

ABCF1 extrinsically regulates retinal pigment epithelial cell phagocytosis

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ABSTRACT Phagocytosis of shed photoreceptor outer segments (POSs) by retinal pigment epithelial (RPE) cells is critical to retinal homeostasis and shares many conserved signaling pathways with other phagocytes, including extrinsic regulations. Phagocytotic ligands are the key to cargo recognition, engulfment initiation, and activity regulation. In this study, we identified intracellular protein ATP-binding cassette subfamily F member 1 (ABCF1) as a novel RPE phagocytotic ligand by a new approach of functional screening. ABCF1 was independently verified to extrinsically promote phagocytosis of shed POSs by D407 RPE cells. This finding was further corroborated with primary RPE cells and RPE explants. Internalized POS vesicles were colocalized with a phagosome marker, suggesting that ABCF1-mediated engulfment is through a phagocytic pathway. ABCF1 was released from apoptotic cells and selectively bound to shed POS vesicles and apoptotic cells, possibly via externalized phosphatidylserine. ABCF1 is predominantly expressed in POSs and colocalized with the POS marker rhodopsin, providing geographical convenience for regulation of RPE phagocytosis. Collectively these results suggest that ABCF1 is released from and binds to shed POSs in an autocrine manner to facilitate RPE phagocytosis through a conserved pathway. Furthermore, the new approach is broadly applicable to many other phagocytes and will enable systematic elucidation of their ligands to understand extrinsic regulation and cargo recognition.

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INTRODUCTION

Phagocytosis of apoptotic cells, also called efferocytosis, is an important biological process for maintaining tissue homeostasis and innate immune balance (Erwig and Henson, 2007; Hochreiter-Hufford and Ravichandran, 2013; Sierra *et al.*, 2013). Phagocytotic defect may lead to debris accumulation, inflammation, autoimmune

disease, and tissue degeneration (Gal *et al.*, 2000; Neumann and Takahashi, 2007; Jonsson *et al.*, 2013). Functional roles of various phagocytes are defined by what deleterious cargoes they clear. Apoptotic cells are by far the most studied phagocytotic cargo. Other known cargoes include stressed/injured cells, aged cells (e.g., aged erythrocytes), cellular debris (e.g., myelin debris), and metabolic products (e.g., amyloid β peptide [A β], and oxidized lipids; Strauss, 2005; Sierra *et al.*, 2013; Brown and Neher, 2014).

Despite our knowledge of different phagocytotic cargoes, much less is known about cargo recognition. Phagocytotic ligands recognize deleterious cargoes, link them to phagocytes, and initiate engulfment by activating cognate receptors (Li, 2012a). Phagocytotic ligands consist of eat-me signals and soluble bridging molecules. Eat-me signals are abnormal molecules displayed on the surface of phagocytotic cargoes but not healthy cells, and are recognized directly by phagocytic receptors or indirectly through bridging molecules. In this regard, these ligands are the key to understanding cargo recognition and phagocyte functions.

Retinal pigment epithelial (RPE) cells are specialized phagocytes that maintain retinal homeostasis and prevent retinal degeneration (Strauss, 2005; Kevany and Palczewski, 2010). Photoreceptor outer

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Abbreviations used: A β , amyloid β peptide; ABC, ATP-binding cassette; ABCF1, ATP-binding cassette subfamily F member 1; AGE, advanced glycation end product; AMD, age-related macular degeneration; DAMP, damage-associated molecular pattern molecule; DAPI, 4',6-diamidino-2-phenylindole; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; HMG, high-mobility group of proteins; IgG, immunoglobulin G; MBP, maltose-binding protein; NGS, next-generation DNA sequencing; OPD, open reading frame phage display; PBS, phosphate-buffered saline; PFC, phagocytosis-based functional cloning; POS, photoreceptor outer segment; RGC, retinal ganglion cell; RPE, retinal pigment epithelium; RT-PCR, reverse transcription PCR.

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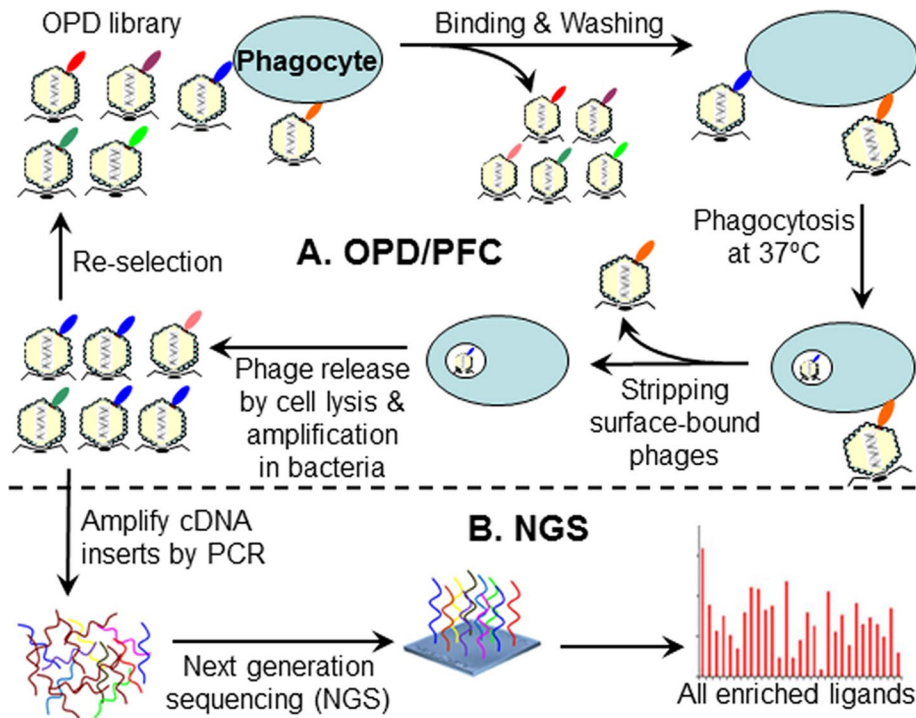


FIGURE 1: Schematic of OPD-NGS for systematically identifying phagocytotic ligands. (A) OPD/PFC selection. The OPD cDNA library bound to D407 RPE cells as phagocytes at 4°C. After being washed, bound phages were phagocytosed at 37°C for 30 min. Surface-bound unphagocytosed phages were removed by stripping with low-pH isotonic buffer. Internalized phages were released, amplified, and used as input for the next round of OPD/PFC selection. (B) NGS analysis. After three rounds of selection, the cDNA inserts of enriched clones were amplified by PCR and identified by NGS.

segments (POs) convert light to electric impulses in the retina but are susceptible to photo-oxidation and metabolic damage. To renew POs, photoreceptors shed old POs at their tips in a diurnal rhythm. Shed POs are phagocytosed by underlying RPEs for nutrient recycling and PO regeneration at their bases. The importance of RPE phagocytosis is highlighted by MerTK, whose mutations cause RPE phagocytotic defect and accumulation of unphagocytosed POS vesicles, thereby leading to retinal degeneration (Dowling and Sidman, 1962; Gal *et al.*, 2000). RPE aging results in reduced phagocytotic activity (Katz and Robison, 1984) and is implicated in age-related macular degeneration (AMD; Li, 2013). Systematic identification of phagocytotic ligands will provide molecular insights into abnormal structures on the cargo surface, cargo recognition, engulfment regulation, and phagocytotic dysfunction in diseases or aging conditions.

