REPORT

Biallelic Mutations in the Autophagy Regulator *DRAM2* Cause Retinal Dystrophy with Early Macular Involvement

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Retinal dystrophies are an overlapping group of genetically heterogeneous conditions resulting from mutations in more than 250 genes. Here we describe five families affected by an adult-onset retinal dystrophy with early macular involvement and associated central visual loss in the third or fourth decade of life. Affected individuals were found to harbor disease-causing variants in *DRAM2* (DNA-damage regulated autophagy modulator protein 2). Homozygosity mapping and exome sequencing in a large, consanguineous British family of Pakistani origin revealed a homozygous frameshift variant (c.140delG [p.Gly47Valfs*3]) in nine affected family members. Sanger sequencing of *DRAM2* in 322 unrelated probands with retinal dystrophy revealed one European subject with compound heterozygous *DRAM2* changes (c.494G>A [p.Trp165*] and c.131G>A [p.Ser44Asn]). Inspection of previously generated exome sequencing data in unsolved retinal dystrophy cases identified a homozygous variant in an individual of Indian origin (c.64_66del [p.Ala22del]). Independently, a gene-based case-control association study was conducted via an exome sequencing dataset of 18 phenotypically similar case subjects and 1,917 control subjects. Using a recessive model and a binomial test for rare, presumed biallelic, variants, we found *DRAM2* to be the most statistically enriched gene; one subject was a homozygote (c.362A>T [p.His121Leu]) and another a compound heterozygote (c.79T>C [p.Tyr27His] and c.217_225del [p.Val73_Tyr75del]). *DRAM2* encodes a transmembrane lysosomal protein thought to play a role in the initiation of autophagy. Immunohistochemical analysis showed DRAM2 localization to photoreceptor inner segments and to the apical surface of retinal pigment epithelial cells where it might be involved in the process of photoreceptor renewal and recycling to preserve visual function.

Retinal dystrophies are a clinically and genetically heterogeneous group of disorders characterized by progressive photoreceptor degeneration.[1] The pattern of visual loss and retinal appearance varies and is related to the degree to which cone and rod photoreceptors are affected. In subjects with retinitis pigmentosa (RP), for example, the rods are affected more severely and earlier than the cones, and the presenting symptoms are typically night blindness and/or visual field loss. Disorders in which the cones are more severely affected than the rods include macular dystrophies (MDs; localized loss of central/macular cones as a primary or secondary event) and cone-rod dystrophies (CRDs; central and peripheral cone involvement). MDs and CRDs show clinical overlap and loss of central vision is often the common presenting symptom. Frequently, subjects with CRDs also report light sensitivity, a symptom that can suggest generalized cone system dysfunction. Assigning a disease category can sometimes be challenging, with confounding factors being inter- and intra-familial phenotypic variability and the presence of age-dependent phenotypic transitions. RP, MDs, and CRDs can be transmitted in a dominant, recessive, or X-linked manner and, to date, variants in 70, 14, and 30 genes, respectively, have been shown to give rise to these conditions (RetNet, accessed February 2015).

The initial aim of this study was to identify the genetic basis of an adult-onset retinal dystrophy with early macular involvement (Figure 1) in a consanguineous Pakistani family with multiple affected members living in the UK (family ES1; Figure 2). Affected individuals became symptomatic early in the third decade of life, describing increasing difficulty with close visual tasks. Neither light sensitivity nor night blindness were significant early symptoms. There was progressive loss of visual acuity in all symptomatic individuals; light sensitivity and difficulty seeing in dim illumination were inconsistent features of advanced disease. Fundus examination revealed maculopathy in all symptomatic individuals tested, with peripheral retinal degeneration being a frequent finding in older subjects. Notably, optical coherence tomography (OCT)

