Studies of the Nuclear Localization Signal and Pathway of E2 Protein of High Risk HPV 16

Author: Veniamin Ilich Slavitskiy

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STUDIES OF THE NUCLEAR LOCALIZATION SIGNAL AND PATHWAY OF E2 PROTEIN OF HIGH RISK HPV 16

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

July 2014
Abstract

Studies of the Nuclear Localization Signal and Pathway of E2 Protein of High Risk HPV 16

Veniamin Slavitskiy

Thesis Advisor: Prof. Junona Moroianu, Ph.D.

Human papillomaviruses (HPVs) are the most common sexually transmitted infection in the United States. High risk HPV types, including HPV 16, can cause cervical carcinomas upon infecting squamous basal epithelial cells. The HPV E2 protein is a multifunctional protein that regulates viral DNA replication and expression of a large number of cellular and viral genes, including the E6 and E7 viral oncogenes.

Previous research in the Moroianu lab has identified a novel alpha-helical nuclear localization signal (NLS) in the C-terminal domain of HPV 16 E2 protein (75). Here, we focused on continuing the dissection of the HPV 16 E2 NLS and on identification of the nuclear import mechanism used by this protein.

We identified several residues in the C-terminal domain of HPV 16 E2 (327KHK329) and within the NLS (K299, C300) that enhance the function of the NLS. Additionally, we determined that dimerization of the C-terminal domain plays an important role in the nuclear import of HPV 16 E2 as a mutation that disrupted it led to a significant decrease in the nuclear localization of the protein.
We discovered that importin 11 karyopherin is a nuclear import receptor for HPV 16 E2. Our data suggest a nuclear import mechanism for HPV 16 E2 whereby UbcM2/UBE2E3 E2-type ubiquitin-conjugating enzyme acts as an adapter to bind HPV 16 E2 to importin 11 karyopherin for its nuclear import. This is a previously undescribed nuclear import mechanism which may have implications for the control of HPV 16 E2 functions.
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Acknowledgements

First and foremost, I would like to thank my thesis advisor, Prof. Junona Moroianu. I would have never gotten to this point without her guidance and support. I know I have not been an ideal graduate student, yet she has always been very patient, understanding, kind and supportive. She is an exemplary advisor, a wonderful person and a true blessing to everyone who has ever worked in her lab.

Many thanks go to my committee members, Dr. Charlie Hoffman, Dr. Marc-Jan Gubbels, Dr. David Newburg and Dr. Anthony Annunziato. I very much appreciate you taking the time and effort to help me with this project. I have learned a lot from all of you.

My time at Boston College would not be the same without teaching. I owe a great deal of gratitude to Dr. Clair O’Connor, Dr. Doug Warner, Dr. Carol Halpern, and Dr. Lisa Nelson. They have taught me how to be a better teacher and supported my passion for teaching.

I would like to thank my labmates and friends, Dr. Jeremy Eberhard and Dr. Zeynep Onder, for their advice, technical support and assistance with experiments; but most of all for their friendship, optimism, smiles, discussions of European soccer and Japanese martial arts, moving furniture up two flights of stairs, sharing their secrets and otherwise making the lab a place that felt like home.
This project owes a lot to the efforts of Kelley Dentino and Erin Groden, the undergraduate researchers whom I had the privilege to mentor. I very much appreciate their hard work.

Many accolades go to the Biology office staff, and especially to Peter Marino, for always being there when help was needed.

I want to thank my friends Dr. Deborah Ritter, Dr. Brooke Anderson-White, Dr. Heather McKay-Gudejko, Andrew Dinninger, Dr. Katie Moorhouse, Dr. Megan Farrell, Dr. Amit Indap, Ana Medeiros, Rashmi Dubey, Sudeshna Saha and many others who have always been there when I needed a shoulder to cry on. I will always remember our lunch conversations and defense celebrations at Sunset.

Last, but not the least, I want to thank my family. No amount of words can express how much I appreciate what they have done for me over the years. Maria Slavitskaya, my wife and my best friend, you are the most loving and patient person I know. Thank you for sticking together with me, I would not have been able to pull this through without you. Alisa, my little collection of “why’s”, you are the reason I live. Thank you for allowing daddy to disappear to the lab and do experiments. I hope one day you will follow this path and go further than I could. My mom and dad, Ilya Slavitskiy and Nina Slavitskaya, I owe you everything that I am. I am here because of your love, support and advice but most of all, because of your continuing belief in me. Finally, my love and gratitude goes to my grandmother, Dr. Tamara Paperno, who introduced me to the world of science when I was five years old, and who never stopped encouraging me. It is to her that I dedicate this thesis.
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<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>6His</td>
<td>Polyhistidine Tag</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase Promoting Complex</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BPV</td>
<td>Bovine Papillomavirus</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent Kinase</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding Domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>HeLa Cytosol</td>
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<td>HDAC</td>
<td>Histone Deacetylase</td>
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<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
<td>HRP</td>
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</tr>
<tr>
<td>HSPG</td>
<td>Heparan Sulfate Proteoglycan</td>
</tr>
<tr>
<td>Imp11</td>
<td>Importin 11</td>
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<tr>
<td>Kap</td>
<td>Karyopherin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani Medium</td>
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<tr>
<td>NE</td>
<td>Nuclear Envelope</td>
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<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
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<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>NPC</td>
<td>Nuclear Pore Complex</td>
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<td>Nup</td>
<td>Nucleoporin</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PV</td>
<td>Papillomavirus</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous Sarcoma Virus</td>
</tr>
<tr>
<td>SC</td>
<td>Synthetic Complete Medium</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Vacuolating Virus 40</td>
</tr>
<tr>
<td>TB</td>
<td>Transport Buffer</td>
</tr>
<tr>
<td>TEM</td>
<td>Tetraspanin-enriched Microdomain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like Particle</td>
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</table>
Chapter 1

Introduction
Human Papillomaviruses

Papillomaviruses (PV) are small (around 60 nm in diameter), nonenveloped viruses with icosahedral capsid and double-stranded circular DNA genome that infect squamous basal epithelial cells causing a wide range of disease, including cancer (1-5). To date, more than 200 types of PVs have been identified (http://pave.niaid.nih.gov) of which the majority are human papillomaviruses (HPV). However the host range of PVs includes many species of mammals, birds and reptiles and it is likely that all amniotes harbor a variety of PV (6, 7). PVs are highly species-specific and cross-species infections are extremely rare (6, 8). It is believed that PVs evolve to occupy novel ecological niches within their host species, rather than adapt to a different host, therefore diversity within PVs infecting any one host species could be quite extensive (8).

PVs are classified based on the sequences of their L1 gene and are divided into genera named after letters of Greek alphabet, from Alpha to Pi (10-12). HPVs are found within Alpha, Beta, Gamma, Mu and Nu genera (10-12) with the majority of them (90%) belonging to Alpha and Beta genera (1). Furthermore, based on their propensity to cause cancer, HPVs are classified into low risk or high risk types. The low risk types (e.g. HPV 6 and 11) cause mostly benign genital warts (condyloma acuminata), while the high risk types (e.g. HPV 16, 18, 31, 45) can induce the development of anogenital cancers, including cervical cancer, upon infection (1, 3, 15, 16). In particular, HPV 16 demonstrates viral fitness and rate
of persistence unmatched by other HPV types (50). It has been demonstrated that high risk HPV types produce fewer virions reducing the per-contact transmission rate but permitting longer infection (thus increasing the chance of a malignant outcome), whereas low risk HPV types produce large number of virions increasing the per-contact transmission rate but allowing for faster clearance by the immune system. The relative evolutionary advantage of each strategy depends on the sexual behaviors in a particular population (16).

Interestingly, most PVs do not optimize their genetic code to align with the host species codon preferences. Since PVs rely on the host for translation, this may seem as a disadvantage, however, at least in the case of HPVs, the virus prefers to use the codons that are most likely to result in amino acid changes due to “unsafe” nucleotides in the wobble position. This could be regarded as an evolutionary mechanism constantly generating novel protein variants and expanding PVs diversity (7, 9).

HPV infections have been causing disease since the ancient times. An HPV-induced wart was found on the foot of the Egyptian mummy from 12 century BCE and in 400 BCE “Father of Medicine” Hippocrates described a way to treat penile warts using immune-stimulating herbs (13). In the early 20th century, Giuseppe Ciuffo demonstrated that the warts had viral origin by injecting himself with a cell-free extract of a warty tissue. Later, in 1933, Richard Shope used extracts from papillomas in cottontail rabbits to show that the virus was species-specific and Peyton Rous could induce malignant transformation of the benign papillomas by
exposing them to chemical agents, thus demonstrating the possibility of HPV to cause cancer (13, 14).

The PV genome is on average 8 kb in size and encodes 8 or 9 open reading frames (ORFs) (1). The viral transcripts are initiated at two major promoters, the early one that initiates upstream of E6 ORF and the late one that initiates transcription at several heterogeneous sites clustered around nucleotide 670 in HPV 16 and 742 in HPV 31 (1, 15). All known PVs encode at least five proteins: L1, L2, E1, E2 and E4. Additionally, many PVs, including the high-risk HPV 16 and 18, encode E5, E6 and E7 proteins (8). The PV genomes do not encode polymerases or other enzymes necessary for viral replication and rely on the host cell’s machinery for DNA replication and protein synthesis (23).

The viral capsid is composed of two proteins: 360 molecules of the major capsid protein L1 are organized into 72 pentameric capsomeres that form an icosahedral lattice. The core of the capsomeres is composed of a conserved antiparallel β-sandwich while the outwards facing loops are highly variable and determine the type specificity of a particular virus. One molecule of a minor capsid protein L2 is present at the center of each capsomere at the virion vertices (1, 2, 17, 18).

The E1 is the most conserved protein among PVs (1, 15) and forms a complex with E2 protein at the origin of replication where E1 acts as a DNA helicase (1, 8, 15, 19, 20). E2 also acts as a transcription factor regulating, among other genes,
the expression of E6 and E7 proteins (1, 8). Additionally, E2 is involved in viral DNA replication, genome maintenance and other functions (21). As E2 protein is the major focus of this work, it will be discussed in more detail in subsequent sections.

The E4 protein is expressed by alternative splicing as a fusion with part of E1 to generate E1^E4 protein. Its role is thought to be in viral egress, regulation of gene expression via interaction with E4-DBD RNA helicase and cell cycle progression control where E1^E4 is able to induce a G_2 arrest thus potentially counteracting the effects of E7 which functions to initiate progression to S phase (1, 15, 22).

The E5 protein is thought to contribute to transforming activities of E6 and E7 oncoproteins. E5 is a transmembrane protein that localizes primarily in the endoplasmic reticulum and the Golgi where it acts as a receptor for growth factors contributing to cell proliferation. Also, E5 may be involved in reducing the surface levels of major histocompatibility complex class I proteins leading to reduced immune response to viral infection (15, 23). The E6 and E7 oncoproteins are the primary cause of high-risk HPVs transforming abilities and their functions will be discussed below in the context of HPV-induced cancer development.
PVs infect through microwounds that expose the basal epithelial layer (1, 15). Most PV types studied to date use heparan sulfate proteoglycans (HSPGs) as the primary attachment receptors (1, 2, 5, 15, 24, 32, 43). The surface-exposed basic residues of L1 mediate the attachment (2, 24). Following the attachment of the viral particle to the target cell’s surface, cyclophilin B, a chaperone protein, is thought to initiate a conformational change affecting L1 and L2 and leading to the exposure of the N-terminal end of L2 which seems to be required for successful internalization of the virus (2, 24). It has also been previously suggested that integrins α6 and α4β6 may play a role as PV cell surface receptors (1, 25). The viral endocytosis is thought to occur via tetraspanin-enriched microdomains (TEMs) for HPV 16 (2, 3), although previously clathrin and calveolin were implicated for this role (2-4, 33). It is possible that individual types of PV use different pathways for viral entry (2). Following their entry into the cell, PV virion particles disassemble in the late endosomes and lysosomes and the viral DNA is transferred to the nucleus by a mechanism involving the minor capsid protein L2 (1, 2, 26-29). First viral transcripts can be detected about 12 hours post infection (1) and E1 and E2 proteins are expressed first followed by other Early genes (1, 15, 21, 23). The action of E6 and E7 oncoproteins prevents the terminal differentiation of the basal epithelial cells (1, 15, 23, 25). Eventually, the infected cell moves up from the stratum basale into the suprabasal layer, the viral DNA is replicated in high copy number and the Late genes are expressed, resulting in the viral particle assembly and release (1, 15, 23, 25).
There is a rather strong association between HPV infection and cancer. Specific types of HPV (HPV 16, 18, 31, 33, 45, 51) are associated with over 95% of all cervical tumors and although the progression to cancer is relatively rare, cervical cancer is the third leading cancer killer of women worldwide (23, 34, 38, 42, 44). While in the United States the number of cervical cancer cases have declined by 80% in the last 50 years due to Pap smear screening, worldwide almost 500,000 new cases are diagnosed yearly (15). Moreover, immunocompromised populations, such as HIV-positive individuals, are at an increased risk for HPV infection and associated cancers (51). Large percentages of oropharyngeal, penile, vulvovaginal and anal cancers have been linked with HPV 16 and 18 as well (25, 44, 45). In addition, benign anogenital warts (condyloma acuminata) also present a significant medical problem and HPV infections make the most common sexually transmitted disease worldwide (46, 47, 48). Recently available vaccines target HPV 6, 11, 16 and 18, and are based upon L1-only virus-like particles (VLPs) (18, 43). A therapeutic vaccine based on HPV 16 and 18 E6 and E7 antigens is currently in clinical trials (133). Since it has been demonstrated that persistent HPV 16 infection results in toll-like receptors (TLRs) downregulation, TLR agonists, such as imiquimod, could be potentially used to treat HPV infections (49) and therapies based on making HPV infected cells sensitive to antiviral drugs via HPV E2-driven expression of herpes simplex virus thymidine kinase have been proposed (134). Additionally, in separate trials viral oncogenes E6 and E7 have been targeted with siRNA (135) and viral E1, E2, E6 and E7 proteins have been targeted with small molecule inhibitors (136).
As mentioned above, during normal progression of HPV infection, viral E2 protein is expressed to regulate the expression of other Early genes, notably E6 and E7. This allows for precise control of viral DNA copy number. However, in some cases, recombination events lead to integration of E6 and E7 oncogenes into the host cell’s genome. Integration usually disrupts E2 gene leading to unregulated expression of E6 and E7. Moreover, the mRNAs expressed from integrated copied of oncogenes have increased stability and copy number which imparts a selective advantage to the cells with integrated oncogenes (23). The integration usually occurs in the vicinity of common fragile sites and may lead to disruption of important cellular genes, for example Notch1 (36, 109, 110). It should be noted that oncogenes integration is incompatible with the normal viral life cycle and confers no evolutionary advantage to the virus, therefore cancer must be considered an unfortunate side effect of the HPV infection (23, 34, 39, 42).

