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1 Cytoplasmic parvovirus capsids recruit importin beta for nuclear delivery 2 3 Elina Mäntylä,^{a,b} Vesa Aho,^c Michael Kann,^{d,e} Maija Vihinen-Ranta^c 4 5 6 BioMediTech, Faculty of Medicine and Health Technology, Tampere University, Tampere, 7 Finland^a; Tampere University Hospital, Tampere, Finland^b; Department of Biological and 8 Environmental Science and Nanoscience Center, University of Jyvaskyla, Jyvaskyla, Finland^c; 9 Department of Infectious Diseases, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden^d; Region Västra Götaland, Sahlgrenska University Hospital, 10 Department of Clinical Microbiology, Gothenburg, Sweden^e. 11 12 13 14 #Address correspondence to Maija Vihinen-Ranta, maija.vihinen-ranta@jyu.fi 15 Department of Biological and Environmental Science and Nanoscience Center, P.O. Box 35, FI-40014 University of Jyvaskyla, Finland. Phone: +358 400 248 118 16 17 18 Running head: Parvoviral capsid interactions with importin beta 19 20 21 ABSTRACT 22 Parvoviruses are an important platform for gene and cancer therapy. Their cell entry and the 23 following steps including nuclear import are inefficient limiting their use in therapeutic applications. Two models exist on parvoviral nuclear entry: classical import of the viral capsid 24 25 using nuclear transport receptors of the importin (karyopherin) family, or direct attachment of the 26 capsid to the nuclear pore complex leading to local disintegration of the nuclear envelope. Here, by

27 laser scanning confocal microscopy and *in situ* proximity ligation analysis combined with co-28 immunoprecipitation we showed that infection requires importin β -mediated access into the nuclear 29 pore complex and nucleoporin 153-mediated interactions on the nuclear side. Importin β -capsid 30 interaction continued within the nucleoplasm, which suggests that a mixed model of nuclear entry 31 in which the classical nuclear import across the nuclear pore complex is accompanied by transient 32 ruptures of the nuclear envelope allowing also passive entry of importin β -capsid complexes into

33 the nucleus.

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36 IMPORTANCE

Parvoviruses are small DNA viruses that deliver their DNA into the post-mitotic nuclei, which is an important step for parvoviral gene and cancer therapies. Limitations in virus-receptor interaction or endocytic entry do not fully explain the low transduction/infection efficiency, indicating a bottleneck after virus entry into the cytoplasm. We thus investigated the transfer of parvovirus capsids from the cytoplasm to the nucleus showing that the nuclear import of parvovirus capsid follows a unique strategy, which differs from classical nuclear import and that of other viruses.

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44 Key words: Parvovirus capsid, importin β , interaction, cytoplasm, nucleoplasm

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50 INTRODUCTION

51 The vast majority of DNA viruses replicate in the nucleus. This requires that the viral genome 52 traverses the cytoplasm toward the nucleus and subsequently passes the nuclear envelope (NE). The 53 viral DNA is surrounded by viral proteins that form a protective capsid around it. After cytoplasmic 54 entry, the DNA-containing capsids exploit active transport toward the NE using cytoplasmic dynein 55 for their movement along microtubules (1-3). A few viruses such as Human T-cell leukemia virus 56 type 1 (HTLV-1) and papillomaviruses (4, 5) need NE degradation to gain access to the nucleus but the vast majority infects non-dividing cells. This requires passage through the nuclear pore 57 58 complexes (NPC), which are the only aqueous connections between the cytoplasm and nucleus. 59 Due to the 39 nm size limit of the pore (6), capsids of most viruses such as the non-enveloped 60 adenoviruses or the herpes simplex virus type 1 (HSV-1) disintegrate at the cytoplasmic side, requiring either direct or indirect interaction with the NPC (7). The released viral genomes mostly 61 62 remain attached to karyophilic viral proteins and are then actively imported (e.g. adenoviral genomes), or the genomes pass the NPC by repulsion forces upon capsid opening (HSV-1) (8–11). 63

Active nuclear import is needed for macromolecules with a size larger than 2.6 - 9 nm (12–15). It is 64 65 mediated by nuclear transport receptors of the importin (Imp) β superfamily (16–18). In the classical pathway, Imp β attaches via Imp α to a nuclear localization signal (NLS) exposed on the 66 67 cargo surface (19, 20). Imp α provides the NLS-binding domain and an Imp β -binding domain, whereas Imp β mediates the subsequent interactions with the NPC that drive translocation through 68 69 the pore into the nuclear basket (21, 22). The basket is a filamentous structure on the nuclear face of 70 the NPC, and in metazoan cells the nuclear basket is composed of three different nucleoporins 71 (Nups): Tpr, Nup50, and Nup153 (23). The import process is terminated at the C-terminus of Nup153 where the import complex is dissociated by the GTP-bound form of ras-related nuclear 72 73 protein (Ran) (24–26), which binds to Imp β . While the Imp β -RanGTP complex becomes directly 74 recycled back to the cytoplasm, the released cargo and Imp α diffuse deeper into the nucleus (21,

