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Review

Intermixing the *OPN1LW* and *OPN1MW* Genes Disrupts the Exonic Splicing Code Causing an Array of Vision Disorders

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Abstract: The first step in seeing is light absorption by photopigment molecules expressed in the photoreceptors of the retina. There are two types of photoreceptors in the human retina that are responsible for image formation, rods and cones. Except at very low light levels when rods are active, all vision is based on cones. Cones mediate high acuity vision and color vision. Furthermore, they are critically important in the visual feedback mechanism that regulates refractive development of the eye during childhood. The human retina contains a mosaic of three cone types, short-wavelength (S), long-wavelength (L) and middle-wavelength (M); however, the vast majority (~94%) are L and M cones. The *OPN1LW* and *OPN1MW* genes, located on the X-chromosome at Xq28, encode the protein component of the light-sensitive photopigments. Here we review mechanism by which splicing defects in these genes cause vision disorders.

Keywords: colorblindness, color vision, myopia, cone photopigment, exon skipping, X-linked cone dysfunction

1. Introduction

Cone photoreceptors contain light sensitive photopigments comprised of an apoprotein component termed opsin, and a covalently bound chromophore, 11-cis retinal. The human S cone opsin gene, OPN1SW resides on chromosome 6, while the L and M cone opsin genes, *OPN1LW* and *OPN1MW*, are located on the X-chromosome at Xq28. Recently, specific haplotypes of exon 3 in the human L and M cone opsin genes have been shown to cause exon 3-skipping [1] and are associated with a variety of vision disorders including red-green color blindness, blue cone monochromacy, X-linked cone dysfunction, X-linked cone dystrophy, and syndromic and non-syndromic high myopia [2-13]. The diversity of exon 3 haplotypes arose through recombination mechanisms that have intermixed the *OPN1LW* and *OPN1MW* genes[14-16] and disrupted sequences within exon 3 that are critical components of the splicing code resulting in the failure of exon 3 to be included in the mature mRNA[1,7,10,11,17]. The predicted consequence for vision depends on how much correctly spliced opsin mRNA is made for a given haplotype, and the relative number of cones expressing the first versus the second opsin gene in the array.

2. *OPN1LW* and *OPN1MW* exon 3 haplotypes associated with exon 3 skipping and vision disorders

The *OPN1LW* and *OPN1MW* genes are each about 40 kilobase pairs in length and are more than 98% identical in nucleotide sequence. Each has six exons[18]. The first and sixth exons are identical among and between the L and M opsin genes. The fifth exon harbors 11 common single nucleotide polymorphism (SNPs) and is the only exon that can be used to distinguish between the *OPN1LW* and *OPN1MW*

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genes because two of the SNPs encode amino acid differences that are responsible for ~21 nanometers (nm) of the ~30 nm difference in the wavelength of peak spectral sensitivity of L versus M cones [19]. There are 17 common SNPs in the other three exons with four in exon 2, eight in exon 3, and five in exon 4. Exon 3 haplotypes that have been identified in patients with cone-photoreceptor-based vision disorders and that exhibit exon 3 skipping in minigene splicing assays are given in Table 1 [1,3-6,8-13,20,21].

Table 1. Exon 3 skipping haplotypes associated with vision disorders

	G/A	C/A	G/C	A/G	T/G	C/T	A/G	T/G	R/R	L/M	V/V	I/V	A/V	I/V	S/A	correctly
Haplotype	c.453	457	465	511	513	521	532	538	p.151	153	155	171	174	178	180	spliced(%)
Hap 1/2	G	C	G/C	A	T	C	G	G		L		I	A	V	A	0
Hap 3	G	C	G	G	G	C	G	G		L		V	A	V	A	6.7
Hap 4	G	C	G	A	T	C	G	T		L		I	A	V	S	20.3
Hap 5/6	A	A	C/G	A	T	C	G	G		M		I	A	V	A	10.4-8.8
Hap 7/8	A	A	C/G	G	G	T	G	G		M		V	V	V	A	80.1-75.6
Hap 9	A	A	C	G	G	C	A	G		M		V	A	V	A	53.0
Hap 10	G	C	G	A	T	C	A	G		L		I	A	I	A	40.8

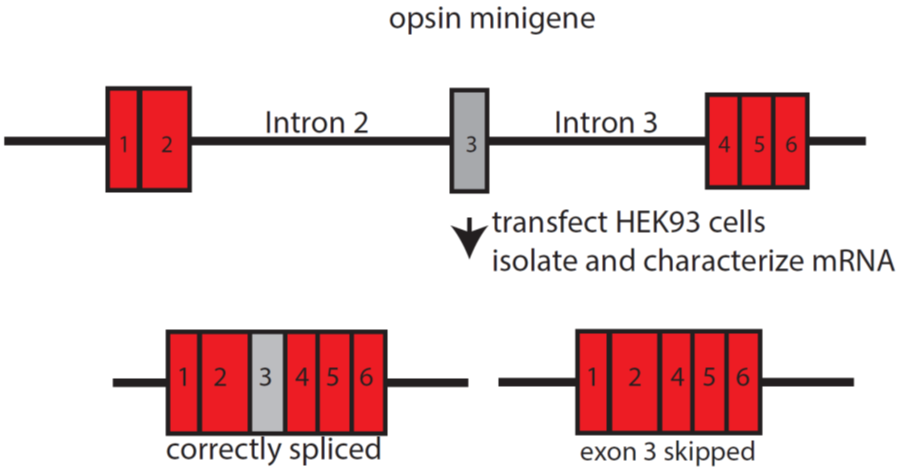

Figure 1. Opsin minigene assay. Top: minigene is *OPN1LW* cDNA with introns 2 and 3. Exons are numbered boxes. Introns are labeled. All minigenes are identical except exon 3 (gray box). mRNA is isolated from HEK293 cells following transient transfection and the amount of correctly spliced mRNA is quantified.

