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REVIEWS

Regulation of the rhythmic diversity of daily photoreceptor outer segment phagocytosis in vivo

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Abstract

Outer segment phagocytosis (OSP) is a highly-regulated, biological process wherein photoreceptor outer segment (OS) tips are cyclically phagocytosed by the adjacent retinal pigment epithelium (RPE) cells. Often an overlooked retinal process, rhythmic OSP ensures the maintenance of healthy photoreceptors and vision. Daily, the photoreceptors renew OS at their base and the most distal, and likely oldest, OS tips, are phagocytosed by the RPE, preventing the accumulation of photo-oxidative compounds by breaking down phagocytosed OS tips and recycling useful components to the photoreceptors. Light changes often coincide with an escalation of OSP and within hours the phagosomes formed in each RPE cell are resolved. In the last two decades, individual molecular regulators were elucidated. Some of the molecular machinery used by RPE cells for OSP is highly similar to mechanisms used by other phagocytic cells for the clearance of apoptotic cells. Consequently, in the RPE, many molecular regulators of retinal phagocytosis have been elucidated. However, there is still a knowledge gap regarding the key regulators of physiological OSP in vivo between endogenous photoreceptors and the RPE. Understanding the regulation of OSP is of significant clinical interest as age-related macular degeneration (AMD) and inherited retinal diseases (IRD) are linked with altered OSP. Here, we review the in vivo timing of OSP peaks in selected species and focus on the reported in vivo environmental and molecular regulators of OSP.

Abbreviations: AMD, age-related macular degeneration; ANXA5, annexin a5; AO-OCT, adaptive optics optical coherence tomography; ATXN3, ataxin-3; Bmal1, brain and muscle ARNT-Like 1; Cav-1, caveolin-1; CD36, cluster of differentiation 36; Cerkl, ceramide kinase-like; Dbp, D-Box Binding PAR BZIP Transcription Factor; DRD2, Dopamine receptor D2; Egr1, early growth response 1; FAK, focal adhesion kinase; Gas6, growth-arrest-specific gene 6; IRD, inherited retinal diseases; Kif17, kinesin family member 17; Klc1, kinesin light chain 1; L:D, Light:Dark; Lamp2, lysosomal associated membrane protein 2; LDL, low-density lipoprotein; MerTK, MER proto-oncogene, tyrosine kinase; MFG-E8, milk fat globule-epidermal growth factor 8; Mreg, melanoregulin; MT_1, melatonin receptor type 1; MT_2, melatonin receptor type 2; Npas2, neuronal PAS domain protein 2; Nr1d1, nuclear receptor subfamily 1 Group D member 1; Nrl, neural retina leucine-zipper; ONL, outer nuclear layer; OS, outer segment; OSP, outer segment phagocytosis; PAI-1, plasminogen activator inhibitor 1; Per1, period circadian regulator 1; Per2, period circadian regulator 2; PI3K, phosphoinositide 3-kinase; ProS, protein S; $PtdIns(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; PtSer, phosphatidylserine; RAC1, Ras-related C3 botulinum toxin substrate 1; Rasgrp3, Ras guanyl-releasing protein 3; RCS, Royal College of Surgeons; Rorc, RAR-related orphan receptor C; ROS, rod outer segment; RPE, retinal pigment epithelium; ZT, Zeitgeber Time; αvβ5, alpha V beta 5 integrin.

KEYWORDS
circadian, cone, in vivo, outer segment phagocytosis, photoreceptor, regulators, retina, retinal disease, rod, RPE

1 | INTRODUCTION-BACKGROUND INFORMATION ON OSP

Outer segment phagocytosis (OSP) is a highly-regulated, biological process wherein photoreceptor outer segment (OS) tips are cyclically phagocytosed by the adjacent cells of the retinal pigment epithelium (RPE) (Figure 1). Often an overlooked retinal process, rhythmic OSP ensures the maintenance of healthy photoreceptors and vision.

To maintain function, photoreceptors renew OS at their base and the RPE phagocytoses the most distal, and likely oldest, OS tips.[1] Light changes often coincide with an escalation of OSP and within hours the phagosomes formed in each RPE cell are resolved (Figure 2). OSP prevents the accumulation of photo-oxidative compounds by breaking down phagocytosed OS tips and recycling useful components to the photoreceptors. The mass of OS removed is significant; 7%–10% of the OS is eliminated daily, meaning an entire OS is replenished every 2 weeks.[2]

OSP has been researched since the latter 20th century. However, over the last two decades, individual molecular regulators were elucidated. Some of the molecular machinery used by RPE cells for OSP is highly similar to mechanisms used by other phagocytic cells for clearance of apoptotic cells.[3] Consequently, in the RPE, many molecular regulators of retinal phagocytosis have been elucidated. However, there is a knowledge gap regarding the key regulators of physiological OSP in vivo, a process involving the cellular interplay between endogenous photoreceptors and the RPE and timed by environmental and intrinsic signals. Understanding the regulation of OSP is of significant clinical interest as age-related macular degeneration (AMD) and inherited retinal diseases (IRD) are linked with altered OSP.[4–6] Failure to phagocytose OS can result in the accumulation of undigested lipids and proteins and the consequential production of toxic compounds, *e.g.* A2E.[7]

As the term OSP has had ambiguous interpretations, we clarify our definition. We consider OSP as a timed, cyclical process encompassing the demarcation of the OS area to be shed, overlapping with phagocytosis (Figure 1). The phagocytosis element itself consists of three distinct phases; binding/recognition, engulfment/internalization, and degradation/digestion (Figure 1). There are many gaps in our knowledge of the diverse complement of molecules coordinating OSP. Here, we review the in vivo timing of OSP peaks

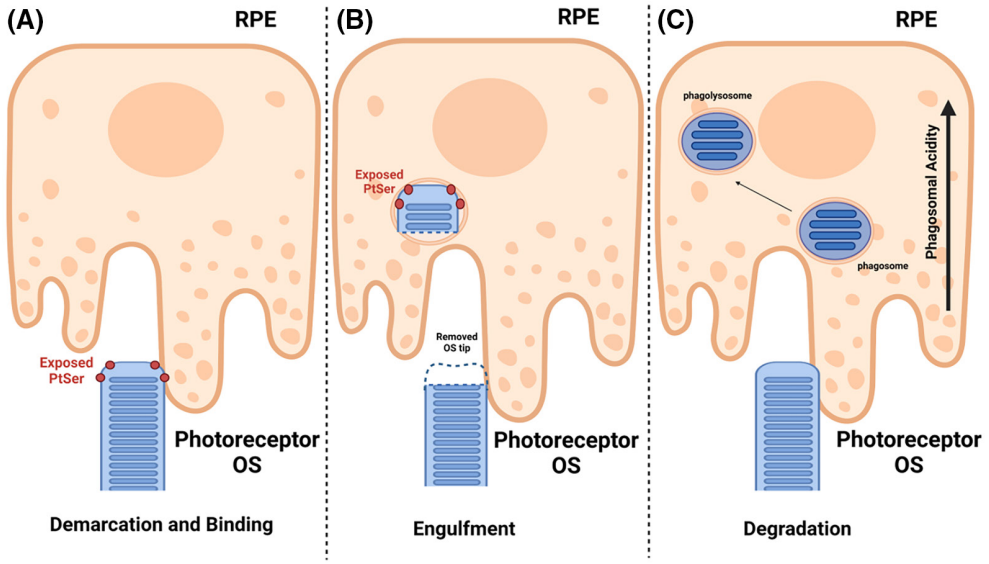

FIGURE 1 Schematic showing the outline of the entirety of the OSP process. (A) Demarcation and binding show exposure of phosphatidylserine (PtSer) at the tip of the photoreceptor outer segment highlighting the portion of the photoreceptor to be removed. Receptors at the apical membrane of the RPE recognize the PtSer signal and allow for binding. (B) Engulfment involves the movement of the demarcated tip into the RPE. (C) The engulfed OS is sealed by a membrane in a nascent phagosome that undergoes maturation while moving from the apical-basal RPE, which involves the graded fusion of endosomes and then lysosomes. Created using BioRender.com.

