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The Retinal Pigment Epithelium in Health and Disease

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Abstract

Retinal pigment epithelial cells (RPE) constitute a simple layer of cuboidal cells that are strategically situated behind the photoreceptor (**PR**) cells. The inconspicuousness of this monolayer contrasts sharply with its importance [1]. The relationship between the RPE and PR cells is crucial to sight; this is evident from basic and clinical studies demonstrating that primary dysfunctioning of the RPE can result in visual cell death and blindness. RPE cells carry out many functions including the conversion and storage of retinoid, the phagocytosis of shed PR outer segment membrane, the absorption of scattered light, ion and fluid transport and RPE-PR apposition. The magnitude of the demands imposed on this single layer of cells in order to execute these tasks, will become apparent to the reader of this review as will the number of clinical disorders that take origin from these cells.

Keywords

Retinal pigment epithelium; visual cycle; phagocytosis; melanin; retinal degeneration; transport; lipofuscin; retinoid

1. A SIMPLE EPITHELIUM

When viewed *en face*, RPE cells exhibit a hexagonal shape. Microvilli extend from the apical surface of the RPE cells to envelop the outer segments of both rod and cone PRs, although the length of outer segment cloaked by the microvilli is greater for rods than cones [2]. The elaboration of these apical microvilli expands the surface area of the RPE 30-fold [3] thereby facilitating the unique functional relationship with the PR cells. As with microvilli associated with other epithelial cells, RPE microvilli are supported by an actin core.

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The lateral membranes of RPE are the sites of cell– cell adhesion and cell communication The apical junctional complexes (Fig. 1) of RPE – a composite of tight, adherens and gap junctions - play a role in maintaining cell polarity by forming a physical barrier that prevents intramembranous diffusion of components between the apical and basolateral membrane domains [4, 5]. These junctional complexes also prevent the passage of molecules and ions through the paracellular space thereby making RPE cells the guardians of the outer blood-retinal barrier [5]. Nevertheless, the tight junctions of RPE do exhibit some leakiness [5] with the trans-epithelial electrical resistance, a measure of the movement of ions across a paracellular pathway, of differentiated human RPE in culture being 834 ohm/cm^2 [6] as compared to 1000–2000 ohm/cm^2 for endothelial cells subserving the blood-brain barrier [5]. Mutations in the CLDN10 gene that encodes the tight junction protein claudin-19 expressed by RPE, results in multiple ocular abnormalities including macular colobomata [7].

The basal plasma membrane of RPE is embellished with complex infoldings, a specialization that is typical of cells involved in transport. The organelles generally exhibit domain specific distribution with the nucleus, Golgi and mitochondria being preferentially localized in the basal cytoplasm while melanosomes are primarily located apically.

2. A PIGMENTED EPITHELIUM

A prominent organelle within the RPE is the melanosome (Fig. 1), a lysosome-related body dedicated to the synthesis and housing of melanin. The contents of the melanosome, including enzymes, structural proteins and ion channels necessary for melanin synthesis, are delivered to the melanosome *via* tightly controlled cellular sorting and trafficking mechanisms [8]. Melanin is synthesized from L-DOPA by human RPE cells during early fetal life but the absence of tyrosinase activity and premelanosomes (partially melanized melanosomes) in adult RPE indicates that melanin synthesis is minimal in the adult and indeed, diminishes with age (reviewed in [9]).

The topographic distribution of RPE melanin is fairly uniform with the exception of a narrow peak at the fovea [10, 11]. It is often assumed, mistakenly, that the concentration of melanin in human RPE varies amongst racial groups; in fact however, while pigmented cells of neural crest origin (melanocytes) exhibit marked racial variability in melanin density, the melanin pigmentation in neuroepithelium-derived RPE does not vary with race [10]. The autofluorescence of the human fundus that can be imaged using near-infrared excitation wavelengths originates in large part from melanin in the RPE and choroid [12].

It is generally accepted that RPE melanin, by absorbing light that has passed through the PR layer of retina, protects against reflected light that would otherwise degrade the visual image. In keeping with this function, melanosomes throughout most of the lifetime of an individual are located apically in the cell. However, this segregation diminishes with age [10] and melanosomes appear to associate with lipofuscin granules in what are referred to as melanolipofuscin complexes [13]. Besides serving as an absorbing pigment, melanin also quenches singlet oxygen, scavenges reactive radical species and harbors metal ions such as iron [9, 14, 15]. Disrupted iron homeostasis is suggested to contribute to oxidative damage

in RPE [16, 17]. RPE cells express ceruloplasmin and hephaestin, proteins that facilitate iron export from cells by oxidizing ferrous to ferric iron, the form that can be bound by the serum transport protein transferrin. Ceruloplasmin and hephaestin deficiency in mice leads to iron build up and death of RPE [18] and retinal degeneration in humans [19].

Myosin VIIa, a molecular motor that uses energy from ATP hydrolysis to move along actin filaments, is involved in melanosome transport. In addition the protein Rab27a links myosin VIIa to the melanosome surface. It is thought that together the proteins constrain the organelle within a zone of filamentous actin [20, 21]. Accordingly, in mice carrying a mutation in myosin VIIa (Shaker-1 mice), melanosomes are absent from the apical actin-rich region of the RPE. In humans, mutations in the gene encoding myosin VII are responsible for Usher type IB, a disorder associated with the loss of both hearing and sight [22]; in retina, loss of myosin VII function could impact RPE, PRs or both.

RPE melanin or elements of its biosynthetic pathway also play an important role in retinal development. Disruptions in melanin or melanosome biogenesis during retinal development, as occurs in the albino eye, is associated with inappropriate routing of ganglion cell axons at the optic chiasm. The result is a decrease in the numbers of ipsilaterally projecting axons and abnormal binocular vision. Importantly, rod PRs are also reduced in number and the fovea fails to form [23]. Types of albinism include various forms of oculo-cutaneous albinism that disrupt melanin synthesis in hair, skin and the eyes and that are inherited as autosomal recessive disorders. On the other hand, in ocular albinism only ocular tissues are hypopigmented. The gene product associated with X-linked ocular albinism 1 (OA1) is known to be a G-protein coupled receptor localized to melanosomes and lysosomes [24]. How melanosome biogenesis and melanin synthesis signals to the developing retina is not known.

3. RPE TOPOGRAPHY

RPE cells in the fovea are taller than in non-foveal areas but their cross-sectional area is also smaller (~14 microns in diameter) than the cells in the periphery (up to 60 microns) [25–27]. In human retina the numbers of PR cells (rods and cones) per RPE, has been reported to be the same in fovea as in peripheral retina [26] although in the non-human primate, foveal RPE were found to have the lowest numbers of PRs per RPE cell (~20:1) with the ratio increasing to ~ 40:1 at an eccentricity of 4° [27]. RPE cells are lost with age, the decline being approximately 2.3% of total RPE per decade of life [28]. Interestingly, however while RPE cell density decreases in peripheral retina with age, an age-associated change in RPE cell density is not observed in the fovea [26], probably because of inward migration of peripheral RPE so as to compensate for loss of foveal RPE [28].

4. RPE AND DISORDERS INVOLVING BRUCH'S MEMBRANE

The basal surface of the RPE rests on a prominent basement membrane – Bruch's membrane (Fig. 1). In the aging eye, Bruch's membrane is of clinical significance since material is deposited there. This material can be located between the RPE plasma membrane and the RPE basal lamina (basal linear deposits) and/or between the RPE basal lamina and the inner collagenous layer (basal laminar deposits) [29]; focal mounds in the latter location are

referred to as drusen. Studies of the constituents of drusen have revealed apolipoprotein B and E, esterified and unesterified cholesterol, amyloid P and several proteins associated with inflammation and/or the complement system including C-reactive protein, immunoglobulin G, vitronectin, clusterin and complement components (C) C3, C5, C9 [30–33]. Large confluent drusen are a risk factor for progression of age-related macular degeneration (AMD) [34] and the presence of complement associated proteins in drusen is consistent with genetic association studies reporting that certain DNA sequence variants in the genes encoding complement factor H, complement factor B/C2 and complement component C3 confer increased risk of AMD [35–41].

Bruch's membrane is the focus of another disease process, that associated with Sorby's fundus dystrophy (Fig. 1). This autosomal dominant disease of central retina is due to mutations in the gene encoding the tissue inhibitor of metalloproteinases-3 (TIMP3) [42], a protein secreted by RPE cells [43, 44]. Sorby's fundus dystrophy manifests as deposits in Bruch's membrane, with choroidal neovascularization and progressive RPE cell loss (geographic atrophy).

5. A POLARIZED MONOLAYER: MOLECULAR AND FUNCTIONAL IMPLICATIONS

As is typical of epithelia, RPE cells exhibit polarity – that is proteins, organelles and some functions are asymmetrically distributed in apical or basolateral domains of the cell [45]. Yet for several proteins, domain-specific expression is the reverse of that typically exhibited by epithelia. One protein exhibiting a reversed polarized expression relative to that typical of epithelia is sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) (Fig. 1). In contrast with other epithelia where Na^+/K^+-ATPase is localized to the basolateral domain of the cell, in the RPE cell, this pump is expressed apically [46]. The apical placement of Na^+/K^+-ATPase reflects the important role played by the RPE cell in controlling ion concentrations in the subretinal space, and the need for active sodium transport across the apical membrane to sustain the dark current of PRs. Ankyrin and fodrin, sub-membrane cytoskeletal proteins known to be associated with Na^+/K^+-ATPase, also exhibit an apical expression [47]. Other proteins exhibiting polarized expression on the basolateral plasma membrane, are bestrophin (hBest1; discussed in Section 6.4) [48] and MCT3 [49].