Of the eight SNPs in exon 3, c.453 and c.465 are silent, c.511 and c.513 both occur within codon 171 and are associated with an amino acid substitution, and the rest, c.457, 521, 532 and 538, specify amino acid substitutions individually. Estimates of the relative amount of correctly spliced mRNA for each haplotype in Table 1 were obtained from a semi-quantitative minigene splicing assay [10]. The assay is illustrated in Figure 1. For each of the haplotypes shown with the exception of haplotypes 7, 8 and 9, the major splicing isoform observed was missing exon 3 and had exon 2 spliced directly to exon 4, which alters the reading frame in codon 138 and introduces a premature translation termination signal at codon 143. For haplotypes 7, 8 and 9 the major splicing isoform observed was the correctly spliced message but ~20 - 47% of the mRNA also lacked exon 3. Thus, one of the major differences

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among these disease-associated haplotypes is the relative amount of functional photopigment that is made in the photoreceptor due to differences in the amount of mRNA encoding the full-length opsin [11].

For the LIAVA variant (Hap1/2 in Table 1), no correctly spliced mRNA was observed[1,7,10,11]. For the LVAVA variant (Hap 3 in Table 1), which differs from LIAVA by two (c.511 and c.513) or three (c.465, c.511, c.513) nucleotides, a small amount of correctly spliced mRNA was observed [1,7,10,11]. The LIAVA and LVAVA opsins were shown to function close to normally in transgenic mice in which cDNAs extending from within exon 2 through exon 6 of the human *OPN1LW* LIAVA and LVAVA variants were fused in frame to the endogenous mouse X-chromosome cone opsin gene [11]. The primary transcript contained only one intron (intron 1), and thus the defect in splicing due to the exon 3 haplotype was circumvented. Visual function in these mice, as measured by the ERG, was compared to control transgenic mice harboring a normal human *OPN1LW* variant encoding LIAIS, that is found in males with normal color vision and no other known vision disorder [14,22]. Cone function and retinal morphology in mice expressing the LIAVA or LVAVA variant were similar to control transgenic mice with LIAIS variant. However, LVAVA showed signs of a slow cone degeneration. Furthermore, using a long-flash electroretinogram (ERG) with custom L/M cone-isolating stimuli to test for cone function in males with only the LIAVA or only the LVAVA variant expressed in all non-S cones revealed ERG b-wave amplitudes indicative of the absence of functional photopigment for males with the LIAVA variant, and with the presence of a small amount of functional photopigment in males with the LVAVA variant [11]. Together, these data support the conclusion that the splicing defect in the LIAVA and LVAVA opsin genes is responsible for the disease phenotypes associated with these haplotypes. However, there are very important differences in the disease phenotypes associated with these two haplotypes. Phenotypes associated with LIAVA are more stationary while LVAVA phenotypes are more progressive presumably because of the small amount of abnormal pigment expressed.

Gardner et al. [7] suggested that the nucleotide difference at c.532 (A/G) is principally responsible for the exon 3 skipping phenotype, however, analysis of the data from Buena-Atienza et al. [10] provides evidence that all of the nucleotide polymorphisms contribute to exon 3 skipping, and that the magnitude of the effect of changing c.532 depends on the identities of the nucleotides at the other polymorphic positions. Position c.532 is clearly important as LIAIA (Hap 10, Table 1) differs from LIAVA only by the c.532 nucleotide yet LIAIA splices correctly ~41% of the time compared to 0% for LIAVA (Table 1). However, the MVAVA variant (Hap 9, Table 1) differs from LVAVA (Hap 3) at three nucleotide positions, c.453, c.457 and c.465, and 53% of the MVAVA mRNA is correctly spliced compared to 6.7% for LVAVA, a difference of 46.3%, which is larger than the effect of c.532. These same three SNPs distinguish LIAVA from MIAVA, but MIAVA only yields 9-10% correctly spliced mRNA compared to 0 for LIAVA, which is a much smaller effect that observed for these three SNPs in LVAVA vs MVAVA, suggesting that the nucleotides in codon 171 may modify the contribution of differences at c.453, 457 and 465. MIAVA (Hap 5/6, Table 1) differs from MVVVA (Hap 7/8, Table 1) at three positions, c.511 and c.513 in codon 171 and c.521 and MVVVA yields 67 to 70% more correctly spliced mRNA than MIAVA, a much larger effect size than observed for c.532. Finally, LIAVS differs from LIAVA at only one position, c.538, yet LIAVS yields 20% more correctly spliced mRNA than LIAVA.

Comparing incremental increases in correct splicing from LIAVA (0%) to MIAVA (10.4%) to MVAVA (53%) to MVVVA (80.1%) considering only haplotypes with c.465C (Table 1) suggests the effects are additive. That is, starting with the LIAVA haplotype, changing c.453, c.457 to create MIAVA improves splicing by about 10.4%.

