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Phagocytosed photoreceptor outer segments activate mTORC1 in the retinal pigment epithelium

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Abstract

The retinal pigment epithelium (RPE) transports nutrients and metabolites between the microvascular bed that maintains the outer retina and photoreceptor neurons. The RPE removes photoreceptor outer segments (POS) by receptor-mediated phagocytosis, a process that peaks in the morning. Uptake and degradation of POS initiates signaling cascades in the RPE. Upstream stimuli from various metabolic activities converge on mechanistic target of rapamycin complex 1 (mTORC1), and aberrant mTORC1 signaling is implicated in aging and age-related degeneration of the RPE. In the current study, we measured mTORC1-mediated responses to RPE phagocytosis in vivo and in vitro. During the morning burst of POS shedding, there was transient activation of mTORC1-mediated signaling in the RPE. POS activated mTORC1 through lysosome-independent mechanisms and engulfed POS served as a docking platform for mTORC1 assembly. The identification of POS as endogenous stimuli of mTORC1 in the RPE provides a mechanistic link underlying the photoreceptor-RPE interaction in the outer retina.

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Author Contributions

YC and JC conceived and coordinated the study and wrote the paper. YC, BY, PX, BL, and ZZ performed the in vivo experiments. YC, BY, AE, RD, PX, and JC performed the in vitro experiments. HM provided support in data analysis and interpretation. RC provided technical support. TGW provided technical support and conceptual advice. All authors reviewed the results and approved the final version of the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

Data and Materials Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

Introduction

Signaling pathways mediated by mechanistic target of rapamycin complex 1 (mTORC1) play central roles in maintaining cellular metabolic homeostasis by balancing catabolic and anabolic activities (1-3). The spatial distribution of mTORC1 components is crucial for their functions (4), and lysosomal surfaces are likely to be major sites of mTORC1 activation (1, 5, 6). Recruitment of mTORC1 to the lysosomal surface is mediated by two protein complexes, Regulator and heterodimer Rag GTPases (7, 8). Regulator has at least five protein subunits: p14, p18, MP1, HBXIP, and C7orf59 (or LAMTOR 1-5) (9). It is a scaffold that tethers Rag GTPases to the lysosomal membrane (8) and also serves as a guanine nucleotide exchange factor for Rags (9). GTP-loaded active heterodimeric Rag GTPase recruits mTORC1 (7,10). Certain lysosomal membrane transporter proteins, such as vacuolar H⁺-ATPase (V-ATPase)(11, 12), SLC38A9 (13, 14), and SLC15A4 (15), function as nutrient sensors and facilitate mTORC1 activation. Localization of mTORC1 to subcellular organelles other than lysosomes has also been reported (4).

mTORC1 plays a key role in controlling organism longevity and tissue aging, particularly in postmitotic cells, such as the retinal pigment epithelium (RPE), which are susceptible to age-related degeneration (16). The RPE is highly metabolically active. It is responsible for transporting nutrients and metabolites between the choroidal blood supply and the neural retina, and is part of the visual cycle, converting all-*trans*-retinol into 11-*cis*-retinal. The 11-*cis*-retinal is transported into the retina and utilized by G-protein coupled receptor, opsin, as the chromophore for phototransduction (17, 18). Furthermore, photoreceptor outer segments (POS) that are shed daily are removed by the RPE through receptor-mediated phagocytosis (17, 19). We have previously reported that aged RPE cells have increased lysosomal mass, increased mTORC1 activity, and decreased ability to degrade phagocytosed POS (20).

RPE phagocytosis follows a diurnal pattern, which peaks at approximately two hours after lights are turned on in the morning because of synchronized disc shedding (21, 22). POS discs are recognized and engulfed by the RPE through receptor-dependent mechanisms (19, 23-26). Internalized POS are transported to lysosomes for maturation and degradation. Renewal of POS by RPE phagocytosis is crucial for visual function, and the process is tightly regulated by the receptor tyrosine kinase MerTK and focal adhesion kinase (26-28). Proteins of the autophagic machinery participate in POS trafficking through LC3-associated phagocytosis (LAP), which is a non-canonical autophagy pathway independent of the pre-initiation unc-51-like kinase 1 (ULK1) complex (29-33).

We sought to determine whether mTORC1 activity was regulated by phagocytosis (the most fundamental function of the RPE), and to explore the underlying mechanisms of mTORC1 activation. We found periodic changes in mTORC1 activity in response to the morning burst of phagocytosis in vivo, as well as increased mTORC1 activity in cultured RPE cells exposed to purified POS. Ingested POS served as a docking platform for recruiting mTORC1 components, and phagocytosis-induced mTORC1 activation occurred independently of lysosomal function. Transient activation of mTORC1 resulted in phosphorylation of ULK1, suggesting potential inhibition of the autophagy pre-initiation complex. Together, these data demonstrate that internalized POS are physiological stimuli of

mTORC1 through a signaling pathway that is distinct from the canonical amino acid-mediated pathway of activation.

Results

Fluctuation of mTORC1 activity in response to retinal pigment epithelium phagocytosis

Synchronized RPE phagocytosis occurs during the morning burst of disc shedding (27). The precisely controlled timing of the two processes provides a unique model system for studying the potential relationship between mTORC1 signaling and RPE phagocytosis in vivo. To validate the time course of the morning burst of phagocytosis in our experimental animals, whole mount tissues of RPE/choroid were prepared from C57 BL/6J mice at 1-hour intervals starting at the onset of light. Ingested POS were measured by immunostaining rhodopsin-positive particles in phalloidin-delineated RPE cells (Fig. 1A). Consistent with previous reports (21, 22), uptake of POS by the RPE increased after light onset and peaked approximately 2 hours later (Fig. 1A and 1B). Next, we examined mTORC1 activity in the tissue at the same times by immunostaining for the phosphorylated form of ribosomal protein S6, a downstream effector of mTORC1 (34). There was a time-dependent change in S6 phosphorylation, which peaked at approximately 1 hour after lights-on, followed by a return to basal levels thereafter (Fig. 1C and 1D). Phagocytosis-induced S6 phosphorylation was further confirmed by western blot analysis of RPE tissues harvested around the time of the morning burst (Fig. 1E and 1F). Maximum phosphorylation of S6 was detected 1 hour after lights-on. The time course was similar using the two methods.

