The kinesin KIF16B mediates apical transcytosis of transferrin receptor in AP-1B-deficient epithelia

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Polarized epithelial cells take up nutrients from the blood through receptors that are endocytosed and recycle back to the basolateral plasma membrane (PM) utilizing the epithelial-specific clathrin adaptor AP-1B. Some native epithelia lack AP-1B and therefore recycle cognate basolateral receptors to the apical PM, where they carry out important functions for the host organ. Here, we report a novel transcytotic pathway employed by AP-1B-deficient epithelia to relocate AP-1B cargo, such as transferrin receptor (TfR), to the apical PM. Lack of AP-1B inhibited basolateral recycling of TfR from common recycling endosomes (CRE), the site of function of AP-1B, and promoted its transfer to apical recycling endosomes (ARE) mediated by the plus-end kinesin KIF16B and non-centrosomal microtubules, and its delivery to the apical membrane mediated by the small GTPase rab11a. Hence, our experiments suggest that the apical recycling pathway of epithelial cells is functionally equivalent to the rab11a-dependent TfR recycling pathway of non-polarized cells. They define a transcytotic pathway important for the physiology of native AP-1B-deficient epithelia and report the first microtubule motor involved in transcytosis.

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Introduction

Endocytosis and recycling of plasma membrane (PM) receptors play a key role in the uptake of many critical cell nutrients and in the relay of extracellular signals (Maxfield and McGraw, 2004). In non-polarized cells (e.g., fibroblasts), most nutrient

receptors (e.g., transferrin receptor (TfR) and low-density lipoprotein receptor (LDLR)) traffic through a single major recycling pathway that involves sequential internalization into peripheral sorting endosomes (SE) and juxtanuclear recycling endosomes (RE) (Hopkins, 1983; Yamashiro *et al*, 1984; Maxfield and McGraw, 2004). In contrast, in epithelial cells, which display polarized apical and basolateral PM domains and receive most of their nutrients and signals from the blood, most endocytic receptors reside at the basolateral PM and must be recycled back to this PM domain after endocytosis (Perret *et al*, 2005; Rodriguez-Boulan *et al*, 2005). In these cells, most nutrient receptors are sequentially internalized into peripheral basolateral sorting endosomes (BSE) and juxtanuclear common recycling endosomes (CRE), where they mix with apical receptors internalized via separate apical sorting endosomes (ASE) (Figure 1A) (Hughson and Hopkins, 1990; Golachowska *et al*, 2010). Whereas in non-polarized cells recycling of nutrient receptors such as TfR along the major recycling route is regulated by the small GTPase rab11a, localized at RE (Ullrich *et al*, 1996; Ren *et al*, 1998) (see Supplementary Table 1), in epithelial cells recycling of nutrient receptors from CRE to the basolateral PM does not require rab11a (Wang *et al*, 2000). Remarkably, in epithelial cells rab11a resides at a more apically localized endosomal compartment inaccessible to TfR, the apical recycling endosomes (ARE) (Hunziker *et al*, 1990; Apodaca *et al*, 1994; Barroso and Sztul, 1994; Casanova *et al*, 1999; Golachowska *et al*, 2010), where it regulates instead the transcytotic route of polymeric IgA receptor (pIgR) and the biosynthetic route of a subset of apical PM proteins (Wang *et al*, 2000; Cresawn *et al*, 2007; Weisz and Rodriguez-Boulan, 2009).

Because in epithelial cells basolateral and apical endocytic receptors are mixed at CRE, they must be sorted by specific signals before they return to their respective surface. Sorting is mediated by apical and basolateral sorting signals structurally similar to those that sort them in the biosynthetic route (Rodriguez-Boulan *et al*, 2005; Weisz and Rodriguez-Boulan, 2009). Sorting of basolateral recycling receptors at CRE is mediated by a sorting machinery that includes clathrin and the epithelial-specific clathrin adaptor AP-1B (Folsch *et al*, 1999; Ohno *et al*, 1999; Gan *et al*, 2002; Folsch *et al*, 2003; Gravotta *et al*, 2007; Deborde *et al*, 2008). Thus, the expression of AP-1B by epithelial cells appears to promote the incorporation of nutrient receptors to a recycling route to the PM that avoids interaction with rab11a.

AP-1B is widely expressed by epithelial cells. However, some epithelial cell lines, e.g., LLC-PK1 (Roush *et al*, 1998; Folsch *et al*, 1999) and some native epithelia, e.g., retinal pigment epithelium (RPE) and kidney proximal tubule, lack AP-1B (Diaz *et al*, 2009; Schreiner *et al*, 2010), which promotes apical expression of many basolateral proteins that are normally sorted by AP-1B (Roush *et al*, 1998; Folsch *et al*, 1999). Importantly, the apical localization of basolateral proteins has important physiological consequences for AP-1B-deficient native epithelia; for example, apical TfR in RPE is

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Figure 1 AP-1B KD MDCK cells display microtubule- and rab11a-dependent transcytosis of basolateral TfR. (A) Model of a WT MDCK cell displaying endosomal compartments and the endosomal itinerary of plgR, TfR and LDLR postendocytic pathways. (B) A SulfoTag-Tf assay. Tf labelled with biotin and the luminophore SulfoTag (SulfoTag-Tf) provides a signal $10 \times$ higher than ^{125}I -Tf, allowing to detect 2×10^{-15} mol per 12 mm filter (the estimated amount of endogenous dog TfR present at the apical membrane of confluent MDCK cells). (C) MT mediate apical transcytosis of TfR. Values of apical transcytosis, basolateral recycling and the intracellular pool of SulfoTag-Tf (60 min) in control WT, nocodazole-treated WT, control AP-1B KD and nocodazole-treated AP-1B KD MDCK cells. (D) Dominant-negative rab11a inhibits apical transcytosis of TfR. A MDCK cell line was generated that stably expresses dominant-negative rab11a fused to monomeric Cherry fluorescent protein (mCh-DN-rab11a) under the control of a tetracycline-repressible promoter. Western blot analysis showed that expression of mCh-DN-rab11a increased four-fold by removing doxycycline for 12 h. (E) RNA levels of $\mu 1\text{B}$ and GAPDH in WT, stable AP-1B KD, transient luciferase KD and transient AP-1B KD MDCK cells. (F) Values of apical transcytosis of SulfoTag-Tf (75 min) in WT and transient AP-1B KD MDCK cells with or without 12-h expression of mCh-DN-rab11a. * $P < 0.05$, ** $P < 0.001$. Bars represent mean $\pm$ standard error. Ap, apical; BL, basolateral; IC, intracellular.

thought to regulate iron levels in the retina (Garcia-Castineiras, 2010). Unfortunately, the mechanisms and pathways that mediate apical localization of basolateral proteins in AP-1B-deficient epithelia are not known.

Here, using ultrasensitive biochemical and microscopic recycling assays, we show that AP-1B-deficient epithelia incorporate two basolateral nutrient receptors, TfR and LDLR into an alternative rab11a-dependent recycling route

to the apical membrane that, like the transcytotic pathway of pIgR, involves microtubule (MT)-mediated trafficking from CRE to ARE. This pathway requires non-centrosomal MTs and the plus-end kinesin KIF16B. The requirement for this kinesin is specific, as it is not shared by pIgR. Other experiments show that expression of AP-1B in fibroblastic CHO cells generates a recycling pathway for TfR independent of rab11a. In summary, our results illustrate for the first time the case of a cargo protein (TfR) that can use both the rab11a-dependent general recycling route of non-polarized cells and the rab11a-dependent apical recycling route of epithelial cells, directly supporting the concept that the apical recycling pathway of epithelial cells is equivalent to the general recycling route of non-polarized cells. They demonstrate that the epithelial-specific adaptor AP-1B can generate signal-dependent recycling routes for TfR in both polarized and non-polarized cells and report the first MT motor involved in transcytosis across epithelia.