ATP-binding cassette subfamily F member 1 (ABCF1, ABC50) is a member of the ATP-binding cassette (ABC) family proteins (Klein *et al.*, 1999). Unlike most ABC proteins, ABCF1 does not possess membrane-spanning domains. Similar to other ABC members, ABCF1 contains two ABCs. The protein was first identified as a tumor necrosis factor α -inducible gene in synoviocytes (Richard *et al.*, 1998), and subsequently copurified with eukaryotic translation initiation factor eIF2 (Tyzack *et al.*, 2000). Compared with other ABC membrane transporters, however, the functional roles of ABCF1 are poorly defined.

In this study, we identified ABCF1 as a putative phagocytotic ligand by a newly developed approach of functional screening. How can intracellular ABCF1 function as an extracellular phagocytotic ligand? To tackle this question, we independently characterized

ABCF1 as a genuine RPE phagocytotic ligand with multiple criteria. The knowledge provides a new insight into phagocytotic regulation and cargo recognition. Finally, the innovative approach developed in this study should be broadly applicable to different phagocytes to advance our understanding of their extrinsic regulation.

RESULTS

Identification of ABCF1 as a new phagocytotic ligand

Phagocytotic ligands are traditionally identified on a case-by-case basis and present technical challenges. We recently developed open reading frame phage display (OPD) as a new technology of functional proteomics (Li, 2012b) and further designed phagocytosis-based functional cloning (PFC) for unbiased identification of phagocytotic ligands (Li, 2012a). The validity of this new approach has been demonstrated by identifying and independently characterizing Tulp1 as an RPE phagocytotic ligand (Caberoy *et al.*, 2010a,c). In this study, we combined OPD/PFC selection with next-generation DNA sequencing (NGS) for efficient identification of phagocytotic ligands (Figure 1). We performed three rounds of OPD/PFC selection with D407 RPE cells (Caberoy *et al.*, 2009, 2010a). The cDNA inserts of enriched clones were amplified by PCR and globally identified by NGS. A total of 6,611,438 valid sequence reads were identified by NGS. All identified sequences were blasted against the National Center for Biotechnology Information CCDS database to identify possible ligands with internalization activity. Among the identified putative ligands with high copy numbers of cDNA inserts were ABCF1, Gas6, and Tulp1. The latter two are known phagocytotic ligands for MerTK receptor (Hall *et al.*, 2005; Caberoy *et al.*, 2010c), suggesting that OPD-NGS is a valid approach for high-throughput identification of phagocytotic ligands.

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Independent validation of ABCF1 as a phagocytotic ligand

To independently verify the finding, we expressed and purified ABCF1 as a maltose-binding protein (MBP) fusion protein (MBP-ABCF1; Supplemental Figure S1). The predicted molecular weight of MBP-ABCF1 is ~138.0 kDa. The purified protein has two bands at ~138 kDa and ~155 kDa. Anti-ABCF1 antibody recognized both bands by Western blot analysis, suggesting that both were MBP-ABCF1. The higher band may have additional modifications.

Bovine POS vesicles were prepared and labeled with pHrodo, a pH-sensitive fluorogenic dye that can be activated in acidic phagosomes to increase its fluorescence intensity (Caberoy *et al.*, 2012b). Coupled with confocal microscopy, pHrodo reliably distinguishes internalized cargoes from surface-bound unphagocytosed cargoes. Our results showed that MBP-ABCF1 stimulated phagocytosis of POSs by D407 RPE cells (Figure 2A). The superimposed confocal z-stack images with cognate bright fields in high magnification indicated that pHrodo signals were inside RPE cell bodies. Engulfed pHrodo signals were partially degraded within the time of phagocytotic assay, as degraded pHrodo was dispensed into the cytoplasm. Some undegraded cargoes inside RPE cells were still visible as

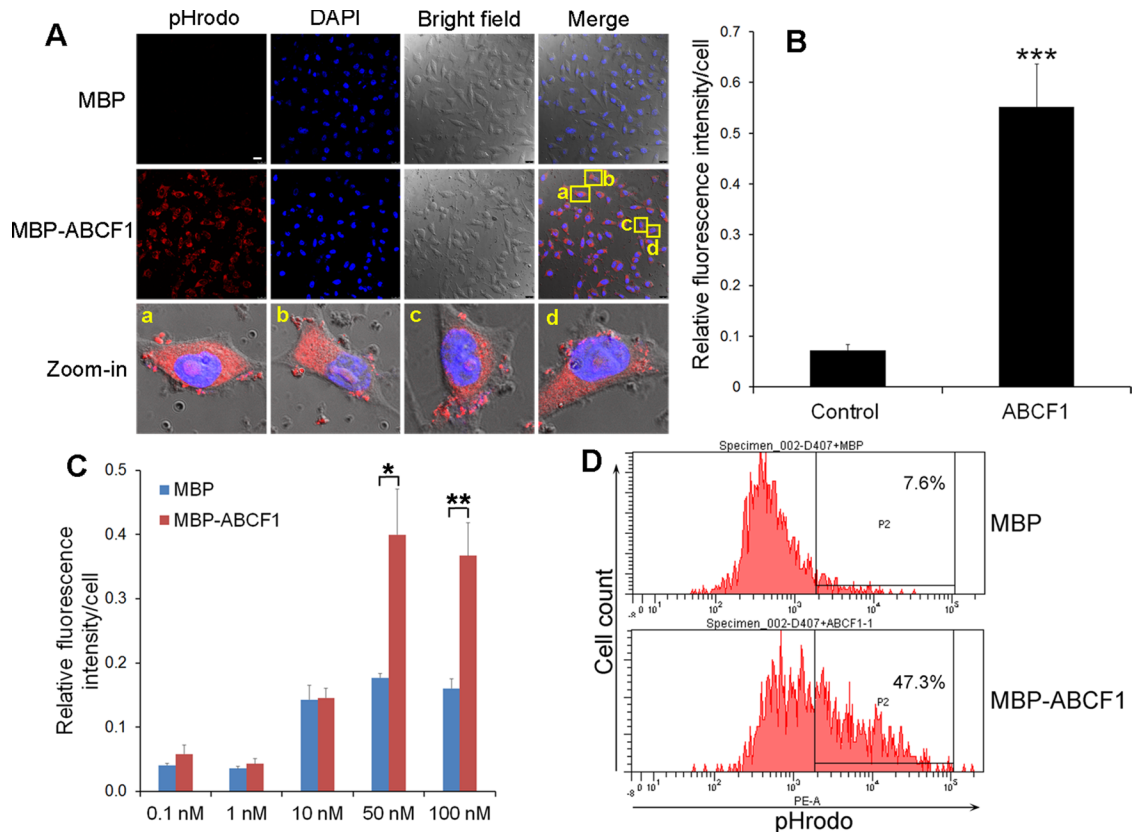


FIGURE 2: ABCF1 facilitates RPE phagocytosis. (A) Phagocytosis of pHrodo-labeled POS vesicles was performed in the presence of MBP-ABCF1 or MBP control (50 nM) with D407 RPE cells. The same z-stack images of internalized POS vesicles (red) and nuclei (blue) were superimposed with the cognate bright fields to show the internalized cargoes. (B) Relative fluorescence intensity of internalized pHrodo in A was quantified ($n = 10$). (C) ABCF1 stimulates RPE phagocytosis in a dose-dependent manner. RPE phagocytosis was performed as in A, with increased concentration of MBP-ABCF1 and MBP control, and quantified as in B ($n = 5$). (D) Quantification by flow cytometry. RPE phagocytosis was performed as in A and analyzed by flow cytometry. Mean \pm SEM, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Scale bar: 50 μ m.