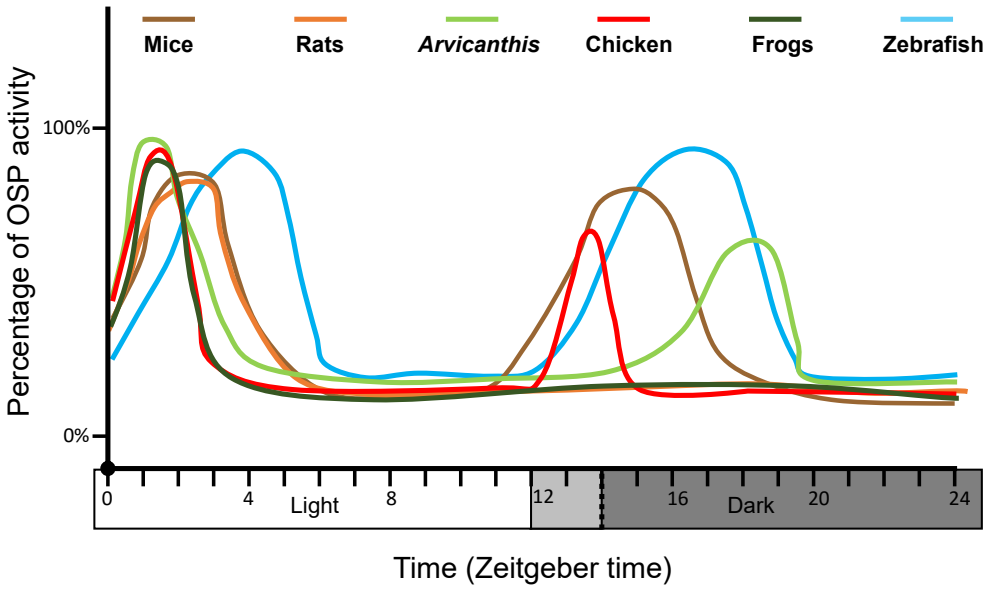

FIGURE 2 Peaks of OSP in different species. Schematic diagram of the percentage of OSP activity in different species at different times in the light–dark cycle. Frogs and zebrafish are maintained on a 14:10 Light:Dark cycle compared with all other animals which are kept on a 12:12 Light:Dark cycle. The time that the lights turn off for frogs and fish are represented by light gray and the light off time for all other species are represented by dark gray. Time is recorded on the *x*-axis in zeitgeber time which starts counting in hours from a zero time of initial light onset.

in selected species and focus on the reported in vivo environmental and molecular regulators of OSP.

2 | DIVERSITY OF RHYTHMIC OSP PEAKS IN DIFFERENT SPECIES

In the field, there has been controversy surrounding (i) the number of OSP peaks that occur daily, (ii) whether rod and cone photoreceptor tips are phagocytosed within the same or different OSP peaks and (iii) whether the OSP process undergoes circadian regulation. Part of the discrepancy arises from the different methodological approaches used to study OSP in vivo and a lack of standardization of time points analyzed. As reviewed recently, OSP is typically analyzed by transmission electron or fluorescence microscopy of OS-laden phagosomes in the RPE.[8–10] Phagocytosis of the distal OS tips of photoreceptor cells has been reported in animal models since the late 1960s.[2,11] Numerous subsequent studies investigating the chronology of OSP report variation amongst, and even conflicting evidence within species. Our review of OSP in different species reveals a rich OSP diversity in nocturnal, diurnal, and crepuscular animals, exemplified by 1–3 OSP peaks; rod or cone only peaks, or mixed rod and cone OSP peaks, and circadian and non-circadian regulation (Figure 2, Table 1).

A study in 1978 assessed OSP in goldfish raised under a 12-h day-night environmental light cycle.[26] The study reported a clear preponderance of rod OSP just after light onset, with a second peak of OSP occurring just after dark onset, dominated by cone OSP, stimulating the idea that rod photoreceptors were solely shed in the morning, and cone photoreceptors at night. However, as explained below, reports have since shown that the ratio of rod:cone shedding at the various peaks is likely species-dependent. A similar profile is observed in zebrafish, albeit raised under a different environmental light–dark cycle of 14 h light: 10 h dark. 2 prominent OSP peaks occur, the first after light exposure, and a second shortly after darkness.[16,28] In contrast to the goldfish study, the zebrafish were shown by immunolabeling to display mixed rod and cone OSP peaks, with the morning peak slightly favoring the phagocytosis of distal rod cell OS tips and the evening peak favoring the phagocytosis of distal cone cell OS tips.[16]

Chickens, rhesus monkeys, and ground squirrels are also reported to display two daily peaks of OSP.[12,21,23] In contrast, a single OSP peak was reported in cats, frogs (*Rana pipiens* and *Xenopus laevis*), and lizards.[14,24,25,29] Studies conducted in chickens, rhesus monkeys, and tree and ground squirrels reported similar patterns of dark-to-light (dawn) associated rod OSP peaks and light-to-dark (dusk) associated cone OSP peaks.[12,21–23] However, further diversity is apparent as in cats, the cone-dominant Nile rat (*Arvicanthis ansorgei*) and in tree shrews, morning

TABLE 1 Summary of what is currently known for OSP in different model species

Species/Strain	Lifestyle	Retina structure	Peaks in LD	Peaks in DD	Response to new light cycle
Rhesus Monkey[12,13]	Diurnal	Cone dominant	After light onset/after lights off	No data	No data
Cat[14]	Crepuscular	Rod dominant	After light onset	No data	No data
Rat[15]	Nocturnal	Rod dominant	After light onset	Circadian, peaks damped but timing is equal to LD conditions	Long entrainment time ~2 weeks
Mice[16,17]	Nocturnal	Rod dominant	After light onset/after lights off	Circadian, peaks happen at the correct times at approximately the same amplitude	No data
Arvicanthis ansorgei[18,19]	Diurnal	Cone dominant	After light onset/slight peak after lights off	Circadian, peaks happen at the correct time but are dampened	Immediate response to new light cycles
Nrl KO mouse[20]	Nocturnal	'Cone-like' dominant	After light onset	Circadian, peak present at the correct time but amplitude reduced	No data
Squirrel[21,22]	Diurnal/crepuscular	50:50 rods: cones	After light onset/after lights off	No data	No data
Chicken[23]	Diurnal	Cone dominant	After light onset/after lights off	No data	No data
Lizard[24]	Diurnal	Only cones	One after light offset	Circadian, peak present at the correct time but amplitude reduced	Immediate response to new light cycles
Rana pipens[25]	Nocturnal/crepuscular	Rod dominant	One after light onset	No OSP peaks	Immediate response to new light cycles
Goldfish[26,27]	Diurnal	Cone dominant	After light onset/after lights off	No OSP peaks	Immediate response to new light cycles
Zebrafish[28]	Diurnal	Cone dominant	After light onset/after lights off	No OSP peaks	No data

and night OSP peaks show both cone and rod OS laden phagosomes.[14,18,19,30]

Studies in mice and rats with rod-dominant retinae, in addition to a nocturnal, *Nrl* knockout mouse dominated with reported cone-like photoreceptors,[20] typically report one distinct OSP peak; shortly after exposure to light.[31–35] However, recently a second OSP peak in mice was reported in two independent studies, which occurs after the onset of darkness.[16,36,37] Both groups observed a distinct increase in phagosome levels by electron microscopy[16] and immunofluorescence[36] in a C57BL/6J background between Zeitgeber Time (ZT) 13–14. The term Zeitgeber refers to environmental variables (e.g., light) capable of acting as time cues to regulate a biological rhythm. Here, Zeitgeber time signifies the number of hours after light onset,[38] typically with ZT 0 representing lights on at the beginning of the day and ZT 12 (or often ZT 14 in zebrafish) representing lights off at the end of the day. Extensively discussed in a recent review, several reasons could account for the discrepancies between murine studies including sample collection times and genetic background.[8]

Additional studies of the molecular and environmental regulation of OSP discussed later in this review, corroborate the complexity and diversity of OSP. Nonetheless, we present a current consensus view of the OSP profile in different species for visual comparison (Figure 2).

3 | REGULATION OF OSP TIMING, AMPLITUDE, AND ENTRAINMENT

With OSP showing heightened activity at specific times of the day, a key question is what external or internal signals orchestrate OSP. Of most interest are the determinants of the timing and amplitude of the OSP peaks and the basal OSP levels at other times. The profile of OSP can be dysregulated in several ways including changes in the amplitude of OSP peaks, changes in the basal levels of OSP, and changes in the entrainment of OSP peaks to a new light: dark cycles (Figure 3). These represent common ways that dysregulated OSP presents itself across separate investigations in different species. In this section, different ways that dysregulated OSP can present itself will be discussed first followed by what is currently known about the regulation of OSP in different species.

