**Late maturation steps in the nucleus preceding prelytic active egress of**

**nonenveloped parvovirus**

Raphael Wolfisberg1, Christoph Kempf1,2 and Carlos Ros1,2\*

1Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern, 2CSL Behring AG, Wankdorfstrasse 10, 3000 Bern 22, Switzerland

Running title: Late maturation and active egress of MVM

Word count for the abstract:

Word count for the text:

\*Corresponding author: Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern. Phone: +41 31 6314349. Fax: +41 31 6314887.

E-mail: carlos.ros@ibc.unibe.ch

**Abstract**

Although not well understood, growing evidence indicates that following genome packaging the nonenveloped parvovirus minute virus of mice (MVM) may actively egress before passive release through cell lysis. We have dissected the final intranuclear steps that lead to the maturation and egress of MVM. By using anion exchange chromatography, intranuclear progeny MVM particles were separated by their net surface charges. Apart from empty capsids, two distinct progenies of DNA-containing particles arose in the nuclei of infected cells. The earliest population of DNA-containing capsids to appear is fully infectious but cannot be actively exported from the nucleus. A further maturation of this early population involving N-VP2 exposure as well as phosphorylations of surface residues gave rise to a second late population with egress potential. While the capsid surface phosphorylations were strictly associated to nuclear export capacity, mutational analysis revealed that the phophoserine-rich N-VP2 was dispensable. During entry, a reverse situation was observed. Inside endosomal vesicles, incoming particles lost both, N-VP2 and the additional surface phosphorylations ***that were acquired in the nucleus to confer nuclear export potential***. Our results reveal the late intranuclear maturation steps of a nonenveloped virus that promote active egress before late release by cell lysis.

**Importance**

In general, the process of egress of enveloped viruses is active and involves host cell membranes, however, release of nonenveloped viruses seems to rely more on cell lysis. At least for some nonenveloped viruses, an active process before passive release by cell lysis has been described, although the mechanisms involved remain poorly understood. By using the nonenveloped model parvovirus, minute virus of mice, which replicates and assembles inside the cell nucleus, we could confirm the existence of an active process of egress that precedes passive release by cell lysis and further characterize the capsid maturation steps involved. Following DNA packaging, capsids required further modifications inside the nucleus involving N-VP2 exposure and phosphorylation of capsid surface residues to acquire export potential. Although N-VP2 sequences were dispensable, capsid surface phosphorylations were always associated to export capacity. Those phosphorylated capsids were segregated from other nuclear pre-mature populations and escaped the cells before the onset of cell lysis.

**Introduction**

The egress of enveloped viruses is well characterized ([32](#_ENREF_32), [64](#_ENREF_64)). Nascent capsids become engulfed in a plasma membrane-derived lipid envelope by hijacking the multivesicular body (MVB) biogenesis machinery ([7](#_ENREF_7)). Current models propose a sequential, virus-induced recruitment of class E vacuolar protein sorting (VPS) proteins which act in four complexes, referred to as endosomal sorting complex required for transport (ESCRT 0-III) ([37](#_ENREF_37)). Direct interaction of retroviruses with ESCRT components via viral late domains (L-domains) results in the ubiquitination of either interaction partner ([3](#_ENREF_3)). Ubiquitination initiates the biogenesis and fission of MVBs and thus, budding of the virions ([31](#_ENREF_31)).

The egress of non-enveloped viruses is less well understood. In general, release of non-enveloped viruses is associated with cellular lysis, thus considered a passive process ([12](#_ENREF_12), [33](#_ENREF_33), [59](#_ENREF_59)). However, there is rising evidence that an active egress precedes virus-induced cell lysis and subsequent passive release. For instance, bluetongue virus (BTV) has been demonstrated to usurp the ESCRT machinery for egress by means of its L-domains ([36](#_ENREF_36), [65](#_ENREF_65)). Similarly, Hepatitis A virus (HAV) release involves ESCRT-associated proteins ([18](#_ENREF_18)). Furthermore, drug-induced stimulation of the autophagy pathway increased non-lytic spread of poliovirus and progeny virions were shown to accumulate unilaterally on the apical surface of polarized and productively infected epithelial cells ([4](#_ENREF_4), [60](#_ENREF_60)). Equally, simian vacuolating virus 40 (SV40) and simian rotavirus (RRV) were almost exclusively recovered from the apical culture fluid of polarized epithelial cells prior to cell lysis. Electron microscopy studies and specific inhibition of vesicular transport pathways indicate a vesicle-associated release of progeny virions ([9](#_ENREF_9), [20](#_ENREF_20)).

Recent studies show increasing evidence for an active egress of parvoviruses (PV), a group of small, non-enveloped viruses. Autonomous rodent PVs, including minute virus of mice (MVM), display a T=1 icosahedral capsid containing a single-stranded DNA genome of about 5 kb ([11](#_ENREF_11)). The compact coding sequence contains two large overlapping open reading frames (ORFs) under the control of an early and a late promoter, termed P4 and P38, respectively. P4 controls the expression of the viral non-structural proteins NS1 and NS2, while P38 drives the expression of the viral capsid proteins VP1 and VP2, as well as the non-structural protein SAT ([45](#_ENREF_45)). Productive PV infection causes dramatic morphological and physiological changes of their host cells, culminating in cell death and lysis ([6](#_ENREF_6), [19](#_ENREF_19)). PV cytotoxicity is mainly associated with the large non-structural protein NS1, an 83-kDa multifunctional protein ([12](#_ENREF_12)).

Besides having the possibility to passively egress the host cell by NS1-induced cellular lysis, the latest data suggest an active, pre-lytic egress for MVM ([1](#_ENREF_1), [2](#_ENREF_2), [30](#_ENREF_30)). Although the mechanism for nuclear export and subsequent release of MVM virions remains elusive, several important viral and cellular effectors involved in PV egress have been identified. MVM is exported from the nucleus by a Crm1 dependent mechanism. Stable interaction of NS2 with Crm1 was successfully demonstrated ([5](#_ENREF_5), [44](#_ENREF_44)). Classical nuclear export signals (NES) exhibit low affinity for Crm1 to prevent from the formation of the Crm1/cargo complex in the cytoplasm where RanGTP is absent ([39](#_ENREF_39)). Surprisingly, the NES of NS2 belongs to the supraphysiological NES which tightly bind to Crm1 regardless of the presence of RanGTP. Therefore, NS2 competitively inhibits Crm1 function by sequestering endogenous nuclear export receptors ([15](#_ENREF_15)). MVM mutant genomic clones generating NS2 proteins harbouring either regular NES, or substitutions which abrogated Crm1 interaction were shown to be compromised in viral nuclear export and productive infection. As expected, NS2-Crm1- mutants showed nuclear accumulation of export deficient NS2 in transfected cells. Surprisingly, the nuclear retention of mutant NS2 proteins came along with a substantial accumulation of progeny virions in the nucleus of infected cells, suggesting a NS2-dependent export of progeny virions. However, NS2 interaction with viral capsid proteins has not yet been demonstrated. Since NS2 exhibits pleiotropic effects, abrogation of the tight NS2-Crm1 interaction might interfere with early functions during a productive infection which indirectly affect progeny maturation and their export from the nucleus.

