Published in final edited form as:

Methods Cell Biol. 2015 ; 130: 271–288. doi:10.1016/bs.mcb.2015.06.019.

Structural and functional analysis of endosomal compartments in epithelial cells

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Abstract

Epithelial cells display segregated early endosomal compartments, termed apical sorting endosomes and basolateral sorting endosomes, that converge into a common late endosomal-lysosomal degradative compartment and common recycling endosomes (CREs). Unlike recycling endosomes of nonpolarized cells, CREs have the ability to sort apical and basolateral plasma membrane proteins into distinct apical and basolateral recycling routes, utilizing mechanisms similar to those employed by the trans Golgi network in the biosynthetic pathway. The apical recycling route includes an additional compartment, the apical recycling endosomes, consisting of multiple vesicles bundled around the basal body. Recent evidence indicates that, in addition to their role in internalizing ligands and recycling their receptors back to the cell surface, endosomal compartments act as intermediate stations in the biosynthetic routes to the plasma membrane. Here we review methods employed by our laboratory to study the endosomal compartments of epithelial cells and their multiple trafficking roles.

INTRODUCTION

Endosomal compartments in epithelial cells participate in many polarized signaling and nutrient delivery functions; to carry out these functions they are endowed with the ability to traffic and sort plasma membrane (PM) receptors, for example, transferrin (TfR), low-density lipoprotein (LDLR), epidermal growth factor receptor (EGFR) and megalin, in the endocytic, recycling, and biosynthetic pathways (Perret et al., 2005). Early studies in nonepithelial cells have shown that these receptors are internalized from the PM into peripheral sorting endosomes (SE), where the acidic environment dissociates the ligands (except for transferrin (Tf), which loses the iron while remaining bound to TfR) (Maxfield & McGraw, 2004). Within minutes, SE cease to receive incoming vesicles from the cell surface and mature into late endosomes (LE), a process characterized by microtubule motor-facilitated translocation toward the cell center and exchange of the small guanine triphosphatase (GTPase) Rab5 by Rab7 (Poteryaev et al., 2010; Rink, Ghigo, Kalaidzidis, & Zerial, 2005). LE then fuse with lysosomes thus exposing the luminal material to hydrolases for degradation (Puertollano et al., 2001). Most membrane receptors in SE (exception EGFR) are incorporated into narrow tubules, in part through the action of sorting nexins (Traer et al., 2007), (1) quick return to the cell surface (fast-recycling loop) or (2)

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microtubule- and dynein-mediated traffic to perinuclear Rab11-positive recycling endosomes (RE^{Rab11}) (Shah, Bateria, Taupin, & Farquhar, 2013), and Rab11/myosin Vb-dependent return to the cell surface (slow recycling loop) (Lapierre et al., 2001; Ren et al., 1998; Ullrich, Reinsch, Urbe, Zerial, & Parton, 1996) (Figure 1(A)).

Because polarized epithelia have distinct apical and basolateral PM domains, separated by tight junctions, their endosomal organization and function are more complex. The basolateral PM displays nutrient receptors, such as TfR and LDLR, which pick up the respective ligands from the blood and are then internalized into basolateral SE (BSE). The apical PM faces the lumen of the organs and participates in organ-specific endocytic processes. For example, epithelial cells in the proximal tubule of the kidney display the fast recycling receptors megalin and cubilin at the apical PM, which act as a Velcro mechanism to recover proteins in the glomerular ultrafiltrate and are internalized into apical SE (ASE), localized immediately under the apical surface. ASE and BSE mature into common LE targeting the fluid phase and luminal ligands for lysosomal degradation (Bomsel, Prydz, Parton, Gruenberg, & Simons, 1989). Apical receptors in ASE and basal receptors in BSE are recycled via complex mechanisms. They are either returned by a fast recycling loop to the respective cell surface or transported via tubular carriers to TfR-rich perinuclear common recycling endosomes (CRE^{TfR}) where they are sorted via specific mechanisms and recycled via distinct carrier vesicles back to the correct PM domain (slow recycling loop) (Hughson & Hopkins, 1990). Basolateral receptors are sorted via basolateral signals similar to endocytic motifs, bound by clathrin adaptors such as AP-1B (Folsch, Ohno, Bonifacino, & Mellman, 1999; Gan, McGraw, & Rodriguez-Boulton, 2002; Gravotta et al., 2007), and incorporated into clathrin-coated vesicles for delivery to the basolateral PM (Deborde et al., 2008). The recycling pathway of apical receptors has not been fully characterized yet. Based mainly on the route of transcytotic receptors such as polymeric IgA receptor (pIgR) (Apodaca, Katz, & Mostov, 1994; Brown et al., 2000; Wang, Kumar, Navarre, Casanova, & Goldenring, 2000), neonatal Fc receptor (FcRn) (Tzaban et al., 2009), and TfR in epithelial cells lacking AP-1B (Perez Bay et al., 2013), they are believed to be sorted at CRE^{TfR} via specific apical signals (e.g., N-glycans, for pIgR and TfR) (Luton et al., 2009; Perez Bay et al., 2014) into vesicles for microtubule-dependent transport to a subapical endosomal compartment enriched in Rab11, the apical recycling endosome (ARE^{Rab11}) (Apodaca et al., 1994; Brown et al., 2000; Golachowska, Hoekstra, & van, 2010; Perez Bay et al., 2013; Perret et al., 2005), and then delivered to the apical PM via a Rab11, myosin Vb-dependent mechanism (Thuenauer, Rodriguez-Boulton, & Romer, 2014; Wang et al., 2000) (Figure 1(B)).

