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The *rd8* mutation of the *Crb1* gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes

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Short running title: *rd8* mutation in vendor B6 mice and ES cells

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ABSTRACT

PURPOSE: The authors noted an unexpected inheritance pattern of lesions in several strains of gene-manipulated mice with ocular phenotypes. The lesions, which appeared at various stages of backcross to C57BL/6, bore resemblance to the *rd8* retinal degeneration phenotype. The authors set out to examine the prevalence of this mutation in induced mutant mouse lines, vendor C57BL/6 mice and in widely used embryonic stem cells.

METHODS: Ocular lesions were evaluated by fundus examination and histopathology. Detection of the *rd8* mutation at the genetic level was performed by PCR with appropriate primers. Data were confirmed by DNA sequencing in selected cases.

RESULTS: Analysis of several induced mutant mouse lines with ocular disease phenotypes revealed that the disease was associated 100% with the presence of the *rd8* mutation in the *Crb1* gene rather than with the gene of interest. DNA analysis of C57BL/6 mice from common commercial vendors demonstrated the presence of the *rd8* mutation in homozygous form in all C57BL/6N substrains, but not in the C57BL/6J substrain. A series of commercially available embryonic stem cells of C57BL/6N origin and C57BL/6N mouse lines used to generate ES cells also contained the *rd8* mutation. Affected mice displayed ocular lesions typical of *rd8* which were detectable by fundoscopy and histopathology as early as 6 wk of age.

CONCLUSIONS: These findings identify the presence of the *rd8* mutation in the C57BL/6N mouse substrain widely used to produce transgenic and knockout mice. The results have grave implications for the vision research community who develop mouse lines to study eye disease, as presence of *rd8* can produce significant disease phenotypes unrelated to the gene or genes of interest. It is suggested that researchers screen for *rd8* if their mouse lines were generated on the C57BL/6N background, bear resemblance to the *rd8* phenotype, or are of indeterminate origin.

INTRODUCTION

The *rd8* mutation is a single nucleotide deletion in the *Crb1* gene, which results in a form of retinal degeneration appearing with distinct clinical appearance including multiple light-colored spots in the fundus of the eye that correspond histologically to retinal folds, pseudorosettes, and focal retinal dysplasia and degeneration [1]. The trait is autosomal recessive and has been identified in several mouse lines [2]. C57BL/6 mice, on which many transgenic strains are generated and are the source of increasingly popular lines of embryonic stem (ES) cells, were not known to carry the mutation. Furthermore, the C57BL/6 background was reported to suppress expression of the *rd8* phenotype [2].

Recent data with induced ocular mutants generated by the authors, or derived from strains available from collaborators, revealed an unexpected inheritance pattern of ocular lesions in several lines of gene-manipulated mice. Specifically, while crossing mice carrying a gene deletion or a transgene designed to confer an ocular phenotype onto the C57BL/6 background, the phenotype being studied was noted in some littermate controls. These findings raised the possibility that the observed phenotypes were not due to the genes of interest but were the result of other unidentified factors. On close examination, the lesions were reminiscent of *rd8* associated retinal degeneration [2] so we conducted a PCR screen for *rd8* and found 100% of mice with disease were homozygous for *rd8*. Since the *rd8* positive mice examined were derived in different institutions, and the ocular phenotype was discovered during backcross to C57BL/6 in littermates that did not carry the gene of interest, it was decided to examine C57BL/6 mice from major commercial vendors and commercially available C57BL/6 ES cells for the *rd8* mutation. These data demonstrated the presence of the *rd8* mutation in homozygous form in all C57BL/6N substrains regardless of the commercial source, but not in the C57BL/6J substrain. Embryonic stem cells of C57BL/6N origin also contained the *rd8* mutation. Affected mice displayed ocular lesions typical of *rd8* detectable by fundus and histological examination as early as at 6 wk of age. These findings have serious implications for the ocular community who develop transgenic and knockout mice using C57BL/6N embryos or C57BL/6N-derived ES cells.

MATERIALS AND METHODS

Mice

HLA-A29 construct was provided by Dr. Jacques Cohen, INSERM, Paris France [3]. Founder mice transgenically expressing this construct were generated by the NEI genetic core facility using embryos derived from female C57BL/6N mice purchased from DCT, Frederick, MD. *Ccl2/Cx3cr1* double-knockout mice were bred from the respective single knockouts obtained from collaborators [4, 5]. Wild type C57BL/6 mice (6-8 wk old) were purchased from several NIH-approved vendors including DCT Frederick (stock# 01CC55), Taconic (TAC stock# B6), Harlan Sprague Dawley (HSD stock# 044), Charles River Laboratories (CRL stock# 027), and Jackson Labs (JAX stock#000664). C57BL/10J mice were purchased from JAX. DCT, HSD, CRL and TAC supplied C57BL/6N mice and JAX supplied C57BL/6J mice. B6Smn.C3-FasL^{gld}/J (B6-*gld*) mice were obtained from Jackson labs, C57BL/6-*Bid*$^{-/-}$ (B6-*Bid*$^{-/-}$) mice were obtained from Dr. Richard Hotchkiss (Washington University School of Medicine, St. Louis, MO), and the C57BL/6-*Apo-2L*$^{-/-}$ (C57BL/6-*Trail*$^{-/-}$ or B6-*Trail*$^{-/-}$) mice were provided by Dr. Thomas Griffith (University of Minnesota, Minneapolis, MN). These lines were crossed to the *Ccl2/Cx3cr1* double knock out mice to obtain triple knockout mice. All mice were maintained and used in compliance with ARVO and Institutional guidelines.

