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HPV entry into cells

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Abstract

Human papillomavirus (HPV) is a sexually transmitted virus responsible for the development of cervical cancer, anal cancer, head and throat cancers, as well as genital area warts. A major focus of current HPV research is on preventing the virus from entering a cell and transferring its genetic material to the nucleus, thus potentially preventing the development of cancer. Although the available HPV vaccines are extremely successful, approximately 15 additional cancer-causing HPVs have been identified that the vaccines do not protect against. Therefore, roughly 150,000 cancer cases will not be prevented annually with the current vaccines. Research efforts focused on the basic cell biology of HPV infection have a goal of identifying common infectious events that may lead to inexpensive vaccines or anti-virals to prevent infection by most, if not all, HPVs. In this review we attempt to summarize what is known regarding the process of HPV binding, entry, and intracellular trafficking.

Keywords

HPV binding entry intracellular trafficking

1. Introduction

Papillomaviruses (PVs) are a large family of non-enveloped, double-stranded circular DNA viruses that infect skin and mucosa. PVs infect most mammals, birds and reptiles and they are highly tissue- and species-specific, with the exception of bovine papillomavirus 1 (BPV1) and BPV2 [1, 2]. Although there are more than 200 PV types, PV infection mechanisms are fundamentally similar: Two viral capsid proteins, the major capsid protein (L1) and minor capsid protein (L2), mediate cellular binding, internalization and trafficking of the virus [3, 4]. Due to this similarity, BPV1 virions that were purified from bovine papillomas had been largely used to study PV infection mechanisms until the development of laboratory techniques to generate PVs *in vitro* [5–7] (see below).

To establish an infection, PV must enter the host cell and deliver its DNA into the nucleus of the cell. Viral entry is achieved via complex interactions of the viral capsid with cellular proteins. The viral capsid is composed of 72 pentameric capsomeres of L1 protein. The

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other capsid protein, L2, is mostly buried inside the capsid surface except for a portion of its N-terminus [8, 9]. Therefore, initial cell surface binding interactions are L1-dependent. PVs infect basal cells of the epithelium and depend on epithelial cell differentiation for replication and virion production. Because of this dependence, it was difficult to efficiently generate PVs *in vitro* and the study of PV infection had been hindered until 1990s. Organotypic raft cultures and mouse xenografts were the first methods that were used to generate PVs [10–13]. These methods were technically demanding and produced relatively low yields of virions. The production of non-infectious virus-like particles (VLPs) and, subsequently, infectious pseudoviruses (PsVs) in heterologous expression systems has been a major breakthrough for PV research [14–17].

VLPs can self-assemble into L1-VLPs when L1 is expressed alone or L1L2-VLPs if both capsid proteins are expressed. VLPs mimic the structure of the native PV virions and are the basis of the current prophylactic vaccines. VLPs are empty capsids; therefore, they are non-infectious and mostly used in binding studies. PsVs harbor reporter plasmids that function as viral “genomes” and can be used to quantify infection levels. Development of these PV virion production systems has greatly increased our understanding of the initial steps of PV infection.

Human papillomaviruses (HPVs) are a large family of viruses consisting of more than 150 types [4]. HPVs with transforming potential are the cause of almost all cases of cervical cancer and a majority of the anal, head and neck cancer cases [1]. The majority of the cervical cancer cases are caused by HPV16; therefore, it is the most studied HPV type [1, 18]. HPV16 infection is a multistep process: the virus takes advantage of a trauma to the epithelium to reach the basal keratinocytes—the only mitotically active cells of the epithelium. *In vivo*, the virus first binds to the basement membrane (BM) and then to the cell surface through interactions between the viral L1 protein and heparan sulfate proteoglycans (HSPGs). Cellular entry requires several conformational changes within the capsid via proteases and chaperones, and interaction of the capsid proteins with different receptors. The virus interacts with an elusive receptor complex and is subsequently internalized via a non-traditional endocytosis mechanism. During intracellular trafficking, the virus has been shown to localize to the endosomal system, trans-Golgi-network (TGN), Golgi complex and endoplasmic reticulum (ER) before delivery to the nucleus, where the viral DNA replicates. In this review, we summarize the knowledge on HPV16 entry and trafficking, and describe the roles of cellular proteins that mediate these events.

2. Binding and cell surface receptors of HPV16

HSPGs are glycoproteins containing one or more covalently bound heparan sulfate (HS) chains, a type of glycosaminoglycan (GAG). They can be located on the cell membrane, such as glypicans and syndecans, or in the extracellular matrix, such as perlecan [19]. The HSPGs on the cell membrane can be shed via proteolytic cleavage of the core protein by matrix metalloproteinases (MMPs) and their HS chains can be cleaved via endoglycosidic cleavage by extracellular heparanase [20, 21]. Early work has shown that cell membrane resident HSPGs are attachment receptors for HPV11 L1-VLPs and they are required for HPV16 PsV and HPV33 PsV infection [22, 23]. In those studies, heparinase or heparin (a

soluble form of heparan sulfate) treatment inhibited binding and infection. Subsequent work has revealed that different preparations of PVs require different HS modifications for successful binding, e.g. HPV33 L1L2-VLPs require O-linked sulfation whereas HPV33 PsVs require N-linked sulfation in addition to O-linked sulfation [24]. Although HS modifications play an important role for HPV binding and infection, it was suggested that the type of HSPG protein core does not. A study conducted with an HSPG-null cell line showed that when stably expressed, different HSPGs can serve as cellular receptors for HPV16 L1-VLP and the level of HSPG on the cell surface correlated with the number of bound viruses [25]. In the same study, syndecan-1 was proposed to be the primary attachment receptor for HPV as its expression is the highest in keratinocytes, and it is upregulated during wound healing [25]. A murine cervicovaginal challenge model has shown that wound formation is required for the virus to have access to the basement membrane (BM) and subsequently to the basal keratinocytes [26]. According to the model, the virus first binds to the BM through interactions with HSPGs and then, during the wound healing process, the virus is transferred to basal keratinocytes as they migrate to the wounded area. Since *in vitro* systems lack the BM, it was shown that the virus binds to the extracellular matrix (ECM) instead of the BM via interactions with HSPGs and laminin-332 [27–30]. Two separate studies suggested that in both *in vivo* and *in vitro* systems HPV uses retrograde filopodia transport to reach the cell body by binding to the leading edge of the migrating cells [31, 32].

Several reports have indicated that upon HSPG binding, the viral capsid goes through conformational changes [23, 24, 33–35]. One model suggests that the purpose of HSPG binding is to induce conformational changes that are required for a secondary binding event, which results in the infectious uptake of the virus through an unidentified receptor or receptor complex [34–36]. One conformational change leads to the exposure of a highly conserved furin convertase site on the L2 N-terminus and its cleavage via furin or proprotein convertase (PC) 5/6. Upon furin cleavage, the RG-1 epitope (residues 17–36) on the L2 protein are exposed [34]. In an *in vitro* study, using furin precleaved virus (FPC), it was shown that FPC PsVs can bind and infect HSPG-null cells, suggesting that the primary role of HSPG binding is to enable cell surface furin cleavage [37]. On the other hand, in the *in vivo* murine cervicovaginal challenge model, FPC PsV infection was inhibited when HSPGs were cleaved with heparinase III treatment [35]. Under heparinase III treatment, it was shown that FPC PsVs still bind to the cell surface, but not to the BM [35]. These results suggest that productive infection occurs through BM-bound FPC PsVs and not through cell surface-bound FPC PsVs. To explain this phenomenon, it was speculated that BM-bound virus can be transferred to a mitotically active epithelial cell, e.g. basal keratinocytes, during wound healing process, whereas direct cell surface binding of the virus might occur on nonpermissive cells, e.g. non-dividing cells. *In vitro* and *in vivo* experimental systems bear differences; therefore findings from these systems can be contradictory, such as with FPC virus and HSPG requirement on the plasma membrane.

Another model suggests that initial HSPG binding of the virus is not a passive event for infection [38]. According to this model HPV binds to the HSPGs on the cell surface (probably to syndecan-1) and when syndecan-1 is shed from the cell membrane as part of normal cell surface HSPG turnover via MMPs, the virus remains bound to shed syndecan-1.

In support of this model, treatment with MMP inhibitors, such as batimastat and marimastat reduced HPV16 release from the plasma membrane and viral infection [38, 39]. Following release, the virus and the shed syndecan-1 were found in high molecular weight complexes with active molecules, e.g. growth factors (GFs). These complexes might be endocytosed via epidermal growth factor receptors (EGFRs) or keratinocyte growth factor receptors (KGFRs). The type of receptor used for the viral uptake is determined by the nature of the active molecules that ‘decorate’ the virus and shed HSPG complex [38].

