Parvovirus infection alters the nucleolar structure

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Running head: Parvovirus infection-induced changes in nucleoli

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ABSTRACT

The nucleolus is a dynamic nuclear structure which plays important roles in ribosome biogenesis and cellular stress response to stressors such as viral infection. The nucleolus and nucleolar proteins are essential for the progression of infection by several viruses. Consequently, viral infection often induces alterations in nucleolar structure and composition. Here, we applied a deep learning algorithm segmentation and nucleolin labeling to analyze the nucleolar changes induced by autonomous parvovirus infection. Our results show that the size of nucleoli decreases and nucleolin is released into the nucleoplasm in late infection. Analyses of ki-67, one of the NS2-associated nucleolar proteins and a key factor in nucleolar organization, showed that the interaction between ki-67 with DNA increases in infection. The infection initiated by a viral clone lacking an intact NS2 failed to decrease the nucleolar size, however, the orientation of the nucleoli was changed. Our results suggest that parvoviruses modify and exploit nucleoli and nucleolar proteins during infection, and NS2 protein might play a role in the regulation of these processes.

(Words 169)

Authors summary

Autonomous parvoviruses with small genomes and only a small number of encoded proteins depend on the cellular nuclear machinery for their efficient replication. The nucleolus is an active nuclear structure involved in the essential functions of cells including synthesis of ribosomal RNA and regulation of cell growth and cellular stress. Many viruses target the nucleoli to manipulate cellular antiviral defense and to facilitate their replication by recruiting nucleolar proteins. Capsids of dependoparvoviruses, such as AAV, are assembled in the nucleolus, however, much less is known about how autonomous parvoviruses interact with the

nucleolus. Our results suggest that the progression of autonomous parvovirus infection results in structural alteration of nucleolar structure and redistribution of nucleolar proteins. Analyses of the nonstructural protein NS2 mutants in comparison to wild-type infection indicated that NS2 might play an important role in viral interactions with the nucleolus.

INTRODUCTION

Parvoviruses are small DNA viruses that undergo replication inside the host cell nucleus. Due to their relatively small genome size parvoviruses depend heavily on cellular factors and pathways for successful replication. Interaction with a multifunctional subnuclear compartment, nucleolus, is crucial for the replication and pathogenesis of several DNA viruses including herpesviruses (Boyne & Whitehouse, 2009; Callé et al., 2008; Pearson et al., 2011) and adenoviruses (Hindley et al., 2007; Lam et al., 2010; Lee et al., 2003; Okuwaki et al., 2001). Moreover, the capsids of adeno-associated viruses (AAV), a helper-dependent parvovirus, are assembled in the nucleolus (Johnson & Samulski, 2009; Wistuba et al., 1997). It is not clear, however, what is the role of nucleoli in autonomous parvovirus life cycle.

The nucleolus is the largest nuclear subcompartment which performs essential cellular functions involved in ribosome biogenesis such as transcription of ribosomal RNA genes, processing of precursor rRNAs, and ribosome subunit assembly (Dubois & Boisvert, 2016; Raška et al., 2006). Nucleoli are formed from specific chromosomal regions of acrocentric chromosomes (HSA13, 14, 15, 21, 22) termed as nucleolar-organizing regions (Henderson et al 1972). The mammalian nucleolus comprises three major morphological components, the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC) (Hernandez-Verdun, 2011; Pederson, 2011; Raška et al., 2006). The FC contains several hundred copies of rRNA genes, arranged in tandem arrays of ribosomal gene repeats (McStay,

2016; Pederson, 2011). FCs and DFCs are involved in rDNA transcription and production of precursor ribosomal RNA (pre-rRNA), whereas further processing of pre-rRNA and assembly of ribosome subunits occur in GC (Hernandez-Verdun, 2011; Pederson, 2011; Raška et al., 2006). The nucleoli are dynamic structures and assemble through liquid-liquid phase separation when their mobile proteins constantly move between the nucleolus and the nucleoplasm (Falahati & Wieschaus, 2017; Feric et al., 2016; Lafontaine et al., 2021). The proteome of the human nucleoli contains approximately 4500 mobile proteins (Ahmad et al., 2009; F. M. Boisvert et al., 2010). The nucleoli have many additional functions besides ribosomal biogenesis including the regulation of mitosis and the cell-cycle, and acting as sensors for stressors such as oxidative stress, heat shock, nutrient deprivation (F.-M. Boisvert et al., 2007; Boulon et al., 2010; Iarovaia et al., 2019; Scherl et al., 2002) and viral infection (Salvetti & Greco, 2014).

Autonomous parvoviruses have non-enveloped icosahedral particles of ∼25 nm in diameter containing a linear single-stranded DNA genome of ∼5.0 kb. The viral DNA encodes two open reading frames for structural and nonstructural proteins (Cotmore et al., 1983). The canine parvovirus (CPV) capsid is composed of viral proteins VP1 and VP2, and capsids with DNA contain VP3, which is formed through the proteolytic cleavage of the VP2 N-terminus (Weichert et al., 1998). Autonomous parvoviruses encode two nonstructural proteins, NS1 and NS2 (Cotmore et al 1983). The CPV NS1 protein is a multifunctional protein that exhibits site-specific DNA binding, ATPase, helicase, and nickase activities (Niskanen et al., 2010, 2013) and is involved in the induction of apoptosis in host cells (Gupta et al., 2016; Saxena et al., 2013). The NS2 protein of an autonomously replicating parvovirus, minute virus of mice (MVM), is involved in capsid assembly, viral DNA replication and translation of viral mRNA (Cotmore et al., 1997; Naeger et al., 1990, 1993). Although the function of CPV NS2 is

currently mostly undefined our recent studies showed that it plays a role in chromatin remodeling and DNA damage response during replication (Mattola et al., 2022).

In this study the structural changes of nucleoli were monitored as CPV infection proceeded, particularly, the role of the NS2 protein in the process was clarified. We discovered that upon infection the nucleolar size was significantly altered and cellular DNA and nucleolar proteins were redistributed, and many of these changes were not detected if the virus lacked an intact NS2. The potential role of NS2 in the interaction between virus and nucleolus is also supported by the recent proximity-dependent biotin identification (BioID) findings showing that NS2 is associated with several nucleolar proteins.

RESULTS

Nucleolar size decreases in late infection

The nucleolus is a highly dynamic structure that responds by profound morphological and compositional alterations to cellular stresses such as viral infections (Lam et al., 2010; D. A. Matthews, 2001; Olausson et al., 2012; Salvetti & Greco, 2014). To characterize the nucleolar response to CPV infection-induced cellular stress, we examined the morphological alterations of nucleoli as CPV infection proceeded.

