Mutation in the guanine nucleotide-binding protein beta-3 causes retinal degeneration and embryonic mortality in chickens

Mutation in the Guanine Nucleotide–Binding Protein β -3 Causes Retinal Degeneration and Embryonic Mortality in Chickens

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Abstract

PURPOSE. To identify the gene defect that causes blindness and the predisposition to embryonic death in the retinopathy globe enlarged (rge) chicken.

METHODS. Linkage analysis, with previously uncharacterized microsatellite markers from chicken chromosome 1, was performed on 138 progeny of an rge/ β and an rge/rge cross, and candidate genes were sequenced.

RESULTS. The rge locus was refined and the gene for guanine nucleotide– binding protein β -3 (GNB3), which encodes a cone transducin β subunit, was found to have a 3-bp deletion (D153del) that segregated with the rge phenotype. This mutation deleted a highly conserved aspartic acid residue in the third of seven WD domains in GNB3. In silico modeling suggested that this mutation destabilized the protein. Furthermore, a 70% reduction was found in immunoreactivity to anti-GNB3 in the rge-affected retina.

CONCLUSIONS. These findings implicate the β -subunit of cone transducin as the defective protein underlying the rge phenotype. Furthermore, GNB3 is ubiquitously expressed, and the c.825C3T GNB3 splicing variant (MIM 139130) has been associated with hypertension, obesity, diabetes, low birth weight, coronary heart disease, and stroke in the human population. It therefore seems likely that the defect underlying these human diseases also causes reduced embryonic viability in the rge chicken, making it a powerful model for studying the pathology involved in these associations. (Invest Ophthalmol Vis Sci. 2006;47:4714–4718) DOI:10.1167/iovs.06-0292

The chicken genome was the first of the taxonomic class Aves (Birds) to be sequenced,1 and now many resources are available to support genetic research in chickens (http:// poultry.mph.msu.edu/). Furthermore, the chicken has served for many years as a useful developmental model, particularly for the formation of the eye (see, for example, Fokina and Frolova[2]). In contrast, the chicken has been underused as a model for human inherited disease. Nevertheless, five forms of hereditary retinal degeneration have been reported in the chicken. These are the Rhode Island Red strain rd,[3] blindness enlarged globe (beg),[4] retinal dysplasia and degeneration

(rdd),[5–7] delayed amelanotic strain DAM,8 and retinopathy globe enlarged (rge).[9,10] The rd phenotype is caused by a null mutation in the photoreceptor guanylate cyclase (Gucy2d) gene and is thus a model for Leber congenital amaurosis.3 Originally reported as a recessive trait, rdd was subsequently mapped to the chicken Z chromosome by linkage analysis,11 and rge was localized to chicken chromosome 1.

The chicken eye differs from that of the human eye in a number of ways. In contrast to the rod-dominated human retina, avian retinas are generally cone dominated and often bifoveate.[12] However, the chicken eye is comparable in size to the human eye, which facilitates pathologic examination and should simplify the testing of experimental therapies. Furthermore, the level of conservation of gene order between the chicken and human genomes is similar to that between humans and mice, in spite of the much greater evolutionary separation.[13]

One of the blind chicken lines, retinopathy globe enlarged (rge), arose spontaneously in commercial chicken flocks in the United Kingdom.[9] By 3 weeks of age, affected chickens exhibit poor pupillary light response and abnormal behavior resulting from loss of vision. All functional vision appears to be lost by 8 weeks, but electroretinogram amplitudes, which are reduced at hatch, are still measurable in 1-year-old birds. The earliest retinal changes, seen at 1 day after hatch, are disorganization of the outer plexiform layer and abnormal location of the endoplasmic reticulum in photoreceptors. These changes are associated with developmental disruption of rod and cone photoreceptor synaptic terminals that progresses with age. Total retinal thickness is normal at hatch but decreases with age, though at a much slower rate than visual loss, suggesting a functional deficit. Older affected birds have generalized secondary globe enlargement and cataracts.[14,15]

