Mechanism of α-defensin HD5 Inhibition of Human Papillomavirus-16

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Abstract

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Human papillomavirus (HPV) is a significant oncogenic virus, but the innate immune response to HPV is poorly understood. Human α-defensin 5 (HD5) is an innate immune effector peptide secreted by epithelial cells in the genitourinary tract. HD5 is broadly antimicrobial, exhibiting potent antiviral activity against HPV at physiologic concentrations; however, the specific mechanism of HD5-mediated inhibition against HPV is unknown. During infection, the HPV capsid undergoes several critical cell-mediated viral protein processing steps, including unfolding and cleavage of the minor capsid protein L2 by furin. Using HPV16 pseudovirus, I show that HD5 interacts directly with the virus and inhibits the furin-mediated cleavage of L2. My data supports a model in which HD5 prevents furin from accessing L2 by occluding the furin cleavage site via direct binding to the viral capsid. This direct binding also results in capsid stabilization and inhibits viral uncoating inside the cell. The stabilized capsid cannot release L2, which is critical for endosomal exit of the viral genome. The HD5-HPV16 complex then traffics to the lysosome where the virus is degraded. These findings are similar to the model of HD5-mediated inhibition of other non-enveloped viruses, suggesting there is a general α-defensin

antiviral mechanism of direct virus binding and capsid stabilization against non-enveloped viruses.

In addition to elucidating the antiviral mechanism against HPV16, I have determined the physical properties of HD5 critical for neutralization of HPV16. These include the importance of both charge and paired arginine residues on one side of the HD5 molecule, as well as the disulfide stabilized structure and ability to dimerize. Overall, residues on one face of the HD5 molecule are necessary for antiviral activity against HPV16. Similar residues are also important for HD5 neutralization of human adenovirus, another non-enveloped virus. These studies may lead to a greater understanding of the interactions between α-defensins and non-enveloped viruses.

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DEDICATION

This thesis is dedicated to my parents, Aletha and LeRoy, who never know what I'm talking about but listen anyway.

Chapter 1. Introduction

1.1 Antiviral activity of Defensins

1.1.1 Defensin Structure, Expression, and Physiologic Concentrations*

Defensins are one of the most abundant classes of antimicrobial peptides in humans and have primarily been studied as effector components of the innate immune response with direct antibacterial activity. However, their antiviral properties were appreciated from the earliest functional studies of α-defensins [1,2]. In fact, their ability to neutralize herpes simplex virus 1 (HSV-1) was one of the defining criteria in the identification and purification of the α-defensins human neutrophil peptide 1 (HNP1), HNP2, and HNP3 from human neutrophils [3]. Defensins are small (~29-42 amino acid) cationic, amphipathic peptides with a predominantly β-sheet structure stabilized by 3 disulfide bonds. They can be broadly classified on the basis of structure and disulfide bond organization into three groups: α-, β-, and θ-defensins [4]. As humans lack θ-defensins, they will not be discussed in detail [4,5]. Humans express six α-defensins and up to 31 β-defensins [6]. The α-defensins can be further subdivided into myeloid (HNP1-4) and enteric [human defensin (HD) 5 and 6] peptides on the basis of both expression patterns and genetic organization [7]. All of the α-defensins have been shown to multimerize into at least dimers either in solution or in crystal structures (Figure 1.1) [8-11]. HNP1-4 are predominantly expressed by neutrophils but can also be found in or expressed by monocyte/macrophages, natural killer (NK)

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* Adapted from Wilson SSW, Wiens ME, and Smith JG. Antiviral mechanisms of human defensins. J Mol Bio 425, 4965-4980 (2013). Reprinted with permission.

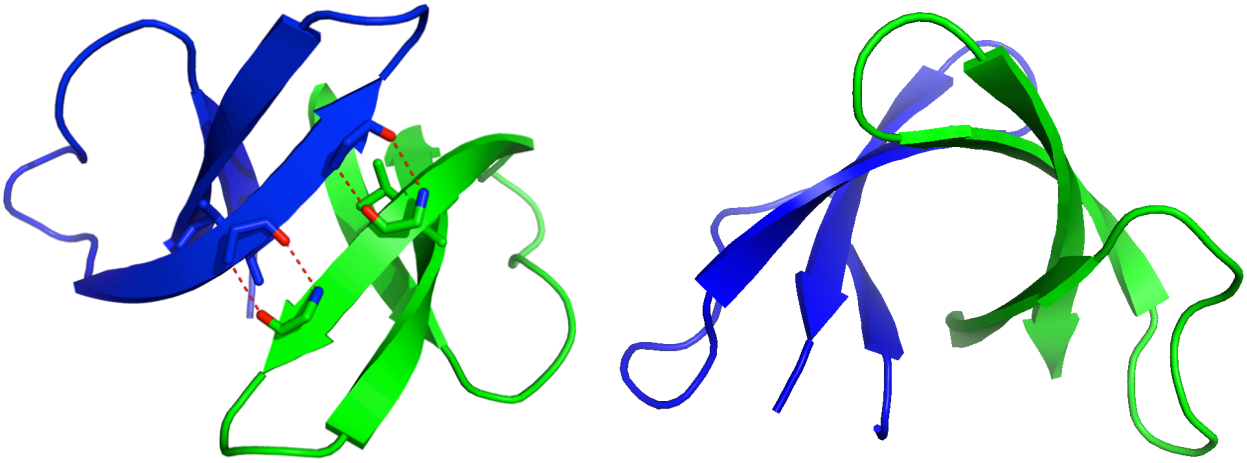

Figure 1.1 Structure of α-defensins.

A representative structure of HD5 as a dimer (PDB:1zmp) is shown from two different angles. Residues contributing to the dimer interface are shown as ball and stick models. Dotted lines indicate hydrogen bonds at the dimer interface. Molecular images were created with the PyMOL Molecular Graphics System (Schrödinger, LLC). doi:10.1371/journal.ppat.1004360. g002

cells, some T cells, B cells, and immature dendritic cells (DCs) [7,12]. HD5 and HD6 are expressed by specialized epithelial Paneth cells of the small intestine [13,14]. HD5 is also expressed by epithelial cells in the male and female genitourinary tract [15-18]. Human β-defensins (HBDs) are more widely expressed by epithelial cells in skin and at mucosal surfaces in contact with the environment [19]. Like the α-defensins, some β-defensins (e.g., HBD3) exist in oligomeric forms, while others, such as HBD1 and HBD2, are monomeric [20]. They are also expressed by monocytes, macrophages, and certain DCs, and a subset of β-defensins are only expressed in the male reproductive tract [19,21]. Although there are commonalities in expression patterns of defensins in humans and other species, one important difference relevant for experimental models of infectious disease is that mice lack myeloid α-defensins [22,23].

Quantification of physiologic defensin concentrations *in vivo* is complex, as defensins are present at high local concentrations within specific cell types or upon release from cells into confined anatomical niches (e.g., crypts of the small intestine) but can become diluted in extracellular fluids. For the myeloid α-defensins, Daher et al. estimated ~3 mM (10 mg/ml) HNPs in neutrophils, with even higher local concentrations in the azurophilic granules in which they are stored [1]. For the enteric α-defensins, Ayabe et al. estimated concentrations of ≥3.5 mM (15-100 mg/ml) in the crypt lumen, the site of Paneth cell degranulation [24]. These concentrations are likely similar in the human small intestine, where HD5 expression exceeds that of HD6 by 6-fold [25]. In healthy patients, epithelial lining fluid of the lung contains 31-79 nM HNP1-3, nasal fluid contains ~2.7 μM HNP1-3, saliva contains 0.3-3 μM HNP1-3, and vaginal secretions contain ~1.5 μM HNPs and 0.3-14 μM HD5 [16,26-31]. For the β-defensins, 5-10 nM HBD2 has been measured in nasal fluid [30,32]. However, in certain diseased states defensin levels can be highly elevated. For example, 57 μM to 2.4 mM concentrations of HNP1 have been found in

epithelial lining fluid of cystic fibrosis patients [29]. Overall, the concentrations of defensins present *in vivo* are generally within the range that is needed for direct antiviral activity.

1.1.2 Antiviral mechanisms through direct interactions between defensins and virus

Modes and determinants of defensin binding to viruses

There are multiple modes of defensin binding to ligands such as viral particles. First, defensins interact with lipid bilayers, which is facilitated by the presence of negatively charged phospholipids [7,12,33]. Second, four of the α-defensins (HNP1-3 and HD5) and HBD3 are lectins capable of binding to glycoproteins and glycolipids [34-37]. Third, defensins can potentially engage in protein-protein or protein-DNA interactions. Because they are cationic and amphipathic, defensins interact with ligands through both charge-charge and hydrophobic interactions. Defensin oligomerization, particularly for α-defensins, and conformational stability imparted by disulfide bonds may further influence binding. Each of these interactions contributes to the antiviral activity of defensins, and their relative importance depends on the specific virus/defensin pair under investigation.

The property of defensins that has been most widely investigated for its contribution to antiviral activity is stabilization of the 3D structural fold through the formation of disulfide bonds. Generally, destabilized or "linear" defensins are generated by substituting the conserved cysteine residues either in toto, individually, or in pairs to natural or non-natural residues that cannot form disulfide bonds such as serine or α-amino-n-butyric acid (Abu). Alternatively, wild type defensins are reduced and chemically modified (alkylated) to prevent disulfide bond formation. All reported studies have shown that the disulfide-stabilized forms of α-defensins are required to either inhibit [HSV-1, human adenovirus serotype 5 (HAdV-5), influenza A virus (IAV), and human immunodeficiency virus-1 (HIV-1)] or enhance (HIV-1) virus infection [1,2,38-

[41]. In two cases, the antiviral activity of β-defensins was unaffected by "linearization" [42,43]. Given the paucity of data in this regard for β-defensins, it is unclear if this is a fundamental difference between α- and β-defensin antiviral activity. Together, these studies suggest that the effects of α-defensins on virus infection are more likely to be due to their amphipathicity or ability to multimerize, which are structurally dependent, rather than the net positive charge of the molecule that is common to both native and "linearized" forms.

The capacity of defensins to function as lectins and bind selectively to sugars contributes to their antiviral properties; however, defensins also bind to host cellular and serum proteins [1,34]. The relative affinity for viral targets versus serum components may explain why some defensins are only antiviral against particular viruses in the absence of serum. Although it has been shown that HD5 binds natural viral glycoproteins, notably HSV-1 glycoprotein D (gD) and HIV-1 gp120, with a higher affinity than bovine serum albumin or fetuin, serum substantially attenuates the antiviral activity of HNP1 against HSV-1, even at low concentrations [1,3,34]. Nonetheless, this effect can be overcome at higher HNP1 concentrations or by pre-incubating the virus with HNP1 before it is added to cells. The addition of serum also abrogates the antiviral activity of HNP1-3, HBD2, and HBD3 against both X4 and R5 tropic HIV-1 [44,45]. Although it has been reported that HD5 enhances HIV attachment and infection of primary T cells in the presence of 5-10% serum, a recent study suggests that HIV-1 infection of these cells is blocked in 0-2% serum [39,46-49]. Upon further investigation, it was revealed that the apparent antiviral activity of HD5 against HIV-1 infection of primary $CD4^+$ T cells in the absence of serum is due to increased HD5-mediated cell toxicity, which is not observed with other cell types [50]. Serum also competes for HD5 binding to and inhibition of HAdV, which lacks viral glycoproteins [51]. Two notable exceptions to the abrogating effects of serum are inhibition of human papillomavirus (HPV) and human BK virus

(BKV) infection by α-defensins, which are not blocked by 10% or 5% serum respectively [52-54]. Rapid inactivation by binding to serum components may protect host cells from damage by defensins; however, defensins likely remain potently antiviral *in vivo* at high local concentrations upon initial secretion and in serum-free anatomical locations (e.g., phagocytic vacuoles or the bowel lumen).

Although much of the antibacterial activity of defensins is attenuated at physiologic salt concentrations, this is not generally true for their antiviral activity [7]. One instance of salt-sensitivity is that HBD2 and HBD3 have attenuated anti-HIV activity in physiological salt concentrations, which is somewhat surprising given that even the antibacterial effects of HBD3 are generally not salt-sensitive [45,55]. Nonetheless, the anti-HIV activity of these β-defensins in low salt and serum-free conditions may reflect the physiological conditions of the oral cavity [56,57]. We have shown that super-physiological concentrations of salt inhibit HD5 binding to HAdV, which implicates charge-charge interactions in HD5 binding to the viral capsid [51,58]. In general, differential salt sensitivity may reflect variation in both the molecular interactions and the mechanisms of defensin-mediated killing or neutralization of viruses versus bacteria.

A limited number of structure-function studies have evaluated the roles of additional features of α-defensins in modulating viral infection. We have shown that the conserved salt bridge stabilizing a loop between two beta strands of HD5 is dispensable for HAdV-5 inhibition [38]. Similarly, mutation of an invariant glycine residue (Gly17) of HNP2 to glutamate severely attenuates the antibacterial activity of HNP2 but results in only a minor loss of antiviral activity against HPV-16 [52,59]. In contrast, specific arginine residues are critical for HD5 binding to HAdV-5 and HPV-16, and this activity is not purely charge-dependent, as lysine substitutions for selected arginine residues did not preserve antiviral activity [58]. Likewise, a double R9H/R13H

mutant of HD5 was attenuated for enhancement of HIV-1 infection in the presence of serum, indicating that this property is also not simply charge-dependent [60]. Furthermore, the capacity of HD5 to self-associate is critical for antiviral activity against both HAdV-5 and HPV-16 and for HIV-1 gp120 and HSV-1 gD binding [34,58,61,62]. These properties were revealed through mutations that disrupt defensin activity. In the converse approach, residues in HD5 under positive selection were mutated in an effort to augment the antiviral activity of HD5 against HSV-2 [48]. One mutant, (HD5 E21R) demonstrated improved anti-HSV-2 and anti-HIV-1 activity in cell culture and was both prophylactically (1 hour before infection) and therapeutically (24 hours after infection) protective against lethal HSV-2 challenge in a mouse model. Collectively, these studies suggest that specific features of viruses are selectively bound by defensins, and that the binding interface of the defensin is sequence-specific and not merely charge-dependent.

The basis for selective recognition of diverse viruses by defensins is largely unknown. Defensins may interact with either the lipid bilayer or envelope glycoproteins of enveloped viruses; however, protein-protein interactions must be critical for binding to non-enveloped viruses (e.g., HPV and HAdV) and likely contribute to binding to enveloped viruses as well. For HIV-1, competition with site-specific antibodies was used to map HNP2 binding sites on gp120 [63]. For species C HAdV (HAdV-C), we have used structural studies and a chimeric approach to identify capsid determinants of HD5 binding [38,64]. Other than for these two viruses, almost nothing is known about specific determinants of the viral particle that dictate defensin binding. Nonetheless, the fact that closely related defensins have differential antiviral (or infection-enhancing) effects indicates selectivity and may inform the design of future studies to elucidate the basis for recognition.

Direct virus inactivation by affecting envelope

Direct interactions between defensins and structural components of the virion, particularly the lipid bilayer of enveloped viruses, could destroy or destabilize the virus and render it non-infectious. This mechanism was proposed in the first studies of the antiviral activity of human and rabbit neutrophil α-defensins [1-3,65]. In support of this hypothesis, HSV-1 inactivation by rabbit NP-2 or HNP1 was impaired at temperatures below 20°C and was more effective at 40°C than 37°C, implicating the fluidity of the membrane rather than binding as an important parameter [1,2]. HNP1 has also been shown to bind directly to HSV-1 and to model membranes containing phosphatidylserine [1]. Nonetheless, the morphology of HSV-1 by electron microscopy was not altered by incubation with a neutralizing concentration of HNP1 [1]. HBD2 and HBD3 bind directly to and irreversibly inactivate HIV-1, but if this interaction affects or damages the viral envelope is unknown [45,66]. Similarly, treatment of HIV-1 with HNP1 in the absence of serum irreversibly decreases infectivity, although a physical change in particle integrity was not determined [46,66]. More recently, the lipid bilayer of respiratory syncytial virus (RSV) exposed to neutralizing HBD2 but not non-neutralizing HBD1 was visibly damaged when assessed by electron microscopy [67]. Although the universality of this phenotype was not quantified, the proteins of the bulk population of the virus were more buoyant in a density gradient, suggesting that most of the viral particles were disrupted. The altered morphology was correlated with a 70% reduction in virus attachment to the cell. This is perhaps the most convincing data for direct viral envelope disruption by a defensin and likely extends to human parainfluenza virus 3 (HPIV-3), which has a profile of defensin sensitivity similar to that of RSV[67].

The broad neutralization of many enveloped viruses supports the hypothesis that the lipid bilayer is the target; however, enveloped viruses are not universally susceptible and their sensitivity to α-defensins can be highly variable. For example, in one study rabbit NP-1 and NP-2 inhibited HSV-1 up to 1000-fold, HSV-2 10-fold, vesicular stomatitis virus (VSV) 100-fold, and IAV 56-fold but had no effect on cytomegalovirus (CMV) [2]. Similarly, HNP1 potently inhibited HSV-1 (1000-fold) and HSV-2 (100-fold) but only weakly inhibited CMV (6-fold), VSV (7-fold), and IAV (6-fold) [1,3]. Defensin perturbation of lipid bilayers is dependent upon their composition. It is favored by negatively charged phospholipids; whereas, neutral bilayers are largely inert to defensin [61,68]. The lipid content of viral envelopes is dependent upon the subcellular location and membrane microdomains from which they bud and likely varies among viral families[69]. If direct membrane perturbation contributes to the antiviral effect of defensins, then differences in the lipid composition of the envelope may in part explain the differential susceptibility of viruses to defensins.

In contrast to disruption of enveloped viruses, increased capsid resistance to mechanical force or heat has been observed upon HD5 binding to non-enveloped HAdV-C [38,51,70]. This effect correlates with the inability of the HD5-bound particle to uncoat, similar to a genetic mutant of HAdV-C that is stabilized by the presence of unprocessed precursor capsid proteins [51,71,72]. A failure to uncoat precludes release of an internal, membrane-permeabilizing capsid protein, thereby blocking HAdV-C escape from the endosome and introduction of the viral genome into the nucleus, its replication niche [51,72-74]. Thus, unlike enveloped viruses where destabilization of the virion impairs infectivity, an opposite, stabilizing interaction with the non-enveloped HAdV-C capsid produces a similar outcome.

Extracellular aggregation

As many defensins form multimeric structures, which have been demonstrated in both crystal structures and in solution, there is the potential that interactions between defensin peptides bound to neighboring viruses will cause virions to aggregate [8-11,20,34]. This has been shown directly for IAV by HNP1 or HNP2 and for HAdV-5 and BK virus by HD5 [54,58,75]. For IAV, it is unclear if defensin-mediated aggregation is required to block infection. Similarly, aggregation of HAdV by HD5 is not sufficient to inhibit infection, as a mutant of HD5 that is able to induce aggregation is non-neutralizing [58]. In contrast, aggregation and a concomitant inability to bind host cells have been shown to be the dominant mechanisms of neutralization for BK virus [54]. The mechanism of inhibition of other polyomaviruses such as JC and SV40, which are also sensitive to HD5, has not been determined [54]. In addition to multivalent binding due to defensin oligomerization, neutralization of capsid charge by defensin binding may reduce repulsion between virions. We have shown this directly for HAdV-C [58], and this effect may facilitate the aggregation of other viruses. These effects are likely interrelated, as mutants of HD5 that are impaired in self-association are incapable of both aggregating HAdV-C and fully neutralizing the capsid charge. Aggregation can impact viral infectivity by directly impeding cell binding or by causing viruses in clumps to enter fewer cells.

Blocking receptor binding

Defensin binding to viral attachment protein(s) could disrupt receptor interactions critical for viral entry into the cell. HNP1-3, HD5, and HBD3 bind a recombinant viral glycoprotein (gB) of both HSV-1 and HSV-2, which correlates with the ability of these defensins to inhibit HSV-1 and HSV-2 entry and adhesion [37,76]. Lectin activity of HNP1 is critical, as deglycosylation of HSV-2 gB abrogates binding [76]. The contribution of glycoprotein binding to

the antiviral mechanism of HD5 was underscored by a direct correlation between the capacity of HD5 mutants to neutralize HSV-2 and their affinity for recombinant gD [48]. HNP4 and HD6 also inhibit HSV-1 and HSV-2 infection, but do not bind to viral glycoproteins [37]. Instead, HNP4 and HD6 have been shown to bind heparan sulfate, the receptor for attachment, as well as other glycosaminoglycans. HBD3 is the only defensin able to bind both host cell receptors and viral glycoproteins [37]. Those defensins that failed to bind gB or heparan sulfate, HBD1 and HBD2, were also unable to neutralize infection. Overall, blocking host cell receptors and binding to viral glycoproteins is a major mechanism by which defensins inhibit HSV-1 and HSV-2 infection.

Similarly, HNP1-4 bind HIV-1 gp41 and gp120 as well as the cell surface receptor CD4 [36,40,77]. The binding sites of HNP1 and HNP2 on gp120 have been mapped in antibody competition assays to the CD4 and co-receptor binding sites [63]. Conversely, the HNP1 and HNP2 binding sites on CD4 have been mapped to the gp120 binding site [63]. The mode of α-defensin binding to gp120 and gp41 may be complex, as both lectin-dependent and -independent binding have been reported. In an early study, deglycosylation of gp120 reduced HNP1 binding and abolished HNP2 and HNP3 binding [36]; however, a more recent paper suggested that deglycosylation of gp120 or gp41 does not affect HNP1 binding[40]. In addition, HNP4 binding is not abolished by deglycosylation of gp120 [36], consistent with the observation that HNP4 binds more weakly to both polysaccharides and serum proteins [77]. Nonetheless, HNP4 is a more potent inhibitor of HIV-1 than are HNP1-3 [77]. In summary, like for HSV, defensins directly interfere with HIV-1 binding and attachment.

In contrast to a block in cell binding, we have observed that receptor-dependent and -independent binding of HAdV-C to the cell is enhanced in the presence of a inhibitory concentration of HD5 [38,58]. This effect may be related to neutralization of the net negative capsid

charge, which promotes aggregation, in a manner comparable to enhancement of retrovirus infections by polybrene or HAdV infections by poly-cations [58,78]. Similarly, HIV-1 binding to cells and infection is enhanced by HD5 and HD6 [39,60]. Thus, although in several cases the net effect of defensin binding to the virus is to block cellular attachment, the opposite effect has also been observed. The balance of these activities *in vivo* is unclear.

Inhibition of viral fusion with or penetration of host cell lipid bilayers

To introduce their genomes into host cells, enveloped viruses must fuse their lipid bilayer with that of the host cell [79]. The fusion protein of the virus mediates this reaction by inserting a hydrophobic stretch of amino acids into the target cell membrane followed by a conformational change to a less energetic state, termed the six-helix bundle [80,81]. The energy for lipid fusion is derived from this conformational change. HIV-1 fusion, mediated by gp41, is inhibited by HNP1 [40,63]. Inhibition requires the disulfide-stabilized form of HNP1 and is abrogated by serum [40]. HNP1 aggregates recombinant peptides of both the carboxyl- and amino-termini of gp41 that comprise the six-helix bundle, suggesting a direct effect on formation of the post-fusion conformation of gp41 [40]. HNP1 also increases the binding and efficacy of neutralizing antibodies specific for the gp41 pre-hairpin conformation, likely due to greater antibody access to hidden neutralizing epitopes as a consequence of slowed refolding kinetics [82]. Thus, HNP1 binding directly alters HIV-1 fusion through interactions with gp41. Whether this mechanism of HIV-1 neutralization extends to other viruses has not been shown; however, a different mechanism of β- and θ-defensin blockade of enveloped virus fusion due to host cell interactions will be discussed in Section 1.1.3.

Rather than fuse with the cell membrane, non-enveloped viruses must penetrate the limiting membrane of the host cell, which is generally mediated by a specific viral protein [8389].

This step may occur at the plasma membrane, but often follows a conformational change leading to uncoating of the viral capsid triggered by a drop in pH or other host factors in the endosomal pathway [84]. For HAdVs, the internal capsid protein VI is the membrane lytic factor [73,74]. Upon binding to the viral capsid, α-defensins, including HNP1 and HD5, stabilize HAdV-C and prevent uncoating, release of protein VI, and subsequent disruption of the endosomal membrane [51,70,72]. To mediate this effect, α-defensins bind directly to the virus, either when mixed together in the absence of cells or when the defensin is added to virus that is pre-bound to receptors on the cell surface [38,51,58,64,72]. Thus, the defensins can recognize the virus in the complexity of host proteins, carbohydrates, and lipids that could potentially compete with the virus for defensin binding. Neutralization of HAdV by α-defensins is restricted to HAdV-B and -C and to a lesser extent to HAdV-A and -E; whereas, HAdV-D and -F infection is either unaffected or enhanced by α-defensins [38,51]. Furthermore, resistance to HD5 neutralization can be conferred to HAdV-C through the replacement of capsid vertex proteins with those from the resistant HAdV-D [38]. The basis for enhanced HAdV-D infection is unknown. Moreover, the extent to which the antiviral mechanism against HAdV-C extends to other non-enveloped viruses has not been determined; however, HPV16 entry is also blocked by α-defensins, possibly at an analogous step [52]. In addition, α-defensins inhibit the membrane penetration of another non-enveloped virus, JC polyomavirus (JC PyV) [85]. JC PyV infection requires trafficking of the virus from the endosome to the endoplasmic reticulum, where the virus uncoats. HD5 treatment reduces the amount of JC PyV that reaches the endoplasmic reticulum, indicating a block in the virus intracellular trafficking. In addition, HD5 appears to inhibit uncoating of the virus, possibly via direct binding and stabilization of the JC PyV capsid. Interestingly, the mechanisms of

inhibition of the two polyomaviruses appear to be distinct, highlighting the apparent specificity of the defensin-virus interactions.

Post Entry Neutralization

Infection is not completed by merely penetrating the host cell membrane. Viral transcription, protein production, assembly, and egress must all occur to complete a replicative cycle. These steps present opportunities for defensins to block viral infection, either by targeting the virus specifically or by targeting the cell. In this regard, HNP1 and HD5 are able to block HSV-1 and HSV-2 when added post-entry [37]. The defensins accumulated intracellularly in the human cervical epithelial cell line used in this study, indicating that they can still come in contact with the viruses in the cell. Hazrati et al. demonstrate that HNP2 and HD5 can bind HSV-2 DNA and speculate that this could contribute to inhibition by blocking gene expression, although a post-transcription block by an unknown mechanism was also suggested by the data [37].

Post-entry neutralization of HIV-1 has also been reported. In one study, HBD2 blocked reverse transcription but had no effect on fusion, although the mechanism of this block was not determined [86]. Additional effects of defensins on HIV-1 infection attributable to disruption of intracellular signaling will be discussed in Section 1.1.3.

Among the non-enveloped viruses sensitive to defensins, inhibition of steps after penetration of the cell membrane is not thought to be important for polyomaviruses or HAdV [51,54]. For multiple types of papillomaviruses that are sensitive to HNP1-4 and HD5, the only step known to be inhibited is the nuclear localization of the HPV-16 genome, the last step in the virus entry pathway [52]. Unlike the case for HAdV-C neutralization, the HPV genome is exposed under conditions of HNP1 and HD5 neutralization, implying virus uncoating in the presence of the

defensins [51,52]. Thus, it is unclear if defensins block papillomavirus penetration of the host membrane or the ability of the genome to traverse the cytoplasm to the nucleus.

No mechanism but antiviral activity reported

We lack mechanistic insight for a variety of viruses that have been shown to be sensitive to defensin neutralization. Purified HBD3 and porcine β-defensin-3 were antiviral against porcine reproductive and respiratory syndrome virus [42]. HBD2 has been shown to reduce the yield of varicella zoster virus at 10 days post-infection, but not at earlier time points [87]. Transduction by recombinant adeno-associated virus is inhibited by relatively high concentrations (100 μM) of HNPs; however, ≥100 μM HNPs were measured in epithelial lining fluids from cystic fibrosis patients, which were also inhibitory in the same study [29]. And, vaccinia virus is inhibited by HBD3 when incubated with the virus for 24 h [88]. Whether previously described or novel mechanisms contribute to the neutralization of these viruses warrants further study.

In addition to purified peptides, virus inhibition by degranulated neutrophils has been noted. When neutrophils degranulate, for example in response to stimulation by leukotriene b4 (LTB4), they release high concentrations of defensins as well as other antimicrobial factors. CMV infection of human peripheral blood leukocytes containing neutrophils is weakly inhibited by LTB4 treatment [89]. A combination of α-defensins (HNP1-3), the human cathelicidin LL-37, and eosinophil-derived neurotoxin mediates much of the effect. Cell-free supernatants from LTB4-stimulated neutrophils mixed with CMV was similarly inhibitory, and anti-HNP1-3 antibodies reduced the observed activity by half. These results corroborate the data on modest CMV neutralization using purified α-defensins, although the antiviral mechanism remains unknown [1,89]. In other studies, supernatants from human neutrophils treated with LTB4 or

stimulated to synthesize LTB4 strongly inhibited human coronavirus and HSV-1 and more modestly inhibited RSV and influenza B virus [90,91]. Collectively, these studies demonstrate that naturally secreted defensins retain antiviral activity. In contrast, HNP1-3 have been shown to interact with and reduce the activity of surfactant protein D (SP-D) and bronchoalveolar lavage fluid containing SP-D against IAV [92,93]. These observations reinforce the importance of studying both purified peptides for their individual biological effects and more complex mixtures in which defensins and other antimicrobial factors are naturally produced, which could demonstrate synergistic or, alternatively, mutually inhibitory effects.

