Nucleus softens during herpesvirus infection

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ABSTRACT

Nuclear mechanics is remodeled not only by extracellular forces but also by internal modifications, such as those induced by viral infections. During herpes simplex virus type 1 infection, the nuclear structures undergo drastic reorganization, but little is known about how nuclear mechanobiology changes as a result. We show that the nucleus softens dramatically during the infection. To understand the phenomenon, we used advanced microscopy and computational modeling. We discovered that the enlarged viral replication compartment had a low biomolecular density, partially explaining the observed nuclear softening. The mobility of the nuclear lamina decreased, suggesting increased rigidity and inability to cause the softening. Computational modeling supported this by showing that decreased outward forces, such as intranuclear osmotic pressure and cytoskeletal pull, explain the decreased nuclear stiffness. Our findings reveal the mechanistic coordination between the infection-induced nuclear deformation and decreased nuclear stiffness.

INTRODUCTION

Mechanical forces have an essential role in many cellular functions. Cells sense and react to external forces, which can lead to various responses, such as changes in gene expression and cell differentiation[1,2]. Cells also generate intracellular forces, for example, to regulate cell shape and motion[3]. Intracellular forces are transmitted via cytoskeletal actin filaments and can be conveyed into the nucleus and to the chromatin via the transmembrane proteins in the nuclear envelope (NE). In herpes simplex virus type 1 (HSV-1)-infected cells, expansion of the viral replication compartment (VRC) and virus-induced manipulation of nuclear structure and

functions most likely lead to changes in the mechanical properties of the nucleus. However, little is known about how the infection-induced changes remodel the mechanical forces in the nucleus.

The main mechanical components of the nucleus are the NE with its underlying nuclear lamina, the chromatin, and the filamentous nucleoskeleton[4,5]. Structural changes in the organization of the NE, chromatin, or both often regulate nuclear stiffness[6,7]. The nuclear lamina, a 14-nm-thick filamentous network associated with the inner nuclear membrane[8], mainly comprises intermediate filament lamins divided into A/C and B types[9]. It reinforces the NE, connects to the cytoskeleton via the linker of the nucleoskeleton and cytoskeleton (LINC) complex, and interacts with the lamina-associated domains (LADs) of the chromatin[10–12]. The nuclear lamina and especially A-type lamins have been shown to respond to mechanical forces structurally, and their amount is co-regulated by tissue rigidity[13,14]. Furthermore, reorganization of the lamina can change the ability of the nucleus to resist deformations and large strains[15]. The rigidity of chromatin, another regulator of nuclear stiffness, is modulated by changes in its compaction state[15–18] and by the chromatin crosslinker HP1α[19]. DNA damage and the LAD connections also contribute to the mechanical strength of the nuclei[7,20]. Furthermore, osmotic pressure in the nucleus has been suggested to contribute to its mechanical response[21].

Viral infections can cause drastic changes in nuclear function and structure. HSV-1 infection leads to the formation and expansion of spherical, membraneless VRCs for viral DNA replication and progeny capsid assembly[22–24], which is accompanied by reorganization of the host chromatin toward the nuclear periphery[25,26]. The VRC formation in HSV-1 and another herpesvirus, human cytomegalovirus, infection has properties similar to liquid-liquid phase separation. However, other compartmentalization mechanisms might also be included[27–29]. The

substantial chromatin condensation toward the nuclear periphery regulates the diffusion of newly formed capsids through the chromatin to the NE[30–33], where the first step of nuclear egress, viral primary envelopment through the inner nuclear membrane, is assisted by the viral nuclear egress complex and disintegration of the nuclear lamina[34,35]. The activity of viral UL34 and US3 proteins leads to the local disintegration of lamin A/C and lamin B[36].

In this study, we analyze HSV-1 infection-induced changes in the nuclear biomechanics in host cells using various experimental methods, including atomic force microscopy (AFM), fluorescence microscopy, volume electron microscopy (EM), and cryo-soft X-ray tomography (SXT), as well as using computational modeling. Our data shows that the nuclei become softer upon the progression of infection as the nuclear structure undergoes profound changes and the nuclear lamina is modified. The modeling suggests that besides these changes, osmotic pressure and cytoskeletal pull to the nucleus might contribute to the softening.

RESULTS

Nuclear stiffness decreases as the infection proceeds

To analyze how HSV-1 infection alters nuclear stiffness, we measured the mechanical response of Vero cell nuclei to compressive force using AFM[37]. The nuclei were subjected to indentation by a spherical tip of the AFM cantilever. The measurement of the bending of the cantilever with a known spring constant provides the force applied to the nucleus (Fig. 1a,b, Supplementary Movie 1), which allows quantification of the elastic modulus of the nucleus, i.e., nuclear stiffness[38,39]. The measurements were conducted in intact cells, so even though almost all the volume under the tip consisted of the nucleus, thin layers of cytoplasm remained above and below the nucleus.

There was a large variation in noninfected cell nuclei stiffness, with values between 200 and 3,000 Pa. Compared to noninfected cells, the mean stiffness during the infection was lower at 4 hours post infection (hpi) and kept decreasing until 8 hpi, when Young's modulus was only about one fourth of the noninfected cell value (Fig. 1c). Finally, after this there was a minor recovery at 10 hpi.

Our findings show that the progression of HSV-1 infection softens the nucleus. To understand this phenomenon, we next studied how the infection modifies the mechanical components of the nucleus, such as the chromatin and the nuclear lamina.

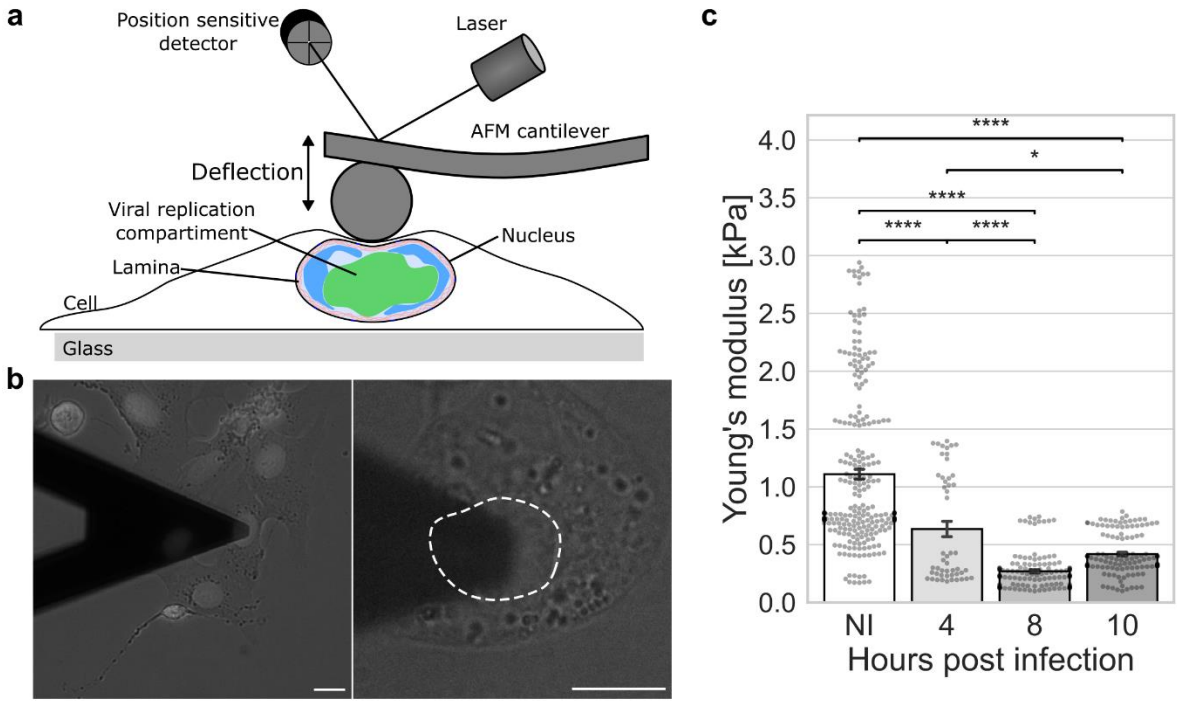

Fig. 1. Nuclear stiffness decreases during the progression of infection.
(**a**) Schematic side view of an atomic force microscope (AFM) cantilever with a colloidal probe over the nuclear region of a cell. (**b**) Brightfield microscopy images of an infected cell underneath the AFM cantilever at 8 hpi. The nuclear periphery is demarcated with a dotted white line. Infected Vero cells were identified with the presence of ICP4 by epifluorescence. See also Supplementary Movie 1. (**c**) AFM analysis of nuclear stiffness performed on noninfected and HSV-1 ICP4-EYFP-infected cells at 4, 8, and 10 hpi (n=255, 50, 129, and 129, respectively). The error bars show the standard error of the mean. Statistical significance was determined using Tukey's test, and the significance values shown are denoted as **** ($p < 0.0001$) and * ($p < 0.05$). Nonsignificant differences ($p \geq 0.05$) are not labeled.