With the use of DNA from 138 progeny of an rge/ + x rge/rge cross, linkage analysis mapped the trait to a 13.7-Mb centromeric interval of chicken chromosome 1 delineated by the polymorphic markers MCW0112 and LEI0101.[14] Results from this cross also suggested that the rge trait affects embryonic viability in chickens. Of 138 progeny, 56 were blind and 82 were sighted. This ratio of 0.41:0.59 is significantly different from the expected 1:1 ratio under a model of simple recessive inheritance ($P = 0.026$). Mortality after hatch was low and was not observed beyond the first 2 weeks of life, before sight was severely compromised. Here, we report that a 3-bp

homozygous deletion in the guanine nucleotide– binding protein β -3 (GNB3) gene causes the rge phenotype.

Methods

Animals

The rge line was maintained at the Roslin Institute, as described,14 under a Home Office project license. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Genotyping

Genomic DNA was extracted from chicken blood using DNAzol (Invitrogen, Paisley, UK). High-resolution genotyping was performed with previously uncharacterized microsatellite markers that had been downloaded as simple repeat sequences from the chicken genome database at UCSC (http://www.genome.ucsc.edu/). Primer sequences were designed (Primer3 program; http://frodo.wi.mit.edu/cgi-bin/primer3/), and primers were labeled with fluorochrome FAM, HEX, or TET (Invitrogen). Primer pairs were optimized, and parent DNAs were genotyped to check for informativity of the microsatellite markers. PCR products were size fractionated on an automated DNA sequencer (ABI377; ABI Prism; Applied Biosystems, Warrington, UK), and fragment length analysis was carried out (GeneScan 3.5 and Genotyper 3.6 software; Applied Biosystems). Polymorphic markers were amplified using the progeny DNA as template, and refinement of the rge critical interval was achieved by constructing a linked haplotype.

Sequencing

PCR was carried out on 50 ng genomic DNA in the presence of master mix solution (Promega, Southampton, UK) for each 25-mL reaction. Primers dATGCAGGATTGGAACCCTTCA and dTGAGATCACACAGCACCCTGA were used to amplify exons 5 and 6 of chicken GNB3. The reaction was performed for 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Reaction products were cleaned up with a purification kit (QIAquick PCR Purification; Qiagen,

Crawley, UK). Ten nanograms purified PCR product and 3.2 pmol of either the forward or the reverse primers used to amplify exons 5 and 6, or the internal sequencing primer dCCCGCTCTCCTATCTACT, were sent to The Sequencing Service at the University of Dundee (http://www. dnaseq.co.uk/) for sequencing on a DNA sequencer (ABI377; ABI Prism), and the generated SCF sequence trace files were analysed (Chromas lite software; http://www.technelysium.com.au/chromas_ lite.html).

PCR-ARMS Analysis

The presence of the D153del mutation in genomic DNA was determined using the PCR-ARMS (amplification refractory mutation system). This procedure relies on two independent reactions that use the same anchor primer but a different discriminatory primer during PCR. Each discriminatory primer differs at the 3 ’-end of the sequence to permit the specific amplification of the wild-type or mutant allele. For each 10- μL reaction, PCR was carried out (PCRx Enhancer Kit; Invitrogen) with 1x enhancer solution, 1x amplification buffer, 1.5 mM $MgSO_4$, 0.2 mM dNTP, and 1.25 U Taq DNA polymerase. Primers used in the reaction were dGCAGGGAACTCTCAGCTCATA (as the anchor primer) and either dAGCTAGTCACAATACTGTTGTGATC (wild-type) or dAGCTAGTCACAATACTGTTGTGAAG (mutant) as the discriminatory primer to give reaction products of 175 bp and 172 bp, respectively. Twenty nanograms DNA was used as a template for each reaction, which was performed for 30 cycles at 94°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds. Reaction products were visualized after agarose gel electrophoresis under ultraviolet illumination.