Based on early experiments, sorting of apical and basolateral PM proteins in the biosynthetic route is believed to occur at the trans Golgi network (TGN) (Matlin et al., 1983; Misek, Bard, & Rodriguez-Boulton, 1984; Rodriguez Boulton & Sabatini, 1978; Rodriguez-Boulton, Paskiet, Salas, & Bard, 1984). Sorting of apical PM proteins at the TGN is mediated by apical sorting signals such as glycosylphosphatidyl inositol anchors (Brown, Crise, & Rose, 1989; Lisanti et al., 1989), transmembrane domains (Kundu et al., 1996; Scheiffele, Roth, & Simons, 1997; Skibbens, Roth, & Matlin, 1989), cytoplasmic determinants (Takeda, Yamazaki, & Farquhar, 2003; Thuenauer et al., 2014), and N- or O-glycans (Delacour et al., 2006; Perez Bay et al., 2014; Stechly et al., 2009). These signals are recognized by

membrane micro-domains termed lipid rafts, galectins, or microtubule motors. Sorting of basolateral proteins at the TGN is mediated by short peptidic sequences often resembling endocytic motifs (Yxx ϕ NPYX), which are recognized by the clathrin adaptors AP-1A or AP-1B (Carvajal-Gonzalez et al., 2012; Folsch, Pypaert, Maday, Pelletier, & Mellman, 2003; Gravotta et al., 2012) for incorporation into clathrin-coated vesicles (Deborde et al., 2008). However, over the past decade it has become evident that, in addition to their specific roles in recycling processes, endosomes are also intermediate compartments in the biosynthetic routes of PM proteins. For example, the basolateral PM protein VSV G traverses CRE^{TfR} en route to the basolateral domain (Folsch et al., 2003; Gravotta et al., 2007), whereas several apical proteins traverse ARE^{rab11} (i.e., endolin and rhodopsin) (Cresawn et al., 2007; Thuenauer et al., 2014) or ASE (i.e., influenza hemagglutinin (HA)) (Cresawn et al., 2007). The functional significance of transendosomal transport pathways remains unclear. Interestingly, the basolateral adhesion molecule E-cadherin also traverses ARE^{rab11} in its biosynthetic pathway (Lock & Stow, 2005), suggesting the emerging concept that ARE^{rab11} might have apical-basolateral sorting roles such as the TGN and CRE.

The exact site of function of AP-1A and AP-1B in basolateral sorting is controversial. Although biochemical sorting assays indicate that AP-1B mediates basolateral sorting at CRE^{TfR} and the highly homologous adaptor AP-1A performs basolateral sorting at the TGN, a recent report, on the basis of microscopy experiments using overexpressed tagged adaptor subunits, has suggested that both adaptors localize and function at the TGN and CRE^{TfR} (Folsch et al., 2003; Gravotta et al., 2012; Guo et al., 2013; Rodriguez-Boulan, Perez-Bay, Schreiner, & Gravotta, 2013). Full elucidation of the basolateral sorting mechanisms of epithelial cells will require to identify the compartments where the endogenous adaptors operate and to determine how these adaptors carry out their complementary sorting functions. Interestingly, AP-1B is an epithelial-specific adaptor and AP-1A is ubiquitous (Ohno et al., 1999), there are some epithelia that lack AP-1B and hence express a subset of cognate basolateral proteins at the apical PM. These include the retinal pigment epithelium (RPE) and the kidney proximal tubule (Diaz et al., 2009; Schreiner et al., 2010). The mechanisms responsible for the apical localization of basolateral proteins in these epithelia have recently been identified. Using the basolateral recycling receptor TfR as a model, it was shown that, in MDCK cells lacking AP-1B and in RPE and kidney proximal tubule cell lines, TfR is internalized into CRE^{TfR} where it is sorted apically by an N-glycan signal and galectin-4 into vesicles that are then transported via Golgi-nucleated microtubules and the kinesin KIF16B to ARE^{rab11}, from where it is delivered to the apical PM by a Rab11-dependent mechanism (Perez Bay et al., 2013, 2014).

