

Review

The early endosome: a busy sorting station for proteins at the crossroads

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Summary. Endocytosis marks the entry of internalized receptors into the complex network of endocytic trafficking pathways. Endocytic vesicles are rapidly targeted to a distinct membrane-bound endocytic organelle referred to as the early endosome. Despite the existence of numerous internalization routes, early endosomes (EE) serve as a focal point of the endocytic pathway. Sorting events initiated at this compartment determine the subsequent fate of internalized proteins and lipids, destining them either for recycling to the plasma membrane, degradation in lysosomes or delivery to the *trans*-Golgi network. Sorting of endocytic cargo to the latter compartments is accomplished through the formation of distinct microdomains within early endosomes, through the coordinate recruitment and assembly of the sorting machinery. An elaborate network of interactions between endocytic regulatory proteins ensures synchronized sorting of cargo to microdomains followed by morphological changes at the early endosomal membranes. Consequently, the cargo targeted either for recycling back to the plasma membrane, or for retrograde transport to the *trans*-Golgi network, localizes to newly-formed tubular membranes. With a high ratio of membrane surface to luminal volume, these tubules effectively concentrate the recycling cargo, ensuring efficient transport out of the EE. Conversely, receptors sorted for degradation cluster at the flat clathrin lattices involved in invaginations of the limiting membrane, associating with newly formed intraluminal vesicles. In this review we will discuss the characteristics of early endosomes, their role in the regulation of endocytic transport, and their aberrant function in a variety of diseases.

Key words: Endocytosis, Golgi, Traffic, Disease

Introduction

The internalization of receptors, lipid membranes, and extracellular fluid at the plasma membrane (PM) of higher eukaryotes is carried out by various endocytic trafficking pathways. These include the well-characterized clathrin-dependent pathway as well as clathrin-independent pathways. Among the latter pathways is the phagocytic uptake of large particles, pinocytosis, raft-mediated endocytosis and Arf6-dependent internalization (Mellman, 1996; Mukherjee et al., 1997; Conner and Schmid, 2003; Maxfield and McGraw, 2004). Many of the cargo molecules internalized through these diverse pathways are either recycled back to the PM, or undergo degradation in late endosomes and lysosomes. Accordingly, it is not surprising to find that eukaryotic cells have evolved mechanisms to converge these differentially internalized proteins to a common sorting station from where the cargo destined for degradation and recycling are separated. This common sorting station is known as the early endosome (EE) or the sorting endosome (Mayor et al., 1993).

The EE is defined as the first endocytic compartment to accept incoming cargo internalized from the PM and is a highly dynamic structure with a high propensity to undergo homotypic fusion (Gruenberg et al., 1989). The EE appears as an elaborately complex pleomorphic compartment in most cell types. It is composed of regions of thin tubular extensions (~60 nm diameter) and large vesicles (~400 nm diameter) that have membrane invaginations and give rise to a multi-vesicular appearance (Gruenberg, 2001). These morphologically distinct EE sub-domains are thought to be functionally important. For example, proteins targeted for recycling may cluster within primarily tubular membranes, whereas the multi-vesicular elements are usually involved in sorting to the degradative pathway (Mellman, 1996). Vesicles generated from these two distinct membrane regions exhibit differences in their

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acidification properties. The pH decreases from 6.2 to ~5.5 in the lumen of multivesicular bodies, whereas it increases in the tubular recycling endosomes to ~6.5 (Mayor et al., 1993; Mellman, 1996). It is interesting to note that such morphologically, chemically and functionally distinct compartments undergo biogenesis from the same EE.

As mentioned above, the primary function of the EE is the sorting of internalized cargo to different intracellular destinations. Within minutes of internalization, the separation of various endocytic cargo molecules occurs within the EE. Although most of the internalized ligands are degraded, their receptors are often recycled back to the cell surface for additional rounds of ligand-binding and internalization (Dunn et al., 1989). For instance, proteins such as transferrin receptor or low density lipoprotein receptor are slated for recycling from the EE, whereas low density lipoprotein itself and the ligand-bound epidermal growth factor receptor (EGFR) are transported to late endosomes/lysosomes for degradation (Dautry-Varsat et al., 1983; Herbst et al., 1994). The moderately acidic pH of the EE lumen (~6.3-6.8) allows for the dissociation of ligand from its receptor. Due to the greater membrane surface area of the tubular domain of the EE, receptors (and other membrane proteins) are concentrated in this region. On the other hand, soluble ligands accumulate in the vesicular regions that ultimately fuse with late endocytic organelles. The tubular endosomes return most of the membrane lipids and proteins back to the cell surface and some of the fluid occupied by the membrane lumen (Mellman, 1996). The critical functional role of the EE necessitates that any sorting occurring in this organelle is a precise and highly controlled process.

The protein and lipid content of EE has been extensively analyzed through a variety of biochemical and fluorescence-based techniques. Below we discuss some of the key protein and lipid components of EE that facilitate the sorting of internalized cargo from this compartment to different intracellular destinations.

Proteins at the early endosome

A critical group of endocytic regulators are the Ras-associated binding (Rab) proteins. Rabs are small GTP-binding proteins that cycle between a GTP-bound active state and a GDP-bound inactive state. In their active state they localize to intracellular membranes where they can interact with and recruit a variety of proteins known as Rab effectors (Grosshans et al., 2006). Indeed, it is through binding to specialized effectors that Rab proteins carry out multiple roles in endocytic trafficking events, including vesicle tethering, fusion, budding and motility (Pfeffer, 2001; Zerial and McBride, 2001; Deneka and van der Sluijs, 2002; Grosshans et al., 2006). The Rab proteins primarily localized to the EE include Rab5 and Rab4, which regulate distinct early endocytic events (Gorvel et al., 1991; Bucci et al., 1992; van der Sluijs et al., 1992a; Daro et al., 1996). In

addition to these two Rab proteins, some of the other less well-characterized Rabs at the EE include Rab10 (Babbey et al., 2006), Rab14 (Junutula et al., 2004; Proikas-Cezanne et al., 2006), Rab21 (Simpson et al., 2004) and Rab22 (Mesa et al., 2001, 2005; Kauppi et al., 2002; Magadan et al., 2006).

