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Tubby and tubby-like protein 1 are new MerTK ligands for phagocytosis

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Tubby and tubby-like protein 1 (Tulp1) are newly identified phagocytosis ligands to facilitate retinal pigment epithelium (RPE) and macrophage phagocytosis. Both proteins without classical signal peptide have been demonstrated with unconventional secretion. Here, we characterized them as novel MerTK ligands to facilitate phagocytosis. Tulp1 interacts with Tyro3, Axl and MerTK of the TAM receptor tyrosine kinase subfamily, whereas tubby binds only to MerTK. Excessive soluble MerTK extracellular domain blocked tubby- or Tulp1-mediated phagocytosis. Both ligands induced MerTK activation with receptor phosphorylation and signalling cascade, including non-muscle myosin II redistribution and co-localization with phagosomes. Tubby and Tulp1 are bridging molecules with their N-terminal region as MerTK-binding domain and C-terminal region as phagocytosis prey-binding domain (PPBD). Five minimal phagocytic determinants (MPDs) of K/R(X)$_{1-2}$KKK in Tulp1 N-terminus were defined as essential motifs for MerTK binding, receptor phosphorylation and phagocytosis. PPBD was mapped to the highly conserved 54 amino acids at the C-terminal end of tubby and Tulp1. These data suggest that tubby and Tulp1 are novel bridging molecules to facilitate phagocytosis through MerTK.

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Introduction

Tubby and tubby-like protein 1 (Tulp1) belong to tubby protein family with four members (tubby, Tulp1, 2 and 3; Tulps), which share the highly conserved C-terminal region of the 'tubby domain' with ~260 amino acids (Carroll *et al*, 2004). A spontaneous mutation in *tubby* gene causes adult-onset obesity, progressive retinal and cochlear degeneration in *Tubby* mice with undefined mechanisms, whereas mutations in Tulp1 associate with only retinal degeneration (Ikeda *et al*, 2002; Abbasi *et al*, 2008). Tubby and Tulp1 have been characterized with multiple intracellular functions. For example, tubby is associated with phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] on the inner leaflet of the plasma membrane through its C-terminal domain (Santagata *et al*, 2001). Receptor-mediated activation of G protein α_q releases tubby from the plasma membrane and triggers its translocation into the nucleus. Despite its nuclear translocation and putative C-terminal binding to double-stranded DNA (Ikeda *et al*, 2002), no target gene transcriptionally regulated by tubby has been identified. Tulp1 was reported to interact with F-actin and GTPase dynamin-1 (Xi *et al*, 2005, 2007). Tulp1$^{-/-}$ mice exhibit mislocalization of rhodopsin in the photoreceptors and malformation of photoreceptor synaptic ribbon (Hagstrom *et al*, 2001; Grossman *et al*, 2009).

We recently demonstrated unconventional secretion of Tulps (Caberoy and Li, 2009), suggesting that tubby and Tulp1 may function extracellularly as well. A new extracellular role of Tulp1 as a phagocytosis ligand for retinal pigment epithelium (RPE) and macrophage phagocytosis was delineated by a novel strategy of phagocytosis-based functional cloning (Caberoy *et al*, 2010a). This further led to the identification of tubby as the second phagocytosis ligand in the same family. The functional cloning strategy creatively exploited the unique functional characteristic of the phagocytes and the versatile application of open reading frame (ORF) phage display as a newly developed technology of functional proteomics (Li and Caberoy, 2010), and is the only available approach for unbiased identification of phagocytosis ligands. However, a potential concern is whether this new strategy is a legitimate approach, namely, whether Tulp1 identified by this new strategy is a genuine phagocytosis ligand. For this reason, we further characterized tubby and Tulp1 as phagocytosis ligands in this study and identified MerTK as a common receptor for both proteins.

MerTK belongs to TAM receptor tyrosine kinase (RTK) subfamily, which includes Tyro3 (Sky), Axl and MerTK (Hafizi and Dahlback, 2006b; Lemke and Rothlin, 2008). Among TAM RTKs, MerTK is the most critical RTK for macrophage and RPE phagocytosis, whereas Tyro3 and Axl have limited roles in phagocytosis (Seitz *et al*, 2007). For example, photoreceptor outer segments (POS) in the retina are susceptible to photooxidative damage. As part of the renewal process, aged or damaged POS are shed at the tip of the outer segments in a diurnal rhythm, and phagocytosed by RPE cells underneath photoreceptors for recycling (Strauss, 2005). Mutations in MerTK cause defective RPE phagocytosis, leading to accumulation of unphagocytosed debris and retinal degeneration (Bok and Hall, 1971; D'Cruz *et al*, 2000).

Gas6 and protein S are the only two known MerTK ligands and function as bridging molecules to facilitate phagocytosis with their C-terminal two globular laminin G-like domains (2 × LG) binding to MerTK and N-terminal Gla domain of

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γ-carboxyglutamic acid residues interacting with phosphatidylserine on apoptotic cells (Hafizi and Dahlback, 2006a; Lemke and Rothlin, 2008). Gas6 and protein S have different receptor-binding specificity. Gas6 binds to all three TAM RTKs, whereas protein S interacts only with MerTK and Tyro3 (Hafizi and Dahlback, 2006b). However, it is unclear whether there is any other unknown MerTK ligand capable of stimulating phagocytosis.

Here, we demonstrate that tubby and Tulp1 are new MerTK ligands and facilitate phagocytosis in a MerTK-dependent manner. Tubby specifically binds to MerTK, whereas Tulp1 interacts with all three RTKs in the TAM subfamily. Both proteins induced MerTK activation with receptor autophosphorylation, leading to myosine II redistribution in RPE cells and co-localization with phagocytosed cargos. Tubby and Tulp1 stimulated phagocytosis as Gas6-like bridging molecules with their N-terminal domain binding to MerTK and C-terminal domain interacting with apoptotic cells. We further defined five 'minimal phagocytic determinants' (MPDs) of $K/R(X)_{1-2}KK$ in the N-terminus of Tulp1 as essential motifs for MerTK binding, activation and phagocytosis. We mapped the 54 amino acids at the C-terminal end of tubby and Tulp1 as 'phagocytosis prey-binding domain' (PPBD). These data suggest that tubby and Tulp1 are new type of MerTK ligands to facilitate phagocytosis. The characterization of tubby and Tulp1 as genuine phagocytosis ligands validates the novel strategy of phagocytosis-based functional cloning as a valuable approach for unbiased identification of phagocytosis ligands.

Results

MerTK is a common phagocytic receptor for tubby and Tulp1

To identify the phagocytic receptor for tubby and Tulp1, we screened their interaction with several known receptors. The results showed that both tubby and Tulp1, but not Tulp2 and Tulp3, bound to Mer-Fc (MerTK extracellular domain fused to human IgG1 Fc domain) (Figure 1A), a profile matching their capacities to stimulate RPE phagocytosis (Caberoy *et al*, 2010a).

We then analysed the role of MerTK in tubby- and Tulp1-mediated RPE phagocytosis. To minimize the interference of endogenous tubby and Tulp1, we expressed recombinant tubby or Tulp1 in Neuro-2a or HEK293 cells, which co-expressed plasma membrane-targeted green fluorescent protein (mGFP). mGFP-labelled plasma membrane vesicles were prepared from tubby-expressing cells (i.e. tubby vesicles) or Tulp1-expressing cells, and analysed for tubby- or Tulp1-mediated RPE phagocytosis in ARPE19 cells in the presence or absence of excessive Mer-Fc. The results revealed that tubby vesicles and Tulp1 vesicles, but not control