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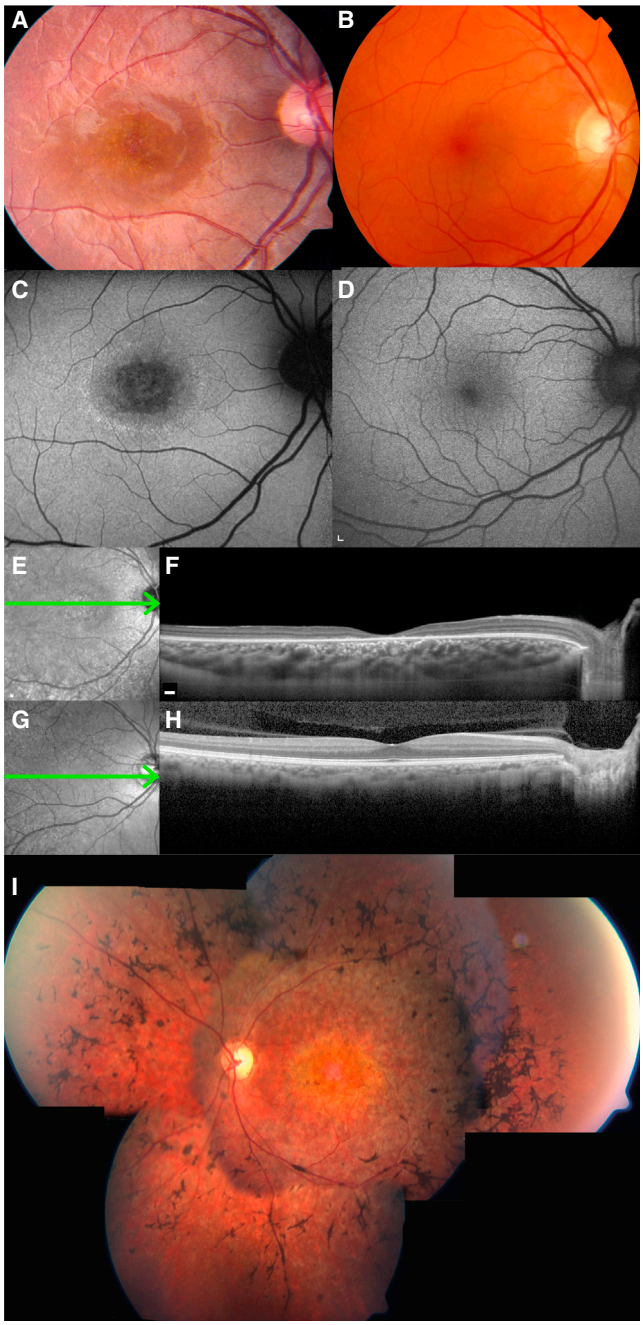

Figure 1. Clinical Features of Individuals from Family ES1 with Retinal Dystrophy and Early Maculopathy Caused by Recessive *DRAM2* Mutations

Color fundus photograph (A), fundus autofluorescence (C), infra-red reflectance (E), and OCT (F) images from the right eye of subject IV.9 at 25 years. Corresponding images from an unaffected individual are provided for comparison (B, D, G, and H). Macular atrophy with white dots at its temporal edge are observed on fundus photography. On autofluorescence imaging, there is a central area of reduced autofluorescence surrounded by a hyperautofluorescent ring. On OCT imaging, there is significant thinning in the foveal region consistent with photoreceptor loss. A composite color photograph from the left eye of case III.1, at the age of 48, is also shown (I). This reveals macular atrophy, mid-peripheral bone-spicule pigmentation, and attenuated retinal vessels. On the infra-red reflectance images, the horizontal green lines indicate the position and direction of the corresponding OCT scan. The scale bars represent 200 μm.

