Title
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Variations in Opsin Coding Sequences Cause X-Linked Cone Dysfunction Syndrome with Myopia and Dichromacy

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PURPOSE. To determine the role of variant L opsin haplotypes in seven families with Bornholm Eye Disease (BED), a cone dysfunction syndrome with dichromacy and myopia.

METHODS. Analysis of the opsin genes within the L/M opsin array at Xq28 included cloning and sequencing of an exon 3-5 gene fragment, long range PCR to establish gene order, and quantitative PCR to establish gene copy number. In vitro expression of normal and variant opsins was performed to examine cellular trafficking and spectral sensitivity of pigments.

RESULTS. All except one of the BED families possessed L opsin genes that contained a rare exon 3 haplotype. The exception was a family with the deleterious Cys203Arg substitution. Two rare exon 3 haplotypes were found and, where determined, these variant opsin genes were in the first position in the array. In vitro expression in transfected cultured neuronal cells showed that the variant opsins formed functional pigments, which trafficked to the cell membranes. The variant opsins were, however, less stable than wild type.

CONCLUSIONS. It is concluded that the variant L opsin haplotypes underlie BED. The reduction in the amount of variant opsin produced in vitro compared with wild type indicates a possible disease mechanism. Alternatively, the recently identified defective splicing of exon 3 of the variant opsin transcript may be involved. Both mechanisms explain the presence of dichromacy and cone dystrophy. Abnormal pigment may also underlie the myopia that is invariably present in BED subjects. (*Invest Ophthalmol Vis Sci.* 2013;54:1361–1369) DOI: 10.1167/iovs.12-11156

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X-linked cone dysfunction syndrome, or Bornholm Eye Disease (BED), was first described in a large family from the Danish island of Bornholm with features of X-linked infantile myopia, astigmatism, hypoplasia of the optic nerve head, reduced visual acuity, deuteranopia, and reduced cone responses on electroretinography (ERG) and nonspecific pigment abnormalities.[1,2] The disorder was shown to be linked to chromosome Xq28.[2] A second BED family was reported[3] but, unlike the original family, all affected individuals were protanopes. Additional BED families were subsequently identified[4] and these also demonstrated protanopia, indicating that either form of dichromacy may be present.

The long wavelength-sensitive (L) and middle wavelength-sensitive (M) pigments that underlie trichromacy in humans are encoded by L and M opsin genes that reside in a tandem array at chromosome Xq28. The two genes are in a head-to-tail configuration with the L gene proximal and the M gene distal. The testis-expressed protein 28 gene (*TEX28*)[5] lies at the distal boundary of the array with a truncated copy between the opsin genes. This genomic structure arose as a consequence of the original duplication of the ancestral long wavelength-sensitive (*LWS*) opsin gene that occurred at the base of the Old World primate lineage and was responsible for the separate L and M opsin genes.[6] Copy number variants (CNVs) for the opsin genes within the array are common,[7,8] with a range of 2 to greater than 5 repeats in normal trichromats. The additional copies arise from mispairing between the highly homologous L and M genes, followed by cross-over within the array. Whilst there may be several opsin genes present in the array, it is generally the case that only the first two genes are expressed.[9] Since *TEX28* is part of the array, the CNV for *TEX28* would be expected to follow that for the opsin genes; an unexplained finding in one of the original BED families was a numerical mismatch with five copies of

TEX28 but only four copies of opsin.[10] However, whether this contributes to the BED phenotype is uncertain.

A study of four United Kingdom (UK) BED families[4] focused on the complement and sequence of the L and M opsin genes in affected individuals. One of these families was shown to have an L opsin mutation that encodes a Cys203Arg substitution, a change that is known to result in a nonfunctional pigment.[11] Expression of this mutant gene would be expected to result in a subset of cones lacking a functional pigment, thereby explaining the dichromacy. The survival of these cones may also be compromised, as demonstrated in a study of two individuals with the Cys203Arg substitution.[12] A further two families possess a hybrid L/M gene that accounted for the protanopia, but not the cone dysfunction, while the fourth family[4] showed no changes that could account for either the dichromacy or cone dysfunction.

Whereas BED manifests as a dichromatic disorder, blue cone monochromacy (BCM) arises from the loss of both L and M cones. The molecular mechanism underlying BCM is either gene loss within the L/M array followed by a point mutation in the single remaining gene,[13,14] exon deletion within the opsin gene,[15] or deletion of the locus control region proximal to the opsin gene array on the X chromosome that is required for opsin gene expression.[13] In addition, however, two coding changes in exon 3 of the L gene, Val171Ile and Ile178Val, have been identified that cosegregate with BCM in one pedigree,[13] indicating that the encoded pigment is nonfunctional. The same rare combination of residues at these sites was found in a family with a novel form of X-linked achromatopsia[16] and in an individual that was reported to display cone loss.[17,18]

In the absence of an explanation for BED in the majority of affected families, we have re-examined the L and M opsin genes present in affected individuals in more detail in order to assess the coding sequences of the L genes, together with a determination of gene number and order within the array. This has been combined with an in vitro assessment of cone opsins carrying these amino acid substitutions to determine whether these variant opsins are trafficked to the cell membrane and generate functional pigments.

METHODS

Origin and Isolation of DNA

The protocol of the study adhered to the provisions of the Declaration of Helsinki and was approved by the local ethics committee at Moorfields Eye Hospital, and the institutional review board of the University of Minnesota. Subjects provided informed written consent for all parts of the study. Blood samples were collected in EDTA tubes. Genomic DNA was extracted using a Nucleon Genomic DNA extraction kit (Tepnel Life Sciences, Manchester, UK) by following the manufacturer's instructions.

L/M Exon 3 to 5 Amplifications

The BIOXACT Long with HiSpec (Bioline UK Ltd., London, UK) protocol was followed based on the manufacturer's guidelines to amplify a fragment of 3809 base pairs (bp) from exon 3 through to exon 5 of the L and M opsin genes from genomic DNA. PCRs were carried out with a 62°C annealing temperature using primers 3F and 5R (Table 1). The resulting amplicons were ligated into pGEM-T Easy Vector (Promega [UK] Ltd., Southampton, UK) and cloned. Thirty colonies were selected and plasmids extracted using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK), followed by sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK).