1.1.3 Antiviral mechanisms targeting the cell

Blocking fusion by crosslinking host proteins

Rather than directly targeting viral fusion proteins to block enveloped virus fusion with the host cell, HBD3 and a synthetic human θ-defensin called retrocyclin 2 (RC2) have been shown to inhibit IAV fusion by cross-linking host glycoproteins [35]. RC2 and HBD3 binding limit the mobility of host surface proteins in the vicinity of the nascent viral fusion pore, restricting its maturation to full fusion. Inhibition is blocked by serum and by deglycosylation (PNGase treatment) of the cells, indicating that this effect is due to the lectin activity of the defensins. In this regard, a non-defensin mannan-binding lectin had a similar effect. This mechanism may be general, as inhibition is independent of direct binding to the viral hemagglutinin glycoprotein and extends to fusion reactions mediated by unrelated proteins from baculovirus and Sindbis virus. More recently for HIV-1, HNP1 treatment has also been shown to decrease the mobile fraction of CD4, CCR5, and CXCR4 receptors in the cell membrane, reflecting the same phenomenon mediated by the lectin activity of HNP1 [40].

Modulation of cell surface receptors

HIV-1 has a complex entry pathway that utilizes several cell surface receptors and co-receptors. The relative ability of defensins to directly modulate host surface receptors important for HIV-1 infection has been debated by various groups. In peripheral blood mononuclear cells (PBMCs) and a human T cell line expressing CXCR4 and CCR5, HBD2 and HBD3 but not HBD1 reduced cell surface expression of CXCR4 but not CCR5 in 0-0.5% serum [45,94]. A subsequent study confirmed CXCR4 downregulation in PBMCs by HBD2 and HNP1 under similar conditions. In contrast, the downmodulation of CXCR4 was not observed in PBMCs treated with HBD2 in the presence of serum [57,88]. Similarly, in primary $CD4^+$ T cells in the presence of 10% serum, HNP1 does not alter CXCR4, CCR5, or CD4 expression on the cell surface [46]; however, this result may be explained by the comparably long (16 hour) period of defensin treatment, which potentially allowed for surface protein turnover. The differences in these studies are likely explained by the use of different cell types and serum concentrations, thus the contribution of changes in cell surface receptor levels to HIV-1 infection remains unresolved.

Changes in intracellular signaling that impact infection

Many viruses regulate protein kinase C (PKC) signaling during entry and infection: HIV-1 requires phosphorylated PKC for viral fusion, transcription, integration, and assembly [46,95,96]. IAV requires PKC for endosomal escape and nuclear entry [97,98]. HSV requires PKC for cell entry as well as for nuclear egress of the viral capsid [99,100]. As HNP1-3 are known to inhibit the activity of PKC *in vitro* [101], altering or inhibiting this cellular signaling pathway may be another defensin-mediated antiviral mechanism, which explains the post-entry block to infection observed for some viruses. HNP1 treatment of cells prior to or during infection with either IAV or HIV-1 reduces the levels of phosphorylated PKC [41,46]. Treatment of $CD4^+$ T cells with a PKC

activator, bryostatin-1, partially rescued HIV-1 infection [46]. In addition, HNP1 and the PKC inhibitor Go6976 had similar inhibition kinetics [46]. Inhibition of PKC by HNP1 explains the observed block in nuclear import of the incoming pre-integration complex as well as transcription of the integrated viral genome in HIV-1 infected cells [46]. Although HSV infection is also inhibited by HNP1 through a post entry mechanism, it is not rescued by pretreatment with bryostatin-1 [37]. In addition, cellular entry of some HAdV serotypes is also sensitive to PKC inhibition [102-104]; however, differential defensin sensitivity of chimeric HAdVs in which only certain capsid proteins are variable argues against a role for cellular targets such as PKC in HAdV neutralization by α-defensins [38]. Together, these data indicate that the PKC signaling pathway is involved in defensin mediated neutralization of HIV and possibly IAV, but is uninvolved in HSV and HAdV neutralization.

Cell signaling pathways mediated by the chemokine receptor CCR6 also play a role in defensin-mediated HIV inhibition. HBD2 is known to bind CCR6 and has been shown to induce expression of host restriction factor apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) which has antiviral activity against HIV [105,106]. Thus, defensins can both inhibit cellular pathways required for viral infection and activate intracellular antiviral mechanisms. The diverse mechanisms by which defensins inhibit viral infection are summarized in figure 1.2.

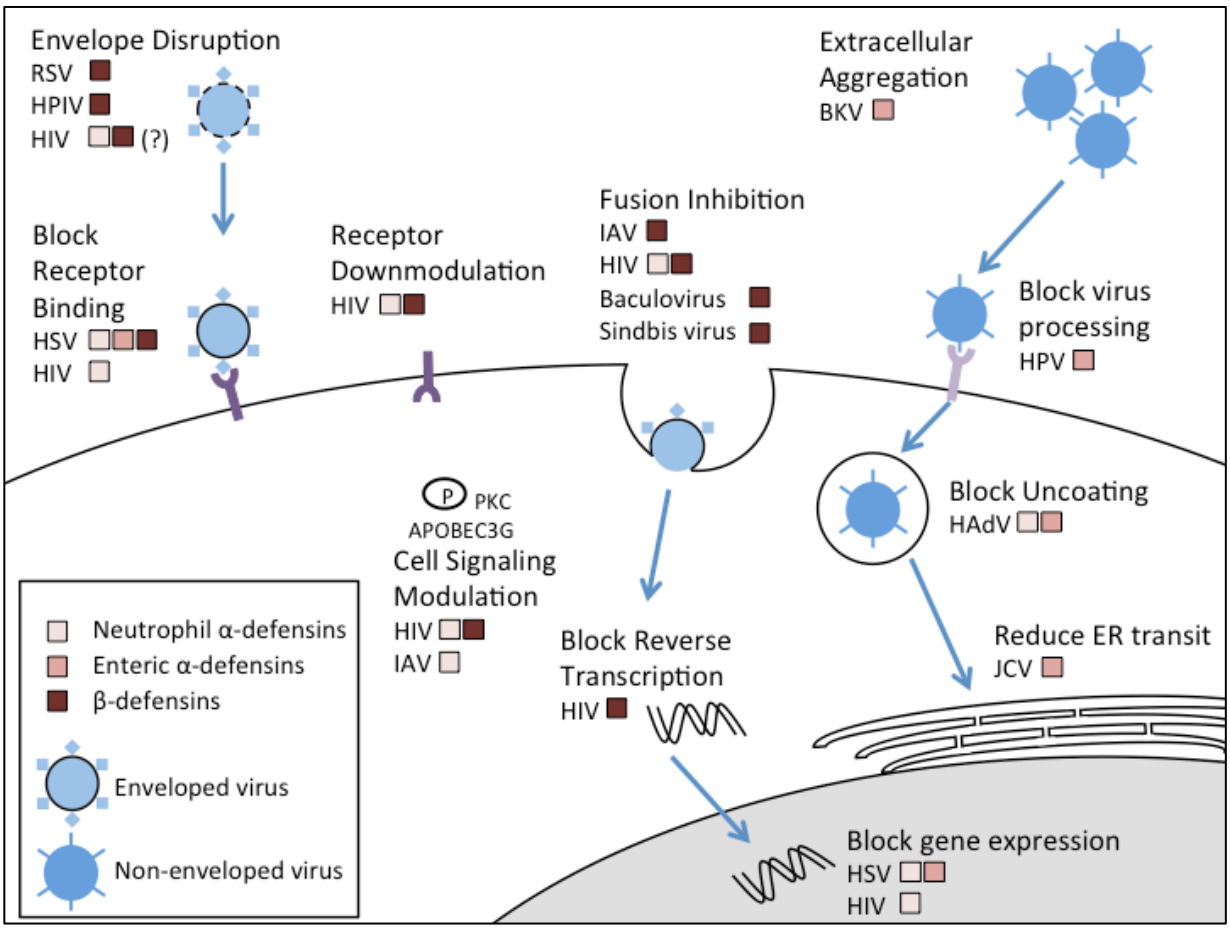

Figure 1.2 Major antiviral mechanisms of defensins

Defensins inhibit many steps in enveloped and non-enveloped viral infection. Mechanistic information is available for respiratory syncytial virus (RSV), human parainfluenza virus (HPIV), human immunodeficiency virus (HIV), herpes simplex virus (HSV), influenza A virus (IAV), BK polyomavirus (BK), human adenovirus (HAdV), JC polyomavirus (JCV) and human papillomavirus (HPV). Most mechanisms impact viral entry, but post-entry effects have been described. Omitted are defensin effects that have been reported but their contribution to blocking infection is in doubt (e.g., aggregation of HAdV by α-defensins). Note that although the defensins relevant for each virus at each step are indicated in broad classifications (e.g., β-defensins), in most cases not all of the defensins within these groups have been tested or have equivalent activity. Adapted in part from Wilson, S.S., Wiens, M.E. and Smith, J.G. Antiviral mechanisms of human defensins. *J Mol Biol* **425**, 4965-4980 (2013).

1.1.4 Defensin-mediated enhancement of viral infection[†]

Although the majority of studies have focused on the antiviral activity of defensins, in some cases α-defensins actually enhance human immunodeficiency virus (HIV) and human adenovirus (HAdV) infection [38,60]. For both viruses, enhancement is not observed with linearized defensins, indicating that structure-dependent interactions are required. Treatment of HIV with HD5 or HD6 substantially increases infection, which for some strains can reach >100-fold [60]. This enhancement is sufficient to overcome the effects of entry and fusion inhibitors and acts primarily by increasing viral attachment to target cells [39]. Naturally produced HD5 from *Neisseria gonorrhoeae*-infected cells also enhances HIV infection, suggesting that it is likely to occur under physiological conditions *in vivo* [60]. We have observed a similar, albeit much more modest, HNP1- and HD5-dependent increase in infection by certain serotypes of HAdV, which is also correlated with increased receptor-dependent and -independent attachment to cells [38,58]. Whereas HIV is sensitive to HNPs but enhanced by HD5 and HD6, HAdV serotypes appear to be more uniformly resistant or sensitive to α-defensins in general. Given that infection by two disparate viral families is enhanced by defensins, it would not be surprising if this were true for other viruses. It also remains to be seen if enhancement occurs *in vivo* and whether enhancement or inhibition predominates.

1.1.5 Defensins expression or secretion elicited by viral infection[‡]

In addition to their direct antiviral properties, many defensins function as cytokines or chemokines to elaborate an antimicrobial immune response, which could indirectly affect viral

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[†] Adapted from Wiens ME, Wilson SS, Lucero CM, Smith JG. Defensins and viral infection: dispelling common misconceptions. PLoS Pathog. 2014 Jul;10(7):e1004186.

[‡] Adapted from Wilson SS, Wiens ME, Smith JG. Antiviral mechanisms of human defensins. J Mol Biol. 2013 Oct 2;425(24):4965–80.

pathogenesis. In this regard, a number of viruses have been shown to stimulate defensin expression or secretion, whether or not the elicited defensins are directly antiviral against that virus. This has primarily been observed for β-defensins, which are often inducibly expressed, rather than the constitutively expressed α-defensins [7]. For example, HBD2 and HBD3 mRNA were upregulated in primary bronchial epithelial cells by human rhinovirus-16 (HRV-16) infection [107]. A subsequent study confirmed the mRNA upregulation and demonstrated increased HBD2 protein production [32]. Both studies found that viral replication was required and that poly(I:C) but not ssRNA could mimic viral infection, implicating a requirement for innate intracellular RNA detection pathways (RIG-I-like receptors) [108]. Consistent with this observation, multiple HRV serotypes that enter via distinct receptors and pathways had similar defensin-inducing properties [32]. Despite potent upregulation of HBD2, direct inactivation of HRV-16 was not observed [32]. IL-17A stimulation of HRV-16 infected bronchial epithelial cells synergistically induces HBD2, although the signaling pathways leading to HBD2 upregulation by these two stimuli only partially overlap [109]. When HBD2 upregulation was monitored in human volunteers following experimental HRV-16 infection, one group found elevated HBD2 message in nasal epithelial scrapings and HBD2 protein in nasal lavage following a one day lag from onset of symptoms [32]; however, a second group observed low HBD2 levels and an increase in HBD2 protein in sputum only on days 15-21 post-infection [110]. These discrepancies may reflect differences in the patient samples that were monitored.

For paramyxoviruses, infection of neonatal lambs with ovine parainfluenza virus 3 (PIV3) elevated sheep β-defensin 1 (SBD1) mRNA in the lung 4-7-fold compared to saline controls. PIV3 causes a lower respiratory infection in lambs similar to that of human PIV in children, and SBD1 is expressed and distributed similarly to HBD1 [111]. Increased inflammation

and PIV3 infection due to concurrent HAdV vector infection led to further increases in SBD1 expression [112]. In contrast, bovine RSV infection of sheep did not significantly affect SBD1 expression [113]. Thus, repair and regeneration during resolution and recovery from inflammation rather than acute inflammation due to viral infection may be more associated with SBD1 upregulation [113]. The direct antiviral activity of SBD1 against these pathogens was not assessed. In studies of RSV infection of human A549 respiratory epithelial cells, HBD2 message and protein were both upregulated in an NF-κB-dependent manner [67]. Increased HBD2 expression resulted from the induction of TNF-α production upon RSV infection and replication. This pathway may be relevant *in vivo*, as mice challenged intranasally with RSV expressed increased amounts of murine β-defensin 4 (MBD4) but not MBD3, both of which are homologs of HBD2 [6,67]. In these studies, the elicited HBD2 was also shown to be directly antiviral by disrupting virion integrity, as discussed in Section 3. In addition, HNP1-3 levels were elevated in tracheal aspirates of infants infected with RSV during acute illness compared to convalescence [114]. By correcting for total phospholipid content in their samples, the authors suggest that increased neutrophil infiltration alone does not explain the elevated α-defensin levels, rather increased production may also contribute [114]. These α-defensins likely contribute to the anti-RSV activity of degranulated neutrophils, but the effect of purified HNPs on RSV was not addressed in either study [90,114].

HIV-1 infection in epithelial cells transcriptionally upregulates HBD2 and 3, which neutralize HIV-1, but not HBD1, which does not block HIV-1 infection [45]. In HaCaT human keratinocytes, vaccinia virus infection has been shown to stimulate the expression of HBD3, to which the virus is sensitive [88]. HSV-2 infection in keratinocytes induces HBD1 and HBD4, and HBD1 does not block HSV-2 infection [115]. Furthermore, human PBMCs, plasmacytoid DCs

(pDCs), and purified monocytes increase HBD1 at the RNA and protein level after HSV-1, IAV and Sendai virus infection [116]. UV inactivated virus is less stimulatory, indicating a role for replication. Similarly, IAV infection of mice induces expression of MBD3 and MBD4 in the nasosinus, trachea, and lungs and MBD1 and MBD2 in the lungs [117,118]. Overexpression of MBD3 in MDCK cells or addition of recombinant MBD2 during infection inhibits IAV infection in cell culture [118,119]. In summary, many studies have documented the induction of defensin expression as a consequence of viral infection, and the induced defensins may play both direct and indirect roles in viral pathogenesis.

1.1.6 Importance of defensins in viral pathogenesis *in vivo*

Most of the work demonstrating the antiviral effect of defensins has been in cell culture. Although there has been one study showing an increase in lethality from IAV infection in a MBD1 knockout mouse, those results are attributed to an increase in inflammation in the knockout mice rather than direct inhibition of viral replication [116]. Similarly, administration of rhesus θ-defensin protected mice from lethal SARS-coronavirus challenge without affecting lung viral titers, likely due to a reduction in immunopathology in the treated animals [120]. In fact, there is no example of a direct role for endogenous defensins in blocking virus infection *in vivo*, in large part due to the lack of a complete defensin knockout animal model. Indirect evidence for the importance of defensins *in vivo* comes from association studies looking at defensin levels during various viral disease states. For example, patients with atopic dermatitis have reduced cathelicidin and β-defensins and are at greater risk of developing eczema vaccinatum [121-124]. And, HIV-1 positive women had lower HNP1-3 levels and lower anti-HSV-2 activity in their cervicovaginal lavage fluid compared to healthy controls[26 33]. Similarly, cervicovaginal lavage fluid levels of HNP1-3 have been shown to correlate with the anti-HIV-1 activity of the fluid [27].

Production of HNP1-3 mRNA and protein in monocyte-derived DCs is higher in HIV-1 infected individuals compared to healthy non-infected controls [125]. And, the $CD4^+$ T cell counts in those individuals with higher levels of defensin decreased more slowly than the individuals with lower levels of defensin. α-defensins are also detectable in the breast milk of HIV-1 infected mothers, although levels vary from approximately 0.1-7 nM, and are positively correlated with levels of HIV-1 RNA; however, the presence of α-defensins is also positively correlated with higher maternal $CD4^+$ T cell counts and a decrease in the risk of HIV transmission from the breastfeeding mother to her infant [126,127]. The apparent contradiction between higher α-defensin levels correlating to higher HIV viral titers and yet lower transmission might be explained by the fact that the defensin levels were also correlated with a healthier maternal immune system, as indicated by the increased $CD4^+$ T cell count. Alternatively, HIV-1 infection may be stimulating α-defensin expression and secretion. Thus, α-defensin levels in HIV-1 infected individuals are correlated with control of viral infection, slowed disease progression, and lower vertical transmission.

In addition to their direct antimicrobial activity, defensins are able to augment and direct the immune response to viruses in ways that impact the outcome and resolution of infection. Neutrophil and enteric α-defensins have been found to selectively chemoattract different subsets of T lymphocytes, macrophages, and immature DCs [8,128-130]. Although the chemokine receptor used by α-defensins has not been identified, the recruitment of these immune cell subsets to the site of viral infection undoubtedly contributes to the outcome of viral infections. For the β-defensins, HBD1-4 induced chemotaxis of macrophages, while HBD1 and HBD2 also induce chemotaxis of immature DCs and memory T cells [105,131]. This recruitment has been shown to occur via direct binding and activation of the chemokine receptors CCR6 and CCR2 [105,132].

However, a conflicting paper indicates that although HBD1-4 can all induce the chemotaxis of macrophages, they only weakly recruit DCs [131]. In addition, they found that this effect is independent of CCR6 [131]. Overall, the ability of HBDs to control immune cell chemotaxis and therefore regulate immune responses to viral infections is well established, even if there remains debate about the specific cell subsets that are recruited.

The recruitment of immune cells has been shown to be relevant *in vivo*, as both recombinant and naturally produced defensins augment adaptive immune responses. Intranasal inoculation of mice with ovalbumin (OVA) in combination with HNP1-3 resulted in an increase OVA specific IgG and IgM titers compared to mice that received OVA alone [133]. However, despite mucosal inoculation, no OVA specific IgA was observed, indicating that the HNP augmentation may not allow for class switching. A subsequent study co-administered OVA intranasally with HNP1, HNP2, HBD1, or HBD2 individually and found that the predominant IgG isotypes and interleukin profiles were unique to the defensin used, highlighting the complexity of the interaction between defensins and immune cells [134]. Finally, the augmented immune response has been shown to be functionally relevant, as intraperitoneal injection of HNPs enhanced the antibody response to a syngeneic tumor challenge and increased the survival time of mice after tumor challenge [135]. Thus, although not yet shown in the context of an infectious challenge, stimulation of the adaptive immune response by defensins likely contributes to antiviral immunity.

In addition to inducing antibody secretion, direct contact with defensins can activate immune cells and induce cytokine secretion. For neutrophils, HNP1-3 and HBD3 suppress apoptosis, which could help to prolong an immune response [136,137]. HNP1, HNP2, and HD5 have also been shown to increase neutrophil uptake of IAV [75]. In regards to DCs, exposure to MBD2

matures and activates DCs through direct binding to TLR4, and HBD3 activates myeloid DCs and monocytes via TLR1 and TLR2 [138,139]. HBD3 can also activate Langerhans cell-like DCs and primary human cutaneous DCs [140]. In addition, HNP1 and HBD1 promote the activation and maturation of monocyte-derived DCs and production of pro-inflammatory cytokines [141]. Defensins can elicit pro-inflammatory cytokine production from treated cells that could then stimulate DCs [4,142,143]. Moreover, HNP1 and HNP2 have also been shown to induce CC-chemokine expression and secretion in macrophages, which block HIV infection [144]. Therefore, some of the activation of antigen expressing cells could be explained through an indirect mechanism.

There is also increasing evidence that defensins can be immunosuppressive. Some defensins, notably HBD1, are constitutively expressed at epithelial surfaces [145]. It has been speculated that this constitutive expression minimizes the effects of exposure to low levels of commensal and pathogenic microbes and allows for the maintenance of a non-inflammatory environment [146]. This idea is supported by the fact that HBD3 can suppress the lipopolysaccharide-induced production of pro-inflammatory cytokines by human and mouse macrophages [147,148]. It is worth noting that the amount of HBD3 used in these studies (1 uM), is lower than the amount of HBD3 that has been shown to activate macrophages via TLRs or neutralize HIV-1 infection (4 uM) [86,139,148]. More recently, naturally expressed and exogenous HBD3 were also shown to reduce the innate immune response to lentiviral vector transduction of muscle cells [149]. Finally, in line with previous observations about the cathelicidin LL37, HBD2 and HBD3 can bind DNA and promote its uptake by plasmacytoid DCs [150,151]. Overall, α- and β-defensins function to augment and alter the immune responses to microbes, and the response

elicited is likely dependent upon the amount of microbial stimuli and the subsequent concentration of defensin that is produced.

1.1.7 α-defensin immunomodulatory activity may be more important than direct anti-viral activity in vivo [§]

In most cases, α-defensin concentrations in vivo can reach levels where antiviral activity has been observed in vitro [152]. Thus, direct antiviral activity in vivo is plausible. In the case of HPV, there has been interest in examining HD5 expression in cervical tissue, the site of HPV infection that leads to cancer [153]. More than 90% of high-grade cervical intraepithelial neoplasias are thought to be due to HPV infection of a population of cells at the squamocolumnar junction, the region between the ectocervix and endocervix [154]. HD5 is expressed in the ectocervix, but not in the squamocolumnar junction or endocervix. It is possible that the absence of HD5 at this site allows for more HPV infection and therefore a greater possibility of cervical cancer progression [153]. However, the squamocolumnar junction is also thought to be more permissive to HPV infection due to a surface exposed population of stem cells [155]. Therefore, to what extent the absence of HD5 at this location plays a role in HPV infection *in vivo* is unclear.

In the sole paper to directly test antiviral activity in a natural infection model, mice were challenged with mouse AdV-1 (MAdV-1), which is sensitive to neutralization by mouse and human α-defensins in cell culture [156]. In susceptible strains of mice, this mouse pathogen crosses the blood brain barrier and causes fatal encephalitis [157]. Upon infection by oral gavage, wild type mice were significantly protected from MAdV-1 compared to mice lacking functional α-defensins due to deletion of matrix metalloproteinase 7 (Mmp7-/-), which is required for the

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[§] Adapted from Wilson SS, Wiens ME, Holly MK, Smith JG. Defensins at the Mucosal Surface: Latest Insights into Defensin-Virus Interactions. Journal of Virology. 2016 Mar 23.

activation of α-defensin precursors [156]. The survival difference was maintained in mice depleted of commensal bacteria but absent upon parenteral infection, arguing for a specific effect of the α-defensins in the gut independent of their effects on the microbiota. A direct antiviral effect of α-defensins during the initial infection should delay or reduce viral dissemination from the gut to the brain; however, a time course quantifying viral genome copies in brain and spleen revealed equivalent dissemination kinetics in both mouse genotypes through day 9 post-infection. Only on day 11 were the viral loads in the brains and spleens of Mmp7-/- mice significantly higher than wild type, coincident with divergence of survival and clinical presentation of Mmp7-/- and wild type mice in survival studies. Thus, rather than a direct antiviral barrier role, the lack of α-defensins appeared to impact pathogenesis relatively late after infection. This is more consistent with an effect on the adaptive immune response to MAdV-1, an idea that was supported by histologic changes indicative of immune stimulation (e.g., germinal center formation and marginal zone thickening) in the spleens of wild type mice but not Mmp7-/- mice. Moreover, neutralizing antibody titers, crucial for protection from MAdV-1 encephalitis, were reduced and delayed in the Mmp7-/- mice, although Mmp7-/-mice do not in general have an impaired humoral response. Thus, this first study to investigate the role of naturally secreted α-defensins in viral pathogenesis supports an adjuvant effect of the α-defensins rather than a direct antiviral effect at the site of initial infection. Similarly, β- and θ-defensins, whether naturally secreted or exogenously administered, have less of an effect as direct antivirals than by limiting immunopathology [116,120]. Human neutrophil α-defensins have been shown previously to function as adjuvants in mice [133,135], but these prior studies did not include enteric α-defensins and did not demonstrate the functional consequences of defensin adjuvant activity in

infection. Continued investigation along these lines will provide a crucial link between the known activities of α-defensins in vitro and their role in host defense in vivo.

1.2 Human Papillomavirus

Human papillomavirus (HPV) is a dsDNA, non-enveloped virus that is the etiologic agent of virtually all cervical cancer, as well as skin warts and throat and other anogenital cancers [158]. Over 150 human serotypes exist, organized into five species (alpha, beta, gamma, mu, and nu) based on the DNA sequence of L1 [158,159]. Alpha-papillomaviruses are divided into mucosal or cutaneous serotypes. The other HPV species infect cutaneous epithelial cells and mainly cause warts, but some serotypes have been associated with skin cancer [158]. Twelve alpha mucosal serotypes are associated with cervical cancer and are termed high-risk HPV. Of these, HPV16, and -31 are the most well studied in terms of entry and infection [160]. HPV16 and -18 together account for approximately 70% of cervical cancer cases worldwide [161].

HPV's circular dsDNA genome encodes eight genes; six early genes responsible for genome maintenance and amplification and two late genes that encode the capsid structural proteins L1 and L2 [158,162]. The capsid is comprised of 360 L1 monomers arranged into 72 pentameric units called capsomers [163-165]. The L2 protein interacts with the L1 capsomers and is largely internal, although short stretches of the N terminus of L2 are surface exposed [166-169]. Although the orientation of L2 in the capsid is unknown, CryoEM reconstructions have proposed that L2 sits at the center of each L1 capsomer, resulting in up to 72 copies of L2 present in the HPV capsid [166,170]. L1 and L2 translocate independently into the nucleus where assembly of the HPV capsid happens[171]. L2 is required for genome encapsidation during particle assembly [166,169,172-174]. However, L2 appears to lack a specific packaging sequence and rather binds circular

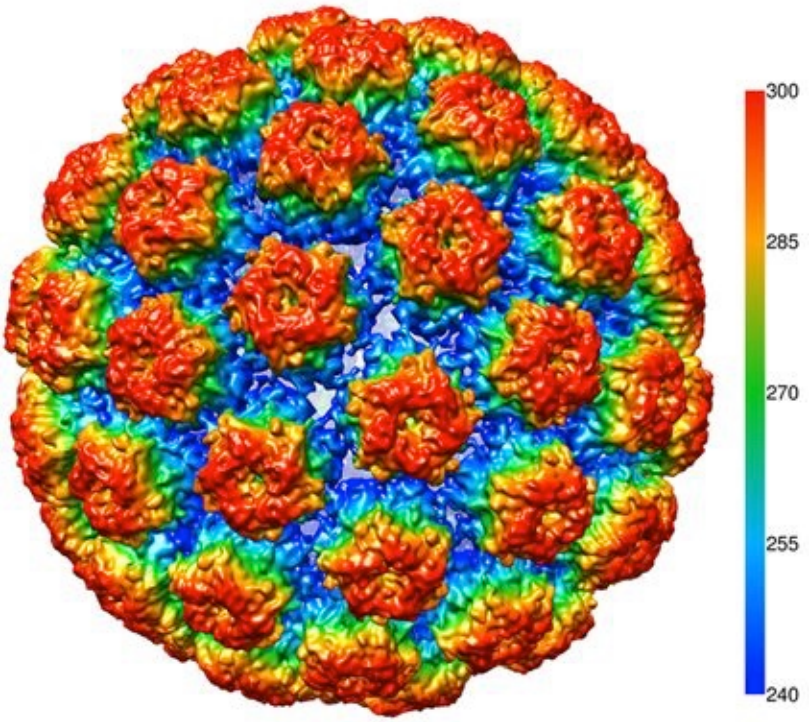

Figure 1.3. Reconstruction of a fully mature HPV16 capsid at 9 Å resolution.

Surface rendering of the capsid, colored according to radius. Radius values (color bar) are in angstroms. Giovanni Cardone et al. mBio 2014; doi:10.1128/mBio.01104-14. Figure and legend reprinted with permission.

DNA of the appropriate size (ie, ~8 kb) [173]. Mature capsids are about 55 nm in diameter (Figure 1.3) [163,175].

