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

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24 February 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now assessed it and their comments are shown below. As you will see while all three referees consider your study as interesting in principle, ref 1 and 3 in particular raise significant issues that lead us to conclude that the current dataset remains too premature for publication in The EMBO Journal. Referee 1 feels strongly that more experiments need to be done with purified tubby and Tulp1 proteins. Furthermore, he/she asks for a deeper analysis under which conditions tubby or Tulp1 are actually secreted as well as for the identity of the determinants on apoptotic cells that bind tubby and Tulp1. Referee 3 feels that the physiological significance of a bridging activity of tubby and Tulp1 between MerTK and apoptotic cells needs to be analysed in considerably more depth and/or that the biological focus of the study should be shifted to the role of tubby/Tulp1 in macrophage-mediated engulfment of apoptotic cells. All in all it becomes clear that that stronger evidence for the interaction and effect of tubby and Tulp1 on MerTK based on purified proteins would be required. Second, the physiological significance of your findings would need to be analysed further to a considerable extent, either in the retinal epithelial cell or the macrophage system. Clearly, the amount of work required to address these two issues in an adequate manner - in particular the second one - would go far beyond the scope and the time frame of a single revision (3 months); and it is our policy to allow a single round of revision only. I am afraid to say that we consequently cannot consider a revision and I thus see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this stage.

Given that the study is an interesting one in principle, we would, however, not exclude the possibility to consider a new submission on the same topic if you can address the two issues detailed above in an adequate manner. We would certainly not require a deeper analysis of how tubby and Tulp1 are released from cells nor ask for the identification of the binding partner for tubby/Tulp1 on
the surface of apoptotic cells. It would, however, be indispensable to develop the study further and to make a considerably stronger case for the physiological significance of your findings, either in the retinal pigment epithelial cell system or with macrophages, however, without fully removing the RPE part/aspect of the study to allow for more general conclusions regarding the function of tubby/Tulp1. To be completely clear, however, I would like to stress that if you wish to send a new manuscript this will be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh, also with respect to the literature and the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript, the authors claim that Tubby and Tubby-like protein work as bridges between apoptotic cells and phagocytes, and activate the phagocytes by binding to Mer receptor. This is an interesting possibility. However, the data are too preliminary to support their conclusion.

1. The authors claim that Tubby and Tubby-like protein binds to Mer. Please use the purified Tubby and Tubby-like protein, and determine Kd (dissociation constant) to Mer by Biacore or a similar technique.

2. The activation of MER by Tubby and Tubby-like protein should be done with the pure protein (not with a mixture with membrane fraction).

3. How are the cytoplasmic proteins of Tubby and Tubby-like protein released from the cells? When are they released, continuously or only after activation? How much percentage proteins are released?

4. Why are the recombinant Tubby and Tubby-like proteins prepared differently for different experiments (in one case from the culture sup or in another case from the cell lysates).

5. Use the purified Tubby and Tubby-like protein to show the binding to apoptotic cells.

6. The authors should show what on the apoptotic cells is recognized by Tubby and Tubby-like protein.

7. How do the authors explain the "obesity" of the tubby-deficient mice?

Referee #2 (Remarks to the Author):

This manuscript communicates novel studies on Tubby and Tulp1 demonstrating that they are functional as bridging molecules, bridging cell-derived vesicles and apoptotic cells to both professional and non-professional phagocytes and stimulating phagocytosis via MerTK signalling.

The work is generally very well performed and includes novel molecular characterization of a
proposed "phagocytosis prey-binding domain" in Tubby and Tulp1 which could be useful in future work.

Two specific points deserve particular attention:

1. Figure 5 demonstrates strong association of Tubby and Tulp1 proteins with apoptotic but not healthy Jurkat cells. At what stage in the apoptosis programme is the association? It would be helpful if the authors included kinetics data of Tubby and Tulp1 protein binding to apoptotic cells showing apoptosis in relation to PS exposure and plasma membrane integrity. Similar criticisms can be made about Figure 6.

2. In addition, although they do show that Tubby doesn't require PtdIns(4,5)P2 to bind to apoptotic cells and it is already known that such binding is PS-independent, it would be worth knowing how conserved the ligands of apoptotic cells for Tubby and Tulp1 are. Thus far the studies of apoptosis in the paper are restricted to Jurkat cells only. What about other cells of other lineages (including retinal epithelial cells)? While it might be unreasonable to require the identification of the apoptotic cell ligand(s) for these bridging molecules, it would be useful to have an indication as to how conserved/ubiquitous they are.

Should the last label on the x axis of Figure 6D read GST-Tulp1+GST?

