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Targeting of viral capsids to nuclear pores in a cell-free reconstitution system

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Abstract

Many viruses deliver their genomes into the nucleoplasm for viral transcription and replication. Here, we describe a novel cell-free system to elucidate specific interactions between viruses and nuclear pore complexes (NPCs). Nuclei reconstituted *in vitro* from egg extracts of *Xenopus laevis*, an established biochemical system to decipher nuclear functions, were incubated with GFP-tagged capsids of herpes simplex virus, an alphaherpesvirus replicating in the nucleus. Capsid binding to NPCs was analyzed using fluorescence and field emission scanning electron microscopy. Tegument-free capsids or viral capsids exposing inner tegument proteins on their surface bound to nuclei, while capsids inactivated by a high-salt treatment or covered by inner and outer tegument showed less binding. There was little binding of the four different capsid types to nuclei lacking functional NPCs. This novel approach provides a powerful system to elucidate the molecular mechanisms that enable viral structures to engage with NPCs. Furthermore, this assay could be expanded to identify molecular cues triggering viral genome uncoating and nuclear import of viral genomes.

Keywords

herpesviruses; Herpes Simplex Virus; Xenopus nuclei; reconstitution; nuclear pore

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Introduction

More than 30 proteins termed nucleoporins (Nups) assemble into NPCs that serve as specialized gateways in the nuclear envelope to allow active and passive bidirectional molecular traffic between the cytosol and the nucleoplasm (1–3). The structural organization of NPCs is similar in humans and amphibians; both possess a central channel with an inner diameter of about 50 nm at their center surrounded by an elaborate scaffold structure with 8-fold rotational symmetry (4, 5). Their nucleoplasmic face is capped by the nuclear basket, while filaments of up to 50 nm in length protrude freely from the cytosolic face. NPCs allow the passage of molecules up to about 5 nm in diameter by diffusion, while shuttling nuclear transport receptors, called importins and exportins, are required for active translocation of cargoes (6–8). A RanGTP/RanGDP gradient across the nuclear envelope and members of the importin β superfamily control the directionality of transport, but the translocation process itself does not require GTP hydrolysis (8–12).

Herpesviruses comprise a large group of DNA viruses that have been identified from humans to mollusks (13). Herpes simplex virus type 1 (HSV1) is a human alphaherpesvirus with a genome of 152 kb that is packaged into an icosahedral capsid of 125 nm in diameter (14). The major capsid protein VP5 forms 150 hexons on the capsid faces and edges and 11 pentons at vertices, while the 12^{th} vertex is occupied by a dodecamer of pUL6 that operates as a portal (15). The capsids are enclosed by an eccentric tegument of about 25 distinct proteins that have been classified into a more ordered inner and a less structured outer layer (16–22). The outer tegument binds to cytosolic domains of viral glycoproteins embedded into the viral envelope, overall forming a virion of about 225 nm in diameter (16, 22). Three types of capsids can be readily purified from the nuclei of infected cells (c.f. Fig. 1, top left): C capsids, which are the heaviest on sedimentation gradients, contain a viral genome; B capsids contain the scaffold proteins but no DNA; and A capsids, which are empty, lack both scaffold proteins and DNA. The packaging of progeny genomes into nuclear, rather spherical pro-capsids results in the formation of C capsids that are then predominantly exported across the nuclear envelope, while the nuclear A and B capsids are considered dead-end products (14, 23). The cytosolic capsids recruit inner tegument proteins such as pUL36 and pUL37 and are transported to cytoplasmic membranes to acquire the outer tegument and their envelopes (22–26). The virions are transported within secretory vesicles to the plasma membrane for release by exocytosis (22–24).

After HSV1 cell entry by fusion with host membranes, inner tegument proteins such as pUL36 and pUL37 remain capsid-associated, while most or all outer tegument proteins detach (25–31). The capsids recruit the motor protein dynein and its cofactor dynactin for transport along microtubules towards the nucleus (32, 33). Electron microscopy images show parental capsids interacting with the cytosolic filaments emanating from NPCs, with one vertex facing the central channel (33–39). While most of these capsids appear empty, fortuitous cross sections seem to visualize the viral DNA genome as it is ejected from one vertex through the NPC (33, 39). Similarly, after a short adsorption to a solid surface, the DNA is also ejected *in vitro* as a single double helix from one vertex, most likely the portal (40). These observations are consistent with the notion that HSV1 utilizes energy from ATP hydrolysis to package genomes into capsids, while a pressure-driven mechanism pushes the

genomes out of the capsids and through the permeability barrier of NPCs (41–43). The capsids are able to withstand this internal pressure by further stabilization of the capsid shell that seems to be initiated by DNA packaging after protein scaffold expulsion (44).

A biochemical cell-free system based on *Xenopus laevis* extracts has greatly aided studies of NPC assembly and nuclear transport (c.f. Fig. 1, right). Adding chromatin to fractionated egg extracts triggers a complex series of events including membrane vesicle recruitment, vesicle fusion, and assembly of NPCs into the newly forming nuclear envelopes (45–48). NPC assembly can be blocked by the addition of Imp $\beta^{45\text{-}462}$, a truncated form of importin β, or by the Ca^{2+}-chelator BAPTA [1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid], resulting in the formation of apparently pore-free nuclei in which the chromatin is fully enclosed by membranes lacking any signs of NPCs (49). These and other biochemical interventions make this *in vitro* system a powerful tool for the analysis of various nuclear functions (50).

Much remains to be learned about capsid docking at NPCs, viral genome uncoating, and viral genome translocation into the nucleoplasm. Since NPCs are essential for cell survival, it is a challenge to elucidate their function in intact cells. Our first *in vitro* study has shown that isolated HSV1 capsids derived from extracellular virions by TX-100 lysis in the presence of 0.5 M NaCl can bind to nuclei isolated from rat liver, and that importin β, the RanGTP/GDP cycle, and capsid-NPC interactions are required to trigger the release of the viral genomes (37). Similarly, capsids isolated from virions by TX-100 lysis in the presence of 0.5 M KCl interact *in vitro* with dynein, its cofactor dynactin, kinesin-1 and kinesin-2 from both, pig brain cytosolic extracts or from *Xenopus* egg extracts, and assemble functional capsid-motor complexes that can translocate along microtubules *in vitro* (summarized in Table 1; (19, 20, 51)).

These studies show that key virus-host interactions are conserved from amphibians to mammals, and that capsids derived from extracellular virions by detergent lysis at 0.5 M NaCl or 0.5 M KCl maintain their affinity for specific host factors. In contrast, capsids isolated from the nuclei of infected cells, or capsids prepared by detergent lysis of extracellular virions in the presence of 0.1 M KCl do not bind to microtubule motors, while viral capsids isolated in the presence of 1 M KCl maintain their ability to interact with dynein, but no longer bind to kinesins (summarized in Table 1; c.f. Fig. 1 left; (19, 20)). Our subsequent immunoblot analysis, quantitative mass spectrometry and quantitative immunoelectron microscopy studies have shown that viral capsids treated with 0.5 M KCl expose the inner tegument proteins pUL36, pUL37 and pUS3 and only low amounts of outer tegument proteins such as VP13/14, VP16, VP22, while capsids treated with 0.1 M KCl harbor in addition to inner tegument high amounts of outer tegument (summarized in Table 1; (19, 20)).

In this report, we describe a novel cell-free assay that measures the binding of HSV1 capsids to reconstituted *Xenopus* nuclei (c.f. Fig. 1, bottom). Our results show that nuclear capsids that display only outer capsid proteins on their surfaces and 0.5 M KCl viral capsids exposing outer capsid proteins in addition to inner tegument proteins, but not the other viral capsid types bind specifically to nuclei harboring functional NPCs. This new biochemical

assay has broad potential for the study of interactions between viral structures with distinct components of the NPC as well as the nuclear import of viral proteins and genomes for transcription and replication.

