Published in final edited form as:
Exp Eye Res. 2014 September ; 0: 51–60. doi:10.1016/j.exer.2014.01.010.

Understanding photoreceptor outer segment phagocytosis: Use and utility of RPE cells in culture

Francesca Mazzoni, **Hussein Safa**, and **Silvia C. Finnemann***
Department of Biological Sciences, Center for Cancer, Genetic Diseases, and Gene Regulation, Fordham University, Bronx, NY 10458, USA

Abstract

RPE cells are the most actively phagocytic cells in the human body. In the eye, RPE cells face rod and cone photoreceptor outer segments at all times but contribute to shedding and clearance phagocytosis of distal outer segment tips only once a day. Analysis of RPE phagocytosis *in situ* has succeeded in identifying key players of the RPE phagocytic mechanism. Phagocytic processes comprise three distinct phases, recognition/binding, internalization, and digestion, each of which is regulated separately by phagocytes. Studies of phagocytosis by RPE cells in culture allow specifically analyzing and manipulating these distinct phases to identify their molecular mechanisms. Here, we compare similarities and differences of primary, immortalized, and stem cell-derived RPE cells in culture to RPE cells *in situ* with respect to phagocytic function. We discuss in particular potential pitfalls of RPE cell culture phagocytosis assays. Finally, we point out considerations for phagocytosis assay development for future studies.

I Insight from investigating outer segment fragment phagocytosis by the RPE *in situ*

I. 1. RPE phagocytosis of shed outer segment fragments is essential for lifelong maintenance of photoreceptors and thus vision

RPE cells perform numerous processes to maintain and support photoreceptors (reviewed by (Strauss, 2005)). Among these, their contribution to the continuous renewal of the light-sensitive outer segment portions of photoreceptors is critical for vision (reviewed by (Ruggiero and Finnemann, 2014b)).

The outer segment of both rods and cones is a dynamic structure that undergoes constant renewal (Young, 1967). Photoreceptors synthesize new outer segment components at a very high rate and form new outer segment disks thereby gradually elongating outer segments. A process commonly termed disk shedding compensates for this addition during which RPE

*Please address correspondence to: Silvia C. Finnemann PhD Larkin Hall 441 East Fordham Road Bronx, NY10458, USA Phone: 001-718-817-3630 Fax: 001-718-817-3514 finnemann@fordham.edu.

cells and photoreceptors collaborate to remove the most distal tip of rod and cone outer segments (Young, 1967; Young and Bok, 1969)). RPE cells contribute to disk shedding and are responsible for the prompt engulfment and degradation of shed tips by phagocytosis. In all vertebrate species tested, photoreceptor outer segment shedding and phagocytosis follow a diurnal rhythm (reviewed by (Ruggiero and Finnemann, 2014a)). In mammals, these processes are controlled by both changes in light conditions and by the circadian rhythm (Goldman et al., 1980; LaVail, 1980). Rod outer segment tip clearance occurs at light onset (Besharse et al., 1977; Fisher et al., 1983; LaVail, 1976), while the timing of cone outer segment clearance varies by species (Bobu and Hicks, 2009; Immel and Fisher, 1985; O'Day and Young, 1978; Young, 1977, 1978). Given that RPE cells are post-mitotic in the mammalian eye and that each RPE cell serves numerous photoreceptors, RPE cells phagocytose more material over a lifetime than any other cell type in the body. Phagocytosis by RPE cells in culture is highly regulated and specific for spent outer segment fragments (Mayerson and Hall, 1986). Altogether, phagocytic activity is a core trait of RPE cells *in vivo* and in culture.

I. 2. *In situ* analysis of RPE phagosomes has identified essential proteins of the RPE phagocytic machinery

The strict rhythm and thus synchronicity of outer segment renewal in the mammalian eye offers the unique opportunity to quantify RPE phagocytosis *in situ* in experimental animals. Shedding and phagocytosis of rod outer segments peak at light onset in mice and rats entrained to a 12-hour light 12-hour dark light cycle (LD) (LaVail, 1976). Quantification of phagosome inclusions containing rod outer segment components in the RPE of animals sacrificed at different times in relation to light onset thus allows precise quantification of RPE phagocytosis. Comparing phagosome load between experimental animals that differ genetically, by age, or by experimental treatment but that were sacrificed at the same time of day allows comparing the phagocytic activity of the RPE dependent on genotype. Comparing RPE phagosome content between animals of the same genotype but sacrificed at different times in relation to light onset allows identifying the timing and capacity of RPE phagocytic in a given experimental strain. For instance, increase in rhodopsin-positive phagosomes from 1 hour prior to 1 hour after light onset is indicative of a synchronized phagocytic burst (Nandrot et al., 2007). Decrease of rhodopsin-positive phagosomes from 1 hour after light onset to 4 hours after light onset is indicative of efficient phagolysosomal digestion (Damek-Poprawa et al., 2009).

Methods for *in situ* phagosome quantification—Phagosomes in the RPE may be identified by light microscopy based on their size and position in the RPE. Phagosomes appear as pale violet inclusions of about 1 μm diameter in the RPE in toluidine blue-stained cross-sections of paraffin-embedded eyecups (Gibbs et al., 2003). Analysis of albino experimental animals greatly facilitates accurate quantification of toluidine blue-stained phagosomes as RPE melanosomes may in part obscure phagosomes in pigmented animals (Gibbs et al., 2003). Electron microscopy allows identifying phagosomes based on their size, location, and content of outer segment disks regardless of RPE pigmentation (Bosch et al., 1993; Damek-Poprawa et al., 2009; Nandrot et al., 2004). Fluorescence microscopy allows unequivocal identification of immunolabeled phagosomes in cross sections (Nandrot et al.,

2007). Immunolabeling any outer segment protein may aid in identification of RPE phagosomes but we suggest that rhodopsin protein is an ideal phagosome marker for three reasons: (i) Rhodopsin is by far the most abundant protein in rod outer segments; (ii) a number of well characterized monoclonal antibodies specific to rhodopsin are commercially available; (iii) RPE cells do not express rhodopsin themselves. Rhodopsin content of the RPE therefore will correlate directly with relative phagocytic load. We recently published a very detailed description of a protocol for quantification of rhodopsin-labeled RPE phagosomes, which has proven highly reliable in our laboratory (Sethna and Finnemann, 2013). Finally, rhodopsin antibody-labeled phagosomes can also be examined in whole mount tissue preparations (Law et al., 2009).

The molecular mechanism of RPE phagocytosis—Using the above approaches, studies over the past 15 years have quantified RPE phagocytosis *in situ* in mutant mice lacking candidate genes/proteins. These studies demonstrated key roles for two receptor ligand pairs, the receptor tyrosine kinase MerTK and its secreted ligands Gas6 and protein S, and the integrin receptor αvβ5 and its secreted ligand MFG-E8. Rat RPE cells lacking MerTK or both of its ligands Gas6 and Protein S do not engulf shed outer segments (Feng et al., 2002; Mullen and LaVail, 1976;Burstyn-Cohen et al., 2012). Mice lacking αvβ5 integrin or its ligand MFG-E8 fail to increase RPE phagocytosis after light onset but maintain a low level of RPE phagocytosis at all times of day (Nandrot et al., 2004;Nandrot et al., 2007). RPE cells lacking $\alpha v\beta$5 integrin or MFG-E8 also show profound abnormalities in cytosolic phagocytic signaling such as lack of activation of focal adhesion kinase (FAK) and MerTK. Activation of Rac1 GTPase, a potent F-actin regulator, is also defective in mice lacking αvβ5 integrin receptors (Mao and Finnemann, 2012). The secreted ligand proteins relevant for RPE phagocytosis share a phosphatidylserine-binding domain. Rods expose this conserved “eat me” signal specifically at their distal tip and at light onset. Notably, phosphatidylserine exposure is not rhythmic in mice lacking the diurnal rhythm of RPE phagocytosis due to lack of αvβ5 integrin or MFG-E8 suggesting that photoreceptor outer segment exposure of eat-me signals is linked to RPE phagocytic activity (Ruggiero et al., 2012).

In addition to the essential two ligand-receptor pairs, the roles of three cytoplasmic proteins in RPE phagocytosis have been assessed exploring mutant mouse strains. These studies also quantified phagosome load of the RPE at different times in relation to light onset using similar phagosome counting approaches. In mice lacking myosin VIIa, an actin motor protein, RPE cells show a normal peak in the phagocytic process after light onset. However, engulfed phagosomes remain abnormally localized in the apical region of cells while they traffic swiftly to the basal region of RPE cells in wild type RPE (Gibbs et al., 2003). This suggests a delay in phagosome trafficking. In mice lacking annexin A2, another cytoplasmic actin-associated protein, RPE cells also show normal peak engulfment but a modest shift in phagosome localization towards the apical region of the cell at early times after engulfment (Law et al., 2009). In mice lacking the lysosomal protein melanoregulin (MREG), in contrast, phagosome numbers after light onset and trafficking in the RPE are normal. However, phagosome numbers do not decline as in wild-type mice at later time points

indicating that phagolysosomal digestion of rhodopsin phagosome content is markedly delayed (Damek-Poprawa et al., 2009).