We further measured POS-induced mTORC1 activation in cultured RPE cells. Confluent ARPE-19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/mL glucose and 1% fetal bovine serum (FBS) overnight to minimize the basal activity of mTORC1. Dose-dependence and time-dependence of mTORC1 activation were measured in response to treatment with purified POS. S6 phosphorylation plateaued at a POS:RPE ratio of approximately 10:1 (Fig. 2A), a ratio routinely used in studies of RPE phagocytosis (35). Exposing RPE cells to POS led to rapid but transient activation of mTORC1 (Fig. 2B), which was similar to the time course observed in vivo (Fig. 1). The level of phosphorylated S6 increased at 30 minutes, was sustained until 2 hours, and returned to the basal level within 6 hours after POS challenge. The mTORC1 inhibitor rapamycin inhibited S6 phosphorylation (Fig. S1 A), consistent with activation of mTORC1 by POS, although rapamycin treatment did not influence the rate of POS degradation (Fig. S1B). The mTORC1 substrates 4E-BP1 and ULK1 were also phosphorylated in ARPE-19 cells upon exposure to POS (Fig. 2A and 2B). Dose-dependent and time-dependent mTORC1 activation by POS were also observed in cultured primary human fetal RPE cells (Fig. S2). Similarly, Muniz-Feliciano *et al.* have reported that POS activated S6K phosphorylation in RPE-J cells (36). Thus, results from in vivo tissues, cultured primary RPE cells, and immortalized cell lines all indicated that POS can activate the mTORC1 pathway in the RPE.

POS phagocytosis is mediated by cell surface receptors, and $\alpha V\beta 5$ -integrin plays a major role in recognition and binding of POS (23). We used small interfering RNAs (siRNAs) to knock down integrin $\beta 5$, which suppressed POS engulfment, mTORC1 activation (Fig. 2C)

and S6 phosphorylation. We also treated RPE cells with latex beads, which are taken up through nonspecific, receptor-independent phagocytosis (37). Consistent with the non-specific nature of this phagocytosis, mTORC1 was not activated in RPE cells that engulfed latex beads (Fig. 2D and Fig. S3). Together, these experiments demonstrated that POS phagocytosis activates mTORC1 in the RPE.

Redistribution of mTORC1 components to photoreceptor outer segments-derived organelles

Subcellular localization has emerged as a key mechanism regulating mTOR activity (4). Thus, we examined the distribution of mTORC1 components in POS-treated RPE cells (Fig. 3). ARPE-19 cells can efficiently take up POS, indicated by the appearance of POS-positive phagosomes as early as 15 minutes after POS challenge (Fig. S4). Immunostaining and imaging of cultured RPE cells showed that, in the absence of POS, mTOR was localized to both the cytosol and the late endosome/lysosome, as indicated by patterns of diffuse and small punctate staining, respectively (Fig. 3) (4, 7). There was apparent co-localization of mTOR with rhodopsin-positive organelles, which was seen as early as 15 minutes after POS exposure and persisted for 4 hours. During the time frame of 4 hours, ingested POS sequentially fused with endosomes and lysosomes (Fig. 3), as indicated by co-localization of rhodopsin with EEA1 (an early endosome marker), Rab5 (an endosome protein crucial for transition of early endosome to late endosome), and LAMP2 (a lysosome marker).

Two protein complexes, the p18-containing scaffold Ragulator complex and Rag GTPases, are responsible for tethering mTORC1 to its activation site (7, 8, 12). Both p18 (Fig. 4A) and RagA (Fig. 4B) co-localized with engulfed POS after 30 minutes. Similar changes were observed for Rheb (Fig. 4C and 4D), the small GTPase (4, 38, 39) essential for mTORC1 activation. The presence of mTOR, Raptor, p18, and RagA in POS-containing structures was further confirmed by western blot analyses of the re-purified POS-enriched fraction (Fig. 4E), whereas Rictor, the unique component of the mTORC2 complex, was not detected in this fraction.

To validate these findings *in vivo*, we co-stained whole mounts of mouse RPE/choroid tissue with antibodies against mTOR, p18, Rag A, and rhodopsin during the morning burst of phagocytosis. Consistent with our *in vitro* observations, mTORC1 components were co-localized with rhodopsin-positive POS phago (lyso)somes (Fig. 4F and 4G) in the RPE. Collectively, our data showed that engulfed POS rapidly interact with organelles of the RPE vesicular trafficking system (40) and likely exchange membrane and protein components with these organelles.

Lysosome-independent mTORC1 activation induced by photoreceptor outer segments

Assembly and activation of mTORC1 has been suggested to occur on the lysosomal membrane (41). Our immunostaining studies showed that redistribution of mTOR to rhodopsin-positive organelles occurred quickly after POS uptake, raising the possibility that mTORC1 was activated before engulfed POS matured into phagolysosomes. This hypothesis was tested using various independent experimental approaches. Kinesin-1 light chain 1 (KLC1), the light chain subunit of microtubule motor kinesin 1, regulates motility of

phagosomes and facilitates interactions between phagosomes and endosomes or lysosomes in the RPE (42). siRNA knockdown of KLC1 suppressed the rate of POS degradation in cultured RPE cells without affecting uptake (Fig. S5) (42). In contrast, POS-stimulated mTORC1 activity was comparable between control and KLC1-depleted cells (Fig. 5A), indicating that the pathway of POS trafficking and degradation can be separated from that of POS-mediated mTORC1 activation.

In addition to KLC1, we found that an unconventional myosin motor protein, myosin VI (Myo6), was involved in POS transport and maturation in the RPE. Myo6 serves as a tethering protein between cargoes and the cytoskeleton, regulating trafficking processes, such as autophagy (43). In both cultured RPE and in vivo RPE tissue, Myo6 co-localized with rhodopsin-positive phagosomes (Fig. 5B – 5D). Downregulation of Myo6, via either CRISPR/Cas9 or siRNA, suppressed POS degradation (Fig. 5E, 5F, and S6A). When mTORC1 activity was assessed, RPE cells with depleted Myo6 continued to respond well to either POS or amino acid stimulation (Fig. 5G, 5H, and S6B). mTORC1 activity was modestly decreased in cells stimulated by both stimuli, indicating that Myo6 has regulatory roles in steps common to both POS- and nutrient-induced mTORC1 activation.

Next, we used chloroquine treatment to disrupt the lysosome pH gradient. Consistent with prior reports (44), chloroquine inhibited amino acid-induced mTORC1 activation and led to LC3-II accumulation (Fig. 6A), indicative of lysosomal dysfunction. However, in the presence of chloroquine, mTORC1 still responded to POS and co-localized with rhodopsin-positive structures in the RPE (Fig. 6A and 6B). Quantification of imaging data regarding co-localization of rhodopsin with mTOR showed no differences between cells exposed or not exposed to chloroquine (Fig. 6B). Furthermore, siRNA knockdown of subunits (ATPv1A and ATPv0C, or ATPv1A and ATPv0D) of lysosomal v-ATPase, which promotes amino acid-induced mTORC1 activation (12), led to decreased S6 phosphorylation in response to amino acids but not to POS (Fig. 6C and Fig. 6D). Collectively, these data demonstrate that POS-induced mTORC1 activation occurred independently of lysosomal function.