Following HSPG interaction and the conformational changes that expose the L2 N-terminus, furin cleaves the L2 protein. The role of furin at the cell surface is still under scientific debate. In an *in vivo* study, furin inhibition resulted in HPV16 PsVs being lost from the BM before reaching the cell surface [35]. It was concluded that furin cleavage is required for the PsVs to be transferred from the BM (where they interact with the HSPGs via L1) to a secondary receptor on the cell surface. It was also indicated that without furin cleavage and the subsequent epitope exposure on the L2 N-terminus, the secondary receptor-binding region on L1 is hindered. Lastly, because the PsVs were lost from the BM under furin inhibition, data suggested that the conformational change that exposed the furin cleavage site on the capsid also resulted in a decreased binding affinity of the viral capsid for the HSPG [35]. An *in vitro* study found that furin cleavage was not required for PsVs to be transferred from the ECM to the cell surface [40]. It was concluded that HS-processing enzymes (heparanase and MMPs) are responsible for the release of ECM-bound virus since ECM binding of the virus is mediated via HS chains of shed syndecan-1 ectodomains [40]. Another *in vitro* study showed that PsVs with a mutated furin/PC cleavage site can bind to cells and be internalized [33]. However, internalization of the PsVs did not progress to infection and the virus particles were retained in late endosomes. Therefore, it was concluded that, *in vitro*, furin activity is not required for the initial steps of infection, i.e. binding and internalization, but required for L2-mediated endosomal escape from the late endosomes [33].

Cyclophilins (CyPs) are a family of peptidyl-prolyl cis trans isomerases and Cyclophilin B (CyPB) is the most abundant member of this family [41]. CyPB has been suggested to interact with HSPGs on the cell surface [42]. An inhibitor-based screen concluded that CyPB facilitates conformational changes in the viral capsid that results in the exposure of the L2 N-terminus with the furin recognition site [43]. Therefore, CyPB-mediated conformational change was thought to be a prerequisite for furin cleavage of L2. Previously, RG-1 exposure was used as an indicator for furin cleavage of L2. However, Campos and colleagues recently showed that CyP inhibition has no effect on furin cleavage of the L2 protein, but still prevents RG-1 exposure, implying that these two events are independent [44]. Furthermore, the recent work of Campos and colleagues showed that siRNA-mediated CyPB depletion does not affect HPV16 infection of HaCaTs [44]. The dissociation of L1 from L2/DNA complex upon endosomal acidification has been suggested as an additional role for CyPs during HPV infection [45].

A recent study showed that a serine protease, Kallikrein-8 (KLK8), cleaves the L1 protein extracellularly [39]. Upon binding to HSPGs on the BM/ECM or plasma membrane, the viral capsid goes through a conformational change that facilitates KLK8 cleavage of the L1

protein. KLK8 cleavage was shown to be required for RG-1 epitope exposure, indicating that L1 cleavage via KLK8 facilitates L2 accessibility. Additionally, siRNA-mediated depletion of KLK8 resulted in defective internalization and trafficking of the virus [39].

Several studies have concluded that a non-HSPG receptor or a receptor complex is needed for the infectious uptake of the virus [30, 37]. To date, proteins that have been shown to be involved in viral uptake include: $\alpha 6$ and $\beta 4$ integrins, tetraspanins CD63 and CD151, annexin A2 heterotetramer (A2t), and EGFR/KGFR.

Integrins are transmembrane cell adhesion receptors. They are obligate heterodimers consisting of an alpha and beta unit [46]. A role for $\alpha 6$ integrin in HPV infection was first described in 1997 [47]. Using HPV6 L1-VLPs, $\alpha 6$ integrin was shown to be a binding receptor in HPV negative HaCaT cells (human basal keratinocytes). It was later demonstrated that $\alpha 6$ integrin knockdown via siRNA transfection significantly reduces HPV16 PsV infection [48]. A comparison of HPV16 PsV infection efficiencies of three keratinocyte cell lines with different $\alpha 6$ integrin expression levels showed that infection is most efficient in the highest $\alpha 6$ integrin expressing cell line [48]. The same study suggested that upon HPV16 PsV binding to the cells, focal adhesion kinase (FAK) is activated through $\alpha 6$ integrin [48]. A significant decrease in HPV16 PsV reporter gene transduction was shown in conditional $\beta 4$ integrin knockout mice [49]. Interestingly, $\alpha 6$ integrin was also lost in the female reproductive tract of those mice together with $\beta 4$ integrin [49]. A similar observation was made in $\beta 4$ integrin knock down HaCaT cells [50]. First, HPV16 PsV infection was significantly reduced in these cells compared to control cells. Second, $\alpha 6$ integrin cell surface expression levels were significantly reduced in $\beta 4$ integrin knock down cells. It has been shown that $\beta 4$ integrin is required for post-translational cleavage of $\alpha 6$ integrin [50]. It was speculated that this cleavage is required for cell surface localization of $\alpha 6$ integrin [50]. While data suggest that $\alpha 6$ integrin is required for HPV16 PsV infection, studies conducted with HPV33 and BPV4 concluded that $\alpha 6$ integrin is not required for infection [23, 51].

The integrin complex $\alpha 6\beta 4$ is the receptor for laminin-332, which is located in the ECM. HPV11 has been shown to transiently interact with laminin-332 in the ECM [28]. Laminin-332-bound virus can infect integrin $\alpha 6$ -expressing cells, but cannot infect cells that lack $\alpha 6$ integrin. Interestingly, certain tetraspanins can interact with integrins that bind laminin-332 [28]. Tetraspanins interact laterally with each other and with other transmembrane proteins to form tetraspanin enriched microdomains (TEMs) in the cell membrane [52]. Tetraspanin interacting proteins include, but are not limited to the $\alpha 6\beta 4$ integrin complex, GFRs and proteases such as MMPs [53]. The first study on tetraspanins and HPV16 infection found that HPV16 PsVs were transported to CD63 positive endosomes, indicating tetraspanin CD63 and HPV16 are internalized together [54]. Confocal and electron microscopy experiments showed that HPV16 PsVs and CD151 associated at the plasma membrane and that they were internalized together during infection [54]. It was found that only CD151-associated PsVs could be internalized. Interestingly, siRNA-mediated knock down of CD151 did not affect binding of the viral particles, but reduced the internalization and disassembly of these particles. Furthermore, knockdown of CD151-interacting integrin subunits, i.e. $\alpha 3$ and $\alpha 6$, significantly reduced HPV16 PsV

infection in HeLa cells [55]. However, $\alpha 3$ integrin was shown to be dispensable for HPV16 PsV infection of HaCaTs [50].

