Mutation-function analysis in vivo of the nuclear localization signals of L2 minor capsid proteins of high risk HPV16 and low risk HPV11

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Mutation-function analysis *in vivo* of the nuclear localization signals of L2 minor capsid proteins of high risk HPV16 and low risk HPV11

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Abstract

During the papillomavirus replication cycle, the L2 minor capsid protein enters the nucleus in the initial phase after uncoating of the incoming virions and in the productive phase when L2 together with L1 major capsid protein mediate the encapsidation of the newly replicated viral genome. L2 proteins of both high risk HPV16 L2 and low risk HPV11 L2 have two nuclear localization signals (NLSs): one at the N-terminus (nNLS) and one at the C terminus (cNLS). The purpose of these experiments is to determine the minimal mutations necessary to inhibit the function of the NLSs. In this study, subcellular localization of enhanced green fluorescent protein (EGFP) fusions with full length L2 and L2 mutants lacking either the cNLS (EGFP-L2ΔC), nNLS (EGFP-L2ΔN), or both NLSs (EGFP-L2ΔNΔC) was analyzed in HeLa cell transfection assays. Full length HPV16 L2 and HPV11 L2 proteins localize to the nucleus. For both HPV16 and 11 L2, each NLS could independently mediate nuclear import in vivo. EGFP fusions were also made with mutated nNLS (EGFP-L2ΔCSbN) or mutated cNLS (EGFP-L2ΔNSbC). Transfected HeLa cells were examined by fluorescence microscopy and quantitative studies were done. In both HPV16 and 11 L2 proteins, mutation of basic residues in either NLS inhibited its nuclear import ability.
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Introduction

Human Papillomavirus

Human papillomaviruses (HPVs) have already been identified in 120 different types, infecting either epithelial cells of the skin or of the anogenital and oropharyngeal mucosa. In the United States, HPV accounts for more infection than all other sexually transmitted diseases combined. HPV infection has been linked to the etiology of numerous types of cancer, most significantly to cervical cancer as well as a large proportion of anal, perianal, vulvar, penile, and oropharyngeal cancers. HPV types are classified as either high-risk or low-risk according to their detection in malignant lesions or genital warts (condylomata acuminata), respectively. HPV high-risk types 16, 18, 31, 33, 39, 45, 52, 58, and 69 and HPV low-risk types 6 and 11 occur with the greatest frequency (zur Hausen et al., 2000). Infection by high-risk strains of HPV accounts for approximately 95% of cervical cancer, the second most common and second leading cause of cancer death among women worldwide (Bosch et al., 1995). Among these high-risk HPV types found in cervical cancer, HPV16 is the most common (50%), with HPV18 (15%), HPV45 (8%), and HPV31 (5%) in succession (Bosch et al., 1995).

Linkage of HPV infection with cervical cancer has been established upon multiple grounds. Strikingly, HPV has been detected in cervical cancer biopsies in 93% of samples (Bosch et al., 1995). Expression of viral oncogenes in tumor material has also been detected. Specifically, oncogenes E6 and E7 have demonstrated transforming properties and are required for maintenance of the malignant phenotype in cervical carcinoma cell lines. Further, viral oncoproteins have been shown to interact with growth-regulating host-cell proteins. Epidemiologic studies have also implicated HPV
infection as the major risk factor for etiology of cervical cancer (zur Hausen et al., 2000). With this mounted evidence, the sexually transmitted HPV has been determined to be the central etiologic factor in cervical cancer, with notable homogeneity among countries worldwide (Bosch et al., 1995).

By virtue of their physical properties, human papillomaviruses have been classified as a taxonomic group known as *Papillomaviridae*. Both low-risk and high-risk types of HPV are specifically classified as Alpha-papillomaviridae (de Villiers et al., 2004). Electron microscopy of negatively-stained HPV virions has determined them to be 55 nm in diameter. Electron microscopy has also revealed a composition of 72 capsomeres arranged on a T=7 icosahedral surface lattice with a dextro (right-handed) skew (Finch et al., 1967). Within these nonenveloped capsids is contained a single molecule of 8-kb double-stranded circular DNA (Galloway et al., 1989). The viral genome encodes the HPV structural proteins, containing two large open reading frames (ORFs), L1 and L2, which are well conserved among all members of the family *Papillomaviridae* (de Villiers et al., 2004). The primary product of the L1 ORF is the major structural protein, with a molecular weight of 57K in HPV1 (Doorbar et al., 1987). Each of the 72 individual capsomeres making up the virion is a pentamer of the L1 major capsid protein (Baker et al., 1991). The product of the L2 ORF is the minor structural protein, with a molecular weight of 78K in HPV1 (Doorbar et al., 1987). Additionally, the viral genome contains open reading frames (ORFs) for three oncoproteins (E5, E6 and E7) and two regulatory proteins (E1 and E2) (de Villiers et al., 2004).

