

# Cone Photoreceptor Function Loss-3, a Novel Mouse Model of Achromatopsia Due to a Mutation in *Gnat2*

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**PURPOSE.** To report a novel mouse model of achromatopsia with a *cpfl3* mutation found in the ALS/LtJ strain.

**METHODS.** The effects of a *cpfl3* mutation were documented using fundus photography, electroretinography (ERG), and histopathology. Genetic analysis was performed using linkage studies and PCR gene identification.

**RESULTS.** Homozygous *cpfl3* mice had poor cone-mediated responses on ERG at 3 weeks that became undetectable by 9 months. Rod-mediated waveforms were initially normal, but declined with age. Microscopy of the retinas revealed progressive vacuolization of the photoreceptor outer segments. Immunocytochemistry with cone-specific markers showed progressive loss of labeling for  $\alpha$ -transducin, but the cone outer segments in the oldest mice examined remained intact and positive with peanut agglutinin (PNA). The *cpfl3* mapped to mouse chromosome 3 at the same location as human *GNAT2*, known to cause achromatopsia. Sequence analysis revealed a missense mutation due to a single base pair substitution in exon 6 in *cpfl3*.

**CONCLUSIONS.** The *Gnat2*<sup>*cpfl3*</sup> mutation leads to cone dysfunction and the progressive loss of cone  $\alpha$ -transducin immunolabeling. Despite a poor cone ERG signal and loss of cone  $\alpha$ -transducin label, the cones survive at 14 weeks as demonstrated by PNA staining. This mouse model of achromatopsia will be useful in the study of the development, pathophysiology, and treatment of achromatopsia and other cone degenerations. The gene symbol for the *cpfl3* mutation has been changed to *Gnat2*<sup>*cpfl3*</sup>. (*Invest Ophthalmol Vis Sci.* 2006;47:5017-5021) DOI:10.1167/iovs.05-1468

Mice are valuable for the identification of novel gene mutations that lead to retinal disease. Mutant retinas are investigated to elucidate the pathophysiology and natural his-

tory of the disorders. During screening for mouse models of ocular diseases at The Jackson Laboratory (TJL; Bar Harbor, ME), a novel mutation, named cone photoreceptor function loss-3 (*cpfl3*), was identified by electroretinography. This mutant appears to be a good model for achromatopsia occurring in humans.

Rod monochromatism or achromatopsia is a group of autosomal recessive human congenital disorders. Achromatopsia (all types) has an estimated incidence of 1 in 30,000.<sup>1</sup> Clinical manifestations usually present in infancy with horizontal pendular nystagmus, photophobia, total color blindness, and poor visual acuity (20/200–20/400), which is slightly improved at dusk and night.<sup>2</sup> The nystagmus often improves as the patient moves from childhood to the teenage years.<sup>3</sup> The primary deficit in achromatopsia is a lack of functional retinal cone photoreceptors. Clinical diagnosis of these conditions relies on electroretinography, as retinal examinations are often normal in early stages of the disease.<sup>4</sup>

Three independent mutations have been identified in humans as causes of achromatopsia (Table 1). Mutations in *CNGB3* (cyclic nucleotide gated channel  $\beta$ -3), which encodes the  $\beta$ -subunit of the cone cyclic GMP-gated cation channel cause approximately 36% to 50% of cases.<sup>5–7</sup> Patients with *CNGB3* mutations present with colorblindness, nystagmus, and subnormal vision and may eventually have myopia. This mutation has been studied primarily among the Pingelap islanders and has been linked to Irish ancestry.<sup>8</sup> Progressive cone dystrophy has also been reported in *CNGB3* mutant families with a history of achromatopsia, linking this gene to multiple diseases.<sup>9</sup> *CNGA3* encodes the  $\alpha$ -subunit of the cone cGMP-gated cation channel, and mutations within this gene account for approximately 41% of cases of achromatopsia.<sup>10,11</sup> *CNGA3* mutations are found in Moroccan, Iraqi, and Iranian Jews, with an increased frequency of cases in Denmark.<sup>12</sup> *CNGA3* mutations may result in complete, incomplete, or (rarely) severe progressive phenotypes. Patients with achromatopsia who have mutations in both *CNGA3* and *CNGB3* present with a similar clinical phenotype, confirming the essential function of both the  $\alpha$ - and  $\beta$ -subunits of the cGMP-gated cation channel for normal cone function.<sup>13</sup> *CNGB3* mutations have also been reported in dogs. The third gene for human achromatopsia is *GNAT2* (guanine nucleotide binding protein [G protein]), which encodes for the  $\alpha$ -subunit of transducin necessary for hyperpolarization of cones. Transducin mediates an initial step in phototransduction, whereas the cGMP-gated channels *CNGA3* and *CNGB3* moderate the final steps in the cascade. Kohl et al.<sup>14,15</sup> reported six separate mutations in *GNAT2*: one nonsense, four small insertions/deletions, and one larger recombination, all of which result in premature termination of translation. This termination prevents the carboxyl terminus of  $\alpha$ -transducin from interacting with the excited photopigment. In addition to these mutations, Aligianis et al.<sup>2</sup> noted a 4-bp insertion in exon 7 of *GNAT2* that results in a frameshift mutation. Finally, one case of achromatopsia was caused by maternal isodisomy of chromosome 14.<sup>16</sup>