Rab5 is the most extensively analyzed Rab of the early endocytic pathway (Gorvel et al., 1991; Bucci et al., 1992; Barbieri et al., 1996; Zerial and McBride, 2001; Grosshans et al., 2006). It regulates entry of the cargo from the plasma membrane to the early endosomes, generation of phosphatidylinositol-3-phosphate (PtdIns(3)P) lipid which is enriched on EE (Christoforidis et al., 1999; Murray et al., 2002), homotypic fusion (Gorvel et al., 1991) and the motility of EE on actin and microtubules tracks (Nielsen et al., 1999; Pal et al., 2006). In addition, it also functions in activating signaling pathways from EE (Benmerah, 2004; Miaczynska et al., 2004; Schenck et al., 2008). The active or GTP-bound Rab5 is generated at the EE by the action of Rabex-5 which acts as a guanine exchange factor (GEF) for Rab5 (Horiuchi et al., 1997). This in turn recruits Rabaptin-5, which binds to the GTP-bound Rab5 and recruits Rabex-5 in a positive feedback loop to maintain and stabilize GTP-bound Rab5 on EE (Stenmark et al., 1995; Lippe et al., 2001). These transient but high levels of active Rab5 are sufficient to recruit effector proteins to the EE where they can carry out their specialized functions in trafficking and sorting (Grosshans et al., 2006). Several key Rab5 effectors and their proposed functions at the EE are outlined below:

PtdIns(3)P-kinase/hVPS34/p150 (VPS34) is thought to be one of the first Rab5 effector proteins to be recruited to the EE (Christoforidis et al., 1999). As suggested by its name, its primary role is to generate PtdIns(3)P, which is the most abundant phosphoinositide in the EE membrane. The concomitant presence of GTP-Rab5 and PtdIns(3)P acts as a signal to recruit effector proteins such as EEA1 (Lawe et al., 2000, 2002; Pfeffer, 2001), Rabenosyn-5 (Nielsen et al., 2000) and/or Sorting nexins (SNXs) (Cozier et al., 2002) that bind to both active Rab5 and PtdIns(3)P simultaneously. Treatment of cells with inhibitors of PtdIns(3)P-kinase such as Wortmannin (Hazeki, 1995) and LY294002 (Vlahos et al., 1994) prevents localization of PtdIns(3)P-binding proteins (such as EEA1) to the EE (Patki et al., 1997) and impairs trafficking from this compartment (van Dam et al., 2002), thus highlighting a crucial role for PtdIns(3)P-kinase in proper functioning of this organelle.

Early endosomal antigen-1 (EEA1) is a well-characterized effector of Rab5 and one of the most widely used markers for EE due to its specific localization to this compartment (Simonsen et al., 1998). EEA1, in coordination with members of the SNARE family, is essential for EE fusion *in vivo* (Mills et al., 1998, 1999). EEA1 can directly interact with syntaxin6 and syntaxin13, two target-SNAREs (t-SNAREs) that

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mediate homotypic fusion of EE (McBride et al., 1999; Simonsen et al., 1999; Mills et al., 2001). EEA1 also contains a FYVE domain that specifically binds to PtdIns(3)P (Raiborg et al., 2001). Thus dual binding of EEA1 to GTP-Rab5 and PtdIns(3)P modulates its localization to EE membranes (Lawe et al., 2002).

Rabenosyn-5 is another FYVE-domain-containing Rab5 effector that localizes to EE (Nielsen et al., 2000). Rabenosyn-5 is regarded as a dual Rab5 and Rab4 effector as it can bind to both of these Rabs in their active states, thus linking these Rab domains at the EE where it can coordinate sorting activity with the fast recycling of cargo directly from EE (de Renzis et al., 2002). In addition, Rabenosyn-5 also interacts with EHD1, a member of the recently discovered C-terminal EHD family of proteins which regulates recycling of cargo from the perinuclear endocytic recycling compartment (ERC) back to the plasma membrane (Naslavsky et al., 2004). Thus Rabenosyn-5 might serve as the Rab5 effector linking sorting events at the EE to recycling of cargo back to the plasma membrane either directly from EE or via the ERC.

APPL1 and APPL2 (Adaptor protein containing PH domain, PTB domain and Leucine zipper motif) are two recently identified homologous Rab5 effectors that are associated with a subset of Rab5-positive EE (Miaczynska et al., 2004). APPL proteins are specialized Rab5 effectors as their primary role appears to be in signaling rather than trafficking. APPL proteins translocate from EE to the nucleus upon stimulation with EGF. Once inside the nucleus, these proteins can interact with the nucleosome remodeling and histone deacetylase multiprotein complex NuRD/MeCP1 which regulates chromatin structure and gene expression (Miaczynska et al., 2004). Accordingly, APPL proteins are essential for cell proliferation and more importantly their binding to Rab5 is crucial for their function in mitogenesis (Miaczynska et al., 2004). A recent study by Zerial and colleagues suggests that APPL proteins also regulate the activity of protein kinase B (Akt) and influence its substrate specificity in a Zebra fish model system (Schenck et al., 2008). Moreover, the endosomal localization of APPL is essential for the activation of Akt. These studies suggest that the EE is not only a sorting station, but a subset of these organelles actively plays a role in signal transduction events initiated at the plasma membrane.