The E7 oncoprotein has multiple binding targets in the host cell including Retinoblastoma protein (pRb), histone deacetylases (HDACs), p21 and p27 CDK inhibitors and cyclins. E6, in turn, targets p53 for degradation, activates telomerase expression and modulates the activities of PDZ domain-containing proteins and tumor necrosis factor receptors. Additionally, E6 and E7 induce genomic instability by inducing mitotic defects and then allowing the cells with abnormal centrosomes to proliferate via relaxation of G2-M checkpoint controls that are normally regulated by p53. E6 and E7 also induce DNA damage via the ATM-ATR pathway. Moreover, the HPV oncogenes target cytokine expression
and interferon response, contributing to immune evasion (1, 15, 23, 25, 34, 35, 37-42, 45, 144).

**Nucleocytoplasmic Transport of Macromolecules**

Eukaryotes, unlike prokaryotes, possess a nucleus meaning that the chromosomes are surrounded by a double membrane called the nuclear envelope (NE). The NE allows for physical separation of DNA and RNA transcription, occurring within the nucleus, from protein synthesis taking place in the cytoplasm. Such separation is crucial to maintain precise control over numerous cell processes; however the cell must also have a way to transport proteins and RNAs across the NE. This transport is accomplished by large protein complexes called nuclear pore complexes (NPCs) that are essentially pores spanning the NE at the points where the two NE membranes join together to form trans-NE channels (52-56).

Structurally, the NPC consists of about 30 different proteins known as nucleoporins (Nups) organized into an octagonally symmetrical cylinder. The NPC is a very large (60-125 MDa in vertebrates) structure of 400-600 protein molecules; however because of the 8-fold symmetry of the complex and because the proteins are present in multiple copies, only about 30 unique Nups are required. The structural Nups form the central core of the NPC. These proteins are organized into three rings (inner, outer and membrane rings) that form the
central channel. The NPC is anchored in the NE by the circular membrane of the nuclear pore passing between the membrane ring and the inner/outer rings and integral membrane proteins, termed Poms. The “FG Nups”, so named because of their phenylalanine-glycine repeats, attach to the inner face of the NPC core structure and form a mesh-like matrix providing attachment sites for the cargoes being transported by the NPC. On the cytoplasmic side of the complex, eight FG Nups provide points of attachment for cargoes, while on the nucleoplasmic side eight of their counterparts, linked by a distal ring, form a basket-like structure (52-58).

The central channel of the NPC has a diameter of about 30 nm which allows free passage of molecules of less than 40 kDa or 5 nm in diameter. Larger macromolecules overcome these limits my either interacting directly with the NPC Nups or by employing soluble transport factors. In this way, molecules up to 39 nm in diameter can be translocated through the NPC (54). The most common group of transport factors is the evolutionary conserved karyopherin-β (Kapβ, importin/exportin) family. Karyopherins interact with their cargoes either directly or indirectly via adapter proteins, for example, karyopherin-β1/importin β interacts via karyopherin-α adapters (52-60).

The proteins destined to be transported into or out of the nucleus possess targeting amino acid sequences: nuclear localization signal (NLS) for nuclear import or nuclear export signal (NES) for nuclear export. These sequences are
required and sufficient to target a protein into and out of the nucleus. To date, many types of NLSs have been identified. The first one to be characterized and one of the most frequent is the so-called “classical” monopartite NLS, a short stretch of basic amino acids (KKKRK, the NLS of SV40 T antigen). More complex classical “bipartite” NLSs consist of two basic clusters separated by a spacer of 10-12 amino acids. While the cargoes containing classical NLS require the aid of karyopherin-α adaptor, most cargoes bind directly to importins. Many different NLSs have been identified, often containing positively charged amino acids. In many cases, the three-dimensional structure of the NLS is critical for its proper function. Kapβ proteins may assume a number of different conformations which allow them to bind and transport cargoes with different NLSs. In this way, a limited number of transport receptors are sufficient to transport thousands of dissimilar cargoes. Some cargoes require only one karyopherin whereas the concerted binding of several karyopherins is required for transport of other cargoes. Cargoes do not always use import receptors exclusively and some cargoes are able to utilize a pool of several karyopherins (52-61, 63-65).

NESs are recognized by exportins. The best characterized nuclear export sequence is the hydrophobic leucine-rich NES that is recognized by exportin CRM-1. CRM-1 is able to transport proteins either by binding to them directly or with the help of adaptors containing NES. Other proteins rely on different exportins. In some cases, phosphorylation of the cargo protein is required for export, while in others phosphorylation inhibits nuclear export (55, 60). It is worth
noting that the “importin”/“exportin” terminology is somewhat vague as there are karyopherins that function in both nuclear import and export, although different cargoes are transported in each direction (54, 62).

The major driving force for the transport of molecules across the NE is a small (25 kDa) GTPase Ran. In its GTP-bound form, RanGTP, it is able to interact with karyopherins and cycles across the NE. In the cytoplasm, RanGAP and RanBP1 amplify the GTPase activity of Ran; in the nucleus, RanGEF (RCC1) acts as a nucleotide exchange factor, converting RanGDP to RanGTP. This results in a gradient of RanGTP across the NE which determines the directionality of the transport. Consequently, import complexes are formed in the cytoplasm where RanGTP concentration is low and dissociate in the nucleus where RanGTP concentration is high, whereas export complexes form in the nucleus in the presence of high concentration of RanGTP and dissociate in the cytoplasm following GTP hydrolysis by RanGAP (54, 55, 59, 60, 63, 64, 66). It should be mentioned that many Ran-independent import and export pathways exist (54, 55, 67) and in addition to the Ran gradient, the nucleocytoplasmic transport is regulated in a variety of ways. This regulation includes inter- or intramolecular masking of the NLS/NES, amplification of the NLS/NES by phosphorylation, retention of a protein in a particular cellular compartment by association with another factor, transport in complex with other proteins and changes in availability of karyopherins or nucleoporins (54, 55, 60).
Nucleocytoplasmic Transport of Papillomavirus Proteins

PVs depend on the host cell for their DNA replication and protein synthesis (23). Consequently, viral proteins and DNA have to enter and exit the nucleus (31). Similar to many other viral proteins (64), PV proteins have evolved to utilize the host cell’s protein transport machinery, as has been demonstrated by a large body of research, including work done in the Moroianu lab.

L1 major capsid protein of HPV 11, 16 and 45 is imported by a classical pathway, using Kapα2β1 heterodimers as import receptors. L1 import is important during the productive phase of the infection when the L1 and L2 proteins are needed for capsid assembly. In addition to the classic import pathway, L1 of HPV 11 interacts with Kapβ2 and Kapβ3 and inhibits the transport of these karyopherins into the nucleus (30, 31, 68-70).

L2 minor capsid protein of HPV 11, 16, 18 and Bovine Papillomavirus (BPV) 1 has two NLSs located at its positively charged termini. These NLSs may function independently and interact with Kapα2β1 heterodimer in a classic import pathway, as well as with Kapβ1, Kapβ2, Kapβ3 (HPV 11); Kapβ2 and Kapβ3 (HPV 16 and 18). The L2 proteins of these HPV types may utilize multiple import strategies. In addition, HPV 16 L2 has an NES mediating its nuclear export and a nuclear retention sequence (NRS) that is essential during the initial phase of infection (26-29, 71).
E1 DNA helicase of HPV 11 contains a CRM-1 binding NES that is dominant over its NLS. The NES is controlled via phosphorylation by cyclin-dependent kinases (CDKs). Similar systems of control exist in HPV 31 and BPV1 E1 but different residues are phosphorylated. BPV1 E1 has been shown to be imported by Kaps α3, α4 and α5 (20, 72-74).

E2 proteins contain different NLSs and NESs depending on the virus type and utilize various transport mechanisms (75-79, Chapter 3 of this thesis). As nuclear import of HPV 16 E2 protein is the topic of this research project, the relevant background information will be considered in detail in subsequent sections. E5 oncoprotein of HPV 16 has been shown to interact with karyopherin β3 (80), while E6 oncoprotein interacts with karyopherins β1 and β2 via its C-terminal NLS and also contains a classical NLS that interacts with Kapα2β1 heterodimer (81, 82). Additionally, a number of putative NLSs have been identified in HPV E6 proteins using a computational biology approach (89).

E7 oncoproteins of HPV 5, 8, 11 and 16 are imported via a Ran-dependent non-classical pathway that involves a c-terminal NLS with a functionally required zinc finger motif. This NLS interacts directly with the FG repeats of Nup62 and Nup153. Also, HPV 11 and 16 E7 oncoproteins contain a leucine-rich NES within the zinc finger domain that mediates nuclear export via a CRM1-dependent pathway (83-88).
In summary, papillomavirus proteins have evolved a variety of different import and export pathways. As such, elucidation the exact mechanism of nucleocytoplasmic transport for a particular PV protein presents an interesting scientific challenge.

**HPV 16 E2 Protein and its Nucleocytoplasmic Transport**

The primary focus of this research project is to elucidate the nucleocytoplasmic transport strategies of the high-risk HPV 16 E2 protein. Consequently, the last section of this introduction reviews the existing body of research on HPV 16 E2’s structure, function and nucleocytoplasmic transport.

**HPV 16E2 structure**

E2 proteins have a molecular weight of about 50 kDa (15, 42, 90). They consist of two conserved domains, each with a distinct function: amino-terminal transactivation domain and carboxy-terminal DNA-binding domain. The domains are connected by a variable hinge (linker) region (21, 42, 75, 90-96, 103, 105).

The structure of the N-terminal domain has been determined by X-ray crystallography for HPV 11, 16, 18 and BPV1. The transactivation domain forms a cashew-shaped structure where the N-terminal half of the domain is comprised of three long alpha helices folded into an anti-parallel bundle, whereas the C-
terminal half of the domain contains anti-parallel beta sheets. These two regions are connected by a two-fold helix and the N-terminal alpha helix tightly packs against the beta sheet (21, 90, 94). Residues important for transactivation (Arg37, Ile73 in HPV 16) and replication (Glu39) are found on the outside, solvent-exposed surfaces of the domain and are highly conserved (21, 90). The intact transactivation domains of HPV 16 E2 dimerize and the same residues that are essential for transactivation are also necessary for dimerization. It has been suggested that HPV 16 E2 N-terminal domains form “interdimers” by bringing together two already dimeric E2 molecules (formed via C-terminal domains, see below) bound to opposite sides of a DNA loop. This helps to stabilize the DNA loops and is essential for transactivation and replication (21, 90, 97). The N-terminal domain dimerization occurs via different residues in BPV 1 E2 and is not strictly required for transactivation in that PV type (21, 98), while in HPV 11 and 18, the N-terminal domain is monomeric (21). This variability may underscore important differences in transactivation and function between various PV types.

The structure of the C-terminal DNA-binding domain has been determined by X-ray crystallography and NMR spectroscopy for HPV 6, 16, 18, 31 and BPV 1 E2 proteins. The domain’s structure is a dimeric eight-stranded anti-parallel beta barrel with two surface alpha helices making up the DNA recognition surface (Fig. 1). The DNA binding site bends smoothly around the C-terminal domain to allow the interaction of the recognition helices with the major grooves of the DNA (21, 91-93, 95, 96). Each monomer of the C-terminal domain is composed of two four-
stranded anti-parallel beta sheets and two alpha helices found on one surface. Extensive hydrophobic interactions between the beta sheet and alpha helices stabilize the structure and these interactions are highly conserved between the E2 proteins of different PV types. The order of secondary structure elements is $\beta_1$-$\alpha_1$-$\beta_2$-$\beta_3$-$\alpha_2$-$\beta_4$; $\alpha_1$ is the recognition helix (91-93, 96). Although all known E2 proteins bind to the same palindromic DNA sequence (ACCgNNNNcGGT) the amino acid composition of the “spacer” (NNNN) varies between PV types and determines the specificity of E2 proteins. Additionally, the 8-residue loop between $\beta_2$ and $\beta_3$ strands is disordered in HPV 16 E2 and it is not conserved, suggesting its function as another specificity determinant (91). Significant differences in DNA binding affinity exist between low and high risk HPV types and may determine the differences in transforming abilities of these viruses (91-93, 96).

Several residues are important in the formation of the E2 C-terminal domain dimer interface. These are hydrophobic residues, mostly Ile, His, Trp and Met. The most important residue is the highly conserved Trp360 in BPV 1 (corresponding to Trp319 in HPV 16) that is called the “tryptophan bridge” and His288 in HPV 16 (conserved in HPV 18 and 33 but not in HPV 11 or BPV 1) (21, 91-93, 99-102). Interestingly, the single chain mutants of HPV 16 E2 C-terminal domain show a 5-fold increase in the strength of binding to specific DNA sites, suggesting that the dimeric form evolved as a mean to precisely control E2 binding to DNA needed for the protein’s functionality (104).
E2 is one of the five proteins encoded in the genomes of all known PVs (8). E2 proteins have been established as the major transcriptional regulators of PVs. They can bind to specific sequences on the viral DNA and activate or repress transcription by recruiting cellular factors or by preventing the binding of cellular factors by steric hindrance, often achieved via short forms of E2 that compete with full length protein for DNA binding sites (21).