Cells and reagents

Mouse ES lines R1, W4 and JM8.N4 were obtained from the NEI Genetics Core. The ES cells characterized in this study were cultured in the absence of feeder cells for several months (at least 3 passages). Mouse strains used to derive the ES cell lines SCC1O, B6-Blu, B6-GFP, EDJ22, R1, SCC10 were tested at the Siteman Cancer Center at Washington University School of Medicine. DNA was prepared by standard protocols and samples were genotyped for the *rd8* mutation by PCR.

Detection of *rd8* mutation by PCR

DNA was isolated from mouse tail biopsy samples using a Qiagen Biosprint instrument, Qiagen reagents and the protocol suggested by the manufacturer. DNA samples isolated from tail biopsies were amplified separately for wild type (*wt*) allele and mutant *rd8* allele using primers specified Mehalow et.al [2]. Primer sequences: mCrb1 mF1: GTGAAGACAGCTACAGTTCTGATC; mCrb1 mF2: GCCCCTGTTTGCATGGAGGAAACTTGGAAGACAGCTACAGTTCTTCTG; mCrb1 mR: GCCCCATTTGCACACTGATGAC. For PCR amplification approximately 25ng DNA was used in a 25µl reaction volume containing 1.5mM MgCl2, 100µM of each dNTP, 1.6µM each of forward and reverse primer for Wt allele and 0.8µM of forward and 1.6µM of reverse primer for *rd8* mutant allele and 0.025U AmpliTaq DNA polymerase. Reactions were initially denatured at 94^{o}C for 5 minutes followed by 35 cycles at 94^{o}C for 30 sec, 65^{o}C for 30 sec, 72^{o}C for 30 sec and a final extension at 72^{o}C for 7 minutes. Amplicons were separated using 3% Nusieve 3:1 (Lonza) agarose gel and visualized under UV light after staining with ethidium bromide. Amplicon sizes are Wt allele = 220bp and *rd8* allele = 244 bp. Alternately, genotyping was performed by a TaqMan allelic discrimination assay with common PCR primer sequences CCCTGTTTGCATGGAGGAAA (forward) and CCTGACCATCCCGAGAGACA (reverse), and the allele-specific probe sequences were [6FAM]AGCTACAGTTCTTATCGG[MGBNFQ] (wild type) and [VIC]CAGCTACAGTTCTTATGGT[MGBNFQ] (*rd8*). The allelic discrimination assay was run on an Applied Biosystems 7900HT running SDS 2.3 software using the protocols specified by the manufacturer.

DNA sequencing for detection of *rd8*-associated nucleotide deletion

Mouse tail DNA from a C57BL/6J (JAX) mouse and a C57BL/6N (DCT) mouse was PCR amplified with the primers GGTGACCAATCTGTTGACAATCC (forward) and GCCCCATTTGCACACTGATGAC (reverse), and the product was cleaned up using a QIAquick PCR Purification Kit (Qiagen). The two samples and two PCR primers were sent to ACGT Inc. (Wheeling, Illinois) for sequencing in both directions. Portions of the sequences were aligned using the MegAlign program (DNAStar, Madison WI).

Fundus photography and optic coherence tomography (OCT)

Mice were anesthetized systemically with 200µl – 300µl of a mixture of Ketamine (7.7mg/ml) and Xylazine (0.45mg/ml) and topically with 0.5% Proparacaine HCl (Alcon Lab, Inc.). The pupil was dilated with 0.5% Tropicamide ophthalmic solution (Bausch& Lomb) and 2.5% Phenylephrin HCl (Bausch& Lomb) drops and the corneas were kept moist with regular application of sterile lubricant GenTeal severe (Novartis). Fundus photographs were taken with Micron-2 camera (Phoenix Research lab, Inc) using StreamPix 5 software.

An ultra-high resolution Spectral-Domain Optic coherence tomography (SD-OCT) system designed for small animal imaging (Bioptigen, Research Triangle Park, NC) was used for *in vivo* imaging of mouse retina. A 1.4 mm linear scan was obtained with 1000 A-Scan per B-scan.

Histopathology

Eyes were enucleated, fixed in 4% phosphate buffered glutaraldehyde for 1 hr and stored in 10% phosphate buffered formaldehyde until processing. Tissues were embedded in methacrylate. Sections 6-8 µm in thickness were stained with hematoxylin and eosin. Eight to ten sections cut at different planes were examined for each eye in a masked fashion by one of us (CCC), an ophthalmic pathologist.

