

Genetic ablation of retinal pigment epithelial cells reveals the adaptive response of the epithelium and impact on photoreceptors

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The retinal pigment epithelium (RPE) plays a critical role in the maintenance of the outer retina. RPE cell death or dysfunction drives the pathophysiology of many retinal diseases, but the physiological response of the retina to RPE cell loss is poorly understood, mainly because of the absence of suitable experimental models. Here, we generated a transgenic mouse in which an inducible Cre recombinase is expressed exclusively in the RPE under the control of the monocarboxylate transporter 3 gene promoter (RPE^{CreER}). This was crossed with a transgenic mouse harboring a diphtheria toxin A (DTA) chain gene rendered transcriptionally silent by a floxed stop sequence. We show that activation of DTA in the double transgenic mouse (RPE^{CreER}/DTA) led to 60–80% RPE cell death, with surviving cells maintaining the integrity of the monolayer by increasing their size. Despite the apparent morphological normality of the enlarged RPE cells in the RPE^{CreER}/DTA mice, functional analysis revealed significant deficits on electroretinography, and retinal histopathology showed regions of photoreceptor rosetting and degeneration although with retention of a normal vascular network. Our study reveals that whilst the RPE monolayer has a remarkable intrinsic capacity to cope with cellular attrition, specific aspects of RPE multifunctionality essential for photoreceptor survival are compromised. The RPE^{CreER}/DTA mouse offers advantages over models that employ chemical or mechanical strategies to kill RPE cells, and should be useful for the development and evaluation of RPE-based therapies, such as stem cell transplantation.

cell death | pigment | retina

The retinal pigment epithelium (RPE) is a monolayer of multifunctional epithelial cells that physically abuts the photoreceptor layer and effectively forms the posterior blood–retinal barrier. RPE cells contain photoprotective pigment granules, phagocytose shed photoreceptor outer segments (POS), recycle 11-cis retinal, and regulate ion and nutrient fluxes between the outer retina and choriocapillaris (1). Defects in any of these essential activities may cause secondary photoreceptor dysfunction, retinal degeneration and blindness. For example, humans and rodents that lack the Mer tyrosine kinase exhibit severely defective phagocytosis of POS and undergo rapid retinal degeneration (2, 3), and mutation or loss of RPE65 in patients with Leber's Congenital Amaurosis leads to failure of the visual cycle and progressive retinal degeneration (4, 5). Although these are relatively rare conditions, the many energy-dependent activities of RPE cells create a metabolic burden in normal individuals that increases with age and is manifested in the accumulation of insoluble waste products, such as the toxic bis-retinoid lipofuscin (6). In humans this leads to a decrease in RPE cell numbers with age (7, 8), whereas age-related photoreceptor loss appears to correlate more specifically with lipofuscin levels in the underlying RPE (9).

Various experimental models have been used to investigate the effects on the retina of RPE loss, stress, or dysfunction. The

principle methods used to kill RPE cells have been chemical ablation using sodium iodate, and mechanical debridement to physically remove the RPE. Several distinct responses to sodium iodate have been reported, including loss of adhesion between the retina and RPE (10), photoreceptor degeneration (11) and atrophy of the choriocapillaris (12). Some of these features have also been reported following surgical debridement of the RPE, for example, pathological changes to the choriocapillaris in rabbits and cats (13–15). Photoreceptor degeneration following loss of RPE occurs regardless of method used, but RPE regrowth has been noted only following debridement in pig eyes (16). Both experimental strategies achieve RPE loss, but may also induce off-target effects or indirect responses such as inflammation following necrotic cell death (11). RPE proliferation observed upon debridement most likely occurs as a consequence of the procedural retinal detachment, as in proliferative vitreoretinopathy, where loss of the intimate physical association with the photoreceptors permits RPE cells, even while contact inhibited, to enter the cell cycle (17, 18).

To circumvent these problems, we have developed a mouse model in which conditional genetic ablation of the RPE leads to a rapid and profound reduction in RPE cell number. We find that the RPE monolayer is highly adaptive in its ability to compensate for significant individual cell loss, maintaining an effective blood–retinal barrier and normal monolayer thickness in the absence of significant cell division. However, other RPE-dependent activities necessary for normal photoreceptor function are impaired leading to anatomical and functional changes in the retina. Our model provides insight into the relationship between RPE cells and retina, and should be useful for studies aimed at repopulating or replacing RPE following death or dysfunction in disease.