The molecular density of the nuclear center decreases in the infection

In the lytic HSV-1 infection, the nuclear import of the viral genomes is followed by the emergence of distinct punctate VRCs at early infection (1–5 hpi)[40]. Later in infection (8–12 hpi), the growth and fusion of the VRC foci result in an enlarged VRC, marginalization of host cell chromatin next to the nuclear envelope, and growth of the nuclear volume[25,26,41].

SXT imaging and analysis of infected mouse embryonic fibroblast (MEF) cells at 8 hpi showed that the biomolecular density (quantified by the linear absorption coefficient, LAC) in the central areas of the nucleus occupied by the VRC (Fig. 2a,b and Supplementary Movie 2) was lower than that in the euchromatin areas of the noninfected cells (Fig. 2c). Notably, the density of the perinuclear chromatin in infected cells remained similar to the density of the heterochromatin in noninfected cells (Fig. 2d).

To further study the three-dimensional distribution and organization of chromatin in the nucleoplasm of MEF cells, we used focused ion-beam scanning electron microscopy (FIB-SEM) and the ChromEM chromatin labeling method[42]. This approach confirmed the presence of dense chromatin close to the NE in infected cells at 8 hpi (Fig. 2e). Notably, finger-like protrusions of dense chromatin towards the center of the nucleus were less pronounced in infected cells. However, small dense chromatin aggregates were still detected in the central area of the nucleus where the VRC and viral capsid were located (Fig. 2f,g and Supplementary Movie 3). This can be cellular chromatin remaining in the VRC region or replicated viral genomes[43]. The ChromEM labeling can detect viral DNA, as shown by the intense labeling of full (DNA-containing) capsids (Supplementary Fig. 1).

In summary, the enlarged low-density region emerging during the infection had a lower biomolecular density than the central parts of noninfected cells. The density decreased as the infection proceeded, which might have implications for the nuclear softness during the infection. The marginalized chromatin of the infected cell was not denser than the control cell heterochromatin.

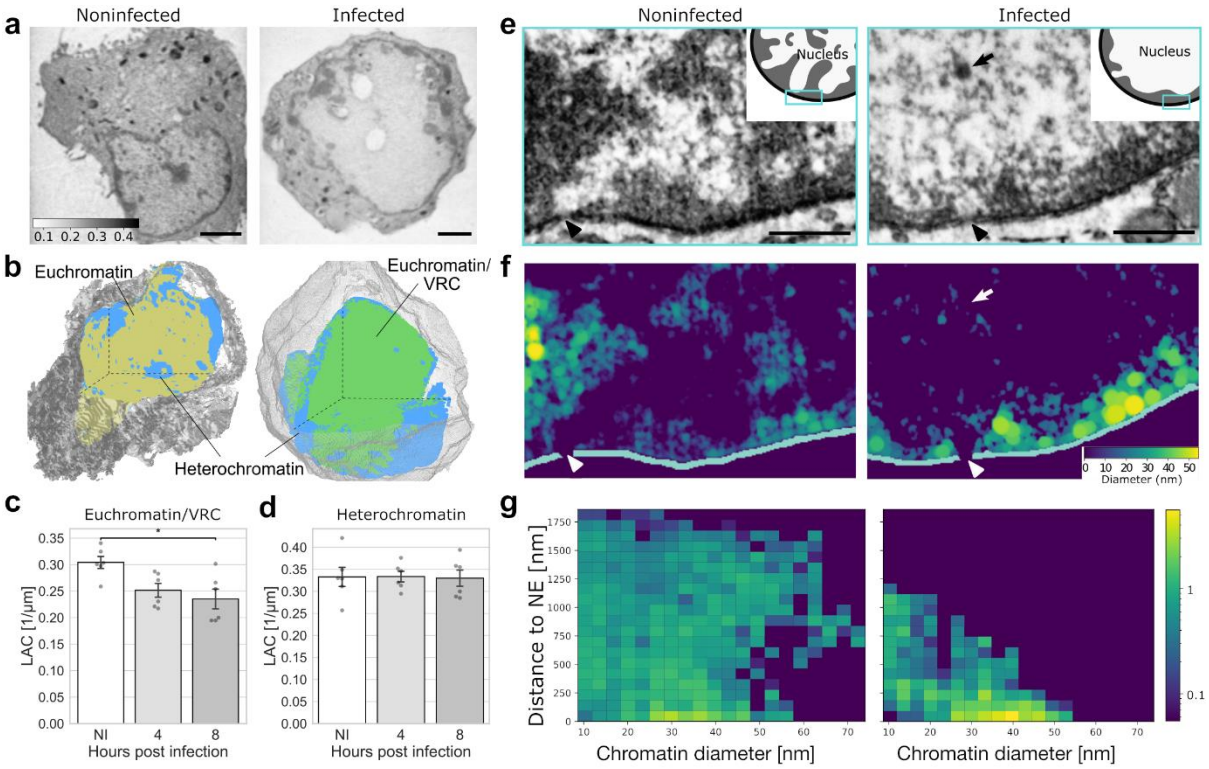

Fig. 2. The enlarged viral replication compartment has a low biomolecular density.
(**a**) Cryo-soft X-ray (SXT) tomography ortho-slices of noninfected (NI) and infected mouse embryonic fibroblast (MEF) cells at 8 hpi. Molecular densities are presented in a continuous grey scale representing linear absorption coefficient values ranging from 0.1 to 0.4 μm^{-1} (bar). Scale bars, 2 µm. (**b**) Cross-sections of 3D SXT reconstructions of noninfected and infected cells. The high-density region (heterochromatin) is shown in blue, the low-density region (euchromatin) in yellow, the viral replication compartment (VRC/euchromatin) area in green, and the cytoplasm in grey. See also Supplementary Movie 2. Quantitative analysis of linear absorption coefficient (LAC) of the (**c**) low-density and (**d**) high-density region in noninfected and infected cells 4 and 8 hpi (n=6). (**e**) Representative cross-sections of focused ion beam scanning electron microscopy (FIB-SEM) images of noninfected and infected MEF cells at 8 hpi. The viral capsid (arrows) and nuclear pore complexes (arrowheads) are shown. Scale bars, 0.5 µm. See also Supplementary Movie 3. (**f**) Reconstruction of chromatin fiber thickness and (**g**) chromatin density as a function of the distance from the nuclear envelope in noninfected and infected cells. The color bar in (f) indicates the high and low chromatin thickness (yellow-green) and in (g) high and low density (yellow-blue). The error bars show the standard error of the mean. Statistical significance was determined using Tukey's test, and the significance values shown are denoted as * ($p<0.05$). Nonsignificant differences ($p \geq 0.05$) are not labeled.

Lamins regulate the nuclear volume

The nuclear volume increases during the progression of HSV-1 infection[26], and previous studies have shown that nuclear lamins control the volume of the nucleus[44]. Here, we analyzed the significance of lamins in the infection-induced nuclear growth of Vero cells by using overexpression of A-type lamins and the presence of truncated and farnesylated lamin A protein, progerin[45]. Confocal microscopy showed that the progression of infection and overexpression of lamin A/C increased nuclear volume, whereas the presence of progerin reduced it (Fig. 3a,b). Lamin A/C was localized to the nuclear envelope. However, the progerin mutation caused it to localize additionally into the cytoplasm in noninfected and infected cells (Fig. 3a). Both the excess of lamin A/C and progerin resulted in a more scattered distribution of ICP4 at 8 and 12 hpi in comparison to the solid VRCs seen in the wild-type cells. The lamin A/C overexpression and progerin also led to more uniform chromatin localization compared to marginalization close to the NE in the infected cells (Fig. 3a). The quantitative data analysis confirmed that in the cells with endogenous expression of lamin A/C, the progression of infection to 12 hpi induced enlargement of the nucleus compared to noninfected cells (Fig. 3b). In lamin A/C overexpressing cells, the infection caused a more significant nuclear volume increase when compared to the cells with endogenous levels of lamin A/C: the nuclear volume change from noninfected cells to 12 hpi was 1.33-fold in lamin A/C overexpressing cells compared to 1.24-fold increase in wild-type cells. In progerin-expressing cells, the nuclear volume decreased as the infection proceeded. The nuclear height remained relatively unchanged in all cases, even in the presence of progerin (Fig. 3c). To summarize, the progression of infection led to an increase in the nuclear volume, and the expansion was enhanced by lamin A/C overexpression and inhibited by progerin.

