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Research Paper

Cellular Autophagy Machinery is not Required for Vaccinia Virus Replication and Maturation

Haiyan Zhang[1,2]
Claude E. Monken[2,3]
Yong Zhang[1,2]
John Lenard[2,4]
Noboru Mizushima[5]
Edmund C. Lattime[2,3]
Shengkan Jin[1,2,*]

[1]Department of Pharmacology; [2]Cancer Institute of New Jersey; [3]Department of Surgery and Molecular Genetics, Microbiology and Immunology; [4]Department of Physiology and Biophysics; University of Medicine and Dentistry of New Jersey; Robert Wood Johnson Medical School; Piscataway, New Jersey USA

[5]Department of Bioregulation and Metabolism; the Tokyo Metropolitan Institute of Medical Science; Tokyo, Japan

*Correspondence to: Shengkan Jin; Department of Pharmacology; UMDNJ-Robert Wood Johnson Medical School; 675 Hoes Lane; Piscataway, New Jersey 08854 USA; Tel.: 732.235.4329; Fax: 732.235.4073; Email: jinsh@umdnj.edu

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KEY WORDS

autophagy, Vaccinia virus, Beclin1, Atg5, electron microscopy, plaque assay

ABBREVIATIONS

Atg	autophagy-related gene
IV	immature virions
MEF	mouse embryonic fibroblast
ES cell	embryonic stem cell
AV	autophagic vacuole
MOI	multiplicity of infection
pfu	plaque formation unit

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ABSTRACT

The origin of the primary membrane of the vaccinia virus, a double-membrane structure that surrounds the immature virions (IV), is not fully understood. Here we investigated whether the primary membrane originates from the autophagic membrane. Morphologic studies by electron microscopy (EM) showed no apparent difference in viral maturation in the autophagy-deficient cell lines, the *atg5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and the *beclin1*$^{-/-}$ embryonic stem (ES) cells, compared to their isogenic wild-type counterparts. Moreover, viral growth curves demonstrated that vaccinia viruses replicate and mature in the autophagy-deficient cell lines as efficiently as they do in their isogenic wild type counterpart cells. This study indicates that the cellular autophagy machinery is not required for the life-cycle of vaccinia virus, suggesting that the primary vaccinia viral membrane does not originate from the autophagic membrane.

INTRODUCTION

Vaccinia virus is the best-studied member of the family *Poxviridae*, which includes the small pox virus. Poxviruses are large DNA viruses and they differ from most other DNA viruses in that their genome replication and virion assembly occur entirely in the cytoplasm. Vaccinia virus matures through a series of intermediate structures that have been visualized by electron microscopy.[1] The viral morphogenesis begins with the formation of a double-layered, crescent-shaped membrane that engulfs dense viroplasm to form spherical immature virions (IV). A series of subsequent events transform the spherical immature virions into the infectious intracellular mature virions (IMV), which further mature to form extracellular cell-associated virions (CEV) that attach to the cell surface as well as free extracellular enveloped virions in the medium.[2,3]

The origin of the first membrane structure, the double-layered membrane surrounding spherical immature virions (also referred to as the primary membrane, see ref. 4), has been intensively investigated.[4] More than 25 years ago, Dales and coworkers[5] considered a de novo origin of this membrane, whereas others[6-9] suggested that it was derived from the ER-Golgi intermediate compartment. However, physical connections between viral crescents and cellular membranes were not found.[10] In addition, recent data demonstrated that cargo transport from the endoplasmic reticulum to the endoplasmic reticulum-Golgi intermediate compartment is not essential for the formation of intracellular mature virions.[11] Therefore, the origin of the primary membrane is still not fully understood.

Macroautophagy, which will be referred to as autophagy in this paper, is a membrane trafficking process that leads to lysosomal degradation.[12] The hallmark of autophagy is the emergence of double-membrane autophagic vacuoles (AV). The autophagic vacuole originates from a crescent-shaped double-layered membrane structure, which engulfs a portion of the cytosol and forms a closed vesicle, the autophagic vacuole. These vesicles then dock and fuse to lysosomes, where the cargos are further degraded. The molecular components that are essential for autophagy were first identified in yeast by genetic screening[13] (named *APG*—autophagy—genes, which were later changed to *ATG* for autophagy-related genes[14]). The functions of those autophagy genes were subsequently characterized.[15]

Most of the yeast autophagy gene product orthologs have been identified in other organisms ranging from worms to mammals,[16] and the autophagy machinery is highly conserved in eukaryotes. Autophagy plays important roles in various physiological processes,[17] including neonatal survival,[18] development and aging (ref. 17 and references wherein), and tumor suppression.[19,20] In addition, autophagy is a part of the innate immune response.

