The Journal of Physiology

TOPICAL REVIEW

Regulation of membrane trafficking by signalling on endosomal and lysosomal membranes

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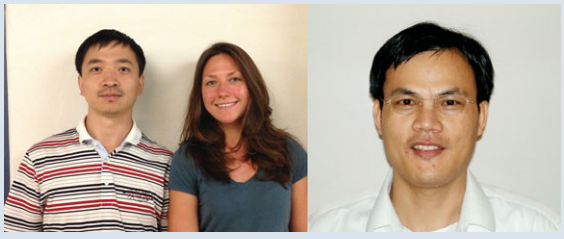
Abstract Endosomal and lysosomal membrane trafficking requires the coordination of multiple signalling events to control cargo sorting and processing, and endosome maturation. The initiation and termination of signalling events in endosomes and lysosomes is not well understood, but several key regulators have been identified, which include small GTPases, phosphoinositides, and Ca^{2+}. Small GTPases act as master regulators and molecular switches in a GTP-dependent manner, initiating signalling cascades to regulate the direction and specificity of endosomal trafficking. Phosphoinositides are membrane-bound lipids that indicate vesicular identities for recruiting specific cytoplasmic proteins to endosomal membranes, thus allowing specificity of membrane fusion, fission, and cargo sorting to occur within and between specific vesicle compartments. In addition, phosphoinositides regulate the function of membrane proteins such as ion channels and transporters in a compartment-specific manner to mediate transport and signalling. Finally, Ca^{2+}, a locally acting second messenger released from intracellular ion channels, may provide precise spatiotemporal regulation of endosomal signalling and trafficking events. Small GTPase signalling can regulate phosphoinositide conversion during endosome maturation, and electrophysiological studies on isolated endosomes have shown that endosomal and lysosomal Ca^{2+} channels are directly modulated by endosomal lipids. Thus trafficking and maturation of endosomes and lysosomes can be precisely regulated by dynamic changes in GTPases and membrane lipids, as well as Ca^{2+} signalling. Importantly, impaired phosphoinositide and Ca^{2+} signalling can cause endosomal and lysosomal trafficking defects at the cellular level, and a spectrum of lysosome storage diseases.



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DOI: 10.1113/jphysiol.2013.258301

(Received 7 May 2013; accepted after revision 16 July 2013; first published online 22 July 2013)
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Abbreviations Atg, autophagy-related gene; EEA1, early endosome antigen 1; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GECIs, genetically encoded Ca^{2+} indicators; GEF, guanine nucleotide exchange factor; KO, knockout; mTOR, mammalian or mechanistic target of rapamycin; NAADP, nicotinic acid adenine dinucleotide phosphate; PATs, proton-assisted amino acid transporters; PI, phosphatidylinositol; SNARE, soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor; TRPML, transient receptor potential cation channel, mucolipin subfamily; TPC, two-pore channel; V-ATPase, vacuolar-type H^+-ATPase; VAMP, vesicle-associated membrane protein.

Introduction

In eukaryotic cells, membrane trafficking through the endocytic pathway (endocytic trafficking) is an ongoing process that requires the cooperation of many proteins, membrane lipids and ions, and defects in trafficking can lead to a number of endosome and lysosome-related human diseases. Endocytic trafficking involves a series of steps including endocytosis, cargo sorting and processing, intracellular membrane fusion and fission, vesicle mobility, and exocytosis (Fig. 1). The purpose of this review is to highlight recent studies and synthesize research findings on how signalling by small GTPases, phosphoinositides, and Ca^{2+} regulate endosomal and lysosomal trafficking events. We regret that we are unable to cite every paper related to the ideas in this review. As a result, we cite only the most recent review papers and primary research findings to provide an update on the topics discussed. We begin with a brief overview of endocytic trafficking before discussing key regulators of membrane trafficking, including small GTPases, phosphoinositides, and Ca^{2+} in more depth.

Early endosomes

Endocytic trafficking begins with the uptake of extracellular substance through the formation of a nascent endocytic vesicle that is excised from the plasma membrane (Fig. 1 (a)). The four types of endocytosis, that is, clathrin-mediated (McMahon & Boucrot, 2011), caveolar (Parton & del Pozo, 2013), phagocytosis (Flannagan *et al.* 2012) and macropinocytosis (Lim & Gleeson, 2011), have been excellently reviewed elsewhere. With the exception of macropinocytosis, endocytic events are mediated by specific receptors on the cell membrane (for review, see Huotari & Helenius, 2011). Nascent endocytic vesicles then undergo maturation and sorting processes to become early endosomes (Fig. 1 (b)). Early endosomes commonly consist of a large vacuolar domain and multiple tubular domains (Cullen, 2011), through which most plasma membrane receptors are sorted and recycled back to the plasma membrane via the pathway of the recycling endosome (Fig. 1 (c); for review, see Grant & Donaldson, 2009; Hsu & Prekeris, 2010). The cargo destined for further transport and/or degradation is retained inside or on the membranes of early endosomes. Besides cargo taken up through endocytosis, the vacuolar domains of early endosomes are also capable of accepting cytosolic or membrane-bound cargo through a protein complex called endosomal sorting complex required for transport (ESCRT) to sort ubiquitinated cargo proteins into multi-vesicular bodies (MVBs; Babst, 2011).

Late endosomes

After sorting in the early endosomes, endocytic vesicles undergo maturation and further acidification into late endosomes (Fig. 1 (d)). Late endosomes are derived from the vacuolar domain of the early endosome and are often referred to as multi-vesicular bodies (MVBs) because they contain many intraluminal vesicles (Fig. 1; Huotari & Helenius, 2011). Transport vesicles from the Golgi apparatus carry membrane receptors that recognize lysosome-destined hydrolytic enzymes, including mannose 6-phosphate receptor (M6PR), sortilin and lysosomal integral membrane protein 2 (LIMP2), from the Golgi to late endosomes. Upon arrival, the hydrolytic enzymes are dissociated from the receptors due to the acidic environment in late endosomes, while membrane receptors undergo retrograde trafficking back to the Golgi for reuse in the next round of delivery (Fig. 1 (e); Braulke & Bonifacino, 2009; Coutinho *et al.* 2012).