HPV infects epithelial basal stem cells. In most cases, access to the basal cells is believed to be due to abrasions or breaks in the epithelial layer of the skin or the mucosa in the male or female genitourinary tract. However, recent work has also identified a region in the female reproductive tract between the ectocervix and the transformation zone of the vagina called the squamocolumnar junction that has surface exposed stem cells [154]. HPV infection of these cells is believed to be the source of HPV infection that leads to cervical cancer. Infection of basal stem cells is critical for HPV as the virus lifecycle is linked to cellular differentiation [158,162]. Assembly of the mature capsid is restricted to terminally differentiated cells due to the dependence of the L1 and L2 late promoter on cellular transcription factors [162].

The infection pathway of HPV is complex [176]. The virus binds to heparin sulfate proteoglycans (HSPGs) or laminin-332 on the cell surface (*in vitro)* or on the extracellular matrix (*in vivo*) and then passes to HSPGs on the cell surface [177-183]. This binding induces a conformational change of L1 that exposed a region of the N terminus of L2 that contains a cyclophilin B binding site [182,184,185]. Cyclophilin B (CyPB) is a peptidylprolyl isomerase, a host chaperone protein that mediates protein folding or unfolding by rotating the peptide bond upstream of a proline residue [186]. During HPV infection, cyclophilin B binds L2 and unfolds it, exposing more of the amino terminus of L2. This exposed region contains a furin cleavage site that is conserved across all papillomaviruses, including non-human papillomaviruses [183,187]. Host furin cleaves the exposed L2. After cleavage the virus capsid is passed to a secondary receptor that mediates internalization [178,188]. This receptor is currently unknown but has been proposed to be α6 integrins, A2t annexin, or CD151 tetraspannin [189-194]. However, the receptor appears to be

serotype specific, and different HPV serotypes are able to infect cell lines lacking one or more of each of these putative receptors [167,189,195]. The cleavage of L2 is not required for internalization, as L1-only capsids or capsids added to cells in the presence of furin inhibitor are still internalized. However, the cleavage of L2 at the cell surface is critical for downstream entry steps, as the addition of furin inhibitor results in a loss of infection [187,196].

Early studies in the papillomavirus field found a variety of different internalization mechanisms, including both clathrin-mediated and caveolin-mediated uptake [197-201]. However, some of this confusion may be due differences in the HPV serotypes studied and the pleiotropic inhibitors used. A recent comprehensive study using HPV16 found that the virus is internalized by an undefined mechanism that is not clathrin or caveolin mediated, and is not macropinocytosis [202]. Instead, the cell appears to form tubes that the capsids enter. This process requires actin polymerization, as cytochalasin D inhibits infection [178,202,203]. The virus particles can spend up to 4 hours at the cell surface before internalization. Infection of HPV induces phosphorylation of the tyrosine kinase FAK within 30 minutes of capsid binding *in vitro*, but the role of the FAK signaling pathway in HPV infection is unclear, as are the roles of protein kinase C and the PI3K pathway which are also activated after HPV infection [176,204,205]. After internalization, the virus colocalizes with the early endosome between 4 and 8 hours after infection. Another tyrosine kinase protein, Pyk2, is required for infection and is thought to be required for retention of the virus in the endosome system, but the specific mechanism is also unknown [206]. As the endosome matures, the virus uncoats in a pH and CyPB dependent manner 6 to 8 hours after entering the cell [185,202,207]. The viral genome remains bound to L2, while L1 and L2 dissociate. L2 interacts with both Sorting Nexin 17 (SNX17)[208] and Sorting Nexin 27 (SNX27)[209], possibly to ensure that the L2 and genome complex is retained in the maturing or

late endosome and not sorted to the lysosome, but how L2 interacts with these cytosolic proteins is unclear. In addition, L2 interacts with members of the Rab family, Rab7b and 9a, again possibly for retention or sorting within the endosomal system [210]. After uncoating, L2 mediates exit of the viral genome from the endosomal pathway. L2 contains two putative transmembrane domains that are required for infection and it is believed that these domains insert into the endosomal membrane [196,211-214]. Furin cleavage of L2 at the cell surface is thought to be required for this interaction with the membrane, as viral particles with uncleaved L2 are unable to escape the endosome [196]. Interaction with the membrane does not induce lysis of the endosome, but rather allows for the interaction of L2 with a cytoplasmic protein complex called the retromer [213-216]. The retromer complex is part of the retrograde transport system of the cell, taking surface exposed proteins or vesicle cargo proteins from the endosome back to the *trans*-Golgi network in order to be repackaged and redelivered to the cell surface [217-219]. L2 interaction with the retromer results in vesicle budding out of the endosome system [215]. The vesicles contain L2 that is interacting with the retromer complex, the viral genome, and a portion of L1 that has either not completely dissociated from L2 or was stochastically included with the vesicle. The majority of L1 remains in the endosomal system and eventually traffics to the lysosome and is degraded [210]. The retromer mediates transport of the vesicle containing the L2 and genome complex to the *trans*-Golgi network 8 to 16 hours after infection [210,215,216]. The movement of the virus through the cell requires dynein-mediated transport along microtubules [220,221]. The mechanism of entry or fusion of the vesicle with the *trans*-Golgi network is still unknown, but requires γ-secretase activity [222,223]. However, no viral protein is cleaved during this step, so the γ-secretase target must be an unknown cellular protein [222]. How the L2 and genome complex traverse the *trans*-Golgi network is currently unknown. It is also unknown if the viral complex

travels through the ER, as there is data suggesting that an ER protein, syntaxin-18, is required for BPV infection [224]. However, there are also conflicting reports of both ER localization of the viral genome and no ER localization of the viral genome with various HPV serotypes or BPV during infection [225,226]. Entry of the viral genome complex into the cell nucleus requires nuclear envelope breakdown during mitosis 20 to 24 hours after infection [214,227]. After entering the nucleus, the L2 and genome complex localize to ND10 regions and the viral genes are expressed [228-230]. Although L2 contains multiple nuclear localization signals, these appear to not be required during infection but are rather used during replication for the newly translated L2 to re-enter the nucleus to encapsidate the viral genome [211,231]. Overall, the HPV16 entry pathway is best characterized (Figure 1.4), and there are multiple indications that some of these steps differ among HPV serotypes [176,189].

1.3 Human Papillomavirus and α-Defensins

There is limited data on the mechanism of α-defensins' antiviral activity against HPV. Buck *et al* found that both cutaneous (HPV5) and mucosal (HPV6, 16, 18, 31) serotypes of HPV were sensitive to HNP1 and HD5 [52]. Two non-human papillomaviruses, bovine papillomavirus and cotton-tail rabbit papillomavirus, were also sensitive to HNP1 and HD5, and the Smith lab has also shown that mouse papillomavirus is also sensitive to HD5 (unpublished data), indicating broad species neutralization by the defensins. HNP2-4 were also tested against HPV16 and found to also be active against HPV16. HD6 was not active against HPV16.

Despite this study, the molecular mechanism of antiviral activity against HPV16 is still unclear. Buck *et al* showed that HD5 only effectively neutralized HPV16 when the defensin and virus interacted at the cell surface [52]. A time course of HD5 treatment showed that pre-treatment of cells with HD5 before HPV16 infection or addition of HD5 after the virus had been

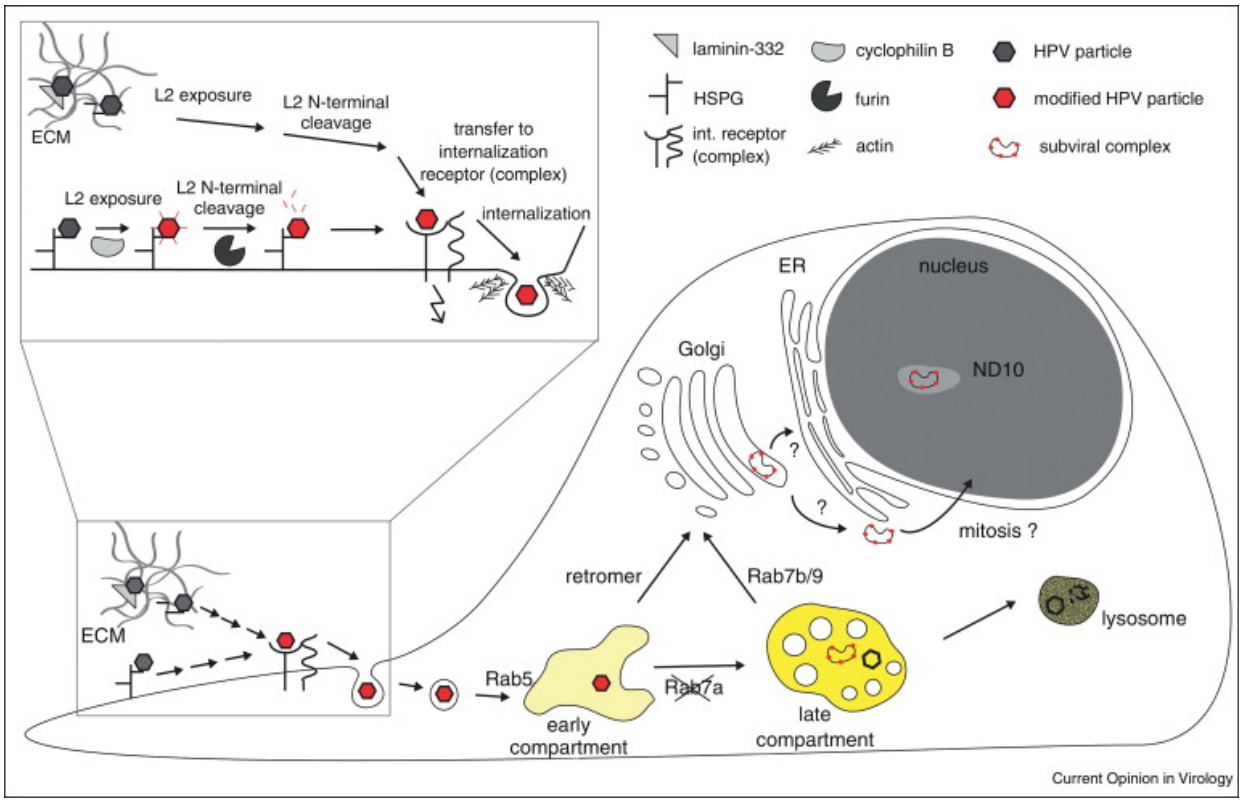

Figure 1.4 Schematic depiction of PV entry.

Depicted are certain steps of PV entry into host cells. The initial interaction with cellular receptors and structural modifications on the cell surface or ECM/basement membrane are enlarged for a better view. Indicated is also the uptake by a novel, ligand-activated endocytic mechanism where actin polymerization assists in vesicle scission from the plasma membrane. After internalization, the structurally modified PV particles are delivered to an early endosomal compartment, that is, early endosome or macropinosome-like endosome. Delivery of a subviral complex consisting of L2 and the vDNA to the TGN occurs after separation of most of the L1 capsomers through Rab7b/Rab9-mediated and/or retromer-mediated vesicular trafficking. After membrane penetration and nuclear import the subviral complex locates to ND10 in the nucleus. Indicated is the need for acidification of endosomal organelles by the yellow shading of organelles. Day PM, Schelhaas M. Concepts of papillomavirus entry into host cells. Curr Opin Virol. 2013 Dec 14;4C:24–31. Figure and legend reprinted with permission.

internalized did not result in effective neutralization of the virus. In addition, both HD5 and HNP1 inhibited nuclear localization of the viral genome in an immunofluorescence assay, but did not inhibit uncoating of the viral particle, as measured by exposure of a BrdU-labeled viral genome. However, a more specific mechanism was not described.

The defensin antiviral mechanism against HPV showed both commonalities and differences with the mechanisms against other non-enveloped viruses, such as HAdV, JC PyV, and BK PyV [38,51,54,85,152]. Against HAdV and BK PyV, the defensin interacts directly with the virus in the extracellular milieu. In the case of both HAdV and JC PyV, the virus is still able to enter the cell but the viral capsids are stabilized and cannot uncoat, while BK JyV is aggregated and cannot bind to its receptor. Interestingly, HPV16 possibly uncoats in the presence of HD5, which is distinct from the antiviral mechanisms against HAdV and JC PyV. Therefore, how HD5 inhibits HPV16 potentially represents a distinct antiviral mechanism.

Chapter 2. HD5 Blocks An Extracellular Step of HPV Entry**

2.1 Introduction

Previous work indicated that HD5 only effectively neutralized HPV16 when the defensin and virus interacted at the cell surface [52]. Thus, I hypothesized that HD5 binding to the virus in the extracellular milieu would alter viral entry, resulting in deleterious effects on infection. Consistent with this model, treatment of cells with HD5 before infection or after the virus has been internalized has minimal effect on infection. I first demonstrated that purified HD5 aggregates HPV16 PsV, confirming a direct interaction that was suggested by previous studies. By assaying the steps in HPV16 entry that occur on the cell surface, I found that furin cleavage and exposure of a specific epitope in L2 is blocked by HD5 binding. Disruption of this conserved, critical step in HPV entry is consistent with the previously described failure of the genome to escape the endosomal pathway due to HD5 inhibition and provides a rationale for the broad activity of HD5 against mucosal and cutaneous HPV types.

2.2 Results

2.2.1 HD5 interacts directly with HPV16 PsV

For other non-enveloped viruses, direct binding of defensins to the viral capsid is required for antiviral activity [51,54,58,232]. As the antiviral activity of HD5 is highest when HD5 and HPV16 PsV are first co-incubated outside of the cell [52], it is likely that inhibition of HPV16 infection is also due to direct binding of the defensin to the viral capsid. To assess this

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**Wiens ME, Smith JG. Alpha-defensin HD5 inhibits furin cleavage of human papillomavirus 16 L2 to block infection. Journal of Virology. 2015 Mar;89(5):2866–74. Reprinted with permission.

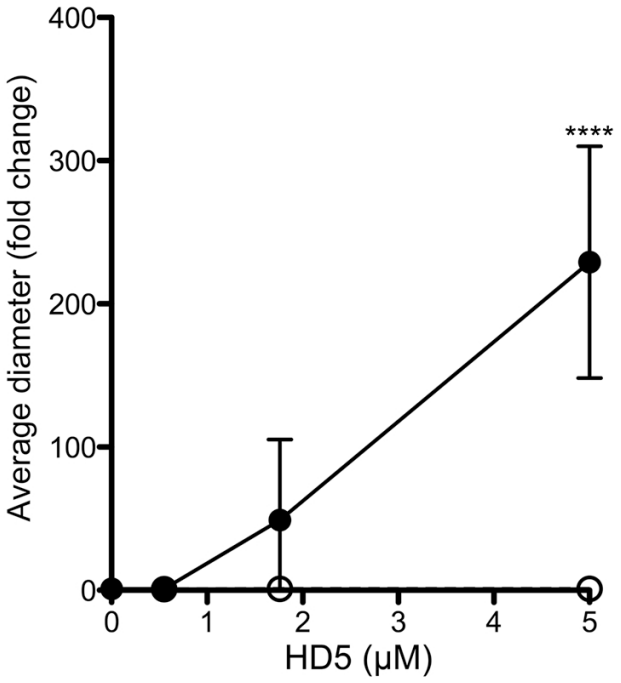

Figure 2.1 HD5 binding aggregates HPV16 PsV.

The mean diameter of HPV15 PsV was measured upon incubation with increasing concentrations of wild type HD5 (black circles), or HD5 Abu (open circles). Data is the average fold increase in diameter for each condition compared to untreated controls of 4 independent experiments ± SD. ****, $p<0.0001$.

interaction, I quantified viral aggregation as a change in mean particle diameter using dynamic light scattering (DLS), which was a correlate for defensin binding in previous studies of HAdV [58]. HD5 treatment increased the average diameter of HPV16 PsV in a dose dependent manner (Figure 2.1). No change in diameter was observed in samples treated with 0.6 μM HD5, consistent with minimal antiviral activity at this concentration (Figure 2.2A). To assess the importance of defensin tertiary structure in HPV binding, I analyzed a linear HD5 mutant (HD5 Abu), which has the same charge as HD5 but no regular structure [38]. Like for HAdV, HD5 Abu has no antiviral activity against HPV16 PsV (Figure 4.2A) and did not aggregate HPV16 PsV (Figure 1.1). These data support a model in which HD5 interacts directly with the HPV16 capsid to neutralize infection.

2.2.2 HD5 inhibits exposure of an L2 neutralizing antibody epitope

To identify the step in HPV entry that is blocked by HD5, I assessed the capacity of the anti-L2 neutralizing antibody RG-1 to bind its epitope. RG-1 is known to bind to L2 only after furin cleavage [233,234]. Accordingly, binding of the RG-1 antibody can be used to assess the completion of necessary processing at the cell surface. HaCaT cells were infected with Alexa Fluor-labeled HPV16 PsV that had been incubated with HD5, RG-1, or HD5 and RG-1 together prior to infection. For these experiments, I used inhibitory concentrations based on the dose responsive effects of these agents on HPV16 infection alone and in combination (Figures 2.2A, 2.2B and 2.2F). Cells were fixed at 12 h post-infection (p.i.), and an Alexa Fluor 488-conjugated secondary antibody was added to visualize RG-1 that was bound before fixation. In the absence of both HD5 and RG-1, HPV16 was well dispersed in the cytoplasm. HD5 treatment alone had no effect on the intracellular distribution of the virus. However, RG-1 treatment aggregated the virus, which remained in proximity to the plasma membrane. Consistent with this effect, most of

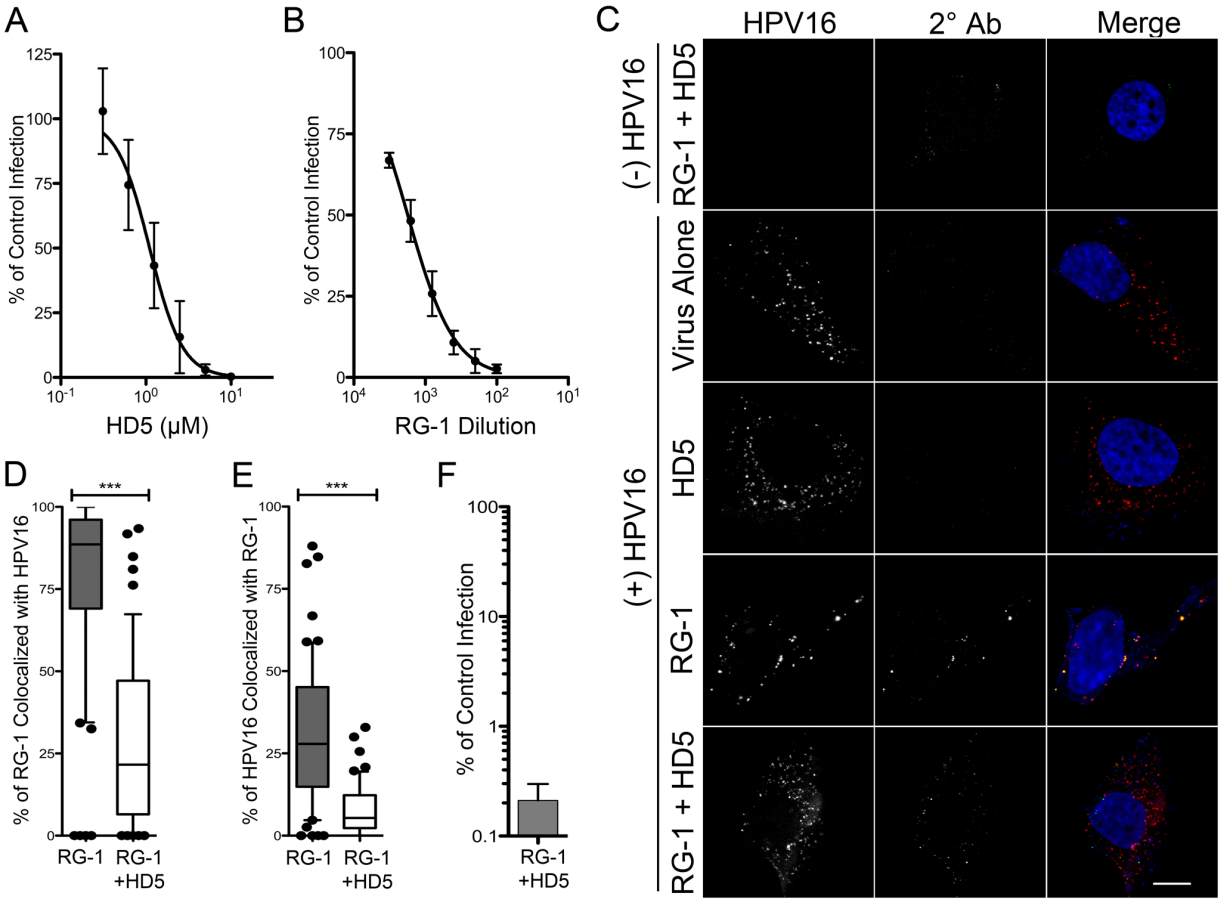

Figure 2.2 HD5 blocks exposure of an L2 antibody epitope.

(A) HD5 neutralizes AF555-HPV16 PsV in complete media. HeLa cells were infected with AF555-HPV16 PsV incubated with increasing concentrations of HD5 in complete media. Data is three independent experiments normalized to control infection in the absence of inhibitor ± SD. IC_{50}=1.1 μM, 95% CI=0.93 to 1.32 μM. (B) RG-1 antibody neutralizes AF555-HPV16 PsV. HeLa cells were infected with AF555-HPV16 PsV incubated with increasing concentrations of RG-1 antibody in complete media. Data is three independent experiments normalized to control infection in the absence of inhibitor ± SD. IC_{50}=1759, 95% CI=1428 to 2167. (C) The presence of HD5 prevents binding of the RG-1 antibody to HPV16 during cell entry. Images of HaCaT cells 12 h p.i. with [(+) HPV16] or without [(-) HPV16] AF555-HPV16 PsV in the presence of no inhibitor (virus alone), 5 μM HD5 (HD5), 5.4 μg/ml RG-1 antibody (RG-1), or 5 μM HD5 and 5.4 μg/ml RG-1 together (RG-1 + HD5). Individual panels depict

maximum intensity z-projections of signal above threshold for images in the z-stack that are co-planar with the nucleus for HPV16 (red) and RG-1 (2° Ab, green). In the merge images, the nucleus is blue. Scale bar is 10 μm. Manders coefficient values M1 (D) and M2 (E) are plotted as percent of RG-1 colocalized with HPV16 and percent of HPV16 colocalized with RG-1, respectively, for 50-60 cells for each condition. Whiskers are 5-95%, the horizontal line is the median, and outliers are depicted as individual points. ***, $p<0.0001$. (F) HPV16 PsV treated with a combination of 5.4 μg/ml RG-1 and 5 μM HD5 is neutralized. Data is the mean of 3 independent experiments normalized to infection in the absence of inhibitor ± SD.

the RG-1 signal colocalized with virus (median ~89%, Figure 2.2D), and a significant portion of the total viral signal colocalized with RG-1 (median 28%, Figure 2.2E). Treatment with both HD5 and RG-1 together resulted in an intracellular distribution of virus comparable to that of HD5 alone. Some RG-1 signal was observed in these samples; however, RG-1 colocalization with the virus (median ~22%, Figure 2.2D) and viral colocalization with RG-1 (median ~5%, Figure 2.2E) were both dramatically decreased upon the addition of HD5. I observed some internalization of RG-1 even in the absence of virus (Figure 2.2C, top), which likely explains the presence of RG-1 that is not co-localized with virus in these samples. Thus, HD5 prevents RG-1 from binding to the virus.

2.2.3 HD5 does not directly interfere with RG-1 binding to L2

One possible reason that RG-1 cannot bind to the virus in the presence of HD5 is that HD5 binds to either RG-1 itself or to the RG-1 epitope on L2 and directly interferes with the antibody-epitope interaction. To address this, I made a C-terminally (6x)His- and HA-tagged recombinant L2 peptide (rL2:13-36) containing the RG-1 epitope (residues 17-36) [233]. RG-1 binds to rL2:13-36 and could be precipitated through the (6x)His tag using TALON beads, while the antibody alone did not bind to the beads (Figure 2.3A). Addition of HD5 did not interfere with the ability of the peptide to precipitate the RG-1 antibody. Importantly, these experiments were performed using HD5, RG-1, and L2 concentrations that were calculated to closely approximate those used in the immunofluorescence studies and under which I have observed neutralization of infection (Figures 2.2A-C). These experiments indicate that HD5 does not directly interfere with the RG-1/L2 interaction.

To confirm this interpretation, I designed an assay to rescue viral infection by competing HD5 or RG-1 with a molar excess of rL2:13-36. The amount of rL2:13-36 was calculated to be

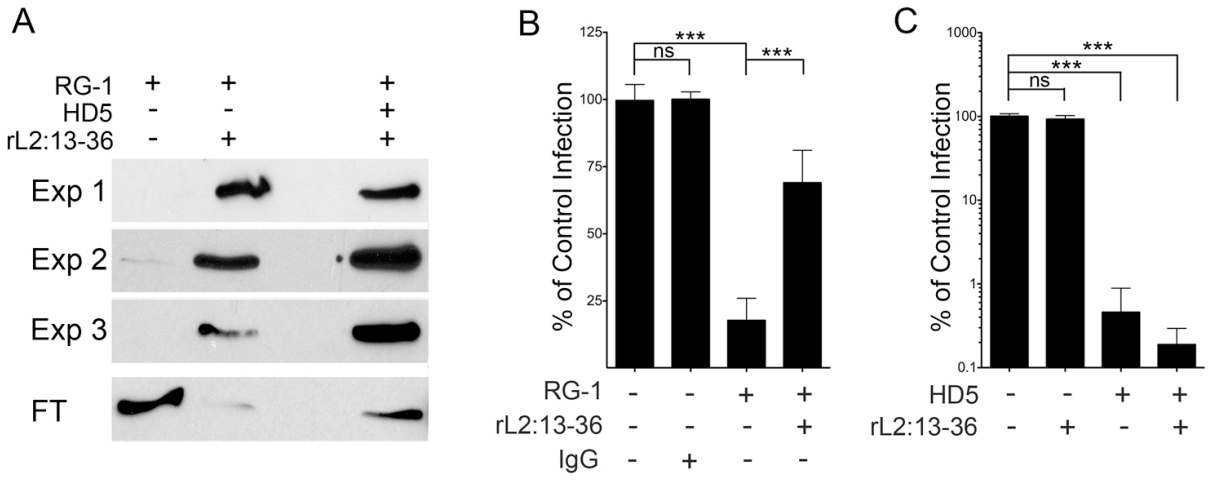

Figure 2.3 HD5 does not block RG-1-L2 epitope binding.

(A) RG-1 was immunoprecipitated by rL2:13-36 in the presence or absence of 5 μM HD5. Shown are bound antibody from three independent experiments (Exp 1-3) and a representative unbound fraction (FT) from one experiment visualized by immunoblot. (B) Excess rL2:13-36 rescues HPV16 from RG-1 neutralization. Infection of HeLa cells by HPV16 PsV incubated with RG-1 alone or in competition with a 500-fold molar excess of rL2:13-36 was quantified relative to infection in the absence of inhibitor. BSA was used to normalize protein levels in all samples, and mouse IgG1 was used as isotype control for RG-1. Data are three independent experiments ± SD. ***, $p<0.0001$. (C) rL2:13-36 does not rescue HPV16 infection from HD5 neutralization. Infection of HeLa cells by HPV16 PsV incubated with 5 μM HD5 alone or in competition with a 500-fold molar excess of rL2:13-36 was quantified relative to infection in the absence of inhibitor. BSA was used to normalize protein levels in all samples. Data are three independent experiments ± SD. ***, $p<0.0001$.

500-fold greater than the maximum amount of L2 that could be present in PsVs in the sample. As proof of concept, I first assessed the ability of the L2 peptide to rescue HPV16 infection in the presence of RG-1. In the absence of rL2:13-36, RG-1 neutralized the virus and decreased infection to approximately 20% of control infection (Figure 2.3B). However, incubation of the RG-1 antibody with the rL2:13-36 peptide before addition of the virus successfully competed the antibody away from the virus and rescued viral infection to ~70%. I then determined whether excess rL2:13-36 could similarly compete with the virus for HD5 binding. As expected, HPV16 PsV infection was unaffected by the presence of excess rL2:13-36, and HD5 alone potently inhibited infection (Figure 2.3C). Incubation of HD5 with excess rL2:13-36 prior to addition of the mixture to HPV16 PsV did not attenuate HD5 anti-viral activity, indicating that HD5 does not bind to residues 13-36 of L2. Taken together, these results indicate that HD5 does not bind to either the antibody or the epitope on L2. Therefore, the defensin may be indirectly inhibiting RG-1 binding to the virus by interfering with exposure of the L2 epitope at the cell surface.

