

Controversial view of a genetically altered mouse model of focal retinal degeneration

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Tuo et al. (2012) demonstrated tumor necrosis factor-inducible gene 6 recombinant protein (TSG-6) arrest of focal retinal lesions on a *Ccl2* and *Cx3cr1* double deficient mouse (DKO) on *rd8* background (hereon referred to as DKO *rd8*). DKO *rd8*, a model of focal retinal degeneration with earlier onset and higher penetrance than *Ccl2* and *Cx3cr1* single knockout strains, demonstrates characteristic features of AMD such as focal photoreceptor atrophy, retinal pigmented epithelium (RPE) degeneration, elevated ocular A2E levels and complement deposition in addition to retinal dystrophy. The discovery of the accidentally introduced *Crb1* mutation (*rd8*) in the C57BL/6N strain has led to the recent opinion that DKO *rd8* is not a model of AMD but solely a model of *Crb1*-associated retinal degeneration. Differences between DKO *rd8* and *Crb1^{rd8}* photoreceptor and RPE pathology, as well as increased A2E and immune dysfunction, show that DKO *rd8* recapitulates some AMD-like features in addition to *rd8* retinal dystrophy. The appearance of *rd8* lesions and *Ccl2/Cx3cr1* lesions and the amelioration of most *Ccl2/Cx3cr1* lesions in intervention studies show DKO *rd8* to be a useful and appropriate model for therapeutic compound screening, such as the case with anti-inflammatory TSG-6.

Introduction

Recently, Tuo et al. (2012) used a *Ccl2^{-/-}/Cx3cr1^{-/-}* double knockout (DKO) on C57BL/6N genetic background to show

tumor necrosis factor-inducible gene 6 recombinant protein (TSG-6) arrest of focal retinal lesions.¹ The DKO strain was generated as a model for focal retinal degeneration after report of senescent *Ccl2* single knockout mice developing key characteristics of human age-related macular degeneration (AMD) and the discovery of an association between *CX3CR1* single nucleotide polymorphisms and AMD.²⁻⁵ The *Ccl2* and *Cx3cr1* deficient DKO strain exhibits AMD-like features such as retinal pigmented epithelium (RPE) alteration, photoreceptor atrophy, immune activation and A2E elevation and thus has been suggested as an appropriate model for human AMD.^{6,7} As compared with independently-generated *Ccl2* and *Cx3cr1* single knockout strains, DKO shows earlier onset, higher penetrance and more lesions (Fig. 1). Therefore, this strain has served as a valuable tool for screening therapies for human AMD.

The recent finding of the accidentally introduced crumbs-like 1 (*Crb1*) mutation and *Crb1 rd8*-associated retinal dystrophy in the C57BL/6N background however, has challenged DKO as an AMD mouse model.^{8,9} CRB1 is thought to be involved in maintaining the integrity of the external limiting membrane and mutations in *CRB1* result in genetic retinal disorders such as retinitis pigmentosa and Leber congenital amaurosis in humans.¹⁰ The *Crb1^{rd8}* mutation results in irregular retinal lesions in the inferior nasal quadrant of the fundus and thus is suggested to have potential implications for all ocular vision research models on C57BL/6N background, including DKO (hereon referred to as DKO *rd8*).

Keywords: age-related macular degeneration, TSG-6, *Ccl2*, *Cx3cr1*, *rd8*, animal model

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Although it is critical to create retinal degeneration on *rd8* background, the DKO *rd8* mice develop not only retinal dystrophy (a few, mild *Crb1^{rd8}* lesions in C57BL/6N mouse) but also additional focal retinal degenerative lesions. While the retinal lesions in these mouse strains may appear clinically similar (Fig. 1), differences in photoreceptor and RPE pathologies, A2E levels and immune response with *Crb1^{rd8}* mice show DKO *rd8* to be more consistent with human AMD than with solely *Crb1^{rd8}*-associated retinal diseases.

