Dynamics and function of PtdIns(3)*P* in autophagy

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Addendum to:

1) Obara K, Noda T, Niimi K, and Ohsumi Y. Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in *S. cerevisiae*. Genes Cells 2008; 13:537-547.
2) Obara K, Sekito T, Niimi K, and Ohsumi Y. The Atg18-Atg2 complex is recruited to autophagic membranes via PtdIns(3)*P* and exerts an essential function. J Biol Chem 2008; in press

Abbreviations PtdIns, phosphatidylinositol; PtdIns(3)*P*, phosphatidylinositol 3-phosphate; PE, phosphatidylethanolamine

Abstract

Phosphorylation of phosphatidylinositol (PtdIns) by PtdIns 3-kinase is essential for autophagy. However, the distribution and function of the enzymatic product, PtdIns 3-phosphate (PtdIns(3)*P*), has been unknown. We monitored PtdIns(3)*P* distribution during autophagy by live imaging, biochemistry, and electron microscopy, and found that PtdIns(3)*P* is massively delivered into the vacuole *via* autophagy.[1] PtdIns(3)*P* is highly enriched as a membrane component of the elongating isolation membranes and autophagosome membranes rather than as an enclosed cargo, implying direct involvement of PtdIns(3)*P* in autophagosome formation. This observation also provides important basic information on the nature of the autophagosome membrane, which is still poorly understood. Notably, PtdIns(3)*P* is highly enriched on the inner (concave) surfaces of the isolation membrane and autophagosome compared to the outer surfaces. PtdIns(3)*P* is also enriched on ambiguous structures juxtaposed to the elongating tips of isolation membranes. We also investigated the function of PtdIns(3)*P* in autophagy, and show that PtdIns(3)*P* recruits the Atg18-Atg2 complex to autophagic membranes through an Atg18-PtdIns(3)*P* interaction.[2] Interestingly, PtdIns(3)*P* is required only for

the association of the Atg18-Atg2 complex to autophagic membranes but not for any subsequent functional activity of the Atg18-Atg2 complex, suggesting that PtdIns(3)*P* does not act allosterically on Atg18. Based on these results we discuss the function of PtdIns(3)*P* in autophagy.

We previously isolated phosphatidylinositol (PtdIns) 3-kinase complexes and demonstrated that one of them (complex I) functions in autophagy.[3] Vps34 is a sole catalytic subunit of PtdIns 3-kinase complexes in yeast, and is also involved in transport of vacuolar proteins *via* endosomes.[4] Complex I contains a unique subunit, Atg14, and associates with autophagic membranes (including the isolation membrane, autophagosome, and the pre-autophagosomal structure) in an Atg14-dependent manner, thereby sorting this complex to act specifically in autophagy.[5] Cells expressing a $Vps34^{N736K}$ variant that harbors a mutation within the lipid-kinase domain[4] have no autophagic activity, indicating that enzymatic activity of PtdIns 3-kinase complex I and, in turn, the product PtdIns 3-phosphate (PtdIns(3)*P*) is essential for autophagy.[1] PtdIns(3)*P* is abundant on endosomes under autophagy-non-inducing conditions.[6,7] In contrast, distribution of PtdIns(3)*P* under autophagy-inducing conditions has been totally unknown. We recently reported a detailed monitoring of PtdIns(3)*P* during autophagy and obtained novel knowledge,[1] which will be introduced in the first part of this addendum.

How PtdIns(3)*P* is involved in autphagy has not been revealed. Among autophagy-related (Atg) proteins[8,9] essential for autophagosome formation, Atg18 is a good candidate for a PtdIns(3)*P*-effector since it binds to phosphoinositides including PtdIns(3)*P in vitro*.[10-12] We focused on the relationship between PtdIns(3)*P*-binding of Atg18 and autophagy *in vivo*, and revealed one of the functions of PtdIns(3)*P* in autophagy.[2] We introduce these results in the second part.