Lysosomes

Late endosomes can mature into lysosomes through direct acidification by the vacuolar-type H^+-ATPase (V-ATPase) proton pump or through fusion with existing lysosomes (Fig. 1 (f)). Proteins found on the membranes of late endosomes and lysosomes are similar. The primary difference between late endosomes and lysosomes is their luminal pH. While late endosomes have a luminal pH of 5.0–6.0, lysosomal pH is 4.5–5.0. The highly acidic pH in the lysosome lumen facilitates efficient hydrolysis of cargo

delivered to lysosomes (Mindell, 2012). Extracellular and plasma membrane-derived cargo is delivered to lysosomes via the endocytic pathway. In addition to late endosomes, lysosomes also acquire cytoplasm-derived cargo from autophagosomes during autophagy (Mizushima & Komatsu, 2011). Autophagy, mediated by a set of autophagy-related genes (Atgs; Mizushima *et al.* 2011), involves the formation of double membrane-bound autophagosomes that contain large cargo such as damaged organelles (Choi *et al.* 2013). Autophagosomes then fuse with lysosomes to form autolysosomes, in which autophagic substrates are broken down for reutilization (Fig. 1 (g); Mizushima & Komatsu, 2011). Subsequently, the digested products from lysosomes are either released into the cytosol via membrane transporters and channels, or transported to the Golgi via retrograde trafficking for reutilization. However, only a few lysosomal transport proteins have been well characterized to date (Schwake *et al.* 2013). For example, lipid and cholesterol export from the lysosome is regulated by lysosomal protein NPC1 (Chang *et al.* 2006). Likewise, proton-assisted amino acid transporters (PATs) on lysosomal membranes couple the H^+ gradient, driven by the lysosomal V-ATPase, to amino acid transport into the cytosol for reutilization by the cell (Boll *et al.* 2004; Thwaites & Anderson, 2011). PAT1, in a complex with Rag GTPases on lysosome membranes, plays important roles in sensing amino acid levels in the lysosome lumen (Ogmundsdottir *et al.* 2012), and can regulate lysosomal recruitment of mammalian or mechanistic target of rapamycin (mTOR) to promote cell growth (Heublein *et al.* 2010). There are still many unanswered questions regarding how lysosomal membrane proteins sense and export degraded products, and is a field ripe with opportunity for future research.

Although conventionally believed to be the 'end point' of endosomal trafficking, membrane fusion and fission events do occur in lysosomes and autolysosomes. First, lysosomes undergo exocytosis in most, if not all, cell types (Fig. 1 (h); Reddy *et al.* 2001). The physiological functions of lysosomal exocytosis may include cell migration (Colvin *et al.* 2010), transmitter release (Dou *et al.* 2012), large

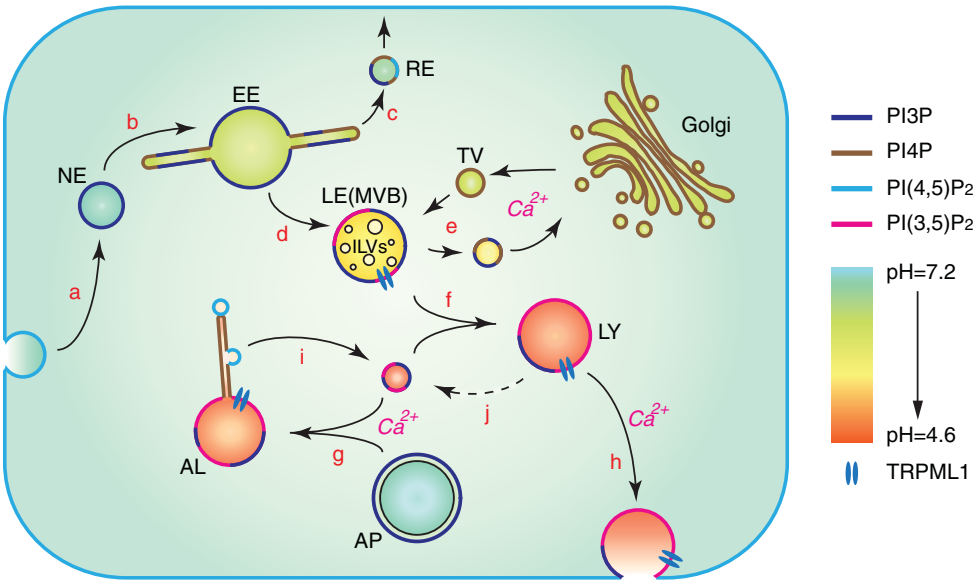

Figure 1. Endosomal trafficking network
A schematic view of the endosomal trafficking network. Vesicular pH and predominant membrane phosphoinositides on different compartments are represented by different colours. During endocytosis, a piece of the plasma membrane is excised and enters the cytosol in the form of a nascent endosome (NE; a). Nascent endosomes fuse with each other (b) and recruit early endosomal proteins to become early endosomes (EE; b). Membrane receptors are sorted and recycled back to the plasma membrane through recycling endosomes (RE; c). Material destined for degradation is passed on to the late endosomes (LE; d), which are also referred to as multi-vesicular bodies (MVB) due to the intraluminal vesicles (ILVs) that contain membrane proteins sorted for degradation. Hydrolytic enzymes are transported to late endosomes through transport vesicles (TV) from Golgi (e). Membrane receptors carrying the enzymes are shuttled back to Golgi through retrograde transport. Late endosomes mature into lysosomes (LY) either through further acidification, or through fusion with existing lysosomes (f). During starvation or when organelles are damaged, lysosomes also accept cargo from autophagosomes (AP) carrying damaged organelles or cytosolic material for degradation (g). The resulting autophagic lysosomes (AL) are usually larger than endocytic lysosomes. Lysosomes can undergo Ca^{2+}-dependent exocytosis (h). Lysosomal membrane proteins are recycled from autophagic lysosomes by fission processes that happen on tubular structures (i). The mechanism of recycling of membrane proteins from endocytic lysosomes has yet to be established (j).