TABLE 1. Primers Used for Screening Proximal and Distal L and M Genes and for qPCR Analysis of Copy Number Variation

Primer Name	Sequence
FG	GAGGCGAGGCTACGGAGT
DG	TTAGTCAGGCTGGTCGGGAACT
E6	GCAGTGAAAGCCTCTGTGACT
L/M5F	TTTGCTGCTGCCAACCCT
L/M5R	TTGCTTACCTGCCGGTTCATAA
L probe	6FAM-CCTGCCGGCCTACTTTGCCAAA
M probe	JOE-CCTGCCGGCCTTCTTTGCCAAA
2F	GCACTGGTATCGACAGGCG
2R	CAGTATATGGATGTGAGGC
3F	CTCAGTCCGTGGAGCCCTGAATTC
3R	ACATTGATAGACATTGCACGCTCA
4F	GGTGACTGCCACAGAATTGAT
4R	CTGATTCTCATCGCTGGATCT
5F	CTATGCCTGGGTCACCTGCCTC
5R	CTTATCAGAGACATGATTCCAGGTCC
PF2	GAGGAGGAGGTCTAAGTCCC
PR	GGCTATGGAAAGCCCTGTCCC

Amplification of the First and Downstream Genes of the L/M Array

The protocol[19] used primer FG (Table 1) that is specific to the upstream promoter region of the first gene in the L/M array. For the amplification of the downstream genes, the forward primer DG was used to bind specifically to the downstream promoter. Both amplifications used the same reverse primer (E6). The amplified products were then used as templates for subsequent PCR amplification of exons 2 to 5 using exon-specific primer pairs (Table 1). Screening of the upstream and downstream promoters was carried out with primers PF2 and PR. All PCR products were sequenced.

Ratio of L to M Genes

A previously described protocol[20] was adopted that used a single primer set (L/M5F and L/M5R) to hybridise to exon 5 of both L and M opsin genes. Fluorescent reporter probes specific for either the L or M exon 5 sequence were then used to detect amplicons. The L and M TaqMan probes (Applied Biosystems) were tagged at their 5′ end with 6FAM and JOE, respectively, and with TAMRA at the 3′ end to act as a quencher. Reactions were run in triplicate with three repeats. Each reaction contained 10 ng of purified genomic DNA, 140 nM L probe, 250 nM M probe, 900 nM of both the forward and reverse primers, and 12.5 μL TaqMan Gene Expression Master Mix (Applied Biosystems). The cycling schedule involved an initial incubation at 50°C for 2 minutes, followed by 95°C for 2 minutes, 3 cycles of 95°C for 30 seconds, and 67°C for 1 minute, and 27 cycles of 95°C for 15 seconds, and 67°C for 1 minutes. The data were analyzed offline using the comparative-Ct method.[21]

Production and Expression of Variant Opsins

Exons 1 to 6 of the common or wild-type (WT) L and M opsin genes were amplified with primers (Table 2) designed specifically for use in SPLICE[22] reactions. The coding L and M exons were amplified separately and their sequences confirmed. Combinations of different exons were then mixed together as template in amplified SPLICE reactions, using HSLM1F and HSLM6R outer primers, to create different full length L and M coding sequences. The clones produced were WT-L, WT-M, LIAVA-L, LVAVA-L, and MVVVA-M. Note that LIAVA, LVAVA, and MVVVA represent the amino acids at sites 153, 171, 174, 178, and 180, respectively, of the L/M opsins. LIAVA changes to the L opsin coding sequence were made using two primer combinations, HSLM1F/LIAVAR

TABLE 2. Primers Designed for the Generation of Full Length L and M Coding Sequences Using SPLICE

Primer Name	Sequence
HSLM1F	GCGCGAATTCCACCATGGCCCAGCAGTGGAGCCTC
HSLM1R	GCCTTCGTTGGGGCCTCTGGTGGAGTTGCT
HSLM2F	CAACTCCACCAGAGGCCCCTTCGAAGGCCC
HSLM2R	AGAGACCTGTGATCCCACACAGGGAGACGG
HSLM3F	CGTCTCCCTGTGTGGGATCACAGGTCTCTA
HSLM3R	CCGTGGGGCCAGTACCTGCTCCAACCAAAG
HSLM4F	CTTTGGTTGGAGCAGGTACTGGCCCCACGG
HSLM4R	CTGCTGCTTTGCCACCGCTCGGATGGCCAG
HSLM5F	CTGGCCATCCGAGCGGTGGCAAAGCAGCAG
HSLM5R	ATGCAGTTTCGAAACTGCCGGTTCATAAAG
HSLMF6	TTTATGAACCGGCAGTTTCGAAACTGCATC
HSLM6R	CGGCGTCGACCGTGCAGGCGATACCGAGGACA
Lex3F	TGGCTGGTGGTGTGCAAGCCCT
Lex3R	AGGGCTTGCACACCACCAGCCA
LIAVAF	TGCCTTCTCCTGGGTCTGGGCTGCTGTGTG
LIAVAR	CAGGAGAAGGCAATGCCCACGATGGCCAGC

and LIAVAF/ HSLM6R, using normal WT-L coding sequence as template. These two amplicons were then used as overlapping templates in a SPLICE reaction with HSLMF1 and HSLM6R primers. LVAVA changes were made in a similar manner, but using Lex3F and Lex3R primers and LIAVA-L coding sequence as template. The MVVVA sequence was already present in the selected genomic DNA sample. All coding sequences were ligated into pMT4, which introduces the bovine rod opsin (1D4) tag at the 3′ end of the opsin coding sequence.

The resulting constructs were transfected into HEK293T cells using the GeneJuice Transfection Reagent (Merck Chemicals Ltd., Nottingham, UK) and the corresponding pigments generated and reconstituted with 11-*cis* retinal, as described previously.[23] Absorbance spectra were recorded in triplicate in the dark using a Shimadzu UV-visible spectrophotometer (UV-2550; Shimadzu UK Ltd., Milton Keynes, UK) and bleached by exposure to broad spectrum, white fluorescent light for 1 hour before a second spectrum was recorded. The peak sensitivity, λ_{max}, for each pigment was determined from the difference spectrum (bleached spectrum subtracted from the dark spectrum) using a standard Govardovskii rhodopsin/vitamin-A$_1$ template.[24] Best-fit curves were obtained using the Solver add-in function in Microsoft Excel (Microsoft, Thames Valley Park, Reading, UK) to determine the λ_{max} by the method of least squares as previously described.[25]

Immunocytochemistry

Plasmids containing the various L and M opsin variants described above were used to transfect a murine neuronal (Neuro2a) cell line following the previously described protocol[26] using the mouse anti-1D4 primary antibody (diluted 1 in 2000 in 10% donkey serum/PBS). Images were taken on a confocal microscope (LSM710 confocal microscope; Zeiss, Cambridge, UK).