In addition to Rab5 and its effectors, **Rab4** is also localized to EE (Van Der Sluijs et al., 1991), as well as to Rab11-positive recycling endosomes (Trischler et al., 1999). Unlike Rab5, which potentiates fusion events of primary endocytic vesicles with EE and homotypic fusion of EE (Gorvel et al., 1991), Rab4 regulates the exit of constitutive recycling cargo from EE directly back to the plasma membrane as well as directing the sorting of cargo to the ERC (van der Sluijs et al., 1992a;

Sheff et al., 1999a). Rab4 also regulates specialized trafficking events such as transport of the glucose transporter GLUT4 upon insulin stimulation from an intracellular storage compartment to the plasma membrane in adipocytes and muscle cells (Aledo et al., 1995; Cormont et al., 1996; Vollenweider et al., 1997). Consistent with this role, Rab4 is also involved in the budding of synaptic-like microvesicles (SLMVs) from EE in PC12 neuroendocrine cells (de Wit et al., 2001). These SLMVs are similar to synaptic vesicles (SVs) of neurons and are involved in endo- and exocytosis of neurotransmitters, suggesting a potential role for Rab4 in SV trafficking as well. Rab4 has also been implicated in the regulation of an essential step in receptor-mediated antigen processing in B lymphocytes (Lazzarino et al., 1998), in regulated exocytosis of dense granules in platelets (Shirakawa et al., 2000), and α -amylase exocytosis in pancreatic cells (Ohnishi et al., 1999). Thus Rab4 is involved in regulating constitutive as well as specialized trafficking/recycling pathways.

SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are highly conserved membrane proteins that mediate fusion events in membrane trafficking pathways (Bennett, 1995; Sollner, 1995). The hallmark of SNARE proteins is the presence of a SNARE motif: an evolutionarily conserved domain of 60-70 amino acids arranged in heptad repeats (Fasshauer, 2003). Monomeric SNAREs are unstructured; however, when appropriate sets of SNAREs interact with one another, their SNARE motifs spontaneously associate to form helical core complexes of extraordinary stability (Chen and Scheller, 2001). The center of the helical core is highly hydrophobic except for a central '0' layer that contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residue. Consequently, the SNARE motifs contributing these residues are classified as Qa-, Qb-, Qc- and R-SNAREs. A functional SNARE complex that drives membrane fusion is formed by four SNAREs, with one member each of Qa-, Qb-, Qc- and R-SNARE subfamilies (Fasshauer et al., 1998; Bock et al., 2001). According to the 'zipper' model of SNARE function, during vesicle fusion, the SNARE motifs of interacting SNAREs assemble into a parallel four-helix bundle. This is initiated by 'zipping' of the membrane-distal N-termini to the membrane-proximal carboxyl-termini, forming a stable complex that can bring the membranes in close apposition and thus overcome the energy barrier to drive fusion of the lipid bilayers (Hanson et al., 1997; Lin and Scheller, 1997).

The key SNARE machinery components known to be involved in homotypic fusion of EE are syntaxin13 (Qa), vti1a (Vps10p tail interactor 1, Qb), syntaxin6 (Qc), and VAMP4 (R) (Brandhorst et al., 2006; Zwilling et al., 2007). The specificity of SNARE fusion is likely determined by upstream targeting proteins that interact and recruit these SNAREs onto specific membranes (Brandhorst et al., 2006). In the case of homotypic EE

fusion, the Rab5 effector and tethering protein EEA1 can directly bind and thus recruit Syntaxin13 and Syntaxin6 to the EE (McBride et al., 1999; Simonsen et al., 1999). Interestingly it has been observed that the PtdIns(3)P-kinase inhibitor, Wortmannin blocks endosome fusion (Patki et al., 1997). This observation conforms to a model whereby EEA1 fails to be recruited to EE upon treatment with Wortmannin, inhibiting targeting of the SNARE machinery to the EE and thus preventing fusion. Subsequent to membrane fusion, SNARE recycling occurs upon dissociation of the helical bundle, which is mediated by the AAA+ (ATPases Associated with diverse cellular Activities) protein, NSF (N-ethylmaleimide-sensitive factor) (Mayer et al., 1996; Hanson and Whiteheart, 2005).

Motor proteins at the early endosome

The movement of intracellular membrane-bound structures in the cell is powered by motor proteins. For EE in particular, the activity of Rab5 and its effector protein PtdIns(3)P-kinase is required for motility on microtubule tracks (Nielsen et al., 1999). One of the motor protein complexes involved in the movement of vesicles budding from EE to the ERC or towards lysosomes is the dynein-dynactin complex which moves along microtubule tracks from the cell periphery towards the microtubule-organizing center (MTOC) (Valetti et al., 1999). A recent study by Buss and colleagues has also shown that the actin-based motor Myosin VI and its interacting partner LMTK2 (lemur tyrosine kinase 2) are involved in cargo delivery from EE to ERC (Chibalina et al., 2007). The movement of EE in the opposite direction (from center of the cell to the periphery) is mediated by the plus-end directed kinesin-3 motor KIF16B, which transports vesicles budding from EE in a process regulated by the small GTPase Rab5 and its effector, the PtdIns(3)P-kinase/hVPS34 (Hoepfner et al., 2005). KIF16B localizes to EE *in vivo* owing to the presence of a PX domain which mediates its recruitment to PtdIns(3)P-enriched EE membranes. Overexpression of KIF16B causes the relocation of EE to the cell periphery, while expression of dominant negative KIF16B or RNAi-mediated knockdown causes EE to cluster in the perinuclear region (Hoepfner et al., 2005). A recent study by Zerial and colleagues has identified a novel effector of Rab5, Huntingtin-associated protein-40 (HAP-40) that regulates the dynamics of EE (Pal et al., 2006). HAP-40 can interact with Huntingtin (Htt) protein and recruit it to EE in a GTP-Rab5 dependent manner. Htt binds to microtubules and is implicated in clathrin-mediated endocytosis. Overexpression of HAP-40 sequesters Htt, causing detachment of EE from microtubules and a preferential association with actin filaments, thus reducing long-range movement on microtubule tracks to short-range movements along actin microfilaments (Pal et al., 2006). Surprisingly, HAP-40 is highly up-regulated in fibroblasts and brain tissue

from human patients affected by Huntington's disease (HD) (see Table I). In cells from diseased patients, the shift of EE from microtubules to actin filaments could be observed, causing a severe decrease in mobility *in vivo* (Pal et al., 2008).