particle phagocytosis (Czibener *et al.* 2006), membrane repair (Reddy *et al.* 2001), and release of hydrolytic enzymes into extracellular space (Czibener *et al.* 2006). Second, with the aid of high-resolution live imaging, lysosomes are also observed to undergo budding off through very dynamic tubular structures (Fig. 1 (i); Yu *et al.* 2010). This type of tubular fission is most active when autophagy is induced upon prolonged starvation, and is referred to as autophagic lysosome reformation. Lysosome reformation rapidly increases the number of lysosomes when there is a high demand for digestion (Chen & Yu, 2013), and is activated when the digested material in auto-lysosomes is released from the lumen through membrane transporters into the cytosol (Yu *et al.* 2010; Rong *et al.* 2011).

Regulators of endosomal trafficking

Although the detailed mechanisms by which cells regulate endocytic trafficking are still being elucidated, several key regulators have been identified. This review will focus on three key regulators, including small GTPases, phosphoinositides and Ca^{2+}.

Small GTPases

Several subfamilies of the Ras small GTP-binding protein superfamily regulate intracellular membrane trafficking, with the Rab and Arf/Sar subfamilies being the most well studied (Mizuno-Yamasaki *et al.* 2012). Rab proteins are the largest subfamily of the Ras GTPases, with more than 60 known Rabs in humans (Rojas *et al.* 2012; see also Galvez *et al.* 2012). Rab GTPases usually regulate sequential events in the endosome maturation process, including transport of vesicles along the cytoskeleton and vesicle fusion (Mizuno-Yamasaki *et al.* 2012). Arf proteins are the most divergent subfamily of Ras GTPases (Rojas *et al.* 2012) and typically control vesicle budding (Mizuno-Yamasaki *et al.* 2012). Ral GTPases are found only in animal cells and play a role in both endocytosis and exocytosis (Wu *et al.* 2008). There are more than 20 known Rho GTPases in humans, which function in a variety of processes including vesicle trafficking, as well as cell polarity, cell migration and virus transport (Chi *et al.* 2013).

The association and dissociation of particular small GTPase proteins with specific vesicle membranes is one method of establishing membrane identity in vesicle trafficking (Pfeffer, 2013). GTPases have two functional states: an active, GTP-bound state, and an inactive, GDP-bound state. Guanine nucleotide exchange factors (GEFs) catalyse the exchange of GDP for GTP which initiates signalling activity. Rab GEFs have been shown to be sufficient to recruit Rab proteins to specific endosomal membranes, suggesting that GEFs play a central role in establishing the localization of Rabs in endosomal trafficking (Blumer *et al.* 2013). After binding to GTP, GTPases bind to effectors that stimulate their activity and/or recruit them to the appropriate site of action. GTPases can then recruit additional effectors that can help to change the functional identity of the membrane and direct trafficking events (Mizuno-Yamasaki *et al.* 2012). GTPase-activating proteins (GAPs) mediate inactivation by catalysing the hydrolysis of GTP to GDP, thus terminating signalling (Stenmark, 2009).

The 'on and off' nature of GTPases makes them ideal regulators of membrane trafficking (Mizuno-Yamasaki *et al.* 2012). Indeed, Rab and Arf GTPases are involved in almost all aspects of vesicular transport. The intracellular location of specific members of the Rab protein family is essential to their regulation of membrane trafficking (Hutagalung & Novick, 2011). For example, the switch of Rab5 to Rab7 is a key initiating event that drives maturation from early endosomes to late endosomes (Poteryaev *et al.* 2010). Rabs also recruit tethering factors that dock vesicles prior to fusion (Fig. 2*A*). Tethering factors accelerate and increase the efficiency of fusion, and regulate the formation of soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) complexes (Yu & Hughson, 2010). The tethering factors early endosome antigen 1 (EEA1) for early endosomes and the multisubunit homotypic fusion and protein sorting (HOPS) complex for late endosomes are recruited by Rab5 and Rab7, respectively (Yu & Hughson, 2010). Conversely, tethering factors can also act as Rab effectors (Yu & Hughson, 2010).

The coordination of GTPase activity with other intracellular signalling events allows for fine-tuned spatiotemporal regulation of trafficking events. GTPases are networked to one another through coordinated hydrolysis and association/dissociation with vesicle membranes, as well as through common effectors (Mizuno-Yamasaki *et al.* 2012). Both Rab GTPases and intracellular phosphoinositides (discussed in detail below) help to maintain the identity of intracellular vesicles in order to recruit specific effectors. The coordination of Rab GTPases and phosphoinositides provides additional specificity and directionality in trafficking steps (Jean & Kiger, 2012). First, GTPases can recruit enzymes that regulate phosphoinositides and phosphoinositides can recruit Rab regulators. Secondly, downstream effectors can have co-requirements for specific Rab GTPase and phosphoinositide combinations. Third, enzymes involved in the synthesis and phosphorylation of phosphoinositides have been shown to have GAP and GEF activity for specific Rab GTPases (Jean & Kiger, 2012). For details regarding the function and regulation of small GTPases in membrane traffic, the reader is encouraged to consult recent extensive and excellent reviews (Stenmark, 2009; Hutagalung &

Novick, 2011; Jean & Kiger, 2012; Mizuno-Yamasaki *et al.* 2012; Pfeffer, 2013).