RESULTS

Bornholm Eye Disease Families

A total of seven families diagnosed with BED were analyzed. These comprise the original Danish[1] and Minnesota[3] families, the four UK families previously reported,[4] and a new UK family. In all cases, data from affected male subjects are reported.

The new UK family originated from Morocco. It comprises three affected male siblings aged 14, 23, and 26 years (Fig. 1). In all three subjects, a color vision defect is present accompanied with poor visual acuity (6/36, 6/24 to 6/24, respectively), high myopia with astigmatism (OD: −10.0/−2.50

× 70, OS: −10.0/−2.50 × 115; OD: −8.00/−1.50 × 30, OS: −9.25/−1.25 × 180; and OD: −7.00/−1.50 × 60, OS: −6.00/−1.00 × 180, respectively), and mild photophobia, but lacking nystagmus. The maternal grandfather was also affected, revealing a clear X-linked pattern of inheritance. ERG analysis showed evidence for cone dysfunction but normal rod function. Psychophysical testing[4] of the 14- and 23-year-old subjects revealed a deutan color vision phenotype.

Overall, two families were determined to be deuteranopic and five families to be protanopic.

Exon 3 to 5 Screening

Exons 3 to 5 of the L/M opsin genes in affected individuals from six of the seven families were PCR amplified using primers designed to intronic flanking regions that are identical for both L and M genes. The PCR products were shotgun cloned and 30 colonies selected for secondary amplification and sequencing of exons 3, 4, and 5. For all but one family, a number of clones contained an L opsin exon 3 sequence that encoded an unusual combination of amino acids across sites 153, 171, 174, 178, and 180 of either Leu, Ile, Ala, Val, Ala (LIAVA), or Leu, Val, Ala, Val, Ala (LVAVA) (Fig. 2, Table 3). The WT haplotype at these sites in L opsin is LVAIS; neither of these variant haplotypes has been seen in normal trichromats[16] or in dichromats that otherwise possess normal vision,[27,28] but were previously identified as the only change in a family with BCM[13] and in a dichromatic individual with loss of cone photoreceptors.[17] As shown in Table 3, in most families, the M gene exon 3 encoded the less common MVVVA haplotype.

The single family which lacks either of the variant haplotypes, was previously shown[4] to have a deleterious Cys203Arg substitution encoded by exon 4. No further analysis of this family was carried out.

Gene Order and Copy Number within the L/M Opsin Array

In order to determine gene order within the opsin gene array of each family, the first and downstream genes of the L/M array were amplified using a long range PCR approach.

Deuteranopic Family 1. This is the original BED family.[1,3] Long range PCR identified three genes, an L gene, an M gene, and a hybrid gene with M exons 1 to 3 and L exons 4 to 6. The hybrid haplotype is abbreviated to M$_{1-3}$L$_{4-6}$, but note that exons
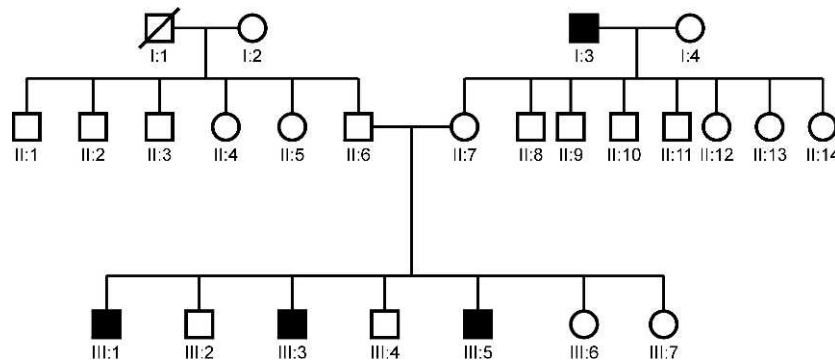
FIGURE 1. UK family pedigree. Affected individual are shown as *filled symbols*. III.1, III.3, and III.5 were aged 26, 23, and 14 years at examination.

1 and 6 of both L and M genes are identical. Exon 3 of the L gene encoded the LVAVA amino acid combination (Table 3).

The exon 3 to 5 sequencing data (Table 3) gave 6 clones with L opsin exon 5 sequences (L plus M_3L_{4-5} hybrid sequences) and 7 clones with M opsin exon 5 sequences. This approximate 1:1 ratio was confirmed using quantitative PCR (qPCR) to amplify exon 5 (Fig. 3, Table 4). Since both the L opsin and $M_{1-3}L_{4-6}$ hybrid genes contain an identical L exon 5 sequence, there must be two M genes present to account for the 1:1 ratio. The first gene in the array was confirmed to be the L gene, which places the $M_{1-3}L_{4-6}$ hybrid gene and two M genes downstream (Table 4). Exon 3 of this $M_{1-3}L_{4-6}$ hybrid encodes the uncommon MVVVA haplotype (Table 3), with both downstream M genes encoding the more common MVAIA haplotype. The affected male in this family is deuteranopic, suggesting that he has no cones expressing a functional pigment with M-like spectral properties. Since the amino acids at sites 277 and 285 encoded by exon 5 are responsible for most of the spectral shift between and L and M pigments, this would indicate that the second expressed gene in the array is

the $M_{1-3}L_{4-6}$ hybrid. Collectively, these data predict that the L opsin gene with the LVAVA haplotype will be dysfunctional, leaving functional cones expressing the $M_{1-3}L_{4-6}$ hybrid only. This hybrid would be expected to be spectrally similar to an L pigment, thereby accounting for the deuteranopia.

Deuteranopic Family 2. This is the new UK family. The opsin gene arrays from two affected males in this family were analyzed. The long range PCR experiments indicated the presence of three distinct genes, L, M, and the hybrid $L_{1-3}M_{4-6}$. Sequencing of the L gene and the $L_{1-3}M_{4-6}$ hybrid showed that exon 3 of both genes encode the rare LIAVA haplotype (Tables 3, 4), whereas exon 3 of the M gene encodes MVVVA. For both affected males of this family, the L gene was identified as the first in the array with the $L_{1-3}M_{4-6}$ and M genes downstream.

