North Carolina Macular Dystrophy Is Caused by Dysregulation of the Retinal Transcription Factor PRDM13

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Purpose: To identify specific mutations causing North Carolina macular dystrophy (NCMD).

Design: Whole-genome sequencing coupled with reverse transcription polymerase chain reaction (RT-PCR) analysis of gene expression in human retinal cells.

Participants: A total of 141 members of 12 families with NCMD and 261 unrelated control individuals.

Methods: Genome sequencing was performed on 8 affected individuals from 3 families affected with chromosome 6-linked NCMD (MCDR1) and 2 individuals affected with chromosome 5-linked NCMD (MCDR3). Variants observed in the MCDR1 locus with frequencies <1% in published databases were confirmed using Sanger sequencing. Confirmed variants absent from all published databases were sought in 8 additional MCDR1 families and 261 controls. The RT-PCR analysis of selected genes was performed in stem cell-derived human retinal cells.

Main Outcome Measures: Co-segregation of rare genetic variants with disease phenotype.

Results: Five sequenced individuals with MCDR1-linked NCMD shared a haplotype of 14 rare variants spanning 1 Mb of the disease-causing allele. One of these variants (V1) was absent from all published databases and all 261 controls, but was found in 5 additional NCMD kindreds. This variant lies in a DNase 1 hypersensitivity site (DHS) upstream of both the PRDM13 and CCNC genes. Sanger sequencing of 1 kb centered on V1 was performed in the remaining 4 NCMD probands, and 2 additional novel single nucleotide variants (V2 in 3 families and V3 in 1 family) were identified in the DHS within 134 bp of the location of V1. A complete duplication of the PRDM13 gene was also discovered in a single family (V4). The RT-PCR analysis of PRDM13 expression in developing retinal cells revealed marked developmental regulation. Next-generation sequencing of 2 individuals with MCDR3-linked NCMD revealed a 900-kb duplication that included the entire IRX1 gene (V5). The 5 mutations V1 to V5 segregated perfectly in the 102 affected and 39 unaffected members of the 12 NCMD families.

Conclusions: We identified 5 rare mutations, each capable of arresting human macular development. Four of these strongly implicate the involvement of PRDM13 in macular development, whereas the pathophysiologic mechanism of the fifth remains unknown but may involve the developmental dysregulation of IRX1. Ophthalmology 2015;1–10 © 2015 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Supplemental material is available at www.aaojournal.org.

Few tissues in the human body are as important to the well-being of a person as the central 3 mm of the human retina. The ability to drive a car, recognize friends in public, and see words on a computer, cell phone or printed page are just a few of the many activities of daily living that depend heavily on the normal function of the macula.

For all but a few people, the macula functions very well for the first 6 or 7 decades of life, but in older individuals, the macula is prone to a genetically and mechanistically diverse group of disorders that are known collectively as age-related macular degeneration (AMD). For many years, the neovascular complications of AMD were the most common cause of irreversible blindness in developed countries. However, the recent advent of anti–vascular endothelial growth factor drugs has dramatically reduced vision loss from neovascularization, thereby increasing the fraction of blindness caused by geographic atrophy of the macula.

There are at least 2 approaches that one could envision for reducing the burden of blindness caused by geographic atrophy of the macula. The first would be to understand the pathophysiologic mechanisms of AMD in sufficient detail that one could detect the disease at a very early stage, perhaps even as an...
asymptomatic genetic predisposition, and deliver a safe and effective preventive therapy to those at risk, much as statins are now used to reduce the risk of heart disease. Another strategy would be to rebuild an injured macula with new stem cell—derived retinal cells. Molecular genetics will play an important role in both of these approaches.