Numerous proteins have been found to be enriched in the apical region of the cell (reviewed in [50]) including the glycoprotein CD147/EMMPRIN (an inducer of metalloproteinase secretion), NCAM (neural cell adhesion molecule) [51], the $\alpha v\beta 5$ receptor that participates in the phagocytosis of shed PR membrane [52] (Section 8.1) and the proton-coupled monocarboxyate transporter 1 (MCT1), a lactate transporter [53]. Due to its association with RPE microvilli, the actin-binding protein ezrin is also more abundant apically. Domain specific expression of the MCTs – MCT1 apically and MCT3 basolaterally – is requisite to the ability of RPE to control lactate levels in retina [53].

Polarity is further reflected in domain specificity of some functions, and extends to the directionality of at least some of the factors secreted by RPE. For instance, neuroprotectin D1, an anti-inflammatory and cell survival factor derived from docosahexaenoic acid (DHA)

is released only from the apical side of RPE cells [54]. Conversely, vascular endothelial growth factor-A (VEGF-A) is secreted by RPE primarily from the basolateral domain [55] (Fig. 1). VEGF is a modulator of vascular permeability and angiogenesis and an important survival factor for the endothelial cells of the choriocapillaris. Therapeutic approaches to the treatment of neovascular AMD include three anti-VEGF agents, ranibizumab (Lucentis, Genentech Inc, San Francisco CA), bevacizumab (Avastin; Genentech Inc) and pegaptanib (Macugen, Eyetech Pharmaceuticals, New York) [56].

6. TRANSPORT FUNCTIONS OF THE RPE

6.1. RPE Cells on the Frontline

Poised at the interface of choroid and neural retina, RPE cells are responsible for the trafficking of nutrients and metabolic endproducts and serve to maintain ion balance in the subretinal space [57]. The vectoral transport that is needed for this function depends on an asymmetric distribution of channels and transporters in the apical and basolateral domains of the RPE cells. That the transport function of RPE is critical to a well-regulated retinal microenvironment may be obvious; less obvious effects may also be important. For instance RPE cells have been implicated in the modulation of eye growth and myopia perhaps by signaling through transport of ions and fluid or the release of other molecules [58].

Central to transport function by the RPE is the sodium gradient established by the electrogenic Na^+/K^+-ATPase (Fig. 1) through the active outward transport of Na^+ across the apical membrane together with K^+ influx [46]. The location of Na^+/K^+-ATPase on the apical membrane together with a polarized distribution of other channels and transporters creates high Na^+ concentration in the subretinal space and high K^+ concentration in the RPE cytosol. The high concentration of Na^+ in the subretinal space is essential for the PR dark current, whereby Na^+ ions enter open cGMP-gated channels and depolarize PRs [5].

In a function critical to phototransduction, the RPE cell is responsible for maintaining the volume and chemical composition of the subretinal space that would otherwise change with the light and dark cycle [59]. For instance, in the dark the concentration of K^+ in the subretinal space is approximately 5 mM. Under these conditions there is a net transepithelial K^+ transport from the subretinal space to the choroid as the ion enters RPE cells through the apically located Na^+/K^+-ATPase and leaves the cells *via* channels in the basolateral membrane. With the transition to light, PRs hyperpolarize due to closure of cyclic-nucleotide gated Na^+ channels in the membrane of outer segments and K^+ efflux is diminished. The result is that K^+ concentration in the subretinal space decreases (2 mM) and the RPE apical membrane hyperpolarizes. In addition, with activation of K^+ channels and reversal of the $Na^+/K^+/2$ chloride (Cl^-) cotransporter, movement of K^+ across the RPE changes direction so as to reestablish subretinal K^+ concentrations.

6.2. Fluid Transport and Retina-RPE Apposition

Proper anatomical apposition between the RPE and PR cells is essential not only to the optics of the eye but also to the health of PR cells since many RPE cell functions are critical to PRs. Indeed studies of experimental retinal detachment have shown that PR outer segments begin to degenerate, some PR cells die and PR synaptic terminals begin to retract

within a few days of RPE-neural retina separation [60, 61].The management of subretinal volume is essential to holding the neural retina in proximity to the RPE and to this end, active water removal from the subretinal space by the RPE is necessary [62].

Indeed, fluid from the subretinal space is transported across the RPE cell toward the choriocapillaris at a rate of several microliters per square centimeter per hour [63] utilizing the RPE cell's water channels, the aquaporins. Aquaporin 1, an integral membrane protein that serves as a channel for water, has been detected in human adult and fetal RPE cells, and in functional assays aquaporin facilitates water movement across cultured RPE. However, a clear polarized distribution of the protein was not observed [64].

The transepithelial transport of ions, particularly Cl^-, in an apical to basal direction drives the movement of water through water channels. This ionic movement is powered by the Na^+ gradient that is maintained by the Na^+/K^+-ATPase electrogenic pump (Fig. 1). Chloride exits across the basolateral membrane *via* a chloride conductance that is modulated by intracellular calcium [59, 65].

Bicarbonate (HCO_3) transport can also provide an impetus for fluid elimination. In the dark, oxygen consumption by PR cells is considerably increased and the carbon dioxide (CO_2) and water that is produced proportionally, is transferred to the choriocapillaris by the RPE so as to prevent acidosis of the subretinal space and alkalinization of the RPE cell. CO_2 can enter the RPE cell from the subretinal space both by diffusion across the RPE apical membrane and by carbonic anhydrase-mediated conversion to HCO_3 in the apical membrane followed by transmembrane transport *via* the electrogenic Na+/2HCO_3 cotransporter [57, 59, 66]. Diffusion of CO_2 across the basolateral membrane is quite limited (due to difference in effective surface area) and thus is dependent on carbonic anhydrase II to hydrate cytosolic CO_2 into protons and HCO_3. Efflux of HCO_3 across the basolateral membrane is then mediated by the Na^+/HCO_3 co-transporter [66]. The efflux of HCO_3 basolaterally is followed by fluid transport from the subretinal space to the choroid [66].

6.3. RPE Contributions to the DC ERG and the EOG

As a consequence of the polarized distribution of ion channels and transport proteins on the apical and basolateral membranes of the cell [67, 68], the RPE generates a spontaneous transepithelial potential (TEP) (5–15 mV) that is primarily responsible for the cornea-positive potential difference that can be recorded across the eye in the dark; this voltage difference increases with a light stimulus [63, 67, 68]. The TEP reflects the difference in membrane potentials between the apical and basolateral domains of the cell. Specifically, due to a large chloride conductance the basolateral membrane is relatively more depolarized (the TEP is positive) than the apical membrane.

Further hyperpolarization of the RPE apical membrane is induced by the light-evoked subretinal decrease in K^+ concentration that follows from closure of cGMP-gated channels in PR outer segments. This response is recorded as the c-wave slow component (seconds after light onset) of the electroretinogram (ERG) obtained using direct current (DC) amplification (DC ERG). DC amplification is necessary because of the slow time course [69]. Recovery of the RPE potential is reflected in the second slow component, the fast

oscillation (~ 1 minute after light onset and of negative polarity). This response involves reduced Cl^- influx across the RPE apical membrane and a hyperpolarization of the basolateral membrane. The third slow component of the DC ERG is the light peak; this response has positive polarity, reaches a maximum at 6–9 minutes and is generated by an increased chloride conductance in basolateral membrane that is depolarizing. Accordingly, the transepithelial potential is augmented.

The TEP can also be measured indirectly by the clinical electrical oculogram (EOG), an electrophysiological test that measures the voltage across the RPE as an indicator of RPE integrity. In recording the EOG, a period of dark-adaptation causes the resting potential to decrease to a minimum value (dark trough) [70, 71]; with onset of ambient light, the potential slowly increases to a peak (light peak). It is generally accepted that the light peak reflects a depolarization of the RPE basolateral membrane due to an increased outward Cl^- conductance.

For the analysis of the EOG, the ratio of the voltages (light peak divided by dark trough) is taken as the Arden ratio; in normal individuals the ratio is usually >2 (Fig. 2). A diagnostic feature of Best vitelliform macular dystrophy (BVMD), a dominantly inherited juvenile form of macular degeneration, is a decreased light peak (Arden ratio < 1.5). The product of the Best disease gene is bestrophin (hBest1) [72] (Fig. 1), and since the protein is expressed on the basolateral membrane of RPE there has been considerable interest in whether hBest1 is a calcium-activated Cl^- channel protein on the basolateral membrane of the RPE [73]. Nevertheless, although there is compelling evidence that hBest1 can flux Cl^- [68], experimental evidence indicates that hBest1 may not act alone with respect to the LP [48]. How exactly hBest1 dysfunction explains hBest1-related disease, including BVMD, adult vitelliform macular dystrophy, autosomal recessive bestrophinopathy [74], autosomal dominant vitreoretinochoroidopathy [75], and dominant retinitis pigmentosa [76] is not known and whether enhanced accumulation of RPE lipofuscin [77, 78] participates in the disease process is not understood.