Results

Cre Recombination Activity in RPE^{CreER} Mice. To genetically target the RPE we made transgenic mice that express a tamoxifen-inducible form of Cre recombinase (iCreER^{T2}) (19–21) under the transcriptional control of a gene that is specifically expressed in the RPE. It has been previously shown that the monocarboxylate transporter 3 (Mct3, also known as Slc16a8) is only expressed in two cell populations, RPE in the eye and choroid plexus epithelium in the brain (22, 23). We therefore obtained a bacterial artificial chromo-

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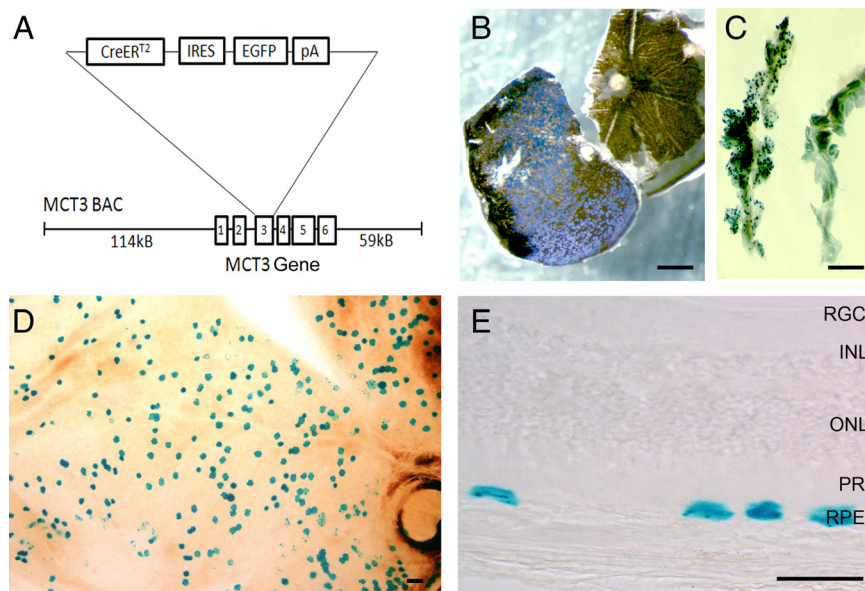


Fig. 1. Generation and testing of RPE-specific CreER mice. (A) Schematic representation of the construction of the BAC incorporating the CreER^{T2} under the expression control of the Mct3 gene. (B) RPE/Sclera from neonate animals with the CreER Lac Z transgene (bottom left) showed approximately 20% of the RPE cells were positive, and an absence of any positive X-gal dye in non-transgene animals (top right). (C) Similarly, a population of X-gal positive cells was found in choroid plexus epithelium of CreER Lac Z transgenics (left) compared to controls (right). (D) Induction of Cre-recombinase activity in adult animals (6 weeks old) was less pronounced. The pigment of the RPE cells has been bleached to enable visualization of the X-gal dye product. (E) A cryosection (20 μm) cut transversely from a Lac Z-Mct3-Cre retina, incubated with X-gal dye, demonstrates the expression of the Lac Z specifically within the RPE cells. RGC-retinal ganglion cells, INL inner nuclear layer, ONL outer nuclear layer, PR photoreceptor layer, RPE retinal pigment epithelium. (Scale bar B and C, 500 μm; scale bar D and E, 100 μm.)

some (BAC) containing the Mct3 gene, placed the coding sequence for iCreER^{T2} into its ORF (Fig. 1A) using homologous recombination in *E. coli* (24) and used it to make transgenic mice by pronuclear injection. The BAC contained about 114kb genomic sequence upstream and about 59kb downstream of the Mct3 gene and, due to its large size, is likely to contain all of the regulatory elements required to replicate endogenous Mct3 expression.

Cre recombination activity in the resulting mouse strain was tested in a ROSA-lacZ reporter background (25). In this reporter strain a loxP-flanked stop cassette normally prevents the expression of lacZ, but upon Cre activation via systemic tamoxifen delivery, the stop cassette is removed and lacZ is expressed, reporting Cre recombination activity. In the first instance, we induced newborn RPE^{CreER} × ROSA-lacZ mice with a single injection of 4-hydroxytamoxifen (the metabolically active form of tamoxifen). After 1 week, lacZ expression was assessed in sclera/RPE whole mounts. We found strong staining in a subpopulation (approximately 20%) of RPE cells in mice containing the iCreER^{T2} transgene but not in iCreER^{T2}-negative littermates (Fig. 1B). We also observed lacZ expression in choroid plexus epithelium in the brain (Fig. 1C), confirming Mct3 specific expression of the transgene. We then induced Cre activity in adult mice (6 weeks old) because an adult experimental paradigm is more relevant for human RPE dysfunction, which typically displays adult onset. Two weeks after tamoxifen administration we found that recombination had occurred in approximately 5% of RPE cells, revealing lower recombination efficiency in adult than in neonatal animals (Fig. 1D). Cross sections demonstrated that recombination activity was restricted to the RPE and completely absent from the retina (Fig. 1E).