Results

HSV1 capsids with defined surface features

To identify the role of HSV1 structures in binding to NPCs, we prepared capsids characterized by different surface features. To isolate nuclear capsids (c.f. Fig. 1, left), we treated infected cells prior to lysis with two different buffers: with TNE containing 0.5 M NaCl as reported by Newcomb and Brown (52, 53), or with MKT containing a physiological concentration of 0.1 M KCl before cell lysis as described in our previous studies (19, 20, 51). Viral capsids were prepared from extracellular virions using TX-100 in the presence of 1 M, 0.5 M or 0.1 M KCl as described in our previous studies (19, 20); c.f. Fig. 1, middle). Immunoblot analysis did not reveal any differences in the protein composition of the different nuclear capsid preparations irrespective of the buffer used prior to cell lysis (Fig. 2). Nuclear capsids contained the capsid proteins VP5, VP19c and VP23 in similar amounts to viral capsids, although there were some minor variations between different preparations. B capsids also contained the scaffold proteins VP21 and VP22a. The amount of pUL25 was similar on A, B and C capsids, but appeared higher on viral capsids than on nuclear capsids. When compared to such nuclear capsids, the three types of viral capsids also contained similar amounts of the inner tegument proteins pUL36 and pUL37. By contrast, the outer tegument protein VP13/14 was most prominent on viral 0.1 M KCl capsids, but not present on nuclear capsids, consistent with our previous analysis (summarized in Table 1; (19, 20, 51)).

We next analyzed the capsid surfaces using electron cryo tomography and sub-volume averaging, and compared the degree of tegumentation of viral capsids to that of nuclear C capsids (Fig. 3). Since viral capsids had a tendency to aggregate, we selected single capsids present in holes of the carbon support film for analysis. In total, we averaged 183, 246 or 198 capsids isolated in the presence of 1 M, 0.5 M or 0.1 M KCl, respectively. The capsid averages were filtered to a resolution of 5.6 nm, and the differences in densities were calculated by subtracting the gray values of nuclear capsids from those of KCl-treated capsids. We detected little differences between viral capsids treated with 1 M KCl and nuclear capsids (yellow in Fig. 3Av, 3Avi). By contrast, there was a clear extra density on viral capsids prepared with 0.5 M KCl (orange in Fig. 3Bv, 3Bvi) and even more pronounced on 0.1 M KCl capsids (red in Fig. 3Cv, 3Cvi). These densities were located at the vertices protruding from the top of the pentons and from one side of the neighboring hexons, but not on the other hexons. Thus, viral and nuclear HSV1 capsids vary substantially in their protein composition and in their surface structure, and may therefore differ in their interactions with shuttling nuclear transport receptors and NPCs.

Nuclei harboring NPCs reconstituted from *Xenopus laevis* egg extracts

To develop a novel method to study capsid interactions with NPCs, we assembled nuclei *in vitro* from *Xenopus laevis* egg extracts. De-membranated sperm chromatin was incubated

with membrane vesicles as a source for nuclear membranes and a cytosolic fraction as a source for Nups (Fig. 1, top left; (46, 54)). Nuclear assembly was validated for each experiment by labeling with the monoclonal antibody mAb414 recognizing FG-repeat Nups in mature NPCs (55). A rim-like signal around the chromatin indicated the formation of nuclei surrounded by nuclear envelopes harboring NPCs (Fig. 4Aii). No NPC staining was observed when either Imp $\beta^{45\text{-}462}$ (Fig. 4Bii) or BAPTA (Fig. 4Cii) had been added to the reconstitution mixture. Furthermore, TRITC-NLS-BSA, a reporter cargo of the classical importin α/β pathway, was imported into functional control nuclei (Fig. 4Aiv), but not into pore-free nuclei (Figs. 4Biv and 4Civ).

An *in vitro* assay to decipher the interactions of viral capsids with NPCs

To detect capsids, we used an HSV1-GFPVP26 strain in which the outer capsid protein VP26 has been replaced by a GFPVP26 fusion protein that is incorporated into virions during capsid assembly (56). Although a fraction of GFPVP26 seems to dissociate during cytosolic passage in epithelial cells, sufficient amounts remain associated with and are detected on HSV1 capsids docked at the nuclei (57). Furthermore, HSV1-GFPVP26 capsids utilize microtubule transport and, like wild-type capsids, recruit dynein from cytosolic extracts of *Xenopus laevis* eggs (20, 57). Thus, the GFP tag on VP26 does not interfere with crucial cytosolic capsid-host interactions. Of the capsids tested here, viral capsids treated with 0.5 M KCl are most similar in terms of protein composition to the ones that bind to NPCs of rat liver nuclei or to microtubule motors (19, 20, 37). To investigate whether such capsids can also bind to *Xenopus* NPCs, we incubated 0.5 M KCl viral capsids of HSV1-GFPVP26 with reconstituted nuclei. To delineate the nuclear envelopes, fluorescent Imp $\beta^{45\text{-}462}$ was added during the last 10 min of the respective binding reaction. Initial time course experiments revealed that the number of bound capsids varied between the different capsid types and preparations, but remained rather constant during a time window of 30 to 90 min (data not shown). While surface views in top and bottom confocal laser microscopy sections showed punctate labelling typical of NPCs (Fig. 4Di, red in Fig. 4Diii) and HSV1 capsids scattered over the nuclear surface (Fig. 4Dii, green in Fig. 4Diii), mid sections revealed capsids (Fig 4Eii, green in Fig. 4Eiii, white arrowheads) closely juxtaposed to the rim labelling of the nuclear envelopes (Fig. 4Ei, red in Fig. 4Eiii).

To obtain further information on the association of capsids with *Xenopus* nuclei, we used high resolution field emission scanning electron microscopy (FESEM) to image the nuclear surface. Samples were labeled with a polyclonal antiserum raised against viral capsids, followed by anti-rabbit-antibodies conjugated to 12 nm colloidal gold particles. The three-dimensional surface topography of reconstituted nuclei, including the cytoplasmic surface of NPCs is seen in the in-lens images (Fig. 4F), while the corresponding backscatter electron detector images reveal the position of gold-conjugated antibodies (Fig. 4G). Thus, HSV1 capsids could be unequivocally identified by multiple gold particles, which often lined up along the capsid edges and vertices (Fig. 4G; white arrowheads). Each capsid blocked a considerable area of the nuclear envelope from view, but depending on the imaging angle, it was possible to obtain partial views of NPCs underneath the capsids (Fig. 4Fi, 4Fii; white arrows). Controls in which either the capsids or the primary antibodies had been omitted

revealed little background labeling (not shown). These experiments demonstrated that HSV1 capsids are capable of docking onto NPCs of *in vitro* reconstituted *Xenopus* nuclei.

Specific capsid features and functional NPCs are required for HSV1 capsid binding to *Xenopus* nuclei

To determine which HSV1 capsid types were targeted to NPCs, we tested nuclear C capsids, as well as viral capsids extracted at 1 M, 0.5 M or 0.1 M KCl, for binding to functional nuclei harboring NPCs or to pore-free nuclei assembled in the presence of Imp $\beta^{45\text{-}462}$ or BAPTA. Several images of HSV1-GFPVP26 capsids were acquired by epi-fluorescence microscopy at different focal planes, merged and superimposed with a middle focal plane image of the DNA-stained nucleus. Only immobile capsids colocalizing with the DNA were scored as bound to nuclei, and their number was determined for 5 to 20 nuclei in each condition. This setup allowed a comparison of several conditions on the same day. However, no binding of HSV1 capsids to *Xenopus* nuclei was observed if either component had been stored for several hours at room temperature, on ice or frozen. Instead, it was mandatory to isolate HSV1 capsids for each experiment and to reconstitute *Xenopus* nuclei just prior to their co-incubation, and only a few parameters could be compared within one given experiment. Therefore, we integrated all experiments into one analysis, although some conditions had been evaluated more often than others (Table 2).

