Published in final edited form as:

Semin Cell Dev Biol. 2011 February ; 22(1): 39–47. doi:10.1016/j.semcdb.2010.09.002.

ARF6-mediated endocytic recycling impacts cell movement, cell division and lipid homeostasis

Jill Kuglin Schweitzer*, Alanna E. Sedgwick*, and Crislyn D'Souza-Schorey

Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556-0369

Abstract

A wide range of cellular activities depends upon endocytic recycling. ARF6, a small molecular weight GTPase, regulates the processes of endocytosis and endocytic recycling in concert with various effector molecules and other small GTPases. This review highlights three critical processes that involve ARF6-mediated endosomal membrane trafficking—cell motility, cytokinesis, and cholesterol homeostasis. In each case, the function of ARF6-mediated trafficking varies—including localization of specific protein and lipid cargo, regulation of bulk membrane movement, and modulation of intracellular signaling. As described in this review, mis-regulation of endocytic traffic can result in human disease when it compromises the cell's ability to regulate cell movement and invasion, cell division, and lipid homeostasis.

Keywords

Endocytic recycling; ARF6; cell motility; cytokinesis; cholesterol trafficking

1. Introduction

ARF6 is a small molecular weight GTPase that localizes to the plasma membrane and endosomal compartments where it regulates endocytic membrane trafficking and actin remodeling (reviewed in [1]). Like other small GTPases, ARF6 cycles between its active-GTP-bound and inactive-GDP-bound conformations. Guanine nucleotide exchange factors, GEFs, activate ARF6 by mediating exchange of GTP for GDP, whereas GTPase activating proteins, GAPs, downregulate ARF6 activity through hydrolysis of GTP to GDP. As ARF6 cycles through its active and inactive conformations it facilitates ligand internalization at the cell surface, further trafficking along the endocytic pathway following internalization, and endosomal recycling and subsequent fusion of an endosomal membrane with the plasma membrane. Distinct effector molecules determine the result of ARF6 GTP/GDP cycling at discrete subcellular locations. At the cell surface ARF6-GTP can facilitate ligand internalization, whereas activation of ARF6 is also required for recycling of membrane back to the cell surface [1]. Recycling to the plasma membrane can occur directly from the sorting endosome, “fast” recycling, or through a pericentriolar endosomal recycling compartment (ERC), “slow” recycling. The longer-lived ERC seems to consist of a

© 2010 Elsevier Ltd. All rights reserved.

Correspondence should be addressed to: Crislyn D'Souza-Schorey, Department of Biological Sciences, University of Notre Dame, Box 369, Galvin Life Sciences Building, Notre Dame, IN 46556-0369. D'Souza-Schorey.1@nd.edu / cdsouzas@nd.edu.

*These authors contributed equally to the review.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

heterogeneous subset of endosomal populations, which may vary among cell types [1,2]. This review focuses on processes involving ARF6-mediated endosomal membrane trafficking.

The family of Rab GTPases also regulates intracellular vesicular trafficking, with distinct Rabs operating at various stages along the endocytic pathway [3]. Rab4, an early endosomal Rab, localizes to sorting endosomes and mediates “fast” endosomal recycling [2]. Some Rabs, namely Rab11 and Rab8, often functionally overlap with ARF6 in mediating endosomal recycling required for the cellular processes described in this review. Rab11 is a resident protein of the ERC and regulates transport from this compartment to various locations, including both the TGN and the plasma membrane. When Rab11 function is perturbed, exit out of the ERC is blocked [2]. Recently, a family of effector molecules, known as arfophilins or FIPs, have been shown to bind to both ARF6 and Rab11 and are involved in some trafficking events mediated by these GTPases [4,5]. The function of Rab8 has been more difficult to identify. In polarized cells, Rab8 appears to mediate the transport of newly synthesized proteins en route from the Golgi to the plasma membrane, using recycling endosomes as an intermediate [6,7]. Recent work in non-polarized cells indicates that Rab8 can regulate the trafficking of newly endocytosed ligands and that Rab8 and ARF6 may sometimes function in a common endocytic recycling pathway [8].

A wide range of cellular activities depends upon endocytic recycling. This review focuses on the ways by which endosomal trafficking directed by ARF6 and other small GTPases impacts cell motility, cell division, and cholesterol homeostasis (Table 1). During cell migration and invasion, ARF6-regulated endosomal traffic promotes loss of cell-cell contacts, changes in cell shape, and proteolysis of extracellular matrix. During cytokinesis, endosomal recycling mediated by ARF6 is required for the completion of abscission. In the regulation of cholesterol homeostasis, endosomal recycling facilitates normal cholesterol efflux and can alleviate aberrant cholesterol accumulation. In each case, the function of ARF6-mediated trafficking varies—including localization of specific protein and lipid cargo, regulation of bulk membrane movement, and modulation of intracellular signaling. As described in this review, mis-regulation of endocytic traffic can result in human disease when it compromises the cell’s ability to regulate cell movement and invasion, cell division, and lipid homeostasis.

2. ARF6 trafficking impacts cell adhesion and motility

2.1 ARF6 regulates epithelial cell-cell adhesion

ARF6-directed trafficking regulates multiple events that impinge upon cell migration and tumor cell invasion. These processes include endocytic membrane recycling, trafficking and targeting of adhesion molecules and proteases, and rearrangements of the actin cytoskeleton. Modulation of ARF6 activity influences the cell’s ability to perform these processes, and thus directly impacts migratory and invasive capacity.

It has been demonstrated that ARF6 regulates the internalization and trafficking of various adhesion molecules, including cadherin-based cell junctions in epithelial cells and integrin receptors[9]. The disassembly of cell-cell contacts is a critical event in an epithelial-to-mesenchymal transition (EMT), an important step in epithelial tumor progression that promotes acquisition of the migratory phenotype necessary for tumor cell invasion. In polarized epithelial cells E-cadherin is constitutively recycled between the basolateral plasma membrane and early endosomes, and studies have shown that ARF6 activity regulates this turnover. In Madin-Darby Canine Kidney (MDCK) cells it has been shown that ARF6-GTP recruits the nucleoside diphosphate kinase Nm23-H1 to the basolateral cell surface, initiating a down-regulation of Rac1 activity and promoting the clathrin-dependent

endocytosis of E-cadherin to the early endosome, both of which facilitate the disassembly of adherens junctions[10]. Upon Src-induced intercellular junction disassembly, internalized E-cadherin is targeted to the lysosome for degradation in an ARF6-dependent manner so that it cannot be recycled to the plasma membrane, thus ensuring that cell-cell contacts will not be reformed[11]. The TBC/RabGAP Armus has recently been shown to play a role in this process, bridging signaling between ARF6, Rac1, and Rab7. Upon interaction with active Rac1, Armus can locally inactivate Rab7 to facilitate lysosome biogenesis and the degradation of E-cadherin[12]. In contrast, dominant-negative ARF6, ARF6(T27N), blocks hepatocyte growth factor (HGF) and Src-induced cell scattering by preventing the internalization of E-cadherin into endosomal compartments, thus avoiding EMT and instead enhancing the epithelial phenotype[11,13]. In addition, recent work has demonstrated a downregulation of ARF6 activity established by a positive feedback loop between EphA2 and E-cadherin that enhances E-cadherin-based cell-cell contacts and the maturation of apical-basal polarity in MDCK cells[14]. The formation of stable adherens junctions during epithelial cell polarization depends upon the spatially regulated activation of ARF6, which is modulated by the formation of a complex between FRMD4A/GRSP-1/PAR3 and the ARF6-GEF cytohesin-1 at cell junctions [15].

The aforementioned effects of ARF6 on the internalization of E-cadherin and other cell surface receptors has been shown to impact epithelial glandular organization. Sustained ARF6 activation in three dimensional basement membrane cultures of epithelial cells has deleterious effects on glandular morphogenesis via its influence over the formation of the cyst and tubule-like structures, the primary building blocks of epithelial organs. Accumulation of growth factor receptors internalized by ARF6-regulated pathways in signaling endosomes leads to the formation of aberrant glandular morphologies, reminiscent of tumorigenic phenotypes seen *in vivo* [16]. Proper functioning of the ARF6 GDP/GTP cycle is also necessary for normal tubule development. Expression of constitutively activated ARF6 leads to the formation of an increased number of immature tubular structures, whereas expression of dominant-negative ARF6 blocks tubule extension in response to HGF stimulation [16].