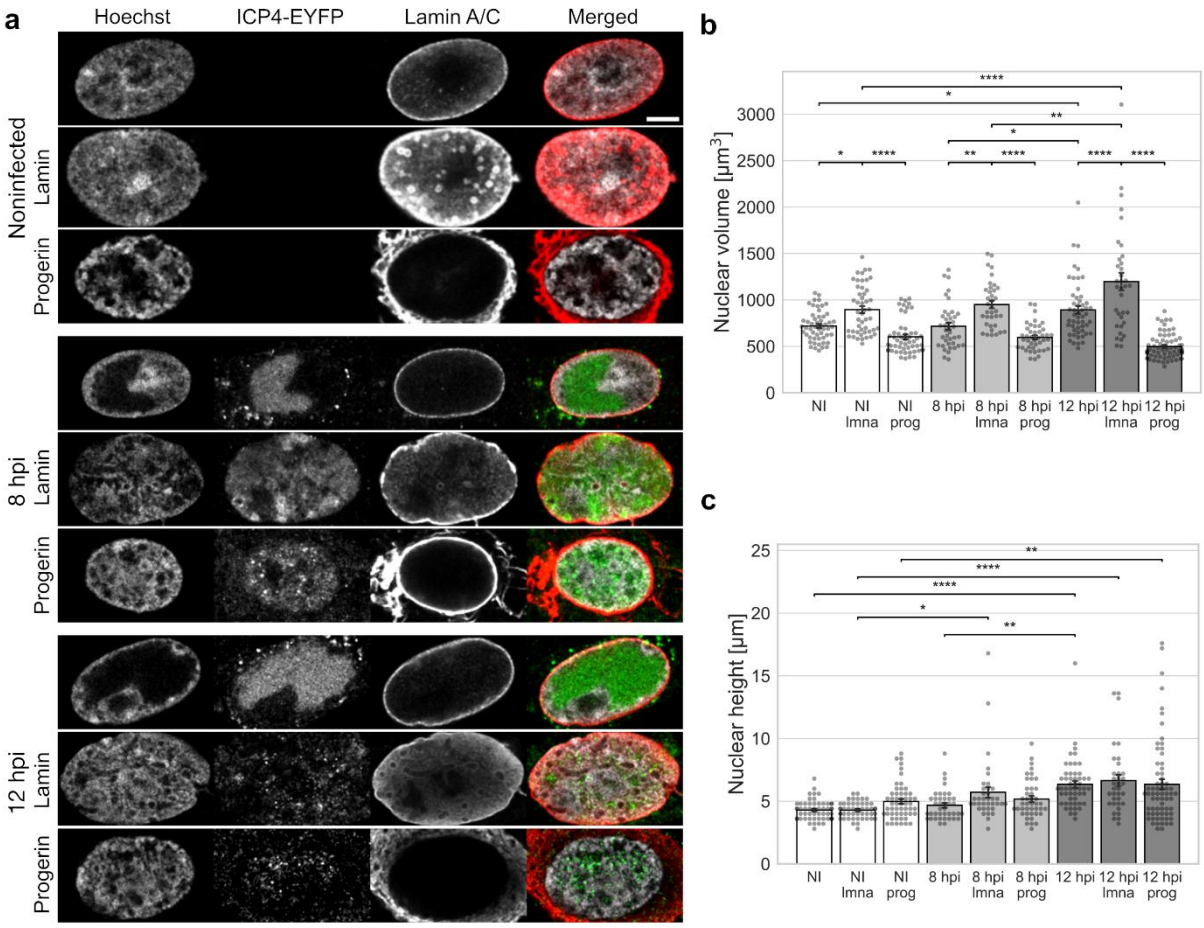

Fig. 3. Overexpression of lamin A enhances nuclear growth.
(**a**) Representative confocal images of noninfected and HSV-1 EYFP-ICP4-infected Vero cells at 8 and 12 hpi together with Vero cells overexpressing lamin A (lmna) and expressing progerin (prog). Lamin A/C was labeled with lamin A/C antibody (red), EYFP-ICP4 (green) shows the localization of the VRC, and the chromatin was labeled with Hoechst 33342 (grey). Quantitative analysis of (**b**) the volume and (**c**) the height of the nucleus in noninfected (NI) and infected cells (n=53, 48, 53, 40, 36, 42, 52, 35, and 67 for the bars from left to right). The error bars show the standard error of the mean. Statistical significance was determined using Tukey's test, and the significance values shown are denoted as **** ($p < 0.0001$), ** ($p < 0.01$), or * ($p < 0.05$). Only values of the same time point or treatment were statistically compared. Nonsignificant differences ($p \geq 0.05$) are not labeled.

The nuclear lamina motion decreases and the nuclear membrane tension increases in the infection

Since nuclear lamina is an essential mechanical structure for the nucleus, we used lamin A/C chromobody to assess differences in the nuclear lamina shape and motion in noninfected and infected living Vero cells[46]. We hypothesized that the mechanical stiffness of the nuclear lamina is connected to its shape and mobility, and the internal pressure increase (or decrease) would cause the nucleus to bulge (or wrinkle), respectively. To analyze the lamina shape, it was segmented, skeletonized, and discretized. Local regions of positive and negative curvature

of the nuclear lamina with respect to the nuclear interior were then quantified (Fig. 4a). The proportion of negative curvature regions was smallest at 8 hpi (Fig. 4b), but the change was not statistically significant. Next, the extent of lamina fluctuations was analyzed by taking the maximum intensity projection of the segmented and skeletonized lamina over the time series and calculating its area (Fig. 4c). The motion of the nuclear lamina, quantified as the area covered by the lamina skeleton during the time series and normalized by the length of the lamina, was significantly smaller at 8 hpi than in noninfected cells (Fig. 4d). This shows that the motion of the lamina decreases in infection, indicating higher tension or reduced mechanical dynamics of the lamina. Finally, the nuclear membrane tension was measured by the ER Flipper-TR probe and fluorescence lifetime imaging microscopy (FLIM)[47]. The changes in the fluorescence lifetime indicated an increase in the tension in the NE at 12 hpi compared to noninfected cells, although at 8 hpi the membrane tension was similar to that in the noninfected cells (Fig. 4e,f).