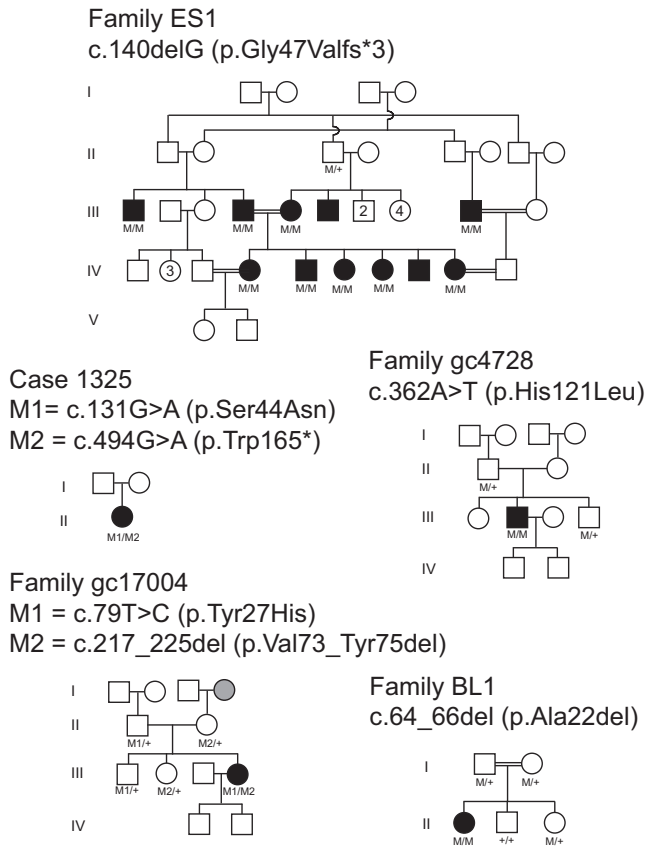

Figure 2. Pedigrees of Families and Case Subjects Reported in This Study and *DRAM2* Mutation Segregation Data

Affected individuals are shaded black. The maternal grandmother of individual gc17004 has age-related macular degeneration in her 90s (shaded gray). The genotypes for all tested family members are shown below each individual, with M representing the mutant allele and + representing the wild-type allele.

imaging in the pre-symptomatic second decade of life (subject IV.9, family ES1; Figure 2) suggested early central photoreceptor cell loss.

This study was approved by the Leeds East (Project number 03/362), Moorfields Eye Hospital, and Ghent University Hospital (PA2015/012) Research Ethics Committees and adhered to the tenets of the Declaration of Helsinki. Individuals participated in the study after giving their informed consent. Peripheral blood was collected from affected individuals, parents, and unaffected relatives where these were available. Genomic DNA was extracted from blood leukocytes according to standard procedures.

Homozygosity mapping was performed with Affymetrix 250K SNP arrays on genomic DNA from seven affected individuals from family ES1. Data were analyzed with the AgileMultiIdeogram software. Two homozygous regions were shared among all seven affected individuals: a 10.1 Mb interval on chromosome 1 (between rs6677953 and rs814987; containing 160 genes) and a 2.9 Mb region on chromosome 7 (between rs17140297 and rs12706292; containing 5 genes) (Figure S1). Given the absence of genes previously reported to be associated with retinal dystrophy