C-terminal EHD proteins at the early endosome

In addition to the well-characterized Rab proteins, another family of proteins recently implicated in endocytic trafficking is the C-terminal Eps15 Homology Domain (EHD) proteins (Naslavsky and Caplan, 2005; Grant and Caplan, 2008). Four highly homologous C-terminal EHD proteins (EHD1-EHD4) have been identified in mammals (Mintz et al., 1999; Pohl et al., 2000). EHD1 is the best characterized of these EHD proteins, and has been implicated in regulating the recycling of various receptors including transferrin (Tf) (Lin et al., 2001; Naslavsky et al., 2004), major histocompatibility complex class I (Caplan et al., 2002) and β 1 integrin receptors (Jovic et al., 2007). Recent studies suggest that both EHD3 and EHD4 are involved in trafficking from EE to the ERC (Naslavsky et al., 2006; Sharma et al., 2008). Depletion of EHD3 or EHD4 prevents the delivery of TfR and other cargo to the ERC, and it accumulates in enlarged EE structures (Fig. 1). In addition, EHD4-depletion also induces enhanced activation of Rab5 (Sharma et al., 2008). EHD proteins also interact with Rab effectors such as Rabenosyn-5 at the EE (Naslavsky et al., 2004) which provides insight as to the mode by which EHD3 and EHD4 can coordinate their function with Rab4 and Rab5 in EE trafficking.

Lipids at the early endosome

Phospholipids play a crucial role in membrane trafficking pathways by recruiting proteins that contain specific domains capable of binding to them (Simonsen et al., 2001; Gruenberg, 2003). Localized synthesis of lipids at distinct cellular locations ensures selective recruitment of endocytic proteins at these sites. The EE compartment and multivesicular bodies in the cell are enriched in PtdIns(3)P (Gillooly et al., 2000). In its active form, the small GTPase Rab5 recruits PtdIns(3)P-kinase/Vps34 to EE membranes where it mediates localized synthesis of PtdIns(3)P (Christoforidis et al., 1999). This in turn can recruit PtdIns(3)P-binding proteins containing a FYVE domain (such as EEA1, Rabenosyn-5 or Hrs), or a PX domain (such as SNX1) (Nielsen et al., 2000; Raiborg et al., 2001; Cozier et al., 2002). Recruitment of EEA1 can assemble the SNAREs Syntaxin13 and Syntaxin6 at EE membranes thus enabling EE fusion (Mills et al., 2001). On the other hand, recruitment of Hrs to EE is essential for the sorting of EGFR into multivesicular bodies, which fuse with lysosomes and leads to EGFR degradation (Morino et al., 2004). In addition, recruitment of sorting nexins to

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the EE is essential for transport of cargo to the Golgi (Carlton et al., 2004). Generation of PtdIns(3)P is thus essential for regulating almost all of the EE sorting events. Interestingly, a study by Petiot et al., has shown that the process of bulk transport (fluid-phase) from EE to late endosomes does not require the activity of PtdIns(3)P-kinase and consequently is insensitive to Wortmanin treatment (Petiot et al., 2003).

One of the proteins recruited by PtdIns(3)P at the EE is PIKfyve, the only known enzyme that synthesizes PtdIns(3,5)P₂ from PtdIns(3)P in mammalian cells (Cabezas et al., 2006). PIKfyve is recruited to EE via its FYVE domain and is sensitive to Wortmanin treatment (Sbrissa et al., 2002). Once localized to the EE, this kinase can phosphorylate PtdIns(3)P to PtdIns(3,5)P₂, a phosphoinositide that is enriched on late endosomal membranes (Ikononov et al., 2002). Knockdown of PIKfyve or expression of a kinase-dead mutant induces EE enlargement and profound cytoplasmic vacuolation

(Ikononov et al., 2001; Rutherford et al., 2006). This is most likely caused due to a lack of PtdIns(3,5)P₂ synthesis which promotes endosomal fission and an increase in the cellular pool of PtdIns(3)P which promotes homotypic EE fusion (Shisheva, 2008). PIKfyve dysfunction causes a delay in fluid-phase transport to late endosomes and also impairs EE-to-TGN retrograde traffic (Ikononov et al., 2003; Rutherford et al., 2006). However, receptor sorting to lysosomes or recycling to the PM remains unaffected (Ikononov et al., 2003). These data suggest a role for PIKfyve in maintaining EE morphology by regulating PI3P levels and the exit of cargo from early endosomes to late endosomes.

In addition to PtdIns(3)P conversion to PtdIns(3,5)P₂, a second mechanism to downregulate PtdIns(3)P at the EE is by the action of the lipid phosphatase MTM1, a member of the myotubularin family of proteins (Blondeau et al., 2000; Begley and

Table 1. Aberrant EE function contributes to multiple diseases.

Disease name	Clinical features	Mutated protein that causes the disease	Effect on early endosome	References
Alzheimer's disease (AD)	Personality changes, gradual decrease in memory function and intellectual ability	?	Increased endocytic uptake and fusion leading to enlargement of EE and increase of Amyloid Precursor Protein (APP) and Apolipoprotein E (ApoE) processing	(Cataldo et al., 1997; Tate and Mathews, 2006)
Down syndrome	Mental retardation, congenital malformations of heart and gastrointestinal tract, etc.	Amyloid Precursor Protein (APP)	Increased APP gene dosage leads to increased endocytic uptake, and enlargement of both EE and late endosomes as well as increased soluble beta-Amyloid peptides in neurons and fibroblasts	(Cataldo et al., 2003, 2008)
Huntington's disease	Dementia (commonly associated with seizures), rigidity, and progressive chorea	Huntingtin (Htt)	Expansion of CAG repeat in the Htt gene leads to upregulation of Htt-associated protein 40 (HAP40), decrease in transferrin uptake and impaired EE motility	(Pal et al., 2006)
Lowe's syndrome	Renal Fanconi Syndrome, mental retardation, congenital cataracts	Oculocerebrorenal syndrome of Lowe (OCRL1)	Mutations in OCRL1 impair its co-localization with APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif 1) and binding with Rab5 on early endosomes. Depletion of OCRL1 changes the rate of transport between early endosomes and Trans-Golgi Network (TGN)	(Choudhury et al., 2005; Erdmann et al., 2007)
Myotubular myopathy	Facial and eye muscle weakness, low muscle tone, and respiratory failure	Myotubularin	Impaired vesicular transport from EE	(Dowling et al., 2008)
Hermansky-Pudlak syndrome types 2, 3, 5, 6, 7 and 8	Albinism, bleeding, and innate immune defects	BLOC-(Biogenesis of lysosomal-related complex)1, BLOC-2, and Adaptor Protein (AP)-3	Surface accumulations of CD63 and tyrosinase-related protein 1 (Tyrp1) due to missorting of Tyrp1 from EE to the plasma membrane	(Di Pietro et al., 2006)