Phosphoinositides

Phosphoinositides are low-abundance membrane lipids that are localized to specific compartments in the endocytic pathway (Di Paolo & De Camilli, 2006). There are seven phosphoinositides based on the combination of phosphorylation at the 3′, 4′ and 5′ positions of the inositol head group: phosphatidylinositol 3-phosphate or PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, and PI(3,4,5)P_3. PI(3)P is found on the cytoplasmic sides of early and late endosomes and newly formed phagosomes and autophagosomes (Poccia & Larijani, 2009). PI(4)P is mainly localized on the cytoplasmic leaflet of Golgi membranes (Santiago-Tirado & Bretscher, 2011). The localization of PI(5)P is the least well studied; however, it has been suggested that it plays a role in nuclear signalling and membrane dynamics (Mayinger, 2012). PI(3,4)P_2 and PI(3,4,5)P_3 are generated transiently upon activation of plasma membrane receptors. PI(4,5)P_2 is relatively abundant on the plasma membrane and participates in a variety of plasma membrane-specific functions (Falkenburger *et al.* 2010). Finally, PI(3,5)P_2 is produced in late endosomes and lysosomes using PI(3)P as the substrate (Y. Zhang *et al.* 2012; Zolov *et al.* 2012).

Phosphoinositides regulate compartment-specific membrane trafficking via at least two distinct mechanisms. First, they recruit a spectrum of cytoplasmic effector proteins that function on specific membrane compartments (Falkenburger *et al.* 2010). Rapid production and elimination of phosphoinositides by their specific regulatory enzymes could recruit phosphoinositide effector proteins necessary for the initiation and termination of membrane trafficking events in a sequential manner. For example, the recruitment of early endosomal tethering factor EEA1 onto early endosomes requires the recognition of PI(3)P by EEA1's FYVE domain (Poccia & Larijani, 2009). Second, phosphoinositides can directly regulate the activity of membrane proteins such as ion channels and transporters (X. Zhang *et al.* 2012). Many plasma membrane ion channels have been shown to be activated or positively regulated by the plasma membrane phosphoinositide PI(4,5)P_2 (Falkenburger *et al.* 2010). Two families of late endosomal and lysosomal cation channels, the transient receptor potential cation channels, mucolipin subfamily (TRPML) and two-pore channels (TPC), both implicated in endosomal and lysosomal membrane trafficking, are activated by the late endosomal and lysosomal phosphoinositide PI(3,5)P_2 (Dong *et al.* 2010; Wang *et al.* 2012). Furthermore, TRPML1 is inactivated by the plasma membrane phosphoinositide PI(4,5)P_2 (X. Zhang *et al.* 2012). Thus, phosphoinositides may have dual functions in recruiting cytoplasmic proteins and providing compartment-specific regulation of membrane proteins in intracellular vesicular compartments.

Genetically encoded fluorescent phosphoinositide probes, constructed from phosphoinositide-binding domains of a variety of proteins, have been generated for at least four of the seven phosphoinositides (Balla, 2007). Phosphoinositide probes allow for the visualization of real-time changes in both the abundance and localization of phosphoinositides, revealing novel aspects of phosphoinositide-mediated regulation of membrane trafficking. For example, a transient, localized increase in the endosomal PI(3,5)P_2 level may induce Ca^{2+} release, which could trigger a membrane fusion event (see Fig. 2). Likewise, localized production of PI(4,5)P_2 on tubular structures of lysosomes may recruit clathrin in a microdomain, which initiates clathrin-mediated membrane fission (Rong *et al.* 2012). Live imaging with

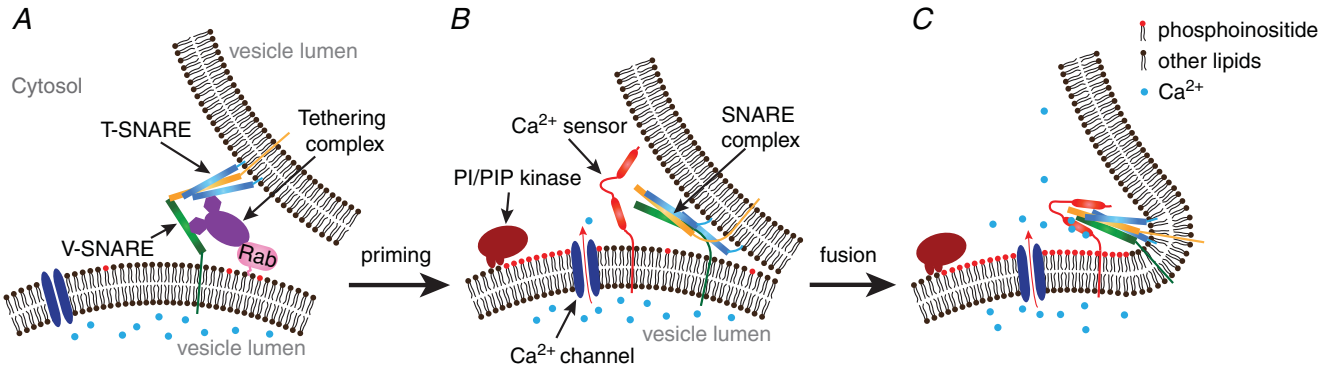

Figure 2. A proposed model of the phosphoinositide–Ca^{2+}–membrane fusion pathway
A, the initiation of vesicle fusion is mediated by the cooperation of Rab proteins and tethering complexes, which coordinate the assembly of the SNARE complex. *B*, after the SNARE complex is assembled, the vesicles are in a ready-to-fuse state. *C*, an increase in the membrane PI(3,5)P_2 concentration activates Ca^{2+} influx into the cytosol, which acts as a trigger for vesicle fusion.

phosphoinositide probes to monitor phosphoinositide levels *in vivo* could directly test such hypotheses. If phosphoinositides also act as triggers, then it is expected that the levels of phosphoinositides will undergo local increases that directly precede or even coincide with membrane fusion/fission events. Optogenetics will allow very precise manipulation of phosphoinositide levels both spatially and temporally, providing a powerful means towards decoding the functions of phosphoinositides in membrane trafficking (Idevall-Hagren *et al.* 2012).