Genetic Ablation of the RPE. To recreate certain aspects of RPE pathology in human eye diseases we utilized the RPE^{CreER} mice to genetically ablate RPE cells. To this end we generated RPE^{CreER}/DTA mice by crossing the RPE^{CreER} strain with mice that conditionally express diphtheria toxin A chain (DTA) under a ubiquitous promoter (26) (Fig. 2A). In this and all subsequent experiments tamoxifen was administered to adult animals (see Table S1 for details of ages). After tamoxifen administration we used an antibody against ZO-1 (visualising tight junctions and the outline of RPE cells) to assess changes in the RPE. Six days after tamoxifen administration numerous individual RPE cells became rounded, lost their well defined ZO-1 staining, and were extruded toward the retina (Fig. 2B). Two weeks after tamoxifen application the re-

maining RPE cells appeared enlarged and irregular in shape with clumps of extruded cells in the subretinal space (Fig. 2C and D and Fig. S1). In comparison to the ROSA-LacZ background the percentage of affected cells was significantly higher in the ROSA-DTA background with an average 68% reduction of RPE cell numbers after 14 days (Fig. 2E). However, after the initial wave of RPE loss cell numbers stabilized and remained unchanged between 14 days and 6 months (Fig. S2). RPE flat mounts from animals 2 weeks post-tamoxifen and wild-type littermates revealed that dead cells did not leave gaps in the continuity of the epithelium. Instead, surrounding cells dramatically increased their size to prevent holes appearing (Fig. 2F). The RPE^{CreER}/DTA cells were found to be more irregularly shaped (Fig. 2G) with significantly longer perimeters (Fig. 2H) and a significant reduction in the total length of junctional interfaces between cells, measured by assessing the perimeter:area ratio (Fig. 2I). The impact on cell-cell junctions was also investigated by Western blotting protein fractions isolated from the RPE cells of both RPE^{CreER}/DTA and wild-type control eyes (Fig. 2J). An average 42% reduction in the amount of ZO-1 was observed (Fig. 2K) ($P < 0.001$), which corresponds to the reduction in the 43% perimeter:area ratio calculated from the RPE flat mount analysis (Fig. 2I). In addition, there were significant reductions in the relative levels of RPE65 and MerTK in the tamoxifen-treated animals (Fig. 2L–O) suggesting possible deficits in the visual cycle and phagocytosis respectively, two aspects of RPE cell function vital for photoreceptor survival.

Ultrastructural Changes in Surviving RPE Cells. Since the average diameter of surviving RPE cells was significantly increased, we tested whether the cell spreading was associated with a reduction in cell thickness. Electron microscopy (EM) was performed on wild-type (Fig. 3A) and RPE^{CreER}/DTA (Fig. 3B) retinas to measure the thickness of the RPE cells. Surprisingly, there was no difference in RPE cell thickness (Fig. 3C) indicating that the fold-increase in cell ‘footprint’ corresponds directly to an increase in cell volume. One of the functions of the RPE cell is to phagocytose shed photoreceptor outer segments. The EM images of the retinas allowed us to quantify the number of phagosomes per unit length of RPE (Fig. 3D) and the cellular localization (apical versus basal) of phagosomes (Fig. 3E). The cellular localization gives an insight into phagosome processing and thus provides an indication of the functionality of the RPE cell. Despite the reduced level of MerTK expression (Fig. 2N), there was no significant difference in the