*Dynamics of PtdIns(3)*P *during autophagy*

We utilized EGFP- and mRFP-2xFYVE domains to monitor intracellular PtdIns(3)*P*. The 2xFYVE domain is known to bind specifically to PtdIns(3)*P* both *in vitro* and *in vivo*.[7] Under nutrient-rich conditions, PtdIns(3)*P* was enriched on endosomes as reported previously. In addition, we found a significant level of PtdIns(3)*P* on the vacuolar membrane and occasionally at the pre-autophagosomal structure where Atg proteins localize. Upon induction of autophagy, PtdIns(3)*P* was massively transported into the vacuole in an autophagy-dependent manner. Interestingly, the mRFP-2xFYVE labeled rims of autophagosomes and autophagic bodies, exhibiting

ring-shaped signals, which indicates that PtdIns(3)*P* is enriched as a membrane component of autophagosomes rather than as an enclosed cargo. This result suggests that PtdIns(3)*P* is directly involved in autophagosome formation. To gain further insights, we performed immunoEM analysis. Similar to the result of fluorescence microscopy, PtdIns(3)*P* was enriched on the membranes of autophagosomes and autophagic bodies (Fig. 1). ImmunoEM also revealed that PtdIns(3)*P* is already enriched on elongating isolation membranes. Morphologically, the autophagosome membrane does not resemble any other intracellular membranes,[13] and its lipid composition has been unknown. Our results provide important basic information on the nature of this mysterious membrane structure. In addition, we observed two striking features of PtdIns(3)*P* distribution on autophagic membranes. First, PtdIns(3)*P* is highly enriched on the inner (concave) surfaces of isolation membranes and autophagosomes compared to the outer surfaces (Fig. 1). This biased distribution might be achieved by biased production and metabolism of PtdIns(3)*P*. Alternatively, a PtdIns(3)*P*-enriched membrane source might be supplied preferentially to the inner surface. Of course we do not completely exclude the possibility that this biased distribution resulted from

technical limitations. Second, PtdIns(3)*P* is enriched on some ambiguous structures around the elongating tips of isolation membranes (Fig. 1). We speculate that these PtdIns(3)*P*-enriched structures supply lipids to isolation membranes. One possible hypothesis would be that PtdIns(3)*P* on the inner isolation membranes and ambiguous structures around the tips are involved in the generation of the negative curve of the inner membranes and maintenance of the edges of isolation membranes.

*Function of PtdIns(3)*P *in autophagy*

What is the function of PtdIns(3)*P* on autophagic membranes? We recently report that one of the roles of PtdIns(3)*P* is to recruit the Atg18-Atg2 complex to autophagic membranes through Atg18-PtdIns(3)*P* interaction.[2] Using an Atg18 variant (Atg18(FTTG)) that cannot bind PtdIns(3)*P*[10,12], we show that Atg18-PtdIns(3)*P* interaction is required for efficient progression of both selective and non-selective autophagy. Next, we dissected Atg18-related processes from a viewpoint of PtdIns(3)*P*-binding of Atg18. Atg18 forms a complex with Atg2.[14] Co-immunoprecipitation and gel-filtration analyses reveal that this complex formation

does not require the Atg18-PtdIns(3)*P* interaction. In contrast, PtdIns(3)*P*-binding of Atg18 is required for association of the Atg18-Atg2 complex to autophagic membranes. Interaction between Atg2 and some component on the autophagic membranes likely acts synergistically in this recruitment process. We connected the 2xFYVE domain to the Atg18(FTTG) variant to restore affinity to PtdIns(3)*P* (*via* the FYVE domain) but not to other phosphoinositides. This fusion protein associated with autophagic membranes and restored normal autophagic activity, highlighting the requirement of the Atg18-PtdIns(3)*P* interaction in autophagy. This result also indicates that the Atg18-PtdIns(3)*P* interaction through its native site is only needed for correct localization of the Atg18-Atg2 complex but not for any subsequent functional activity of Atg18, since Atg18(FTTG) was functional once the localization role was bypassed by fusion with the 2xFYVE domain. In other words, PtdIns(3)*P* may not act allosterically on Atg18. Taken together, one of the functions of PtdIns(3)*P* in autophagy is to associate the Atg18-Atg2 complex with autophagic membranes.