In human transformed cells, NS2 was dispensable for infection (ref) and progeny export was not affected by treatment with the antifungal antibiotic leptomycin B (LMB), a drug which inhibits Crm1-dependent nuclear export (ref). For these cells an alternative export mechanism was proposed involving the unordered N-terminus of VP2 (N-VP2).

~~Productive MVM infections in either permissive human cells or restrictive murine cells disclosed a cell specific use of alternative strategies for nuclear export. It became apparent when different cell types were treated with the antifungal antibiotic leptomycin B (LMB), a drug which inhibits Crm1-dependent nuclear export. LMB treatment of susceptible murine cells resulted in a significant inhibition of nuclear export whereas transformed human cells were much less affected even at high doses of LMB. This indicates that Crm1 is not essentially involved in the nuclear export of MVM in the latter cell line (~~[~~30~~](#_ENREF_30)~~). The observed differences may result from a cell-type dependent phosphorylation status of MVM. Generally, MVM capsids derived from permissive human cells displayed more prominent phosphorylation compared to that of capsids isolated from restrictive murine fibroblasts (~~[~~29~~](#_ENREF_29)~~).~~ Site-directed mutagenesis of the three distal serine residues at position 2, 6, and 10 of the ~~unordered N-terminus of VP2 (~~N-VP2~~)~~ revealed an important role of these phosphorylations in the Crm1-independent nuclear export of MVM in permissive human cells. When the N-terminal phosphorylations were mutated, progeny virions were predominantly retained in the nucleus and the corresponding mutants displayed a small plaque phenotype, indicating the importance of those phosphorylations in viral spread ([30](#_ENREF_30)).

Currently, the detailed mechanism for nuclear export of MVM progeny virions still remains unclear. Further investigations are required to understand how MVM is exported from the nucleus and initiates final egress towards the cell periphery.

~~MVM transport from the nucleus to the cell periphery is associated with the degradation of actin fibers. These alterations to the filamentous network were attributed to a virus-induced imbalance between the actin polymerization factor N-WASP (Wiscott-Aldrich syndrome protein) and gelsolin, a member of the actin-severing protein family (~~[~~40~~](#_ENREF_40)~~). Indeed, the MVM titer in the medium following MVM infection drastically declined when gelsolin function was diminished. During MVM infection, gelsolin activity is regulated by the CKIIα/NS1 complex which was demonstrated to be capable of phosphorylating gelsolin. Consequentially, inhibition of CKIIα correlated with prolonged persistence of actin fibers (~~[~~1~~](#_ENREF_1)~~,~~ [~~42~~](#_ENREF_42)~~).~~ Several lines of evidence indicate an active, vesicle-associated, gelsolin-dependent egress of MVM following nuclear export. Progeny virions were shown to co-localize with exocytic, endosomal, and lysosomal markers in immunofluorescent experiments ([1](#_ENREF_1), [13](#_ENREF_13)). Cell fractionation experiments confirmed this observation by demonstrating a co-migration of viral particles with cytosolic vesicles rather than free, vesicle-independent localization in the soluble cytosolic fraction. A cooperative cross-talk between actin- and microtubule dependent transport might be involved in MVM transport from the nucleus to the cell periphery ([46](#_ENREF_46), [52](#_ENREF_52), [54](#_ENREF_54)).

The secretory pathway has been proposed as the route for active egress of MVM. Progeny virions would become engulfed by COPII-vesicle formation in the perinuclear ER where they accumulated with dynamin. Accordingly, a dramatic retention of virions in the perinuclear area and inhibition of virion release into the medium was observed in cells lacking functional effectors of the secretory pathway ([2](#_ENREF_2)). In addition, members of the ERM family, such as radixin and moesin, were shown to play a role in virus maturation and spreading capacity, as judged by their impact on MVM plaque morphology ([41](#_ENREF_41)). Consequentially, dominant negative radixin or moesin mutants failed in wrapping progeny virions into transport vesicles, resulting in a marked reduction of egressed virions in the medium. Finally, active egress promotes cellular lysis as demonstrated by the prolonged viability of cells in which vesicular transport was either inhibited or by-passing the Golgi apparatus. Besides, the involvement of progeny particles in cytolysis was demonstrated by the prolonged survival of murine cells transduced with a viral vector deficient for the production of progeny virion particles ([2](#_ENREF_2)).

Documentation of active egress requires accurate demonstration that no cell lysis occurred during the experiment. However, it is challenging to exclude the possibility that lysis of a few cells may passively release progeny virions, which could additionally contribute to uncontrolled second rounds of infection. In tissue culture, passive egress considerably contributes to viral spread. However, its importance in animal infection might be largely limited by clearance of virus-infected cells by components of the immune system. Parvoviruses, particularly MVM, are highly robust and persist as intact particles mainly in the lysosomes of infected cells where they are slowly degraded ([28](#_ENREF_28)). Since the endosomal pathway is highly dynamic and partially overlaps with the egress route of progeny viruses, the discrimination of incoming from progeny virions represents a major challenge.

The present investigation aims to characterize the final maturation steps leading to nuclear export and egress of MVM. Using anion exchange chromatography (AEX) in combination with quantitative PCR (qPCR) we demonstrate that two distinct populations of DNA containing progeny particles co-exist in the nucleus of infected murine cells. *De novo* synthesized capsids undergo a maturation step in the nucleus that involves surface phosphorylation(s) and exposure of N-VP2. Only fully infectious virions were able to exit the nuclei and egress from the cells prior to cell lysis, confirming an active egress of parvovirus MVM.

**Materials and Methods**

**Cells and viruses.** A9 mouse fibroblasts ([57](#_ENREF_57)) and NB324K cells ([51](#_ENREF_51)), were routinely propagated under a minimal number of passages in DMEM supplemented with 5 % FCS at 37 °C in 5 % CO2 atmosphere. Stocks of MVM were propagated on A9 cells. As soon as the cytopathic effect was complete, the supernatant was collected, pre-cleared from cell debris by low-speed centrifugation and the virus pelleted through 20 % sucrose cushion. The virus pellet was washed and resuspended in PBS and the titers were determined by qPCR as DNA-containing particles per microliter. DNA-containing (full capsids; FC) and empty capsids (EC) were separated by CsCl gradient as previously described (ref). CsCl was removed by size-exclusion chromatography through PD-10 desalting columns (GE Healthcare, Chalfont St Giles, UK) and when required, the capsids were concentrated in Amicon® centrifugal filter devices (Merck Millipore, Billerica, MA).