While the organization and number of L1 major capsid proteins present in the HPV virion has largely been elucidated, similar data for the L2 minor capsid proteins...
remains unclear, especially for those of genital HPVs (Buck et al., 2008). The tight coupling of HPV propagation to the differentiation of the host epithelial cells, and simultaneous difficulty in creating a stratified model, has significantly impeded in vivo study of virion assembly (Doorbar et al., 2005; Hagensee et al., 1993). In vitro, capsids have been shown to self assemble into icosahedral 55 nm particles even when containing L1 alone. However, these capsids were considerably fewer and more variable in morphology than those containing both L1 and L2 (Hagensee et al., 1993). Current literature conflicts over the estimated number of L2 molecules per capsid, ranging from 12 to 36 to recent estimates of up to 72 L2 binding sites in an assembled virion (Roden et al., 1996; Kirnbauer et al., 1993; Buck et al., 2008). L2 has been proposed to interact in the capsid hollow with the L1 capsomers at an L2-specific density button beneath each unit (Buck et al., 2008).

**Human Papillomavirus Life Cycle**

The life cycle of HPV is tightly linked to the differentiation of the host epithelial cell. For initial infection to be achieved, infectious particles must reach the cells of the undifferentiated basal epithelial layer, often through a break in the stratified epithelium. Upon recognition by a cell surface receptor, endocytosis of the virus occurs through clathrin coated vesicles. The virus is subsequently uncoated and viral DNA is transported into the nucleus. In the basal layer, the nonproductive stage occurs via selective expression of the early genes E1 and E2. In this infective state, the virus utilizes the host DNA replication machinery to synthesize its own DNA as a low-copy-number episome.
The productive stage occurs as the basal cells proliferate and migrate into upper layers of stratified squamous epithelia. While uninfected basal cells reach terminal differentiation shortly after migrating to suprabasal cell layers, those expressing the E6 and E7 oncoproteins experience a loss of cell cycle progression restraint and do not undergo normal terminal differentiation. E6 and E7 stimulate cell cycle progression as they associate with cell cycle regulators. In the mid or upper epithelial layers, expression of all viral early gene products occurs and the viral genome is amplified to a high-copy-number.

Once viral genome amplification is complete in the upper layer, the structural proteins L1 and L2 are expressed. The L2 minor capsid protein is expressed before the major capsid protein, L1. L1 and L2 proteins must be imported into the nucleus, where they form the icosahedral shell and encapsidate the newly replicated viral DNA to complete assembly of the virus. Virions are eventually released with the shedding of the upper layer epithelium and must then survive extra-cellularly until re-infection (Doorbar et al., 2004).

**L2 Minor Capsid Protein**

While the role of L2 in the HPV life cycle has yet to be completely clarified, certain studies have shed light upon the functions of the minor capsid protein. Organotypic raft culture studies have shown that L2 participates in at least two steps in the life cycle of high risk strain HPV31. First, L2 deficient HPV31 virions showed a 10-fold reduction in the amount of encapsidated viral DNA, revealing the critical role L2 plays in the encapsidation of the viral genome. Second, those virions lacking L2 also
exhibited over a 100-fold reduction in infectivity, lending to L2’s involvement in virion assembly and infectivity (Holmgren et al., 2005).

L2’s role in encapsidating the viral genome may be due to its known association with the early protein E2. It has previously been demonstrated that L2 localizes to punctuate nuclear regions known as promyelocytic leukemia (PML) oncogenic domains (PODs), or nuclear domains 10 (ND10). When coexpressed with either L1 or E2, L2 colocalizes and shifts their locality to the PODs as well (Day et al., 1998). This colocalization of L2 with E2, a facilitator of viral DNA replication, may indicate L2’s ability to modulate its function in the productive phase of the viral life cycle (Doorbar et al., 2004; Heino et al., 2000). L2 has more specifically been shown to bind to E2TA, a transcriptional transactivator, and relocalize it to these nuclear subdomains. In binding to E2TA, L2 inhibits its function as a transcriptional transactivator but does not disrupt its replication function. L2 also relocalizes E2TR, a transcriptional repressor, to the PODs (Heino et al., 2000). It has been suggested that the PODs to which L2 and its associated proteins localize may play a positive role in viral replication (Day et al., 1998). L2 may be recruiting the viral genome to the site of virion assembly, as E2 binds specifically to the viral genome (Holmgren et al., 2005; Androphy et al., 1987). Alternatively, the PODs may play a role in cellular antiviral defense, as viral infection disrupts PODs (Ahn et al., 1998).

One essential role of L2 in the infectivity of the virus has been connected to its membrane-penetrating activity. A C-terminal 23-amino-acid peptide from the L2 protein was identified as essential for transit of the viral DNA across the endosomal membrane during infection. Though L2 is not required for initial uptake, it is required for egress of
the viral genomes from the endocytic compartment after viral uncoating (Kamper et al., 2006). Further, a cell-surface binding motif in HPV16 L2, which is conserved in BPV1 L2, binds to HeLa cell receptors and facilitates infection. This binding motif is thought to function in the infectious process after initial surface binding (Yang et al., 2003).

L2 minor capsid protein also has the ability to both bind DNA nonspecifically and may translocate the viral genome to the nucleus. In bovine papillomavirus type 1 (BPV1), L2 functions as an adapter between viral DNA via its nuclear localization signal (NLS) at the C terminus (cNLS) and the Kaps involved in nuclear import via its NLS at the N terminus (nNLS), thus facilitating nuclear import of the DNA during infection (Fay et al., 2004). Self-assembling virus-like particles (VLPs) containing only L1 of BPV1 have been found to be noninfectious. Contrarily, when L1 and L2 are coexpressed, viral DNA is detected in the VLPs, indicating that genome encapsidation requires the presence of L2. These results were found in both BPV1 and HPV16 (Roden et al., 1996).