Defects in RPE phagocytosis impair retinal function—In addition to discovering a contribution of individual genes/proteins to the phagocytic pathway, the *in vivo* studies have also illustrated the importance of efficient, synchronized RPE phagocytosis for retinal health and function. Deficiency in MerTK or its ligands causes rapid and complete retinal degeneration in the RCS rat and in mutant mice (Bok and Hall, 1971; Burstyn-Cohen et al., 2012; Duncan et al., 2003). Moreover, mutations in MerTK cause retinitis pigmentosa in human patients (Gal et al., 2000; McHenry et al., 2004; Ostergaard et al., 2011). Lack of the synchronizing receptor α v β5 integrin causes age-related accumulation of outer segment remnants and oxidative protein modification in the RPE (Nandrot et al., 2004). Ultimately, the RPE's F-actin cytoskeleton is significantly destabilized, and photoreceptors no longer respond to light resulting in blindness (Yu et al., 2012). Even at 12 months of age, mice lacking myosin VIIa or annexin A2 show normal retinal histology and only very slight aberration in retinal function due to myosin VIIa loss in photoreceptors suggesting that the subtle defects in RPE phagosome transportation does not cause pathology during the relatively short lifespan of the mouse (Colella et al., 2013). However, myosin VIIa mutation in human patients causes a form of Usher syndrome, a severe deafness-blindness disorder (Millan et al., 2011). Also of note, annexin A2 expression has been reported to increase with aging in the RPE/choroid and to accumulate in drusen in the aging human eye (Crabb et al., 2002; Ida et al., 2003). Thus, lack of obvious retinal pathology in model mice in response to mutation or loss of genes encoding aspects of the RPE phagocytic pathway should not be interpreted as irrelevance for the phagocytic clearance pathway.

II Insight from investigating outer segment fragment phagocytosis by RPE cells in culture

Studying RPE phagocytosis *in situ* continues to be a powerful methodology to discover new molecular components of the RPE phagocytosis pathway and their functional crosstalk. However, it is challenging to dissect the distinct steps of the phagocytic process in animal models. Furthermore, defects in RPE phagocytosis detected *in situ* may be secondary effects of changes in photoreceptor outer segment composition, dynamics, or changes in photoreceptor-RPE interaction. Studies of RPE phagocytosis in a controlled *in vitro* model system are thus a necessary complement to *in vivo* studies of RPE phagocytosis. Only they can assign specific functions in the RPE's phagocytic pathway to candidate molecules.

II. 1. Different types of RPE cells in culture are avid professional phagocytes

Since the 1970s, scientists have dissected RPE cells from eyes of experimental animals or from human cadaver eyes to test their survival, phenotype, and function after re-attachment and proliferation to some extent in cell culture (Albert et al., 1972; Mannagh et al., 1973). To date, numerous RPE cell culture protocols have been developed independently of each other and are in use in many laboratories worldwide to assess a variety of the RPE's functions including but certainly not limited to phagocytosis. Of particular note, the Williams laboratory has published detailed protocols of the methodologies they developed to

prepare polarized mouse primary RPE and to analyze RPE phagocytic capacity and mechanism (Gibbs et al., 2010; Gibbs et al., 2003; Gibbs and Williams, 2003). We have recently published an in depth description of the POS phagocytosis assays and quantification methods routinely performed in our laboratory (Mao and Finnemann, 2013). Here, we will distinguish three different types of RPE cell cultures: 1) primary cultures of RPE that are either unpassaged or used at early passage and that are not immortalized; 2) immortalized cell lines, some of which are available from cell depositories such as ATCC or from commercial sources; and 3) human stem cell-derived RPE cells including embryonic or adult stem cells and induced pluripotent stem cells.

In general, these three types of RPE cell populations in culture retain phagocytic activity toward isolated photoreceptor outer segment fragments (POS). In our opinion, no model is clearly superior to all others for phagocytosis studies. However, different cell models differ greatly in efficiency and speed of phagocytosis (Finnemann et al., 1997; Mao and Finnemann, 2012, 2013). Furthermore, RPE cells in culture differ in gene expression and hence functionality depending on cell passage and degree of epithelial polarization (Singh et al., 2013a; Strunnikova et al., 2010). Additionally, environmental factors including type of tissue culture plastics used for plating, media composition, and frequency of medium addition will affect cell physiology and cell function (Ahmado et al., 2011; Carr et al., 2011; Luo et al., 2006). Therefore, it is very important during experimental design to establish and consider the specific characteristics of the cell model to be used and to carefully optimize conditions. Conditions will always differ somewhat between researchers/laboratories. We do not suggest that a single best model and protocol could or even should be agreed upon and used among different laboratories. However, we wish to advocate keeping variations in materials, protocols and procedures to a minimum within each study. This will greatly increase reproducibility of series of experiments and thus strengthen study outcome.

Primary cultures of rat or mouse RPE cells—Primary RPE cells from mouse or rat have been shown to retain avid phagocytic activity toward isolated POS particles with fast kinetics of binding, engulfment, and digestion, similar to the prompt uptake of shed photoreceptor outer segment tips by RPE cells in the eye. Rat primary RPE cells have been especially well characterized and they bind POS within 30 minutes, engulf within 2 hours, and digest within 6 to 8 hours after an initial POS challenge (Mao and Finnemann, 2013; Mayerson and Hall, 1986). In our experience, generating polarized primary RPE cells is best achieved by plating the cells at high density from the beginning. Figure 1 illustrates that low initial cell density can compromise the final maturation of the polarized RPE monolayer. Here, we purified wild-type mouse RPE before plating cells at normal density (purified RPE of 2 mouse pup eyes per well) or 8-fold diluted. After 6 days in culture, cells plated at the higher density have attached and spread to form a confluent monolayer that expresses critical RPE and phagocytic proteins (Fig. 1, A and B). Even after having been plated in diluted format, cells have reached confluence within 6 days and they express the same proteins albeit at lower abundance (Fig. 1, A and D). However, their shape is irregular and ZO-1 does not assemble into continuous lateral tight junctions (Fig. 1 D). Hexagonal cells originally plated at higher density possess well-organized cortical F-actin while cells plated a low density retain abundant stress fibers (Fig. 1, C and E). Quantifying RPE phagocytosis

by cells in such dedifferentiated state is questionable, as these differences in F-actin would be expected to affect availability of actin for phagocytic cup formation. Furthermore, the cells may have lost expression or assembly of proteins of the phagocytic pathway as well.

In our hands, unpassaged mouse and rat primary RPE seeded at high density as patches of cells on glass coverslips and maintained in culture between 4 and 8 days after purification show negligible cell-to-cell variability with regard to expression of αvβ5 integrin POS binding receptors or MerTK engulfment receptors (Finnemann, 2003a; Mallavarapu and Finnemann, 2010; Nandrot et al., 2004). In these primary cultures, only very limited proliferation takes place after isolation at borders of cell patches isolated from eyes and plated as groups of cells. These cultures exhibit very high phagocytic activity toward POS with most cells in the culture taking up particles (Figure 2, A and B) and phagocytosis of 5 POS on average per cell within 30 min (Nandrot et al., 2007). Due to their post-mitotic nature, RPE cells isolated from adult mice or rats tend to proliferate slowly and often fail to form functional monolayers. We caution against studying single, spread RPE cells growing without cell-cell contacts a these cells do not mimic well differentiated RPE in the eye and their gene and protein expression may lack RPE and phagocyte characteristics. On the other hand, mouse and rat RPE cells lack phagocytic ability at birth (Philp and Bernstein, 1981). During postnatal retinal maturation, the RPE extends long, apical microvilli to ensheath developing photoreceptor outer segments. We detect apical αvβ5 integrin *in vivo* in rat RPE by postnatal day (PN) 5 (Finnemann et al., 1997). Daily POS shedding and RPE phagocytosis commence at around PN 12 in the rat eye (Ratto et al., 1991). We find that RPE cells isolated from eyes of rats and mice at PN 8 to 12 reliably provide high quality phagocytic cells. These exhibit pronounced pigmentation, polarity, cell-cell junctions including tight junctions and they assemble a phagocytic machinery including αvβ5 integrin and MerTK at their apical surface (Nandrot et al., 2004). Cell yield from these juvenile rodent eyes is low such that a single rat eye or 2 mouse eyes provide only enough RPE cells to be seeded into a single well of a 96-well plate. However, such unpassaged rat and mouse primary RPE cells can be isolated from mutant mice or rats allowing investigators to directly determine how specific molecular defects alter RPE phagocytosis.

Primary cultures of human RPE cells—Over the past ten years, detailed protocols have been developed and published to generate populations of primary human fetal- and adult donor-derived RPE cells in culture that very well retain appearance and marker expression of human RPE in the eye (Hu and Bok, 2001, 2010; Maminishkis et al., 2006; Sonoda et al., 2009). These cells are generally allowed to proliferate in culture and are thus passaged primary cells (non-transformed cell strains). These RPE cells have been demonstrated to phagocytose POS over a period of several hours and are thus significantly slower than unpassaged primary rat or mouse RPE (Liao et al., 2010; Westenskow et al., 2012). They generally require careful control of culture conditions to achieve re-polarization in culture, which occurs only over a relatively long period (often several weeks) in culture. The complexity of cell culture protocols may be the reason that few studies have been published exploring human RPE cells for mechanistic studies of the phagocytic pathway. We anticipate that this will change in the near future. Furthermore, our knowledge of genetic factors playing a role in human eye diseases is rapidly advancing. RPE cells may thus

increasingly be derived from human patients for study of specific diseases and specific haplotypes (Hu and Bok, 2013). Studies exploring such donor materials mimicking aspects of RPE-based diseases *in vitro* have enormous potential in contributing to our understanding of how defined genotypes alter RPE functionality and specifically phagocytosis.

