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AMPK-activated ULK1 phosphorylates PIKFYVE to drive formation of PtdIns5P-containing autophagosomes during glucose starvation

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ABSTRACT

The induction of macroautophagy/autophagy upon glucose deprivation can occur independently of the PIK3C3/VPS34 complex. Recently, we described a non-canonical signaling pathway involving the kinases AMPK, ULK1 and PIKFYVE that are induced during glucose starvation, leading to the formation of PtdIns5P-containing autophagosomes, resulting in increased autophagy flux and clearance of autophagy substrates. In this cascade, the activation of AMPK leads to ULK1 phosphorylation. ULK1 then phosphorylates PIKFYVE at S1548, leading to its activation and increased PtdIns5P formation, which enables the recruitment of machinery required for autophagosome biogenesis.

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Autophagy is a conserved, intracellular degradation mechanism essential for maintaining cellular energy levels through the concerted action of numerous proteins that lead to the turnover of old or damaged proteins and organelles. Recently, increasing evidence has suggested that autophagosome formation may be induced without employing the complete set of proteins that were initially considered a part of the autophagy canon. We have been particularly interested in the findings showing that autophagy can be upregulated independently of the PIK3C3/VPS34 complex and PtdIns3P lipids. It appears that the conventional steps of autophagosome formation involving the PIK3C3/VPS34 complex can be bypassed during glucose starvation, as PtdIns5P-containing autophagosomes are formed, offering a possible explanation for the presence of autophagosomes in *PIK3C3/VPS34*-null cells. In the conventional pathway, PtdIns3P-containing autophagosomes are formed on the RAB11A-positive recycling endosome compartment. We found that this compartment is similarly associated with PtdIns5P-containing autophagosomes and the lipid kinase responsible for the synthesis of PtdIns5P lipids, PIKFYVE, suggesting that PtdIns5P may act similarly to PtdIns3P. Indeed, a previous study in our lab had shown that PtdIns5P lipids can recruit downstream autophagy effectors required for the biogenesis of phagophores, such as WIPI2.

In our recent study [1], we elucidated the mechanism leading to the formation of PtdIns5P-containing autophagosomes, specifically during glucose starvation. We identified a new signaling axis that involves the master autophagy regulator AMP-activated protein kinase (AMPK), the key autophagy protein ULK1 and PIKFYVE – a kinase mostly studied in the context of PtdIns(3,5)P_2 lipid formation, lysosomal function and endocytic trafficking.

We used a variety of pharmacological and genetic tools and could corroborate that glucose starvation leads to STK11/LKB1-mediated activation of AMPK, which, in turn, activates ULK1 by phosphorylation of multiple serine residues. As we were interested in the induction of autophagosome formation, we transfected cells with a mutant form of ATG4B that prevents the lipidation of autophagosomes and a bio-probe that allows the microscopy-based visualization of PtdIns5P lipids in cells. The bio-probe consists of a GFP vector with three tandem repeats of the plant homeodomain (PHD) of the protein ING2, which has a strong preference for PtdIns5P. Next, by staining cells for ATG16L1, a marker for phagophores, we were able to specifically visualize early autophagic structures that contained PtdIns5P lipids. Using this approach, we found that the activation of AMPK with multiple AMPK activators or glucose starvation lead to an increase in the formation of autophagosomes that contain PtdIns5P in a variety of cell lines. This was not observed when treating cells with the AMPK inhibitor compound C or in *PRKAA*-null cells or when transfecting cells with a dominant-negative, kinase-deficient AMPK construct. The increase in the PtdIns5P-containing autophagosomes correlates with an increase in LC3 puncta and autophagy flux, which was measured by transfecting cells with an mRFP-GFP-LC3 construct. Similarly, overexpression of active human or mouse ULK1 also leads to an increase in the formation of PtdIns5P-containing autophagosomes. Interestingly, expression of active ULK1 in *PRKAA*-null cells is sufficient to induce and rescue the formation of the autophagosomes, whereas pharmacological ULK1 inhibition prevents the increase in PtdIns5P-containing autophagosomes observed when activating AMPK, thus putting AMPK upstream of ULK1 in this pathway. However, neither AMPK or ULK1 activation can

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induce the formation of PtdIns5P-containing autophagosomes without a functional PIKFYVE kinase, which was tested genetically by expression of a kinase-deficient PIKFYVE^{K1831E} or pharmacologically using a PIKFYVE inhibitor (YM201636).

