Focus Review

Endosome maturation

Jatta Huotari and Ari Helenius*
Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

Being deeply connected to signalling, cell dynamics, growth, regulation, and defence, endocytic processes are linked to almost all aspects of cell life and disease. In this review, we focus on endosomes in the classical endocytic pathway, and on the programme of changes that lead to the formation and maturation of late endosomes/multivesicular bodies. The maturation programme entails a dramatic transformation of these dynamic organelles disconnecting them functionally and spatially from early endosomes and preparing them for their unidirectional role as a feeder pathway to lysosomes.

Keywords: degradation; endocytosis; intraluminal vesicle (ILV); multivesicular body (MVB); sorting

Introduction

Endocytosis is the general term for internalization of fluid, solutes, macromolecules, plasma membrane components, and particles by the invagination of the plasma membrane and the formation of vesicles and vacuoles through membrane fission. In metazoan cells, endocytosed cargo includes a spectrum of nutrients and their carriers, receptor-ligand complexes, fluid, solutes, lipids, membrane proteins, extracellular–matrix components, cell-debris, bacteria, viruses, etc. By sorting, processing, recycling, storing, activating, silencing, and degrading incoming substances and receptors, endosomes are responsible for regulation and fine-tuning of numerous pathways in the cell.

Having left endosome research after the early discovery period in the 1980s, and returning to it only recently, one of us (AH) has been impressed by the amount of information that has become available in the meantime on most aspects of endocytosis. Also, it is evident that the central role of endocytosis in cell life and pathogenesis is now much more fully appreciated. The reason for concentrating again on this topic is our interest in host cell entry of animal viruses, the majority of which enter cells by endocytosis and exploit endosomes and the endocytic pathways for penetration into the cytosol (Marsh and Helenius, 1989; Mercer et al., 2010). While virus particles themselves are relative simple and in many cases well characterized, the challenge is to understand the cellular processes used by them, and the responses of the cell to the invasion. Deeper knowledge of endocytosis is urgently required. Incoming viral particles also provide a tool to learn more about the endocytic machinery.

In this review, we focus on a relatively narrow topic; the formation and maturation of late endosomes (LEs) in mammalian cells. They are also known as multivesicular bodies, because—although heterogeneous and variable in size and composition—most LEs have a multivesicular morphology, that is, they contain intraluminal vesicles (ILVs). There are many excellent reviews to recommend dealing with endosomes and different aspects of LE maturation. These include the following (Mellman, 1996; Zerial and McBride, 2001; Maxfield and McGraw, 2004; Piper and Katzmann, 2007; Luzio et al., 2007b; van Meel and Klumperman, 2008; Woodman and Futter, 2008; Saftig and Klumperman, 2009; Jovic et al., 2010; Von Bartheld and Altwick, 2011).

The logistics of the endosome system

In a basic, ‘stripped-down’ representation, the classical endocytic pathway has only a few elements (Figure 1). The elements include a recycling circuit for plasma membrane components and their ligands, a degradative system for digestion of macromolecules, and a connecting, unidirectional feeder pathway for transport of fluid and selected membrane components from the recycling circuit to the degradative system. The feeder function is mediated by LEs. LEs also function as a system for mediating transport of lysosomal components from the trans-Golgi network (TGN) to lysosomes. This allows maintenance, diversification, and expansion of the recycling and degradative systems. Finally, the cytosol must be included among the essential elements, because it provides a spectrum of transiently associated, peripheral membrane components that support, regulate, and define the pathway (Figure 1).

Of the cargo internalized by ongoing endocytosis in mammalian cells, the majority is recycled back to the plasma membrane via early endosomes (EEs) (Figure 2). In typical mammalian cells, the equivalent of 50–180% of the surface area of the plasma membrane is cycled in and out of the cell every hour (Steinman et al., 1983). The amount of fluid internalized by macrophages corresponds to some 30% of cell volume per hour of which about two-thirds are returned to the extracellular space within about 10–15 min (Steinman et al., 1976, 1983; Besterman and Low, 1983).
One of the consequences of the active recycling is that transport to lysosomes via LEs is a side pathway limited to a relatively small fraction of internalized fluid and especially membrane components. To enter this side pathway, membrane components undergo stringent selection so that only a specific cohort is transported to lysosomes and degraded. The majority of large particles (such as viruses and ILVs) are also targeted to LEs. The bulk fluid and solutes diverted into this side pathway are not specifically sorted.

In addition to ferrying cargo for degradation, LEs transport new lysosomal hydrolases and membrane proteins to lysosomes for the maintenance and amplification of the degradative compartment. Lysosomes depend on the influx of new components, because without incoming endosomal traffic, they lose their intactness, acidity, and perinuclear localization (Bucci et al., 2000). It is evident that the magnitude of the side pathway from EEs to lysosomes is under regulation through some of the cargo. Thus, formation of LEs and inward vesiculation to form ILVs has been shown to increase upon signalling via growth factor receptors. This suggests that the cell adjusts the use of this side pathway according to need (White et al., 2006). In doing so, it probably also adjusts the size of the degradative compartment. How such adjustment is achieved and which factors influence it, is an interesting question that deserves careful study.

Unlike the secretory pathway, the endocytic pathway has the advantage that the starting compartment, the extracellular space, is open and accessible. This means that a variety of ligands, fluid, solutes, and particles can be added to cells, and their fate after endocytosis followed in different ways. In mammalian tissue culture cells, the most commonly used cargo markers today are transferrin (Tf) and its receptor (TfR), which faithfully follow the recycling pathway, and epidermal growth factor (EGF) and its receptor (EGFR), which after ubiquitination of the receptor’s cytosolic domain and inclusion in ILVs take the pathway to lysosomes for inactivation and degradation. Fluid uptake is usually followed using fluorescently-labelled dextran and other fluorescent solutes that do not adsorb to the cell surface. The membrane as such can be followed using fluorescent lipid markers (Maier et al., 2002). Viruses and bacterial toxins are also useful tools.

In addition to tissue culture cells, the most valuable system for endocytosis studies has been yeast, which seems to have endosomal compartments comparable to animal cells (Lewis et al., 2000; Pelham, 2002). More recently, filamentous fungi such as Aspergillus nidulans, have proven to be excellent systems to study endosomes and their maturation (Penalva, 2010). Caenorhabditis elegans and Drosophila melanogaster also have an impact given the background of strong genetics and the possibility of in situ studies in a multicellular organism (Grant and Sato, 2006; Michelet et al., 2010; Poteryaev et al., 2010). Endocytosis in plant cells is actually quite active (Irani and Russinova, 2009). The most important difference is the apparent lack of an independent EE compartment. The functions of EEs are carried out by an organelle that combines EEs and the TGN (Dettmer et al., 2006; Niemes et al., 2010). Conceptually, the situation in plants and fungi implies that by participating actively in secretory functions, endosomes can be viewed as an extension of the TGN.

**Early endosomes**

EEs provide the starting point for LE maturation. Defined initially as the compartment that first receives incoming cargo and fluid (Helenius et al., 1983), EEs are now recognized as the main sorting station in the endocytic pathway. Exactly how EEs arise is not entirely clear, but the membrane and volume is mainly derived from primary endocytic vesicles that fuse with each other. EEs receive endocytic cargo not only through the clathrin-mediated pathway but several other pathways including caveolar-, GEEC-, and ARF6-dependent pathways (Mayor and Pagano, 2007). Typically, an EE accepts incoming vesicles for about 10 min during which time mem-

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**Figure 1** The basic elements of the endocytic machinery. The membrane organelles involve a *recycling circuit* (the plasma membrane (PM), the EEs, the recycling endosomes, and a variety vesicular carriers), a *degradation cycle* (lysosomes), and a connecting ‘*feeder* pathway’ (LEs) from the recycling circuit to the degradative system. The main interacting partner in the Golgi providing lysosomal components is the TGN, which communicates with the PM, EEs and LEs. The recycling circuit has functions independent of the degradative cycle. The degradative cycle is, in turn, a shared ‘facility’ for degradation in the cell and is not only used for substrates delivered via endosomes. The cytosol has a central role by providing peripheral proteins to all the membrane compartments. These proteins define functions such as molecular sorting, membrane fusion and fission, compartment identity, and organelle motility.
brane and fluid is rapidly recycled away, while some of the incoming cargo is retained and accumulates over the lifetime of the EE to be included in the LEs (Maxfield and McGraw, 2004).