7. THE RPE, VISUAL CYCLE AND ASSOCIATED DISEASES

The visual cycle (Figs. 1 and 2) is the metabolic process through which the visual pigment (rhodopsin in rods and cone-opsins in cones) is regenerated after light activation, in order to maintain the light sensitivity of the opsin pigments [79]. Light isomerizes 11-*cis* retinal, the unique chromophore of all vertebrate opsins, into all-*trans* retinal, changing the conformation of the visual pigment which becomes activated. Subsequent inactivation of the visual pigment through phosphorylation and steric hindrance of the transducin binding site yields apo-opsin and all-*trans* retinal. All-*trans* retinal must then be isomerized back to 11-*cis* retinal through several steps occurring in the PRs, adjacent RPE and Muller cells [80]. The kinetics of the visual cycle are controlled by several key enzymes and binding proteins. Defects in these proteins can slow down or interrupt the visual cycle causing a lack of chromophore, which as a consequence leads to various retinal diseases including relatively moderate functional visual impairment and severe blindness [81, 82]. In some instances, toxic byproducts accumulate with pathogenic consequences.

7.1. Molecular Actors in the Rod Visual Cycle

After rhodopsin decay, all-*trans* retinal is released into the lumen of the rod outer segment disc where it can combine with membrane phosphatidylethanolamine (PE) to form N-retinylidene-phosphatidylethanolamine (N-*ret*-PE). N-*ret*-PE is then flipped from the luminal side of the rod outer segmen discs to the cytoplasmic face by ABCA4, a large 12-transmembrane domain protein belonging to the ATP-binding cassette family [83–88] (Fig. 2). In the cytoplasm, all-*trans* retinal then dissociates from PE [89]. Some all-*trans* retinal molecules can freely diffuse through the disc membrane. Mutations that impair the ABCA4 transporter slow down the extrusion of all-*trans* retinal from the outer segment disc lumen, therefore leading to accelerated formation of the bisretinoid precursors of RPE lipofuscin *via* non-enzymatic pathways (Section 9). ABCA4-related disorders include recessive Stargardt disease, the most frequent inherited macular dystrophy [90], a form of cone-rod dystrophy and retinitis pigmentosa [91, 92] (Fig. 1).

In the rod outer segment cytoplasm, all-*trans* retinal is converted to all-*trans* retinol by reduction that is catalyzed by members of the subfamily of serine alcohol dehydrogenases (retinol dehydrogenases; RDH) namely RDH8 and possibly RDH12 [93, 94] (Fig. 1). After enzyme-mediated reduction, all-*trans* retinol is taken up by a large binding protein of the interphotoreceptor matrix, the interphotoreceptor retinol binding protein (IRBP), which allows it to cross the matrix and to reach the membrane of RPE microvilli [95, 96]. More recently, mutations in *RBP3*, the gene encoding IRBP have recently been shown to cause severe retinitis pigmentosa with high myopia [97].

In the RPE, all-*trans* retinol is tightly bound to the cellular retinol binding protein (CRBP) (Figs. 1, 2) which brings it to the endoplasmic reticulum where the subsequent steps of the visual cycle occur. In addition, all-*trans* retinol can be directly internalized by the RPE through phagocytosis of the outer segment tips (Section 8). It is also provided to RPE by serum retinol binding protein (SRBP) through the choroidal blood flow and subsequent interactions with the STRA6 receptor at the basal side of the RPE [98]. Impairment of SRBP and STRA6 causes various forms of developmental diseases. In the endoplasmic reticulum, lecithin:retinol acyl transferase (LRAT) (Figs. 1, 2) catalyzes the esterification of all-*trans* retinol in palmitate or stearate retinyl esters, through the transfer of an acyl group from membrane phosphatidylcholine to the retinol [99]. LRAT works closely with an abundant RPE protein, RPE65 [100, 101] (Figs. 1, 2). Through an unresolved mechanism, RPE65 hydrolyzes and isomerizes all-*trans* retinyl esters in 11-*cis* retinol [102–104]. Mutations in both LRAT and RPE65 genes are causes of Leber congenital amaurosis [105–107] (discussed below). By binding to 11-*cis* retinol, cellular retinaldehyde binding protein (CRALBP) affects the kinetics of isomerohydrolysis [108] (Figs. 1, 2). Newly formed 11-*cis* retinol can be stored as 11-*cis* retinyl esters through LRAT esterification, which are in turn hydrolyzed by unknown 11-*cis* retinyl ester hydrolases to restore 11-*cis* retinol. RPE endoplasmic reticulum also contains an opsin-like RPE-retinal G protein receptor (RGR) [109]. Light activation of RGR photoisomerizes bound all-*trans* retinal into 11-*cis* retinal. There is also some evidence that light activation of RGR could result in mobilization of all-*trans* retinyl esters from lipid droplet storage pools towards LRAT and RPE65 in the endoplasmic reticulum, where all-*trans* retinyl esters would undergo hydrolysis to all-*trans*

retinol [110, 111]. CRALBP binds 11-*cis* retinal with greater affinity than does 11-*cis* retinol, thereby facilitating the oxidation of 11-*cis* retinol into 11-*cis* retinal by 11-*cis*-specific RDHs [112]. RDH5 is the main 11-*cis*-specific RDH of the RPE [113], but RDH11 could also contribute to this activity [93]. Both RDH5 and CRALBP cause retinal dystrophies (Fig. 1) characterized by punctate lesions; retinitis punctata albescens, Bothnia Dystrophy and Newfoundland rod cone dystrophy in the case of CRALBP, and fundus albipunctatis in the case of RDH5 [114–116]. CRALBP then translocates to the RPE apical microvilli where 11-*cis* retinal is handed off to IRBP (Fig. 2); the latter protein binds 11-*cis* retinal with greater affinity than it binds all-*trans* retinol. 11-cis retinal is subsequently delivered to apo-rhodopsin to form a regenerated rhodopsin molecule.

7.2. Cone Visual Cycle

There is another visual cycle which does not go through RPE but rather cycles retinoids through cones and Muller cells [117]. Although cones probably require chromophore synthesized by the RPE since several visual cycle genetic defects of the RPE cause cone degeneration in humans [118, 119], the need to provide rapid visual pigment regeneration in day light conditions may explain the existence of this cone visual cycle [120]. Briefly, all-*trans* retinal is reduced to all-*trans* retinol by undefined RDHs in cones. All-*trans* retinol is then taken up by Muller cells where it is directly converted to 11-*cis* retinol by an unknown isomerase [81, 121, 122], a reaction driven by 11-*cis* retinyl ester synthesis through an acyl-Coenzyme A:retinol acyl transferase activity, then hydrolyzed to 11-*cis* retinol which finally goes to cones where the RDHs oxidize it to 11-*cis* retinal.

7.3. Genetic Diseases Associated with RPE Proteins of the Visual Cycle

Impairment of some RPE genes involved in the visual cycle leads to a spectrum of conditions ranging from stationary visual defects to very severe forms of progressive retinal dystrophies. Yet, all these conditions have in common an impairment of dark adaptation experienced by patients as congenital night blindness due to the congenital metabolic defect in the RPE. In comparison, night blindness in other conditions such as retinitis pigmentosa is caused by progressive rod cell death and therefore often appears more progressively during the first decade of life, or even later. Additionally, in RPE disorders, pigment deposits in retina are scarce, even at late disease stages. This feature could reflect impaired RPE migration and proliferation, while with primary defects in PR cells the RPE could be healthier.

RPE65 and LRA—*RPE65* was the first RPE gene to be reported to be involved in human retinal dystrophy. The condition is usually severe and congenital and is described as Leber congenital amaurosis (LCA) [105], or severe early-onset retinal dystrophy [106] (Figs. 1; 3A,B). However, milder phenotypes can be encountered and have been reported as retinitis pigmentosa or retinal dystrophy [123, 124]. Accordingly, noticeable variations have been found in visual acuity and in the amount of PR loss among patients, even amongst members of the same family [125, 126]. Nonetheless, these are descriptions of the same clinical entity albeit with varying degrees of severity. The clinical presentations have been previously reported [81, 122, 126–131].

RPE65 is estimated to account for 5 to 10 % of LCA and 1–2 % of RP cases [132–134]. Many mutations have been described including amino acid changes or C-terminal truncating mutations [122, 126, 127, 129–131, 135–154]. Most of these mutations totally abrogate isomerase activity but a few are associated with residual function [104, 143, 155–160]. RPE65 patients usually present with a nystagmus in the first months of life, either pendular (large eye rolling movements) or fine [122, 129], sometimes with no eye contact. Very characteristically, the visual behavior of ffected babies improves after 6 months to one year of life [130]. They search for light (tendency to light fixation) in contrast to other genetic forms of LCA in which they are often photophobic. During the second year of life, they show an almost normal walking behavior in daylight while they cannot move in dimlight, indicating profound, congenital night blindness. Thus nystagmus, night blindness and behavioral improvements are all indicative of *RPE65* or *LRAT* mutations. Refractive error, either myopia or hyperopia, is moderate. On average, visual acuity is low at 20/200 but it can vary from hand motion to 30/60 [161]. Funduscopy shows a slightly grayish retina with moderately attenuated retinal vessels. Importantly, there are no pigment deposits or macular atrophy at this stage. Scotopic ERG responses are absent, but photopic responses can be present, although very attenuated. After age 5, additional clinical indicators can be observed. For instance, there is absent or minimal fundus autofluorescence, due to the absence of lipofuscin in the RPE (Section 9), a pathognomonic sign of RPE65 or LRAT defect [162]. OCT (optical coherence tomography) scans performed in patients as early as 6 years of age reveal an early 30–50 % loss of cones [163] that could even be congenital [164, 165], reminiscent of the early cone degeneration observed in *Rpe65*$^{-/-}$ mice [166]. There is a concomitant loss of rods, more prominently in peripheral macula and peripheral retina [167]. Affected children have color vision defects, in blue hues rather than red/green. The peripheral visual field is restricted but can be drawn. Dark adaptometry reveals an absence of dark adaptation [168], and the transient pupillary light reflex is elevated by 3 to 6 log units [169]. This reflects the virtual absence of rhodopsin in rods. This clinical presentation results from both the metabolic defect (disruption of visual cycle) and the relatively slow neuronal degeneration (loss of PRs). However, within the second decade of life, PR degeneration usually predominates. Thereafter visual acuity and peripheral visual fields decline progressively. Scattered bone spicule shaped pigment deposits appear in the peripheral retina as well as pigment mottling and atrophy of the macula [147]. Patients usually become legally blind by the third decade but light perception can persist until 50–60 years of age. Briard dogs, which carry a truncating *RPE65* mutation [170, 171], show striking similarities with the human RPE65 dystrophy [172, 173]. These dogs have congenital night blindness slowly progressing towards total blindness by the end of life. Because of slow progression of the disease in both dogs and humans, gene therapy trials using AAV2 vectors have been performed on Briard dog pups. These studies have resulted in partial restoration of visual function and long term expression of the transduced RPE65 mRNA [174–178]. More recently, human trials have lead to reports of improvement in retinal sensitivity to light; better visual restoration can be expected with treatment of young children [179–183].