1 . OBJECTIVES AND RATIONALE

Based on the discussion above, it is clear that a full understanding of the recycling, transcytotic, and biosynthetic pathways of epithelial cells requires the appropriate methodology to study the transport of cargo proteins along these routes. Here we discuss assays used by our laboratory to study endosomal compartments in epithelial cells and the polarized recycling and transcytotic routes of apical and basolateral receptors. Microscopic and biochemical recycling and transcytotic assays provide complementary information. Microscopy assays inform on endosomal localization and morphology as well as the kinetics

with which internalized cargo proteins traverse specific endosomes. Biochemical assays also provide kinetic data on the endocytic and transcytotic pathways, but in a much higher quantitative manner, since this approach allows one to analyze millions of cells at the same time. Here we provide protocols for microscopy and biochemical assays that allow one to (1) specifically label the multiple endosomal compartments of epithelial cells, (2) determine the trafficking kinetics of internalized cargo proteins to these compartments, and (3) determine the trafficking kinetics of cargo proteins to the apical and basolateral PM in the recycling and transcytotic pathways.

2 . EXPERIMENTAL PROTOCOLS

2.1 MICROSCOPY-BASED PROTOCOLS FOR ENDOSOMAL LABELING IN POLARIZED EPITHELIAL CELLS

The endosomal compartments of epithelial cells can be labeled with different approaches:

1. Internalization of fluorescently labeled antibodies that recognize a luminal epitope of a receptor or internalization of fluorescently labeled specific ligands (e.g., Tf for TfR). In order to label the desired compartment, fluorescent antibodies/ligands are applied to the apical and/or basolateral PM domain for specific times.
2. Immunofluorescence of endosomal markers (e.g., proteins highly enriched in specific endosomes) or expression of these markers tagged with fluorescent proteins (FP) (Table 1).

Internalized antibodies/ligands are usually more specific than endosomal markers, for example, 5 min basolateral internalization of Tf labels BSE and 5 min apical internalization of wheat germ agglutinin (WGA) labels ASE, whereas immunofluorescence of the early endosomal antigen EEA1 labels both BSE and ASE. On the other hand, expression of endosomal markers fused to FP are specially useful for life imaging experiments, as opposed to fluorescent ligands that usually require to fix the cells at a specific time to prevent them from labeling other compartments. Therefore, fluorescent ligands should be used to achieve higher specificity and FP-tagged endosomal markers for high-temporal resolution kinetic experiments.

Section 2.1 describes protocols for fluorescent ligand-based endosomal labeling and provides a list of endo/lysosomal markers for immunofluorescence and FP-tagged expression (Table 1).

2.1.1 Apical sorting endosomes

2.1.1.1 Background and objective: ASE in polarized MDCK cells appear as small vesicular compartments located immediately beneath the apical PM (Figure 2(A), green fluorescent signal). ASE rapidly exchange apical PM proteins and lipids with the apical PM domain (~2 min for internalization and ~5 min for recycling). This can be exploited by adding fluorescent WGA (Flu-WGA, Sigma–Aldrich, Saint Louis, MO, USA), which binds to highly glycosylated proteins abundant in the apical PM (Cresawn et al., 2007; Perez Bay et al., 2013).

2.1.1.2 Buffers composition: Hanks balanced salt solution (HBSS): 1.26 mM CaCl₂, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 5.3 mM KCl, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 137.9 mM NaCl, 0.338 mM Na₂HPO₄, 5.55 mM dextrose.

Phosphate buffer saline (PBS): 137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄.