Results

Ultrasensitive assay to measure TfR recycling and transcytosis

Most studies on TfR recycling and transcytosis with ^{125}I -Tf (transferrin) in MDCK cells were carried out overexpressing the human TfR because of the low amount of Tf transcytosed to the apical PM (Odorizzi and Trowbridge, 1997; Brown *et al*, 2000; Gravotta *et al*, 2007). Indeed, apical transcytosis of endogenous TfR requires long incubation times (i.e., 4–6 h) to allow accumulation of ^{125}I -Tf in the apical medium (Fuller and Simons, 1986). To stay as close as possible to the physiological situation, we carried out our studies focusing on the endogenous (dog) TfR. Hence, we developed an ultrasensitive method to measure TfR recycling, that utilizes as a ligand dog Tf double-labelled with biotin and the luminophore SulfoTag (SulfoTag-Tf). This method proved to be 10 times more sensitive than ^{125}I -Tf (Supplementary Figure S1A,B and Materials and methods). Titration curves of SulfoTag-Tf and ^{125}I -Tf showed that only the former can detect 2×10^{-15} mol of Tf, which is the estimated amount of apical TfR present in confluent MDCK monolayers grown on a 12-mm Transwell filter (Figure 1B) (Fuller and Simons, 1986).

Using this method, we showed that wild-type (WT) MDCK cells recycle most of the basolaterally internalized SulfoTag-Tf to the basolateral membrane (85%) and transcytose a small amount of SulfoTag-Tf to the apical PM (4%) (Figure 1C). MDCK cells lacking AP-1B by stable knockdown of its medium subunit $\mu 1\text{B}$ (AP-1B KD), which exhibit normal levels of transepithelial electrical resistance (Gravotta *et al*, 2007), display $4 \times$ higher apical transcytosis of basolaterally internalized SulfoTag-Tf (16% versus 4%, $P < 0.001$) (Figure 1C, left) and lower basolateral recycling (71% versus 85%, $P < 0.001$), compared to WT MDCK cells (Figure 1C, middle) (see recycling kinetics in Supplementary Figure S1C).

Apical transcytosis of TfR is microtubule- and rab11a-dependent

MTs play important roles in apical biosynthetic trafficking of PM proteins (Rodriguez-Boulán *et al*, 2005; Weisz and Rodriguez-Boulán, 2009) and transcytosis of pIgR (Hunziker *et al*, 1990; Apodaca *et al*, 1994). Hence, we studied whether apical transcytosis of TfR is MT dependent. Indeed,

depolymerization of MTs by cold and nocodazole significantly inhibited apical transcytosis of TfR in AP-1B KD MDCK cells (from 16% to 8%, $P < 0.001$) (Figure 1C left) and increased basolateral recycling (from 71% to 77%, $P < 0.05$) (Figure 1C middle). As transcytosis of pIgR also depends on rab11a, we studied whether this was also the case for the transcytosis of endogenous TfR in AP-1B KD MDCK cells. To this end, we generated a tetracycline-repressible MDCK cell line stably expressing a GTP binding-deficient mutant of rab11a (S25N) tagged with monomeric Cherry fluorescent protein (mCh-DN-rab11a). This approach allows efficient overexpression of mCh-DN-rab11a only after the monolayer polarity is established, thus circumventing the disrupting effects of this protein during polarity development (Datta *et al*, 2011). In the presence of doxycycline, mCh-DN-rab11a expression was much lower than that of endogenous rab11a, whereas removal of the drug induced a $4 \times$ increase of mCh-DN-rab11a expression to a level comparable to the endogenous protein, thus avoiding non-specific effects associated with massive overexpression (Figure 1D). We transiently knocked down $\mu 1\text{B}$ in these cells using electroporation of siRNA. RT-PCR analysis showed that transient knockdown of $\mu 1\text{B}$ -KD was effective, albeit less efficient than in permanently transfected MDCK cells (Figure 1E), resulting in a smaller but statistically significant apical transcytosis of SulfoTag-Tf (Figure 1F). Importantly, expression of mCh-DN-rab11a caused a significant reduction of apical transcytosis of SulfoTag-Tf in transiently AP-1B KD MDCK cells (from 7% to 5%, $P < 0.05$) (Figure 1E), suggesting that rab11a regulates this pathway. Expression of mCh-DN-rab11a did not impair the accuracy of basolateral recycling of SulfoTag-Tf in WT MDCK cells (Supplementary Figure S1D) or the transepithelial electrical resistance (not shown), indicating that mCh-DN-rab11a did not affect establishment of membrane polarity.

These experiments demonstrate that polarized AP-1B KD MDCK cells transport basolateral TfR to the apical PM via a transcytotic route that depends on MTs and rab11a.

Apical transcytosis of TfR in AP-1B KD MDCK cells involves both ARE and ASE

To identify the compartments involved in the transcytotic pathway of TfR, we carried out basolateral uptake experiments with fluorescent Tf and studied its colocalization with specific endosomal markers in WT and AP-1B KD MDCK cells. In order to facilitate detection of endogenous TfR, dog Tf was labelled with CF594 (594-Tf), which yielded 3 to > 10 times stronger signal than the routinely used Alexa488-Tf in the relevant concentration range (10^{-14} to $\sim 10^{-12}$ mol per well), as revealed by spectrofluorometry analysis (Supplementary Figure S2A). The specificity of 594-Tf for dog TfR was confirmed by colocalization experiments with anti-TfR antibody (Supplementary Figure S2B) and by competition experiments with unlabelled Tf (not shown).

AP-1B KD MDCK cells incubated basolaterally with 594-Tf for 15 min displayed strong colocalization of 594-Tf with immunostained rab11a at ARE, which typically appeared as a bright spot under the apical PM (Figure 2A, column 3). This colocalization was not observed in AP-1B KD MDCK cells pretreated with nocodazole/cold (Figure 2A, column 4). In contrast, basolaterally internalized 594-Tf was not detected in ARE in WT MDCK cells in the absence or presence of

Figure 2 Microtubules mediate transport of basolateral TfR to both ARE and ASE in AP-1B KD MDCK cells. (A) MDCK cells were incubated from the basolateral surface with fluorescent 594-Tf for 15 min and immunostained with anti-rab11a. From left to right, columns show: control WT, nocodazole-treated WT, control AP-1B KD and nocodazole-treated AP-1B KD MDCK cells. (A') Cells from experiments represented in (a) were quantified for the percentage of pixels of rab11a colocalizing with 594-Tf (top) and the percentage of pixels of 594-Tf colocalizing with rab11a (bottom). Circles correspond to individual cells obtained from different experiments and red lines indicate the median value. (B) MDCK cells were incubated with fluorescent 594-Tf as in (a) and stained for ASE with incubation of 488-WGA from the apical surface for 5 min at 37°C and washed with NADG at 4°C. (B') Cells from experiments represented in (b) were quantified for the percentage of pixels of 488-WGA colocalizing with 594-Tf (top) and the percentage of pixels of 594-Tf colocalizing with 488-WGA (bottom). ** $P < 0.001$. Red line represents the median. Scale bar, 10 μ m.

