Altered photoreceptor metabolism in mouse causes late stage age-related macular degeneration-like pathologies

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Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly. While the histopathology of the different disease stages is well characterized, the cause underlying the progression, from the early drusen stage to the advanced macular degeneration stage that leads to blindness, remains unknown. Here, we show that photoreceptors (PRs) of diseased individuals display increased expression of two key glycolytic genes, suggestive of a glucose shortage during disease. Mimicking aspects of this metabolic profile in PRs of wild-type mice by activation of the mammalian target of rapamycin complex 1 (mTORC1) caused early drusen-like pathologies, as well as advanced AMD-like pathologies. Mice with activated mTORC1 in PRs also displayed other early disease features, such as a delay in photoreceptor outer segment (POS) clearance and accumulation of lipofuscin in the retinal-pigmented epithelium (RPE) and of lipoproteins at the Bruch’s membrane (BrM), as well as changes in complement accumulation. Interestingly, formation of drusen-like deposits was dependent on activation of mTORC1 in cones. Both major types of advanced AMD pathologies, including geographic atrophy (GA) and neovascular pathologies, were also seen. Finally, activated mTORC1 in PRs resulted in a threefold reduction in di-docosahexaenoic acid (DHA)-containing phospholipid species. Feeding mice a DHA-enriched diet alleviated most pathologies. The data recapitulate many aspects of the human disease, suggesting that metabolic adaptations in photoreceptors could contribute to disease progression in AMD. Identifying the changes downstream of mTORC1 that lead to advanced pathologies in mouse might present new opportunities to study the role of PRs in AMD pathogenesis.

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Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss in the elderly (1, 2). Early disease stages are defined by the formation of drusen, which are lipid-rich deposits located between the retinal-pigmented epithelium (RPE) and the underlying Bruch’s membrane (BrM) (1). Advanced disease stages are characterized by confluent RPE atrophy, referred to as geographic atrophy (GA), and/or edemas that result from choroidal and/or retinal neovascularization (1). Both advanced stages cause blindness through extensive secondary loss of photoreceptors (PRs). Notably, 20% of individuals with early stage drusen progress to the advanced disease forms. Although the RPE is the primary cell type affected in advanced AMD, what causes disease progression remains unclear (2).

Two factors that contribute to drusen genesis and disease progression in AMD are genetic variations in genes of the innate immune system and poor diet (3–7). Dysregulation of complement components such as C3 and CFH, which are central to the activation and inhibition of the complement system, respectively, is thought to contribute to RPE atrophy (3). Excessive consumption of saturated dietary fats also influences drusen formation as drusen are rich in lipoproteins that are partially derived from the circulation (8). In contrast, foods enriched in polyunsaturated fatty acids, such as the omega-3 fatty acid docosahexaenoic acid (DHA), have been linked through many epidemiological studies to a risk reduction for advanced AMD (9, 10). Although DHA is highly enriched in photoreceptor outer segments (POs), the mechanism of protection remains unknown (11).

The role of PRs in disease pathogenesis remains to be determined as PRs have been associated with both the early and late disease stages (8, 12–14). For example, macular translocation procedures revealed that PRs can cause the late disease stage of GA. Patients whose retina was rotated to move macular cones away from an area of GA to an area of healthy RPE redeveloped GA where the cones were translocated (12, 13). Similarly, analyses on the distribution of soft drusen and subretinal drusen deposits revealed that their preferential location mirrors the density of PRs (1). The mechanism of protection remains unknown (11).

The main cause for blindness in the elderly worldwide is age-related macular degeneration (AMD). What causes AMD remains unknown. The high metabolic demands of photoreceptors are thought to contribute to disease pathogenesis, yet whether photoreceptor metabolism differs in individuals with AMD has not been determined. Here, we show that photoreceptor metabolism does differ between diseased and non-diseased individuals. Mimicking the metabolic profile of diseased individuals in mouse resulted in the similar advanced pathologies as those that cause blindness in humans. A disease model with photoreceptors as a contributing factor explains also why AMD affects preferentially the macula; it is the region of highest photoreceptor density. The data might open new avenues to study the role of PRs in disease pathogenesis.
cones and rods, respectively (8, 14). In both cases, the high and differential metabolic demands of cones and rods are thought to contribute to disease pathogenesis (8, 12–14). However, whether the metabolic demands of PRs differ in individuals with AMD has not been investigated. This question is particularly pertinent since glucos e shortage in PRs of AMD patients has been predicted by several models of disease pathogenesis. For example, the mitochondrial dysfunction seen in RPE cells of AMD patients has been proposed to reduce glucose transfer from the RPE to PRs. The idea here is that, to compensate for the energy shortfall caused by dysfunctional mitochondria, the RPE starts utilizing glucose itself, rather than providing it to PRs and use PR-derived lactate (15). The finding of a lipid wall at the BrM that forms during the early drusen stage of the disease (oil spill model) is also predicted to cause a glucose shortage in PRs as this hydrophobic barrier should reduce the transfer of hydrophilic molecules like glucose from the choroidal vascular to the RPE and thus PRs (8). These models are not mutually exclusive; rather they represent sequential disease stages that once superimposed could compound the glucose shortage in PRs even more. Thus, if these models are correct, one would expect to find adaptive changes in PRs of AMD patients that allow PRs to cope with a glucose shortfall.