2.2 ARF6 impacts integrin-dependent cell migration and cell shape

ARF6 activity also regulates the internalization and trafficking of integrins, which strongly impacts the cell's migratory and invasive capabilities. Actively migrating and invading cells display an increased requirement for the internalization and recycling of integrins from the retracting edge of the cell to the leading edge and sites of invasive structures. ARF6 regulates the intracellular trafficking and recycling of integrins, thereby impacting the cell's migratory and invasive capabilities. $\beta 1$ integrin has been shown to co-localize in an ARF6-regulated recycling endosome alongside internalized transferrin receptor, major histocompatibility class I molecules, and Rab11[17]. Expression of dominant-negative ARF6 abrogates the cell's ability to recycle this compartment to the plasma membrane[17]. In addition, it has been noted that an ARF6-GAP, ACAP1, is involved in the recycling of $\beta 1$ integrin, and the inhibition of ACAP1 or its kinase Akt inhibits integrin recycling and cell migration[18]. It has recently been discovered that the actin-modifying protein supervillin, which regulates multiple processes including invadopodia-mediated invasion and migration, also regulates the actin-dependent recycling of integrins through an ARF6-positive early/sorting endosomal recycling pathway[19]. The mechanisms controlling the ARF6-regulated delivery of endosomal membranes to the plasma membrane appear to be mediated by its activation of phospholipase D (PLD)[20], which is an intermediary in several ARF6 regulated functions, and by its interaction with the exocyst[21], the octameric complex involved in endocytic recycling and anchoring secretory vesicles to the plasma membrane[21,22]. Along with phosphatidic acid, a product of PLD activity which activates

type I phosphatidylinositol-4-phosphate 5-kinase (PIP5K), ARF6 can directly activate PIP5K for the production of phosphatidylinositol 4,5-bisphosphate (PIP₂), important for clathrin-dependent endocytosis[23], as well as other activities at the cell periphery including regulated exocytosis[24], regulation of actin dynamics[25-28], tethering of the exocyst to the plasma membrane[29-31], and membrane attachment to the cytoskeleton[32]. ARF6 has been shown to be necessary for the recycling of Rac1 from the recycling endosome to the plasma membrane upon integrin engagement, a step important for modulation of the actin cytoskeleton[33]. It has been suggested that the recruitment of Rac1 to the plasma membrane is due to the ARF6 regulated trafficking of lipid raft components to the cell surface, thus providing membrane binding sites for Rac1[33].

ARF6's ability to regulate remodeling of the actin cytoskeleton is central to its role in cell migration and tumor cell invasion, influencing the formation of many structures involved in motility and invasion, including lamellipodia[34], membrane ruffles [35,36], podosomes[37], and invadopodia[38,39]. ARF6 activity induces alterations in the actin cytoskeleton via its influences on Rac1, Rac1 effectors, and phospholipid modulation[1]. For example, a recent study showed that ARF6 can induce the trafficking of Rac1 to the plasma membrane and can regulate the activation of Rac1 via its activation of ERK [40], thereby impacting Rac1-mediated rearrangements of the actin cytoskeleton. The migratory ability of cells is also modulated by engagement with the extracellular matrix and requires ARF6-mediated activation of Rac1 for the formation of migratory structures. The formation of an α_4 integrin-paxillin-Arf-GAP complex at the trailing edge of the cell assists in directional migration by inhibiting ARF6 activity, thereby blocking adhesion-dependent Rac activation and the extension of lamellipodia[41]. Slit2-Robo signaling, important in cell migration, can block the ARF6 and Rac1 activation induced by integrin engagement, important in pathologies of endothelial cell protrusive activity [42].

2.3 ARF6 regulates tumor cell invasion via local and distal proteolysis

The ability to form functional invadopodia—membrane protrusions extending from the cell's adherent face which serve as nucleating sites for protease deposition—demonstrates a cell's acquisition of an invasive phenotype. Studies investigating the role of ARF6 in melanoma and breast tumor cell invasion have shown that endogenous ARF6 localizes to invadopodia[39] and that ARF6 activity is required for invadopodia formation. Expression of siRNA targeting ARF6 or a dominant-negative ARF6 construct abrogates the ability of tumor cells to form functional invadopodia[39,43]. This regulation is likely to be multifaceted. First, it has been suggested that ARF6's ability to regulate endosomal membrane recycling may control the amount of bulk membrane available for delivery to the leading edge of the cell [1,44,45], as well as for the formation of invadopodia, where membrane protrusions into the extracellular matrix must be initiated. Second, as mentioned above, ARF6 influences the deposition and distribution of membrane proteins, including the availability of integrins for adhesion to the matrix. The matrix engagement of $\alpha_6\beta_1$ integrin has been shown to be important for the initiation of invadopodia[46], and integrins have also been demonstrated to play an important role in the regulation of protease deposition from invading cells. $\alpha_v\beta_3$ integrin has been shown to be involved in the recruitment of matrix metalloproteinase -2 (MMP-2) to the cell surface[47], as well as in MMP-2 maturation[48], and integrin $\alpha_3\beta_1$ has been shown to be involved in docking the gelatinolytic enzyme seprase at invadopodia[49].

In addition to the local, pericellular proteolysis initiated at invadopodia, tumor cells can also increase their migratory potential through the release of “invasive”, protease-loaded microvesicles for focused, distal proteolysis, thereby facilitating cell migration by preparing a cleared path. A variety of cell types release microvesicles—a selectively enriched, heterogeneous population of vesicles pinched off from the plasma membrane for the transfer

of selected intracellular proteins, transmembrane receptors, and nucleic acids to neighboring and distant cells[50]. While microvesicles are present in bodily fluids under normal physiological conditions, their prevalence is increased in pathophysiological states such as cardiovascular disease, inflammation, diabetes, renal failure, and cancer[51]. Since the profile of microvesicle contents may correspond to disease progression, they are an active area of interest for the development of diagnostic and prognostic indicators of disease. Invasive tumor cells can exploit these vesicles for the transfer of oncogenic molecules and evasion of the immune system, as well as for the release of proteases in order to degrade the extracellular matrix and clear a path for cell migration. For example, it has been shown that aggressive glioma cells that express an oncogenic mutant form of the epidermal growth factor receptor (EGFR), known as EGFRvIII, can release microvesicles into the pericellular space and bloodstream for the transfer of this receptor to indolent cancer cells, facilitating their transformation to a more aggressive phenotype[52]. It has also been demonstrated that melanoma[53], ovarian [54][55], colorectal[56], and oral squamous cell carcinoma[57] cells are able to release microvesicles containing molecules such as Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), capable of inducing apoptosis in activated T cells, presumably as a mechanism of impeding an immune attack. The proteolytic capabilities of these microvesicles appears to be central to their role in tumor cell invasion, and a correlation has been demonstrated between the proteolytic contents of microvesicles and the invasiveness of the cells from which they are derived [58].

There are conserved signaling pathways involved in invadopodia formation and microvesicle release, with ARF6-regulated ERK activation being necessary for both[39,59]. It has been demonstrated that Rac1 activation lies downstream of ERK, necessary for invadopodia formation[60]. A recent study has shown that ARF6 activation is critical for the release of invasive microvesicles from the surface of melanoma cells[59]. ARF6-GTP activates PLD, which in turn recruits extracellular signal-regulated kinase (ERK) to the plasma membrane. ERK can then activate myosin light-chain kinase, which phosphorylates and activates the myosin light chain, facilitating actomyosin contraction at the neck of microvesicles and allowing their abscission from the plasma membrane[59]. Invasive melanoma cells expressing constitutively active ARF6, ARF6(Q67L), show a substantial increase in the number of microvesicles shed, and a corresponding increase in matrix degradation[59]. Released microvesicles contain functional proteases and actively degrade an extracellular gelatin matrix. Just as in invadopodia, it appears that engagement of integrin receptors is necessary for the interaction of microvesicles with the extracellular matrix (ECM), as the addition of a $\beta 1$ integrin-blocking antibody abrogates their ability to release their proteolytic contents and degrade the ECM[59]. On the other hand, melanoma cells expressing dominant-negative ARF6 exhibit a block in cell invasion and a plasma membrane studded with microvesicles that cannot be released due to a blockade of the abscission pathway[59].

The association between ARF6 and tumor cell invasion and metastasis has been well documented, and involves a complex array of downstream effectors[61]. For instance, studies have revealed a direct correlation between increased levels of ARF6 protein, as well as increased expression of the ARF6-GEF GEF100, with increased invasiveness in mammary cancers (reviewed in [62]). Recently ARF6 has been found to be required during glioma cell invasion in the brain [63]. Suppression of ARF6 expression inhibits glioma cell invasion both *in vitro* and in *ex vivo* brain slices and produces less invasive tumors *in vivo* in mice brains [63]. ARF6 stimulates glioma cell motility through the formation of a complex with Rac1 and the IQ-domain GTPase-activating protein 1, IQGAP1, for the formation of protrusions from the plasma membrane [63]. Furthermore, the brain exclusive ARF6-GEF, EFA6A, has been shown to promote cell motility and invasion through ARF6/MEK/ERK signaling cascades[64]. The influence of ARF6 on tumor cell invasion has also

been demonstrated in an *in vivo* study by Muralidharan-Chari *et al.* looking at melanoma tumor growth in nude mice subcutaneously injected with cells from the invasive LOX line expressing constitutively active ARF6 [60]. While showing decreased tumor size, the cells displayed a much higher level of invasiveness compared to the control, with a greater number of cells breaching the tumor border and infiltrating the surrounding tissue. Cells expressing the dominant-negative ARF6 showed a decrease in invasive capacity [60].