**Antibodies, chemicals and enzymes**. Rabbit anti-VPs (polyclonal against MVM structural proteins), rabbit anti-N-VP2 (polyclonal against the N-terminus of VP2), and mouse anti-capsid (monoclonal against intact capsids; clone B7) antibodies have been previously described ([26](#_ENREF_26), [30](#_ENREF_30)). Fluorescent-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and horseradish peroxidase-conjugated antibodies were purchased from DakoCytomation (Glostrup, DK). Bafilomycin A1 (BafA1), chymotrypsin and chymostatin were obtained from Sigma-Aldrich (St. Louis, MO) and reconstituted in ethanol at 0.1 mg/mL or in DMSO at 10 mM, respectively. To avoid enzymatic digestion or dephosphorylation during the processing of cell extracts, the lysis buffer was supplemented with protease inhibitors (Roche, Basel, CH); 1 mM sodium orthovanadate (Na3VO4), and 1 mM sodium fluoride (NaF) (Sigma-Aldrich).

**Virus infection.** A9 or NB324K cells (105 for qPCR or 3 × 106 for AEX) were infected with MVM (5’000 DNA-containing particles per cell, corresponding to approximately 10 PFU/cell ([56](#_ENREF_56))) for 1 h at 4 °C for binding. Unbound virus was removed by washings and the cells were incubated at 37 °C to initiate infection. At progressive times post-internalization total cellular DNA was extracted for qPCR analysis or cells were fractionated and subjected to AEX.

**Cell fractionation**. A9 and NB324K cytoplasmic fractions were extracted in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 1 mM Na3VO4, 1 mM NaF, protease inhibitor cocktail (Roche), pH 7.2 at 4 °C for 30 min. Following vortexing, intact nuclei and cell debris were removed by high-speed centrifugation at 4 °C. Isolation of nuclei was performed by using the Nuclei EZ Prep Nuclei Isolation Kit (Sigma-Aldrich) following the manufacturer’s instructions. In order to obtain highly pure nuclear fractions, the isolated nuclei were further processed by centrifugation at 500 × g for 10 min through a sucrose gradient. The integrity of the isolated nuclei was examined by light microscopy after trypan blue staining. The purity of the nuclei and the absence of the outer nuclear membrane were evaluated with lamin A/C (inner membrane) and serca 2 ATPase (outer membrane) antibodies. Purified nuclei were lysed in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 1 mM Na3VO4, 1 mM NaF, protease inhibitor cocktail (Roche), pH 7.2 at 4 °C for 30 min. Following vortexing, the nuclear lysate was passed 10 times through a 27 G needle and nuclear debris was removed by high-speed centrifugation at 4 °C.

**Quantitative PCR (qPCR).** Template DNA was extracted by using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, DE) following the manufacturer’s guidelines. Amplification and real-time detection of PCR products was performed by using the CFX96TM Real-Time System with the iTaqTM Universal SYBR® Green Supermix (Biorad, Hercules, CA). Primers for MVM DNA amplification were: forward (5’-GACGCACAGAAAGAGAGTAACCAA-3’; nucleotides 231 to 254) and reverse (5’-CCAACCATCTGCTCCAGTAAACAT-3’; nucleotides 709 to 732). Specificity of the amplification was determined by melting curve analysis. As external standard, an infectious clone of MVM ([34](#_ENREF_34)) was used in 10-fold serial dilutions.

**Anion exchange chromatography (AEX).** The Mono Q HR 5/5 column (5 x 50 mm; Pharmacia, Uppsala, SW) was connected to the ÄKTAmicro chromatography system operated by the UNICORN control software (GE Healthcare). The Mono Q column was equilibrated with five column volumes (CV) starting buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.2). Viruses (at least 108 virus particles) diluted in 1 mL starting buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) were applied to the Mono Q column trough a 2 mL injection loop, rinsed with six CV of starting buffer and eluted by a linear salt gradient (0-2 M NaCl) in 20 mM Tris-HCl, 1 mM EDTA, pH 7.2. The flow rate was constantly kept at 1.5 mL/min and salt concentration was monitored by electrical conductivity. Viruses in each fraction (185 µl) were quantified by qPCR.

**Immunofluorescence microscopy.** A9 cells (3 × 105) were seeded onto coverslips within 12-well plates. After 24 h, the cells were infected with 2’500 DNA-containing particles per cell, corresponding to approximately 5 PFU/cell ([48](#_ENREF_48)), for 1 h at 4 °C. Subsequently, the cells were washed to remove unbound virus, and incubated at 37 °C. At different times, cells were washed and processed for immunofluorescence as previously described ([24](#_ENREF_24), [25](#_ENREF_25)) with fluorescent-conjugated secondary antibodies. Cells were mounted with Mowiol (Calbiochem, Billerica, MA) containing 30 mg/ml of DABCO (Sigma-Aldrich, St. Louis, MO) as an anti-fading agent and examined by laser scanning microscopy (LSM 510 Meta; 100 x magnification objective, Carl Zeiss).

**Immunoprecipitation.** Viruses were incubated with specific antibodies inside LoBind eppendorf tubes pre-blocked with PBSA (PBS-1 % BSA) for 1 h at 4 °C. Subsequently, 20 μL protein G-agarose beads were added and the samples were further incubated overnight at 4 °C. The beads were washed with PBSA. To remove residual BSA an additional washing step was carried out with PBS.

**Enzymatic reactions**. All enzymatic reactions were performed in a 50 µL reaction volume. Viruses diluted in PBS (108 virus particles) were incubated for 1.5 h at 37°C with chymotrypsin (0.5 mg/mL) and the reaction was stopped by adding 100 μM chymostatin. Lambda phosphatase treatment (40000 U/mL) was performed in 50 mM Tris-HCl, 100 mM NaCl, 2 mM MnCl2, 5 mM DTT, pH 7.8 for 3 h at 37 °C. Phosphatase was inactivated by adding 1 mM Na3VO4 and 1 mM NaF. Free DNA was digested using 50 DNase I (50U) at 37°C for 1.5 h. DNase I was inhibited by incubation at 75 °C for 15 min.

**Results**

**Two distinct populations of *de novo* DNA-containing particles are detectable in the nucleus of MVM infected cells.** Progeny viral particles released in the supernatant of infected cell cultures were collected 7 days post infection (dpi), when cytopathic effect was complete. Cell debris was excluded by low speed centrifugation. The collected viral capsids were analysed by anion exchange chromatography (AEX), which can separate particles based on their net surface charges, followed by quantitative PCR (qPCR). Two distinct virus populations of DNA-containing particles (full capsids; FC) were separated and their relative amount quantified by qPCR (Fig. 1A). A second AEX-qPCR analysis was performed in parallel, which included both medium and cell lysate, obtained by freeze and thaw cycles to fully release intracellular viruses. By including intracellular viruses, the same two populations were detected but their proportion was different. The more positively charged population (referred to as FC-P1) was predominantly associated with cells, thus it increased when intracellular viruses were included. In contrast, the more negatively charged population (referred to as FC-P2) was the predominant population in the supernatant when intracellular viruses were mostly excluded.

In order to verify the integrity of the two DNA-containing viral populations, we collected supernatant and intracellular viruses and subjected them to nucleolytic digestion. As shown in Fig. 1B, both virus populations were resistant to nuclease digestion and their AEX profile did not change, indicating that both particle types represent fully assembled DNA-containing particles.

