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Focus Review

Endosome maturation

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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.

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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 intralumenal 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 Altick, 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).

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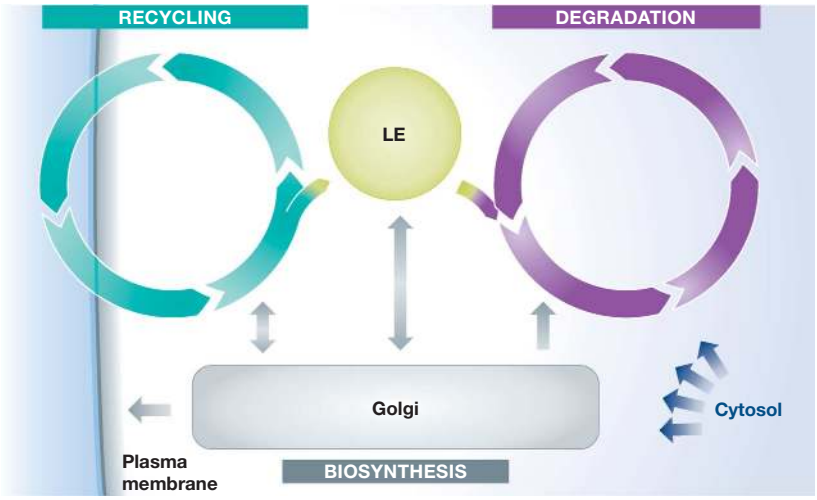

Figure 1 The basic elements of the endocytic machinery. The membrane organelles involve a *recycling circuit* (the plasma membrane (PM), the EEs, the recycling endosomes, and a variety vesicular carriers), a *degradation cycle* (lysosomes), and a connecting '*feeder*' *pathway* (LEs) from the recycling 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.

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 loose 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-