27) from where Imp α becomes exported into the cytoplasm in a complex with the Cellular Apoptosis Susceptibility gene protein (CAS) and RanGTP (28, 29). This pathway exists also in an Imp α-independent form in which the cargo exposes an Imp β-binding domain allowing direct Imp β-binding. Furthermore, nuclear import can be mediated by other functional Imp β homologues such as transportin (30, 31), and nuclear import of macromolecules and viruses independent of transport receptors has been described (32–35).

81 Parvoviruses (18-26 nm) and hepatitis B virus (HBV) capsids (36 nm) are smaller than the physical 82 diameter of the NPC, which allows the passage of intact capsids across the NPC. HBV capsids use 83 Imp α/β -mediated nuclear import (36), but instead of diffusing deeper into the nucleoplasm the 84 capsids remain attached to Nup153 (37, 38). Parvoviruses comprise protoparvoviruses such as the 85 canine parvovirus (CPV) and parvovirus H1, the latter used in the first phase I/IIa clinical trial of 86 glioblastoma (39, 40). The second group of parvoviruses, constituting the genus 87 dependoparvoviruses, includes adeno-associated viruses which are widely used as gene therapy 88 vectors (AAV) (41).

89 Most parvoviruses are composed of two viral proteins (VP) VP1 and VP2 and of non-structural 90 protein 1 (NS1), which is bound to the viral DNA. They enter cells via clathrin-mediated 91 endocytosis requiring acidification in late endosomes for productive infection (42-45). The 92 acidification changes the parvovirus structure leading to the exposure of the N-terminus of VP1, 93 which is hidden in the virion. This sequence comprises a cluster of four basic amino acids 94 (MAPPAKRARRGLV in CPV) fulfilling the minimal sequence requirement for a classical NLS 95 (K-K/R-X-K/R) (21, 46–49). It further exhibits a phospholipase A2 domain, which is essential for 96 endosomal release and progress of infection (2, 50-54) but is dispensable for the disintegration of 97 the nuclear envelope in AAV (55).

98 Parvoviruses reach the perinuclear region of the nucleus by dynein-mediated transport ~ 1 hour post 99 infection (pi) (56, 57). The subsequent steps of their NPC interaction have remained controversial. 100 The exposure of a putative NLS supports a classical nuclear transport using Imp α/β , which 101 suggests nuclear entry of the intact capsid. This conclusion is supported by analyses of fluorescence 102 fluctuations showing correlation of CPV capsid- and Imp β -movement across the NE (56). 103 However, the need of importins is not unequivocally clear as an unconventional Imp α /Imp β -104 independent nuclear import motif was found on the minute virus of mice (MVM) mediating the 105 nuclear import of at least preassembled capsid subunits (58, 59). In contrast, Porwal et al. reported 106 that a direct attachment of PV-H1 and AAV2 to Nups caused local NE degradation activating key 107 enzymes in mitosis (60). NE fenestration allows entry of macromolecules such as IgG (60) but also 108 of papillomaviruses, which have a diameter of 55 nm (5).

109 To differentiate between these scenarios, which are important for understanding the restricting 110 factors of parvovirus infection, we investigated the CPV capsid interplay with Imp β and NPC at early infection. Our studies demonstrated the need of Imp β for infection but also showed NE 111 112 disintegration, allowing us to propose a mixed model of nuclear import, which is consensual with 113 previously contradictious observations.