RPE cell lines—Over the past 20 years, numerous RPE cells lines have been generated and reported to retain some aspects of RPE functionality. Among RPE cell lines, we will briefly discuss human ARPE-19 cells and rat RPE-J cells because both have been used in many published RPE phagocytosis studies and both are readily available from the ATCC depository. They both phagocytose POS but at a much slower rate compared to unpassaged primary rat or mouse RPE cells (Finnemann et al., 1997; Finnemann and Rodriguez-Boulan, 1999). These cells will bind POS within 1 to 2 hours, engulf POS within 4 to 6 hours, and digest engulfed components within 16 to 20 hours following initial POS challenge (Finnemann et al., 2002; Lakkaraju et al., 2007).

ARPE-19 cells arose spontaneously from RPE of a 19-year-old human donor with no known eye disease (Dunn et al., 1996). ARPE-19 cells can be propagated in culture over extended periods of time but they are not transformed and thus may become senescent (Dunn et al., 1996). ARPE-19 cells readily proliferate to confluence and they exhibit contact inhibition (Dunn et al., 1996). They may develop morphological and some functional polarity after at least 4 weeks in culture but this is dependent on the cell culture medium used (Dunn et al., 1996). Cells are highly variable with respect to shape and protein expression depending on culture conditions and even within the same population (Ahmado et al., 2011; Luo et al., 2006; Pasovic et al., 2013; Turowski et al., 2004). However, ARPE-19 cells phagocytose isolated POS prepared from cow or pig eyes (Finnemann et al., 1997). ARPE-19 cells have been demonstrated to use the MFG-E8-αvβ5-integrin-FAK mechanism to recognize and bind POS (Chowers et al., 2004; Finnemann et al., 1997; Olchawa et al., 2013; Qin and Rodrigues, 2012). They assemble annexin A2 beneath bound POS (Law et al., 2009). They engulf bound POS but there has been some controversy as to whether ARPE-19 cells express and utilize the engulfment receptor MerTK, as MerTK may be undetectable in these cells under some culture conditions (Carr et al., 2009a). This illustrates the enormous importance of specific and constant cell culture conditions for phagocytosis experiments. It is essential to ensure at the onset of any phagocytosis study that the model RPE cells to be used possess at their phagocytic surface the molecular machinery of αvβ5 integrin and MerTK that mediates RPE phagocytosis *in vivo*.

RPE-J cells are a clonal derivative of Long Evans rat primary RPE immortalized by infection with a temperature-sensitive SV40 virus (Nabi et al., 1993). As a result, RPE-J cells will continue to proliferate only if kept at 32°C and they will terminally differentiate and exhibit contact inhibition if shifted to 39°C for two days in the presence of retinoic acid. Like ARPE-19 cells, RPE-J cells readily phagocytose POS (Finnemann et al., 1997). Efficient phagocytic activity by RPE-J cells requires cell confluence but it does not require temperature shift and terminal differentiation (Finnemann and Rodriguez-Boulan, 1999). Notably, RPE-J cells may change with respect to their proliferative characteristics, phenotype and phagocytic function at extended passages after thaw. Furthermore, RPE-J cells may decrease phagocytic function if plated at low density after passaging. Based on our

extensive experience using RPE-J cells we recommend diluting the cells four-fold in each passage and passaging every seven days exactly, and using cells only up to approximately passage 10 after thaw for phagocytosis experiments. If maintained under these conditions, RPE-J cells reproducibly polarize on glass or transwell filter support to the extent that most if not all cells assemble an active phagocytic machinery for POS at their apical surface. There is little heterogeneity between cells with respect to surface receptor expression although cells will vary in shape. The majority of cells in a given sample phagocytose POS (Fig. 2 C) and these cells use the MFG-E8-α v β5 integrin-FAK-MerTK/Rac1 pathway to bind and engulf POS. More recently, we found that RPE-J cells also recruit myosin II and annexin A2 to the site of phagocytosis (Bulloj et al., 2013). Altogether, all published evidence to date indicates that RPE-J cells employ the same set of molecules to phagocytose POS that have been demonstrated to mediate phagocytosis by the RPE in the eye. Finally, using rat rather than human RPE-derived model cells is a clear advantage for studies that also explore mouse or rat experimental animals because one can use species-specific reagents such as antibodies interchangeably for both *in vivo* and *in vitro* assays.

Stem cell-derived RPE cells—A number of protocols have been developed that yield differentiated RPE from pluripotent precursor populations including human embryonic stem (hES) cells, human adult stem cells, and induced pluripotent stem (iPS) cells (reviewed by (Carr et al., 2013)). Like primary human RPE cells, RPE cell cultures derived from stem cell sources offer the opportunity to assess the phagocytic function of RPE cells carrying specific disease-associated haplotypes (Gamm et al., 2013). An exciting recent study compared functionality of iPS-derived RPE cells in culture derived from skin biopsies obtained from two patients with Best Disease caused by distinct single amino acid substitutions in Bestrophin-1 in and an unaffected sibling (Singh et al., 2013b). Daily incubation with isolated POS led to increasing accumulation of undigested autofluorescent material in cells from Best patients indicating that mutant Bestrophin-1 directly or indirectly impairs the phagolysosomal function of RPE cells.

Aside from disease modeling, stem cell-derived RPE cells promise new therapeutic options for retinal degenerations caused by or involving early RPE atrophy (reviewed by (Ramsden et al., 2013; Rowland et al., 2012)). Clinical and translational studies are under way aiming to eventually rescue photoreceptor function and thus vision after replacement of RPE with stem cell-derived RPE transplants (reviewed by (Melville et al., 2013)). Maintenance by grafted RPE of overlying photoreceptor cells must include participating in the synchronized shedding and phagocytosis program that is driven by photoreceptor-RPE interactions in intact retina. Published results to date show phagocytosis by all types of stem cell-derived RPE cells in principle but suggest a slow rate similar to immortalized cells and low efficiency both with respect to percent of cells in a culture taking up POS and numbers of POS taken up by individual cells (Carr et al., 2009a; Carr et al., 2009b; Cuenca et al., 2013; Liao et al., 2010; Subrizi et al., 2012; Westenskow et al., 2012). Recently, Westenskow and colleagues for the first time directly compared both phagocytosis and surface levels of α v β 5 and MerTK phagocytic receptors of ARPE-19 cells, human fetal RPE and iPS-derived RPE after different times in culture. They used flow cytometry to quantify specifically bound and internalized POS or surface receptor levels with higher accuracy than had been

done in any earlier study of RPE phagocytosis. Remarkably, they revealed that little more than half of the RPE population of any of the RPE models they studied carry αvβ5 integrin or MerTK (Westenskow et al., 2012). Lengthening the period of *in vitro* differentiation from 10 to 18 weeks in culture allowed iPS-derived RPE cells to adopt a highly regular hexagonal shape and increased pigmentation. These changes correlated with significantly increased phagocytic capacity of iPS-derived RPE cells while αvβ5 integrin and MerTK expression remained largely unchanged. This illustrates well the benefit of RPE differentiation/polarization for the phagocytosis function of the RPE: RPE cells may assemble proteins into functional phagocytic machineries with increasing efficiency as they adopt a more differentiated, polarized phenotype. As this study used flow cytometry for receptor analysis, receptor polarity or colocalization could not be assessed. Furthermore, expression of phagocytic receptors was analyzed in separate experiments and it remains to be shown if cells expressing the binding receptor αvβ5 integrin also tend to express the engulfment receptor MerTK. It is possible that even less than half of the cells tested form a complete phagocytic machinery capable of both POS binding and engulfment. Besides lack of a functional phagocytic machinery, technical issues such as characteristics of POS preparation and lack or excess of bridge ligands may also be in part responsible for the lack of vigorous uptake by stem cell-derived RPE. Certainly, the phagocytic function of stem cell-derived RPE deserves further experimental scrutiny.

II. 2. Studies of RPE cells in culture have confirmed and expanded insight gained from studies of RPE phagocytosis *in situ*.

Despite their considerable differences, the above-mentioned approaches exploring different types of RPE cells in culture agree that the two ligand-receptor pairs, MFG-E8-αvβ5 integrin and Gas6/Protein S-MerTK, are essential for POS phagocytosis. Furthermore, they demonstrated that αvβ5 integrin acts during POS recognition and upstream of MerTK (Finnemann, 2003a). They have further shown that MerTK in turn mediates a negative feedback mechanism to limit αvβ5 activity (Nandrot et al., 2012). None of these important mechanistic details could have been shown by examining RPE phagocytosis *in situ*. In addition to clarifying the specific functions of proteins already identified through *in vivo* studies, cell culture studies have also revealed contributions of additional proteins for which *in vivo* analysis proved ambiguous due to compensation, redundancy, or effects of gene/protein deficiency on the retina unrelated to RPE phagocytosis.

