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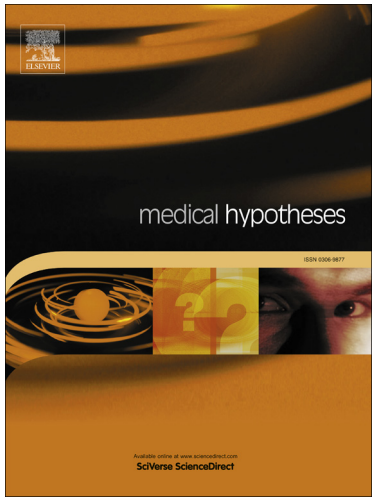

Well begun is half done: rubella virus perturbs autophagy signalling, thereby facilitating the construction of viral replication compartments

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TITLE: Well begun is half done: rubella virus perturbs autophagy signalling, thereby facilitating the construction of viral replication compartments

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ABSTRACT

The rubella virus is the causative agent of postnatal German measles and the congenital rubella syndrome. The majority of the rubella virus replication complexes originate from the endomembrane system. The rubella virus perturbs the signalling pathways regulating the formation of autophagic membranes in the infected cells, including the Ras/Raf/MEK/ERK and PI3K/Akt pathways. It is widely accepted that these pathways inhibit autophagy. In contrast, the class III PI3K enzymes are essential for autophagy initiation. By manipulating the Ras/Raf/MEK/ERK, class I PI3K/Akt and class III PI3K axes of signal transduction, the rubella virus may differentially regulate the autophagic cascade, with consequent stimulation of the initiation and strong suppression of the later phases. Dysregulation of autophagy by this virus can have a significant impact on the construction of replication compartments by regulating membrane trafficking. We hypothesize that the rubella virus perturbs the autophagic process in order to prevent the degradation of the virus progeny, and to ensure its replication by hijacking omegasomes for the construction of the replication complexes. The virus is therefore able to utilize an antiviral mechanism to its own advantage. Therapeutic modalities targeting the autophagic process may help to ameliorate the serious consequences of the congenital rubella syndrome.

INTRODUCTION

The rubella virus (RV), a member of the *Togaviridae* family, is the causative agent of postnatal German measles and the congenital rubella syndrome (CRS) [1]. The slow multiplication of the virus is related to the intracellular endomembrane system. The RV enters the host cells by endocytosis. As early endosomes mature into late endosomes, acidification triggers the fusion of the viral envelope to endosomal membrane and uncoating [1]. Following decapsidation, the establishment of RV replication complexes is initiated in the cytoplasm [2]. The replication compartments have a complex structure, the central elements of which are modified endosomes, called virus factories. The RV then triggers the formation of bends and intrusions on the endosomal membrane, leading to the formation of spherules that provide a protective environment for viral RNA replication (Fig. 1) [2]. These modified endosomes subsequently recruit small endoplasmic reticulum (ER) fragments, and mitochondria, collectively termed replication complexes (Fig. 1) [2].

Although the structure of the RV replication complexes has already been fully clarified, the biogenesis of these organelle compartments remains unclear. Strikingly, the majority of the RV replication complexes originate from the endomembrane system [2]. Several steps of the RV replication cycle are closely associated with membrane-bound organelles, and this virus therefore has the possibility to alter the structure and function of the intracellular membranes in either a direct or an indirect manner. During its multiplication, the RV inhibits autophagy, triggers apoptosis, and causes a slowdown in cell cycle progression [3,4]. Among the RV-mediated cellular effects, the dysregulation of autophagy may have the most significant impact on the construction of replication compartments via the regulation of membrane trafficking.