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115 **RESULTS**

116 **Cytoplasmic microinjection of Imp** β **antibodies inhibits CPV infection.** To determine whether 117 Imp β transport is important for capsid nuclear import and progression of infection, we studied the 118 effect of cytoplasmically co-microinjected anti-Imp β monoclonal antibody (MAb) on infection. 119 Infected cells were detected by nuclear emergence of CPV capsid proteins. Microinjected cells were 120 identified by co-microinjection with fluorescent dextran (150 kDa, radius of gyration $r_g = 13$ nm) 121 that remains cytoplasmic (61). Imaging at 16 and 24 h pi demonstrated that the proportion of 122 nuclear capsid protein-positive cells in the presence of anti-Imp β MAb was strongly reduced (Fig. 123 1A and B). In the non-microinjected control cells, capsid proteins accumulated in the nucleus 124 excluding the nucleoli (62). Finally, in microinjected cells infected for 4 h, viral proteins were 125 detected in the cytoplasm. At this early stage of infection, before expression of viral proteins, 126 cytoplasmic viral proteins are most likely associated with the entering viral capsids (Fig. 1C). 127 Microinjection of control Ab, anti-mouse IgG, does not affect the nuclear import of capsids (2). 128 Fluorescent dextran was not observed in the nucleoplasm, and we conclude that neither the 129 microinjection of the antibodies nor infection induced significant damage to the NE.

In the non-microinjected cells, Imp β located in the cytoplasm and at the NE, while the microinjection of anti-Imp β MAb led to its nuclear localization (**Fig. 1A and B**, grey and white panels). This is in agreement with an earlier study showing that Imp β antibodies preclude the formation of carrier-cargo complexes by preventing Imp β and Imp α interaction and subsequent reexport from the nuclear basket (63).

135 The number of microinjected and non-microinjected cells with nuclear capsid proteins was 136 analyzed. The occurrence of nuclear capsid proteins in anti-Imp β -microinjected cells was 77% 137 lower at 16 h pi and 74% lower at 24 h pi compared to that in control cells (**Fig. 1D**). This suggests 138 that the anti-Imp β MAb-induced inhibition of nuclear import of capsids and/or capsids proteins 139 inhibited the progression of infection. 140

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141 Nuclear entry of CPV is NPC-dependent. Next, we investigated whether CPV infection requires 142 Imp β -dependent nuclear import for passage across the NPC. To address this question we microinjected HBV capsids and wheat germ agglutinin (WGA) into the cytoplasm prior to 143 144 infection. HBV capsids block in particular Imp α/β -dependent nuclear import via their interaction 145 with Nup153 (38), and WGA completely blocks active nuclear transport for an hour with 146 decreasing blocking efficiency after that (about 50% at 4 hours after microinjection) (64). We 147 analyzed the effect of NPC blockage on the progression of CPV infection and initiation of 148 replication by detecting the nuclear presence of the viral replication protein NS1. Immunostaining 149 revealed a significant suppression or complete lack of nuclear NS1 proteins in the presence of HBV 150 capsids at 16 h pi (Fig. 2A and 2B). Quantitative analysis showed an 86% decrease in the number 151 of nuclear NS1-positive HBV-microinjected cells and a 67% decrease in the number of nuclear NS1-positive WGA-microinjected cells, which suggests that CPV has to pass the NPC in order to 152 153 initiate infection. Since the NPC blockage by WGA occurs only at the beginning of the infection 154 before significant viral protein production takes place, the observed decrease of nuclear NS1 later at infection is probably due to the blockage of capsid entry into the nucleus and not due to the 155 156 blockage of de novo synthesized capsid proteins or nonstructural proteins.

157 Based on approximately 4000 NPCs per cell (65-68), the number of microinjected HBV capsids 158 corresponded to approximately 1:1 ratio of HBV/NPC. This concentration was chosen as previous studies showed that it leads to a reduction of nuclear import of Imp α/β -dependent karyophilic 159 160 cargoes by 70% (38). This occurs via capsid binding to the nuclear basket-localized Nup153, which 161 is crucial for importin β -cargo dissociation. Transportin-mediated nuclear import, which is essential 162 for cell viability, remains in contrast unchanged (38). Higher capsid concentrations would have 163 caused the blockage of the central channel as we showed earlier by electron microscopy (38). In 164 contrast, 4000 HBV capsids is far below the number of karyophilic cargoes per cell, which

practically excludes indirect effects by competing with physiological nuclear import. As HBV capsids in a concentration of one per NPC mainly localize in the nuclear basket and not within the channel (38), our finding further indicates that the inhibition of infection was not caused by sterical block of the NPC but rather by blocking the access of CPV capsids to Nup153.

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Intracellular capsid interactions with Imp β . According to the classical nuclear import of karyophilic cargoes, CPV capsids should bind Imp α/β in the cytoplasm and pass through the pore into the nuclear basket where Imp β dissociates followed by direct recycling into the cytosol. We first verified the capsid-Imp β -interaction by co-immune precipitation showing that CPV from infected cells is in fact bound to Imp β (**Fig. 3A**). As the precipitation was done at 1 h post infection, which is before viral proteins are made (69), we conclude that Imp β interacted with incoming capsids and not with progeny ones.

