Regulation of LC3 lipidation by the autophagy-specific class III phosphatidylinositol-3 kinase complex

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ABSTRACT Autophagy is a conserved eukaryotic pathway critical for cellular adaptation to changes in nutrition levels and stress. The class III phosphatidylinositol (PI)3-kinase complexes I and II (PI3KC3-C1 and -C2) are essential for autophagosome initiation and maturation, respectively, from highly curved vesicles. We used a cell-free reaction that reproduces a key autophagy initiation step, LC3 lipidation, as a biochemical readout to probe the role of autophagy-related gene (ATG)14, a PI3KC3-C1-specific subunit implicated in targeting the complex to autophagy initiation sites. We reconstituted LC3 lipidation with recombinant PI3KC3-C1, -C2, or various mutant derivatives added to extracts derived from a CRISPR/Cas9-generated ATG14-knockout cell line. Both complexes C1 and C2 require the C-terminal helix of VPS34 for activity on highly curved membranes. However, only complex C1 supports LC3 lipidation through the curvature-targeting amphipathic lipid packing sensor (ALPS) motif of ATG14. Furthermore, the ALPS motif and VPS34 catalytic activity are required for downstream recruitment of WD-repeat domain phosphoinositide-interacting protein (WIPI)2, a protein that binds phosphatidylinositol 3-phosphate and its product phosphatidylinositol 3,5-bisphosphate, and a WIPI-binding protein, ATG2A, but do not affect membrane association of ATG3 and ATG16L1, enzymes contributing directly to LC3 lipidation. These data reveal the nuanced role of the ATG14 ALPS in membrane curvature sensing, suggesting that the ALPS has additional roles in supporting LC3 lipidation.

INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is essential for maintaining cellular homeostasis as it is activated by nutritional stress to induce bulk turnover of cytoplasmic contents (Mizushima et al., 2008; Choi et al., 2013; Mercer et al., 2018). Upon activation, a cup-like structure called the phagophore forms and elongates, engulfing bulk cytoplasm or targeted cargo using membrane derived from cellular sources that have been identified as the endoplasmic reticulum (ER), Golgi apparatus intermediate compartment (ERGIC), Golgi apparatus, recycling endosomes, mitochondria, plasma membrane, ER-mitochondria contact sites, or the dynamic autophagy-related gene (ATG)9 compartment (Ge et al., 2014a; Mercer et al., 2018). After the growing phagophore engulfs and seals cargo into a double membrane autophagosome, it travels to the lysosome into which it deposits its cargo for degradation (Rubinsztein et al., 2012; Lamb et al., 2013; Ge et al., 2014a).

Because it is a fundamental homeostasis control mechanism,
autophagy has been implicated in a variety of human disorders, yet
the molecular mechanisms by which mammalian autophagy activa-
tion signals are translated into phagophore formation are still
emerging.

More than two decades of research has identified and character-
ized the hierarchy of machinery that initiates and organizes autopha-
gosome formation (Behrends et al., 2010; Hurley and Schulman,
2014; Young and Hurley, 2017; Mercer et al., 2018). Briefly, upon
autophagy initiation, the ULK1 kinase complex is activated and
translocates to the phagophore. This activated kinase complex then
recruits ATG9, a multispansing transmembrane protein, and the
class III phosphatidylinositol (PI)-3 kinase complex I (PI3KC3-C1) that
produces the signaling lipid, phosphatidylinositol 3-phosphate
(PtdIns3P) to the phagophore membrane. Next, PtdIns3P can be
converted to phosphatidylinositol 3, 5-bisphosphate (PtdIns(3,5)P2)
by additional lipid kinase activity, and the two phospholipids can
recruit effectors such as the double-FYVE containing protein 1, the
tyrosphospho-aspartic acid (WD)-repeat domain phosphoinositide-
interacting proteins (WIPI) family proteins and their binding
partners, such as ATG2A, to the phagophore. Downstream of ULK1
and PI3KC3-C1 activity, two ubiquitin-like enzymatic cascades work
to form the autophagic marker and cargo tether, LC3-II, by conju-
gating the cytosolic LC3-I protein to phosphatidylethanolamine
(PE). The final LC3 lipiddation reaction is orchestrated by ATG7,
ATG3, and a complex consisting of ATG12–ATG5 and ATG16L1
serving as E1, E2, and E3-like enzymes, respectively, to form LC3-II
by conjugating the cytosolic LC3-I protein to PE.

