Protective responses to sublytic complement in the retinal pigment epithelium

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The retinal pigment epithelium (RPE) is a key site of injury in inherited and age-related macular degenerations. Abnormal activation of the complement system is a feature of these blinding diseases, yet how the RPE combats complement attack is poorly understood. The complement cascade terminates in the cell-surface assembly of membrane attack complexes (MACs), which promote inflammation by causing aberrant signal transduction. Here, we investigated mechanisms crucial for limiting MAC assembly and preserving cellular integrity in the RPE and asked how these are compromised in models of macular degeneration. Using polarized primary RPE and the pigmented *Abca4*^{−/−} Stargardt disease mouse model, we provide evidence for two protective responses occurring within minutes of complement attack, which are essential for maintaining mitochondrial health in the RPE. First, accelerated recycling of the membrane-bound complement regulator CD59 to the RPE cell surface inhibits MAC formation. Second, fusion of lysosomes with the RPE plasma membrane immediately after complement attack limits sustained elevations in intracellular calcium and prevents mitochondrial injury. Cholesterol accumulation in the RPE, induced by vitamin A dimers or oxidized LDL, inhibits these defense mechanisms by activating acid sphingomyelinase (ASMase), which increases tubulin acetylation and derails organelle traffic. Defective CD59 recycling and lysosome exocytosis after complement attack lead to mitochondrial fragmentation and oxidative stress in the RPE. Drugs that stimulate cholesterol efflux or inhibit ASMase restore both these critical safeguards in the RPE and avert complement-induced mitochondrial injury in vitro and in *Abca4*^{−/−} mice, indicating that they could be effective therapeutic approaches for macular degenerations.

macular degeneration | inflammation | cholesterol | mitochondria | drug targets

The complement system is an important regulator of immunity and inflammation. Complement activation by the classical, alternative, or lectin pathways results in the assembly of C5b-9 membrane attack complexes (MACs), which form membrane pores and cause target cell lysis. Within the eye, a low level of constant complement activity is necessary for immune surveillance, and several membrane-bound and soluble regulators prevent excessive complement activation (1). An imbalance between complement activation and inhibition in the retina is implicated in the pathogenesis of age-related macular degeneration (AMD), which causes central vision loss in more than 30 million people globally (reviewed in refs. 2–4).

Evidence suggests that the retinal pigment epithelium (RPE), which helps maintain ocular immune privilege, is the first line of defense against unregulated complement activation in the retina (5). The complement cascade is activated by insults that disturb RPE homeostasis, including defective clearance of phagocytosed photoreceptor outer segments (6) and abnormal accumulation of vitamin A dimers (7–9) or oxidized lipids (10) in the RPE. Studies also show that exposure to sublytic levels of MAC alters RPE barrier integrity, causes mitochondrial stress, and induces secretion of VEGF and proinflammatory cytokines (11, 12). Thus, RPE dysfunction activates the complement cascade, which can promote

further RPE damage and establish a vicious cycle of chronic inflammation within the retina (13).

How the RPE counters MAC formation and eliminates MAC pores to preserve cell integrity is not well understood (14). The alternative pathway of complement activation begins with the spontaneous hydrolysis of C3 and sequential recruitment of C5b, C6, C7, C8, and finally C9, which polymerizes to form the MAC (2). The membrane-bound complement regulator CD59, which binds C8 and C9, prevents recruitment of C9 to the C5b-8 complex and thus inhibits the final step of MAC assembly (15). Immunostaining of human donor tissue shows that CD59 is predominantly found on the apical surface (16). Decreased CD59 expression (16) and increased MAC deposition in sub-RPE drusen and at the RPE-choroid interface (13, 17) have been detected in AMD patient tissues, pointing to a breakdown in mechanisms that prevent MAC assembly. Activated complement components have also been detected in retinal tissue from the *Abca4*^{−/−} mouse model of Stargardt macular dystrophy, characterized by lipofuscin accumulation and accelerated formation of vitamin A dimers, such as A2E, in the RPE (7, 8). There appears to be a direct correlation between levels of vitamin A dimers in the RPE and complement activation, because inhibiting vitamin A dimerization decreases both A2E levels and complement activity in *Abca4*^{−/−} mice (7). How the accumulation of vitamin A dimers leads to complement activation in Stargardt disease remains unclear.

