VIRAL INTERACTIONS WITH THE NUCLEUS

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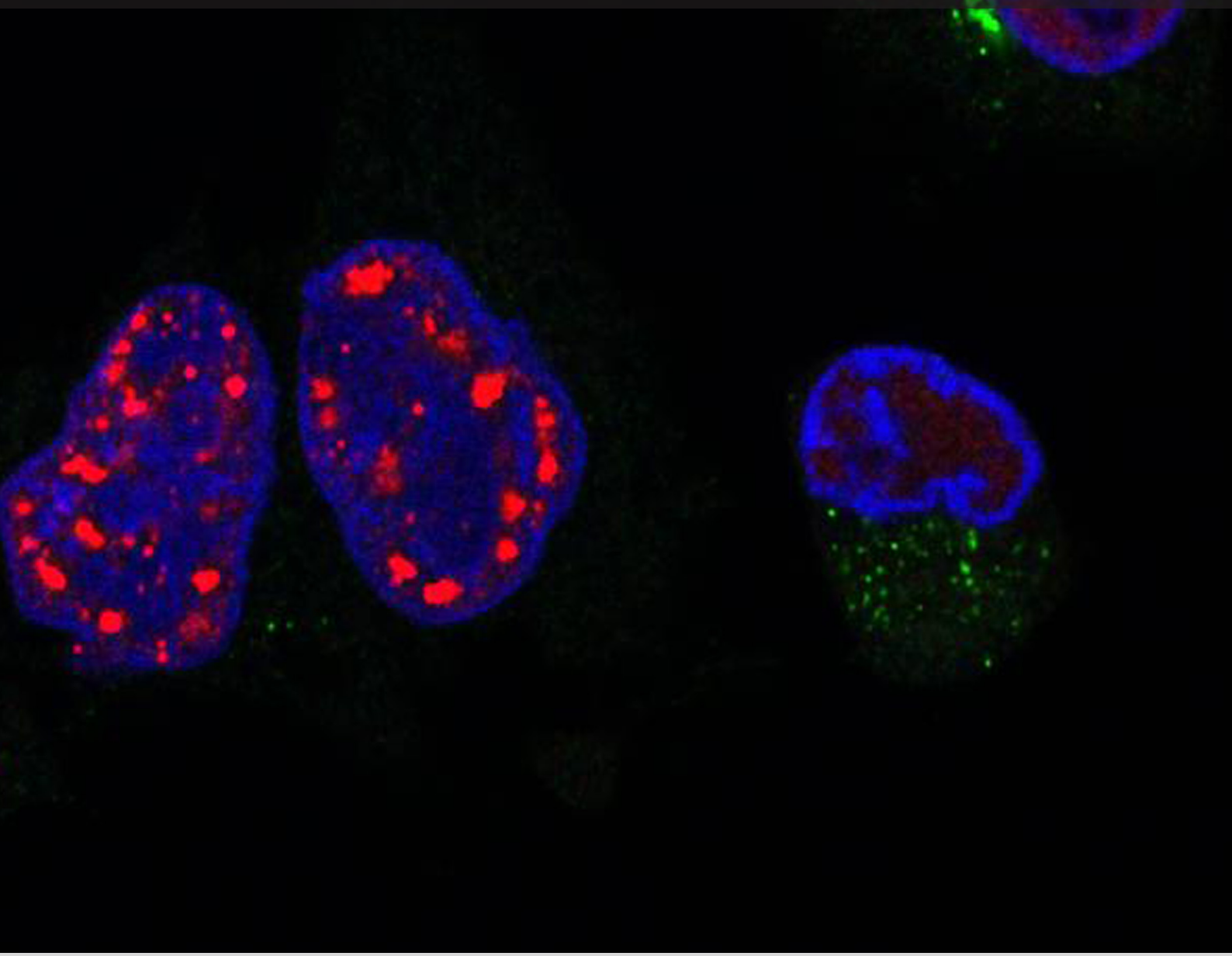

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VIRAL INTERACTIONS WITH THE NUCLEUS

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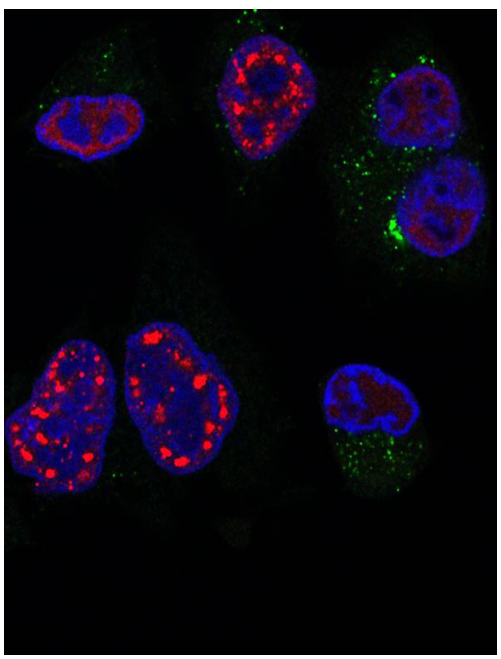

Ohio-HeLa cells grown on coverslips were infected with HRV2 and fixed at 9 h.p.i. Cells were permeabilized then probed with anti-dsRNA to indicate infected cells and anti-SC35 antibodies, followed by CF488 and Alexa-568 conjugated secondary antibodies, respectively. Coverslips were mounted in ProlongGold mounting media with DAPI. Fluorescence was imaged by CLSM. Infected cells (indicated by green cytoplasmic spots) show diffusion of the nuclear speckle protein SC35 (indicated in red). Image by Erin Joanne Walker

Viruses cause numerous medically important diseases, affecting developing, developed, rich and poor alike. The diseases vary in severity, including chickenpox, smallpox, influenza, shingles, herpes, rabies, polio, Ebola, hanta fever, AIDS and the common cold, amongst others. Regardless of the type of tissue or organ affected, all viruses follow the same basic steps to infect host cells. Once in contact with host cells viruses release their genetic material into the cell followed by genome replication, production of viral proteins, assembly of the virus particle and egress from

the infected cell. Viruses disrupt normal host cell processes in order to facilitate their own replication/assembly by re-directing cellular machinery for viral transcription, translation, assembly, release and by inhibiting antiviral responses.

Regulated nuclear transport of macromolecules through the nuclear pore complex, the only means of transport across the nuclear membrane, is essential for normal cell function and an effective antiviral response. Many viruses disrupt or exploit the nucleocytoplasmic trafficking pathways in host cells. Cytoplasmic viruses exploit the host cell nucleocytoplasmic trafficking machinery to access nuclear functions and/or disrupt nuclear transport, while several DNA viruses use the trafficking pathways to enable export of their components into the cytoplasm; yet others complete their assembly within the nucleus and use nuclear export pathways to access the cytoplasm. Indeed, the many and varied interactions of viruses and viral proteins with nucleocytoplasmic trafficking components have been invaluable in pathway discovery. Importantly, mounting evidence suggests that these interactions play essential roles in virus replication/assembly and hence may be key to understanding pathophysiology of viral diseases.

This Frontiers Research Topic is dedicated to the importance of nucleocytoplasmic trafficking to viral pathogenesis.

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EDITORIAL
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Editorial: Viral Interactions with the Nucleus

Erin J. Walker and Reena Ghildyal *

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Keywords: DNA viruses, RNA viruses, nuclear pore complex, viral nuclear interactions, nuclear transport

Editorial on the Research Topic

Viral Interactions with the Nucleus

The eukaryotic genetic material is sequestered within the nucleus bound by the nuclear envelope (NE), separating the genetic material, and its functions from the surrounding cytoplasm. Regulated transport of macromolecules through the nuclear pore complex (NPC), the only means of transport across the nuclear envelope, is essential for normal cell function and effective antiviral responses. Many viruses disrupt or exploit the host cell nucleocytoplasmic trafficking pathways in order to access nuclear functions.

This research topic has assembled reviews and original research articles demonstrating the diversity and importance of viral interactions with the nucleus. The viruses range from DNA, RNA viruses, to enveloped, non-enveloped viruses, and include retroviruses, demonstrating that exploitation of the host nuclear process is a common theme across diverse virus families. All articles in this topic address viruses of veterinary and/or medical importance.

The importance of nuclear entry is addressed in a review focusing on enveloped and non-enveloped DNA viruses (Fay and Panté), which deliver their genome into the nucleus for replication. Intact capsids of hepatitis B virus (HBV) are transported into the nucleus via translocation through the NPC. In contrast, herpes simplex virus 1 (HSV1) docks on the cytoplasmic side of the NPC, enabling release of the viral genome into the nucleus. Non-enveloped DNA viruses deliver their genomes into the nucleus in diverse ways, including docking at the NPC (adenovirus), disruption of the NE (parvovirus), and accessing the nucleus during breakdown of the NE in mitosis (human papillomavirus).

Most RNA viruses do not strictly require nuclear entry of their genome to replicate, but may require RNA-binding proteins found in the nucleus for replication. Entry of RNA virus proteins such as proteases into the nucleus also enables disruption of host-cell transcription and innate anti-viral responses, as discussed in the review on picornaviral nuclear interactions (Flather and Semler). The picornaviral theme is developed further by Walker et al. who describe the nuclear effect of two serotypes of human rhinovirus (HRV), a member of the picornavirus family (Walker et al.). While both serotypes lead to changes in NPC proteins and redistribution of nuclear proteins, this study demonstrates the variation that occurs between family members.

Influenza virus, HRV, and respiratory syncytial virus (RSV) are the main viral causative agents of respiratory infectious disease and all three exploit the nucleocytoplasmic trafficking mechanisms, albeit in different ways (Caly et al.). Influenza virus replication occurs at viral ribonucleocapsid complexes in the nucleus. HRV disrupts nucleocytoplasmic trafficking by directed cleavage of NPC proteins by viral proteases, disrupting nuclear transport, and providing viral access to nuclear proteins required to support HRV replication. RSV uses nuclear transport pathways to move its matrix protein into and out of the nucleus at specific times in infection to facilitate virus replication and assembly.

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Nucleocapsid proteins of some enveloped RNA viruses localize to the nucleus of infected cells; this is intriguing as these proteins are essential for virus replication and assembly in the cytoplasm. A review of the nucleocytoplasmic transport of these proteins and their proposed nuclear functions is provided by the Ghildyal group, detailing the nuclear transport signals present in members of the *Flaviviridae*, *Coronaviridae*, *Arteriviridae,* and *Paramyxoviridae* families (Wulan et al.).

As well as the nucleocapsid (or core protein) of the hepatitis C virus (HCV), a number of non-structural proteins carry nuclear localization signals and are found in the nucleus during infection, as reviewed by Bonamassa et al. The functional roles of non-structural protein NS5A are varied, including biogenesis of viral replication factories, viral replication and assembly of viral particles (Bonamassa et al.), potentially acting as a switch between viral replication, and particle assembly. As described for many viruses, the HCV NS5A protein affects host interferon responses by interfering with transcriptional regulation of relevant genes.

The host cell nucleus is essential to retrovirus replication as the reverse transcribed DNA is integrated into the host genome where it is transcribed by the host machinery. Rice et al. show that the Gag protein may co-opt cellular splicing signals to ensure encapsidation of the unspliced genomic viral RNA (gRNA). Previous work by the group had shown nuclear trafficking of Gag is required for efficient encapsidation of gRNA. The current article (Rice et al.) takes their work and sheds light on the nuclear functions of Gag. Their work raises the possibility that Gag localizes to the nucleus and associates with transcription factories to gain access to nascent unspliced viral RNA in order to capture it for encapsidation.

Herpes viruses, enteroviruses, and flaviviruses comprise majority of the known neurotrophic viruses. Interestingly, despite their very different genomes and diverse replication cycles, many have very similar pathogenic mechanisms. In their review, Hill et al. discuss the induction of a key micro-RNA (miRNA) by several neurotrophic viruses. miRNA-146a is induced early in infection via an NF-κB dependent pathway. As NF-κB is a mediator of proinflammatory and antiviral pathways, the question arises as to whether the induction of miRNA-146a is a protective host response or supports the virus infection; this is not yet known. It is however clear that miRNA-146a is involved in the observed pathogenic responses to infection and may have a role in the observed modulation of inflammation in antiviral treatment (Hill et al.).

The article by Tu and Rao addresses the important topic of immunosenescence in T cells. We have known for some time that the immune system undergoes cumulative age-associated changes, which produce a progressive deterioration in the ability to respond to infections and to develop immunity after vaccination. Modification of nuclear functions via epigenetic changes induced by latent cytomegalovirus infection is implicated in pathological changes in T cells that accelerate age-associated immunosenescence (Tu and Rao). Vaccine efficiency in the elderly may be enhanced by targeting the epigenetic changes induced by cytomegalovirus infection.

Together these papers demonstrate the importance of interactions with the nucleus across a range of different viruses of medical importance; whether by hijacking nuclear transport pathways, disrupting host nuclear transport to limit immune responses, or access specific nuclear proteins.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Nuclear entry of DNA viruses

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DNA viruses undertake their replication within the cell nucleus, and therefore they must first deliver their genome into the nucleus of their host cells. Thus, trafficking across the nuclear envelope is at the basis of DNA virus infections. Nuclear transport of molecules with diameters up to 39 nm is a tightly regulated process that occurs through the nuclear pore complex (NPC). Due to the enormous diversity of virus size and structure, each virus has developed its own strategy for entering the nucleus of their host cells, with no two strategies alike. For example, baculoviruses target their DNA-containing capsid to the NPC and subsequently enter the nucleus intact, while the hepatitis B virus capsid crosses the NPC but disassembles at the nuclear side of the NPC. For other viruses such as herpes simplex virus and adenovirus, although both dock at the NPC, they have each developed a distinct mechanism for the subsequent delivery of their genome into the nucleus. Remarkably, other DNA viruses, such as parvoviruses and human papillomaviruses, access the nucleus through an NPC-independent mechanism. This review discusses our current understanding of the mechanisms used by DNA viruses to deliver their genome into the nucleus, and further presents the experimental evidence for such mechanisms.

Keywords: Nuclear import, nuclear envelope, nuclear pore complex, nucleoporins, virus nuclear entry, virus nuclear import, DNA virus

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Introduction

Viruses are opportunistic pathogens that infect a host by attacking their cells and hijacking the cellular machinery to replicate, produce progeny virus particles, and spread infection. A few RNA viruses and almost all DNA viruses replicate themselves in the nucleus of their host cells. To accomplish this, their viral genome must enter the host nucleus. Entry into the nucleus is also achieved by many viral proteins (reviewed by Fulcher and Jans, 2011), which either assist in viral replication or are required for the formation of progeny viral subparticles or capsids. Nuclear export is also at the basis of viral infections because many viral pathogens assemble in the nucleus and must then exit the nucleus. Thus, viral hijacking of nuclear transport is important for several viruses to complete their infection cycle.

Physiological transport of macromolecules between the cytoplasm and the nucleus is highly selective and occurs through nuclear pore complexes (NPCs), large protein assemblies embedded in the nuclear envelope (NE). The NPC allows passive diffusion of ions and small molecules through aqueous channels with a diameter of about 9 nm. Nuclear import or export of molecules larger than this diffusion limit, and up to 39 nm in diameter (Pante and Kann, 2002), is a highly selective process that requires a signal residing on the transported molecule and soluble import or export receptors that recognize these signals (Wente and Rout, 2010). Few years ago it was largely assumed that viruses or viral capsids were either small enough to enter the nucleus through the NPC or disassemble in the cytoplasm releasing subviral particles that then enter the NPC. However,

recent progress in the characterization of the nuclear import of several viruses has drastically changed the perception of how viruses use the cellular machinery for nuclear import. The emerging picture is that each virus has evolved a unique strategy to deliver its genome into the nucleus. These strategies include the use of all or some of the components of the cellular nuclear transport machinery, but also unexpected mechanisms such as the use of the NPC for capsid disassembly or viral nuclear entry through the nuclear membranes instead of the NPC.

Retroviruses, such as the human immunodeficiency virus type 1, and orthomyxoviruses, such as influenza A virus, are RNA viruses that also deliver their genomes into the nucleus of the cells they infect. Since these viruses are significant human pathogens, their mechanisms of nuclear transport have been the focus of intense research, and several excellent reviews describing their nuclear transport have been published in recent years (Boulo et al., 2007; Di Nunzio, 2013; Hutchinson and Fodor, 2013; Matreyek and Engelman, 2013; Eisfeld et al., 2015). Thus, we do not cover nuclear import of RNA viruses in this review, and instead present the current knowledge and recent advancements in studies of the nuclear entry mechanisms used by DNA viruses. We first briefly summarize the principles of cellular nuclear transport, discuss the experimental approaches used to study viral nuclear import, and then discuss the nuclear entry strategies used by several DNA viruses, including hepatitis B virus (HBV), herpes simplex virus 1 (HSV1), baculoviruses, adenoviruses, parvoviruses, the polyomavirus simian virus 40 (SV40), and human papillomavirus (HPV). The key features of these viruses are summarized in **Table 1**.

Overview of the Cellular Nuclear Transport Machinery

Although several macromolecules enter the nucleus (e.g., spliceosomal and 5S ribosomal ribonucleoprotein complexes, and mitochondrial DNA), the nuclear import of proteins is the best-characterized process. Key players involved in this process are: the NPC, nuclear localization sequences (NLSs) on the transported proteins, nuclear transport receptors (NTRs) that recognize the NLSs in the transported proteins, and the small Ras-like GTPase Ran (reviewed by Wente and Rout, 2010).

The NPC is a large proteinaceous structure (125 MDa in vertebrates; Reichelt et al., 1990), made of only 30 different proteins called nucleoporins (Nups). These are present in multiple copies and are arranged in a structure of 120 nm in diameter and 70 nm in height. The NPC has eightfold rotational symmetry, and structurally is described as having three rings: a central framework ring embedded in the NE, which is sandwiched between a cytoplasmic and a nuclear ring. Extending from the cytoplasmic ring are eight cytoplasmic filaments, and attached to the nuclear ring is a basket-like structure, termed the nuclear basket (see NPC diagram in **Figure 1**). The central framework ring contains a large central channel of about 50 nm in diameter through which molecules enter or exit the nucleus. Nups contain hydrophobic phenylalanine glycine (FG) sequence repeat motifs that line this channel. NTRs bind to these FG-Nups to transiently melt the barrier at the center of the NPC, thus allowing nuclear transport (reviewed by Wente and Rout, 2010).

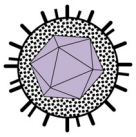
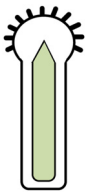
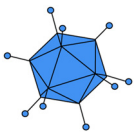
Proteins destined to enter the nucleus bear a biochemical "pass code" – one or more short stretches of specific amino acid sequences termed NLSs (Lange et al., 2010; Marfori et al., 2011). There are many types of NLSs and each one is recognized by a specific NTR – with the exception of the NTR importin-β, which recognizes the classical NLS through an adaptor protein, importin-α. The NTRs belong to a superfamily of proteins termed the karyopherin-β-family; there are over 20 β-karyopherins in human cells (Chook and Suel, 2011). The GTPase Ran regulates the association and dissociation of the NTR with the NLS-containing cargo; NTR binds to NLS-cargo in the presence of Ran-GDP, and dissociates from it in the presence of RanGTP (Gorlich et al., 1996; Izaurralde et al., 1997; Fried and Kutay, 2003).

In contrast to nuclear import of proteins, less is know about nuclear import of DNA, even though studies on nuclear transport of DNA have already been published decades ago (Capecchi, 1980). Nevertheless, two recent studies addressed the role of NTR in the nuclear import of exogenous and endogenous DNA. The first found that nuclear import of exogenous DNA is mediated by the NTR transportin-1, with histones acting as an adaptor between tranportin-1 and the DNA (Lachish-Zalait et al., 2009). The second study, reported that the NTR importin-7 mediates nuclear import of mitochondrial DNA (Dhanoya et al., 2013), which is transported from mitochondria to the nucleus in eukaryotic cells. It is not clear whether importin-7 binds directly to the DNA or uses an adaptor protein. Since some DNA viruses transport their genome into the nucleus, studies on nuclear import of DNA viruses will help unveil the cellular mechanism for endogenous DNA.

Experimental Approaches to Study Viral Nuclear Import

Two well-established systems of studying nuclear import have provided much of our insight into its mechanisms, and these systems have been adapted for the study of nuclear import of viruses. These are the *Xenopus* oocyte nuclear import assay and the *in vitro* nuclear import assay with digitonin-permeabilized cells. In the oocyte system, the substrate to be studied (protein, virus, or viral capsid) is introduced by microinjection into the cytosol of the oocyte, and nuclear import is monitored by electron microscopy (EM; Pante, 2006; Au et al., 2010). In the digitonin-permeabilized cell system, the plasma membrane but not the NE becomes permeabilized with the detergent digitonin (Adam et al., 1990; Cassany and Gerace, 2009), which preferentially extracts cholesterol present in the plasma membrane but not in the nuclear membranes. This treatment and subsequent washing releases soluble cytosolic components; thus for this assay the permeabilized cells are incubated with the fluorescently labeled substrate, exogenous cytosol or purified recombinant NTRs and Ran, and an energy-regenerating system. Nuclear import is then monitored by fluorescence microscopy or indirect immunofluorescence microscopy if the import cargo is

TABLE 1 | Summary of key features of the DNA viruses discussed in this review.

Envelope	Enveloped			Non-enveloped			
Capsid symmetry	Icosahedral		Rod-shaped	Icosahedral			
Family name	Hepadna	Herpes	Baculo	Adeno	Parvo	Polyoma	Papilloma
							
Virus	Hepatitis B virus (HBV)	Herpes simplex virus 1 (HSV1)	*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)	Adenovirus	Parvovirus	Simian virus 40 (SV40)	Human papilloma virus (HPV)
Virus/capsid size (nm)	42–47/32 or 36	180–225/120	60 × 300/30 × 250	90–100	18–26	45	50–55
Genome architecture	Double-stranded circular	Double-stranded linear	Double-stranded circular	Double-stranded linear	Single-stranded linear	Double-stranded circular	Double-stranded circular
Genome size	3.2 kbp	152 kbp	90–180 kbp	36–38 kbp	5 kb	5 kbp	8 kbp

not fluorescently labeled. Other systems used for the study of nuclear import of viruses are microinjection of tissue culture cells and *in vitro* nuclear import assays with purified nuclei. However, because viruses are often modified during cellular entry, results obtained using microinjection or *in vitro* nuclear import assays are then validated with experiments in infected cells.

To study the involvement of the NPC in viral nuclear import a common experimental strategy is the use of the lectin wheat germ agglutinin (WGA), which binds to the *N*-acetylglucosamine residues found on Nups (Finlay et al., 1987; Dabauvalle et al., 1988), or anti-Nup antibodies. Both treatments block the NPC and inhibit nuclear transport. Antibodies against a particular Nup or depletion of a particular Nup by RNA interference (RNAi) have also been used to study the role of a particular Nup in the mechanism of nuclear import of viruses. Finally, to determine the role of viral components, virus or viral capsids carrying mutations of a particular protein are used.

Nuclear Entry of Enveloped DNA Viruses

The first barrier viruses have to overcome to infect a host cell is the plasma membrane. Enveloped viruses often have a fusion protein that promotes fusion of the viral envelope with the plasma membrane or with the endosomal membrane after their uptake by endocytosis. This results in the release of viral capsids into the cytoplasm of the host cell. Subsequently the capsid uses the host cytoskeleton to travel to the nuclear periphery. Although enveloped viruses may share a similar cell entry mechanism due to the existence of their membranes, their nuclear entry mechanisms are very unique, most likely due to their differing features, such as size and architecture of the virus, and genome size. As summarized in **Figure 1**, we have now come to understand the nuclear entry strategy used by three enveloped viruses to deliver their genome into the nucleus.

Hepatitis B Virus

Human HBV is an enveloped virus with a diameter of 42–47 nm, containing an icosahedral capsid with a partially double-stranded DNA genome of 3.2 kbp (Seeger et al., 2013). The capsid is composed of a single protein called the core protein (185 amino acids, 21 kDa); there are two types of capsids: one with diameters of 32 nm and containing 180 copies of the core protein, and the second with diameter of 36 nm and containing 240 copies of the core protein (Vanlandschoot et al., 2003). Studies of the early HBV infection steps have been challenging because human hepatocytes, the physiological host, are difficult to maintain in tissue culture. Nevertheless, the recent development of alternative liver-derived cell lines able to support HBV infection *in vitro*, has considerably advanced our understanding of HBV cell entry in the last few years. Thus, it has only recently been established that HBV enters cells by caveolin-1- or clathrin-mediated endocytosis depending on the cell line used (Macovei et al., 2010; Huang et al., 2012). The DNA-containing capsid is then released into the cytoplasm by the fusion of the viral envelope with endosomal membranes, and travels along the microtubule network toward the nucleus (reviewed by Kann et al., 2007). The HBV capsid then uses a unique mechanism to deliver its genome into the nucleus: the intact capsid crosses the NPC, in a phosphorylation- and importin-α/β-dependent manner, and undergoes complete disassembly at the NPC nuclear basket releasing the uncoated genome into the nucleus (**Figure 1A**).

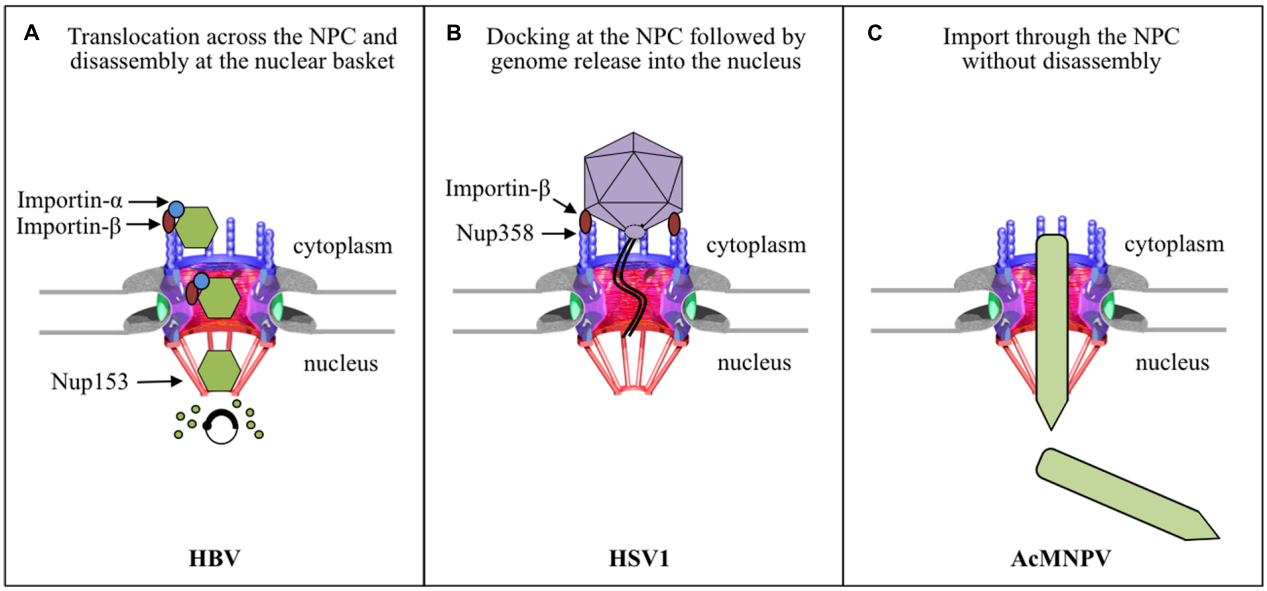


FIGURE 1 | Strategies used by enveloped viruses for nuclear entry of their genomes. (A) HBV capsids bind and cross the NPC in an importin-α and -β dependent manner. The capsid then binds directly to Nup153 at the nuclear basket, which somehow triggers disassembly of the capsid and nuclear entry of the viral genome. **(B)** The HSV1 capsid docks with a distinct orientation to the NPC cytoplasmic filament by binding to Nup358 in an importin-β and Ran dependent manner. The genome is delivered into the nucleus through the NPC by a unique mechanism of genome release through the capsid portal, and the empty capsid remains at the cytoplasmic side of the NPC. **(C)** The intact nucleocapsid of the baculovirus AcMNPV enters the nucleus through the NPC, but the roles of any viral or cellular components remain to be determined.

Due to the unavailability of an *in vitro* infection system that allows the study of the early steps in HBV infection, studies of nuclear import of HBV have used nuclear import assays with digitonin-permeabilized cells and *Xenopus* oocyte microinjection. Using the former assay and recombinant HBV capsids obtained by expressing the core protein in *Escherichia coli,* Kann et al. (1999) first demonstrated that these capsids bind to the NE of digitonin-permeabilized cells in the presence of cytosol and an energy-regenerating system, yielding a characteristic fluorescent rim staining of the nucleus only when the capsid is phosphorylated *in vitro* prior to the assay (Kann et al., 1999). The core protein has two classical NLSs at its arginine rich C-terminus (Yeh et al., 1990; Eckhardt et al., 1991; Li et al., 2010). Because the C-terminus is not exposed on the capsid (Zlotnick et al., 1997), it has been suggested that phosphorylation of the core protein exposes these NLSs, which then mediates the binding of the capsid to the NPC (Kann et al., 1999). In agreement with this, binding of the recombinant capsids to the NE of digitonin-permeabilized cells is inhibited by synthetic peptides containing these NLSs (Kann et al., 1999). Binding of the capsid to the NPC was confirmed by blocking the NPC with WGA and antibodies against Nups, and was reconstituted by performing the nuclear import assay in the presence of recombinant importin-α and β (Kann et al., 1999). Since in the semipermeabilzed cells the HBV capsid binds to the NPC without migration into the nucleus, EM of *Xenopus* oocytes microinjected with phosphorylated recombinant capsids was used to visualize where at the NPC the capsid binds (Pante and Kann, 2002). It was shown that HBV capsids not only bind to the NPC, but are able to cross the NPC intact, without disassembly (Pante and Kann, 2002; **Figure 2**). However, although these recombinant capsids are able to cross the NPC, they are unable to be released into the nucleus, and instead get arrested within the NPC nuclear basket (**Figure 2**).

In addition to the experiments with recombinant HBV capsids, further experiments were performed with isolated HBV capsids from cells transfected with a plasmid containing the HBV genome (Rabe et al., 2003). HBV contains a DNA genome, but replicates by means of an RNA pregenome (Seeger et al., 2013). Thus during infection, HBV capsids in the cytoplasm of infected cells have their genome at different stages of maturation (immature capsids), but only those with complete genome maturation (mature capsids) form progeny viruses that are released from the infected cell (Gerelsaikhan et al., 1996). Rabe et al. (2003) purified immature capsids from the cytosol of HBV-transfected cells and extracellular mature capsids, and used these in digitonin-permeabilized cells in combination with fluorescence *in situ* hybridization (FISH) to detect the viral genome. It was found that mature capsids enter the nucleus and are able to undergo genome uncoating, DNA nuclear import, and replication (Rabe et al., 2003). Immature capsids on the other hand, were able to bind to the NE of semipermeabilized cells, but did not show release of the immature genome (Rabe et al., 2003). Repeating the digitonin-permeabilized assay with mature capsids in the presence of recombinant importin-α and β, and Ran, it was determined that genome uncoating from the mature capsid is Ran-independent (Rabe et al., 2003). Using

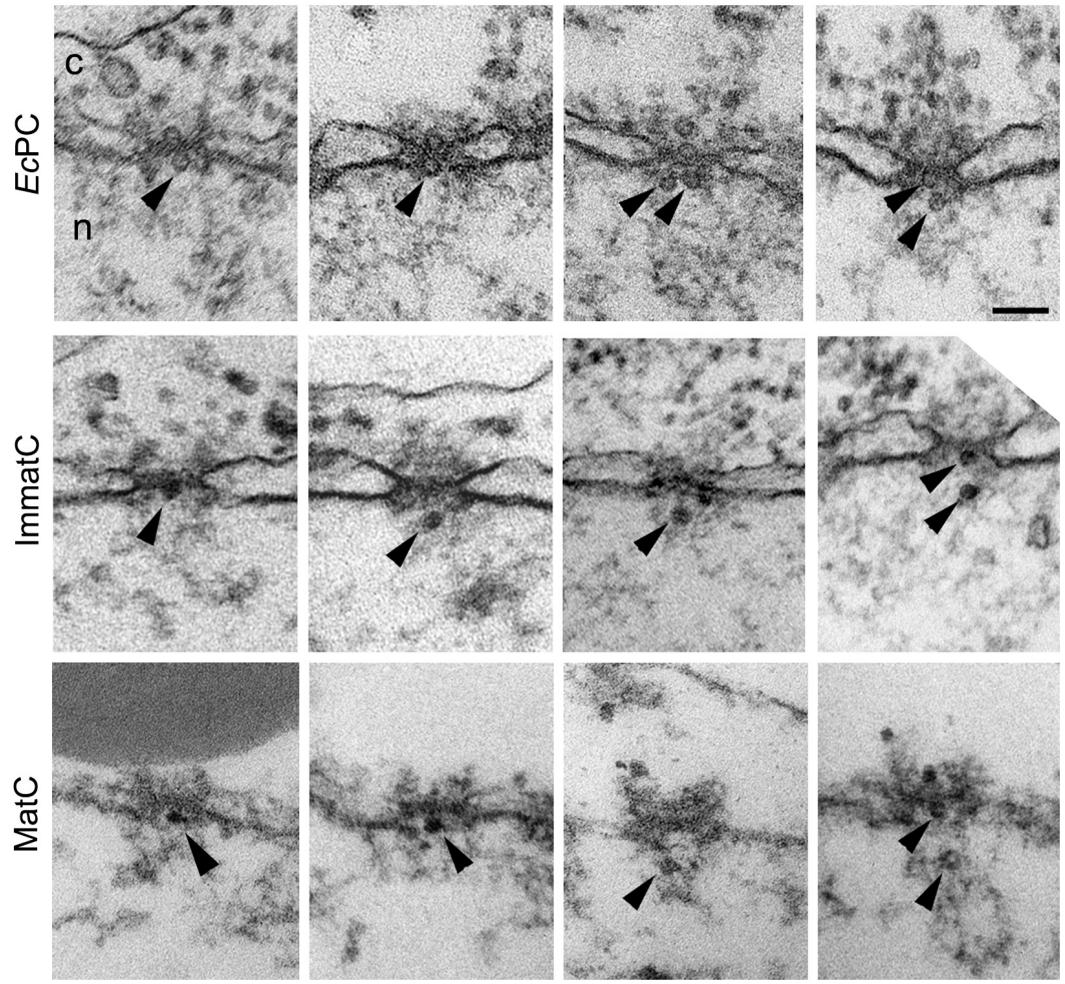


FIGURE 2 | Hepatitis B virus capsids cross the NPC intact and get arrested within the NPC nuclear basket, depending on genome maturation. Electron micrographs of NPC cross-sections from *Xenopus* oocytes microinjected with phosphorylated recombinant HBV capsids (*Ec*PC), HBV capsids containing the mature viral genome (MatC), or HBV capsids that did not contain the mature viral genome (ImmatC). While all three types of capsids cross the NPC intact, recombinant capsids and immature capsids remain arrested within the NPC nuclear basket, while mature capsids are released into the nucleus. Scale bar, 100 nm; n, nucleus; c, cytoplasm. Arrowheads point to capsids associated with the nuclear face of the NPC. Some of the micrographs have been reproduced with permission from Rabe et al. (2003).

the EM *Xenopus* nuclear import assay it was further demonstrated that, similar to the recombinant capsids, both the mature and immature capsids are translocated through the NPC into the nuclear basket; however, immature capsids remain arrested within the nuclear basket, while mature capsids are released into the nucleus (Rabe et al., 2003; **Figure 2**). These results reveal that HBV uses a well-coordinated strategy to deliver only mature viral DNA into the nucleus. Capsids containing immature viral genomes cross the NPC but are trapped at the NPC nuclear basket and are not released into the nucleus until the genomic double stranded DNA is completed. These results also show that HBV capsids are an exception from other nuclear import cargos that use the cellular transport receptors importin-α and β, in that the NPC translocation of HBV capsid is mediated by these importins but not by Ran.

To determine which components of the NPC are involved in HBV capsid retention at the NPC nuclear basket, and in capsid disassembly, Schmitz et al. (2010) performed immunoprecipitation assays with different type of capsids and rat liver nuclear extracts, and found that all type of capsids precipitated the nuclear basket protein Nup153 (Schmitz et al., 2010). Evidence of a direct interaction of the capsid with Nup153 was demonstrated by performing *in vitro* binding assays with purified capsids and recombinant Nup153, as well as with different fragments of Nup153 (Schmitz et al., 2010). Interestingly, binding of capsids is to the importin-β binding region of Nup153, implying that HBV capsids and importin-β compete for Nup153 binding (Schmitz et al., 2010). Indeed, competition experiments suggested that Nup153 is responsible for releasing importin-β from the capsids (Schmitz et al., 2010). Since all these were results from *in vitro* biochemical assays, the authors further

determined the involvement of Nup153 in capsid arrest at the nuclear basket by performing experiments with cells in which Nup153 has been partially depleted by RNAi, and found that a significant portion of recombinant phosphorylated capsids were no longer retained in the nuclear basket, but instead entered the nucleus of these cells (Schmitz et al., 2010). Furthermore, cross-linked mature capsids do not interfere with capsid binding to the NPC but prevent their entry into the nucleus (Schmitz et al., 2010). Therefore, based on these results the authors proposed a mechanism for capsid disassembly in the nuclear basket whereby mature HBV capsid uses importin-α and β to cross the NPC, where, upon reaching the nuclear basket, dissociation of the importins from the capsid allows it to directly bind to Nup153, with some core proteins binding all available Nup135 sites, resulting in capsid disassembly and release of the uncoated DNA and the remaining core proteins into the nucleus (Schmitz et al., 2010).

In summary, HBV capsids use the cellular nuclear import machinery (NPC, and importin-α and β) to deliver their genome into the nucleus, but in contrast to cellular cargo, the capsid is not released into the nucleus but binds directly to Nup153 at the nuclear basket, and this somehow triggers disassembly of the capsid and nuclear entry of the viral genome (**Figure 1A**). The disassembly step is genome-dependent: while mature capsids disassemble, immature capsids remain arrested in the nuclear basket. However, it remains to be determined how Nup153 triggers capsid disassembly and preferentially disassembles mature capsids instead of immature capsids.

Herpes Simplex Virus 1

Herpesviruses are a large family of viruses, of which HSV1 is the best characterized for its nuclear import strategy. HSV1 is an enveloped virus containing an icosahedral capsid (120 nm), a double-stranded DNA genome of 152 kbp, and a proteinacious layer (called the tegument) between the capsid and envelope (Roizman et al., 2013). HSV1 enters the host cell by either fusion of the viral envelope with the plasma membrane or endosomal membrane after endocytosis (reviewed by Campadelli-Fiume et al., 2012). The DNA-containing capsid and its surrounding tegument is then released into the cytoplasm, with some of the tegument proteins immediately dissociating from the capsid and others remaining more tightly bound to the capsid, and is transported along microtubules, using dynein, to the nuclear periphery (Sodeik et al., 1997; Dohner et al., 2002). The capsid with some of its tegument proteins then docks at the NPC, and this binding somehow triggers ejection of the genome from the capsid. The DNA is subsequently transported to the nucleus through the NPC and the capsid devoid of DNA (empty capsid) is left at the NPC cytoplasmic periphery and eventually is released into the cytoplasm (**Figure 1B**).

Several studies have shown that the HSV1 capsid binds to the NPC cytoplasmic filaments. These use HSV1 infected cells, *in vitro* binding assays with HSV1 capsids and isolated rat liver nuclei, and injection of HSV1 into *Xenopus* oocytes (Sodeik et al., 1997; Ojala et al., 2000; Shahin et al., 2006). Binding of purified

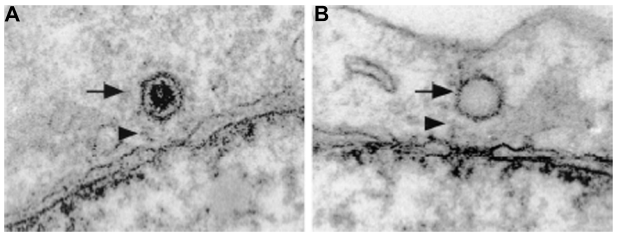


FIGURE 3 | Herpes simplex virus 1 capsids dock at the cytoplasmic side of the NPC. Electron micrographs of NE cross-sections from Vero cells infected with HSV1. Both DNA-containing capsids **(A)** and empty capsids **(B)** are located in close proximity to the NPC. HSV1 capsids bind to the NPC with a distinct orientation with one of the vertices facing the NPC. Arrows point to HSV1 capsids and arrowheads point to NPC cytoplasmic filaments. Reproduced with permission from Ojala et al. (2000).

HSV1 capsids to isolated nuclei is blocked by anti-Nups antibodies and by WGA, confirming the EM observations that the capsid docks to the NPC (Ojala et al., 2000). In cross-section EM pictures the capsid is depicted bound to NPCs at a distance of about 50 nm away from the center of the NPC and it has a distinct orientation with one of the vertices facing the NPC (**Figure 3A**; Sodeik et al., 1997; Ojala et al., 2000). It was thus speculated that the HSV1 capsid binds to the cytoplasmic filaments of the NPC. More recently, EM of cells infected with HSV1 clearly shows the binding of the capsid to the NPC cytoplasmic filament (Bauer et al., 2013). In order to determine the specific NPC binding partners, Copeland et al. (2009) performed experiments with cells either preloaded with an anti-Nup358 antibody or depleted of Nup358 by RNAi and found reduced capsid binding to the NPC of HSV1 infected cells (Copeland et al., 2009). Reduced capsid binding to the NPC was also reported in cells depleted of Nup214 by RNAi (Copeland et al., 2009; Pasdeloup et al., 2009), but an antibody against Nup214 did not inhibit this binding (Copeland et al., 2009). Thus, incoming HSV1 capsids dock at the NPC is via binding to Nup358 and possibly Nup214, which are components of the NPC cytoplasmic filaments.

Two studies have addressed the cellular factors and viral proteins involved in the binding of the HSV1 capsid to the NPC (Ojala et al., 2000; Copeland et al., 2009). To determine cellular factors, Ojala et al. (2000) first performed *in vitro* binding assays with purified capsids and isolated nuclei in the presence or absence of cytosol, in the presence of anti-importin-β antibodies, or in the presence or absence of recombinant importin-β, and determined that capsid binding to the NPC is importin-β dependent. Moreover, performing similar experiments but adding RanQ69L, a dominant-negative mutant of Ran unable to hydrolyze GTP, it was found that the docking of the capsid to the NPC is also Ran dependent (Ojala et al., 2000). The purified capsids used in these assay were associated with tegument proteins; therefore, to test whether teguments proteins were involved in the docking of the capsid to the NPC, Ojala et al. (2000) performed an *in vitro* binding assay with trypsin-treated capsids, which have the tegument proteins removed, and found reduced binding of the trypsin-treated capsids to the NPC (Ojala

et al., 2000). More recently, Copeland et al. (2009) found that microinjection of antibodies against the tegument protein VP1/2 (UL36) into cells prior to HSV1 infection inhibits the binding of the HSV1 capsid to the NPC, and Pasdeloup et al. (2009) showed that the minor capsid protein UL25 immunoprecipitates Nup214. Taken together, these data suggest that HSV1 capsid docking at the NPC is importin-β- and Ran-mediated, and involves the tegument protein VP1/2 and the capsid protein UL25. However, it remains undetermined whether VP1/2 and UL25 binds to importin-β directly.

The next step in the nuclear transport mechanism for the HSV1 genome is the ejection of the DNA from the capsid. In contrast to the capsid docking at the NPC step, less is known about this step. Initial studies showed that empty capsids are associated with the NPC (**Figure 3B**), presumably after DNA release (Sodeik et al., 1997), and that DNA release from the capsid requires the presence of cytosol and energy (Ojala et al., 2000). Although the exact trigger for DNA release has yet to be determined, Nup214 has been hypothesized as the possible cue for this because Nup214 depletion by RNAi results in a delay of viral genome delivery into the nucleus (Pasdeloup et al., 2009). In terms of which viral proteins are required, the involvement of the tegument protein VP1/2 in DNA release has long been known since a HSV1 temperature-sensitive mutant with a single amino acid change on VP1/2 (Abaitua et al., 2011) binds to the NPC, but does not release its DNA at the non-permissive temperature (Batterson et al., 1983). Furthermore, proteolytic cleavage of VP1/2 is required for the release of the DNA into the nucleus (Jovasevic et al., 2008). The capsid protein UL25 is also involved in facilitating nuclear import of the HSV1 genome, as overexpression of UL25 impairs DNA nuclear entry by competing with the UL25 protein in the virus (Rode et al., 2011). Interestingly, an *in vitro* EM study of HSV1 DNA uncoating demonstrated that DNA release occurs through the capsid portal (Newcomb et al., 2001), a ring structure formed by 12 copies of the tegument protein UL6 located at one of the capsid vertices (Trus et al., 2004; Cardone et al., 2007). Although the current data does not provide a full picture for the DNA release step, the evidence suggests that the interactions between VP1/2 and Nup358 first bring the capsid and NPC closer together, leading to an interaction between UL25 and Nup214, which then ultimately leads to the ejection of the viral DNA through the capsid portal.

How the HSV1 DNA translocates through the NPC is also unclear. However, based on the similarities of the mechanism of viral genome packing between HSV1 and bacteriophages it has been hypothesized that translocation through the NPC does not depend on NTRs, but instead the DNA is expelled from the capsid by pressure, and this pressure is the force that propels the DNA through the NPC (reviewed by Liashkovich et al., 2011). In support of this hypothesis it has been shown that similar to bacteriophages, the DNA end that is packed last, is the first to be ejected, and immediately transcribed (Newcomb et al., 2009). The DNA has also been visualized by atomic force microscopy as a condensed rod-like structure coming out of the nuclear basket (Shahin et al., 2006), thus possibly the ejection force makes the DNA cross the NPC as a rod-like structure. Moreover, by osmotically suppressing DNA release from HSV1 capsids, Bauer et al. (2013) showed for the first time that capsids have high internal pressure. Thus, it is possible that the pressure-driven DNA ejection pushes the DNA through the NPC.

In summary, HSV1 uses a unique mechanism to deliver its genome into the nucleus, which involves capsid binding to the NPC, DNA release from the capsid through the capsid portal, and transport of the DNA through the NPC possible by the pressure of DNA ejection from the capsid (**Figure 1B**). Much is left to be determined in this mechanism, including which viral proteins interact with importin-β, the exact trigger for genome release, and the exact mechanism for DNA translocation through the NPC.

Baculoviruses

Baculoviruses are rod-shaped (30–60 × 250–300 nm), enveloped viruses with circular double-stranded DNA genomes ranging in size from 80 to 180 kbp that infect insects and other arthropods (Rohrmann, 2013). Baculoviruses are unique compared to other virus families because they have two infectious forms: the occlusion-derived virion (ODV), comprising enveloped virions embedded within a crystalline matrix of protein, and the budded virus (BV), comprising a capsid containing the genome (referred to as the nucleocapsid) surrounded by a membrane envelope acquired by budding at the plasma membrane (Friesen, 2007; Rohrmann, 2013). ODVs are involved in virus transmission between insect larvae and the BV is the infection form responsible for cell-to-cell transmission within the insect and in tissue culture cells. Although both the ODV and BV forms contain nucleocapsids enclosed within envelopes of different origins, it is the nucleocapsid that eventually gets released into the cytoplasm, moves within the cytoplasm using actin-polymerization, and delivers the genome into the nucleus. Despite the popular use of baculoviruses as protein expression vectors in biomedical research and its potential to be developed as a biopesticide, the detailed molecular mechanism by which this virus delivers its genome into the nucleus remains to be established.

Baculoviruses are classified based on their DNA sequence into four genera: *Alpha-*, *Beta-*, *Gamma-*, *and Deltabaculovirus* (Jehle et al., 2006; Herniou et al., 2011). *Autographa californica* nucleopolyhedrovirus (AcMNPV), the archetype species of the *Alphabaculovirus* genus, is the most studied baculovirus and the most commonly used viral vector for protein expression. In tissue culture cells, budded virions of AcMNPV enter the cell by endocytosis, and the low pH of endosomes triggers a conformational change in the viral fusion protein GP64, resulting in fusion of the viral and endosomal membranes, releasing the nucleocapsid into the cytoplasm (Rohrmann, 2013). In the cytoplasm, the WASP-like protein VP78/83, located at one end of the nucleocapsid, induces polymerization of actin, which then looks like a comet tail that propels the baculovirus toward the cell nucleus (Goley et al., 2006; Ohkawa et al., 2010). An earlier EM study using tissue cultured insect cells infected with AcMNPV found intact nucleocapsids in the nucleus [(Carstens et al., 1979), reviewed by (Au et al., 2013)]. However, it was not

clear whether the nucleocapsids entered the nucleus at mitosis during NE breakdown. EM of larvae infected with AcMNPV also revealed intact nucleocapsids in the nucleus and associated with the NPC (Granados and Lawler, 1981), but it was unclear whether these were newly synthesized nucleocapsids. These caveats were addressed in a study using non-dividing mammalian cells infected with AcMNPV, which revealed nucleocapsids both in the nucleus and at the cytoplasmic side of the NPC (van Loo et al., 2001). These studies indicate that the intact nucleocapsid of AcMNPV enters the nucleus through the NPC (**Figure 1C**).

Two recent studies, one using fluorescently labeled AcMNPV and fluorescence microscopy (Ohkawa et al., 2010), and the second using the *Xenopus* oocyte nuclear import assay (Au and Pante, 2012), convincingly demonstrated that indeed the nuclear entry mechanism of the AcMNPV nucleocapsid involves the NPC. To determine that the NPC is the route of nuclear entry of the AcMNPV nucleocapsid, Ohkawa et al. (2010) used two experimental approaches: infection of cells pre-injected with WGA and infection of cells that expressed a dominant-negative mutant of importin-β, which binds to the NPC and blocks nuclear import (Gorlich et al., 1996). Both experiments yielded significantly less nucleocapsids in the nucleus than in the control untreated cells, but still few nucleocapsids were found in the nucleus (Ohkawa et al., 2010). Microinjection of *Xenopus* oocytes with purified AcMNPV nucleocapsids in combination with EM and electron tomography showed nucleocapsids vertically traversing the NPC and midway through the NPC (Au and Pante, 2012; **Figure 4**). At early time of injection the nucleocapsids were interacting with the NPC cytoplasmic filaments, and at late time of infection they were found in the nucleus. Furthermore, injecting WGA into the oocytes before injection of the nucleocapsid did not yield nucleocapsids in the nucleus, confirming that the only route for nuclear entry of the nucleocapsid is through the NPC (Au and Pante, 2012).

Although there is much experimental evidence demonstrating that intact AcMNPV nucleocapsids enter the nucleus through the NPC, early EM studies of *Betabaculovirus*-infected larvae revealed nucleocapsids docking at the NPC at different stages of releasing their genome, but not in the nucleus [(Summers, 1969, 1971), reviewed by Au et al., 2013]. This suggests a mechanism of DNA nuclear import similar to that used by HSV1, which attaches to the cytoplasmic side of the NPC and ejects its nucleic acid into the nucleus through the NPC, leaving empty capsids at the NPC.

In summary, viruses from the large *Baculoviridae* family can use different mechanisms for delivering their genome into the nucleus. Two mechanisms have been unveiled thus far. The first, used by *Alphabaculoviruses*, indicates that the intact nucleocapsid enters the nucleus through the NPC (**Figure 1C**). The second, used by *Betabaculoviruses*, involves docking of the nucleocapsid at the NPC, followed by ejection of the nucleic acid through the NPC leaving an intact empty capsid at the cytoplasmic side of the NPC. However, for both types of mechanisms further studies are required to determine the cellular and viral components involved.

Nuclear Entry of Non-Enveloped DNA Viruses

Non-enveloped viruses in general enter their host cells through an endocytic pathway and must escape from endosomes into the cytoplasm, or they risk degradation in the host lysosome. Viruses such as, adenoviruses and parvoviruses, have enzymes in their capsids that become exposed or active in the acidic environment of endosomes, ultimately leading to the disruption of endosomal membranes and the release of the virus into the cytoplasm. SV40 on the other hand, has a unique strategy for cytoplasmic release: instead of escaping from endosomes, it enters the ER, from which it travels to the cytoplasm or possibly to the nucleus. Interestingly, HPV either requires the acidic endosomes in order to escape to the cytoplasm or is routed to the Golgi in order to then travel to the nucleus. As summarized in **Figure 5**, although these four non-enveloped viruses may share some structural features or similar entry pathways, each virus uses a distinct mechanism for the delivery of its genome into the nucleus.

Adenoviruses

Adenoviruses are one of the largest (90–100 nm) and most complex non-enveloped viruses. They contain a double-stranded linear DNA genome of about 36 kbp that encodes more than 40 proteins, but only 13 of these are found in the virion (Benevento et al., 2014). The DNA is condensed by viral proteins, V, VII, and

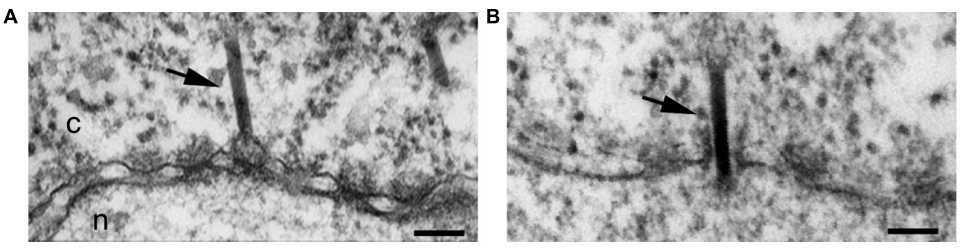


FIGURE 4 | Baculovirus AcMNPV capsids cross the NPC intact. Electron micrographs of NE cross-sections from *Xenopus* oocytes microinjected with baculovirus AcMNPV capsids. Intact capsids (arrows) are detected docked at the NPC prior to nuclear import **(A)** and midway through the NPC **(B)**. Scale bars, 100 nm; n, nucleus; c, cytoplasm. Arrows point to capsids. Reproduced with permission from Au et al. (2013).

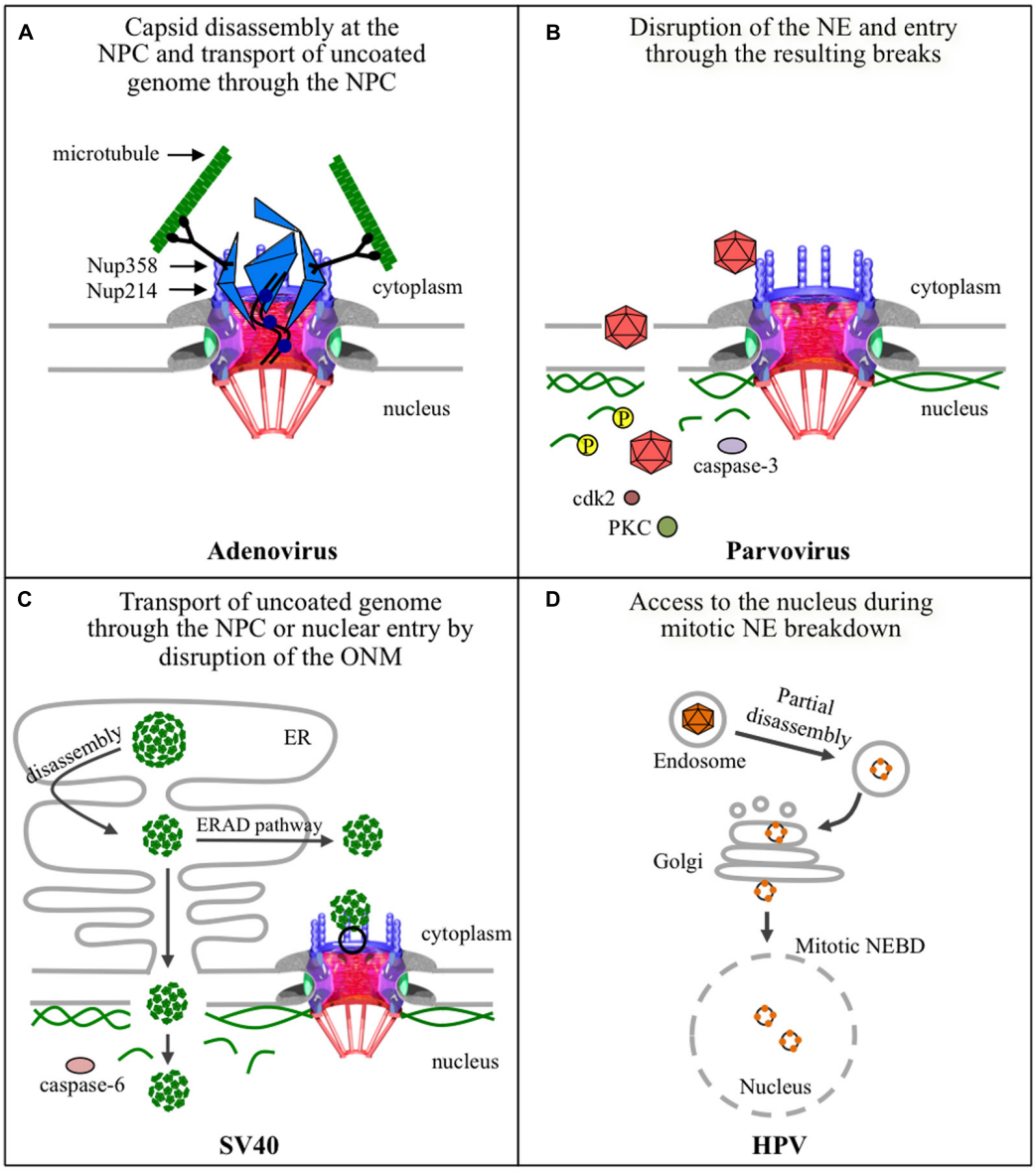


FIGURE 5 | Strategies used by non-enveloped viruses for nuclear entry of their genomes. (A) The adenovirus capsid docks at the NPC via binding of hexon protein to Nup214, and uses Nup358-bound kinesin-1 via its heavy chain to completely disassemble the capsid that is also bound to kinesin-1, via its light chain, and deliver the uncoated genomes into the nucleus through the NPC. Kinase-1 binds to the capsid via its light chains and to Nup358 via its heavy chains. Movement of kinase-1 along microtubules exerts a pulling action that disassembles the capsid. **(B)** Parvoviruses enter the nucleus by transiently disrupting nuclear membranes, by a yet unknown mechanism, and disassembling the nuclear lamina. The latter involves phosphorylation of lamin A/C by PKC and cdk2, and cleavage of lamin-B by caspase-3. **(C)** SV40 partially disassembles inside the endoplasmic reticulum (ER) and the subviral particles could follow two different pathways to deliver the genome into the nucleus. The first involves exit of the subviral particle into the cytoplasm using the cellular ERAD pathway and cellular chaperones, disassembly at the NPC, and import of the uncoated genome through the NPC. The second is directly from the ER to the nucleus by direct disruption of the inner nuclear membrane and underlying nuclear lamina using caspase-6. **(D)** HPV partially disassembles in endosomes, and a possible route through the Golgi apparatus, and gains access to the nucleus during mitosis when the NE breaks down.