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Substituting position c.511 and c.513 into MIAVA to create MVAVA increases correct splicing by ~42.6%. and altering MVAVA to create MVVVA increases correct splicing by about 27.1%. The sum of the three stepwise changes is 80.1, which is the difference observed between LIAVA and MVVVA. This is also true for substitutions required to go from LIAVS to LIAVA to LIAIA. The c.538 change needed to convert LIAVS to LIAVA reduces the amount of correctly spliced mRNA by about 20.3%. Converting LIAVA to LIAIA increases correct splicing by 40.8%. The difference between LIAVS and LIAIA is 20.5% which is the sum of the effects at c.532 and c.538 individually.

In human males with the LIAVA, LVAVA, LIAVS or MIAVA opsin who have been studied, there is clear evidence of cone dysfunction, in part because it has been possible to investigate cone function in individuals who have one of these haplotypes in all of the expressed X-chromosome cone opsin genes so each haplotype has been studied in isolation [1,3-6,8-13,20,21], however this is not true for the MVVVA, MVAVA or LIAIA haplotypes. The MVVVA haplotype has been commonly observed in normally functioning cones [5,14,23,24] and it is commonly found in exon 3 in human *OPN1MW* genes in people with no known vision problems. It has been implicated in vision disorders through its occurrence in the *OPN1MW* genes of males who have another *OPN1LW* or *OPN1MW* gene with the LIAVA, MIAVA or LVAVA haplotype and the observation that it does not include exon 3 in the mature mRNA 100% of the time. However, to what degree exon 3 skipping can be tolerated without causing disease or under what circumstances the degree of exon 3 skipping exhibited by the MVVVA haplotype is disease causing are all unknown. Likewise, the association of the MVAVA haplotype with disease is due to its occurrence in males with another *OPN1LW* or *OPN1MW* gene having the LVAVA haplotype and the fact that only about 50% of the mature mRNA from the minigene assay includes exon 3; however, it has been observed in normally functioning cones [5]. The LIAIA haplotype is quite rare, so whether the disease phenotype observed in a patient with this haplotype is indeed due to the haplotype is uncertain.

3. The architecture of the opsin gene array and the importance of gene order

In humans, non-reciprocal recombination mechanisms have generated gene rearrangements that underlie the common inherited color vision deficiencies and copy number variation in people with normal color vision [25,26], as well as the relatively rare disorder, blue cone monochromacy[27]. Normal trichromatic color vision requires short-wavelength (S), long-wavelength (L) and middle-wavelength (M) cones (commonly referred to as blue, red and green cones). Figure 2A illustrates the most common arrangement in individuals with normal color vision, which is an *OPN1LW* gene followed by one or more *OPN1MW* genes [18,25]; however, only the first two genes in the array are expressed [28]. Upstream of the opsin gene array is a locus specific enhancer termed the locus control region (LCR) [29]. The *OPN1LW* and *OPN1MW* genes share the same LCR, thus in each cone photoreceptor only one gene is expressed at a time and epigenetic mechanisms [30] ensure that *OPN1LW* and *OPN1MW* genes are each expressed in separate populations of cone photoreceptors, as required to confer trichromatic color vision. The choice of which opsin gene to express, the first or second in the array, is stochastic and there are no known molecular distinctions between L and M cones besides the opsin gene they express [31].

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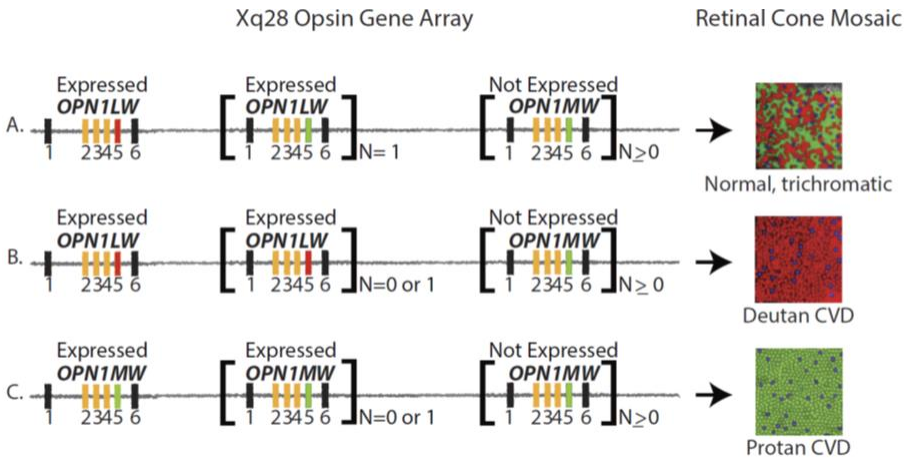

Figure 2. Opsin gene array structure, cone mosaic and color vision phenotype. *OPN1LW* and *OPN1MW* gene exons are depicted as numbered boxes. Black boxes are exons 1 and 6. Yellow boxes depict exons 2, 3, and 4. Red boxes indicate exon 5 of *OPN1LW* genes which encode tyrosine and threonine at amino acid positions 277 and 285, respectively. Green boxes indicate exon 5 of *OPN1LW* genes which encode phenylalanine and alanine at amino acid positions 277 and 285, respectively. **A**. Opsin gene array with the two expressed positions occupied by *OPN1LW* and *OPN1MW* genes giving rise to retinal cone mosaic with L cones (red circles), M cones (green circles), S cones (blue circles) and normal trichromatic color vision. **B.** Arrays with only one *OPN1LW* gene (second and third genes N=0) or the two expressed positions occupied by *OPN1LW* genes (second gene N = 1) underly deutan CVD and retina mosaic has L and S cones. **C**. Arrays with one *OPN1MW* gene (second and third genes N=0) or with both expressed positions (second gene N=1) both occupied by an *OPN1MW* gene underly protan CVD and retina mosaic has M and S cones.