2.2.4 Bypassing the CyPB-mediated unfolding of L2 does not relieve the HD5 block

Prior to RG-1 epitope exposure, the virus undergoes an L1 conformational change [184]. Inhibition of this step by neutralizing antibodies blocks viral internalization [235]. Defensin-treated HPV16 is still able to enter the cell, suggesting that the defensin-dependent block is after this L1 conformational change [52]. The next step is a L2 conformational change mediated by host CyPB that results in exposure of L2 for subsequent furin cleavage [185]. Accordingly, inhibition of CyPB with cyclosporin A (CsA) blocks furin cleavage. CsA inhibition is bypassed by mutation of the CyPB binding site in L2 (G99A and P100A), likely by altering the flexibility of L2 and increasing exposure of the furin cleavage site, as demonstrated by detection of the RG-1 epitope during infection [185]. To assess the possibility that HD5 inhibits the CyPB-mediated unfolding of

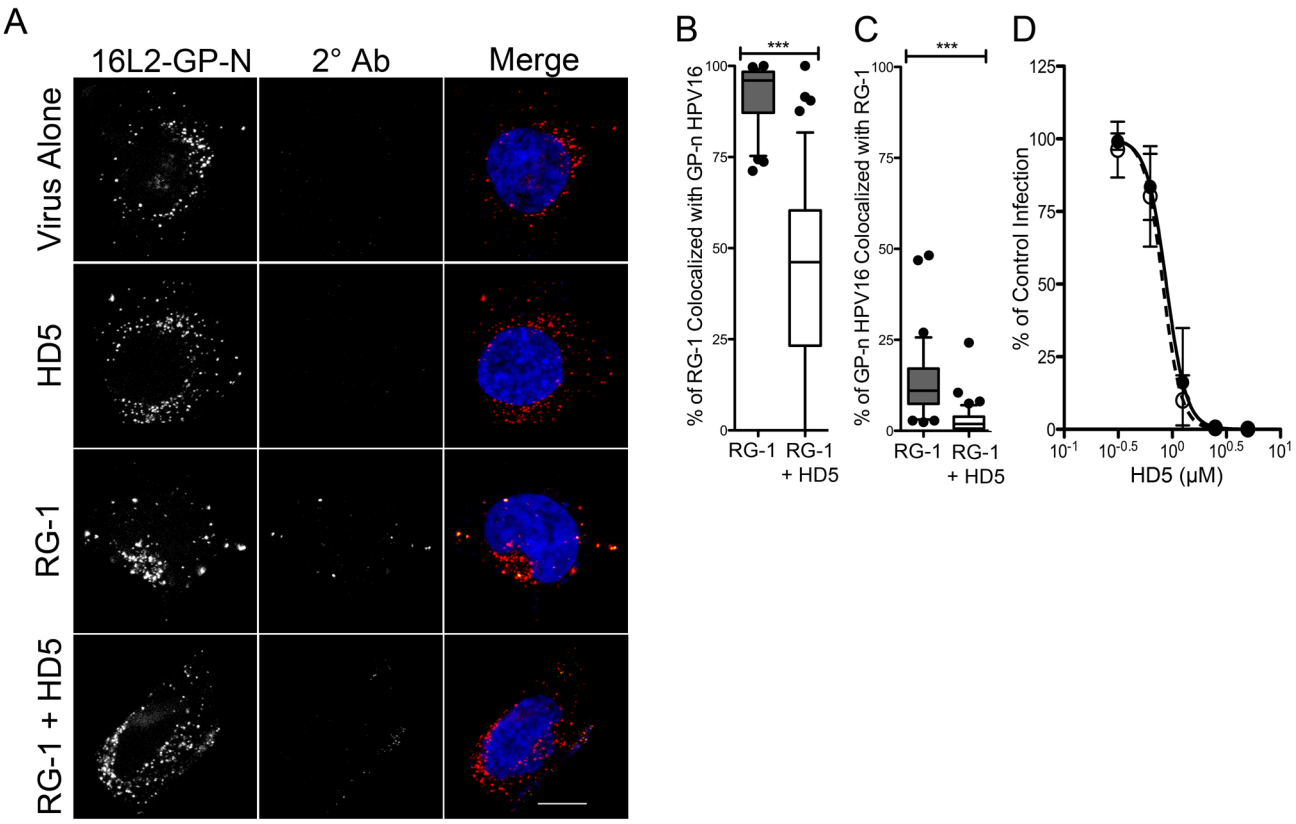

Figure 2.4 Cyclophilin B-independent HPV16 mutant (16L2-GP-N) remains sensitive to HD5 neutralization.

(A) Images of HaCaT cells 12 h p.i. with AF555-16L2-GP-N PsV in the presence of no inhibitor (virus alone), 5 μM HD5 (HD5), 5.4 μg/ml RG-1 antibody (RG-1) or 5 μM HD5 and 5.4 μg/ml RG-1 together (RG-1 + HD5). Individual panels depict maximum intensity z-projections of signal above threshold for images in the z-stack that are co-planar with the nucleus for 16L2-GP-N (red) and RG-1 (2° Ab, green). In the merge images, the nucleus is blue. Scale bar is 10 μm. Manders coefficient values M1 (B) and M2 (C) are plotted as percent of RG-1 colocalized with 16L2-GP-N and percent of 16L2-GP-N colocalized with RG-1, respectively, for 45-60 cells for each condition. Whiskers are 5-95%, the horizontal line is the median, and outliers are depicted as individual points. ***, $p<0.0001$. (D) HD5 neutralizes 16L2-GP-N. HeLa cells were infected with WT HPV16 PsV (black circles, IC_{50}=0.88 μM, 95% CI=0.78 to 0.99 μM) or 16L2-GP-N PsV (open circles, IC_{50}=0.89 μM, 95% CI=0.72 to 0.92 μM) incubated with increasing concentrations of HD5. Data is the mean of three independent experiments compared to control infection in the absence of inhibitor ± SD.

L2, I engineered these mutations into HPV16 PsVs to make them CyPB-independent (16L2-GP-N PsVs). I first found that the mutant 16L2-GP-N is as sensitive to HD5 as WT HPV16, with an almost identical IC_{50} (Figure 2.4D), indicating that the mutations in L2 were unable to bypass the HD5 block. Next, I repeated the RG-1/HD5 competition assay to determine the state of L2. I found that the intracellular distribution of Alexa Fluor 555 labeled 16L2-GP-N in the presence of HD5, RG-1, or both HD5 and RG-1 was equivalent to WT under each condition (compare Figures 2.2C and 2.4A). Furthermore, the ability of HD5 to reduce RG-1 colocalization with virus (Figure 2.4B) and virus colocalization with RG-1 (Figure 2.4C) was unchanged in 16L2-GP-N compared to WT. Thus, mutation of the virus to bypass the requirement for a CyPB-mediated conformational change in L2 at the cell surface was not sufficient to alleviate the HD5 block in infection, suggesting that this block was at a subsequent step.

2.2.5 HD5 inhibits cleavage of L2

Cleavage of L2 by cellular furin occurs after the CyPB induced conformational change [187]. In order to directly assess the cleavage state of L2 during infection, I made an L2 construct containing a Myc tag on the N terminus and an HA tag on the C terminus. The HA tag facilitates detection of L2 in cellular lysates, while the Myc tag extends the N terminus of L2 and increases the ability to resolve the cleaved and uncleaved forms of L2 by SDS-PAGE. I generated PsVs incorporating this L2 construct in place of WT L2 (Myc-16L2-HA). The sensitivity of Myc-16L2-HA PsV to HD5 inhibition was equivalent to WT (Figure 2.5A). I then assessed the effect of HD5 on the cleavage state of L2 during infection of HeLa cells. As this biochemical assay required ~10x more input virus ($4.8x10^{10}$ particles/sample) than I used in previous experiments, I verified the IC_{50} of HD5 in complete media against this higher virus concentration. Although the dose-response of HD5 was slightly altered by the higher viral concentration, 10 μM HD5 was

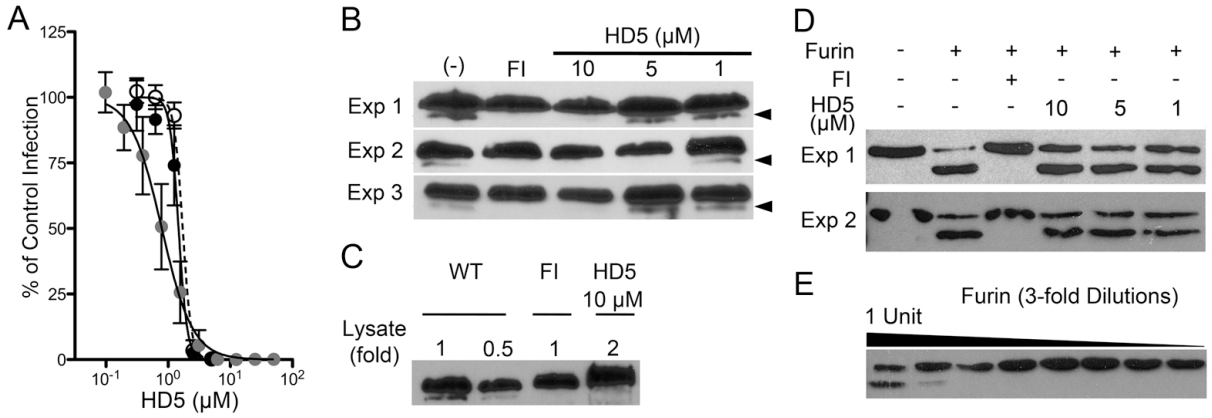

Figure 2.5 HD5 Inhibits cleavage of HPV16 L2.

(A) Myc-L2-HA HPV16 PsV is sensitive to HD5. HeLa cells were infected with WT HPV16 PsV in SFM (black circles, IC_{50}=1.47 μM, 95% CI=1.35 to 1.6 μM), Myc-L2-HA PsV in SFM (open circles, IC_{50}=1.71 μM, 95% CI=1.6 to 1.8 μM), or ten times as much Myc-L2-HA PsV in complete media (gray circles, IC_{50}=0.79 μM, 95% CI=0.69 to 0.9 μM) incubated with increasing concentrations of HD5. Data is three independent experiments normalized to infection in the absence of inhibitor, ± SD. (B) HD5 inhibits furin cleavage of L2. HeLa cells were infected with Myc-16L2-HA HPV16 PsV in the presence of 40 μM furin inhibitor (FI), 1-10 μM HD5, or no inhibitor (-). L2 cleavage was assessed by immunoblot of cell lysates 16 h p.i. using an anti-HA antibody. Cleaved L2 (arrow) is visible as a faster migrating band below uncleaved L2. Shown are three independent experiments. (C) Analysis of a greater amount of lysate confirms the inhibition of furin cleavage. Different amounts of HD5-treated HPV16 PsV lysate, indicated by fold change relative to the amounts loaded in panel B, were assessed by immunoblot with anti-HA antibody. (D) HD5 does not directly affect the enzymatic activity of furin. 1.8 ng rL2:1-160 was digested with 1 U of furin in the presence or absence of the indicated inhibitors for 1 h at 30 °C. Samples were immunoblotted using an anti-His antibody. Cleaved rL2:1-160 is the faster migrating band. Shown are two independent experiments. (E) Titration of furin required for rL2:1-160 cleavage. rL2:1-160 was digested with a three-fold dilution series of furin, starting at 1 U of total furin. Samples were resolved on a reducing gel and immunoblotted using anti-His antibody. Cleaved rL2:1-160 is the faster migrating band.

still inhibitory (Figure 2.5A). PsV was incubated with or without increasing concentrations (1 – 10 μM) of HD5, and the mixture was added to HeLa cells. Furin inhibitor (40 μM) was used as a positive control for inhibition of L2 cleavage. Cells were lysed 16 h p.i., and clarified lysates were resolved by SDS-PAGE and immunoblotted for L2. In untreated samples, a minor band with faster mobility was observed, consistent with removal of the N-terminus of L2 including the Myc tag (Figure 2.5B). In samples treated with furin inhibitor, this cleavage product was absent. HD5 also completely inhibited cleavage of L2, and this effect was dose responsive in three independent experiments. To confirm the disappearance of the L2 cleavage product, I ran twice the amount lysate from the 10 μM HD5 sample of experiment 1 and did not detect the cleaved L2 band (Figure 2.5C). Thus, HD5 blocks L2 cleavage by furin during cell entry.

I surmised that HD5 was either blocking the furin enzyme directly or interfering with the ability of furin to access the L2 cleavage site on the virus. To investigate the first possibility, I purified a truncated L2 protein comprising the first 160 residues of L2 (rL2:1-160). Using a minimal amount of furin and an amount of rL2:1-160 (1.8 ng) that on a molar basis was below the total L2 content of the PsVs in the infection assay, I found that anti-viral concentrations of HD5 had no effect on furin cleavage of this unencapsidated L2 (Figure 2.5D). Importantly, the assay remained sensitive to inhibition by furin inhibitor. And, L2 remained largely uncleaved in samples treated with three-fold less furin, suggesting that even small changes in furin activity would be detectable in this assay (Figure 2.5E). Thus, HD5 does not directly attenuate the protease activity of furin. Rather, HD5 obstructs L2 cleavage during infection, likely through steric hindrance of L2 cleavage sites on the virus.

2.3 Discussion

I have uncovered a unique mechanism by which α-defensin binding to a viral capsid at the cell surface alters host-mediated processing of viral capsid proteins, which manifests as a block in cellular trafficking further downstream in the viral entry pathway. Furin cleavage of L2 is a key step in HPV entry, as inhibition of furin cleavage during infection results in retention of PsVs in the endosomal pathway [187]. My finding that HD5 binds to HPV16 PsV and directly and specifically blocks L2 furin cleavage is consistent with the initial description of α-defensin inhibition of HPV16, which resembled the uncleaved PsV phenotype [52]. This is an appealing model that may explain the broad inhibitory activity of defensins against both mucosal and cutaneous PV serotypes from both humans and other mammals, as the requirement for furin cleavage is widely conserved, although the generalization of this mechanism to other HPV serotypes remains to be formally demonstrated. My data exclude an effect of HD5 on the enzymatic activity of furin itself. Rather they suggest that the ability of furin to access L2 in the context of the incoming capsid is compromised through steric hindrance, likely imposed by HD5 binding to L1. Although the molecular mechanisms differ, my data supports a general theme of α-defensin-mediated alteration in intracellular trafficking as an inhibitory mechanism of diverse non-enveloped viruses.

The most probable scenario is that the antiviral activity of HD5 is due to a direct interaction between the virus and HD5. My current data (Figure 2.1) and previous studies strongly support this notion. First, HD5 aggregates HPV16 PsV, indicating a direct interaction between the defensin and virus. In addition, α-defensin mutants that were attenuated for anti-HAdV activity due to direct alterations in capsid binding were also deleterious for anti-HPV16 activity [58,232]. Finally, pretreatment of cells with HD5 was not sufficient to neutralize infection,

arguing against a cellular rather than a viral target that mediates inhibition [52]. I speculate that HD5 likely binds L1 specifically and blocks access of the furin enzyme to the cleavage site on L2 via steric hindrance, as my data shows that HD5 does not interfere with the interaction between the RG-1 antibody and the epitope on L2 nor does a molar excess of rL2:13-36 rescue viral infection, making L2 itself an unlikely binding partner. This peptide comprises a significant amount of L2 that is thought to be exposed at the capsid surface even after the L1 conformational change [211]. Moreover, rL2:1-160 was also unable to compete with HD5 for binding to the capsid in experiments analogous to those in Figure 2.3 (data not shown). While my data indicate that L2 is not the sole binding partner, the N terminus of L2 may still comprise part of the interaction site with HD5, as the exact binding site on the capsid is still unknown. It remains to be determined exactly how the defensin interacts with the capsid to block furin cleavage at the molecular level. One possibility is that the defensins bind to an element of the HPV capsid that facilitates multimeric defensin-defensin interactions, effectively blanketing the virus and blocking access of furin to the N terminus of L2 that is exposed following the CyPB interaction. This would be similar to the AdV-HD5 interaction, which is highly multivalent [51,232]. Indeed, mutations that affect defensin-defensin interactions are markedly detrimental for defensin activity against both HAdV and HPV16 [58,232]. Alternatively, the specific location of the defensin interaction with the capsid may be deleterious to furin access. These models might be distinguished by future biophysical measurements of defensin binding or by high-resolution structures of defensins in complex with HPV.

Beyond a molecular explanation for inhibition of furin cleavage, a more detailed delineation of the defensin-virus interface may provide an explanation for the ability of defensins to bind to capsids of unrelated viruses. On the virus side of the interaction, it is unclear if the

defensins recognize a conserved sequence or structural element common to multiple viruses. Previous studies have identified critical determinants at the junction of two capsid proteins of AdV that dictates HD5 binding [38]. Analogous experiments using HPV16 or PyVs have not yet been performed. On the defensin side, much more is known about the specific molecular features of both HD5 and HNP1 that dictate their antiviral activity against AdV and HPV16. Alanine scan mutagenesis studies of HD5 and HNP1 have shown that neutralization of AdV and HPV16 is reliant on sequence, charge, and specific hydrophobic residues of each defensin [58,232]. Importantly, most HD5 mutants that alter binding to AdV capsomers are also attenuated for HPV16 neutralization, indicating that HPV16 inhibition is likely dependent on a similar binding principle. Thus, future studies of HPV and other viral systems may reveal common principles that dictate α-defensin activity against non-enveloped viruses, defining a mechanism distinct from the more completely characterized activity of defensins against bacteria and enveloped viruses [152,236].

I note a discrepancy between the concentration of HD5 that completely blocks furin cleavage and the concentration required to maximally block infection. This may be explained by differences in the sensitivities of the two assays or by differences in the total amount of virus in each assay. Alternatively, partial prevention of L2 cleavage may be sufficient to completely block infection, since the proportion of L2 in each virion that must be cleaved by furin for effective endosome lysis is unknown. A third possible explanation is that HD5 inhibits the virus via a secondary mechanism, possibly by altering intracellular trafficking. Consistent with this hypothesis, α-defensins do not block HPV16 uncoating, perhaps because the virus has entered a non-infectious pathway where the uncoated virion is unable to deliver its genome to the nucleus [52]. HD5 could mediate this effect via targeting the virus to defensin-specific receptors,

analogous to binding of serum growth factors to HPV16 that allow the virus to enter cells through growth factor receptors [237]. Alternatively, defensins could block viral protein interactions with cellular proteins involved in endosomal function such as the retromer complex and γ-secretase, which were recently shown to be critical for moving the viral genome through the endosomal system to the nucleus [210,215,222,223]. Thus, future studies to address the impact of HD5 on intracellular trafficking of HPV are warranted.

My data reveal a striking contrast between the inhibitory mechanisms of a secreted innate immune effector, α-defensins, and secreted effectors of the adaptive immune response, neutralizing antibodies. The major difference is that α-defensins block a critical step in the viral entry pathway but still allow virus internalization, whereas most neutralizing antibodies specific to L1 or L2 inhibit viral entry altogether [234,235,238]. Indeed, the very fact that defensin-treated HPV still enters cells indicated to us that the defensin acts after the L1 conformational change induced by HSPG binding and through a mechanism distinct from those previously described for neutralizing antibodies. This difference likely has ramifications for subsequent immune control of infection, as internalized virus may be exposed to intracellular sensors of the innate immune response (e.g., toll-like receptors, the inflammasome, and interferon stimulatory DNA pathways) inaccessible to antibody-neutralized virus, leading to the induction of an antiviral state in neighboring cells [239-241].

α-defensin mediated control of HPV infection in the female and male genitourinary tract may play a direct and indirect role in preventing HPV-associated cancers, by both inhibiting virus infection and modulating the anti-viral immune response. HPV is associated with roughly 5% of all cancers worldwide, and is thought to be the predominant cause of cervical cancer, with 60% of cervical carcinomas associated with HPV16 alone [242]. Increasingly, HPV has also been

linked to anal, oral, and throat cancers [243]. New data suggests that cervical carcinoma originates at cells of the squamocolumnar junction [154]. Recent evidence for reduced HD5 expression at these sites could render them more permissive to HPV infection [153]. Moreover, α-defensins are known to act as cytokines and chemokines, and immune responses to HPV at these sites could be attenuated [52,128,244]. Thus, better understanding of the molecular mechanisms underlying the inhibition of HPV infection and the potential role for α-defensins in HPV infection *in vivo* may lead to improvements in antiviral therapies and vaccine design against these clinically relevant viruses.

Chapter 3. HD5 Alters the Intracellular Trafficking of HPV16

3.1 Introduction

In the previous chapter I found that HD5 affects L2 cleavage, a critical extracellular step of the HPV entry pathway. To confirm this action as the mechanism of defensin-mediated neutralization of HPV, I next assessed the effect of HD5 on furin precleaved HPV16 (fcHPV16 PsV). This is a modified PsV in which the L2 is cleaved during production, resulting in PsV that is capable of infecting cells lacking HSPGs or in the presence of furin inhibitor [245]. It is thought that PsV with cleaved L2 can bind directly to the secondary receptor that mediates internalization [188]. If HD5's main mechanism of inhibitory action against papillomaviruses is to inhibit the cleavage of L2 during infection, this cleaved virus should be resistant to the defensin. However, I found that HD5 also efficiently neutralized fcHPV16 PsV (Figure 3.1A), indicating that the defensin has additional activity against HPV beyond inhibition of L2 cleavage. This is not without precedence in both the defensin and HPV literature. HNP1 has been shown to have multiple antiviral mechanisms against HIV and HSV, including inhibition of membrane fusion, disruption of the viral envelope, and inhibition of viral RNA or DNA transcription [152]. In the HPV literature, host cyclophilin B is known to be required at both the cell surface, to unfold L2 and expose the furin cleavage site, and in endosomal compartments, to mediate L1 and L2 dissociation [185,246]. Since HPV capsids isolated from *in vivo* infections are comprised of mixed populations of cleaved and uncleaved virions multiple neutralization mechanisms may be required to account for the variation in wild type HPV capsids during *in vivo* infection [247].

To assess the effect of HD5 on the intracellular entry pathway of HPV16, I studied HPV16 genome and L2 trafficking in the cell using PsV containing either labeled viral genome or tagged L2 and their colocalization with cellular markers in immunofluorescence assays. I also

assessed the stability of L1 during infection after treatment with HD5. I found that while the HD5-treated virus entered through the endosomal system and possibly uncoated, HD5 treatment dramatically changed the trafficking of the viral genome after the early endosome, redirecting the genome to the lysosome. In accordance with this, L1 and L2 are also degraded faster during infection in the presence of HD5. This redirection of the viral genome accounts for the inhibition of the fcHPV16 PsV as well as wild type HPV16 PsV.

3.2 Results

3.2.1 fcHPV16 PsV is sensitive to HD5

To confirm the previous finding that the antiviral mechanism of HD5 against HPV16 is inhibition of the furin cleavage of HPV16 L2, I made fcHVP16 PsV. PsV made in 293TT cells overexpressing furin (293TTF) is cleaved during maturation by the overproduced furin [245,248]. Cleavage of L2 can be verified by resistance of the resulting fcHPV16 PsV to furin inhibitor. To test the fcHPV16 PsV sensitivity to HD5, WT HPV16 or fcHPV16 was incubated with or without increasing concentrations of HD5. Furin inhibitor was added to the fcHPV16 to ensure that only cleaved virus would infect cells. Infection was measured by eGFP reporter gene expression ~40 h p.i (Figure 3.1A). Interestingly, HD5 neutralized both WT and fcHPV16 PsV at similar IC_{50}s, indicating that while furin cleavage at the cell surface is inhibited by HD5, there is a secondary mechanism by which HD5 inhibits HPV16 infection.

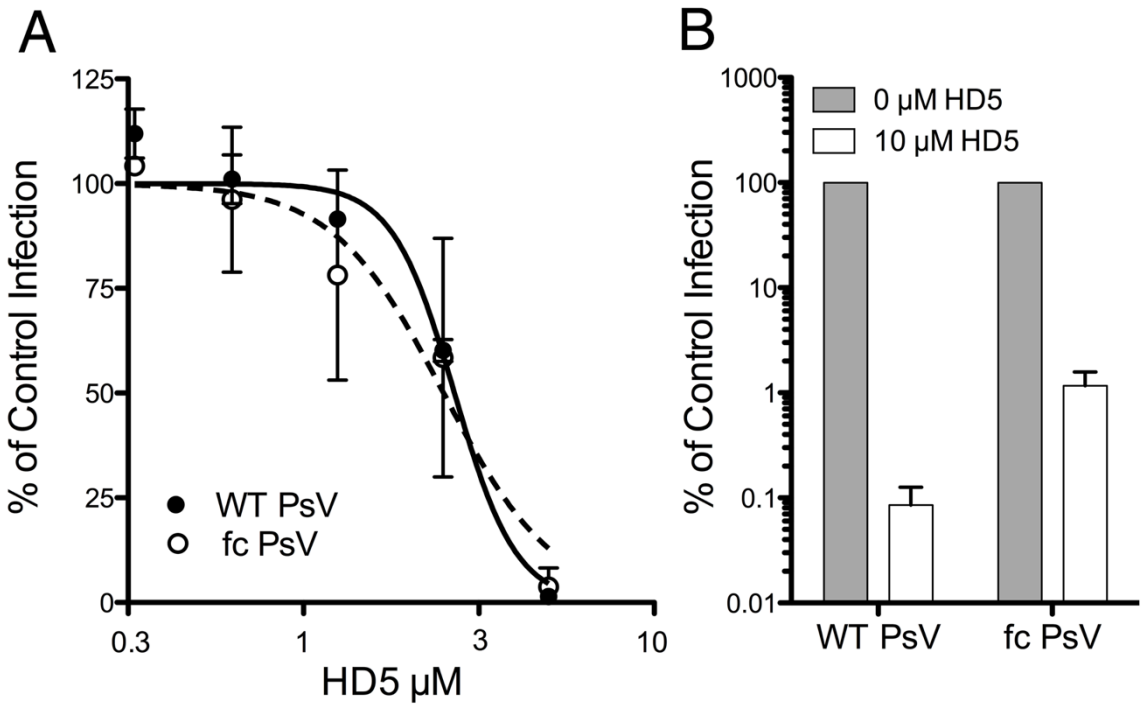

Figure 3.1 Furin precleaved HPV16 (fcHPV16) PsV is sensitive to HD5

(A) HeLa cells were infected with WT HPV16 PsV (black circles) or fcHPV16 PsV (open circles) with 20 µM furin inhibitor incubated with increasing concentrations of HD5 in SFM. Data is three independent experiments normalized to infection in the absence of inhibitor, ± SD. (B) WT HPV16 PsV or fcHPV16 PsV were incubated with HeLa cells in serum complete media. After unbound virus was washed off, 0 or 10 µM HD5 was added in complete media. Data is three independent experiments normalized to infection in the absence of inhibitor, ± SD.

3.2.2 HD5 does not alter early endosome colocalization

I hypothesize that HD5 alters the intracellular entry of HPV16. I therefore performed immunofluorescence assays of the virus in the presence of HD5 to determine any changes in the virus entry pathway after internalization. To assess any changes in the virus entry pathway due to HD5, I used fcHPV16 PsV with a 5-ethynyl-2'-deoxyuridine (EdU) labeled genome. EdU is a thymidine analogue that can be incorporated into DNA during DNA synthesis. An azide group on the EdU can form a bond with fluorescent alkyne substrates via ClickIT chemistry, allowing for visualization of the labeled DNA. fcHPV16 EdU PsV was prebound to HeLa cells for 1 hr at °C, unbound virus washed off and cells further incubated for 1 hr at 4°C in the presence of absence of HD5. Samples were then shifted to 37°C for 0, 4, 8, 12, or 16 hours and stained for the viral EdU genome and the early endosomal marker EEA1 (Figure 3.2A). Preliminary studies showed that treatment of HPV16 with HD5 significantly increases viral binding to cells (unpublished data). In an effort to equalize the EdU signal in HD5 treated and untreated samples, 3-fold less virus was used in the HD5 samples. Confocal z-series images were analyzed for colocalization of the viral genome and EEA1 using ImageJ. At the early time points (4 and 8 hours) the addition of HD5 did not alter the colocalization of the viral genome and the early endosome (Figure 3.2B) (median: 10% to 20%). These observations suggest that HD5 did not induce viral uptake by a completely alternative pathway. By 16 h p.i. in the untreated samples the viral genome colocalizes with the nucleus (median nuclear signal ratio: 0.65) (Figure 3.2C). However, the addition of HD5 resulted in decreased colocalization of the vial genome and the nucleus (median nuclear signal ratio: 0.25), implying sequestration of the genome outside of the nucleus. Therefore, although HD5-treated fcHPV16 enters the early endosome with the same kinetics as untreated virus, it has decreased nuclear entry and cannot complete the viral infection.