In the 1990s, scientists sought the genetic causes of several Mendelian forms of human macular disease for at least 2 reasons. First, it was possible that mild mutations in the genes responsible for these early-onset conditions might prove to be responsible for a significant subset of the age-related forms of the disease. Second, it was thought that by discovering how relatively minor alterations of individual genes could cause clinical findings similar to AMD, one would gain valuable insight into the normal function of the macula. Twenty years later, it is clear that none of the genes that cause the classic Mendelian macular dystrophies cause a significant fraction of the late-onset disease, and none of the genes that have been shown to predispose people to typical AMD cause any meaningful fraction of early-onset Mendelian macular disease.

The first of the classic macular dystrophies to have its gene mapped to a chromosome, North Carolina macular dystrophy (NCMD), is the last to have its specific disease-causing mutations identified. The reason for this delay—the unusual developmental mechanism of this disease—may ultimately make NCMD the most relevant of the Mendelian macular dystrophies to the treatment of AMD. North Carolina macular dystrophy first described in a large kindred from North Carolina by Lefler et al and later described in more detail by Frank et al. The cross-sectional nature of these studies led the investigators to believe that the disease was slowly progressive. However, Small reexamined the original Lefler kindred approximately 20 years later and realized that NCMD is in fact a nonprogressive developmental disorder with widely variable expressivity. In the decades since the MCDR1 locus was mapped, many additional families with NCMD have been described, including 2 families that link to a separate locus on chromosome 5 (MCDR3). The critical region on chromosome 6 has been considerably narrowed, and all of the coding regions of genes within this interval have been exhaustively studied by us and other investigators. The failure of these experiments to identify plausible disease-causing mutations in any of these kindreds suggested that the mutations were likely to exist in nonexonic DNA and to affect the expression of a nearby gene or genes rather than the structure of its gene product. The purpose of this study was to take advantage of recent advances in whole-genome sequencing to comprehensively screen the nonexonic sequences within the MCDR1 and MCDR3 loci to identify disease-causing mutations in families affected with these diseases.

Methods

Human Subjects

All subjects provided written informed consent for this research study, which was approved by the institutional review board of the University of Iowa and adhered to the tenets set forth in the Declaration of Helsinki. Blood samples were obtained from all subjects, and DNA was extracted using a nonorganic protocol, as previously described.

Next-Generation Sequencing of MCDR1 Patients

A targeted genome capture of the linked region was performed on 3 members of family A (2 affected and 1 unaffected), 2 members of family K, and 1 member of family B. Libraries prepared from these captures were sequenced on an Illumina HiSeq. In addition, whole genomes were obtained from 5 affected individuals: 2 from family A, 1 from family K, and 2 from family L. These libraries were sequenced on an Illumina HiSeqX. All of these individuals are noted in blue in Supplemental Figure 1 (available at www.aaojournal.org).

Bioinformatic Analysis of Next-Generation Sequencing Data

Sequences were analyzed as described previously. Briefly, sequences were aligned to the reference genome using BWA-mem, and single nucleotide variants and small insertions/deletions were identified using a GATK-based pipeline. Variants mapping outside the MCDR1-linked region and those found at a frequency of 1% or greater in public databases were removed. Variants were then filtered, requiring that all affected individuals with a given haplotype share the heterozygous variant; all other individuals did not share the variant.

Copy number variants were investigated using Pindel and manual inspection of the aligned sequence data using the Integrative Genome Viewer. As a control, the identified genes were screened for copy number variants using Conifer in an internal database of 953 whole exomes of patients with eye disease.

Confirmation of Whole-Genome Sequencing

Variants identified by whole-genome sequencing were confirmed using automated bidirectional DNA sequencing with dye termination chemistry on an ABI 3730 sequencer (ThermoFisher Scientific, Foster City, CA).

Screening of Control Subjects

A total of 261 normal control subjects were screened for the presence of V1 to V3 (Table 1) using unidirectional automated DNA sequencing. To evaluate these controls for the presence of V4 and V5 (Table 1), oligonucleotide primers were designed to amplify across the novel junctions created by these tandem duplications (Supplemental Table 1, available at www.aaojournal.org), and the products of these amplifications were evaluated by electrophoresis on 6% nondenaturing polyacrylamide gels followed by silver staining, as previously described.