The manuscript needs serious attention to English throughout and the discussion should be shortened and more incisive.

Referee #3 (Remarks to the Author):

In General

The paper by Caberoy and colleagues represents data which indicate that tubby and tubby-like protein-1 are new MerTK ligands. The binding of tubby and tubby-like-protein-1 results in the phagocytosis of these proteins. Furthermore, an amino acid sequence could be identified which binds to debris or apoptotic cells with which tubby or tubby-like protein-1 can function as a bridge for phagocytosis for other substances. The experiments are well elaborated and a large part of the data is sound and provides strong evidence for some of the conclusions. The data present the scientific novelty that tubby and tulp-1 are ligands of MerTK. However, regarding the functional relevance and the role of MerTK for the phagocytosis process the data are incomplete. Furthermore, the discussion is not clearly differentiating between the several types of phagocytic activity (POS phagocytosis versus apoptotic cell phagocytosis or RPE versus macrophage phagocytosis).

Specific comments

1. Role of MerTK: The authors clearly demonstrated that tubby and tulp-1 bind to MerTK receptors and that that binding leads to activation of this receptor by autophosphorylation. Furthermore, it was shown that tubby and tulp-1 were identified as phagocytosed material. The evidence of the involvement of MerTk relies only on the effects of Mer-FC. The effect of Mer-Fc was quantified by fluorescence per cell. However, there is still phagocytosis measurable. The fluorescence looks different in control cells and in Mer-Fc treated cells. That the material is ingested was shown by co-staining against organelles of the phagocytic pathway. This should be shown for the Mer-Fc effects too. Moreover it would be good to to show that tubby and the ligand of tubby is not phagocytosed in the absence of MerTK. Furthermore, it is needed to show that debris or apoptotic cells are not phagocytosed in the absence of tubby.

2. The authors showed that tubby appears in the cytosol after phagocytosis. Is MerTK also ingested?

3. According to the paper from Finnemann (EMBO J 2003) alone the presence of photoreceptor outer membranes is sufficient to induce phosphorylation of MerTK via focal adhesion kinase which results from activation of alphaV-integrins. This is in contrast to the data presented here: showing that the intrinsic content of tubby in photoreceptor outer membranes alone is sufficient.
4. ARPE-19 cells: this is a RPE cell line which not necessarily shows all features of native RPE cells. The authors should show at least that these cells express MerTK.

5. Physiology of phagocytosis: The phagocytosis of shed photoreceptor outer membranes by the retinal pigment epithelium requires at least three receptors: CD36, MerTK and alphaV-integrins. Based on the analysis of phagocytosis by retinal pigment epithelial cells from RCS rats, alphaV-/ mice and other work it is clear that the three receptors interact in the initialization of phagocytosis. In this network alphaV-integrin enables binding of the photoreceptor outer segments (RCS rat which does not have an active MerTK can bind outer segments but is unable to ingest the outer segments although cells from the RCS rat shows unspecific phagocytosis of beads and other material). Binding to alphaV-integrin activates FAK which phosphorylates MerTK which subsequently can be activated by Gas6. Thus, the model proposed here is not in accordance with the literature about photoreceptor phagocytosis by the retinal pigment epithelium. Also the experiments shown here do not take the current knowledge into account.

6. The Discussion mixes the mechanisms which control the phagocytosis by the retinal pigment epithelium and that by macrophages. Furthermore, the authors do not differentiate between phagocytosis of photoreceptor outer segments and apoptotic cells/debris. The phagocytosis of photoreceptor outer membranes is a highly regulated process and not only the removal of debris. The process must be tightly regulated because it would change the structure of photoreceptors and therefore alter vision. If phagocytosis activity is too large the photoreceptor outer segments would become too short. On the other and it is shown that during retinal degeneration in general macrophages are activated. However, this is more a secondary effect in the chain of events leading to degeneration of the retina. The primary effects are gene mutations leading to cell apoptosis in either photoreceptors or cells of the retinal pigment epithelium. Thus, also the text about the role of tubby in phagocytosis by macrophages is mixed up with the different mechanisms involved in retinal degeneration. I don't think that the data can generalized as it is done on page 21.

7. Tubby as eat me signals: The authors argue that tubby is secreted by the photoreceptors to initialize the phagocytosis of photoreceptor outer segments. This model is not well elaborated because also the secretion of this protein is not shown in the retina and it is not clear where tubby is secreted. The phagocytosis takes place at the outer segment whereas it is likely that the inner segments secret tubby which makes a rather long diffusion way for a tight regulation of photoreceptor outer membrane phagocytosis.