2.1.1.3 Experimental protocol

1. Plate MDCK cells at 300,000 cells/cm² on 12-mm polycarbonate Transwell filters[®] (Corning, Union City, CA, USA), in Dulbecco Modified Eagle Medium (Corning, Union City, CA, USA), supplemented with 5% fetal bovine serum (5% FBS DMEM). Culture MDCK cells for 4 days, to allow polarization.
2. Rinse twice (apically and basolaterally) with HBSS buffer, supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (37 °C).
3. Add apically 25 µg/mL Flu-WGA in prewarmed HBSS supplemented with 20 mM HEPES (37 °C, 5 min). This step allows Flu-WGA internalization into ASE.
4. Incubate three times with 100 mM N-acetyl-D-glucosamine (Sigma–Aldrich, Saint Louis MO, USA) in HBSS (4 °C, 10 min). This step strips Flu-WGA bound to the apical PM.
5. Rinse with HBSS (4 °C), fix with 4% paraformaldehyde in PBS (4 °C, 10 min), and process for immunofluorescence.

2.1.2 Basolateral sorting endosomes

2.1.2.1 Background and objective: Like ASE and the apical PM, BSE are small vesicular compartments intimately related to the basolateral PM (Figure 2(B), red fluorescent signal) with which it exchanges components at a very high rate. Since TfR is strictly basolateral in most epithelial cells (i.e., MDCK, CaCo, CaLu, and FRT cells), BSE can be efficiently labeled by exposure of MDCK cells grown on Transwell filters[®] to fluorescent TfR (Flu-Tf, Sigma–Aldrich, St Louis, MO, USA) added to the basolateral compartment. This approach is also achievable in AP-1B-deficient RPE cells, since a large fraction of TfR remains basolateral in these cells. It is worth noting that MDCK cells express low levels of TfR, compared to the extensively used TrvBi cells (McGraw, Greenfield, & Maxfield, 1987); therefore, overexpression of TfR might be necessary to perform these experiments. Alternatively, labeling Tf with bright and photostable fluorophores yields enough signal to detect endogenous Tf in MDCK cells (in our laboratory, we have successfully used the fluorophores CF594 (Biotium, Hayward, CA, USA) and SeTau647 (Seta Biomedicals, Urbana, IL, USA), among others (Perez Bay et al., 2013, 2014).

2.1.2.2 Experimental protocol

1. Plate MDCK cells at 300,000 cells/cm² density on 12-mm polycarbonate Transwell filters[®] (Corning, Union City, CA, USA), in 5% FBS DMEM (Corning, Union City, CA, USA) and culture for 4 days to allow polarization.

2. Rinse twice (apically and basolaterally) with HBSS buffer supplemented with 20 mM HEPES (37 °C).
3. Incubate with HBSS buffer supplemented with 20 mM HEPES (37 °C, 60 min). This step removes unlabeled Tf from TfR.
4. Add basolaterally 5–10 µg/mL Flu-Tf in HBSS supplemented with 20 mM HEPES and 1% bovine serum albumin (BSA) (4 °C, 60 min). This step allows for basolateral surface binding of Flu-Tf. Avoid concentrations higher than 20 µg/mL of Flu-Tf, since this increases the likelihood of fluid phase endocytosis.
5. Incubate three times with HBSS buffer supplemented with 20 mM HEPES and 1% BSA (4 °C, 5 min).
6. Incubate with prewarmed HBSS buffer supplemented with 20 mM HEPES and 1% BSA (37 °C, 5 min). This allows Flu-Tf internalization into BSE.
7. Rinse (HBSS, 4 °C), fix with 4% paraformaldehyde in PBS (4 °C, 10 min) and process for immunofluorescence.

2.1.2.3 Other markers of ASE and BSE: Several additional markers, such as early endosome antigen 1 (EEA1) and the small GTPases Rab4 and Rab5, are used to label SE in nonpolarized cells, either by immunofluorescence or by tagging them with FP. However, polarized epithelial cells display segregated sets of ASE and BSE, which are specifically labeled with apical internalization of Flu-WGA and basolateral internalization of Flu-Tf, as described above.

2.1.3 Common recycling endosomes

2.1.3.1 Background and objective: Notably, there is currently no specific marker for CRE^{TfR}. These endosomes have been originally defined by electron microscopy experiments as a peri/supranuclear compartment where apically internalized concanavalin A and basolaterally internalized Tf mix, 10–15 min after internalization (Hughson & Hopkins, 1990). Although Flu-Tf is the gold standard for CRE^{TfR} labeling, continued basolateral incubation of Flu-Tf for 10 or more minutes labels both BSE and CRE^{TfR}. Therefore, we developed a two-color Flu-Tf approach to dissect CRE^{TfR} and BSE in the same WT MDCK cell (Perez Bay et al., 2013). As shown in Figure 2(B), CRE^{TfR} appear as large tubule-vesicular structures concentrated in the supranuclear region in WT MDCK cells (green fluorescent signal).