Induced Pluripotent Stem Cell Generation and 3-Dimensional Differentiation

Human dermal fibroblasts were isolated from skin biopsies obtained from normal individuals after informed consent. Cultured fibroblasts were reprogrammed via viral transduction of the transcription factors OCT4, SOX2, KLF4, and c-MYC, as previously described. Human induced pluripotent stem cells (iPSCs) were maintained in Essential 8 media (Life Technologies, Carlsbad, CA) on Laminin 521−coated plates (Corning Life Sciences, Tewksbury, MA). To initiate differentiation, iPSCs were removed from the culture substrate via incubation with TrypLE Express Enzyme (Life Technologies) dissociated into a single cell suspension and subsequently differentiated via the 3-dimensional (3D) differentiation protocol previously published by Eiraku et al.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from normal human iPSCs isolated at 0, 30, 60, and 100 days after differentiation using the
RNAse Mini-kit (Qiagen, Germantown, MD), per the manufacturer’s instructions, and 100 ng of RNA was amplified via SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Life Technologies) using the gene-specific primers described in Supplemental Table 1 (available at www.aaojournal.org).

Immunocytochemistry of 3-Dimensional Induced Pluripotent Stem Cell–Derived Eyecups. Three-dimensional iPSC-derived eyecups were embedded in 4% agarose, sectioned at a thickness of 100 μm using a Leica VT1000 S vibratome (Leica Microsystems, Wetzlar, Germany) and labeled with primary antibodies targeted against mouse anti-SOX2 (#MAB2018; 1:1000; R&D Systems, Minneapolis, MN), rabbit anti-biotinylated-OTX2 (#BAF1979; 1:500; R&D Systems), mouse anti-HuC/D (#A-21271; 1:500; Thermo Fisher Scientific, Waltham, MA), and rabbit anti-recoverin (#AB5585; 1:2000; EMD Millipore, Billerica, MA). To detect F-actin, eyecups were stained with Alexa Fluor 488 Phalloidin (#A12379; 1:500; Life Technologies). Cell nuclei were counterstained using 4,6-diamidino-2-phenylindole. Sectioned eyecups were imaged using a Leica DM 2500 SPE confocal microscope (Leica Microsystems, Wetzlar, Germany).

### Results

Twelve families manifesting the clinical features of NCMD were studied, all but 1 of whom have been described elsewhere. Six of these families share a haplotype of short tandem-repeat polymorphisms in the MCDR1 locus on chromosome 6, suggestive of a common founder, whereas 5 others have been linked to MCDR1 but exhibit a different marker haplotype. The remaining family has been linked to the MCDR3 locus on chromosome 5. DNA samples from 102 affected and 39 unaffected members of these families were available for this study. The family structures and specific individuals included in this study are shown in Supplemental Figure 1 (available at www.aaojournal.org).

Subject 7043 in family A has been observed by us for more than 30 years and is an excellent example of the cardinal clinical features of NCMD. She was first seen at 2 years and 9 months of age and displayed a visual acuity of 20/40 in the right eye and 20/60 in the left eye with line pictures. Fundus examination revealed small areas of atrophy surrounded by drusen-like deposits in both eyes. A prism cover test revealed unmaintained fixation in the left eye, and a trial of part-time occlusion was begun. Two months later, her vision had improved to 20/40 in both eyes, and patching was discontinued. At age 6 years, her acuity had decreased slightly to 20/50 in both eyes. Two small red dots suggestive of hemorrhage were observed on the nasal edge of the atrophy in the left eye (Fig 1A, B), but fluorescein angiography revealed no evidence of active neovascularization at that visit (Fig 1C, D and Supplemental Fig 2, available at www.aaojournal.org). At age 8 years, her acuity remained 20/50 in both eyes and a new subretinal fibrotic scar was noted in the left eye, extending from 6 o’clock to 7 o’clock around the central patch of atrophy (Fig 1E). Two years later, the acuity and fundus appearance were unchanged (Fig 1F), but the following year, at age 11 years, the scar in the left eye had extended another 3 clock hours (Fig 1G) with little change in acuity (20/50-2). When last seen at age 33 years, her visual acuity was 20/60-1 in the right eye and 20/70-1 in the left eye. The fundus appearance (Fig 1H) was similar to that at her visit 22 years earlier. Optical coherence tomography of the left eye revealed an abrupt termination of the photoreceptors, retinal pigment epithelium, and choroid at the 1 edge of the atrophic lesion that was not distorted by the fibrotic scar (Fig 1I, J).