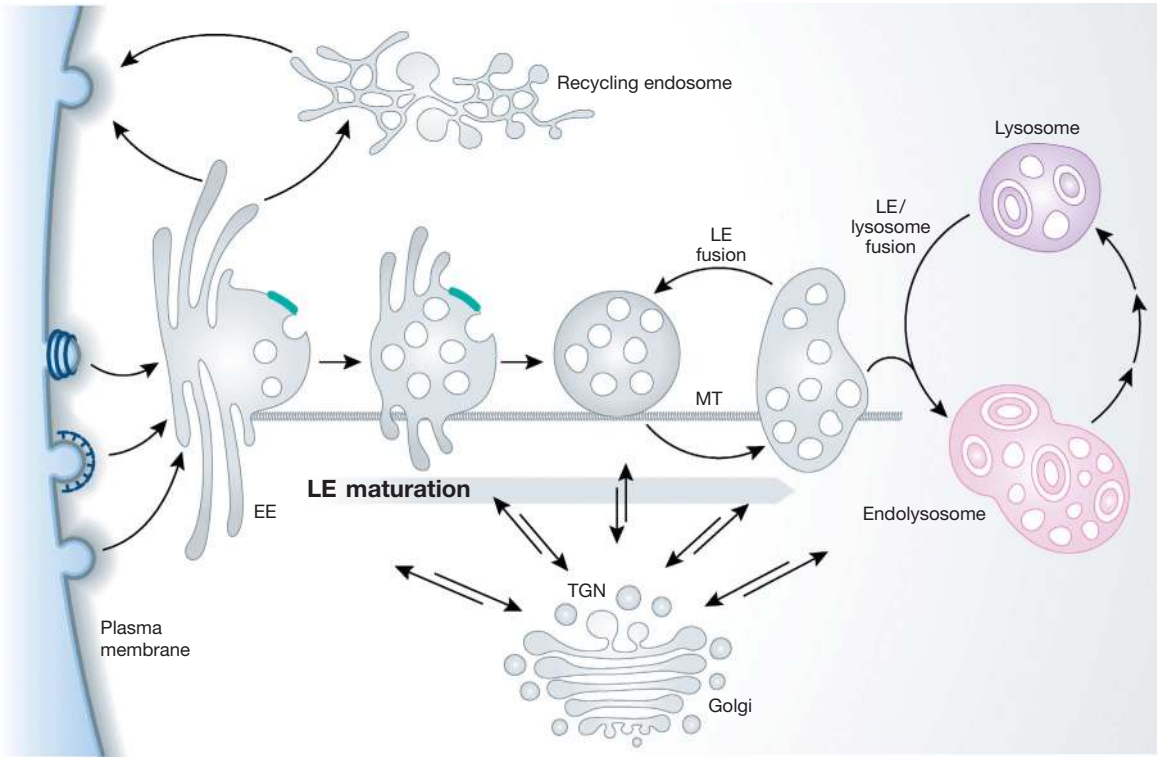

Figure 2 The endosome/lysosome system. The primary endocytic vesicles deliver their contents and their membrane to EEs in the peripheral cytoplasm. After a period of about 8–15 min during which the EEs accumulate cargo and support recycling to the plasma membrane (directly or via recycling endosomes in the perinuclear region), conversion of the EEs to LE takes place. Thus, as the endosomes are moving towards the perinuclear space along microtubules (MT), the nascent LE are formed inheriting the vacuolar domains of the EE network. They carry a selected subset of endocytosed cargo from the EE, which they combine *en route* with newly synthesized lysosomal hydrolases and membrane components from the secretory pathway. They undergo homotypic fusion reactions, grow in size, and acquire more ILVs. Their role as feeder system is to deliver this mixture of endocytic and secretory components to lysosomes. To be able to do it, they continue to undergo a maturation process that prepares them for the encounter with lysosomes. The fusion of an endosome with a lysosome generates a transient hybrid organelle, the endolysosome, in which active degradation takes place. What follows is another maturation process; the endolysosome is converted to a classical dense lysosome, which constitutes a storage organelle for lysosomal hydrolases and membrane components.

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 heterogenous 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

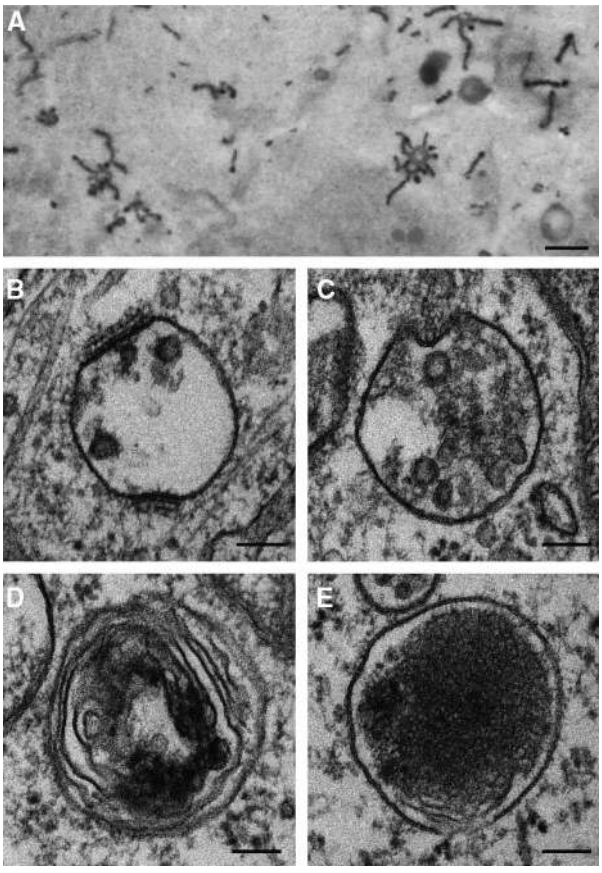

Figure 3 Morphologies of endosomes and lysosomes at the ultrastructural level. (**A**) Electron micrographs of peripherally located EEs containing HRP-conjugated Tf. They contain vacuolar and tubular domains. Courtesy of Tooze and Hollinshead (1992). Electron micrographs of (**B**) EE with clathrin lattices and a few ILVs; (**C**) LE, containing numerous ILVs; (**D**) endolysosome, with partial electron dense areas; and (**E**) lysosomes, with electron dense lumen. Images are all from HeLa cells that had been processed for thin section EM. Scale bars in (**A**): 500 nm and (**B**–**E**): 100 nm. Figure 3A is reproduced with kind permission from Rockefeller University Press;©2009 Rockefeller University Press. Originally published in *J Cell Biol* 118: 813–830. doi: 10.1083/jcb.118.4.813.

occurs at the level of EEs, maturing LEs, and possibly for some time after the fusion of LEs with lysosomes. At the endosome level, the sorting and vesicle formation for transport to the TGN depends on factors such as Rab7, Rab9, and the retromer complex (Bonifacino and Hurley, 2008; Pfeffer, 2009). The retromer is a multimeric complex composed of sorting nexins and other proteins recruited to the cytosolic surface of EEs and maturing LEs.

