

Endosomal Phosphatidylinositol-3-phosphate is Essential for the Final Assembly of CMV Virions

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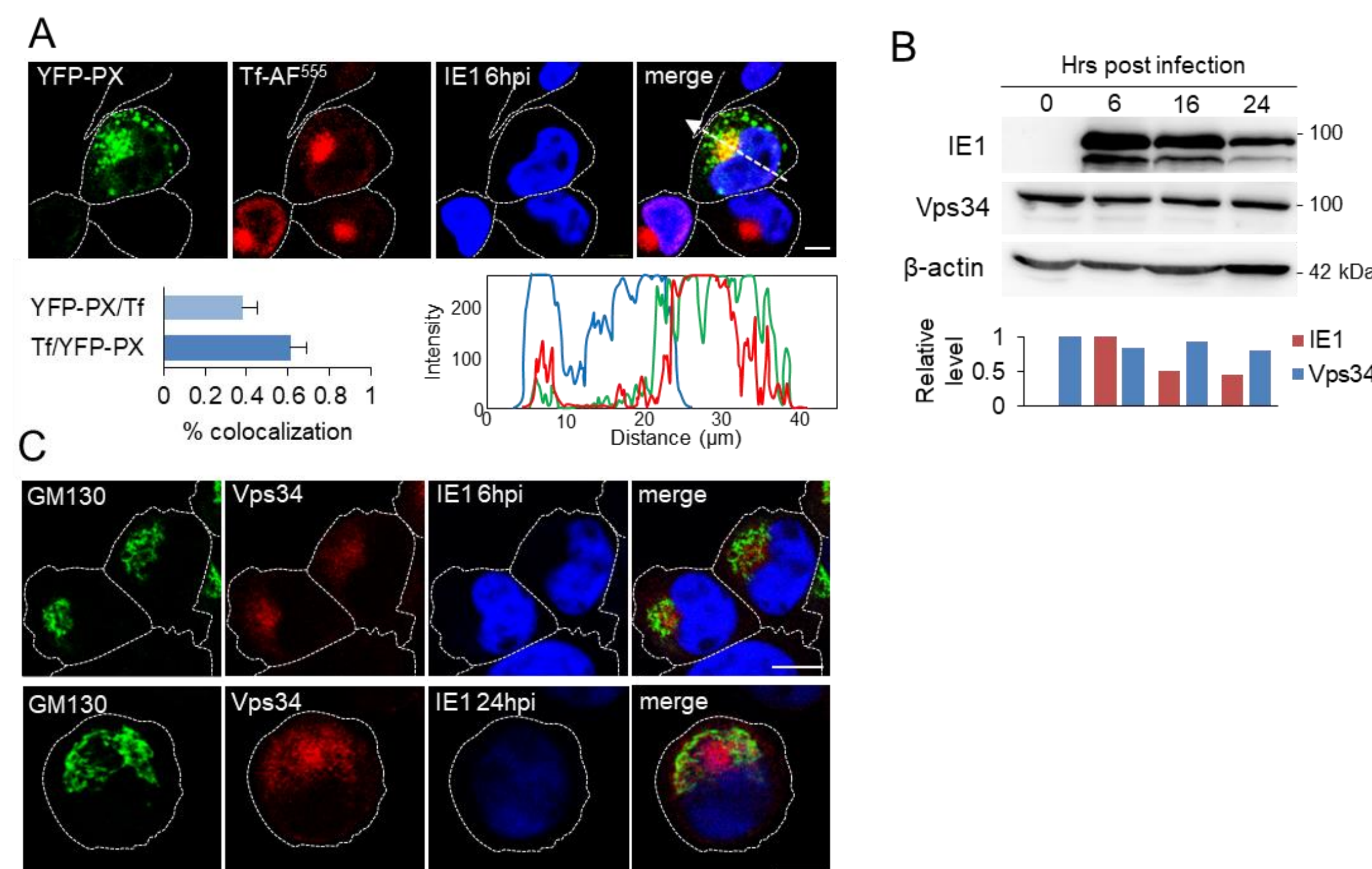
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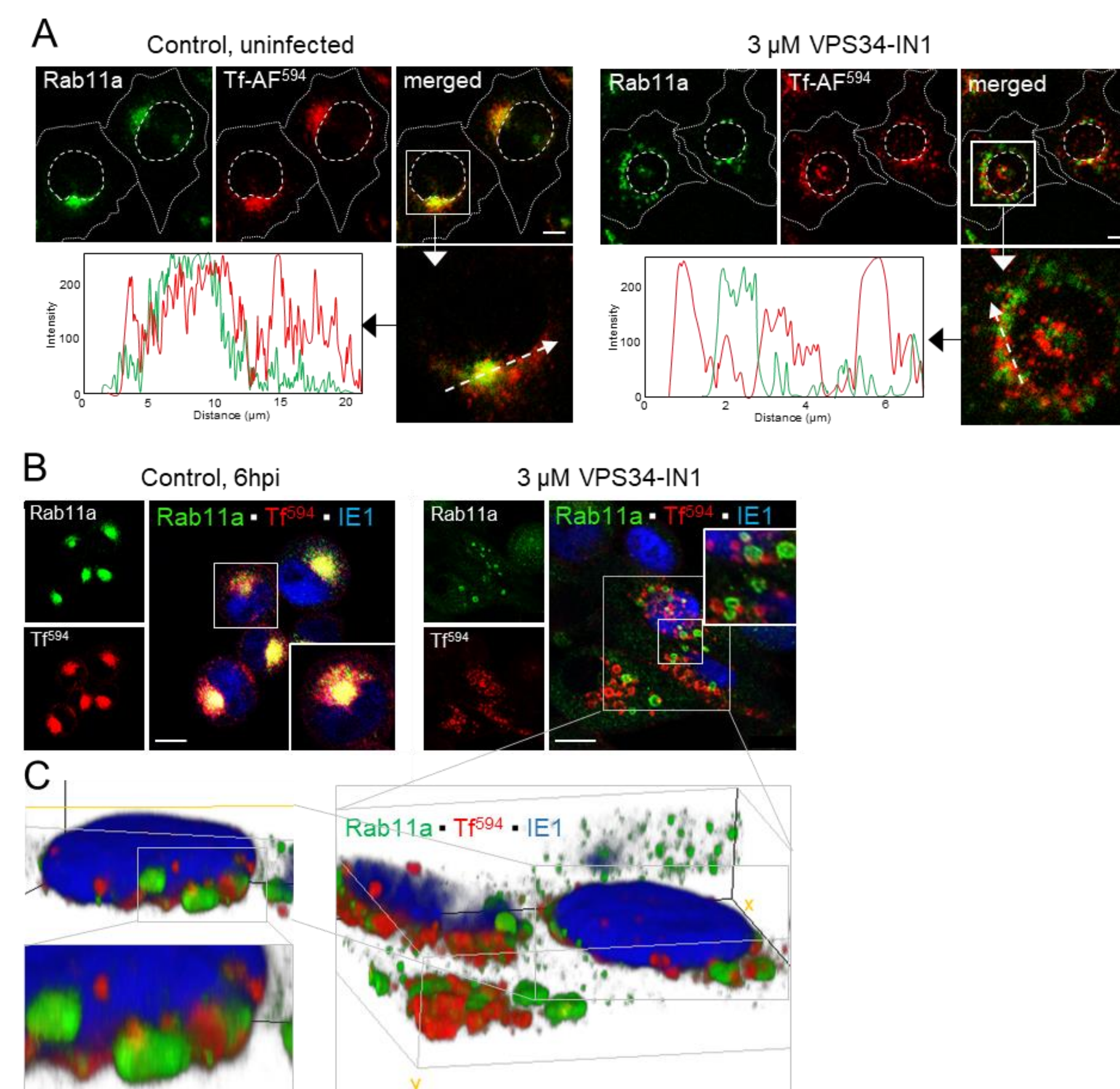
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Marcelić Marina¹, Mahmutefendić Lučin Hana^{1,3}, Jurak Begonja Antonija², Blagojević Zagorac Gordana^{1,3}, Viduka Ivona¹, Lučin Pero^{1,3}¹ Department of Physiology and Immunology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia² Department of Biotechnology, University of Rijeka, Rijeka, Croatia³ University North, University Center Varaždin, Varaždin, Croatia**Figure 1. High PI(3)P production and enrichment of PI(3)P membranous domains in the pre-cVAC and cVAC of MCMV infected cells.**