RESULTS

Detection of *rd8* in HLA-A29 transgenic mice.

The human uveitic disease known as birdshot chorioretinopathy (BC, also known as birdshot retinochoroidopathy) is very strongly associated with the HLA-A29 gene [6]. A model for this disease had been generated in transgenic mice by Szpak et al. [3] using an HLA-A29 gene sequence cloned from a BC patient, which developed BC-like ocular lesions between 8–12 months of age. The strain had been lost so one of us (RRC) obtained the original HLA-A29 DNA construct and generated founder mice by microinjection into embryos of C57BL/6N females purchased from DCT, Frederick MD. The founders were bred to C57BL/6N mice from the same source to produce F1 mice. F1 mice were either intercrossed, or bred to C57BL/10J (in preparation for subsequent crossing to human β2m transgenic mice which are on the C57BL/10J background). All founders and progeny were examined periodically for ocular lesions by fundus examination. Ocular lesions were observed in all HLA-A29 founders and in many of their progeny, suggesting the original findings of Szpak et al.[3] may have been recapitulated.

However, some inconsistencies became apparent that questioned the association of the observed lesions with the HLA-A29 transgene: First, unlike BC in patients and the original mouse line of Szpak et al. [3], the ocular lesions in HLA-A29 transgenic mice appeared to be degenerative, not inflammatory, and appeared soon after weaning age. Second, mice did not have the human β2 microglobulin gene needed to associate with the A29 chain for expression on the cell surface; therefore, expression of HLA-A29 molecules in these mice was so low as to be undetectable by conventional assays. This suggested that the HLA-A29 may not be responsible for the observed phenotype. Third and most significant, similar lesions were observed in the littermates not carrying the A29 transgene (Table 1 and Figure 1).

Careful genotyping of phenotype-positive *vs*. -negative mice, performed independently by one of us (TAF) and by Dr. Bo Chang at Jackson labs and confirmed by the NEI Genetic Core Facility, revealed that (a) all founders were homozygous for the *rd8* mutation and (b) the various progeny displayed a complete correlation between the ocular phenotype and presence of homozygous $Crb1^{rd8}$ allele (Table 1). Notably, of the F2 progeny that were crossed to C57BL/10J and were heterozygous for *rd8*, about 20% also displayed similar retinal lesions, whether or not they carried the A29 transgene. Since disease was independent of the A29 genotype, we conclude that the observed phenotype was either caused by the *rd8* mutation or was heavily influenced by its presence.

<u>The presence of the *rd8* mutation in mice with AMD-like phenotypes</u>

In 2007, one of us (CCC) published an AMD-like model in *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice [7]. These mice were produced by crossing the single knockout *Ccl2*$^{-/-}$ [4] and *Cx3cr1*$^{-/-}$ [5] strains, both with reported AMD like features that developed by 1 yr of age. The *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ double knockout mice develop ocular spots observed by fundoscopic examination (Fig 2A) and retinal lesions upon histological examination as early as 6-8 wk of age (Figure 2B). In the process of exploring the mechanisms of disease in the *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ by one of us (TAF) the *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ line was crossed to several other mouse lines (B6-*gld*, B6-*Bid*$^{-/-}$, and B6-*Trail*$^{-/-}$) to develop triple knockout mice. During this process a number of heterozygous littermate controls were generated (see Table 2). Significant disease (see Figure 2A and B) was noted that was independent of the genotype. A more complete list of mouse lines is shown in Table 2 where disease correlated 100% with the presence of the *rd8* mutation.

The *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice were derived from *Ccl2*$^{-/-}$ (obtained from Bao Lu and Barrett J. Rollins of Children's Hospital, Harvard Medical School) [4] and *Cx3cr1*$^{-/-}$ (obtained from Philip Murphy of the National Institute of Allergy and Infectious Diseases, National Institutes of Health) [5]. Notably, the founder *Cx3cr1*$^{-/-}$ mice had been bred to C57BL/6N mice from DCT Frederick, MD for 13 generations, and the *Ccl2*$^{-/-}$ founder mice had been bred to C57BL/6N mice obtained from Charles River for 6–7 generations before we received them (Philip Murphy and Bao Lu, personal communication). Analysis of these parental lines (Table 2) revealed that the *Ccl2*$^{-/-}$ mice were either heterozygous for the *rd8* mutation or wild type at the *Crb1* locus. The *Cx3cr1*$^{-/-}$ parental mice were all homozygous *rd8*. Interestingly only 50% *Cx3cr1*$^{-/-}$ mice developed retinal lesions at 6 wk and no *Ccl2*$^{-/-}$ mice developed retinal lesions at 6 wk of age. This analysis suggests that the *rd8* mutation is likely to have entered these mouse lines during the creation of the single knockout mice. Furthermore, as with the B6-HLA-A-29 transgenic mice discussed above, the pathological features in *Ccl2*$^{-/-}$, *Cx3cr1*$^{-/-}$, and *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice either depend on *rd8* or are modified by the presence of this mutation.

