 offspring. If, only after 3 months from ENU administration, mutagenized G0 mice were able to sire offspring, the mutagenesis was considered successful. For recessive mutations, a three-generation screen was used in which male G1 mice from successfully mutagenized G0 males were mated to wild-type female mice of the same strain to produce G2 offspring. Female G2 mice were backcrossed to their G1 sire to produce G3 offspring. G3 mice were screened by indirect ophthalmoscopy. Once a potential mutant was identified, affected G3 mice were crossed with wild-type mice of the same genetic background to generate F1 offspring, and the F1 mice were intercrossed. If the F1 offspring were unaffected and one-fourth of the individuals in the F2 generation were affected, the mutation was considered to be heritable in a recessive fashion. The mutant mouse colonies were maintained by heterozygote \times heterozygote, mutant \times heterozygote and mutant \times mutant matings. To genetically map the mutations, affected mutants were mated with DBA/2J mice, and the resulting F1 progeny were intercrossed to produce F2 offspring or backcrossed to the affected parental strain to produce a BC population. In the subsequent high-resolution intercrosses, uninformative recombinant mice were progeny-tested by crossing them to mice homozygous for the mutation to confirm that the recombinant animal carried the mutant allele.

**Chromosomal localization, fine structure mapping and genotyping**

Tail DNA was isolated from mice generated in the intercrosses described above, according to Buffone and Darlington (33). DNA pools of affected and unaffected F2 offspring were subjected to a genome-wide scan with 80 robust SSLP markers known to differ between the parental strains, A.B6-\textit{Tyr}\textsuperscript{+/J} or C57BL/6J, and DBA/2J (34). Once a significant skewing in allele abundance between the two DNA pools was observed, DNA samples were tested individually using SSLP MIT and SNP markers (Supplementary Material, Table S1) to confirm the linkage.

Figure 8. (A) TUNEL staining and western analyses using anti-cleaved caspase-3 antibody in retinas from A.B6-\textit{Tyr}\textsuperscript{+/J}-Pde6anmf282\textsuperscript{+/+} and A.B6-\textit{Tyr}\textsuperscript{+/J}-Pde6anmf282\textsuperscript{+/+} mice at postnatal day (P) 11. Few nuclei that were positive both in TUNEL staining and anti-cleaved caspase-3 staining are seen in the inner nuclear layer (indicated by arrows) of A.B6-\textit{Tyr}\textsuperscript{+/J}-Pde6anmf282\textsuperscript{+/+} (indicated by arrowheads). All sections were photographed at the same magnification (200\times). (B and C) Immunoblots using anti-\textalpha-\textit{ fodrin, anti-cleaved caspase-3 and anti-\textbeta-actin antibodies. Calpain-cleavage product (140–150 kDa) and caspase-3-cleavage product of \textalpha-\textit{ fodrin (120 kDa) and cleaved caspase-3 were not seen in A.B6-\textit{Tyr}\textsuperscript{+/J}-Pde6anmf282\textsuperscript{+/+} A.B6-\textit{Tyr}\textsuperscript{+/J}-Pde6anmf282\textsuperscript{+/+} and C57BL/6J-Pde6anmf282\textsuperscript{+/+} at P11 (B), and C57BL/6J-Pde6anmf282\textsuperscript{+/+} and C57BL/6J-Pde6anmf282\textsuperscript{+/+} at P14 (C).
To screen for a mutation within the coding region of the candidate gene, *Pde6a*, which mapped to the region, we designed PCR primers based on complementary and genomic DNA sequences of the gene. Both cDNA and genomic DNA were used for templates in PCR reactions. To extract total RNA, wild-type, heterozygous and mutant

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retinas at postnatal day (P) 10 were excised and treated with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Respective cDNA pools were generated by reverse transcription from total RNA using the Retroscript kit (Ambion, Austin, TX, USA) as instructed by the manufacturer. For PCR amplifications, 25 ng of DNA was used in a 25 μl volume using AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), Advantage 2 polymerase mix (Clontech, Mountain View, CA, USA) or HotMaster Taq (Eppendorf AG, Hamburg, Germany) according to the manufacturer’s instruction. PCR products were either separated by electrophoresis on a 1% agarose gel or 4% MetaPhor (Cambrex, East Rutherford, NJ, USA) agarose gel, and visualized under UV light after staining with ethidium bromide, or used for direct sequencing. DNA sequencing was performed using the Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) by the DNA sequencing service in The Jackson Laboratory. After the mutation was identified, mutagenically separated PCR (35) assays were carried out to determine the genotype of the animals. Table 1 shows the sequences of the primer sets and annealing temperatures used.

