Published in final edited form as:

Methods Cell Biol. 2015 ; 130: 127–138. doi:10.1016/bs.mcb.2015.04.004.

Rab and Arf G Proteins in Endosomal Trafficking

Dipannita Dutta and Julie G. Donaldson

Cell Biology and Physiology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

Endocytosis is a fundamental process that cells use to remove receptors, extracellular material, plasma membrane proteins and lipids from the cell surface. After entry into cells the cargo proteins are subsequently trafficked to late endosomes and lysosomes for degradation, to the Golgi complex or to recycling endosomes for return to the plasma membrane. Small G proteins in the Rab and Arf family are present on endosomes and coordinate the trafficking of cargo proteins. Here we describe some basic experimental approaches to begin to study the endosomal trafficking of a given cell surface protein.

INTRODUCTION

Endocytosis is an important phenomenon where cells internalize different extracellular molecules, ligands, membrane proteins, and lipids. Endocytosis occurs by different mechanisms that may be dependent on or independent of the clathrin coat (Conner & Schmid, 2003). Clathrin-independent modes of entry include macropinocytosis, phagocytosis, caveolar and a variety of other possibly distinct forms (Mayor, Parton, & Donaldson, 2014). Whatever the different entry mechanism, the cargo molecules are captured in the endosomes that are pinched off from the plasma membrane and then typically these endosomes fuse with early or sorting endosomes to begin their intracellular journey to the final destination.

Collectively, “endosomes” consist of different heterogeneous compartments that can be designated as incoming endosome, early endosome, late endosome, or recycling endosome depending on the kinetics with which they are loaded with endosomal cargo (Maxfield & McGraw, 2004). Within this endosomal system, the incoming cargo are sorted for transport to the *trans* Golgi network (TGN), degradation in lysosomes or recycling back to the plasma membrane depending on cytoplasmic sorting sequences on the cargo molecules and the machinery assembled around it (Figure 1A). This sorting of membrane and cargo is a crucial step in the proper functioning of endosomal systems in the cell. In the degradative pathway, membrane proteins are often recognized by ubiquitination of residues in their cytoplasmic domains that facilitates sorting into multi-vesicular bodies and then to the lysosomes where they are degraded (Piper & Katzmann, 2007). Recycling pathways are numerous, may vary with the type of cargo and become quite complex in polarized cells. The transferrin receptor (TfR) is a prototypical cargo protein that enters through clathrin-mediated endocytosis (CME) to deliver, via its bound-transferrin, iron to the cytoplasm and is then recycled back to the cell surface via a rapid recycling pathway or a slower pathway that emanates from the

juxtannuclear endocytic recycling compartment. The recycling route can also be different for cargo proteins that enter the cells by clathrin-independent endocytosis (CIE); in many cells CIE cargo proteins recycle back to the plasma membrane via specialized recycling tubules (Grant & Donaldson, 2009). Endosomal compartments are defined by their protein and lipid composition. This includes differences in phosphoinositides (PIPs) and association with Ras family GTP-binding proteins (G proteins) (Di Paolo & De Camilli, 2006).

In eukaryotic cells, the formation of transport vesicles and their specific delivery to target membranes are often regulated by different small G proteins mainly of the Rab and Arf families. There are over 70 mammalian Rab proteins and more than half of them are involved in regulating endosomal membrane traffic (Wandinger-Ness & Zerial, 2014). Rabs define compartment identity and are involved in vesicle formation, transport and fusion to the target compartment. These varied roles are accomplished through the many Rab effector proteins. Rab5 is a central endosomal Rab that defines the initial sorting compartment or early endosome. Rab5, through its effectors, regulates endosomal fusion, signaling, sorting and transport along cytoskeletal elements (Zeigerer et al., 2012). Rabs 4, 7, 9, 11, 22, and 35 have all been known to play important roles in the endosomal systems. Rab4, Rab35, Rab11 and Rab22 are known to play a role in the recycling of cargo back to the plasma membrane through recycling endosomes (Kouranti, Sachse, Arouche, Goud, & Echard, 2006; van der Sluijs et al., 1992; Weigert, Yeung, Li, & Donaldson, 2004). Rab7 has been shown to be important for endosomal fusion and maintenance of lysosomal structures whereas Rab9 regulates cargo transport from the late endosomes to the trans-Golgi network (Wandinger-Ness & Zerial, 2014). Rab G proteins act as molecular switches in regulating different cellular events by their activation and inactivation.