We assume that this is not the sole function of PtdIns(3)*P* in autophagy. Localization of the Atg12–Atg5-Atg16 complex and Atg8 to autophagic membranes is

lost in *atg14*Δ cells.[14] A similar result was obtained in cells expressing Vps34^{N736K}, suggesting that PtdIns(3)*P* is involved in association of these molecules with autophagic membranes (our unpublished data). Since localization of these proteins is not dependent on Atg18, and *vice versa*,[14] PtdIns(3)*P* likely recruits these proteins and the Atg18-Atg2 complex independently. Given that the Atg12–Atg5-Atg16 complex has an E3-like activity upon Atg8–PE formation[15] and thereby regulates the site of Atg8 lipidation[14], PtdIns(3)*P* likely affect the site of Atg8–PE formation indirectly through Atg12–Atg5-Atg16. These putative PtdIns(3)*P*-effectors are known to localize to isolation membranes and are considered to be involved directly in the membrane formation process.[16-19] In contrast, deletion of *ATG14* does not affect localization of Atg17 and its associated proteins that are thought to function in earlier processes. Thus, PtdIns(3)*P* seems not to act in an early signaling process but functions to spatially coordinate early signaling molecules and proteins that are directly involved in membrane formation (Fig. 2). It is also possible that concentrated PtdIns(3)*P* itself affects membrane curvature without the assistance of binding proteins. We are now investigating these issues. Accordingly, the function(s) of PtdIns(3)*P* in autophagy is

just beginning to be unveiled.

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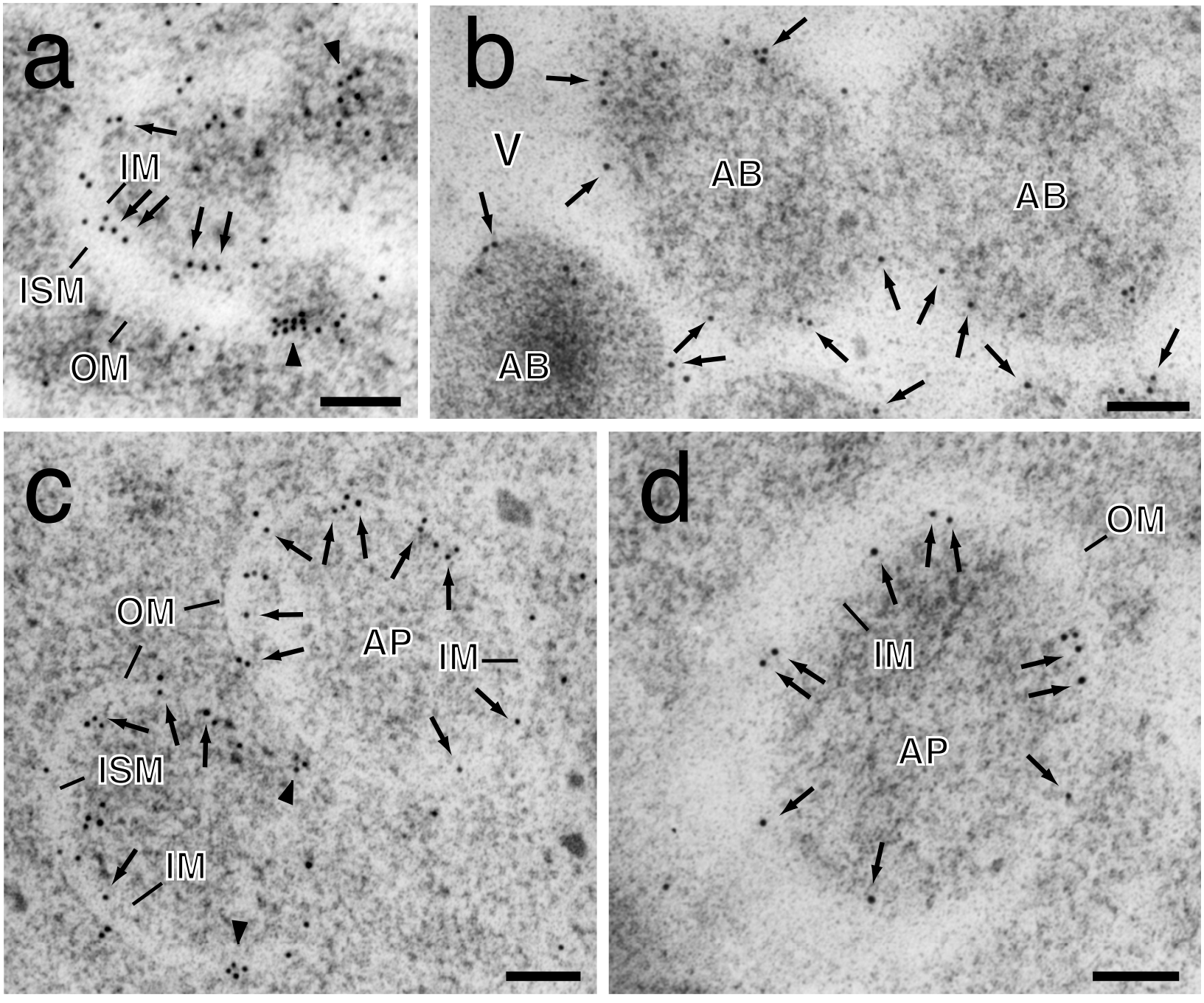
Figure Legend