8. Probably the authors should concentrate their paper on the role of tubby or tulp-1 on macrophages and their role in removing debris or apoptotic cells. The data and conclusions concerning the phagocytosis by the retinal pigment epithelium are less convincing. This can be seen for example in the discussion part of the paper on page 20. This part states at the beginning that tubby and tulp-1 play a critical role in retinal homeostasis but at the end of that paragraph it is concluded that tubby and tulp-1 are unlikely essential eat-me signals for retinal homeostasis.

Minor comments:

- Figure 4E: the blot shows no size markers and strongly differs from that shown in Figure 1D.
- "phagocytosis through MerTK": The role of MerTK is possibly more to trigger the endocytosis machinery and the ingestion process is more due to other receptors such as CD36. It should be noted that activation of MerTK results in the formation of inositol-1,4,5-trisphosphate as second-messenger in RPE cells.
- Several papers which report basic mechanisms of photoreceptor phagocytosis and data from RCS rat are not cited and not put into account neither for experimental design nor in the discussion.

Resubmission 11 August 2010

We are grateful for reviewers’ valuable comments and the opportunity to resubmit our manuscript. The following is our point-to-point answers to the comments. All revisions are underlined in the manuscript.
Reviewer #1

1. “The authors claim that Tubby and Tubby-like protein binds to Mer. Please use the purified Tubby and Tubby-like protein, and determine Kd (dissociation constant) to Mer by Biacore or a similar technique.”

**Answer:** The University of Miami lost its only Biacore instrument several years ago. Although we located several Biacore instruments in other institutes, the core facility at the University of Florida at Gainesville is the only one willing to help us. However, the service fee is prohibitively expensive and beyond the reach of our current budget. As a result, we hope the reviewer will understand that we are unable to perform this assay at this time and will look forward to this excellent experiment when the budget situation is improved.

2. “The activation of MER by Tubby and Tubby-like protein should be done with the pure protein (not with a mixture with membrane fraction).”

**Answer:** We expressed FLAG-tagged tubby and Tulp1 in HEK293 cells and purified the recombinant proteins using anti-FLAG monoclonal antibody (mAb) affinity columns (See #5 below). Purified proteins were used to validate MerTK activation in D407 cells. The following sentence has been added to the Results.

“The results were validated with purified tubby and Tulp1 (Supplementary Figure 3).”

3. “How are the cytoplasmic proteins of Tubby and Tubby-like protein released from the cells? When are they released, continuously or only after activation? How much percentage proteins are released?”

**Answer:** Tubby and Tulp1 are secreted continuously through unconventional pathway without the requirement of cell activation (Caberoy et al. 2009b). We quantified the relative signal intensities of extracellular and intracellular tubby in our previous study (Figure 2 in Caberoy et al, 2009b), and added the following sentence to the Discussion.

“Based on our recent data (Caberoy et al., 2009b), we calculated that ~3.5% of endogenous tubby are secreted in a period of 24 h.”

4. “Why are the recombinant Tubby and Tubby-like proteins prepared differently for different experiments (in one case from the culture sup or in another case from the cell lysates).”

**Answer:** Supplementary Figure 4 is the only case to use tubby and Tulp1 in the conditioned medium. The main reason of using the conditioned medium was to demonstrate that secreted tubby and Tulp1 can function as bridging molecules by binding to membrane vesicles.

5. "Use the purified Tubby and Tubby-like protein to show the binding to apoptotic cells.”

**Answer:** As suggested, we expressed FLAG-tagged tubby, Tulp1 and their mutants in HEK293 cells. The recombinant proteins were purified with anti-FLAG mAb affinity columns, analyzed by SDS-PAGE (shown below), and used to characterize their binding to apoptotic cells (new Figure 5B and 5C). The results were similar to original data obtained with cell lysates (not shown in the revised manuscript).

Data not shown in the manuscript. Purification of FLAG-tagged tubby, Tulp1 and their mutants. FLAG-tagged proteins were expressed in HEK293 cells, purified using anti-FLAG mAb affinity columns, eluted with FLAG peptide, dialyzed and analyzed by SDS-PAGE. Estimated ~2 – 3 µg protein/lane. (A) Tulp1 and its mutants; (B) Tubby and its mutants.
6. “The authors should show what on the apoptotic cells is recognized by Tubby and Tubby-like protein.”

Answer: As suggested by reviewer #2, identification of the unknown ligands on the apoptotic cells recognized by tubby and Tulp1 is a daunting challenge and will require considerable time. Both proteins (e.g. calreticulin, Gardal et al., 2005) and non-protein molecules (e.g. PS) may serve as ligands. It is difficult to solve all the puzzles in a single study and publication. We are working on identification of the unknown ligands, and we hope the reviewer will allow us to publish the work in a future paper.

