Published in final edited form as:

Virus Res. 2017 March 02; 231: 1–9. doi:10.1016/j.virusres.2016.10.015.

Cruising the cellular highways: how human papillomavirus travels from the surface to the nucleus

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Abstract

The non-enveloped human papillomaviruses (HPVs) specifically target epithelial cells of the skin and mucosa. Successful infection requires a lesion in the stratified tissue for access to the basal cells. Herein, we discuss our recent progress in understanding binding, internalization, uncoating, and intracellular trafficking of HPV particles. Our focus will be on HPV type 16, which is the most common HPV type associated with various anogenital and oropharyngeal carcinomas. The study of HPV entry has revealed a number of novel cellular pathways utilized during infection. These include but are not restricted to the following: a previously uncharacterized form of endocytosis, membrane penetration by a capsid protein, the use of retromer complexes for trafficking to the trans-Golgi network, the requirement for nuclear envelope breakdown and microtubule-mediated transport during mitosis for nuclear entry, the existence of membrane-bound intranuclear vesicles harboring HPV genome, and the requirement of PML protein for efficient transcription of incoming viral genome. The continued study of these pathways may reveal new roles in basic biological cellular processes.

Graphical abstract

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Keywords

HPV binding; receptor; endocytosis; uncoating; microtubule; intracellular transport; mitosis; nuclear vesicles

1.1 Introduction

In this article, we are reviewing the binding and entry process of human papillomaviruses (HPVs) with a focus on HPV type 16. This is the best-studied type due to its association with the majority of HPV-induced cancers. Even though we will cover every aspect of binding, internalization, and intracellular trafficking, our focus is on the most recent findings regarding the late trafficking events of HPV16. Over the years, HPV entry has been very contentious and many conflicting reports have been published. However, more recently a consensus view has emerged. We are refraining from covering all articles disputing this consensus view and redirect those interested to a number of excellent recent reviews (Day and Schelhaas, 2014; Raff et al., 2013; Sapp and Bienkowska-Haba, 2009; Sapp and Day, 2009).

1.2 HPV capsid

The HPV capsid is comprised of two proteins, the major capsid protein L1 and the minor capsid protein L2. There are 360 molecules of L1 monomers arranged into 72 pentamers, also called capsomeres, forming a $T=7$ icosahedral lattice (Baker et al., 1991; Finch and Klug, 1965; Liddington et al., 1991). Computer reconstructions of cryoelectron micrographs demonstrated that twelve capsomeres are pentavalent, which means they contact five other capsomeres whereas the remaining sixty capsomeres are hexavalent contacting six other capsomeres (Baker et al., 1991; Trus et al., 1997). Crystal structure determined by X-ray crystallography demonstrated that L1 folds into a ‘jelly roll’ β sandwich (Chen et al., 2000). The intimate contacts between adjacent L1 monomers makes the pentamer form a tightly packed donut-like shape with a conical hollow opening on the top. Protruding from the exterior surface are a number of poorly conserved exposed loop domains (Chen et al., 2000). The very C-terminus of L1 is α -helical in nature, which makes it both disordered and flexible. This allows for the C-terminus of the L1 protein to act as an “invading arm” that invades neighboring capsomeres (Modis et al., 2002; Wolf et al., 2010). These intercapsomeric interactions are further stabilized by L1 disulfide bonds that covalently link highly conserved cysteine residues resulting in the formation of L1 dimers and trimers (Buck et al., 2005; Fligge et al., 2001; Li et al., 1997; Sapp et al., 1998; Sapp et al., 1995; Volpers et al., 1994). The minor capsid protein, L2, is present in an undetermined number of copies, but it is estimated that each capsid can accommodate up to 72 molecules (Buck et al., 2008; Doorbar and Gallimore, 1987; Komly et al., 1986). Conservative estimates for the number of L2 molecules per capsid range between 12-36 copies per capsid (Roden et al., 1996; Volpers et al., 1994). However, the exact conformation of the L2 protein in the capsid still remains mostly a mystery. It has been shown that the majority of the L2 protein is hidden within the mature capsid, whereas only a portion of the N-terminus at residues 60-120 is surface exposed (Liu et al., 1997). A C-terminal peptide of L2 (residues 384-460

for BPV-1 or 396-439 for HPV11) has been shown to interact with the C-terminus of L1 via mostly hydrophobic interactions (Finnen et al., 2003; Okun et al., 2001). Upon infectious entry, the L2 protein undergoes conformational changes and emerges (Day et al., 2008; Richards et al., 2006).