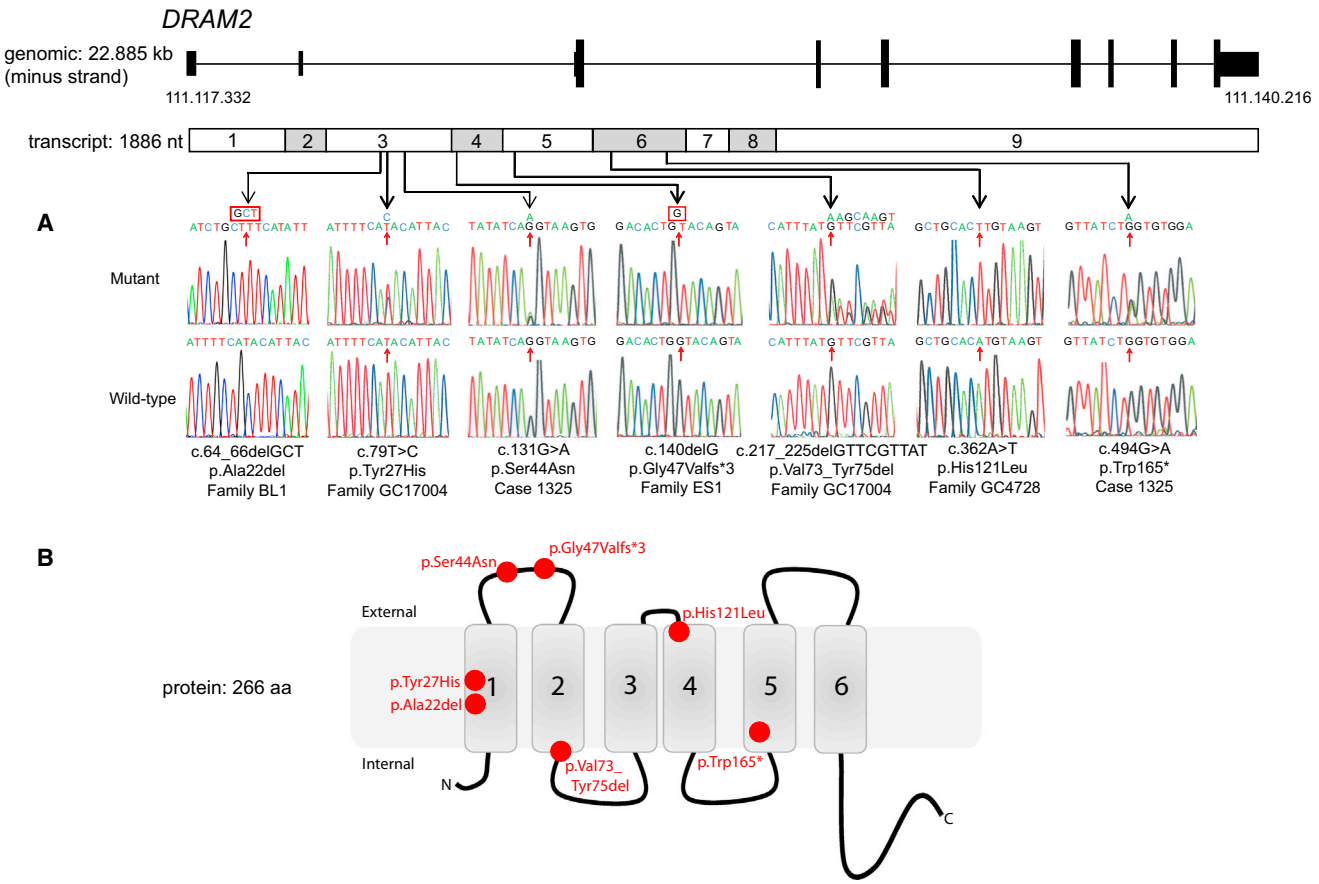

Figure 3. Variants in *DRAM2* Cause Retinal Dystrophy
(A) Schematic representation of the *DRAM2* genomic structure and major transcript (GenBank: NM_178454.4) showing the location and sequence traces of the seven disease-causing variants identified in this study.
(B) Schematic diagram of DRAM2 showing the location of the affected amino acids within the protein domains.