In sum, these findings demonstrate that ARF6-regulated intracellular trafficking can regulate a cell's migratory and invasive potential through several mechanisms, including the trafficking of adhesion molecules, invadopodia formation, microvesicle release, and rearrangements of the actin cytoskeleton. Its dynamic behavior and strong influence over these processes presents it as an important focus for future research in this area.

3. ARF6 directs endosomal recycling required for the completion of cytokinesis

3.1 Abscission requires endocytic recycling

As the final step of mitosis, cytokinesis completes cell division and produces two new daughter cells. During telophase the formation and action of the actomyosin contractile ring begins to divide the elongating cell into two daughter cells and the plasma membrane ingresses at a region called the cleavage furrow. After the cleavage furrow ingresses, forming a deep invagination, the two new daughter cells remain connected by a thin intercellular bridge. At the center of the intercellular bridge lies the midbody—a region dense with cytoskeletal, protein and membrane components. During late cytokinesis, the connection is eventually severed (abscission) and the midbody remnant is inherited by one of the daughter cells.

Successful cytokinesis is important for normal development and can cause disease when improperly regulated. Exquisite control of cytokinesis throughout development allows for changes in cell fate that lead to cell differentiation, such as asymmetric cell division and polyploidization [65]. For example, in the hematopoietic cell lineage, regulation of late cytokinesis events is crucial for the normal formation of polyploid megakaryocytes, from which platelets arise [66]. On the other hand, defects in cytokinesis appear to promote progression of at least one disease—cancer. Defects in cytokinetic events can promote proliferation potential when they disrupt asymmetric cell division needed for cellular differentiation or when a block in cytokinesis results in the abnormal formation of polyploid cells—a highly unstable genomic situation that has been shown to lead to tumor development [65].

Over the past decade, endocytic transport—which was once thought to be inactive during mitosis—has emerged as a key contributor to the process of cytokinesis (recently reviewed in [67,68]). Proteins that regulate endocytic traffic, in particular the ARF6 and Rab11 GTPases, direct endosome recycling required for successful cytokinesis. ARF6 was first identified to function during cytokinesis in mammalian cells where ARF6-GTP levels increase transiently during cytokinesis and constitutively active ARF6 localizes to the cleavage furrow and midbody [69]. Furthermore, expression of ARF6 mutants gives rise to binucleate cells and other cytokinesis defects, while depletion of ARF6 leads to cell division failure during late cytokinesis [69-71]. Although ARF6 is not an essential gene in *Drosophila*, it is required for spermatocyte cell division where it plays a role during cleavage furrow ingression and late cytokinesis [72]. During *Drosophila* spermatocyte division ARF6 mediates rapid membrane addition during cleavage furrow ingression [72]. In *C. elegans* embryos, Rab11 is thought to contribute to membrane addition at the cleavage furrow since suppression of Rab11 expression leads to cleavage furrow regression [73]. In both cases,

evidence points to recycling endosomes as a crucial source for increasing the surface area of the plasma membrane during cytokinesis. A study in mammalian cells shows that transferrin receptors (a marker for recycling endosomes) are substantially trapped in recycling endosomes during the early stages of mitosis when endosomal recycling is depressed [74]. During late telophase, endosomal recycling allows these vesicles to fuse with the plasma membrane and help to increase the surface area of the dividing cell [74]. Thus, recycling of endocytic vesicles may supply extra membrane needed to maintain proper plasma membrane surface area during cytokinesis.

In addition to the ingressing cleavage furrow, endocytic vesicles localize to the midbody region during late cytokinesis and abscission. When abscission is inhibited, mitosis fails—even at this late stage—and a binucleate cell results. Although the precise mechanism of abscission remains unclear, vesicular trafficking pathways play a key role. Both endocytic and secretory (Golgi-derived) vesicles traffic to the cleavage furrow and midbody area during cytokinesis [67,75]. Trafficking of vesicles to the midbody region likely serves many purposes during abscission, including the localization of machinery necessary for abscission as well as active participation in the membrane fusion and fission events required for abscission (recently reviewed in [76,77]).

3.2 ARF6 regulates endosome movement at the midbody

ARF6 appears to be important for targeting recycling endosomes to the cleavage furrow and midbody. Studies in mammalian cells as well as *Drosophila* and *C. elegans* have also identified a role for Rab11 during cytokinesis [73,78,79]. However, in *Drosophila* spermatocytes, the population of recycling endosomes at the central spindle during cytokinesis preferentially contain ARF6, regardless of whether they are Rab4- or Rab11-positive [72]. This finding suggests that ARF6 plays a specific, vital role in membrane trafficking at the furrow during cytokinesis. Both ARF6 and Rab11 also interact with FIP3 and FIP4, effector molecules that are required for late cytokinesis in mammalian cells [79,80]). In this case, loss of ARF6, but not Rab11, prevented the localization of FIP3-positive endosomes at the cleavage furrow and midbody area [79,80]). Depletion of ARF6 inhibits cytokinesis in mammalian cells and leads to a two-fold reduction in the amount of recycling endosomes at the midbody area during late cytokinesis [70,71]. Furthermore, by interacting with its effector JIP4, ARF6 appears to function as a switch to regulate the directionality of endosome movements out of the midbody area via kinesin-directed trafficking. Depletion of JIP4 delays abscission and reduces the number of endosomes at the midbody area [71]. The interaction of ARF6 with JIP4 promotes dynein/dynactin-directed trafficking of recycling endosomes out of the midbody area [71]. In this regard, ARF6 may regulate movement of endosomal vesicles out of the midbody area to facilitate a downregulation of cytokinesis and completion of abscission.

At the midbody recycling endosomes likely deliver membrane via interaction with the exocyst. The exocyst is a multi-protein complex involved in both secretory and recycling vesicle delivery to specific plasma membrane domains [22]. Several studies have shown that the exocyst is required for abscission [80-82]. GTPases interact with exocyst subunits to help regulate vesicle tethering and delivery via the exocyst. For example, ARF6 interacts directly with Sec10 during endosome recycling and Rab11 and FIP3 have also been shown to interact with exocyst subunits [21,80]. During cytokinesis, interaction with the exocyst may tether recycling endosomes to the midbody area and allow for delivery of membrane and/or protein cargo from the recycling endosomes.

3.3 Roles for varied types of recycling endosomes at the midbody

Multiple endocytic recycling pathways likely participate in the completion of cytokinesis. Evidence suggests that “fast” endosomal recycling Rab proteins are also important for cytokinesis. For example, Rab5 is important for cellularization, a process related to cytokinesis, during *Drosophila* development and for cytokinesis in cultured *Drosophila* S2 cells [78,83]. In addition, studies show that Rab4-positive recycling endosomes localize to the cleavage furrow in *Drosophila* spermatocytes and participate in membrane addition [72]. Rab35, a recently identified mammalian Rab protein, resides on clathrin-coated vesicles and may regulate the fast recycling of transferrin in mammalian cells [83]. Knockdown of Rab35 or use of dominant negative Rab35, Rab35(S22N), in mammalian cells delays the “fast” recycling of transferrin [83]. Loss of Rab35 activity also blocks cytokinesis in both *Drosophila* and mammalian cells. Specifically, Rab35 is required for stabilization of the intercellular bridge and abscission in mammalian cells [83]. Thus, recycling endosomes transiting through both the “fast” and “slow” recycling pathways likely play a role during cleavage furrow ingression and late cytokinesis.

Recycling endosomes may also mediate phospholipid changes at the cleavage furrow and midbody during cytokinesis. Local accumulation of PIP2 at the cleavage furrow has been observed in mammalian cells and other organisms and is important for proper cytokinesis [84]. The cleavage furrow undergoes changes in lipid composition, including an accumulation of PIP2. Such polarization of the lipid domains at the plasma membrane of the cleavage furrow is likely important for protein localization and signaling cascades. Specifically, accumulation of PIP2 at the cleavage furrow may facilitate interaction with pleckstrin homology (PH) domain- and basic domain-containing proteins, such as septins, that are important for cytokinesis and may be important for actin remodeling at the cleavage furrow [85,86]. The mechanism for PIP2 accumulation is not clear but may be mediated in part by GTPases. For example, cells expressing Rab35(S22N) display decreased PIP2 accumulation at the cleavage furrow/midbody region during cytokinesis [83]. Rab35 may direct the trafficking of recycling endocytic vesicles containing PIP5K, the enzyme that produces PIP2, to the cleavage furrow [83]. Also, ARF6 is a known activator of PIP5K activity [28], but it remains to be determined whether or not ARF6 facilitates PIP2 production at the cleavage furrow.