The original linkage of the NCMD phenotype to chromosome 6q11 and the subsequent narrowing of the MCDR1 interval depended heavily on families A and J. Detailed genotyping of additional members of these families revealed an unaffected recombinant individual (Supplemental Fig 1K, available at www.aaojournal.org) that narrowed the centromeric end of the interval to the genetic marker D6S1717 (Fig 2). A genomic fragment capture of the narrowed disease interval and next-generation sequencing were then performed in 1 unaffected and 2 affected members of family A. However, only 85% of the nucleotides in the disease interval were successfully sequenced in this experiment, and therefore 2 additional
affected members of the same family were subjected to whole-genome sequencing. Analysis of the sequence data from these 4 affected individuals (noted in blue in Supplemental Fig 1A, available at www.aaojournal.org) revealed a haplotype of 14 rare variants that spanned 1 Mb of the disease-causing allele (Fig 2). One of these variants (V1; Table 1) was absent from all published databases and 261 normal controls, but was found in all affected members of 5 of 10 additional NCMD kindreds (families B–F; Supplemental Fig 1, available at www.aaojournal.org) that were known or suspected to map to MCDR1. This variant lies in a DNase 1 hypersensitivity site (DHS) upstream of both the PRDM13 and CCNC genes (Fig 2). Sanger sequencing of 1000 base pairs (bp) centered on V1 was performed in the probands of the remaining 5 NCMD families, and 2 additional novel single nucleotide variants (V2 in families G–I and V3 in family J; Table 1; Supplemental Fig 1, available at www.aaojournal.org) were identified within 134 bp of the location of V1 (Fig 2). Whole-genome sequencing of an affected individual from the remaining MCDR1 family (family K; Supplemental Fig 1, available at www.aaojournal.org) was performed, and a 123-kb tandem duplication (V4; Table 1) containing the entire coding sequence of PRDM13 was identified (Fig 2 and Supplemental Fig 3A, available at www.aaojournal.org). Collectively, V1 to V4 were present in 91 of 91 affected members of these 11 families, absent from 38 of 38 unaffected members, and absent from 261 unrelated control individuals (522 chromosomes). In addition, a review of the Database of Genome Variants revealed no instances of duplication of the entire PRDM13 coding sequence in normal individuals.

To determine whether PRDM13 and CCNC are expressed during retinal development, iPSCs were used to generate retinal tissue via 3D differentiation. After 30 days of differentiation (D30), 3D iPSC-derived eyecup-like structures are polarized with highly organized filamentous actin (F-actin) networks composed of actively proliferating Ki67-positive cells (Fig 3A). At this stage of development, 3D eyecups predominantly contain cells that express early retinal-specific markers: SOX2, PAX6, and OTX2 (Fig 3B). PAX6, a master regulator of retinal development, is expressed throughout the eyecup and helps to drive the expression of the photoreceptor precursor cell-specific transcription factor OTX2. PAX6 and OTX2 are co-expressed in most cells at this stage of development (Fig 3B). After 60 days of differentiation, PAX6 expression becomes restricted to presumptive retinal pigment epithelium cells, and pockets of presumptive photoreceptor cells that express OTX2 independent of PAX6 arise (Fig 3C). After 100 days of differentiation, 3D eyecups are laminated with an inner layer containing retinal neurons that express the ganglion cell-specific marker HuC/D and an outer layer containing photoreceptor cells that robustly express the phototransduction protein recoverin (Fig 3D). Analysis of RNA isolated from iPSCs at 0, 30, 60, and 100 days after differentiation revealed that expression of PRDM13 is negatively correlated with retinal development (Fig 4). Specifically, as cells progress from a pluripotent stem cell state to mature retinal neurons, PRDM13 transcript is downregulated. Of note, CCNC is consistently expressed across all developmental time points (Fig 4).