X, forming a nucleoprotein core that is packed into an icosahedral capsid of about 90 nm in diameter (Berk, 2013). The most abundant protein of the capsid is the hexon protein (protein II), with 720 copies in each virion (Benevento et al., 2014). Although there are over 57 human adenovirus serotypes, the most studied are serotype 2 and 5. One unique feature of adenovirus is the presence of fibers protruding from the 12 vertices of the capsid. For cell entry, the knobs at the end of these fibers attach to cellular receptors in the plasma membrane of host cells; this is followed by clathrin-mediated endocytosis of the virus and escape from endosomes by the lytic activity of protein VI found inside the capsid (reviewed by Leopold and Crystal, 2007; Smith et al.,

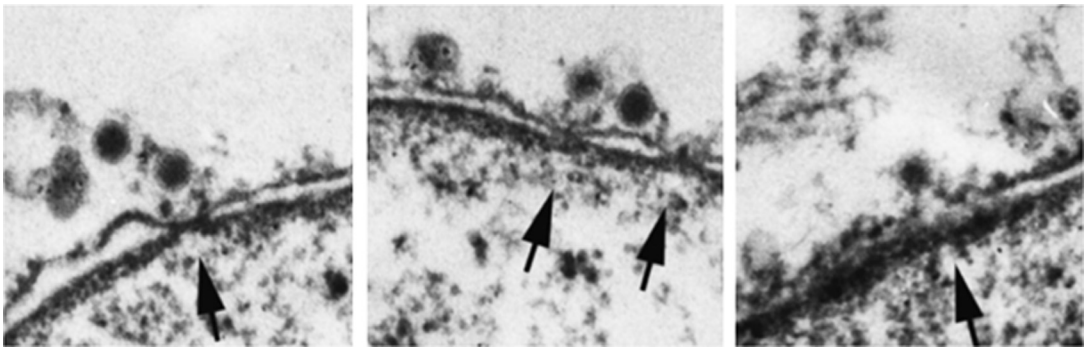


FIGURE 6 | Adenovirus capsids dock at the NPC cytoplasmic face before disassembly. Electron micrographs of NE cross-sections from digitonin-permeabilized cells incubated with adenovirus capsids and cytosol. Arrows point to NPCs. Reproduced with permission from Saphire et al. (2000).

2010). During cellular uptake the fibers dissociate from the capsid (Greber et al., 1993; Nakano et al., 2000), which disassembles further in the endosome and during endosomal escape (reviewed by Suomalainen and Greber, 2013). After being released into the cytoplasm, the partially disassembled capsids are transported along microtubules to the nuclear periphery where they dock at NPCs (reviewed by Leopold and Crystal, 2007; Smith et al., 2010). Capsid binding to the NPC (**Figure 6**) results in complete capsid disassembly and subsequent nuclear import of the viral genome (**Figure 5A**).

Docking of the partially disassembled adenovirus capsid at the NPC is via the binding of hexon protein to the NPC cytoplasmic filament protein Nup214. This was first demonstrated by Trotman et al. (2001), who used an *in vitro* binding assay incubating isolated nuclei or purified NE with adenovirus capsids or purified adenovirus proteins. In this assay neither cytosol nor importin-α/β are required for the binding of the adenovirus capsid to purified NE. To show that the NPC was involved in the *in vitro* binding of the capsids to the NE, experiments were performed in the presence of several anti-Nup antibodies, and it was found that antibodies against Nup214 block the binding of the adenovirus capsid to purified NE (Trotman et al., 2001). Furthermore, detection of hexon protein (to assess capsid disassembly) by immunofluorescence and viral DNA by FISH (to detect the viral genome) in cells microinjected with anti-Nup214 antibodies and infected with adenovirus showed that these antibodies prevent capsid disassembly and nuclear import of the adenoviral genome (Trotman et al., 2001). Binding of partially disassembled adenovirus capsid and purified hexon to Nup214 has recently been confirmed, using the nuclear import assay with digitonin-permeabilized cells depleted of Nup14 by RNAi (Cassany et al., 2015). These authors also studied adenovirus DNA nuclear import combining the digitonin-permeabilized assay with FISH, and found that Nup214, but not Nup358, was required for binding of adenovirus to the NE, for capsid disassembly, and for efficient nuclear entry of the adenovirus genome (Cassany et al., 2015). Moreover, these authors narrowed down the Nup214 binding site to a 137-amino-acid segment in the N-terminal domain of Nup214 by performing several experiments, including rescue experiments with the digitonin-permeabilized Nup214-depleted cells in the presence of several fragments of recombinant Nup214, adenovirus infection of cells overexpressing different fragments of Nup154, and *in vitro* binding assays with purified hexon and recombinant Nup214 fragments.

The disassembly of adenovirus capsids at the NPC has been shown to be mediated by kinesin-1 and to require the NPC cytoplasmic filament protein Nup358 (Strunze et al., 2011). Indirect immunofluorescence microscopy yields cytoplasmic co-localization of Nup358, Nup214, and Nup62 with disassembled virus particles 3 h post-infection (Strunze et al., 2011). Co-localization of Nups with disassembled capsids was also observed at the cell periphery of adenovirus-infected cells. To explain how the disassembled capsids, which are too large to diffuse through the cytoplasm, reach the cell periphery, Strunze et al. (2011) performed immunolocalization of the anterograde microtubule motor kinesin-1, and found that cytoplasmic disassembled capsids, detected with an antibody against disrupted but not intact capsids, indeed co-localize with kinesin-1 light chain in infected cells. Moreover, kinesin-1 light chain was also detected at the cytoplasmic periphery of the NPC together with capsids by immuonogold, and both overexpression of the C-terminus of kinesin-1 light chain, which is involved in binding to the transported cargo, and RNAi-mediated knockdown of kinesin-1 light chain reduce the amount of disassembled capsids found in the cytoplasm and adenovirus infection (Strunze et al., 2011). Further biochemical experiments demonstrated direct binding of the kinesin-1 light chain with adenovirus protein IX (Strunze et al., 2011). Since kinesin-1 heavy chain interacts with Nup358 (Cai et al., 2001), Strunze et al. (2011) studied adenovirus infection in both cells depleted of Nup358 by RNAi and cells overexpressing the kinesin-1 heavy-chain, which binds to Nup358 but not to the adenovirus capsid, and found that both conditions reduce adenovirus infection. Thus, it was proposed that disassembly of the adenovirus capsid at the NPC is mediated by the pulling action of kinesin-1, which is bound to Nup358 through its heavy chains and to the Nup214-docked capsid via its light chains (Strunze et al., 2011), when it moves along microtubules.

In addition to the displacement of Nups, Strunze et al. (2011) also found that the NPC permeability of adenovirus-infected cells increases; infection of cells that had been microinjected with large fluorescent dextrans, which are normally excluded from the nucleus, yielded nuclear localization of the dextran at 3 h post-infection (Strunze et al., 2011). Thus, they proposed that, in addition to disassembling the capsid, the movement of the Nup358- and capsid-bound kinesin-1 along microtubules also dissociates Nups from the NPC resulting in an increase of the NPC permeability (Strunze et al., 2011). The authors suggest that this increase in the NPC permeability may facilitate entry of the uncoated viral DNA into the nucleus (Strunze et al., 2011). However, several cellular factors and NTRs are involved in the nuclear import of the adenoviral genome, including hsp70, histone H1, importin-β, importin-7, and transportin-1 (Saphire et al., 2000; Trotman et al., 2001; Hindley et al., 2007). As these NTRs are known to bind to proteins and not to nucleic acids, and since adenoviral DNA is associated with several viral core proteins, the major core protein VII has been proposed as an adaptor between the viral genome and the NTRs (Wodrich et al., 2006). Indeed, transportin-1 mediates the nuclear import of recombinant mature protein VII in the digitonin-permeabilized cell nuclear import assay (Hindley et al., 2007). Thus, most likely the adenovirus genome enters the nucleus as a nucleoprotein using several NTFs, and the increased permeability of the NPC may speed this process.

In summary, adenovirus docks at the NPC by binding of hexon protein with the N-terminal domain of Nup214. Once docked at the NPC the capsid binds to the light chains of kinesin-1, which also binds Nup358 via its heavy chains. Movement of kinesin-1 along microtubules leads to capsid disassembly and NPC disruption through the pulling action of kinesin-1 (**Figure 5A**). Subsequently the uncoated viral genome associated with viral core proteins enters the nucleus through the NPCs that have increased permeability using several NTRs. More studies are needed to determine the actual mechanism of translocation of the viral DNA through the NPC.

Parvoviruses

Parvoviruses are the smallest DNA animal virus, consisting of a single-stranded DNA genome of about 5 kb that is protected by a icosahedral capsid of 18–26 nm in diameter (reviewed by Berns and Parrish, 2013). The capsid is assembled from 60 copies of two (or three in some parvoviruses) size variants of the structural proteins VP1 and VP2, which are identical, except for the unique N-terminal sequence (140 amino acids) of VP1 (reviewed by Parrish, 2010). Parvoviruses in general use receptor-mediated endocytosis and escape from endocytic compartments into the cytoplasm by means of the enzymatic action of a phospholipase A2 (PLA2) motif in the unique region of VP1 (reviewed by Vihinen-Ranta and Parrish, 2006; Cotmore and Tattersall, 2007). Due to their small size, it was initially thought that parvoviruses enter the nucleus via the NPC. EM studies using the parvovirus minute virus of mice (MVM), however, have shown no evidence of entry through the NPC. Instead, a novel nuclear entry mechanism involving small disruptions of the NE was identified (**Figure 5B**; Cohen and Pante, 2005; Cohen et al., 2006).

Several studies using different parvoviruses and different experimental approaches have indicated that parvoviruses use an NPC-independent nuclear entry mechanism. A first study used incubation of purified nuclei with adeno-associated virus type 2 (AAV2) and found the virus in the nucleus even when the experiments were performed in the presence of WGA or antibodies against Nups, which both block the NPC (Hansen et al., 2001). Subsequent *Xenopus* oocyte import assays using MVM showed virions in the perinuclear space along with small (100–300 nm) disruptions of the NE (Cohen and Pante, 2005; Cohen et al., 2006; **Figure 7**). These NE disruptions were still seen even with WGA blocking the NPC, suggesting that disruptions in the NE are independent of the NPC (Cohen and Pante, 2005). Similar results were found by EM of MVM-infected cells, which also revealed virions in the perinuclear space and alterations in nuclear morphology (Cohen et al., 2006). Moreover, *Xenopus* oocyte import assays with other parvoviruses including canine parvovirus (CPV; Cohen et al., 2011a) and rat parvovirus H1 (Porwal et al., 2013) yielded similar results, indicating a possible conserved mechanism for nuclear entry among the parvovirus family. In addition to EM detection of NE disruptions, immunofluorescence microscopy and western blot analyses of MVM-infected cells show that the nuclear lamina immunostaining is disrupted (Cohen et al., 2006) and that lamin-B is cleaved (Cohen et al., 2011b). Interestingly, infected fibroblast cells show intact nuclear lamina by 21 h post-infection (Cohen et al., 2006, 2011b), indicating that the MVM-induced NE disruptions are transient. More recently, Porwal et al. (2013) developed a confocal microscopy method to quantifying chromatin release from the nuclei of digitonin-permeabilized cells that were incubated with several parvoviruses, including AAV2, CPV, and H1. This study confirmed that parvoviruses cause NE disruptions. Interestingly, AAV2 and H1 seem to differ in their mechanism for NE disruption, since for AAV2 but not H1, NE disruption was limited to virions that were exposed to pH 5.2 (Porwal et al., 2013).

More recently, scientist have tried to identify the mechanism by which parvovirus disrupt the NE. Since the only lipolytic enzyme of parvoviruses is the PLA2 activity found in the unique region of VP1, Cohen et al. (2011b) microinjected *Xenopus* oocytes with a mutated MVM for PLA2 activity (H42R, Farr et al., 2005) or with MVM treated with a drug that inhibits the PLA2 activity, and found NE disruptions in both experiments (Cohen et al., 2011b), indicating that the viral PLA2 is not responsible for MVM-induced NE disruption. Similar conclusions were drawn from the results of experiments performed by incubating digitonin-permeabilized cells with an AAV2 mutant with an inactivated PLA2 motif (Porwal et al., 2013). Since an enzyme of the virus did not cause the NE disruptions, attention was turned to cellular factors implicated in NE breakdown in cellular processes such as apoptosis and mitosis. Cohen et al. (2011b) adapted an *in vitro* NE breakdown assay (Muhlhausser and Kutay, 2007), which uses semipermeabilized cells expressing GFP-lamina-associated polypeptide 2β (GFP-LAP2β) as a NE marker, to screen for

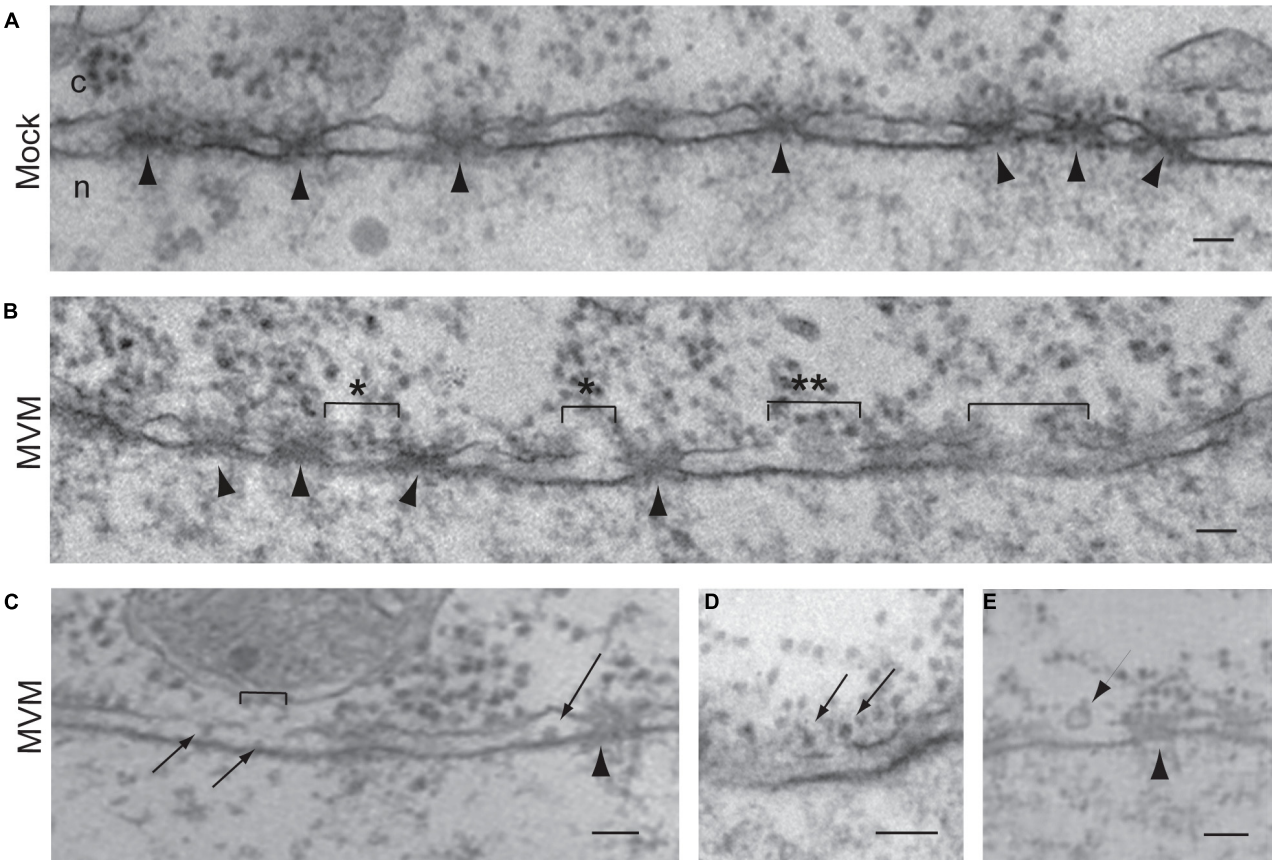


FIGURE 7 | Parvoviruses cause disruption of the NE for nuclear import. Electron micrographs of NE cross-sections from *Xenopus* oocytes mock-injected **(A)** or injected with the parvovirus MVM **(B–E)**. Arrowheads point to NPCs. Brackets indicate breaks in the NE caused by MVM, which are often found close to NPCs (indicated by *). Virions are found close to the breaks (**B**, indicated by **), associated with the outer nuclear membrane (**D**, arrows), and in the perinuclear space (**C**, arrows). Small vesicles are found close to the breaks (**E**, arrow). Scale bars, 100 nm; n, nucleus; c, cytoplasm. Reproduced with permission from Cohen and Pante (2005).

the effect of caspases inhibitors in MVM-induced NE disruption. In this assay, the permeability of the NE is measured as a nuclear influx of a fluorescently labeled 155-kDa dextran, which is too large to diffuse through the NPCs (Muhlhausser and Kutay, 2007). It was found that semipermeabilized cells showed a lack of dextran influx into the nucleus in the presence of caspase inhibitors and MVM, with the highest effect seen using a caspase-3 inhibitor (Cohen et al., 2011b). Subsequent studies co-injecting *Xenopus* oocytes with MVM and caspase inhibitors, and infecting cells with MVM in the presence of caspase inhibitor led to the conclusion that caspase-3 is involved in the unusual MVM nuclear entry mechanism (Cohen et al., 2011b). The role of caspase-3 is most likely in the proteolytic cleavage of lamin-B and not in the direct disruption of the nuclear membranes.

Similar studies using digitonin-permeabilized cells with their nucleus preloaded with a fluorescent 100 kD cargo, and measuring the nuclear fluorescence over time after incubation of the cells with the parvovirus H1 and inhibitors of mitotic enzymes involved in NE breakdown concluded that protein kinase C (PKC) and cyclin-dependent kinase 2 (cdk2) are involved in the mechanism of NE disruption induced by parvoviruses (Porwal et al., 2013). Furthermore, it was shown that PKC and cdk2 were activated with the addition of H1 (Porwal et al., 2013). Thus, it was proposed that the activated PKC phosphorylates lamin A/C, which activates cdk2 (further activated by caspase-3), leading to hyper phosphorylation of lamin A/C and disassembly of the nuclear lamina (Porwal et al., 2013). Thus, again the involvement of these enzymes explains the disassembly of the nuclear lamina that occurs during parvovirus infection, but not how the virus disrupts the nuclear membranes.

It has been proposed that although parvoviruses do not cross the NPC, they might interact with the NPC to cause NE disruption. This is based on indirect evidence for an interaction between Nups and parvoviruses: both H1 and AAV precipitate several Nups from a purified preparation of Nups (Porwal et al., 2013). Moreover, plugging the NPC with recombinant HBV capsids that attach to the nuclear basket without being released into the nucleus, inhibits H1-induced NE disruption (Porwal et al., 2013), arguing that the NPC is needed for parvovirus to cause NE ruptures. However, when the NPCs are blocked with WGA, H1-induced NE disruption was still present (Porwal et al., 2013).

Thus, a direct interaction between parvovirus and the NPC during virus infection, and whether the NPC plays any role in the unusual mechanism of parvovirus nuclear entry, remain to be established.

A recent study using recombinant AAV2 (rAAV2) proposes that this virus enters the nucleus through the NPC (Nicolson and Samulski, 2014). This study found that blocking the NPC by microinjection of WGA into tissue culture cells partially inhibited the nuclear import of Cy5-labeled rAAV2 particles. This is in contrast to results with wild-type AAV2, for which WGA completely blocks the nuclear import of the virus (Hansen et al., 2001). The difference may be due to exposure of putative NLSs in recombinant but not wild-type virus. Several potential NLSs are located in the capsid proteins of AVV2 (Grieger et al., 2006) and other parvoviruses (Vihinen-Ranta et al., 1997; Lombardo et al., 2000; Pillet et al., 2003). However, since newly synthesized capsid proteins must reach the nucleus to permit assembly of new virus particles, these NLSs may be involved in the nuclear import of newly synthesized capsid proteins and not in the nuclear import of intact capsids. More studies are needed to explain why rAAV2 and wild-type AAV2 use different mechanisms for nuclear entry.

In summary, parvoviruses enter the nucleus by transiently disrupting nuclear membranes (**Figure 5B**), by a yet unknown mechanism that possibly involves Nups, followed by phosphorylation of lamin A/C by PKC and cdk2, and cleavage of lamin-B by caspase-3, ultimately causing disassembly of the nuclear lamina and nuclear entry of the virus. The potential contributions of Nups and the mechanism for NE disruption are two aspects that deserve more investigations.

Simian Virus 40

Simian virus 40, the prototype of the *Polyomaviridae* family, is a small (45 nm in diameter) non-enveloped virus with a circular double-stranded DNA genome of about 5 kbp (DeCaprio et al., 2013). The genome is wrapped with cellular histone into a minichromosome, which is enclosed by the icosahedral capsid composed of 72 VP1 pentamers that are stabilized by interpentamic disulfide bonds (DeCaprio et al., 2013). Beneath the VP1 outer shell are the hydrophobic VP2 and VP3, which are not exposed on the virus surface. To enter the host cell, SV40 binds to gangliosides on the cell surface and is internalized in vesicles that then fuse with the ER delivering the virus to the ER lumen, where the capsid begins to disassemble using host disulfide isomerases (reviewed by Inoue and Tsai, 2013). From the ER, two nuclear entry pathways for SV40 have been described: one that involves the NPC after the virus escapes from the ER to the cytoplasm and another that is through direct disruption of the inner nuclear membrane from the ER lumen to the nucleus (**Figure 5C**).

There are several lines of evidence that provide support for both nuclear entry pathways. An initial study, using cells microinjected with SV40, found that blocking the NPC by co-injection of WGA or antibodies against Nups prevents nuclear accumulation of VP1 and the viral large T-antigen (early gene product), suggesting that the nuclear entry process is NPC-dependent (Clever et al., 1991). Similar microinjection experiments combined with EM depicted virion at the NPC (Yamada and Kasamatsu, 1993; **Figure 8**). Because microinjection bypasses the normal entry route of the virion, it was not clear whether the virus crosses the NPC during an actual infection. Subsequently, it was demonstrated that when antibodies against the viral capsid protein VP2/3 are microinjected into the cytoplasm of SV40-infected cells there is a lack of nuclear accumulation of the viral T-antigen (Nakanishi et al., 1996), demonstrating that during an infection the virus enters the nucleus from the cytoplasm and suggesting that VP2/3 contains NLSs mediating the nuclear import of the virion. Indeed, VP3 contains an NLS that can bind to importin-α/β (Nakanishi et al., 2002). The role of this NLS in the nuclear import of the viral genome was tested in experiments using mutated virus-like particles that were formed from NLS defective VP3 in which all basic residues of the NLS were altered (Nakanishi et al., 2002). In these experiments cells infected with wild-type or VP3 mutated virus-like particles were subcellular fractionated at different hours post-infection, and the amount of full-length viral DNA accumulated in the cytoplasmic and nuclear fractions were measured. It was found that the DNA of the wild-type virus accumulates in the nucleus, whereas that of the VP3 mutated virus-like particles do not (Nakanishi et al., 2002). Similarly, virus particles that do not contain the coding region of VP2/3 do not transport their DNA into the nucleus (Nakanishi et al., 2007). Since VP2/3 is not found on the virus surface, it was important to determine whether VP2/3 is exposed after cell entry in order to

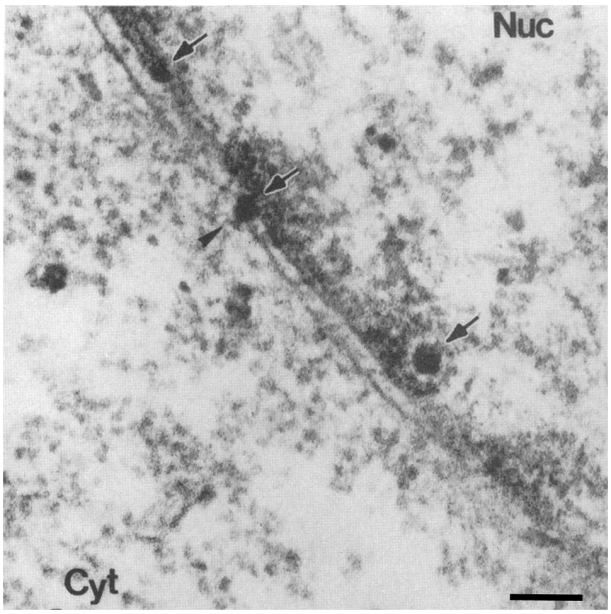


FIGURE 8 | Simian virus 40 is depicted at the NPC after microinjection. Electron micrographs of a cross-section through the NE of a cell injected with SV40. Scale bars, 100 nm; Nuc, nucleus; Cyt, cytoplasm. Arrows point to virions, arrowhead points to a virion at the NPC. Reproduced with permission from Yamada and Kasamatsu (1993).

then mediate nuclear entry. Co-immunoprecipitation experiments showed that the virion undergoes partial disassembly during viral entry, exposing the VP2/3 and allowing the VP3 NLS to then mediate nuclear entry of the viral DNA complexed to VP1-VP2/3 via cellular importins (Nakanishi et al., 2002). However, recent findings show that VP2/3 may not enter the nucleus along with the genome (Kuksin and Norkin, 2012). In this study, cells were infected with 5-Bromo-2-deoxyuridine-(BrdU) labeled DNA SV40, and the BrdU and VP2/3 were detected by indirect immunofluorescence microscopy (Kuksin and Norkin, 2012). Only SV40 DNA and not VP2/3 were found in the nucleus 12 h post-infection. Therefore, it was proposed that the viral DNA-protein complex might disassemble at the NPC before nuclear entry of the genome (Kuksin and Norkin, 2012).

Since evidence for the involvement of the NPC in nuclear entry of the viral genome requires that the virus be in the cytoplasm, several studies have elucidated the mechanism of viral escape from the ER to the cytoplasm. Virus disassembly first occurs within the ER using protein-disulfide isomerases and molecular chaperones, resulting in the exposure of VP2/3 (Schelhaas et al., 2007). The N-terminus of VP2 appear to be directly involved in the translocation of the virus across the ER membrane, since it is required for viral infection, creates pores within membranes, and therefore integrates into the ER membrane (Giorda et al., 2013). On the other hand, cellular proteins belonging to the ER-associated degradation (ERAD) pathway of misfolded proteins, BiP and BAP13, have also been implicated in translocation of the virus across the ER membrane (Geiger et al., 2011). This study showed that BiP and BAP13 are needed for VP2 mediated tethering of viral particles to the ER membrane (Geiger et al., 2011; reviewed by Byun et al., 2014). Furthermore, in support of this finding, other potential interaction partners were identified using immunoprecipitation, siRNA, and immunofluorescence microscopy techniques, including the cytosolic chaperone SGTA (small glutamine-rich tetratricopeptide repeat-containing protein α) and the two J-proteins, DnaJB14 and DnaJB12 (Walczak et al., 2014). Thus, there is adequate evidence for a model by which the partially disassembled virions traffic from the ER to the cytoplasm using the ERAD pathway.

The evidence suggesting direct entry of SV40 from the ER into the nucleus came from early EM studies showing NE disruptions near SV40 particles in the nucleus (Hummeler et al., 1970; Mackay and Consigli, 1976). Additional support for this model came recently with a study that found disruption to the nuclear lamina with SV40 infection (Butin-Israeli et al., 2011). In this study, immunofluorescence microscopy of non-dividing cells infected with SV40 shows gaps in the lamin-A/C immunostaining as early as 2 h post-infection (Butin-Israeli et al., 2011). Furthermore, fluctuations in the levels of lamin-A/C were also found by western blot within 10 h of SV40 infection. In order to determine the mechanism of nuclear lamina disruption, cells were infected with SV40 in the presence of several caspase inhibitors. It was found using immunofluorescence microscopy and western blot that a caspase-6 inhibitor prevents nuclear lamina disruptions and viral protein expression (Butin-Israeli et al., 2011). It had previously been found that caspase-6 is also activated during early infection (Butin-Israeli et al., 2010). Combining these findings confirms the model whereby SV40 leads to disruption of the nuclear lamina by activated caspase-6, ultimately leading to direct entry into the nucleus without leaving the ER. Thus, similar to parvoviruses, it appears that SV40 can also disrupt the nuclear lamina for nuclear entry of its viral genome; however, the molecular mechanism appears to be different. For example, SV40 infection involves lamin-A/C cleavage by caspase-6, whereas parvovirus infection involves lamin-B cleavage by caspase-3. Either way, similar to parvovirus infection, the molecular mechanism by which SV40 disrupts the nuclear membrane remains to be further elucidated.

In summary, the SV40 viral genome gains access to the nucleus through two potential pathways (**Figure 5C**). The first involves an NPC-dependent pathway, in which the partially disassembled virion exists the ER using the ERAD pathway, and through interaction between the VP3 NLS and importin-α/β is targeted to the NPC, completely disassembles at the NPC, and the uncoated viral DNA enters the nucleus through the NPC. The second pathway is through direct perforation of the inner nuclear membrane by partial disassembled subviral particles in the ER, followed by cleavage of lamin-A/C by caspase-6 and disruption of the nuclear lamina. Both pathways are possible and deserve further investigation to elucidate their molecular mechanisms.

Human PapillomaVirus

Papillomaviruses are a large family of viruses of which the majority are HPVs (Bernard et al., 2010). The 55-nm icosahedral capsid is formed by 360 copies of the major structural protein L1 that assembles into pentamers, and variable amounts of the minor structural protein L2 (Howley et al., 2013). The genome is circular, double-stranded DNA of ∼8 kbp. In general, studies on early steps of HPV infection, especially the mechanism for nuclear entry, have been challenging and limited (reviewed by Day and Schelhaas, 2014). This is in part because viral production in cell culture systems yields limited amount of virions, due to the restriction of HPV productive life cycle to terminally differentiating keratinocytes (reviewed by Day and Schelhaas, 2014). Therefore, many studies use surrogate viral particles, either non-infectious virus-like particles (Kirnbauer et al., 1993) or pseudovirions that contain a plasmid encoding a reporter gene (Buck and Thompson, 2007; reviewed by Day and Schelhaas, 2014). Nevertheless, it has been established that L1 mediates the binding of the virus to the host receptor heparan sulfate proteoglycans, which initiates conformational changes in L1 and exposes L2 leading to internalization of the virus using multiple cellular pathways, including clathrin- and non-clathrin mediated endocytosis (reviewed by Sapp and Bienkowska-Haba, 2009; Day and Schelhaas, 2014). HPV virions then partially disassemble in the acidic environment of endosomes, leading to separation of L1 and L2, and the subviral particle consisting of L2 and the genome is then either

routed to the Golgi apparatus or L2 disrupts the endosomal membrane and the subviral particle is released in the cytoplasm (reviewed by Sapp and Bienkowska-Haba, 2009; Day and Schelhaas, 2014).

Up until recently, very little was known about the nuclear entry of HPV. Several studies have documented that L2 from HPV11 and HPV16 contains two NLSs that mediate nuclear import when fused with GFP and interact with NTFs (Darshan et al., 2004; Bordeaux et al., 2006; Mamoor et al., 2012). In addition, L2 and the viral genome co-localize in the nucleus of cells infected with HPV, suggesting that they enter the nucleus as a complex (Day et al., 2004). However, no direct evidence for the use of the L2 NLSs during nuclear import of the viral genome has been shown. Instead, a second scenario has been proposed that involves NE breakdown during mitosis for the virus to access nuclear components required for viral replication (**Figure 5D**). The initial evidence for this mechanism came from a screen of a large library of bioactive compounds, which found that cell cycle inhibitors completely blocked HPV infection (Pyeon et al., 2009). Subsequently, it was showed that cell cycle arrest by serum starvation or using drugs inhibits HPV infection (Pyeon et al., 2009). More specifically, early prophase rather than late prophase or metaphase was found to be important for HPV infection (Pyeon et al., 2009). A CDK1 inhibitor was also found to block HPV infection in a dose-dependent manner, suggesting that phosphorylation of NE components by CDK1 and NE breakdown during early prophase is important for HPV nuclear entry and ultimately infection (Pyeon et al., 2009).

Further evidence for the dependence of HPV infection on NE breakdown during mitosis came from a recent high-throughput RNAi screen that found host mitotic and cell cycle regulator factors involved in HPV infection (Aydin et al., 2014). In this study, the requirement for mitosis during HPV infection was further tested, by infecting interphase cells with HPV16 pseudovirus containing pseudogenomes encoding GFP and monitoring early steps of infection by immunofluorescence microscopy. In interphase cells, the virus was endocytosed, moved to the perinuclear area, and partially uncoated, but only when mitosis was initiated would the viral DNA enter the nucleus (Aydin et al., 2014). More importantly, co-infection of S-phase-arrested cells with HPV and the parvovirus H1, which induces NE disruption, yield a detectable number of cells infected with HPV, suggesting that the NE disruption by H1 is sufficient for nuclear entry of HPV DNA (Aydin et al., 2014). Thus, the authors proposed that NE breakdown during mitosis is the step required for nuclear entry of HPV. Furthermore, because L2-GFP associate with host cell condensed chromatin from metaphase plate formation through cytokinesis, it was proposed that the subviral complex waits in the perinuclear area until NE breakdown occurs in order to enter the nucleus and gain access to nuclear components (Aydin et al., 2014).

In summary, NE breakdown during mitosis might facilitate the nuclear entry of HPV (**Figure 5D**). Unlike parvoviruses, however, HPV does not induce NE disruption, but rather must wait for the cell to disassemble the NE during mitosis in order to access nuclear components and replicate its genome. This strategy for nuclear entry is also used by retroviruses, such as the murine leukemia virus (reviewed by Cohen et al., 2011a).

Concluding Remarks

Even though all known DNA viruses use very similar mechanisms to enter their host cells, they have evolved unique mechanisms to deliver their genome into the nucleus of their host cells. Recent studies on nuclear import of viruses have drastically changed the perception of how viruses use the cellular machinery for nuclear import. What was once thought to be just two or three common mechanisms for several viruses has now revealed to include a completely different and unique strategy for each virus to deliver its genome into the nucleus. This is in part due to the use of more advanced and correlative techniques, the emergence of alternative cell lines, and the development of more consistent wild-type and mutant virus production protocols between research groups. It is intriguing that not only the strategies between virus families are different, but that some viruses within the same family, as is the case for baculoviruses, may even use different strategies. It is fascinating that even some individual viruses, such as SV40, may use several different nuclear import strategies.

From comparison of some of the best-characterized viral nuclear import pathways, it is also evident that although some viruses use a similar strategy, the cellular components used could be different. For example, parvoviruses and SV40 both disrupt the nuclear lamina to deliver their genomes into the nucleus, however, parvoviruses use both phosphorylation and proteolytic cleavage of nuclear lamins, which require PKC, cdk2, and caspase-3, whereas SV40 uses proteolytic cleavage of lamin-A/C by caspase-6. It is also becoming evident that some viruses use the NPC and binding to Nups as a cue for disassembly. This is the case of the HBV capsid that disassembles at the nuclear basket after binding to Nup153, or HSV1 that ejects its genome by a possible binding of the capsid to Nup214. Although the picture for nuclear entry mechanisms is now clearer for many viruses, much remains to be explored. For example, the cellular factors and viral proteins involved in baculovirus nuclear entry, the factors that triggers HSV1 genome release from the capsid, how the different viral DNA translocate across the NPC for viruses that uncoat their genome before nuclear entry, and how parvoviruses and SV40 initially disrupt the NE, are among the many aspects of nuclear entry that remain undetermined.

Viruses are excellent models to understand cellular processes. The study of nuclear import of viruses could lead to new insights into the detailed mechanism by which molecules, including endogenous DNA, translocate through the NPC central channel, which is still unclear and a much-debated topic in the field of nuclear transport. Moreover, a detailed characterization of the nuclear import of viruses is an important step in the development of antiviral therapy that may successfully resolve viral diseases by interrupting entry of the viral genome into the nucleus of infected cells. Additionally, since viral vectors – containing a gene

of interest – must also enter the cell's nucleus to allow for gene expression, studies of nuclear import of viral genomes could help in the design of more efficient vectors for gene therapy. Thus, by learning about viruses and how they target their genome into the cell nucleus, we can learn about cell biology mechanisms, find antiviral targets for some viruses, and improve therapeutic potential for others.

Acknowledgments

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Picornaviruses and nuclear functions: targeting a cellular compartment distinct from the replication site of a positive-strand RNA virus

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The compartmentalization of DNA replication and gene transcription in the nucleus and protein production in the cytoplasm is a defining feature of eukaryotic cells. The nucleus functions to maintain the integrity of the nuclear genome of the cell and to control gene expression based on intracellular and environmental signals received through the cytoplasm. The spatial separation of the major processes that lead to the expression of protein-coding genes establishes the necessity of a transport network to allow biomolecules to translocate between these two regions of the cell. The nucleocytoplasmic transport network is therefore essential for regulating normal cellular functioning. The *Picornaviridae* virus family is one of many viral families that disrupt the nucleocytoplasmic trafficking of cells to promote viral replication. Picornaviruses contain positive-sense, single-stranded RNA genomes and replicate in the cytoplasm of infected cells. As a result of the limited coding capacity of these viruses, cellular proteins are required by these intracellular parasites for both translation and genomic RNA replication. Being of messenger RNA polarity, a picornavirus genome can immediately be translated upon entering the cell cytoplasm. However, the replication of viral RNA requires the activity of RNA-binding proteins, many of which function in host gene expression, and are consequently localized to the nucleus. As a result, picornaviruses disrupt nucleocytoplasmic trafficking to exploit protein functions normally localized to a different cellular compartment from which they translate their genome to facilitate efficient replication. Furthermore, picornavirus proteins are also known to enter the nucleus of infected cells to limit host-cell transcription and down-regulate innate antiviral responses. The interactions of picornavirus proteins and host-cell nuclei are extensive, required for a productive infection, and are the focus of this review.

Keywords: picornavirus, enterovirus, cardiovirus, nucleus, nucleocytoplasmic trafficking, viral RNA replication, IRES

Introduction

Overview

In this section, we will first provide a brief review of nucleocytoplasmic trafficking in uninfected eukaryotic cells, followed by an outline of the salient features of picornavirus gene expression and replication. Refer to **Table 1** for acronyms used in this article.

The Nucleus and Nucleocytoplasmic Transport

The nucleus is bound by a double membrane of phospholipids termed the nuclear envelope. The inner nuclear membrane is associated with a network of the scleroprotein lamin, comprising the nuclear lamina, and the outer nuclear membrane is an extension of the endoplasmic reticulum (Callan et al., 1949). The nuclear envelope functions as a physical barrier between the cytoplasm and the nucleus and is selectively permeable via nuclear pores, which average in number between 2000 and 5000 per nucleus in vertebrate cells (Grossman et al., 2012). Macromolecules traffic between the nucleus and cytoplasm through these pores that fuse the inner and outer nuclear envelope. Protein complexes known as the nuclear pore complex (NPC) are integrated within the nuclear pores and act as gates that restrict the diffusion of larger biomolecules across the nuclear envelope. With an approximate mass of 125 MDa, the NPC is one of the largest and most complex assemblages of proteins in the eukaryotic cell and is composed of approximately 30 different nucleoporin (Nup) proteins, with ∼500–1000 individual Nups comprising a single NPC (Reichelt et al., 1990; Cronshaw et al., 2002; Hoelz et al., 2011). The NPC is a dynamic and modular structure with eight-fold rotational symmetry and can be divided into three recognizable ring-like structures surrounding the central channel of the nuclear pore: the cytoplasmic ring, the central spoke ring, and the nuclear ring (which make up the symmetrical portion of NPC) (Frenkiel-Krispin et al., 2010). Attached to the cytoplasmic ring and nuclear ring are 8 proteinaceous filaments which extend into the cytoplasm and nucleus, respectively, with the nuclear filaments converging to form the nuclear basket (Cautain et al., 2015). These extended structures, together, make up the asymmetric portion of the NPC. Nups are categorized as transmembrane, barrier, or scaffold Nups based upon location within the NPC, amino acid sequence motifs, and structure (Grossman et al., 2012). Transmembrane Nups anchor the NPC to the nuclear envelope pores, barrier Nups facilitate active transport of cargoes, and scaffold Nups link the transmembrane Nups to the barrier Nups, providing the structural framework of the NPC (**Figure 1**).

Barrier Nups contain repeated phenylalanine-glycine-rich (FG) sequences that form intrinsically disordered motifs and act as the major impediment to free diffusion through the main channel of the NPC (Cautain et al., 2015). Concomitantly, these FG-Nups provide the only route for active transport of cargo biomolecules between the cytoplasm and nucleus by providing binding sites for nuclear transport receptors, within the NPC, through multiple low-affinity interactions (Ben-Efraim and Gerace, 2001; Ribbeck and Görlich, 2001). The translocation

TABLE 1 | Acronyms used in this article.

Acronym	Definition
NPC	Nuclear pore complex
Nup	Nucleoporin
FG	Phenylalanine-glycine-rich
NLS	Nuclear localization signal
NES	Nuclear export signal
NCR	Non-coding region
IRES	Internal ribosome entry site
S-L	Stem-loop
ITAF	IRES trans-acting factors
RNP	Ribonucleoprotein
EMCV	Encephalomyocarditis virus
FMDV	Foot and mouth disease virus
HRV	Human rhinovirus
CVB3	Coxsackievirus B3
EV71	Enterovirus 71
TMEV	Theiler's murine encephalomyelitis virus
HAV	Hepatitis A virus
EGFP	Enhanced green fluorescent protein
PAMP	Pathogen associated molecular pattern
ISG	Interferon-stimulated gene
Pol	RNA polymerase

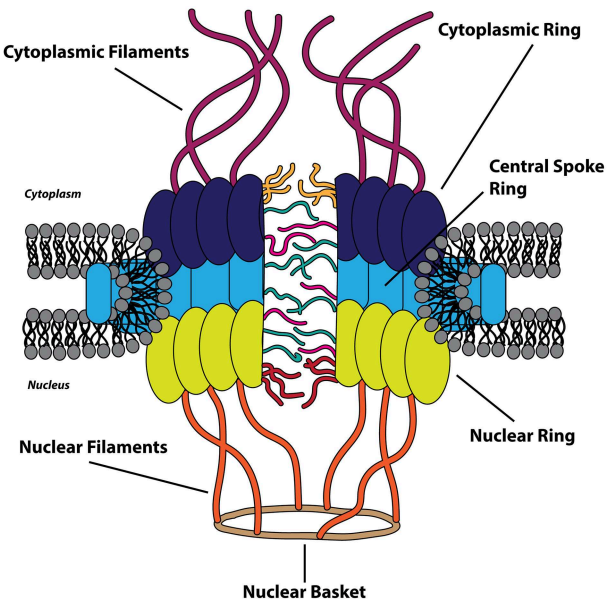

FIGURE 1 | The nuclear pore complex. The cytoplasmic (dark blue), central spoke (light blue), and nuclear ring (chartreuse) structures constitute the symmetric portion of the nuclear pore complex (NPC) that surrounds the central channel. The asymmetric portion of the NPC is composed of cytoplasmic filaments (purple) on the cytoplasmic side and the nuclear filaments (orange) and nuclear basket (brown) on the nuclear side of the nuclear envelope. Transmembrane and scaffold nucleoporins are found within the three symmetric ring-like structures of the NPC, and the FG-repeat containing barrier nucleoporins are depicted as filaments within the central channel.

of complexes through the NPC is energy-independent as GTP hydrolysis is required only as a final step in the transport process (Schwoebel et al., 1998). The efficiency of nucleocytoplasmic transport is staggering: a single NPC has been proposed to be capable of transporting a 100 kDa protein at an average rate of 800 translocation events per second (Ribbeck and Görlich, 2001; Fried and Kutay, 2003).

Small molecules including ions, metabolites, and proteins less than ∼40 kDa are able to translocate between the cytoplasm and nucleus via passive diffusion, perhaps through channels peripheral to the major channel of the NPC (Hinshaw et al., 1992). In addition to allowing this energy-independent diffusion, the NPC simultaneously facilitates the selective, energy-dependent nucleocytoplasmic trafficking of large cellular molecules. This is generally accomplished via specific amino acid sequences present on cargo proteins known as nuclear localization signals (NLSs) or nuclear export signals (NESs), depending on the directionality of transport. These signal sequences are recognized by the soluble nuclear transport receptors that bind cargo proteins and actively transport these molecules through the NPC. Many nuclear transport receptors belong to the karyopherin protein family, known as importins or exportins, and bind specific cargo proteins directly or through adaptor molecules to shuttle proteins from one side of the nuclear envelope to the other. The energy required for this process is provided by GTP hydrolysis carried out by the GTPase Ran, and the concentration gradient of Ran bound to GTP (Ran-GTP) imparts the directionality needed for the proper segregation of nuclear and cytoplasmic functions. Ran-GTP is abundant in the nucleus due to the presence of chromatin-bound Ran-guanine nucleotide exchange factor (Ran-GEF). Conversely, Ran-GDP is more abundant on the cytoplasmic side of the nuclear envelope as a result of the cytoplasmic filament-bound Ran-GTP-activating protein (Ran-GAP), which increases the GTPase activity of Ran, rapidly hydrolyzing bound GTP to GDP (Grossman et al., 2012). Accordingly, the Ran-GTP gradient provides directionality to nucleocytoplasmic transport because importins and exportins utilize the Ran-GTP gradient in a complementary fashion. Nuclear import complexes (importin(s)/cargo) associate at low Ran-GTP concentrations in the cytoplasm, traverse the NPC through transient association-dissociation between importin and FG-Nups. The cargo is then released by the interaction between the import complex and Ran-GTP in the nucleus (Rexach and Blobel, 1995; Görlich et al., 1996). Conversely, trimeric nuclear export complexes (exportin/cargo/Ran-GTP) associate at high Ran-GTP concentrations in the nucleus, traverse the NPC, and dissociate upon interconversion of Ran-GTP to Ran-GDP in the cytoplasm. Both importins and exportins bind Ran-GTP directly and utilize the metabolic energy provided by the Ran-GTPase system to relate directionality to transport (**Figure 2**). Nucleocytoplasmic transportation is a highly regulated and effective process necessary for cellular homeostasis and, correspondingly, is the target of perturbation by many viral pathogens, including the picornaviruses.

Picornaviruses

The picornaviruses are a large group (26 genera currently recognized) of non-enveloped, small (∼30 nm in diameter) viruses containing a positive-polarity, single-stranded RNA genome of ∼7–9 kb in length with a viral protein (VPg) covalently attached to the 5′-terminus of the genome. These RNA molecules contain both 5′ and 3′ untranslated regions that function, in association with viral and host cell proteins, to facilitate both translation of the single open reading frame flanked by these regions, as well as RNA replication for genomic amplification. The long (∼600–1500 nucleotide, including up to a ∼500 nucleotide poly(C) tract for some aphthoviruses), highly structured, 5′-noncoding region (NCR) contains an internal ribosome entry site (IRES) that directs the cap-independent translation of a large polyprotein from the viral genome (Racaniello, 2013; Martínez-Salas et al., 2015). This precursor polyprotein is co- and post-translationally processed by viral proteinase 3CD (as well as enteroviral 2A and leader proteinase L for aphthoviruses and erboviruses) to generate intermediate and mature viral proteins with distinct functions. In addition to the highly structured IRES region, many picornaviruses also contain stem-loop (S-L) structures important for protein interactions that promote genome replication. On the other terminus of the viral genome, the shorter (∼50–350 nucleotide) 3′-NCR contains structured regions involved in viral RNA synthesis (though non-essential for infectivity), as well as an essential poly(A) tract (Racaniello, 2013). The RNA-dependent RNA polymerase, 3D, functions to replicate the viral genome through a negative-stranded intermediate, and is encoded within the P3 region of the polyprotein (**Figure 3**).

The infectious cycles of picornaviruses are initiated following viral attachment to specific cellular receptors. The RNA genome is then released from the virion capsid and enters the cytoplasm of the infected cell. Once in the cytoplasm, the picornavirus RNA molecule is used as a template for IRES-driven viral protein production. An incompletely defined set of events that likely involve local concentrations of viral proteins and cleavage of specific host factors allows the same RNA template used for translation to be cleared of ribosomes and utilized for the production of a complementary, intermediate negative-sense (anti-genomic) RNA molecule, producing a double-stranded RNA structure called the replicative form. This opposite polarity molecule is, in turn, used for the production of positive-sense RNA molecules, generating a multiple-stranded RNA complex composed of negative-sense RNA templating multiple genomic RNA molecules simultaneously, called the replicative intermediate. These nascently produced molecules are then recycled back through the translation/replication process or packaged into progeny virions. Importantly, proteins predominantly localized to the nucleus in uninfected cells are utilized by these cytoplasmic viruses from the very primary steps of the replication process.

Nuclear-Resident Proteins Are Hijacked For Picornavirus Translation

The positive-sense RNA genome of a picornavirus is competent for immediate IRES-driven translation upon uncoating. In addition to picornavirus RNAs, it has been suggested that up to 10% of cellular mRNAs contain an IRES element (Spriggs

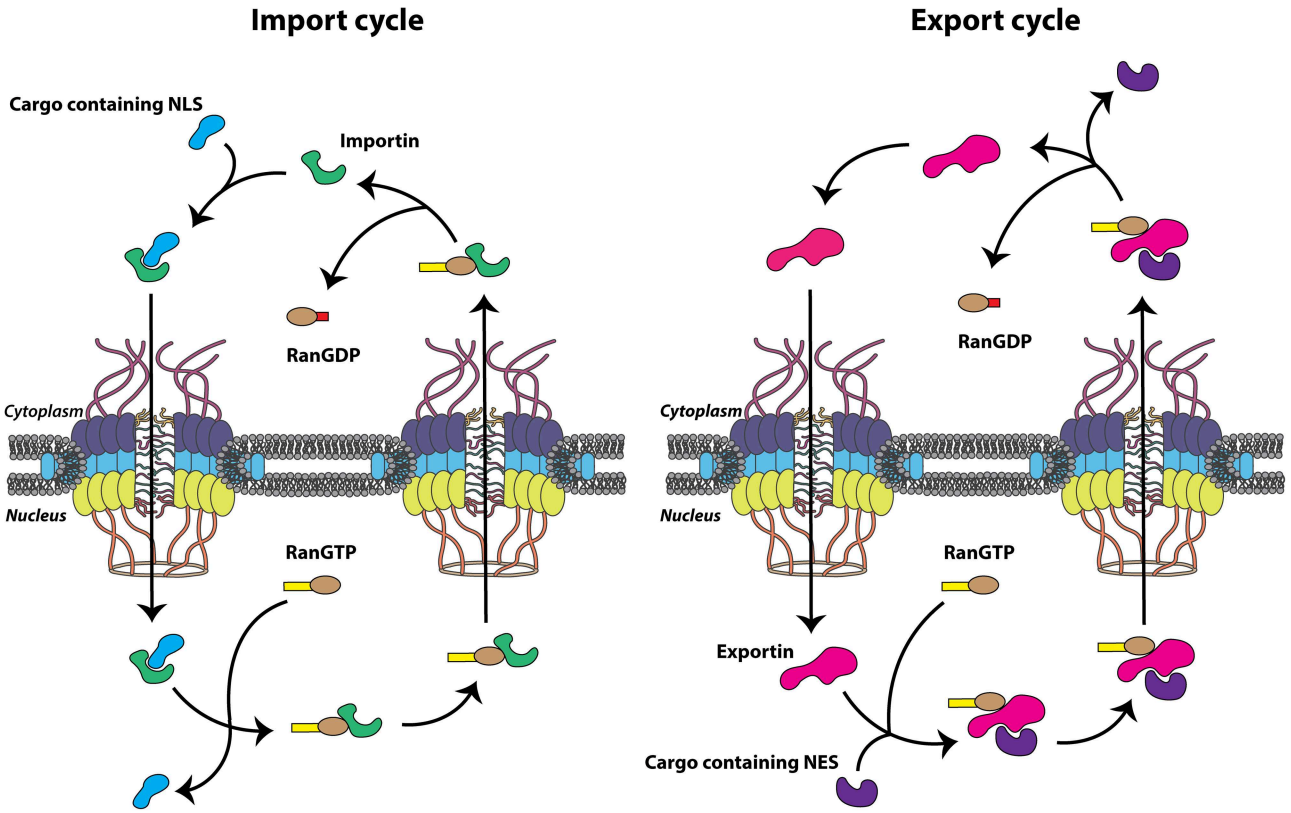

FIGURE 2 | Nuclear transport cycles. Nuclear import and export cycles function in complementary fashion to recycle nuclear transport receptors, importins and exportins (green and pink, respectively), through the nuclear pore complex (NPC). Transport of biomolecules (cargo) containing a nuclear localization signal (NLS) (light blue) or nuclear export signal (NES) (purple) through the NPC itself is energy independent but the movement of nuclear transport receptors is dependent upon the hydrolysis of GTP.

et al., 2005). Somewhat counterintuitively, many of the proteins that mediate IRES-dependent translation of cellular and viral mRNAs, known as IRES trans-acting factors or ITAFs, are compartmentalized in the host cell nucleus or shuttle between the nucleus and the cytoplasm (Semler and Waterman, 2008). It is currently unclear whether ITAFs associate with cellular IRES elements during the biogenesis of mRNA transcripts in the nucleus and are subsequently transported to the cytoplasm as ribonucleoprotein (RNP) complexes, or whether these ITAFs are redistributed to the cytoplasm, where IRES-containing mRNAs are already present, in response to signals stimulating IRES-driven translation. Regardless of the mechanism in which ITAFs associate with cellular IRES elements, the ITAFs utilized by picornaviruses are available at sufficient concentration in the cytoplasm upon infection, at least for the initial rounds of viral protein production. Importantly, picornaviruses subvert the host protein synthesis machinery by cleavage of canonical translation factors, inhibiting cellular translation and releasing ribosomes and associated proteins from their roles in cap-dependent translation. For the purpose of discussion, nuclear-resident proteins will be defined as those that are normally more concentrated in the nucleus than the cytoplasm, because all cellular proteins can be found to some extent in the cytoplasm during biogenesis and many shuttle between the nucleus and cytoplasm to perform their functions. Importantly, these nuclear-resident/shuttling proteins often have RNA-binding capabilities and control many features of RNA biology and gene expression including: splicing, mRNA transport out of the nucleus, and mRNA stability. Furthermore, mammalian cells encode nearly 1000 RNA-binding proteins (although not all of these are nuclear-resident) and as a result, the viral mRNAs of picornaviruses employ the functions of several of these RNA-binding and RNA-chaperone proteins to facilitate translation (Castello et al., 2012).

While all picornaviruses contain IRES elements within the 5′-NCR of their genomes to facilitate ribosome association, these elements are categorized into four separate types, I–IV, for the 12 best studied picornavirus genera, depending upon primary RNA sequence, secondary RNA structure, location of translation initiation codon, and phylogeny. Type I structures are found in the genomes of enteroviruses; Type II in aphthoviruses, cardioviruses, erboviruses, kobuviruses, and parechoviruses; Type III in hepatoviruses; and Type IV in avihepatoviruses, sapeloviruses, senecaviruses, teschoviruses, and tremoviruses (Palmenberg et al., 2010; Martínez-Salas et al., 2015). The categorization of picornavirus IRES types is somewhat arbitrary and flexible, especially as work related to cap-independent translation from these viruses continues. Recently, it has been proposed that the Kobuvirus genus contains a distinct, fifth type

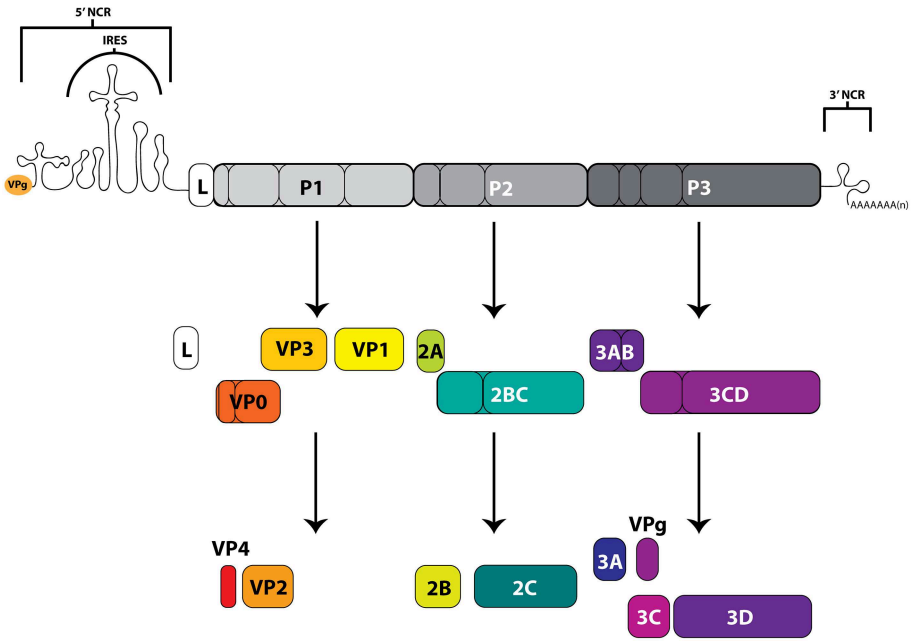

FIGURE 3 | Picornavirus genome map and polyprotein cleavage cascade. The positive-sense RNA genomes of picornaviruses contain an internal ribosome entry site (IRES), within a 5′-noncoding region (5′NCR), which drives the cap-independent translation of the downstream open reading frame. Viral gene products include functional precursors (some of which are depicted here) that are further processed by viral proteinases to produce mature viral proteins. The precursor protein 3AB associates with membranes and stimulates the function of 3CD and the RNA-dependent RNA polymerase 3D. The proteolytically active 3CD precursor functions in VPg uridylylation and viral RNA replication through the formation of the ternary complex on the 5′-terminus of viral genomic RNA molecules. Protein 3C functions in viral protein maturation through its proteinase activity. VPg (3B) acts as a protein primer for initiation of viral RNA synthesis. The 2A protein of enteroviruses has proteinase activity, but the 2A of cardioviruses does not. Proteins that form the viral capsid are encoded in the P1 region. Leader protein (L) is not encoded by all picornaviruses, and in some genera (including aphthoviruses), L has proteinase activity. The 3′-terminus of the genome contains a 3′-noncoding region as well as a genetically encoded poly(A) tract.

of IRES, but there will be no further discussion within this review, as no nuclear-resident ITAFs have been reported for this IRES type (Sweeney et al., 2012). There is little sequence homology across the four IRES types and, as a result, picornaviruses harboring different IRES structures likely utilize slightly different cohorts of ITAFs and in different ratios. However, there is at least some overlap in the identity of those nuclear proteins that are used as ITAFs for general picornavirus translation. ITAFs often have functions in the uninfected cell related to transcriptional regulation, splicing, and RNA transport and stability (Martínez-Salas et al., 2015).

