Université de Montréal

The Role of Poly(C)-binding protein 1 in HSV-1 Infection

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Ce mémoire intitulée

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A été évaluée par un jury composé des personnes suivantes

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Résumé

Lors de l'infection par le virus herpès simplex de type 1 (VHS-1), quatre types de capsides nucléaires sont créés : les procapsides et les capsides A, B, et C. Sur les quatre capsides, seules les capsides C contiennent de l'ADN viral et deviendront des particules infectieuses. Un niveau de régulation se produit lors de la sortie du noyau qui favorise la sortie d'es capsides C du noyau. Le mécanisme qui sous-tend ce phénomène est actuellement inconnu. Les recherches actuelles suggèrent que l'interaction entre la protéine virale pUL25 modifie la conformation de la couche hexamérique plane du complexe de sortie nucléaire (NEC) pour y introduire des pentamères et donc causer un arrondissement de la membrane et le bourgeonnement des capsides. Cependant, des questions subsistent quant à la manière dont les capsides A, B et C sont différenciées au sein du noyau pour assurer une sortie spécifique de la capside C puisque pUL25 se retrouve dans tous les types de capsides. Nous étudions ici comment les protéines de l'hôte peuvent agir dans la sortie nucléaire des capsides C. En se basant sur une étude précédente du laboratoire où la protéine hôte poly(C)-binding protein 1 (PCBP1) a été trouvée spécifiquement sur les capsides C par spectrométrie de masse, nous explorons le rôle de la PCBP1 dans l'infection par le VHS-1. À l'aide d'essaies de plaques, nous montrons que la PCBP1 est importante pour l'infection virale, car en son absence, les titres diminuent et lorsque la PCBP1 est sur-exprimée, les titres augmentent. Ce résultat ne semble pas être dû au fait que les PCBP1 affectent l'expression génique de sous-ensembles de gènes viraux immédiats précoces, précoces ou tardifs, ni qu'ils affectent la réplication du génome ou son encapsidation. La réduction des PCBP1 ne provoque pas d'accumulation de capsides ou de particules matures tel qu'évalué par la microscopie électronique, mais elle augmente le nombre de capsides B enveloppées dans l'espace périnucléaire (PNS). L'inhibition de PCBP1 diminue également le niveau de protéine pUL24, une protéine virale importante pour la sortie du virus du noyau. Nos résultats démontrent que la PCBP1 pourrait réguler l'activité de pUL24, de sorte que lorsque la PCBP1 est épuisée, pUL24 permet à plus de capsides B de se rendre dans l'espace périnucléaire. Cette recherche constitue un point de départ pour une analyse plus approfondie du mécanisme exact des PCBP1 dans les infections à HSV-1. En outre, elle pourrait fournir des indices importants pour élucider comment le pUL24 favorise la sortie du nucléaire.

Mots-clés : Virus de l'herpès simplex de type 1, PCBP1, hnRNP E1, sortie nucléaire

Abstract

During herpes simplex virus type 1 (HSV-1) infection, four types of nuclear capsids are made: procapsids and A-, B- and C-capsids. Of the four capsids, only C-capsids contain the viral DNA and will become infectious progeny. A level of regulation occurs during nuclear egress that ensures only C-capsids exit the nucleus. The mechanism that underlies this phenomenon is presently unknown. Current research suggests the viral protein pUL25 alters the conformation of the viral nuclear egress complex (NEC) that forms a flat hexameric coat on nuclear membranes by the introduction of pentamers and therefore the induction of membrane rounding and viral budding. However, questions remain for how A-, B-, and C-capsids are differentiated within the nucleus to ensure C-capsid specific egress since pUL25 is found on all capsid types. Here we investigate how host proteins may play a role in nuclear egress of C-capsids. Based on the lab's previous study where host protein poly(C)-binding protein 1 (PCBP1) was found specifically on C-capsids via mass spectrometry, we explore the role of PCBP1 in HSV-1 infection. Using plaque assays we show that PCBP-1 is important for viral infection, as in its absence titers decrease and when PCBP1 is over expressed titers increase. This result does not seem to be due to PCBP1 affecting gene expression of immediate early, early, or late viral gene subsets, nor does it seem to affect genome replication or encapsidation. PCBP1 knockdown does not cause an accumulation of capsids or mature particles as assessed by electron microscopy, but it does increase the number of enveloped B-capsids observed in the perinuclear space (PNS). Depletion of PCBP1 also decreases the level of pUL24, a viral protein implicated in viral nuclear egress. Our results suggest that PCBP1 could be regulating pUL24 for proper activity in nuclear egress, such that when PCBP1 is depleted, more B-capsids are able to bud through the PNS. This research constitutes a starting point for further analysis into the exact mechanism of PCBP1 in HSV-1 infections. In addition, it may provide important clues to elucidate how pUL24 supports nuclear egress.

Keywords: Herpes simplex virus type 1, PCBP1, hnRNP E1, nuclear egress

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Abbreviations

BAT: TGF-β-activated translation

BIP: 2,2, bypyridal

CHMP: Charged multivesicular body protein

CSFV: Classical swine fever virus

CVSC: Capsid vertex specific component

Dab2: Disabled-2

DNA H/P: DNA helicase/primase

DOHH: Deoxyhydrogenase hydroxylase

dsDNA: Double-stranded DNA

E: Early

EIF4E: Eukaryotic initiation factor 4E

EM: Electron microscopy

EMT: Epithelial-mesenchymal transition

eNOS: Endothelial nitric oxide synthase

ER: Endoplasmic reticulum

ESCRT: Endosomal complexes required for transport

ESS: Exon splicing silencer

EV71: Enterovirus 71

GH: Growth hormone

GHR: Growth hormone receptor

GSK3β: Glycogen synthase kinase 3β

GUV: Giant unilamellar vesicles

HCV: Hepatitis C Virus

HEV: Hepatitis E virus

HHV-6: Human herpes virus 6

HIFα: Hypoxia inducible factor α

HIV: Human immunodeficiency virus

hnRNA: Heterogeneous nuclear RNA

hnRNP: Heterogeneous nuclear ribonucleoproteins

Hpi: hours post infection

HPV: Human papilloma virus

HR: Homologous recombination

HSPG: Heparan sulphate proteoglycan

HSV-1: Herpes simplex virus type 1

HVEM: Herpesvirus entry mediator

IE: Immediate early

IF: Immunofluorescence

IFN: Interferon

ILE2: Interleukin-like EMT inducer

INM: Inner nuclear membrane

KD: Knockdown

KH: hnRNP K-homology

KSHV: Kaposi's sarcoma-associated herpesvirus

L: Late

LAP: Lamina-associated protein

LUV: Large unilamellar vesicles

MAVS: Mitochondrial anti-viral signaling

MOR: μ-opioid receptor

MTOC: Microtubule-organizing centre

NEC: Nuclear egress complex

NHEJ: Non-homologous end joining

NLS: Nuclear localization signal

NPC: Nuclear pore complex

NT: non-targeting

OBP: Origin binding protein

OMN: Outer nuclear membrane

ORF: Open reading frame

$P21^{waf}$: WT p53 activated fragment

Pak-1: p21-activated kinase

PAMPs: Pathogen-associated molecular patterns

PBS: Phosphate-buffered saline

PCBP1: Poly (C)-binding protein 1

PHD2: Prolyl hydroxylase 2

PKC: Phospho-kinase C

PM: Plasma membrane

PNS: Perinuclear space

PNUTs: Phosphatase regulatory subunit 10

PRL-3: Phosphatase of regenerating liver

PRR: Positive regulatory region

RLR: RIG-I like receptors

RNAP II: RNA polymerase II

RNP: Ribonuclear protein

RT: Room temperature

SSA: Single strand annealing

ssDNA: Single-stranded DNA

TGN: Trans-Golgi network

UL: Unique Long

US: Uniqu Short

UTR: Untranslated region

VAMP: Vesicle-associated membrane protein

vhs: viral host shutoff

VP: Viral protein

VSV: Vesicular stomatitis virus

WT: Wild-type

XIAP: X-linked inhibitor of apoptosis

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1. Herpesvirus Biology

1.1 Introduction

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA (dsDNA) virus that belongs to the family Herpesviridae. Within this large family of viruses, nine of them cause a wide range of infections in humans from mild to severe. A key commonality between all herpesviruses is the ability to switch between lytic and latent life cycles. All herpesviruses cause active lytic infection upon first infection, then go latent, a process defined by minimal gene expression where only the genome is maintained, and no viral particles are made (1). Throughout the life of infected individuals, certain stresses can cause reactivation from latent to lytic. HSV-1 is an alphaherpesvirus, defined by the propensity to go latent in neurons (reviewed in (2)). Primary infection occurs in epithelial cells, then the virus travels through sensory neurons to become latent in ganglia (3). HSV-1 most commonly causes herpes labialis (cold sores) and genital herpes but can cause more serious diseases such as encephalitis and keratitis in rare cases (4). Lytic HSV-1 can be treated with the antiviral drug Acyclovir and other related nucleotide analogs, but there is no cure or vaccine currently available (5).

As HSV-1 can cause life-threatening disease, and the common mild manifestations of the virus can cause psychological stress, it is important to find a way to prevent the negative consequences of the virus. Using biochemical, molecular biological and classical virology techniques I explore the virus-host protein interaction of poly(C)-binding protein 1 (PCBP1) on the infection of HSV-1. These studies will hopefully define new pathways for drug action and increase our overall knowledge of HSV-1 biology.

1.2 HSV-1 Structure

HSV-1, consistent with all herpesviruses, is made up of four main parts: The DNA core, an icosahedral capsid, a protein-rich tegument, and a lipid envelope spiked with viral glycoproteins (Figure 1). This section will describe the structure of each element.

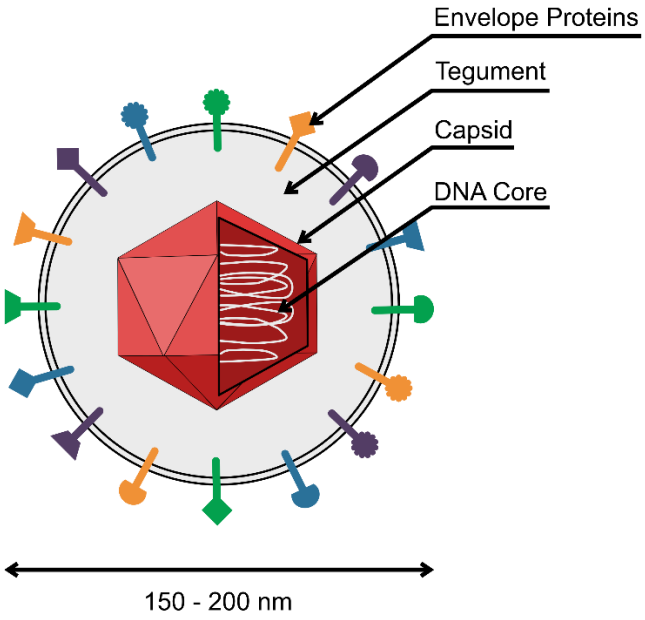

Figure 1. – The HSV-1 Virion Structure

A stylized virion showing the 4 main structural sections of HSV-1: DNA core, capsid, tegument, and envelope. Adapted with permission from Kukhanova *et al.*, 2014 (6).

1.2.1 DNA

The HSV-1 genome consists of dsDNA spooled within the icosahedral capsid (7, 8). The genome is roughly 150,000 base pairs in length, with a GC content of 68% (9). The structure of the DNA is made up of two unique regions, unique long (UL) and unique short (US), which are flanked and separated by inverted repeats (Figure 2) (10). The terminal repeats designated DR1 are direct repeats of each other and facilitate circularization of the genome once in the nucleus of a host (Figure 2) (11). HSV-1 encodes approximately 80 protein-coding open reading frames (ORFs), which can be found across the UL and US regions accommodating 65 and 15 genes, respectively (8). A list of all genes and their protein products can be found in annex 1.

1.2.2 Capsid

The mature HSV-1 capsid is 125 nm in diameter and made up of 162 capsomers consisting of 150 hexon faces and 12 penton vertices which yield an icosahedron with a triangulation number of T=16 (12). The major capsid protein VP5 makes up both the pentons and the hexons, but the hexons are decorated with a ring of VP26 in a hexamer (13). A triplex made up of one copy of VP19c and two copies of VP23 connects the capsomeres (14). One of the twelve vertices is unique

and designated as the ‘polar vertex,’ where DNA enters and exits the capsid. Most vertices bind the tegument proteins pUL17 and pUL25, also called the capsid-vertex specific component (CVSC), and the polar vertex is no different. However, the polar vertex is unique in that 12 copies of pUL6 protein arrange to form a ring around the vertex, and pUL25 is in different conformation compared to other vertices (7, 15, 16). Mature capsids also contain VP24 which is one of three HSV-1 scaffold proteins, but unlike VP21 and VP22a which are expelled upon DNA packaging, VP24 stays within the capsid (17).

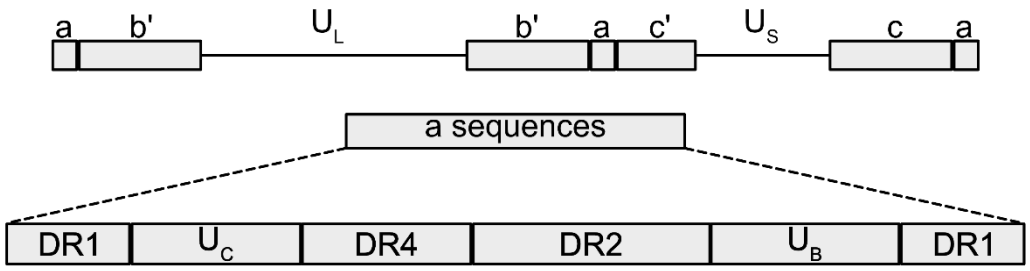

Figure 2. – Structure of the HSV-1 Genome.

Structure and sequence arrangement of the HSV-1 genome, including the ‘a’ sequence elements. Adapted with permission from Heming *et al*. 2017 (18).

1.2.3 Tegument

The tegument contains approximately 23 viral proteins and 49 host proteins (19). The viral proteins arrange in a complex network of protein:protein interactions of which pUL36 is a scaffold. This network of proteins is asymmetrical and amorphous concentrating near the distal pole, the envelope pole furthest from the capsid (20). Tegument proteins pUL36, pUL37, pUS3, ICP0, ICP4, VP22 and virion host shut off (vhs) are initially added in the nucleus (19, 21–23) and continue to be added with the rest of the tegument in the cytoplasm (24, 25). Many tegument proteins are released upon viral envelope fusion with the host cell membrane and can act immediately without needing HSV-1 gene expression. The role of host proteins in the HSV-1 tegument is unclear, but the available studies suggest an important role in viral infection (26, 27).

1.2.4 Envelope

Like the tegument, the concentration of membrane-bound proteins in the viral lipid envelope is not symmetrical, as they concentrate around the distal pole (20). HSV-1 has 15 proteins in the lipid envelope, 12 glycosylated viral glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, gN) and 3 non-glycosylated (US9, UL20, UL45) (28, 29). Of these 15 proteins, four are essential for entry in cell culture: gH/gL, gB and gD (30, 31). The 11 other 'non-essential' envelope proteins help mediate entry in cells, increase cell-to-cell spread and facilitate syncytia during infection (32–34). The current favoured hypothesis suggests the trans-Golgi network (TGN) as the point of secondary envelopment, however, there is some debate on whether the virus gains its envelope at the TGN, endosomal vesicles, or both (35–38).

1.3 HSV-1 Life cycle

1.3.1 Attachment and Entry

A summary of the HSV-1 life cycle can be seen in Figure 3. HSV-1 first binds to cells by attaching to heparan sulphate proteoglycans (HSPGs) present on the surface of cellular plasma membranes (PMs). HSV-1 attachment and entry rely on five viral glycoproteins: gB, gC, gD, gH, and gL, with only gC being non-essential to the process. Attachment to the PM requires gC or gB to bind to HSPGs, gD then must bind to one of three types of receptors: nectin-1 or -2, herpesvirus entry mediator (HVEM), or a modified heparan sulphate. This induces a conformational change in gD, which sends a signal to gH/gL activating gB to facilitate the formation of the fusogenic complex of gH/gL and gB commencing the process of fusing viral and host membranes mediated by gB (39, 40). Once the membranes have fused, the bulk of the tegument of the virus gets released into the cytoplasm and the capsid is transported to the nucleus by microtubules (41, 42). Once at the nucleus, the capsid interacts with the nuclear pore complex (NPC), specifically with the NPC cytosolic tails. The proteins crucial for this binding are host proteins Importin-β, Nup358, and Nup214, and viral proteins pUL6, pUL25, pUL36, VP16, VP22 and VP13/14) (43–48). Once bound to the capsid, the HSV-1 genome is injected through the nuclear pore in a rod-like conformation (49). The process of DNA translocation from the capsid to the nucleus is thought to be dependent on the C-terminus of pUL25 (50), on pUL36 cleavage (51), and the difference between the pressurized capsid and unpressurized nucleus (44, 52). While Ojala *et al.* showed that

ATP is needed for total ejection of viral DNA (43), it is thought that the initial force difference is enough for passive ejection of DNA translocation, but once the pressure difference decreases as DNA is emptied out of the capsid, a more active, ATP-dependent process may take over (53).