In 2010, Rosenberg et al mapped the disease-causing mutation of a Danish kindred (family L; Supplemental Fig 1, available at www.aaojournal.org) with an NCMD phenotype to an 8-cM interval on chromosome 5 (MCDR3) (Fig 5A) that had been...
Previously identified by Michaelides et al., we performed whole-genome sequencing of 2 affected individuals in this family and identified a 900-kb tandem duplication (V5) (Table 1) that included the entire coding sequence of IRX1 (Fig 5A and Supplemental Fig 3B, available at www.aaojournal.org). This duplication was present in all 11 affected members of the family, absent from 1 unaffected member, absent from the Database of Genome Variants, and absent from 1 unaffected member, absent from the Database of Genomic Variants, and absent from 261 unrelated controls. This novel variant falls within a DNase hypersensitivity site (pink) upstream of the PRDM13 gene (green) that was later found to include other rare variants in NCMD families (V2 and V3). In addition, a 123-kb tandem duplication containing the PRDM13 gene (yellow, V4) was discovered in 1 NCMD family. bp = base pairs.

![Figure 2. Discovery of North Carolina macular dystrophy (NCMD)—causing variants in MCDR1. The critical region of MCDR1 was narrowed to 883 kb by a single, unaffected recombinant individual (recombinant denoted by asterisks here and in Supplemental Fig 1A, asterisk, available at www.aaojournal.org). Genome sequencing revealed 14 rare variants (violet vertical bars) across this region, 1 of which (V1) has never been observed in normal individuals. This novel variant falls within a DNase hypersensitivity site (pink) upstream of the PRDM13 gene (green) that was later found to include other rare variants in NCMD families (V2 and V3). In addition, a 123-kb tandem duplication containing the PRDM13 gene (yellow, V4) was discovered in 1 NCMD family. bp = base pairs.](image-url)

Discussion

The technologic advancements that have occurred in the field of human genomics since the NCMD locus on chromosome 6 was first identified have been breathtaking. Few investigators who studied inherited eye diseases in the 1990s would have imagined that in less than 25 years, whole-genome sequencing of individual patients would be commonplace that the sequence of thousands of unrelated individuals would be freely available in public databases and that the President of the United States would launch a precision medicine initiative based on these new molecular capabilities and data. However, the most valuable data in both the original linkage study and the present study were not molecular; the most valuable data were the detailed clinical observations that allowed several families with a rare and unusual phenotype to be correctly separated from thousands of other members of hundreds of other families with similar diseases caused by genes at other loci.

Although counterintuitive to many people, it is a fact that as genomic tools become more powerful and less expensive, accurate and detailed clinical information become more necessary for the correct interpretation of the resulting molecular data. There are both quantitative and qualitative reasons for this. Now that tens of thousands of genes can be assessed in a single patient, there are tens of thousands of additional opportunities to observe a plausible disease-causing variant by chance than if one investigated only a single gene. By using clinical data to focus the hypothesis to just a few genes, one can overcome the large multiple measurements problem inherent in whole-genome data.

The qualitative reason that molecular data have become more difficult to interpret as they have become easier and less expensive to acquire is embodied in the difference between the coding and noncoding portions of genes. Coding sequences exist in groups of 3 nucleotides, known as codons, that each specify a single amino acid in the resulting proteins. The universality of the genetic code allows one to predict the structural effect of a given coding sequence mutation on the resulting protein with greater accuracy than one could if the same mutation occurred in the noncoding portion of a gene, where its effect would be tempered by the actions of DNA binding proteins, DNA methylation, noncoding RNA molecules, the proximity to coding sequences, and other factors that are incompletely understood at the present time.