LEs and lysosomes

Mature LEs are typically round or oval and have a diameter of 250–1000 nm (Figure 3C). They have a low bouyant 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 $\geqslant$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, macropinosomes, 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 lumenal 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 tubules 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. Gruenberg and Stenmark (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 dynein-mediated pulling forces are critical for the separation of endosomal elements containing the recycling ligand Tf from the lysosome-targeted ligand 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 lumenal 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 Rabs, 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 lumenal pH drops from values above pH 6 to 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 Rab5/7 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 lumenal ionic environment. In addition to the drop in pH, there is an increase in Cl^-, and changes in Ca^{2+}, 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, phosphatydylinositide 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 (GDIs), 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 (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 Rabex-5 itself is monoubiquitinated and it is thought that a cycle of Ub binding and monoubiquitination regulates its recruitment. The GEF activity of Rabex-5 is promoted by 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.

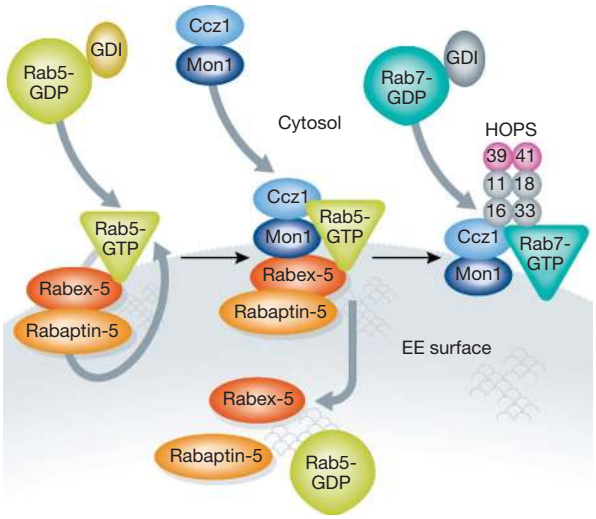

Figure 4 The Rab5/Rab7 switch during endosome maturation. Normally, Rab5-GDP and Rab7-GDP reside in the cytosol bound to its GDI. Rab5 is activated to its GTP-bound and membrane-associated form by the GEF Rabex-5 on EE membranes. The Rab5 effector Rabaptin-5 binds to Rabex-5 and promotes the activation of Rab5, thus forming a positive feedback loop in which more Rab5 molecules are activated and recruited. To initiate the Rab switch, Mon1/SAND-1 complexed with Ccz1 binds to Rab5, PtdIns(3)P, and Rabex-5, causing disassociation of Rabex-5 from the membrane. This in turn terminates the feedback loop, resulting in Rab5 inactivation and disassociation. The Mon1/SAND-1–Ccz1 complex promotes (directly or indirectly) the recruitment and activation of Rab7. Members of the HOPS complex (Vps11, Vps16, Vps18, Vps33, Vps39, and Vps41) are able to bind both Rab7 and the Mon1/SAND-1–Ccz1 complex. The HOPS complex mediates membrane tethering, needed for fusion with other LEs and lysosomes. Adapted from Cabrera and Ungermann (2010).

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* have 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 PtdInd(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 (Rab7) (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 RN-Tre and RAB GAP-5 (Lanzetti *et al*, 2000; Haas *et al*, 2005). However, the target of RN-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 PLEKHM1, 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 mammalians. 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-