We studied the size and number of nucleoli in NLFK cells at 8, 12 and 24 hours post infection (hpi) by segmenting the labeled nucleolin signal using a deep learning algorithm (Fig. S1). The analyses showed that at 8 and 12 hpi the nucleolar volume was not significantly different from that in noninfected cells. However, a significant decrease was detected at 24 hpi (Fig. 1A and 1B). Simultaneously, the average number of nucleoli per nucleus remained similar to

noninfected cells at 8 and 12 hpi, however, a slight but statistically insignificant increase was detected at 24 hpi (Fig. 1C).

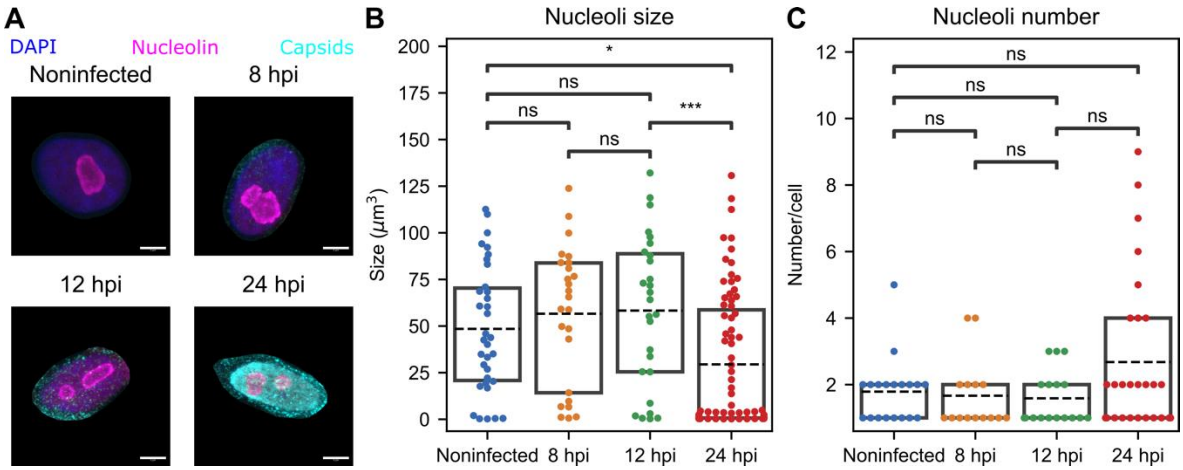

Figure 1. Progression of infection leads to changes in size and structure of nucleoli. (A) Representative confocal images show the localization of the nucleoli (magenta) in noninfected and infected NLFK cell nuclei at 8, 12, and 24 hpi. Only nuclei are shown. The cells were immunolabeled with antibodies against nucleolin (magenta) and intact capsids (green). Blue corresponds to DAPI staining. Scale bars, 5 µm. The box-swarm plots represent (B) size defined by nucleoli volume and (C) number of nucleoli in noninfected and infected cells (n = 34, 25, 27, and 75 for noninfected, 8, 12, and 24 hpi, respectively). The 3D nucleoli were segmented using a deep learning algorithm. The error bars show the standard error of the mean. Statistical significances were determined using Student´s t-test. The significance values shown are denoted as *** ($p<0.001$), * ($p<0.05$), or ns (not significant).

Altogether, our findings demonstrated a reduced nucleolar size and a slightly increased number of nucleoli in response to progression of infection, suggesting that infection induces important nucleolar alterations typical to nucleolar stress (Yang et al., 2018).

Nucleolin is released into the nucleoplasm from the nucleolus in late infection

The multifunctional phosphoprotein nucleolin is one of the most abundant nucleolar proteins (Biggiogera et al., 1990). Nucleolin has important roles in rDNA transcription, rRNA maturation, in the assembly and transport of ribosomes (Ginisty et al., 1999; Srivastava & Pollard, 1999). To participate in these processes nucleolin shuttles between the nucleolus and nucleoplasm (Chen & Huang, 2001). Relocalization of nucleolin out of the nucleoli is also triggered by cellular stress (Daniely & Borowiec, 2000; Jia et al., 2017) and by viral infection,

e.g. with herpes simplex virus type 1 (HSV-1) (Callé et al., 2008; Greco et al., 2012; Lymberopoulos & Pearson, 2007).

To gain insight into CPV infection-induced changes in the distribution of nucleolin, we analyzed noninfected and infected NLFK cells at 8, 12, and 24 hpi. Compared to noninfected cells, the total amount of intranuclear nucleolin was higher at 8and 12 hpi, but not at 24 hpi (Fig 2A). The nuclear distribution of nucleolin in infected cells was relatively similar to noninfected cells, however, nucleolin was slightly more concentrated close to the nuclear envelope (NE) at 12 hpi (Fig 2B). The amount of nucleolin in the segmented nucleoli was similarly significantly higher at 8 hpi and 12 hpi, but not at 24 hpi, in comparison to noninfected cells (Fig. 2C). Notably, the comparison of the ratios of nucleolar nucleolin to nuclear nucleolin in revealed a significant decrease the relative amount of nucleolar nucleolin at 24 hpi. This indicated a shift in the nucleolin distribution from the nucleolus to the nucleoplasm (Fig 2D).