If MACs and other complement proteins are predominantly found in the sub-RPE region in macular dystrophies, what is the role of CD59 on the apical surface of the RPE? Recent studies suggest that complement activation in the subretinal space also plays a role in AMD: Oxidative modifications of photoreceptor lipids such

Significance

The complement system regulates immune defense and inflammation. Abnormal complement activation in the retina is associated with blinding macular degenerations, which have limited treatment options. The retinal pigment epithelium (RPE) is an initial site of injury in macular degenerations, but mechanisms that protect the RPE from complement-mediated damage are unclear. Here, we identify two critical responses to complement in the RPE: accelerated recycling of the complement inhibitor CD59 and lysosome-mediated membrane repair. We show that in models of macular degeneration, excess cholesterol impairs both defense mechanisms, resulting in mitochondrial damage. Drugs that decrease RPE cholesterol restore these mechanisms and help the RPE combat complement attack. Our studies identify promising drug targets to preserve RPE health and function in macular degenerations.

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Conflict of interest statement: L.X.T., K.A.T., and A.L. are listed on a patent application filed by the Wisconsin Alumni Research Foundation at the University of Wisconsin–Madison on the use of acid sphingomyelinase inhibitors to treat macular degenerations. This patent has not yet been issued.

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Fig. 3. Exposure to complement stimulates lysosome exocytosis. (A) Stills from TIRF imaging of pHluorin-Syt7 (green) in RPE exposed to 10% NHS. Images shown were captured immediately after NHS was added ($t = 0$ s) and 300 s later. See [Movie S1](#) (control cells) and [Movie S2](#) (cells with A2E). Arrowheads and dashed ellipses denote areas with increased pHluorin signal indicating lysosome fusion. Cumulative number of events (B) and event frequency in control RPE and RPE with A2E (C). Mean $\pm$ SEM, *** $P < 0.0001$. (D) Surface LAMP2 immunolabeling (green) after 10% NHS for 10 min. ZO-1 is in red. (E) β -Hex activity in apical and basal media from cells exposed to 10% NHS or heat-inactivated NHS (HI-NHS). Mean $\pm$ SEM, $n = 5$. ** $P < 0.001$ relative to all other conditions, one-way ANOVA with Bonferroni's post hoc test. (F) Number of lysosomes within 500 nm of the plasma membrane in primary RPE, $n = 30$ cells per group. ** $P < 0.005$.

treatment with PI-PLC in control RPE monolayers. In contrast, in cells with A2E, surface CD59 levels remained significantly less than controls even after 120 min (Fig. 1C).

Structural studies show that C5b-8 on the membrane induces conformational changes in CD59 that enable the latter to bind to C9 and inhibit MAC formation (15). Thus, only CD59 on the cell surface can respond to C5b-8, suggesting that sublytic complement attack could modulate CD59 recycling. To test this theory, we exposed RPE monolayers for 10 min to either 10% normal human serum (NHS) or 60 μ M purified C9 protein (mean physiological C9 concentration) (11) reconstituted in 10% C9-depleted serum. Analysis of lactate dehydrogenase (LDH) activity in the apical and basolateral media confirmed that both treatments were sublytic, i.e., caused <5% cytotoxicity (24) (Fig. S1C and D). Exposure to NHS (Fig. 1C) or C9 (Fig. S1E) caused an $\sim$ threefold increase in CD59 recycling within 15 min after PI-PLC treatment, compared with baseline recovery in untreated cells (Fig. 1C) or cells exposed to C9-depleted serum alone (Fig. S1E). This accelerated CD59 recycling in response to complement was not observed in cells with A2E (Fig. 1C). Acute treatment with A2E, as in the CD59 recycling experiments, did not alter CD59 protein levels (Fig. S1F); however, CD59 protein was significantly reduced after chronic treatment with A2E (50 nM for 3 wk) (Fig. S1F) and in 6-mo-old *Abca4*^{-/-} mice RPE (Fig. S1G; antibody validated in Fig. S2). Because CD59 gene expression is not altered in pigmented *Abca4*^{-/-} mice RPE (7), the data in Fig. 1A and Fig. S1F and G suggest that A2E-induced cholesterol storage shunts CD59 toward lysosomal degradation, resulting in decreased CD59 protein levels over time.