Successful autophagosome formation requires an orchestrated
manipulation of highly curved membrane vesicles, tubules, and
sheets. Because it is one of the first complexes recruited to the
phagophore, creates an essential signaling lipid, PtdIns3P, and
contains several domains that preferentially bind highly curved
membranes (Fan et al., 2011; Nguyen et al., 2017; Young and
Hurley, 2017), the autophagy-specific PI3KC3-C1 is a key regulator of
autophagosome formation. However, the direct mechanism by
which the complex and its product, PtdIns3P, orchestrate autopha-
gosome formation by coordinating recruitment and activation of
autophagic machinery on the phagophore membrane in vivo is
unclear.

There are at least two forms of the PI3KC3 heterotetramer in-
volved in distinct stages of mammalian autophagy (Itakura and
Mizushima, 2009; Matsunaga et al., 2009). Both contain the core
machinery of vacuole protein sorting (VPS) 15, VPS34, and BECN1.
Complex one (PI3KC3-C1), primarily involved in autophagosome
formation, is defined by the presence of the autophagy-specific
ATG14 (Itakura et al., 2008; Sun et al., 2008; Matsunaga et al., 2009;
Zhong et al., 2009) and the metabolic switch, NRBF2 (Zhong et al.,
2014; Young et al., 2016; Ma et al., 2017). Complex two (PI3KC3-
C2), which replaces ATG14 with UVRAG, is principally involved in
later stages of autophagosome and endosome maturation (Itakura
and Mizushima, 2009; Matsunaga et al., 2009).

As an autophagy-specific factor of PI3KC3-C1, ATG14 is likely
the key regulator of autophagy-induced translocation of this com-
plex to the phagophore. Previous work has demonstrated that
ATG14 contains an ATG14/Barkor autophagosome targeting
sequence (BATS) domain that is required for recruiting the PI3KC3-
C1 complex to the autophagosome membrane by sensing curva-
ture in these membranes (Fan et al., 2011; Wilz et al., 2011; Hurley
and Schulman, 2014). The BATS domain includes a predicted
amphiphatic lipid packing sensor (ALPS) motif on its extreme C-
terminal (Drin et al., 2007; Gautier et al., 2008; Fan et al., 2011; Wilz
et al., 2011). We predict that the ATG14 ALPS motif serves as a
membrane curvature sensor responsible for targeting the PI3KC3-
C1 complex to the highly curved phagophore membrane as the
BATS domain membrane association capabilities are abolished
when the bulky hydrophobic residues within the ALPS motif are mu-
tated (Fan et al., 2011).

Our group has established a cell-free reaction that faithfully
reconstitutes LC3 lipiddation and early stages of phagophore forma-
tion (Ge et al., 2013). We adapted this assay to examine the PI3KC3-
C1 complex by employing reconstructions of CRISPR/Cas9-generated
ATG14 knockout (KO) cytosolic fractions and purified recombinant
PI3KC3-C1. We used this biochemical reconstitution assay to di-
rectly assess how the PI3KC3-C1, particularly its PI3K catalytic
activity and membrane curvature sensing capabilities, regulate aut-
ophagy through LC3 lipiddation and membrane association of au-
tophagy machinery. We found that PI3KC3-C1 catalytic activity and
the ATG14 ALPS domain were directly responsible for LC3 lipida-
tion, the recruitment of WIPI2 and ATG2A, and PtdIns3P produc-
tion. The PI3KC3-C2 complex by itself did not overcome ATG14
depletion. However, the membrane association of ULK1 complex
components and critical LC3 lipiddation machinery was not affected
by defects in PI3KC3-C1 activity despite the requirement of this
activity to promote the production of lipiddated LC3-II.

RESULTS

ATG14 is required for LC3 lipiddation

To probe the role of the PI3KC3-C1 in autophagy initiation, we
adapted our previously established cell-free LC3 lipiddation reaction
that combines membrane fractions from ATG5 KO mouse embry-
onic fibroblast (MEF) cells, which cannot support LC3 lipiddation, with
cytosolic fractions from autophagy-induced cells (Ge et al., 2013).
After the addition of recombinant T7-LC3-I, GTP, and an ATP regen-
eration system, the resultant autophagy activity was observed by
quantifying the conversion of precursor LC3-I to LC3-II product in the
membrane fraction by SDS–PAGE and immunoblot (IB) (Supple-
mental Figure S1) (Ge et al., 2013).