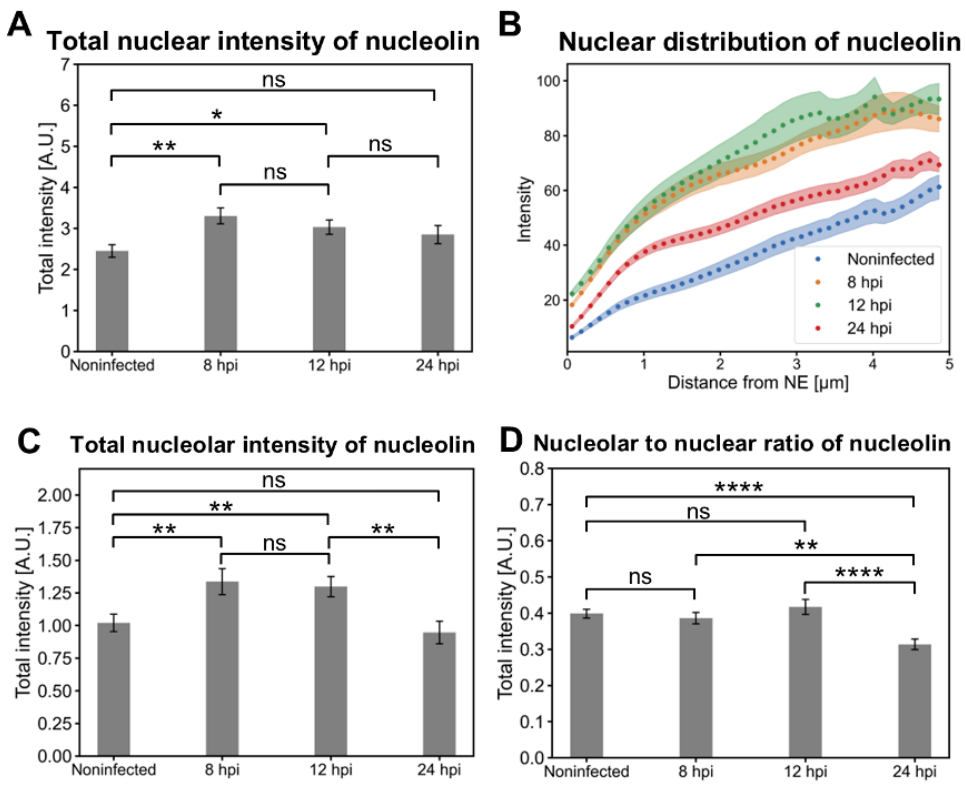

Figure 2. Distribution of nucleolin changes as infection proceeds.
Analyses of temporal changes in infected cells at 8, 12 and 24 hpi (see Fig. 1A). (A) Total nuclear intensities of nucleolin, (B) nuclear distributions of nucleolin as a function of distance from the NE, (C) total nucleolar intensities of nucleolin in the segmented nucleoli, and (D) the nucleolar to the nuclear ratios of nucleolin are shown (n=19, 15, 17, and 28 for noninfected, 8, 12, and 24 hpi, respectively). The shaded areas in distribution graphs represent the standard error of the mean. The error bars show the standard error of the mean. Statistical significances were determined using Student´s t-test. The significance values shown are denoted as **** ($p<0.0001$), ** ($p<0.01$), * ($p<0.05$) or ns (not significant).

To characterize potential structural alterations in nucleolar structure during CPV infection, we examined the changes in the chromatin organization inside the nucleolus. As expected, DNA was accumulated toward the NE at late stages of infection (Fig. 3A). Next, the analysis of segmented nucleolar region revealed that the amount of nucleolar DNA was significantly increased in comparison to noninfected cells at 8 and 12 hpi and decreased from 12 hpi to 24 hpi (Fig. 3B). To examine the organization of chromatin around and inside the nucleoli, we analyzed the distribution of DNA on both sides of the nucleolar border. In noninfected cells the DNA was distributed both outside and inside the nucleolus. In infected cells, nucleolin was distributed more to the nucleolar interior at 8 and 12 hpi, and to the exterior at 24 hpi (Fig. 3C).

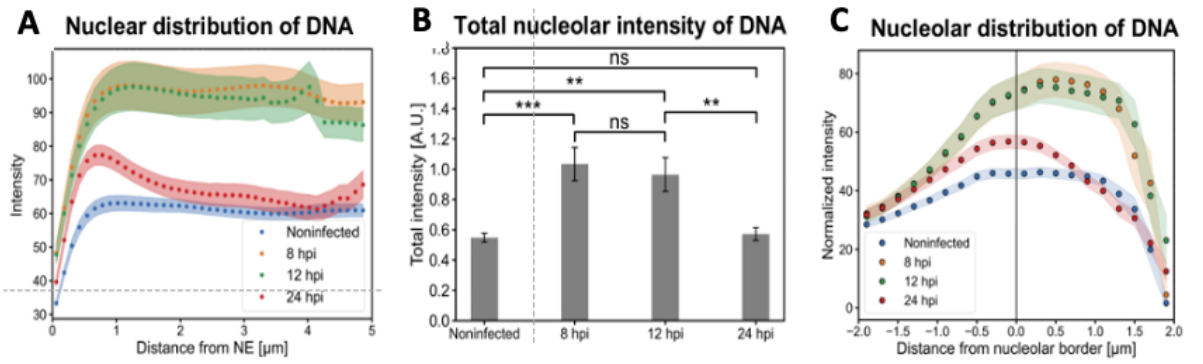

Figure 3. Nucleolar chromatin organization is changed in infected cells.
Analyses of time-dependent changes in DNA intensity and distribution of infected cells (see Fig. 1A). A) Nuclear distribution of DNA in noninfected and infected NLFK cell nuclei at 8, 12, and 24 hpi (n =19, 15, 17, and 28, respectively). (B) The total nucleolar intensities of DNA in segmented nucleoli and (C) distribution of DNA close to the segmented nucleolar edge as a function of increasing distance from the nucleolar border are shown. The error bars and the shaded areas in the distribution graphs represent the standard error of the mean. The error bars show the standard error of the mean. Statistical significances were determined using Student´s t-test. The significance values shown are denoted as *** ($p<0.001$), ** ($p<0.01$) or ns (not significant).

Taken together, these findings suggest that the shuttling of nucleolin from nucleoli to nucleoplasm is increased upon progression of infection, possibly due to the infection-induced cellular stress. Moreover, the infection-induced redistribution of DNA in close proximity and inside the nucleolus suggest that nucleolar structure changes during infection.

NS2 interactomes are localized to multiple nucleolar regions

Our previous BioID analyses revealed 122 high-confidence interactor proteins of NS2 (Bayesian false discovery rate, FDR ≤0.05) in infected and noninfected cells (Mattola et al., 2022). Comparison of the NS2 interacting proteins with the identified nucleolar proteins from the Human Protein Atlas (HPA) database (www.proteinatlas.org; Uhlen et al. 2010; Thul et al. 2017) revealed 26 nucleolar NS2 interactors in infected cells. The database is based on a study which identified 1,318 nucleolar proteins mostly located in FC, DFC or nucleolar rim in the interphase cells (Fig. 4A) (Stenström et al., 2020). Our analysis of the distribution of NS2-associated proteins demonstrated that 13 proteins were localized to the whole nucleolus, five proteins to the fibrillar center, and two proteins to the nucleolar rim. One protein showed dual localization to both the fibrillar center and nucleoplasm, and five proteins to the nucleolar rim and nucleolus (Fig. 4B). Further identification of NS2-associated proteins indicated that they have a role in multiple biological processes including transcription, RNA processing, DNA damage response and cell cycle (Fig. 4C, Table S1). The proteins involved in RNA processing were localized to the whole nucleolus, whereas proteins of DNA damage response were found from the fibrillar centers. Many of the proteins had multiple functions within several nucleolar regions. The number of nucleolar NS2-associated proteins was smaller in infected cells compared to that in NS2 transfected noninfected cells which suggest that actual number of interactors during infection is smaller than the possible number of interactors. Consequently, it is likely that some of these associated proteins are temporally linked to NS2 at a specific time

of the infection (Fig. S2A). In noninfected cells, NS2-associated proteins were localized to the whole nucleolus (21), to nucleolar rim (3), and five proteins had dual localization to both the fibrillar center and nucleoplasm, and five proteins to the nucleoli rim and nucleolus. The interactors associated to fibrillar centers were specific to that localization only (Fig. S2B). The top putative interactor of NS2 identified by BioID in infected cells was the proliferation marker protein Ki-67 (MKI67) (Mattola et al., 2022).