To summarize the data of many experiments in a comparative manner, we set the mean number of the 0.5 M KCl viral capsids (14.8 capsids) bound per functional nucleus to 100% (Table 2, Fig. 5). All types of viral and nuclear C capsids were targeted to *Xenopus* nuclei to some extent, with the viral 0.5 M KCl capsids and the nuclear capsids showing the highest degree of binding. While the number of viral 0.5 M KCl capsids was comparable to that of nuclear capsids, 0.1 M and 1 M capsids bound significantly less efficiently ($p = 0.001$). Furthermore, the binding of all capsid types was substantially reduced for nuclei that had been reconstituted in the presence of Imp $\beta^{45\text{-}462}$ or BAPTA. Since the binding of 1 M viral capsids to BAPTA-treated nuclei was the lowest of all conditions tested, it may be considered as nonspecific background. These results show that HSV1 capsids docked specifically onto NPCs of *in vitro* reconstituted *Xenopus* nuclei, and that this interaction was mediated by outer capsid and/or inner tegument proteins exposed in a functional conformation on the surface of 0.5 M KCl and nuclear C capsids.

Discussion

A cell-free assay to analyze viral interactions with NPCs

Very few biochemical studies have been able to address an association of viral structures with NPCs (37, 58–61). To study the nuclear targeting of mammalian viruses, we have harnessed *Xenopus* nuclei reconstituted *in vitro* that have been used extensively to study nuclear assembly and nuclear transport (48, 54, 62, 63). HSV1 viral capsids, isolated from virions with TX-100 in the presence of 0.5 M KCl, as well as nuclear C capsids interacted specifically with nuclei harboring functional NPCs. High resolution FESEM imaging demonstrated that capsids bound specifically to the cytoplasmic surface of NPCs. In cells, incoming HSV1 capsids require microtubule motors for their transport towards NPCs, and

indeed viral 0.5 M KCl capsids, but not nuclear C capsids recruit microtubule motors from *Xenopus* egg and pig brain cytosol (19, 20). Notably, both 0.5 M KCl and nuclear C capsids were shown here to bind specifically to NPCs.

Xenopus reconstitution reactions can be fine-tuned to block nuclear assembly at specific stages (64, 65). The addition of Imp $\beta^{45\text{-}462}$ or BAPTA results in the formation of membrane-sealed assembly intermediates that lack any architectural signs of NPCs and are thus considered to be pore-free (49). We observed a residual affinity of HSV1 capsids to such nuclei, which most likely indicates the level of background binding to nuclear envelopes. The preferential binding of 0.5 M KCl and nuclear C capsids cannot be solely attributed to the fact that nuclei assembled in the presence of Imp $\beta^{45\text{-}462}$ or BAPTA do not expand as much as in the controls (Fig. 4; (49, 54)). While they present a reduced surface area, there was little specificity among the four capsid types in binding to Imp $\beta^{45\text{-}462}$ or BAPTA nuclei. In contrast, 0.5 M KCl and nuclear capsids had a significantly higher affinity for nuclei harboring functional NPCs.

Surface features on HSV1 capsids

Using electron cryo tomography, we have identified substantial differences on the surface of the different capsid types. There were pronounced extra densities on 0.5 M KCl capsids and even more prominent densities on 0.1 M capsids when compared to nuclear C capsids. The shape of these densities is similar to those on cytosolic capsids imaged *in vivo* in the axons of neurons (66), and on viral capsids extracted with detergent and 0.5 M NaCl (67). Previous protein analyses have also revealed differences: whereas nuclear capsids lack tegument proteins, viral 0.5 M KCl capsids contain mainly inner tegument proteins, and viral 0.1 M KCl capsids maintain both inner and outer tegument proteins (summarized in Table 1; (19, 20)). Similar to viral 0.5 M KCl capsids, viral 0.5 M NaCl capsids also contain pUL36 and pUL37 and only low amounts of outer tegument proteins (68).

These data are consistent with the notion that viral capsids extracted with TX-100 and 0.5 M salt maintain additional proteins around their pentons, which most likely include inner tegument proteins. By contrast, we detected only minor structural differences between 1 M KCl viral and nuclear capsids, although the former contain more tegument proteins than the latter (c.f. Fig. 2; (19, 20)). Apparently these proteins do not form an ordered density on the capsid surface and were therefore not revealed in the icosahedrally symmetrized averages.

HSV1 proteins interacting with Nups

The outer capsid protein pUL25, and the inner tegument protein pUL36, are likely candidates to target incoming capsids to NPCs.

pUL17 and pUL25 form complexes that connect the pentons and possibly also the portal with the surrounding hexons (69, 70). Furthermore, capsid-associated pUL25 is required for proper genome uncoating at the NPC, and biochemical assays have shown that pUL25 can interact with Nup214 and hCG1, two Nups facing the cytosol (39, 71–73). pUL25 epitopes are accessible on the surface of the four capsid types tested here, as shown by immunoelectron microscopy of isolated capsids (c.f. Table 1; (19)).

pUL36 and pUL37 form complexes that are presumably located at the pentons, and that remain capsid-associated during transport to the nucleus (25, 26, 28, 67, 68, 71). Antibodies directed against pUL36 reduce nuclear targeting, incoming capsids lacking pUL36 or lacking the N-terminal nuclear localization sequence in pUL36 are not targeted to NPCs, capsids with a point-mutation in pUL36 fail to release their genomes, and proteolysis of pUL36 seems to be a prerequisite for the nuclear import of viral DNA (34, 74–77). Polyclonal antibodies have access to more pUL36 epitopes on the surface of 0.5 M KCl capsids than on 1 M or 0.1 M capsids, whereas pUL36 cannot be detected on nuclear capsids by these antibodies or by mass spectrometry (c.f. Table 1; (19)).

Thus, surface exposed pUL25 could mediate the interaction of both viral 0.5 M KCl and nuclear capsids with NPCs, while surface exposed pUL36 could only be involved in the association of viral 0.5 M KCl capsids with NPCs. However, additional pUL36 on the 0.5 M KCl capsids when compared to nuclear capsids did not stimulate NPC association. Furthermore, the treatment with 0.1 M or 1 M KCl apparently impaired HSV1 factors contributing to NPC binding. The treatment with 1 M salt might perturb the conformation of pUL36, pUL25 or another viral cofactor, while the treatment with 0.1 M KCl might prevent the removal of a putative inhibitor involved in NPC binding. Thus, we were able to demonstrate that the surface of the four HSV1 capsid types harbor structural differences that modulate their capability to interact with *Xenopus* NPCs as well as their affinity to antibodies directed against structural HSV1 proteins. However, so far the molecular and structural details of these changes remain elusive, and could not yet be used to unequivocally pinpoint to specific proteins mediating the interaction of HSV1 capsids with NPCs.

Clearly, more studies are required to characterize the interactions of viral capsids with NPCs. With this new approach, protein domains and peptide motifs suggested by protein-protein interaction assays using isolated proteins could be tested in the context of the complex capsid and NPC structures. Furthermore, this cell-free system may be used to determine the requirements for genome release from the capsids and their import into the nucleoplasm. It provides many advantages: nuclei harboring NPCs but lacking a particular Nup can be generated (62, 63), and even for mutants that do not form virions, cell entry can be bypassed if sufficient amounts of viral particles can be generated in heterologous systems. Further insight into capsid-docking and viral genome uncoating may be obtained by modulating other host factors such as kinases and using nuclei lacking specific Nups or capsids harboring subtle mutations. Such studies can identify molecular interactions essential for nuclear targeting, which should be maintained in viral vectors but could be targeted in antiviral therapy.

Materials and Methods

HSV1 virion preparation

Extracellular virions of HSV1 strain F (ATCC VR-733) or HSV1-GFPVP26 strain KOS (K26GFP; (56)) were prepared as before (19, 33, 57). BHK cells (ATCC CCL-10) grown to a density of 1 to 2 x 10^7 cells/175 cm^2 flask in MEM (Cytogen, Sinn, Germany) containing 10% fetal calf serum (PAA, Pasching, Austria) were infected with an MOI of 0.01 to 0.02 pfu/cell for 2 to 3 days. The media containing extracellular virions and infected cells were

centrifuged at 3,000 g for 10 min at 4°C. The cell pellet was suspended in an equal volume of TNE (0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) to prepare TNE-nuclear capsids, or of MKT (0.1 M KCl, 30 mM MES, 20 mM Tris, pH 7.4) to prepare MKT-nuclear capsids. The cells were aliquoted, frozen in liquid N_2, and stored at -80°C. Extracellular viral particles were sedimented from the supernatant at 12,000 rpm for 90 min (Type19 rotor, Beckman Coulter, Fullerton, USA), and the pellet was suspended in MKT buffer, aliquoted, frozen in liquid N_2, and stored at -80°C.

