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Phosphoinositides in the mammalian endo-lysosomal network

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Abstract

The endo-lysosomal system is an interconnected tubulo-vesicular network that acts as a sorting station to process and distribute internalised cargo. This network accepts cargoes from both the plasma membrane and the biosynthetic pathway, and directs these cargoes either towards the lysosome for degradation, the peri-nuclear recycling endosome for return to the cell surface, or to the *trans*-Golgi network. These intracellular membranes are variously enriched in different phosphoinositides that help to shape compartmental identity. These lipids act to localise a number of phosphoinositide-binding proteins that function as sorting machineries to regulate endosomal cargo sorting. Herein we discuss regulation of these machineries by phosphoinositides and explore how phosphoinositide-switching contributes toward sorting decisions made at this platform.

Keywords

Endosome; membrane traffic; phosphoinositide; endosomal sorting

1. Introduction - the endo-lysosomal network

The endo-lysosomal network begins at the plasma membrane after transmembrane cargo becomes internalized through the process of endocytosis (Mayor and Pagano, 2007). After internalization, cargo proteins enter the early endosome, an endomembrane compartment characterised by interconnected morphologically distinct vacuolar and tubular elements. Here cargo sorting occurs (Cullen, 2008). Cargoes destined for degradation, such as the epidermal growth factor receptor (EGFR), are sorted onto intraluminal vesicles (ILVs) of endosomes, which, through a process of compartment maturation give rise to the late endosome/multivesicular body (MVB) (Piper and Katzmann, 2007, Williams and Urbe, 2007, Hurley and Hanson, 2010). In parallel, other cargoes are retrieved from this pathway. Transferrin receptors (TfnRs), integrins and a variety of other cargo undergo sorting into tubular-branched structures from where they are recycled back to the plasma membrane via a direct fast recycling route or, more slowly, through the juxtanuclear endocytic recycling compartment (ERC) (Grant and Donaldson, 2009, Hsu and Prekeris, 2010). Yet other cargoes, such as cation-independent mannose 6-phosphate receptors (CI-MPRs) are sorted into distinct tubular sub-domains for retrieval to the *trans*-Golgi network (TGN) (Bonifacino and Rojas, 2006, Johannes and Popoff, 2008). When all recycled material has been removed,

late endosomes/MVBs fuse with lysosomes thereby degrading cargoes associated with the intraluminal vesicles (Saftig and Klumperman, 2009) (Figure 1a).

Interest in this membranous network stemmed from academic curiosity into how the complexities of the network are established and maintained. Subsequent medical importance of endo-lysosomal sorting has emerged from an ever-growing body of evidence implicating adverse sorting as an underlying mechanism for a number of diseases. Moreover, adventitious viruses, pathogens and toxins exploit this pathway in order to enter target cells and gain access to the cytosol (Gruenberg and van der Goot, 2006, Johannes and Romer, 2010, Mercer et al., 2010). Research into this network is therefore moving increasingly towards defining the molecular mechanisms of endosomal related diseases, and nowhere is this more evident than in the study of endosomal phosphoinositides (Nicot and Laporte, 2008, McCrea and De Camilli, 2009, Liu and Bankaitis, 2010).

2. Subcellular distribution of phosphoinositides

Phosphoinositides are not uniformly distributed throughout cellular membranes rather they adopt a compartmentalised distribution which is dependent on the spatial and temporal regulation of specific kinases and phosphatases. While the exact phosphoinositide composition of individual membrane compartments is not comprehensively understood, imaging based studies employing fluorescently-tagged isomer-specific phosphoinositide biosensors have described a general map of phosphoinositide distributions (Balla and Várnai, 2009, Cooke, 2009). For the endo-lysosomal network this is defined by the enrichment of $\text{PtdIns}(4,5)\text{P}_2$ at sites of clathrin-mediated endocytosis (Haucke, 2005), while $\text{PtdIns}(3)\text{P}$ is localized to the cytosolic face of the limiting membrane of early endosomes and those intraluminal vesicles contained within MVBs (Gillooly et al., 2000a). $\text{PtdIns}(3,5)\text{P}_2$ on the other hand has been proposed to be primarily localised to the limiting membrane of late endosomes although the evidence for this remains incomplete (Michell et al., 2006). Finally, membranes of the ERC, Golgi apparatus and cytoplasmic vesicles are thought to contain $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ (De Matteis et al., 2005, Hammond et al., 2009, Jovic et al., 2009) (Figure 1b).

Such a phosphoinositide map should always be taken as a generality. It is clear that under certain physiological conditions specific phosphoinositide can be generated on membranes other than those described within the map. Take for example the early endosomal pool of $\text{PtdIns}(3)\text{P}$. It can be generated on the inner leaflet of the plasma membrane during insulin stimulation (Maffucci et al., 2003), appears to be associated with the outer plasma membrane leaflet (Kale et al., 2010), and is present in the endoplasmic reticulum during the formation of autophagosomes (Axe et al., 2008).

3. Endosomal phosphoinositide kinases and phosphatases

A vital element in the biology of phosphoinositide is their rapid inter-conversion through the actions of specific kinases and phosphatases (see (Balla and Balla, 2006, Robinson and Dixon, 2006, Ooms et al., 2009, van den Bout and Divecha, 2009, Liu and Bankaitis, 2010, Vanhaesebroeck et al., 2010) for detailed reviews). Briefly, mammalian cells express families of specific kinases and phosphatases which add or remove phosphates from the D3,

D4 and D5 position of the inositol head-group of specific phosphoinositide isomers. Thus, Class I PI 3-kinase generates PtdIns(3,4,5)P₃ by catalysing the addition of a phosphate to the 3-position of PtdIns(4,5)P₂, while PTEN is a 3-phosphatase that dephosphorylates PtdIns(3,4,5)P₃ re-generating PtdIns(4,5)P₂ (Chalhoub and Baker, 2009). Broadly speaking, the molecular architecture of phosphoinositide kinases and phosphatases are organised so as to target the catalytic region to different subcellular membranes through mechanisms that, for the majority of enzymes, remain to be completely defined. This has the effect of spatially restricting the activity of individual kinases and phosphatases to the membrane:cytosol interface of specified compartments. If one adds to this the fact that these enzymes are also designed to respond to changes in the cellular state, for example receptor activation and changes to the cell cycle, then a picture emerges in which the metabolic turnover of specific phosphoinositides can be spatially and temporal restricted to unique membrane compartments (i.e. the phosphoinositide map, Figure 1b).

To further illustrate and expand this, consider the endo-lysosomal network. The central axis of this pathway classically begins with the formation of PtdIns(4,5)P₂-enriched clathrin-coated endocytic pits, proceeds through PtdIns(3)P-containing early endosomes before ending with PtdIns(3,5)P₂-containing late endosomes. Importantly, the transition from clathrin-coated pits to late endosomes occurs through compartment maturation – clathrin-coated pits mature and then fuse with early endosomes, which in turn mature into late endosomes (Rink et al., 2005, Poteryaev et al., 2010). Along this maturation axis therefore, there appears to be three phosphoinositide switches – PtdIns(4,5)P₂-to-PtdIns(4)P, PtdIns-to-PtdIns(3)P and PtdIns(3)P-to-PtdIns(3,5)P₂ (Joly et al., 1995, Dove et al., 1997, Shin et al., 2005, Zoncu et al., 2009).

3.1. PtdIns(4,5)P₂-to-PtdIns(4)P switch

The formation of plasma membrane PtdIns(4,5)P₂ through the actions of PtdIns(4)P 5-kinases is an essential localisation cue required for clathrin-mediated endocytosis (Jost et al., 1998, Wenk et al., 2001). Early genetic studies in mice revealed the importance of Type I PtdIns(4)P 5-kinase- γ for this process during synaptic transmission (Paolo et al., 2004). This class of phosphoinositide kinases are able to directly associate with the clathrin-binding cargo adaptor AP-2 (Krauss et al., 2006, Nakano-Kobayashi et al., 2007, Thieman et al., 2009), an intriguing observation given that initial endocytic assembly zones are nucleated at the plasma membrane through low affinity interactions between AP-2 and discrete, localised patches of plasma membrane PtdIns(4,5)P₂ (Traub, 2009b) (Figure 2a, b) (see section 5.1. for more detail).

As for inositol 5-phosphatases, a number, including two isoforms of synaptojanin 1 (synaptojanin 1-170 and synaptojanin 1-145), synaptojanin 2, SHIP2, INPP5B and OCRL (McPherson et al., 1996, Shin et al., 2005, Hyvola et al., 2006, Perera et al., 2006, Erdmann et al., 2007, Nakatsu et al., 2010), are recruited to forming clathrin-coated endocytic structures, further establishing the importance of PtdIns(4,5)P₂ turnover in the dynamics of this process. While there is no evidence to suggest a direct association between Type I PtdIns(4)P 5-kinases and PtdIns(4,5)P₂ 5-phosphatases, synaptojanin 1-170 does associate with the AP-2 adaptor (Haffner et al., 2000, Jha et al., 2004), arguing for a close spatial

relationship between these two catalytic activities. Interestingly, while synaptojanin 1-170 remains associated with clathrin-coated pits from early nucleation through to clathrin uncoating after fission (Perera et al., 2006), the association of other 5-phosphatases is dependent on the maturation state of the clathrin-coated pit (Nakatsu et al., 2010). SHIP2 associates at early stages during nucleation and growth but dissociates prior to fission (Perera et al., 2006, Nakatsu et al., 2010), and synaptojanin 1-145 and OCRL both arrive at late stages of pit formation. After clathrin uncoating, an event triggered by PtdIns(4,5)P₂-to-PtdIns(4)P switching, only OCRL remains associated with the newly formed endosomal vesicle (Perera et al., 2006, Erdmann et al., 2007) (see section 5 for a discussion of clathrin-mediated endocytosis).

Overall, the requirement for Type I PtdIns(4)P 5-kinases and the sequential association of multiple 5-phosphatases is consistent with the continuous turnover of PtdIns(4,5)P₂ and PtdIns(4)P during maturation of clathrin-coated pits and vesicles helps create a plastic system allowing for regulation of the maturation process (Figure 2a, b). One interpretation of these data is that inclusion of individual enzymes within discrete interaction networks within the global clathrin coat interactome may, at specified times during the maturation pathway, modify on a local scale the level of PtdIns(4,5)P₂.