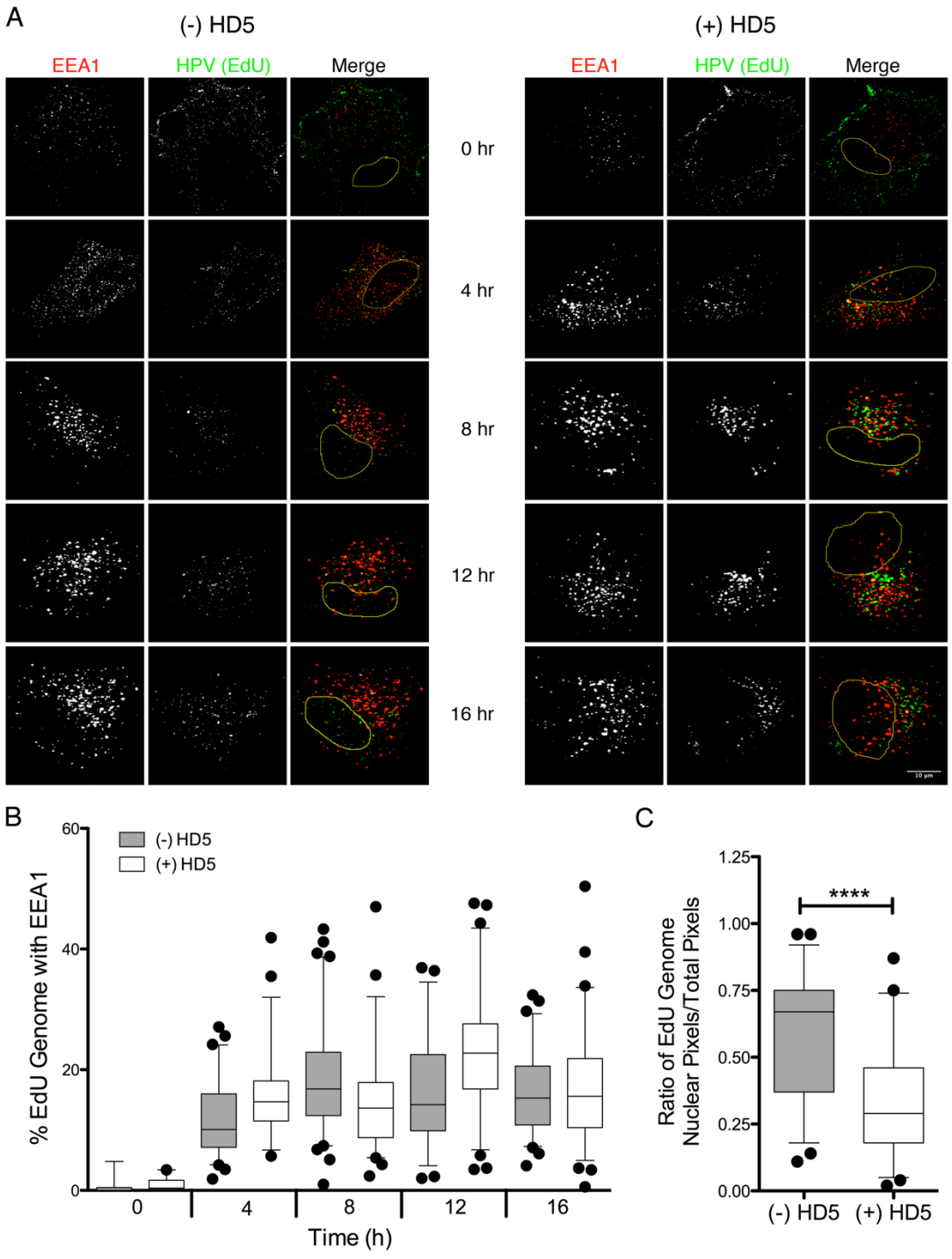

Figure 3.2 fcHPV16 traffics to the early endosome after HD5 treatment

(A) Images of cells costained for EEA1 and the EdU labeled fcHPV16 genome at the indicated time points post infection in the presence (+) or absence (-) of HD5. Individual panels depict

signal above threshold for images in the z-stack that are co-planar with the nucleus for EEA1 (red) and fcHPV16 EdU genome (green). In the merged images, the nucleus is outlined in yellow. Scale bar is 10 μm. (B) Manders coefficient values M1 (fraction of EdU genome colocalized with EEA1) are plotted as a percent for 40-60 cells for each condition. (C) HD5 blocks nuclear colocalization of the fcHPV16 genome. The ratio of EdU pixels in the nucleus to total EdU pixels in the cell is plotted at 16 h p.i. for 40-60 cells. Cell and nuclei borders were determined by a bright field image. (B) and (C) Whiskers are 5-95%, the horizontal line is the median, and outliers are depicted as individual points. ****, $p<0.0001$.

To confirm that HD5 still neutralized the fcHPV16 PsV in these assays, I repeated the HD5 neutralization studies by binding WT and fcHPV16 PsV to HeLa cells for 1 hr at 4°C. Unbound virus was washed off and 0 μM or 10 μM HD5 in complete media was added for an additional hour. Samples were shifted to 37°C for ~40 hours and eFGP reporter genome expression was quantified (Figure 3.1B). HD5 neutralization was determined by comparison to the 0 μM HD5 sample. 10 μM HD5 neutralized both WT and fc HPV16 PsV in complete media.

3.2.3 HD5 inhibits L1 and viral genome dissociation and uncoating

HD5 is known to block the uncoating of other non-enveloped viruses, specifically HAdV and JC PyV. In both cases, this HD5-mediated inhibition of uncoating is believed to be the major mechanism of neutralization. However, an initial study found that HPV16 still uncoated after treatment by both HD5 and HNP1 based on exposure of a BrdU epitope in the HPV16 genome [52]. BrdU is a thymidine analogue, similar to EdU, that allows for antibody staining of DNA. To confirm this finding, I assessed the uncoating of fcHPV16 EdU after HD5 or NH_4Cl treatment. NH_4Cl was used as a negative control as it is a weak base that inhibits endosome acidification and is known to inhibit viral uncoating and HPV16 infection. HeLa cells were infected with fcHPV16 EdU in the presence of 10 μM HD5, 20 μM NH_4Cl, both inhibitors, or no inhibitor. Samples were fixed at 1 or 6 h p.i for immunofluorescence (Figure 3.3A). The alkyne substrate in the EdU reagent buffer is a small molecule and can pass through the virus capsid even before uncoating. Therefore, to assess uncoating I used an HPV L1 antibody 33L1-7 that has previously been used as a marker for HPV16 uncoating [185,222,246,249]. The 33L1-7 epitope is on the internal facet of L1 and is only accessible after viral uncoating [246,250]. ClickIT chemistry required for EdU staining is also known to induce conformational changes in HPV that can result in exposure of the 33L1-7 epitope [246].

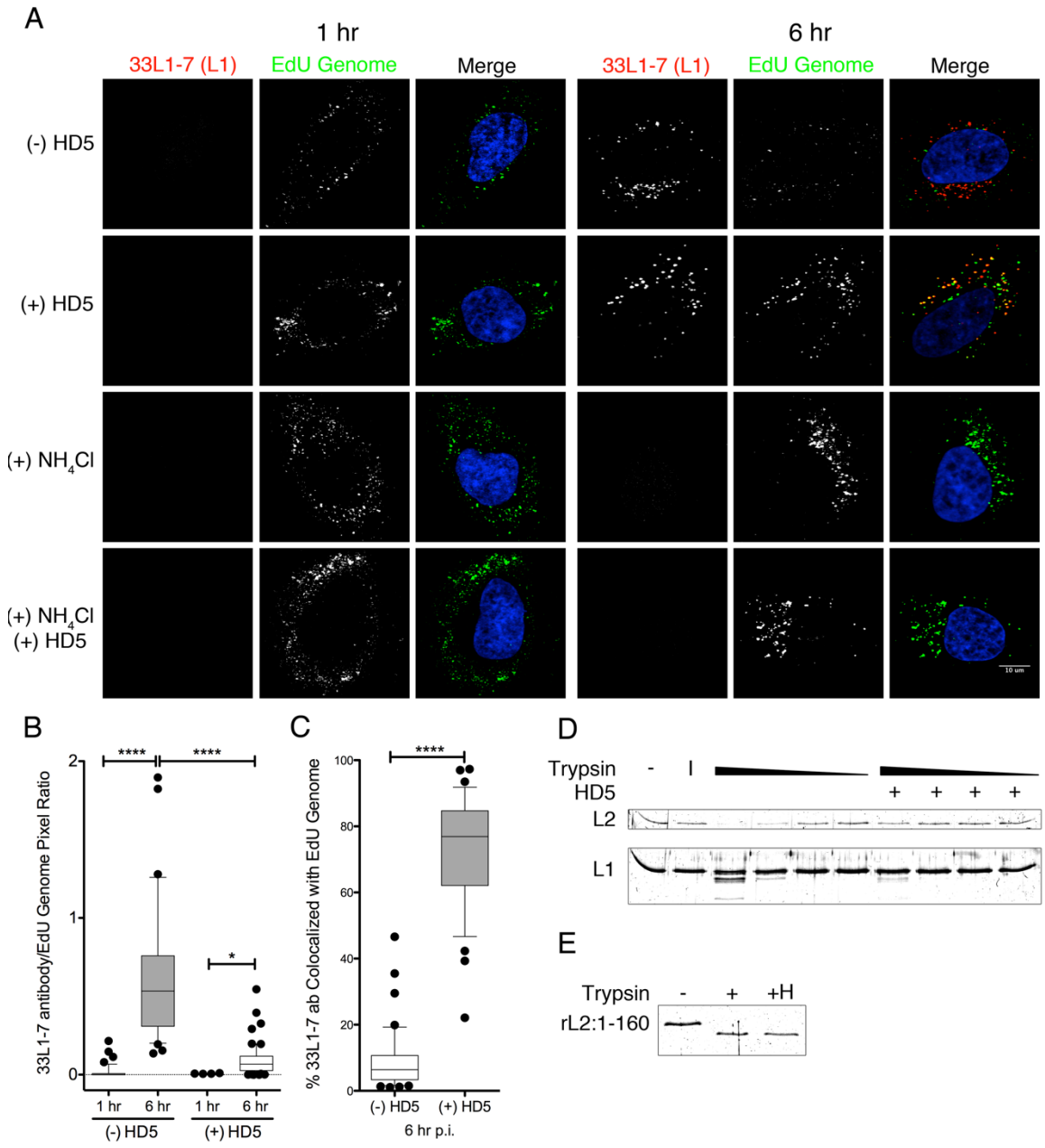

Figure 3.3 HD5 inhibits L1 and viral genome dissociation

(A) Images of cells costained for L1 (33L1-7 antibody) and the EdU labeled fcHPV16 genome at the indicated time points post infection in the presence of no inhibitor, 10 µM HD5, 20 µM NH4Cl or both 10 µM HD5 and 20 µM NH4Cl. Individual panels depict signal above threshold for images in the z-stack that are co-planar with the nucleus for L1 (red) and fcHPV16 EdU genome (green). In the merged images, the nucleus is blue. Scale bar is 10 µm. (B) The amount of uncoated L1 is plotted as the ratio of the pixel area of 33L1-7 staining divided by the pixel

area of EdU genome staining for 40-60 cells for each condition at 6 h p.i. (C) Manders coefficient values M1 (L1 colocalized with EdU genome) are plotted as a percent for 40-60 cells for each condition at 6 h p.i. (B) and (C) Whiskers are 5-95%, the horizontal line is the median, and outliers are depicted as individual points. ****, $p<0.0001$. (D) HD5 protects the HPV16 capsid from trypsin degradation. Purified HPV16 PsV was digested with 4-fold increasing amounts of trypsin in the presence of trypsin inhibitor (I), 10 μM HD5, or no inhibitor. The highest concentration of trypsin used was added to the trypsin inhibitor sample. (E) HD5 does not affect trypsin enzymatic activity. 50 ng rL2:1-160 was digested with trypsin in the presence or absence of 10 μM HD5. (D) and (E) Samples were separated via SDS-PAGE and total protein stained using SYPRO Ruby.

Accordingly, all samples were stained with 33L1-7 and a fluorescent secondary antibody before EdU staining. TOPRO-3 was used to visualize the nucleus (Figure 3.3B). There is no antibody staining at 1 h p.i. in all samples as expected, as HPV16 is not in an acidified cellular compartment at that time to induce capsid uncoating. To quantify the amount of uncoating, I measured the area of EdU genome pixels and 33L1-7 antibody pixels in ImageJ in the untreated and HD5 treated samples and graphed the ratio of 33L1-7 pixels/EdU genome pixels for at least 40 cells in each condition. This quantification allowed me to normalize the amount of L1 staining to input viral genome signal. As expected, in the untreated sample the virus uncoats at 6 hours as indicated by the 33L1-7 antibody staining (antibody to genome pixels ratio median: 0.5). Addition of NH_4Cl resulted in no antibody staining, indicating no capsid uncoating, regardless of the addition of HD5. HD5 treated samples still had 33L1-7 staining. However, there is less antibody staining compared to the untreated sample (antibody to genome pixels ratio median: 0.1), indicating that HD5 affected the amount of L1 antibody epitope exposure, suggesting a decrease in capsid uncoating.

During infection, L1 and the genome dissociate after uncoating and the L1 remains in the endosomal system. As L1 and genome dissociation is a necessary step in the viral entry pathway, I next assessed if HD5 inhibited this separation. Confocal z-series images were analyzed for colocalization of the viral genome and L1 using ImageJ. As well as decrease the amount of 33L1-7 epitope exposed, HD5 also inhibited the dissociation of the EdU genome and L1 (Figure 3.3C). L1 and the HPV genome dissociate at 6 hr p.i. in the absence of HD5 (median: 5%), but remain colocalized after HD5 treatment (median: 75%), indicating that L1 and the viral genome do not completely dissociate after HD5 treatment.

The decrease in 33L1-7 epitope exposure and the inhibition of L1 and genome dissociation suggest that HD5 may stabilize the capsid via direct interaction. To confirm that HD5 treatment affected the integrity of the HPV16 capsid, I next assessed the ability of trypsin to degrade the virus in the presence or absence of HD5. Purified HPV16 PsV was incubated with 0 μM HD5, or 10 μM HD5, or trypsin inhibitor on ice for 45 minutes, then decreasing amounts of trypsin were added, and the samples were incubated at 37°C for 15 minutes. Cleavage of the HPV16 capsid proteins was assessed via SDS-PAGE and SYPRO Ruby staining (Figure 3.3D). Addition of the trypsin inhibitor protected both L1 and L2 from degradation, while the untreated virus was degraded. Interestingly, the addition of HD5 protected the virus capsid from degradation, as seen by retention of full length L2 even in the highest amount of trypsin. As a control to make sure that HD5 did not affect the trypsin enzymatic activity, a recombinant L2 peptide comprising the first 160 residues of L2, rL2:1-160, was incubated with or without HD5 and trypsin and assessed for cleavage in a parallel experiment (Figure 3.3E). Addition of HD5 did not affect the ability of trypsin to cleave the rL2:1-160 peptide. Although this experiment was performed using purified HPV16 PsV and not in the context of infection, it does suggest that HD5 binds to and stabilizes the HPV16 capsid. Taken together with the uncoating data, these experiments suggest that HD5 does not allow complete uncoating and dissociation of the HPV16 capsid proteins during entry, a step that is required for viral infection.

3.2.4 HD5 does not interfere with L2 interacting with the viral genome

L2 remains bound to the genome during infection. Without L2 interactions with cellular proteins, the viral genome cannot traffic to the *trans*-Golgi network and the virus is non-infectious [215,216]. I next assessed if HD5 alters the interaction of the viral genome and L2. I used HPV16 L2-FLAG EdU PsV, an uncleaved EdU labeled PsV in which the L2 has a carboxyl

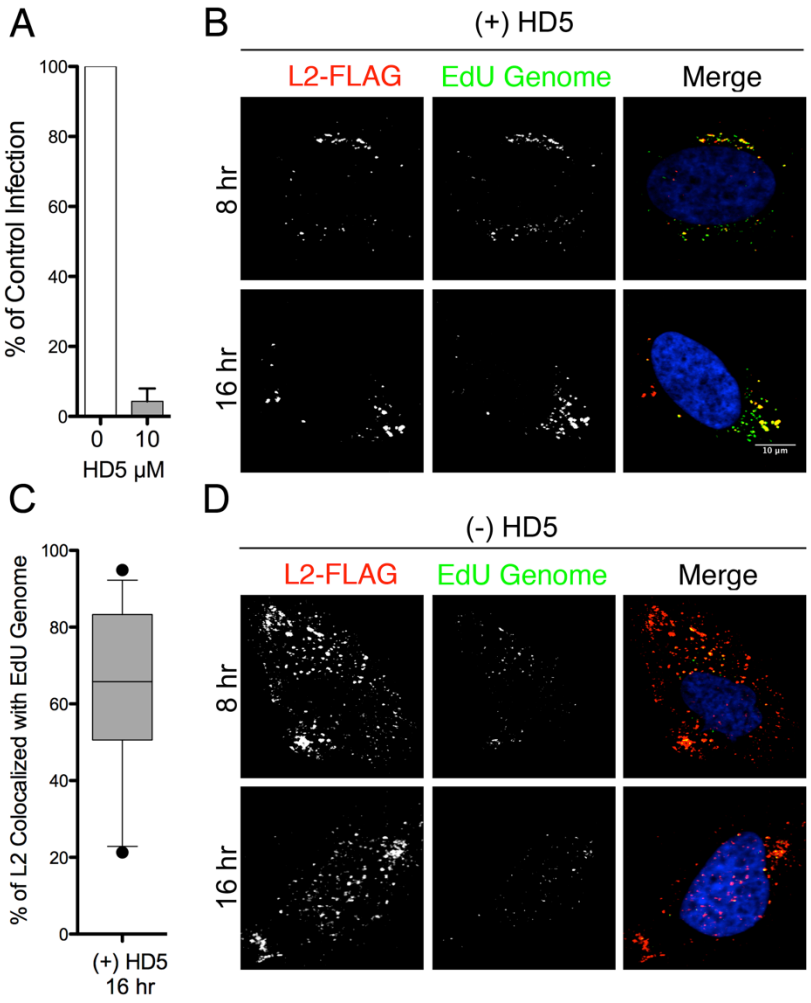

Figure 3.4 HD5 does not induce dissociation of L2 and the viral genome

(A) WT HPV16 L2-FLAG is sensitive to HD5. WT HPV16 L2-FLAG PsV was incubated with HeLa cells in complete media. After unbound virus was washed off, 0 or 10 µM HD5 was added in complete media. Data is three independent experiments normalized to infection in the absence of inhibitor, ± SD. (B) and (D) Images of cells costained for L2-FLAG and the EdU labeled HPV16 L2-FLAG genome at the indicated time points post infection in the presence of 10 µM HD5 (B) or 0 µM HD5 (D). Individual panels depict signal above threshold for images in the z-stack that are co-planar with the nucleus for L2-FLAG (red) and HPV16 EdU genome (green). In the merged images, the nucleus is blue. Scale bar is 10 µm. (C) Manders coefficient values M1 (L2 colocalized with EdU genome) are plotted as a percent for 30 cells at 16 h p.i. after 10 µM HD5 treatment. Whiskers are 5-95%, the horizontal line is the median, and outliers are depicted as individual points.

terminal FLAG tag. This tag has been shown to be accessible before uncoating, allowing for the visualization of L2 via immunofluorescence at early time points during infection [223]. I first repeated the HD5 neutralization assay to confirm that the FLAG-L2 PsV is still sensitive to HD5 (Figure 3.4A). Then, HPV16 L2-FLAG EdU PsV was bound to cells for 1 hour at 4°C, unbound virus was washed off, and the samples were incubated with or without 10 μM HD5 for an additional hour before shifting to 37°C to allow virus internalization. Samples were fixed at 8 and 16 h p.i. and stained for L2 and the EdU genome (Figure 3.4B). Confocal z-series images of the HD5 treated samples were analyzed for colocalization of the viral genome and L2 using ImageJ. However, the HD5 treated samples lost L2 staining dramatically at 8 and 16 hours compared to the untreated samples. Due to this loss of staining, the colocalization of L2 and genome in the HD5 treated and untreated samples cannot be compared, as there is not equal L2 signal in both samples. Despite this limitation, L2 and genome remain colocalized to each other at 16 h p.i. even after HD5 treatment (Figure 3.4C). Samples infected for 8 or 16 h p.i. in the absence of HD5 are shown for comparison of L2 staining and genome colocalization (Figure3.4D). Therefore, HD5 does not induce the dissociation of L2 and the viral genome. Combined with my previous data showing that HD5 inhibits L1 and genome dissociation, this data suggests that HD5 inhibits the separation of the viral capsid during entry. Therefore, L1 and L2 likely remain associated with each other and the genome during viral entry.

3.2.5 HD5 directs the viral genome to the lysosome

Although HD5 did not appear to alter the early steps in viral entry, the viral genome has reduced nuclear localization after treatment with HD5, indicating that the genome trafficking was altered. The lysosome is the last step in the endosomal pathway, so if HD5 affected

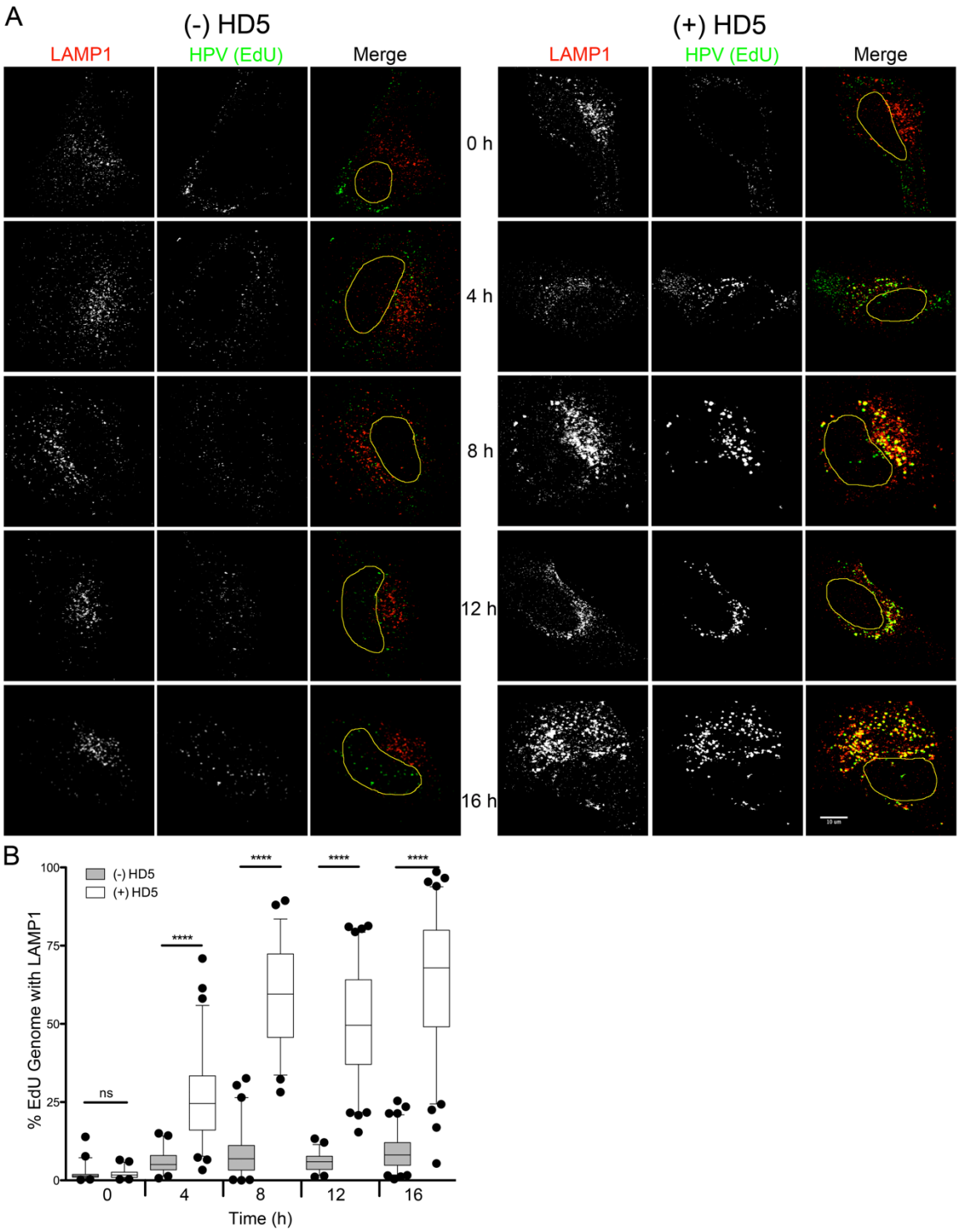

Figure 3.5 HD5 increases the lysosomal colocalization of fcHPV16 genome

(A) Images of cells costained for LAMP1 and the EdU labeled fcHPV16 genome at the indicated time points post infection in the presence (+) or absence (-) of HD5. Individual panels depict signal above threshold for images in the z-stack that are co-planar with the nucleus for EEA1 (red) and fcHPV16 EdU genome (green). In the merged images, the nucleus is outlined in

yellow. Scale bar is 10μm. (B) Manders coefficient values M1 (EdU genome colocalized with LAMP1) are plotted as a percent for 40-60 cells for each condition. Whiskers are 5-95%, the horizontal line is the median, and outliers are depicted as individual points. ****, $p<0.0001$.

endosomal exit of HPV16, the virus would traffic to the lysosome. To test this hypothesis, I infected cells with EdU labeled fcHPV16 PsV in the presence and absence of HD5 and stained the cells for LAMP1, a marker of the lysosome (Figure 3.5A). As early as 4 hours post infection the HD5-treated virus genome was colocalized with the lysosome (median: 25%), and this colocalization increased to 70% of the viral genome by 16 h p.i. (Figure 3.4B). In contrast, the lysosomal colocalization of the untreated samples remained low (median: 1-8%). Routing of the fcHPV16 genome to the lysosome in the presence of HD5 may be the major antiviral mechanism, as the viral genome is sequestered in the lysosome and can never travel to the nucleus to express viral genes.

3.2.6 HD5 treatment results in increased L1 and L2 degradation

As an alternative approach to examine the effect of HD5 on HPV during entry, I assessed the stability of L1 and L2 during infection with and without HD5 via immunoblot. HeLa cells were infected with fcHPV16 EdU PsV in the presence of various inhibitors and harvested at 0, 6, and 8 h p.i and assayed by immunoblot for L1. An anti-β actin antibody was used as a loading control. The addition of 10 μM HD5 resulted in increased L1 degradation at both 6 and 8 h p.i (Figure 3.6A). This degradation is consistent with the observation that HD5 decreased the dissociation of L1 and the genome and increased the localization of the viral genome to lysosome, suggesting that L1 also travels to the lysosome after HD5 treatment. To determine which cellular proteases may be involved in the degradation of HPV16 after HD5 treatment, I used two different protease inhibitors with and without HD5. NH_4Cl inhibits pH-dependent proteases in the lysosome, while MG132 is a proteasome inhibitor. 20 μM NH_4Cl protected L1 from degradation, but the addition of HD5 still resulted in increased L1 degradation at both 6 and 8 h p.i. Similar results were seen with 5 μM MG132 with and without HD5, where the addition

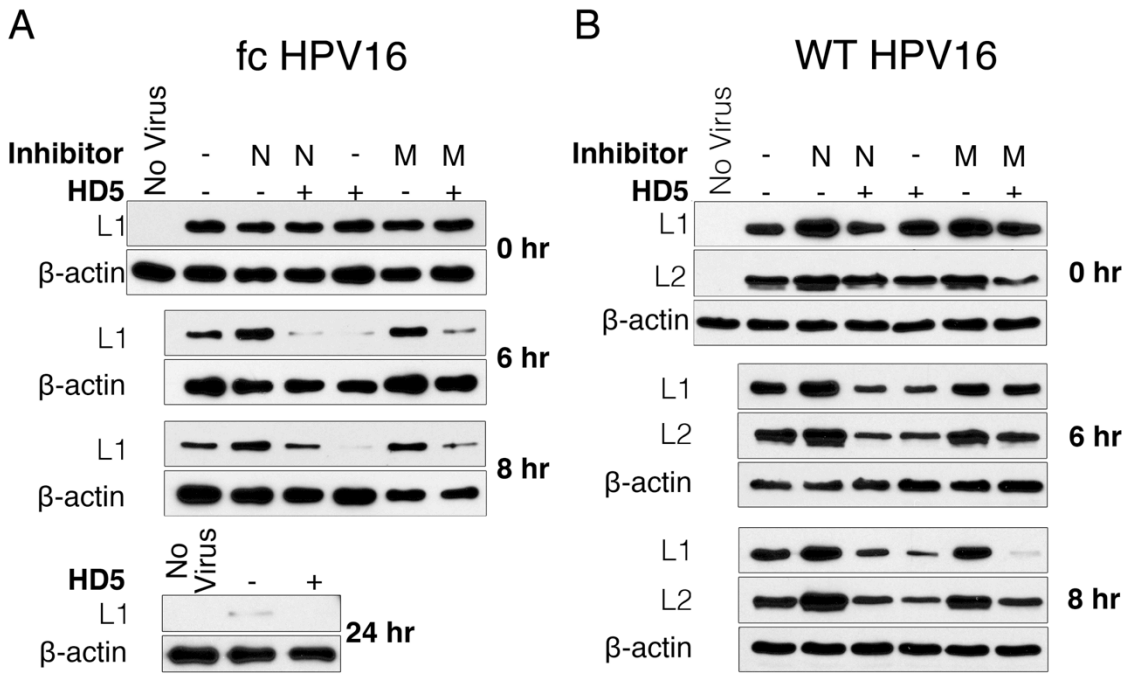

Figure 3.6 HD5 treatment increases the degradation of fcHPV16 and WT HPV16 capsid proteins

(A) HeLa cells were infected with fcHPV16 or WT HPV16 MycL2-HA PsV with no inhibitor (-), NH_4Cl (N), 20 μM NH_4Cl and 10 μM HD5 (N+), 10 μM HD5 (+), 5 μM MG132 (M) or 5 μM MG132 and 10 μM HD5 (M+). Samples were harvested with NP40 lysis buffer at the indicated times. L1 and L2 protein degradation was assessed via immunoblot of cell lysates using anti-camvir (L1) and anti-HA (L2) antibodies. Anti-β-actin antibody was used as a loading control.

of HD5 resulted in increased L1 degradation. Therefore, the HD5-mediated degradation is not due to proteases in the proteasome or pH-dependent proteases in the lysosome.