highly condensed pHrodo signals. Quantification of internalized pHrodo signals on confocal images revealed that ABCF1 significantly induced RPE phagocytosis (Figure 2B). Control MBP elicited minimal phagocytosis. ABCF1 facilitated RPE phagocytosis in a dose-dependent manner (Figure 2C). Additional quantification by flow cytometry confirmed that ABCF1 facilitated RPE phagocytosis (Figure 2D). Moreover, ABCF1 was independently verified as a phagocytosis ligand with binding activity to D407 RPE cells by flow cytometry (Supplemental Figure S2). Tubby was previously characterized as an RPE phagocytotic ligand (Caberoy *et al.*, 2010c) and showed a capacity to stimulate RPE phagocytosis (Supplemental Figure S3). Tubby was reported to synergistically stimulate Tulp1-mediated RPE phagocytosis (Caberoy *et al.*, 2010a). However, no synergy was observed between tubby and ABCF1 (Supplemental Figure S3).

We further verified ABCF1 as a phagocytotic ligand with primary RPE cells. The results showed that MBP-ABCF1 preferentially stimulated POS phagocytosis by primary RPE (Figure 3A). Quantitative analysis indicated a statistical difference in RPE phagocytosis for ABCF1 versus control (Figure 3B).

It is possible that long-term culture of the D407 cell line and primary RPE cells *in vitro* may alter RPE phenotype and phagocytotic behavior. To minimize the effect of long-term culture, we prepared fresh RPE explants, in which RPE cells remained attached to Bruch's membrane *in situ*. The results showed that ABCF1 induced

POS phagocytosis by RPE explants with statistical significance (Figure 3, C and D).

Engulfment through the phagocytic pathway

To verify that ABCF1-mediated ingestion of pHrodo-labeled POSs was via a phagocytic pathway, we analyzed the colocalization of the internalized vesicles with the phagosome marker Rab7. Immunocytochemistry showed that internalized POS vesicles in D407 RPE cells were colocalized with Rab7 (Figure 4). A similar colocalization pattern of pHrodo signal and Rab7 was observed for tubby-mediated RPE phagocytosis. These results suggest that the engulfed cargoes were targeted to a phagocytic pathway.

Extracellular trafficking

ABCF1 is a cytoplasmic protein without a classical signal peptide. Indeed, Figure 5A shows that ABCF1 was not detected in the conditioned medium of healthy cells. A critical question is how an intracellular protein can access extracellular cargoes and phagocyte surface receptors. Previous studies indicated that cytoplasmic proteins can be secreted through conventional/unconventional pathways or released from apoptotic cells to function as phagocytotic ligands (Arur *et al.*, 2003; Li, 2012a). Figure 5A shows that ABCF1-FLAG expressed in HEK293 cells was released into culture medium from apoptotic but not healthy cells. As a control, intracellular green fluorescent protein (GFP)-FLAG was also released only from apoptotic cells.

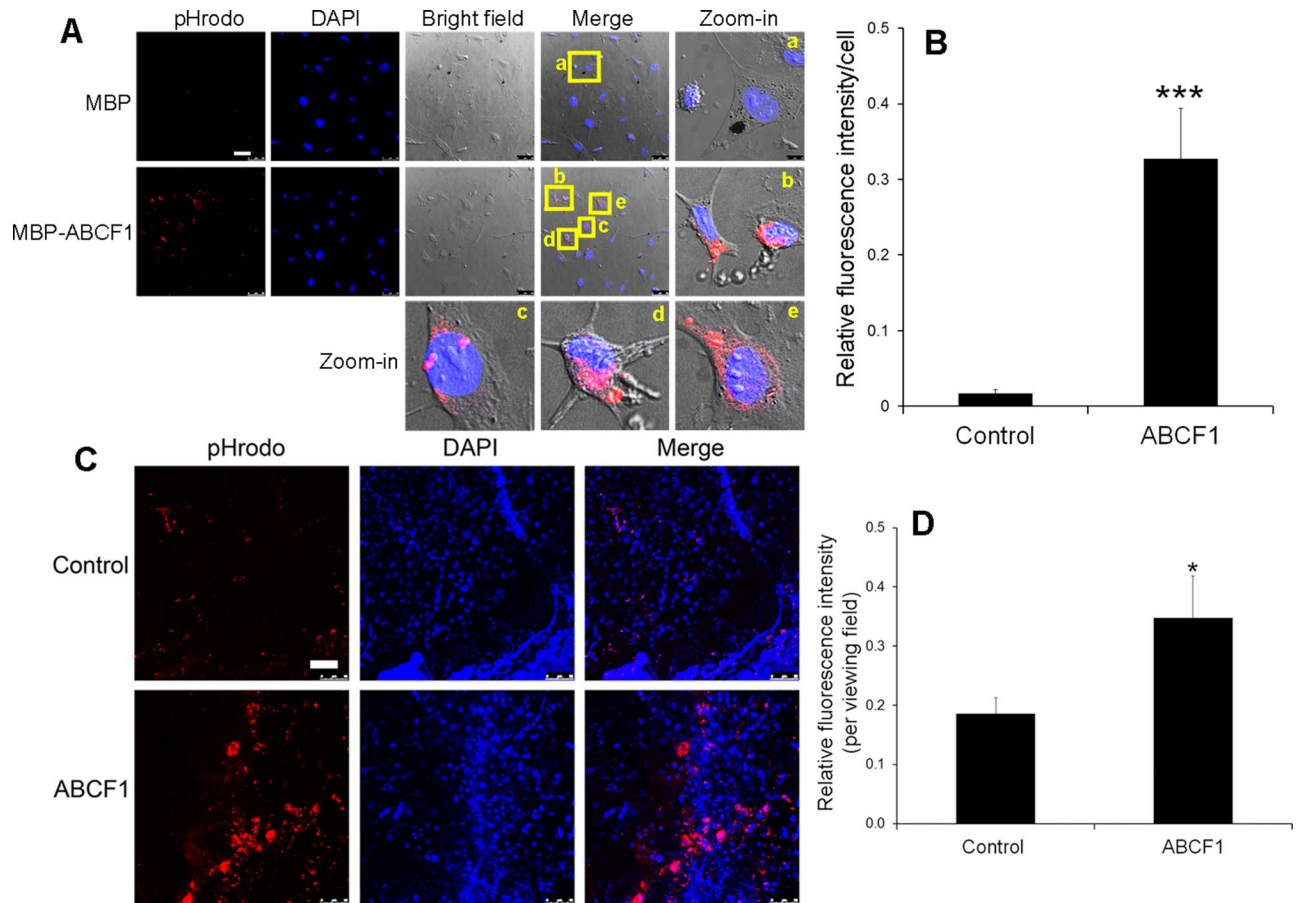


FIGURE 3: ABCF1 stimulation of RPE phagocytosis is independently validated with primary RPE cells and RPE explants. (A) Mouse primary RPE cells were prepared from neonatal mouse retina and incubated with pHrodo-labeled POS vesicles for phagocytosis assay in the presence of MBP-ABCF1 or MBP (50 nM). After washing, phagocytosed pHrodo signals were analyzed by confocal microscopy. (B) Relative fluorescence intensity of internalized pHrodo in A was quantified ($n = 10$). (C) RPE explants were used for phagocytosis assay, as in A. Highest pHrodo signals in each viewing field are presented. (D) Quantification of internalized pHrodo signal in C ($n = 5$). Mean \pm SEM, *, $p < 0.05$; ***, $p < 0.001$. Scale bar: 50 μm .