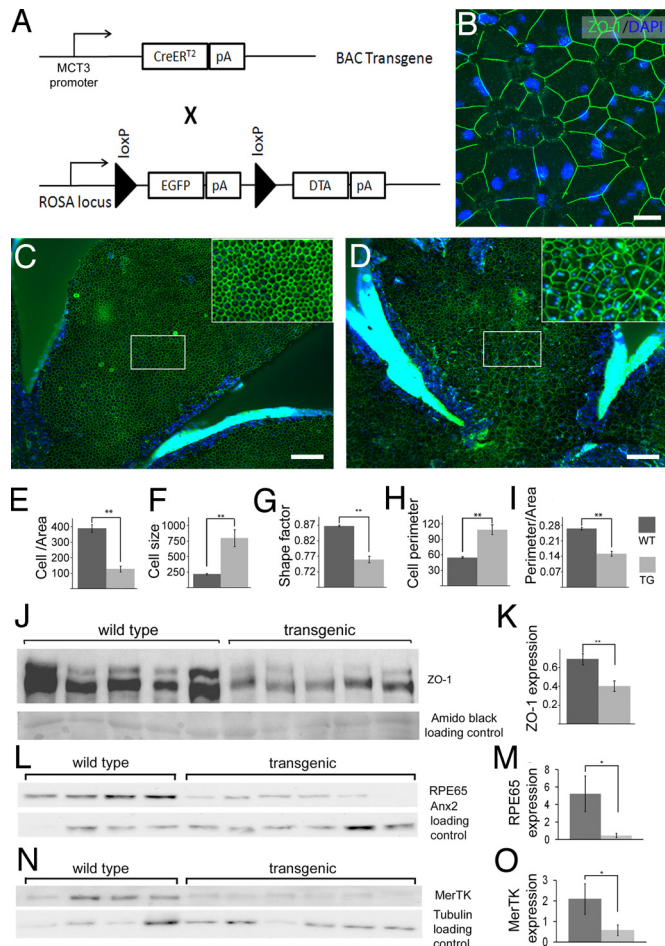


Fig. 2. RPE flat mount morphological analysis. (A) The cross of Mct3-CreER² and the ROSA-DTA-loxP cross animals schematic is shown. RPE flat mounts stained with DAPI (Blue) and ZO-1 (green) from animals expressing the DTA 1 week (B) and 2 weeks [C (WT) and D (TG)] post-tamoxifen administration. The insets (C and D) show a higher magnification of the area in the corresponding white rectangle, allowing better visualization of the change in morphology of the RPE cells in the transgenic (TG) animals. Morphometric analysis revealed, in the transgenic animals, a significant decrease in the number of cells per unit area [(E), $P < 0.000005$], an increase in cell size [(F), $P < 0.0018$], a reduction in shape factor [(G), $P < 0.000001$], an increase in average cell perimeter [(H), $P < 0.0002$] and a reduction in total length of junctional interface (perimeter) per unit area [(I), $P < 0.0000038$]. The amount of ZO-1 protein in the RPE fraction from TG retina was measured by Western blotting (J) (5WT, 5TG retinas blotted), densitometric scanning revealed a significant reduction in the amount of ZO-1 protein in the TG RPE fractions (K, $P < 0.001$). RPE65 (L) and MerTK (M) protein levels in the RPE fractions were also measured by Western blotting (four WT, six TG retinas blotted), densitometric scanning revealed a significant reduction in the amount of both RPE65 protein [(M), $P < 0.009$] and MerTK [(O), $P < 0.02$] in the TG RPE fractions. (All error bars S.E.M., ** indicates $P < 0.001$). (Scale bar B, 20 μ m; scale bar C and D, 200 μ m).

number of phagosomes per unit length of RPE. However, there was an increase in the proportion of phagosomes in the basal half of the RPE cells in the RPE^{CreER}/DTA retinas when compared to wild-type littermate controls, suggesting that phagolysosomal fusion and processing may be defective in these animals.

Morphological Changes in the Retina. Semithin sections and immunohistochemical techniques were used to assess the retinal morphology of the RPE^{CreER}/DTA mice. These analyses revealed varying degrees of morphological abnormality in the RPE^{CreER}/DTA mice, the major change being the appearance of retinal folds termed rosetting. The extent of rosetting varied between animals,

with some severely (Fig. 3H) and some mildly affected (Fig. 3G) in comparison to a wild-type (Fig. 3F) retina. We also immunostained sections of retina using protein markers whose expression lies both within and outside the areas that are morphologically altered in the RPE^{CreER}/DTA retinas. Phalloidin was used to visualize the F-actin distribution throughout all of the layers of the retina (Fig. S3A and B). The distribution of F-actin, although altered at the anatomical level, was consistent at the cellular level with the pattern of F-actin expression in wild-type retinas. Thus, regions of enrichment are still aligned with the same cellular boundaries, principally flanking the photoreceptor inner segments. PKC α (Fig. S3C), cone transducin (Fig. S3D), and mGluR6 staining were unaffected in the RPE^{CreER}/DTA retinas (Fig. S3E). There was however a change in the expression of Müller cell glial fibrillary acidic protein (GFAP), with an increase in staining in the inner plexiform and inner nuclear layers of the retina (Fig. S3F), consistent with retinal stress (27). We also observed staining for macrophages/microglia in the RPE^{CreER}/DTA retinas especially in the subretinal space and outer retina (Fig. S3G). This, however, did not correlate with the appearance of deep leakage (Fig. S3H) and indicates that RPE barrier function is maintained even during significant cell loss. Histological assessment of the retinas of the RPE^{CreER}/DTA mice at 6 months revealed no vascular anomalies despite clear alterations to the RPE and outer nuclear layer, and a normal vascular network in the RPE^{CreER}/DTA mice was confirmed in retinal flat mounts (Fig. S4).

RPE Stress Reduces Visual Function. To assess the effects of RPE cell death on photoreceptors, we measured rhodopsin levels and investigated visual function in RPE^{CreER}/DTA mice 2 weeks following tamoxifen induction. Rhodopsin levels were measured by spectral analysis of pre- and postbleach visual pigment absorbance, which revealed no significant difference in the amount of pigment extracted from the RPE^{CreER}/DTA animals in comparison to control animals (Fig. 4). Electroretinography (ERG) was used to assess the functional responsiveness of the neural retina of the RPE^{CreER}/DTA mice, using full-field flash stimuli under scotopic and photopic conditions. Representative traces from wild-type (Fig. 5A and D) and RPE^{CreER}/DTA mice [Fig. 5B and E (1 animal) and Fig. 5C and F (1 animal)] illustrate the range of responses observed following RPE cell ablation. Under scotopic conditions the ERG responses are principally produced by the rods, which in the RPE^{CreER}/DTA mice revealed significantly reduced a wave ($P < 0.011$) and b wave ($P < 0.005$) amplitudes in comparison to wild-type controls (Fig. 5G and H). After a period of light adaptation, cone function was assessed under photopic conditions (Fig. 5I), but there was no significant difference in the b wave amplitudes in the two experimental groups. At the highest light intensity tested scotopically, log intensity 1, the initial a wave slope, which was taken within the first 10 ms of the ERG response, was significantly reduced in the RPE^{CreER}/DTA animals (Fig. 5J). These changes in ERG amplitudes were neither a transient response to RPE cell loss nor a prelude to further decline, remaining stable for at least a further 6 months (Fig. S5). Scotopic responses from the wild-type animals were of comparable amplitudes to those previously reported in C57B/6 animals (28). The reduction in scotopic amplitude and a wave gradient indicate that the functionality of the rod photoreceptors is compromised, although in many animals this was in the absence of any detectable histological changes within the retina and normal levels of visual pigment.