Here, we show that the expression of two genes that are characteristic of PR metabolism and cell growth (16–20), namely pyruvate kinase muscle isoenzyme M2 (Pkm2) and hexokinase-2 (Hk2), is increased in individuals with AMD. HK2 improves glucose retention and PKM2 allows for a more efficient diversion of glycolytic intermediates into the pentose phosphate pathway (PPP). Because the PPP promotes cellular NADPH synthesis and NADPH is a reducing agent that is also required for fatty acid synthesis, the PPP helps reduce cellular oxidative stress and maintain the renewal of the lipid-rich POSs (16, 17, 19). HK2 and PKM2 have both been implicated during secondary cone death in retinitis pigmentosa where cone death was found to be in part due to a glucose shortage in cones (18, 21, 22). One of the adaptive responses of cones in retinitis pigmentosa is to increase the expression of these two proteins through increased activity of the mammalian target of rapamycin complex 1 (mTORC1), a key regulator of cell metabolism and cell growth (22–25). Loss of mTORC1 activity or HK2 accelerates cone death in retinitis pigmentosa while constitutive activation of mTORC1 promotes cone survival (18, 22). Similarly, loss of mTORC1 activity in the sodium iodate model of GA accelerates PR death (26). Therefore, to mimic the adaptive responses seen in PRs of AMD patients and study their effect on retinal and RPE health, we generated a mouse model with constitutive activation mTORC1 in PRs of wild-type mice. This was achieved by deletion of the tuberous sclerosis complex 1 (Tsc1) gene, a negative regulator of mTORC1 (27). We found an age- and mTORC1-dependent onset of advanced AMD pathologies, including GA and neovascular pathologies, at a frequency similar to that seen in humans (2). Mice with activated mTORC1 also displayed early disease features, such as accumulation of lipoproteins at the BrM (8, 14) and changes in complement C3 and CFH deposition (3). These changes occurred uniformly across the tissue independent of the presence of any advanced pathology. Finally, we also found a reduction in 1,2-docosahexaenoyl sn-glycero-3-phospholipid species in retinas of these mice. Feeding mice a DHA-enriched diet alleviated most pathologies (9, 10). The data mimic most aspects of the human disease, suggesting that metabolic adaptations in PRs could be a contributing factor for disease progression in AMD.

Results

**HK2 and PKM2 Expression Are Increased in PRs of AMD Patients.** HK2 and PKM2 are two glycolysis enzymes that are generally associated with cell growth during development, as well as tumorigenesis, as they promote progrowth pathways such as the PPP (28, 29). While normally not expressed in most postmitotic neurons, both genes are a key signature of PR metabolism (16–20). This is because PRs are in a constant phase of cell growth, due to the need to replace the daily shed POSs. Both genes thus play an important role in POS renewal, function, and survival, in particular under nutrient stress (16–22). To determine whether PR metabolism differs in individuals with AMD, we analyzed the expression of these two key metabolic genes in human donor eyes with or without AMD. On retinal sections, we found increased expression of PKM2 and HK2 in PRs of AMD patients (n = 3), with the highest increase found in cones (Fig. L4 and SI Appendix, Fig. S1A and B). Interestingly, expression in nondiseased retinas was quite low for PKM2 as those sections required up to five times longer exposure to the histochemical reagent in order for a strong signal to emerge (Fig. L4). To allow for a more linear comparison between samples, we repeated the experiments using immunofluorescence (SI Appendix, Fig. S1A). A twofold scaling of the signal between nondiseased and diseased tissue was sufficient to reveal a PR signal in nondiseased tissue without causing exposure of the signal in diseased retinas. Interestingly, the expression of both genes in mouse has been shown to decline with age (SI Appendix, Fig. S1C) (18, 30). This could in part account for the stark difference in signal intensity seen between diseased and nondiseased individuals. In summary, the data show that levels of HK2 and PKM2 increase in PRs of individuals with AMD, suggesting that glucose availability is reduced in diseased individuals.