(A) For direct visualization of the PI(3)P-containing membrane domains on the predecessor of the cVAC (pre-cVAC) we transfected cells with the YFP-PX MSCV vector, infected them 24 hours after transfection and analyzed by confocal microscopy 6 hours post infection (hpi) when the membranous organelle reorganization is initiated. The transfected cell displayed green fluorescence of PI(3)P enriched vesicles mainly localized in the perinuclear area that largely colocalized with internalized transferrin (Tf-AF⁵⁵⁵) which represents EE/ERC compartments in the inner part of the pre-cVAC. (B) Given that more than 90% of endosomal PI(3)P is produced by Vps34, we performed Western blot analysis of Vps34 expression during MCMV life cycle and showed that MCMV infection, followed by viral intermediate early protein 1 (IE1), did not alter Vps34 levels. (C) Confocal immunofluorescence analysis of Vps34 distribution 6 hpi showed that it is mainly concentrated in the inner pre-cVAC, as its product PI(3)P, within the established GM130-positive organelles that represent reorganized cis-Golgi or outer pre-cVAC. This distribution was also maintained 24 hpi when the cVAC was fully formed.

**Figure 2. PI(3)P depletion affects membrane flow from EE to ERC.**

To monitor the effect of PI(3)P depletion on endosomal flow, we treated the cells with VPS34-IN1 (3 μ M) and after 1 hour we analyzed Tf internalization (Tf⁵⁹⁴) into Rab11a positive compartments (ERC) by confocal microscopy. VPS34-IN1 treatment enlarged, vacuolized, and displaced Rab11a positive compartments from the perinuclear area in uninfected (A) and MCMV infected cells (B) as well as abolished recycling cargo sorting (Tf) in this pathway. (C) The 3D imaging analysis using Image J program demonstrated the segregation of Tf loaded and enlarged Rab11a domains within the same compartment.

**Conclusion**

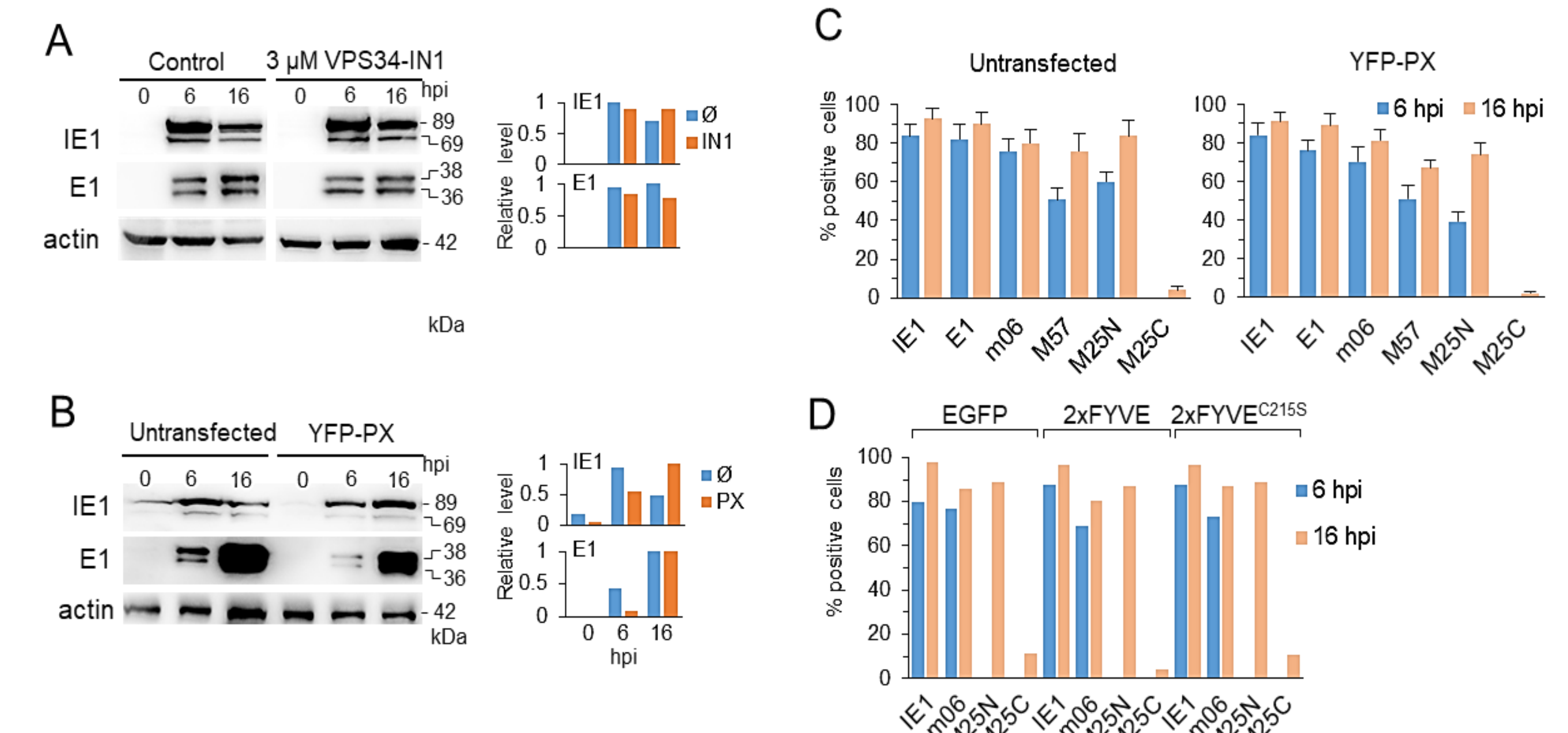
PI(3)P associated processes in the reorganization of the membranous system and cellular signaling during cytomegalovirus infection are insufficiently understood. Our results demonstrate that PI(3)P enriched membranes represent substantial part of the cVAC, especially at the inner cVAC membranous organelles, and that despite some alterations of membrane flow from EEs to the ERC after PI(3)P depletion, PI(3)P membrane domains are not required for the initial steps in the biogenesis of cVAC, but intact PI(3)P associated pathways are essential for the final formation of new virions.