Co-localization of mTOR and mTORC1 components with rhodopsin-positive structures (Fig. 3 and 4F) suggested that POS or POS-containing early phagosomes can act as an activation site of mTORC1. To test this possibility, we performed a cell-free reconstitution assay using purified POS and lysosome-depleted cytosolic fractions and assessed mTORC1 signaling. The cytosol/membrane (C/M) fraction of ARPE-19 cells was prepared by differential centrifugation (Fig. 7A). To remove lysosomes, the C/M fraction was subjected to either immunodepletion with an antibody specific to LAMP2 (C/M-Lyso) or ultracentrifugation at 100,000g (S100). Efficient removal of lysosomes was confirmed in both fractions by immunoblotting analysis (Fig. 7B). When incubated with purified POS for the in vitro reconstitution and kinase assay, the C/M fraction exhibited increased mTORC1 activity. The phosphorylation of S6K at Thr³⁸⁹, the mTORC1-dependent phosphorylation site (45), was 1.61-fold higher in the C/M fraction containing POS than in the C/M fraction without POS (Fig. 7C). Furthermore, addition of POS to C/M-Lyso also led to an increase in S6K phosphorylation. The ability of POS to activate mTORC1 was similar in the C/M fractions with or without lysosomes (Fig. 7D). p85 S6K, rather than p70, appeared to be the major form of S6K that was phosphorylated. S6, a protein substrate of S6K, normally has

more robust activation than S6K in cultured RPE cells. In the reconstitution assay, however, S6 only exhibited modest phosphorylation after POS addition (Fig. 7C and 7E). This may indicate that subcellular organelles, such as lysosomes, facilitate the transduction of an initial activation signal to more robust downstream responses. The S100 fraction did not reliably lead to mTORC1 activation in the presence of POS (Fig. 7C). We also used liposomes and the subcellular fractions to perform the reconstitution assay. Incubation of C/M-Lyso fraction with liposomes activated the mTORC1 pathway (Fig. 7F and 7G), suggesting that lipid membrane structures were sufficient to initiate mTORC1 activation in the in vitro reconstitution system.

Discussion

Upstream stimuli of mTORC1 are diverse and can include growth factors, nutrients, changes in cellular energy level, or stress. We demonstrated that phagocytosis of POS can also serve as a physiological stimulus of mTORC1 in the RPE. Both in vivo and in vitro studies showed increased phosphorylation of downstream substrate proteins, such as S6, 4E-BP1, and ULK1. Components of the mTORC1 pathway, including mTOR, RagA, p18, and Rheb, were recruited to POS-derived structures. Synchronized photoreceptor disc shedding and RPE phagocytosis led to changes in mTORC1 activity around the time of the morning burst. Receptor-mediated phagocytosis was required for mTORC1 activation, which could not be achieved by phagocytosis of latex beads. During the revision of this manuscript, Ferguson's group similarly reported that S6K phosphorylation can be stimulated by POS phagocytosis in RPE-J cells (36). Their data independently validate POS-induced mTORC1 activation in the RPE.

Effective POS phagocytosis is a major task of the RPE, and dysregulated phagocytosis leads to retinal degeneration (26). Rapid activation, followed by inactivation, of mTORC1 during the morning burst of phagocytosis is critical for RPE and retinal homeostasis. RPE cells use multiple intracellular trafficking systems, such as endocytosis and LAP, to ensure prompt removal and recycling of ingested POS (40) (33). LAP and autophagy both utilize LC3-containing membranes. In contrast to autophagy, however, POS trafficking and degradation are not merely catabolic processes. RPE phagocytosis stimulates β -oxidation of fatty acids and ketogenesis (46). High mTORC1 activity supports aerobic glycolysis (47) and suppresses ketogenesis (48), which may serve as an off signal after the cells have used free fatty acids derived from POS as metabolic substrates. When mTORC1 activity returns to basal level, utilization of glucose through glycolysis will be reduced, and the RPE can export glucose to support photoreceptor function (49). Thus, the rhythmic activation and inactivation of mTORC1 in response to phagocytosis is likely essential for metabolic coupling between the RPE and photoreceptor neurons.

Starvation stimulates RPE autophagy in mice and suppresses POS degradation (36), suggesting that autophagy and LAP may counteract each other. mTORC1 directly phosphorylates ULK1 at Ser⁷⁵⁸(50) to suppress macroautophagy. Conditions that stimulate mTORC1 activity have been reported to inhibit autophagy in different cell lines, including the RPE (51). Unlike classic autophagy, LAP does not require ULK1 complex formation (33). When activated by POS, mTORC1 may suppress macroautophagy and reserve

autophagic machinery for LAP, ensuring efficient POS degradation following the morning burst. However, the roles of mTOR in phagocytosis are not limited to its effects on autophagy initiation. We have previously shown that cells with persistent mTORC1 activation have impaired POS degradation (20). Indeed, with aging and/or degeneration, RPE cells exhibit increased mTORC1 activity (52), which correlates with the accumulation of undigested POS. Future studies will be necessary to further elucidate the downstream signaling events of mTORC1 that control vesicular trafficking and lysosome-mediated degradation via RPE-specific mechanisms.

Activation of mTORC1 requires spatial redistribution and re-assembly of the protein complex and interaction with downstream substrates. In addition to localization to late endosomes and lysosomes, components of the mTORC1 pathway have been detected in other organelles, such as nuclei and mitochondria (4,53-57). However, it is unclear whether mTORC1 in various subcellular locations responds to different stimuli and/or regulates distinct substrates (4). Findings from our current study suggest that POS-stimulated mTORC1 activation occurred independently of lysosomal function and distribution. Inhibiting lysosome functions blocked amino acid-induced, but not POS-induced, mTORC1 activation. Similarly, siRNA-mediated depletion of components of the lysosomal v-ATPase complex reduced mTORC1 activation by amino acids but not by POS. mTOR colocalized with POS-derived structures and in vitro reconstitution assays further showed that POS-containing structures, most likely early phagosomes, served as a docking platform for mTORC1. Initial mTORC1 activation in the early phase of phagocytosis could be further amplified when phagosomes fuse with lysosomes. This later step could lead to more robust mTORC1 activation, which can be effectively transduced to downstream proteins and is important for promptly turning off the signal. A major mechanism of mTORC1 inactivation is through redistribution of the TSC1/2 complex to lysosomes (1, 5). Although the detailed mechanisms of activation remain elusive, utilization of early phagosomes of POS as activation sites may allow mTORC1 to respond rapidly during phagocytosis to meet cellular metabolic needs (Fig. S7).