The nucleus represents the site of MVM capsid assembly and morphogenesis. It was therefore of interest to verify the presence of both virus populations in the nucleus early at the onset of assembly and packaging. Viruses were collected from isolated nuclei of infected murine fibroblasts early after infection and subjected to AEX-qPCR. As shown in Fig. 1C, by 18h pi both DNA-containing viral populations co-existed in the cell nucleus in similar amounts.

Besides DNA-containing capsids, MVM infection results in the accumulation of empty capsids (EC), which represent assembled capsid precursors that have not packaged viral genomes ([38](#_ENREF_38)). To verify their AEX profile, EC precursors were purified by CsCl, subjected to AEX and visualized by dot blot using an antibody against assembled capsids (Mab B7; ([26](#_ENREF_26))). As illustrated in Fig. 1D, EC had an AEX profile resembling that of the FC-P1 population. The fact that FC-P1 and EC are predominantly cell-associated and have the same AEX profile would suggest that they represent immature particles without nuclear export potential, whereas FC-P2 would represent particles displaying a further maturation step conferring nuclear export potential.

**FC-P1 and FC-P2 are infectious but differ in N-VP2 conformation.** In order to further characterize the two FC populations, we separated them by AEX, pooled the fractions corresponding to each population and performed a second AEX. In Fig. 2A the chromatograms of purified FC-P1 and FC-P2 are shown. The purified viral populations were used to investigate their capacity to initiate the infection in A9 cells. To this end, we quantitatively analyzed the ability of each FC populations to initiate DNA replication. As demonstrated in Fig. 2B, both virus populations were able to reach the nucleus and their genomes were efficiently replicated without significant differences.

In MVM virions the N-terminal region of the VP2 (N-VP2) occupies an external positon in the capsid, however, during entry N-VP2 is cleaved by endosomal proteases to render a shorter protein named VP3 ([58](#_ENREF_58), [62](#_ENREF_62)). The function of N-VP2 cleavage is not fully understood, but is seems to be required to allow the exposure of the N-terminal region of VP1 (N-VP1) ([10](#_ENREF_10), [16](#_ENREF_16)), which harbors important functional motifs essential for infection ([61](#_ENREF_61)), particularly endosomal escape ([17](#_ENREF_17)) and nuclear targeting ([25](#_ENREF_25)). We analyzed the surface conformation of N-VP2 in the two populations of FC by immunoprecipitation with a specific antibody raised against this region ([30](#_ENREF_30)). As demonstrated in Fig. 2C, N-VP2 occupies a surface position in FC-P2 but is predominantly sequestered in FC-P1. Exposure of FC-P1 to temperature (50 °C) or to acidic conditions (pH 4.5) resulted in a significant externalization of N-VP2 (Fig. 2D). Accordingly, FC-P1 resembles to EC also in the sequestered N-VP2 conformation. Chymotrypsin (CHT) has been previously demonstrated to mimic the *in vivo* cleavage of N-VP2. In contrast, EC do not expose the N-VP2 termini on the capsid surface and thus they cannot be cleaved. The different AEX-purified capsid populations were subjected to proteolytic digestion by CHT under neutral and acidic conditions. As shown in figure 2E, FC-P2 particles were completely processed under all tested conditions. In contrast, the N-VP2 of FC-P1 was only marginally accessible to CHT under neutral conditions and required acidification to improve the cleavage rate, confirming its predominant internal conformation. The substantial amount of VP2 that remained unprocessed originates from EC, which elute in the same AEX fractions as the FC-P1.

When incubated with A9 cells, both virion progenies bound similarly to cells as shown by immunofluorescence. The N-VP2 from FC-P2 was detectable on the surface of the cells and was fully processed by 4 hpi. In contrast and as expected, the N-VP2 from FC-P1 was not visible at binding but became exposed after internalization and a proportion remained detectable for several hours pi (Fig. 2F), indicating a slower or less efficient VP2 to VP3 processing.

The prominent differences in N-VP2 conformation in the two FC populations could account for the different AEX profile. In order to investigate whether the different AEX profile between the two FC populations is caused by the different N-VP2 conformation, FC-P1 were exposed to acidic conditions to provoke the exposure of N-VP2 (as shown in figure 2D) and subjected to AEX. As shown in figure 2G, exposure of N-VP2 did not change the AEX profile of FC-P1. Accordingly, the distinct N-VP2 conformation is not responsible for the different AEX profile. The results also suggest that packaging does not directly result in N-VP2 externalization and further emphasize similarities between the FC-P1 and EC populations.

**The surface phosphorylation status of the capsid is a key determinant of the different AEX profile.** In order to further examine biochemical and structural differences between the two FC populations, both capsid species were isolated from the nuclei of infected A9 cells, treated with CHT or with lambda phosphatase and subsequently analyzed by AEX-qPCR. As expected, only FC-P2 capsids were sensitive to CHT resulting in a prominent shift in the AEX profile, in contrast, FC-P1 remained unaffected (Fig. 3A). When treated with lambda phosphatase, FC-P2 changed its AEX profile to that of FC-P1, which remained unchanged. These results suggest that the differences between the two FC populations are due to a distinct surface phosphorylation status, other than the distal phosphoserines in N-VP2 ([29](#_ENREF_29)). To confirm this, we used a MVM mutant, in which all distal serine residues on N-VP2 were substituted by glycine (referred to as 4SG). The AEX profile of 4SG virions, untreated or treated with lambda phosphatase, was similar to that of the wild type (Fig. 3C and D), confirming that additional phosphorylation(s), other than the distal phosphoserines in N-VP2, are present exclusively in the FC-P2 population and are responsible for their specific AEX profile.

**Only FC-P2 can actively egress from the infected host cell.** The subcellular distribution of the two full capsid progenies was examined. A9 cells were infected with MVM and at progressive times post-infection, progeny viruses were collected from nuclear, cytoplasmic and supernatant fractions and subjected to AEX-qPCR. While in the nucleus, both FC populations emerged with similar kinetics, in the cytoplasm and in the supernatant, the accumulation of FC-P2 preceded that of FC-P1 (Fig. 4A-C). The FC-P2 egress started largely before the onset of cell lysis, which occurred from 30 hpi (Fig. 4E) and resulted in the appearance of FC-P1 in the cytoplasmic and supernatant fractions. The strong segregation of the two initially mixed nuclear full capsid progenies at increasing post-infection times reveals the existence of an active mechanism of nuclear export and egress involving FC-P2 and preceding the passive release of FC-P1 and EC through late virus-induced cell lysis.