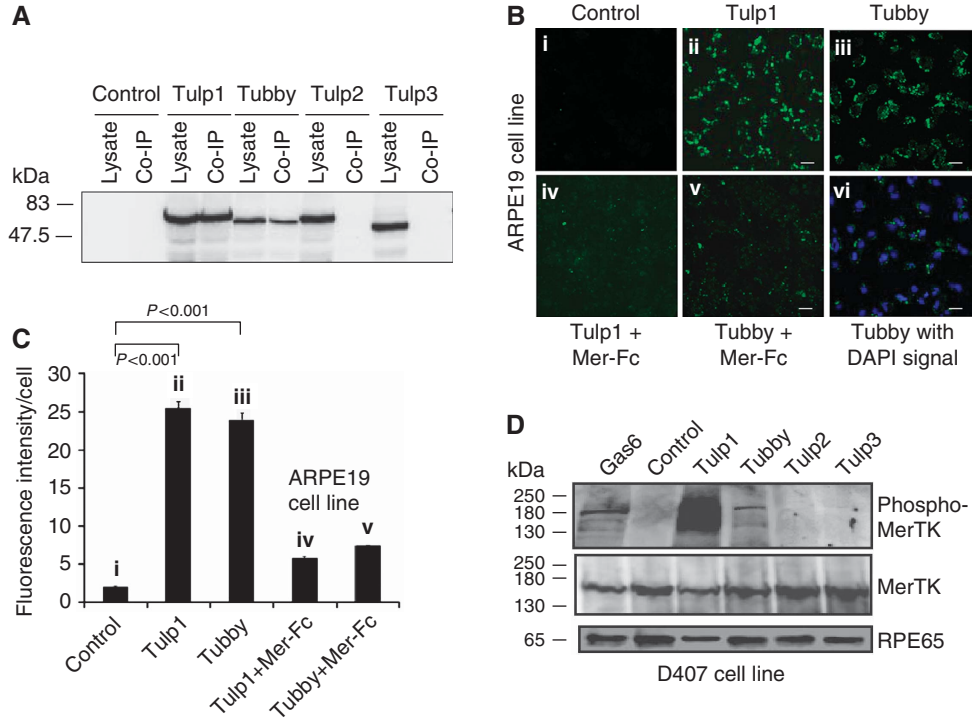

Figure 1 MerTK as a common phagocytic receptor for tubby and Tulp1. (**A**) Co-immunoprecipitation. FLAG-tagged Tulps were expressed in HEK293 cells. The cell lysates were prepared, incubated with Mer-Fc, co-immunoprecipitated with protein A resin and analysed by western blot using anti-FLAG mAb. (**B**) Tubby and Tulp1 stimulation of RPE phagocytosis is blocked by Mer-Fc. mGFP-labelled plasma membrane vesicles were prepared from tubby- or Tulp1-expressing Neuro-2a cells, preincubated in the presence or absence of Mer-Fc and analysed for phagocytosis in ARPE19 cells by confocal microscopy. Membrane vesicles prepared from cells transfected with pcDNA3 plasmid were included as a negative control. The panel (vi) is identical to (iii), but has DAPI signals for nuclei. DAPI signals in other confocal images are removed for better visibility of GFP signals. Similar results were obtained with HEK293 cells (not shown). Bar = 10 μm. (**C**) Relative fluorescence intensity per cells in (**B**) was quantified in >100 cells per group (± s.e.m., $n > 100$). (**D**) Tubby and Tulp1 induce MerTK phosphorylation. Phagocytosis was performed as in (**B**) in D407 RPE cells. MerTK phosphorylation was directly analysed by western blot using anti-phospho-MerTK, anti-MerTK or anti-RPE65 Ab. Gas6 (50 nM) was included as a positive control. The bottom two panels are sample loading controls.

vesicles, induced vigorous RPE phagocytosis (Figure 1B and C), as described (Caberoy *et al*, 2010a). Excessive Mer-Fc blocked RPE phagocytosis of tubby vesicles and Tulp1 vesicles. MerTK expression in RPE cell lines was verified by RT–PCR and western blot analyses (Supplementary Figure S1). Moreover, MerTK knockdown by small-hairpin RNA (shRNA) in J774 macrophages blocked tubby- and Tulp1-mediated phagocytosis of apoptotic cells (Supplementary Figure S2). These data suggest that both proteins stimulate RPE and macrophage phagocytosis in a MerTK-dependent manner.

Proteins capable of binding to receptors may not always be genuine ligands. Ligands bind to receptors through ligand-binding pockets and are able to trigger receptor activation and signalling cascades. However, false-positive binding to receptors elsewhere should not activate receptors. MerTK activation leads to phosphorylation of receptor tyrosine residues, which has been widely used as a surrogate marker for MerTK activation. To analyse whether tubby and Tulp1 are genuine ligands for MerTK, we performed phagocytosis in D407 RPE cells as in Figure 1B with tubby vesicles or Tulp1 vesicles, and analysed MerTK phosphorylation by western blot using anti-phospho-MerTK antibody (Ab). The results showed that tubby and Tulp1, but not Tulp2 and Tulp3, activated the receptor, leading to MerTK autophosphorylation (Figure 1D). Tulp1 induced more vigorous MerTK phosphorylation than tubby. Gas6 was included as a positive control. These results were validated with purified tubby and Tulp1 (Supplementary Figure S3).

Tubby and Tulp1 induce myosin II redistribution

Phagocytosis is initiated by ligands with receptor activation and signalling cascades, followed by cytoskeletal rearrangement and engulfment. For example, RPE phagocytosis of POS has been recently demonstrated through MerTK by directly recruiting non-muscle myosin II-A (NMMII-A) heavy chain to the engulfment site, leading to the redistribution and co-localization of NMMII-A with ingested POS (Strick *et al*, 2009). To further investigate MerTK-dependent signalling cascade induced by tubby- and Tulp1-mediated phagocytosis, we characterized the redistribution of NMMII-A heavy chain. mGFP-labelled tubby vesicles and Tulp1 vesicles induced NMMII-A redistribution and co-localization with phagocytosed vesicles, whereas the control vesicles with minimal phagocytosis had no effect on myosin II redistribution (Figure 2). Blockade with excessive Mer-Fc reduced tubby- or Tulp1-mediated co-localization of NMMII-A with the phagocytosed vesicles in ARPE19 cells. These data suggest that MerTK activation induced by tubby and Tulp1 facilitate MerTK-dependent cytoskeletal reorganization.

Cargo trafficking of tubby- and Tulp1-mediated internalization through phagocytic pathway

Tubby and Tulp1 are new phagocytosis ligands identified by phagocytosis-based functional cloning strategy. The question is whether tubby and Tulp1 facilitate the internalization of membrane vesicles via phagocytic pathways or non-specifically via other internalization pathways. Phagosome maturation is a highly conserved biological process and has been well characterized with various biomarkers for early and late phagosomes (Scott *et al*, 2003). We analysed the subcellular distribution of Lamp-1, a late phagosome marker, during tubby- or Tulp1-mediated vesicle internalization. The results showed that internalized tubby vesicles and Tulp1 vesicles were co-localized with Lamp-1 (Figure 3). Blockade with excessive Mer-Fc reduced tubby- or Tulp1-mediated co-localization of Lamp-1 with the phagocytosed vesicles. These data suggest that tubby and Tulp1 facilitate vesicle internalization via phagocytic pathway.

Minimal phagocytosis determinants as receptor-binding motifs

All four Tulps share highly conserved C-terminal tubby domain with diverse N-terminal regions (Ikeda *et al*, 2002). Given the failure of Tulp2 and Tulp3 to stimulate RPE phagocytosis (Caberoy *et al*, 2010a), we speculated that the divergent N-termini of tubby and Tulp1 may have unique sequence motif(s) for MerTK binding and phagocytosis. To define the unique structural basis in tubby and Tulp1, we constructed a series of phage clones expressing Tulp1 N-terminus with deletions and/or mutations and analysed the phagocytosis activity of the phage clones in ARPE19 cells (Figure 4A and B), as described (Caberoy *et al*, 2009). The results led to the prediction of five 'minimal phagocytosis determinants' of $K/R(X)_{1-2}KKK$ in Tulp1 N-terminus. Sequence analysis revealed that tubby has one MPD, whereas Tulp2 and Tulp3 have none, a pattern that matches to the profiles of their stimulation of RPE phagocytosis (Caberoy *et al*, 2010a).

To verify the importance of MPDs, we analysed MerTK-binding activity of MPD-null Tulp1, in which all five MPDs were mutated to $K/R(X)_{1-2}AAA$. We prepared membrane vesicles from Neuro-2a cells expressing individual FLAG-tagged Tulps or MPD-null Tulp1, and analysed Mer-Fc binding to the membrane vesicles immobilized on ELISA plates. The bound Mer-Fc was detected by horseradish peroxidase (HRP)-labelled goat anti-human IgG and quantified by colorimetric assay. The results showed that Mer-Fc bound to immobilized tubby vesicles, Tulp1 vesicles, but not to Tulp2 vesicles, Tulp3 vesicles and MPD-null Tulp1 vesicles (Figure 4C), indicating that MPDs are important for MerTK binding. The critical role of MPDs in Tulp1 interaction with MerTK was further verified with MPD-null Tulp1 by co-immunoprecipitation (Figure 4D). MPD-null Tulp1 failed to induce MerTK autophosphorylation (Figure 4E). Furthermore, the importance of MPDs was ultimately validated by drastic reduction in RPE phagocytosis of MPD-null Tulp1 vesicles (Figure 4F and G).

Receptor-binding specificity

To analyse the receptor-binding specificity of tubby and Tulp1, we characterized Tulps and MPD-null Tulp1 binding to Axl-Fc and Tyro3-Fc by co-immunoprecipitation. The results showed that Tulp1 interacted with MerTK, Axl and Tyro3 and that tubby bound only to MerTK (Figures 1A and 4H). MPD-null Tulp1 failed to bind to all TAM RTKs (Figure 4D and H), suggesting that five MPDs are essential for Tulp1 receptor-binding activity. Interestingly, Tulp2 was partially co-precipitated with Axl-Fc and Tyro3-Fc, but not with Mer-Fc (Figures 1A and 4H). These data suggest that tubby and Tulp1 have distinct receptor-binding specificity.