Defective early endosomal proteins can lead to a variety of diseases. Most of these diseases are neurological-related illnesses, likely resulting from impaired neuron function in rapid signal transmission from one neuron to another, which requires intact synaptic vesicle transport. Among the additional diseases related to genes encoding early endosomal proteins are schizophrenia and bipolar disorder; the dysbindin subunit of BLOC1 (encoded by gene DTNBP1) is a candidate gene for schizophrenia (Di Pietro et al., 2006), whereas a rare variant in the promoter region of hVps34 gene is related to bipolar disorder and schizophrenia (Di Pietro et al., 2006; Nicot and Laporte, 2008). Early endosomal proteins also play an important role in the replication of bacteria and viruses. Perskvist and co-workers reported that Rab5 binds tightly to phagosomes containing *Mycobacterium tuberculosis*, which causes delayed phagolysosome maturation (Perskvist et al., 2002). The SpoE (*Streptococcus pneumoniae*) protein of Salmonella recruits non-prenylated Rab5 on Salmonella-containing phagosomes to induce fusion of early endosomes, thus preventing phagolysosome maturation by acting as a Rab5-specific guanine exchange factor (Mukherjee et al., 2001). Rab5 and other early endosomal proteins have also been reported to associate with Hepatitis C virus (HCV) NS4B (one of the non-structural proteins) (Stone et al., 2007).

Dixon, 2005). MTM1 primarily localizes to Rab5 and EEA1 positive EE (Cao et al., 2007). In this recent study, it was demonstrated that MTM1 is the major phosphatase regulating PtdIns(3)P levels, since overexpression of MTM1 causes depletion of PtdIns(3)P from EE and multivesicular bodies (Cao et al., 2007). Conversely, depletion of MTM1 leads to increased PtdIns(3)P levels in the cell (Cao et al., 2008). Interestingly, in either scenario EGFR sorting to the late endocytic pathway is impaired (Cao et al., 2008). This highlights the importance of maintaining the overall balance of PtdIns(3)P levels in the cell for proper sorting to occur and suggests that a highly coordinated mechanism of PtdIns(3)P-kinases and phosphatases must exist at the EE. Indeed, MTM1 directly interacts with the Vps15/Vps34 PtdIns(3)P-kinase complex and this interaction might regulate the activity of the phosphatase at the EE (Cao et al., 2007).

Regulation of endocytic trafficking at the early endosome

Sorting for degradation

The sorting of receptors from EE to the degradation

pathway depends upon the presence of sorting signals. Signaling receptors en route to lysosomes, including the epidermal growth factor receptor (EGFR), contain cytosolic domains necessary for recruitment of sorting machinery. Mono-ubiquitination of one or more lysine residues in receptor tyrosine kinases is critical for this process (Haglund et al., 2003; Mosesson et al., 2003). Initial recognition of the mono-ubiquitinated cargo on the EE is carried out by the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) via its ubiquitin interacting motif (UIM) (Raiborg et al., 2002). Hrs also associates with clathrin found in flat clathrin lattices through its C-terminal clathrin box motif (Raiborg et al., 2002). Flat clathrin lattices are present only on select EE membranes involved in the sorting of cargo to the degradation pathway (Raiborg et al., 2002). Binding of Hrs to ubiquitin and clathrin thus results in formation of a unique sorting microdomain that actively concentrates and sorts cargo into intraluminal vesicles and away from the tubule-forming, actively recycling membranes. However, the weak binding of Hrs to ubiquitin necessitates the presence of additional ubiquitin-binding proteins. Two additional UIM-containing proteins, Eps15 and signal transducing adaptor molecule 2 (STAM2), stabilize the association with ubiquitinated