7. “How do the authors explain the "obesity" of the tubby-deficient mice?”

Answer: Tubby is expressed in the retina and several brain areas, including the hypothalamus. Unknown tubby function(s) in the hypothalamus, rather than in the retina, may be responsible for adult-onset obesity associated with tubby mutation. Tubby is distributed in several subcellular compartments, including the cytoplasm, the nucleus and the extracellular space, and is likely to have multiple functions. This is supported by identification of 16 new tubby-binding proteins in our recent study (Caberoy et al., 2010b). The current manuscript only characterized one of tubby functions with a primary focus on its role in RPE phagocytosis with the discussion of possible relevance to retinal degeneration. It is difficult to explain all tubby-associated diseases, such as obesity, with only one characterized function.

Reviewer #2

1. “Figure 5 demonstrates strong association of Tubby and Tulp1 proteins with apoptotic but not healthy Jurkat cells. At what stage in the apoptosis programme is the association? It would be helpful if the authors included kinetics data of Tubby and Tulp1 protein binding to apoptotic cells showing apoptosis in relation to PS exposure and plasma membrane integrity. Similar criticisms can be made about Figure 6.”

Answer: To tackle the question, we induced apoptosis of Jurkat cells by different reagents, including staurosporine for 3 h (early apoptosis) or etoposide for 16 h (later apoptosis), and analyzed the binding of tubby and Tulp1 to apoptotic cells with purified FLAG-tagged tubby, Tulp1 and their mutants. The results in new Figure 5B and 5C (early apoptosis) are similar to those obtained with later apoptotic cells in original Figure 5B and 5C (not shown in the revised manuscript). Apoptotic cells were characterized by flow cytometry using phosphatidylserine-binding annexin V in Supplementary Figure 5. These data suggest that tubby and Tulp1 bind to cells at both early and later stages of apoptosis induced by different reagents. Similar results to those plotted in Figure 6C (later apoptosis) are shown in Supplementary Figure 2 with staurosporine (early apoptosis).

2. “In addition, although they do show that Tubby doesn’t require PtdIns(4,5)P2 to bind to apoptotic cells and it is already known that such binding is PS-independent, it would be worth knowing how conserved the ligands of apoptotic cells for Tubby and Tulp1 are. Thus far the studies of apoptosis in the paper are restricted to Jurkat cells only. What about other cells of other lineages (including retinal epithelial cells)? While it might be unreasonable to require the identification of the apoptotic cell ligand(s) for these bridging molecules, it would be useful to have an indication as to how conserved/ubiquitous they are.”

Answer: We appreciate the reviewer’s recognition of the difficulties in identifying apoptotic cell ligand(s) for tubby and Tulp1. We agree that it is important to determine the cell specificity of the ligand(s). We chose HEK293 cells, rather than retinal epithelial cells, to investigate the cell specificity, because apoptotic RPE cells may have not only the ligand(s) for PPBD but also MerTK for MPD. The dual binding activities may complicate the data analysis. Our new data showed that HEK293 cells do not express MerTK (Supplementary Figure 1). The results revealed that PPBD of tubby or Tulp1 is also capable of binding to apoptotic HEK293 cells, but not healthy cells (Supplementary Figure 6).

3. “Should the last label on the x axis of Figure 6D read GST-Tulp1+GST?”
Answer: The label is corrected.

4. “the discussion should be shortened and more incisive.”

Answer: As suggested, we shortened the Discussion section by removing the subsection entitled “Global elucidation of unknown eat-me signals, which is less relevant to this study.

Reviewer #3

General comments

“…However, regarding the functional relevance and the role of MerTK for the phagocytosis process the data are incomplete. Furthermore, the discussion is not clearly differentiating between the several types of phagocytic activity (POS phagocytosis versus apoptotic cell phagocytosis or RPE versus macrophage phagocytosis).”

Answer: We agree with the reviewers that although our work provides sufficient evidence of tubby and Tulp1 as new MerTK ligands, their functional roles in RPE and macrophage phagocytosis are far from complete. This is particularly true to their roles in the maintenance of retinal homeostasis as elaborated in the Discussion. Since the identification of Gas6 and protein S as the ligands of TAM RTK family in 1995, many studies have investigated their signaling pathways and functional roles in various phagocytes by different groups. Similarly, it is difficult to provide all the answers for the functional roles of tubby and Tulp1 as new phagocytosis ligands in a single paper. But we believe that solid evidence of tubby and Tulp1 as MerTK ligands in RPE and macrophage phagocytosis will stimulate future investigation of their functions with joint endeavors from other groups. Differences between RPE and macrophage phagocytosis are discussed below in “specific comment” #6.

