The Kinesin KIF16B mediates Apical Transcytosis of Transferrin Receptor in AP-1B deficient epithelia

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Editor: David del Alamo

1st Editorial Decision 13 July 2012

Thank you for sending me your manuscript and for your interest in The EMBO Journal. I have read it, discussed with the rest of the editorial team and further contacted two external advisors, experts in the field.

We all highly appreciate that your manuscript directly demonstrates with a very solid and convincing set of evidences, including in vivo assays, that proteins that would normally take a basolateral recycling pathway, invade the apical recycling/transcytotic pathway in the absence of AP-1B. However, we also agree that your study does not explore the mechanisms involved beyond what it is already known about the apical recycling pathway. We have to conclude that, although we actually acknowledge that this is the first formal description of the sorting pathway used by basolateral proteins in the absence of AP-1B, this would not provide a sufficient conceptual advance to justify the publication of your manuscript in The EMBO Journal.

I would like to thank you again for your interest in our journal. I am really sorry to disappoint you and I sincerely hope that we can work together in the future under more favorable conditions. I wish you luck with the rapid publication of this study.
I enclose a manuscript that we would like to have considered for EMBO J. This manuscript developed from a ms you monitored last summer. Since then, we have added important mechanistic insights.

We show that the plus-end kinesin KIF16B mediates selectively the transcytosis of TfR to the apical surface. This is the first description of a MT motor involved in transcytosis. Other experiments indicate that KIF16B does not mediate transcytosis of polymeric IgA receptor, indicating that there are multiple motors involved in transcytosis. We show experiments that suggest that KIF16B operates on non-centrosomal microtubules. We show that apical trafficking of TfR is mediated by its N-glycans as tunicamycin blocks its transcytosis. Other experiments not yet included in the ms show that one of the three glycans is critical for transcytosis of TfR, but not the other two. Finally, we show that TfR is also segregated from Rab11a endosomes in sub confluent MDCK cells, but this segregation is broken in the absence of AP-1B.

We believe that, together with the first solid demonstration that basolateral proteins invade the apical pathway using a transcytotic route through two different types of apical endosomes, our manuscript represents a solid and innovative contribution to epithelial cell biology and polarized trafficking.

I hope you agree with us. Please let me know if we may go along with a formal submission.

Thank you for the submission of your manuscript entitled "The kinesin KIF16B mediates apical transcytosis of transferrin receptor in AP-1B deficient epithelia" and please accept my apologies for the delay in the decision but we have only now received the full set of reports from the referees, which are copied below. As all three referees agree on the high interest of your manuscript and their comments are in general positive, I would like to invite you to revise it.

Without going into details that you will find below, referees #1, #2 consider that your manuscript should be published in The EMBO Journal and propose a number of points for you to consider in order to improve your message before final acceptance can be granted. As you will also see, referee #3 is less enthusiastic about your work but still proposes some experiments to certainly reinforce the conclusiveness of your data. Although the referees are rather explicit in their queries, I would like to draw your attention to certain points that I consider of particular relevance. Referee #2 argues that given the fact that cell lines that naturally lack AP-1B are readily available, at least key experiments should be performed in these cell lines (ARPE-19 or LLC-PK1). Additionally, in agreement with referee #3, s/he considers that loss of function analyses should be performed, instead of using dominant-negative constructs that might be the cause of unwanted secondary effects.

Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will essentially depend on the completeness of your responses included in the next version of the manuscript. Do not hesitate to contact me by e-mail or on the phone in case you have any questions, you need further input or you anticipate any problem during the revision process.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let me know in advance and I may be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the 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:
Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

Epithelia use the specialized medium adaptor subunit AP-1B to sort membrane proteins to the basolateral cell surface. Several epithelial cell lines including LLC-PK1 for which it was first demonstrated, however do not express AP-1B. As a consequence of which, they now target many basolateral proteins apically. The precise intracellular itineraries for these cargo molecules and the underlying molecular sorting and traffic mechanisms are long standing questions in which limited progresss has been made since the initial description.

Authors used knockdown of AP1B in MDCK cells and an AP-1B-deficient retinal pigment epithelial cell line for their experiments. They show here that basolaterally endocytosed transferrin is transcytosed via common recycling endosomes to apically localized endosomes via a pathway that requires microtubules, the plus end-directed KIF16B motor, N-linked glycosylation of the cargo and is dependent of rab11 GTPase (unlike the transcytotic pathway taken by Tf in epithelia expressing AP1B).

Comments:
The main progress of the paper is the demonstration of a KIF16B requirement in vesicular transport from common recycling endosomes to apical endosomes along non-centrosomal microtubules. This motor was already shown by Zerial's lab to be involved in regulating transport from sorting endosomes in fibroblasts. Although these observations are interesting, I find the general gain of knowledge limited as long as we do not learn more about the proteins (except rab11, KIF16B, and AP-1B) that are involved in apical transcytosis in epithelial cells with and without AP-1B expression.

Authors show that pIgAR segregates from TfR at the level of common recyling endosomes and does not follow the AP-1B pathway. What other receptors that normally recycle basolaterally, now go together with TfR to the plasma membrane in the absence of AP-1B.

Tunicamycin is a general inhibitor of N-linked glycosylation. It is therefore less suitable to establish the significance of glycosylation for a particular protein. Instead it is more appropriate to mutate the Asn-X-Thr/Ser glycosylation consensus sequence(s) of TfR, and then determine the consequences in the transport assays.