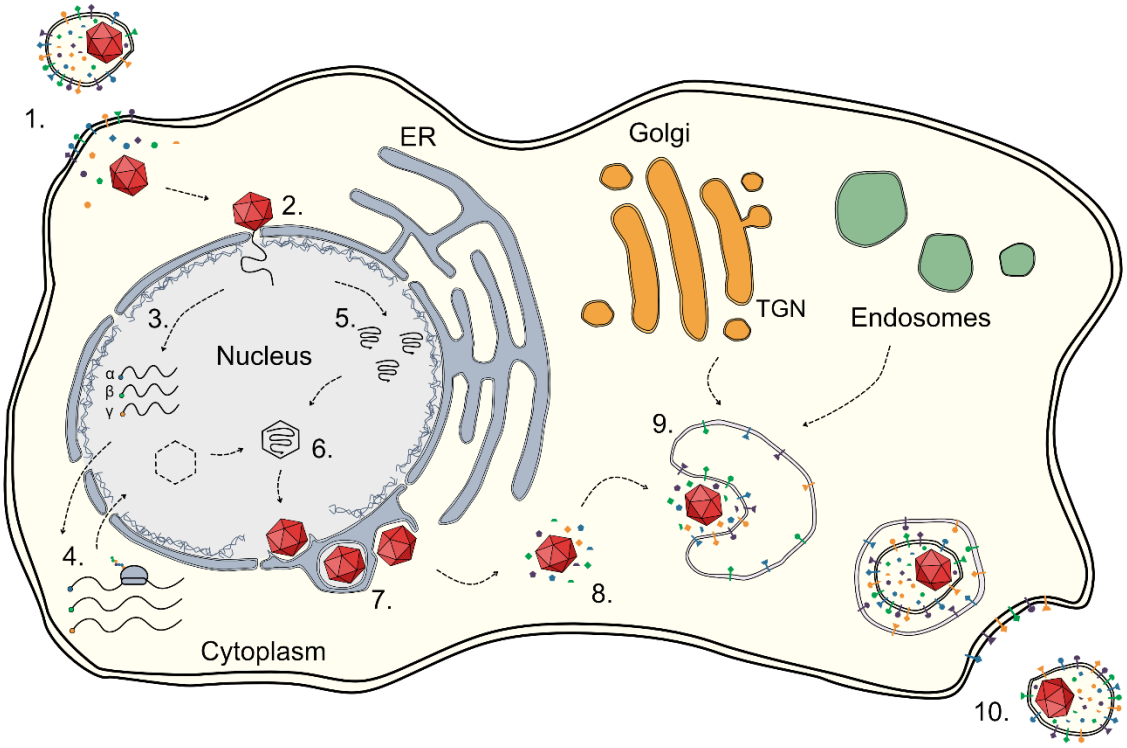

Figure 3. – Life cycle of HSV-1

1. Binding of HSV-1 glycoproteins to host cell receptors resulting in the fusion of the viral membrane with the plasma membrane and the release of the capsid and tegument into the host cell. **2**. The capsid travels to an NPC through interactions with microtubules allowing the release of DNA into the nucleus. **3.** Temporal activation and transcription of α, β, γ genes results in mRNAs that are exported from the nucleus and **4**. translated. **5.** Genome replication occurs. **6.** Capsid maturation and encapsidation of the HSV-1 genome occurs resulting in a mature nucleocapsid. **7**. Through the envelopment-deenvelopment-reenvelopment model, the nucleocapsid buds into the PNS and fuses with the ONM to be released as a naked capsid in the cytoplasm. **8**. The majority of tegumentation occurs in the cytoplasm. **9.** The interaction of tegument proteins and envelope proteins present on envelopment membranes drives secondary envelopment. **10.** Particles released from the reenvelopment compartment fuse with the plasma membrane resulting in the release of HSV-1.

1.3.2 Gene Expression

Once in the nucleus, the viral DNA is transcribed in a temporal cascade. Three groups of viral genes exist: Immediate early (IE, α), early (E, β) and late (L, γ) (54). IE genes are defined as genes that can be expressed without *de novo* viral protein synthesis and include five genes: infected cell protein 0 (ICP0), ICP4, ICP22, ICP27, and ICP47 (reviewed in (55)). Transcription of all HSV-1 genes is done by the cellular RNA polymerase II (RNAP II) (56). IE genes are activated by tegument protein VP16 delivered by incoming virus in complex with two cellular proteins, OCT-1 and HCF, which bind slightly upstream of IE gene promoters on the viral genome (57–59). One role of IE proteins is induction of E gene expression (55). There are 12 early genes, all of which are involved in DNA replication and nucleic acid metabolism (reviewed in (55)). The bulk of early gene expression is promoted through interactions with ICP0, ICP4 and ICP27 (60–62). The promoters for both IE and E contain cis-acting elements such as SP1 and OCT-1 binding motifs, but no cis-acting motifs are found upstream of the TATA-element in the L promoters (63).

Late genes are divided into two groups: γ1 and γ2 (leaky-late and true-late). Both subsets are abundantly expressed after DNA replication, but true-late genes absolutely require it. Leaky-late genes have low-level expression after E gene transcription but before DNA replication (64, 65). This reliance on DNA replication with no obvious cis element in the γ gene promoters has puzzled researchers for many years. Recent research from the Deluca lab has shown that replication of DNA causes a decrease in the transcription factor concentration which in turn decreases β gene transcription but allows for γ gene transcription (63). It is thought this increase of γ gene transcription is due to sheer increase in available genomes after DNA replication (63). While the exact mechanism is unclear, this study complements previous research that *cis*-acting elements were only found upstream of the IE and E, but not upstream of L genes (66). More research needs to be done but it seems that parental HSV-1 genomes require enhancers to ensure transcription while L genes need only rely on newly synthesized genomes and strong initiator elements (63). After DNA replication, the transcription of HSV-1 genes moves from ND10 foci to replication compartments which may also play a role in availability of transcription factors, and early-to-late switch (67).

After transcription and mRNA processing, which is similar for viral transcripts as it is for host transcripts, viral mRNA must be exported into the cytoplasm for translation which is where viral

transcripts differ from cellular mRNA. For cellular mRNAs, export from the nucleus relies on ribonucleoproteins (RNPs) involved in RNA splicing, an activity not needed by most HSV-1 genes, and actively suppressed during infection by multifunctional ICP27 (68). HSV-1 exports unspliced viral mRNA by usurping host mechanisms through ICP27 binding of TAF/NXF1 (69). TAF/NXF1 is a nuclear export receptor critical for interacting with the nucleoporin allowing RNP cargo to exit the nucleus into the cytoplasm (70). Once in the cytoplasm, HSV-1 transcripts are translated by host ribosomes. Here lies another point of regulation for late gene expression. The viral protein vhs is an RNase that degrades both viral and host mRNA which increases polysome accessibility to L transcripts therefore facilitating E to L gene switch through translation regulation (71).

1.3.3 Genome Replication

HSV-1 has three origins of replication, one in the UL region and two in the US region, which are all recognized by pUL9, the origin binding protein (OBP) (72–74). OBP is one of seven essential viral proteins needed for HSV-1 genome replication. The others include single stranded DNA (ssDNA) binding protein ICP8, DNA polymerase pUL30, processivity protein UL42, and three proteins that make up the DNA helicase/primase complex (DNA H/P) (reviewed in (75)). When DNA enters the nucleus, it translocates to ND10 foci (76). Once at these foci, the linear genome circularizes due to the higher concentration of recombination machinery present (77, 78). Once the genome is circularized, pUL9 and ICP8 destabilize it at one of the three origins of replication (79). pUL9 has helicase and NTPase activity resulting in separation of the dsDNA to single-stranded DNA which is stabilized by ICP8 (80). Once enough ssDNA accumulates, ICP8 becomes a single-stranded binding protein, preventing the DNA from reannealing and allowing the HSV-1 DNA H/P to be recruited through pUL9 (81, 82). The DNA H/P complex is made up of three viral proteins: pUL8, pUL5, and pUL52. pUL5 and pUL52 are the enzymatically active components of the complex, while pUL8 has no enzymatic activity alone, but stimulates primer synthesis in cooperation with pUL5 and pUL52 (83–85). Catalytically active pUL52 recruits the DNA polymerase to the replication fork (86). Like a eukaryotic polymerase, the HSV-1 DNA polymerase pUL30 also has proofreading activity through its 3’-5’ exonuclease activity, while the processivity factor pUL42 stabilizes pUL30 on the DNA, preventing dissociation much like eukaryotic clamp proteins (87, 88). It is thought that HSV-1 replicates its DNA in two stages, the

first stage was described above and was originally believed to yield concatemeric DNA longer than an individual HSV-1 genome that would later be cleaved, through rolling circle replication (89). However, due to observations of branched replication intermediates, it is now believed that a second stage of replication may require recombination (90, 91). Recombination through single strand annealing (SSA) would recover replication at stalled replication forks and facilitate the production of concatemers to prepare for encapsidation (92). This is consistent with the findings that HSV-1 increases SSA recombination while decreasing non-homologous end joining (NHEJ) and homologous recombination (HR), a phenomenon dependent on expression of viral exonuclease pUL12 and ICP8 (92–94).

1.3.4 Capsid Assembly and DNA Packaging

The majority of L genes encode for structural proteins and once those are made, the procapsid can start to assemble. The minimum proteins required for assembly of the procapsid *in vitro* include VP5, VP19, VP23, pre-VP22a, VP26 and UL26 gene products (95, 96), but many others are involved in a wild-type (WT) HSV-1 infection. The process begins in the nucleus, where VP5, pUL26 and pre-Vp22a scaffolding proteins bind to begin the formation of the procapsid (97). VP5 makes up the pentons and hexons which account for the bulk of the capsid, and triplets containing minor capsid proteins VP19c and VP23 connect them together further building the procapsid (97). Twelve molecules of pUL6 oligomerize to form the singular portal structure on one of the 12 vertices (98) and six molecules of VP26 form a hexamer ring around each hexon (13), all of which results in a spherical procapsid arranged in a T=16 lattice. The transition from unstable procapsid to mature capsid occurs when pre-VP22 scaffolding is cleaved by the protease pUL26, resulting in a quick change to a more stable icosahedral shape (99). While the timing of this maturation event is unclear, the presence of defective B-capsids that are icosahedral in shape but contain only cleaved scaffolding protein and the protease VP24 suggest maturation would have to occur sometime before DNA encapsidation (Figure 4) (100).

As discussed above, DNA replication occurs in ND10 foci within the nucleus and therefore capsids must move to encounter DNA for packaging to occur. Of the seven required proteins for DNA packaging (pUL6, pUL15, pUL17, pUL25, pUL28, pUL32 and pUL33(101–103)), pUL32

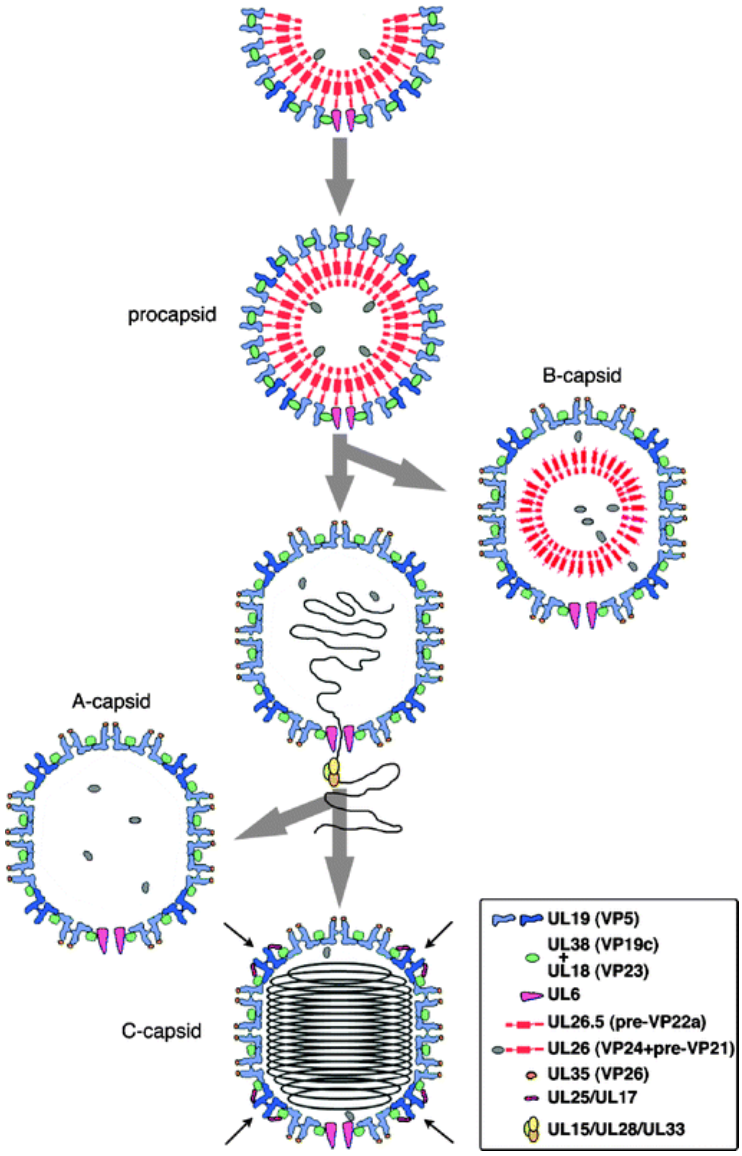

Figure 4. – Assembly and Maturation of the HSV-1 Nucleocapsid

Successive states of capsid maturation are shown schematically in cross sections. Figure from Cardone *et al.,* 2012 (104), used with permission.

acts in directing capsids towards replication centres (105). The terminase complex, made of UL15, UL28 and UL33, interacts with the pUL6 portal allowing the binding, scanning, and cleaving of HSV-1 DNA at specific sequences (106, 107). Structural studies have observed that the viral DNA is in a left-handed spool arranged in concentric layers inside the capsid (16). To make room for the DNA, the scaffolding protein must be cleaved, as described in the paragraph above. The exact sequence of this is still unknown but it thought the terminase complex associates with procapsids before the scaffold is cleaved and the capsid becomes an icosahedral (108). The components of the CVSC, pUL25 and pUL17, are needed to ensure DNA stability within the capsid (50, 109). New research has shown that pUL25 and pUL17 are present on the portal vertex, but the C-terminus of pUL25 is in a unique conformation to the other 11 vertices (16). If DNA encapsidation is successful, the mature nucleocapsid will leave the nucleus to become a mature viral particle. However, if DNA encapsidation is unsuccessful, two defective capsids are made: A-capsids and B-capsids. B-capsids contain cleaved scaffolding protein but no DNA, which may be due to lack of initiation of DNA packaging (104). A-capsids are formed due to an abortive DNA packaging event where scaffolding has been expelled, but the genome was not packaged resulting in an empty capsid shell (Figure 4) (110).

1.3.5 Nuclear Egress

Capsid egress occurs at the nuclear envelope; therefore, capsids must move from the replication compartments to the nuclear periphery. HSV-1 infection causes a complete re-shaping of the nucleus, including the moving of dense chromatin to the periphery to make way for the expanding replication compartment (111). The movement of nucleocapsids in the nucleus is most likely passive (112–114), but what permits the passage through dense chromatin is the presence of virally induced channels (115–117). Once at the nuclear membrane the nucleocapsid must exit the nucleus. There are three proposed mechanism for how this occurs: 1) The single envelopment model, 2) The nuclear pore model and 3) The envelopment-deenvelopment-reenvelopment model.

The single envelope model suggests that once the nucleocapsids bud into the perinuclear space (PNS), they travel into the endoplasmic reticulum (ER) which is contiguous with the PNS to continue through the secretory system (118, 119). However, electron micrographs of virions within the Golgi have not been seen (120, 121). This is an issue because the 12 glycoproteins the virus encodes must be glycosylated within, and mature through, the Golgi (122). Finally, the

pUL31 and pUL34 form a coat on the inside of the primary envelopment vesicle, something that is not seen on the final envelope of the mature virus (123, 124). The second hypothesis proposes that the flexibility of the nuclear pore could allow nuclear egress directly through the NPC (125). This hypothesis has been opposed due to the 125 nm size of nucleocapsids (126). The initial research on the NPC described the maximum cargo size as ~26 nm (127). However new research has shown that the NPC central channel is ~50 nM wide at its narrowest point (128) and through 3D correlative light-electron microscopy 60 nm human immunodeficiency virus type 1 (HIV-1) capsids were observed in the NPC during import into the nucleus, suggesting some flexibility (129). However, no evidence has been found for HSV-1 capsids at this point in time. Finally, the third model is the envelopment-deenvelopment-reenvelopment model where the nucleocapsid buds through the inner nuclear membrane (INM), fuses through the outer nuclear membrane (ONM) and gets enveloped in the cytoplasm for maturation. This is the model that is widely supported and will be expanded on in the paragraph below.

1.3.6 Envelopment-deenvelopment-reenvelopment model

The envelopment-deenvelopment-reenvelopment model was initially prosed by Stackpole in 1969 after observing capsids budding into the PNS and deenvelopment into the cytoplasm of the Ranid herpesvirus 1 in frog kidneys (130). Since then, many lanes of evidence supporting this model have been elucidated. Two of the strongest pieces of evidence are that 1) the nuclear egress complex (NEC) forms a coat on the inside of the vesicles that envelope capsids in the PNS as an artifact of membrane budding that is not observed in mature virions but is essential to production of viral progeny (124, 131, 132). 2) When an ER retention signal is added to envelope proteins, keeping them present in the ER and nuclear membranes, they no longer accumulate on mature particles (133, 134). Other observations such as the different protein and lipid composition of mature virions and nuclear membranes (135, 136), and the presence of naked capsids in the cytoplasm (38), add to the evidence that suggests HSV-1 loses its primary envelope and must mature through a second envelopment stage in the cytoplasm.

