**Results**

**Two distinct populations of DNA containing particles accumulate during the productive infection of MVM in murine fibroblasts.** 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 analyzed by anion exchange chromatography (AEX), which separates 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 repeated freeze and thaw cycles to 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 18 hpi 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 (ref?). To verify their AEX profile, EC precursors were purified by CsCl gradient centrifugation, subjected to AEX, and visualized by dot blot using an antibody against assembled capsids (Mab B7; ref?). As illustrated in Fig. 1D, ECs had an AEX profile resembling that of the FC-P1 population. The fact that FC-P1 capsids and EC are predominantly cell-associated, and have the same AEX profile, would suggest that they represent immature particles unable of being exported from the nucleus, 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 population 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 structural protein VP2 (N-NP2) occupies an external position in the capsid. However, during entry N-VP2 is cleaved by endosomal proteases to render a truncated protein referred to as VP3. The function of N-VP2 cleavage is not fully understood, but it seems to be required to allow the exposure of the N-terminal region of VP1 (N-VP1) which harbors important functional motifs which are essential for infection, particularly endosomal escape and nuclear targeting (ref?). We analyzed the surface conformation of N-VP2 in the two populations of FC by immunoprecipitation (IP) with a specific antibody raised against this region (ref?). 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, ECs 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 Fig. 2E, FC-P2 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 position. 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 progeny types bound similarly to cells as shown by immunofluorescence (IF). The N-VP2 of 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 of 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 predominant 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 Fig 2D) and subjected to AEX. As shown in Fig. 2G, exposure of N-VP2 did not change the AEX profile of FC-P1. Although clearly distinct between the two FC populations, the N-VP2 conformation is not responsible for the different AEX profile. The results also emphasize further similarities between the FC-P1 and EC populations.

**Capsid surface phosphorylation 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 sub-sequentially analyzed by AEX-qPCR. As expected, only FC-P2 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 (ref). 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 identical 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 characteristic AEX profile.

**Only FC-P2 can actively egress from infected host cells.** The subcellular distribution of the two FC progeny types was examined. A9 cells were infected with MVM and at progressive times pi, progeny viruses were collected from nuclear, cytoplasmic, or 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. 4D) and resulted in the appearance of FC-P1 in the cytoplasmic and supernatant fractions. The strong segregation of the initially mixed nuclear full capsid progeny FC populations 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.

**FC-P1 represents the direct precursor of FC-P2.** Transfection experiments were performed in order to bypass early trafficking of incoming virions during entry which is giving rise to virus degradation in the lysosomes. Reinfection through *de novo* synthesized progeny virions was prevented by supplementing the media with 330 U/mL neuraminidase and **xy** ug/mL B7 antibody. The quantity FC-P1 and FC-P2 progeny virions was analyzed by AEX-qPCR at 24 and 48 hpt when viral steady-state levels were achieved in transfected cells in the absence of a considerable degradation of DNA-containing virus particles (Fig. 5A). As shown in Fig. 5B, FC-P1 was the predominant virus population at 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.

Assuming that there is no considerable degradation of MVM progeny virions in transfected cells, these results indicate that FC-P1 particles are quantitatively transferred to FC-P2 nuclear export competent virions, thus supporting the hypothesis that there is an almost dynamic, sequential arrangement between both DNA-containing progeny populations.

**N-VP2 is not involved in the nuclear export of MVM**. The role of N-VP2 and its distal phosphorylations in the nuclear export of MVM was investigated. We used a mutant lacking the five most distal serine phosphorylations on the N-VP2 terminus, called 5SG. Additionally, we used a MVM mutant containing a bulky phenylalanine residue at position 33 within the flexible poly-glycine stretch of the VP2 protein sequence, referred to as G33F. Due to this substitution the mutant progeny particles were unable to externalize N-VP2 following DNA packaging. As previously reported, N-VP2 cleavage was abolished and VP1u externalization and consequentially infectivity was prevented (ref). However, though being non-infectious, DNA-containing progeny particles of G33F progressively accumulated in the media at increasing times post-transfection to similar quantities as 5SG and 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, substantiating that N-VP2 and/or its distal phosphorylations are not responsible for the two distinct AEX profiles of FC-P1 and FC-P2. To examine whether the observed accumulation of G33F, 5SG, and WT progeny particles in the media resulted from an active nuclear export and subsequent egress we analyzed the intra- and extracellular FC-P1/FC-P2 ratios 24 hours post-transfection using WT and G33F plasmids. FC-P1, the predominant population at 24 hpt (Fig. 5B), serves as a marker for lytic passive egress since it did not accumulate in the media in the absence of cellular lysis (Fig. 4 C and D). Although more FC-P1 was present in the cellular extracts, the accumulation of FC-P2 particles in the media exceeds the one of FC-P1 24 hpT (Fig. 5B). This only can be explained by a predominant active egress of FC-P2 particles in the presence of a marginal passive release of virus progeny induced by viral cytotoxicity as well as detrimental impacts caused by the transfection.

In summary these results rule out any direct involvement of the N-VP2 sequence or its distal phosphorylations in the nuclear export and active egress of MVM.

**During endocytosis acidic phosphatases remove the FC-P2 surface phosphorylations and change its AEX profile to that of FC-P1.**

Incoming FC-P2 become cleaved by endosomal proteases (Fig. 2F). As previously demonstrated in Fig. 2G, 3A, and 3B, externalized N-VP2 which contains four distal serine phosphorylations is not responsible for the distinct AEX profile of the two FC populations. Nevertheless, when MVM was internalized in A9 cells, the AEX profile of the initially mixed FC populations became homogenized in a way that all virions displayed a FC-P1 AEX profile (Fig. 7A). As already demonstrated *in vitro* (Fig. 3B), the different AEX profiles of the two FC populations result from different surface phosphorylations which give rise to distinct surface net charges. Accordingly, the critical phosphorylation(s) is removed from the surface of FC-P2 during early trafficking by an endolysosomal phosphatase. In order to challenge this hypothesis, the lysosomotropic drug Bafilomycin A1, a vacuolar-type H+-ATPase inhibitor which raises the endosomal pH (ref?), was applied to A9 cells (…at 150 nM). As shown in Fig. 7A, Bafilomycin A1 is able to prevent dephosphorylation of the FC-P2 population. This result indicates that a pH-dependent, acidic endosomal phosphatase removes the phosphorylation(s) from the surface of incoming FC-P2 virions.

Interestingly, when progeny virions were analyzed at increasing times p.i., FC-P1 was the first population to occur in the nuclei of infected A9 or NB cells. At later times, A9 and NB cells showed important differences. Progeny virions completely inverted their AEX profile from FC-P1 to FC-P2 when isolated from the nuclei of infected NB cells. In contrast, the transfer from FC-P1 to FC-P2 was not complete in A9 cells. Consequentially, both FC populations co-existed in similar amounts in the nuclei of infected A9 cells. However, whether FC-P1 is a direct precursor of FC-P2 remains unclear.