Differences in DKO *rd8* and *Crb1^{rd8}* Pathology

Analysis of C57BL/6 mouse lines and embryonic stem cells revealed the presence of the *Crb1* mutation in homozygous form in the C57BL/6N strain, but not the C57BL/6J strain.⁸ This finding indicates a “founder effect” mutation in C57BL/6N, suggesting that transgenic and knockout models created on the C57BL/6N background carry the *Crb1* mutation and thus may present *rd8*-associated pathology.⁸ *Crb1^{rd8}*-associated pathology is characterized by fundus lesions in the inferior nasal quadrant corresponding to abnormal Müller cells, retinal folds and pseudorosettes between the inner and outer nuclear layers. Within these retinal dystrophic lesions there is shortening and disarray of focal photoreceptor inner and outer segments (Fig. 2C).¹¹ It is thought that the *rd8* mutation results in truncation of the transmembrane and cytoplasmic domain of Crb1, disrupting the adhesion of adherens junction proteins to the external limiting membrane (ELM, comprised of zonula adherens that link photoreceptors and Müller cells) and affecting the development of photoreceptor inner and outer segments.¹¹ In the *Crb1^{rd8}* mouse, loss of adherens junctions and the Müller cell apical processes underlying the junctions of the ELM is observed. Crb1 is largely absent or diffuse in Müller cell apical processes, which are aberrantly prominent in the inner nuclear layer. Preservation of normal inner and outer segment length is seen in areas adjacent to the region of degeneration.¹¹ These

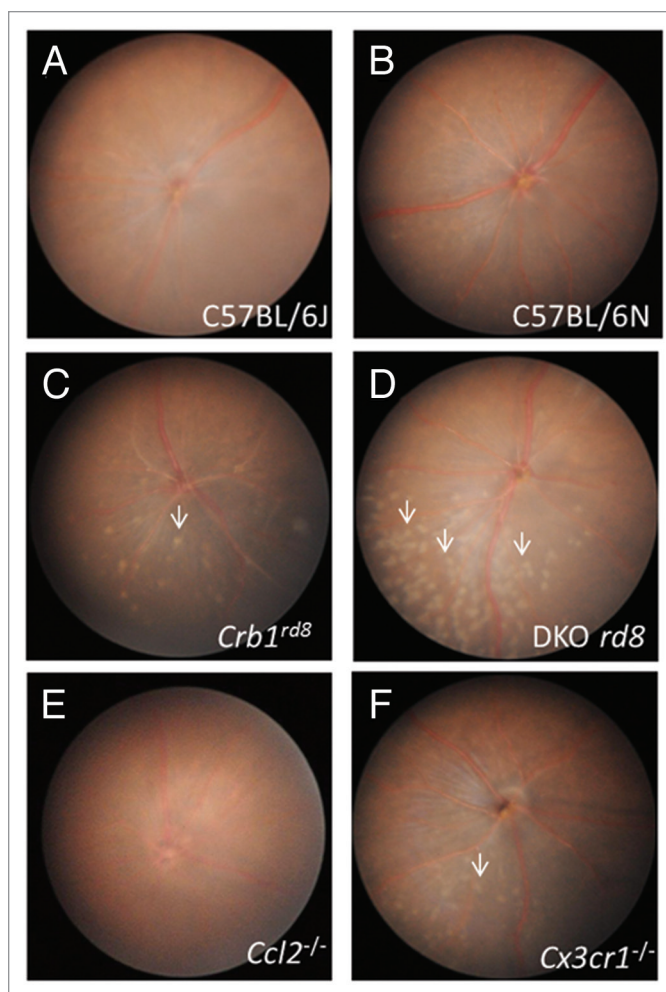


Figure 1. Fundus photos of C57BL/6J, C57BL/6N, *Crb1^{rd8}*, DKO *rd8*, *Ccl2^{-/-}*, *Cx3cr1^{-/-}* at two months. (A) C57BL/6J shows no phenotype, (B) C57BL/6N shows very little phenotype, (C) *Crb1^{rd8}* displays a few, small retinal lesions in the inferior nasal quadrant. (D) DKO *rd8* shows focal retinal lesions at a greater number than *Crb1^{rd8}* and the *rd8*-containing C57BL/6N. (E) *Ccl2^{-/-}* exhibits no lesions. (F) *Cx3cr1^{-/-}* has retinal lesions that are greater in number than *Crb1^{rd8}*, but markedly less than DKO *rd8*.

findings suggest the primary pathology of *Crb1^{rd8}* mice to be developmental defects in Müller cells and photoreceptor inner and outer segment shortening, resulting in the secondary pathology of retinal dysplasia and degeneration of focal photoreceptors. Thus, *Crb1^{rd8}* is a model of retinal dystrophy resulting from malformed Müller cell apical villi.¹²

DKO *rd8* mice also show photoreceptor pathology (Fig. 2D).⁶ However, in addition to photoreceptor dystrophic lesions, RPE degeneration appears to be a featured pathological event in DKO *rd8*. Whereas retinal folds and pseudorosettes are frequently seen in *Crb1^{rd8}* mice (Fig. 2C), these retinal lesions are seldom found in DKO *rd8* (Fig. 2D).