2.1.3.2 Experimental protocol

1. Plate WT MDCK cells at 300,000 cells/cm² density on 12-mm polycarbonate Transwell filters® (Corning, Union City, CA, USA), in 5% FBS DMEM (Corning, Union City, CA, USA) and culture for 4 days to allow polarization.
2. Rinse twice (apically and basolaterally) with HBSS buffer supplemented with 20 mM HEPES (37 °C).
3. Incubate with HBSS buffer supplemented with 20 mM HEPES (37 °C, 60 min).

4. Add basolaterally 5–10 µg/mL Flu-Tf in HBSS supplemented with 20 mM HEPES and 1% BSA (4 °C, 60 min).
5. Incubate with prewarmed HBSS buffer supplemented with 20 mM HEPES and 1% BSA (37 °C, 15 min).
6. Add basolaterally 5–10 µg/mL Flu-Tf (labeled with a different fluorophore than the one used in step 4) in HBSS supplemented with 20 mM HEPES and 1% BSA (4 °C, 60 min).
7. Incubate three times with HBSS buffer supplemented with 20 mM HEPES and 1% BSA (4 °C, 5 min).
8. Incubate with prewarmed HBSS buffer supplemented with 20 mM HEPES and 1% BSA (37 °C, 5 min).
9. Rinse (HBSS, 4 °C), fix with 4% paraformaldehyde in PBS (4 °C, 10 min) and process for immunofluorescence.

2.1.4 Apical recycling endosome

2.1.4.1 Background and objective: In contrast to ASE, BSE, and CRE^{TfR}, ARE^{Rab11} are best labeled with immunofluorescence of the endosomal marker Rab11 or by expressing Rab11-FP fusion protein. Immunofluorescence conditions should be optimized according to the cell type and antibody used. As shown in Figure 2(A), ARE^{rab11} appear as a characteristic large spot located just beneath the center of the apical PM in MDCK cells. The appearance of ARE^{rab11} may vary in different epithelial cells.

2.1.4.2 Experimental protocol: The following is one of many possible immunofluorescence protocols.

1. Plate MDCK cells at 300,000 cells/cm² density on 12-mm polycarbonate Transwell filters[®] (Corning, Union City, CA, USA), in 5% FBS DMEM (Corning, Union City, CA, USA) and culture for 4 days to allow polarization.
2. Rinse twice (apically and basolaterally) with HBSS buffer supplemented with 20 mM HEPES (37 °C).
3. Fix with 4% paraformaldehyde in PBS (room temperature, 10 min).
4. Quench paraformaldehyde with 50 mM ammonium chloride in PBS (room temperature, 15 min).
5. Block with 1% BSA-PBS (room temperature, 40 min).
6. Incubate with rabbit anti-Rab11 (Invitrogen, Carlsbad, CA, USA) in 1% BSA-PBS (1 in 100 dilution; room temperature, 60 min).
7. Wash three times with 1% BSA-PBS (room temperature, 5 min).
8. Incubate anti-rabbit fluorescently labeled secondary antibody in 1% BSA-PBS (room temperature, 45 min).
9. Wash three times with 1% BSA-PBS (room temperature, 5 min).

2.1.5 Late endosomes and lysosomes

2.1.5.1 Background and objective: LE/Lys can be labeled with immunofluorescence of the endo/lysosomal markers Rab7 (LE) and Lamp1 (Lys) or expression of these proteins tagged with FP; however, these markers are not completely specific since small amounts of Rab7 can be found in Lys and Lamp1 in LE. Lys can be selectively labeled with internalization assays of fluorescent fluid phase solutes, such as fluorescently labeled low-molecular-weight dextrans (10 kDa Flu-Dx; Invitrogen, Carlsbad, CA, USA). There exist other commercially available probes to label Lys based on a pH-sensitive dye, which is almost nonfluorescent at neutral pH and fluoresces in the acidic environment of the Lys. This section describes a classical protocol for Lys labeling with 10 kDa Flu-Dx.