RT-PCR Analysis

Reverse transcription of chicken RNA isolated from various tissues was performed using oligo $(dT)_{15}$ primer and reverse transcriptase (Superscript II; Invitrogen). Amplification of 373 bp GNB3 cDNA was carried out with the primers dCTGGTCAGTGCCTCACAAGA and dGGGAAACTGCCAAGCTCATA. As a normalization control, amplification of 237 bp glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was performed with the primers dGGAAAGTCATCCCTGAGCTG and dCATCAAAGGTGGAGGAATGG.

Bioinformatics

Multiple sequence alignments were performed (ClustalW; http:// www.ebi.ac.uk/clustalw/index.html). Alignments were viewed (Jalview; Java; Sun Microsystems, Santa Clara, CA) and were saved in MSF format. The MSF file was then opened in GENDOC (http://www. psc.edu/biomed/genedoc/). Selected blocks of the generated alignment were saved in rtf format and edited (Word; Microsoft, Redmond, WA).

Structural Prediction

The D153del GNB3 mutation was modeled in Bos taurus GNB-1 (1B9X) using the "What If" program

(http://btcpxx.che.uni-bayreuth.de/ COMPUTER/Software/WHATIF/html/).

Immunoblotting

Retinas were isolated from the eyes of a 5-week-old normal and rgeaffected bird and were frozen in liquid nitrogen before storage at –80°C. Thawed retinas were weighed and placed in tissue extraction buffer (Invitrogen) containing a protease inhibitor cocktail (kindly donated by Cyclacel, Dundee, UK) at a concentration of 10 mL extraction buffer for each milligram of tissue. Retinal samples were then homogenized and centrifuged at 8000g for 5 minutes at 4°C to pellet the tissue debris. The extracted protein supernatant was collected, aliquoted, and frozen at -80°C. Protein extracts were subsequently quantified with the use of a spectrophotometer, with BSA as a standard.

For slot blotting, 15 μ L equal amounts of normal and rge-affected protein extracts were blotted, in triplicate, on a nitrocellulose membrane (Hybond ECL; GE Healthcare Life Sciences, Little Chalfont, UK) with a slot blot apparatus (PR600; Hoefer Scientific Instruments, San Francisco, CA). Blots were then incubated with blocking solution (Upstate, Southampton, UK) for 1 hour at room temperature before the addition of a 1:1000 dilution of rabbit anti–GNB3 polyclonal antibody (Merck Biosciences, Nottingham, UK) or rabbit anti–PDE6α polyclonal antibody (Merck Biosciences) and incubation for another 2 hours. Blots were washed three times for 15 minutes each with wash buffer (Upstate). A 1:1000 dilution of horseradish peroxidase– conjugated secondary antibody (Merck Biosciences) was then added to each blot and incubated for 1 hour with agitation. Membranes were washed three times with wash buffer before they were treated with the colorimetric substrate 3,3' ,5,5' -tetramethylbenzidine (TMB; Upstate). Resultant bands were photographed and quantified by densitometry analysis (Gelpro software; Image Processing Solutions, North Reading, MA).

Results

Further Refinement and Candidate Gene Screening

The first draft of the chicken genome sequence is available through the Ensembl (http://www.ensembl.org) and UCSC (http://www.genome.ucsc.edu/) databases. Further testing of previously uncharacterized microsatellite markers in the rge interval refined the locus to a 3.8-Mb region spanning the centromere between 68.1 and 71.9 Mb (data not shown). Significant chromosomal synteny exists between the human and chicken genomes, so the synteny view function of Ensembl (http://www.ensembl.org/Gallus_gallus/syntenyview) provides a powerful tool for identifying orthologous genes within syntenic chromosomal regions. The largest contiguous syntenic region identified in this way within the rge interval extended from the TAPBL gene to the Pex5 gene, a region located at 6.4 Mb and 7.2 Mb on human chromosome 7 and at 71.0 Mb and 71.5 Mb on chicken chromosome 1, respectively.

Chicken GNB3 and its human ortholog map within this region at 71.3 Mb on chicken chromosome 1 and at 6.8 Mb on human chromosome 7. GNB3 is ubiquitously

expressed but is highly expressed in mammalian cones,[16] and, given that the avian eye is cone dominated, it was considered a strong candidate rge gene. We therefore sequenced GNB3 in wild-type and rge birds and identified an in-frame 3-bp deletion spanning codons 153 and 154 (Fig. 1), which segregated with the rge phenotype in all birds tested (Fig. 2).