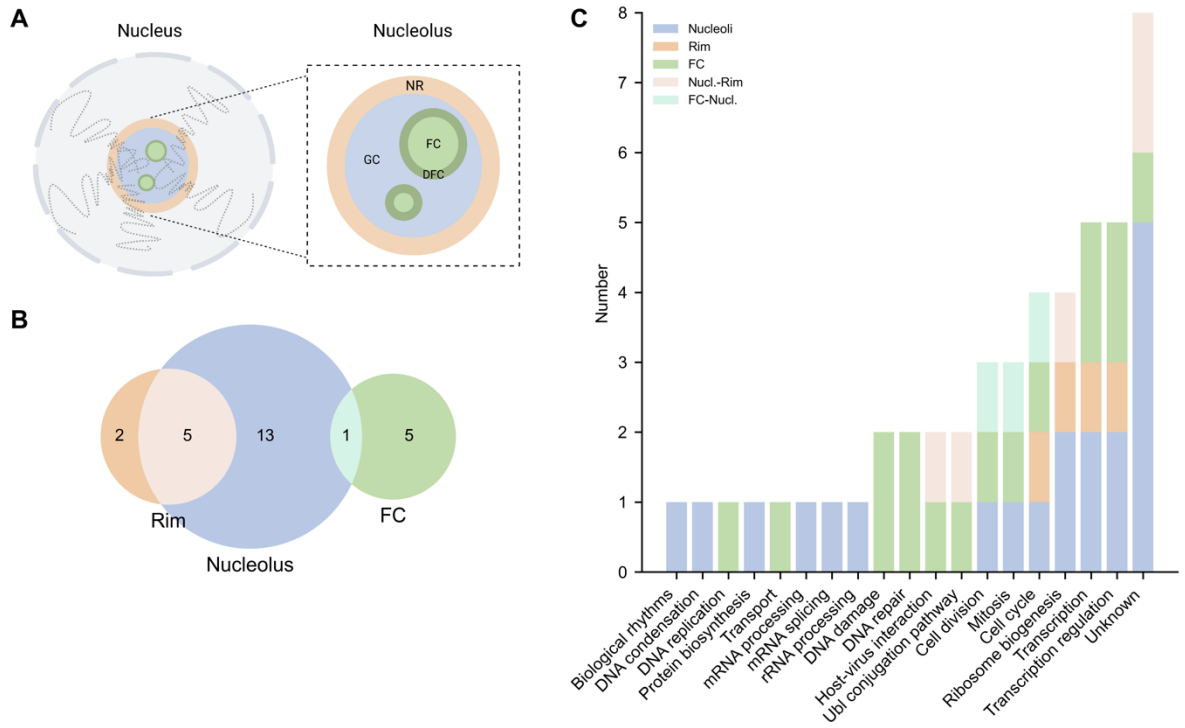

Figure 4. NS2-associated proteins have multiple localization sites in the nucleoli.
(A) A schematic image of the organization of the nucleolus and its subcompartments: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). (B) A Venn diagram showing the nucleolar localization of NS2-associated proteins identified by BioID in infected cells. The numbers of proteins localized into the whole nucleolus (blue), fibrillar center including FC and DFC (green), nucleolar rim (orange), or dual localization (light brown, light green) are shown. (C) The cumulative plot shows the enriched Gene Ontology (GO) terms for biological processes and nucleolar localization of NS2-associated proteins.

Altogether, proteins associated with NS2 are localized to multiple regions of the nucleolus most likely reflecting their multiple structural and functional features during infection.

Interaction between chromatin and ki-67 increases in late infection.

The ki-67 protein is a nucleolar heterochromatin anchoring protein which is specifically localized to the nucleolar rim of interphase cells (Stenström et al., 2020). Ki-67 is involved in the structural organization of chromatin and the nucleolus (Booth et al., 2014; Cuylen et al., 2016; MacCallum & Hall, 2000; Sobecki et al., 2016). As described previously, ki-67 is a prominent interactor for CPV NS2 (Mattola et al., 2022).

To examine virus-induced changes in nucleolar organization, we analyzed the distribution of ki-67 in noninfected and infected NLFK cells at 24 hpi. In noninfected cells ki-67 was accumulated in nucleoli, whereas in infected cells ki-67 was located at the perinuclear region, around the nucleoli and in the nucleoplasm (Fig. 5A). Both DNA and ki-67 showed similar nuclear distribution in infected cells including accumulation close to the NE (Figs 5B and 5C). Total intensities of DNA and ki-67 were significantly increased in comparison to noninfected cells (Fig 5D and 5E). Notably, the correlation between DNA and ki-67 increased significantly suggesting that infection facilitated tethering of ki-67 to the DNA (Fig 5F).

Taken together, we demonstrated that infection leads to an increase in the amount of ki-67, enhancement of ki-67 association with cellular DNA, and redistribution of ki-67 to the perichromosomal layer around the nucleoli and close to the NE. This supports a model where ki-67 is involved in the virus-induced change of DNA organization in nuclear and nucleosomal periphery.

