

## Pairing phosphoinositides with calcium ions in endolysosomal dynamics

Phosphoinositides control the direction and specificity of membrane trafficking by regulating the activity of calcium channels in the endolysosomes

Dongbiao Shen, Xiang Wang and Haoxing Xu\*

The direction and specificity of endolysosomal membrane trafficking is tightly regulated by various cytosolic and membrane-bound factors, including soluble NSF attachment protein receptors (SNAREs), Rab GTPases, and phosphoinositides. Another trafficking regulatory factor is juxta-organellar  $\text{Ca}^{2+}$ , which is hypothesized to be released from the lumen of endolysosomes and to be present at higher concentrations near fusion/fission sites. The recent identification and characterization of several  $\text{Ca}^{2+}$  channel proteins from endolysosomal membranes has provided a unique opportunity to examine the roles of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  channels in the membrane trafficking of endolysosomes. SNAREs, Rab GTPases, and phosphoinositides have been reported to regulate plasma membrane ion channels, thereby suggesting that these trafficking regulators may also modulate endolysosomal dynamics by controlling  $\text{Ca}^{2+}$  flux across endolysosomal membranes. In this paper, we discuss the roles of phosphoinositides,  $\text{Ca}^{2+}$ , and potential interactions between endolysosomal  $\text{Ca}^{2+}$  channels and phosphoinositides in endolysosomal dynamics.

### Keywords:

■  $\text{Ca}^{2+}$ ; lysosomes; membrane trafficking; mucolipin transient receptor potential channel;  $\text{PI}(3,5)\text{P}_2$

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Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI, USA

### \*Corresponding author:

Haoxing Xu  
E-mail: haoxingx@umich.edu

### Introduction: Overview of endolysosomal dynamics

Mammalian endosomes and lysosomes (collectively referred to as endolysosomes) frequently exchange their contents, or cargos, with each other or with other intracellular organelles/vesicles through membrane fusion and fission processes. These events are essential for many cellular functions including protein transport, signal transduction, transmitter release, autophagy, and lysosomal degradation of macromolecules [1, 2]. Based on their membrane composition and function, endosomes can be classified as early endosomes, recycling endosomes, or late endosomes [3]. Upon endocytosis, plasma membrane lipids and proteins are first delivered to early endosomes, where they are either sorted to recycling endosomes to be trafficked back to the plasma membrane, or are processed through the late endosome and lysosome (LEL) pathway for degradation [1, 4].

Endolysosomal membrane trafficking is a spatiotemporally regulated process that ensures proper delivery of cargo via the endolysosomal pathway. Both early and late endosomes can bud inwardly from their limiting membranes to form luminal vesicles, which are referred to as multi-vesicular bodies (MVBs). These vesicles contain ubiquitinated membrane proteins, which are eventually sorted into lysosomes that contain acidic hydrolases for macromolecular digestion [3]. However, lysosomes can also fuse with late endosomes to acquire macromolecules that are sorted for lysosomal degradation, or to obtain newly synthesized hydrolytic enzymes from the *trans*-Golgi network (TGN)

### Abbreviations:

**ALS**, amyotrophic lateral sclerosis; **ciM6PR**, cation-independent mannose-6-phosphate receptor; **CMT**, Charcot-Marie-Tooth; **ESCRT**, endosomal sorting complex required for transport; **LEL**, late endosome and lysosome; **MVB**, multi-vesicular body; **NAADP**, nicotinic acid adenine dinucleotide phosphate; **NSF**, *N*-ethylmaleimide-sensitive fusion protein; **PIP**, phosphoinositide; **SNARE**, soluble NSF attachment protein receptors; **TGN**, *trans*-Golgi network; **TPC**, two-pore channel; **TRP**, transient receptor potential; **TRPML**, mucolipin TRP channel.

pathway [2, 5]. When lysosomes fuse with autophagosomes or phagosomes, fusion events mediate the degradation of obsolete cytoplasmic components/organelles or invading pathogens, respectively [2, 6]. In addition, lysosomes have also been shown to mediate plasma membrane repair via the exocytosis of lysosomal contents, i.e. lysosomal exocytosis [7]. On the other hand, lysosomes can be regenerated from LEL hybrids based on a membrane fission event, which is a process commonly referred to as lysosome biogenesis [2, 8, 9]. Membrane fission also facilitates the recycling of materials obtained from late endosomes or lysosomes to the TGN via retrograde transport vesicles [2, 10]. When lysosomal trafficking is interrupted or compromised, a variety of neurological or lysosomal storage diseases can develop [2, 11–14].

Endolysosomal trafficking, i.e. membrane fusion/fission, is regulated by sequential and stepwise recruitment of a variety of cytosolic and membrane-bound proteins and factors [13, 15–17]. Extensive studies of exocytosis, particularly neurotransmission, have elucidated many of the essential components of membrane fusion events that take place during exocytosis [16]. For example, soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptors (SNAREs) have been identified as the basic structural components needed for membrane fusion events [16, 18]. For the regulation of pre-fusion steps (i.e. priming, tethering, and docking), Rab small GTPases, Rab effectors, tethering factors, and phosphoinositides (PIPs) have been found to be important factors [13, 15, 16, 18, 19]. Numerous *in vitro* and *in vivo* studies suggest that intracellular membrane fusion events in the endolysosomal pathway are regulated by mechanisms similar to those used in exocytosis [15, 16, 20]. Loss-of-function studies in yeast and mammalian cells have identified a similar, yet unique, set of proteins mediating endolysosome trafficking, i.e. endolysosome-specific SNAREs/tethering factors/Rabs/PIPs. Likewise, while previous studies have also demonstrated that  $\text{Ca}^{2+}$  influx through voltage-gated  $\text{Ca}^{2+}$  channels triggers the final step in exocytotic membrane fusion events during neurotransmission [16], levels of juxta-organellar  $\text{Ca}^{2+}$  are hypothesized to regulate endolysosomal dynamics [2, 20–22]. With membrane fission events generally viewed as the reverse of membrane fusion events, fission and fusion events would be expected to share many common mechanisms, including regulation by Rabs, PIPs, and  $\text{Ca}^{2+}$  levels near the fission site [2, 13, 20, 23–26].