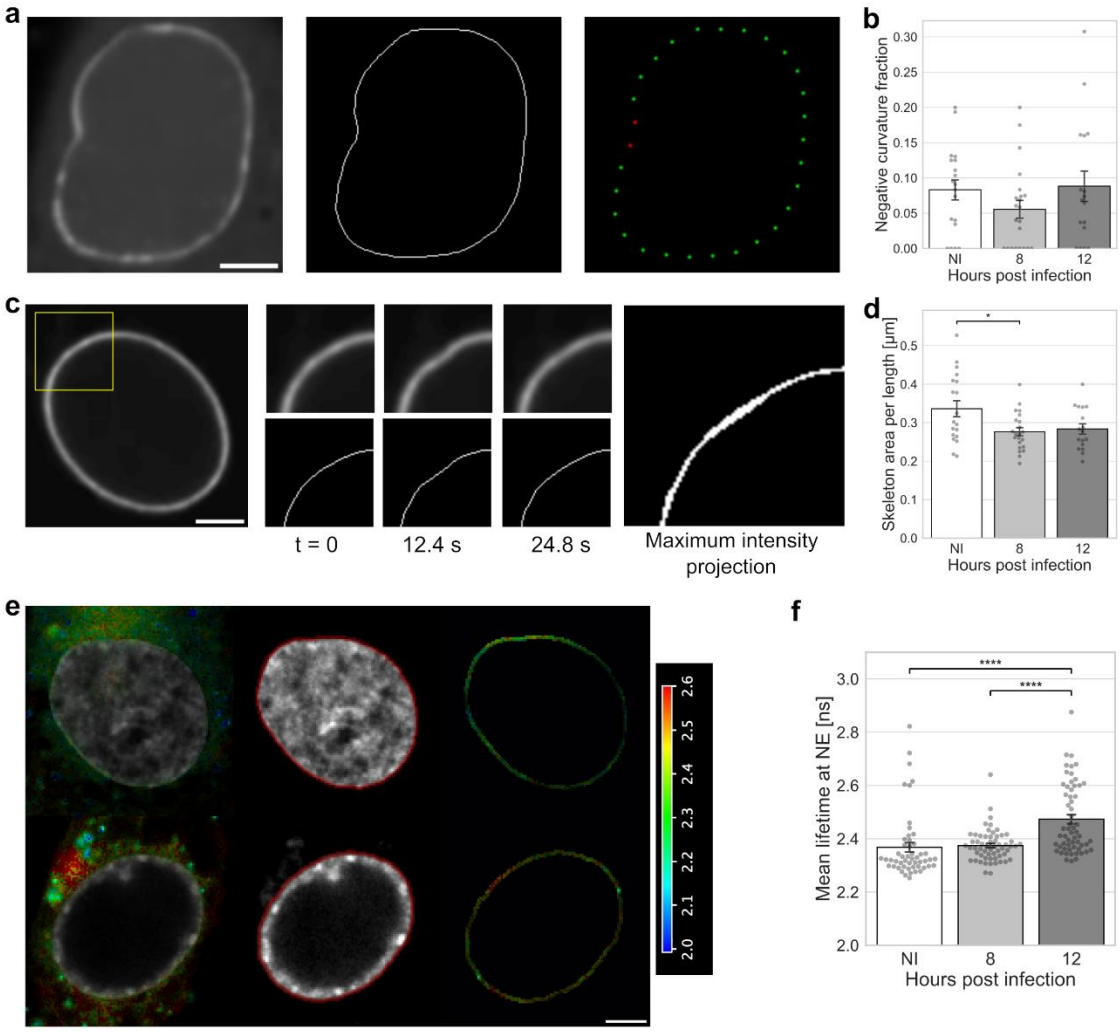

Fig. 4. The motion of nuclear lamina decreases in infection.
(**a**) Nuclear lamina labeled by lamin A chromobody, skeletonization of the lamina, and discretized lamina points showing regions of positive (green) and negative curvature (red) in infected Vero cells at 8 hpi. (**b**) The fraction of negative curvature points for noninfected cells and cells imaged 8 and 12 hpi (n=19, 22, and 17, respectively). **(c)** A zoom-in of the nuclear lamina, showing its motion and maximum intensity projection of the skeleton during 24.8 s. Scale bars, 5 µm. (**d**) Nuclear lamina mobility, measured as the area covered by the skeleton during one minute for noninfected cells and cells imaged 8 and 12 hpi (n=19, 22, and 17, respectively). (**e**) Nuclear envelope (NE) tension analysis by fluorescence lifetime imaging microscopy (FLIM) of noninfected and HSV-1 17+ wild-type virus-infected Vero cells at 12 hpi using fluorescence lifetime reporting membrane tension probe ER Flipper-TR. A fast FLIM image presented in a pseudocolor scale representing average photon arrival times in each pixel ranging from 2.0 to 2.6 ns (color bar). Representative cells with average lifetime distribution, segmented NE (red) around the Hoechst signal (grey), and average lifetime at the NE region are shown. Scale bar, 10 µm. (**f**) The longer lifetime component of the ER Flipper probe, responsive to the membrane tension, in noninfected and infected cells at 8 and 12 hpi (n=51, 58, and 59, respectively). The error bars show the standard error of the mean. Statistical significance was determined using Tukey's test, and the significance values shown are denoted as * ($p<0.05$) or **** ($p<0.0001$). Nonsignificant differences ($p \geq 0.05$) are not labeled.

Transcription of nuclear lamins and SUN2 changes in infection

The nuclear lamina is one of the major determinants of the mechanical behavior of nuclei. Thus, to explore the transcriptional activity of lamina-associated proteins during infection, we

employed a global run-on sequencing (GRO-Seq) analysis in Vero cells. The gene ontology analysis (GO) of GRO-Seq data according to the PANTHER classification system for the GO biological process (http://www.pantherdb.org) identified three main categories: protein localization to the NE (GO:0090435), nuclear migration (GO:0007097), and NE organization (GO:0006998) (Fig. 5a,b and Supplementary Table 1).

At 4 hpi, the genes encoding lamins A/C and B2 were upregulated, whereas at 8 hpi, the genes encoding both lamin B1 and B2 were downregulated compared to noninfected control cells. Notably, transcription of SUN2, one of the essential proteins of the LINC complex and the key regulator of nucleus-cytoskeleton connections[48], was strongly downregulated at 8 hpi. The zinc metallopeptidase STE24 (ZMPSTE24), a protein needed during the preprocessing of lamin A[49], was upregulated at 4 and 8 hpi. This protein has a strong antiviral activity inhibiting the replication of many enveloped DNA and RNA viruses[50,51]. At 4 and 8 hpi, torsin-1A-interacting protein 1 (TOR1AIP1, also known as LAP1), a protein involved in the connection between the nuclear membrane and the nuclear lamina[52,53], was upregulated. The downregulation of SUN2 at 8 hpi could decrease the overall interaction between SUN2 and LINC complex components, nesprin family proteins, encoded by SYNE 1–4 genes[54]. The clustering of genes (https://string-db.org/) (Fig. 5c and Supplementary Table 1) confirmed an interaction between SUN2 and lamin A/C, which further interacted with lamin B2. In addition, lamin A/C interacted with the upregulated TOR1AIP1.

Confocal imaging of lamin A/C and B showed that they were distributed to the nuclear rim in noninfected and infected Vero cells (Fig. 5d). In infected cells, the total nuclear intensity of immunolabeled lamins, especially lamin A/C, increased at 8 hpi compared to noninfected cells. The total amount of lamins was highest at 12 hpi due to the increase in lamin B from 8 to 12

hpi (Fig. 5e). The amount of lamins analyzed near the nuclear envelope followed a similar pattern to the amount in the whole nucleus (Supplementary Fig. 2). However, the epitope accessibility of lamins depends on lamin filament organization regulated by nuclear volume, chromatin condensation, and cytoskeletal tension[14]. Therefore, infection-induced modification in the nuclear organization most likely affects the conformational epitope of lamins, which may impact the amount of lamins detected by antibodies. Altogether, our findings show that the transcription of key regulators of the cytoskeleton–nucleus force transmission, B-type lamins and LINC complex protein SUN2, was downregulated at 8 hpi.

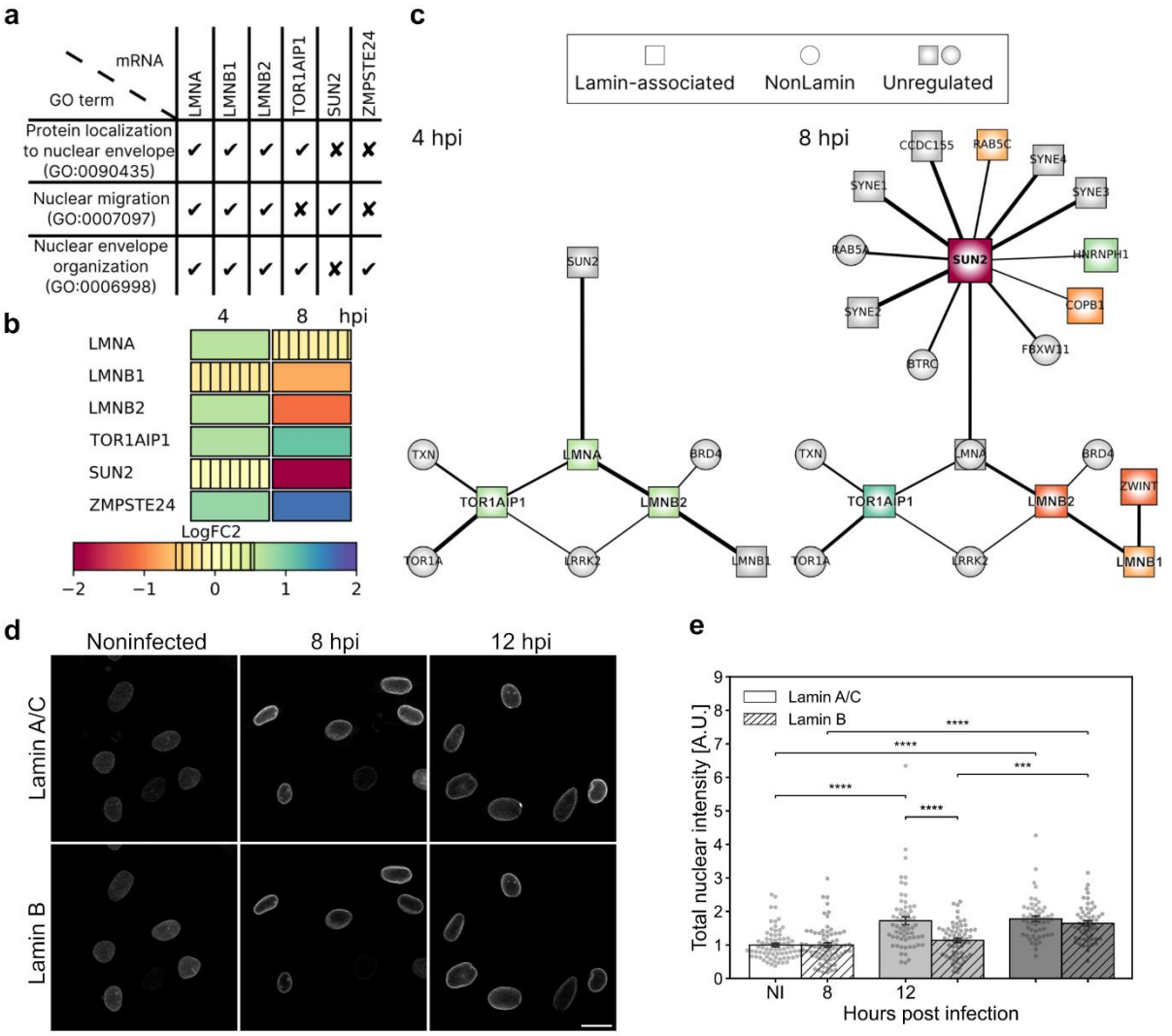

Fig. 5. The amount of lamins increases in infection.
Global run-on sequencing (GRO-seq) analysis of nascent RNA levels of nuclear lamins and lamin-associated proteins in infected Vero cells at 4 and 8 hpi. (**a**) GO term classification for lamin-associated processes and (**b**) regulation of proteins. The color bar indicates upregulation (green-blue), downregulation (orange-red), or low change of gene transcription (vertical stripes) compared to noninfected control cells. (**c**) The genes encoding for

lamins and lamin-associated proteins (square nodes) and cellular interactor proteins not associated with lamins (circular nodes) are shown. The upregulated (green-blue) or downregulated (red-orange) transcripts in response to the infection are visible together with unregulated interacting transcripts (grey). The node size correlates with a logarithmic fold change (logFC) of regulation, and the thickness of black lines between the nodes is proportional to the interaction in the STRING database (https://string-db.org/). (**d**) Distribution of immunolabeled lamin A/C and B in noninfected and HSV-1 ICP4-EYFP-infected Vero cells at 8 and 12 hpi. (**e**) Normalized fluorescence intensity of lamin A/C and B in the nuclei of noninfected and infected cells at 8 and 12 hpi (n=74, 62, and 54, respectively). The error bars show the standard error of the mean. Statistical significance was determined using Tukey's test, and the significance values shown are denoted as **** ($p<0.0001$) or *** ($p<0.001$). Nonsignificant differences ($p \geq 0.05$) are not labeled.