mTORC1 has been reported to be activated during phagocytosis and/or entosis of cell corpses in macrophages (55). There are differences between engulfed POS-induced and cell debris-induced mTORC1 activation. The latter occurs late in phagocytosis and depends on the degradation of engulfed cargoes. In POS-induced mTORC1 activation, increased mTORC1 activity occurs shortly after phagocytosis. When lysosomal functions were impaired by a chemical inhibitor, mTORC1 was still stimulated by POS ingestion. Reducing the expression of v-ATPase, which is critical for amino acid-stimulated mTORC1 activity, did not affect POS-induced activation. Together, these data suggest that POS-activated mTORC1 is not a direct result of nutrient stimulation, in contrast to the situation with phagocytosis and degradation of cell debris.

In summary, we have demonstrated that POS internalization stimulated mTORC1 activity in the RPE and identified a mechanism of mTORC1 activation that is independent of lysosomal function and location. Because the RPE is the primary site of age-related macular degeneration and mTOR is a key regulator of aging, delineating the signaling pathways of

mTORC1 in RPE should lead to improved strategies for intervention in age-related retinal diseases.

Materials and Methods

Antibodies and chemicals

Primary antibodies used in the study are listed in Supplementary Table 1. Secondary antibodies were obtained from Life Technologies or LI-COR Biosciences. Chloroquine was obtained from Sigma-Aldrich. Latex beads were purchased from Life Technologies.

Cell culture and transduction with CRISPR/Cas system

ARPE-19 cells (American Type Culture Collection) were grown and passaged in DMEM/Ham's F12 50/50 mix (Mediatech), supplemented with 10% FBS (Sigma-Aldrich) (59). Primary cultures of human fetal RPE cells were established and cultured, as described previously (20). Cells were grown and passaged in alpha-modified Eagle's medium (α -MEM), containing 10% FBS, 10 mL/L N1 supplements, 10 mL/L MEM nonessential amino acid (100X), 1 mM sodium pyruvate, and 2 mM glutamine on collagen-coated plates and wells (Sigma-Aldrich). Cells at passages 3 to 7 were used for the experiments. Cells were maintained in a humidified carbon dioxide tank at 37°C and passaged at 90% to 100% confluence. Cas9 virus (lentiCas9-Blast) was purchased from Addgene. Lentivirus-expressing guide RNA (gRNA) targeting Myo6 9 (pLV[gRNA]-Puro-U6>hMYO6) or scrambled gRNA (pLV[gRNA]-Puro-U6>Scramble_gRNA) were designed, cloned, and packaged by VectorBuilder. To establish ARPE-19 cells stably expressing Cas9 endonuclease, ARPE-19 cells were transduced with lentiCas9-Blast viral particles at a multiplicity of infection of 10:1 and selected with 20 μ g/mL blasticidin for 7 days. ARPE-19 cells expressing Cas9 (ARPE-19 Cas9) were further transduced with lentiviral particles expressing either gRNA targeting human Myo6 or scrambled gRNA and selected with 10 μ g/mL puromycin for 10 days.

Mice

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch. Male C57 BL/6J mice (Jackson Laboratory) were housed under cyclic 12-light light/dark conditions (7 a.m. lights-on/7 p.m. lights-off). Routine screening did not reveal the Rd8 mutation in the *Crb1* gene (60). Euthanasia was performed by exposure to carbon dioxide delivered at 2 L/min through a three-stage gas regulator. All procedures were conducted in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Immunofluorescence microscopy

Whole mount tissues of RPE/choroid were prepared from 3- to 4-month-old mice, as described previously (20). The tissues were fixed in 4% paraformaldehyde and permeabilized with ice-cold acetone. After blocking in 10% (v/v) FBS/0.5% (v/v) Triton X-100/1 $\times$ phosphate buffered saline (PBS), RPE flat mounts were stained with different antibodies. Nuclei were counterstained with Hoechst 33342 (Life Technologies). The F-actin-specific dye phalloidin (Life Technologies) was used to delineate the RPE boundaries.

In some experiments, control RPE autofluorescence was determined using unstained whole mount tissues from the contralateral eye of the same animal.

For cell staining, cultured RPE cells were seeded on cover glass (20). After fixation, permeabilization, and blocking, cells were labeled with primary antibodies, followed by labeling with the appropriate secondary antibodies. After nuclear counterstaining, samples were mounted on glass slides with mounting medium (Electron Microscopy Service). Images were acquired with a Carl Zeiss LSM 510 confocal microscopy system. Data were analyzed with ImageJ software or Zen software.

Small interfering RNA (siRNA).

siRNAs were synthesized by Integrated DNA Technologies. The targeting sequences were as follows: GAA TAA ATC TGC TCA GTC TTT GG (Myosin VI siRNA #1), GGA GGA ATC CCA ACA GCA AGC AG (Myosin VI siRNA #2), GAA CGA ACA GCC TGA CAC CG (ATP6V0C siRNA #1), GTC CAT CAT CCC AGT GGT CAT GG (ATP6V0C siRNA #2), GAA GCA ATC AGT AGA GAA TTC AG (ATP6V1A siRNA #1), GAC ATT TCA CCC TGC CTG CTC GTT CAT (ATP6V1A siRNA #2), GTC GCA ACA TCG TGT GGA TCG C (ATP6V0D), CAA TGA GGA AGT TCG GAA ACA GA (Integra β 5 siRNA #1), and GTT GTC CCA ATG AAA TAC TGA GAT G (Integrin β 5 siRNA #2). Predesigned Dicer-Substrate siRNAs specific for human KLC1 (hs.Ri.KLC1.13.2, hs.Ri.KLC1.13.2, and hs.Ri.KLC1.13.4) were purchased from Integrated DNA Technologies. Scrambled siRNA was obtained from Life Technologies. Cells were transfected with 100 pmole of siRNA duplex by electroporation (Neon® transfection system, Life Technologies), following the manufacturer's instructions. The parameters used were pulse voltage 1,200 V, pulse width 10 ms, and a pulse number of 2. Forty-eight hours after transfection, cells were used for further functional analyses.