Tubby and Tulp1 as bridging molecules

As MerTK-binding MPDs are all located in the N-terminal region of tubby and Tulp1, the question is why mutations in

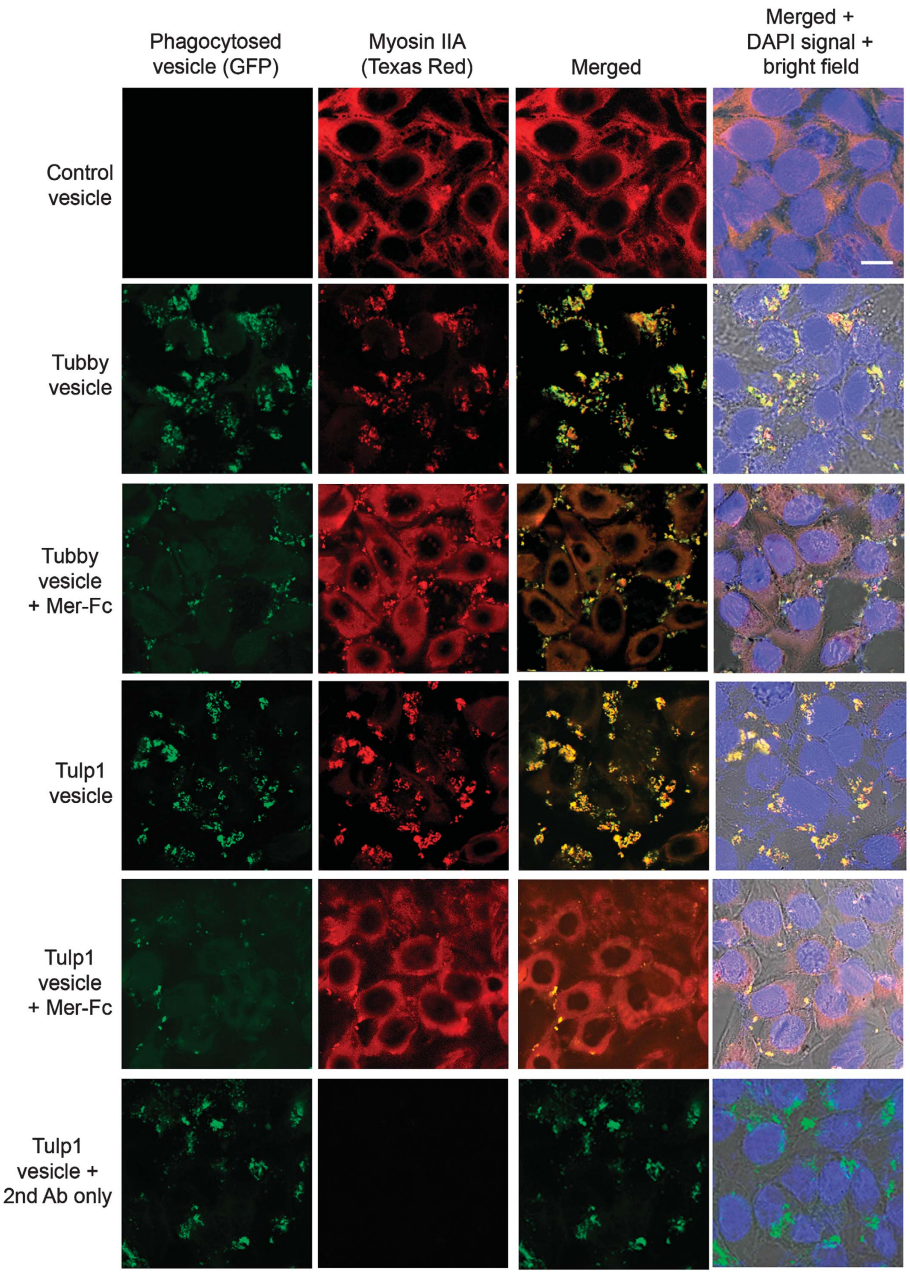

Figure 2 Myosin II-A redistribution and co-localization with phagocytosed vesicles. ARPE19 cells were incubated with mGFP-labelled tubby vesicles, Tulp1 vesicles or control vesicles in the presence or absence of Mer-Fc, as described in Figure 1B, and detected by immunocytochemistry using anti-NMMII-A Ab, followed by Texas Red-labelled secondary Ab. Intracellular fluorescence signals were analysed by confocal microscopy. Intracellular confocal images are superimposed with cognate bright fields to reveal the phagocytosed cargos. Bar = 10 μm for all.

the C-terminal region of tubby and Tulp1 abolish their capacity to stimulate RPE phagocytosis (Caberoy *et al*, 2010a). Perhaps Gas6 and protein S, two well-characterized MerTK ligands, could serve as guides. Both proteins facilitate MerTK-dependent phagocytosis as bridging molecules with their N-terminus Gla domain interacting with phosphatidylserine on apoptotic cells and the C-terminal 2 × LG domains binding to MerTK on phagocytes. We hypothesized that tubby and Tulp1 will stimulate phagocytosis as Gas6-like bridging molecules with their C-terminal domain binding to phagocytosis preys, such as apoptotic cells or membrane vesicles, and N-terminal MPD(s) binding to MerTK.

The association of tubby and Tulp1 with membrane vesicles was suggested by the results in Figure 4C. To further test the hypothesis, we collected the conditioned medium of Neuro-2a cells expressing FLAG-tagged tubby or Tulp1, which had been previously characterized for their unconventional secretion (Caberoy and Li, 2009). Control Neuro-2a membrane vesicles were incubated with the conditioned medium, then extensively washed and analysed for RPE phagocytosis of the membrane vesicles. The results showed that tubby or Tulp1 medium, but not control medium, stimulated RPE phagocytosis (Supplementary Figure S4). These findings suggest that both proteins were secreted, bound to the membrane vesicles, survived extensive washing and stimulated RPE phagocytosis as bridging molecules.

Phagocytosis bridging molecules normally bind to apoptotic cells, but not healthy cells. This discriminative binding

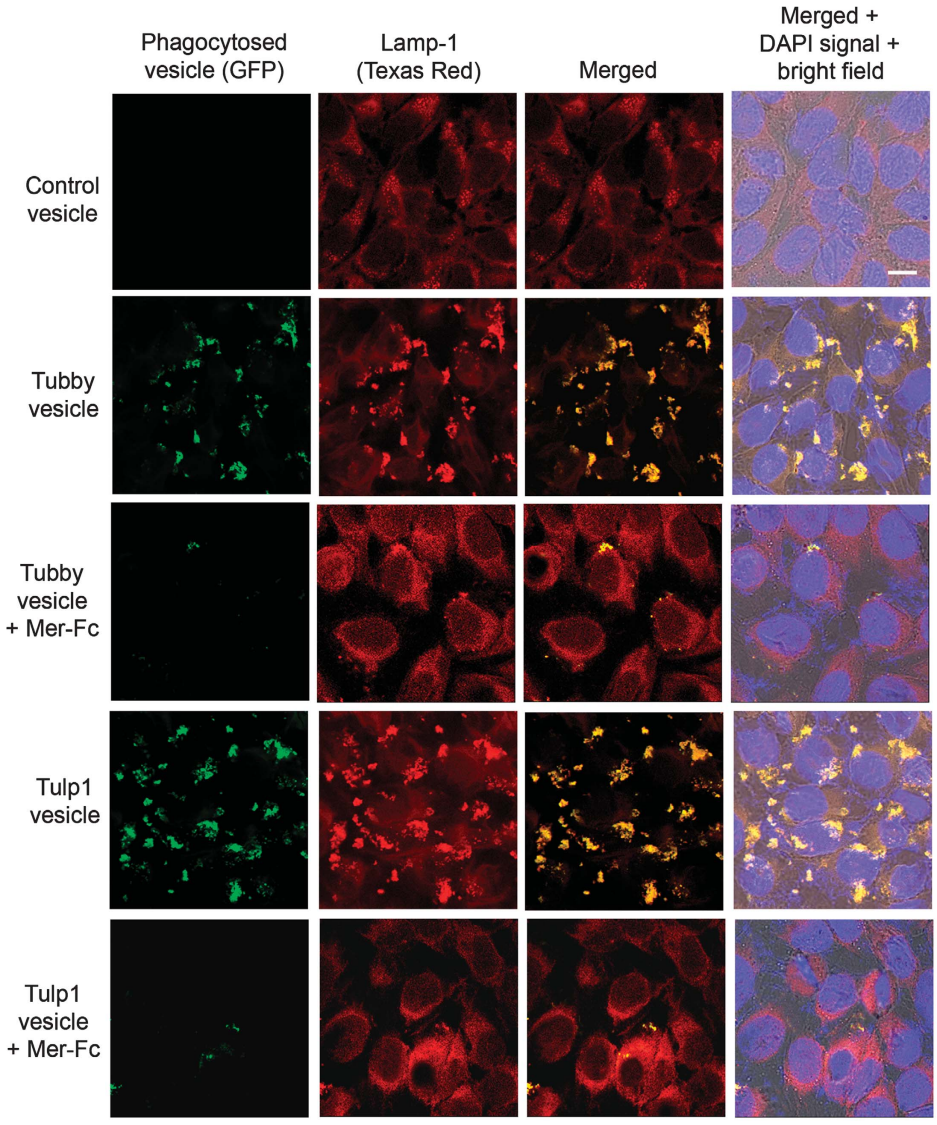

Figure 3 Tubby and Tulp1 facilitate cargo internalization through phagocytic pathway. Phagocytosis was performed as in Figure 2, detected by immunocytochemistry using anti-Lamp-1 Ab and Texas Red-labelled secondary Ab and analysed by confocal microscopy. Intracellular confocal images are superimposed with cognate bright fields to reveal the phagocytosed cargos. Bar = 10 μm for all.

activity is essential for selective phagocytic clearance of apoptotic cells. To investigate whether tubby and Tulp1 possess similar discriminative binding activity, we analysed their binding to apoptotic cells versus healthy cells. Purified FLAG-tubby was incubated with apoptotic or healthy Jurkat cells, and analysed for cell surface-bound FLAG-tubby by flow cytometry with FITC-labelled anti-FLAG monoclonal antibody (mAb). The results showed that tubby specifically bound to apoptotic cells, but not to healthy cells (Figure 5A and B). Interestingly, the C-terminal domain of mouse tubby (243Val–505Glu) had much better binding activity to apoptotic cells than the N-terminal domain (1M-242Pro), suggesting that tubby C-terminus is critical for its binding to phagocytosis preys.