3.1 Blue cone monochromacy (BCM)

Vision in blue cone monochromats is mediated solely by S cones and rod photoreceptors. Cones mediate visual acuity, and in humans ~94% of the cone photoreceptors are L or M cones, thus blue cone monochromats have poor visual acuity because they have no functional L or M cones. If the LCR is deleted, none of the X-chromosome cone opsin genes is expressed, and this is a common cause of blue cone monochromacy [27]. BCM is also caused by the combined deletion of all but one opsin gene on the X-chromosome and the presence of inactivating mutation in the remaining gene. The most common inactivating mutation underlying blue cone monochromacy is a missense mutation substituting arginine for cysteine at amino acid position 203. Exon 3-skipping haplotypes of the *OPN1LW* or *OPN1MW* gene also cause blue cone monochromacy, as will be described in more detail below.

3.2 Red-green color vision deficiency

About 8% of males (1 in 12) and 0.4% of females (1 in 230) are affected by some form of inherited red-green color vision deficiency (CVD). Whereas normal color vision is mediated by three cone types, L, M and S (Figure 2A), inherited red-green color vision deficiency is mediated by just two cone types, S and L or S and M (Figure 2B and 2C, respectively). The term for CVD mediated by S and L cones is deutan from Greek for "the second type" and it is the most common form, accounting for about 6% of males. Deutan CVD is dichromatic (termed deuteranopia) if there is only one opsin gene in the array or if the first and second genes specify L cone photopigments that are identical in their wavelengths of peak sensitivity. Deutan CVD can also be

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anomalous trichromatic (termed deuteranomaly) if the first and second genes specify L cone photopigments that differ in the wavelength of peak sensitivity (Figure 2B). The term for inherited CVD mediated by S and M cones is protan from Greek for "the first type" and it affects about 2% of males. Protan CVD is dichromatic (protanopia) if there is only one opsin gene in the array or if the first and second genes specify M cone photopigments that are identical in their wavelengths of peak sensitivity. Protan CVD can also be anomalous trichromatic (protanomaly) if the first and second genes specify M cone photopigments that differ in their wavelengths of peak sensitivity (Figure 2C). More rarely, red-green color vision deficiency is caused by inactivating mutations in one of the first two genes in the array. For recent reviews on common causes of inherited defective red-green color vision see references [17,32]. Exon 3-skipping *OPN1LW* and *OPN1MW* haplotypes have been identified as a cause of red-green color vision deficiency, as will be described in more detail below.

