Intracellular Interplay Between Canine Parvovirus with Nuclear Pore Complex and Effect of Infection on RNA Synthesis

Master's Thesis
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Alkusanat

Tämän pro gradu -tutkielman kokeellinen osuus suoritettiin Jyväskylän yliopiston molekyylibiologian osastolla. Haluan kiittää dosentti Maija Vihinen-Rantaa mahdollisuudesta osallistua tutkimukseen tieteellisesti merkittävän, sekä kiinnostavan aiheen parissa. Kiitokset ohjaajaalleni FM Olli Kalliolinnalle, jonka avustamana pääsin nopeasti vauhtiin kokeiden suorittamisessa. Kiitokset kuuluvat myös FT Einari Niskaselle, joka ehti kiireistään huolimatta opastamaan minua sekä kokeiden suoritukseen liittyvissä kysymyksissä että kuvankäsittelyssä Ollin ollessa sidottuna muiden velvollisuuksien johdosta. Lisäksi haluan kiittää FM Sami Willmania asiantuntevasta opastuksesta virtaussytometria-mittauksissa. Lopuksi kiitokset kaikille niille henkilöille, jotka tavalla tai toisella auttoivat ja tukivat minua tämän työn eri vaiheissa.

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Tiivistelmä:

Koiran parvovirus (CPV) on halkaisijaltaan 26 nm vaipaton virus. Ikosahedraalinen kapsidi sisältää yksijuosteisen ~5kb DNA-genomin. CPV sisälleotetaan soluun endosytoosilla, jonka jälkeen se kulkeutuu endosomin sisällä kohti tumaa. CPV vapautuu solulimaan tumaa ympäröivistä lysosomeista. Genomin replikaatio ja uusien virionien kasautuminen tapahtuvat tumassa. Aikaisemmin on oletettu, että parvovirukset todennäköisesti pääsisivät tumaan tumahuokosen (NPC) kautta. Uudet tutkimukset kuitenkin viittaavat siihen että siirtyminen tumaan voisi tapahtua toista reittiä käyttäen.

CPV ja NPC proteiinien (Nup) kolokalisaatio-kokeissa selvitettiin voidaanko tiettyjä vuorovaikutuksia havaita ja missä mahdolliset vuorovaikutukset sijaitsevat. Näissä kokeissa käytettiin uutta in situ proximity ligation assay (PLA) -menetelmää, joka tuottaa fluoresenssi-signaalin kahteen eri kohdeproteiineihin sitoutuneiden vasta-aineiden sijaitessa lähellä toisiaan. Bromouridiini (BRU) on uridiinin analogi, joka voidaan tunnistaa spesifisti vasta-aineilla. Soluja viljeltiin BRU-mediumissa, jolloin BRU saatiin liitettyä kehittyviin RNA-molekyyleihin. Tämän jälkeen oli mahdollista arvioida infektiosta johtuvia muutoksia RNA-synteesissä. Fluoresenssia mitattiin virtaussytometrian ja konfokaalimikroskopian avulla.

Erityistä kolokalisaatiota ei havaittu tumakalvolla Nup358 ja viruskapsidin välillä. Sen sijaan signaali esiintyi levinneenä sytoplasmassa ja näytti siirtyvän kohti tumaa infektion edetessä. Samankaltaista sytoplasmista kolokalisaatiota nähtiin infektoimattomissa soluissa Nup153 ja Nup358 vasta-aineilla suoritetuissa kokeissa. Tämä signaali oli kuitenkin joissain soluissa vahvimmillaan tuman välittömässä läheisyydessä. Infektion myöhäisessä vaiheessa sytoplasmassa oli havaittavissa vahva signaali CPV kapsidi ja NS1 proteiinien välillä. Immunoleimaus ja virtaussytometri mittaukset osoittavat RNA-synteesin vaimentumista infektion seurauksena.

Avainsanat: Koiran Parvovirus (CPV), tumahuokonen (NPC), nukleoporiini (Nup), proximity ligation assay (PLA).

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Abstract of Master's thesis

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Abstract:

Canine parvovirus (CPV) is a non-enveloped virus with a 26 nm diameter icosahedral capsid. The capsid holds a ~5kb single-stranded DNA genome. CPV is internalized by endocytosis followed by intracellular trafficking inside an endosome towards the nucleus. CPV is released from perinuclear lysosomes followed by replication of the genome and assembly of progeny virions inside the nucleus. It has been previously assumed that parvoviruses are likely to enter the nucleus through the nuclear pore complex (NPC). Recent studies however suggest that entry to the nucleus might take place by an NPC-independent mechanism.

Colocalization of CPV and NPC proteins (Nups) were examined to find out if specific interactions could be detected and if so, where these interactions would occur. Experiments were carried out by a novel in situ proximity ligation assay (PLA) method that generates fluorescence only when two antibodies bound to different target proteins are situated at close proximity. Bromouridine (BRU) is an uridine analog that can be specifically bound by antibodies. Cells were cultured in the presence of BRU that became incorporated into nascent RNA molecules. It was then possible to estimate changes in RNA synthesis as a result of infection. The fluorescence was measured by flow cytometry and visualized by confocal microscopy.

Colocalization at the nuclear envelope was not detected between Nup358 and CPV capsid. Instead, the signal was spread across the cytoplasm and appeared to be shifting towards the nucleus in time-dependent manner. Cytoplasmic colocalization, sometimes located close to perinuclear region, was detected between Nup153 and Nup358 in noninfected cells. In a late phase of infection a very strong colocalization signal was seen between CPV capsid and NS1 proteins. Immunolabeling and flow cytometry assays suggest a decrease in the rate of RNA synthesis as a result of infection.

Keywords: Canine Parvovirus (CPV), nuclear pore complex (NPC), nucleoporin (Nup), proximity ligation assay (PLA).

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Abbreviations

BrdU	Bromodeoxyuridine antibody
BRU	5-Bromouridine 5'-triphosphate sodium salt
BSA	Bovine serum albumin
CPV	Canine parvovirus
EM	Electron microscope
FPV	Feline parvovirus
INM	Inner nuclear membrane
MVM	Minute virus of mice
NE	Nuclear envelope
NL	Nuclear lamina
NLFK	Nordern Laboratory feline kidney cells
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NS	Non-structural protein
Nup	Nucleoporin
ONM	Outer nuclear membrane
PCNA	Proliferating cell nuclear antigen
p.i.	Post-infection
PLA	Proximity ligation assay (in situ PLA)
TfR	Transferrin receptor

1 INTRODUCTION

1.1 Taxonomy of parvoviruses

The *Parvoviridae*-family is divided into two subfamilies classified by host species: *Densovirinae* and *Parvovirinae* infecting arthropods and vertebrates, respectively. The *Parvovirinae* subfamily was previously divided into three separate genus, but has been recently reclassified and now contains eight genus. Meanwhile, *Parvovirus* genus was renamed as *Protoparvovirus*. *Carinvore protoparvovirus 1* species belongs to the *Protoparvovirus* genus and contains four variants: canine parvovirus (CPV), feline parvovirus (FPV), mink enteritis virus and racoon parvovirus. Viruses in genus *Dependoparvovirus* such as adeno-associated viruses are able to integrate into the host cell genome and require a helper virus for replication. With this exception, rest of the species in the *Parvovirinae* subfamily are all autonomous (Cotmore et al., 2014).

1.2 Canine parvovirus

1.2.1 Emergence, evolution, host range and symptoms

CPV was recognized in 1978 as a novel virus infecting canine animals and was designated as CPV type-2 to distinguish it from distantly related minute virus of canines. CPV is thought to be derived from either FPV or from one of several closely related carnivore viruses (for review see Hueffer and Parrish, 2003; Hoelzer and Parrish, 2010). Analyzing sera collected from domestic dogs and wild coyotes revealed that a new antigenic variant replaced CPV2 between 1979 and 1983. The new strain was designated as CPV type-2a to distinguish it from CPV2. Dogs infected with CPV2 are immune to infection by CPV-2a. (Parrish et al., 1988). New variants, CPV-2b and CPV-2c appeared after subsequent mutations. These strains currently co-exist around the world, while specific regions vary in prevalence of different strains (for review see Hoelzer and Parrish, 2010). CPV2 is unable to efficiently replicate in cats unlike the new strains derived from CPV2 that can infect cats as well as canine animals (Truyen et al., 1996). There are only few amino acid replacements in the capsid sequence between FPV and CPV (Reed et al., 1988). Specific structure of the CPV capsid interacts with canine and feline transferrin receptor (TfR) and variations at this capsid region determines the host range of different strains (Parker et al.,

2001; Hueffer et al., 2003). Structures at separate regions on threefold spike around residues 93, 300 and 323 of VP2 control binding to canine TfR and the structure around residue 300 is also involved in CPV binding to feline TfR. More recent isolates also have substitutions at other residues near the threefold spike indicating continuing evolution involving the surface structure (for review see Hueffer and Parrish, 2003).

CPV can be spread by direct or indirect contact involving fomites. Symptoms differ in relation to age of the host. In animals older than four weeks mostly actively dividing cells are affected. These include intestinal and hematopoietic cells expressing high levels of TfR such as: bone marrow, lymphoid tissue, thymus and intestinal progenitor cells. Symptoms can manifest as hemorrhagic enteritis with associated leukopenia similar to infection caused by FPV, but severity of the disease vary from severe to subclinical. In endemic populations mostly young pups with expired maternal antibodies are affected. In addition, infection of the myocardium in fetuses and neonatal puppies is possible after expiration of maternal immunity (for review see Hueffer and Parrish, 2003; Hoelzer and Parrish, 2010).