3.2. PtdIns-to-PtdIns(3)P switch

Clathrin derived endocytic vesicles (and also those from clathrin-independent endocytosis) on entering the endosomal network contain Rab5 and APPL, but lack detectable levels of PtdIns(3)P (Zoncu et al., 2009): presumably these vesicle also contain PtdIns and residual PtdIns(4)P from dephosphorylation of PtdIns(4,5)P₂. Conversion of these intermediate endosomes to PtdIns(3)P-positive early endosomes correlates with displacement of APPL and the acquisition of PtdIns(3)P-binding proteins such as EEA1 (Zoncu et al., 2009). How this conversion is controlled is unclear although competition with Rab5 may be one possibility (Zoncu et al., 2009): APPL directly binds Rab5 (Miaczynska et al., 2004) and also the 5-phosphatase OCRL which, as described above, remains associated with the newly formed endosomal vesicle after clathrin-mediated endocytosis (Hyvola et al., 2006, Perera et al., 2006, Erdmann et al., 2007).

Active GTP-bound Rab5 is a key regulatory of the principal PtdIns 3-kinase in mammalian cells, VPS34 (Christoforidis et al., 1999, Backer, 2008). VPS34 is associated with a HEAT domain-containing regulatory subunit VPS15, and it is the interaction with Rab5 that targets this dimer to endosomes catalysing the formation of PtdIns(3)P (Christoforidis et al., 1999). The antagonistic PtdIns(3)P 3-phosphatases are members of the myotubularin family: a large group of lipid phosphatases, mutations in which are linked to various genetic diseases affecting skeletal muscle and the neuronal system (Robinson and Dixon, 2006, Liu and Bankaitis, 2010). Myotubularin 1 (MTM1) (defective in X-linked myotubular myopathy) and myotubularin-related 2 (MTMR2) (defective in Charcot-Marie-Tooth disease type 4B (CMT4B1)) are both able to associate with the VPS15:VPS34 complex through an interaction with VPS15 (Cao et al., 2007, Cao et al., 2008). In these complexes both 3-kinase and 3-phosphatase activities are inactivated, effectively shutting off the turnover of PtdIns(3)P (Cao et al., 2007, Cao et al., 2008) and stabilising domains of this lipid upon

endosomes. Rab5 and Rab7 however compete with MTM1 and MTMR2 for binding to the VPS15, releasing the inhibition and allowing PtdIns(3)P turnover (Cao et al., 2007, Cao et al., 2008). Imaging studies are consistent with the VPS15:VPS34:MTM1 complex residing at the Rab5 early endosome and the VPS15:VPS34:MTMR2 complex localising to Rab7 late endosomes (Cao et al., 2007, Cao et al., 2008). Perhaps competition of Rab5 with the VPS15:VPS34:MTM1 complex on APPL-labelled intermediate endosomes may be one component in switching to a PtdIns(3)P positive early endosomes. Understanding how the activation statuses of Rab5 (and Rab7) modulate the assembly of these PtdIns 3-kinase:PtdIns(3)P 3-phosphatase complexes will surely reveal interesting insight into the co-ordination of PtdIns(3)P turnover and early-to-late endosomal maturation.

3.3. PtdIns(3)P-to-PtdIns(3,5)P₂ switch

In mammalian cells the PtdIns(3)P-to-PtdIns(3,5)P₂ switch is catalysed by the PtdIns(3)P 5-kinase PIKfyve (Sbrissa et al., 1999, Shisheva et al., 1999), an enzyme localised to early and late endosomes (Cabezas et al., 2006, Ikononov et al., 2006, Rutherford et al., 2006) through its PtdIns(3)P-binding FYVE domain. The reverse reaction, converting PtdIns(3,5)P₂-to-PtdIns(3)P, is driven by the 5-phosphatase mFig4/Sac3 (Sbrissa et al., 2007). These two enzymes associate in a complex alongside the associated regulator of PIKfyve (ArPIKfyve/hVac14) (Sbrissa et al., 2004, Jin et al., 2008), a HEAT domain-containing scaffold protein which regulates the catalytic activities of both PIKfyve and mFig4 (this complex is evolutionarily conserved being also present in yeast (Bonangelino et al., 2002, Dove et al., 2002, Gary et al., 2002, Rudge et al., 2004, Duex et al., 2006, Botelho et al., 2008)). Like many other examples where antagonistic or complementary enzymatic activities are associated within a single complex, the PIKfyve/mFig4/hVac14 complex appears designed to allow co-ordination and fine regulation of the relative 5-kinase versus 5-phosphatase activities during the PtdIns(3)P-to-PtdIns(3,5)P₂ switch (Botelho, 2009). Again, how this regulation is co-ordinated with endosomal sorting and maturation will be an interesting area for further research.

That this complex is of central importance for normal endo-lysosomal function is however clear (see section 7.3. for more detailed discussion). Perturbing PtdIns(3,5)P₂ leads to a variety of phenotypes one of which is the striking formation of numerous swollen endosomal vacuoles that arise from a defect in the biology of late endosomes and lysosomes (Michell et al., 2006). In mice the loss of Fig4 or Vac14 leads to vacuolation of neurons and neurodegeneration (Chow et al., 2007, Zhang et al., 2007). Moreover, mutations in Fig4 are observed in patients with recessive peripheral-nerve disorder CMT4J, amyotrophic lateral sclerosis (ALS) and primary lateral sclerosis (PLS) (Chow et al., 2007, Zhang et al., 2008, Chow et al., 2009), in which at least for CMT4J patients, isolated fibroblasts have swollen vacuoles and impaired trafficking (Zhang et al., 2008).

3.4. Other phosphoinositide switches

Besides these switches one needs to consider that PtdIns(3,4,5)P₃ also plays an important role during certain endocytic events including, albeit perhaps in a more minor capacity than PtdIns(4,5)P₂, clathrin-mediated endocytosis (Shin et al., 2005, Nakatsu et al., 2010). Here a Rab5-GTP regulated phosphatase cascade acts to sequentially convert PtdIns(3,4,5)P₃ to

PtdIns(3,4)P₂ before dephosphorylation of PtdIns(3,4)P₂ generates PtdIns(3)P – respective type II inositol 5-phosphatase and type 1α PI 4-phosphatase activities (Shin et al., 2005). Rab5 therefore controls the generation of PtdIns(3)P through two distinct routes – direct activation of the VPS34 Class III PI 3-kinase and indirect switching of PtdIns(3,4,5)P₃-to-PtdIns(3)P.

Away from the central axis of the endo-lysosomal degradative pathway, other endosomal trafficking routes require phosphoinositide switches for their maturation. Switching of PtdIns-PtdIns(4)P-PtdIns(4,5)P₂ is necessary for recycling through the slow juxtanuclear endocytic recycling compartment. Here, the activity of a type II PtdIns 4-kinase generates PtdIns(4)P, and the ARF6-mediated activation of PtdIns(4)P 5-kinase leads to the production of PtdIns(4,5)P₂ in order to regulate exit from the compartment back to the plasma membrane (Brown et al., 2001, D'Angelo et al., 2008, Grant and Donaldson, 2009, Jovic et al., 2009). Finally, one can speculate that retrograde retrieval pathways will also require phosphoinositide switching as membrane containing carriers are transported from the donor endosomes to the target membranes. For example, retrograde carriers en route to the *trans*-Golgi network will, depending on whether they originated from early or late endosomal donor compartments, be predicted to contain respectively PtdIns(3)P or PtdIns(3,5)P₂. As the *trans*-Golgi network is not enriched in these phosphoinositides, switching must occur to maintain phosphoinositide compartmental identity.

In summary: to maintain compartmental identity the phosphoinositide signature of the proceeding compartment must be erased while the new phosphoinositide identity is developed (Botelho, 2009). These switches appear tightly regulated. Indeed, as a general concept one can argue that an inability to efficiently maintain phosphoinositide switching, and the resultant effects on compartment identity may well underlie many of the diseases that are linked to endosomal phosphoinositides.

4. Sensing the phosphoinositide identity code

With the distinction between phosphoinositides residing mostly in the organization of phosphate groups on the inositol ring, the ability of proteins to bind these lipids is mediated primarily through electrostatic interactions with basic amino acids. The ways in which these amino acids are presented to the phosphoinositide are however distinct, ranging from structurally well organized binding pockets (e.g. PH (pleckstrin homology) domains, FYVE (Fab1, YOTB, Vac1, EEA1 homology) domains, PX (phox homology) domains, ENTH (epsin N-terminal homology) and ANTH (AP180 N-terminal homology) domains) through to unstructured surfaces defined by amino acid clusters (e.g. MARCKS proteins (McLaughlin et al., 2002)) (for structural details of these and other phosphoinositide-binding domains, plus broader discussion of their roles in signaling and membrane trafficking see (Cullen et al., 2001, Balla, 2005, Lemmon, 2008)).

Phosphoinositide binding sites and surfaces are associated with integral proteins such as ion channels or, more commonly, peripheral cytosolic proteins. For the later, binding is often characterized by relatively low (often micromolar) affinities (Carlton and Cullen, 2005). This favors a rapid, reversible mode of binding where the phosphoinositide-binding protein

is constantly sampling the membrane surface for the presence of the corresponding phosphoinositide, enriching at sites where the lipid resides (Carlton and Cullen, 2005). By itself such binding of individual molecules to membranous phosphoinositides may not be of sufficient strength to lead to stable membrane association. Here the affinity for phosphoinositide recognition needs to be enhanced by combination with another effect to drive productive membrane localization. This additional localization signal can simply be interaction with a pre-localised binding partner (Carlton and Cullen, 2005), or can be the avidity-mediated enhancement in affinity that occurs through generation of multivalent interactions with membranes. To refer back to a previously described analogue (Carlton and Cullen, 2005) – this avidity-mediated binding can be likened to the ability of Velcro™ to generate a high-strength bond even though the interaction of each individual hook and loop is relatively weak. Thus, although phosphoinositides are commonly regarded as membrane association cues that target peripheral proteins to membrane surfaces, it is increasingly clear that other interactions besides recognition of phosphoinositides function to restrict the association of phosphoinositide-binding proteins to cellular membranes. Such avidity-dependent, phosphoinositide-mediated membrane association can take multiple forms. For example, the PH domain of dynamin has a low millimolar affinity for membranes containing PtdIns(4,5)P₂ (Klein et al., 1998, Lemmon, 2008). However, upon self-assembly into oligomers the apparent affinity shifts towards the micro/nanomolar range leading to stabilisation of dynamin oligomers to PtdIns(4,5)P₂ enriched regions of the plasma membrane during the process of endocytic scission (Figure 3a) (Klein et al., 1998, Lemmon, 2008). Other modes of avidity-based membrane association include, where a given phosphoinositide-binding protein can associate with another membrane-bound protein such as integral cargo protein or a lipid-modified small GTPase (eg, PTB domains from Dab1/2, numb and ARH all bind both NPXY-containing cargo and PtdIns(4,5)P₂ (Traub, 2003)), with an additional membrane lipid (e.g. phosphatidic acid or phosphatidyl serine in the case of the PX domain of p47phox) (Figure 3b) (Karathanassis et al., 2002, Stahelin et al., 2003), or can sense the geometry of the membrane surface (e.g. the combination of membrane curvature sensing BAR domain and PtdIns(3)P-binding PX domain of the sorting nexins SNX1 and 2 (Figure 3c) ((Carlton et al., 2004, Carlton et al., 2005) see section 7.1.1.)). An intriguing example of how these associations can be regulated is observed for HIV-1 Gag; here a polybasic region drives association with PtdIns(4,5)P₂ (Ono et al., 2004, Chukkapalli et al., 2008) for viral assembly at the plasma membrane. Association with PtdIns(4,5)P₂ induces exposure of a sequestered myristate moiety (Saad et al., 2006) and is thought to stabilize Gag associated at the plasma membrane. Similarly, in the case of p47phox's PX domain, phospholipids binding sites are masked by self-interaction and phospho-mimic mutations that relieve this self-interaction greatly enhance membrane association, suggesting that this interaction is regulatable (Stahelin et al., 2003).