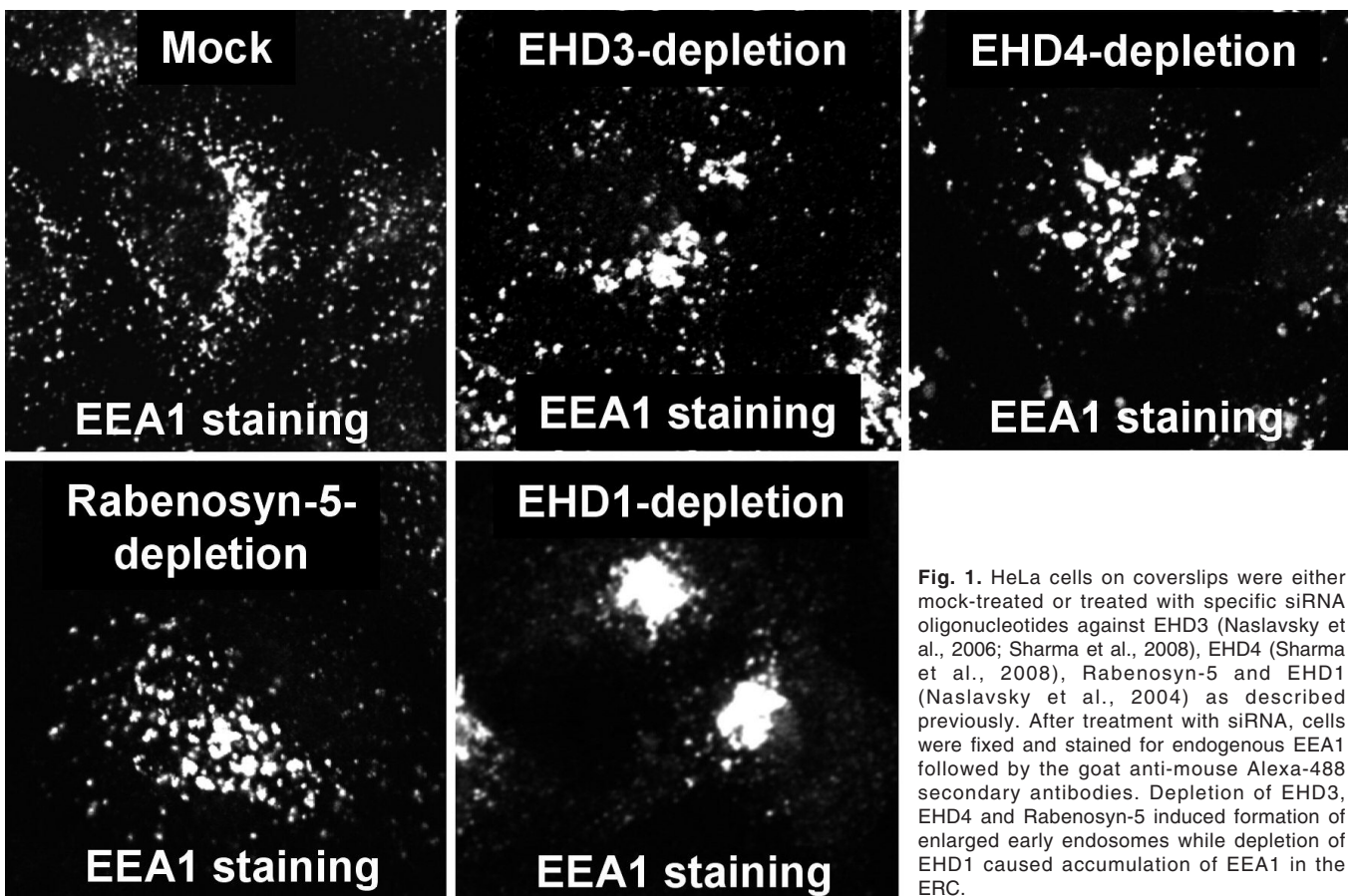


Fig. 1. HeLa cells on coverslips were either mock-treated or treated with specific siRNA oligonucleotides against EHD3 (Naslavsky et al., 2006; Sharma et al., 2008), EHD4 (Sharma et al., 2008), Rabenosyn-5 and EHD1 (Naslavsky et al., 2004) as described previously. After treatment with siRNA, cells were fixed and stained for endogenous EEA1 followed by the goat anti-mouse Alexa-488 secondary antibodies. Depletion of EHD3, EHD4 and Rabenosyn-5 induced formation of enlarged early endosomes while depletion of EHD1 caused accumulation of EEA1 in the ERC.

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cargo and in concert with Hrs form a functional sorting complex (Bache et al., 2003b). Furthermore, Hrs interacts with the Tsg101 subunit of the endosomal sorting complex required for transport (ESCRT-I), thus recruiting it to late endosomal membranes (Bache et al., 2003a). Subsequently ESCRT-II complex binds to ESCRT-I, followed by recruitment of ESCRT-III, leading to the onset of membrane invagination and formation of multivesicular bodies (MVBs) in a yet poorly understood process (Babst et al., 2002a,b; Bonifacino and Hurley, 2008). Ultimately, fusion of such MVBs with the lysosomal vesicles carrying proteolytic enzymes results in degradation of EGFR and other sorted receptors.

Sorting for Recycling

Upon delivery to the EE, internalized receptors can be sorted into one of at least two distinct recycling pathways (Fig. 2). Efficient sorting of receptors undergoing recycling is achieved through extensive tubulation of the EE membranes in a process of "geometry-based sorting" (Maxfield and McGraw, 2004), whereby receptors that are sorted into the newly-formed tubular membranes recycle back to the PM. In parallel with tubulation, Rab4 targets receptors to the ERC where they proceed via a 'slow-recycling' route back to the plasma membrane (Deneka et al., 2003). However, Rab4 can also sort receptors directly back to the plasma membrane in a "fast recycling" route (van der Sluijs et al., 1992b; Sheff et al., 1999b). Classical

kinetic studies measuring the recycling rates of the canonical recycling receptor, the transferrin receptor (TfR), confirmed the existence of two routes with differing kinetics; the 'fast' recycling pathway ($t_{1/2}=5$ min) and the 'slow' recycling pathway ($t_{1/2}=15-30$ min) (Hopkins and Trowbridge, 1983).

For transferrin to undergo fast recycling, it needs to be quickly sorted away from an early endosomal Rab5-containing microdomain into a Rab4 microdomain on the same vesicle. Alternatively, transferrin is delivered to a Rab4/Rab11-containing ERC in a trafficking step that exhibits significantly slower kinetic rates (Sonnichsen et al., 2000). Perturbation of the Rab4 GTPase activity in GDP-locked dominant-negative mutants results in reduced exit of TfR from the EEs and subsequent accumulation of TfR (McCaffrey et al., 2001). However, although the constitutively-active Rab4 mutant induces a very modest increase in TfR recycling, the overexpression of this mutant led to a dramatic increase in TfR localization to the tubular membranes. An identical effect has been observed upon wild-type Rab4 overexpression, implying the role of Rab4 in budding and formation of recycling tubules at the level of EE.