Emerging evidence suggests that proper chromosome segregation may be functionally linked to cytokinesis via recycling endosome traffic. Separase, a protein that regulates chromosome segregation during mitosis, also appears to function during cytokinesis. Work in *C. elegans* showed that during mitosis, separase localized to chromosomes, spread along the anaphase spindle, and later appeared on the cleavage furrow during cytokinesis [87]. Loss of separase inhibited cytokinesis directly and caused an accumulation of Rab11-positive vesicles at the cleavage furrow and midbody [87]. Separase seems to be important for regulation of endosomal traffic at the midbody after the targeting of Rab11-positive endosomes. The effect of separase knock-out on endocytic vesicles at the midbody did not result simply from defective chromosome separation [87]. RACK1, an adaptor protein that may anchor proteins at various locations within the cell, is essential for cytokinesis in *C. elegans* and regulates localization of Rab11-positive vesicles during mitosis [88]. The presence of lagging chromosomes during mitosis—a defect in chromosome segregation—increases in the absence of RACK1 [88]. Future studies are needed to determine whether chromosome segregation helps to regulate the timing of cytokinesis via communication through endocytic vesicles.

4. Cholesterol homeostasis relies on endosomal recycling mediated by ARF6 and other GTPases

4.1 ARF6-regulated endosomal recycling alleviates NPC1 disease phenotype

Cholesterol homeostasis relies on multiple feedback mechanisms that sense cholesterol levels and modulate uptake or efflux accordingly. Vesicular trafficking impacts the fate of cholesterol within the cell [89]. Previous work has shown that cholesterol traffics through the ARF6-regulated endosomal pathway [90]. The functional importance of maintaining cholesterol homeostasis is manifested by the variety of disorders, including neurodegenerative and cardiovascular diseases, which result when cholesterol levels cannot be regulated properly [91,92]. The following examples demonstrate how endocytosis and endocytic recycling, mediated by ARF6 and other GTPases, impact cholesterol homeostasis.

Within the cell, cholesterol enters the ERC, a major repository of free cholesterol, via endocytosis and non-vesicular mechanisms [93]. Cells acquire exogenous cholesterol primarily through low-density lipoprotein (LDL)-mediated endocytosis. The LDL particle transits along the endosomal network to the late endosome/lysosome (LE/LY), where it is hydrolyzed by acid lipase. Then, the free cholesterol moves to the endoplasmic reticulum (ER) or returns to the plasma membrane (PM) via vesicular and non-vesicular transport. In addition, LDL-derived free cholesterol may move to the plasma membrane without transiting to LE/LY since acid lipase is also present in early endocytic organelles [94]. In the neurodegenerative disease Niemann-Pick Type C (NPC), LDL-cholesterol as well as endogenous cholesterol and other lipids accumulate aberrantly in LE/LY compartments and efflux of cholesterol, particularly LDL-derived cholesterol, is greatly diminished [95-99]. This phenotype results from defects in intracellular lipid trafficking and not from changes in cholesterol uptake via LDL.

NPC arises from mutations in either NPC1 or NPC2, proteins that localize to the LE/LY at steady state and are believed to play a role in cholesterol and/or lipid transport [94]. Recent work on these proteins suggests a model whereby NPC1 and NPC2 work in tandem to mediate the transfer of free cholesterol out of the late endosome/lysosome. The model suggests that NPC2 transfers cholesterol from the limiting membrane of LE/LY to NPC1 and then NPC1 transfers it to a different acceptor, such as the membrane lipid transporter ATP-binding cassette transporter A1 (ABCA1) [100]. Modulation of late endosomal trafficking has been shown to reduce cholesterol accumulation in NPC1 mutant cells. Rabs 7 and 9 regulate transport to the late endosome and lysosome (Rab7) and from the late endosome to the Golgi (Rab9). Overexpression of either Rab7 or 9 restored normal lipid trafficking and reduced cholesterol accumulation in NPC cells [101-103]. In this way, stimulation of traffic out of the late endosome and lysosome by overexpression of Rabs7 and 9 may bypass the block caused by NPC1 mutations.

Emerging evidence suggests that defects in endocytic recycling contribute to cholesterol storage in NPC. Although cholesterol enters the ERC via vesicular and non-vesicular mechanisms, its exit relies on vesicular traffic ([104,105]. Endocytosis, *per se*, is generally not affected in NPC mutant cells, but endosomal recycling of lipid, protein, and fluid-phase markers to the PM is significantly suppressed [106-109]. In NPC1 fibroblasts rapid recycling from Rab4-positive early endosomes is inhibited [108]. In NPC1-deficient CHO cells, endosomal recycling of several protein and lipid components is inhibited and a portion of internalized transferrin receptor, a classic resident of the endosomal recycling pathway, mis-localized to the LE/LY [109]. Since inhibition of endosomal recycling in NPC cells may further enhance accumulation of cholesterol and other lipids, stimulation of endosomal recycling may increase cholesterol efflux out of the cell and alleviate the NPC phenotype.

Studies in our lab showed that expression of constitutively activated ARF6, ARF6(Q67L), reduced cholesterol accumulation in fibroblasts derived from NPC patients and in HeLa cells treated with the drug U18666A to mimic the NPC phenotype [110]. ARF6-GTP expression also increased cholesterol efflux from U18666A-treated HeLa cells and restored normal glycosphingolipid trafficking in NPC fibroblasts [110]. In addition, depletion of ARF6 led to increased intracellular cholesterol accumulation [110]. These findings demonstrate that stimulation of endosomal recycling can impact cholesterol homeostasis by increasing cholesterol efflux [110,111]. In further support of this hypothesis, Linder and colleagues found that overexpression of Rab8 increased cholesterol efflux in NPC fibroblasts, decreased cholesterol accumulation and restored normal lipid trafficking [112]. The Rab8 GTPase mediates traffic from the Golgi to the PM with recycling endosomes as an intermediate [7]. Knockdown of Rab8 expression also induced cholesterol accumulation in normal fibroblasts [112].

4.2 Cholesterol efflux depends upon endosomal recycling

Cholesterol removal, or efflux, from cells involves ATP-binding cassette protein A1 (ABCA1)-mediated transfer of cellular free cholesterol to apolipoprotein A-I (apoA-I), an extracellular acceptor in plasma, to ultimately form high-density lipoprotein (HDL) [113]. Formation of HDL allows excess cholesterol to be eliminated from peripheral cells and plasma HDL levels correlate inversely with coronary heart disease [113]. In Tangier Disease—caused by mutations in ABCA1—patients exhibit extremely low serum HDL levels, abnormal cholesterol accumulation in a variety of tissues, and frequently neurological symptoms [114]. It is unclear whether cholesterol efflux occurs exclusively at the cell surface or involves intracellular lipidation of ABCA1/apoA-I [113]. A recent study supports the importance of the internalization and recycling of ABCA1 and apoA-I for HDL formation [115]. This study demonstrates that ABCA1 is internalized via clathrin-dependent endocytosis in the presence or absence of apoA-I and that at least 30% of ABCA1 is recycled to the PM [115]. Under basal conditions, endocytosis and recycling of ABCA1 was not required for HDL formation. However, when cells accumulated intracellular cholesterol due to incubation with acetylated-LDL (ac-LDL), inhibition of ABCA1 internalization (and subsequent recycling) inhibited HDL formation [115]. When clathrin-dependent endocytosis was blocked, ABCA1 and apoA-I were trapped at the PM and could not undergo internalization and recycling necessary to mobilize endosomal cholesterol for HDL formation at the plasma membrane. These findings suggest that recycling of ABCA1 to the PM is required specifically to move cholesterol to the PM for HDL formation.