within these intervals, a whole exome sequencing (WES) strategy was utilized to identify the molecular pathology in the family. DNA from one affected family member (subject IV.6, family ES1; Figure 2) was analyzed with a HiSeq2000 system (Illumina). After aligning the sequencing data output against the reference genome (hg19/GRCh37) as well as variant calling and filtration steps, a list of 33 homozygous variants was generated (Table S1). Only one of these sequence alterations mapped within the shared regions of homozygosity identified in family ES1. This was a homozygous single-base deletion in *DRAM2* (DNA-damage regulated autophagy modulator protein 2 [MIM: 613360; GenBank: NM_178454.4]) that creates a frameshift and is predicted to lead to premature truncation of the protein (c.140delG [p.Gly47Valfs*3]). Segregation of this variant with the disease in the family (Figure 2) was confirmed by Sanger sequencing of *DRAM2* exon 4 (Figure 3A; primer pairs are shown in Table S2). This change was excluded from 159 ethnically matched control individuals and was not present in the dbSNP and EVS databases. It was found once in heterozygous state in WES data from 61,486 unrelated individuals sequenced as part of various disease-specific and population genetic studies (accessed via the Exome Aggregation Consortium [ExAC] browser, v.0.2). Notably, no homozygous presumed loss-of-function variant in *DRAM2* was present in the ExAC dataset. A maximum two-point LOD score of 2.4 was obtained between c.140delG and the disease in nine genotyped family members using Superlink.[2] For this analysis the c.140delG change was treated as a genetic marker with a MAF of 0.01%, and the disease was assumed to segregate in the family in a recessive fashion with full penetrance.

In an attempt to identify additional families with *DRAM2*-associated retinopathy, the seven coding *DRAM2* exons and flanking splice sites were PCR amplified and Sanger sequenced in 74 individuals diagnosed with RP, 154 with CRD or MD, and 94 with infantile-onset retinal dystrophy (Leber congenital amaurosis) (primer pairs are shown in Table S2). This screen identified an isolated female case (subject 1325) of European ancestry in the CRD/MD panel who was compound heterozygous for a nonsense variant in exon 6 (c.494G>A [p.Trp165*]) and for a missense change in exon 3 (c.131G>A [p.Ser44Asn]). The latter affects a serine residue that is conserved from human to nematodes (Figures 3 and S2). This missense change was predicted to be pathogenic by a number of bioinformatics prediction tools (Table S3) and was not present

in the dbSNP, EVS, or ExAC databases. The c.494G>A change is an annotated variant in dbSNP (rs201422368) with a MAF of 0.008% (1/13,003) in EVS and 0.003% (3/118,572) in ExAC; it is reported only in heterozygous state in these databases. Subject 1325 experienced blurred vision at age 29 and was soon after found to have maculopathy on fundus examination. At age 35, she also complained of night vision problems and sensitivity to light; fundus examination revealed mild peripheral retinal degeneration in addition to the maculopathy. At the age of 47, she had acuity of 1.0 logMAR in each eye and electrophysiology revealed severely attenuated or absent full-field electroretinograms (ERGs) and pattern ERGs (Figure S3).

Meanwhile, interrogation of previously generated WES data from unsolved cases with retinal dystrophy led to the identification of a homozygous variant in exon 3 of *DRAM2* (c.64_66del [p.Ala22del]) in a subject of Indian origin. This change, which removes an alanine residue from the first transmembrane domain of the molecule, was predicted to be pathogenic by bioinformatics prediction tools (Table S3) and was absent in the dbSNP, EVS, and ExAC databases. The variant was confirmed by Sanger sequencing (Figure 3) and segregated in family BL1 with the disease as expected in a recessive manner (Figure 2). The affected subject, a woman in her early forties, has maculopathy with a normal full-field ERG indicating absence of a generalized retinal dysfunction. She also suffers from iron-deficiency anemia, which might have been brought on by a vegetarian diet or might be the result of a genetic factor resulting from parental consanguinity.