Rab4 exerts its endocytic regulatory role through interaction with various effector proteins, including the previously discussed Rabenosyn-5 and Rabaptin-5/Rabex-5 complex (Vitale et al., 1998; de Renzis et al., 2002; Mattera and Bonifacino, 2008). Immunodepletion of Rabaptin-5/Rabex-5 in permeabilized, cytosol-free cells resulted in stimulation of the recycling vesicle formation *in vitro*, while the addition of purified

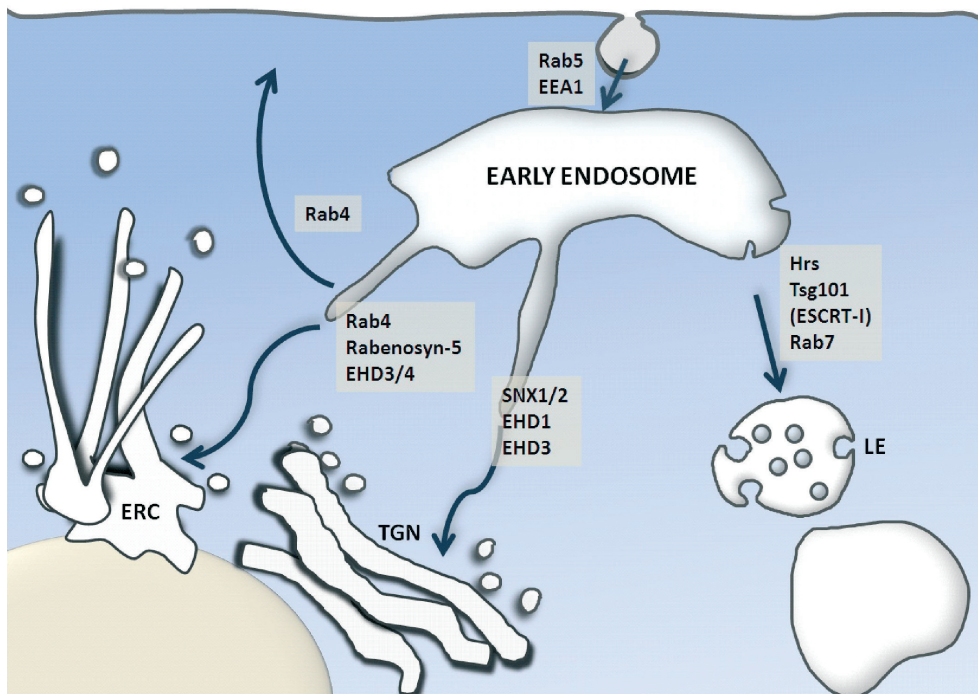


Fig. 2. Endocytic sorting at the EE. Delivery of internalized cargo molecules to EE is mediated by GTP-Rab5 and its effector EEA1 in a series of homotypic fusion events. Cargo can then either be sorted for recycling back to the plasma membrane in a fast (Rab4-mediated) or slow (Rab4, Rabenosyn-5, EHD3 and/or EHD4-mediated) pathway. Alternatively, in the case of mono-ubiquitinated cargo, proteins are sorted for lysosomal degradation (via Hrs, Tsg101, ESCRT complex, Rab7) or to the Golgi (via SNX1/2 and EHD1/3).

Rabaptin-5/Rabex-5 had the opposite effect (Pagano et al., 2004). Although both proteins also serve as Rab5 effectors, the process of recycling vesicle formation appears to be only Rab4-mediated since the immunodepletion of Rab5 itself had no effect. Interestingly, a study by Deneka et al. previously identified Rabaptin-5 as a linker protein between Rab4 and the γ 1-adaptin subunit of AP-1 (Deneka et al., 2003). Therefore the binding of Rabaptin-5/Rabex-5 to AP-1 could potentially have a regulatory role in preventing the association of clathrin with AP-1 γ 1-adaptin, resulting in inhibition of coat formation and budding at the EE membranes. Subsequent formation of the recycling vesicles, however, likely requires the presence of a nexus protein linking Rab4 on EE to proteins at the ERC. Rabenosyn-5, a multivalent Rab4/Rab5 effector, may act as such a linker between Rab4 and EHD1, an endocytic regulator found primarily on the tubulo-vesicular ERC membranes (Naslavsky et al., 2004). Depletion of Rabenosyn-5 leads to accumulation of transferrin in an EE compartment containing EEA1 and Rab5, indicating its role in mediating the delivery of recycling cargo to the ERC. On the other hand, depletion of EHD1 results in cargo accumulation at the ERC (Naslavsky et al., 2004).

A fork in the road

Until recently, most models proposed that receptors lacking a degradative or biosynthetic targeting signal would by default undergo recycling due to lipid-based sorting and subsequent tubulation (reviewed by (Gruenberg, 2001; Maxfield and McGraw, 2004)). Accordingly, truncation of the TfR cytosolic tail did not affect its recycling rate (Johnson et al., 1993), while a more recent study showed that the fusion of a mono-ubiquitin moiety to the TfR cytosolic tail induced its sorting into the degradative pathway, further supporting the precedence of degradative sorting signals over the signal-independent recycling events (Raiborg et al., 2002). However, a recent finding has challenged this model. The Arf6 GTPase-activating protein, ACAP1, serves as a sorting molecule involved in direct binding to two phenylalanine-containing clusters on the TfR (Dai et al., 2004). Mutation of these critical residues abolishes the ACAP1 association and delays TfR recycling, although the lack of a complete block in recycling indicates the existence of an alternative recycling route(s) (Dai et al., 2004). Other receptors also contain sorting motifs that can be transplanted to a non-recycling receptor and induce recycling (Pattni and Stenmark, 2006). Although there is no single consensus motif, phosphorylation of serine 411 of the β 2-adrenergic receptor recruits the EBP50 (ezrin-radixin-moesin (ERM)-binding phosphoprotein-50) to regulate recycling of this receptor (Cao et al., 1999). Hrs also binds to the cytosolic tail of both the β 2-adrenergic and μ -opioid receptors and directs them to a recycling pathway, thus exerting a critical role in receptor recycling independent

of its function as a lysosomal sorting determinant (Hanyaloglu et al., 2005).