ABCA1-mediated cholesterol efflux to apoA-I also impacts the development of atherosclerosis. The initial stage of atherosclerosis, formation of a fatty streak, involves recruitment of macrophages and uptake of modified LDLs to produce cholesterol-laden macrophages called foam cells. Macrophages rely on cholesterol efflux to prevent their transformation into foam cells [116]. A recent study shows that endosomal recycling, mediated in part by Rab8, impacts cholesterol accumulation in foam cells [117]. In human atherosclerotic lesional macrophages, Rab8 was abundantly expressed and in isolated primary macrophages Rab8 protein levels were upregulated when intracellular cholesterol was increased. When Rab8 was overexpressed in primary human macrophages, ABCA1 protein levels increased, as did cholesterol efflux [117]. Depletion of Rab8 in primary macrophage cells led to a decrease in the amount of ABCA1 at the cell surface and a concomitant decrease in cholesterol efflux after prior loading with ac-LDL. These findings show that cholesterol efflux depends on endosomal recycling in macrophage foam cells. The inability of macrophages at a vascular lesion to efflux cholesterol promotes maintenance of the foam cell phenotype and progression of atherosclerosis.

5. Conclusion

Here we have highlighted the ways by which ARF6-directed membrane trafficking allows cells to become migratory and invade extracellular matrix, to complete the process of cytokinesis, and to modulate intracellular cholesterol levels. Although we focused our discussion on vesicular trafficking, the effects of ARF6 action are not limited to membrane traffic. Certainly, its ability to induce cortical actin rearrangements impacts cell migration and invasion as well as other cellular activities. The diverse cellular events described here all impact human disease. *In vivo* studies attest to the increased invasive nature of tumor cells displaying increased ARF6 activity. A lack of endosomal recycling during cytokinesis blocks cell division and produces binucleate cells—a condition known to promote cancer development. Finally, stimulation of endosomal recycling can alleviate aberrant cholesterol accumulation observed in NPC1 disease and in atherosclerotic lesions. Future studies need to address how modulation of endosomal recycling and other types of ARF6-directed traffic can be exploited to impact disease progression.

Acknowledgments

We apologize to those investigators whose work was not cited or indirectly cited, due to space constraints. We thank Christine Monteleon for critical reading of the manuscript. Research in the D'Souza-Schorey Laboratory on the topics described here has been supported in part by the American Cancer Society, the National Cancer Institute, the Leukemia and Lymphoma Society of America, the Walther Foundation and the Leda J. Sears Trust.

References

- [1]. D'Souza-Schorey C, Chavrier P. ARF proteins: roles in membrane traffic and beyond. *Nat.Rev.Mol.Cell Biol.* May; 2006 7(5):347–58. [PubMed: 16633337]
- [2]. Maxfield FR, McGraw TE. Endocytic recycling. *Nat.Rev.Mol.Cell Biol.* Feb; 2004 5(2):121–32. [PubMed: 15040445]
- [3]. Zerial M, McBride H. Rab proteins as membrane organizers. *Nat.Rev.Mol.Cell Biol.* Feb; 2001 2(2):107–17. [PubMed: 11252952]
- [4]. Shin OH, Couvillon AD, Exton JH. Arfophilin is a common target of both class II and class III ADP- ribosylation factors. *Biochemistry (N.Y.).* 2001; 40(36):10846–52.
- [5]. Hickson GR, Matheson J, Riggs B, Maier VH, Fielding AB, Prekeris R, et al. Arfophilins are dual Arf/Rab 11 binding proteins that regulate recycling endosome distribution and are related to *Drosophila* nuclear fallout. *Mol.Biol.Cell.* Jul; 2003 14(7):2908–20. [PubMed: 12857874]
- [6]. Ang AL, Taguchi T, Francis S, Folsch H, Murrells LJ, Pypaert M, et al. Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells. *J.Cell Biol.* Nov 8; 2004 167(3):531–43. [PubMed: 15534004]
- [7]. Henry L, Sheff DR. Rab8 regulates basolateral secretory, but not recycling, traffic at the recycling endosome. *Mol.Biol.Cell.* May; 2008 19(5):2059–68. [PubMed: 18287531]
- [8]. Hattula K, Furuholm J, Tikkanen J, Tanhuanpaa K, Laakkonen P, Peranen J. Characterization of the Rab8-specific membrane traffic route linked to protrusion formation. *J.Cell.Sci.* Dec 1; 2006 119(Pt 23):4866–77. [PubMed: 17105768]
- [9]. D'Souza-Schorey C. Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol.* Jan; 2005 15(1):19–26. [PubMed: 15653074]
- [10]. Palacios F, Schweitzer JK, Boshans RL, D'Souza-Schorey C. ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. *Nat.Cell Biol.* 2002; 4:929–36. [PubMed: 12447393]
- [11]. Palacios F, Tushir JS, Fujita Y, D'Souza-Schorey C. Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions. *Mol.Cell.Biol.* Jan; 2005 25(1):389–402. [PubMed: 15601859]

- [12]. Frasa MA, Maximiano FC, Smolarczyk K, Francis RE, Betson ME, Lozano E, et al. Armut is a Rac1 effector that inactivates Rab7 and regulates E-cadherin degradation. *Curr.Biol.* Feb 9; 2010 20(3):198–208. [PubMed: 20116244]
- [13]. Palacios F, Price L, Schweitzer J, Collard JG, D'Souza-Schorey C. An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *EMBO J.* 2001; 20(17):4973–86. [PubMed: 11532961]
- [14]. Miura K, Nam JM, Kojima C, Mochizuki N, Sabe H. EphA2 engages Glt1 to suppress Arf6 activity modulating epithelial cell-cell contacts. *Mol.Biol.Cell.* Apr; 2009 20(7):1949–59. [PubMed: 19193766]
- [15]. Ikenouchi J, Umeda M. FRMD4A regulates epithelial polarity by connecting Arf6 activation with the PAR complex. *Proc.Natl.Acad.Sci.U.S.A.* Jan 12; 2010 107(2):748–53. [PubMed: 20080746]
- [16]. Tushir JS, Clancy J, Warren A, Wrobel C, Brugge JS, D'Souza-Schorey C. Unregulated ARF6 Activation in Epithelial Cysts Generates Hyperactive Signaling Endosomes and Disrupts Morphogenesis. *Mol.Biol.Cell.* May 26.2010
- [17]. Powelka AM, Sun J, Li J, Gao M, Shaw LM, Sonnenberg A, et al. Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. *Traffic.* Jan; 2004 5(1):20–36. [PubMed: 14675422]
- [18]. Li J, Ballif BA, Powelka AM, Dai J, Gygi SP, Hsu VW. Phosphorylation of ACAP1 by Akt regulates the stimulation-dependent recycling of integrin beta1 to control cell migration. *Dev.Cell.* Nov; 2005 9(5):663–73. [PubMed: 16256741]
- [19]. Crowley JL, Smith TC, Fang Z, Takizawa N, Luna EJ. Supravillin reorganizes the actin cytoskeleton and increases invadopodial efficiency. *Mol.Biol.Cell.* Feb; 2009 20(3):948–62. [PubMed: 19109420]
- [20]. Jovanovic OA, Brown FD, Donaldson JG. An effector domain mutant of Arf6 implicates phospholipase D in endosomal membrane recycling. *Mol.Biol.Cell.* Jan; 2006 17(1):327–35. [PubMed: 16280360]
- [21]. Prigent M, Dubois T, Raposo G, Derrien V, Tenza D, Rosse C, et al. ARF6 controls post-endocytic recycling through its downstream exocyst complex effector. *J.Cell Biol.* Dec 8; 2003 163(5):1111–21. [PubMed: 14662749]
- [22]. He B, Guo W. The exocyst complex in polarized exocytosis. *Curr.Opin.Cell Biol.* Aug; 2009 21(4):537–42. [PubMed: 19473826]
- [23]. Paleotti O, Macia E, Luton F, Klein S, Partisani M, Chardin P, et al. The small G-protein Arf6GTP recruits the AP-2 adaptor complex to membranes. *J.Biol.Chem.* Jun 3; 2005 280(22):21661–6. [PubMed: 15802264]
- [24]. Micucci F, Capuano C, Marchetti E, Piccoli M, Frati L, Santoni A, et al. PI3KI-dependent signals are critical regulators of the cytolitic secretory pathway. *Blood.* Apr 15; 2008 111(8):4165–72. [PubMed: 18073347]
- [25]. Lassing I, Lindberg U. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature.* Apr 4-10; 1985 314(6010):472–4. [PubMed: 2984579]
- [26]. Janmey PA, Stossel TP. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. *Nature.* Jan 22-28; 1987 325(6102):362–4. [PubMed: 3027569]
- [27]. Fukami K, Furuhashi K, Inagaki M, Endo T, Hatano S, Takenawa T. Requirement of phosphatidylinositol 4,5-bisphosphate for alpha-actinin function. *Nature.* Sep 10; 1992 359(6391):150–2. [PubMed: 1326084]
- [28]. Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, et al. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell.* 1999; 99(5):521–32. [PubMed: 10589680]
- [29]. Zhang X, Orlando K, He B, Xi F, Zhang J, Zajac A, et al. Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. *J.Cell Biol.* Jan 14; 2008 180(1):145–58. [PubMed: 18195105]
- [30]. He B, Xi F, Zhang X, Zhang J, Guo W. Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. *EMBO J.* Sep 19; 2007 26(18):4053–65. [PubMed: 17717527]