2.1.5.2 Experimental protocol

1. Plate MDCK cells at 300,000 cells/cm² density on 12-mm polycarbonate Transwell filters® (Corning, Union City, CA, USA), in 5% FBS DMEM (Invitrogen, Carlsbad, CA, USA) and culture for 4 days to allow polarization.
2. Rinse twice (apically and basolaterally) with HBSS buffer supplemented with 20 mM HEPES (37 °C).
3. Add apically and basolaterally 0.1 mg/mL 10 kDa Flu-Dx in HBSS supplemented with 20 mM HEPES and 1% BSA (37 °C, 60 min). Note that high concentration of the fluorophore is used to maximize fluid phase endocytosis.
4. Rinse three times with HBSS buffer supplemented with 20 mM HEPES and 1% BSA (37 °C).
5. Incubate with HBSS buffer supplemented with 20 mM HEPES and 1% BSA (37 °C, 60 min). This step allows the accumulation of internalized 10 kDa Flu-Dx into Lys and the clearance from ASE and BSE.
6. Rinse (HBSS, 4 °C), fix with 4% paraformaldehyde in PBS (4 °C, 10 min), and process for immunofluorescence.

2.2 MICROSCOPY-BASED TRAFFICKING ASSAYS IN POLARIZED EPITHELIAL CELLS

This section describes assays to determine the time of arrival of a basolaterally internalized PM protein to a specific endosomal compartment (2.2.1) and the transcytosis of a basolaterally internalized PM protein in polarized MDCK cells with a microscopy-based approach (2.2.2).

2.2.1 Trafficking of internalized cargo proteins to specific endosomal compartments

2.2.1.1 Background and objective: This assay requires the combination of an internalization assay for the PM cargo protein and an endo/lysosomal labeling protocol. As an example, this section describes a protocol to study the trafficking of basolaterally internalized TfR to ARE^{Rab11} in WT and AP-1B KD MDCK cells. As we had previously reported (Perez Bay et al., 2013), Figure 3 shows that CF594-Tf internalized from the basolateral side for 10 min avoids ARE^{Rab11} in WT MDCK cells and reaches this

compartment in AP-1B KD MDCK cells. For protocols on TfR trafficking to other compartments such as ASE or Lys, please see the original papers (Perez Bay et al., 2013, 2014).

2.2.1.2 Experimental protocol

1. Plate MDCK cells at 300,000 cells/cm² density on 12-mm polycarbonate Transwell filters® (Corning, Union City, CA, USA), in 5% FBS DMEM (Corning, Union City, CA, USA) and culture for 4 days to allow polarization. Prepare one filter for each time point to be studied in one experiment.
2. Rinse twice (apically and basolaterally) with HBSS buffer supplemented with 20 mM HEPES (37 °C).
3. Incubate with HBSS buffer supplemented with 20 mM HEPES (37 °C, 60 min).
4. Add basolaterally 5–10 µg/mL Flu-Tf in HBSS supplemented with 20 mM HEPES and 1% BSA (4 °C, 60 min).
5. Add basolaterally 5–10 µg/mL Flu-Tf in prewarmed (37 °C) HBSS supplemented with 20 mM HEPES and 1% BSA (add the same medium without Flu-Tf to the apical chamber).
6. Incubate for different times. Typical incubation times are 0, 5, 10, 15, and 30 min.
7. Rinse (HBSS, 4 °C), fix with 4% paraformaldehyde in PBS (4 °C, 10 min).
8. Immunostain ARE^{Rab11} using Rab11 as endosomal marker and the immunofluorescence protocol described above.
9. In order to estimate the time of arrival of TfR to ARE^{Rab11}, carry out colocalization analysis of Rab11 with TfR in the different time points studied. For an excellent review on colocalization analysis, please see (Dunn, Kamocka, & McDonald, 2011).

2.2.2 Microscopy-based apical transcytosis assay

2.2.2.1 Background and objective: This section describes a protocol to measure apical transcytosis of TfR luminally tagged with green fluorescent protein (GFP) (TfR-GFP), in WT and AP-1B KD MDCK cells. We have used this approach to study apical transcytosis of transiently transfected TfR-GFP mutated in each of TfR glycosylation sites (Perez Bay et al., 2014). Briefly, a fluorescently labeled primary rabbit anti-GFP antibody (MBL International, Des Plaines, IL, USA) is applied to the basolateral side and a fluorescently labeled secondary anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA) to the apical side. Therefore, basolateral TfR-GFP internalization will be proportional to the fluorescent signal of the primary antibody and apical transcytosis will be proportional to the fluorescent signal of the secondary antibody.