**Nuclear Localization Signals**

The L2 minor capsid protein must enter the nucleus twice during viral infection, both during the initial phase after virion uncoating and the productive phase of new viral DNA encapsidation (Doorbar et al., 2004). HPV1 L2 localizes to the nucleus when expressed alone, signifying that the protein itself contains sequences which direct nuclear localization (Hagensee et al., 1993). It has been determined that nuclear localization of both HPV11 L2 and HPV16 L2 is achieved through pathways mediated by two such nuclear localization signals (NLSs), one in the N terminus (nNLS) and one in the C terminus (cNLS) (Bordeaux et al., 2006; Darshan et al., 2004). These classical NLSs are
composed of basic amino acids, often arginine and lysine, and can each independently mediate nuclear import (Moroianu et al., 1999). Studies on HPV18 L2 have shown this high-risk type to also contain an nNLS and cNLS, each of which is capable of independently mediating nuclear import (Klucevsek et al, 2006). An NLS in the middle region (mNLS) of HPV16 L2 has previously been proposed, although unsuccessful in \textit{in vitro} import assays at achieving nuclear localization (Darshan et al., 2004). The NLSs of HPV11 L2, HPV16 L2, and HPV18 L2 also appear to be DNA binding sites, with binding occurring without nucleotide sequence specificity (Bordeaux et al., 2006; Klucevsek et al., 2006).

**Nuclear Import Pathways**

In order for macromolecules to enter the nucleus, they must undergo mediated transport through the nuclear pore complex (NPC) by soluble transport receptors. Nuclear localization signals (NLSs) located on the macromolecules allow recognition, either directly or indirectly, by the transport receptors which shuttle between the cytoplasm and the nucleus. Specifically, these NLSs are recognized by import receptors of the karyopherin \( \beta \) (Kap \( \beta \) (importin) family. The general nuclear import pathway consists of the NLS-protein cargo bound directly, or indirectly via an adapter, by an import receptor in the cytoplasm. This complex is then transported through the NPC, the cargo is released in the nucleus, and the receptors and adaptors are exported back to the cytoplasm. Directionality of the Kap \( \beta \) import pathways is partially defined by a concentration gradient of RanGTP across the NPC. Distribution of RCCI, the nucleotide exchange factor of Ran, inside the nucleus maintains high levels of nuclear RanGTP.
Oppositely, RanGAP, a GTPase-activating protein, and RanBP1, a Ran-binding protein, are mainly cytoplasmic and thus maintain low levels of RanGTP in the cytoplasm (Moroianu et al., 1999).

The classical import pathway is for proteins containing classic NLSs. In this specific pathway, the NLS-protein cargo requires the involvement of an adaptor, importin α, to bind to the import receptor (Moroianu et al., 1999). Importin α associates both with the classical NLS of the cargo and with the importin β binding domain (IBB) of Kap β (Fried et al., 2003). Thus, the NLS-protein/importin α (Kap α)/importin β (Kap β) trimeric complex is translocated into the nucleus via interaction of Kap β with cytoplasmic nucleoporins of the nuclear pore complex (NPC). Affinity of the import receptors with the NLS-protein cargo is dictated by the concentration gradient of RanGTP. A low concentration of RanGTP in the cytoplasm allows the formation of a stable cargo/receptor complex (Moroianu et al., 1999). A high level of RanGTP in the nucleus, however, leads to release of the cargo. Nuclear RanGTP binds to Kap β/importin β to cause dissociation of Kap β from the importin α/cargo complex. Importin α’s IBB, without importin β binding, then causes release of the NLS-protein cargo from importin α through autoinhibition. Finally, Kap β and Kap α are separately exported back into the cytoplasm to be reused (Fried et al., 2003).

The interaction of various import receptors and adapter/receptor heterodimers with the L2 minor capsid protein of multiple HPV types has been analyzed. Specifically, HPV11 L2 interacts with Kap β₁, Kap β₂, and Kap β₃ and complexes with the Kap α₂β₁ heterodimer (Bordeaux et al., 2006). HPV16 L2 interacts with Kap β₂ and Kap β₃ as well as the Kap α₂β₁ heterodimer (Darshan et al., 2004). Finally, HPV 18L2 interacts with the
Kap $\alpha_2$ adaptor, Kap $\beta_2$, and Kap $\beta_3$ and also complexes with the Kap $\alpha_2\beta_1$ heterodimer (Klucevsek et al., 2006).

**Research on Nuclear Localization of L2 Minor Capsid Proteins**

Both HPV11 L2 and HPV16 L2 have been analyzed *in vivo* and *in vitro* for nuclear localization. The nNLS and cNLS of L2 proteins of both HPV types 11 and 16 have been shown to be capable of independently mediating nuclear import of L2. However, deletion of both NLSs results in mostly cytoplasmic localization (Bordeaux et al., 2006; Darshan et al., 2004). Research on the mNLS of HPV6b L2 has suggested a role in nuclear retention (Sun et al., 1995). Consequently, the proposed mNLS in HPV16 L2 may have a similar role (Darshan et al., 2004).