Exposure of RPE to either NHS or C9 caused MAC deposition, which was significantly increased in cells with A2E (Fig. 1D and E and Fig. S1H), likely due to less cell surface CD59. We have shown that excess cholesterol in RPE with vitamin A dimers activates ASMase, which interferes with organelle traffic by acetylating microtubules (MT) (20). To investigate how excess cholesterol and MT acetylation impacts CD59 trafficking, we treated RPE monolayers with either U18666A to increase cholesterol (25) and, thereby, acetylated MT (20), or with the HDAC6 inhibitor tubacin, which prevents MT deacetylation (26). Similar to A2E, both drugs decreased cell surface CD59 and increased MAC deposition after complement exposure (Fig. S1I). Thus, cholesterol-induced MT acetylation in RPE with vitamin A dimers prevents rapid CD59 recycling in response to complement and enables MAC assembly on the cell surface.

RPE Cholesterol Modulates Susceptibility to Sublytic MACs. To confirm that excess cholesterol promotes MAC deposition after complement attack, we used oxidized LDL (OxLDL) to increase RPE cholesterol independent of vitamin A dimers (Fig. 2A). OxLDL also increased acetylated tubulin (Fig. 2B) and decreased

cell surface CD59 (Fig. 2C). Complement attack resulted in more MAC deposition in OxLDL-treated cells, compared with RPE treated with LDL (Fig. 2D). Because vitamin A dimers, oxidized lipids, and lipoproteins are all implicated in the pathogenesis of macular dystrophies (8, 10, 18), our data point to excess cholesterol as a common etiological factor that could link these insults with abnormal complement activation in the outer retina.

Sublytic Complement Attack Induces Rapid Lysosome Exocytosis. One of the earliest detectable events after complement exposure is an increase in intracellular calcium ($[Ca^{2+}]_i$) via influx through MAC pores (24) and/or activation of endogenous ion channels (27). In many cell types, elevated $[Ca^{2+}]_i$ has been shown to trigger fusion of lysosomes with the plasma membrane (25, 28). Lysosome exocytosis, mediated by the lysosomal calcium sensor synaptotagmin 7 (Syt7), is vital for preserving membrane integrity after exposure to pore-forming toxins (29). We used live-cell imaging by total internal reflection fluorescence (TIRF) microscopy and complementary biochemical assays to determine whether sublytic MACs stimulate lysosome exocytosis in the RPE. We transfected primary RPE with Syt7 tagged with pHluorin, whose fluorescence is quenched in acidic lysosomal pH and relieved upon exposure to neutral pH after exocytosis (30). Individual exocytic events after addition of 10% NHS were detected as bright flashes of unquenched pHluorin in control cells (Fig. 3A and Movie S1). Cells with A2E had significantly fewer detectable exocytic events (Fig. 3A and Movie S2): an average of 9 events per min compared with 93 events per min in control cells (Fig. 3C).

Lysosome exocytosis causes transient appearance of lysosomal membrane proteins (e.g., LAMP2) on the plasma membrane and release of lysosomal hydrolases such as β -hexosaminidase (β -hex) into the extracellular medium (25). We detected LAMP2 on the RPE cell surface after a 10-min exposure to either 10% NHS (Fig. 3D) or 60 μ M C9 (Fig. S3A). Live-cell TIRF imaging showed that lysosome-plasma membrane interactions are transient with both full and partial kiss-and-run fusions; therefore, we were able to detect LAMP2 mostly on the apical surface of a subset of cells. β -hex activity was detected in both the apical and basolateral media after complement attack in control cells, suggesting that lysosomes fuse with both domains of the RPE (Fig. 3E). No β -hex activity was detected after exposure to heat-inactivated NHS; thus, only active complement components stimulate lysosome exocytosis. Consistent with TIRF imaging, RPE cells with A2E had significantly less surface LAMP2 and β -hex activity in the medium after complement attack (Fig. 3D and E).