While differences in mouse and human physiology limit the formation of drusen proper,¹³ infrequent appearance of drusen-like lesions in DKO *rd8*, which has not been reported in *Crb1^{rd8}* mice, demonstrate distinct retinal pathology between the two strains.¹⁴ In general, C57BL/6N mice seem to have much less severe retinal phenotypes with exception of the foci of mild retinal dystrophy (Fig. 2B) compared with the *Crb1^{rd8}* mice (Bo Chang, personal communication).

In addition to photoreceptor degeneration, RPE alteration is a cardinal feature of human AMD.^{15,16} One of the prominent histopathologies in DKO *rd8* is abnormal RPE cells, which display hypopigmentation, depigmentation, vacuolation and

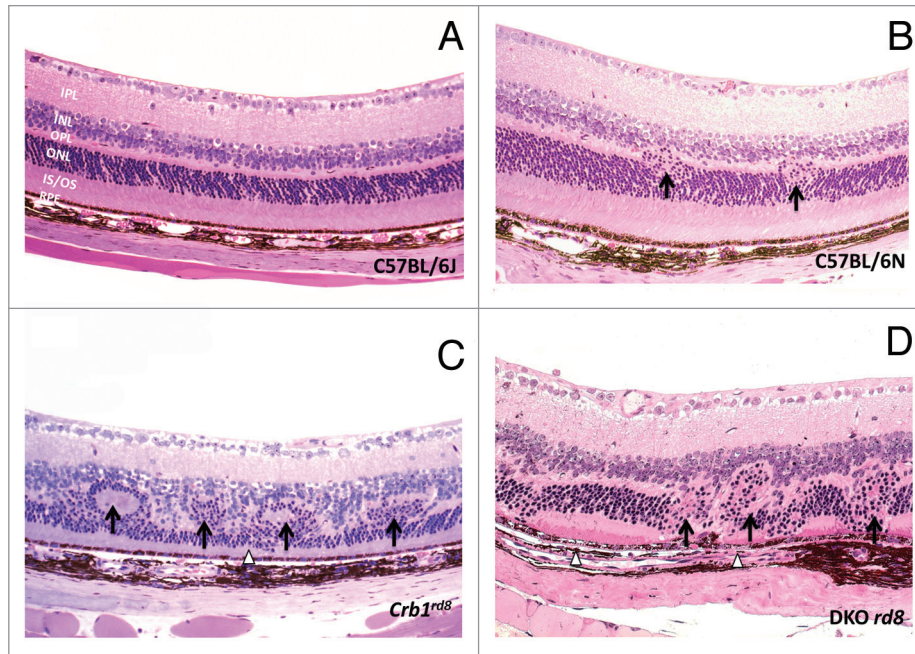


Figure 2. Photomicrographs of C57BL/6J, C57BL/6N, *Crb1^{rd8}* and DKO *rd8* retina. (A) C57BL/6J strain that does not carry the *rd8* mutation shows normal ocular architecture. (B) The C57BL/6N strain displays small *rd8*-associated dystrophic lesions showing migration of the ONL into the OPL (arrows), preserved photoreceptor IS/OS, and intact RPE. (C) *Crb1^{rd8}* displays pseudorosettes and retinal folds in addition to retinal dystrophy involving the inner and ONL (arrows) and thinning of IS/OS (arrowhead). (D) In addition to the *rd8*-associated lesions observed in C57BL/6N, DKO *rd8* shows severe ONL disorganization (arrows), focal loss of IS/OS, and hypopigmentation and vacuolation of RPE (arrowheads). IPL, innerplexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS/OS, inner segments and outer segments; RPE, retinal pigmented epithelium. (hematoxylin and eosin, original magnification, 200 \times).