Why incorporate phosphoinositide-binding into a membrane association system that is based on avidity? Low affinity gives rise to a highly plastic system, defined by dynamic instability, that allows rapid re-modelling in response to, for example, fluctuations in the level of phosphoinositides. Moreover, the fact that membrane association depends upon multivalent interactions, builds into the system a property referred to as co-incidence detection (Carlton and Cullen, 2005). Here, two or more signals, one of which is phosphoinositide recognition,

are required to be present at the same time within the same membrane in order to achieve membrane association of the phosphoinositide-binding protein. In turn, co-incidence detection affords a greater element of regulation as manipulation of signals, other than the level of phosphoinositides, can have pronounced effects on the membrane association and hence the function of phosphoinositide-binding proteins. Avidity-based membrane association thereby gives rise to a greater spatial and temporal regulation of phosphoinositide-mediated cellular regulation. In the following we expand on the importance of avidity in phosphoinositide biology by discussing the four major steps within the endo-lysosomal network: endocytosis, the biogenesis of MVBs, and cargo sorting into the retrograde endosome-to-Golgi and endosomal recycling pathways.

5. PtdIns(4,5)P₂ in endocytosis

The function of PtdIns(4,5)P₂ in clathrin-mediated endocytosis can be viewed as a paradigm for the role of phosphoinositide in membrane trafficking. All membrane trafficking pathways follow a similar series of co-ordinated events. At the donor membrane, coat complexes package with high fidelity selected transmembrane spanning cargo into membrane regions that they deform into vesicular or tubular shaped buds. Bud fission generates coated cargo-enriched carriers, which undergo uncoating, either prior to or after, short or long range movement towards their target membrane. The correct target membrane is detected through interaction with tethering complexes before SNARE-mediated membrane fusion allows lateral movement of the cargo into the target compartment.

Mechanistically, clathrin-mediated endocytosis can be considered to comprise three overlapping stages: (i) co-ordination of cargo selection and sorting with the assembly of the coat complex; (ii) generation and stabilization of membrane curvature; and (iii) fission to liberate the cargo-enriched clathrin-coated vesicle.

5.1. Initiation and assembly of clathrin-coat pits

As discussed previously, formation of endocytic assembly zones appears to occur through low affinity interactions between AP-2 and discrete, localised patches of plasma membrane PtdIns(4,5)P₂ (Figure 2a, b) (Traub, 2009b). Actual nucleation of these assembly zones may however be dependent on the F-BAR domain-containing protein family FCHo1/2 (Fer/Cip4 homology domain-only proteins 1 and 2) (Henne et al., 2010). FCHo proteins, which associate with the plasma membrane through binding to PtdIns(4,5)P₂ (Henne et al., 2010), contain an F-BAR domain that is distinct to other BAR domains in being able to recognise less extreme membrane curvature (Henne et al., 2007, Shimada et al., 2007). Importantly, overexpression of FCHo1/2 increases the rate of clathrin-coated pit nucleation (Henne et al., 2010), and FCHo2 associates with eps15 and intersectin, proteins that directly interact with AP-2 but not clathrin (Henne et al., 2010). FCHo1/2 proteins therefore drive the initial membrane sculpturing required for nucleating pit formation prior to the recruitment of AP-2 (Henne et al., 2010). Once recruited to these nucleation 'hot-spots', AP-2 leads to clathrin recruitment and self-assembly and mediates cargo capture (Traub, 2009b).

A key feature in the formation of clathrin-coated pits is the reliance on multiple low affinity interactions in order to generate the necessary avidity to drive stable coat assembly (Schmid

and McMahon, 2007). A consequence of avidity is the generation of dynamic instability, leading to the formation of pathway checkpoints (Loerke et al., 2009). For example, there is a dependence on a threshold of PtdIns(4,5)P₂ being breached to ensure sufficient AP-2 is membrane associated to engage enough cargo. Indeed, a significant number of initial assemblies, rather than proceeding to productive clathrin-coated pits, undergo early or late abortive events (Loerke et al., 2009, Saffarian et al., 2009). Furthermore, manipulating the level of cargo can alter the lifetime and shift the relative levels of abortive to productive assemblies (Mettlen et al., 2010). A beautiful study suggests that abortive events may correspond to an incomplete conformation opening of the AP-2 complex, necessary for multiple simultaneous PtdIns(4,5)P₂-binding events and cargo recognition through linear sorting motifs present on the cytosolic tail of cargo proteins (Jackson et al., 2010).

5.2. Maturation of clathrin-coated pits

Co-ordinated with the initial assembly of the clathrin-coated pit, the association of a large number of accessory proteins (many of which associate with PtdIns(4,5)P₂) act to constrain clathrin self-assembly and further sculpture membrane bending (Figure 2a). Interacting with the core clathrin coat components are proteins such as dynamin, amphiphysin, epsin and sorting nexin-9 (SNX9), which through the presence of BAR and ENTH (epsin N-terminal homology) domains, sense and/or generate membrane curvature (Schmid and McMahon, 2007). These drive and/or stabilize further membrane re-modelling, allowing maturation towards highly invaginated clathrin-coated pits. Again, recruitments are based on multivalent interactions, which generate dynamic instability, allowing for pathway progression (Schmid and McMahon, 2007). Within these accessory proteins, the GTPase dynamin appears to play a key role in monitoring the maturation state of the clathrin-coated pit (Loerke et al., 2009). Dynamin itself is PtdIns(4,5)P₂-regulated, and its association with PtdIns(4,5)P₂ containing membranes is driven by avidity-mediated oligomerisation (Figure 3a) (Klein et al., 1998). Manipulating dynamin levels has the effect of either enhancing or decreasing the turnover rate of abortive clathrin-coated pit assemblies (Loerke et al., 2009).

5.3. Formation of clathrin-coated vesicles

Fission of the mature invaginated clathrin coated pit requires GTP hydrolysis and the self-assembly of a dynamin spiral at the collar of the pit (Hinshaw and Schmid, 1995, Takei et al., 1995) (see (Mettlen et al., 2009, Pucadyil and Schmid, 2009, Traub, 2009a, Traub, 2009b) for extensive reviews on dynamin regulation and function). Dynamin is recruited at late stages of clathrin-coated vesicles formation through an interaction of its PH domain with PtdIns(4,5)P₂ (Salim et al., 1996, Merrifield et al., 2002, Rappoport et al., 2008). In solution, dynamin can spontaneously assemble into helices and rings, and in the presence of protein-free, PtdIns(4,5)P₂-containing liposomes can drive the formation of membrane tubules (Hinshaw and Schmid, 1995, Sweitzer and Hinshaw, 1998). *In vivo* however, it is predicted that with the level of membrane tensions observed in cellular membranes, dynamin may not deform membranes into tubules (Roux et al., 2010b). Rather, dynamin is recruited to the neck of clathrin-coated pits only when the curvature of the neck is sufficiently high (Roux et al., 2010b). Subsequent polymerisation of dynamin constricts the neck further down to a radius of approximately 10 nm (Roux et al., 2010b). Assembly of the dynamin collar also triggers cooperative GTP hydrolysis. This is argued to lead to fission

either via mechanochemical constriction, extension and twisting of the collar (Stowell et al., 1999, Roux et al., 2006), or through a regulated cycle of dynamin assembly and disassembly which, through imposing curvature stress, allows spontaneous stochastic fission (Bashkurov et al., 2008).

Also aiding the sculpturing and fission of mature clathrin-coated pits is the regulated polymerisation of actin (Orth and McNiven, 2003, Schafer, 2004). Indeed, a number of dynamin interacting proteins are actin-regulatory proteins (Orth and McNiven, 2003, Schafer, 2004). An example is the BAR domain-containing protein sorting nexin-9 (SNX9). This PtdIns(4,5)P₂-binding protein directly binds dynamin, aiding its recruitment to the highly invaginated necks of mature clathrin-coated pits (Lundmark and Carlsson, 2003, Lundmark and Carlsson, 2004, Lundmark and Carlsson, 2009). SNX9 also associates with and activates, in a membrane-dependent manner, N-WASP (Shin et al., 2007, Yasar et al., 2007, Shin et al., 2008, Yasar et al., 2008), thereby driving actin polymerisation via the Arp2/3 actin nucleating complex. The localised, co-ordinated burst of actin polymerisation may assist in the efficiency of dynamin-mediated fission through the generation of longitudinal force (Figure 2a) (Ferguson et al., 2009, Lundmark and Carlsson, 2009).

Following liberation from the plasma membrane, the cargo-enriched clathrin-coated vesicles undergo uncoating prior to delivery to early elements of the endosomal network. Here, one needs to overcome the increased stability arising from the avidity-based interactions that have developed during maturation of the clathrin-coated vesicle, in order to allow recycling of the molecular components for further rounds of endocytosis (Schmid and McMahon, 2007). For clathrin-coated vesicles, uncoating is triggered by recruitment of the auxilin and Hsc70 ATPase (Fotin et al., 2004) along with the switching of PtdIns(4,5)P₂ to PtdIns(4)P (Figure 2a, b) (see section 3.1).