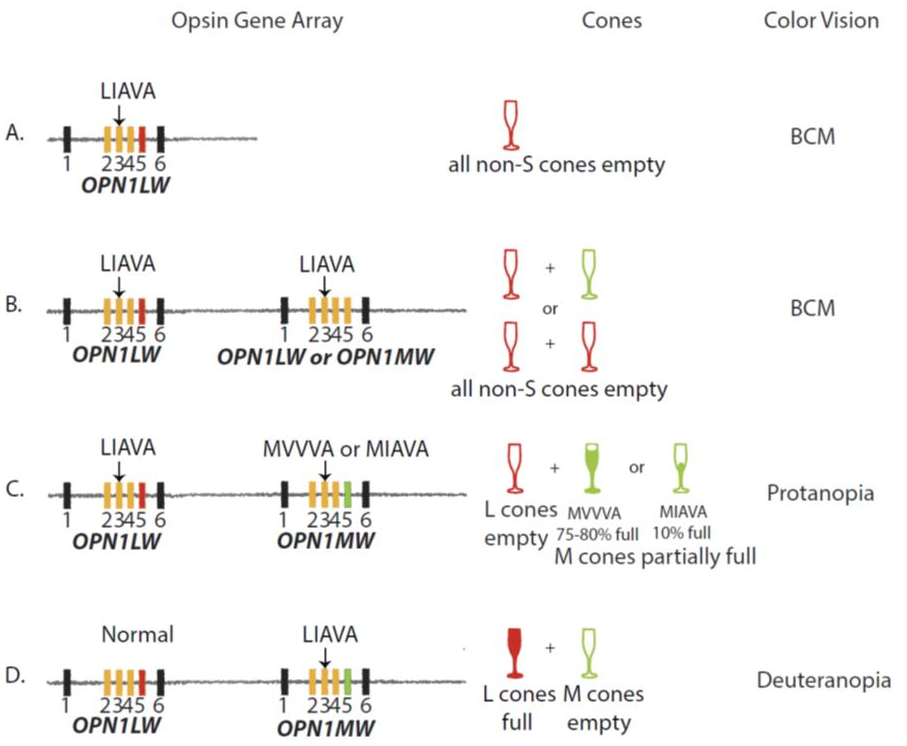

Figure 3. A. Arrays with a single *OPN1LW* gene (see Figure 2 caption for color coding of boxes representing exons) that specifies that *OPN1LW* genes makes only exon 3-skipped mRNA so all non-S cones are devoid of pigment, and a male with this array is an obligate BCM. B. Array in which first two genes have the LIAVA exon 3 haplotype will cause BCM regardless of whether the second gene encodes and *OPN1LW* or *OPN1MW* genes (indicated by yellow box for exon 5) because all cones will be devoid of photopigment. C. Arrays in which the *OPN1LW* genes has the LIAVA haplotype and the second gene has the MVVVA haplotype or the MIAVA haplotype both of which make some amount of full length mRNA will give rise to protanopia because only functional M cones and S cones will be made. D. Arrays in which the *OPN1LW* has a normal (non-exon 3 skipping) haplotype and the second gene is an *OPN1MW* gene that has the LIAVA haplotype will cause deuteranopia because only L cones and S cones will contain functional photopigment.

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4. The LIAVA haplotype and its consequences for vision

The LIAVA haplotype was initially identified in studies aimed at understanding the molecular genetics of inherited red-green color vision deficiencies [1,3,5], and blue cone monochromacy[27], thus the initial observations were in individuals with the color vision phenotypes recruited for the studies. The LIAVA haplotype was also identified in an early clinical study of individuals with X-linked cone dysfunction and protanopia, with moderate to high myopia, astigmatism, reduced cone function measured with the photopic electroretinogram, and moderately reduced visual acuity[33]. In this study, the LIAVA haplotype was not recognized as deleterious because it represents a combination of known polymorphisms that are individually benign. The subsequent discovery that the LIAVA haplotype causes complete exon 3 skipping provided an explanation for the color vision phenotype [1] and provided insight into the mechanism by which this and other exon 3 skipping haplotypes might cause nearsightedness.

4.1 The LIAVA haplotype and blue cone monochromacy

Males who have X-chromosome opsin gene arrays that comprise one and only one opsin gene, and the gene has the LIAVA haplotype or arrays in which both the first and second genes specify LIAVA will not have any L or M cones that contain functional photopigment (Figure 3A and 3B). Thus, genetically they are obligate blue cone monochromats. Key features of BCM caused by an LIAVA opsin gene haplotype expressed in all L/M cones include pathological myopia, very poor visual acuity, and a severely reduced or absent cone function measured using the photopic electroretinogram (ERG) (Table S1) [6,7,10].

The fovea is the center of the field of vision and is the location where cone photoreceptors are concentrated and where visual acuity is the highest. In conventional charts for measuring visual acuity, normal acuity corresponds to being able to resolve 1 minute of arc of visual angle, and the size of each letter is such that its strokes will subtend 1 minute of arc at a specified distance. In conventional notation, visual acuity is expressed as a fraction where the numerator indicates the viewing distance, and the denominator indicates the size of the letter. An observer who from 20 feet away can just recognize the line on the chart with letters having strokes of 1 minute of arc has a visual acuity of 20/20, an observer who requires letters twice that size has acuity of 20/40, and so forth. Under ideal conditions an observer with excellent vision can just resolve the fine detail for which the angular subtense approaches that of a single cone [34]. L- and M-cones mediate high-acuity achromatic spatial vision. Blue cone monochromacy is characterized by poor visual acuity because affected males lack functional L and M cones.

After birth, human eyes undergo a controlled axial elongation (termed emmetropization) that is governed by a feedback mechanism in which L-and M-cones play a critical role so that the eye stops growing when the length is optimally matched to the power of the optical components (lens and cornea) for high acuity vision. Nearsightedness (myopia) results if the eye grows too long. When emmetropization occurs normally, changes in the pattern of light and dark in the image that characterize blurred versus sharply focused images are monitored by the retina to control eye growth so that its adult length matches the focal length of its optics. Presumably, the high myopia associated with the LIAVA opsin genes can be explained by a disruption of the signals that guide emmetropization, which are initiated by light absorption in the photopigments expressed in the L and M cones.

4.2 The LIAVA haplotype and inherited red-green color vision deficiency

Males missing an opsin gene at either one of the two expressed positions are obligate dichromats. Males with an array in which the *OPN1LW* gene has the LIAVA haplotype

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and the *OPN1MW* gene has a haplotype that allows functional photopigment to be made will have deuteranopia because all L cones will be devoid of photopigment, and only S and M cones will contribute to vision (Figure 3C). Likewise, males with an array in which the OPNLW gene has a haplotype that allows functional pigment to be made and an *OPN1MW* gene that has the LIAVA haplotype will have protanopia (Figure 3D) because M cones will be devoid of photopigment, and S and L cones will contribute to color vision. Males with these array types whose clinical characteristics have been reported in the literature are listed in Table S2.

Results from color vision testing performed under typical clinical conditions are not reliable. Clinical color vision testing is often not performed under valid conditions or with appropriate tests, and the interpretation of the test results is often not performed by a knowledgeable person. In Table S2, color vision is listed as impaired if color vision testing was performed but did not distinguish between protan and deutan defects. In Table S2, members of the MOL0152 family were diagnosed with blue cone monochromacy in the absence of color vision testing. All three have a genotype consistent with protanopia, not blue cone monochromacy. Three other subjects received a clinical diagnosis of blue cone monochromacy (BCM160-23130, ZD314-18057, BCM51-12359) but all three have genotypes consistent with protanopia, not blue cone monochromacy, and color vision tests performed were not adequate to reliably detect BCM.