PI(3)P dynamics in early and late endosomes. PI(3)P is the predominant phosphoinositide on early endosomes and autophagosomes, and is responsible for the localization of a spectrum of otherwise cytosol-localized proteins to these compartments (Noda *et al.* 2010). PI(3)P is produced from phosphatidylinositol (PI) by the class III phosphatidylinositol 3-kinase (class III PI3K, also known as Vps34) complex (Lindmo & Stenmark, 2006). PI(3)P can be converted back to PI by lipid phosphatases of the myotubularin/myotubularin-related-protein family (MTM/MTMR; Shen *et al.* 2011). Alternatively, PI(3)P can be converted into $PI(3,5)P_2$ by the phosphatidylinositol 5-kinase PIKfyve complex (Gillooly *et al.* 2000). PI(3)P effector proteins, for example, EEA1 and hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), contain the PI(3)P-binding FYVE domains, which have been used to generate PI(3)P probes (Gillooly *et al.* 2000). FYVE-containing PI(3)P probes exhibit vesicular membrane localization that is consistent with early endosomal markers such as EEA1 (Fig. 3*A*).

PI(3)P probes are recruited to the membranes of newly formed endosomal compartments (including nascent phagosomes, macropinosomes, and autophagosomes) within minutes after endocytosis, but are also visible on lysosomal-associated membrane protein 1 (LAMP1)-positive compartments, suggesting that PI(3)P may play a role in the entire endosomal maturation process (Kerr *et al.* 2010). Vsp34 knockout (KO) mice, which lack the enzyme to make PI(3)P, are defective in autophagy and normal physiological functions in a spectrum of tissues and organs, indicating the importance of PI(3)P signalling for normal cell functions (Jaber *et al.* 2012). Hence PI(3)P signalling may carry important information for the maturation of endosomes.

$PI(3,5)P_2$ dynamics in late endosomes and lysosomes. $PI(3,5)P_2$ is produced from PI(3)P by PIKfyve (Zolov *et al.* 2012). PIKfyve exists in a complex with the scaffold protein Vac14 and the phosphoinositide 5-phosphatase Fig4, and is the only enzyme found in mammalian cells to produce $PI(3,5)P_2$ (Duex *et al.* 2006*b*; Zhang *et al.* 2007). Knockout of PIKfyve in mice results in embryonic lethality (Ikonomov *et al.* 2011), and knockout of Vac14 or Fig4, which results in a roughly 50% decrease in total $PI(3,5)P_2$ levels, leads to severely enlarged vacuoles of late-endocytic origin (Chow *et al.* 2007; Zhang *et al.* 2007). It is still unclear how $PI(3,5)P_2$ regulates late endocytic trafficking, however. Atg18 is the first identified $PI(3,5)P_2$ effector protein, but is localized in the autophagic compartments (Obara *et al.* 2008), suggesting that it may not be responsible for the primary effect of $PI(3,5)P_2$ in late endosomes and lysosomes. Patch-clamp recordings of endolysosomes have demonstrated that $PI(3,5)P_2$ in a nanomolar range robustly activates TRPML1, a late-endosomal and lysosomal channel (Dong *et al.* 2010). In addition, cells that lack the TRPML1 channel share a similar vacuolar phenotype to Vac14 and Fig4 KO cells (Dong *et al.* 2010). Taken together, these findings suggest that $PI(3,5)P_2$ may regulate endosomal trafficking through endolysosomal ion channels. However, the cellular and animal phenotypes of TRPML1 KO mice are much less severe than $PI(3,5)P_2$-deficient Vac14 or Fig4 KO mice, suggesting that there are additional unidentified $PI(3,5)P_2$ effector proteins that regulate late endosomes and lysosomes (Shen *et al.* 2011).

The level of $PI(3,5)P_2$ and its recruitment dynamics in the late endosomes and lysosomes is poorly understood, largely due to the lack of a widely-accepted $PI(3,5)P_2$ probe. Biochemical studies have revealed that the $PI(3,5)P_2$ level in the endosome and lysosome is regulated by serum factors, insulin and osmotic stress in some mammalian cell types (Poccia & Larijani, 2009; Zolov *et al.* 2012). However, the connection of these conditions to endosomal and lysosomal trafficking has not been established. The most well-understood example of $PI(3,5)P_2$ dynamics is from yeast studies. Deletion mutants of Vac14 or Fab1 (the yeast orthologues of mammalian Vac14 and PIKfyve, respectively) lead to a single huge vacuole in yeast cells (Odorizzi *et al.* 1998; Bonangelino *et al.* 2002; Dove *et al.* 2002), which could result from increased fusion or decreased fission of vacuoles. Additionally, a rapid, more than 10-fold increase in $PI(3,5)P_2$ levels in yeast cells caused by hyperosmotic shock is required for the synchronized vacuole fission in response to hyperosmolarity, because in deletion mutants fission is impaired (Duex *et al.* 2006*a*,*b*). These findings suggest that the production of $PI(3,5)P_2$ is required for vesicle membrane fission, at least in yeast.