TIRF imaging showed that lysosomes exocytose within seconds of complement exposure, indicating that a pool of lysosomes pre-docked at the plasma membrane are rapidly mobilized in response

Fig. 4. Persistent sublytic MAC causes mitochondrial damage in the RPE. (A) Surface LAMP2 labeling (green) after exposure to 10% NHS in control cells and after lysosome ablation. Phalloidin (red) labels the actin cytoskeleton and demarcates cell boundaries. (B) Representative single-cell recording of intracellular calcium measured by Fluo-4 NW fluorescence in control (green curve) and lysosome-ablated (red curve) cells immediately after addition of 10% NHS. (C) Surface rendering of mitochondrial volume in RPE-expressing mito-RFP. Control and lysosome-ablated cells were exposed to 10% NHS. Color bar shows decreasing mitochondrial volume from red to violet (indicating increasing mitochondrial fragmentation). (D) Quantification of the number of mitochondrial fragments after NHS exposure. $^{**}P < 0.005$, 25 cells per condition. (E) Surface rendering of mitochondrial volume as in C. (F) Quantification of the number of mitochondrial fragments after NHS exposure. $^{*}P < 0.05$, $n \geq 31$ cells. (G) Representative immunoblot and quantification of OPA1 protein levels normalized to β -tubulin in primary polarized RPE after complement attack. Mean $\pm$ SEM, $n = 3$. $^{**}P < 0.005$. (H) Representative immunoblot and quantification of OPA1 protein levels normalized to RPE65 in RPE from wild-type and *Abca4*^{-/-} mice. Mean $\pm$ SEM, $n = 3$. $^{*}P < 0.05$. (I) Stills from live imaging of CellROX labeling to monitor reactive oxygen species (green) in polarized primary RPE after exposure to 10% NHS for 10 min. (J) Quantitation of CellROX fluorescence. Mean $\pm$ SEM, $n = 3$ independent experiments. $^{*}P < 0.05$.

to elevated $[Ca^{2+}]_i$ (28). Analysis of the subcellular distribution of LAMP2-labeled lysosomes showed that there were significantly fewer lysosomes within 500 nm of the cell membrane in RPE with A2E ($49.43 \pm 1.8\%$) compared with control cells ($58.43 \pm 1.9\%$) (Fig. 3F). Taken together, these data demonstrate that sublytic complement attack stimulates lysosome exocytosis in the RPE. However, in cells with A2E, this is impaired because excess cholesterol and tubulin acetylation prevent MT-based transport of lysosomes to the cell periphery (31, 32).

Defective Lysosome Exocytosis Promotes Mitochondrial Damage After Complement Attack. To determine whether lysosome exocytosis is a critical response to sublytic MACs, we loaded RPE lysosomes with horseradish peroxidase (HRP) and ablated them with diamminobenzidine (DAB) (33). Inactivation of lysosomes was confirmed by loss of Lysosensor Blue labeling (Fig. S4B), whereas recycling endosomes labeled with Alexa-Transferrin were unaffected (Fig. S4C). Lysosome ablation prevented the appearance of LAMP2 on the RPE cell surface after exposure to NHS (Fig. 4A) or C9 (Fig. S4D). Live-cell imaging showed sustained elevation of $[Ca^{2+}]_i$ in lysosome-ablated RPE exposed to NHS compared with control cells whose $[Ca^{2+}]_i$ returned to baseline levels within 60 s (Fig. 4B and Fig. S4E).

Exposure to MACs over 12–24 h causes mitochondrial stress due to persistent increase in $[Ca^{2+}]_i$ (11, 24). Here, we asked whether defective lysosome exocytosis leads to mitochondrial damage in the RPE after a short exposure to complement. Live-cell imaging showed that the RPE mitochondrial network is remarkably robust with no appreciable fragmentation after exposure to 10% NHS for 10 min (Fig. 4C). However, in RPE with ablated lysosomes, complement attack increased mitochondrial fragmentation (Fig. 4C and D). The mitochondrial network in RPE cells with A2E was similarly susceptible to fragmentation after two exposures to NHS over 48 h (Fig. 4E and F), likely due to impaired lysosome exocytosis (Fig. 3A–E).