Given the previous research on the location and independent functioning of the NLSs of HPV11 L2 and HPV16 L2, further mutation-function analysis *in vivo* has been conducted. Single NLS deletions in HPV11 L2 have supported the findings that the nNLS and cNLS function independently. *In vivo* transfection studies of single NLS deletions of HPV16 L2 have provided corroborating evidence with previous studies *in vitro* that its nNLS and cNLS function independently. Further, while the deletion of both the nNLS and cNLS in HPV11 L2 has resulted in mostly cytoplasmic localization, HPV16 L2 still retains some nuclear localization. Thus, the mNLS previously suggested in HPV16 L2 may be weakly functional *in vivo*. Novel analysis of L2 localization with deletion of one NLS and mutation of basic residues in the second NLS has shown a complex phenotype in both HPV11 L2 and HPV16 L2. However, quantitation has shown a definite decrease in nuclear localization of these L2 mutants. Further analysis of L2
mutants will then lead to the determination of the minimal mutation necessary to inhibit function of the NLSs. Such L2 mutants will prove vital in analyzing the role of the L2 protein in nuclear import of viral DNA.

**Materials and Methods**

**Cell Culture**

The ATCC HeLa cell line was cultured in Dulbeccos Modified Eagle medium (DMEM+) and supplemented with 5% fetal bovine serum (FBS) and Penstrep. The cells were maintained in culture at 37 degrees C and 5 percent CO$_2$.

**Preparation of HPV16 and HPV11 EGFP-L2 and mutant EGFP-L2 plasmids**

PCR was used with the pProEX HTb plasmids containing the wild-type HPV16 and HPV11 L2 and L2ΔN, L2ΔC, and L2ΔNΔC mutants with sequence specific oligonucleotides incorporating an EcoRI restriction site to the 5’ end of the sequence and a BamHI restriction site to the 3’ end of the sequence. The PCR products were extracted from a 0.7% agarose gel using the QIAquick Gel Extraction Kit and double digested with EcoRI and BamHI. The pEGFP-C1 vector (EGFP = enhanced green fluorescence protein) with a kanamycin resistance gene (Clontech) was also double digested with the restriction enzymes EcoRI and BamHI, run on an agarose gel and the linearized vector was extracted in the same manner. The double digested vector and L2 insert were ethanol precipitated with a 1:3 vector:insert, 3 M ammonium acetate in a 1:10 of DNA volume and 2.5x the total volume of 95% EtOH (-20°C) and incubated overnight at -20°C, then centrifuged for 30 min (13,000 RPM, 4°C), supernatant was removed, 200 µl of 70%
EtOH (4°C) was added and centrifuged for 30 min, supernatant removed and DNA was air dried at R.T. for 10 min. The insert and vector were resuspended in 15 µl DNase free distilled water, 4 µl Ligate-IT 5x buffer, 1 µl Ligase (USB), and ligated for 5 min at R.T. on rotator. The HPV16 and HPV11 EGFP-L2, EGFP-L2ΔN, EGFP-L2ΔC, and EGFP-L2ΔNΔC constructs were transformed into XL1 blue bacteria and confirmed by automated sequence analysis (MGH DNA Sequencing Department).

*Procedure and preparation courtesy of Jennifer Bordeaux

**Transfection Assay**

HeLa cells were plated on poly-L lysine coated coverslips to 50-60% confluency 24 hrs prior to use and incubated under normal cell conditions. For each plasmid to be transfected, the following mix was prepared per well in the order listed: 97 ul DMEM (-) (Dulbecco's Modified Eagle Media, warmed to 37°C), 4 ul FUGENE 6 (placed at room temperature 15 minutes prior to preparation and vortexed), and 0.4-0.8 ug plasmid DNA. This sample was mixed and allowed to incubate at least 20 min at room temperature. The plated cells were then washed with 500 ul DMEM (-) per well and 500 ul fresh DMEM (-) was added. Each prepared mix was then added to wells, the plate was swirled, and then placed back into the incubator. After 6 hours, the media was changed from DMEM (-) to DMEM (+) (DMEM + 10% FBS and 5 mL Pen-Strep). The transfection mixture was then incubated overnight. The cells were fixed on glass slides 24 hours post initial transfection. Plates were placed on ice and covered from light. Cells were washed three times with 500 ul PBS per well, 5 minutes each wash, and covered in between washes. Cells were then incubated 10 minutes covered on ice with 500 ul per well PFA. Cells
were washed again three times with 500 ul PBS per well, 5 minutes each. After all washes, coverslips were drained and fixed on glass slides with dapi-vectashield and glue. Finally, localization of the EGFP-L2 fusion proteins was analyzed via fluorescence microscopy.

**Results**

**EGFP-16L2 is localized in the nucleus in vivo.**

An EGFP reporter protein was used to investigate the localization of HPV16 L2 in vivo by constructing a recombinant EGFP-16L2 plasmid to be used in transfection assay. HeLa cells were transfected with the EGFP-16L2 plasmid and subcellular localization was visualized 24h post transfection. Transfection with the EGFP plasmid serves as the control, as EGFP is capable of passive diffusion through the nuclear pore complex and therefore localizes diffusely throughout the cell (Figure 3, panel B) when compared with nuclear DAPI staining (Figure 3, panel A). The wild-type HPV16 L2 localizes to the nucleus (Figure 3, panel D), as made clear in comparison to the visualization of the nucleus with the DAPI staining (Figure 3, Panel C). Quantitation of 80 HeLa cells shows EGFP-16L2 to localize in the nucleus in 55% of cells, with 45% of cells showing diffuse localization throughout the cell (Table 2). Graphic representation of the quantitative analysis of subcellular localization is shown (Figure 6).