Association of proteins from the cytosol to the cytosolic surface of the EE membrane defines many of its functional attributes. Rab5 is a key component together with its effector VPS34/p150, a phosphatidylinositol 3-kinase (PI(3)K) complex that generates the phosphoinositide (PI) PtdIns(3)P and thus helps to manifest the identity of the organelle (Christoforidis et al, 1999; Zerial and McBride, 2001; Behnia and Munro, 2005). Rab5 follows the endocytic membrane from the beginning through various stages of EE maturation, and is later the main regulator of the conversion to LEs. EEs communicate with the TGN through bidirectional vesicle exchange. The arrival of hydrolases gives them an initial degradative identity further strengthened during maturation of LEs.

EEs are heterogeneous in terms of morphology, localization, composition, and function (Miaczynska et al, 2004; Lakadamyali et al, 2006; van Meel and Klumperman, 2008). Most of them are relatively small and patrol the peripheral cytoplasm close to the plasma membrane through saltatory movement along microtubules (Nielsen et al, 1999; Hoepfner et al, 2005). The overall distribution of EEs is cell-type dependent.

Individual EEs have a complex structure with tubular and vacuolar domains (Figure 3A). Most of the membrane surface area is in the tubules, and much of the volume in the vacuoles. The limiting membrane contains a mosaic of subdomains that differ in composition and function (Zerial and McBride, 2001). They include domains enriched in Rab5, Rab4, Rab11, Arf1/COPI, retromer, and caveolae (Vonderheit and Helenius, 2005; Rojas et al, 2008; Hayer et al, 2010). Many of the domains are located in the tubular extensions where they provide for molecular sorting and generate vesicle carriers targeted to distinct organelles, including the plasma membrane, the recycling endosomes, and the TGN (Bonifacino and Rojas, 2006) (Figure 2).

The formation of ILVs begins already in EEs. For this the cytosolic surface of the EE membrane has characteristic ‘plaques’ containing clathrin and components of the endosomal sorting complex required for transport (ESCRT), machinery responsible for sorting of ubiquitinated membrane proteins into ILVs (Raiborg et al, 2002; Sachse et al, 2002) (Figure 3B). The lumen of the vacuolar EE domains often contains several ILVs; in HepG2 cells there are 1–8 ILVs (van Meel and Klumperman, 2008). EEs are weakly acidic (pH 6.8–5.9) (Maxfield and Yamashiro, 1987), and contain a relatively low Ca\(^{2+}\) concentration (Gerasimenko et al, 1998).

The traffic between endosomes and the TGN is a continuously ongoing process that has been extensively studied. It is responsible for the delivery of lysosomal and removal of endosomal components during endosome maturation. It
LEs and lysosomes

Mature LEs are typically round or oval and have a diameter of 250–1000 nm (Figure 3C). They have a low buoyant density, and a high negative surface charge (Bayer et al., 1998; Falguieres et al., 2008). The limiting membrane contains lysosomal membrane proteins such as LAMP1 and the lumen contains a complement of acid hydrolases. The lumen also has numerous ILVs (often up to \( \geq 30 \)) with a diameter of about 50–100 nm. The pH ranges between pH 6.0–4.9 (Maxfield and Yamashiro, 1987).

LEs formed in the peripheral cytoplasm move to the perinuclear area of the cell where they fuse with each other to form larger bodies and to undergo transient (‘kiss-and-run’) fusions and eventually full fusions with lysosomes and pre-existing hybrid organelles between endosomes and lysosomes (Luzio et al., 2007b) (Figure 2). By fusing with lysosomes, the LEs follow what is essentially a unidirectional, dead-end pathway. Whereas most of the components of LEs are degraded in the lysosomal environment, others are longer lived and contribute, as already mentioned, to the maintenance and generation of lysosomes. It has been suggested that a few components such as mannose-6-phosphate receptors, tetraspanins, and SNAREs may escape degradation through vesicle trafficking after LE fusion with lysosomes, but this pathway is not well characterized.

To determine whether a multivesicular organelle is a LE, or a fusion product between LEs and lysosomes is not always straightforward because the hybrid organelles contain components from both fusion partners (Figure 3D and E). We will call these hybrid organelles endolysosomes, to distinguish them from classical dense, primary lysosomes and from lysosomes generated through fusion with phagosomes, macro- and autophagosomes. It is in the endolysosomes that most of the hydrolysis of endocytosed cargo takes place.

The lysosomal compartment as a whole consists of a collection of vacuoles of heterogeneous composition, morphology, location, and density. The heterogeneity is due to the diversity of cargo and the existence of several feeder pathways of which the classical endosome pathway is a major one. Heterogeneity is also generated by the varying degree of cargo degradation within individual vacuoles, and by fusion events between the various vacuoles. The classical lysosomes of high buoyant density and high hydrolase content correspond to the end point of the degradation process. They serve in part as storage vacuoles for lysosomal components ready to be redeployed. These components include the hydrolases, the limiting membrane protected by LAMPs, and other substances resistant to degradation. They also contain slowly degraded luminal lipids often present as multilamellar membrane whirls.

Taken together, the EEs, LEs, endolysosomes, and lysosomes provide a dynamic and adaptable continuum (Von Bartheld and Altick, 2011). The pathway is elusive because the organelles are scattered, and undergoing continuous maturation, transformation, fusion, and fission. Specific protein and lipid components are only partially useful as molecular markers because the majority is either transiently associated with the organelles or follow the organelles through several steps of transformation. In the pathway, events occur, moreover, non-synchronously. A cohort of cargo molecules simultaneously internalized from the plasma membrane may, for example, arrive at a certain acidic pH value in a time window spread over hours (Kielian et al., 1986). The ambiguity, heterogeneity, and lack of synchrony in the endocytic system may, in part, explain a certain lack of common, universally agreed concepts and models for endosome function and maturation.

**Formation of LEs**

LEs are derived from the vacuolar domains of EEs. By itself this constitutes molecular sorting because in addition to a
large fraction of the fluid, the vacuolar domains of EEs have a different composition than the tubules. In the lumen, they contain ligands dissociated from their receptors as well as proteins and solutes internalized as components of the bulk fluid. The ILVs, and other large particles such as incoming viruses, are also present in the vacuolar elements of EEs. The membrane probably contains most of the cholesterol and sphingolipid-rich lipid rafts, membrane protein aggregates, V-ATPases, clathrin, ESCRT complexes, and a selected spectrum of membrane proteins destined for degradation (Ukkonen et al., 1986; Mukherjee et al., 1999; Mukherjee and Maxfield, 2004).

The formation of a new LE is preceded by the generation of a Rab7 domain (Rink et al., 2005; Vonderheit and Helenius, 2005). This leads to the transient formation of a hybrid Rab5/ Rab7 endosome. As discussed below, Rab7 is recruited to the EE by Rab5-GTP. How the tubes and the rest of the EE domains are lost during the formation of an LE is not entirely clear, but there are two possibilities. Evidence obtained by analysing large, spherical, juxtanuclear Rab5-positive endosomes supports a mechanism in which Rab5 after recruiting Rab7 is converted to the GDP-bound form and dissociates together with its effectors. A process like this was observed by live microscopy (Rink et al., 2005). Rab5 was lost within a few minutes, and replaced with Rab7.

Another mechanism envisages a fission event that separates parts of the hybrid endosome containing the nascent Rab7 domain from the rest. Grunenberg and Stemmark (2004) call the newly formed LEs endosomal carrier vesicles (ECVs), and suggest that these serve as transporters of cargo to a stable LE compartment before delivery to lysosomes. While following viruses after endocytosis in small peripheral endosomes by live microscopy, we found support for such splitting of the endosomes. We observed microtubule-dependent events in which the virus and the Rab7 domain separated from the rest of the endosome containing Rab5, Rab4, and Arf1 (Vonderheit and Helenius, 2005). Consistent with this, it has been shown that dynenin-mediated pulling forces are critical for the separation of endosomal elements containing the recycling ligand Tf from the lysosome-targeted EGF (Driskell et al., 2007). Similar observations were recently made by a group that proposed a role for dynamin in fission of LEs from EEs (Mesaki et al., 2011). It is possible that different mechanisms exist for the peripheral and perinuclear populations of EEs, with gradual maturation occurring in perinuclear endosomes and a fission-based mechanism in peripherally located, relatively small and motile endosomes.

**Maturation of LEs**

Regardless of the mechanism of initial formation, the newly formed LEs continue to undergo a multitude of changes (Table I). As a result, by the time they fuse with lysosomes some 10–40 min later, they have completed a remarkable transformation leaving them with few similarities to EEs. The maturation process involves exchange of membrane components, movement to the perinuclear area, a shift in choice of fusion partners, formation of additional ILVs, a drop in luminal pH, acquisition of lysosomal components, and a change in morphology (Table I). The programme is closely coordinated and regulated by factors recruited to the surface of the limiting membrane from the cytosol.