ATG14 is required for PI3KC3-C1 complex membrane targeting
and autophagy initiation (Sun et al., 2008; Matsunaga et al., 2010;
Fan et al., 2011). Therefore, to directly study this complex in autopha-
ygenesis initiation, we focused on ATG14 activity by depleting it
from cells. We used CRISPR/Cas9 (Cong et al., 2013; Jinek et al.,
2013) to target the first exon of ATG14 in HEK293T cells creating an
ATG14 KO cell line from which a cytosol fraction was isolated
(Figure 1A and Supplemental Table S1). To remove active PI3KC3-
C1 from the membrane fraction, we treated ATG5 KO MEF cells
with the PI3K inhibitor, wortmannin (Blommaart et al., 1997).
Wortmannin-inactivated VPS34 is undetectable by IB in cellular
membrane fractions of starved cells (Figure 1B). Wild-type cytosol
combined with the wortmannin-treated membrane fraction
supported LC3 lipiddation (Figure 1C, lane 1). Conversely, lipiddation
efficiency with ATG14 KO cytosol dropped by more than 80%
compared with wild-type cytosol (Figure 1C, lane 2).

To confirm that the decline in LC3-II production observed with
ATG14 KO cytosol was specific, we complemented the reaction with
purified recombinant PI3KC3-C1 (Figure 1D) (Baskaran et al., 2014)
at levels comparable to wild-type levels of VPS34 (Supplemental
Figure S2). Recombinant PI3KC3-C1 stimulated and fully comple-
mented LC3 lipiddation in the ATG14-depleted system in a dose-
dependent manner (Figure 1C). Lipidation complementation was a
direct result of increased VPS34 activity as the addition of either
wortmannin or a GST-FYVE peptide that binds and sequesters avail-
able PtdIns3P (Ridley et al., 2001; Ge et al., 2013) reduced lipiddation
levels to that observed without recombinant PI3KC3-C1 (Figure 1E).

Volume 30 April 15, 2019 P13KC3 is required for LC3 lipiddation | 1099
Lipidation defects in the ATG14-depleted system were fully complemented by the addition of 100 μM PtdIns3P. Consequently, PtdIns3P production is necessary and sufficient in fulfilling the role of ATG14 in stimulating LC3 lipidation in the reconstituted autophagy system.

**PI3KC3-C2 does not complement ATG14-depletion defects**

There are two PI3KC3 subcomplexes in mammalian cells: C1 regulates autophagy initiation and contains ATG14; C2 is primarily involved in late endosome and autophagosome maturation and contains UVRAG in the active complex (Liang et al., 2008; Itakura and Mizushima, 2009; Zhong et al., 2009; Kim et al., 2013). We also purified recombinant PI3KC3-C2 (Supplemental Figure S3B) to probe its ability to stimulate LC3 lipidation and complement the defect associated with ATG14 depletion. First, we tested the relative catalytic activity of these two complexes based on membrane curvature using either large unilamellar vesicles (LUVs) or highly curved SUVs incorporated with the PtdIns3P precursor, PI. We found that C2 was 50% less active than C1 on SUVs, suggesting that C1 contains an additional factor that increased its activity on highly curved membranes (Figure 2D). We generated SUVs and LUVs and found that the catalytic activity of C1 and C2 was highly dependent on membrane curvature (Figure 2D). In contrast to a finding in a previous report (Rostislavleva et al., 2015), the activity of both complexes decreased as vesicle diameter increased. We then compared the LC3 lipidation activity of these complexes and found that C1 robustly stimulates LC3 lipidation in a dose-dependent manner in incubations containing wild-type cytosol, whereas C2 does not (Figure 2, E and F). Additionally, C2 only modestly stimulates LC3 lipidation in a dose-dependent manner compared with C1 in reactions containing ATG14-KO cytosol (Figure 2, G and H). Thus, although the kinase complexes contain the same core machinery, the limited catalytic activity of PI3KC3-C2 on small vesicles cannot replace the C1 complex in stimulating lipidation of LC3, possibly due to a failure to target the C2 enzyme to a highly curved early autophagosome membrane template.