Color vision testing indicated that both affected members of this family are deuteranopes. Since the proximal L opsin gene and the downstream $L_{1-3}M_{4-6}$ hybrid gene both encode LIAVA, the pigments from both genes would be expected to be nonfunctional. As both affected males are deuteranopes, however, it would appear that one of the expressed opsins
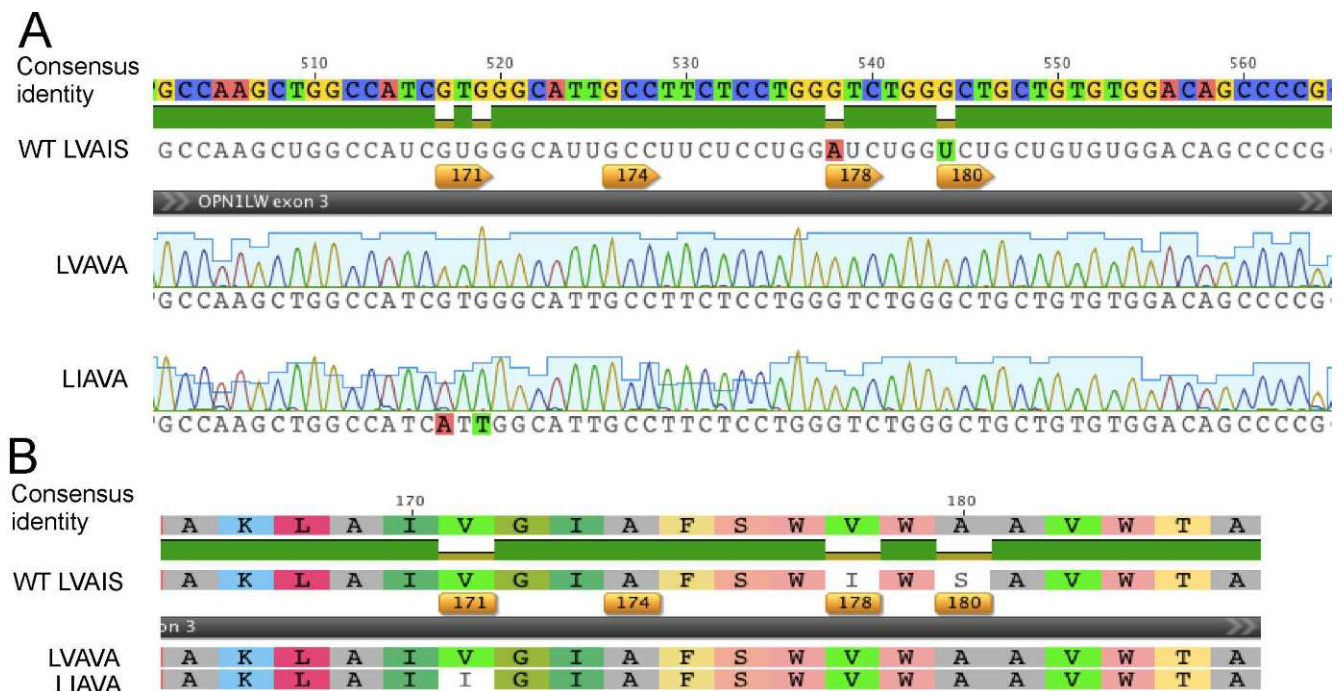

FIGURE 2. Exon 3 sequence of L opsin. (**A**) Electropherograms of both LVAVA and LIAVA haplotypes aligned with WT sequence. (**B**) Amino acid sequence for both LVAVA and LIAVA haplotypes aligned with WT (LVAIS) sequence. Numbers at the *top* of the figure correspond to nucleotides within the opsin coding sequence.

TABLE 3. Sequence Analysis of Cloned PCR Products Encompassing Exons 3, 4, and 5 of the L/M Opsin Genes

	Opsin Genes	Number of Clones Analyzed	Exon 3 Haplotype
Deuteranopia			
Family 1	L	1	LVAVA
	$M_3L_{4\text{-}5}$	5	MVVVA
	M	7	MVAIA
Family 2			
Affected 1	L	4	LIAVA
	$M_{3\text{-}4}L_5$	2	MVVVA
	$L_3M_{4\text{-}5}$	11	LIAVA
	M	6	MVVVA
Affected 2	L	5	LIAVA
	$M_{3\text{-}4}L_5$	1	MVVVA
	$L_3M_{4\text{-}5}$	10	LIAVA
	M	8	
Protanopia			
Family 1			
	L		LVAVA
	$M_3L_{4\text{-}5}$		MVVVA
	M		MVAIA
Family 2			
Affected 1	L	5	LIAVA
	M	16	MVVVA
Affected 2	L	5	LIAVA
	M	14	MVVVA
Family 3			
Affected 1	$L_{3\text{-}4}M_5$	10	LIAVA
	$M_3L_4M_5$	4	MVVVA
	M	10	MVVVA
Affected 2	$L_{3\text{-}4}M_5$	9	LIAVA
	$M_3L_4M_5$	4	MVVVA
	M	3	MVVVA
Family 4	ND	ND	LIAVA
			MVVVA

Exons are classified as L or M and identified by a subscript number. ND, not determined.

FIGURE 3. Quantitative PCR data showing the L:M opsin exon 5 ratios of male subjects from four BED families. L opsin, *solid columns*; M opsin, *open columns*. P, protanopic; D, deuteranopic. *Error bars* represent SEM.

must be generating a pigment that is functionally and spectrally similar to an L cone pigment. This suggests that another gene in the array is expressed. The amplification of exons 3 to 5 as a single fragment revealed a small number of clones with an $M_{1\text{-}4}L_{5\text{-}6}$ hybrid sequence (Table 3). This variant was identified in the mother and both offspring, but at a much lower frequency than L, M, and the $L_{1\text{-}3}M_{4\text{-}6}$ hybrid. It is probable, therefore, that this gene is responsible for providing a functional pigment with an absorbance peak similar to an L cone pigment. However, the long range PCRs and subsequent screening of upstream and downstream genes failed to identify this gene, so the basis for the deuteranopia in this family remains uncertain. An interesting observation in these males is that while they show apparent deuteranopia, they also make protanomolous matches with the anomaloscope, a finding that has been previously reported in deuteranopes.[29] It may be that the LIAVA combination has a more detrimental effect in the hybrid $L_{1\text{-}3}M_{4\text{-}6}$ opsin than in a WT L opsin, leading to affected males being deuteranopic with protanomolous matches as opposed to being protanopic with deuteranomolous matches.