My immunofluorescence data suggests that HD5 treatment inhibits the dissociation of L1 and L2 during infection. Since L1, but not L2, is normally degraded in the lysosome during infection, I next assessed if HD5 treatment also resulted in increased L2 degradation. To assess L2 degradation I repeated the assay using WT HPV Myc-16L2-HA PsV (Figure 3.6B). As before, I observed the same increased degradation of L2 in the samples with HD5. In addition, I assessed L1 degradation to confirm that the previous degradation was not an artifact of the fcHPV16 PsV system. As with the fcHPV16 PsV, the WT L1 was also degraded after HD5 treatment. L1 and L2 degradation is consistent with increased lysosomal trafficking of the HPV16 particle to the lysosome after HD5 treatment. In addition, this degradation is consistent the observation that HD5 decreased L1 and L2 staining in immunofluorescence.

3.2.7 HD5 blocks the phosphorylation of FAK during infection

HPV16 has been shown to induce phosphorylation of FAK at early times (<30 min) during infection [204,206]. This phosphorylation is required for viral infection, as siRNA knockdown of FAK and addition of FAK inhibitors both decrease HPV infection. Since HD5 interacts directly with the HPV capsid at the cell surface, I hypothesized that the defensin may interfere with the virus-mediated induction of cellular signaling pathways such as FAK. I next assessed if HD5 treatment affected the induction of FAK phosphorylation (Figure 3.7A). FcHPV16 PsV was incubated with HeLa cells for 1 h at 4°C. Unbound virus was washed off and media containing 0 μM or10 μM HD5 was added for an additional hour. 0 h time point samples were collected in NP40 buffer and the samples shifted to 37°C for 15 or 30 minutes. Samples assayed via

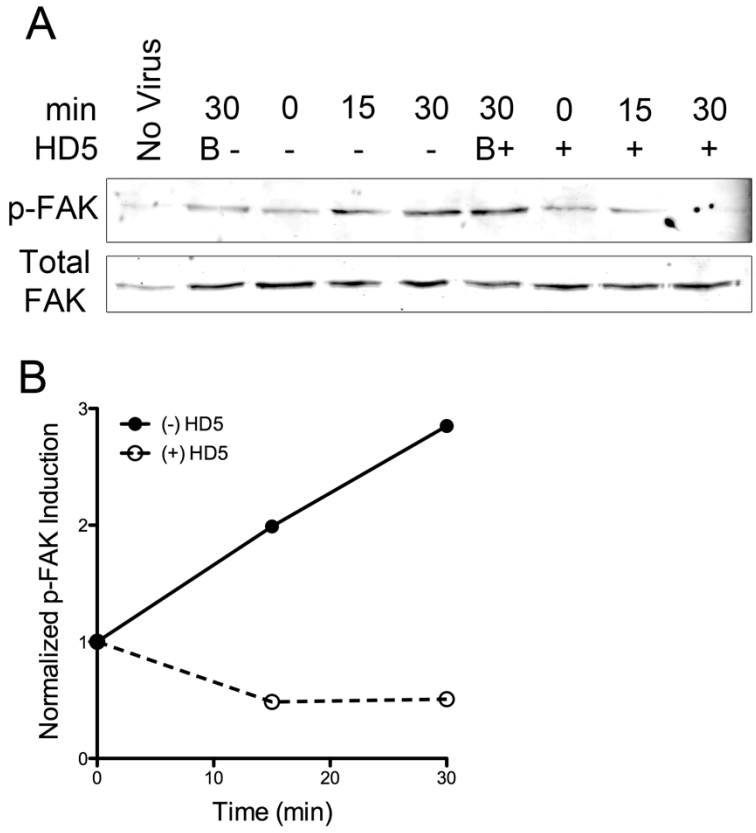

Figure 3.7 HD5 blocks the phosphorylation of FAK during fcHPV16 infection

(A) HeLa cells were infected with fcHPV16 PsV with no inhibitor (-) or 10 μM HD5. 10 nm bombesin was added to one (-) HD5 (B-) and one (+) HD5 (B+) sample as a control for FAK activation. Samples were harvested with NP40 lysis buffer at the indicated times and FAK phosphorylation assessed via immunoblot of cell lysates using anti-p-FAK (Y397) and total FAK antibodies. (B) The ratio of p-FAK signal to total FAK signal normalized to the 0 min time point of either the (-) HD5 samples or (+) HD5 samples. The bombesin signal is not graphed.

immunoblot for p-FAK (Y397) or total FAK (Figure 3.7A). As previously described, HPV16 quickly induced FAK phosphorylation, with increased p-FAK staining at 15 min p.i. and 30 min p.i. However, HD5 treatment completely abrogated the phosphorylation of FAK during infection. This decrease in FAK induction may be due to either the defensin blocking HPV from interacting with the FAK receptor, or by the defensin directly modulating the activation of the FAK pathway. To distinguish between these two possibilities I added a FAK agonist, bombesin, to one HD5 treated or untreated sample for 30 minutes. The addition of bombesin to the HD5 treated sample still induced FAK phosphorylation, indicating that HD5 is affecting HPV16 induction of the FAK pathway, and not directly inhibiting the cellular pathway. To quantify the amount of phosphorylation, I quantified the ratio of p-FAK to total FAK for each sample and normalized them to the 0 min ratio of either the HD5 untreated or treated sample (Figure 3.7B).

3.3 Discussion

My data provides a further elucidation of the mechanism by which the α-defensin HD5 inhibits HPV16. My previous work showed that HD5 inhibits the cleavage of L2, a critical step in the HPV16 entry pathway at the cell surface [53]. Although loss of this cleavage is known to have deleterious effects on HPV infection, I found that virus particles with cleaved L2 (fcHPV16) are still sensitive to HD5[187,188]. Upon examining the effect of HD5 on the intracellular trafficking of HPV16 I found that HD5 did not alter virus entry via the early endosome, an early step in infection. However, HD5 treatment did decrease the amount of HPV16 uncoating, and, more strikingly, result in a dramatic trafficking of the virus to the lysosome. In agreement with trafficking of the viral genome to the lysosome, the viral capsid proteins were degraded faster after HD5 treatment in an immunoblot assay. Additionally, L2 protein staining was greatly decreased in an immunofluorescence assay. Taken together, this data

indicates that HD5 redirects the viral capsid to lysosome during infection where the viral proteins are degraded.

My 33L1-7 antibody staining data is in contrast to the previous finding that HD5 did not affect HPV16 capsid uncoating [52]. The differences in these findings may be due to use of different reagents to assess capsid uncoating; the BrdU staining protocol typically requires a denaturing step to expose the BrdU epitope in the labeled DNA, a step which can denature proteins as well. In my data the NH_4Cl treated samples function as a control for the EdU staining procedure, as there is no 33L1-7 binding in those samples although there is EdU genome staining. Therefore, the decreased 33L1-7/EdU staining ratio in the HD5 treated sample is likely due to differences in the 33L1-7 epitope accessibility during infection and not an artifact of sample processing. It is also important to note that 33L1-7 staining is an indirect proxy for HPV16 uncoating. Although the epitope is on the bottom of L1, and thus the inside of the viral capsid, it is unclear how much epitope exposure truly reflects complete capsid uncoating [246,250]. It is possible that the epitope is accessible to the 33L1-7 antibody before L1 and L2 fully dissociate. Therefore, the 33L1-7 staining in the HD5 treated sample in the uncoating assay may reflect partial or incomplete uncoating of the capsid. Alternatively, the 33L1-7 antibody staining in the HD5 treated sample may be due to exposure of the epitope during lysosomal degradation. The uncoating immunofluorescence assay was performed 6 h p.i., as that is when HPV16 has reached an acidified endosomal compartment. However, the lysosomal colocalization assay shows that HD5 treatment results in 25% of HPV16 genome colocalized with the lysosome at 4 h p.i., increasing to 60% at 8 h p.i. Therefore, a significant amount of HPV16 is already in the lysosome at 6 h p.i. and being degraded. And, the immunoblot of HPV16 proteins shows a dramatic loss of both L1 and L2 at 6 h p.i. Because of protein degradation, it is possible that not

all of the 33L1-7 staining in the HD5 treated sample is due to capsid uncoating in the endosome, but rather due to staining of partially degraded capsids.

In general, I note a discrepancy between capsids that are positive for both L1 and genome, and L1 staining without EdU staining or vice versa. This may be due to multiple reasons based on the biology of the EdU labeling of HPV genome and technical limitations of fluorescent microscopy in general. 1) Not every capsid has encapsidated EdU labeled DNA, in fact empty HPV particles are believed to outnumber DNA filled particles by ten or twenty to one. In addition, not all of the DNA may contain an EdU nucleotide. So there may be a large number of viral particles that stain for L1 but not genome. 2) The EdU staining reagent is small enough to pass through the HPV capsid before uncoating, as confirmed by EdU staining of untreated samples at 0 or 1 h p.i. and the NH_4Cl treated samples. Therefore some EdU signal in the uncoated samples is independent of HPV16 uncoating. 3) There is a limit of detection in fluorescent microscopy, so it is possible that there is genome or L1 signal in the cells that is not detectable. And 4) although I attempted to synchronize the HPV16 PsV infections by prebinding virus at 4°C, the kinetics of entry are slow and may become asynchronous quickly, with some virus particles reaching the acidified endosome and dissociating before other particles. Therefore, some of the EdU signal may be genome that has already trafficked out of the endosome towards the *trans*-Golgi network.

Pilot studies with HD5 and HPV16 found that HD5 treatment increased the amount of virus that entered cells in immunofluorescence assays, probably due to HD5-induced aggregation of HPV16. Therefore, to account for this increase in binding I used 3-fold less virus in the HD5 treated samples for the immunofluorescence assays. This aggregation may also result in more

viral particles in any given cellular compartment, which could explain the stronger EdU staining signal in the HD5 treated samples as more EdU signal is above the microscope limit of detection.

My previous work showed that HD5 binds directly to HPV16 via an indirect assay: by using dynamic light scattering I showed that HD5 caused the virus to aggregate [53]. The trypsin protection assay confirms this finding. The addition of HD5, which is resistant to trypsin, protected both L1 and L2 from degradation, indicating that the defensin physically blocked the trypsin cleavage sites on the viral capsid. However, HD5 did not affect the enzymatic activity of trypsin, as a purified recombinant peptide of L2, rL2:1-160, was still sensitive to trypsin digest in the presence of HD5. Taken together with my previous data this further confirms that HD5 interacts directly with the HPV16 capsid. Also, my L1 and genome colocalization and L2 and genome colocalization data suggest that L1 and L2 do not dissociate after HD5 treatment. Therefore, HD5 binding may result in increased capsid stability and decreased capsid uncoating.

In addition to altering the viral internalization pathway, HD5 may also interfere with HPV16-mediated induction of cellular signaling pathways. HPV16 requires FAK activation for infection, although its function in the HPV lifecycle is unclear [204]. HD5 inhibits phosphorylation of FAK by HPV16, but not by a FAK agonist, bombesin. Therefore, HD5 likely does not directly suppress FAK signaling but rather blocks the interaction of HPV16 with FAK that results in induction. Furthermore, another tyrosine kinase, Pyk2, is required for HPV16 retention in the endosomal system [206]. The effect HD5 has on activation of Pyk2 activation during HPV16 is unknown. Further studies to elucidate the effect HD5 has on the cellular signaling required for HPV16 infection is warranted.

Combined with my previous work I speculate that HD5 interacts directly with the HPV16 capsid in the extracellular milieu, resulting in both steric hindrance of the furin enzyme to block the cleavage of L2, and stabilization of the capsid such that L2 and the genome cannot be released from L1 in the endosome. The HD5-bound capsid may be able to undergo a limited conformational change that allow for exposure of an epitope inside the capsid, but not enough to release L2. Due to this stabilized capsid, L2 cannot interact with the retromer complex to induce trafficking out of the endosome and to the *trans*-Golgi network. The HD5-HPV16 complex therefore remains in the endosomal system and is directed to the lysosome where the viral proteins are degraded and the viral genome is sequestered. This may explain why HPV16 particles with cleaved L2 are still sensitive to HD5.

Furthermore, this antiviral mechanism is similar to the antiviral activity of HD5 against other non-enveloped viruses. Five non-enveloped viruses are known to be sensitive to HD5; HADV, AAV, BK PyV, JC PyV, and HPV [152]. Of these, only BK PyV does not enter the cell after HD5 binding, rather HD5 induces aggregation of BK PyV that blocks virus binding to the cellular receptor and thus blocks virus entry [54]. HD5 is known to interact directly with the other three viruses, HAdV, JC PyV, and HPV, but still allow the viruses to bind and enter cells during infection [38,51-53,85]. However, the defensin alters their intracellular trafficking. HD5 is known to redirect HAdV to the lysosome for degradation, similar to this study [38,51]. The redirection of JC PyV is still unclear, but the defensin does inhibit trafficking of JC PyV to the endoplasmic reticulum, a step in the viral entry pathway [85]. In addition, HD5 is believed to stabilize both HAdV and JC PyV as the molecular mechanism for the alterations in viral entry [51,85]. The antiviral mechanism against AAV has not been studied. Therefore, the general antiviral mechanism against these diverse non-enveloped viruses appears to be direct interaction that leads

to viral capsid stabilization and redirection of the viral capsid inside the cell. It is very interesting that a small peptide like HD5 can function in a broadly similar way against unrelated non-enveloped viruses. Further work to identify the defensin binding sites on these viruses may lead to a better understanding of the molecular mechanisms of the broad α-defensin neutralization of these diverse pathogens.

Chapter 4. HD5 Sequence and Structure Required for HPV16 Neutralization

4.1 Introduction[††]

I have shown that HD5 binds to HPV16 and redirects the virus to the lysosome for degradation [53]. However, the precise molecular basis for the HD5-HPV16 interaction is still largely unknown. The structural basis of antibacterial activity of α-defensin has been extensively studied [62,251-254]. However, the importance of each physical property of the α-defensin is specific to each bacterial species. For several α-defensins, activity is dependent on structure, hydrophobicity, and charge. This is not surprising as α-defensins have a conserved beta-sheet fold, also shared by β-defensins, which is stabilized by three disulfide bonds [255,256]. α-defensins are also cationic due to multiple positively charged arginine residues. Interestingly, human α-defensins show a strong preference for arginine residues over lysines [252]. HD5 has six arginines, one of which forms a salt bridge with Glu-14, and an invariant glycine shared by all α-defensins at residue 18 to form a β-bulge [8,59,257]. α-defensins have also been shown to form dimers in crystal structures and are believed to form higher order multimers [8,61,254,255]. Using a structure-function approach, I found that neutralization of HPV16 is dependent on HD5 dimerization and high order multimerization and is mediated by critical residues on one face of the HD5 monomer that delineated an interface between the defensin and virus. These results were consistent with HAdV studies performed in our lab in parallel with my analyses.

[††] Adapted from Gounder AP, Wiens ME, Wilson SS, Lu W, Smith JG. Critical Determinants of Human α-Defensin 5 Activity against Non-enveloped Viruses. Journal of Biological Chemistry. 2012 Jul 13;287(29):24554–62 and Tenge VR, Gounder AP, Wiens ME, Lu W, Smith JG. Delineation of interfaces on human alpha-defensins critical for human adenovirus and human papillomavirus inhibition. PLoS Pathog. 2014 Sep;10(9):e1004360. Reprinted with permission.

4.2 Results

4.2.1 HD5 neutralization of HPV16 is residue specific, not charge dependent

To examine the critical charged residues required for antiviral activity, I tested HD5 mutants with individual or pairwise substitutions of arginines to alanine or lysine (Figure 4.1). Four of the six arginines in HD5 are paired on opposite sides of the defensin monomer (Arg-9 and Arg-28, or Arg-13 and Arg-32). The other two arginine residues are either unpaired (Arg-25) or form a salt bridge with Glu-14 (Arg-6). Increasing concentrations of HD5 mutants were incubated with HPV16 PsV on ice before the mixture was added to HeLa cells. Infection was quantified ~40 h p.i. via eGFP reporter gene expression. Wild type HD5 had an IC_{50} between 0.63 and 1.25 μM. Of the single arginine mutants, R28A and R32A had the most deleterious effect, attenuating HD5 by ~4 fold, while R13A and R25A had intermediate antiviral activity, ~2 fold less than wild type HD5. R9A had a minor decrease in antiviral activity compared to wild type HD5. In the pairwise substitutions, Arg-13 and Arg-32 or Arg-9 and Arg-28 were substituted with either lysines, to assess the sequence specificity of HD5 neutralization (R13-32K and R9-28K), or alanine, to assess the role of charge. Interestingly, the R13-32K mutant, which maintains positive charge, retained antiviral activity against HPV16 at the higher concentrations used in the assay, while the similar mutant R9-28K had the least activity of all the mutants against HPV16, with an IC_{50} of more than 5 μM. In both cases, however, the alanine mutants R13-32A and R9-28A were also highly attenuated against HPV16. In total, this data shows that HD5 activity is residue specific and that R28 and R32 are critical residues for the neutralization of HPV16. In addition this interaction is not simply charge-dependent, as the substitution that maintains the positive charge of the peptide, R9-28K, resulted in attenuation of the antiviral activity of HD5.

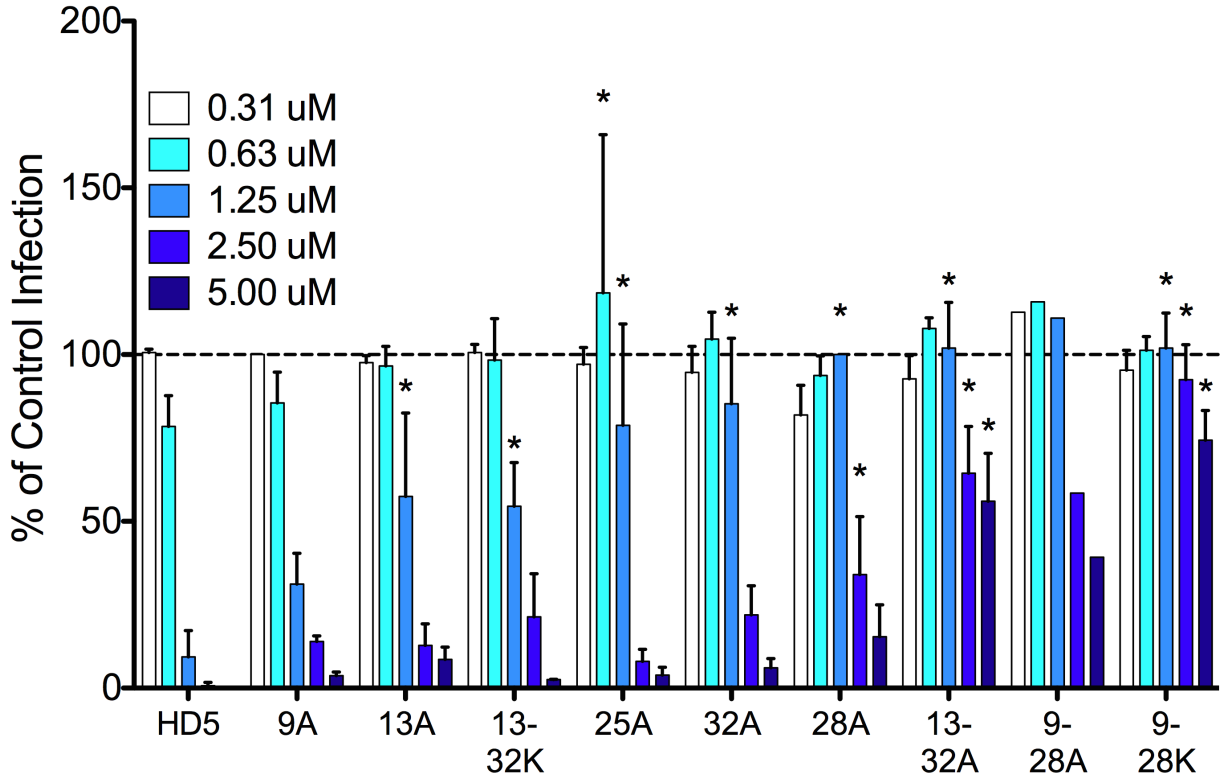

Figure 4.1 Antiviral activity of HD5 Arginine mutants against HPV16

Infection of HeLa cells by HPV16 PsV prebound to the indicated concentrations of WT HD5 or HD5 mutants relative to a control infection in the absence of HD5. Arginine substitutions are labeled by the position of the arginine residue and the single letter code for the substituted amino acid. Double mutants are indicated by a dash. Data are the mean of at least three independent experiments ± S.D. except for R9-28A, which is one replicate, $p<0.05$.

4.2.2 HD5 activity requires a disulfide stabilized structure

Defensins have a very strongly conserved fold that is stabilized by disulfide bonds between the six cysteine residues; a defining characteristic between α- and β-defensins is the order of the disulfide bonds between these cysteine residues [255].To test the requirement of structure on the antiviral activity of HD5 I used an isoform of HD5 in which the cysteines have been replaced by α-aminobutyric acid (HD5 Abu) (Figure 4.2). The resulting peptide can no longer form disulfide bonds, resulting a loss of stabilized secondary structure. At all concentrations tested, HD5 Abu had completely abrogated antiviral activity to HPV16, indicating that a stabilized secondary structure is critical for HD5's antiviral activity. This is consistent with other studies, including viruses and bacteria [152,255].

4.2.3 Hydrophobicity of residue 29 is correlated with antiviral activity

Two previous studies on the antibacterial activity of α-defensins identified Trp-26 in HNP1 and Leu-29 in HD5 as critical residues [62,254]. Based on the crystal structures of both HNP1 and HD5, these residues appear to form a hydrophobic pocket in the dimer interface. To test the importance of hydrophobicity at this residue, I assessed the antiviral activity of HD5 mutants with a variety of natural and artificial side chains at position 29 (Figure 4.3). I found a positive trend between the hydrophobicity of the side chain and antiviral activity. Norleucine (Nle) attenuated HD5 by ~ 2 fold, while both norvaline and α-aminobutyric affected the activity ~4 fold. The alanine substitution had the most deleterious effect, although L29A retained some antiviral activity at the highest concentration (5 μM) tested. Thus, in concordance with the antibacterial studies, hydrophobicity at L29 is required for HD5 antiviral activity.

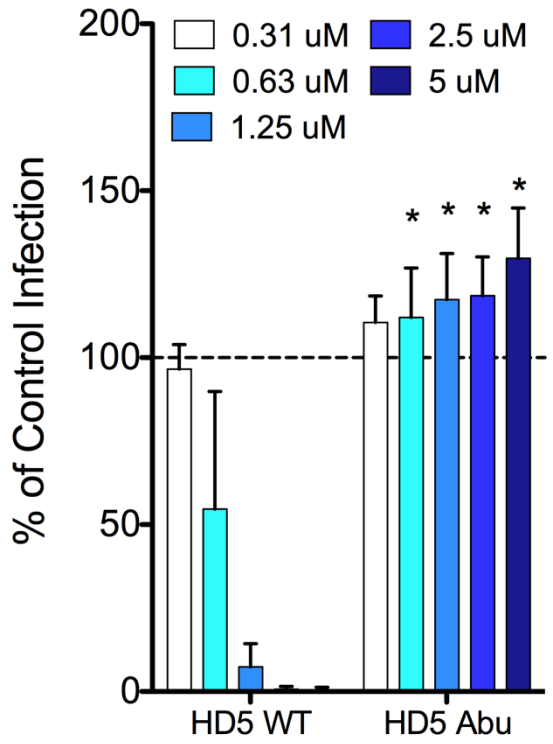

Figure 4.2 HD5 requires a stabilized structure for antiviral activity

Infection of HeLa cells by HPV16 PsV prebound to the indicated concentrations of WT HD5 or HD5 Abu, in which the cysteine residues have been modified to α-amino-n-butyric acid and cannot form disulfide bonds, relative to a control infection in the absence of HD5. Data are the mean of at least three independent experiments ± S.D., $p<0.05$.

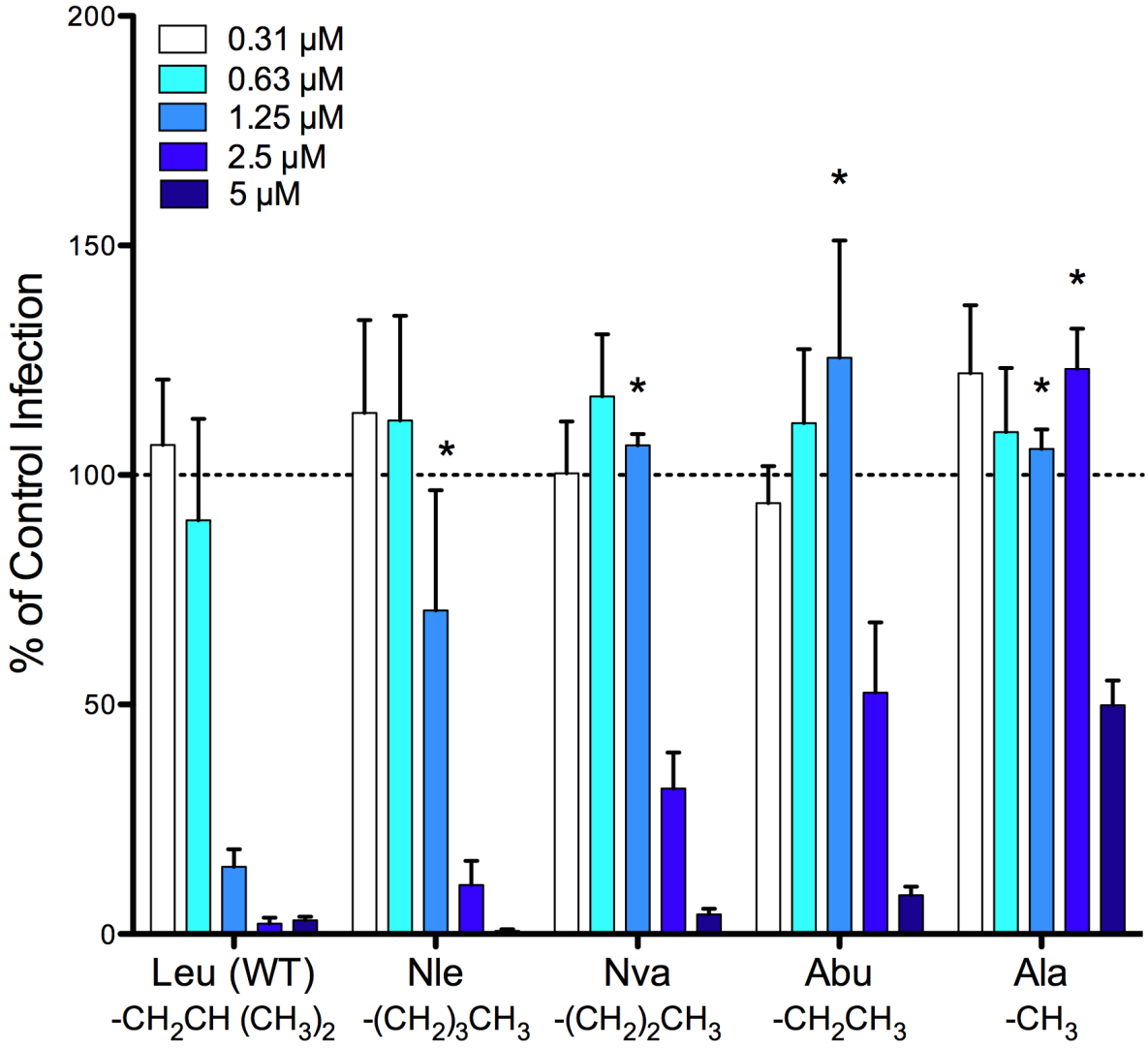

Figure 4.3 Effects of the hydrophobicity of HD5 Leucine 29 on antiviral activity

Infection of HeLa cells by HPV16 PsV prebound to the indicated concentrations of WT HD5 or HD5 Leu-29 mutants relative to a control infection in the absence of HD5. The structure of each side chain is shown. Nle, norleucine; Nva, norvaline; Abu, α-aminobutyric acid. Data are the mean of at least three independent experiments ± S.D., $p<0.05$.

4.2.4 HD5 multimerization is required for antiviral activity

It is known that HD5 forms dimers in solution and higher order multimers after ligand binding [34]. These interactions are mediated by hydrogen bonds between the backbones of residues in the beta sheet of the defensin. *N*-methylation of the peptide backbone at residue 21 (E21me) disrupts this hydrogen bonding [61,254]. HD5 E21me has the same sequence, structure, and charge as wild type HD5, but has been shown to crystalize as a monomer [254]. To test the requirement of dimerization and high order multimerization for HD5 antiviral activity, I repeated the antiviral assay with HD5 E21me and HPV16 PsV and found that HD5 E21me was completely non-functional against HPV16 (figure 4.4). Together with the L29 hydrophobicity data, this indicates that dimerization and multimerization are critical for the antiviral activity of HD5 against HPV16.