Most importantly, E2 regulates the expression of E6 and E7 viral oncogenes via its transactivation domain and consequently suppresses the uncontrolled cell growth that may potentially lead to cancer (106, 107). Also, E2 is able to directly bind to E7 and thus inactivate the former protein by competing with E7’s natural targets (108). The loss of E2 gene that occurs when HPV genome is integrated into the host cell’s chromosome leads to uncontrolled expression of E6 and E7 oncoproteins and the development of associated cancers (21, 35, 37).

E2 directly binds to E1 helicase via E2’s N-terminal domain (111) and loads E1 onto the viral origin of replication thus serving as an enhancer in the initiation of viral replication (21). A form of E2, called E8^E2C, where the N-terminal domain is replaced with a product of E8 viral gene, functions as a transcriptional repressor by competing with active form of E2 for DNA binding sites (21, 112).
The PV genome needs to be tethered to the host chromosomes in mitosis for retention, maintenance and partitioning. The E2 performs this tethering by binding to the viral DNA with its DNA-binding domain and to mitotic host chromosomes with its transactivation domain via protein adapters, the best studied of which is Brd4 protein (21). E2 proteins of HPV 16, 31, BPV 1 and other (but not all) PV types interact with Brd4 and form stable heterotetramers via the same residues that are involved in formation of N-terminal E2 dimers (21, 90, 113, 114, 116). The interaction between E2 and Brd4 is enhanced by C-terminal dimerization of E2, thus making the formation of C-terminal dimers essential for E2 tethering function (115).

E2 proteins are known to induce growth inhibition and apoptosis in host cells (21). The exact mechanism of this in HPV negative cells is unknown, however p53 dependent pathways appear to be involved (21, 117, 118). Other mechanisms of E2 induced apoptosis may involve caspase 8 (118). In HPV positive cells (e.g. HeLa cells), E2 down-regulates E6 and E7 expression and, since HPV positive cells are dependent on the viral oncogenes expression for their sustained growth, expression of E2 leads to increased apoptosis (21, 106). This appears to happen via pRb and p21\textsuperscript{CIP} dependent pathways (119). The rate of apoptosis in HeLa cells is increased from 6% to 20% following the expression of E2 in HeLa cells (117).
E2 is a rather short-lived protein, with half-life around 45-50 minutes (121, 122). It is degraded via a proteasome degradation pathway involving SCF\textsuperscript{Skp2} ubiquitin ligase at the end of G1 phase. The ubiquitination and degradation are preceded by phosphorylation at specific serine residues (298 and 301 in BPV 1) by casein kinase II (120, 121, 123, 124). The degradation occurs via interactions in the transactivation domain and deletion of this domain increases the proteins half-life more than 6-fold to 6 hours (121).

The stabilization of E2 may happen via phosphorylation by Cdk2 in S phase of the cells cycle (122). Also, NRIP (nuclear receptor interaction protein) binds the transactivation domain of E2 and stabilizes it by recruiting calmodulin which activates the phosphatase calcineurin to dephosphorylate E2. Since Ca\textsuperscript{2+} acts as a regulator of keratinocyte differentiation, it stands to reason that Ca\textsuperscript{2+} levels are used to regulate E2 expression (125).

E2 protein is generally viewed as a repressor of oncogenic transformation because it regulates the expression of E6 and E7 oncogenes and because E2 is generally lost in HPV-induced cancers. However, E2 proteins of high-risk HPVs have been shown to promote polyploidy, chromosomal mis-segregation and centrosome amplification leading to genomic instability. The interactions between high-risk E2 and activators of the anaphase promoting complex (APC), Cdc20 and Cdh1, have been implicated in these processes (126). One hypothesis proposes that E2 brings the viral DNA close to the cellular DNA by binding to
both as described above and through E2-induced genomic instability and DNA
breaks facilitates integration of viral DNA into the cellular genome. In this view,
high-risk E2 could be considered an oncogene (127). In fact, E2 has been
proposed as an early marker of viral infection as its expression has been
detected in the cervical intraepithelial neoplasia (CIN), the stage preceding the
invasive cervical carcinoma (128).

Several studies have looked at interactions of E2 proteins with cellular molecules
in order to elucidate how E2 might influence cellular physiology. The results
indicate that E2 proteins act on a variety of cellular targets independently of E6
and E7 viral oncoproteins. The cellular processes regulated by E2 include
apoptosis, cell proliferation and differentiation, gene transcription, RNA
processing, ubiquitination and degradation of proteins and intracellular transport.
While E2 proteins of different PV types vary in their interaction networks, the end
result is leading the cell towards a convenient environment for a replicative viral
cycle (129-132).

In summary, E2 is a versatile multifunctional protein that controls viral DNA
replication and expression of a multitude of viral and cellular genes. The
complete understanding of E2’s functions remains a scientific challenge for the
future.
Nucleocytoplasmic Transport of HPV E2

E2 proteins are synthesized outside of the nucleus and they need to be transported into the nucleus and, at least in some HPV types, out of the nucleus to bind targets in the cytoplasm. As described above, all E2 proteins share the general domain structure and significant sequence homology; however their nucleocytoplasmic transport mechanisms differ.

BPV 1 E2 has two non-classic NLS sequences, one in the transactivation domain $(107^{PKRCF\text{K}GAV_{117}})$ and the other in the DNA-binding domain $(339^{KCYRFRV\text{K}KNHRHR_{352}})$ (76). Partially conserved homologous sequences exist in other PV types (75) and in HPV 18 E2 a dominant NLS has been shown to be located in the C-terminal region (78). At the same time, HPV 18 E2 contains a NES in its N-terminal domain that is absent in HPV 16 E2 (78).

HPV 11 E2, in contrast, contains a classic monopartite NLS in its hinge region $(236^{PPRKR\text{A}RPG_{244}})$ while the C-terminal NLS is much less conserved (75, 77).

Previous research in the Moroianu lab has identified the NLS in the C-terminal domain of HPV 16 E2 as $298^{LKCL\text{R}YRFKKH_{308}}$ (75). This NLS overlaps with the alpha helix involved in DNA binding (75, 91). This NLS is partially conserved in HPV 18 E2 (high risk HPV) but not in HPV 11 E2 (low risk HPV). No N-terminal NLS or NES exists in HPV 16 E2 (75). Therefore, it appears that differences exist between low and high-risk HPV E2 proteins in terms of their NLSs and moreover,
significant variability in the NLS types suggests that E2 proteins of different PV types use different karyopherins (75).

It has been demonstrated that HPV 16 E2 is able to preferentially bind importin α5 \textit{in vitro}, similar to BPV 1 E2, but HPV 11 E2 bound importin α3 and α5 with similar affinity, although these interactions have not been confirmed \textit{in vivo} (79, 129). No functional assay demonstrating the requirement for these importins has been performed and only five importins (α1, α3, α4, α5 and α7) have been tested for their binding to E2 proteins, leaving open the possibility that other importins may interact.

The alpha-helical NLS of HPV 16 E2 is rather unusual; however Gag protein of Rous sarcoma virus (RSV) contains a similar alpha-helical NLS. Gag is imported by importin α and importin 11 karyopherins, with importin 11 binding to the alpha-helical NLS in the MA domain, while importin α binds to the second NLS in NC domain to initiate import via a classical import pathway (137-139).

This research project has continued the analysis of HPV 16 E2 NLS to identify additional residues important for NLS function. Several such residues were identified, including Trp319 residue previously known to be involved in dimerization of E2 C-terminal domain (100, 102). In the second part of my research project, I have demonstrated that importin 11 is required for the nuclear transport of HPV 16 E2 protein and that additionally, E2 appears to bind importin
11 via an adapter, UbcM2/UBE2E3, which has been previously known as a cargo of importin 11 (140). In summary, my research demonstrates that nuclear import of HPV 16 E2 protein occurs via a non-classical and previously undescribed pathway and several residues outside of the previously described NLS appear to be important for efficient import.
Chapter 2

Materials and Methods
Plasmids and Mutagenesis

EGFP-E2 and EGFP-cE2 plasmids were obtained previously (75). The following mutants were generated with QuickChange site-directed mutagenesis kit (Stratagene) using the mutagenesis primers shown below. The constructs were used to transform *E. coli* XL-1 Blue competent cells (Stratagene) per manufacturer’s protocol. The plasmids were purified using Quantum MidiPrep Kit (BioRad) per manufacturer’s protocol and verified by sequencing (Eurofins MWG). Plasmids were stored at -80°C in bacterial stock cultures supplemented with 15% glycerol.

*Mutagenesis primers (5’-3’):*

**K299A**

Forward:

GGTGATGCTAATACTTTTAGCCTGTTTAAGATATAG

Reverse:

CTATATCTTAAACAGGCTAAAGTATTAGCATCACC

**C300A**

Forward:

GGTGATGCTAATACTTTAAAAGCCTTAAGATATAG

Reverse:

CTATATCTTAAAGCCTTTAAAGTATTAGCATCACC
327KHK/AAA329
Forward:
GGACAGGACATAATGTAGCGGCGCCAGTGCAATTGTTACAC
Reverse:
GTGTAACAATTGCACTGGCGGCGGCTACATTATGTCTGTCC
W319G
Forward:
GCAGTGTCGTCTACAGGGCATTGGACAG
Reverse:
CTGTCCAATGCCCTGTAGACGACACTGC
L301A
Forward:
GGTGATGCTAATACTTTAAAATGTGCAGATATAGA
Reverse:
TCTATATCTCGCAGCATTATTAAAGTATTAGCATCACC
F305A
Forward:
GTTTAAGATATAGAGCGAAAAAGCATTG
Reverse:
CAATGCTTTTCCGCTCTATATCTTTAAC
V326STOP
Forward:
CGTCTACATGGCATTGGACAGGACATAATTGAAAACATAAAAGTGC
Reverse:
GCACTTTTATGTTTTCAATTATGTCCTGTCCAATGCCATGTAGACG

A331STOP

Forward:
GGACAGGACATAATGTAAAACATAAAAGTTGAATTGTTACAC

Reverse:
GTGTAACAAATTCAACTTTTATGTTTTACATTATGTCCTGTCC

**Cell Culture**

HeLa cells (American Type Culture Collection, ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Sigma) in a 5% carbon dioxide environment at 37°C.

**Transient Transfections**

*Analysis of Mutant Intracellular Localization Phenotypes*

HeLa cells were plated on 12 mm poly-L-lysine coated glass coverslips in 24-well plates and grown to approximately 60% confluency. The cells were transfected with 0.6 – 0.8 µg of EGFP plasmid construct using Fugene 6 transfection reagent (Roche) according to manufacturer’s protocol. Briefly, 4 µl of Fugene 6 were
mixed with the 0.6-0.8 μg of plasmid DNA in 97 μl of DMEM, incubated at room temperature for 20 minutes and added to the cells in 500 μl of DMEM (without supplements). The cells were incubated for 6 hours at 37°C in DMEM without supplements at which point the media was changed to DMEM supplemented with FBS and penicillin/streptomycin and the cells were incubated for additional 18 hours. Following this, the cells were washed 3 times with phosphate buffered saline (PBS) on ice and fixed by incubating for 10 min in ice cold 3.7% paraformaldehyde (PFA) in PBS on ice followed by 3 washes with PBS on ice. The cells were mounted on glass microscope slides and stained with DAPI-Vectashield (Vector Labs) to identify the nuclei. The cells were imaged using Leica TCS Sp5 broadband confocal microscope under 630X magnification using LAS AF software (Leica Microsystems) to obtain the images. The localization of the EGFP fusion proteins was analyzed using MetaMorph software (Molecular Devices) and the data were graphed using Prism 4 (GraphPad) and BoxPlotR software (141).

**Immunoblotting**

To ensure that the EGFP fusion proteins are intact and expressed at similar levels, the cells grown and transfected as above were solubilized with standard SDS-PAGE loading buffer and boiled for 10 minutes to lyse. The lysates were subjected to SDS-PAGE on a 12% polyacrylamide gel at 45 mA followed by a transfer to a nitrocellulose membrane at 75V for 45 minutes. The membrane was
stained with Ponceau stain to verify transfer, washed with dH$_2$O and blocked in 5% non-fat milk/0.1% Tween20 in PBS for 1 hour at room temperature on a rocker. The membrane was then incubated with a 1:1000 dilution of a primary mouse anti-EGFP antibody (Clontech) in blocking buffer for 1 hour at room temperature on a rocker, washed 3 times in the blocking buffer and incubated with a 1:1000 dilution of a secondary goat anti-mouse HRP-conjugated antibody (Santa Cruz Biotech) for 30 minutes at room temperature on a rocker, followed by 2 washes in the blocking buffer and 1 wash in PBS. The blots were developed using HyGLO Chemiluminescent HRP antibody detection reagent (Denville Scientific) per manufacturer’s protocol, exposed to HyBlot CL autoradiography film (Denvile Scientific) and detected on a Kodak X-Omat developer.

*Generation of HeLa Cell Lysates for Binding and Immunoprecipitation Experiments*

To obtain lysates of HeLa cells expressing EGFP fusion proteins for binding assay analysis, HeLa cells were grown in 75 cm$^2$ flasks to ~60% confluency and transfected with 4-8 µg of plasmid DNA using 20 µl of Fugene 6 transfection reagent (Roche) mixed into 600 µl of DMEM, incubated for 20 minutes at room temperature and added to the cells in 10 ml of DMEM. Following a 6 hour incubation at 37°C, the media was changed to DMEM supplemented with FBS and penicillin/streptomycin and the cells were incubated for additional 18 hours.
The cells were collected by treating them with trypsin for 2 minutes at 37°C, washing with DMEM and centrifugation at 3000xG for 5 minutes. The cell pellets were washed in PBS and resuspended in 100 µl of lysis buffer (0.1% Triton X-100 (Sigma), 5mM EDTA supplemented with protease inhibitors (0.05 mg/ml PMSF, 0.1 mg/ml leupeptin, 0.1 mg/ml aprotinin.) The cells were incubated under rotation for 30 minutes at 4°C and the cellular debris were collected by centrifugation at 13000 rpm in a microcentrifuge. The resulting extracts of soluble cellular components were stored at -80°C until needed.