177 To further analyze the temporal and spatial location of capsid Imp β -interactions, we used *in situ* 178 proximity ligation assay (PLA) (70). This method allows detection of single intermediate or direct 179 interactions between two proteins in their native form. The interactions, even weak or transient, are 180 detectable when the distance between the proteins is less than 40 nm. We analyzed the capsid-Imp β PLA signal distribution at 1, 2, 4 and 6 h pi, considering that newly synthesized viral capsid 181 182 proteins can be disregarded for at least 4 h pi (69). Fig. 3B shows that the majority of signals were 183 located in the perinuclear cytoplasm but a few signals were also found in the nuclear area. 184 Quantitative analysis at various times after infection showed a temporal increase in the number of 185 PLA signals (Fig. 3C). At 1 h pi, the PLA signal density in the 2D maximum intensity projections of the cells was 0.025 ± 0.008 PLA/µm² (mean PLA signal/area ± standard deviation, STDEV) with 186 187 19.0 ± 1.4 signals per cell (PLA/cell). At 2 and 4 h pi an increase in interaction was detected (0.06 \pm 0.020 PLA/um^2 , $60 \pm 30 \text{ PLA/cell}$; $0.12 \pm 0.04 \text{ PLA/um}^2$, $100 \pm 50 \text{ PLA/cell}$, respectively). At 6 h 188 pi the mean PLA signal density slightly decreased ($0.08 \pm 0.04 \text{ PLA/um}^2$, $90 \pm 8 \text{ PLA/cell}$), which 189

190 was, however, within the range of the variability between the individual cells. The positive control 191 of PLA signal between antibodies against VP2 capsid protein and intact capsids at 1 h pi verified 192 the specificity of the PLA signal (Fig. 3D). When the capsids were labeled with antibodies against capsid proteins and intact capsids at 1 h pi the total number of signals was 0.09 ± 0.04 PLA/um² 193 and 88 ± 30 PLA/cell (n = 9 cells). The negative viral capsid antibody control in non-infected cells 194 195 (Fig. 3D) and technical controls with PLA probes only in 1 h pi infected cells (Fig. 3E) indicated 196 that the background was low (approximately 7.18 PLA signals/cell, n = 170, and 0.09 PLA 197 signals/cell, n = 40, respectively).

198 To study the nuclear and cytoplasmic distribution of interaction, we analyzed 3D confocal 199 microscopy images of PLA signals at 1 h pi. The analysis showed that the majority of the PLA 200 signals was located in the cytoplasm, but some of the signals were located inside the nucleus (Fig. 201 4A). To quantitate the signal localization, the PLA signals were segmented and their lateral 202 distances to the NE, defined by the border of DAPI-staining, were calculated (Figs. 4B & 4C). The 203 analysis showed that at 1 h pi on average 13 ± 7 signals were located in the nucleus and 90 ± 50 204 signals in the cytoplasm. The number and density of cytoplasmic signals increased from the cell 205 periphery toward the NE. This is in agreement with earlier studies showing that parvovirus capsids 206 are transported into the nucleus in a similar time frame after infection (2, 57, 58, 71). The 207 accumulation of PLA signals in the nuclear periphery further implies that structural change in the 208 acidic environment, which allows importin-binding, is preserved after capsid neutralization. 209 Ouantitative analysis showed that within the nucleus the highest number and density values were 210 found close to the NE, but interestingly, a small portion of signals was located also deeper, within 211 $0.75 - 6.0 \ \mu m$ from the NE.

212 In summary, these findings indicate that the nuclear entry of capsids is preceded by the cytoplasmic 213 interaction of capsid with Imp β and is followed by the nuclear access of capsids, which in contrast 214 to classical karyophilic cargos, were still interacting with Imp β .

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216

217 **DISCUSSION**

The nuclear entry of parvoviral capsids is controversial as previous observations indicate a classical nuclear import via transport receptors or by transient permeabilization of the NE following attachment to the NPC.

221 The first model requires a structural change upon virus entry leading to exposure of a classical NLS 222 on the N-terminus of VP1. As all parvoviruses exhibit diameters below the transport limit of the 223 nuclear pore, it was hypothesized that the capsid-Imp β -complex follows the classical nuclear 224 import pathway, which comprises dissociation of the complex in the nuclear basket after binding to Nup153 (72–74). This would result in capsid entry into the nucleus while Imp β becomes recycled 225 226 back to cytoplasm. In agreement with this model, our results show that anti-Imp β antibody 227 microinjection strongly inhibited infection. Inhibition was not complete indicating the presence of 228 functional Imp β molecules, which in turn are required to maintain cell viability. Noteworthy, the 229 need of Imp β for CPV infection does not exclude the requirement of Imp α as an adaptor molecule. 230 Further support for this model comes from our observation that no significant entry of 150 kDa 231 Dextran was observed, indicating that the NE remained intact. The conclusion that CPV capsids 232 need direct or indirect access to Nup153 for infection is also congruent, as Imp β dissociates from 233 cargos after interaction with Nup153 within the basket (73).