**Table I** The endosome maturation programme

| Rab switch. Rab5 is exchanged for Rab7. This switch reprograms the association of effector proteins from the cytosol and redefines many of the properties of the endosomes. Other Rab, such as Rab4, Rab11, are Rab22 also lost, while Rab9 is added. |
| Formation of ILVs. Ubiquitinated cargo recruits machinery from the cytosol (ESCRT and other factors) that induce inward-budding of the limiting membrane, and thus the formation of ILVs containing membrane proteins and lipids targeted for lysosomal degradation. Capacitation of microphagocytosis-like mechanisms for the inclusion of cytoplasmic proteins and RNAs, of ILV backfusion with the limiting membrane, and exosome release. |
| Acidification. The luminal pH drops from values above pH 6.0–4.9. |
| PI conversion. PtdIns(3)P is converted to PtdIns(3,5)P(2), and some of the PtdIns(3)P is sorted into the ILVs. |
| Change in size and morphology. The tubular extensions present on EEs are lost and the endosomes acquire a round or oval shape and grow in size. |
| Loss of recycling with the plasma membrane. Recycling receptors are lost from the organelle and recycling of membrane and fluid to the cell surface stops. |
| Gain of lysosomal hydrolases and membrane proteins. These lysosomal components are transported mainly from the TGN. Some of them are active already in the maturing endosomes. |
| Switch in fusion specificity. The endosomes can no longer fuse with EEs. Instead, they acquire the necessary tethering complexes and SNAREs to fuse with each other, with lysosomes, and with autophagosomes. The conversion of CORVET to HOPS complex on membranes. The HOPS/CORVET complexes are involved in a number of processes, including membrane tethering, the Rab7 switch, and mediating SNARE assembly. |
| A switch in cytoplasmic motility. The endosomes associate with a new set of microtubule-dependent motors that allow them to move into the perinuclear region of the cell. |
| Changes in luminal ionic environment. In addition to the drop in pH, there is an increase in Cl−, and changes in Ca2+, Na+, K+ concentrations. |
| Change in temperature sensitivity. Unlike earlier steps in endocytosis, LE formation or their fusion with lysosomes is blocked at temperatures below 19–20°C. |
| Decrease in buoyant density and increase in negative surface charge. These properties are used to isolate LEs. |

Before considering the conversion in more detail, it is important to ask why LEs are subject to such a dramatic transformation. What is the reason for this complex maturation programme? The general answer is that the programme is in place to close down recycling and other functions of EEs and to allow the union of LEs with the degradative compartment. Similar maturation using some of the same factors occurs in phagosomes, autophagosomes, and probably macropinosomes before they fuse with LEs and lysosomes (Eskelinen, 2008; Kinchen and Ravichandran, 2008; Kerr and Teasdale, 2009).

Since lysosomes constitute a point-of-no-return for most macromolecules and lipids, the cargo contents of LEs must be narrowed down to molecules and particles that need to be degraded, and to cargo that the LEs and lysosomes require for their functionality. Some of the membrane-bound cargo, moreover, needs to be presented in a form easily digested by hydrolases, which may explain in part the formation and
the composition of ILVs. The limiting membrane of the LEs must, on the other hand, be rendered resistant to the hydrolases by inclusion of lysosomal glycoproteins such as LAMPS. Moreover, interactions with the cytoskeleton have to be altered so that the LEs can move to the region of the cytoplasm where the lysosomes are localized. LEs also need new tethering factors and SNAREs to be able to fuse with each other, with lysosomes, and perhaps with macropinosomes and autophagosomes.

In the following sections, we will focus on some of the events and factors involved in endosome maturation. These include the Rab GTPase switch, phosphatidylinositol conversion, Arf1/COPI association, ILV biogenesis, acidification, and LE motility. Finally, we will discuss some of the roles that LEs have beyond the degradative function.

The Rab switch

Rab GTPases, especially Rab5 and Rab7 (Vps21p and Ypt7p in yeast, respectively), provide the most important organelle identity markers and master regulators in the endocytic pathway. Rab5 itself, its guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), GDP dissociation inhibitors (GDI), and GDI displacement factors (GDFs) as well as its effectors determine the functions of EEs (Chavrier et al., 1990; Ullrich et al., 1994; Rink et al., 2005; Jovic et al., 2010). Rab7 and its corresponding factors have a similar role in LEs and lysosomes (Chavrier et al., 1990; Meresse et al., 1995; Tjelle et al., 1996).

Endosome maturation involves a conversion from Rab5 to Rab7 (Rink et al., 2005; Vonderheit and Helenius, 2005; Poteryaev et al., 2010). The conversion can be blocked by expressing a constitutively active mutant of Rab5 (Q79L), and by depletion of VPS39, a subunit of the HOPS complex (Rink et al., 2005). This results in the formation of hybrid endosomal compartments with markers for both EEs and LEs. These abnormal hybrid compartments seem to arise from homotypic fusions of EEs and heterotypic fusions with LEs or lysosomes, and are found to accumulate ILVs from homotypic fusions of EEs and heterotypic fusions with LEs and lysosomes (Chavrier et al., 1990). Endosome maturation involves a conversion from Rab5 to Rab7 (Rink et al., 2005; Vonderheit and Helenius, 2005; Poteryaev et al., 2010). The conversion can be blocked by expressing a constitutively active mutant of Rab5 (Q79L), and by depletion of VPS39, a subunit of the HOPS complex (Rink et al., 2005). This results in the formation of hybrid endosomal compartments with markers for both EEs and LEs. These abnormal hybrid compartments seem to arise from homotypic fusions of EEs and heterotypic fusions with LEs or lysosomes, and are found to accumulate ILVs (Rosenfeld et al., 2001; Hirota et al., 2007; Wegner et al., 2010). Expression of the Rab5(Q79L) mutant results in sorting defects of both recycling and degradative cargo. Lysosome biogenesis is also affected as the amount of lysosomes in dense Percoll gradient fractions is greatly reduced (Rosenfeld et al., 2001).

Initially, Rabex-5, a GEF for Rab5, is recruited to EEs where it activates Rab5 (Horiuchi et al., 1997; Barr and Lambright, 2010). Interestingly, in addition to its GEF activity, Rabex-5 also possesses ubiquitin (Ub) E3 ligase activity and can bind to ubiquitinated proteins, which is indeed required for its association with EE membranes (Mattera and Bonifacino, 2008). In the cytosol Rab5 itself is monoubiquitinated and it is thought that a cycle of Ub binding and monoubiquitination regulates its recruitment. The GEF activity of Rab5 promotes the recruitment of Rabaptin-5, a Rab5 effector with which it forms a complex (Horiuchi et al., 1997) (Figure 4). This complex is required to establish a feedback loop, whereby Rab5-GTP promotes further Rab5 binding (Lippe et al., 2001). This causes a rapid recruitment of numerous Rab5 effectors including the VPS34/p150 complex that produces PtdIns(3)P (Christoforidis et al., 1999; Jovic et al., 2010), which in turn promotes the binding of a spectrum of proteins with specific PI-binding domains.

Removal of Rab5 and its replacement with Rab7 is an essential step in LE formation and in the transport of cargo to lysosomes. Rab5 removal requires both inhibition of the feedback loop for Rab5 binding as well as a GAP activity to activate Rab5’s GTP hydrolysis activity. On the basis of theoretical and experimental approaches, Del Conte-Zerial and colleagues have proposed a ‘cutoff switch’ model to explain the paradox of rapid initial growth of Rab5 domains and their sudden disassembly during Rab conversion (Del Conte-Zerial et al., 2008). In this model, Rab5 activates Rab7, and Rab7 displays autoactivation and suppresses Rab5. In this way, Rab5 orchestrates its own removal from the endosomes and at the same time initiates the takeover by Rab7.

Additional key players in the Rab5/Rab7 conversion are starting to emerge. According to recent data, these include SAND-1/Mon1 and Ccz1, two factors recruited from the cytosol to endosomal membranes. They were first identified as factors required for vacuole fusion in yeast, and in transport of yolk proteins from EEs to LEs in C. elegans (Wang et al., 2003; Poteryaev et al., 2007). Recent work focusing on phagosomes in C. elegans has provided evidence that SAND-1/Mon1 and Ccz1 are involved in the Rab conversion in endosomes and phagosomes (Kinchen and Ravichandran, 2010; Poteryaev et al., 2010).