Mapping of PPBD

Unlike Gas6, tubby lacks phosphatidylserine-binding activity, but is capable of associating with PtdIns(4,5)P_2 on the inner leaflet of the plasma membrane through its highly conserved C-terminal domain (Santagata *et al*, 2001). Although PtdIns(4,5)P_2 has not been reported to be selectively displayed on apoptotic cell surface as phosphatidylserine (Ravichandran and Lorenz, 2007), it is appealing to speculate that PtdIns(4,5)P_2 may be a molecular anchor for tubby binding to apoptotic cells. Tubby with double mutations at K330A and R332A (Tubby-2M) was demonstrated to abolish its binding activity to the phospholipid (Santagata *et al*, 2001). However, our data showed that Tubby-2M remained capable of binding to apoptotic cells and stimulating phagocytosis (Figure 5B, D and E), suggesting that tubby PtdIns(4,5)P_2-binding activity is irrelevant to prey binding and phagocytosis.

Deletion mutation of the 44 amino acids at tubby C-terminal end (Tubby-ΔC44) causes adult-onset obesity, retinal and cochlear degeneration in *Tubby* mice with unknown pathological mechanisms (Ikeda *et al*, 2002). Intriguingly, this domain was essential for tubby-mediated phagocytosis with undefined molecular mechanism (Figure 5D) (Caberoy *et al*, 2010a). Furthermore, our results revealed that Tubby-ΔC44 abolished its binding activity to apoptotic cells (Figure 5B), indicating that this domain is

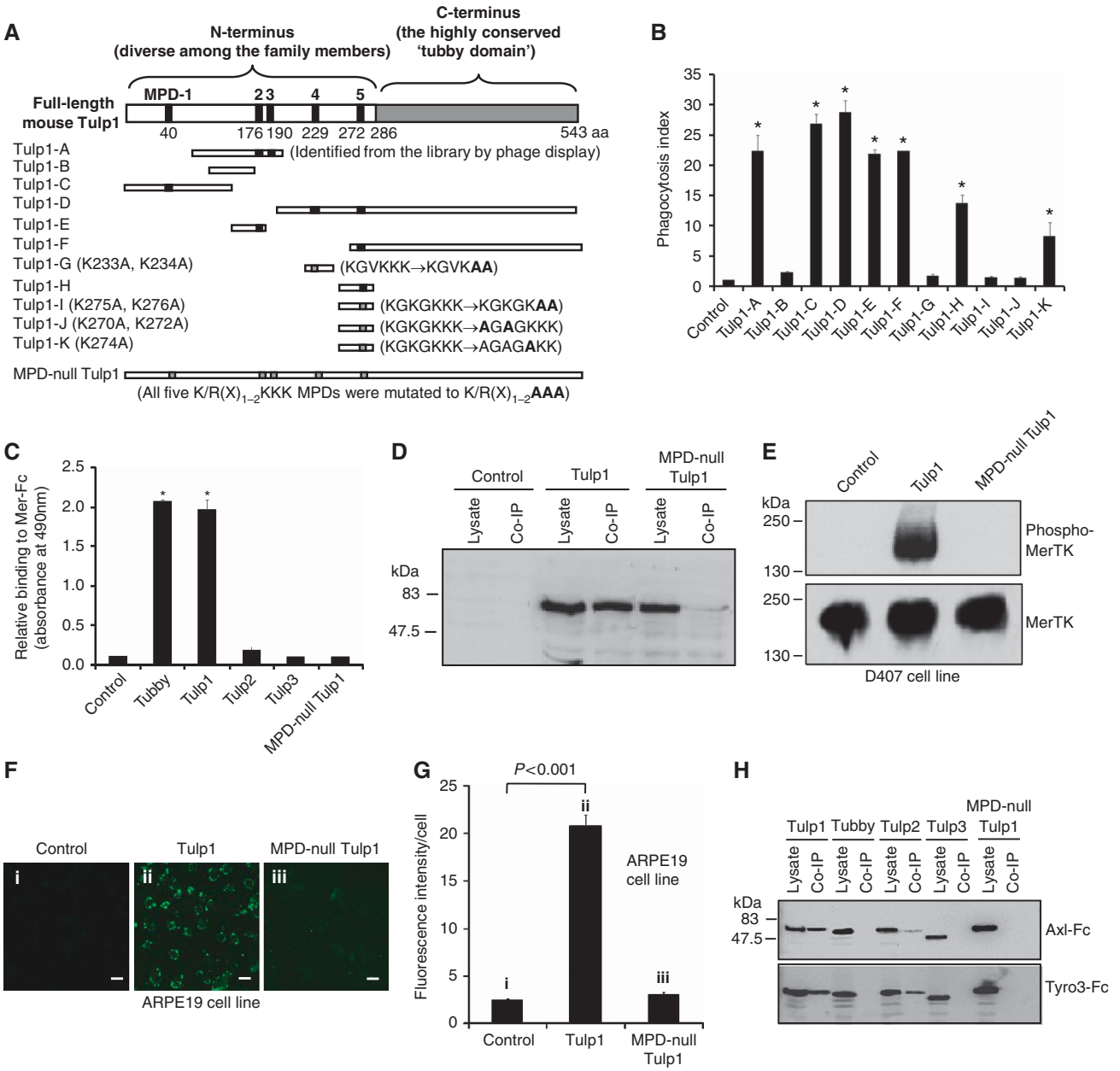

Figure 4 Mapping of N-terminal MPDs. (**A**) Structure of mouse Tulp1 and its mutant constructs. Different phage clones were constructed to express Tulp1 with deletions or mutations as indicated. (**B**) Mutant phage clones were analysed for RPE phagocytosis by phage phagocytosis assay. Total phagocytosed phages were quantified by plaque assay and expressed as phagocytosis index (±s.e.m., $n=3$, $^*P<0.001$, versus control phage). (**C**) MerTK does not bind to membrane vesicles of MPD-null Tulp1. Membrane vesicles were prepared from Neuro-2a cells expressing Tulps or MPD-null Tulp1 with all five K/R(X)$_{1-2}$KKK MPDs mutated to K/R(X)$_{1-2}$AAA, immobilized on ELISA plates, incubated with Mer-Fc. Bound Mer-Fc was detected with HRP-conjugated secondary Ab, followed by colorimetric assay (±s.e.m., $n=3$, $^*P<0.001$, versus control) (**D**) MPD-null Tulp1 does not bind to MerTK. Co-immunoprecipitation with Mer-Fc was performed as in Figure 1A. (**E**) MPD-null Tulp1 fails to induce MerTK phosphorylation. After the induction of MerTK phosphorylation in D407 cells as described in Figure 1D, MerTK was immunoprecipitated with anti-MerTK Abs, followed by western blot analysis with anti-phospho-MerTK or anti-MerTK Abs. Wild-type Tulp1 was included as a positive control. (**F**) MPD-null Tulp1 is incapable of stimulating RPE phagocytosis. mGFP-labelled plasma membrane vesicles were prepared from Neuro-2a cells expressing wild-type or MPD-null Tulp1 and analysed for RPE phagocytosis as in Figure 1B. Bar = 10 μm. (**G**) Relative fluorescence intensity per cells in (**F**) was quantified in >100 cells per group (±s.e.m., $n>100$). (**H**) Tulp1, but not tubby and MPD-null Tulp1, binds to Axl and Tyro3. Co-immunoprecipitation was performed with Axl-Fc and Tyro3-Fc, as described in Figure 1A.

critical for tubby binding to apoptotic cells. We termed this C-terminal domain as PPBD (Figure 5A).

PPBD was further verified with human Tulp1. Similar to tubby, previous studies demonstrated that deletion mutation of the 44 amino acids at human Tulp1 C-terminal end (Tulp1-ΔC44) not only associated with retinal degeneration (Banerjee *et al*, 1998), but also abolished its stimulation of phagocytosis (Caberoy *et al*, 2010a). Our data revealed that Tulp1 bound to apoptotic cells, but marginally to healthy cells (Figure 5C), and that Tulp1-ΔC44 lost nearly all its binding activity to apoptotic cells. Similar results were obtained with apoptotic HEK293 cells (Supplementary Figure S6). These

findings suggest the importance of PPBD for Tulp1 binding to phagocytosis preys.