POS phagocytosis assays

POS were isolated from fresh porcine eyes obtained at a local slaughter house, as described previously (20). Cholesterol and phospholipid content was measured by the Amplex™ Red Cholesterol Assay Kit (Invitrogen) and Phospholipid Assay Kit (Sigma-Aldrich), respectively. POS were used at a 10:1 POS:RPE ratio, unless otherwise specified. After cells reached confluence, they were incubated in low-glucose DMEM medium supplemented with 10% FBS for 16 hours. POS were then added to the culture, and the cells were incubated for specific times. At the end of incubation, the cells were washed with ice-cold PBS containing 1 mM MgCl₂ and 0.2 mM CaCl₂ to remove unbound POS before use in downstream assays.

Isolation of phagosome-enriched fraction

Cells were treated with POS for 3 hours, and unbound POS were removed as described above. Cells were then resuspended in sucrose buffer (250 mM sucrose, 20 mM Tris-C1, 1 mM EDTA, pH 7.4), supplemented with protease inhibitors (Sigma-Aldrich). Digitonin (1 mg/mL) was added to the cells (1/10 V/V) to promote selective breakdown of the plasma membrane (61). Nuclei and intact cells were removed by centrifugation at 1,000 g for 5 minutes. The supernatant was further centrifuged at 5,000 g for 10 minutes to obtain a

phagosome pellet. The supernatant was designated as the cytosolic fraction. The pellet was washed with PBS once and collected as the POS-enriched fraction.

Western blot analysis

Cells were lysed in buffer containing CelLytic™ M cell lysis Reagent (Sigma-Aldrich) and 2× Laemmli Sample Buffer (Bio-Rad) at a 1:1 ratio, 10 mM glycerophosphate, 10 mM pyrophosphate, 1 mM NaF, 1 mM Na₃VO₄, and protease inhibitor cocktails. After sonication, samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with specific antibodies, and signals were detected by Odyssey Infrared Imaging System (LI-COR). For tissue samples, RPE/choroid cells were harvested from mice eye cups and lysed.

In vitro reconstitution assay

Confluent ARPE-19 cells were cultured in complete medium for 16 hours before subcellular fractionation. Cells were lysed in subcellular fractionation buffer (250 mM Sucrose, 2 mM MgCl₂, 10 mM KCl, 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, pH7.4), supplemented with protease inhibitor cocktails. The lysate was passed through a 26-G needle 10 times, followed by incubation on ice for 20 minutes. The whole cell lysate (WL) was centrifuged at 300 g for 5 minutes, and the supernatant was removed and centrifuged at 20,000 g for 10 minutes. The resultant supernatant was designated as the cytosol/membrane (C/M) fraction. To remove the lysosomes, the C/M fraction was centrifuged at 100,000 g for 30 minutes, and the supernatant (S100) was used as the lysosome-free fraction for the reconstitution assay. Alternatively, the C/M fraction was subjected to immunodepletion by incubating with antibody against lysosome membrane protein LAMP2 and protein G agarose beads to obtain a lysosome-free fraction (C/M-Lyso). Purified POS were incubated with different cellular fractions (including WL, C/M, C/M-Lyso, and S100) at a ratio of 20:1 POS:starting cell number for 25 minutes at 37°C in kinase buffer (25 mM Tris-Cl, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂, 2 mM ATP, pH 7.5). At the end of incubation, the reaction was stopped by adding 4× SDS sample buffer and then analyzed by western blotting. Liposomes were prepared using the Liposome Kit (Sigma-Aldrich), following the manufacturer's instructions. The lipid components were 10% cholesterol, 70% phosphatidylcholine, and 20% stearylamine. Liposomes with lipid contents comparable to those of POS were used for the in vitro reconstitution assay.

Statistical analysis.

Between-group differences were assessed by Student's t-test or Mann-Whitney test, with the level of significance presented as P values. Normality of the data distribution was evaluated using the Kolmogorov-Smirnov test. For multiple group comparison, if data passed the normality test ($P > 0.05$), one-way analysis of variance (ANOVA) was used, followed by Dunnett's post-tests or Tukey-Kramer Multiple comparisons Test. If the data were not normally distributed, Kruskal-Wallis test (a non-parametric alternative to ANOVA) was used, followed by Dunn's multiple comparisons tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and Notes

- Menon S, Dibble CC, Talbott G, Hoxhaj G, Valvezan AJ, Takahashi H, Cantley LC, Manning BD, Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. *Cell* 156, 771–785 (2014). [PubMed: 24529379]
- Yip CK, Murata K, Walz T, Sabatini DM, Kang SA, Structure of the human mTOR complex I and its implications for rapamycin inhibition. *Molecular cell* 38, 768–774 (2010). [PubMed: 20542007]
- Zoncu R, Efeyan A, Sabatini DM, mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular cell biology* 12, 21–35 (2011). [PubMed: 21157483]
- Betz C, Hall MN, Where is mTOR and what is it doing there? *J Cell Biol* 203, 563–574 (2013). [PubMed: 24385483]
- Demetriades C, Doumpas N, Teleanu AA, Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. *Cell* 156, 786–799 (2014). [PubMed: 24529380]
- Kim SG, Hoffman GR, Poulgiannis G, Buel GR, Jang YJ, Lee KW, Kim BY, Erikson RL, Cantley LC, Choo AY, Blenis J, Metabolic stress controls mTORC1 lysosomal localization and dimerization by regulating the TTT-RUVBL1/2 complex. *Molecular cell* 49, 172–185 (2013). [PubMed: 23142078]
- Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM, The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320, 1496–1501 (2008). [PubMed: 18497260]
- Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM, Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* 141, 290–303 (2010). [PubMed: 20381137]
- Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM, Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. *Cell* 150, 1196–1208 (2012). [PubMed: 22980980]
- Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL, Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol* 10, 935–945 (2008). [PubMed: 18604198]
- Zhang CS, Jiang B, Li M, Zhu M, Peng Y, Zhang YL, Wu YQ, Li TY, Liang Y, Lu Z, Lian G, Liu Q, Guo H, Yin Z, Ye Z, Han J, Wu JW, Yin H, Lin SY, Lin SC, The lysosomal v-ATPase-Ragulator complex is a common activator for AMPK and mTORC1, acting as a switch between catabolism and anabolism. *Cell metabolism* 20, 526–540 (2014). [PubMed: 25002183]
- Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM, mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. *Science* 334, 678–683 (2011). [PubMed: 22053050]
- Rebsamen M, Pochini L, Stasyk T, de Araujo ME, Galluccio M, Kandasamy RK, Snijder B, Fauster A, Rudashevskaya EL, Bruckner M, Scorzoni S, Filipek PA, Huber KV, Bigenzahn JW, Heinz LX, Kraft C, Bennett KL, Indiveri C, Huber LA, Superti-Furga G, SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature*, (2015).
- Wang S, Tsun ZY, Wolfson RL, Shen K, Wyant GA, Plovanich ME, Yuan ED, Jones TD, Chantranupong L, Comb W, Wang T, Bar-Peled L, Zoncu R, Straub C, Kim C, Park J, Sabatini