**The phosphoserine-rich N-VP2 is dispensable for active egress.** In sharp contrast to FC-P1 and empty particles, FC-P2 capsids are exported from the nucleus and subsequently released from the host cell prior to cell lysis. Distinct from empty or from FC-P1, N-VP2 of FC-P2 is external and the capsids have additional capsid surface phosphorylations. These features represent a late maturation step and might confer export potential. N-VP2 is heavily phosphorylated at serine residues, which has been previously suggested to assist nuclear export in a cell-specific manner. Mutants lacking N-VP2 distal phosphoserines were deficient in nuclear export and egress in NB324K cells, a human transformed cell line, but not in A9 murine cells ([30](#_ENREF_30)). We used a mutant lacking all the distal serine phosphorylations on the N-VP2 terminus, referred to as 5SG. Additionally, we used a MVM mutant containing a bulky phenylalanine residue at position 33 within the flexible poly-glycine stretch near the N-VP2 sequence, referred to as G33F. Due to this substitution the mutant progeny particles were unable to externalize N-VP2 following DNA packaging. Transfection with this mutant generated DNA-containing particles that were not infectious due to failure to expose and process N-VP2 and to externalize VP1u during entry ([8](#_ENREF_8)). Upon transfection, DNA-containing progeny particles of 5SG and G33F progressively accumulated in the cell culture medium to similar quantities as the wild-type (WT) virions (Fig. 6A). AEX analysis revealed that the G33F, as well as the 5SG progeny, consisted of both FC-P1 and FC-P2 particles (data not shown), substantiating that N-VP2 and/or its distal phosphorylations are not responsible for the two distinct AEX profiles of FC-P1 and FC-P2. Transfection results in an increased cell lysis due to cell damage and therefore it is expected to increase passive release. To examine whether the observed extracellular accumulation of G33F, 5SG, and WT progeny particles resulted mostly from active egress, we analyzed the intra- and extracellular FC-P1/FC-P2 ratios 24 hours post-transfection (hpt). At this time post-transfection, FC-P1 population exceeded that of FC-P2 in the nucleus (Fig. 5B and 6B), however, in the extracellular milieu, the ratio was inversed (Fig. 5B). The inverted ratios can only be explained by the existence of an active egress of FC-P2 particles in the presence of a marginal passive release of virus progeny induced by viral cytotoxicity and cell damage caused by the transfection. These results emphasize that N-VP2 sequences or its distal phosphorylations do not play a direct role in the nuclear export and active egress of MVM.

**FC-P1 is the precursor of FC-P2**. During productive in the presence of neuraminidase and B7 antibody to prevent reinfections, FC-P1 population was the first to appear in the nucleus of A9 and NB324K cells. While in NB324K cells, FC-P1 disappeared to give rise to FC-P2, in A9 cells this transfer was less efficient leading to the accumulation of both populations(Fig. 4A and Fig. 6A). In order to clarify whether FC-P1 is the precursor of FC-P2,transfection experiments were performed in the presence of neuraminidase and B7 antibody. FC-P1 and FC-P2 progeny virions were quantitatively analyzed by AEX-qPCR at 24 and 48 hpT when no significant *de novo* production or degradation of viral progeny particles were observed (Fig. 6B). As shown in Fig. 6C, FC-P1 was the predominant virus population 24 hpT, representing approximately two third of the total progeny virions. However, 48 hpT the total amount of FC-P1 virions significantly declined representing only one third of the whole virus progeny and giving rise to a significant increase in the amount of FC-P2 DNA-containing particles. These results indicate that FC-P1 particles are the precursors of FC-P2 virions. The maturation of FC-P1 would be more efficient in the human transformed cells than in the A9 murine fibroblasts.

**Nuclear FC-P2 represents the ultimate maturation step of MVM in terms of egress potential and infectivity.** We next examined whetherthe nuclear FC-P2 population represents the final maturation step in MVM morphogenesis or whether further maturation steps occur during the process of egress required for infectivity. To this end, nuclear and extracellular FC-P2 particles were purified by AEX and their infectivity quantitatively compared. Apart from having the same AEX profile, both particles were equally infectious (Fig. 7A). Therefore, virus egress does not involve further maturation steps required for infection.

**During entry, acidic phosphatases remove the FC-P2 surface phosphorylations and change its AEX profile to that of FC-P1.** In the nucleus, FC-P1 particles maturate to generate FC-P2, which are particles with nuclear export potential. During entry a reverse situation was observed, FC-P2 particles were processed to generate FC-P1 (Fig. 8). The N-VP2 of incoming FC-P2 became cleaved by endosomal proteases (Fig. 2F). However, as previously shown in Fig. 2G, 3A, and 3B, the presence or absence of N-VP2 with its distal phosphoserines has no influence in the AEX profile. As already demonstrated *in vitro* (Fig. 3B), the different AEX profiles results from the presence of capsid surface phosphorylations. Accordingly, these critical surface phosphorylations are removed from the incoming FC-P2 early during entry by endosomal acid phosphatases. In order to confirm the involvement of acid phosphatases in the processing of incoming particles, bafilomycin A1 (BafA1), which raises the endosomal pH and inhibit acid phosphatases, was applied to A9 cells. As shown in Fig. 8, BafA1 totally blocked the dephosphorylation of the incoming FC-P2 population.

**Discussion**

The active egress of enveloped viruses from infected cells is well documented and involves budding through host cell membranes. The egress of nonenveloped viruses is generally thought to be the result of a lytic burst occurring at the end of the infection. However, there is growing evidence that egress of nonenveloped viruses may not be a mere consequence of the passive virus release induced by cell lysis but it may involve a prelytic active transport of the progeny virions ([2](#_ENREF_2), [4](#_ENREF_4), [9](#_ENREF_9), [18](#_ENREF_18), [20](#_ENREF_20), [60](#_ENREF_60), [65](#_ENREF_65)). The proof of an active prelytic egress in nonenveloped viruses is challenging since the lysis of few cells can passively release mature virions complicating the discrimination between active and passive release. Therefore, it is not sufficient to detect progeny virions in the culture media prior to significant cell lysis. In addition, the identification of an active mechanism of virus egress is necessary. By using anion-exchange chromatography (AEX) we confirmed the existence of an active egress for the model parvovirus minute virus of mice (MVM) prior to passive release by cell lysis. Additionally, we identified late capsid maturation steps occurring in the cell nucleus preceding virus egress.

Following trimerization of translated viral structural proteins in the cytoplasm, the trimeric assembly intermediates translocate to the nucleus where they assemble to generate icosahedral empty capsids (ECs) ([47](#_ENREF_47)). Nuclear ECs are filled with the viral single-stranded DNA genome to generate full capsids (FCs) ([22](#_ENREF_22)). The amino-terminal domain of the VP2 major structural protein (N-VP2) is internal in ECs, but it becomes exposed outside of the shell through the fivefold axis of symmetry in FCs ([11](#_ENREF_11), [58](#_ENREF_58)). The exposed phosphoserine-rich N-VP2 has been suggested to mediate the export of the FCs out of the nucleus ([30](#_ENREF_30)), followed by virus egress, which was proposed to occur by vesicular transport through the endoplasmic reticulum and Golgi ([2](#_ENREF_2)).