- [31]. Liu J, Zuo X, Yue P, Guo W. Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. *Mol.Biol.Cell.* Nov; 2007 18(11):4483–92. [PubMed: 17761530]
- [32]. Nebt T, Oh SW, Luna EJ. Membrane cytoskeleton: PIP(2) pulls the strings. *Curr.Biol.* May 4; 2000 10(9):R351–4. [PubMed: 10801429]
- [33]. Balasubramanian N, Scott DW, Castle JD, Casanova JE, Schwartz MA. Arf6 and microtubules in adhesion-dependent trafficking of lipid rafts. *Nat.Cell Biol.* Dec; 2007 9(12):1381–91. [PubMed: 18026091]
- [34]. Santy LC, Casanova JE. Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J.Cell Biol.* Aug 6; 2001 154(3):599–610. [PubMed: 11481345]
- [35]. Zhang Q, Calafat J, Janssen H, Greenberg S. ARF6 is required for growth factor- and rac-mediated membrane ruffling in macrophages at a stage distal to rac membrane targeting. *Mol.Cell.Biol.* Dec; 1999 19(12):8158–68. [PubMed: 10567541]
- [36]. Radhakrishna H, Al-Awar O, Khachikian Z, Donaldson JG. ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. *J.Cell.Sci.* Mar; 1999 112(Pt 6):855–66. Pt 6. [PubMed: 10036235]
- [37]. Svensson HG, West MA, Mollahan P, Prescott AR, Zaru R, Watts C. A role for ARF6 in dendritic cell podosome formation and migration. *Eur.J.Immunol.* Mar; 2008 38(3):818–28. [PubMed: 18286566]
- [38]. Hashimoto S, Onodera Y, Hashimoto A, Tanaka M, Hamaguchi M, Yamada A, et al. Requirement for Arf6 in breast cancer invasive activities. *Proc.Natl.Acad.Sci.U.S.A.* Apr 27; 2004 101(17):6647–52. [PubMed: 15087504]
- [39]. Tague SE, Muralidharan V, D'Souza-Schorey C. ADP-ribosylation factor 6 regulates tumor cell invasion through the activation of the MEK/ERK signaling pathway. *Proc.Natl.Acad.Sci.U.S.A.* Jun 29; 2004 101(26):9671–6. [PubMed: 15210957]
- [40]. Tushir JS, D'Souza-Schorey C. ARF6-dependent activation of ERK and Rac1 modulates epithelial tubule development. *EMBO J.* Apr 4; 2007 26(7):1806–19. [PubMed: 17363898]
- [41]. Nishiya N, Kiosses WB, Han J, Ginsberg MH. An alpha4 integrin-paxillin-Arf-GAP complex restricts Rac activation to the leading edge of migrating cells. *Nat.Cell Biol.* Apr; 2005 7(4):343–52. [PubMed: 15793570]
- [42]. Jones CA, Nishiya N, London NR, Zhu W, Sorensen LK, Chan AC, et al. Slit2-Robo4 signalling promotes vascular stability by blocking Arf6 activity. *Nat.Cell Biol.* Nov; 2009 11(11):1325–31. [PubMed: 19855388]
- [43]. Hashimoto S, Onodera Y, Hashimoto A, Tanaka M, Hamaguchi M, Yamada A, et al. Requirement for Arf6 in breast cancer invasive activities. *Proc.Natl.Acad.Sci.U.S.A.* Apr 27; 2004 101(17):6647–52. [PubMed: 15087504]
- [44]. D'Souza-Schorey C, van Donselaar E, Hsu VW, Yang C, Stahl PD, Peters PJ. ARF6 targets recycling vesicles to the plasma membrane: insights from an ultrastructural investigation. *J.Cell Biol.* 1998; 140(3):603–16. [PubMed: 9456320]
- [45]. Song J, Khachikian Z, Radhakrishna H, Donaldson JG. Localization of endogenous ARF6 to sites of cortical actin rearrangement and involvement of ARF6 in cell spreading. *J.Cell.Sci.* Aug; 1998 111(Pt 15):2257–67. Pt 15. [PubMed: 9664047]
- [46]. Nakahara H, Mueller SC, Nomizu M, Yamada Y, Yeh Y, Chen WT. Activation of beta1 integrin signaling stimulates tyrosine phosphorylation of p190RhoGAP and membrane-protrusive activities at invadopodia. *J.Biol.Chem.* Jan 2; 1998 273(1):9–12. [PubMed: 9417037]
- [47]. Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, et al. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell.* May 31; 1996 85(5):683–93. [PubMed: 8646777]
- [48]. Deryugina EI, Ratnikov B, Monosov E, Postnova TI, DiScipio R, Smith JW, et al. MT1-MMP initiates activation of pro-MMP-2 and integrin alphavbeta3 promotes maturation of MMP-2 in breast carcinoma cells. *Exp.Cell Res.* Feb 15; 2001 263(2):209–23. [PubMed: 11161720]

- [49]. Mueller SC, Gherzi G, Akiyama SK, Sang QX, Howard L, Pineiro-Sanchez M, et al. A novel protease-docking function of integrin at invadopodia. *J.Biol.Chem.* Aug 27; 1999 274(35): 24947–52. [PubMed: 10455171]
- [50]. van Doormaal FF, Kleinjan A, Di Nisio M, Buller HR, Nieuwland R. Cell-derived microvesicles and cancer. *Neth.J.Med.* Jul-Aug;2009 67(7):266–73. [PubMed: 19687520]
- [51]. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. *Blood Rev.* May; 2007 21(3):157–71. [PubMed: 17118501]
- [52]. Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat.Cell Biol.* May; 2008 10(5):619–24. [PubMed: 18425114]
- [53]. Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J.Exp.Med.* May 20; 2002 195(10):1303–16. [PubMed: 12021310]
- [54]. Meng Y, Kang S, Fishman DA. Lysophosphatidic acid stimulates fas ligand microvesicle release from ovarian cancer cells. *Cancer Immunol.Immunother.* Aug; 2005 54(8):807–14. [PubMed: 15662527]
- [55]. Abrahams VM, Straszewski SL, Kamsteeg M, Hanczaruk B, Schwartz PE, Rutherford TJ, et al. Epithelial ovarian cancer cells secrete functional Fas ligand. *Cancer Res.* Sep 1; 2003 63(17): 5573–81. [PubMed: 14500397]
- [56]. Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology.* Jun; 2005 128(7):1796–804. [PubMed: 15940614]
- [57]. Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. *Clin.Cancer Res.* Feb 1; 2005 11(3):1010–20. [PubMed: 15709166]
- [58]. Ginestra A, La Placa MD, Saladino F, Cassara D, Nagase H, Vittorelli ML. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer Res.* Sep-Oct;1998 18(5A):3433–7. [PubMed: 9858920]
- [59]. Muralidharan-Chari V, Clancy J, Plou C, Romao M, Chavrier P, Raposo G, et al. ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr.Biol.* Dec 1; 2009 19(22):1875–85. [PubMed: 19896381]
- [60]. Muralidharan-Chari V, Hoover H, Clancy J, Schweitzer J, Suckow MA, Schroeder V, et al. ADP-ribosylation factor 6 regulates tumorigenic and invasive properties in vivo. *Cancer Res.* Mar 15; 2009 69(6):2201–9. [PubMed: 19276388]
- [61]. Premont RT, Schmalzigaug R. Metastasis: wherefore art thou? *Curr.Biol.* Dec 1; 2009 19(22):R1036–8. [PubMed: 19948141]
- [62]. Sabe H, Hashimoto S, Morishige M, Ogawa E, Hashimoto A, Nam JM, et al. The EGFR-GEP100-Arf6-AMAP1 signaling pathway specific to breast cancer invasion and metastasis. *Traffic.* Aug; 2009 10(8):982–93. [PubMed: 19416474]
- [63]. Hu B, Shi B, Jarzynka MJ, Yiin JJ, D'Souza-Schorey C, Cheng SY. ADP-ribosylation factor 6 regulates glioma cell invasion through the IQ-domain GTPase-activating protein 1-Rac1-mediated pathway. *Cancer Res.* Feb 1; 2009 69(3):794–801. [PubMed: 19155310]
- [64]. Li M, Ng SS, Wang J, Lai L, Leung SY, Franco M, et al. EFA6A enhances glioma cell invasion through ADP ribosylation factor 6/extracellular signal-regulated kinase signaling. *Cancer Res.* Feb 1; 2006 66(3):1583–90. [PubMed: 16452216]
- [65]. Li R. Cytokinesis in development and disease: variations on a common theme. *Cell Mol.Life Sci.* Dec; 2007 64(23):3044–58. [PubMed: 17882379]
- [66]. Geddis AE, Fox NE, Tkachenko E, Kaushansky K. Endomitotic megakaryocytes that form a bipolar spindle exhibit cleavage furrow ingression followed by furrow regression. *Cell.Cycle.* Feb 15; 2007 6(4):455–60. [PubMed: 17312391]
- [67]. Montagnac G, Echard A, Chavrier P. Endocytic traffic in animal cell cytokinesis. *Curr.Opin.Cell Biol.* Aug; 2008 20(4):454–61. [PubMed: 18472411]
- [68]. Ai E, Skop AR. Endosomal recycling regulation during cytokinesis. *Commun.Integr.Biol.* Sep; 2009 2(5):444–7. [PubMed: 19907714]