2.1 Binding

In the past, the study of HPV entry was hindered due to the lack of a system to generate efficient quantities of infectious viral particles. The development of pseudoviral vectors, also termed “pseudoviruses” (PsVs), has been used to overcome this roadblock (Buck et al., 2004; Buck et al., 2005; Kawana, 1998; Rossi et al., 2000; Touze and Coursaget, 1998; Unckell et al., 1997). PsVs are generally considered indistinguishable from native virions with only few reported differences with regard to entry (Biryukov and Meyers, 2015). PsVs are generated by co-transfecting expression plasmids that encode codon-optimized L1 and L2 genes that allow for high-level expression (Leder et al., 2001; Zhou et al., 1999) together with a reporter plasmid. The pseudoviral capsids are composed of both structural proteins exhibiting the proper disulfide bondages (Buck and Thompson, 2007; Buck et al., 2005) and encapsidate the plasmid vector as a pseudogenome. This system is very flexible as there is no specific packaging sequence required. Packaging of the pseudogenome seems to only be limited to abundance and size exclusion, about 8 kb (see review by Cerqueira and Schiller in this issue). Successful delivery of the pseudogenome to the nucleus offers an easily measurable readout for infectivity using a chosen reporter. The pseudoviral system also offers the ability to utilize reverse genetics while generating efficient quantities of mutant pseudoviruses used for entry assays; a mutational approach is very restricted using native virions. Furthermore, the availability of monoclonal antibodies and DNA-labeling techniques has allowed us to investigate the trafficking of each individual component of the HPV capsid. Therefore, PsVs have been critical for our success in the last decade to tease apart how HPV virions bind to and enter keratinocytes during a primary infection.

During a primary infection in cell culture, it was demonstrated that HPV capsids preferentially bind to components of the extracellular matrix (ECM). The ECM is a network of secreted molecules that support the cell in adhesion, cell-to-cell communication, differentiation, and structure (reviewed in (Mouw et al., 2014)). In cell culture models, the ECM mimics the basement membrane, which separates the dermis from the epidermis. The ECM is rich in proteoglycans, particularly heparan sulfate proteoglycans (HSPGs), which are glycoproteins that contain one or more covalently attached heparan sulfate chains (Esko and Lindahl, 2001). The HPV capsid directly engages these molecules on the ECM and cell surface (Giroglou et al., 2001; Johnson et al., 2009; Joyce et al., 1999; Knappe et al., 2007; Selinka et al., 2007). This engagement is largely attributed to the L1 protein involving the sequential engagement of three heparan sulfate-binding sites, but triggers specific conformational changes in both L1 and L2 proteins (Dasgupta et al., 2011; Richards et al., 2013). In addition, several groups have shown that ECM-resident laminin 332 (also known as laminin 5) can also function as an additional attachment receptor for HPV11 and HPV16 but not HPV18 and related types of species 7 and may even contribute to anatomical-site specificity (Culp et al., 2006; Richards et al., 2014; Selinka et al., 2007). After this engagement, cell surface-resident host cell cyclophilin B, a peptidyl-prolyl *cis-trans*

isomerase, facilitates the exposure of the very N-terminus of the L2 protein (Bienkowska-Haba et al., 2009). This finding was recently challenged by Campos and coworkers, who claimed that exposure of L2 does not depend on cyclophilin B (Bronnimann et al., 2016). However, the authors of this study used a L2 protein harboring a large tag at the N-terminus for their studies, which can be expected to alter the conformation of L2 protein within the capsid. More subtle alterations, such as the introduction of point mutations to the N-terminus already pre-expose the L2 protein (Bienkowska-Haba et al., 2009; Bienkowska-Haba et al., 2012). Exposure of the N-terminus, which can be measured by accessibility to monoclonal antibodies, is followed by proteolytic processing by the pro-convertase enzyme furin or closely related proteases, which cleaves off the first 12 amino acids of the L2 protein at a highly conserved cleavage motif site (R-X-K/R-R) (Richards et al., 2006). These events occur on the cell surface and are essential downstream for uncoating in the endocytic compartment and subsequent trafficking. Following these specific conformational changes, which likely reduce the affinity to heparan sulfate, the virion associates with subsequent non-HSPG uptake receptor(s) (Day et al., 2008). Numerous candidates of the non-HSPG receptors have been identified including: integrins, tetraspanins, growth factor receptors, and annexin A2, (Abban et al., 2008; Dziduszko and Ozbun, 2013; Evander et al., 1997; Scheffer et al., 2013; Spoden et al., 2008; Spoden et al., 2013; Surviladze et al.; Woodham et al., 2012). Among these, tetraspanins are the best studied. They form tetraspanin-enriched microdomains (TEM) in the plasma membrane, which may serve as an entry platform where many of the other putative uptake receptors have been shown to localize (reviewed in (Scheffer et al., 2014)). The ECM and cell surface events are mainly mediated by L1 protein and do not require L2. However, the L2 protein is absolutely required for infection, as post-internalization events seem to rely heavily on identified regions of the L2 protein to facilitate intracellular trafficking.