One way the profile of OSP can be dysregulated is OSP peaks occurring at the correct times but with a reduced amplitude (Figure 3A). This was initially found when probing circadian control of rod OSP in rats wherein rats were maintained in constant darkness and sampled at times of expected OSP peaks and troughs. Despite the peaks occurring at the correct times, their amplitude is reduced.[15] This dysregulation of OSP also occurs in genetic models. In *Rab28* and Kif17 knockout zebrafish and mice, the peak levels of OSP become reduced.[16,28] OSP peaks can also be increased in amplitude. In goldfish and *Rana pipens*, exposure to constant light inhibits OSP peaks from occurring.[27,40] When these constant light-treated frogs or goldfish are brought from constant light into darkness, a small amount of shedding occurs. However, if the frogs are exposed to constant light for several days followed by darkness and then light again, OSP peaks occur at a much higher amplitude compared to controls. The factors mediating this dysregulation can be thought of as important for setting the amplitude of a peak but are potentially distinct from factors that regulate the timing of OSP peaks.

Another way OSP can become dysregulated is when peaks are completely absent and the basal level of OSP is apparently increased (Figure 3B). This phenomenon may occur in genetic models including the alpha-V beta-5 integrin (αvβ5), brain and muscle ARNT-like 1 (Bmal1), and dopamine receptor D2 knockout mice.[35–37] Very interestingly, in all these models, it appears that basal levels of OSP increase in compensation such that the total amount of OSP throughout the day is roughly equal between the WT and knockouts. In the *Bmal1* RPE-specific KO mouse this increase in the basal level of OSP is only seen during the diurnal period of approximately ZT 3-ZT 11, while the total amount of OSP (basal and peak) over the total 24-h period remains unchanged.[37] In the study with the αvβ5 knockout mouse and the dopamine receptor D2 knockout mice, the statistical analysis carried out did not focus on basal levels. This prevents us from making definitive conclusions about the potential increased basal level of OSP. However, in the *Bmal1* knockout mouse, the basal level of OSP is significantly higher in knockout compared to the WT. It is worth noting that in the period circadian regulator 1 (Per1) and period circadian regulator 2 (Per2) there is a defect that prevents an OSP peak from occurring but this does not result in an increase in the basal OSP level.[17] However, this study looked at a peak happening under circadian control in constant dark conditions. The studies that report an increase in basal levels of OSP in response to the removal of peaks were carried out in animals maintained on a normal light–dark cycle.[35–37] Therefore, despite these limitations, we consider elevated basal OSP levels an interesting, understudied phenomenon, which should inform future investigations in the field.

Another pattern of OSP dysregulation, which can be observed in species with dysregulated circadian control of OSP, is the inability to entrain to new environmental light:dark cycles. Rats with bilateral severed optic nerves maintain OSP peaks with the original rhythmic timing and amplitude but do not entrain to peak at shifted light cycles over time, unlike their control counterparts.[39] A separate way that the timing of OSP can become dysregulated is

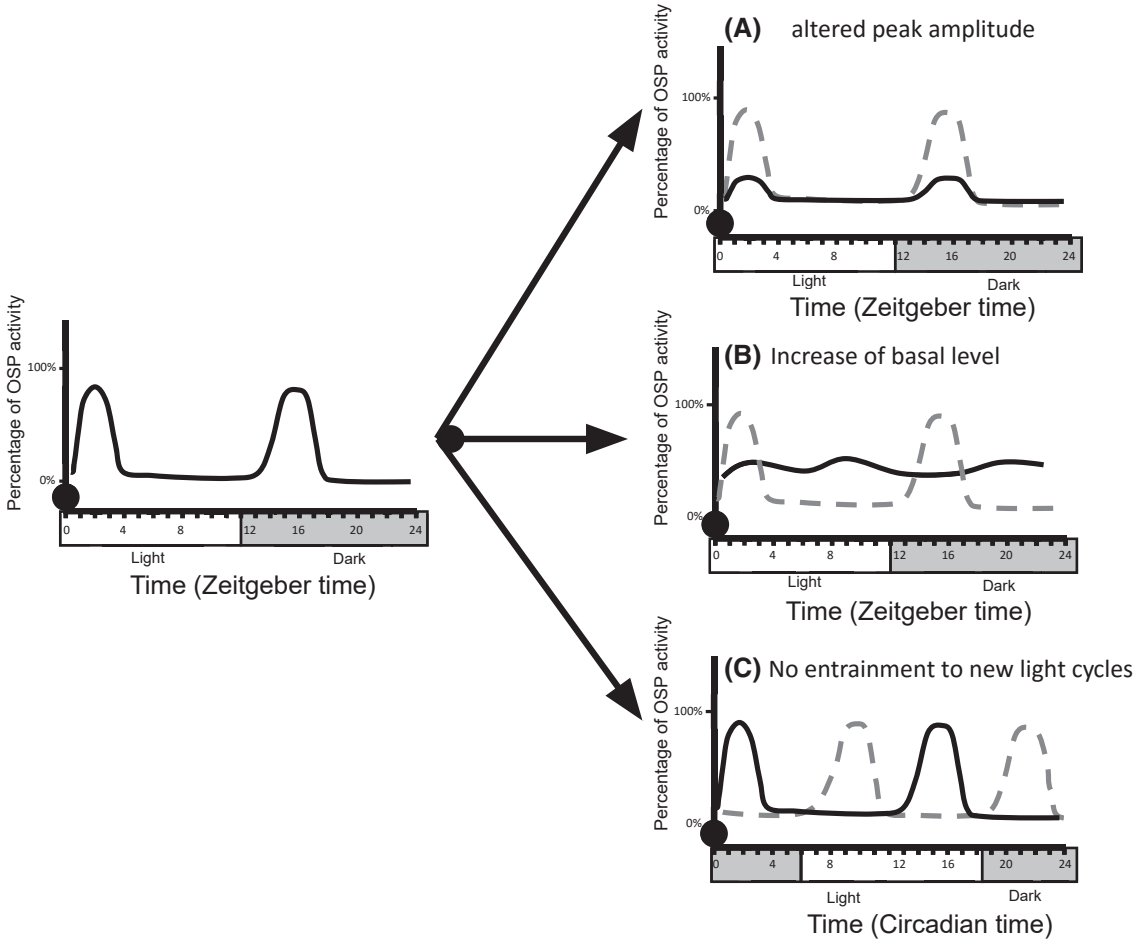

FIGURE 3 Examples of ways that OSP peaks can become dysregulated by different environmental or molecular regulators. Three schematic examples of ways that dysregulated OSP can present itself are shown. These include: (A) Altered peak amplitude (e.g., *rab28* KO zebrafish[28]). (B) Increase of basal OSP level and removal of peaks (e.g., dopamine receptor D2 knockout mice[36]), and (C) No entrainment to new light cycles (e.g., rats with severed optic nerves[39]). The gray dashed line represents the pattern of how OSP should look under normal conditions and the solid black line represents how it has become dysregulated. Circadian time is used as the measurement of time in example C. This is the equivalent of zeitgeber time but is based on the initial Light:Dark cycle before the cycle was shifted.

when the peak OSP times happen under a normal light–dark cycle, but the peaks do not occur at the correct time. An example of this is in the melatonin receptor type1 (MT_1) or type 2 (MT_2) KO mouse. When this KO mouse is held in a standard light–dark cycle there is a 3-h anticipatory shift in the OSP peak.[41]

A key initial question in understanding OSP was if its timing was controlled by environmental factors or internal circadian regulators. Studies of rod photoreceptor OSP suggest a strong circadian control in rats.[15,31] When maintained in constant darkness, rats still display OSP peaks at presumptive light onset and this persists for up to 12 days.[15,31] After prolonged dark exposure, the time of OSP peak activity diverged slightly from the presumptive light onset time suggesting that this process behaves as a free-running circadian rhythm.[15] For example, LaVail was able to shift the peak OSP time backward by exposing rats to altered light–dark cycles with a 3-h delay in light onset. However, this entrainment took at least 4 weeks which is comparatively long compared to the time needed to entrain other circadian rhythms to new cycles.[15]