- BL, Sabatini DM, Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science* 347, 188–194 (2015). [PubMed: 25567906]
15. Kobayashi T, Shimabukuro-Demoto S, Yoshida-Sugitani R, Furuyama-Tanaka K, Karyu H, Sugiura Y, Shimizu Y, Hosaka T, Goto M, Kato N, Okamura T, Suematsu M, Yokoyama S, Toyama-Sorimachi N, The histidine transporter SLC15A4 coordinates mTOR-dependent inflammatory responses and pathogenic antibody production. *Immunity* 41, 375–388 (2014). [PubMed: 25238095]
 16. Costa-Mattioli M, Monteggia LM, mTOR complexes in neurodevelopmental and neuropsychiatric disorders. *Nat Neurosci* 16, 1537–1543 (2013). [PubMed: 24165680]
 17. Strauss O, The retinal pigment epithelium in visual function. *Physiological reviews* 85, 845–881 (2005). [PubMed: 15987797]
 18. Takahashi Y, Moiseyev G, Ma JX, Identification of key residues determining isomerohydrolase activity of human RPE65. *The Journal of biological chemistry* 289, 26743–26751 (2014). [PubMed: 25112876]
 19. Kevany BM, Palczewski K, Phagocytosis of retinal rod and cone photoreceptors. *Physiology* 25, 8–15 (2010). [PubMed: 20134024]
 20. Yu B, Xu P, Zhao Z, Cai J, Sternberg P, Chen Y, Subcellular distribution and activity of mechanistic target of rapamycin in aged retinal pigment epithelium. *Investigative ophthalmology & visual science* 55, 8638–8650 (2014). [PubMed: 25491300]
 21. LaVail MM, Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. *Science* 194, 1071–1074 (1976). [PubMed: 982063]
 22. Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC, Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking α v β 5 integrin. *J Exp Med* 200, 1539–1545 (2004). [PubMed: 15596525]
 23. Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulton E, Phagocytosis of rod outer segments by retinal pigment epithelial cells requires α v β 5 integrin for binding but not for internalization. *Proceedings of the National Academy of Sciences of the United States of America* 94, 12932–12937 (1997). [PubMed: 9371778]
 24. Ryeom SW, Sparrow JR, Silverstein RL, CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium. *Journal of cell science* 109 (Pt 2), 387–395 (1996). [PubMed: 8838662]
 25. Chaitin MH, Hall MO, Defective ingestion of rod outer segments by cultured dystrophic rat pigment epithelial cells. *Investigative ophthalmology & visual science* 24, 812–820 (1983). [PubMed: 6345445]
 26. D'Cruz PM, Yasumura D, Weir J, Matthes MT, Abderrahim H, LaVail MM, Vollrath D, Mutation of the receptor tyrosine kinase gene *Mertk* in the retinal dystrophic RCS rat. *Hum Mol Genet* 9, 645–651 (2000). [PubMed: 10699188]
 27. Finnemann SC, Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. *The EMBO journal* 22, 4143–4154 (2003). [PubMed: 12912913]
 28. Duncan JL, LaVail MM, Yasumura D, Matthes MT, Yang H, Trautmann N, Chappelov AV, Feng W, Earp HS, Matsushima GK, Vollrath D, An RCS-like retinal dystrophy phenotype in mer knockout mice. *Investigative ophthalmology & visual science* 44, 826–838 (2003). [PubMed: 12556419]
 29. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, Hengartner MO, Green DR, Microtubule-associated protein 1 light chain 3 α (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proceedings of the National Academy of Sciences of the United States of America* 108, 17396–17401 (2011). [PubMed: 21969579]
 30. Florey O, Kim SE, Sandoval CP, Haynes CM, Overholtzer M, Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. *Nat Cell Biol* 13, 1335–1343 (2011). [PubMed: 22002674]
 31. Sanjuan MA, Dillon CP, Tait SW, Moshiah S, Dorsey F, Connell S, Komatsu M, Tanaka K, Cleveland JL, Withoff S, Green DR, Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 450, 1253–1257 (2007). [PubMed: 18097414]

32. Henault J, Martinez J, Riggs JM, Tian J, Mehta P, Clarke L, Sasai M, Latz E, Brinkmann MM, Iwasaki A, Coyle AJ, Kolbeck R, Green DR, Sanjuan MA, Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. *Immunity* 37, 986–997 (2012). [PubMed: 23219390]
33. Kim JY, Zhao H, Martinez J, Doggett TA, Kolesnikov AV, Tang PH, Ablonczy Z, Chan CC, Zhou Z, Green DR, Ferguson TA, Noncanonical autophagy promotes the visual cycle. *Cell* 154, 365–376 (2013). [PubMed: 23870125]
34. Castro AF, Rebhun JF, Clark GJ, Quilliam LA, Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *The Journal of biological chemistry* 278, 32493–32496 (2003). [PubMed: 12842888]
35. Nandrot EF, Silva KE, Scelfo C, Finnemann SC, Retinal pigment epithelial cells use a MerTK-dependent mechanism to limit the phagocytic particle binding activity of alphavbeta5 integrin. *Biology of the cell / under the auspices of the European Cell Biology Organization* 104, 326–341 (2012).
36. Muniz-Feliciano L, Doggett TA, Zhou Z, Ferguson TA, RUBCN/rubicon and EGFR regulate lysosomal degradative processes in the retinal pigment epithelium (RPE) of the eye. *Autophagy* 13, 2072–2085 (2017). [PubMed: 28933590]
37. Ershov AV, Bazan NG, Photoreceptor phagocytosis selectively activates PPARgamma expression in retinal pigment epithelial cells. *Journal of neuroscience research* 60, 328–337 (2000). [PubMed: 10797535]
38. Inoki K, Li Y, Xu T, Guan KL, Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* 17, 1829–1834 (2003). [PubMed: 12869586]
39. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL, Thomas G, Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Molecular cell* 11, 1457–1466 (2003). [PubMed: 12820960]
40. Gordiyenko NV, Fariss RN, Zhi C, MacDonald IM, Silencing of the CHM gene alters phagocytic and secretory pathways in the retinal pigment epithelium. *Investigative ophthalmology & visual science* 51, 1143–1150 (2010). [PubMed: 19741243]
41. Laplante M, Sabatini DM, mTOR signaling in growth control and disease. *Cell* 149, 274–293 (2012). [PubMed: 22500797]
42. Jiang M, Esteve-Rudd J, Lopes VS, Diemer T, Lillo C, Rump A, Williams DS, Microtubule motors transport phagosomes in the RPE, and lack of KLC1 leads to AMD-like pathogenesis. *J Cell Biol* 210, 595–611 (2015). [PubMed: 26261180]
43. Tumbarello DA, Waxse BJ, Arden SD, Bright NA, Kendrick-Jones J, Buss F, Autophagy receptors link myosin VI to autophagosomes to mediate Tom1-dependent autophagosome maturation and fusion with the lysosome. *Nat Cell Biol* 14, 1024–1035 (2012). [PubMed: 23023224]
44. Rocznia-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, Walther TC, Ferguson SM, The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. *Science signaling* 5, ra42 (2012). [PubMed: 22692423]
45. Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J, Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. *The Journal of biological chemistry* 273, 16621–16629 (1998). [PubMed: 9632736]
46. Reyes-Reveles J, Dhingra A, Alexander D, Bragin A, Philp NJ, Boesze-Battaglia K, Phagocytosis-dependent ketogenesis in retinal pigment epithelium. *The Journal of biological chemistry* 292, 8038–8047 (2017). [PubMed: 28302729]
47. Makinoshima H, Takita M, Saruwatari K, Umemura S, Obata Y, Ishii G, Matsumoto S, Sugiyama E, Ochiai A, Abe R, Goto K, Esumi H, Tsuchihara K, Signaling through the Phosphatidylinositol 3-Kinase (PI3K)/Mammalian Target of Rapamycin (mTOR) Axis Is Responsible for Aerobic Glycolysis mediated by Glucose Transporter in Epidermal Growth Factor Receptor (EGFR)-mutated Lung Adenocarcinoma. *The Journal of biological chemistry* 290, 17495–17504 (2015). [PubMed: 26023239]
48. Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM, mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature* 468, 1100–1104 (2010). [PubMed: 21179166]