X-chromosome opsin gene arrays underlying the common forms of inherited red-green color vision deficiency are not associated with a reduction in the number of functional cones, just in the relative number of L versus M cones, thus individuals with inherited red-green CVD usually have normal acuity [3,35]. Even in the case were there is a single opsin gene on the X-chromosome, all non-S cones express the available X-chromosome opsin gene, and the total number of cones and density of cones does not differ from that observed for normal trichromats. However, deuteranopia and protanopia caused by the LIAVA haplotype are associated with a reduced number of functional cones [3], and there is tremendous variability in visual acuity (Table S1).

For males with arrays such as those illustrated in Figure 3C and 3D in which one of the first two genes has the LIAVA haplotype and the other has a "normal" haplotype, depending on the fraction of cones that are devoid of photopigment, visual acuity may be reduced to a greater or lesser extent. Variation in the ratio of L:M cones in males with normal color vision reflects the relative number of cones that express the first versus the second opsin gene in the X-chromosome array. Among males with normal color vision there is tremendous variability in the relative ratio of L to M cones, with a mean of 2L:1M cones for males of European ancestry [36,37], which corresponds to 67% of L and M cones being L cones. The mean for males of African or African American ancestry is 1 L for every 1 M cone, or about 50% L cones[38]. A corresponding variation in the relative number of cones expressing the first versus downstream genes in arrays such as those in Figure 3C and 3D, would correspond to variability in the fraction of empty vs functioning cones, and this likely contributes to variability in visual acuity [9]

High resolution adaptive optics imaging indicates that both the degree of myopia as indicated by axial length of the eye and as indicated by spherical equivalent refraction is greater when the larger fraction of cones expresses the LIAVA haplotype [9,13]. For example, two brothers (Table S2, JC_0195 and JC_0196) differ in the relative fraction of cones that are dysfunctional due to the lack of photopigment, and they differ dramatically in the degree of myopia with JC_0195 having a much larger fraction of cones expressing the LIAVA opsin gene and having much more severe nearsightedness. His visual acuity is also worse than his brother JC_0196. Also, subjects MM_0142 and MM_0145 both have a much smaller fraction of cones expressing the LIAVA haplotype compared to subjects JC_0609 and JC_0195. MM_0142 is only slightly myopic where MM_0145 is not myopic and both have much better visual acuity than JC_0609 and JC_0195 (Table

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S2). Adaptive optics imaging for subject JC_0084 suggests that about one-third of his cones express the LIAVA opsin gene, and he has normal visual acuity [3].

Results in Table S2 demonstrate that the LIAVA haplotype is often associated with reduced visual acuity, and such individuals often receive a clinical diagnosis of blue cone monochromacy, even in the absence of definitive color vision test results. Blue cone monochromacy is usually associated with decreased acuity. Thus, the decreased acuity in the individuals with array structures illustrated in Figure 3C or 3D may contribute to their misdiagnosis as BCM rather than red-green color vision deficient with reduced numbers of L/M cones.

5. The MIAVA haplotype

The MIAVA haplotype is an exon 3 skipping haplotype, but unlike LIAVA, a small amount of correctly spliced mRNA is made (Table 1). Patients with arrays containing an opsin gene with the MIAVA haplotype in one of the expressed positions have been seen in two scenarios (Table S3). In one scenario, both of the expressed positions contain genes with the MIAVA haplotype and both are *OPN1LW* genes. Because the cones expressing the MIAVA haplotype have a small amount of functional photopigment and will have the spectral sensitivity of L cones, the patients are expected to have red-green color vision deficiency (deutan, Table S3); however, color vision testing was not performed on the subjects. The two subjects that have been described in the literature are related, and were found to have pathological myopia and moderate to severely reduced cone function [7]. In the second scenario the first gene has the LIAVA haplotype, and the second gene has the MIAVA haplotype (Table S3). Some of these patients received clinical diagnoses of blue cone monochromacy in the absence of color vision testing and have poorer acuity than the two subjects with MIAVA in all non-S cones, likely due to the fraction of cones expressing the LIAVA opsin. The subjects with an *OPN1LW*$_{\text{LIAVA}}$ gene and an *OPN1MW*$_{\text{MIAVA}}$ gene have a genotype consistent with protanopia with decreased L/M cone numbers, not BCM. Likewise, the subjects with *OPN1LW*$_{\text{MIAVA}}$ genes in both expressed positions have a genotype consistent with deuteranopia with decreased L/M cone function, not BCM. Several of these subjects exhibit degenerative changes associated with myopia[39].

Subjects that have an opsin gene with the MIAVA haplotype in both of expressed positions have better visual acuity than subjects with LIAVA in the first gene and MIAVA in the second gene. This is likely due at least in part to all cones having some amount of functional photopigment in the former, versus a fraction cones being completely devoid of photopigment in the latter. All subjects in Table S3 were found to have absent or severely reduced cone electroretinograms, consistent with having cones that either completely lack or have a severely reduced amount of cone photopigment.