The Arf family of G proteins also control membrane traffic through a regulated cycle of GTP-binding to activate and GTP hydrolysis to inactivate the Arf protein. Arfs localize to membranes throughout the cell, including the plasma membrane and the membranes of the secretory, endosomal and lysosomal pathways (Donaldson & Jackson, 2011). Arfs function by recruiting coat proteins onto membranes and through activation of enzymes that alter membrane lipid composition (phospholipase D and phosphatidylinositol 4-phosphate 5-kinase). The five Arf proteins in humans are ubiquitously expressed. Arfs 1, 3, 4 and 5 are mainly observed at the Golgi complex but they also are released into the cytoplasm and have been observed to function in the periphery (Caviston, Cohen, & Donaldson, 2014; Kumari & Mayor, 2008). Arf6 is present at the plasma membrane and influences both the cortical actin cytoskeleton and endosomal membrane trafficking (Boshans, Szanto, van Aelst, & D'Souza-Schorey, 2000; Radhakrishna & Donaldson, 1997). Arf6, through its activation of PIP5-kinase, has been observed in clathrin-coated vesicles and has been suggested to facilitate the rapid recycling of the transferrin receptor back to the plasma membrane through interaction with the microtubule motor adaptor protein JNK-interacting protein 4 (JIP4) after the uncoating of clathrin from the vesicle (Montagnac et al., 2011).

Studying the localization and the activation of the Rab and Arf proteins helps in understanding compartment identity and how cargo proteins are sorted and trafficked to their final destinations. In this chapter we describe a series of experimental approaches to study the roles of Rab and Arf proteins in regulating endosomal membrane traffic. These

approaches can assist the investigator who is trying to identify the endosomal compartments that their protein of interest passes through and to understand how that protein is sorted to reach its final destination.

OBJECTIVES AND RATIONALE

Rab and Arf proteins cycle between a GDP-bound, inactive or 'OFF' and a GTP-bound, active or 'ON' conformation. The GDP/GTP exchange reaction is catalyzed by guanine nucleotide exchange factors (GEFs) and GTP hydrolysis is catalyzed by GTPase activating proteins (GAPs) (Fig. 1B). Rab and Arf proteins are generally not abundantly expressed; plus good immunological reagents are generally not available for localization of the endogenous proteins. Thus, investigators typically express exogenous, epitope-tagged Rab and Arf proteins. For expression of Rabs, the green fluorescent protein (GFP) or other genetically-encoded fluorescent protein is appended to the amino terminus, as Rabs are prenylated at the carboxyl terminus. For Arfs, GFP or other tags are appended to the carboxyl terminus, as Arfs are amino terminally myristoylated.

Studying the localization and phenotype of overexpression of the wild type, GTP-binding-defective or GTP-hydrolysis-resistant mutants of G proteins has been useful in understanding the trafficking of proteins in the endosomal system. Typically, expression of the GTP-binding-defective (often called "dominant negative") forms of G proteins bind to and sequester the GEFs and thereby block the ability of the endogenous G protein to become activated. In many cases, the resulting phenotype is similar to what is observed when expression of the endogenous G protein is diminished by siRNA targeting. This was the case for Rab22, which is required for the formation of recycling endosomal tubules; expression of dominant negative Rab22 mutant, S19N, or depletion of Rab22 from cells leads to a loss of these tubules and inhibition of recycling (Weigert et al., 2004). On the other hand, expression of the GTP-hydrolysis-defective, constitutively active mutants of G proteins can also lead to an over-active phenotype or inhibition of trafficking. Expression of the active mutant of Rab5 (Q79L) stimulates fusion of early endosomes forming large endosomal compartments (Wandinger-Ness and Zerial, 2014). For Arf6, expression of its active mutant Q67L leads to the accumulation of early endosomes containing plasma membrane proteins that enter cells by CIE (Fig. 1A); failure to inactivate ARF6 blocks further trafficking of this membrane towards recycling or to other destinations (Eyster et al., 2009; Naslavsky, Weigert, & Donaldson, 2003). In addition to expressing the G proteins, in some cases the identity of specific GEFs and GAPs is also known and exogenous expression of these regulatory proteins can also alter the extent of activation or inactivation of the G proteins.