**rodTsc1−/−** Mice Develop Advanced AMD Pathologies. We previously showed that nutrient-deprived cones have higher mTORC1 activity and that further activation of mTORC1 promotes the survival of nutrient-deprived cones in part through increased expression HK2 and PKM2 (21, 22, 26). To determine the effect of such metabolic changes on retinal and RPE health in wild-type mice, we constitutively activated mTORC1 in rods by deletion of the Tsc1 gene (henceforth referred to as *rodTsc1−/−*) using the Cre/lox system (31). Increased mTORC1 activity was confirmed by immunofluorescence and Western blot analyses for phosphorylated ribosomal protein S6 (p-S6) (Fig. 1B and C) (24). Similarly, changes in PR metabolism were confirmed by quantifying retinal PKM2, lactate, and NADPH levels (Fig. 1C–E).

To determine whether *rodTsc1−/−* mice develop advanced AMD-like pathologies, we followed the mice over a period of 18 mo (18M) by funduscopy and fluorescein angiography (Fig. 2A). We found, at 2M, migration and accumulation of microglia into the subretinal space and, at 4M, formation of retinal folds, some of which were filled with microglia (SI Appendix, Fig. S3) (32). Flat mount and section analyses revealed highly autofluorescent RPE cells opposing these folds (Fig. 3A and B), which in mice may be a direct consequence of acutely compromised or lost RPE cells (33, 34).

GA was seen in 5% of mice at 6M and 25% of mice at 18M (Fig. 2C). Interestingly, while GA did also overlap with areas of retinal folds, the presence of these folds was not required for GA to develop. Generally, pathologies worsened within the same animal with age (SI Appendix, Fig. S2). To confirm that areas of GA correlate with regional PR atrophy and that RPE atrophy precedes PR atrophy, we compared the RPE and corresponding retina by flat mount analyses (Fig. 3A–C), identified intermediate RPE pathologies (Fig. 3D), and performed semithin sectioning through regions of GA that were identified by optical coherence tomography (OCT) (Fig. 3E and F).

Neovascular pathologies reaching a frequency of 7% by 18M were seen less frequently than GA (Fig. 2C) although most coincided with regions of GA. While we readily detected retinal neovascular pathologies on semithin sections (Fig. 3F), choroidal neovascular pathologies were not evident on RPE flat mounts. However, given the overall low frequency of neovascular pathologies, we cannot conclude that choroidal neovascular pathologies do not occur. Except for the accumulation of subretinal pathologies, such as the formation of retinal folds, some of which were filled with microglia (SI Appendix, Fig. S3) (32). Flat mount and section analyses revealed highly autofluorescent RPE cells opposing these folds (Fig. 3A and B), which in mice may be a direct consequence of acutely compromised or lost RPE cells (33, 34).

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microglia, none of the heterozygous rodTsc1+/− mice nor any of the Cre− littermate control mice (rodTsc1+/−) developed advanced pathologies (Fig. 2 B and C). Consistent with this, activation of mTORC1 and the increase in PKM2 expression levels were both minimal in rodTsc1+/− mice (Fig. 1C).

To determine if RPE stress and atrophy also occurred outside regions of GA, we determined the percentage of polynucleated RPE cells and measured changes in RPE cell size in non-GA areas. At 18M, we found a significant increase in polynucleated RPE cells and measured changes in RPE cell size in non-GA regions of GA, we determined the percentage of polynucleated RPE cells increased dramatically with age (SI Appendix, Fig. S5). This increase likely reflects an increase in the number of sick disease, except for areas where advanced pathologies precipitate. To confirm that GA was not caused by aberrant CRE recombinase expression in the RPE, we stained RPE flat mounts for p-S6 and PKM2 with retinas from 2M-old mice (16, 24). Further studies are warranted to determine what specifically causes these higher a-wave amplitudes in rods (16, 24). Interestingly, c-wave amplitudes, which reflect in part RPE health, did not differ between rodTsc1−/− and controls (SI Appendix, Fig. S4D) (20). Here, higher retinal lactate levels may have benefited a sick RPE (15, 20). Overall, the data indicates that loss of Tsc1 in rods leads to a slow progressive disease, except for areas where advanced pathologies precipitate.