In contrast to RPE65, LRAT mutations are a very rare cause of severe retinal dystrophy. To date, only 3 mutations in 7 patients have been reported [107, 137, 184–186]. The

presentation is very similar to that of RPE65, and the reported phenotype (4 out of 7 cases) is typically of hyperopic (from +4 to +9 diopters) children who are night blind from early infancy. The fundi show only minimal changes in childhood but at adulthood scattered pigment deposits are present. The electroretinogram shows no response. The peripheral visual field is reduced and visual acuity decreases progressively. In adulthood, patients become blind with retinal pigment deposits and macular involvement.

CRALBP and RDH5—To date, 11 mutations in RLBP1 (CRALBP) have been reported [114, 115, 134, 187–192] and 17 mutations in RDH5 [116, 193–203] (Fig. 1). The phenotypes of CRALBP (*RLBP1)* and RDH5 mutations are quite similar, although with some differences. For both genes, night blindness is present from the first years of life and dark adaptation thresholds are elevated [114, 204]. The presence of tiny, whitish dotlike deposits in the retinal periphery is a hallmark of these conditions (Fig. 3C–F). These lesions are readily visible in young adults and tend to progressively disappear after 50 years of age [205, 206]. In cases of *RDH5* mutations the white deposits are often radially distributed as in classic fundus albipunctatus (FA) while with *RLBP1* mutations, the distribution is somewhat irregular and is thus described as retinitis punctata albescens (RPA). Importantly, FA is considered as a stationary condition whereas RPA is a form of retinitis pigmentosa. Indeed, in RPA there is progressive thinning of retinal vessels, round patches of atrophy with few pigment deposits which appear in the retinal periphery in adults, and the ERG responses disappear rapidly, first in scotopic conditions and later in photopic conditions. In FA, retinal vessels remain normal, there are no pigment deposits and the ERG responses, although attenuated, are recordable after prolonged dark adaptation. Nevertheless, some patients with FA undergo cone dystrophy with age as reflected in decreased ERG cone responses and macular dystrophy while RPA can be slowly evolving and mild [198, 207–209]. It is therefore sometimes difficult to distinguish between these phenotypes [191, 210]. *RLBP1* mutations are also associated with phenotypes similar to RPA including Newfoundland rod-cone dystrophy in which there is no macular atrophy [188] and Bothnia dystrophy [115]. The latter disorder is largely due to a homozygous R234W mutation [211], presenting as a macular atrophy by the age of 30 and is associated with decreased visual acuity and central scotoma. In an animal model of *RDH5* mutation, one function was abnormal [212]. The biochemical nature of the dot-like deposits is not known, but since they are not autofluorescent they are not lipofuscin [213]. They could more probably be 11-*cis* retinyl ester accumulations in the RPE. Some patients were recently given algae extracts containing 9-cis beta-carotene with improvement of rod recovery rates [214].

RGR—RGR can act *in vitro* as a photoisomerase [109, 215, 216] (Fig. 2). $Rgr^{-/-}$ mice exhibit decreased regeneration of visual pigment after light exposure, but they do not undergo PR degeneration [215]. In humans, only 2 mutations in *RGR* have been reported, Ser66Arg (recessive) and a 1-bp insertion in codon Gly275 (possibly dominant) [134]. Patients have a rather severe, atrophic form of RP, in which the choroidal vasculature becomes visible, and possess sparse bone spicule-shaped pigment deposits in the mid-peripheral retina. There are no flecks. To date, there have been no other reports of RGR mutations.

STRA6 and SRBP—Less than 20 mutations have been described in STRA6 (Fig. 2), the gene encoding the receptor for serum retinol binding protein (SRBP). These mutations are responsible for developmental abnormalities referred to as Matthew-Wood, Spear, PDAC or MCOPS9 syndrome, alternative names for clinical entities characterized by microphtalmia/anophtalmia, malformative cardiac defects, pulmonary dysgenesis and diaphragmatic hernia [217, 218]. Most cases are lethal but there are rare viable forms [219]. The deficiency in SRBP is very rare and reported cases are much less severe than for STRA6 [220, 221]. Two sisters, 13- and 17-years of age, having compound heterozygous mutations in *RBP4*, presented with mild iris coloboma and moderate RPE atrophy. The patients complained of congenital night blindness (dark adaptation was severely impaired) and moderate decreases in visual acuity. ERG responses were unrecordable for low stimulation levels in scotopic conditions, and were reduced by 20 % for high stimulation levels in photopic conditions.

Other RPE Genes—Both RDH10 and RDH11 were shown to be capable of oxidizing 11-*cis* retinol to 11-*cis* retinal [222, 223]. These proteins are expressed in many tissues and have not been found to be mutated in retinal diseases [185, 224].

8. THE PHAGOCYTIC FUNCTION OF THE RPE

One of the most important functions of the RPE is to participate actively in PR membrane turnover. It has been calculated that each rod regenerates its outer segment (ROS) within 7–12 days [225]. PR turnover is composed of two phases, necessarily balanced in order to preserve cellular integrity: an anabolic aspect involving RNA transcription, protein synthesis, transport, and generation of new membranes; and a catabolic aspect consisting of removal of effete membrane, digestion and recycling of some components to the PR [226]. It is this catabolic aspect that corresponds to the process of PR phagocytosis by the RPE. In the rat each RPE cell contacts 250–300 ROS, and engulfs 20–30,000 shed distal ROS discs every day [227]. It has been calculated that in humans by the age of 80, every RPE will have ingested and processed some 100 million such discs [228]. Why do PRs undergo such rapid and complete membrane turnover? One possibility [229] is that phagocytosis has evolved as a defence against chronic oxidative stress to which PR are vulnerable because of exposure to light radiation and nearly arterial levels of oxygen, and because they contain a very high proportion of polyunsaturated fatty acids (of which the major species is DHA). Importantly, the complete replacement of the OS every 10–14 days serves to prevent accretion of the bisretinoid compounds that are the precursors of RPE lipofuscin [230] (Fig. 1; Section 9). These bisretinoid compounds are photosensitizers with the capacity to oxidatively damage protein and lipid [231]. Furthermore, PRs do not contain the widespread detoxifying enzyme glutathione reductase, which is postulated to have disappeared from these cells by selective pressure [229], so they are under constant stress from free oxygen radical attack. Membrane turnover through phagocytosis thus represents a method of continual self-repair, the removal of oxidised membrane being exactly compensated by the addition of newly synthesized cellular components which maintains the cell at a constant overall length. It has also been suggested that this process of membrane turnover is essential to the survival of both RPE and PR since from the ingested DHA, RPE synthesize Neuroprotectin 1, a bioactive substance providing protection from oxidative insult [232–234].Overall the molecular processes of phagocytosis in RPE cells are quite analogous to those involved in recognition

and elimination of apoptotic cells by circulating macrophages [235, 236]. RPE cell phagocytosis has also been the subject of a recent review [237].

8.1. Cellular and Molecular Aspects of Phagocytosis

Membrane-Bound Receptors—The early studies by Hall and colleagues suggested that PR phagocytosis involved a ligand-receptor mediated interaction, since *in vitro* analyses demonstrated that OS binding and internalisation by RPE monolayers were temperature dependent, saturable and specific [238, 239]. Different receptors were identified by different workers: a mannose-6-phosphate receptor [240], CD36 [241, 242], αVβ5 integrin [243] and a number of glycoproteins expressed on the RPE surface [244]. A real breakthrough was the identification of the c-mer (mertk) (Fig. 1 and 4) transmembrane tyrosine kinase receptor as responsible for OS internalisation, since mutations in the gene coding for this protein led to failure of phagocytosis and retinal degeneration in the Royal College of Surgeon's (RCS) rat [245]. As definitive proof of its central role, the same group showed that expression of the full length normal protein in RCS rat RPE both *in vivo* [246] and *in vitro* [247] partially restored normal phagocytosis. Furthermore, mutations in the MERTK gene have been linked to sub-groups of patients with inherited retinal degeneration [248]. Mertk is one of three members of the "TAM" family (Tyro 3, Axl, and Mer), preferentially expressed in the nervous, immune, and reproductive systems [249]. The members are characterized by a conserved sequence in the kinase domain and homology to cell adhesion molecule motifs in the extracellular domains. In addition to their roles in phagocytosis, TAM receptors are involved in immune regulation and cancer [250]. Within the RPE, Tyro 3 co-localises with mertk in apical microvilli, and is rapidly down-regulated in mertk knockout mice. However, Tyro 3 is not necessary for PR phagocytosis since retinal degeneration does not occur in the Tyro $3^{-/-}$ mouse [251]. The manner in which mertk activation participates in phagocytosis was recently clarified by Strick and Vollrath [252], showing that it associates with non-muscle myosin IIA (and thereby actin) to reorganise cell shape and form phagocytic cups. It is still not known whether this association is direct or indirect, as evidence exists in other systems for both possibilities. The importance of downstream cytoskeletal involvement for continued proteolytic digestion and degradation of ingested OS debris is underscored by myosin VIIA defects in the *shaker1* mutant mouse, an animal model of the deafblind syndrome Usher type 1B [253].