**The ATG14 ALPS is critical for PI3KC3-C1 membrane targeting**

ATG14 is responsible for targeting the PI3KC3-C1 to the autophagic membrane through its BATS domain (Fan et al., 2011). The BATS domain preferentially binds highly curved membrane and encompasses a predicted ALPS motif (Gautier et al., 2008; Antonny, 2011; Fan et al., 2011; Wilz et al., 2011). ALPS motifs are short 20–40 amino acid–long peptides that sense membrane curvature by inserting an alpha helix into lipid-packing defects inherent in highly curved membrane bilayers (Antonny, 2011).

We designed a PI3KC3-C1 mutant lacking the final 25 residues of ATG14 encompassing the ALPS motif (∆ALPS) (Figure 3A and}
PI3KC3 activity is required for LC3 lipitation

For targeting a stable PI3KC3-C1 to the early autophagic membrane.

We then tested the LC3 lipitation activity of the \( \Delta \text{ALPS} \) PI3KC3-C1 mutant and found that it did not complement ATG14-depleted cytosol defects (Figure 3E). Therefore, although the \( \Delta \text{ALPS} \) PI3KC3-C1 mutant retained half of wild-type catalytic activity, it neither bound to membranes nor stimulated LC3 lipitation.

**PI3KC3-C1 activity is required for WIPI2 and ATG2A membrane association**

Imaging studies have uncovered that PI3K activity is required for stable phagophore localization of upstream autophagy machinery, such as the ULK1 kinase complex, and also downstream machinery, such as the LC3 lipitation machinery DFCP1, WIPI2, and ATG2A (Mizushima et al., 2001; Axe et al., 2008; Itakura and Mizushima, 2010; Koyama-Honda et al., 2013; Kishi-Itakura et al., 2014). However, because only WIPI proteins and DFCP1 contain a known PtdIns3P effector, the mechanism by which the PI3KC3-C1 regulates association of other factors and subsequent autophagy activity is unknown.

We isolated membrane fractions from our cell-free reaction to directly assess how the PI3KC3-C1 regulates the membrane association of the autophagy machinery. First, we treated the reactions with wortmannin to probe how membrane association differed after chemical PI3KC3-C1 inhibition. Wortmannin treatment drastically depleted membrane association of the PtdIns3P effector, WIPI2, and ATG2A, which binds another PtdIns3P effector, WIPI4 (Bakula et al., 2017) (Figure 4, A and B). In contrast, although LC3 lipidation was inhibited by wortmannin, there were no significant changes in the membrane association of the lipidation components, ATG3 and ATG16L1, or of subunits of the ULK1 complex, FIP200 and ATG13 (Figure 4, A and B).

We next tested how ATG14 depletion and complementation affected the membrane association of components of the autophagy machinery. WIPI2 and ATG2A membrane association increased at least three-fold in incubations containing a concentration of PI3KC3-C1 that complemented ATG14-depletion defects in the lipidation reaction (Figure 4, C and D). Although PI3KC3-C1 complementation stimulated LC3 lipitation activity, it did not significantly alter the membrane association of ATG16L1 and ATG3 or FIP200 and ATG13 (Figure 4, C and D).

PI3KC3-C2 and PI3KC3-C1 mutants lacking VPS34 catalytic activity or the ALPS motif did not complement ATG14-depletion lipitation defects (Figures 2 and 3). To determine whether these lipitation defects are a result of aberrant autophagy machinery membrane association, we examined their effect in the membrane association of components of the autophagy machinery. WIPI2 and ATG2A membrane association increased at least three-fold in incubations containing a concentration of PI3KC3-C1 that complemented ATG14-depletion defects in the lipidation reaction (Figure 4, C and D). Although PI3KC3-C1 complementation stimulated LC3 lipitation activity, it did not significantly alter the membrane association of ATG16L1 and ATG3 or FIP200 and ATG13 (Figure 4, C and D).
recruitment assay. First, we tested the differences in C1 and C2 and found that C2 did not stimulate WIPI2 and ATG2A membrane association in an ATG14-depleted reaction (Figure 5, A and B), suggesting that PI3KC3-C2 failed to produce PtdIns3P in a location competent to recruit WIPI2 or ATG2A and to stimulate autophagosome formation.