1.2.2 Genome

The ~5.3-kb negative-sense, single-stranded DNA genome of CPV contains two large open reading frames in the positive sense strand. Left hand open reading Frame A encodes nonstructural NS1 and NS2 proteins. Right hand open reading frame B contains the sequence for capsid proteins VP1 and VP2. Promoters for open reading frames A and B are located at map positions 4 (promoter P04) and 38 (promoter P38), respectively. Each NS and VP protein is produced from the two primary transcripts with alternative splice sites and the mRNA have coterminal poly(A) addition sites. Terminal ends of the DNA contain palindromic sequences base paired into hairpin structures. The genome shares a high overall homology of ~98% with FPV and besides the sequence encoding VP2, the only other apparent difference between CPV and FPV is reiteration of untranslated DNA. Regions of the genome coding NS1 and NS2 proteins share a homology of ~73% with Minute virus of mice (MVM) strains and hamster H1 virus, but is significantly smaller with only ~23% homology when compared with human parvovirus B19. MVM and H1 share ~50% homology with CPV in VP1 and VP2 coding regions. Translated amino acid sequence between CPV and rodent parvoviruses has less variation (Reed et al., 1988).

1.2.3 Capsid structure and assembly

CPV capsid contains 60 structural (VP) proteins arranged into T=1 icosahedral symmetrical structure, with a size of ~26 nm. Most of the capsid is composed of VP2 that forms an eight-stranded antiparallel beta barrel structure. Long 22 ångstrom protrusions (spikes) important for the antigenic properties are found at threefold axes of the capsid. Depressions are found at twofold axes and surrounding the fivefold axes (Tsao et al., 1991). Two areas at the threefold spike region are important for antigenicity of the capsid. One is at the tip of the threefold spike and the other one on the shoulder region, around VP2 residue 300 (Strassheim et al., 1994). Large portion of the outer surface of the capsid is hydrophobic and this could further in the binding to cellular membranes. A curious feature about the internal surface of the capsid is an unexpected scarcity of positively charged residues that would be expected to interact and assist in the packaging of the viral genome. Instead the region interacting with the DNA is mostly polar and hydrophobic (Xie and Chapman, 1996). Equal number of empty and full capsids are produced during infection. Most profound conformational variation between full and empty capsids is found at the area where DNA is bound to the full capsid (Wu and Rossmann, 1993). N-terminal region of VP2 protrudes outside from full capsids, where it can be cleaved to VP3 and incorporated into the mature virion (Tsao et al., 1991; Weichert et al., 1998).

Details about parvovirus capsid assembly are not yet clear. The first step might be a formation of either dimer, trimer or pentamer intermediates (Xie and Chapman, 1996). Such complexes of VP2 can be released by treating purified capsids with high or low pH or with urea. Trimeric form of VP2 can also be isolated from cell lysates (Yuan and Parrish, 2001). It has been suggested that the capsid proteins VP1 and VP2 form oligomeric intermediates in the cytoplasm to be co-imported into the nucleus for final assembly as full capsids. Mutations in VP2 of MVM affecting correct folding of the protein prevents import of capsid intermediates into the nucleus (Lombardo et al., 2000). However, in the case of CPV, mutating VP2 to prevent the formation of oligomers with VP1 does not prevent the transport of VP2 into the nucleus as a monomer. Without capsid assembly VP2 is not efficiently retained inside the nucleus, but also found throughout the cytoplasm (Yuan and Parrish, 2001).

1.2.4 Replication

Autonomous parvoviruses require S-phase cells for successful production of progeny virions. The single-stranded-DNA genome must enter the nucleus where it is converted to double-stranded-DNA before replication and transcription of viral proteins can proceed. Terminal hairpin structures protect the genome from degradation and also function as primers. NS proteins of several autonomous parvoviruses are phosphorylated and play a vital role in both gene expression and DNA replication. Replication is carried out by modified rolling-hairpin model and proceeds by single-strand displacement (for review see Berns, 1990). Multifunctional NS1 with an ATPase activity is involved in replication and gene expression. It controls transcription from both NS and VP genes from promoters P04 and P38, respectively (Doerig et al., 1990; Rhode et al., 1985; Rhode and Richard, 1987). Mutations of CPV NS1 at ATP-binding residues prevents its binding to DNA, efficient transcription from the P38 promoter and genome replication (Niskanen et al., 2010).

NS1 binds to viral DNA at two origins of replication together with endogenous proteins: glucocorticoid modulatory binding proteins at the left hand side and high-mobility group proteins at the right hand side, and the binding leads to nicking of the DNA (Christensen, et al., 1997; Cotmore and Tattersall, 1998). NS1 is then covalently attached to the 5' end of replicative-form of viral DNA and subsequently stays attached to progeny single-strand DNA (Cotmore and Tattersall, 1988). NS1 also functions as helicase during replication resolving terminal hairpin structures of the DNA (Willwand, et al., 1997). Several endogenous factors involved in replication and signaling have been identified inside parvovirus-induced replication bodies (for review see Schmid et al., 2014). Polymerase δ seems to be responsible for the replication of the genome, although polymerase α also colocalizes within sites of replication together with NS1 and replication protein A (Bashir et al., 2001). Similar colocalization can be seen for NS1 and proliferating cell nuclear antigen (PCNA) in CPV infected, but not in noninfected cells transfected with a fluorescent NS1 construct (Ihalainen et al., 2007). Identical binding times for NS1 and PCNA to viral DNA suggests the replication to lasts for 83 seconds (Ihalainen et al., 2009). Possible functions of CPV NS2 are not well understood, but it does not seem to be necessary for efficient infection or replication of the genome (Wang et al., 1998).

1.3 Infection of cells by animal viruses

Typical animal virus has either a helical or an icosahedral capsid surrounding a genome consisting of either RNA or DNA. Depending on the type of virus, variety of enzymes and other proteins essential for viral life cycle can be contained within the capsid. For example, viruses replicating in the cytoplasm need to carry polymerases required for genome replication, while retroviruses depend on reverse transcriptase and integrase for integration of the genome into the host cell DNA. Some viruses contain an additional layer of lipid molecules surrounding the capsid. This envelope contains glycoprotein spikes necessary for binding to cell surface receptors. Non-enveloped viruses have projections or indentations on the capsid surface for similar function. Different types of cell surface molecules such as proteins, lipids, carbohydrates can be used by viruses for binding and attaching to the cell surface, while accessory molecules are sometimes required for internalization (for review see Smith and Helenius, 2004; Mercer et al., 2010).

Entry of both enveloped and non-enveloped viruses into cells may rely on lipid raft microdomains on the plasma membrane (for review see Chazal and Gerlier, 2003). These rafts concentrate and anchor proteins required for several different cell functions such as endocytosis and signal transduction pathways. Initial attachment to cell surface molecules can modify the virus or the cell to allow the virus to bind additional receptors or to fuse with plasma membrane. Also, binding can lead to clustering of cell surface receptors, endocytosis of the virus and activation of signaling pathways required by the virus later during infection. Most enveloped viruses such as human immunodeficiency virus 1 and herpes simplex virus can enter cells by membrane fusion. In this process viral envelope fuses with the cell membrane and a naked capsid is pushed into the cytosol (for review see Smith and Helenius, 2004; Mercer et al., 2010). Other enveloped viruses and non-enveloped viruses are internalized by endocytotic mechanisms such as: phagocytosis, macropinocytosis, clathrin-mediated endocytosis into coated vesicles, caveolar pathway or less well defined lipid raft-dependent pathways. Viruses entering cells by endocytosis are contained inside bilayered lipid structures called endosomes. Together with the cytoskeleton, endosomes can be utilized by the virus as native intracellular transports to the relevant site of replication (for review see Radtke et al., 2006). Some viruses can also attach directly to motor proteins.

Viruses can also utilize the cytoskeleton for transporting viral components into different locations inside the cell during gene expression, replication, assembly and egress of the virion. Endosomes vary on pH scale in relation to the function and location inside the cell. These pH changes are detected and an important cue for several viruses to initiate subsequent phases of infection and to escape (penetrate) from endosomes by different methods specific for each virus (for review see Lozach et al., 2011). Enveloped viruses can penetrate endosomes by membrane fusion, whereas non-enveloped viruses either form a pore and squeeze through or cause a complete lysis of the vesicle. Whether cytoplasmic or nuclear, viruses induce formation of replication bodies, consisting of viral and cellular proteins (for review see Schmid et al., 2014). These compartments maximize efficient replication of the genome, assembly of the virus and also help to conceal viral components from detection by cellular defense mechanisms. Infection by cytoplasmic viruses leads to extensive relocalization and reorganization of organelles and cytoplasmic membranes, while nuclear viruses cause reorganization of chromatin and nuclear domains. Enveloped viruses are usually released from the cell by budding or secretion, while lysis of the cell is the most significant type of release mechanism for non-enveloped viruses.

2 THE NUCLEAR BARRIER

2.1 Structure and function

2.1.1 Envelope

The nuclear envelope (NE) defines nuclear space and restricts free passage of large molecules between the cytoplasm and the nucleoplasm. The NE consists of double lipid bilayer separated by 40-50 nm wide perinuclear space. The inner nuclear membrane (INM) contains embedded integral membrane proteins, whereas the outer nuclear membrane (ONM) holds ribosomes and is continuous with endoplasmic reticulum (for review see Stewart et al., 2007). Mammalian nuclear lamina (NL) is a 15-20 nm protein meshwork located at the nuclear side of INM. It consists of type V intermediate filaments called lamins. divided into A-type, lamins A and C, and B-type, lamins B1 and B2 (for review see Burke and Stewart, 2013). Mutations in lamin genes causes a diverse group of diseases called laminopathies (for review see Stewart et al., 2007). The lamin network is phosphorylated and disassembled during normal cellular processes such as mitosis and apoptosis, but rupturing of the NE can also be caused by laminopathy, cancer and viral infection (for review see Hatch and Hetzer, 2014). Besides structural function, the NL provides anchoring sites for signaling proteins and transcription factors. The NL is attached to cytoskeletal proteins by linker of the nucleoskeleton and cytoskeleton complexes. These connections are important for determining cytoplasmic organization and cytoskeletal interactions. The NL also modulates transcription by attaching to chromatin domains. (for review see Burke and Stewart, 2013).