PI(4)P and $PI(4,5)P_2$ dynamics in lysosomes. Current findings suggest that $PI(4,5)P_2$ is localized mainly on the plasma membrane (Fig. 3*B*). PI(4)P is most abundant on the Golgi. Recent studies have demonstrated that both PI(4)P and $PI(4,5)P_2$ are also probably generated on lysosomal membranes, in particular on lysosomes and autolysosomes with tubular structures (Yu *et al.* 2010; Rong *et al.* 2012; Sridhar *et al.* 2013). Upon completion of

autophagy, degradation of the cargo delivered by autophagosomes to lysosomes triggers the reactivation of mTOR. mTOR reactivation in turn triggers the formation of proto-lysosomal tubules from lysosomes that reform to regenerate functional lysosomes (Yu *et al.* 2010). The tubular structures undergo very quick elongation and retraction, and proto-lysosomes have been observed to bud off (membrane fission) from tubular structures (Rong *et al.* 2012). Lysosomal tubular structures are infrequently observed in healthy, well-fed cells, but their number greatly increases in conditions like prolonged serum starvation, when there is a high demand for digestion. The tubular domains of lysosomes contain many lysosomal membrane proteins, while cargo and luminal enzymes are restrained in the vacuolar domains of the lysosomes to complete degradation (Yu *et al.* 2010; Sridhar *et al.* 2013). At least three phosphoinositide kinases have been suggested to regulate this lysosomal reformation process. In cells lacking phosphatidylinositol 4-phosphate 5-kinase 1A (PI4P5K1A) or PI4P5K1B, two enzymes catalysing the generation of $PI(4,5)P_2$ (Rong *et al.* 2012), or phosphatidylinositol 4-kinase IIIβ (PI4K-IIIβ), an enzyme catalysing the generation of PI(4)P (Sridhar *et al.* 2013), the lysosome reformation process is defective. Using the PI(4)P probe and $PI(4,5)P_2$ antibody, it was shown that the levels of PI(4)P and $PI(4,5)P_2$ are elevated in the tubular structures of autolysosomes (Rong *et al.* 2012). The production of $PI(4,5)P_2$ on tubular structures is considered to be important for the initiation of clathrin-mediated membrane fission (Rong *et al.* 2012), but exactly how PI(4)P and $PI(4,5)P_2$ coordinate the tubular-lysosomal fission process is still unclear. It seems likely that the conversion of PI(4)P into $PI(4,5)P_2$ on autophagic lysosomes can initiate tubule formation, while the production of $PI(4,5)P_2$ on elongated tubules can promote final fission (Rong *et al.* 2012; Sridhar *et al.* 2013).

Calcium

Ca^{2+} serves as a multifunctional signal in a variety of intracellular processes. Ca^{2+} signalling can occur in a wide range of spatial (highly localized *vs.* synchronized release) and temporal (transient *vs.* sustained release; microseconds to hours) ways (Berridge *et al.* 2003). The endoplasmic reticulum (ER) is appreciated as a significant source of Ca^{2+} in the cell, and ER-mediated Ca^{2+} signalling is integral to numerous intracellular processes (Berridge, 2002), including autophagy and membrane trafficking (Smaili *et al.* 2013). Lysosomes are beginning to be appreciated as an additional store of Ca^{2+} (Morgan *et al.* 2011), and some recent reports suggest that Ca^{2+} signalling can occur between the ER and lysosomes (Kilpatrick *et al.* 2013; Lopez-Sanjurjo *et al.* 2013; Morgan *et al.* 2013). However, while the mechanisms by which the ER maintains its Ca^{2+} stores are beginning to be elucidated (Lewis, 2011), less is currently known about how lysosomal Ca^{2+} stores are regulated. After endocytosis, early

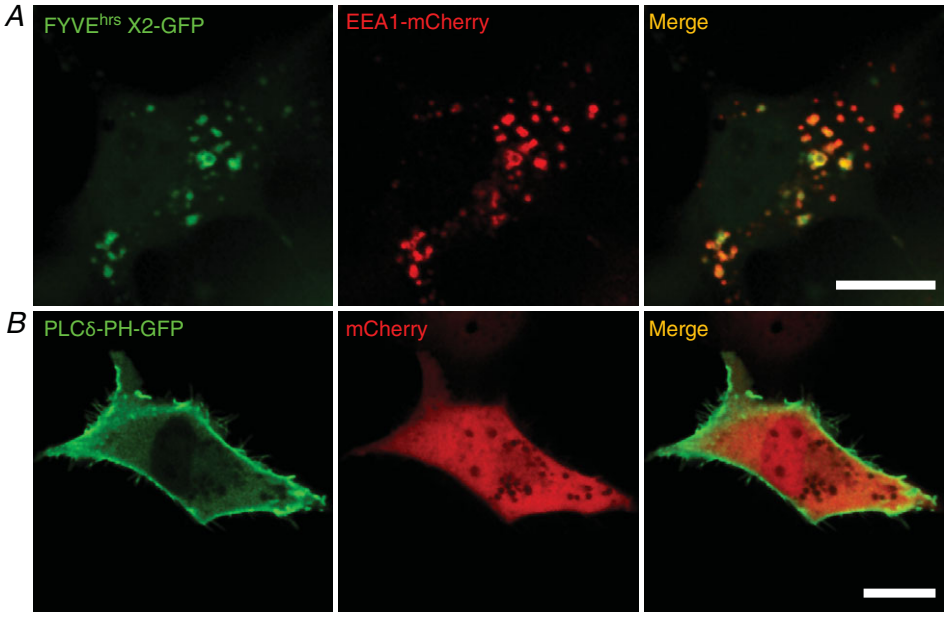

Figure 3. Fluorescently tagged phosphoinositide probes
Cos7 cells are transfected with green fluorescent protein (GFP)-tagged phosphoinositide-binding domains. *A*, a PI(3)P probe, $FYVE^{hrs}X2$-GFP, is concentrated in EEA1-positive vesicles. mCherry-EEA1 is co-transfected to visualize early endosomes. *B*, the $PI(4,5)P_2$ probe, PLCδ-PH-GFP, is mainly localized in the plasma membrane at rest. mCherry is co-transfected to define cell morphology. Scale bars represent 20 μm.