Several other mutations of human Tulp1 have been characterized for their effects on phagocytosis (Caberoy *et al*, 2010a) and were analysed for their impact on prey binding. Mutation of R420P or I459K with no effect on phagocytosis was fully capable of binding to apoptotic cells (Figure 5C). However, K489R or F491L mutation, which completely abolished Tulp1 stimulation of phagocytosis (Caberoy *et al*, 2010a), had partial reduction in Tulp1-binding activity to the phagocytosis preys. The latter two mutations are located <10 amino acids from ΔC44, suggesting that the PPBD should include the C-terminal 54 amino acids. Mouse tubby and human Tulp1 share $>85\%$ of amino acid identity in this region. Taken together, these data suggest that the C-terminal PPBD of tubby and Tulp1 is an essential domain for their binding to apoptotic cells. However, both Tubby-ΔC44 and Tulp1-ΔC44 bound to apoptotic cells better than healthy cells (Figure 5B and C). Thus, it is possible that other regions of tubby and Tulp1 have additional binding activity to apoptotic cells.

N-terminal competitive blockade to disrupt 'bridges'

Our results demonstrated that tubby and Tulp1 bind to MerTK through their N-terminal MPD(s) and associate with phagocytosis preys through their C-terminal PPBD, suggesting that both proteins function as Gas6-like bridging molecules to facilitate phagocytosis. If so, we hypothesized that excessive amount of tubby N-terminal domain (Tubby-N) or Tulp1-N without PPBD may competitively block tubby- or Tulp1-mediated phagocytosis as a dominant-negative competitor by disrupting the 'bridge'. Our recent study showed that POS vesicles prepared from pig retina associated with endogenous tubby and that endogenous tubby or Tulp1 was essential for efficient RPE phagocytosis of POS vesicles (Caberoy *et al*, 2010a). This was further confirmed by two different anti-tubby Abs (Supplementary Figure S7).

To further investigate tubby and Tulp1 as bridging molecules, we analysed endogenous tubby- and Tulp1-mediated RPE phagocytosis with fluorescence-labelled POS vesicles in the presence or absence of excessive Tubby-N or Tulp1-N. The results showed that Tubby-N or Tulp1-N significantly inhibited POS phagocytosis (Figure 6A and B), suggesting that excessive Tubby-N or Tulp1-N was capable of disrupting the 'bridging' function of endogenous tubby or Tulp1.

We analysed the competitive blockade of macrophage phagocytosis by tubby-N and Tulp1-N. Fluorescence-labelled apoptotic and healthy Jurkat cells were preincubated with purified tubby and Tulp1, washed, and analysed for their phagocytosis by J774 macrophages in the presence or absence of excessive Tubby-N or Tulp1-N (Figure 6C and D). In the absence of tubby or Tulp1, macrophage preferentially phagocytosed apoptotic cells, but not healthy cells, possibly because of other eat-me signals exposed on apoptotic cells, such as phosphatidylserine (Ravichandran and Lorenz, 2007). However, tubby and Tulp1 vigorously stimulated macrophage phagocytosis of apoptotic cells but not healthy cells. Many individual macrophages phagocytosed more than three apoptotic cells. Excessive tubby-N or Tulp1-N significantly blocked tubby- or Tulp1-induced macrophage phagocytosis of apoptotic cells, respectively. Together with the RPE phagocytosis data, these results suggest that tubby and Tulp1 function as bridging molecules to facilitate macrophage and RPE phagocytosis.

Blockade of RPE phagocytosis by antibodies

Finally, we analysed the competitive blockade of RPE phagocytosis with anti-tubby Abs. Polyclonal Ab against tubby N- or C-terminal peptide recognized full-length recombinant tubby and endogenous tubby in POS vesicles (Supplementary Figure S7). Excessive amounts of both Abs partially blocked tubby-mediated RPE phagocytosis of POS vesicles (Supplementary Figure S8). However, the C-terminal specific Ab was far less effective than the N-terminal specific Ab, possibly due to incomplete steric hindrance or insufficient affinity of the Ab to fully disrupt endogenous tubby binding to POS vesicles.

Discussion

Tubby and Tulp1 as new MerTK ligands

In this study, we identified MerTK as a common receptor for tubby and Tulp1. MerTK is a well-characterized phagocytic receptor, essential for the maintenance of retinal homeostasis and the prevention of autoantibody production and autoimmunity (D'Cruz *et al*, 2000; Seitz *et al*, 2007). It belongs to TAM RTK subfamily, which share structural similarity (Hafizi and Dahlback, 2006a; Lemke and Rothlin, 2008), but distinct roles in macrophage and RPE phagocytosis. Elimination of MerTK completely abolishes macrophage phagocytosis of apoptotic cells, whereas ablation of Axl and Tyro3 reduces macrophage phagocytosis by only approximately one half (Seitz *et al*, 2007). MerTK-deficient mice develop retinal degeneration due to defective RPE phagocytosis, whereas ablation of Axl or Tyro3 appears to have minimal impact on retinal homeostasis (Seitz *et al*, 2007).

Gas6 and protein S were the only two known MerTK ligands identified $\sim$15 years ago (Stitt *et al*, 1995), with different binding specificities to TAM RTKs (Hafizi and Dahlback, 2006b). Gas6 binds to all three TAM RTKs, whereas protein S only interacts with MerTK and Tyro3. Besides their roles in MerTK-mediated phagocytosis, both proteins also involve in coagulation, atherosclerosis, glomerular pathophysiology, innate immunity and cellular homeostasis (Yanagita, 2004; Hafizi and Dahlback, 2006a; Fernandez-Fernandez *et al*, 2008; Lemke and Rothlin, 2008).

Similar to Gas6 and protein S, tubby and Tulp1 have distinct binding specificities to TAM RTKs. Tulp1 binds to all three RTKs, whereas tubby only recognizes MerTK. These findings suggest that Tulp1 could regulate other cellular functions through Tyro3 or Axl. Unlike Gas6 and protein S, however, tubby and Tulp1 lack the well-characterized $2 \times$ LG domains for receptor binding. Instead, MPD(s) within their diverse N-terminal region is a new type of MerTK-binding motif. Mouse Tulp1 with five MPDs elicits more robust MerTK phosphorylation than tubby with only one MPD, possibly due to more efficient receptor homodimer and/or heterodimer formation by binding to TAM RTKs (Lemke and Rothlin, 2008). However, it remains unknown whether a single Tulp1 with five MPDs is capable of binding to one or multiple receptors. Thus, the stoichiometry of MPDs and TAM RTKs are yet to be determined. Moreover, the relative importance and receptor-binding specificity of individual MPDs needs to be characterized.