To confirm that GA was not caused by aberrant CRE recombinase expression in the RPE, we stained RPE flat mounts for p-S6. While occasional p-S6 positive cells were seen in both rodTsc1−/− and controls at 2M (SI Appendix, Fig. S5A), we did not find CRE recombinase expression in p-S6–positive cells (SI Appendix, Fig. S5B). Additionally, the number of p-S6–positive cells increased dramatically with age (SI Appendix, Fig. S5 A and C). This increase likely reflects an increase in the number of sick leads to a reduction of the scotopic response and a reduction in retinal lactate and NADPH levels (18). Thus, the early higher amplitude may reflect higher energy availability. Alternatively, increased transcription or translation of phototransduction genes due to increased PKM2 expression or increased mTORC1 activity, respectively, could also account for higher a-wave amplitudes in rods (16, 24). Further studies are warranted to determine what specifically causes these higher a-wave amplitudes in rods (16, 24). Interestingly, c-wave amplitudes, which reflect in part RPE health, did not differ between rodTsc1−/− mice and controls (SI Appendix, Fig. S4D) (20). Here, higher retinal lactate levels may have benefited a sick RPE (15, 20). Overall, the data indicates that loss of Tsc1 in rods leads to a slow progressive disease, except for areas where advanced pathologies precipitate.
RPE cells in rodTsc1−/− mice as increased mTORC1 activity in the RPE has been associated with RPE dysfunction, senescence, and cell loss (35–37). Moreover, a recent study that deleted Tsc1 from all RPE cells did not report any advanced AMD pathologies (37).

rodTsc1−/− Mice Also Display Early Disease Features. The metabolic demands of PRs have been proposed to contribute to lipoprotein accumulation and drusen formation (8). To determine if the metabolic changes induced in PRs also contribute to lipoprotein accumulation, we analyzed the distribution of ApoB and ApoE at the BrM. We found accumulation of both lipoproteins at the RPE basal lamina and BrM, independent of any advanced pathology (SI Appendix, Fig. S6A). Electron microscopy (EM) analyses revealed neutral lipids within the BrM, as well as basal laminar deposits and thickened BrM in areas of GA (SI Appendix, Fig. S6B). However, drusen-like deposits were not seen; rather, basal mounds were quite common (SI Appendix, Fig. S6C). Additionally, we found increased autofluorescence in the RPE of rodTsc1−/− mice, indicative of increased lipofuscin accumulation (SI Appendix, Fig. S6D).

The complement system plays an important role in AMD pathogenesis (3). In particular, C3 activation is thought to contribute to RPE atrophy and GA (3). We found a uniform down-regulation of C3 at the BrM and a uniform up-regulation of CFH in rodTsc1−/− mice (SI Appendix, Fig. S6A). In all, the findings show that these early disease features, which are induced by activation of mTORC1 in rods, occur uniformly across the tissue independent of the presence of any advanced pathology.

AMD-Like Pathologies Are Dependent on the Dose of Activated mTORC1. To test the requirement of mTORC1 to the pathologies seen, we generated mice with simultaneous deletion of Tsc1 and the mTORC1 adaptor protein Raptor (referred to rodTsc1−/− rodRaptor−/− mice). Fundus imaging revealed no pathology, except for the accumulation of microglia in 76% of mice aged between 12M and 18M (Fig. 4 A and B). Even heterozygous Raptor mice (rodTsc1−/− rodRaptor+/−) did not develop any GA or neovascular pathologies by 12M (Fig. 4B). However, retinal folds were present, albeit at lower frequency. The absence of any severe pathology was in line with the quantification of polynucleated RPE cells and RPE cell size, which revealed no substantial difference among these lines at 12M (Fig. 4C). Western blot analyses for p-S6 and PKM2 confirmed the reduction in mTORC1 activity (Fig. 4E). Interestingly, while p-S6 levels in rodTsc1−/− rodRaptor−/− showed a dose-dependent decline when compared to in rodTsc1−/− mice, PKM2 levels remained similar to PKM2 levels in rodTsc1−/− (compare Fig. 4D with Fig. 1C). In contrast, lactate and NADPH levels remained at the levels of Cre− controls in heterozygous rodTsc1−/− rodRaptor+/− mice (Fig. 4 E and F). To determine to which extend this affected the early pathologies, we reanalyzed the accumulation of ApoB, ApoE, C3, and CFH. While accumulation of these markers was restored to normal in rodTsc1−/− rodRaptor−/−, heterozygous rodTsc1−/− rodRaptor+/− mice displayed a more intermediate phenotype (Fig. 4G).