Computational modeling explains the nuclear softening

Our experimental data indicated that the amount of lamin proteins increases, the nuclear lamina becomes less mobile, and its outward curvature increases in HSV-1-infected cells. These changes suggest that the nuclear envelope becomes stiffer during the infection. However, AFM indentation measurement suggested that nuclear stiffness reduces during the infection. To understand this seeming contradiction, we built a computational model of the nucleus to simulate the possible scenarios leading to the observed changes. The model consisted of the two components that determine the main mechanical properties of the nucleus: the NE, which is considered to include the nuclear lamina, and the chromatin. Furthermore, the connections between the two, LADs, and transient crosslinks within the chromatin were included in the model. The NE was modeled as a thin 3D triangulated shell, whereas the chromatin was represented by a polymer model. Additionally, an outward force was included, describing a combination of outward osmotic pressure and cytoskeletal forces on the NE. Finally, to model the VRC, another shell, similar to the NE, was added inside the nucleus and grown to an appropriate size to marginalize the chromatin towards the periphery (Fig. 6a and Supplementary Movie 4). Model parameters for the noninfected nucleus were taken from the literature or fitted by replicating the AFM experiments (Supplementary Movie 5).

To study the nuclear mechanics during HSV-1 infection, various model parameters were iteratively adjusted for observation of their impact on the simulated AFM results. Based on the observed low molecular density (Fig. 1a-c), we hypothesized that the VRC is very soft and disregarded its mechanical role in the infected cell AFM simulations. Additionally, we postulated that most of the changes seen in Young's modulus were due to the morphological and structural alterations of the nucleus rather than the cytoplasm. First, we examined the effect of the nuclear volume and height change on the simulated AFM results while keeping everything else constant (Fig. 6b). While there was no change in the simulated Young's moduli between the noninfected and 8 hpi similar-sized nuclei, the increase in the nuclear size at 12 hpi led to a significant increase in the simulated stiffness compared to the noninfected nuclei. Furthermore, since the amount of lamins in the nuclear lamina approximately doubled at late infection (Supplementary Fig. 2), we simulated the infected cases with doubled values for the lamina mechanical parameters. These changes led to further increased stiffness compared to the simulations with the base lamina parameters. Next, we simulated the effect of chromatin marginalization on the simulated Young's moduli and found no significant change (Fig. 6c). Finally, we investigated the importance of lamina-chromatin tethering by simulating the 8 hpi nuclei with and without LADs. We found that the omission of LADs significantly reduced Young's modulus (Fig. 6d). However, this decrease in stiffness was relatively minor compared to the experimentally observed change between noninfected and 8 hpi nuclei. Therefore, since these changes in the nucleus could not explain the experimentally observed reduction in nuclear stiffness, other mechanical changes must occur during HSV-1 infection.

To understand the changes in nuclear mechanics that could reduce nuclear stiffness, we focused on two key changes: decreased outward force and lamina disintegration (Fig. 6e,f and Supplementary Fig. 3). The first change includes the decrease in the osmotic pressure

difference between the cytoplasm and the nucleus or the decrease in cytoskeletal force on the nucleus via the LINC complexes. Lamina disintegration, a phenomenon observed during HSV-1 infection, was implemented by reducing the mechanical strength of a given percentage of connections in the lamina without changing the number of LADs. In general, the decrease in the outward forces had the most significant effect, as shown by the reduction of the simulated Young's moduli. Finally, lamina disintegration was generally insignificant when the outward forces were high but became more important at lower strengths of the outward forces. Thus, our computational data suggested that the outward force, including osmotic pressure and cytoskeletal forces, had the most prominent effect on the nuclear stiffness. Analysis of cell adhesion revealed that the cell contact area to the substrate, occupied by the actin-labeled cells, was reduced in infection (Supplementary Fig. 4). This suggests that the decreased outward forces by reduced actin cytoskeleton pull occurs in the infection.

Next, we used the model to study if the experimentally observed increase in NE membrane tension during the infection can co-occur with decreased outward forces and disintegration of the lamin network. We found that tension in the nuclear membrane is affected by both lamina disintegration and the decrease in outward forces (Fig. 6g and Supplementary Fig. 3). Lamina disintegration led to a significant increase in the nuclear membrane tension, while a reduction of outward forces decreased the tension significantly. The increase in membrane tension during lamina disintegration can be explained by the larger share of the outward forces falling on the membrane rather than the lamina. Importantly, these results show that the combined effect of lamina disintegration and the reduction of the outward forces can lead to a constant or increased NE membrane tension during infection. Furthermore, we compared the distribution of lamina tension in the NE as the lamina was disintegrated (Fig. 6h,i). There were more local high-tension spots on the top surface of the nucleus as the lamina disintegrated, which led to an

overall increase in the nuclear lamina tension. In all cases, the tension was higher on the sides of the nucleus (Fig. 6h), which is explained by the fact that there is no actin cap pushing against the nuclear outward forces in these regions. Taken together, the simulation results indicate that the decrease in nuclear stiffness during HSV-1 infection is a consequence of the reduced nuclear outward forces and not as significantly of the lamina disintegration. Also, the reduced nuclear stiffness can coincide with increases in the nuclear membrane and local lamina tension. Lastly, based on our assumptions and simulations, chromatin marginalization did not affect the nuclear stiffness during the infection.

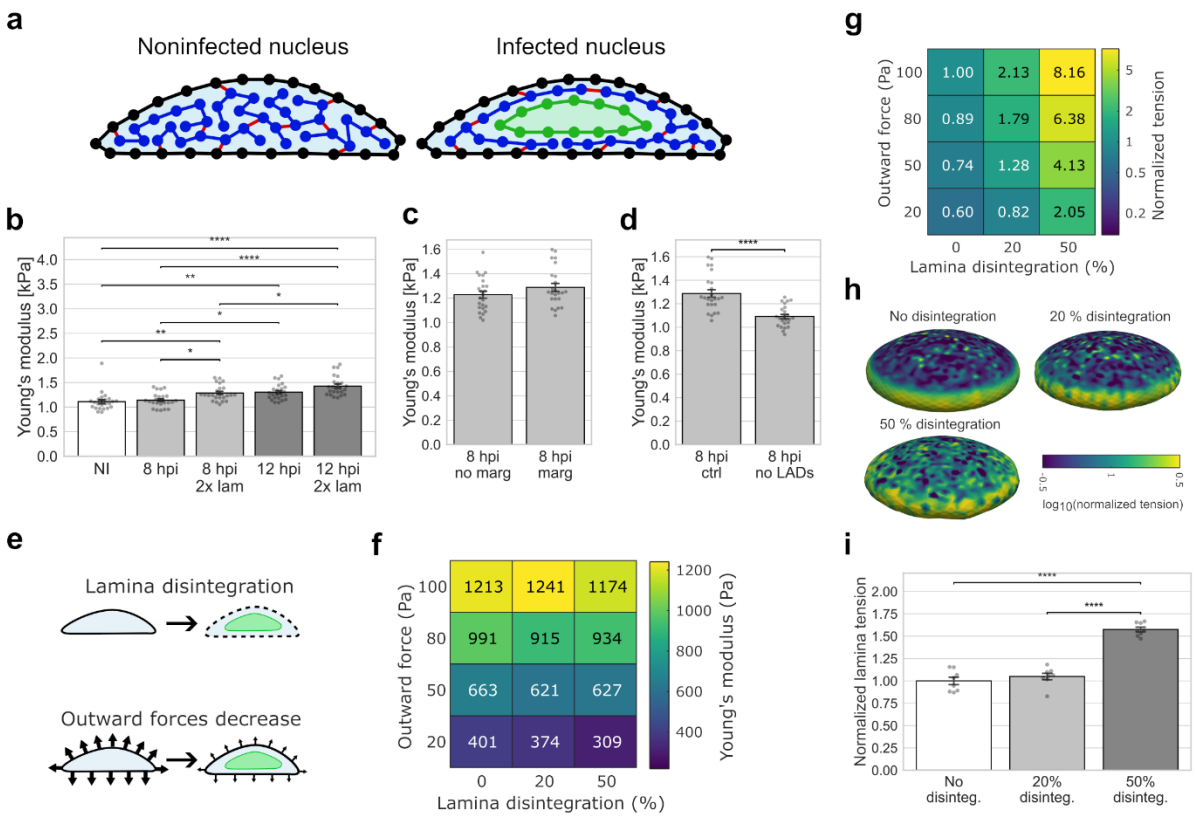

Fig. 6. Changes in mechanical parameters alter the nuclear stiffness.
(a) The setup for the computational model shows a noninfected and an infected cell nucleus. The nuclear envelope (black line), chromatin polymers (blue), envelope-chromatin and chromatin-chromatin connections (red lines), and the viral replication compartment (green line) are shown. (**b**) Simulated Young's moduli for the noninfected (NI) and infected cells (8 and 12 hpi), (**c**) for the infected nuclei (8 hpi) without and with chromatin marginalization close to the nuclear envelope, and **(d)** for the infected nuclei with and without lamina-associated domains (LADs) (n=24). The lamina strength was doubled for (b) and (c) compared to NI. (**e**) Simulation of two changes in the nuclear mechanical parameters: lamina disintegration and decrease in the outward forces. **(f)** Simulated Young's moduli as a function of parameters in infected cell nuclei at 8 hpi with double the lamina strength compared to NI (n=8). Significances are shown in Supplementary Fig. 3. **(g)** Normalized membrane tension as a function of lamina disintegration and outward forces (n=8). Significances are shown in Supplementary Fig. 3. (**h**) Representative examples of lamina forces with no lamina disintegration and disintegration of 20% and 50%. (**i**) The sum of normalized lamina forces on the top surface of the nuclei without or with 20% or 50% disintegration (normalized