2.2 Internalization

HPV entry into cells is generally considered asynchronous. Internalization of the bulk of particles have been reported to occur with a half-time of up to 12 hours but the leading edge may reach the nucleus in as little as 2 hours yielding measurable viral transcripts at 4 hours post infection (Broniarczyk et al., 2015; Christensen et al., 1995; Selinka et al., 2007; Selinka et al., 2002). The virion is internalized by a previously unknown uptake pathway, which has the most commonalities with micropinocytosis resulting in the formation of HPV-harboring small, smooth endocytic vesicles (Schelhaas et al., 2012; Spoden et al., 2008; Spoden et al., 2013). Uptake does not depend on clathrin, caveolin, dynamin, or flotillin but requires reorganization of the actin cytoskeleton and may involve the cytoskeletal adaptor obscurin-like 1 (OBSL1) (Schelhaas et al., 2008; Schelhaas et al., 2012; Smith et al., 2008b; Wustenhagen et al., 2016). Activation of PI3 kinase has been implicated, likely participating in reorganization of the actin cytoskeleton during uptake (Fothergill and McMillan, 2006; Schelhaas et al., 2012; Surviladze et al., 2013). Additional signaling factors have also been implicated in early events including EGFR, phosphatases, PKC, PAK-1, and FAK, although their exact contribution to the viral infection is still unclear (Abban and Meneses, 2010; Schelhaas et al., 2012; Smith et al., 2008b; Surviladze et al., 2013).

2.3 Uncoating

Early HPV-harboring endocytic vesicle trafficking requires the tetraspanin CD63, syntenin-1, and ESCRT-associated ALIX and results in delivery of HPV particles to multivesicular endosomes (Grassel et al., 2016; Schelhaas et al., 2012; Spoden et al., 2008). These recent findings strengthen the argument for TEMs serving as entry platforms. Acidification of endocytic vesicles has long been known to trigger disassembly of the viral capsid (Day et al., 2003; Selinka et al., 2002; Smith et al., 2008a; Spoden et al., 2008; Spoden et al., 2013). It was demonstrated that host cell cyclophilins, likely interacting with a binding site located on the C-terminus of the L2 protein in the vicinity of the region that was shown to mediate hydrophobic interactions with the inner surface of capsomers, facilitates the dissociation of most of the L1 protein from the L2 protein, which remains in complex with the viral DNA (Bienkowska-Haba et al., 2012). While the dissociated L1 protein seems to be targeted to the lysosome for degradation, a subset of L1 protein does in fact remain associated with both L2 and the viral DNA (DiGiuseppe et al., 2014). This viral protein/DNA complex is rescued from degradation by being diverted away from the lysosomal compartment by the retromer complex and trafficked to the trans-Golgi network (TGN) (Day et al., 2013; Lipovsky et al., 2013). Retromer is a cytosolic protein complex that facilitates recycling of transmembrane receptors from the endosomes to the TGN. Another factor, γ -secretase, a protease that cleaves single-pass transmembrane proteins, is also essential for infection, however the specific substrate required for the HPV infection has yet to be identified (Huang et al., 2010; Karanam et al., 2010; Zhang et al., 2014). Inhibition of either cyclophilin or γ -secretase activity results in endosomal retention and diversion to lysosomes whereby illustrating the importance of both activities as prerequisites for trafficking to the TGN (DiGiuseppe et al., 2014; Zhang et al., 2014).