**The nNLS of HPV16 L2 can mediate nuclear localization of HPV16 L2 in vivo.**

An EGFP reporter protein was used to investigate the role of the nNLS of HPV16 L2 in vivo by constructing recombinant EGFP-L2, EGFP-L2ΔC and EGFP-L2ΔNΔC
plasmids. HeLa cells were transfected with the plasmids and subcellular localization was visualized 24h post transfection. EGFP-16L2∆C clearly localizes to the nucleus (Figure 4, Panel D), shown in correlation with the visualization of the nuclei with DAPI staining (Figure 4, Panel C). Quantitation has shown that approximately 98% of cells exhibit nuclear localization when transfected with the EGFP-16L2∆C mutant (Table 2). The nuclear localization by the nNLS alone is comparable to that of the full length EGFP-16L2 (Figure 4, panel B). With the deletion of both the nNLS and cNLS in the EGFP-L2∆N∆C mutant, however, nuclear localization was abolished (Figure 4, Panel H), resulting in mostly cytoplasmic localization of the protein. Quantitation studies have shown that while approximately 31% of cells retain nuclear localization of L2∆N∆C, 56% show localization throughout the cell and about 12.5% show mostly cytoplasmic localization (Table 2). DAPI nuclear staining is also shown (Figure 4, panels A and G). Thus, the nNLS is capable of mediating nuclear localization of HPV16 L2 and deletion of both the nNLS and cNLS results in clear loss of nuclear localization. Quantitative analysis of subcellular localization of HPV16 L2 and L2 mutants has been represented graphically (Figure 6).

**Mutation of basic residues in the nNLS of HPV16 L2 inhibits its nuclear import ability.**

A mutant EGFP-16L2∆CsbN plasmid was created in which the wild-type sequence of the nNLS, MRHKRSAKRTKR, was changed to MRHKRSAAAATKR (Table 1). The basic residues arginine and lysine were therefore changed to two neutral alanine residues. The EGFP reporter protein was used to investigate the nuclear import ability of
the mutated nNLS of HPV16 L2 in vivo by transfecting HeLa cells with the recombinant plasmid and visualizing subcellular localization 24h post transfection. The nuclei have been visualized using DAPI staining (Figure 4’, panels A, C, E and G). Compared to the strong nuclear localization of both EGFP-16L2 (Figure 4’, panel B) and EGFP-16L2ΔC (Figure 4’, panel D), the nuclear localization of EGFP-16L2ΔCsbN was largely disrupted (Figure 4’, panel F). The level of EGFP-16L2ΔCsbN protein remaining in the cytoplasm more closely resembles that of the EGFP-16L2ΔNΔC double mutant (Figure 4’, panel H). As such, the mutation of basic residues in the nNLS resulted in a complex phenotype. While nuclear localization still occurs in approximately 51% of cells (Table 2), this localization is much less defined, with more protein localized to the cytoplasm than seen with the EGFP-16L2ΔC mutant. Further, a substantial 36% of cells showed localization throughout the cell and about 13% were mostly cytoplasmic in the localization of the protein (Table 2). Thus, it has been shown that the mutation of basic residues in the nNLS of HPV16 L2 inhibits its nuclear import ability. Quantitative analysis of subcellular localization has been visualized graphically (Figure 6).

The cNLS of HPV16 L2 can mediate nuclear localization of HPV16 L2 in vivo.

An EGFP reporter protein was used to investigate the role of the cNLS of HPV16 L2 in vivo by constructing recombinant EGFP-L2, EGFP-L2ΔN and EGFP-L2ΔNΔC plasmids. HeLa cells were transfected with the plasmids and subcellular localization was visualized 24h post transfection. EGFP-16L2ΔN shows strong nuclear localization (Figure 5, Panel D), with 98% of cells showing nuclear localization in quantitative analysis (Table 2). As compared to nuclear visualization through DAPI staining (Figure
5, panels A and C), the single deletion mutant EGFP-16L2ΔN shows nuclear localization similar to that of the full length EGFP-16L2 (Figure 5, panel B). The EGFP-L2ΔNΔC mutant, having deleted both the nNLS and cNLS, lost this strong nuclear localization (Figure 5, Panel H). DAPI nuclear staining for the double mutant is also shown (Figure 5, panel G). The data suggests that the cNLS is capable of independently mediating nuclear localization of HPV16 L2. Graphic representation of the quantitative analysis of subcellular localization is shown (Figure 6).