Discussion

In this study, we report the development of a transgenic mouse in which inducible expression of the DTA gene was used to specifically kill RPE cells. Cell type-specific targeting of the transgene was achieved by integration of the Cre recombinase into a BAC containing the Mct3 gene, a proton-coupled monocarboxylate transporter, which in the eye is expressed uniquely

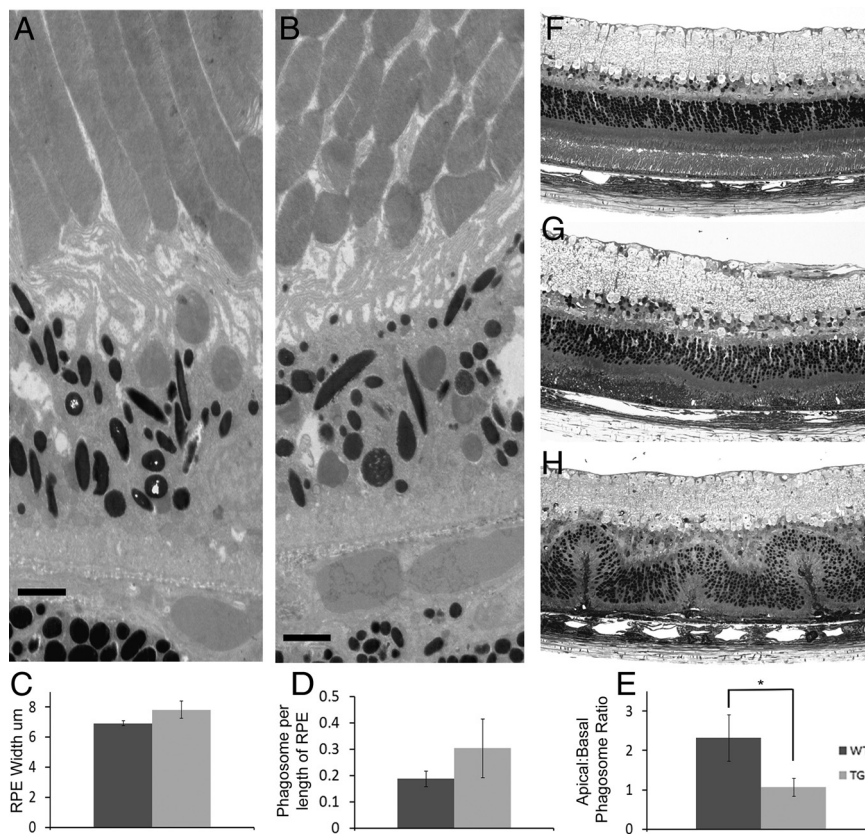


Fig. 3. Ultrastructural changes in the RPE^{Cre}/DTA retina. Representative EM images are shown from wild-type (A) and transgenic (B) retinas. Images from 6 different animals in each group were analyzed, and measurements taken. The width of the RPE cell layer was measured across the whole of the retina in each animal. (C) The average RPE thickness was not significantly different between the WT and TG retinas. The total number of phagosomes per unit length of RPE was quantified (D) and the distribution of phagosomes within the RPE cell, apical or basal was analyzed (E). Although total phagosome numbers were not significantly different, there was a change in the intracellular profile of phagosome distribution, which is shown by a significant difference ($P < 0.036$) (Student's *t* test, 1 tailed, unpaired) in the apical:basal phagosomes ratio. Semithin (0.5 μm) toluidene blue-stained sections are shown from a wild-type control retina (F) and from a mildly affected (G) and a severely affected transgenic (H). (All error bars S.E.M., * indicates $P < 0.05-0.019$). (Scale bar A and B, 2 μm; scale bar H, 50 μm.)

in the RPE (22, 29). Activation of DTA led to a phase of cell death lasting a few days, during which individual dying cells appeared to be extruded from the monolayer with concomitant enlargement of the surrounding cells. The behavior of the RPE in vivo is therefore consistent with that observed in studies on epithelial monolayers in culture, in which cells primed for apoptosis undergo an actin and myosin dependent 'purse-string' expulsion (30). Such a mechanism would explain the appearance in certain retinal sections of clumps of pigmented cells at the

RPE/photoreceptor interface, although since RPE cells are inherently phagocytic it is also likely that some of the dead cells were ingested both by their surviving neighbors, as well as invading macrophages.