Here, we describe basic approaches for studying endosomal trafficking in cells. These protocols involve ways to follow endosomal trafficking of cell surface cargo proteins to determine their itinerary and final destination. In addition, we show the use of transient expression of Rab and Arf proteins to determine whether their activities are involved in the trafficking of the cargo protein of interest. Following transfection, immunofluorescence staining can reveal the distribution of the Rab and Arf proteins with respect to the cargo proteins that are allowed to enter the cells by internalization assays.

MATERIALS AND INSTRUMENTS

Materials

HeLa cells

Media containing DMEM plus 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution

Fugene X-tremegene 9 DNA transfection agent (Roche) or other transfection reagent

Mammalian expression plasmids encoding Arf and Rab proteins of interest with an epitope tag

Prewashed, 12 mm diameter round glass coverslips (#1.5). (Acid wash cover slips by immersing them in 1M HCl for 6 to 8 h at 65°C. Rinse them three times in distilled water and two times in 70% ethanol. Store the coverslips in 70% ethanol at room temperature).

6-well and 12-well tissue culture plates

15 cm tissue culture dish

Watchmaker forceps

Parafilm

1.2 mm thick glass slides

Kimwipes

5 ml round bottom polystyrene tubes

Primary antibody against the endocytic cargo of interest e.g., mouse anti-MHCI (clone W6/32), mouse anti-CD98, Alexa-fluor 488 or 594 or 633 conjugated Transferrin

2% formaldehyde solution in PBS

Phosphate buffered saline (PBS)

Acid wash containing 0.5% acetic acid, 0.5M NaCl, pH 3.0

PBS/FBS: 10% (v/v) FBS in PBS and 0.02% (w/v) sodium azide

Primary antibody against the epitope tag or Arf protein (e.g., anti-Arf6)

10% saponin

Secondary antibody: Alexa Fluor 488, 594 or 633 conjugated goat-anti-mouse and goat-anti-rabbit antibodies

Fluoromount-G

Clear nail polish

Instruments

Fluorescence microscope: epifluorescence or confocal microscope

METHODS

This protocol describes methods to study the itinerary or trafficking of cell surface proteins that enter cells by endocytosis. Included are ways to identify internal compartments by examining the presence of Rab and Arf G proteins and their activities in HeLa cells. This method can be adapted to study endosomal protein trafficking in other systems as well.

Antibody internalization assays

Cell plating—HeLa cells are maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a 5%-CO₂ incubator. One day prior to the experiment, cells are plated on pretreated glass cover slips and allowed to grow overnight at 37°C. The following day when cells reach a desirable confluency of 60–70%, they are used for experiments. Cells are washed with media twice and incubated for another 1–2 h before using them for internalization assays.