- [69]. Schweitzer JK, D'Souza-Schorey C. Localization and activation of the ARF6 GTPase during cleavage furrow ingression and cytokinesis. *J.Biol.Chem.* 2002; 27730:27210–6. [PubMed: 12016212]
- [70]. Schweitzer JK, D'Souza-Schorey C. A requirement for ARF6 during the completion of cytokinesis. *Exp.Cell Res.* Nov 15; 2005 311(1):74–83. [PubMed: 16181626]
- [71]. Montagnac G, Sibarita JB, Loubery S, Daviet L, Romao M, Raposo G, et al. ARF6 Interacts with JIP4 to control a motor switch mechanism regulating endosome traffic in cytokinesis. *Curr.Biol.* Feb 10; 2009 19(3):184–95. [PubMed: 19211056]
- [72]. Dyer N, Rebollo E, Dominguez P, Elkhatab N, Chavrier P, Daviet L, et al. Spermatocyte cytokinesis requires rapid membrane addition mediated by ARF6 on central spindle recycling endosomes. *Development.* Dec; 2007 134(24):4437–47. [PubMed: 18039970]
- [73]. Skop AR, Bergmann D, Mohler WA, White JG. Completion of cytokinesis in *C. elegans* requires a brefeldin A-sensitive membrane accumulation at the cleavage furrow apex. *Curr.Biol.* May 15; 2001 11(10):735–46. [PubMed: 11378383]
- [74]. Boucrot E, Kirchhausen T. Endosomal recycling controls plasma membrane area during mitosis. *Proc.Natl.Acad.Sci.U.S.A.* May 8; 2007 104(19):7939–44. [PubMed: 17483462]
- [75]. Goss JW, Toomre DK. Both daughter cells traffic and exocytose membrane at the cleavage furrow during mammalian cytokinesis. *J.Cell Biol.* Jun 30; 2008 181(7):1047–54. [PubMed: 18573914]
- [76]. Steigemann P, Gerlich DW. Cytokinetic abscission: cellular dynamics at the midbody. *Trends Cell Biol.* Nov; 2009 19(11):606–16. [PubMed: 19733077]
- [77]. Gould GW, Lippincott-Schwartz J. New roles for endosomes: from vesicular carriers to multi-purpose platforms. *Nat.Rev.Mol.Cell Biol.* Apr; 2009 10(4):287–92. [PubMed: 19277045]
- [78]. Pelissier A, Chauvin JP, Lecuit T. Trafficking through Rab11 endosomes is required for cellularization during *Drosophila* embryogenesis. *Curr.Biol.* Oct 28; 2003 13(21):1848–57. [PubMed: 14588240]
- [79]. Wilson GM, Fielding AB, Simon GC, Yu X, Andrews PD, Hames RS, et al. The FIP3-Rab11 protein complex regulates recycling endosome targeting to the cleavage furrow during late cytokinesis. *Mol.Biol.Cell.* Feb; 2005 16(2):849–60. [PubMed: 15601896]
- [80]. Fielding AB, Schonteich E, Matheson J, Wilson G, Yu X, Hickson GR, et al. Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. *EMBO J.* Oct 5; 2005 24(19):3389–99. [PubMed: 16148947]
- [81]. Gromley A, Yeaman C, Rosa J, Redick S, Chen CT, Mirabelle S, et al. Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. *Cell.* Oct 7; 2005 123(1):75–87. [PubMed: 16213214]
- [82]. Chen XW, Inoue M, Hsu SC, Saltiel AR. RalA-exocyst-dependent recycling endosome trafficking is required for the completion of cytokinesis. *J.Biol.Chem.* Dec 15; 2006 281(50):38609–16. [PubMed: 17028198]
- [83]. Kouranti I, Sachse M, Arouche N, Goud B, Echard A. Rab35 regulates an endocytic recycling pathway essential for the terminal steps of cytokinesis. *Curr.Biol.* Sep 5; 2006 16(17):1719–25. [PubMed: 16950109]
- [84]. Field SJ, Madson N, Kerr ML, Galbraith KA, Kennedy CE, Tahiliani M, et al. PtdIns(4,5)P2 functions at the cleavage furrow during cytokinesis. *Curr.Biol.* Aug 9; 2005 15(15):1407–12. [PubMed: 16085494]
- [85]. Janetopoulos C, Devreotes P. Phosphoinositide signaling plays a key role in cytokinesis. *J.Cell Biol.* Aug 14; 2006 174(4):485–90. [PubMed: 16908667]
- [86]. Echard A. Membrane traffic and polarization of lipid domains during cytokinesis. *Biochem.Soc.Trans.* Jun; 2008 36(Pt 3):395–9. [PubMed: 18481967]
- [87]. Bembenek JN, White JG, Zheng Y. A role for separase in the regulation of RAB-11-positive vesicles at the cleavage furrow and midbody. *Curr.Biol.* Feb 9; 2010 20(3):259–64. [PubMed: 20116245]
- [88]. Ai E, Poole DS, Skop AR. RACK-1 directs dynactin-dependent RAB-11 endosomal recycling during mitosis in *Caenorhabditis elegans*. *Mol.Biol.Cell.* Mar; 2009 20(6):1629–38. [PubMed: 19158384]