Sorting to the TGN

In addition to its role as a diverging point between the degradative and recycling pathways, the EE also serves as a junction between the endocytic and biosynthetic routes. Such a diverse role of the EE as a critical sorting organelle is reflected in its complex morphology. Distinct membrane subdomains mediate sorting of cargo into various pathways. While the formation of endosomal tubular structures facilitates efficient recycling of receptors back to the plasma membrane, retromer-mediated tubulation is essential for retrograde transport of cargo from EE to the trans-Golgi network (TGN) (Bonifacino and Hurley, 2008). A defining property that differentiates these two tubulation events is the maturation state of the EE. Sorting of the recycling cargo into endosomal tubular structures precedes the retromer-mediated formation of the tubules recently described as SNX1/sortilin-enriched endosome-to-TGN transport carriers (ETCs) (Mari et al., 2008). Retromer machinery is preferentially recruited to maturing EEs containing increasing concentrations of PtdIns(3,5)P₂ generated by PIKfyve kinase (Rutherford et al., 2006) and an increased number of intraluminal vesicles (Arighi et al., 2004), both hallmarks of maturation into late endosomes.

Instrumental to the understanding of retrograde transport were the trafficking studies conducted using lysosomal/vacuolar acid-hydrolase receptors as prototypical retromer cargos. *S. cerevisiae* hydrolase receptor Vps10p and mammalian cation-independent mannose 6-phosphate receptor (CI-MPR) directly associated with the sorting subunit of the retromer complex, Vps35p (Nothwehr et al., 2000) and mammalian hVps35 (Arighi et al., 2004), respectively. This interaction in *S. cerevisiae* is followed by the direct binding of Vps29p to Vps35p/Vps10p that gives rise to a stable retromer subcomplex responsible for cargo sorting (Seaman et al., 1998). In addition, a second retromer subcomplex involved in membrane association is assembled through dimerization of the two members of the sorting nexin family of proteins, Vps5p and Vps17p (Horazdovsky et al., 1997; Seaman et al., 1998). Both proteins contain a PtdIns(3)P-binding Phox (PX) domain, essential for association with EE membranes (Burda et al., 2002), as well as a curvature-sensing Bin Amphiphysin Rvs (BAR) domain, essential for retromer-regulated formation of tubular membrane structures necessary for efficient retrograde transport (Carlton et al., 2004; Bonifacino and Rojas, 2006). Final assembly of the two subcomplexes into a functional retromer requires the interaction of Vps5p/Vps17p with Vps35p and is facilitated by an additional retromer subunit, Vps29p (Reddy and Seaman, 2001). Depletion of Vps26p by SiRNA and knock-out approaches rendered the retromer inactive, resulting in significant defects in

Sorting at early endosomes

the retrograde transport of cargo, including the CI-MPR (Seaman, 2004).

Recent studies have identified two additional sorting nexins as critical components of the mammalian retromer. An RNAi-mediated loss-of-function screen of the entire SNX family of proteins has yielded a retrograde trafficking role for SNX5 and SNX6 (Wassmer et al., 2007). Interestingly, endogenous SNX6 associates with endogenous SNX1, while both SNX5 and SNX6 co-localize with SNX1 on tubular and vesicular EE structures. Furthermore, knockdown of either SNX5 or SNX6 results in destabilization and dramatic loss of endogenous SNX1, most likely through degradation (Wassmer et al., 2007). Collectively these recent findings have attributed a more complex composition of mammalian retromer than that observed in yeast. The current model for the assembly of the membrane-bound subcomplex proposes that it consists of SNX1 and SNX2 as the interchangeable Vps5p orthologues, and SNX5 and SNX6 as the functional orthologues of Vps17p (Bonifacino and Hurley, 2008; Collins, 2008; Cullen, 2008). However, the exact modality of the SNX subcomplex assembly as well as existence of additional components remains to be determined.

A recent study by Rojas et al. has shed a new light on the mechanism of retromer recruitment to the EE through the coordinate action of Rab5 and Rab7, with the VPS26/29/35 subcomplex acting as a Rab7 effector (Rojas et al., 2008). Once the GTP-bound active Rab5 associates with EE, numerous effectors are recruited, including a class III PtdIns(3)-kinase that generates PtdIns(3)P at these membranes. Subsequently the enrichment of EE membranes with PtdIns(3)P results in the increased recruitment of the SNX subcomplex. Nevertheless, due to the weak interaction of two retromer subcomplexes, the association of SNX subcomplex with the EE is insufficient for the recruitment of the cargo-sorting subunits (Rojas et al., 2008). Instead, Rab5 binds to a known Rab7 GEF, the VPS C-homotypic fusion and vacuole protein sorting (HOPS) complex, resulting in Rab7 activation and association with the EE membranes (Rink et al., 2005). Rab7-GTP can then serve as a nexus between the two subcomplexes through direct association with the cargo-sorting subcomplex. This interaction is then sufficient to bring the two subcomplexes in close proximity to generate a more stable association, leading to retromer assembly (Rojas et al., 2008).

In addition to its central role in retromer assembly, VPS35 also mediates cargo recognition by low affinity binding to a conserved cargo sorting motif Trp/Phe-Leu-Met/Val at the cytosolic tail of CI-MPR (Arighi et al., 2004; Gokool et al., 2007). Furthermore, studies aimed at identifying retromer-binding proteins have proven instrumental to the efforts of dissecting the cargo selection. Yeast sorting nexin Grd19 associates with retromer and mediates its binding to the C-terminal sorting sequence GHLPFTKNLQ of the Ftr1/Fet3

reductive iron transporter (Voos and Stevens, 1998; Strohlic et al., 2007). Other endocytic proteins, such as the C-terminal EHD protein, EHD1, can also bind the retromer (Gokool et al., 2007). Although it remains to be determined whether EHD1 can bind retromer directly, it plays a role in facilitating efficient retrograde transport and tubulation of the retromer-associated membranes. Moreover, it has been recently demonstrated that EHD3, the closest mammalian EHD1 paralog, regulates endosome-to-Golgi transport and retromer subunit localization (Naslavsky et al., 2009).

Proper sorting of endocytic cargo at the EE depends on the coordinate action of numerous endocytic regulatory molecules. Loss of regulation of sorting machinery can therefore result in aberrant protein localization and potentially lead to the onset of various pathological conditions (Table 1). However, in spite the apparent significance of this process, our understanding of EE sorting is still evolving as we further dissect the protein and lipid interactions occurring at the EE.

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