Taken together, the results indicate that SAND-1/Mon1 (or the SAND-1/Mon1 complexed with Ccz1) can (1) bind...
to Rab5-GTP; (2) recruit Rab7 (and bind Rab7 when both SAND-1/Mon and Ccz1 are present); (3) bind to PtdIns(3)P; (4) interact with components of the HOPS complex; and (5) displace Rabex-5 from the endosomal membrane. In this way, SAND-1/Mon1 could (together with Ccz1) promote Rab7 binding by potentially displacing the GDI from Rab7 and/or by activating the VPS39 subunit of the HOPS complex, which in yeast has been proposed to act as a GEF for Ypt7p (Wurmser et al., 2000). Moreover, by interacting with Rabex-5 and displacing it from the membrane, it could interrupt the positive feedback loop that enhances Rab5 binding to EEs (Poteryaev et al., 2010). As a result, SAND-1/Mon1 (or the SAND-1/Mon1 complexed with Ccz1) would turn off Rab5 at the same time as it would turn on Rab7 (Figure 4).

The timing of SAND-1/Mon1 association with EEs is critical because the Rab conversion should not occur until the endosome has accumulated cargo for degradation. In this context, it was found that SAND-1/Mon1 recognizes PtdIns(3)P, and that the PI(3)K inhibitor wortmannin prevented SAND-1/Mon1 binding to endosomes and thus Rab5 inactivation (Vieira et al., 2003; Poteryaev et al., 2010). This suggests that the concentration of PtdIns(3)P on endosomes could be a determinant defining the timing of the Rab conversion.

Many molecular details in this interesting model remain unclear. For example, it has been proposed that the HOPS complex component VPS39 serves as a GEF for Rab7 (Rink et al., 2005) because the yeast orthologue Vps39p/Vam6p possesses GEF activity towards Ypt7p in yeast (Wurmser et al., 2000). However, it was recently reported that in mammalian cells, VPS39 does not possess GEF activity towards Rab7 (Peralta et al., 2010). The GEF might, instead, be the Mon1–Ccz1 complex itself as recently shown in yeast (Nordmann et al., 2010). Another question is whether SAND-1/Mon1 directly interacts with the GDI of Rab7 or whether the reported effects of SAND-1/Mon1 on the disassociation of the GDI from Rab7 are indirect (Cabrera and Ungermann, 2010). In mammals, there are two SAND-1 orthologues, Mon1a and Mon1b. It is unclear what their differences are and how redundantly they function.

To terminate Rab5 activation on endosomes, a GAP has to activate Rab5-mediated hydrolysis of bound GTP into GDP. Several GAPs have been assigned to Rab5, including RAB GAP-5 (Lanzetti et al., 2000; Haas et al., 2005). However, the target of RAB-Tre may actually be Rab41 (Haas et al., 2005). A recent addition to the list of Rab5 GAPs is TBC-2, which in C. elegans was shown to be required both during endosome and phagosome maturation (Li et al., 2009; Chotard et al., 2010). It is recruited to endosomes only when Rab7 is already present, thereby allowing coordination of Rab5 inactivation with Rab7 activation. Confusion is caused by the observation that the closest human homologue of TBC-2, Armus, possesses GAP activity towards Rab7 (Frasa et al., 2010). Earlier studies have recognized a role for the yeast Gyp7p and its human orthologue TBC1D15 as Ypt7p/Rab7 GAPs (Vollmer et al., 1999; Zhang et al., 2005; Peralta et al., 2010).

Once established as a domain in the hybrid endosome, Rab7-GTP recruits its own effectors. These include factors such as RILP, a protein that connects LEs to dynein motors; components of the retromer complex that support vesicle traffic to the TGN; components of the HOPS complex serving as a tether for LE fusion; and Rabring7, a RING-type E3 ligase (Zhang et al., 2009). In contrast to these, Rubicon and PLEKH1, recently identified Rab7 effectors, act as negative regulators of endosome maturation (Sun et al., 2010; Tabata et al., 2010).

One crucial role of the Rab5/Rab7 conversion is to exchange the fusion machinery on the endosomal membrane so that LEs can only fuse with other LEs, lysosomes, and possibly autophagosomes. Two complexes that are thought to regulate the fusion machinery are the class C VPS CORVET and HOPS complexes, for EEs and LEs, respectively (Nickerson et al., 2009). They both have a core of four proteins (Vps11, Vps16, Vps18, and Vps33), but differ in accessory proteins; CORVET is associated with Vps3 and Vps8 and HOPS with Vps39 and Vps41. The composition and function of these complexes have mainly been studied in yeast, where they were first identified. Differences to mammalian cells may exist; it is still uncertain whether for instance VPS39 occurs in both CORVET and HOPS complexes in mammals. Nevertheless, as Rab5/Vps21p can interact with the CORVET and Rab7/Ypt7p conversely with the HOPS complex, the current model suggests that the Rab5/Rab7 and the CORVET/HOPS conversion are interlinked and coordinately regulated.

Early studies in yeast showed that the Rab7 orthologue, Ypt7p, was required for delivery of degradative cargo to the vacuole, that is, to the yeast lysosome (Wichmann et al., 1992; Schimmoller and Riezman, 1993). Subsequent studies in mammalian cells demonstrated that expression of dominant negative mutants of Rab7 results in dispersal of endosomes, a block in cargo trafficking to the lysosomes, and the deterioration of lysosome acidification and functionality (Bucci et al., 2000). By recruiting a variety of effector proteins on LEs, Rab7/Ypt7p coordinates various maturation processes, and renders the organelle capable of fusion with other LEs as well as lysosomes (or the vacuole in yeast). In addition, in yeast, Ypt7p is known to be directly involved in the assembly of the fusion machinery at both the LEs and the vacuole membrane (Haas et al., 1995; Ostrowicz et al., 2008).

In this context, it is interesting that Ypt7p has been found among ubiquitinated proteins in vacuoles and may be a substrate for proteasomal degradation during vacuole fusion (Kleijnen et al., 2007). A role for the proteasome in the degradative pathway has also been suggested on several occasions in mammalian cells (van Kerkhof and Strous, 2001; Longva et al., 2002; Khor et al., 2003; Geetha and Wooten, 2008), although the functions and molecular details remain elusive. Interestingly, in mammalian cells, Rab7 interacts with a subunit of the proteasome (Dong et al., 2004). However, no evidence for the proteasomal degradation of Rab7 in mammalian cells was found. One proposed function for the proteasome at endosomes is regulation of cargo deubiquitination and sorting into ILVs (Longva et al., 2002; Alwan et al., 2003; Geetha and Wooten, 2008). Interactions between a proteasome accessory protein, Ecm29 and LE resident proteins, including components of ESCRT, retromer and motor proteins have also been reported (Gorbea et al., 2010), suggesting that proteasomes have perhaps multiple functions at LEs.

### The PI conversion

Like the Rab GTPase switch, the PI conversion plays an indisputable role in endosome maturation. The two impor-
tantal PIs are PtdIns(3)P and PtdIns(3,5)P(2), two lipids that contribute to the identity of EE and LE membranes. As mentioned earlier, PIs recruit a number of effector proteins with PI-binding domains including FYVE, PH, PX, and GRAM. The PI species are synthesized locally in organelle membranes by the action of specific kinases and phosphatases allowing tight control of compartmentalization (Vicinanza et al., 2008).

PtdIns(3)P is mostly found on the cytosolic leaflet of EE membranes, generated mainly by a class III PI(3)K, VPS34 (Schu et al., 1993), which is recruited by Rab5-GTP through direct interaction with its partner, p150 (Christoforidis et al., 1999; Murray et al., 2002). Inhibition of VPS34 activity causes enlarged LEs with fewer ILVs, and EGFR accumulates in the limiting membrane (Futter et al., 2001). Dephosphorylation of PtdIns(3)P is executed by the myotubularin family of 3-phosphatases (Robinson and Dixon, 2006).

VPS34 acts in concert with other proteins that regulate its kinase activity; p150 (Vps15 in yeast) and Beclin-1. Together, these form a core complex (Funderburk et al., 2010), which on endosomes associates with a protein called UVRAG (Itakura et al., 2008), an activator of the HOPS complex. Recent findings indicate that a Rab7 effector, Rubicon, acts as a negative regulator of UVRAG by sequestering it away from the HOPS complex (Sun et al., 2010). Upon activation, Rab7 competes for binding with Rubicon, causes the release of UVRAG, and allows it to associate with the HOPS complex (Figure 5).

PtdIns(3,5)P(2) is important later in the degradative pathway. It is generated by a phosphatidylinositol 3-phosphate 5-kinase, PIKfyve (Fab1p in yeast). Originally characterized in yeast (Gary et al., 1998), PIKfyve/Fab1p was later found to play an essential role in the endolysosomal system of C. elegans, D. melanogaster, and mammalian cells. This is highlighted by the finding that PtdIns(3,5)P(2) depletion leads to highly vacuolated phenotypes (Ikonomov et al., 2001; Nicot et al., 2006; Rusten et al., 2006; Jeffery et al., 2008).