Figure 1 PtdIns(3)*P* is enriched on autophagic membranes.

BJ2168 cells expressing mRFP-2xFYVE were cultured in SD(-N) medium for 3.5 h and subjected to immunoelectron microscopy with affinity-purified anti-FYVE antibody. Gold particles on the inner membrane (IM) of the isolation membrane (ISM in a and c), autophagosome (AP in c and d), and autophagic body (AB in b) membranes are indicated by arrows. Arrowheads in a and c indicate gold particles near the tips of isolation membranes. Ribosomes appear more hazy than gold particles, and can therefore be distinguished. OM, outer membrane. V, vacuole. Bars, 100 nm. Reproduced from Obara K, Noda T, Niimi K, Ohsumi Y. Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in *S. cerevisiae*. Genes Cells 2008; 13:537-547 (Copyright 2008 by the Molecular Biology Society of Japan/Blackwell Publishing Ltd).

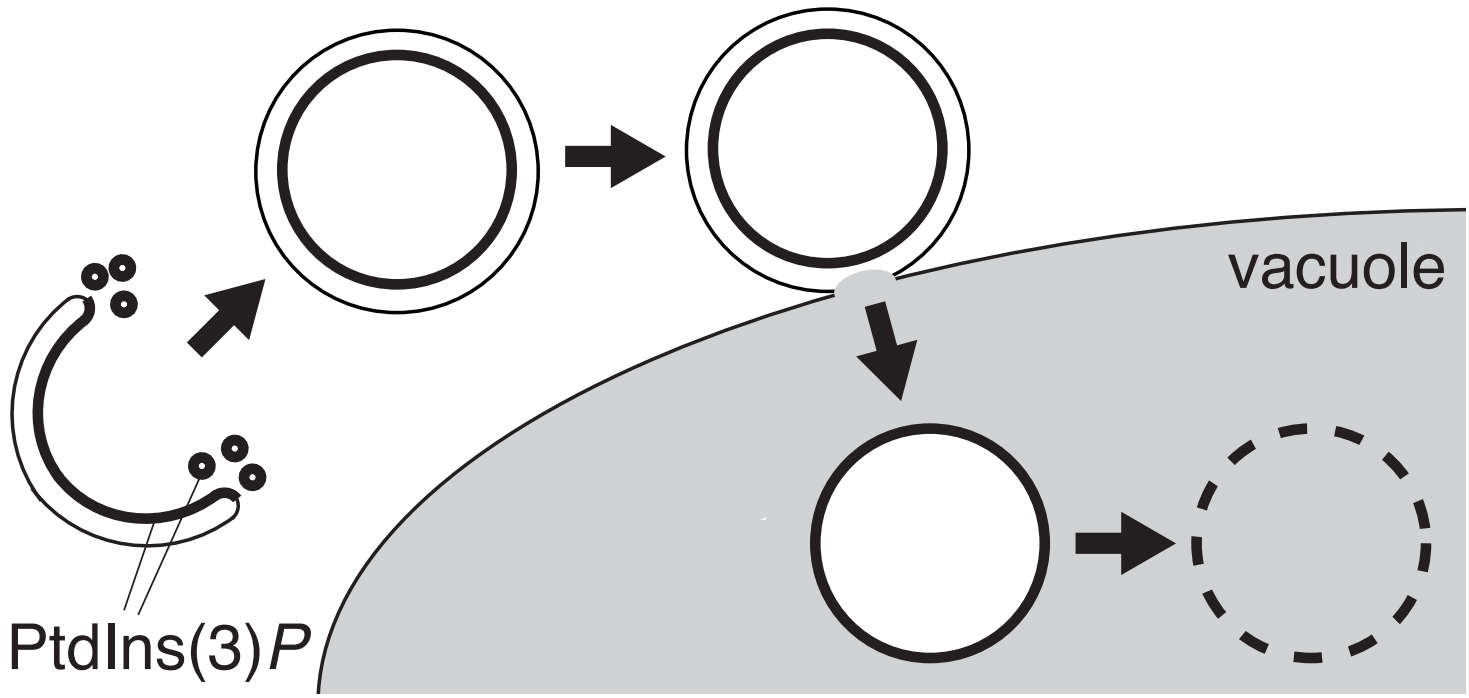
Figure 2 Summary of dynamics and function of PtdIns(3)*P* in autophagy.