Currently, the most challenging question in the field of endolysosomal trafficking is how the aforementioned factors are coordinated to control the direction and specificity of trafficking processes. While many components of trafficking pathways have been readily assigned to one of the following steps, i.e. priming, tethering, docking, or fusion [15, 18], there may be additional roles mediated by these factors that remain unknown. For example, recent evidence suggests that in addition to their roles as structural determinants of fusion, SNAREs can also regulate Rab effectors [13] and plasma membrane  $\text{Ca}^{2+}$  channels [27]. In yeast, *trans*-SNARE complex formation may directly induce  $\text{Ca}^{2+}$  release from the lumen of the vacuole [28]. Similarly, PIPs have been shown to be important not only for the recruitment of Rabs and tethering factors, but also for interactions with SNAREs [29, 30] and  $\text{Ca}^{2+}$  sensors [29]. In addition, recent results from our laboratory have suggested that PIPs directly activate  $\text{Ca}^{2+}$ -release chan-

nels [31]. In this paper, the roles of PIPs and  $\text{Ca}^{2+}$  in regulating endolysosomal dynamics are discussed and analyzed.

## **Trans-SNARE complexes in endolysosomes: Structural components of the fusion machinery**

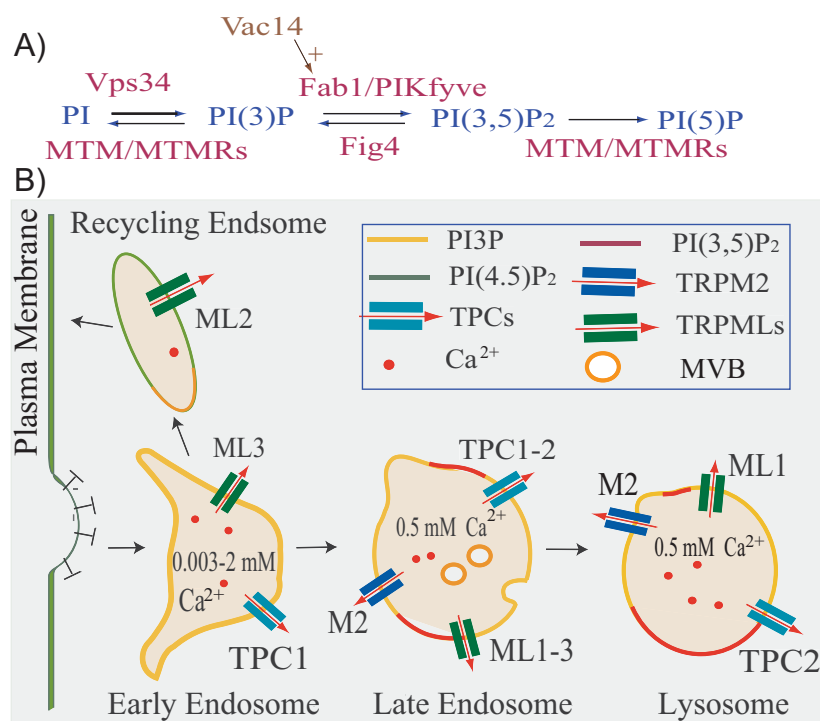
After tethering factors make contact with two endolysosomal membranes, a *trans*-SNARE complex must be formed prior to membrane fusion [32]. Tethering factors that have been identified include EEA1 for early endosomes [15], and HOPS for late endosomes [33]. The resulting SNARE complex that is formed is a highly stable, four-helix bundle formed by three Q-SNAREs on one membrane, and one R-SNARE on the opposing membrane [15]. In lysosomes, Vamp7 has been identified as an R-SNARE, and Syntaxin-7 and -8 are Q-SNAREs present on LELs [2]. After a *trans*-SNARE complex is formed, a bilayer mixing reaction is initiated [2, 15]. However, SNARE interactions alone are not sufficient for membrane fusion to efficiently occur [15]. Additional regulatory proteins are required, and these include Rab effectors and  $\text{Ca}^{2+}$  sensor synaptotagmins, which facilitate interactions between the fusing membranes and SNAREs [15, 18, 29]. To read more on this topic, there are several excellent reviews available [15, 18, 19].

## **Rab small GTPases: Primitive organelle tags and coordinators of endolysosomal trafficking**

Rab proteins are compartmentalized in specific organelle membranes, establishing the basic tags to define organelle identity [13]. Rab proteins can recruit additional effectors, and, depending on the types of Rab proteins and effectors present, an organelle's identity and transport specificity is further determined [13]. For example, while Rab5 is localized in the early endosome, Rab7 is localized to LELs. Rab effectors include sorting adaptors, tethering factors, lipid kinases, and lipid phosphatases [13]. Tethering factors mediate vesicle tethering by interacting with molecules on the acceptor membrane [19], while lipid kinases produce PIPs (see below) to further define vesicular identity and the direction of membrane trafficking [17, 25, 34]. Importantly, it is the interactions between Rab proteins and lipid kinases/phosphatases that form a functional interaction loop to regulate membrane trafficking [34]. While PIPs can recruit PIP-binding proteins to regulate the activity of GTPases, GTPases can in turn control the activity of PIP-metabolizing enzymes [34]. Rabs and Rab effectors can also interact with SNAREs and their accessory proteins to regulate the formation of SNARE complexes, thus modulating membrane fusion processes [13]. This topic has also been excellently reviewed elsewhere [13].