Internalization assays—Cells grown on cover slips are used in internalization assays one day after plating. Ideally cells are 60–70% confluent on the day of the experiment. To follow the endocytosis of cell surface proteins, monoclonal antibodies directed to the extracellular portion of the proteins can be added to the media at a concentration between 5–20 µg/ml. Excellent antibodies are available that label clathrin-independent cargo proteins like the major histocompatibility complex Class I protein (MHCI), CD59, CD98, and CD147 in human and mouse cells. To follow a typical CME cargo protein, fluorescently labeled transferrin (Alexa-488-Tfn, 5 µg/ml) is often used. Growth factor receptors, such as EGF receptor, are often labeled with a fluorescently tagged ligand and for G-protein-coupled receptors, epitope-tagged receptors are followed with antibodies to the tag. Antibodies or fluorescent ligands are diluted in pre-warmed media. 50 µl drops containing primary antibodies are deposited on Parafilm placed in 15 cm diameter dishes that have been pre-warmed in the incubator for 15 min. Note that when transferrin endocytosis is examined with fluorescently tagged transferrin, media without serum is typically used since serum contains (unlabeled) transferrin that could compete with the fluorescently labeled transferrin. Coverslips are gently transferred to the pre-equilibrated drops with cells facing the medium and incubated for 30 min at 37°C to allow endocytosis. This time point allows surface-bound antibodies to enter by endocytosis and subsequently enter sorting and recycling endosomes. Cells are then rinsed with PBS at RT and then surface bound MHCI, CD98 antibodies can be removed with a brief low pH rinse (0.5% acetic acid, 0.5M NaCl, pH 3.0) of 20 s, followed by two washes with PBS. Cells are then fixed with 2% formaldehyde in PBS at RT for 10 min. Cells are then rinsed twice with PBS (5 min wash each). Cells are then immersed in blocking solution (PBS containing 10% serum) for 20 min before labeling them with secondary antibodies. Not all antibodies will be released from the cell surface by the acid wash. In these instances, surface antibodies bound to CD147 are masked by incubating fixed cells with 0.1 mg/ml unlabeled goat anti-mouse antibody in the absence of saponin for 1.5 h at room temperature. Internalized antibodies are then visualized by probing with Alexa 594-

or 488-conjugated goat anti-mouse antibodies (Molecular Probes, Eugene, OR) dissolved in blocking solution plus 0.2% saponin for 45 min. Cells are washed twice with blocking solution and with two final wash with PBS with each wash for 5 min. Coverslips are mounted on slides with 1 drop of Fluoromount-G as mounting media on the slides and sealed with a ring of clear nail polish. Mounted slides are imaged with a confocal or epifluorescence microscope.

Cell Imaging—Images of cells on coverslips are taken at room temperature using a confocal LSM 780 microscope (Carl Zeiss) with a 63× 1.4-NA Plan-Apochromat oil immersion objective lens and different 488-, 532, and 633-nm laser excitation. Imaging conditions are chosen upon calibration of laser power to avoid minimal loss of fluorescence signal due to photobleaching. The laser power, exposure, contrast are adjusted such that none of the pixels are saturated during the collection of images. Sorting endosomes and early endosomes are best imaged with confocal slices taken from the middle of the cells. Recycling tubular endosomes often lay flat at the bottom of the cell and images of the recycling endosomes are taken focusing at the bottom. Images are acquired using ZEN 2011 software and processed using Photoshop CS6 (Adobe). Alternatively, cells can also be imaged with a conventional epifluorescence microscope.

Expression of wild type and mutant Rab and Arf proteins and effects on endosomal trafficking

Due to a lack of good immunological tools to detect endogenous Rab proteins by immunofluorescence, we overexpress a series of green fluorescent protein (GFP)-tagged Rabs (Rab5, Rab22a, Rab11) to screen for their localization to different endosomes. These overexpressed, wild type Rab proteins generally do not alter the morphology of the endosomal system when expressed at low levels. The localization of endogenous Rab22 is very similar to that of the overexpressed wild type GFP-Rab22 (Weigert et al., 2004). Overexpression of GTP locked mutants of Rab proteins (like GFP-Rab22a Q64L and GFP-Rab5 Q79L) are impaired in GTP-hydrolysis and often lead to the alteration in the morphology of the endosomal system. Similarly overexpression of mutants defective in GTP binding (like GFP-Rab22a S19N) impairs the endosomal system in a different way. The block of cargo protein trafficking by expression of the different forms of GTP and GDP locked mutants helps in studying the function of the Rabs on endosomal trafficking. Similar to the Rabs, expression of an epitope tagged wild type Arf proteins at low levels does not usually cause a change in the phenotype by itself and can be used to study the localization of Arf proteins. However, epitope tags are not benign and care should be taken in each case to be sure that the tag does not affect the function of the G protein.