This led us to further explore the relationship between AMPK, ULK1 and PIKFYVE. First, we corroborated existing evidence that AMPK activates PIKFYVE by phosphorylating it on serine 307, leading to an increase in PtdIns(3,5)P_2 lipids. To this end, we used a bio-probe that contains GFP bound to tandem repeats of ML1N, the cytosolic N-terminal polybasic domain of the MCOLN1/TRPML1 (mucolipin 1 TRP cation channel 1) channel, which is known to be bound by PtdIns(3,5)P_2. However, by using a phospho-mutant PIKFYVE^{S307A}, we showed that the increase in PtdIns(3,5)P_2 lipids caused by AMPK activation does not play a role in the formation of autophagosomes upon glucose starvation, but appears to regulate lysosome and endosome abundance. We found that PIKFYVE binds ULK1 on its kinase domain by using ULK1 deletion mutants and performing immunoprecipitation experiments. By mass spectrometry and immunoprecipitation studies, we showed that ULK1 phosphorylates PIKFYVE on multiple residues. We performed an *in vitro* lipid kinase assay and showed that the ULK1-mediated phosphorylation of serine 1548, in particular, leads to an increase in the levels of PtdIns5P lipids, resulting in the formation of PtdIns5P-containing autophagosomes and autophagy flux, while it did not affect the formation of PtdIns(3,5)P_2, suggesting that the ULK1-mediated activation of PIKFYVE serves to specifically induce the PtdIns5P-related activities of PIKFYVE.

Finally, we found that phospho-mimic PIKFYVE^{S1548D} drives autophagy upregulation and lowers autophagy substrate levels, such as the neurodegeneration-associated mutant polyQ-HTT (huntingtin). Thus, our study describes how ULK1 upregulates autophagy upon glucose starvation and induces the formation of PtdIns5P-containing autophagosomes by activating PIKFYVE (Figure 1), revealing a mechanism by which autophagy is induced by glucose deprivation and identifying new roles for both ULK1 and PIKFYVE in autophagy signaling.

These findings may have particular importance for the use of autophagy upregulation in the treatment of neurodegenerative diseases and other situations where PtdIns3P-dependent autophagy is compromised, and where clearance of toxic proteins or other autophagy substrates may function as a therapeutic strategy. Therefore, overcoming BECN1-PIK3C3/VPS34-dependent autophagy downregulation by upregulating autophagy in a PIK3C3/VPS34-independent manner could evade the involvement of functionally impaired proteins and open a new avenue for therapeutic interventions.

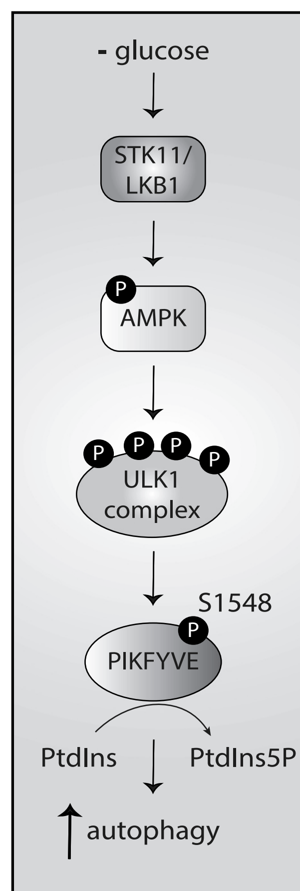

Figure 1. Schematic overview of non-canonical autophagy signaling during glucose starvation. STK11/LKB1-mediated activation of AMPK leads to phosphorylation and activation of ULK1. ULK1 phosphorylates PIKFYVE at S1548, causing an increase in the PIKFYVE-mediated synthesis of PtdIns5P phospholipids and resulting in increased formation of PtdIns5P-containing autophagosomes and autophagy flux.

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Disclosure statement

DCR is a consultant for Aladdin Healthcare Technologies SE, PAQ Therapeutics Inc. and Nido Biosciences. CK has no conflicts of interest.

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