49. Kanow MA, Giarmarco MM, Jankowski CS, Tsantilas K, Engel AL, Du J, Linton JD, Farnsworth CC, Sloat SR, Rountree A, Sweet IR, Lindsay KJ, Parker ED, Brockerhoff SE, Sadilek M, Chao JR, Hurley JB, Biochemical adaptations of the retina and retinal pigment epithelium support a metabolic ecosystem in the vertebrate eye. *Elife* 6, (2017).
50. Shang L, Chen S, Du F, Li S, Zhao L, Wang X, Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK. *Proceedings of the National Academy of Sciences of the United States of America* 108, 4788–4793 (2011). [PubMed: 21383122]
51. Golestaneh N, Chu Y, Xiao YY, Stoleru GL, Theos AC, Dysfunctional autophagy in RPE, a contributing factor in age-related macular degeneration. *Cell Death Dis* 8, e2537 (2017). [PubMed: 28055007]
52. Zhao C, Yasumura D, Li X, Matthes M, Lloyd M, Nielsen G, Ahern K, Snyder M, Bok D, Dunaief JL, LaVail MM, Vollrath D, mTOR-mediated dedifferentiation of the retinal pigment epithelium initiates photoreceptor degeneration in mice. *The Journal of clinical investigation* 121, 369–383 (2011). [PubMed: 21135502]
53. Saci A, Cantley LC, Carpenter CL, Rac1 regulates the activity of mTORC1 and mTORC2 and controls cellular size. *Molecular cell* 42, 50–61 (2011). [PubMed: 21474067]
54. Schieke SM, Phillips D, McCoy JP, Jr., Aponte AM, Shen RF, Balaban RS, Finkel T, The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *The Journal of biological chemistry* 281, 27643–27652 (2006). [PubMed: 16847060]
55. Desai BN, Myers BR, Schreiber SL, FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction. *Proceedings of the National Academy of Sciences of the United States of America* 99, 4319–4324 (2002). [PubMed: 11930000]
56. Goh ET, Pardo OE, Michael N, Niewiarowski A, Totty N, Volkova D, Tsaneva IR, Seckl MJ, Gout I, Involvement of heterogeneous ribonucleoprotein F in the regulation of cell proliferation via the mammalian target of rapamycin/S6 kinase 2 pathway. *The Journal of biological chemistry* 285, 17065–17076 (2010). [PubMed: 20308064]
57. Kantidakis T, Ramsbottom BA, Birch JL, Dowding SN, White RJ, mTOR associates with TFIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proceedings of the National Academy of Sciences of the United States of America* 107, 11823–11828 (2010). [PubMed: 20543138]
58. Krajcovic M, Krishna S, Akkari L, Joyce JA, Overholtzer M, mTOR regulates phagosome and entotic vacuole fission. *Mol Biol Cell* 24, 3736–3745 (2013). [PubMed: 24088573]
59. Chen Y, Sternberg P, Cai J, Characterization of a Bcl-XL-interacting protein FKBP8 and its splice variant in human RPE cells. *Investigative ophthalmology & visual science* 49, 1721–1727 (2008). [PubMed: 18385096]
60. Mattapallil MJ, Wawrousek EF, Chan CC, Zhao H, Roychoudhury J, Ferguson TA, Caspi RR, The Rd8 mutation of the *Crb1* gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. *Investigative ophthalmology & visual science* 53, 2921–2927 (2012). [PubMed: 22447858]
61. Chen Y, Cai J, Murphy TJ, Jones DP, Overexpressed human mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. *The Journal of biological chemistry* 277, 33242–33248 (2002). [PubMed: 12032145]

Figure 1: Diurnal variation of mTORC1 activity in the RPE during morning burst of POS shedding.

(A and C) Images of immunostained RPE/choroid whole mounts with antibodies against rhodopsin (A) or phosphorylated ribosome protein S6 (C) at the indicated times after light-on. (B) and (D) Quantification of the imaging data from three independent experiments (n=3 mice per condition), presented as mean $\pm$ SEM. (E) and (F) Western blot analysis of phosphorylated S6 levels in RPE/choroid tissues. Data are means of five independent experiments (mean $\pm$ SEM). **P<0.01 relative to lights-on (one-way ANOVA and Dunnett's post-hoc test). Scale bar: 20 μm .

Figure 2. mTORC1 activation by POS in cultured RPE cells.