3.1 Membrane penetration and the L2 protein

Like other non-enveloped viruses, it was assumed that endosomal egress was facilitated by membrane disruption and subsequent release of the viral DNA into the cytosol after uncoating. Indeed, a membrane-destabilizing peptide of HPV16 and HPV33 was identified on the C-terminus of L2 (residues 454-473) (Kämper et al., 2006). Further sequence analysis of the HPV16 L2 protein predicted another transmembrane-like domain located on the N-terminus (residues 45 to 67) that was characterized by the Campos group (Bronnimann et al., 2013). Both domains were indispensable for HPV infection and have the propensity to insert into cellular membranes. However, it was really the DiMaio group's demonstration that the very C-terminus of L2 protein (aa 446-455) interacted directly with components of retromer that suggested at least partial cytosolic residence of the L2 protein during infection (Lipovsky et al., 2013; Popa et al., 2015). Based on these findings and others, it was reasoned that L2 adopts a transmembranous conformation post entry. In support of these findings, our group demonstrated using epitope-mapped monoclonal antibodies and trypsin sensitivity in selectively permeabilized cells that 40 some N-terminal amino acids of L2 remain luminal while portions of the C-terminal L2 protein are likely cytosolic during infectious entry (DiGiuseppe et al., 2015). Interestingly, numerous cytosolic factors have been identified as putative binding partners for the L2 protein during infection. The Banks group demonstrated that sorting nexin 17, a retrograde sorting protein that binds to the

cytoplasmic tails of cargo, interacts with a highly conserved motif (NPxY) on the HPV16 L2 protein (residues 254-257) (Bergant and Banks, 2013; Bergant Marusic et al., 2012). Following up this study, they also showed that sorting nexin 27 interacts in the central region of L2 (residues 192-292), independent of the NPxY motif (Pim et al., 2015). C-terminal 40 amino acids of the HPV16 and HPV33 L2 protein have been shown to also interact with the dynein light chains DYNLT1 and DYNLT3, an interaction that seems to be important for nuclear delivery (Florin et al., 2006; Schneider et al., 2011). Recently, it was identified that obscurin-like 1 protein, a cytosolic cytoskeletal adaptor protein, forms complexes with the C-terminus of L2 (residues 280-473), which is important for efficient endocytosis (Wustenhagen et al., 2016). Currently, it is unknown precisely when the L2 protein becomes transmembranous. However, blocking acidification during infectious entry resulted in increased resistance of the L2 protein to trypsin digestion (DiGiuseppe et al., 2015). This suggests that membrane penetration likely occurs right after uncoating within the endosomes. This would make sense, as the L2 protein would have to become partially cytosolic to directly engage the retromer complex to traffic to the TGN.

3.2 Viral protein/DNA complex after uncoating

After membrane penetration, it can be observed that L2 protein remains associated with the viral genome, which is oriented on the luminal side of intracellular membranes such as the TGN. How the L2 protein remains associated with the viral genome after uncoating remains unclear. The only putative DNA binding domain on the luminal portion of L2 is cleaved off by furin convertase before internalization (residues 1-13) (Sun et al., 1995). Since the L2 protein has multiple domains capable of interacting with L1 capsomeres, including at the N-terminus of L2, and there is a well-characterized DNA binding domain on the C-terminus of L1, we find it attractive to speculate that L1, likely arranged as capsomeres, may serve as a bridge between L2 and the viral DNA (Finnen et al., 2003; Okun et al., 2001; Sapp et al., 1995; Schäfer et al., 2002). It was previously reported that a subset of the L1 protein accompanies L2/DNA complex to the TGN (DiGiuseppe et al., 2014). In fact, L1 protein was even observed associated with condensed chromosomes during mitosis (DiGiuseppe et al., 2016a; DiGiuseppe et al., 2016b). Interestingly, unpublished data from our lab shows that this subset of L1 protein associated with the viral genome when localized to the TGN and during mitosis is still conformationally intact, indicative of capsomeres, thus supporting our proposal (unpublished). Unfortunately, the nature of the capsid protein/DNA interactions within the capsid is severely lacking. While contributions of L1 has been thoroughly investigated on the cell surface and early on in the infection, it is completely unknown whether L1 plays additional roles later in the entry process and should be considered for future studies. However, this will be difficult to achieve due to L1's role early in binding and entry.

3.3 TGN and the ER

Several studies have concluded that the HPV viral genome traffics to the TGN during infectious entry (Day et al., 2013; DiGiuseppe et al., 2014; Lipovsky et al., 2013). Why HPV must traffic to the TGN has not yet been elucidated. However, it is known that inhibition of TGN dynamics greatly reduces infectivity whereby emphasizing just how important