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The second model, based on the NE permeabilization, neither requires nor excludes VP1 N-terminal domain exposure during early infection. Co-immunoprecipitation studies showed that parvoviruses interact directly with some NPC proteins enhancing exposure of the N-terminal VP1 domain. The identified nucleoporins localize in all parts of the NPC, including cytoplasmic filaments (Nup358), the central channel (Nup62) and the nuclear basket (Nup153) (60). These findings, however, do not

240 exclude that Imp β could mediate the attachment, possibly prior to direct capsid-interaction with the 241 NPC. This would be similar to that occurring during transport of HBV capsids (36, 37) where Imp β allows the passage of the capsid through the central cannel of the NPC, which is a requirement for 242 243 its interaction with Nup153. In the experiments leading to this model, nuclear permeabilization was 244 not only observed after cytoplasmic microinjection of different parvoviruses (AAV2, PV-H1), but also after infection (PV-H1) allowing nuclear entry of polyomaviruses (5). Evidently, our 245 246 observation that at least no significant amounts of 150 kDa dextran entered the nucleus seemingly 247 contradicts pore formation in the NE in particular since antibodies (a typical IgG is ~14.2 nm in 248 diameter) entered the nucleus after parvoviral microinjection (60, 75, 76). It must be considered 249 however that the number of microinjected capsids in these experiments were much higher than what 250 can be achieved with infections, and that the nuclear permeabilization is transient allowing 251 maintenance of the nuclear cytoplasmic gradient of e.g. RanGTP, which is crucial for cell viability 252 (77, 78). In support of nuclear permeabilization, we observed a fraction of nuclear CPV capsids 253 associated with Imp β . This observation indicates that at least these capsids had not interacted with

Nup153, which is a prerequisite for Imp β -dissociation.

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256 Collectively, our data allow proposing a composite model combining the key observations, which 257 led to the previously proposed nuclear entry models (Fig. 5). After cell entry (Fig. 5A), 258 acidification in the endosomes (Fig. 5B) triggers structural changes leading to the exposure of the 259 VP1 N-terminal domain, which comprises the phospholipase A2 and the NLS on the capsid surface 260 (2, 79). The phospholipase activity disintegrates the endosomal membrane allowing the interaction 261 of NLS with Imp α and β after endosomal opening (Fig. 5C) or after capsid release from the 262 endosome during capsid transport using microtubules (80–83) (Fig. 5D). Imp β then allows binding 263 to the NPC and passage through the pore (Fig. 5E), which is in agreement with the concomitant movement of Imp β and CPV we observed earlier (54). The parts of the capsid that are not masked 264

by Imp β interact with Nup358, Nup62 and Nup153 (**Fig. 5F**). Once the translocation is terminated in the nuclear basket by interaction of Imp β with Nup153, RanGTP dissociates the import complex, leading to recycling of Imp β into the cytoplasm (**Fig. 5G**). Concomitantly, the NPCbound capsids disintegrate the NPC/NE (**Fig. 5H**) probably by permeabilizing the nuclear membrane, which triggers Ca²⁺ release, a known initiator of NE degradation in mitosis (60). The holes in the NE then allow passive entry of cytosolic capsid-Imp-complexes into the nucleus (**Fig. 5I**).

While our data and the resulting model allow consistent interpretation of previous findings, we must admit that we cannot conclude whether the NPC-bound or the free nuclear capsids initiate infection. The data, however, support a new and unique model of virus transport across the NE combining features of phylogenetically distant viruses such as HTLV-1 and papillomaviruses, which need NE permeabilization during mitosis for nuclear entry, with e.g. adeno- and herpesviruses, which bind to the NPC triggering genome release and subsequent passage of the genome through the nuclear pore.