Membrane localization of PIKfyve/Fab1p is brought about by its PtdIns(3)P binding FYVE domain, which links the production of PtdIns(3,5)P(2) to existing membranes rich in PtdIns(3)P. PIKfyve activity is regulated by its activator ArPIKfyve/Vac14p and the PtdIns(3,5)P(2) phosphatase Sac3/Fig4p (Shisheva, 2008), with which it forms a stable complex critical for both lipid kinase and phosphatase activities (Sbrissa et al., 2008; Ikonomov et al., 2009) (Figure 5). PIKfyve is also able to contribute to the production of PtdIns(5)P (Sbrissa et al., 2002). The functional significance of this in the endocytic system is, however, unclear. In addition to its lipid substrates, PIKfyve has been found to possess kinase activity towards proteins, exemplified by p40, an effector of Rab9 (Ikonomov et al., 2003b). This activity might be partially coupled to PIKfyve’s proposed role in LE transport to the TGN, as perturbation of PIKfyve activity results in a block of endosome–TGN transport of both retromer-dependent and -independent cargo (de Lartigue et al., 2009).

Open questions still remain regarding the various functions of PIKfyve/Fab1p (Shisheva, 2008; Dove et al., 2009). For instance, acidification of LEs and lysosomes/vacuoles is dependent on PIKfyve/Fab1p activity in lower organisms, such as yeast, C. elegans and D. melanogaster (Gary et al., 1998; Nicot et al., 2006; Rusten et al., 2006), but in mammalian cells this is not entirely clear (Ikonomov et al., 2003a; Jeffery et al., 2008; de Lartigue et al., 2009). Enlarged vacuoles induced by inhibition of PIKfyve are not acidic in mammalian cells. However, in the same cells a fraction of endosomes is found to accumulate dyes that mark acidic LE or lysosomal compartments. How acidification is controlled by PIKfyve/Fab1p is not understood.

PIKfyve has also been linked to the regulation of ion channels, mostly by controlling their localization at the plasma membrane by exocytosis (Shisheva, 2008). In addition, PIKfyve may directly regulate the activity of channels in LEs. The calcium permeable cation channel TRPML1 was recently demonstrated to be directly activated by the presence of PtdIns(3,5)P(2) enabling the efflux of Ca$^{2+}$ (Dong et al., 2010). Ca$^{2+}$ is known to have an important regulatory function in homotypic and heterotypic fusions of LEs and lysosomes/vacuoles as well as in reformation of lysosomes from endolysosomes (Luzio, 2007a). The localized production of PtdIns(3,5)P(2) on endosomal membranes thus allows the control of channel activity in confined regions and may enable regulation of LE fusion/fission in a spatio-temporal manner. It is plausible that the highly vacuolated phenotype of PIKfyve/Fab1p mutants is partially the result of osmotic swelling or deregulated fusion/fission processes induced by the misregulation of endosomal ion channels (Shisheva, 2008).

PI conversion seems to be tightly linked to other endosome maturation steps. Parts of the ESCRT machinery responsible for ILV formation are recruited by both PtdIns(3)P and
PTDIns(3,5)P(2) (Katzmann et al., 2003; Whitley et al., 2003; Teo et al., 2006). This probably allows ILV formation and cargo sorting to be coordinated with the rest of the maturation programme. Likewise, interactions occur between RabGTPases and the PI converting machinery: VPS34 is known to interact with both Rab5 and Rab7. The difference is the activation state of the Rab: Rab5 recruits VPS34 more prominently in the GTP-bound form (Christofori et al., 1999), while interaction with Rab7 is more prominent when Rab7 is in its inactive GDP-bound form (Stein et al., 2003). Therefore, activation of Rab7 reduces interaction of VPS34 on membranes providing a feedback control to PTDIns(3)P synthesis.

**COPI and Arf1**

A role for the coatomer COPI in endosome maturation was first suggested by the effects of brefeldin A, a drug that prevents Arf1 activation and thus COPI binding to membranes (Hunziker et al., 1992). It was found to cause massive tubulation and redistribution of endosomes as well as loss of ILVs (Lippincott-Schwartz et al., 1991; Tooze and Hollinshead, 1992). COPI and Arf1 are both present on EEs where they form a domain (Aniento et al., 1996).

The most convincing evidence for a role of COPI in endosome maturation comes from the analysis of a temperature-sensitive mutant of CHO cells called IdIF, which looses COPI subunit epsilon at non-permissive temperature (Guo et al., 1996). In addition to defects in the secretory pathway, IdIF cells are impaired in the endocytic pathway (Daro et al., 1997; Gu et al., 1997): EEs take the form of tubular clusters without vacuolar domains. Formation of ILVs is inhibited, and fluid phase accumulation is reduced. Sorting of EGF and lysosomal proteins to lysosomes is inefficient, and the sensitivity to acid-activated bacterial toxins and viruses is lost (Daro et al., 1997; Abrami et al., 2004).

A model to explain these effects has been proposed by Gruenberg and coworkers, who suggest that Arf1 binding to EEs is regulated by an unidentified membrane protein activated by the low lumenal pH (Clague et al., 1994; Aniento et al., 1996; Gu and Gruenberg, 1999). Arf1 in turn recruits the COPI subunits, which promote molecular sorting of membrane proteins, the formation of ILVs, and the ECVs. Now when it is clear that the main mediator for ILV formation is the ESCRT complex and that formation of LEs is driven by other mechanisms, it would seem important to revisit the role of Arf1 and COPI in endosome maturation.

**The formation of ILVs**

One of the most important processes in LE biogenesis is the formation of ILVs. It is critical for the selective sorting of membrane-associated cargo for degradation in lysosomes, and for the maturation and fate of LEs. There are several reasons for ILV formation. One is that by inclusion in ILVs, signalling receptors are inactivated because they are deprived of contact with the cytosol (Figure 7A). Lipids and membrane proteins are, moreover, delivered to lysosomes in a form easily accessible to the hydrolyases. Unlike the limiting membrane, which is covered by a coat of highly glycosylated proteins, such as LAMPS, the ILV membrane is devoid of a protective glycocalyx. In addition, ILVs in late compartments contain a phospholipid bis(monoacylglycerophosphate)-lysobisphosphatidic acid (BMP/LBPA), which is thought to promote lipid hydrolysis (Kobayashi et al., 1998). BMP/LBPA is negatively charged and therefore contributes to the recruitment of hydrolyases that are positively charged in an acidic milieu. An example is acid sphingomyelinase, which converts sphingomyelin in ILVs to ceramide (Kolter and Sandhoff, 2010). That yeast does not possess LAMPS or LBPA/BMP implies that there are other mechanisms to ensure coordinated degradation of ILVs and their contents.

Among the main players in ILV biogenesis are the ESCRT complexes (ESCRT-0, -I, -II, -III) and a number of accessory proteins such as the AAA-type ATPase VPS4 and Alix. These are individually recruited from the cytosol to the surface of endosomes. Since the ESCRT machinery and its functions has been reviewed on many occasions (Babst, 2005; Eden et al., 2009; Raiborg and Stenmark, 2009; Woodman, 2009; Hurley, 2010; Hurley and Hanson, 2010; Roxrud et al., 2010), we will only discuss some of the general aspects.

The role of the ESCRT machinery is to sort out among the cargo that enter EEs, a cohort of pre-tagged membrane proteins and their ligands, to segregate them into domains within the limiting membrane, and to generate inward-budding vesicles. Sorting is based on the presence of Ub-tags in the cytosolic domains of membrane proteins recognized by components of the ESCRT machinery through their various Ub-binding domains. Parts of the ESCRT machinery bind in addition to clathrin and PIs, which help to recruit the complexes to endosomal membranes. Ub-tagged cargo molecules include a variety of activated growth factor and other signalling receptors, adhesion molecules such as integrins, ion channels, and other proteins such as unassembled caveolin-1 oligomers (Staub et al., 1997; Vaccari and Bilder, 2005; Sorkin and Goh, 2008; Hayer et al., 2010; Lobert et al., 2010). Before vesicle closure, the Ub-tag is removed by deubiquitinating enzymes (DUBs) (Clague and Urbe, 2006).