nocodazole/cold treatment (Figure 2A, columns 1 and 2). As previously reported, MT disruption dispersed ARE in MDCK cells, but did not inhibit endocytosis of TfR (Apodaca *et al*, 1994; Daro *et al*, 1996; Casanova *et al*, 1999; Babbey *et al*, 2006). Quantitative analysis using the Mander's colocalization coefficient (see Materials and methods) showed that the percentage of rab11a colocalizing with 594-Tf increased from $0 \pm 0.1\%$ in WT MDCK to $34 \pm 3\%$ in AP-1B KD ($P < 0.001$) and was reduced to $0.3 \pm 0.2\%$ by nocodazole/cold treatment ($P < 0.001$) (Figure 2A' top). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from $0 \pm 0.1\%$ in WT MDCK cells to $7 \pm 0.7\%$ ($P < 0.001$) in AP-1B KD MDCK cells and was reduced to $1.4 \pm 0.3\%$ ($P < 0.001$) after nocodazole/cold treatment (Figure 2A' bottom). That the subapical rab11a-positive spot was indeed ARE was confirmed by the displacement of this spot to the apicolateral junction upon treatment with the MT-stabilizing drug taxol (Supplementary Figure S3A), a typical behaviour of ARE (Casanova *et al*, 1999; Lapierre *et al*, 2003). Notably, taxol treatment did not prevent transport of 594-Tf to the laterally

displaced ARE in AP-1B KD MDCK cells (Supplementary Figure S3A,A'). Taken together, these results demonstrate that AP-1B KD MDCK cells carry out MT-mediated transcytosis of basolateral TfR to ARE.

Apical sorting endosomes (ASE) appear as multiple small dots located immediately under the apical membrane upon incubation for 5 min with apical 488-WGA (Leung *et al*, 2000; Cresawn *et al*, 2007) (Figure 2B). Similar experiments to those performed for ARE, indicated that AP-1B KD MDCK cells transport significantly more basolaterally internalized 594-Tf than WT MDCK cells to ASE (Figure 2B, columns 1 and 3). Transport of basolaterally internalized 594-Tf to ASE was inhibited by nocodazole/cold treatment (Figure 2B, columns 3 and 4). Quantitative analysis showed that the percentage of 488-WGA colocalizing with 594-Tf increased from $1 \pm 0.1\%$ in WT MDCK cells to $14 \pm 1\%$ ($P < 0.001$) in AP-1B KD MDCK cells and was reduced to $5 \pm 1\%$ ($P < 0.001$) after nocodazole/cold treatment (Figure 2B' top). Accordingly, the percentage of 594-Tf colocalizing with 488-WGA increased from $0.6 \pm 0.1\%$ in WT MDCK cells to $7 \pm 0.8\%$ ($P < 0.001$)

Figure 3 LDLR undergoes apical transcytosis to ARE in AP-1B KD MDCK. MDCK cells were transiently transfected with Tfr-GFP (A) LDLR (B) or NBC1 (C) and immunostained for rab11a. (A', B', C') Cells from experiments represented in (a), (b) and (c) were quantified for the percentage of pixels of rab11a colocalizing with the cargo (left) and the percentage of pixels of the cargo colocalizing with rab11a (right). NS, no significance. ** $P < 0.001$. Red line represents the median. Scale bar, 10 μ m.

in AP-1B KD MDCK cells and was reduced to $3.6 \pm 0.4\%$ ($P < 0.001$) by nocodazole/cold treatment (Figure 2B' bottom).

Control experiments indicated that basolaterally internalized 594-Tf also reached ASE in the presence of the iron chelator deferoxamine added to the apical medium (Supplementary Figure S3B,B'), ruling out the possibility that 594-Tf was delivered to the apical membrane and subsequently re-internalized into ASE. The amount of 594-Tf transported to ARE and ASE did not increase with longer incubation times (e.g., 30 min) indicating that the transport of basolateral 594-Tf to ARE and ASE reached steady state after ~15 min (Supplementary Figure S3C,C' and D,D').

The experiments reported in this section demonstrate that the transcytotic route of Tfr in AP-1B KD MDCK cells involves MT-mediated transport to both classes of apical endosomes, ARE and ASE. In contrast, pIgR displayed an extensive colocalization with ARE, as previously reported (Apodaca *et al*, 1994; Brown *et al*, 2000), but colocalized poorly with ASE (Supplementary Figure S4D,D'). Our experiments demonstrate that the transcytotic pathways of Tfr in AP-1B KD MDCK cells and pIgR involve different endosomal itineraries and unravel a novel role for ASE in apical transcytosis.

LDL receptor undergoes apical transcytosis to ARE in AP-1B KD MDCK

We next studied whether the apical transcytotic route utilized by Tfr in AP-1B KD MDCK cells is also utilized by other AP-1B cargoes. Like Tfr, LDLR is basolateral in WT MDCK and non-polar in AP-1B KD MDCK cells and LLC-PK1 cells (Folsch *et al*, 1999; Gan *et al*, 2002; Gravotta *et al*, 2007). In contrast, the bicarbonate transporter NBC1 localizes basolaterally in kidney

proximal tubule cells indicating that its basolateral localization mechanism is independent of AP-1B (Li *et al*, 2007).

For these experiments, we studied the expression of basolateral proteins tagged with GFP. Tfr-GFP colocalized poorly with rab11a in WT MDCK cells and extensively in AP-1B KD MDCK cells (Figure 3A), confirming the results obtained with endogenous Tfr. LDLR-GFP also extensively increased its colocalization with rab11a in AP-1B KD MDCK cells (Figure 3B). In contrast, NBC1-GFP colocalized poorly with rab11a in both WT and AP-1B KD MDCK cells. Quantitative analysis showed that the percentage of rab11a colocalizing with Tfr-GFP increased from $4 \pm 1\%$ in WT MDCK to $42 \pm 7\%$ in AP-1B KD ($P < 0.001$). The percentage of rab11a colocalizing with LDLR-GFP also increased from $6 \pm 2\%$ to $50 \pm 8\%$ ($P < 0.001$), whereas the percentage of rab11a colocalizing with NBC1-GFP did not increase ($6 \pm 4\%$ and $5 \pm 3\%$, NS). Accordingly, the percentage of Tfr-GFP colocalizing with rab11a increased from $2 \pm 0.8\%$ to $13 \pm 2\%$ ($P < 0.001$). The percentage of LDLR-GFP colocalizing with rab11a also increased from $5 \pm 1\%$ to $24 \pm 5\%$ ($P < 0.001$), whereas the percentage of NBC1-GFP colocalizing with rab11a did not increase ($4 \pm 3\%$ and $3 \pm 1\%$, NS) (Figure 3A',B' and C').

These results demonstrate that AP-1B cargoes undergo transcytosis via ARE in AP-1B KD MDCK cells, whereas AP-1B independent cargoes remain strictly basolateral and avoid ARE in AP-1B KD MDCK cells.

Transport of basolateral Tfr to ARE requires the plus-end microtubule motor KIF16B

To date, the MT motor(s) that mediate transcytosis across epithelia remain unknown. We used a candidate approach to search for MT motors involved in the transcytosis of Tfr.