6. PtdIns(3)P and MVB biogenesis

Cargos targeted for lysosomal degradation are tagged with ubiquitin after receptor activation at the plasma membrane, suggesting cargo fate is determined early in its endocytic itinerary. Ubiquitinated cargos are retained within specialized subdomains of the endosome and, through the actions of endosomal sorting machinery, become incorporated onto intraluminal endosomal vesicles (ILV) to generate an organelle called a multivesicular body (MVB) (Figure 1a) (Gruenberg and Stenmark, 2004, Piper and Katzmann, 2007). Subsequent fusion of MVBs with the yeast vacuole or mammalian lysosome results in the delivery of these cargoes to the hydrolytic and proteolytic interior. A key event in degradative sorting is the separation of cargoes destined for degradation onto ILVs, preventing them from being recycled. ILV biogenesis requires deformation of the limiting endosomal membrane into the endosomal lumen with transfer of sequestered ubiquitinated cargoes onto the developing bud (Gruenberg and Stenmark, 2004, Piper and Katzmann, 2007). Extension of these buds leads to the development of intraluminal membrane invaginations and scission of the necks of these invaginations leads to the release of nascent ILV into the MVB interior. Importantly, these activities are in topological apposition to other cellular budding event (Figure 4a) and must be effected through the actions of a cytoplasmic machinery.

Isolation of the yeast Vacuolar Protein Sorting (VPS) mutants provided initial insights into mechanisms of degradative sorting (Bankaitis et al., 1986, Robinson et al., 1988, Raymond et al., 1992). The Class E subset displayed clear defects in ILV biogenesis and accompanying failure to properly sort cargo to the vacuole. Proteins encoding these genes were subsequently found to form three macromolecular complexes, termed ESCRT-I, -II and -III (Katzmann et al., 2001, Babst et al., 2002a, Babst et al., 2002b) (Table 1). Genetic evidence suggested that these complexes acted sequentially in the trafficking of ubiquitinated cargo onto the yeast vacuole and homologues of these proteins were identified in mammalian cells and an evolutionarily conserved activity of degradative protein sorting was ascribed to these complexes (Williams and Urbe, 2007, Carlton, 2010). In addition to a role for ESCRT proteins in regulating degradative sorting, ESCRT-proteins were shown to be required for the release of retroviruses such as HIV-1 (Morita and Sundquist, 2004, Carlton and Martin-Serrano, 2009). Here, they are recruited to sites of particle assembly at the plasma membrane through direct interaction with viral structural proteins and provide an activity allowing scission of membranous stalks connecting the viral particles to the cell and subsequent release of the viruses (Morita and Sundquist, 2004, Carlton and Martin-Serrano, 2009, Carlton, 2010). Further, the ESCRT-machinery has been shown to be important for the terminal phase of cell division where it plays a topologically equivalent role in severing the membranous stalk connecting daughter cells at the end of cytokinesis (Carlton and Martin-Serrano, 2007, Morita et al., 2007, Carlton et al., 2008). The topological equivalence of the membrane scission event required for ILV biogenesis, viral particle release and cytokinesis suggests a common mechanism is involved (Figure 4a). That all three processes require the ESCRT-machinery to effect this scission suggests further that this machinery provides this membrane fission activity. Thus, a picture emerges of the ESCRT-machinery as a transplantable membrane deformation and scission apparatus, able to impose its topologically unique activity upon cellular membranes.

In the context of endosomal sorting, the *vps34* and *vps15* mutants in yeast displays clear defects in the trafficking hydrolases to the yeast vacuole (Schu et al., 1993, Stack et al., 1993). Vps34p is the sole PI 3-kinase in yeast, and Vps15p its regulatory subunit, suggesting that 3-phosphoinositides are important in regulating degradative sorting. In mammalian cells, treatment with PI 3-Kinase inhibitors such as wortmannin or microinjection of antisera targeting the Class-III PI 3-kinase produce a striking defect in MVB biogenesis with endosomes lacking ILVs (Fernandez-Borja et al., 1999, Futter et al., 2001). These data suggested that products of this Class 3 PI 3-kinase controlled key events allowing MVB biogenesis and subsequent degradative sorting, findings confirmed by an analysis of sorting in cells in which endosomal PtdIns(3)P was sequestered (Petiot et al., 2003). This endosomal PI 3-kinase activity is important for generating a localization cue for a variety of endosomal regulators. As described previously, Rab5-GTP can associate with the endosomal PI 3-kinase complex, coupling Rab5 activity to PtdIns(3)P generation (Christoforidis et al., 1999). Further, PtdIns(3)P-binding Rab5 effectors such as EEA1 and Rabenosyn5 can be recruited to these microdomains, and their associated proteins such as the Rab5 GEF Rabex-5 can further stimulate Rab5 activation and thus PI 3-kinase activity, suggesting how microdomains of PtdIns(3)P may be generated upon the endosomal membrane (Zerial and McBride, 2001). These microdomains have been visualized in mammalian cells (Gillooly et

al., 2000b, Gillooly et al., 2003) and act as localization signals for PtdIns(3)P-binding endosomal regulators. Generation of these domains appears dependent upon the localised activation of PI 3-kinases and 3'-phosphatases, which, as discussed earlier are subject to feed-forward control through the actions of Rab5. These findings support the generation of highly plastic endomembrane domains to support nucleation of the ESCRT-machinery. Here we will examine 3-phosphoinositide binding domains present within distinct ESCRT-complexes and will consider how these endosomal lipids may regulate ESCRT-dependent cargo sorting to the MVB.

6.1. ESCRT-0

ESCRT-0 is a complex of Hrs, Stam-1 and -2 in mammalian cells (Vps27p and Hse1p in yeast) (Williams and Urbe, 2007). A FYVE domain within Hrs confers high affinity PtdIns(3)P-binding upon this complex (Table 1) (Burd and Emr, 1998, Kutateladze et al., 1999, Raiborg et al., 2001, Sankaran et al., 2001), directing ESCRT-assembly to PtdIns(3)P-enriched microdomains (Figure 4b). Multiple VHS (Vps27, Hrs, Stam) and UIM domains within ESCRT-0 components bind strongly and co-operatively to ubiquitinated cargo (Ren and Hurley), ensuring that these cargoes and downstream ESCRT-complexes are retained within the ESCRT-0 containing PtdIns(3)P microdomain. Subsequent interactions of ESCRT-0 with deubiquitinating enzymes such as AMSH or UBPY (McCullough et al., 2004, McCullough et al., 2006, Mizuno et al., 2006), explain how ubiquitin removal and recycling is coupled to cargo sequestration. ESCRT-0 thus appears a PtdIns(3)P-localised endosomal adaptor for subsequent ESCRT-recruitment.

6.2. ESCRT-I and -II

ESCRT-I and -II play important roles in linking ESCRT-III with upstream adaptors: in the case of endosomal sorting – the ESCRT-0 complex, and in the case of viral release or cytokinesis, viral Gags or the cytokinetic apparatus respectively (Carlton and Martin-Serrano, 2009). ESCRT-I functions as a 1:1:1:1 heterotetramer (Vps23, Vps28, Vps37, Mvb12 in yeast; paralogues in mammalian cells) that comprises a ubiquitin-binding UEV-domain from TSG101/Vps23p (Sundquist et al., 2004, Teo et al., 2004) linked to an elongated stalk made from all 4 ESCRT-I subunits (Kostelansky et al., 2006, Teo et al., 2006, Gill et al., 2007, Kostelansky et al., 2007). This stalk terminates in a headpiece that connects to ESCRT-II via interactions between Vps28 and the GLUE domain of Vps36/EAP45 within ESCRT-II (Teo et al., 2006, Gill et al., 2007, Im and Hurley, 2008). The presence of ubiquitin binding domains within ESCRT-I and -II (the UEV domain of Tsg101 (Sundquist et al., 2004, Teo et al., 2004), non-canonical Ub-binding domain within Mvb12p (Shields et al., 2009), the GLUE domain of ESCRT-II (Slagsvold et al., 2005, Alam et al., 2006, Hirano et al., 2006)) suggest how ubiquitinated cargo can be transported through these complexes). No defined phosphoinositide binding domains have been observed within ESCRT-I and consistent with this, the recombinant ESCRT-I complex binds only to modestly to acidic membranes with moderate enhancement given by the presence of PtdIns(3)P (Kostelansky et al., 2007). Whilst unable to bind PtdIns(3)P when in isolation, mixture of ESCRT-I and -II produced a PtdIns(3)P-binding complex, suggesting that interaction with ESCRT-II is sufficient to drive ESCRT-I onto PtdIns(3)P containing membranes (Figure 4b) (Teo et al., 2006).

The ESCRT-II core binds to acidic membranes in a manner requiring the GLUE domain of Vps36. This domain was found to bind PtdIns(3)P (Table 1), suggesting how ESCRT-II could localize independently to PtdIns(3)P enriched endosomes (Figure 4b) (Teo et al., 2006). Indeed, in mammalian cells, ESCRT-II localizes to endosomal membranes and could bind 3-phosphoinositides (Slagsvold et al., 2005) (although in this case, interaction with PtdIns(3,4,5)P₃ was observed via dot blot). The GLUE domain has a PH-domain like fold (Slagsvold et al., 2005, Teo et al., 2006). In fungi, two NZF fingers make a large 150-residue insertion into this fold, suggesting that it may be a split PH domain. The 1st NZF-finger binds Vps28 in ESCRT-I, the second NZF-finger binds ubiquitinated cargo (Teo et al., 2006). Split phosphoinositide binding domains comprise a relatively new class of PI binding module, formed by intra- or inter-molecular interactions of half-domains that preclude their identification by sequence-based searching (van Rossum et al., 2005). Crystallisation of the yeast Vps36 GLUE domain lacking this insertion reveals a PH domain with a non-canonical PtdIns(3)P binding pocket, which is responsible for endosomal localization (Teo et al., 2006). The spatial relationship between the NZF-fingers and the PtdIns(3)P binding surfaces is unclear in the context of holo-Vps36, although interactions between lipid, ESCRT-I and ubiquitinated cargo can be simultaneously observed, suggesting that the ESCRT-II GLUE domain is a master regulator of ESCRT-dependent traffic and that these co-incident localization cues may act as foci for ESCRT-assembly (Figure 4b). Indeed, expression of a Vps36 mutant rendered unable to bind PtdIns(3)P induced a severe defect in degradative sorting, emphasizing the critical role 3-phosphoinositides play in this process (Teo et al., 2006). Importantly, mammalian Vps36 lacks these NZF fingers, yet retains an ability to bind ESCRT-I and ubiquitinated cargo, suggesting a functional conservation of ESCRT-II GLUE domain activity in the process of lysosomal sorting (Slagsvold et al., 2005). In addition to the 3'-phosphoinositide binding GLUE domain, the mammalian ESCRT-II subunit Vps22 contains an acidic lipid-binding N-terminal helix. This helix is conserved from yeast to humans and cooperates with the 3-phosphoinositide binding GLUE domain to allow full binding of ESCRT-II to cellular membranes (Im and Hurley, 2008).