The control of cone photoreceptor OSP timing has been studied in the diurnal mammal *Arvicanthis ansorgei* and in the neural retina leucine-zipper (*Nrl*) knockout mouse wherein rods are replaced by cone-like photoreceptors.[42] In *Nrl* knockouts, the reported peak of cone-like OSP is shortly after light onset.[20] This peak time appears to be under circadian regulation as it is maintained at presumptive light onset, even when the mice are kept in constant darkness.[20] In *Arvicanthis*, peak cone and rod OSP is reported shortly after light onset and is maintained in animals housed in constant darkness, suggesting circadian control.[19] However, when *Arvicanthis* is shifted to a 6-h light advance or delay, OSP peaked slightly after the new light onset time, on the first day of exposure to the new light cycle.[19] This is in stark contrast to the several

weeks required to entrain rats to a new lighting cycle.[15] It is worth noting that there is a large difference in the light intensities in these two studies as *Arvicanthis ansorgei* are maintained at 300 lux vs the rats which were maintained at 5.4–54 lux.[15,19]

Unlike rodents, in frog models (*Rana pipens*), OSP peak timing is not controlled by internal circadian regulators.[25] Exposure to constant darkness or constant light eliminates OSP peaks at presumptive light onset times.[40,43] OSP always occurs shortly after exposure to light.[43] Less is known about the control of OSP timing in fish models. Early studies using goldfish confirmed that OSP occurs shortly after light onset and shortly after the lights turn off when goldfish is maintained in cyclic lighting.[26] However, rhythmic timing of OSP peaks is lost when the goldfish is maintained in constant darkness, suggesting that peak OSP timing in goldfish is light-driven.[27] Like frogs, goldfish OSP is inhibited by constant light and a massive shedding event happens after constant light treated animals are exposed to a dark period and then back to light.[27] In zebrafish, morning and night peaks of OSP, quantified by transmission electron microscopy (TEM), are inhibited in animals held in constant darkness suggesting a lack of circadian control.[28]

Beyond environmental or circadian control, researchers have sought to elucidate molecular mechanisms by which OSP peak timing is regulated. Initial work showed that the rat pineal gland, pituitary, parathyroid complex, or suprachiasmatic nucleus were not involved in the maintenance of OSP peaks in constant darkness or entrainment of OSP peaks to new light cycles.[32,33,44] One study suggested against a humoral regulator, as rats with one eye occluded retrained OSP peaks to a new light cycle, but only in the open eye. The occluded eye maintained its original circadian OSP peaks.[39] Also, this study focused on the central control of OSP through experiments severing the optic nerve. Rats with bilateral severed optic nerves maintained OSP peaks as in control animals but could not be retrained to new light cycles. This suggested that central control is needed for the process of entrainment of OSP to new rhythms.[39]

Frogs taken from constant light to darkness experience a small amount of OSP but a much larger amount is observed when animals maintained in constant light are exposed to a small dark period before being exposed to light again.[40] This suggests that a dark priming period is required to cause a change in the retina that enables OSP.[40] OSP peaks can be initiated unilaterally in frog eyes by exposing one eye to light suggesting against humoral regulation of OSP peak timing.[45] Furthermore, localized light stimulation was sufficient to induce whole retina OSP peaks in frogs. Use of a fundus lens to illuminate specific regions of the retina resulted in observed OSP in regions far from the stimulated site.[46] The color of the light was reported to influence efficiency to stimulate OSP peaks and it was suggested that this effect may be mediated by the 575-nm-cone.[47] Aside from control of OSP peaks via light cycles, temperature cycles also proved enough to stimulate rhythmic OSP in frogs held in constant darkness. Frogs held at a constant temperature in constant darkness do not exhibit OSP peaks. However, peaks are observed in frogs held in constant darkness when held for 14 h at 26°C and 10 h at 15°C.[43]

More recent studies advanced our understanding of molecular regulators involved in controlling the timing of OSP. Early studies using mice incapable of melatonin synthesis showed that OSP still occurred but with dampened peaks.[48] Further work in melatonin receptor knockout mice reported a 3-h anticipatory shift in peak OSP activity when mice were held in cyclic lighting.[41] Dopamine was also flagged as a potential regulator of OSP peak timing as pharmacological inhibition of dopamine synthesis dampens OSP peaks in rats.[49] This is unsurprising as dopamine is a common mediator of circadian control.[50] In a separate study using a dopamine receptor D2 knockout mouse, rhythmic bursts of OSP peaks were not observed; however, the total number of phagosomes over the course of the day was equal to the wildtype control.[36] Transcriptomics revealed that expression patterns of core circadian clock genes were unchanged, but the integrin signaling pathway was downregulated in the KO mouse relative to the wildtype.[36] Newer studies used transcriptomics to identify differentially expressed transcripts in photoreceptors and the RPE at peak and through OSP times. One study in mice presented an elegant model in which the phosphoinositide signaling pathway interacts with core circadian clock machinery to mediate rhythmic OSP.[51] This study identified several core circadian genes and downstream interactors being differentially expressed at peak and through OSP times including *Bmal1*, *Clock*, D-Box binding PAR bZIP transcription factor (*Dbp*), early growth response 1 (*Egr1*), *Fos*, neuronal PAS domain protein 2 (*Npas2*), nuclear receptor subfamily 1 group D member 1 (*Nr1d1* [*Rev-Erbα*]), and RAR-related orphan receptor C (*Rorc*). In addition to this key OSP regulators plasminogen activator inhibitor 1 (*Pai-1*), Ras guanyl-releasing protein 3 (*Rasgrp3*), and MER proto-oncogene, tyrosine kinase (*MerTK*) contain motifs in their promoters that could allow regulation via core circadian clock genes.

A separate transcriptomics-based study in mice sought to identify differentially expressed genes under circadian control. This was accomplished by keeping mice in constant dark conditions for 3 days and collecting samples at times with a circadian peak or trough of OSP.[52] Genes for several important OSP pathways including integrin signaling and actin cytoskeletal signaling showed differential expression

at the times sampled. This suggests that these pathways are under circadian control. Notably, this paper did not identify enrichment of genes involved in phosphoinositide signaling as in the previous transcriptomics study, which may be due to differences in study design from using mice raised on cyclic lighting compared to mice kept in constant darkness.

Subsequent studies investigated the profile of OSP in rodent models with core clock genes disrupted. One group reported that *Bmal1* KO in the RPE disrupts the daily rhythm of OSP in cyclic lighting.[37] The KO mice have an equal daily total level of OSP but it is distributed throughout the day as opposed to peaks and troughs in the WT. *Bmal1* KO mice had reduced amounts of the dopamine receptor D2 protein which is suggested as the mechanism by which *Bmal1* KO disrupts OSP. This disruption of OSP was only seen in *Bmal1* RPE-specific KO and not in neuroretina KO.[37] In a separate study, OSP was examined in mice lacking the *Per1* and *Per2* genes.[17] When these mice were held in constant darkness the *Per1/Per2* KO mouse failed to show a peak in OSP activity at the presumptive light onset time. In this KO mouse, there was a large transcriptional change in the RPE. This, taken with the findings from the *Bmal1* KO mouse, suggest that the peak of OSP activity in rodents is mediated by the RPE and is under circadian control.[17,37]

Much less is known about the molecular regulators that regulate OSP in animals that display light-driven control of OSP. In Xenopus, expression of leukotriene C_4 was correlated with increased OSP activity.[29] One study in zebrafish using a bespoke reporter system to measure rod outer segment (ROS) growth/removal identified the phosphodiesterase inhibitors sildenafil and vardenafil as inhibitors of OSP. The authors suggest the ability of these drugs to inhibit OSP involves the phototransduction machinery in signaling an OSP peak at light onset in zebrafish.[53]

Due to the limitations of experimental methods used to study OSP, there is no understanding of the influence of circadian control in human OSP. Knowing the influence of circadian control on human OSP would be a key step in understanding which model systems may prove most useful for studying the process. There is some evidence that the human retina possesses a circadian clock and carries out some processes with a circadian rhythm but there is no evidence linking this circadian clock to human OSP.[54,55]

4 | MOLECULAR REGULATORS OF OUTER SEGMENT PHAGOCYTOSIS—WHAT DO WE KNOW FROM IN VIVO STUDIES?