KIF5B mediates transport of the apical protein p75-neurotrophin receptor (p75) in MDCK cells (Jaulin *et al*, 2007). KIF16B was discovered in a search for kinesins that bind the endosomal lipid PI3P and found to mediate TfR recycling in fibroblastic cells (Hoepfner *et al*, 2005). Transfection of a truncated, motor-less version of KIF5B (DN-KIF5B-CFP) that blocks biosynthetic trafficking of p75 did not prevent transport of basolaterally internalized 594-Tf to ARE in AP-1B KD MDCK cells (Figure 4B). In contrast, transfection of a motor-less form of KIF16B (DN-KIF16B-YFP) that inhibits TfR recycling in HeLa cells (Hoepfner *et al*, 2005) prevented transport of basolaterally internalized 594-Tf to ARE in AP-1B KD MDCK cells (Figure 4A). Quantitative analysis showed that the percentage of rab11a colocalizing with 594-Tf increased from $0.3 \pm 0.2\%$ in untransfected WT MDCK cells to $32 \pm 3\%$ ($P < 0.001$) in untransfected AP-1B KD MDCK cells and was reduced to $12 \pm 3\%$ ($P < 0.001$) by the expression of DN-KIF16B-YFP, but not by the expression of DN-KIF5B-CFP (Figure 4C, left). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from $0 \pm 0.1\%$ in untransfected WT MDCK cells to $8 \pm 1\%$ ($P < 0.001$) in untransfected AP-1B KD MDCK cells and was reduced to $2 \pm 1\%$ ($P < 0.05$) by expression of DN-KIF16B-YFP, but not by DN-KIF5B-CFP in AP-1B KD MDCK cells (Figure 4C, right).

Because antibodies against canine KIF16B are not available, we investigated the expression of KIF16B in MDCK cells by RT-PCR analysis using two different sets of primers. These experiments showed that KIF16B is endogenously expressed by both WT and AP-1B KD MDCK cells; furthermore, the sequence of the amplified product blasted exclusively with the canine KIF16B mRNA (Supplementary Figure S5).

We next investigated whether knockdown of endogenous KIF16B in MDCK cells inhibits the apical transcytosis of basolaterally internalized TfR. Treatment of both WT and AP-1B KD MDCK cells with a pool of four siRNAs against KIF16B decreased its expression by 78% (Figure 4D'' and Supplementary Figure S5C). KD of KIF16B in AP-1B KD MDCK cells caused a significant reduction in the colocalization of 594-Tf with immunostained rab11a at ARE after incubation with basolateral 594-Tf for 15 min (Figure 4D, columns 3 and 4), although this reduction was less pronounced than that observed after treatment with DN-KIF16B-YFP. In contrast, 594-Tf was virtually not detected in ARE in WT MDCK cells transfected with luciferase or KIF16B siRNA (Figure 4D, columns 1 and 2). Quantitation of these experiments showed that the percentage of rab11a colocalizing with 594-Tf increased from $3 \pm 0.4\%$ in WT-Luc KD to $29 \pm 2\%$ in AP-1B KD-Luc KD ($P < 0.001$) and was reduced to $11 \pm 1\%$ in AP-1B KD-KIF16B KD MDCK cells ($P < 0.001$) (Figure 4D' left). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from $0.4 \pm 0.1\%$ in WT-Luc KD to $6 \pm 0.6\%$ in AP-1B KD-Luc KD ($P < 0.001$) and was reduced to $4 \pm 0.4\%$ in AP-1B KD-KIF16B KD MDCK cells ($P < 0.05$) (Figure 4D' right).

These results show that KIF16B mediates transcytosis of TfR to ARE in AP-1B KD MDCK cells.

KIF16B mediates trafficking of TfR from CRE to ARE using non-centrosomal microtubules

From which compartment does KIF16B mediate apical transcytosis of TfR to ARE? Experiments in non-polarized cells have shown that KIF16B regulates TfR recycling at the level of

SE (Hoepfner *et al*, 2005); however, apical transcytosis of pIgR occurs from CRE to ARE (Apodaca *et al*, 1994; Brown *et al*, 2000). Hence, we designed experiments to determine which candidate endosomal compartments contain KIF16B.

As CRE has no specific marker, we identified this compartment from BSE using a 2-colour pulse-chase protocol using 594-Tf (red) and 633-Tf (green) (Supplementary Figure S6). MDCK cells were exposed basolaterally to 633-Tf for 25 min and to 594-Tf for 5 additional minutes. Under these conditions, 633-Tf labelled preferentially CRE and 594-Tf labelled preferentially BSE. Using this protocol, we observed that WT-KIF16B colocalized to a significantly higher extent with CRE than with BSE (Figure 5A arrows and quantification analysis in Figure 5A'), suggesting that KIF16B operates at CRE. Importantly, DN-KIF16B did not inhibit the segregation of 633-Tf and 594-Tf during the 5-min chase (Figure 5B), indicating that KIF16B is not involved in traffic of TfR from BSE to CRE. DN-KIF16B, which lacks the motor domain and therefore can attach to the proximal compartment (through the C-terminus region) but cannot traffic to the distal compartment, colocalized preferentially with CRE (Figure 5B arrows and Figure 5B'), suggesting that CRE is the proximal compartment of this motor. Furthermore, DN-KIF16B did not alter the morphology of BSE and CRE. Similar results were obtained in experiments carried out in AP-1B KD MDCK cells (Figures 5C, C', D and D').

Given that KIF16B is a plus-end kinesin and that the centrosome and apical endosomes localize more apically than CRE in polarized MDCK cells, transport of basolateral TfR to apical endosomes must rely on non-centrosomal MTs. The Golgi acts as an MT-organizing centre (Chabin-Brion *et al*, 2001) and is in intimate spatial relationship with CRE in polarized MDCK cells (our own unpublished observations) and with RE in non-polarized cells (REs have been denominated the para-Golgi compartment) (Yamashiro *et al*, 1984). We therefore carried out MT regrowth assays (by adding and removing nocodazole) in polarized WT MDCK cells (see Materials and methods) to determine whether the Golgi apparatus acts as a MT organizing centre in polarized MDCK cells. These experiments showed MT growing from Golgi nucleation sites (Figure 5E, arrowhead) more basally localized than the centrosome (Figure 5E, arrow). As these non-centrosomal MTs grow with their plus ends oriented mainly towards the subapical cytoplasm, they are likely to be involved in KIF16B-mediated transport of TfR from juxta-Golgi CRE to subapical ARE.

Taken together, these experiments support a model in which AP-1B KD MDCK cells transcytose basolateral TfR from CRE to ARE utilizing the plus-end kinesin KIF16B, likely using non-centrosomal MTs emerging from the Golgi complex (see model in Figure 8A). As parallel experiments showed that KIF16B does not mediate transcytosis of pIgR (Supplementary Figures S4A,B,C), our results indicate that parallel transcytotic routes exist in MDCK cells, with remarkable selectivity for different cargoes.

AP-1B-deficient RPE displays apical transcytosis of TfR

The RPE constitutes the main barrier between the blood and the retina and hence performs many functions essential for survival of the retinal photoreceptors (Strauss, 2005). Indeed, RPE cells take up iron from the neural retina through their apical membrane, thus preventing the accumulation of toxic

Figure 4 Transport of basolateral TfR to ARE requires the plus-end microtubule motor KIF16B. (A, B) WT and AP-1B KD MDCK cells transiently transfected with DN-KIF16B-YFP (a) or DN-KIF5B-CFP (b) were incubated from the basolateral surface with fluorescent 594-Tf for 15 min and immunostained with anti-rab11a. Transfected and non-transfected cells can be identified in the bottom panel by the signal of DN-KIF16B-YFP or DN-KIF5B-CFP. (C) Cells from experiments represented in (a) and (b) were quantified for the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (D) WT and AP-1B KD MDCK cells nucleofected with either luciferase or KIF16B siRNA were incubated from the basolateral surface with fluorescent 594-Tf for 15 min and immunostained with anti-rab11a. (D') Cells from experiments represented in (d) were quantified for the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (D'') RNA levels of KIF16B and GAPDH in AP-1B KD cells nucleofected with luciferase or KIF16B siRNA. NS, no significance, * $P < 0.05$, ** $P < 0.001$. Red line represents the median. Scale bar, 10 μ m.