to the mean value with no disintegration) (n=8). The error bars show the standard error of the mean. Statistical significances were determined using Tukey's test (b, i) or Student's t-test (c, d), and the significance values shown are denoted as **** ($p<0.0001$), *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$). Nonsignificant differences ($p \geq 0.05$) are not labeled.

DISCUSSION

Previous studies performed in isolated nuclei showed that nuclear stiffness increases after the nuclear injection of HSV-1 genome[55]. Our data shows that this is a transitory phenomenon or specific to isolated nuclei since the progression of the infection led to nuclear softening. The observed softening could be explained by changes in the nuclear structure caused by the enlarged VRC appearing at the center of the nucleus, changes in the nuclear lamina and chromatin, a decrease in the osmotic pressure, or a decrease in the external pull by the cytoskeleton.

The progression of HSV-1 infection leads to an enlarged nuclear VRC, and at the late stages of infection, the VRC can occupy one-third of the nuclear volume[26]. Our SXT results indicate that the VRC region is less dense than the euchromatin region in noninfected cells and the marginalized chromatin in infected cells. This shows that HSV-1 infection forms an expanded low-density nuclear center surrounded by chromatin. It has been demonstrated that particle density is proportional to stiffness in many materials, such as collagen-based hydrogels[56,57]. While other factors, such as crosslink density between hydrogel fibers[58], also influence the stiffness, the lower density of the VRC may indicate a lower stiffness compared to chromatin and contribute to nuclear softening during the infection. It has remained unclear how the infection and enlarged VRC affect the intranuclear ion and osmotic concentration. Modeling has stated that nuclear osmotic pressure is proportional to its macromolecular

concentration[59,60]. Thus, the reduced macromolecular concentration in the VRC region likely participates in regulating nuclear stiffness via osmotic pressure decrease.

One of the major regulators of nuclear stiffness is the nuclear lamina associated with peripheral heterochromatin, particularly lamins A and C[10,12,61–64]. Studies with lamin A/C-deficient yeast cells have shown that the chromatin tethering to the nuclear membrane increases the mechanical stiffness of the NE and untethering of the chromatin leads to a softer nucleus[7]. We showed that HSV-1 infection leads to a lamin-regulated nuclear volume increase and chromatin marginalization near the NE. Notably, the density of marginalized chromatin remains similar to the heterochromatin of the control cells. This might partially explain why chromatin translocation close to the NE does not increase the nuclear stiffness. We observed an increase in the expression and amount of lamin A, which has been linked to increased nuclear stiffness[65], contradicting our findings of nuclear softening. However, epitope accessibility might change in infection[14], and in a previous study, the expression of lamins remained unchanged during HSV-1 infection[66].

In HSV-1 infection, the nuclear softness is possibly altered by the virus-induced modification of the nuclear lamina. Herpesvirus infection results in local dispersion of the nuclear lamina by phosphorylation and redistribution of lamins A/C and B. Various viral proteins and kinases (UL13, UL34, ICP34.5, and Us3) mediate these processes[36,67–70]. We showed how the mobility of the nuclear lamina decreases in infection, suggesting a possible increase in its tension or rigidity. However, it cannot be ruled out that infection-induced changes in the microtubules might also play a role in nuclear envelope mobility[7]. Our simulations showed that the effect of lamina disintegration alone was not significant on simulated nuclear stiffness. Still, it became relevant when the cytoskeletal outward forces, described as the combined effect of cytoskeletal

forces and the osmotic pressure on the NE, were reduced. Finally, we showed that the nuclear membrane tension increases at late infection simultaneously with the expansion of the nuclear volume. This could be explained by earlier studies showing that nuclear swelling induces mechanical stretching forces that lead to elevated membrane tension[71]. Membrane tension could also be affected by changes in lipid deformation during the formation of the viral egress complex[72]. Our simulation showed that the disintegration of the lamina led to increased membrane tension even when the outward forces were reduced, as more of the outward forces were subjected to the membrane rather than the lamina.

Nuclear elasticity is partly regulated by the cytoskeletal tension mediated by the LINC complex, which links the nucleo- and cytoskeleton[10,64,12]. Suppressing LINC protein SUN2 changes nuclear mechanical properties and causes nuclear softening[73]. In macrophages, a deficiency of SUN1 and SUN2 proteins causes a decrease in nuclear size and an increase in nuclear elasticity[74]. Our data show that the progression of HSV-1 infection leads to a strong downregulation of SUN2. SUN2 is a short-lived protein with a degradation half-life of 240 min[75], and temporal proteomics analysis has confirmed the reduced amount of SUN2 at late HSV-1 infection[66]. This most likely regulates the mechanical properties of the nucleus and decreases the nuclear stiffness as the infection proceeds. However, it cannot be the sole effector since the nuclear stiffness is lower already at 4 hpi when the SUN2 has not been downregulated yet. SUN2 depletion also modifies the NE double membrane and the perinuclear space[75,76]. In HSV-1 infection, decreased expression of SUN2 supports the progression of infection and results in increased production of HSV-1 extracellular capsids, possibly by enhancing the NE modifications during the nuclear egress of capsids[77].

Our simulations were conducted to explain the experimental softening of the nucleus. They indicated that the reduction of the outward forces caused the most significant decrease in nuclear stiffness, suggesting that this could be the primary mechanism for the experimentally observed change in stiffness during the infection. The outward forces described the combined effect of cytoskeletal forces on the NE as well as the osmotic pressure. The observed decrease in the cell-substrate contact area suggests that the actin cytoskeleton forces might be altered. Additionally, it is known that HSV-1 rearranges the actin cytoskeleton via cofilin-1[78]. In addition, the low-density VRC leading to decreased osmotic pressure in the nuclear center could also lead to the measured reduction of stiffness observed in the AFM measurements.

Herpesvirus infection and the emergence of enlarged low-density VRC lead to changes in nuclear volume, the organization of chromatin, nuclear lamina, and the connection to the cytoskeleton. Virus-induced nuclear deformation modulates the physical nuclear environment and triggers changes in intrinsic forces acting on the nucleus (Fig. 7). Altogether, these profound changes cause nuclear softening. Our simulations show that the measured changes in the nuclear lamina and size have quite a minor effect on stiffness compared to the nucleocytoplasmic forces, namely cytoskeletal-nuclear connections and osmotic pressure. While the lamina reorganization is known to be essential for the nuclear egress of the new HSV-1 viral capsids, the role of the predicted reduction in the nucleocytoplasmic forces in the infection remains elusive. Changes in the osmotic balance between the nucleus and the cytoplasm can, for example, originate from changes in nuclear transport, which HSV-1-induced changes in the nuclear pore function could cause[79]. The impact of HSV-1 infection on nucleocytoplasmic osmotic balance merits further experimental investigation to clarify its role in the progression of the infection. In summary, these emerging insights shed new light on the

virus infection-induced fundamental nuclear modifications and their roles in cellular mechanobiology.