To begin primary envelopment, the NEC must form along the nuclear side of the INM. pUL34 is membrane bound and therefore travels from the ER to the INM, pUL31 is a soluble protein and must be imported into the nucleus from the cytoplasm. Research has shown that the N-terminus of pUL31 is important for inhibiting a cytosolic interaction between pUL34 and pUL31, which is

relieved through an unknown mechanism once pUL31 is in the nucleus (137). Once bound in a heterodimer, pUL31 and pUL34 interact through two different interfaces to form a 2D hexagonal lattice when crystallized (138). Before the NEC can prepare the INM for budding, the nuclear lamina, made of Lamin A, B, and C and control nuclear size and stability, must be rearranged to allow for capsid docking and increased flexibility of the membrane. The viral kinase pUL13 activates another viral kinase pUS3 through phosphorylation allowing the phosphorylation of Lamin A/C, Lamina-associated protein (LAP) emerin, and pUL31 resulting in the rearrangement of the nuclear lamina and the proper localization of the NEC (139–141). Experiments where late viral protein $\gamma_1$34.5 is mutated at the amino terminus cause the retention of capsids within the nucleus compare to WT virus due to lack of Lamin A/C rearrangement (142). $\gamma_1$34.5 has also been shown to bind phospho-kinase C (PKC) and mitochondrial protein p32 and shuttle them to the nucleus, leading Wu *et al*. to hypothesize that $\gamma_1$34.5 and p32 form a complex to regulate the kinase activity of pUS3 and PKC in the phosphorylation and rearrangement of Lamins A, B, and C. (142–145).

Expression of the NEC without any other viral or host proteins is sufficient to produce budding in synthetic vesicles (123, 146), but exactly how a hexagonal lattice, which is flat, gains curvature is still unknown. Recently, Draganova *et al.* used Cryo-EM and Cryo-ET on synthetic vesicles to show that pUL25 can bind lipid-bound NECs, and that this binding causes the NEC to form pentagons rather then hexagons (147). This is consistent with the observation that docking of the capsid to the NEC is mediated by an interaction between the 20 amino acids on the C-terminal of pUL25 (148). However, the evidence that NEC can produce membrane budding without other viral proteins is clear, despite not occurring *in vivo,* suggesting a yet unknown inhibitory mechanism regulating the NEC. After budding has started, many groups have reported for HSV-1 and the related alphaherpesvirus PRV-1 that the NEC is sufficient to mediate scission of the vesicle into the INM (123, 146). One group has even shown that pUL31 is sufficient for membrane scission when attached to a membrane tether in PRV-1 (149). All three of these studies used synthetic vesicles like giant and large unilamellar vesicles (GUVs, LUVs) as well as the overexpression of pUL34 and pUL31 which, while important to help our understanding of the mechanism, may be overlooking important viral-viral and host-viral protein interactions. Recent research in cell culture has shown that endosomal complexes required for transport (ESCRT)-III machinery is important in membrane scission of primary envelopes (150). Previously implicated

in the secondary envelopment of HSV-1 (151, 152), knock out of ESCRT-III machinery caused both an accumulation of capsids in aberrant membrane invaginations within the nucleus, and membrane budding stalled with only a small channel connecting the vesicle and the nucleus (150). The ability of ESCRT-III proteins to mediate budding was dependent on its recruitment to the nuclear membrane by the NEC complex. It is likely that while the NEC complex is sufficient for membrane budding and scission of synthetic vesicles, the nucleus of a cell requires a regulated process for control of budding, a process ESCRT-III could mediate.

Once the nucleocapsid is enveloped in the INM, it must fuse through the ONM to be released into the cytoplasm. Deletion experiments of both viral and host proteins have resulted in the accumulation of nucleocapsids in the PNS and aberrant membrane invaginations within the nucleus. The viral proteins involved in de-envelopment are pUS3, and gH and gB combined (153, 154). For host proteins, tandem affinity mass spectrometry found CD98 heavy chain (CD98hc) to be an interactor of gB which forms a complex made of gB, gH, CD98hc, β-integrin, pUL34 and pUL31 (155). Host protein VAPB, which is a vesicle-associated membrane protein (VAMP), a class of proteins involved in vesicle fusion, also likely enhances fusion and viral deenvelopment as knockdown causes accumulation of nucleocapsids in the PNS (156). The role of host proteins in enhancing fusion of the primary envelope with the ONM in the presence of gB, the viral fusogenic protein, is unsurprising due to the need of gB activation to function as seen during entry (40). However, unlike entry, gD does not seem involved in viral nuclear egress (157).

1.3.7 Tegumentation

The tegument is the protein-rich layer between the capsid and the lipid envelope. At least 23 viral proteins are present in the tegument of HSV-1, which are mainly acquired in the cytoplasm and during second envelopment where electron micrographs have observed an increase in electron dense material around the capsid (24). However, some tegument proteins such as pUL36, pUL37, pUS3, ICP0, ICP4, VP22 and vhs have been found to associate with nuclear capsids (19, 21–23, 25). pUL36 is critical for the recruitment of other tegument components, it binds pUL25 and pUL17 as well as VP5 and pUL37 and acts as a scaffold for other protein-protein interactions in the tegument (158, 159). This binding is so essential to the function of the CSVC that some argue it should be a member of the complex (159). Once this scaffold is formed, proteins continue to build a network until outer tegument proteins like pUL11, pUL16, and pUL51 interact with

glycoproteins or the membrane directly (160–163). This tethers the tegument to the lipid envelope of the virus and plays a role in secondary envelopment.

1.3.8 Secondary Envelopment

The precise location of secondary envelopment is unclear. The current view is that the main secondary envelopment compartment is the TGN. Seminal research in the field has shown that the lipid composition of mature virions mimicked Golgi-derived membranes (164) which has been supported by many other studies (reviewed in (38, 165)). However, a few studies have shown that the membranes can be derived from endocytic vesicles or multivesicular bodies in the cytoplasm (166–168). While these experiments seemingly oppose each other, the rearrangement of membrane-bound subcellular compartments in HSV-1 infection is so severe that classic definitions may not be useful in discussion of where HSV-1 secondary envelopment occurs (169).

Regardless of the exact location of HSV-1 secondary envelopment, 15 viral membrane-bound proteins must traffic to those sites for proper envelope composition. The virus has at least two ways of mediating the trafficking of these proteins. First, some viral proteins like gB and gE have trafficking motifs which transport them to the TGN (170, 171). The second method relies on viral proteins to shuttle them to the location of envelopment. This has been observed for the TGN localization of gI, which is dependent on gE (171) and while pUL20 and gK rely on each other for proper trafficking (172), gD and gH/L in turn rely on gK/UL20, or gM to get to the site of secondary envelopment (173, 174). The process of proper glycoprotein trafficking is not fully elucidated, but it is clear that it does not require the presence of a capsid as L-particles with normal composition of envelope proteins, and no capsids are observed during infection (36, 175, 176).

To get the capsid to the site of secondary envelopment, HSV-1 reorganizes the microtubule-organizing centres (MTOCs) within the cell and facilitates the creation of new ones throughout the cytoplasm (177). While the exact mechanism is unclear, it seems that pUS3 stabilizes new microtubules by inactivating glycogen synthase kinase 3β (GSK3β), a protein responsible for the regulation of microtubule stability through phosphorylation of MAP1β (177, 178). VP22 has also been shown to mediate the induction and stability of microtubules through acetylation (179, 180). This reorganization of microtubules facilitates anterograde transport of cytoplasmic capsids mediated by kinesin motors through an interaction with pUL36 and pUL37 (181, 182). Once kinesin motors move cytoplasmic capsids towards the site of envelopment, capsid docking is

thought to occur through pUL37. Due to the role of pUL37 as a tegument scaffold, it was hard to draw conclusions as to whether this was a direct or indirect interaction. It was not until recent studies discovered the interaction between membrane proteins gK/pUL20 and pUL37 that the mechanism of capsid docking became clearer (183, 184).

Once at the site of secondary envelopment, the formation and scission of the secondary envelope occurs through the involvement of ESCRT-III machinery (151). The ESCRT-III machinery consists of 12 proteins that fall into the Charged Multivesicular Body Protein 1-8 (CHMP1-8) families that require a nucleation event to begin polymerizing on a membrane (185). The two main nucleation proteins are ESCRT-II and ALIX, yet HSV-1 does not require either of them for secondary envelopment (186, 187). Interestingly, pUL51 is structurally similar to CHMP4B and can form filaments *in vitro*, like ESCRT-III filaments (188). The heterotrimeric complex that pUL51 forms with pUL7 could be evidence of a control mechanism for envelopment. The binding of pUL7 to pUL51 prevents the polymerization seen by unbound pUL51, and therefore the complex must be broken for nucleation of membrane curvature to occur (188). pUL51, while not membrane bound, associates to membranes through a palmitoylation site directly, or through interaction with gE (162, 189). The similarity of pUL51 to ESCRT-III proteins and its ability to associate to membranes makes pUL51 a likely player in secondary envelopment nucleation. After nucleation, ESCRT-III machinery drives the formation of a vesicle, pinching off the membrane through scission and finally being disassembled by AAA ATPase Vps4 (185). The enveloped virion within a vesicle is then trafficked to the plasma membrane through the secretory pathway and is released from the cell through exocytosis (190, 191).

2. Poly(C)-binding Protein 1

2.1 hnRNPs

PCBP1 is an RNA-binding protein, which belongs to the family of poly(C)-binding proteins that include the heterogeneous nuclear ribonucleoproteins (hnRNP) K and hnRNP E1-4 (PCBP1-4) (Figure. 5) (192). hnRNPs were originally discovered as large complexes associated with heterogeneous nuclear RNA (hnRNA), more often called pre-mRNA, through dynamic and transcript specific interactions (193, 194). Since their discovery, 20 abundant hnRNPs have been found and termed hnRNP A1-U and many other minor proteins have been elucidated (195, 196).

While sequence specificity seems to drive association with pre-mRNA transcripts, transcript abundance and post-translational modifications of hnRNPs can also affect these RNA interactions (197). hnRNPs are ubiquitous across tissue types and while many are localized in the nucleus, half of the major hnRNPs can shuttle between the nucleus and cytoplasm (198, 199). hnRNPs are mainly involved in RNA metabolism such as RNA splicing, 3' processing and transcriptional regulation, but the ability of some to shuttle into the cytoplasm suggests a greater breadth of multi-functionality (199).

2.2 PCBP1

PCBP1, also called hnRNP E1, is an hnRNP defined by its ability to bind poly(C) RNA through K-homology (KH) domains. KH domains were originally found in hnRNP K and later discovered in PCBP1-4 as well as other proteins like FMR1 involved in Fragile X syndrome (200, 201). The KH domains are roughly 70 amino acids in length arranged in a βααββα fold where the triple β pleated sheet platform supports three α-helices (201). KH domains can be divided into two groups based on their terminal extensions: Type I has a C-terminal βα extension and type II has an N-terminal αβ extension (202). PCBP1 has three type I KH domains, of which only KH1 and KH3 have been shown to bind RNA (201, 203, 204). PCBP1 is also one of the ten hnRNPs that shuttle between the nucleus and the cytoplasm, containing a nuclear localization signal (NLS) between the KH2 and KH3 domain (205). PCBP1 is multifunctional within the cell exhibiting classic hnRNP activity but also some less classical activity such as in iron transport, modulating the immune system, in cancer, and viral propagation which will be expanded on in the following sections.

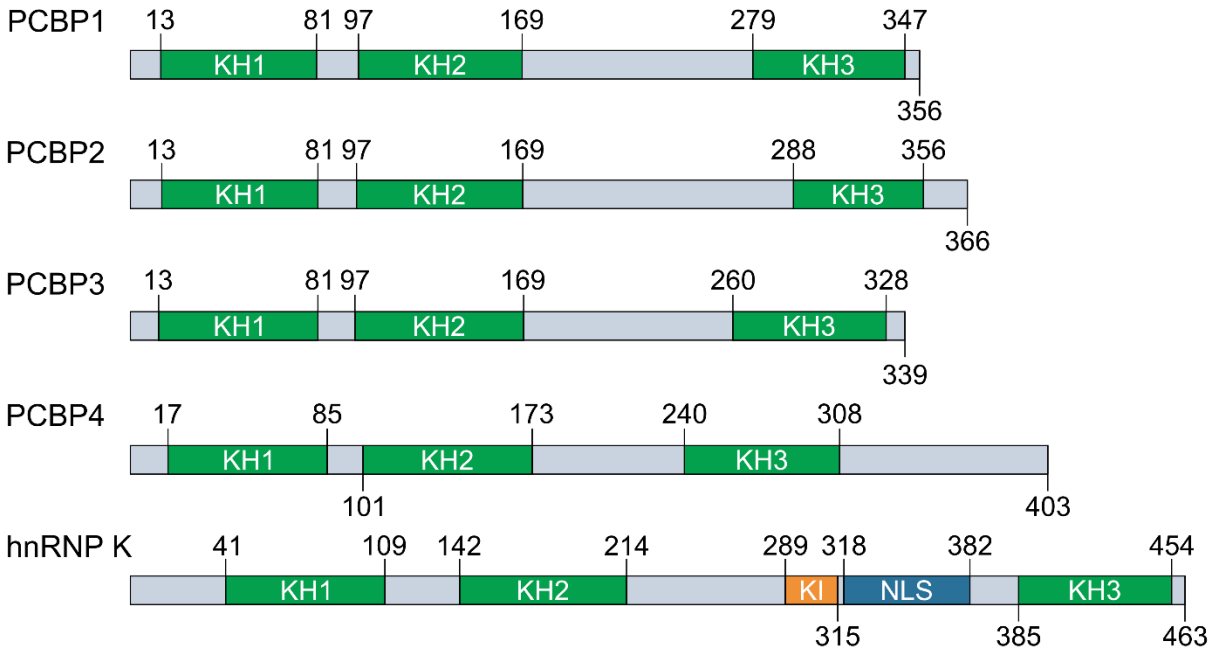

Figure 5. – Multidomain Structure of Poly(C)-binding Proteins

The five major members of the PCBP family are shown. Numbers of amino acid residues are indicated for each respective human sequence. The conserved KH domains (1, 2, and 3) are shown in green. The sequences between KH2 and KH3 are the most variable in length and primary sequence. KI = Kinase interactor domain, NLS= nuclear localization sequence. Adapted with permission from Makeyev and Lieberhaber, 2002 (192).

2.2.1 Transcription

While PCBP1 was originally described as an RNA-binding protein, evidence suggests it can also bind ss- and dsDNA (203). The DNA-binding capacity has been important for translational control of BRCA1, the μ-opioid receptor (MOR), and eukaryotic initiation factor 4E (eIF4E). PCBP1 binds the promoter of these genes resulting in the activation of transcription (206–208). In the BRCA1 gene, PCBP1, along with other proteins like hnRNP K, binds the polypyrimidine/polypurine region of the promoter termed the positive regulatory region (PRR) to mediate activation (206). Furthermore, of the proteins that bind the PRR, only PCBP1 was found to be decreased in breast cancer cell lines where the BRCA1 gene is nonfunctional (206). PCBP1, and PCBP2-4, binds a C-rich 26-nucleotide single-stranded segment of the MOR promoter (207). While exogenous overexpression of each PCBP individually was sufficient to activate the promoter, all four PCBPs must be depleted to see a decrease in promoter activity suggesting some redundancy (207). Finally, PCBP1 has been shown to specifically aid in the transcriptional control of eIF4E by binding to its promoter (208, 209). This mechanism requires PCBP1 to be phosphorylated by p21-activated kinase 1 (Pak-1), which facilitates PCBP1 dissociation from cytoplasmic mRNAs and its nuclear retention, therefore increasing access to promoters (208).

2.2.2 mRNA Stability

PCBP1 was discovered as a component of the α-globin mRNA stability complex that binds to C-rich elements in the 3’ UTR of α-globin mRNA (210, 211). The proteins involved include hnRNP D, PABP, PCBP1 and PCBP2, of which PCBP1 and 2 are essential to the complex (210–213). The interaction of the α-globin mRNA stability complex may protect the mRNA from deadenylation, therefore extending its half-life (214). Since those initial studies, other research has shown that PCBP1 stabilizes other mRNAs, suggesting the stability complex is not specific to α-globin mRNA. For instance, p63 and endothelial nitric oxide synthase (eNOS) both bind PCBP1 through a C-rich element (215, 216). While the mechanism behind p63 has not been elucidated, the binding of eNOS by PCBP1 protects eNOS mRNA from microRNA specific repression (216). The interaction of the PCBP1-rich RNP complex that binds eNOS did not contain PCBP2, unlike the RNP complex found on α-globin mRNA. PCBP1 and PCBP2 have also been observed in an RNP that binds the C-rich 3’UTR of sortilin and extending its mRNA half-life (217, 218). However, in this case, increased intracellular zinc disrupts the interaction with PCBP1, removing

the RNP and the mRNA stability it conferred (217, 218). Sortilin is present in immune cells and is involved in the exocytosis of IFN-α. Interestingly, the interaction of TLRs with pathogen-associated molecular patterns (PAMPs) results in a release of intracellular zinc, suggesting that the PCBP1/2 stability complex binding to sortilin is part of negative regulation of the innate immune response (217, 218).

While the majority of cases report that PCBP1 RNP complexes bind mRNA to confer stability, there is one case where PCBP1 destabilizes mRNA. WT p53 activated fragment ($p21^{WAF}$) is a moderator of the cell cycle and its expression is associated with G_1 arrest (219). Co-depletion of PCBP1 and PCBP2 results in increased mRNA stability of $p21^{WAF}$ by interaction with C-rich sequences in its 3' UTR (219). The sequences PCBP1 and PCBP2 bind in $p21^{waf}$ mRNA are similar to those found in α-globin mRNA, and yet the effect of binding is the opposite. The diverse effects of PCBP1-containing RNPs on mRNA stability may point to PCBP1 as an adapter protein. PCBP1, and perhaps PCBP2, may simply bind C-rich RNA creating a platform for other proteins to affect mRNA stability. More work needs to be done to be sure of the role of PCBP1 in mRNA stability.