endosomes rapidly lose their Ca^{2+} as endosomes mature and acidify (Gerasimenko *et al.* 1998), a process driven by the V-ATPase on the lysosome (Gerasimenko *et al.* 2001). Conversely, increasing lysosomal pH results in a significant loss of lysosomal Ca^{2+} (Christensen *et al.* 2002), which suggests that the ionic balance in the lysosome is important in maintaining lysosomal Ca^{2+} stores. Thus, the high proton gradient (pH_{lumen} 4.6 *vs.* pH_i 7.2) in the lysosome probably drives a putative Ca^{2+}/H^+ pump to regulate Ca^{2+} in the lysosome. Furthermore, it is likely that the concentration of various intracellular ions including Na^+, Zn^{2+} and Ca^{2+} in endosomes and lysosomes changes throughout endosome maturation and that the balance of these ions is important; however, this possibility remains to be explored. The role of Ca^{2+} in trafficking and signalling is the most well understood and will be discussed here; however, other ions such as Na^+ (Wang *et al.* 2012) and Zn^{2+} (Aballay *et al.* 1995) may also be involved in trafficking. For example, Na^+ efflux may regulate the fusogenic potential of the lysosome by causing membrane depolarization (Wang *et al.* 2012), or affecting Ca^{2+} uptake (Morgan *et al.* 2011). However, further studies are necessary to test these possibilities.

Neurotransmitter exocytosis as a model for membrane fusion events. The relationship between Ca^{2+} and membrane trafficking has been best characterized in the case of neurotransmitter release, which serves as a reference model for the less well understood case of intracellular membrane trafficking. Neurotransmitter release involves very rapid (<1 ms) membrane fusion (exocytosis) between synaptic vesicles and the plasma membrane of synaptic terminals. Membrane fusion requires the binding of a variety of SNARE proteins found on the vesicle (v-SNAREs) and target (t-SNAREs) membranes (Sudhof & Rothman, 2009). SNAREs are a protein superfamily sharing a coiled-coil homology domain (Jahn & Fasshauer, 2012). Fluorescence resonance energy transfer (FRET) at synapses has allowed for precise visualization of the assembly, rearrangements and disassembly of SNARE proteins (Degtyar *et al.* 2013). Fusion of a synaptic vesicle requires the binding, or 'zippering', of a vesicle-associated membrane protein (VAMP, also known as synaptobrevin) with plasma membrane-bound syntaxin and SNAP-25 to form a SNARE complex (Gao *et al.* 2012). SNARE complexes are competent to form a fusion pore between membranes (Domanska *et al.* 2009), but fast membrane fusion requires the interaction between the SNARE complex and the Ca^{2+} sensor synaptotagmin, and is triggered by the binding of Ca^{2+} ions to the cytoplasmic C2 domains of synaptotagmin (Tucker *et al.* 2004). During an action potential, Ca^{2+} enters the synaptic terminal through voltage-gated Ca^{2+} channels and binds to synaptotagmin on the pre-docked synaptic vesicles (Xu *et al.* 2007). Synaptotagmin undergoes a conformational change upon Ca^{2+} binding and the C2 domains interact directly with the SNARE complex (van den Bogaart *et al.* 2011) and phospholipids on the plasma membrane to trigger SNARE-mediated membrane fusion.

Intracellular membrane trafficking. Membrane trafficking involves the fusion and fission of endosomes and lysosomes, as well as lysosomal exocytosis. Like neurotransmitter release, intracellular vesicle fusion requires SNARE proteins. After membrane tethering, membrane fusion is mediated by endosomal SNARE proteins, which include v-SNAREs such as VAMP4 (Tran *et al.* 2007) and VAMP7 (Fraldi *et al.* 2010), and t-SNAREs such as syntaxin6 (Jung *et al.* 2012) and syntaxin7 (Ward *et al.* 2000; Fig. 2*A*). Hence, similar machinery is employed for general trafficking and neurotransmitter release.

Ca^{2+} as a trigger for membrane fusion. Similar to neurotransmitter release, the Ca^{2+} release that mediates endosomal trafficking events is likely to come from a local source, as membrane trafficking steps can be efficiently blocked by the fast, membrane-permeable Ca^{2+} chelator

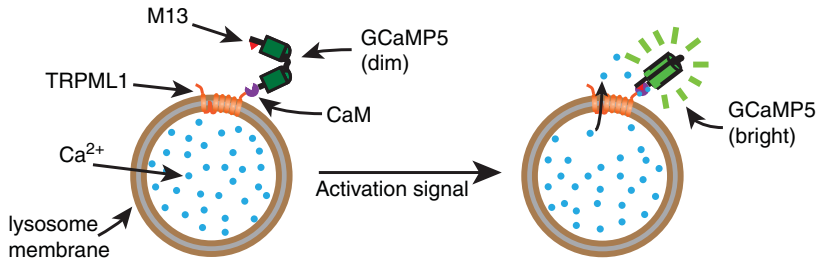

Figure 4. A lysosome-targeted genetically encoded Ca^{2+} sensor
GCaMP5 contains a circularly permutated enhanced GFP (EGFP). The N-terminal of the fragment containing residues 149–238 is linked to the M13 peptide, while the C-terminal of the fragment containing residues 1–144 is linked to calmodulin (CaM). The binding of Ca^{2+} ions to calmodulin causes a conformational change and calmodulin then binds to the M13 peptide, bringing the two parts of the permutated EGFP together, reconstituting a functional EGFP. When GCaMP5 is attached to a lysosomal membrane protein (e.g. TRPML1), it works as a sensor for local Ca^{2+} release.