279 MATERIALS AND METHODS

280 Cell lines and viruses

Norden laboratory feline kidney (NLFK) cells were cultured in Dulbecco's modified Eagle medium 281 (DMEM) with GlutamaxTM supplemented with 10% fetal bovine serum (FBS), 1% non-essential 282 amino acids and 1% penicillin-streptomycin (Gibco, Thermo Fischer Scientific, Waltham, MA) at 283 37 °C in the presence of 5% CO₂. Canine parvovirus (CPV) type 2 was a generous gift from Colin 284 285 Parrish (Cornell University, Ithaca, NY) and derived from an infectious plasmid clone p265 by transfection of NLFK as previously described (84, 85). The viruses were grown, isolated and 286 287 concentrated as described earlier (83). For infection, the cells were inoculated with CPV (MOI 1-2) 288 and incubated at 37°C in a humidified incubator (5% CO₂) for the duration of the infection time.

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290 Antibodies

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291 CPV VP2 proteins were detected with a rabbit antibody (Ab) and intact capsids with a mouse 292 monoclonal antibody (MAb) from Colin Parrish (Cornell University, Ithaca, NY). Viral non-293 structural protein NS1 was localized by a MAb obtained from Caroline Astell (86). Primary 294 antibodies were detected with Alexa 488, 555 or 633-conjugated anti mouse/rabbit IgGs (Thermo 295 Fisher Scientific). In microinjection studies, an Imp β 1 MAb (anti-KPNB1 [3E9], ab2811, Abcam, 296 Cambridge, UK) was used to interfere the docking of the Imp/substrate complex to the NPC.

297 Confocal microscopy of fixed cells

For laser scanning confocal microscopy, cells were grown on glass cover slips, fixed at set time 299 300 intervals with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100 in 301 phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin and 0.01% sodium azide. The cells were embedded with ProLong Gold antifade reagent with 4',6-diamidino-2-302 303 phenylindole (DAPI, Thermo Fischer Scientific). Images were acquired with an Olympus FV-1000 confocal microscope with a UPLSAPO $60 \times$ oil immersion objective (numerical aperture =1.35). 304 305 Images of size 800 by 800 pixels were acquired with line averaging of 2 with voxel size 88 nm in 306 the x and y, and 150 nm in the z dimension (zoom factor 2). 500 kDa fluorescein isothiocyanate 307 (FITC)-dextran was excited with a 488 m argon laser, and the fluorescence was collected with a 510 308 to 540 nm band-pass filter, Alexa 555 and PLA probes conjugated with Alexa 594 were excited 309 with a 543 m He-Ne laser, and the fluorescence was collected with a 570 to 620 nm band-pass filter; Alexa 633 was excited with a 633 nm He-Ne laser, and the fluorescence was collected with a 310 311 647 nm long-pass filter. DAPI was excited by a 405 nm diode laser and monitored with a band-pass 312 filter of 460 to 500 nm.

313 Immunoblotting and co-immunoprecipitation

Cells were cultured on 56 cm² dishes. Infected and mock-infected NLFK cells were lysed with icecold hypotonic gentle lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.1%

Triton-X 100) supplemented with protease inhibitor (250 μ l/5 ml CEB, P-2714, Merck KGaA, Darmstadt, Germany) on ice for 30 min. Suspension was centrifuged (10 000 g, 15 min, +4°C) and the supernatant decanted. Nuclei were resuspended in 0.5% Tween-20 in PBS with 500 mM NaCl, incubated on ice for 15 min and centrifuged (10 000 g, 15 min, +4°C). Collected supernatants were combined to produce a total cell lysate.

321 For co-immunoprecipitation assays, the total cell extracts (200 µl) were incubated with rotation in RT for 1 h with 50 μ l (1.5 mg) Protein G Dynabeads® (Thermo Fischer Scientific) coated with BS³ 322 323 (Thermo Fiscer Scientific) cross-linked anti-VP or anti-Imp β (10 µg) antibodies freshly bound to 324 beads in 200 µl PBS with Tween-20 for 10 min in RT with rotation. After washes the Dynabeads-325 Ab-antigen complexes were resuspended in 100 µl washing buffer and transferred into a new tube. 326 Target antigens were eluted by resuspending the complexes directly in preheated SDS sample 327 buffer and heated for 10 min at 70°C. The eluates were boiled for 10 min and cooled to RT prior to 328 gel (12%) loading. After the electrophoresis, the proteins were transferred to nitrocellulose 329 membranes. The membranes were blocked for 16 h at 4°C with 5% BSA in 1% Tween-20/1X-TEN. 330 For immunoblotting, primary antibodies against Imp β and capsid proteins were diluted in 1% 331 Tween-20/1X-TEN and incubated at RT for 1 h. For detection, horseradish peroxidase-conjugated 332 goat anti-rabbit or anti-mouse IgG (Bio-Rad) were diluted in 0.1% Tween-20/1X-TEN (1:2000) and 333 incubated at RT for 1 h. SuperSignal® West Pro Pico chemiluminescent detection kit (Thermo 334 Fisher Scientific) was applied in detection of the proteins. Signal was collected quantitatively with a 335 Chemidoc XRS hood (Bio-Rad, UK) equipped with a CoolSNAP HQ2-CCD camera. Saturation of 336 the signal was avoided with exposure time adjustment.