## **Phosphoinositides: Organelle tags and regulators of endolysosomal trafficking**

PIPs are another class of molecules that determine the vesicular identity and direction of membrane trafficking [12, 17, 25]. Of the



**Figure 1.** PIPs and Ca<sup>2+</sup> channels in endolysosomes. **A:** An overview of PI(P) synthesis in endolysosomes. PI(3)P is synthesized from PI via a PI 3-kinase (Vps34) present in endosomes, while PI(3,5)P<sub>2</sub> is synthesized from PI(3)P via a PI 5-kinase (PIKfyve/Fab1). PIKfyve is a PI(3)P effector that is associated with LELs, and is positively regulated by Vac14. PI(3,5)P<sub>2</sub> can be dephosphorylated by Fig4 or MTM/MTMRs to generate PI(3)P or PI(5)P, respectively. **B:** The distribution of PI(3)P and PI(3,5)P<sub>2</sub> associated with the membranes of early and late endosomes, as well as lysosomes, are represented by yellow and red outlines at the membrane of these vesicle types, respectively [35]. TRPML1-3, TRPM2, and TPC1-2 are putative Ca<sup>2+</sup> release channels associated with endolysosomal membranes that are also labeled in this diagram [69, 75, 76]. TRPML1-3 channels are predominantly localized to LELs, while TRPML2 and TRPML3 are also associated with recycling and early endosomes, respectively. TPC2 is mainly localized in the LELs, and TPC1 is mainly associated with early and late endosomes. TRPM2 is expressed in specific cell types, and is associated with LELs as well as the plasma membrane. The concentration of Ca<sup>2+</sup> stores associated with each vesicle type is also indicated, with luminal [Ca<sup>2+</sup>] estimated to be in the micro- to millimolar ranges.

seven PIP isoforms known, PI(3)P and PI(3,5)P<sub>2</sub> are exclusively localized to the endolysosomal system. PI(3)P is mainly localized on the cytoplasmic side of endosomes, while PI(3,5)P<sub>2</sub> is believed to localize exclusively to LELs [25, 35] (see Fig. 1). Overall, both PI(3)P and PI(3,5)P<sub>2</sub> have multiple roles that are essential for endolysosomal trafficking.

### PI(3)P in early endosomes

PI(3)P is essential for almost every single aspect of membrane trafficking of early endosomes: fusion, fission, sorting, and maturation. PI(3)P is mainly synthesized from PI (see Fig. 1A) by PI 3-kinase Vps34 (Vps34p in yeast), which localizes to endosomes and is expressed in all eukaryotes [25]. Correspondingly, yeast cells lacking Vps34p are defective in endosomal sorting; homotypic fusion of early endosomes is sensitive to the broad spectrum PI 3-kinase inhibitor, wortmannin [36]. These defects cannot be rescued by constitutively active Rab5, suggesting that Vps34 functions downstream of Rab5, and is an effector of Rab5 [13]. PI(3)P has more than 70 effector proteins in mammalian cells, all of which contain PI(3)P-binding motifs such as FYVE, PX, or PH domains [37]. Selective recruitment of these effectors by PI(3)P may provide a mechanism by which the directionality for incoming vesicles and endosomes is established. For example, PI(3)P is a critical recognition marker for many tethering/regulatory factors such as EEA1 [38]. Moreover, acute enzymatic depletion of PI(3)P from Rab5-positive endosomes has been shown to disrupt the maturation of endosomes [39]. Thus, PI(3)P is critical for the maturation of endosomes, and for fusion events with other intracellular organelles.

PI(3)P is also important for fission events of endosomes and essential for the formation of MVBs [40], a process facili-

tated by endosomal sorting complex required for transport (ESCRT) machinery. ESCRT sorts proteins that are intended to be degraded in lysosomes into the LEL pathway [4]. Since multiple components of the ESCRT complex contain PI(3)P binding modules, interactions with PI(3)P are essential for the recruitment of these proteins to endosomes [4]. Correspondingly, disruption of PI(3)P synthesis by wortmannin affects the formation of internal vesicles within MVBs [40].

### PI(3)P in autophagosomes and phagosomes

PI(3)P is also important for the formation of autophagosomes, playing an essential role in autophagy [41]. For example, during amino acid starvation, autophagy-specific proteins are specifically recruited to PI(3)P-enriched structures [42]. Indeed, Vps34 is an essential component of the core complex involved in autophagy [43]. Inhibition of PI 3-kinase with wortmannin prevents fusion of autophagosomes with LELs [44].

PI(3)P also has a role in phagocytosis, and is important for the formation and maturation of phagosomes [45–47]. Upon phagocytosis, invading microorganisms or latex beads are packaged into phagosomes, which then recruit various trafficking regulators to modify themselves and eventually fuse with lysosomes to form phago-lysosomes, a process referred to as phagosome maturation [47]. Inhibition of Vps34 with wortmannin prevents fusion of the phagosomes with late endosomes [48]. Recruitment of PI(3)P effectors such as EEA1 is necessary for phagosomal maturation [49, 50]. Pathogenic bacteria such as *Mycobacterium tuberculosis* prevent phagosomal maturation (commonly referred to as phagosome maturation arrest) by disrupting Rab, PtdIns(3)P, or Ca<sup>2+</sup> signaling [46]. Although it is described in more detail below, PI(3)P is an essential substrate for the synthesis of PI(3,5)P<sub>2</sub>

(see Fig. 1A) [17], and therefore, the observed effects could be due to insufficient synthesis of PI(3,5)P<sub>2</sub>.

### PI(3,5)P<sub>2</sub> in late endosomes and lysosomes

*PI(3,5)P<sub>2</sub> is a low-abundance PIP in the late endosome and lysosome*

PI(3,5)P<sub>2</sub> only represents ~0.05% of the total cellular PIPs (PI(3)P, PI(4)P, PI(5)P, PI(3,4)P<sub>2</sub>, PI(4,5)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub>) present in non-stimulated cells under basal conditions [35, 51]. This is ~10-fold less than the levels of PI(3)P that are normally maintained in cells [52]. Due to its low abundance and lack of a specific detection probe [37], the majority of PI(3,5)P<sub>2</sub> studies have focused on PI(3,5)P<sub>2</sub>-metabolizing enzymes, including PIKfyve/Fab1 and its regulators [52–55]. PI(3,5)P<sub>2</sub> is generated from PI(3)P via PIKfyve/Fab1 (see Fig. 1), a PI 5-kinase that localizes to endolysosomes in both yeast and mammalian cells [17, 35, 51, 56–58]. Therefore, PI(3,5)P<sub>2</sub> is hypothesized to predominantly localize to the LELs [35, 52, 55, 59]. The activity of PIKfyve/Fab1 has been shown to be positively regulated by several associated proteins such as Fig4 and Vac14 [35, 52, 54, 55]. However, PI(3,5)P<sub>2</sub> can be hydrolyzed to PI(5)P by the myotubularin (MTM/MTMR) family of PI-3 phosphatase [17, 35, 60]. Furthermore, although the global concentration of PI(3,5)P<sub>2</sub> is very low, the local concentration in PIKfyve-enriched microdomains is likely to increase in response to stimuli [58, 61], and can reach levels similar to those of PI(4,5)P<sub>2</sub> at the plasma membrane (~10 μM) [56, 57, 62]. Correspondingly, mutations in PI(3,5)P<sub>2</sub>-metabolizing enzymes have been shown to be responsible for a variety of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and Charcot-Marie-Tooth (CMT) disease in humans [35, 55, 63].