Flow of the experiment

Transfection

1. Place 4 pre-washed coverslips in a 6-well dish containing 2 ml of culture media. Plate 3×10^5 cells into each well and incubate in a 37°C, 5%-CO₂ incubator overnight.

(Cells should be 40–60% confluent the day after plating for optimal transfection efficiency).

2. Transfect cells with 1 µg DNA for all the Rab and Arf constructs. During transfection, the authors follow the instruction protocol of Eugene-X-tremegene 9 from Roche, but other transfection reagents and protocols can also be used. With HeLa cells, we typically obtain 60–70% transfection efficiency for most constructs.
3. Incubate cells for 18–24 h at 37°C to allow expression of proteins from plasmids.

Internalization assay

4. After the transfection period, on the following day, change the media (no serum if transferrin endocytosis will be assessed) and allow the plate to incubate for 1–2 h in a 37°C incubator.
5. Dissolve the primary antibodies against the endocytic cargo in pre-warmed media (no serum if using transferrin) at a concentration of 5–20 µg/ml.
6. Place a piece of parafilm in a 15 cm tissue culture dish. Place a 50 µl aliquot of the antibody onto the parafilm.
7. Carefully place the coverslips on the drop, with cells facing down to the media. Carefully place the coverslips on the parafilm back into the incubator for 30 min.
8. After desired time, take out the coverslips and place each coverslip into 1 ml of PBS (cells facing up) in a 12-well plate. Wash the coverslips twice with PBS at room temperature.
9. Treat the cells with 1 ml of acid wash (0.5% acetic acid, 0.5M NaCl, pH 3.0) for 20 s. Thoroughly rinse the cells with 1 ml of PBS three times.

Fix cells

10. Fix cells by adding 1 ml of 2% formaldehyde to the wells. Incubate for 10 min at room temperature.
11. Remove formaldehyde and wash the cells thoroughly with 1 ml of PBS twice.
12. Add 1 ml of blocking solution (PBS/FBS) to each well and incubate for 20 min at room temperature.

Stain cells

13. Primary antibodies to epitope tags on Arf or Rab proteins or to other cellular proteins (e.g., EEA1, a Rab5 effector protein and marker for the early endosome) are diluted in blocking solution containing 0.2% saponin to permeabilize the cell for access to the interior. Antibodies to GFP can sometimes be used here to amplify low expressing GFP-tagged Rab and Arf proteins. Place 25 µl of the diluted antibody onto parafilm in 15 cm dishes. Invert coverslips over the drop. A 15 cm plate can hold up to 12 such coverslips. Incubate for 1 h at room temperature.

14. Carefully place the coverslips back into the 12 well dishes (cells facing up) and wash them thoroughly with 1 ml of PBS/FBS. Repeat the wash three times (5 min each).
15. Dilute fluorescently-conjugated secondary antibodies against the primary antibodies (e.g., goat-anti-mouse or goat-anti-rabbit IgG) in PBS/FBS with 0.2% saponin. Place the coverslips on 25 μ l of secondary antibodies as in Step 13 and incubate for 1 h at room temperature.
16. Place the coverslips back into the wells with cells facing up. Wash two times with 1 ml of PBS/FBS and two times in PBS, incubating 5 min for each wash.
17. Label slides and wipe with a clean Kimwipe. Place a drop of the Fluoromount-G mounting medium into the middle of the glass slide. Invert coverslip on top of the drop with cells facing down. Gently press the coverslips with 2–3 Kimwipes to remove excess mounting media.
18. Allow the coverslips to partially dry and then seal the edges with clear nail polish.
19. Allow time to dry the nail polish before taking the slides for imaging.

Slides can be stored in covered boxes for several months at 4°C.