4.2.5 Alanine scan of HD5 reveals clustering of critical residues on the HD5 dimer

In conjunction with the alanine scan of the arginines in HD5, I also tested HD5 mutants in which every residue was substituted with alanine, except for the cysteines required for structure, the residues involved in the salt bridge (Arg-6 and Glu-14) and the invariant Gly-18 residue. Most mutations had less than a two-fold effect (figure 4.5A). However, substitution of hydrophobic residues (Ile-22, Leu-16, Leu-26, Leu-29, Val-19) or residues with bulky side chains (Tyr-27) were correlated with ~3-4 fold attenuation of antiviral activity. Interestingly, with the exception of Leu-29, the residues required for viral neutralization are different than the residues important for bacterial killing, implying a distinct method of interaction of the defensin with bacteria and non-enveloped viruses [254].

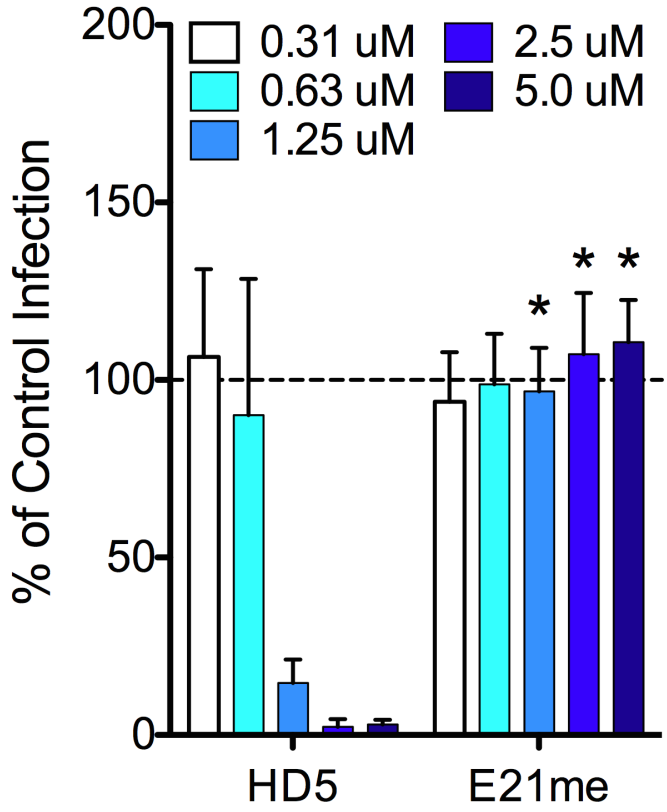

Figure 4.4 Effect of disrupting HD5 multimerization on antiviral activity

Infection of HeLa cells by HPV16 PsV prebound to the indicated concentrations of WT HD5 or HD5 E21Me mutant relative to a control infection in the absence of HD5. Data are the mean of at least three independent experiments ± S.D., $p<0.05$.

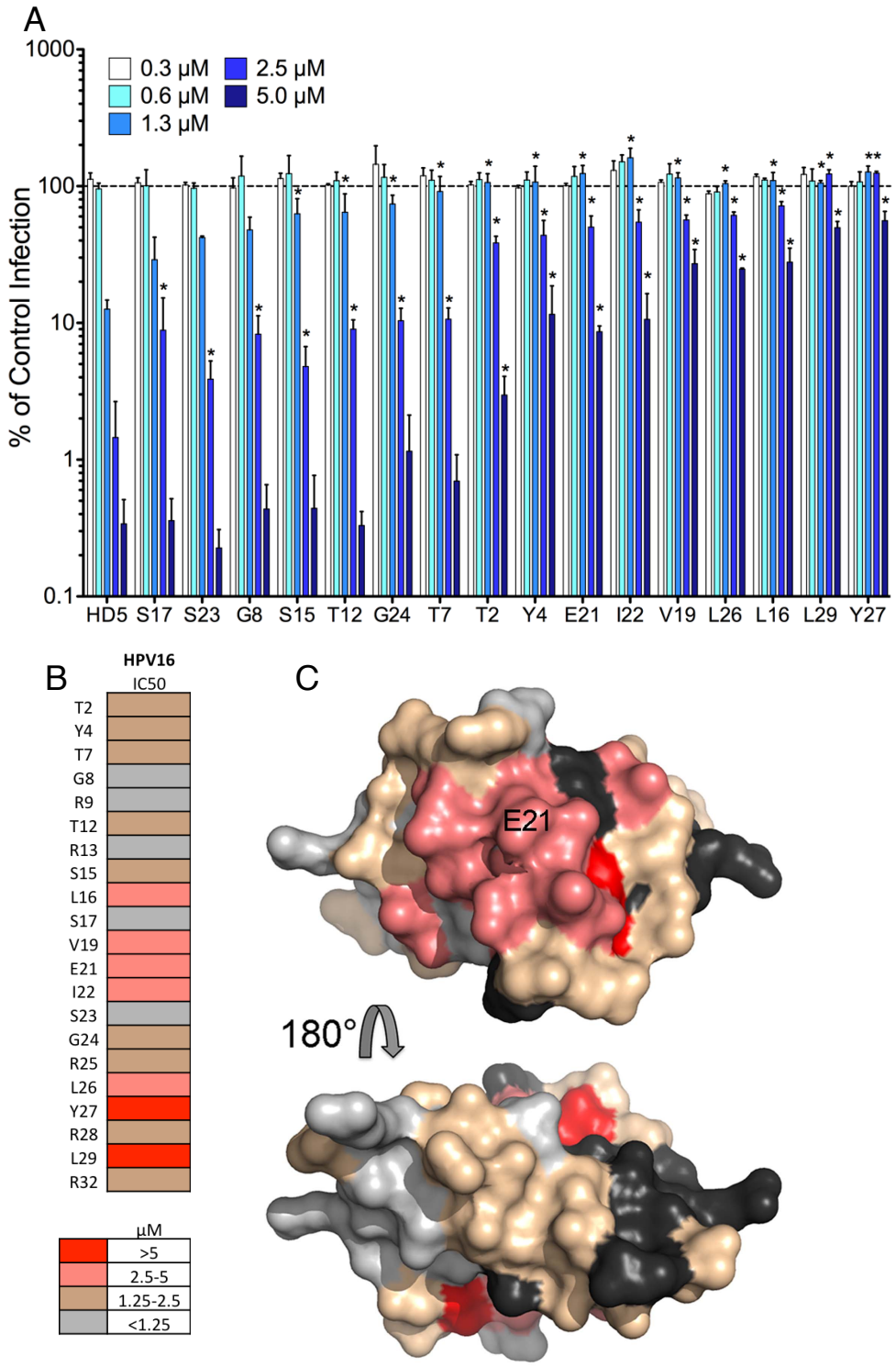

Figure 4.5 Antiviral activity of HD5 alanine scan mutants

(A) Infection of HeLa cells with HPV16 PsV prebound to the indicated concentrations of HD5 WT or HD5 alanine mutants relative to a control infection in the absence of HD5. Alanine was

substituted for HD5 residues that are indicated by position and single letter code. Data are the means of three independent experiments ± S.D., $p<0.05$. (B) Heat map of the effect of alanine substitutions on the IC50 of HD5 against HPV16 PsV according to the color key below. (C) Surface rending of the HD5 dimer (PDB: 1zmp) with residues colored as in B according to their effect on the IC50 of HD5 against HPV16. Black and light grey are equivalent and used to distinguish the HD5 monomers comprising the dimer. Molecular images were created with the PyMOL Molecular Graphics System (Schrödinger, LLC). doi:10.1371/journal.ppat.1004360.g002

When the most critical residues were mapped on a space-filling model of the HD5 dimer, the residues that had the greatest effect on antiviral activity, L16, V19, E21, and L26, are clustered together on one exposed surface of HD5 (Figure 4.5C). The L29 and Y27 residues are both buried in the dimer interface. Mutation of the residues on the other face of the HD5 dimer had minimal effect on antiviral activity.

4.3 Discussion

These studies are part of a larger body of work in our lab identifying the critical determinants of HD5 mediated neutralization of non-enveloped viruses. The initial assumption of defensin method of neutralization was that the interaction of the virus and defensin would be predominantly based on charge. Accordingly, the arginine residues were significant for the antiviral activity, as substitutions of most of these arginines with alanine had modest to significant attenuation on activity. Interestingly, the identity of these residues was also critical, as in some cases pairwise replacement of the arginines with lysine that changed the residue but retained the charge were deleterious to viral inhibition. Therefore, both charge-charge and other non-covalent interactions are required for antiviral activity. It is important to note that the structures of most of the HD5 mutants have been obtained, and the changes in the side chains of the residues have not been shown to change the fold or structure of the defensin [254]. Therefore the changes in antiviral activity between the wild type HD5 and these mutants is unlikely to be due to misfolding of the defensin. In addition, human α-defensins have a strong selective pressure for arginine over lysine, which may reflect the importance of the arginine-mediated interactions with viruses [252]. Arginine and lysine have different capacities to form hydrogen bonds, so the preference for arginine may be due to its ability to be a better hydrogen bond partner, as well as differences in their hydrophobicity [258,259].

The requirement for a secondary structure stabilized by cysteine bonds for antiviral activity is not surprising, as antimicrobial activity against other viruses, as well as bacteria, has previously been shown to be structure-dependent [152,255]. What is surprising is the identification of Leu-29 as a critical residue for the neutralization of HPV. This residue in particular has been shown to be necessary for antibacterial neutralization and lectin activity [254]. However, the HPV capsid is not glycosylated, so the lectin-like activity of HD5 should not be a defining antiviral mechanism against HPV. However, reduction of hydrophobicity at Leu-29 both adversely affected HPV16 neutralization and the ability of HD5 to dimerize in solution [254]. In addition, the arginine to alanine mutations that most affected the ability of HD5 to neutralize HPV16, R13-32A, and R9-28A, also impaired the self-dimerization of HD5 in solution [34]. Therefore, the abilities of the defensin to both bind to the virus capsid and self-associate are critical for viral neutralization. However, because these HD5 mutants had both binding and self-association impairment, separating the importance of each function to HPV16 neutralization is difficult. To address the self-association requirement for antiviral activity, I used the HD5 E21Me mutant. This HD5 mutant has the same primary sequence as wild type HD5, but methylation of the Glu-21 residue results in a monomeric form of HD5 [254]. This HD5 E21Me mutant is completely non-functional against HPV16, indicting that the self-association of HD5 into dimers and higher order monomers is a critical part of the defensin's antiviral activity. In combination with other studies on bacteria and viruses, this data indicates that multimerization of HD5 may be a general feature of HD5 antimicrobial action.

After assessing the role of specific residues in HD5 activity, I determined the role of each residue by testing a complete alanine scan mutagenesis of HD5. In agreement with the previous data showing that hydrophobicity at Leu-29 is important, alanine substitution of other

hydrophobic residues, Ile-22, Leu-16, Leu-26, and Val-19, and Tyr-27 had the most deleterious effect of HD5 activity against HPV16. These residues cluster, along with Glu-21, another critical residue, on one face of the HD5 dimer. Interestingly, many of the same residues were also found to be critical for HAdV neutralization by HD5, suggesting that this patch of residues on HD5 is critical for antiviral activity against non-enveloped viruses [232].

These neutralization studies, along with the dynamic light scattering aggregation data in chapter two, indicate that HD5 directly binds to the HPV16 capsid through specific residues and charge-charge interactions. In addition, the ability of HD5 to self-associate into dimers and higher order multimers is critical for viral neutralization. However, the exact binding region or viral sequence on the HPV16 capsid is still unknown. Unfortunately, I cannot perform comparison studies of sensitive and resistant papillomavirus species, as the Smith lab has done with HAdV, as there are no resistant papillomaviruses known yet. In addition, I have still not determined the capsid protein that HD5 is binding. However, the sensitivity of recombinant L2 to furin and trypsin cleavage in the presence of HD5 in chapters two and three, as well as the fact that the majority of the HPV protein that is surface exposed is L1, suggests that L1 is highly likely to be the capsid subunit that HD5 is interacting with during infection. Identification of the HPV capsid region and sequence that HD5 is interacting with may allow for comparison studies of non-enveloped virus binding determinants.

Chapter 5. Optimization of Sample Preparation for CryoEM studies of HPV16 and HD5

5.1 Introduction

Although there is strong evidence that HD5 interacts directly with HPV16, it is unclear where the defensin binds to the viral capsid. A previous CryoEM study with AdV have defined some of the defensin binding regions on the AdV capsid [38]. This data in turn has informed studies to determine the viral capsid elements that result in resistance or sensitivity to HD5. An ongoing project in the Smith lab is to explore the specific regions of the AdV capsid that confer resistance to HD5 by making chimeric viruses that contain regions of the viral capsid from resistant serotypes. This approach is not possible for HPV as there are no known serotypes of HPV or other non-human papillomavirus species that are resistant to defensins. However, a CryoEM study of HD5 bound to HPV16 is possible and would inform the antiviral mechanism by defining a specific region on the viral capsid to which the defensin binds.

A CryoEM structure of HPV16 has already been determined and a well-established protocol for producing HPV16 capsids for CryoEM studies is publicly available [175]. However, in order for the viral capsids to be stable for CryoEM studies, the virus is made in a high salt buffer (500 mM NaCl). HD5 neutralization of AdV is known to be sensitive to salt content [51]. Therefore, HD5 may not bind to HPV in this high salt buffer. In addition, my collaborators for this project are at a different institution, and so the virus must be shipped over night on ice before CryoEM grids can be prepared. My aim for this project was three-fold: 1) determine if the virus must be shipped in 500 mM NaCl or if it was stable in physiological salt, 2) determine if the virus was still stable after storage on ice, and 3) determine if HPV16 is still sensitive to HD5 at increasing NaCl concentrations.

5.2 Results

5.2.1 HPV must be produced and shipped at high salt concentrations

The HPV16 capsid production protocol optimized by Buck and Cardone first purifies the viral capsids in OptiPrep with 800 mM NaCl, then gel filters the capsids into a DPBS buffer with 500 mM NaCl. This is important for CryoEM samples for two reasons: 1) OptiPrep is optically dense and will adversely affect the imaging of virus and 2) salt affects the freezing of solutions required for CryoEM grid preparation. In addition, increasing the salt concentration of the virus sample buffer may also affect the defensin binding. However, higher salt concentrations can be beneficial for capsid stability and decrease virus particle adsorption to plastic during purification and shipping. As I need to ship my samples to a collaborator at Case Western University, I first assessed if the higher salt concentration was required for shipping. I made a sample of HPV16 capsids and purified it via OptiPrep. I then split the sample and gel filtered half of it into DPBS/ 500 mM NaCl buffer, and the other half into 25 mM Tris-HCl/150 mM NaCl. Gel filtered fractions were screened for DNA content using Picogreen, a fluorescent substrate that binds to DNA. The sample that had been gel filtered into the 25mM Tris/150 mM NaCl buffer had lower viral DNA signal than the sample using the traditional DPBS/500 mM NaCl buffer, suggesting the vral particles had adsorbed to the gel filtration resin or column (Figure 5.1). Samples were shipped to my collaborators for CryoEM analysis. Upon analysis by negative stain electron microscopy the Tris/150 mM NaCl samples appeared to have completely degraded in transit while the DBPS/500 mM NaCl samples still had complete virus capsids (collaborator's data, not shown). Therefore, the higher salt concentration in the DPBS buffer was required for both better virus recovery during gel filtration and capsid stability during shipping.

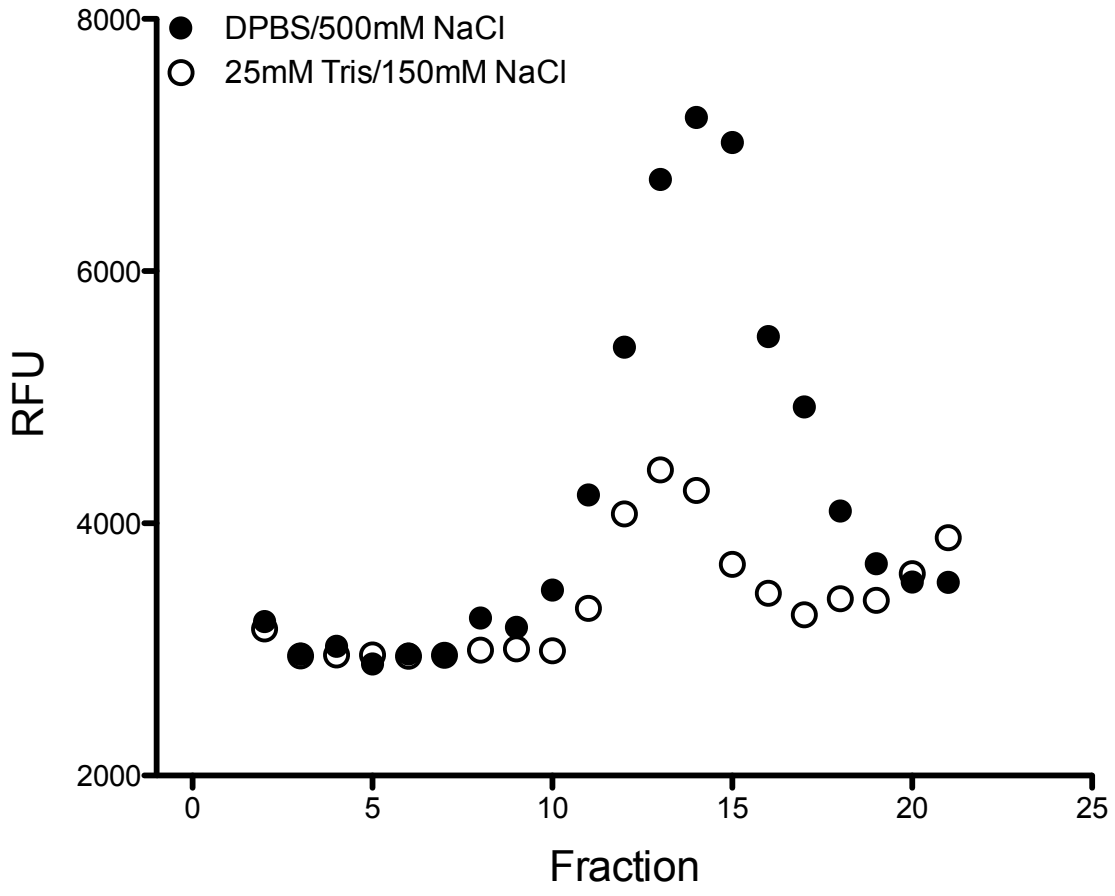

Figure 5.1. Gel filtration into Tris buffer results in lower viral capsid recovery

OptiPrep purified virus was buffer exchanged into either DPBS/500 mM NaCl buffer or 25 mM Tris/150 mM NaCl buffer using by gel filtration. Gel filtered fractions were collected and DNA content was measured via Picogreen. Relative fluorescence units (RFU) of each fraction are shown.

5.2.2 HPV16 is stable up to 5 days after purification in 0.5 M NaCl buffer

Since the CryoEM grids cannot be prepared on the day of the virus purification, it was important to confirm that the virus was stable for a few days after preparation. To assess virus stability, I made a sample of HPV16 capsids as I would for CryoEM, including gel filtration into DPBS/500 mM NaCl buffer, and stored it on ice for 5 days. Each day I snap froze a sample, with Day 1 being the virus preparation day. I then titered each sample by infecting HeLa cells with s dilution series of each for ~48 hours before the cells were fixed and eGFP expression quantified via FACS (Figure 5.2A). After an initial 40% loss of infectivity between Day 1 and Day 2 that is probably in part due to adsorption of the virus to the plastic sample tube, the virus lost about 15% of infectivity per day from Day 2-4. The final sample, Day 5, was about 20% as infective as the Day 1 sample (Figure 5.2B). Thus, although there was a loss of infectivity, probably due to loss of virus stability, the majority of the viral particles were still infectious from Day 2-4. Therefore, the time required for shipment of the virus to my collaborator likely does not result in a significant loss of virus capsid stability.

5.2.3 HD5 Neutralizes HPV16 in buffer containing up to 850 mM NaCl

Previous work with HAdV and HD5 showed that HD5 neutralization of the virus was ion concentration sensitive [51]. Increasing the salt concentration of the HD5-HAdV mixture resulted in greatly decreased defensin binding and antiviral activity. However, in order for the HPV16 samples to be stable for shipping, I produce them in a high salt buffer. To confirm that HPV is still neutralized at higher salt concentrations, I performed an HD5 neutralization assay in increasing concentrations of NaCl and quantified infection by eGFP reporter genome expression.

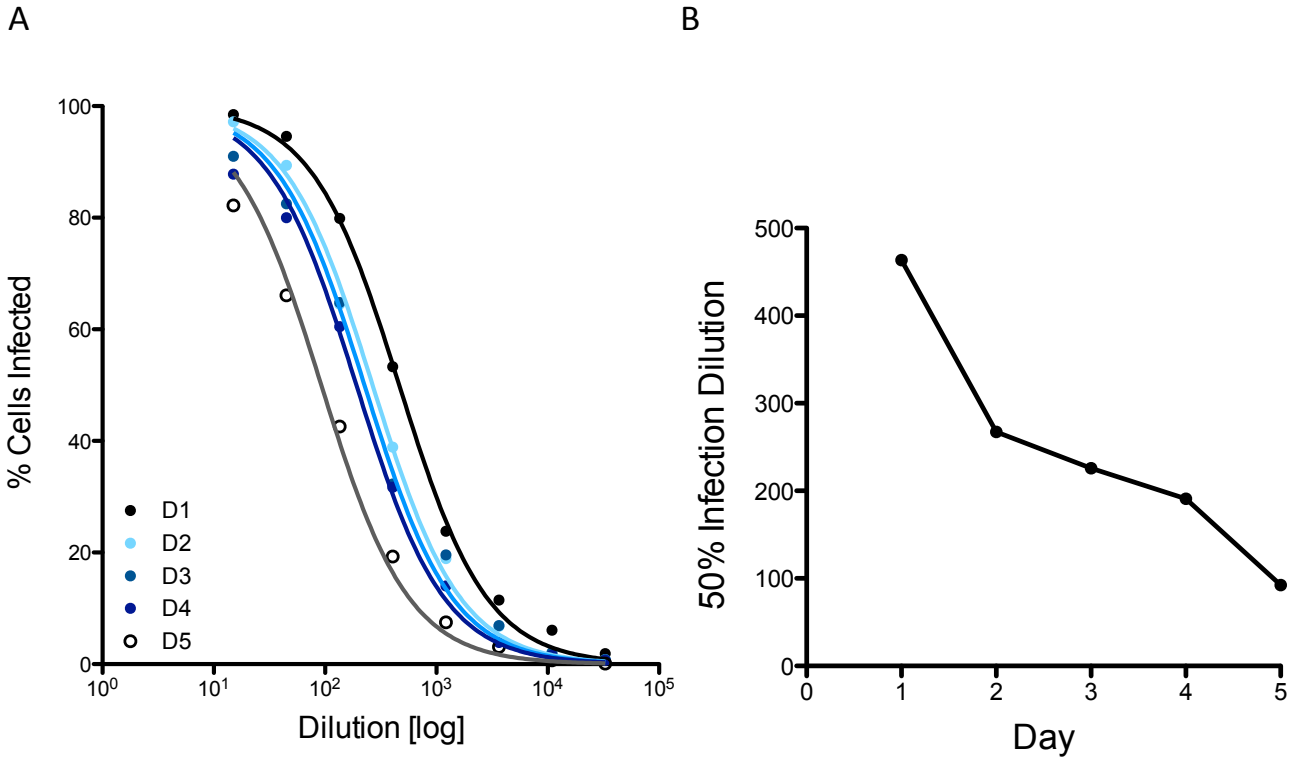

Figure 5.2. HPV16 is stable up to 5 days after purification in 0.5 M NaCl buffer

(A) Purified HPV16 was stored on ice for 5 days and samples snap frozen each day. Each sample was diluted in complete media and used to infect HeLa cells. eGFP expression of the HPV16 reporter genome was quantified via FACS 48 h p.i. (B) The dilution factor required to infect 50% of the cells for each sample is graphed. A lower dilution factor means more virus is required for infection. By Day 5 post purification, ~4.5 fold more virus is required to infect the same number of cells as the Day 1 sample.

As higher salt concentrations kill cells, I could not simply repeat the defensin neutralization assay in differing salt concentrations, as it includes a 4-hour cell-binding step. Therefore, I bound HPV16 to HeLa cells at 4°C for 1 hour, washed off unbound virus, and added HD5 that had been diluted in increasing NaCl concentrations. Cells were incubated with the HD5-NaCl mixture for an additional 45 minutes. As a control for the effects of the salt on the viral infection, cells were incubated in parallel with the same NaCl conditions and no HD5. The salt mixture was removed and replaced with media and the cells were incubated for ~48 hours. Infection was quantified by eGFP reporter genome expression (Figure 5.3). I found that while increasing the salt concentration decreased infection, probably by adversely affecting the binding of the virus to the cells, HD5 still neutralized HPV16 up to 850 mM M NaCl, indicating that the defensin still interacts with the virus beyond the salt concentration required for capsid stability. Therefore, CryoEM samples of the HD5-HPV16 complex can be prepared in 500 mM NaCl without affecting binding of the defensin to the virus.

5.3 Discussion

Determining the HD5 binding region on HPV16 is an important aspect of understanding the neutralization of non-enveloped viruses by defensins. Our lab has undertaken a variety of directed mutation and evolution studies of HAdV to understand the binding sites on the virus that are critical for HD5 neutralization or resistance. However, unlike HAdV, there are no human or non-human papillomavirus serotypes that are resistant to HD5. Crosslinking studies of defensin-virus complexes are possible but technically challenging. Therefore, we chose to collaborate with a CryoEM lab to resolve the structure of the HD5-HP16 complex. Although a high resolution CryoEM HPV16 structure exists, various production and handling questions remained, including

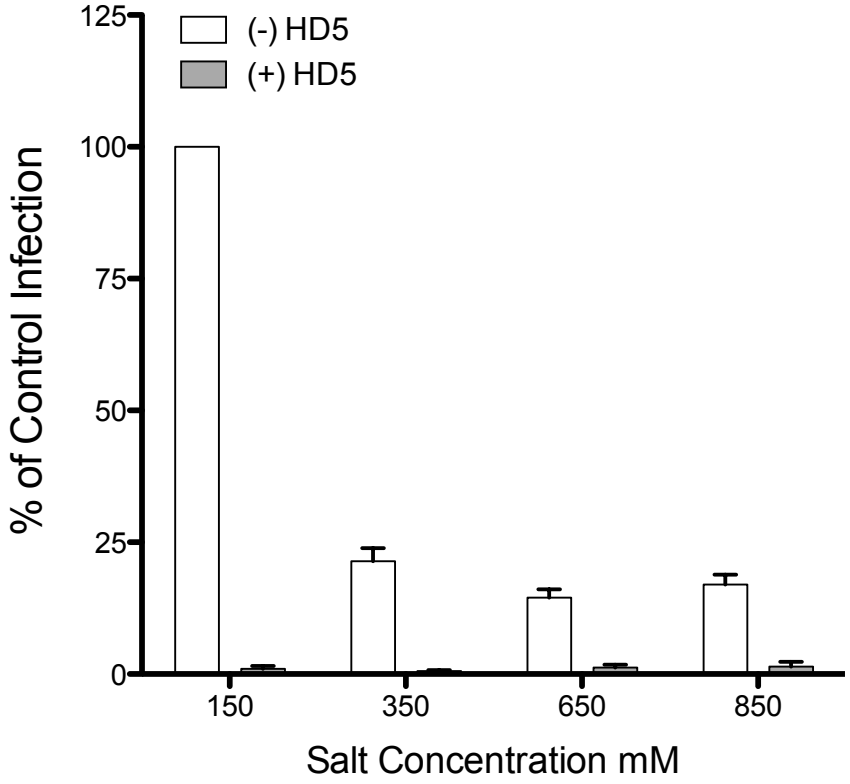

Figure 5.3 HD5 neutralizes HPV16 in up to 850 mM NaCl

HPV16 PsV was incubated HeLa cells. Unbound virus was washed off and cells were incubated with varying concentrations of NaCl or NaCl and 10 μM HD5 for an additional 45 minutes at 4°C. The salt media was replaced with complete. eGFP expression of the HPV16 reporter genome was quantified. Data is three independent experiments normalized to infection in physiological salt in the absence of HD5, ± SD.

the best shipping conditions and the requirement for high ion concentration to stabilize the viral capsid that may interfere with the defensin's binding ability. I have shown that the HPV16 capsid is stable for up to 5 days post production when stored on ice, increasing the time available for preparing CryoEM grids. In addition, while a high salt concentration is required for HPV16 capsid stability, it does not affect HD5's ability to neutralize the virus. Therefore, HPV16 and HD5 can be incubated together in the high salt buffer without having to be filtered or dialyzed into lower salt buffer, manipulations that increase the time between virus capsid production and CryoEM grid preparation. These protocol optimizations will be necessary for the continuation of this project.

The ability of HD5 to still neutralize HPV16 at high salt concentrations is interesting, implying that the HPV16-HD5 complex is not as ion-sensitive as the HAdV-HD5 complex [51]. In addition to high salt, HD5 also retains its antiviral activity against HPV16 in serum-containing media, a stark contrast to HD5 neutralization of HAdV, which is very sensitive to serum in neutralization assays. Additionally, the IC_{50} of HD5 against HPV16 is >two fold lower (~1 μM) than HAdV-5 (~2.5 μM) [58]. Overall, HPV16 is much more sensitive to HD5 in a variety of conditions than HAdV. Unfortunately, the binding affinity and kinetics of the HD5-HPV interaction are still unknown, but these indirect pieces of data may point to a stronger or more stable interaction between HD5 and HPV16 than between HD5 and HAdV. More direct studies of the binding affinity of HPV16 and HD5 would potentially clarify this point.