The *rd8* mutation is present in C57BL/6 vendor lines

On the basis of the data described above, it seemed unlikely that *rd8* appeared spontaneously in the unrelated HLA-A29 transgenic and *Ccl2-Cx3cr1* knockout mouse lines. Rather, the source was more likely a mouse strain common to their genetic history. Although it was not possible to trace the complete lineage of all strains involved, we thought it reasonable to examine strains common to the ancestry of many transgenic and knockout mice used in vision research. We obtained C57BL/6 mice from the major vendors including Harlan, DCT Frederick, Charles River, and Taconic Farms. These suppliers all maintain the C57BL/6N substrain which was derived after 1951 from C57BL/6J that had been maintained at Jackson labs since the early part of the 20th century [8]. We also obtained C57BL/6J and C57BL/10J mice from Jackson labs. Genotyping revealed that the C57BL/6N substrain, regardless of the vendor, uniformly carried the *rd8* gene (Fig 3A) while C57BL/6J and C57BL/10J were wild type at the *Crb1* locus. The location and identity of the mutation found in C57BL/6N was confirmed by sequencing through the *Crb1* gene, demonstrating the expected deletion in the C57BL/6N mouse line (Fig. 3B). Fundus and histological examination revealed that C57BL/6N mice from all four suppliers demonstrated typical *rd8* lesions to a variable extent, which were well developed as early as 6 weeks of age (Fig. 4 A–D). Notably, C57BL/6J mice appear normal, as did control *rd8* negative C57BL/10J mice (Fig 4 E, F). Histology was performed on affected eyes demonstrating typical lesions with non-inflammatory retinal degeneration involving the inner and outer nuclear layers, the outer plexiform layer, as well as the photoreceptor outer segments (Fig. 4 G).

The *rd8* mutation is present in ES cells of C57BL/6N origin

C57BL/6N mice are the source of many ES cell lines used to generate gene targeted mice. Consequently we obtained a number of ES cell lines from the NEI Genetic Engineering Core as well as the ES cell core lab in the Siteman Cancer Center at Washington University in St. Louis. PCR analysis revealed that all ES cells derived from C57BL/6N were uniformly homozygous for the *rd8* mutation, in keeping with the vendor C57BL/6N results. ES lines derived from C57BL/6J or the 129 strain had the wild type *Crb1* allele (Table 3).

DISCUSSION

Crb1^{rd8} is a recessive mutation and the C57BL/6 background was reported by Mehalow et al. to suppress expression of the phenotype [2]. These authors demonstrated that *rd8*-positive mice on a mixed background lost the phenotype upon extensive backcrossing of the mutation to C57BL/6J. Interestingly, these investigators noted what they called "founder effects" in mice that expressed the phenotype and considered the gene to have originated from C57BL/6 (of unspecified substrain). The findings of our study indicate that the C57BL/6N background not only harbors the mutation in its homozygous state but also allows for its full expression. The C57BL/6N substrain is offered by many major vendors and it seems that neither the vendors, nor the eye research community, are aware of this issue. The fact that C57BL/6N from all of the major US mouse vendors that were examined carry the *rd8* allele suggests that this mutation occurred and became fixed in the C57BL/6N stock after 1951 when C57BL/6 was originally transferred from JAX to NIH [8]. Furthermore, it remained in this mouse substrain when the C57BL/6N line was transferred from NIH to Charles River labs in 1974 and subsequently was disseminated to additional mouse vendors [8]. Notably, C57BL/6NJ strain (JAX #005304) maintained at Jackson Labs from NIH embryos that had been cryopreserved in 1984, also carries the *rd8* mutation (B. Chang, Jackson Labs, personal communication). While C57BL/6N mice available from vendors in Europe have not yet been examined, numerous substrains of C57BL/6N available in Japan, including the C57BL/6ByJ strain (derived from C57BL/6N around 1960 by Jackson Labs), were found to be homozygous for *Crb1^{rd8}*. C57BL/6J mice available in Japan did not harbor *rd8* (Atsushi Yoshiki, RIKEN BioResource Center, Ibaraki, Japan, personal communication). The wide prevalence of the rd8 mutation seems reminiscent of the previously described *rd1* mutation that was identified in many common laboratory mouse strains [9].

Importantly, ES cells used to generate gene-targeted mice are typically derived from the C57BL/6N strain including the widely used KOMP (Knockout Mouse Project) and EUCOMM (European Conditional Mouse Mutagenesis Program) cell lines. We found the *rd8* mutation in KOMP as well as EUCOMM ES cells that we tested. Although we did not examine ES cells from TIGM (Texas A&M Institute for Genomic Medicine) or NorCOMM (Canadian Mouse Mutant Repository), these lines were all derived from C57BL/6N mice and likely harbor the *rd8* mutation. This raises the possibility that the undiscovered presence of *rd8* in induced mutant strains might account for some or all of the published ocular phenotypes. We are currently reexamining all our mutant lines for presence of the *rd8* gene. Notably, unlike C57BL/6J, at least some investigator-generated mice on C57BL/6 background distributed by Jackson Labs carry the *rd8* mutation (e.g. IFN-$\gamma^{-/-}$, IL-$10^{-/-}$) (data not shown). It is likely that these lines were generated using C57BL/6N ES cells and/or were crossed onto the B6 background using C57BL/6N mice by the investigator who deposited them at Jackson Labs.