However, mertk is not the only actor involved, and extensive studies by the laboratory of Finnemann have also revealed the importance of αVβ5 integrin. Her biochemical studies have revealed that αVβ5 (Fig. 4) is involved in recognition and binding of OS packets, and that this activates downstream signalling pathways that in turn recruit mertk and set into motion the complete phagocytic process [52, 254, 255]. $\alpha V\beta 5^{-/-}$ mice do not exhibit retinal degeneration [256], in contrast to RCS rats [257] and $mertk^{-/-}$ mice [258]. However, they lack the daily morning burst in phagosome formation seen in wild type retinas (see below), and demonstrate an age-related accumulation in lipid deposits associated with a decline in visual activity [256]. An alternative pathway seems to be involved in PR phagocytosis under more pathological conditions: when PR breakdown is induced by intense light exposure, leading to generation of oxidized lipids and massive shedding of OS material, an uptake process mediated by the scavenger receptor CD36 (Fig. 4) comes into play [259]. This

receptor was initially identified in RPE by Ryeom *et al*. [241, 242], and shown to be involved in OS uptake by competition and antibody blocking experiments. A mannose receptor has also been implicated in the binding phase of phagocytosis [240, 260], although its precise role in OS uptake is less sure. Two other membrane proteins have recently been shown to be involved in PR phagocytosis: CD81, a tetraspannin which appears to form a functional complex with αVβ5 to facilitate OS binding [261]; and L type calcium channels [262] which display a bidirectional interaction with αVβ5 and whose inhibition using pharmacological blockers decreases phagocytosis in cultured RPE. The role of melatonin in phagocytosis is controversial, since data exist which argue both for and against such a property (see below). Assuming it does exert an effect, this would be mediated by one or both of the high affinity melatonin receptor (MT) (Fig. 4) subtypes present on the RPE surface [263]. Both MT1 and MT2 are members of the G protein-coupled receptor superfamily, with seven transmembrane domains [264]. Their relationship to the other receptors involved in phagocytosis is currently not known (but see below).

Potential Ligands—The TAM family members were originally classified as orphan receptors since their natural ligands were not known [249]. Using mouse Tyro 3 and mouse Axl as affinity probes, Stitt and colleagues [265] identified the closely related proteins Gas6 and Protein S (ProS) (Fig. 4) as preferred ligands for Axl and Tyro 3, respectively [250]. Hall *et al*. showed that Gas6 stimulated OS uptake by RPE in culture, and postulated that it represents the principal physiological ligand for phagocytosis [266]. In addition, using an antibody-blockade approach Karl *et al*. [262] showed that Gas6 is synthesized by RPE in culture, and that it stimulates phagocytosis under *in vitro* conditions. However, $Gas6^{-/-}$ mice do not exhibit retinal degeneration, and additional studies have demonstrated a clear role for ProS as well [251, 267]. A third soluble ligand has been identified, milk fat globule-EGF 8 (MFG-E8) (Fig. 4), which exhibits high-affinity binding for αVβ5 [268]. Both *in vivo* and *in vitro* studies have shown that MFG-E8 is necessary to initiate the integrin-dependent activation of mertk phosphorylation, and as in the $\alpha V\beta 5^{-/-}$ mouse the normal morning peak in phagocytosis is absent from MFG-E8−/− mice [269]. However, another study indicated that MFG-E8 was ineffective as a ligand [270]. In all three cases the mode of ligand-receptor interaction is suggested to be autocrine/paracrine in nature, since RPE cells express both the high-affinity membrane-bound receptors and the ligands. But the real endogenous source(s) of these ligands is uncertain, since they are widely distributed within the retina and elsewhere. Currently, how this scheme would function *in vivo* to synchronise OS shedding and phagocytosis, is not clear (see below).

Intracellular Pathways—Activation of different membrane-bound receptors triggers a variety of downstream signalling pathways, which play either direct or indirect roles in the phagocytic process (Fig. 4). It was shown many years ago that increases in intracellular cAMP levels in cultured RPE through the use of non-hydrolysable analogues reduced phagocytosis of ROS, while similar increases in cGMP had no effect [271]. Subsequent work using drugs that also act to increase cAMP levels also inhibited ROS ingestion (but not binding) [272]. Although the molecular mechanism is not clear, melatonin is suggested to play a role in the phagocytic burst observed in early morning, since sub-cutaneous implants containing melatonin increase phagosome numbers [273]. On the other hand, strains of mice

which do not synthesize melatonin in the retina continue to exhibit normal cyclic phagocytosis [274]. MT1 activation leads to decreases in cAMP through inhibition of adenylyl cyclase, while MT2 inhibits both adenylyl cyclase and the soluble guanylyl cyclase pathways [264].

Stimulation of integrin αVβ5 leads to activation of the focal adhesion kinase (FAK) (Fig. 4) pathway: ROS binding increases FAK complex formation with αVβ5 at the RPE apical surface and activates FAK. Subsequent ROS internalisation coincides with dissociation of activated FAK from αVβ5. Mutant FAK (retaining focal adhesion targeting but lacking kinase activity) competes with recruitment of full length FAK to αVβ5 and abrogates FAK activation in response to RPE phagocytic challenge. Such inhibition of FAK signaling has no effect on αVβ5-dependent binding of particles but inhibits their uptake. Expression of FAK in fibroblasts lacking FAK promotes particle engulfment. Selective ligation of αVβ5 at the apical RPE surface is sufficient to phosphorylate and mobilize FAK, and FAK signalling is independent of mertk. By contrast, inhibition of FAK signalling decreases mertk phosphorylation [275]. Hence the FAK pathway forms an essential link between ROS binding and the ensuing internalisation mechanisms of PR phagocytosis. In addition, the src-kinase pathway is stimulated by integrin activation, and it can lead to phosphorylation of both mertk and L type calcium channels [276]. Very recently, Law *et al*. [277] have shown the involvement of annexin A2 in later stages of phagocytosis, since this downstream intracellular signalling molecule is necessary for phagosome processing to proceed. These molecular details are only known with certainty for rod uptake.

8.2. Physiological and Integrated Aspects

Compared to the important advances in identification of molecular components of the phagocytic process, less progress has been made in recent years concerning the general environmental and physiological control of PR phagocytosis, and the integration of the phagocytic process within the overall retinal metabolism. In respect to environmental factors, this concerns especially the role of ambient light (and dark) as an essential trigger, but also temperature for poikylotherms.

Essentially two methodological approaches have been used to quantify phagocytosis: *in vivo* studies rely principally on histology-based analyses, using semithin or ultra-thin sections to identify phagosomes by their size and structure, eg. [278]. Since structurally identifiable phagosomes persist for several hours within the RPE cytoplasm, as a consequence positive scoring of phagocytic activity is seen for protracted periods. On the contrary, immunodetection-based methods give narrower peaks since the epitopes are degraded rapidly by proteases active within the phagosomes [279]. Immunocytochemical techniques are also mostly employed in culture studies, for their rapidity and simplicity. Irrespective of the technical approach used, rod phagocytosis exhibits a marked morning rise in activity in every species examined: eg., fish [280], frogs [281], birds [282], and mammals such as rats [278], cats [283] and monkeys [284]. In rats, this daily rhythm is established by one month of age, and is irrespective of the lighting conditions during development [285]. There is often a secondary smaller peak occurring during the night [278, 283, 286], and seems to correspond to clock activity “priming” the system to prepare for light onset and dawn.

The timing and magnitude of cone shedding are more variable, since in many species [282, 284, 287–289] there is a distinct peak temporally removed from that of rods, occurring during the early night time. It has been suggested, based on these inversed rod and cone peaks, that phagocytic activity may be linked to PR physiology, rod shedding occurring at the beginning of the light period in which rod PR are less solicited, and cone shedding taking place during their resting period (darkness) [289]. However, in several species rod and cone OS shedding occur simultaneously and maximally just after light onset [283, 290, 291]. These differences cannot easily be explained by differences in behaviour, since both groups are diurnally active, but indicate that timing of phagocytosis is not simply related to visual activity. It is noteworthy that maximal rod phagosome numbers exceed by tenfold those of cones in mammalian species in which both have been compared [283, 291]. This may reflect a different turnover rate of rods versus cones, since rod membranes may be renewed more rapidly than cones.