- [89]. Schmitz G, Grandl M. The molecular mechanisms of HDL and associated vesicular trafficking mechanisms to mediate cellular lipid homeostasis. *Arterioscler.Thromb.Vasc.Biol.* Nov; 2009 29(11):1718–22. [PubMed: 19846837]
- [90]. Naslavsky N, Weigert R, Donaldson JG. Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements. *Mol.Biol.Cell.* Aug; 2004 15(8):3542–52. [PubMed: 15146059]
- [91]. Daniels TF, Killinger KM, Michal JJ, Wright RW Jr, Jiang Z. Lipoproteins, cholesterol homeostasis and cardiac health. *Int.J.Biol.Sci.* Jun 29; 2009 5(5):474–88. [PubMed: 19584955]
- [92]. Liu JP, Tang Y, Zhou S, Toh BH, McLean C, Li H. Cholesterol involvement in the pathogenesis of neurodegenerative diseases. *Mol.Cell.Neurosci.* Jan; 2010 43(1):33–42. [PubMed: 19660552]
- [93]. Chang TY, Chang CC, Ohgami N, Yamauchi Y. Cholesterol sensing, trafficking, and esterification. *Annu.Rev.Cell Dev.Biol.* 2006; 22:129–57. [PubMed: 16753029]
- [94]. Liscum L, Sturley SL. Intracellular trafficking of Niemann-Pick C proteins 1 and 2: obligate components of subcellular lipid transport. *Biochim.Biophys.Acta.* Oct 11; 2004 1685(1-3):22–7. [PubMed: 15465423]
- [95]. Cruz JC, Chang TY. Fate of endogenously synthesized cholesterol in Niemann-Pick type C1 cells. *J.Biol.Chem.* Dec 29; 2000 275(52):41309–16. [PubMed: 11013265]
- [96]. Chen W, Sun Y, Welch C, Gorelik A, Leventhal AR, Tabas I, et al. Preferential ATP-binding cassette transporter A1-mediated cholesterol efflux from late endosomes/lysosomes. *J.Biol.Chem.* Nov 23; 2001 276(47):43564–9. [PubMed: 11559713]
- [97]. Choi HY, Karten B, Chan T, Vance JE, Greer WL, Heidenreich RA, et al. Impaired ABCA1-dependent lipid efflux and hypoalphalipoproteinemia in human Niemann-Pick type C disease. *J.Biol.Chem.* Aug 29; 2003 278(35):32569–77. [PubMed: 12813037]
- [98]. Ikonen E, Holtta-Vuori M. Cellular pathology of Niemann-Pick type C disease. *Semin.Cell Dev.Biol.* Aug; 2004 15(4):445–54. [PubMed: 15207834]
- [99]. Wang MD, Franklin V, Sundaram M, Kiss RS, Ho K, Gallant M, et al. Differential regulation of ATP binding cassette protein A1 expression and ApoA-I lipidation by Niemann-Pick type C1 in murine hepatocytes and macrophages. *J.Biol.Chem.* Aug 3; 2007 282(31):22525–33. [PubMed: 17553802]
- [100]. Subramanian K, Balch WE. NPC1/NPC2 function as a tag team duo to mobilize cholesterol. *Proc.Natl.Acad.Sci.U.S.A.* Oct 7; 2008 105(40):15223–4. [PubMed: 18832164]
- [101]. Choudhury A, Dominguez M, Puri V, Sharma DK, Narita K, Wheatley CL, et al. Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J.Clin.Invest.* Jun; 2002 109(12):1541–50. [PubMed: 12070301]
- [102]. Walter M, Davies JP, Ioannou YA. Telomerase immortalization upregulates Rab9 expression and restores LDL cholesterol egress from Niemann-Pick C1 late endosomes. *J.Lipid Res.* Feb; 2003 44(2):243–53. [PubMed: 12576506]
- [103]. Narita K, Choudhury A, Dobrenis K, Sharma DK, Holicky EL, Marks DL, et al. Protein transduction of Rab9 in Niemann-Pick C cells reduces cholesterol storage. *FASEB J.* Sep; 2005 19(11):1558–60. [PubMed: 15972801]
- [104]. Mukherjee S, Maxfield FR. Lipid and cholesterol trafficking in NPC. *Biochim.Biophys.Acta.* Oct 11; 2004 1685(1-3):28–37. [PubMed: 15465424]
- [105]. Hao M, Lin SX, Karylowski OJ, Wustner D, McGraw TE, Maxfield FR. Vesicular and non-vesicular sterol transport in living cells. The endocytic recycling compartment is a major sterol storage organelle. *J.Biol.Chem.* Jan 4; 2002 277(1):609–17. [PubMed: 11682487]
- [106]. Pagano RE. Endocytic trafficking of glycosphingolipids in sphingolipid storage diseases. *Philos.Trans.R.Soc.Lond., B, Biol.Sci.* May 29; 2003 358(1433):885–91. [PubMed: 12803922]
- [107]. Wastney ME, Pentchev PG, Neufeld EB. Fitting a mathematical model to biological data: intracellular trafficking in Niemann-Pick C disease. *Adv.Exp.Med.Biol.* 2003; 537:63–75. [PubMed: 14995028]
- [108]. Choudhury A, Sharma DK, Marks DL, Pagano RE. Elevated endosomal cholesterol levels in Niemann-Pick cells inhibit rab4 and perturb membrane recycling. *Mol.Biol.Cell.* Oct; 2004 15(10):4500–11. [PubMed: 15292453]

- [109]. Pipalia NH, Hao M, Mukherjee S, Maxfield FR. Sterol, protein and lipid trafficking in Chinese hamster ovary cells with Niemann-Pick type C1 defect. *Traffic*. Feb; 2007 8(2):130–41. [PubMed: 17156101]
- [110]. Schweitzer JK, Pietrini SD, D'Souza-Schorey C. ARF6-mediated endosome recycling reverses lipid accumulation defects in Niemann-Pick Type C disease. *PLoS One*. 2009; 4(4):e5193. [PubMed: 19365558]
- [111]. Schweitzer JK, Krivda JP, D'Souza-Schorey C. Neurodegeneration in Niemann-Pick Type C disease and Huntington's disease: impact of defects in membrane trafficking. *Curr. Drug Targets*. Jul; 2009 10(7):653–65. [PubMed: 19601769]
- [112]. Linder MD, Uronen RL, Holtta-Vuori M, van der Sluijs P, Peranen J, Ikonen E. Rab8-dependent recycling promotes endosomal cholesterol removal in normal and sphingolipidosis cells. *Mol. Biol. Cell*. Jan; 2007 18(1):47–56. [PubMed: 17050734]
- [113]. Cavelier C, Lorenzi I, Rohrer L, von Eckardstein A. Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1. *Biochim. Biophys. Acta*. Jul; 2006 1761(7):655–66. [PubMed: 16798073]
- [114]. Kolovou GD, Mikhailidis DP, Anagnostopoulou KK, Daskalopoulou SS, Cokkinos DV. Tangier disease four decades of research: a reflection of the importance of HDL. *Curr. Med. Chem*. 2006; 13(7):771–82. [PubMed: 16611066]
- [115]. Azuma Y, Takada M, Shin HW, Kioka N, Nakayama K, Ueda K. Retroendocytosis pathway of ABCA1/apoA-I contributes to HDL formation. *Genes Cells*. Feb; 2009 14(2):191–204. [PubMed: 19170766]
- [116]. Pennings M, Meurs I, Ye D, Out R, Hoekstra M, Van Berkel TJ, et al. Regulation of cholesterol homeostasis in macrophages and consequences for atherosclerotic lesion development. *FEBS Lett*. Oct 9; 2006 580(23):5588–96. [PubMed: 16935283]
- [117]. Linder MD, Mayranpaa MI, Peranen J, Pietila TE, Pietiainen VM, Uronen RL, et al. Rab8 regulates ABCA1 cell surface expression and facilitates cholesterol efflux in primary human macrophages. *Arterioscler. Thromb. Vasc. Biol*. Jun; 2009 29(6):883–8. [PubMed: 19304576]

Table 1
Endocytic recycling contributes to critical cellular activities

Endocytic recycling mediated by ARF6 and other small molecular weight GTPases contributes to the processes of cell motility and invasion, cytokinesis, and cholesterol homeostasis in various ways. Specific events, or steps, involved in these processes are listed here as well as the GTPases and downstream effector proteins, as described in this review.

CELL MOTILITY AND INVASION		
Event	GTPases and downstream effectors	Reference(s)
Adherens Junction disassembly via E-cadherin internalization and degradation	<i>Upregulation of ARF6 activity via:</i> Nm23-H1, Rac1	9-11,13
	Armus, Rac1, Rab7	12
Adherens Junction stabilization	<i>Downregulation of ARF6 activity via:</i> EphA2 and E-cadherin	14
	FRMD4A/GRSP-1/PAR3 and cytohesin-1	15
Disruption of glandular epithelial organization	Sustained ARF6 activation, growth factors in signaling endosomes	16
β 1 integrin recycling needed for cell migration	ARF6, Rab11	17
	ARF6, Akt, ACAP1	18
	ARF6, supervillin	19
Remodeling of actin cytoskeleton during cell migration and invasion	ARF6, ERK, Rac1, phospholipid changes	1, 33
	a4 integrin-paxillin-Arf-GAP (to inhibit ARF6)	41
	Slit2-Robo (to inhibit ARF6)	42
Invadopodia formation and activity	ARF6, integrins, proteases	39,43,46-49
Protease-loaded microvesicle release	ARF6, PLD, ERK, Rac1 and MLCK	59, 60
Glioma cell invasion (<i>in vitro</i> and <i>in vivo</i>)	ARF6, Rac1, and IQGAP1 ARF6-GEF	63
	EFA6A and ARF6, MEK, ERK	64
CYTOKINESIS		
Event	GTPases and downstream effectors	Reference(s)
Membrane addition during cleavage furrow ingression	ARF6	72
	Rab11	73, 78
	recycling endosomes (transferrin-positive)	74
	Rab4	72
Abscission	ARF6	69-72
	Rab11, FIP3, FIP4	79,80
	Rab35	83
Endosome localization at cleavage furrow or midbody	ARF6	71,72,79,80
	Rab11	78
Endosome movement into and out of midbody area	ARF6, JIP4	71
Tethering endosomes at midbody	exocyst and its interaction with ARF6 and Rab11	21,80-82
Localized PIP2 accumulation	Rab35	83
CHOLESTEROL HOMEOSTASIS		

	CELL MOTILITY AND INVASION	
Event	GTPases and downstream effectors	Reference(s)
Event	GTPases and downstream effectors	Reference(s)
Cholesterol uptake/endocytosis	ARF6	90
Cholesterol efflux to relieve NPC phenotype	ARF6	110
	Rab8	112
ABCA1-mediated cholesterol efflux for HDL formation	clathrin-dependent endocytosis and endosomal recycling (Rab5, Rab4)	115
Cholesterol efflux in foam cells	Rab8	117

Abbreviations: ACAP1, arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein; ERK, extracellular signal-regulated kinase; MLCK, myosin light-chain kinase; MEK, MAPK/ERK kinase; FIP3/FIP4, family of Rab11-interacting proteins 3 and 4; JIP4, c-Jun-N-terminal-kinase interacting protein 4