6.3. ESCRT-III

ESCRT-III has long been considered the machinery providing membrane fission activity. ESCRT-III subunits are all highly related Charged Multivesicular Body Proteins (CHMP) with a highly basic N-terminus and a highly acidic C-terminus (Williams and Urbe, 2007). They are thought to fold back into a 'closed' or autoinhibited state, rendering them inactive. Like other ESCRT-subunits, ESCRT-III proteins are mostly peripheral membrane proteins. The exception to this Vps20p/CHMP6; this protein is myristolated and thought to be pre-localised to endosomal membranes. Subsequent interaction of CHMPs with membranes or other CHMP proteins has been postulated to drive them into an active conformation, allowing them to drive membrane fission (Williams and Urbe, 2007, Teis et al., 2008, Saxena et al., 2009). ESCRT-III proteins are able to bind to membranes and deform them into inward invaginations, recapitulating key features of ESCRT-activity (Hanson et al., 2008, Saxena et al., 2009). The ESCRT-III subunit CHMP3 was isolated by virtue of its ability to bind 3-phosphoinositides including PtdIns(3,5)P₂ (Figure 4b, Table 1) (Whitley et al., 2003). Again, no detectable phosphoinositide-binding domains were observed, suggesting that this interaction occurred through a non-canonical PI-binding motif. Indeed,

other ESCRT-III proteins that are thought to form part of the core membrane fission machinery (CHMP2a, CHMP4b) could be isolated from cytosolic extracts upon PtdIns(3,5)P₂ liposomes (Catimel et al., 2008), suggesting that PtdIns(3,5)P₂ recognition may be a conserved, if unexplained, feature of this complex that tallies with the predicted location of this lipid (Figure 1b). Crystallization of the central region of CHMP3 demonstrated this protein folded into a multimeric helical arrangement with a flat membrane targeting face (Muziol et al., 2006). Both basic membrane targeting and multimerisation activities were required for CHMP3 function, and sequence analysis of other ESCRT-III subunits revealed similar electrostatic potentials, suggesting that these proteins function as a membrane bound lattice to effect ESCRT-activity (Muziol et al., 2006). Thus, whilst CHMP3 and possibly other CHMPs can bind to 3-phosphoinositides, it is possible that the ESCRT-III complex acts simply on cellular membranes to effect membrane scission with upstream ESCRT-complexes (ESCRT-0 and ESCRT-II) directing its assembly in the endosomal context to regions of endosomal PtdIns(3)P. Consistent with this possibility is the finding that ESCRT-III can function effectively at the plasma membrane – usually devoid of PtdIns(3)P – to effect viral particle release. However, it should be noted that PtdIns(3)P has been detected at the midbody of dividing cells and localizes a PtdIns(3)P-binding protein called FYVE-CENT to this structure (Sagona et al., 2010). FYVE-CENT is required for cytokinesis and can interact with ESCRT-III via TTC19 (Sagona et al., 2010). These data suggest that under some conditions, PtdIns(3)P may be generated upon non-endosomal membranes where it may assist ESCRT-dependent events.

6.4. In vitro reconstitution of ESCRT function

Recent elegant biophysical studies have reconstituted ESCRT-activity upon artificial giant unilamellar vesicles (GUVs) *in vitro* (Wollert et al., 2009, Wollert and Hurley, 2010). Here, ordered addition of ESCRT-0, -I, -II and -III acted to sequester ubiquitinated cargo, generate invaginations and effect ILV release from the limiting membrane of GUVs containing small molar percentages of PtdIns(3)P. These data recapitulated numerous observed functions of the ESCRT-machinery, with ESCRT-0 required for cargo sequestration, ESCRT-I and -II acting to produce inward invaginations and ESCRT-III acting to release the ILV. An isolated study of ESCRT-III-dependent ILV release revealed that PtdIns(3)P or PtdIns(3,5)P₂ provided only a 2-fold enhancement of ILV release, suggesting that 3-phosphoinositides are not absolutely required for ESCRT-III activity (Wollert et al., 2009) and may instead play regulatory roles in this activity and/or specifying endosomal localisation of this machinery through coordination of upstream regulators.

6.5. ESCRT-independent, phosphoinositide-mediated sorting into MVBs

Traffic to the lysosome appears PtdIns(3)P-dependent (Petiot et al., 2003) and the ESCRT proteins are localized to endosomes in a 3-phosphoinositide-dependent manner. However, ESCRT-dependent routes are not the only way of generating MVBs; Gruenberg and colleagues have recently demonstrated that the PtdIns(3)P-binding protein SNX3 is required for MVB biogenesis, suggesting either that SNX3 may coordinate the formation of a distinct class of MVB from those formed downstream of Hrs, or that SNX3 acts synergistically with the ESCRTs to produce ILVs. Yet other data have shown that MVBs are still present in cells undergoing long term depletion of numerous ESCRT-proteins (Stuffers et al., 2009), that

some proteins can access the MVB lumen independently of ESCRTs (Theos et al., 2006) and that enveloped viruses such as influenza (Bruce et al., 2009, Rossman et al., 2010) can exit cells in an ESCRT-independent manner. All of these data point to the existence of ESCRT-independent mechanism for ILV biogenesis and membrane fission. Whether SNX3 co-ordinates such an activity remains an exciting area of future study.

7. Phosphoinositides and retrograde transport

Retrograde trafficking is the process by which proteins and lipids are transported between endosomes and the biosynthetic/secretory compartments including the *trans*-Golgi network (TGN) and Golgi apparatus (Figure 5a) (Bonifacino and Rojas, 2006, Johannes and Popoff, 2008). It defines not a single pathway, but a number of mechanistically distinct transport routes that may function in parallel or sequentially. Retrograde transport has a variety of roles in normal physiology, which include the retrieval of receptors and cargoes from the endo-lysosomal degradative pathway, cellular adaptation to fluctuations in the nutrient environment and the establishment of morphogenic gradients during tissue patterning (Johannes and Popoff, 2008). Moreover, endosome-to-Golgi transport is perturbed in various diseases and is required for the cellular entry of pathogens and pathogenic toxins (Johannes and Popoff, 2008). Defining the mechanistic basis of retrograde transport is therefore important in understanding human disease and constitutes a novel avenue for therapeutic development.

A number of molecular entities play important roles in retrograde transport (see Table 2 in (Johannes and Popoff, 2008) for an extensive list). Included are clathrin (Lauvrak et al., 2004, Saint-Pol et al., 2004, Esk et al., 2010), clathrin-binding proteins such as epsinR, AP-1, AP-3, and OCRL, the clathrin uncoating ATPase Hsc70 (Folsch et al., 2001, Medigeshi and Schu, 2003, Saint-Pol et al., 2004, Choudhury et al., 2005, Popoff et al., 2009, Shi et al., 2009), and various small GTPases including ARF1 and Rab9 along with their regulatory proteins (Lombardi et al., 1993, Diaz et al., 1997, Shiba et al., 2010). In addition, the VPS34 PtdIns 3-kinase (Burda et al., 2002), the PtdIns(3)P 5-kinase PIKfyve (Rutherford et al., 2006), the PtdIns(4,5)P₂ 5-phosphatase OCRL (Choudhury et al., 2005) have all been implicated in retrograde transport as have the PtdIns(4)P-binding proteins AP-1 (Wang et al., 2003) and epsinR (Mills et al., 2003) and a number of PtdIns(3)P and PtdIns(3,5)P₂-binding proteins that make up components of the multimeric retromer complex (Cozier et al., 2002). That a number of these phosphoinositide regulatory and binding proteins also associate with clathrin and small GTPases hints at a complex level of communication between multiple retrograde transport pathways.

7.1. The retromer complex

A major advance in our understanding of the role played by phosphoinositides in the regulation of retrograde transport came with the identification of the yeast retromer complex (Seaman et al., 1998). Here retromer is composed of two sub-complexes: a stable cargo-binding Vps26p-Vps29p-Vps35p trimer and a membrane deforming coat comprising the PtdIns(3)P-binding proteins Vps5p and Vps17p (Figure 5b) (Burda et al., 2002). Retromer is evolutionarily conserved, being present in the most primitive of eukaryotic ancestors (Dacks et al., 2009), and functions to regulate endosome-to-Golgi retrograde transport of cargo

proteins including the yeast carboxypeptidase Y receptor Vps10p (reviewed in (Bonifacino and Hurley, 2008, Verges, 2008, Attar and Cullen, 2010)). The mammalian retromer is more complex and comprises a VPS26-VPS29-VPS35 trimer (including two isoforms of VPS26) (Edgar and Polak, 2000, Haft et al., 2000, Kerr et al., 2005) and gene duplication appears to have resulted in two Vps5p's, sorting nexin-1 (SNX1) and SNX2, and two, possibly three Vps17p's, SNX5 and SNX6 (and possibly SNX32) (Teasdale et al., 2001, Wassmer et al., 2007, Wassmer et al., 2009). Like its yeast counterpart, mammalian retromer regulates retrograde endosome-to-Golgi transport of an increasingly diverse group of cargo proteins which include the cation-independent mannose 6-phosphate receptor (CI-MPR) (Arighi et al., 2004, Carlton et al., 2004, Seaman, 2004), sortilin (Seaman, 2007, Mari et al., 2008), the Wntless receptor/chaperone for Wnt developmental morphogens (Belenkaya et al., 2008, Franch-Marro et al., 2008, Pan et al., 2008, Port et al., 2008), polymeric immunoglobulin receptor (Verges et al., 2004), the iron transporter DMT1-II (Tabuchi et al., 2010), amyloid precursor protein (He et al., 2005, Small et al., 2005) and Shiga toxin (Bujny et al., 2007, Popoff et al., 2007). Retromer has also been linked with trafficking between mitochondria and peroxisomes (Braschi et al., 2010), and in the clearance of apoptotic bodies in *C. elegans* (Chen et al., 2010).