2.2.3 PCBP1 and Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) plays an important role in development but can be reactivated inappropriately as part of the progression of cancer to a metastatic phenotype (220). PCBP1 is an important player in regulating EMT through both translation and alternative splicing. Transforming growth factor β (TGF-β) is one of the many growth factors that can induce EMT. Under normal conditions PCBP1 binds to a subset of mRNA with TGF-β-activated translation (BAT) elements in the 3'UTR, silencing the translation of these mRNA (221). This silencing occurs through binding of eEF1A1 that forms a bridge between the 3' UTR and the elongating ribosome causing the elongation to stall because eIF1A1 cannot be removed (222). However, the expression of TGF-β activates Akt2 which phosphorylates PCBP1 to disrupt its interaction with the BAT element, allowing eEF1A1 to be released from the ribosome derepressing translation (221). So far only disabled-2 (Dab2) and interleukin-like EMT inducer (ILE2) mRNA have described BAT elements involved in TGF-β-induced EMT (220).

PCBP1 also uses a different mechanism to repress the translation of an inducer of EMT, phosphatase of regenerating liver 3 (PRL-3). PRL-3 is a phosphatase that induces both EMT and tumor angiogenesis contributing to the progression of many cancers (223). Through binding to a

GC-rich sequence in the 5'UTR of PRL-3, PCBP1 prevents the incorporation of PRL-3 in heavier polysomes regardless of the amount of mRNA present (224). Heavier polysomes are more translationally active, so PCBP1 is somehow mediating translational repression of oncogenic PRL-3 through an unknown mechanism.

Alternative splicing is a process important for forming fully functional proteins. When errors in alternative splicing occur, it can lead to the expression of non- or semi-functional pseudogenes that can mediate disease and disrupt cellular functions (225). PCBP1 has been implicated in the regulation of alternative splicing for genes involved in EMT such as CD44, phosphatase regulatory subunit 10 (PNUTs), and growth hormone receptor (GHR). PCBP1 prevents the isoform switching of CD44 associated with EMT and progression of cancer, identifying it as a negative regulator of CD44 (226, 227). These authors hypothesize PCBP1 binds through an exonic splicing silencer (ESS) present on the pre-mRNA, which is supported by previous research on the GHR (228). PCBP1 was observed to bind to GHR pre-mRNA alongside U1 small nuclear ribonucleoprotein (snRNP), a component of the pre-splicing silencing complex. This complex was found to suppress the inclusion of a pseudo-exon within GHR, an effect that was reversed upon the depletion of PCBP1 (228). While pseudo-exons of GHR have not been implicated in EMT, expression of both GHR and its ligand growth hormone (GH) have been shown to mediate it (229). Finally, PCBP1 has been shown to bind an alternative splice site within PNUTs pre-mRNA. When PCBP1 is depleted, an alternative splice site is used to produce a pseudogene termed lncRNA-PNUTs (long non-coding RNA PNUTs) (230). Through the chelation of microRNA-205, lncRNA derepresses ZEB mRNAs that are integral for EMT (230). Therefore, PCBP1 negatively regulates the alternative splicing of at least three mRNAs involved in EMT. This suggests an important role for PCBP1, not only in the progression of cancer, but also in development where EMT is required.

2.2.4 PCBP1 in Iron Transport

Ferritin is the main iron storage molecule in cells that accumulates iron through chaperones (231). PCBP1 has been implicated as a cellular iron transport chaperone for ferritin. PCBP1 binds three molecules of ferrous iron and PCBP1 depletion decreases the amount of iron bound to ferritin in Huh7 cells (231). Later work determined that PCBP2 and PCBP1 were both required for efficient iron loading of ferritin (232). Since its discovery in mediating iron transport to ferritin,

PCBP1 and PCBP2 have been described as iron chaperones for both prolyl hydroxylase 2 (PHD2) and deoxyhydrogenase hydroxylase (DOHH) (233, 234).

Hypoxia inducible factor α (HIF α) is a conserved protein that binds HIFα response elements turning on hundreds of genes during hypoxic events (235). PHD2 is required to add hydroxyl groups to prolines on HIFα facilitating ubiquitination and proteasomal degradation to regulate HIFα levels (233). PHD2 needs iron to function, and PCBP1 and PCBP2 supplies the iron through direct binding to PHD2 (233). Similarly, DOHH also requires both PCBP1 and PCBP2 to transport iron in order to enable the conversion of lysine to hypusine, an uncommon amino acid found only in eIF5A, a protein critical to eukaryotic function (234). It is interesting that all cases require both PCBPs 1 and 2, concluding that they have non-redundant properties in iron transport. This has led some researchers to hypothesize that this could be a more general iron chaperone mechanism used by many iron-dependent enzymes (233).

2.2.5 PCBP1 and Antiviral Immunity

PCBP1 has been implicated in two aspects of antiviral immunity, specifically through the detection of PAMPs by RIG-I like receptors (RLR) and cGAS/STING. PCBP1 acts as an adaptor protein that binds both mitochondrial antiviral signaling (MAVS) and the E3 ubiquitin ligase AIP4, facilitating the ubiquitination and downstream proteasomal degradation of MAVS (236). Interestingly, PCBP2 also exhibits this behavior, although it is only triggered upon viral infection of Sendai virus (236). PCBP1 therefore acts as a housekeeper of MAVS protein, while PCBP2 is part of a negative feedback response regulating IFN-β response after infection (236). A recent study has also demonstrated PCBP1 as an adaptor protein enabling the interaction of DNA and cGAS (237). When PCBP1 was depleted from the system, viral infection increased in an interferon-independent manner (237). These two studies show an important role of PCBP1 in cytosolic DNA sensing, through both IFN-β dependent and independent methods.

2.2.6 PCBP1 in Viral Infection

Besides being involved in antiviral immunity, PCBP1 has specific roles in many different viral infections. Previous research has shown PCBP1 is antiviral for hepatitis E virus (HEV), vesicular stomatitis virus (VSV), HIV-1, and human papilloma virus type 16 (HPV-16) (238–241), while it is pro-viral for enterovirus 71 (EV71), poliovirus, classical swine fever virus (CSFV), and

Kaposi's sarcoma-associated herpesvirus (KSHV) (242–245). While many of the exact mechanisms have not been elucidated, those that have been demonstrated show a wide range in activities, consistent with the multifunctional nature of PCBP1. This next section will go into details on both the antiviral and pro-viral nature of PCBP1.

2.2.6.1 PCBP1 as an Anti-Viral Protein

PCBP1 is multifunctional within cellular host roles, and likewise is multifunctional within viral infection. PCBP1 has been shown to hinder translation and transcription through specific interaction with viral proteins. For VSV, a negative-sense RNA virus, PCBP1 overexpression inhibited primary transcription as measured by the level of nucleoprotein (N) mRNA (238). The depletion of PCBP1 also increased the RNA levels and viral replication of HEV, a positive sense RNA virus (241). This effect was mediated through a RNP complex made of at least PCBP1, PCBP2 and hnRNP H that bound to the genomic promoter of HEV (241).

For HIV-1, PCBP1 interacts with the exon splicing silencer within the tat/rev exon 3 (ESS3) (239, 246, 247). This is part of an RNP complex that also binds PCBP2, hnRNP A1, Q, K, U and R (239). Interestingly, the overexpression of PCBP1 decreased the p24, p5 and gp160/120 viral proteins but caused a much smaller reduction at the RNA level. Conversely, depletion of PCBP1 led to an increase in protein levels, but no change in RNA (239). This effect was mediated by the C-terminal end of PCBP1 interacting with the ESS3 sequence on rev. Even though PCBP2 was involved in this process, switching the C-terminus of PCBP1 with PCBP2 reduced the effect overexpression of PCBP1 had on protein levels, suggesting a non-redundant role for the two PCBPs (240).

Translation of the late expressing genes of HPV-16 is also inhibited by PCBP1 (240). Expression of L1 and L2 mRNA is observed only in terminally differentiated squamous epithelium, and therefore a mechanism must exist to inhibit expression in non-differentiated cell types. PCBP1, PCBP2, and hnRNP K were all observed to bind the 3' end of the L2 RNA *in vitro*, which caused almost a four-fold decrease in L2 mRNA (240, 248). The effect of PCBP1 and PCBP2 binding was not additive or synergistic since translation was similarly reduced whether they were individually bound or bound together. When tested in cell lines, PCBP1 inhibited L2 mRNA translation, consistent with patient observations where PCBP1 expression decreases with

the progression from low-grade squamous intraepithelial lesions to cancer (249). This supports the role for PCBP1 inhibition of L2 mRNA in HPV-16.

2.2.6.1 PCBP1 as a Pro-Viral Protein

The pro-viral aspect of PCBP1 seems to be through enhancement of IRES activation and moderating the immune system. Picornaviruses such as EV71 and Poliovirus are two viruses that cause hand-foot-mouth-disease and poliomyelitis, respectively. Both viruses have single-stranded RNA genomes with a highly structured 5' UTR required for replication and IRES-mediated translation. PCBP1 was observed to bind stem loops I and IV of the EV71 and poliovirus IRESs (242, 245). In EV71, the KH1 domain of PCBP1 is responsible for the interaction with the IRES, and when PCBP1 was depleted, viral titers decreased (245). For poliovirus, PCBP2 was also involved in IRES binding. PCBP2 had a stronger affinity for stem loop IV, and depletion of PCBP1 consequently decreased genome replication, but depletion of PCBP2 depletion resulted in lower levels of both translation and genome replication (244).

PCBP1 mediates a second type of IRES interaction, outside of picornaviruses. PCBP1 binds to KSHV protein ORF57 (244). ORF57 is a homolog of HSV-1 ICP27, a multifunctional protein involved in regulation of gene expression. The binding of PCBP1 to ORF57 occurs through a sequence that extends from the KH1 domain to the beginning of the KH2 domain (244). This interaction enhances the IRES-mediated translation of host protein X-linked inhibitor of apoptosis (XIAP) but does not affect translation of KSHV IRES-mediated vFLIP (243). The role of PCBP1 seems to be preventing apoptosis upon reactivation of KSHV infection. However, the intricacies of IRES recognition and enhancement by PCBP1 is currently unknown.

CSFV is a flavivirus from the genus pestivirus that causes a highly contagious disease in pigs. Pestiviruses exclusively contain a cysteine-like autoprotease called N^{pro} that, in CSFV, has been shown to bind to PCBP1 (243). This interaction requires the KH3 domain, and, in the absence of PCBP1, CSFV infection results in reduced titers (243). Oddly, the pro-viral effect of PCBP1 bound to N^{pro} may be due to the reduction of the IFN-β response, a reduction that is independent of proteasomal degradation (243). As discussed above, PCBP1 is a housekeeper of MAVS, mediating its proteasomal degradation (236). This observation that PCBP1, N^{pro} and PCBP1+N^{pro} reduced IFN-β response suggests that PCBP1 may play a role in immune regulation independent of MAVS signaling that is amplified by its interaction with N^{pro} (250). Another flavivirus, hepatitis

C virus (HCV), interacts with PCBP through the 5'UTR of its genome but the nature of that interaction has yet to be elucidated (251, 252).

3.1 Research Objectives

As mentioned in the above text, questions remain whether the nuclear egress of C-capsids is mediated through the physical interaction of the CVSC and the NEC, or if there is another protein, or subset of proteins, bridging the interaction with C-capsids and the NEC to facilitate specific egress of C-capsids. The Lippé lab previously performed a proteomic analysis of the nuclear capsids which resulted in a list of host and viral proteins associated with C capsids (253). The list of host protein hits, specifically PCBP1, is what motivated this master's thesis.

The overall research objectives of this project were to determine the role of PCBP1 in HSV-1 infection through molecular biology techniques. We aimed to report the changes in viral titers when PCBP1 was knocked down or overexpressed using RNAi knockdown (KD) or lentivirus overexpression, respectively. We also designed experiments to investigate the specific role of PCBP1 depletion on viral capsid maturation and localization using both immunofluorescence (IF) and electron microscopy (EM). Finally, we focused on the RNA binding capability of PBCP1, analysing the HSV-1 genome for PCBP1 consensus sequences.

3. Methods

3.1 Cell Lines

HeLa cells (cervical adenocarcinoma) and Vero cells (African Green Monkey cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Wisent Bio Products) supplemented with 2 mM L-Glutamine (Sigma-Aldrich), 5 % bovine growth serum (BGS, GE Healthcare) and 1 % penicillin and streptomycin (Sigma-Aldrich). Cells were routinely tested for mycoplasma contamination using the e-myco mycoplasma detection kit (iNtRON Biotechnology).

3.2 Viruses

3.2.1 Viruses and Propagation

Two strains of HSV-1 were used throughout this dissertation. 1) HSV-1 Strain 17+ was acquired from Dr. Beate Sodeik (Institute of Virology, Hannover Medical School, Germany) and used for routine infections. 2) HSV-1 17+ VP16-GFP was a gift from Dr. Peter O'Hare (Imperial College London, Great Britain) and was used in immunofluorescence and flow cytometry analyses. Stocks of both viruses were generated by infecting monolayers of Vero cells with small amounts of virus. Upon observing cytopathic effect, cells and supernatant were harvested, centrifuged, and resuspended in MNT (30 mM morpholinoethanesulfonic acid, 100 mM NaCl, and 20 mM Tris, pH 7.4). The cell fraction went through three cycles of freeze-thaw and all samples were sonicated briefly before being placed at -80 °C for storage.

3.2.2 Infection

Prior to the infection, HeLa cells were seeded in the appropriate dish for 24 hours at 37 °C, 5 % CO_2 so that they reached a confluency of 80-90 % for the next day. The day of the infection, one well of cells was trypsinized, suspended in culture medium, and counted. The number of cells was used to determine the amount of virus in plaque forming units (PFUs) to reach a multiplicity of infection (MOI) of 5. The probability of an infectious agent interacting and infecting a target can be described by Poisson distribution, such that when five particles of HSV-1 are added per cell (MOI of 5), 99% of the cells are infected (8). The viral stock was diluted in Roswell Park Memorial Institute (RPMI, Wisent Bio Products) media containing 0.1 % bovine serum albumin (BSA, Sigma-Aldrich) and added to the cell monolayer. Cells were returned to the 37 °C

incubator and were rotated gently every 15 min for 1 hr to ensure equal coverage cell monolayer with the viral inoculum. After 1 hour of absorption, complete 1x DMEM was added to the infected cells. Eight to forty-eight hours post infection (hpi), the supernatant and the cell lysates were harvested for subsequent analysis. The supernatant was harvested and centrifuged at 500 x*g* for 10 min at 4 °C to remove cell debris. The cell lysate was harvested by scraping the monolayer of infected cells in 1x phosphate-buffered saline (PBS), pelleting at 500 x*g* for 10 min at 4 °C and resuspending in 1x PBS. One-third of the lysate, along with the supernatant, was flash-frozen in liquid nitrogen and thawed three times before being stored at -80 °C as the intracellular and extracellular viral fraction, respectively. The remaining two-thirds were pelleted, then lysed by addition of Radioimmunoprecipitation assay buffer (RIPA buffer) containing a cocktail of proteases (CLAP) for 30 min on ice. Lysates were sheared through a 27-gauge needle before being centrifuged at 12,000 x*g* for 10 min at 4 °C. The supernatant was harvested and stored at -20 °C for further analysis.

3.3 RNA Interference

HeLa cells were seeded at $1x10^5$ cells per well in a 6-well dish. The next day dsiRNA transfection was performed with LipoJet (SynGen Laboratories) according to manufacturer's instructions. Briefly, 25 nM of a non-targeting dsiRNA (NC1, Integrated DNA Technologies (IDT)) or 25 nM of dsiRNA targeted to PCBP1 (hs.Ri.PCBP1.13.1, IDT) was added to 100 µL of 1x LipoJet buffer, mixed, and 2 µL of LipoJet reagent was added. The solution was incubated for 10 min at room temperature (RT) before being added to cells dropwise. Twenty-four hours after transfection, 2 mL of DMEM was added to the cells. Forty-eight hours after transfection cells were either harvested or infected, as described above.

3.4 Viability Test

HeLa cells were seeded at $8x10^3$ cells per well in a 96-well Greiner black plate 24 hours prior to the viability test. Cells were treated with 25 nM of control dsiRNA or dsiRNA directed at PCBP1 as described above. Forty-eight hours post transfection, 10 µL of alamar blue (Bio-Rad) was added per well and incubated for 4 hrs at 37 °C. Alamar blue alone was diluted 1:10 and autoclaved to reduce completely, before being added to the plate as a positive control. After incubation, the plate was read with the SpectraMax Gemini EM spectrophotometer without the lid of the plate.

3.5 Plaque Assay

Viral titers of extracellular and intracellular fractions were determined by plaque assay. The day before infection Vero cells were seeded to be confluent the next day. Virus samples were serially diluted in RPMI medium supplemented with 0.1 % BSA. Two wells of a confluent six well plate had 200 µl of virus dilution per well, or RPMI alone, added to it for 1 hr at 37 °C, shaking every 15 min. After absorption, 5 mL of a 1:1 mix of 2 % Agarose (Fisher BioReagents) and 2X DMEM were added to the cells. The plates were placed back at 37 °C for 72 hours. After 72 hpi, the agarose plugs were removed and the cell monolayer was fixed with 100 % methanol at -20 °C. Crystal violet was used to stain the monolayer, and the least diluted samples containing 20-200 plaques were counted.

3.6 Lentiviral Transfection and Transduction

3.6.1 Cloning of the lentiviral vector

The plasmid pHAGE-EF1a-MCS-IRES-zsGREEN was a gift from Dr. Etienne Gagnon (Institute for Research in Immunology and Cancer (IRIC) of the Université de Montréal, Montreal, Quebec, Canada). Using Gibson assembly, PCBP1 was amplified from pT7-V5-SBP-C1-HshnRNPE1 (Addgene, plasmid #64921) and cloned into pHAGE-EF1a-MCS-IRES-zsGREEN with the primers shown below. The forward primer contains 40 bp homology to the pHAGE plasmid and the reverse primer contains 40 -bp of homology in addition to the P2A peptide sequence. The P2A peptide was placed in between the PCBP1 and the zsGREEN coding sequences to facilitate 1:1 expression through ribosomal ‘skipping’ (254). This allows a marker of gene expression (zsGreen) to be added without introducing the possible complications of a fusion protein, such as preventing protein function at the tagged protein terminus.