**GST Fusion Proteins Purification**

GST-E2 and GST-cE2 plasmids were obtained previously (75). The plasmids were transformed into *E. coli* BL-21 CodonPlus cells (Agilent Technologies) per manufacturer’s protocol and the stock cultures were stored at -80°C in 15% glycerol. To purify the GST fusion proteins, the stock cultures were used to inoculate LB-ampicillin 10 ml starter culture overnight at 30°C. The starter culture was used to inoculate a 250 ml LB-ampicillin culture which was incubated at 30°C until it reached OD$_{600}$ of 0.6. GST protein expression was induced with 1 mM IPTG for 2-3 hours at 30°C, bacterial cells were collected by centrifugation and the GST proteins were purified on glutathione-sepharose beads (GE Healthcare). Briefly, the bacterial cells were resuspended in 20-40 ml of lysis buffer (0.1 mg/ml lysozyme, 10% glycerol, 0.1 mg/ml aprotinin, 0.1 mg/ml leupeptin in PBS) and lysed by sonication using Branson sonifier 450. The
lysates were centrifuged for 60 minutes at 10500 x G to remove the insoluble debris. The cleared lysates were incubated with glutathione sepharose beads for 60 minutes at 4°C under rotation. The beads were washed with wash buffer (1% glycerol in PBS) and the bound proteins were eluted with elution buffer (20 mM reduced glutathione, 0.1 mM EDTA, 100 mM Tris, pH 8, 1 mM MgCl₂, 20 mM DTT, 10% glycerol) for 60 minutes at 4°C under rotation. The purified proteins were dialyzed into transport buffer (20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DDT, supplemented with protease inhibitors (aprotinin and leupeptin at 0.1 mg/ml). Protein purity and size were verified by SDS-PAGE and the purified proteins were stored at -80°C until needed.

**In vitro Nuclear Import Assay**

The nuclear import assays were performed essentially as previously described (70). Briefly, HeLa cells were grown to 60-70% confluency on poly-L-lysine coated glass coverslips in 24-well plates. The cells plasma membranes were permeabilized with 70 µg/µl digitonin for 5 minutes on ice and washed 3 times with cold transport buffer (described above) to remove the cytoplasmic components. The permeabilized cells were then incubated with import mixes containing an energy regenerating system (1 mM ATP, 1 mM GTP, 5 mM phosphocreatine, 0.4 U creatine phosphokinase), 10 µl HeLa cytosol extract (ATCC) and 0.25-0.5 µg of various GST fusion proteins. Additionally, antibodies
against various import receptors (karyopherins) were added to the import mixes as appropriate by the experimental design (1 mg per import mix). In several experiments, the HeLa cytosol was depleted of various import receptors by incubating it with 0.5 µg of antibody per 1 µl of cytosol for 1 hour at 4°C under rotation followed by incubation with 100 µl (50 µg) of protein A sepharose beads (Sigma) for 2 hours at 4°C under rotation and centrifugation for 2 minutes at 500 rpm in the microcentrifuge to remove the beads. The depletion of karyopherins was verified by western blot.

Following the incubation with the import mixes, the cells were fixed by incubating for 10 min in 3.7% paraformaldehyde (PFA) in PBS on ice and then the nuclear membranes were permeabilized by incubating in -20°C cold methanol for 3 minutes at -20°C. In order to visualize the localization of GST fusion proteins, the fixed and methanol-permeabilized cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature, followed by incubation with a 1:1000 dilution of anti-GST antibody (GE Healthcare) in 3% BSA for 1 hour at room temperature, 3 washes with 3% BSA in PBS and incubation with 1:100 dilution of FITC conjugated rabbit anti-goat secondary antibody (Sigma) in 3%BSA in PBS for 30 minutes at room temperature. The cells were then washed 3 times with 3% BSA in PBS and 1 time with PBS. The cells were mounted onto microscope slides in DAPI-Vectashield (Vector labs) and allowed to dry. The images of the cells were taken on the Leica TCS Sp5 confocal microscope as described above or on the Nikon Eclipse TE300 microscope at 600x magnification.
**In vitro Isolation Assay**

The GST fusion proteins were immobilized on glutathione sepharose beads by following the procedure for GST fusion proteins purification above, except the final elution step was omitted. The beads with immobilized proteins were equilibrated in transport buffer (described above). Fifty µl of beads with immobilized proteins were incubated with 1 ml of HeLa cytosol extracts (ATCC) with 0.1% Tween20 (Sigma) for 2 hours at 4°C under rotation. The beads were then washed 3 times with transport buffer and the bound proteins were eluted by boiling the beads in standard 2x SDS-PAGE loading buffer for 5 minutes. The elutions were analyzed by immunoblot using primary antibodies against karyopherins of interest and secondary antibodies conjugated to HRP. The presence of GST fusion proteins on the beads was verified by immunoblot with anti-GST antibody and also by SDS-PAGE followed by Coomassie blue staining.

**In vitro Immunoprecipitation**

HeLa cells were transfected with EGFP fusion plasmids, grown and lysed as described above but the lysis buffer volume was reduced to 30 µl to increase protein concentration and 1 mM GDP was added to the lysis buffer to stabilize the import complexes. The lysates were incubated with 20 µl (5 mg) of swollen protein A sepharose beads (Sigma) for 1 hour at 4°C under rotation to remove nonspecifically binding proteins. The beads were removed by centrifugation for 5
minutes at 13000 rpm in microcentrifuge. The cleared lysates were incubated with 2.5 µg of anti-EGFP antibody (Clontech) for 2 hours at 4°C under rotation. Following this, 20 µl (5 mg) of protein A sepharose beads were added and the incubation continued for an additional 1 hour. The beads with bound antibody-protein complexes were collected by centrifugation at 500 rpm in the microcentrifuge for 3 minutes and washed 4 times with PBS. The bound proteins were eluted by boiling the beads in 10 µl of standard 4x SDS-PAGE loading buffer. The presence of putative EGFP-fusion protein-importin 11 binding was detected by immunoblot using the procedure described above; with anti-importin 11 primary antibody (Novus) diluted 1:1000 and goat anti-rabbit-HRP secondary antibody (Santa Cruz Biotech) diluted 1:1000. Alternatively, the cleared lysates were incubated with 2.5 µg of anti-importin 11 antibody and the putative interaction was detected by immunoblot with anti-EGFP primary antibody (Clontech) diluted 1:2000 and goat anti-mouse-HRP secondary antibody (Santa Cruz Biotech) diluted 1:1000.

**HPV 16 E2 and Importin 11 Binding Assay**

GST fusion proteins (GST-E2, GST-UbcM2, GST) were purified as described above and either left bound to glutathione sepharose beads or eluted and then rebound to the beads at the ratio of 0.2 µg of protein per 1 µl of beads. The beads with bound GST fusion proteins were incubated with lysate of *E. coli* culture expressing induced 6His-importin 11 protein overnight at 4°C under
rotation. The beads were washed 3 times with wash buffer (1% glycerol in PBS) and the bound proteins were eluted by boiling the beads in standard 2x SDS-PAGE loading buffer for 10 minutes. The presence of importin 11 in the elutions was detected with anti-importin 11 primary antibody (Life Span Biosciences) used at 1:250 dilution and goat anti-rabbit-HRP secondary antibody (Santa Cruz Biotech) used at 1:1000 dilution. The procedure for immunoblotting is described above. The pGex-UbcM2 and pET30-Importin 11 plasmids were kind gifts of Dr. Scott Plafker (Oklahoma Medical Research Foundation, OMRF) (140).

**HPV 16 E2 and UbcM2 Isolation Assay**

EGFP-E2 or EGFP-cE2 and EGFP-C1 (negative control) plasmids were transfected into HeLa cells for transient protein expression as described above. GST-UbcM2 and GST proteins were purified as described above and rebound to glutathione sepharose beads at the ratio of 0.2 µg of protein per 1 µl of beads. The HeLa cells lysates were prepared as described above and 50 µl of lysate was incubated with 40 µl of beads with bound GST fusion proteins overnight at 4°C under rotation. The beads were collected by centrifugation at 500 rpm for 2 minutes in microcentrifuge, washed 3 times with wash buffer (1% glycerol in PBS) and the bound proteins were eluted by boiling in standard 2x SDS-PAGE loading buffer for 10 minutes. The presence of bound EGFP fusion proteins was detected by primary anti-EGFP antibody (Clontech) diluted 1:5000 and a secondary goat anti-mouse-HRP antibody (Santa Cruz Biotech) diluted 1:1500.
Yeast Two-hybrid Binding Assay

Yeast two-hybrid bait plasmids, pGBT10-importin 11 and pGBT10-importin 11 (78-975) were kind gifts of Dr. Scott Plafker (OMRF). The yeast two-hybrid prey plasmid pACTII template was a kind gift of Dr. Charles Hoffman (Boston College).

To generate pACTII-E2 and pACTII-cE2 plasmids, the appropriate fragments were created by PCR using pEGFP-E2 plasmid as a template and the following primers to amplify the E2 and cE2 gene sequences while introducing SmaI restriction site into the PCR products.

Forward E2 primer:
GATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGACTCTTTGCCAAC
GTTTAAATG

Forward cE2 primer:
GATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGACACCC
ATAGTACATTAAAAAGGTG

Reverse primer:
CACAGTTGAAGTGACTTGGGATTTTCAGTATCTACGATTCATATAGAC
ATAAACATCCAGTAGACAC

The PCR reactions were set up using 6 ng of template plasmid DNA, 1.5 µg of forward and reverse primers, 50 mM MgSO₄, 10 mM dNTP mix, 10X Pfx amplification buffer, 1 U of Pfx DNA polymerase in a final volume of 50 µl. The PCR reaction proceeded for 2 minutes at 94°C, 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 68°C for 90
seconds, followed by a final extension at 68°C for 10 minutes. The PCR products were verified on a 1% agarose gel.

The pACTII plasmid was digested with Smal restriction enzyme (NEB) and transformed into *Saccharomyces cerevisiae* YRG2 competent cells along with either E2 or cE2 PCR products.

To generate the competent yeast cells, an overnight culture of YRG2 cells grown at 30°C was diluted to 5x10⁶ cells/ml and allowed to grow at 30°C until it reached 10⁷ cells/ml. The cells were pelleted for 5 minutes at 2000 rpm in a tabletop centrifuge and resuspended in 10 ml of sterile water, pelleted again and resuspended in 1 ml of sterile water and finally pelleted for 5 seconds and resuspended in 1x lithium acetate – TE buffer to wash, then pelleted and resuspended in 1x lithium acetate – TE at 2x10⁹ cells/ml. 50 µl of competent cells were used per transformation reaction and mixed with 10 ng of digested pACTII plasmid and 5 µl of either E2 or cE2 PCR product. 50 µg of single stranded salmon sperm carrier DNA was added to each transformation reaction, the contents were mixed with 300 µl of lithium acetate – TE – PEG and incubated for 3 hours at 30°C. Following this, 35µl of DMSO was added to each reaction, the reaction were mixed and heat-shocked in 42°C water bath for 15 minutes followed by incubation on ice for 10 minutes. The transformed cells were plated on SC medium lacking leucine (SC-leu) to allow selection for successful transformants and incubated at 30°C until colonies appeared (3 days). The colony PCR was performed on the resulting colonies to ensure the correct ligation of the insert into the plasmid. Fail Safe DNA polymerase (Epicentre) and
pACTII forward and reverse sequencing primers (IDT) were used for the colony PCR. Additionally, pACTII-E2 and pACTII-cE2 plasmids were purified using “Smash and Grab” protocol (142) and the plasmids were sequenced (Eurofins MWG).

Either pGBT10-importin 11 of pGBT10-importin 11 (78-975) plasmids were transformed into the yeast cells containing pACTII-E2 or pACTII-cE2 plasmids using the protocol above, except the transformants were plated on SC medium lacking leucine and tryptophan (SC-leu-trp) to allow for selection of double transformants. Some transformants were patched to SC-leu plate and allowed to grow for 24 hours at 30°C. This plate was then replica plated onto SC-leu-trp-his (histidine) plates containing various concentration of 3-aminotriazol as well as to SC-leu-trp plates as positive controls and to use for filter lift β-galactosidase activity assay performed as described (143).
Chapter 3

Investigation of the C-terminal NLS of HPV 16 E2 Protein
As mentioned in Chapter 1, previous research in the Moroianu lab has identified the NLS in the C-terminal domain of HPV 16 E2 ($^{298}$LKCLRYRFKKH$^{308}$) that overlaps with the alpha helix involved in DNA binding (75, 91). The original study that identified the NLS had established that RYR and KK sequences are required for nuclear import of HPV 16 E2 (75). However, other residues within the NLS were not analyzed in detail. Additionally, we hypothesized that several residues outside of the C-terminal NLS might be important for successful nuclear import based on either their similarity with established NLS sequences or their role in the maintenance of the protein’s quaternary structure (dimerization). Site directed mutagenesis was used to create several mutants, as shown in Fig. 1, and nuclear localization of these mutants was investigated by transient transfections in HeLa cells.
Figure 1: Schematic Overview of HPV16 E2 Protein

The two domains are shown. The amino acid sequence of the DNA-binding domain is shown with the wild type sequence on top and mutant sequence on bottom. Individual mutants are differentiated by colors. The α-helical NLS is underlined. The position of α1 recognition helix is shown with the red box; the position of the disordered loop is shown with the blue box. The 3-dimentional diagram of the DNA-binding domain is shown in the bottom panel (diagram is adapted from Hegde and Androphy, J. Mol. Biol. (1998) 284, 1479.)
The Role of K299 and C300 in Nuclear Import of HPV 16 E2

K299 and C300 residues are located at the N-terminal end of the HPV 16 E2 NLS. Both lysine and cysteine residues have been known to be involved in the function of NLSs of HPV proteins (see Chapter 1). The role of K299 and C300 has been investigated in the context of EGFP-16E2 and EGFP-16cE2 (residues 286-365) fusion proteins. The representative images of transiently transfected HeLa cells are shown in Fig. 2, while the quantification of the results is presented in Fig. 5 and Table 1. The results show that while for the wild type EGFP-16E2, 81% of the protein, as measured by EGFP signal, is localized to the nuclei. The K299A mutation reduces in the percentage of nuclear localization to 62.3%, a statistically significant decrease (as determined by two-tailed t-test). The C300A mutant shows a 73.4% nuclear localization, a very modest, yet also statistically significant decrease. In the case of EGFP-16cE2, the wild type protein shows an almost exclusively nuclear localization (92.7%), while for both K299A and C300A mutants, the percentage decreases by a very small amount (87.8% and 88.3%, respectively).
Figure 2: Effects of K299A and C300A Mutations on the Localization of EGFP-16E2 and EGFP-16cE2

A. HeLa cells were transfected with either EGFP-16E2 wild type (panels A and E), EGFP-16E2 K299A (panels B and F), EGFP-16E2 C300A (panels C and G), or EGFP-C1 negative control (panels D and H) plasmids.