Autophagy is an evolutionarily conserved, cell-autonomous catabolic and defence mechanism, through which eukaryotic cells are able to recycle the long-lived cytosolic components and to degrade intracellular pathogens [5,6]. The autophagic capture and delivery of microorganisms to the lysosomes serves as an important cellular defence mechanism, xenophagy [7]. Autophagy-inducing signals are mainly sensed and coordinated by the mammalian target of rapamycin complex 1 (mTORC1) [5]. mTORC1 inactivation leads to activation of the core autophagy machinery, the most important morphological characteristic of which is the formation of double membrane-vesicles, autophagosomes (Fig. 2) [5]. The development of the autophagosomes starts at the ER in the form of small membrane protrusions, omegasomes [8]. Following the induction of autophagy, inhibition of the mTOR leads to activation of the ULK kinase complex (UKC) at the ER membrane. The UKC then recruits the class III phosphatidylinositol 3-kinase (PI3K) complex, promoting formation of the omegasome from which the isolation membrane appears to be generated [5,8]. The isolation membrane provides a platform for two ubiquitin-like conjugation systems involved in the elongation step of the autophagic process. The first leads to the formation of a supramolecular complex composed of Atg5/Atg12/Atg16L, while the second generates microtubule-associated protein 1 light chain 3-I (LC3-I). The Atg5/Atg12/Atg16L complex elicits the covalent conjugation of phosphatidylethanolamine to LC3-I, giving rise to the formation of LC3-II. LC3-II translocates to the isolation membrane, and facilitates its elongation. The cargo designated for degradation (e.g. a virus particle) is bound by adaptor molecules to the LC3B-II on the inner wall of the autophagosomes [5]. Autophagosomes mature into autolysosomes by sequential fusion with lysosomes in the endocytic pathway. In the developing autolysosomes, the content is degraded by hydrolases and then recycled (Fig. 2) [5]. It has been clearly demonstrated that many RNA viruses have the ability to counteract

or exploit the autophagic process in order to alter the cellular physiology and metabolism for the benefit of their own replication [9].

HYPOTHESIS

We hypothesize that the RV-mediated perturbation of autophagy may facilitate the construction of viral replication compartments.

Basis for the hypothesis

The RV is known to inhibit the autophagic flux, suppress the level of Atg12–Atg5 conjugation, impair LC3B lipidation, decrease both the average number and the size of autophagosomes per cell and impede the formation of acidic vesicular organelles [3]. Together, these data indicate that the RV perturbs autophagy without causing a complete block.

The RV is also endowed with the ability to manipulate cellular signal transduction pathways, which allows it to gain control over a number of physiological processes and to fine-tune various elements of the intracellular environment so as to support certain steps in its replication cycle [10]. The RV has been shown to activate the Ras/Raf/MEK/ERK and PI3K/Akt pathways [10]. These signalling branches elicit diverse cellular effects, and regulate both membrane trafficking and autophagy. It is widely accepted that the Ras/Raf/MEK/ERK and class I PI3K/Akt pathways inhibit autophagy by activating mTORC1 [11]. In contrast, class III PI3K displays strikingly different effects, as it is essential for autophagy initiation and omegasome formation [8]. By manipulating the Ras/Raf/MEK/ERK, class I PI3K/Akt and class III PI3K axes of signal transduction, the RV may differentially regulate the

autophagic cascade, with consequent stimulation of the initiation and strong suppression of the later phases (Fig. 3).

Together, RV infection perturbs the signalling pathways regulating the formation of autophagic membranes [2,10]. Thus, it is conceivable that the dysregulated autophagic process may be involved in the construction of the replication compartment composed of altered endomembranes.

EVALUATION OF THE HYPOTHESIS

In order to determine whether perturbed autophagy contributes to the biogenesis of RV replication complexes, it is essential to demonstrate that modulation of the class III PI3K activity affects the formation of omegasomes in RV-infected cells.

To verify that class III PI3K is activated by RV infection, we plan to infect SIRC corneal cells with the To336 strain of RV at a multiplicity of infection of 5. The expression level of class III PI3K will be determined by Western blot analysis. The filters will be developed by using a chemiluminescence detection system. The autoradiographs will be scanned, and the relative band intensities will be quantified. The subcellular distribution of class III PI3K will be determined through the use of confocal microscopy. The fluorescence intensity of class III PI3K will be measured in RV-infected cells by using the line scan analysis function of the Image J software and compared with that in mock-infected control cultures.

To decrease the expression level of class III PI3K, two approaches can be applied. The expression of class III PI3K will be suppressed at the RNA level by using siRNA technology in the first approach. Chemically synthetized siRNA targeting class III PI3K and non-silencing control siRNA will be obtained. Transient transfections will be performed by using the siPORT amine reagent, with a final siRNA concentration of 50 nM. The effect of

silencing will be analysed at the protein level by Western blot assay. The transfected SIRC corneal cells will then be infected, and the generation of omegasomes and the assembly of viral factories will be visualized by using transmission electron microscopy. SAR405, a highly potent and selective chemical inhibitor of class III PI3K will be applied in the second approach [12]. SIRC cells will be treated with SAR405 at a final concentration of 1 μM. The SAR405-treated SIRC cultures will then be infected, and the formation of omegasomes will be evaluated by means of transmission electron microscopy.