Figure 5 KIF16B mediates trafficking of TfR from CRE to ARE. WT MDCK cells transiently transfected with the WT-KIF16B-YFP (full-length KIF16B) (**A**) or the DN-KIF16B-YFP (motorless KIF16B) (**B**) were incubated for 25 min from the basolateral surface with 633-Tf (green) followed by a 5 min chase, in which 594-Tf (red) was added to the basolateral medium. The left panel displays the signal of 594-Tf and 633-Tf and the right panel displays the signal of KIF16B-YFP from the same field. Arrows emphasize endosomes containing KIF16B and 633-Tf, but not 594-Tf. (**A'**, **B'**) Cells from experiments represented in **A** and **B** were quantified for the percentage of pixels of the kinesin colocalizing with 633-Tf or 594-Tf (top) and the percentage of pixels of 633-Tf or 594-Tf colocalizing with the kinesin (bottom) (**C**, **C'**, **D**, **D'**). The same experiment described in **A**, **A'**, **B** and **B'** was carried out in AP-1B KD MDCK cells. (**E**) WT MDCK cells were treated with nocodazole/cold at 4°C, incubated at 37°C in the absence of the drug for MTs regrowth (see Methods), fixed and immunostained with markers of MTs (α -tubulin), golgi (giantin) and centrosomes (γ -tubulin). * P <0.05, ** P <0.001. Red line represents the median. Scale bar, 10 μ m.

levels of this metal (Yefimova *et al*, 2000; He *et al*, 2007; Garcia-Castineiras, 2010). As we have recently shown that RPE constitutively lacks μ 1B (Diaz *et al*, 2009), we

investigated whether RPE cells transcytoses TfR to the apical PM through the apical transcytotic route we demonstrated in AP-1B KD MDCK cells. For these

Figure 6 AP-1B-deficient retinal pigment epithelium displays apical transcytosis of TfR. (A) Values of apical transcytosis, basolateral recycling and the intracellular pool of SulfoTag-Tf after 60 min of recycling in WT MDCK and ARPE-19 cells. (B) WT MDCK or ARPE-19 cells were incubated from the basolateral side with fluorescent 594-Tf for 30 min and immunostained with anti-rab11a. (B') Cells from experiments represented in (b) were quantified for the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (C) WT MDCK or ARPE-19 cells were incubated with fluorescent 594-Tf as in (b) and stained for ASE with incubation of 488-WGA from the apical surface for 5 min at 37°C and washed with NADG at 4°C. (C') Cells from experiments represented in C were quantified for the percentage of pixels of 488-WGA colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with 488-WGA (right). (D) ARPE-19 fully polarized in Transwell filters were electroporated with DN-KIF16B-YFP (Deora *et al*, 2007), incubated from the basolateral surface with fluorescent 594-Tf for 15 min and immunostained with anti-rab11a. Transfected and non-transfected cells can be identified in the bottom panel by the signal of DN-KIF16B-YFP. (D') Cells from experiments represented in (d) were quantified for the percentage of pixels of rab11a colocalizing with the 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). Bars represent mean $\pm$ standard error. * $P < 0.05$, ** $P < 0.001$. Red line represents the median. Scale bar, 10 μ m.

Figure 7 Expression of AP-1B in fibroblastic CHO cells generates a rab11a-independent recycling route. (A) WT and AP-1B KD subconfluent MDCK were incubated with fluorescent (dog) 594-Tf for 30 min and immunostained for rab11a. (A') Quantification of the percentage of pixels of rab11a colocalizing with 594-Tf (left) and the percentage of pixels of 594-Tf colocalizing with rab11a (right). (B) WT and AP-1B (+) CHO cells were incubated with fluorescent (human) 594-Tf for 30 min and immunostained with anti-rab11a and anti-HA (μ1B-HA). (B') Quantification of the pixels of rab11a occupied by 594-Tf (left) and the pixels of 594-Tf occupied by rab11a (right). (C) Western blot analysis showing expression of stably transfected μ1B-HA and/or transiently transfected mCh-DN-rab11a in CHO cells. (C') TfR recycling assay: after 60 min uptake of SulfoTag-Tf, cells were either allowed to recycle for 56 min or lysed immediately afterwards (0-min recycling) (see Methods). Retention was calculated as 'retention after 56-min recycling/retention after 0-min recycling'; and normalized to 'control WT CHO'. Bars represent mean ± standard error. NS, no significance, * $P < 0.05$, ** $P < 0.001$. Red line represents the median. Scale bar, 10 μm.

experiments, we utilized monolayers of the human RPE cell line ARPE-19, which develop tight junctions and a polarized distribution of the monocarboxylate transporters MCT1 and MCT4 (Supplementary Figures S7A,B) similar to that observed in RPE *in vivo*. Biochemical SulfoTag-Tf recycling assays (50 min) showed that, compared to WT MDCK cells, RPE cells displayed significantly higher apical transcytosis (15% versus 3%, $P < 0.001$), lower basolateral recycling (32% versus 76%, $P < 0.001$) and higher intracellular retention (52% versus 22%, $P < 0.05$) (Figure 6A). Microscopic uptake assays using human 594-Tf showed that after 30 min of basolateral internalization, RPE cells display a large amount of 594-Tf in ARE (which in these cells appear as a bright spot under the apical surface), where it colocalizes with rab11a (Figure 6B). Colocalization of 594-Tf with rab11a was significantly higher than that observed in control WT MDCK cells (Figure 6B'). Similarly, after 30 min, RPE cells displayed extensive colocalization of 594-Tf with apically internalized 488-WGA (Figure 6C), a marker of ASE, significantly higher than that of WT MDCK cells (Figure 6C'). Transfection of DN-KIF16B-YFP (Hoepfner *et al*, 2005) prevented transport of

basolaterally internalized 594-Tf (15 min) to ARE in ARPE-19 cells (Figure 6D), as previously shown for AP-1B KD MDCK cells. Quantification of these experiments showed that the percentage of rab11a colocalizing with 594-Tf decreased from $34 \pm 4\%$ to $10 \pm 2\%$ ($P < 0.001$) by the expression of DN-KIF16B-YFP in ARPE-19 cells. Accordingly, the percentage of 594-Tf colocalizing with rab11a decreased from $8 \pm 1\%$ to $1 \pm 0.1\%$ ($P < 0.001$) by expression of this mutant (Figure 6D').

These results show that constitutively μ1B-deficient RPE cells transcytose a substantial fraction of basolateral TfR to ARE in a KIF16B-dependent manner, as we have shown in AP-1B KD MDCK cells. This route is likely responsible for the presence of high levels of TfR in the apical PM of RPE, necessary for iron homeostasis in the retina.