It is not clear what rab11 mutant was used throughout these experiments. On page 6, line 3 authors mention a GTPase-deficient mutant. Such a mutant very slowly hydolyzes GTP and is therefore mostly in an active form. Dominant negative rab mutants usually are the ones that are in a GDP or empty state.

Referee #2

The paper presented by Perez-Bay and coworkers shows a new transcytotic pathway to target AP-1B-dependent cargoes from the basolateral membrane to the apical membrane in absence of the AP-1B adaptor complex. The authors show that this pathway is Rab11-dependent, and requires N-glycan signals, microtubules and the kinesin KIF16B. They also demonstrate the relevance of the pathway for the physiology of native AP-1B deficient epithelial cells, using as the retinal pigmented epithelial cells as a model. Of special note, the authors develop a novel, highly sensitive, technique to quantify transferrin endocytosis and trafficking. Using this technique, they demonstrate the existence of a transcytotic pathway working in absence of AP-1B. The experiments were performed in µ1B-depleted-MDCK cells, as well as in ARPE-19 and µ1B-expressing CHO cell lines. Altogether the proposed hypothesis seems to be correct, and the experimental data sufficiently
validated for the most part. The article is well and concisely written and the experimental design is accurate and clear. However, there are certain gaps in the mechanism proposed by the authors that should be addressed before publication. In fact the model would benefit from further evidence to support it more solidly. For instance, the precise function of KIF16B is far from clear. This is of special importance since the title of the work highlights the role of KIF16B in this new pathway, and there are very few experiments that contribute to elucidate this mechanism.

Major Concerns

1-A general criticism to the article is the extensive use of µ1b-depleted MDCK cells, especially when the authors propose that the new pathway they describe function physiologically in epithelial cells that natively lack AP-1B (i.e. ARPE-19 or LLC-PK1). Especially surprising is the lack of experiments with LLC-PK1 cells, which is a AP-1B deficient model used by the authors before (Yan et al. NCB 2002). Considering that ARPE-19 cells take 6 weeks to properly polarize, the authors should consider performing some key experiments on LLC-PK1 cells, or at least explain why the experiments cannot be done using this cell line.

2-The authors remark the importance of the role of KIF16B in transcytosis. Indeed, this is the first evidence of a kinesin that functions in transcytosis, and more importantly, it seems to be specific for Tfr, or at least does not play a role in pIgAR transcytosis. However, the manuscript does not sufficiently address the role of KIF16B. The major caveat is that all the experiments on KIF16B function are done only using MDCK AP-1B KD cells. This is an important gap that should be addressed: the authors need to prove that natively AP-1B-deficient epithelia express KIF16B, and characterize its function in these cells (expression of DN-KIF16B or KIF16B silencing in a natively AP-1B-deficient epithelial cell line).

3-The authors suggest that the mechanistic function of KIF16B is to target TfR from the CRE to the ARE through microtubules. However, the fact that MT disorganization leads to a mistargeting of TfR to the ARE in AP-1B-KD cells could also be explained by a traffic defect at basolateral sorting endosomes (Rab4+), instead of at the CRE (Figure 2). Perhaps a key question in this matter is whether Tf reaches the CRE in the presence of nocodazole, which the authors could address using longer incubation times of labeled Tf.

4-The KIF16B experiments were all performed using a dominant negative (DN) constructs, and only in transient expression. In order to demonstrate that Tf cannot reach the apical surface in KIF16B DN cells, the authors should prove the effect of the DN using an inducible expression system, and then use the Sulfotag-Tf assay to quantify what they observe by IF. Additionally, these experiments should be complemented with experiments silencing endogenous KIF16B, and if possible with the correspondent rescue experiment (Figure 3).

5-It has been shown that KIF16B participates in the transport and distribution of early endosomes (Hoepfner et al., 2005). Thus, the defect found in TfR trafficking to the ARE may be explained by a defect in early endosomes BL distribution instead of a defect in trafficking from CRE to ARE, as the authors suggest. In fact Figure 3a shows what seems to be a scattered distribution of TIR+ endosomes in DN-KIF16B expressing cells compared with controls. To tackle this, the authors should prove that early endosomal Tf (5 min Tf-chase) or other early-endosomal markers are not affected by KIF16B-DN and normally localize in WT and AP-1B-deficient cells.

Furthermore, another important question is whether KIF16B-DN affects BSE-to-CRE trafficking of Tf (instead of CRE-to-ARE). In this regard, the authors should characterize the localization of Tf at 5-vs-30 min (using dual Tf labeling, as in Figure 2) in WT cells transfected with DN-KIF16B. This experiment is crucial to address whether KIF16B specifically controls transcytosis of Tf in AP-1B-deficient cells, or if it is involved in Tf trafficking towards the RE.

6-Concerning the localization of KIF16B in the CRE endosomes (Figure 3d and 3e). Here, the main difficulty is the poor definition of what represents the CRE. The authors should clearly show the localization of KIF16B and late-endocytosed Tf in this compartment using 3D reconstructions (and an x-z projection of a whole cell). It would also be interesting to show a colocalization with µ1B. Also, it would be important to show if this localization is MT-dependent to complement the present data.
Minor Points

1- Rab11 has a very important role in polarity establishment, junctional recycling, and apical trafficking. The authors should analyze if Cherry-Rab11-DN cells have general defects in cell polarity that may affect the Sulfotag-Tf assay (Figure 1c-e). This is especially important since treatment with Dox 20 ng/ml does not completely block Ch-Rab11-DN expression. In addition, the authors should address what happens with BL recycling of Tf in Rab11-DN cells. Is it reduced or enhanced? What happens with the internal pool?