BAPTA-AM, but not the slow chelator EGTA-AM (Chen *et al.* 2002; Czibener *et al.* 2006; Shen *et al.* 2011). In late endosomes and lysosomes, there are two potential known candidates: TRPMLs (Cheng *et al.* 2010) and TPC channels (Grimm *et al.* 2012). TRPML1 is a Ca^{2+}-permeable cation channel that is ubiquitously expressed in most cell types. TRPML1 is primarily localized in the late endosome and lysosome and has been shown to mediate the release of Ca^{2+} from the lysosome lumen to the cytosol (Dong *et al.* 2009, 2010). Mammalian cells lacking TRPML1 exhibit multiple membrane trafficking defects in the late-endocytic pathway including altered autophagosome–lysosome fusion and lysosome-to-Golgi trafficking (Shen *et al.* 2011, 2012). TPC channels are a family of ion channels that reside on endocytic compartments, and were reported to mediate the nicotinic acid adenine dinucleotide phosphate (NAADP)-evoked Ca^{2+} release from late endosomes and lysosomes (Zong *et al.* 2009). However, whole-endolysosome patch-clamp analyses indicate that TPC channels exhibit little Ca^{2+} permeability and are essentially $PI(3,5)P_2$-activated sodium-selective channels (Wang *et al.* 2012). Therefore, TPCs may not be the direct targets of NAADP. Instead, NAADP may bind to a unidentified 23 kDa protein (Lin-Moshier *et al.* 2012) to regulate the activity of a variety of Ca^{2+} release channels (Guse, 2012). However, the role of NAADP is still debated. Collectively, these findings suggest that TRPML1 plays a role in lysosomal membrane trafficking similar to voltage-gated Ca^{2+} channels in neurotransmission. Interestingly, overexpression of both TRPML1 and TPC channels leads to enlarged endolysosomes, indicating that both channels could be up-regulating fusion events (Dong *et al.* 2010; Wang *et al.* 2012). TFEB, a transcription factor and master regulator for lysosome biogenesis and autophagy, has been suggested to induce lysosomal exocytosis in a TRPML1-dependent manner (Medina *et al.* 2011). Hence, TRPML1 is a strong candidate in mediating Ca^{2+} release in general vesicular trafficking events in late endosomes and lysosomes.

The majority of the aforementioned evidence supporting Ca^{2+} as a triggering event in membrane trafficking remains inconclusive. Cell-free vesicle fusion assays do not fully mimic *in vivo* situations. Intracellular vesicle fusion has been mainly studied using Ca^{2+} chelators such as BAPTA-AM (Chen *et al.* 2002), which also chelates other cations in *in vitro* assays and may have additional actions that are not specific to Ca^{2+} (Starai *et al.* 2005). While EGTA-AM has a slower time course than BAPTA-AM and probably does not chelate transient Ca^{2+} increases (making it a good negative control for BAPTA-AM), many studies would benefit from the direct measurement of Ca^{2+} release from Ca^{2+} channels located in endosomal membranes. The use of high resolution, genetically encoded Ca^{2+} indicators (GECIs) has aided the study of local Ca^{2+} release from ion channels in endosomal membranes (Tian *et al.* 2009). For example, fusing the GECI GCaMP3 to the cytoplasmic amino acid terminus of TRPML1 has enabled the direct measurement of Ca^{2+} release from the lysosome using Ca^{2+} imaging (Fig. 4; Shen *et al.* 2012). Furthermore, ratiometric lysosome-targeted GECIs may prove useful in monitoring Ca^{2+} dynamics in moving vesicles (McCue *et al.* 2013). Whether Ca^{2+} acts as a trigger for trafficking events, or is more upstream from direct vesicle fusion and fission is unknown. If Ca^{2+} is the trigger for vesicle fusion, then the local Ca^{2+} increase revealed by vesicularly targeted Ca^{2+} indicators should coincide with, or directly precede, membrane fusion or fission (Fig. 2*C*).

Ca^{2+} sensors in membrane trafficking. Distinct Ca^{2+} sensors are implicated in different membrane fusion and fission events (Fig. 2*B*; Ghislat & Knecht, 2013). Lysosomal exocytosis is mediated by synaptotagmin-VII with VAMP7 as the major v-SNARE (Reddy *et al.* 2001; Czibener *et al.* 2006). It is not known whether synaptotagmin-VII also regulates the fusion between lysosomes and other endocytic vesicles. There are at least 16 members of the synaptotagmin family, and many of them sense and respond to different ranges of Ca^{2+} (Bhalla *et al.* 2005). This suggests that different synaptotagmins may be expressed in different cell types and may reside on different intracellular vesicles to promote vesicle fusion and fission. Apart from synaptotagmins, calmodulin (CaM) has been shown to be important for vesicle fusion in yeast studies and *in vitro* reconstitution systems (Peters & Mayer, 1998; Pryor *et al.* 2000), but its involvement in intact mammalian cells is still unclear. Additionally, other Ca^{2+}-binding proteins such as ALG-2 may serve as Ca^{2+} sensors in the late endosomal and lysosomal membranes. Importantly, ALG-2 binds to the N-terminal cytosolic tail of TRPML1, probably to modulate its function in trafficking events (Vergarajauregui *et al.* 2009). Future studies examining the recruitment kinetics of Ca^{2+} sensors in vesicular membranes will provide a greater understanding of Ca^{2+} signalling in membrane trafficking.

Concluding remarks

With the recent development of high-resolution live imaging methods, we are just beginning to understand some aspects of membrane signalling and trafficking. The coordinated interactions between small GTPases, lipids and Ca^{2+} signalling are essential; however, other signalling events should not be underestimated. For example, luminal pH is known to affect endosomal and lysosomal membrane trafficking, and acidification is tightly associated with endosome maturation. In addition, the lysosome has a high Na^+ content, and Na^+-selective TPC channels are found in lysosomal membranes.

This suggests that endosomal membrane potential may undergo large regulatory changes; however, this possibility has yet to be explored. Continued advances in live imaging, molecular probes and electrophysiological methods to measure endosomal membrane potential will allow us to continue to elucidate how membrane trafficking is regulated.

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Additional information

Competing interests

None declared.

Funding

This work in the author's laboratory is supported by NIH grants (NS062792, MH096595 and AR060837 to H.X.).

Acknowledgements

We thank Dr Harald Stenmark for the FYVE∗2-GFP construct. We appreciate the encouragement and helpful comments from other members of the Xu laboratory.