Depletion of components of the ESCRT machinery results in fewer ILVs, and accumulation of cargo in endosomes with abnormal morphology (Rieder et al., 1996; Doyotte et al., 2005; Razi and Futter, 2006). EGF-induced formation of LEs is strongly reduced by the depletion of TSG101 (ESCRT-I), whereas the effect of Hrs (ESCRT-0) depletion is milder (Razi and Futter, 2006). In TSG101-depleted cells, unlike Vps24 (ESCRT-III)-depleted cells, the acidification of EGF-containing endosomes is perturbed (Bache et al., 2006). In mammalian cells and in yeast, the loss of TSG101/Vps23p induces formation of multicytanel endosomes, the so-called class E compartments (Rieder et al., 1996; Doyotte et al., 2005). Altogether, it is evident that among the ESCRT components TSG101 has a central role in the biogenesis of LEs.

Although in yeast the ESCRT machinery is essential for ILV biogenesis (Odorizzi et al., 1998), in higher organisms, ESCRT-independent mechanisms also exist. Depletion of factors of all four ESCRTs simultaneously causes a reduction in ILV filled LEs (Stoffers et al., 2009). However, this only affects the EGF-induced population, an EGF-independent population of LEs is largely unaffected. An example of ESCRT-independent ILV cargo is the melanosomal protein Pmel17, which relies on a luminal domain that enables its sorting and inclusion into ILVs, without cargo ubiquitination (Theos et al., 2006). Likewise, major histocompatibility complex
class II (MHC II) molecules in activated dendritic cells (DCs) are inserted into ILVs independently of ubiquitination (Buschow et al., 2009).

Factors proposed to be involved in ESCRT-independent ILV biogenesis include the lipids BMP/LBPA and ceramide, both of which have been demonstrated to spontaneously induce formation of internal vesicles in liposomes in vitro in the absence of any proteins (Matsuo et al., 2004; Trajkovic et al., 2008). For this, BMP/LBPA requires an acidic environment. Although not belonging to the ESCRT machinery, GGA proteins and the Tom1–Tollip complex have been implicated in the sorting of ubiquitinated cargo into LEs (Katoh et al., 2004; Puertollano and Bonifacino, 2004; Berger et al., 2007; Lauwers et al., 2009). However, due to similarity in structure to the ESCRT-0 component Hrs, and their capacity to interact with the ESCRT-I protein TSG101 (Puertollano and Bonifacino, 2004), it is likely that they act in a manner redundant with Hrs or that their role is upstream of the ESCRT machinery (Puertollano and Bonifacino, 2004).

ILV biogenesis is only partially dependent on factors that drive other events during endosome maturation. Lack of acidification does not seem to perturb ILV biogenesis, as in the absence of V-ATPase activity LEs are found to accumulate high numbers of ILVs (Vacca et al., 2010) (our unpublished observations). The increased number of ILVs, in this case, is probably due in part to decreased degradation. As mentioned earlier, overexpression of the constitutively active Rab5(Q79L) mutant does not block ILV formation or sorting of cargo into ILVs (Wegner et al., 2010). Similarly, Rab7 activity is not required as Rab7-depleted cells tend to have LEs with high numbers of ILVs (Vanl{"a}ndingham and Ceresa, 2009).

In contrast, the absence of RILP, a Rab7 effector, causes a dramatic decrease in ILV formation. There is evidence that RILP interacts with ESCRT-II, but the molecular consequences are inadequately understood (Progida et al., 2006, 2007; Wang and Hong, 2006). Although more prominently known for its role in establishing contact with dynactin/dynein motors and thereby regulating LE motility (Jordens et al., 2001; Johansson et al., 2007), RILP may have additional Rab7-independent functions. Possibly, it coordinates ILV biogenesis with endosome motility (Progida et al., 2007).

Deletion of the PI kinases VPS34 and PIKfyve/Fab1p both result in enlarged early/late hybrid endosomes with few ILVs (Odorizzi et al., 1998; Futter et al., 2001; Ikonomov et al., 2003a). Recruitment of some of the ESCRT proteins to endosomal membranes is mediated in part by their PtdIns(3)P and PtdIns(3,5)P(2) binding domains (Raiborg et al., 2001; Whitley et al., 2003; Slagsvold et al., 2005). However, the highly enlarged endosomes observed especially in PIKfyve mutant cells are not similar to those seen after depletion of the ESCRT machinery. For instance, depletion of a component of ESCRT-III, VPS24, which binds to PtdIns(3,5)P(2), causes a decrease in the size of LEs (Bache et al., 2006). Therefore, it is likely that the effects of PtdIns(3)P and PtdIns(3,5)P(2) depletion on ILV biogenesis extend beyond ESCRT function.

The lipids of ILVs are different from the limiting membrane. ILVs contain more cholesterol, sphingolipids, PtdIns(3)P, and BMP/LBPA. Cholesterol and sphingolipids are especially enriched in ILVs at early stages, whereas ILVs in older LEs and lysosomes contain high concentrations of BMP/LBPA and ceramide (Mobius et al., 2003). The decrease in cholesterol is due to extraction and transport to the ER through the action of NPC1 and NPC2, mutant forms of which cause Niemann-Pick disease, a lysosomal storage disorder in which cholesterol accumulates in LEs and endolysosomes (Karten et al., 2009). NPC2 is a soluble protein that extracts cholesterol from ILVs, while NPC1 resides in the limiting membrane and transfers cholesterol across.

Cholesterol extraction from ILVs is also dependent on sphingolipid degradation and formation of ceramide (Kolter and Sandhoff, 2010). Therefore, enrichment of sphingolipids also leads to cholesterol accumulation. The amount of BMP/LBPA correlates with the total level of cholesterol in cells, presumably due to the role of BMP/LBPA in controlling endosomal storage of cholesterol (Chevallier et al., 2008). The accumulation of cholesterol and sphingolipids causes severe disturbances in the endolysosomal pathway (Lebrand et al., 2002; Sobo et al., 2007; Vittier et al., 2010).

PtdIns(3)P in ILVs disappears during later phases most probably due to degradation or modification by kinases/phosphatases (Gillooly et al., 2000; Wurmser and Emr, 1998). ILVs that contain EGFR and PtdIns(3)P occur in LEs distinct from those that have BMP/LBPA (Gillooly et al., 2000; Kobayashi et al., 2002). This suggests that different populations of LEs exist, supported by the observation that in the absence of ESCRT machinery, a population of LEs still contains ILVs, devoid of EGFR (Stuffers et al., 2009).

Acidification

The luminal pH of endocytic organelles and lysosomes is acidic, and acidification and its regulation constitute an important part of endosome maturation. EEIs have a pH in the 6.8–6.1 range, LEs in the 6.0–4.8 range, and in lysosomes the pH can drop to values around 4.5 (Maxfield and Yamashiro, 1987). The low pH not only provides a better environment for hydrolytic reactions, but it is also essential for membrane trafficking, for the sorting and routing of cargo, for the inactivation of internalized pathogens, etc. The difference in extracellular and EE pH (or between the TGN and LEs) provides the asymmetry needed to allow receptors to bind ligands in one compartment and release them in the other. Thus, the progressively decreasing pH in the endocytic pathway can provide incoming cargo a ‘sense’ as to their location within the pathway. This is most clearly illustrated by viruses and bacterial toxins that have a relative sharp pH threshold adjusted so that they are specifically activated when they arrive in EEIs, in LEs, or in lysosomes (Mercer et al., 2010).

The V-ATPases are large, complex proton pumps with a membrane-associated V0 complex that serves as a transmembrane pore for protons, and a soluble cytosolic V1 complex that binds and hydrolyzes the ATP (Forgac, 2007; Marshansky and Futai, 2008). The regulation of luminal pH involves adjustment of the V-ATPase concentration in the membrane, selection of V-ATPase isoforms, as well as the association and dissociation of the V0 and V1 complexes (Trombetta et al., 2003; Kane, 2006; Lafourcade et al., 2008). Since V-ATPases generate a positive inside membrane potential, acidification is also affected by a variety of independent factors such as Na⁺/K⁺ ATPase, and channels for the influx of counter ions such as Cl⁻ or efflux of cations such as Ca²⁺, Na⁺, and K⁺ (Huynh and Grinstein, 2007;
Jentsch, 2008; Marshansky and Futai, 2008). Since V-ATPase partition into detergent resistant membrane domains, its location is sensitive to the concentration of cholesterol (Marshansky and Futai, 2008).

The pH drops rapidly by about 0.5 pH units after LE formation and keeps decreasing until fusion occurs with a lysosome. It is possible that one reason for the initial drop is that V-ATPases are predominantly localized in the vacuolar domains of EEs and therefore become concentrated in LEs. There is also evidence that the V1 complexes interact more efficiently with V0 in LEs generating a larger fraction of functional pumps (Lafourcade et al., 2008).