337 In Situ proximity ligation assay

For in *situ* proximity ligation assay (PLA) (70), cells were grown on 8-well chamber slides (Nunc[™]
Lab-Tek[™] II Chamber Slide[™] System, Thermo Fischer Scientific) to 80-90% confluence. The
cells were fixed with 4% PFA in PBS and permeabilized with 0.1% Triton X-100 in PBS

341 supplemented with 1% bovine serum albumin and 0.01% sodium azide. Analysis was performed with Duolink[®]II kit (Olink Bioscience, Uppsala, Sweden). Anti-mouse PLA probes were diluted in 342 343 3% bovine serum albumin in PBS and incubated on cells for 1 h at 37°C in a humidified chamber 344 followed by ligation and amplification according to manufacturer's protocol. The samples were 345 embedded in ProLong Gold antifade with DAPI (Thermo Fisher Scientific). The signal was 346 detected with the Olympus FV-1000 confocal microscope and UPLSAPO 60× oil immersion 347 objective (NA = 1.35). The images of single cells were of size 512 by 512 pixels with a voxel size 348 of 69 or 82 nm in the x and y and 150 nm in the z dimension (line averaging 2).

349 The intracellular distribution of PLA signals was analyzed by segmenting the nucleus and PLA 350 signals from the fluorescence microscopy images using the minimum cross entropy segmentation 351 (87). The geometric centers of PLA signals were calculated, and their lateral (xy-) distances to the 352 border of the segmented nucleus were determined. The distance values of the centroids were sorted 353 into 750 nm wide bins and the mean number and density of PLA signals for each bin were then 354 calculated resulting in a graph of PLA signal numbers and densities as a function of the distance 355 from the NE. Because the axial resolution of confocal microscopy images is much lower than the 356 lateral resolution, it was difficult to determine if PLA signals located near the top and bottom of the 357 nucleus were inside the nucleus or not. For this reason, the lateral distance to the NE was selected 358 as the distance metric and only those PLA signals that were within 750 nm from the plane of 359 geometric center of the nucleus were accepted into the analysis.

360

361 Microinjection

For microinjection, the cells were cultured on 21.5 cm² glass-bottom culture dishes (MatTek Cultureware, Ashland, MA) or on cover glasses (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) to 80-90% confluency. Microinjection was accomplished with a system comprised of Transjector 5246 and a Micromanipulator 5171 (Eppendorf, Hamburg, Germany) on a 366 Zeiss LSM510 confocal microscope (equipped with Normarski DIC). Microinjection capillaries 367 (FemtotipsTM) were purchased from Eppendorf AG (Hamburg, Germany). The injection pressure 368 used was 250 hPa leading to the cytoplasmic injection volume of 0.01 to 0.05 pl (88). Imp β MAb 369 combined with injection buffer (phosphate-buffered saline, PBS) was concentrated to 2.5 mg/ml. 370 Based on previous estimation of the number of NPCs (~ 4000 NPC/HeLa cell nucleus) (69, 89, 90) 371 the cells were microinjected with HBV capsids with a ratio of 1-2 capsids/NPC. Alexa Fluor 488 372 conjugate of WGA was diluted in the injection buffer and used at a concentration of 2.5 mg/ml. To 373 identify the microinjected cells, 4 μ l HBV capsids or anti-Imp β were combined with 1 μ l of 500 374 kDa FITC-dextran, 12.5 mg/ml, Molecular Probes, Thermo Fisher Scientific) and centrifuged for 15 375 minutes at 10 000 x g before filling them into the capillaries and injecting them into cells. HBV 376

microinjected cells were incubated at 37 °C with 5% CO₂ for 30 min prior to infection. After the 377 duration of infection, cells were fixed with paraformaldehyde (4% in PBS), immunostained and 378 embedded in ProLong Gold antifade with DAPI (Thermo Fisher Scientific). The effects of 379 microinjection-induced inhibition of nuclear transport were analyzed by calculating from confocal 380 microscopy images the proportion of infected and non-infected cells, with or without 381 microinjection, based on the presence of nucleoplasmic NS1 or VP2 antibody signal.