Polypyrimidine Tract-Binding Protein 1 (PTBP1)

Type I and Type II IRESs have been the most extensively studied of the picornavirus IRES elements and correspondingly, have been shown to associate with the greatest number of nuclear-resident proteins compared to the other IRES Types. Polypyrimidine tract-binding protein 1 (PTBP1 also known as hnRNP I) was the first host protein shown to interact with, and promote translation from, the IRES regions of encephalomyocarditis virus (EMCV), foot and mouth disease virus (FMDV), poliovirus, and human rhinovirus (HRV) 2 (Jang and Wimmer, 1990; Luz and Beck, 1991; Hellen et al., 1993; Kaminski et al., 1995; Niepmann, 1996; Hunt and Jackson, 1999). PTBP1 contains four RNA recognition motifs distributed across a flexible structure, functions in the regulation of pre-mRNA splicing and transport, and has been shown to be predominantly localized to the nucleus while also shuttling to the cytoplasm (Ghetti et al., 1992; Oh et al., 1998; Sawicka et al., 2008). This protein is hypothesized to promote translation initiation on Type I IRESs by modulating an interaction between domain V of these structures and the C-terminal portion of translation initiation factor eIF4G, which is cleaved during infection with enteroviruses but retains some RNA binding capability, with the C-terminal fragment utilized for cap-independent translation (Buckley and Ehrenfeld, 1987; Ohlmann et al., 1995, 1996; Kafasla et al., 2010). Coxsackievirus B3 (CVB3), which contains a Type I IRES element, also utilizes PTBP1 as an ITAF, and because it has been shown to bind both the 5′- and 3′-NCRs of CVB3 RNA, has been proposed to facilitate circularization of the RNA molecule to promote efficient translation (Verma et al., 2010). Furthermore, EMCV and FMDV Type II IRESs appear to require the binding of two copies of PTBP1, at two distinct regions, for maximal IRES activity, at least *in vitro* (Kolupaeva et al., 1996; Kafasla et al., 2009). PTBP1 likely acts as an RNA chaperone, stabilizing viral IRES structures, since poliovirus and EMCV translation is dependent upon the simultaneous interaction of three of the four RNA-binding domains found within this protein, and FMDV

requires two of the four RNA-binding domains of PTBP1 for efficient IRES activity (Song et al., 2005; Kafasla et al., 2011). PTBP1 has been demonstrated to be the only ITAF (non-canonical translation factor) that is required for the translation of EMCV transcripts *in vitro* (Pestova et al., 1996).

Lupus La Protein (La)

The Lupus La protein (La, also known as La autoantigen) has also been implicated in having a role in the cap-independent translation of type I and II IRESs. La has been shown to bind a portion of the poliovirus IRES as a dimer and enhance the production of poliovirus proteins (Meerovitch et al., 1989, 1993; Craig et al., 1997). Similarly, La protein stimulates the translation of both CVB3 and EMCV RNA (Kim and Jang, 1999; Ray and Das, 2002). La functions to stabilize nascent RNA produced in cells. It binds the 3′ poly(U) termini of RNA polymerase III transcripts to protect and promote maturation of these RNA molecules, and as a result is generally confined to the nucleus (Stefano, 1984). La has been proposed to mediate an interaction between the 40S ribosomal subunit and the poliovirus IRES *in vivo* (Costa-Mattioli et al., 2004).

Poly(rC)-Binding Protein 2 (PCBP2)

Poly(rC)-binding protein 2 (PCBP2) binds single-stranded nucleic acids through three hnRNP K-homologous domains (KH domains) and is involved in the stabilization of several cellular mRNAs (Siomi et al., 1994; Holcik and Liebhaber, 1997). The predominantly nuclear PCBP2 binds to both stem-loop IV (S-L IV) and stem-loop I (S-L I) of the 5′-NCR within the poliovirus and CVB3 genomic RNA (Blyn et al., 1995, 1996; Leffers et al., 1995; Gamarnik and Andino, 1997; Parsley et al., 1997; Zell et al., 2008a,b; Sean et al., 2009). S-L IV is located in the central portion of Type I IRES elements and alterations to the nucleic acid sequence identified as important for PCBP2 association decrease poliovirus translation *in vitro* (Blyn et al., 1995). Moreover, depletion of PCBP2 from cellular extracts results in inefficient poliovirus translation (Blyn et al., 1997). Although PCBP2 is required for translation of poliovirus, coxsackievirus, and HRV, it is not necessary for the translation of the type II IRES-containing RNAs of EMCV and FMDV (Walter et al., 1999). PCBP2 is the only ITAF shown to be required for the translation of poliovirus, enterovirus 71 (EV71), and bovine enterovirus (i.e., Type I IRESs) by *in vitro* reconstitution of translation initiation (Sweeney et al., 2014). However, as with PTBP1 and the EMCV IRES, whether these *in vitro* systems are representative of the conditions encountered within the cellular milieu during infection, or if other, non-essential, ITAFs enhance viral IRES-driven translation, remains to be elucidated.

Serine/Arginine-Rich Splicing Factor 3 (SRSF3)

Type I IRES structures also utilize serine/arginine-rich splicing factor 3 (SRSF3 or SRp20) to promote cap-independent translation. The SR proteins comprise a group of splicing factors with a multitude of functions related to gene expression including: constitutive and alternative splicing, mRNA export and stability, and translation (Graveley, 2000; Huang and Steitz, 2001; Huang et al., 2003; Sanford et al., 2004). As a result of these functions, a subset of SR proteins including SRSF3 are considered shuttling proteins, although they most often accumulate in the cellular nucleus (Cáceres et al., 1998). Depletion of SRSF3 from cells or cellular extracts decreases the protein production from a reporter construct containing the poliovirus IRES (Bedard et al., 2007). In addition, SRSF3 and PCBP2 have been shown to act synergistically to increase the efficiency of IRES-mediated translation *in vitro* and in poliovirus-infected cells, with SRSF3 associating with S-L IV of the IRES via a PCBP2 bridge. Specifically, this enhancement in non-canonical translation is a result of SRSF3 interacting with the KH3 domain of PCBP2 to directly or indirectly recruit ribosomes to the viral RNA. Furthermore, both are found associated with translation initiation complexes in poliovirus-infected cells (Bedard et al., 2007; Fitzgerald and Semler, 2011). CVB3 and HRV 16 also likely utilize SRSF3 to promote translation, as this protein is relocalized in cells expressing the 2A proteinase of these viruses (Fitzgerald et al., 2013).

Proliferation-associated Protein 2G4 (PA2G4)

The SRSF3/PCBP2 cooperative enhancement to poliovirus IRES-driven translation initiation is mirrored by the interaction between PTBP1 and proliferation-associated protein 2G4 (PA2G4 or EBP1) with the FMDV IRES element. A chimeric Theiler's murine encephalomyelitis virus (TMEV, which also utilizes a Type II IRES) containing the FMDV IRES element in place of the TMEV IRES was unable to replicate in mouse neurons, suggesting the absence of a necessary ITAF for minimal FMDV translation. This ITAF was identified as PA2G4 ("ITAF 45") in assaying for the formation of 48S initiation complexes through biochemical reconstitution and was shown to bind directly to viral RNA corresponding to the FMDV IRES through UV cross-linking (Pilipenko et al., 2000). PA2G4 and PTBP1 bind to distinct sites within the FMDV IRES, causing localized structural changes within these regions, thereby enhancing binding of the eIF4G/4A complex to the IRES structure. It has been proposed that unlike in the case of the TMEV IRES, PTBP1 alone is unable to promote the RNA structural modifications to the FMDV IRES necessary for ribosome association and translation initiation. This is likely due to differences in the nucleotide sequence within these Type II IRES structures and associated differences in the way in which PTBP1 binds and arranges these regions, forcing FMDV to rely on PA2G4 to shape a functionally competent IRES structure (Pilipenko et al., 2000). PA2G4 was also shown to interact with TMEV and EMCV RNA, so this protein likely binds RNA non-specifically. However, EMCV IRES-driven translation has been shown to be unaffected by the presence of PA2G4, and PA2G4 does not interact with PTBP1, corroborating the fact that translation initiation from the EMCV IRES is independent of PA2G4 (Monie et al., 2007). Interestingly, despite the fact that FMDV and EMCV have different ITAF requirements, experiments comparing the sites of hydroxyl radical cleavage within the IRES structures from the eIF4G hub demonstrated that when these Type II IRESs interact with their cognate ITAFs, similar structural conformations are adopted (Yu et al., 2011). This suggests that although these IRES sequences can vary by ∼50%, their shared requirement for

PTBP1 seems to lie in the fact that it acts as versatile adaptor protein, whether alone or in combination with other ITAFs, in translation initiation form Type II IRESs.

Minimal vs. Stimulatory ITAFs

It is important to note that while there has been rather extensive study of the Type I and Type II IRESs and their associated ITAFs, there is some variability in the reported ITAF requirements across particular viral species. For the Type I IRES elements tested (poliovirus, HRV 2, and CVB3), PTBP1 has been shown to be both required or unnecessary (Hunt and Jackson, 1999; Verma et al., 2010; Sweeney et al., 2014). Similarly, for viruses containing Type II IRES elements, there is discrepancy in the obligatory ITAFs reported. PTBP1 is required for FMDV translation, but the requirement for this ITAF in EMCV translation appears conditional upon the reporter and IRES variant utilized in experiments (Kaminski and Jackson, 1998). Additionally, translation from the TMEV IRES has been shown to be independent of, as well as strongly dependent on, PTBP1 (Kaminski et al., 1995; Pilipenko et al., 2001). As mentioned previously, the inconsistencies in reported ITAFs is likely a result of the assay used (*in vitro* compared to experiments in cells) as well as the use of different strains of virus/sequences of reporter constructs. These apparently different results likely point to the fact that there are some minimal ITAFs required for Type I and Type II IRES elements but a multitude of ITAFs that play some stimulatory or translation-enhancing role depending on the specific context of an infection. For example, it is possible that different ITAFs are utilized by viral IRES elements depending on the cell type infected (i.e., cell-type-specific ITAFs), as the availability of particular proteins that function in this regard likely dictate whether viral protein production and growth are supported, and to what extent (Chang et al., 1993; Wimmer et al., 1993). Cell-type-specific IRES function is exemplified by the fact that viral RNAs that initiate translation from the HRV 2 IRES, but not the poliovirus IRES, are excluded from neuronal cell polysomes but not from those of glioma cells. This is thought to be the result of a specific protein heterodimer that inhibits HRV 2 IRES-driven translation in neuronal but not glioma cells, as discussed below (Merrill and Gromeier, 2006).

Other ITAFs of Type I IRESs

Other nuclear-resident proteins that function as ITAFs of Type I IRESs have also been proposed, but less completely characterized. Nucleolin, which relocalizes to the cytoplasm of poliovirus-infected cells, has been shown to stimulate translation from constructs containing poliovirus and rhinovirus IRES structures *in vitro* and in cells, and the amino-terminal domain of this protein is important for this activity (Waggoner and Sarnow, 1998; Izumi et al., 2001). hnRNP A1 shuttles between the nucleus and the cytoplasm, functions in both pre-mRNA splicing and nuclear export of mRNA molecules, and has been shown to interact with the EV71 IRES via electrophoretic mobility shift assay. Furthermore, when hnRNP A1 and hnRNP A2 are knocked down in cells, there is a decrease in translation of a reporter gene containing the EV71 IRES sequence, and there is an overall reduction in viral replication in these cells (Lin et al., 2009b). Similar results have also been observed with the EV71 IRES and far upstream element-binding protein 1 (FBP1) (Huang et al., 2011). It has also been reported that the Type II IRES-containing FMDV may utilize the nuclear KH domain-containing, RNA-binding, signal transduction-associated protein 1 (Sam68) to promote IRES-dependent protein production following L-protein dependent cleavage and subsequent cytoplasmic localization (Lawrence et al., 2012).

Nuclear-resident Proteins That Inhibit Picornavirus Translation

In contrast to the discussion thus far, there are also examples of nuclear-resident proteins that act to inhibit enterovirus translation. Double-stranded RNA binding protein 76 (DRBP76, also known as interleukin enhancer-binding factor 3) heterodimerizes with nuclear factor of activated T cells, 45 kDa (NF45, also known as interleukin enhancer-binding factor 2), and inhibits translation initiation from the HRV 2 IRES in neuronal cells. The recombinant oncolytic poliovirus PVS-RIPO exploits the incorporated HRV 2 IRES to permit attenuated neurovirulence in the treatment of malignant glioma, possibly due to the presence of DRBP76:NF45 heterodimers in the cytoplasm of neuronal cells (Merrill et al., 2006; Merrill and Gromeier, 2006). Similarly, far-upstream element-binding protein 2 (FBP2 or KSRP), which is involved in splicing and mRNA trafficking, inhibits EV71 IRES-driven translation and is cleaved during infection (Lin et al., 2009a; Chen et al., 2013). Cytoplasmic proteins that function as ITAFs for picornavirus RNA translation have also been reported, as well as canonical elongation factors, but these are outside the scope of this review (for a recent review of picornavirus translation including the role of cytoplasmic proteins, see Martínez-Salas et al., 2015).

ITAFs of Type III and Type IV IRESs

There has been comparatively little study of type III and IV IRES elements, but several of the same nuclear-resident proteins involved in IRES-driven translation of type I and type II IRES structures have been implicated for type III structures as well. The type III IRES structure found in the genome of hepatitis A virus (HAV) interacts with, is stabilized by, and is stimulated by PTBP1 (Chang et al., 1993; Gosert et al., 2000a). Similarly, HAV IRES activity is increased in the presence of PCBP2. However, although this protein interacts with the 5′-NCR of the HAV genome, it does not bind to regions that correspond to the IRES structure (Graff et al., 1998). Interestingly, in contrast to its role in the functions of type I and type II IRESs, La suppresses translation from the HAV IRES (Cordes et al., 2008). Even less is known about the ITAF requirements of picornavirus type IV IRES elements, but as these IRES elements are very similar to those found in some Flaviviruses, it is expected that at least some of the same ITAFS utilized by hepatitis C virus, for example, also enhance the translation of sapelovirus, senecaviruses, teschoviruses, and tremoviruses.

Other Nuclear-resident Proteins That Interact with Viral RNA Molecules

Large-scale proteomic studies have identified a multitude of RNA-binding proteins that interact with poliovirus RNA isolated

from infected cells as well as FMDV RNA *in vitro*, but the specific role of each of these identified proteins remains to be elucidated (Pacheco et al., 2008; Lenarcic et al., 2013). One protein identified as interacting with the FMDV IRES element through large-scale proteomic studies is Gem-associated protein 5 (Gemin5), which was shown to inhibit FMDV translation, likely by competitively inhibiting PTBP1 binding (Piñeiro et al., 2013). Although those proteins that act as ITAFs to mediate the translation of picornavirus RNA templates warrant further investigation, one commonality among the proteins discussed above is that they are all RNA-binding proteins with the ability to form multimers. This suggests that they are able to interact with the viral IRESs in multiple locations and perhaps stabilize the structures of their associated IRESs to promote recognition by the translation machinery (Jackson et al., 1995; Kafasla et al., 2009) (**Table 2**).

Nuclear-resident Proteins Function in Template-usage Switching

In addition to the roles that nuclear RNA binding proteins play in viral translation, they may also govern the template usage switch that occurs following the production of picornavirus proteins to transition to viral RNA replication. The same genomic template is used for both translation and RNA replication; however this RNA but cannot be traversed simultaneously by ribosomes and the viral polymerase, which travel in opposite directions on the template. Thus, the RNA must be "reset" prior to RNA replication (Barton et al., 1999). The regulation of this switch is dependent, in part, upon the sufficient production of viral proteins, specifically proteinases, which subsequently target the ITAFs that initially promoted the translation of the proteinases themselves. As mentioned previously, PCBP2 binds to both S-L I and S-L IV of poliovirus genomic RNA, but with much greater affinity to S-L IV in isolation (Gamarnik and Andino, 2000). However, upon cleavage by the viral 3CD/3C proteinase, the cleaved PCBP2 is unable to stimulate IRES-driven translation. 3CD/3C liberates the C-terminal KH3 domain, leaving the N-terminal portion of the protein unable to form a complex with S-L IV and therefore unable to aid in ribosome recruitment (Perera et al., 2007). The N-terminal portion of PCBP2 is, however, still capable of interacting with S-L I RNA structures, an interaction that is enhanced when the viral proteinase precursor 3CD is present on this 5′-terminal RNA structure (Gamarnik and Andino, 1998; Perera et al., 2007). The resulting ternary complex formed between S-L I RNA, PCBP2, and 3CD promotes viral RNA synthesis (Parsley et al., 1997; Gamarnik and Andino, 2000). Although the interaction between PCBP2 and S-L I may aid in the stimulation of viral protein synthesis early during infection, it is the presence of 3CD and subsequent interactions with PCBP2 that allows the viral RNA replication process to proceed (Kempf and Barton, 2008). Adding further regulation to the template usage switch orchestrated by poliovirus is the fact that PTBP1, another ITAF, is cleaved by 3CD/3C leading to inhibition of poliovirus translation (Back et al., 2002). Similarly, cleavage of the nuclear shuttling polyadenylate-binding protein 1 (PABP1), which functions in regulation of mRNA metabolism and is closely associated with RNA and mRNP complexes, has been proposed to be involved in the inhibition of poliovirus translation because expression of PABP1 resistant to poliovirus 3C mediated cleavage during infection increases viral protein synthesis from non-replicating reporter RNAs and reduces viral RNA accumulation, compared to wild type PABP1 expression (Afonina et al., 1998; Bonderoff et al., 2008). HAV likely utilizes a similar method to proceed from translation to RNA replication, since the 3C proteinase of this virus has been shown to cleave PCBP2 and PTBP1, resulting in reduced protein-RNA affinity and suppression of translation (Zhang et al., 2007; Kanda et al., 2010). Increased viral protein accumulation and alterations to nuclear alterations to nuclear RNA-binding proteins is the mechanism by which poliovirus and HAV RNA replication is able to proceed, but whether this mechanism to induce a template usage switch is broadly applicable to other picornaviruses is not clear. Indeed, a recent report demonstrated that three HRV serotypes do not induce the cleavage of PCBP2 or PTBP1 during infection of a human lung cell line, suggesting alternative mechanisms must be used under theses experimental conditions (Chase and Semler, 2014).

A Nuclear-resident Protein Removes VPg From Viral Genomic RNA Molecules

Finally, in addition to the nuclear-resident proteins utilized by picornaviruses to promote IRES-mediated protein production, another nuclear-resident protein functions in RNA processing of poliovirus RNA. VPg, the viral protein covalently attached to the 5′-terminus of the viral RNA, is cleaved from viral RNAs found on polysomes (Ambros et al., 1978). This cleavage is performed by 5′-tyrosyl–DNA phosphodiesterase-2 (TDP2), which normally functions to remove covalent adducts from DNA via hydrolysis of 5′-phosphodiester bond (Virgen-Slane et al., 2012). VPg serves as the protein primer for viral RNA synthesis and is present on encapsidated RNA molecules, but the functional role of TDP2 in viral infection and its VPg cleavage capability is not completely clear. Independent of understanding the precise role of TDP2 during infection, this protein provides an additional example of nuclear-resident proteins being used in diverse ways, even during the very initial steps in the infectious cycles of picornaviruses.

During the initial rounds of IRES-mediated translation, picornaviruses co-opt nuclear shuttling proteins that are encountered in the cytoplasm of an infected cell. As genome amplification proceeds and further rounds of translation are initiated however, increasing amounts of nuclear-resident proteins are required in the cytoplasm of cells to facilitate a productive infectious cycle. To provide these critical nuclear factors to the sites of viral replication, alterations to nucleocytoplasmic trafficking and the normal compartmentalization of cellular proteins occurs, resulting in a large cytoplasmic stock of nuclear factors. How this is achieved is the focus of a subsequent section of this review. So, despite the fact that the early rounds of viral translation and genomic replication can proceed utilizing the limited supply of nuclear-resident proteins already in the cytoplasm, successive rounds require the selective loss of cellular protein compartmentalization allowing cellular factors to be available

TABLE 2 | Nuclear-resident proteins involved in picornavirus translation.

Nuclear-resident protein	Picornavirus IRES type	Effect	References
Polypyrimidine tract-binding protein 1 (PTBP1)	I, II, III	Promotes translation through stabilization of IRES structure	Jang and Wimmer, 1990; Luz and Beck, 1991; Chang et al., 1993; Hellen et al., 1993; Kaminski et al., 1995; Kolupaeva et al., 1996; Niepmann, 1996; Pestova et al., 1996; Hunt and Jackson, 1999; Gosert et al., 2000a; Song et al., 2005; Kafasla et al., 2009, 2010; Verma et al., 2010; Kafasla et al., 2011
Lupus La protein (La)	I, II, III	Stimulates translation from Type I and Type II IRESs; suppresses translation from Type III IRES structures	Meerovitch et al., 1989, 1993; Craig et al., 1997; Kim and Jang, 1999; Ray and Das, 2002; Costa-Mattioli et al., 2004; Cordes et al., 2008
Poly(rC)-binding protein 2 (PCBP2)	I, III	Stimulates translation from Type I and Type III IRESs but does not bind Type III IRES element	Blyn et al., 1995, 1996; Gamarnik and Andino, 1997; Parsley et al., 1997; Graff et al., 1998; Zell et al., 2008a,b; Sean et al., 2009; Sweeney et al., 2014
Serine/arginine-rich splicing factor 3 (SRSF3 or SRp20)	I	Acts synergistically with PCBP2 to increase efficiency of poliovirus translation	Bedard et al., 2007; Fitzgerald and Semler, 2011
Proliferation-associated protein 2G4 (PA2G4 or EBP1)	II	Necessary for FMDV translation but not required for TMEV or EMCV translation	Pilipenko et al., 2000; Monie et al., 2007
Nucleolin	I	Stimulates translation	Waggoner and Sarnow, 1998; Izumi et al., 2001
Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1)	I	Interacts with EV71 IRES, depletion results in reduced reporter gene translation	Lin et al., 2009b
Far upstream element-binding protein 1 (FBP1)	I	Interacts with EV71 IRES	Huang et al., 2011
KH domain-containing, RNA-binding, signal transduction-associated protein 1 (Sam68)	II	Promotes FMDV translation	Lawrence et al., 2012
Double-stranded RNA binding protein 76 (DRBP76)	I	Heterodimerizes with nuclear factor of activated T-cells, 45 KDa (NF45) and inhibits HRV 2 translation	Merrill et al., 2006; Merrill and Gromeier, 2006
Far-upstream element-binding protein 2 (FBP2)	I	Inhibits EV71 IRES-driven translation	Lin et al., 2009a; Chen et al., 2013
AU-rich binding factor 1 (AUF1 or hnRNP D0)	I	Binds IRES element and inhibits translation	Cathcart et al., 2013; Wong et al., 2013; Lin et al., 2014
Gem-associated protein 5 (Gemin5)	II	Likely inhibits FMDV translation through competitive inhibition of PTBP1 binding	Piñeiro et al., 2013

for viral replication processes. It is clear that nuclear resident proteins play a critical role in the regulation of picornavirus translation, despite that fact that these positive-sense RNA viruses complete their replication cycle in the cytoplasm of the infected cell.

Nuclear-resident Proteins Utilized in the Process of Picornavirus RNA Replication

Although picornaviruses encode their own RNA-dependent RNA polymerase (3D), they utilize host cell factors in order to

augment the function of this replicase enzyme. As with IRES-dependent translation, most of the factors utilized in the process of RNA synthesis are nuclear-resident proteins with RNA-binding functions that can be used by the virus to facilitate the replication of viral RNA molecules. These host proteins act in the context of RNP complexes they form with picornavirus RNAs to impart replication specificity to the polymerase, as 3D is able to replicate RNA non-specifically when provided with a primed template *in vitro* (Tuschall et al., 1982). During an infection, however, 3D solely replicates picornavirus RNA despite the large excess of cellular mRNA present. To make use of picornavirus RNA templates exclusively, complexes of picornavirus RNA, host nuclear-resident proteins, and viral proteins are thought to act as recognition elements that enable template recognition by 3D and initiate RNA replication. Particularly critical in the regulation of 3D appears to be 5′-3′ intramolecular interactions that yield circularized templates. This section will focus on those nuclear-resident proteins that are known to promote either the production of intermediate negative-sense RNA molecules or the amplification of positive-sense RNA molecules from this template.

As discussed previously, a template usage switch from translation to RNA replication occurs as viral proteins accumulate and, in some cases, proteinases cleave host cell factors functioning as ITAFs. Prior to this transition, it has been proposed that optimal translation of poliovirus RNA requires the circularization of the RNA molecule, likely allowing ribosomes to be efficiently reloaded on the template (Ogram et al., 2010). This finding supports the fact that picornavirus RNA molecules that are to be replicated must first be translated (Novak and Kirkegaard, 1994). Host proteins present on the viral RNA that allow for the initial circularization of the translation-competent template could enhance the efficiency of the circularization of the replication-competent template. This coupling between translation and RNA replication has been suggested to be promoted by at least two common proteins: PABP1 and PCBP2.

The Possible Role of Nuclear-resident Proteins in Promoting Enterovirus Genomic RNA Circularization and Negative-sense RNA Production

The 5′ non-coding regions of picornavirus genomes contain RNA structural elements that are required for the replication of these genomes by acting as scaffolds for protein interactions (Andino et al., 1990; Barton et al., 2001; Nateri et al., 2002; Nagashima et al., 2008). Electrophoretic mobility shift assays incorporating recombinant proteins and subgenomic portions of poliovirus RNA molecules have been instrumental in identifying the components of RNP elements *in vitro*, that may be important for the process of enterovirus RNA replication. The 5′ terminal structure of the poliovirus genome, known as S-L I or cloverleaf, has been shown to be critical for the formation of RNP complexes that function in the initiation of RNA synthesis (Andino et al., 1993). One of the proteins involved in this RNP formation is the nuclear-resident PCBP2, which binds to the S-L I structure with increased affinity when the viral polymerase precursor, 3CD, is also present near the 5'-terminus of the RNA, forming a ternary complex (Gamarnik and Andino, 1997, 2000; Parsley et al., 1997). On the opposite terminus of the genome, PABP1 associates with the genetically encoded poly(A) tract of the poliovirus genome. Through co-immunoprecipitation using antibodies directed against PABP1, it has been demonstrated that 3CD and PABP1 directly interact in poliovirus-infected cells. As a result, it has been proposed that PABP1 acts as a bridge to link both ends of the viral genome because it is able to simultaneously interact with the 3′-terminus of poliovirus genomic RNA as well as both 3CD and PCBP2, which are present on the 5′-terminus of the same RNA molecule (Herold and Andino, 2001). More recently it has been shown that PCBP2 binds to both the S-L I structure and to a C-rich spacer region that is found between S-L I and the IRES element (Toyoda et al., 2007). Based on similar interactions between PCBP2 and CVB3 RNA, it is likely that PCBP2 modulates the RNA replication of this closely related virus, and the poly(C) binding protein hnRNP K may be exploited in place of PCBP2 by EV71 (Lin et al., 2008; Zell et al., 2008a,b) (**Figure 4A**).

The nuclear Sam68 which is a putative regulator of mRNA stability and mRNA nuclear export, has been identified as interacting with poliovirus 3D through yeast two-hybrid assays; however, the role this protein plays in the viral RNA replication cycle is not clear (McBride et al., 1996; Coyle et al., 2003). Non-POU domain-containing octamer-binding protein (NONO), identified as interacting with poliovirus RNA through thiouracil cross-linking mass spectrometry, also impacts the generation of positive-sense RNA during infection (Lenarcic et al., 2013). Another nuclear-resident protein with a proposed role in picornavirus replication is RNA Helicase A (RHA), which binds the 5′-NCR of FMDV genomic RNA (Lawrence and Rieder, 2009).

A Nuclear-resident Protein Promotes Enterovirus Genomic RNA Production

The production of negative-sense viral RNA from genomic templates results in the formation of a double-stranded RNA molecule called the replicative form. The replicative form structure contains the template for the production of genomic RNA, and therefore the duplexed RNA strands within this structure must be separated at the 3'-terminus of the negative-strand RNA to allow for 3D association and the initiation of RNA synthesis. A single host-cell protein, heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP C1/C2), has been demonstrated to promote the amplification of positive-strand poliovirus RNA from the negative-strand template and, as with the majority of other host proteins involved in the infectious cycles of picornaviruses, is a nuclear-resident protein. hnRNP C1 and C2 are produced by alternative splicing, with the C2 isoform containing 13 additional amino acids (Koloteva-Levine et al., 2002). Together, these hnRNP C1 and C2 proteins form a heterotetramer containing three copies of C1 and a single copy of C2 that bind pre-mRNA, regulate splicing, and nucleate the formation of 40S hnRNP particles (Barnett et al., 1989; Huang et al., 1994). Each C protein contains an RNA recognition motif, an oligomerization domain, a nuclear localization signal, and a nuclear retention signal (Görlach et al., 1992; McAfee et al.,

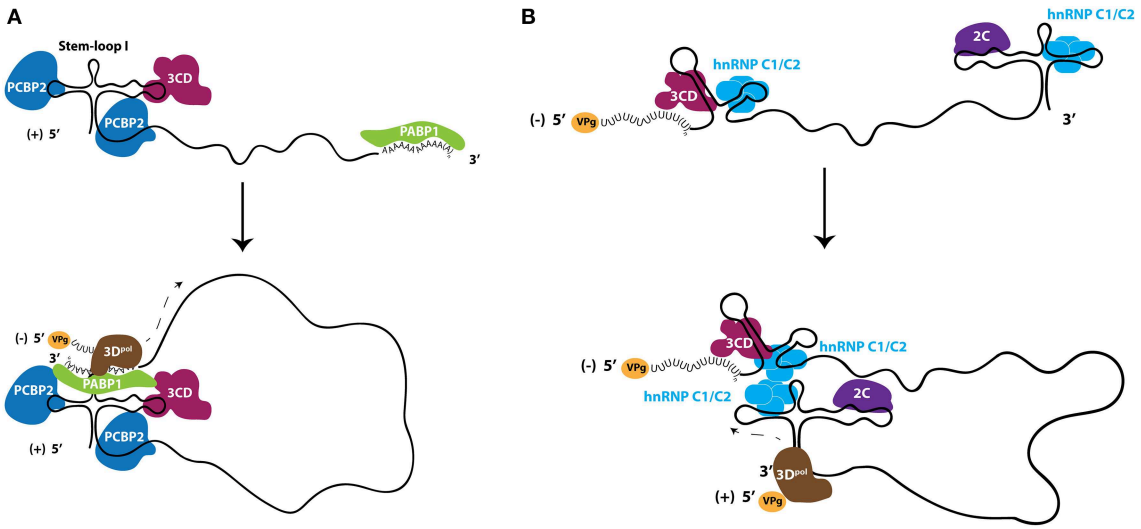

FIGURE 4 | Ribonucleoproteins (RNPs) comprised of nuclear-resident proteins facilitate enterovirus RNA replication. (A) Nuclear-resident proteins PCBP2 (dark blue) and PABP1 (green) act in conjunction with viral protein 3CD (fuchsia) to circularize genomic RNA for use as templates to produce negative-sense RNA intermediates. **(B)** Nuclear protein hnRNP C1/C2 (light blue) interacts with both termini of negative-sense RNA molecules and is hypothesized to circularize the negative-sense template to promote genomic RNA production. Although likely in the form of double-stranded RNA, the negative-sense RNA is shown here as single stranded for clarity. Viral protein 2C (purple) interacts with the 5′-terminus of negative-sense RNA, although the direct function of this protein in viral RNA replication is unclear. The viral RNA-dependent RNA polymerase $3D^{pol}$ (brown) is recruited to these circularized templates and initiates viral RNA synthesis. VPg (yellow), the viral protein that primes RNA synthesis, is found on RNA molecules that have not been translated.

1996; Nakielny and Dreyfuss, 1996; Wan et al., 2001). In contrast to many other hnRNP proteins, hnRNP C1/C2 appears to be restricted to the nucleus and does not shuttle to the cytoplasm in complex with mRNA (Piñol-Roma and Dreyfuss, 1992, 1993). hnRNP C1/C2 can bind both the 3′- and 5′-termini of poliovirus negative-sense RNA intermediates (regions complementary to the 5′NCR and 3′NCR of genomic RNA, respectively) and has been proposed to play a role in poliovirus RNA replication by facilitating and/or stabilizing the terminal strand separation required for replication of this template (Roehl and Semler, 1995; Brunner et al., 2005; Ertel et al., 2010). The association of hnRNP C1/C2 with both termini of the negative-sense RNA molecule may also allow for the end-to-end linkage of this RNA template via the multimerization of hnRNP C1/C2 tetramers, since the multimerization domain of this protein is required for efficient *in vitro* replication of poliovirus RNA (Ertel et al., 2010). Recombinant hnRNP C1/C2 is able to rescue positive-strand RNA synthesis in cellular extracts depleted of endogenous hnRNP C1/C2, supporting a critical role for this protein in the production of poliovirus genomic RNA (Brunner et al., 2005). It has also been demonstrated that hnRNP C1/C2 interacts with the poliovirus protein 3CD (the polymerase precursor) through glutathione S-transferase pull-down assays; therefore, hnRNP C1/C2 may aid in the recruitment of the 3D polymerase to the replication template (Brunner et al., 2005). Furthermore, reduced cellular levels of hnRNP C1/C2 cause a decrease in the kinetics of poliovirus RNA synthesis during infection (Brunner et al., 2010). Combined with the finding that an intact 3′ NCR of poliovirus genomic RNA contributes to positive-strand RNA synthesis efficiency through complementary elements conserved at the 5′ end of negative-sense strand, a model for positive-sense RNA synthesis has been proposed. This model is reminiscent of negative-sense RNA production, with both ends of the RNA template in close proximity, albeit in the form of a predominantly dsRNA molecule (Ertel et al., 2010). Due to the proximity of the ternary complex found on the 5′-end of the positive-sense poliovirus RNA molecule, it is possible that at least one polymerase utilized in the synthesis of the positive-sense RNA is recruited directly from the ternary complex on the genomic RNA molecule to the negative-sense template, as suggested by the trans-initiation model (Vogt and Andino, 2010) (**Figure 4B**).

The circularization of RNA templates proposed to promote poliovirus RNA replication is made possible by nuclear-resident proteins. Circularized templates may serve to function as a fidelity check on the RNA itself, to act *in lieu* of a true promoter region to enhance the initiation of RNA synthesis, and to provide a mechanism by which 3D specifically recognizes a polyadenylated mRNA of viral origin in an infected cell containing abundant polyadenylated mRNA transcripts. While there has been little exploration of the RNA replication process in genera beyond the enteroviruses, it is possible that, as with the handful of nuclear proteins that are considered picornavirus-general ITAFs, there is a minimal requirement of particular nuclear-resident proteins to promote picornavirus RNA replication (at present the three major players appear to be PCBP2, PABP1, and hnRNP C1/C2), with other cellular proteins that may be species-specific that act to further enhance the efficiency of RNA replication. As with translation, the cohort of proteins utilized for picornavirus RNA replication may also be dependent upon the availability of particular proteins within

the context of the cellular microenvironment where the viral RNA molecules are located. The different levels of enhancement provided to picornavirus RNA replication through the activities of different (or available) nuclear-resident host-cell proteins may contribute to the variable ratios of positive- to negative-sense RNA ratios, which have been reported to range from 30:1 to 70:1 for poliovirus (Andino et al., 1990; Giachetti and Semler, 1991; Novak and Kirkegaard, 1991).

Alteration to Nucleocytoplasmic Trafficking Causes the Loss of Normal Subcellular Localization of Nuclear-resident Proteins and Facilitates Picornavirus Replication

As discussed previously, the initial rounds of picornavirus translation and RNA replication are dependent on nuclear-resident proteins that are present in the cytoplasm of the infected cell as a result of their shuttling function or nascent biogenesis. As the replication process continues, there is an amplification in both viral protein production and RNA replication as the number of viral RNA templates grows. The low concentration of nuclear proteins normally present in the cytoplasm is no longer sufficient to meet the increased demand for these proteins. As a result, picornaviruses alter nuclear-cytoplasmic trafficking to provide the functions of normally nuclear-resident proteins to the cytoplasm where viral replication takes place.

Enterovirus Proteinases Degrade the Nucleoporin Proteins of the NPC

Because the NPC is the main route by which the nucleus and cytoplasm exchange material, picornaviruses target the NPC specifically to disrupt normal protein trafficking pathways resulting in the cytoplasmic accumulation of cellular proteins. Enterovirus (poliovirus or HRV 14) infection alters both the classical import pathway, which relies on a heterodimer consisting of an importin-α (karyopherin α) adaptor protein that binds the arginine-lysine-rich NLS of the cargo protein and transport receptor importin-β1 (karyopherin β1), as well as the transportin-1 (karyopherin β2) pathway in which the import receptor transportin recognizes a glycine-rich motif known as the M9 NLS (reviewed in Cautain et al., 2015). In uninfected cells expressing enhanced green fluorescent protein (EGFP) linked to either a classical NLS derived from the large T antigen of simian virus 40 (SV40) or the M9 NLS of hnRNP A1, EGFP localizes to the nucleus. However, upon infection with poliovirus, an accumulation of EGFP protein in the cytoplasm is observed by 4.5 h post-infection, demonstrating a disruption in these two import pathways as a consequence of infection (Gustin and Sarnow, 2001). Additionally, hnRNP K, which contains a unique 40-amino acid motif NLS, known as KNS, is also relocalized to the cytoplasm of poliovirus infected cells, suggesting the import of proteins through the KNS-mediated pathway is also prevented during infection (Michael et al., 1997; Gustin and Sarnow, 2001). HRV 14 infection also causes cytoplasmic localization of EGFP fusion proteins containing a classical or M9 NLS, albeit at later times during infection than observed for poliovirus (Gustin and Sarnow, 2002). Conversely, an EGFP fusion protein containing an NLS that mediates nuclear import through a hormone-dependent but unknown importin-α-independent pathway remains localized to the nucleus upon poliovirus infection, suggesting that specific import pathways are targeted by poliovirus, while some import pathways remain functional. Furthermore, an EGFP fusion protein containing a leucine-rich NES recognized by the chromosome region maintenance 1 (CRM1) export receptor is localized to the cytoplasm during infection, and a small molecule inhibitor of CRM1 causes retention of the EGFP fusion protein in the nucleus, suggesting that this export pathway is unaltered in poliovirus infected cells (Gustin and Sarnow, 2001).

Like poliovirus, CVB3 also causes the relocalization of GFP fused with a classical NLS, indicating that particular alterations to nuclear-cytoplasmic transport pathways is a general feature of enteroviruses (Belov et al., 2000). However, the use of Timer proteins that change emission fluorescence based on their age has shown that the accumulation of nuclear proteins in the cytoplasm of enterovirus infected cells is the result of increased efflux of these proteins from the nucleus as a result of NPC degradation, rather than simply disruption of the import of newly-synthesized proteins into the nucleus (Belov et al., 2004).

The finding that nuclear-resident proteins accumulate in the cytoplasm of cells upon picornavirus infection as a result of increased protein efflux from the nucleus appears at odds with the observation that picornavirus-induced disruptions in nuclear import cause cytoplasmic retention of newly synthesized nuclear-resident proteins. However, these seemingly disparate findings can be reconciled upon closer examination of the particular alterations made to the NPC during infection. The increased permeability of, and inability to import proteins through the NPC during enterovirus infection is the result of changes made to Nup proteins that comprise the NPC itself. Electron microscopy of poliovirus-infected cells shows structural alterations to the nuclear envelopes and nuclear pores, specifically the loss of an obstructing bar-like structure in the central channel, which is at least partially caused by the viral proteinase 2A. General inhibitors of the 2A proteinase suppress the efflux of marker proteins from the nucleus during infection. In addition, transfection of a wild type 2A expression construct, but not a construct encoding an inactive 2A, into cells yields cytoplasmic re-localization of stably expressed GFP-NLS proteins (Belov et al., 2004). Like enteroviruses, the cardiovirus EMCV breaks down the specificity of bidirectional protein traffic through the NPC in infected cells by directly modifying the architecture of the NPC (Lidsky et al., 2006).

Structural data from electron microscopy studies showing destruction of the NPC and products of proteolysis within the pore bolster biochemical data that demonstrates the degradation of Nup 153 and Nup 62 in cells infected with either poliovirus or rhinovirus. Moreover, immunofluorescence microscopy indicates a decrease in overall levels of these Nups as the course of an enterovirus infection proceeds (Gustin and Sarnow, 2001, 2002). Prior to the proteolysis of Nup 153 and Nup 62, Nup 98 is degraded by 2A in poliovirus-infected cells. The cleavage of Nup 98 is insensitive to guanidine hydrochloride

treatment, which inhibits enterovirus RNA replication and results in reduced viral protein production, whereas the cleavage of Nup 153 and Nup 62 is sensitive to the presence of guanidine. In conjunction with the fast kinetics of this cleavage (within 1 h post-infection), this suggests that Nup 98 is cleaved even when there is a very low concentration of viral protein present within the infected cell. This also suggests that poliovirus (and perhaps other enteroviruses) may target specific Nups and trafficking pathways at different times in the infectious cycle to facilitate viral replication (Park et al., 2008). The addition of purified HRV 2 2A to whole cell lysates causes the cleavage of Nup 98 while the expression of poliovirus 2A in cells results in the degradation of Nup 62, Nup 98, and Nup 153, demonstrating that 2A is able to alter components of the NPC (Park et al., 2008; Castelló et al., 2009). Furthermore, purified HRV 2 2A is able to cleave recombinant Nup 62 *in vitro*. This cleavage event liberates an FG-rich region, a domain important for nuclear transport receptor association during transport through the NPC, of Nup 62 during infection (Park et al., 2010). Interestingly, experiments utilizing recombinant 2A from the three different HRV clades demonstrated that these proteinases cleave Nup 62, Nup 98, and Nup 153 at distinct sites and with variable rates (Watters and Palmenberg, 2011).

Enterovirus 2A has an obvious role in nucleoporin proteolysis during infection; however, two additional nucleoporins, Nup 214 and Nup 358, as well as the previously mentioned Nup 153, are degraded in cells transfected with HRV 16 3C or 3CD expression constructs, suggesting that the proteolytic activity of 2A alone does not account for all Nup degradation in enterovirus-infected cells (Ghildyal et al., 2009). In support of this suggestion, Nup 62 does not contain a 2A specific Tyr-Gly cleavage site and the size of Nup 153 cleavage products from poliovirus-infected cells do not correspond to those expected if 2A does degrade this nucleoporin (Belov et al., 2004). Moreover, high concentrations of purified HRV 14 3C are able to induce the partial cleavage of Nup 62 *in vitro* (Park et al., 2010). Enterovirus-induced alterations to the NPC cause the loss of normal protein partitioning not only for nuclear-resident proteins but also for cytoplasmic-resident proteins, demonstrated by the distribution of normally cytoplasmic-resident proteins such as GAPDH and cyclin-B1 throughout the cytoplasm and nucleus of enterovirus-infected cells (Belov et al., 2004).

Cardiovirus Infection Induces the Hyper-phosphorylation of Nucleoporin Proteins

The 2A proteins of cardioviruses lack proteinase activity, and Nup 62 as well as Nup 153 are stable in mengovirus infected cells (Lidsky et al., 2006). Despite the lack of degradation of these nucleoporins, mengovirus and EMCV promote the redistribution of stably-expressed EGFP proteins containing the classical NLS of the SV40 large T antigen to the cytoplasm and normally cytoplasmic-resident proteins such as cyclin-B1 to the nucleus (Lidsky et al., 2006). The normal subcellular partitioning of proteins in cardiovirus-infected cells, like that of enterovirus-infected cells, is disrupted by dysregulation of bidirectional nucleocytoplasmic trafficking. Unlike enteroviruses, however, cardioviruses achieve this dysregulation through the action of the leader (L) protein. Mengovirus mutants lacking the leader protein coding sequence or encoding an L protein with a mutated zinc finger domain are unable to trigger the cytoplasmic redistribution of a stably-expressed GFP-NLS fusion protein in cells. Furthermore, phosphorylation of a threonine residue at position 47 of the L-protein of mengovirus has also been suggested to play a functional role in L-dependent alterations to nuclear-resident protein localization (Lidsky et al., 2006). Mutations to the L protein of TMEV, specifically disruptions made to the zinc-finger domain of this protein, also fail to facilitate the relocalization of endogenous nuclear proteins to the cytoplasm that are relocalized during infection with wild type TMEV (Delhaye et al., 2004). Protease inhibitors fail to suppress the cytoplasmic redistribution of stably expressed EGFP-NLS fusion proteins in EMCV-infected cellular extracts. Additionally, EMCV replicons containing mutations to the 2A coding sequence do not affect the nuclear envelope leakiness observed during EMCV infection. Taken together, these studies demonstrate that cardioviruses do not utilize a proteinase or viral protein 2A to promote alterations to nucleocytoplasmic trafficking.

In the absence of other cardioviral proteins, recombinant EMCV L alone is able to disrupt normal nuclear localization of a transiently transfected GFP-NLS, and an intact zinc-finger domain within the L protein is specifically required for the observed increase in permeability of the nuclear envelope. A cellular phosphorylation pathway is also required to induce nuclear envelope leakiness, because L protein does not possess kinase activity. Additionally, the protein kinase inhibitor staurosporine can rescue nuclear import/export activity from L-dependent inhibition (Porter and Palmenberg, 2009). Nup 62, Nup 153, and Nup 214 each become hyperphosphorylated in an L-dependent manner as shown by phosphoprotein staining during infection with EMCV, and similarly, Nup 62 and Nup 98 are hyperphosphorylated in mengovirus and TMEV infected cells, respectively, as shown by assaying for gel migration shifts following alkaline phosphatase treatment (Bardina et al., 2009; Porter and Palmenberg, 2009; Ricour et al., 2009b). The phosphorylation of Nup 62 and 98 in mengovirus and TMEV infected cells, respectively, is also dependent upon the zinc-finger domain of L (Bardina et al., 2009; Ricour et al., 2009b). Interestingly, there is no phosphorylation level change of Nup 358 during cardiovirus infection, a Nup that is cleaved during enterovirus infection, (Porter and Palmenberg, 2009).

Direct architectural changes to the NPC can be observed through electron microscopy of NPC cross-sections from cardiovirus infected cells. The central channel of nuclear pores is less electron dense in infected compared to uninfected cells, similar to what is observed in poliovirus-infected cells. How phosphorylation of Nups achieves un-blocking of these pores is not clear (Lidsky et al., 2006; Bardina et al., 2009). One possibility is that the increased negative charge present on FG-motif containing fibrils, as a result of phosphorylation, within the pores that normally act to block passive diffusion of macromolecules, could promote retraction of fibrils to the NPC scaffold leaving the pore empty (Cohen et al., 2012). Through screening a panel of kinase inhibitors, Nup hyperphosphorylation appears to be

carried out via two mitotic terminal kinase effectors within the mitogen activated protein kinase cascade: extracellular signal-regulated receptor kinase (ERK) and p38 mitogen-activated protein kinase (p38), although the exact mechanism by which L co-opts these kinases is not known (Porter et al., 2010). A C-terminal acidic domain within the L protein is also important for the Nup hyperphosphorylation, perhaps via interactions with MAPK pathway regulatory proteins (Porter et al., 2010). Another kinase that may have a role in L-dependent alterations to nucleocytoplasmic trafficking is casein kinase II (CK-2), as it has been shown to phosphorylate Thr-47 of mengovirus L, a phosphorylation event that has a functional role in nuclear-protein efflux (Zoll et al., 2002; Lidsky et al., 2006). The L protein of EMCV has also been shown to bind directly to Ran-GTPase, the concentration of which provides the gradient that imparts directionality to transport, suggesting cardioviruses may utilize multiple strategies to inhibit homeostatic nucleocytoplasmic trafficking (Porter et al., 2006). mRNA export has also been reported to be inhibited in cells expressing TMEV L protein by assaying for poly(A) transcript retention in the nucleus of cells via *in situ* hybridization (Ricour et al., 2009b).

FMDV Infection Does Not Cause Dysregulation of Nucleocytoplasmic Trafficking

Interestingly, another picornavirus of the aphthovirus genus, FMDV, which encodes a proteolytic L-protein, does not appear to target nucleoporins for degradation (Castelló et al., 2009). Moreover, infection with FMDV has not been reported to alter general nucleocytoplasmic trafficking, although some nuclear-resident proteins are redistributed to the cytoplasm of infected cells, likely through a more cellular protein-specific directed approach (Lawrence and Rieder, 2009; Lawrence et al., 2012).

Alterations to NPC Components Are Not a Result of Apoptosis

Apoptotic cell death has been shown to cause damage to the nuclear envelope barrier, including cleavage of Nup 153, through the actions of cellular caspase-9 (Buendia et al., 1999; Faleiro and Lazebnik, 2000). Because enteroviruses can promote apoptotic cell death, it is theoretically possible that the increases in NPC permeability observed during picornavirus infection are due to caspase-9 induction rather than the direct actions of viral proteins themselves. Indeed, poliovirus can cause the initiation of an apoptotic program through caspase-9, and expression of 2A alone can cause cell death through apoptosis (Tolskaya et al., 1995; Agol et al., 1998; Goldstaub et al., 2000; Belov et al., 2003). However, cells deficient in caspase-9 (as well as associated caspase-3) did not show differences in NPC permeability compared to cells expressing normal levels of caspases when infected with poliovirus, suggesting that the increased permeability of the NPC during picornavirus infection is independent of the action of pro-apoptotic caspases (Belov et al., 2004). Moreover, Nup 62 destruction is a marker of picornavirus-infected cells but not apoptotic cells (Buendia et al., 1999; Gustin and Sarnow, 2001, 2002). HAV, CVB3, and TMEV induce apoptosis as well, but these same picornaviruses can also inhibit apoptosis, indicating that the consistent relocalization of cellular proteins to different cellular compartments seen during infection is likely not attributable to pathways involved in programmed cell death (Tolskaya et al., 1995; Gosert et al., 2000b; Henke et al., 2001; Jelachich and Lipton, 2001; Neznanov et al., 2001; Belov et al., 2003; Romanova et al., 2009).

Although both increased efflux of nuclear resident proteins as a result of the dysregulation of the barrier function of the nuclear envelope as well as impediments to nuclear import of nascently produced cellular proteins in the cytoplasm (i.e., prior to host protein translation inhibition by viral infection) have been demonstrated to occur in picornavirus infected cells, these mechanisms are not mutually exclusive. Enterovirus-induced cleavage of Nups 62, 98, 153, 214, and 358 by the 2A and 3CD/3C proteinases, or cardiovirus-induced hyper-phosphorylation of Nups 62, 98, 153, and 214 through actions of the Leader protein, is directly responsible for both the increased "leakiness" of the nuclear envelope and the lack of nuclear import receptor–cargo complex docking at the cytoplasmic face of the NPC. Nucleocytoplasmic trafficking is an intricate and tightly regulated process allowing for precise control of gene expression. As a result, drastic alterations to the components of the NPC gateway between the two major compartments of the eukaryotic cell will have diverse and far-reaching consequences on trafficking pathways. The picornaviruses have evolved to take advantage of this important regulatory node to provide the functions of nuclear-resident proteins to the cytoplasm to promote their replication.

Picornavirus Induced Alterations to Nucleoporin Proteins Result in the Redistribution of Cellular Proteins

The five Nup proteins that are the targets of alteration by picornavirus proteins have critical roles in shuttling macromolecules through the NPC (**Figure 5**). Nup 214 and 358 are positioned on the cytoplasmic side of the NPC, with Nup 358 making up cytoplasmic filaments of the NPC, and Nup 214 residing on the cytoplasmic face of the NPC. Nup 62 is localized to the central pore of the NPC and Nup 153 is a component of the nuclear basket. Nup 98 is found within and on both sides of the NPC and can function, to some degree, independently of the NPC due to its mobile nature (Griffis et al., 2002). All five of these nucleoporins contain FG-repeat domains, indicative of their direct role in nucleocytoplasmic transport (reviewed in Chatel and Fahrenkrog, 2011). Nup 62, in association with other Nups, forms a central "plug" in the channel of the NPC, and the cleavage of this protein by enteroviral proteinases has been suggested to account for the loss of the electron-dense material within the NPC and appearance of "granules" likely corresponding to Nup cleavage products, as observed via electron microscopy (Bardina et al., 2009). This particular alteration could allow for the diffusion of proteins in and out of the nucleus, accounting for the increased "leakiness" of the nuclear envelope observed as a result of infection (Belov et al., 2004). Interestingly, it appears that the alterations made to these proteins individually are not sufficient to promote a loss of normal cellular protein partitioning and that inhibition of nuclear import can only occur in cells with a composite of nucleoporin alterations (Park et al., 2008).

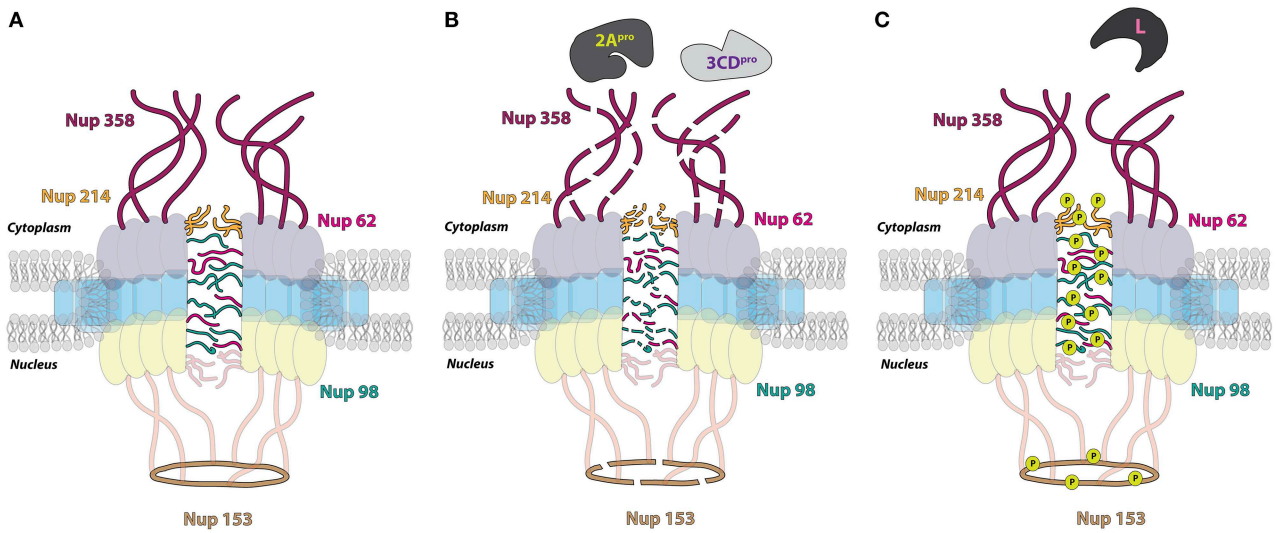

FIGURE 5 | Picornavirus-induced alterations to the nuclear pore complex (NPC). (A) Regions of the NPC where the five nucleoporin (Nup) proteins targeted by picornavirus proteins during infection are located. **(B)** Enterovirus proteinase 3CD/3C ($3CD^{pro}$) cleaves Nup358 (fuschia) and Nup153 (brown), which are components of the cytoplasmic filaments and nuclear basket, respectively. The 2A proteinase ($2A^{pro}$) of enteroviruses degrades FG-containing barrier Nups of the central channel, including the cytoplasmic Nup214 (yellow), as well as Nup62 and Nup98 (pink and green, respectively). **(C)** Cardiovirus infection induces the hyper-phosphorylation (P within chartreuse circle) of the same Nups targeted by enteroviruses, excluding Nup358, through the actions of the Leader protein (L).

Nup 153 has been reported to interact with importin α/β and transportin; therefore, alterations to Nup 153 are at least partially able to account for the inhibition of the nucleocytoplasmic trafficking pathways that rely on these receptors (Shah et al., 1998; Nakielny et al., 1999). Although Nup 98 has been implicated in mRNA export from the nucleus, alterations made to this nucleoporin during enterovirus infection do not inhibit cellular mRNA export, in contrast to the mRNA export inhibition observed in cardiovirus infected cells (Powers et al., 1997; Griffis et al., 2003; Porter et al., 2006; Park et al., 2008; Ricour et al., 2009b). Notably, however, a study in which an enteroviral 2A expression construct was electroporated into cells demonstrated that nuclear export of mRNAs, U snRNAs, and rRNAs but not tRNAs was blocked when 2A was expressed (Castelló et al., 2009). In addition to FG domains present in Nup 358 and Nup 153, these nucleoporins also contain Ran binding domains and as a result, picornavirus targeting of these proteins could conceivably have profound consequences on the compartmentalization of cellular proteins by disruption of the Ran gradient (Wente and Rout, 2010). The L-protein of EMCV, and likely cardioviruses in general, is simultaneously able to disrupt the differential Ran gradient across the nuclear envelope by directly binding and sequestering Ran (Porter et al., 2006).

The picornavirus-mediated changes to components of the NPC critical to nucleocytoplasmic trafficking increase the bidirectional transport of proteins within the infected cell, thus causing a massive dysregulation of the transport process as a whole. However, there appears to be some level of specificity to which import and export pathways are affected, as a subset of nuclear-resident proteins relocalize while others do not. For example, nuclear-resident proteins fibrillarin, TATA-box-binding-protein 1 (TBP1), and serine/arginine-rich splicing factor 2 (SRSF2 or SC35) do not relocalize from the nucleus to the cytoplasm during poliovirus infection (Meerovitch et al., 1993; McBride et al., 1996; Waggoner and Sarnow, 1998; Gustin and Sarnow, 2001). SRSF2 is imported into the nucleus through a unique import receptor called transportin-SR. So it is possible that this pathway is unaffected by picornavirus infection (Kataoka et al., 1999). However, because picornavirus infection halts cellular translation in the early stages of infection, the persistence of some nuclear-resident proteins in the nucleus cannot be attributed to continued transport of newly synthesized proteins to this subcellular compartment. Nuclear-shuttling proteins, however, could be inhibited from re-entering the nucleus by picornavirus-mediated disruptions to trafficking pathways, trapping these proteins in the cytoplasm. Protein-specific nuclear retention signals (NRSs) may allow some proteins to maintain normal localization throughout the course of infection, although not all proteins that contain NRSs remain in the nucleus, as even the strongly nuclear-resident hnRNP C1/C2 is relocalized to the cytoplasm between 3 and 4.5 h following infection with enteroviruses (Gustin and Sarnow, 2001). Further complicating attempts to fully explain how picornavirus infection affects the subcellular localization of endogenous proteins is the fact that some normally-nuclear-resident proteins such as nucleolin, La, Sam68, hnRNP A1, hnRNP K, and hnRNP C1/C2 are almost completely relocalized to the cytoplasm upon poliovirus infection (as observed by immunofluorescence microscopy), even though a general increase in bidirectional trafficking through the nuclear envelope would predict a uniform distribution of these proteins between the nucleus and cytoplasm (Gustin and Sarnow, 2001). It is

possible that the apparent uneven redistribution of some proteins between the nucleus and cytoplasm as a result of picornavirus infection can be interpreted as these proteins being associated with specific cellular or viral structures that do not change their subcellular location upon infection. For example, retention of nuclear-resident proteins within the nucleus following infection could be explained by these proteins having strong associations with DNA, which remains in the nucleus regardless of virus-induced nuclear pore modifications.