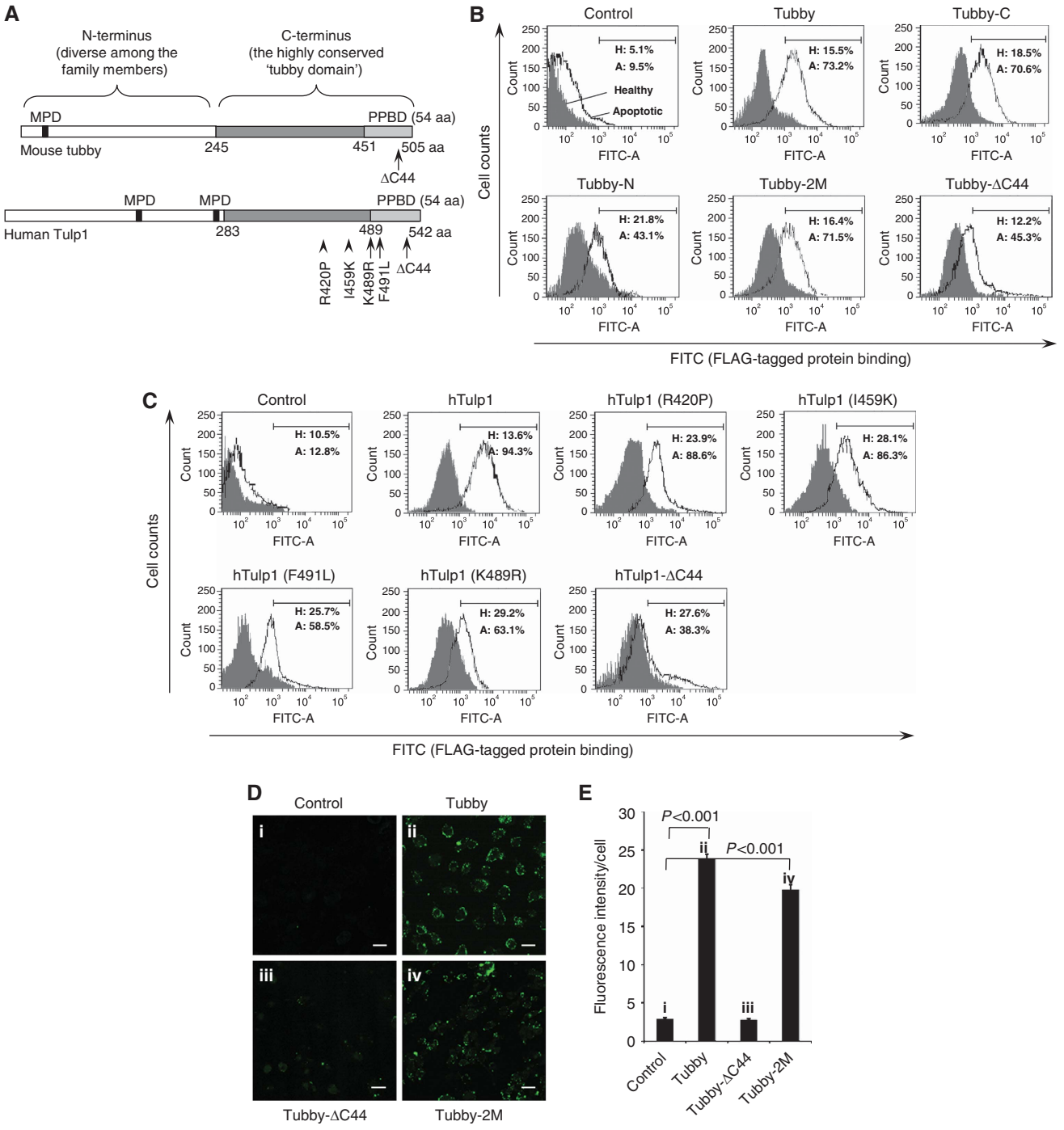

Figure 5 Mapping of C-terminal PPBD. (**A**) Maps of mouse tubby, human Tulp1 and their mutants. Mouse tubby has one MPD, and human Tulp1 has two MPDs. PPBD includes ΔC44 domain. Arrows indicate mutations that abolish stimulation of phagocytosis. Arrowheads indicate mutations without effect on phagocytosis. (**B**) Binding of tubby and its mutants to apoptotic or healthy Jurkat cells. Purified FLAG-tagged tubby and its mutant proteins were incubated with apoptotic or healthy Jurkat cells. Apoptosis was induced by staurosporine for 3 h. Cell surface-bound proteins were detected by anti-FLAG mAb and FITC-labelled secondary Abs, and the cells were analysed by flow cytometry. H, healthy Jurkat cell; A, apoptotic Jurkat cell. (**C**) Binding of Tulp1 and its mutants to apoptotic or healthy Jurkat cells. Similar results were observed for tubby, Tulp1 and their mutants with apoptotic Jurkat cells induced by etoposide for 16 h with cell lysate expressing FLAG-tagged proteins (not shown). Apoptosis was analysed in Supplementary Figure S5. (**D**) Stimulation of RPE phagocytosis by tubby and its mutants. Membrane vesicles were prepared from Neuro-2a cells expressing tubby or its mutants. RPE phagocytosis was analysed as in Figure 1B. (**E**) Relative fluorescence intensity per cells in (**D**) was quantified in >100 cells per group ($\pm$s.e.m., $n>100$).

Tubby and Tulp1 as new bridging molecules for phagocytosis

A limited number of known phagocytosis ligands have been identified and can be classified into two categories: direct phagocytosis stimulators and phagocytosis bridging molecules (Ravichandran and Lorenz, 2007). For example, phosphatidylserine is a well-characterized direct stimulator, which is normally located on the inner leaflet of the plasma

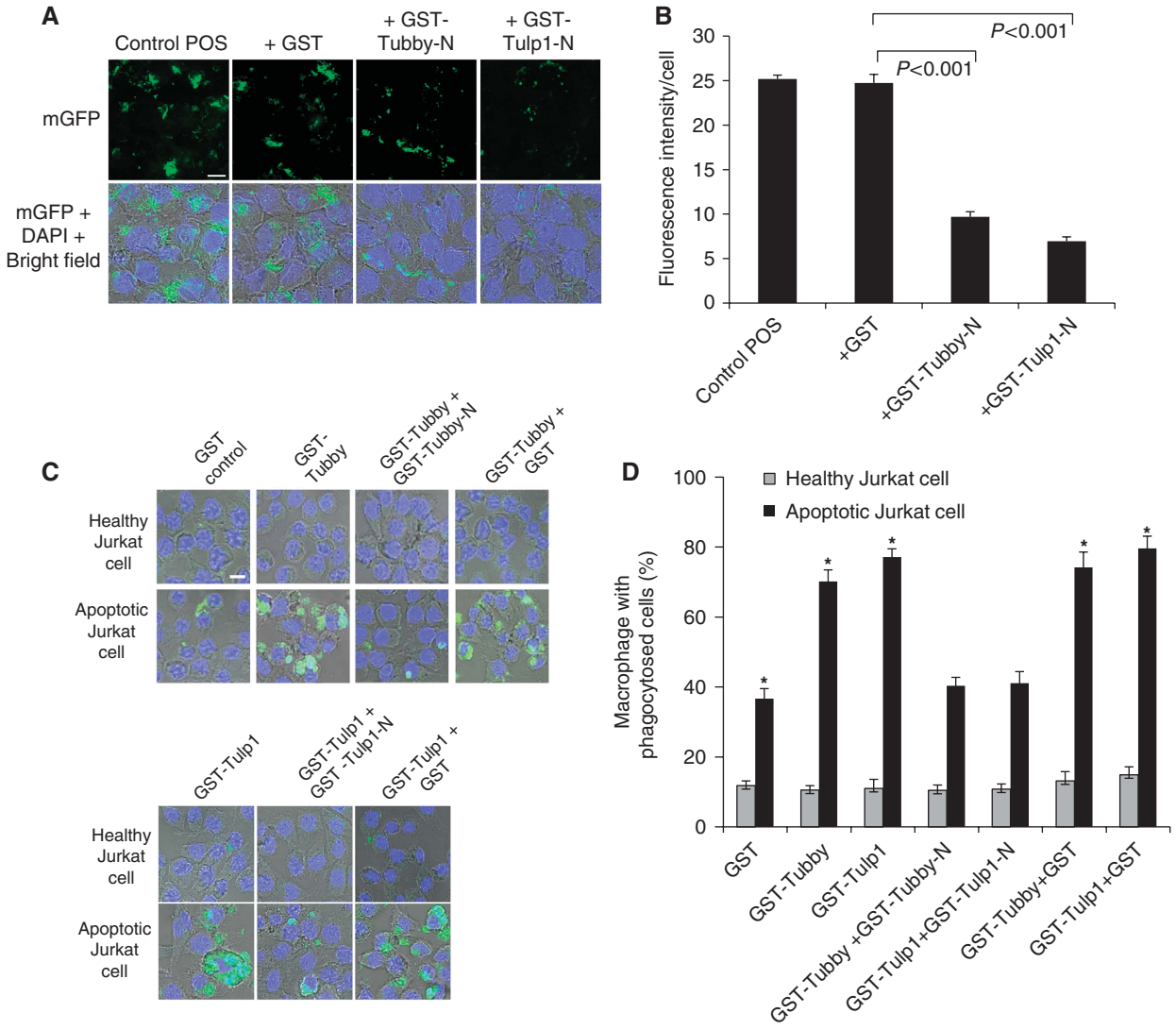

Figure 6 Tubby and Tulp1 function as bridging molecules to facilitate phagocytosis. (**A**) Excessive Tubby-N or Tulp1-N blocks RPE phagocytosis of POS vesicles. Fluorescence-labelled POS vesicles with endogenous tubby and Tulp1 were analysed for phagocytosis in ARPE19 cells in the presence or absence of excessive GST-Tubby-N, GST-Tulp1-N or GST control (4 μM), as described in Figure 1B. (**B**) Relative fluorescence intensity per cells in (**A**) was quantified in >100 cells per group (± s.e.m., $n>100$). (**C**) Excessive Tubby-N or Tulp1-N blocks macrophage phagocytosis of apoptotic Jurkat cells. Fluorescence-labelled apoptotic Jurkat cells were preincubated with GST-Tubby, GST-Tulp1 or GST, washed and analysed for phagocytosis in J774 macrophage cells in the presence or absence of excessive GST-Tubby-N, GST-Tulp1-N or GST, as described in (**A**). Confocal images of phagocytosed green fluorescence signals, DAPI signals and bright fields are superimposed. (**D**) Percentage of macrophages with phagocytosed Jurkat cells in (**C**) was quantified (± s.e.m., $n>17$, $^*P<0.001$, apoptotic versus healthy in GST group, all others versus GST apoptotic). Bar = 10 μm for all.

membrane in healthy cells to serve as a co-factor for intracellular signalling (Stace and Ktistakis, 2006). During apoptosis, phosphatidylserine flips across the membrane bilayer to be displayed on apoptotic cell surface and serves as a direct stimulator to facilitate phagocytic clearance of apoptotic cells (Ravichandran and Lorenz, 2007). Phosphatidylserine receptors, including Tim4, BAI1 and stabilin-2, have been recently identified on the surface of phagocytes (Miyanishi *et al*, 2007; Park *et al*, 2007, 2008). Proteins upregulated on the surface of apoptotic cells, such as calreticulin, may also serve as direct stimulator by binding to phagocytic receptors like LDL-receptor-related protein (Gardai *et al*, 2005). In contrast, phagocytosis bridging molecules are soluble extracellular proteins that simultaneously bind to molecules on phagocytosis preys and phagocytic receptors on phagocytes to facilitate phagocytosis. For example, Gas6 and protein S are well characterized as bridging molecules that simultaneously bind to MerTK and phosphatidylserine. Another example is MFG-E8 with N-terminal RGD motif binding to αvβ3 or αvβ5 integrin and C-terminal discoidin-like domain recognizing phosphatidylserine (Hanayama *et al*, 2002).