More recently research has shifted to elucidating molecular regulators of the OS tip demarcation and phagocytosis phases of OSP. Whilst many molecular regulators have been identified in vitro, their significance remains unknown in vivo, as there are few models available to study the entire process of OSP. While cell culture and in vitro systems provide in-depth insights into the molecular regulation of specific aspects of OSP (reviewed in Lakkaraju et al., Kwon et al., Mazzoni et al.[9,10,56]), they are unable to provide a system-wide, physiologically relevant understanding of OSP in an integrated biological process. Therefore, here, we focus on studies involving in vivo models to dissect molecular regulators of (1) demarcation/binding, (2) engulfment, and (3) degradation[1] (Figure 4).

Since early studies on OSP, there have been differing opinions on whether the tip of the photoreceptor is shed following demarcation and subsequently encompassed by the RPE, or whether demarcation initiates a single-step engulfment process of the designated tip fraction by the extending RPE microvilli.[9,13] Whilst there are no definitive studies, we lean toward the latter mechanism. First, from a cellular perspective there have been no reports of "shed" discs or lamellae floating in the intracellular space, but rather the opposite, where RPE removal halts OS tip removal.[57] Indeed, for OSP, there must be an interaction between receptors and ligands on the RPE and the OS tip simultaneously.[58] Lastly, in genetic mutants where phagosome degradation is affected, the levels of "shed" OS not yet part of a nascent phagosome do not increase.[59,60] Thus, we try to avoid using the term "*shed*" OS where possible, and propose the term pruning, as this may be a more accurate description of the OSP process. Further studies are required to elucidate the exact mechanisms involved.

4.1 | Demarcation of OS area to be phagocytosed

Studies in rodent models demonstrate the externalization of phosphatidylserine (PtSer) as an initiation step, presenting an "eat me" signal on OS tips, similar to apoptotic cells.[58,61,62] PtSer is an anionic phospholipid, normally restricted to the cytosolic leaflet of the plasma membrane due to the continuous activity of flippases, also known as phospholipid translocases.[63] During apoptosis, the flippases are cleaved by caspases while activating scramblases in parallel. Such activation/inactivation mechanisms can randomly flip membrane phospholipids across the cytosolic membrane, signaling phagocytosis. Using annexin V, PtSer binding reagents, and a PtSer biosensor, Ruggiero et al.[58] and Mazzoni et al.[64] show using real-time imaging that PtSer exposure on OS tips occurs prior to the dawn burst of OSP in wildtype mice. It is assumed that decoration of the OS tips with externalized PtSer is necessary to signify the part of the OS to be engulfed and degraded.

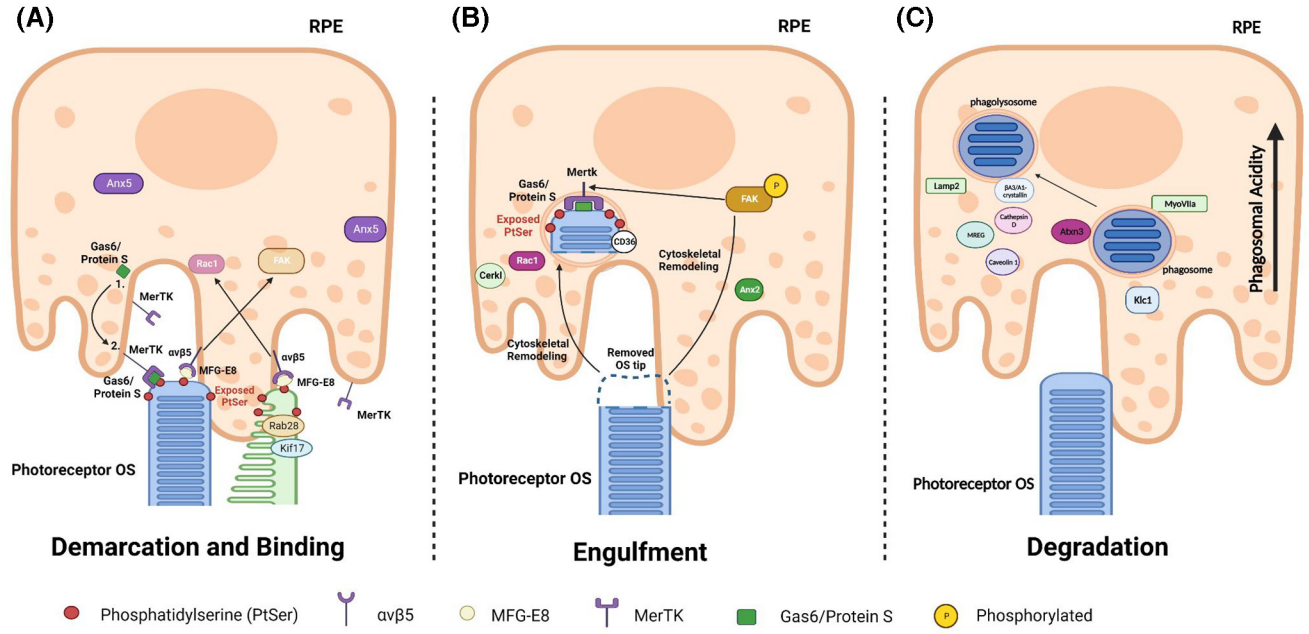

FIGURE 4 Components identified in vivo which regulate OSP at the various stages. (A) Demarcation, and binding of photoreceptor OS by the RPE. Phosphatidylserine (PtSer) localizes to the outer membrane of the photoreceptor OS to initiate the OSP process. MFG-E8 acts as an opsonization agent allowing for effective binding to the αvβ5 integrin receptor and binds directly to the outer leaflet of the photoreceptor outer segment upon exposure of phosphatidylserine on the outer leaflet. Other regulators involved in the first phase of OSP are Annexin 5 (Anx5) which is present in the RPE, and the ciliary proteins Rab28 and Kif17, which are thought to carry out their role in the cone photoreceptor OS. MER proto-oncogene, tyrosine kinase (MerTK), and the ligand Gas6 and Protein S are present on the apical membrane of the RPE (1). MertTK is activated by either the Gas6 or Protein S ligand at the apical membrane to initiate OS tip engulfment (2). αvβ5 also activates G-protein Rac1 and Focal adhesion kinase (FAK). (B) The engulfment stage of OSP. The next stage of OSP involves Cerkl, CD36, activated Rac1, and Annexin 2 (Anx2). Focal adhesion kinase (FAK)-non receptor tyrosine kinase, and MER Proto-Oncogene, Tyrosine Kinase (MerTK), are essential for RPE phagosome engulfment in vivo. Phosphorylated FAK is essential for the cytoskeletal remodeling of the RPE to physically engulf the OS tip. Gas6 and Protein S work as interchangeable ligands for MerTK. The dashed line on the photoreceptor OS highlights the portion of the OS which has been removed. (C) Phagosome maturation and degradation within the RPE. As the phagosome matures in the RPE and moves toward the basal side of the RPE, where the pH gradient of the phagosome becomes more acidic. The phagosome interacts with lysosomal vesicles and fuses to form phagolysosomes, to begin degradation. Some of the in vivo regulators identified in this stage of OSP include Cathepsin D, Lamp2, Myosin 7a (VIIa), Ataxin 3 (Atxn3), Caveolin 1, Mreg, βA3/A1-crystallin, and kinesin light chain 1 (Klc1). Created using BioRender.com.

4.2 | Binding

When PtSer is flipped to the external membrane, this results in opsonization of Milk Fat Globule-Epidermal growth factor 8 (MFG-E8), an αv ('VN binding') integrin (αvβ5) ligand, and initiation of the MFG-E8-αvβ5-MerTK pathway.[61] PtSer externalization of OS tips is lacking in models deficient for the RPE receptor αvβ5 integrin or its extracellular ligand MFG-E8,[61] which is also expressed in the RPE and the subretinal space, as well as the rest of the neural retina.[65] This analysis revealed in vivo regulators of OSP but also identified that the RPE and photoreceptors do not work autonomously to regulate OSP. Via their phagocytic machinery, the RPE and photoreceptors contribute to PtSer exposure by POS tips.[58] However, the MFG-E8–αvβ5 receptors do not physically move the designated portions of the OS into the RPE, but rather are signal regulators.[61] Thus, later studies focused on elucidating downstream components of the signaling pathway controlling reorganization of the RPE plasma membrane and subsequent engulfment of the OS tip.