2.1.2 Pore complex

Nuclear pore complexes (NPC) are gated channels connecting cytoplasmic and intranuclear space by penetrating the NE at the site where the INM and the ONM are fused and similarly to the NL, disassembled and reassembled during mitosis. The NPC is an eightfold-symmetrical structure with an inner and an outer ring connected by a central channel. Both rings have eight filaments attached. Cytoplasmic filaments mainly composed of Nup358 (RanBP2) have loose ends, whereas at the nuclear side join to form a structure

called a nuclear basket. The NPC contains ~30 different proteins called nucleoporins (Nups) in copies or multiples of eight, totaling to ~500-1000 Nups per pore with a mass of ~60-125 MDa in higher eukaryotes. Few Nups are required to anchor NPCs to the NE, but most are soluble. Scaffolding Nups such as Nup 107-160 complex are very stable in interphase cells, but peripheral Nups such as Nup50 and Nup153 contained within the nuclear basket have been shown to be dynamic. It has been suggested that dynamic Nups might assist in transporting of cargo to the NPC, while changes at conformation of other Nups could regulate the NPC permeability by controlling the central channel diameter (for review see D'Angelo and Hetzer, 2008; Tran et al., 2014).

Nup153 can dissociate from the NPC (Daigle et al., 2001; Griffis, et al., 2004), but has also been shown to be an essential structural element of the pore. The nucleus can be reconstructed from *Xenopus* oocyte extracts in vitro with Nup153 depleted NPCs. Several Nups from the nuclear basket appear to be missing from these nuclei. In addition, Nup153 depleted NPCs are not anchored to nuclear lamina and instead float around the NE, with a tendency to cluster (Walther et al., 2001). On the other hand, Nup358 has been found to be distributed throughout cytoplasm where it seems to control microtubule stability in interphase cells (Joseph and Dasso, 2008). Nucleoporins also have other transport independent functions such as: role in NE breakdown, modulating activity of sumoylating enzymes and at least in lower eukaryotes regulating chromatin activity by reorganization (for review see D'Angelo and Hetzer, 2008).

2.2 Macromolecular transport

The NPC can mediate ~1000 transport events with a mass of ~100 MDa every second. Small molecules diffuse freely between the cytoplasm and the nucleus, but larger molecules >40kDa require to be actively carried through the NPC by transport proteins called importins and exportins in mammalian cells. Importin-β binds cytoplasmic proteins containing either a classical or non-classical nuclear localization signals (NLS). In the case of classical NLS that contains a stretch of basic amino acids, importin-α is required as an adaptor. Exportins recognize a nuclear export signal containing 4-5 hydrophobic residues. Direction of transport is determined by RanGTP gradient across the nuclear envelope. The central channel of the NPC is lined with Nups with phenylalanine-glycine repeat

containing domains, functioning as a sieve-like barrier to restrict the passage of molecules through weak hydrophobic interactions. Transport receptors overcome this restriction by interacting with the phenylalanine-glycine domains to reduce energy loss of large proteins moving through the entropic barrier (for review see Fried and Kutay, 2003, D'Angelo and Hetzer, 2008; Tran et al., 2014)

Nup358 has been shown to be involved in NPC-mediated transport of cargo. Preventing hydrolysis of Ran-GTP by Ran-GTPase activating protein associated with Nup358 leads to inhibition of nuclear import (Mahajan et al., 1997). Interestingly, the cytoplasmic filaments of the NPC and proteins therein including Nup358 do not seem to be always essential for NLS-mediated import. In vitro experiments with *Xenopus* oocyte extracts show no reduction of import in Nup358 depleted nuclei (Walther et al., 2002). Nup153 is required for NLS / importin-β mediated import and export of several types of RNA (Shah et al., 1998; Walther et al., 2001; Ullmann et al., 1999). Nup153 might assist in the transport of nascent mRNA to the NPC. Association and release of Nup153 from the NPC depends on RNA transcription and the release can be prevented by Pol I and Pol II inhibitors. Fluorescence recovery after photobleaching experiments show that large fraction of Nup153s are released from NPCs during transcription, but about a quarter seems to be immobile, bound into a stable structure at the basket (Griffis, et al., 2004).

2.3 Translocation of viruses

2.3.1 Entry to the nucleus

Most RNA viruses prefer to replicate in the cytoplasm, whereas the nucleus is almost always an obligatory target for viruses with a DNA genome. The NE however presents another barrier that needs to be overcome for a successful infection. Retroviruses such as pre-integration complex of murine leukemia virus overcomes this obstacle during mitosis when the NE is disassembled (for review see Cohen et al., 2010). Entry inside the nucleus of an interphase cell is usually dependent on NPCs. Some viruses are small enough to pass through the NPC intact, while larger viruses either have to change conformation or uncoat to release the genome through the NPC. Many viruses or components released from the capsid interact with different Nups and transport proteins during entry. Viruses can take

advantage of importins and target NPCs with an NLS. For example lentivirus human immunodeficiency virus 1 interacts with several import proteins and nucleoporins including Nup153 and Nup358 (for review see Cohen et al., 2010). Human immunodeficiency virus 1 and influenza A virus are disassembled in the cytoplasm and released components contain an NLS required for the NPC-mediated import. Herpes simplex virus 1 is another example of uncoating at the cytoplasmic side, where the capsid interacts with Nup358 (Copeland et al., 2009). Mature hepatitis B virus is small enough to be imported through the NPC intact, after which uncoating takes place inside the nuclear basket, where the capsid is bound by Nup153 (Schmitz et al., 2010). Recent research on parvoviruses points to a novel mechanism for NPC independent nuclear entry. Even though small enough to theoretically fit inside the NPC, parvoviruses have been shown to transiently disrupt the NE and at least MVM seems to cross these gaps into the nucleoplasm (Cohen and Panté 2005; Porwal et al., 2013).

2.3.2 Escape from the nucleus

Release of the components or fully assembled viruses from the nucleus also depends on different strategies that may or may not involve NPCs. These mechanisms are not well understood, although some insights have been gained in recent years. Eight genome segments of influenza virus are exported through the NPC in the presence of M1 protein, which also directs the assembly and budding of the virus. Human immunodeficiency virus 1 RNA genome interacts with Rev protein that drives export also through the NPC. The genome is then encapsidated in the cytoplasm (for review see Whittaker et al., 1998). Export of both Rev and viral RNA can be prevented by Nup153 antibodies (Ullman et al., 1999). Besides NPC-mediated export, disruption of the NL has been demonstrated during escape of herpesvirus, Epstein-Barr virus and some cytomegaloviruses. Herpesvirus capsids assemble in the nucleus, bud through the INM and fuse with the ONM releasing naked nucleocapsids to the cytoplasm. The virus then interacts with Golgi membranes or endoplasmic reticulum acquiring a new envelope (for review see Cibulka et al., 2012). It has been previously assumed that non-enveloped DNA viruses maturing inside the nucleus are released at a late stage of infection when the NE breaks down during apoptosis of the cell. This view has been challenged by increasing evidence of parvovirus escape prior to disassembly of the NE (Maroto et al., 2004; Bär et al., 2008).

3 CANINE PARVOVIRUS INFECTION

3.1 Binding and internalization

Many details about the life cycle of parvoviruses still remain as mysteries. However, in recent years there have been several advances elucidating the early phases of infection including intracellular trafficking and interactions of CPV with cellular components (for review see Harbison et al., 2008). CPV binds to TfR at the cell surface and the receptor is then internalized by endocytosis along with the virion. CPV does not bind or enter TfR negative cells, but can successfully infect TfR expressing cells. Infection can be blocked when polyclonal antibodies are added at the same time with virus inoculation, but not after 2 h p.i. Microinjection of an antibody specific against the cytoplasmic tail of TfR 2 h before virus inoculation could almost completely block the infection. Mircoinjections are effective up to 4 h p.i., but at 7 h p.i. have significantly reduced effect. Endocytosis is not affected, but virus-containing vesicles are larger and more widely dispersed throughout the cytoplasm when compared noninjected cells (Parker et al., 2001). Electron microscope (EM) images demonstrate CPV virions initially concentrating to clathrin coated pits, rapidly internalized inside coated vesicles and finally only noncoated virion-containing vesicles can be identified. Overexpression of mutated dynamin blocks clathrin-mediated endocytosis of TfR and retains receptors at the vicinity of plasma membrane. Although virion movement towards the nucleus is significantly reduced, it is not completely prevented (Parker and Parrish, 2000).