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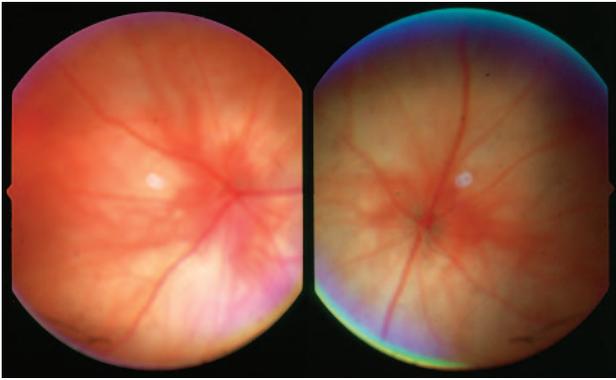
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**FIGURE 2.** Fundus photography at 2 (*left*) and 8 (*right*) months for ALS/LtJ mice. The retinas appear normal for a mouse with an albino background. The retinal veins in the 8-month-old mouse were slightly dilated, whereas the arterioles were slightly constricted, a sign of early retinal degeneration in murine models.

### Gene Mapping and Sequencing

To determine the chromosomal location of the *cpfl3* gene, ALS/LtJ-*cpfl3* mice were mated to CAST/EiJ mice. The F1 mice, which exhibit no retinal abnormalities, were backcrossed to ALS/LtJ-*cpfl3* mice (Fig. 1). Amplification was performed on isolated tail DNAs, using 25 ng of DNA in a 10- $\mu$ L volume containing 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 0.2 mM oligonucleotides, 200  $\mu$ M dNTP, and 0.02 U DNA polymerase (*AmpliTaq*; Applied Biosystems, Foster City, CA). The reactions were initially denatured for 3 minutes at 94°C and then subjected to 40 cycles of 15 seconds at 94°C, 1 minute at 51°C, 1 minute at 72°C, and a final 7-minute extension at 72°C. PCR products were separated by electrophoresis on 3% agarose gels (MetaPhor; FMC, Rockland, ME) and visualized under UV light after staining with ethidium bromide. Initially, a genome scan of microsatellite (Mit) DNA markers was performed on pooled DNA samples. After detection of linkage on chromosome 3, the microsatellite markers *D3Mit6*, *D3Mit49*, *D3Mit286*, *D3Mit11*, *D3Mit288*, and *D3Mit350* were scored in individual DNA samples. To test the *Gnat2* gene as a candidate, we designed two pairs of PCR primers based on the mouse coding sequence to amplify overlapping cDNA fragments. For direct sequencing, the PCR reaction was scaled up to 30  $\mu$ L. Amplification was performed for 36 cycles with a 15-second denaturing step at 94°C, a

2-minute annealing step at 60°C, and a 2-minute extension step at 72°C. PCR products were purified from agarose gels with a kit (Qiagen, Valencia, CA). Sequencing reactions were performed with automated fluorescence tag sequencing. Total RNA was isolated from retinas of newborn mice (TRIzol LS Reagent; Invitrogen-Gibco, Grand Island, NY) and a preamplification system (SuperScript; Invitrogen-Gibco) was used to make first-strand cDNA. The following primer pairs were used to amplify overlapping cDNA fragments and sequence directly: (Gnat2-1F)-AATGGGGAGTGGCATCAGTGCTG and (Gnat2-to 1R)-TAGCCGCAAAGAAGTGTGG; (Gnat2-2F)-ATCGCATGCACGAGTCTTTG and (Gnat2-2R)-CTAAACAGAACCAGCCTTGG.