#### *What are cellular consequences of PI(3,5)P<sub>2</sub> deficiency?*

At the cellular level, PI(3,5)P<sub>2</sub>-deficient cells, such as fibroblasts from Fig4 or Vac14 KO mice, exhibit enlarged endolysosomes/vacuoles and trafficking defects in the late endocytic pathways, which include retrograde late endosome/lysosome-to-TGN trafficking, MVB formation, autophagosome-lysosome fusion, and exocytosis [52, 54, 55, 64, 65]. In studies of Vac14<sup>-/-</sup> cells, immunoreactivity of a cation-independent mannose-6-phosphate receptor (ciM6PR), a membrane protein that shuttles between TGN and the LEL, was detected in Lamp1-positive compartments rather than in the TGN [52], suggesting that reformation of ciM6PR-positive transport vesicles from LELs was compromised [52]. PI(3,5)P<sub>2</sub> has also been shown to have an essential role in the fusion of late endosomes or lysosomes with other organelles, including autophagosomes [65]. Consistent with a role for PI(3,5)P<sub>2</sub> in exocytosis, PIKfyve has been implicated in the translocation of several ion channels and transporters to the plasma membrane [66].

#### *What are the effectors of PI(3,5)P<sub>2</sub>?*

Like PI(3)P, PI(3,5)P<sub>2</sub> also exerts its effect through the recruitment of effector proteins that bind PI(3,5)P<sub>2</sub>, although the number of effectors associated with PI(3,5)P<sub>2</sub> is much smaller

than the number of PI(3)P effectors [35]. Atg18 is a protein that potentially mediates retrograde trafficking following its binding of PI(3,5)P<sub>2</sub> in yeast [67]. Similarly, WIP1 has been shown to bind both PI(3)P and PI(3,5)P<sub>2</sub> to affect ciM6PR recycling in mammalian cells, thereby suggesting that it is an effector of PI(3,5)P<sub>2</sub> [68]. Finally, mucolipin subfamily of transient receptor potential (TRP) channels (TRPMLs) were recently identified as novel PI(3,5)P<sub>2</sub> effectors that mediate the release of Ca<sup>2+</sup> from endolysosomes [31] (see details below). Overall, the identification of multiple PI(3,5)P<sub>2</sub> effectors is consistent with the multifaceted actions of this lipid in membrane trafficking.

## Ca<sup>2+</sup> and Ca<sup>2+</sup> channels in endolysosomal trafficking

### Ca<sup>2+</sup>-dependent fusion and fission

The transient release of Ca<sup>2+</sup> from endosomes/lysosomes in close proximity to fusion/fission sites is hypothesized to be important for the trafficking of endolysosomal membranes [2, 20, 22, 69]. In vitro membrane fusion assays using cell extracts from yeast or mammalian cells have shown that both homotypic and heterotypic fusion events between LELs are inhibited by BAPTA, but not by EGTA [21, 23]. Although both BAPTA and EGTA are Ca<sup>2+</sup> chelators, the former binds to Ca<sup>2+</sup> 10–100 times faster than the latter. The most plausible explanation for this distinct difference in sensitivity to BAPTA versus EGTA, is that the putative fusion site is in close proximity to the Ca<sup>2+</sup>-release site [20, 23, 70]. By using membrane-permeable forms of Ca<sup>2+</sup> chelators, i.e. BAPTA and EGTA labeled with acetoxymethyl ester (AM), additional studies of intact cells have further demonstrated that distinct BAPTA/EGTA sensitivities are also associated with multiple steps during endolysosomal transport, including endolysosomal fusion and retrograde trafficking from endolysosomes to the TGN [20, 22, 24, 70]. Thus, both in vitro and in vivo evidence suggests that Ca<sup>2+</sup> release from endolysosomes participates in most, if not all, fusion and fission events along the endolysosomal pathway [69].

### Endolysosomes as Ca<sup>2+</sup>-release stores

Based on the ionic composition and electrical properties of endolysosomes, the capacity for endolysosomes to release Ca<sup>2+</sup> during membrane trafficking has been considered [69]. The luminal Ca<sup>2+</sup> concentration of lysosomes is estimated to be ~0.5 mM [11, 71], while Ca<sup>2+</sup> concentrations associated with endosomes have been shown to vary from 0.003 to 2 mM [72]. Thus, both endosomes and lysosomes may represent sources of Ca<sup>2+</sup> release along the endolysosome trafficking pathway (Fig. 1B). Dysregulation of luminal Ca<sup>2+</sup> concentration is known to cause a block of phagosome maturation in macrophages [73], and trafficking defects in neurons and subsequent neurodegenerative diseases [11, 74].

### Candidate Ca<sup>2+</sup>-release channels in endolysosomes

Recently, several putative endolysosome Ca<sup>2+</sup>-release channels have been identified using Ca<sup>2+</sup> imaging and a lysosomal



patch-clamp technique (for reviews see [69, 75, 76](Fig. 1B). Previous studies have also functionally characterized TRPMLs to be inward-rectifying  $\text{Ca}^{2+}$ -permeable channels [75]. In addition, loss-of-function studies in *Caenorhabditis elegans*, *Drosophila*, and mammalian cells suggest that TRPMLs are required for multiple steps in the fusion/fission processes of endolysosomes [75, 77–81]. TRPML1 has been shown to be ubiquitously expressed, while TRPML2 and TRPML3 are associated with a more restricted expression profile: TRPML2 is expressed in thymus, kidney, spleen, and other tissues; TRPML3 is expressed in cochlea, kidney, skin, and other tissues [75]. Furthermore, while all three TRPMLs are primarily associated with the LELs, TRPML2 and TRPML3 have also been associated with recycling endosomes and early endosomes, respectively [75].