Many proteins, including nucleolin, hnRNP A1, PTBP1, La, Sam68, hnRNP K, hnRNP C1/C2, SRSF3, and TDP2, that are relocalized as a result of infection have known functions in the replication cycle of picornaviruses (Meerovitch et al., 1993; McBride et al., 1996; Waggoner and Sarnow, 1998; Gustin and Sarnow, 2001, 2002; Back et al., 2002; Ricour et al., 2009a; Fitzgerald and Semler, 2011; Virgen-Slane et al., 2012; Fitzgerald et al., 2013). Aside from direct roles in picornavirus replication, redistribution of nuclear proteins likely has consequences on cellular homeostasis. For example, because Sam68 functions in cell cycle transitions, the redistribution of this protein as a result of infection could disrupt the cell cycle. Other proteins, including various splicing factors which appear to have no role in viral replication, have also been shown to redistribute to the cytoplasm of poliovirus 2A expressing cells, likely as a mechanism to further disrupt host-cell gene expression (Alvarez et al., 2013). While the relocalization of most cellular proteins utilized by picornaviruses during infection can be attributed to disruption of homeostatic nucleocytoplasmic trafficking, some proteins are relocalized, at least partially, through direct cleavage by viral proteinases and the subsequent loss or alteration of functional NLS regions within these proteins. Cleavage of PTBP1 and La, both of which contain a bipartite NLS, by poliovirus 3CD/3C exemplifies this phenomenon (Simons et al., 1996; Romanelli et al., 1997; Shiroki et al., 1999; Back et al., 2002). RHA and Sam68, which likely function in aphthovirus RNA replication and translation, respectively, are mislocalized in FMDV infected cells. This relocalization has been attributed to the demethylation of RHA and liberation of NLS containing-regions of Sam68 through cleavage by FMDV L proteinase (Lawrence and Rieder, 2009; Lawrence et al., 2012). Furthermore, the nuclear efflux of RHA occurs with a concomitant influx of Jumonji C-domain containing protein 6, a demethylating protein that may act on RHA. This suggests that because FMDV does not degrade nucleoporin proteins directly, it may have more precise control over cellular protein trafficking compared to general disruptions in trafficking pathways observed with other picornaviruses (Lawrence et al., 2014).

Virus-induced NPC Alterations Lead to Interference with Host Antiviral Defenses

In addition to concentrating proteins with functions that are co-opted by picornaviruses for replication in the cytoplasm, alterations to nucleocytoplasmic trafficking can also disable innate antiviral signaling cascades in infected cells. One of the earliest host responses to viral infection, the innate antiviral response, is mediated primarily by the action of Type-I interferons (IFNs). This response is activated by the cellular recognition of viral molecules called pathogen associated molecular patterns (PAMPs). One major receptor of intracellular PAMPs that recognize picornavirus family members is interferon-induced helicase C domain-containing protein 1 (also known as melanoma differentiation-associated protein 5 or MDA-5) (Kato et al., 2006). Upon PAMP recognition, cytoplasmic receptors such as MDA-5 initiate a cascade of events that results in the activation of transcription factors such as IFN regulatory factor 3 (IRF-3), nuclear factor (NF)-κ-B, and transcription factor AP-1. Importins then act in transporting these transcription factors through the NPC to the nucleus, which, in turn, activates the transcription of type I interferons (IFN-α/β) and interferon-stimulated genes (ISGs). The resultant IFN mRNA is exported to the cytoplasm where it is translated and then secreted, inducing a secondary response in an autocrine and paracrine process. This results in the activation of a second signaling cascade involving many effectors and transcription factors such as signal transducer and activator of transcription (STAT) proteins, which translocate to the nucleus. Within the nucleus, these proteins activate transcription of additional ISGs. These IFN stimulated gene products target the pathogen for destruction (Younessi et al., 2012).

Changes made to the NPC by picornaviruses can lead to an attenuation in ISG expression and the associated antiviral response. Nup 98 is cleaved much more rapidly than the other Nup targets in poliovirus infected cells, and this has been suggested to be an early target of picornavirus proteins to diminish the transcriptional-induction of antiviral genes. Experiments utilizing guanidine hydrochloride, which inhibits enterovirus RNA replication and, as a result, normal levels of viral protein production, have shown that cleavage of Nup 98 in poliovirus-infected cells is not sufficient to cause relocalization of proteins like nucleolin and that high concentrations of viral proteins are needed to degrade other nucleoporin targets to allow the widespread redistribution of nuclear-resident proteins to the cytoplasm (Park et al., 2008). Nup 98 is a component of the NPC but can also be found dissociated from this complex both in the nucleus and the cytoplasm. Enteroviral 2A and cardioviral L may be capable of targeting free Nup 98 prior to direct transcriptional shut-off, which occurs when 3C precursors enter the nucleus of infected cells (discussed in the subsequent section), and interfere with signaling to the nucleus and/or the export of ISG transcripts. As a result, the virus may be able to avoid inducing an early antiviral response within the cell and promote maximal viral amplification (Park et al., 2008). Interestingly, Nup 98 has recently been shown to act as a transcription factor and induce the expression of antiviral defense genes in *Drosophila* (Panda et al., 2014). Therefore, the rapid degradation of Nup 98 in infected cells could allow enteroviruses to limit innate antiviral responses as well as set the stage for dysregulation of nucleocytoplasmic trafficking early during infection. The importance of restricting the production of IFN-α/β during picornavirus infection is demonstrated by the fact that pre-treatment of cells with IFN-α/β inhibits picornavirus replication, confirming that once ISG products are expressed they cannot be overcome (Chinsangaram et al., 2001).

As mentioned above, the cardioviral TMEV L protein prevents export of cellular mRNAs from the nucleus. Furthermore, the TMEV L protein inhibits the transcription of cytokine and chemokine genes that are ordinarily activated upon viral infection (Van Pesch et al., 2001). This inhibition can be attributed to the fact that TMEV infection disallows the formation of IRF-3 dimers, which normally translocate to the nucleus to regulate transcription of antiviral genes (IFN-α/β as well as ISGs) in response to infection. Because infection with TMEV containing a mutation in the zinc finger motif of L or a partial deletion of L allows the dimerization of IRF-3 but wild type TMEV infection does not, the inability of this nuclear translocation and subsequent antiviral protein production can be attributed to antagonizing this pathway by the L protein. Moreover, disruptions in nucleocytoplasmic trafficking and the block to mRNA export from the nucleus correlate with Nup 98 hyper-phosphorylation (Ricour et al., 2009a). The leader protein of mengovirus has also been demonstrated to inhibit IFN-α/β expression in infected cells by suppressing the activation of NF-κB, a suppression dependent upon phosphorylation of Thr-47 in the L protein (Zoll et al., 2002). Interestingly, negative-sense RNA complementary to the L coding region of the EMCV genome was recently shown to be a determinant of MDA-5 mediated interferon production (Deddouche et al., 2014). Finally, the L proteinase of FMDV functions in the inhibition of IFN-β mRNA induction. However, this inhibition is not dependent upon the dysregulation of cellular nucleocytoplasmic trafficking because FMDV does not target these pathways. Instead, the FMDV L proteinase enters the nucleus and promotes the degradation of an NF-κB component (De Los Santos et al., 2006, 2007).

The Redistribution of Cellular Proteins as a Result of Infection Is Not Necessarily Specific

Because fundamental components and regulatory mechanisms of nucleocytoplasmic trafficking are disrupted during picornavirus infection, some proteins that relocalize to the cytoplasm of infected cells have no known function in viral replication, and in some instances even have antiviral roles. AU-rich binding factor 1 (AUF1, also known as hnRNP D0) binds with high affinity to RNA molecules containing AU-rich elements (usually in the 3′ non-translated region), is a predominantly nuclear-resident protein that shuttles to the cytoplasm, and promotes mRNA turnover (reviewed in Gratacós and Brewer, 2010). AUF1 relocalizes from the nucleus to the cytoplasm of poliovirus, HRV 14, and HRV 16 infected cells in a 2A-driven manner and binds to S-L IV within the IRES of poliovirus RNA (Rozovics et al., 2012; Cathcart et al., 2013). The presence of this protein inhibits poliovirus translation in a dose dependent manner *in vitro,* and cells lacking AUF1 produce higher titers of poliovirus, CVB3, and HRV 1a (Cathcart et al., 2013). The enteroviral proteinase precursor 3CD and mature 3C cleave AUF1 *in vitro*, leading to a decrease in the affinity of AUF1 for poliovirus S-L IV (Cathcart et al., 2013; Wong et al., 2013). Consequently, enteroviral infection and resultant NPC degradation cause the relocalization of this negative regulator of viral replication, but subsequent action by viral proteinases diminishes the antiviral effect of AUF1. Similar results have been observed with EV71 (Lin et al., 2014). Interestingly, AUF1 also relocalizes from the nucleus during cardiovirus infection but does not have a negative effect on EMCV amplification and is not degraded during infection (Cathcart and Semler, 2014). The fact that the anti-enteroviral AUF1 is relocalized in enterovirus-infected cells demonstrates that nucleocytoplasmic trafficking disturbances can cause relocalization of a broad subset of nuclear-resident proteins that do not necessarily benefit viral replication.

Although cardioviruses and enteroviruses target many of the same nucleoporin proteins, the mechanisms that cause cytoplasmic accumulation of nuclear proteins are distinct. Enteroviral proteinases degrade FG-repeat containing Nups while cardioviruses promote phosphorylation of these Nups. Nonetheless, the consequences of Nup alterations by picornaviruses are equivalent: they provide the proteins that facilitate viral replication in the cytoplasm. Additionally, degradation of NPC components functions as a mechanism to limit signal transduction to the nucleus and thereby attenuate host antiviral defense pathways. Although to what degree picornaviruses are able to specifically orchestrate different nucleocytoplasmic trafficking pathways is unclear, increased NPC leakiness (i.e., increased export of outbound macromolecules from the nucleus and the restriction of inbound cargo from the cytoplasm) is a functionally significant event that many picornaviruses have evolved to accomplish in order to mount effective cytoplasmic replication strategies.

Picornavirus Proteins Enter the Nucleus to Limit Host-cell Gene Expression

In addition to shutting down most host-cell translation through actions in the cytoplasm, some picornaviruses also target the transcriptional components of cellular gene expression. Picornavirus infection has long been known to greatly reduce the initiation rate of cellular RNA synthesis (Baltimore and Franklin, 1962). This occurs despite the fact that cellular DNA-dependent RNA polymerase II (Pol II) itself is functional during infection. However, at least one factor required for Pol II transcription is deficient in picornavirus-infected cells (Apriletti and Penhoet, 1978; Crawford et al., 1981). Viral protein synthesis is required to induce this cellular transcription inhibition, and cytoplasmic extracts of poliovirus-infected cells inhibit RNA synthesis in isolated nuclei (Franklin and Baltimore, 1962; Balandin and Franklin, 1964; Bossart et al., 1982). This early work prompted the question of whether picornavirus proteins are able to enter the infected cell nucleus to carry out this inhibitory task. Subsequent studies demonstrated that radiolabeled viral proteins do in fact enter the cell nucleus (Bienz et al., 1982; Fernández-Tomás, 1982).

Picornavirus Infection Results in the Termination of Cellular Transcription

Enterovirus infection interferes with transcription driven by all three DNA-dependent RNA polymerases of mammalian cells. RNA polymerase I (Pol I) synthesizes ribosomal RNA (except 5S rRNA), which accounts for over half the RNA produced in the cell, and is carried out in the nucleolus (Russell and

Zomerdijk, 2006). The Pol I transcription machinery is made up of two major transcription factor complexes: upstream binding factor (UBF) and selectivity factor 1 (SL1). Poliovirus infection was first shown to alter Pol I driven transcription using *in vitro* transcription systems in combination with uninfected or poliovirus-infected cellular lysates. Oligonucleotides containing the Pol I promoter element in combination with poliovirus-infected HeLa cell extracts results in faster migrating complexes by electrophoretic mobility shift assays compared to the same oligonucleotides incubated with uninfected extracts. Incubation of poliovirus 3C with mock-infected extracts recapitulated this result, suggesting that this viral proteinase is responsible for cleavage of host factors required for Pol I driven transcription. This claim was verified when incubation of purified poliovirus 3C with cellular extracts was shown to produce near total inhibition of Pol I transcription (Rubinstein et al., 1992). The inhibition of Pol I transcription during poliovirus infection has been attributed to the inactivation of both UBF and SL1, two components of the Pol I transcription initiation complex. *In vitro* restoration of rRNA transcription requires the addition of both UBF and SL1 to extracts from poliovirus-infected cells. Poliovirus 3C cleaves TATA box-binding protein-associated factor RNA polymerase I subunit C (TAF 1C or TAF110), a component of SL1. Although UBF is not targeted by 3C *in vitro*, it does appear to be modified by a 3C-dependent mechanism within poliovirus infected cells (Banerjee et al., 2005).

The Pol II transcription system is responsible for the production of messenger RNA within eukaryotic cells. TFIID binding to the TATA box DNA sequence is the initial transcriptional step in the formation of the preinitiation complex and is performed by TBP1, a component of TFIID. TFIID activity in poliovirus-infected cells is greatly decreased compared to mock-infected cells and only TFIID is capable of restoring basal Pol II transcription *in vitro* (Kliewer and Dasgupta, 1988; Yalamanchili et al., 1996). The decrease in TFIID activity during poliovirus infection has also been attributed to 3C, as extracts from cells infected with a poliovirus encoding 3C with reduced activity is less effective at inhibiting Pol II transcription *in vitro*. Further experiments involving the co-transfection of constructs encoding poliovirus 3C and plasmids competent in Pol II transcription demonstrated that 3C is sufficient to inhibit Pol II transcription within cells (Yalamanchili et al., 1996). Poliovirus proteinases target TBP1 for cleavage during infection, and incubation of TBP1 with poliovirus 3C and 2A recapitulate the degradation of TBP1 *in vitro* (Clark et al., 1993; Das and Dasgupta, 1993; Yalamanchili et al., 1997a). However, only 3C is capable of inhibiting Pol II transcription within these cellular extracts. Furthermore, poliovirus inhibits activator-dependent Pol II transcription by cleaving and promoting the dephosphorylation of cyclic AMP-responsive element-binding protein 1 (CREB-1), leading to inhibition of CREB-1-activated transcription in poliovirus infected cells (Kliewer et al., 1990; Yalamanchili et al., 1997b). POU domain, class 2, transcription factor 1 (known as octamer-binding transcription factor 1 or Oct-1) is also specifically cleaved in poliovirus-infected cells and by 3C *in vitro*, and cleaved Oct-1 is unable to support activated transcription (Yalamanchili et al., 1997c).

Finally, the RNA polymerase III (Pol III) system, which controls the production of tRNA and 5S rRNA within eukaryotic cells, is also altered in poliovirus-infected cells. Studies similar to those discussed above demonstrate that 3C is responsible for cleaving the general transcription factor IIIC polypeptide 1 (TFIIIC box B-binding subunit or TFIIICα), leading to reduced activity of this transcription factor and subsequent inhibition of Pol III transcription (Fradkin et al., 1987; Clark and Dasgupta, 1990; Clark et al., 1991; Shen et al., 1996).

Although most work focusing on enterovirus-induced alterations to cellular transcription has implicated 3C, 2A also functions in this regard. Transient expression of poliovirus 2A in cells leads to reductions in DNA replication, Pol II transcription, as well as cap-dependent translation demonstrating that both enteroviral proteinases function in transcriptional repression (Davies et al., 1991). Probable ATP-dependent RNA helicase DDX20 (also known as Gemin3), a protein found in both the nucleus and cytoplasm of cells and which functions in biogenesis of spliceosomal complexes, is also proteolyzed in poliovirus-infected cells. The cleavage of this protein can be recapitulated *in vitro* using poliovirus 2A, and transfection of 2A results in Gemin3 cleavage in cells. Although the purpose of this cleavage event during infection is not clear, the assembly of spliceosomal complexes is reduced in infected cells and correlates with Gemin3 degradation and the loss of Gemin3 localization to the nucleus. Therefore, it is likely that cellular splicing is also targeted by enteroviruses during infection to further inhibit cellular gene expression (Almstead and Sarnow, 2007).

Picornavirus Proteins Translocate to the Nucleus of Infected Cells

To enter the nucleus of an infected cell and alter cellular transcription, poliovirus proteinase 3C utilizes an NLS present within the 3D polymerase domain and thus accesses the nucleus in the form of the 3CD precursor protein. The single, basic-type NLS present in 3D consists of amino acids 125–129, with the sequence KKKRD, which is a motif that is highly conserved within the enteroviruses. Interestingly, although 3D contains an NLS, this signal is necessary but not sufficient to allow the transport of 3CD into the nucleus. Constructs encoding poliovirus 3C, 3D, 3CD, or a mutated 3CD that is not capable of autoproteolysis, fused to EGFP, were transfected into cells and these cells were then infected with poliovirus or mock-infected. Despite the presence of the NLS in 3D, 3D-containing proteins were only relocalized to the nucleus of infected cells (Sharma et al., 2004). Mutation of the putative NLS sequence in 3D eliminates the nuclear localization of 3D-containing proteins, even in infected cells, demonstrating that the NLS as well as additional alterations to nucleocytoplasmic trafficking resulting from poliovirus infection are necessary for 3CD entry into the nucleus. Mutation of the 3D NLS results in cytoplasmic retention of transfected 3CD-EGFP expression constructs following infection, suggesting that this viral precursor protein does not enter the nucleus as a result of passive diffusion. Autoproteolysis of 3CD or trans-cleavage of one 3CD molecule by another, once within the nucleus, liberates 3C which then targets various transcription factors as

discussed above. It should be noted that because 3CD is an active proteinase, this precursor form is also likely involved in the cleavage of host-cell proteins in the nucleus. Importantly, an NLS is required for 3CD to translocate to the nucleus, suggesting that the nuclear import pathway utilized by this viral precursor protein remains operational even in the face of the multiple alterations made to the NPC and nucleocytoplasmic trafficking in general during poliovirus infection. This provides further evidence that not all nuclear-resident proteins relocalize to the cytoplasm of poliovirus infected cells, including those which utilize transportin-SR import receptors (Gustin and Sarnow, 2001). The fact that the NLS of 3D most closely resembles a classical NLS while the import of proteins containing this type of NLS is altered in picornavirus-infected cells remains to be reconciled.

Recent evidence demonstrates that poliovirus 3D also functions in alterations of host cell gene expression within the nucleus. Once 3CD enters the nucleus and self-cleaves, 3D targets the pre-mRNA processing factor 8 (Prp8), a central component of the spliceosome, causing interference with pre-mRNA splicing and mRNA synthesis (Liu et al., 2014). Specifically, 3D associates with the C-terminal region of Prp8, resulting in lariat forms of the splicing intermediate accumulating within poliovirus infected cell nuclei.

In addition to inhibiting cellular transcription upon nuclear entry, poliovirus 3CD also targets components important for antiviral response and apoptotic pathways. Poliovirus infection initially leads to the activation of NF-κB, but the antiviral response is subsequently curtailed by 3C-mediated cleavage of the transcription factor p65 (p65/RelA) subunit of NF-κB (Neznanov et al., 2005). This alteration to cellular induction of antiviral pathways was shown to be independent of caspase-mediated cleavage, as the activation of caspase-3, the hallmark of apoptosis, was not observed in poliovirus-infected cells. Furthermore, the enteroviruses HRV 14 and Enteric Cytopathic Human Orphan virus-1 (ECHO virus-1), demonstrated a similar degradation of the NF-κB subunit p65/RelA. Poliovirus 3C also induces the degradation of the transcriptional activator and tumor suppressor protein p53 in a manner dependent upon the cellular protein promyelocytic leukemia protein (PML) during infection (Weidman et al., 2001; Pampin et al., 2006). Poliovirus infection regulates the intranuclear movement of PML from the nucleoplasm to the nuclear matrix, as well as recruitment of E3 ubiquitin-protein ligase MDM2, leading to the proteasome-dependent degradation of p53. This activity is hypothesized to counteract p53-target gene activation and ensuing induction of apoptosis (Pampin et al., 2006).

Studies focused on other enteroviruses have revealed similar insights into the translocation of viral proteins to the nucleus during infection. Cells infected with HRV 16, probed with antibodies against 3C, then imaged via confocal microscopy demonstrated that proteins containing HRV 16 3C accumulate in the nucleus of infected cells between 2 and 4 h post-infection, similarly to poliovirus 3C (Amineva et al., 2004). The NLS of HRV16 3D is likely located in the N-terminal portion of the polymerase, since a transfected RNA construct encoding 3D with a 371 amino acid deletion from the C-terminus is still able to localize to the nucleus. However, the nuclear localization of the truncated 3CD and full-length 3CD was observed in the absence of infection, contradicting previous work with poliovirus. Much like poliovirus, HRV 16 3CD degrades Oct-1 in infected cells (Yalamanchili et al., 1997c; Amineva et al., 2004). Cells infected with EV71 show decreased expression of cleavage stimulation factor subunit 2 (also known as CstF-64), which correlates, with the production of EV71 3C. Furthermore, 3C cleaves recombinant CstF-64 and inhibits cellular 3′-end pre-mRNA processing and polyadenylation *in vitro*. The accumulation of unprocessed pre-mRNA and reductions in mature mRNA are also observed in EV71-infected cells, suggesting that host-cell polyadenylation is targeted during EV71 infection (Weng et al., 2009).

Cardioviral proteins also enter the nuclei of infected cells. Indirect immunofluorescence and confocal microscopy revealed that 2A, VPg (3B), 3C, and 3D localize to the nucleus, and specifically to the nucleolus, of EMCV and mengovirus infected cells (Aminev et al., 2003a). Cardioviral 2A contains a short motif (KRVRPFRLP) that closely resembles an NLS found in yeast ribosomal proteins, and deletions within this sequence result in the loss of nucleolar localization (Svitkin et al., 1998; Aminev et al., 2003a; Groppo et al., 2011). Similar to observations of rhinoviral 3CD protein translocations to the nucleus, cardioviral 2A is capable of entering the nucleus in the absence of infection. Furthermore, cardioviral 2A colocalizes with nucleophosmin (nucleolar phosphoprotein B23), a nuclear shuttling chaperone protein that, among other things, functions in ribosome biogenesis. The nucloeolar localization motif present within cardioviral 2A and the consistent colocalization of 2A and nucleophosmin has led to the hypothesis that nucleophosmin associates with 2A and aids in the nucleolar localization of this picornavirus protein. This would allow cardioviral 2A to enter the nucleus camouflaged as a ribosomal protein, where Pol I transcribes rRNA genes and ribosomal subunit assembly occurs (Aminev et al., 2003a). 2A may target the nucleolus to alter ribosomal biogenesis in some way, promoting viral IRES-dependent translation and/or 2A inclusion within ribosomes (Medvedkina et al., 1974). The EMCV 3BCD minor precursor protein has also been shown to associate with nucleophosmin and enter the nucleolar compartment via an NLS present in the 3D amino acid sequence, independent of infection (Aminev et al., 2003b). In agreement with studies of HRV 16, this NLS is located at the very N-terminus of 3D and mimics a yeast ribosomal protein NLS rather than the basic NLS suggested for poliovirus.

FMDV Has Unique Interactions with the Nucleus

FMDV has distinct interactions with the transcription apparatus of cells. In contrast to other picornaviruses, FMDV modulates cellular transcription by targeting the regulation of transcriptionally active chromatin through the cleavage of the nucleosome component histone H3 (Griger and Tisminetzky, 1984). The FMDV 3C proteinase is capable of histone H3 cleavage *in vitro,* thus inhibiting euchromatin formation and subsequent transcription (Falk et al., 1990; Tesar and Marquardt, 1990). Transient transfection of FMDV 3ABC precursor protein expression constructs causes histone H3 degradation; however,

a recent report suggests that the NLS motif MRKTKLAPT present in FMDV 3D is responsible for transporting 3CD to the nucleus, as well as GFP fused to this NLS, in the absence of infection (Capozzo et al., 2002; Sanchez-Aparicio et al., 2013). It is possible that the cleavage observed upon transfection of FMDV 3ABC was attributable to infection with vaccinia virus encoding a T7 RNA polymerase prior to transfection to facilitate 3ABC expression from the T7 promoter-containing plasmid. Nonetheless, it appears that similar to the enteroviruses, FMDV 3CD enters the nucleus via an NLS present in the 3D amino acid sequence, allowing proteinase functions to target cellular proteins within the nucleus. The L proteinase of FMDV has also been shown to localize to the nucleus of porcine cells during wild type FMDV infection (De Los Santos et al., 2007). As noted above, FMDV L entry into the nucleus correlates with a decrease in the transcription of antiviral IFN-β mRNA (De Los Santos et al., 2006). The resulting decrease in IFN production is not a result of FMDV infection causing inhibition of NF-κB translocation to the nucleus, reductions in NF-κB mRNA transcription, or a result of widespread degradation of nuclear proteins, but instead is a consequence of decreases in the nuclear levels of the p65/RelA protein subunit of NF-κB, as has also been observed during enterovirus infection. The L proteinase appears to be necessary and sufficient for the degradation of p65/RelA, as infection with the murine-specific TMEV encoding FMDV L also leads to FMDV L nuclear localization and disappearance of p65/RelA in the nucleus of infected cells (De Los Santos et al., 2007). Furthermore, L proteinase catalytic activity is required for p65/RelA degradation. The motif present within FMDV L responsible for imparting nuclear localization was mapped to a SAF-A/B, Acinus, and PIAS (SAP) domain within L, a protein domain that is associated with nuclear retention of some proteins involved in transcriptional control. Virus containing a double mutation in the SAP region of L showed altered nuclear localization upon infection (De Los Santos et al., 2009) (**Figure 6**).

The inhibition of cellular gene expression by picornaviruses is carried out in diverse ways that target translation as well as splicing and transcription. To affect the latter two processes, some picornavirus proteins enter the nucleus of the infected cell. The pro-viral effects of reduced cellular gene expression are two-fold: to liberate cellular proteins with functions advantageous

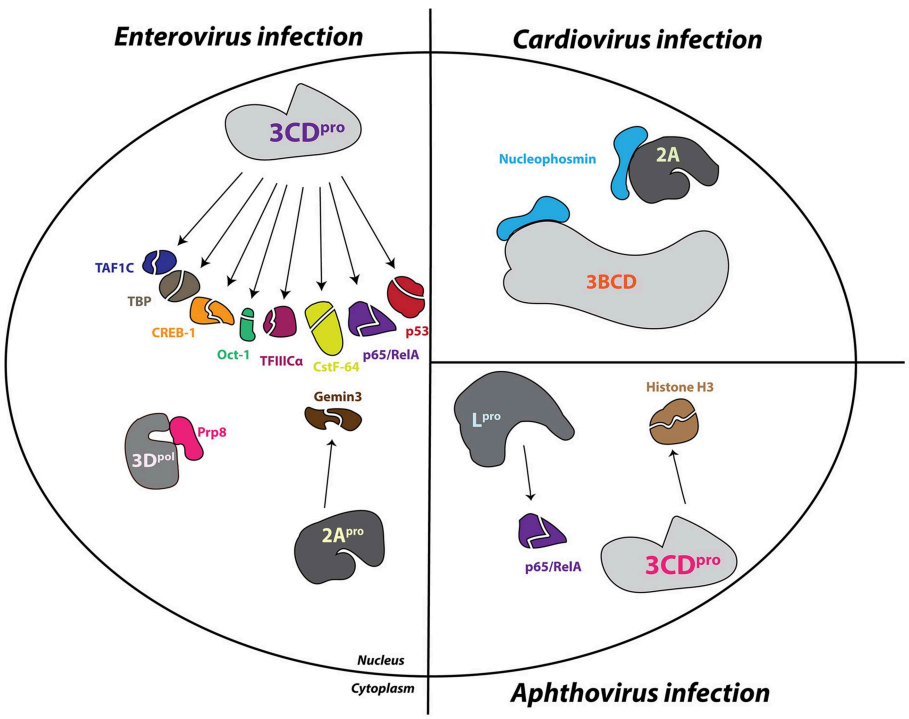

FIGURE 6 | Picornavirus proteins enter the nucleus and alter nuclear-resident proteins. During enterovirus infections, the viral proteinase 3CD/3C (3CD^{pro}) enters the nucleus and degrades TATA box-binding protein-associated factor RNA polymerase I subunit C (TAF 1C), leading to inhibition of RNA Polymerase I (Pol I) transcription. Pol III-driven transcription is also inhibited by 3CD^{pro}, through cleavage of general transcription factor IIIC polypeptide 1 (TFIIICα). Pol II transcription, including cellular mRNA production, is terminated in enterovirus infected cells through cleavage of TATA-box-binding-protein 1 (TBP1), cyclic AMP-responsive element-binding protein 1 (CREB-1), and POU domain, class 2, transcription factor 1 (Oct-1) by 3CD^{pro}. The transcription factors cellular tumor antigen p53 (p53), cleavage stimulation factor subunit 2 (CstF-64), and the NF-κB subunit p65/RelA (p65/RelA) are also degraded in a 3CD^{pro} dependent manner during enterovirus infections. Furthermore, the enteroviral polymerase 3D (3D^{pol}) associates with the splicing factor pre-mRNA processing factor 8 (Prp8), causing dysregulation of splicing, and the 2A proteinase (2A^{pro}) cleaves Probable ATP-dependent RNA helicase DDX20 (Gemin3). Cardiovirus infection causes the nuclear localization of both 2A and precursor protein 3BCD, both of which may associate with nucleophosmin. Infection with FMDV causes the cleavage of both Histone H3 as well as p65/RelA within the nucleus, following the entry of both L proteinase (L^{pro}) and 3CD^{pro}.

to viral replication and to re-route metabolic energy from cell-specific to viral-centric functions. It is somewhat surprising that the RNA-dependent RNA polymerase, 3D, of many picornaviruses becomes localized to the nucleus of infected cells, because it would seem that maximizing viral RNA production in the cytoplasm would be prioritized during the infectious cycle. The fact that many picornavirus 3D proteins contain an NLS suggests that there are specific functions of 3D and/or the precursor 3CD in the nucleus, some of which remain to be uncovered, which are balanced with the polymerase function of 3D in the cytoplasm. It is a given that further experimentation will continue to reveal the elegant ways in which picornaviruses alter the cellular functions that occur in the nucleus and the way in which viral proteins are able to infiltrate the command center of the cell to appropriate cellular components and functions for their benefit.

Conclusions

Picornaviruses have traditionally been labeled as "cytoplasmic" RNA viruses as a result of the subcellular region in which these viruses produce viral proteins and replicate their genomic RNA molecules. Indeed, picornaviruses are able to complete the replicative cycle and produce infectious progeny in nucleus-free cytoplasts and cytoplasmic extracts (Pollack and Goldman, 1973; Follett et al., 1975; Molla et al., 1991; Barton and Flanegan, 1993; Svitkin and Sonenberg, 2003). However, it is not clear that enucleation treatments, e.g., with cytochalasin B, do not disrupt the nuclear envelope and allow the escape of nuclear-resident proteins or that cytoplasmic extracts do not contain significant concentrations of nuclear-shuttling or nuclear-resident proteins as a result of nuclear envelope leakage during cellular fractionation. It is significant that following cytochalasin B enucleation treatment, poliovirus capsid synthesis and virus growth are less efficient compared to infection of nucleated, untreated cells with a final yield from enucleated cells one-fifth of that from nucleated cells (Pollack and Goldman, 1973). Some studies have even provided evidence of a nuclear requirement of poliovirus early in infection, as virion RNA is able to replicate in the absence of a cell nucleus but transfection of replicative form RNA into enucleated cells produced no detectable viral progeny (Detjen et al., 1978). As discussed in this review, it is clear that picornaviruses make extensive use of nuclear-resident and nuclear-shuttling proteins to promote viral replication. The nuclear shuttling proteins PCBP2 and PTBP1 are particularly important for enterovirus translation as well as mediating the template usage switch required for RNA replication to proceed. Furthermore, PCBP2 and PABP1 may function in the circularization of genomic RNA templates to facilitate negative-sense intermediate RNA production. Additionally, the nuclear-resident hnRNP C1/C2 is necessary for the efficient synthesis of poliovirus genomic RNA molecules from negative-sense intermediate RNA forms. Following the early rounds of translation in which the limited quantities of nuclear proteins in the cytoplasm are sufficient for viral protein production, picornavirus proteins 3CD/3C, 2A, and L induce alterations to the NPC. Cleavage of nucleoporins by enteroviral 2A and 3CD/3C or hyperphosphorylation of nucleoporins triggered by cardioviral L results in dysregulation of nucleocytoplasmic trafficking and subsequent loss of nuclear compartmentalization of particular proteins within picornavirus infected cells. The presence of these nuclear proteins with functions critical to viral replication (including RNA-binding capabilities) allows for the amplification of viral progeny. Also as a result of deviations from standard nucleocytoplasmic trafficking, picornaviruses are able to disrupt the proper signaling pathways that allow for a strong innate immune response, including interferon production. Finally, picornavirus proteins enter the nucleus of infected cells to carry out functions related to host-cell transcription inhibition, such as degradation of transcription factors, allowing metabolic energy and proteins sequestered in cellular roles to be redirected to virus-centric demands. Picornaviruses display a prominent level of direct and indirect interactions with the nucleus, interactions that have the potential to reveal pathogenic mechanisms and unique antiviral strategies as well as insights into cell biology through more detailed study. The fact that viruses of the *Picornaviridae* family have evolved to orchestrate a well-balanced promotion of viral replication with host-cell attenuation of nucleocytoplasmic trafficking demonstrates that interactions with the nucleus are functional and that these coding-capacity-limited cytoplasmic RNA viruses are master manipulators of even the most complex of cellular processes.

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Variation in the nuclear effects of infection by different human rhinovirus serotypes

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Human rhinovirus (HRV) is a positive sense RNA virus, which, despite replicating in the cytoplasm, has a significant impact on nuclear transport and nuclear localization of host proteins. A number of studies have identified differences between HRV serotypes, with respect to host response, protease activity and replicative ability. Here we report the sero-specific effects of two group-A HRV serotypes, the minor group HRV2 and the major group HRV16, on nuclear transport and nuclear protein localization. Using Western analysis, immunofluorescence and real time PCR, we show that HRV2 replicates at a faster rate than HRV16, which correlates with earlier production of viral proteases and disruption of host nuclear transport. There is significant variation in the nuclear effects of different rhinovirus species, which in turn may impact disease progression and patient response.

Keywords: human rhinovirus, nuclear transport, nucleoporin, serotype, viral proteases

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Introduction

Human rhinovirus (HRV) is a positive sense RNA virus within the *Enterovirus* genus, which belongs to the *Picornaviridae* family. There are >100 strains of HRV, which have variously been categorized by their response to anti-viral compounds (Andries et al., 1990), the specific cell receptor used for entry into host cells [major group: intercellular adhesion molecule 1 (ICAM1) or minor group: low-density lipoprotein receptor (LDLR)] (Vlasak et al., 2005), or more recently, by genomic sequence analysis (currently categorized into HRV-A, HRV-B, and HRV-C) (Palmenberg et al., 2009).

Despite being a positive sense RNA virus with a wholly cytoplasmic replication cycle, HRV proteins are known to interact significantly with the host nucleus, both to alter and utilize host proteins for viral polyprotein production, as well as subverting the host immune response and shutting down host transcription and translation. These processes are achieved via the activity of the virally encoded proteases, $2A^{pro}$ and $3C^{pro}$, and involve the cleavage of specific host nuclear and nuclear-pore proteins (Bushell et al., 2001; Gustin and Sarnow, 2002; Amineva et al., 2004; Watters and Palmenberg, 2011; Walker et al., 2013).

A number of studies have examined the differences between HRV serotypes, considering both protease activity as well as host cytokine response. A study of recombinant $2A^{pro}$ activity *in vitro* from different serotypes demonstrated that the protease activity of 2A against specific host cell proteins varies with HRV-A > HRV-C >> HRV-B (Watters and Palmenberg, 2011). In addition, *in vitro* cleavage of host nuclear proteins during infection with HRV16 (Group A) occurred earlier than cleavage by HRV14 (Group B) (Watters and Palmenberg, 2011). Others have examined the effect of HRV species on cytokine response, identifying

significant variation in cytokine production associated with HRV serotype (Nakagome et al., 2014; Rajan et al., 2014). Furthermore, there is some clinical data to support the notion that HRV-A viruses cause more severe clinical disease compared to HRV-B viruses (Iwane et al., 2011; Lee et al., 2012).

The few published studies comparing major and minor HRV serotypes within the same group have identified reduced replicative ability in minor HRV serotypes compared to major HRV serotypes, as well as variation in disease severity and cytokine response (Wark et al., 2009; Schuler et al., 2014). Since it is evident that significant variation exists between HRV serotypes even within the same group, both in terms of viral protease activity and host response, we examined the effect of a major and minor group HRV-A virus on host nuclear transport. We found infection with the minor group HRV2 resulted in fully processed viral proteases evident earlier during infection compared to HRV16, leading to earlier cleavage of host nuclear pore proteins. Interestingly, we found that infection with HRV2 results in cleavage of nucleolin as well as the relocalization of SC35, as has been previously described for HRV16. Finally we found the HRV2-induced relocalization of hnRNP-C1/C2 occurs at least 3 h prior to that induced by HRV16 infection, timing that correlates with more complete disruption of nuclear pore components.

Materials and Methods

Antibodies

The primary antibodies for the following proteins were used for Western analysis and immunofluorescence (IF): anti-Nup62 (BD Biosciences #610497, used at 1:2000 for Western), anti-Nup98 (Abcam #45584, used at 1:1000 for Western), anti-Nup153 (Abcam #96462, used at 1:1000 for Western), anti-eIF4G (BD Biosciences #610536, used at 1:1000 for Western), anti-PABP (Cell Signaling Technology #4992, used at 1:1000 for Western), anti-nucleolin (Abcam #22758, used at 1:3000 for Western), anti-hnRNP-C1/C2 (Santa Cruz #32308, used at 1:500 for IF), anti-SC35 (Sigma #4045, used at 1:500 for IF), anti-Sam68 (Santa Cruz #sc333, used at 1:500 for IF), and anti-α/β-tubulin (Cell Signaling Technology #2148, used at 1:1000 for Western). Antibodies to $3C^{pro}$ were kindly provided by S. Amineva (Madison, WI, USA; Amineva et al., 2004) and antibodies to dsRNA were kindly provided by S. Bowden (VIDRL, Melbourne, VIC, Australia).

Cell Culture and Infection

Ohio-HeLa cells (provided by Bo Lin, Biota Holdings) and A549 cells (ATCC) were grown in high glucose DMEM supplemented with 10% heat inactivated Foetal Bovine Serum (FBS) and antibiotics (penicillin, streptomycin, neomycin) at 37°C in a humidified atmosphere of 5% CO_2. For HRV infection of A549 cells, 20 mM $MgCl_2$ was added to the infection media. Rhinovirus serotype 16 (HRV16) was a gift from E. Dick and W. Busse (Madison, WI, USA) and Rhinovirus serotype 2 (HRV2) was provided by Biota Holdings. Viral stocks were prepared by infecting subconfluent monolayers of Ohio-HeLa cells at a multiplicity of infection (MOI) of 1 by absorption for 1 h with occasional rocking, followed by replacement of the medium with fresh DMEM supplemented with 2% FBS and antibiotics. Once extensive cytopathic effects were observed, infected cultures were frozen at −80°C to release virus (Ghildyal et al., 2005). Cultures were thawed, vortexed and clarified of cellular debris by centrifugation for 15 min at 3,500 rpm. Infectious virus was titrated on Ohio-HeLa cells by standard TCID50 protocol and titre calculated using the Spearman–Karber equation (Mahy and Kangro, 1996).

Western Analysis

Overnight subconfluent cultures of Ohio-HeLa or A549 cells with or without infection with HRV2 or HRV16 at an MOI of 1 (Ohio-HeLa cells) or 5 (A549 cells) were lysed at different times by incubation in RIPA buffer containing protease and phosphatase inhibitors (Roche) for 30 min on ice (Walker et al., 2013), prior to heating at 100°C for 5 min in Laemmli buffer (Hames, 1998). Cell lysates were subjected to SDS-polyacrylamide electrophoresis using pre-cast gradient (4–20%) or 10% acrylamide gels (Bio-Rad, TGX gels) followed by Western transfer to nitrocellulose membranes in Tris-Glycine-ethanol buffer (25 mM Tris-HCl, 192 mM Glycine, 20% Ethanol) for 90 min at 400 mA. Blots were stained with Ponceau S (Sigma) to confirm transfer and blocked for 1 h in 4% skim milk (Diploma) in PBS (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.2, 2.7 mM KCl, 137 mM NaCl), prior to incubation with different primary antibodies diluted in 1% skim milk in PBS-T (PBS containing 0.1% Tween 20) overnight at 4°C with rocking. After washing in PBS-T, blots were incubated with species specific secondary antibodies conjugated to horseradish peroxidase diluted 1:5000 in 1% skim milk in PBS-T, followed by washing and detection of bound antibodies with Enhanced Chemiluminescence (ECL, Perkin Elmer). Protein bands were detected using the Licor OdysseyFc, and captured digital images were analyzed using ImageStudio to quantitate relative protein levels. Where required, blots were stripped to remove bound antibodies (2% SDS, 62.5 mM Tris-HCl pH 6.8, 114.4 mM β-mercaptoethanol) at 50°C for 10 min, washed in PBS-T, blocked in 4% skim milk in PBS and reprobed using different primary antibodies as required.

Immunofluorescence

Overnight subconfluent monolayers of Ohio-HeLa or A549 cells grown on glass coverslips (Proscitech, #1) were infected with HRV16 or HRV2 at an MOI of 1 (Ohio-HeLa cells) or 5 (A549 cells) or left uninfected (mock) and fixed with 4% formaldehyde in PBS followed by permeabilization of cell membranes with 0.2% Triton X-100 in PBS at various times post infection (p.i.) (Ghildyal et al., 2005). Cells were incubated with primary antibodies diluted in PBS for 30 min, washed twice in PBS, incubated with species specific secondary antibodies conjugated to CF 488 (Biotium) or Alexa Fluor 568 diluted 1:1000 in PBS for 30 min, and then washed twice in PBS and mounted using ProLong Gold mounting media with DAPI (Invitrogen). Digitized fluorescent cell images were collected using a Nikon Ti Eclipse confocal laser-scanning microscope (CLSM) with Nikon 60×/1.40 oil immersion lens (Plan Apo VC OFN25 DIC N2; optical section of 0.5 μm) and the NIS Elements AR software.

Quantitative analysis of the fluorescence signal in the nucleus (Fn) and cytoplasm (Fc) was performed using ImageJ.

RNA Isolation and Real Time PCR Analysis

Overnight subconfluent cultures of Ohio-HeLa or A549 cells were infected as described above and RNA was collected using Tri-Reagent (Sigma). Two samples were collected for each infection time point. At the indicated times, media was completely removed and 500 μL of Tri-Reagent was added to each 35 mm dish. Cell lysate was collected in 1.5 mL Eppendorf tubes and frozen at –80°C for subsequent analysis. Samples were thawed on ice, 100 μL of chloroform was added and samples were vortexed briefly, then incubated on ice for 2 min. Samples were then centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous layer was transferred to a new tube and an equal volume of isopropanol was added. Samples were vortexed and the RNA allowed to precipitate overnight at –20°C. The next day, RNA samples were centrifuged at 10,000 rpm for 10 min at 4°C to pellet the RNA. The pellets were washed with 1 mL of 75% ethanol in DEPC water, vortexed and centrifuged at 7,500 rpm for 5 min at 4°C. All ethanol was removed and pellets were left to dry at room temperature. Dried pellets were resuspended in 15–30 μL of DEPC-water, depending on the size of the pellet. RNA concentration was determined using a NanoDrop 1000 instrument. To generate cDNA, 1 ug from each sample/time point was combined to transcribe a total of 2 ug of RNA per time point. Reverse transcription reactions were performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), following the manufacturer's instructions. Real time PCR was performed on a Bio-Rad CFX96 instrument, using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) as per the manufacturer's instructions, with the following additions: cDNA samples were diluted 1:50 prior to analysis, and the standard curve was generated by combining cDNA from each sample, then diluted fivefold to generate a 5 sample standard curve. Samples were amplified in duplicate and resultant real time PCR data was analyzed using CFX Manager (Bio-Rad). PCR primer sequences for viral RNA and GAPDH are available on request.

Statistical Analysis

GraphPad Prism 6 was used for analyses; a two-tailed Mann–Whitney test was used to assess significant differences in Fn/c values for hnRNP-C1/C2 and Sam68 localization, compared to mock samples.

Results

Production of HRV2 Viral Proteins Occurs Earlier during Infection Compared to HRV16

The production of the viral proteases 2A and 3C during the course of Ohio-HeLa infection with HRV2 and HRV16 was assessed by Western blot (**Figure 1**), with the percentage of full length protein remaining relative to 0 h.p.i (hours post infection) shown under the appropriate lane. Quantitation for HRV2 infection at 24 h.p.i is not shown, as there was a significant decrease in tubulin which caused skewing of the results. However, the overall trend is apparent. The production and activity of $3C^{pro}$ was assessed by direct detection with an anti-3C antibody as well as by monitoring the appearance of cleavage products for a known target of $3C^{pro}$, poly(A)-binding protein (PABP). The production and activity of $2A^{pro}$ was assessed by monitoring the appearance of cleavage products of a known $2A^{pro}$ target, eukaryotic initiation factor 4G (eIF4G).

Despite infecting Ohio-HeLa cells at the same MOI, production of active viral proteases was noticeably different between HRV2 and HRV16. In HRV2 infected cells, $3C^{pro}$ was detectable by 6 h.p.i, with the greatest amount of HRV2 $3C^{pro}$ present at 9 h.p.i; this coincides with the level of full length PABP, where 55% remains at 6 h.p.i and only 19% at 9 h.p.i. In HRV16 infected cells, $3C^{pro}$ was detectable from 6 h.p.i, though at lower levels compared to HRV2 infected cells at the same time p.i. The greatest amount of HRV16 $3C^{pro}$ is observed at 24 h.p.i, which coincides with the greatest quantity of PABP cleavage products.

A similar difference in also observed for $2A^{pro}$, where the appearance of eIF4G cleavage products (as a proxy for detecting $2A^{pro}$ directly) was observed from 3 h.p.i in HRV2 infection, while for HRV16 infection these cleavage products were observed from 6 h.p.i. In addition, the rate of eIF4G cleavage appeared to be faster in HRV2 infection, with only 4% of full length eIF4G remaining at 9 h.p.i, while 26% of full length eIF4G still remained at 24 h.p.i after HRV16 infection. Similar results were observed for A549 cells at 24 h.p.i, where 3C and 3CD can be detected for both HRV2 and HRV16 infection. Cleavage of eIF4G was only observed for HRV2 infection, a result that mirrors the time difference seen in Ohio-HeLa infection (**Supplementary Figure S1A**).

HRV Protease Activity Against Host Nuclear Pore Proteins is Delayed in HRV16 Infection

Since there were obvious differences in the rate of protease production as well as in the proteolytic activity of the proteases from different serotypes, we next examined the effect of HRV2 and HRV16 infection on the cleavage of specific nucleoporins (Nups) in Ohio-HeLa cells, as well as on a nucleolar protein, nucleolin. Representative Western blots are shown in **Figure 2**, with the percentage of full length protein remaining relative to 0 h.p.i shown under the appropriate lane. HRV16-mediated cleavage of Nups 62, 98, 153, and nucleolin was delayed compared to HRV2. Analysis of Nup62 in HRV2 infected cells demonstrated that 55% of the protein remained at 6 h.p.i, and only 28% at 9 h.p.i. In contrast, HRV16-mediated degradation of Nup62 began between 9 and 24 h.p.i, as 79% of Nup62 was still apparent at 24 h.p.i.

Cleavage of Nup98 also began earlier in HRV2 infected cells, with results similar to those observed for Nup62, where approximately 50% of the full length protein had been cleaved by 6 h.p.i and only 21% remained at 9 h.p.i. In cells infected with HRV16, cleavage of Nup98 was apparent by 6 h.p.i, however, at 24 h.p.i, more than 50% of full length protein remained.

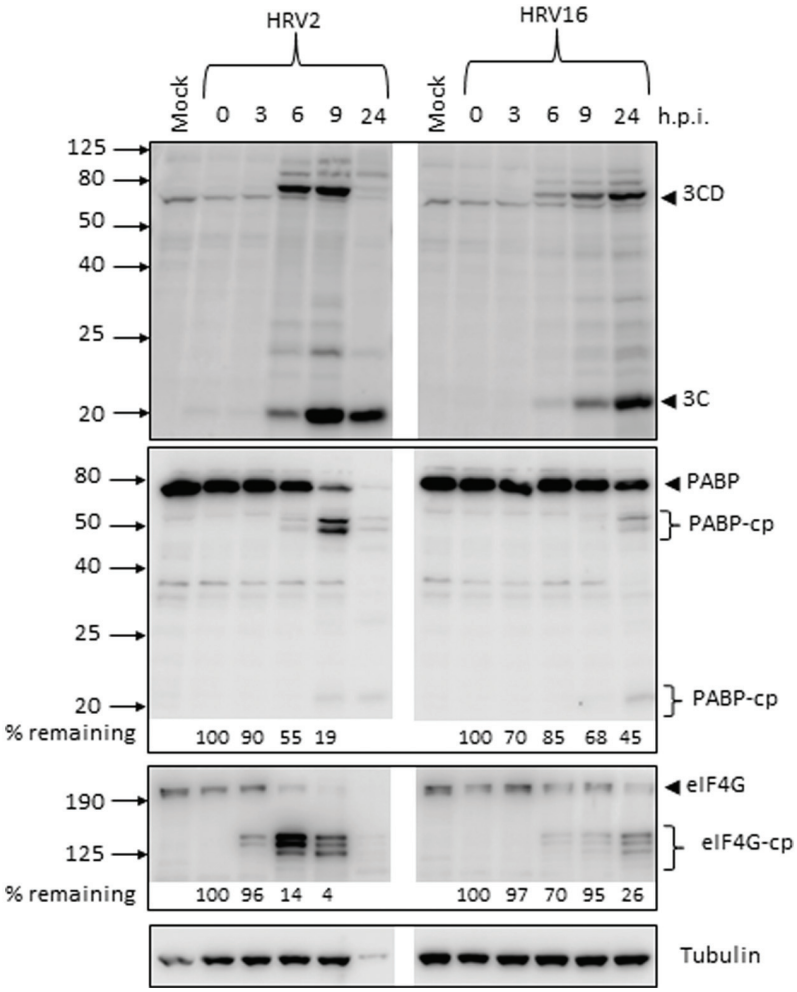

FIGURE 1 | Active proteases are produced at different times after infection with human rhinovirus 2 or 16 (HRV2 or HRV16). Ohio-HeLa cells were infected without (mock) or with HRV2 or HRV16 (MOI of 1) and cells lysed using RIPA buffer containing protease and phosphatase inhibitors at the time points shown. Cell lysates were subjected to SDS-PAGE on 4–20% gradient gels (for anti-3C detection) or 10% gels (remaining antibodies) and Western analysis using the indicated primary antibodies/horseradish peroxidise-conjugated secondary antibodies and enhanced chemiluminescence (Perkin Elmer). The approximate protein size (kDa) is shown on the left and the specificity of the antibodies is indicated on the right. Where cleavage products are observed, bands corresponding to full length proteins are indicated with arrowheads and cleavage products are indicated with brackets. cp, cleavage products; h.p.i, hours post-infection. Results for densitometric analysis of protein bands are shown below the relevant blots, where data were normalized to the corresponding values for tubulin, and are shown as the percent protein remaining relative to the corresponding values for the 0 h sample.

HRV2 appears to cleave Nup153 at a faster rate than HRV16, as approximately 70% of Nup153 was cleaved in HRV2 infected cells at 9 h.p.i, compared to less than 20% in HRV16 infected cells at the same time. Indeed, by 24 h.p.i, 27% of Nup153 (73% remaining) was cleaved in HRV16 infected cells. Together, these results demonstrate significant differences in the proteolytic activities of different HRV serotypes against specific host targets.

Finally, analysis of nucleolin showed that although there was no significant loss of full length protein after infection with either HRV2 or HRV16, a cleavage product was detected from 9 h.p.i in HRV2 and at 24 h.p.i in HRV16 infected cells. A similar result was observed for A549 cells at 24 h p.i., where a cleavage product can be observed in HRV2 infected cells, but not in HRV16 infected cells, again demonstrating the difference in proteolytic activity between the two viruses (**Supplementary Figure S1B**). The apparent increase in nucleolin during the course of Ohio-HeLa infection can be explained by our previous results (Walker et al., 2013), which showed that nucleolin is mislocalized from nucleoli into the nucleus during infection with HRV16 and therefore may be more easily detected.

HRV Infection and Mislocalization of Nuclear Proteins

We next examined the effect of HRV2 and HRV16 infection on the nuclear localization of specific proteins, using IF to identify changes in localization. Ohio-HeLa cells were grown on coverslips and infected with HRV2 or HRV16 or mock infected, then fixed at indicated times post-infection. Cells were co-stained

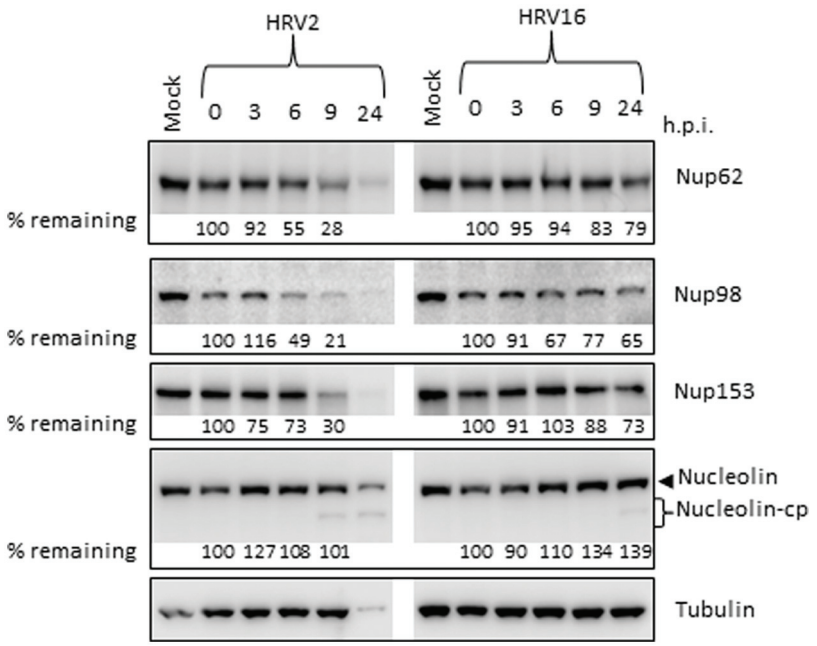

FIGURE 2 | Human rhinovirus protease activity occurs at different times after infection with HRV2 or HRV16. Cell lysates were prepared and subjected to Western analysis as for **Figure 1**. The specificity of the antibodies is indicated on the right. Where cleavage products are observed, bands corresponding to full length proteins are indicated with arrowheads and cleavage products are indicated with brackets. cp, cleavage products; h.p.i, hours post-infection. Results for densitometric analysis of protein bands are shown below the relevant blots, where data were normalized to the corresponding values for tubulin, and are shown as the percent protein remaining relative to the corresponding values for the 0h sample.

for dsRNA to identify infected cells and specific nuclear proteins as indicated (**Figure 3**). **Figure 3A** shows the effect of HRV infection on nuclear speckle protein SC35. At 6 h after HRV2 infection, the SC35 staining was fainter and less obvious, though the protein was still present in small nuclear speckles. By 9 h.p.i, SC35 had become diffuse throughout the nucleus. A similar effect was observed in HRV16 infected cells, where at 6 h.p.i SC35 speckles were fainter than mock infection, and by 9 h.p.i SC35 was diffuse throughout the nucleus. Thus, in contrast to results observed for HRV14 (Gustin and Sarnow, 2002), cleavage or degradation of the nuclear speckle protein SC35 was observed in both HRV2 and HRV16 infected cells.

Examination of hnRNP-C1/C2 by IF (**Figure 3B**) showed limited nuclear mislocalization of hnRNP-C1/C2 at 6 h.p.i in HRV2 infected cells. However by 9 h.p.i, there was significant mislocalization of hnRNP-C1/C2 into the cytoplasm. Quantitation of nuclear compared to cytoplasmic fluorescence (Fn/c, **Figure 4A**) confirmed a moderately significant difference in localization at 6 h.p.i ($p < 0.01$) and a highly significant difference at 9 h.p.i ($p < 0.0001$) compared to mock infection. A similar trend was also observed for cells infected with HRV16, however, quantitation showed there was a significant decrease in Fn/c only at 9 h.p.i ($p < 0.001$) when compared to mock (**Figure 4A**).

Figure 3C depicts HRV2 and HRV16 infected cells stained for the nuclear protein Sam68. Mislocalization of Sam68 from the nucleus into the cytoplasm was apparent at all timepoints, and quantitation of nuclear compared to cytoplasmic fluorescence (Fn/c, **Figure 4B**) confirmed this observation, with a highly significant decrease in Fn/c ($p < 0.0001$) seen for both HRV2 and HRV16, at 6 and 9 h.p.i.

Together these results suggest that disruption of nuclear trafficking leading to mislocalization of nuclear proteins occurs early during infection with both HRV2 and HRV16, as SC35 and Sam68 were affected by 6 h.p.i in cells infected with either serotype. Disruption leading to mislocalization of hnRNP-C1/C2 appears to occur later during infection, as infection with HRV2 at 6 h.p.i led to a small change in nuclear localization compared to the more significant difference observed at 9 h.p.i for HRV2; infection with HRV16 had a more subtle effect on hnRNP-C1/C2 localization, with significant changes compared to mock only apparent at 9 h.p.i. While it could be argued that nuclear protein mislocalization is a result of apoptosis, we have treated Ohio-HeLa cells with Actinomycin D (5 μg/ml) to induce apoptosis, followed by staining for SC35, hnRNP-C1/C2 and Sam68 as described above; no changes in nuclear protein localization were observed during this treatment (data not shown).

We also examined whether HRV2 and 16 could replicate in A549 cells, and observed dsRNA in cells infected with both serotypes at 24 h p.i., indicating replication of the viral genome (**Supplementary Figure S1C**).