Results

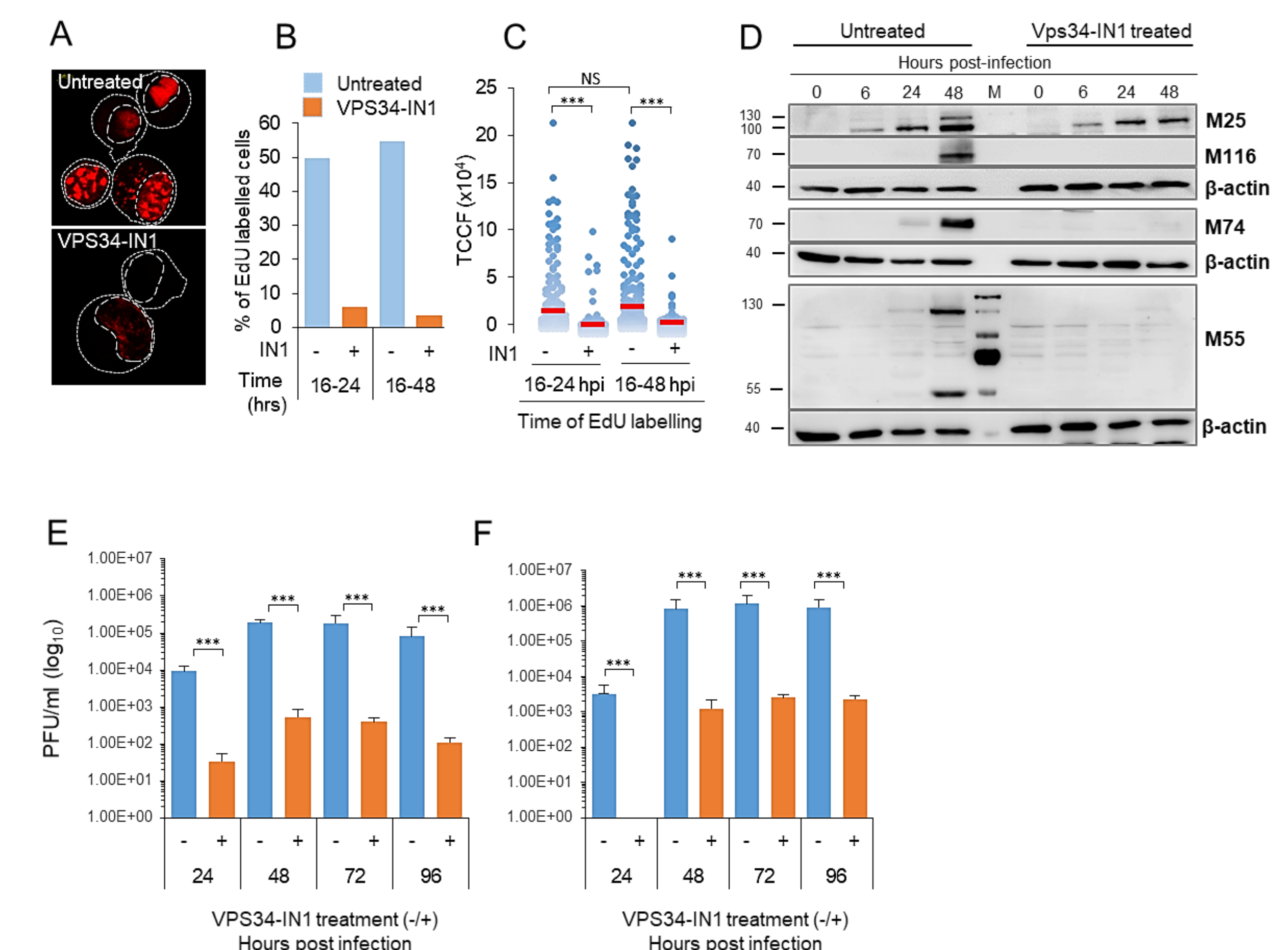
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Figure 3. PI(3)P depletion and PI(3)P saturation do not alter the establishment of MCMV infection and progression through the early phase of infection.