There are at least four more cation channels present in the endolysosome. Two-pore channels (TPC1 and TPC2) are another type of  $\text{Ca}^{2+}$  channel that are ubiquitously expressed and are associated with endolysosomes. Recent studies have shown that TPCs may mediate nicotinic acid adenine dinucleotide phosphate (NAADP)-activated  $\text{Ca}^{2+}$  release from endolysosomes [76], while overexpression of TPCs has been shown to cause defective membrane trafficking in endolysosomes [82]. Finally, TRPV2 and TRPM2 were detected as cell-specific  $\text{Ca}^{2+}$  permeable channels present in endolysosomes [83, 84]. However, characterization of these channels in the membrane trafficking of endolysosomes is ongoing.

### PI(3)P, PI(3,5)P<sub>2</sub>, and Ca<sup>2+</sup> in endolysosomal trafficking

Unlike neurotransmission where the stimulus to trigger  $\text{Ca}^{2+}$  flux (i.e. membrane depolarization) is known, the activation and regulation of vesicular  $\text{Ca}^{2+}$  channels remain largely unexplored. However, due to the specificity involved in endolysosomal trafficking processes,  $\text{Ca}^{2+}$  release needs to be closely regulated [20]. Given that plasma membrane channels are known to be regulated by Rab proteins [85], SNAREs [27], and PIPs [86], one possible regulatory mechanism for endolysosomal  $\text{Ca}^{2+}$  release channels is modulation by these trafficking players. However, this hypothesis is difficult to test due to the inaccessibility of endolysosomal channels to conventional functional assays [87].

Based on the co-localization of endolysosomal channels and PI(3)P/PI(3,5)P<sub>2</sub> in endolysosomes [69, 75, 76], it is possible that PI(3)P and PI(3,5)P<sub>2</sub> have the capacity to modulate the function of endolysosomal channels such as TRPMLs. Interestingly, PI(3,5)P<sub>2</sub>-deficient cells and TRPML1<sup>-/-</sup> cells share a number of similarities in the trafficking defects exhibited by these two cell types [35, 75]. For example, PI(3,5)P<sub>2</sub>-deficient cells and TRPML1<sup>-/-</sup> cells exhibit enlarged endolysosomes/vacuoles and trafficking defects in the late endocytic pathway, and these include defects in LEL-to-TGN retrograde trafficking, autophagosome-lysosome fusion, and exocytosis [31]. In TRPML1<sup>-/-</sup> cells, the processes of retrograde trafficking from LEL to TGN, and the reformation of lysosomes from LEL hybrid organelles, are both impaired [77, 78, 88]. Similarly, these same defects are also associated with

PI(3,5)P<sub>2</sub>-deficient cells [52, 55]. Moreover, fusion of LELs with other intracellular compartments has been shown to be compromised in both TRPML1<sup>-/-</sup> and PI(3,5)P<sub>2</sub>-deficient cells [35, 65, 75]. In combination, these observations suggest that PI(3,5)P<sub>2</sub> and TRPML1 modulate multiple endolysosomal trafficking processes coordinately.

### PI(3,5)P<sub>2</sub> binds to and activates TRPML1

Using direct patch-clamping of endolysosomal membranes, Dong et al. [31] have reported that PI(3,5)P<sub>2</sub> is able to robustly activate TRPML1 in both heterologous and endogenous systems. In contrast, decreases in PI(3,5)P<sub>2</sub> levels by chelation [i.e. using a PI(3,5)P<sub>2</sub> antibody or poly-lysine], or by overexpression of PI-3 phosphatase (MTM1), has been shown to suppress the basal current associated with TRPML1 overexpression [31]. Based on these results, PI(3,5)P<sub>2</sub> would appear to be the primary determinant of the channel activity of TRPML1 in endolysosomes [31]. Correspondingly, none of the other PIPs, including PI(3)P and PI(5)P, have been shown to activate TRPML1 [31]. Therefore, TRPML1 activation is hypothesized to be restricted to PI(3,5)P<sub>2</sub>-enriched LEL membranes.

An additional consideration is whether PI(3,5)P<sub>2</sub> induces TRPML1-mediated  $\text{Ca}^{2+}$  release from endolysosomes. While PI(3)P levels have been reported to increase in autophagosomes of autophagic cells [17], direct evidence to indicate that local PI(3,5)P<sub>2</sub> levels are affected in endolysosomes of mammalian cells is still lacking. However, a transient increase in levels of PI(3,5)P<sub>2</sub> in mammalian endolysosomes has been associated with recruitment of PIKfyve in response to insulin and osmotic stress [66]. Furthermore, in yeast, hyper-osmotic stress has been shown to induce a dramatic increase (>10-fold) in global PI(3,5)P<sub>2</sub> levels [89], and to induce the release of  $\text{Ca}^{2+}$  from vacuoles [90]. Thus, it is possible that mammalian cells are also responsive to physiologically relevant acute stimuli, or constitutive signals, which result in the activation and recruitment of PIKfyve to induce  $\text{Ca}^{2+}$  release in TRPML1 endolysosomes.

### Mechanisms associated with interactions between PI(3,5)P<sub>2</sub> and TRPML1

PI(3,5)P<sub>2</sub>-dependent modulation of TRPML1 appears to be physiologically significant. For example, the enlarged endolysosome phenotype associated with Vac14<sup>-/-</sup> fibroblasts can be largely rescued by overexpressing wild-type TRPML1, but not by a PI(3,5)P<sub>2</sub>-insensitive mutant TRPML1 [31]. A possible reason for this observation is that overexpression of TRPML1 can increase PI(3,5)P<sub>2</sub>-dependent, TRPML1-mediated  $\text{Ca}^{2+}$  release, thereby rescuing trafficking defects of PI(3,5)P<sub>2</sub>-deficient cells [31]. Alternatively, the basal activity of TRPML1 resulting from overexpression may alter the ionic composition of the vacuoles, thereby affecting their morphology. A third possible explanation is that PI(3,5)P<sub>2</sub>-dependent fusion/fission processes are restored based on an increase in basal TRPML1 channel activity, which may compensate for the decrease in activation signals, including activation by PI(3,5)P<sub>2</sub> [31]. It is anticipated that the recently identified TRPML chemical agonists may prove helpful in distinguishing these possibilities [91].