CONSEQUENCES OF THE HYPOTHESIS AND DISCUSSION

The establishment of viral factories that originate from membranous intracellular organelles is essential for the multiplication of RNA viruses that have dsRNA replicative intermediates in their life cycle [13]. Many of these viruses have been shown to activate the class I PI3K/Akt signalling pathway, and to manipulate the autophagic process for their own benefit [14,15]. These cellular effects of RNA viruses offer versatile support for viral multiplication by providing a protective environment for replication of the genomic RNA, by hindering the activation of dsRNA-dependent antiviral defence mechanisms, and by preventing the autophagic degradation of progeny virions. Some RNA viruses, such as coxsackievirus B3 and perhaps the RV, may even usurp autophagic membranes to construct their replication complexes, while they also block subsequent phases of autophagy, and thereby escape from degradation (Fig. 4) [16]. Precise recognition of the unique molecular strategies involved in the biogenesis of membranous viral factories is of pivotal importance for a better understanding of infections and the identification of new therapeutic targets.

The most serious complication of RV infection is the CRS [1,2]. Patients born with the CRS exhibit ocular symptoms (cataract, congenital glaucoma and retinitis), congenital heart defects (patent ductus arteriosus and pulmonary artery stenosis), a sensorineural hearing loss

and pigmentary neuropathy [1]. The exact molecular mechanism of the teratogenic effect caused by the RV is not yet fully known. However, it is reasonable to infer that the apoptosis induction and autophagy inhibition elicited by the virus play pivotal roles in the pathogenetic mechanism of the CRS. The function of apoptosis in the foetal development is widely accepted [17]. The implication of autophagy likewise appears to be important in certain phases of ontogeny, especially in the differentiation of blastocytes and during organogenesis [18]. Recent studies have clearly revealed that autophagy plays an essential and conserved role in cardiac and neural morphogenesis during vertebrate development [19,20]. In light of these findings, we suggest that RV-mediated autophagy plays a part in the pathogenesis of the organ malformations observed in CRS patients.

Another important stage of ontogeny in which autophagy is involved is the first few days of extrauterine life. Until the evolvement of regular breastfeeding activity, autophagy ensures the energy for survival [21]. A perturbation of autophagy can therefore irreversibly harm the neonate at this time. Although the only acceptable way to prevent the CRS at present is vaccination, there may be other options to improve the quality of life of CRS patients. Parenteral feeding might moderate the starvation that arises as a severe consequence of the decreased autophagic activity in the first few days of life by normalizing the serum amino acid levels and reducing the rate of tissue acidosis, thereby decreasing the perinatal mortality in CRS patients by alleviating the degree of organ damage.

Chemical inducers of autophagy have proved effective in the therapy of various neurodegenerative diseases [22]. CRS patients often display neurological symptoms (mental retardation, spastic diplegia, conduct disorders and neuropsychiatric problems) that may also be late-onset sequelae [2]. The RV persists and replicates in the central nervous system for years after birth, and causes neural damage [1]. It is therefore reasonable to infer that the

restoration of autophagic activity may improve the quality of life by preventing the neurological defects that arise after the birth of CRS patients.

Overall, our hypothesis suggests that RV perturbs the autophagic process to prevent degradation of the virus progeny, and to ensure its replication via the hijacking of omegasomes for the construction of the replication complexes (Fig. 4). The virus is thereby able to utilize an antiviral mechanism to its own advantage. Therapeutic modalities targeting the autophagic process may help to ameliorate the serious consequences of CRS.

CONFLICT OF INTEREST STATEMENT

The authors do not have any financial or personal conflict of interest to declare.

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FIGURE LEGENDS

Figure 1: Structure of the RV replication complex. The replication complex of the RV comprises a modified endosome which gathers ER fragments and mitochondria. The modified endosome contains spherules protruding into the lumen. The spherules are connected to the vacuolar membrane via thin membranous necks, and are the sites of viral RNA synthesis.

Figure 2: Mechanism of autophagy. Autophagy inducers trigger the inactivation of mTORC1. Following the induction of autophagy, inhibition of mTORC1 leads to activation of the UKC, which in turn undergoes translocation to a certain domain of the ER. Atg14 directs class III PI3K, where it is phosphorylated by the UKC. Phosphorylation activates the class III PI3K, and induces the formation of phosphatidylinositol 3-phosphate (PI3P) at the ER membrane. This event drives the recruitment of PI3P effectors, such as DFCP1 and WIPI, that enable the omegasome to emerge. Omegasomes are sources of the isolation membranes, which incorporate LC3B-II produced by two conjugation systems. The localization of the conjugation machinery on the isolation membrane requires UKC activity. The formation of LC3B-II ensures the elongation of the isolation membrane and the cargo connection to the double membrane through various adaptor molecules. Evolving autophagosomes fuse with lysosomes to generate autolysosomes. Hydrolases located in the autolysosomes degrade the cargo.