Histological methods

Eyes from heterozygous and mutant mice sacrificed at P11 through P36 by carbon dioxide asphyxiation were enucleated, and fixed overnight at 4°C in a solution of methanol:acetic acid (3:1) diluted to 50% with 1X phosphate buffered saline (PBS). Fixed eyes were embedded in paraffin, and sectioned at 4 μm intervals. After staining with hematoxylin and eosin, the sections were examined for photoreceptor morphology and scored for degree of photoreceptor degeneration by assessing the number of photoreceptor cell bodies present. A Polaroid DMC1 digital camera (Polaroid, Waltham, MA, USA) connected to a Leica DM LB microscope (Leica Microsystems,
Wetzlar, Germany) was used to photograph retinal sections to quantitate and compare the thickness of the inner and OSs and the ONL cell body loss between the mutants and heterozygous controls from P11 to P36. TUNEL staining was performed to detect apoptotic cells using ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) according to the manufacturer’s instruction. For immunohistochemical analysis, tissue sections were rehydrated and subsequently treated for antigen retrieval by boiling samples for 10 min in 10 mM sodium citrate (pH 6.0). Sections were blocked with 5% normal goat serum in PBS, and then incubated in rabbit polyclonal anti-cleaved caspase-3 antibody (1:200; Cell signaling, Danvers, MA, USA) or mouse monoclonal anti-rhodopsin antibody (1:50; Leinco Technologies, St Louis, MO, USA) overnight at 4°C. Finally, sections were treated with Alexa Fluor® 488 Goat Anti-rabbit IgG (1:200, Invitrogen) or Alexa Fluor® 488 Goat Anti-mouse IgG (1:200, Invitrogen) for 1 h at room temperature. Nuclear counterstaining was performed with 4,6-diamidine 2-phenylindoledihydrochloride (DAPI; VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA, USA). Images of TUNEL staining and anti-cleaved caspase-3 staining were collected on a Leica DM LB fluorescent microscope (Leica Microsystems, Wetzlar, Germany) equipped with a SPOT® CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA) using appropriate bandpass filters for each fluorochrome.

Immunoblotting

After sacrifice, retinas from enucleated eyes were dissected using a dissection microscope. When cytosolic and crude membrane fractions were used for samples, retinas were homogenized with PDE assay buffer comprised of 40 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1 mM PMSF and 1 mM leupeptin. The homogenized samples were then centrifuged at 16 000 g for 30 min at 4°C. The supernatant was considered the cytosolic fraction and the pellet resuspended in the PDE assay buffer was considered the crude membrane fraction. When total lysate was used, retinas were homogenized with lysis buffer containing detergents: 50 mM Tris–HCl (pH 7.4), 0.15 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1 mM PMSF and 1 mM leupeptin. The homogenized samples were then centrifuged at 16 000 g for 30 min at 4°C and the supernatant was used as total lysate. Total protein in samples was determined with a micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots of each fraction containing equal quantities of protein were separated on NuPAGE® Novex® 4–12% Bis-Tris Mini Gels and NuPAGE® Novex® 3–8% Tris-Acetate Mini Gels (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The separated proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, MA, USA) using a NuPAGE transfer buffer (Invitrogen, Carlsbad, CA, USA) with 10% methanol, blocked by 3% Perfect-block (MoBiTec, Göttingen, Germany) and probed with anti-PDE6α (Abcam, Cambridge, UK), anti-PDE6β (Abcam), anti-PDE6γ (Abcam), anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), anti-PDE6b (Abcam), anti-

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anti-α-fodrin (Biomol, Plymouth Meeting, PA, USA), anti-guanylate cyclase type E (PGCE) (Abcam) or anti-β-actin antibodies (Sigma, St Louis, MO, USA). Western Lightning chemiluminescence reagent Plus (Perkin-Elmer, Norwalk, CT, USA) was used to visualize immunoreactions on a standard medical X-ray film (Kodak, Rochester, NY, USA). The band density was measured with ImageJ software (National Institute of Health, Bethesda, MD, USA).