Forward: 5’
TCCATTTCAGGTGTCGTGAAGCGGCCGCACCGGTCTGCAGATGGATGCCGGTGTGAC
TGA 3’

Reverse: 5’
ATGGTCATCTCCTTGGTCAGGCCGTGCTTGGACTGGGCCATGGGACCGGGGTTTTCT

TCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGCTGCACCCCATGCCC TTCT 3’

pHAGE-EF1a-MCS-IRES-zsGREEN was linearized using NheI and NdeI restriction enzymes, mixed with the PCBP1 PCR product at a molar ratio of 1:3 and 2X Gibson Reagent as described (255). Briefly, the reaction was incubated at 50 °C for 1 hr before being diluted 1:5 and adding to 50 µl of chemically competent JM109 *Escherichia coli* (*E. coli*) cells for transformation. Positive colonies were grown overnight in a 37 °C incubator with agitation and the plasmid was extracted using Nucleobond Midi Prep kit (Macherey-Nagel) according to manufacturers instructions.

3.6.2 Transfection

The production of these cell lines was performed in collaboration with the Gagnon Lab. The cell lines were made using a third generation lentivirus system (256).The day before transfection, a confluent dish of Hek 293 clone 17 cells was split into two wells of a 6-well dish. The next day, 375 µL of RPMI was mixed with 1.25 µg of pHAGE-PCBP1-P2A-zsGreen, 0.3125 µg each of packaging plasmids pMD2-pRRE and pRSV-REV, and 0.625 µg of pMD2-VSVg plasmids which encode Gag and Pol, Rev, and VSVg envelope protein, respectively. The plasmid solution was vortexed, and 7.5 µl of TransIT (Mirus Bio) was added. The transfection mixture was incubated at RT for 15 min before being added dropwise to the cells, which were returned to 37 °C for 12 hrs. Supernatant was removed from the cells and 4.5 mL of DMEM supplemented with 15 % fetal bovine serum (FBS) and 25 mM HEPES was added. After 36 hrs the supernatant was collected and used immediately for transduction.

3.6.3 Transduction

The collected virus was centrifuged at 2000 x*g* for 5 min to remove any cellular debris and the virus was diluted 1:3 in DMEM before being added to a 70 % confluent well of HeLa cells in a 6-well dish. The cells were incubated at 37 °C for 12 hrs at which time the inoculum was removed and placed in standard DMEM. The cells were kept in cell culture for 1 week before used for experiments to the ensure the absence of active lentivirus.

3.7 Western Blotting

Samples were collected and processed as previously described in 3.2.2 Infection. Protein samples were quantified using the Pierce BCA Protein Assay Kit (ThermoFisher). Typically, 25 µg - 35 µg were loaded on 10 % acrylamide SDS-PAGE gels in protein sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 2% β-mercaptoethanol). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad), which were incubated for 1 hr in blocking buffer (5% non-fat dry milk, PBST (1x PBS and 0.1 % Tween 20)). The membranes were treated with primary antibodies overnight at 4 °C or 1 hr at RT. All antibodies were diluted in 2.5 % non-fat milk in PBST, specific dilutions can be found in Table 1. Membranes were washed three times with PBST before secondary antibody was added for 1 hr at RT. Protein bands were visualized with the ChemiDoc XRS+ system (Bio-Rad) or the G:Box Chemi XRQ (Syngene) after addition of enhanced chemiluminescence substrate (ECL, Bio-Rad).

Protein	Antibody	Dilution	Secondary
PCBP1	Abcam (ab74793)	1:500	Rabbit
hnRNP-E1 (E2)	Santa Cruz (sc-137249)	1:500	Mouse
VP5	East Coast Bio (HA018)	1:1000	Mouse
GAPDH	Millipore Sigma (MAB374)	1:10000	Mouse
β-Actin	Abcam (ab6276)	1:5000	Mouse
UL25	Dr. William Newcomb	1:5000	Mouse
UL24	Dr. Angela Pearson	1:500	Rat
Remus V	Dr. Beate Sodeik	1:1000	Mouse

Table 1. Primary Antibodies

3.8 Immunofluorescence

Cells seeded on coverslips were fixed with at RT with a 4 % paraformaldehyde (PFA) solution and placed in blocking buffer for 1 hr (1x PBS, 5 % FBS, 0.3 % Triton™ X-100). Afterwards coverslips were placed directly in primary antibody in antibody dilution buffer (1x PBS, 1 % BSA, 0.3 % Triton™ X-100) overnight at 4 °C, or for 2 hr at RT. After three washes with 1x PBS, the secondary antibody donkey anti-mouse Alexa 647 (Molecular Probes, 1:1000) was added to the cells for 1 hr. Coverslips were mounted

in Hoechst-Dako (Agilent) on a microscope slide, and images of the cells were captured with a Leica DMi8 wide-field microscope and analyzed with LAS X software (Leica).

3.9 qPCR

3.9.1 RT-qPCR

RNA from HeLa cells was extracted using the SV Total RNA Isolation System (Promega Corporation) per manufacturer's instructions. After extraction, RNA was measured using a Nanodrop-1000 spectrophotometer (ThermoFisher) so that 1 µg of RNA was used to make cDNA with the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosciences). cDNA was diluted 1:2 with nuclease-free water (NFW). All reverse-transcriptase quantitative polymerase chain reactions (RT-qPCRs) were done with Perfecta Syber Green supermix (Quanta Bioscience) and analyzed by a Lightcycler 96 (Roche). Primers were designed on SnapGene (GSL Biotech LLC) (Table 2). All cDNA levels were normalized to an internal control (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)).

3.9.2 Total Viral Genome Copies

Quantification of the viral genomic DNA in the intracellular fraction was performed by qPCR using the Perfecta Sybr Green supermix. Viral gene gB was used as a proxy for genome copies. Briefly, viral genomic DNA was extracted from each sample using the GenElute mammalian genomic DNA miniprep kit without the use of DNase (Sigma). Sybr Green was mixed with the extracted DNA and primers to amplify gB using a Lightcycler 96. To convert the Cq value into absolute DNA copy numbers, a standard curve was also performed using a known amount of a plasmid (pCyto-gBFull) containing the sequence for gB. The plasmid pCyto-gBFull was a generous gift from Dr. Michel Desjardins (Department of Pathology and Cellular Biology, University of Montreal).

3.10 Flow Cytometry

Confluent 10 cm dishes of cells were trypsinized and collected in culture media. Cells were centrifuged at 300 x*g* for 5 min at 4 °C and washed twice with 1 mL of 1x PBS. Cells were pelleted as before, and the supernatant was removed. The cells were fixed by resuspending the pellet in 2 % PFA for 10 min at RT. Samples were spun as before, washed twice with 1x PBS, and resuspended in PBS + 1 % BGS. Cells were vortexed well and transferred to 5 mL polystyrene

tubes. These samples were analyzed by an LSR Fortessa Flow Cytometer (BD Biosciences) with the GFP channel to detect zsGreen.

Primer Sequences		
Gene	Protein	Sequence (5'-3')
PCBP1	PCBP1	F: GGAAGCATCATTGGGAAGAA R: TCTTCCTCCAGCTTGTCGAT
IFN-β	IFN-β	F: AAACTCATGAGCAGTCTGCA R: AGGAGATCTTCAGTTTCGGAGG
RL2	ICP0	F: CTGTCGCCTTACGTGAACAA R: CATCCAGAGGCTGTTCCACT
RS1	ICP4	F: CGACACGGATCCACGACCC R: GATCCCCCTCCCGCGCTTCGTCCG
UL29	ICP8	F: ACATTACGTTCACGGCCTTC R: GGCCATCGACACGATAGACT
UL23	TK	F: GTAATGACAAGCGCCCAGAT R: ATGCTGCCCATAAGGTATCG
UL48	VP16	F: GGACGAGCTCCACTTAGACG R: AGGGCATCGGTAAACATCTG
UL31	pUL31	F: GTGAAGACCACTCCCGTCTC R: ATCGTGTTGATCTGCTGCAC
UL55	gB	F: TTTGTGTACATGTCCCCGTTTTAC R: AGAAGCCGTCGACCTGCTT
UL19	VP5	F: CTTCTGCGAGACGAGCTTTT R: CCACTTTCAGGAAGGACTGC
GAPDH	GAPDH	F: GAGTCAACGGATTTGGTCGT R: TTGATTTTGGAGGGATCTCG

Table 2. Primer Sequences

3.10.1 Fluorescent-associated cell sorting (FACS)

Confluent 10 cm dishes of cells were trypsinized, collected in a 15 mL tube and placed on ice immediately. Cells were washed twice by centrifuging at 500 x*g* for 5 min at 4 °C and gently resuspended in 1x PBS. Supernatant was removed and cells were resuspended in 500 µL/10 cm dish in sort buffer (1x PBS, 1 mM EDTA, 25 mM Hepes pH 7.0, 1 % FBS). The cells were transferred into 5 mL polystyrene tube through a 35 µM cell strainer cap. The cell strainer cap was

then replaced with a standard cap. All samples were sorted on a FACs Aria II Cell Sorter (BD Biosciences) at the Cytometry Platform at CR CHU-Ste Justine. After sorting, samples were transferred to a 15 mL tube and DMEM was added for a total volume of 10 mL. The samples were spun at 500 x*g* for 5 min at 4 °C and resuspended in sufficient media depending on size of the culture dish.

3.10.2 FACS with Immunolabeling

Cells were treated with dsiRNA as described above and infected with HSV-1 VP16-GFP at an MOI of 5. After 18 hpi, cells were trypsinized, counted and re-pelleted by centrifuging at 500 x*g* for 5 min at 4 °C. After fixing, cells were treated with BD Cytoperm/Cytofix kit (BD Biosciences) following manufacturer's instruction. Briefly, cells were blocked in 10% fetal calf serum (FCS) + 1x PBS solution for 30 min at RT. Cells were pelleted as above and resuspended in 100 µL of primary antibody in BD PERM wash buffer at a 1:100 dilution for anti-VP5. This solution was incubated overnight at 4 °C. After the incubation cells were washed three times with 1x PBS and resuspended in secondary antibody goat anti-mouse Alexa 568 (Molecular Probes) at 10 µg/mL for 2 hours at 4 °C. Cells were washed twice with 1x PBS and resuspended in 500 µL PBS+ 1 % FCS solution. These samples were analyzed by LSR Fortessa Flow Cytometer (BD Biosciences) with the GFP channel for VP16-GFP the PE-CF549 channel for VP5.

3.11 DNA Packaging Assay

A DNA packaging assay was performed as previously described by Hodge *et al.*(257). HeLa cells were transfected with non-targeting dsiRNA or dsiRNA against PCBP1 as described above. After 48 hrs post transfection, cells were infected with HSV-1 Strain 17 at an MOI of 5 for 16 hrs. At this time, cells were harvested and resuspended in 400 µL of resuspension buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$) with NP-40 at 1 % and divided into two samples: DNase I treated and DNase I untreated. DNase I treated cells were mixed with 200 µg of DNase I and were incubated for 20 min at 37 °C. After incubation, 200 µL of lysis buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 1.2 % SDS) with 1 mg of protease K was added to both DNase I treated and untreated samples which were then incubated at 37 °C for 1 hr. After 1 hr incubation with lysis buffer, DNA was extracted using phenol-chloroform. Briefly, 500 µL of Ultrapure phenol:chloroform:isoamyl alcohol (25:24:1, v/v, Fisher Scientific) was added to the samples

which were then vortexed for 10 s and centrifuged at 12, 000 x*g* for 15 min at 4 °C. The aqueous phase was transferred to a clean 1.5 mL Eppendorf tube and the process was repeated with chloroform only (Fisher Scientific). To the extracted aqueous phase, 1 mL of 95% ethanol was added, and samples were placed at -80 °C for 15 min to facilitate nucleic acid precipitation. Afterwards, the samples were spun at 12,000 x*g* for 15 min at 4 °C. Supernatant was carefully removed and the pellet was resuspended in 300 mM Sodium Acetate. To remove RNA, 20 µL of 1 mg/mL RNase A (Roche) was added and the samples were incubated at 37 °C for 45 min. DNA was precipitated by the addition of 750 µL of 95 % ethanol and incubation at -80 °C as described above. The pellet was washed with 70 % ethanol and then 95 % ethanol to removes salts before being dried and resuspended in nuclease-free water. Total genome copies were calculated by qPCR as described as above.

3.12 Statistics

Two-tailed student T-tests were performed to determine all statistical significances of our data using Prism8 (Graphpad). Difference between the control and the experimental condition was considered statistically significant when *p* value was smaller or equal to 0.05.

4. Results

4.1 PCBP1 depletion decreases HSV-1 titers independently of IFN-β

Mass spectrometry data from a previous study in the Lippé Lab revealed the presence of PCBP1 on HSV-1 C-capsids, leading us to hypothesize it may play a role in C-capsid nuclear egress (253). To test the importance of PCBP1 during infection, RNA interference was used to deplete PCBP1 from the same cells as the above study, i.e. HeLa cells. Three separate dsiRNAs were tested for efficacy in PCBP1 KD at 10 nM and 25 nM concentrations (Figure 6A). As dsiRNA against PCBP1 at best minimally decreased mRNA levels by 20 % at 10 nM, we moved forward with dsiRNA at concentrations of 25 nM since they efficiently reduced PCBP1 transcript levels by 63 % to 80 % depending on the dsiRNA. Cells were depleted for PCBP1 and were evaluated for change in viability, of which none showed a decrease (Figure 6B). The first dsiRNA, siPCBP1 #1 had the highest KD efficiency at an 80 % reduction of PCBP1 compared to cells treated with a non-targeting (NT) dsiRNA (Figure 6A). The KD of PCBP1 mRNA was also reflected at the protein level as seen by western blot (Figure 6C). As such, we used the PCBP1 #1 dsiRNA at concentrations of 25 nM in all subsequent experiments.

To determine the impact of PCBP1 reduction on viral propagation, PCBP1 depleted cells were infected with HSV-1 at a MOI of 5 and the supernatants and cell fractions were collected at various times across infection. PCBP1 KD decreased extracellular HSV-1 titers significantly with an average inhibition of 55 %, 61 % and 47 % for 12 hpi, 24 hpi and 48 hpi, respectively (Figure 7A). However, while no statistically significant decrease was observed for intracellular fractions in cells depleted of PCBP1 compared to NT dsiRNA, a slight and reproducible reduction of 24%, 45%, and 25% was noted for 12 hpi, 24 hpi, and 48 hpi, respectively (Figure 7B). As discussed above, PCBP1 binds to MAVS and shuttles it to the proteasome for degradation (236). The reduction of PCBP1 may cause an increase in MAVS which would increase IFN-β production and inhibit HSV-1. To explore this affect, IFN-β expression was examined by RT-qPCR during infection. IFN-β mRNA increased 56 % in mock infected PCBP1 KD cells which aligns with the previous data that PCBP1 depletion increases signaling through MAVs (236). In contrast, there were no significant differences between NT dsiRNA and siPCBP1 conditions in HSV-1 infected cells, with

an average increase of a modest 14 % (Figure 7C). Together these data suggest depleting PCBP1 inhibits overall production of HSV-1 in an IFN-β independent mechanism.

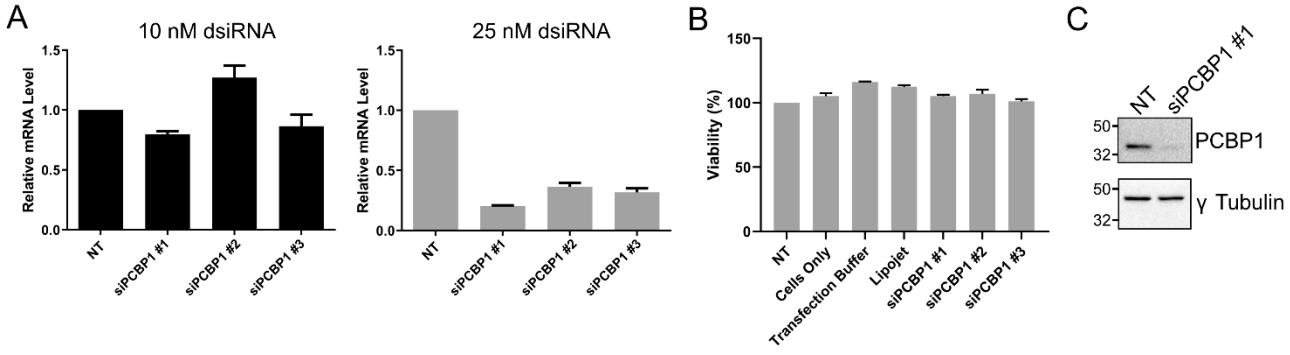

Figure 6. – Efficient depletion of PCBP1 by RNA interference

A) HeLa cells were transfected with three separate dsiRNA targeting PCBP1 at 10 nM or 25 nM for 48 hrs. RNA was harvested and RT-qPCR was performed to measure PCBP1 mRNA levels. **B)** HeLa cells were transfected with three separate dsiRNA targeting PCBP1 at 25 nM for 48 hrs and an alamar blue viability assay was performed. A and B were repeated for three technical replicates. **C)** HeLa cells were transfected with siPCBP1 or a NT dsiRNA for 48 hrs and cell lysates were collected and used for immunoblotting against PCBP1 to monitor protein inhibition and γ tubulin was used as loading control.