In brief, RPE cell culture assays have been instrumental in demonstrating a pre-formed apical surface complex of αvβ5 integrin receptors with the membrane organizer tetraspan CD81 and FAK that is stabilized by reversible anchorage to the F-actin cytoskeleton (Chang and Finnemann, 2007; Finnemann and Rodriguez-Boulan, 1999). Phagocytic ligands MFG-E8, Gas6 and Protein S possess phosphatidylserine-binding domains and MFG-E8 binds to phosphatidylserine exposed by POS (Ruggiero et al., 2012). Engagement of αvβ5 receptors by MFG-E8-opsonized POS triggers two independent downstream signals activating MerTK via FAK and activating Rac1 GTPase. Inhibition of Rac1 has no effect on tyrosine kinase signaling and vice versa during POS challenge (Mao and Finnemann, 2012). Both signaling pathways are essential for internalization of integrin-bound POS by RPE cells. Rac1 activation is required for recruitment of F-actin into phagocytic cups beneath

bound particles. PI3 kinases and, independently, AKT kinases also contribute to formation of functional phagocytic cups, which also contain annexin A2 and myosin II (Bulloj et al., 2013; Law et al., 2009). FAK activation by phosphorylation at several tyrosine residues promotes complex formation of its substrate $p130^{CAS}$ with the adaptor protein CrkII (Finnemann, 2003a). Active FAK directly or indirectly increases phosphorylation and hence activity of MerTK in an inside-out signaling pathway. A role in MerTK dependent POS engulfment for several other cytoplasmic signaling and adaptor proteins that possess Src-homology 2 domains has also been suggested (Shelby et al., 2013). Ultimately, phagocytic cups must contract to promote membrane fusion and completing engulfment. This process depends on myosin II activity and localization, which is in part dependent on MerTK (Strick et al., 2009). In addition to the constitutive αvβ5-FAK-MerTK pathway, CD36 scavenger receptors can recognize oxidized phospholipids on POS and enhance the phagocytic capacity of RPE cells possibly to promote clearance of excess damaged outer segment debris following retinal light damage (Finnemann and Silverstein, 2001; Sun et al., 2006). To date, the essential digestion phase of RPE phagocytosis remains less well understood than the recognition and engulfment phases. Individual POS components must either be degraded or processed and trafficked back to photoreceptors for re-use (likely for retinoids). Phagolysosomal maturation in RPE cells is a step-wise process (Bosch et al., 1993). Rhodopsin digestion also occurs in stages with cathepsins S and D playing major roles (Rakoczy et al., 1997; Rakoczy et al., 1998; Rakoczy et al., 2002). The small, acidic protein MREG associates with lysosomal membranes in RPE cells and is required for prompt rhodopsin digestion (Damek-Poprawa et al., 2009). Its precise role in POS phagolysosomal processing is still under investigation. Figure 3 summarizes our current knowledge of the RPE phagocytic mechanism for POS clearance.

II. 3. RPE phagocytosis belongs to a conserved form of clearance phagocytosis mechanisms but its strict rhythmicity sets it apart even in cell culture

RPE phagocytosis is a form of non-inflammatory clearance phagocytosis that shares many aspects with phagocytosis mechanisms used by other cell types to take up apoptotic cells or debris. These forms of phagocytosis are commonly non- or even anti-inflammatory, they use αv integrin receptors for binding, they recognize phosphatidylserine eat-me signals on debris and spent cells, and they rely on complex tyrosine kinase signaling for engulfment (reviewed by (Hochreiter-Hufford and Ravichandran, 2013)). Purified outer segment fragments and apoptotic cells compete quantitatively for recognition and surface tethering by both RPE and macrophages (Finnemann and Rodriguez-Boulan, 1999). We can thus learn from studies on clearance phagocytosis in other tissues and in invertebrate organisms. Furthermore, in cases where genetic modification of bona fide RPE cells in culture is not possible for instance because of cytotoxic effects, other phagocytic cells such as mouse embryonic fibroblasts, may be substituted for phagocytosis assays. For instance, FAK's cytosolic tyrosine kinase activity in RPE cells is essential not only for phagocytosis at the apical surface but also for basal substrate adhesion. Constitutive lack of FAK results in RPE detachment precluding phagocytosis assays. FAK null embryonic fibroblasts bind and engulf POS using an αv integrin-MerTK dependent pathway and can be used to re-express FAK or mutant forms of FAK to further dissect the roles of FAK in POS phagocytosis (Finnemann, 2003a).

While a one-time phagocytic event of the RPE shares many similarities with other phagocytic mechanisms, only RPE cells phagocytose daily for life in a strict diurnal rhythm. We therefore speculate that they possess molecular mechanisms, which they employ to control their phagocytic activity allowing efficient shed outer segment clearance after light onset but suppressing uptake activity at other times. We recently observed that unpassaged primary rat RPE cells and RPE-J cells in culture will express but will not activate excess αvβ5 integrin receptors in acute over-expression experiments (Nandrot et al., 2012). Cells prevent excess αvβ5 integrin binding of POS in a negative feedback mechanism that requires specifically the engulfment receptor MerTK. Furthermore, we have long observed that RPE cells in culture inhibit their recognition/binding activity for POS following an initial phagocytic meal (Finnemann, 2003b). RPE cells in culture fail to efficiently bind POS for many hours and long after bound POS of the previous phagocytic challenge have been cleared from the surface. These and other mechanisms used by RPE cells to control their phagocytic activity are still poorly understood and remain under active investigation.

III. Considerations for RPE phagocytosis assay development

III. 1. RPE cells in culture need appropriate levels of species-matched bridge ligand proteins to engage their POS specific phagocytosis pathway

RPE cells *in vivo* and in culture recognize experimental POS using their integrin receptor αvβ5. However, αvβ5 receptors do not recognize POS components directly. Instead, shedding outer segments or experimental POS must expose phosphatidylserine, the only well characterized eat-me signal for clearance phagocytosis regardless of cell type (Ruggiero et al., 2012). The secreted, soluble glycoprotein MFG-E8 contains both phosphatidylserine and αv integrin binding domains (Hanayama et al., 2004). In the retina, MFG-E8 localizes to the interphotoreceptor matrix (Burgess et al., 2006). Upon phosphatidylserine externalization at outer segment tips at light onset, MFG-E8 acts to opsonize tips and promote recognition by αvβ5 receptors of the RPE (Nandrot et al., 2007). Receptor engagement triggers the complex signaling including MerTK activation, which promotes engulfment. Additionally, MerTK ligands Gas6 and Protein S in the interphotoreceptor matrix likely further trigger MerTK activation.

During phagocytosis experiments using RPE cells in culture, the same molecular players interact in principle. RPE cells possess apical αvβ5 and MerTK receptors and they make and secrete apically MFG-E8. However, cell culture experiments commonly involve removal of apical supernatant and replacement with purified POS. During the short time frame for the assay, cells may not secrete sufficient MFG-E8 to opsonize all POS. If bridge ligand is limiting in the phagocytosis assay, RPE cells will bind and engulf less POS than they are capable of (Nandrot et al., 2007). Experiments during which POS binding is artificially capped in this manner risk ambiguous results because changes in phagocytosis activity due to treatment or genetic manipulation may be missed. Conversely, since each molecule of MFG-E8 must bridge phosphatidylserine on POS and αvβ5 receptor on RPE very high levels of MFG-E8 will also decrease POS binding and uptake as excess MFG-E8 will block receptors without binding to POS. It is therefore critically important to establish the effective concentration of MFG-E8 ligand during phagocytosis assays, which depends

on the model RPE cells and POS used. Similar considerations may apply to the addition of MerTK ligands during POS engulfment assays. It has long been known that engulfment by RPE cells in culture is greatly enhanced in the presence of low but not high concentrations of heat inactivated fetal bovine serum, which contains both MFG-E8 and the MerTK ligand protein S and thus supplies ligands for both receptors. However, for signaling experiments, additional activities also present in serum may make serum addition problematic. Addition of purified MFG-E8 and MerTK ligand protein (both available from several commercial sources) ensures specifically activating the POS phagocytic machinery. We consider this a clear advantage.