7.1.1. Membrane deformation within the retromer pathway—The retromer sorting nexins all share a common domain architecture defined by an amino-terminal phosphoinositide-binding PX domain (which in most cases binds the early endosomal phosphoinositide PtdIns(3)P but can also associated with PtdIns(3,5)P₂ (Cozier et al., 2002)) and a carboxy-terminal BAR domain (Cullen, 2008): a domain that through dimerisation generates a rigid 'banana-shaped' structure, the concave face of which preferentially associates with membranes of high positive curvature (Frost et al., 2009). The combined ability to bind PtdIns(3)P and the curvature sensing mode of the BAR domain generates a co-incidence signal that effectively targets retromer sorting nexins to high curvature sub-domains of the PtdIns(3)P-enriched early endosome (Figure 3c) (Carlton et al., 2004).

Like other BAR domain-containing proteins, retromer sorting nexins have an inherent potential to drive the formation of membrane tubules (Carlton et al., 2004, Gokool et al., 2007). Here, as co-incidence detection increases their effective membrane concentration, retromer sorting nexins undergo spontaneous assembly into a helical coat (Frost et al., 2008). In forming this coat, the rigid nature of the BAR domain imposes membrane curvature which is accommodated by the formation of membrane tubules. In other words, on increasing their effective membrane concentration retromer sorting nexins may switch from a curvature sensing to curvature inducing mode (Roux et al., 2010a). That said, although these proteins can induce tubulation in a protein-free reconstituted membrane system (Carlton et al., 2004), nucleation *in vivo* may require additional factors.

One factor that lies upstream of retromer and therefore may play a role in retromer nucleation *in vivo* is clathrin (Johannes and Popoff, 2008). Evidence from endosome-to-TGN transport of Shiga toxin and CI-MPR, suggests that clathrin and retromer act sequentially in sorting from endosomes (Popoff et al., 2007, Popoff et al., 2009). Indeed, a two-step model for retrograde sorting has been presented in which retromer drives the formation of a membrane tubule from the edge of a flat, cargo-enriched clathrin containing

lattice or, alongside other clathrin adaptors such as AP-1 and epsinR, clathrin generates a dynamic flat, cargo-enriched lattice that nucleates the formation of high membrane curvature which is subsequently stabilized and processed into cargo-enriched carriers by retromer (Figure 5a) (Popoff et al., 2007, Johannes and Popoff, 2008). Irrespective of the subtleties of these models, evidence that retromer sorting nexins can form complexes with the clathrin-binding protein Hrs (Chin et al., 2001, Popoff et al., 2009), the clathrin uncoating ATPase Hsc70 (Popoff et al., 2009, Shi et al., 2009), and clathrin heavy chain 22 (Towler et al., 2004, Esk et al., 2010), all point to a dynamic relationship between clathrin and retromer. Clarifying the precise nature of these relationships will be an important area for future research into retrograde transport.

7.1.2. Cargo selection – the mammalian VPS26-VPS29-VPS35 sub-complex—

One route for endosomal association of the trimeric cargo-selective retromer sub-complex is through binding to the GTP-bound form of Rab7 (Figure 5b) (Rojas et al., 2008, Seaman et al., 2009): this is entirely consistent with retromer-decorated tubules occurring at a relative late stage of early endosome maturation close to the transition with Rab7-labeled late endosome (Mari et al., 2008). The VPS35 subunit has been implicated in direct recognition of sorting motifs within the cytosolic region of cargo proteins: for CI-MPR and sortilin this comprises a linear tripeptide motif of $[FM]L[MV]$ with similar motifs being present in the cytosolic tails of sorLA, Wntless and DMT1-II (Seaman, 2007, Tabuchi et al., 2010). Regions lying upstream and/or downstream of these motifs also appear important for efficient cargo binding and, at least for CI-MPR and sortilin, palmitoylation by DHHC-15 is required for the interaction with VPS35 (McCormick et al., 2008). Structurally, VPS35 is arranged as an α -solenoid composed of a predicted series of 17 HEAT-like α -helical repeats (Hierro et al., 2007). Onto this core component the VPS29 and VPS26 subunits bind independently at respectively the carboxy and amino distal ends to form an extended trimeric complex (Hierro et al., 2007, Norwood et al., 2010). VPS29 itself displays a phosphatase-like fold, although this appears to be non-functional (Collins et al., 2005, Wang et al., 2005, Damen et al., 2006, Hierro et al., 2007), and VPS26 has an arrestin-like fold, leading to the suggestion that it may also play a role in cargo recognition (Shi et al., 2006, Collins et al., 2008). Currently there is no data to support this conclusion.

Away from the process of cargo selection, the VPS26-VPS29-VPS35 heterotrimer also functions as a hub for the scaffolding of additional complexes that play important regulatory roles in endosomal sorting (Harbour et al., 2010). Interacting proteins include TBC1D5, a Rab GTPase-activating protein that probably functions on Rab7 (Harbour et al., 2010), FKBPI5 (Harbour et al., 2010), and, most notably, the WASH complex. In humans this is composed of WASH and a core unit, recently termed the 'WASH regulatory complex' (Jia et al., 2010), comprising FAM21, SWIP, Strumpellin and CCDC53 (coiled-coil domain containing protein 53) (Derivery et al., 2009, Gomez and Billadeau, 2009, Harbour et al., 2010, Jia et al., 2010). VPS26-VPS29-VPS35 associates through direct binding of VPS35 to FAM21 (Harbour et al., 2010): additional binding of VPS35 and SNX1/SNX2 to WASH and FAM21 may stabilize the interaction (Gomez and Billadeau, 2009, Harbour et al., 2010). Importantly, while FAM21 does bind to a variety of phosphoinositides including PtdIns(3)P and PtdIns(3,5)P₂ (Jia et al., 2010) (a note of caution here – data derived using

fat blots), the presence of VPS26-VPS29-VPS35 is necessary for the association of the WASH complex to endosomes (Harbour et al., 2010).

Functionally, suppression of the WASH complex results in the formation of extended and exaggerated retromer-labelled tubules leading to a defect in retrograde sorting of the CI-MPR (Derivery et al., 2009, Gomez and Billadeau, 2009, Harbour et al., 2010). WASH itself is a member of the Wiskott-Aldrich syndrome protein (WASP) family member, and shares with these proteins an ability to regulate the actin nucleating ability of the Arp2/3 complex (Derivery et al., 2009, Gomez and Billadeau, 2009, Jia et al., 2010) (there are obvious parallels here with the role of actin polymerization in clathrin-mediated endocytosis). Importantly, while isolated WASH is constitutively active in driving Arp2/3-dependent actin nucleation, the addition of the regulatory complex is inhibitory (Jia et al., 2010). Moreover, the WASH regulatory complex also interacts, although more weakly, with CAPZ (Harbour et al., 2010, Jia et al., 2010). This binds to the barbed ends of actin filaments, preventing further elongation and promoting increased branch density – characteristics required for force generation. A model emerges therefore where the VPS26-VPS29-VPS35 sub-complex regulates the association of the WASH complex to sites of endosomal tubulation (Harbour et al., 2010). From here, Arp2/3-dependent nucleation leads to actin polymerization suitable for generating longitudinal force, thereby aiding the efficiency of tubule scission (Figure 5a) (Derivery et al., 2009, Gomez and Billadeau, 2009). Overall, given that the VPS26-VPS29-VPS35 sub-complex is also required for the endosomal association of TBC1D5 and FKBP15 (Harbour et al., 2010), one should consider that, besides a role in cargo selection, this sub-complex acts to scaffold the recruitment of accessory proteins that regulate the dynamics of tubular profiles during retromer-mediated endosomal transport.

Modes of cargo selection that are independent of the VPS26-VPS29-VPS35 are also evident in the retromer pathway. In yeast the PtdIns(3)P-binding sorting nexin, Grd19/Snx3p, acts as a cargo-specific adaptor, which, by ‘piggy-backing’ on the retromer sorts cargo to the Golgi (Strochlic et al., 2007). Whether the equivalent proteins in mammalian cells, sorting nexin-3 (SNX3) and sorting nexin-12 (SNX12), function in a similar manner remains to be determined. However, if this does prove to be the case, and as SNX3 has been implicated in the biogenesis of MVBs ((Pons et al., 2008), see section 6.5), this sorting nexin may form a nexus between PtdIns(3)P-mediated endo-lysosomal sorting and retrograde transport. Finally, retromer sorting nexins also directly associate with cargoes including EGFR, TGF β R and various G-protein-coupled receptors (Kurten et al., 1996, Otsuki et al., 1999, Parks et al., 2001, Pons et al., 2003, Heydorn et al., 2004, Gullapalli et al., 2006, Yoon et al., 2006, Nisar et al., 2010). Together, these VPS26-VPS29-VPS35-independent routes for cargo selection allow for a far greater repertoire of retromer-dependent cargoes.

7.1.4. Processing of retromer tubules—As the retromer sorting nexins, SNX1 and SNX2, can associate with VPS35 and VPS29 (Haft et al., 2000, Collins et al., 2005), the classic model describing retromer function argues that it acts by co-ordinating cargo selection with an ability to drive membrane tubulation, thus enriching cargo into tubular carriers destined for retrograde transport. Once cargo has been enriched into the tubular profiles membrane scission occurs via a mechanism that has yet to be described, but may require the interaction of retromer with dynamin and/or the dynamin-like protein EHD1

(Figure 5a) (Daumke et al., 2007, Gokool et al., 2007). However, what is emerging is that the generation of longitudinal tension is an important element for efficient processing of retromer tubules. As discussed above, retromer associates with the WASH complex thereby driving the polymerization of actin (Derivery et al., 2009, Gomez and Billadeau, 2009), and SNX5 and SNX6 are able to bind the p150^{glued} component of the dynein-dynactin minus-end directed microtubule motor complex (Hong et al., 2009, Wassmer et al., 2009). In both cases, uncoupling of retromer from actin polymerization or motor function leads to the formation of extend tubular profiles that appear unable to undergo efficient processing into tubular carriers (Gomez and Billadeau, 2009, Wassmer et al., 2009).