A, summary of PtdIns(3)*P* distribution on autophagic membranes. PtdIns(3)*P*-enriched sites are shown in bold lines. B, functions of PtdIns(3)*P* in autophagy. PtdIns(3)*P* recruits the Atg18-Atg2 complex to autophagic membranes. Interaction between Atg2 and some factor (x) on the autophagic membranes acts synergistically. Localization of the Atg12–Atg5-Atg16 complex is likely regulated by PtdIns(3)*P* and some factor (y).



Obara K and Ohsumi Y Fig.1

A



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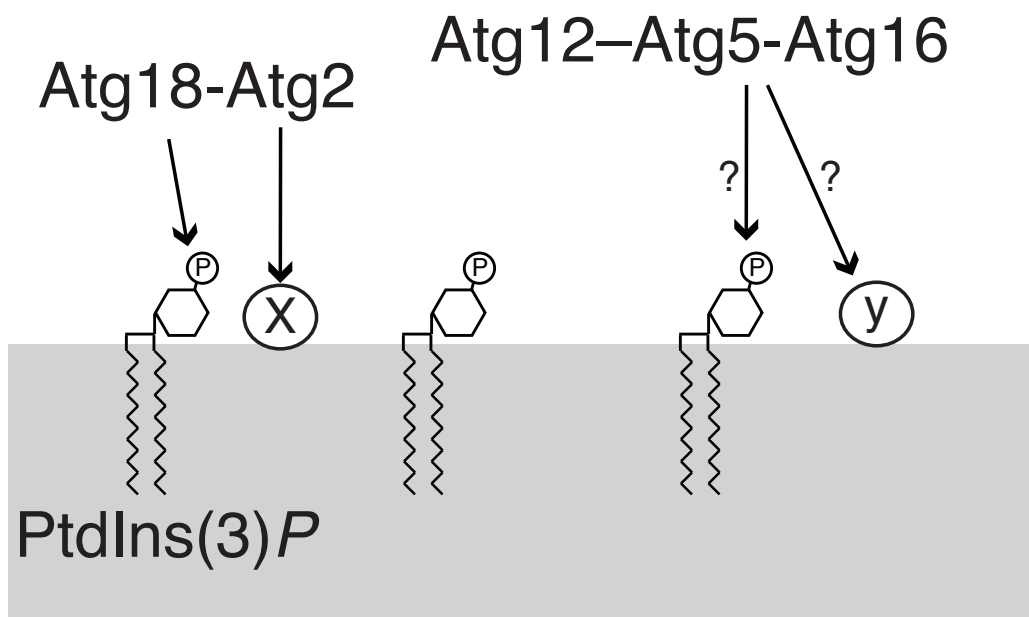

Obara K and Ohsumi Y Fig.2