ARPE-19 cells were treated with purified POS at the indicated ratios for 1 hour (A) or at a 10:1 POS: RPE ratio for the indicated times (B). Phosphorylation status of S6, 4E-BP1, and ULK1 were examined by western blot analyses. Data are means from five independent experiments (mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$ relative to cells without POS exposure (one-way ANOVA and Dunnett's post-hoc test). (C) mTORC1 activation in cells depleted of $\beta 5$ integrin using small interfering RNA (siRNA) or cells exposed to control scrambled (Scr) siRNA. POS were added at a 10:1 POS: RPE ratio for 1 hour. Blots are representative of three independent experiments. (D) mTORC1 activity in cells exposed to latex beads for the indicated times. Data are means from three independent experiments (mean $\pm$ SEM). No statistically significant differences were detected between control and bead-loaded RPE cells (one-way ANOVA).

Figure 3. Localization of mTOR protein on POS-containing organelles.

ARPE-19 cells were incubated with purified POS for up to 4 hours. Co-localization of POS with mTOR or endosome and lysosome marker proteins was monitored by co-staining with antibodies against rhodopsin and mTOR, EEA1 (early endosome marker), Rab5 (early endosome marker), or LAMP2 (lysosomes) at the indicated times. Co-localization of each protein to rhodopsin-positive phagosomes was analyzed by determining Pearson's correlation coefficients. Thirty rhodopsin-positive particles were analyzed for each protein from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to cells exposed to POS for 15 minutes (one-way ANOVA and Dunnett's post-hoc test). Scale bar: 20 μm .

Figure 4. Localization of mTORC1 pathway proteins on POS-containing organelles.

(A to C) Co-localization of rhodopsin with p18 (A), RagA (B), or Rheb (C) in cells exposed or not exposed to POS for 30 minutes. Scale bar: 20 μ m. Images are representative of three independent experiments. (D) Quantification of data in A-C. Twenty fields with an area of $50 \times 50\text{-}\mu\text{m}^2$ were analyzed for each protein. (E) Western blot analyses of POS-enriched organelles from ARPE-19 cells exposed to POS for 3 hours. Blots are representative of three independent experiments. (F) Representative images of immunostaining of flat-mounted RPE/choroid tissues with antibodies against mTOR, RagA, p18, and rhodopsin. Small panels are enlarged images of white insets. Arrows indicate proteins associated with rhodopsin-positive phagosomes. Samples were prepared 1 hour after lights-on. Images are representative of three independent experiments. Scale bar: 20 μ m. (G) Quantification of data in F. Six fields with an area of $100 \times 100\text{-}\mu\text{m}^2$ were analyzed for each protein.

Figure 5. POS-stimulated mTORC1 activation in cells with reduced expression of KLC1 or myosin 6.

(A) POS-induced mTORC1 activation in ARPE-19 cells transfected with either KLC1 small interfering RNA (siRNA) or control scrambled (Scr) siRNA. S6 phosphorylation and KLC1 knockdown were analyzed by Western blots. Quantification data are means from five independent experiments (mean $\pm$ SEM). * P <0.05 (Kruskal-Wallis test and Dunn's multiple comparisons test). (B) and (C) Co-immunostaining of rhodopsin and myosin 6 (Myo6) in POS-treated ARPE-19 cells and RPE/choroid whole mounts, respectively. Images are representative of three independent experiments. Scale bar: 5 μ m. (D) Quantitation of the data in B and C. Twenty fields with an area of 50 $\times$ 50 μ m² were analyzed. (E) Depletion of Myo6 in ARPE-19 cells expressing Cas9 endonuclease and transfected with gRNA targeting Myo6. Blots are representative of three independent experiments. (F) Degradation of POS in cells depleted of Myo6 by CRISPR/Cas9. Quantification data are means from six independent experiments (mean $\pm$ SEM). * P <0.05 (Kruskal-Wallis test and Dunn's multiple comparisons test). (G) POS-induced mTORC1 activation in cells depleted of Myo6 by CRISPR/Cas9. Quantification data are means from five independent experiments (mean $\pm$ SEM). *** P <0.001 (one-way ANOVA and Tukey-Kramer Multiple comparisons Test). (H) Amino Acid (AA)-induced mTORC1 activation in cells depleted of Myo6 by CRISPR/Cas9. Data are means of five independent experiments (mean $\pm$ SEM). *** P <0.001 (one-way ANOVA and Tukey-Kramer Multiple comparisons Test).

Figure 6. POS-induced mTORC1 activation occurred independently of lysosomal functions.

(A) Amino acid- or POS-induced mTORC1 activity in cells cultured in the absence or presence of 20 μ M chloroquine (CQ). Blots are representative of three independent experiments. (B) Immunofluorescence with or without chloroquine treatment. Blue: nuclei (N). Scale bar: 10 μ m. Co-localization of mTOR and rhodopsin-positive phagosomes was analyzed by determining Pearson's correlation coefficients. Thirty rhodopsin-positive particles were included from three independent experiments for each condition. No statistically significant differences were detected between samples with or without chloroquine treatment (Student's t-test). (C) Western blot analysis of amino acid-induced mTORC1 activation. Cells were transfected with small interfering RNAs (siRNAs) against subunits of V-ATPase (V1A + V0D or V1A + V0C) or control scrambled (Scr) siRNA and then stimulated with amino acids. Blots are representative of three independent experiments. (D) Western blot analysis of POS-induced mTORC1 activation. Quantification data are means from seven independent experiments (mean $\pm$ SEM). * $P < 0.05$; *** $P < 0.001$ (Kruskal-Wallis test and Dunn's multiple comparisons test).

Figure 7. In vitro reconstitution of POS-mediated mTORC1 activation.

(A) Schematic illustration of preparation of cell extracts depleted of lysosomes. WL: whole cell lysate; C/M: cytosol/membrane fraction; C/M-Lyso: C/M fraction immunodepleted of lysosomes. (B) Fractions prepared according to scheme illustrated in A were separated on SDS-PAGE and labeled with antibodies against lysosomal proteins LAMP1, LAMP2, and Cathepsin B. EEA1: early endosome marker; HSC70: cytosolic marker. Blots are representative of three independent experiments. (C) mTORC1 activity in different fractions. Blots are representative of four independent experiments. Quantification of phosphorylation of S6K (D) and S6 (E) in C/M and C/M-Lyso fractions (mean $\pm$ SEM). * $P < 0.05$ (Student's t-test). (F) mTORC1 activity in different fractions with or without incubation with liposomes. Blots are representative of three independent experiments. (G) Quantification of phosphorylation of S6K in the C/M-Lyso fraction in the presence or absence of liposomes (Lipo) (mean $\pm$ SEM). * $P < 0.05$ (Student's t-test).