Functional Consequences of GNB3 D153del

RT-PCR of the GNB3 transcript in a range of normal chicken tissues confirmed its ubiquitous expression and relatively high expression in the retina, but comparison of the transcript level in normal and rge retinas revealed no significant difference, implying that the mutated transcript escapes nonsense-mediated decay (Fig. 3). The in-frame deletion in GNB3, designated D153del, deletes one of two highly conserved aspartic acid residues in the third of seven WD domains in the GNB3 protein (Fig. 4).[17] Each WD domain consists of a stretch of approximately 40 amino acids that usually end with tryptophan and aspartic acid (WD) residues and that forms 4 β strands in the protein structure. The seven WD domains within GNB3 permit the formation of a stable platform onto which protein scaffolds can form and multiple protein–protein interactions can occur.[18,19] Hence, it seems likely that the D153del mutation, which lies between two β strands in the third WD domain, disrupts the spatial configuration of the folded protein and prevents its optimal function.

Effect of GNB3 D153del on Protein Structure

The structure of B. taurus GNB1, which is a close homolog of human GNB3, has been described by Sondek et al.20 Modeling the equivalent mutation in GNB1 using the "What If" computer program[21] suggests that this deletion abolishes β sheets in propellers 1 and 5 of the GNB protein (Fig. 5). On submitting this structure to the CASP5 committee (Critical Assessment of Methods for Protein Structure Prediction[22]), it was predicted that this GNB protein would be unstable and liable to premature proteolysis. This was verified by comparing levels of GNB3 protein in retinal cell extracts from wild-type and rge birds. A 70% reduction in GNB3 protein immunoreactivity was seen in affected retinas compared with age- and sex-matched

normal retinas (Fig. 6). This decrease in GNB3 protein level in rge birds provides further proof that D153del is the rge mutation and strongly suggests haploinsufficiency caused by protein instability as the primary disease mechanism. A similar outcome has been reported for two Drosophila missense mutations in the retina-specific G-□ protein Gbe, each of which causes marked reductions (95% and 99.5%) in the amount of protein produced, resulting in a dramatic loss in light sensitivity.[23]

Discussion

Guanine nucleotide– binding proteins, also known as G-proteins or transducins, mediate signal transduction triggered by hormones, neurotransmitters, and sensory stimuli and are found in all eukaryotes. They are composed of three protein subunits— α, β, and γ. In mammals, 16 α subunits and 11 γ subunits have been documented, many with a high degree of tissue specificity. Only five β subunits have been reported, and GNB3 is one of them.[24] It is ubiquitously expressed but is particularly highly expressed in cone photoreceptors, [16] where it functions as the transducin β subunit in cone phototransduction.

This study shows that a mutation in the chicken cone β transducin, GNB3, causes retinal degeneration, implying that GNB3 is also a candidate human retinal dystrophy gene. However, a previous screen of the human GNB3 gene for mutations in 164 patients with cone-rod and macular dystrophy revealed no sequence change that could be unequivocally linked to human disease.25

In contrast, defective splicing of human GNB3 caused by the c.825C→T substitution has been associated with hypertension,[26] obesity,[27] coronary heart disease,[28] stroke,[29] depression,[30] and diabetes.[31] Homozygotes for c.825T still produce a proportion of normal-sized mRNA in all tissues but also produce a splice variant that lacks 123 nucleotides.[26] The latter deletes 41 amino acids (one complete propeller domain) and produces a protein that cannot form heterotrimers or modulate ion channel activity at the cell surface.[32] However, these studies do not report a defect of vision in c.825T homozygotes, and an independent study found no association between the c.825T allele and diabetic retinopathy.[33] To date, it remains to be investigated whether rge