III. 2. RPE cells in culture phagocytose POS regardless of species of origin but can phagocytose latex beads using pathways irrelevant to POS uptake

As all evidence indicates that POS are not recognized directly by RPE receptors, the species of origin of the POS particle is less important for phagocytosis than the species specificity of the ligands used (Hall et al., 2005; Hall et al., 2002; Mayerson and Hall, 1986). Indeed, human, rat, and mouse RPE cells in culture phagocytose POS isolated from cow, pig or rat retina (Finnemann et al., 1997; Mayerson and Hall, 1986). Mouse or human MFG-E8 protein binds efficiently to phosphatidylserine exposed by POS or apoptotic cells of bovine, porcine, rat, mouse, or human origin (Finnemann and Rodriguez-Boulan, 1999; Nandrot et al., 2007; Ruggiero et al., 2012). However, human and mouse MFG-E8 are only 52% identical at the amino acid level. It is thus not surprising that human MFG-E8 does not bind efficiently to mouse or rat α v β5 integrin and vice versa (our unpublished results). Phagocytosis assays must therefore use appropriate ligands that match the RPE species, not the species of origin of POS. Coincidentally, bovine serum proteins can promote phagocytosis by both human and rodent RPE cells (Finnemann et al., 1997; Mayerson and Hall, 1986; Miceli et al., 1997). As mentioned above, we suggest substituting specific ligands for the undefined mix of serum during POS phagocytosis assays. POS size is an important factor. The diameter of outer segment fragments engulfed by RPE cells *in vivo* is in the order or 1 μm in diameter. We find that POS of similar size are bound and engulfed efficiently via the POS specific phagocytosis pathway. If particles are considerably smaller, they may be taken up via endocytic pathways instead. If particles are much larger or aggregate, they will remain tethered to the RPE surface. After preparation of POS and frozen storage at −80°C using well-established, published procedures (Mao and Finnemann, 2013), we suggest utmost care to prevent aggregation and mechanical shear forces during manipulations such as centrifugation (5 min at 2,400 × g is safe and effective, immediately gently resuspend POS in room temperature solution) and avoiding temperature shifts (up to 8 hours at room temperature in the dark and in suspension is safe). Covalent pre-staining of POS with a variety of commercially available dyes and tracers has been described by numerous investigators and does not impact uptake of POS if above guidelines are followed (Carr et al., 2009a; Mao and Finnemann, 2013; Shelby et al., 2013).

We can use our knowledge of the RPE's phagocytic mechanism for POS to substitute particles other than POS for phagocytosis assays. It is well known that RPE cells avidly take up latex beads. However, latex beads may be engulfed via a distinct pathway that is unrelated to the POS uptake pathway. Indeed, RCS RPE cells that completely lack

phagocytic activity toward POS will efficiently phagocytose latex beads (Edwards and Szamier, 1977). This illustrates well the risks of using bead substitutes for phagocytosis experiments. It should be possible to opsonize beads with phagocytic ligands MFG-E8 and Gas6 or Protein S triggering uptake by the RPE's specialized phagocytic pathway for POS, but this requires careful consideration of ligand load and control experiments proving that beads quantitatively compete for both binding and engulfment with *bona fide* POS. Lack of digestibility of beads can give rise to additional complications during phagocytosis assays of long duration. As bead content of phagosomes fails to be processed by the RPE cells' lysosomal system, organelle dynamics and energy metabolism may become abnormal and stress responses and secondary effects on cell functions and even cell survival are possible. We therefore suggest extreme caution when using beads as phagocytic particles in RPE phagocytosis experiments.

III. 3. Synchronized phagocytosis assays using limited POS challenge and extended chase periods render phagocytosis by RPE cells in culture more similar to phagocytosis *in situ*

Rod POS phagocytosis by RPE cells *in situ* occurs in a synchronized burst after light onset. Incubating RPE cells in culture continuously for many hours with POS does not mimic well this process. Challenge with POS for only a brief period of time reduces the discrepancy between POS but does not eliminate it: some POS will bind early during POS challenge and be engulfed early while others will bind at the end of the POS challenge period. RPE cells in culture bind POS via α v β5 integrin at 20°C (but not at 4°C, at which temperature macrophages bind particles via Fc receptors) (Finnemann and Rodriguez-Boulan, 1999). In contrast, engulfment does not take place at this restricted temperature (Mayerson and Hall, 1986). We have recently started to exploit this characteristic differential temperature dependence of binding and engulfment phases of RPE phagocytosis to truly synchronize the process. Specifically, challenge of POS at 20°C in the presence of MFG-E8 will yield efficient binding of POS to the RPE's apical surface. After removing excess POS in two brief rinses in serum free assay medium, we shift the assay temperature to the permissive temperature (37°C for most mammalian cells, 32°C for RPE-J cells) and add MerTK ligands (purified Gas6, purified protein S, or if not available 4% fetal bovine serum containing both). During the engulfment phase, three distinct methods can be used to quantify the fraction of POS engulfed (Mao and Finnemann, 2013): 1- selective surface immunofluorescence of bound POS after non-permeabilizing fixation (Chaitin and Hall, 1983); 2- wash with 2 mM EDTA to remove bound but not engulfed POS before quantification of engulfed POS based on fluorescence or rhodopsin content immunoblotting (Mao and Finnemann, 2012); 3- quenching of surface-bound FITC-labeled POS by incubation with trypan blue followed by quantification of remaining FITC fluorescence indicative of engulfed POS only (this method unlike the others requires FITC-labeled POS) (Finnemann et al., 1997). For further details, we refer to our recently published protocol of POS phagocytosis assays in culture (Mao and Finnemann, 2013). Quantification of the fraction of POS engulfed after several time periods of chase at permissive temperature allows quantifying both speed of phagocytosis and total POS engulfment capacity. We suggest exploring such or similar pulse-chase POS phagocytosis experiments for studies focusing on the two later phases of POS phagocytosis, engulfment and digestion.

IV Perspective

The value of any specific RPE cell population in culture for a research question depends on the ability to recapitulate functional and genetic characteristics of the native tissue. Classically, RPE cell characteristics can be summarized as the “five p’s”: post-mitotic, polygonal, polarized, pigmented, and phagocytic. These remain most important in our assessment of any cell culture with respect to overall “like-ness” to native RPE. Here, we suggest that, in any study with specific focus on the RPE’s phagocytic function, it is essential to determine expression and distribution of the known phagocytic machinery at the phagocytic (apical) surface of model RPE cells. Other characteristics of RPE such as pigmentation and a post-mitotic state are less critical for the phagocytic process itself but variability in these characteristics may influence the phagocytic rate or capacity. Therefore, we propose utmost diligence to ensure that the four other “p’s” remain constant and do not vary during the duration of any phagocytosis study. For example, proliferating cells may be used but cells should always be subjected to phagocytosis challenge at constant times after plating at specific density on the same attachment matrix. With such careful experimental planning, we do not need to restrict new studies to select gold standard forms of vetted RPE cell models and protocols. Rather, we encourage exploring the wide range of different sources of RPE cells available for phagocytosis research while considering the guidelines and notes of caution we discussed. Whenever feasible, cell culture and *in situ* studies of RPE phagocytosis should continue to go hand-in-hand and confirm validity of each other’s results. We anticipate that this will accelerate the discovery process and further our understanding of this fascinating and complex molecular mechanism.

Acknowledgments

National Institutes of Health

R01-EY13295

References

Ahmado A, Carr AJ, Vugler AA, Semo M, Gias C, Lawrence JM, Chen LL, Chen FK, Turowski P, da Cruz L, Coffey PJ. Induction of differentiation by pyruvate and DMEM in the human retinal pigment epithelium cell line ARPE-19. Invest. Ophthalmol. Vis. Sci. 2011; 52:7148–7159. [PubMed: 21743014]

Albert DM, Tso MO, Rabson AS. *In vitro* growth of pure cultures of retinal pigment epithelium. Arch. Ophthalmol. 1972; 88:63–69. [PubMed: 5049457]

Besharse JC, Hollyfield JG, Rayborn ME. Photoreceptor outer segments: accelerated membrane renewal in rods after exposure to light. Science. 1977; 196:536–538. [PubMed: 300504]

Bobu C, Hicks D. Regulation of retinal photoreceptor phagocytosis in a diurnal mammal by circadian clocks and ambient lighting. Invest. Ophthalmol. Vis. Sci. 2009; 50:3495–3502. [PubMed: 19234351]

Bok D, Hall MO. The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. J.Cell Biol. 1971; 49:664–682. [PubMed: 5092207]

Bosch E, Horwitz J, Bok D. Phagocytosis of outer segments by retinal pigment epithelium: phagosome- lysosome interaction. J. Histochem. Cytochem. 1993; 41:253–263. [PubMed: 8419462]

Bulloj A, Duan W, Finnemann SC. PI 3-kinase independent role for AKT in F-actin regulation during outer segment phagocytosis by RPE cells. Exp. Eye Res. 2013; 113:9–18. [PubMed: 23669303]

Burgess BL, Abrams TA, Nagata S, Hall MO. MFG-E8 in the retina and retinal pigment epithelium of rat and mouse. Mol. Vis. 2006; 12:1437–1447. [PubMed: 17167398]

Burstyn-Cohen T, Lew ED, Traves PG, Burrola PG, Hash JC, Lemke G. Genetic dissection of TAM receptor-ligand interaction in retinal pigment epithelial cell phagocytosis. Neuron. 2012; 76:1123–1132. [PubMed: 23259948]

Carr AJ, Smart MJ, Ramsden CM, Powner MB, da Cruz L, Coffey PJ. Development of human embryonic stem cell therapies for age-related macular degeneration. Trends Neurosci. 2013; 36:385–395. [PubMed: 23601133]

Carr AJ, Vugler A, Lawrence J, Chen LL, Ahmado A, Chen FK, Semo M, Gias C, da Cruz L, Moore HD, Walsh J, Coffey PJ. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Mol. Vis. 2009a; 15:283–295. [PubMed: 19204785]

Carr AJ, Vugler AA, Hikita ST, Lawrence JM, Gias C, Chen LL, Buchholz DE, Ahmado A, Semo M, Smart MJ, Hasan S, da Cruz L, Johnson LV, Clegg DO, Coffey PJ. Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. PLoS One. 2009b; 4:e8152. [PubMed: 19997644]