There are 10 genes in the MCDR1 locus, and individuals from multiple unrelated kindreds affected with MCDR1-linked NCMD have been extensively screened for mutations in the coding sequences of these genes, with no plausible disease-causing variants identified. Therefore, we expected that NCMD-causing mutations would eventually be found in the noncoding portions of the MCDR1 locus, and we took advantage of 2 valuable resources and 1 genetic fact to detect these mutations among the many functionally neutral polymorphisms that exist in the noncoding sequences of all individuals: (1) multiple unrelated families exhibiting a classic NCMD phenotype, (2) public genome databases with sequences of thousands of individuals, and (3) the fact that mutations that cause high-penetration autosomal-dominant diseases should be no more common in the general population than the disease itself.

The data supporting the pathogenicity of V1-V4 are compelling. In family A, the original NCMD family and the largest one ascertained to date, V1 is the only nucleotide in the 883-kb MCDR1 locus that is absent from all public databases and therefore of similar population frequency to NCMD itself. It is noteworthy that PRDM13 is the only gene in the MCDR1 critical region that is solely expressed in the neural retina. DNase I hypersensitivity is an indicator of development (Fig 5B).
of chromatin accessibility that is often associated with transcription factor binding sites. V1 was later found in 5 independently ascertained NCMD kindreds, shown to segregate perfectly among 65 affected and 29 unaffected members of these 6 families, and shown to be absent from 261 unrelated individuals ascertained in Iowa. The latter individuals were sequenced just to make sure that there was not an artifactual gap in the public genome data. Conventional sequencing of this DHS in 5 V1-negative NCMD families revealed that 4 harbored point

Figure 3. Using normal human induced pluripotent stem cells (iPSCs) to model retinal development. A–D. Immunocytochemical analysis of iPSC-derived eyecup-like structures targeted against F-actin, SOX2, PAX6, OTX2, HuC/D, and recoverin (RCVRN). After 30 days of differentiation (D30), polarized neural epithelia (A, F-Actin in green) composed of proliferating cells (A, Ki67 in red) that are positive for the early retinal progenitor cell markers SOX2 (B, green), PAX6 (B, red), and OTX2 (B, white) are present. After 60 days of differentiation (D60), PAX6 (C, red) expression is restricted to OTX2-negative presumptive retinal pigment epithelium, whereas OTX2 (C, white) is restricted to PAX6-negative photoreceptor precursor cells. After 100 days of differentiation (D100), eyecups are laminated with HuC/D-positive (D, green) ganglion cell–like neurons in the inner layer and recoverin-positive (D, red) photoreceptor precursor cells in the outer layer. Insets depict individual fluorescent channels. A and C, 20× magnification. B and D, 40× magnification. DAPI = 4’6-diamidino-2-phenylindole.
mutations (V2 and V3) within 134 bp of V1. Whole-genome sequencing of the fifth V1-negative family revealed a tandem duplication containing the DHS and the entire coding sequence of PRDM13 (V4). V2 to V4 were found to segregate perfectly among the 26 affected and 9 unaffected members of these 5 families and were absent from all public databases and the 261 control individuals from Iowa. Although the association between these variants and the disease phenotype is extraordinarily strong ($P < 10^{-20}$, by Fisher exact test), the mechanism by which they cause disease is far from established. For example, the gene CCNC, which encodes a ubiquitous cell cycle controller, lies in the opposite orientation of PRDM13 on the opposite side of the DHS and thus could, in principle, also be affected by these mutations and therefore be involved in the pathogenesis of NCMD. One argument against CNCC as an NCMD gene, in addition to its ubiquitous expression, is the configuration of the DHSs in the tandem duplication of family K. The entire coding region of PRDM13 is duplicated in this mutation, and both DHSs are immediately adjacent to a PRDM13 gene. In contrast, only 1 of the DHSs is adjacent to the unduplicated CCNC gene (Supplemental Fig 4, available at www.aaojournal.org).