Specific comments

1. “…..That the material is ingested was shown be co-staining against organelles of the phagocytic pathway. This should be shown for the Mer-Fc effects too. Moreover it would be good to to show that tubby and the ligand of tubby is not phagocytosed in the absence of MerTK. Furthermore, it is needed to show that debris or apoptotic cells are not phagocytosed in the absence of tubby.”

Answer: As suggested, we analyzed the co-localization of NMIIA and Lamp-1 with phagocytosed cargos in the presence of Mer-Fc and have added the data to Figure 2 and 3, respectively. The results showed that Mer-Fc blocked the co-localization of both biomarkers with phagocytosed tubby- or Tulp1-vesicles.

Moreover, we knocked down MerTK expression in J774 macrophage cells by small hairpin RNA (shRNA)). We chose macrophages for this study because of Reviewer #3’s suggestion in Specific Comment #8. The results indicated that MerTK expression was substantially reduced at mRNA and protein levels (Supplementary Figure 2B). Phagocytosis assay showed that MerTK knockdown reduced tubby- or Tulp1-mediated macrophage phagocytosis (Supplementary Figure 2C and 2D).

Our recent published study showed that retinal vesicles (i.e. debris) prepared from Tubby mice, which have spontaneous deletion mutation of C-terminal 44 amino acids (i.e. PPBD domain), had reduced RPE phagocytosis (Caberoy et al., 2010a).

2. “The authors showed that tubby appears in the cytosol after phagocytosis. Is MerTK also ingested?”

Answer: Commercially-available anti-MerTK antibodies only work for Western blot and immunoprecipitation, and are not recommended for immunocytochemistry. In fact, Western blot analyses of MerTK with cell lysates in Figure 1D and Supplementary Figure 2B were technically challenging with high background. These antibodies have not been documented for immunocytochemistry. In our hand, none of these antibodies detected MerTK in D407 or ARPE19 cells above the background level by immunocytochemistry.

3. “According to the paper from Finnemann (EMBO J 2003) alone the presence of photoreceptor outer membranes is sufficient to induce phosphorylation of MerTK via focal adhesion kinase which
results from activation of alphaV-integrins. This is in contrast to the data presented here: showing that the intrinsic content of tubby in photoreceptor outer membranes alone is sufficient.”

**Answer:** MerTK is a well-characterized receptor tyrosine kinase with extracellular ligand-binding domain and intracellular domain. It is well-known that MerTK ligands, such as Gas6, bind to the extracellular domain to induce receptor autophosphorylation and phagocytosis. On the other hand, it is not surprising that the intracellular domain may also be regulated by other kinases, such as focal adhesion kinase (FAK), at the horizontal cellular signaling level. Thus, our results of tubby and Tulp1 as new MerTK ligands to induce phagocytosis do not contradict the regulation of MerTK phosphorylation by other intracellular kinases, including FAK by Finnemann.

4. “ARPE-19 cells: this is a RPE cell line which not necessarily shows all features of native RPE cells. The authors should show at least that these cells express MerTK.”

**Answer:** Our data showed that MerTK mRNA is expressed in D407 and APRE19 cells, but not in HeLa and HEK293 cells (Supplementary Figure 1). In addition, Western blot verified MerTK expression in ARPE19 and D407 cells.

5. “Physiology of phagocytosis: The phagocytosis of shed photoreceptor outer membranes by the retinal pigment epithelium requires at least three receptors: CD36, MerTK and alphaV-integrins. Based on the analysis of phagocytosis by retinal pigment epithelial cells from RCS rats, alphaV-/- mice and other work it is clear that the three receptors interact in the initialization of phagocytosis. In this network alphaV-integrin enables binding of the photoreceptor outer segments (RCS rat which does not have an active MerTK can bind outer segments but is unable to ingest the outer segments although cells from the RCS rat shows unspecific phagocytosis of beads and other material). Binding to alphaV-integrin activates FAK which phosphorylates MerTK which subsequently can be activated by Gas6. Thus, the model proposed here is not in accordance with the literature about photoreceptor phagocytosis by the retinal pigment epithelium. Also the experiments shown here do not take the current knowledge into account.”

**Answer:** We have revised Figure 7 by incorporating the integrin pathway accordingly. It is worth noting that the Figure is a summary of the current study, rather than a comprehensive roadmap of all signaling pathways as in a review article. Otherwise, the diagram will be too busy to highlight the results generated from this study.