The qPCR data for the two affected siblings differed, with L:M exon 5 ratios of 1:4 and 1:2, respectively (Fig. 3). This implies that the number of downstream M genes differs between the two siblings, indicating that an intergenic recombination event has occurred that placed an additional two copies of the M gene into the array of subject one (Table 4).
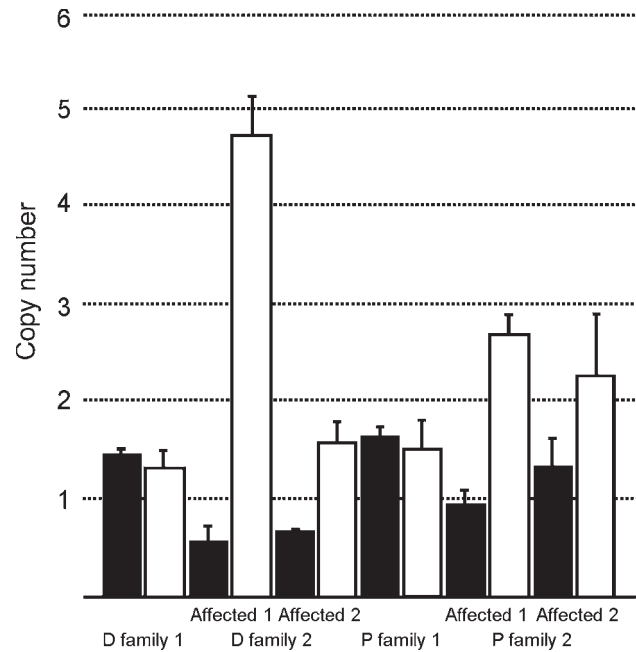
Protanopic Family 1. This is the family originally identified in Minnesota.[3] The long range PCR data consistently identified an L opsin gene that encodes for LVAVA as the upstream gene, with $M_{1\text{-}3}L_{4\text{-}6}$ hybrid (encoding MVVVA) and M (encoding MVAIA) opsin genes downstream (Table 4). The qPCR analysis revealed a 1:1 ratio of L to M exon 5 sequences, indicating the presence of a total of four genes in the array comprising L, $M_{1\text{-}3}L_{4\text{-}6}$, and two M genes (Fig. 3). The protanopia in this family is predicted to be due to a proximal L opsin gene that would generate a nonfunctional LVAVA pigment, followed by an intact M gene, with two unexpressed genes located further downstream, namely an $M_{1\text{-}3}L_{4\text{-}6}$ hybrid and a second M gene.

The following data were derived from the remaining three UK families previously reported.[4]

Protanopic Family 2. Exon 3 to 5 amplification and cloning identified only two gene sequences in this family, an L opsin that encoded for LIAVA and an M opsin that encoded for MVVVA (Table 3). Long range PCR identified the first gene in the array as an L opsin with the LIAVA sequence (Table 4). The qPCR analysis gave an L:M exon 5 ratio of around 1:2 for both affected males, suggesting that there are two M genes both encoding MVVVA downstream of the L gene that encodes for LIAVA.

Protanopic Family 3. Long range PCR and qPCR were not carried out with this family. Exon 3 to 5 PCR analysis, however, identified three genes in both affected siblings, $L_{1\text{-}4}M_{5\text{-}6}$ and $M_{1\text{-}3}L_4M_{5\text{-}6}$ hybrids, and a WT M opsin gene (Table 4). Whereas the $L_{1\text{-}4}M_{5\text{-}6}$ hybrid encodes LIAVA, all of the M opsin exon 3 sequences encode MVVVA. As L opsin genes are generally placed first in the array, it is likely that the hybrid $L_{1\text{-}4}M_{5\text{-}6}$ gene will be in the proximal position. The phenotypes of the two affected individuals suggest that this gene would encode for a nonfunctional pigment. Thus, the protanopia most probably arises in this family from a single expressed M or hybrid $M_{1\text{-}3}L_4M_{5\text{-}6}$ gene, the latter of which would encode for a pigment that is spectrally similar to a WT M opsin.

TABLE 4.   The Order and Number of Opsin Genes in the L/M Arrays of Affected Individuals

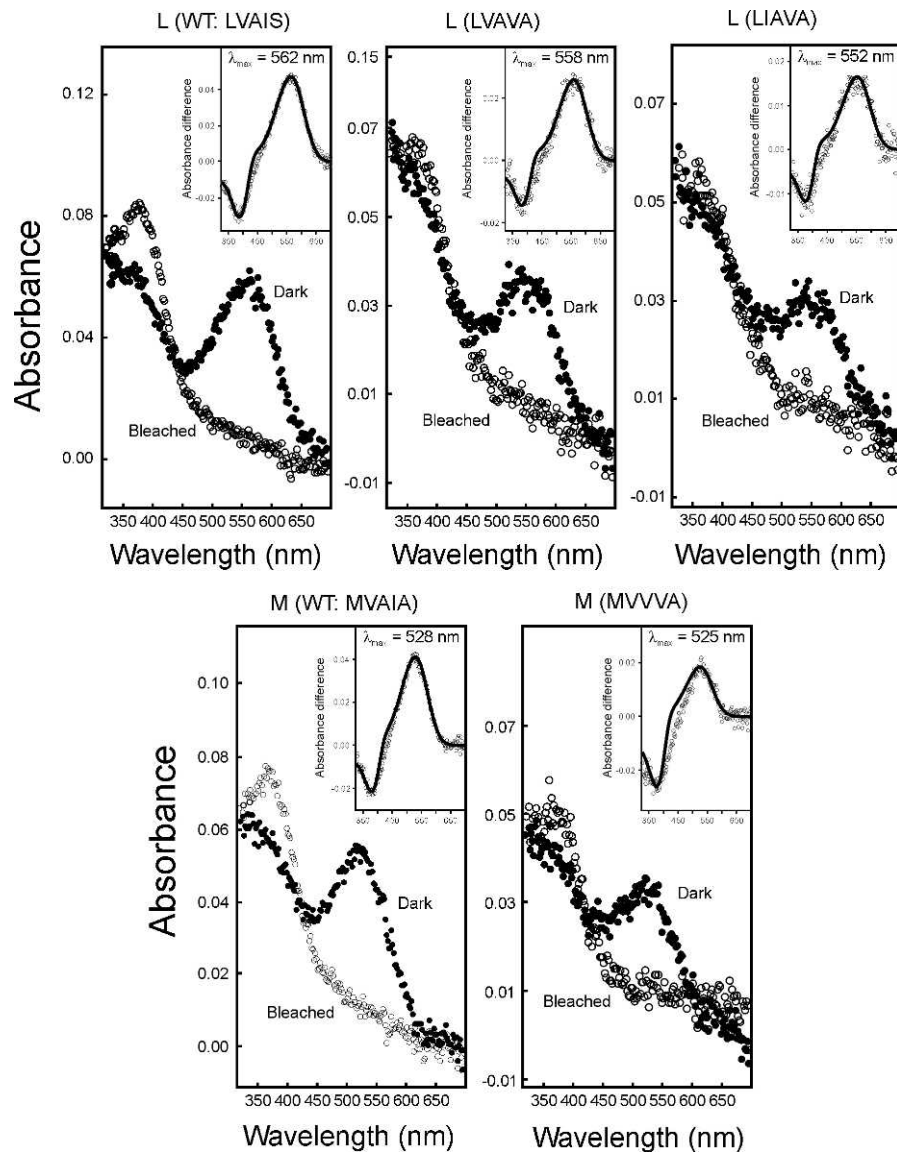|	First Gene in Array	L Opsin Exon 3	Downstream Genes	Exon 5 L:M Ratio
Deuteranopia				
Family 1	L	LVAVA	$M_{1-3}L_{4-6}/M/M$	1:1
Family 2				
Subject 1	L	LIAVA	$L_{1-3}M_{4-6}/M_{1-4}L_{5-6}/M/M/M$	1:4
Subject 2	L	LIAVA	$L_{1-3}M_{4-6}/M_{1-4}L_{5-6}/M$	1:2
Protanopia				
Family 1	L	LIAVA	$M/M_{1-3}L_{4-6}/M$	1:1
Family 2				
Subjects 1 and 2	L	LIAVA	M/M	1:2
Family 3	ND	LVAVA		ND
Family 4	ND	LIAVA		ND