It must be taken into account that although full expression of an *rd8* phenotype was reported to require homozygosity, it is difficult to exclude the possibility that a heterozygous *rd8* might modify expression of other ocular phenotype genes, such that a phenotype might be observed that would not be present without *rd8*. Moreover, because retinal lesions were seen in *rd8*-heterozygous animals with C57BL/6N x C57BL/10J parentage (Table 1), it must be considered that some genetic backgrounds might allow heterozygous expression of the *rd8* phenotype. In addition, since ocular phenotypes are not completely uniform between mutant mouse lines examined, we also cannot exclude the possibility that background genes (or even the genes of interest) might modify the *rd8* phenotype. Similarly it is possible that a mutation in the *Crb1* gene, which is involved in regulating cell polarity in epithelial cells, might influence phenotypes in inflammatory or injury-induced ocular disease models. This will require further analysis. However, on the basis of the data presented here, it is highly recommended that researchers working with knockout or transgenic mice with retinal phenotypes should genotype their mice for presence of the *rd8* mutation.

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FIGURE LEGENDS

Figure 1: *Ocular findings in WT littermates of HLA A29 Tg mice compared to healthy eyes.* Fundus photographs were taken at 9-10 weeks of age (A) and SDOCT at 18 weeks of age (B). Healthy retinas are depicted on the right. (A) Fundus lesions appeared as multiple bright spots in the retina, coalescing to form diffuse lesions or large patches. (The three bright spots visible in the same position on all fundi and the central black spot are a camera artifact.). (B) SDOCT of eyes in shown in panel A: linear scans of retina showed localized areas of hyperreflectivity and thin retina with loss of inner and outer segment layers, or loss of normal architecture of the inner and outer nuclear layers at focal areas away from the optic nerve; note similarity of these lesions to the ones described by Aleman et al. [10] in mouse *CRB1* associated retinal degeneration. (C) Histology – note foci of total photoreceptor atrophy with loss of outer and inner segments, outer nuclear and outer plexiform layers (left) multiple focal photoreceptor dystrophy without RPE abnormalities (center) and. (D). Sample gel showing resolution of the *rd8* homozygous (244bp), *rd8* heterozygous and WT (220bp) genotypes. GC – ganglion cells; INL – inner nuclear layer; ONL – outer nuclear layer; PIS/OS – photoreceptor inner segments/outer segments; RPE – retinal pigment epithelium.

Figure 2: *Ocular finding in AMD-like mice.* A. Fundus photographs from selected mice displaying multiple spots typical of the *rd8* phenotype. All mice with the genotype *rd8/rd8* had a similar appearance while wt/wt mice were identical to the $Ccl2^{-/-}Cx3Cr1^{-/-}gld$ mice shown. B. Histological finding in selected mice *rd8/rd8* and wt/wt mice. Note the retinal folding, dysplasia of the nuclear layers, retinal degeneration, and RPE vacuolation. Lesions of this type are typically focal in the eyes examined.

Figure 3: *Genotyping for rd8* (A) Genotyping of vendor mice by PCR for *rd8*. Bands were resolved by capillary electrophoresis. Mice are from Harlan Sprague-Dawley (HSD), DCT Frederick (DCT), Charles River (CRL), Taconic Farms (TAC) and Jackson (JAX). Genotyping for *rd8* was performed by PCR. Two representative mice out of 7 obtained from each vendor in 2 separate shipments are shown. (B) Sequencing of *Crb1* gene shows a single base deletion in the N substrain at the expected position in the aligned sequences (MegAlign program). N-MF, N substrain sequenced with forward primer; N-MR, N

substrain sequenced with reverse primer; J-MF, J substrain sequenced with forward primer; J-MR, J substrain sequenced with reverse primer.

Figure 4: *Vendor C57BL/6N mice, but not C57BL/6J mice, have fundus lesions.* (A–E) Fundus photographs of mice whose genotyping is shown in Fig 3A that were obtained from the specified vendors. Shown is one representative mouse out of 7 obtained from each vendor in 2 separate shipments. Note spotty lesions of varying extent in all mice except C57BL/6J. (F) C57BL/10J mice with normal fundi serve as control. (G) Histopathology. Shown are sections from affected DCT and Charles River C57BL/6N mice compared to normal JAX C57BL/6J mice. Note retinal lesions marked by arrows. Histology of other lesion-positive mice looked essentially identical.