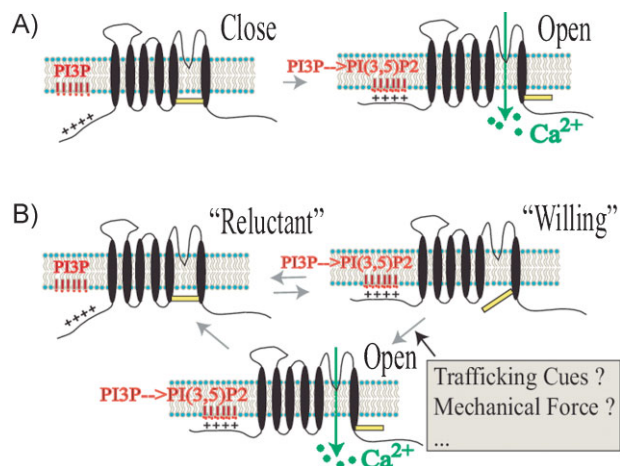
The exact concentration of local PI(3,5)P<sub>2</sub> in the LEL under basal conditions, or upon cellular stimulation, is not known. Therefore, the mechanisms by which PI(3,5)P<sub>2</sub> modulates TRPMLs under physiological conditions remains unclear. A key question is whether PI(3,5)P<sub>2</sub> is the sole activator of TRPML1, or whether other factors are involved. It is likely that constitutive signals, or acute stimuli, recruit/activate PIKfyve present in microdomains, thereby resulting in a rapid and transient increase in PI(3,5)P<sub>2</sub> and TRPML1-mediated Ca<sup>2+</sup> release (Fig. 2A). However, although the EC<sub>50</sub> for recombinant TRPML1 is only ~50 nM [31], higher concentrations of PI(3,5)P<sub>2</sub> are required to activate endogenous TRPML1 (Wang, Dong, and Xu; unpublished data). Thus, it remains unclear whether physiological levels of PI(3,5)P<sub>2</sub>, which are presumably in the low nanomolar range, are sufficient to activate endogenous TRPML1 in endolysosomes.

It is also possible that, *in vivo*, additional regulatory mechanisms for TRPML1 include factors other than PI(3,5)P<sub>2</sub>. For example, PI(4,5)P<sub>2</sub> has been shown to be a “permissive” factor in the activation of voltage-gated Ca<sup>2+</sup> channels in response to depolarization [92]. Similarly, PI(3,5)P<sub>2</sub> may “sensitize”, yet not fully “activate” TRPML1. Increases in levels of PI(3,5)P<sub>2</sub> may also promote Ca<sup>2+</sup> channels to be maintained in a “willing state”, thereby facilitating channel activation in the absence of secondary activation factors (see Fig. 2B). Conversely, in the absence of PI(3,5)P<sub>2</sub>, activation of Ca<sup>2+</sup> channels may require much higher concentrations or stronger binding interactions by activation factors

that exceed the physiologically permitted range. Another activation factor to consider is that of mechanical force, which may be generated by the membrane curvature achieved prior to fusion [15]. For example, since the Ca<sup>2+</sup>-release site is hypothesized to be in close proximity to the fusion/fission sites [20, 23, 70], such mechanical force may be sensed by Ca<sup>2+</sup>-release channels. Correspondingly, YVC1/TRPY1, the TRPML homolog in yeast, has been shown to be directly gated by mechanical force [93]. Further studies will be needed to test whether TRPML1 is synergistically gated by PI(3,5)P<sub>2</sub> and mechanical force. However, other activation factors should be evaluated as well, including proteins that are recruited to interact with TRPML1 following transient elevations in levels of PI(3,5)P<sub>2</sub>. These studies would also test the hypothesis that PI(3,5)P<sub>2</sub>, in coordination with these “cofactors”, can achieve specific activation of endolysosomal Ca<sup>2+</sup>-release channels.

### Local and transient increases in levels of PI(3,5)P<sub>2</sub> and Ca<sup>2+</sup>

The ability of PI(3,5)P<sub>2</sub> to specifically activate TRPML1 suggests that acute regulation of Ca<sup>2+</sup> release via TRPMLs can be achieved by regulating PI metabolizing enzymes, such as PIKfyve, or Fig4 and MTM/MTMR phosphatases. These PI-metabolizing enzymes are usually localized to the cytosol [58]. However, these enzymes can be recruited to endolysosomal membranes based on the binding of PIPs to their PI-binding domains [37], or by interacting with key components of the trafficking pathways [58]. For example, PI(3)P can recruit Rab5, and activation of Rab5 can further stimulate the activity of Vps34 PI-3 kinase [94]. Similar mechanisms may also be employed by PI(3,5)P<sub>2</sub>, Rab7, and PI(3,5)P<sub>2</sub>-metabolizing enzymes [58]. For TRPML1, it is not inactivated by voltage, or desensitized by Ca<sup>2+</sup> [31]. Therefore, to avoid excessive Ca<sup>2+</sup> release from endolysosomes, activation of TRPML1 needs to be transient. It has been hypothesized that this can be achieved if the synthesis and turnover of PI(3,5)P<sub>2</sub> is actively and tightly regulated [58]. This has been shown to be the case in yeast where Vac14 physically associates with Fig4, Vac7, and Fab1 [54, 58, 95], and the formation of the Vac14-Fig4 complex is required for rapid increases in the synthesis and turnover of PI(3,5)P<sub>2</sub> in response to hyperosmotic stress [56]. The proximity of PI(3,5)P<sub>2</sub>-metabolizing enzymes to each other further supports this hypothesis, and has been shown to facilitate the ability of complexes to form near fusion/fission sites [96].



**Figure 2.** Two working models for PI(3,5)P<sub>2</sub>-dependent TRPML1 activation in endolysosomes. **A:** “Activation” model. Physiological levels of PI(3,5)P<sub>2</sub> activate TRPML1 by binding to the poly-basic domain in the N terminus of TRPML1, leading to the release of Ca<sup>2+</sup>. **B:** “Sensitization” model. In this model, physiological levels of PI(3,5)P<sub>2</sub> are not sufficient to activate TRPML1. Instead, PI(3,5)P<sub>2</sub> is hypothesized to sensitize TRPML1 by converting the channel from a “reluctant” state to a “willing state” [92]. Channel opening/activation and Ca<sup>2+</sup> release are then induced by unidentified trafficking cues, one of which may be the mechanical force generated during pre-fusion membrane curvature/bending. This model confers multiple layers of regulation on TRPML1 activity. Under stress conditions that are analogous to hyper-osmotic shock in yeast, acute stimuli may induce high levels of PI(3,5)P<sub>2</sub> to sufficiently activate TRPML1 as shown in (A).