2- Do AP-1B deficient MDCK cells have defects in tight junction assembly or have increased junction leakiness that might affect data interpretation in figure 2a? The authors previous showed that general distribution of polarity markers is not affected (Gravotta et al 2012), but the TER was not tested.

3- AP-1B-KD MDCK cells when expressing DN-KIF16B seem to have apical Tf, although not reaching the ARE (Figure 3a). Are these WGA-positive Tf-loaded endosomes? The authors should show that the endosomes are WGA-negative to support their model. Also, it would be interesting to show μ1B localization in WT-MDCK expressing KIF16B-DN to prove that KIF16B does not affect localization of the CRE, and only affects trafficking of Tf.

4- The KIF-16B endogenous localization images are not very clear in Figure 3D. Better images should be provided. A quantification of KIF-16B colocalization with BSEs vs. CRE should also be provided (as it was done throughout the manuscript). It would be also very interesting if the authors could address the localization of AP-1B in these experiments.

5- Why do the authors use transiently transfected TfR-GFP instead of endogenous TfR in the experiment described in figure 4? There are indeed monoclonal antibodies suitable for MDCK endogenous TfR imaging commercially available from Zymed.

6- The fact that BL recycling is so much lower in RPE cells than in μ1B-KD MDCK cells in figure 5 should be discussed. Why is the intracellular pool so much larger? It would be also clarifying to show x-z reconstructions of ARPE-19 cells (as it is done in the rest of the manuscript).

7- Figures 6-7 could be merged in only one figure.

Referee #3

While the role of microtubules in transcytosis has long been known, the exact nature of microtubule motors involved has remained mysterious. The major advance in this paper is the identification of KIF16B as a candidate regulator of this pathway. KIF16B has already been implicated in endocytic trafficking, so the novelty and importance of this work depends largely on whether the role of KIF16B is different from its previously reported function in TfR recycling. As it stands this is not adequately addressed, and none of the data show a direct role for endogenous KIF16B in transcytosis in this system.

The initial part of the manuscript focuses on the role of Rab11 and microtubules. The data presented in Figure 1 show that a dominant negative form of rab11 inhibits apical transcytosis of TfR. Exactly why this effect occurs is debatable, and does not prove that endogenous rab11 is involved. Other dominant negative rabs might give the same effect, but none are tested - rab4 and 14 seem like obvious choices. Dominant negative rabs are most likely acting by blocking the function of a cellular exchange factor, and therefore perturbing the endogenous rab. For rab11 it still isn't known what the exchange factor is, so this is somewhat of a black box experiment. A better strategy to show that rab11 has a function in apical transcytosis when AP-1b is absent would be to knock down the endogenous pool of rab11. For this multiple si/shRNAs would need to be used and blotting performed to confirm efficient depletion.

I have some additional queries about Figure 1 that should be addressed by Western blots:
1. How much Rab11 is endogenous and how much is overexpressed?
2. Are endogenous Rab11 levels the same in all KD cell lines?

There are similar issues with the experiments investigating the function of KIF16B, which have to be viewed as rather preliminary in their present form. Dominant negative kinesin constructs are used, but no attempt is made to look at endogenous protein. A first step would be to establish if KIF16B is expressed in these cells, and then test if its knock down has any effect on transcytosis. Localisation of endogenous KIF16B would also be valuable, since it may be present on moving transcytotic vesicles.

This first part of the work if fully developed could make an important contribution to the literature on transcytosis, but my view of the current manuscript is that it is too preliminary for publication.

The later section of the work is an addition rather than a core part of the study. In Figure 4 the role of glycosylation in TfR traffic is investigated. While tunicamycin causes altered transport of TfR this does not in itself prove that glycosylation of TfR is the cause. To make this point a TfR mutant lacking N-glycosylation sites would need to be used. While the role of N-glycans in sorting is an interesting issue it could form a study in its own right and doesn't seem to be that relevant unless a direct link to KIF16B is made.

Finally, the data on CHO cells does not relate to transcytosis and could be omitted entirely without changing any of the main conclusions. If AP-1b expression creates a novel recycling pathway the obvious question is which rab is required, and this isn't followed up.

Minor points
In the introduction both Hunziker et al & Matter et al (EMBOJ 1990 9(11):3515-25; 3163-70) should be cited in addition to the Apodeca 1994 paper when introducing the role of microtubules in transcytosis.

Typo in Figure 3d. "length".

Referee #1

Epithelia use the specialized medium adaptor subunit AP-1B to sort membrane proteins to the basolateral cell surface. Several epithelial cell lines including LLC-PK1 for which it was first demonstrated, however do not express AP-1B. As a consequence of which, they now target many basolateral proteins apically. The precise intracellular itineraries for these cargo molecules and the underlying molecular sorting and traffic mechanisms are long standing questions in which limited progress has been made since the initial description.