tant 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

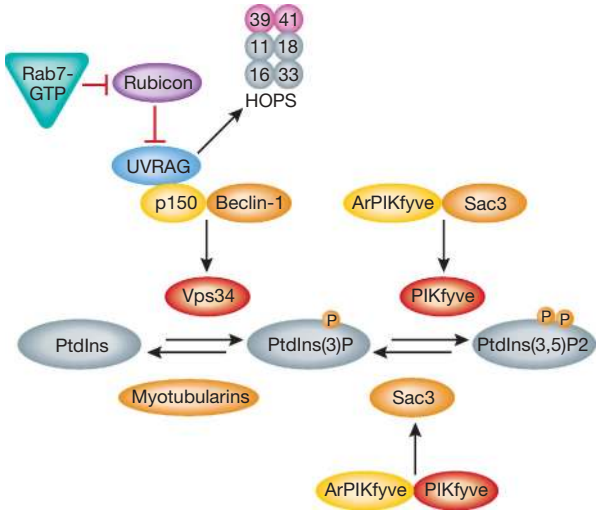

Figure 5 Phosphatidylinositide regulation on endosomes. On EEs, PtdIns(3)P is synthesized by the kinase VPS34, which forms a core complex together with p150 and Beclin-1. The complex binds on endosomes to UVRAG (Itakura *et al*, 2008), which is normally inhibited by a Rab7 effector, Rubicon (Sun *et al*, 2010). Once activated, Rab7 sequesters Rubicon from UVRAG, allowing it to activate the HOPS complex. Dephosphorylation of PtdIns(3)P is catalyzed by members of the Myotubularin family. The kinase responsible for conversion of PtdIns(3)P to PtdIns(3,5)P(2) is PIKfyve (Fab1p) (Gary *et al*, 1998; Ikonomov *et al*, 2002). It forms an active complex with its activator ArPIKfyve (Vac14p) and the phosphatase Sac3 (Fig4p). This complex is required for both the kinase and the phosphatase activities (Sbrissa *et al*, 2008; Ikonomov *et al*, 2009).

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; Jefferies *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; Jefferies *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 *et al*, 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 spatiotemporal 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 (Christoforidis *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 ldlF, which looses COPI subunit epsilon at non-permissive temperature (Guo *et al*, 1996). In addition to defects in the secretory pathway, ldlF 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 hydrolases. 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(monoacylglycero)phosphate/lysobisphoshatidic 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 hydrolases 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 multicisternal 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 (Stuffers *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 (Vaccari *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 (Vanlandingham 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; Vitner *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 lumenal pH of endocytic organelles and lysosomes is acidic, and acidification and its regulation constitute an important part of endosome maturation. EEs 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 EEs, 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 lumenal 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 Ca^{2+}, 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_4Cl, 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 lumenal 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 lumenal 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 lumenal 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 GTPase) 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 lumenal 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 (Vonderheit 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

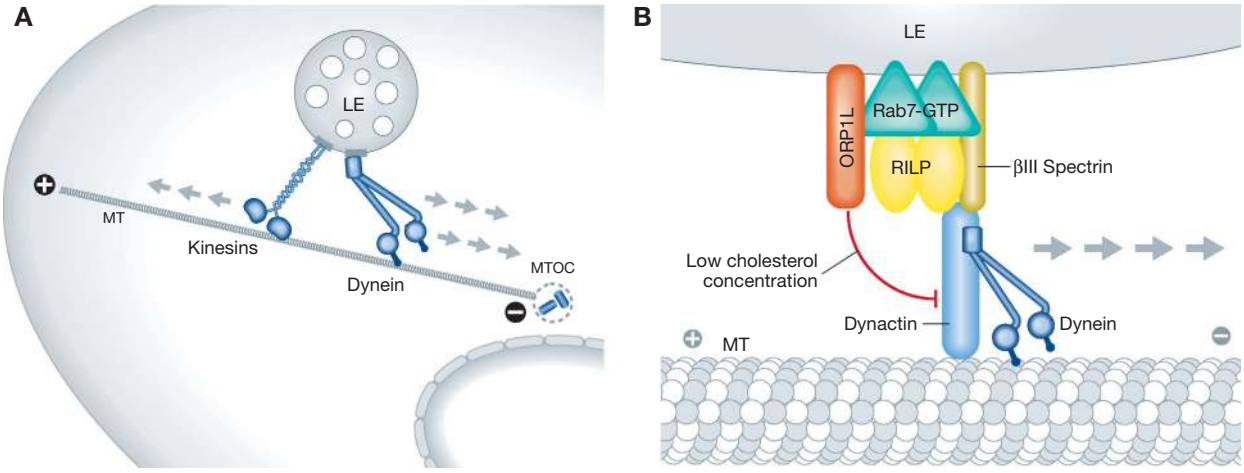

Figure 6 LE motility. (**A**) LEs are transported on microtubules (MT) in a bidirectional manner, by the help of both kinesin and dynein motor proteins. Net movement is towards the microtubule organizing centre (MTOC), located in the perinuclear region of the cell, where most of the LEs and lysosomes 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).

stages of maturation (Bomsel *et al*, 1990; Aniento *et al*, 1993; Driskell *et al*, 2007).