By using AEX, proteins can be separated based on their net surface charges. Therefore, AEX can be used to separate virus populations displaying different protein surface configurations. Apart from ECs, the AEX profile of intranuclear MVM progeny revealed not one but two well-defined DNA-containing progeny populations, here named FC-P1 and FC-P2 (Fig. 1). FC-P1 progeny share many characteristics with the EC precursors. They appeared early, had a similar surface phosphorylation pattern, N-VP2 was internal and they were unable to be exported from the nucleus (Fig. 1D, 2C-F, and 4). FC-P2 virions appeared later, featured additional surface phosphorylations, N-VP2 was exposed and they showed nuclear export potential (Fig. 2C-F, 3, and 4). FC-P1 would represent a previously unrecognized stage in the MVM morphogenesis, intermediate between ECs and the late FC-P2 virions. The internal conformation of N-VP2 in FC-P1 indicates that, in contrast to the general assumption, DNA packaging alone is not sufficient to trigger the externalization of N-VP2. The AEX profile of the different virus progeny populations was exclusively determined by the phosphorylation status of highly surface capsid residues and not by the different conformations of the phosphoserine-rich N-VP2 (Fig. 3C and 3D).

Heat treatment or incubation at low pH did not change the AEX profile of FC-P1 even though these treatments externalized their N-VP2 termini (Fig. 2D, E, and G). Therefore, we conclude that the transfer of FC-P1 to FC-P2 is caused by a nuclear kinase rather than by structural rearrangements exposing capsid phosphorylations. The nuclear export competent FC-P2 virions represent the fully mature infectious progeny. The infectivity of FC-P2 progeny isolated from the nuclei (pre-egress) or actively released from the cells (post-egress) was the same (Fig. 7). Hence, during active egress no further maturation steps were required to acquire full infectivity. Interestingly, the efficiency to achieve this late nuclear maturation stage was cell type dependent. The kinetics for FC-P1 and FC-P2 virions in the murine A9 fibroblasts or in the human transformed NB324K cells differed significantly (Fig. 5A). In NB324K cells, FC-P1 virions were predominant early after the infection. At later times, FC-P2 virions, were the only DNA-containing progeny detectable in the nucleus. In A9 cells, FC-P1 were also the first population to appear but they did not mature efficiently to FC-P2 particles. Consequently, both populations accumulated at similar levels in A9 cells.

The efficient FC-P1 to FC-P2 conversion in the nucleus of NB324K cells indicates a high kinase activity for this cell type. The reduced kinase activity observed for murine cells is in accordance with previous studies reporting lower overall capsid phosphorylation levels in murine cells compared to transformed human cells ([29](#_ENREF_29)). An up-regulated activity of the respective kinase may contribute to enhanced nuclear export and active prelytic virus egress.

*~~Upregulation of nuclear kinases in cancer cells has been previously reported (ref). Therefore, the identified nuclear phosphorylation might contribute to the oncolytic capacity of MVM since virus progeny appears to better mature in transformed cells enhancing active virus egress and thus contributing to an improved cell to cell spread. (do not over-estimate…)~~*

The N-VP2 termini, particularly their distal serine phosphorylations, have been previously suggested to play a crucial role in the nuclear export of *de novo* synthesized virion progeny ([30](#_ENREF_30)). This data is in line with our findings which demonstrate that following packaging, FC-P1 particles with internal N-VP2 did not have nuclear export capacity. In order to challenge a possible involvement of N-VP2 and its prominent distal phosphorylations in the export of the late progeny population, we used two mutants. The first mutant, referred to as 5SG, lacks the five most distal serine phosphorylations within the N-VP2 termini. The second mutant, referred to as G33F, is unable to externalize the N-VP2 sequence on the surface of the capsid due to the insertion of a bulky phenylalanine residue at position 33 of its poly-glycine stretch within the VP2 protein sequence (Fig. ). As expected, removal of the distal serine phosphorylations of N-VP2 or prevention of its externalization had no influence in the different AEX profiles, which was exclusively defined by additional surface phosphorylations (Fig. 3C and 3D). Following transfection in NB324K cells, both mutants were able to generate the early FC-P1 and the late FC-P2 progeny populations in the nucleus and accumulated in the culture media with similar kinetics and quantities as observed for the WT (Fig. 3C and 6A). Compared to infection, transfection is expected to boost cell lysis and consequently increase passive release. Therefore, it is necessary to demonstrate the active segregation of the two nuclear populations. Analysis of the intra- and extracellular AEX profiles following transfection revealed that, equally to the WT virions, mainly FC-P2 particles accumulated in the media, indicating an active prelytic egress (Fig. 6B). These results confirm that the N-VP2 termini and their distal serine phosphorylations are not key players in virus egress. Parvoviruses display a high mutation rate comparable to RNA viruses ([49](#_ENREF_49), [50](#_ENREF_50)). Accordingly, genetic substitutions that interfere with crucial stages of the viral life cycle result in reversions after only a few rounds of infections. The distal S/G substitutions in N-VP2 were highly stable and no genetic reversions were observed following several passages (data not shown). In agreement with our findings, a deletion of seven amino acids within the sequence of N-VP2 with the intention to disturb its function did not affect egress of progeny particles. The only limitations reported for this truncation were a slight impairment for binding and nuclear targeting and thus a delayed time course of infection ([62](#_ENREF_62)).

The requirement of NS2 in progeny egress has already been demonstrated to be indispensable for murine A9 cells but it is not a prerequisite in transformed NB324K cells ([14](#_ENREF_14), [35](#_ENREF_35)). These results explain the previously observed cell type specific inhibition of nuclear export by leptomycin B (LMB) ([30](#_ENREF_30)). Despite extensive attempts, demonstration of a direct or indirect interaction between N-VP2 or other capsid regions and Crm1 failed. The dependence of progeny egress on the Crm1 export pathway may rather be indirect via the supraphysiological interaction between NS2 and Crm1 ([15](#_ENREF_15)).

Apart from the N-VP2 conformation, the surface phosphorylation pattern is the second prominent difference between FC-P1 and FC-P2 (Fig. 3). Therefore, it is tempting to speculate that the acquirement of additional surface phosphorylation(s) confers nuclear export potential to the late progeny population. There are alternative nuclear export routes that function in higher eukaryotes independently of the Crm1/exportin1 pathway involving the prototypic leucine-rich NES ([23](#_ENREF_23), [55](#_ENREF_55)); reviewed in reference ([27](#_ENREF_27)). These export mechanisms are predominantly regulated by protein phosphorylation ([21](#_ENREF_21), [43](#_ENREF_43)). Accordingly, the additional capsid surface phosphorylations in FC-P2 may explain their nuclear export potential. In line with this notion, these surface phosphorylations were efficiently removed by acidic endosomal phosphatases during entry of incoming virions, resulting in a complete reversion to FC-P1 particles (Fig. 8). Together with N-VP2 cleavage and N-VP1 externalization, the dephosphorylation of surface residues would represent a novel processing step during parvovirus cell entry which could be critical to confer nuclear import potential to the incoming capsids. In line with this concept, it has been previously shown that the endocytic route is required for nuclear targeting of CPV and AAV. Particles microinjected into the cytoplasm to bypass the endocytic route failed to target the nucleus, even when pretreated under acidic conditions ([53](#_ENREF_53), [63](#_ENREF_63)). These temporally controlled changes in capsid surface phosphorylation would provide nuclear import and export potential required to complete the life cycle of the karyophilic virus. Further studies are required to identify the corresponding phosphorylations on the capsid surface and to demonstrate their specific role in the active egress of the nonenveloped parvovirus.