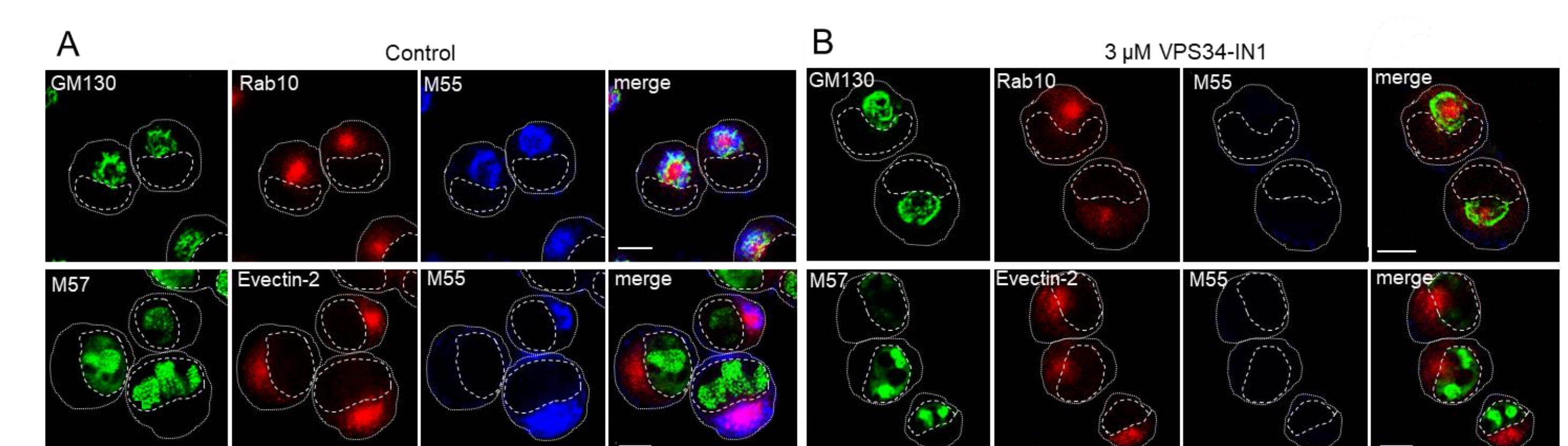
To test the effect of PI(3)P depletion and saturation of PI(3)P on the establishment of MCMV infection and its progression through the early phase of infection, we monitored the expression of MCMV proteins by immunofluorescence and Western blot analysis in VPS34-IN1 treated and 2xFYVE and PX expressing cells. (A) Cells were infected and 4 hpi treated with VPS34-IN1 until 6 and 16 hpi when they were analyzed by Western blot. There was no significant difference in the amounts of the MCMV proteins that characterize the immediate early (IE1) and early (E1) phase of infection in VPS34-IN1 treated cells, compared to untreated (control). The same was observed when comparing untransfected and YFP-PX transfected cells that were infected 42-48 hrs after transfection (B). (C-D) Cells were transfected with PI(3)P binding vectors, infected 42-48 hrs after transfection and immunofluorescence analysis was performed. The overexpression of YFP-PX did not affect the establishment of the infection and progression through the early phase as determined by immunofluorescence quantification of immediate early (IE1) and early (E1, m06, M57, M25N) MCMV proteins. The same was observed with EGFP-2xFYVE, its PI(3)P non-binding mutated form 2xFYVE^{C215S} and EGFP alone.