In the final steps of retromer-mediated retrograde transport, coupling to the dynein-dynactin motor allows for long-range movement of cargo-enriched carriers from the cell periphery towards the *trans*-Golgi network (Figure 5a) (Wassmer et al., 2009). The carriers themselves, referred to as ETC's (endosome-to-TGN transport carriers), are devoid of clathrin and appear as non-branched tubules on average 170-230 nm in length and 20-50 nm in diameter (Mari et al., 2008). Recognition of ETCs at the TGN is mediated, at least in part, through the binding of SNX1 and SNX2 by Rab6IP1 (Figure 5a) (Wassmer et al., 2009): a TGN resident protein proposed to act as a tethering factor for carriers from the endosomal network (Miserey-Lenkei et al., 2007). That components of the retromer coat are required during the tethering process suggests that uncoating of ETCs occurs after arrival at the TGN: a scenario similar to the recently described interaction of the COPII coat with the TRAPPI tethering complex (Cai et al., 2007). The nature of the signal(s) that leads to uncoating remains to be defined, although based on analogue with clathrin uncoating during endocytosis, one can speculate on the involvement of phosphoinositide phosphatases and the need for energy input to destabilize the assembled sorting nexin coat complex.

7.2. Phosphoinositide regulation of retromer-independent retrograde transport

Other phosphoinositide-binding sorting nexins have been implicated in retrograde transport. RNAi suppression of early endosomal associated sorting nexin-8 (SNX8) leads to an inhibition of retrograde ricin transport whilst elevating endosome-to-TGN transport of Shiga toxin (Dyve et al., 2009). In addition, suppression of sorting nexin-4 (SNX4) leads to an inhibition of retrograde ricin transport, which is enhanced by retromer co-suppression (Skanland et al., 2007) (see 8.2 for more detailed discussion of SNX4). Finally, a paralog of SNX9, sorting nexin-18 (SNX18), has been localised to early endosomes positive for the AP-1 adaptor and PACS1 (Haberg et al., 2008). This sorting nexin displays an *in vitro* binding preference for PtdIns(4,5)P₂, has the ability to drive membrane tubulation (it contains a BAR domain) and forms complexes with dynamin-2 and AP-1 (Haberg et al., 2008). SNX18 may therefore scaffold a membrane-fission unit that mediates carrier formation during AP-1-positive endosomal trafficking (Haberg et al., 2008). That said, others have questioned this interpretation, preferring a model in which SNX18 plays a redundant function with SNX9 in modulating endocytosis at the plasma membrane (Park et al., 2010).

7.3. PtdIns(3,5)P₂ in retrograde transport

To completely define the role of phosphoinositides in endosomal sorting, one needs to complement the growing repertoire of molecular complexes regulated by PtdIns(3)P, with those regulated by PtdIns(3,5)P₂. As eluded to previously, this phosphoinositide clearly plays a key role in endosomal homeostasis: perturbing PtdIns(3,5)P₂ levels in a variety of organisms results in multiple endosomal phenotypes including endosomal swelling, and defects in acidification, MVB sorting, endosome-to-Golgi transport and the late endosome-to-lysosome axis (Michell et al., 2006, Jefferies et al., 2008, Shisheva, 2008).

A major hurdle in our appreciation of the role played by this phosphoinositide is the lack of well defined, *bona-fide* PtdIns(3,5)P₂ effectors. While mVps24, Ent3p and Ent5p have been suggested as PtdIns(3,5)P₂-binding proteins their true phosphoinositide specificity remains controversial (Michell et al., 2006). The best characterized specific PtdIns(3,5)P₂-binding protein is yeast Svp1p/ATG18 which regulates traffic from the vacuole (the yeast equivalent of the mammalian lysosome) back to the endosome (Dove et al., 2004). A mammalian ATG18 is WIPI49 which binds PtdIns(3)P and PtdIns(3,5)P₂, is localized to TGN/endosomes and regulates endosome-to-TGN transport of the CI-MPR (Jefferies et al., 2004). Since the retromer sorting nexins, SNX1 and SNX2 also bind with similar affinities PtdIns(3)P and PtdIns(3,5)P₂ (Cozier et al., 2002), these proteins have been independently isolated in a PtdIns(3,5)P₂ interactome screen (Catimel et al., 2008), the role of PtdIns(3,5)P₂ in mammalian retromer biology remains controversial, as does retromer's relationship with WIPI49. However, it is clear that defective retrograde endosome-to-TGN transport, through retromer-dependent and -independent pathways is observed upon manipulation of PtdIns(3,5)P₂ (Rutherford et al., 2006, Zhang et al., 2007, de Lartigue et al., 2009). Some of the phenotypes observed upon perturbing the level of this phosphoinositide may arise indirectly from the miss-sorting of important components in multiple pathways.

Moving away from retrograde transport, recent studies have linked PtdIns(3,5)P₂ with promoting the targeting of CaV1.2 channels to lysosomes, thereby promoting their degradation and protecting neuronal cells from excitotoxicity (Tsuruta et al., 2009), regulating the open probability of single RyR2 channels and hence modulating cardiac contractility (Touchberry et al., 2010), and perhaps most intriguingly, activating the endo-lysosome-localized mucolipin TRP (TRPML) channel (Dong et al., 2010). Interestingly, in cells lacking TRPML1, late endocytic trafficking defects are observed along with the generation of swollen endo-lysosomes (Dong et al., 2010). The PtdIns(3,5)P₂-mediated regulation of the TRPML1 channel is evolutionarily conserved being observed in yeast upon hyper-osmotic shock (Dong et al., 2010). As TRPML1 channels regulate Ca²⁺ release from the endo-lysosomal network, an enticing aspect of PtdIns(3,5)P₂ biology may be the regulation of localized Ca²⁺ levels required for fusion and fission reactions during membrane trafficking (Dong et al., 2010).

8. Phosphoinositides in endosomal recycling

Cargos that are to be returned to the cell surface are rescued from the degradative route via endosomal recycling pathways. Recycling cargoes are returned to the plasma membrane from the early endosome either via a rapid recycling route or transit more slowly through a

tubular juxtannuclear endocytic recycling compartment (the ERC) (Figure 1a) (Maxfield and McGraw, 2004). By virtue of their high surface area to volume ratio, recycling tubules are thought to effectively partition transmembrane receptors whilst capturing little luminal content (Maxfield and McGraw, 2004). Although recycling has long been assumed to constitute a default sorting mechanism occurring by virtue of iterative geometric sorting, recent data have identified new protein and lipid regulators of this pathway, suggesting that machineries exist to recognize, retain and sort cargoes into these recycling routes. Endosomal recycling may therefore not be as simple as was once thought.

8.1. EHD proteins and recycling tubules

Perhaps the clearest example of regulators of endosomal recycling is found in *C. elegans*. Here, Receptor-Mediated Endocytosis-1 (Rme-1) was identified as a regulator of endosomal recycling in the worm (Grant et al., 2001, Lin et al., 2001). Mammalian cells express four orthologues of Rme-1 – the Eps15 homology domain (EHD) containing proteins EHD1-4 and of these, EHD-1 is the best characterized, being required for endosomal recycling of cargoes including the TfnR receptor, MHC-I molecule and numerous other recycling cargoes (Lin et al., 2001, Caplan et al., 2002, Grant and Caplan, 2008). EHD-1 is a peripheral membrane protein that localizes to membrane tubules exiting the recycling endosome (Caplan et al., 2002). EHD-1 localisation to these tubules is abolished either upon expression of a PIP5K1 γ , a PtdIns(4)P 5-kinase, or upon expression of SacI-1, a PtdIns(4)P 4'phosphatase, suggesting that PtdIns(4)P controls localisation of EHD-1 to these tubules. The EHD proteins have a broad phosphoinositide-binding specificity in both lipid overlay and liposome-based assays (Blume et al., 2007, Daumke et al., 2007, Naslavsky et al., 2007), binding promiscuously to many phosphoinositides, with some preference for the 4'-position. NMR-based analysis of the EHD-1 EH domain have demonstrated it can indeed bind PtdIns(4)P and a targeted mutation within this domain abolished EHD-1's ability to localize to endosomal recycling tubules and, importantly, prevented it from rescuing transferrin and β 1 integrin recycling in EHD-1 depleted cells (Jovic et al., 2009). Interestingly, this mutant EHD-1 still localized to punctate endosomal structures, suggesting that EH-domain-independent mechanisms of endomembrane binding exist, but that 4'-phosphoinositide binding is important for recruitment to recycling tubules. In worm and mammals, association of EHD1/Rme1 with BAR-domain containing proteins (AMPH-1 in *C-elegans*, BIN-1 in mammals) and Rab8 effectors such as MICAL-L1 (Sharma et al., 2009) also assisted the tubular localization of these proteins and their function in endocytic recycling (Pant et al., 2009). Indeed, the localization of this complex to phosphoinositide-enriched tubules courtesy of coincident phosphoinositide:EH domain interactions and BAR domain:membrane interactions has parallels with the localization of endosomal retrieval complexes such as retromer ((Carlton and Cullen, 2005), see section 7.1.1.) and suggest that cells may employ common mechanisms of localizing sorting complexes to endosomal exit sites via coincident localization signals.

Other EHD proteins also regulate various aspects of early endosome trafficking; EHD3 assists cargo retrieval to the Golgi (Naslavsky et al., 2009), (a pathway that EHD1 participates in through interactions with retromer (Gokool et al., 2007)), EHD1 and 3 interact with the Rab11-FIP2 proteins to regulate export from early endosomes to the

endocytic recycling compartment (Naslavsky et al., 2006) and EHD4 is involved in the trafficking of proteins at the early endosome (Sharma et al., 2008). Given the role of the EH-domain in binding phosphoinositides, these data define the family of EHD proteins as important regulators of phosphoinositide-mediated endosomal trafficking,

8.2. Sorting nexins – another route for the generation of recycling tubules

PtdIns(3)P is another phosphoinositide important for traffic through recycling endosomes: for example, PtdIns(3)P consumption through overexpression of the 3'-phosphatase MTMR4 inhibits Tfn recycling (Naughtin et al., 2010). Here, an important component of the PtdIns(3)P-dependent recycling pathway is the sorting nexin, sorting nexin-4 (SNX4). Localised to both the early endosome and to membranes of the juxtanuclear ERC, SNX4 acts to regulate the formation of membrane tubules required for the geometric based recycling of TfnR away from the degradative route, allowing their return to the cell surface (Traer et al., 2007). Like the previously described role of retromer sorting nexins (see 7.1.1), SNX4 contains a PtdIns(3)P-binding PX domain and a curvature-sensing BAR domain, which can switch to a curvature inducing mode, driving the formation of recycling membrane tubules (Traer et al., 2007). Furthermore, nucleation of SNX4 tubules may again be intimately linked with clathrin dynamics, given that this sorting nexin interacts with clathrin (Skanland et al., 2009). Again, as with the retromer SNX4 also associates with the minus-end directed microtubule motor protein dynein to aid the efficiency of tubule fission (Traer et al., 2007, Skanland et al., 2009), although whether it also interacts with elements that regulate actin polymerization remains to be established.