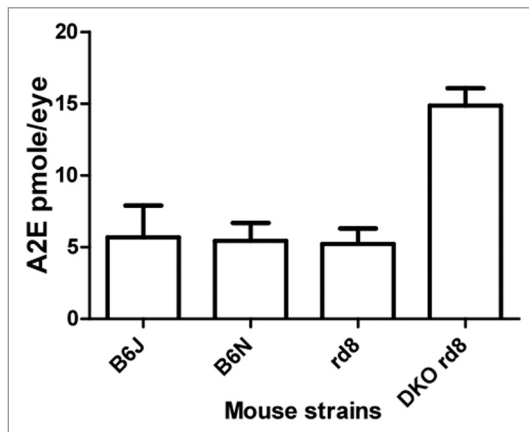


Figure 3. Quantification of A2E in C57BL/6J, C57BL/6N, *Crb1^{rd8}* and DKO *rd8* strains at 2 mo. DKO *rd8* shows an A2E increase of more than 3-fold compared with the other three strains. * $p < 0.05$ in DKO *rd8* compared with age-matched C57BL/6J, C57BL/6N and *Crb1^{rd8}*.

atrophy.^{6,14} Contrastingly, RPE histology is relatively preserved in *Crb1^{rd8}* mice and in patients with Leber congenital amaurosis (LCA), a disease associated with *Crb1* mutation, even in the advanced stages.¹⁰ These observations suggest that *Crb1^{rd8}*-associated and DKO *rd8*-associated pathologies may not operate through identical mechanisms.

Elevated Lipofuscin Biochemical Marker A2E in DKO *rd8*

Accumulation of the lipofuscinfluorophore A2E, a well-known biochemical marker of human AMD, is seen in DKO *rd8*. A2E accumulation is associated with retinal aging and is known to activate the complement system as well as induce

RPE apoptosis and neurodegeneration.¹⁷⁻¹⁹ Compared with age-matched C57BL/6N mice, a 3- to 4-fold increase of ocular A2E is seen in DKO *rd8*.^{6,14} Contrastingly, *Crb1^{rd8}* and C57BL/6J mice do not show elevated A2E levels (Fig. 3). The generation of A2E is considered to be the consequence of burst oxidative stress that is not related in mechanism to the function of Crb1, a component of the molecular scaffold.²⁰

Immunopathology of DKO *rd8*

The DKO *rd8* model expresses a spectrum of immunological features of AMD. Increased deposition of complement factors, altered secretion of chemokines and cytokines, and the accumulation of activated microglia and macrophages in retinal lesions have been linked to human AMD and can correspondingly be demonstrated in the DKO *rd8*. Expression of CD46, a regulatory protein of complement proteins C3 and C4, suggests the presence of activated complement.⁶ Elevated expression of C3d is seen in

RPE and photoreceptors of DKO *rd8* and the recruitment of microglia and macrophages in subretinal and retinal lesions is also observed.²¹ Consistent with AMD patients, DKO *rd8* mice demonstrate increased expression of anti-retinal autoantibodies.²¹

Moreover, the DKO *rd8* strain shows greater macrophage infiltration and complement factor deposition than *Crb1^{rd8}* or C57BL/6N (Fig. 4A and B), suggesting that immune dysfunction or activation of innate immunity, which is documented in human AMD, seen in DKO *rd8* cannot reasonably be accounted for by the *rd8* mutation alone. These results suggest that the immunopathology seen in DKO *rd8* should not be thought of as solely secondary immune response to *rd8*-associated retinal dystrophy, and indicate that DKO *rd8* recapitulation of AMD-like immunopathology is not the same as *rd8*-mediated immunopathology.

Ccl2^{-/-}/Cx3cr1^{-/-} and *Crb1^{rd8}* Interaction

As evidenced by differences in photoreceptor atrophy and RPE degeneration, DKO and *Crb1^{rd8}* pathology are distinct. *Ccl2^{-/-}/Cx3cr1^{-/-}* deficiency and the *rd8* mutation thus produce two different types of lesions, which, despite arising from distinct mutations, nonetheless interact and modify each other at some level. Independently established *Ccl2^{-/-}/Cx3cr1^{-/-}* double deficient mice on B6J background could not replicate the retinal phenotypes observed in our DKO *rd8*; interestingly, these mice can develop retinal AMD-like lesions under oxidative stress (HepingXu, EVER abstract, 2012). However, it has been reported that *Ccl2* and *Cx3cr1* single deficiency generate a retinal AMD phenotype in aging mice and additionally, that only 81% of *rd8* mice show a retinal phenotype compared with 100% of DKO *rd8* mice.^{6,8,14} This suggests that *Ccl2* and *Cx3cr1* double deficiencies may add other lesions to the early onset retinal dystrophy caused by *rd8* alone. A recent study of *Ccl2* and *Cx3cr1* single knockouts reports that despite similar accumulation of subretinal macrophages in both strains, *Ccl2* and *Cx3cr1* differentially modulate AMD-like lesions. Particularly, it is suggested that