3.2 From plasma membrane to the nucleus

CPV is carried from early to perinuclear endosomes along the microtubule network in dynein-dependent manner. Depolymerizing microtubules with nocodazole or lowering temperature to +18 °C disrupts normal transport of virion containing endosomes toward the perinuclear region and prevents release from endosomes (Vihinen-Ranta et al., 1998; Vihinen-Ranta et al., 2000; Suikkanen et al., 2002; Suikkanen et al., 2003a). In addition, recent experiments have identified vimentin intermediate filament network to be also essential for MVM infection. Virion containing vesicles fail to accumulate at perinuclear region in vimentin negative cells or when the vimentin network has been artificially

disrupted (Fay and Panté, 2013). Fluorescence microscopy studies carried out in the presence antibodies against CPV capsid illustrate the movement of virions inside the infected cell. At 5 min p.i. capsids can first be seen at the cell surface and inside early endosomes at the cellular periphery. From 30 min p.i. and onward virion containing vesicles are transported towards the nucleus, with an increasing proximity to nuclear space from 1 to 3 h p.i. (Suikkanen et al., 2002).

Virions are released from endosomes slowly and colocalize with TfR at perinuclear-late endosomes and lysosomes for up to 10 h p.i., after which capsid antigens are also found inside the nucleus (Weichert et al., 1998; Parker and Parrish, 2000; Suikkanen et al., 2002; Suikkanen et al., 2003a). Capsid antibodies microinjected into the cytoplasm or into the nucleus can successfully block infection and only after 8 h significant increase in the number of infected cells can be observed (Vihinen-Ranta et al., 2000). Virions can also be seen to directly associate with recycling endosomes and at the proximity of mitochondrial membranes (Suikkanen et al., 2002; Nykky et al., 2014). The capsid has an affinity to interact with different types of lipids found on the surface of several endocytic vesicles. These includes lipids within clathrin coated vesicles, and early-, late- and recycling endosomes (Suikkanen et al., 2003b).

Low pH is required in the endosomal comparartment for successful escape of virions as treatment with lysomotropic bases leads to accumulation of virions inside large endosomal vesicles (Basak and Turner 1992). Phospholipase A2 activity located in several parvoviruses at VP1 N-terminal region is required, but not sufficient for escape from vesicles and successful transport of viral DNA into the nucleus (Zadori et al., 2001; Suikkanen et al., 2003b). Release into the cytoplasm is not likely dependent of endosomal membrane lysis as α-sarcin and large dextrans and are retained inside endosomes (Parker and Parrish, 2000; Suikkanen et al., 2003b). Smaller dextrans however manage to leak into the cytoplasm suggesting modification of endosomal membrane permeability (Suikkanen et al., 2003b). Parvovirus genomes can be released by heat treatment without complete disassembly of the capsid and this appears to be a common feature between distantly related parvoviruses. Mildly acidic conditions mimicking that of an endosomal compartment exposes the genome of parvovirus B19, but the genome remains associated

with the capsid. However, same treatments have no effect on the release of MVM genome (Ros et al., 2006). It seems that capsids remain at least mostly intact during transport and release from endosomes, and uncoating to occur at a later stage. Proteasomes have been shown to be required for endosomal trafficking of some parvoviruses such as MVM and CPV. Accumulation of virions to the perinuclear region can be prevented for up to 6 h p.i. by a reversible proteasome inhibitor. Proteasomes are not involved in proteolytic processing of VP2 in MVM or externalization of VP1 N-terminus. No ubiquitination or degradation of capsids was observed so the function proteasomes during infection remains to be further explored (Ros and Kempf 2004).

3.3 Entry to the nucleus

Clustered basic residues in CPV capsid protein VP1 constitute a classical NLS. A sequence of ten amino acids at the N-terminal residues 4-13 is recognized by a nuclear import protein and is required during infection (Vihinen-Ranta et al., 1997). The N-terminal region is buried inside the capsid and can be exposed by either urea or heat treatments and is required for nuclear import of the capsids (Vihinen-Ranta et al. 2002). Microscopic studies conducted with antibodies only recognizing full capsids suggests that intact capsids may enter the nucleus and uncoating to follow entry. Minor localization of capsids inside the nucleus can be detected already 3 h after microinjections, while after 6 h localization increases significantly (Vihinen-Ranta et al., 2000). In terms of size, parvoviruses are small enough to fit inside the NPC, but no direct evidence of NPC-mediated entry has been found. However, a novel method has been discovered that is likely to be utilized by at least some parvoviruses for gaining access to the host species nucleus.

EM sections of *Xenopus* oocytes, microinjected with MVM, reveal ~100-200 nm gaps 1 h p.i. at the ONM near NPCs (Cohen and Panté 2005). These gaps are large enough to allow leakage of chromatin into the cytoplasm (Porwal et al., 2013). Virions can be found near these disruptions and occasionally between the ONM and INM. The gaps increase in both size and frequency in respect to time elapsed from the beginning of infection. Disrupting effect might be specific to the NE as the membranes of mitochondria are not affected (Cohen and Panté 2005). Similar gaps in the NE and in addition, disruption of the NL network can be observed by infecting mouse fibroblasts with MVM, providing evidence

that this mechanism is also active inside cells of the host organism. The nuclei of infected cells appear shriveled and irregularly shaped. Large gaps appear in lamin A/C immunostaining and MVM virions colocalize within the gaps. Besides breaks at the ONM, invaginations of the NE with associated virions was also detected by EM (Cohen et al., 2006). When NPCs are blocked by wheat germ agglutinin and cells injected with bovin serum albumin (BSA) conjugated gold particles, the particles localized inside the nucleus, demonstrating that another route independent of NPCs is accessible (Cohen and Panté 2005). However, direct attachment to the NPC by parvoviruses might be required for the NE disruption. At least adeno-assosicated virus 2 and parvovirus H1 interact with three Nups in vitro. These include peripheral Nup358 and Nup153, and Nup68, an internal component of the hydrophobic mesh. Interaction of the capsid with Nups exposes an undefined domain of VP1 required for permeation of the NE. Following the breach of the ONM, signaling pathways are initiated, resulting in lamin phosphorylation and depolymerization (Porwal et al., 2013).

During this process caspase-3 is relocated to the NE where it facilitates transient disruptions. Caspase-3 and caspase-6 are proteases involved in apoptosis and simultaneous breakdown of the NE. Nuclear entry and gene expression of MVM is reduced by caspase-3 inhibition, but not by caspase-6 inhibition. Activation of caspase-3 by MVM did not lead to apoptosis and dsDNA breaks in chromatin were not detected. The structure of the NE was also found intact later after virus entry. Lamin A/C cleaved by caspase-3 is located at the INM, suggesting a yet to be defined method utilized by MVM to facilitate access of caspase-3 to the INM. However, phospholipase A2 activity is not required by MVM for the NE disruption, since inhibition of the phospholipase activity or mutation of VP1 active site was sufficient at preventing infection (Cohen et al., 2011). The NE breakdown seems to be a common feature during infection by different parvoviruses including adeno-associated virus 2 and CPV. Although kinetics of the NE breakdown appear similar, CPV causes slightly delayed response compared to other parvoviruses (Porwal et al., 2013). The NLS of VP1 might be required to direct incoming virions to the NPC and facilitate entry through the NE in the vicinity of NPCs. However, the entry mechanism of CPV into the nucleus still remains to be confirmed and possible import through the NPC has not yet been excluded.

3.4 Inside the nucleus

Fluorescence inside the nucleus can be detected at 8 h p.i. with antibodies targeted against CPV NS1, while the NS1 mRNA can be detected as early as 4 h p.i. and to increase in time-dependent manner (Suikkanen et al., 2002; Ihalainen et al., 2012). NS1 does not diffuse freely inside the nucleus, but is contained to specific virus-induced replication compartments (Ihalainen et al., 2007; Ihalainen et al., 2009; Schmid et al., 2014 review). Live cell imaging reveals formation and enlargement of these areas, followed by uniform nuclear distribution of NS1 excluding the nucleoli. However, photobleaching techniques indicate shuttling of NS1 between the nucleus and the cytoplasm (Ihalainen et al., 2007). Osmotic pressure caused by enlargement of replication bodies is followed by chromatin marginalization into highly condensed state near the nuclear periphery. While protein mobility is increased in infected cells, capsids tend to cluster at the proximity of the NE, even though in terms of size would be small enough to fit inside replication bodies. This can be interpreted in two possible ways: either capsid assembly takes place near the NE or possibly release of virions is slower than the rate of assembly (Ihalainen et al., 2009).

3.5 Apoptosis and modification of the cytoskeleton

Progeny virions continue to accumulate inside the cell during the infection and are released by apoptosis. At the early stage of infection mitochondrial damage and depolarization can be observed (Nykky et al., 2010; (Nykky et al., 2014). Furthermore, damaging of mitochondrial membranes leads to the release of reactive oxygen species. At the same time anti-apoptotic ERK1/2 signaling is activated (Nykky et al., 2014). Number of cells arrested into S phase of the cell cycle increases progressively during infection. Only a minority of CPV infected Nordern Laboratory feline kidney cells (NLFK) cells show signs of apoptosis at 24 h p.i., but at 48 h p.i. most of the cells show morphological changes such as cell shrinkage and detachment. Apoptosis follows activation of caspases 9, 8 and 3/7 and later during infection cells become necrotic with associated plasma membrane damage (Nykky et al., 2010). No changes to the expression of p53 or anti-apoptotic Bcl-2 occurs indicating that apoptosis is not affected by these signaling pathways (Saxena et al., 2013).

Prior to lysis, dramatic changes to the cytoskeleton can be observed in MVM infected cells. Vimentin fibers are degraded and re-organized into a perinuclear ring structure. The amount of gelsolin, a negative regulator of F-actin increases, while polymerisation activator Wiscott–Aldrich syndrome protein is diminished. Furthermore, the location of F-actin processing is altered and actin fibers are rearranged into distinct “patches” within the cytoplasm. On the contrary, microtubules become more resistant to nocodazole induced depolymerisation as a result of altered phosphorylation (Nüech et al., 2005).