RPE Phagocytosis Is Perturbed in rodTsc1−/− Mice. Impaired RPE lysosomal activity has been associated with AMD (38–40). The uniform nature of RPE cell stress led us to investigate if POS clearance was perturbed in the rodTsc1−/− mice. Since shedding of rod POSs is circadian, clearance can be monitored over time on RPE flat mounts stained for the rhodopsin protein. We found that rod POS clearance was already significantly slowed at 2M in
Fig. 3. Histological analyses of advanced AMD-like pathologies. (A) RPE and corresponding retinal flat mount of the same eye, showing autofluorescent RPE cells and the corresponding area with retinal folds marked with the letter b, and an area of GA and corresponding PR atrophy marked with the letter c. RPE whole mount is shown in the Left Half, and the corresponding retina in the Right Half of the panel. (Scale bar: 300 μm.) (B) Higher magnification of the region in A marked with letter b showing autofluorescent RPE cells (arrowhead: Left) that correspond to retinal folds (arrowhead: Middle). Right shows higher magnification of a fold (different eye) with Iba-1 staining (red) marking microglia (arrows). (Scale bars: 50 μm.) (C) Higher magnification of area of GA marked in A with the letter c showing in gray scales loss of RPE cells (Left) and retinal PRs (Right; PR side up showing reduced nuclear DAPI density). Note that no folds are visible in the area of GA in A (letter c), meaning that folds are not required for the formation of GA. (Scale bar: 50 μm.) Colors in A–C are as indicated by labels in panels. Annotation of colors for A is indicated in the first two images of B (blue, nuclear DAPI; green, autofluorescence [AF] or cone sheets marked by peanut agglutinin lectin [PNA]; red, RPE boundaries marked by ZO1, cones marked by cone arrestin [CA] or microglia marked by Iba-1). (D) Semithin section through intermediate stage of GA showing RPE atrophy with PRs still present. No fold is present in this area of RPE atrophy (image size: ~100 μm). (E) Consecutive OCT images through area of GA identified by fundus (same eye as shown in Fig. 2 A and B: 18M with GA), showing collapse (between arrowheads) of the outer nuclear layer (ONL) (between dotted lines). (F) Semithin sections of eye with GA shown in E showing multilayered RPE (white asterisk), RPE migration into the retinal proper (arrow), RPE atrophy (between arrowheads), and retinal angiogenesis (red arrows). As PRs die, retinal folds flatten if they overlap with areas of GA. Reminiscence of retinal folds is indicated by dotted lines. (Scale bars: 20 μm.) (G) RPE polynucleation and hypertrophy analyses. Top shows representative RPE image of cell boundaries marked by ZO1 (red signal) used for quantification analyses with output from the IMARIS software on the Right to identify cell shape, size, and nuclei (blue signal, nuclear DAPI). Bottom shows quantification of RPE polynucleation (Left) and distribution of RPE cell size (Right). Bars show mean ± SEM (n = 4 RPE flat mounts; *P < 0.05). (Scale bar, 10 μm.)
rodTsc1−/− mice and was rescued in rodTsc1−/− rodRaptor−/− mice, indicating that the effect was due to increased mTORC1 activity in rods (Fig. 5A–C).