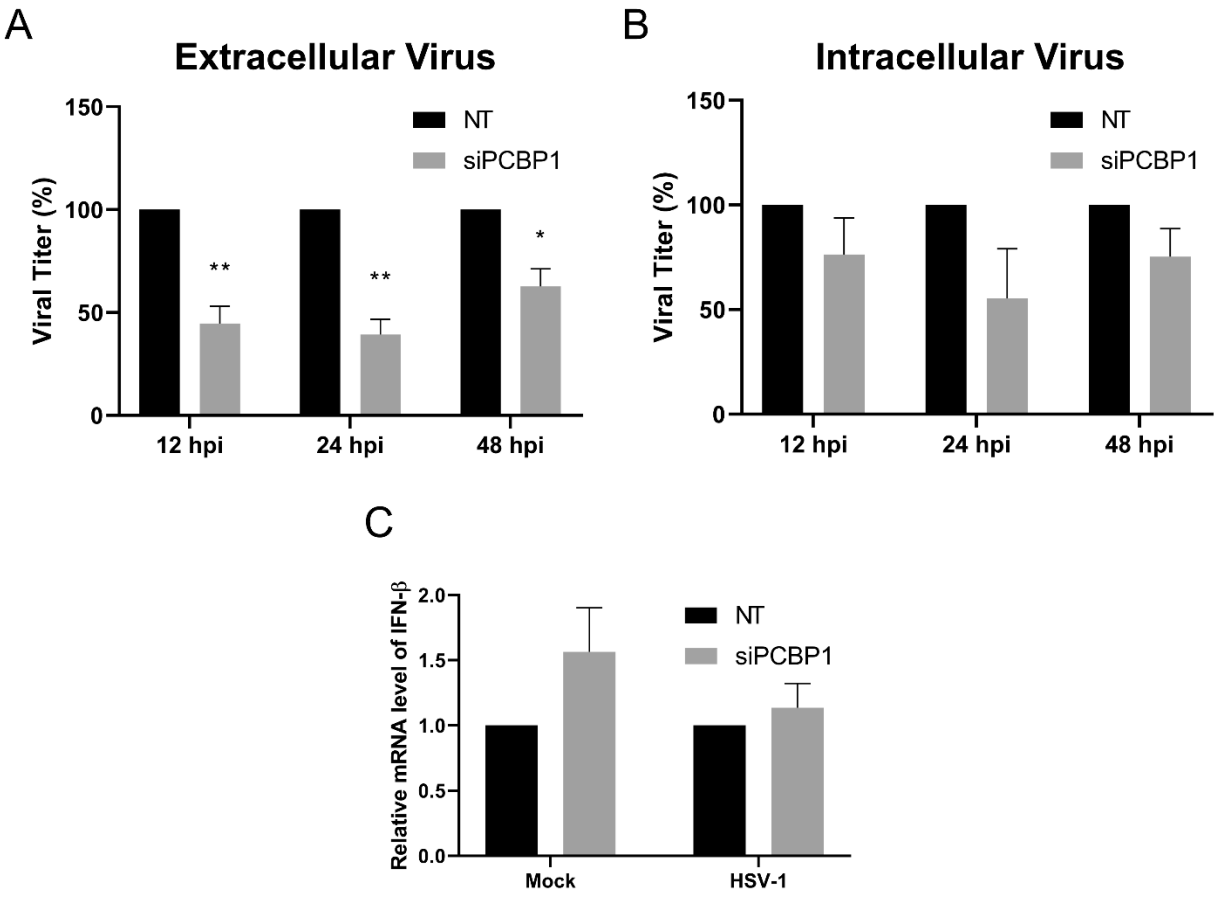

Figure 7. – PCBP1 depletion decreases viral titers independently of IFN-β

HeLa cells transfected with siPCBP1 or a control dsiRNA were infected with HSV-1 Strain 17 at a MOI of 5 for the indicated amounts of time. **A)** Supernatants or **B)** cells were harvested and titered by plaque assay on a monolayer of Vero cells. n=3 **C)** HeLa cells were infected as above, then cells were harvested 8 hpi. RNA was extracted and RT-qPCR was performed probing for IFN β. n=3. Error bars represent SEM and data was analyzed using unpaired student T-tests.

4.2 Overexpression of PCBP1 increases viral titers

As depletion of PCBP1 had a negative effect on HSV-1 propagation, we wanted to determine if overexpression of PCBP1 would increase HSV-1 titer. To test this, HeLa cell lines overexpressing PCBP1-ZsGreen was generated and used to assess the effect of PCBP1 overexpression on HSV-1. The PCBP1-ZsGreen cell line was analysed by flow cytometry and IF to ensure overexpression (Figure 8AB). The HeLa-PCBP1-zsGreen population was 88.5 % positive for GFP (Figure 8A). Both HeLa WT and HeLa-PCBP1-zsGreen cells were infected with HSV-1 at a MOI of 5 for 24 hrs. The supernatant and cell fractions were collected and titered by plaque assays. The viral titers in the extracellular fraction were increased 56 % compared to WT when PCBP1 was overexpressed (Figure 8C). Similarly, the intracellular fraction from PCBP1 overexpressing cells had a 59 % increase in viral titers from WT (Figure 8D). While both extra- and intracellular observations were not found to be statistically significant, the increase of viral titers was reproducible across three experiments, aligning with our knockdown data that suggests HSV-1 viral titers are positively correlated with PCBP1 expression.

4.3 Depletion of PCBP1 does not specifically inhibit VP5 expression

Since PCPB1 is a known regulator of gene expression and it favors HSV-1 propagation, we asked whether it might impact capsid assembly. To this end, VP5, the major capsid protein, was probed by IF to study its localization. HSV-1 capsids are too small to be seen individually by epifluorescence, requiring confocal or deconvolution microscopy (258, 259), however large changes in protein distribution are noticeable. After imaging PCBP1 depleted HeLa cells, no changes in localization were detected, but VP5 was found to be less bright in PCBP1 than in control cells (Figure 9A). When quantified over two experiments, VP5 was found to be significantly decreased by 65.4 % in the PCBP1 depleted cells when compared to control dsiRNA (Figure 9B).

To determine if the decrease of VP5 reflected a capsid assembly defect or an impact on viral gene expression, flow cytometry was used to quantify both the level of VP5 and the level of VP16, using a VP16-GFP tagged virus. VP16 is an important player in initiating the viral expression cascade and is not related to capsid structure allowing us to clarify whether PCBP1 causes an effect on capsid assembly, or a more general effect. In agreement with the above observations, a decrease in VP5 fluorescence was noted in PCBP1 depleted cells (Figure 10, right).

Interestingly, a similar decrease was also observed in VP16-GFP intensity suggesting that a general effect on viral gene expression rather than an impact on capsid assembly (Figure 10, left).

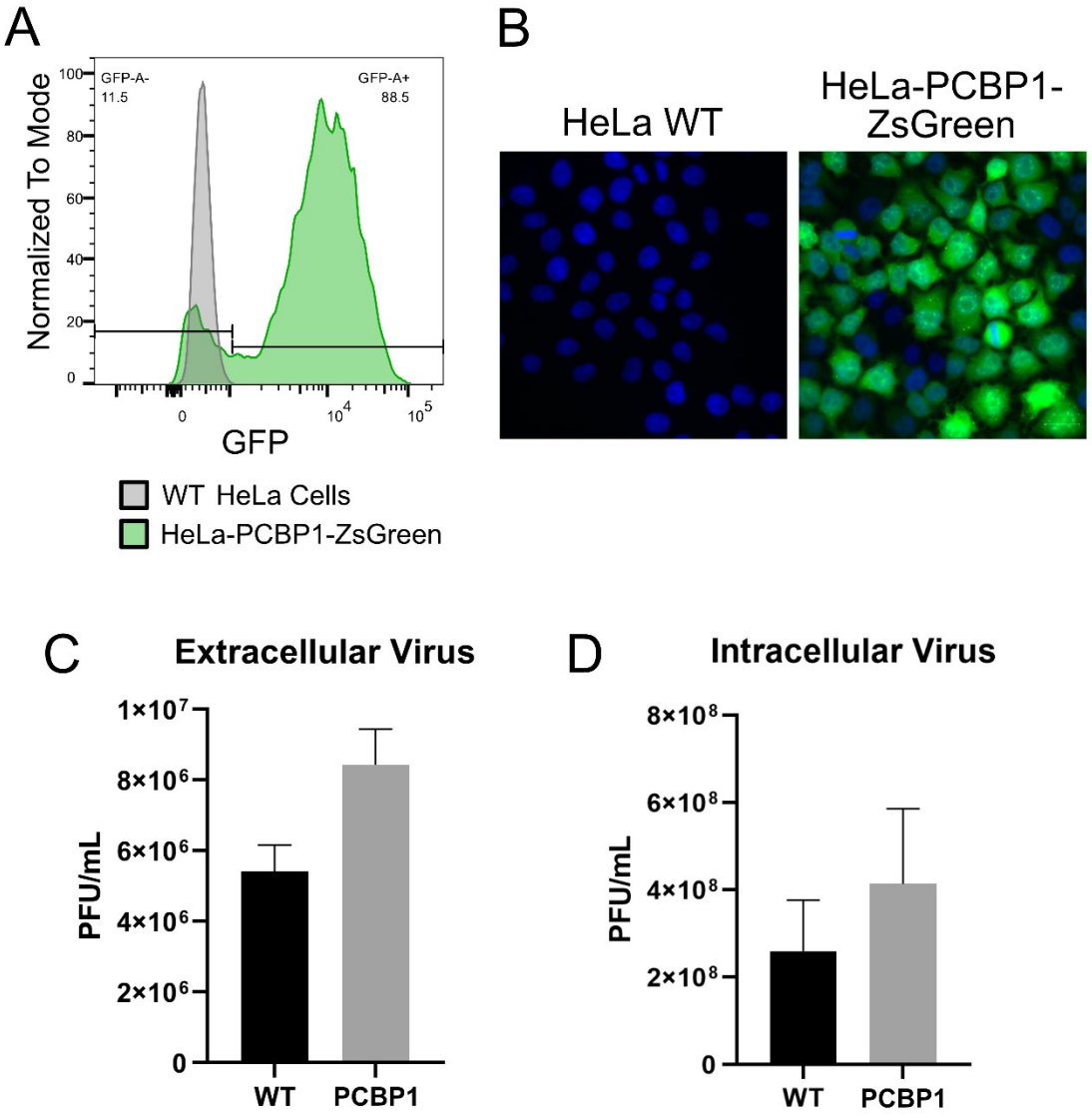

Figure 8. – Overexpression of PCBP1 increases HSV-1 viral titers

A) HeLa-PCBP1-ZsGreen cells were sorted on GFP **B)** Immunofluorescence of HeLa WT and HeLa-PCBP1-ZsGreen cell lines. PCBP1 is in a 1:1 ratio with GFP due to the presence of a P2A peptide between the coding sequences. **C-D)** HeLa WT and HeLa-PCBP1-ZsGreen cells were infected with HSV-1 strain 17 at a MOI=5 for 24 hrs. **C)** Extracellular fraction and **D)** intracellular fraction were titered by plaque assay on a monolayer of Vero cells. Data was analyzed with a student's T-test. n=3

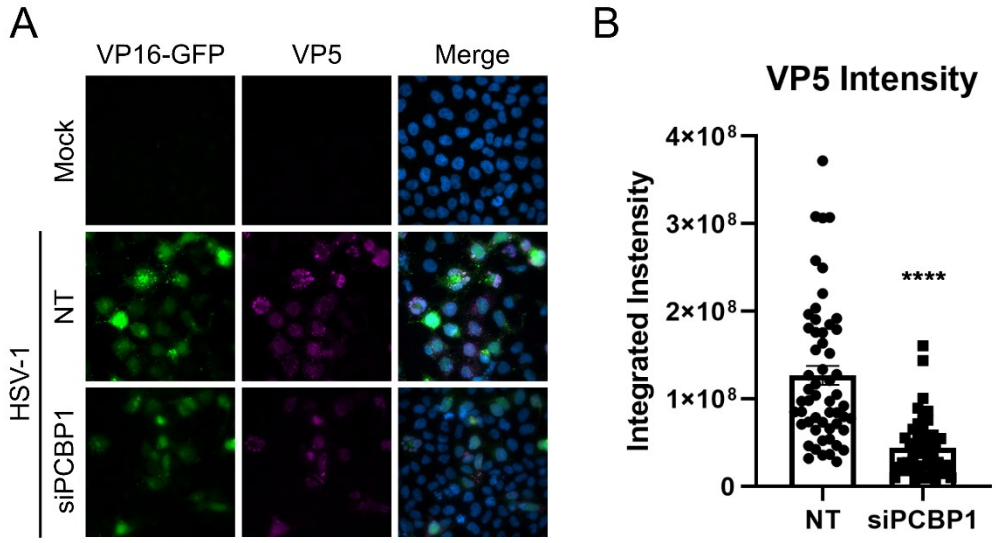

Figure 9. – PCBP1 knockdown decreases VP5 intensity in immunofluorescence

A) HeLa cells transfected with siPCBP1 or NT dsiRNA were infected with a MOI=5 of HSV-1 VP16-GFP for 18 hpi. Coverslips were fixed and probed with mouse anti-VP5 primary antibody and goat anti-mouse Alexa-568 secondary antibody. All images were taken on a Leica DMi8 wide field microscope. Representative images are shown **B)** Using Las X software, integrated intensity was measured, and a student's T-test was performed on PRISM. n=2.

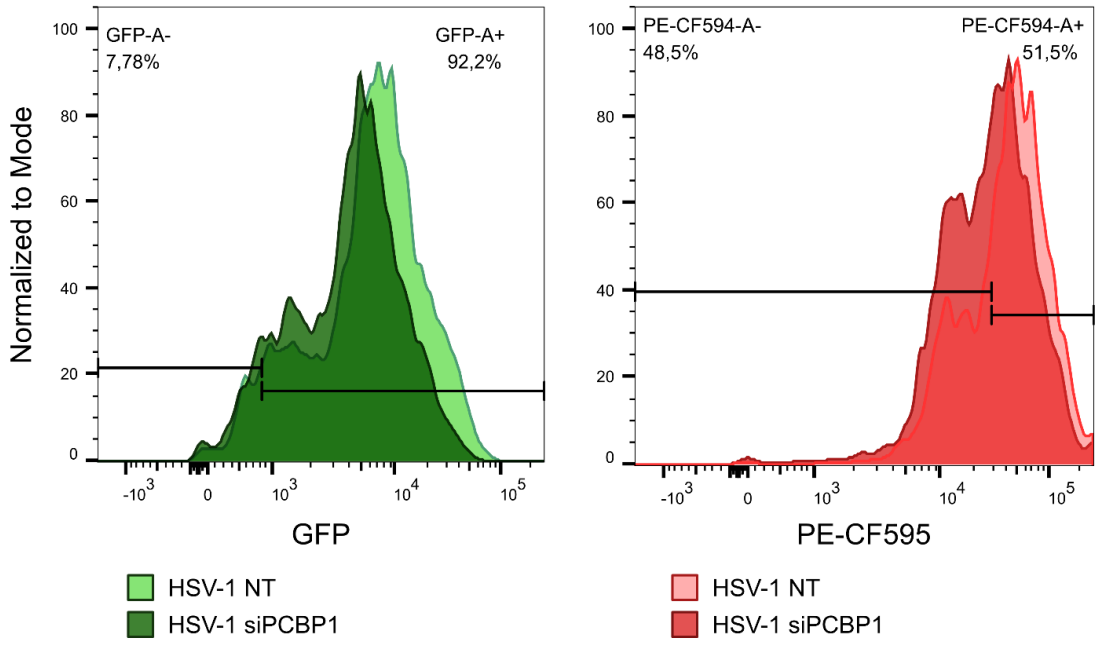

Figure 10. – Decrease in VP5 fluorescence is a non-specific effect

HeLa cells transfected with siPCBP1 or NT dsiRNA were infected with a MOI=5 of HSV-1 VP16-GFP for 18 hpi. Cells were fixed and probes with Anti-VP5 antibodies and cells were analyzed on a BD LSRFortessa. The baseline was set with no first antibody mock controls. Left panel is the GFP channel and the Right panel is the PE-CF595 channel.

4.4 PCBP1 only minimally impacts HSV-1 genome replication

Given the lack of effect on IFN-β production, we aimed to determine where and when PCBP1 was affecting the viral life cycle. To assess when PCBP1 is interacting with the genome replication machinery, viral genome copies in PCBP1 depleted or control non-depleted cells were detected by qPCR. HeLa cells with PCBP1 KD or control dsiRNA were infected with HSV-1 at a MOI of 5 for 12, 24 or 48 hpi, at which time DNA was extracted and prepared for qPCR. A plasmid coding for glycoprotein B was used to define absolute genome copy numbers. Despite the lack of statistically significant differences between the control dsiRNA and siPCBP1 treated cells, there was a clear and reproducible trend that genome copy numbers decreased with PCBP1 knockdown (Figure 11A). To determine if the infectivity of the newly assembled viral particles was affected by PCBP1 depletion, the ratio of PFU to genome copies was calculated and normalized to cells treated with control dsiRNA. There were no significant differences between PFU/genome copy ratios in PCBP1 depleted cells and control cells (Figure 11B). We conclude that PCBP1 only moderately impacts genome replication without an added effect on the infectivity of newly synthesized viral progeny.