B. HeLa cells were transfected with either EGFP-16cE2 wild type (panels A and E), EGFP-16cE2 K299A (panels B and F), EGFP-16cE2 C300A (panels C and G), or EGFP-C1 negative control (panels D and H) plasmids.

All cells were examined by confocal fluorescence microscopy 24 hours post transfection. Panels A-D represent the fluorescence of EGFP, panels E-H represent the DAPI staining of the nuclei.
W319 residue is essential for the formation of the tryptophan bridge that is a critical element in the dimerization of the DNA-binding domains of HPV 16 E2 protein (see Chapter 1). As such, W319G mutant was created in order to investigate the role of this important residue in nuclear import of HPV 16 E2. Glycine was chosen because it is non-hydrophobic and W360G mutation of the tryptophan bridge residue in BPV 1 resulted in the complete loss of dimerization (100). The representative images of HeLa cells transiently transfected with EGFP-16E2, EGFP-16cE2 or W319G mutants are shown in Fig. 3, while the quantitative data are presented in Fig. 5 and Table 1. The data show that the W319G mutation results in a very modest decrease in the nuclear localization of EGFP-16E2 (81% of the wild type protein localized to the nucleus vs. 73.3% for the mutant protein). However, in the context of EGFP-16cE2, the W319G mutation results in a significant shift from almost exclusively nuclear localization (92.7% nuclear) to pancellular localization (59.7% nuclear). It is worth noting that at 42 KDa the size of EGFP-16cE2 monomer is at the edge of the exclusion limit of the NPC, whereas EGFP at 32.7 KDa is well below the exclusion limit and, as such, is able to freely enter and exit the nucleus regardless of the presence of any NLS and NES. In this regard, it is important to notice that only 37.6% of EGFP negative control protein had nuclear localization, suggesting that EGFP-16cE2 W319G monomeric proteins do not enter the nucleus by passive diffusion.
The Role of $^{327}$KHK$^{329}$ Sequence in Nuclear Import of HPV 16 E2

The $^{327}$KHK$^{329}$ sequence is located 19 residues downstream of the C-terminal NLS of HPV 16 E2, outside of the DNA-binding alpha-helix (Fig. 1). Because HPV 16 E2 NLS, as well as several other known NLSs, contain critical lysine residues, we decided to investigate whether $^{327}$KHK$^{329}$ had a role in the nuclear import of HPV 16 E2. Fig. 3 shows the representative images of HeLa cells transiently transfected with EGFP-16E2, EGFP16cE2 or KHK327AAA mutants and Fig. 5 and Table 1 show the quantitative data. In the context of both EGFP-16E2 and EGFP-cE2, the $^{327}$KHK$^{329}$ mutant displays a less nuclear localization than the wild type protein. For EGFP-E2, 57.6% of the mutant protein is nuclear vs. 81% for the wild type protein; for EGFP-cE2, 78.9% of the mutant protein is nuclear vs. 92.7% for the wild type protein. The p-values are similar in both cases (7.07 E-38 and 3.11 E-33 for EGFP-16E2 and EGFP-16cE2, respectively). These p-values indicate a statistically significant reduction in the nuclear localization of HPV-16 E2 protein resulting from mutating $^{327}$KHK$^{329}$ sequence to AAA.

In order to verify that all of the mutant proteins described above were properly expressed in HeLa cells, the cellular lysates of transiently transfected cells were investigated by immunoblot. The results demonstrated that all of the EGFP fusion proteins were properly expressed at similar concentrations and were not degraded (Fig. 4).
Figure 3: Effects of W319G and KHK327AAA Mutations on the Localization of EGFP-16E2 and EGFP-16cE2

A. HeLa cells were transfected with either EGFP-16E2 wild type (panels A and E), EGFP-16E2 W319G (panels B and F), EGFP-16E2 KHK327AAA (panels C and G), or EGFP-C1 negative control (panels D and H) plasmids.

B. HeLa cells were transfected with either EGFP-16cE2 wild type (panels A and E), EGFP-16cE2 W319G (panels B and F), EGFP-16cE2 KHK327AAA (panels C and G), or EGFP-C1 negative control (panels D and H) plasmids.

All cells were examined by confocal fluorescence microscopy 24 hours post transfection. Panels A-D represent the fluorescence of EGFP, panels E-H represent the DAPI staining of the nuclei.
Figure 4: EGFP-16E2 and EGFP-16cE2 Mutant Proteins are Properly Expressed in HeLa Cells

A. HeLa cells were transfected with EGFP-cE2 wild type (lane 1), EGFP-cE2 C300A (lane 2), EGFP-cE2 K299A (lane 3), EGFP-cE2 KHK327AAA (lane 4), EGFP-cE2 W319G (lane 5), or EGFP-C1 plasmids (lane 6). Cell lysates were prepared 24 hours post transfection, subjected to Western blot and probed with anti-EGFP antibody.

B. HeLa cells were transfected with EGFP-E2 wild type (lane 1), EGFP-E2 C300A (lane 2), EGFP-E2 K299A (lane 3), EGFP-E2 KHK327AAA (lane 4), or EGFP-E2 W319G (lane 5) plasmids. Cell lysates were prepared 24 hours post transfection, subjected to Western blot and probed with anti-EGFP antibody.
In addition to the mutants described in this chapter, several other HPV 16 E2 mutants were generated during the course of this research project which are described in Appendix A.

To summarize, the work presented in this chapter continued the analysis of the previously identified C-terminal NLS of HPV 16 E2. We discovered that K299 and C300 residues contribute to the function of the NLS. Additionally, several residues outside of the NLS (327KHK329 and W319) were analyzed and found to play a role in the nuclear import of HPV 16 E2.
Figure 5: Effects of Mutations on Cellular Distribution of EGFP-16E2 and EGFP-16cE2

The Y-axis shows the percentage of EGFP fusion protein that is localized to the cell nucleus as determined by MetaMorph software. The data represent a cumulative average of 10 independent transfections (at least 100 individual cells) graphed as a Box plot with Tukey-defined whiskers using BoxPlot software. Boxes represent the interquartile range (IQR), or the middle 50% of the data values. The whiskers extend to the data values that are less than 1.5 IQR. The circles represent outlier data points. The solid horizontal bars represent the median values.
Table 1: Statistical Data on the Effects of Mutations on Cellular Distribution of EGFP-16E2 and EGFP-16cE2

<table>
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<th></th>
<th>WT</th>
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<th>WG</th>
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<th>cCA</th>
<th>cKA</th>
<th>cKHK</th>
<th>cWG</th>
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<td>P value</td>
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<td>3.26 E-19</td>
<td>7.07 E-38</td>
<td>1.06 E-05</td>
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<td>2.83 E-04</td>
<td>3.16 E-05</td>
<td>3.11 E-33</td>
<td>1.08 E-08</td>
<td>5.9 E-126*</td>
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<tr>
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<td>108</td>
<td>144</td>
<td>109</td>
<td>302</td>
<td>148</td>
<td>156</td>
<td>153</td>
<td>109</td>
<td>203</td>
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</tbody>
</table>

The mean values represent the percentage of EGFP fusion protein that is localized to the nucleus as determined by MetaMorph software. The P value measures significance of the data, as determined by two-tailed t-test, compared to wild type protein (either WT or cWT); smaller values represent more statistically significant change.

(*) The P value for EGFP-C1 negative control is shown vs. EGFP-16E2 (WT); the P value vs. EGFP-16cE2 (cWT) is 1.50 E-242.
Chapter 4

Investigation of the Role of Importin 11 in the Nuclear Import of

HPV 16 E2 Protein
As stated in Chapter 1, the nuclear import of E2 protein of high risk HPV 16 is dependent upon a NLS \( ^{298} \text{LKCLRYRFKKH}_{308} \) in the DNA binding (C-terminal) domain of the protein. This NLS is only partially conserved in high risk HPV 18 (LKCLRYRLRKH) and not conserved in low risk HPV 11 (LKCFRYRLNDK) (75). Unlike the classical NLS, which binds karyopherins in an extended conformation and therefore is not dependent on the secondary structure elements for its function, the 16E2 NLS overlaps with the DNA binding alpha helix (75, 91).

Literature analysis before the beginning of this research project revealed that MA domain of Gag protein of Rous sarcoma virus (RSV) contained an alpha-helical NLS that was similar in structure to the C-terminal NLS of HPV16 E2 (137). The MA domain of Gag was found to be imported in \textit{Saccharomyces cerevisiae} with the aid of Kap120 karyopherin, which is homologous to importin 11 in humans, and in avian cells via mammalian importin 11 (137, 138). Based on this, we hypothesized that importin 11 is the most likely candidate for the role of the import receptor of HPV 16 E2.

\textit{Nuclear Import Assays Demonstrate that Importin 11 is Required for the Nuclear Import of HPV 16 E2}

To analyze the importance of importin 11 for nuclear import of HPV 16 E2, anti-importin 11 antibody was used to block importin 11 during an import assay. As shown in Fig. 6, when anti-importin 11 antibody was added to the import mix, localization of GST-16cE2 changed from nuclear to cytoplasmic (panels B and C).
Adding anti-Kapβ2 antibody had no effect on the localization of GST-16cE2 (panel D). On the other hand, addition of anti-Kapβ2 to the import mix changed the localization of M9-GST from nuclear to cytoplasmic (panels F and H), whereas anti-importin 11 antibody had no such effect (panel G). GST localized to cytoplasm regardless of the presence of HeLa cytosol extracts, energy mix or antibodies. This experiment demonstrates that importin 11 is required for nuclear import of HPV 16 E2 protein and that the requirement is specific as blocking a different karyopherin had no effect on GST-16cE2 localization. The nuclear import of M9-GST control was inhibited in the presence of anti-Kapβ2 antibody which specifically blocked the karyopherin known to import the M9 NLS.
Figure 6: Nuclear Import of GST-16cE2 is Inhibited in the Presence of Anti-importin 11 Antibody

Digitonin-permeabilized HeLa cells were incubated with either GST-16cE2 (panels A-D), M9-GST (panels E-H), or GST (panels I-L) in the presence of transport buffer (TB, panels A, E, I), HeLa cytosol and energy mix (HC, panels B, F, J), HeLa cytosol, energy mix and anti-importin 11 antibody (panels C, G, K), or HeLa cytosol, energy mix and anti-Kapβ2 antibody (panels D, H, L). Following the incubation, cells were fixed with paraformaldehyde and the localization of GST fusion proteins was detected with anti-GST primary antibody and FITC-conjugated secondary antibody and examined by confocal fluorescence microscopy.
To confirm the observations made in the previous experiment, anti-importin 11 antibody was used in combination with protein A sepharose to deplete HeLa cytosol extracts of importin 11. Protein A sepharose binds IgGs, and therefore is able to precipitate any antibody bound protein out of solution. As can be seen in Fig. 7A, importin 11 was successfully depleted from HeLa cytosol. This depleted cytosol was then used in a nuclear import assay to test whether the absence of importin 11 had an effect on the nuclear localization of E2. As Fig. 7B shows, immunodepletion of importin 11 resulted in the change of localization of GST-16cE2 from nuclear to cytoplasmic, similar to negative control where only transport buffer was used (compare panels A, B and C). At the same time, nuclear localization of M9-GST, which uses karyopherin β2 as an import receptor (145), was unaffected by immunodepletion of importin 11 (panels D, E and F). GST was used as a negative control and localized in the cytoplasm regardless of the presence or absence of karyopherins or energy mix demonstrating that NLS is required for a successful nuclear import (panels G and H). The results of this experiment are in agreement with the nuclear import assay presented in Fig. 6 and demonstrate that the presence of importin 11 is required for HPV 16 E2 to localize in the nucleus.
Figure 7: Depletion of HeLa Cytosol of Importin 11 Inhibits Nuclear Import of GST-16cE2

A. HeLa cytosol used in the import assay is depleted of importin 11 following the incubation with anti-importin 11 antibody and protein A sepharose (left lane, (−)). Importin 11 is present in the HeLa cytosol used for positive control (right lane, (+)). Anti-importin11 antibody was used for detection. IPO11, importin 11; PA, protein A.