Annexin A2 tetramer (A2t) has been shown to facilitate HPV16 infection [56]. Identification of several neutralizing epitopes on the L2 N-terminus that are able to inhibit HPV16 infection implied the presence of an L2-specific receptor [57–60]. One of those epitopes, amino acids 108–126, of L2 was shown to interact with A2t [56]. Experiments showed that HPV16 bound to A2t on the cell surface of HeLa cells. HPV16 harboring mutations in the L2_{108–126} region had reduced A2t binding and infectivity in HaCaT cells. shRNA-mediated knockdown of Annexin A2 (AnxA2) resulted in decreased internalization of HPV16 L1L2 VLPs, but not HPV L1VLP internalization in HeLa cells [56]. It was shown that AnxA2-shRNA mediated knockdown of A2t significantly reduced HPV16 pseudoinfection of HeLa cells. A2t consists of two monomers of AnxA2 and an S100A10 dimer [61, 62]. Using biochemical analyses, it was shown that the L2_{108–126} region interacts specifically with S100A10 subunit of A2t [56]. A2t is a calcium-binding protein and is localized to the inner leaflet of the plasma membrane. Src-mediated phosphorylation of AnxA2 results in extracellular translocation of A2t [63]. The knowledge that HPV16 binding activates EGFR signaling in cells and Src is activated as a result of EGFR activation was the basis for further work [38, 64, 65]. It was shown that HPV16 binding leads to EGFR-dependent Src activation. Subsequently, activated Src phosphorylates AnxA2 and induces its extracellular translocation [65]. Following this translocation, HPV16 and A2t associate at the cell surface. To distinguish the roles of AnxA2 and S100A10 during HPV16 infection, the cells were treated with antibodies against these two proteins. Both antibodies effectively reduced HPV16 PsV infection of HaCaTs. AnxA2-antibody treatment caused retention of the virus particles on the plasma membrane. Surprisingly, S100A10 antibody treatment did not prevent viral entry, but the virions were trapped in late endosomes/lysosomes. Evidence was also provided for EGFR-AnxA2-HPV16 interaction at the cell surface via co-immunoprecipitation experiments. It was further shown that AnxA2 interacts with HSPGs both in the presence and absence of HPV16 PsVs. The authors proposed the following model for AnxA2 function in HPV16 infection: after binding to the cell surface via HSPGs, HPV16 is released from the cell surface via MMP action on the HSPGs, and is decorated with EGFs; the EGF-decorated form of HPV16, together with the HSPGs, binds to EGFR on the cell surface [38]. The binding induces EGFR-dependent Src kinase activation, and the active Src subsequently phosphorylates AnxA2. Phosphorylation of AnxA2 in the A2t complex results in extracellular translocation of A2t. Therefore, HPV16 can bind to AnxA2 on the cell surface. HSPGs may bridge the HPV16 and AnxA2 interaction. The study provides evidence for AnxA2's involvement in HPV16 entry and trafficking. The model suggests that Src-mediated phosphorylation of AnxA2 might facilitate its recruitment to lipid microdomains to initiate endocytosis of HPV16 [65]. Recent work took advantage of the availability of small molecule inhibitors of A2t (A2ti) that disrupt the interaction between AnxA2 and S100A10 [66]. It was found that A2ti completely inhibits HPV16 PsV infection of HeLa and HaCaT cells. Internalization assays resulted in a significant decrease (65%) in HPV16 PsV entry when the cells were treated with A2ti [66].

3. Endocytosis of HPV16

Upon cell binding, HPV16 needs to be internalized into the cells to transport its DNA to the nucleus for a productive infection. Early studies on HPV16 endocytosis mostly utilized inhibitors against the key players in common endocytosis pathways. However, this resulted in blocking major endocytic pathways used by cells. Furthermore, under these inhibitor treatments, the virus could have used an alternative pathway for entry. Establishment of specific gene-targeting methods, e.g. siRNA mediated knock down technology, has made it possible to limit off-target effects that may have been caused by inhibitors. Additionally, because the protocol to generate high-titer PsVs was not available until 2004, early studies used VLPs instead of PsVs. VLP studies focused on internalization assays, whereas PsV studies focused on infection assays to validate their findings on endocytosis. The diversity in the techniques yielded contradicting results about the route the virus takes after its internalization; therefore, our knowledge of viral endocytosis is not yet complete. Although the details of HPV endocytosis are not fully defined, it has been shown that the virus particles enter the cells asynchronously and the half times of internalization ranges from 4 to 12 hours post infection (hpi) [67].

Two well-studied endocytic pathways are clathrin- and caveolin-mediated endocytosis. These are two commonly used pathways by non-enveloped viruses [68]. Dynamin is responsible for pinching off the endocytic vesicles, which form via clathrin- or caveolin-mediated endocytosis, from the plasma membrane. Early work suggested that HPV16 used clathrin-mediated endocytosis to invade the host cells [69–71]. One study supported the idea that HPV16 internalization is both clathrin- and caveolin-mediated [72]. Later, using dominant-negative mutants of clathrin- and caveolin-mediated endocytosis and dynamin function and siRNA knock down of clathrin, caveolin and dynamin in HeLa cells, one study concluded that HPV16 entry occurs via a clathrin and caveolin independent mechanism [54]. The study suggested that HPV16 internalization is instead mediated by tetraspanins, CD63 and CD151, at TEMs. The study provided evidence for the formation of a complex with CD63, CD151 and HPV16 PsVs at the cell surface. When the cells were pre-treated with CD63- and CD151- specific antibodies, HPV16 PsV infection was reduced. Similarly, siRNA-mediated knock down of CD151 resulted in reduced HPV16 PsV infection. It was concluded that HPV16 PsV entry was inhibited in the cells that are pre-treated with CD151 antibody and also in CD151 depleted cells. Therefore, the authors suggested that HPV16 entry occurs at the TEMs and HPV16 is internalized into the cells together with CD151 [54]. In subsequent work, several CD151 mutants were generated to better understand the role of CD151 in HPV16 endocytosis [55]. Expression of CD151 mutants that are unable to bind to laminin-binding integrins ($\alpha 3\beta 1$ and $\alpha 6\beta 1/\beta 4$) and palmitoylation-deficient CD151 that cannot be recruited to TEMs could not rescue HPV16 internalization in CD151 depleted cells. The results suggested that recruitment of CD151 to the TEMs and its interaction with laminin-binding integrins are required for HPV16 endocytosis. Using live cell imaging, the authors showed that HPV16 associates with CD151 and the complex moves laterally at the cell surface before internalization. This observation suggested that CD151 directs the virus to the entry sites on the cell surface. The authors showed that only CD151-bound virus can enter the cells [55].

Another study using live microscopy imaging showed that once the lateral movement of the virus particle stops, HPV16 internalization occurs within 2 minutes [67]. The study described a novel endocytic pathway for HPV16 internalization, which is clathrin-, caveolin-, flotillin-, dynamin- and cholesterol-independent. This novel pathway is related to macropinocytosis, as it involves actin dynamics, Na^+/H^+ exchangers, tyrosine kinase, p21-activated kinase and protein kinase C signaling; yet it is distinct from macropinocytosis due to lack of membrane protrusions and presence of small, evenly shaped vesicles rather than large and irregularly shaped vacuoles upon HPV16 endocytosis [67].

4. Intracellular trafficking of HPV16

Trafficking of the viral DNA (or pseudogenome in PsVs) to the nucleus of a target cell is a critical stage in the infectious pathway of HPVs. Transcription by the host cell requires the viral DNA to travel from the plasma membrane to the nucleus. The current model of the intracellular trafficking of HPV16 during infection has been shown to involve the movement of the virus through early endosomes, late endosomes/lysosomes, TGN, ER, and access to the nucleus. Host cellular components and mechanisms are described to play roles in the viral trafficking.

It is well-established that HPV16 PsVs localize to early endosomes during infection. In HaCaT cells, the L1 capsid protein of HPV16 PsVs has been shown to colocalize with the early endosome marker early endosome antigen 1 (EEA1) as early as 5 minutes post-infection and through to 4 hours via immunofluorescence [48, 72]. AF488-labeled HPV16 PsVs colocalized with EEA1 at 2.5 hours post-infection in HaCaT cells [73]. Proximity ligation assays (PLA) in HaCaT cells showed colocalization between EEA1 and L1 (HPV16 PsVs) at 8 hours and showed a decrease in colocalization between the two proteins at 16 hours [74]. The EdU-labeled pseudogenome of HPV16 PsVs colocalized with EEA1 in HaCaT cells at 2 hours post-infection [75]. In HeLa cells, HA-tagged L2 in HPV16 PsVs colocalized with EEA1 at 12 hours post-infection [76]. In contrast, another group found no significant colocalization between HPV16 PsVs (AF448-labeled) and EEA1 in HeLa cells, but did find that the PsVs colocalized briefly with Rab5 in live microscopy at 3.5 hours post-infection [67]. Rab5 is a GTPase known to function in the fusion and movement of early endosomes [77]. It was shown that HPV16 PsV infection in HeLa cells required Rab5 function through the use of DN and CA Rab5 constructs [78]. Similarly, HPV31 was shown to require Rab5 for infection of HaCaT cells through the expression of a DN form of Rab5 [79].

Many labs have shown the movement of HPV16 particles from early endosomes to late endosomal/lysosomal compartments via colocalization of the viral capsid proteins or pseudogenome with the late endosome/lysosome markers LAMP1 and LAMP2 (lysosome-associated membrane protein 1 and 2). In early studies of papillomavirus trafficking, the L1 protein in BPV1 VLPs colocalized with LAMP2 in C127 cells at 4 hours post-infection [71]. Colocalization of the HPV L1 protein with LAMP1 seems to occur slightly later; starting at approximately 6–8h post-infection [54]. The level of colocalization between HPV L1 and LAMP1 increases as infection progresses, which suggests the majority of the L1 protein is retained in the lysosome for degradation [75, 79]. AF488-labeled HPV16 PsVs colocalized

with LAMP1 at 8h post-infection in HeLa cells [67]. In HaCaT cells, HPV16 PsV L1 (using the conformational-dependent antibody H16.V5 against L1 [80, 81]) colocalized with LAMP1 starting at 8 h post-infection and increased at 16h [75]. AF488-labeled HPV31 virions colocalized with LAMP1 at 16h and 24h post-infection in HaCaT cells [79]. One potential tactic for viral movement between the endosome and lysosome is the use of components in the cellular endosomal sorting complex required for transport (ESCRT). The interaction of the HPV16 L2 protein with ESCRT machinery has been shown to be necessary for viral infection [82]. The ESCRT machinery sorts cargoes during endosomal/lysosomal maturation and could play a role in sorting L2/pseudogenome complex from dissociated L1 protein.