Viral RNA Replication Occurs Earlier during Infection with HRV2

To assess the rate of virus replication, the relative amount of viral RNA in cells infected with HRV2 or HRV16 at specified

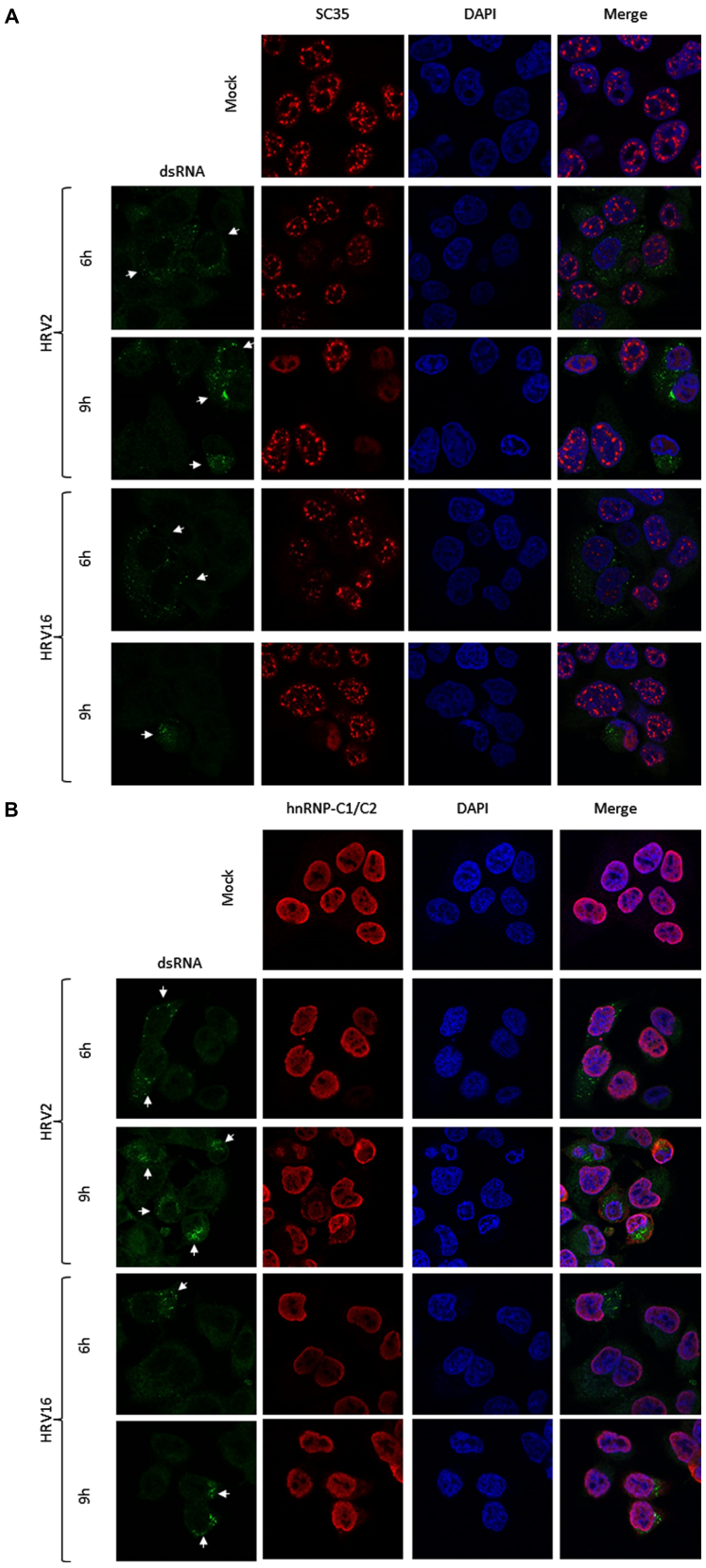

FIGURE 3 | Continued

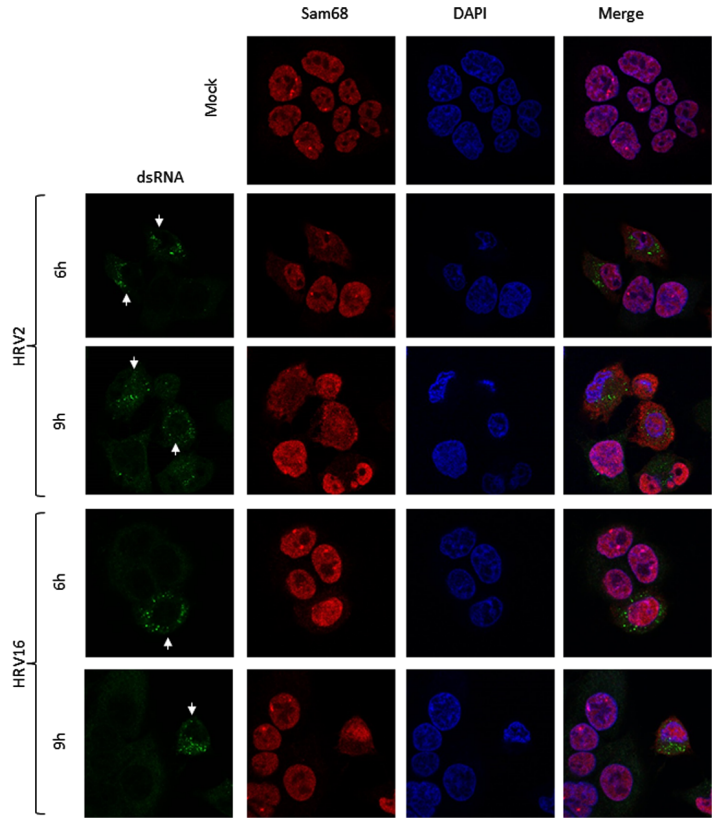

FIGURE 3 | Continued
HRV2 and HRV16 infection lead to mislocalization of nuclear proteins. Ohio-HeLa cells grown on coverslips were infected without (mock) or with HRV2 or HRV16 as for **Figure 1**; cells were fixed at the indicated times and permeabilized, and then probed with **(A)** anti-dsRNA and anti-SC35 antibodies, **(B)** anti-dsRNA and anti-hnRNP-C1/C2 antibodies, or **(C)** anti-dsRNA and anti-Sam68 antibodies, followed by CF488 and Alexa-568 conjugated secondary antibodies. Coverslips were mounted in ProlongGold mounting media with DAPI. Fluorescence was imaged by CLSM (see Materials and Methods). White arrows indicate infected cells.

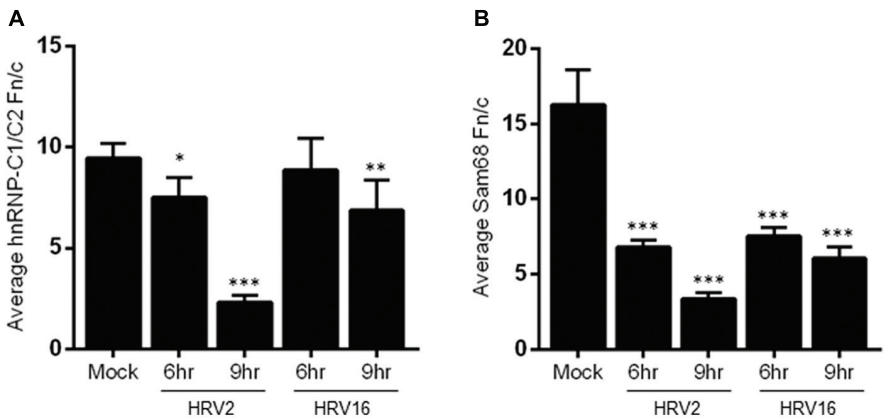

FIGURE 4 | Infection with HRV2 or HRV16 leads to significant mislocalization of nuclear proteins. The nuclear and cytoplasmic fluorescence of infected and mock infected cells prepared as in **Figure 3** was quantitated using ImageJ, and the mean Fn/c values were plotted for **(A)** hnRNP-C1/C2 and **(B)** Sam68. Values are shown ±SEM. Significance compared to mock values are shown as $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

time points was measured. RNA was collected from HRV2 or HRV16 infected Ohio-HeLa cells, reversed transcribed and used in real-time PCR reactions, using primers specific for HRV viral RNA (Dagher et al., 2004) as well as GAPDH as a loading control. Data is shown as arbitrary units of viral RNA relative to GAPDH (**Figure 5**). These results show a steady

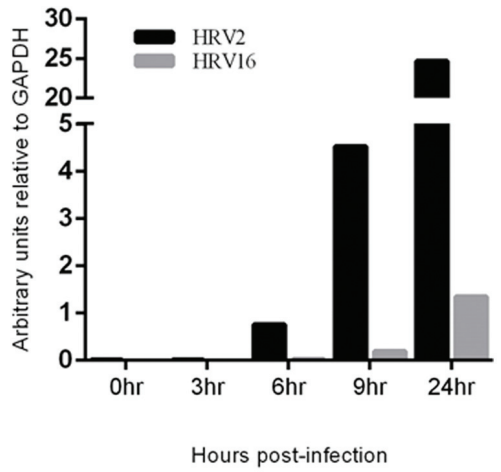

FIGURE 5 | Viral RNA replication is detectable earlier in HRV2 infection compared to HRV16 infection. Real-time PCR was used to assess the quantity of viral RNA present in infected cells at the specified time points for HRV2 and HRV16 infection. Data is shown as arbitrary units relative to GAPDH.

increase in HRV2 viral RNA from ∼1 unit at 6 h.p.i to ∼4.5 units at 9 h.p.i and ∼25 units at 24 h.p.i. In contrast, viral replication after infection with HRV16 appeared to lag behind HRV2, with <1 unit detected at 9 h.p.i and ∼1.5 units detected at 24 h.p.i; these results correlate with the lag observed for protease activity against host proteins (**Figures 1** and **2**). Given the results shown in **Supplementary Figure S1**, we predict that it should also be possible to detect HRV genomes in infected A549 cells. Furthermore, since it appears that HRV2 is able to produce viral proteins and potentially assemble new virions more quickly than HRV16, we may also expect to observe less HRV2 viral RNA compared to that generated in HRV16 infected cells. Indeed, analysis of infected A549 cells at 24 h p.i. shows both HRV2 and HRV16 are able to replicate in these cells (**Supplementary Figure S2**) and there is more HRV16 viral RNA in cells compared to HRV2 viral RNA.

Discussion

In the current study, we found the minor group HRV2 was able to replicate more efficiently than the major group HRV16 (**Figure 5**), which resulted in earlier production of viral proteases, concomitant with earlier cleavage of the target proteins PABP and eIF4G (**Figure 1**). In addition, the earlier production of viral proteases led to earlier and more complete cleavage of host nuclear pore proteins (Nups) as well as cleavage of the nucleolar protein, nucleolin (**Figure 2**). Despite the apparent lag in replication and therefore protease activity between HRV2 and HRV16, analysis of IF results (**Figures 3** and **4**) indicate that changes occur in the appearance of nuclear speckle protein SC35 by 6 h.p.i with HRV16, as well as significantly altering the nuclear localization of Sam68 at this time. In contrast, the mislocalization of nuclear protein hnRNP-C1/C2 occurred later, by 9 h.p.i for HRV2 infection and >9 h.p.i for HRV16 infection, possibly once complete disruption of the NPC has been achieved.

While picornavirus protease cleavage of PABP, eIF4G, and Nups is well established (Lamphear et al., 1993; Liebig et al., 1993; Gustin and Sarnow, 2002; Amineva et al., 2004; Kuyumcu-Martinez et al., 2004; Watters and Palmenberg, 2011), the effect on nucleolin beyond mislocalization (Gustin and Sarnow, 2002) has not been explored in detail. Here we show that infection with either HRV2 or HRV16 can lead to the presence of a cleavage product, similar to our previously reported results for HRV16 (Walker et al., 2013). The exact reason for nucleolin cleavage in HRV infection is unclear, as there are no reports of this event during infection with other viruses. Indeed, nucleolin has been reported as the cellular receptor for respiratory syncytial virus (Tayyari et al., 2011) and has been implicated as a required interacting protein in a number of other virus infections, including human cytomegalovirus (Strang et al., 2012), rabies virus (Oksayan et al., 2015), herpes simplex virus 1 (Greco et al., 2012), and a member of the picornavirus family, enterovirus 71 (Su et al., 2015). One possibility is that HRV infection initiates the apoptotic cascade in such a manner that cleavage of nucleolin is a downstream result, as nucleolin at the approximate size we observed has been reported to be cleaved by okadaic acid-induced apoptosis in cell lines (Kito et al., 2003), however, further investigation is required.

The mislocalization of Sam68 and alteration in the appearance of SC35 occurred as an early event during infection with both HRV2 and HRV16, before there was significant disruption of nuclear pore components in either HRV2 or HRV16 infection, suggesting that either a very small amount of viral protease activity is required to initiate these changes, or that early infection events prompt an additional pathway that contributes to the observed changes. That Sam68 is mislocalized during picornavirus infection has been well established (McBride et al., 1996; Gustin and Sarnow, 2002; Walker et al., 2013), however, HRV-initiated changes in SC35, an essential component of the spliceosome, have only been reported for HRV16 (Walker et al., 2013). In the current paper we also describe the same changes in HRV2 infection; this is in contrast to HRV14 or poliovirus infection (Meerovitch et al., 1993; Gustin and Sarnow, 2001; Gustin and Sarnow, 2002), where no changes in SC35 appearance were observed. While there is precedent for viruses that undergo alternative splicing, such as human papilloma virus (McFarlane and Graham, 2010) and human immunodeficiency virus (Maldarelli et al., 1998), to induce expression of SC35, the reason for HRV disruption of SC35 is unclear, beyond general disruption of the host cellular transcription machinery. It remains to be determined whether alterations in SC35 occur more generally in HRV infection, or if they are group specific and do not occur in Group B HRV.

The mislocalization of hnRNP-C1/C2 in picornavirus infection has been reported previously (Gustin and Sarnow, 2001; Gustin and Sarnow, 2002) and our results are consistent with these reports. Interestingly and in contrast to Sam68 and SC35, the timing of hnRNP-C1/C2 mislocalization varied between HRV2 and HRV16 by at least 3 hours. This delay in hnRNP-C1/C2 mislocalization better reflects the

observed disruption of nuclear pore components, which are cleaved much earlier in HRV2 infected cells. Thus it is likely that complete disruption of the nuclear pore and nuclear trafficking is required before hnRNP-C1/C2 is displaced into the cytoplasm. The reason for this requirement is unclear, since hnRNP-C1/C2, at ∼40 kDa, is a similar size to both SC35 (35 kDa) and Sam68 (68 kDa) so all might be expected to diffuse into the cytoplasm at a similar time. Alternatively it is possible the strong nuclear retention signal carried by hnRNP-C1/C2 (Nakielny and Dreyfuss, 1996) enables these proteins to maintain their nuclear localization for longer during infection.

The impact of HRV serotype on pathogenesis has been examined by a small number of studies, which have found differences in the host immune response to major vs minor group HRV, as well as reduced cell viability after infection with minor group HRV (Wark et al., 2009; Schuler et al., 2014); we also observe greater cell death in cells infected with minor group HRV2 compared to major group HRV16 (**Supplementary Figure S3**). A study of asthma exacerbation has suggested exposure to minor Group A HRV is significantly associated with exacerbations (Denlinger et al., 2011), and a recent study by Schuler et al. (2014) suggests the differential receptor usage between major and minor Group A HRV may be a mechanism for the observed variation in response to different HRV serotypes, though additional studies of multiple HRV serotypes are required.

Conclusion

This study demonstrates there are clear differences in the timing of viral protein production, host protein cleavage and host protein mislocalization, as well as in the amount of viral RNA produced, during HRV2 and HRV16 infection. The mislocalization of some nuclear proteins (SC35 and Sam68) occurs early after the initial infection, prior to the complete disruption of the nuclear pore. Other nuclear proteins (hnRNP-C1/C2) seem to require complete disruption of the nuclear pore, which may be a consequence of this protein carrying a strong nuclear retention signal. Future work is aimed at further investigating the differences between different HRV serotypes and determining whether the variation observed between HRV2 and HRV16 is similar for other major and minor Group A HRV serotypes.

Funding

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00875

FIGURE S1 | Human rhinovirus effects in A549 cells. (A,B) A549 cells were infected without (mock) or with HRV2 or HRV16 (MOI of 5) for 24 h and cells lysed collected and subjected to Western analysis as described in **Figure 1**. The approximate protein size (kDa) is shown on the left and the specificity of the antibodies is indicated on the right. Where cleavage products are observed, bands corresponding to full length proteins are indicated with arrowheads and cleavage products are indicated with brackets. cp – cleavage products. **(C)** A549 cells were grown on coverslips and infected without (mock) or with HRV2 or HRV16 (MOI of 5). At 24 h p.i. cells were fixed, permeabilized and probed with anti-dsRNA antibodies, followed by CF488 secondary antibodies. Coverslips were mounted in ProlongGold mounting media with DAPI. Fluorescence was imaged by CLSM (see Materials and Methods).

FIGURE S2 | HRV2 and HRV16 replicate in A549 cells. Real-time PCR was used to assess the quantity of viral RNA present in infected cells at 24 h p.i. for HRV2 and HRV16 infection. Data is shown as arbitrary units relative to GAPDH.

FIGURE S3 | Cell death after HRV2 or HRV16 infection. Ohio-HeLa cells were grown in six well plates and were mock infected, or infected with HRV2 or HRV16, at an MOI of 1. Live cells were imaged at 24 h p.i. using a Leica DMIL light microscope.

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REVIEW
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Respiratory virus modulation of host nucleocytoplasmic transport; target for therapeutic intervention?

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The respiratory diseases caused by rhinovirus, respiratory syncytial virus, and influenza virus represent a large social and financial burden on healthcare worldwide. Although all three viruses have distinctly unique properties in terms of infection and replication, they share the ability to exploit/manipulate the host-cell nucleocytoplasmic transport system in order to replicate effectively and efficiently. This review outlines the various ways in which infection by these viruses impacts on the host nucleocytoplasmic transport system, and examples where inhibition thereof in turn decreases viral replication. The highly conserved nature of the nucleocytoplasmic transport system and the viral proteins that interact with it make this virus–host interface a prime candidate for the development of specific antiviral therapeutics in the future.

Keywords: rhinovirus, nuclear transport, importins, anti-viral strategies, influenza, respiratory syncytial viruses, exportins

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Introduction: the Global Health Problem

Viral respiratory disease (VRD) results in the hospitalization and deaths each year of millions of people worldwide, representing a large social and financial burden on healthcare globally. Although 100s of viruses can potentially cause VRD, the main causative agents are, Influenza virus, RSV, and HRV. Influenza virus, an orthomyxovirus, is responsible for an estimated 3–5 million cases of severe illness and 250–500 thousand deaths worldwide per year, with an economic impact of $87.1 billion in the US alone (Molinari et al., 2007). During an epidemic/pandemic year, such as the recent 2009 H1N1 outbreak, these figures can rise dramatically; an estimated 42–86 million cases of infection were reported in 2009 (Centers for Disease Control and Prevention, 2010).

The pneumovirus RSV is the single greatest cause of lower respiratory tract illness (LRTI) and bronchiolitis in infants and the elderly, with an estimated 64 million infectious cases and 160,000–600,000 deaths recorded worldwide each winter (Falsey et al., 1995; Law et al., 2002; Simoes, 2008; WHO, 2009; Krilov, 2011). RSV related disease represents a cost of US$2.4 billion in the US alone (Tran et al., 2013). The Picornavirus HRV, is the primary causative agent of the "common cold," resulting in upper respiratory tract infection (URTI) that is generally cleared; although severe

Abbreviations: DAF, decay accelerating factor; Flu, influenza; HRV, human rhinovirus; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IMP, importin; ISG, interferon stimulated gene; ISRE, interferon stimulated response element; LDLR, low density lipoprotein receptor; LRTI, lower respiratory tract infection; MDA-5, melanoma-differentiation-associated gene 5; NE, nuclear envelope; NES, nuclear export signal; NPC, nuclear pore complex; NLS, nuclear localization signal; Nup, nucleoporin; PAMPs, pathogen associated molecular patterns; RIG-I, retinoic-acid-inducible protein; RSV, respiratory syncytial virus; SINE, selective inhibitors of nuclear export; TLR, toll-like receptors; URTI, upper respiratory tract illness; VRD, viral respiratory disease; vRNA, viral RNA; vRNP, viral ribonucleoprotein complex; XPO, exportin.

complications can arise in vulnerable individuals including the elderly, but especially those with underlying respiratory conditions such as asthma (Nicholson et al., 1993; Costa et al., 2014), where HRV has been identified as the causative agent in 50–85% of virally induced asthma hospitalization cases, costing billions of dollars (Costa et al., 2014).

Influenza virus, RSV, and HRV alone represent a huge burden of disease and economic strain worldwide. Unlike influenza virus, where a seasonal vaccine is available, there are currently no efficacious vaccines or treatments for either RSV (Bawage et al., 2013) or HRV (Jacobs et al., 2013). Research over the past decade indicates that many cytoplasmically replicating RNA viruses, such as RSV and HRV, utilize and/or manipulate the host-cell nuclear transport machinery to their benefit, either by transporting specific viral proteins into the nucleus to modulate cellular function/minimize the host antiviral response, or by inhibiting host nuclear transport itself and thereby dampening innate immune responses (Alvisi et al., 2007; Fulcher and Jans, 2011). In the case of influenza virus, nuclear transport, and localization of key viral proteins is required for vRNA replication to occur, which is subsequently exported from the nucleus (Whittaker et al., 1996; Neumann et al., 1997; Samji, 2009; Huet et al., 2010). The importance of the host-cell nucleocytoplasmic transport machinery to viral infection makes it a therapeutic target of great potential for development of anti-viral agents in the future (Perwitasari et al., 2014).

Nuclear Transport

Gaining Access to the Nucleus

The nucleus is a specialized compartment within eukaryotic cells where the genetic information is contained, surrounded by the lipid double membrane structure of the NE representing the boundary between the genome and the cytoplasm (Dingwall and Laskey, 1992). Specific mechanisms are required to effect the transport of proteins, mRNA, and protein–RNA complexes between the cytoplasm and the nucleus in a controlled and regulated manner (Jans and Hubner, 1996). In order to permit the necessary passage of proteins and mRNA into and out of the nucleus, the NE is perforated by a series of NPCs through which all transport into and out of the nucleus occurs. These channels comprise up to 50 different nup proteins (8–32 copies of each; Rout et al., 2000) resulting in a super-protein-complex of around 125 MDa (Reichelt et al., 1990). Specific nups harbor hydrophobic (Phe-Gly or FG) repeat sequences, which are believed to function as transient binding sites for complexes passing through the NE. The NPC acts as a molecular sieve, enabling the passage of molecules <50 kDa in molecular weight into or out of the nucleus by passive diffusion (Talcott and Moore, 1999). Larger molecules can only be transported through the NPC in an active energy-dependent mechanism requiring specific targeting signals, NLS and NES, which mediate transport into and out of the nucleus, respectively.

Signal-dependent protein nuclear import (see **Figure 1**) is mediated by members of the IMP superfamily of transporters, of which various α and β subtypes exist that recognize and bind to specific and highly conserved NLSs on their respective cargo proteins (Conti et al., 1998); generally through either the classical IMPα/β1 heterodimer (see **Figure 1ia**) or one of the IMPβs alone (**Figure 1ib**; Jans and Hubner, 1996; Alvisi et al., 2007; Fulcher and Jans, 2011). The NLS-cargo/IMP complex then docks to nups at the cytoplasmic side of the NPC (**Figure 1ii**), before translocating through the pore via transient and sequential interactions between IMPβ and the nups (**Figure 1iii**; Bednenko et al., 2003). Once within the nucleus, RanGTP binds to IMPβ (**Figure 1iv**) resulting in NLS-cargo release (Stewart, 2007). The nuclear IMPs are then recycled to the cytoplasm where they are available for subsequent rounds of import (Kutay et al., 1997). In analogous fashion to import, the nuclear export of NES-containing proteins is mediated by the XPO family of homologs of IMPβ1, of which XPO1 is the best-characterized (Hutten and Kehlenbach, 2007). Briefly, RanGTP binding to the XPO (**Figure 1v**) is required to allow potential cargo proteins to bind. The RanGTP/XPO/cargo trimeric complex then passes through the NPC (**Figure 1vi**) to the cytoplasm via sequential interactions between the XPO and nups of the NPC. Once in the cytoplasm, hydrolysis of RanGTP to RanGDP (**Figure 1vii**) effects release of the NES containing cargo protein into the cytoplasm.

Nucleocytoplasmic Transport and the Innate Immune Response to Viral Infection

Viral respiratory tract infections represent one of the most common types of infectious disease(s) encountered, so it is imperative that the host innate immune response is able to identify and eliminate these threats efficiently. During viral infection, the family of TLRs (Thompson and Locarnini, 2007) in concert with the cellular helicases RIG-I and MDA-5 recognize different viral by-products, or PAMPs, which initiates a cascade of events resulting in the activation of transcription factors such as IFN response factor 3 (IRF3; Hiscott, 2007), nuclear factor κB (NF-κB; Fagerlund et al., 2005) or activating protein 1 (AP1). These transcription factors are transported to the nucleus through their interaction with specific IMPs (Torgerson et al., 1998), resulting in the initiation of IFN-β transcription. Newly translated IFN-β protein is then secreted from the cell to work in an autocrine and paracrine matter, whereby binding to the IFN-α/β receptor leads to a secondary cascade of events involving multiple proteins and transcription factors such as the STAT (signal transducer and activator of transcription) proteins. These proteins are transported to the nucleus in an IMP mediated manner where they interact with IFN-sensitive response elements (ISRE), activating the transcription of numerous anti-viral IFN stimulated genes (ISGs). Clearly, it is imperative that host-cell nuclear import remains functional during a viral infection for a concerted immune response to be initiated. With regulated host-cell nuclear transport playing such a critical role in the innate immune response, this system is therefore ripe for attenuation/modulation by infectious pathogens.

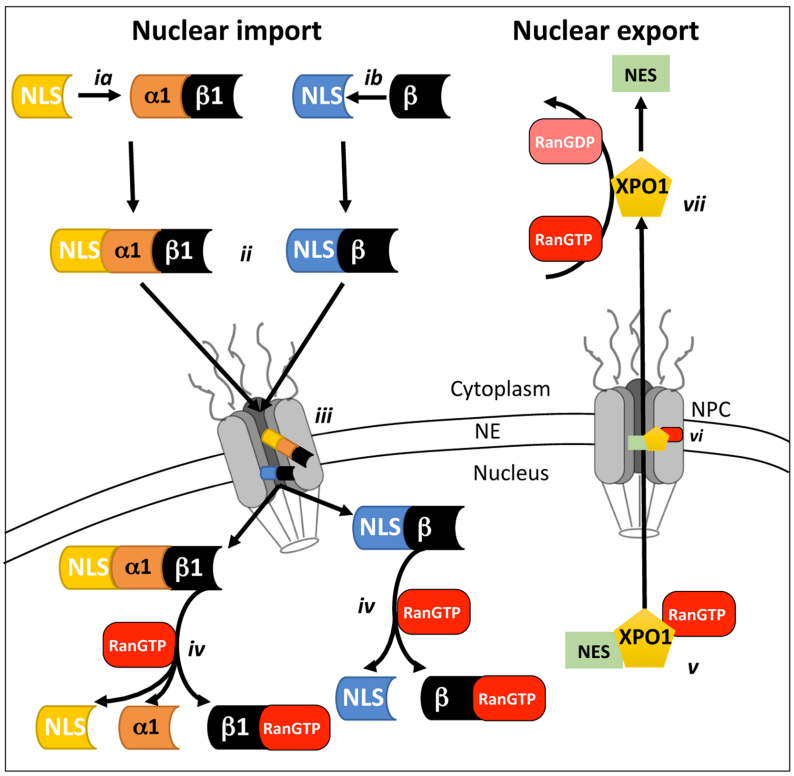

FIGURE 1 | Schematic representation of mammalian cell nucleocytoplasmic transport. Nuclear transport across the NE requires the recognition and binding of NLS-containing cargo proteins by either the IMPα/β1 heterodimer **(ia)** or IMPβ homologues alone **(ib)** on the cytoplasmic side of the NPC. Once bound **(ii)**, the transport complex is believed to dock to the cytoplasmic side of the NPC and then move through the NPC **(iii)** via a series of transient interactions between IMPβ and the nups that comprise the NPC. Once within the nucleus, binding of RanGTP **(iv)** to Impβ causes dissociation of the transport complexes and release of the cargo to perform its nuclear function. Nuclear export **(v)** requires the recognition of a nuclear export sequence (NES)-containing cargoes by an XPO such as XPO1 in complex with RanGTP. The trimeric RanGTP/XPO1/cargo complex passes through the NPC via a series of transient interactions **(vi)** with nups within the NPC. Once within the cytoplasm, hydrolysis of RanGTP to RanGDP **(vii)** results in dissociation of the XPO1/cargo complex.

Viral Replication and Interaction(s) with Host-Cell Nucleocytoplasmic Transport

Human Rhinovirus (HRV)

Human rhinovirus, a member of the *picornaviridae* family, is a non-enveloped icosahedral virus that at its core possesses a positive sense single-stranded RNA (+ssRNA) genome that encodes 11 proteins initially expressed as a single polyprotein. To date there have been 156 different HRV serotypes identified (divided into serotypes A–C based on the phylogenetic relationship of the respective VP1 and VP2/4 genes; McIntyre et al., 2013). Current understanding is that the majority of HRV A/B serotypes can bind specifically to ICAM-1 on the host cell surface (Greve et al., 1989), with approximately 10% utilizing the LDLR (Hofer et al., 1994). A very small subset appear able to utilize the DAF protein (Blomqvist et al., 2002) to bind target cells, while the Cadherin-related family member 3 (CDHR3) has only recently been identified as the cell surface receptor used by HRV-C serotypes (Bochkov et al., 2015). Viral binding and attachment to the host-cell has traditionally been viewed as a viable target for drug development, but the fact that at least four different cell-surface receptors are used by HRV serotypes means that a pan-serotype inhibitor of HRV binding is unlikely to be a realistic possibility in the near future. Rhinovirus infection is initiated by inhalation of HRV into the nasal passage whereby the virions make their way to the rear of the nose where they bind one of the respective cell surface receptors. Upon binding the virions are internalized by either clathrin-dependent endocytosis or macropinocytosis (reviewed in Fuchs and Blaas, 2010), after which viral uncoating occurs, and the +ssRNA genome is released into the cytoplasm where it is translated on entry to produce a single polyprotein. The polyprotein undergoes self-proteolysis during translation by the viral proteases 2A and 3C (Skern et al., 1985; Cordingley et al., 1990) to generate the structural (VP1, VP2, VP3, VP4) and non-structural (2A, 2B, 2C, 3A, 3B, 3C, 3D) proteins required for virion assembly, meaning that the full-length product is rarely observed.

In recent years, a hallmark of picornavirus, and thus HRV infection, is the shutdown of regulated host-cell nucleocytoplasmic transport (see **Figure 2**), contributing to

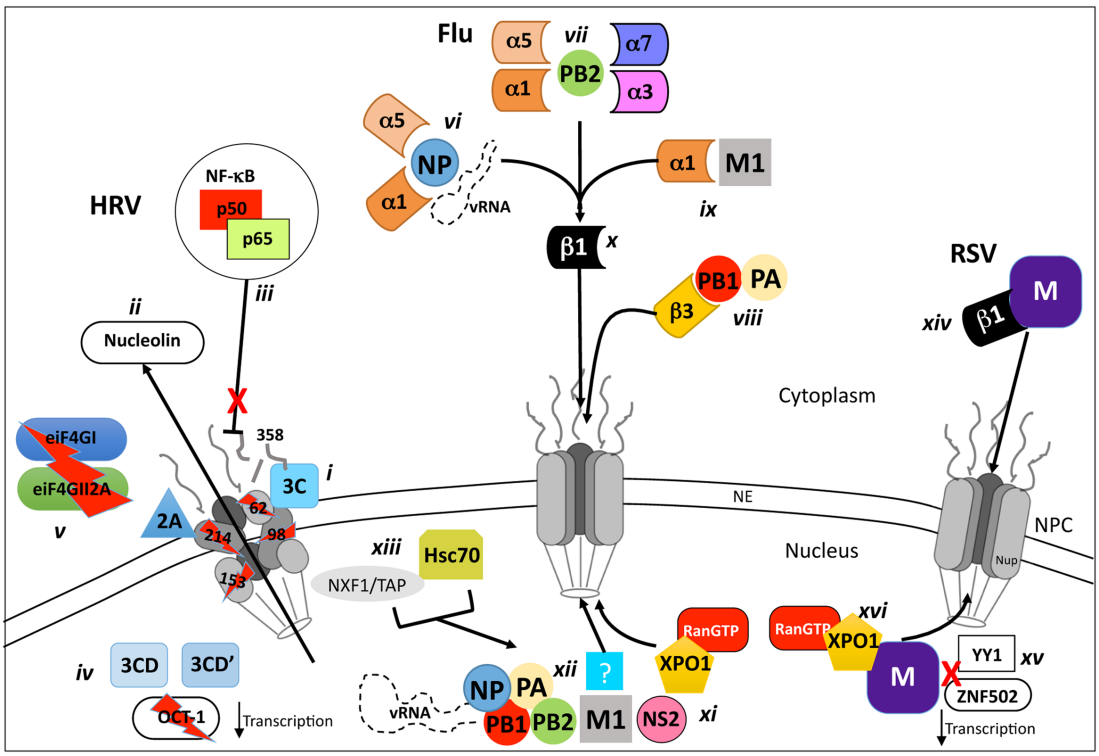

FIGURE 2 | Schematic representation of VRD modulation and/or exploitation of host nucleocytoplasmic transport processes. Inhibition and/or utilization of host-cell nucleocytoplasmic transport are key features of infection by Rhinovirus (HRV), Influenza virus and RSV. During HRV infection, the viral proteases 2A and 3C localize to the NPC **(i)** and degrade nups 62, 98, 153, 214, and 358, causing mislocalization of nuclear proteins such as nucleolin **(ii)** and preventing nuclear import of complexes such as the anti-viral NF-κB transcription factor **(iii)**. Host-cell transcription/translation is severely reduced by the NLS of the 3CD and 3CD' proteases which degrade the general transcription factor OCT-1 **(iv)** in concert with 2A, which also degrades the cytoplasmic translation elongation factors eIF4GI and eIF4GII2A **(v)**. Efficient influenza virus replication requires the transport of the viral genome and proteins required for its replication (PB1, PB2, PA, and NP) to the nucleus where they form a vRNP complex. The vRNA is transported to the nucleus through binding to NP, which is recognized by IMPα1 or α5 **(vi)** in complex with Impβ1 **(x)**, is transported through the NPC, as is PB2 **(vii)**, which is recognized by either IMPα7, α5, α3, or α1 in complex with IMPβ1 **(x)**. The PB1/PA heterodimer is transported to the nucleus by interaction with the IMPβ1 homologue IPO5 **(viii)** which can bind the NPC directly. The M1 protein, critical for the nuclear export of the vRNP complex is imported to the nucleus via IMPα1/β1 **(ix,x)**. The newly synthesized vRNA (part of the vRNP-N1-NS2 complex) is exported from the nucleus by XPO1 interaction with NS2 **(xi)**. An unknown exporter **(xii)** that interacts with M1 has been implicated in this process, as have the proteins Hsc70 and NXF1/TAP **(xiii),** which are postulated to act as cofactors via an undefined mechanism. The RSV M protein relies on interaction with Impβ1 **(xiv)** early during infection to localize to the nucleus where it suppresses host-cell transcription by potentially blocking the activity of transcription factors such as ZNF502 and YY1 **(xv)**. M is exported to the cytoplasm later in infection by XPO1 **(xvi),** where it is critical for pro-virion assembly.

reduced cellular transcription and translation, although viral transcription/translation continues unabated. The disruption of host-cell nuclear transport has been attributed to the specific proteolysis and degradation of the FG-containing nups 62, 98, and 153 within the NPC by the viral proteases 2A and 3C (Ghildyal et al., 2009b; Park et al., 2010; Watters and Palmenberg, 2011; Walker et al., 2013; see **Figure 2i**), leading to disruption of classical nucleocytoplasmic shuttling (Gustin and Sarnow, 2002; see **Table 1**). The general disruption of nuclear transport can be observed early in HRV infection whereby endogenous nuclear proteins such as the RNA associated La and Sam68 proteins (Itoh et al., 2002; Wolin and Cedervall, 2002) are mislocalised to the cytoplasm, along with the critical ribosome maturation factor, nucleolin (**Figure 2ii**; Gustin and Sarnow, 2002) leading to cell-cycle arrest and subsequent apoptosis (Ugrinova et al., 2007). In an *in vitro* semi-intact cell system, GFP-tagged 3C was found to disrupt both active (IMP-mediated) and passive (size exclusion) nuclear transport through degradation of nups 358, 214, and 153 (Ghildyal et al., 2009b). Interestingly, nup62 was not degraded, implying that proteolysis of specific nups within the NPC may be through the concerted action of 2A and 3C.

In addition to its role in nup degradation, 3C in the context of the larger 3CD and 3CD' precursors appears to localize in the nucleus and degrade the general transcription factor OCT-1 (see **Figure 2iv**; Amineva et al., 2004), leading to a rapid loss of host-cell transcription early in infection (2–4 h). In parallel, the eukaryotic initiation factors (eIFs) eIF4GI and eIF4GII2A, which form part of the eIF4F complex that recognizes capped-RNA, are degraded by 2A (**Figure 2v**) further contributing to halting host-cell translation (Liebig et al., 2002) but not IRES-mediated HRV RNA translation.

TABLE 1 | Summary of respiratory virus protein interactions with components of the host nucleocytoplasmic transport system.

Virus	Viral Protein	Host protein	Effect of interaction	Effect of disrupting interaction on virus titre	Reference
Rhinovirus	2A	nup62	Disruption of host nucleocytoplasmic transport	N/A	Gustin and Sarnow (2002)
	2A	nup98			
	2A/3C	nup153			
	2A/3C	nup214/358			
RSV	M	IMPβ1	IMPβ1 transports M to nucleus to initiate host-cell transcriptional inhibition and increase virus production	Mutation of M NLS results in 20-fold reduction in virus titre	Ghildyal et al. (2005a)
	M	XPO1	Nuclear export of M by XPO1 is absolutely essential to initiate virion formation	Mutation of M NES abolishes RSV virus production; inhibition of XPO1 mediated nuclear export using the XPO1 inhibitor LMB reduces RSV titre > 10-fold	Ghildyal et al. (2009a)
Influenza	NP	IMPα1, IMPα5	NP binding to α1 or β5 allows nuclear import of NP through Impβ1	Cells treated with peptides that compete with NP binding for Impα show 2–5 log reduction in Flu virus titre. Granzyme K mediated proteolysis of Impα/β reduced NP nuclear import and subsequent Flu viral titre twofold	O'Neill et al. (1995), Cros et al. (2005)
	PB1	IMPβ3	PB1 (heterodimer with PA) binds to IMPβ3 and is trafficked to the nucleus	Mutation of the IMPβ3 recognised NLS in PB1 results in a 4-$\log_{10}$ reduction in virus titre	Hutchinson et al. (2011)
	PB2	IMPα1, α3, α5, α7	PB2 shows preference for IMPβ7 in mammalian cells *in vivo*, but it can bind to IMPα3 and α5	Mutation of PB2 NLS results in 100-fold reduction in virus titre	Resa-Infante et al. (2008), Boivin and Hart (2011), Pumroy et al. (2015)
	PA	IMPβ3	Requires heterodimerization with PB1 to undergo nuclear import by IMPβ3	Mutation of the IMPβ3 recognised NLS in PB1 results in a 4-$\log_{10}$ reduction in virus titre	Hutchinson et al. (2011)
	NS2 (NEP)	XPO1	Allows nuclear export of vRNP-M1-NS2 complex from nucleus to cytoplasm where viral budding occurs	The XPO1 inhibitor LMB almost completely suppresses influenza virus levels in infected MDCK cells. XPO1 inhibitor Verdinexor inhibits influenza virus A and B replication in tissue culture and a mouse model.	Watanabe et al. (2008), Brunotte et al. (2014), Gao et al. (2014), Perwitasari et al. (2014)
	M1	IMPα1 (porcine)	M1 shows interaction with porcine IMPα1, but this has yet to be confirmed in human cells	N/A	Liu et al. (2014)
		Exporter (?)	Leucine-rich NES region identified in M1. Mutation causes accumulation of vRNP in the nucleus, even in the presence of NS2	Alanine mutation of the M1 NES reduced Flu viral titre 200–300-fold	Cao et al. (2012)
	vRNP-M1-NS2	Hsc70	Hsc70 has been shown to interact with M1 and may help mediate vRNP nuclear export in the absence of NS2		Watanabe et al. (2014)
		NXF1/TAP	NXF1/TAP is required for the nuclear export of viral mRNA encoding HA, NA, M1, NS1, and M2	siRNA depletion of NXF1 reduced Flu viral titre 100-fold	Read and Digard (2010)

IMP, importin; nup, nucleoporin; LMB, leptomycin B.

During HRV infection, the production of INF-β mRNA through the Type 1 IFN response is attenuated, leading to dampening of the antiviral response (Kotla et al., 2008). Although the precise mechanism leading to the cause of this reduced response has yet to be elucidated, it is entirely plausible that the deregulation of host nucleocytoplasmic transport by the 2A and/or 3C proteases may be responsible, by preventing the nuclear import of activated NF-kB (**Figure 2iii**). Although the

precise role(s) and kinetics of 2A and 3C protease-mediated nup degradation remain to be determined *in vivo*, it is clear that disruption and degradation of the NPC and cleavage of essential transcription factors by 3C is central to host cell shutdown, through deregulation of host cell nucleocytoplasmic transport to prevent the infected cell mounting an antiviral response.

Respiratory Syncytial Virus (RSV)

In a similar fashion to HRV, the paramyxovirus RSV replicates entirely within the cytosol of infected cells. The outer surface of the RSV virion is comprised of a lipid-rich membrane that encompasses the nucleocapsid within which resides the ssRNA viral genome that encodes all 11 viral proteins (Ogra, 2004). Although glycosaminoglycans are utilized in cell culture (Hallak et al., 2000a,b), clinical RSV infection is initiated by binding of the large glycoprotein (G; Levine et al., 1987) on the virion surface to as yet uncharacterized cell-surface receptor(s). A potential candidate in this regard appears to be the nucleolar protein nucleolin, believed to be present on the apical surface of lower respiratory tract epithelia (Tayyari et al., 2011); see also (Holguera et al., 2014). Following attachment, the viral fusion (F) protein causes both viral and cellular membranes to fuse, subsequently releasing the ssRNA containing nucleocapsid core into the host cell cytoplasm and allowing viral transcription/translation to proceed.

A key pathogenic factor and component of RSV is the virally derived Matrix (M) protein, which associates with the nucleocapsid and envelope glycoprotein complexes within the virion, and is believed to be a key driver of virus assembly in the infected cell (Ghildyal et al., 2006, 2009a). In addition to this important role, M is also able to traffic early in infection to the host cell nucleus (**Figure 2xiv**), dependent on interaction with IMPβ1 (Ghildyal et al., 2005a; see **Table 1**). Nuclear M inhibits host cell transcription (**Figure 2xv**; Ghildyal et al., 2003), potentially by targeting transcription factors such as the Zinc finger-containing ZNF501 and ZNF502, or the ubiquitous YY1 (Yin Yang 1; Kipper et al., 2015).

Later in infection, M is ferried to the cytoplasm by XPO1 (**Figure 2xvi**; Ghildyal et al., 2009a), where it localizes to viral inclusion bodies (IBs; Lifland et al., 2012), and functions as an adaptor bringing together newly formed nucleocapsids and envelope glycoproteins F and G (Ghildyal et al., 2002, 2005b, 2006) to effect virus assembly. Although RSV replication and assembly occurs exclusively within the cytoplasm, RSV virus with mutations within M's IMPβ1-recognized NLS are approximately 20-fold attenuated in terms of virus production (Ghildyal et al., 2009a), indicating that M nuclear import through IMPβ1 is central to RSV infection, and represents a viable target for the development of agents to combat RSV infection. Analogously, M nuclear export through XPO1 is an interesting target based on the fact that RSV mutated in the XPO1-recognized NES of M is not viable, presumably due to the critical requirement for M in the cytoplasm later in infection for RSV virion assembly (Ghildyal et al., 2009a); inhibition of XPO1 using the XPO1 specific inhibitor leptomycin B (LMB) added later in infection reduces RSV virus production 20-fold, underlining the utility of targeting M nuclear export as an approach to inhibit RSV (Ghildyal et al., 2009a).

Influenza (Flu)

Unlike RSV and HRV, which replicate their viral genomes within the host-cell cytoplasm, Influenza virus must transport its genome, in the form of a RNP complex, into the host-cell nucleus in order for replication to occur. The key components of the RNP are the vRNA binding nucleoprotein (NP; O'Neill et al., 1995) and the vRNA-dependent RNA polymerase (vRdRp), which comprises the three subunits, PB1, PB2 (protein basic 1 and 2) and polymerase acidic (PA). NP-dependent nuclear import of vRNA appears to be through interaction of NP with either IMPα1/β1 or α5/β1 (**Figure 2vi**; O'Neill et al., 1995; Wu et al., 2007). Nuclear import of the PB1/PA heterodimer is mediated through recognition of PB1 by IMPβ3 (IPO5; **Figure 2vii**; Deng et al., 2006; Hutchinson et al., 2011). Finally, PB2 is able to interact with a number of different IMPs, including IMPα7/β1, α1/β1, α3/β1, and α5/β1, all of which can transport it into the nucleus (**Figure 2vii**; Resa-Infante et al., 2008; Boivin and Hart, 2011; Pumroy et al., 2015). Thus, various IMPs are responsible for nuclear import of the various proteins that make up the mature vRNP complex, meaning that there are potentially many candidate NLS:IMP targets for therapeutic intervention early in the viral lifecycle (see **Table 1**).

Once the vRNP is localized within the host-cell nucleus, the vRNA initially undergoes a round of replication resulting in the production of 5′ capped and 3′ poly(A) viral mRNA which is exported to the cytosol utilizing the host-cells mRNA transport machinery to undergo translation to produce new viral proteins. A second round of replication then proceeds, whereby positive sense RNAs are produced to serve as templates for the production of negative sense vRNA genomes, which in turn combine with newly synthesized and nuclear localized NP, PB1, PB2, and PA proteins to form new vRNPs (Josset et al., 2008). These vRNPs undergo nuclear export through the action of the viral NS2 (NEP) and M1 proteins that enter the nucleus either via passive diffusion, or potentially through IMPα1 in the case of M1 (**Figure 2ix**; **Table 1**; Liu et al., 2014), and recruit the host export protein XPO1. XPO1 recognizes the vRNP-M1-NS2 complex (**Figure 2xi**) via 2 NESs on NS2, which helps effect transport of the complex out of the nucleus via the NPC. M1 also appears to contribute to vRNP nuclear export through a NES that would appear to be recognized by an export protein other than XPO1 (**Figure 2xii**; see **Table 1**); mutation of the NES results in vRNPs nuclear accumulation (Cao et al., 2012). Additionally, two recent studies have identified additional cellular proteins as co-factors (**Figure 2xiii**) for vRNP-M1-NS2-XPO1 export (**Table 1**); the heat shock protein Hsc70 (Watanabe et al., 2014), which is believed to play a key role in calcium-/calmodulin-dependent nuclear import of SOX proteins nuclear import (Kaur et al., 2013), and NXF1/TAP (Read and Digard, 2010), integrally involved in the nuclear export of cellular mRNA. These may also represent potential candidates for antiviral intervention.

Clearly, the host-cell nucleocytoplasmic transport machinery is absolutely required during multiple stages of the Influenza virus life cycle, with both nuclear import and export of vRNPs presenting targets for potential antiviral intervention (Perwitasari et al., 2014).

Current Therapeutics

Although there are currently a number of therapeutic and prophylactic approaches to manage HRV, RSV, and Flu, there is an ever-present need for new specific and low toxicity treatments for all three.

A number of different treatment regimens targeting the HRV viral capsid and protease proteins have been trialed in the past (De Palma et al., 2008), but none have had any appreciable effect on HRV disease severity (Jacobs et al., 2013). Vapendavir, a capsid binding small molecular inhibitor is currently undergoing Phase 2b clinical trials, with encouraging preliminary data (Matz, 2013), however, as for previous drugs targeting the viral capsid, which is less highly conserved across HRV strains than the other non-structural proteins, there remains a strong likelihood of selection for viral escape mutants (Thibaut et al., 2012). In contrast, the viral proteases 2A and 3C are highly conserved between HRV serotypes (Tapparel et al., 2007) and represent the most likely candidates for successful therapeutic intervention. The most promising HRV protease inhibitor thus far has been Rupintrivir, which specifically recognizes and irreversibly binds a domain within protease 3C that is highly conserved in all picornavirus species (Binford et al., 2007). Although promising results have been obtained *in vitro* and in early challenge trials, a significant reduction in viral load was not achieved during trials with natural infection due to the requirement for the drug to be taken immediately prior or just after infection (Patick et al., 2005); thus further development in regards to HRV was ceased. Recently the enterovirus 3C protease inhibitor SG85 was shown to effectively inhibit HRV infection, with little evidence for resistance arising under long-term serial passage of virus in a tissue culture system in the presence of the drug (Lacroix et al., 2015). Although few small molecular inhibitors have been developed targeting proteases 3C or 2A (Patick et al., 2005), recent studies show that these may prove be a promising avenue in the future.

There is no vaccine currently available against RSV, with previous approaches having caused increased sensitivity and morbidity of premature babies to RSV (Fulginiti et al., 1969). There are currently two prophylactics available, which are able to be safely administered to individuals identified as high-risk of complications if infected with RSV. The monoclonal antibody Palivizumab targets the RSV F protein, and can be administered to pre-identified high-risk infants such as those with chronic lung/heart disease or premature babies (Chavez-Bueno et al., 2007; American Academy of Pediatrics Committee on Infectious and American Academy of Pediatrics Bronchiolitis Guidelines, 2014). Palivizumab can be administered monthly for a maximum of 5 months to maintain therapeutic levels over winter (Saez-Llorens et al., 1998), but although it has helped reduce hospitalization of "at-risk" individuals, it is unsuitable as a large-scale, community wide prophylactic, due to logistic issues and high cost due to the frequency of dosing required (La Via et al., 2013). A more potent derivative, Motavizumab was initially thought to reduce RSV hospitalizations by 25%, compared to those treated with Palivizumab (Feltes et al., 2011), but a recent study of 118 RSV-infected infants has shown that Motavizumab had no appreciable effect on the duration of hospitalization, severity of illness, or wheezing episodes compared to placebo (Ramilo et al., 2014).

The nucleoside Ribavirin in aerosol form has also been prescribed to treat RSV, but appears to have only a moderate effect in terms of reducing days of hospitalization and recurrent wheezing post RSV infection (Ventre and Randolph, 2007). Ribavirin is also extremely costly (∼US $14,000 per treatment) in aerosol form, making it unsuitable for use in impoverished countries (Pelaez et al., 2009). Finally, apart from being carcinogenic and gonadotoxic (Narayana et al., 2005), it appears to be a potential teratogen for women of childbearing age who may come into contact with the drug via secondary exposure (e.g., those caring for children receiving Ribavirin treatment; Krilov, 2002). Clearly, the lack of a vaccine, and limitations of current antiviral prophylactics and therapeutics underline the need for safer, low toxicity and low cost antivirals for RSV.

In contrast to RSV, current options to treat Flu include vaccines and anti-virals. Vaccines, although highly successful, are only able to protect against those viruses that are antigenically close to the vaccine viral reference strain used. Thus, in cases such as the 2009 H1N1 pandemic, vaccine production and lead time was 3–6 months, requiring the use of antiviral agents in the interim (Organization, 2009). The two main classes of anti-influenza virus compounds commercially available are neuraminidase (NA) inhibitors and M2 ion channel blockers. The glycoprotein NA is located within the viral envelope and plays a crucial role in viral particle release from infected cells and prevents virus self-aggregation (Itamura, 1997; Yano et al., 2008; Shtyrya et al., 2009). Historically, NA has been a viable, druggable target due to its high conservation amongst influenza virus strains (Yen et al., 2006), surface accessibility and low rate of mutation compared to other viral proteins, but resistant viral strains have started to appear in the last few years (Garcia et al., 2009).

Influenza virus M2 protein forms a transmembrane pore that helps control the acidity of the virion interior and facilitate viral fusion and release of the genome-containing core. Numerous drugs of the adamantine family have been developed over the last five decades (Davies et al., 1964; Grunert et al., 1965; Tyrrell et al., 1965) that block the action of this pore, although their use is falling out of favor due to the rapid occurrence of resistant viral strains (Leonov et al., 2011). Thus, with use of the two major classes of influenza anti-virals becoming more and more problematic, it is imperative that new, broad-spectrum anti-viral agents be developed.

As indicated above, nucleocytoplasmic transport is integral to the majority of the influenza virus replicative cycle. Previous

studies have looked at the effect of attenuating the nuclear transport of various influenza virus proteins as an antiviral approach. Peptides derived from IMPα have been used in a competition approach to reduce the level of nuclear NP, resulting in a dose-dependent reduction in viral titre by decreasing influenza vRNA nuclear import (Cros et al., 2005). In addition, results from a reverse genetics system indicate that mutation of the well-conserved IMPβ3-recognized NLS1 and NLS2 region of PB1 results in severely attenuated/impaired viral replication (Hutchinson et al., 2011). Finally, specific proteolysis of Impα/β1 by Granzyme K in influenza virus-infected A549 cells both impaired NP nuclear import and reduced viral titres by 50% (Zhong et al., 2012), highlighting that the nuclear import of the vRNP can be a viable target for therapeutic intervention.

Exportin1-mediated nuclear export has been identified as critical for efficient influenza virus replication to occur. Influenza virus infected cells treated with LMB, an irreversible inhibitor of XPO1, show a dose-dependent reduction in viral titre, with complete inhibition of viral replication at 10 ng/ml (Watanabe et al., 2008). LMB is an unsuitable drug candidate, however, due to its irreversible binding to XPO1 and associated potential toxicity. Recently a new class of orally available SINE (Lapalombella et al., 2012) have been engineered which form a slowly *reversible* covalent bond with XPO1, minimizing cytotoxicity. The SINE compound Verdinexor has recently been found inhibit the replication of various influenza A and B virus strains in cell culture and reduce lung virus titres and associated disease pathology in a mouse model with minimal cytotoxicity (Perwitasari et al., 2014).

Screening for Inhibitors

The main challenge in looking for inhibitors that target ubiquitous systems such as nucleocytoplasmic transport for therapeutic intervention is cytotoxicity. Strategies to overcome this include those where potential hit compounds can be identified that specifically target the interface between viral and host-cell proteins (e.g., IMP-viral protein or XPO-viral protein) rather than the IMP or XPO directly, which would potentially block transport of all host cell proteins that use the IMP/XPO for normal trafficking. High-throughput screening where a counterscreening strategy has been incorporated has proved efficacious in identifying compounds that have proved to be specific inhibitors (Wagstaff et al., 2011; Fraser et al., 2014), with low toxicity and strong antiviral activity (**Figure 3**; Wagstaff et al., 2011, 2012, Fraser et al., 2014). Primary screening (**Figure 3i**) is performed on a compound library to identify molecules that disrupt the interaction between the viral protein-IMP/XPO of interest, followed by specificity counter-screening (**Figure 3iii**) to identify compounds that directly inhibit IMP/XPO function. Only compounds shown to specifically inhibit viral protein-IMP/XPO are selected for further cell based antiviral activity analysis (**Figure 3iv**) and structural refinement (**Figure 3v**; structure/activity determination). Refined molecules are re-screened (**Figure 3vi**) to confirm activity and specificity before evaluation in animal models (**Figure 3viii**). This strategy has been used successfully to identify antivirals targeting nuclear import for HIV and DENV (Wagstaff et al., 2011, 2012; Fraser et al., 2014) – see next section – underlining its intrinsic

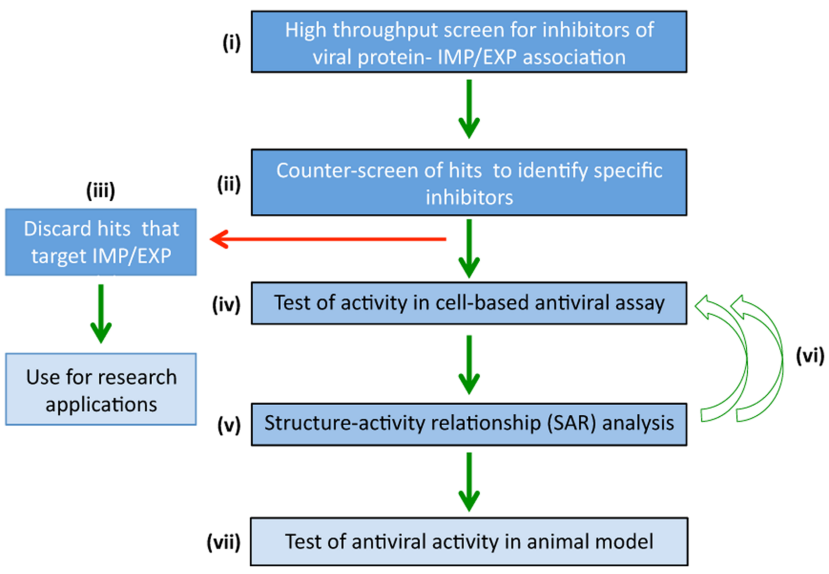

FIGURE 3 | High-throughput screening to identify specific agents targeting nucleocytoplasmic transport that have antiviral activity. High-throughput screening strategies to rapidly identify antiviral compounds that specifically block viral:IMP/XPO/host protein interaction. **(i)** Primary screening is performed using a chemical library to identify molecules that inhibit viral protein-IMP/XPO interaction. **(ii)** Hits are counterscreened to identify compounds that target IMP/XPO function directly, which although of interest for research applications, are discarded from further examination in the pipeline toward specific antivirals. **(iii)** Compounds not targeting IMP/XPO function but rather specifically inhibiting viral protein:IMP/XPO interaction are screened in cell-based assays **(iv)** to confirm antiviral activity. **(v)** Structure-activity relationship analysis/focused library screening) is performed to optimize properties of the inhibitor (e.g., pharmacodynamics) in multiple iterations **(vi)**, prior to evaluation of lead compounds in animal models of viral infection **(vii)**.

utility for future endeavors to develop efficacious, non-toxic antivirals.