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617 Figure legends

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620 FIG 1

621 Effect of importin β inhibition on the progress of infection.

622 The importin β (Imp β) -mediated nuclear transport was inhibited by cytoplasmic microinjection of 623 importin β antibody (Imp β MAb). Confocal microscopy images of microinjected cells at (A) 16, 624 (B) 24 and (C) 4 h pi. The infected cells were detected with an anti-VP2 Ab followed by anti-rabbit 625 Alexa 633 (white), and microinjected cells were identified by co-injected 500 kDa FITC-dextran 626 (green). Distribution of endogenous Imp β immunostained with Imp β 1 MAb and anti-mouse Alexa 627 555 is shown as inverted greyscale images in the third column from the left. Scale bars, 10 µm. (D) 628 The fraction of Imp β antibody-microinjected cells containing nuclear VP2 at 16 (n=18) and 24 h pi 629 (n=33) normalized by the fraction of nuclear VP2-positive non-microinjected cells (n=23 at 16 h pi, 630 n=62 at 24 h pi). Error bars present the standard deviations.

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632 FIG 2

633 Effect of HBV capsid- or WGA-induced blockage of NPCs on the progress of infection.

Proceeding of infection after NPC blockage caused by cytoplasmic microinjection of HBV capsids 634 635 and WGA. (A) Confocal images show the emergence of replication protein NS1 recognized with an 636 anti-NS1 MAb (white) in cells microinjected with (A) HBV capsids and (B) WGA-Alexa 488 at 16 637 h pi. Cells microinjected with HBV capsids were identified by co-injection of 500 kDa FITC-638 dextran (green). Scale bars, 10 µm. (C) The fraction of HBV antibody-microinjected (n=16 639 microinjected and n=19 non-microinjected cells) and WGA-microinjected cells (n=96 640 microinjected and n=142 non-microinjected cells) containing nuclear NS1 at 16 h pi normalized by 641 the fraction of nuclear NS1-positive non-microinjected cells. Error bars present the standard 642 deviations.

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646 FIG 3

647 **Capsid-Imp** β interactions.

The interaction of capsid and Imp β analyzed by coimmunoprecipitation at 1 h pi. (A) Immunoblot 648 649 detection of viral capsid proteins precipitated with Imp β MAb (left) and Imp β precipitated with 650 capsid protein Ab (right). Lysates were from infected and non-infected cells (mock). The arrows 651 indicate positions of the VP2 capsid protein (67 kDa, left) and Imp β (97 kDa, right). The proximity 652 of capsid and Imp β was detected with a PLA assay. (B) Views of cellular middle sections of 653 confocal images showing infected cells labeled with antibodies against capsid proteins and Imp β at 654 1, 2, 4, and 6 h pi. (C) Quantitative analysis of the time-dependent change in the number of PLA 655 signals detected at 1, 2, 4 and 6 h pi (n=17, 12, 5, 2). The error bars represent the standard error of 656 the mean. (D) Positive and negative controls showing PLA signal of the VP2 capsid proteins and 657 intact capsids in non-infected (left) and infected cells at 1 (middle) and 4 h pi (right). (E) Technical 658 controls using the two PLA probes without (left) and with Imp β MAb (middle) and capsid Ab 659 (right). Scale bars, 10 µm.

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662 FIG 4

663 Cellular distribution of capsid-Imp β interactions

Visualization of capsid and Imp β interactions in infected cells. A) Confocal sections of DAPI and PLA signals at 1 h pi. The intranuclear localization of PLA signals is shown by xy- and yz-slices of the segmented nucleus and PLA signals, obtained by visualizing the PLA signals with black color in the white nucleus. The yz-slice is taken along the line shown in red color. Scale bars, 5 µm. B) The number of segmented PLA signals at 1 h pi as a function of the lateral (xy) distance from the nuclear envelope (n=16). C) The number density of PLA signals at 1 h pi as a function of the lateral

cytoplasmic side and positive values to the nuclear side of the nuclear envelope. The error bars 671 672 show the standard error of the mean. 673 674 675 Figure 5. Schematic presentation of the entry and nuclear import model of CPV capsids. 676 677 Grey: cytoplasm, white nucleus. The magnification shows events in the nuclear pore. Capsids are 678 shown as blue hexagons containing the viral ssDNA genome (green circle). Imp α : orange, Imp β : 679 dark blue, nucleoporins: green ellipses. The dashed lines indicate degradation. Further details are 680 given in the text.

distance from the nuclear envelope (n=16). The negative distance values denote the distance to the

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