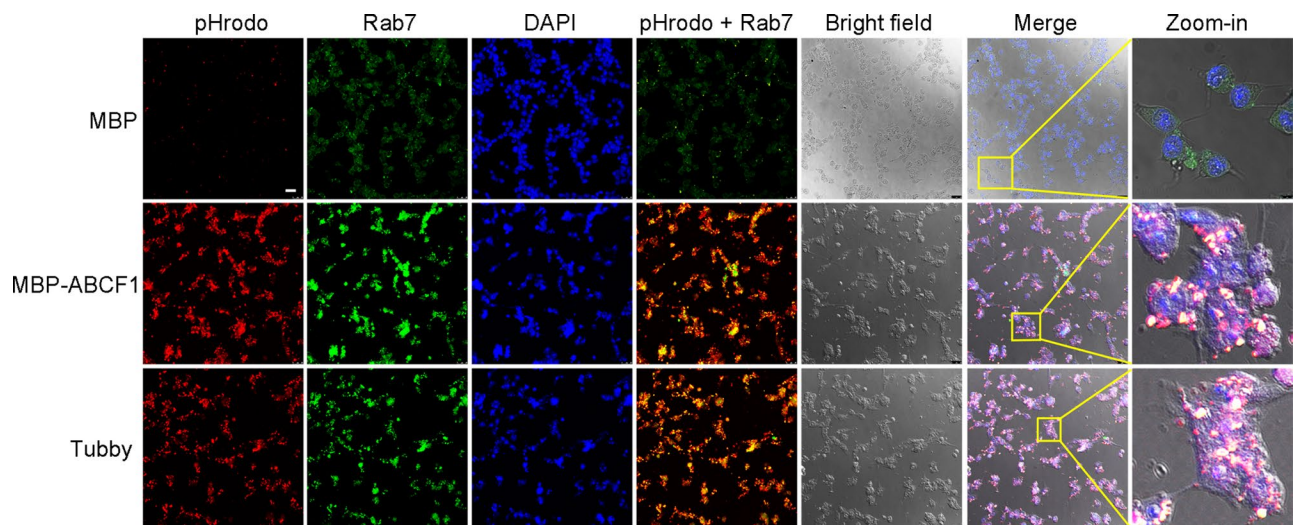


FIGURE 4: ABCF1 induces RPE engulfment of POSs via a phagocytic pathway. D407 RPE cells were incubated with pHrodo-labeled POSs for phagocytosis in the presence or absence of MBP-ABCF1, tubby, or MBP (50 nM), as in Figure 2A. Phagosome marker Rab7 was detected by immunocytochemistry and colocalized with ingested pHrodo signals. Scale bar: 25 μm .

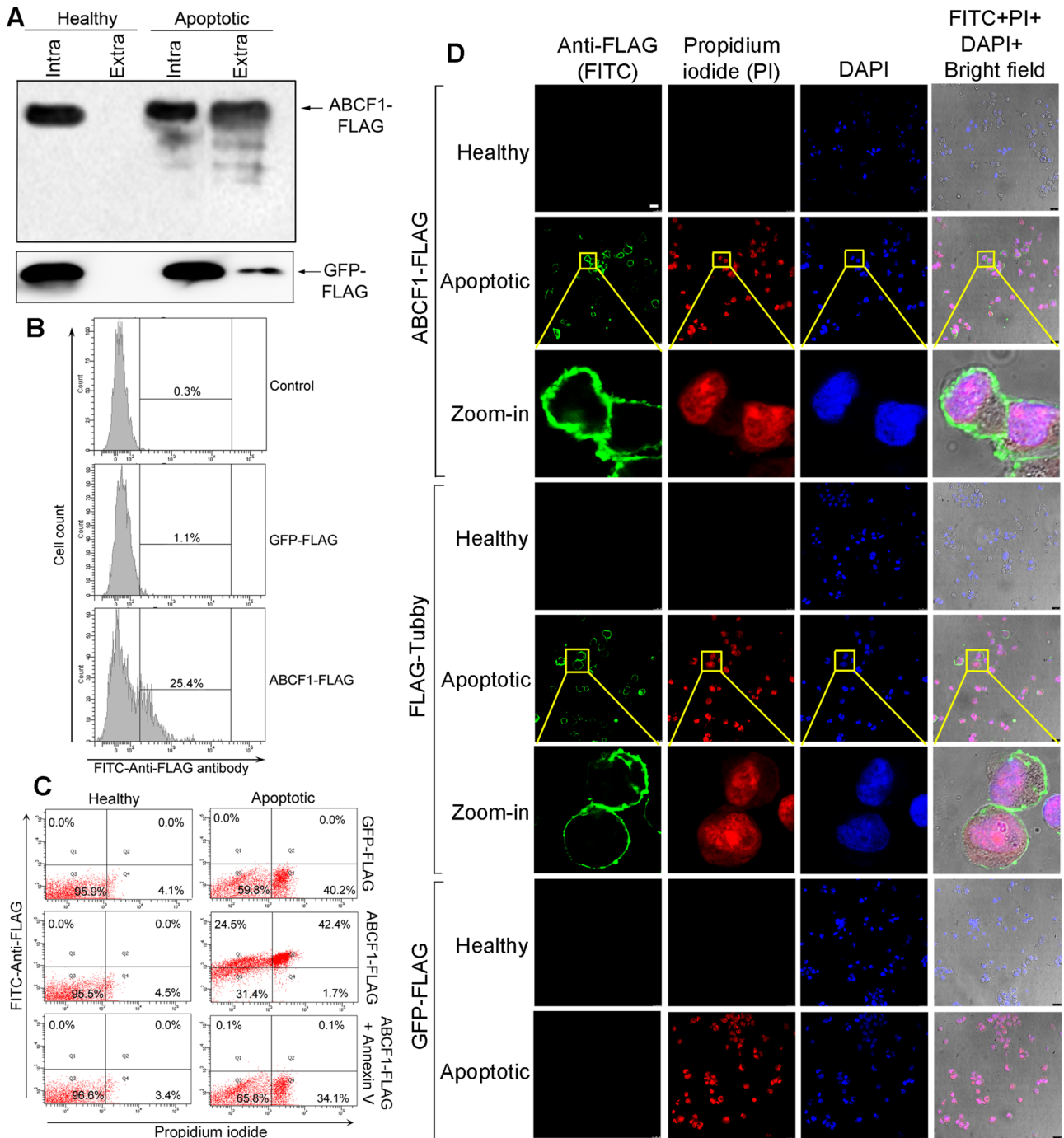


FIGURE 5: ABCF1 selectively binds to phagocytotic cargoes but not healthy cells. (A) ABCF1 is released from apoptotic cells. HEK293 cells were transfected with ABCF1-FLAG or GFP-FLAG plasmid and induced for apoptosis. The conditioned medium was collected, concentrated, and analyzed by Western blotting using anti-FLAG antibody. (B) ABCF1-FLAG binding to POS vesicles was detected by flow cytometry using FITC-labeled anti-FLAG antibody. GFP-FLAG was a negative control. (C) ABCF1 binds to phosphatidylserine on apoptotic cells. ABCF1-FLAG was incubated with apoptotic or healthy cells in the presence or absence of annexin V (10 μ g/ml) and analyzed by flow cytometry. (D) ABCF1 selectively binds to the surface of apoptotic cells but not healthy cells. ABCF1-FLAG, FLAG-Tubby, or GFP-FLAG lysate was incubated with apoptotic and healthy HEK293 cells, washed, and detected by confocal microscopy using FITC-anti-FLAG antibody. Apoptotic cells were labeled with propidium iodide. Scale bar: 25 μ m.