Authors used knockdown of AP1B in MDCK cells and an AP-1B-deficient retinal pigment epithelial cell line for their experiments. They show here that basolaterally endocytosed transferrin is transcytosed via common recycling endosomes to apically localized endosomes via a pathway that requires microtubules, the plus end-directed KIF16B motor, N-linked glycosylation of the cargo and is dependent of rab11 GTPase (unlike the transcytotic pathway taken by Tf in epithelia expressing AP1B).

Comments:
The main progress of the paper is the demonstration of a KIF16B requirement in vesicular transport from common recycling endosomes to apical endosomes along non-centrosomal microtubules. This motor was already shown by Zerial's lab to be involved in regulating transport from sorting endosomes in fibroblasts. Although these observations are interesting, I find the general gain of knowledge limited as long as we do not learn more about the proteins (except rab11, KIF16B, and AP-1B) that are involved in apical transcytosis in epithelial cells with and without AP-1B expression.

1) Authors show that pIgAR segregates from TfR at the level of common recycling endosomes and
does not follow the AP-1B pathway. What other receptors that normally recycle basolaterally, now go together with TfR to the plasma membrane in the absence of AP-1B.

Like TfR, LDLR is a fast-recycling receptor and an AP-1B cargo (Folsch et al., 1999; Gan et al., 2002; Gravotta et al. 2007). Additional experiments (new Fig. 3) show that exogenous LDLR-GFP but not NBC1-GFP (a protein that is basolateral in AP-1B deficient proximal tubule cells) follow the same transcytotic pathway as TfR-GFP in AP-1B KD MDCK cells.

2) Tunicamycin is a general inhibitor of N-linked glycosylation. It is therefore less suitable to establish the significance of glycosylation for a particular protein. Instead it is more appropriate to mutate the Asn-X-Thr/Ser glycosylation consensus sequence(s) of TfR, and then determine the consequences in the transport assays.

We have performed experiments with five TfR mutants lacking either each or all four glycosylation sites. These experiments confirmed our tunicamycin experiments and revealed differential effects of each glycan on apical sorting. However, as suggested by reviewer #3 and to keep the focus of the manuscript on KIF16B, we have removed these data from this manuscript.

3) It is not clear what rab11 mutant was used throughout these experiments. On page 6, line 3 authors mention a GTPase-deficient mutant. Such a mutant very slowly hydrolizes GTP and is therefore mostly in an active form. Dominant negative rab mutants usually are the ones that are in a GDP or empty state.

We apologize for not including this information in the original manuscript and we thank the reviewer for pointing out this deficiency. The mutant utilized in this work is the S25N mutation, which is the GTP-binding deficient form of rab11a (Ren et al, 1998; Wang et al, 2000). We have corrected the original phrase on page 6, line 3 “GTPase-deficient mutant” to “GTP binding-deficient mutant of rab11a (S25N)”.

Referee #2

The paper presented by Perez-Bay and coworkers shows a new transcytotic pathway to target AP-1B-dependent cargoes from the basolateral membrane to the apical membrane in absence of the AP-1B adaptor complex. The authors show that this pathway is Rab11-dependent, and requires N-glycan signals, microtubules and the kinesin KIF16B. They also demonstrate the relevance of the pathway for the physiology of native AP-1B deficient epithelial cells, using as the retinal pigmented epithelial cells as a model. Of special note, the authors develop a novel, highly sensitive, technique to quantify transferrin endocytosis and trafficking. Using this technique, they demonstrate the existence of a transcytotic pathway working in absence of AP-1B. The experiments were performed in µ1B-depleted-MDCK cells, as well as in ARPE-19 and µ1B-expressing CHO cell lines.

Altogether the proposed hypothesis seems to be correct, and the experimental data sufficiently validated for the most part. The article is well and concisely written and the experimental design is accurate and clear. However, there are certain gaps in the mechanism proposed by the authors that should be addressed before publication. In fact the model would benefit from further evidence to support it more solidly.

For instance, the precise function of KIF16B is far from clear. This is of special importance since the title of the work highlights the role of KIF16B in this new pathway, and there are very few experiments that contribute to elucidate this mechanism.

We fully appreciate this comment and have performed several additional experiments on the endogenous expression, function and localization of KIF16B and experiments in native AP-1B-deficient RPE cells (new Figs 4, 5 and 6 and supplementary figures 5 and 6).

Major Concerns

1-A general criticism to the article is the extensive use of µ1b-depleted MDCK cells, especially when the authors propose that the new pathway they describe function physiologically in epithelial cells.
that natively lack AP-1B (i.e. ARPE-19 or LLC-PK1). Especially surprising is the lack of experiments with LLC-PK1 cells, which is a AP-1B deficient model used by the authors before (Yan et al. NCB 2002). Considering that ARPE-19 cells take 6 weeks to properly polarize, the authors should consider performing some key experiments on LLC-PK1 cells, or at least explain why the experiments cannot be done using this cell line.

We appreciate the point of the reviewer, therefore we added additional experiments in ARPE-19 cells, as it will be described bellow. Major reasons for using these cells are: (1) research on RPE is a central interest of our laboratory and (2) In the RPE, apical targeting of TfR serves a physiological function (i.e. it is thought to remove excess iron from the retina).

