Discussion:

The study of viral egress is of interest since it directly affects the transmission and proliferation of virus progeny trough the host’s tissue. The egress of enveloped viruses is well documented. At the end of their replication cycle they form vesicles at the host’s plasma membrane and egress via budding. The egress of non-enveloped viruses is poorly understood. It has long been thought that the egress of non-enveloped viruses is the result of a lytic burst occurring at the end of a virus infection. However, there is growing evidence that egress of non-enveloped viruses is not a mere consequence of the passive virus release induced by cell lysis but involves a prelytic active vesicular transport of the progeny virions (Nüesch..).

In the case of enveloped viruses, actively egressing progeny acquires a host cell membrane derived envelope and thus it is simply distinguishable from passively released viruses lacking such envelope. The documentation of an active prelytic egress of non-enveloped viruses is complex since the lysis of only few cells can passively release virions that might be misinterpreted as actively egressing progeny. Therefore, it is of importance to not only demonstrate the presence of virions in the culture media prior to cytolysis but to directly show an active retrograde transport of virus progeny. By using anion-exchange chromatography (AEX) we were able to identify a late nuclear maturation step leading to a segregation of two DNA-containing virion populations. Only one population was able to actively egress the cell prior to cell lysis, confirming an active egress for MVM, a commonly accepted model parvovirus.

The two DNA-containing progeny populations differed in their net surface charges in consequence of distinct phosphorylation states resulting in distinctive AEX profiles. Interestingly, ECs displayed an identical AEX profile as the FC-P1 population, indicating a similar phosphorylation pattern on their capsid surface. Significantly, both ECs and FC-P1 were unable of being exported from the nucleus. In contrast, the nuclear export competent FC-P2 virions featured a more negatively charged capsid surface caused by additional phosphorylations. Moreover, the N-VP2 conformation represented a major structural difference between FC-P1 and FC-P2. While externalized in FC-P2, the N-VP2 termini of FC-P1 were not accessible to antibodies or proteolytic digestion as previously observed for ECs (ref). This result clearly demonstrates that, opposite to the common perception, DNA packaging is not sufficient to trigger the externalization of N-VP2. Additional maturation steps are required to provoke N-VP2 exposure. These observations encouraged us to investigate a potential involvement of N-VP2 in the nuclear export of MVM.

The N-VP2 termini, particularly their distal phosphorylations, have been previously suggested to play a crucial role in the nuclear export of *de novo* synthesized virion progeny (4SG, Almendral). This data is in line with our findings that nuclear export capacity was observed only for the N-VP2 exposing FC-P2 virions. In order to challenge a possible involvement of N-VP2 and its prominent distal phosphorylations in the export of FC-P2 virions, we used two genomic 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. Our results revealed that removal of the distal serine phosphorylations of N-VP2 or prevention of its externalization had no influence in the different AEX profiles. Both mutants were still able to generate FC-P1 and FC-P2 virions. These findings indicate that other surface phosphorylations distinct from the distal N-VP2 serine phosphorylations determine the two characteristic AEX profiles.

Unexpectedly, these N-VP2 defective mutants were able to accumulate in the culture media to similar quantities as observed for the WT. Analysis of the intra- and extracellular AEX profiles following transfection in transformed human fibroblasts revealed that, equally to the WT virions, mainly FC-P2 particles accumulated in the media, indicating an active prelytic egress. These results are not in agreement to previous studies suggesting a role of the N-VP2 termini and their distal serine phosphorylations in egress (4SG, Almendral). However, importance of the distal phosphorylations of N-VP2 for nuclear export has been only demonstrated for transformed human fibroblasts. In addition, no genetic reversions were observed for mutants harboring the distal S/G substitutions within the N-VP2 termini. For genetic substitutions interfering with crucial stages of the viral life cycle, reversions are commonly observed after only a few rounds of infections with parvoviruses which were affirmed to display similar high mutation rates to RNA viruses (ref). In murine fibroblasts but not in transformed human fibroblasts, progeny egress was sensitive to inhibition of Crm1 by LMB and required the infection process itself. 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 (ref, Almendral 4SG). The requirement for NS2 in progeny egress has already been demonstrated to be indispensable for murine cells (Pintel, Salomé), explaining the cell type specific inhibition of nuclear export by LMB treatment (Almendral, 4SG). Moreover, a 7 aa deletion in the trypsin sensitive RVER region within the sequence of N-VP2 with the intention to affect 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 (Pintel, own observations).

Apart from the N-VP2 conformation, the surface phosphorylation pattern is the second prominent difference between FC-P1 and FC-P2. The surface phosphorylations responsible for the characteristic AEX profiles for FC-P1 and FC-P2 changed during a productive infection. Following pre-assembly of ECs, FC-P1 was the first progeny virion population to occur in the nucleus and was transferred into FC-P2 particles in corresponding stoichiometric amounts. FC-P2 was the only population able to be exported from the nucleus. Therefore, it is tempting to speculate that the acquirement of additional surface phosphorylation(s) confers a nuclear export potential to the FC-P2 population. Moreover, these surface phosphorylations providing a putative nuclear export capacity to FC-P2 particles were efficiently removed by acidic endosomal phosphatases during endocytic trafficking of incoming FC-P2 virions, resulting in a complete reversion towards FC-P1 particles. These changes in surface phosphorylation would provide nuclear export and import potential required to complete the life cycle of karyophilic viruses. Previously, there have been described alternative nuclear export routes that function in higher eukaryotes independently of the Crm1/exportin1 pathway involving the prototypic leucine-rich NES (ref 29, 62; reviewed in reference 36 🡪refs with respect to 4SG paper). These export mechanisms are predominantly regulated by protein phosphorylation (25, 46 🡪with respect to 4sg). 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 can 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 (ref).

Interestingly, the kinetics for FC-P1 and FC-P2 virions in murine fibroblasts or human transformed cells differed significantly. In human transformed cells, FC-P1 virions appeared early in the nucleus but never accumulated in large quantities. In clear contrast, FC-P2 virions accumulated in the nuclei of infected cells at late times post-infection. The efficient and complete transfer of FC-P1 to FC-P2 in the nucleus of transformed human cells indicates a high kinase activity for this cell type. In restrictive murine cells, both populations continuously increased to similar amounts. The FC-P1 precursors were not completely transferred to FC-P2, indicating a lower activity of the corresponding kinase in the nuclei of these cells. 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 (4SG; Almendral). 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 reported previously (ref). Therefore, the identified nuclear phosphorylation might contribute to the oncolytic capacity of MVM since virus progeny appears to mature better in transformed cells enhancing active virus egress and thus contributing to an improved cell to cell spread.

The nuclear export competent FC-P2 virions represent the fully mature progeny of a productive infection. FC-P2 progeny isolated from the nuclei of infected cells was equally infectious as actively released FC-P2 virions in the media. Hence, no further maturation steps are required with regards to infectivity. However, we do not exclude other maturations required for active transport towards the cell periphery or improving capsid stability in order to better resist environmentally induced stress.

In summary, this study clearly demonstrates the existence of an active prelytic egress for a non-enveloped virus. Late nuclear maturation involves capsid surface phosphorylations that were related to nuclear export and active progeny egress. However, the detailed mechanism for progeny egress remains elusive. Further studies are required to identify the corresponding surface phosphorylations and to demonstrate their direct or indirect implications in the egress of non-enveloped viruses.