Chapter 6. Significance and Future Perspectives

6.1 Similarities between the mechanisms of HD5 inhibition of non-enveloped viruses

In order to fully understand α-defensins' methods of antiviral activity, it is important to elucidate the specific mechanisms by which various viruses are neutralized. Together, this data facilitates the synthesis of a general antiviral mechanism. The work presented in this thesis expands the known antiviral mechanism of HD5 to HPV16 [53]. The data presented in Chapters 2 and 3 elucidate the molecular mechanisms by which HD5 inhibits HPV16. The mechanism can be broken down into events that happen extracellularly and events that happen intracellularly. In both cases, there are similarities and differences with the methods by which HD5 inhibits HPV16 and other non-enveloped viruses.

Extracellular Mechanism

For all of the non-enveloped viruses sensitive to HD5, the defensin is known to interact directly with the virus in the extracellular milieu [152]. Against JC PyV and HAdV, HD5 binds to the virus capsid and causes viral aggregation, but the virus is still internalized [38,51,85]. Against BK PyV, however, HD5 induces viral aggregation that inhibits internalization [54]. The interaction of HD5 with HPV16 is like that of JC PyV and HAdV: HD5 interacts with HPV16 and causes aggregation but the virus is still internalized. However, in the case of HPV16, HD5 binding also prevents a critical cleavage step of the viral capsid protein L2 [53]. This cleavage event is known to be required for virus genome exit from the endosomal pathway [196]. This inhibition of a viral protein cleavage step appears to be unique in the defensin literature. Although the specific binding region on the virus capsid is still unknown, it is likely that HD5 binds to the major capsid protein L1 and occludes the cleavage site on L2. However, it inhibition of this cleavage

event is only one aspect of the antiviral mechanism of HD5, as HPV16 with cleaved L2 is not resistant to HD5, which would be predicted if the primary mechanism of HD5 is to prevent the cleavage of L2. In addition, recent work has shown that the HPV16 virus capsids produced during wild type infections are a mixed population of cleaved and uncleaved particles [247]. Differences in the amount of capsids with cleaved and uncleaved L2 are likely due to the cell culture pseudovirus production system, which does not recapitulate the slow maturation of HPV capsids in tissue. Thus, in *in vivo* infections, it is likely important that HD5 functions against both forms of the virus capsid in the genitourinary tract in order to protect the host against HPV infection.

Intracellular mechanism

Many non-enveloped viruses entry cells via endocytosis [84]. However, the virus must uncoat and escape the endosomal system to deliver its genome to the cytoplasm or nucleus. HAdV, JC PyV, and HPV16 all utilize different endosomal escape mechanisms: HAdV uncoating releases a viral protein that lysis the endosome [260], HPV16 uncoating releases L2 which inserts itself into the endosomal membrane and interact with the cellular retromer complex to mediate vesicle travel to the *trans*-Golgi network[216], and JC PyV uses retrograde transport to travel from the endosome to the ER where the virus partially uncoats [261]. Therefore, entry into the endosomal system and viral uncoating is the last stage in entry these viruses have in common, and may represent a bottleneck that defensins can target. The interaction of HD5 with HAdV and JC PyV capsids outside the cell has been shown to stabilize the virus capsid and inhibit uncoating[51,85]. Due to this uncoating inhibition, both viruses are directed to the lysosome and degraded, thereby protecting the cell from infection [152]. Similar to this activity, HD5 treatment still results in HPV16 internalization via the endosomal pathway. In contrast to HAdV

and JC PyV, however, HPV16 does appear to undergo some amount of uncoating, enough to expose an epitope on the inside of the capsid that has been used as a proxy for uncoating in other HPV16 studies. However, the HD5 treatment results in incomplete dissociation of L1 from a complex of L2 and the genome, indicating that the viral capsid is somewhat stabilized by the defensin. The result of this capsid stabilization is that L2 and genome complex travels with L1 to the lysosome and is degraded. Thus, while inhibiting the cleavage of HPV16 L2 as an antiviral mechanism is unique in the defensin field, the second mechanism of capsid stabilization and lysosomal trafficking is shared by the other sensitive non-enveloped viruses. It is interesting that a small peptide like HD5 can affect such diverse non-enveloped viruses by the same molecular mechanism, while also inhibiting enveloped viruses and bacteria by other distinct mechanisms.

6.2 Critical determinants of HD5 antiviral activity against non-enveloped viruses

In combination with my studies on the mechanism of HD5 neutralization, I also studied the HD5 determinants required for antiviral activity against HPV16[58,232]. These studies were performed in parallel with studies against HAdV and were the first systemic investigation into the HD5 determinants for activity against non-enveloped viruses. Although there were some differences between the two viruses, the HD5 determinants critical for neutralization of HPV16 were largely the same as those for HAdV, including charge-charge interactions, similar arginine residues, and dimerization and higher order multimerization. In fact, the residues important for both HAdV and HPV16 neutralization were grouped in the same area on one face of the HD5 dimer. Therefore, HD5 likely interacts with both viruses through a common mechanism. The only comprehensive studies of the α-defensin antiviral determinants are the one published by the Smith lab. Other studies have assessed the roles of limited aspects of HD5 or HNP1, such as the disulfide stabilized structure, or certain arginine residues. However, a complete alanine scan

similar to the data presented in chapter 4 has not been published for the polyomaviruses or for any enveloped virus. Expanding these studies to the other non-enveloped viruses that are sensitive to HD5 would allow for more general conclusions of the antiviral mechanism to be drawn. In addition, identifying the HD5 determinants required for enveloped viruses may help elucidate the differences in the mechanisms by which HD5 neutralizes enveloped and non-enveloped viruses. Perhaps one face of HD5 is required for interactions with non-enveloped viruses and another for enveloped viruses? More studies elucidating the specific determinants required for each class of microbe may allow for more comprehensive comparison studies.

6.3 Future perspectives

Virus Binding Regions

It is interesting to note that while there is some variation in the specifics of the antiviral mechanisms between the four non-enveloped viruses known to be sensitive to HD5, the general mechanism by which HD5 inhibits viral infection is the same: direct binding outside the cell, capsid stabilization, and inhibition of viral uncoating. These viruses are all evolutionarily distinct from each other, yet HD5 recognizes them and inhibits them in largely the same way. Unfortunately, the viral determinants by which HD5 recognizes and binds each virus are still unknown. It is unclear if there is a common sequence, structure, or region on each of these diverse viruses that the defensin interacts with. Elucidating the regions of each virus that HD5 interacts with is important to more clearly understand how the defensin has such broad antiviral and antimicrobial activity. A current project in the Smith lab is to identify the sequence differences between the capsid proteins of resistant and sensitive HAdV serotypes, anticipating that this information can be combined with future studies on other viruses.

Unfortunately, similar studies cannot be completed for HPV as there are no known human or animal papillomavirus that are resistant to HD5. HD5-sensitive papillomaviruses include human mucosal (HPV 6, 16, 18 and 31) and cutaneous (HPV 5) serotypes, as well as bovine, cotton-tailed rabbit, and mouse species; a very diverse set of papillomaviruses [52]. Interestingly, these papillomavirus genera all have L1 DNA sequence identity of less than 60%, yet HD5 still neutralizes all of them [262]. It is possible that the defensin is binding to a strongly conserved region on the capsid or to a sequence-independent structure. Perhaps sequence alignment studies of these human and non-human serotypes may identify targets for directed mutational studies to determine the HD5 binding site on HPV. Alternatively, the CryoEM project is attempting to characterize the HD5 binding region on the HPV16 capsid. Once this region has been identified, a combination of HD5-HPV16 crosslinking and HPV16 capsid mutational studies may identify the HD5 binding site on HPV16. Use of the HD5 lysine mutant R13-32K, which retains antiviral activity against HPV16, would increase the available amines for crosslinking studies. It would be interesting to known if there are any sequence or structure similarities between the HD5 binding sites on HAdV and HPV, which may explain the broad antiviral activity of HD5.

Determining the binding region of HD5 on HPV16 may also lead to better understanding of the HPV16 capsid itself. It is currently unclear how L2 is structured in the capsid [170]. My data suggests that HD5 binds to HPV16 in a way that both stabilizes the capsid and occludes the furin cleavage site on L2. Determining the site of HD5 binding may indicate where L2 emerges from the interior of the capsid. Understanding how L1 and L2 are arranged in the capsid may help with the development of antiviral drugs or antibodies against HPV.

Effects of HD5 on cell signaling during HPV16 infection

It is unknown if HD5 alters HPV16-induced signaling during infection. HPV16 infection is known to induce various cellular signaling pathways, including FAK and PI3K [202,204,205]. However, the role these pathways play in HPV16 infection is still unclear. In addition, recent work has shown that a FAK related non-receptor tyrosine kinase called Pyk2 is required for retention of the HPV16 capsid in the endosomal system. Retention may be required for the virus to complete uncoating and interact with the cellular retromer complex [206]. Knockdown of Pyk2 results in increased lysosomal localization of the HPV16 genome during infection, similar to HD5 treated HPV16. Perhaps HD5 binding inhibits the cellular interactions that lead to activation of these pathways. Interestingly, HD5 treatment inhibits FAK phosphorylation during infection, but still allows the virus to be internalized, in direct contrast with a FAK antagonist, TAE226, that inhibits virus internalization [206]. How HD5 blocks FAK phosphorylation but still allows HPV16 internalization is unknown, as is the requirement of FAK phosphorylation for internalization. Perhaps HD5 blocks FAK phosphorylation and induces viral entry in a non-canonical pathway by bridging a cellular HD5 receptor that still results in entry to the endosomal system. The use of HD5 as an inhibitor of these signaling pathways that still allows virus internalization may help to further elucidate the role of these cellular pathways in HPV infection. Further studies of the effect of HD5 on the signaling pathways HPV16 induces during infection are warranted.

Chapter 7. Materials and Methods

Cell Culture and Pseudovirus production

293TT cells were a kind gift from Denise Galloway (University of Washington, Seattle, WA). 293TTF cells were a kind gift from Richard Roden (Johns Hopkins University). HaCaT cells were a kind gift from Paul Nghiem (University of Washington, Seattle, WA). HeLa cells were purchased from American Type Culture Collection (ATCC). Cell culture reagents were purchased from Corning CellGro. All cells were cultured in DMEM containing 10% fetal bovine serum (Sigma-Aldrich), 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, and 0.1 mM non-essential amino acids (complete media). 293TT culture media was supplemented with 0.4 mg/ml Hygromycin B (Sigma-Aldrich). 293TTF culture media was supplemented with 2 µg/ml puromycin (Sigma-Aldrich).

HPV16 pseudovirus (PsV) was made as previously described via viral propagation and the improved maturation method (38, 39) (http://home.ccr.cancer.gov/lco/ImprovedMaturation.htm). PsV seed stock was made by co-transfecting 293TT cells with plasmids encoding codon optimized HPV16 L1 and L2 (p16L1L2, kind gift of Martin Müller, GCRC) and an eGFP reporter (pfwB, kind gift of John Schiller, National Cancer Institute, Bethesda, MD). Cleared lysate from this transfection contained mature PsV and was then used to infect additional 293TT cells. Mature PsV from the lysate of these cells was purified by ultracentrifugation on a discontinuous Optiprep (Sigma-Aldrich) density gradient (27%-33%-39%). To make 16L2-GP-N HPV16 PsV (18), L2 G99A and P100A mutations were introduced by short overlap extension PCR into the L2 ORF of the p16L1L2 vector. The mutated plasmid was then used to create PsVs by viral propagation as described above. Similarly, Myc-16L2-HA HPV16 PsV was made by fusing a Myc epitope tag

(EQKLISEEDL) to the amino terminus and an HA tag (YPVYDVPDYA) to the carboxyl terminus of L2 via PCR in the p16L1L2 vector. The expression plasmid for L2-FLAG HPV16 was a kind gift from Daniel DiMaio (Yale University). fcHPV16 PsV was produced by the same protocol with the following changes: 293TTF cells, a furin overproducing cell line, were used for the transfection, the lysate was allowed to mature for 48 hours, no ammonium sulfate was added to the lysate, and $CaCl_2$ was added to the lysate to a final concentration of 5 μM. For fcHPV16 EdU PsV production, 25 μM EdU was added to the cells 6 hours post transfection. In all cases, the L1 content of purified PsVs was quantified by BioRad BCA protein assay. L1 protein content was converted to particle number using a conversion factor of $3.0x10^7$ particles/ng L1.

Dynamic Light Scattering

Folded HD5 was made from a synthesized 80% pure linearized peptide (CPC Scientific, Sunnyvale, CA) and purified by reverse-phase high-pressure liquid chromatography; and an HD5 derivative containing L-α-aminobutyric acid in place of cysteine (HD5 Abu) was chemically synthesized, as previously described (11, 40). HD5, HD5 Abu, proHD5, Crp2, and Crp23 were serially diluted in 10 mM Tris, 150 mM NaCl, pH 7.5 and mixed with $1.3x10^{10}$ p/ml of HPV16 PsV, MAdV-1, or MAdV-2 in a final volume of 50 μl in disposable cuvettes (Malvern Instruments). Control samples of HPV16 PsV, MAdV-1, or MAdV-2 alone were diluted in the same buffer. Samples were incubated on ice for 45 min and equilibrated at 37°C for three minutes before analysis. Each sample was measured using a Zetasizer Nano ZS (Malvern Instruments), and the volume mean diameter (HPV16 PsV) or the Z-average diameter (MAdV-1 and MAdV-2) were obtained from the manufacturer's software. The fold change was calculated by dividing the average diameter of the defensin treated samples by the average diameter of the untreated controls for each of four independent replicates. Experiments were analyzed by two-

way ANOVA with Bonferroni post test to compare each concentration of HD5 or Crp isoform to the control sample using Prism (version 5.0d, GraphPad Software, Inc., La Jolla, CA). For all tests, $p < 0.05$ was considered significant.

Neutralization assays

Serial dilutions of HPV16 PsVs in serum free DMEM (SFM) were used to infect HeLa cells in 96 well plates for 4 h. fcHPV16 PsV was titered on HeLa cells in the presence or absence of 20 μM furin inhibitor (dec.-RVKR-cmk, Calbiochem) to assess the efficacy of the furin cleavage of L2. The cells were washed and cultured in complete media for ~44 h. Total eGFP expression was quantified 48 h post-infection with a Typhoon 9400 variable mode imager (GE Healthcare) and ImageJ software. A standard curve of infection was constructed in Prism software. A virus concentration resulting in ~80% total signal was used in inhibition studies.

To determine antiviral concentrations of HD5 or RG-1, increasing concentrations of either inhibitor were incubated with HPV16 PsV on ice for 45 min in SFM (Figures 2.4D and 2.5A) or complete media (Figures 2.2A, 2.2B, and 2.5A). 20μM furin inhibitor was also added to the fcHPV16 PsV samples. The mixture was added to HeLa cells in a 96 well plate and incubated at 37°C for 4 h. Cells were washed and cultured with complete media for ~44 h. Total eGFP fluorescence was quantified as above and normalized to a control sample infected without inhibitors using ImageJ. Fifty percent inhibitory concentrations (IC_{50}) were determined using non-linear regression in Prism.

To confirm the antiviral activity of HD5 in serum containing media, cells were incubated for 1 hour at 4°C with HPV16, fcHPV16, and HPV16 Myc-16L2-HA PsV diluted in serum containing media. Unbound virus was washed off and the cells incubated with 0 or 10 μM HD5 in serum complete media for an additional hour at 4°C. The samples were shifted to 37°C for 4

hours and the HD5 removed Total eGFP fluorescence was quantified as above and normalized to a control sample infected without HD5 using ImageJ and graphed in Prism.

Immunofluorescence microscopy

To label PsV with Alexa Fluor 555 (AF555), the standard PsV maturation and lysis buffers were altered to omit ammonium sulfate. After PsV maturation, cleared lysate (750 µl) was diluted with an equal volume of Dulbecco's phosphate buffered saline (DPBS), 1 M NaH_3CO_2 was added to a final concentration of 100 mM, and 35 µg of Alexa Fluor 555 dye dissolved in DMSO were added. After 1 h incubation at RT, the labeling reaction was neutralized by the addition of 13 mM $NaPO_4$. Labeled PsV was purified from the lysate by ultracentrifugation through an Optiprep gradient, and HPV16 particle number was determined as described above. $2.5x10^9$ particles of AF555-HPV16 PsV were incubated with or without 5 µM HD5 or 5.4 µg/ml RG-1 (43)(kind gift of Richard Roden, Johns Hopkins University, Baltimore, MD) alone or in combination for 45 min on ice in complete media. As a control, 5 µM HD5 and 5.4 µg/ml RG-1 were also incubated together in complete media without AF555-HPV16 PsV. The samples were then added to HaCaT cells plated on coverslips (seeded at $3x10^5$ cells/coverslip 24 h prior to infection) and allowed to infect for 12 h. Assuming that the cells doubled overnight, this is equivalent to a multiplicity of infection (MOI) of $4x10^3$ PsV particles/cell. Cells were fixed using 4% paraformaldehyde (PFA) then quenched and permeabilized in 20 mM glycine/0.5% Triton X-100 in PBS. All samples were stained using goat anti-mouse Alex Fluor 488 (Life Technologies) in blocking buffer (1% BSA/0.05% Tween 80) for 45 min and TO-PRO (Life Technologies) in PBS/1% Tween 20. Samples were mounted using ProLong Gold (Life Technologies). Three fields of view were captured for each sample on a Zeiss 510 Meta laser scanning confocal microscope.

Image analysis was performed using ImageJ of maximum intensity z-profiles of cells, excluding images in the z-series above or below the plane of the nucleus. Cell boundaries were defined using bright field images. Thresholds for green (antibody) and red (PsV) channels above background were determined using uninfected cells. Manders coefficients were obtained using the JaCoP plugin of ImageJ for 40-50 individual cells per condition. M1 coefficients for individual cells were converted to percentages for Figures 2.2D and 2.4B. M2 coefficients for individual cells were converted to percentages for Figures 2.2E and 2.4C. Experiments were analyzed by unpaired t test using Prism. For all tests, $p < 0.05$ was considered significant.

For colocalization studies in chapter 3, $6x10^4$ HeLa cells were seeded the day before the infection on glass cover slips. Cells were infected with $2.35x10^9$ particles of fcHPV16 with EdU labeled genome, for an MOI of approximately $1.95x10^4$ particles per cell, assuming a doubling of the cells overnight. The virus was incubated with the cells at 4°C to allow binding, unbound virus was washed off with cold media, and the cells were further incubated at 4°C for an additional hour with media, 10 μM HD5 or 20 μM NH_4Cl. Cells were washed with cold PBS and fixed in 2% PFA for the 0 hour time points or shifted to 37°C for the indicated times and then fixed. Cells were permeabilized with 20 mM glycine/0.5% Triton X-100 in PBS for 20 minutes, then stained with antibodies for cellular markers or HPV16 L1. Anti-EEA1 (1:250, Cell Signaling), LAMP1 (1: 250, AbCam), or 33L1-7 (1:100, kind gift from Martin Sapp, LSU Shreveport) were diluted in 1% BSA/0.05% Tween 80/PBS. AlexaFluor 488, 555, or 647 (Life Technologies) were used at 1:1000. EdU labeled virus genome was stained with the ClickIT kit according to the manufacturer's instructions (Life Technologies). Cell nuclei were stained with TOPRO-3 (1:1000, Life Technologies). Coverslips were mounted in ProLong Gold (Life

Technologies). Z-stack images of least 3 fields of view for each sample were taken on a Zeiss 510 Meta laser scanning confocal microscope.

Images analysis was performed using ImageJ. Individual cell borders were defined using brightfield images. Thresholds for PsV and cell marker channels above background were determined using uninfected cells. For colocalization studies Manders coefficients were obtained using the JaCoP plugin of ImageJ of at least 40 individual cells per condition. For uncoating studies, in addition to colocalization using the JaCoP plugin, total pixel area of the EdU genome signal and 33L1-7 antibody signal of at least 40 cells were quantified in ImageJ using the pixel area tool. Experiments were analyzed by one-way ANOVA with Bonferroni post tests to compare selected pairs of samples using Prism software. For all tests, $p < 0.05$ was considered significant.

rL2:13-36 and rL2:1-160 production

To generate rL2:13-36, an ORF encoding amino acids 13-36 of HPV16 L2 with a C-terminal HA epitope tag, ProTEV enzyme cleavage site, and 6x[His] epitope tag was cloned into pRSET-A (Life Technologies) . The rL2:1-160 construct encoded an N-terminal Myc tag and residues 1-160 of HPV16 L2 with a C-terminal 6x[His] tag. 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce protein expression in BL21 CodonPlus (DE3)-RIPL or BL21-Gold (DE3) *E. coli* (Stratagene), and the protein was purified using TALON resin (Clontech) according to the manufacturer's instructions. Purified protein was exchanged into 50 mM $NaPO_4$/130 mM NaCl via dialysis and stored at -80°C.

RG-1 immunoprecipitation

10.8 µg/ml RG-1 was incubated alone, with 14 ng of rL2:13-36, or with 14 ng of rL2:13-36 and 5 µM HD5 in binding buffer (50 mM $NaPO_4$/150 mM NaCl/1 mM PMSF pH 8, 50 µl

total) at 4°C for 1.5 h. These concentrations were calculated to approximate the conditions under which HD5 neutralizes viral infection in our immunofluorescence competition assay. 20 µl 50% TALON resin in binding buffer was added to each sample and incubated at 4°C for an additional 1 h. Flow through was saved, and beads were washed 3x with wash buffer (50 mM $NaPO_4$/150 mM NaCl/1 mM PMSF/0.1% Triton X-100/10 mM imidazole). Samples were eluted using wash buffer without 0.1% Triton X-100 and containing 250 mM imidazole. Samples were resolved on a 15% SDS-PAGE gel. Antibody heavy chain was visualized using goat anti-mouse HRP (1:5000, Thermo-Fisher) and enhanced chemiluminescence (BioRad).

rL2:13-36 competition assay

I first calculated the concentration of HPV16 L2 molecules in the control wells of my infection assay, assuming a maximal 72 copies of L2 per PsV. Based on this number, we incubated a 500-fold molar excess of rL2:13-36 with or without 5.4 µg/ml RG-1, 5 µg/ml mouse IgG1 (Sigma-Aldrich), or 5 µM HD5 in serum free media for 1 h on ice. Total protein in all samples was equalized using bovine serum albumin (BSA). HPV16 PsV was then added to the samples and incubated on ice for an additional hour. These mixtures were added to HeLa cells that had been washed with SFM and allowed to infect at 37°C for 4 h. Inoculum was removed, cells were washed once, and complete media was added. Infection was quantified 48 h post-infection as above. Treated samples were normalized to control infections in the absence of inhibitors. Experiments were analyzed by one-way ANOVA with Bonferroni post tests to compare selected pairs of samples (Figure 2.3B) or with Dunnett post tests to compare each sample to control (Figure 2.3C) using Prism software. For all tests, $p < 0.05$ was considered significant.

Myc-16L2-HA HPV16 PsV cleavage assay

$4.8x10^{10}$ particles of Myc-16L2-HA HPV16 PsV were incubated with or without 1, 5, or 10 μM HD5 or 40 μM furin inhibitor (dec.-RVKR-cmk, Calbiochem) in complete media for 1 h on ice. Samples were added to HeLa cells, and infection was allowed to proceed at 37°C for 16 h. Media was removed, and cells were washed with serum free media. Samples were lysed with 2X SDS loading buffer (BioRad) with β-mercaptoethanol and heated at 95°C. Samples were resolved on 7.5% SDS-PAGE gels and immunobloted using mouse anti-HA (1:1000, Thermo-Fisher) and goat anti-mouse HRP (1:5000, Thermo-Fisher).

Furin cleavage assay

1.8 ng of rL2:1-160 was incubated with or without 1, 5, or 10 μM HD5 or 40 μM furin inhibitor in 20 μl furin cleavage buffer (100 mM HEPES/1 mM $CaCl_2$, pH 7.4) on ice for 45 min. 1 Unit of furin (NEB) was added, and the samples were incubated at 30°C for 1 h. Reduced, denatured samples were resolved on 15% SDS PAGE gels and analyzed by immunoblot with mouse anti-His antibody (1:1000, Thermo-Fisher) and goat anti-mouse HRP (1:5000, Thermo-Fisher).

Trypsin cleavage assay

$3x10^9$ particles of HPV16 PsV were diluted in PBS and incubated with or without 10 μM HD5 on ice for 45 minutes. 2.5% tryspin (Thermo Fisher) was diluted in PBS and added to samples for final dilutions of 1:2000, 1:8000, 1:32000, and 1:128000. Samples were incubated at 37°C for 15 minutes and SDS loading buffer and DTT added. 50 ng of rL2:1-160 with or without 10 μM HD5 was incubated with trypsin at a final dilution of 1:2000 in parallel. Samples were separated on 10% (PsV) or 15% (rL2:1-160) SDS-PAGE gels and protein visualized with SYPRO Ruby total protein stain (Thermo Fisher).

L1 and L2 degradation immunoblotting

HeLa cells were plated in 48 well plates to confluence. $7x10^8$ particles of fcHPV16 EdU or uncleaved HPV Myc-16L2-HA (WT) were added to cells and allowed to bind at 4°C for 1 hour. Unbound virus was removed and cells incubated with at 4°C for an additional hour with media or 10 μM HD5, 20 μM NH_4Cl, or 5 μM MG132 alone or in combination. Samples were shifted to 37°C for the indicated times. Samples were collected by washing with cold PBS and then lysed with NP40 buffer (150 mM NaCl, 1% NP40, 10 mM Tris pH 8.0) and HALT protease inhibitor (Sigma). SDS loading buffer with bromophenol blue and DTT was added and samples heated at 95°C. Samples were run on 10% SDS-PAGE and transferred to nitrocellulose. Blots were blocked with 5% NFDM in 0.1% Triton X-100/PBS. L1 was immunoblotted using anti-camvir (1:1000, Millipore), L2 was immunoblotted with anti-HA (1:1000, Thermo Scientific), mouse anti-HRP secondary (1:5000, Thermo-Fisher), and enhanced chemiluminescence (BioRad). Anti-beta actin (1:5000, Sigma) was used as a loading control.

FAK immunoblotting

HeLa cells were plated in 48 well plates to confluence. $1.6x10^9$ particles of fcHPV16 PsV were added to cells and allowed to bind at 4°C for 1 hour. Unbound virus was removed and cells incubated with at 4°C for an additional hour with media or 10 μM HD5. 10 nm bombesin was added to one HD5 treated and one untreated sample immediately prior to shift to 37°C. Samples were shifted to 37°C for the indicated times. Samples were collected by washing with cold PBS and then lysed with NP40 buffer (150 mM NaCl, 1% NP40, 10 mM Tris pH 8.0) and HALT protease inhibitor (Sigma). SDS loading buffer with bromophenol blue and DTT was added and samples heated at 95°C. Samples were run on 10% SDS-PAGE and transferred to nitrocellulose. Blots were blocked with 5% BSA in 0.1% Triton X-100/TBS. p-FAK was immunoblotted using

anti-p-FAK (Y397) (1:1000, Sigma), total FAK was immunoblotted with anti-FAK (1:1000, Santa Cruz Biotechnology), rabbit Alexa Fluor 488 secondary (1:1000, Life Technologies), and scanned on a Typhoon 9400 variable mode imager (GE Healthcare).

Statistical analysis

For Figures 2.1, 2.3B, 3.3B, 3.5B, and 4.1-4.4A experiments were analyzed by two-way analysis of variance (ANOVA) with Bonferroni post-tests to compare each HD5 treated condition to the control samples using Prism (version 5.0d; GraphPad Software, Inc., La Jolla, CA). Figure 2.3D was analyzed by two-way analysis of variance (ANOVA) with Dunnets post-tests to compare each HD5 treated condition to the control sample using Prism. For Figures 2.2D, 2.2E, 2.4B, 2.4C, 3.2C, and 3.3C experiments were analyzed by unpaired *t* test. For all tests, *P* values of <0.5 were considered significant.

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VITA

EDUCATION

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PUBLICATIONS

1. Wilson SS, **Wiens ME**, Holly MK, Smith JG. Defensins at the Mucosal Surface: Latest Insights into Defensin-Virus Interactions. Journal of Virology. 2016 Mar 23.
2. Gounder AP, Myers ND, Treuting PM, Bromme BA, Wilson SS, **Wiens ME**, et al. Defensins Potentiate a Neutralizing Antibody Response to Enteric Viral Infection. PLoS Pathog. 2016 Mar;12(3):e1005474.
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CONFERENCE PRESENTATIONS

1. Wiens ME and Smith JG. (2014) Alpha-defensin HD5 inhibits furin cleavage of human papillomavirus 16 L2 to block infection. International Papillomavirus Conference, Seattle WA. **Oral presentation**

2. Wiens ME and Smith JG. (2014) Alpha-defensin HD5 inhibits furin cleavage of human papillomavirus 16 L2 to block infection. Northwest chapter of the American Society for Microbiology, Seattle, WA. **Oral presentation.**

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