Upon viral or bacterial infection, autophagy is activated. The autophagic vacuoles sequester the microbes and deliver them to the lysosome for degradation.[21,22] This is an important cellular defense mechanism against the infection of Sindbis virus,[23] tobacco mosaic virus,[24] as well as Mycobacterium tuberculosis and group A Streptococcus in various host organisms.[25,26] Moreover, the autophagy-mediated lysosomal degradation of viruses contributes to the MHC-II antigen presentation.[27] Apparently, some microbes have evolved to evade autophagy. For example, Shigella secrets a protein called IcsB to escape the entrapment inside autophagic vacuoles.[28] Interestingly, some viruses even take advantage of the autophagy system. For example, the double membrane structures associated with the replication complex of the poliovirus[29,30] and coronavirus mouse hepatitis virus[31] originate from autophagic membranes and the cellular autophagy machinery is required for the efficient replication and maturation of these viruses.

The morphological resemblance between the maturation of the autophagic vacuole and the formation of vaccinia immature virons, as well as the involvement of the autophagic membrane in the replication and maturation of a number of viruses, prompted us to investigate whether the vaccinia primary membrane originates from autophagic membrane and whether the cellular autophagy machinery is required for the replication and maturation of the vaccinia virus. In the current study, morphological analysis by electron microscopy showed no apparent difference in terms of vaccinia viral maturation between the autophagy deficient cells, the *atg5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) or the *beclin1*$^{-/-}$ mouse embryonic stem (ES) cells, and their wild type counterpart cells. Further viral growth analysis indicated that the kinetics of viral replication and infection in the autophagy deficient cells is similar to that in the wild type counterparts. Our results demonstrated that the cellular autophagy machinery is not required for the vaccinia virus replication and maturation, which suggests that the membrane of the vaccinia virus does not originate from autophagic membrane.

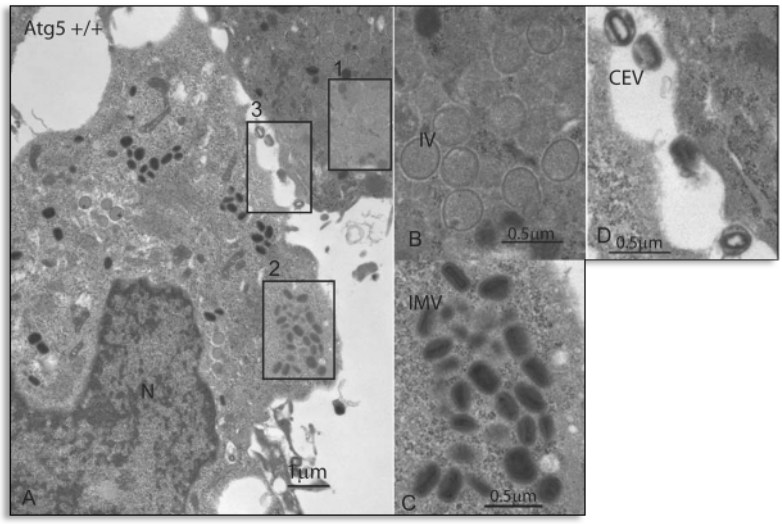

Figure 1. The morphogenesis of vaccinia virus in atg5$^{+/+}$ MEFs. Representative electron microscopy pictures of atg5$^{+/+}$ MEFs 24 h after vaccinia virus infection (MOI=2.0). Three morphologically distinct forms of virons, the immature viron (IV), the intracellular mature viron (IMV), and the exracellular cell-associated viron (CEV) are shown in Frame 1, 2, and 3, respectively in (A), and enlarged to see the detail in (B), (C), and (D), respectively. Scale bars were included in each panel; N, nucleus.