2.2.2.2 Experimental protocol

1. Plate MDCK cells at 300,000 cells/cm² density on 12-mm polycarbonate Transwell filters® (Corning, Union City, CA, USA), in 5% FBS DMEM (Corning, Union City, CA, USA) and culture for 4 days to allow polarization. Prepare one filter for each time point to be studied in one experiment and two additional filters for negative controls.
2. On day 3, transfect TfR-GFP (2 µg DNA and 2 µL Lipofectamine in 200 µL OptiMem (Invitrogen, Carlsbad, CA). Apply the mix overnight.
3. On day 4, rinse twice (apically and basolaterally) with HBSS buffer supplemented with 20 mM HEPES (37 °C).
4. Incubate with HBSS buffer supplemented with 20 mM HEPES (37 °C, 60 min).
5. Add basolaterally 2.5 µg/mL rabbit 647-αGFP in HBSS supplemented with 20 mM HEPES (4 °C, 120 min).
6. Rinse three times with HBSS supplemented with 20 mM HEPES (4 °C).
7. Incubate apically with 2.5 µg/mL 568-αRabbit in prewarmed (37 °C) HBSS supplemented with 20 mM HEPES for different times (add the same medium without 568-αRabbit to the basolateral chamber). This step promotes the apical transcytosis of rabbit 647-αGFP in AP-1B KD MDCK cells, which is subsequently detected by 568-αRabbit.
8. Rinse three times (apically and basolaterally) with HBSS supplemented with 20 mM HEPES (4 °C).
9. Fix with 4% paraformaldehyde in PBS (4 °C, 10 min).
10. Apical transcytosis can be estimated in several ways: (1) mean intensity fluorescence (MIF) of 568-αRabbit, (2) 568-αRabbit MIF/647-αGFP MIF, or (3) Manders colocalization coefficient I, which reports the percentage of 647-αGFP pixels occupied by 568-αRabbit pixels. For detailed information on quantification and colocalization analysis, please see the original papers (Perez Bay et al., 2013, 2014).
11. Appropriate negative controls for this experiment include: (1) apical application of secondary 568-αRabbit without basolateral application of rabbit 647-αGFP primary antibody and (2) basolateral application of rabbit 647-αGFP and apical application of 568-αRabbit antibodies at 4 °C.

2.3 BIOCHEMICAL-BASED ASSAY

2.3.1 Sulfotag biotin-Tf recycling and transcytotic assay—In this assay, Tf is labeled with both the luminophore Sulfotag and biotin (sb-Tf). To measure TfR recycling and transcytosis in polarized MDCK cells, sb-Tf is applied basolaterally until achieving steady-state occupancy of the TfR in endosomes. After stripping sb-Tf from the basolateral surface, sb-Tf released to the apical and baso-lateral medium is collected at different time points. The collected mediums are applied to streptavidin-coated 96-well plates (Meso Scale

Discovery, Rockville, MD, USA), which allows the capture of sb-Tf to the bottom of the wells and the detection of Sulfotag luminescence using SI2400 Multiplex analyzer (Meso Scale Discovery, Rockville, MD, USA). This assay is similar to the radioactivity-based biochemical assay reported previously (McGraw & Subtil, 2001) except for the fact that Tf is labeled with Sulfotag/biotin instead of ^{125}I . In our hands sb-Tf is detected with higher sensitivity than ^{125}I -Tf.

2.3.1.1 Experimental protocol

2.3.1.1.1 Tf labeling with Sulfotag and biotin

1. Dissolve NHS-Sulfotag (Meso Scale Discovery, Rockville, MD, USA) in dimethyl sulfoxide (DMSO) and mix with apo-Tf. Use 5X NHS-Sulfotag/apo-Tf molar ratio.
2. Rotate (room temperature, 60 min).
3. Purify Sulfotag-Tf using PD10 columns (GE Healthcare, Piscataway, NJ, USA).
4. Prepare NHS-biotin (Pierce, Waltham, MA, USA) in DMSO and mix with Sulfotag-Tf. Use 5X NHS-biotin/Sulfotag apo-Tf molar ratio.
5. Rotate (room temperature, 60 min).
6. Purify sb-Tf using PD10 columns (GE Healthcare, Piscataway, NJ, USA).
7. Iron load sb-Tf using standard protocol described previously (McGraw & Subtil, 2001).