Table 1

Association of ocular phenotype with *rd8* genotype in HLA-A29 Tg mice

Mouse line	**Number examined**	**Number with disease (percent)**	***rd8* genotype**[†]
HLA-A29 Tg Founders (B6N)*	5	5 (100%)	*rd8/rd8*
HLA-A29 Tg (B6NxB6N)	18	17 (94%)	*rd8/rd8*
HLA-A29 WT littermates (B6Nx B6N)	9	8 (89%)	*rd8/rd8*
HLA-A29 Tg (B6NxB10J)	20	5 (25%)	*rd8/wt*
HLA-A29 WT littermates (B6NxB10J)	25	5 (20%)	*rd8/wt*

*B6N – C57BL/6N; B10J – C57BL/10J

[†]Genotype shared by all mice within each category (phenotype-positive and negative)

TABLE 2

Association of ocular phenotype with *rd8* genotype in AMD-like model mice

Mouse line	Number examined	Disease*	*rd8* genotype†
Ccl2$^{-/-}$ *Cx3cr1*$^{-/-}$	80	Yes	*rd8/rd8*
Ccl2$^{-/-}$*Cx3cr1*$^{+/-}$	5	Yes	*rd8/rd8*
Ccl2$^{-/-}$	4	No	*wt/rd8*
Ccl2$^{-/-}$ (Jax)‡	3	No	*wt/wt*
Cx3cr1$^{-/-}$	6	Yes	*rd8/rd8*
Cx3cr1$^{+/-}$	2	No	*wt/rd8*
Ccl2$^{+/-}$*Cx3cr1*$^{+/-}$	4	No	*wt/rd8*
Ccl2$^{+/-}$ *Cx3cr1*$^{+/-}$	5	No	*wt/wt*
Ccl2$^{-/-}$ *Cx3cr1*$^{-/-}$ *Bid*$^{-/-}$	5	Yes	*rd8/rd8*
Ccl2$^{-/-}$ *Cx3cr1*$^{-/-}$ *Bid*$^{-/-}$	4	No	*wt/wt*
Ccl2$^{-/-}$ *Cx3cr1*$^{+/-}$ *Bid*$^{+/-}$	3	Yes	*rd8/rd8*
Ccl2$^{-/-}$ *Cx3cr1*$^{-/-}$ *Trail*$^{-/-}$	5	Yes	*rd8/rd8*
Ccl2$^{-/-}$ *Cx3cr1*$^{-/-}$ *Trail*$^{-/-}$	5	No	*wt/wt*
Ccl2$^{-/-}$ *Cx3cr1*$^{-/-}$ *gld/gld*	50	No	*wt/wt*
Ccl2$^{+/-}$ *Cx3cr1*$^{+/-}$ *gld/*$^{+}$	3	No	*wt/rd8*
B6-*gld* (Jax)‡	15	No	*wt/wt*
B6-*Trail*$^{-/-}$	10	No	*wt/wt*
B6-*Bid*$^{-/-}$	5	No	*wt/wt*
Ccl2$^{-/-}$ *Cx3cr1*$^{-/-}$ (C57BL/6J)°	10	No	*wt/wt*

*Disease criteria included 2 or more of the following: spots on fundus, retinal folds, photoreceptor degeneration, focal retinal lesions such as retinal angiomatous proliferation, photoreceptor dystrophy, RPE vacuolation, RPE degeneration.
†Genotype shared by all mice within each category

‡Purchased from Jackson labs.
°Backcrossed to C57BL/6J and determined to be 100% C57BL/B6J by microsatellite (SNP) analysis.

Table 3

Genotyping of C57BL/6N-derived ES cells

A. ES cells tested at NEI genetic core facility

Mouse	**ES cell line**	***Rd8* status**
129Sv hybrid	R1	*wt/wt*
129S6/SvEvTac	W4	*wt/wt*
C57BL/6N	JM8.N4 (KOMP)	*rd8/rd8*

B. ES cell lines tested at the Siteman Cancer Center (Washington University)

Mouse	**ES cell line**	***Rd8* status**
129Sv/J*	SCC10	*wt/wt*
C57BL/6N*	B6-Blu	*rd8/rd8*
C57BL/6N	EUCOMM	*rd8/rd8*
C57BL/6J*	B6-GFP	*wt/wt*
129svEv/6J*	EDJ22	*wt/wt*
129x129 hybrid*	R1	*wt/wt*
MJC (129)*	SCC10	*wt/wt*

* Mice used to generate ES cell lines for the Siteman Cancer Center ES Core were screened for the *rd8* mutation.