chickens are also predisposed to the diseases associated with c.825T in humans. Interestingly, homozygous mice with disruptions of the other guanine nucleotide–binding protein β subunits, GNB5 and GNB1, have abnormal rod transduction physiology and high infant mortality[34] or embryonic lethality[35] rates, respectively. GNB1 heterozygotes do survive but undergo retinal degeneration, as demonstrated by abnormal electroretinogram responses.[35] Moreover, when the Caenorhabditis elegans gpb-1 (GNB1 ortholog) is knocked down by RNAi, it causes 50% to 80% embryonic lethality and an uncoordinated phenotype in surviving adult worms.[36] Evidence of early (stage 10) embryonic expression of GNB3 in chickens from EST data (est 603499939F1; http:// www.chick.umist.ac.uk/) may also help to explain the high embryonic mortality rate in rge chickens.

This finding is the first use of Mendelian genetics in chickens to highlight a gene of potential medical significance. The eye phenotype observed shifts interest in GNB3 back to a potential human retinal phenotype and illustrates the strength of the chicken as a model organism for studying human cone disease, as previously suggested.[37] The absence of GNB3 mutations in patients with cone-rod and macular dystrophy in a previous study,[25] together with the relatively severe phenotype in the rge chickens, could imply stationary achromatopsia or early-onset Leber amaurosis phenotype. Furthermore, systemic hypertension, obesity, and diabetes, which have been associated with the GNB3 c.825T variant, are also significantly associated with age-related macular dystrophy (AMD),[38,39] making evaluation of c.825C→T and other GNB3 changes in AMD a priority. However, the reduced embryonic viability seen in rge chickens and similar observations in other GNB mutant animal models suggest that GNB mutations in humans may also predispose to pregnancy loss.

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Fig.1

GNB3 mutation analysis in wild-type and rge chickens.

Upper: homozygous D153del GNB3 mutation present in rge chickens.

Lower: corresponding normal sequence.