The localization of viral particles to late endosomes and lysosomes is accompanied by viral capsid disassembly and/or degradation. In this review we use the terms “disassembly” and “degradation” to describe trafficking events that involve the viral capsid. Here we consider “disassembly” an unfolding of the viral capsid, aided by cellular molecules such as chaperones, and “degradation” as a falling apart of the capsid, due to pH or cellular proteases and cleavage of L1 capsid proteins. Both capsid disassembly and degradation result in a loosening of the viral capsid that allows the pseudogenome (complexed with the L2 capsid protein) to continue trafficking to the nucleus for infection. The use of the L1–7 antibody, which recognizes an epitope of L1 located in the interior of intact viral capsids, has been used to assess the disassembly/degradation of viral capsids during infection [45, 83, 84]. The L1–7 epitope begins to become accessible approximately 4 hours post-infection, increasing in accessibility over the next 12 hours of infection in HaCaT and HeLa cells, and colocalizes with LAMP1 in HaCaTs [45, 54, 75, 85].

It is thought that a combination of pH reduction during endosomal to lysosomal maturation and various cellular host proteins facilitate the disassembly and/or degradation of the viral capsid, which appears to be a necessary step for the L2/pseudogenome complex to successfully enter the nucleus [43, 73, 86]. Reduced pH in the late endosome/lysosome is necessary for the degradation of the viral capsid during infectious entry. However, it is unclear whether low pH is directly responsible for capsid degradation or has an indirect effect, such as activation of cellular proteases necessary for viral capsid degradation. Many studies have used the weak base NH_4Cl to neutralize acidic late endosome and lysosome compartments and Bafilomycin A (which inhibits vacuolar ATPase and endosomal trafficking) and have shown a resulting decrease in viral infection. In studies with HPV16 PsVs in HeLa and HaCaT cells, HPV16 PsVs accumulated in the late endosome with NH_4Cl or Bafilomycin A1 treatment, but when the agents were removed, infection was able to proceed [67]. It was found that for HPV16 PsVs to be infectious, they must be exposed to low pH for several hours (or, as the authors called attention to, exposed to enzyme/s that require low pH to function) [67]. Bafilomycin A treatment prevented the dissociation of the L1 capsid protein from the EdU-labeled pseudogenome in HPV16 PsV-infected HaCaT cells [45]. In 293 cells, NH_4Cl treatment reduced HPV16 PsVs infection and prevented the movement of the PsVs from early endosomes into Caveolin1+ (Cav1+) vesicles, possibly by inhibiting the fusion of vesicles [72].

The negative effect that inhibiting endosome acidification has on HPV16 infection extends to other types of PVs. NH_4Cl or Bafilomycin A1 treatment blocked BPV1 infection in C127 cells when added to the cells up to 4 hours post-infection [71]. The treatment of COS-7 cells with bafilomycin A1, NH_4Cl , or chloroquine (to raise endosomal and lysosomal pH) resulted in decreased HPV33 infection [87]. In HaCaT cells, NH_4Cl treatment reduced HPV31 PsV (AF594-labeled) infection and resulted in accumulation of the PsVs in LAMP1+ vesicles [79]. An *in vitro* study convincingly demonstrated the effect of low pH on HPV31 PsV viral capsid conformation and accessibility to the viral genome [79]. These *in vitro* experiments showed that encapsidated viral DNA became accessible to digestion by DNase after a 2 hour incubation of viral preparations at reduced pH. At neutral pH of 7.6, >75% of the viral genome was resistant to DNase treatment. In contrast, 98% of the viral genomes were digested after incubation at pH of 5 (comparable to the pH of lysosomes) and 60% of the viral genomes were digested at a pH of 6 (comparable to the pH in endosomes). The DNase results, along with visualization by TEM, suggested that the conformation of the HPV31 viral capsid loosens when exposed to an environment with reduced pH.

Several host cellular factors have been shown to be necessary for viral capsid disassembly while the virus is in the endolysosomal system. The host cell chaperones CyPs have been shown to facilitate viral capsid disassembly during intracellular trafficking in HPV16 PsV infection [43, 45]. Using HPV16 PsVs produced with a mutant L2 protein (to bypass the potential requirement of CyP at the plasma membrane), it was shown that inhibiting CyP function resulted in increased colocalization of the L1 capsid protein with the EdU-labeled pseudogenome and decreased infection in HaCaT cells [45]. *In vitro* experiments showed the ability of CyP to catalyze the separation of L1 and L2 proteins. These data suggested that inhibiting intracellular CyP prevented the separation of the L1 protein from the pseudogenome, and that the dissociation between L1 and the pseudogenome was necessary for successful infection. Further work showed that CyP activity was necessary for the L2/pseudogenome complex to localize to the TGN during HPV16 PsV infection of HaCaT cells, presumably due to the lack of L1 separation from the L2/pseudogenome complex in the endosomal system [88]. Recent work has shown that siRNA-mediated knockdown of CyPB has no effect on HPV16 infection of HaCaT cells, and it was suggested that CyPA is involved in the later infectious route of the virus [44].

The non-receptor tyrosine kinase Pyk2 has been suggested to play a role in HPV16 PsV capsid disassembly [75]. Pyk2 depletion via siRNA resulted in decreased viral infection but accelerated localization of the virus to LAMP1+ vesicles and accelerated disassembly of the viral capsid. These results suggested that endogenous Pyk2 delays virus movement through the endolysosomal system. Further work is needed to understand Pyk2's function in endolysosomal trafficking of the virus.

The studies summarized above suggest that disassembly/degradation of the viral capsid and separation of the L1 capsid protein from the L2/pseudogenome complex are necessary steps during trafficking for viral infection to occur. However, research has shown the HPV16 PsV L1 protein both remaining in the lysosome for degradation, and accompanying the L2/vDNA complex to the TGN and ER [72, 76, 88, 89]. In HaCaT and HeLa cells, the majority of HPV16 PsV L1 colocalized with LAMP1 at late time points post-infection (24h), while the

L2/pseudogenome complex colocalized with TGN markers and PML in the nucleus [89]. In contrast, another study showed that a portion of L1 (using HPV16 PsVs) colocalized with TGN and Golgi markers at 16 h post-infection in HeLa cells via IF and PLA [76]. Similarly, a study using mutant HPV16 PsVs that are retained in the TGN during infection showed that a small portion of L1 traffics with the L2/pseudogenome complex to the TGN via colocalization between the L1 protein and TGN markers at 18 h post-infection in HaCaT cells [88]. In HPV16 PsV infection of HaCaT cells, L1 colocalized with ER markers starting at 4 hours post-infection and colocalization increased at 12 hours [72]. It is unclear what role, if any, L1 plays in accompanying the L2/vDNA complex to these later cellular compartments.

Data have suggested that HPV16 PsVs traffic first to early endosomes and then to Cav1+ vesicles in 293 cells [72]. In these immunofluorescence studies it was shown that HPV16 PsVs colocalized with Cav1 at later time points post-infection than with early endosome marker EEA1. Treatment with NH₄Cl resulted in a decrease in HPV16 colocalization with Cav1, but also resulted in a decrease in overall Cav1+ vesicles. It is unclear whether the neutralization of lysosomes with NH₄Cl inhibited the formation of Cav1+ vesicles, thereby inhibiting movement of the PsVs from the early endosomes. While there are reports of HPV16 PsVs colocalizing with Cav1 [72, 88], whether or not Cav1 plays a role during infection is unclear. One study used shRNA to reduce Cav1 protein levels in HaCaT cells and showed a 60% decrease of HPV16 PsV infection via flow cytometry [72]. In contrast, Caveolin-dependent entry of HPV16, 18, and 31 PsVs into HaCaT cells has been ruled out through the use of inhibitors such as filipin and nystatin, which prevent Caveolin-dependent entry at the plasma membrane [54]. A study conducted in HeLa and 293TT cells showed no effect on HPV16 PsV infection using a DN form of Cav1 (GFP-tagged on the N-terminus) and even an increase in infection when targeting Cav1 using siRNA [54]. In the same study, immunofluorescence results showed no colocalization between the L1 protein (using the L1-7 antibody; the epitope only becomes exposed during capsid disassembly/degradation) and Caveolin-1 (GFP-tagged on the C-terminus, which does not affect Cav1 function) 10 hours post infection. Perhaps the virus detected in Cav1+ vesicles is non-infectious if the capsid is unable to loosen in these compartments.