**Figure 4. Long term PI(3)P depletion inhibits viral DNA replication, late protein expression and virion production.**

For the analysis of the late phase infection that starts with viral DNA replication 16 hpi, we incubated untreated or VPS34-IN1 treated cells 16 hpi with Edu and analyzed them by confocal microscopy 24 or 48 hpi (A). PI(3)P depletion abolished Edu incorporation and resulted in the reduction of Edu signal, as shown by a lower percentage of Edu-positive cells when treated with VPS34-IN1 (C) as well as with the quantification of Edu signal on confocal images using ImageJ program (B) (dots represent the Total Corrected Cell Fluorescence (TCCF) of individual cells in the representative experiment (N=3) and horizontal bars the median value). PI(3)P depletion also inhibited the expression of late viral glycoproteins (M116, M74, M55.2) and late tegument protein M25 (130 kDa) as shown by Western blot analysis (D). In accordance with the obtained results, after the plaque assay was performed on samples of infected cells and their supernatants (1-4 days of infection), a significantly reduced number of cell-associated (E) and extracellular (F) virions were detected in cells treated with VPS34-IN1.

**Figure 5. PI(3)P depletion does not prevent the membranous organelle reorganization associated with cVAC development.**

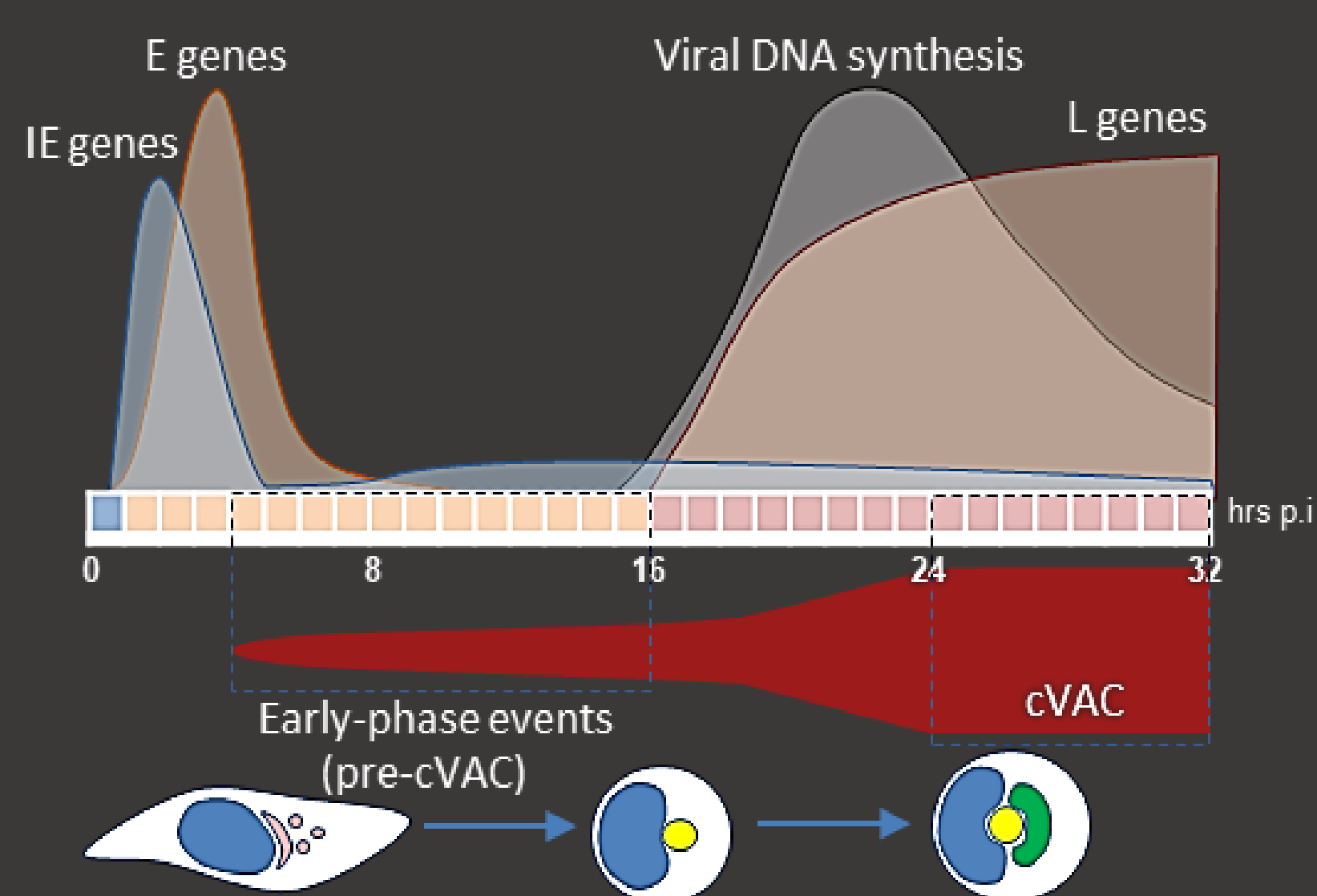
(A-B) To investigate the PI(3)P depletion effect on membrane rearrangement related to cVAC development, 4 hpi cells were treated with VPS34-IN1 and 30 hpi immunofluorescence analysis was performed. With anti-GM130 antibody cis-Golgi membranes within the outer pre-AC were displayed and with anti-Rab10 and anti-Evctn-2 membranes of the inner pre-AC at the EE-ERC interface were detected. The infection was monitored by staining the late M55 protein. The presence of VPS34-IN1 neither prevented the Golgi compacting and displacement nor the inner accumulation of Rab10 and Evctn-2. According to the results in Figure 4.D, expression of late MCMV M55 protein was reduced after PI(3)P depletion.