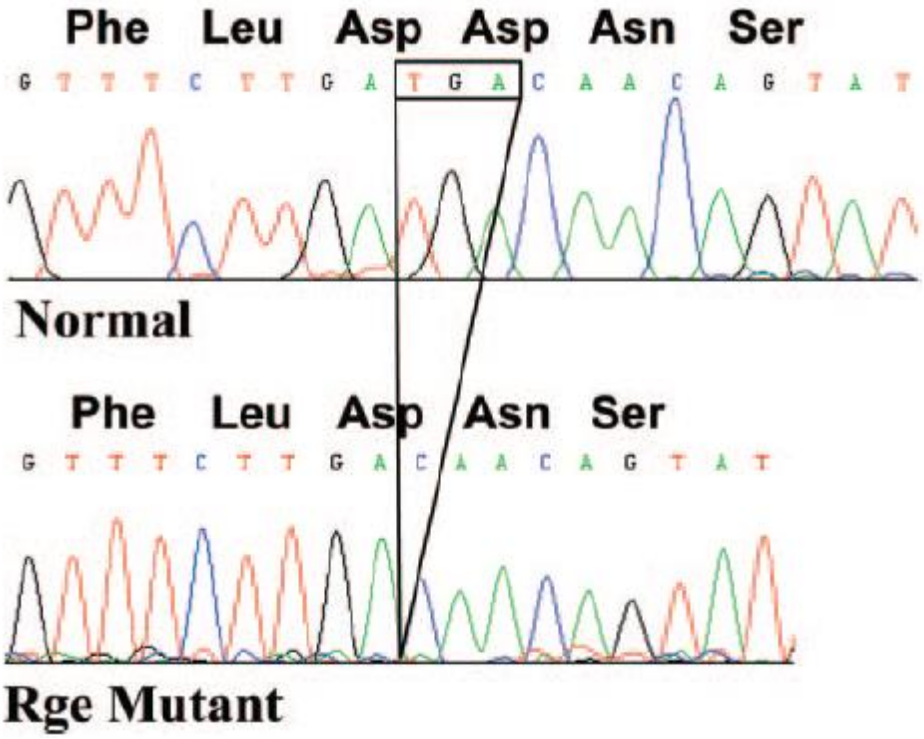

Fig.2

PCR-ARMS analysis of the GNB3 mutation D153del in two rge flocks. Lane 1: banding pattern in a White Leghorn (wild-type) bird. Lanes 2, 28: asymptomatic heterozygous female parents that gave rise to flocks 1 and 2. Lane 15: banding pattern of the homozygous D153del male parent of the flocks. Lanes 3–8, 16–21: banding patterns for the asymptomatic offspring from flocks 1 and 2, respectively. Lanes 9–14, 22–27: pattern for the rge-affected birds. Lane 29: no DNA control.

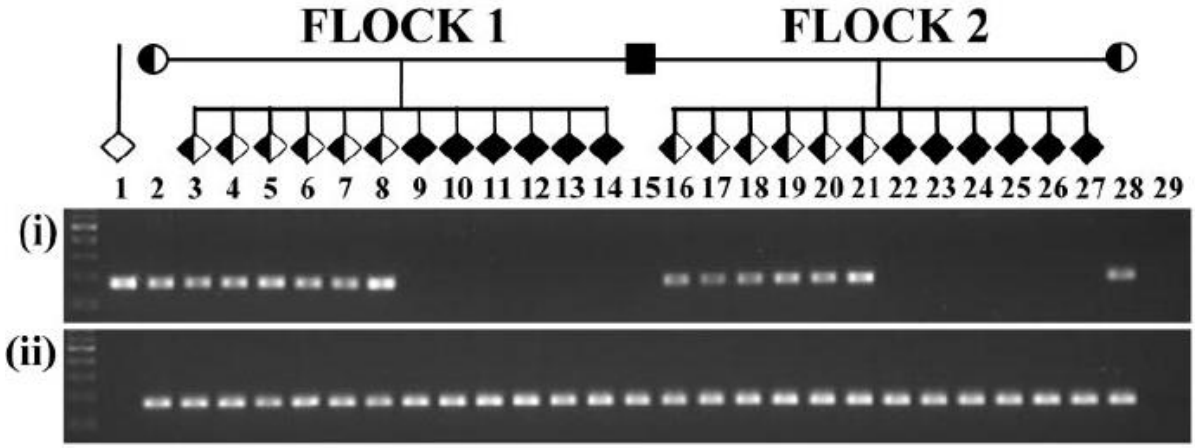

Fig.3

RT-PCR analysis of GNB3 expression in multiple chicken tissues and in normal and rge retinas. Lanes 1–10: GNB3 expression levels in tissues derived from a normal bird. Lanes 11–12: level of GNB3 expression in the retina of a White Leghorn and an rge-affected bird, respectively. Lane 13: no DNA control.