### PI(3)P and endolysosomal Ca<sup>2+</sup> channels

Like PI(3,5)P<sub>2</sub> activation of TRPML1 in the LEL, PI(3)P in the early endosome may also modulate Ca<sup>2+</sup> channels in the early endosome, for example, TRPV2, TPC1, and TRPML3. However, because the concentration of PI(3)P in the early endosome under resting conditions might be already high [52], it is likely that PI(3)P plays a “permissive” role in channel activation, similar to the effect of PI(4,5)P<sub>2</sub> on plasma membrane channels [86]. These possibilities remain to be tested. It is worth mentioning that both PI(3)P production and intracellular Ca<sup>2+</sup> release are inhibited during phagosome maturation arrest upon phagocytosis of pathogenic bacteria [45–47].

## Cross-talk between $\text{Ca}^{2+}$ signaling and PIP effectors

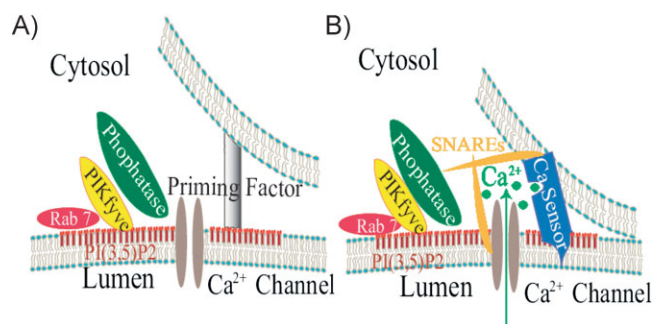
*Are there synergistic actions of trafficking regulators?*

Activation of more than one trafficking factor simultaneously appears to provide specificity for the membrane trafficking that takes place in a cell [15]. For example, the functional interplay between PIPs and small GTPases has been well characterized, with PIPs shown to be important for recruiting GTPases, and GTPases being able to activate PIP-metabolizing enzymes [34]. Therefore, although both PIPs and GTPases can recruit effectors to specific organelles, the binding affinity for each class of interactions is low. To compensate for this, a combination of PIPs and GTPases have high-affinity interactions with their effectors, which facilitates a coincidence detection mechanism [34].

*Are there interactions between endolysosomal  $\text{Ca}^{2+}$  channels and other  $\text{PI}(3,5)\text{P}_2$  effectors?*

$\text{PI}(3,5)\text{P}_2$ -deficient cells have been observed to have more defects in their membrane trafficking processes than  $\text{TRPML1}^{-/-}$  cells [31], suggesting that  $\text{Ca}^{2+}$  is not the only downstream effector of  $\text{PI}(3,5)\text{P}_2$ .  $\text{PI}(3,5)\text{P}_2$  has been shown to recruit the necessary cytosolic proteins for membrane fusion and fission, to activate  $\text{TRPML1}$ , and may regulate the functions of other endolysosomal membrane proteins to determine the physical properties and fusogenic potential of endolysosomal membranes [17, 31, 35]. It is also possible that  $\text{Ca}^{2+}$  and its effectors (i.e.  $\text{Ca}^{2+}$  sensors) may in turn regulate the activities of  $\text{PI}(3,5)\text{P}_2$ -metabolizing enzymes and functions of other  $\text{PI}(3,5)\text{P}_2$  effectors.

Pre-fusion mechanisms have been established for PIPs such as  $\text{PI}(4,5)\text{P}_2$  and  $\text{PI}(3)\text{P}$  [17, 18, 30]. Therefore, it has also been hypothesized that  $\text{PI}(3,5)\text{P}_2$  participates in endolysosomal pre-fusion steps. For exocytosis,  $\text{PI}(4,5)\text{P}_2$  has been shown to be a critical cofactor that recruits protein priming factors to facilitate SNARE-dependent fusion [97], and is required for generating the membrane curvature necessary for membrane fusion [29]. In reconstituted proteoliposome studies, it has been demonstrated that the yeast vacuole Q-SNARE protein, Vam7p, binds to  $\text{PI}(3)\text{P}$  and promotes the formation of a SNARE complex [18, 30]. Studies of pre-fusion steps (i.e. tethering and docking) have led a testable hypothesis that increases in levels of  $\text{PI}(3,5)\text{P}_2$  may lead to the direct activation of  $\text{TRPML1}$  to elevate levels of juxta-organellar  $\text{Ca}^{2+}$ , thereby triggering fusion events (Fig. 3). Moreover, regarding the hypothesis that  $\text{PI}(3,5)\text{P}_2$  configures  $\text{TRPML1}$  in a “willing” state, the assembly of SNARE complexes and the membrane curvature involved in this process may provide a necessary “activation factor” for  $\text{TRPML1}$ -mediated  $\text{Ca}^{2+}$  release (Fig. 2B). Indeed, *trans*-SNARE complex formation is shown to promote  $\text{Ca}^{2+}$  release from yeast vacuoles [28]. In the vertex of fusion-ready vesicles, there are rapid changes in the concentrations of both  $\text{PI}(3,5)\text{P}_2$  and proteins involved in pre-fusion and fusion steps. If these proteins can affect the gating of  $\text{TRPML1}$  through protein-protein interaction,  $\text{TRPML1}$ -mediated  $\text{Ca}^{2+}$  release could be readily triggered in the presence of high  $\text{PI}(3,5)\text{P}_2$ . In combination, these roles for  $\text{PI}(3,5)\text{P}_2$  indicate that  $\text{PI}(3,5)\text{P}_2$  may function as a PIP switch that controls the function and maturation of organelles enriched with  $\text{PI}(3,5)\text{P}_2$ , i.e. LELs.