Future Prospects

Since many RNA viruses rely on nuclear import of specific viral gene products for efficient replication, nucleocytoplasmic transport of viral proteins represents a viable target for the development of anti-virals, with all of the various interactions listed in **Table 1** thus at least in theory representing potential targets for drug development. Targeting the host cell nuclear import/export machinery itself can clearly have an effect on virus production (Ghildyal et al., 2009a; Prasetyo et al., 2009; Rawlinson et al., 2009; Wagstaff et al., 2012), but it is important to recognize that, in general, targeting host cell proteins directly can lead to toxicity (Newlands et al., 1996),. However, recent work utilizing lower toxicity inhibitors of XPO1 (SINE compounds) against influenza virus (Perwitasari et al., 2014) has progressed into Phase I clinical trails, representing landmark studies in the pursuit of antivirals directed at host nuclear transport components. Drugs targeting viral proteins directly can select for mutations that can reduce binding of anti-viral agents without diminishing viral protein function too drastically (Johnson et al., 2011; Kuritzkes, 2011; Skar et al., 2011; Lundgren and Lazarus, 2012). Targeting the viral protein:host protein interface (Loregian et al., 2002) may be the most profitable in terms of avoiding issues of cytotoxicity, as well as limiting the possibilities for viruses to mutate and still maintain viability. The recognition and subsequent transport of cargo proteins across the NE requires recognition of an NLS by its cognate IMP, with only very minor changes in the NLS tolerated before nuclear transport rates decline (Yang et al., 2010). This makes nucleocytoplasmic transport an attractive therapeutic target, since selective pressure to alter the NLS/NES to prevent drug binding are likely to result in NLSs that are no longer able to be recognized by the host-cell IMP/XPOs and thus fail to mediate efficient nuclear transport.

Mifepristone is an example of a compound that specifically inhibits HIV-1 IN:IMPα/β1 interaction (Wagstaff et al., 2011), and can inhibit HIV infection (Wagstaff et al., 2012), whilst recent work for dengue virus (DENV) shows that a specific inhibitor [*N*-(4-hydroxyphenyl) retinamide] of DENV non-structural protein five nuclear import through IMPα/β1 can protect against infection by all four serotypes of DENV, including severe, antibody-dependent enhanced disease in a lethal mouse model (Fraser et al., 2014). Clearly, targeting the host-pathogen interface in terms of nucleocytoplasmic transport represents an exciting and viable avenue for the future development of novel anti-viral drugs that are likely to be efficacious and importantly, highly specific (Perwitasari et al., 2014).

The approaches of Wagstaff et al. (2011) and Fraser et al. (2014) to derive specific inhibitors targeting the host-pathogen interface used a counterscreening approach (**Figure 3**) to identify all inhibitors that were likely to be directed against IMPs rather than the host-virus interface; only compounds specifically disrupting viral:host protein interaction were pursued, resulting in successful and rapid identification of the inhibitors that ultimately proved to be highly specific for the host-virus interface. This represents an exciting strategy in the future, potentially for HRV 2A/3C nuclear import, for RSV M nuclear import and export, and nuclear import/export of the various influenza virus proteins. Targeting the viral protein-IMP (or viral protein-XPO) interface in the case of these various proteins would appear to be an exciting possibility, with great potential to generate novel, specific antivirals with low toxicity, and little risk of selecting for viral resistance.

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REVIEW
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Nucleocytoplasmic transport of nucleocapsid proteins of enveloped RNA viruses

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Most viruses with non-segmented single stranded RNA genomes complete their life cycle in the cytoplasm of infected cells. However, despite undergoing replication in the cytoplasm, the structural proteins of some of these RNA viruses localize to the nucleus at specific times in the virus life cycle, primarily early in infection. Limited evidence suggests that this enhances successful viral replication by interfering with or inhibiting the host antiviral response. Nucleocapsid proteins of RNA viruses have a well-established, essential cytoplasmic role in virus replication and assembly. Intriguingly, nucleocapsid proteins of some RNA viruses also localize to the nucleus/nucleolus of infected cells. Their nuclear function is less well understood although significant advances have been made in recent years. This review will focus on the nucleocapsid protein of cytoplasmic enveloped RNA viruses, including their localization to the nucleus/nucleolus and function therein. A greater understanding of the nuclear localization of nucleocapsid proteins has the potential to enhance therapeutic strategies as it can be a target for the development of live-attenuated vaccines or antiviral drugs.

Keywords: enveloped RNA viruses, nucleocapsid protein, nuclear localization, nucleolar localization, antiviral responses

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Introduction

The nucleocapsid protein of RNA viruses is essential for virus assembly, encapsidating the genomic RNA in preparation for packaging in the virion (Hunter, 2007). In positive strand RNA viruses (PSVs), the capsid or nucleocapsid protein is the major, often the only structural protein. In negative strand RNA viruses (NSVs), the nucleocapsid protein has an essential role in viral replication and transcription (Whelan, 2013) in addition to its role in assembly. For most enveloped RNA viruses, both replication and assembly occur in the cytoplasm (with the exception of orthomyxovirus, bornavirus, retroviruses, and hepatitis delta virus whose replication occurs in the nucleus; Whelan, 2013). Interestingly, the nucleocapsid protein (variously named N, NP, NC, or C protein) of many cytoplasmic RNA viruses (PSVs and NSVs) transiently localizes to the nucleus and/or nucleolus. There is limited information about the nuclear function of cytoplasmic RNA virus nucleocapsid proteins, but evidence suggests supporting roles in successful viral replication by interfering with or inhibiting the host antiviral response (Ahmed and Lyles, 1998; Ahmed et al., 2003; Lee et al., 2006; Takayama et al., 2012) as has been described for several RNA virus structural proteins.

The nucleocapsid protein of mouse hepatitis virus (MHV, *Coronaviridae*) and infectious bronchitis virus (IBV, *Coronaviridae*) localizes to the nucleolus of infected cells where it may regulate/delay cell cycle progression (Wurm et al., 2001), while nuclear localization of vesicular stomatitis virus (VSV, *Rhabdoviridae*) M, rabies (*Rhabdoviridae*) P, and Measles virus (MV, *Paramyxoviridae*) N protein (Ahmed et al., 2003; Schnell et al., 2010; Takayama et al., 2012) is associated with the pathogenesis of viral infection. Nuclear localization of the nucleocapsid protein of porcine respiratory and reproductive syndrome virus (PRRSV, *Arteriviridae*) appears to be essential for optimal virus replication and inhibition of cellular antiviral processes (Lee et al., 2006); in an analogous fashion, the successful production of infectious progeny of respiratory syncytial virus (RSV, *Paramyxoviridae*) and Nipah virus (*Paramyxoviridae*) requires nuclear localization of the matrix (M) protein (Ghildyal et al., 2005, 2009; Wang et al., 2010).

The aim of this review is to summarize current literature on the role of nuclear/nucleolar localization of nucleocapsid proteins of cytoplasmic PSVs and NSVs, which may lead to similar investigations in other related virus families. Most of our knowledge in this area derives from studies of viruses belonging to the *Coronaviridae*, *Arteriviridae, Flaviviridae,* and *Paramyxoviridae* families and these will be the main focus (**Table 1**), with reference to other virus families when required. *Coronaviridae* and *Arteriviridae* are classified together into the order *Nidovirales*; for the sake of simplicity and due to the close relationship among these viruses, both families will be discussed together.

The location of the nucleocapsid gene within the genome of selected viruses from the named families is shown in **Figure 1**. The nucleocapsid [N, NC, or C (core or capsid)] gene is in the first position (3′ end) in Paramyxovirus genomes and (5′ end) in Flavivirus and Hepacivirus genomes. The nucleocapsid gene of Coronavirus and Arterivirus is in the last position (3′ end) of these genomes, but the first gene to be copied into a negative sense subgenomic mRNA template for mRNA transcription.

The Cytoplasmic Role of RNA Virus Nucleocapsid Protein

The nucleocapsid protein of RNA viruses is a structural protein found in a complex with the genomic RNA. The role of genome encapsidation varies across PSVs and NSVs, but usually comprises one or more of the following functions; [1] to protect the genome from cellular nucleases, [2] to form the ribonucleoprotein transcriptase complex, and [3] to package the genome into new infectious particles prior to budding.

Nucleocapsid Protein of NSVs

The NSV nucleocapsid protein protects genomic viral RNA against ribonuclease degradation and high salt concentration, as well as maintaining the rigidity of the RNA structure following cycles of folding/dissociation/refolding during the replication cycle (Lynch and Kolakofsky, 1978; Mir and Panganiban, 2006; Raymond et al., 2010). The nucleocapsid protein is the driving force for the formation of ribonucleoprotein (RNP) complexes that are the replicative/transcriptive unit, as well as for assembly and packaging into the virion. These functions are facilitated through its propensity to self-associate and ability to interact with other RNP components, including RNA and viral polymerase subunits (Becker et al., 1998; DiCarlo et al., 2007; Green et al., 2011; Sun et al., 2012). Nucleocapsid protein has an inherent ability to interact with the phosphoprotein (P; polymerase subunit and cofactor). This interaction is particularly important because association between nucleocapsid and RNA is not specific; instead, specificity is conferred via the nucleocapsid-phosphoprotein interaction, such that RNA associated with the nucleocapsid-phosphoprotein complex is the form recognized by RNA polymerase (Myers et al., 1999; Longhi, 2009). Additionally, the NSV phosphoprotein acts as a chaperone for the nucleocapsid protein restricting non-specific encapsidation of non-viral RNA (Curran et al., 1995; Errington and Emmerson, 1997). The nucleocapsid protein of VSV, in association with the P protein, is required for genome encapsidation and transcription (Zhang et al., 2008; Morin and Whelan, 2014) with mutations in its C-terminus resulting in reduction of viral particle production in cell culture (Heinrich et al., 2012). In RSV and Sendai virus (*Paramyxoviridae*), it is the N-terminal of the nucleocapsid protein that plays a similar role in genome encapsidation (Buchholz et al., 1993; Myers and Moyer, 1997; Khattar et al., 2000).

The Nucleocapsid/Capsid/Core Protein of PSVs

Similar to the NSV nucleocapsid protein, the nucleocapsid/capsid/core protein of PSVs binds tightly to the genomic RNA, drives RNA packaging and sometimes, serves as a nucleic acid chaperone. Flavivirus (*Flaviviridae*) C protein plays an important role during assembly and budding of infectious particles. Mutations in C protein lead to the inhibition of infectious particle release while causing increased production of defective particles (Jones et al., 2011). The hepatitis C virus (HCV, *Flaviviridae*) core protein and the N protein of coronaviruses severe acute respiratory syndrome coronavirus (SARS-CoV) and transmissible gastroenteritis virus (TGEV) are potent RNA chaperones that can resolve RNA misfolding and promote annealing of complementary sequences (Rajkowitsch et al., 2007; Zuniga et al., 2007; Sharma et al., 2010). Most of the evidence for chaperone activity is derived from *in vitro* investigations and although these proteins clearly have the ability to function as RNA chaperones, the importance of this function in infection is not clear.

Non-Encapsidation Roles of Nucleocapsid Proteins

The N/NC/C proteins of both NSVs and PSVs have functions in addition to their RNA stabilizing, encapsidating and, in

TABLE 1 | Classification of enveloped RNA viruses discussed in the review.

Family	Sub-family	Genus	Species
Flaviviridae		Flavivirus	Dengue virus, Japanese Encephalitis virus (JEV), West Nile virus
		Hepacivirus	Hepatitis C virus
Coronaviridae	*Coronavirinae*	Alphacoronavirus	Transmissible gastroenteritis virus (TGEV)
		Betacoronavirus	Severe acute respiratory syndrome coronavirus (SARS-CoV), mouse hepatitis virus
		Gammacoronavirus	Infectious bronchitis virus
Arteriviridae		Arterivirus	Lactose dehydrogenase elevating virus, Equine arterivirus, Porcine respiratory and reproductive syndrome virus
Paramyxoviridae	*Paramyxovirinae*	Morbillivirus	Measles virus, Canine distemper virus (CDV), Rinderpest virus
		Henipavirus	Nipah virus
		Respirovirus	Sendai virus
	Pneumovirinae	Pneumovirus	Respiratory syncytial virus

the case of NSVs, viral transcriptase activities. The flavivirus core protein interacts with several host cell proteins and is implicated in disease development through its roles in apoptotic pathways as well as regulation of the innate immune response (Urbanowski et al., 2008). Similarly, the coronavirus N protein interferes with cellular antiviral responses (McBride et al., 2014). The N protein of morbilliviruses (*Paramyxoviridae*) has been shown to regulate interferon signaling via interference with the signal transducer and activator of transcription (STAT) signaling pathway (Takayama et al., 2012). Increasing literature suggests that at least some of the non-encapsidating functions of the nucleocapsid proteins of NSVs and PSVs derive from their ability to localize to the nucleus or nucleolus of the infected cells.

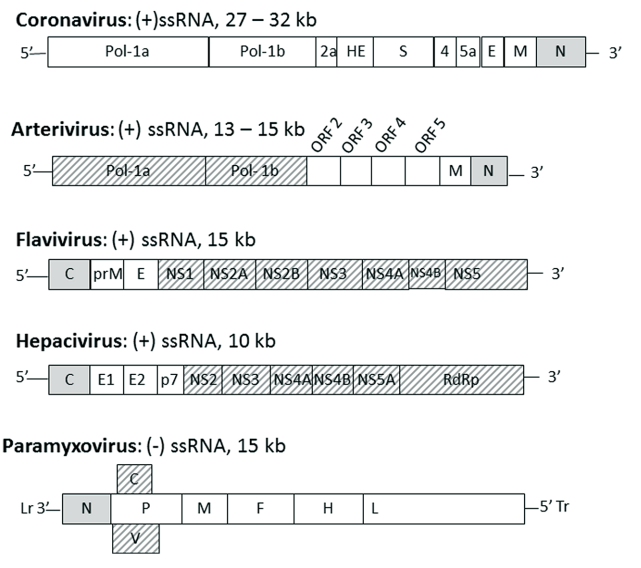

FIGURE 1 | The position of nucleocapsid protein in the genome of selected enveloped RNA virus families. Genomes of the named virus families are indicated schematically with each gene indicated by a box with family name, genome type and length indicated above each schematic. The gene for nucleocapsid protein (N or C) is indicated by a gray box, other structural proteins are indicated by white boxes and non-structural proteins are indicated by hashed boxes. Lr, leader; Tr, trailer.

Nuclear Localization of Nucleocapsid Proteins of RNA Viruses

Despite undergoing replication in the cytoplasm, the nucleocapsid protein of several NSVs and PSVs localizes in the nucleus or nucleolus of infected cells during infection. This nuclear localization usually takes place early in the infectious cycle as soon as the nucleocapsid protein is translated, possibly to perform non-structural functions, followed by return to the cytoplasm in the late stage of infection to participate in assembly (Tijms et al., 2002; Yoo et al., 2003; Sato et al., 2006). Although some progress has been made in our understanding of the nucleocytoplasmic shuttling of several nucleocapsid proteins, thus far, no definitive single molecule studies have been undertaken; such studies will be important to elucidate the finer details of nuclear transport pathways in the future.

Nucleocytoplasmic Transport

Eukaryotic cells sequester their genome in the nucleus, which is surrounded by the double lipid bilayer structure of the nuclear envelope (NE). The only avenue for transport into and out of the nucleus is via the NE-embedded nuclear pore complexes (NPCs) that are made up of over 40 different proteins called nucleoporins (Nups). Although diffusion of molecules <55 kDa can occur, most transport through the NPC is mediated by members of the importin superfamily, which recognize nuclear localization sequences (NLSs) or nuclear export sequences (NESs) on cargo molecules for transport into and out of the nucleus, respectively (Ghildyal et al., 2005, 2009; Cardarelli et al., 2008; Cohen et al., 2011). NLSs are usually short stretch(es) of basic residues (monopartite NLS, e.g., that of the simian virus 40 T antigen) which may be separated by 10–15 amino acids (bipartite NLS, e.g., that of nucleophosmin), while the most well characterized NES is the Leucine-rich motif recognized by the exportin CRM-1 (e.g., the NES in HIV Rev protein). Importins function by binding NLSs and docking transiently at various "FG" (Phenylalanine–Glycine repeat containing) Nups within the NPC to effect translocation through it, followed by release within the nucleus facilitated by the guanine nucleotide binding protein

Ran (Gorlich and Kutay, 1999). The best studied nuclear import pathway is mediated by the importin-α/β1 heterodimer, where importin-α recognizes and directly binds to the NLSs of the cargo, and importin-β1 mediates binding of the import complex to Nups (Macara, 2001). However, nuclear import can be effected by direct action of importin-β1 or homologs thereof, without a requirement for importin-α (Fulcher and Jans, 2011). In all cases, release within the nucleus occurs through binding of Ran, in its guanosine triphosphate (GTP)-bound form, to importin-β1, or homologs, to effect dissociation of the import complex. Nuclear export is analogous to nuclear import, wherein cargo molecules containing NESs bind importin-β homologs, such as CRM-1, in complex with Ran in its GTP bound state and are transported out of the nucleus; release in the cytoplasm is facilitated by Ran hydrolysis of GTP to guanosine diphosphate (GDP), which leads to dissociation of the export complex (Macara, 2001).

Localization to the nucleolus is less well defined, but is most likely regulated by cargoes interacting with nucleolar residents, possibly the so-called hub proteins (Emmott and Hiscox, 2009). Nucleolar localization signals (NoLSs), similar to NLSs, are short stretches of basic proteins, however, there is a high level of variability in the NoLSs described to date (de Melo et al., 2013). NoLSs are not capable of directing cargo into the nucleus, although very often they overlap the NLS sequences. Interestingly, CRM-1 has been shown to play an important role in nucleolar localization of small nuclear and nucleolar RNA molecules via complex binding mechanisms that lead to masking/unmasking of the NoLS (Verheggen and Bertrand, 2012).

Coronaviridae and Arteriviridae

Viruses in the *Coronaviridae* family possess a positive (+) ssRNA genome and cluster into two subfamilies including *Coronavirinae* which includes the human coronaviruses, and six genera. The viruses of *Arteriviridae* family are also positive (+) ssRNA viruses and grouped into one genus, Arterivirus.

The nucleocapsid proteins of viruses belonging to genus Arterivirus localize to the nucleus, as has been shown for lactate dehydrogenase-elevating virus (LDV; Mohammadi et al., 2009), equine arteritis virus (EAV; Tijms et al., 2002) and PRRSV (Rowland et al., 1999). Nuclear import of the LDV nucleocapsid protein is facilitated by a monopartite NLS motif, ^{38}KKKK^{41} (**Figure 2A**), probably mediated by the importin-α/β complex (Mohammadi et al., 2009), and whilst the EAV nuclear import has not been clearly defined, nuclear export is facilitated by CRM-1 (Tijms et al., 2002). PRRSV nucleocapsid protein possesses NLS, nucleolar retention/localization signal (NoRS/-LS), and NES motifs that facilitate independent nucleocytoplasmic transport and accumulation in the nucleoli of infected cells (Rowland et al., 1999; Yoo et al., 2003). The NLS motifs that are located in amino acid residues 10–13 (NLS1) and 41–47 (NLS2) facilitate nuclear import via interaction with importin-α/β, while the NES motif is located in amino acid residues 106–117 and works with CRM-1 to facilitate nuclear export (Pei et al., 2008). Disruption of nuclear/nucleolar localization of PRRSV nucleocapsid protein has been shown not to affect the capability to produce infectious particles in MARC-145 cells (monkey kidney cells permissive to PRRSV infection), however, it did result in attenuated viral replication and induced a higher titer of neutralizing antibody in pigs. This suggests the nucleocytoplasmic localization of PRRSV nucleocapsid protein has an important role in pathogenesis of PRRSV infection (Lee et al., 2006; Pei et al., 2008).

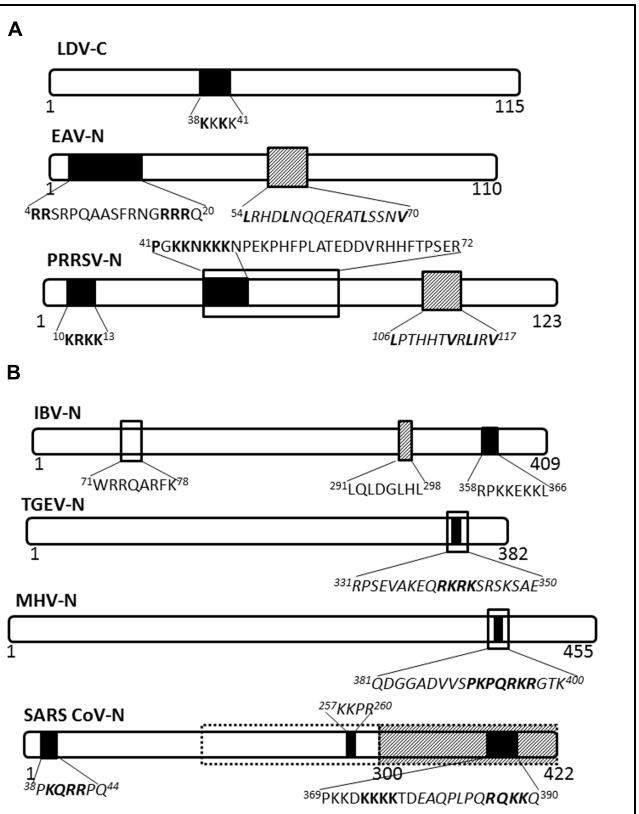

FIGURE 2 | Nuclear/nucleolar transport signals in nucleocapsid proteins of *Arteriviridae* and *Coronaviridae*. The nucleocapsid proteins of representatives of named virus families are shown schematically. Numbers under the schematics indicate position of amino acid residues, nuclear localization signals (NLSs) are indicated by solid black filled boxes, nuclear export signals (NESs) are indicated by hatched boxes and nucleolus localization/retention signals (NoLS/RS) are indicated by black open boxes. The sequence of the transport signals is shown in single letter format, with essential residues in bold; sequences in italics are predicted but not yet confirmed. **(A)** *Arteriviridae*: LDV, lactate dehydrogenase virus; EAV, equine arteritis virus; PRRSV, porcine respiratory and reproductive syndrome virus. **(B)** *Coronaviridae:* Broken black line indicates predicted NoLS/RS. IBV, infectious bronchitis virus; TGEV, transmissible gastroenteritis virus; MHV, mouse hepatitis virus; SARS-CoV, severe acute respiratory syndrome coronavirus.

Nucleocapsid proteins of coronaviruses IBV and MHV localize to the nucleolus of infected cells facilitated by NLSs, NoRS/-LS, and an NES (Hiscox et al., 2001; Wurm et al., 2001; Chen et al., 2002; Reed et al., 2006; **Figure 2B**). In the nucleolus the nucleocapsid protein may modulate cell division, as evidenced by the fact that cells expressing coronavirus nucleocapsid protein appear to be arrested in the G2/M phase of the cell cycle (Chen et al., 2002; Ding et al., 2014a; McBride et al., 2014). Delay in the cell cycle may promote conditions suitable for virus replication and assembly.

The nucleocapsid protein of IBV has been shown to associate with fibrillarin and nucleolin in the nucleolus (Hiscox et al., 2001). In particular, as also observed for MHV and TGEV nucleocapsid proteins, it causes G2 arrest of the cell cycle that leads to reorganized fibrillarin distribution, where the interaction and/or redistribution are associated with a delay in cell growth (Hiscox et al., 2001; Chen et al., 2002; Ding et al., 2014a).

The nucleolus is a multifunctional nuclear substructure that is central to the normal operations of a cell, with important roles in ribosome synthesis and assembly, cell cycle regulation, transcription regulation, cell senescence and sensing and response to stress (Bartova et al., 2010; Shaw and Brown, 2012; Golomb et al., 2014). The nucleolar proteome consists of more than 500 proteins identified so far; some are long term residents, while others shuttle between the nucleolus, nucleoplasm, cytoplasm, and cell membrane (Andersen et al., 2002; Lam et al., 2005) depending on the metabolic state of the cell. Fibrillarin, nucleolin, and nucleophosmin are major nucleolar proteins that have been studied extensively. Fibrillarin is involved in many post-transcription processes and ribosome assembly; nucleolin serves crucial roles in pre-rRNA processing and may regulate transcription; nucleophosmin is implicated in ribosome assembly, nucleocytoplasmic shuttling and may regulate rDNA transcription [(Hiscox, 2002) and references therein].

The SARS-CoV nucleocapsid protein is predominantly cytoplasmic in infected cells and in cells transfected to express the full length protein. Interestingly, the protein sequence contains three putative NLSs and a putative NES (**Figure 2B**); the latter is non-functional (You et al., 2005, 2007). A bipartite NLS and putative NoLS appear to be functional but are masked in the context of the full length protein by a dominant CRM-1-independent NES in the C-terminus. Additionally, SARS CoV nucleocapsid cytoplasmic localization is facilitated by interaction with 14-3-3 (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; Macara, 2001; Surjit et al., 2005). Thus, the SARS-CoV nucleocapsid protein, to the best of our knowledge, does not localize to the nucleus or the nucleolus of infected cells, in contrast to the nucleocapsid proteins of other coronaviruses studied.

Paramyxoviridae

Nucleocapsid proteins of some viruses in the *Paramyxoviridae* family have been shown to undergo transient nuclear localization. The nucleocapsid proteins of canine distemper virus (CDV) and rinderpest virus (RPV) possess conserved NLS and NES motifs; the NES is CRM-1 independent, despite being a Leucine-rich motif (Sato et al., 2006; **Figure 3**). MV infected cells contain nuclear inclusion bodies (composed of N, phosphoprotein and large polymerase; Robbins and Eagle, 1985). More importantly, the nuclear translocation of MV nucleocapsid protein also affects the host immune response by inhibiting the nuclear translocation of STATs, causing disruption of the IFN-α/β and IFN-γ signaling pathways (Takayama et al., 2012). The NLS motif is contained within ^{70}TGILISILSLF^{80}; while the NES is contained within residues ^{425}SENELPRLGGKEDRRV^{440} (Sato et al., 2006).

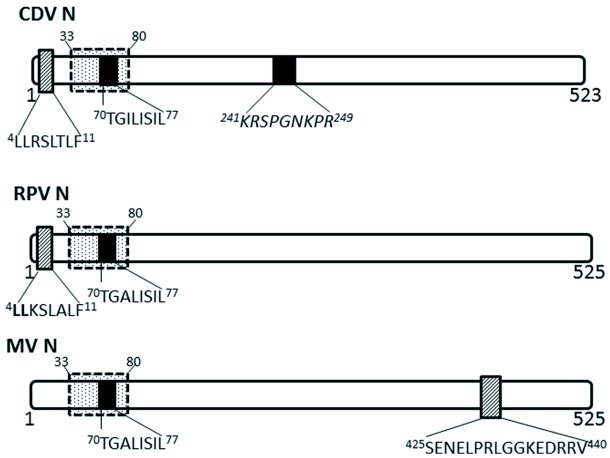

FIGURE 3 | Nuclear transport signals in nucleocapsid proteins of *Paramyxoviridae*. The NLSs and NESs are indicated as in **Figure 2**; a dashed-line box with dots indicates flanking sequences that have been shown to have a role in nuclear import and retention. CDV, canine distemper virus; RPV, Rinderpest virus; MV, measles virus.

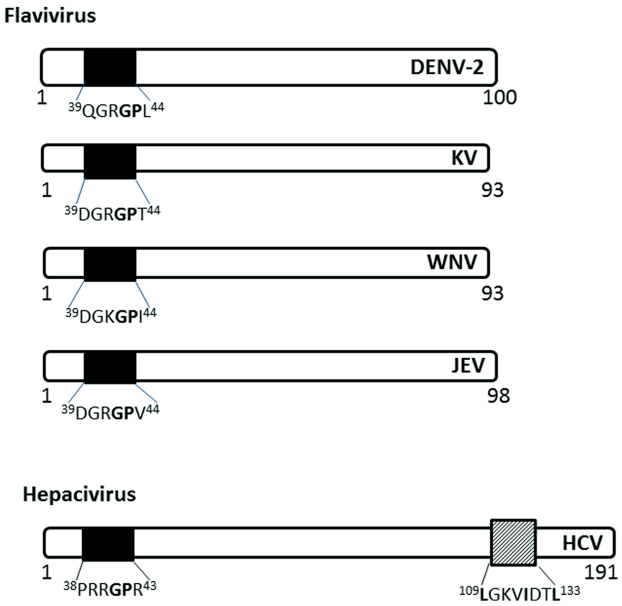

FIGURE 4 | Nuclear localization signals in C (capsid or core) proteins of *Flaviviridae*. The NLSs for each viral protein and the NES for HCV are indicated as in **Figure 2**. DENV-2, Dengue virus serotype 2, KV, Kunjin virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; HCV, hepatitis C virus.

Flaviviridae

The capsid (C) protein of several members of the *Flaviviridae* family localize to the nucleus of infected cells using NLSs (**Figure 4**). The Flavivirus C protein is small enough to diffuse across the NE; however, studies have shown that it utilizes active nuclear transport pathways to localize to the nucleus.

The C protein of Dengue virus localizes to the nucleus of infected mammalian cells, starting at 6 h post-infection (h.p.i)

and remains at high levels throughout the course of infection (up to 72 h.p.i; Sangiambut et al., 2008). The C protein does not localize to the nucleus of mosquito cells, possibly due to the different availability of carrier proteins or the cellular components involved in nuclear translocation.

The C (core) protein of the Japanese encephalitis virus (JEV) has been shown to localize into the nucleus and associate with the phosphoprotein B23 in the nucleolus (Tsuda et al., 2006). The nuclear localization is required for successful replication in mammalian cell lines and is determined by amino acid residues ^{42}GP^{43} of the NLS, with G^{42} being the major determinant (Mori et al., 2005). ^{42}GP^{43}substitution into A$^{42-43}$ eliminates the nuclear localization ability in both mosquito and mammalian cell lines and decreases infectious virus production in mammalian cell lines and in infected mice. The GP motif is conserved in Dengue, Kunjin, and Hepatitis C virus C proteins (Mori et al., 2005).

Hepatitis C virus C protein has been found in the nucleus in the C-terminally truncated form, particularly in hepatocytes isolated from chronic HCV patients (Falcon et al., 2003), suggesting a possible role in persistence of HCV infection, which often leads to hepatocellular carcinoma. The nuclear localization of HCV C is dependent on importin-α, which recognizes NLS motifs contained within the residues 5–13 (PKPQRKTKR), 38–43 (PRRGPR), and 58–71 (PRGRRQPIPKARRP; Suzuki et al., 2005). HCV C also has a functional CRM-1 dependent NES in residues 109–133 (Cerutti et al., 2011). The nuclear localization has been observed early in infection (∼ from 6 h.p.i), similar to that of Dengue C protein, and disruption of the nucleocytoplasmic translocation process reduces the production of infectious particles (Cerutti et al., 2011).

Nuclear Functions of the Nucleocapsid Proteins of RNA Viruses

Based on current literature and our understanding of nucleocytoplasmic trafficking in the context of virus infection, it can be proposed that the nuclear accumulation of nucleocapsid proteins may serve two functions. Nucleocapsid proteins may have non-structural roles in modulation of nuclear processes in order to maximize virus replication or to inhibit IFN induction and signaling pathways. Alternatively, nucleocapsid proteins may localize coincidentally to the nucleus/nucleolus due to molecular mimicry.

Virus Replication

The nuclear/nucleolar translocation of the nucleocapsid protein is probably most often associated with optimal virus replication. For example, IBV nucleocapsid redistributes fibrillarin and delays cytokinesis to divert biosynthetic resources from the dividing nucleus to the cytoplasm, where viral replication takes place (Wurm et al., 2001; Chen et al., 2002). The resultant cell cycle arrest/delay would be conducive to higher virus replication through both redirection of biosynthetic machinery for viral replication and delay in cell division. A similar cell cycle arrest has also been observed in other coronaviruses and may be a common consequence of the nucleolar localization of the nucleocapsid protein (Wurm et al., 2001). In nature, these viruses infect terminally differentiated epithelial cells which do not actively divide; it is unclear how the cell cycle modulation observed in cell culture would be useful in a natural infection. The nuclear/nucleolar localization of JEV C protein is also required for optimal virus replication in mammalian cells (Mori et al., 2005). The importance of the nuclear functions of the core protein in flavivirus infection is demonstrated by the inability of some flaviviruses to replicate in enucleated cells (Kos et al., 1975; Lad et al., 1993). Both the Dengue and HCV C protein interact with heterogeneous nuclear RNP K, possibly to regulate host cell transcription, thereby freeing cellular machinery for viral RNA synthesis (Hsieh et al., 1998; Chang et al., 2001).

Interferon Antagonism

Nucleocapsid proteins of some RNA viruses function in the nucleus to inhibit the potent antiviral IFN response. The innate antiviral response, mediated principally by the action of Type-I IFNs, is one of the earliest responses of the host to viral infection (Conzelmann, 2005). Virus specific intracellular pathogen associated molecular patterns (e.g., viral RNA or dsRNA) are recognized by cellular helicases retinoic-acid-inducible protein I and melanoma-differentiation-associated gene 5 (See and Wark, 2008) initiating a cascade of events resulting in the activation of transcription factors including IFN response factor 3 (IRF3), nuclear factor (NF) κB and activating protein 1 that are subsequently transported into the nucleus to activate transcription of IFN-β. IFN-β mRNA is exported out of the nucleus, where it is translated into protein which is secreted from the cell to induce a secondary cellular response in an autocrine and paracrine manner, by binding to the IFN-α/β receptor. This in turn leads to activation of a second cascade of events involving several effectors and transcription factors such as the STAT proteins. These proteins translocate into the nucleus, interact with IFN-sensitive response elements and activate transcription of a broad range of IFN stimulated genes (ISG). The ISG products mount a concerted immune response which prevents virus replication (See and Wark, 2008).

As mentioned already, MV nucleocapsid protein inhibits the nuclear translocation of STATs, causing disruption of the IFN-α/β and IFN-γ signaling pathways (Takayama et al., 2012). Coronavirus nucleocapsid protein has been shown to have potent IFN antagonistic activity; however, the mechanism varies in different virus species. The MHV nucleocapsid protein interferes with the IFN induced 2′-5′ oligoadenylate synthetase RNase L pathway and can functionally replace the IFN antagonist activity of E3L protein in a recombinant vaccinia virus (Ye et al., 2007), thus contributing to the IFN resistance exhibited by MHV (Rose and Weiss, 2009). The SARS-CoV nucleocapsid protein is also a potent IFN antagonist, however, it is mostly cytoplasmic (discussed above) and inhibits IFN production (Kopecky-Bromberg et al., 2007)

rather than IFN signaling. Similar to SARS-CoV, the porcine epidemic diarrhea virus nucleocapsid protein inhibits IFN production via direct interaction with TRAF-associated NF-kB activator binding kinase (TBK1), thus interfering with the TBK1 mediated phosphorylation of IRF3, required for IRF3 dimerization and subsequent nuclear localization, which is critical to IFN activation (Zhao, 2013; Ding et al., 2014b). Nucleocapsid protein's IFN antagonist activity may not always be dependent on its nuclear localization.

Molecular Mimicry

The nucleocapsid protein generally has Lysine (K) or Arginine (R) rich RNA binding domains (to associate with viral genome), which resemble nuclear transport signals and these motifs may serve the dual purpose of mediating nuclear localization of nucleocapsid protein coincidental to their main function. This is the basis for molecular mimicry, wherein the K/R-rich RNA binding domains resembling NLS/NoLS motifs, allow the nucleocapsid protein to be transported into the nucleus by cellular nucleocytoplasmic trafficking machinery (Rowland and Yoo, 2003). Other possibilities include association with ribosomal proteins, and passive diffusion. Association with ribosomal proteins in the cytoplasm might result in nucleocapsid protein being coincidentally translocated into the nucleolus, where the ribosomal proteins associate with rRNA to form ribosomal subunits (Tijms et al., 2002). Although termed "coincidental," nuclear localization of nucleocapsid protein might serve a specific viral purpose by maintaining the nucleocapsid protein:ribonucleotides stoichiometry in the cytoplasm. This is essential in order to produce functional genomes, such that transient nuclear/nucleolar localization of excess nucleocapsid protein can be a means to keep the appropriate concentration of nucleocapsid protein molecules in the cytoplasm (Tijms et al., 2002).

Potential for Future Therapeutic Strategies

As discussed above, N/NC/C proteins of cytoplasmic RNA viruses have an important nuclear role in addition to their essential cytoplasmic role in virus assembly. This presents an opportunity for a broad ranging antiviral that may inhibit nuclear transport of these proteins, thus indirectly reducing virus assembly, leading to reduced infectious virus production and hence reduced disease. New generations of specific inhibitors of nuclear export (SINE) are well characterized compounds that have low toxicity and good bioavailability and are currently in clinical trials for various cancers and could potentially be re-purposed for virus infections (Azmi et al., 2013a,b; Gao et al., 2014). However, targeting host–pathogen interface is preferable to targeting of cellular processes. Elucidation of the NLS/NoLS motifs of the nucleocapsid proteins and their mechanism of localization should identify interactions that may be modeled for development of small molecule inhibitors (Lundberg et al., 2013); indeed, given the vast array of small molecule inhibitors currently available, it may be possible to identify inhibitors via high throughput screening assays (Wagstaff et al., 2011). Ivermectin and Mifepristone are two such compounds shown to reduce infectious virus titers of several viruses that exploit host nuclear transport machinery (Tay et al., 2013). Determination of the NLS/NoLS also presents an opportunity for development of attenuated vaccine candidates. Specific mutations that disable the nuclear/nucleolar localization function, without hampering the cytoplasmic function of nucleocapsid proteins, should result in viruses that replicate, albeit to lesser degree due to reduced ability to inhibit antiviral responses; mutating the NLS of RSV M protein leads to reduced replication fitness, supporting the viability of such an approach (Ghildyal et al., 2009). That targeting the nucleocapsid protein for attenuation is a viable strategy has been demonstrated for VSV (Sato et al., 2011).

Summary

Structural proteins of several enveloped RNA viruses localize into the nucleus and/or nucleolus at certain times of the viral life cycle, primarily early in infection. Their nucleocytoplasmic transport is facilitated by nuclear localization/export signals in association with cellular transport proteins. The nucleocapsid protein is a major structural protein of enveloped RNA viruses which serves a structural function in virus assembly. Its function includes protecting the genome against ribonucleases, regulating the fidelity of the replication template, forming the replication complex, and packaging of the genomic RNA for assembly, budding, and particle release. The nucleocapsid proteins of several viruses belonging to *Coronaviridae, Arteriviridae, Flaviviridae*, and *Paramyxoviridae* possess NLS/NoLS and transiently localize into the nucleus/nucleolus during the viral life cycle. In some cases the nuclear localization appears to support viral proliferation by interfering with the host's immune response or other mechanisms including maintaining the balance of nucleocapsid protein:RNA molecules for optimum production of the functional viral genome. As such, the nuclear localization of nucleocapsid protein of enveloped RNA viruses has potential as a target for the development of live-attenuated vaccines or antiviral drugs, as inhibition of its nuclear localization could negatively impact on the production of infectious viral particles and cell-to-cell spread.

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Hepatitis C virus and host cell nuclear transport machinery: a clandestine affair

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There is growing evidence that factors encoded by cytoplasmic replicating viruses functionally interact with components of the nucleocytoplasmic transport apparatus. They do so either to access the cell nucleus, thus affecting genes expression, or to interfere with nuclear transport functionality, hindering host immune response. Recent studies revealed that the hepatitis C virus (HCV) makes no exception, interacting with the host cell nuclear transport machinery at two different levels. On the one hand, small amounts of both core and NS5A localize within the host cell nucleus during productive infection, modulating gene expression and signaling functions to promote persistent infection. On the other hand, HCV infection causes a profound redistribution of certain nucleoproteins to the close proximity of endoplasmic reticulum membrane-derived viral replication factories, where viral RNA amplification occurs. These nucleoporins are believed to form nuclear pore complex-like structures, as suggested by their ability to recruit nuclear localization sequence-bearing proteins. Thus, both processes are linked to virus-induced persistence and pathogenesis, representing possible targets for the development of novel anti-HCV therapeutics.

Keywords: nucleoporin, importin, exportin, Ran, nuclear import, caspase, replication factories, NPC

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Nuclear Transport

The eukaryotic nucleus is separated from the cytoplasm by a double membrane called nuclear envelope (NE). Nucleocytoplasmic transport occurs through NE-embedded nuclear pore complexes (NPCs). Cargoes >9 nm in diameter (~60 kDa) are actively transported through NPCs by kariophilic transporters (Kaps) in a signal-mediated process. Such signals can be either nuclear localization or nuclear export sequences (NLSs and NESs), responsible for targeting into and out of the nucleus as mediated by importins (IMPs) or exportins (EXPs), respectively (Tran et al., 2007). Several types of NLSs exist, differing in their sequence and IMP-binding properties. Short, basic NLSs similar to that of the Simian Virus 40 Large Tumor Antigen (Tag) are referred to as "classical" NLSs (Kalderon et al., 1984). Such signals are recognized by IMPβ1 (IPO1) *via* one of the seven IMPα family members (IPOA1, IPOA3-8), which are classified in three subgroups according to their homology and cargo-binding specificity (Alvisi and Jans, 2015). Alternatively, IMPβ1 and its homolog can also bind cargoes directly without the need of IMPαs (Truant and Cullen, 1999; Fontes et al., 2000; Chook and Suel, 2011). These include IMPβ2 (IPO2), mainly responsible for importing RNA-binding proteins, and IMPβ3 (IPO5), involved in transport of core histones and ribosomal proteins (Jakel and Gorlich, 1998). All IMPβ homolog share similar mechanisms for cargo

nuclear transport and release. Assembly of cargo-IMP complexes results in docking to the NPC, as mediated by IMPβ interaction with specific nucleoporins (Nups), followed by NPC translocation. Once inside the nucleus, binding of Ran-GTP to IMPβ mediates a conformational change, resulting in the dissociation of the complexes (Cansizoglu et al., 2007; Imasaki et al., 2007). Conversely, EXPs such as CRM-1 (XPO1) recognize NES-bearing cargoes in the nucleus upon binding to Ran-GTP, and translocate to the cytosol.

Nuclear replicating viruses interact with the nuclear transport apparatus to ensure nuclear targeting of viral replicating enzymes (Alvisi et al., 2011b, 2013). Strikingly, several proteins encoded by cytoplasmic-replicating viruses similarly interact with the nuclear transport apparatus, mainly with the aim to interfere with host cell functionality, promoting their self-propagation (Camus-Bouclainville et al., 2004; Zhang et al., 2007; Alvisi et al., 2008; Wang et al., 2012). Among these, the hepatitis C virus (HCV) makes no exception.

Hepatitis C Virus

Hepatitis C virus is a cytoplasmic-replicating RNA virus belonging to the *Flaviviridae* family. Once inside the host cell, its ∼9.6 Kbp genome is translated on the rough endoplasmic reticulum (ER) into a single polyprotein of about 3000 amino acids, co- and post-translationally cleaved by viral and host proteases into three structural proteins (core, E1, and E2), the viroporin p7, and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B; Kato, 2000). Core, E1 and E2 constitute the viral particle, p7 and NS2 are involved in virion assembly and release, while NS3 to NS5B proteins are sufficient for RNA replication (Lohmann et al., 1999; Jones et al., 2007; Steinmann et al., 2007). Expression of the HCV polyprotein induces profound membrane rearrangements, including the formation of double membrane vesicles (DMVs) where viral proteins and host factors are recruited to replicate viral RNA (Romero-Brey et al., 2012). Such membrane alterations, i.e., viral replication factories (VFs), are common amongst positive-stranded RNA viruses, and are surrounded by autophagosomes and lipid droplets (LDs) in the case of HCV infection. Although their precise structure and function are still not completely clear, they support viral infection by a variety of mechanisms (Paul and Bartenschlager, 2013), such as by tethering the viral RNA during unwinding (Lyle et al., 2002) and by ensuring compartmentalization and concentration of viral products (Paul and Bartenschlager, 2013). VFs also provide lipids essential for replication (Ahola et al., 1999), and facilitate avoidance of host cell cytoplasmic pattern recognition receptors crucial for initiating antiviral immune responses (Overby et al., 2010). Furthermore, HCV-induced VFs have been recently shown to protect viral RNA from double strand RNA-induced host defenses and nucleases (Paul et al., 2013). Upon HCV infection, development of VFs is mainly dependent on NS4B and NS5A, the latter being responsible for DMV biogenesis *via* stimulation of the type III phosphatidylinositol 4-kinase α (PI4KIIIα; Reiss et al., 2011; Berger et al., 2014). Activated PI4KIIIα causes a profound redistribution of phosphatidylinositol-4 phosphate from the plasma membrane to VFs, where RNA polymerase NS5B-mediated HCV RNA replication occurs (Lohmann et al., 1999; Bianco et al., 2012). Newly synthesized viral genomes are subsequently delivered by NS5A on the surface of core-decorated LDs to be packaged in nucleocapsids, before being secreted through the very low density lipoprotein pathway (Miyanari et al., 2007; Gastaminza et al., 2008; Alvisi et al., 2011a). Although HCV life cycle has no clear nuclear intermediate, certain HCV proteins contain multiple functional NLSs and NESs, and interact with several Kaps (**Figure 1**; Chung et al., 2000; Germain et al., 2014; Levin et al., 2014). This redundancy of signals probably reflects the need for certain HCV proteins to be functionally targeted to different subcellular compartments according to specific stages of HCV life cycle. This would be consistent with the very recent discovery that HCV infection causes partial redistribution of certain Nups to the close proximity of VFs that are the sites of NLS-bearing cargo recruitment (Neufeldt et al., 2013; Levin et al., 2014).

Functional Role of NS5A and Core Within the Cell Nucleus

So far, among all the HCV proteins bearing functional NLSs/NESs, insights into nuclear function has been delineated only for the NS5A and the core protein. NS5A is a multifunctional phosphoprotein directly involved in multiple stages of viral life cycle, including VFs biogenesis, viral RNA replication and particle assembly (Shimakami et al., 2004; Masaki et al., 2008; Reiss et al., 2011). Consequently, NS5A has been proposed to act as a master regulator of HCV life cycle, controlling the switch between viral replication and particle assembly through a still uncharacterized phosphorylation-dependent mechanism (Masaki et al., 2008). Additionally, NS5A affects a number of host cell signaling pathways, as exemplified by its ability to interact with several cellular proteins and regulate their activities (Pichlmair et al., 2012; Eberle et al., 2014). NS5A nuclear role seems to be primarily linked to HCV ability to establish a persistent infection, avoiding the host cell response and simultaneously enhancing the cellular survival ability (**Figure 2**). Indeed, nuclear NS5A impairs both interferon (IFN) antiviral and apoptotic pathways (Khabar et al., 1997; Ghosh et al., 1999; Majumder et al., 2001). NS5A bears a C-terminally-located NLS (**Figure 1**), which interacts with IPO5 and confers nuclear localization when fused to heterologous proteins (Ide et al., 1996; Chung et al., 2000; Levin et al., 2014). However, full-length NS5A is tethered to the ER by its N-terminal amphipathic α-helix (AH), which prevents nuclear translocation (Elazar et al., 2003). Importantly, NS5A contains two caspase cleavage sites at residue position 154 and 389 (**Figure 1**). In HCV-infected cells, basal caspase activity generates low levels of NS5A truncated forms in the absence of apoptotic stimuli. These include nuclear-accumulating fragments containing the NLS but lacking the AH, which bind the promoters of interleukin-8 (a negative regulator of the IFN pathway), as well as of the apoptosis inhibitors lymphotoxin beta and NUAK2, up-regulating their transcription (Satoh et al., 2000; Legembre et al., 2004; Haybaeck et al., 2009; Sauter et al., 2009; Maqbool et al., 2013). NS5A ability to migrate to the nucleus and modulate host gene expression

A

Viral protein	*NTS type*	*NTS sequence*	*Kaps*
core*	*NLS*	PK**P**Q**RK**T**KR**-13	IPOA5; IPO5
	NLS	P**RR**GP**R**-43	IPOA5; IPO5
	NLS	P**R**G**RR**QPIP**KARR**P-117	IPOA5; IPO5
	NLS	P**R**H**RS**RNV-119	IPO5
	NES	LALLSCITV-187	XPO1
	NES	KVIDTLTCGFADLMG-135	XPO1
NS2	*NLS*	PIIFSPME**KK**VIVWG-991	IPOA5
NS3*	*NLS*	PA**RRR**GD**K**-1152	IPO5
	NLS	PQDAVS**R**SQ**RR**G**R**T-1495	IPOA5; IPO5
	undefined region		IPO1,2; XPO1,5
NS5A*	*NLS*	PP**RKKR**TVV-2333	IPO5
NS5B	*undefined region*		IPO7

(Chang et al., 1994; Ide et al., 1996; Cerutti et al., 2011; Germain et al., 2014; Levin et al., 2014)

B

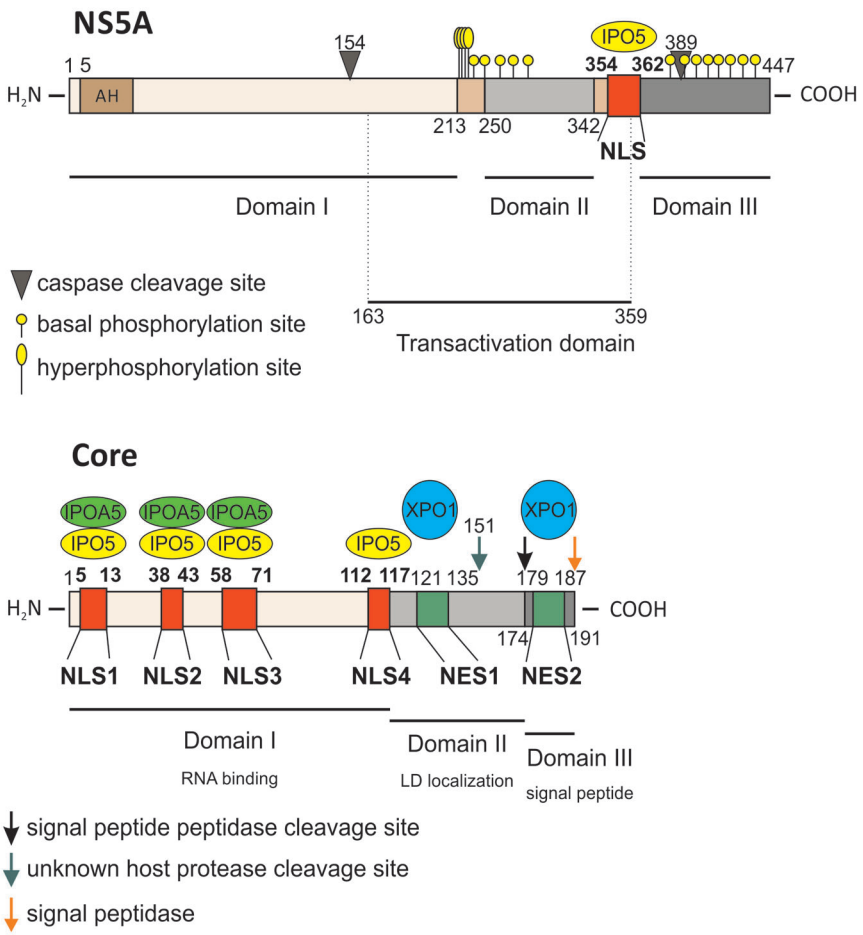

FIGURE 1 | HCV proteins contain functional nuclear transport signals (NTSs). (A) A summary of HCV proteins binding to Kaps, as well as the sequences of identified NTSs. Basic residues within NLSs are in boldface; hydrophobic residues within NESs are underlined; the single letter amino acid code is used; *protein that can be detected within the host cell nucleus during viral infection. **(B)** Schematic representation of core and NS5A proteins, their functional domains as well as their NTSs and the Kap-binding sites.

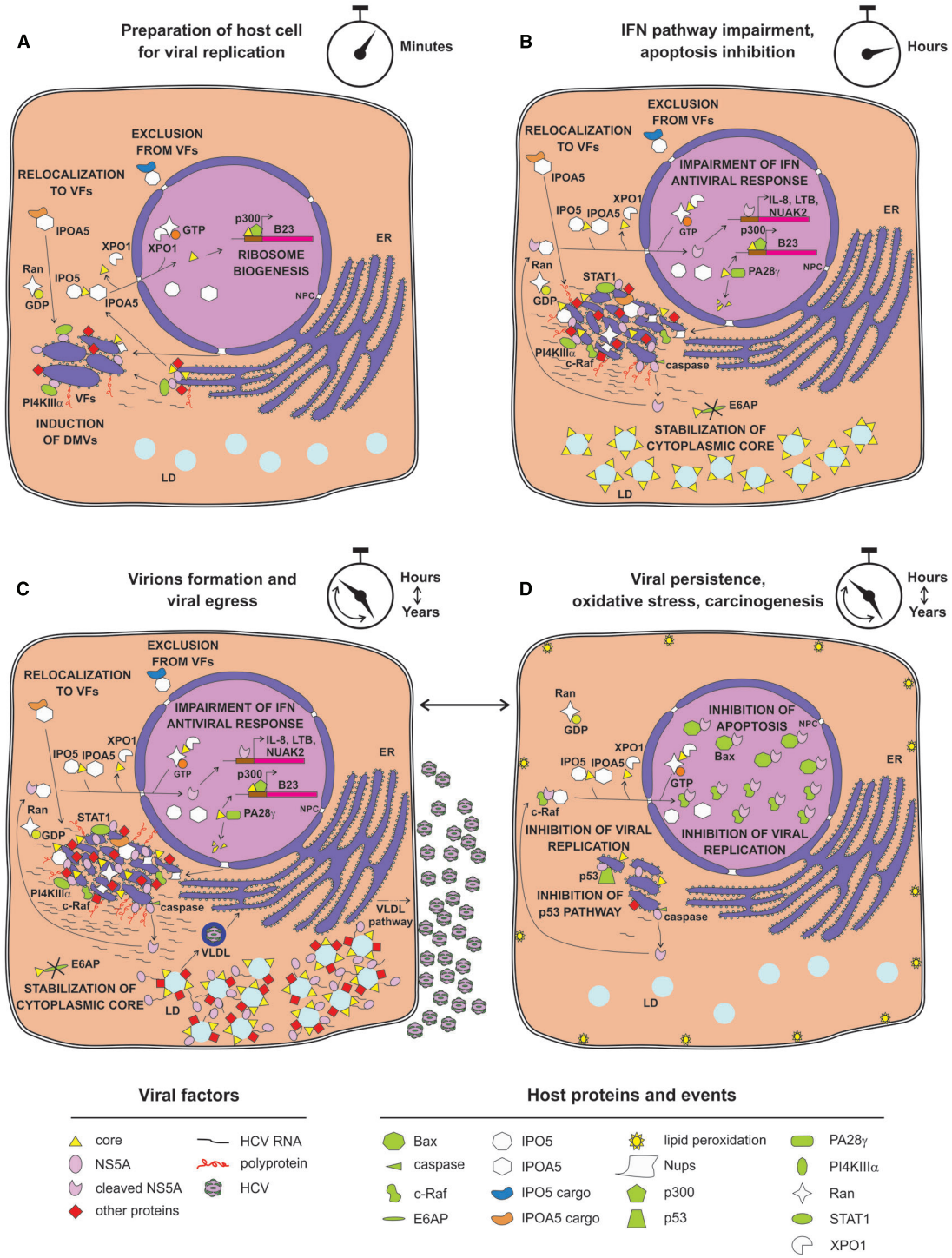

FIGURE 2 | Possible relationship between nuclear transport apparatus and HCV life cycle. (A) Soon after HCV infection, core is transported into the nucleus to promote ribosome biogenesis. Newly synthesized viral proteins promote VF development, depending on NS5A-mediated recruitment of PI4KIIIα. Simultaneously, specific Nups are recruited by core and NS5A to VFs. **(B)** Small amounts of NS5A reach the nucleus after being processed by basal caspase activity, resulting in activation of a specific transcriptional program aimed at inhibiting IFN response and apoptosis. Core migrates to the nucleus to be degraded by PA28γ instead, thus stabilizing cytoplasmic core. **(C)** Viral replication progresses and specific host factors are recruited to VFs, either directly by viral proteins or indirectly by IMPs recognizing IPO5-dependent NLSs. **(D)** Strong viral replication and production eventually causes oxidative stress and lipid peroxidation that is detrimental to host cells, leading to activation of caspase activity and NS5A nuclear accumulation, thereby sequestering the host dependency factor c-Raf from VFs. This impairs viral replication and the pro-apoptotic factor Bax, thus promoting infected cells survival, viral reactivation, and liver damage.

appears to be important for HCV life cycle. Indeed, the degree of transcriptional activation mediated by different NS5A variants correlates with the levels of viral replication in the HCV replicon system. Furthermore, pharmacological ablation of caspase activity negatively affected HCV genome replication, preventing NS5A nuclear translocation (Maqbool et al., 2013). Besides transcriptional regulation, nuclear NS5A promotes cell survival also by preventing translocation of the apoptotic activator Bcl-2 associated X protein (Bax) to the nuclear membrane in response to cellular stress, thus mitigating its pro-apoptotic function (Chung et al., 2003; Ruggieri et al., 2012; Lindenboim et al., 2014). Furthermore, it has been recently shown that overexpression of NS5A N-terminally truncated forms impairs HCV replication, by relocalizing the host dependency factor c-Raf from VFs to cell nuclei (Sauter et al., 2009). Since c-Raf facilitates viral replication through attenuation of the IFN pathway, its withdrawal from VFs impairs HCV replication (Zhang et al., 2012). These findings probably reflect an auto-inhibitory mechanism evolved by HCV to reduce its own replication in case of virus-induced cellular stress, thus preventing apoptosis and promoting persistent infection. This is consistent with the ability of several HCV genotypes to decrease viral replication levels once viral-induced lipid peroxidation threatens cell viability (Yamane et al., 2014).

As mentioned above, important nuclear functions are also emerging for the core protein, a small structural protein forming the viral nucleocapsid and undergoing multiple maturation steps. Upon translation on the rough ER, core protein is initially cleaved from the nascent polyprotein by the host cell signal peptidase. This immature form of core is anchored to the ER-membrane by a C-terminal signal peptide, which facilitates translocation of the polyprotein into the ER lumen. Subsequently, it is cleaved by the intermembrane host cell signal peptide peptidase between residue 177 and 178 to enable the mature form of core to migrate on LDs (Ogino et al., 2004; Hope et al., 2006; Okamoto et al., 2008). Once there, core encapsidates newly generated viral genomes in concerted action with other viral proteins such as NS5A, NS2 and p7 (Appel et al., 2008; Stapleford and Lindenbach, 2011). In contrast to its predominantly cytoplasmic localization upon overexpression in cell culture or in productively infected cells *in vitro*, the core protein has been detected in the nucleus of hepatocytes of chronically infected patients and core transgenic mice (Moriya et al., 1997). Indeed, it contains four N-terminally located NLSs and two C-terminally located NESs (**Figure 1**). Such signals are functional, conferring both nuclear shuttling and Kap-binding abilities to reporter proteins (Cerutti et al., 2011; Levin et al., 2014). Importantly, immuno-electron microscopic analysis of HCV-infected hepatocytes allowed detection of small amounts of core into the cell nucleus 20 min post-infection. Additionally, core could also be observed in the nucleus later on, upon pharmacological inhibition of CRM-1, indicating that both NES and NLS are functional in the context of productively infected hepatocytes. Intriguingly, core could be detected in the nucleus in the absence of CRM-1 inhibition also upon knock-down of the proteasome activator PA28γ, which is responsible for ubiquitin-independent degradation of core in the cell nuclei. PA28γ inactivation also caused increase in the ubiquitination of cytoplasmic core, and impaired viral assembly (Moriishi et al., 2010; Cerutti et al., 2011). Indeed, core can be degraded both in the nucleus in an ubiquitin-independent manner, and in the cytosol through an ubiquitin-dependent process involving the E3 ubiquitin ligase E6AP (Suzuki et al., 2009; Shoji, 2012). Therefore, nuclear activation of PA28γ by core inhibits E6AP, resulting in increased overall core stability and promoting viral assembly and release (Moriishi et al., 2010). Additionally, core is endowed with transcriptional activity, and represents the only HCV protein globally regulating host cell transcription, by directly binding the TATA-binding protein (Moriishi et al., 2003; Kao et al., 2004). This transcriptional stimulation is further supported by the ability of core to promote ribosome biogenesis, supporting cell growth and viral replication through epigenetic enhancement of the nucleolar protein B23 transcription. Indeed, core can recruit the histone acetyltransferase p300 at the B23 promoter (Mai et al., 2006). In addition, nuclear core also inhibits transcription of genes linked to apoptosis, by up-regulating the expression of inhibitor of caspase-activated DNase (Sacco et al., 2003).