Independently, in a study designed to identify novel genes associated with retinal disease, 28 families from the inherited retinal disease clinics at Moorfields Eye Hospital (London) were ascertained. Details on this cohort have been previously reported.[3] The main inclusion criteria included a CRD or MD phenotype and an absence of retinal imaging findings suggestive of *ABCA4* retinopathy. Genomic DNA from the probands was analyzed by WES and variant filtering was performed as previously described.[3] The molecular diagnosis was identified in 10 of 28 families.[3] On the 18 unsolved case subjects, a gene-based case-control association analysis was performed utilizing WES data generated by a consortium of UK-based researchers ("UCL-exomes," Table S4). Aiming to minimize bias,[4] UCL-exome control subjects were initially split into two sets. The first set of 500 randomly selected samples was used in conjunction with EVS to determine variant frequency for inclusion in case-control tests. In that context, "rare" variants are those with MAF $< 0.5\%$ in EVS and no more than two occurrences in this first set of 500 UCL-exome control samples. The second set of 1,917 unrelated UCL-exome control subjects was used to directly compute gene-based association p values, using a recessive disease mode, i.e., samples were labeled as potential carriers only if they carried at least two rare (using the definition stated above) and potentially functional (presumed loss-of-function, non-synonymous, or splice site altering) variants. A binomial test was used for excess of such potential biallelic variants in the 18 case subjects compared to the 1,917 control subjects (Table S4).

The most significant gene-based p value was obtained for *DRAM2* (Table S4). Two of the 18 case subjects were found to harbor likely disease-associated variants in this gene. A 37-year-old female proband (family gc17004, Figure 2) of European ancestry was a compound heterozygote for a missense variant (c.79T>C [p.Tyr27His]) and an in-frame deletion (c.217_225del [p.Val73_Tyr75del]). Furthermore, a 47-year-old male proband of South Asian origin (family gc4728, Figure 2; parents not knowingly related) was homozygous for a missense change (c.362A>T [p.His121Leu]). None of these three changes which are reported to be pathogenic by a number of prediction tools (Table S3) exist in dbSNP, EVS, or ExAC. Both missense variants, p.Tyr27His and p.His121Leu, affect a tyrosine and histidine residue, respectively, that are evolutionarily conserved from human to nematodes (Figure S2). All changes were confirmed by Sanger sequencing (Figure 3) and segregated with the disease phenotype in the family as expected for a recessive condition (Figure 2).

Both probands presented with central visual loss (at age 29 for the proband of family gc17004 and at age 34 for the proband of family gc4728). At presentation, fundus examination and retinal imaging revealed macular photoreceptor loss with an apparently normal peripheral retina. These observations were consistent with electrophysiological findings. Notably, 8 years after presentation, the central areas of atrophy have expanded and peripheral changes were observed. The phenotype was notably similar to the affected members of families ES1 and BL1 and to subject 1325 described above.

Given that affected members of family ES1 are homozygous for a *DRAM2* variant that is likely to lead either to nonsense-mediated decay of the encoded mRNA or to a truncated protein of only 47 amino acids, the molecular pathology of the disease is likely to be loss of DRAM2 function. This speculation is further supported by the biallelic state and predicted severity of the additional six likely disease-causing variants identified as well as by the similar phenotype in all five families.

DRAM2, also known as TMEM77 (transmembrane protein 77), encodes a 266-amino-acid protein containing six putative transmembrane domains (Figure 3B). Previous overexpression studies in HEK293 cells localized it to lysosomal membranes[5,6] where it initiates the conversion of endogenous LC3-I (microtubule-associated protein light chain 3) to the general autophagosome marker protein, LC3-II (LC3-1/phosphatidylethanolamine conjugate). This suggests that DRAM2 induces the autophagy process.[5] Autophagy is a natural cell survival mechanism triggered in response to stress stimuli such as nutrient starvation or the accumulation of damaged organelles. It is responsible for degrading and recycling cytoplasmic proteins and lipids as well as organelles within the cell.[7] This usually begins with isolation of the macromolecules and