6. “The Discussion mixes the mechanisms which control the phagocytosis by the retinal pigment epithelium and that by macrophages. Furthermore, the authors do not differentiate between phagocytosis of photoreceptor outer segments and apoptotic cells/debris. The phagocytosis of photoreceptor outer membranes is a highly regulated process and not only the removal of debris. The process must be tightly regulated because it would change the structure of photoreceptors and therefore alter vision. If phagocytosis activity is too large the photoreceptor outer segments would become too short. On the other and it is shown that during retinal degeneration in general macrophages are activated. However, this is more a secondary effect in the chain of events leading to degeneration of the retina. The primary effects are gene mutations leading to cell apoptosis in either photoreceptors or cells of the retinal pigment epithelium. Thus, also the text about the role of tubby in phagocytosis by macrophages is mixed up with the different mechanisms involved in retinal degeneration. I don’t think that the data can generalized as it is done on page 21.”

**Answer:** We agree that the length of POS must be precisely maintained by a set of biological processes, including shedding, phagocytosis, recycling and regeneration of the PS discs. Several earlier studies of MerTK have provided new insights into molecular regulations of RPE phagocytosis. However, we have to be careful to claim a uniqueness of these molecular regulations in RPE phagocytosis, because some of them have not been characterized for macrophage phagocytosis. To strike a balance on this concern, we included the following two paragraphs to meet the reviewer’s request to discuss the importance of RPE phagocytosis to maintain retinal homeostasis and the difference between RPE and macrophage phagocytosis.

“Another important issue is whether tubby and Tulp1 are essential eat-me signals to maintain retinal homeostasis. POS function as power plants by converting light into electrochemical impulses and undergo a constant renewal process to repair photo-damage (Strauss, 2005). The tips of the POS...”
contain the highest concentration of radicals, photo-damaged 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.”

“Current knowledge reveals that many phagocytosis signaling 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. 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 signaling pathways, and define the similarities and differences between RPE and macrophage phagocytosis.”

We agree with the reviewer that macrophage recruitment is the secondary effect of the retinal degeneration and have revised the Discussion to “macrophages have been demonstrated to be recruited into the retina during retinal degeneration caused by genetic mutations or photo-damage.”

Tubby most likely has multiple functional roles as described in the Discussion. We discuss the role of tubby in RPE and macrophage phagocytosis in one subsection entitled “Biological relevance” and the retinal degeneration associated with tubby mutations in another subsection entitled “Disease relevance”. I hope this arrangement helps to separate its different roles.

The discussion on original page 21 was the subsection entitled “Global elucidation of unknown eat-me signals”, which was removed in the revised manuscript as suggested by reviewer #2 (Comment #4). However, a related discussion of “systematic identification of phagocytosis ligands” by our new strategy is included as above. We believe that this strategy can be “generalized” because we are working on a project to globally identify cell-wide phagocytosis ligands by combing our ORF phage display technology with phagocytosis-based functional selection (Caberoy et al., 2010a) and next generation DNA sequencing.

7. “Tubby as eat me signals: The authors argue that tubby is secreted by the photoreceptors to initialize the phagocytosis of photoreceptor outer segments. This model is not well elaborated because also the secretion of this protein is not shown in the retina and it is not clear where tubby is secreted. The phagocytosis takes place at the outer segment whereas it is likely that the inner segments secret tubby which makes a rather long diffusion way for a tight regulation of photoreceptor outer membrane phagocytosis.”

**Answer:** Given that tubby association with POS as in Supplementary Figure 7, we have added the following sentence in the Discussion.

“Tubby in the POS (Supplementary Figure 7) could be directly secreted into the interface of RPE and POS.”

8. “Probably the authors should concentrate their paper on the role of tubby or tulp-1 on macrophages and their role in removing debris or apoptotic cells. The data and conclusions concerning the phagocytosis by the retinal pigment epithelium are less convincing. This can be seen for example in the discussion part of the paper on page 20. This part states at the beginning that tubby and tulp-1 play a critical role in retinal homeostasis but at the end of that paragraph it is concluded that tubby and tulp-1 are unlikely essential eat-me signals for retinal homeostasis.”

**Answer:** The critical roles of tubby and Tulp1 in the maintenance of retinal homeostasis are manifested by retinal degeneration associated with their mutations. The question is whether RPE phagocytosis mediated by tubby and Tulp1 plays an essential role in retinal homeostasis. Our discussion addresses this question. Although the results of this study provide sufficient evidence for tubby and Tulp1 as new MerTK ligands for phagocytosis, their roles in retinal homeostasis are yet to be further determined by in vivo studies.