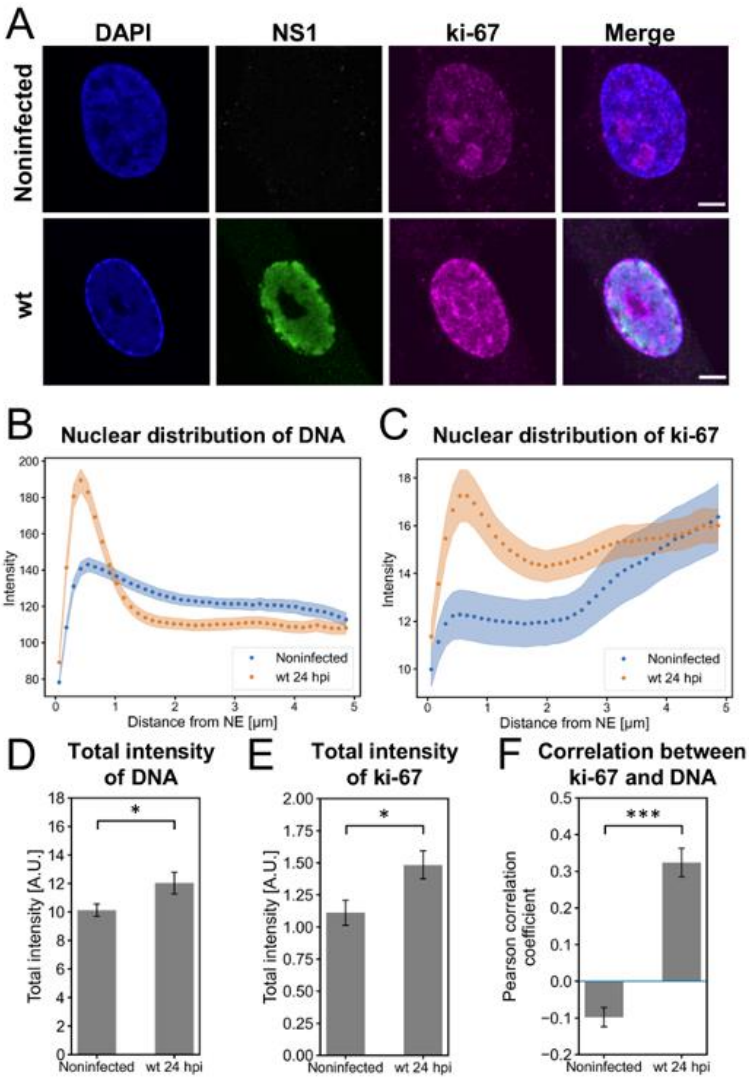

Figure 5. Association of ki-67 with DNA increases in infection
(A) Representative confocal images showing the localization of DNA (blue), NS1 (green), and ki-67 (magenta) in noninfected and infected NLFK cells at 24 hpi. The cells were immunolabeled with antibodies against NS1 and ki-67. DNA was labeled with DAPI. Scale bars, 5 µm. The nuclear distributions of DNA (B) and ki-67 (C) as a function of increasing distance from the NE in noninfected and infected cells (n = 30). The shaded areas represent the standard error of the mean. Total intensities of DNA (D) and ki-67 (E) are shown together with the Pearson correlation coefficients between ki-67 and DNA (F). The error bars show the standard error of the mean. Statistical significances were determined using Student´s t-test. The significance values shown are denoted as *** ($p<0.001$) and * ($p<0.05$).

NS2 plays a role in the infection-induced changes of nucleoli

Our previous BioID analysis suggested an association between CPV NS2 and several nucleolar proteins (Mattola et al., 2022). To further assess the role of NS2 in the nucleolar changes, we compared the nucleolar size and number in cells transfected with wild type (wt) infectious clone or with NS2 mutant clones. These mutants were designed to disrupt the expression of NS2 but not to affect sequence of NS1 (Wang et al., 1998). The NS2 mutants contained termination codons in the NS2 coding sequence of the infectious clone either upstream of the common NS1/2 splice donor or downstream of the NS2-specific splice acceptor. The original publication indicated that the NS2 splice donor mutant led to significantly deficient production

of viral capsids, capsid proteins and viral DNA. However, analyses of the splice acceptor indicated that the C-terminal domain of NS2 could be largely deleted with only modest effects on the viral replication in tissue culture cells and animals (Wang et al., 1998).

The previously shown nucleolar localization of NS2 in infected cells and NS2 association with nucleolar proteins (Mattola et al., 2022) suggest that NS2 might have a potential role in the infection-induced modification of the nucleoli described above. Here, we assessed how NS2 mutations affected the virus-induced changes of nucleoli in NLFK cells. Similarly, to infected cells at 24 hpi, the average size of segmented nucleoli was decreased and the number of nucleoli was increased in wt infectious clone transfected cells in comparison to nontransfected cells at 24 hours post transfection (hpt) (Fig. 6A). The quantitative analysis confirmed these changes in wt transfected cells and showed a similar decrease in nucleolar size in the NS2 splice acceptor mutant transfected cells. Notably, the size of nucleoli and their number did not change in the NS2 splice donor mutant transfected cells in comparison to nontransfected cells. (Figs 6B and C).

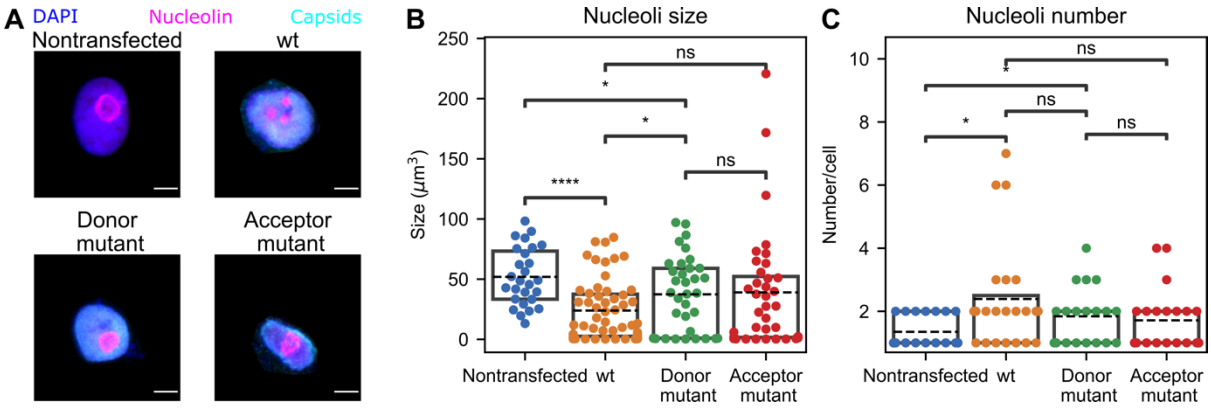

Figure 6. Intact NS2 is required for nucleolar changes
(A) Representative confocal images show nucleoli in nontransfected NLFK cells, and cells transfected with wt, NS2 splice donor and acceptor mutants at 24 hpt. The cells were immunolabeled with antibody against nucleolin (magenta) and intact viral capsids (green), and stained with DAPI (blue). Scale bars, 5 µm. The box-swarm plots represents (B) size and (C) number of nucleoli in nontransfected and transfected cells (n = 26, 47, 31 and 26 nucleoli for nontrasnfected, wt, and splice donor mutant and splice acceptor mutant, respectively). The 3D nucleoli were segmented using deep learning algorithm. The error bars show the standard error

of the mean. Statistical significances were determined using Student´s t-test. The significance values shown are denoted as **** ($p<0.0001$), * ($p<0.05$) or ns (not significant).

Taken together, our results suggest that NS2 may play a role in the infection-induced change of nucleoli. However, the mechanisms by which NS2 is associated with the nucleolar alterations remains to be determined.

Nucleolar distribution of chromatin is affected by NS2 mutation

As shown above, infection induces changes in chromatin distribution in close proximity and within the nucleolus (Fig. 3). To further assess the possible role of NS2 in nucleolar organization of chromatin, we analyzed DNA distribution in cells transfected with wt or NS2 mutants.