**Figure 3.** A proposed model for  $\text{PI}(3,5)\text{P}_2$ - and  $\text{Ca}^{2+}$ -dependent endolysosomal membrane fusion. Microdomain-specific recruitment of an array of tethering and priming factors has the potential to bring into close proximity these factors between an endolysosomal membrane (lower membrane illustrated) and a membrane of another vesicular compartment (upper membrane illustrated). **A:** Recruited proteins that may be involved include Rab GTPases (Rab7 for LEL), lipid kinases (PIKfyve for LEL), and several phosphatases (Fig4 and MTM/MTMRs). Activation of the assembled  $\text{PI}(3,5)\text{P}_2$ -metabolizing complex would ensure a transient and local increase in the level of  $\text{PI}(3,5)\text{P}_2$ .  $\text{PI}(3,5)\text{P}_2$  effectors would also be subsequently recruited and/or activated. **B:** The release of  $\text{Ca}^{2+}$  from the lumen of endolysosomes would transiently elevate the levels of juxta-organellar  $\text{Ca}^{2+}$  to activate a putative  $\text{Ca}^{2+}$  sensor protein, such as ALG-2/synaptotagmin/calmodulin, to promote the fusion of the lipid bilayers. Both SNARE complex formation and  $\text{PI}(3,5)\text{P}_2$  may facilitate  $\text{Ca}^{2+}$  release. An increase in  $\text{PI}(3,5)\text{P}_2$  levels could also alter the physical properties of the membranes involved, and regulate the interactions between SNARE complexes and  $\text{Ca}^{2+}$  sensor proteins.

For endolysosomes, membrane fusion/fission events may be triggered by  $\text{Ca}^{2+}$  released from  $\text{TRPML1}$ -containing LELs following PIKfyve-mediated synthesis of  $\text{PI}(3,5)\text{P}_2$  from  $\text{PI}(3)\text{P}$ , and subsequent recruitment of trafficking factors (see Fig. 3). Moreover, regulation of this process could be provided by dephosphorylation of  $\text{PI}(3,5)\text{P}_2$  by Fig4 or MTMR phosphatases.

*What are the  $\text{Ca}^{2+}$  sensors in the endolysosome system?*

Given the specificity of endolysosomal trafficking processes, compartment-specific  $\text{Ca}^{2+}$  sensors might also be involved. The best candidate is ALG-2, a penta-EF-hand protein that physically associates with  $\text{TRPML1}$  in a  $\text{Ca}^{2+}$ -dependent manner [98]. Calmodulin has also been implicated in endolysosomal trafficking processes [21–23], with vacuolar fusion being found to be defective in loss-of-function calmodulin mutants in yeast [21]. Synaptotagmins are another type of  $\text{Ca}^{2+}$  sensor that have been shown to be essential for exocytosis [86]. Since lysosomes also undergo  $\text{Ca}^{2+}$ -regulated exocytosis for plasma membrane repair [7], synaptotagmins may play a role in the fusion of lysosomes with other membranes. In addition, recent studies have demonstrated that Doc2b, a high-affinity  $\text{Ca}^{2+}$  sensor, is important for the spontaneous release of neurotransmitters, and may play a role in endolysosomal trafficking [99].

*How do  $\text{Ca}^{2+}$  sensors transduce an increase in levels of juxta-organellar  $\text{Ca}^{2+}$  to affect fusion/fission events?*

One possibility is that upon binding of  $\text{Ca}^{2+}$ , a putative  $\text{Ca}^{2+}$  sensor may exhibit high-affinity associations with phospholi-



pids such as PI(3,5)P<sub>2</sub>, thus bringing two membranes into closer proximity. This has been observed during the release of neurotransmitters using functional assays of synaptotagmins and PI(4,5)P<sub>2</sub> [100]. Another possibility is that a putative Ca<sup>2+</sup> sensor may interact with SNARE proteins to facilitate the formation of a SNARE complex, thus promoting membrane fusion. Correspondingly, calmodulin has been shown to interact with SNAREs and promote intracellular membrane fusion [22]. A third possibility is that Ca<sup>2+</sup> signaling may regulate other trafficking factors, including PIP effectors. For example, TRPML2 and Ca<sup>2+</sup> have been shown to promote the activity of Arf6 GTPase and other ArfGAPs, respectively [101]. Ca<sup>2+</sup>/calmodulin is shown to be a positive regulator of Vps34 in phagosomes [47]. Thus, functional cross-talk between endolysosomal Ca<sup>2+</sup> channels, PIPs, and other PIP effectors may regulate the release of Ca<sup>2+</sup> in a spatiotemporal manner, thus ensuring the specificity of endolysosomal membrane trafficking.

## Conclusions and prospects

Studies of PI(3,5)P<sub>2</sub> and TRPMLs have provided valuable insight into the gating mechanisms that regulate Ca<sup>2+</sup> channels along the endolysosomal pathway. Endolysosomal Ca<sup>2+</sup> channels may also be modulated by lipids enriched in endolysosomes, similar to various channels at the plasma membrane whose functions are modulated by phospholipids. Correspondingly, PIPs such as PI(3)P and PI(3,5)P<sub>2</sub> are hypothesized to modulate other Ca<sup>2+</sup> channels, such as TPCs and TRPM2, in the endolysosomal system. Many neurodegenerative diseases, including Alzheimer's and Parkinson's disease, as well as lysosomal storage diseases, exhibit abnormal lipid accumulation, and defective endolysosomal trafficking and autophagy [102]. While several lysosomal storage diseases have been characterized by a progressive accumulation of sphingolipids, mucopolysaccharides, and oligosaccharides in endolysosomes, it is an intriguing possibility that abnormal lipid accumulation may lead to trafficking defects and the compromise of PIP-regulated Ca<sup>2+</sup> channels.

It is anticipated that future studies would address the following areas of interest:

- (i) Identification of other intracellular Ca<sup>2+</sup> channels that are involved in endolysosomal trafficking, and their possible activation mechanisms. These studies will help further characterize the roles of endolysosomal Ca<sup>2+</sup> channels in membrane trafficking.
- (ii) The use of high resolution imaging to monitor Ca<sup>2+</sup> transients during both constitutive and regulated membrane trafficking, including membrane fusion and fission events.
- (iii) An examination of downstream events following the release of Ca<sup>2+</sup> that facilitate fusion and fission events involving endolysosomal membranes.

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