The observation that NCMD is a developmental abnormality is also consistent with PRDM13 being the responsible gene. PRDM13 is a member of a large family of “helix-loop-helix” DNA-binding proteins that play key roles in controlling gene expression during development.31 Because the formation of the macula is accompanied by differential expression of an array of genes involved in axon guidance and inhibition of angiogenesis,32 this process likely relies on a precise interaction between transcription factors (like PRDM13) and their target genes. Thus, a change in the abundance of a transcription factor due to mutations in its own regulatory regions could plausibly lead to impaired cell fate specifications in the developing macula. Therefore, it is notable that both PRDM13 and IRX1 are proteins with important roles in regulating gene expression.

One of the great advantages of iPSCs is their ability to differentiate ex vivo into any cell type of the 3 embryonic germ layers. For many organ systems, iPSC differentiation faithfully recapitulates the various cell fate decisions made during embryonic development.33–36 Being able to obtain embryonic tissue from adult somatic cells affords researchers with the ability to determine if and when in cellular development specific genes are expressed. In this study, human iPSC-derived retinal tissue was used to demonstrate that PRDM13 is developmentally regulated while other genes in the MCDR1 locus (i.e., CCNC) are not. To demonstrate this finding in the absence of the pluripotent stem cell technology, one would have to obtain retinal tissue from human fetuses at different points in development, an approach that would be logistically difficult and raise serious ethical concerns. The capability of iPSCs to generate otherwise inaccessible tissues such as the retina also gives researchers the ability to investigate the pathophysiology of newly identified gene defects on cell health and function. This will be especially useful in the
modern gene-sequencing era when trying to determine the mechanistic effects of noncoding genetic variants such as those identified in this study. In future studies, it will be interesting to generate retinal tissue from patients with each of the mutations described in this study and to determine their effect on gene expression, as well as cellular differentiation, maturation, health, and function.

The tandem duplication in the MCDR3 locus is very likely to be the disease-causing mutation in family L simply because it is very unlikely that the largest duplication involving IRX1 currently known to exist among the thousands of currently available human genome sequences would occur by chance in the very small portion of the genome that has been implicated in the disease. However, unlike MCDR1, no additional mutations have been identified in different MCDR3 families to corroborate this finding and to narrow the mechanistic possibilities. Also unlike MCDR1, where PRDM13 exhibits dramatic expression differences in the first 100 days of retinal development, IRX1 is constitutively expressed in normal individuals. Perhaps the large duplication alters the evolutionarily conserved chromosome conformation of the IRXA gene cluster. Future experiments with retinal cells generated from patients with NCMD themselves will likely significantly clarify the mechanism of both MCDR loci.

A practical outcome of this work is that one can detect every mutation reported in this article using only 3 polymerase chain reaction—based sequencing reactions (Supplemental Table 1, available at www.aaojournal.org). The availability of a simple genetic test for this disease will likely result in the diagnosis of many additional individuals, which not only will allow physicians to provide more accurate genetic and prognostic information than was possible before but also will likely accelerate the discovery of additional disease-causing variants and additional clinical manifestations of the known mutations. Both of these will help unravel the precise mechanisms through which these loci contribute to the formation of the normal macula.

In conclusion, we identified 5 rare mutations that each are capable of arresting the development of the human macula. Four of these strongly implicate the involvement of the gene PRDM13 in macular development, whereas the pathophysiologic mechanism of the fifth remains unknown but may involve the developmental dysregulation of IRX1.

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Abbreviations and Acronyms:
AMD = age-related macular degeneration; bp = base pairs; DHS = DNase I hypersensitivity site; iPSC = induced pluripotent stem cell; NCMD = North Carolina macular dystrophy; 3D = 3-dimensional.

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