Therefore, both core and NS5A can localize in the cell nucleus and modulate transcription, promoting cell survival. In addition, NS5A also promotes immune evasion, while core specifically increases the translation rate of infected cells via increased ribosome biogenesis.

Cytosolic Relocalization of Nups Upon HCV Infection

Hepatitis C virus VFs are a separate compartment from the cytoplasm (Miyanari et al., 2003; Hsu et al., 2010). Although the existence of a selective permeable barrier between the VF lumen and the surrounding cytoplasm has been postulated long ago, functional proof lacked until very recently (Neufeldt et al., 2013). Eventually, its definition has allowed reconciling the lack of HCV nuclear replication intermediate with the presence of functional NLSs and NESs within HCV proteins that are not observed in the nucleus during viral life cycle (Levin et al., 2014).

Various HCV proteins interact with high affinity nuclear transport factors (NTFs), although only core and NS5A functionally localize, to some extent, in the cell nuclei during viral infection (Moriya et al., 1997; Sauter et al., 2009; Cerutti et al., 2011; Maqbool et al., 2013; Germain et al., 2014; Levin et al., 2014). These findings suggested that HCV protein interaction with NTFs might be broader than originally thought. Mounting experimental evidence suggests that functional NPC-like structures could be recruited by HCV within the cytoplasmic VFs to build up a permeability barrier that improves the viral fitness (Neufeldt et al., 2013). While similar structures have been previously described within several cell types and are induced by other viruses, their functional characterization has begun only recently, using the HCV infectious system (Merisko, 1989; Marshall et al., 1996; Wang et al., 1997; Neufeldt et al., 2013). In particular, Nups representing most of the major NPC subcomplexes formed cytoplasmic foci upon HCV infection, and either co-localized or physically interacted with core and NS5A. Interestingly, this redistribution is associated with up-regulation at both the mRNA and protein level of specific Nups, further supporting the previously described HCV transcriptional regulation ability.

Similarly, both IPOA5 and IPO5 partially relocalized to VFs upon HCV infection and interacted with several HCV proteins, although recruitment of Kaps to sites of HCV replication did not correlate with their over-expression, as in the case of Nups. Importantly, addition of NLS-mimicking peptides was sufficient to prevent the interaction of core and NS5A with IPO5/IPOA5 but not with Nups, suggesting that the interaction of viral proteins with Nups was NLS- and Kap-independent (Neufeldt et al., 2013). Knock-down of Nups and Kaps revealed that different NTFs play specific roles in HCV life cycle. Indeed, depletion of Nup98 and 153 strongly impaired viral replication, whereas down-regulation of Nup155 and IPO5 specifically affected viral particle assembly. As mentioned above, several HCV proteins have functional NLSs capable of interacting with different Kaps, including IMPα and IMPβ homolog such as IPOA5 and IPO5 (Germain et al., 2014; Levin et al., 2014). Intriguingly, addition of cell permeable HCV NLS-mimicking peptides binding to specific Kaps differently affected the outcome of viral infection. While IPOA5-binding peptides specifically impaired viral replication, IPO5-binding ones interfered with both viral replication and assembly (Levin et al., 2014). Importantly, functionality of the HCV-induced cytoplasmic Kap-Nup network was demonstrated by the delocalization of the small GTPase Ran to NS5A positive foci, and by the fact that such complexes were able to recruit a specific subset of cargoes (Neufeldt et al., 2013; Levin et al., 2014). Indeed, reporter proteins fused to IPOA5-binding NLSs (such as Tag NLS) were efficiently transported to core positive foci during productive infection, while IPO5-binding NLSs (such as Rev NLS) were not. This raises the possibility that IPOA5 and IPO5 may contribute to distinct processes during the HCV life cycle, respectively allowing and restricting trafficking of specific cargoes in and out of VFs (Levin et al., 2014).

Overall, these findings would support the redirection of Nups to cytoplasmic NPC-like structures and movement of viral and host NLS-bearing proteins from the cytoplasm to the lumen of VFs with the purpose to create isolated and protected niches of viral replication and assembly (**Figure 2**). The VF-associated NPCs could also be able to discriminate between NTFs, importing IPOA5-shuttled proteins in a selective way and excluding IPO5 cargos as well as NLS-lacking proteins from the HCV replication complexes. Therefore, it is possible that HCV exploits the nucleocytoplasmic transport system to target specific cargoes of viral and cellular origin to the nucleus and VFs. In this context, NS5A interaction with IPO5 would suggest that NPC-like structures play a role in triggering the switch between viral replication and assembly by restricting NS5A from entering the VFs. Since infection causes a decrease in the levels of nuclear accumulation of IPOA5-transported cargoes such as GFP-Tag NLS by partially relocalizing them to core positive foci, it is also possible that HCV-induced NPCs similarly impair nuclear targeting of specific host factors such as p53 or STAT1, thus interfering with their functions (Germain et al., 2014). Indeed, altered host cell nuclear transport and functions are not uncommon upon viral infections (Yarbrough et al., 2014).

It remains to be investigated how the two Kap-mediated transport systems affect each other in HCV-infected hepatocytes and whether trafficking to VFs differs from nuclear import at the molecular level. For sure, the striking news about HCV life cycle is that Nups do work outside the physiological environment of the NE during viral infection. The molecular basis responsible for functional and regulatory differences between the NPC-dependent transport systems located on VFs *or* NE are elusive at present (including the role of the GTPase Ran in VF import), which requires further experimental efforts.

Conclusion

Detection of core- and NS5A-dependent transcriptional regulation and of cellular NTF-HCV protein interactions suggests that HCV exploits previously unidentified and nucleus-linked mechanisms to regulate both its survival and liver damage. These findings are consistent with the functionality of numerous NLSs found on HCV proteins. On the one hand, these NLSs are employed for protein shuttling to the nucleus. Consistently, core and NS5A and/or their variants regulate the cellular transcriptional environment, making it conductive to cell survival and prone to persistent viral infection by promoting ribosome biogenesis, and inhibiting IFN antiviral pathway and apoptotic cell death. On the other hand, NPC-like structures are proposed to form channels across the VF double membrane structures, which arise upon the HCV-induced cytoplasmic redistribution of Nups and Kaps. Selective trafficking across them regulates viral infection during distinct phases of HCV life cycle, with IPOA5- and IPO5-cargos being allowed or denied VF entry, respectively.

The further characterization of HCV trafficking signals has the potential to support the design of selective viral NLS/NES-targeted molecules with pangenotypic antiviral activity, targeting conserved regions among the HCV genotypes. Potentially, the same holds true for the discovery of new pieces of the cellular transcriptional puzzle altered by HCV, which may further help to forecast therapeutic outcomes after antiviral treatment.

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Interplay between the alpharetroviral Gag protein and SR proteins SF2 and SC35 in the nucleus

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Retroviruses are positive-sense, single-stranded RNA viruses that reverse transcribe their RNA genomes into double-stranded DNA for integration into the host cell chromosome. The integrated provirus is used as a template for the transcription of viral RNA. The full-length viral RNA can be used for the translation of the Gag and Gag-Pol structural proteins or as the genomic RNA (gRNA) for encapsidation into new virions by the Gag protein. The mechanism by which Gag selectively incorporates unspliced gRNA into virus particles is poorly understood. Although Gag was previously thought to localize exclusively to the cytoplasm and plasma membrane where particles are released, we found that the Gag protein of Rous sarcoma virus, an alpharetrovirus, undergoes transient nuclear trafficking. When the nuclear export signal of RSV Gag is mutated (Gag.L219A), the protein accumulates in discrete subnuclear foci reminiscent of nuclear bodies such as splicing speckles, paraspeckles, and PML bodies. In this report, we observed that RSV Gag.L219A foci appeared to be tethered in the nucleus, partially co-localizing with the splicing speckle components SC35 and SF2. Overexpression of SC35 increased the number of Gag.L219A nucleoplasmic foci, suggesting that SC35 may facilitate the formation of Gag foci. We previously reported that RSV Gag nuclear trafficking is required for efficient gRNA packaging. Together with the data presented herein, our findings raise the intriguing hypothesis that RSV Gag may co-opt splicing factors to localize near transcription sites. Because splicing occurs co-transcriptionally, we speculate that this mechanism could allow Gag to associate with unspliced viral RNA shortly after its transcription initiation in the nucleus, before the viral RNA can be spliced or exported from the nucleus as an mRNA template.

Keywords: splicing factors, nuclear trafficking, SC35, SF2, retroviral Gag proteins, nuclear bodies

Introduction

Retroviruses are significant human and animal pathogens, causing cancer and immunodeficiency syndromes in a wide variety of species. Most well-known is the human immunodeficiency virus type 1 (HIV-1), the etiological agent of acquired immunodeficiency syndrome (AIDS). Many retroviruses that infect animals have served as important model systems for unraveling the mechanisms of retroviral replication, pathogenesis, and host defense. The first retrovirus discovered, the avian alpharetrovirus Rous sarcoma virus (RSV), has proven to be

among the most valuable, launching challenges to existing dogmas that led to the discovery of reverse transcription and cellular oncogenes (reviewed in Parent, 2012).

Retroviruses are enveloped, positive-sense, single-stranded RNA viruses that package two copies of their genomes into virions. Following viral entry, the retroviral genomic RNA (gRNA) undergoes reverse transcription to generate a complementary, double-stranded DNA that integrates into the host cell genome to form the provirus. The integrated provirus is used as a template for the cellular RNA polymerase II to direct the synthesis of retroviral RNA. The genome-length retroviral transcript may be spliced to create subgenomic mRNAs, which are exported from the nucleus to synthesize other viral proteins. Alternatively, the full-length RNA may remain unspliced with two potential outcomes: it may serve as the mRNA template for the translation of the Gag and Gag-Pol structural proteins or it may be bound by the Gag protein for packaging into new virions as the gRNA. The Gag protein selects the gRNA for encapsidation through a high-affinity interaction between the nucleocapsid (NC) domain of Gag and the psi (Ψ) packaging sequence in the 5′ untranslated region of the viral RNA (Shank and Linial, 1980; Aronoff and Linial, 1991; Aronoff et al., 1993; Berkowitz et al., 1995, 1996; Butsch and Boris-Lawrie, 2002; Lee et al., 2003; Lee and Linial, 2004; Zhou et al., 2005, 2007).

Historically, it was thought that the initial Gag-gRNA interaction occurred in the cytoplasm or at the plasma membrane, where budding virions are released. Mounting evidence, including recent studies using sensitive microscopic imaging techniques, indicates that the Gag proteins of several retroviruses including HIV-1, RSV, mouse mammary tumor virus (MMTV), feline immunodeficiency virus (FIV), prototype foamy virus (PFV), Mason-Pfizer monkey virus (MPMV), and murine leukemia virus (MLV) undergo nuclear localization (Nash et al., 1993; Schliephake and Rethwilm, 1994; Amendt et al., 1995; Risco et al., 1995; Scheifele et al., 2002; Tobaly-Tapiero et al., 2008; Prizan-Ravid et al., 2010; Müllers et al., 2011; Renault et al., 2011; Elis et al., 2012; Kemler et al., 2012; Beyer et al., 2013; Lochmann et al., 2013). In the case of RSV, a connection has been established between Gag nuclear trafficking and gRNA incorporation. Genetic experiments demonstrated that targeting an RSV Gag mutant strongly to the plasma membrane reduced its nuclear trafficking, leading to the production of virus particles that encapsidate significantly reduced levels of gRNA (Scheifele et al., 2002). However, inserting an exogenous nuclear localization signal (NLS) into this Gag mutant restores gRNA packaging to nearly normal levels (Garbitt-Hirst et al., 2009). These results raise the intriguing possibility that nucleocytoplasmic transport of RSV Gag is required for proficient packaging of gRNA.

Treatment of RSV Gag expressing cells with the CRM1 inhibitor leptomycin B (LMB) traps Gag in the nucleus, and genetic mapping studies revealed a nuclear export signal (NES) in the p10 domain (**Figure 1A**). Mutation of hydrophobic residues within the NES causes Gag to accumulate in numerous, discrete nucleoplasmic foci and within nucleoli (Scheifele et al., 2002, 2005; Kenney et al., 2008; Lochmann et al., 2013). These nucleoplasmic foci are also observed at a lower frequency in the nuclei of cells expressing the wild-type Gag protein in the

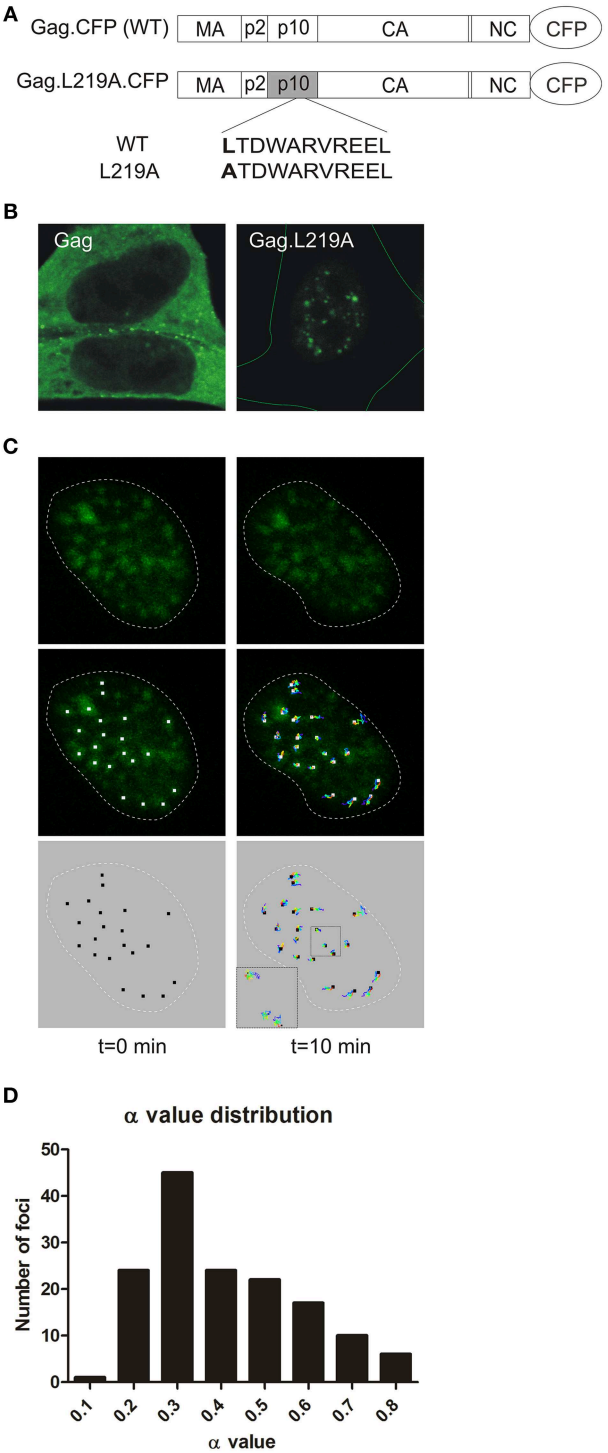

FIGURE 1 | Characterization of Gag.L219A nuclear foci. (A) Schematics of the CFP-tagged wild-type RSV Gag protein (top) and the Gag.L219A nuclear export mutant (bottom), with leucine 219 in the p10 domain mutated to alanine. **(B)** Confocal micrographs of fluorescent protein-tagged wild-type Gag (left panel) and Gag.L219A (right panel) in QT6 cells. On the right hand image, the entire cell is outlined with a thin green line. **(C)** QT6 cells expressing Gag.L219A-CFP were imaged using time-lapse 3D confocal microscopy. A series of single optical slices through the nucleus were captured every 8 s for

(Continued)

FIGURE 1 | Continued
10 min. After acquisition, the images were reconstructed as a 3D time-course using Imaris imaging software, and a single representative nucleus is shown with time = 0 on the left and $t = 10$ min on the right. The nucleus of each cell is outlined by a white dashed line. In the top panels, Gag foci (green) are shown. In the middle panels, the particle tracks were superimposed on the Gag foci, with white squares placed at the center of each focus (left) and tracks colored from blue (time = 0) to red ($t = 10$ min) in the middle panels. The particle tracks alone are shown in the bottom panel. In the lower left corner of the right image, a higher magnification of the particle tracks shows the course of the particles over the entire time period. **(D)** A histogram representing the anomalous diffusion coefficient α values for 149 nuclear foci is provided.

absence of LMB treatment (**Figure 1B**), providing evidence that formation of nuclear foci cannot be completely attributed to drug treatment or mutation. Furthermore, we demonstrated that Gag NES mutant proteins remain assembly-competent, as they interact with wild-type Gag proteins and can be rescued into virus particles (Kenney et al., 2008). The number and size of Gag nuclear foci increase with higher protein expression levels of the NES mutant Gag protein (data not shown), therefore it is possible that smaller accumulations of wild-type Gag proteins may form at lower expression levels, but these small foci are not readily detected by imaging studies.

To characterize the intranuclear population of RSV Gag proteins, we undertook the present studies to determine whether Gag nuclear foci share properties with host proteins that accumulate in nuclear bodies. These well-characterized subnuclear bodies are dynamic, non-membrane bound structures where nuclear proteins that perform specific functions are concentrated (Dundr and Misteli, 2010; Mao et al., 2011), including nuclear speckles, paraspeckles, and promyelocytic leukemia (PML) bodies. Nuclear speckles store and modify splicing factors that process pre-mRNAs (Mintz and Spector, 2000; Spector and Lamond, 2011). Paraspeckles are nucleated by the binding of the PSP1 protein to the long noncoding RNA NEAT1 and function in the retention of incompletely or aberrantly processed mRNAs (Fox et al., 2002; Prasanth et al., 2005; Bond and Fox, 2009; Clemson et al., 2009; Fox and Lamond, 2010; Souquere et al., 2010; Naganuma and Hirose, 2013; Yamazaki and Hirose, 2015). PML bodies form in response to DNA damage, stress, and viral infection (Dundr and Misteli, 2010; Mao et al., 2011). In this report, we examined whether the nuclear foci formed by nuclear-restricted Gag proteins have features in common with nuclear bodies and whether Gag localizes to any of the same nucleoplasmic sites as components of host nuclear bodies.

Materials and Methods

Expression Vectors

RSV Gag expression plasmids: pGag-GFP (Scheifele et al., 2002), pGag.L219A-CFP, pGag.L219A-YFP were described previously (Kenney et al., 2008). Expression plasmids used to encode human nuclear body proteins PSF and p54/nrb were constructed using PCR to exchange YFP for GFP from plasmids pGFP-PSF and pGFP-p54nrb, which were gifts from Dr. James Patton (Dye and Patton, 2001; Peng et al., 2002); human pSC35-YFP and human pYFP-SF2/ASF were gifts from Dr. David Spector (Prasanth et al., 2003); human pYFP-SUMO1 and human pCFP-PML were gifts from Dr. Mary Dasso (Ayaydin and Dasso, 2004); human pYFP-PSP1 was a gift from Dr. Angus Lamond, University of Dundee, UK; and murine pGFP-Clk1 was a gift from Alan Cochrane (Wong et al., 2011) (with permission from John Bell, University of Ottawa), in which GFP was exchanged with mCherry using PCR amplification and restriction fragment exchange.

Cells, Transfections, Fixation, and Immunofluorescence

QT6 cells, chemically transformed quail fibroblasts (Moscovici et al., 1977), were maintained as described (Craven et al., 1995), seeded at 0.2×10^6 on coverslips in 35 mm dishes containing glass coverslips and transfected using the calcium phosphate method (Fujiwara et al., 1988) with the following plasmids: pGag.L219A-CFP (1.5 μg), pGag-CFP (500 ng), pCMV.SC35-YFP (100 and 125 ng), pCMV.YFP-SF2 (125 ng), pYFP-PSP1 (100 and 125 ng), pYFP-Nrb (100 ng), and pYFP-PSF (100 ng). Cells were fixed 16 h post-transfection in 3.7% PFA in PHEM buffer (120 mM PIPES, 55 mM HEPES, 20 mM EGTA, and 16.5 mM $MgSO_4$, pH to 7.0) (Matic et al., 2008) for 10 min, incubated with DAPI at 5 μg/ml for 1 min and mounted on slides in SlowFade Antifade mounting medium (Invitrogen).

HeLa cells (Azad et al., 1993) were maintained as described (Lochmann et al., 2013), seeded at $0.4–0.5 \times 10^6$ on coverslips in 35 mm dishes containing glass coverslips, and transfected using Lipofectamine 2000 (Invitrogen) with the following plasmids: pGag.L219A-CFP (4 μg), pCMV.SC35-YFP (1 μg), pCMV.YFP-SF2 (500 ng), pYFP-PSP1 (250 ng), pYFP-nrb (250 ng), and pYFP-PSF (250 ng). Cells were fixed between 18 and 23 h post-transfection with 3.7% PFA in PHEM for 10 min at room temperature, stained with DAPI at 5 μg/ml for 1 min, and mounted on slides in SlowFade Antifade mounting medium (Invitrogen). To detect endogenous phosphorylated RS domain proteins, cells were fixed in 3.7% PFA in PHEM for 10 min, permeabilized with 0.25% Triton X-100 for 10 min at room temperature, blocked with 10% BSA in PBS for 1 h, incubated for 1 h with mouse anti-SC35 (Sigma S4045) antibody at a dilution of 1:1000, which recognizes the phosphorylated RS domains of the splicing factors SC35 and SF2, in 3% BSA/0.01% Tween-20 in PBS, and incubated with donkey anti-mouse Alexa 647 (Invitrogen) at a 1:1000 dilution for 1 h at room temperature.

Laser-scanning Confocal Microscopic Imaging

Cells were imaged using a Leica SP8 TCS scanning confocal microscope equipped with a White Light Laser (WLL) and argon laser using a 63X oil immersion objective. Sequential scanning between frames was used to average four frames for each image. DAPI was excited with the 405 nm UV laser at 10% laser power and emission detection between 415 and 450 nm using a PMT detector. CFP was imaged using the WLL excited with the 470 nm laser line and a hybrid detector window of 475–500 nm. YFP was imaged using the WLL with a laser line excitation of 514 nm and a hybrid detector window of 518–650 nm. Alexa 647 was

imaged using the WLL with the 647 laser line and the hybrid detector window ranged from 652 to 775 nm. mCherry was imaged using the 587 nm laser line and the hybrid detector window of 592–637 nm. All channels using the hybrid detectors had a time gating of 0.3 to 6.5 ns.

Particle Tracking in Living Cells

QT6 cells seeded at a density of 0.2×10^6 were cultured in 35-mm glass-bottomed dishes (MatTekCorporation) and transfected with 1.5 μg pGag.L219A-CFP. Cells were imaged on a live cell stage equilibrated to 38.5°C with 5% CO_2 at 16 h post-transfection using a Leica SP8 TCS scanning confocal microscope to capture 3D time-lapse images with a 63X water objective. Imaging was performed with a line average of two using the WLL with a laser line excitation of 470 nm and a hybrid detector window of 475–601 nm. A series of 0.3 μm ocular slices were captured to create a z-stack encompassing the entire nucleus approximately every 8 s for 10 min at 1400 Hz. The captured data was imported into Imaris analysis software (v8.0 Bitplane) to create a 3D volume rendering. Using the Imaris spot tool, Gag.L219A-CFP foci that measured ≤650 nm in diameter were identified in 3D space and tracked using Brownian motion detection. Correction for 3D drift was applied within the software, and only foci that were identified during the entire 10 min time-lapse period were analyzed. After focal drift compensation was performed, a line representing the movement of each individual particle was then superimposed onto the 3D time-lapse images. The Imaris software reported statistics for individual and average particle movement, and the x, y, z positions for each individual particle at each time point was exported from Imaris into MatLab using a script publically available at https://bitbucket.org/tim_lochmann/imaris-parser-for-msdanalyzer. The mean squared displacements (MSD) over time and diffusion coefficients were analyzed using the MSD analyzer script package (Tarantino et al., 2014). The MSD over time for each focus was calculated, and curves were fitted to the data. The α (anomalous diffusion coefficient) was calculated for 149 foci that had a curve fit of $R^2 > 0.8$. The binned α values were displayed as a histogram. The value for α was used to determine the type of mobility of each particle as defined by these parameters: $\alpha < 0.1$, confined diffusion; $0.1 \leq \alpha < 0.9$, obstructed diffusion; $0.9 \leq \alpha < 1.1$, simple diffusion; and $\alpha \geq 1.1$, directed motion (Bacher et al., 2004).

Quantitative Image Analyses

To quantitatively analyze co-localization of fluorescent proteins in cells, ImageJ (v1.49p, Schindelin et al., 2012) was used to calculate Mander's statistics using the Just Another Colocalization Plugin (JACoP) (Bolte and Cordelières, 2006). A minimum of 8 QT6 cells and 4 HeLa cells were analyzed per condition, the mean ± standard error of the mean for each Mander's score was calculated, and the values were analyzed statistically using a two-tailed, unpaired *t*-test. Outliers were determined and removed using the Grubbs test using $\alpha = 0.05$. (http://graphpad.com/quickcalcs/Grubbs1.cfm). Anti-Phospho RS domain staining was quantitatively analyzed using ImageJ by measuring the mean signal intensity of the nucleus for the antibody channel in cells with or without Gag.L219A and statistical analysis was performed as described above.

For quantification of nuclear foci, QT6 cells were seeded on coverslips at 0.4×10^6 and transfected with 1.5 μg of pGag.L219A-CFP and 125 ng of plasmids expressing YFP-tagged SF2, SC35, or PSP1 using the calcium phosphate method. DF1 cells (Himly et al., 1998) were seeded on coverslips at a density of 0.6×10^6, and co-transfected with 4 μg of pGag.L219A-CFP and 1 μg of each plasmid expressing a YFP-tagged host nuclear factor (SC35, SF2, or PSP1) using FuGene HD (Promega). Sixteen hours post-transfection, cells were fixed with 3.7% paraformaldehyde in PHEM buffer for 10 min at room temperature and mounted on slides with SlowFade Antifade mounting medium. Images were captured with a Deltavision DV Elite microscope (Applied Precision) using a 60 × (DF1 cells) or 100 × (QT6 cells) oil immersion objective with $N = 1.514$ oil using a CoolSNAPHQ2 (Photometrics) camera. Images were deconvolved and composite channel images were exported as RGB TIFF files post-acquisition using softWorx (v5.5.1, Applied Precision). ImageJ (v1.49o) (Schneider et al., 2012) was used for downstream export and analysis. A macro (modified from Alex Herbert, "ImageJ Batch Processing." http://www.sussex.ac.uk/gdsc/intranet/pdfs/ImageJBatchProcessing.pdf) was used to split, recolor, and save the composite channel image into the constituent CFP and YFP channel images. The section through the widest diameter of the nucleus was cropped from a single optical slice obtained from a minimum of 20 DF-1 cells and 16 QT6 cells. The number of Gag.L219A-CFP nuclear foci were counted using the same modified macro, which first automatically adjusted the histogram of each image from 1 to the maximum pixel intensity value of that image, and then applied a pixel intensity threshold determined empirically for each slide. Foci were identified with a size constraint of ≥4 pixels squared and with no circularity constraint. Outliers were removed, as determined by the Grubbs test using $\alpha = 0.05$. Prism statistical package (GraphPad Software 5.04) was used to create scatter plots showing the mean and standard error of the mean for the number of Gag nuclear foci, and a two-tailed, unpaired *t*-test was performed.

Results

RSV Gag.L219A Nuclear Foci Exhibit Obstructed Diffusion

The fluorescently-tagged wild-type RSV Gag protein (Gag-GFP) localizes primarily to the plasma membrane with the visualization of several small foci in the nucleus when examined using confocal microscopy (**Figures 1A,B**, top left panel). By contrast, the Gag L219A mutant (Gag.L219A-CFP), which contains a single amino acid change that inactivates the NES in the p10 domain, is predominantly localized to the nucleus within numerous, discrete, punctate foci (**Figures 1A,B**, top right panel) (Scheifele et al., 2005). As previously described, Gag.L219A also concentrates in the nucleolus in a subset of cells and undergoes rapid exchange with Gag proteins in the nucleus (Lochmann et al., 2013). However, we had not reported the characteristics of Gag proteins localized to nucleoplasmic puncta. To examine the kinetic properties of Gag.L219A localized within nucleoplasmic

foci, we used live-cell confocal imaging to examine the movement of the foci over a 10 min time period with z-stacks acquired every 8 s (**Figure 1C**). Following acquisition, the x, y, and z coordinates of each of 149 foci that were tracked during the entire 10 min imaging period were analyzed to measure particle movement. The mean square displacement (MSD) over time of each particle track was analyzed and a curve was fitted to the data (Tarantino et al., 2014). Using stringent parameters for curve fitting ($R^2 > 0.8$) of each particle track, the data from 149 Gag nuclear foci were further analyzed for their distance of diffusion. The average anomalous diffusion coefficient (α) of these tracked particles was found to be 0.46 ± 0.16 with high significance for α values < 1.0 ($p = 1.4 \times 10^{-82}$), indicating a pattern of obstructed diffusion based on definitions previously described by Bacher and colleagues (confined diffusion, $\alpha < 0.1$; obstructed diffusion, $0.1 \leq \alpha < 0.9$; simple diffusion, $0.9 \leq \alpha < 1.1$; directed motion, $\alpha \geq 1.1$) (Bacher et al., 2004). The α values were binned to plot a histogram (**Figure 1D**), which indicated that 100% of nuclear Gag foci display obstructed diffusion, indicating that the movement of each Gag focus has a limited range of movement, suggesting that Gag molecules may be tethered to a cellular partner within the nucleus.

Analysis of Gag.L219A Co-localization with Host Proteins in Subnuclear Bodies

The particle tracking data suggest that Gag.L219A foci appeared to be tethered in foci which resemble subnuclear bodies. To determine whether Gag.L219A co-localized with host proteins in subnuclear bodies, we expressed fluorescently-tagged splicing factors that are components of speckles (SC35 and SF2); paraspeckles (PSP1, PSF, and p54nrb); or PML bodies (PML and SUMO1). Plasmids expressing these nuclear body components tagged with YFP were used to co-transfect avian (QT6) and human (HeLa) cells with pGag.L219A-CFP. We expected human SC35 and SF2 to form characteristic splicing speckles in QT6 cells due to the high level of conservation between human and chicken orthologs (98.19 and 98.79% amino acid identity, respectively). However, SC35 and SF2 appeared more diffuse in QT6 cells even when a low amount of plasmid DNA was used for transfection (100 ng), although there were areas of consolidation where the protein was concentrated (**Figure 2A**). Of interest, both SC35 and SF2 co-localized with Gag.L219A foci to a high degree. Quantitative Mander's analysis performed in 8 cells revealed that a mean of 69.8 ± 4.7% of Gag.L219A co-localized with SC35 and 61.5 ± 5.2% of Gag.L219A with SF2 (**Figure 2C**). To determine whether co-localization was present in 3-dimensions, z-stacks were obtained and reconstructions were performed using Imaris imaging analysis software (Supplemental Movie S2). Rendering of the Gag.L219A (red) and SC35 (green; **Figure 2D**, left) or SF2 (**Figure 2D**, right) signals revealed that Gag.L219A/SC35 and Gag.L219A/SF2 co-localized in the x, y, and z dimensions and appear to be in close proximity, at least based on the limits of resolution of the microscopic images obtained (theoretical resolution 250 nm in the x and y planes and 600 nm in the z plane). For Gag.L219A/SC35 and Gag.L219A/SF2, the Mander's co-localization values were statistically significantly higher ($p < 0.0001$ in both cases) than the quantitative co-localization measured between Gag.L219A and proteins that reside in paraspeckles (p54nrb, PSF, and PSP1) or PML bodies (PML and SUMO1) (**Figures 2B,C**). Together, these data suggest that Gag.L219A protein accumulated at subnuclear locations enriched in splicing speckle components SC35 and SF2.

Next, because we observed co-localization between Gag.L219A and human SC35 and SF2 expressed in avian cells, we wanted to determine whether similar co-localization patterns would be observed in human cells, in which the localization of splicing factors has been more extensively studied. When expressed alone, SC35 adopted its characteristic speckled appearance; however, co-expression with Gag.L219A resulted in a more diffuse pattern, and there was a high degree of co-localization between the proteins (79.7 ± 2.5% of Gag co-localized with SC35; **Figures 3A,B**). SF2 accumulated in speckles when expressed alone, and SF2 also showed a high degree of co-localization with Gag.L219A (60.6 ± 6%). Gag co-localization was significantly higher with SC35 compared to SF2 ($p = 0.0192$) and both SC35 and SF2 were more strongly associated with Gag.L219A compared to p54nrb, PSF or PSP1 ($p < 0.0001$). Analysis of cells co-expressing Gag.L219A and PML or SUMO1 could not be performed due to cell toxicity (data not shown).

To determine whether Gag.L219A foci co-localized with endogenous nuclear splicing speckles, we performed immunofluorescence using an antibody that recognizes the phosphorylated RS domains of SF2 and SC35 (**Figure 4A**). Gag.L219A accumulated in nuclear foci in transfected HeLa cells that appeared similar to those observed in QT6 cells (**Figure 4B**). In cells expressing Gag.L219A, overlapping signals were observed at the intersection of the phosphorylated SR domain proteins and Gag.L219A foci, which appeared to be juxtaposed (see enlarged image in bottom row of **Figure 4C**), suggesting that Gag foci form near accumulations of splicing speckle components. Of note, in HeLa cells expressing Gag.L219A, the amount of endogenous SC35/SF2 staining was dramatically reduced, as indicated by the statistically significant ($p < 0.001$) decrease in the mean signal intensity of the anti-phospho RS antibody channel (**Figure 4C**, solid arrowhead) compared to cells in which there was no Gag expression (open arrowhead). This result suggests that expression of Gag.L219A interferes with staining of endogenous phospho RS domains of splicing factors, although the mechanism remains unclear.

Co-expression of Clk1 Enhances Co-localization of Gag.L219A with SC35 and SF2

Phosphorylation is a major mechanism for regulating the localization of SR proteins in the nucleus (Yeakley et al., 1999), therefore we examined whether the degree of phosphorylation of splicing factors SC35 and SF2 influenced their association with Gag.L219A. To that end, murine Clk1, an SR protein kinase (SRPK) that phosphorylates the RS domains of SC35 and SF2 (Gui et al., 1994; Colwill et al., 1996; Nayler et al., 1997; Koizumi et al., 1999; Aubol et al., 2002; Ngo et al., 2005) was expressed as an mCherry fusion protein in QT6 cells, either alone (**Figure 5A**) or in conjunction with Gag.L219A-CFP, SC35-YFP or YFP-SF2 (**Figure 5B**). When co-expressed with Gag.L219A, there was no significant co-localization with Clk1-mCherry (**Figure 5B**, upper

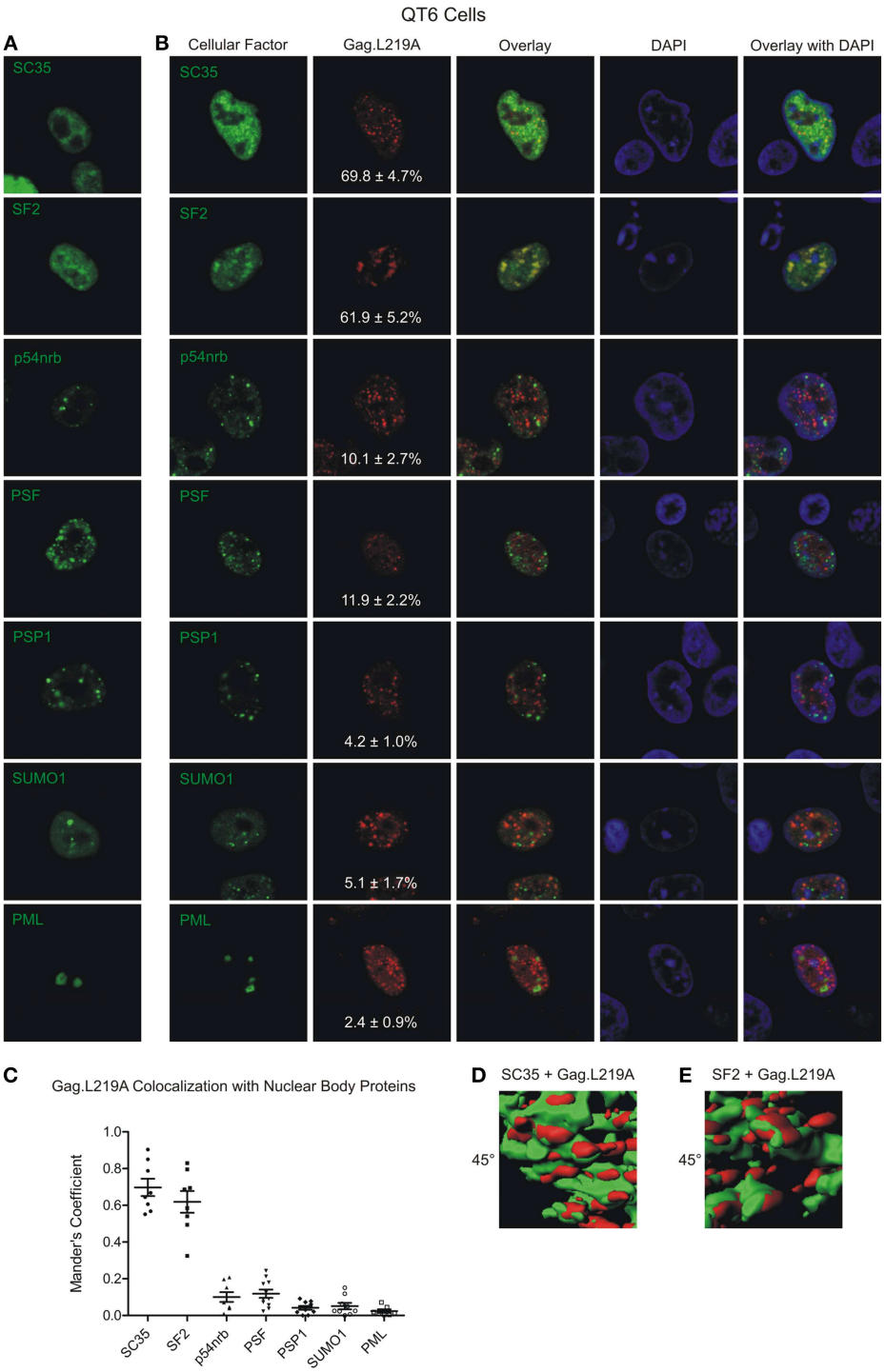

FIGURE 2 | Localization of Gag.L219A with host nuclear body proteins in QT6 cells. (A) Localization of Gag.L219A and nuclear body proteins in singly transfected QT6 cells. **(B)** Co-localization analysis between Gag.L219A and the indicated nuclear body proteins in co-transfected QT6 cells. Merging of Gag.L219A and nuclear body marker protein channels is displayed (Overlay). The DAPI channel is also depicted. The percentage of Gag.L219A with each factor is depicted in the Gag.L219A channel with the standard error of the mean. **(C)** Scatterplot depicting the mean and standard error of the mean of Gag.L219A co-localization with each of the nuclear body protein. **(D)** Still image from Supplemental Video 1 that closely examines a surface rendering of SC35 (green) and Gag.L219A (red). **(E)** Still image from Supplemental Video 2 that closely examines a surface rendering of SF2 (green) and Gag.L219A (red).

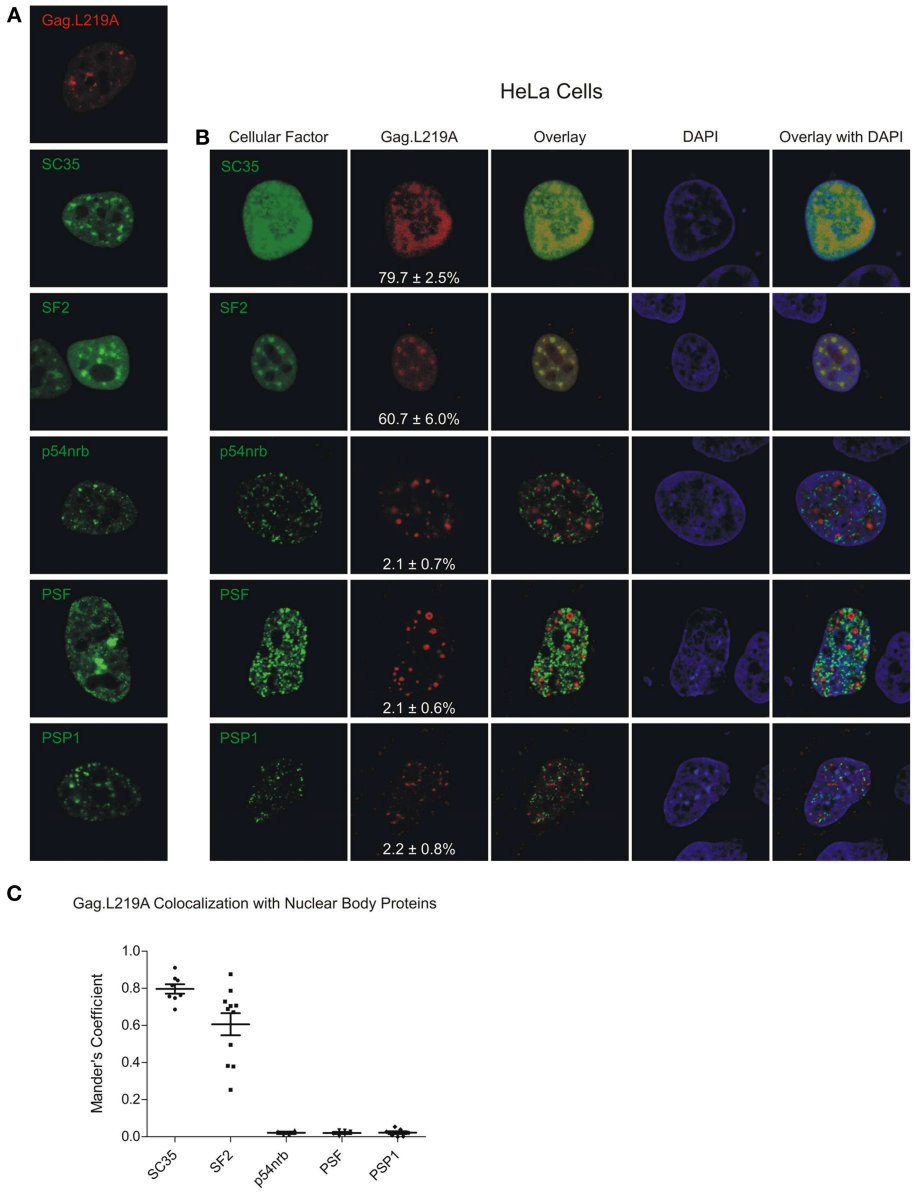

FIGURE 3 | Localization of Gag.L219A with host nuclear body proteins in HeLa cells. (A) Localization of Gag.L219A and nuclear body proteins in singly transfected HeLa cells **(B)** Co-localization analysis between Gag.L219A and the indicated nuclear body proteins in co-transfected HeLa cells. Merging of Gag.L219A and nuclear body marker protein channels is displayed (Overlay). The DAPI channel is also depicted. The percentage of Gag.L219A with each factor is depicted in the Gag.L219A channel with the standard error of the mean. **(C)** Scatterplot depicting the mean and standard error of the mean of Gag.L219A co-localization with each of the nuclear body proteins.

row). However, as expected, there was co-localization between Clk1/SC35 and Clk1/SF2 (**Figure 5B**, lower rows) because Clk1 phosphorylates both SC35 and SF2.

In cells expressing Gag.L219A/Clk1/SC35 (**Figure 5C**, upper row), the degree of Gag co-localization with SC35 increased to 73.5% ± 5% (compared to 69.8% without Clk1, **Figure 2**), although the increase was not statistically significant. However, Clk1 co-expression did significantly increase the co-localization of Gag.L219A with SF2 to 84% ± 2.2% (compared to 62.9% without Clk1, **Figure 2**; $p = 0.0066$). To assess whether Clk1 hyperphosphorylated YFP-SF2 and SC35-YFP in QT6 cells, we performed Western blotting of nuclear lysates (Supplemental Figure 1). For both SF2 and SC35, there was a change in the migration of the hyperphosphorylated proteins (red asterisks) in cells co-expressing Clk1-mCherry compared to the position of YFP-SF2 and SC35-YFP isolated from cells not expressing Clk1-mCherry (red circles). Treatment of the nuclear lysates with calf intestinal phosphatase dephosphorylated the proteins, as demonstrated by the faster migration of phosphatase-treated forms of SF2 and SC35, consistent with previous reports

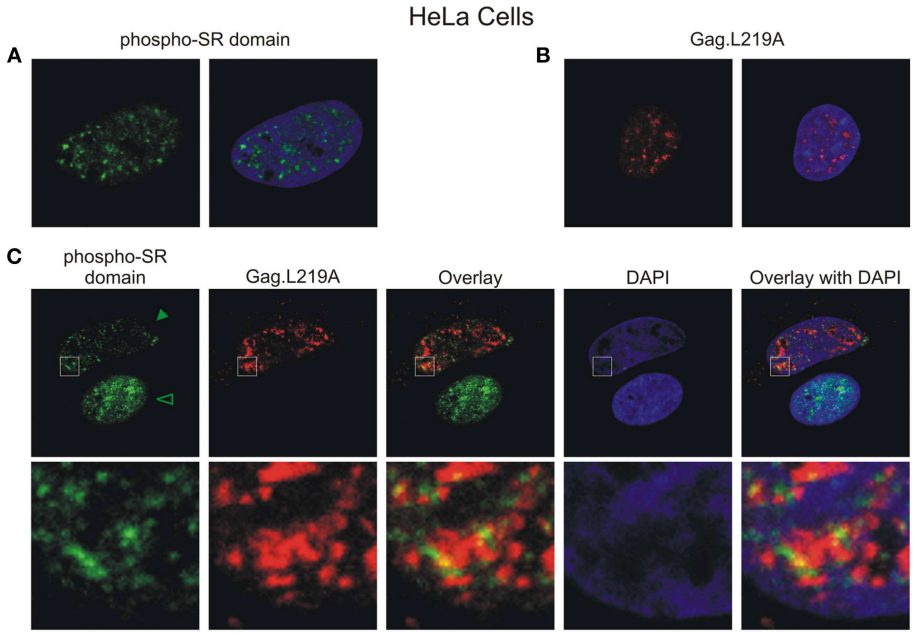

FIGURE 4 | Gag.L219A localization with endogenous splicing speckles in HeLa cells. (A) Visualization of endogenous splicing speckles in HeLa cells using immunofluorescence with α-phospho RS antibody (also called α-SC35 antibody; see Materials and Methods). **(B)** Localization of Gag.L219A in unstained HeLa cells fixed and permeabilized using the conditions outlined in Materials and Methods used to visualize endogenous splicing speckles. **(C)** Gag.L219A transfected HeLa cells were stained with α-phospho RS antibody. The Gag.L219A and α-phospho RS channels were combined (Overlay). This merged image was overlaid with the DAPI channel (Overlay with DAPI). The same region from each channel denoted by the white box was cropped, enlarged, and displayed in the bottom panel. The intensity of α-phospho RS antibody decreased in the presence of Gag.L219A (indicated by solid arrowhead) compared to cells in which Gag.L219A was not expressed (indicated by open arrowhead) ($p < 0.001$; 6 cells were analyzed per condition).

(Ngo et al., 2005). Together, these results suggest that the association of Gag.L219A with splicing factors, particularly SF2, was enhanced by hyperphosphorylation of the RS domain.

The Number of Nuclear Gag.L219A Foci Increases with SC35 Overexpression

During the course of our imaging studies, we noticed that the number of nuclear Gag.L219A-CFP foci appeared to increase in cells that co-expressed SC35-YFP. To determine whether this effect was specific for SC35, we compared the number of nuclear Gag.L219A foci in cells expressing Gag.L219A-CFP alone compared to cells transfected with equal amounts of pSC35-YFP, pYFP-SF2, or pYFP-PSP1 (**Figure 6A**). In cells expressing Gag.L219A alone, the average number of Gag foci was approximately 22 per nucleus, whereas upon co-expression of SC35-YFP, the average number of Gag nucleoplasmic foci increased significantly to 36 (**Figure 6B**, $p = 0.0003$). By contrast, co-transfection of equal amounts of pGag.L219A with pYFP-SF2 or pYFP-PSP1 did not lead to a significant change in the number of nuclear Gag foci. This experiment was repeated in DF1 cells with the same outcome, indicating that the result was not specific to QT6 cells (data not shown).

Discussion

The biological role of retroviral Gag proteins in the nucleus is not well understood, even though several Gag proteins have been observed within the nucleus (Parent, 2011; Stake et al., 2013). RSV is unique among retroviral Gag proteins because the protein accumulates in the nucleus when nuclear export is blocked by treatment of cells with LMB and a CRM1-mediated nuclear export signal (NES) was identified in the p10 region of Gag (Scheifele et al., 2002, 2005). The NES mutant Gag.L219A is a very informative tool, permitting the examination of the intranuclear activities of Gag, which are difficult to study using the wild-type Gag protein because a small amount of Gag protein is present in the nucleus. In this work, we focused on understanding the characteristics of the nuclear Gag population and to identify potential interacting partners within the nucleus.

With careful inspection, the wild-type Gag protein can be detected within small nucleoplasmic foci (**Figure 1B**). The Gag.L219A protein accumulated in similar nuclear puncta, although the foci were larger and more numerous. The foci formed by nuclear-restricted Gag proteins exhibited obstructed diffusion with an average anomalous diffusion coefficient of 0.46, suggesting that Gag.L219A proteins are tethered to another molecule within the nucleus. In an attempt to identify the tethering partner, when we examined whether Gag.L219A was associated with other subnuclear bodies that form intranuclear foci. We found a high degree of co-localization with splicing factors SC35 and SF2, although the co-localization was partial (∼70%), suggesting that either Gag proteins move dynamically between SF2/SC35 nuclear aggregates and other nucleoplasmic sites, or a different molecule could be the Gag anchor.

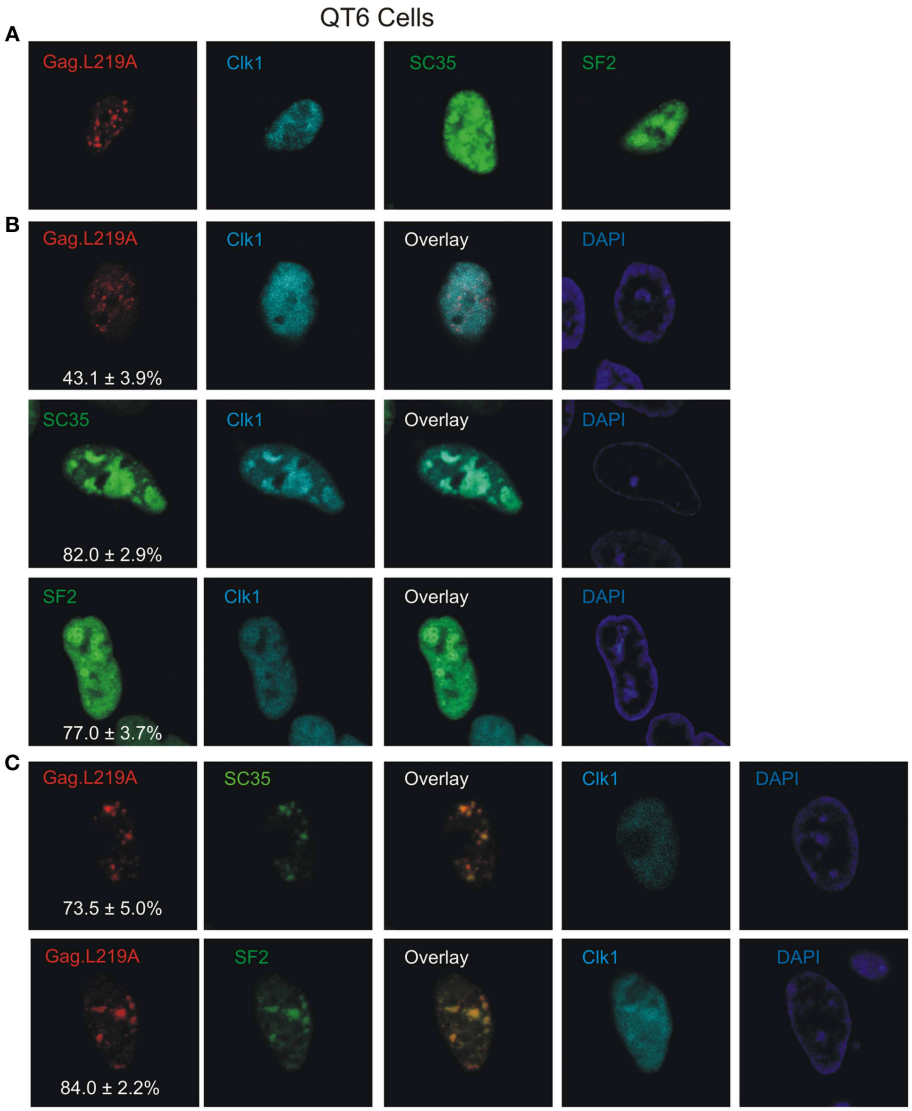

FIGURE 5 | The effect of Clk1 on Gag.L219A localization in QT6 cells. (A) Localization of Gag.L219A, Clk1, SC35, or SF2 in singly transfected QT6 cells. **(B)** Co-expression of Clk1 with Gag.L219A, SC35, or SF2 in QT6 cells. "Overlay" depicts a merge of the Clk1 and Gag.L219A or splicing factor channels. The DAPI channel is also displayed. The percentage of Gag.L219A, SC35, or SF2 co-localizing with Clk1 is depicted in the corresponding Gag.L219A, SC35, and SF2 channels with the standard error of the mean. **(C)** QT6 cells co-transfected with Gag.L219A, Clk1, and SC35 (top panel) or SF2 (bottom panel). Merging the Gag.L219A and splicing factor channels results in the "Overlay" channel. The DAPI channel is also displayed. The percentage of Gag.L219A co-localization with SC35 or SF2 in the presence of Clk1 overexpression is depicted in the corresponding Gag.L219A channel with the standard error of the mean.

Finding that increased expression of SC35 induced an increase in the number of Gag foci suggests that SC35 may facilitate the formation of Gag.L219A nuclear puncta (**Figure 6**). Additional experiments will need to be performed to examine whether SC35 is required for the formation of Gag.L219A foci, whether Gag interacts with SC35 and if so, whether the interaction is direct or indirect. SF2 also co-localized with Gag foci (**Figure 2**), and the degree of co-localization increased with co-expression of the with SR protein kinase Clk1, which phosphorylates the RS domain of splicing factors (Colwill et al., 1996). These data suggest that Gag.L219A interacts more efficiently with phosphorylated splicing factors, although alternatively, it remains possible that Clk1 has pleiotropic effects on cellular proteins that result in increased co-localization of Gag.L219A with SF2 and SC35. Furthermore, we noted that SC35 and SF2 appeared more diffuse in the nuclei of QT6 cells rather than forming discrete puncta characteristic of splicing speckles in HeLa cells (**Figure 3**). The localization of SR proteins is dependent on phosphorylation of their RS domains by SRPKs, including Clk1. Hyperphosphorylation of the RS domain of SF2 by Clk1 relocalizes SF2 from discrete nuclear speckles to the nucleoplasm (Ngo et al., 2005), and other work has shown that

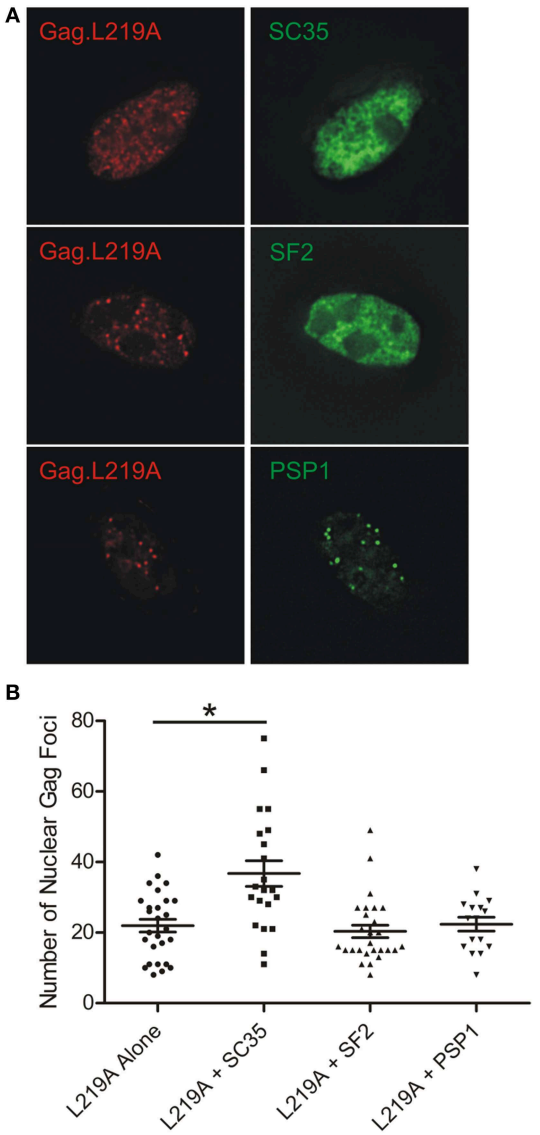

FIGURE 6 | Quantification of Gag.L219A nuclear foci number with co-expression of SC35, SF2, or PSP1. (A) Representative images of QT6 cells co-transfected with Gag.L219A and SC35, SF2, or PSP1. **(B)** Quantification of the number of Gag.L219A nuclear foci in QT6 cells expressing Gag.L219A alone or Gag.L219A co-transfected with SC35, SF2, or PSP1. Mean and standard error of the mean are indicated for each transfection. Nuclear foci in at least 16 QT6 nuclei were counted. *denotes $p = 0.0003$.

phosphorylation of the SR domain targets splicing factors to nascent RNA transcripts (Misteli et al., 1998; Yeakley et al., 1999). Therefore, the increased association of Gag.L219A with hyperphosphorylated SR proteins implies that Gag.L219A may preferentially associate with SR proteins that are primed for splicing nascent transcripts at sites of transcription.