**Mutation of basic residues in the cNLS of HPV16 L2 inhibits its nuclear import ability.**

The wild-type sequence of the cNLS of HPV16 L2, RKRRKR, contains basic residues implicated in its function as a classical NLS. A mutant EGFP-16L2ΔNsbc plasmid was created in which the cNLS sequence was changed to RKEEKR (Table 1), exchanging arginine and lysine for two glutamic acid residues. The EGFP reporter protein was used to investigate the nuclear import ability of the mutated cNLS of HPV16 L2 in vivo by transfecting HeLa cells with the recombinant plasmid and visualizing subcellular localization 24h post transfection. The nuclei have been visualized using DAPI staining (Figure 5’, panels A, C, E, and G). As opposed to the very clear nuclear localization seen for EGFP-16L2ΔN (Figure 5’, panel D), a far greater amount of the EGFP-16L2ΔNsbc mutant is localized to the cytoplasm (Figure 5’, panel F). Quantitative analysis has shown a retention of nuclear localization in approximately 77% of cells. However, the EGFP-16L2ΔNsbc mutant nuclear localization was generally accompanied by higher levels of protein in the cytoplasm than those with only a single
deletion of the nNLS. Approximately 16% of cells transfected with this mutant showed localization throughout the cell, and about 1.3% showed mostly cytoplasmic localization (Table 2). The data reveals a complex phenotype, yet shows some inhibition in the ability of the cNLS to mediate nuclear import with the mutation of basic residues. Quantitative analysis of subcellular localization of HPV16 L2 and mutants has been represented graphically (Figure 6).

The nNLS and cNLS of HPV11 L2 can independently mediate nuclear localization of HPV11 L2.

An EGFP reporter protein was used to investigate the role the nNLS and cNLS of HPV11 L2 in vivo by constructing recombinant EGFP-11L2, EGFP-L2ΔN, EGFP-L2ΔC, and EGFP-L2ΔNΔC plasmids, transfecting HeLa cells and visualizing subcellular localization 24h post transfection. The wild-type EGFP-11L2 clearly localizes to the nucleus (Fig. 7A, panel A) when compared to the localization of EGFP throughout the cell (Fig. 7A, panel C). The nuclei were also visualized with DAPI staining (Fig. 7A, panels B and D). Both the EGFP-L2ΔN (Fig. 7B, panel A) and the EGFP-L2ΔC (Fig. 7B, panel C) proteins also localize primarily to the nucleus, however not as efficiently as the wild-type EGFP-L2, as some of the protein was still located in the cytoplasm for each single mutant when compared with the nuclear DAPI staining (Fig. 7B, panels B and D). With the EGFP-L2ΔNΔC double deletion mutant (Fig. 7B, panel E) nuclear localization was no longer seen and the majority of the protein was cytoplasmic when compared to the nuclear DAPI staining (Fig. 7B, panel F). Graphic representation of the quantitative analysis of subcellular localization is shown (Figure 10).

*Figure and results taken from Jennifer Bordeaux (Bordeaux et al., 2006).
Mutation of basic residues in the nNLS of HPV11 L2 inhibits its nuclear import ability.

A mutant EGFP-H11L2ΔCsbN plasmid was created in which the wild-type sequence of the nNLS, MKPRARRRKRA, was changed to MKPRARAAKRA (Table 1). The basic residues arginine and lysine were thus changed to two neutral alanine residues. The EGFP reporter protein was used to investigate the nuclear import ability of the mutated nNLS of HPV11 L2 in vivo by transfecting HeLa cells with the recombinant plasmid and visualizing subcellular localization 24h post transfection. The nuclei have been visualized using DAPI staining (Figure 8, panels A, C, E, and G; Figure 8`, panels A, C, E, and G). Compared to the strong nuclear localization of both EGFP-11L2 (Figure 8, panel B; Figure 8`, panel B) and EGFP-11L2ΔC (Figure 8, panel D; Figure 8`, panel D), the nuclear localization of EGFP-11L2ΔCsbN was abolished (Figure 8, panel F; Figure 8`, panel F). Quantitative analysis revealed that only 28% of cells retained nuclear localization when transfected with the EGFP-11L2ΔCsbN plasmid (Table 2). Rather, about 43.5% of cells showed the protein to be localized throughout the cell while a considerable 28.5% of cells showed the protein to be localized cytoplasmically (Table 2). The resultant phenotypes have shown that mutation of basic residues in the nNLS of HPV11 L2 clearly inhibits its nuclear import ability. Graphic representation of the quantitative analysis of subcellular localization of HPV11 L2 is shown (Figure 10).

Mutation of basic residues in the cNLS of HPV11 L2 inhibits its nuclear import ability.
A mutant EGFP-16L2ΔNsbC plasmid was created in which the wild-type cNLS sequence was changed from RRRRKRR to RRAAKR (Table 1), exchanging arginine and lysine for two neutral alanine residues. The EGFP reporter protein was used to investigate the nuclear import ability of the mutated cNLS of HPV16 L2 in vivo by transfecting HeLa cells with the recombinant plasmid and visualizing subcellular localization 24h post transfection. The nuclei have been visualized using DAPI staining (Figure 9, panels A, C, E, and G; Figure 9’, panels A, C, E, and G). While very clear punctate nuclear localization is seen for HPV11 L2 (Figure 9, panel B; Figure 9’, panel B) and EGFP-11L2ΔN (Figure 9, panel D; Figure 9’, panel D), cells expressing the EGFP-11L2ΔNsbC mutant clearly show a disruption in nuclear import (Figure 9, panel F; Figure 9’, panel F), although multiple phenotypes do occur. Quantitative analysis shows only 32% of cells transfected with the EGFP-11L2ΔNsbC plasmid to exhibit nuclear localization (Table 3). Further, 28.5% of cells had localization throughout the cell and about 39% showed cytoplasmic localization (Table 3). The data has shown that the mutation of basic residues in the cNLS of HPV11 L2 disrupts the ability to mediate nuclear import. Graphic representation of the quantitative analysis of subcellular localization is shown (Figure 10).