It has been shown that after the late endosome, the L2/pseudogenome complex (and a portion of L1) traffic to the TGN, Golgi, and ER before entering the nucleus during infection [72, 76, 88, 89]. Viral trafficking from the endosomal system to the TGN/Golgi and subsequent compartments has been shown to be facilitated by the viral L2 protein and a variety of host cellular factors including Sorting Nexin 17 (SNX17), Sorting Nexin 27 (SNX27), retromer complex components, and γ -secretase [73, 76, 86, 88, 90, 91].

L2 has been shown to be necessary for the pseudogenome to traffic to the TGN [88, 89]. L1-only PsVs are non-infectious and the EdU-labeled pseudogenome does not colocalize with TGN markers, suggesting that without L2, the pseudogenome is unable to traffic to the TGN [89]. Studies investigating L2-mediated trafficking have shown that mutating L2 in two transmembrane regions resulted in the L2/pseudogenome complex being retained in the late endosome and failing to enter the nucleus and colocalize with PML bodies [86, 90]. It has been proposed that L2 mediates the trafficking of the L2/pseudogenome complex out of

endosomal compartments via the ability of transmembrane domains at both the L2 C- and N-termini to destabilize membranes [86, 90]. Additionally, a central nuclear retention sequence (amino acids 291–316) in the HPV16 L2 protein has been shown to be necessary for L2 retention in the nucleus and is necessary for HPV16 infection in HaCaT cells and mouse model [88, 92]. Mutations made to the central NRS (named mNLS by DiGiuseppe et al.) in the L2 protein resulted in the retention of the L2/pseudogenome complex in the TGN [88].

Cleavage of L2 by several cellular proteases has been shown to be necessary for post-endosomal viral trafficking. It was shown that cleavage of L2 by cellular furin (a proprotein convertase) was necessary for BPV1 PsVs to traffic from endosomal compartments and enter the nucleus in HeLa cells [33]. Likewise, furin cleavage of L2 in HPV16 PsVs was found to be necessary for L2/pseudogenome localization to the TGN [89]. One study suggested that cathepsin B could possibly compensate for loss of furin function by using HPV16 PsVs pretreated with cathepsin B protease, but further investigation is needed to confirm this finding [72]. The activity of the cellular membrane-associated protease γ -secretase has been shown to be necessary for HPV infection [93, 94]. γ -secretase inhibitor treatment prevented infection of HaCaT cells and mouse female reproductive tract by diverse papillomavirus types [93, 94]. Inhibiting γ -secretase activity prevented the BrdU-labeled pseudogenome and L2 protein from entering the nucleus during HPV16 PsV infection of HeLa cells [85]. In further studies, it was found that γ -secretase activity is necessary for the trafficking of HPV16 PsVs after exit from the endosomal pathway and before entry into the Golgi and ER in HaCaT and HeLa cells [74]. Using PLA, L2 and the EdU-labeled pseudogenome were shown to be in close proximity to TGN and Golgi markers (TGN46 and GM130) at 16 h and 18 h post-infection and ER markers (BiP and PDI) at 20 h post-infection. γ -secretase inhibition prevented the PLA signal in these experiments, indicating γ -secretase activity is required for the L2/pseudogenome complex to localize to the TGN, Golgi, and ER.

The cellular protein SNX17 is involved in the trafficking of the L2/pseudogenome complex from late endosome/lysosome compartments [73]. SNX17 interacts with L2 through a highly conserved binding motif in L2 ($^{254}\text{NPxY}^{257}$) and the SNX17/L2 interaction facilitates viral escape from late endosomes [73]. Depletion of SNX17 increased lysosomal degradation of L2 and decreased infection. It was proposed that the interaction of L2 with SNX17 prevents the virus from being routed to the lysosome and being degraded. The use of HPV16 PsVs containing a mutant L2 that is unable to bind SNX17 and siRNA-mediated depletion of SNX17 both resulted in reduced viral infection in HaCaT cells. AF488-labeled HPV16 PsVs colocalized with LAMP2 earlier in SNX17 knockdown HaCaT cells than in untreated cells. AF488-labeled mutant L2 HPV16 PsVs (deficient in binding SNX17) exhibited a similar pattern of accelerated and increased colocalization with LAMP2 as compared to the control cells. SNX17 was found to increase the stability of the L2 protein by delaying the trafficking through the endosomal compartments. Furthermore, it was found that L2 from multiple types of papillomaviruses interact with SNX17 and that the interaction was necessary for infection [95]. These studies suggested that the interaction between L2 and SNX17 is required for trafficking of the L2/pseudogenome complex from the late endosome/lysosome. A second sorting nexin, SNX27, was found to interact with HPV16 L2

via a PDZ domain on SNX27 and a central region of L2 [91]. The siRNA-mediated knockdown of SNX27 alone only moderately reduced HPV16 infection, but simultaneous knockdown of SNX27 and SNX17 reduced infection to a greater degree.

Various cellular factors have been found to be involved in the retrograde transport of HPV, including retromer complex components and Rab GTPases [67, 76, 79, 89, 96]. siRNA-mediated depletion of retromer components resulted in decreased HPV16 PsV infection in HaCaT cells [76]. Binding of HPV16 L2 to the retromer complex was shown to be necessary for HPV to traffic from the endosomal system and localize to the TGN in HeLa and HaCaT cells [96]. The authors proposed that L2 may interact with the retromer complex through the endosomal membrane, a hypothesis that is supported by the study that demonstrates the C-terminal transmembrane domain of L2 has membrane penetrating ability [86]. In fact, the mutations to the C-terminus of HPV16 L2 that Kämper et al. generated are located directly downstream from the retromer recognition sites identified by Popa et al. The deletion of the C-terminal residues from L2 disrupted the ability of L2 to penetrate membranes, which could in turn have prevented exposure of the retromer binding sites from the endosomal membrane. Rab GTPases mediate retrograde trafficking from the endosome to the TGN. The expression of DN forms of Rab7b and Rab9a resulted in decreased HPV16 PsV infection in 293TT cells [89].

The movement of HPVs through the cytoplasm has been shown to depend on microtubules and the interaction of the viral protein L2 with the motor protein, dynein, and L2 interaction with β -actin [67, 71, 87, 97–99]. Pre-treatment of HeLa cells with nocodazole, which disrupts microtubule polymerization, effectively reduced AF594-labeled HPV16 PsV infection without disrupting virus internalization [67]. Nocodazole treatment was shown to greatly reduce HPV33 PsV infection of COS-7 cells in a dose-dependent manner [87]. Nocodazole addition at 6–12 hours post-infection showed the greatest reduction of viral infection [87], indicating that intact microtubules were required at a time post-infection when the virus is thought to traffic from late endosomal/lysosomal compartments to the TGN. Nocodazole treatment was similarly found to inhibit BPV1 infection of C127 cells [71]. However, in these studies the inhibitor only reduced BPV1 infection when added during 0 – 6 hours post-infection and failed to inhibit infection when added 8 hours after the infection start point, indicating that perhaps BPV1 infection occurs at a different rate than HPV infection. It is unclear whether the virus stays contained within vesicles while traversing the cytoplasm between the endosomal system and the TGN, or if the L2/pseudogenome complex exits the late endosome and traffics to the TGN via direct interaction with the microtubule transport system. The L2 proteins from HPV16 and HPV33 have been shown to interact with dynein, the main motor protein that drives minus-end-directed (retrograde) transport along microtubules, specifically with the dynein light chains DYNLT1 and DYNLT3 [97, 99]. The dynein interaction site on L2 was mapped to the C-terminal 40 residues, which has also been characterized as a transmembrane domain capable of penetrating membranes [86], such as that of endosomes. The close proximity of these two domains indicates the possibility that L2 could interact with dynein through a vesicular membrane while trafficking to subsequent compartments during infection. The N-terminal residues 25–45 of HPV16 and BPV1 L2 have been shown to interact with β -actin *in vitro* and in HeLa, SiHa, and C33A cells [98]. HPV16 PsVs containing an L2 protein that lacked

residues 25–45 displayed reduced infectivity in a focus-forming assay in C127 cells. Incidentally, the N-terminal transmembrane domain described by Bronnimann et al. is located directly downstream (residues 45–67) from the actin-binding motif [90], suggesting the possibility that the N-terminus of L2 could interact with β -actin through an endosomal membrane while trafficking to subsequent compartments.