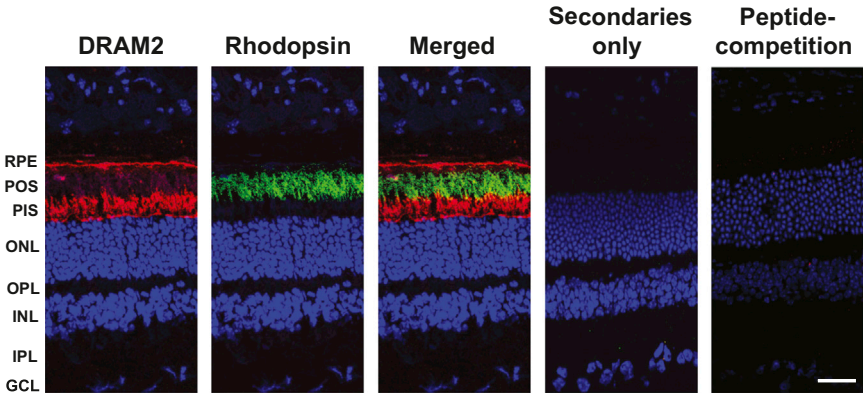

Figure 4. Localization of DRAM2 to the Photoreceptor Inner Segments and Retina Pigment Epithelium

Radial 6 μm cryosections of mature mouse retina (P30) were labeled with anti-DRAM2 (M-12, Santa Cruz Biotechnology) and anti-Rhodopsin (Sigma-Aldrich) followed by the secondary antibody Alexa Fluor 568-conjugated donkey anti-goat immunoglobulin (red) (Molecular Probes Incorporation) and Alexa Fluor 488-conjugated chicken anti-rabbit immunoglobulin (green) (Molecular Probes Incorporation), respectively, and the nuclei were counterstained with DAPI (Vector Laboratories). An independent section stained with both secondary antibodies only and another with peptide (Santa Cruz Biotechnology, sc-241077-P)-competition against the DRAM2 primary antibody served as negative controls in the experiment. Immunofluorescence was analyzed with an Eclipse TE2000-E inverted confocal microscope (Nikon Instruments) and shows localization of DRAM2 to the inner segment of the photoreceptor layer (PIS) and the retinal pigment epithelium (RPE). Rhodopsin localizes to the outer segment of the photoreceptor layer (POS). The other layers are the outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and the ganglion cell layer (GCL). Scale bar represents 50 μm.

organelles within the cytoplasm into single membrane vesicles, which fuse together to produce an autophagosome. These autophagosomes subsequently fuse with lysosomes containing acid hydrolases and form a double-membrane autolysosome.[8,9] Although the aim of autophagy is to relieve cellular stress, its excessive induction can in some cases lead to apoptosis rather than protection from cell death.[10]

There is also some evidence to suggest that DRAM2 might have tumor-suppressor capability. DRAM2 transcript and protein expression are reduced in ovarian tumors compared to normal matched tissues.[5] Also, siRNA knockdown of endogenous DRAM2 results in reduced conversion to LC3-II in cells subject to starvation-induced autophagy[11] and increased survival in deoxyrubicin-treated cells that would normally undergo p53-mediated apoptosis.[5] We note that examination of medical histories in the reported subjects with *DRAM2*-associated retinal dystrophy provided no evidence of increased susceptibility to cancer.

Although *DRAM2* is transcribed ubiquitously (Figure S4),[6] in light of the finding that human *DRAM2* variants cause retinal dystrophy, we investigated the precise distribution of the normal protein in the mouse retina. Serial sections were taken from mouse eyes at postnatal day 30 and were stained with a goat polyclonal antiserum against DRAM2 (Figure 4). Confocal immunofluorescence microscopy showed that DRAM2 localized to the inner segment of the photoreceptor layer and the apical surface of the retinal pigment epithelium (RPE), which are located at the basal and distal ends of the outer segment, respectively. This coincides with the primary pathology observed on pre-symptomatic OCT analysis in which the photoreceptor layer appeared specifically affected.