The αvβ5 integrin is the sole integrin family receptor at the apical surface of the RPE in both rodent and human retinae.[66,67] Akin to other clearance phagocytosis mechanisms, αvβ5 integrin is required for efficient binding of the RPE cells to the POS tip, as knockout of αvβ5 in mice shows an absence of photoreceptor engagement and binding of the RPE to the demarcated OS tip.[61] The importance of the αvβ5 receptor and MFG-E8 ligand are highlighted in loss of function models which present with loss of rhythmic OSP, and elimination of the OSP peak. In both cases, this results in the gradual accumulation of the unwanted visual cycle by-product A2E.[35] In a time-course

of daily phagocytosis using opsin antibody-labeled retinal cross-sections, the *αvβ5*$^{-/-}$ and *MFG-E8*$^{-/-}$ mouse models show consistent basal levels of RPE phagosomes, but at a higher basal level (>2 fold increase) than their wildtype counterparts.[61] Analysis of whole retinal extracts did not reveal any significant alterations in αvβ5 or MFG-E8 protein levels at all time points tested throughout the day from 30 min prior to light onset to 3 h after light offset and did not peak in correlation with the burst of OSP after light onset.[61] However, it has been postulated by the Finneman group that the αvβ5 receptor responsiveness and/or MFG-E8 ligand availability could change right at the photoreceptor/RPE interface given their role in phagocytosis.

The OSP peak elimination in the *αvβ5*$^{-/-}$ and *MFG-E8*$^{-/-}$ eyecups, is similar to results observed in rodent models lacking annexin A5 (*Anxa5*), an additional molecule recently shown to regulate the binding capacity of photoreceptor OS destined to be phagocytosed. Silencing *Anxa5* is sufficient to reduce levels of *αvβ5* receptors at the apical phagocytic surface of RPE cells, with the association of cytosolic annexin A5 and αvβ5 integrin in RPE cell culture and in vivo supporting their functional interdependence.[68] Interestingly, and an important point to emphasize in relation to POS binding in rodent models, is the absence of functional redundancy and compensation. Loss of each regulator identified to date shows a complete ablation of the diurnal peak experienced under normal conditions.

To date, the majority of OSP regulators characterized, carry out their primary function in the RPE. However, in recent years several studies began to elucidate the role of photoreceptor signaling in the promotion and regulation of OSP.[4,16,28,69] A study focusing on ciliary protein Kif17 (kinesin family member 17) shows a potential role for Kif17 phosphorylation in both OS localization as well as promoting phagocytosis of zebrafish and mouse cone photoreceptors in a cell-autonomous manner.[16] By electron microscopy and immunogold labeling, this study showed that expression of a phospho-mimetic Kif17, solely in cone photoreceptors, was sufficient to induce a threefold increase in the levels of RPE phagosomes at the morning peak of phagocytosis. In parallel, a similar electron microscopy assay revealed mutant *kif17* strains showed significantly fewer RPE phagosomes throughout the day compared to wildtype siblings, in particular at the 2 identified OSP peaks.[16] More recently, two studies focusing on another ciliary protein, Ras-related protein Rab28, have similarly shown that loss of Rab28 is sufficient to significantly diminish levels of RPE phagosomes at peak OSP times.[28,69] At the 2 established peak times for OSP, the amount of RPE phagosomes is maintained at basal levels. Notably, transgenic overexpression of Rab28, solely in zebrafish cones, rescues the OSP defect in *rab28* KO fish, suggesting that *rab28* gene replacement in cone photoreceptors is sufficient to regulate Rab28-OSP.[28] In mice, loss of Rab28 results in defects in the OSP process. Using electron microscopy and immunofluorescence, defective removal and phagocytosis of outer segment (OS) material from the tips of mouse cones, but not rods, was observed, with a subsequent loss of visual function. Failure to remove old lamellae led to the accumulation of membranous material at cone tips and eventual degeneration and death of the cones, followed by rods.[4]

4.3 | Engulfment

RPE cells extend apical processes into the matrix adjacent to the photoreceptors creating cellular interdigitations with the distal outer segments.[70] Recognizing the externalized PtSer decoration, the actin-rich apical processes of the RPE begin ensheathment of the photoreceptor OS tips long before phagocytosis occurs. It is postulated that this recognition may occur in a similar manner as in other phagocytic processes,[1] and although many bridging molecules have been suggested, there is currently no conclusive data.[10]

These apical processes are essential for forming the nascent phagocytic cup and subsequent phagosome membrane fusion as a result of actin reorganization. PS recognition also leads to recruitment of the small GTPase RAC1 (Ras-related C3 botulinum toxin substrate 1), which following activation by avβ5 and MFG-E8 regulates cytoskeletal rearrangement and promotes the formation of the phagocytic cup.[71] GTP-RAC1 levels, quantified by GLISAs (a small GTPase activating assay), increase 2.3-fold in wildtype mouse eyes an hour on either side of light onset, correlating with peak OSP times, and decrease by ZT 3. Notably, although RAC1 is downstream of avβ5 and MFG-E8, GTP-RAC1 expression levels appear unchanged at any time point studied in *avβ5*$^{-/-}$ or *Mfg-E8*$^{-/-}$ eyes, in which the OSP peak amplitude is diminished.[71]

In relation to retinal cytoskeleton reorganization, two components integral for OSP are focal adhesion kinase (FAK)-non receptor tyrosine kinase, and MER Proto-Oncogene, Tyrosine Kinase (MerTK), a member of the Tyro/Axl/Mer family of receptor tyrosine kinases. Lack of either the avβ5 receptor or MFG-E8 ligand leads to a failure of the retina to stimulate the tyrosine kinase MerTK after light onset.[72] This suggests maximal stimulation of MerTK requires functional avβ5 and subsequently its ligand MFG-E8.

A lot of the initial studies on OSP engulfment were carried out on the Royal College of Surgeons (RCS) rat. Since its generation in the 1930's the RCS rat has been utilized to identify MerTK as the crucial receptor for POS tip

engulfment.[60,73–75] The RCS rat is perhaps the most well-studied in vivo model of retinal phagocytosis and retinal degeneration.[76] The model shows a defect in the RPE cells, where the destined POS phagosomes are unable to be ingested, subsequently leading to large accumulations of photoreceptor debris in the subretinal space.[76]

In contrast to the integrin receptors, focal adhesion kinase (FAK) and MerTK are not required for the initial binding of the demarcated POS tips to be phagocytosed by the RPE, but rather are essential for the engulfment of OS by the RPE. *Nandrot* et al. show that the levels of FAK-non receptor tyrosine kinase and phosphorylation of MerTK peaked in succession in wildtype eyecups correlating with the daily phagocytic burst of RPE due to activation by αvβ5.[77] Work carried out initially in RPE cell culture was reaffirmed in a rodent study, showing that FAK lies upstream of MerTK in the hierarchy of signaling molecules.[77,78] The cytoplasmic non-receptor tyrosine kinase FAK transduces signaling pathways downstream of integrin activation at RPE focal contacts, allowing for essential cytoskeletal reorganization, in a reversible manner.[79] Additionally, activation of MerTK by the αvβ5 integrin is dependent on the presence of FAK.[80] As mentioned in an earlier section of this review, loss of the dopamine receptor D2 in a mouse model also shows ablation of an OSP peak.[36] Proteomic profiling of the knockout model suggests that the reduced peak may be due to reduced expression of FAK in the *DR2* mutant model, positing a role for the dopamine receptor D2 in controlling integrin signaling.[36]

Just as MFG-E8 is a ligand for αvβ5, similar bridging molecules exist for MerTK, with 2 of the most important being the vitamin K-dependent factors Protein S (ProS) and growth-arrest-specific gene 6 (Gas6). Protein S and Gas6 stimulate OSP, through a similar Mer-dependent mechanism, as shown in vitro.[81] Though Gas6 and Protein S are well studied in other processes, their characterization as regulators of OSP in the rat retina was the first demonstration of a common role for Gas6 and ProS in any biological process. The existence of these as redundant ligands for Mer-dependent OSP also implies a critical role of OSP in the maintenance of retinal function. However, as Gas6 mutants have normal retinal morphology, due to compensation by Protein S,[81,82] it was suggested that Gas6 and Protein S are interchangeable for OSP in vivo. This hypothesis was validated by a double knockout model described by Burstyn- Cohen et al., which recapitulated the phagocytosis and retinal degeneration phenotypes observed in *MERTK* mutants.[83]

Studies in zebrafish show *cerkl* (ceramide kinase-like), a gene linked to retinitis pigmentosa, as a potential regulator of MerTK expression.[84] The *cerkl* KO shows rod-cone degeneration from 2 months old. EM analysis shows an accumulation of phagosomes in the interphotoreceptor matrix, although it is likely these samples were not collected at peak OSP times. Subsequent analysis by RT-qPCR and western blotting identified that *cerkl* deficiency causes an age-dependent decrease in the expression level of MerTK in the zebrafish retina, although the molecular mechanism is yet to be uncovered.