Selective binding to phagocytotic cargoes but not healthy cells

Phagocytotic ligands should recognize their cargoes. Shed POSs are the intended cargoes for RPE. We analyzed ABCF1 binding to shed POS vesicles by flow cytometry. The results showed that

ABCF1-FLAG but not GFP-FLAG bound to shed POSs (Figure 5B), suggesting that ABCF1 can recognize POS vesicles.

An important criterion of phagocytotic ligands is their selective recognition of phagocytotic cargoes but not healthy cells. This selective binding is necessary to avoid “friendly fire”—the

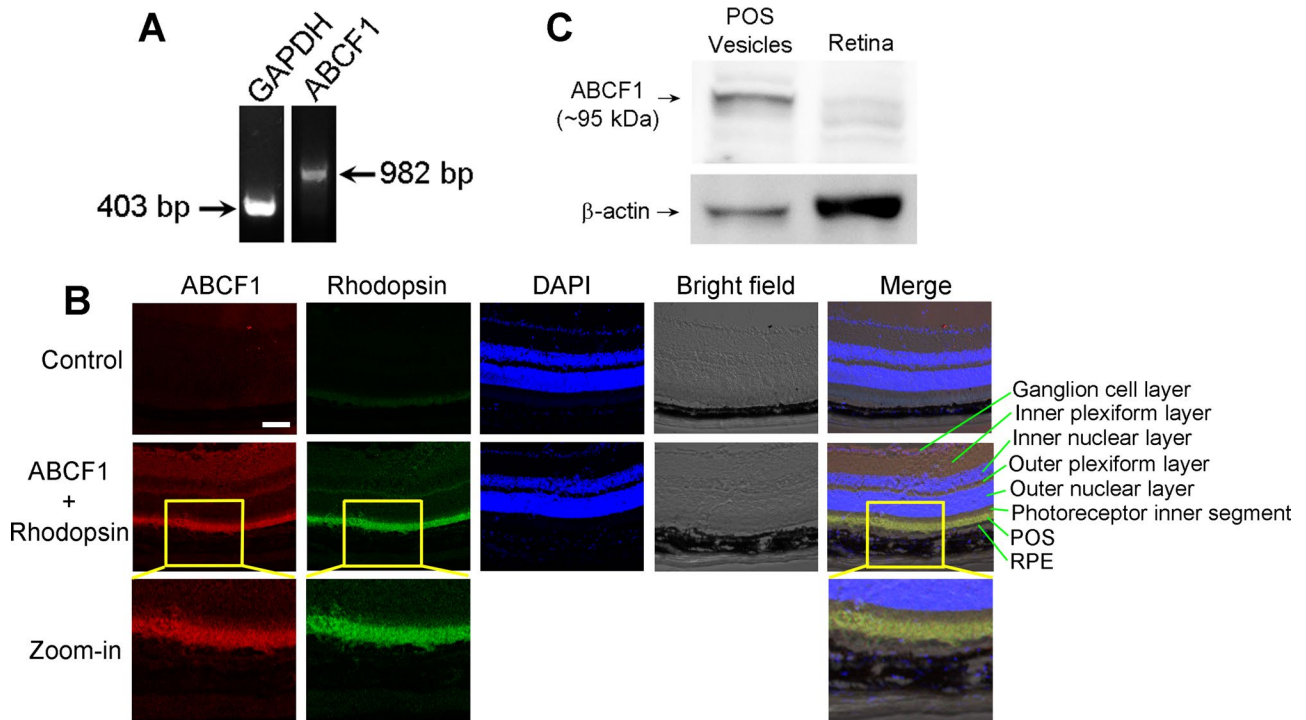


FIGURE 6: ABCF1 has the access to RPE phagocytotic site. (A) ABCF1 expression in the retina was detected by RT-PCR. GAPDH was a positive control. (B) ABCF1 is predominantly expressed in POSs and colocalized with the POS marker rhodopsin. Low levels of ABCF1 were also detected in the retinal ganglion cells, inner plexiform layer, outer plexiform layer, and photoreceptor inner segments. No ABCF1 staining was found in the inner and outer nuclear layers. (C) Expression of ABCF1 in the retina and shed POSs was detected by Western blotting (50 μ g protein/lane). Scale bar: 50 μ m.

indiscriminate engulfing of healthy, live cells (Brown and Neher, 2014). Because POS vesicles are the plasma membrane shed from photoreceptors, we investigated whether ABCF1 can selectively recognize apoptotic cells. Indeed, our results confirmed that ABCF1-FLAG bound only to apoptotic but not healthy cells (Figure 5C). GFP-FLAG bound to neither apoptotic nor healthy cells. Excessive annexin V blocked ABCF1-FLAG binding to apoptotic cells, suggesting that ABCF1 recognizes externalized phosphatidylserine during apoptosis. Selective binding of ABCF1-FLAG to apoptotic cells was further verified by confocal microscopy and detected as ring-like z-stack images (Figure 5D, ABCF1/Anti-FLAG/Zoom-in), which perfectly aligned with the RPE plasma membrane. Few intracellular fluorescein isothiocyanate (FITC) signals of ABCF1-FLAG were detected. In contrast, engulfed pHrodo signals in Figures 2A, 3A, and 4 and Supplemental Figure S3 were located inside RPE cells. As a positive control, tubby showed a similar ring-like binding pattern to the surface of apoptotic cells but not healthy cells (Figure 5D, tubby/Anti-FLAG/Zoom-in). GFP-FLAG did not bind to apoptotic or healthy cells. Taken together, the results seen in Figure 5, A–D, suggest that ABCF1 can be released from and selectively bind to apoptotic cells in an autocrine manner to facilitate phagocytosis.

Local expression of ABCF1 for access to the RPE phagocytotic site

Phagocytotic ligands should have access to their phagocytotic receptors and cargoes. To prove that ABCF1 has access to RPE and shed POSs, we characterized ABCF1 expression in the retina. ABCF1 expression in the retina was verified by reverse transcription PCR (RT-PCR; Figure 6A). Immunohistochemistry revealed that ABCF1 is expressed in the retinal ganglion cells (RGCs), inner plexiform layer,

outer plexiform layer, photoreceptor inner segments, and POSs, with the highest expression in POSs (Figure 6B). ABCF1 is minimally expressed in the inner and outer nuclear layers. The predominant expression of ABCF1 in POSs was further verified by Western blot (Figure 6C). It is worth noting that POSs as a special cellular compartment have a different level of β -actin compared with retinal homogenate. Although the same amount of protein was loaded for POSs and retina in Figure 6C, much less β -actin was detected in POSs than in the retina. Nonetheless, the high level of ABCF1 in POSs was demonstrated in Figure 6C. The predominant expression of ABCF1 in POSs provides a convenient means for the phagocytotic ligand to access to shed POSs and RPE cell surface. The importance of ABCF1 expression in POSs in its biological relevance as an RPE phagocytotic ligand is elaborated in the *Discussion*.