Carr AJ, Vugler AA, Yu L, Semo M, Coffey P, Moss SE, Greenwood J. The expression of retinal cell markers in human retinal pigment epithelial cells and their augmentation by the synthetic retinoid fenretinide. Mol. Vis. 2011; 17:1701–1715. [PubMed: 21738400]

Chaitin MH, Hall MO. Defective ingestion of rod outer segments by cultured dystrophic rat pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 1983; 24:812–820. [PubMed: 6345445]

Chang Y, Finnemann SC. Tetraspanin CD81 is required for the αvβ5 integrin-dependent particle-binding step of RPE phagocytosis. J. Cell Sci. 2007; 120:3053–3063. [PubMed: 17684062]

Chowers I, Kim Y, Farkas RH, Gunatilaka TL, Hackam AS, Campochiaro PA, Finnemann SC, Zack DJ. Changes in retinal pigment epithelial gene expression induced by rod outer segment uptake. Invest. Ophthalmol. Vis. Sci. 2004; 45:2098–2106. [PubMed: 15223782]

Colella P, Sommella A, Marrocco E, Di Vicino U, Polishchuk E, Garrido MG, Seeliger MW, Polishchuk R, Auricchio A. Myosin7a deficiency results in reduced retinal activity which is improved by gene therapy. PLoS One. 2013; 8:e72027. [PubMed: 23991031]

Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, Kamei M, Hasan A, Yan L, Rayborn ME, Salomon RG, Hollyfield JG. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:14682–14687. [PubMed: 12391305]

Cuenca N, Fernandez-Sanchez L, McGill TJ, Lu B, Wang S, Lund R, Huhn S, Capela A. Phagocytosis of photoreceptor outer segments by transplanted human neural stem cells as a neuroprotective mechanism in retinal degeneration. Invest. Ophthalmol. Vis. Sci. 2013; 54:6745–6756. [PubMed: 24045996]

Damek-Poprawa M, Diemer T, Lopes VS, Lillo C, Harper DC, Marks MS, Wu Y, Sparrow JR, Rachel RA, Williams DS, Boesze-Battaglia K. Melanoregulin (MREG) modulates lysosome function in pigment epithelial cells. J. Biol. Chem. 2009; 284:10877–10889. [PubMed: 19240024]

Duncan JL, LaVail MM, Yasumura D, Matthes MT, Yang H, Trautmann N, Chappelow AV, Feng W, Earp HS, Matsushima GK, Vollrath D. An RCS-like retinal dystrophy phenotype in mer knockout mice. Invest. Ophthalmol. Vis. Sci. 2003; 44:826–838. [PubMed: 12556419]

Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp. Eye Res. 1996; 62:155–169. [PubMed: 8698076]

Edwards RB, Szamier RB. Defective phagocytosis of isolated rod outer segments by RCS rat retinal pigment epithelium in culture. Science. 1977; 197:1001–1003. [PubMed: 560718]

Feng W, Yasumura D, Matthes MT, LaVail MM, Vollrath D. Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J. Biol. Chem. 2002; 277:17016–17022. [PubMed: 11861639]

Finnemann SC. Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. EMBO J. 2003a; 22:4143–4154. [PubMed: 12912913]

Finnemann SC. Role of α v β5 integrin in regulating phagocytosis by the retinal pigment epithelium. Adv. Exp. Med. Biol. 2003b; 533:337–342. [PubMed: 15180282]

Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E. Phagocytosis of rod outer segments by retinal pigment epithelial cells requires α v β5 integrin for binding but not for internalization. Proc. Natl. Acad. Sci. U. S. A. 1997; 94:12932–12937. [PubMed: 9371778]

Finnemann SC, Leung LW, Rodriguez-Boulan E. The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:3842–3847. [PubMed: 11904436]

Finnemann SC, Rodriguez-Boulan E. Macrophage and retinal pigment epithelium phagocytosis: apoptotic cells and photoreceptors compete for αvβ3 and α v β5 integrins, and protein kinase C regulates α v β5 binding and cytoskeletal linkage. J. Exp. Med. 1999; 190:861–874. [PubMed: 10499924]

Finnemann SC, Silverstein RL. Differential roles of CD36 and α v β5 integrin in photoreceptor phagocytosis by the retinal pigment epithelium. J. Exp. Med. 2001; 194:1289–1298. [PubMed: 11696594]

Fisher SK, Pfeffer BA, Anderson DH. Both rod and cone disc shedding are related to light onset in the cat. Invest. Ophthalmol. Vis. Sci. 1983; 24:844–856. [PubMed: 6683265]

Gal A, Li Y, Thompson DA, Weir J, Orth U, Jacobson SG, Apfelstedt-Sylla E, Vollrath D. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat. Genet. 2000; 26:270–271. [PubMed: 11062461]

Gamm DM, Phillips MJ, Singh R. Modeling retinal degenerative diseases with human iPS-derived cells: current status and future implications. Expert Rev. Ophthalmol. 2013; 8:213–216. [PubMed: 24039627]

Gibbs D, Diemer T, Khanobdee K, Hu J, Bok D, Williams DS. Function of MYO7A in the human RPE and the validity of shaker1 mice as a model for Usher syndrome 1B. Invest. Ophthalmol. Vis. Sci. 2010; 51:1130–1135. [PubMed: 19643958]

Gibbs D, Kitamoto J, Williams DS. Abnormal phagocytosis by retinal pigmented epithelium that lacks myosin VIIa, the Usher syndrome 1B protein. Proc. Natl. Acad. Sci. U. S. A. 2003; 100:6481–6486. [PubMed: 12743369]

Gibbs D, Williams DS. Isolation and culture of primary mouse retinal pigmented epithelial cells. Adv. Exp. Med. Biol. 2003; 533:347–352. [PubMed: 15180284]

Goldman AI, Teirstein PS, O'Brien PJ. The role of ambient lighting in circadian disc shedding in the rod outer segment of the rat retina. Invest. Ophthalmol. Vis. Sci. 1980; 19:1257–1267. [PubMed: 7429762]

Hall MO, Obin MS, Heeb MJ, Burgess BL, Abrams TA. Both protein S and Gas6 stimulate outer segment phagocytosis by cultured rat retinal pigment epithelial cells. Exp. Eye Res. 2005; 81:581–591. [PubMed: 15949798]

Hall MO, Obin MS, Prieto AL, Burgess BL, Abrams TA. Gas6 binding to photoreceptor outer segments requires gamma-carboxyglutamic acid (Gla) and Ca(2+) and is required for OS phagocytosis by RPE cells *in vitro*. Exp. Eye Res. 2002; 75:391–400. [PubMed: 12387786]

Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, Nagata S. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. Science. 2004; 304:1147–1150. [PubMed: 15155946]

Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. Cold Spring Harb. Perspect. Biol. 2013; 5:a008748. [PubMed: 23284042]

Hu J, Bok D. A cell culture medium that supports the differentiation of human retinal pigment epithelium into functionally polarized monolayers. Mol. Vis. 2001; 7:14–19. [PubMed: 11182021]

Hu J, Bok D. Culture of highly differentiated human retinal pigment epithelium for analysis of the polarized uptake, processing, and secretion of retinoids. Methods Mol. Biol. 2010; 652:55–73. [PubMed: 20552421]

Hu J, Bok D. The use of cultured human fetal retinal pigment epithelium in studies of the classical retinoid visual cycle and retinoid-based disease processes. Exp. Eye Res. 2013 doi: 10.1016/j.exer.2013.09.009.

Ida H, Boylan SA, Weigel AL, Hjelmeland LM. Age-related changes in the transcriptional profile of mouse RPE/choroid. Physiol. Genomics. 2003; 15:258–262. [PubMed: 14519767]

Immel JH, Fisher SK. Cone photoreceptor shedding in the tree shrew (Tupaia belangerii). Cell Tissue Res. 1985; 239:667–675. [PubMed: 3986885]

Lakkaraju A, Finnemann SC, Rodriguez-Boulan E. The lipofuscin fluorophore A2E perturbs cholesterol metabolism in retinal pigment epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 2007; 104:11026–11031. [PubMed: 17578916]

LaVail MM. Rod outer segment disk shedding in rat retina: relationship to cyclic lighting. Science. 1976; 194:1071–1074. [PubMed: 982063]

LaVail MM. Circadian nature of rod outer segment disc shedding in the rat. Invest. Ophthalmol. Vis. Sci. 1980; 19:407–411. [PubMed: 7358492]

Law AL, Ling Q, Hajjar KA, Futter CE, Greenwood J, Adamson P, Wavre-Shapton ST, Moss SE, Hayes MJ. Annexin A2 regulates phagocytosis of photoreceptor outer segments in the mouse retina. Mol. Biol. Cell. 2009; 20:3896–3904. [PubMed: 19587120]

Liao JL, Yu J, Huang K, Hu J, Diemer T, Ma Z, Dvash T, Yang XJ, Travis GH, Williams DS, Bok D, Fan G. Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. Hum. Mol. Genet. 2010; 19:4229–4238. [PubMed: 20709808]