Responses of phagocytosis to changes in ambient lighting have been widely studied. La Vail [278] showed that a morning shedding peak was retained in rats placed in permanent darkness (DD). A subsequent study examined the effects of multiple lighting paradigms on the shedding response in albino rats [292],and obtained similar resultsn DD, persisting even after two weeks of such conditions. By contrast, the phagocytic response in *Rana pipiens* seems to be purely light driven, since it disappears in DD conditions [281]. Effects of constant light (LL) have also been investigated, demonstrating that the shedding peak is abolished rapidly [292]. In the Nile Rat, LL induced complete loss in rhythmicity of rod and cone PR phagocytosis, which instead showed a broadly uniform activity [279]. In addition cone but not rod PR phagocytosis was strongly up-regulated, perhaps linked to the constant activation of the former by ambient light levels. Indeed rod phagosome activity dropped to comparable levels to that of cones, fitting broadly with previous observations in rats [292] which concluded that prolonged maintenance in LL abolishes the burst of shedding. On the other hand, these findings contrast sharply with previous studies on nocturnal mice [293] which showed no changes. The apparent increase in cone phagosome numbers in LL may reflect either increased shedding and/or uptake, or decreased degradation.

Recent studies using mouse mutants have identified specific components of the circadian phagocytic pathway. Among the different molecules that have been identified as crucial for rod phagocytosis, the αVβ5 integrin receptor present at the RPE surface is critically involved in binding shed outer segment packets [243]. Interestingly, β5 knockout mice lack the normal morning peak but instead exhibit a uniform pattern [256]. Knockout mice further develop age-related autofluorescent inclusions within the RPE, and exhibit age-related decline in visual responses. Subsequent studies highlighted the role of MFG-E8 as a candidate PR-bound ligand for this integrin, and revealed that mice with a gene deletion for this protein also showed uniform uptake profiles, although there was no age-related accumulation of lipofuscin [269]. The same authors also identified a second distinct role for αVβ5 within the retina, in that it also mediates rhythmic variations in retinal adhesion [294]. These data suggest ligand-receptor interactions may constitute one of the retinal circadian clock “outputs” or checkpoints that control rhythmic phagocytosis (and adhesion), although the authors did not detect any rhythmic changes in either αVβ5 or MFG-E8. There are so far

no data linking specific clock genes to retinal phagocytosis, or whether similar mechanisms underlie cone phagocytosis.

8.3. When Phagocytosis Fails

The RCS rat is a commonly used animal model of inherited retinal degeneration. Known since 70 years, and well described nearly 50 years ago [295], it was long ago realised through studies using chimeric animals derived from combinations of control and affected embryos that the disease state correlated with RPE rather than neural retina [256]. Tissue culture studies demonstrated that RCS RPE were defective in OS internalisation, although they were still capable of binding normally [296, 297]. But the difficulties associated with performing rat genome searches hindered identification of the mutation responsible. This was finally achieved ten years ago, when the laboratory of Vollrath showed through positional cloning that the orphan tyrosine kinase receptor c-mer (or mertk) was a strong candidate [245]. This was proven beyond doubt when the same group showed that replacement of a normal mertk gene into RCS RPE partially rescued the disease phenotype *in vivo* [246]. Consistent with the degeneration observed in the rat and mouse [257] models, mutations in the human *MERTK* gene are responsible for a sub-group of early-onset retinitis pigmentosa characterized by severe rod-cone dystrophy [248]. Mertk mutations have also been linked with a rare retinal dystrophy [154] and severe rod-cone dystrophy [298].

9. RPE LIPOFUSCIN

The membrane renewal process engaged in by PR cells involves the adjacent cell monolayer – the RPE – in the disposal of spent OS membrane components. The consequence of the phagocytotic load born by the RPE is apparent from the abundant lipofuscin (Fig. 1) that accumulates with age in the lysosomal compartment (lipofuscin granules) of these cells [10, 299]. For most non-dividing cell types, lipofuscin probably originates internally by autophagy [300–302]. However, in the case of the RPE, these compounds also accumulate as a consequence of the cell's role in phagocytosing PR OS membrane [303, 304]. Despite speculation that RPE lipofuscin is protein-based, an amino acid analysis of purified lipofuscin granules, revealed only 2% amino acid content while parallel HPLC analysis demonstrated the presence of previously identified bisretinoid compounds originating from PR cells [305]. Several bisretinoids of RPE lipofuscin have been identified; these compounds include the founding members of 3 families (A2E, A2-DHP-PE and all-*trans*-retinal dimer) various isomers and photo-oxidized forms and conjugated and unconjugated members in the case of the all-*trans*-retinal dimer series [230, 306–312].

It has been shown by a number of studies that the bisretinoid lipofuscin of RPE exerts adverse effects on RPE, acting both as a photosensitizer to damage cellular organelles [313, 314] and exhibiting detergent properties which destroy membranes [315]. *In vivo*, the regional lipofuscin content correlates directly with adjacent PR death [316].

9.1. RPE Lipofuscin in Retinal Disorders and Disease Models

The lipofuscin that is amassed by RPE cells is the source of fundus autofluorescence, the natural autofluorescence [317] that is imaged clinically by confocal scanning laser

ophthalmoscopy (cSLO) [77] and fundus camera-based system [318]. Given that the autofluorescent pigments of RPE lipofuscin are produced by reactions of all-*trans*-retinaldehyde it is not surprising that when the 11-*cis*- and all-*trans*-retinal chromophores fail to be generated *via* the visual cycle, as in early-onset retinal dystrophy associated with mutations in RPE65, fundus autofluorescence is not present [162].

While the accumulation of lipofuscin pigments is characteristic of normal RPE, this autofluorescent material is amassed in particular abundance in retinal disorders associated with mutations in the *ABCA4* gene (Fig. 1) including recessive Stargardt macular degeneration, recessive cone-rod dystrophy and recessive retinitis pigmentosa [319, 320] (Section 7.1). Individuals heterozygous for some disease-causing mutations in ABCA4 may also exhibit increased susceptibility to AMD [321]. The link between excessive bisretinoid formation and PR cell death is demonstrated by the finding that PR cell death is progressive in the *Abca4*$^{-/-}$ mouse, a model characterized by prodigious accumulation of the bisretinoids of lipofuscin [89, 311, 312, 322]. Therapeutic strategies aimed at alleviating vision loss in ABCA4-diseased have been shown to suppress the formation of the bisretinoids in *Abca4*$^{-/-}$mice. These approaches include ABCA4 gene replacement [323, 324] and systemic administration of compounds that limit the visual cycle [325–329].

There is also considerable interest in the role of RPE lipofuscin in age-related macular degeneration. Complement activation that is insufficiently regulated is considered to underlie the susceptibility to age-related macular degeneration that occurs with certain genetic variants in complement factors [35–38] (Section 4). In this regard it is notable that photooxidation products of A2E and all-*trans*-retinal dimer can activate complement while depletion of factor B reduces complement activation as does an inhibitor of C3 [330, 331]. Complement activation triggered by photooxidation products of RPE bisretinoid lipofuscin could generate chronic inflammatory processes that gradually predispose the macula to disease.

In mutant mice lacking the retinol dehydrogenase Rdh8, clearance of all-trans-retinal from OS following photobleaching, is significantly delayed and the mice exhibit several fold increases in A2E relative to wild-type mice [325]. Another retinol dehydrogenase RDH12 is expressed in inner segments of PR where it may be involving in the reduction of not only all-*trans*-retinal but other damaging compounds [332, 333]; a null mutation in this gene also results in elevated levels of A2E [334]. In humans, mutations in RDH12, cause a severe form of autosomal recessive retinal degeneration characterized by early-onset and progressive rod and cone degeneration [335, 336].

Another juvenile-onset form of maculopathy with dominant inheritance is associated with enhanced RPE lipofuscin accumulation [337–339]. This retinal disorder occurs due to mutations in *ELOVL4* (elongation of very long fatty acids-4) and is commonly referred to as dominant Stargardt-like macular degeneration (STGD3) (Fig. 1). The ELOVL4 protein serves to synthesize C28 and C30 saturated fatty acids and in the synthesis of C28-C38 very long chain polyunsaturated fatty acids, the latter being abundant in retina [340]. The link between ELOVL4-mediated fatty acid production and bisretinoid formation in PR cells is not completely understood.

10. RPE AND PROLIFERATIVE DISEASE

In the healthy developed eye, RPE do not undergo mitosis; however, under some conditions, including complicated cases of retinal detachment [341] and retinitis pigmentosa [342], RPE cells both proliferate and migrate. When the the neural retina detaches from RPE, the latter cells can be dispersed into the vitreous cavity where they undergo a epithelium-to-mesenchyme transition to myofibroblasts that proliferate on the surface of the retina and *via* their contractile properties can re-detach a repaired retinal detachment [341, 343]. Known as proliferative vitreoretinopathy, this condition is a result of the phenotypic regression of the cells that follows from a loss of junctional complexes [45].

11. CONCLUSIONS AND PERSPECTIVES

Mutations in several genes expressed in RPE lead to retinal degeneration. It is thus significant that RPE cells are also an attractive target for gene therapy in part because the cells are readily accessible. Another rapidly growing area of research involves cell-based therapies aimed at the replacement of defective RPE. Success in this area could be curative for conditions such as AMD. RPE cell proliferation in the form of PVR is the major cause of failure of retinal detachment surgery and yet this condition remains difficult to treat. Improved understanding of RPE biology may be key here.