B. Digitonin-permeabilized HeLa cells were incubated with either GST-16cE2 (panels A-C), M9-GST (panels D-F), or GST (panels G and H) in the presence of transport buffer (TB, panels A, D, G), HeLa cytosol and energy mix (HC, panels B, E, H), or HeLa cytosol depleted of importin 11 (panels C and F). Following the incubation, cells were fixed with paraformaldehyde and the localization of GST fusion proteins was detected with anti-GST primary antibody and FITC-conjugated secondary antibody and examined by fluorescence microscopy.
In order to confirm that the proteins used in nuclear import assays were properly expressed and purified, SDS-PAGE and Coomassie staining were used (Fig. 8). The results showed that GST-16cE2, M9-GST and GST used in nuclear import assays were pure and present at relatively similar concentrations.

In summary, nuclear import assays indicated that importin 11 was required for nuclear localization of GST-16cE2 protein. This observation led to analysis of binding of E2 protein to importin 11 since many known NLSs directly interact with karyopherins.
Figure 8: Proteins Used in Nuclear Import Assays are Properly Expressed and Purified

Proteins expressed in BL-21 CodonPlus cells, GST-16cE2 (lane 1), M9-GST (lane 2), and GST (lane 3), were purified, run on a 12% SDS-PAGE gel and visualized by Coomassie Brilliant blue staining.
In order to analyze the binding of HPV 16 E2 protein to importin 11, isolation assay was used in which GST-16cE2 was immobilized on glutathione sepharose beads and incubated with HeLa cytosol extracts. Analysis of the proteins bound to the beads after the procedure showed that no importin 11 could be detected in the elutions after incubation with either GST-16cE2 or GST (Fig. 9A; lanes 1 and 2, respectively) and furthermore, importin 11 was not depleted from HeLa cytosol extract following the incubation with GST-16cE2 or GST (Fig. 9A; lanes 4 and 5, respectively), remaining at similar concentration as it was before the procedure (compare the importin 11 band at 112.5 KDa in lanes 3, 4 and 5 of Fig. 9A). This result shows that the binding of importin 11 to GST-16cE2 could not be detected using the isolation assay. As a control for this assay, another isolation assay was performed adding M9-GST as a control and using anti-kapβ2 antibody for detection. Kapβ2 binds strongly to M9-GST, its specific cargo (Fig. 10A; lane 3) while at the same time analysis of HeLa cytosol extract after the isolation procedure shows that Kapβ2 is depleted from HeLa cytosol after incubation with M9-GST (Fig. 10A, lane 7) but not after incubation with GST-16cE2 or GST (Fig. 10A, lanes 5 and 6, respectively). The presence of Kapβ2 band at 102 KDa in lanes 1 and 2 indicates non-specific binding of Kapβ2 to GST-16cE2 and GST; however, given the fact that the Kapβ2 band in lane 3 is stronger and Kapβ2 is depleted from HeLa cytosol following the incubation with M9-GST, it could be concluded that the isolation assay procedure was valid and specific karyopherins
could be isolated from HeLa cytosol extracts using GST fusion proteins with cargoes or NLSs. As an additional control, the GST fusion proteins used in isolation assays were analyzed by SDS-PAGE and Coomassie Brilliant blue staining. As shown in Fig. 9B and 10B, the proteins are expressed at relatively similar concentrations and were present on glutathione sepharose beads used in these experiments. Overall, isolation assay experiments fail to answer the question of whether importin 11 binds HPV 16 E2.
Figure 9: Isolation Assay Does Not Show Binding of Importin 11 to GST-16cE2

A: GST-16cE2 (lane 1) or GST (lane 2) were immobilized on glutathione sepharose beads and incubated with HeLa cytosol extracts. Bound proteins were eluted and analyzed via immunoblotting with anti-importin 11 antibody. Importin 11 present in the HeLa cytosol extract before the procedure is shown in lane 3, while importin 11 remaining in the unbound fraction after binding to GST-16cE2 or GST is shown in lanes 4 and 5, respectively.

B: GST-16cE2 (lane 1) and GST (lane 2) bound to glutathione sepharose beads used in importin 11 isolation experiment are detected with Coomassie Brilliant blue staining.
Figure 10: Isolation Assay Demonstrates Binding of Karyopherin β2 to M9-GST

A: GST-16cE2 (lane 1), GST (lane 2) or M9-GST (lane 3) were immobilized on glutathione sepharose beads and incubated with HeLa cytosol extracts. Bound proteins were eluted and analyzed via immunoblotting with anti-kapβ2 antibody. Karyopherin β2 present in the HeLa cytosol extract before the procedure is shown in lane 4, while karyopherin β2 remaining in the unbound fraction after binding to GST-16cE2, GST, or M9-GST is shown in lanes 5, 6 and 7, respectively. Note the depletion of Kapβ2 from HeLa cytosol after incubation with M9-GST (red arrow).

B: GST-16cE2 (lane 1), GST (lane 2) and M9-GST (lane 3) bound to glutathione sepharose beads used in karyopherin β2 isolation experiment are detected with Coomassie Brilliant blue staining.
In Solution Binding Assay to Test the Binding of Importin 11 to HPV 16 E2

This assay was another way to test the binding of HPV 16 E2 and importin 11 in vitro. The advantage of this method over the isolation or immunoprecipitation assay is that since importin 11 is being expressed as a 6His-tagged fusion protein in E. coli cells, it could not have bound any of its natural cargoes that would normally be present in HeLa cells or HeLa cytosol extracts. In theory, competition with natural cargoes could have prevented the binding of importin 11 to HPV 16 E2.

The results of this experiment show that GST-E2 does not bind 6His-importin 11 (Fig. 11, lane 1); however, GST-UbcM2 did (lane 2, importin 11 band at 112 KDa). UbcM2 is the natural cargo for importin 11 (140) and provides a positive control for this experiment. All GST fusion proteins used in the binding experiment with 6His-importin 11 were properly expressed and immobilized on glutathione sepharose beads at approximately similar concentrations (Fig. 12).
Figure 11: In Solution Binding Assay Does not Show Binding of GST-16E2 to 6His-importin 11 but Confirms Binding of GST-UbcM2 to 6His-importin 11

GST-16E2 (lane 1), GST-UbcM2 (lane 2), or GST (lane 3) were immobilized on glutathione sepharose beads and incubated with lysates of *E. coli* cells expressing 6His-importin 11. Bound proteins were eluted and importin 11 was detected by immunoblot with anti-importin 11 antibody. Lanes 4 and 5 show importin 11 present in *E. coli* lysates and HeLa cytosol extracts, respectively.
Figure 12: GST Fusion Proteins Used in GST-16E2 and 6His-importin 11 Binding Assay are Properly Expressed and Bound to Glutathione Sepharose Beads

GST-16E2 (lane 1), GST-UbcM2 (lane 2) and GST (lane 3) bound to glutathione sepharose beads used in binding assay with 6His-importin 11 are detected with Coomassie Brilliant blue staining.
Immunoprecipitation Assays to Test the Binding of Importin 11 to HPV 16 E2

These assays were another way to test the binding of HPV 16 E2 and importin 11 \textit{in vitro}. The advantage of this method is that HPV 16 E2 protein is expressed in HeLa cells, its native environment, as opposed to being expressed in the bacterial expression system. In immunoprecipitation assay, HeLa cells were transiently transfected with EGFP-16cE2, EGFP-16E2 or EGFP-C1 plasmids to allow the expression of viral proteins. Cells were lysed and lysates were analyzed for binding of EGFP-16cE2 or EGFP-E2 to importin 11 by using either anti-EGFP or anti-importin 11 antibody along with protein A sepharose beads to precipitate binding complexes out of solution.

In the first immunoprecipitation experiment, potential binding complexes were immunoprecipitated with anti-EGFP antibody and proteins bound to protein A sepharose beads were analyzed with anti-importin 11 antibody (Fig. 13). The results show that neither EGFP-16cE2 (lane 1), nor EGFP-16E2 (lane 7) bound importin 11, similarly to EGFP negative control (lane 3). Importin 11 was expressed in transiently transfected HeLa cells (lanes 9, 10 and 11), although at lower levels than in untransfected HeLa cells or commercially available HeLa cytosol extract (lanes 4 and 5). Since transfection reagents and conditions can slow down the rate of cell culture growth and cellular metabolism, lower levels of protein expression could be expected. Mock immunoprecipitation reactions, where no anti-EGFP antibody was added, were included to control for non-
specific binding of importin 11 to protein A sepharose beads. No non-specific binding was detected (lanes 2 and 8).
Figure 13: Immunoprecipitation with Anti-EGFP Antibody Does not Demonstrate Binding of Importin 11 to EGFP-16cE2 or EGFP-16E2

HeLa cells were transfected with EGFP-16cE2, EGFP-16E2 or EGFP-C1 plasmids. Cell lysates were prepared 24 hours post transfection and incubated with anti-EGFP antibody and protein A sepharose beads. Bound proteins were eluted and elutions (EGFP-16cE2 (lane 1), EGFP-16E2 (lane 7), EGFP-C1 (lane 3) were probed with anti-importin 11 antibody to detect potential importin 11 binding. Control for non-specific binding to protein A sepharose beads where no anti-EGFP antibody was added are shown in lanes 2 (EGFP-16cE2) and 8 (EGFP-16E2). Lanes 9, 10 and 11 show importin 11 present lysates of HeLa cells transfected with EGFP-16E2, EGFP-16-cE2 and EGFP-C1, respectively. Lanes 4 and 5 show importin 11 present in lysate of untransfected HeLa cells and in HeLa cytosol extract (ATCC), respectively. Lane 6 shows protein A band resulting from binding of secondary antibody to protein A.
In the second immunoprecipitation experiment, potential binding complexes were immunoprecipitated with anti-importin 11 antibody and proteins bound to protein A sepharose beads were analyzed with anti-EGFP antibody (Fig. 14). Since different antibodies have different affinities for their antigens, performing this “reversed” version of immunoprecipitation could provide results missed by the first experiment. The data show that EGFP-16cE2 and EGFP proteins are expressed very well in transiently transfected HeLa cells (lanes 4 and 5). However, EGFP-16cE2 appears to bind protein A sepharose beads in a non-specific manner, as evidenced by the presence of EGFP-16cE2 in elutions regardless of whether anti-importin 11 antibody was added to the binding assay or not (lanes 1 and 2).

Taken together, the results of the experiments described indicate the need for a different approach to answer the question of whether importin 11 binds HPV 16 E2, provided such an interaction exists. Other researchers have used yeast two-hybrid assays to study interactions of karyopherins with their cargoes. For example, one of importin 11’s natural cargoes, ribosomal protein L12, was identified in this manner (146). As such, it was decided to use yeast two-hybrid assay to study the putative interaction of importin 11 with HPV 16 E2.
Figure 14: Immunoprecipitation with Anti-importin 11 Antibody Does not Demonstrate Binding of Importin 11 to EGFP-16cE2 Due to Non-specific Interaction of EGFP-16cE2 with Protein A Sepharose Beads

HeLa cells were transfected with EGFP-16cE2 or EGFP-C1 plasmids. Cell lysates were prepared 24 hours post transfection and incubated with anti-importin 11 antibody and protein A sepharose beads. Bound proteins were eluted and elutions (EGFP-16cE2 (lane 1), EGFP-C1 (lane 3) were probed with anti-EGFP antibody to detect potential binding of EGFP-16cE2 or EGFP to importin 11. Control for non-specific binding of EGFP-16cE2 to protein A sepharose beads where no anti-importin 11 antibody was added is shown in lane 2. Lanes 4 and 5 show EGFP-16cE2 and EGFP, respectively, present in lysates of transfected HeLa cells, while lane 6 shows protein A band resulting from binding of secondary antibody to protein A.

Note the presence of EGFP-16cE2 band in lane 2, indicating non-specific interaction of EGFP-16cE2 with protein A sepharose beads.
Yeast Two-Hybrid Assays

Yeast two-hybrid assay is based on the principle that if two proteins of interest interact, two domains of the transcription factor, one of which is fused to each of the proteins of interest, come close together to produce a functional transcription factor that activates the expression of the reporter gene to produce a reporter protein that confers a new quality to the yeast cell in which the interaction is taking place. For example, reporter proteins may allow the cell to grow in the absence of certain nutrients or the yeast colony might change color.

To investigate the interaction of HPV 16 E2 with importin 11 using yeast two-hybrid assay, *Saccharomyces cerevisiae* YGR2 yeast cells were transformed with GBT10-importin 11 “bait” and pACTII-16cE2 “prey” plasmids and grown on SC-leu-trp media that allowed selection for double transformants (Fig. 15, panel A). Yeast cells transformed with GPA2 bait and fragment of adenylate cyclase prey, which are known to interact in a two-hybrid assay (147), were used as a positive control. The successfully transformed yeast colonies were replica plated onto SC-leu-trp-his plates. Since His3 enzyme was used as a reporter in this assay, cells would only grow on the medium lacking histidine if interaction between HPV 16 E2 and importin 11 took place. Additionally, 3-aminotriazol (3AT), which inhibits His3 enzyme, was added to the growth media to require the cells to express more selection marker.

As can be seen in Fig. 15 (panels B-G), GBT10-importin 11/pACTII-16cE2 double transformants failed to grow on the media lacking histidine, similar to the
negative control, whereas the positive control cells grew even in the presence of relatively high concentrations of 3AT. This indicates the lack of interaction between importin 11 and HPV 16cE2 proteins.