Following the interaction of MerTK with either ligand, Gas6, or Protein S, the kinase domain of MerTK initiates multimerization, leading to pseudopod extension facilitated by recruited adaptors and effectors. Studies in vitro suggest MerTK can stimulate Rac activation by recruiting the p130Cas/CrkII/Dock180 GEF complex.[85] However, so far related rodent studies are inconclusive. At peak OSP times, levels of GTP-RAC1 in wildtype and RCS mice were not significantly different. Interestingly, GTP-Rac1 levels subsequently decreased less in RCS eyes, remaining elevated several hours after the window for peak OSP.[71] Rac 1 activation is also supported by MerTK indirectly, as the tyrosine kinase activates phosphoinositide 3-kinase (PI3K). In turn PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PtdIns[4,5]P_2) leading to a linear generation of further phosphoinositides.[86]

As highlighted throughout this review, OSP is an extremely regulated process, in terms of time, and localization. MertTK, in addition to its crucial role in POS internalization, is also involved in the negative regulation of OSP, controlling the amount of OS destined to be engulfed.[87] Cleavage of MerTK releases a soluble form of MerTK (sMerTK) into the RPE which can inhibit the function of Gas6. This release is rhythmic in vivo and postulated to regulate OSP peak duration.[87]

Annexin A2 is known to be a predominant Src substrate, a proto-oncogene tyrosine-protein kinase, with phosphorylation regulating cytoskeleton dynamics and actin remodeling.[88] Retinal section analysis of an annexin A2 knockout mouse($AnxA2^{-/-}$) shows impeded phagocytosis, establishing that annexin A2 present in RPE cells function as a signaling intermediate in the uptake of photoreceptor outer segments. Probing of eye cup lysates revealed a delay in the rhythmic activation of FAK, suggesting a model in which recruitment of anx A2 to the forming phagosome leads to recruitment of c-Src, highly similar to what has been observed by F actin described in vitro.[78]

One of the traditional trademarks of phagocytosis is the exquisitely localized actin polymerization, directly below the phagocytic target. Actin polymerization drives phagosome formation, and in other phagocytic cells, F-actin begins to depolymerize at or before the point at which phagosome closure is complete. Whilst there are not many in vivo studies, there have been extensive studies carried out in cell culture models as reviewed in Ref. [9,10,56].

An interesting scenario where in vitro models of OSP differ from in vivo studies is exemplified with CD36 (cluster of differentiation 36). CD36 is associated with many types of phagocytosis and had first been described as having a role in OSP from RPE cell culture work, and was shown to be involved in OS tip engulfment by recognizing phospholipid OS ligands.[89] However, the retinal histology and OSP patterns in the *Cd36* mutant mice initially did not differ significantly from those of their wildtype counterparts under standard conditions. Rather an age-related phenotype was observed wherein *Cd36* mutant mice revealed a thickened Bruch's membrane by 12 months old, and a build-up of harmful oxidized LDL (low-density lipoprotein particles), suggesting that CD36 could be involved in the removal of retinal deposits in vivo.[90,91]

4.4 | Degradation

Mechanistically, many molecular mechanisms of degradation of phagosomes in RPE cells are well conserved with other cell types involved in phagocytosis of apoptotic cells or debris. As such, OS-containing phagosomes now within the depths of the RPE cells interact with lysosomal vesicles and fuse to form phagolysosomes. The phagolysosomes move from the apical to the basal region of the RPE, where the pH gradient becomes more acidic.[92–94] The phagolysosomes achieve the enzymatic degradation of the engulfed OS material. However, our knowledge on the maturation process of phagolysosomes and how the digestive hydrolases efficiently degrade POS components so rapidly remains incomplete.

A mouse model (Shaker1) for Usher Syndrome 1B, a syndrome with associated retinal degeneration and vestibular deficit, exhibits a delay in phagosome movement from the apical region of the RPE to the more acidic environment of the cell body. The lack of the affected protein, Myosin 7a, leads to a lower number of melanosomes in the RPE apical processes. In addition, decreased phagosome clearance was observed in conjunction with reduced levels of total phagosomes by analyzing samples at various time points spanning ZT 0–3.5 using TEM. The extended half-life of the phagosomes may be causative of deleterious effects within the RPE and on photoreceptor integrity.[59] Mutations in the microtubule-based motor Kinesin-1, also affect the degradation of phagosomes. Initial analysis carried out in RPE cells, led to investigations into the loss of the axonal transporter using a *Klc1* (kinesin light chain 1) knockout mouse model. From 2 months old, retinae of knockout mice maintained under a normal light–dark cycle showed double the amount of RPE phagosomes 3 h after light onset when compared to wildtype controls, suggesting a defect in phagosome processing. By 18 months old, this phenotype was accompanied by photoreceptor degeneration predominantly in the central retina and an abundance of lipofuscin accumulation in the RPE.[95] Earlier work in cell culture allowed for the postulation that Myosin 7a and Kinesin-1 work sequentially, with Myosin 7a facilitating transport through an actin filament network, superseded by Kinesin-, and microtubule motor transit toward the cell body.[95] A similar mouse model for phagosome maturation studies was observed in a melanoregulin (MREG) knockout mouse.[96] The $Mreg^{-/-}$ mouse exhibits the characteristic phagocytic burst at light onset, however, phagosomes quantified by immunofluorescent staining showed that levels remain elevated for up to 8 h when compared with control C57Bl6/J RPE.[96] More recently, a mouse model lacking Ataxin-3 ($Atx3^{-/-}$) also showed dysfunctional phagosome maturation and transport. At 2 years old, electron microscopy analysis revealed an accumulation of phagosomes in the apical zone in *Atxn3* KO compared to the wildtype control which showed phagosomes localizing to the basal zone. The overall number of phagosomes quantified was similar between genotypes, showing that the initial stages of OSP occur as normal.[97]

Analysis of mouse retinae revealed that for the phagosome maturation process to be completed, the cytoplasmic rhodopsin epitope must be removed from the phagocytic load and precedes movement from the apical to basal regions of the RPE cell. Double labeling of retinal sections showed that only loss of the C-terminal cytoplasmic rhodopsin epitope is necessary for the OSP process to continue,[98] as the progression through the maturation process showed a time lapse of doubly labeled to single labeled phagosomes. This loss must occur before complete maturation to allow for phagolysosome fusion, an observation uncovered by co-staining for cathepsin D and rhodopsin.[98]

The soluble lysosomal aspartic protease cathepsin D is necessary to break down opsin and Rhodopsin from the phagosome. Cathepsin D is a ubiquitously expressed lysosomal marker associated with phagocytosis in many cell types. Multiple in vivo studies in mouse, *drosophila*, and zebrafish uncovered a conserved role for cathepsin D in both organ development and tissue homeostasis. A global knockout of cathepsin D in a mouse model was lethal by 21 days post-natal, underscoring its vital role in survival and homeostasis.[99,100] The mouse model shows rapid retinal degeneration, accompanied by a build-up of an autofluorescent lipopigment, or ceroid lipofuscin, in the retina and other CNS neurons,[101] with a lack of cathepsin D processing in a caveolin-1 (*Cav-1*) knockout model showing similar phenotypes.[102] The main retinal atrophy observed in knockout mice was a result of degenerating photoreceptor cells[101] in the outer nuclear layer (ONL), with a lesser effect on the retinal ganglion cell layer and inner nuclear

layer, despite ubiquitous levels of expression. This was attributed to a lack of lysosomal degradation and subsequent activation of caspase 3 and 9.[100] Immunofluorescence microscopy of tissue sections stained for cathepsin D, carried out in an independent study by Sethna et al. in 2016, shows increased levels between ZT 0.5 and 2 amidst the phagocytic burst in comparison to later stages of day.[102]