2-The authors remark the importance of the role of KIF16B in transcytosis. Indeed, this is the first evidence of a kinesin that functions in transcytosis, and more importantly, it seems to be specific for TfR, or at least does not play a role in pIgAR transcytosis. However, the manuscript does not sufficiently address the role of KIF16B. The major caveat is that all the experiments on KIF16B function are done only using MDCK AP-1B KD cells. This is an important gap that should be addressed: the authors need to prove that natively AP-1B-deficient epithelia express KIF16B, and characterize its function in these cells (expression of DN-KIF16B or KIF16B silencing in a natively AP-1B-deficient epithelial cell line).

We completely agree with the reviewer and have performed the following additional experiments:

In ARPE-19 cells:
1) We confirmed expression of KIF16B by RT-PCR (new Supplementary Fig 4).
2) We showed that DN- KIF16B inhibited traffic of TfR to ARE (similar to AP-1B KD MDCK cells) (new figure 6d)

In MDCK cells (WT and AP-1B KD):
1) We confirmed expression of KIF16B by RT-PCR (new Supplementary Fig 4).
2) We knocked down endogenous KIF16B in MDCK with siRNA (new Fig 4 and Suppl Fig 4).
3) We showed that knock-down of KIF16B inhibited traffic of TfR to ARE (similar to experiments with DN-KIF16B) (new Fig 4).

3-The authors suggest that the mechanistic function of KIF16B is to target TfR from the CRE to the ARE through microtubules. However, the fact that MT disorganization leads to a mistargeting of TfR to the ARE in AP-1B-KD cells could also be explained by a traffic defect at basolateral sorting endosomes (Rab4+), instead of at the CRE (Figure 2). Perhaps a key question in this matter is whether Tf reaches the CRE in the presence of nocodazole, which the authors could address using longer incubation times of labeled Tf.

We thank the reviewer for this important suggestion. However, Daro et al (1996) have already shown that nocodazole does not inhibit traffic of internalized Tf from sorting endosomes to recycling endosomes in CHO cells. Furthermore, we have shown that in polarized MDCK cells nocodazole does not alter basolateral recycling (Fig 1), further supporting the idea that normal basolateral recycling through BSE and CRE does not require microtubules

4-The KIF16B experiments were all performed using a dominant negative (DN) constructs, and only in transient expression. In order to demonstrate that Tf cannot reach the apical surface in KIF16B DN cells, the authors should prove the effect of the DN using an inducible expression system, and then use the Sulfotag-Tf assay to quantify what they observe by IF. Additionally, these experiments should be complemented with experiments silencing endogenous KIF16B, and if possible with the correspondent rescue experiment (Figure 3).

As suggested by the reviewer, we performed additional experiments (new Fig 4) that show that silencing endogenous KIF16B with siRNA inhibited transcytosis of TfR to ARE in MDCK cells. These experiments were carefully quantified for over 50 cells in different experiments, using confocal stacks representing the entire cell, and the Manders colocalization coefficient. These results were highly statistically significant. We clearly established that transport to ARE was an important aspect of apical transcytosis (Fig 1 and 2). Given this information, although the other
experiment proposed by the reviewer is interesting, we considered that the effort of preparing inducible dominant negative KIF16B cell lines was disproportionate to the possible benefits provided by these experiments.

5-It has been shown that KIF16B participates in the transport and distribution of early endosomes (Hoepfner et al., 2005). Thus, the defect found in TfR trafficking to the ARE may be explained by a defect in early endosomes BL distribution instead of a defect in trafficking from CRE to ARE, as the authors suggest. In fact Figure 3a shows what seems to be a scattered distribution of TfR+ endosomes in DN-KIF16B expressing cells compared with controls. To tackle this, the authors should prove that early endosomal Tf (5 min Tf-chase) or other early-endosomal markers are not affected by KIF16B-DN and normally localize in WT and AP-1B-deficient cells.

In our original manuscript we had labeled BSE and CRE (using dual-color Tf labeling) in WT and AP-1B KD MDCK cells expressing only the wild type KIF16B. As suggested by the reviewer, we now include equivalent experiments with the DN-KIF16B (new Fig 5). We show that DN-KIF16B did not affect the normal localization of BSE (small peripheral dots close to the basolateral membrane) or CRE (deeper spots located just above the nucleus). This was true in both WT and AP-1B KD MDCK cells.

Furthermore, another important question is whether KIF16B-DN affects BSE-to-CRE trafficking of Tf (instead of CRE-to-ARE). In this regard, the authors should characterize the localization of Tf at 5-vs-30 min (using dual Tf labeling, as in Figure 3) in WT cells transfected with DN-KIF16B. This experiment is crucial to address whether KIF16B specifically controls transcytosis of Tf in AP-1B-deficient cells, or if it is involved in Tf trafficking towards the RE.

To study the role of KIF16B in BSE-to-CRE trafficking, we performed additional experiments using dual color Tf uptake and co-localization with KIF16B (new Fig 5 and supplementary Fig 5). These experiments showed the following:
1) Basolateral incubation of MDCK cells with 633-Tf and 594-Tf for 5 min showed colocalization in peripheral sorting endosomes (BSE) (Supplementary Fig 5)
2) Basolateral incubation of MDCK cells with 633-Tf and 594-Tf for 30 min showed colocalization in peripheral (BSE) and juxta-nuclear (CRE) endosomes. (Supplementary Fig 5).
3) Basolateral incubation of MDCK cells with 633-Tf for 25 min followed by a 5 min chase in the presence of 594-Tf showed that these probes did not colocalize with each other, with 633-Tf localizing to CRE and 594-Tf localizing to BSE (Fig 5).
4) Expression of DN-KIF16B did not inhibit the segregation of 633-Tf and 594-Tf, suggesting that KIF16B does not work in in BSE-to-CRE trafficking. (Fig 5).