We then complemented the ATG14-depleted reconstitution with wild-type, ΔALPS, and KD PI3KC3-C1 and found that only the wild-type PI3KC3-C1 stimulated WIPI2 and ATG2A membrane association (Figure 5, C and D). In contrast, ATG3 and ATG16L1 membrane association did not significantly change upon the addition of wild-type or mutant PI3KC3-C1 complexes, even though they displayed distinct defects of lipidation activity.

The ΔALPS mutant retained roughly 60% of wild-type kinase activity, enough to permit a test of the role for ALPS in PtdIns3P production. We assessed the production of PtdIns3P in the lipidation reaction by detecting the association of GST-FYVE peptide on membranes isolated from the reconstitution by buoyant density flotation. Changes in both GST-FYVE and WIPI2 membrane association may be used as reporters for the production of overall and autophagy-specific PtdIns3P, respectively. The addition of wild-type PI3KC3-C1 increased both GST-FYVE and WIPI2 membrane association, indicating an increase in PtdIns3P production (Figure 5E). The ΔALPS PI3KC3-C1 mutant did not stimulate WIPI2 or GST-FYVE association; therefore deleting the ATG14 ALPS motif abolished PI3KC3-C1 catalytic activity on isolated membranes.

DISCUSSION
In this study, we employed a biochemical approach to assess the roles that PI3KC3-C1 catalytic activity and the ability to associate with highly curved membranes have in autophagy factor membrane association and LC3 lipidation activity. We confirm through the use of a cell-free assay that PI3KC3-C1 activity and PtdIns3P production are necessary for LC3 lipidation. In addition, we uncovered that the ALPS motif of ATG14 is directly required for LC3 lipidation, PtdIns3P production, and the association of WIPI2 and ATG2A with
membranes. The PI3KC3-C1 contains additional membrane association factors, such as a lipid anchor on VPS15 and the VPS34 C-terminal helix, which are also required for catalytic activity (Miller et al., 2010). Therefore, the ALPS motif is likely critical for targeting PI3KC3-C2 to the appropriate membrane microenvironment for phagophore expansion and elongation, such as the highly curved, PtdIns3P-enriched tips of the growing phagophore, where imaging studies have localized the PI3KC3-C1 complex in the ERGIC or ER-exit sites where PI3K activity is required for producing small autophagic precursor vesicles (Graef et al., 2008; Suzuki et al., 2008; Nath et al., 2010; Velikkakath et al., 2012). However, other studies have demonstrated that FIP200 and WIPI2 (and their representative homologues in S. cerevisiae) do not directly affect LC3 lipidation to the same extent as other components of the autophagic machinery (Proikas-Cezanne et al., 2004; Suzuki et al., 2007; Polson et al., 2010; Mauthe et al., 2011; Velikkakath et al., 2012). Therefore, as this study and others have demonstrated the importance of VPS34 activity for LC3 lipidation, it is likely that either WIPI family proteins or ATG2A regulates the lipidation machinery. Studies have suggested that WIPI2 and ATG2A (and their representative homologues in S. cerevisiae) do not directly affect LC3 lipidation to the same extent as other components of the autophagic machinery (Proikas-Cezanne et al., 2004; Suzuki et al., 2007; Polson et al., 2010; Mauthe et al., 2011; Velikkakath et al., 2012). However, other studies have demonstrated that FIP200 and WIPI2 bind ATG16L1 isoforms and that this interaction and subsequent membrane association is responsible for ATG16L1-dependent lipidation of LC3 (Gammoh et al., 2012; Nishimura et al., 2013; Proikas-Cezanne et al., 2014). In this study, we found that membrane association capabilities of LC3 lipidation machinery components ATG3 and ATG16L1, which have been shown to dictate the site of LC3 lipidation (Fujita et al., 2008; Nath et al., 2014), are not regulated by PI3KC3-C1 activity. However, as our study uses a cell-free approach to directly assess differences in membrane association, these distinctions in the role of FIP200 and WIPI2 in ATG16L1 recruitment may be a result of the sensitivity of different technical approaches.