Three classes of inhibitors are frequently used to elevate the pH in endosomes and other acidic organelles. Lysosomotropic weak bases (NH$_4$Cl, chloroquine, methylamine, etc.) elevate the pH by entering the acid compartment in their unprotonated form and titrating the pH upwards (Ohkuma and Poole, 1978). Carboxylic ionophores, such as monensin and nigericin, cause electroneutral exchange of protons across the membrane against other monovalent cations (Reijngoud et al., 1976). They do not affect the membrane potential, but like lysomotropic weak bases, they cause vacuolization. Finally, bafilomycins and concanamycins are potent inhibitors of the V-ATPase (Bowman et al., 1988). Like the other agents, they rapidly elevate the pH in acidic organelles of the cell.

In addition to elevating the luminal pH, these agents have other effects, which renders interpretation of results often complicated (Weisz, 2003). Although causing tubulation of EEs, bafilomycin A usually has no dramatic effect on the recycling pathway through EEs unless recycling depends on acid-mediated dissociation of receptor/ligand complexes (Baravalle et al., 2005). However, transfer of cargo from EEs to LEs is blocked in most cases indicating a defect in endosome maturation (Clague et al., 1994; D’Arrigo et al., 1997; Bayer et al., 1998; Baravalle et al., 2005). This effect may be somewhat cell-type dependent because one study concluded that the block occurs in LE to lysosome fusion stage (van Weert et al., 1995).

Gruenberg and colleagues have proposed that the detachment of LEs (or ECVs) from EEs requires low luminal pH. The suggested mechanism involves regulated binding of Arf1, which in turn mediates binding of COPI to the cytosolic surface of endosomes (Aniento et al., 1996; Gu and Gruenberg, 1999). There is independent evidence that Arf1 binding to microsomes in vitro increases when the luminal pH is reduced (Zeuzem et al., 1992). Moreover, as already mentioned, it was recently reported that isolated endosomes can form ILVs in vitro, a process reduced by both bafilomycin A and monensin (Falguieres et al., 2008).

It remains unclear, however, how the pH in the lumen can be sensed on the cytosolic surface of the endosome. The presence of a trans-membrane pH-sensor has been proposed. One possible sensor is the V-ATPase itself. It is involved in recruiting ARNO (a GEF for Arf6 GTase) to the cytosolic surface of endosomes (Hurtado-Lorenzo et al., 2006). It is interesting in this context that bafilomycin A, which inhibits the V-ATPase directly, blocks formation of LEs. Lysosomotropic weak bases and carboxylic ionophores have a similar effect on luminal pH, but generally do not have dramatic effects on the generation of LEs or on the transport of cargo to lysosomes (Ascoli, 1984; Ukkonen et al., 1986; Clague et al., 1994).

In addition to acidification, the ionic environment in endosomes undergoes other changes that may be important for maturation and function. These have been recently reviewed (Scott and Gruenberg, 2011). The calcium concentration drops initially, but seems to recover late in the pathway. There is evidence that a calcium channel and the efflux of calcium may be important both for fusion with lysosomes and for reformation of lysosomes from endolysosomes (Pryor et al., 2000; Luzio et al., 2007a). The sodium concentration drops while potassium increases. Serving as a counter ion during acidification, chloride ions increase during maturation. For a detailed review of organelle acidification and related topics, the reader is referred to Weisz (2003).

**Endosome motility**

Endosomes are motile, and their movements tightly linked to their stage of maturation and function. In addition, it is clear from recent data that location in the cytoplasm, measured as distance from the nucleus, is a key parameter that influences number, size, and cargo contents of endosomes consistent with the spatio-temporal progression of endosome maturation (Collinet et al., 2010). Many small endosomes in the periphery are replaced later by fewer, larger endosomes close to the nucleus. During formation in the cell periphery, EEs are subject to slow, short-range back and forth movements, but as they mature, many of them undergo rapid long-range saltatory oscillations with a net movement towards the perinuclear region, where the majority of lysosomes are localized. The centripetal movement is mediated by both kinesin and dynein motor proteins along microtubules that radiate out from the microtubule organizing centre. Conversion to LEs can occur in the periphery, in transit, or in the perinuclear region (Nielsen et al., 1999; Bananis et al., 2000; Hoepfner et al., 2005; Soldati and Schliwa, 2006; Driskell et al., 2007; Loubery et al., 2008).

Disruption of microtubules causes the dispersal of LEs and lysosomes throughout the cytoplasm (Bayer et al., 1998). At the same time, maturation of endosomes is delayed, as Rab5 persists on endosomes together with Rab7 (Vonderheide and Helenius, 2005). Inhibiting dynein has a similar effect; Tf and EFG separation and loss of Rab5 is delayed (Driskell et al., 2007). Interestingly, although endosome acidification to pH 6.0 has been reported to occur mainly at the perinuclear area after microtubule-mediated transport (Lakadamyali et al., 2003), disruption of microtubules by nocodazole treatment does not seem to inhibit initial acidification of endosomes (~pH 6.0) (Mesaki et al., 2011). However, it does prevent cargo from reaching compartments with lower pH (~pH 5.0) found in mature LEs and lysosomes (Bayer et al., 1998). Consistent with this, nocodazole treatment delays cargo degradation (Mesaki et al., 2011).

Motility of endosomes depends on dynein and kinesin motors, providing opposing forces that move endosomes in opposite directions (Murray et al., 2000; Soppina et al., 2009) (Figure 6A). Why do endosomes undergo such oscillatory motion on microtubules? Studies indicate that kinesin and dynein motor activity is required for fission of EEs and sorting of cargo into degradative and recycling compartments (Bananis et al., 2000; Hoepfner et al., 2005; Driskell et al., 2007). Evidence also points to a role for these motor proteins in fusion of endosomes with each other during different
LEs and lysosomes localize. (A) LEs are transported on microtubules (MT) in a bidirectional manner, by the help of both kinesin and dynein motor proteins. Net movement is towards the microtubule organizing centre (MTOC), located in the perinuclear region of the cell, where most of the LEs and lysosomes localize. (B) Schematic of LE attachment to dynein motor through RILP. Activated Rab7 binds to its effectors RILP and ORP1L. Homodimeric RILP then recruits dynein–dynactin motor complex through the p150(Glued) subunit. The complex has to additionally interact with the dynein membrane receptor βIII spectrin (Johansson et al., 2007). This allows transport of cargo towards the MTOC. In the presence of low cholesterol on LE membranes, sensed by ORP1L, the complex is disassembled due to a conformational change of ORP1L, and resulting in inhibition of motility towards the MTOC (Rocha et al., 2009).

Kinesins constitute a large family of mainly plus-end-directed motor proteins (Hirokawa et al., 2009). Some of them have been implicated in EE and LE motility. KIF16B is a kinesin-3 motor protein responsible for plus-end-directed movement of EEs (Hoepfner et al., 2005). It attaches to EEs through its PtdIns(3)P binding PX domain when Rab5 is activated. KIF16B seems to alter the balance between degradation and recycling of cargo; depletion leads to a block of cargo recycling and an increase in cargo degradation whereas overexpression has the opposite effect. The kinesin-2 motor protein subunit KIF3A has been implicated in LE trafficking (Brown et al., 2005). Impairment of its activity results in clustering of LEs in the perinuclear region.

LE motility that brings the cargo to the vicinity of lysosomes in the perinuclear area is thought to be mainly dependent on minus-end-directed, dynein-dependent transport (Aniento et al., 1993; Bananis et al., 2005). In contrast to the large number of different kinesin motors, dynein is the only member of its family (Kardon and Vale, 2009). Organelles bind to dynein motors either directly or through adaptor protein complexes. Direct binding is mediated by the intermediate, light intermediate, and light chains of dynein. Indirect binding can be accomplished through the dynactin multiprotein complex. Attachment of LEs through dynactin is dependent on Rab7 activity (Jordens et al., 2001). Upon activation, Rab7-GTP binds its effectors RILP and the oxysterol-binding protein ORP1L that in turn recruit the dynactin subunit p150(Glued) through the dynactin receptor βIII spectrin (Johansson et al., 2007) (Figure 6B). This interaction mediates the minus-end-directed movement of LEs. Another Rab7 effector, FYCO1, has been reported to have the opposite function and to counteract the effects of RILP by mediating plus-end-directed LE and autophagosome motility (Pankiv et al., 2010). The authors propose that FYCO1 links Rab7-positive vesicles to kinesin motors, although direct interaction with kinesins is yet to be shown.