A key question in diseases such as AMD, in which RPE cell death or dysfunction leads to secondary photoreceptor degeneration (31), is whether or not the surviving RPE cells have any capacity for self-renewal that might be stimulated to therapeutic benefit. A subsidiary issue is whether, if the intrinsic barriers to RPE cell proliferation can be overcome, problems may be encountered in repopulating an age-modified Bruch's membrane that has been shown to be hostile both to cell attachment in vitro (32, 33) and to autologous RPE in vivo following surgical translocation (34, 35). In the RPE^{CreER}/DTA mice, RPE cell death is induced in a healthy retina in which we assume Bruch's membrane offers a favorable substrate for cell growth, yet in both newborns and adults there was no evidence of cell proliferation as judged by incorporation of BrdU or phosphohistone staining. Indeed a striking feature of the cell-depleted RPE monolayer was the increase in 'footprint' of the surviving cells, in which the largest cells expanded to almost 20 times their normal size. Increases in RPE cell size and irregularity have also been observed in the retinas of patients with AMD (36), particularly in association with Drusen, thus the RPE^{CreER}/DTA mice may to some extent recapitulate the pigment epithelial cell response in AMD. These data are also consistent with the idea that in geographic atrophy the exposed extracellular matrix is prohibitive to RPE cell attachment and/or survival as it is unable to accommodate RPE cell expansion from the surrounding population.

Despite maintaining a functionally intact monolayer, RPE cell function was clearly compromised. Several animals exhibited rosetting or retinal folds, a characteristic of several human retinal diseases that has also been described in various models of retinal degeneration. Although the mechanisms that drive rosette forma-

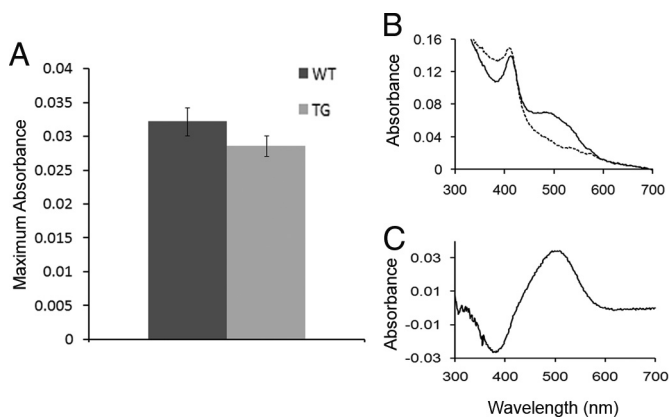


Fig. 4. Rhodopsin quantification. Rhodopsin quantification was performed by retinal extraction and spectral analysis. (A) Represents the average amount of pigment extracted from the two eyes of RPE^{Cre}/DTA transgenic ($n = 5$) and wild-type ($n = 6$) animals. There was no significant difference in the amount of pigment in the RPE^{Cre}/DTA and wild-type mice (Student's *t* test). A representative spectrum from one eye is shown (B) pre (solid) and post (dashed) bleach. The amount of pigment in each eye was calculated from the peak absorbance of the difference spectrum (C). (All error bars S.E.M.)