HSV1 capsid preparation

Nuclear capsids were isolated from infected cells that were homogenized in H_2O with 10 mM DTT and protease inhibitors (PI; 10 μg/ml antipain, 2 μg/ml bestatin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, 10 μg/ml E-64, 2 μg/ml leupeptin, 160 μg/ml PMSF; Sigma-Aldrich), and the nuclei were sedimented at 230 g and 4°C for 7 min and lysed in 1% Triton X-100 in TNE supplemented with 10 mM DTT and PIs. These lysates were layered onto linear sucrose gradients (20% to 50% sucrose in TNE), and the capsids were banded at 67,000 g for 80 min at 4°C (cf. Fig. 1, top left; (19, 20, 51, 53)). The fractions containing the A, B or C capsids were diluted 1 to 4 with TNE buffer with DTT and PIs, and sedimented at 50,000 rpm (TLA 100.2 rotor, Beckman) for 20 min and at 4°C.

Viral capsids were prepared as described before (19, 20, 37, 51). To isolated viral capsids, we mixed the sediment of the medium of infected cells with an equal volume of a two-fold capsid lysis buffer (2% [w/v] Triton X-100 in 0.2 M, 1 M or 2 M KCl respectively, 20 mM MES, 30 mM Tris, pH 7.4, 20 mM DTT, PIs), incubated for 30 min on ice, and layered onto sucrose cushions (20% [w/v] sucrose in 0.1 M, 0.5 M or 1 M KCl respectively, 20 mM MES, 30 mM Tris pH 7.4, 10 mM DTT, PIs). The capsids were separated from solubilized envelope and tegument proteins by ultracentrifugation at 110,000 g for 15 min at 4°C (TLA 100.2 rotor), suspended in BRB80 (80 mM PIPES, pH 6.8, 12 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT, PIs), and treated with 0.1 to 0.2 U/μl DNase I (Promega, Madison, USA or Roche, Basel, Switzerland) and 100 μg/ml RNase (Roth GmbH, Karlsruhe, Germany) for 30 min at 37°C to facilitate suspension. After an overnight incubation at 4°C, the capsids were sedimented at 110,000 g for 15 min at 4°C (TLA 100.2 rotor) and suspended in ELB-S buffer (250 mM sucrose, 50 mM KCl, 10 mM HEPES, pH 7.6, 10 mM DTT, 2.5 mM $MgCl_2$, 1 mg/ml BSA, PIs) using a tip sonifier (Sonic B-12; Branson, Danbury, USA) at 40 W for initially 3 and up to 10 pulses of 1 to 2 seconds. If the capsids still appeared clumped by fluorescence microscopy (BX61TRF, 20x objective, Olympus, Melville, USA), further pulses were applied. The extent of sonication required for dispersion increased from C-capsids to viral capsids isolated at 1 M, 0.5 M or 0.1 M KCl.

Antibodies

VP5 was detected by mouse monoclonal antibodies (mAb) H1.4 (Biodesign International - Meridian Life Science, Memphis, USA), VP19c by rabbit polyclonal antibodies (pAb) NC2 (78, 79), VP21/22a by pAb NC3/4 (78), VP23 by pAb NC5 (78), pUL25 by mAb #166 (80), pUL36 by pAb #147 (25), pUL37 by pAb pUL37 (26, 81), and VP13/14 by pAb R220 (82). NPCs were visualized with fluorescently labeled mAb 414 (A488-120L; Covance, Princeton, NJ, USA; (55)), and DNA with Hoechst 33258 (2 μg/ml; Sigma-Aldrich, St.

Louis, MO). HSV1 structural proteins were detected by pAb Remus that has been raised against tegumented capsids (37).

Immunoblot analysis

Nuclear or viral capsids suspended in sample buffer [1% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, 5% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 0.001% (w/v) bromphenol blue] were loaded onto 6 to 16% linear gradient SDS-PAGE gels, and the proteins were transferred to nitrocellulose membranes (Pall Corporation, NY). After blocking with 5% (w/v) fat-free milk in PBS-T (0.1% [v/v] Tween-20, 2.7 mM KCl, 137.9 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), the membranes were probed with primary antibodies and IRDye anti-rabbit or anti-mouse antibodies (LI-COR Biosciences, Bad Homburg, Germany), and imaged using an Odyssey® Infrared Scanner (LI-COR Biosciences).

Electron cryo tomography

Viral capsids were sonicated for 3 times 10 seconds (BB6 cup horn, Sonoplus HD3200, 60% maximal output, Bandelin, Berlin, Germany) directly before adding them onto the grids, and processed for electron cryo tomography as described before (66). 5 µl of a capsid preparation were added together with 2 µl of a homemade colloidal gold suspension (10 nm gold coated with BSA in PBS) onto the holey carbon support film of electron microscopy grids (R2-1, Cu 200 mesh, Quantifoil, Jena, Germany), and excess liquid was removed by blotting with filter paper. Specimens were vitrified by plunge-freezing into liquid ethane and stored in liquid N_2.

Data were collected using transmission electron microscopy; some of the 1 M KCl viral capsid tilt series were collected with a CM300 (43 of the 183 subvolumes; FEI-Philips, Eindhoven, The Netherlands), while the rest including the tilt series from other capsid types were collected with a Tecnai Polara (FEI, Eindhoven, The Netherlands). The microscopes were operated at 300 kV; the pixel size was 0.68 nm (CM300) or 0.81 nm (Polara) at the specimen level, and tilt series were collected with an angular increment of 2° or 3° from -60° to 60°. Defocus was measured along the tilt axis after each tilt and automatically maintained at -8 µm +/- 0.5µm to gain phase contrast. The total electron dose received at the specimen level was kept between 60 and 90 electrons/$Å^2$. The applied electron dose was kept proportional to 1/cosα of the tilt angle (α). Images were acquired on a 2Kx2K Multiscan CCD camera (GIF 2002 post-column energy filter, Gatan, Pleasanton, CA).

Image processing and averaging of tomographic sub-volumes

Alignment of tilted projection image series was performed using 10 nm gold beads as fiducial markers. Three-dimensional reconstructions were calculated using the software IMOD (83), and subsequent processing using Bsoft as described before (66, 84). Capsids were located in unbinned tomograms, and sub-volumes with a size of 180x180x180 pixels were extracted. The 3D orientation of all sub-volumes was determined using a 22 Å resolution structure of the HSV1 capsid (85) as a template. The oriented sub-volumes were averaged and icosahedral symmetry was applied. Symmetrized averages were used as templates for the next iteration of orientation refinement. Three iterations were performed.

The resolution of the averages was determined by Fourier shell correlation using the 0.5 criterion, after splitting the data in two halves, calculating two separate averages and imposing icosahedral symmetry. To calculate the difference map of the two averages gray values were scaled to the same radial density maximum within the capsid and minimum just outside of the capsid. The difference of densities was then calculated by subtracting the capsids without tegument from the capsids with tegument. All capsid reconstructions were first scaled against the nuclear C capsid reconstruction. Magnification differences up to 3.5% were detected and these were compensated for by creating up-scaled or down-scaled maps.

Reconstitution of nuclei

Xenopus laevis egg extracts, cytosolic and membrane fractions, demembranated sperm chromatin, and an ATP regeneration system were prepared, and nuclei reconstituted within 50 to 60 min at RT (47, 64). To assemble pore-free nuclei, 8 μM of recombinant Imp $\beta^{45\text{-}462}$ or 5 mM BAPTA (Calbiochem, La Jolla, CA, USA) were added to the mixture and incubated for 60 min (48). In each experiment the functionality of the reconstituted nuclei was tested by removing a fraction of the nuclei for staining with mAb 414 and Hoechst 33258, and nuclear import analysis with bovine serum albumin coupled to a nuclear localization sequence and to tetramethyl-rhodaminyl-isothiocyanate (TRITC-NLS-BSA; (48, 50)).