We believe that RPE phagocytosis should be included for two reasons: (a) Mutations in MerTK, tubby or Tulp1 cause retinal degeneration; and (b) Tulp1 is specifically expressed in the retina, and tubby is expressed in the retina and brain, but not in other immunological tissues, such as thymus or spleen. In this regard, an exclusive focus on macrophage phagocytosis is not appropriate and might
raise new questions about their functional roles in RPE phagocytosis from other reviewers or readers.

Minor comments:

9. "Figure 4E: the blot shows no size markers and strongly differs from that shown in Figure 1D."

**Answer:** The size markers have been added to Figure 4D.

MerTK in Figure 1D and Supplementary Figure 2B was analyzed by “direct” Western blot with cell lysates using different antibodies. Figure 4E and Supplementary Figure 1B were “indirect” Western blot analyses, which were immunoprecipitated with anti-MerTK antibodies, followed by Western blot using anti-phospho-MerTK or anti-MerTK antibodies. Direct Western blot had higher background than indirect Western blot, which already removed majority of irrelevant proteins by immunoprecipitation. Direct Western blot of MerTK is technically difficult and was used only for quantitative analyses, whereas indirect Western blot of MerTK is more convenient and was used for qualitative analyses. The procedures for all these MerTK analyses are described in the Figure legends.

10. "phagocytosis through MerTK": The role of MerTK is possibly more to trigger the endocytosis machinery and the ingestion process is more due to other receptors such as CD36. It should be noted that activation of MerTK results in the formation of inositol-1,4,5-trisphosphate as second messenger in RPE cells. Several papers which report basic mechanisms of photoreceptor phagocytosis and data from RCS rat are not cited and not put into account neither for experimental design nor in the discussion."

**Answer:** We have included inositol triphosphate (IP3) as the second messenger in MerTK signal pathways in Figure 7 and cited previous studies of RCS RPE cells in the Figure legend.

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2nd Editorial Decision 21 September 2010

Thank you for sending us a new version of your original manuscript EMBOJ-2010-73783 as a new submission. Let me apologise for the exceptionally long delay in getting back to you with a decision. This was caused by difficulties with the availability of our original referees during the past summer holiday season.

Our original referees have now finally seen it again (see below). Referees 1 (original Ref. 2) and 3 are now in favour of publication of the paper here. Referee 2 (original Ref. 1) still raises a number of concerns that would require additional experimentation. Given the more positive vote by the other two referees I have come to the conclusion that the remaining issues raised by referee 2 should be addressed where appropriate or at least responded to in an amended version of the manuscript. I would also like to ask you to deal with the remaining minor point suggested by referee 3.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your amended manuscript.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #2 (Remarks to the Author):
In this manuscript, the authors claim that proteins called "Tubby" and "Tubby-like protein" work as bridging molecules between apoptotic cells and phagocytes, and stimulate the engulfment of apoptotic cells. The results are of some interest. However, the results are too preliminary to support their conclusion.

1. Since all the experiments were performed with the crude vesicle fractions, I asked to carry out the experiments with the purified protein. They claim that the recombinant proteins were purified, and the key experiments were validated. Please describe the detailed procedure how the recombinant proteins were purified, and show the data in the manuscript (not just in rebuttal). The authors claimed that "Tubby" and "Tubby-like protein" are associated with vesicles. I want to ask whether these recombinant proteins could be recovered as soluble protein or not?

2. Since the authors claim that "Tubby" and "Tubby-like protein" are ligands for MER, I asked to determine their dissociation constant. The authors reply that they do not have Biacore, and it is very expensive. There are many methods to determine Kd. If the authors want to claim that "Tubby" and "Tubby-like protein" are ligands for MER, they should determine the Kd. From the Kd value, we can judge whether it has a physiological role or not. For example, the authors use 5 µg/ml the Flag-tagged Tubby in Figure 5 for the binding to apoptotic cells. This concentration is 20 times higher than that used for the recombinant MFG-E8 to bind apoptotic cells (Nature 417, 182, 2002). If such high concentration of Tubby is necessary for the recognition of apoptotic cells, it is difficult to think of any physiological roles of Tubby in the engulfment of apoptotic cells.

3. In Figure 5B, the authors point out that Tubby binds to 73.2% of the apoptotic cells. The FACS profile indicates that all cells underwent apoptosis, and Tubby homogeneously binds to them. Judging from the percentage, the authors conclude that the N-terminal half of Tubby is responsible for recognition of the apoptotic cells. However, it is not clear how this percentage is determined. In contrast to the statement in the text, the FACS profile indicates that the mutants (Tubby N and Tubby-ΔC44) still bind to the apoptotic cells. Figure 5C has the same problem.