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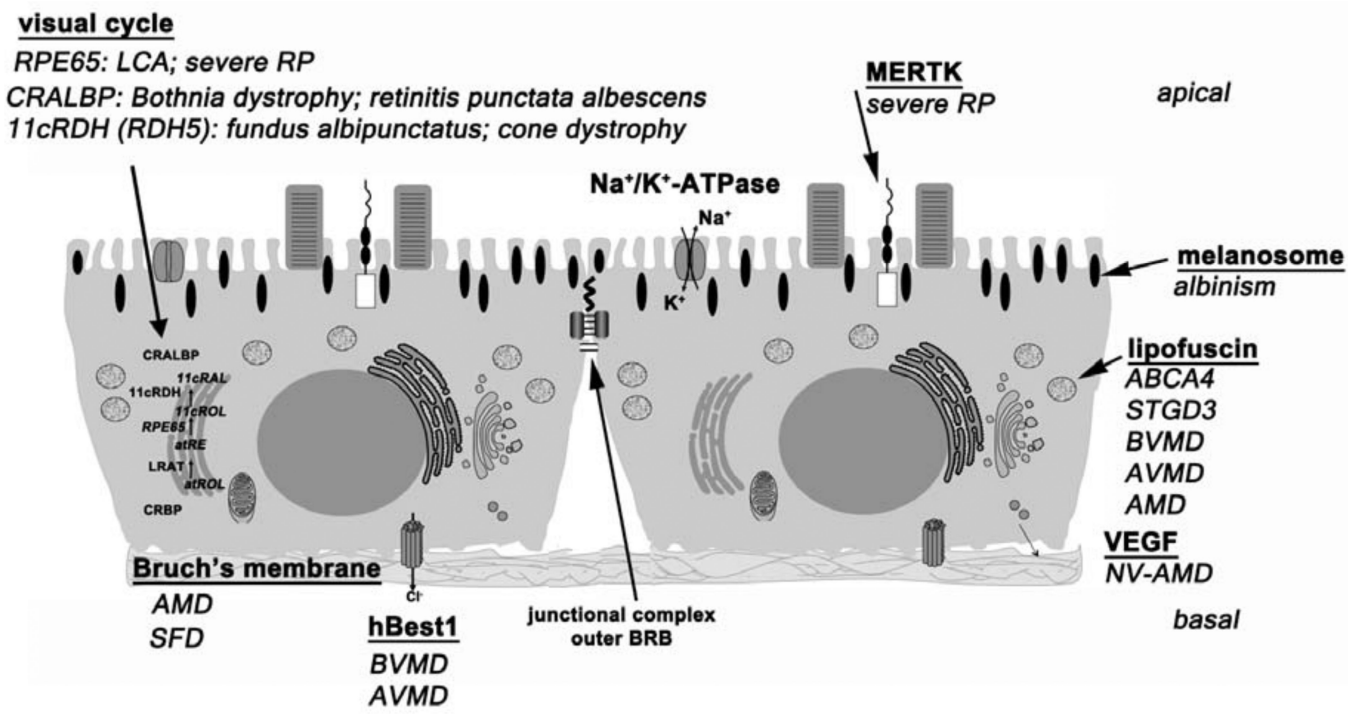

Fig. (1). Schematic depiction of RPE cell structure / functions with associated retinal disorders

Cellular retinol binding protein (CRBP) binds all-trans-retinol (atROL) and directs it the smooth endoplasmic reticulum where lecithin:retinol acyltransferase (LRAT) converts all-*trans*-retinol to all-*trans*-retinyl ester which is then converted to 11-*cis*-retinol (11cROL) by RPE65 and to 11-cis retinal (11cRAL) by 11-cis-retinol dehydrogenase (11cRDH). Visual cycle proteins associated with forms of retinal degeneration include RPE65 (Leber congenital amaurosis, LCA; severe retinitis pigmentosa, RP); CRALBP and 11cRDH (encoded by gene retinol dehydrogenase 5, RDH5). The sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) mediates outflux of sodium (Na^+) and influx of potassium (K^+). The c-mer tyrosine kinase (MERTK) receptor is responsible for internalization of shed out segment membrane. Mutations in MERTK are associated with severe retinitis pigmentosa (RP). Melanin pigment is contained within melanosomes; disruptions in melanin or melanosome biogenesis leads to albinism. Lipofuscin accumulates with excess in ABCA4-related disease, Stargardt-like macular dystrophy (STGD3), Best vitelliform macular dystrophy (BVMD) and adult-onset vitelliform macular dystrophy (AVMD); lipofuscin is also implicated in the pathogenesis of AMD. RPE cell secretion of vascular endothelial growth factor (VEGF) is implicated in neovascular age-related macular degeneration (NV-AMD). Junctional complexes between adjacent RPE cells constitute the outer blood-retinal barrier. Mutations in the protein bestrophin (hBEST1) are associated with Best vitelliform macular dystrophy (BVMD) and adult-onset vitelliform macular dystrophy (AVMD). The basal surface of the RPE rests on a basement membrane - Bruch's membrane - that is implicated in age-related macular degeneration (AMD) and Sorby's fundus dystrophy (SFD). See text for details.

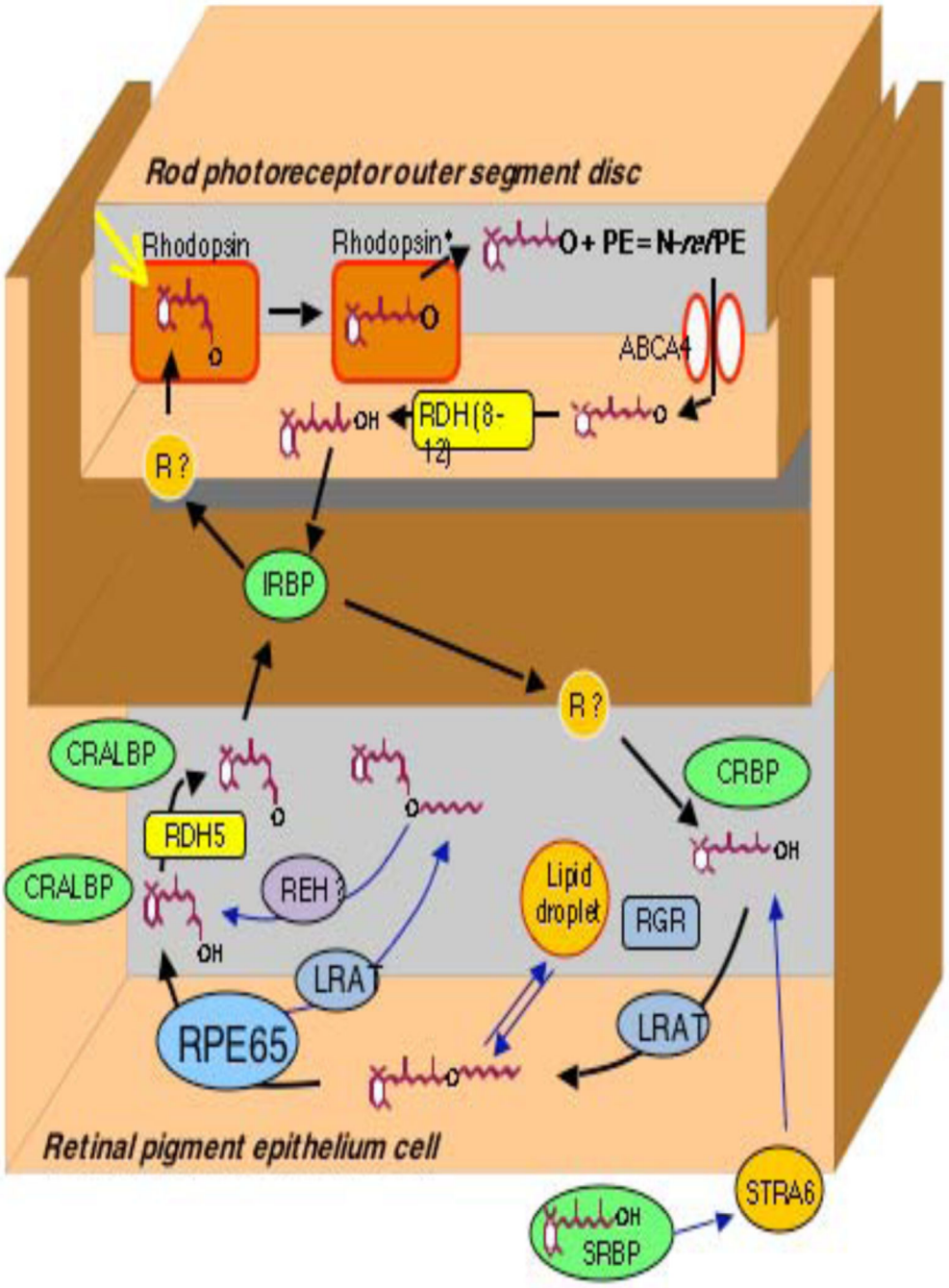

Fig. (2). The visual cycle

Schematic of the visual cycle in rod photoreceptor outer segments and retinal pigment epithelium. Binding proteins are in green (interphotoreceptor retinol binding protein, IRBP; cellular retinol binding protein, CRBP; cellular retinaldehyde binding protein, CRALBP; serum retinol binding protein, SRBP); retinol dehydrogenases (RDH) in yellow (11-cis retinol dehydrogenase, RDH5); retinylester hydrolase (REH) in purple and other enzymes in blue (lecithin:retinol acyltransferase, LRAT; retinal G protein receptor, RGR; RPE65;

membrane receptors (R) are brown (stimulated by retinoic acid gene 6, STRA6). N-retinylidene-phosphatidylethanolamine, N-*ret*-PE. See text for details.