**References**

1. **Bar, S., L. Daeffler, J. Rommelaere, and J. P. F. Nuesch.** 2008. Vesicular egress of non-enveloped lytic parvoviruses depends on gelsolin functioning. Plos Pathogens **4**.

2. **Bar, S., J. Rommelaere, and J. P. F. Nuesch.** 2013. Vesicular Transport of Progeny Parvovirus Particles through ER and Golgi Regulates Maturation and Cytolysis. Plos Pathogens **9**.

3. **Bieniasz, P. D.** 2006. Late budding domains and host proteins in enveloped virus release. Virology **344:**55-63.

4. **Bird, S. W., N. D. Maynard, M. W. Covert, and K. Kirkegaard.** 2014. Nonlytic viral spread enhanced by autophagy components. Proc Natl Acad Sci U S A **111:**13081-6.

5. **Bodendorf, U., C. Cziepluch, J. C. Jauniaux, J. Rommelaere, and N. Salome.** 1999. Nuclear export factor CRM1 interacts with nonstructural proteins NS2 from parvovirus minute virus of mice. J Virol **73:**7769-79.

6. **Caillet-Fauquet, P., M. Perros, A. Brandenburger, P. Spegelaere, and J. Rommelaere.** 1990. Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. EMBO J **9:**2989-95.

7. **Calistri, A., C. Salata, C. Parolin, and G. Palu.** 2009. Role of multivesicular bodies and their components in the egress of enveloped RNA viruses. Reviews in Medical Virology **19:**31-45.

8. **Castellanos, M., R. Perez, A. Rodriguez-Huete, E. Grueso, J. M. Almendral, and M. G. Mateu.** 2013. A slender tract of glycine residues is required for translocation of the VP2 protein N-terminal domain through the parvovirus MVM capsid channel to initiate infection. Biochemical Journal **455:**87-94.

9. **Clayson, E. T., L. V. Brando, and R. W. Compans.** 1989. Release of simian virus 40 virions from epithelial cells is polarized and occurs without cell lysis. J Virol **63:**2278-88.

10. **Cotmore, S. F., A. M. D'Abramo, C. M. Ticknor, and P. Tattersall.** 1999. Controlled conformational transitions in the MVM virion expose the VP1 N-terminus and viral genome without particle disassembly. Virology **254:**169-181.

11. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv Virus Res **33:**91-174.

12. **Daeffler, L., R. Horlein, J. Rommelaere, and J. P. F. Nuesch.** 2003. Modulation of minute virus of mice cytotoxic activities through site-directed mutagenesis within the NS coding region. J Virol **77:**12466-12478.

13. **Di Piazza, M., C. Mader, K. Geletneky, Y. C. M. Herrero, E. Weber, J. Schlehofer, L. Deleu, and J. Rommelaere.** 2007. Cytosolic activation of cathepsins mediates parvovirus H-1-induced killing of cisplatin and TRAIL-resistant glioma cells. J Virol **81:**4186-98.

14. **Eichwald, V., L. Daeffler, M. Klein, J. Rommelaere, and N. Salome.** 2002. The NS2 proteins of parvovirus minute virus of mice are required for efficient nuclear egress of progeny virions in mouse cells. J Virol **76:**10307-10319.

15. **Engelsma, D., N. Valle, A. Fish, N. Salome, J. M. Almendral, and M. Fornerod.** 2008. A supraphysiological nuclear export signal is required for parvovirus nuclear export. Molecular Biology of the Cell **19:**2544-2552.

16. **Farr, G. A., S. F. Cotmore, and P. Tattersall.** 2006. VP2 cleavage and the leucine ring at the base of the fivefold cylinder control pH-dpendent externalization of both the VP1 N terminus and the genome of minute virus of mice. J Virol **80:**161-171.

17. **Farr, G. A., L. G. Zhang, and P. Tattersall.** 2005. Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. Proc Natl Acad Sci U S A **102:**17148-17153.

18. **Feng, Z., L. Hensley, K. L. McKnight, F. Hu, V. Madden, L. Ping, S. H. Jeong, C. Walker, R. E. Lanford, and S. M. Lemon.** 2013. A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. Nature **496:**367-71.

19. **Herrero, Y. C. M., J. J. Cornelis, C. Herold-Mende, J. Rommelaere, J. R. Schlehofer, and K. Geletneky.** 2004. Parvovirus H-1 infection of human glioma cells leads to complete viral replication and efficient cell killing. Int J Cancer **109:**76-84.

20. **Jourdan, N., M. Maurice, D. Delautier, A. M. Quero, A. L. Servin, and G. Trugnan.** 1997. Rotavirus is released from the apical surface of cultured human intestinal cells through nonconventional vesicular transport that bypasses the Golgi apparatus. J Virol **71:**8268-8278.

21. **Kaffman, A., N. M. Rank, E. M. O'Neill, L. S. Huang, and E. K. O'Shea.** 1998. The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature **396:**482-486.

22. **King, J. A., R. Dubielzig, D. Grimm, and J. A. Kleinschmidt.** 2001. DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. Embo Journal **20:**3282-3291.

23. **Kutay, U., F. R. Bischoff, S. Kostka, R. Kraft, and D. Gorlich.** 1997. Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell **90:**1061-1071.

24. **Lombardo, E., J. C. Ramirez, M. Agbandje-McKenna, and J. M. Almendral.** 2000. A beta-stranded motif drives capsid protein oligomers of the parvovirus minute virus of mice into the nucleus for viral assembly. J Virol **74:**3804-3814.

25. **Lombardo, E., J. C. Ramirez, J. Garcia, and J. M. Almendral.** 2002. Complementary roles of multiple nuclear targeting signals in the capsid proteins of the parvovirus minute virus of mice during assembly and onset of infection. J Virol **76:**7049-7059.

26. **Lopez-Bueno, A., M. G. Mateu, and J. M. Almendral.** 2003. High mutant frequency in populations of a DNA virus allows evasion from antibody therapy in an immunodeficient host. J Virol **77:**2701-2708.

27. **Macara, I. G.** 2001. Transport into and out of the nucleus. Microbiology and Molecular Biology Reviews **65:**570-+.

28. **Mani, B., C. Baltzer, N. Valle, J. M. Almendral, C. Kempf, and C. Ros.** 2006. Low pH-dependent endosomal processing of the incoming parvovirus minute virus of mice virion leads to externalization of the VP1 N-terminal sequence (N-VP1), N-VP2 cleavage, and uncoating of the full-length genome. J Virol **80:**1015-24.

29. **Maroto, B., J. C. Ramirez, and J. M. Almendral.** 2000. Phosphorylation status of the parvovirus minute virus of mice particle: mapping and biological relevance of the major phosphorylation sites. J Virol **74:**10892-902.

30. **Maroto, B., N. Valle, R. Saffrich, and J. M. Almendral.** 2004. Nuclear export of the nonenveloped parvovirus virion is directed by an unordered protein signal exposed on the capsid surface. J Virol **78:**10685-10694.