In another experiment, filter lift β-galactosidase activity assay, was used to assess the same interaction using replica plates of the plate shown in panel A of Fig. 15. This assay (Fig. 16) shows lack of color change, suggesting the absence of interaction, for GBT10-importin 11/pACTII-16cE2 double transformants, while the positive control cells turned blue indicating production of X-gal.
Figure 15: Yeast Two-Hybrid Binding Assay Does not Show Binding of HPV 16 cE2 and Importin 11

Saccharomyces cerevisiae YRG2 yeast cells were transformed with GBT10-importin 11 bait plasmid and pACTII-16cE2 prey plasmid. The yeast were plated on –leu –trp +his SC plate to allow selection of double transformants (panel A) and then replica plated to –leu –trp –his SC plates with various concentration of 3-aminotriazol (3AT) as indicated and grown at 30°C for 3 days (panels B-G). HIS3 reporter gene allows growth in the absence of histidine if interaction between the two proteins in question is present. 3AT inhibits HIS3 gene and requires the cells to express more selection marker.
Figure 16: Yeast Two-Hybrid Filter Lift β-galactosidase Activity Assay Does not Show Binding of HPV 16 cE2 and Importin 11

*Saccharomyces cerevisiae* YGR2 yeast were transformed with plasmids as shown, grown on SC –trp-leu media for 24 hours at 30°C, transferred to a nitrocellulose filter and assessed for β-galactosidase activity. Blue color shows X-gal production as an indicator of protein interaction.
In an attempt to promote a more stable interaction between importin 11 and HPV 16 E2, we used importin 11 (78-975) N-terminal deletion mutant. This mutation was known to promote stable interactions between importin 11 and UbcM2, one of its natural cargoes, by preventing the binding of RanGTP to importin 11, thus allowing import complexes to remain in the nucleus where they could be successfully detected by yeast two-hybrid assay (140).

Two-hybrid assays were performed with importin 11 (78-975) and either HPV 16 cE2 (Fig. 17) or HPV 16 E2 (Fig. 18). Similarly to the two-hybrid assays with full length importin 11, the GBT10-importin 11 (78-975)/pACTII-16cE2 or pACTII-16E2 double transformants failed to grow on the media lacking histidine, while the positive control cells grew successfully.

In order to verify that importin 11 was properly expressed in S. cerevisiae, the cells were lysed and subjected to immunoblot with anti-importin 11 antibody. The results indicated that importin 11 (78-975) was expressed somewhat better than full length importin 11 (Fig. 19).

Overall, the results of yeast two-hybrid assays did not provide evidence to support the direct interaction HPV 16 E2 protein and importin 11 karyopherin.
Figure 17: Yeast Two-Hybrid Binding Assay Does not Show Binding of HPV 16 cE2 and Importin 11 (78-975)

Saccharomyces cerevisiae YRG2 yeast cells were transformed with GBT10-importin 11 (78-975) bait plasmid and pACTII-cE2 prey plasmid. The yeast were plated on –leu –trp +his SC plate to allow selection of double transformants (panel A) and then replica plated to –leu –trp –his SC plates with various concentration of 3-aminotriazol (3AT) as indicated and grown at 30°C for 3 days (panels B-G). HIS3 reporter gene allows growth in the absence of histidine if interaction between the two proteins in question is present. 3AT inhibits HIS3 gene and requires the cells to express more selection marker. Importin 11 (78-975) does not bind Ran-GTP and therefore allows potential import complexes to remain in the nucleus for better detection by two-hybrid assay.
Figure 18: Yeast Two-Hybrid Binding Assay Does not Show Binding of HPV 16 E2 and Importin 11 (78-975)

_Saccharomyces cerevisiae_ YRG2 yeast cells were transformed with GBT10-importin 11 (78-975) bait plasmid and pACTII-16E2 prey plasmid. The yeast were plated on –leu –trp +his SC plate to allow selection of double transformants (panel A) and then replica plated to –leu –trp –his SC plates with various concentration of 3-aminotriazol (3AT) as indicated and grown at 30°C for 3 days (panels B-G). HIS3 reporter gene allows growth in the absence of histidine if interaction between the two proteins in question is present. 3AT inhibits HIS3 gene and requires the cells to express more selection marker. Importin 11 (78-975) does not bind Ran-GTP and therefore allows potential import complexes to remain in the nucleus for better detection by two-hybrid assay.
Figure 19: Importin 11 is Properly Expressed in *S. cerevisiae* Used in Yeast Two-Hybrid Assays

*S. cerevisiae* YGR cells used in yeast two-hybrid assays with either importin 11 (A) or importin 11 (78-975) (B) were grown in culture overnight at 30°C, pelleted and lysed in SDS-PAGE loading buffer by boiling for 10 minutes. The lysates were analyzed by immunoblot with anti-importin 11 antibody. Lane 1, cells transformed with pACT-16cE2 prey plasmid and pGBT10-importin 11 or pGBT10-importin 11 (78-975) bait plasmid; Lane 2, cells transformed with pACT-16E2 prey plasmid and pGBT10-importin 11 or pGBT10-importin 11 (78-975) bait plasmid; Lane 3, non-transformed cells; Lane 4, HeLa cytosol extract. (*) indicates nonspecific interaction; red arrows indicate BD-importin 11 fusion proteins.
To summarize, the results presented in this chapter led to two conclusions: a) importin 11 is necessary for nuclear import of HPV 16 E2, and b) importin 11 does not seem to directly bind HPV 16 E2. To explain these observations, we hypothesized that HPV 16 E2 bound to importin 11 via an adapter protein. Such trimeric complexes are not uncommon; for example, in the classic nuclear import pathway, Kapα2β1 heterodimers are used as import receptors (30, 31). Moreover, several proteins have been described that use piggyback mechanism of nuclear import, whereby they bind to an adapter protein that, in turn, binds to the import receptor (152-154). Some NLS-defective mutants of HPV 11 E2 have been suggested to use piggybacking on HPV 11 E1 for their import (77). The question, then, was to identify putative candidates for the role of the adapter between HPV 16 E2 and importin 11.
As mentioned before, one of the natural cargoes of importin 11 is murine E2-type ubiquitin-conjugating enzyme UbcM2. The human homolog of UbcM2 is UBE2E3 and these two enzymes share 100% sequence homology (140, 148). HPV 16 E2 protein is known to interact with UBE2K ubiquitin-conjugating enzyme (129). UbcM2/UBE2E3 and UBE2K share approximately 50% sequence homology and general tertiary structure (148, 149). Because of these similarities, we hypothesized that UbcM2/UBE2E3 could serve as an adapter protein in binding of HPV16 E2 to importin 11 during nuclear import of HPV 16 E2. Therefore, we tested whether HPV 16 E2 could bind UbcM2.

The binding of HPV 16 E2 to UbcM2 was tested in the context of EGFP-16E2 (Fig. 20) or EGFP-16cE2 (Fig. 22) and GST-UbcM2, with EGFP and GST serving as negative controls. The results revealed that EGFP-16cE2 specifically bound to GST-UbcM2 \textit{in vitro} (Fig. 22, lane 1). The binding of EGFP-16E2 to GST-UbcM2 was not detected; however, the level of expression of EGFP-16E2 in HeLa cells was much lower than the level of expression of EGFP-16cE2 (compare lanes 5 and 7 in Fig. 20 and Fig. 22). Therefore, it is possible that the apparent difference in binding might have been caused by insufficient amounts of EGFP-16E2 in the HeLa lysates to detect binding. The amount of GST fusion proteins immobilized on glutathione sepharose beads was similar for GST-UbcM2 and GST in both experiments (Fig. 21 and 23). Overall, the results of these binding experiments strongly suggest that UbcM2/UBE2E3 can serve as an adapter protein in the
binding of HPV 16 E2 to importin 11 and that HPV 16 E2 is imported into the nucleus as a trimeric complex with importin 11 and UbcM2/UBE2E3.

In summary, the results presented in this chapter revealed that a) the nuclear import of HPV 16 E2 protein requires importin 11 karyopherin and b) the import is most likely to occur as a trimeric complex where HPV 16 E2 binds to UbcM2/UBE2E3 ubiquitin-conjugating enzyme that binds to importin 11.
Figure 20: Low Expression of EGFP-16E2 May Interfere with Binding of EGFP-16E2 to GST-UbcM2

GST-UbcM2 (lanes 1 and 2) or GST (lanes 3 and 4) were immobilized on glutathione sepharose beads and incubated with lysates of HeLa cells transfected with either EGFP-16E2 (lanes 1 and 3) or EGFP-C1 (lanes 2 and 4). Bound proteins were eluted and analyzed by immunoblot with anti-EGFP antibody. Lanes 5-8 are controls showing the presence of EGFP-E2 (lanes 5 and 7) or EGFP (lanes 6 and 8) in HeLa cell lysates used in binding assays in lanes 1-4, respectively. EGFP-16E2 bands are shown with the red arrow.
Figure 21: GST Fusion Proteins Used in GST-UbcM2 and EGFP-E2 Binding Assay are Properly Expressed and Bound to Glutathione Sepharose Beads

GST-UbcM2 (lane 1) and GST (lane 2) bound to glutathione sepharose beads used in binding assay with EGFP-E2 or EGFP are detected with Coomassie Brilliant blue staining.
Figure 22: Isolation Assay Demonstrates Binding of GST-UbcM2 to EGFP-16cE2

GST-UbcM2 (lanes 1 and 2) or GST (lanes 3 and 4) were immobilized on glutathione sepharose beads and incubated with lysates of HeLa cells transfected with either EGFP-16cE2 (lanes 1 and 3) or EGFP-C1 (lanes 2 and 4). Bound proteins were eluted and analyzed by immunoblot with anti-EGFP antibody. Lanes 5-8 are controls showing the presence of EGFP-cE2 (lanes 5 and 7) or EGFP (lanes 6 and 8) in HeLa cell lysates used in binding assays in lanes 1-4, respectively.
Figure 23: GST Fusion Proteins Used in GST-UbcM2 and EGFP-cE2 Binding Assay are Properly Expressed and Bound to Glutathione Sepharose Beads

GST-UbcM2 (lane 1) and GST (lane 2) bound to glutathione sepharose beads used in binding assay with EGFP-E2 or EGFP are detected with Coomassie Brilliant blue staining.
Chapter 5

Discussion
Papillomaviruses (PV) are small (55-60 nm in diameter), nonenveloped viruses with icosahedral capsid and double-stranded DNA genome of about 8 kb. PVs infect squamous basal epithelial cells with each type of PV exhibiting specific preference for a particular host organism and tissue type. Human papillomaviruses (HPV) are very diverse and cause a wide range of disease, most importantly, cancer. Most of HPVs belong to Alpha and Beta genera and they are commonly classified into low risk and high risk groups based on their propensity to cause cancer. The low risk types (e.g. HPV 6 and 11) cause mostly benign genital warts, whereas the infection with high risk types (e.g. HPV 16, 18, 31, 45) often results in the development of anogenital cancers, such as cervical cancer.

This work focused on HPV E2 protein. This protein is the major transcriptional regulator that is involved in the regulation of expression of viral E6 and E7 oncogenes and the loss of E2 contributes to progression of the infected cell towards cancer. E2 functions to tether the viral genome to the host chromosomes in mitosis thus allowing for the proper retention, maintenance and partitioning of the viral genome. In addition, E2 interacts with a multitude of host cell’s gene products, including those involved in apoptosis, cell proliferation and differentiation, gene transcription, RNA processing, ubiquitination and degradation of proteins and intracellular transport. Overall, E2 is a potent regulatory protein that allows the cells to maintain an environment conducive to a successful replicative viral cycle.
Since E2 proteins are synthesized outside of the nucleus, they need to be transported into the nucleus to perform their functions. The present study focused on the nuclear import of high risk HPV 16 E2 protein. Previous research in the Moroianu lab has identified an alpha-helical NLS in the C-terminal (DNA-binding) domain of HPV 16 E2 (75). This NLS, 298LKCLRYRFKKH308, overlaps with the alpha helix involved in DNA binding and it is partially conserved in high risk HPV 18 and not conserved in low risk HPV 11. Here, we continued the analysis of the nuclear import pathway(s) used by HPV 16 E2.

In the first part of this research project, mutational analysis of several residues within the C-terminal NLS and elsewhere in the C-terminal domain of HPV 16 E2 was performed in order to determine if the residues in question played a role in the function of the NLS. The K299A mutant demonstrated less robust nuclear import ability that the wild type protein. This residue is highly conserved among the PV E2 proteins; including both high and low risk HPV types and non-human PVs (92). Lysines and arginines, positively charged residues, are typically found in NLSs, therefore it is reasonable to assume that K299 may be contributing its charge to the NLS’s receptor-binding interface. Also, high degree of K299 conservation suggests that this residue could be functionally important and, given its location within the DNA-binding alpha-helix, contribute to the formation of E2-DNA contact interface. Since the alpha-helical NLS of HPV 16 E2, unlike classical NLS, must maintain proper secondary structure in order to function (75),
mutations of the conserved residues that undermine the helical folding are expected to reduce the NLS’s functional fitness.

The K299A mutation affected the nuclear import of EGFP-16E2 more than the nuclear import of EGFP-16cE2. This could be explained by the fact that EGFP-16cE2 is smaller and has a much longer half-life due to the absence of the transactivation domain that is involved in E2 degradation (121). As such, EGFP-16cE2 is generally imported into the nucleus more efficiently than EGFP-16E2 (compare the import of wild type proteins, Table 1). Therefore, the absence of an extra lysine with its positive charge plays a less important role in the context of EGFP-16cE2 than in the context of EGFP-16E2. However, even for the import of EGFP-16cE2, a modest reduction in the NLS function was observed for K299A mutant.