Luo Y, Zhuo Y, Fukuhara M, Rizzolo LJ. Effects of culture conditions on heterogeneity and the apical junctional complex of the ARPE-19 cell line. Invest. Ophthalmol. Vis. Sci. 2006; 47:3644–3655. [PubMed: 16877439]

Mallavarapu M, Finnemann SC. Neural retina and MerTK-independent apical polarity of αvβ5 integrin receptors in the retinal pigment epithelium. Adv. Exp. Med. Biol. 2010; 664:123–131. [PubMed: 20238010]

Maminishkis A, Chen S, Jalickee S, Banzon T, Shi G, Wang FE, Ehalt T, Hammer JA, Miller SS. Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. Invest. Ophthalmol. Vis. Sci. 2006; 47:3612–3624. [PubMed: 16877436]

Mannagh J, Arya DV, Irvine AR Jr. Tissue culture of human retinal pigment epithelium. Invest. Ophthalmol. 1973; 12:52–64. [PubMed: 4570989]

Mao Y, Finnemann SC. Essential diurnal Rac1 activation during retinal phagocytosis requires αvβ5 integrin but not tyrosine kinases FAK or MerTK. Mol. Biol. Cell. 2012; 23:1104–1114. [PubMed: 22262456]

Mao Y, Finnemann SC. Analysis of photoreceptor outer segment phagocytosis by RPE cells in culture. Methods Mol. Biol. 2013; 935:285–295. [PubMed: 23150376]

Mayerson PL, Hall MO. Rat retinal pigment epithelial cells show specificity of phagocytosis *in vitro*. J. Cell Biol. 1986; 103:299–308. [PubMed: 3522605]

McHenry CL, Liu Y, Feng W, Nair AR, Feathers KL, Ding X, Gal A, Vollrath D, Sieving PA, Thompson DA. MERTK arginine-844-cysteine in a patient with severe rod-cone dystrophy: loss of mutant protein function in transfected cells. Invest. Ophthalmol. Vis. Sci. 2004; 45:1456–1463. [PubMed: 15111602]

Melville H, Carpiniello M, Hollis K, Staffaroni A, Golestaneh N. Stem cells: a new paradigm for disease modeling and developing therapies for age-related macular degeneration. J. Transl. Med. 2013; 11:53. [PubMed: 23452406]

Miceli MV, Newsome DA, Tate DJ. Vitronectin is responsible for serum-stimulated uptake of rod outer segments by cultured retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 1997; 38:1588–1597. [PubMed: 9224287]

Millan JM, Aller E, Jaijo T, Blanco-Kelly F, Gimenez-Pardo A, Ayuso C. An update on the genetics of usher syndrome. J. Ophthalmol. 20112011:417217. [PubMed: 21234346]

Mullen RJ, LaVail MM. Inherited retinal dystrophy: primary defect in pigment epithelium determined with experimental rat chimeras. Science. 1976; 192:799–801. [PubMed: 1265483]

Nabi IR, Mathews AP, Cohen-Gould L, Gundersen D, Rodriguez-Boulan E. Immortalization of polarized rat retinal pigment epithelium. J. Cell Sci. 1993; 104:37–49. [PubMed: 8383696]

Nandrot EF, Anand M, Almeida D, Atabai K, Sheppard D, Finnemann SC. Essential role for MFG-E8 as ligand for α v β5 integrin in diurnal retinal phagocytosis. Proc. Natl. Acad. Sci. U. S. A. 2007; 104:12005–12010. [PubMed: 17620600]

Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC. Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking α v β5 integrin. J. Exp. Med. 2004; 200:1539–1545. [PubMed: 15596525]

Nandrot EF, Silva KE, Scelfo C, Finnemann SC. Retinal pigment epithelial cells use a MerTK-dependent mechanism to limit the phagocytic particle binding activity of α v β5 integrin. Biol. Cell. 2012; 104:326–341. [PubMed: 22289110]

O'Day WT, Young RW. Rhythmic daily shedding of outer-segment membranes by visual cells in the goldfish. J. Cell Biol. 1978; 76:593–604. [PubMed: 632320]

Olchawa MM, Herrnreiter AM, Skumatz CM, Zareba M, Sarna TJ, Burke JM. Photosensitized oxidative stress to ARPE-19 cells decreases protein receptors that mediate photoreceptor outer segment phagocytosis. Invest. Ophthalmol. Vis. Sci. 2013; 54:2276–2287. [PubMed: 23449722]

Ostergaard E, Duno M, Batbayli M, Vilhelmsen K, Rosenberg T. A novel MERTK deletion is a common founder mutation in the Faroe Islands and is responsible for a high proportion of retinitis pigmentosa cases. Mol. Vis. 2011; 17:1485–1492. [PubMed: 21677792]

Pasovic L, Utheim TP, Maria R, Lyberg T, Messelt EB, Aabel P, Chen DF, Chen X, Eidet JR. Optimization of Storage Temperature for Cultured ARPE-19 Cells. J. Ophthalmol. 2013; 2013:216359. [PubMed: 24251032]

Philp NJ, Bernstein MH. Phagocytosis by retinal pigment epithelium explants in culture. Exp. Eye Res. 1981; 33:47–53. [PubMed: 7250231]

Qin S, Rodrigues GA. Roles of α v β5, FAK and MerTK in oxidative stress inhibition of RPE cell phagocytosis. Exp. Eye Res. 2012; 94:63–70. [PubMed: 22138557]

Rakoczy PE, Lai CM, Baines M, Di Grandi S, Fitton JH, Constable IJ. Modulation of cathepsin D activity in retinal pigment epithelial cells. Biochem. J. 1997; 324:935–940. [PubMed: 9210419]

Rakoczy PE, Lai MC, Baines MG, Spilsbury K, Constable IJ. Expression of cathepsin S antisense transcripts by adenovirus in retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 1998; 39:2095–2104. [PubMed: 9761288]

Rakoczy PE, Zhang D, Robertson T, Barnett NL, Papadimitriou J, Constable IJ, Lai CM. Progressive age-related changes similar to age-related macular degeneration in a transgenic mouse model. Am. J. Pathol. 2002; 161:1515–1524. [PubMed: 12368224]

Ramsden CM, Powner MB, Carr AJ, Smart MJ, da Cruz L, Coffey PJ. Stem cells in retinal regeneration: past, present and future. Development. 2013; 140:2576–2585. [PubMed: 23715550]

Ratto GM, Robinson DW, Yan B, McNaughton PA. Development of the light response in neonatal mammalian rods. Nature. 1991; 351:654–657. [PubMed: 2052091]

Rowland TJ, Buchholz DE, Clegg DO. Pluripotent human stem cells for the treatment of retinal disease. J. Cell Physiol. 2012; 227:457–466. [PubMed: 21520078]

Ruggiero, L.; Finnemann, SC. Rhythmicity of the retinal pigment epithelium. In: Tosini, G.; Iuvone, PM.; McMahon, DG., editors. The Retina and Circadian Rhythms. Springer Press; 2014a. Springer Series in Vision Research. © 2014

Ruggiero, L.; Finnemann, SC. Photoreceptor-RPE Interactions: Diurnal Phagocytosis. In: Werner, JS.; Chalupa, LM., editors. The New Visual Neurosciences. MIT press; 2014b. © 2014

Ruggiero L, Connor MP, Chen J, Langen R, Finnemann SC. Diurnal, localized exposure of phosphatidylserine by rod outer segment tips in wild-type but not Itgb5$^{-/-}$ or Mfge8$^{-/-}$ mouse retina. Proc. Natl. Acad. Sci. U. S. A. 2012; 109:8145–8148. [PubMed: 22566632]

Sethna S, Finnemann SC. Analysis of photoreceptor rod outer segment phagocytosis by RPE cells *in situ*. Methods Mol. Biol. 2013; 935:245–254. [PubMed: 23150373]

Shelby SJ, Colwill K, Dhe-Paganon S, Pawson T, Thompson DA. MERTK interactions with SH2-domain proteins in the retinal pigment epithelium. PLoS One. 2013; 8:e53964. [PubMed: 23390493]

Singh R, Phillips MJ, Kuai D, Meyer J, Martin JM, Smith MA, Perez ET, Shen W, Wallace KA, Capowski EE, Wright LS, Gamm DM. Functional analysis of serially expanded human iPS cell-derived RPE cultures. Invest. Ophthalmol. Vis. Sci. 2013a; 54:6767–6778. [PubMed: 24030465]

Singh R, Shen W, Kuai D, Martin JM, Guo X, Smith MA, Perez ET, Phillips MJ, Simonett JM, Wallace KA, Verhoeven AD, Capowski EE, Zhang X, Yin Y, Halbach PJ, Fishman GA, Wright LS, Pattnaik BR, Gamm DM. iPS cell modeling of Best disease: insights into the pathophysiology of an inherited macular degeneration. Hum. Mol. Gen. 2013b; 22:593–607. [PubMed: 23139242]

Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. Nat. Protoc. 2009; 4:662–673. [PubMed: 19373231]

Strauss O. The retinal pigment epithelium in visual function. Physiol. Rev. 2005; 85:845–881. [PubMed: 15987797]