### PCR Methods for Genotyping *cpfl3* Mutations

One pair of primers (Gnat2-dF CATCGAGACCAAGTTTTCTG; Gnat2-dR ACCATGTCGTAGGCACTGAG) was used to genotype and confirm the *cpfl3* mutation. A 362-bp fragment was amplified for mutant and wild-type *Gnat2*. There is an *MseI* recognition site in the *cpfl3* mutant *Gnat2* that is not found in wild-type *Gnat2*. PCR amplification was performed in 36 cycles with a 15-second denaturing step at 94°C, a 1-minute annealing step at 51°C, and a 1-minute extension step at 72°C. *MseI* digestion was performed directly in a 10- $\mu$ L volume by adding 8  $\mu$ L of PCR products, 1  $\mu$ L of 10  $\times$  buffer (NE Buffer 2), and 2 to 5 units of *MseI* (NE Enzyme).

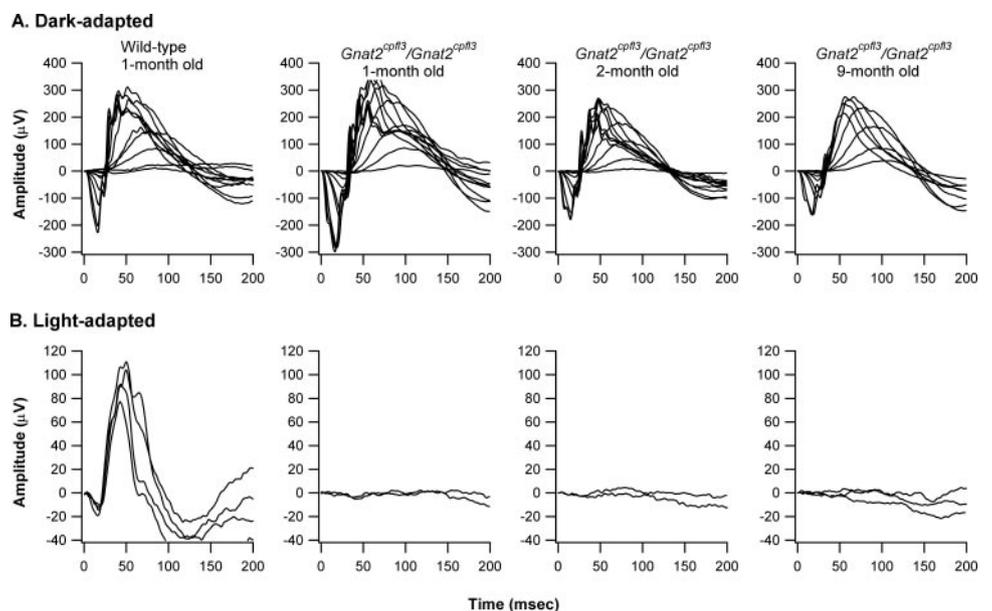
### Complementation Tests

Complementation tests between the *cpfl3* strain and other strains with similar phenotype were performed. Affected homozygotes were mated to ALS/LtJ mice that were homozygous for the *cpfl3* mutation. The cone function of F1 offspring was tested by ERG.

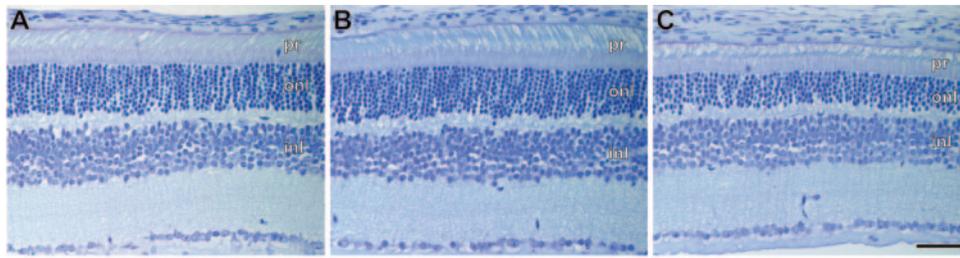
## RESULTS

### Genetics

One hundred twenty-three mice derived from a cross of *cpfl3/cpfl3* and CAST/EiJ were phenotyped and genotyped. Genetic analysis revealed that the functional loss with the cones is due to a mutation in mouse chromosome 3, closely linked to *D3Mit286* (Fig. 2). This location suggests that the corresponding human homolog is located on chromosome 1p13, the same location as the human *GNAT2* gene. On sequence analysis, the mutation was identified as a missense mutation in exon 6 of the *GNAT2* gene. Specifically, in the *cpfl3/cpfl3* mice, a single-base



**FIGURE 3.** Electroretinogram of normal control mouse (an ALR/LtJ) at 30 weeks, and *Gnat<sup>cpfl3</sup>* mice at 1, 2, and 9 months. Shown are intensity response tracings in (A) dark-adapted and (B) photopic conditions.