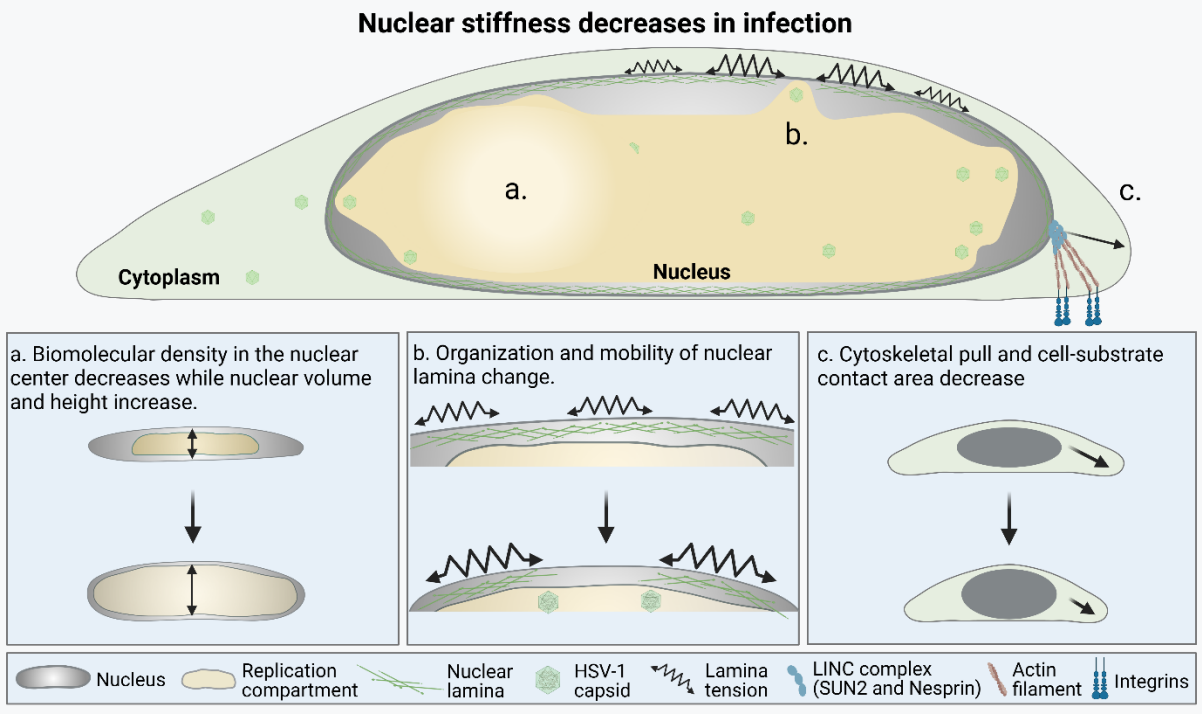

Fig. 7. Virus-induced changes in mechanical forces regulating nuclear stiffness.
Virus infection leads to extensive changes in nuclear organization and mechanical forges. (**a**) The infection results in an enlarged nuclear viral replication compartment, a low-density area surrounded by a condensed chromatin layer. (**b**) Changes in the organization and mobility of lamina accompany the increase in nuclear volume and height. (**c**) The nuclear stiffness is affected by cytoskeletal pull and osmotic pressure. The key regulator of cytoskeletal tension of the nucleus, LINC complex protein SUN2, is strongly downregulated in infection. Virus-induced alterations control the mechanical properties of the nucleus and lead to nuclear softening.

MATERIALS AND METHODS

Cells and viruses

Mouse embryonic fibroblast cells (MEF, ATCC® CRL-2991™) and African green monkey kidney cells (Vero, ATCC® CCL-81™) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine and penicillin-streptomycin (Gibco-Invitrogen, Carlsbad, CA) at 37 °C in the presence of 5% CO_2. HA-tagged progerin, a truncated version of lamin A[80], was expressed in the Vero cells using adenovirus[81]. Lamin A

adenovirus was used to overexpress wild-type lamin A in Veros. Lamin A adenovirus (based on RefSeq BC014507) was purchased from Vector Biolabs and used as previously described[81]. The cells were infected 4, 8, or 12 hours before imaging and measurements with wild-type 17+ strain, EYFP-ICP4 (vEYFP-ICP4[82]), or VP26-mCherry virus (HSV1(17^+)Lox-CheVP26[83–85]) using a multiplicity of infection of 5.

Atomic force microscopy (AFM)

Force spectroscopy AFM experiments were performed on noninfected and infected Vero cells at 4, 8, and 10 hpi using a NanoWizard 4 XP instrument (JPK BioAFM, Bruker Nano GmbH, Berlin, Germany) mounted on an inverted microscope (Nikon Ti-U, Nikon Instruments Europe B.V., Amsterdam, the Netherlands) equipped with a standard monochrome CCD camera (ProgRes MFCool, Jenoptik, Jena, Germany). Fluorescence imaging was performed by wide-field illumination (Intensilight Hg lamp, Nikon) with a 100X objective (Nikon CFI Apo VC, 1.4 NA, oil immersion) and the appropriate filter cube for EYFP fluorescence. A software module (DirectOverlay, JPK Bio-AFM, Bruker Nano GmbH, Berlin, Germany) calibrated the tip position with the optical image. Nanomechanical measurements were performed in spectroscopy mode using a 6.62 µm probe (CP-PNPL-SiO-C from SQube, mean cantilever spring constant $k_{cant} = 0.06$ N/m). Before each set of acquisitions, the sensitivity and spring constant of the cantilever was calibrated (thermal noise method). The measurements were performed in the buffered culture medium at 37°C with an injection of 5% CO2, with a relative setpoint of 2 nN, a Z-length of 12 µm, and a constant extend velocity of 1 µm/s. The center of the nucleus of a cell was visualized by fluorescence, and ten successive force curve measurements were taken. Raw data was cleaned manually by removing any aberrant contact point, and each force curve was fitted with a Hertz model to calculate Young's modulus using

JPK data Processing software (7.0.181). The average measures were plotted by cell (one point is one cell) after filtering out outliers (z-score>3).

Cryo-soft X-ray tomography (SXT) of suspended cells in capillaries

MEF cells were seeded into culture flasks (Corning, Corning, NY) and infected with HSV-1 vEYFP-ICP4 at an MOI of 5 for 4 and 8 hours at 37°C. Noninfected and infected cells were detached with trypsin-EDTA, pelleted by centrifugation (300x*g*), and washed with PBS. Cells were then fixed with glutaraldehyde (0.5%) and paraformaldehyde for 10 minutes at RT and 35 minutes at 4°C (4%, cat. No. 15713-S; Electron Microscopy Sciences, Hatfield, PA), washed once with PBS, pelleted by centrifugation (300x*g*), re-suspended in the PBS buffer, filtered, loaded into thin-walled cylindrical borosilicate glass capillaries (Hilgenberg GmbH, Hilgenberg, GER, Cat.No. 4023088), and then vitrified by quickly plunging them into liquid-nitrogen-cooled (~90 K) liquid propane. SXT tomographic data were collected by full-rotation imaging with a soft X-ray microscope (XM-2) in the National Center for X-ray Tomography at the Advanced Light Source (http://www.als.lbl.gov) of Lawrence Berkeley National Laboratory (Berkeley, CA). Cells were kept in a stream of liquid-nitrogen-cooled helium gas to avoid radiation damage during data collection. For each data set, 180° rotation tomographs (1 projection image per 2° angle) were collected[86,87]. Projection images were aligned by tracking fiducial markers and reconstructed using automatic reconstruction software[88]. The segmentation of the high- and low-density chromatin was realized using the histogram separation into two classes by Dragonfly (Comet Technologies Canada Inc., Montreal, Canada).

Focused Ion Beam Scanning Electron Microscopy (FIB-SEM)

The volume imaging data set of MEF cells having enhanced contrast on chromatin was generated by FIB-SEM. MEFs, noninfected or HSV-1 infected at MOI of 5 for 8 h, were processed for imaging as described earlier[89]. The Chrom EM method was used to enhance the contrast of DNA[42]. The deposition of the osmiophilic polymer of diaminobenzidine, specifically on the DNA, was carried out using the fluorescent emission of DRAQ5. For this, the cells were placed on a cooled stage of a Leica DM IL LED microscope and exposed to 620 $\pm$ 30 nm light in the presence of diaminobenzidine, inducing its local polymerization over DRAQ5-bound DNA. The cells were treated with osmium tetroxide, followed by dehydration with a graded series of ethanol and embedding in resin. One set of cells was thin-sectioned and then poststained with 2% uranyl acetate and lead citrate. Another set of cells was coated with platinum. The sections were imaged using a Jeol JEM – 1400HC transmission electron microscope with an Olympus SIS Quemesa bottom-mounted CCD camera (80 kV). The datasets were acquired in a Zeiss Crossbeam 500 using backscattered and secondary electron signals. The area of interest was exposed by a milling beam (0.7 nA at the accelerating voltage of 30 kV) and imaged by a scanning electron beam (0.31 or 0.50 nA, 1.5 or 1.6 keV). The milling and scanning process on the cross-section of the cell created an image volume having a voxel size of 2.5 x 2.5 x 5 nm^3. The datasets were collected and aligned using Zeiss Atlas 5 software with a 3D tomography module. Microscopy Image Browser[90] was used to segment the capsids, chromatin, and nuclear envelope. The chromatin segmentation was done semi-automatically using MIB (https://mib.helsinki.fi/) and cleaned using iterative intensity-based erosion. The local thickness of the chromatin was measured using ImageJ (https://imagej.net/imagej-wiki-static/Local_Thickness) in Python, the local maxima were identified, and the local thickness value was extracted. The distance of these local maxima to the nuclear envelope was then calculated using the Euclidean distance transform.