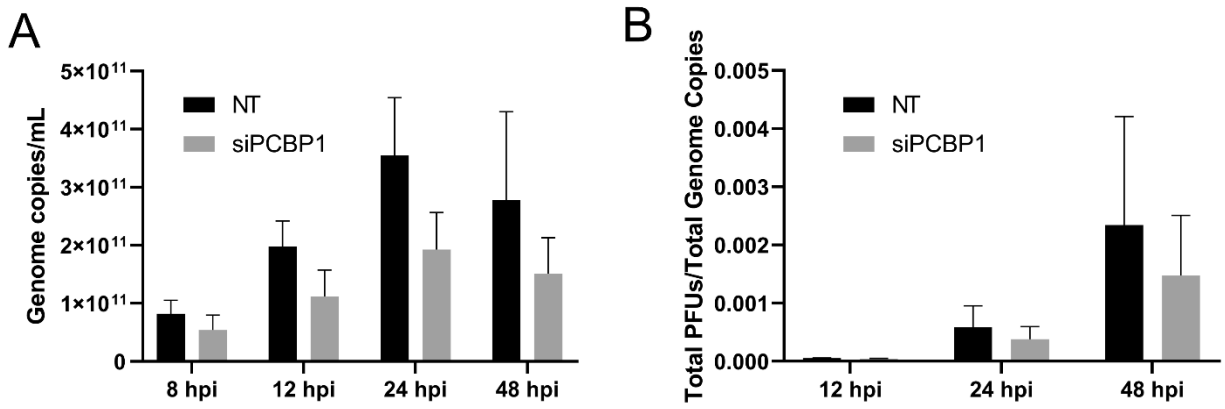

Figure 11. – Depletion of PCBP1 only minimally impacts genome replication

A) HeLa cells with or without PCBP1 KD, were infected as above and at the hours indicated on the graph, cells were harvested, and their genomes extracted. Genome count was assessed by qPCR, using gB as a proxy for genomes. n=3. **B**) Ratio of pfu: genome copy was calculated. n=3. Error bars represent the SEM and data was analyzed by an unpaired student's T-test.

4.5 PCBP1 KD does not specifically target IE, E or L gene groups

Knowing that PCBP1 was not limiting infection through the IFN-β pathway or through genome replication, but affected VP5 and VP16 gene expression, we wanted to assess the overall effect on viral gene expression by probing immediate early, early, and late genes. HeLa cells were depleted for PCBP1 and infected with HSV-1 at a MOI of 5. Two candidate gene products from each temporal gene group were analyzed by RT-qPCR to determine if PCBP1 depletion targeted a specific group. Immediate early gene candidates ICP0 and ICP4, early genes ICP8 and UL23, and late genes UL48 and UL31 were consequently monitored. Though not statistically significant, our RT-qPCR data in PCBP1 depleted HeLa cells show a slight and reproducible decrease in all genes tested (Figure 12). This data suggests that PCBP1 may enhance HSV-1 gene expression generally, as depletion of PCBP1 decreases expression all six genes tested.

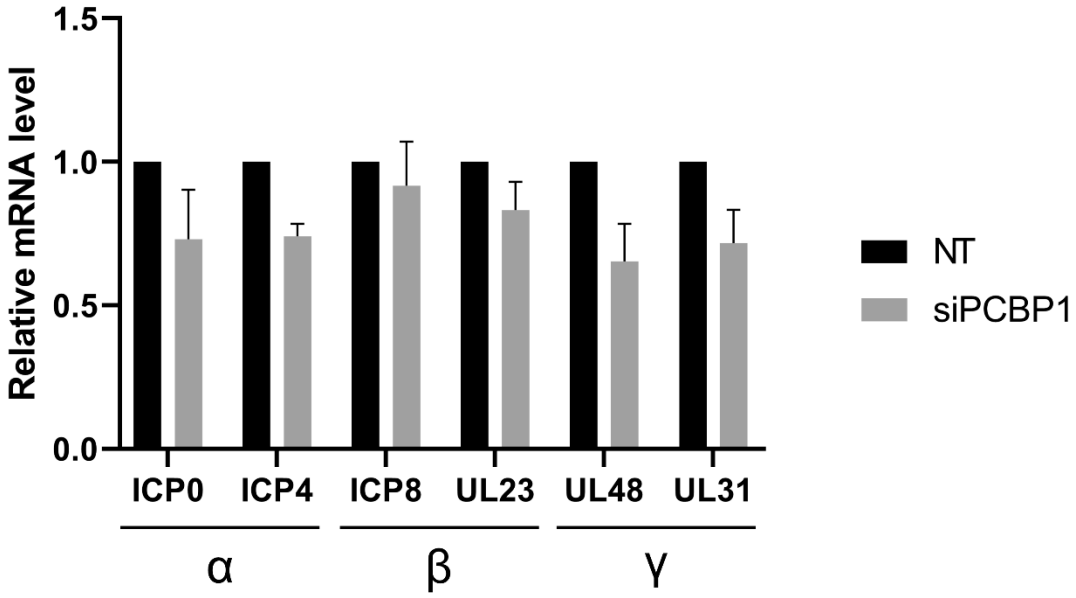

Figure 12. – PCBP1 does not specifically target IE, E or L gene groups

HeLa cells with or without PCBP1 KD, were infected with HSV-1 Strain 17 at a MOI=5 for 8 hr. At that time, RNA was harvested. RT-qPCR was done on cDNA for representative members of the three gene groups of HSV-1; α, β, γ. n=3. Error bars represent the SEM and data was analyzed by an unpaired student's T-test.

4.6 PCBP1 depletion decreases expression of 28 kDa viral protein

After determining the effect of PCBP1 KD was minimal on genome replication, and gene expression, we wanted to test the effect of PCBP1 depletion on protein levels. Using a polyclonal antibody that detects many proteins from the HSV-1 capsid, tegument, and envelope, for general changes in protein expression were analyzed (Figure 13). While no general decrease was observed with PCBP1 depletion, a clear decrease of a protein ~28 kDa in size after PCBP1 KD was detected (Figure 13). This protein was decreased by 31 %, 67 % and 53 % at 8 hpi, 12 hpi and 24 hpi, respectively. While there is no HSV-1 protein that corresponds to 28 kDa exactly, pUL24 is 30 kDa in size and pUL51 is a phosphoprotein with species present at 27, 29, and 30 kDa (260, 261). Interestingly, these proteins are both late proteins which matches well with our results that show lower expression at 8 hpi (Figure 13).

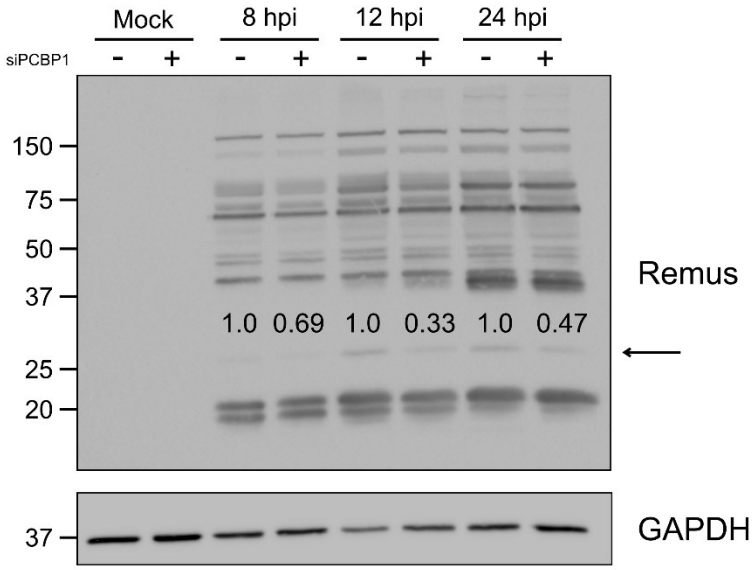

Figure 13. – PCBP1 depletion decreases expression of ~28 kDa viral protein

HeLa cells transfected with siPCBP1 or a control dsiRNA were infected with a MOI=5 of HSV-1 Strain 17 for the hours indicated. Cells were harvested and lysed in RIPA buffer before being run on an SDS-PAGE gel and transferred to PDVF for immunoblotting. This is a representative blot of n=3 experiments. Black arrow indicates the 28 kDa protein of interest. The numbers on the image represent the expression ratio between control dsiRNA and cells transfected with siPCBP1 for each time point.

4.7 PCBP1 may impact viral particle nuclear egress

Depletion of PCBP1 caused a decrease in viral titers of 50%, which is not fully explained by the modest decrease in viral mRNA and genome copies. Since previous work in our lab showed the presence of PCBP1 on C-capsids (253), we investigated nuclear egress and the proportions of A-, B- and C- capsids during PCBP1 KD. To do so we transfected HeLa cells with siPCBP1 or control dsiRNA as described above and infected with HSV-1 at a MOI of 5. At 16 hpi cells were fixed and prepared for transmission EM. Our analysis of 16 cells, eight per independent experiment, from NT dsiRNA or siPCBP1 treated cells determined that there were no significant differences in the relative expression of A-, B-, and C- nuclear capsids (Table 3). There was, however, a statistically significant decrease in extracellular virions for PCBP1-depleted cells, with a reduction from 20.4% to 9.2% of total viral particles (Table 3). This reduction of roughly half supports our initial observation that titers decrease ~50 % with knockdown of PCBP1 (Figure 7A).

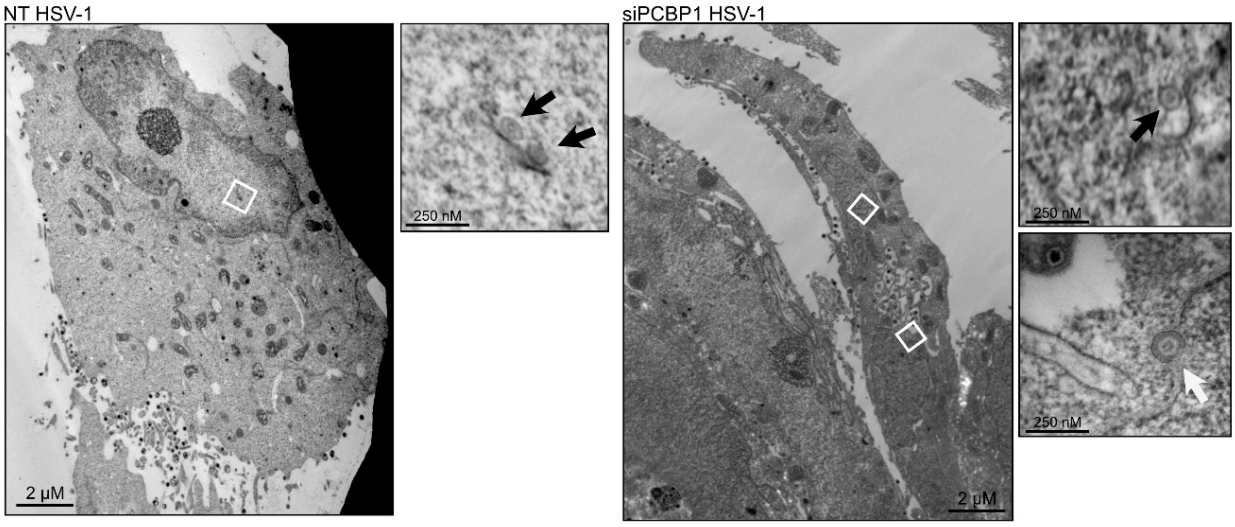

Figure 14. – Electron microscopy of PCBP1 depleted cells

HeLa cells treated with control dsiRNA (left-panel) or siPCBP1 (right-panel) were infected at an MOI=5 for 16 hr. Cells were fixed and prepared for transmission electron microscopy. Black arrows point to unenveloped B-capsids in the nucleus. White arrows point to enveloped B-capsids in the PNS. The white boxes encompass the area of the magnified images.

While electron micrographs that capture perinuclear virions are rare owing to their transient nature, we unexpectedly observed more enveloped B-capsids in the PNS in of PCBP1 KD cells than those treated with a control siRNA (Figure 14). B-capsids in the PNS are rare, but observable events in normal infection (262). B-capsids in the PNS made up 0.1 % of total viral particles in control cells, while they made up 0.6 % in PCBP1 depleted cells (Table 3). The increase of enveloped B-capsids may suggest a defect in DNA packaging resulting in more B-capsids therefore increasing the rare chance of B-capsid egress, or it could point to a lack of C-capsid specificity in nuclear egress. Our data from EM shows an increase of B-capsids in siPCBP1 cells of 20 % from control cells (Table 3), however this observation is not statistically significant as the increase in B-capsids was not as substantial in the second repeated experiment. Overall, we conclude that the EM data matches our titer data that less viral particles are being made in PCBP1 depleted cell, it also reinforces the hypothesis that PCBP1 could be acting at the nuclear egress step in the HSV-1 life cycle as we observe an increase in enveloped B-capsids in the PNS.

		NC1			siPCBP1			
		Total Count			Total Count			p-Value
	Types of Particles	N1	N2	% of Total Particles	N1	N2	% of Total Particles	
Nuclear	A-capsids	50	87	9.4 ± 2.9	39	97	6.9 ± 1.9	0.543257
	B-capsids	355	292	43.8 ± 2.5	591	530	62.5 ± 14.2	0.333287
	C-capsids	20	48	4.7 ± 2.1	33	42	4.1 ± 0.2	0.78587
Mature	Perinuclear viral particles							
	A-capsids	1	0	0.07 ± 0.07	3	0	0.09 ± 0.1	0.396756
	B-capsids	1	1	0.1 ± 0.006	6	4	0.6 ± 0.2	0.171908
	C-capsids	25	7	2.1 ± 1.1	17	14	1.7 ± 0.5	0.783093
	Cytoplasmic virions	150	136	19.4 ± 0.2	26	306	15.6 ± 12.1	0.779886
	Extracellular virions	165	136	20.4 ± 1.1	61	115	9.2 ± 1.3	0.021825

Table 3. Distribution of HSV-1 Particles in control and PCBP1 siRNA treated cells

4.8 PCBP1 depletion minimally affects genome encapsidation

The observation that PCBP1 depletion increased B-capsids in the nucleus and increased enveloped B-capsids in the PNS suggests that PCBP1 may be acting in viral nuclear egress or in viral genome encapsidation (Table 3). More enveloped B-capsids in the PNS may represent less stringent specificity in nuclear egress. It could also mean that less genomes are being incorporated into capsids due to a defect in genome encapsidation. A larger proportion of B-capsids in general may translate to an increase in the rare event of B-capsid nuclear egress. To investigate genome encapsidation, HeLa cells were treated with control dsiRNA or siPCBP1 and were infected with HSV-1 at an MOI of 5. At 16 hpi cells were harvested, lysed, and divided into two samples, one of which was treated with DNase I, and the other without. The initial lysis method breaks open both the cell and nucleus but leaves the capsids intact. This allows quantification of capsid-protected genomes by qPCR as DNase I treatment degrades replicating and unprotected viral DNA. When protected genomes from siPCBP1 treated cells were compared to control dsiRNA treated cells, a slight decrease of 32 % was observed in PCBP1 depleted samples (Figure 15). Total genome copies decrease 55 % in PCBP1 depleted cells compared to the cells treated with control dsiRNA (Figure 15). While neither of these results are statistically significant, the total genome copy data supports our previous results that PCBP1 depletion minimally affects genome replication (Figure 11A). The minor decrease of protected genomes in PCBP1 depleted cells suggests that DNA encapsidation is not the subject of PCBP1's pro-viral mechanism. However, it does not rule out the potential for PCBP1 to effect viral nuclear egress.

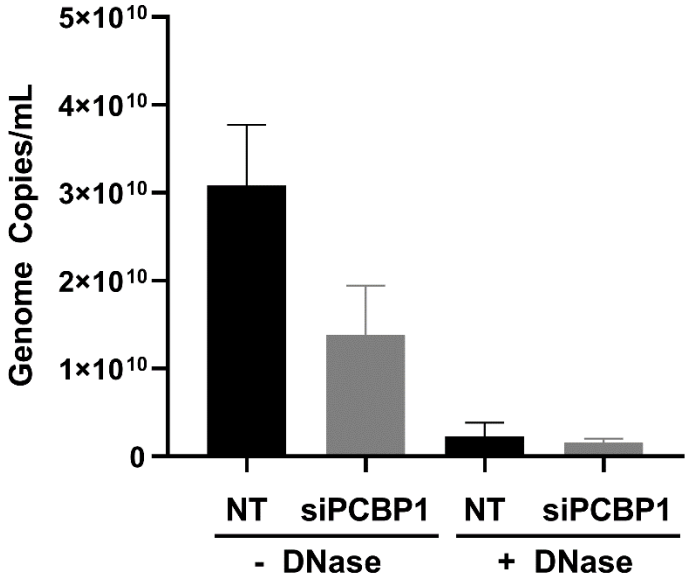

Figure 15. – PCBP1 depletion minimally affects genome encapsidation

HeLa cells treated with control dsiRNA or siPCBP1 were infected at an MOI of 5 for 16 hrs. Cells were harvested and divided into two sample which were either treated with DNase I or left untreated. DNA was extracted from these samples and qPCR was done to calculate the number of total genomes in each sample. n=3. Error bars represent SEM and data was analyzed using an unpaired student's T-test.

4.9 PCBP1 knockdown decreases pUL24 protein expression

The modest effect of PCBP1 on overall viral gene expression observed in Figure 12 was not overly surprising given PCBP1 tends to act on specific genes containing C-rich consensus sequences (263). Thus, we explored whether particular HSV-1 genes contain consensus sequences, such as the C-rich elements found in the promoter region of the MOR gene, in the 3' UTR of Sortilin, or in the 5' UTR of mRNA of PRL-3 (217, 264). Based on previous literature on PCBP1 consensus sequences, the analysis of the HSV-1 genome revealed two sequences of interest: 5' GCCCAG 3' which confers translational repression when bound to PCBP1 by preventing incorporation of the mRNA into polysomes (224), and 5' CCCCUCCCCC 3' that when bound by PCBP1 causes the stabilization of mRNA (217)(Table 4). Since our previous data suggests a pro-viral role for PCBP1, it was hypothesized that PCBP1 may be stabilizing viral mRNA which contain the 5' CCCCUCCCCC 3' sequence in the 3' UTR. Upon a bioinformatics analysis of the viral genome, one viral gene was identified with such a sequence in the 3' UTR, namely UL24 (Table 4).