Expression of AP-1B in CHO cells generates a rab11a-independent recycling route

The experiments described above demonstrate that lack of expression of AP-1B promotes incorporation of TfR into a rab11a-dependent transcytotic route to the apical surface in MDCK and RPE cells and suggest that AP-1B plays a major

Figure 8 Model. (A) WT MDCK cells recycle most TfR to the basolateral membrane through BSE and CRE. In contrast, in AP-1B KD MDCK cells a substantial fraction of TfR is trafficked from CRE to ARE by KIF16B and non-centrosomal MTs and delivered to the apical PM in a rab11a-mediated manner. (B) WT CHO cells recycle TfR through perinuclear RE in a rab11a-mediated manner. In contrast, CHO cells stably transfected with AP-1B generate a rab11a-independent route, likely mediated by AP-1B.

role in generating a rab11a-independent recycling route for TfR in polarized epithelia. Is this also true for non-polarized MDCK cells? Experiments following the internalization of 594-Tf for 30 min in subconfluent MDCK cells (Figure 7A) showed that indeed, expression of AP-1B prevents access of TfR to rab11a-positive endosomes, which are localized to the perinuclear region of these cells. Quantitative analysis indicated that the percentage of rab11a colocalizing with 594-Tf increased from $6 \pm 1\%$ in non-polarized WT MDCK cells to $42 \pm 3\%$ ($P < 0.001$) in non-polarized AP-1B KD MDCK cells (Figure 7A', left). Accordingly, the percentage of 594-Tf colocalizing with rab11a increased from $5 \pm 1\%$ in non-polarized WT MDCK cells to $15 \pm 3\%$ ($P < 0.05$) in non-polarized AP-1B KD MDCK cells (Figure 7A', right). These results clearly show that the presence of AP-1B is sufficient to restrict recycling TfR from reaching rab11a-positive endosomes even before polarity develops and suggest that subconfluent MDCK cells recycle different cargoes through AP-1B and rab11a recycling endosomes.

We speculated that expression of AP-1B might also be sufficient to generate an alternative recycling route for TfR in fibroblastic CHO cells, which normally recycle TfR along a rab11a-dependent general recycling pathway. To test this hypothesis, we expressed the epithelial-specific subunit $\mu 1B$ tagged with a HA epitope ($\mu 1B$ -HA) in a CHO cell line in which hamster TfR had been replaced for human TfR (McGraw *et al*, 1987). As CHO cells express the ubiquitous adaptor AP-1A, which shares three of its four subunits ($\beta 1$, $\sigma 1$ and $\gamma 1$), these cells were able to assemble AP-1B as demonstrated by the almost complete colocalization of $\mu 1B$ -HA

with endogenous $\gamma 1$ subunit (Supplementary Figure S8A,A'). In addition, WT and AP-1B (+) CHO cells expressed similar levels of rab11a (Supplementary Figure S9).

Expression of AP-1B in fibroblastic CHO cells did not reduce the colocalization of internalized 594-Tf with rab11a (Figure 7B arrows, Figure 7B'), indicating that AP-1B is not sufficient to divert these proteins in CHO cells. However, $\mu 1B$ -HA colocalized extensively at perinuclear RE with internalized 594-Tf (Supplementary Figure 8B,B'), suggesting that AP-1B might be functionally involved in TfR recycling. To test this hypothesis, mCh-DN-rab11a was transiently transfected in WT and AP-1B (+) CHO cells (Figure 7C) and recycling assays were carried out. As previously reported (Ullrich *et al*, 1996; Ren *et al*, 1998), mCh-DN-rab11a inhibited TfR recycling by 60% in WT CHO cells ($n = 6$, $P < 0.001$). However, mCh-DN-rab11a did not inhibit TfR recycling in AP-1B (+) CHO cells (Figure 7C').

These results suggest that expression of AP-1B in CHO cells is sufficient to generate a rab11a-independent TfR recycling pathway but not sufficient to generate geographically segregated TfR and rab11a endosomes (see model in Figure 8B). They also suggest that the AP-1B-dependent pathway in CHO cells is equivalent to the basolateral pathway of epithelial cells, whereas the rab11a pathway is equivalent to the apical-recycling pathway of epithelial cells.

Discussion

It is well known that epithelial cell lines constitutively lacking AP-1B or silenced of the epithelial-specific subunit $\mu 1B$

express basolateral proteins at the apical PM (Folsch *et al*, 1999; Gan *et al*, 2002; Gravotta *et al*, 2007); however, the route and mechanisms responsible for the apical 'missorting' of basolateral proteins are still unknown. Elucidating the nature of this route and mechanisms has become more urgent with the discovery of two native epithelia, the RPE and the kidney proximal tubule, that lack AP-1B and depend on apically expressed basolateral proteins to perform their normal physiological functions (Diaz *et al*, 2009; Schreiner *et al*, 2010). Using the sensitive biochemical and microscopic techniques that we developed here to study the recycling of endogenous dog TfR, we show that AP-1B KD MDCK cells divert TfR from its basolateral recycling circuit into an apical transcytotic pathway with distinctive features that differentiate it from the well-studied transcytotic pathway of pIgR. The transcytotic route of TfR, like that of pIgR, traverses ARE and depends on rab11a. Surprisingly (and in contrast to pIgR), TfR traverses ASE, an apical endocytic compartment (Bomsel *et al*, 1989) previously shown to participate in the biosynthetic route of raft-associated PM proteins (Cresawn *et al*, 2007; Weisz and Rodriguez-Boulán, 2009). The 'missorting pathway' is also utilized by other AP-1B-dependent basolateral cargoes (e.g., LDLR), but not by AP-1B independent basolateral cargoes (NBC1) (Figure 3).

We found that MT integrity is necessary for transcytosis of TfR to ARE, as previously shown for pIgR (Apodaca *et al*, 1994; Brown *et al*, 2000). Strikingly, we also found that the plus-end kinesin KIF16B transports TfR from CRE (where the bulk of it resides) to ARE, likely utilizing MT nucleated in the Golgi, which is in intimate relationship with the CRE. As this motor does not mediate transcytosis of pIgR to ARE, our results indicate that different apical transcytotic pathways exist in epithelial cells, with remarkable selectivity for different cargoes (Figures 4, 5 and 8).

KIF16B was previously shown by the Zerial group to be capable of binding, through a Px domain, the early endosomal lipid PI3P and to regulate the recycling of TfR in fibroblastic cells (Hoepfner *et al*, 2005). More recently, Hirokawa and coworkers showed that KIF16B binds to and traffics FGF receptor vesicles to the PM and that rab14 might be necessary for both processes (Ueno *et al*, 2011). As Wilson and coworkers have shown that rab14 regulates apical trafficking of the raft-associated protein MAL (Cheong *et al*, 1999; Puertollano *et al*, 1999; Kitt *et al*, 2008), future work is necessary to determine whether rab14 participates in the transcytotic route of TfR in AP-1B KD MDCK cells. Rab4 might also be involved in the pathway reported here, as it has been previously shown to increase apical transcytosis of TfR when overexpressed in WT MDCK cells (Mohrmann *et al*, 2002). However, the mechanism involved is unclear given that rab4 is believed to function at BSE, which are proximal to the site of action of AP-1B.

Recent observations from our laboratory suggest that AP-1B mediates basolateral recycling of TfR from CRE to the basolateral membrane through the binding of its medium subunit μ 1B to a sorting signal in a 40-amino-acid stretch in the cytoplasmic domain of TfR (Odorizzi and Trowbridge, 1997; Gravotta *et al*, 2012). Because loss of AP-1B results in trafficking of TfR to the apical membrane rather than retention at CRE, TfR might express recessive apical signals. Previous work has shown that apical transcytosis of the pIgR in WT MDCK cells is mediated by N-glycans acting as apical

signals (Luton *et al*, 2009). TfR contains three N-glycosylation and one O-glycosylation sites and our preliminary results (Perez Bay, Schreiner and Rodriguez-Boulán) suggest that N-glycans are involved in guiding its apical transcytosis in AP-1B-deficient epithelia.