Strick DJ, Feng W, Vollrath D. Mertk drives myosin II redistribution during retinal pigment epithelial phagocytosis. Invest. Ophthalmol. Vis. Sci. 2009; 50:2427–2435. [PubMed: 19117932]

Strunnikova NV, Maminishkis A, Barb JJ, Wang F, Zhi C, Sergeev Y, Chen W, Edwards AO, Stambolian D, Abecasis G, Swaroop A, Munson PJ, Miller SS. Transcriptome analysis and molecular signature of human retinal pigment epithelium. Hum. Mol. Gen. 2010; 19:2468–2486. [PubMed: 20360305]

Subrizi A, Hiidenmaa H, Ilmarinen T, Nymark S, Dubruel P, Uusitalo H, Yliperttula M, Urtti A, Skottman H. Generation of hESC-derived retinal pigment epithelium on biopolymer coated polyimide membranes. Biomaterials. 2012; 33:8047–8054. [PubMed: 22892561]

Sun M, Finnemann SC, Febbraio M, Shan L, Annangudi SP, Podrez EA, Hoppe G, Darrow R, Organisciak DT, Salomon RG, Silverstein RL, Hazen SL. Light-induced oxidation of photoreceptor outer segment phospholipids generates ligands for CD36-mediated phagocytosis by retinal pigment epithelium: a potential mechanism for modulating outer segment phagocytosis under oxidant stress conditions. J. Biol. Chem. 2006; 281:4222–4230. [PubMed: 16354659]

Turowski P, Adamson P, Sathia J, Zhang JJ, Moss SE, Aylward GW, Hayes MJ, Kanuga N, Greenwood J. Basement membrane-dependent modification of phenotype and gene expression in human retinal pigment epithelial ARPE-19 cells. Invest. Ophthalmol. Vis. Sci. 2004; 45:2786–2794. [PubMed: 15277505]

Westenskow PD, Moreno SK, Krohne TU, Kurihara T, Zhu S, Zhang ZN, Zhao T, Xu Y, Ding S, Friedlander M. Using flow cytometry to compare the dynamics of photoreceptor outer segment phagocytosis in iPS-derived RPE cells. Invest. Ophthalmol. Vis. Sci. 2012; 53:6282–6290. [PubMed: 22871841]

Young RW. The renewal of photoreceptor cell outer segments. J. Cell Biol. 1967; 33:61–72. [PubMed: 6033942]

Young RW. The daily rhythm of shedding and degradation of cone outer segment membranes in the lizard retina. J. Ultrastruct. Res. 1977; 61:172–185. [PubMed: 562420]

Young RW. The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina. Invest. Ophthalmol. Vis. Sci. 1978; 17:105–116. [PubMed: 624604]

Young RW, Bok D. Participation of the retinal pigment epithelium in the rod outer segment renewal process. J. Cell Biol. 1969; 42:392–403. [PubMed: 5792328]

Yu CC, Nandrot EF, Dun Y, Finnemann SC. Dietary antioxidants prevent age-related retinal pigment epithelium actin damage and blindness in mice lacking αvβ5 integrin. Free Radic. Biol. Med. 2012; 52:660–670. [PubMed: 22178979]

- RPE cells in culture phagocytose photoreceptor outer segment fragments (POS).
- Capacity and rate of POS phagocytosis depends on the specific type of cultured RPE.
- Capacity and rate of POS phagocytosis depends on exact cell culture conditions.
- RPE cells *in situ* and in culture use the same mechanism for POS phagocytosis.

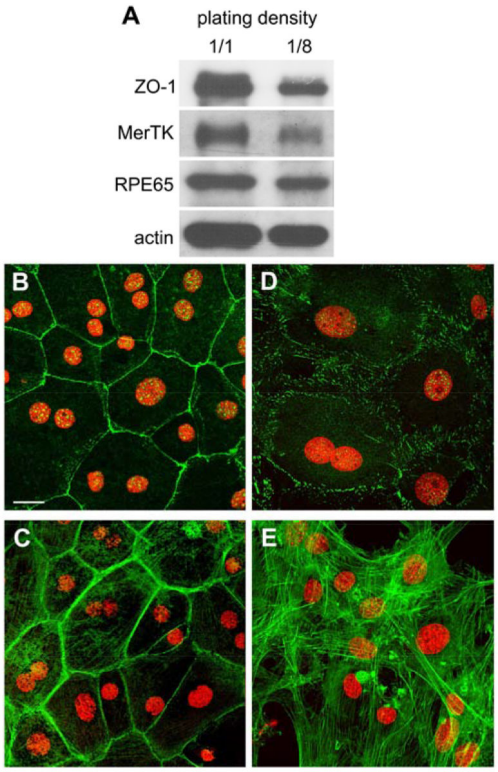

Figure 1. Primary mouse RPE cells seeded at high and low density after purification significantly differ in cell shape and F-actin organization at confluence.

RPE cells were purified according to published procedures from PN8 wild-type mice (strain 129SvEms/J) and seeded at a density of yield of 2 eyes per well (A, lane 1/1, B and C) or diluted 8-fold (A, lane 1/8, D and E). Cells were analyzed after 6 days in culture. (A) Immunoblotting of protein content of one well of each cell population reveals that RPE cells seeded at either density express the tight junction protein ZO-1, the engulfment receptor MerTK, actin, and the visual cycle enzyme RPE65 (lanes as indicated). Cells seeded at lower density express lower levels of ZO-1, MerTK and actin. (B to E) Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton × 100 and labeled with antibodies to ZO-1 (B and D, green) or Alexafluor488 conjugated phalloidin (C and E, green). Nuclei were counterstained with DAPI (shown in red). Maximal projections of image stacks acquired on a Leica TSP5 confocal microscopy system are shown. Scale bar: 20 μm.

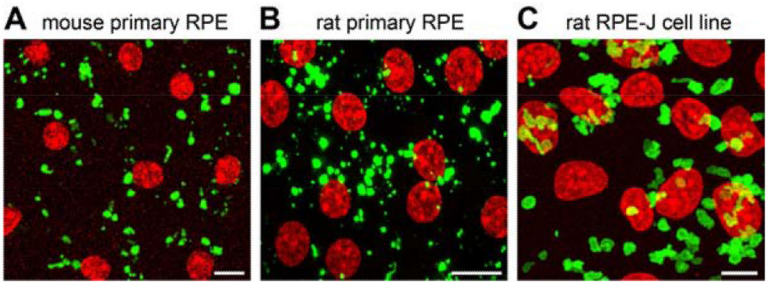

Figure 2. Unpassaged primary mouse and rat RPE cells in culture and immortalized RPE-J cells are avid phagocytes.

RPE cells were purified according to published procedures from PN8 wild-type mice (A) or PN8 Long Evans rats (B) and seeded at a density of 2 eyes per well (A) and 1 eye per well (B). Immortalized rat RPE-J cells were seeded at 25% confluence (¼ dilution at split) on glass coverlips (C). All cells were challenged with 10 FITC-POS/cell 6 days after plating in DMEM supplemented with 0.2 ug/ml mouse MFG-E8 and 4% delipidated heat inactivated fetal bovine serum. Primary cells were challenged for 1.5 hours and RPE-J cells were challenged for 5 hours before fixation with ice-cold methanol. Nuclei were counterstained with DAPI. Images show representative maximal projections of image stacks of bound plus internalized FITC-POS (green) and RPE cell nuclei (red) obtained on a Leica TSP5 confocal microscopy system. For details of methods please see (Mao and Finnemann, 2013). Scale bars: 10 μm.

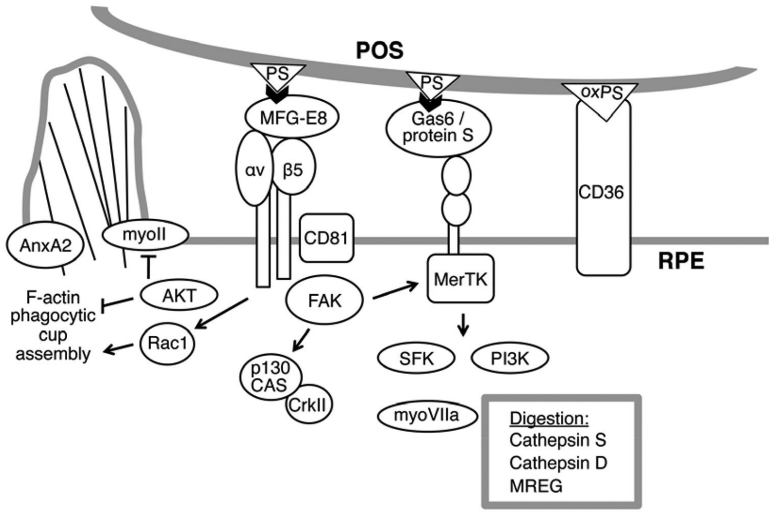

Figure 3. POS phagocytosis by RPE cells requires coordinated activities of numerous cell surface and cytosolic proteins.

The scheme illustrates our current knowledge on proteins involved in POS uptake gained from studies of both RPE phagocytosis *in situ* and in cell culture. Abbreviations: AnxA2, annexin A2; myoII, myosin II; myoVIIa, myosin VIIa; PI3K, PI3 kinases; SFK, Src family kinases. Adapted from (Mao and Finnemann, 2012). For details and references please see text.