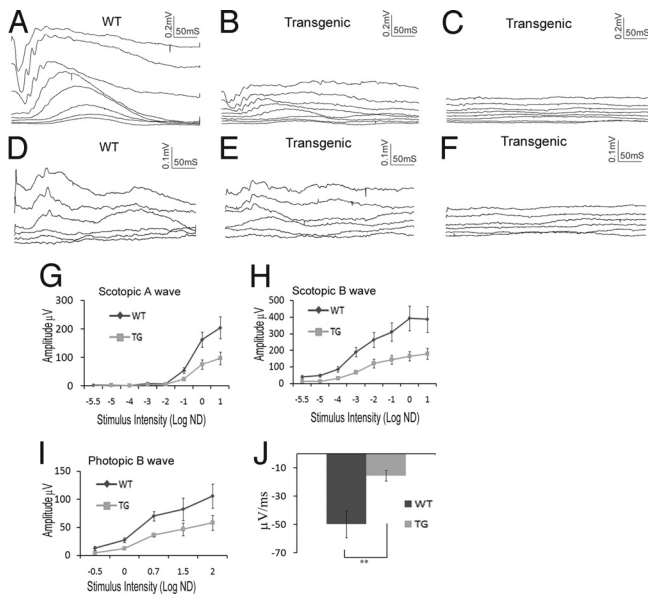


Fig. 5. Electrophysiological assessment of the RPE^{Cre}/DTA transgenic mice and WT control animals. Representative ERG traces are shown for scotopic (A–C) and photopic (D–F) responses from a wild-type control animal (A and D) and two different transgenic animals (B and E; C and F) to illustrate the range of responses evoked from the transgenic animals. ERG was used to assess the responses of the neural retina to light flashes of increasing intensity under both scotopic and photopic conditions. Under scotopic conditions the TG animals ($n = 12$) displayed significantly reduced a wave (G) and b wave (H) amplitudes, $P < 0.011$ and $P < 0.005$ respectively, tested by performing an ANOVA with repeated measures (SPSS, GLM repeated measures) in comparison to WT control animals ($n = 11$). Under photopic conditions the b wave (I) showed no significant difference in the transgenic animals in comparison to control responses. The initial slope of the scotopic a wave was measured over 5 ms (J) from the brightest flash, log intensity 1. The transgenic animals have a significantly reduced gradient in comparison to wild-type $P < 0.00097$ (one-tailed Student's t test). (All error bars S.E.M., ** indicates $P < 0.01$.)

tion are not understood, it may occur as a consequence of a loss of close interdigitation between the RPE and outer segments. We also observed deficits in scotopic ERG a and b wave amplitude in RPE-depleted animals, irrespective of the presence or absence of retinal structural abnormalities. This suggests that even in anatomically normal photoreceptors, changes in the depleted epithelial monolayer influence phototransduction. This could occur through alterations in ion and electrolyte flow between the RPE and retina, or possibly through disruption to the visual cycle. In addition, retrieval of photopigment from phagocytosed outer segments may be compromised in the RPE^{Cre}/DTA mice given the accumulation of phagosomes in the basal RPE. This is suggestive of a delay in phagolysosomal fusion and is consistent with defects in phagosome motility reported in a model of sublethal photic RPE stress (37).

In summary, we have shown that although the RPE can accommodate a significant level of cell loss, at least with regard to maintaining a confluent and intact monolayer, it is unable to fully support the functional demands of the outer retina. Thus in aged humans, loss of RPE may place an excessive burden on the remaining population and hence predisposition to retinal dysfunction in the area of greatest metabolic demand.

Materials and Methods

Generation of RPE^{Cre}/DTA Mice and Tamoxifen Administration. A plasmid coding for iCreER^{T2}-IRES-EGFP-pA and a FRT flanked kanamycin resistance cassette (19) was used for recombinant targeting of a BAC containing the Mct3 gene, Slc16a8 (RP24–149-K18, provided by the U.K. HGMP Resource Centre). The 5' and 3' homologues for recombinant targeting were created by PCR amplification from

the same BAC and cloned into the targeting plasmid. The iCreER^{T2}-IRES-EGFP-pA sequence was placed at the start of the ORF of the Mct3 gene (removing exon 3) in the BAC by homologous recombination in EL250 bacteria (24) (kindly provided by N. Copeland) using the kanamycin resistance cassette to screen for clones that have undergone recombination. The resistance cassette was then removed by arabinose-induced Flp recombinase activity in EL250 bacteria (24). The resulting BAC was purified and used for mouse transgenesis in a C57Bl6 X CBA F2 background as described (38). Offspring were genotyped using PCR with the primers 5'-GGGTGTCCTGGGCTGTTTCTTT-3' and 5'-CTCCCAGCATCCACATTCTCCTT-3'. Heterozygous, male RPE^{Cre} animals were then crossed with homozygous female conditional DTA animals (26) resulting in offspring that contained a conditional DTA allele in 100% and the Cre allele in 50% of the pups. OHT was administered via i.p. injection (20 µg per newborn pup) and tamoxifen via gavage (3 mg per adult, 2–5 months old). In vivo tamoxifen is converted to OHT, which is the biologically active form of tamoxifen.

Histology. For X-gal staining tissue was dissected and fixed in 0.2% glutaraldehyde, 2 mM MgCl₂, 5 mM EGTA in PBS for 30 min at room temperature. Following incubation in wash buffer (PBS containing 5 mM EGTA, 0.01% sodium deoxycholate and 0.02% Nonidet P-40) tissue was incubated in wash buffer also containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ and 1 mg/mL X-gal overnight at 37 °C. To better visualize X-gal staining in the RPE, pigment was bleached (after X-gal staining) by incubation in KMnO₄ (0.25% in water) for 10 min at room temperature and subsequent incubation in oxalic acid (1% in water) for 20 min at room temperature.

Retinal whole mount preparations were dissected from whole eyes after brief fixation in 2% (wt/vol) paraformaldehyde in PBS and then processed for immunohistochemistry, as previously described (39). RPE whole mount preparations were dissected from unfixed eyes and then fixed in cold (–20 °C) methanol for at least 30 min. Cryo-sections were blocked with 0.1% BSA, 2% goat serum, and 0.1% Triton-X in PBS for 10 min and incubated with primary antibodies diluted in the blocking solution for 2 h at room temperature or overnight at 4 °C.