Cx3cr1 results in more pronounced phenotype of *rd8*-associated degeneration, a finding that was maintained in the *Ccl2* and *Cx3cr1* double knockout (HepingXu,

EVER abstract, 2012, Ulrich F. Luhmann, EVER abstract 2012).

Crb1^{rd8} mice primarily show retinal lesions at the ELM whereas DKO *rd8* show

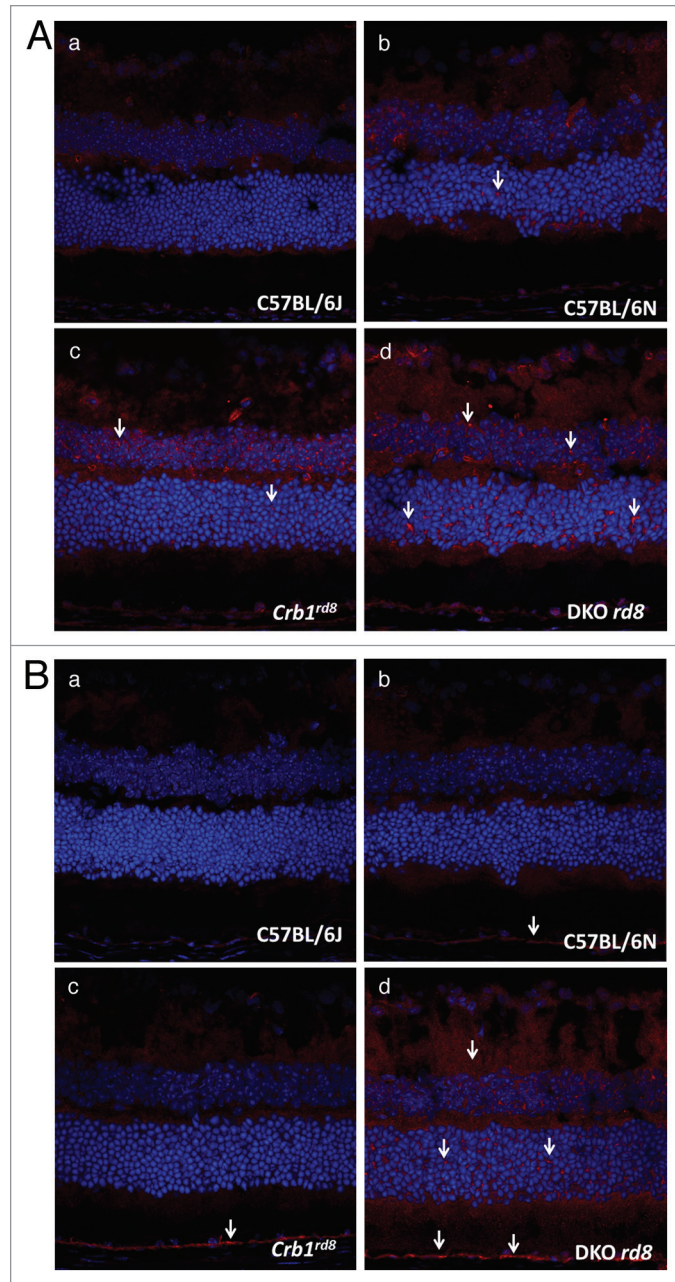


Figure 4. (A) Photomicrographs of macrophages (arrows) in C57BL/6J, C57BL/6N, *Crb1^{rd8}* and DKO *rd8* retina detected by immunohistochemistry and confocal microscopy. (A) There are few macrophages in C57BL/6J wild-type mouse. (B) C57BL/6N shows increased macrophages compared with wild-type. (C) *Crb1^{rd8}* displays more macrophage infiltration relative to C57BL/6N. (D) DKO *rd8* exhibits greater macrophage infiltration than *Crb1^{rd8}* and C57BL/6N. (original magnification, 400x). (B) Photomicrographs of complement C3d (arrows) in C57BL/6J, C57BL/6N, *Crb1^{rd8}* and DKO *rd8* retina detected by immunohistochemistry and confocal microscopy. (A) No complement C3d in C57BL/6J wild-type mouse. (B) C57BL/6N shows minimal C3d deposition in RPE compared with wild-type. (C) *Crb1^{rd8}* displays elevated C3d deposition in RPE relative to C57BL/6N. (D) DKO *rd8* exhibits greater C3d deposition than *Crb1^{rd8}* and C57BL/6N, significantly in the RPE and neuroretina. (original magnification, 400x).