While gelsolin is not required for productive infection, it appears to be involved in transport of MVM modified endosomes towards the cellular periphery and subsequent release of virions from the cell. Dynamin can also be seen to accumulate and colocalize with progeny virions at the perinucler region. (Bär et al., 2008). The cytotoxic effects of MVM are caused by NS1 complexed with a catalytic subunit of an endogenous protein casein kinase II. The complex is able to phosphorylate both VP1 and VP2 polypeptides of the capsid. Infected cells retain normal morphology at least several days when NS1 binding to casein kinase II is prevented by mutation of NS1 (Nüech and Rommelaere, 2006).

4 AIMS OF THE STUDY

Two themes were explored in this work. The first objective was to elucidate interactions of CPV and intracellular proteins in NLFK cell line. Especially, the interaction of CPV capsid with nuclear pore protein Nup358 was of major focus. Proximity ligation assay is a method for examining protein colocalization. It is based on fluorescent signal amplification so it allows detection of weak interactions. It was interesting to see if this method could be utilized during infection for detection of specific interactions directly by confocal microscopy. Binding of two primary antibodies to different target proteins is followed by binding of secondary antibodies conjugated to oligonucleotide sequences. Oligonucleotides in close proximity hybridize and seal by ligation, forming a closed circle. It can then be amplified by a polymerase to create a repeated sequence of nucleotides by rolling-circle replication. The amplified sequence is detected by binding of fluorescent oligonucleotides.

Second goal was to find out if CPV infection has a measurable impact on the rate of RNA synthesis. NLFK cells were cultured in a medium containing modified uridine analog to be incorporated into nascent RNA molecules. The uridine analog contained in the RNA could then be specifically bound by antibodies and conjugated to secondary fluorescent antibodies. The fluorescence was assessed by confocal microscopy and flow cytometry.

5 MATERIALS AND METHODS

5.1 Cell culturing and infections

NLFK cells were grown in Dulbecco's modified Eagle medium (Gibco) containing 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, and 1% non-essential amino acids. Cells were cultured at +37 °C in a CO^2 incubator, in 75 cm^2 flasks and divided 1:3 – 1:6 every 2 – 3 days. Cells (not counted) were transferred to 3,5 cm^2 culture dishes and incubated at +37 °C for 1-2 days before infections. Dishes selected for confocal imaging contained 10 mm Ø glass slips. Cells were infected with 100 µl of 6 x CPV at +37 °C from 10 min to 24 h for PLA (Table 1) and 24 h for immunolabeling and flow cytometry. During 10 min infections, 6 x CPV was diluted 1:1 into growth medium and 30 µl was added directly over the slip. Dishes were rinsed with 1 x PBS, fixed with 4 % PFA for 20 min and stored at +4 °C in 1 x PBS.

5.2 Proximity ligation assay

Protein colocalization was studied by proximity ligation assay (PLA). Duolink Fluorescence kit (Olink Bioscience) was used according to manufacturer's reagents and protocol. All reagents were diluted into H_2O and wash buffers prepared according to instructions. Primary antibodies (Table 1) were diluted into antibody diluent and once into 3 % BSA – PBS. Slip were placed into separate wells at 6-well plates and a clean plate was used after each wash step, while keeping slips separated at all times. Humidity incubator at +37 °C was used during reactions and each wash step was done with TBS–Tween. Before each new reaction a thin blade was placed under the slip and it was tapped sideways against an absorbent paper to remove most of wash solution. Open droplet reaction volumes were initially 40 µl and later reduced to 30 µl.

Slips were first blocked with a blocking solution to avoid non-specific binding and incubated for 30 min. Antibody solution containing the primary antibody pair (Table 2) for different protein interactions to be studied, was added to slips and left at RT, outside humidity incubator for 1 h. Slips were rinsed for 3 x 5 min in a low turning rate swing, then incubated for 2 h with a PLA probe solution containing oligonucleotide conjugated

secondary antibodies. Slips were rinsed for 2 x 5 min and incubated for 15 min with a hybridization solution. Slips were rinsed for 1 min before adding a ligation solution with ligase (1 U/µl) and incubation was resumed for 15 min. After 2 x 2 min rinse, incubation was resumed for 90 min with an amplification solution containing polymerase (10 U/µl). Another 2 x 2 min rinse was done before 60 min incubation with a detection solution containing labeled nucleotide probes. Downgrade concentrations of SSC from 2 x to 0,02 x were used for final washes according to instructions, each step lasting at least 2 min. Last 1 min wash was done with 70 % EtOH after which slips were mounted with Mowiol-Dabco to microscope slides. Reagents in the kit contained Hoechst color for nuclear staining. New version of the kit contains mounting medium with DAPI and the protocol is also shorter.

Table 1: Antibodies used for PLA, immunolabeling and flow cytometry

Antibody	Host	Source	Dilution
α-Tubulin	rabbit	Abcam	1:1000
A3B10	mouse	Colin Parrish, Cornell University, Ithaca, USA	1:200
BrdU	mouse	Santa Cruz Biotechnology	1:100
Cornell#2	rabbit	Colin Parrish, Cornell University, Ithaca, USA	1:200
Lamp1	rabbit	Abcam	1:50
NS1	mouse	Caroline Astell, University of British Columbia, Vancouver, Canada	1:70
Nup153	mouse	Abcam	1:50
Nup358	rabbit	Abcam	1:200
PCNA	rabbit	Abcam	1:500
Alexa 488 anti-mouse IgG	goat	Molecular Probes, Invitrogen	1:200
Alexa 555 anti-rabbit IgG	goat	Molecular Probes, Invitrogen	1:200

Table 2: Antibody pairs and infection times used for PLA

Antibody pairs	Infection times
A3B10 + Nup358	cpv-, 10m, 30m, 1h, 3h
Cornell#2 + NS1	cpv-, 24h
NS1 + PCNA	cpv-
Nup153+ Nup358	cpv-
α Tubulin + Nup153	cpv-
Lamp1 + Nup358	cpv-
Control (no antibodies)	cpv-

5.3 Bromouridine treatment and immunolabeling

Cells were infected for 24 h after which dishes were rinsed with 1 x PBS. 2 ml of 20 mM 5-Bromouridine 5'-triphosphate sodium salt (Sigma) (BRU) medium was added to each dish. Infected and noninfected control cells were incubated at +37 °C for 15 min, 30 min or 60 min, rinsed with 1 x PBS and fixed with 4 % PFA for 20 min at RT. Slips were rinsed with 1 x PBS and then with a permeabilization buffer (1 % BSA, 0,1 % Triton-X-100, 0,01 % NaN_3) for 15 min. Slips were transferred to a 6-well plate. Mouse monoclonal bromodeoxyuridine (BrdU) and rabbit polyclonal PCNA antibodies (Table 1) were diluted to 3 % BSA / PBS and 30 µl was added over the slip for 1 h reaction at RT. Permeabilization buffer was added to each well, then rinsed with 1 x PBS and rinsed again with the permeablization buffer, each wash step lasting for 15 min. Slips were transferred to a clean 6-well plate and 30 µl of secondary antibody solution with anti-mouse and anti-rabbit antibodies (Table 1) diluted to 3 % BSA / PBS was added and incubated for 30 h at RT. Slips were rinsed in permeablization buffer, then in 1 x PBS and mounted to microscope slides with DAPI.

5.4 Flow cytometry

Cells infected for 24 h and noninfected cells were labeled with BRU for 30 min with the same protocol as during immunolabeling. Control cells were neither infected nor received BRU treatment. BRU containing medium was replaced by 750 µl trypsin and incubated for 5 min at +37 °C. Cells were pelleted by centrifugation at 500 g for 5 min, after which supernatant was removed. Centrifugation and supernatant removal was repeated after each step. Pellet was suspended into 200 µl 4% PFA and vortexed for 15 min. Without pelleting 100 µl 1 M glycine was added and the suspension vortexed for another 5 min. Pellet was suspended in permeabilization buffer (see above) and vortexed for 15 min. Pellet was suspended into 3 % BSA / PBS with mouse monoclonal BrdU primary antibody (Table 1) and vortexed for 1 h. Pellet was suspended in permeabilization buffer and vortexed for 15 min. Pellet was suspended into 3 % BSA / PBS with Alexa 488 anti-mouse secondary antibody (Table 1) and vortexed for 30 min. Pellet was suspended into permeabilization buffer and vortexed for 20 min. Final pellet was suspended into 1 x PBS and analyzed in a FACSCalibur flow cytometer (Becton Dickinson).

First cell size and granularity were determined by examining forward scatter / side scatter of the sample. Fluorescence was then measured with a 488 nm laser and collected with FL1 detector equipped with a 530/30 nm filter. Total of 3 x 10^4 cells were analyzed in each assay. Mean fluorescence intensity values were taken from CellQuest Pro software (Becton Dickinson).

5.5 Confocal microscopy and image processing

Fixed cells were visualized and imaged with Olympus Fluoview 1000 confocal microscope using UPL SAPO 60x oil immersion objective. Fluorophores were excited with 488, 543 and 568 nm (for 555 nm fluorophores) lasers. Samples were scanned to Z-plane image stacks with a variable number of slices adjusted for the thickness of each imaged cell. 640x640 or 800x800 resolutions were selected for images. Image stacks were processed into maximum intensity projections with a non-commercial ImageJ software. Brightness and contrast were adjusted and occasionally Gaussian filtering (radius = 1) was applied to reduce background noise.