In yeast, Snx4p is part of a complex with two additional sorting nexins, Snx41p and Snx42p, and acts to retrieve Snc1p from a post-Golgi endosome (Hettema et al., 2003), although in this case, the complex functions in parallel with retromer, returning cargoes to the yeast Golgi. As discussed previously (see 7.2), mammalian SNX4 is also thought to play a role in endosome-to-Golgi trafficking of toxins such as ricin (Skanland et al., 2007), suggesting that it may bisect both recycling and retrieval pathways. Recent data suggesting that the endocytic recycling compartment lies on a route between early endosomes and the Golgi may reconcile these itineraries (Ang et al., 2004). Whether mammalian cells also contain a similar SNX4 complex, the most closely related proteins to Snx41p and Snx42p would appear to be SNX7 and SNX30, remains to be explored. Given that SNX7 and SNX30 each contain predicted phosphoinositide-binding PX domains, studying their function alongside SNX4 may reveal exciting new insight into phosphoinositide-mediated endosomal recycling.

8.3. Sorting nexins and cargo recognition

Besides the role of SNX4 in driving the formation of endosomal recycling tubules (Traer et al., 2007), other members of the sorting nexin family have emerged as cargo adaptors in endosomal recycling. Sorting nexin-17 (SNX17), which contains a PtdIns(3)P-binding PX domain and a truncated FERM (4.1, ezrin, radixin, and moesin) domain (it lacks a BAR domain), is associated with recycling tubules emanating from the early endosome (Florian et al., 2001, van Kerkhof et al., 2005). As an adaptor, SNX17 associates directly with a number of recycling proteins including low-density lipoprotein (LDL) receptor-related protein (LRP)

(here binding is via an NPxY motif in the cytosolic domain of the receptor) (Stockinger et al., 2002, Burden et al., 2004, van Kerkhof et al., 2005), amyloid precursor protein (APP) (recognition via YXNPXY motif) (Lee et al., 2008), the scavenger receptor FEEL-1/stabilin-1 (possible involvement of an NPxF motif) (Adachi and Tsujimoto, 2010), and P-selectin (recognition motif FxNaa(F/Y)) (Florian et al., 2001, Williams et al., 2004, Knauth et al., 2005). Where trafficking itineraries have been explored, data is consistent with SNX17 sorting receptors into an endosomal recycling pathway (*e.g.* see (van Kerkhof et al., 2005)). Whether this is achieved by 'piggy-backing' onto pre-existing SNX4 derived tubules, in a manner that would be analogous to the role of SNX3 in Fet3p-Ftr1p sorting onto the retromer pathway (Strochlic et al., 2007), is an interesting possibility that certainly merits exploration.

Another PtdIns(3)P-binding sorting nexin with a role in endosomal recycling is sorting nexin-27 (SNX27) (Lunn et al., 2007, Rincon et al., 2007). In addition to the early endosomal targeting PX domain, SNX27 contains a PDZ domain and, possibly, an association domain for the small GTPase Ras (Fujiyama et al., 2003, Kajii et al., 2003). In the case of the β 2-adrenoreceptor (β 2-AR), the PDZ domain of SNX27 associates with the β 2-AR tail sequence and is required for the sorting of the receptor into the endosome-to-plasma membrane recycling pathway (this is also dependent on the ability of SNX27 to bind PtdIns(3)P) (Lauffer et al., 2010). SNX27 also associates, again via its PDZ domain, with other cargoes, including G protein-gated potassium (Kir3) channels (Lunn et al., 2007), and the α -variant of the 5-hydroxytryptamine type 4 receptor (5-HT4R) (Joubert et al., 2004). With regard to these cargoes, the interaction with SNX27 is argued to regulate endocytosis or endo-lysosomal sorting rather than recycling (Joubert et al., 2004, Lunn et al., 2007). Clarifying these somewhat distinct conclusions will require further studies. In addition, as with the role of SNX17, it will be interesting to determine whether the adaptor function of SNX27 in cargo recognition is co-ordinated to transport through other phosphoinositide-mediated endosomal recycling pathways.

8.4. Carrier movement and fusion with the plasma membrane

From the growing body of molecular evidence, it is clear that PtdIns(3)P can regulate recycling pathways that exit the early endosome, and it is possible that transition of PtdIns(3)P to PtdIns(4)P may allow for co-ordination of separable machineries along the endocytic recycling pathway(s). Alternatively, these phosphoinositides may sequentially regulate an individual protein. In this regard, the ACCH (Amot coiled-coil homology) domain-containing Amot family of proteins may be of relevance (Heller et al., 2010). Amot associates with both PtdIns(3)P and PtdIns(4)P, is localized to the juxtanuclear ERC and, through its ACCH domain, has the ability to sense membrane curvature and drive membrane tabulation (Heller et al., 2010). Moreover, Amot can re-model the juxtanuclear endocytic recycling compartment (Heller et al., 2010). These data are entirely consistent with an important role for Amot family members in phosphoinositide-mediated endosomal recycling.

Whilst phosphoinositides function in endosomal recycling pathways and the formation of carriers carrying recycling cargoes, it is currently unknown what signals are required to allow

fusion of these endosomal recycling carriers with the plasma membrane. Identification of targeting and fusion machineries that regulate this step will be instrumental in our understanding of mechanisms underlying endosomal recycling. The PX domain-containing kinesin, KIF16B regulates the microtubule plus-end-directed movement of endosomal recycling carriers towards the plasma membrane through binding to PtdIns(3)P (Hoepfner et al., 2005). As for fusion, some insight has come from depleting cellular levels of PtdIns(4,5)P₂ through overexpression of the 5'-phosphatase domain of synaptojanin, depletion of PtdIns(4)P 5'-kinases or induced PtdIns(4,5)P₂ hydrolysis via recruitment of a rapamycin-sensitive PtdIns(4,5)P₂ 5'-phosphatase to the plasma membrane. In all cases, recycling of Tfn was slowed (Abe et al., 2008) (Kim et al., 2006). Interestingly, in the latter, Tfn accumulated beneath the plasma membrane (Abe et al., 2008) suggesting that PtdIns(4,5)P₂ may regulate fusion of these recycling carriers with the plasma membrane.

9. Future perspectives

The embryonic lethality that occurs upon manipulation of enzymes that regulate endosomal phosphoinositide, and the increasing number of genetic diseases linked to mutations in these enzymes, clearly highlights the importance of phosphoinositides for the function of the endo-lysosomal network. Several of these genetic disorders are linked to defects in the sculpturing of membranes that occur during all aspects of endosomal sorting. Further analysis of these, and related proteins, will certainly extend our appreciation of the basic mechanisms through which membrane re-modelling is co-ordinated with the process of cargo capture and sorting. Moreover, characterizing additional phosphoinositide-binding proteins, in particular those for PtdIns(3,5)P₂, will inevitably generate greater insight to human disease and may identify new targets for therapeutic intervention.

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Figure 1. The endo-lysosomal network

A. The interconnected membranous network that constitutes the endo-lysosomal network.

B. Steady state distribution of phosphoinositide isomers within the network: the 'phosphoinositide map'.

Figure 2. Clathrin-mediated endocytosis

A. Schematic depicting stages of clathrin-coated vesicle (CCV) formation.

B. Phosphoinositide kinases and phosphatases involved in shaping the phosphoinositide profile of the nascent CCV and predicted PtdIns(4)P – PtdIns(4,5)P₂ transition during this process.

Figure 3. Mechanisms of enhancing the strength of interaction between phosphoinositides and membranes

A. Oligomerisation of dynamin's PH domain strengthens its interaction with membranes through avidity-mediated enhancement of affinity.

B. The PX-domain of p47phox binds to both PtdIns(3)P and acidic phospholipids such as PtdSer/PtdOH. Ligation of both classes of lipid enhances membrane association.

C. Sorting nexins contain tandem membrane-binding PX and BAR domains. Membrane association requires co-incident recognition of both PtdIns(3)P and curved membranes.

Figure 4. Phosphoinositide-dependent regulation of ESCRT activity

A. Topological equivalence of sites of ESCRT-activity. The ESCRT-machinery regulates membrane fission events leading to MVB biogenesis, cytokinesis and viral release, indicated by the red asterisks. Note topological equivalence of these events and contrast with topology of internalisation.

B. Known phosphoinositide-binding activities within discrete ESCRT-complexes. Yeast nomenclature used for simplicity (barring mVps24). It is unknown whether yeast Vps24 shares PtdIns(3,5)P₂ binding specificity

Figure 5. Retromer-mediated retrograde endosome-to-TGN transport

A. Retrieval pathways in mammalian cells. TGN resident cargos (CI-MPR) are retrieved from endosomes through the actions of the retromer complex. WASH and EHD-1 assist this process. F-actin polymerisation may drive carrier biogenesis, Directed movement to the TGN is accomplished through dynein-mediated transport to the perinuclear region where Rab6IP1 acts to tether incident cargo and dephosphorylation of PtdIns(3)P is thought to allow removal and recycling of retromer components.

B. Schematic depiction of retromer assembly. Retromer comprises 2 subcomplexes – a membrane deforming SNX-BAR protein containing subcomplex and a cargo-binding

VPS26:VPS29:VPS35 subcomplex. Rab7-GTP is thought to help localise the cargo-binding subcomplex. 3'-phosphoinositides are thought to localise the SNX-BAR subcomplex.

Table 1
ESCRT-subunits in mammals and yeast

Complex	Yeast	Mammals
ESCRT-0	Vps27p	HRS
	Hse1p	STAM1, STAM2
ESCRT-I	Vps23p	TSG101
	Vps28p	VPS28
	Vps37p	VPS37a, VPS37b, VPS37c, VPS37d
ESCRT-II	Vps22p	EAP30
	Vps25p	EAP20
	Vps36p	EAP45
ESCRT-III	Did2p/Vps46p	CHMP1a, CHMP1b
	Vps2p	CHMP2a, CHMP2b
	Vps24p	CHMP3
	Snf7p	CHMP4a, CHMP4b, CHMP4c
	Vps60p	CHMP5
	Vps20p	CHMP6
	?	CHMP7
ESCRT-associated	Vps4p	VPS4a, VPS4b
	Vta1p	LIP5
	Ist1p	IST-1
	Bro1p	ALIX, HD-PTP