This localization is consistent with a role for DRAM2 in photoreceptor autophagy. Photoreceptor outer segments are in a constant state of renewal by ciliogenesis in response to light-induced damage. Recent studies have suggested that there is interplay between ciliogenesis and autophagy. In one study it was shown that disruption of ciliogenesis partially inhibited autophagy, whereas blocking autophagy enhanced primary cilia growth and cilia-associated signaling during normal nutritional conditions. The authors therefore proposed that basal autophagy regulated ciliary growth through the degradation of proteins required for intraflagellar transport.[12] In another study, the protein OFD1 (oral facial digital syndrome 1), which accumulated at centriolar satellites located close to the base of the cilium, was rapidly degraded by serum starvation-induced autophagy. This led to ciliary growth, suggesting that OFD1 normally inhibited ciliogenesis.[13]

A high level of autophagy is also expected to take place in the RPE. These cells have a key role in processing shed photoreceptor outer segment discs and consequently in removing toxic metabolites and recycling phototransduction components. This process, which involves RPE phagocytosis, causes up to 10% photoreceptor volume loss each day and is entrained to the circadian rhythm.[14,15] Indeed there is increasing interest in the role of autophagy in preserving photoreceptor function in connection with the circadian cycle,[16] the aging process,[17] and retinal disease pathology.[18] It is therefore likely that the absence of DRAM2 in the retina reduces the efficiency of autophagy in recycling cell components, which in turn reduces photoreceptor renewal, leading to the thin photoreceptor layer observed on OCT, which is the first presenting feature in pre-symptomatic patients.

To summarize, we have shown that biallelic missense, nonsense, and frameshift variants in *DRAM2* cause retinal dystrophy with early macular cone photoreceptor involvement. The clinical features and course of retinal degeneration were highly similar among affected individuals from the five reported families. Our findings suggest that DRAM2 is essential for photoreceptor survival and further

studies are expected to provide important insights into its precise role in the retina.

Accession Numbers

The variants reported in this paper have been deposited into the ClinVar database at the National Centre for Biotechnology Information under accession numbers ClinVar: SCV000222243-49.

Supplemental Data

Supplemental Data include four figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2015.04.006.

Consortia

The UK Inherited Retinal Disease Consortium includes Graeme Black, Georgina Hall, Stuart Ingram, Rachel Gillespie, Simon Ramsden, Forbes Manson, Panagiotis Sergouniotis, Andrew Webster, Alison Hardcastle, Michel Michaelides, Vincent Plagnol, Michael Cheetham, Gavin Arno, Niclas Thomas, Shomi Bhattacharya, Tony Moore, Chris Inglehearn, Carmel Toomes, Manir Ali, Martin McKibbin, James Poulter, Emma Lord, Andrea Nemeth, Susan Downes, Stefano Lise, and Veronica van Heyningen.

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Web Resources

The URLs for data presented herein are as follows:

AgileMultiIdeogram, http://dna.leeds.ac.uk/agile/AgileMultiIdeogram/
ANNOVAR, http://www.openbioinformatics.org/annovar/
CADD, http://cadd.gs.washington.edu/
ClustalW, http://www.ebi.ac.uk/Tools/msa/clustalw2/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
ExAC Browser, http://exac.broadinstitute.org/
Galaxy, https://usegalaxy.org/
GATK, http://www.broadinstitute.org/gatk/
IGV, http://www.broadinstitute.org/igv/
Macular Society, http://www.macularsociety.org/
MutationTaster, http://www.mutationtaster.org/
MutPred, http://mutpred.mutdb.org/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
Picard, http://picard.sourceforge.net/
PolyPhen-2, http://www.genetics.bwh.harvard.edu/pph2/
Primer3, http://bioinfo.ut.ee/primer3
PROVEAN, http://provean.jcvi.org/index.php
RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq
RetNet – Retinal Information Network, https://sph.uth.edu/retnet/home.htm
SIFT, http://sift.bii.a-star.edu.sg/
Superlink Online SNP, http://cbl-hap.cs.technion.ac.il/superlink-snp/

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