**FIGURE 4.** Histologic section through the retinas of *Gnat2<sup>cpfl3</sup>* homozygotes. (A–C) Plastic sections at 6, 15, and 27 weeks of age stained with toluidine blue. There was increasing vacuolization and shortening of outer segments with time. pr, photoreceptor layer; onl, outer nuclear layer; inl, inner nuclear layer. Scale bar, 50  $\mu$ m.

substitution at position 598 (G→A) in exon 6 was found that changes codon 200 from GAT to AAT (aspartic acid to asparagine; amino acid change Asp200Asn). As the functional loss within the cones is caused by the missense mutation in *GNAT2* designated *cpfl3*, the gene symbol for the *cpfl3* mutation has been changed to *GNAT2<sup>cpfl3</sup>*. The *cpfl3* mutation results in a new *MseI* site that enabled us to genotype the *cpfl3* mutation by PCR-RFLP (polymerase chain reaction and restriction fragment length polymorphism; data not shown). To confirm the presence of the missense codon in the *cpfl3* *Gnat2* gene, we re-examined 123 DNAs (58 affected and 65 unaffected mice) from our linkage analysis for the *MseI* RFLP. We amplified a *Gnat2*-dF/*Gnat2*-dR 362-bp genomic fragment that contains two *MseI* sites in the normal allele and three in the *cpfl3* allele. Digestion of the PCR-amplified products with *MseI* from wild-type, homozygous, and heterozygous *cpfl3* DNA revealed the predicted RFLP pattern (wild-type: three bands of 257, 85, and 20 bp; homozygous *cpfl3*: four bands of 156, 101, 85, and 20 bp; heterozygous *cpfl3*: five bands of 257, 156, 101, 85, and 20 bp). This analysis showed that there was absolute concordance between the *cpfl3/cpfl3* phenotype and the missense mutation. The RFLP pattern thus provides a tool for verification of the presence or absence of the *cpfl3* allele in genetic analysis.

Other strains of mice at TJL have been found with the *Gnat2* mutation. Because we screen with electroretinography, other mice originally were found with cone abnormalities. Complementation tests in the following stocks or strains: stock Tg(Fos-lacZ)34Efu/J 004623; stock Tg(Trp53A135V)L3Ber/J 003262; stocks Tb(ACTB-EGFP)/Nagy/J 003773, SENCARA/PtJ 002746, SENCARB/PtJ 002,747, and SENCARC/PtJ 002448 were positive, and genotyping these stocks or strains by PCR-RFLP confirmed that all these stocks or strains have the same missense mutation as the *cpfl3* in ALS/LtJ mice. One more strain, N/nBSwUmabJ 005052 was identified with the *Gnat2* mutation by complementation test only.

### Fundus Photography

Photographs of the fundi of ALS/LtJ- *Gnat2<sup>cpfl3</sup>* homozygotes were taken from birth to 8 months of age and two are shown in Figure 2 at 2 and 8 months of age (Fig. 2). The only noticeable findings were dilated retinal vessels at age 8 months, which is common in early retinal degenerations in mice.

### ERG Phenotype

Mice homozygous for *Gnat2<sup>cpfl3</sup>* were examined with ERG at age 4 weeks. The initial results showed normal rod-mediated responses and abnormal photopic responses, which were approximately 25% of normal and were extinguished by 9 months (Fig. 3). Scotopic responses showed some diminution with time but were near normal at age 9 months.