**Measurement of cGMP content in retina**

After mice were dark-adapted for at least 2 h, they were sacrificed by carbon dioxide inhalation, and their eyes were enucleated. Retinas were dissected under dim red illumination and homogenized in a 6% trichloroacetic acid solution to extract cGMP. The samples were centrifuged at 16 000g for 10 min at 4°C. Both the supernatant and the pellet were saved for the cGMP and protein assays, respectively. Trichloroacetic acid in the supernatant was then removed with water-saturated diethyl ether. The levels of cGMP in the samples were determined by radioimmunoassay using a cGMP assay kit (RPA525, GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. The total protein in the retinas precipitated by trichloroacetic acid was determined using a micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

**Measurement of cGMP-PDE activity**

Retinas were dissected and immediately homogenized in 500 μl of PDE assay buffer comprised of 40 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1 mM PMSF and 1 mM leupeptin. The homogenate was centrifuged at 16 000g for 30 min at 4°C. The pellet resuspended in 200 μl of PDE assay buffer served as retinal crude membrane fraction. For trypsinization experiments, the samples were incubated with 10 μg/ml L-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at 4°C for 30 min, and the reactions were stopped with the addition of 60 μg/ml soybean trypsin inhibitor (Sigma). PDE activity was measured using an assay mixture containing 0.4 mM [³H]-cGMP and 10–30 μg of cytosolic and retinal crude membrane fraction in a total volume of 100 μl. Samples were incubated for 15 min at 37°C and the reaction was terminated by incubation at 100°C for 3 min. Calf intestine alkaline phosphatase, 0.4 U/sample (New England Biolabs, Ipswich, MA, USA) was added to each sample to cleave the phosphate group from [³H]-5’-GMP generated by PDE. After incubation for 10 min at 37°C, 1.0 ml of a suspension of AG1-X2 resin (50–100 mesh, Cl⁻ form, mixed 1:3 with distilled water; Bio-Rad, Hercules, CA, USA) was added to each sample to remove unreacted [³H]-cGMP and tubes were first rotated for 1 h at room temperature, and then centrifuged for 5 min at 3500g. The radioactivity contained in 500 μl of supernatant was assayed in a liquid scintillation counter. A micro BCA protein assay kit (Pierce Biotechnology) was used to determine the total protein contained in each sample. PDE activity was calculated using the retention rate of guanosine determined by spectrophotometry.

**Guanylate cyclase assay**

Retinas were dissected and immediately homogenized in 500 μl of GC homogenizing buffer comprised of 50 mM Tris–HCl (pH 7.4) and 3.3 mM MnSO₄. The homogenate was centrifuged at 16 000g for 30 min at 4°C. The pellet resuspended in 200 μl of GC homogenizing buffer served as the crude retinal membrane fraction. To measure GC basal activity, 100–300 μg of crude retinal membrane was incubated in 50 mM Tris–HCl (pH 7.4), 3.3 mM MnSO₄, 100 μM 3-isobutyl-1-methylxanthine (Sigma), 40 mM phosphocreatine (Sigma), 75 μg creatine phosphokinase (Sigma) and 1 mM GTP. Total assay volume was 100 μl. To measure background levels of cGMP, samples in which 20 μl of 30 mM EDTA was added were also prepared. Samples were incubated for 10 min at 37°C and the reaction was terminated by adding 400 μl of 6% trichloroacetic acid. The samples were centrifuged at 16 000g for 10 min at 4°C. Both the supernatant and the pellet were saved for cGMP and protein assays, respectively. Trichloroacetic acid in the supernatant was then removed with water-saturated diethyl ether. The levels of cGMP in the samples were determined by radioimmunoassay using a cGMP assay kit (RPA525, GE Healthcare) according to the manufacturer’s instructions. The total amount of the pellet precipitated by trichloroacetic acid was determined using a micro BCA protein assay kit (Pierce Biotechnology). The GC activity was calculated as follows: the activity (pmol/mg protein/min) = ([cGMP content in the intact sample/protein content in the intact sample] – background GMP content/protein content in the background sample)/10.

**Electroretinography**

After dark-adaptation for at least 2 h, mice were anesthetized with ketamine (80 mg/kg) and xylazine (16 mg/kg). The pupils were dilated by the administration of 1% cyclopentolate. Body temperature was maintained at 37°C using a heating pad. Retinal responses were recorded as described previously (36).

**Statistical analysis**

Values from the quantitative assays are represented as mean ± SEM. Student’s t-test was used to determine if the differences between experimental groups were significant. Tukey–Kramer test was used for multiple comparisons. A statistical difference was considered significant at P < 0.05.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at *HMG* Online.

**ACKNOWLEDGEMENTS**

We thank Dr Wesley G. Beamer (The Jackson Laboratory) for careful review of the manuscript and the Fine Mapping Service for carrying out the initial mapping of both mutants.

*Conflict of Interest statement.* None declared.
FUNDING
This research was supported by National Institutes of Health grants, EY016501 and NS41215, Foundation for Fighting Blindness and an institutional grant, CA34196 to The Jackson Laboratory.

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