residence in the TGN is to an HPV infection (Day et al., 2013; Lipovsky et al., 2013). It is also unclear whether or not the viral genome egresses from the TGN during interphase. A recent study suggests that egress from the TGN may involve Pyk2 signaling in cells. While the exact contribution of Pyk2 signaling during intracellular trafficking remains to be determined (Gottschalk and Meneses, 2015), it would be interesting for future studies to investigate whether Pyk2 plays a role in trafficking following the onset of mitosis. There is also some evidence to support the idea that the viral genome traffics to the ER. A genome-wide siRNA screen implicated several ER proteins were required for HPV infection (Lipovsky et al., 2013). Using the proximity ligation assay, the DiMaio group showed a positive signal for tagged L2 protein and ER resident chaperone proteins thus suggesting possible residence in the ER (Zhang et al., 2014). Offering some support for this notion, it was previously shown that HPV16 L1 protein co-localized with ER markers after 4 hours post infection (Laniosz et al., 2009). However, a more recent study reported that there is no clear association between the viral genome and ER (Day et al., 2013). In our hands, we observe some degree of co-localization between viral genome and ER markers, but since the ER signal is so pronounced throughout the cytoplasm, we are concerned that co-localization may be coincidental (unpublished). Future studies using high-resolution microscopy would be necessary to continue the investigation into this possible association. Whether the TGN and/or ER are just pit stops or more of an extended stay during interphase remains to be determined.

4. Nuclear translocation

While most studies have focused on HPV entry into resting cells, it was recently demonstrated that cell division plays a significant role in the HPV infection. It was shown that nuclear envelope breakdown during mitosis was a rate limiting step in delivery of the viral genome to the nucleus (Aydin et al., 2014; Pyeon et al., 2009). While the incoming viral genomes of other DNA viruses egress into the cytoplasm and utilize nuclear pore complexes (NPCs) for nuclear translocation, genome-wide siRNA screens failed to identify NPC proteins as being required for the HPV infection (Aydin et al., 2014; Lipovsky et al., 2013). Instead, HPV relies on nuclear envelope breakdown during mitosis for nuclear translocation (Aydin et al., 2014; Pyeon et al., 2009). The nuclear envelope consists of an outer nuclear membrane that is contiguous with the ER (Hetzer, 2010). The inner nuclear membrane is distinct and houses proteins used for attachment to heterochromatin and nuclear lamina, which makes up the meshwork of filaments that provide nuclear structure during interphase (Lippincott-Schwartz, 1997). During mitosis, it is thought that the nuclear envelope fragments into tubules and vesicles that are released into the cytoplasm following breakdown of the lamina network (Hetzer, 2010). Reformation of the nuclear envelope is thought to be the reverse process. There is a coordinated reorganization of precursor vesicles containing nuclear envelope membrane proteins that target and bind to decondensing chromatin then fuse together. This model ensures proper segregation of nuclear envelope and ER membranes during mitosis to each newly formed daughter cell. Data suggests that during mitosis the HPV genome resides in the lumen of transport vesicles, which likely bud from the TGN, line up along microtubules, and traffic to the condensed chromosomes (DiGiuseppe et al., 2016a; DiGiuseppe et al., 2016b). The viral genome can be detected by

immunofluorescent confocal microscopy throughout mitosis and within the newly formed nuclei of cells in telophase as the nuclear envelope is still forming around the condensed chromosomes (unpublished). Surprisingly, the nuclear localized viral genome at this time was still not accessible to cell impermeable low molecular weight dyes in selectively permeabilized cells treated with a low concentration of digitonin (DiGiuseppe et al., 2016b). Therefore, these data may provide the first evidence of intranuclear membrane-bound vesicles, albeit indirectly. Currently, a marker for HPV-harboring vesicles has yet to be identified. Whether or not these vesicles are virally induced or, rather, exist to serve a function during cell division in uninfected cells also remains to be determined. While other DNA viruses must protect their viral genome in the cytoplasm from innate immune sensors, HPV seems to have evolved a way to keep its genome safe from detection within membranous compartments until the viral genome reaches its final destination. Hiding from cytosolic innate immune sensors by residing in transport vesicles during mitosis is a hitherto unrecognized form of immune evasion. This also may explain why we are unable to detect the induction of innate immune responses in cell culture upon infection (unpublished).