Previously, interaction between the BPV1 L2 protein and syntaxin 18 (a protein that mediates vesicular trafficking to and from the ER) was demonstrated to be necessary for BPV1 infection [100]. Trafficking of HPV16 PsVs to the ER via detection of L1 was suggested by Laniosz et al. 2009 [72]. Immunofluorescence studies showed colocalization of L1 with antibodies against ER markers Calnexin and ERp29 in HaCaT cells. Additionally, treatment with Brefeldin A, an inhibitor of COP1-mediated vesicle formation involved in ER export and import, reduced HPV16 PsV infection of HaCaT cells. Since that initial study, two HPV entry siRNA screens have identified ER components that may be involved in infection [76, 101]. Furthermore, a study using PLA showed that in HeLa and HaCaT cells the L2 protein and EdU-labeled pseudogenome are in close proximity (within 40 nm distance) to ER markers BiP and PDI [74]. Although multiple studies have shown the L2/vDNA complex, and occasionally L1, localizing to the TGN, Golgi, and ER, subsequent steps to the nucleus remain unclear. Studies have suggested that entry of the L2/pseudogenome complex into the nucleus is dependent on nuclear envelope breakdown, which occurs during cell cycle progression [101, 102]. It is well-established that the pseudogenome and L2 protein enter the nucleus, as research has shown colocalization of viral DNA and L2 with the PML protein in the nucleus of cells [88, 103].

5. Conclusions

In this review we aim to summarize the current knowledge on HPV16 entry and trafficking. Decades of research on PVs have provided valuable and practical information on infection mechanisms. One of the major breakthroughs in the field was the development of prophylactic vaccines against several high- and low-risk HPV types, which was made possible by research that showed the ability of the L1 protein to self-assemble into highly immunogenic VLPs. From the 1980s, when the first association between HPV and cancer was shown, HPV research has increased our understanding of the underlying mechanism of several types of cancer, including cervical, genital, head and neck cancers.

Over the last thirty years we have gained considerable insight into the HPV infectious process. However, in spite of extensive efforts, we still lack a clear, widely accepted, consensus model for viral entry and trafficking. Having said that, we know that initial binding of HPV16 is L1 and HSPG mediated. This interaction induces conformational changes in both capsid proteins that facilitate productive infection. Subsequently the virus is transferred to a receptor complex and it is internalized via a novel endocytic pathway. After internalization, the virus traffics through the endosomal system where the capsid disassembles and L2 remains associated with the vDNA. The L2/vDNA complex travels to the TGN, possibly through the ER, and finally to the nucleus during nuclear envelope breakdown during mitosis. Despite progress, key mechanisms in HPV entry and trafficking are still under scientific debate and studies on HPV infection mechanisms produce diverse

and contradicting results. The discrepancies might partially be attributable to the different virus genotypes, cell lines, methods of virus production that have been used for the experimental setups and to different observations from *in vivo* and *in vitro* models.

There is much left for us to discover in this field and molecular biology and virology tools that have been developed during these years have enabled researchers to shed light on previously unknown HPV biology. The development of high-titer pseudovirus production protocols and mouse models allow for better studies of HPV infection *in vivo*. High-throughput screening technologies and imaging techniques that provide relatively higher resolution will continue to accelerate HPV research. Although our understanding of HPV infection has come a long way, considering the recent developments in the field, one expects that the current unknowns and discrepancies will be resolved in the near future.

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References

1. Doorbar J, et al. The biology and life-cycle of human papillomaviruses. *Vaccine*. 2012; 30(Suppl 5):F55–70. [PubMed: 23199966]
2. Munday JS. Bovine and human papillomaviruses: a comparative review. *Vet Pathol*. 2014; 51(6): 1063–75. [PubMed: 24981715]
3. Campo MS, Roden RB. Papillomavirus prophylactic vaccines: established successes, new approaches. *J Virol*. 2010; 84(3):1214–20. [PubMed: 19906917]
4. Bernard HU, et al. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology*. 2010; 401(1):70–9. [PubMed: 20206957]
5. Dvoretzky I, Shober R, Chattopadhyay SK, Lowy DR. A quantitative in vitro focus assay for bovine papilloma virus. 1980
6. Law MF, Lowy DR, Dvoretzky I, Howley PM. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. 1981
7. Watts SL, Phelps WC, Ostrow RS, Zachow KR, Faras AJ. Cellular transformation by human papillomavirus DNA in vitro. 1984
8. Buck CB, et al. Arrangement of L2 within the papillomavirus capsid. *J Virol*. 2008; 82(11):5190–7. [PubMed: 18367526]
9. Kondo K, et al. Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. *Virology*. 2007; 358(2):266–72. [PubMed: 17010405]
10. Dollard SC, Wilson JL, Demeter LM, Bonnez W, Reichman RC, Broker TR, Chow LT. Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures.pdf. 1992
11. Meyers C, Frattini MG, Hudson JB, Laimins LA. Biosynthesis of Human Papillomavirus from a Continuous Cell Line Upon Epithelial Differentiation.pdf. 1992
12. Bonnez W, DaRin C, Borkhuis C, de Mesy Jensen K, Reichman RC, Rose RC. Isolation and Propagation of Human Papillomavirus Type 16 in Human Xenografts Implanted in the Severe Combined Immunodeficiency Mouse.pdf. 1998
13. Sexton CJ, Williams AT, Topley P, Shaw RJ, Lovegrove C, Leigh I, Stables JN. Development and characterization of a novel xenograft model permissive for human papillomavirus DNA amplification and late gene expression.pdf. 1995

14. Zhou J, SX, Stenzel DJ, Frazer IH. Expression of Vaccinia Recombinant HPV 16 L1 and L2 ORF Proteins in Ep&heMt Cetis Is Sufficient for Assembly of HPV Virion-like Particles. 1991
15. Kirnbauer R, BF, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic.pdf. 1992
16. Rose RC, BW, Reichman RC, Garcea RL. Expression of human papillomavirus type 11 L1 protein in insect cells- in vivo and in vitro assembly of viruslike particles..pdf. 1993
17. Hagensee ME, YN, Galloway DA. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins.pdf. 1993
18. Clifford GM, et al. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer*. 2003; 88(1):63–73. [PubMed: 12556961]
19. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. *Cold Spring Harb Perspect Biol*. 2011; 3(7)
20. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 2007; 446(7139):1030–7. [PubMed: 17460664]
21. Fitzgerald ML, WZ, Park PW, Murphy G, Bernfield M. Shedding of syndecan-1 and-4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase.pdf. 2000
22. Joyce JG, TJ, Przysiecki CT, Cook JC, Lehman ED, Sands JA, Jansen KU, Keller PM. The L1 Major Capsid Protein of Human Papillomavirus Type 11 Recombinant Virus-like Particles Interacts with Heparin and Cell-surface Glycosaminoglycans on Human Keratinocytes.pdf. 1999
23. Girolglou T, FL, Schäfer F, Streeck RE, Sapp M. Human Papillomavirus Infection Requires Cell Surface Heparan Sulfate.pdf. 2001
24. Selinka HC, GT, Nowak T, Christensen ND, Sapp M. Further evidence that papillomavirus capsids exist in two distinct conformations.pdf. 2003
25. Shafti-Keramat S, et al. Different Heparan Sulfate Proteoglycans Serve as Cellular Receptors for Human Papillomaviruses. *J Virol*. 2003; 77(24):13125–13135. [PubMed: 14645569]
26. Roberts JN, et al. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nat Med*. 2007; 13(7):857–61. [PubMed: 17603495]
27. Culp TD, Budgeon LR, Christensen ND. Human papillomaviruses bind a basal extracellular matrix component secreted by keratinocytes which is distinct from a membrane-associated receptor. *Virology*. 2006; 347(1):147–59. [PubMed: 16376962]
28. Culp TD, et al. Keratinocyte-secreted laminin 5 can function as a transient receptor for human papillomaviruses by binding virions and transferring them to adjacent cells. *J Virol*. 2006; 80(18): 8940–50. [PubMed: 16940506]
29. Cerqueira C, et al. Heparin increases the infectivity of Human Papillomavirus type 16 independent of cell surface proteoglycans and induces L1 epitope exposure. *Cell Microbiol*. 2013; 15(11): 1818–36. [PubMed: 23601855]
30. Selinka HC, et al. Inhibition of transfer to secondary receptors by heparan sulfate-binding drug or antibody induces noninfectious uptake of human papillomavirus. *J Virol*. 2007; 81(20):10970–80. [PubMed: 17686860]
31. Smith JL, Lidke DS, Ozbun MA. Virus activated filopodia promote human papillomavirus type 31 uptake from the extracellular matrix. *Virology*. 2008; 381(1):16–21. [PubMed: 18834609]
32. Schelhaas M, et al. Human papillomavirus type 16 entry: retrograde cell surface transport along actin-rich protrusions. *PLoS Pathog*. 2008; 4(9):e1000148. [PubMed: 18773072]
33. Richards RM, et al. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. *Proc Natl Acad Sci U S A*. 2006; 103(5):1522–7. [PubMed: 16432208]
34. Day PM, et al. Mechanisms of human papillomavirus type 16 neutralization by l2 cross-neutralizing and l1 type-specific antibodies. *J Virol*. 2008; 82(9):4638–46. [PubMed: 18305047]
35. Kines RC, et al. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. *Proc Natl Acad Sci U S A*. 2009; 106(48):20458–63. [PubMed: 19920181]