More recently a zebrafish morpholino knockdown of cathepsin D highlighted its role in the retina of a cone-dominant vertebrate. While this study did not specifically focus on the process of OSP, retinal imaging and immunofluorescence showed the knockdown retinae were reduced in size with RPE cells lacking microvilli.[103] Another regulator of OSP identified in vivo is the βA3/A1-crystallin gene. *Nuc* mouse models, which have a mutated βA3/A1-crystallin, identified its role in phagosome degradation, as it is required to localize to the lysosome. Lack of βA3/A1-crystallin results in lower levels of cathepsin D, further impairing phagosome degradation.[104] Consistent with the above, lysosomal degradation deficiency also observed in a *Lamp2* (lysosomal-associated membrane protein 2) knockout mouse, was shown to affect phagosome degradation, and subsequently lead to the accumulation of sub-RPE deposits, with mutant mice showing a phenotype similar to AMD.[105]

Rab proteins play a large role in phagocytosis, regardless of cell type. The most well-studied interactions involve the sequential recruitment and replacement of Rab5 and Rab7a respectively, pushing forward the maturation process of the nascent phagosome. Rab7 is essential for phagosome-lysosome fusion (in addition to autophagosomes and endosomes) by recruiting tethering factors.[106] However, studies showing their direct involvement in OSP in vivo are unavailable.

An exciting study by the Finneman group in 2016 revealed a method of investigating POS phagosome acidification, trafficking, and quantification of an in vivo system ex situ. Their new imaging approach directly analyzed live rat RPE tissue ex situ.[107] This live approach using an acidified biosensor is a complementary approach to using fixed process tissue to rapidly analyze the phagocytic load in the RPE and will allow for further specification of the factors involved in phagosome maturation and degradation. Several results from this assay have since been described in Vargas et al.[8]

5 | CONCLUSIONS AND KNOWLEDGE GAPS

The relationship between the photoreceptors and the supporting RPE is an intriguing example of cross-cellular cooperation to regulate a rhythmic biological process. It will be fascinating to elucidate more deeply the in vivo factors orchestrating this process. The RPE's phagocytic function is integral for photoreceptor health and vision. Many of the molecular regulators discussed above play a role in inherited retinal degenerations, further highlighting the necessity of the *pruning*, *waste disposal*, and *recycling* circuitry phases of OSP.

Mutations in human *MERTK*, *CERKL*, *MYO7A*, and *RAB28*, result in inherited retinal disorders including, but not limited to, retinitis pigmentosa, cone-rod dystrophy, and Usher Syndrome.[108] Notably, to date, no effective therapies targeting OSP are available. Additionally, although the effect of reduced OSP on the aging retina is not fully appreciated, several studies hypothesize it leads to drusen deposition and increased lipofuscin accumulation, resulting in RPE death and acquired macular degenerations.[6,109,110]

As mentioned, a deficiency in many of the molecular regulators involved in the engulfment and degradation stages of OSP results in retinal degeneration in in vivo systems.[4,60,83,84,100,102] However, in more subtle presentations that solely lack peak OSP amplitudes in vivo, there is no translation into rapid degeneration of the retina or RPE.[28,36,61,72] In this context, vision loss and late-onset retinal degeneration are observed in $\alpha v\beta 5^{-/-}$ knockouts but not in *MFG-E8*$^{-/-}$ knockouts, demonstrating that loss of phagocytic rhythm alone is not sufficient to cause age-related blindness.[36,72] Notably, $\alpha v\beta 5$ also plays a role in retinal adhesion, independent of its role in phagocytosis.[72,111] Thus, the potential clinical consequences of affecting the precise temporal regulation of POS phagocytosis by the RPE have yet to be determined. But certainly, the mechanisms by which the basal levels of OSP are increased in numerous models lacking characteristic phagocytic peaks are appealing therapeutic targets for impaired vision linked to defective waste removal and recycling in the retina.

Enhancing OSP may be of therapeutic relevance to many retinal degenerative diseases and macular degenerations, which could be administered as a stand-alone therapy or in combination with gene- and cell-based therapies to accentuate their outcomes. Currently, trials and studies are in place to investigate the potential of a therapeutic option directed toward replacing *MERTK*. Preclinical studies which injected an AAV2 vector with functional human MerTK into the RCS rat showed positive results, providing rescue of OSP for all time points examined up to 6.5 months, in addition to an improvement in ERG-response amplitude.[112] Initial Phase I clinical trials also showed that this vector was well tolerated in patients with MERTK-related RP using subretinal injection and that rAAV2-VMD2-h*MERTK* is not associated with major side effects. However, the improvements seen in

visual acuity waned entirely in the 2 years following treatment.[113] Another study has also highlighted the use of subretinal lavage and sparse photocoagulation to remove lipid deposits, which preserved photoreceptors for up to 6 months of age in RCS rats.[114]

More recently in vitro systems revealed potential therapeutic options to enhance OSP. A high-throughput, orthogonal assay, aimed to discover therapeutics at a broad patient spectrum, targeting the OSP process rather than specific regulators.[115] The top hit reported in the 2020 study, ramoplanin (a lipoglycodepsipeptide antibiotic), was shown to not affect phagocytosis in healthy RPE cells but was capable of rescuing OSP in RPE cells with defective *MERTK*. However, in vivo validation is yet to be reported.[115] Another study has also shown that a translational readthrough inducing drug named PTC124 was also able to partially restore phagocytosis in RPE cells with *MERTK* mutations.[116] Also, a phosphomimetic Dbl3 molecule was sufficient to rescue phagocytosis in *MERTK* defective RPE cells.[117] It will be fascinating to determine if the in vitro findings in iPSC translate into therapeutic effects in vivo.

Such advances are promising, nonetheless, to fully comprehend the OSP process and its effect on retinal health, extended research into the area is required. As described throughout the review, many animal models have aided in the elucidation of the molecular machinery implicated in OSP, its patterns, and the consequences of its failures, providing a greater understanding of various retinal pathologies. Little is known, however, about the regulation of OSP in the human eye. The use of in vivo systems allows for investigation into the relationship between OSP regulators in the RPE and the photoreceptors compared to in vitro cell culture models, and certainly helps us to better understand the initial triggers which spark the daily burst of OSP. Predominantly, the mechanisms identified to date have been studied in nocturnal rod-dominant rodents. This highlights the need for further studies into the crucial, perhaps still unknown regulators of cone phagocytosis, which could also be essential for the maintenance of human OSP.

Perhaps most scientifically challenging, there is still a large void in our analysis of OSP in real-time. Although studies have been carried out using in vivo animal models and post-mortem human eyes, a question remains as to how OSP occurs in a living human eye. The methods currently used can introduce aspects of intra-organismal variation which may lead to outliers and discrepancies when assessing OSP. A recent publication from the Finneman group provides excellent detail on such potential discrepancies.[8]

These limitations highlight the need for techniques that assess OSP activity in real-time. Kocaoglu et al. utilized adaptive optics optical coherence tomography (AO-OCT) to dynamically assess individual cone cells in the intact living human eye.[118] This technique utilizes scanning deformable mirrors to orient a probing beam onto the retina by shining it through the pupil of the individual and measuring the relative difference in interference caused by phagosome activity in real-time, revealing a peak of cone OSP in the morning time.[118] Although this method is constrained in resolution due to the finite size of the human pupil, it will be extremely interesting to see how future studies incorporating this technique can be adapted and applied to healthy retinae, and those of animal and human patients with inherited retinal disease age-related macular degenerations.

Despite exponential progress in our knowledge of OSP regulation in vivo, there is still much research to be done to fully comprehend the circuitry between the RPE, photoreceptors and external, and internal regulators which allow for efficient waste removal, and maintenance of photoreceptor integrity. This review provides a concise overview of the current landscape of OSP research undertaken on the environmental and molecular regulators of various in vivo systems and hopefully will inform future investigations.

AUTHOR CONTRIBUTIONS

Ailis L. Moran was the primary author of this review. John D. Fehilly and Breandán N. Kennedy contributed significantly to preparing sections for this review. Daniel Floss Jones was involved in the initial draft of this review. Ailis L. Moran and John D. Fehilly prepared the figures. Ross Collery contributed significant intellectual input to the review. All authors edited the final draft.

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