Expected Results

The antibody internalization assay is a powerful tool for studying the endocytosis of endogenous cell surface proteins into cells. With it, one can follow the route taken by the protein over time and determine which intracellular compartments it visits while trafficking to its final destination. We often use this assay to study the effect of expression of the different regulators of trafficking. For instance, in the experiment shown in Figure 2, we overexpress GFP-Rab5 Q79L or Arf6 Q67L and study the trafficking of endogenous MHCI and TfR, CIE and CME cargo respectively, under these conditions. Expression of the active mutant of Rab5, Rab5 Q79L promotes enlargement of early endosomes and GFP-Rab5 Q79L is present on those enlarged endosome. Upon allowing MHCI and Transferrin (Tfn) to enter the cells by internalization assay, we see them accumulate in the Rab5 positive enlarged endosomes. Similar studies with other Rab proteins and mutants of Rab proteins can be helpful in studying their function on trafficking. Similarly, overexpression of wild type and mutant forms of Arf6 can be performed to examine the function of Arf6 in cells. The GTP-hydrolysis-defective mutant of Arf6, Arf6 Q67L, can be used to mimic the activated state. We overexpress Arf6 Q67L and study the trafficking of MHCI and Tfn under these conditions. Expression of Arf6 Q67L leads to formation of enlarged vacuoles (see Figure 1A and 2B) and MHCI, a CIE cargo protein, gets trapped in these clustered vacuoles whereas Tfn, which enters bound to the TfR via CME, is not trapped on the vacuoles. Rather it is dispersed and appears to be close to the vacuoles but not on them. Thus the active form of Arf6 traps CIE cargo proteins in a unique early endosome that is distinct from the enlarged endosome formed upon expression of active Rab5 that traps both CIE and CME cargo in a shared early endosome.

SUMMARY

Here we provide a basic protocol for studying the role of Rab and Arf proteins in the endosomal trafficking of cargo proteins entering cells by either CME or CIE. One example is shown using constitutively active forms of Rab5 and Arf6, but the reader can expand their studies to other Rab proteins (4, 7, 11, 22, 35, etc) as well as expression of the GTP-binding-defective, dominant negative forms of these Rabs to ascertain which G proteins are influencing the trafficking of their cargo protein. In addition to examining the Rabs themselves, downstream effectors as well as the consequences of expression or depletion of known GEFs and GAPs can be added into the experimental protocols.

REFERENCES

- Boshans RL, Szanto S, van Aelst L, & D'Souza-Schorey C (2000). ADP-ribosylation factor 6 regulates actin cytoskeleton remodeling in coordination with Rac1 and RhoA. *Mol Cell Biol*, 20(10), 3685–3694. [PubMed: 10779358]
- Caviston JP, Cohen LA, & Donaldson JG (2014). Arf1 and Arf6 promote ventral actin structures formed by acute activation of protein kinase C and Src. *Cytoskeleton (Hoboken)*, 71(6), 380–394. doi: 10.1002/cm.21181 [PubMed: 24916416]
- Conner SD, & Schmid SL (2003). Regulated portals of entry into the cell. *Nature*, 422(6927), 37–44. [PubMed: 12621426]
- Di Paolo G, & De Camilli P (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature*, 443(7112), 651–657. [PubMed: 17035995]
- Donaldson JG, & Jackson CL (2011). ARF family G proteins and their regulators: roles in membrane transport, development and disease. *Nature reviews. Molecular cell biology*, 12(6), 362–375. doi: 10.1038/nrm3117 [PubMed: 21587297]
- Eyster CA, Higginson JD, Huebner R, Porat-Shliom N, Weigert R, Wu WW, ... Donaldson JG (2009). Discovery of New Cargo Proteins that Enter Cells through Clathrin-Independent Endocytosis. *Traffic*, 10(5), 590–599. [PubMed: 19302270]
- Grant BD, & Donaldson JG (2009). Pathways and mechanisms of endocytic recycling. *Nat Rev Mol Cell Biol*, 10(9), 597–608. [PubMed: 19696797]
- Kouranti I, Sachse M, Arouche N, Goud B, & Echard A (2006). Rab35 regulates an endocytic recycling pathway essential for the terminal steps of cytokinesis. *Curr Biol*, 16(17), 1719–1725. [PubMed: 16950109]
- Kumari S, & Mayor S (2008). ARF1 is directly involved in dynamin-independent endocytosis. *Nat Cell Biol*, 10(1), 30–41. [PubMed: 18084285]
- Maxfield FR, & McGraw TE (2004). Endocytic recycling. *Nat Rev Mol Cell Biol*, 5(2), 121–132. [PubMed: 15040445]
- Mayor S, Parton RG, & Donaldson JG (2014). Clathrin-independent pathways of endocytosis. *Cold Spring Harb Perspect Biol*, 6(6), 93–112. doi: 10.1101/cshperspect.a016758
- Montagnac G, de Forges H, Smythe E, Gueudry C, Romao M, Salamero J, & Chavrier P (2011). Decoupling of Activation and Effector Binding Underlies ARF6 Priming of Fast Endocytic Recycling. *Curr Biol*. 10.1016/j.cub.2011.02.034
- Naslavsky N, Weigert R, & Donaldson JG (2003). Convergence of Non-clathrin- and Clathrin-derived Endosomes Involves Arf6 Inactivation and Changes in Phosphoinositides. *Mol Biol Cell*, 14(2), 417–431. [PubMed: 12589044]
- Piper RC, & Katzmann DJ (2007). Biogenesis and function of multivesicular bodies. *Annu Rev Cell Dev Biol*, 23, 519–547. [PubMed: 17506697]
- Radhakrishna H, & Donaldson JG (1997). ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *J Cell Biol*, 139(1), 49–61. [PubMed: 9314528]