5. Trafficking during mitosis

While most studies have focused on HPV entry into resting cells, it was recently demonstrated that cell division plays a significant role in the HPV infection. It was shown that nuclear envelope breakdown during mitosis was a rate limiting step in delivery of the viral genome to the nucleus (Aydin et al., 2014; Pyeon et al., 2009). Following the onset of mitosis, the cell undergoes extreme structural reorganization. For example, the TGN fragments and the vesicles migrate along microtubules congregating at each of the two centrosomes. This ensures proper segregation of these compartments to each new daughter cell after division occurs (Jongsma et al., 2015; Shima et al., 1997). Interestingly, HPV seems to have evolved a mechanism to take advantage of this structural reorganization during mitosis by becoming cargo for microtubule-associated transport. Following the onset of mitosis, the viral genome dissociates from the TGN, lines up along astral microtubules, and localizes in close proximity to centrosomes. In the later stages of mitosis, such as meta- and anaphase, the viral genome associates with spindle microtubules and/or directly with condensed chromosomes. During this time, data suggest that the L2 protein is still transmembranous, while the L1 protein and the viral genome are situated on the luminal side of intracellular membranes (DiGiuseppe et al., 2016b). These data point to the conclusion that the HPV genome is residing in a transport vesicle and utilizing microtubule-associated transport during mitosis, which in a way curious resembles the process of nuclear envelope disassembly and reformation.

Microtubules are a network of cellular highways used to transport cargo back and forth across the cell. To achieve this, microtubules have directionality, which allows for directed transport via two classes of motor proteins, dynein and kinesins (Sharp et al., 2000; Welte, 2004). Dynein facilitates transport of cargo towards the minus ends of the microtubules, which converge at the microtubule-organizing center, also called centrosomes. Kinesins transport cargo towards the growing plus ends of microtubules, which are often at the cell periphery or attached to kinetochores during mitosis (Walczak and Mitchison, 1996). During mitosis, dynein is enriched on astral microtubules while mitotic kinesins are enriched on

spindle microtubules (Busson et al., 1998). Since the HPV genome is observed associated with astral microtubules during prophase and spindle microtubules at later stages, this suggests that HPV may be cargo for both motor proteins, at different times, during mitosis. It can be presumed from recent data that HPV-harboring vesicles likely bud or take advantage of fragmentation of TGN components during prophase and traffics to the centrosome, likely mediated by dynein. Once at the centrosome, we speculate that HPV-harboring vesicles may get picked up by mitotic kinesins and traffic along spindle microtubules towards kinetochores. Since L2 is still accompanying viral genome at this time, it is tempting to speculate that L2 would be able to engage these motor proteins directly or through secondary protein interactions. A direct interaction between L2 and dynein would be feasible, because L2 was previously shown to interact with components of the dynein motor protein complex on the C-terminus, which would be facing the cytosol at this time (DiGiuseppe et al., 2016a; DiGiuseppe et al., 2016b; Florin et al., 2006; Schneider et al., 2011). Currently, there is no direct evidence to suggest that HPV can be cargo for kinesins and that L2 protein mediates this putative interaction. However, a number of mitotic kinesins were recently identified through siRNA screens as being required for HPV infection although no assigned role has been determined (Aydin et al., 2014; Lipovsky et al., 2013). Unfortunately, using our current approaches to image HPV particles during an infection, we are unable to directly test whether there is in fact a switch in directionality. Until we are able to infect live cells and image the viral particles in real time, this question cannot be thoroughly examined.

6. Establishment of infection

Currently, it is unknown how the viral genome egresses the intranuclear membrane-bound vesicle. It was observed that release of the viral genome within the newly formed nuclei was estimated to be delayed up to several hours following the completion of mitosis and nuclear envelope reformation (DiGiuseppe et al., 2016b). Therefore, egress seems to be a time-dependent step possibly involving degradation of the intranuclear membrane bilayer. Since the nuclear envelope must reassemble in a coordinated fashion of fusion events, we speculate that degradation pathways may exist to remove vesicles that either purposefully or fortuitously end up in the nucleus after the completion of mitosis. Numerous phospholipases have been identified to localize in both the cytoplasm and the nucleus but their exact role within the nucleus is completely unknown (Faenza et al., 2013). Curiously, siRNA screens identified several phospholipases as being required for the HPV infection, however, the role these enzymes play in the HPV infection has not yet been investigated beyond the initial screens (Aydin et al., 2014; Lipovsky et al., 2013). If phospholipases are necessary for egress, this may explain the transient nature of the HPV-harboring intranuclear vesicle after the completion of mitosis.