Consistent with our infection data, the total nuclear intensity of DNA was increased in wt infectious clone transfected cells and also in wild-type-like splice acceptor mutant transfected NLFK cells at 24 hpt in comparison to nontransfected cells. The most significant increase in nuclear DNA was detected in the splice donor mutant transfected cells (Figs 7A). DNA showed a similar nuclear distribution in all transfected cells including accumulation close to the NE (Fig. 7B). The total amount of nucleolar DNA was most significantly increased in the splice donor transfected cells, and more moderately in wt and the splice acceptor transfected cells compared to nontransfected cells (Fig. 7C). Distribution analyses showed that consistently with 24 hpi (Fig. 3D), DNA was localized both to the inner and outer side of the nucleolar border in wt and the splice acceptor mutant transfected cells. In the splice donor mutant transfected cells, the DNA distribution was slightly shifted towards the nucleolar interior (Fig. 7D).

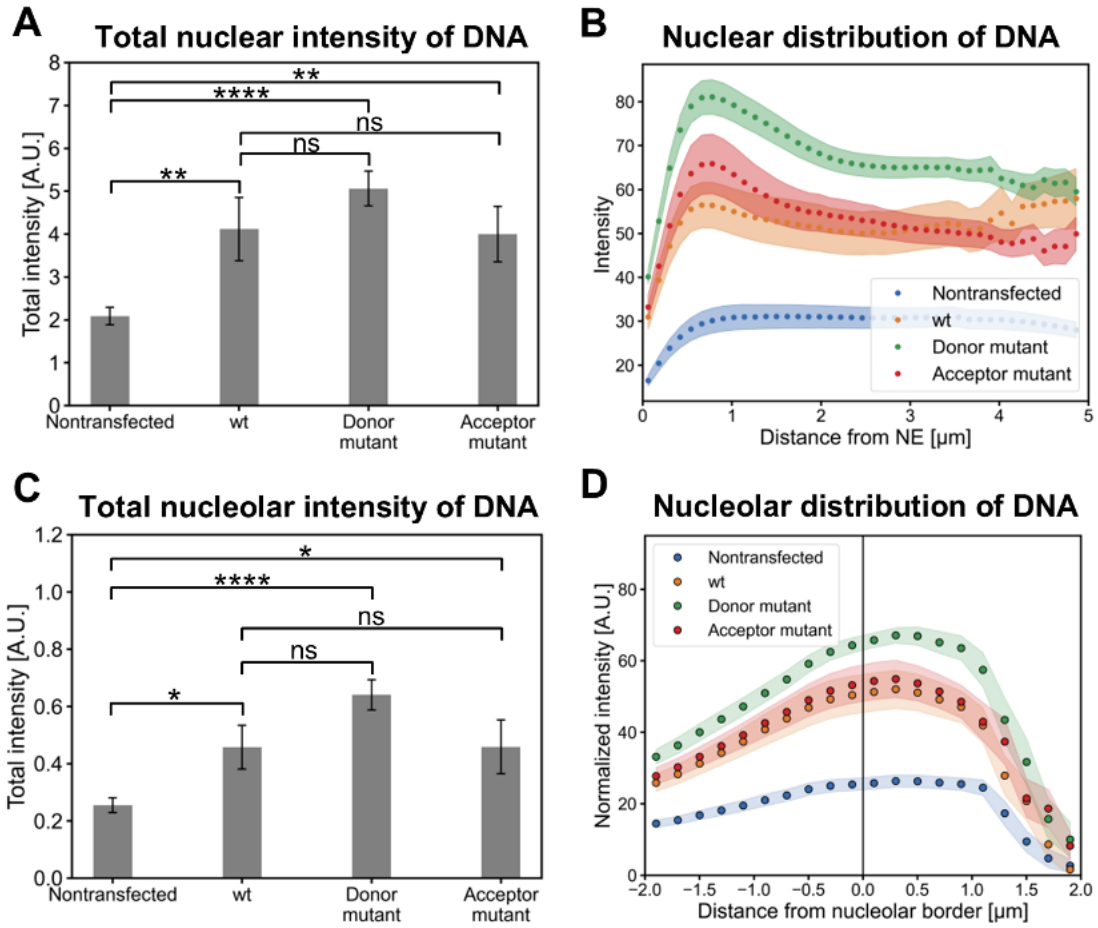

Figure 7. Chromatin organization close to and within the nucleolus is affected by NS2 mutation

(A) Total nuclear intensities of DNA and (B) nuclear distribution of DNA as a function of distance from the NE in nontransfected, wt, and NS2 splice donor and acceptor mutant transfected NLFK cells at 24 hpt (n =20, 19, 18, and 16, respectively). (C) Total nucleolar DNA intensities in segmented nucleoli and (D) distribution of DNA close to the segmented nucleolar edge are shown. The error bars and the shaded areas in the distribution graphs represent the standard error of the mean. The error bars show the standard error of the mean. Statistical significances were determined using Student´s t-test. The significance values shown are denoted as **** ($p<0.0001$), ** ($p<0.01$), * ($p<0.05$) or ns (not significant).

We showed previously that CPV NS2 has a potential role in controlling chromatin remodeling and DDR response (Mattola et al., 2022). Our current observations suggest that NS2 might be needed to organize chromatin packaging on the nucleolar interior and exterior.

Orientation of the nucleoli is altered in NS2 mutant infection

The nucleolus-nucleoplasm interface is known to be maintained by processes dependent on ATP consumption, and impacted by changes in the chromatin packaging and active transcription. In ATP depletion, the curvature and shape of the nucleoli are affected but not the average nucleolar area, eccentricity, or orientation. Orientation is unaffected by a wide range of biochemical perturbations (Caragine et al., 2019). Viral replication of autonomous parvoviruses is a highly ATP-dependent process, overseen by the NS1 protein. The ATP binding pocket of NS1 residing in the middle of the protein, a region not shared with the NS2 (Niskanen et al., 2010).

To further examine the participation of NS2 in the nucleolar organization we determined the orientation of the nucleoli in NS2 mutants transfected cells compared to wt transfected and nontransfected cells. Orientations of the nucleoli were calculated between the major nucleolar and nuclear axes and were given the values from 0 to 90 degrees (Fig 8A). Interestingly, we noticed that the orientation of nucleoli was often perpendicular to the orientation of the nucleus in the replication deficient splice donor mutant transfected NLFK cells (Fig. 8B). This observation was supported by our findings of the orientation of segmented nucleoli that the average nucleolar angle in the splice donor mutant transfected cells was significantly changed in comparison to nontransfected and wt transfected cells (Fig. 8C).