4. In Figure 5, the authors prepare apoptotic cells by treating human Jurkat cells with staurosporine or etoposide. The FACS profile indicate that all the cells are apoptotic. This is difficult to believe. Please show the Annexin V- and PI-staining profile. I want to know that Tubby binds to Annexin V-positive but not PI-positive cells.

5. "Eat me" signal is the signal on apoptotic cells. The Tubby" and "Tubby-like protein"should not be called "eat me" signal.

Referee #3 (Remarks to the Author):

The authors have successfully addressed all requests and concerns made by the referees. The data now convincingly support the conclusions made by the authors.

Minor point:
- The authors used several different cell lines for their experiments, sometimes in the same figure. I would suggest that the authors indicate in the figures for every set of data with which cells these data were made.

Revision - authors' response 22 September 2010

We are grateful for reviewers’ valuable comments and the opportunity to resubmit our manuscript. The following is our point-to-point answers to the comments. All revisions are highlighted in the manuscript.

Referee #1:
1. We have included the detailed protocol for the purification of FLAG-tagged recombinant proteins in the Materials and methods. The SDS-PAGE for the purified proteins is included as Supplementary Figure 9. The association of endogenous tubby with photoreceptor outer segments (POS) vesicles has been demonstrated in Supplementary Figure 7. However, purification of endogenous tubby from the vesicles requires anti-tubby antibody affinity column, which is not available at this time. Moreover, both tubby and Tulp1 are susceptible to degradation after acidic buffer treatment for non-detergent protein stripping. Therefore, recovery of tubby protein is technically difficult at this time.

2. We used excessive amount of tubby or Tulp1 (5 µg/ml) for the binding assay with apoptotic cells in Figure 5B and 5C. Excessive unbound proteins were washed away. The previous study (Nature 417, 182, 2002, Fig. 2b) used 1.25 µg/ml of MFG-E8-L for binding to apoptotic cells and flow cytometry analysis. Thus, the amount in our study was only about 4-fold higher than the previous study. The critical issue actually is whether tubby and Tulp1 associated with phagocytosis preys are capable of stimulating phagocytosis. Our previous study (Exp. Cell Res. 316: 245, 2010, Fig. 4A and 4B) showed that POS vesicles prepared from wild-type mouse retinas, but not the retinas of tubby-deficient or Tulp1-deficient mice, stimulated RPE phagocytosis, suggesting that endogenous tubby and Tulp1 associated with POS vesicles are capable of stimulating RPE phagocytosis. Similar results are shown in Figure 6.

3. Tubby and Tubby-C in Figure 5B had similar high binding activity to apoptotic cells, whereas Tubby-N and Tubby-ΔC44 had similar low binding activity. These results led us to conclude that that tubby C-terminal domain, but not the N-terminal domain, is important for the recognition of apoptotic cells. All the cells in the range of the indicated bars in the FACS panels (Figure 5B and 5C) are counted as positive and used to calculate the percentage of positive cells labeled with tubby or Tulp1. We acknowledge that Tubby-N, Tubby-ΔC44 and Tulp1-ΔC44 also had higher binding activity to apoptotic cells than healthy cells. Thus, we have added the following sentence to the Results. “However, both Tubby-ΔC44 and Tulp1-ΔC44 bound to apoptotic cells better than healthy cells (Figure 5B and 5C). Thus, it is possible that other regions of tubby and Tulp1 have additional binding activity to apoptotic cells.”

4. All apoptotic cells for Figure 5B and 5C were prepared by treating Jurkat cells with staurosporine for 3 h and analyzed with purified FLAG-tagged proteins. In a separate study, we treated Jurkat cells with etoposide for 16 h and analyzed their binding with FLAG-tagged tubby, Tulp1 and their mutants in HEK293 cell lysates. Similar results of flow cytometry analysis were obtained (data not shown for etoposide-treated cells due to similarity). The flow cytometry profiles of apoptotic cells treated with staurosporine (3 h) and etoposide (16 h) were analyzed in Supplementary Figure 5. Majority of staurosporine-induced apoptotic cells (95.8%) were Annexin V-positive. Only 2.5% of staurosporine-induced apoptotic cells were propidium iodide (PI)-positive. Thus, Figure 5B and 5C are the results of tubby and Tulp1 binding to Annexin V-positive, but not PI-positive cells.

5. We have changed “eat-me signal” to phagocytosis ligand.

Referee #3:

Both ARPE19 and D407 cell lines are used in Figure 1 and 4. We have labeled the relevant panels in both figures with the name of the cell lines, as suggested. All other figures and supplementary figures have only one phagocyte cell line, which is described in the figure legends to minimize distraction.