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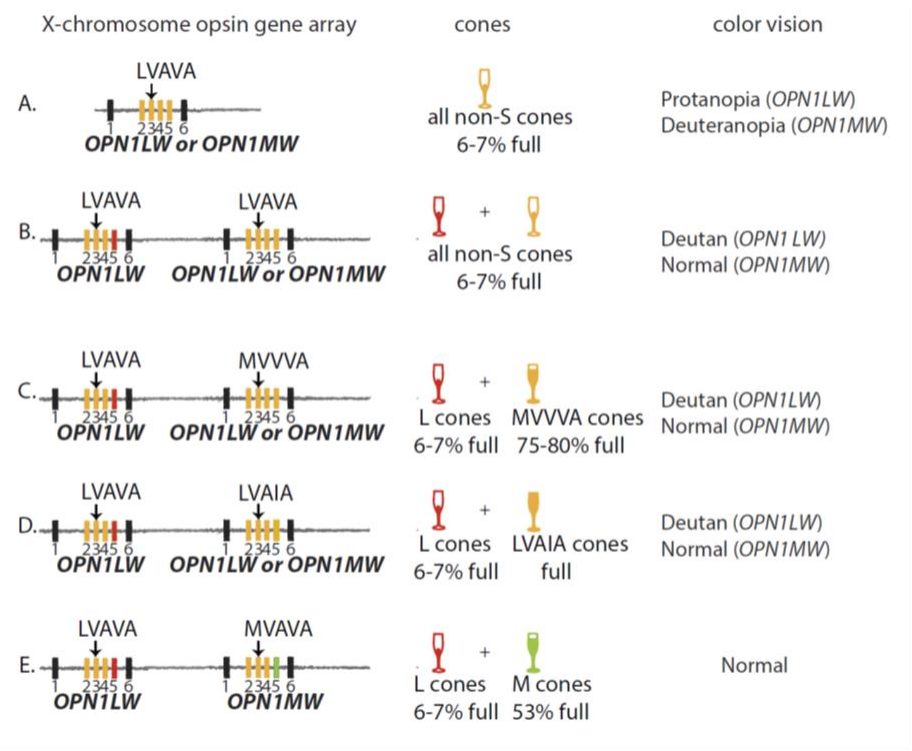

Figure 4. Opsin gene arrays with LVAVA haplotype and associated color vision. Boxes indicated exons, see Figure 2 caption for color coding. Yellow box for exon 5 indicates may encode *OPN1LW* or *OPN1MW*. **A.** Array with a single opsin gene that is either *OPN1LW*$_{\text{LVAVA}}$ or *OPN1MW*$_{\text{LVAVA}}$. Males with an array like this will be an obligate deuteranope (a person with deuteranopia) if the gene is *OPN1LW* or protanope (a person with protanopia) if the gene is *OPN1MW* because retinas will have functional S and L or S and M cones. **B.** Array in which both expressed positions have the LVAVA haplotype. Males with these arrays, have retinal mosaics in which all non-S cones have small amount of functional photopigment and color vision depends on whether second gene is L or M. **C.** and **D.** Array with *OPN1LW*$_{\text{LVAVA}}$ and the second gene having a relatively normally spliced haplotype (MVVVA in C or LVAIA in D). Males with this array will have CVD or normal color vision depending on whether second gene is L or M. **E.** Array with *OPN1LW*$_{\text{LVAVA}}$ and *OPN1MW*$_{\text{MVAVA}}$. Males with this array will have functional L and M cones, and thus normal color vision.

6. The LVAVA haplotype

The LVAVA haplotype was initially reported in an individual with red-green color vision deficiency [2], and it was present in subjects identified with Bornholm Eye Disease and X-linked cone dysfunction with high myopia [40] but was not recognized as deleterious because it represents a combination of individually benign, normal polymorphisms. Bornholm Eye Disease is characterized by red-green color vision deficiency, high myopia, and X-linked cone dysfunction [41].

6.1 LVAVA and red-green color vision deficiency

Unlike the LIAVA haplotype, the LVAVA haplotype is not expected to cause red-green color vision deficiency on its own because it produces a small amount of correctly spliced mRNA that in turn gives rise to functional photopigment [11]. The arrays illustrated in Figure 4A and 4B are expected to cause color vision deficiency either because there is a single opsin gene on the X-chromosome (Figure 4A) or because the first and

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second opsin genes in the array encode the same class of opsin, both L or both M. Because all non-S cones have a very small amount of functional photopigment, these arrays are expected to be associated with severely reduced cone function.

Table S4 lists subjects that have been reported in the literature who have array structures like those illustrated in Figure 4A, 4B and 4C. All individuals who have either a single *OPN1LW* gene that encodes the LVAVA variant, or the first two genes both encode L cone photopigment have genotypes consistent with deutan color vision deficiency. The defect can either be deuteranopia if all L cones have the same spectral sensitivity, or deuteranomalous if the two *OPN1LW* genes specify opsins that differ in peak sensitivity or optical density [42]. For example, the LVAVA and MVVVA photopigments (JC_0758 and JC_0683) have the same peak sensitivity but are expected to differ dramatically in optical density due to the greater extent of exon 3 skipping in the LVAVA variant compared to MVVVA. Individuals who behave as anomalous trichromats due to differences in optical density will behave as dichromats under bleaching conditions that equalize the optical densities. Thus, the color vision test results depend on the testing conditions.

Subjects with single-gene arrays that specify the LVAVA haplotype (Figure 4A, Table S4) and those with LVAVA haplotypes for both opsin genes in the expressed positions (S26 in Table S4) tend to have much worse visual acuities than subjects who have a fraction of cones expressing a haplotype such as MVVVA that produces a much greater amount of functional photopigment.