The apical transcytotic pathway for cognate basolateral proteins we have identified in AP-1B KD MDCK cells, is likely to play an important role in the organization and function of native epithelia that lack AP-1B, for example, RPE and kidney proximal tubule (Diaz *et al*, 2009; Schreiner *et al*, 2010), due to the apical expression of basolateral proteins that perform important physiological functions for the host organs. Indeed, this pathway is present in RPE cells (ARPE-19 cells), which constitutively lack AP-1B and display a similar, although more pronounced, phenotype as AP-1B KD MDCK cells. Notorious features of this phenotype were the reduction of basolateral recycling and the increased intracellular pool of TfR, which support the role of AP-1B in promoting recycling of this receptor back to the (basolateral) PM. The stronger phenotype of ARPE-19, compared to AP-1B KD MDCK cells, might be due to incomplete KD of AP-1B in MDCK cells or a compensatory mechanism that occurs in AP-1B KD cells, but not in cells that constitutively lack this adaptor.

A long standing question in the epithelial trafficking field is: which pathway (apical or basolateral) is mechanistically equivalent to the general trafficking route of fibroblastic cells? The basolateral biosynthetic route was initially hypothesized to fulfil this role because many receptors that mediate exchanges with the blood on the PM of fibroblastic cells localize to the basolateral PM of epithelial cells (Simons and Fuller, 1985; Fuller and Simons, 1986). However, the discovery that rab11a, a major regulator of the general recycling route of non-polarized cells, localizes to subapical ARE, which, like the RE of fibroblastic cells, is clustered by MT motors around the centrosome, suggested the opposite hypothesis, that is, that the apical route of epithelial cells is equivalent to the general trafficking route of fibroblastic cells (Zacchi *et al*, 1998; Wang *et al*, 2000). However, a critical piece of functional evidence in support of this hypothesis has been missing up to date, that is, that a protein recycled by a rab11a-dependent mechanism in fibroblastic cells can traffic along the rab11a-dependent apical route in epithelial cells. Our results, based on the structural and functional analysis of TfR recycling pathways in AP-1B (+) and AP-1B (–) epithelia, support this hypothesis as they demonstrate that apically recycling TfR in AP-1B KD MDCK cells traverses rab11a-positive recycling endosomes and depends on rab11a for surface delivery, exactly as TfR utilizing the general recycling pathway of fibroblastic cells (Ullrich *et al*, 1996; Ren *et al*, 1998).

Materials and methods

Reagents

The following antibodies were used in this study: rabbit anti-rab11a (Invitrogen, Carlsbad, CA), mouse anti-TfR (Zymed, San Francisco, CA), mouse anti-HA (Roche, Indianapolis, IN), rat anti- γ adaptin (BD Biosciences, San Jose, CA), rat anti- α tubulin (kindly provided by Dr Geri Kreitzer), rabbit anti- γ tubulin (Sigma-Aldrich, Saint Louis, MO), mouse anti-Giantin (kindly provided by Dr Hauri), sheep anti-secretory component (kindly provided by Dr Mostov). 488-WGA was purchased from Sigma-Aldrich and 647-WGA from Invitrogen. Nocodazole was from Sigma-Aldrich.

Plasmids

Generation of monomeric Cherry-tagged dominant-negative rab11a (mCh-DN-Rab11a): first, we obtained the tetracycline-responsive promoter from the pTRE-tight vector (Clontech, Mountain View, CA) with *NruI* and *EcoRV*. Second, we removed the CMV promoter from the pcDNA3.1 hygro (+) vector (Invitrogen) and inserted the tetracycline-responsive promoter with *XhoI*. Third, we inserted the Ch-DN-Rab11a (kindly provided by Dr Ching-Hwa Sung) into the above vector using *KpnI* and *XbaI* sites. The NBC1-GFP plasmid was generated by cloning kidney NBC1 (kindly provided by Manoocher Soleimani) with *NheI* and *KpnI* into pEGFP-N1 (Clontech).

The DN-KIF16B-YFP and DN-KIF5B-CFP plasmids were kindly provided by Marino Zerial and Geri Kreitzer.

Cell culture

MDCK cells were maintained in DMEM (Thermo Scientific, Pittsburgh, PA) supplemented with 5% FBS (Invitrogen). Trv-b1 cells (CHO strain expressing the human TfR, kindly provided by Dr Tim McGraw) were maintained in 10% FBS DMEM. Human ARPE-19 cells (ATCC, Bethesda, MD) were maintained in RPE-media: MEM- α (Sigma-Aldrich) supplemented with 1% FBS, N1 supplement (100 $\times$, Sigma-Aldrich), glutamine-penicillin-streptomycin (100 $\times$, Sigma-Aldrich), nonessential amino-acids (100 $\times$, Sigma-Aldrich), hydrocortisone (20 μ g/l), taurine (250 mg/l), and triiodothyronin (0.013 μ g/l) (THT). Polarized MDCK and ARPE-19 cells were plated at 3×10^5 cells/cm² on 12 mm Transwell chambers and used 4 days or 4–6 weeks after plating, respectively. Non-polarized CHO cells were plated at 1×10^5 cells/cm² on 12-well plates and used after 2–3 days; whereas subconfluent MDCK cells were plated at 3×10^4 cells/cm² on 12-well plate and used after 1 day.

Transgenic cell lines

MDCK cells stably knocked down for μ 1B (Gravotta *et al*, 2007) were grown in the presence of 2.5 μ g/ml of puromycin (Sigma-Aldrich). The mCherry-dominant-negative rab11a mCh-DN-Rab11a MDCK cell line was generated by transfection of a T23 MDCK strain expressing the tetracycline repressor (kindly provided by Dr. Keith Mostov) and maintained with 1 mg/ml hygromycin B (Invitrogen) 1 mg/ml G418 (Mediatech, Manassas, VA) and 2.5 μ g/ml puromycin. The AP-1B (+) CHO cell line was generated by transfection of Trv-b1 cells with a plasmid expressing human μ 1B tagged with influenza HA (gift from Dr Heike Folsch and Dr Ira Mellman) and maintained with 1 mg/ml hygromycin for μ 1B expression and G418 for human TfR expression.

siRNA transfection and RT-PCR

To knockdown μ 1B transiently, we used previously published protocol and siRNAs (Gravotta *et al*, 2007). Briefly, WT MDCK cells in suspension culture were treated with 5 μ l of 40 mM siRNA and subjected to three rounds of electroporation with Amaxa Nucleofector kit V, spaced every 3 days, and plated after the last round on Transwell chambers. The extent of μ 1B silencing in cells transfected with siRNA or in the stable μ 1B-KD cell line was assessed by RT-PCR. A one-step RT-PCR (Qiagen, Valencia, CA) was run under standard conditions for a total of 30 cycles with 200 ng of mRNA per 50-ml reaction. To knockdown the canine KIF16B, we used the following siRNAs (designed with an algorithm from Dharmacon (Waltham, MA, USA)): 5'-GGTGAAGGATAA TGAGAAATT-3', 5'-AGGCAGAATTGGAGCGATT-3', 5'-GGGAGAA GGATGAGCAGTATT-3', 5'-AGAAAGAGATGGAAGAAAATT-3'. siRNAs were nucleofected using the protocol described above. KIF16B expression was analysed by RT-PCR with the following primers: canine primer set 1: 5'-AAATGCCGTGTGAAACCGTG-3' and 5'-GCTATTTCAGCAGCGAGCTC-3', canine primer set 2: 5'-GAGCTGCTGTGCTGAAATAGC-3' and 5'-TGCAGTGCTCACTCTCCAAG-3'; human primer set 1: 5'-AGTCCGTGAGCATCCCAAAG-3' and 5'-CCTTGAGC AAGCAGCGTTTT-3'; human primer set 2: 5'-TGACCTCTCTGTGTGCTCC TGA-3' and 5'-ACCTCAGGTGCGACATTCTG-3' (see Supplementary Figure 5a).