lesions in retinal inner and outer segment layer, suggesting that *Ccl2* and *Cx3cr1* double deficiencies could play a role in the high penetrance of rapid photoreceptor and RPE degeneration seen in DKO *rd8*. Intervention studies conducted on DKO *rd8* show reduction or stabilization of these deeper retinal lesions but may have little or no effect on *rd8* lesions in the ELM.^{13,22,23} Study of an independently-generated *Ccl2*^{-/-}/*Cx3cr1*^{-/-}/*Crb1*^{rd8/rd8} strain by Luhmann et al. discredited DKO *rd8* as a model for AMD, citing the primary role of *rd8* in the observed early onset retinal degeneration.⁹ While the *rd8* mutation may contribute critically to photoreceptor degeneration, the RPE pathology, immune dysfunction, and A2E accumulation as manifested in DKO *rd8* still require further explanation. Thus, interaction between *Ccl2*, *Cx3cr1* and *rd8* originated from C57BL/6N is possible and further investigation is needed to elucidate the exact mechanisms and how they fit in to a model for AMD.

DKO *rd8* as a Model for AMD

When discussing the implications of the *rd8* mutation in the DKO *rd8* mouse model, consideration must be given to the influence of the genetic background of any engineered mouse model for disease. The generation of knockout mice using 129 and C57BL/6N strains exposed the model to the possibility of “passenger” genes, residual regions of 129-derived genetic

material that were transported along with the “knocked out” gene onto C57BL/6N background, having created potential sites of genetic variability.²⁴ Genetic confounders arising from traditional knockout technology using 129 and C57BL/6 may have substantial impact on phenotype, as the two mouse strains have significant genetic and physiological differences and 129 is known to have a multitude of its own mutations.²⁴

Currently, there is no clear model for recapitulating the full clinical features of AMD.⁷ It must be taken into consideration that mice lack macula and thus, no murine model can ever fully recapitulate human AMD. Oxidative damage models dependent on phototoxicity or high fat diet alone or in combination do not show all characteristic RPE changes, increased levels of A2E or complement deposition.^{25,26} Induced choroidal neovascularization using matrigel, laser photocoagulation or elevated VEGF expression fail to model mechanisms behind the presentation of disease and thus do not result in increases of A2E or complement deposition.²⁷⁻³⁰ Immunization with carboxyethylpyrrole (CEP)-adducted protein in mice shows RPE alteration and complement deposition, yet retinal lesions independent of macrophage accumulation are observed and choroidal neovascularization does not develop in this model.^{31,32} Nonetheless, a growing body of evidence implicating AMD as an immunological

disease necessitates that any mouse model feature immunopathological mechanisms.^{33,34} While the genetic roles and interactions of *Ccl2*, *Cx3cr1* and *rd8* are not fully elucidated, the recapitulation of certain features of human AMD can be demonstrated in DKO *rd8*. Within known AMD mouse models, DKO *rd8* is a model to date that demonstrates focal RPE alteration, photoreceptor atrophy, enhanced A2E accumulation, and dysregulation of the immune system.^{6,14,34}

While the identification of *rd8* and *rd8*-associated retinal phenotype does impact the power of DKO *rd8* as a model for AMD, the use of the model as self-control by treating one eye and using the contralateral eye as the control allows for appropriate experimental interventional studies. Thus, DKO *rd8* may still serve as a suitable and relevant model for compound screening on focal retinal degenerative diseases including AMD. In intervention studies on DKO *rd8*, treatment is shown to alleviate photoreceptor and RPE lesions that can be attributed to *Ccl2* and *Cx3cr1* double deficiencies while leaving *rd8* lesions unaffected. Such is the case with the report by Tuo (2012) that TSG-6 arrested lesions in the retina.

Disclosure of Potential Conflicts of Interest

The authors state no conflicts of interest.

Acknowledgments

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