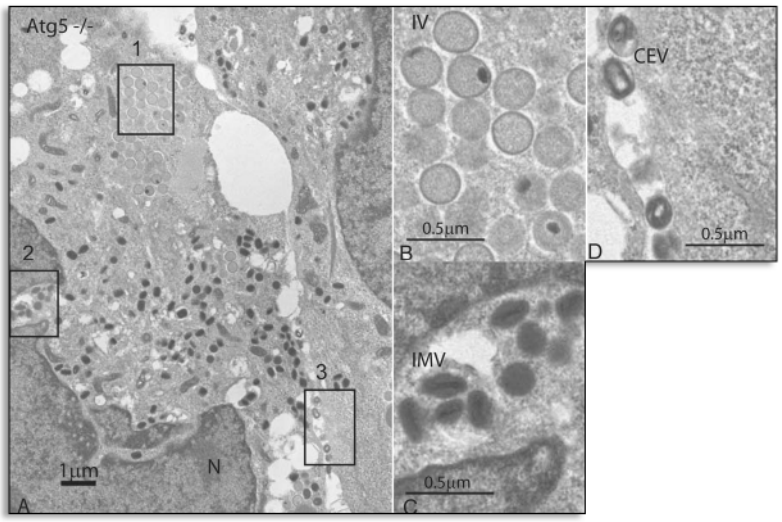

Figure 2. The morphogenesis of vaccinia virus in atg5$^{-/-}$ MEFs. Representative electron microscopy pictures of atg5$^{-/-}$ MEFs 24 h after vaccinia virus infection (MOI=2.0). Three morphologically distinct forms of virons, the immature viron (IV), the intracellular mature viron (IMV), and the exracellular cell-associated viron (CEV) are shown in Frame 1, 2, and 3, respectively in (A), and enlarged to see the detail in (B), (C), and (D), respectively. Scale bars were included in each panel; N, nucleus.

MATERIALS AND METHODS

Viruses and cell lines. The vaccinia virus is a modified version of a wild type virus (New York City Board of Health strain of vaccinia (Centers for Disease Control, Atlanta, GA)), in which a β-galactosidase gene is inserted into the viral thymidine kinase gene for easy plaque analysis.[32] *Atg5*$^{+/+}$ and *atg5*$^{-/-}$ murine embryonic fibroblast (MEF) cells were generated from C57BL/6 wild type or C57BL/6 *Atg5* knockout mice,[18] transformed with SV-40 T antigen as described previously.[18] They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. *Beclin*$^{-/-}$ embryonic stem (ES) cells were generated as described.[20] The wild type or the *beclin1*$^{-/-}$ ES cells were cultured in gelatin coated plates in complete ES medium: DMEM supplemented with 15% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 mM MEM nonessential amino acids, 1X nucleosides, and 1X 2-mercaptoethanol (diluted from 100X stock, Specialty Media, NJ) and 1000 U/ml LIFC leukemia inhibitory factor, (Chemicon, Inc., CA).

Electron microscopy. Electron microscopy was performed by the University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School electron microscopy core facility. Cells were inoculated with the vaccinia virus at multiplicity of infect (MOI) of 2 pfu/cell for 1 h, then normal medium was added. Twenty four h after viral infection, cells were then fixed and embedded. Thin sections (90nm) were cut on a Reichert Ultracut E microtome. Sections were examined at 80 kV with a JEOL 1200EX transmission electron microscope.

Viral growth analysis. Cells were seeded in 6-well plates at 1.5 x 10^6 cells/well 8 h prior to infection and inoculated with the vaccinia virus for 2 h at MOI = 0.1 pfu/cell for multiple-step growth curve (complete growth curve) or at MOI = 5 pfu/cell for one-step curve growth. Medium was then removed and replaced with normal medium. Cells were collected with 2 ml