ABCF1 stimulates microglial phagocytosis of apoptotic but not healthy neurons

RPE shares many conserved pathways with other phagocytes. To investigate whether ABCF1 is capable of promoting microglial phagocytosis, we labeled apoptotic and healthy neurons with pHrodo and incubated them with BV-2 microglial cells in the presence of MBP-ABCF1 or MBP for phagocytosis. MBP-ABCF1 significantly induced microglial phagocytosis of apoptotic neurons but not healthy neurons (Figure 7, A and B). These findings were confirmed by flow cytometry (Figure 7C). These results suggest that the ABCF1 pathway is highly conserved among different phagocytes.

DISCUSSION

In this study we identified and characterized intracellular ABCF1 as a novel ligand that extrinsically regulates RPE phagocytosis. Copurification of ABCF1 with eIF2 (Tyzack *et al.*, 2000) suggests that

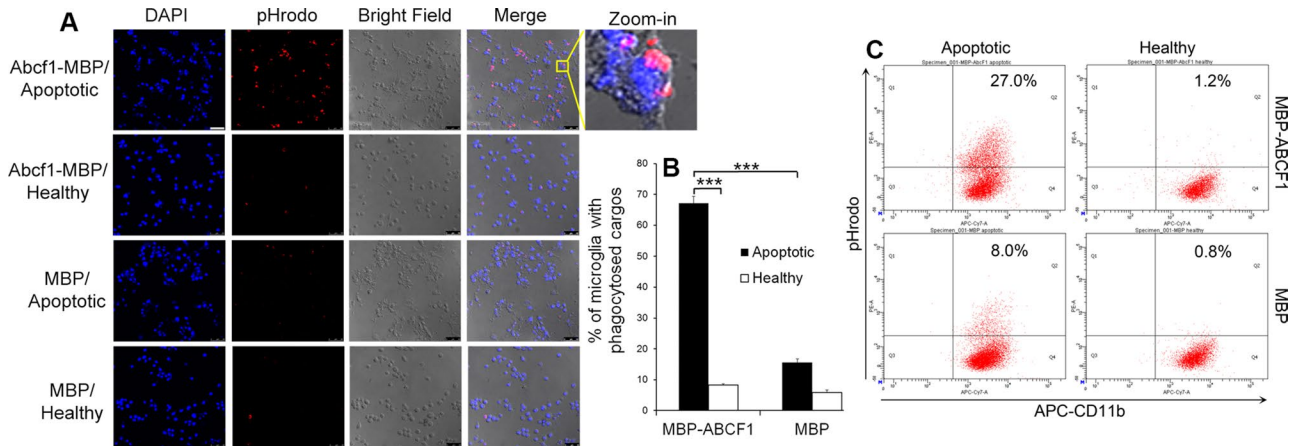


FIGURE 7: ABCF1 induces microglial phagocytosis of apoptotic but not healthy cells. BV-2 microglial cells were incubated with pHrodo-labeled apoptotic or healthy Neuro-2A cells in the presence of MBP-ABCF1 or MBP (50 nM). Phagocytosed pHrodo signals were analyzed by confocal microscopy (A) and quantified (B) as in Figure 2. (C) Microglial phagocytosis was performed as in A and quantified by flow cytometry. Mean \pm SEM, ***, $p < 0.001$, $n = 3$. Scale bar: 50 μ m.

ABCF1 is a cytoplasmic protein. Indeed, no ABCF1 was detected in a culture medium of healthy cells (Figure 5A). Immunohistochemistry further showed that ABCF1 is expressed only in retinal cytoplasmic compartments, such as the inner plexiform layer, outer plexiform layer, photoreceptor inner segments, and POSs, but not in the inner and outer nuclear layers (Figure 6B). Many proteins in photoreceptors, such as rhodopsin, are translated in the inner segments and transported into POSs through the connecting cilia (Deretic, 1998). Protein translation in POSs, if any, should be minimal. It is unlikely that ABCF1 predominantly expressed in POSs is for regulation of protein translation via eIF2.

Unlike mobile professional phagocytes, such as macrophages and microglia, RPE is a stationary nonprofessional phagocyte (Strauss, 2005). POSs are shed directly from photoreceptors into the pockets of RPE microvilli for phagocytosis (Figure 8). The geographical location of phagocytotic ligands is of critical importance to their biological relevance. Conceivably, a ligand exclusively expressed in RGCs or the inner plexiform layer may not be physiologically relevant for regulation of RPE phagocytosis for rapid clearance of shed POSs due to the distance and barrier of multiple retinal cell layers (Figure 6B). The predominant expression of ABCF1 in POSs provides geographical relevance to RPE phagocytosis.

This study demonstrated that intracellular ABCF1 can be released from apoptotic cells (Figure 5A). The Finnemann lab reported that phosphatidylserine is externalized and displayed only on the surface of POS tips as on apoptotic cells (Ruggiero *et al.*, 2012), suggesting that the tips of POSs before shedding may go through apoptosis-like membrane structural changes. These results imply that intracellular ABCF1 may be released through POS tips or from shed POS vesicles. Furthermore, our data showed that ABCF1 can bind to shed POSs and facilitate their phagocytosis by RPE. Given these data, we propose that ABCF1 is released from and binds to shed POSs in an autocrine manner to facilitate their clearance by RPE as a phagocytotic ligand (Figure 8).

A recent study characterized ABCF1 as a regulator of the innate immune response by binding to DNA-sensing HMGB2 (Lee *et al.*, 2013), one of the high-mobility groups of proteins (HMGs) belonging to damage-associated molecular pattern molecules (DAMPs) (Amin and Islam, 2014). ABCF1 was previously reported as one of

the two critical genes in the human leukocyte antigen region associated with susceptibility to autoimmune pancreatitis (Ota *et al.*, 2007). Rapid clearance of apoptotic cells by phagocytes is critical for preventing the release of intracellular contents, such as self-antigens and DAMPs, to trigger inflammation and autoimmune response (Erwig and Henson, 2007; Hochreiter-Hufford and Ravichandran,

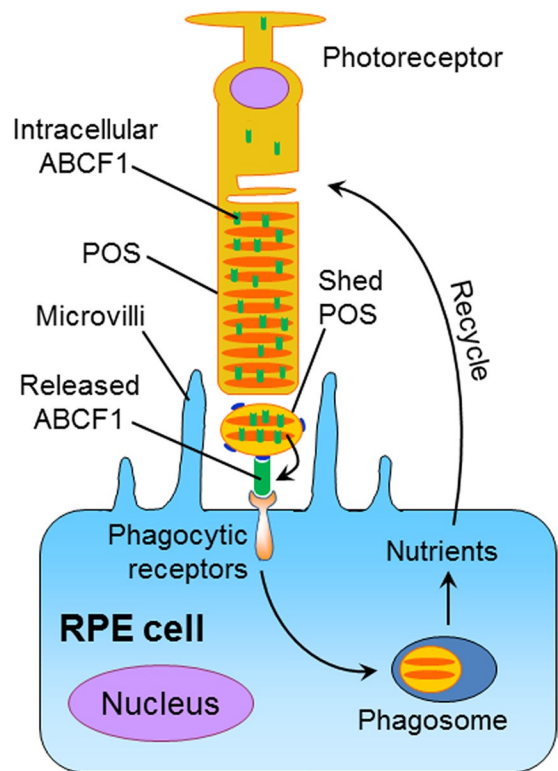


FIGURE 8: Cartoon model to illustrate how intracellular ABCF1 extrinsically regulates RPE phagocytosis. ABCF1 is predominantly expressed inside POS and released from shed POS vesicles. Released ABCF1 binds to POS vesicles and facilitates their engulfment by RPE in an autocrine manner.