RPE flat mount morphological analysis was performed in Metamorph analysis software (Molecular Devices) from images of flat mounted eye cups where the neuroretina layers had been removed leaving the RPE cell layer exposed. RPE cells were stained with a rabbit antibody against ZO-1 (Invitrogen, 1:100) and Hoechst 33342, and images captured on an Olympus Axiovert microscope. The perimeters of 150 cells (three different images) from each eye were traced, and the area, perimeter, and shape factor [$4\pi A/P^2$ ($A = \text{area}$, $P = \text{perimeter}$)] were calculated. The closer to 1, the more circular/regular the cell. In the same three images, the numbers of cells in a standard ROI were counted, to give an average number of cells within a set reference area for each animal. In total, six wild-type (WT) animals and six transgenic animals (TG) from three different litters were analyzed 2 weeks post-tamoxifen dose. Student's t test was performed for each morphological property analyzed.

Vascular endothelial cells were stained using FITC-conjugated isolectin B4 (Sigma, 1:50). Pericytes/smooth muscle cells were stained with a Cy3-conjugated monoclonal anti-ASMA antibody (Sigma, 1:100). Secondary Alexa-488 and Alexa-546 antibodies (Molecular Probes, 1:500) were applied to the sections for 1 h at RT and the sections were mounted in ProLong Gold anti-fade reagent containing DAPI (Molecular Probes) and analyzed by fluorescence microscopy. Further immunohistochemical techniques are detailed in *SI Materials and Methods*.

Western Blotting. RPE protein fractions were isolated in SDS/PAGE sample buffer, resolved by 6% SDS/PAGE and transferred to nitrocellulose membranes. Membranes were washed in Amido black (BD staining solution for 2 min), then washed in destain solution (10% methanol, 10% acetic acid). A loading control image was taken at this stage in PBS. Membranes were further washed in PBS and then blocked and incubated overnight in primary antibodies to ZO1 (gift of K. Matter), MerTK (R&D Systems) and RPE65 (Chemicon International). HRP conjugated secondary (DAKO) and ECL reagent (Amersham Biosciences) was used for visualization. Analysis was performed in Metamorph imaging software.

Ultrastructure Analysis. Eyes from six WT and six TG mice were emersion-fixed and prepared for transmission electron microscopy. Ultra thin sections were viewed on a JEOL 1010 transmission electron microscope and images captured using Gatan Micrograph software and an Orius camera. RPE cell thickness and phagosome number and cellular localization were analyzed in imageJ 1.41 software (National Institutes of Health). The thickness of the RPE was averaged for each animal from measurements made from 5–8 EM images. The averages for all of the animals were then included in the statistical analysis by Student's t test. Phagosomes were counted per length of RPE and relative cellular localization analysis was carried out from the same 5–8 EM images.

Electroretinography. Twelve transgenic (TG) and 11 wild-type (WT) mice from six different litters were dark-adapted overnight before ERG on the left eye 2 weeks post-tamoxifen. Animals were anesthetized by Ketamine/Domitor (0.75 mL Ketamine, 0.5 mL Domitor, 0.75 mL sterile water at 0.2 mL/100 g, i.p. supplemented as required). Under red-light conditions, pupils were dilated with topical atropine and phenylephrine. ERG recordings were carried out via platinum loop electrodes placed on the cornea and a reference platinum electrode in the scalp of the animal. A platinum earth electrode was placed s.c. on the lower back of the animal. The animal was placed on a heated pad (37 °C) in a light-tight box for the recordings. Flash stimuli (3- μ s to 1-ms duration, repetition rate 0.17–0.67 Hz) were presented via an LED stimulator, log intensity -5.5 to $+1$, under scotopic conditions. After scotopic testing was completed, animals were light-adapted to 20 cd/m² background for 20 min. Following light adaption photopic responses to flash stimuli (1–3 ms duration, repetition rate 0.5 Hz), log intensities -1.4 to $+2$ and flicker stimuli (up to 40 Hz) were measured. Statistical analysis using specialized software, SPSS, where an analysis of general linear means was performed examined the trends within the populations.

Quantitation of Rhodopsin. TG ($n = 5$) and WT control ($n = 6$) animals were placed in darkness overnight and all subsequent procedures performed under dim red light. They were killed by cervical dislocation and visual pigments were extracted by homogenizing whole individual eyes in 0.5 mL PBS and 100 μ L of 200 mM *n*-dodecyl *O*- β -1-maltoside. Homogenates were rotated for 2 h at room temperature followed by 10 min centrifugation (4 °C, 23,000 \times g). The resulting supernatant was scanned (300–700 nm) in a quartz cuvette using a Shimadzu UV-2101 PC spectrophotometer. After the initial scan of the unbleached extract it was exposed to white light for 3 min and rescanned. The maximum absorbance of the difference spectra obtained by subtracting these two spectra indicated the total amount of photolabile pigment extracted. The maximum absorbances of the extracts from the two eyes of each animal were averaged. The average amounts for each animal were used for statistical analysis by Student's *t* test.

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