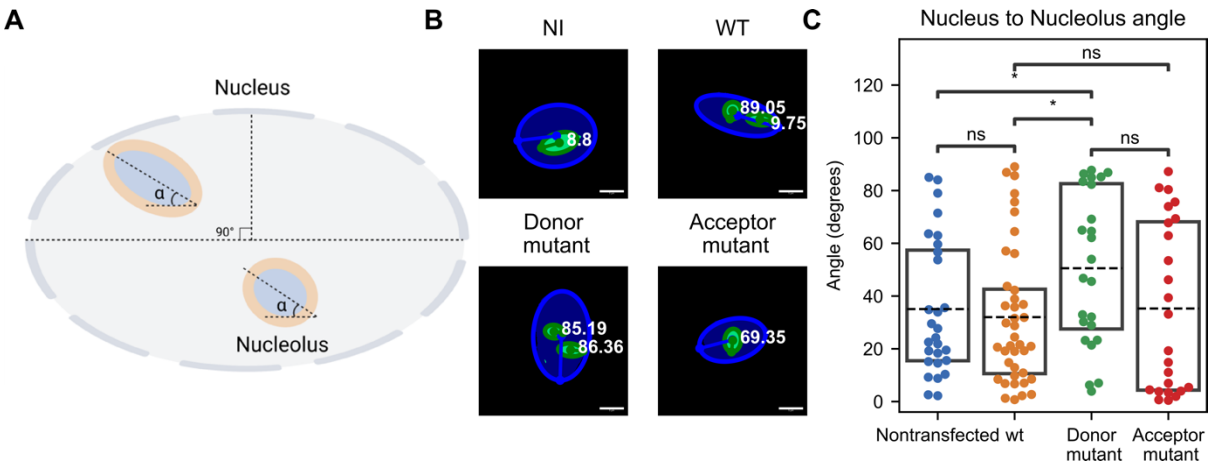

Figure 8. The orientation of the nucleoli is affected in NS2 mutant transfected cells.
(A) Schematic image describing the angle measurement between the nucleus and the nucleolus. (B) Representative segmented images with the represented nucleus direction (blue), nucleolus direction (green) and the angle (white). (C) The angle between the major nuclear and nucleolar axes in nontransfected, wt and NS2 splice donor and acceptor mutants transfected cells (n = 26, 47, 31 and 26 nucleoli respectively). Statistical significances were determined using Student´s t-test. The significance values shown are denoted as * ($p<0.05$) or ns (not significant).

The change in the orientation of nucleoli in the NS2 splice donor transfected cells is an unexpected finding. However, as shown previously the nucleoli are affected by the state of the surrounding nuclear DNA (Caragine et al., 2019). Altogether, consistent with our previous notion that NS2 affects the nuclear chromatin organization (Mattola et al., 2022), our observations support a model where the lack of intact NS2 leads to previously unreported changes in the nucleolar orientation upon CPV.

DISCUSSION

Many DNA viruses, such as HSV-1, adenovirus and AAV, target the nucleolus to interfere with host cell function and to recruit its proteins to facilitate viral infection (Callé et al., 2008; Lee et al., 2003; D. Matthews et al., 2011; Salvetti & Greco, 2014; Wistuba et al., 1997). The nucleolus plays currently mostly undefined roles during the autonomous parvovirus infection. Our findings demonstrated that CPV infection is able to induce temporal changes in the structure and protein content of the nucleolus by a process which is likely mediated by the viral NS2 protein.

The nucleolus is a dynamic structure, and the number and size of nucleoli change during the cell cycle (Caragine et al., 2019; Hernandez-Verdun, 2011). The size of nucleoli is also linked to nucleolar activity under stress (Boulon et al., 2010). Viral infection may also lead to reorganization of nucleolar architecture and change in its size (Greco, 2009; Hiscox, 2007).

Our findings demonstrated that, in addition to changing nucleolar size, CPV infection induced alterations in nucleolar chromatin organization and resulted in nucleolin dispersal from the nucleoli. The nucleolus is widely affected by viral infection and many viral proteins, including CPV NS2, localize into the nucleolus (Mattola et al., 2022), while various nucleolar proteins of infected host cell undergo redistribution (Salvetti & Greco, 2014). Nucleolin is a prominent marker for the nucleoli with as much as 90% of the protein localizing to the nucleoli and 5% to the nucleoplasm (Scherl et al., 2002). The nucleolar-nucleoplasmic shuttling of nucleolin is induced in cellular functions related to stress (Daniely et al., 2002; Daniely & Borowiec, 2000; Jia et al., 2017; Kim et al., 2005). The nuclear distribution of nucleolin is affected by virus infection. For example, nucleolin relocates to the cytoplasm in poliovirus infection (Waggoner & Sarnow, 1998) and adenovirus infection leads to viral protein V-mediated transport of nucleolin to the cytoplasm (Matthews, 2001). In HSV-1 infection, the viral protein UL24 is suggested to be involved in nucleolin dispersal (Lymberopoulos & Pearson, 2007). Nucleolin is also required for proper localization of human cytomegalovirus replication protein UL84 (Bender et al., 2014). Nucleolin promotes binding of HSV-1 and influenza A virus to the cell membrane (Chan et al., 2016; Greco et al., 2012; Kumar et al., 2016). The release of nucleolin from the nucleolus might be a result of a virus-induced heat shock response. This is supported by findings that oncolytic parvovirus H1 infection causes a release of inducible heat-shock protein HSP72 in susceptible human tumor cells (Moehler et al., 2003). The nucleolus is embedded in the chromatin network, which is suggested to support the formation and stabilization of its structure by maintaining the nucleolus-nucleoplasm interface (Caragine et al., 2019; Padeken & Heun, 2014; Qi & Zhang, 2021). It is highly likely that chromosomal organization in the vicinity of nucleolus is regulated by nucleolar DNA sequences of nucleolar-organizing regions (Németh & Längst, 2011). In HSV-1- and CPV -infected cells the formation of viral replication compartments is accompanied by extensive spatial reorganization of

chromatin (Ihalainen et al., 2009; Myllys et al., 2016) which might also affect the structure of nucleoli. Altogether, our data demonstrate that autonomous parvoviruses induce changes in the structure and protein content of nucleolus. We also speculate that the infection-induced changes of chromatin distribution inside and outside of the nucleoli might be due to infection-induced altering of the state of DNA in the surroundings of nucleolus or due to changes in nucleolar interactions with the chromatin. However, further experiments are required to explore these processes.