2013). RPE shares many conserved phagocytotic pathways, including ABCF1, with macrophages and microglia (Figure 7). It is possible that ABCF1 regulates the immune response by facilitating clearance of apoptotic cells and HMGB2 by professional phagocytes.

Phagocytotic ligands are the key to defining functional roles of phagocytes. For example, galectin-3, a well-known binding protein of advanced glycation end products (AGEs), was recently identified by OPD as a bridging molecule for MerTK receptor (Caberoy *et al.*, 2012a). This implies that increased AGE modifications on proteins or the cell surface due to aging or diabetes with hyperglycemia may serve as eat-me signals for phagocytic clearance through the galectin-3-MerTK pathway. Other metabolic molecules, such as A β in Alzheimer's patients and oxidized lipids in patients with smoking or oxidative stress, may also function as eat-me signals in various physiological and pathological conditions (Sun *et al.*, 2006; Lee and Landreth, 2010; Sierra *et al.*, 2013). These abnormal molecules on cargo surfaces highlight the fact that the challenge in phagocytosis research is not only to determine what cargoes are cleared by phagocytes but, more importantly, to understand how these cargoes are recognized. The latter knowledge will shed new light on eat-me signals in various diseases with different profiles of metabolic modifications and provide molecular insight for therapeutic interventions, including future ligand-based phagocytotic therapy.

The question, then, is how to identify unknown eat-me signals. Phagocytotic ligands such as bridging molecules are valuable molecular probes for elucidating unknown eat-me signals but are traditionally identified in individual cases with technical challenges. OPD/PFC was recently developed as a new technology of functional proteomics for unbiased identification of phagocytotic ligands (Caberoy *et al.*, 2010a; Caberoy and Li, 2012; Li, 2012a). The main distinction between OPD and conventional phage display is that OPD can identify real endogenous ligands, as opposed to out-of-frame unnatural peptides (Li and Caberoy, 2010; Li, 2012b). However, manual screening of individual enriched clones is a bottleneck for efficient identification of unknown ligands (Caberoy *et al.*, 2010a). The combination of OPD with NGS in this study enables high-throughput identification of phagocytotic ligands for the first time.

Like many other proteomics technologies, however, OPD-NGS is not a perfect technology and has several limitations. OPD-NGS can identify only protein ligands but not nonprotein ligands, such as phosphatidylserine or AGEs. Similar to OPD, OPD-NGS as a bacterial display system cannot identify some ligands requiring posttranslational modifications for receptor binding. OPD-NGS may miss some true positives due to possible protein misfolding or inadequate representation in the OPD cDNA library. Owing to possible false positives, not all enriched clones with internalization activity encode and display genuine phagocytotic ligands. We propose the following criteria for phagocytotic ligands: 1) an ability to activate their cognate receptors on phagocytes for cargo engulfment; 2) selective recognition of phagocytotic cargoes but not healthy cells; 3) ability to link the cargoes to phagocytes; 4) extracellular trafficking via conventional or unconventional secretion from healthy cells or passive release from apoptotic cells; and 5) access to phagocytotic cargoes and phagocyte surface receptors. Therefore all putative ligands identified by OPD-NGS should be independently validated for their biological relevance with these criteria. ABCF1 meets all these criteria as a phagocytotic ligand, even though its phagocytic receptor(s) on RPE and eat-me signal(s) on POSs have yet to be identified.

Despite the above limitations, OPD-NGS is the only available technology for high-throughput identification of unknown phagocytotic

ligands in the absence of receptor information. The validity of this new approach is supported not only by Gas6 and Tulp1 but also by independent characterization of ABCF1. This will lead to systematic elucidation of molecular phagocyte biology, as discussed in our previous reviews (Caberoy and Li, 2012; Li, 2012a). More importantly, the copy number of cDNA inserts identified by OPD-NGS may reflect the relative abundance of enriched clones or the internalization activity of displayed ligands. We are currently investigating the reliability of OPD-NGS for activity quantification of the entire phagocytotic ligand profile. This new approach of quantitative functional proteomics may enable activity comparison of entire ligand profiles for diseased or aged phagocytes to systematically elucidate mechanistic details of RPE phagocytotic dysfunction in diseases or aging conditions. The new approach is broadly applicable to many other phagocytes, such as macrophages, microglia, and Sertoli cells.

MATERIALS AND METHODS

Cell culture

Human D407 RPE cells, HEK293, BV-2, and Neuro-2A cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1 mM L-glutamine.

Primary RPE cells

Primary RPE cells were prepared as previously described (Caberoy *et al.*, 2010a) with the following modifications. The eyes of killed C57BL/6 mice at postnatal day 10 were isolated. After removal of the cornea, lens, and retina, the RPE cups were digested with trypsin for 3 min at 37°C. RPE cells were collected by pipetting; washed; and cultured in Minimum Essential Medium Eagle (MEM) Alpha Modification (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS, 2 mM L-glutamine, 1 \times nonessential amino acids, 1 \times penicillin/streptomycin, basic fibroblast growth factor (bFGF, 10 ng/ml), epidermal growth factor (EGF, 1 ng/ml), 1 \times N1 supplement, and THT (taurine, 210 ng/ml; hydrocortisone 1.2 μ g/ml; tri-iodo-thyronine, 60 ng/ml; Sigma-Aldrich; Salero *et al.*, 2012). The medium was replaced every 2–3 d until pigmented RPE spheres grew out. The spheres were dissociated with trypsin, washed, and cultured as a monolayer in the same medium without bFGF and EGF for 3 d before phagocytosis assay.

All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Miami and complied with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH).

OPD-NGS analysis

OPD/PFC selection was done as previously described with minor modifications (Caberoy *et al.*, 2010a). Briefly, the OPD cDNA library of mouse eyes (Caberoy *et al.*, 2010b) was amplified in bacteria, precipitated with polyethylene glycol, resuspended in the above complete medium, and incubated with D407 RPE cells at 95% confluence for 30 min at 4°C. After being washed, the cells were incubated at 37°C for 30 min to allow bound phages to be phagocytosed. Surface-bound unphagocytosed phages were removed by stripping with low pH isotonic buffer for 2 min twice at room temperature. After washing, internalized phages were released by cell lysis in a hypotonic buffer (1 mM triethylamine, 0.5% Triton X-100), neutralized with phosphate-buffered saline (PBS), amplified in bacteria, and used as the input for the next round of OPD/PFC selection. After three rounds of selection, the cDNA inserts of enriched phages were amplified by PCR with primers 1 and 2 (Supplemental Table S1) and identified by NGS.