6 RESULTS

6.1 Protein colocalization

6.1.1 Interactions of cellular and viral proteins

Interactions of different cellular and viral components was examined by PLA. It was first determined how much background signal would be observed from non-specific reactions when no interactions were expected. There was barely detectable signal in noninfected cells when primary antibodies were excluded from the reaction (images not shown). Also, no significant signal was produced when noninfected cells were probed with an antibody combination against lysosomal protein Lamp1 and Nup358 as expected (Figure 1). However, when interaction of PCNA and viral NS1 was examined in noninfected cells there was noticeable signal spread across the cytoplasm without any apparent localization. One of the colocalization experiments between cellular proteins was to see if there would be any detectable interactions between α-Tubulin and Nup153. Some cytoplasmic signal was detected (Figure 1), but it was quite weak when compared to controls and did not localize near the NE.

Interaction of Nup358 and Nup153 was examined to see if the two components at opposite sides of NPCs would be found close enough to allow detection by PLA. Sometimes the signal appeared to concentrate near the NE, but also significant signal far away from the nucleus was observed (Figure 1). Equally strong localization near the nucleus was not observed in every cell and instead the signal was sometimes spread across the cytoplasm more evenly (images not shown). Very strong colocalization was detected 24 h p.i. for NS1 and Cornell#2 capsid antibodies (Figure 2) and the signal was significantly weaker when primary antibodies were emitted from the reaction. However, the signal in control cells was similarly apparent when compared to the reaction with PCNA and NS1 antibodies in noninfected cells.

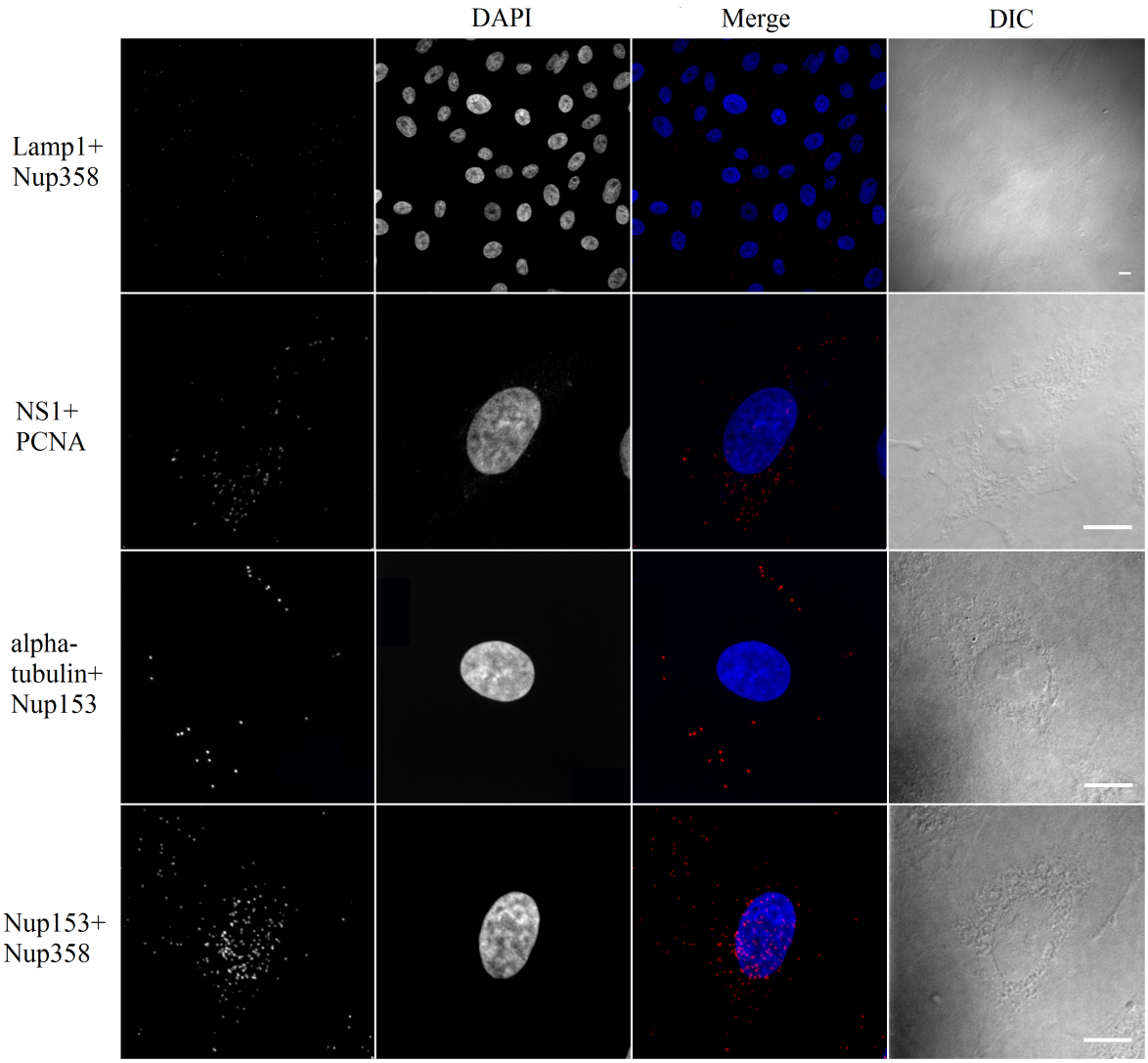

Figure 1: Noninfected NLFK cells after PLA reaction with different combinations of primary antibodies. No significant signal was observed for Lamp1+Nup358 interaction. However, NS1+PCNA produced considerable signal. α-Tubulin+Nup153 also produced some signal beyond the NE periphery. Nup153+Nup358 signal was significant and occasionally localized mostly near the NE. Lamp1+Nup358 was imaged as a single section. Rest of the images are maximum intensity Z-projections. Scale bar 10 µm.

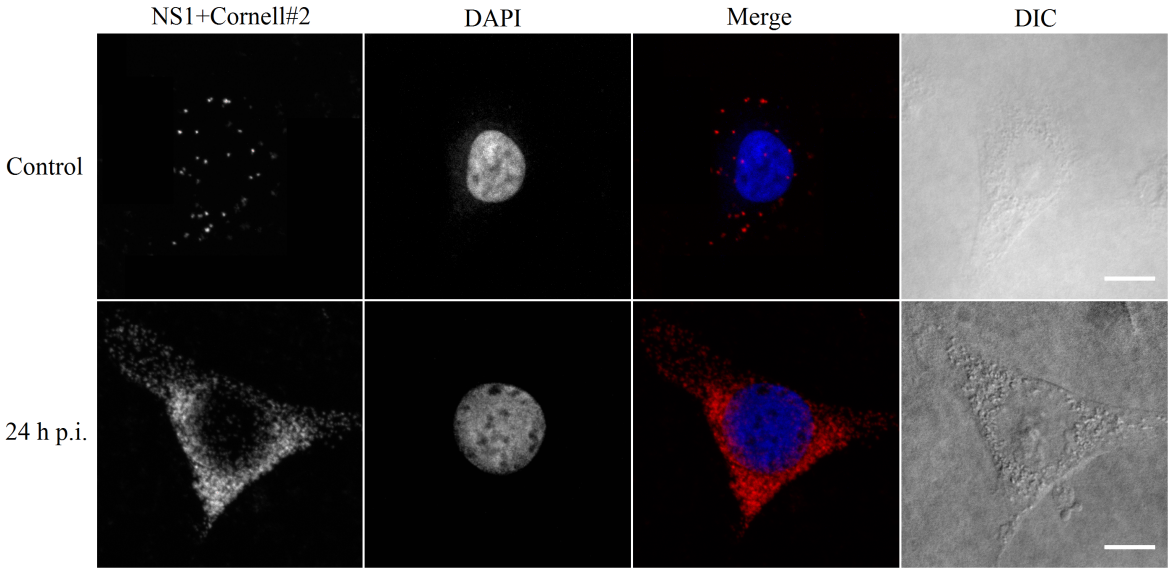

Figure 2: Noninfected and 24 h p.i. NLFK cells after PLA reaction with NS1+Cornell#2 primary antibody pair. Some signal was observed in control cells, but it was much weaker when compared to infected cells. The entire cytoplasm of infected cells exhibited fluorescence, but the signal was absent from the nuclear region. Images were processed into maximum intensity Z-projections. Scale bar, 10 µm.

6.1.2 Interaction of CPV capsid with Nup358

Lastly it was determined if CPV capsids could be detected to colocalize with Nup358, possibly in contact with NPCs. NLFK cells were infected for various durations from 10 min to 3 h. Then PLA reaction was carried out with Nup358 and capsid A3B10 primary antibodies. Only a weak signal was detected in controls and 10 min p.i. (Figure 3). From 30 min p.i. and onward the signal increased quickly in time-dependent manner and 3 h p.i the signal had filled most of the cytoplasm. There was no apparent localization to any specific area in the cytoplasm, but until 3 h p.i. the signal was mostly excluded from the perinuclear region. At that stage, the signal was more evenly spread across the cytoplasm and included the perinuclear region.