6-Concerning the localization of KIF16B in the CRE endosomes (Figure 3d and 3e). Here, the main difficulty is the poor definition of what represents the CRE. The authors should clearly show the localization of KIF16B and late-endocytosed Tf in this compartment using 3D reconstructions (and an x-z projection of a whole cell).

We appreciate the concern of the reviewer, hence we have replaced images of old Fig 3d with new images (new Fig 5), which provide a better definition of the CRE.
We have also added to the new Fig 5:
1) a x-z projection of a whole cell
2) Quantifications of the colocalization of WT-KIF16B with CRE, WT-KIF16B with BSE, DN-KIF16B with CRE, DN-KIF16B with BSE.
These experiments were performed in both WT and AP-1B KD MDCK cells. They show that KIF16B and DN-KIF16B colocalized significantly more with CRE than with BSE, supporting a role of KIF16B in traffic from CRE to ARE rather than from BSE to CRE.

It would also be interesting to show a colocalization with µ1B. Also, it would be important to show if this localization is MT-dependent to complement the present data.

We agree with the reviewer that m1B might colocalize with KIF16B at CRE. However, our laboratory has recently shown that m1B colocalizes with TIR in perinuclear endosomes in MDCK cells (Gravotta et al 2012) and, along with other groups (Folsch et al, 2003, Gravotta et al, 2007), that AP-1B functions in CRE. This indicates that AP-1B localizes at CRE, like KIF16B.
We believe that the dual-color Tf labeling utilized here is the best approach to determine the localization of KIF16B at CRE, since CRE is operationally defined as a juxta-nuclear compartment enriched with TIR internalized from the basolateral side for more than 10-15 minutes. We do not think that microtubules might affect the localization of KIF16B (or m1B) at CRE, since nocodazole does not affect the traffic of TIR to recycling endosomes (Daro et al, 1996) and does not impair basolateral recycling (Fig 1).

Minor Points

1- Rab11 has a very important role in polarity establishment, junctional recycling, and apical trafficking. The authors should analyze if Cherry-Rab11-DN cells have general defects in cell polarity that may affect the Sulfotag-Tf assay (Figure 1c-e). This is especially important since treatment with Dox 20 ng/ml does not completely block Ch-Rab11-DN expression.

Data not shown, now mentioned in the text, show that expression of mCh-DN-rab11a does not affect the TER. Furthermore results shown in Fig 1 (now mentioned in the text) show that expression of mCh-DN-rab11a does not affect the basolateral polarity of TIR in WT MDCK cells

New western blot data in the mCh-DN-rab11a MDCK cell line (new Fig 1c) show that in the presence of Dox 20 ng/ml this cell line displays much lower expression of mCh-DN-rab11a than of endogenous rab11a.

In addition, the authors should address what happens with BL recycling of Tf in Rab11-DN cells. Is it reduced or enhanced? What happens with the internal pool?

Experiments in Ch-DN-rab11a MDCK cells (new supplementary Fig 1d) show that mCh-DN-rab11a caused a small increase in both basolateral recycling and the internal pool of TIR; however, the differences were not statistically significant.

2- Do AP-1B deficient MDCK cells have defects in tight junction assembly or have increased junction leakiness that might affect data interpretation in figure 2a? The authors previous showed that general distribution of polarity markers is not affected (Gravotta et al 2012), but the TER was not tested.

TER is not affected in AP-1B KD MDCK cells (Gravotta et al., PNAS, 2007). We tested the TER of this cell line many times during this work and did not observe TER defects; this is now mentioned in the text.

3- AP-1B-KD MDCK cells when expressing DN-KIF16B seem to have apical Tf, although not reaching the ARE (Figure 3a). Are these WGA-positive Tf-loaded endosomes? The authors should show that the endosomes are WGA-negative to support their model.

Analysis of the images show a small amount of apical Tf in AP-1B-KD MDCK cells expressing DN-KIF16B; however, the amount is small and equivalent to that found in the relevant controls (Fig 4a). Furthermore, our model implicates MT in transport to ASE and does not exclude a role of KIF16B or other kinesin in this process.

Also, it would be interesting to show μ1B localization in WT-MDCK expressing KIF16B-DN to prove that KIF16B does not affect localization of the CRE, and only affects trafficking of Tf. Results in new Fig 5 show that KIF16B does not affect the localization of the CRE (see also major concern #5).

4-The KIF-16B endogenous localization images are not very clear in Figure 3D. Better images should be provided. A quantification of KIF-16B colocalization with BSEs vs. CRE should also be provided (as it was done throughout the manuscript). It would be also very interesting if the authors could address the localization of AP-1B in these experiments.

We have replaced the original images by new images with a better definition of the CRE (new Fig 5). We also added a quantification of the colocalization of KIF16B with either CRE or BSE (see also major concern #6).
5-Why do the authors use transiently transfected TfR-GFP instead of endogenous TfR in the experiment described in figure 4? There are indeed monoclonal antibodies suitable for MDCK endogenous TfR imaging commercially available from Zymed.