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Introduction

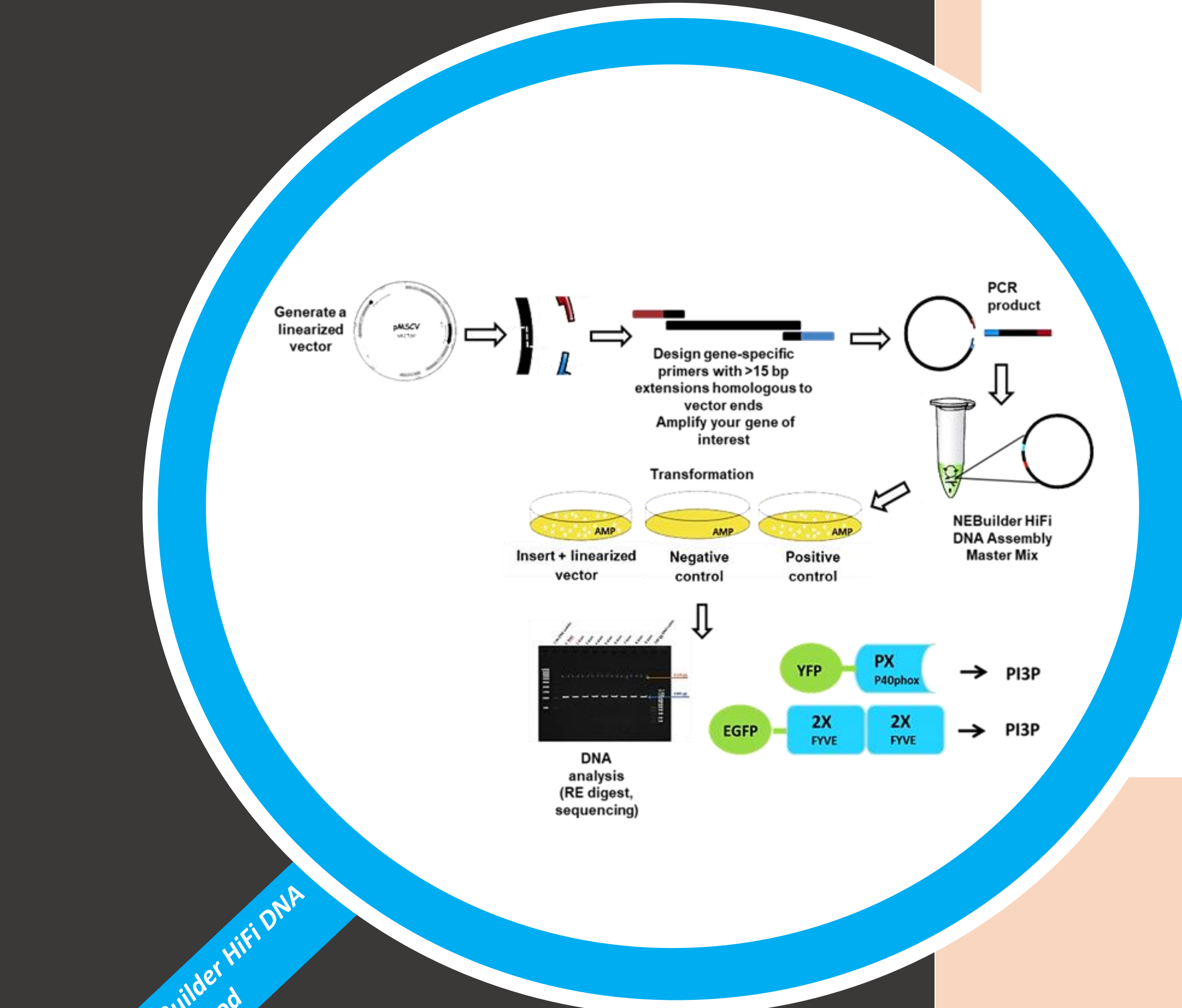
Cytomegalovirus (CMV), a member of the Herpesviridae family, begins with the rearrangement of the host cell membranous organelles early in the infection to create an environment for virion production, known as cytoplasmic virion assembly compartment (cVAC). One of the earliest event identified in the development of the cVAC is the expansion of the early endosome (EE)/endosomal recycling compartment (ERC) interface and relocation of the Golgi complex. These membranous organelle reorganizations are accompanied by changes in membrane phosphoinositides (PIs), especially phosphatidylinositol 3-monophosphate (PI(3)P), as PI(3)P is the master regulator of the EE pathway. Therefore, our goal was to investigate the contribution of PI(3)P-dependent EE domains to cVAC biogenesis and their role in the consequent progression of the CMV life cycle.

**Kinetics of Murine Cytomegalovirus (MCMV) gene expression and development of cytoplasmic virion assembly compartment (cVAC).**

Early phase (E) infection events lead to reorganization of the EE/ERC/TGN interface, which at the end of E-phase (16 hpi) forms a compact juxtanuclear structure that represents the core of the cVAC (yellow). Reorganized Golgi complex that also begins to form very early in the infection, together with viral tegument proteins and glycoproteins that are accumulated after the viral DNA synthesis and expression of late genes (L), form a cap (green) that surrounds the core at 24 hpi and later.

Experimental approach

Our functional analysis of PI(3)P was based on rapid depletion of PI(3)P production at EE membranes using VPS34-IN1, a specific and reversible inhibitor of the major PI(3)P producer on EE (Vps34), and on saturation of PI(3)P by overexpression of recombinant PI(3)P-binding domains (EGFP 2xFYVE^{Hrs}, YFP-PX⁴⁰) that were previously produced by gene cloning techniques (NeBuilder HiFi DNA Assembly). Under such conditions, we monitored changes in the physiology of the EE pathway and the process of cVAC development through the expression of its markers (GM130, Rab10, Evctn-2) by confocal microscopy. With Western blot analysis, viral DNA synthesis (incorporation of 5-ethynyl-2'-deoxyuridine (Edu)), and CMV growth analysis (plaque assay) we examined CMV replication cycle. Experiments were performed on Balb 3T3 murine fibroblast cells, infected with murine cytomegalovirus (MCMV) (recombinant virus Δ m138-MCMV).



Overview of the NEBuilder HiFi cloning method