To identify the mechanism underlying mitochondrial fragmentation, we measured the expression of optic atrophy 1 (OPA1), which mediates mitochondrial inner membrane fusion to maintain cristae architecture (34). Primary RPE with A2E (Fig. 4G) and RPE from *Abca4*^{-/-} mice (Fig. 4H) had significantly less OPA1 protein compared with control RPE and RPE from wild-type mice, respectively. Mitochondrial fragmentation induced by sublytic MACs increased generation of reactive oxygen species (ROS) only in RPE with A2E, but not in control cells (Fig. 4E). These data provide strong support for the hypothesis that impaired CD59 recycling and lysosome exocytosis in RPE with vitamin A dimers make these cells vulnerable to complement-induced mitochondrial damage.

Strategies To Prevent Complement-Induced Injury in the RPE. In *Abca4*^{-/-} RPE and primary RPE with A2E, excess cholesterol results in MT acetylation by activating ASMase (20). To establish that cholesterol-mediated activation of ASMase is responsible for impaired CD59 recycling and lysosome exocytosis, we treated polarized primary RPE monolayers with the liver X receptor α (LXR α) agonist TO901317 (TO; 1 μ M, 16 h) to increase cholesterol efflux, or with the Food and Drug Administration-approved ASMase inhibitor desipramine (Des; 10 μ M, 3 h) to decrease acetylated tubulin in RPE with A2E (20, 21). Both drugs restored surface CD59 (Fig. 5A and Fig. S4A) and lysosome exocytosis (Fig. 5B and Fig. S4B) in cells with A2E to levels seen in control RPE. Treatment with TO or Des also decreased ROS generation after complement exposure in RPE with A2E (Fig. S4C). Administration of TO to 5-mo-old *Abca4*^{-/-} mice by intraperitoneal injections for 4 wk significantly decreased RPE cholesterol (Fig. 5C), restored CD59 to the apical surface of the RPE (Fig. 5D), and increased OPA1 expression (Fig. 5E).

Discussion

To maintain retinal homeostasis and limit inflammation in the face of aberrant complement activation, the RPE needs robust defense mechanisms that rapidly prevent MAC assembly and

Fig. 5. Cholesterol removal or ASMase inhibition restores protective responses in the RPE. (A) Representative x-z scans of surface CD59 immunostaining (green) in polarized primary RPE cells (±A2E) untreated, or treated with either TO901317 (TO) or desipramine (Des). ZO-1 is in red. (B) Appearance of LAMP2 (green) on the plasma membrane after exposure to 10% NHS alone or after treatment with TO or Des. (C) Total cholesterol in the RPE of *Abca4*^{−/−} mice administered either vehicle or TO (20 mg/kg, i.p.). Mean ± SEM, *n* = 4 animals per condition. **P* < 0.05. (D) Single-plane en face and x-z confocal images of CD59 (blue) immunostaining in RPE flatmounts at the apical (Ap) surface in vehicle- or TO-treated *Abca4*^{−/−} mice. (E) Representative immunoblot and quantification of OPA1 protein levels normalized to RPE65 in RPE from *Abca4*^{−/−} mice administered either vehicle or TO. Mean ± SEM, *n* = 4 animals per condition. **P* < 0.05.

eliminate MAC pores. Here, we identified two such responses occurring on timescales of seconds to minutes in the RPE—CD59 recycling and lysosome exocytosis—that are critical for countering sublytic MAC injury. Using pigmented *Abca4*^{−/−} mice as a model for the early stages of Stargardt disease (35), our data also elucidate how vitamin A dimers compromise these rapid protective responses, resulting in RPE mitochondrial damage after sublytic MAC attack.

The membrane-bound complement regulator CD59 inhibits the final step of MAC formation, i.e., recruitment of C9 to the C5b-8 complex. Our data suggest that complement modulates CD59 trafficking to the RPE cell surface because exposure to C9 accelerated CD59 recycling. Nascent C5b-8 complexes cause conformational changes in CD59 necessary for it to bind C9 (15); therefore, increasing the amount of CD59 on the RPE plasma membrane could be a rapid way to combat assembly of functional MAC pores after complement attack. Live-cell imaging showed that complement exposure increased [Ca²⁺]_i and stimulated lysosome exocytosis. Lysosomal hydrolases and lipids released during exocytosis have been proposed to remodel the cell surface and limit membrane damage caused by pore-forming toxins (29). We show here that inactivating lysosomes causes sustained [Ca²⁺]_i elevation and mitochondrial fragmentation, indicating that lysosome exocytosis is a key mechanism for curtailing the downstream consequences of complement attack in the RPE.