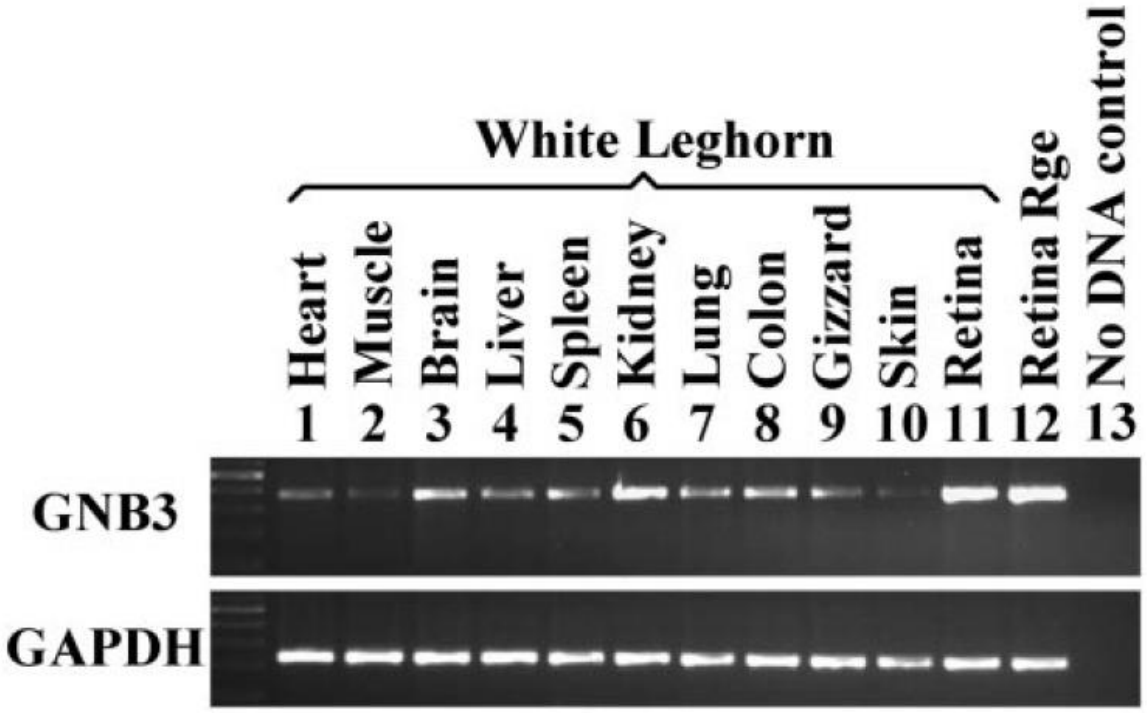

Fig.4

Alignment of the third WD domain of GNB proteins in various eukaryotic species using the program ClustalW. Bt, B. taurus; ce, C. elegans; cf, Canis familiaris; dm, Drosophila melanogaster; dr, Danio rerio; fr, Fugu rubripes; gg, Gallus gallus; hs, Homo sapiens; mm, Mus musculus; rn, Rattus norvegicus; sc, Saccharomyces cerevisiae; tn, Tetraodon nigroviridis; xt, Xenopus tropicalis. *Position of the highly conserved aspartic acid residue at codon 154 in chicken (gg) GNB3. Deletion of this or of the adjacent residue at codon 153, which is also an aspartic acid in chicken GNB3, causes the rge trait. Consistent with the mutation nomenclature criteria by den Dunnen and Antonarakis,[17] we have designated this mutation D153del.