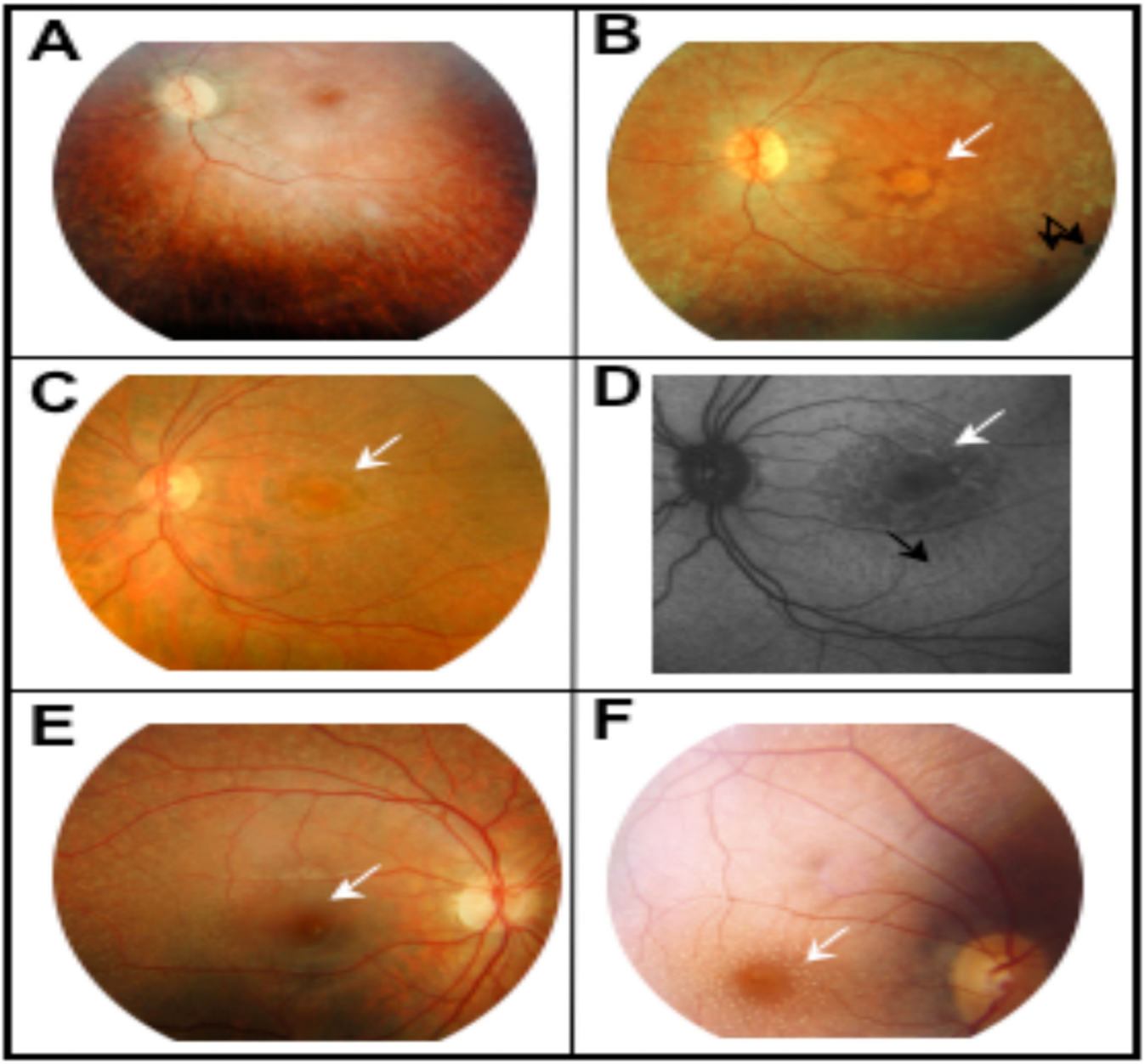

Fig. (3). Fundi of patients with various conditions related to cycle visual genes

A, B: Left eye of a patient with Leber congenital amaurosis caused by mutations in *RPE65* at 13 (**A**) and 22 (**B**) years of age. Note that fundus looks quite normal at 13 except for slight narrowing of retinal vessels and loss of the normal retinal reflex, while at 22 macular mottling (white arrow) and a few pigment deposits in periphery (black arrows) are present. **C, D**: Left eye of a 58 year-old female with fundus albipunctatus caused by mutations in *RDH5*. On fundus (**C**), note the presence of radially distributed, evenly shaped, dot-like white deposits that are characteristic of the disease, and macular pigment mottling (white arrow) which indicates macular involvement; on autofluorescence test (**D**), there is perifoveal hypofluorescence (white arrow) and the dot-like deposits which are not fluorescent appear as dark small spots (black arrow). **E, F**: Right eye of 13 year-old (**E**) and

24 year-old (**F**) males with retinitis punctata albescens caused by mutations in *RLBP1*. Note the presence of unevenly shaped and distributed, dot-like white deposits while there is normal macular reflex (white arrows).

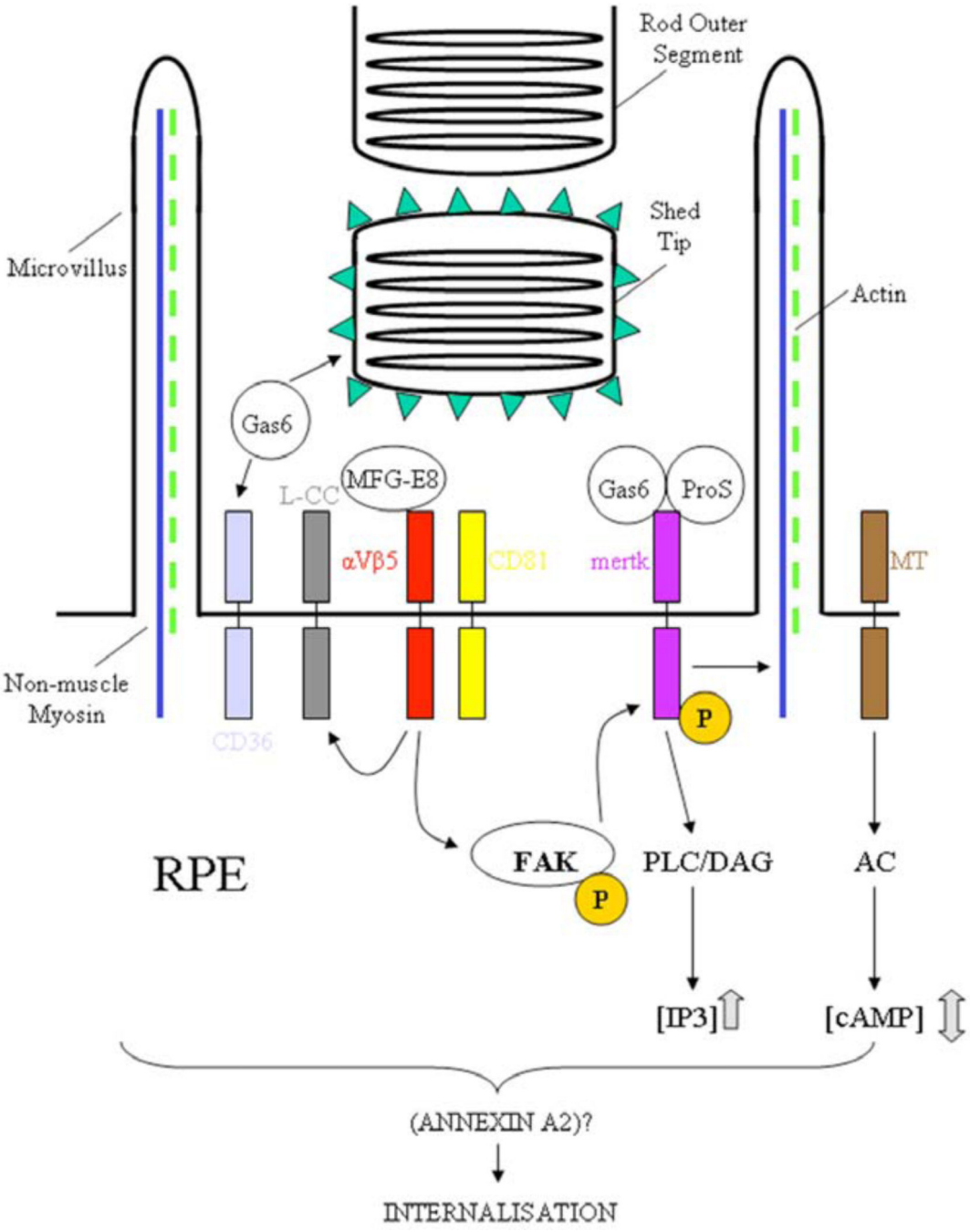

Fig. (4). RPE phagocytosis

Schematic flow chart depicting the ligands (Gas6; protein S, ProS; milk fat globule-EGF 8, MFG-E8), receptors (c-mer tyrosine kinase, mertk; αVβ5; CD36; CD81; melatonin receptor, MT; and L-type calcium channels, L-CC) and intracellular messenger elements (adenylyl cyclase, AC; focal adhesion kinase, FAK; phospholipase C, PLC; diacylglycerol, DAG; inositol trisphosphate, IP3). P, phosphorylated sites; triangles on shed ROS surface represent phosphatidyl serine.