36. Richards KF, et al. Multiple heparan sulfate binding site engagements are required for the infectious entry of human papillomavirus type 16. *J Virol.* 2013; 87(21):11426–37. [PubMed: 23966387]
37. Day PM, Lowy DR, Schiller JT. Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids. *J Virol.* 2008; 82(24):12565–8. [PubMed: 18829767]
38. Surviladze Z, Dziduszko A, Ozbun MA. Essential roles for soluble virion-associated heparan sulfonated proteoglycans and growth factors in human papillomavirus infections. *PLoS Pathog.* 2012; 8(2):e1002519. [PubMed: 22346752]
39. Cerqueira C, et al. Kallikrein-8 Proteolytically Processes Human Papillomaviruses in the Extracellular Space To Facilitate Entry into Host Cells. *J Virol.* 2015; 89(14):7038–52. [PubMed: 25926655]
40. Surviladze Z, Ozbun MA, Sterkand RT. Interaction of human papillomavirus type 16 particles with heparan sulfate and syndecan-1 molecules in the keratinocyte extracellular matrix plays an active role in infection. *Journal of General Virology.* 2015; 96(8):2232–2241. [PubMed: 26289843]
41. Fischer G, W-LB, Lang K, Kiefhaber T, Schmid FX. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. 1989
42. Pakula R, et al. Syndecan-1/CD147 association is essential for cyclophilin B-induced activation of p44/42 mitogen-activated protein kinases and promotion of cell adhesion and chemotaxis. *Glycobiology.* 2007; 17(5):492–503. [PubMed: 17267519]
43. Bienkowska-Haba M, Patel HD, Sapp M. Target cell cyclophilins facilitate human papillomavirus type 16 infection. *PLoS Pathog.* 2009; 5(7):e1000524. [PubMed: 19629175]
44. Bronnimann MP, et al. Furin Cleavage of L2 during Papillomavirus Infection: Minimal Dependence on Cyclophilins. *J Virol.* 2016; 90(14):6224–34. [PubMed: 27122588]
45. Bienkowska-Haba M, et al. Cyclophilins facilitate dissociation of the human papillomavirus type 16 capsid protein L1 from the L2/DNA complex following virus entry. *J Virol.* 2012; 86(18):9875–87. [PubMed: 22761365]
46. Takada Y, Ye X, Simon S. The integrins. *Genome Biol.* 2007; 8(5):215. [PubMed: 17543136]
47. Evander M, et al. Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *J Virol.* 1997; 71(3):2449–56. [PubMed: 9032382]
48. Abban CY, Meneses PI. Usage of heparan sulfate, integrins, and FAK in HPV16 infection. *Virology.* 2010; 403(1):1–16. [PubMed: 20441998]
49. Huang HS, Lambert PF. Use of an in vivo animal model for assessing the role of integrin alpha(6)beta(4) and syndecan-1 in early steps in papillomavirus infection. *Virology.* 2012; 433(2):395–400. [PubMed: 22995187]
50. Aksoy P, et al. HPV16 infection of HaCaTs is dependent on beta4 integrin, and alpha6 integrin processing. *Virology.* 2014; 449:45–52. [PubMed: 24418536]
51. Sibbet G, Romero-Graillet C, Meneguzzi G, Campo MS. $\alpha 6$ integrin is not the obligatory cell receptor for bovine papillomavirus type 4.pdf. 2000
52. Levy S, ST. Protein-protein interactions in the tetraspanin web. 2005
53. Scheffer KD, Berditchevski F, Florin L. The tetraspanin CD151 in papillomavirus infection. *Viruses.* 2014; 6(2):893–908. [PubMed: 24553111]
54. Spoden G, et al. Clathrin- and caveolin-independent entry of human papillomavirus type 16--involvement of tetraspanin-enriched microdomains (TEMs). *PLoS One.* 2008; 3(10):e3313. [PubMed: 18836553]
55. Scheffer KD, GA, Spoden GA, Zhang XA, Lambert C, Berditchevski F, Florin L. Tetraspanin CD151 Mediates Papillomavirus Type 16 Endocytosis.pdf. 2013
56. Woodham AW, et al. The S100A10 subunit of the annexin A2 heterotetramer facilitates L2-mediated human papillomavirus infection. *PLoS One.* 2012; 7(8):e43519. [PubMed: 22927980]
57. Slupetzky K, et al. A papillomavirus-like particle (VLP) vaccine displaying HPV16 L2 epitopes induces cross-neutralizing antibodies to HPV11. *Vaccine.* 2007; 25(11):2001–10. [PubMed: 17239496]
58. Kawana K, YH, Taketani Y, Yoshiike K, Kanda T. Common Neutralization Epitope in Minor Capsid Protein L2 of Human Papillomavirus Types 16 and 6. 1999