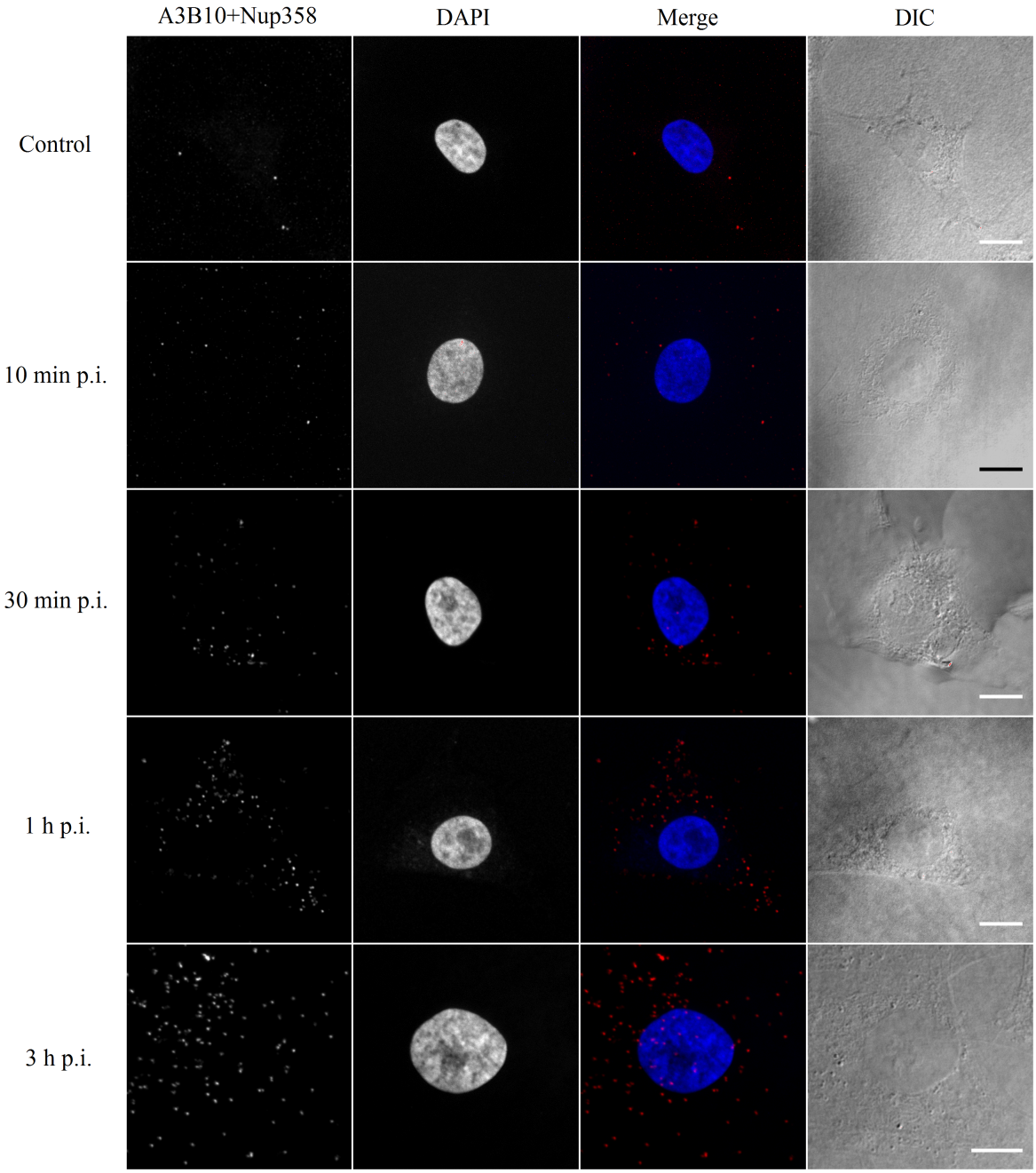

Figure 3: NLFK cells were infected for various durations and imaged after PLA reaction with A3B10+Nup358 antibody pair. The signal appeared to increase and shift towards the perinuclear region in respect to time elapsed from infection. Maximum intensity Z-projections. Scale bar, 10 µm.

6.2 RNA synthesis

6.2.1 Immunolabeling

Noninfected cells and cells infected for 24 h were treated with BRU to be incorporated into nascent RNA molecules. Cells were immunolabeled with BrdU and PCNA antibodies to find out if BRU treatment had been successful and to estimate if infection had a visually noticeable impact on RNA synthesis. Cells were initially treated with BRU for 15 min and this produced a slightly weak signal (images not shown). Raising incubation period to 30 min increased the signal adequately to allow proper visualization (Figure 4). PCNA staining gave a clear picture of the nucleus and infection could be confirmed by alteration of the intranuclear organization. BrdU stain could also be clearly seen spread across the cytoplasm indicating that BRU had been incorporated into nascent RNA. The BrdU signal appeared slightly stronger in noninfected cells when examined visually.

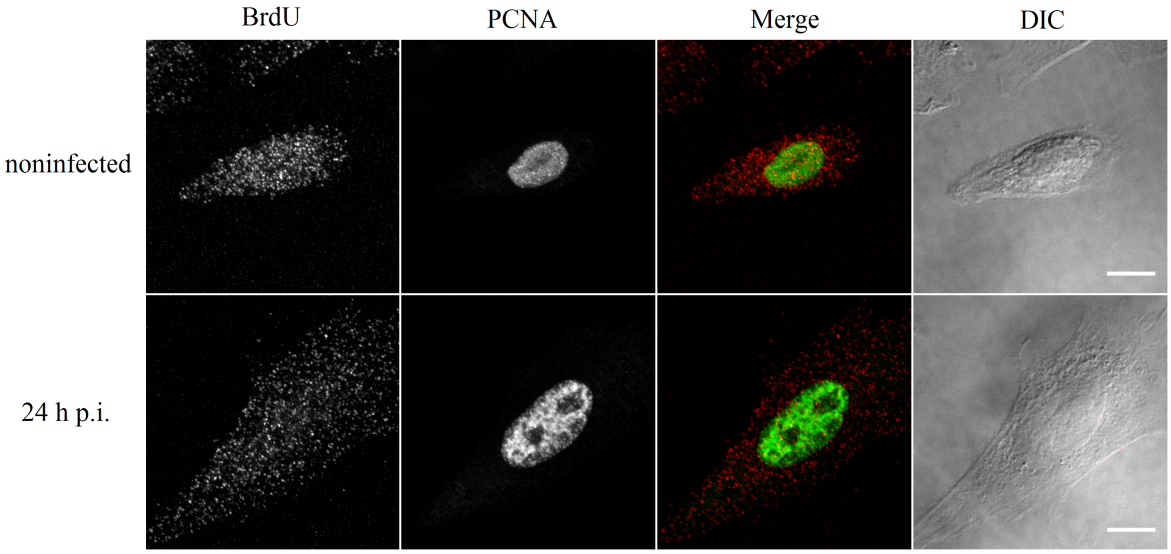

Figure 4: NLFK cells infected for 24 h and noninfected cells were treated with 20 mM BRU for 30 min and immunolabeled with BrdU and PCNA antibodies. The nucleus was clearly distinguished with PCNA staining. Nascent RNA could be seen evenly spread across the cytoplasm without noticeable localization to any specific area. Signal appeared to be slightly weaker in infected cells. Max intensity Z-projections. Scale bar, 10 µm.

6.2.2 Flow cytometry

Cytometric experiments were initially done by fixing cells with PFA and then scraping from dishes (results not shown). This procedure caused non-uniform granule size and dispersal of counts from average values at forward- / side scatter plot. Later experiments were done without fixation. Instead cells were detached with trypsin and labeled in suspension instead of dishes. This method produced more reliable results and a tight population of counts along the scatter plot. Even then some difficulties occurred when trying to get enough BRU treated and CPV infected cells for repeated runs. Cell suspension containing infected cells was few times depleted before reaching 3 x 10^4 cell counts. Similar problems did not occur with control or only BRU treated cells. However, after initial difficulties reliable results were obtained and a strong signal was detected in both infected and noninfected cells when compared to cells that did not receive the BRU treatment. This indicates that BRU had been successfully incorporated into nascent RNA molecules. Mean fluorescence values were determined for control and BRU treated cells with and without an infection. Signal coming from infected cells was clearly reduced during all measurements, with an average decrease of ~32 % (Figure 5).

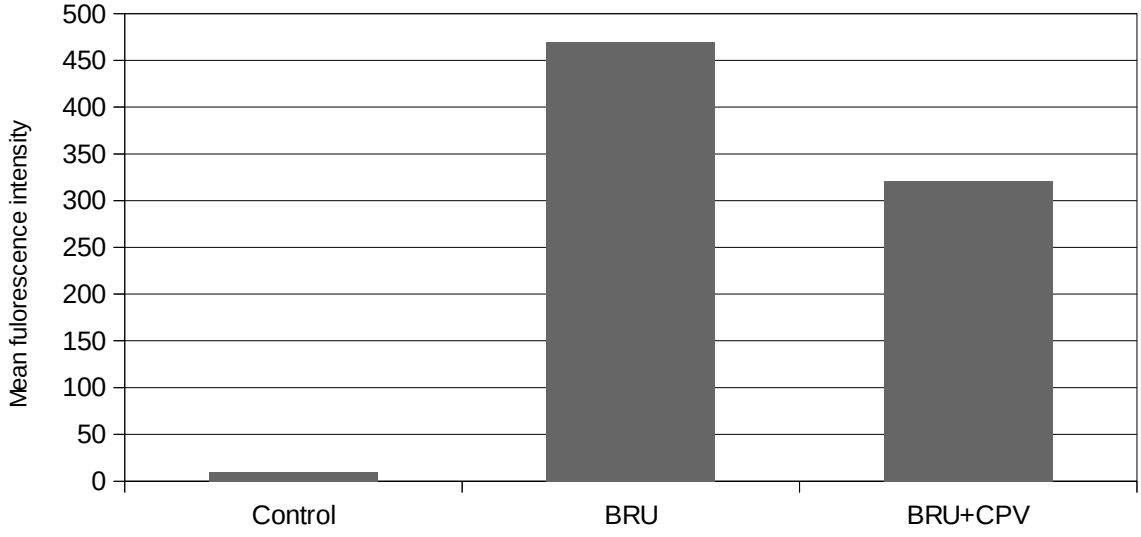

Figure 5: Mean fluorescence intensity values of FACS experiments. Three times 10^4 cells were counted for each separate condition during each assay. Control cells were not infected or treated with BRU.