Based on these data, we propose that the RPE has at least two distinct responses to sublytic MACs that differ primarily in the kinetics of their execution: CD59 recycling and lysosome exocytosis occur within minutes, as we have shown here. Endocytosis and lysosomal degradation of MACs by the RPE (11) and shedding of MACs into the extracellular medium (36) have been reported after tens of minutes or hours of exposure to complement; whether these mechanisms also occur over faster timescales remains to be determined. Data presented here and in other studies (11) show that defects in any of these mechanisms converge on a common pathway of mitochondrial damage in the RPE.

Despite increasing evidence that implicates the complement pathway in the etiology of Stargardt disease (7, 8) and AMD (3, 4), the mechanisms involved remain unclear. Our data show that in RPE with vitamin A dimers, cholesterol-induced MT acetylation (20) interfered with both CD59 recycling and lysosome exocytosis, leading to mitochondrial damage and oxidative stress after complement attack. Because oxidative stress affects mitochondrial fusion/fission dynamics, this could establish a self-propagating cycle of mitochondrial damage, inflammation, and dysfunction in the RPE. Finally, our experiments identify two therapeutic strategies to prevent complement-induced RPE damage: increasing cholesterol efflux by activating LXRα and decreasing MT acetylation by inhibiting ASMase (20). These data provide proof-of-concept

validation of LXRα and ASMase as potential drug targets for Stargardt disease and other retinopathies associated with the accumulation of vitamin A dimers.

To date, approaches to target the complement pathway in macular degenerations have focused on complement proteins, with varying success (37). Our studies move beyond this narrow focus by providing insight into how the RPE deals with complement at a cellular level and by identifying drugs that can strengthen innate mechanisms essential for preserving RPE health.

Materials and Methods

Mice. Wild-type (Jackson Labs; 129S1/SvImJ) and *Abca4*^{−/−} mice (Jackson Labs; *Abca4*^{tm1Ght/J}) were raised under 12-h cyclic light with standard diet. Mice were killed ~4–6 h after light onset, eyes were removed, and eyecups were processed for immunohistochemistry or immunoblotting (38). Studies were approved by the University of Wisconsin–Madison animal care and use authorities.

Primary Cell Culture. RPE were isolated from porcine retinas and plated at ~300,000 cells per square centimeter onto collagen-coated Transwell filters (Corning) to generate polarized RPE monolayers (20, 38).

Microscopy and Image Analysis. Imaging was performed on the Revolution XD dual spinning disk confocal/TIRF system (Andor). Images for a specific experiment were acquired using the same settings and analyzed using Imaris Spots and Surfaces modules (Bitplane) (20, 38).

Sublytic Complement Attack. Polarized RPE were exposed to either 10% NHS or 60 μg/mL purified C9 reconstituted in 10% C9-depleted serum (Quidel) for 10 min (11, 12). Heat-inactivated NHS (56 °C for 30 min) and C9-depleted sera were used as negative controls.

Treatments and Assays. Polarized RPE were treated with A2E (acute: 10 μM, 6 h followed by 48-h chase in fresh medium; chronic: 50 nM, 3 wk) (20). Other reagents used were LDL or OxLDL [both 50 μg/mL, 16 h (10); Athens Research & Technology], the ASMase inhibitor desipramine (10 μM, 3 h; Sigma) and the LXRα agonist TO901317 (1 μM, 16 h; Cayman) (20, 21). Cholesterol levels were measured with the Amplex Red cholesterol assay (ThermoFisher) (20).