Our earlier BioID data linked CPV NS2 to several nucleolar proteins with multiple biological functions (Mattola et al., 2022). Spatial mapping of associated proteins has revealed their localization to nucleolar subcompartments such as the whole nucleolus, fibrillar center, and nucleoli rim. One of the most prominent interaction partners of NS2, ki-67 (Mattola et al., 2022), is located in the nuclear rim (Stenström et al., 2020). ki-67 is involved in the structural organization of chromatin and the nucleolus (Booth et al., 2014; Cuylen et al., 2016; MacCallum & Hall, 2000; Sobecki et al., 2016). The rim localization of ki-67 allows its interaction with the heterochromosomal layer around the nucleoli in interphase cells (Stenström et al., 2020). The expression and the functions of ki-67 are cell cycle dependent, with the protein level peaking in mitosis as ki-67 relocates to condensed chromosomes to facilitate the formation of the perichromosomal layer (Booth et al., 2014; Cuylen et al., 2016; Gerdes et al., 1984; Sobecki et al., 2017; Stamatiou & Vagnarelli, 2021). Our studies showed that interaction between ki-67 and chromatin is increased, and ki-67 was relocated to the heterochromosomal layer around the nucleoli and close to the NE at late stages of CPV infection. We speculate that the redistribution and increased association of ki-67 with chromatin might reflect the virus-induced regulation of cell cycle progression towards mitosis. This model is supported by our recent findings that infected cells escape from the virus-induced G2/M arrest and move to the

early stages of mitosis (Mattola et al., unpublished). Our studies of NS2-deficient mutant demonstrated that intact NS2 is required for the regulation of nucleolar size, organization of nucleolar chromatin and orientation of the nucleoli. Altogether these findings suggest that CPV NS2 could have a role in viral interactions affecting nucleolar structure and association with cellular chromatin.

Given that nucleolar proteins have many functions in RNA metabolism and chromatin organization, these proteins are usable for facilitation of the viral replication pathway. Our results suggest that autonomous parvoviruses such as CPV recruit nucleolar proteins in a process which is mediated by the NS2 protein and leads to alteration of the nucleolar structure. Our studies provide novel insights into interactions between autonomous parvoviruses and nucleoli which are potentially essential for the progression of infection.

MATERIALS AND METHODS

Cell lines, plasmids and viruses

Norden laboratory feline kidney (NLFK) cells were propagated as previously described (Mattola et al., 2022). CPV type 2 was generated from an infectious plasmid clone p265 (Parrish, 1991) and virus was produced as described earlier (Suikkanen et al., 2002). NS2 splice donor and acceptor mutants have been previously described (Wang et al., 1998). Transfection of NLFK cells was performed using JetOptimus reagent (Polyplus transfection, Illkirch, France).

Antibodies

The following primary antibodies against viral antigens and cellular proteins were used for confocal microscopy analyses: MAb anti-NS1 was a generous gift from Dr. Caroline Astell (University of British Columbia, Vancouver, Canada) (Yeung et al., 1991). MAb Anti-A3B10 antibody was used to detect intact capsids (Weichert et al., 1998). Anti-nucleolin rAb was used to label the nucleoli (Abcam, ab22858) (Cambrigde, UK). Ki-67 was detected with rAb anti-MKI-67 antibody (Abcam, ab15580). The primary antibodies were followed by goat anti-mouse or anti-rabbit Alexa448, Alexa546, and Alexa647 conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA).

Immunolabelling and confocal microscopy

For microscopy sample preparation NLFK cells were cultured on coverslips at 37°C with 5 % CO2. Cells were either infected with CPV or transfected with CPV infectious clone or NS2 splice mutants and fixed at 8, 12 or 24 hpi and 24 hpt in 4 % paraformaldehyde for 12 minutes. Following fixation, cells were permeabilized with 0.1 % Triton X-100 in phosphate buffered saline supplemented with 0.1 % bovine serum albumin. After primary antibody immunolabeling cells were labeled with mouse or anti-rabbit secondary fluorescent antibodies. DNA was stained with Pro-Long Diamond anti-fade media with DAPI (Thermo Fisher Scientific).

Microscopy of immunolabeled samples was performed with Leica TCS SP8 FALCON (Leica microsystems, Mannheim, Germany) and Nikon A1R (Nikon, Tokyo, Japan) laser scanning confocal microscopes. The parameters for images acquired with Leica TCS SP8 FALCON were as follows: DAPI was excited with a 405-nm diode laser and the fluorescence was collected between 415-495 nm. Alexa488 and Alexa546 were excited with 499 nm and 557 nm wavelengths, respectively, of pulsed white light laser (80 MHz). The emission detection range

was adjusted to 505-561 nm for Alexa488 and 568-648 nm for Alexa546. Microscopy images with Nikon were acquired as follows: DAPI was excited with a 405 nm diode laser and collected with a 450/50 nm band-pass filter. Alexa488 was excited with a 488 nm argon laser, and fluorescence was collected with a 515/30 nm band-pass filter. Alexa 647 was excited with a 642 nm diode laser and detected with a 660 long-pass filter. Resolution for all images was 512 x 512 nm, with a pixel size between 60 nm in the x- and y-directions and 200 nm in the z-direction. For image analysis purposes a fixed optical configuration was used for all images of the same sample set.

Machine learning based segmentation

Human in the loop (HITL) based deep learning method was utilized for the nucleoli segmentations based on the nucleolin labeling (Bobes-Bascarán et al., 2021; Patel et al., 2019). Here we used a U-shape algorithm for the deep neural network (DNN). First, few cell nuclei were manually segmented to train the DNN. The DNN was used to predict the segmentations, based on images from the same dataset but including all planes of the microscopy stack into the segmented area. The mistakes of the algorithm were manually corrected, and the DNN was retrained. By each iteration, more images were added to the analysis, until the output was satisfying (DNN loss function). At this stage, the DNN was used to segment the nucleoli.

Image analyses

The nuclei were segmented from DAPI stain using Otsu's method (Otsu, 1979). Total fluorescence intensities in the nucleus and nucleoli were calculated by summing the pixel intensity values within the segmented regions. Label intensity distributions with respect to the nuclear or nucleolar border were determined by calculating the shortest distance of each pixel to the border and calculating mean intensity as a function of the distance using 2-pixel wide

distance bins for averaging. Due to the flat shape of fixed cells, the confocal section with the largest nuclear area was selected for the analyses on intensity with respect to the nuclear border and 2D Euclidean distance was used as a metric. Since there can be many nucleoli in one cell and the nucleoli can lie in various confocal planes, the whole 3D stack was used in the analyses with respect to the nucleolar border and 3D Euclidean distance was used as a metric. Correlations between DNA staining and antibody labels were determined by using Pearson correlation coefficients. For the nucleolar orientation analyses first the diameters of the nuclei in all the directions were determined, and the longest direction of the nuclei was designated as the zero degrees angle. The angles of the nucleoli were determined by determining the longest sections of the nucleoli and the orientations were compared to the orientation of the zero degrees diameter of the nuclei. The difference between the nucleoli and the zero degrees angle calibration point were represented as the orientation angles of the nucleoli. The number and size analyses of the nucleoli were based on machine learning based segmentations of the nucleoli. For all the analyses, the given statistics were calculated over cells.

BioID and analyzes

BioID of CPV NS2 protein was performed as previously descripted (Mattola et al., 2022).

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