ND, not determined.

FIGURE 4.   Spectra for in vitro generated WT and variant opsin pigments. The *inset graph* for each pigment shows the difference spectra used to determine the spectral sensitivity peak (λ_{max}).
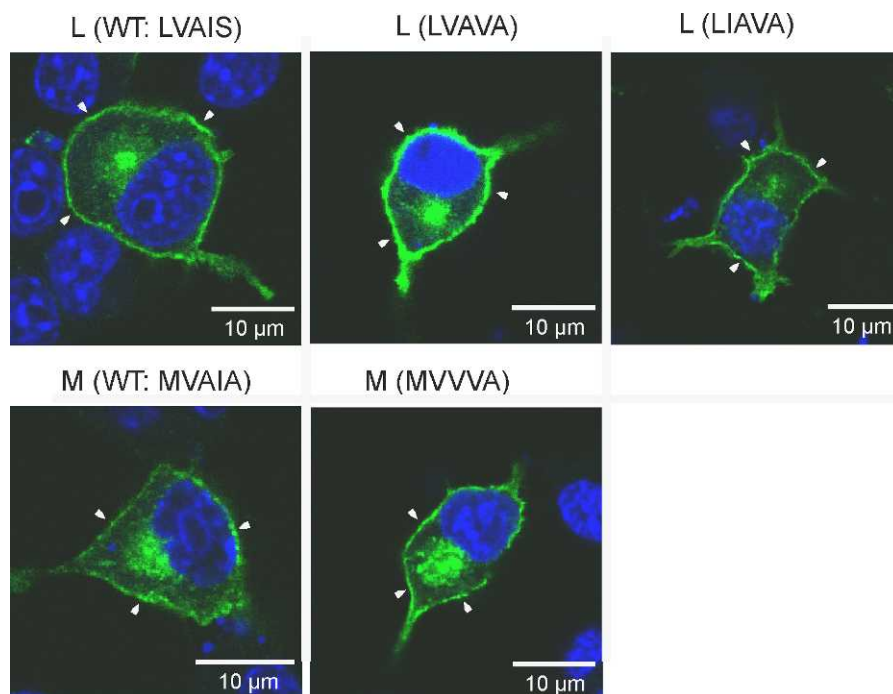
FIGURE 5.   Confocal microscopy images of transfected Neuro2a cells expressing WT and variant opsins. In all cases, the opsin protein was visualized by immunocytochemistry, and shown to be present at the membrane in all cells (*white arrows*).

Protanopic Family 4. A single individual was analyzed. Screening was limited to exon 3, which revealed the LIAVA and MVVVA combinations in L and M opsins, respectively. Given that an L opsin with a combination of LIAVA is predicted to encode a nonfunctional pigment, it would be expected that protanopia in this individual would arise from the expression of a functional M pigment with the MVVVA haplotype.

Functional Analysis of Opsins

The ability of the different opsin variants to form functional pigments was investigated in vitro, first to determine the peak spectral sensitivity of the corresponding pigment and second to obtain an estimate of the in vitro stability of the opsin protein. The SPLICE method[22] was used to generate full length constructs that exactly replicated the WT and variant opsin gene sequences identified in the BED families. Spectra for WT and the different variant pigments are shown in Figure 4. The λ_{max} for the WT LVAIS pigment at 562 nm is almost identical to the predicted value of 560 nm,[30,31] whereas the variant L pigments with LIAVA and LVAVA exon 3 haplotypes yielded spectral peaks at 558 and 552 nm, respectively, that are short wavelength shifted compared with the LVAIS pigment. These variant pigments also showed a consistent reduction in yield compared with WT; the L opsin variant with LVAVA as found in deuteranopic families 1 and 3 and in protanopic family 3 showed an average reduction to 63% of WT L, and the L opsin variant with LIAVA found in deuteranopic family 2 and in protanopic families 1, 2, and 4, an average reduction to 52% of WT L opsin.

A feature of the BED families examined in this study is the presence of an uncommon M exon 3 haplotype that encodes for MVVVA rather than the more common MVAIA combination across the sites that are involved in the L variant opsins. In vitro generation of the uncommon MVVVA variant yielded a pigment with a λ_{max} at 525 nm, which is almost identical to the WT MVAIA pigment at 528 nm.

In order to determine whether the variant sequences interfered with the intracellular trafficking of the pigment to the cell membrane, WT L and M opsins, the two variant L opsins and the uncommon MVVVA M opsin were expressed in the murine neuroblastoma cell line Neuro2a and investigated by immunocytochemistry (Fig. 5). In all cases, the opsin proteins were successfully transported to the cell membranes. These data suggested, therefore, that the reduction of spectrally viable pigment in the in vitro regeneration experiments discussed above was unlikely to be primarily due to a lower concentration of membrane-bound opsin protein.