Binding of HSV1 capsids to *Xenopus* nuclei

The reconstitution reactions were diluted threefold with 1x ELB-S-buffer (pH 7.6, supplemented with 2.5 mM $MgCl_2$), 10 μl of nuclear or viral HSV1 capsids were added to 10 μl of the nuclei, and the reactions were agitated at 7 rpm for 20 to 90 min at room temperature (Fig. 1, bottom). Small aliquots were stained with Hoechst 33258 in 12% [w/v] para-formaldehyde, 250 mM sucrose, 10 mM HEPES, pH 7.5, 0.4% [w/v] N-propylgalate for 5 min. Capsid binding to nuclei was analyzed by epifluorescence microscopy (UPlanSApo 100x NA 1.4 oil immersion, Olympus BX61TRF equipped with a DP70 digital camera), or by laser scanning confocal fluorescence microscopy (63x NA 1.4 oil immersion, ZEISS LSM 510 META, Oberkochen, Germany). 10 to 25 nuclei of each experimental condition were randomly chosen, and the number of capsids bound to each nucleus was quantified over all focal planes.

Field Emission Scanning Electron Microscopy (FESEM)

In vitro reconstituted intact *Xenopus* nuclei incubated with HSV1 capsids for 60 min were labeled with Remus serum. Its dilution was optimized by fluorescence microscopy and adjusted for immunogold labeling. Colloidal gold of 12 nm diameter and coated with goat-anti-rabbit antibodies (Jackson Immuno-Research, West Grove, USA) was titrated to obtain a minimal background signal in the absence of primary antibodies (64, 65). To facilitate the capture of nuclei, they were sedimented at 1,000 g for 10 min onto silicon chips (Ted Pella, Irvine, USA) that had been coated with 0.2 mg/ml poly-lysine for 15 min. The chips were transferred to 24-well plates, washed in 1x ELB-K (100 mM KCl, 10 mM HEPES, pH 7.6, 2.5 mM $MgCl_2$, 1 mM DTT, 5 μg/ml cycloheximide, 5 μg/ml cytochalasin B, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and fixed in 3.7% para-formaldehyde with 0.2% glutaraldehyde (Electron Microscopy Sciences; Hatfield, PA, USA) in 150 mM sucrose, 80

mM PIPES, pH 6.8, 1 mM MgCl2 for 30 min at RT. All subsequent steps were as described in (86), with post-fixation in aqueous 0.5% osmium tetroxide, critical-point drying on a CPD030 apparatus (Bal-Tec AG, Liechtenstein), and sputter coating with 2 nm of chromium (K575X coater, Emitech, London, England). The samples were imaged using a FESEM (Ultra plus, ZEISS, Oberkochen, Germany) with an in-lens detector for secondary electrons to reveal surface structures, and an energy selective backscatter electron detector to localize gold particles.

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Abbreviations

BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
FESEM	field emission scanning electron microscopy
GFP	green fluorescence protein
HSV1	herpes simplex virus type 1
NLS	nuclear localization signal
NPC	nuclear pore complex
Nup	nucleoporin
PI	protease inhibitors
pULX	viral protein encoded by the gene ULX
RT	room temperature
VPX	viral protein X

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Synopsis

A novel assay based on nuclei reconstituted *in vitro* from *Xenopus* egg extracts was used for studying the interactions between viruses and nuclear pore complexes (NPCs). The targeting of GFP-tagged herpes simplex virus capsids to NPCs was followed by fluorescence and scanning electron microscopy. Surface features of different capsid types were analyzed by electron cryo tomography and compared to their targeting efficiency in the *in vitro* assay. Capsids exposing outer capsid and inner tegument proteins are targeted preferentially to NPCs.

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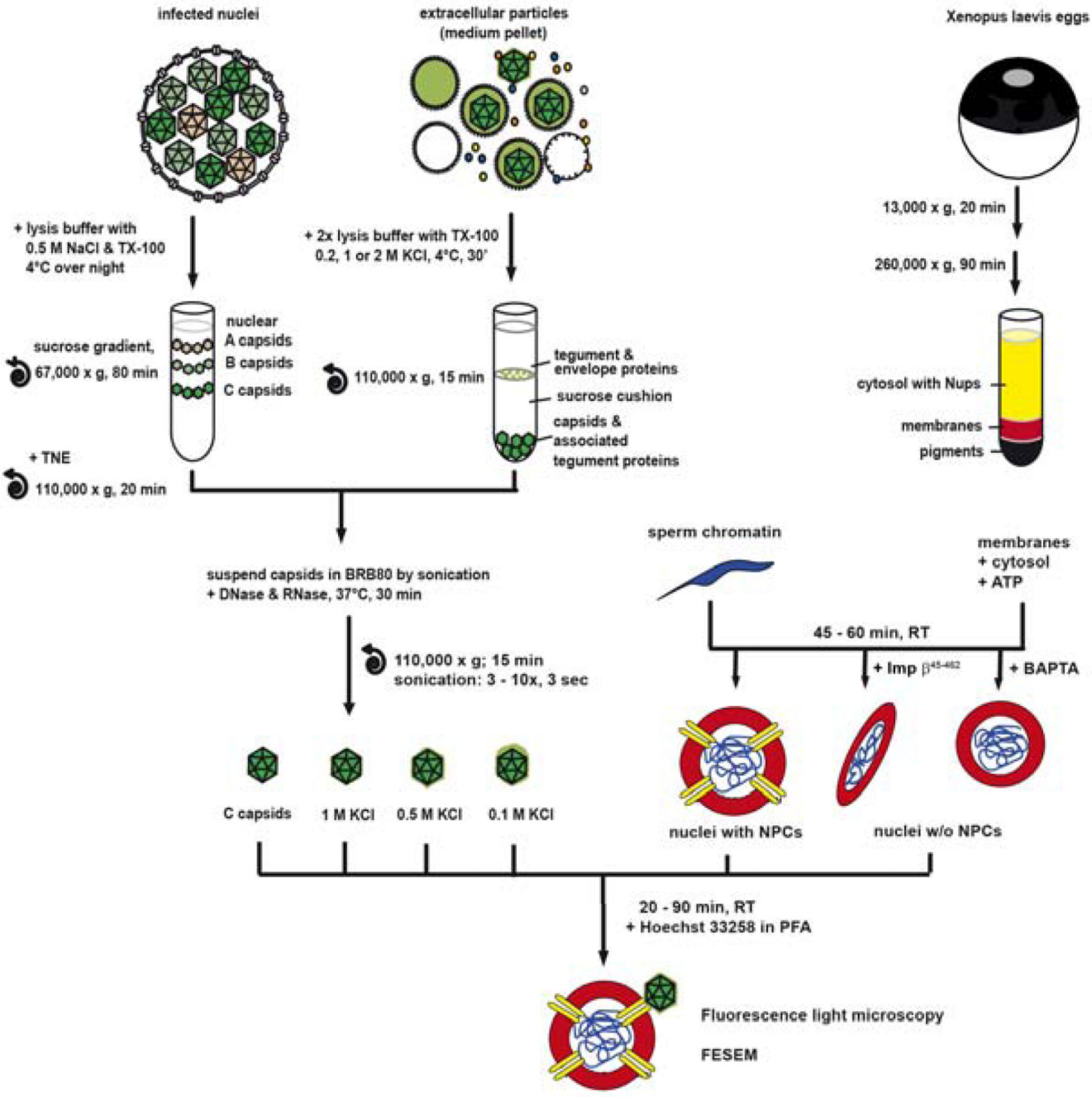

Figure 1. An *in vitro* assay to analyze the binding of viral capsids to NPCs.