Figure 1 – HLA A-29 Tg mice (Birdshot-like)

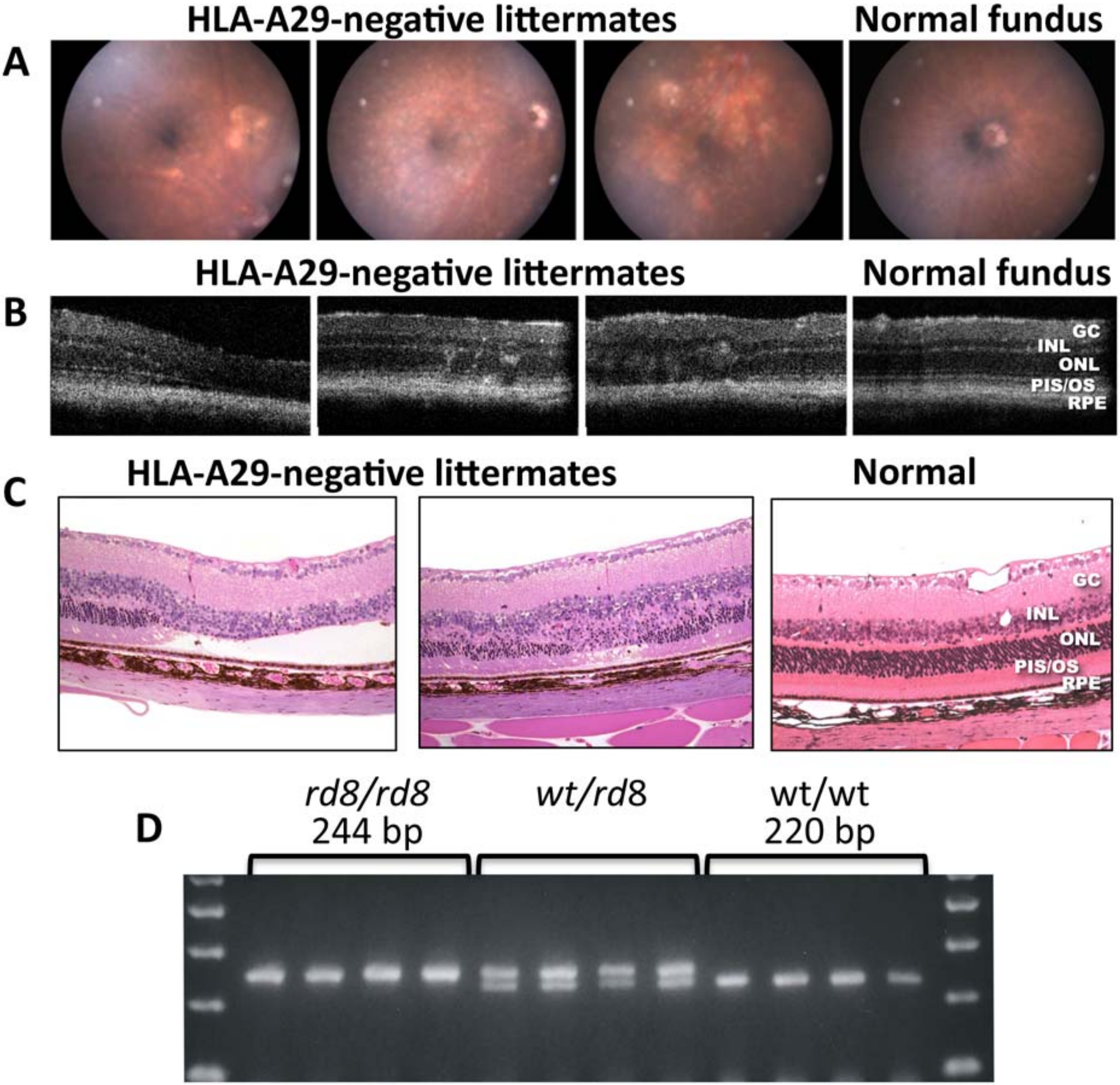


Figure 2 – CCL2/CX3CR1 KO (AMD-like mice)