Tubby and Tulp1 were characterized in this study as bridging molecules with their N-terminal MPD(s) for MerTK binding and C-terminal PPBD for prey binding. The highly conserved C-terminal tubby domain was previously described with binding activity to PtdIns(4,5)P_2, but not to phosphatidylserine (Santagata *et al*, 2001). However, because tubby and Tulp1 bind to apoptotic cells through a phosphatidylserine- and PtdIns(4,5)P_2-independent mechanism, the

molecule(s) on apoptotic cells, but not on healthy cells, recognized by PPBD remains to be delineated. The C-terminal PPBD could be used as a molecular bait to identify binding partner(s) or anchoring molecule(s) on apoptotic cells by technologies of functional proteomics, including our newly developed ORF phage display (Caberoy *et al*, 2010b). As the PPBD sequence is highly conserved among Tulps, delineation of the anchoring molecule(s) on apoptotic cells will also facilitate the delineation of functional roles of the tubby family.

Biological relevance

Tubby and Tulp1 with no signal peptide were previously characterized as intracellular proteins by immunohistochemistry (Ikeda *et al*, 1999). Although Tulp1 is specifically expressed in photoreceptors with the highest level in the inner segments (Milam *et al*, 2000), tubby is expressed in the neurons in the retina, inner ear and brain (Ikeda *et al*, 1999). How can intracellular Tulp1 in the inner segments interact with MerTK on RPE cell surface to facilitate phagocytosis? One possible explanation is that tubby and Tulp1 may function intracellularly under the normal physiological condition, but are released into the extracellular space during photoreceptor degeneration induced by constant light exposure or by genetic mutations (Paskowitz *et al*, 2006). Macrophages have been demonstrated to be recruited into the retina during retinal degeneration caused by genetic mutations or photodamage (Wenzel *et al*, 2005), and their phagocytic clearance of degenerated retinal cells or cellular debris may be facilitated by tubby and Tulp1 released from damaged photoreceptors. These distinct functions under different conditions and cellular compartments have been well documented for phosphatidylserine (Ravichandran and Lorenz, 2007). Our data showed that tubby and Tulp1 are capable of stimulating macrophage phagocytosis.

An alternative explanation for tubby and Tulp1 to function as phagocytosis ligands is our recent characterization of their unconventional secretion (Caberoy and Li, 2009). We demonstrated that tubby and Tulp1 are expressed both intracellularly and extracellularly, and that their extracellular expression is not blocked by brefeldin A and monensin, which specifically inhibit protein transport through the endoplasmic reticulum-Golgi pathway. Moreover, extracellular tubby was not due to protein leakage from apoptotic cells, suggesting that tubby is actively secreted through a non-classical pathway. Based on our recent data (Caberoy and Li, 2009), we calculated that $\sim$3.5% of endogenous tubby are secreted in a period of 24 h. Similar to the well-characterized unconventional secretion of fibroblast growth factor-2 (Temmerman *et al*, 2008), tubby C-terminal binding to PtdIns(4,5)P_2 is partially responsible for its secretion (Caberoy and Li, 2009). An additional secretory signal is mapped to tubby N-terminal region between Asn51 and Arg100, which is essential for its extracellular trafficking. Tubby in the POS (Supplementary Figure S7) could be directly secreted into the interface of RPE and POS. Unconventional secretion has been reported for a number of proteins without a classical signal peptide (Nickel and Seedorf, 2008).

However, an additional question is why earlier immunochemistry studies failed to detect extracellular tubby and Tulp1 at the interface of RPE and photoreceptors. Immunohistochemical analyses of protein expression require negative references for comparative detection in the same image field. Because of diffusion, the extracellular concentrations of soluble proteins are often much lower than their intracellular concentrations. Consequently, it is difficult to sensitively detect soluble extracellular proteins by immunohistochemistry. Thus, other detection methods, such as western blot in our recent study (Caberoy and Li, 2009), are more appropriate to detect diffused soluble extracellular proteins.

The unconventional secretion of tubby and Tulp1 indicates that they may be present in the circulation, as are Gas6 and protein S, to remotely modulate other phagocytes, such as macrophages. However, tubby and Tulp1 are mainly expressed in restricted retinal and neural tissues in adults. The level of circulating tubby and Tulp1 could be too low to remotely regulate other extraocular phagocytes in non-neural tissues. Although tubby expression in lymphocytes has been reported (Giannaccini *et al*, 2007), the level of circulating tubby and Tulp1 is yet to be quantified.

Tubby and Tulp1 have been characterized with a number of intracellular functions (Carroll *et al*, 2004; Xi *et al*, 2007; Grossman *et al*, 2009). An interesting question is whether tubby and Tulp1 can have additional extracellular functions. In fact, it is not uncommon that individual proteins have multiple functional roles both intracellularly and extracellularly. One of the well-characterized proteins with unconventional secretion is galectin-3 with a number of intracellular and extracellular functions (Hughes, 1999; Dumic *et al*, 2006), suggesting that a protein without signal peptide can simultaneously have multiple intracellular and extracellular functions.

Disease relevance

Another important issue is whether tubby and Tulp1 are essential phagocytosis ligands to maintain retinal homeostasis. POS function as power plants by converting light into electrochemical impulses and undergo a constant renewal process to repair photodamage (Strauss, 2005). The tips of the POS contain the highest concentration of radicals, photodamaged proteins and lipids, and are shed from the photoreceptors in a diurnal rhythm. Shed POS are phagocytosed by RPE cells with nutrients recycled back to the photoreceptors for regeneration of the POS at the base (i.e. connecting cilium). Maintenance of the appropriate length of the POS through diurnal shedding and regeneration is critical for visual function. RPE phagocytosis is a key process for the removal and recycling of shed POS and must be precisely regulated to maintain retinal homeostasis.

Our recent study showed that pathogenic mutations in tubby or Tulp1 are partially correlated with retinal degeneration. For example, deletion mutation of the C-terminal 44 amino acids in tubby and Tulp1 not only associates with retinal degeneration (Noben-Trauth *et al*, 1996; Banerjee *et al*, 1998), but also abolishes their stimulation of RPE phagocytosis (Caberoy *et al*, 2010a). These findings suggest that tubby and Tulp1 have a critical function as phagocytosis ligands in the maintenance of retinal homeostasis. Interestingly, this domain is a part of the PPBD, further implicating their important roles as phagocytosis ligands. On the other hand, MerTK mutation in Royal College of Surgeons (RCS) rats leads to retinal degeneration with accumulation of

unphagocytosed debris in the outer segments (Bok and Hall, 1971; D'Cruz *et al*, 2000), which is not observed in *Tubby* mice and Tulp1$^{-/-}$ mice with distinctively different phenotypes of retinal degeneration (Ohlemiller *et al*, 1997; Hagstrom *et al*, 1999; Ikeda *et al*, 2000). Moreover, unlike the retinal degeneration in RCS rats and MerTK-deficient mice, ablation of Gas6 results in minimal defect in phagocytosis and retinal homeostasis (Hall *et al*, 2005), possibly due to functional compensation by other MerTK ligands such as protein S. Similarly, Gas6 and protein S may compensate for the loss of tubby or Tulp1. Thus, the roles of tubby and Tulp1 as phagocytosis ligands for retinal homeostasis need to be further investigated in *in vivo* studies.

Current knowledge reveals that many phagocytosis signalling pathways are highly conserved between RPE and macrophages, including their common receptors (MerTK, integrins and CD36) and ligands (Gas6 and MFG-E8) (Ravichandran and Lorenz, 2007), as tubby and Tulp1 from this study. Perhaps, tissue-specific ligands are keys for in-depth understanding of differential regulation of phagocytosis between RPE and macrophages, as photoreceptor-specific Tulp1 may preferentially regulate local RPE phagocytosis in healthy retina. Moreover, systematic identification of phagocytosis ligands in large scales by our phagocytosis-based functional selection strategy (Caberoy *et al*, 2010a) will facilitate determination of phagocyte-specific ligands and signalling pathways, and define the similarities and differences between RPE and macrophage phagocytosis.

In summary, this study demonstrates that tubby and Tulp1 are capable of binding to MerTK, activating the receptor and inducing MerTK-dependent phagocytosis (Figure 7). Tulp1 binds to all three RTKs in TAM family, whereas tubby only interacts with MerTK. Both proteins function as bridging molecules with their N-terminal MPD(s) interacting with MerTK and C-terminal PPBD binding to apoptotic cells through a phosphatidylserine and PtdIns(4,5)P_2-independent mechanism. Therefore, tubby and Tulp1 are a new type of MerTK ligands to facilitate phagocytosis.