Nuclear capsids (dark green) were isolated from the nuclei of HSV1 infected cells by gradient sedimentation (top left). Viral capsids were generated from mature extracellular particles released from HSV1 infected cells by lysis with 1% TX-100 in the presence of 1 M, 0.5 M or 0.1 M KCl (light and dark green), and purified through sucrose cushions (top middle). The four different capsid types were re-suspended in BRB80 buffer before tip sonication and DNase/RNase treatment. Nuclei harboring functional NPCs were generated *in vitro* from *Xenopus* egg extracts around a chromatin template. The formation of NPCs

was inhibited in some reactions by the addition of Imp β^{45-462} or BAPTA to the assembly reaction to create nuclei enclosed by continuous membranes lacking functional NPCs (top right). After nuclear assembly and re-sedimentation the nuclear or viral 1 M, 0.5 M or 0.1 M capsids were incubated with different nuclei for 20 to 90 min at room temperature. Nuclear assembly and capsid binding were analyzed by fluorescence light microscopy or field emission scanning electron microscopy (FESEM). Modified from Figure 1 of (19).

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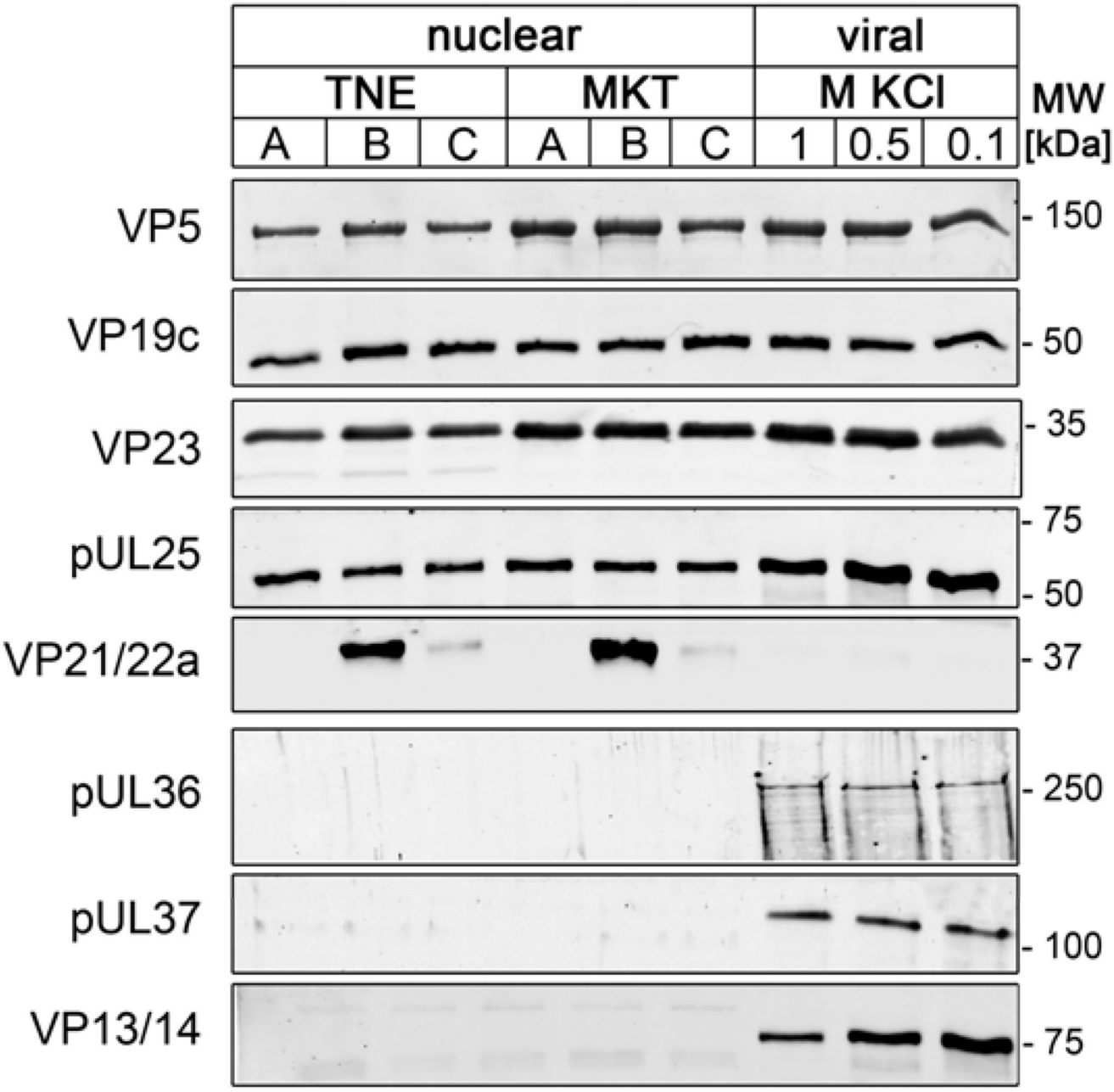

Figure 2. Protein composition of different HSV1 capsids types.

Nuclear capsids were isolated from BHK cells infected with HSV1-GFPVP26 using TNE- or MKT-buffer for cell suspension, and their protein composition was compared to that of viral capsids treated with TX-100 and 1.0 M, 0.5 or 0.1 M KCl by immunoblotting with antibodies raised against the capsid proteins VP5 (mAb H1.4), pUL25 (mAb #166), VP19c (pAb NC-2), VP21/VP22a (pAb NC3/4), or VP23 (pAb5) or the tegument proteins pUL36 (pAb #147), pUL37 (pAb anti-pUL37), or VP13/14 (pAb R220).

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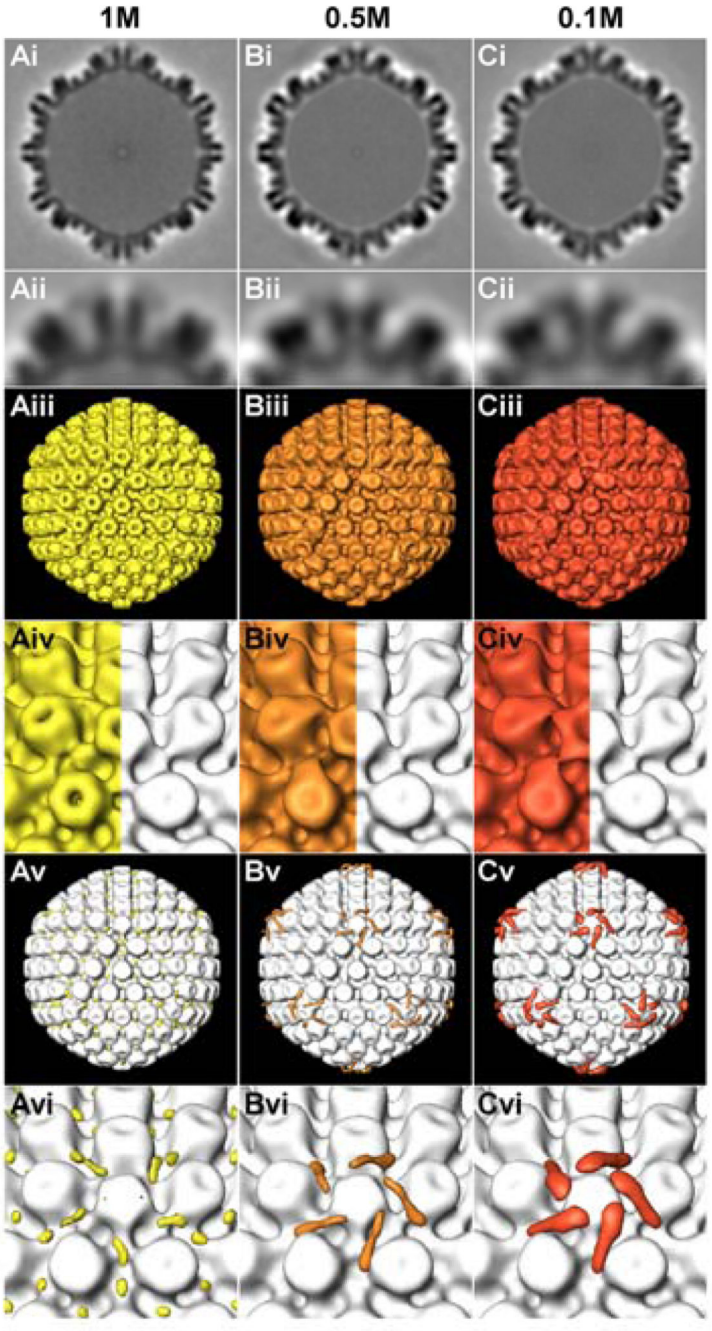

Figure 3. Electron cryo tomography reveals surface features of different HSV1 capsids types.