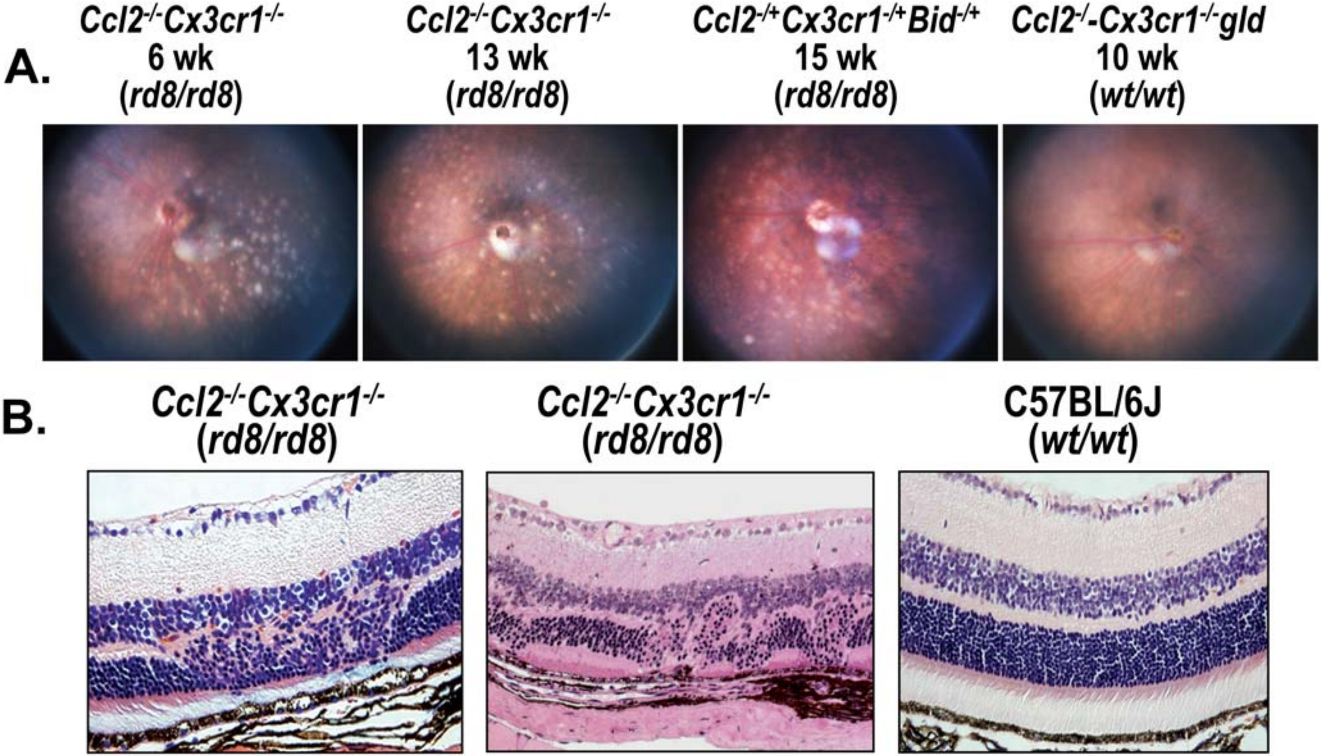
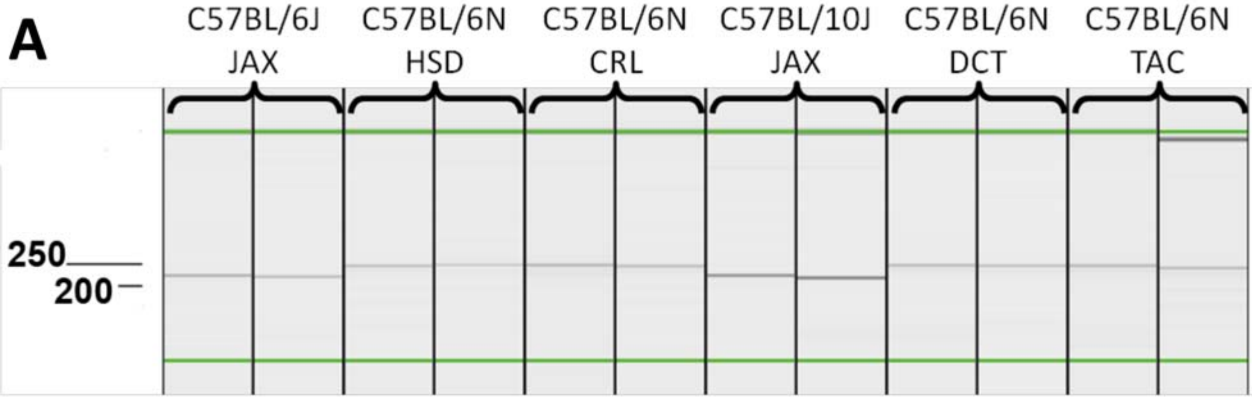


Fig 3: Vendor mice – genotyping for rd8

A



WT Allele 220 bp
rd8 allele 244 bp

B

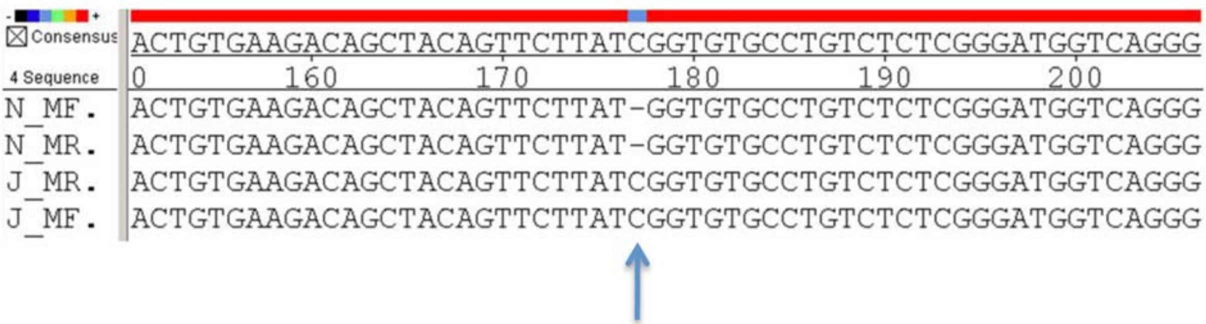


Figure 4 – vendor mice: fundus appearance & pathology