Consensus Sequences	Activity	Reference	Binding Location	HSV-1 Genes
5' GCCCAG 3'	Translational Repressor	Wang *et al*., 2010	5' UTR	UL22 UL32
5' CCCCUCCCCC 3'	mRNA Stabilizer	Yabe-Wada *et al*., 2016	3' UTR	UL24

Table 4. PCBP1 Consensus Sequences

To determine if PCBP1 had a stabilizing effect on UL24 mRNA, cells were depleted of PCBP1 and UL24 mRNA expression and pUL24 protein levels were measured. As before, HeLa cells were transfected with NT dsiRNA, or dsiRNA specific to PCBP1. Two days after transfection, cells were infected, and lysates were harvested at 8, 12 or 24 hpi. At 8 hpi, pUL24 was poorly detected by Western blotting, but this is expected as it is a leaky-late protein (Figure 16A). At 12 hpi and 24 hpi, pUL24 protein levels were detected but decreased by 63 % and 40 % with PCBP1 knockdown, respectively (Figure 16A). When mRNA level of UL24 was measured by RT-qPCR, a modest average decrease of 24 % was observed that was not statistically significant (Figure 16B). The UL25 gene is directly downstream of the UL24 gene in the HSV-1 genome. As

pUL25 does not decrease with PCBP1 depletion, we also conclude that PCBP1 is acting on pUL24 specifically and does not act on neighbouring genes, if it interacts at the gene level (Figure 16A). Overall, these data reflect that PCBP1 influences pUL24 protein expression and has a modest affect on UL24 mRNA abundance.

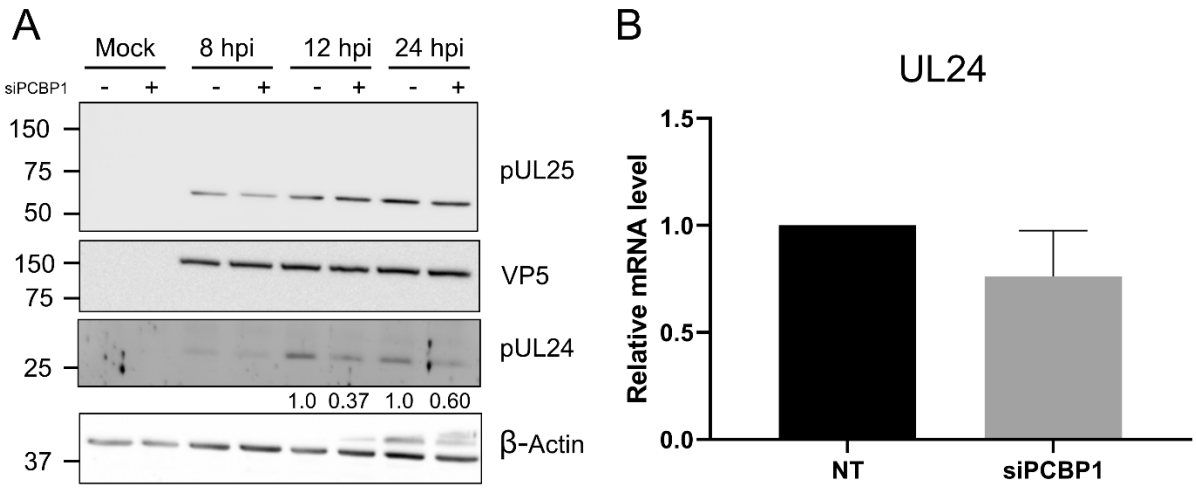

Figure 16. – PCBP1 decreases protein expression of pUL24

A) HeLa cells transfected with siPCBP1 or a control dsiRNA were infected with a MOI=5 of HSV-1 Strain 17 for the hours indicated. Cells were harvested and lysed in RIPA buffer before being run on an SDS-PAGE gel and transferred to PDVF for immunoblotting. **B)** HeLa cells transfected with siPCBP1 or a control dsiRNA were infected with a MOI=5 of HSV-1 Strain 17 for 8 hrs when RNA was extracted, and RT-qPCR was performed probing for UL24. n=3 for RT-qPCR, n=1 for western blot. Data was analyzed by an unpaired student's T-test. The numbers on the image represent the expression ratio between control dsiRNA and cells transfected with siPCBP1 for each time point.

5. Discussion

Previous work in our lab has shown that PCBP1 is present on C-capsids, but not on A- or B-Capsids, suggesting specificity (253). The current model for C-capsid-specific nuclear egress is based on the pUL25 and pUL17 population on capsid vertices: C-capsids have the majority of their vertices covered by the CVSC complex, while A- and B-capsids have less (265). This observation has been corroborated by new evidence that the interaction of pUL25 with the NEC is what instigates curvature of the inner nuclear membrane facilitating budding (147). However, this model does not fully explain the CSVCs found on A- and B- capsids, nor does it eliminate the possibility of protein interactors aiding or mediating C-capsid specificity. Taking our previous study and the gaps in knowledge for nuclear egress, this dissertation explored the role of PCBP1 in HSV-1 infection.

When cells were depleted of PCBP1 a significant decrease in extracellular viral titers was observed (Figure 7A). This supports the idea that PCBP1 is important for HSV-1 infection. Overexpression of PCBP1 also increased extracellular and intracellular HSV-1 titers further strengthening the pro-viral role of PCBP1 in HSV-1 (Figure 8C). PCBP1 is a known transcriptional regulator so to determine how PCBP1 was exerting a pro-viral effect on HSV-1, the temporal gene expression of HSV-1 was examined. All genes tested showed a slight decrease in expression in PCBP1 depleted cells, suggesting that PCBP1 may be important for general viral gene expression, however, it does not target a specific temporal group (Figure 12). The genomic copies of the virus were measured to determine if PCBP1 depletion affected viral DNA replication. As with the temporal genes, all time points reflected a decrease in genome copies with PCBP1 depletion (Figure 11A). Interestingly, the general protein expression was not affected by PCBP1 as seen by western blot (Figure 13), however, both VP5 and VP16 expression were lower in PCBP1 depleted cells when measured by FACS (Figure 10). FACS is more sensitive than western blotting and therefore the FACS data may be able to resolve smaller differences between PCBP1 KD and control cells. In fact, the mRNA expression data supports the FACs data as the UL48 transcript encodes the protein VP16 and both exhibit lower expression with PCBP1 depletion (Figure 10, Figure 12).

With a general pro-viral phenotype to PCBP1 but no overt answer on the exact mechanism, its role on capsid egress was investigated. PCBP1 was observed to be specifically associated with

C-capsids during HSV-1 infection, and therefore may act in capsid maturation or nuclear egress (253). When EM on PCBP1 depleted cells was performed, no retention of C-capsids was observed in the nucleus (Figure 14, Table 3). An increase in nuclear B-capsids and enveloped B-capsids in the PNS was observed (Figure 14, right panel). Of 16 PCBP1 depleted cells, 5 cells contained a combined 10 B-capsids in the PNS while 2 control cells had a single B-capsids each in the PNS, 0.6 % and 0.1 % of total viral particles, respectively. One explanation for this difference was investigated using a DNA encapsidation assay. It was hypothesized that the increase of nuclear B-capsids and enveloped B-capsids in the PNS when PCBP1 is depleted may be explained through a defect in viral genome encapsidation where more B-capsids are made resulting in an increase in overall B-capsid nuclear egress. However, when tested, the amount of protected, encapsidated genomes did not differ greatly between control and PCBP1 depleted cells, a modest decrease of 32 % in PCBP1 knockdown cells (Figure 15). This result is also supported by our EM data that reflects no difference in the number of C-capsids across control dsiRNA or siPCBP1 treated cells (Table 3). Another explanation for the increase in enveloped B-capsids in the PNS is that PCBP1 plays a role in regulating the nuclear egress of C-capsids, as in its absence we observe less stringent C-capsid specificity for nuclear egress (Figure 14, right panel).

Although it is not clear if, or how, PCBP1 is regulating capsid nuclear egress, a significant decrease in viral titers was observed in its absence. The EM data showed that PCBP1 depleted cells had less extracellular particles than cells treated with an NT dsiRNA, a decrease of roughly 50 % (20.4 % to 9.2 %, Table 3). This data is consistent with our extracellular titer experiment where a 55 % decrease of titers at 12 hpi and 61 % decrease at 24 hpi in PCBP1 depleted cells was seen (Figure 7A). The lack of change in PFU/genome copies between PCBP1 depleted cells and control cells suggests that this is not due to an increase in defective particles, but due to less viral particles being made (Figure 11B).

To further explore the mechanism behind how PCBP1 depletion is decreasing titers, the HSV-1 genome for PCBP1 consensus sequences was analyzed. Two different PCBP1 consensus sequences within the HSV-1 genome were found: one that conferred mRNA stability when PCBP1 was bound to it(217), and another that induced translational repression through exclusion in polysomes (224)(Table 4). Since our data reflected a pro-viral role for PCBP1, the gene harbouring an mRNA stability consensus motif, pUL24, was investigated. pUL24 is a leaky-late viral protein

that has been implicated in nuclear egress through mediating the dispersal of nucleolar proteins nucleolin and nucleophosmin (B23) (266–268). When PCBP1 was depleted from cells, a decrease in pUL24 protein of 63 % and 40 % at 24 and 48 hpi was observed, respectively (Figure 16A). To determine if the mRNA stability was being affected, RT-qPCR was performed on cDNA from PCBP1-depleted, and control cells. A very modest decrease of 24 % was observed in PCBP1 depleted cells at the mRNA level (Figure 16B). However, RT-qPCR may not be the most effective way to measure mRNA stability, as it simply reflects a snapshot of how much mRNA is present at a certain time, this is discussed below in section 5.2 'Future Studies'.

The protein level decrease of pUL24 does suggest a specific effect on pUL24 as VP5 and pUL25 protein levels were unaffected (Figure 16A). Also, the general protein blot probed with the polyclonal antibody Remus showed a protein around 28 kDa that was decreased with PCBP1 KD (Figure 13). While it cannot be concluded that this protein is pUL24, it is at the same molecular weight as the protein revealed by a monoclonal pUL24 blot (Figure 16A). The other candidate for this ~28 kDa band is pUL51. However, pUL51 is a phosphoprotein with three species at 27, 29 and 30 kDa and on the Remus blot we only see one species which further suggests the protein identity of pUL24 (Figure 13). Mass spectrometry would be useful to resolve the issue. Interestingly, pUL24 has a PD-(D/E)XK endonuclease domain which has yet to be linked to DNA cleavage during infection (269). Conserved residues within this endonuclease domain are important for redistribution of nucleophosmin and nucleolin (266, 268). Depletion of either pUL24 from the virus or nucleolin from the cells disrupts nuclear egress and lowers viral titers which could link PCBP1 as a regulator of nuclear egress through stabilization of pUL24 supporting our EM observations (259, 268, 270).

While this is the first description of PCBP1 influencing HSV-1, other family members of the KH-domain hnRNPs have been implicated in herpesvirus infections. hnRNP K interacts with ICP27 in HSV-1 and IE 2 in human herpes virus 6 (HHV-6), while PCBP1 interacts with ICP27-homolog ORF57 in KSHV (244, 271, 272). All these studies hypothesize that hnRNP K or PCBP1 are acting as docking adapters, helping viral proteins with the regulation of gene expression. PCBP1 binds ORF57 to enhance IRES translation of XIAP to prevent apoptosis during infection (244). While the downstream effects of the hnRNP K interaction with ICP27 or IE 2 have yet to be elucidated, another study has shown that hnRNP K is important for HSV-1 infection as its

depletion causes a reduction of HSV-1 titers (273). Due to the decrease in HSV-1 titers seen in our study when PCBP1 is depleted, and the Schmidt *et al*. study when hnRNP K is depleted, it is possible that this family of proteins displays some redundancy or work together in a complex as seen in BRAC1 or HPV-16 L2 regulation (206, 240).

The iron transfer quality of PCBP1 may also contribute to the proviral effect that was see with HSV-1. The herpes simplex viruses encode a ribonucleotide reductase enzyme that requires iron to function efficiently (274). One study has shown that when the ferrous iron chelator 2,2, bipyridyl (BIP) is added during HSV-1 infection, titers drop by 50 % (275). While PCBP1 has been shown to transport ferrous iron to specific enzymes such as DOHH and ferritin (231, 234), the lack of relatedness between these enzymes suggests that PCBP1 is a more general iron chaperone (233). The hypothesis that PCBP1 is an iron chaperone for the HSV-1 ribonucleotide reductase is consistent with the titer data (Figure 7A), but it is not consistent with our genome replication data (Figure 9A). If PCBP1 were needed for ribonucleotide reductase, one would expect a decrease in viral DNA output with the depletion of PCBP1. While a trend of viral genome reduction is seen, the data is not statistically significant due to the variability in our results yielding large standard error. PCBP1 as an iron chaperone for the viral ribonucleotide reductase would also not explain its involvement in C-capsid specificity previously published on by the Lippé Lab (253). More research must be done to tease apart the multifunctional roles of PCBP1 in the context of HSV-1 infection.

5.2 Future Studies

The future studies inspired by this dissertation is further investigation into mRNA stability and identifying the molecular partners that lead to PCBP1 capsid recruitment. Specifically, to further investigate the role of PCBP1 in regulating mRNA stability of UL24, I propose an actinomycin D assay. Actinomycin D inhibits transcription through intercalation with the DNA creating a stable complex preventing DNA-dependent RNA polymerase from transcribing mRNA (276). For this reason, it is commonly added to determine mRNA half-life over a time course. If PCBP1 stabilizes UL24 mRNA, we would expect a decrease in UL24 transcripts in PCBP1 depleted cells compared to control cells. Studies into the molecular partners of PCBP1 in the context of HSV-1 infection, through mass spectrometry or co-immunoprecipitation could help answer questions about whether PCBP1 interacts with known actors in viral nuclear egress and

which proteins may be involved in the addition of PCBP1 on C-capsids. This information would inform the next steps into the investigation into the pro-viral role of PCBP1 in HSV-1 infection. Future studies to elucidate the exact mechanism of PCBP1 activity in HSV-1 will not only procure knowledge for herpesvirus biology, but also extend our knowledge of cell biology in general.

6. Conclusions

As an obligate parasite, viruses must usurp the host machinery to replicate and create new progeny. In the case of herpesviruses, this seizure of cell biology is sophisticated and complex. After over 100 years of research, we are still learning about the virus and the exact mechanisms behind every step of the replication cycle. With every new study we not only become closer to understanding the virus, we also become closer to knowing the host cells. HSV-1 has evolved alongside humans for millennia and as such they are some of the best-trained cell biologists (277). This is one of the advantages of working with HSV-1.

The purpose of this study was to explore the role of PCBP1 in HSV-1 infection. Based on previous research out of the Lippé Lab connecting PCBP1 to C-capsid specific egress (253), we designed RNAi experiments to determine the effect of PCBP1 depletion on HSV-1 infection. Our results demonstrate that PCBP1 could be regulating UL24 mRNA transcripts for proper activity in nuclear egress, such that when PCBP1 is depleted, pUL24 is destabilized preventing proper regulation of nuclear egress and therefore more B-capsids are able to bud into the PNS. This research is a starting point for further analysis into the exact mechanism of PCBP1 in HSV-1 infections. In addition, it may provide important clues to elucidate how pUL24 supports nuclear egress.

7. Acknowledgements

The creation of the PCBP1-zsGreen HeLa cell line by lentiviral transduction was performed by Jordan Quenneville in Dr. Gagnon's Laboratory. PCBP1-zsGreen HeLa cells were sorted by Ines Boufaied at the Cytometry Platform at CR CHU-Ste Justine. Diane Gingras (Department of Pathology, Université de Montréal) prepared and imaged all electron micrographs for this dissertation.

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Annex 1

Gene	Protein
STRUCTURAL	
Capsid	
UL17	pUL17
UL18	VP23
UL19	VP5
UL25	pUL25
UL26	VP24, VP21
UL26.5	VP22a
UL35*	VP26
UL38	VP19c
Tegument	
RL1*	ICP34.5
RL2*	ICP0
RS1	ICP4
UL7*	pUL7
UL11*	pUL11
UL13*	UL13 PK
UL14*	pUL14
UL16*	pUL16
UL20*	pUL20
UL21*	pUL21
UL23*	TK
UL41*	vhs
UL46*	VP11/12
UL47*	VP13/14
UL48	VP16
UL49*	VP22
UL50*	pUL50
UL51*	pUL51
UL55*	pUL55
US2*	pUS2
US3*	US3 PK
US10*	pUS10
US11*	pUS11
Envelope	
UL1	gL
UL10*	gM

UL20*	pUL20
UL22	gH
UL27	gB
UL44*	gC
UL45*	pUL45
UL49.5	gN
UL53*	gK
UL56*	pUL56
US4*	gG
US5*	gJ
US6	gD
US7*	gI
US8*	gE
US9*	pUS9
NON-STRUCTURAL	
UL2*	pUL2
UL3*	pUL3
UL4*	pUL4
UL5	pUL5
UL8	pUL8
UL9	pUL9
UL12*	pUL12
UL15	pUL15
UL24*	pUL24
UL28	pUL28
UL29	pUL29
UL30	pUL30
UL31*	pUL31
UL32	pUL32
UL33	pUL33
UL34*	pUL34
UL36	ICP1/2
UL37	pUL37
UL39	pUL39
UL40*	RIR2
UL42	pUL42
UL43*	pUL43
UL52	pUL52
UL54	ICP27

UL55*	pUL55
UL56*	pUL56
US1*	ICP22
US8.5*	pUL8.5
US9*	pUS9
US12*	ICP47

Table 5. HSV-1 genes and proteins

Asterisk indicates genes that are not needed for replication in cell culture.