Discussion
The L2 minor capsid protein of high-risk HPV16 and low-risk HPV11 contains two nuclear localization signals, the nNLS and the cNLS, which mediate nuclear import of the protein. Previous in vitro assays have identified the capacity of the NLSs to bind both to specific nuclear import adaptors and receptors and to nonspecific DNA (Darshan et al., 2004; Bordeaux et al., 2006). Novel in vivo nuclear localization studies on HPV16 L2 have been conducted in this research, as well as in vivo investigations on the effect which specific NLS mutations have on the nuclear localization of HPV16 L2 and HPV11 L2.

The full-length HPV16 L2 protein was shown to localize to the nucleus. Though quantitation showed nuclear localization in only 55% of cells, it is important to note that 0% of cells had mostly cytoplasmic localization of the protein. Overexpression of the protein has been proposed to account for the excess of the L2 minor capsid protein remaining in the cytoplasm in the resultant 45% of cells with diffuse localization. Without overexpression, HPV16 L2 would be expected to localize to the nucleus in the majority of cells, similar to the subcellular localization of HPV16 L2ΔN and L2ΔC mutants.

Given the nuclear localization of HPV16 L2, the capacity of each NLS to independently mediate nuclear import was studied. It was shown that the deletion of a single NLS, either the nNLS or the cNLS, still resulted in efficient nuclear localization of the protein. Deletion of both NLSs, however, significantly disrupted nuclear localization. The retention of some nuclear localization in cells expressing the HPV16 L2ΔNΔC double mutant suggests the possibility of a third moderately functioning NLS. An mNLS in HPV16 L2 has previously been studied and found nonfunctional in vitro. However,
the mNLS may be playing a role in nuclear retention in vivo as previously suggested (Darshan et al., 2004). Thus, it has been shown that both the nNLS and the cNLS are capable of independently mediating nuclear import.

Previous research has shown that HPV11 L2 localizes to the nucleus in vivo. This investigation also revealed that the nNLS and cNLS of HPV11 L2 can independently mediate nuclear import. Further, deletion of both the nNLS and the cNLS abolishes nuclear localization (Bordeaux et al., 2006). Quantitation of transfection assays using the same HPV11 L2 full-length and mutant plasmids has supported these findings.

As both HPV16 L2 and HPV11 L2 have been shown to contain NLSs capable of independently mediating nuclear import, further investigation seeks to identify the minimal mutation necessary to abolish the nuclear import ability of a given NLS. As classical NLSs are defined by their composition of basic amino acids, HPV16 and 11 L2 proteins with NLS mutations to either neutral or acidic residues were analyzed for nuclear localization in vivo. A complex phenotype was revealed in the mutation-function analysis of subcellular localization for each mutant. The substitution in the nNLS of HPV16 L2 proved to be more effective at inhibiting nuclear localization than the substitution in the cNLS. While cells transfected with the HPV16 L2ΔCSbN and L2ΔNSbC mutants showed all three phenotypes of nuclear, diffuse, and cytoplasmic localization, the NLS mutations undoubtedly disrupted nuclear import. The HPV11 L2ΔCSbN and L2ΔNSbC mutants were shown to be more effective in their inhibition of nuclear import. Both mutants substituted a neutral amino acid for a basic amino acid, and inhibited nuclear localization to a similar degree. Cells expressing the HPV11 L2ΔNSbC protein showed a higher rate of mostly cytoplasmic localization. Further mutation-
function analysis of new L2 mutants may reveal mutations with even higher efficiency at preventing nuclear localization.

Having shown that mutation of basic residues in the NLSs of HPV16 L2 and HPV11 L2 inhibits nuclear import ability, further study of these L2 mutants will help to elucidate the role of the L2 minor capsid protein in the HPV life cycle. The punctate pattern of L2 localization within the nucleus previously reported was also seen in this study for both types HPV16 and 11. Investigation of these L2 mutants in association with the nuclear domains 10 may also be made in the future (Day et al., 1998).

Immunoblot assays will be necessary to ensure the proteins expressed are not degraded in the transfections with L2 plasmids. *In vitro* import assays of the HPV16 and 11 L2 substitution mutants will also be necessary to substantiate *in vivo* findings. Further investigation *in vivo* into the possible mNLS of HPV16 L2 may also be made in the future. As the minimal mutation necessary to abolish the nuclear import ability of the HPV16 and 11 L2 NLSs is clarified, these L2 mutants may be used to further understand the functions of the L2 minor capsid protein and to potentially disrupt its role in the infectivity of the virus.
Figure 1.
Illustration of the HPV16 genome. The image illustrates the open reading frames for the early genes (E1, E2, E4, E5, E6, and E7) and the late genes (L1 and L2). The proteins are conserved between high-risk and low-risk strains.
Taken from <http://www.microbiologybytes.com/virology/Papillomaviruses.html>
Figure 2.
Model for nuclear import of classic NLS-containing proteins. Low levels of RanGTP in the cytoplasm allow for the stable formation of a trimeric complex consisting of the NLS-protein cargo and the Kap αβ1 heterodimer. The complex is translocated through the nuclear pore complex and high levels of RanGTP in the nucleus dissociate the Kap αβ1 complex, releasing the NLS-protein cargo. Kap α and Kap β1/Ran GTP are then exported via separate pathways back into the cytoplasm. (Moroianu, J., Nuclear import and export pathways. J Cell Biochem, 1999. Suppl 32-33: p. 76-83.)
### NLSs and mutated NLSs of HPV16/11 L2 proteins