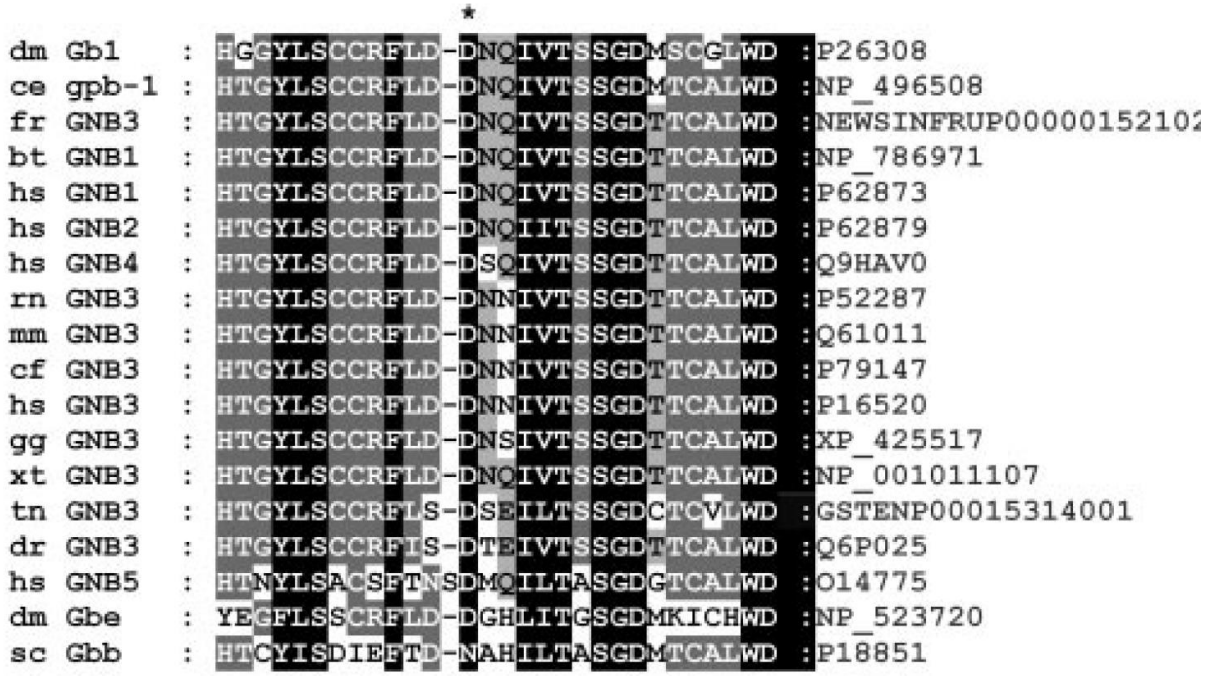

Fig.5

(A) Normal structure of B. taurus (bt) GNB1 (PDB ID: 1B9XA) showing the standard sevenpropeller structure20 in which each propeller domain consists of four anti–parallel β -pleated sheets. (B) A “What If” prediction of a B. Taurus D153del GNB mutation deletes two β -pleated sheets in propellers 1 and 5 of GNB proteins.

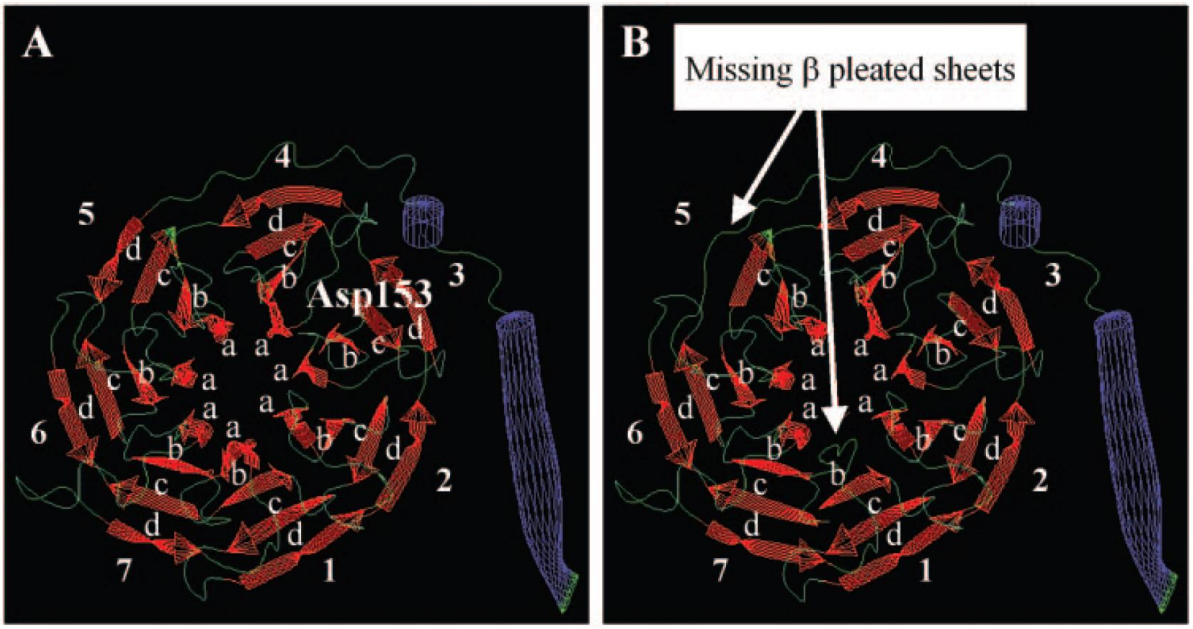

Fig.6

(A) Wild-type (upper) and rge (lower) retinal cell extracts slot blotted and hybridized with anti-GNB3, and densitometric scans showing absolute levels of immunoreactivity. (B) The same slot blots probed with a PDE6α antibody.