WPI2 and ATG2A are the only critical components of autophagy initiation machinery outside of the PI3KC3-C1 that change
membrane association in correlation with lipidation activity in our recombinant reconstitution. However, either PtdIns3P concentrations higher than that necessary to stimulate LC3 lipidation may be required to stimulate this association, or additional lipid kinase activity may be required to produce PtdIns(3,5)P2 from PtdIns3P for robust membrane recruitment, as we could not reconstitute WIPI2 or ATG2A association with membranes in incubations containing exogenous PtdIns3P (unpublished data). Because the PI3KC3-C1 is required to produce small lipidation-competent vesicles and wortmannin-treated cells do not form a phagophore (Ge et al., 2014b; Kishi-Itakura et al., 2014), PI3KC3-C1 activity may indirectly stimulate LC3 lipidation by elongating the phagophore that can then support LC3 lipidation. Phagophore elongation and closure may be dependent on ATG2A, as cells lacking this factor accumulate LC3-II and form large LC3 positive puncta (Kishi-Itakura et al., 2014) with autophagic cargo accessible to exogenous protease attack (Velikkakath et al., 2012; Kishi-Itakura et al., 2014; Zhang et al., 2015). More studies are necessary to uncover this intriguing relationship among PI3KC3-C1 activity, WIPI2 and ATG2A membrane association, phagophore elongation, and LC3 lipidation.

Our study suggests a direct relationship among PI3KC3-C1 catalytic activity, LC3 lipidation, and autophagy activity. However, although PI3KC3-C1 activity regulates WIPI2 and ATG2A membrane association and LC3 lipidation, it does not directly regulate membrane association of components of the lipidation machinery. Therefore, the mechanism by which PtdIns3P production stimulates LC3 lipidation activity remains unknown. As the PI3KC3-C1 is a critical regulator of an important cellular homeostasis pathway, a more detailed understanding of its role in autophagy initiation may be critical in an effort to control autophagy for therapeutic purposes, particularly because highly specific VPS34 inhibitors fail to discriminate kinase activities of the C1 and C2 enzymes (Ronan et al., 2014; Pasquier et al., 2015). Here, we report that deleting a 25-residue-long membrane-targeting motif on ATG14 completely abolishes all

**FIGURE 5:** Only wild-type PI3KC3-C1 can produce PtdIns3P and stimulate WIPI2 and ATG2A recruitment in an ATG14-depleted system. (A) Membrane-buoyant density flotation performed with ATG14 KO cytosol and complemented with 1 nM of noted PI3KC3 complex. (B) Quantification of A. n = 4. **p < 0.01. (C) Experiment performed as in A with ΔALPS and KD mutants. (D) Quantification of C, n = 5, **p < 0.01. (E) Membrane-buoyant density flotation performed as in A but after the lipidation reaction, GST-FYVE peptide was added at 0.08 mg/ml and incubated for an additional 1 h at 30°C before density gradient sedimentation.
PI3KC3-C1 activity, thereby suggesting a potential target to control PI3K activity in the initiation of autophagy.

MATERIALS AND METHODS

Cytosol preparation

Cytosol fractions prepared from HEK 293T cells was conducted as previously described (Ge et al., 2013) with adaptations. Confluent cells in 15-cm-diameter culture dishes were starved in 15 ml Earle’s balanced saline solution (EBSS) for 1 h. After clarification by sedimentation 3 × 60 min at 165,000 × g, 4°C, the supernatant cytosol concentration was determined using protein-assay dye reagent (Bio-Rad, Hercules, CA). Samples were snap-frozen, stored at −80°C, and subjected to no more than one freeze-thaw cycle.

Membrane preparation

Membrane preparations from ATG5 KO cells were isolated as described in Ge et al. (2013) with adaptations to deplete PI3KC3-C1. ATG5 KO MEFS were grown to confluency in 15-cm-diameter culture dishes and washed 1 × with sterile phosphate-buffered saline (PBS). Cells were then starved for 1 h in 15 ml EBSS with 20 nM wortmannin, collected, and lysed in 2.7 × cell pellet volume of membrane lysis buffer (20 mM HEPES, pH 7.2, 1.0 mM EDTA, 250 mM sorbitol, 0.3 mM dithiothreitol [DTT], 1 × Roche Protease Inhibitor Tablet, 1 × Roche Phosphatase Inhibitor Tablet) by passing through a 22-G needle to achieve ~85% lysis, as assessed by trypan blue staining. To reduce lipid loss, all membrane was handled using low-retention pipette tips (Axygen). Cellular debris was sedimented by centrifugation for 10 min at 3000 × g, 4°C. After removing the supernatant fraction, the pellet was washed and sedimented one more time. The two supernatant fractions were combined and the membrane fraction was isolated by sedimentation for 30 min at 25,000 × g, 4°C. The pellet was gently resuspended and washed one time in B88 lysis buffer (20 mM HEPES, pH 7.2, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, 0.3 mM dithiothreitol, 1 × Roche Protease Inhibitor Tablet, 1 × Roche Phosphatase inhibitor Tablet). Phosphatidylcholine (PC) concentration was used as a read-out for overall lipid concentration and determined using the PC assay described in Ge et al. (2011). Membrane fractions were stored at 4°C and used within 24 h of preparation.