Figure 3: The potential effects of the RV on signalling pathways that regulate autophagosome formation. The RV activates class I PI3K-Akt, the Ras-Raf-MEK-ERK pathways, and potentially class III PI3K. Class I PI3K-Akt and the Ras-Raf-MEK-ERK branches activate mTORC1. Active mTORC1 binds UKC and inhibits its activity. Without

active UKC, the elongation of the autophagosomes does not occur, and the late phases of autophagy are therefore inhibited. In contrast, activation of class III PI3K is necessary for the generation of omegasomes at the ER membrane. Omegasomes function as membrane platforms for autophagosome formation.

Figure 4: Biological consequences of RV infection on the regulation of autophagosome formation. In the proposed model, the increased omegasome formation induced by class III PI3K activation, and the decreased elongation of the isolation membrane caused by the activation of class I PI3K-Akt and the Ras-Raf-MEK-ERK pathways, take place simultaneously in the RV-infected cells. This sophisticated manner of autophagy dysregulation allows RV to hijack ER membrane fragments in order to construct viral replication complexes and to avoid autophagic degradation.

Figure 1

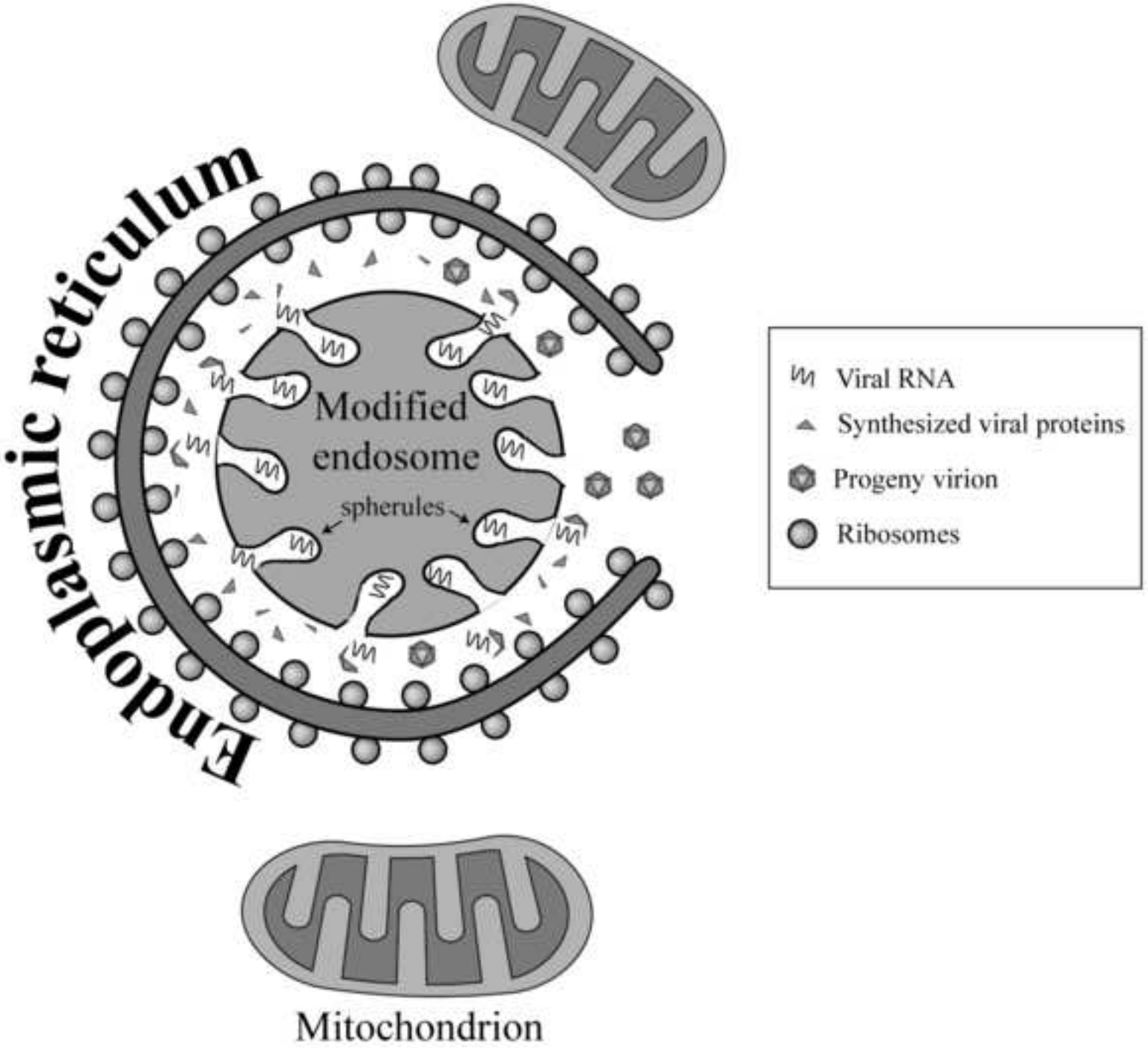

Figure 2

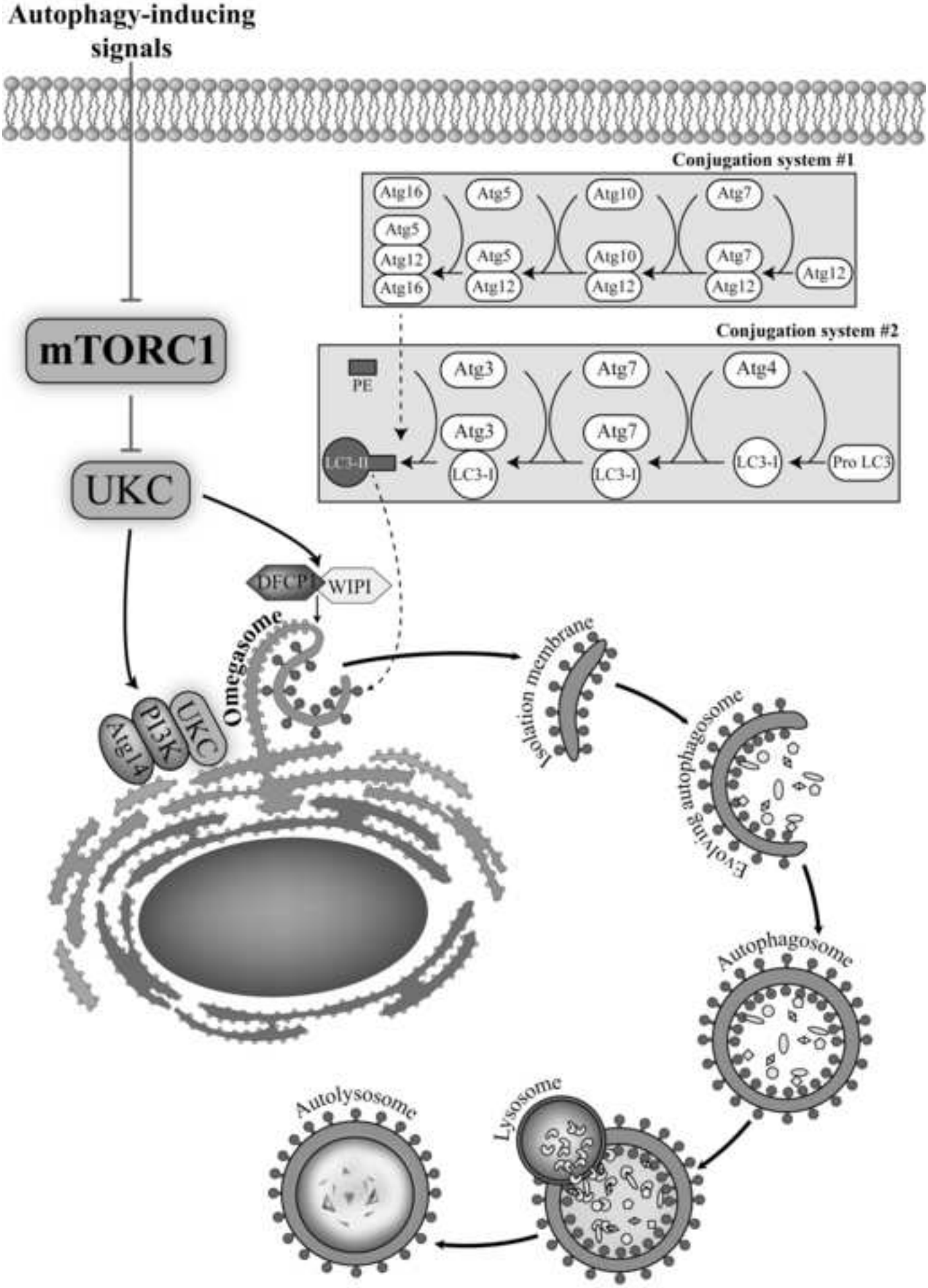

Figure 3

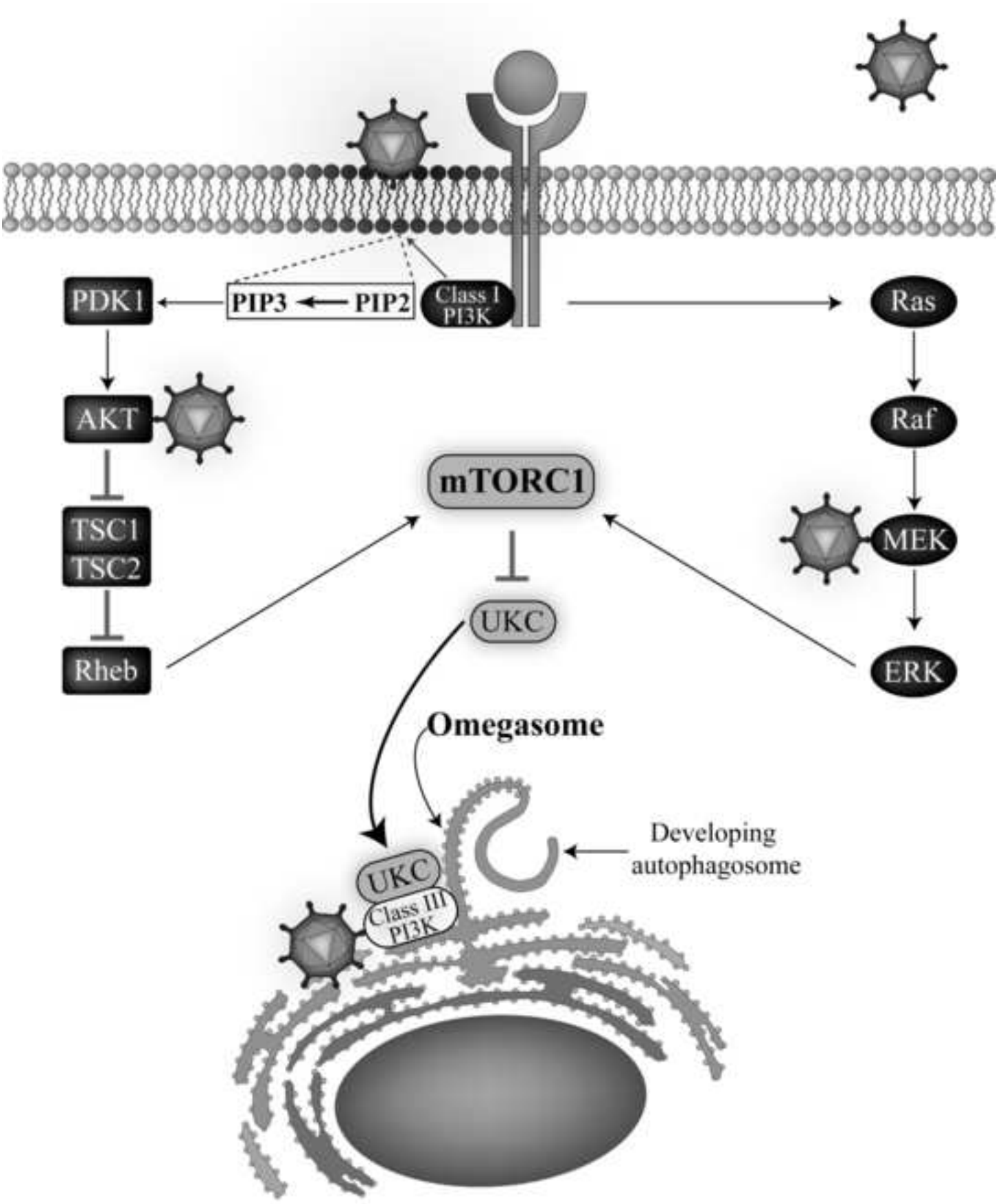

Figure 4