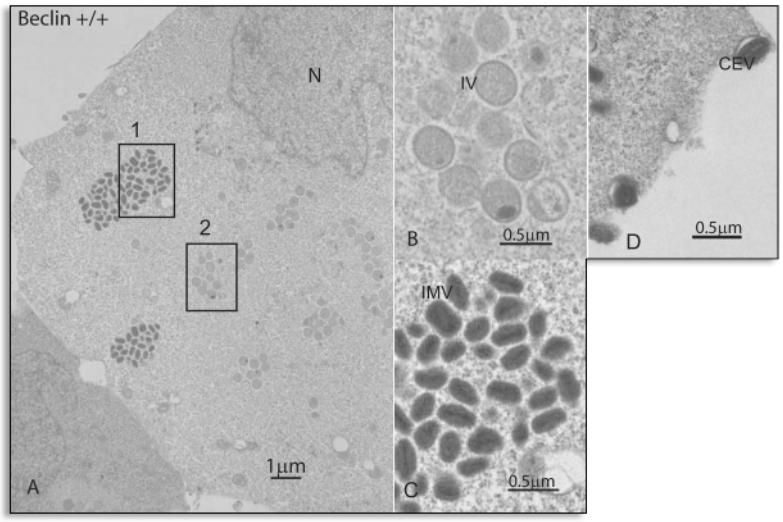

Figure 3. The morphogenesis of vaccinia virus in beclin1$^{+/+}$ ES cells. Representative electron microscopy pictures of beclin1$^{+/+}$ ES cells 24 h after vaccinia virus infection (MOI=2.0). The immature viron (IV) and the intracellular mature viron (IMV) are shown in Frame 1 and 2, respectively in (A), and enlarged to see the detail in (B) and (C), respectively. The exracellular cell-associated viron (CEV), which is from a different cell, is shown in (D). Scale bars were included in each panel; N, nucleus.

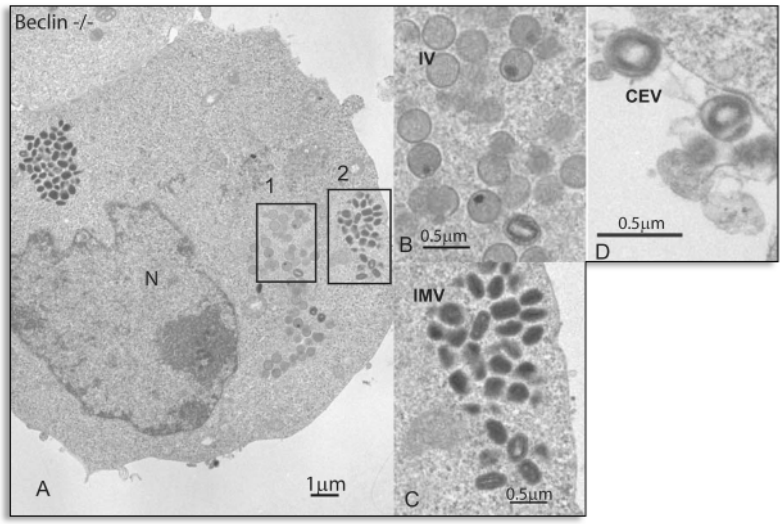

Figure 4. The morphogenesis of vaccinia virus in beclin1$^{-/-}$ ES cells. Representative electron microscopy pictures of beclin1$^{-/-}$ ES cells 24 h after vaccinia virus infection (MOI=2.0). The immature viron (IV) and the intracellular mature viron (IMV) are shown in Frame 1 and 2, respectively in (A), and enlarged to see the detail in (B) and (C), respectively. The exracellular cell-associated viron (CEV), which is from a different cell, is shown in (D). Scale bars were included in each panel; N, nucleus.

PBS/BSA every 4 h consecutively for a total of 48 h and viruses were released by three rounds of freezing and thawing cells followed by sonication. The viral titer was determined by a plaque assay. Briefly, the viruses were diluted in series. One-hundred-microliter viral samples were added into a well of 6-well plates coated with 143B cells, a human osteosarcoma line.[32] The medium was removed the next day, 1 ml 2X DMEM with 1ml 1.8% agarose was added into each well, and cells were further incubated for two days. One-mililiter β-galactosidase substrate-X-Gal (300 μg/ml) was added, which produces a dark blue precipitate on enzymatic hydrolysis, and the colored plaques were counted. Each dilution of a sample was measured in triplicate.