DISCUSSION

BED presents with moderately reduced visual acuity combined with full dichromacy; the latter can be either deuteranopia or protanopia, which indicates that only L or M cones, respectively, together with S cones, are present in the retinae of affected individuals. In our previous study,[4] we noted that one of the BED families segregated for the known deleterious Cys203Arg substitution encoded by exon 4.[11] This mutation could account, therefore, for the observed cone dysfunction if expressed in a subset of cones in the affected individuals. In contrast, we were unable to find any deleterious point mutations in the L and M opsin genes in the remaining families examined. The present study has not only re-examined the opsin genes in these families, but has extended the analysis to the original BED family,[4] the Minnesota family,[3] and a new UK family; in all cases, a rare combination (haplotype) of amino acids, either LVAVA or LIAVA specified by codons 153, 171, 174, 178, and 180 of exon 3 of the L opsin gene, is present, a combination that has not been previously reported in more than 300 L or M opsin genes[16] or in separate studies of the L and M genes in a large number of dichromats.[27,28] These two variant haplotypes would appear to be restricted to individuals

that have been diagnosed with either BED[3,4] or a similar cone dysfunction.[17]

Sequence and gene order analysis of the L and M genes present in the different BED families investigated in this study demonstrates that in many cases, multiple genes are present within the array. However, where studied, the upstream or proximal gene encodes one or other of the variant haplotypes. An earlier study of BCM[13,14] implied that these variant combinations of amino acids give rise to nonfunctional pigments; if this is also the case for BED, then dichromacy could arise from the expression of a nonfunctional pigment from the proximal opsin gene in the array, with the type of dichromacy dependent on the spectral characteristics of the functional pigment expressed by the second gene in the array.

In vitro analysis showed that functional pigments are produced from the variant opsins, with spectral peaks consistent with the particular combination of amino acids at the key tuning sites known for L/M pigments.[30,31] The levels of spectrally viable protein for both of the two variant pigments, however, were significantly reduced. The loss of cone function in these BED individuals may arise, therefore, from a reduced production and function of the variant pigments. Immunocytochemisty using the neuroblastoma cell line Neuro2a revealed that neither of the variant haplotypes appears to alter transport of the opsin to the cell membrane. Collectively, these data suggest that the variant haplotype may interfere with retinal chromophore handling.

A recent study[32] has proposed an alternative explanation for the cone dysfunction associated with these rare L opsin gene haplotypes, where the variant nucleotide sequence of exon 3 interferes with the normal splicing process, such that splicing occurs between exons 2 and 4 with the elimination of exon 3. The two individuals studied both have the LIAVA haplotype, whereas 3 of the 7 families reported here have the variant LVAVA haplotype. Nucleotide changes in codons 151, 153, and 178 were identified as potentially having the greatest impact on splicing; the same novel combination of nucleotides across these three sites is also seen in the LVAVA haplotype. If correct, the removal of exon 3 would result in a change in codon frame, with just six amino acids added to those encoded by exon 2 before a new stop codon is encountered. The truncated product arising from such an aberrant transcript would almost certainly be unable to form a pigment although nonsense-mediated decay[33] would be expected to remove the defectively spliced transcript, resulting in little or no defective protein.

The five exon 3 sites that distinguish the variant L opsin haplotypes also differ amongst M exon 3 sequences. The common M haplotype is M153, V171, A174, I178, and A180 (MVAIA). This haplotype is found, however, in only three of the BED families, whereas the uncommon MVVVA haplotype is found in all six BED families analyzed, and in three of the families would appear to be the only M exon 3 sequence present. In vitro expression confirms that the M opsin containing the MVVVA variant forms a functional pigment, so it is unlikely that this combination contributes to the BED phenotype. The presence of this uncommon M haplotype in the BED families is unexpected and may reflect either a common origin for each of the variant L opsin haplotypes or a role for the uncommon M opsin gene sequence in promoting intra-exonic crossing over in the generation of the variant L haplotypes.

A constant feature in all BED families is myopia. The genetic basis for myopia has been the subject of several recent studies. A number of genetic loci for high grade and moderate myopia have been identified, mostly from studies of family pedigrees,[34] and many of these have been replicated in an international collaborative whole genome study of myopia.[35] Amongst these is the *MYP1* locus (OMIM number 310460), which maps to the tip of the X chromosome at Xq28. It was first identified in the

original BED family[2,3] and a recent study in China[36] has confirmed its location to chromosome Xq28 within 6.1 cM region between marker DXS8069 and Xqter. However, the study failed to find mutations in four candidate genes selected on the basis of expression in the eye and absence of other disease associations; the opsin genes that also map to this region of the X chromosome were not screened. In the present study, we have now established that opsin gene variation is responsible for both cone dysfunction and the color vision defect. Since myopia is an integral component of the BED syndrome, it would seem likely that this must also arise from the dysfunctional cone photopigment and the disruption of the normal cone mosaic,[37] but how this results in myopia remains uncertain.

In conclusion the analysis of the L/M opsin arrays in several BED families has revealed a novel mechanism of dichromacy, whereby the lack of a functional pigment is not caused by an overtly mutant opsin, but instead by a particular combination of nucleotide differences across several codons in exon 3.