2.3.1.1.2 Recycling/transcytosis assay

1. Plate MDCK cells at 300,000 cells/cm² density on 12-mm polycarbonate Transwell filters[®] (Corning, Union City, CA, USA), in 5% FBS DMEM (Corning, Union City, CA, USA) and culture for 4 days to allow polarization. Prepare 6 filters for each time point to be studied in one experiment: three replicas for recycling/transcytosis determination and three replicas for background determination.
2. Rinse twice (apically and basolaterally) with HBSS buffer supplemented with 20 mM HEPES (37 °C).
3. Incubate with HBSS buffer supplemented with 20 mM HEPES (37 °C, 60 min).
4. Add basolaterally 5–10 µg/mL sb-Tf in HBSS supplemented with 20 mM HEPES and 1% BSA (37 °C, 60 min).

For background determination apply the same solution, with 5–10 µg/mL sb-Tf and 200X unlabeled Tf.
5. Rinse three times with HBSS buffer supplemented with 20 mM HEPES and 1% BSA (4 °C).
6. Incubate with pH 5 buffer (200 mM NaCl, 50 mM 2-(N-morpholino) ethane-sulfonic acid, pH 5) plus 50 µM deferoxamine (4 °C, 30 min).

7. Rinse with HBSS buffer supplemented with 20 mM HEPES (4 °C).
8. Incubate with HBSS buffer supplemented with 20 mM HEPES and 200X unlabeled Tf (4 °C, 30 min).
9. Rinse three times with HBSS buffer supplemented with 20 mM HEPES and 1% BSA (4 °C).
10. Incubate (apically and basolaterally) with 200 µL prewarmed (37 °C) HBSS buffer supplemented with 20 mM HEPES, 1% BSA, 50 µM deferoxamine and 200X unlabeled Tf.
11. Collect apical and basolateral medium at the desired time points.
12. Remove filter from the plastic case by cutting through the edges of the case and incubate the filter in 200 µL lysis buffer (40 mM Tris pH 7.6, 150 mM NaCl, 1.5% Triton X-100) with protease inhibitors.
13. Collect lysate in a tube (do not transfer the filter to the tube).
14. Centrifuge (4 °C, 10 min, 16,000 g) and transfer to new tubes.
15. Apply samples to 96-well streptavidin-coated plates (Meso Scale Discovery, Rockville, MD, USA).
16. Incubate in orbital shaker (room temperature, 60 min).
17. Quantify luminescence in SI2400 Multiplex analyzer (Meso Scale Discovery, Rockville, MD, USA).

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FIGURE 1. Model

This cartoon displays the various endosomal compartments and endosomal trafficking pathways in nonepithelial (A) and polarized epithelial (B) cells. The basolateral pathways and mechanisms of polarized epithelial cells are shown in red and the apical in blue. (See color plate)

FIGURE 2.

(A) ASE and ARE^{Rab11} labeling. Polarized WT MDCK cells were incubated for 5 min with Alexa488-WGA (green) from the apical surface, washed with N-methyl-D-glucosamine (NMDG) and immunostained for Rab11. (B) BSE and CRE^{TfR} labeling. Polarized WT MDCK cells were incubated for 15 min from the basolateral surface with Alexa633-Tf (green) followed by a 5 min chase, with CF594-Tf (red). Orthogonal view (top) and two confocal sections at the supranuclear (middle) and nuclear (bottom) regions are shown. Scale: 10 μ m. (See color plate)

FIGURE 3. Trafficking of basolaterally internalized TfR to ARE^{Rab11}

Polarized WT and AP-1B KD MDCK cells were incubated for 10 min with CF594-Tf (red) from the basolateral surface and immunostained with anti Rab11 antibody (green).

Orthogonal view (top) and two confocal sections at the supranuclear (middle) and nuclear (bottom) regions are shown. Scale: 10 μ m. (See color plate)

Table 1

Ligands and Endosomal Markers to Label Endosomal Compartments in Polarized Epithelial Cells

Endosomal Compartment	Fluorescent Ligand	Endosomal Marker (Immunofluorescence)	Endosomal Marker (FP- Fusion Protein Expression)
ASE	Flu-WGA (apical uptake. 5')	EEA1, Rab4, Rab5	EEA1-FP, Rab4- FP, Rab5-FP
BSE	Flu-Tf (basolateral uptake. 5')	TfR	TfR-FP
CRE ^{TfR}	Flu-Tf (basolateral uptake. 10+5')	Flu-Tf (Basolateral uptake. 15')	
ARE ^{Rab11}	–	Rab11	Rab11-FP
LE	–	Rab7	Rab7-FP
Lys	10 kDa Flu-Dx (Uptake. 60+60')	Lamp1	Lamp1-FP