LC3-II lipidation reaction

The LC3-II lipidation reaction was adapted from Ge et al. (2013). For each lipidation reaction, a final concentration of 2.4 mg protein/ml cytosol, 0.1 mg/ml (PC content final concentration) of ATG5 KO membrane, ATP regeneration system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 1 mM ATP), 0.15 mM GTP, and 10.0 μg protein/ml 3 × T7-LC3 were added to the membrane mixture to a final volume of 18 μl in B88 lysis buffer. In experiments containing recombinant PI3KC3K, we first diluted protein stocks to equivalent concentrations and 3 × T7-LC3 were added to the membrane mixture to a final volume of 18 μl and incubated for 70 min at 30°C. Lipidation reaction samples were prepared and analyzed as described above.

PtdIns3P incorporated LC3-II lipidation reaction

To prepare liposomes, we dried 40 μl of a 1.0 mg/ml stock of 18:0 PtdIns3P (Avanti Polar Lipids) in chloroform overnight under a vacuum. The lipid was resuspended in 160 μl B88 by rotating for 1 h at 25°C. After resuspension, liposomes were extruded through a B88 equilibrated 100 nM Whatman Nucleopore Track Etch Membrane filter (Sigma) 30x. Lipids were then added to a mix of 0.1 mg/ml (PC content final concentration) of ATG5 KO membrane, ATP regeneration system, and 0.15 mM GTP and incubated for 1 h at 30°C.

After PtdIns3P incorporation, 2.4 mg/ml cytosol and 0.1 mg/ml 3 × T7-LC3 were added to the membrane mixture to a final volume of 18 μl and incubated for 70 min at 30°C. Lipidation reaction samples were prepared and analyzed as described above.

ADP-Glo kinase assay

The ADP-Glo kinase assay (Promega, Madison, WI) protocol was adapted from previous protocols (Baskaran et al., 2014) and performed in 96-well PS F-Bottom white plates (Greiner Bio-One, Frickenhausen, Germany). The 25-μl reaction consisted of 20 μl of 125 μM Pi: phosphatidylycerine (PS) (Avanti Polar Lipids, Alabaster, AL) SUVs in PI/PS buffer (50 mM HEPES, pH 7.0, 50 mM MnCl2, 0.5 mM TCEP), and 2.5 μl of 100 nM recombinant PI3KC3 in PI3K freezing buffer and was initiated by adding 2.5 μl of 500 mM ATP. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 25 μl ADP-Glo Reagent supplemented with 10 mM MgCl2 for 40 min at 25°C. Then, 50 μl of Kinase Detection Reagent was added to wells and incubated for 30 min at 25°C. Luminescence was measured using an Infinite M1000 system (Tecan, Research Triangle Park, NC) with a 1000-ms integration time.

Buoyant density membrane recruitment assay

Membrane recruitment assays were conducted using a 48-μl LC3 lipidation reaction from which 2.5% input samples were taken after incubation at 30°C. Samples were gently mixed with 50% OptiPrep in B88 to a final concentration of 40% Opti Prep in 200 μl. Layers of 130 μl of 35%, 120 μl of 32%, and 20 μl of 0% Opti Prep in B88 were added above the sample. Density gradients were centrifuged in a swinging bucket TLS-55 rotor (Beckman Coulter, Pasadena, CA) for 90 min at 165,000 × g, 4°C with reduced braking. Fractions (90 μl) were removed by pipette from the top. Samples were mixed with 4x SDS and prepared for SDS-PAGE and IB. Densitometry was calculated using ImageJ band analysis.

Statistical analysis

Unless noted, data are presented as the mean ± SEM and are representative of experiments conducted in triplicate at minimum. Two-tailed Student’s t tests were conducted when indicated.

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Volume 30 April 15, 2019 PI3KC3 is required for LC3 lipidation | 1105
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