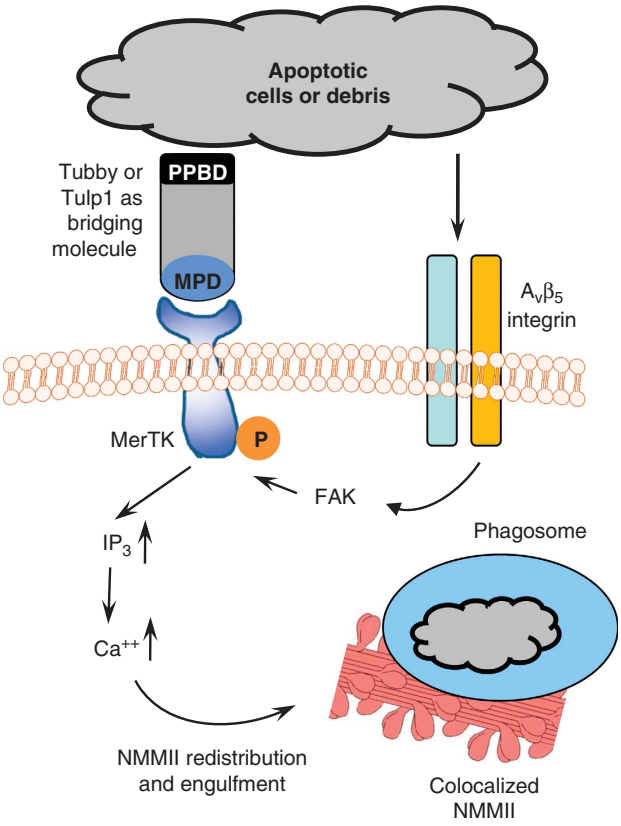

Figure 7 Tubby and Tulp1 facilitate phagocytosis as MerTK-dependent bridging molecules. Tubby and Tulp1 function as bridging molecules by simultaneously binding to phagocytosis preys, such as apoptotic cells or POS vesicles, through the C-terminal PPBD and to MerTK through the N-terminal MPD(s). MerTK activation induces receptor tyrosine phosphorylation, NMMII redistribution and engulfment of phagocytosis preys. Inositol 1,4,5-triphosphate (IP_3) and calcium influx are regulated by MerTK (Heth and Marescalchi, 1994; Strauss *et al*, 1996) and may have important functions in NMMII rearrangement. Intracellular focal adhesion kinase (FAK) activated by $\alpha_v\beta_5$ integrin is capable of phosphorylating MerTK (Finnemann, 2003).

Materials and methods

Cells, plasmids and reagents

ARPE19, D407, HEK293 and Neuro-2a cell lines were cultured as described (Caberoy *et al*, 2009, 2010a). Plasmids for N-terminal FLAG-tagged full-length Tulps, various mutants and mGFP were described (Caberoy *et al*, 2010a). Tubby with double mutations at K330A and R332A (Tubby-2M) was generated by PCR with missense mutations imbedded in the primers, as described (Caberoy *et al*, 2010a). All FLAG-tagged proteins were verified by sequencing and western blot using anti-FLAG M2 mAb (Sigma). FLAG-tagged tubby, Tulp1 and their mutants were expressed in HEK293 cells. The cell lysates were prepared at 48 h post-transfection, bound to anti-FLAG mAb column (Sigma), washed, eluted with FLAG peptide (100 μg/ml) as described (Caberoy *et al*, 2010b), dialysed against PBS and analysed by SDS–PAGE (Supplementary Figure S9). Recombinant glutathione S-transferase (GST), GST-tubby, GST-Tulp1, GST-Tubby-N and GST-Tulp1-N were prepared as described (Caberoy *et al*, 2010b). Gas6, Mer-Fc, Axl-Fc and Tyro3-Fc were purchased from R&D Systems. Anti-Tubby-N and anti-Tubby-C rabbit polyclonal Abs were from Santa Cruz Biotechnology. Fresh pig eyes were obtained from Department of Surgery, University of Miami, as residual organs of surgical trainings.

Co-immunoprecipitation

HEK293 cells were transfected with plasmids expressing FLAG-tagged Tulps or pcDNA3 and collected at 48 h post-transfection. Cell lysates were prepared and incubated for 1 h with Mer-Fc, Axl-Fc or Tyro3-Fc (1 μg) at 4°C followed by protein A resin. The resin was washed and analysed by western blot using anti-FLAG mAb.

RPE and macrophage phagocytosis

RPE phagocytosis was performed as described (Caberoy *et al*, 2010a). Briefly, plasma membrane vesicles were prepared from Neuro-2a cells co-expressing mGFP and individual Tulps, purified by sucrose gradient centrifugation and used for RPE phagocytosis. Alternatively, POS were prepared from fresh pig retina by sucrose gradient centrifugation and labelled by CFSE. RPE phagocytosis with plasma membrane or POS vesicles were performed in ARPE19 cells and analysed by confocal microscopy. Relative fluorescence intensity per cell was quantified as described (Caberoy *et al*, 2010a). More than 100 cells per group were analysed. For blockade study, excessive Mer-Fc (2.5 μg/ml), GST-Tubby-N (4 μM), GST-Tulp1-N (4 μM), polyclonal anti-Tubby-N Ab (40 μg/ml) and anti-Tubby-C Ab (40 μg/ml) were added to RPE cells immediately before the addition of labelled POS or membrane vesicles. Anti-Tubby-N and anti-Tubby-C Abs were extensively dialysed against PBS to remove NaN_3 for the blockade study.

Macrophage phagocytosis of apoptotic or healthy Jurkat cells was performed as described (Caberoy *et al*, 2010a). CFSE-labelled apoptotic or healthy Jurkat cells were preincubated with 200 nM of GST, GST-Tubby or GST-Tulp1 for 30 min, washed and incubated with J774 macrophage cells in the presence of GST, GST-Tubby-N or GST-Tulp1-N (4 μM) for phagocytosis assay. The percentage of

macrophages with phagocytosed Jurkat cells in every viewing field was analysed with a minimal of 17 viewing fields per group.

MerTK phosphorylation

MerTK phosphorylation was analysed as previously described (Sather *et al*, 2007). Briefly, D407 cells were cultured in 293 SFM II medium (Invitrogen) for 2 h to reduce the background of MerTK activation, followed by incubation with membrane vesicles (50 μg membrane protein/ml) expressing different Tulps or control pcDNA3 plasmid in the same medium for 30 min at 37°C. Gas6 (50 nM) was added to control vesicles as a positive control. After washing, the cells were lysed and directly analysed by western blot. Alternatively, MerTK was immunoprecipitated with anti-MerTK Ab (Fabgennix) and analysed by western blot. The immunoblots were probed with primary Ab against phospho-MerTK (Fabgennix), MerTK or RPE65 (Novus Biologicals), followed with HRP-conjugated secondary Ab. The signals were detected by chemiluminescence detection.

Mer-Fc binding

Plasma membrane vesicles were prepared from Neuro-2a cells expressing individual Tulps, immobilized onto ELISA plates (10 μg/ml, 100 μl/well in PBS), blocked and incubated with Mer-Fc (0.5 μg/well). After washing, bound Mer-Fc was detected with HRP-conjugated goat anti-human IgG, and quantified by colorimetric assay, as described (Caberoy *et al*, 2010b).

Immunocytochemistry

ARPE19 RPE cells were incubated with mGFP-labelled membrane vesicles in the presence or absence of Mer-Fc for 3 h, washed, fixed with 4% buffered formalin, permeabilized with 0.5% Triton X-100 in PBS, and incubated with rabbit anti-myosin II-A or anti-Lamp-1 Ab (Sigma). Bound Abs were detected by Texas Red-labelled secondary Abs and analysed by confocal microscopy for co-localization of mGFP and Texas Red signals.

Flow cytometry

Purified FLAG-tagged tubby, Tulp1 and their mutants (5 μg/ml) were incubated with healthy or apoptotic Jurkat cells, washed, stained with anti-FLAG M2 mAb (Sigma), and followed by FITC-labelled goat anti-mouse IgG. The cells were analysed by flow cytometry. Alternatively, HEK293 cell lysates expressing FLAG-tagged proteins were directly used for flow cytometry analysis. Apoptosis of Jurkat cells was prepared by incubating the cells with staurosporine (1 μg/ml) for 3 h or etoposide (40 μM) for 16 h and verified by staining with Annexin V and propidium iodide, as described (Caberoy *et al*, 2010a).

Phage-based phagocytosis assay

Phage clones displaying human Tulp1 or its mutants with missense mutation or deletion were constructed by PCR, cloned into T7Bio3C phage vector and verified by sequencing, as described (Caberoy *et al*, 2010b). Control phage had no foreign cDNA insert. Phage phagocytosis assay were described previously (Caberoy *et al*, 2009). Phagocytosed phages were quantified by plaque assay. The data were expressed as phagocytosis index, which is the plaque forming unit ratio of (total phagocytosed clonal phage)/(total phagocytosed control phage).

MerTK knockdown

MerTK expression in J774 cells was silenced using RNA interference. shRNA constructs against mouse MerTK were purchased from Open Biosystems (Silva *et al*, 2005). J774 cells were transfected with shRNA. After 24 h in culture, the cells were selected with puromycin (1 μg/ml). Puromycin-resistant cells were analysed for MerTK expression by RT–PCR using the primers of 5′-TGTTCATCATCCTCGGCTGCTTC-3′ and 5′-ACCATGGGCTTCGGGATGCCT-3′, or by western blot. The cells were used for phagocytosis assay.

Data analysis

All experiments were repeated at least three times independently. One-way analysis of variance followed by *post hoc* Tukey–Kramer pairwise comparisons was used to determine statistical significance among different groups.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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