POSs are rich in lipids (11, 41), and mTORC1 is known to regulate lipid synthesis (24, 25). To determine a cause for the delayed POS clearance by the RPE, we profiled the retinal lipid composition of rodTsc1−/− mice. We found an approximately three-fold decrease in di-DHA (44:12) containing phosphatidylethanolamine (PE) and phosphatidylcholine (PC) lipids in total retinal (Fig. 5D) and POS preparations (Fig. 5E). To test if this drop in di-DHA PE and PC lipids contributes to the delay in POS clearance, we fed rodTsc1−/− mice a diet enriched with 2% DHA. The 2% value was based on a study that showed a beneficial effect with DHA supplementation on delaying disease progression in humans (42). Feeding rodTsc1−/− mice a 2% DHA-enriched diet from weaning onwards improved POS clearance at 2M (Fig. 5F). To test if delayed POS clearance can also be improved once the delay has occurred, we fed 6M-old rodTsc1−/− mice the DHA-enriched diet for 2 wk. This had an even more pronounced effect as POS clearance was more affected at 6M (Fig. 5G). To determine if dietary DHA also affected overall RPE health, we kept mice on the DHA diet from weaning onwards until 6M. This reduced the percentage of polynucleated RPE cells (Fig. 4H), improved fundus pathologies (Fig. 5I), prevented the accumulation of ApoB, ApoE,
and CFH, and restored C3 expression (Fig. 5). Differences in RPE hypertension were not evident, likely because, in younger mice, hypertrophy is not as pronounced yet. None of 12 DHA-fed RPE hypertrophy were not evident, likely because, in younger mice that were fed a DHA or control diet between weaning to 6M. Proteins of interest indicated on top are shown in green. Higher magnification of the RPE cell over the course of the day from 2M-old mice of genotypes indicated, obtained from immunofluorescence images as shown in Fig. 5. (A) Representative immunofluorescence images of RPE whole mounts from 2M-old rodTsc1−/− mice at time of day indicated showing delayed POS clearance by RPE cells (Bottom Row) when compared to control mice (Top Row). POSs are shown in red stained for ZO1 expression. (Scale bars: 10 μm.) (B) Quantification of the number of Rho-positive dots per RPE cell over the course of the day from 2M-old mice of genotypes indicated, obtained from immunofluorescence images as shown in A. Bars show mean ± SEM (n = 6–8 RPE flat mounts; ***P < 0.001; ****P < 0.0001). (C) Delay of POS clearance shown as percentage of remaining dots 3 h after peak shedding (ratio between 11 AM and 8 AM) in 2M-old mice of genotypes indicated. Bars show mean ± SEM (n = 6–8 RPE flat mounts; ***P < 0.001; ****P < 0.0001). (D) Relative percentage of di-DHA PE (44:12) and PC (44:12) lipids from total retinal extracts of genotypes indicated at 2M. Bars show mean ± SEM (n = 6–9 mice, two retinas per mouse; ****P < 0.0001). (E) Same as in D with purified POSs pooled from six retinas per genotype. (F) POS clearance shown as percentage of remaining dots 3 h after peak shedding (ratio between 11 AM and 8 AM) in 2M-old mice that were fed a DHA or control diet between weaning to 2M. Shown are mean ± SEM (n = 6 RPE flat mounts; *P < 0.05; **P < 0.01; ns: not significant). (G) Same as in F with 6M-old mice that were fed a DHA diet for only 2 wk. Shown are mean ± SEM (n = 6 RPE flat mounts; ***P < 0.001; ****P < 0.0001). (H) RPE polynucleation (Left) and hypertrophy (Right) analyses of rodTsc1−/− mice that were fed a DHA or control diet between weaning to 6M. Bars are mean ± SEM (n = 6 mice RPE flat mounts; *P < 0.05; **P < 0.01). (I) Representative fundus images of rodTsc1−/− mice that were fed a DHA or control diet between weaning to 6M. Proteins of interest indicated on top are shown in green. Higher magnification of the region between arrowheads is shown on Top of each panel (blue, nuclear DAPI; red, cone sheets marked peanut agglutinin lectin [PNA]; magenta, ZO1 marking RPE boundaries for ApoE and C3 panels and Phalloidin marking boundaries for ApoB and CFH panels). (Scale bars: 20 μm.) GCL, ganglion cell layer; RPE, retinal-pigmented epithelium. Images are representative of three independent experiments on three different animals per genotype. (K) Same experiment as in D after feeding mice a DHA diet from weaning onwards for 10 wk. Bars show mean ± SEM (n = 3 mice, two retinas per mouse; *P < 0.05; **P < 0.01; ****P < 0.0001).

Cones Contribute Differently than Rods to Disease. Differences in the metabolic needs of rods and cones have been proposed to contribute to different kinds of deposits (8, 14). To more closely mimic the human retina outside the fovea and study the contribution of cones to advanced pathologies, we generated a line of mice with a cone-specific deletion of Tsc1 (coneTsc1−/−) and one with a rod-and-cone deletion (roconedTsc1−/−). Funduscopy and angiography revealed that coneTsc1−/− mice develop similar pathologies without the formation of retinal folds (Fig. 6A). Combining the metabolic changes in rods and cones did not increase the overall frequency of advanced pathologies by 12M. However, advanced pathologies started to occur already at 4M (Fig. 6B). Choroidal neovascular pathologies in coneTsc1−/− mice were easier to identify on RPE flat mounts when compared to roconedTsc1−/− mice (Fig. 6B), coneTsc1−/− and roconedTsc1−/− mice also developed...
Age-related macular degeneration is a multifactorial disease of unclear etiology. Here, we show that altering the metabolic profile of PRs is sufficient to cause 1) changes in ApoB, ApoE, C3, and CFH accumulation at the BrM, 2) formation of drusen-like deposits, and 3) GA and neovascular pathologies. The fact that these mice develop advanced AMD-like pathologies at the same frequency as humans, and that pathologies can be ameliorated by dietary DHA supplementation, suggests that our mouse model may be a valuable tool to study the progression of human AMD. How do our data complement the current understanding of AMD disease progression?