31. **Martin-Serrano, J.** 2007. The role of ubiquitin in retroviral egress. Traffic **8:**1297-303.

32. **Martin-Serrano, J., and S. J. Neil.** 2011. Host factors involved in retroviral budding and release. Nat Rev Microbiol **9:**519-31.

33. **Maul, G. G.** 1976. Fibrils attached to the nuclear pore prevent egress of SV40 particles from the infected nucleus. J Cell Biol **70:**714-9.

34. **Merchlinsky, M. J., P. J. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an Infectious Molecular Clone of the Autonomous Parvovirus Minute Virus of Mice. J Virol **47:**227-232.

35. **Miller, C. L., and D. J. Pintel.** 2002. Interaction between parvovirus NS2 protein and nuclear export factor Crm1 is important for viral egress from the nucleus of murine cells. J Virol **76:**3257-3266.

36. **Mohl, B. P., and P. Roy.** 2014. Bluetongue virus capsid assembly and maturation. Viruses **6:**3250-70.

37. **Morita, E.** 2012. Differential requirements of mammalian ESCRTs in multivesicular body formation, virus budding and cell division. FEBS J **279:**1399-406.

38. **Muller, D. E., and G. Siegl.** 1983. Maturation of Parvovirus Luiii in a Subcellular System .2. Isolation and Characterization of Nucleoprotein Intermediates. Journal of General Virology **64:**1055-1067.

39. **Nachury, M. V., and K. Weis.** 1999. The direction of transport through the nuclear pore can be inverted. Proc Natl Acad Sci U S A **96:**9622-9627.

40. **Nuesch, E. R., S. Lachmann, and J. Rommelaere.** 2005. Selective alterations of the host cell architecture upon infection with parvovirus minute virus of mice. Virology **331:**159-174.

41. **Nuesch, J. P., S. Bar, S. Lachmann, and J. Rommelaere.** 2009. Ezrin-radixin-moesin family proteins are involved in parvovirus replication and spreading. J Virol **83:**5854-63.

42. **Nuesch, J. P., and J. Rommelaere.** 2006. NS1 interaction with CKII alpha: novel protein complex mediating parvovirus-induced cytotoxicity. J Virol **80:**4729-39.

43. **Ohno, M., A. Segref, A. Bachi, M. Wilm, and I. W. Mattaj.** 2000. PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation. Cell **101:**187-198.

44. **Ohshima, T., T. Nakajima, T. Oishi, N. Imamoto, Y. Yoneda, A. Fukamizu, and K. Yagami.** 1999. CRM1 mediates nuclear export of nonstructural protein 2 from parvovirus minute virus of mice. Biochem Biophys Res Commun **264:**144-50.

45. **Pintel, D., D. Dadachanji, C. R. Astell, and D. C. Ward.** 1983. The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. Nucleic Acids Res **11:**1019-38.

46. **Praefcke, G. J., and H. T. McMahon.** 2004. The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol **5:**133-47.

47. **Riolobos, L., J. Reguera, M. G. Mateu, and J. M. Almendral.** 2006. Nuclear transport of trimeric assembly intermediates exerts a morphogenetic control on the icosahedral parvovirus capsid. Journal of Molecular Biology **357:**1026-1038.

48. **Rubio, M. P., A. Lopez-Bueno, and J. M. Almendral.** 2005. Virulent variants emerging in mice infected with the apathogenic prototype strain of the parvovirus minute virus of mice exhibit a capsid with low avidity for a primary receptor. J Virol **79:**11280-11290.

49. **Shackelton, L. A., and E. C. Holmes.** 2006. Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. J Virol **80:**3666-3669.

50. **Shackelton, L. A., C. R. Parrish, U. Truyen, and E. C. Holmes.** 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc Natl Acad Sci U S A **102:**379-384.

51. **Shein, H. M., and J. F. Enders.** 1962. Multiplication and Cytopathogenicity of Simian Vacuolating Virus 40 in Cultures of Human Tissues. Proceedings of the Society for Experimental Biology and Medicine **109:**495-&.

52. **Slepchenko, B. M., I. Semenova, I. Zaliapin, and V. Rodionov.** 2007. Switching of membrane organelles between cytoskeletal transport systems is determined by regulation of the microtubule-based transport. J Cell Biol **179:**635-41.

53. **Sonntag, F., S. Bleker, B. Leuchs, R. Fischer, and J. A. Kleinschmidt.** 2006. Adeno-associated virus type 2 capsids with externalized VP1/VP2 trafficking domains are generated prior to passage through the cytoplasm and are maintained until uncoating occurs in the nucleus. J Virol **80:**11040-11054.

54. **Stamnes, M.** 2002. Regulating the actin cytoskeleton during vesicular transport. Curr Opin Cell Biol **14:**428-33.

55. **Stuven, T., E. Hartmann, and D. Gorlich.** 2003. Exportin 6: a novel nuclear export receptor that is specific for profilin center dot actin complexes. Embo Journal **22:**5928-5940.

56. **Tattersa.P.** 1972. Replication of Parvovirus Mvm .1. Dependence of Virus Multiplication and Plaque-Formation on Cell-Growth. J Virol **10:**586-&.

57. **Tattersall, P., and J. Bratton.** 1983. Reciprocal Productive and Restrictive Virus-Cell Interactions of Immunosuppressive and Prototype Strains of Minute Virus of Mice. J Virol **46:**944-955.

58. **Tattersall, P., A. J. Shatkin, and D. C. Ward.** 1977. Sequence Homology between Structural Polypeptides of Minute Virus of Mice. Journal of Molecular Biology **111:**375-394.

59. **Tucker, S. P., and R. W. Compans.** 1993. Virus infection of polarized epithelial cells. Adv Virus Res **42:**187-247.

60. **Tucker, S. P., C. L. Thornton, E. Wimmer, and R. W. Compans.** 1993. Vectorial release of poliovirus from polarized human intestinal epithelial cells. J Virol **67:**4274-82.

61. **Tullis, G. E., L. R. Burger, and D. J. Pintel.** 1993. The Minor Capsid Protein Vp1 of the Autonomous Parvovirus Minute Virus of Mice Is Dispensable for Encapsidation of Progeny Single-Stranded-DNA but Is Required for Infectivity. J Virol **67:**131-141.

62. **Tullis, G. E., L. R. Burger, and D. J. Pintel.** 1992. The Trypsin-Sensitive Rver Domain in the Capsid Proteins of Minute Virus of Mice Is Required for Efficient Cell Binding and Viral-Infection but Not for Proteolytic Processing Invivo. Virology **191:**846-857.

63. **VihinenRanta, M., A. Kalela, P. Makinen, L. Kakkola, V. Marjomaki, and M. Vuento.** 1998. Intracellular route of canine parvovirus entry. J Virol **72:**802-806.

64. **Votteler, J., and W. I. Sundquist.** 2013. Virus Budding and the ESCRT Pathway. Cell Host & Microbe **14:**232-241.

65. **Wirblich, C., B. Bhattacharya, and P. Roy.** 2005. Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101. J Virol **80:**460-73.