We used transient transfection of TfR-GFP to ensure that the pools of TfR studied in these experiments were properly deglycosylated. To this end, tunicamycin was added two hours before and during expression of TfR-GFP.

6-The fact that BL recycling is so much lower in RPE cells than in µ1B-KD MDCK cells in figure 5 should be discussed. Why is the intracellular pool so much larger?

We have added a paragraph on this point in Discussion.

The increase in the intracellular pool of TfR in RPE cells supports a role of AP-1B in promoting recycling back to the (basolateral) plasma membrane. Consistently with this, we have observed a trend towards intracellular retention in AP-1B KD MDCK cells. This trend is confirmed by results in RPE cells that show a statistically significant increase in intracellular retention of TfR in RPE cells.

This could be due to incomplete KD of AP-1B in MDCK cells or a compensatory mechanism that occurs in cells knocked down for AP-1B, but not in cells that constitutively lack AP-1B.

It would be also clarifying to show x-z reconstructions of ARPE-19 cells (as it is done in the rest of the manuscript).

We have added X-Z views of ARPE-19 cells (new Fig 6).

Discuss why there is more retention in ARPE-19 cells.

See minor concern #6. We have added a paragraph on this point in Discussion.

7-Figures 6-7 could be merged in only one figure.

We have condensed old Figures 6 and 7 into new Fig 7 and sent some of the results to supplementary materials.

Referee #3

While the role of microtubules in transcytosis has long been known, the exact nature of microtubule motors involved has remained mysterious. The major advance in this paper is the identification of KIF16B as a candidate regulator of this pathway. KIF16B has already been implicated in endocytic trafficking, so the novelty and importance of this work depends largely on whether the role of KIF16B is different from its previously reported function in TfR recycling. As it stands this is not adequately addressed, and none of the data show a direct role for endogenous KIF16B in transcytosis in this system.

The initial part of the manuscript focuses on the role of Rab11 and microtubules. The data presented in Figure 1 show that a dominant negative form of rab11 inhibits apical transcytosis of TfR. Exactly why this effect occurs is debatable, and does not prove that endogenous rab11 is involved. Other dominant negative rabs might give the same effect, but none are tested - rab4 and 14 seem like obvious choices.

We agree with the reviewer that rab14 is an interesting candidate to play a role in apical transcytosis of TfR. This is suggested by data in the literature showing that (i) rab14 binds to KIF16B directly (Ueno et al, 2010), (ii) the rab14/KIF16B complex is essential for delivery of FGFR to the plasma membrane in fibroblastic cells (Ueno et al., 2010) and (iii) rab14 regulates apical trafficking in MDCK cells.

We have reproduced results by Wilson and coworkers (Kitt et al., 2008) regarding the cellular distribution of WT and DN rab14 and we found a strong colocalization with TfR. Unfortunately, we
obtained negative preliminary data showing that transfection of DN rab14 does not impair apical transcytosis of TfR. However, the data in the literature suggests that we should look more deeply into this issue. We are currently working in this direction. Our hypothesis is that rab14 might be part of a machinery that recognizes apical signals of TfR in AP-1B KD MDCK cells, but not in WT MDCK cells.

To keep our manuscript focused on KIF16B and due to an issue of length, we prefer to address this issue in separate studies.

Rab4 might also be a potential interesting candidate to play a role in apical transcytosis of TfR. However, van der Sluijs and coworkers have already reported that rab4 promotes apical transcytosis of TfR in MDCK cells (Mohrmann et al, 2002). The mechanism involved is unclear because rab4 is expressed in BSE. We now discuss this issue in Discussion.

Dominant negative rabs are most likely acting by blocking the function of a cellular exchange factor, and therefore perturbing the endogenous rab. For rab11 it still isn't known what the exchange factor is, so this is somewhat of a black box experiment. A better strategy to show that rab11 has a function in apical transcytosis when AP-1b is absent would be to knock down the endogenous pool of rab11. For this multiple si/shRNAs would need to be used and blotting performed to confirm efficient depletion.

Our experiments using mCh-DN-rab11a were designed to establish a parallelism with the apical transcytosis of the pIgR, before focusing on the more novel aspect of this pathway, the role of KIF16B.

The dominant negative rab11a used in this work (S25N) has been used to study protein trafficking by several other laboratories, i.e. Goldenring, Sabatini, Parton, Apodaca and Stow (Wang et al 2000; Ren et al, 1998; Ulrich et al, 1996, Khandelwal et al, 2008; Lock and Stow, 2005). To our knowledge, none of these studies used siRNA, so this would be a completely novel undertaking. Hence, although these studies may contribute additional mechanistic information on rab11a, we feel they are beyond the scope of our manuscript.

I have some additional queries about Figure 1 that should be addressed by Western blots:
1. How much Rab11 is endogenous and how much is overexpressed?

In response to this comment, we have added the western blot for endogenous rab11a (new Fig 1c).

These new data indicate that the mCh-DN-rab11a cell line displayed:
(i) low leakage (much lower expression of mCh-DN-rab11a than of endogenous rab11a),
(ii) strong response of mCh-DN-rab11a upon activation (4 fold compared to pre-activation expression levels, about the same level of endogenous rab11a).

These moderate expression levels of DN rab11a were low enough to minimize undesired effects associated with massive overexpression but high enough to see a statistically significant effect.