Histo-pathological analyses indicate that the early disease stages start with the formation of a lipid wall at the choroid–retinal blood barrier, affecting primarily the BrM and the RPE basal lamina (oil spill model) (8, 14). This likely impedes the transfer of hydrophilic molecules such as glucose from the choroidal circulation to PRs. From our studies on retinitis pigmentosa, we know that PRs respond to a reduction in glucose availability by increasing the expression of HK2 and PKM2 through increased mTORC1 activity (18, 21, 22, 43). This helps improve glucose uptake and to redirect a larger percentage of the remaining glucose into the anabolic pathway, which helps maintain POS growth (18, 21, 22, 43). Indeed, we found increased expression of PKM2 and HK2 in PRs of AMD patients. Additionally, PKM2 autoantibodies have been found in AMD patients, independent of the disease stage (30), suggesting that this PR adaptive response is an early reaction of PRs to the formation of the initial lipid wall. By activating mTORC1 in PRs, we thus mimic the adaptive response of PRs to the formation of the initial lipid wall. Since lipid accumulation occurs in all of our models, the metabolic adaptations by PRs further contribute to drusen growth. However, large drusen (>60 μm in diameter) as in humans were not seen in our mice. This is likely because the initial lipid wall, which serves as a foundation for further drusen growth, is not present at the start of our experiments. Additionally, large soft-drusen are preferentially associated with the metabolism of foveal cones (8, 14), which are absent in mouse. Based on our findings, we propose a two-stage process of AMD pathogenesis. In the first stage (SI Appendix, Fig. S8), environmental and/or genetic risk factors, such as diet, exercise, and metabolic risk genes, lead to the formation of a lipid wall at the BrM, as proposed by others (8, 44, 45). In the second stage, PR adaptations to the reduction in nutrient transfer further enhance lipid accumulation, while also causing excessive RPE stress. This causes RPE atrophy, GA, and neovascular pathologies, as seen in our mouse models and in humans (15, 39, 40, 46). While this second stage continues to be affected by the same risk factors as the first stage, other risk genes, such as the ones of the innate immune system like C3 and CFH, increase the risk for disease progression (3, 8, 44, 45). The involvement of PRs in disease pathogenesis, which is also supported by a recent RNA sequencing study (47), may also explain why AMD preferentially affects the macular region; it is the area of highest PR density.

The absence of drusen-like deposits in rodTsc1−/− mice suggests that drusen are not required for RPE atrophy or neovascular pathologies to occur. This suggests that there are PR-intrinsic changes that affect RPE health besides the aggregation of lipoproteins at the BrM. In this regard, we found the drop in di-DHA PE and PC lipid species interesting. DHA-containing phospholipids provide an optimal lipid environment for phototransduction (48) and neuroprotective functions (49, 50). In the retina, these phospholipids account for approximately 15 to 20% PC, 20 to 35% PE, and 30 to 50% PS, with POSs that are replenished daily by the RPE having greater levels (51). The finding that dietary DHA supplementation did not restore retinal di-DHA PE and PC levels suggests that the enzymes required for the synthesis of these lipids were down-regulated as a result of increased mTORC1 activity. mTORC1 is a key regulator of lipid metabolism, in part through regulation of the sterol regulatory element-binding protein 1 and 2 (SREBP-1 and SREBP-2) (25). Further support for altered lipid metabolism comes from a human study that profiled total retinal and RPE/choroid fatty acids. The study found that total retinal and RPE/choroid DHA levels in humans with AMD were reduced by ~29% and ~45%, respectively (52). Together, these findings may explain why, contrary to the prediction of many epidemiological studies (9, 10), the AREDS2 study (53) did not find an association between risk reduction and DHA supplementation. If, in AMD patients, DHA acts directly on the RPE to reduce RPE stress, then the nutritional intake of DHA may need to be high in order to benefit the RPE; in particular, the RPE may need to be protected. This is because rod siphon off most of the free DHA that enters the eye (11). Additionally, if the RPE prefers these two specific di-DHA phospholipids as a DHA source for proper cellular function, then exogenous levels that are not PR-derived may need to be higher in order to have a beneficial effect on RPE health. Our study was based on a study that showed a protective effect with five times higher levels of omega-3 fatty acids (42) than the AREDS2 study. It is important to note that we do not think that the reduction in these di-DHA phospholipid species is the sole underlying cause for disease progression; rather, by reducing overall RPE stress, they reduce the risk for progression. The role of these two di-DHA phospholipids in RPE health remains to be investigated. Similarly, how DHA supplementation reduced RPE stress remains to be determined. However, DHA serves as a precursor for the synthesis of RPE-derived neuroprotectin D1, and PE lipids have been found to positively regulate autophagy and reduce age-related oxidative stress and cell death (49–51). Additionally, DHA has been found to have anti-inflammatory properties regulating many aspects of the immune system (54). Thus, the protective effect of DHA seen here could be occurring at multiple levels.