Plasmids

A cDNA clone of ABCF1 (GenBank accession #BC063094) was obtained from Open Biosystems/GE Healthcare (Lafayette, CO). For construction of the ABCF1-FLAG plasmid, the C-terminal region of ABCF1 was amplified by PCR with primers 3 and 4 (Supplemental Table S1), digested with *Clal* and *NotI*, and cloned into the same plasmid at *Clal* and *NotI* sites. For construction of a plasmid expressing MBP-ABCF1 fusion protein, the ABCF1 coding sequence was amplified with primers 5 and 6 (Supplemental Table S1), digested with *EcoRI* and *XhoI*, and cloned into pMAL-c4E plasmid (New England Biolabs, Ipswich, MA) at the *EcoRI* and *Sall* sites. The resulting plasmids were verified by sequencing. GFP-FLAG and FLAG-tubby plasmids were described previously (Caberoy *et al.*, 2010a).

ABCF1 purification

MBP-ABCF1 and control MBP were expressed in BL21(DE3) and purified using amylose columns, as previously described (Kim *et al.*, 2011). Purified MBP-ABCF1 and MBP were dialyzed against PBS and analyzed by SDS-PAGE and Western blotting.

POS vesicles

POS vesicles were prepared from bovine retinas as previously described (Caberoy *et al.*, 2010a). Briefly, fresh bovine eyes within 24-h postmortem were purchased from Pel-Freez Biologicals. POSs were detached from isolated retinas by gentle shaking at 4°C for 15 min in PBS containing 2.5% sucrose. After removal of the retinas, detached POS vesicles were collected and washed twice by centrifugation at $38,700 \times g$ for 30 min. Purified vesicles were labeled with pHrodo succinimidyl ester (Life Technologies, Grand Island, NY), as previously described (Caberoy *et al.*, 2012b). Briefly, POS vesicles (500 μ g protein) were incubated with pHrodo (20 ng/ml in PBS, stock 1 mg/ml in dimethyl sulfoxide) for 30 min at room temperature; this was followed by incubation with 1% bovine serum albumin in PBS for 15 min. The labeled vesicles were washed twice with PBS by centrifugation at $16,000 \times g$ for 30 min before the phagocytosis assay.

Phagocytosis by RPE cells

D407 RPE or primary RPE cells were seeded on coverslips precoated with poly-L-lysine (Sigma-Aldrich) in 12-well plates and cultured overnight. pHrodo-labeled POSs (50 μ g/ml) were added to RPE cells for phagocytosis in the presence of MBP-ABCF1 or MBP control at indicated concentrations for 3 h at 37°C. After being washed, the cells were fixed with 4% buffered paraformaldehyde for 10 min, mounted with 4',6-diamidino-2-phenylindole (DAPI), and analyzed by confocal microscopy or flow cytometry, as previously described (Caberoy *et al.*, 2010a). Intracellular pHrodo signals of confocal images were quantified by ImageJ software (NIH) and normalized against the cell number (i.e., DAPI spot number) per viewing field.

Phagocytosis by RPE explants

Eyes were enucleated from killed mice (4–5 wk old). After removal of the cornea, lens, and retina, RPE explants were incubated in the above primary RPE culture medium without bFGF and EGF for 24 h. Phagocytosis of pHrodo-labeled POS vesicles by RPE explants was performed as above for 16 h. After washing, RPE explants were fixed, stained with DAPI, flat-mounted on slides with coverslips, and analyzed by confocal microscopy for three-dimensional scanning. Z-stack images with the highest pHrodo signals for five nonoverlapping fields were taken for each group in a blind manner and quantified by ImageJ.

Phagocytosis by microglia

Neuro-2A cells were treated with or without etoposide (200 μ M) for 16 h to induce apoptosis (Caberoy *et al.*, 2010c), washed, and labeled with pHrodo as above. BV-2 cells were seeded on coverslips precoated with poly-L-lysine in 12-well plates, cultured to ~80% confluence, and incubated with pHrodo-labeled apoptotic and healthy Neuro-2A cells in the presence of MBP-ABCF1 or MBP for 3 h. After being washed, BV-2 cells were fixed, stained with DAPI, and analyzed by confocal microscopy or flow cytometry, as described in *Phagocytosis by RPE cells*.

Immunohistochemistry

Mice (C57BL/6, 6–8 wk of age) under anesthesia were intracardially perfused with 10% Formalin. After the mice were killed, the eyes were nucleated and fixed with the same solution overnight at 4°C. After removal of the cornea and lens, the eye cups were incubated with sucrose gradient solutions (10 and 20% for 3 h each; 30% for overnight) at 4°C; this was followed by three rounds of freeze-thaw and OCT (optimal cutting temperature) embedding. Frozen tissue sections of 7- μ m thickness were incubated with rabbit anti-ABCF1 (Abcam, Cambridge, MA) and mouse anti-rhodopsin antibodies (Millipore, Billerica, MA), followed by Alexa Fluor 594-labeled goat anti-rabbit immunoglobulin G (IgG) and FITC-conjugated goat anti-mouse IgG antibodies. The nuclei were visualized with DAPI. The fluorescence signals were analyzed using a fluorescence microscope.

RT-PCR

Total RNA was prepared from fresh mouse retinas (6 to 8 wk old). RT-PCR was performed as previously described (Caberoy *et al.*, 2010a) with primers 7 and 8 for ABCF1 and primers 9 and 10 for GAPDH (Supplemental Table S1). The PCR products were analyzed on a 1% agarose gel.

Western blotting

Bovine retinal homogenate and POS vesicles (50 μ g/sample) were analyzed by Western blotting using anti-ABCF1 antibody, as previously described (Li and Handschumacher, 2002).

ABCF1 extracellular trafficking

ABCF1-FLAG plasmid was transfected in HEK293T cells using jetPRIME reagents (Polyplus Transfection, Illkirch, France). After 48 h, the cells in 293SFM II medium (Life Technologies) were treated with or without etoposide for 16 h to induce apoptosis. The conditioned medium was collected from the apoptotic or healthy cells and centrifuged at $200 \times g$ for 10 min. The cell-free supernatant was concentrated by filter concentrator units (Pierce Biotechnology, Rockford, IL; 9-kDa molecular weight cutoff) and analyzed by Western blotting using anti-FLAG M2 monoclonal antibody (Sigma-Aldrich).

ABCF1 binding to phagocytotic cargoes

ABCF1-FLAG, FLAG-tubby, and GFP-FLAG were transfected into HEK293 cells as above. After 48 h, cell lysates were prepared without any detergent by three cycles of freeze-thaw, followed by centrifugation at $16,000 \times g$ for 20 min and filtration through a 0.2- μ m filter. New HEK293 cells were treated with or without etoposide to induce apoptosis as above. The cell lysates were incubated with the apoptotic or healthy cells for 1 h at 4°C. Excessive annexin V was added to block ABCF1 binding to the cell surface as indicated. After washing, cell-bound FLAG-tagged proteins were detected by FITC-labeled anti-FLAG antibody and analyzed by fluorescence microscopy or flow cytometry. Apoptotic cells were labeled with propidium iodide.

Alternatively, POS vesicles were incubated with ABCF1-FLAG cell lysate. After washing, bound ABCF1-FLAG was detected by flow cytometry using FITC-labeled anti-FLAG antibody.

ABCF1 binding to phagocytes

The cell lysate of ABCF1-FLAG or GFP-FLAG was incubated with D407 RPE cells at 4°C for 1 h. After being washed, cells were analyzed by flow cytometry using FITC-anti-FLAG antibody.

Statistical analysis

Data are expressed as means ± SEM and analyzed by unpaired Student's *t* test. Data were considered significant when $p < 0.05$.

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