2. Are endogenous Rab11 levels the same in all KD cell lines?

WT and AP-1B KD MDCK cells displayed similar amounts of rab11a. The same was true for WT and m-1B(+) Trvb1 cells (new Supplementary Fig 9).

There are similar issues with the experiments investigating the function of KIF16B, which have to be viewed as rather preliminary in their present form. Dominant negative kinesin constructs are used, but no attempt is made to look at endogenous protein. A first step would be to establish if KIF16B is expressed in these cells, and then test if its knock down has any effect on transcytosis.

(These issues were also raised by reviewer 2. Hence we reproduce below the same reply to his/her comment.)

We completely agree with the reviewer and have performed the following additional experiments:

In MDCK cells (WT and AP-1B KD):
1) We confirmed expression of KIF16B by RT-PCR (new Supplementary Fig 4).
2) We knocked down endogenous KIF16B in MDCK with siRNA (new Fig 4).
3) We showed that KD of KIF16B inhibited traffic of TfR to ARE (similar to experiments with DN-KIF16B) (new Fig 4).

In ARPE-19 cells:
4) We confirmed expression of KIF16B by RT-PCR (new Supplementary Fig 4).
5) We showed that DN-KIF16B inhibited traffic of TfR to ARE (similar to AP-1B KD MDCK cells) (new figure 6d).

Localisation of endogenous KIF16B would also be valuable, since it may be present on moving transcytotic vesicles.

(Similar information was also requested by reviewer 2)

We have been unable to find good antibodies to endogenous KIF16B, hence we have performed additional experiments to further characterize the localization of WT-KIF16B-YFP and DN-KIF16B-YFP (new Fig 5 and new supplementary Figs 5):

Using a dual color Tf uptake protocol that labels BSE and CRE in the same cell (new supplementary Fig 5), we showed that WT-KIF16B-YFP colocalized with both types of endosomes, but to a significantly larger extent with CRE (new Fig 5). DN-KIF16B-YFP, which lacks the motor domain and therefore can attach to the proximal compartment but cannot traffic to the distal compartment, also colocalized preferentially with CRE (new Fig 5).

Importantly, DN-KIF16B-YFP did not inhibit the traffic of fluorescent Tf from BSE to CRE (new Fig 5).

Together with results from Fig 4, these data indicate that KIF16B resides preferentially at CRE, where it mediates CRE-to-ARE trafficking, rather than BSE-to-CRE trafficking of TfR.

These experiments also showed that DN-KIF16B-YFP did not alter the morphology or localization of BSE and CRE.

This first part of the work if fully developed could make an important contribution to the literature on transcytosis, but my view of the current manuscript is that it is too preliminary for publication.

The later section of the work is an addition rather than a core part of the study. In Figure 4 the role of glycosylation in TfR traffic is investigated. While tunicamycin causes altered transport of TfR this does not in itself prove that glycosylation of TfR is the cause. To make this point a TfR mutant lacking N-glycosylation sites would need to be used. While the role of N-glycans in sorting is an interesting issue it could form a study in its own right and doesn’t seem to be that relevant unless a direct link to KIF16B is made.

We have performed experiments with five TfR mutants lacking each or all four glycosylation sites. These experiments confirmed our previous tunicamycin experiments and unveiled differential effect of each glycans on apical sorting (see above).

However, as suggested by the reviewer, to keep our manuscript focus on KIF16B, we have removed the glycosylation data from this manuscript.

Finally, the data on CHO cells does not relate to transcytosis and could be omitted entirely without changing any of the main conclusions. If AP-1b expression creates a novel recycling pathway the obvious question is which rab is required, and this isn’t followed up.

We respectfully disagree with the reviewer on this particular point. A main conclusion of our manuscript is that loss of AP-1B promotes incorporation of TfR into an apical recycling route that involves rab11a and therefore resembles the general recycling route of non-polarized cells. This observation begs for the reverse experiment, i.e., does addition of AP-1B to non polarized cells promote rab11a independent recycling to the PM? The answer is yes. Expression of AP-1B is sufficient to generate a rab11a-independent route to the PM but is not sufficient to generate geographically separated recycling endosomes, as in confluent of subconfluent MDCK cells.

Another conclusion emphasized by these experiments is that the general recycling pathway of non polarized cells is equivalent to the apical recycling pathway of epithelial cells. This conclusion has
been proposed before by Goldenring based on the fact that both the ARE of epithelial cells and the RE of non-polarized cells express Rab11a and are organized around the centrosome. However, a key experiment is still missing to fully demonstrate this point, i.e., that a protein recycled by a rab11a-dependent mechanism in non-polarized cells can use the rab11a-dependent apical route in epithelial cells. Our observation that in the absence of AP-1B, TfR recycles through the apical rab11a-dependent route in epithelial cells allows us to demonstrate this important point.

We realize that this point was not well presented in the original manuscript. We have improved the discussion of this point in the current version of the manuscript.

Hence, we prefer to keep these data in this manuscript. However, to keep our focus on KIF16B, we have combined all experiments on non-polarized cells in one figure (now figure 7).

Minor points

In the introduction both Hunziker et al & Matter et al (EMBOJ 1990 9(11):3515-25; 3163-70) should be cited in addition to the Apodeca 1994 paper when introducing the role of microtubules in transcytosis.

Typo in Figure 3d. "length".

We have added the Hunziker reference and corrected the typo. We thank the reviewer for pointing out these deficiencies.