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K: Lysine (basic)  
R: Arginine (basic)  
A: Alanine (neutral)  
E: Glutamic acid (acidic)

**Table 1.**  
**NLSs and mutated NLSs of HPV16/11 L2 proteins.** In order to disrupt the nuclear import ability of the classical nuclear localization signals of HPV16 L2 and HPV11 L2 proteins, basic residues were changed to neutral or acidic residues in either the nNLS or cNLS.
Figure 3. EGFP-16L2 is localized in the nucleus in vivo. HeLa cells were transfected with either EGFP (panels A and B) or EGFP-L2 (panels C and D) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h post transfection. The DAPI (4’, 6’-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
Figure 4. The nNLS of HPV16 L2 can mediate nuclear localization of HPV16 L2 in vivo. HeLa cells were transfected with either EGFP-L2 (panels A and B), EGFP-L2ΔC (panels C and D), EGFP-L2ΔCsbN (panels E and F), or EGFP-L2ΔNΔC (panels G and H) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4′, 6′-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
Mutation of basic residues in the nNLS of HPV16 L2 inhibits its nuclear import ability. HeLa cells were transfected with either EGFP-L2 (panels A and B), EGFP-L2ΔC (panels C and D), EGFP-L2ΔCsbN (panels E and F), or EGFP-L2ΔNΔC (panels G and H) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4', 6'-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
Figure 5. The cNLS of HPV16 L2 can mediate nuclear localization of HPV16 L2 in vivo. HeLa cells were transfected with either EGFP-L2 (panels A and B), EGFP-L2ΔN (panels C and D), EGFP-L2ΔNsbC (panels E and F), or EGFP-L2ΔNΔC (panels G and H) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4’, 6’-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
Figure 5.`
Mutation of basic residues in the cNLS of HPV16 L2 inhibits its nuclear import ability. HeLa cells were transfected with either EGFP-L2 (panels A and B), EGFP-L2ΔN (panels C and D), EGFP-L2ΔNsbC (panels E and F), or EGFP-L2ΔNΔC (panels G and H) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4’, 6’-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
Table 2.
Quantitation of subcellular localization of HPV16 L2 and mutants. Localization of HPV16 L2 full length and mutant proteins was quantitated in multiple transfection assays. The mean and standard deviation for protein localization is shown.

<table>
<thead>
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<th>Protein</th>
<th>Mostly Nuclear (%)</th>
<th>Throughout the Cell (%)</th>
<th>Mostly Cytoplasmic (%)</th>
<th>Total Cell Number</th>
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Figure 6.
Subcellular localization of HPV16 L2 minor capsid protein and mutants.
Quantitative analysis data from Table 2 has been represented graphically.
Figure 7.
The nNLs and cNLS of HPV11 L2 can independently mediate nuclear localization of HPV11 L2. HeLa cells were transfected with either EGFP (A, panels A and B), EGFP-L2 (A, panels C and D), EGFP-L2ΔN (B, panels A and B), EGFP-L2ΔC (B, panels C and D), or EGFP-L2ΔNΔC (B, panels E and F) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4’, 6’-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
Figure 8.
Mutation of basic residues in the nNLS of HPV11 L2 inhibits its nuclear import ability. HeLa cells were transfected with either EGFP-L2 (panels A and B), EGFP-L2ΔC (panels C and D), EGFP-L2ΔCsbN (panels E and F), or EGFP-L2ΔNΔC (panels G and H) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4’, 6’-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
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**Figure 9.**
*Mutation of basic residues in the cNLS of HPV11 L2 inhibits its nuclear import ability.* HeLa cells were transfected with either EGFP-L2 (panels A and B), EGFP-L2ΔN (panels C and D), EGFP-L2ΔNsbC (panels E and F), or EGFP-L2ΔNΔC (panels G and H) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4’, 6’-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
Figure 9.
Mutation of basic residues in the cNLS of HPV11 L2 inhibits its nuclear import ability. HeLa cells were transfected with either EGFP-L2 (panels A and B), EGFP-L2ΔN (panels C and D), EGFP-L2ΔNsbC (panels E and F), or EGFP-L2ΔNΔC (panels G and H) plasmids, using FUGENE 6, and were examined by fluorescence microscopy at 24 h posttransfection. The DAPI (4’, 6’-diamidino-2-phenylindole) staining of the nuclei is shown in the left column and the fluorescence of the EGFP is shown in the right column.
### Table 3.
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Figure 10.
Subcellular localization of HPV11 L2 minor capsid protein and mutants.
Quantitative analysis data from Table 3 has been represented graphically.
Works Cited