6.2 The LVAVA haplotype and normal red-green color vision

Subjects who have arrays with an *OPN1LW* gene followed by an *OPN1MW* gene where one specifies the LVAVA haplotype and the other specifies a more normally spliced variant (Figure 4C and 4D) or whose *OPN1LW* and *OPN1MW* genes both specify the LVAVA haplotype (Figure 4B) will have retinas that have functional L and M cones, albeit with reduced function for the LVAVA cones, and are expected to have normal trichromatic color vision. This was found to be the case for members of two Chinese families with high grade myopia (Table S5) [8]. Three subjects identified in reference [10] were identified as blue cone monochromats in the absence of appropriate color vision testing, likely due to their reduced visual acuity and reduced cone function. All subjects in Table S5 except the 1-year-old had pathological myopia, but this subject is expected to become pathologically myopic as axial elongation continues well beyond 1 year of age.

Subjects with an *OPN1LW*$_{\text{LVAVA}}$ and *OPN1MW* with the MVVVA or LVAIA haplotypes (Table S5) have much better visual acuity in general than subjects with single gene opsin arrays (Table S5, Figure 4A) or arrays in which all non-S cones express the LVAVA haplotype. The exception is people over the age of 40 (Table S5), which may be due to progressive degeneration associated with the LVAVA haplotype.

7. The MVAVA haplotype

Like the LVAVA haplotype, the MVAVA haplotype is not expected to cause red-green color vision deficiency on its own because about 50% of the mRNA is correctly spliced and would give rise to functional photopigment (Table 1). This haplotype has only been observed in *OPN1MW* genes in conjunction with *OPN1LW* genes that specify the LVAVA haplotype [12]. This genotype, *OPN1LW*$_{\text{LVAVA}}$ – *OPN1MW*$_{\text{MVAVA}}$, is expected to underly normal red-green color vision. Patients with this genotype have relatively preserved visual acuity (Table S6) but tend to have pathological myopia. The visual acuity and high myopia appear to be similar to what is observed in subjects in Table S4 and Table S5 with LVAVA and a more normally spliced opsin.

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8. The LIAVS haplotype

About 20% of the mRNA for the LIAVS haplotype correctly splices exon 3 and gives rise to functional photopigment. Thus, like the MIAVS, MVAVA, and LVAVA haplotypes, the LIAVS haplotype will not cause color vision deficiency on its own. Nonetheless, all individuals who have LIAVS have been reported to have X-chromosome opsin gene arrays with either a single *OPN1LW* gene with the LIAVS haplotype and no other opsin gene or have a second opsin gene that specifies the LIAVA haplotype (Table S7). All of these individuals are obligate deuteranopes because they have only S cones and L cones. Due to the reduced amount of functional photopigment in their L cones, they all have reduced cone function as measured by the photopic ERG [6,7]. Two of the three individuals with this haplotype who have been studied were myopic (Table S7), and one was reported as being hyperopic (BCM72-16874, Table S7). However, the hyperopic patient was 71 years of age, and it is unclear whether he had undergone cataract surgery in which case his hyperopia would be attributable to an intraocular lens implant. The LIAVS haplotype is associated with poor visual acuity, and in subject MOL0250 IV:3, visual acuity worsened with age.

9. Summary and Conclusions

Exon 3 skipping haplotypes LIAVA, LVAVA, MIAVA, LIAVS are expected to give rise to cone dysfunction because of drastically reduced amount or absence of functional photopigment. As can be seen in Tables S1-S8, myopia due to excessive axial elongation is associated with these haplotypes that give rise to cones with reduced photopigment amounts. People with axial myopia are at risk for retinal detachment and myopic maculopathy, both of which are degenerative and would further reduce cone function. The risk for these degenerative conditions increases with increasing myopia [39]. Cone function may be further reduced as a consequence of degenerative changes. Although the retinal and vision phenotypes among patients with these exon 3 skipping haplotypes are often reported to be "non-progressive" the time frame over which the phenotypes have been monitored are relatively short term. In patients with longer follow up, there is evidence of disease progression. Thus, progression appears to be relatively slow below the age of 40.

How do exon 3 skipping haplotypes cause severe myopia? We have argued that retinal cone mosaics where a fraction of the L/M cones have a dramatically reduced amount of photopigment compared to the other cones, as would be the case, for example for people who have an *OPN1LW*$_{LVAVA}$ and *OPN1MW*$_{MVVVA}$, signal the presence of constitutive contrast and that this is the stimulus for axial elongation of the eye. Because the contrast signal generated is due to differences in the amount of photopigment in adjacent cones and is independent of the image, this interferes with the emmetropization process leading to very high degrees of myopia [11]. This "contrast hypothesis" as a cause of myopia led to the development of spectacle lenses designed to reduce contrast and the spectacles are currently being evaluated in a multisite clinical trial (NCT03623074). Based on 12-month data [43], the spectacles have been given regulatory approval and are authorized for sale as "myopia control" medical devices in the European Union and Canada.

Females have two X-chromosomes, and it would be exceptionally rare for a female to have exon 3 skipping haplotypes in opsin genes on both X-chromosomes. Thus, female carriers of exon 3 skipping *OPN1LW* and *OPN1MW* haplotypes are protected from the severe myopia, reduced visual acuity, and reduced cone function due to the presence of a second X-chromosome; however, carriers have not been studied extensively.

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