RESULTS

Vaccinia virus replicates and matures in both wild type and autophagy-deficient cells. To investigate a possible role of cellular autophagy machinery in the vaccinia virus life cycle, we inoculated autophagy-deficient cells as well as their isogenic wild type cells with vaccinia virus. Two different types of autophagy-deficiency cells were chosen, one was the transformed *atg5*$^{-/-}$ MEFs and the other was the *beclin1*$^{-/-}$ ES cells. The ATG5 protein is conjugated to ATG12, an ubiquitin-like protein, upon autophagy activation.[33] The ATG12—ATG5 conjugation is required for the activation of the ATG8 ubiquitin-like protein conjugation system,[34,35] which is essential for AV formation.[36,37] In the *atg5*$^{-/-}$ cells, the ATG8 (MAP-LC3 in mammals) conjugation is impaired and no autophagic vacuoles can be formed.[18,34] The Beclin 1 protein is the mammalian ortholog of the yeast ATG6, which forms a complex with VPS34. Deletion of the *beclin 1* gene also severely impairs autophagic vacuole formation.[20] The viral life cycle (maturation) in these cells was then examined with electron microscopy. As a control, Figure 1A shows a typical EM picture of *atg5*$^{+/+}$ MEFs infected with the vaccinia virus. The three morphological distinct viron forms representing different stages during viral morphogenesis can be readily detected, which include the spherical immature virons (Fig. 1A, frame 1, and enlarged in Fig. 1B), the intracellular mature viron (Fig. 1A, frame 2, and enlarged in Fig. 1C), and the extracellular cell-associated virions (Fig. 1A, frame 3, and enlarged in Fig. 1D). Similarly, in typical vaccinia virus infected *atg5*$^{-/-}$ MEFs, as shown in Figure 2, all three morphologically-distinct viron forms are present (Fig. 2A, frames 1–3 and Fig. 2B–D). Moreover, there is no apparent difference in terms of the cellular distribution of virons and viral load in an individual cell between the *atg5*$^{+/+}$ and the *atg5*$^{-/-}$ MEFs. The viral maturation was also analyzed in the *beclin1*$^{+/+}$ and the *beclin1*$^{-/-}$ ES cells. Again, all three morphologically distinct forms of virons were detected in both the *beclin1*$^{+/+}$ ES cells (Fig. 3) and the *beclin1*$^{-/-}$ ES cells (Fig. 4). No apparent differences in terms of the cellular distribution of virons and the viral load were detected between the *beclin1*$^{+/+}$ cells and the *beclin1*$^{-/-}$ cells. Clearly, the morphogenesis of vaccinia virus is normal in both types of autophagy deficient cells. Thus, cellular autophagy is not essential for the maturation of vaccinia virus in these cells.

Viral production kinetics is similar in wild type and the autophagy-deficient cells. To further address the possibility that vaccinia viral replication and maturation are quantitatively affected by autophagy, the viral growth was measured in the autophagy deficient cells, the *atg5*$^{-/-}$ MEFs and the *beclin1*$^{-/-}$ ES cells, as well as their isogenic wild type counterparts. As shown in Figure 5A, a viral growth curve at a low multiplicity of infection (a multiple-step growth curve using an MOI of 0.1) was obtained in the *atg5*$^{+/+}$ MEFs as well as in the *atg5*$^{-/-}$ MEFs. The two growth curves show no significant difference. Moreover, single-step growth curves using an MOI of 5 were also measured in the pair of MEFs (Fig. 5B) and these two curves are almost identical. Similarly, the multi-step viral growth curves (MOI = 0.1) as well as the single-step growth curves (MOI = 5) were determined in the *beclin1*$^{+/+}$ and *beclin1*$^{-/-}$ ES cells, as shown in Figure 5C and D respectively. The ES cells are more sensitive to the vaccinia virus infection and an increasing number of ES cells died over the time after infection. Consequently, the viral yield was lower in the ES cells compared to that in the MEFs. Nevertheless, the viral production kinetics in the *beclin1*$^{+/+}$ ES cells is the same as that in the *beclin1*$^{-/-}$ ES cells. Taken together, these results demonstrate that vaccinia viruses are able to replicate and mature into infectious virons in the autophagy deficient cells