Confocal imaging and analysis

Wild-type Vero cells, Vero cells expressing HA-tagged progerin, or overexpressing wild-type lamin A were grown on glass coverslips for microscopy to analyze the nuclear volume. The cells were infected with HSV-1 EYFP-ICP4 and fixed at 8 and 12 hpi with 4% PFA, and DNA was labeled with DAPI. Viral EYFP-ICP4 was boosted with an anti-GFP antibody. Cells were embedded into VECTASHIELD® PLUS Antifade Mounting Medium (H-1900) (Vector Laboratories). The cells were imaged using a Leica SP8 confocal microscope with UPLSAPO 63x oil immersion objective (NA 1.35). DAPI (Invitrogen) was excited with a 405 nm diode laser, and a 410-439 nm band-pass filter was used to detect the fluorescence. EYFP was excited with a 488 nm laser, and the fluorescence was collected with a 519-540 nm bandpass filter. Lamin A/C was excited with a 653 nm laser, and fluorescence was collected with a 661-839 nm filter. Stacks of 1024 x 1024 pixels were collected with a 120 nm/pixel pixel size in the x- and y- directions and 400 nm in the z-direction. The nuclei were segmented using the minimum cross-entropy threshold algorithm[91] for the DAPI channel.

For analyses of the nuclear intensity of lamins, Vero cells infected with HSV-1 EYFP-ICP4 and fixed at 8 and 12 hpi were labeled with antibodies against lamin A/C (mouse monoclonal antibody MAb; ab8984, Abcam, Cambridge, UK) and Lamin B (rabbit polyclonal antibody, rAb; ab16048, Abcam). The primary antibodies were followed by goat anti-rabbit or anti-mouse Alexa 488- and 647-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). DNA was labeled with Hoechst 1:10000 in PBS for 10 min. Cells were embedded into VECTASHIELD® PLUS Antifade Mounting Medium (H-1900) (Vector Laboratories). The immunolabeled cells were imaged using a Leica TCS SP8 FALCON laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany). Alexa 546 and

Alexa 647 were excited with 557 nm and 653 nm wavelengths of pulsed white light laser (80 MHz), respectively. The emission detection range was 568-648 nm for Alexa 546 and 663-776 nm for Alexa 647. DAPI was excited with a 405 nm diode laser, and emission between 415-495 nm was detected. Laser powers for all channels were fixed. Stacks of 1024 x 1024 pixels were collected with 120 nm/pixel in the x- and y- directions and 240 nm in the z-direction. The nuclei were segmented using a minimum cross-entropy threshold algorithm[91] for the DAPI channel. The total nuclear intensity was calculated for the pixels belonging to the segmented nucleus, and the intensity near the NE was calculated by using only pixels having an Euclidean distance of 1 μm or less from the NE.

The distribution and mobility of lamins were imaged in living noninfected and HSV-1 mCherry-infected (8 and 12 hpi) Vero cells transfected with lamin A/C-Chromobody-GFP2 (Proteintech, Manchester, UK) using Leica HC PL APO CS2 1.2NA 63X water immersion objective. During the imaging with Leica SP8 Falcon, the lamin A/C-Chromobody-GFP2 was excited with a 488 nm wavelength of pulsed white light laser (80 MHz). The emission detection range was 495-560 nm. DAPI was excited with a 405 nm diode laser, and 412-475 nm emission was detected. The image stack size was 296 x 296 pixels with a 100 nm/pixel size in the x- and y-directions. The lamina was analyzed by first removing its translational and rotational motion. The lamina was skeletonized at each time point, and then the maximum intensity projection of the skeletons was taken. The area of the maximum intensity projection was calculated and divided by its length. The curvature was defined by discretizing the nuclear lamina using 16-pixel (1.6 μm) long line segments and calculating the angles between consecutive segments.

Nuclear membrane tension was analyzed by fluorescent lifetime imaging (FLIM) using fluorescent membrane tension probe ER Flipper-TR® (Spirochrome) in noninfected and HSV-

1 17+ wt infected live Vero cells at 8 and 12 hpi. Measurements were performed with Leica SP8 Falcon with Leica HyD SMD detectors and Leica HC PL APO CS2 1.2NA 63X water immersion objective. The ER Flipper probe was used in 1 µM concentration, and the cells were also labeled using Hoechst 33342 in 1 µM concentration. One plane at the center of the nucleus, where the nuclear edges were clearly visible, was first acquired with 405 nm excitation and 415-465 nm emission to visualize DNA with Hoechst. Immediately after, the ER Flipper was imaged using 477 nm excitation and 550-650 nm emission and line accumulation of 16. The scanning speed was 200 Hz, the repetition rate was 80 MHz, and the image size was 328x328 pixels with 81x81nm/pixel for both Hoechst and ER Flipper imaging. For tension analysis, pixel binning of 3 was used resulting in approximately 500-1500 photons per pixel. After binning, a pixel-wise two-component fitting was performed, and the longer component describing the membrane tension was extracted. Using the Hoechst data, a 486 nm wide ring just outside the cellular DNA was used as a mask to obtain the nuclear membrane area from the tension data. A mean of the longer ER Flipper lifetime along the nuclear envelope was calculated for each cell.

To measure the contact area of the cells during the infection, ICP4-EYFP-infected cells were labeled using a mouse Anti-beta Actin antibody ([mAbcam 8226], Abcam). The contact area of the cells during the infection was analyzed by taking the sum projection of the actin channel in the z-direction, thresholding the sum projection using the minimum cross-entropy algorithm[91], and calculating the area of the segmented region.

Global Run-On and sequencing (GRO-seq)

GRO-seq analysis was performed as described previously[89]. Briefly, to prepare nuclei, noninfected and infected Vero cells (at 4, 8 hpi) were swollen, and the nuclei were collected

and stored at -80°C. The preparation of GRO-Seq libraries followed the protocol described previously[89,92]. The library quantification was performed using a Qubit fluorometer (Thermo Fischer Scientific) and readied for 50 bp single-end sequencing on an Illumina Hi-Seq2000 platform (GeneCore, EMBL Heidelberg, Germany). The raw sequencing reads were assessed by the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). GRO-seq reads were aligned to the ChlSab1.1 genome using the bowtie-0.12.7 software[93]. The best alignment was reported while allowing up to two mismatches and up to three locations per read. Differentially expressed genes were detected using ChlSab1.1.95.gtf coordinates, and the RNA.pl workflow of HOMER 4.3 and edgeR v3.2.2 was analyzed. Genes were considered differentially expressed using the following cutoffs: log2 fold change 0.585, RPKM 0.5 in at least one sample, and adjusted p-value of 0.05. GRO-seq reads were mapped to the human protein atlas (https://www.proteinatlas.org/) to select lamins and nuclear lamina-related proteins. The interaction of these proteins was further mapped using the String database (https://string-db.org/) by selecting experimentally shown interactions using a threshold of 500. The non-interacting proteins were identified in the same String database with the condition that they interact with at least one detected protein. The resulting graph was generated in Python using the module networkx and manually adjusted.

Computational modeling

The developed computational model described the nucleus as a NE, which was considered to include the lamina, and chromatin components in three dimensions. The main model assumptions were: (1) the mechanical properties of the nucleus are dictated by the nuclear envelope, chromatin, and outward forces, including actin cytoskeleton prestress and osmotic pressure difference; (2) no changes in the mechanical properties occur during the short AFM

measurement; and (3) the VRC has low density and has no effect on the AFM measurement. The envelope was triangulated into a viscoelastic shell, and self-avoiding polymer chains described the chromatin. Furthermore, additional interactions were defined to describe various connections, such as the LADs between the chromatin and the lamina and those between different parts of the chromatin. The herpesvirus VRC was described as a single coherent entity within the nucleus. It was represented similarly to the nuclear envelope as a triangulated shape that repulses the chromatin while it grows.

The model was simulated by calculating a force affecting each vertex originating from various, usually Hookean, interactions and moving them using the dampened equation of motion:

$$\gamma \vec{v}_i + \sum_j c_{ij} \left(\vec{v}_i - \vec{v}_j \right) = \vec{F}_i + \eta,$$

where γ is the system viscosity, $\vec{v}_i$ is the velocity of vertex i, c_{ij} is the friction between vertices i and j, $\vec{F}_i$ is the total force affecting vertex i, and η is the random thermal motion. The equation was solved using the conjugate gradient and the vertex movements using the forward Euler method.

To simulate the AFM experiments, a cantilever with properties mirroring those of the one used in the experiments was used to push down on the nucleus, and the resulting force and indentation depth data was used to calculate the Young's modulus using the Hertz model. The computational model was implemented using Julia programming language (v1.10.2), and the model and the parameters are described in more detail in Supplementary Text 1. The code is available on GitHub (https://github.com/atervn/nuclear_mech) and Zenodo (https://zenodo.org/records/14512358). The data fitting for the Hertz model was done using

Matlab (The MathWorks Inc, R2021a, Natick, Massachusetts) and 3D visualization using ParaView[94].

Data availability

The authors declare that the GRO-Seq data were deposited into the Gene Expression Omnibus database under accession number GSE243613 and are available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE243613. All other relevant data are in the manuscript and its supporting information files.

Contributions

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Ethical statement

The experiments were done using commercially available cell lines without ethical challenges.

Competing interests

The authors have declared that no competing interests exist.

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