Results from sub-volume averaging are presented for capsids isolated from intact virions (HSV1 strain F) and extracted with TX-100 and 1 M (A), 0.5 M (B) or 0.1 M (C) KCl. Row (i): cross sections of the averages from 183 (1 M), 246 (0.5 M) or 217 (0.1 M) capsid sub-volumes. Row (ii): close-up view of the top vertex in row (i). Row (iii): iso-surface representation of the averages. Row (iv): close-up view of a salt treated capsid vertex (left), compared to nuclear C-capsids (66). Row (v): difference map between the respective salt treated capsid group and the nuclear C-capsids average, superimposed onto the nuclear C-

capsids average. Row (vi): close-up view of a vertex from row (v). The capsids have a diameter of 125 nm.

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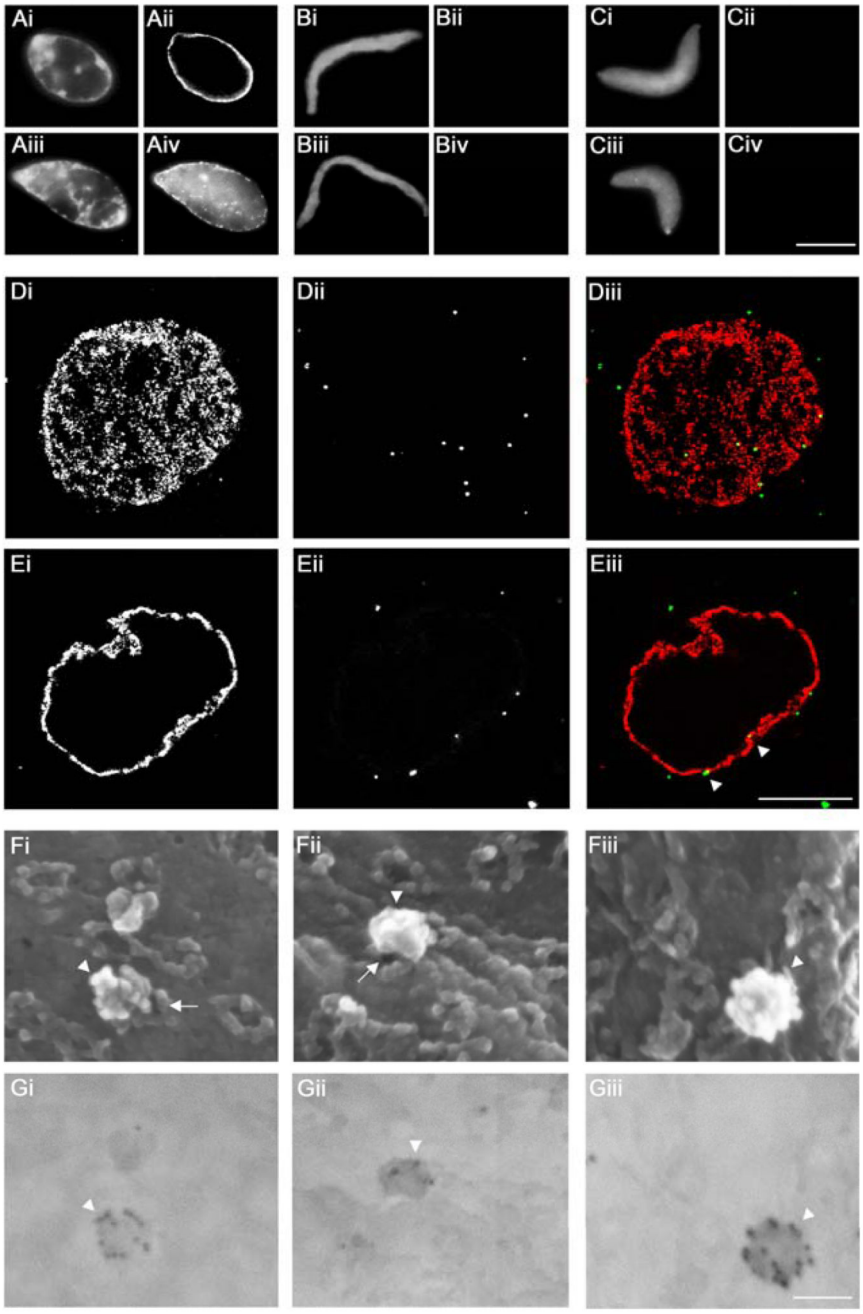

Figure 4. Targeting of HSV1 capsids to NPCs on *Xenopus* nuclei *in vitro*.

A to C. Functional (A), Imp $\beta^{45\text{-}462}$ (B) or BAPTA (C) nuclei were reconstituted *in vitro.* Completion of nuclear assembly was monitored by epi-fluorescence microscopy by DNA staining with Hoechst 33258 (i and iii), NPC staining with fluorescently labeled mAb414 (ii) and the transport capacity of the assembled NPCs was confirmed by the nuclear import of TRITC-NLS-BSA (iv). Scale bar: 10 μm. **D, E.** Binding of viral 0.5 M KCl capsids extracted from extracellular particles of HSV1-GFPVP26 (ii and iii, green, white arrowheads) to *Xenopus* nuclei was analyzed using confocal fluorescence laser scanning

microscopy and a serial z-slicing of 0.37 μm optical sections. A surface view (D) and one midsection (E) of a representative nucleus are shown. NPCs were labeled with Imp $\beta^{45\text{-}462}$-TRITC (i and iii, red). Scale bar: 10 μm. **F, G.** HSV1-GFPVP26 viral 0.5 M capsids were incubated with *Xenopus* nuclei harboring functional NPCs for 45 min, labeled with a polyclonal rabbit antiserum raised against intact HSV1 capsids (Remus, bleed V) and colloidal gold with a diameter of 12 nm coated with anti-rabbit antibodies. The specimens were analyzed by field emission scanning electron microscopy. (F) 3D surface topography of reconstituted nuclei from different views in the in-lens images (Fig. 4Fi-iii). The same areas were imaged through a backscatter electron detector revealing the positions of gold-conjugated antibodies (Fig. 4Gi-iii; inverted color mode). HSV1 capsids (white arrowheads) are bound to the cytoplasmic face of the NPCs (white arrows). Scale bar: 100 nm.