59. Conway MJ, et al. Cross-neutralization potential of native human papillomavirus N-terminal L2 epitopes. *PLoS One*. 2011; 6(2):e16405. [PubMed: 21346798]
60. Gambhira R, et al. A protective and broadly cross-neutralizing epitope of human papillomavirus L2. *J Virol*. 2007; 81(24):13927–31. [PubMed: 17928339]
61. DMW. Annexin II tetramer: structure and function. 1995
62. Rescher U, Gerke V. S100A10/p11: family, friends and functions. *Pflugers Arch*. 2008; 455(4): 575–82. [PubMed: 17638009]
63. Deora AB, et al. An annexin 2 phosphorylation switch mediates p11-dependent translocation of annexin 2 to the cell surface. *J Biol Chem*. 2004; 279(42):43411–8. [PubMed: 15302870]
64. Oshero N, LA. EGFR dependent activation of the src-family kinases.pdf. 1994
65. Dziduszko A, Ozbun MA. Annexin A2 and S100A10 regulate human papillomavirus type 16 entry and intracellular trafficking in human keratinocytes. *J Virol*. 2013; 87(13):7502–15. [PubMed: 23637395]
66. Woodham AW, et al. Small molecule inhibitors of the annexin A2 heterotetramer prevent human papillomavirus type 16 infection. *J Antimicrob Chemother*. 2015; 70(6):1686–90. [PubMed: 25712315]
67. Schelhaas M, et al. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. *PLoS Pathog*. 2012; 8(4):e1002657. [PubMed: 22536154]
68. Pelkmans L, Helenius A. Insider information: what viruses tell us about endocytosis. *Current Opinion in Cell Biology*. 2003; 15(4):414–422. [PubMed: 12892781]
69. Bousarghin L, TA, Sizaret PY, Coursaget P. Human Papillomavirus Types 16, 31, and 58 Use Different Endocytosis Pathways To Enter Cells.pdf. 2003
70. Smith JL, Campos SK, Ozbun MA. Human papillomavirus type 31 uses a caveolin 1- and dynamin 2-mediated entry pathway for infection of human keratinocytes. *J Virol*. 2007; 81(18):9922–31. [PubMed: 17626097]
71. Day PM, Lowy DR, Schiller JT. Papillomaviruses infect cells via a clathrin-dependent pathway. *Virology*. 2003; 307(1):1–11. [PubMed: 12667809]
72. Laniosz V, et al. Human papillomavirus type 16 infection of human keratinocytes requires clathrin and caveolin-1 and is brefeldin A sensitive. *J Virol*. 2009; 83(16):8221–32. [PubMed: 19494002]
73. Marušić MB, et al. Human Papillomavirus L2 facilitates viral escape from late endosomes via Sorting Nexin 17. *Traffic (Copenhagen, Denmark)*. 2012; 13(3):455–467.
74. Zhang W, et al. Vesicular trafficking of incoming human papillomavirus 16 to the Golgi apparatus and endoplasmic reticulum requires gamma-secretase activity. *MBio*. 2014; 5(5):e01777–14. [PubMed: 25227470]
75. Gottschalk EY, Meneses PI. A Dual Role for the Nonreceptor Tyrosine Kinase Pyk2 during the Intracellular Trafficking of Human Papillomavirus 16. *J Virol*. 2015; 89(17):9103–14. [PubMed: 26109718]
76. Lipovsky A, et al. Genome-wide siRNA screen identifies the retromer as a cellular entry factor for human papillomavirus. *Proc Natl Acad Sci U S A*. 2013; 110(18):7452–7. [PubMed: 23569269]
77. Bucci C, et al. The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell*. 1992; 70(5):715–728. [PubMed: 1516130]
78. Schelhaas M, et al. Entry of Human Papillomavirus Type 16 by Actin-Dependent, Clathrin- and Lipid Raft-Independent Endocytosis. *PLoS Pathog*. 2012; 8(4):e1002657. [PubMed: 22536154]
79. Smith JL, et al. Caveolin-1-dependent infectious entry of human papillomavirus type 31 in human keratinocytes proceeds to the endosomal pathway for pH-dependent uncoating. *J Virol*. 2008; 82(19):9505–12. [PubMed: 18667513]
80. Christensen ND, et al. Surface Conformational and Linear Epitopes on HPV-16 and HPV-18 L1 Virus-like Particles as Defined by Monoclonal Antibodies. *Virology*. 1996; 223(1):174–184. [PubMed: 8806551]
81. Roden RB, et al. Characterization of a human papillomavirus type 16 variant-dependent neutralizing epitope. *J Virol*. 1997; 71(8):6247–6252. [PubMed: 9223527]
82. Broniarczyk J, et al. Human papillomavirus infection requires the TSG101 component of the ESCRT machinery. *Virology*. 2014; 460–461:83–90.

83. Sapp M, et al. Analysis of type-restricted and cross-reactive epitopes on virus-like particles of human papillomavirus type 33 and in infected tissues using monoclonal antibodies to the major capsid protein. *J Gen Virol.* 1994; 75(12):3375–83. [PubMed: 7996132]
84. Chen XS, et al. Structure of Small Virus-like Particles Assembled from the L1 Protein of Human Papillomavirus 16. *Mol Cell.* 2000; 5(3):557–567. [PubMed: 10882140]
85. Campos SK, et al. Opposing effects of bacitracin on human papillomavirus type 16 infection: enhancement of binding and entry and inhibition of endosomal penetration. *J Virol.* 2012; 86(8): 4169–81. [PubMed: 22345461]
86. Kämper N, et al. A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. *J Virol.* 2006; 80(2):759–68. [PubMed: 16378978]
87. Selinka H-C, Giroglou T, Sapp M. Analysis of the Infectious Entry Pathway of Human Papillomavirus Type 33 Pseudovirions. *Virology.* 2002; 299(2):279–287. [PubMed: 12202231]
88. DiGiuseppe S, et al. The nuclear retention signal of HPV16 L2 protein is essential for incoming viral genome to transverse the trans-Golgi network. *Virology.* 2014; 458–459:93–105.
89. Day PM, et al. Identification of a role for the trans-Golgi network in human papillomavirus 16 pseudovirus infection. *J Virol.* 2013; 87(7):3862–70. [PubMed: 23345514]
90. Bronnimann MP, et al. A Transmembrane Domain and GxxxG Motifs within L2 Are Essential for Papillomavirus Infection. *J Virol.* 2013; 87(1):464–473. [PubMed: 23097431]
91. Pim D, et al. A Novel PDZ Domain Interaction Mediates the Binding between Human Papillomavirus 16 L2 and Sorting Nexin 27 and Modulates Virion Trafficking. *J Virol.* 2015; 89(20):10145–10155. [PubMed: 26202251]
92. Mamoor S, et al. The High Risk HPV16 L2 Minor Capsid Protein Has Multiple Transport Signals which Mediate its Nucleocytoplasmic Traffic. *Virology.* 2012; 422(2):413–424. [PubMed: 22154072]
93. Karanam B, et al. Papillomavirus infection requires gamma secretase. *J Virol.* 2010; 84(20):10661–70. [PubMed: 20702627]
94. Huang HS, Buck CB, Lambert PF. Inhibition of gamma secretase blocks HPV infection. *Virology.* 2010; 407(2):391–6. [PubMed: 20875908]
95. Bergant M, Banks L. SNX17 Facilitates Infection with Diverse Papillomavirus Types. *J Virol.* 2013; 87(2):1270–1273. [PubMed: 23115288]
96. Popa A, et al. Direct binding of retromer to human papillomavirus type 16 minor capsid protein L2 mediates endosome exit during viral infection. *PLoS Pathog.* 2015; 11(2):e1004699. [PubMed: 25693203]
97. Florin L, et al. Identification of a dynein interacting domain in the papillomavirus minor capsid protein l2. *J Virol.* 2006; 80(13):6691–6. [PubMed: 16775357]
98. Yang R, et al. Interaction of L2 with beta-actin directs intracellular transport of papillomavirus and infection. *J Biol Chem.* 2003; 278(14):12546–53. [PubMed: 12560332]
99. Schneider MA, et al. Identification of the dynein light chains required for human papillomavirus infection. *Cell Microbiology.* 2011; 13(1):1462–5822.
100. Bossis I, et al. Interaction of tSNARE Syntaxin 18 with the Papillomavirus Minor Capsid Protein Mediates Infection. *J Virol.* 2005; 79(11):6723–6731. [PubMed: 15890910]
101. Aydin I, et al. Large scale RNAi reveals the requirement of nuclear envelope breakdown for nuclear import of human papillomaviruses. *PLoS Pathog.* 2014; 10(5):e1004162. [PubMed: 24874089]
102. Pyeon D, et al. Establishment of human papillomavirus infection requires cell cycle progression. *PLoS Pathog.* 2009; 5(2):e1000318. [PubMed: 19247434]
103. Day PM, et al. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proc Natl Acad Sci U S A.* 2004; 101(39):14252–7. [PubMed: 15383670]

Figure 1. Our current understanding of HPV16 binding, entry, and trafficking during infection of a basal keratinocyte

In the extracellular matrix (ECM) or basement membrane (BM), HPV16 transiently binds to Laminin-332. Kallikrein-8 (KLK8) cleaves the L1 capsid protein causing a conformational change in the viral capsid. HPV16 binds to heparin sulfate proteoglycans (HSPGs) on the cell surface. Conformational changes expose the N-terminal region of L2 in the viral capsid, and may be facilitated by cyclophilins. Furin/proprotein convertases (PCs) cleave the exposed N-terminus of L2, causing decreased affinity of the virus for the HSPGs. This allows for movement of the virus to an uptake receptor complex, whose identity is unknown. A) Integrins and tetraspanins are candidates for the uptake receptor complex. B) Alternatively, HSPGs with bound virus can be cleaved by matrix metalloproteases (MMPs) during normal cell surface HSPG turnover, which results in virus-HSPG complexes being shed into the ECM. A growth factor (GF)/HSPG/HPV16 complex can subsequently interact with growth factor receptors (GFRs) on the cell surface. Activation of GFRs can cause the translocation of Annexin A2 tetramer (A2t) from the inner leaflet of the plasma membrane to the outer leaflet. The HPV16/HSPG/GFR/A2t complex can then be internalized by the cell. C) Endocytosis occurs via a macropinocytosis-like mechanism involving actin. The virus traffics through the endolysosomal system where L1 mostly dissociates from the viral genome/L2 complex, facilitated by cyclophilins. The viral genome/L2 complex traffics through the Golgi and may enter the ER, or be in an ER cisternae, before gaining access to the nucleus when the nuclear envelope breaks down during mitosis.