Promyelocytic leukemia (PML) nuclear bodies (NBs) are targeted by many viruses and are often initial sites of viral transcription, especially for DNA viruses (reviewed in (Everett and Chelbi-Alix, 2007)). This involves disruption of PML NBs mediated by viral proteins followed by reassembly of modified PML NBs often requiring exclusion of specific PML isoforms. While it is necessary that most DNA viruses target and disrupt PML NBs to enhance infection, it seems to be the opposite case for HPV infection. It was demonstrated

that PML protein was actually required for efficient establishment of HPV infection (Day et al., 2004). PML NBs are subnuclear structures that recruit a large variety of proteins to multiple concentrated locations. The composition of PML NBs is diverse, numbering up to 100 different proteins, but the essential structural component is PML protein. It is thought that these NBs are organized into super structures via posttranslational protein modifications, most notably SUMOylation, which via SUMO interacting motifs (SIMs) allows the formation of higher order structures (reviewed in (Lallemand-Breitenbach and de The, 2010)). PML NBs are not only targeted by many viruses they have also been implicated to play roles in numerous cellular processes such as transcriptional regulation, innate immune response, cell cycle arrest, and apoptosis. While the exact function of PML NBs is unclear, they have been proposed to serve as a nuclear depot for quick access to a variety of proteins. Notable proteins that reside at PML NBs in addition to PML protein are the transcriptional repressor proteins, Sp100 and Daxx.

In case of HPV infection, incoming L2 and viral DNA associate with PML NBs following completion of mitosis and nuclear envelope reformation (Day et al., 2004). The exact mechanism of how L2/DNA associates with PML NBs is unclear, but it has been proposed that L2 may facilitate this interaction. Indeed, several putative SIMs were previously identified on the L2 protein (residues 105-109, 145-148, and 284-289) (Bund et al., 2014). This notion is supported by the fact that L2 protein accumulates at PML NBs upon over-expression and possibly also in differentiated keratinocytes of natural lesions (Day et al., 1998; Florin et al., 2002). During mitosis, PML NBs disassemble and PML protein forms large cytoplasmic aggregates. PML NBs reassemble in the nucleus after mitosis is completed (Lallemand-Breitenbach and de The, 2010). This poses the question of whether the viral genome is targeted to pre-existing PML NBs or rather PML protein accumulating where the viral genome resides. We think it is quite feasible that L2 mediates the recruitment of PML protein, as the putative SIMs on L2 are accessible during this time based on our current understanding of L2's topology. It would be interesting for future studies to determine the exact timing of when PML protein is recruited to the L2/DNA complex and whether recruitment of PML protein would occur before or after release of the viral genome in the nucleus. It was recently proposed that association with cytoplasmic PML aggregates during mitosis might be responsible for nuclear retention of the L2/viral genome complex after division (Broniarczyk et al., 2015). In our hands, we also observe some co-localization of viral genome and cytoplasmic PML aggregates, but these seem to be rare events. Additionally, we did not observe co-localization of PML protein with the L2/viral genome present on spindle microtubules and condensed chromosomes throughout mitosis (unpublished). Since PML protein enters the nucleus through NPCs in the absence of HPV infection, we think it is unlikely that PML facilitates this event because HPV does not seem to require NPCs for nuclear translocation (Dellaire et al., 2006; Jul-Larsen et al., 2009; Jul-Larsen et al., 2010).

The exact role of PML NBs during the establishment of HPV infection remains obscure. Curiously, it has been demonstrated that the presence of PML protein dramatically increases early viral gene expression suggesting PML NBs function to provide an environment that is favorable for viral transcription (Day et al., 2004; Stepp et al., 2013). In contrast, Sp100 has been shown to restrict HPV transcription in primary keratinocytes in line with its

transcriptional repressor function previously identified and this may be regulated through interferon (Habiger et al., 2015; Stepp et al., 2013). It will be interesting to test whether PML NB components are involved in establishing infection by initially favoring viral transcription (PML protein) followed by repression of viral transcription for a shift to latency (Sp100). This would require a sequential recruitment of PML NB components and/or posttranslational modifications. Given the technological advances of recent years in studying HPV entry, this assumption should now be testable.