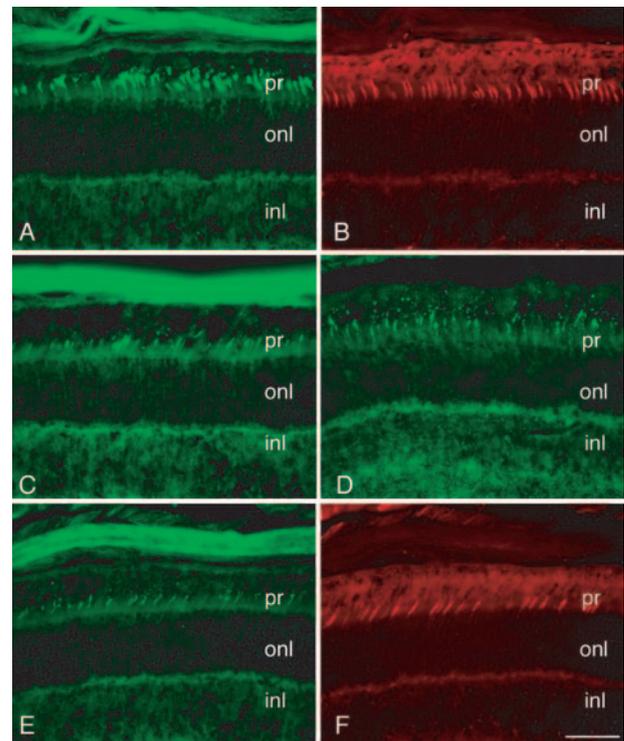
### Histopathology

Toluidine blue-stained sections from mutant retinas at ages 6 and 15 weeks revealed the normal 8 to 10 rows of rod nuclei (Fig. 4), consistent with the normal scotopic ERGs. At 27 weeks, the outer segments of the mutant *Gnat2<sup>cpfl3</sup>* homozy-

gotes were vacuolated (Fig. 4C). Immunofluorescence with anti-cone  $\alpha$ -transducin revealed reduced labeling of cone outer segments in mutant retinas by age 4 weeks (Fig. 5) compared with wild-type retina. PNA staining revealed a normal number of cone outer segments in the oldest *Gnat2<sup>cpfl3</sup>* retinas examined (Fig. 5). Thus, cone outer segment structure was retained even as cone  $\alpha$ -transducin levels appeared reduced and the photopic ERG were extinguished.

### DISCUSSION

Although cone dystrophy has been studied in a mouse model,<sup>20</sup> this is the first naturally occurring mouse model specifically for achromatopsia—in this case resulting from mutations in *Gnat2*. This model is based on a spontaneously occurring G→A mutation in exon 6 of *Gnat2*, which results in a missense mutation of the  $\alpha$ -subunit of the protein transducin, thereby preventing photoreceptor hyperpolarization in the phototrans-



**FIGURE 5.** Immunolabeling of cone transducin in the retinas of wild-type and *Gnat2<sup>cpfl3</sup>* homozygotes. (A) Cryosection from a 4-week-old ALR/LtJ wild-type animal immunostained with antibodies against cone transducin. (B) Same section as in (A), labeled with rhodamine-conjugated PNA for cones. (C–E) Cryosections from *Gnat2<sup>cpfl3</sup>* mice at 4, 9, and 14 weeks of age, respectively, with anti-cone transducin. (F) Same section as in (E), labeled with rhodamine-conjugated PNA. pr, photoreceptor layer; onl, outer nuclear layer; inl, inner nuclear layer. Scale bar, 50  $\mu$ m.

duction cascade, causing the cone function loss in *cpfl3/cpfl3* mice. The carboxyl terminus of *GNAAT2* miscoding (aspartic acid to asparagine; amino acid change Asp200Asn) may eliminate important functional domains of  $\alpha$ -transducin, which have been shown to interact with rhodopsin and phosphodiesterase  $\gamma$ -subunits,<sup>21,22</sup> and this loss of cone transducin function was documented in this study by lack of cone function on ERG. The *cpfl3* mutation in mouse chromosome 3 has significant homology to human chromosome 1, region p13, in which the *GNAT2* gene is located.

In humans, seven mutations have been documented in the *GNAT2* gene, including small insertions and deletions,<sup>10</sup> a nonsense mutation, a larger recombination, and frameshift mutations.<sup>2,12</sup> This heterogeneity is also found in mutations causing achromatopsia in *CNGA3* and *CNGB3*. In particular, *CNGA3* mutations can cause complete, incomplete, or progressive phenotypes. Achromatopsia has been defined as complete at birth and nonprogressive,<sup>10</sup> although the senior author (JRH) has observed a progressive loss of photopic ERG in some cases diagnosed in early childhood. Eksandh et al.<sup>13</sup> noted slight remaining cone responses in younger patients with achromatopsia and midperipheral pigmentary degenerations in the oldest patient examined. This finding indicates that some progressive retinal dysfunction may occur in patients with achromatopsia.

This novel mouse mutant provides a model for future experimental treatments as well as more detailed examination of disease mechanisms. As cones initially appear to retain structural integrity in this model, at least by PNA staining, this disease may be amenable to gene therapy or similar molecular interventions.

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