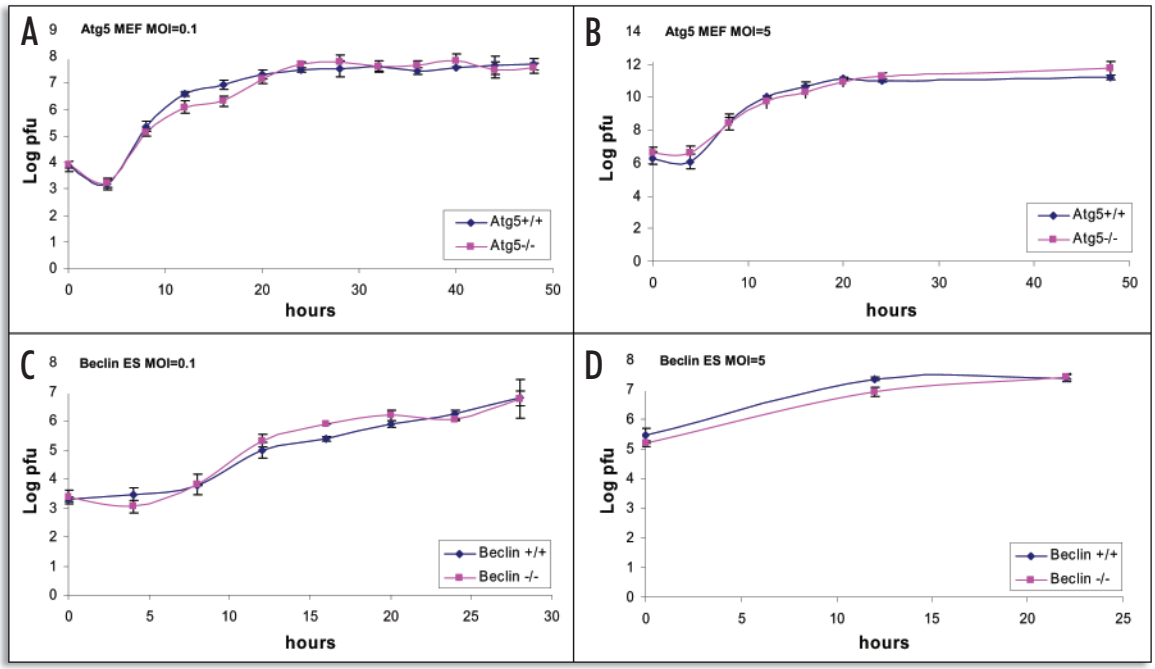

Figure 5. Vaccinia viral growth curves in wild type and the autophagy deficient cells. (A) Multi-step viral growth curves (MOI = 0.1) in the *atg5*$^{+/+}$ and *atg5*$^{-/-}$ MEFs. (B) Single-step viral growth curves (MOI = 5) in the *atg5*$^{+/+}$ and *atg5*$^{-/-}$ MEFs. (C) Multi-step viral growth curves (MOI = 0.1) in the *beclin1*$^{+/+}$ and *beclin1*$^{-/-}$ ES cells. (D) Single-step viral growth curves (MOI = 5) in the *beclin1*$^{+/+}$ and *beclin1*$^{-/-}$ ES cells. Cells were infected with vaccinia viruses at different multiplicity of infection (MOI), as indicated. The surviving cells were harvested at different time points and the viral production was analyzed by plaque assay.

and the kinetics of production of the infectious virons in the autophagy deficient cells is similar to that in the isogenic wild type cells.

DISCUSSION

The origin of the primary membrane of the poxviruses has been a mystery, and so is the origin of the autophagic membrane. The morphologic resemblance between the maturation of vaccinia IV and the maturation of autophagic vacuoles led us ask whether the virus has hijacked the cellular autophagy machinery to make their primary membrane. We tested this hypothesis in two well-characterized genetic model systems, the *atg5* knockout MEFs and the *beclin1* knockout ES cells, by asking whether the cellular autophagy machinery is essential for the life cycle of vaccinia virus. Surprisingly, both morphologic studies and viral growth assays indicate that vaccinia virus replicated and matured indistinguishably in the autophagy-deficient cells, both the MEFs and the ES cells, as compared to in their isogenic wild type counterpart cells. The ATG5 and Beclin 1 proteins are involved in processes that are independently required for autophagy[15,17] and they have no apparent homologs in the vaccinia viral genome, which encodes around 200 proteins. Our results strongly argue that the cellular autophagy machinery is not involved in the vaccinia life cycle; hence the primary vaccinia viral membrane does not originate from autophagic membrane. However, we cannot rule out the possibility that the viral genome might encode some functional homologs of both ATG5 and Beclin1 proteins.

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