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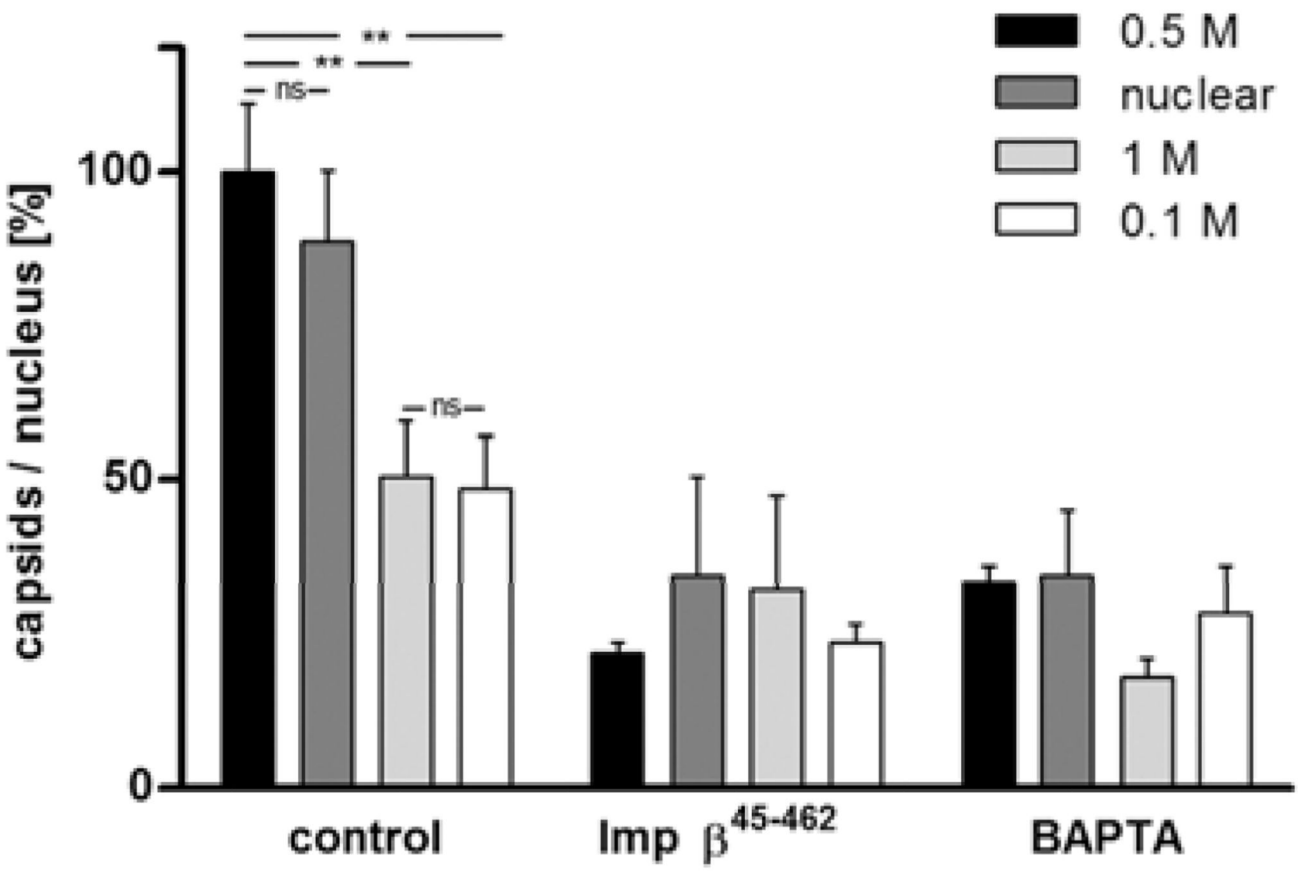

Figure 5. HSV1 capsid binding to nuclei requires specific capsid surface features and NPCs.

Viral capsids isolated from viral particles with TX-100 and 0.1, 0.5 or 1.0 M KCl or nuclear C capsids were incubated with *Xenopus* nuclei reconstituted *in vitro*. The number of HSV1 capsids bound to nuclei harboring functional NPCs (control), or to nuclei assembled in the presence of Imp β^{45-462} or BAPTA was determined by epi-fluorescence microscopy. Images were acquired at different focal planes, merged, bound capsids were counted, and the data normalized to 100% for 0.5 M viral capsids binding to functional nuclei. The error bars indicate the standard error of the mean. Asterisks indicate $p<0.0015$ and "ns" $p>0.05$ as determined in two-tailed student's t-test.

Table 1
Protein Characterization of isolated HSV1 Capsids.

HSV1 capsids were isolated from extracellular virions using TX-100 in the presence of 0.1 M, 0.5 M or 1 M KCl or from infected nuclei (nuclear) and the association of microtubule-associated factors was analyzed by immunoblot and quantitative electron microscopy (19). Furthermore, the composition of structural HSV1 proteins was analyzed by immunoblot, quantitative mass spectrometry and quantitative immunoelectron microscopy (summarized in (51). The signal intensities of the immunoblots for each protein were evaluated qualitatively (+++ for the strongest band, ++, +, and – if there was no or only a negligible band) (19). For mass spectrometry data, the amount of the respective protein in gradient-purified extracellular virions was set to 100%, and the amount of the proteins present on the respective viral capsids were normalized accordingly (19). For the immunoelectron microscopy, the labelling intensity of the capsid type with the highest amount of surface labelling was set to 100%, and the labelling intensities on the other capsids were normalized accordingly. The information on the estimated copy number per virion (copies / virion) was compiled from several reports (17, 21, 68, 87–89).

Association of Host Factors	**Detection Method**	**viral 0.5 M KCl**	**nuclear IB on B and C IEM on B and C**	**viral 1 M KCl**	**viral 0.1 M KCl**
Dynein	IB IEM [%]	+++ 100	- 0	+++ 98	- 33
Dynactin	IB IEM [%]	+++ 100	- 0	++ 66	- 3
Kinesin-1	IB IEM [%]	+++ 100	- 0	- 19	- 4
Kinesin-2	IB IEM [%]	+++ 100	- 35	++ 43	+ 0
HSV1 proteins (copies/virion)	**Detection Method**	**viral 0.5 M KCl**	**nuclear** IB on B and C MS on B IEM on C	**viral 1M KCl**	**viral 0.1 M KCl**
Capsid Proteins		+++	+++	+++	+++
VP5 (955)	MS [%]	100	100	100	100
VP19c (320)	MS [%]	100	100	100	100
VP23 (640)	MS [%]	100	100	100	100
VP24 (150)	MS [%]	100	100	100	100
VP26 (600)	MS [%]	100	100	100	100
pUL17 (60)	IB MS [%]	+++ 100	+++ 100	+++ 100	+++ 100
pUL25 (60)	IB MS [%] IEM [%]	+++ +++ ++	+++ +++ ++	+++ +++ +++	+++ +++ ++
Inner Tegument Proteins		+++	-	+++/-	+++
pUS3 (?)	IB IEM [%]	+++ 90	+ 19	+++ 75	+++ 100
pUL36 (150)	IB middle	++	-/+	++	+++
	MS [%]	28	-	18	20
	MS – Nterm [%]	189	-	183	161

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Association of Host Factors	Detection Method	viral 0.5 M KCl	nuclear IB on B and C IEM on B and C	viral 1 M KCl	viral 0.1 M KCl
	IEM - middle Ab [%]	100	1	81	76
	IEM - Cterm Ab [%]	100	36	2	58
pUL37 (150)	MS [%] IEM (GFP) [%]	49 94	- 1	21 32	48 100
ICP0 (?)	IB	+++	-	++	+++
pUL14 (?)	IB	+++	+	+++	+++
pUL16 (?)	MS [%]	45	-	21	42
pUL21 (?)	MS [%]	55	-	19	50
Outer Tegument Proteins	++	-	+	+++	
pUL41 - vhs (?)	IB	++	-	+	+++
pUL11 (?)	IB	+	-	+	+++
ICP4 (?)	IB	++	-	+	+++
ICP34.5 (?)	IB	++	-	++	+++
VP13/14 (1300-1800)	MS [%] IEM [%]	65 71	- -/+	12 56	124 100
VP16 (700-2000)	MS [%] IEM [%]	41 89	- -/+	19 65	72 100
VP22 (700-1500)	MS [%] IEM [%]	55 72	- -/+	23 24	143 100

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Table 2

Binding of HSV1 capsids to NPCs of *Xenopus* nuclei reconstituted *in vitro*.

HSV1 capsids were isolated from extracellular virions using TX-100 in the presence of 0.1 M, 0.5 M or 1 M KCl (viral capsids) or from infected nuclei (nuclear capsids) and incubated with functional *Xenopus* nuclei or with nuclei assembled in the presence of Imp $\beta^{45\text{-}462}$ or BAPTA. The samples were fixed, and bound capsids were counted. The number of experiments and the number of capsids bound per nucleus with the standard error of the mean have been tabulated for the different conditions.

HSV1 capsid	Functional		Imp $\beta^{45\text{-}462}$		BAPTA	
	# of experiments	# of capsids per nucleus	# of experiments	# of capsids per nucleus	# of experiments	# of capsids per nucleus
0.1 M	22	7.2 ± 1.3	4	4.6 ± 0.6	2	5.5 ± 1.5
0.5 M	24	14.8 ± 1.6	4	4.2 ± 0.4	2	6.5 ± 0.5
1 M	22	7.5 ± 1.3	3	6.3 ± 3.0	10	3.7 ± 0.6
nuclear	25	13.1 ± 1.7	3	6.7 ± 3.2	11	6.7 ± 2.1