The finding that heterozygous Raptor mice with intermediate mTORC1 activity did not develop advanced pathologies suggests that these pathologies are driven in a dose-dependent manner by mTORC1. Consistent with this, some of the early pathologies displayed a more intermediate phenotype. Interestingly, heterozygous Raptor mice (rodTsc1−/− rodRaptor+/−), PKM2 levels...
remained high while lactate and NADPH levels dropped to Cre− control levels. Therefore, it remains to be investigated to which extent changes in the energy ecosystem between PRs and RPE contribute to advanced pathologies (15, 20, 55, 56). However, it is important to note that the circumstances in which PRs find themselves in AMD retinas and in our mouse model differ since...
glucose is not limiting in our experiments due to the absence of a lipid wall. Nonetheless, our mice develop the same intermediate and advanced pathologies as seen in humans with AMD. This suggests that changes in the exchange of glucose and lactate between the retina and RPE are less likely to be the driving force for the development of advanced pathologies as these differ between our mouse models and AMD patients. Rather, the data suggest that gene expression changes that accompany the adaptations to a glucose shortage cause disease progression (e.g., down-regulation of enzymes that cause drop in di-DHA phospholipids). In line with this, DHA feeding was able to ameliorate most pathologies. While we did not measure lactate and NADPH levels in DHA-fed nrsTSC1−/− mice, one would not expect that this feeding regimen would restore lactate and NADPH levels to Ccr− control levels.

The disrupted in the energy ecosystem between the PRs and the RPE in nrsTSC1−/− mice is thus unlikely to be the driving force for the pathologies seen in these mice. It remains to be investigated which changes downstream of mTORC1 lead to disease progression in our mouse models. Identifying these changes is important since these changes may not require high mTORC1 activity in humans to cause disease. Once identified, they would allow us to study how much of our mouse model parallels the human disease. Moreover, it might also offer new therapeutic targets to delay disease progression in humans if such changes are confirmed in humans. There are already several changes that are required to occur, either simultaneously or sequentially, in order for advanced pathologies to develop. This means that preventing one of the changes to occur might suffice to delay disease progression. Because advanced pathologies are dependent on the dose of activated mTORC1 in mouse, one might also consider decreasing mTORC1 activity. In this regard, mTORC1 inhibitors have already been used in several clinical trials on patients with advanced AMD (57, 58). However, these trials reported either no effect or even some adverse effects resulting in reduced vision. The likely explanation for this is that inhibiting mTORC1 activity in patients with advanced disease affects PR survival as mTORC1 activity is required to help PRs adapt to the nutrient stress that they are experiencing due to lost or dysfunctional RPE cells. We previously reported that mTORC1 activity is required for the survival of metabolically stressed PRs (18, 22, 43), in particular in areas of GA (26). Reducing mTORC1 activity during the early disease stages might be more beneficial. In line with this, a retrospective study found that the antidiabetic drug Metformin, which reduces mTORC1 activity by activating AMP kinase, reduces the risk for developing AMD (59). More studies are needed to determine the correct time window and dose of mTORC1 inhibition in humans. Additionally, identifying the downstream factors that lead to advanced disease may allow one to block disease progression without preventing PRs from adapting to the nutrient shortage. In all, our findings complement the current model of AMD pathogenesis, showing in mouse that activation of mTORC1 in PRs leads to the development of a complex pathology that affects overall RPE health over time.

**Materials and Methods**

All procedures involving animals were in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees of the University of Massachusetts Medical School. Procurement of human samples by B.P.H. was approved by the Partners Institutional Review Board. The Institutional Review Board of the University of Massachusetts issued a waiver to R.N.K. to work with deidentified human tissue sections since he was not involved in procuring human tissue. Similarly, the Institutional Review Board of the University of Connecticut issued a waiver to R.N.K. to work with deidentified tissue samples. Tissue sample contributed by R.N.K. was procured under an approved protocol by the University of Connecticut through the University of Connecticut Health Center. R.N.K. received only deidentified tissue that was contributed to this study. Extensive materials and methods regarding all procedures are provided in SI Appendix, Materials and Methods.

**Data Availability.** All data and experimental parameters related to this paper are available in the main text and SI Appendix.

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