Immunohistochemistry and Immunofluorescence. RPE flatmounts were fixed, incubated with primary antibodies [CD59 (1:50; LS Bio) and LAMP1 (1:100; Sigma)] overnight at 4 °C, followed by secondary antibodies for 2 h. Polarized primary porcine RPE were fixed, blocked with 1% BSA, and incubated with primary antibodies in 0.1% saponin (1 h) and secondary antibodies (30 min). For surface immunostaining, live cells were incubated with primary antibodies (30 min, 4 °C), fixed, permeabilized, and processed as above for ZO-1 labeling. Antibodies used were as follows: CD59 (1:100; ThermoFisher), LAMP2 (1:500; AbDSerotec), acetylated tubulin (1:1000; Sigma), C5b-9 (1:100; Novus), and ZO-1 (1:3000) (25). Other reagents were as follows: rhodamine-phalloidin (1:200, PHDR1; Cytoskeleton), Alexa-conjugated secondary antibodies (1:500), and DAPI (1:200), both from ThermoFisher.

Immunoblotting. RPE lysates were resolved using SDS/PAGE, proteins transferred to nitrocellulose membranes (ThermoFisher), and probed with rabbit anti-OPA1 (1:500; Novus), followed by HRP-conjugated secondary antibodies. β -Tubulin (1:500; Novus) and RPE65 (1:5000; Novus) were used as loading controls. Blots were visualized by ECL (ThermoFisher) and quantified with Image Studio (LI-COR).

CD59 Recycling. Cells were incubated with 1 U/mL PIPLC (ThermoFisher) for 1 h at 37 °C to cleave surface CD59, exposed to complement, and returned to normal growth medium (with 100 μ g/mL cycloheximide to inhibit new protein synthesis) for indicated times. Cells were fixed, immunostained for surface CD59, and imaged as above.

TIRF Microscopy. RPE expressing pHluorin-Syt7 (30) were imaged at 37 °C immediately after addition of 10% NHS. TIRF angle was selected for the shallowest evanescent illumination, and images were captured every 10 s for 5 min.

Lysosome Exocytosis and Localization. RPE monolayers were exposed to complement, immediately transferred to ice and processed for surface LAMP2 staining. β -Hex activity in the media was measured as described previously (25) with 4-methyl-umbelliferyl-*N*-acetyl- β -D-glucosaminide (Sigma) as the substrate. For quantification of peripheral lysosomes, cells were transduced with CellLight Plasma Membrane-RFP (ThermoFisher), fixed, permeabilized, and stained for endogenous LAMP2. Cell surface and lysosomes were reconstructed in Imaris. Lysosomes within $\leq$ 500 nm from the plasma membrane were counted as peripheral lysosomes.

Lysosome Ablation. RPE cells were incubated with 5 mg/mL horseradish peroxidase (HRP; Sigma) in DMEM + 12 mM Hepes (1 h, 37 °C), rinsed, and incubated in fresh DMEM + 12 mM Hepes (2 h, 37 °C) to allow HRP uptake and transport to lysosomes (33). Cells were treated with 150 μ g/mL DAB + 0.03% H_2O_2 in HBSS (1 h, 25 °C) to cross-link lysosomal proteins and ablate lysosomes. Control cells were similarly processed except for DAB treatment.

Calcium Imaging. Cells were loaded with Fluo-4 NW (ThermoFisher) for 30 min at 37 °C according to the manufacturer's protocol. Fields of interest were determined and imaging was initiated immediately after addition of 10% NHS. Images were captured at 37 °C at 1 frame per s for 2 min with 30 ms of exposure time. Mean intensity of Fluo-4 over time was calculated using Imaris.

Mitochondrial Dynamics and ROS. CellLight Mitochondria-RFP-expressing RPE were exposed to NHS and imaged live. Mitochondrial fragments were quantified and color-coded in Imaris. For ROS measurements, cells were incubated with 5 μ M CellRox (37 °C, 30 min) and imaged live. CellROX fluorescence was measured at 640/665 excitation/emission (Ex/Em; Tecan) and normalized to DAPI fluorescence (Ex/Em: 360/460).

In Vivo Drug Treatments. *Abca4*^{-/-} mice (males and females) were intraperitoneally injected three times a week for 4 wk with either 100 μ L of 0.9% sodium chloride (Hospira) or 20 mg/kg T0901317 (Cayman) in 0.9% sodium chloride.

Statistics. Data were analyzed by using either a two-tailed *t* test or one-way ANOVA with the Bonferroni post hoc test (GraphPad Prism). Unless otherwise stated, data are presented as mean $\pm$ SEM of $\geq$ 3 independent experiments, with at least three to four replicates per condition per experiment.

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