7 DISCUSSION

Inside the cell thousands of molecules interact every second. Some interactions are very stable whereas others are more transient. In this respect it is of particular importance to be able to detect these interactions specifically without the interference of non-interacting molecules. By examining the colocalization of cellular and viral proteins, not only the locations of interactions are revealed, but also valuable clues to the function of these interactions can be discovered. PLA is a promising method to be harnessed for this purpose, because it can be very specific in optimal conditions and results obtained quickly.

It has been show that many Nups and components of the NE control and even directly interact with microtubules (for review see D'angelo and Hetzer 2008; Burke and Stewart, 2013). Therefore we examined if an interaction between Nup153 and α-Tubulin could be detected. Some signal was seen in the cytoplasm, but it was limited to only few spots. The signal appeared comparable to control experiments and was likely not produced by an actual interaction between the two proteins. These results suggest, in short, if there is an interaction between Nup153 and α-Tubulin, it is not likely to be efficiently detected by PLA. Another interaction between cellular proteins to be examined was between Nup153 and Nup358. These two Nups share similar structural elements, but also interact with same proteins and binding of both to transport receptors is regulated by RanGTP (Nakielny et al., 1999 and references within). The best characterized location of the two Nups are at the opposite sides of the NPC. However, Nup153 has been shown to be mobile within the NPC and one of its domains appears to transiently localize at the cytoplasmic side. Antibodies against Nup153 microinjected into the cytoplasm concentrate at the rim of the nucleus, but binding of secondary antibody requires a strong permeabilization agent such as Triton X-100 (Nakielny et al., 1999).

In the present study, sometimes preferential localization near the NE appeared. However, most of the signal in all cells was seen in the cytoplasm instead of the NE. As previously mentioned, Nup358 is found in the cytoplasm of interphase cells where it interacts with microtubules (Joseph and Dull, 2008). Nup153 is also a diffuse protein and not as stably bound to interphase NPCs when compared to some of the other Nups and during

metaphase it is also mobile within the cytoplasm (Daigle et al., 2001). Not much is known about interactions of Nup153 with Nup358 or other proteins in the cytoplasm. In the results presented here it appears not only that Nup153 is found in significant enough quantity in the cytoplasm during interphase, but also that it comes into close enough proximity with Nup358 to be detectable by PLA. The preferential colocalization near the NE in some cells might indicate interactions of Nups during disassembly / assembly of NPCs.

Parvovirus virions are composed of only few proteins. NS1 is the most important multifunctional regulatory protein involved in several key steps of the infection. The best characterized location where NS1 employs its many functions is inside the nucleus, most notably inside the virus-induced replication compartments. These areas are distinct from other nuclear structures in early phases of the infection before NS1 distribution becomes homogenous. In addition, as previously mentioned, NS1 also shuttles between the cytoplasm and the nucleus (Ihalainen et al., 2007). Mature capsids inside the nucleus are excluded from the replication compartments and are located close to the nuclear membrane instead (Ihalainen et al., 2009). Therefore it was of interest to see if colocalization between the CPV capsid and NS1 could be detected and if so, where it would be most prominent. It has been shown that interactions of viral components at intranuclear foci during viral infection can be detected by PLA (Belzile et al., 2010).

In this study there appeared no localization inside the nucleus when the interaction of the CPV capsid and NS1 was examined. However, a very strong cytoplasmic signal was detected at a late stage of infection. Considering that the colocalization signal filled the entire cell, but was absent in the nucleus, it seems likely that there should have been at least some colocalization inside the nucleus as well. Based on these observation it would appear that some of the reagents did not enter the nucleus, possibly because of incomplete permeabilization of the NE. It has been previously assumed that parvoviruses are released from the nucleus during apoptosis of the cell (for review see Whittaker et al., 1998). However, it has been reported that at least MVM may escape the nucleus by active translocation through the NPC. When N-terminal region of VP2 in full capsids is phosphorylated it appears to function as nuclear export signal driving capids out of the nucleus (Maroto et al., 2004). Keeping in mind the NE disruptions caused by parvoviruses

during entry, it might not be completely unexpected if mature virions could also use a similar mechanism to escape from the nucleus. The strong cytoplasmic signal observed in these results imply that significant number of CPV virions are released from the nucleus prior to lysis of the cell and interaction of the capsid with NS1 can be detected in the cytoplasm.

Early steps of parvovirus infection are now relatively well understood so in recent years the research focus has shifted into revealing the events at the NE and inside the nucleus. Early studies suggested that the NLS of CPV might mediate import to the nucleus via the NPC (Vihinen-Ranta et al., 1997; Vihinen-Ranta et al., 2002). Recent research on MVM and other parvoviruses however suggest a possible NPC-independent route into the nucleus directly through the NE (Cohen and Panté 2005; Porwal et al., 2013). Even if this might also true for CPV, the NLS might still be required to direct incoming virions to the NPC before entry to the nucleus by another mechanism. Therefore it was interesting to examine if colocalization of CPV capsid with the peripheral cytoplasmic nucleoporin Nup358 could be detected. There did not seem to be any preferential colocalization between CPV and Nup358 at the NE, but instead the signal was spread across the cytoplasm. Considering that CPV trafficking is microtubule-dependent process, it would seem plausible to expect such cytoplasmic colocalization of the capsid with Nup358 associated with microtubules (Vihinen-Ranta et al., 1998; Joseph and Dull, 2008).

Also, the signal did seem to increase during progression of the infection, while the number of virons entering the cytoplasm is expected to be simultaneously increasing. These results suggest that the capsid can be detected in the cytoplasm and interacting with Nup358, but not at the NPC. It is also possible that an interaction at the NPC does exists, but it might be too transient to be detected by PLA, while in comparison trafficking of virions is a slow process. It is quite possible that virions might localize at the NPCs and such interaction should not be excluded based on these results. It would also be interesting to see if blocking transport through NPCs could prevent CPV infection similarly to MVM (Cohen and Panté 2005).

PLA control experiments produced mixed results. Sometimes a barely detectable signal was seen, but at other times the signal was strong. The unexpected signal created by what is expected to be non-specific interactions between the antibodies and not by colocalized target proteins makes assessing the results difficult. This is especially the case when certain interactions were examined. Non-specific binding was perhaps most obvious in the case of NS1 interactions with Nup153 or CPV capsid. A noticeable signal was detected when interactions in noninfected cells were examined. Based on these observations the results of this study can provide framework for further experiments, but require validation by other methods.

Updated version of the PLA-kit has a shorter protocol that should help to reduce possible variation of conditions during the preparation of samples for imaging. In addition, it should also make it easier to adjust the protocol to produce more reliable and quantifiable results. Immunolabeling and other traditional methods such as co-immunoprecipitation are important for providing verification and comparative analysis to the results obtained by PLA. Interactions and alterations induced by parvoviruses at the NE remain an important focus in further research. In addition to colocalization experiments, other methods such as fluorescence recovery after photobleaching can be harnessed during viral infection to study localization of viral components and dynamic changes in the structure of the NE, including individual Nups, lamins and other associated proteins within the NE.

Viruses can use different approaches to increase the production of viral proteins at the expense of the host cell. Specific host cell functions can be targeted and regulated to inhibit export of host mRNA from the nucleus. Not much is known how DNA viruses achieve this, although adenoviruses seem to degrade cellular proteins involved at host mRNA export. This leads to preferential export of viral mRNA, while host mRNA accumulates inside the nucleus. (for review see Kuss et al., 2013). One mechanism utilized by parvoviruses to increase viral protein synthesis is to inhibit the activation of heterologous promoters. Yet another activity controlled by NS1 of several parvoviruses including parvovirus H1, MVM and adeno-associated viruses (Rhode and Richard, 1987; Legendre and Rommelaere, 1992; Berns, 1990 review). CPV infection regulates transcription by alternating binding and release kinetics of transcription associated proteins. Similar levels

of total cellular mRNA between noninfected cells and 24 h p.i. have been reported by spectrophotometric quantification, while cytoplasmic RNA was demonstrated to decrease as a result of infection (Ihalainen et al., 2012). The results obtained in this study by immunolabeling and flow cytometry both indicate a noticeable overall reduction in RNA synthesis as a result of infection. Nascent RNA was seen to be evenly spread throughout the cell with similar distribution patterns between infected and nonifected cells. No preferential accumulation of RNA inside the nucleus could be observed in infected cells.

In this work it was revealed that the two nuclear pore proteins, Nup153 and Nup358 interact in the cytoplasm and sometimes preferentially at the vicinity of the nuclear membrane. There appeared to be no significant colocalization between Nup153 and the microtubule protein α-Tubulin. When interaction between CPV capsid and NS1 was examined, a strong colocalization signal in the cytoplasm was detected. These results suggest that the entire cytoplasm was occupied by the virus and NS1 during late phases of the infection. Colocalization of the capsid with Nup358 at the nuclear pore complex was not detected. However, cytoplasmic colocalization was seen between the two proteins, possibly when both come to close contact at the microtubule network during trafficking of virions towards the nucleus. The colocalization signal appeared to increase and shift towards the nucleus in time dependent manner. Control samples sometimes produced a strong signal when colocalization was not expected so these results should be examined cautiously. Immunolabeling and flow cytometry quantification indicate a significant decrease in the synthesis of nascent RNA molecules as a result of infection. These results provide further evidence on the modification of cellular transcription patterns by parvoviruses.

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