References

1. Haim M, Fledelius HC, Skarsholm. X-linked myopia in Danish family. *Acta Ophthalmol (Copenh)*. 1988;66:450–456.
2. Schwartz M, Haim M, Skarsholm D. X-linked myopia: Bornholm eye disease. Linkage to DNA markers on the distal part of Xq. *Clin Genet*. 1990;38:281–286.
3. Young TL, Deeb SS, Ronan SM, et al. X-linked high myopia associated with cone dysfunction. *Arch Ophthalmol*. 2004;122:897–908.
4. Michaelides M, Johnson S, Bradshaw K, et al. X-linked cone dysfunction syndrome with myopia and protanopia. *Ophthalmology*. 2005;112:1448–1454.
5. Hanna MC, Platts JT, Kirkness EF. Identification of a gene within the tandem array of red and green color pigment genes. *Genomics*. 1997;43:384–386.
6. Dulai KS, von Dornum M, Mollon JD, Hunt DM. The evolution of trichromatic color vision by opsin gene duplication in New World and Old World primates. *Genome Res*. 1999;9:629–638.
7. Drummond-Borg M, Deeb SS, Motulsky AG. Molecular patterns of X chromosome-linked color vision genes among 134 men of European ancestry. *Proc Natl Acad Sci U S A*. 1989;86:983–987.
8. Neitz M, Neitz J. Numbers and ratios of visual pigment genes for normal red-green color vision. *Science*. 1995;267:1013–1016.
9. Winderickx J, Battisti L, Motulsky AG, Deeb SS. Selective expression of human X chromosome-linked green opsin genes. *Proc Natl Acad Sci U S A*. 1992;89:9710–9714.
10. Metlapally R, Michaelides M, Bulusu A, et al. Evaluation of the X-linked high-grade myopia locus (MYP1) with cone dysfunction and color vision deficiencies. *Invest Ophthalmol Vis Sci*. 2009;50:1552–1558.
11. Kazmi MA, Sakmar TP, Ostrer H. Mutation of a conserved cysteine in the X-linked cone opsins causes color vision deficiencies by disrupting protein folding and stability. *Invest Ophthalmol Vis Sci*. 1997;38:1074–1081.
12. Carroll J, Baraas RC, Wagner-Schuman M, et al. Cone photoreceptor mosaic disruption associated with Cys203Arg mutation in the M-cone opsin. *Proc Natl Acad Sci U S A*. 2009;106:20948–20953.

13. Nathans J, Davenport CM, Maumenee IH, et al. Molecular genetics of human blue cone monochromacy. *Science*. 1989; 245:831–838.

14. Nathans J, Maumenee IH, Zrenner E, et al. Genetic heterogeneity among blue-cone monochromats. *Am J Hum Genet*. 1993;53:987–1000.

15. Gardner JC, Michaelides M, Holder GE, et al. Blue cone monochromacy: causative mutations and associated phenotypes. *Mol Vis*. 2009;15:876–884.

16. Crognale MA, Fry M, Highsmith J, et al. Characterization of a novel form of X-linked incomplete achromatopsia. *Vis Neurosci*. 2004;21:197–203.

17. Carroll J, Neitz M, Hofer H, Neitz J, Williams DR. Functional photoreceptor loss revealed with adaptive optics: an alternate cause of color blindness. *Proc Natl Acad Sci U S A*. 2004;101: 8461–8466.

18. Neitz M, Carroll J, Renner A, Knau H, Werner JS, Neitz J. Variety of genotypes in males diagnosed as dichromatic on a conventional clinical anomaloscope. *Vis Neurosci*. 2004;21: 205–216.

19. Oda S, Ueyama H, Nishida Y, Tanabe S, Yamade S. Analysis of L-cone/M-cone visual pigment gene arrays in females by long-range PCR. *Vision Res*. 2003;43:489–495.

20. Neitz M, Neitz J. Molecular genetics of color vision and color vision defects. *Arch Ophthalmol*. 2000;118:691–700.

21. Davies WL, Cowing JA, Carvalho LS, et al. Functional characterisation and regulation of visual pigment gene expression in an anadromous lamprey. *FASEB J*. 2007;21: 2713–2724.

22. Davies WL, Carvalho LS, Hunt DM. SPLICE: a technique for generating in vitro spliced coding sequences from genomic DNA. *Biotechniques*. 2007;43:785–789.

23. Davies WL, Carvalho LS, Tay BH, Brenner S, Hunt DM, Venkatesh B. Into the blue: gene duplication and loss underlie color vision adaptations in a deep-sea chimaera, the elephant shark *Callorhinchus milii*. *Genome Res*. 2009;19:415–426.

24. Govardovskii VI, Fyhrquist N, Reuter T, Kuzmin DG, Donner K. In search of the visual pigment template. *Vis Neurosci*. 2000;17:509–528.

25. Davies WL, Cowing JA, Carvahlo LS, et al. Functional characterisation and regulation of visual pigment gene expression in an anadromous lamprey. *FASEB J*. 2007;21: 2713–2724.

26. Davies WI, Zheng L, Hughes S, et al. Functional diversity of melanopsins and their global expression in the teleost retina. *Cell Mol Life Sci*. 2011;68:4115–4132.

27. Ueyama H, Kuwayama S, Imai H, et al. Analysis of L-cone/M-cone visual pigment gene arrays in Japanese males with protan color-vision deficiency. *Vision Res*. 2004;44:2241–2252.

28. Sharpe LT, Stockman A, Jagle H, et al. Red, green, and red-green hybrid pigments in the human retina: correlations between deduced protein sequences and psychophysically measured spectral sensitivities. *J Neurosci*. 1998;18:10053–10069.

29. Smith VC, Pokorny J, Newell FW. Autosomal recessive incomplete achromatopsia with deutan luminosity. *Am J Ophthalmol*. 1979;87:393–402.

30. Asenjo AB, Rim J, Oprian DD. Molecular determinants of human red/green color discrimination. *Neuron*. 1994;12: 1131–1138.

31. Merbs SL, Nathans J. Role of hydroxyl-bearing amino acids in differentially tuning the absorption spectra of the human red and green cone pigments. *Photochem Photobiol*. 1993;58: 706–710.

32. Ueyama H, Muraki-Oda S, Yamade S, et al. Unique haplotype in exon 3 of cone opsin mRNA affects splicing of its precursor, leading to congenital color vision defect. *Biochem Biophys Res Commun*. 2012;424:152–157.

33. Conti E, Izaurralde E. Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. *Curr Opin Cell Biol*. 2005;17:316–325.

34. Hornbeak DM, Young TL. Myopia genetics: a review of current research and emerging trends. *Curr Opin Ophthalmol*. 2009; 20:356–362.

35. Li YJ, Guggenheim JA, Bulusu A, et al. An international collaborative family-based whole-genome linkage scan for high-grade myopia. *Invest Ophthalmol Vis Sci*. 2009;50: 3116–3127.

36. Guo X, Xiao X, Li S, Wang P, Jia X, Zhang Q. Nonsyndromic high myopia in a Chinese family mapped to MYP1: linkage confirmation and phenotypic characterization. *Arch Ophthalmol*. 2010;128:1473–1479.

37. Wagner-Schuman M, Neitz J, Rha J, Williams DR, Neitz M, Carroll J. Color-deficient cone mosaics associated with Xq28 opsin mutations: a stop codon versus gene deletions. *Vision Res*. 2010;50:2396–2402.