To explain the difference in appearance of nuclear foci formed by SF2 and SC35 in avian cells compared to human cells, one possibility is that avian SRPKs do not properly phosphorylate human splicing factors (Gui et al., 1994; Colwill et al., 1996; Nayler et al., 1997; Koizumi et al., 1999), causing them to adopt a more diffuse localization. This idea is supported by the observation that expression of Clk1 in QT6 cells was associated with a more focal consolidation of Gag.L219A with SC35 and SF2 (**Figure 5C**). However, we cannot rule out the possibility that SC35 and SF2 require another host factor (protein or RNA) to form splicing speckles in human cells (e.g., the long noncoding RNA MALAT1) (Tripathi et al., 2010; Nakagawa et al., 2012), which may not be present in avian cells. Whereas our data suggests that Gag may co-localize with splicing factors SC35 and SF2, whether these associations are at canonical speckles near sites of transcription will require further examination.

Using a monoclonal antibody directed against the phosphorylated RS domains of splicing factors to stain for endogenous proteins, we observed that Gag.L219A and endogenous phosphorylated splicing factors appeared to be juxtaposed in HeLa cells (**Figure 4C**). Strikingly, immunostaining with the anti-phospho RS domain antibody was markedly reduced in HeLa cells that also co-expressed Gag.L219A (**Figure 4C**). These findings combined with the 3-dimensional reconstructions (shown in **Figure 2** and Supplemental Movies S1, S2) showing the close proximity of Gag.L219A with SC35 and/or SF2 raises the possibility that Gag may associate with splicing factors in splicing speckles, although more experiments need to be performed to test this idea. The NC domain of Gag.L219A is required for Gag to form intranuclear foci and NC also mediates Gag-Gag and Gag-RNA interactions (Kenney et al., 2008). Therefore, we must consider the idea that the Gag nuclear tether could be a host RNA; thus, it is possible that Gag.L219A interacts with SC35 and/or SF2 through an RNA-mediated association.

Why might RSV Gag interact with splicing factors in the nucleus? Considering our previous data linking nuclear localization of Gag with efficient genomic RNA packaging (Scheifele et al., 2002; Garbitt-Hirst et al., 2009), we hypothesize that RSV Gag might enter the nucleus to package the viral unspliced RNA genome shortly after it is synthesized. This strategy would target Gag to the transcription site, which is where the highest concentration of genome-length RNA is present in the cell. Additionally, Gag would have access to viral RNA before it could be spliced and could select the unspliced RNA as genome rather than permitting its use as an mRNA. We propose that RSV Gag could enter the nucleus, localizing at the periphery of speckles near transcription factories (Sutherland and Bickmore, 2009) to gain access to nascent unspliced viral RNA to capture it for packaging into virions. Other potential reasons for Gag to localize near splicing factors include altering the splicing pattern of host or viral RNAs or modifying nuclear export of viral or host RNAs.

As a means to target the unspliced RSV RNA for packaging by Gag, we propose that the NRS (negative regulator of splicing), a cis-acting element in the *gag* coding region, may play an important role. The RSV NRS regulates the balance between spliced and unspliced RSV RNA (Arrigo and Beemon, 1988; McNally and Beemon, 1992) by binding to SFp30a/b (a complex of SC35 and SF2) and U11/U12 snRNPs to form a nonfunctional spliceosome that inhibits the upstream RSV 5′ splice site (Gontarek et al., 1993; McNally and McNally, 1996).

Interestingly, the RSV psi packaging sequence is located on both the spliced and unspliced viral RNA, yet the unspliced RNA is preferentially packaged by Gag into new virions. Thus, we speculate that the downstream pseudo-spliceosome assembled on the NRS could "mark" the unspliced RNA as a potential genome. Gag could interact with SC35 and SF2 bound to the NRS, scan the RNA for the high affinity psi sequence, and ultimately select an RNA containing both an NRS and psi as the genomic RNA. Additionally, it is feasible that RSV Gag associates with splicing factors to influence the global splicing program of the cell to benefit virus replication.

Interestingly, numerous splicing factors were identified as potential binding partners of the HIV-1 Gag protein using mass spectrometry (Engeland et al., 2011, 2014), including PRPF3 and PRPF4 (components of the U4/U5/U6 tri-snRNP complex), SFRS1 (SF2), SFRS2 (SC35), SFRS3 (SRp20), SRSF5 (SRp40), SRSF6 (SRp55), SFSR7 (9G8), SFRS9 (SRp30c), SRPK1 (an SR Protein Kinase). Additional investigation is required to address whether HIV-1 Gag associates with these factors in cells and whether splicing factors play a functional role in the replication cycle beyond their influence on regulating alternative splicing of retroviral RNAs.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00925

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Induction of the pro-inflammatory NF-kB-sensitive miRNA-146a by human neurotrophic viruses

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A remarkably wide variety of human neurotrophic viruses—ranging from herpes simplex 1 (HSV-1; *Herpesviridae*; dsDNA genome) to Hantavirus (HTV; *Bunyaviridae;* (−)ssRNA genome) to human immunodeficiency virus (HIV; *Retroviridae*; (+)ssRNA genome) are associated with the rapid up-regulation of the NF-kB-sensitive pro-inflammatory microRNA-146a (miRNA-146a) in the host shortly after infection. This significant miRNA-146a up-regulation appears to be beneficial to the infecting virus as part of an immune-evasion strategy. Interestingly, miRNA-146a is also significantly up-regulated in several human central nervous system (CNS) disorders. These include Alzheimer's disease (AD) and prion disease where miRNA-146a participates in pro-inflammatory and innate-immune signaling. This opinion paper will comment on some recently clarified roles for the NF-kB-regulated, pro-inflammatory miRNA-146a in viral-induced cellular dysfunction, and how anti-miRNA-146a and/or related therapeutic strategies may be beneficial in the clinical management of a broad spectrum of viral-mediated CNS disease.

The 22 nucleotide, non-coding, single stranded RNA (ssRNA) miRNA-146a (5′-UGAGAACUGAAUUCCAUGGGUU-3′; 41% C+G; NR_029701) lies at the crossroads of multiple biological processes involved in the innate-immune response, viral-infection and inflammatory disease (Lukiw and Pogue, 2007; Cui et al., 2010; Lukiw, 2012; Saba et al., 2014). miRNA-146a, encoded at chromosome 5q33.3 (chr 5q33.3) in humans, is a rapidly induced, NF-kB-sensitive pro-inflammatory miRNA with a relatively short half-life of about ∼2 h in the human CNS (Taganov et al., 2006; Lukiw et al., 2008; Sethi and Lukiw, 2009; Li et al., 2010; Kroesen et al., 2015). Initially described as being significantly up-regulated after microbial lipopolysaccharide (LPS) stimulation of monocytes and under transcriptional control by NF-κB, miRNA-146a was subsequently found to be: (i) up-regulated by pro-inflammatory cytokines (such as IL-1β and TNFα; Taganov et al., 2006; Lukiw et al., 2008; Cui et al., 2010); (ii) induced by metal sulfate-generated reactive oxygen species (ROS; Pogue et al., 2009); (iii) up-regulated by neurotoxic 42 amino acid amyloid beta (Aβ42) peptides in human primary brain cells (Li et al., 2010; Alexandrov et al., 2011); and (iv) implicated as a key regulator of innate-immune signaling in part through interleukin receptor-associated kinase (IRAK) activation (Cui et al., 2010; Saba et al., 2014). Subsequent sequencing across the chr 5q33.3 locus indicated the presence of 3 tandem, canonical NF-kB-binding sites in the 5′ regulatory-region of the miRNA-146a gene (Sethi and Lukiw, 2009; Cui et al., 2010). Combined with functionality and NF-kB-inhibition assays miRNA-146a was the first NF-κB-regulated, pro-inflammatory miRNA identified in the human CNS (Li et al., 2010, 2011). The most significant miRNA-146a abundances to date have been found in astroglial and microglial cells, the later representing the "resident scavenging-macrophages" of the CNS, and key participants in the brain's innate-immune surveillance and inflammatory response-systems (Li et al., 2010, 2011; Saba et al., 2012). While only basally expressed in the CNS, miRNA-146a can be induced 2- to 25-fold or higher in cultured human primary brain cells after the application of several different classes of physiological stressors including treatment with (i) neurotropic virus (Hill et al., 2009; Lukiw et al., 2009; Li et al., 2010); (ii) neurotoxic metal sulfates (such as aluminum sulfate at low nanomolar concentrations; Pogue et al., 2009); (iii) microbial endotoxins including LPS (Taganov et al., 2006); and (iv) pro-inflammatory cytokines and Aβ peptides, either alone or in combination (Taganov et al., 2006; Lukiw et al., 2008, 2010; Li et al., 2010). While the mechanism for miRNA-146a-mediated immune-evasion is still not fully understood, in humans this process appears to require the activation of NF-kB; other transcription factors such as AP1 may be used in miRNA-146a activation in mice (Tung et al., 2010; Ho et al., 2014; Wang et al., 2014).

A surprisingly large number of different types of potentially incapacitating or lethal viruses, possessing either DNA or RNA genomes, have been shown to significantly induce miRNA-146a in the human CNS, immune, lymphatic, hepatic or circulatory systems, and these include (alphabetically-ordered): (i) Chikungunya virus (CHIKV; *Togaviridae;* (+)ssRNA genome; Selvamani et al., 2014); (ii) enterovirus 71 (EV71; *Picornaviridae*; (+)ssRNA genome; Ho et al., 2014); (iii) Epstein-Barr virus (EBV; *Herpesviridae*; dsDNA genome; Jonigk et al., 2013); (iv) Hantavirus (HTV; *Bunyaviridae;* (−)ssRNA genome; Shin et al., 2013); (v) hepatitis C virus (HCV; *Flaviviridae*; (+)ssRNA genome; Joshi et al., 2013); (vi) herpes simplex virus-1 (HSV-1; *Herpesviridae*; dsDNA genome; Higaki et al., 2003; Hill et al., 2009; Lukiw et al., 2010); (vii) Henipavirus (Hendra) virus (HeV; *Paramyxoviridae*; (−)ssRNA genome; Stewart et al., 2013); (viii) human influenza A viruses (H1N1/H3N2; *Orthomyxoviridae*; (+)ssRNA genome; Chen et al., 2012; Terrier et al., 2013); (ix) hepatitis B virus (HBV; *Hepadnaviridae*; dsDNA genome; Liu et al., 2009); (x) human immunodeficiency virus (HIV; *Retroviridae*; (+)ssRNA genome; Duskova et al., 2013; (xi) human T-cell leukemia (lymphotropic) virus type 1 (HTLV-1; *Retroviridae*; (+)ssRNA genome; Pichler et al., 2008); and (xii) Japanese encephalitis virus (JEV; *Flaviviridae;* (+)ssRNA genome; Pareek et al., 2014). Note that (i) this viral-miRNA-146a-induction/association are all relatively recent discoveries with more than three quarters identified within the last 22 months; (ii) all of the most recent viral-host miRNA-nucleoplasmic signaling studies indicate the up-regulation of miRNA-146a; and (iii) viral infection involving *each virus mentioned above* is associated with progressive neuropathological change. Interestingly (i) miRNA-146a up-regulation has been associated with common age-related, human inflammatory degenerations such as sporadic Alzheimer's disease (AD), and the rare sporadic prion diseases Creutzfeldt-Jakob disease (sCJD) and Gerstmann-Straussler-Scheinker (GSS) syndrome; and (ii) the etiopathogenesis of AD has recently been associated with multiple viral infections, and most recently with latent HCV, HIV-1 or HSV-1 reactivation (Hill et al., 2009, 2014; Lukiw et al., 2011; Ball et al., 2013; Alexandrov et al., 2014).

Under suitable physiological conditions, often within minutes after viral infection, signaling via the pre-existing, heterodimeric transcription factor NF-kB is accomplished by complex, highly interdependent, viral-mediated regulatory mechanisms. These involve protein-protein interactions, phosphorylation, ADP-ribosylation, nucleocytoplasmic-trans-location, ubiquitination and proteolytic-degradation (Vallabhapurapu and Karin, 2009; Cui et al., 2010; Lee and Covert, 2010; Yarbrough et al., 2014; Di Girolamo, 2015). The most ubiquitous NF-κB members in non-stimulated cell cytoplasm are the p50 and p65 (RelA) subunits forming the heterotypic p50/p65 NF-kB dimer complexed with members of the IκB-inhibitor family (which prevents nuclear translocation; Zanella et al., 2013; Di Girolamo, 2015). Typically, after viral-mediated phosphorylation of IκBα at specific serine residues, IκBα dissociates from the p50/p65 dimer, is ubiquitinated and degraded by the proteasome, allowing the majority of NF-κB complexes to translocate through the nuclear pore complex (NPC; typically 10,000 nuclear pores/neuron; Threadgold, 1976). Here NF-kB subsequently recognizes genomic NF-kB binding sites in target gene regulatory regions, to transiently activate RNA Pol II-mediated transcription (Vallabhapurapu and Karin, 2009; Cui et al., 2010; Zanella et al., 2013; Di Girolamo, 2015). The miRNA-146a gene for example may be up-regulated 10-fold or more within minutes of viral infection; importantly NF-κB activation is usually terminated via IκB protein re-synthesis and NF-kB-re-inhibition (Schmid and Birbach, 2008; Hill et al., 2009, 2014; Cui et al., 2010; Lukiw et al., 2010). Gel shift assays and live-cell fluorescence microscopy indicate that NF-κB activation may exhibit oscillatory patterns, with levels of nuclear NF-κB alternately increasing-and-decreasing; this suggests the intriguing possibility that NF-kB-based signaling might exploit the timing of protein-modification and nucleocytoplasmic shuttling to regulate gene expression (Spiller et al., 2010; Kodaman et al., 2014). Oscillatory variation in miRNA-146a abundance is not well understood, indeed viral-mediated phosphorylation of IkB and NF-kB activation and nucleocytoplasmic trafficking is complicated as different viruses may recruit different viral or host proteins to target different signaling components of the NF-kB pathway using multiple strategies. For example polyubiquitination of the (+)ssRNA HTVL-1 virus encoded Tax protein activates IkB kinase resulting in NF-kB activation and nucleocytoplasmic translocation, while the dsDNA EBV-encoded latent membrane protein 1 (LMP1) not only activates IkB kinase to induce nucleocytoplasmic trafficking of NF-kB but also appears to be involved in additional mechanisms including LMP1-mediated interaction with nuclear proteins (Currer et al., 2012; Ersing et al., 2013). Interestingly, many neurotrophic viruses inhibit nucleocytoplasmic trafficking of host mRNAs to promote cytoplasmic viral replication and disrupt expression of anti-viral factors by the host (Yarbrough et al., 2014). What is remarkable is that despite a tremendous variation in their biophysical and genomic structure, nucleic acid type, size and life-cycle, in humans all miRNA-146a-inducing neurotrophic viruses appear to share the common capabilities: (i) to target NF-kB-mediated gene expression; (ii) to induce complex nuclear and/or nucleocytoplasmic signaling that processes miRNA-146a precursors to export mature miRNA-146a back into the cytoplasm; and (iii) to drive a miRNA-146a-mediated arachidonic acid signaling cascade with subsequent pro-inflammatory and pathogenic consequences (Hill et al., 2009; Lukiw et al., 2010; Alexandrov et al., 2014; Yarbrough et al., 2014).

Out of about 24,000 miRNAs so far identified in all species, only about 300 are encoded by viruses (miRBase v.20; Liu, 2014). There is evidence that viral-encoded miRNAs regulate the expression of their own genes or the host's genes, or both (Liu, 2014; Yao and Nair, 2014). dsDNA viruses encode most of the viral-encoded miRNAs, with members of the family *Herpesviridae* accounting for the vast majority, indicating the significance of viral miRNA-mediated gene regulation in the biology of HSV infection (see above; Yao and Nair, 2014). In general

DNA viruses that contain miRNA encoded in their viral DNA require access to the RNA polymerase II and miRNA processing machinery located within the nucleus in order to express that miRNA. In contrast, RNA viruses can replicate in the cytoplasm and, therefore, rarely encode miRNA (Liu, 2014; Swaminathan et al., 2014; Yao and Nair, 2014). There are, however, notable exceptions - for example infection with Ebola virus [EBOV; *Filoviridae*; (−)ssRNA genome] that causes a highly lethal hemorrhagic fever syndrome in humans rapidly induces 3 EBOV genome-derived miRNAs that subsequently target host mRNA (Liang et al., 2014). Indeed, perhaps as part of complex survival and immune-evasion strategies, neurotrophic viruses may modulate host miRNA precursor processing to favor viral miRNA production, thus contributing to viral-disease pathogenesis via multiple and highly interactive mechanisms (Conrad and Niepmann, 2014; Liu, 2014; Yao and Nair, 2014; Yarbrough et al., 2014).

In summary, viruses have evolved multiple and complex strategies to subvert and evade the host immune-response to ensure their own replication and survival (Hill et al., 2014; Kodaman et al., 2014; Yarbrough et al., 2014). While there is still debate as to whether up-regulated miRNA-146a is beneficial to the infecting virus or a protective host innate-immune response, at least 7 recent observations suggest that a virally-induced NF-kB-mediated up-regulation of miRNA-146a is significantly pathogenic and disruptive to homeostatic CNS function: (i) the antiviral acycloguanosine acyclovir prevents an HSV-1-induced miRNA-146a-activated pro-inflammatory cell-death program in human CNS cells via reduction in miRNA-146a abundance (Lukiw et al., 2010); (ii) up-regulated miRNA-146a has been shown to significantly down-regulate expression of complement factor-H to induce a progressive and lethal pro-inflammatory degeneration in stressed human primary brain cells (Cui et al., 2010; Alexandrov et al., 2014); (iii) both viral and cytokine (IL-1β, TNFα) induced up-regulation of miRNA-146a triggers a chronic human retinal-degeneration (Kutty et al., 2013; Alexandrov et al., 2014; Hill et al., 2014); (iv) a progressive up-regulation of miRNA-146a accompanies pro-inflammatory neuropathology in lethal human CNS disorders including sporadic AD and the human-prion diseases GSS and sCJD (Lukiw et al., 2011; Saba et al., 2012); (v) a progressive up-regulation of miRNA-146a accompanies AD-type neuropathology in several transgenic animal models of AD (including Tg2576 and 5xFAD; Alexandrov et al., 2011, 2014; Li et al., 2011); (vi) quenching of miRNA-146a using anti-miRNA-146a strategies restores homeostatic immune signaling in CHIKV-infected human fibroblasts (Selvamani et al., 2014); and (vii) inhibition of EV71-induced miRNA-146a-upregulation employing anti-miRNA-146a strategies has been observed to inhibit viral propagation and improve survival rates in mouse models (Ho et al., 2014). It is our opinion: (i) that NF-kB inhibition may not be an effective therapeutic strategy for neurotrophic viral infections because NF-kB is a ubiquitous transcription factor with large potential for off-target effects; and (ii) that virally-induced miRNA-146a excess could be effectively neutralized using perfectly complementary locked nucleic acid-stabilized anti-miRNA-146a oligonucleotides, and thereby act as an anti-viral agent for a wide variety of DNA- and RNA-virus-induced disease (Lukiw, 2013; Maguire et al., 2014). Indeed, a major advancement in antiviral therapy might involve a broad-spectrum, anti-miRNA-146a strategy which, perhaps in combination with antivirals such as acyclovir and/or the recently described gene editing methods using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/caspase 9)-mediated or other gene therapy technologies (Doudna and Charpentier, 2014; Maguire et al., 2014; Hochstrasser and Doudna, 2015). We envision these to have considerable therapeutic potential in the future clinical management of viral infections where miRNA-146a up-regulation appears to play a pathogenic role.

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REVIEW
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Mechanisms Underlying T Cell Immunosenescence: Aging and Cytomegalovirus Infection

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The ability of the human immune system to protect against infectious disease declines with age and efficacy of vaccination reduces significantly in the elderly. Aging of the immune system, also termed as immunosenescence, involves many changes in human T cell immunity that is characterized by a loss in naïve T cell population and an increase in highly differentiated CD28- memory T cell subset. There is extensive data showing that latent persistent human cytomegalovirus (HCMV) infection is also associated with age-related immune dysfunction in the T cells, which might enhance immunosenescence. Understanding the molecular mechanisms underlying age-related and HCMV-related immunosenescence is critical for the development of effective age-targeted vaccines and immunotherapies. In this review, we will address the role of both aging and HCMV infection that contribute to the T cell senescence and discuss the potential molecular mechanisms in aged T cells.

Keywords: immunosenescence, naive and memory T cells, aging, HCMV infection, epigenetic regulation, vaccination

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THE AGING IMMUNE SYSTEM

The human immune system must fight diverse pathogens and provide sufficient host protection throughout life. Memory T cells, which differentiate from naïve T cells upon primary antigenic stimulation and enable a rapid and robust response to previously encountered pathogens, are key players in adaptive immunity. The generation and maintenance of pathogen-specific memory T cells is crucial for life-long immune protection and effective vaccination (Farber et al., 2014). However, profound changes occur in the human immune system over time, known as immunosenescence. These age-related changes contribute to decreased immune protection against infections and diminished responses to vaccination in the elderly. Changes in T cell immunity appear to be have the most impact (Miller, 1996; Cambier, 2005).

Although T cell numbers remain more or less constant over the human lifespan, pronounced age-associated changes occur in T cell composition (naïve vs. memory T cell subsets). It is well accepted that the functional naïve T cell output decreases after puberty due to thymic involution, resulting in increased homeostatic proliferation of existing naïve T cells and eventually phenotypic conversion of naïve T cells into virtual memory cells (Nikolich-Žugich, 2008, 2014; Goronzy et al., 2015; Jacomet et al., 2015). In contrast to the shrinking naïve compartment and its impaired ability to activate and differentiate with age, the proportion of memory T cells increases during early life, remains stable throughout adulthood, but starts to show senescent changes after about 65 years (Farber et al., 2014). In humans, circulating memory T cells can be subdivided into two major phenotypically and functionally distinct populations: central memory T cells

(T_{CM}; $CD45RA^-CCR7^+CD62L^+$), which are largely confined to secondary lymphoid tissues, and effector memory T cells (T_{EM}; $CD45RA^-CCR7^-CD62L^-$), which can traffic to multiple peripheral compartments (Sallusto et al., 1999; Mueller et al., 2013; Farber et al., 2014). T_{CM} cells are enriched for $CD4^+$ T cells, while T_{EM} cells are predominantly $CD8^+$ T cells in human blood (Moro-García et al., 2013).

One of the most prominent T cell changes to occur with age is the loss of the co-stimulatory molecule CD28 and the progressive accumulation of highly differentiated $CD28^-$ T_{EM} cells ($CD45RA^+CD28^-CCR7^-CD62L^-$), mainly in the $CD8^+$ T cell population (Koch et al., 2008). These cells are characterized by decreased proliferative capacity, shortened telomeres, a reduced TCR repertoire, and enhanced cytotoxic activity. As CD28 is crucial for complete T cell activation, CD28 loss is associated with increased susceptibility to infections and a weakened immune response to vaccination in older people (Saurwein-Teissl et al., 2002; Almanzar et al., 2005; Sansoni et al., 2008; Moro-García et al., 2013). However, $CD28^-$ T cells are not anergic, so they might also play a role in tissue-mediated immunity ($CD8^+CD28^-$ T cells) (Flavell et al., 2013) and cytomegalovirus (CMV) infection control ($CD4^+CD28^-$ T cells) (Moro-García et al., 2013). Further studies to explore the generation and maintenance of $CD28^-$ T cells, especially in different disease states, will help establish their immune function and enhance our understanding of human T cell aging.

It is thought that the memory T cells generated in youth are well preserved and remain strongly protective over decades (Hammarlund et al., 2003, 2005), while T cell memory responses first derived in old age are severely impaired (Haynes et al., 2003; Weinberger et al., 2008; Nikolich-Žugich and Rudd, 2010; Valkenburg et al., 2012). Therefore, age-targeted vaccines and immunotherapies are required.

The ability to generate protective immune responses largely depends on the generation and maintenance of a diverse and well-balanced T cell repertoire. Several studies have shown contraction in T cell diversity corresponding to a shrinkage in the naïve T cell compartment in elderly individuals due to thymic involution (Naylor et al., 2005; Britanova et al., 2014). However, these studies do not take the dramatic influence of latent persistent infection into account, particularly CMV infection, which is known to be associated with age-related alterations in the T cell pool and function. Recent evidence suggests that homeostatic proliferation maintains the naïve $CD4^+$ T cell compartment and its diverse repertoire, but not naïve $CD8^+$ T cells, in CMV-negative individuals. A decline in naïve $CD4^+$ T cell subsets occurs in the presence of CMV, but there is no depletion of naïve $CD8^+$ T cells (Wertheimer et al., 2014). In principle, thymic involution should have an equal impact on both $CD4^+$ and $CD8^+$ T cells. Therefore, the differences seen between the two subsets suggest that shrinkage of the naïve $CD8^+$ T cell pool is more likely to be due to increased development of virtual memory T cells (which are well characterized in murine models Sprent and Surh, 2011; Renkema et al., 2014) than the defective regeneration ability of an aged thymus. Moreover, unprimed "innate/memory-like" $CD8^+$ T cells have recently been identified in humans (Jacomet et al., 2015). Taken together, these data imply that thymic involution might be less important for maintaining T cell diversity than previously thought. Characterization of the epigenetic signatures and transcriptional profiles of these virtual memory T cells will help explain how antigen-inexperienced T cells acquire the memory phenotype and how they contribute to the aging immune system.

Given that polyfunctional T cells confer a more effective immune response to infections (Yamamoto et al., 2009; Boyd et al., 2015; Snyder et al., 2016), it is important to investigate the impact of aging on T cell polyfunctionality. Van Epps et al. demonstrated enhanced polyfunctionality in $CD8^+$ T_{EM} cell subsets in older individuals, but both $CD4^+$ and $CD8^+$ T_{CM} cells exhibited an age-associated decline in polyfunctionality (Van Epps et al., 2014). However, the CMV-seropositive reaction was again not examined, and the possibility that increased polyfunctional $CD8^+$ T_{EM} cells may be due to repeated antigenic stimulation could not, therefore, be excluded.

Despite intensive studies of T cells providing some insights into immune system aging, they have a number of limitations that need to be taken into consideration in future investigations. First, most of our current knowledge on T cell aging is based on studies of circulating peripheral blood T cells, which only represent 2% of the total T cell pool (Farber et al., 2014). Circulating memory T cells predominantly reside in tissues other than the blood (Mueller et al., 2013). In addition, a non-circulating subset of memory T cells has recently been identified and designated tissue-resident memory T cells (T_{RM}; $CD45RA^-CCR7^-CD62L^-CD69^+$), which permanently reside in peripheral tissues and enhance local immunity during infections (Mueller et al., 2013; Farber et al., 2014; Clark, 2015). However, age-related changes in naïve and memory T cells in distinct human organs have only rarely been studied (Lazuardi et al., 2005; Herndler-Brandstetter et al., 2012; Sathaliyawala et al., 2013). Second, most studies are limited to the total $CD4^+$ T cell pool with only a few studies conducted in Th1 and Th2 $CD4^+$ T cell subsets, and even less is known about the impact of aging on other lineages, such as Th17 cells (Huang et al., 2008; Tesar et al., 2009; Lee et al., 2011; Lim et al., 2014), regulatory T cells (Tregs) (Fessler et al., 2013; Garg et al., 2014), and Tfh cells (Winkler and Waisman, 2014; Zhou et al., 2014). Finally, human memory T cells are generated and maintained in the context of exposure to diverse viral infections throughout life, particularly CMV infection (over 90% of young people in developing countries) (Arens et al., 2015). It is well-known that CMV plays an important role in human memory T cell function with aging. Therefore, distinguishing CMV seropositive individuals from others is important to provide a more accurate understanding of age-related memory T cell immunity. The enormous impact of CMV infection on T cell function with aging is further discussed below.

MOLECULAR MECHANISMS OF T CELL SENESCENCE

Although the impact of aging on T cells is relatively well described at the cellular level, the molecular mechanisms of

aging are rather less well understood. There is growing evidence that altered transcription (Chen et al., 2013) and epigenetic regulation (Ponnappan and Ponnappan, 2011) are involved in T cell senescence.

A major feature of immunological memory is the ability of T cells to remember their previous transcriptional profile and propagate their gene expression pattern to progeny, termed "adaptive transcriptional memory" (Turner, 2003; Kundu and Peterson, 2009). In transcriptional or epigenetic memory, genes are marked with active epigenetic signatures that are maintained throughout multiple cell divisions. These marks facilitate more efficient and robust transcription upon encountering a secondary stimulus (Zediak et al., 2011).

Chen et al. provided an excellent overview of the impact of aging on genome-wide transcriptional profiles in $CD4^+$ and $CD8^+$ T cells (Chen et al., 2013). For example, $CD8^+$ T cells (Fann et al., 2005), and to a lesser degree $CD4^+$ T cells (Czesnikiewicz-Guzik et al., 2008), decrease CD28 gene expression with age, with $CD28^+$ and $CD28^-$ $CD8^+$ memory T cells showing different transcriptional profiles. There was elevated expression of several transcription factors including T-bet, eomesodermin (EMOES), and MYC (Fann et al., 2005) in $CD28^-$ memory T cells. T-bet and EMOES play crucial roles in the acquisition of T cell effector functions and memory development in $CD8^+$ T cells and regulate IFN-γ, granzyme B, and perforin expression (Angelosanto and Wherry, 2010), suggesting that they might critically mediate the enhanced cytotoxicity of $CD28^-CD8^+$ memory T cells. In addition, mammalian target of rapamycin (mTOR) has been shown to modulate memory $CD8^+$ T cell formation by regulating T-bet and EMOES gene expression. mTOR inhibition promotes T cell memory by repressing T-bet and inducing EOMES (Rao et al., 2010). Remarkably, the mTOR inhibitor RAD001 has recently been reported to improve immune responses to influenza vaccination in elderly volunteers, but the underlying mechanism remains obscure (Mannick et al., 2014). Two possible mechanisms might explain this observation. First, T-bet expression is higher in terminally differentiated T_{EM} cells (Joshi et al., 2011), while EOMES is highly expressed in persistent T_{CM} cells (Banerjee et al., 2010). Therefore, RAD001 might restrain the conversion of memory T cells to a highly differentiated phenotype, such as $CD28^-$ T_{EM} cells, by reducing T-bet expression in the elderly. As a consequence, T cell senescence is suppressed and the immunological function of memory T cells enhanced. Second, T-bet is also the master regulator of Th1 cell differentiation but is inhibitory in Th2 and Tfh cell lineage development (Lazarevic et al., 2013). Zang et al. found a Th1 $CD4^+$ subset shift and reduced Th2/Tfh cell differentiation in aged mice (Zhang et al., 2014). Hence, the decrease in T-bet caused by RAD001 might enhance Th2 and/or Tfh $CD4^+$ cell development, which is crucial for B cell immunity and antibody production. This latter hypothesis is further supported by clinical observations of an increased antibody response in elderly volunteers receiving the influenza vaccination (Mannick et al., 2014). Further studies of cellular and molecular changes occurring in these different subsets are needed.

Numerous studies have shown that epigenetic mechanisms including DNA methylation, histone modifications, chromatin structure alterations, and microRNA regulation play an essential role in transcriptional memory by regulating gene expression in memory T cells (Gibney and Nolan, 2010). It is well-known that genome-wide decreases in the methylation of repetitive elements (particularly Alu sequences) and gene coding regions occur during aging (Bollati et al., 2009; Heyn et al., 2012; Salpea et al., 2012). These age-dependent DNA methylation changes also occur in immune-specific genes that contribute to T cell senescence (Shanley et al., 2009; Fernández-Morera et al., 2010; Hongdong et al., 2015; Tserel et al., 2015).

Although the age-associated DNA methylation signatures that mark immune responsive genes are well documented, little is known about the contribution of site-specific histone modifications to immunosenescence. Histone acetylation, methylation, phosphorylation, and ubiquitination all regulate transcription by altering chromatin accessibility (Strahl and Allis, 2000; Kouzarides, 2007). Early epigenetic studies in rat livers showed significant decreases in histone acetylation (H3K9ac) and H3 phosphorylation (H3S10ph) contributing to age-associated decreases in transcription (Kawakami et al., 2008). More recently, Sidler et al. demonstrated profound age-dependent changes in gene expression accompanied by decreases in DNA methylation, H3K9me3, and H4K16ac in the rat spleen and thymus, suggesting increased chromatin instability with age. Moreover, several transcription factors (BCL6, MYC, TCF7, and ETS1) with decreased expression in the thymus and increased expression in the spleen are thought to be associated with immunosenescence (Sidler et al., 2013). Since these transcription factors play crucial roles in homeostatic proliferation, differentiation, and activation in T lymphocytes (Muthusamy et al., 1995; Angelosanto and Wherry, 2010), their opposing transcriptional profiles in the thymus and spleen might contribute to the distinct senescence phenotype observed during aging, i.e., the reduced thymic naïve T cell output and increased homeostatic proliferation of peripheral T cells. Chromatin immunoprecipitation (ChIP) studies to identify genome-wide epigenetic signatures with characterization of chromatin accessibility in these regions might provide insights into the epigenetic regulation of immunosenescence.

Several recent studies have explored microRNA regulation in T cell aging. For example, miR-92a expression significantly declined in $CD8^+$ T cells during aging, which also correlated with reduced naïve $CD8^+$ T cell numbers. It has also been suggested that the progressive decline in miR-92a expression may be associated with a reduced naïve T cell repertoire due to repeated stimulus exposure (Ohyashiki et al., 2011). Moreover, an age-dependent decline in miR-181a, an intrinsic regulator of TCR signaling (Li et al., 2007), was observed in aged $CD4^+$ T cells (Li et al., 2012). miR-181a loss increased DUSP6 activity, which eventually impaired TCR sensitivity in elderly $CD4^+$ T cells (Li et al., 2012). Compared to $CD8^+CD28^+$ T cells, highly differentiated $CD8^+CD28^-$ T cells overexpressed miR-24, which was associated with downregulation of the histone variant H2AX and consequent impairment of the response to DNA damage and increased susceptibility to apoptosis in $CD8^+CD28^-$ T cells

(Brunner et al., 2012). Taken together, these studies suggest that microRNAs are causative in immunosenescence and might be useful biomarkers of T cell aging. However, a systematic survey of microRNAs, their direct targets, and function in immunosenescence are needed. Comprehensive studies in well-defined human T cell subsets in both health and disease will enhance our understanding of the molecular mechanisms responsible for age-related T cell impairment so that effective interventional strategies against age-associated diseases can be developed.

Apart from genetic/epigenetic inheritance, lifestyle and environmental factors, such as nutritional status and metabolism also have a significant impact on the human immune system and lifespan (Fuente, 2014). There is good evidence that diets that contain particular nutrient or non-nutrient components can significantly enhance immune function in the elderly (Lesourd et al., 1998; Pae et al., 2011; Maijó et al., 2014). For example, dietary manipulation of the availability of methyl donors can extend and maintain immune function by epigenetically modulating methylation in mice (Miller et al., 2005). The mTOR pathway has provided a focus for understanding how metabolism regulates T cell immunity: upon growth factor stimulation, the PI3K/Akt/mTOR pathway is activated that, in turn, promotes nutrient uptake, enhances many metabolic activities, and results protein biosynthesis in T cells (Jones and Thompson, 2007). The mTOR inhibitor rapamycin promotes $CD8^+$ T cell memory development (Araki et al., 2009), which might be due to metabolic changes in $CD8^+$ T cells (Peng et al., 2002; Sipula et al., 2006; Brown et al., 2007). Moreover, mTOR plays a crucial role in the regulation of effector and regulatory T cell lineage commitment (Delgoffe et al., 2009), since it not only acts as a regulator of translation but also functions as an intracellular energy sensor that positively and directly regulates mitochondrial respiration (Desai et al., 2002; Ramanathan and Schreiber, 2009). A study of energy metabolism has shown that highly differentiated $CD8^+CD28^-$ T cells utilize different metabolic strategies than normal $CD8^+$ memory T cells (Henson et al., 2014); in contrast to the quiescent catabolic metabolism in $CD8^+CD28^+$ memory T cells, $CD8^+CD28^-$ T cells have lower mitochondrial mass and respiration, high basal glycolysis levels, and impaired metabolism due to increased ROS levels (Henson et al., 2014). Further, $CD8^+CD28^-$ T cells favor the use of the cytosolic glycolytic pathway over mitochondrial respiration for energy production. Aerobic glycolysis is a critical metabolic pathway required for the production of the effector cytokine IFN-γ, consistent with the enhanced cytotoxic activity of $CD8^+CD28^-$ T cells (Chang et al., 2013; Gubser et al., 2013). As mitochondrial function is compromised during aging (Ron-harel et al., 2015), a switch to energy generation by glycolysis may produce a population of $CD8^+CD28^-$ T cells with the metabolic advantage to outcompete other T cell subsets competing for nutrients and factors required for survival and function. These data highlight the important role played by nutrient status and metabolic regulation in enhancing T cell immunity and might be a promising approach to promote healthy aging through nutritional and metabolic interventions.

HUMAN CMV INFECTION, MEMORY INFLATION, AND IMMUNOSENESCENCE

Cytomegalovirus (CMV) is an ubiquitous β-herpesvirus with a double-stranded DNA genome that has co-evolved with humans over millions of years (Sinclair and Sissons, 2006; Gibson, 2008). Human CMV (HCMV) is a prevalent human pathogen, infecting 40–100% of world's population. CMV has the capacity to induce both lytic and latent infections to establish lifelong persistence in human hosts following primary infection (Cannon, 2009; Ludwig and Hengel, 2009; Cannon et al., 2010). In lytic infection, HCMV undergoes temporally active replication that can be divided into immediate early (IE), early (E), and late (L) phases. The most crucial viral lytic gene products are viral IE genes, which control subsequent viral gene expression and virus replication. Thus, these major IE gene products essentially determine the HCMV infection states: latency (IE genes suppressed) or reactivation (IE genes expressed) (Sinclair and Sissons, 2006; Paulus and Nevels, 2009). The latent phase of HCMV infection is characterized by viral quiescence, in which the viral genome is maintained as an extra-chromosomal plasmid in the absence of detectable production of infectious virions but able to reactivate to specific stimuli (Goodrum et al., 2002; Reeves et al., 2005; Sinclair and Sissons, 2006). Despite infecting a broad range of host cells, HCMV usually establishes viral latency at specific cellular sites, predominantly $CD34^+$ progenitors and myeloid lineage cells (Hahn et al., 1998; Goodrum et al., 2002).

Primary HCMV infection elicits extensive innate and adaptive immune responses. Thus, to escape host antiviral responses, HCMV has developed diverse immune evasion strategies to alter host immune recognition during both lytic and latent infection. In particular, HCMV restricts major histocompatibility complex (MHC) class I and II antigen presentation, which allows the virus to survive, disseminate, and persist in infected individuals (Noriega et al., 2012). In immunocompetent individuals, both primary and lifelong persistent HCMV infections generally remain subclinical and well controlled by the host immune system. T cell responses are particularly important for controlling viral latency in infected individuals (Hanley and Bollard, 2014; Klenerman and Oxenius, 2016). During the early phases of primary infection, the initial T cell response to HCMV is dominated by circulating HCMV-specific $CD4^+$ T cells that produce the Th1 cytokines IFN-γ and TNF-α (Rentenaar et al., 2000; Gamadia et al., 2003). Highly cytotoxic HCMV-specific $CD8^+$ T cells can be detected in the blood several days after the initial $CD4^+$ T cell response, and they are maintained with an effector phenotype during the latent phase to prevent HCMV reactivation and to protect the host from re-infection (Polić et al., 1998; Kuijpers et al., 2003; Mackus et al., 2003). In addition, the humoral response to HCMV also contributes to controlling the viral load and preventing primary infection in humans (Gerna et al., 2008; Genini et al., 2011; Jackson et al., 2011; Alonso Arias et al., 2013). After primary infection, B cells are activated by antigens with the help of HCMV-specific $CD4^+$ T cells, mainly Th2 cells, leading to the production of antibodies specific for a number of HCMV proteins (Wang and Shenk, 2005; Gerna et al.,

2008; Macagno et al., 2010; Genini et al., 2011). These HCMV-specific antibodies can block viral dissemination and control the infection by neutralizing extracellular virions, a mechanism that is particularly important for protecting the fetus from congenital HCMV infection (Fowler et al., 1992; Revello and Gerna, 2002; Schleiss, 2013).

However, long-term HCMV persistence has a profound impact on the immune system's composition and function, even in healthy HCMV-infected individuals, especially with respect to $CD8^+$ T cells. One hallmark of latent HCMV infection is the progressive and substantial expansion of HCMV-specific memory $CD8^+$ T cells over time, with HCMV-specific memory $CD4^+$ T cells accumulating to a lesser extent (Klenerman and Oxenius, 2016; Weltevrede et al., 2016). This accumulation of HCMV-specific memory T cells during viral persistence is termed "memory inflation," first defined in the mouse CMV (MCMV) infection model (Karrer et al., 2003). HCMV-specific memory T cells tend to gradually increase in number with age: in HCMV-infected elderly individuals, the $CD8^+$ T cell response to HCMV antigens occupies nearly 50% of the entire memory $CD8^+$ T cell compartment in peripheral blood, while approximately 30% of total circulating $CD4^+$ T cells can be HCMV responsive (Sylwester et al., 2005; Pourgheysari et al., 2007; Li et al., 2014).

Human cytomegalovirus (HCMV) persistence is thought to be a driver of immunosenescence in humans (Koch et al., 2007). The majority of HCMV-specific inflationary T cells are T_{EM} cells with the typical age-related senescent T cell phenotype. These terminally differentiated HCMV-specific T cells generally acquire CD57 and CD45RA expression but lack CD28 and CCR7 expression ($CD45RA^+CD57^+CD28^-CCR7^-$) (Gamadia et al., 2001; Appay et al., 2002; Kuijpers et al., 2003). It is widely accepted that late-stage differentiated $CD28^-$ T cells are a major characteristic of T cell aging, suggesting that persistent HCMV infection is associated with immunosenescence. This is further supported by the fact that the large population of HCMV-specific $CD8^+CD28^-$ T_{EM} cells that usually accumulate during HCMV persistence are absent in HCMV-seronegative elderly individuals, even those infected with other persistent herpes viruses (Chidrawar et al., 2009; Derhovanessian et al., 2010). Several studies have shown that acute viral infections generate polyfunctional memory $CD8^+$ T cells with re-expressed CD45RA (Precopio et al., 2007; Miller et al., 2008; Akondy et al., 2009). Live yellow fever vaccine (YF-17D)-specific memory $CD8^+$ T cells exhibited a terminally differentiated $CD45RA^+CCR7^-$ phenotype but remained polyfunctional with robust proliferative potential 5–10 years post vaccination, which may suggest that re-expressed CD45RA biomarks highly functional memory T cells after acute viral infections rather than senescent T cells (Akondy et al., 2009). However, these YF-17D-specific memory cells were not "true" late-stage differentiated $CD28^-$ T cell subsets but $CD27^+CD28^+$ cells. Therefore, the use of CD45RA alone as a marker of T cell differentiation and function is questionable. In fact, the $CD45RA^+CCR7^-CD8^+$ subtype represented resting memory T cells that could be re-activated upon antigenic stimulation. It has also been shown that CD45RA re-expression on memory T cells can occur in the absence of further antigenic stimulation, which is indicative of the time elapsed since previous viral infection rather than the functional potential of $CD8^+$ T cells (Carrasco et al., 2006). In addition, $CD45RA^+CD8^+$ memory T cells did not accumulate with age and showed no correlation with the $CD28^-CD8^+$ T_{EM} subset (Mahnke et al., 2013). Thus, CD28 is a more reliable surface marker for the phenotypic and functional definition of human T cell differentiation. In contrast to age-dependent T cell senescence, increased HCMV-specific memory $CD8^+$ profiles (up to 10% of the total $CD8^+$ T cell memory pool) were observed even in young hosts, suggesting that progressive clonal expansion may lead to immunosenescence at an earlier age in HCMV-infected individuals (Khan et al., 2004; Chidrawar et al., 2009). However, the high frequency of HCMV-responsive T cells may be a "necessary evil" to restrain HCMV re-activation and maintain control of latent HCMV throughout life (Pawelec, 2005).

There is increasing evidence to suggest that memory inflation in HCMV infection is associated with impaired T cell immunity in elderly hosts. Despite the $CD8^+$ T cell repertoire being diverse enough to recognize different viral epitopes soon after primary HCMV infection, clonal diversity starts to shrink with age, with a large proportion of the repertoire limited to a few high-avidity clones with a replicative senescent phenotype (Day et al., 2007). In particular, T cell responses specific to an individual immunodominant HCMV epitope [such as 65kDa phosphoprotein (p65) and 55kDa IE protein 1 (IE1)] can comprise over 25% of the total $CD8^+$ T cell population in elderly individuals (Khan et al., 2002; Sylwester et al., 2005). Thus, the excess expansion of a single HCMV-specific repertoire in memory inflation may compromise immune protection in response to novel and vaccine antigens by decreasing TCR diversity in the elderly. This renders individuals with only limited virus-specific T cell clones at risk of life-threatening diseases as they get older (Klenerman and Oxenius, 2016). Additionally, memory inflation in latent HCMV infection may also impact the balance between Th1 and Th2 cytokine production by $CD4^+$ T cells in aged individuals, shifting it in favor of Th1 responses (Saurwein-Teissl et al., 2002; Pawelec et al., 2005). Most inflationary memory $CD4^+$ T cells are IFN γ-producing Th1 cells, which might explain the poor antibody-mediated immune responses seen in the elderly (Rentenaar et al., 2000; Bitmansour et al., 2002; van Leeuwen et al., 2006). Indeed, HCMV-specific $CD4^+$ T cells were negatively associated with humoral responses to influenza vaccination (Derhovanessian et al., 2013). However, the precise mechanism underlying memory inflation is not fully understood, the current hypothesis being that continuous repetitive antigen exposure during latent HCMV infection contributes to immune system reshaping and enhances the age-related changes in T cell compartments in older adults (Pawelec et al., 2009; O'Hara et al., 2012).

Decreased levels of naïve T cells are also a hallmark of immunosenescence. Several cross-sectional studies have indicated that HCMV has much less of an impact on the naïve T cell pool than on memory T cells (Mekker et al., 2012; Wertheimer et al., 2014), but HCMV status is significantly associated with changes in naïve $CD4^+$ T cells (Wertheimer et al., 2014). Lower levels of naïve $CD4^+$ T cells appear to

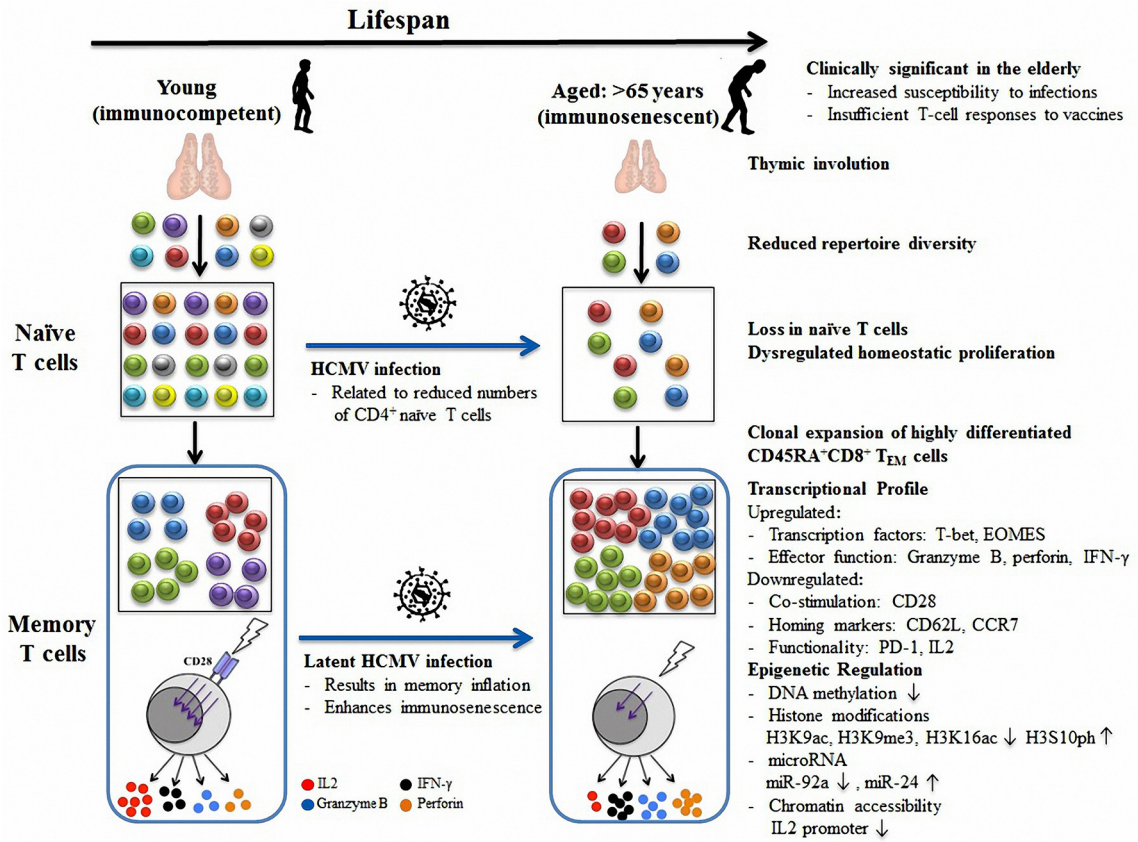

FIGURE 1 | Model of age- and HCMV-related immunosenescence. Thymic involution contributes to reduced naïve T cell export and T cell diversity with age. To compensate for inadequate thymic export, existing naïve T cells increase homeostatic turnover. Upon antigen stimulation, a large number of terminally differentiated CD45RA$^+$CD8$^+$ TEM cells clonally expand in the elderly, which dominate the memory pool and further restrict repertoire diversity. These senescent CD45RA$^+$ memory T cells typically have diminished T cell responses to stimulation in the absence of CD28 co-stimulatory signaling pathways and are characterized by a variety of altered transcriptional profiles which are epigenetically regulated (including by DNA methylation, histone modifications, microRNAs, and chromatin remodeling). In addition, HCMV infection can result in CD4$^+$ naïve pool depletion and memory inflation, which further accelerate immunosenescence in aged individuals. Together, age and HCMV infection contribute to the overall decline in immune function decline and impair the T cell response to vaccines in the elderly.

be HCMV-seropositivity dependent rather than age related, suggesting differential effects of aging and HCMV infection on T cell subsets (Wertheimer et al., 2014).

The "immune risk profile" (IRP) was developed as part of a longitudinal study to predict mortality and morbidity in the aged (Wikby et al., 2005, 2008). HCMV seropositivity was identified as one of the immune parameters of the IRP, which also included an inverted CD4/CD8 ratio, accumulation of CD8$^+$CD28$^-$ T cells, and a lower proportion of naïve cells. However, HCMV infection was detected in very old people, compatible with the extended lifespan observed in studies of individuals at extreme old age in Japan. Plasma HCMV titers were not inversely correlated with the proportion of CD28$^+$ T cells in (semi-)supercentenarians (105 years or older), suggesting that HCMV titers might not be a powerful predictor of T cell senescence in successful aging (Arai et al., 2015). Therefore, it remains controversial whether prediction of mortality can be used as a direct indicator of immunosenescence.

Even though pronounced memory inflation is thought to result in the accumulation of dysfunctional CMV-specific T cells, several studies have shown that HCMV-specific CD8$^+$ T cells are polyfunctional in young and middle-aged hosts and can produce multiple cytokines and induce strong effector immune responses to staphylococcal enterotoxin B (SEB) (Solana et al., 2012a; Pera et al., 2014; Sansoni et al., 2014; Hassouneh et al., 2016). With increasing age, T cell exhaustion, another form of T cell dysfunction, can arise during chronic viral infections, such as with HIV, in which T cells are constantly stimulated by highly replicating viruses (Wherry, 2011). Typically, exhausted T cells fail to control viral infection as they have effector defects (Wherry, 2011) including loss of proliferative potential, decreased cytotoxicity, impaired ability to secrete cytokines (Frebel et al., 2010; Wherry and Kurachi, 2015), and sustained high expression of several inhibitory receptors (e.g., PD1, KLRG1, and CD57) (Blackburn et al., 2009). Although HCMV-specific CD8$^+$ T cells also have low proliferative capacity and express senescence markers, such as KLRG1 and CD57 (Vieira Braga et al., 2015), they are not exhausted as they are still highly cytotoxic and produce Th1 cytokines in response to sporadic viral re-activation (Klenerman

and Oxenius, 2016). In addition, molecular profiling of HCMV-specific $CD8^+$ T cells has demonstrated that PD-1, an inhibitory receptor associated with T cell dysfunction, is expressed at very low levels in healthy individuals (Vieira Braga et al., 2015). Therefore, persistent HCMV infection does not induce massive exhaustion of the T cell repertoire in most immunocompetent individuals. Indeed, HCMV is rarely reported in elderly individuals, suggesting HCMV-specific T cells may be able to control pathological HCMV re-activation during healthy aging. However, the possibility remains that HCMV infection can eventually drive the functional exhaustion of T cells and their extensive accumulation may accelerate immunosenescence in immunocompromised and immunosuppressed individuals (Papagno et al., 2004; Chou and Effros, 2013; Effros, 2016).

Taken together, HCMV infection in the elderly is implicated in immunosenescence and might have a deleterious impact on host immunity and enhance the aging process. Nevertheless, there remains considerable uncertainty regarding the causative role of CMV in immunosenescence. Although it is well-known that HCMV is a common cause of severe morbidity and mortality in immunocompromised individuals (Reeves and Sinclair, 2008), we cannot exclude the possibility that HCMV might improve the polyfunctionality of $CD8^+$ T cells and consequently benefit the host immune system, at least in young healthy individuals. Moreover, it is still unclear whether HCMV re-activation occurs more frequently in the elderly than in younger individuals. Hence, whether expansion of HCMV-specific $CD8^+$ T cells over time is really deleterious in old age remains unknown. It also remains to be seen to what extent accumulated dysfunctional inflationary memory T cells cause immunosenescence and how deleterious these cells are on other immune components, such as B cells, γδ T cells, and NK cells. Finally, the host immune system might inefficiently control latent HCMV reinfection during aging, thereby allowing the large-scale expansion of virus-specific T-cell clones and further enhancing the immunosenescent profile (Klenerman and Oxenius, 2016; Weltevrede et al., 2016). Thus, both HCMV status and HCMV control must be taken into account in future studies on immune aging.

VACCINATION IN THE ELDERLY

Vaccination is the most cost-effective and efficient strategy for improving immune responses and protecting humans from infections and other emerging diseases. However, both the efficacy and effectiveness of vaccination decrease in the elderly (Jefferson et al., 2005; Chen et al., 2009; Boraschi and Italiani, 2014; Haussig et al., 2014). Given the rapidly aging population in both developed and developing countries, improving vaccination efficacy to promote healthy aging is a priority, not only for individual well-being, but also for public health.

In the aged population, infectious diseases are a major cause of morbidity and mortality, mainly because the host immune system cannot generate adequate adaptive immune responses to infections (Chen et al., 2009). As discussed above, aging and latent CMV infection are closely associated with immunosenescence by reshaping the host immune repertoire. This can lead to increased susceptibility to severe infections due to the progressive impairment of innate and adaptive immunity in the elderly. In particular, significantly decreased levels of naïve T cells with a restricted TCR repertoire and expansion of highly differentiated memory T cells that gradually polarize to a specific virus can compromise host immune responses to novel virus vaccine antigens, leading to suboptimal vaccination. In addition, impaired immune responses accompanied by repetitive antigen exposure can result in chronic inflammation in the elderly and are thought to contribute to the defective immune response to vaccination by further enhancing immunosenescence (Franceschi et al., 2000; Freund et al., 2010; Solana et al., 2012b). Therefore, a more advanced understanding of both the cellular and molecular basis of immunosenescence is required to develop efficient vaccinations that better protect elderly individuals from infections and age-related diseases.

CONCLUSIONS AND PERSPECTIVE

The adaptive immune system protects the host from numerous pathogens over the course of human life. However, aging is associated with a quantitative decline in immunity, particularly in T cells, referred to as immunosenescence. Age-related T cell senescence has been attributed to thymic involution, contraction in the T cell repertoire, and accumulation of highly differentiated $CD28^-$ T cells. There is growing evidence that latent HCMV infection might accelerate immunosenescence since it causes similar detrimental effects on T cell phenotype and function to those found in age-associated immunological defects. Thus, age-related and HCMV-related immunosenescence - either together or separately - might contribute to increased susceptibility to infectious disease and impaired immune responses to vaccination in the elderly (**Figure 1**). To prevent severe infections and promote healthy aging, efforts have been made to improve the efficacy of vaccination in the elderly including increased vaccine doses, prime-boost immunization strategies, and the use of adjuvants (Couch et al., 2007; Brown, 2010; Khurana et al., 2011; Dorrington and Bowdish, 2013). Unfortunately, these approaches have yet to significantly improve vaccination outcomes in the older population.

There is also a considerable body of evidence to suggest that epigenetic changes play a crucial role in natural and pathological immune aging (Grolleau-Julius et al., 2009) and a variety of human diseases (Barros and Offenbacher, 2009; Hewagama and Richardson, 2009; Invernizzi, 2009; Wells, 2009). Our unpublished preliminary data suggest that the chromatin accessibility status of certain regulatory elements, in particular that of IL-2, alter over the human lifespan in naïve and memory T cells. This is further supported by findings that epigenetic regulation plays a prominent role in immune responses and age-related differential gene expression of IL-2 (Bruniquel and Schwartz, 2003) and IFN-γ (Yano et al., 2003). A sound knowledge of age-dependent epigenetic gene regulation is essential for optimizing vaccination, perhaps by therapeutically restoring immune function in the elderly. Furthermore, given the

crucial role of HCMV in host immune function during aging, an increased understanding of the impact of latent HCMV infection on epigenetic signatures across regulatory elements might provide a novel avenue for overcoming immune defects and improving vaccine efficiency in the elderly. Integrating cellular and epigenetic insights of immunology and virology during aging is essential for the development of age-targeted vaccines and age-dependent immunotherapies that exploit sustained memory responses to pathogens.

AUTHOR CONTRIBUTIONS

WT participated in drafting the article. SR helped to revise and edit the manuscript and acted as corresponding author.

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