7. Concluding remarks

The cytoplasm of the host cell is an inhospitable place for uninvited guests. Since the host's DNA is carefully maintained within the nucleus, cells have sophisticated machinery used to detect foreign DNA that doesn't belong in the cytoplasm (Chan and Gack, 2016). Once detected, an alarm is set off triggering an antiviral response. This acts as the first line of defense by generating an intracellular environment that has both antiproliferative and immunomodulatory activities that can restrict viral replication (Grandvaux et al., 2002). Unfortunately for DNA viruses that do not bring in their own replication factors, they must use the host machinery within the nucleus to replicate. To this end, DNA viruses are challenged with the task of evading immune detection while safely delivering their genome to the nucleus of the host cell (reviewed in (Fay and Pante, 2015)). To accomplish this feat, the enveloped herpesviruses use a viral fusion protein to fuse with the plasma or endocytic membrane and release an intact viral capsid into the cytoplasm (Le Sage and Mouland, 2013). The capsid protects the viral genome from detection until it docks at the NPC and injects directly into the nucleus through the pore. Adenovirus, a non-enveloped virus, disrupts endocytic membranes, release an intact capsid into the cytoplasm, and injects viral genome directly into the nucleus through the NPC (Le Sage and Mouland, 2013). Instead of escaping directly into the cytoplasm after endocytosis, the small, non-enveloped polyomaviruses must first traffic to the ER. Once in the ER, the viral capsid proteins undergo conformational changes and resident chaperone proteins translocate the viral capsid across the ER membrane. In a similar manner to the other DNA viruses mentioned, the polyomavirus capsid protects the genome within the cytoplasm and injects the viral genome through the pore of the NPC (Fay and Pante, 2015). Since the HPV capsid uncoats in the endosome and does not require the NPC, this would put HPV in danger if it used a similar mechanism. Escape of an uncoated capsid into the cytoplasm would render the viral genome susceptible for cytosolic DNA sensors and trigger an immune response (Chan and Gack, 2016). Therefore, HPV seems to have evolved a unique strategy to keep its viral genome protected within the lumen of various membranous compartments during intracellular trafficking. The HPV genome is likely retained within the TGN after uncoating, where it lies in wait for the host cell to lower its shields following the onset of mitosis. Structural reorganization during cell division requires coordinated trafficking of vesicles from various compartments and enrichment of mitotic associated proteins along microtubules. Meanwhile, HPV seems to take advantage of this event to hitch a ride along microtubules to traffic to the condensed chromosomes. Utilizing vesicular trafficking for delivery of the viral genome directly to the nucleus during mitosis is an underappreciated form of immune

evasion. As we continue to develop new techniques to image viruses during an infection, we may be able to address many of the unanswered questions presented herein.

Acknowledgments

This work was supported by grants from the National Institutes of Allergy and Infectious Diseases and the National Institutes of Dental and Cranofacial Research (R01AI081809 and R01DE0166908S1) to MS and in part by grants from the National Institute of General Medical Sciences (P20GM103433). Further support was provided by the Feist Weiller Cancer Center. SD was supported by a Carroll Feist predoctoral fellowship.

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Highlights

- HPV16 virions preferentially bind to components of the extracellular matrix
- Internalization requires conformational changes in both capsid proteins
- Uncoating within acidified endosomes triggers membrane penetration by the minor capsid protein to interact with cytosolic factors for transport to the trans-Golgi network (TGN)
- Viral particle-harboring vesicles segregate from the TGN for microtubule-mediated transport to the condensed chromosomes during mitosis
- Release of viral genome occurs after nuclear reformation and results in the association with PML nuclear bodies

Figure 1. Overview of HPV entry from the surface to the TGN

HPV virions attach to heparan sulfate proteoglycan (HSPGs) on the extra cellular matrix (ECM). Alternatively, laminin-332 may also serve as a primary attachment receptor on the ECM. HPV virions bind to HSPG on the cell surface. Binding triggers conformational changes (CC) in both L1 and L2. Host cell cyclophilin B (CyPB) facilitates the exposure of the N-terminus of L2. Furin convertase cleaves 12 amino acids from the N-terminus. HPV virions associate with the uptake receptor complex and are internalized. Low pH in the endosome triggers uncoating of the capsid whereupon host cell CyPs facilitate release of the L1 protein from the L2 protein. While most of the L1 protein is targeted to the lysosome for degradation, a subset remains in complex with L2 and the viral genome. The majority of the L2 protein penetrates intracellular membranes while only a small portion of the N-terminus remains luminal. The cytosolic portion of the transmembranous L2 protein engages cytosolic factors required for trafficking to the trans-Golgi network (TGN) during interphase.

Figure 2. Overview of intracellular trafficking of HPV throughout mitosis

Following the onset of mitosis, (prophase to pro-metaphase) HPV-harboring vesicles dissociate from the TGN and line up along astral microtubules (MTs) and coalesce in the vicinity of the microtubule-organizing center (MTOC). In later stages of mitosis (metaphase to anaphase), HPV-harboring vesicles line up along spindle microtubules and associate with condensed chromosomes. During this time, both capsid proteins and the viral DNA still remain in a complex. After the completion of mitosis and the nuclear envelope has been reformed (early to late interphase), viral DNA is released from intranuclear vesicles, viral proteins dissociate, and the viral DNA associates with PML nuclear bodies (PML NBs). Annotations not described: outer nuclear membrane (ONM); inner nuclear membrane (INM); nuclear pore complex (NPCs); trans-Golgi network (TGN)