Protein labelling

Fluorescent Tf. Dog or human Tf (Sigma-Aldrich) were conjugated with the fluorophores CF594 (Biotium, Hayward, CA) SeTau647, SeTau667 (SETA Biomedicals, Urbana, IL) or HiLyte665 (AnaSpec,

Fremont, CA) using NHS chemistry. Different dye/protein molar ratios were tested following manufacturers' indications. Subsequently, fluorescent Tfs were purified with PD10 columns (GE Healthcare, Piscataway, NJ), iron loaded, purified again with PD10 columns and with 50 kDa cut-off filters (Milipore). The brightest reagent (Tf labelled with CF594 (594-Tf)) was identified with serial dilutions and measurements in a fluorometer. 594-Tf was validated as a ligand for TfR through fluorescence microscopy experiments showing its colocalization with anti-TfR (Supplementary Figure S1B) and through competition experiments that showed inhibition of 594-Tf uptake by the presence of 200 $\times$ unlabelled Tf.

SulfoTag-Tf. Dog or human Tf were sequentially conjugated with the luminophore SulfoTag (Mesoscale Discovery, Gaithersburg, MD) and biotin (Sigma-Aldrich), using NHS chemistry and purified with PD10 columns after each reaction. Then, SulfoTag-Tfs were iron loaded, purified again with PD10 columns and with 50 kDa cut-off filters. Different molar ratios (luminophore/protein and/or biotin/protein) were tested following manufacturers' indications. The best-performing reagent was identified by highest signal/noise ratio in surface binding assays of subconfluent MDCK cells. Non-specific binding was blocked with 200 $\times$ unlabelled Tf.

The physiologically relevant linear range of SulfoTag-Tf detection was estimated as follows. MDCK cells express 165 000 copies of endogenous TfR, (Fuller and Simons, 1986), 12 mm Transwell filter (where biochemical assays were conducted) contain 300 000 MDCK cells and one TfR binds two Tf molecules. Assuming that $\sim 2\%$ of TfR resides in the apical PM (Odorizzi and Trowbridge, 1997), it would be necessary to detect 2×10^9 molecules of TfR (3×10^{-15} mol). For this value, the signal of SulfoTag-Tf was 10 times higher than that of ¹²⁵I-Tf (Figure 1B).

Uptake assays and immunofluorescence

For 594-Tf uptake assays, cells were starved for 60 min at 37°C in HBSS containing 20 mM HEPES (standard buffer) and incubated for 60 min at 4°C with 10 mg/ml 594-Tf in 1% BSA standard buffer. Then, temperature was shifted to 37°C and, after the indicated time, cells were immediately rinsed (with ice-cold HBSS) and fixed (ice-cold 4% PFA in PBS). After quenching PFA (50 mM NH₄Cl in PBS) for 15 min, cells were permeabilized with 0.01% triton X-100 in PBS for 7 min, incubated with the corresponding primary antibodies for 45 min and secondary antibodies for 30 min in 1% BSA PBS, and washed three times after each incubation.

To label ASE, cells were exposed from the apical side to fluorescent WGA for 5 min at 37°C (25 μ g/ml in 1% BSA standard buffer). Residual fluorescent WGA was stripped from the PM by washing three times with 100 mM N-acetyl-D-glucosamine in HBSS for 10 min at 4°C and then cells were fixed.

When utilized, nocodazole (33 μ M), taxol (5 μ M) or apical defer-oxamine was applied during starvation until fixation. Tunicamycin (2 μ M) was applied 2 h before and during transfection with TfR-GFP (~ 20 h). For MT re-growth assays, cells were chilled at 4°C for 60 min and treated with nocodazole at 4°C for additional 120 min. Then cells were permeabilized with 0.5% Triton X-100 in HBSS at 37°C for 3 min, incubated in HBSS at 37°C for additional 2 min, fixed with methanol at -20°C for 30 s and processed for immunofluorescence.

Microscopy

Images were collected with a Zeiss Axio Observer inverted microscope equipped with a Yokogawa Confocal Scanner Unit CSU-X1 and a Photometrics Cool Snap XQ2 Camera using a Zeiss planapochromat 40X/1.4 oil-immersion objective. Acquisition and analysis were performed with Axiovision Rel. 4.8 (Zeiss, Oberkochen, Germany) software.

Colocalization analysis and statistics

Pearson's and Manders' coefficients are the two most accepted tools to measure colocalization between different cellular markers A and B. Advantages of the Pearson's coefficient are its simplicity and its independence of signal levels and signal offset. However, it requires the two markers to co-occur in a fixed proportion to one another. This is usually not the case for a cargo reaching a compartment, such as TfR, which localizes in various endosomal compartments at

various levels, not necessarily colocalizing with endosomal markers in a fixed proportion. On the other hand, the Manders' colocalization coefficient does not have this requirement and, importantly, it provides information on two useful components: the fraction of marker 'A' occupied by marker 'B' and the fraction of marker 'B' occupied by marker 'A'. To determine the area of marker 'A' (i.e., rab11a) occupied by marker 'B' (i.e., 594-Tf), we quantified the pixels of marker 'A' and the fraction of pixels of marker 'A' colocalizing with marker 'B'. This operation was applied for all the confocal sections of a region of interest (i.e. one cell). Then, the area of marker 'A' occupied by marker 'B' was calculated by the formula 'pixels of marker 'A' colocalizing with marker 'B'/pixels of marker 'A'. The corresponding formula was utilized to calculate the area of marker 'B' occupied by marker 'A'.

We used this method to assess colocalization of the following marker pairs: TfR and rab11a; TfR and WGA; μ 1B and γ 1 subunit; μ 1B and rab11a; μ 1B and TfR; KIF16B and fluorescent Tf.

Statistics: Values were expressed as mean $\pm$ standard error. The statistical method used was the Student's *t*-test. The levels of statistical significance are specified for each comparison in the respective figure.

Biochemical assays

Recycling/transcytosis. Polarized MDCK or ARPE-19 cells were starved with standard buffer for 60 min at 37°C and incubated with 10 μ g/ml SulfoTag-Tf (dog and human, respectively) in 1% BSA standard buffer for 60 min at 37°C from the basolateral side only. SulfoTag-Tf was removed from the PM at 4°C with: (i) three rinses (1% BSA standard buffer), (ii) 30 min incubation with pH 5 buffer (200 mM NaCl, 50 mM 2-(N-morpholino) ethanesulphonic acid, pH5) plus 50 μ M deferoxamine (EMD Bioscience, Rockland, MA), (iii) 30 min incubation with 200X unlabelled Tf in 1% BSA standard buffer and (iv) three additional rinses with 1% BSA standard buffer. Cells were incubated for the indicated times at

37°C with recycling buffer (200 $\times$ unlabelled Tf in 1% BSA standard buffer). Samples were collected from the basolateral and apical chambers and from cell lysates, and quantified in a SI2400 Multiplex analyser (Meso Scale Discovery). Non-specific binding was determined by incubating the cells with SulfoTag-Tf in the presence of 200 $\times$ excess unlabelled human Tf. When specified, nocodazole (Sigma-Aldrich) (33 μ M) was applied during the whole experiment.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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