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*, 2004). 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 sensing 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.

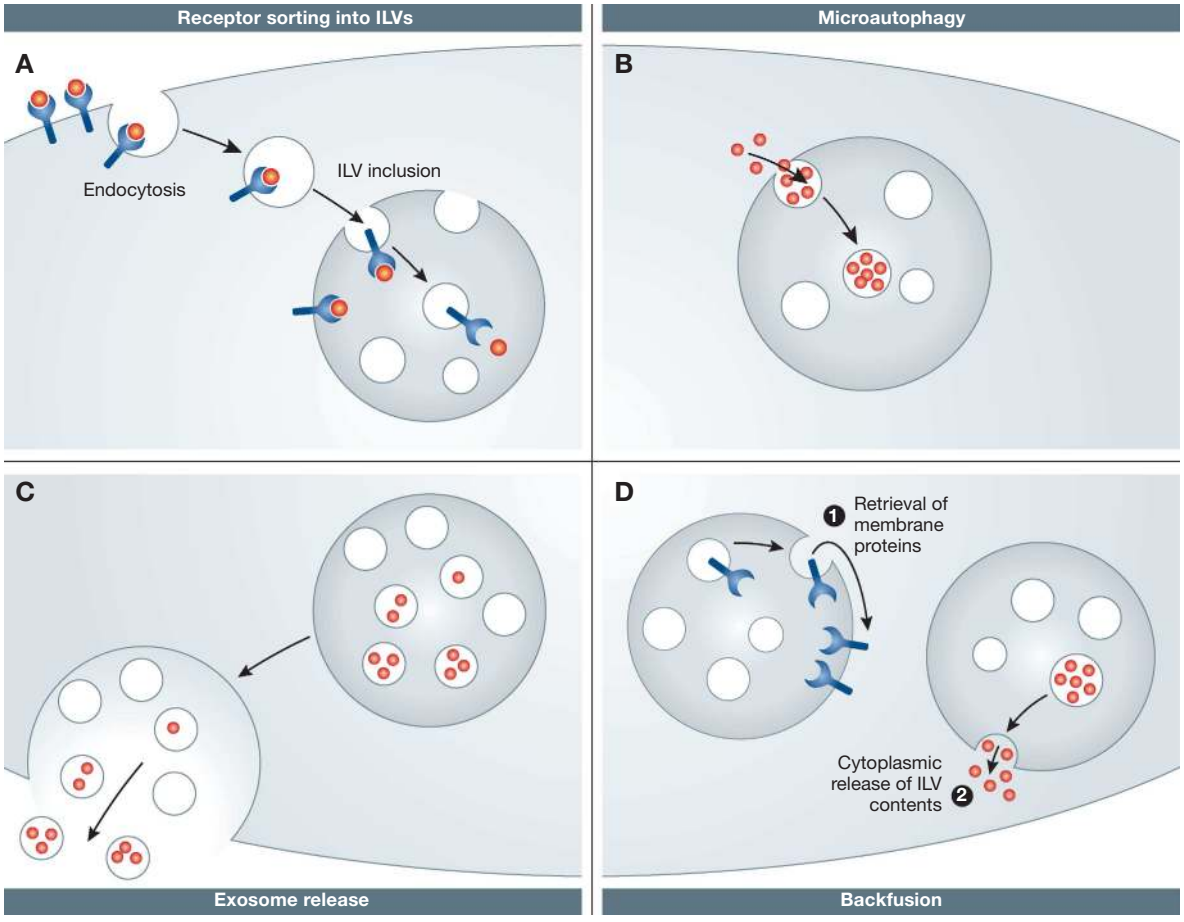

Figure 7 Function of LEs. (**A**) Cargo that needs to degraded is endocytosed and sorted into ILVs. This can be also used to silence signalling from receptors, normally exposed to the cytosol. (**B**) Microautophagy is used to bring cytosolic contents into LEs. (**C**) ILVs can be secreted as exosomes 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 tetraspannin 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 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).

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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