Recent findings indicate a dynactin independent linkage between LEs and dynein through a protein called Snapin, originally identified as a neuronal SNARE-binding protein and implicated in synaptic vesicle fusion (Ilardi et al., 1999; Pan et al., 2009). Snapin links LE cargo by binding directly to dynein intermediate chains. It has a crucial role in controlling LE retrograde transport and maturation in neurons (Cai et al., 2010). Deletion of Snapin in neurons results in accumulation of immature LEs, further highlighting the role of motility in coordinating endosomal maturation. The relationship between Snapin- and the dynactin/RILP-mediated LE transport remains unclear.

Cholesterol has been shown to have a role in the regulation of LE motility (Lebrand et al., 2002; Sugii et al., 2006). Accumulation of cholesterol, as seen in cells from Niemann-Pick C patients, impairs the bidirectional movement of LEs, and leads to their concentration in the perinuclear area of cells. This is partially explained by the cholesterol sensitizing ability of ORP1L, a LE-associated protein that dictates LE binding to the dynactin complex (Rocha et al., 2009).

A recent study contradicts the view of dynein as the major motor protein in transport of LEs towards the minus end of microtubules (Loubery et al., 2008). The authors argue for an indirect role in regulating the actin cytoskeleton. The actin cytoskeleton, well known for its various roles in endocytosis (Kaksonen et al., 2006), seems indeed to have a role in the endosomal degradative pathway. It has been long known that disruption of the actin cytoskeleton results in a block of cargo transport from EEs to LEs (Gruenberg et al., 1989); however, the actual function of actin has remained unclear. A series of recent studies have, however, shed some light on to the matter by indicating that Arp2/3-driven actin nucleation occurs on EEs and is required for a number of membrane fission processes (Derivery et al., 2009; Gomez and Billadeau, 2009; Morel et al., 2009; Duleh and Welch, 2010). These include sorting and vesicle fission in the recycling pathway, and cargo transport to the TGN and lysosomes. Members of the annexin family of proteins, such as annexins A2 and A8,
link actin to endosome membranes (Goebeler et al., 2008; Morel et al., 2009). Annexin A2 was shown to have a role in LE biogenesis, perhaps by regulating fission from EEs.

There is also evidence in both yeast and mammalian cells that actin plays a role in fusion processes that occur between LEs and lysosomes/vacuoles, phagosomes and lysosomes, as well as between autophagosomes and lysosomes (van Deurs et al., 1995; Jahraus et al., 2001; Eitzen et al., 2002; Kjeken et al., 2004; Lee et al., 2010). In yeast, where this process has been studied in more detail, it has been demonstrated that actin nucleation initiated during tethering/docking and persisting through membrane fusion, is induced by the Rho GTPases Rho1p and Cdc42p, which themselves are activated by the yeast Rab7 homologue, Ypt7p (Eitzen et al., 2001).

**Microautophagy, expulsion of exosomes, and backfusion**

The inwards budding of the endosomal membrane and the formation of ILVs not only detaches membrane components from the limiting membrane (Figure 7A), but also traps a small volume of cytosol. Is this used for targeting cytosolic components to the lysosome? Recent studies indicate that cells have indeed evolved different mechanisms for selective uptake of cytosolic proteins and nucleic acids into ILVs during LE formation.

Microautophagy is a process in which cytosolic components are taken up through invaginations of LEs, in both a selective and unselective manner (Figure 7B). Selective microautophagy involves the cytosolic chaperone hsc70 and the ESCRT-I and ESCRT-III complexes, which together allow uptake of cytosolic proteins that bear a KFERQ sequence motif (Sahu et al., 2011). This is thought to rely on the electrostatic association of hsc70 with phosphatidylserine in the cytosolic leaflet of the LE membrane. A similar process has, interestingly, been proposed for the inclusion of certain RNAs into ILVs (Valadi et al., 2007; Gibbings and Voinnet, 2010).

Exosomes are vesicles found in various body fluids that have their origin in LEs (Figure 7C). They are small (40–100 nm in diameter), and contain a single membrane. They are released when LEs fuse with the plasma membrane (Simons and Raposo, 2009; Von Bartheld and Altick, 2011). Their composition reflects that of ILVs, and their biogenesis is linked to endosome maturation. They have received considerable attention in recent years because there is increasing evidence that they may have a role in intercellular communication.

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**Figure 7** Function of LEs. (A) Cargo that needs to degraded is endocytosed and sorted into ILVs. This can be also used to silence signalling from receptors, normally exposed to the cytosol. (B) Microautophagy is used to bring cytosolic contents into LEs. (C) ILVs can be secreted as exosomes to the extracellular environment upon fusion of LEs with the plasma membrane. (D) ‘Backfusion’ of ILVs with the limiting membrane of LEs allows retrieval of membrane cargo or release of ILV contents into the cytosol.
The formation of exosomes depends on ESCRT function, although it is apparent that ESCRT-independent mechanisms may also exist. In immature DCs, MHC II is ubiquitinated, enters ILVs, and is targeted for degradation, while in mature DCs the MHC II containing ILVs are secreted as exosomes (Buschow et al., 2009). How the LEs fuse with the plasma membrane has been the focus of recent studies. In HeLa cells, Rab27a, Rab27b and their effectors Slp4 and Slac2b were found to promote fusion (Ostrowski et al., 2010). In oligodendrocytes, Rab35 and its GAPs, TBC1D10A-C have been implicated (Hsu et al., 2010).

There is also evidence, although still indirect, that ILVs can fuse with the limiting membrane of the LEs (Figure 7D). As a result of such 'backfusion', the membrane proteins in an ILV may become part of the limiting membrane of an endosome or lysosome from which they can recycle to the TGN or the plasma membrane and escape lysosomal degradation (van der Goot and Gruenberg, 2006). This phenomenon has been proposed for the recycling of MHC II in DCs, mannose-6-phosphate receptors, and tetranspannin proteins, all of which are found to be enriched in ILVs (Kobayashi et al., 1998, 2002; Kleijmeer et al., 2001; Trombetta and Mellman, 2005). Backfusion has also been suggested as a mechanism for delaying the release of anthrax toxin and incoming vesicular stomatitis virus capsids into the cytosol (van der Goot and Gruenberg, 2006). The fusion of ILVs with the limiting membrane of the LE or endolysosome is thought to be enhanced by BMP/LBPA and the ESCRT accessory protein Alix (Kobayashi et al., 2002; Abrami et al., 2004; Le Blanc et al., 2005).

In addition to regulating cargo trafficking, storage, and degradation, endosomes are used as signalling compartments (Sorkin and von Zastrow, 2009; Platta and Stenmark, 2011). Signalling occurs in early as well as late compartments. Organelle segregation allows spatio-temporal regulation, and endosome maturation is used for silencing and activation. K-Ras/MAPK and mTORC1-dependent signalling cascades are examples that occur on LEs (Teis et al., 2002; Nada et al., 2009; Flinn et al., 2010). Some signalling molecules undergo proteolytic activation in the degradative environment of LEs. For example, TLR9 requires cathepsin-mediated cleavage of its ectodomain to initiate signalling (Ewald et al., 2008). To what extent such signalling activities affect endosome maturation is receiving increasing attention (Sorkin and von Zastrow, 2009; Collinet et al., 2010). For instance, MAPK signalling on LEs has been found to regulate LE dynamics and cargo degradation (Teis et al., 2006).

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Perspectives

Endosomes have proven infinitely more complex than ever imagined in the early 1980s when their existence as a universal, pre-lysosomal compartment network was first demonstrated. Based on a wealth of studies in many different cell types and organisms, one can now appreciate these multifunctional organelles as a dynamic, highly adaptable system deeply embedded within the core fabric of cell life. As research in the field moves forward, it will be important to define the common mechanisms and concepts underlying endosome function and regulation. The maturation programme discussed above is coordinated so that many of the various changes are interdependent and cooperative. These and other central aspects of the endosome machinery can now be approached at the molecular as well as at a systems level in different organisms.

More work will be required to map the fate of incoming cargo including the activation and inactivation of receptors and signalling proteins. The endosomal system is vital as an extension of the plasma membrane in signalling and cell regulation. Endosomes play a fundamental role in numerous diseases and pathogenic states, and are therefore of critical medical significance. To us, the analysis of viruses and toxins that traverse the pathway is especially interesting, because these exploit the properties of the pathway in many elaborate ways. By the use of perturbants to modify endosome behaviour, it may possible to prevent infection and intoxication. Furthermore, the endosomes provide a much needed entry port for the introduction of membrane impermeant drugs, proteins, and nucleic acids into cells, a function that is increasingly important in gene therapy and medicine.

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Conflict of interest

The authors declare that they have no conflict of interest.
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