- van der Sluijs P, Hull M, Webster P, Male P, Goud B, & Mellman I (1992). The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. *Cell*, 70(5), 729–740. [PubMed: 1516131]
- Wandinger-Ness A, & Zerial M (2014). Rab proteins and the compartmentalization of the endosomal system. *Cold Spring Harb Perspect Biol*, 6(11), a022616. doi: 10.1101/cshperspect.a022616 [PubMed: 25341920]
- Weigert R, Yeung AC, Li J, & Donaldson JG (2004). Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. *Mol Biol Cell*, 15(8), 3758–3770. [PubMed: 15181155]
- Zeigerer A, Gilleron J, Bogorad RL, Marsico G, Nonaka H, Seifert S, ... Zerial M (2012). Rab5 is necessary for the biogenesis of the endolysosomal system in vivo. *Nature*, 485(7399), 465–470. doi: 10.1038/nature11133 [PubMed: 22622570]

Figure 1.

Rab and Arf G proteins in endosomal trafficking.

A. Cartoon depicting entry of proteins via Clathrin-mediated Endocytosis (CME) (blue cargo) and Clathrin-independent Endocytosis (CIE) (red cargo). All incoming cargo reaches an Early Endosome that is associated with Rab5 and its effector EEA1 (early endosomal antigen 1). From there cargo moves on toward late endosomes and lysosomes for degradation, to the Golgi complex (TGN) or to recycling back to the cell surface. Approximate location and site of action for some of the different Rab proteins and Arf6 is indicated.

B. GTP-binding and GTP hydrolysis cycle for Rab (and Arf) G proteins is shown along with the GEFs and GAPs in promoting these two activities, respectively. Expression of the GTP-binding-defective (dominant negative) mutant forms can lock the Rab in the inactive, GDP-bound state; this state may also be observed upon siRNA depletion of the Rab in question. By contrast, expression of the GTP-hydrolysis resistant (constitutive active) mutant forms can lock the Rab in the active, GTP-bound state. The active state can promote activities (ie. Effector function) but in many cases, expression can impair function since the G protein functions best when it cycles between the two states.

Figure 2.

Effect of expression of active forms of Rab5 or Arf6 on cargo entering cells via CME and CIE. HeLa cells expressing GFP-Rab5 Q79L (A) or Arf6 Q67L (B) were allowed to internalize Alexa-594-Tfn and antibody-bound MHCI for 30 min prior to removal of surface antibody and then fixation. Cells were then labeled in the presence of saponin with primary rabbit antibodies to Arf6, followed by fluorescent goat-anti-rabbit and then goat-anti-mouse antibodies to detect the internalized antibodies to MHCI.