The C300A mutation resulted in a modest decrease in the nuclear import of EGFP-16E2 and almost no change in the nuclear import of EGFP-16cE2. This residue, like K299, is highly conserved among a variety of PVs (92) and could be involved in the maintenance of the proper alpha-helical structure and in the contacts between E2 and DNA. If this mutation results in the somewhat distorted alpha-helix, it is reasonable to expect a less functional NLS than in the case of a wild type protein. The more pronounced effect of C300A mutation in the context of EGFP-16E2 vs. EGFP-16cE2 could be explained by the same reasons as for the K299A mutants above.
The $327$KHK$_{329}$ sequence is located 19 residues downstream of the C-terminal NLS of HPV 16 E2. Structurally, it is a part of the disordered loop between $\beta 2$ and $\beta 3$ strands of the C-terminal domain (Fig. 1). The K329 residue is fairly conserved among multiple PV E2 proteins, either remaining unchanged or being replaced by arginine, another positively charged amino acid. The other two residues of the $327$KHK$_{329}$ are not conserved (92). As mentioned earlier, lysine residues are often found in NLSs and histidine residue with its imidazole R group is often involved in protein-protein interactions. As such, it was reasonable to assume that KHK sequence could be important for the nuclear import of HPV 16 E2. Indeed, when KHK was mutated to AAA, there was a significant drop in the nuclear localization of the HPV16 E2 protein, both in the context of EGFP-16E2 and EGFP-16cE2. As with the mutants described before, the EGFP-16cE2 fusion proteins localized to the nucleus more than the EGFP-16E2 proteins. It appears that the $327$KHK$_{329}$ acts as an “enhancer” sequence for the C-terminal NLS of HPV 16 E2, providing additional positively charged residues to the receptor-binding interface. The sequence’s position within a flexible and disordered loop potentially allows it to bind to a different location on the import receptor that the main NLS binding site increasing the strength of the interaction.

Papillomavirus E2 proteins normally exist in solution as dimers. As described in Chapter 1, dimerization occurs via both N-terminal and C-terminal domain residues. In the case of C-terminal domain, dimerization is required for proper DNA binding. A critical tryptophan residue, W319 in HPV 16 E2, is called the
“tryptophan bridge” and mutations in this residue have been known to disrupt the dimerization of the C-terminal domain (100). We hypothesized that if the HPV 16 E2 dimers form in the cytoplasm, prior to the protein’s entry into the nucleus, the two NLSs of the dimeric protein could act cooperatively in the nuclear import of HPV 16 E2. The results show that the W319G mutant demonstrates a significant mislocalization in the context of EGFP-16cE2 but the localization is only modestly affected in the context of EGFP-16E2. This could be explained as follows. In the EGFP-16E2 W319G mutant, the dimerization via the transactivation domain is unaffected, and the two NLSs are able to act cooperatively, similar to the wild type protein. On the other hand, in the EGFP-16cE2, the disruption of the dimer interface leaves only one NLS per molecule. This may lead to decreased affinity of the protein for the import receptor and mislocalization to the cytoplasm. Additionally, it is possible that the monomeric EGFP-16cE2 is unable to maintain the proper secondary and/or tertiary structure. Since the alpha-helical NLS of HPV 16 E2 depends on its structure for proper function, even a partially misfolded mutant should have its nuclear import negatively affected.

EGFP-16cE2 monomer at 42 KDa is almost at the exclusion limit of the NPC. However, the nuclear entry of the monomeric EGFP-16cE2 W319G mutant into the nucleus by passive diffusion seems unlikely in the light of the fact that only 37.6% of EGFP negative control protein localizes to the nucleus, indicating that passive diffusion through the NPC is a rather slow and inefficient process compared to karyopherin-driven transport. If EGFP-16cE2 W319G proteins were in fact entering the nucleus by passive diffusion, the percentage of them
localizing to the nucleus would be similar to that of EGFP negative control, and perhaps even lower, given the fact that EGFP-16cE2 monomer is larger than EGFP (42 v. 32.7 KDa, respectively). Since the observed percent of nuclear localized EGFP-16cE2 W319G is 59.7%, it is unlikely that it enters the nucleus by passive diffusion, but rather it utilizes karyopherin-driven import pathway, albeit with much less efficiency than the wild type protein for the aforementioned reasons. As such, the data strongly suggest that dimerization plays an important role in the nuclear import of HPV 16 E2 protein. In the future, 2xEGFP-16cE2 W319G fusion protein could be generated that would clearly be too large to enter the nucleus by passive diffusion. Also, the disruption of dimerization could be verified by bimolecular fluorescence complementation technique (BiFC) whereby two non-fluorescent halves of a green fluorescent protein (GFP) variant are fused to two monomers. The dimerization leads to the reconstitution of the GFP to a fluorescent molecule. This technique has been successfully used to study the formation of homodimers (159, 160). Also, other residues that had been determined to be involved in the formation of C-terminal dimers (see Chapter 1) could be mutated and studied for their role in the nuclear import of HPV 16 E2.

In addition to the analysis of the HPV 16 E2 mutants mentioned before, other mutants were generated and analyzed. These included the mutations of the hydrophobic residues within the NLS (L301A and F305A). These residues are partially conserved (92) and, in some known NLSs, hydrophobic residues were shown to be critical for the NLS function (155, 156). However, in the case of HPV
16 E2, the hydrophobic residues within the NLS did not appear to play a role in nuclear import. The results of experiments with these mutants, as well as with other mutants, are described in Appendix A.

To summarize, in the first part of this research project, we have identified several residues within the C-terminal domain of HPV 16 E2 protein that play an important role in the protein’s nuclear import. We have shown that the K299 residue increases the propensity of HPV 16 E2 to enter the nucleus, presumably by providing an additional positive charge to the import receptor binding interface. The C300 residue was found to be modestly important to the function of the NLS; presumably by contributing to the maintenance of the proper alpha-helical shape of the NLS. We have identified the NLS “enhancer” sequence, 327KHK329, which significantly increases the robustness of the nuclear import of HPV 16 E2 presumably by acting as an additional element in the import receptor binding interface. Finally, this research demonstrated the importance of dimerization for the nuclear import of HPV 16 E2.

In the second part of this research project, we focused on identifying the nuclear import receptor for the alpha-helical C-terminal NLS of HPV 16 E2 protein. Because of the structural similarity between the HPV 16 E2 NLS and the alpha helical NLS of MA domain of Gag protein of Rous sarcoma virus (RSV) (137), we focused on importin 11, which was found to be an import receptor for the MA domain (138).
The nuclear import assays demonstrated that importin 11 was required for the nuclear import of GST-16cE2 in vitro. At the same time, various in vitro assays, including isolation assays, in solution binding assays, immunoprecipitation assays and yeast two-hybrid assays, failed to demonstrate the direct binding of HPV 16 E2 to importin 11.

As described in Chapter 4, based on the results of nuclear import assays and in vitro assays, we hypothesized that an adapter protein was involved in the nuclear import of HPV 16 E2. Furthermore, based on the analysis of the literature, in particular based on the results of the studies of the binding interactions of importin 11 and HPV 16 E2 (129, 140), we identified UbcM2/UBE2E3 E2-type ubiquitin-conjugating enzyme as a putative adapter protein for the nuclear import of HPV 16 E2. The binding assays revealed that EGFP-16cE2 bound GST-UbcM2 in vitro suggesting that our hypothesis was correct. Moreover, UbcM2/UBE2E3 is present in HeLa cells (157). However, several questions remain.

First, the existence of trimeric complex of HPV 16 E2, UbcM2/UBE2E3 and importin 11 will need to be demonstrated. In vitro, this could be done by immobilizing GST-16cE2 (or GST-16E2) on glutathione sepharose beads and incubating the beads with untagged UbcM2, followed by incubation with 6His-importin 11 and detection with anti-importin 11 antibody. Also, the requirement for the trimeric complex could be demonstrated in a nuclear import assay with
importin 11 and UbcM2 used in the presence of RanGDP. In vivo, a cell line that stably expresses HPV 16 E2 could be used to study its interactions. Our attempts to create a HeLa cell line stably expressing EGFP-16E2 were not successful; however, U2OS cell line stably expressing HPV 16 E2 exists (122).

Second, the use of UbcM2/UBE2E3 as an adapter is interesting because it is not a karyopherin. As such, the import mechanism of HPV 16 E2 does not resemble the classic import pathway. However, the presence of a well-defined NLS in HPV 16 E2 differentiates its import from described “piggyback” import mechanisms wherein a protein that lacks the NLS of its own piggy backs on a NLS-containing protein. Therefore, HPV 16 E2 seems to be using a unique and previously undescribed import mechanism.

Why would such a mechanism exist? As described in Chapter 1, papillomavirus proteins use a variety of import pathways, both classic and non-classic, because they evolved to adapt the host cell’s molecular machinery to successfully propagate. In this regard, the fact that HPV 16 E2 uses a previously undescribed pathway is not surprising.

The evolution of HPV 16 E2 to use UbcM2/UBE2E3 for its nuclear import may not be entirely random. HPV 16 E2 is an important regulator of DNA replication and gene expression. As such, its presence in the nucleus must be precisely controlled. Using UbcM2/UBE2E3 as a part of the import complex allows for a
control mechanism since UbcM2/UBE2E3 is imported only in ubiquitinated state (157) and it is conceivable that HPV 16 E2’s nuclear entry is controlled by alternating the ubiquitination state of UbcM2/UBE2E3. Binding of ubiquitinated UbcM2/UBE2E3 to HPV 16 E2 may lead to specific ubiquitination of E2 that favors its interaction with importin 11. Also, PV E2 proteins are targeted for degradation by SCF^Skp2 ubiquitin ligase which acts on the residues in the transactivation domain. Although highly speculative, it is intriguing to think that a coupling of a ubiquitin-charged ubiquitin-conjugating enzyme (UbcM2/UBE2E3) as an import receptor binding the C-terminal NLS and ubiquitin ligase binding the N-terminal domain could provide a way to precisely control the amount of HPV 16 E2 entering the nucleus. In this view, the deletion of the transactivation domain in 16cE2 would remove this control and lead to more protein localizing to the nucleus, a result that was observed in transient transfections described in Chapter 3.

In addition to the regulation of the nuclear import of HPV 16 E2, the trimeric complex of importin 11 and UbcM2/UBE2E3 may perform a chaperone function to protect HPV 16 E2 during import and to ensure it is not misfolded. Karyopherins are known to have chaperone functions for proteins with exposed basic domains (158).

Our results do not contradict the data by Bian and Wilson (79) who found that HPV 16 E2 bound preferentially to importin α5 in vitro, because it is possible for a
protein to bind more than one karyopherin and also because those researchers did not perform any functional assays demonstrating the requirement for importin α5 for nuclear import of HPV 16 E2 and did not detect the presence of importin α5 in the HeLa cell extract (79). If it was present in cells it could transport HPV 16 E2 into the nucleus in the presence of importin β/karyopherin β1.

Looking into the future, several avenues of research may arise from these results. First of all, the existence of the trimeric complex of HPV 16 E2, importin 11 and UbcM2/UBE2E3 will need to be determined, as described above. Second, it would be interesting to test the mutant forms of HPV 16 E2 and cE2 generated during this project and other mutants that alter nuclear import of HPV 16 E2 (75) for their ability to bind UbcM2/UBE2E3 and/or importin 11 to determine the specific molecular interactions. Third, it would be intriguing to study the nuclear import pathways of E2 proteins of other PV types. Since they have different NLSs, they are likely to bind different karyopherins and/or use different adapters. Finally, it is likely that there is a link between the regulation of the nuclear import of HPV 16 E2 and its function as a transcriptional regulator. It would be interesting to investigate the putative relationship between the nuclear import of HPV 16 E2 and the regulation of the cell cycle. HPV 16 E2 has been found to bind cyclin-dependent kinase-like3 protein (132) and it is a regulator of viral oncogene E7 that is involved in cell cycle control via its interaction with cyclins, CDKs, HDACs and other cellular targets as discussed in Chapter 1. E7-induced destabilization of pRb, for example, is dependent upon the involvement of E2-25K ubiquitin
carrier protein, also known as UBE2K, which is one of the proteins HPV 16 E2 is known to interact with (129, 150). Additionally, importin α and proteins containing classical bi-partite NLSs are known to be involved in G1/S cell cycle progression in S. cerevisiae (151), which opens the possibility that importin 11 and HPV 16 E2 might also be involved in the cell cycle control in some capacity.

In conclusion, this research has contributed to the understanding of the mechanisms of nuclear import of high risk HPV 16 E2 protein. The mutational analysis has continued the past research to further elucidate the properties of the novel, alpha-helical, C-terminal NLS and also looked at the role of the dimerization state of HPV 16 E2 protein in its nuclear import. As described in Chapter 1, PV proteins use a variety of nuclear import pathways. While some of them (for example, L1, L2 and E6) use the classical import pathway involving Kapα2β1 heterodimer, others rely upon non-classical import pathways and use a variety of karyopherins in the alpha and beta karyopherin families (for example, L2, E1 and E6) or bind FG nucleoporins directly (for example, E7). In comparison with previously described import pathways of PV proteins, HPV 16 E2 uses a different import strategy. The search for the HPV 16 E2 import receptor resulted in the discovery of importin 11 karyopherin. The data suggest a previously undescribed import pathway whereby UbcM2/UBE2E3 E2-type ubiquitin-conjugating enzyme is used as an adapter between HPV 16 E2 and importin 11 karyopherin. To our knowledge, this is the first description of a nuclear import
mechanism where a NLS-containing protein binds an ubiquitin-conjugating enzyme adapter in a complex with an importin.
Appendix A

Investigation of the Role of L301A, F305A, V326STOP and V331STOP Mutations in the Nuclear Import of HPV 16 E2 Protein
L301 and F305 are hydrophobic residues that are located within the previously established C-terminal NLS of HPV 16 E2 (75) (Fig. 1). The role of these residues in the nuclear import of HPV 16 E2 protein was investigated using transient transfections in HeLa cells in the context of EGFP-16E2 and EGFP-16cE2 L301A and F305A mutants. These experiments were performed by Erin Groden under my supervision. The results indicated that neither of the two mutations affected the nuclear localization of HPV 16 E2 as both mutants displayed nuclear localization similar to wild type proteins (Fig. A1). These results determined that these hydrophobic residues do not play a role in the nuclear import of HPV 16 E2.

V326STOP and A331STOP mutants were generated in order to investigate putative requirement for 327KHK329 sequence in the nuclear import of HPV 16 E2. As can be seen in Fig. 1, these mutants generated truncated proteins that either included the 327KHK329 sequence or excluded it. The results demonstrated that these mutations resulted in pancellular localization similar to EGFP negative control, both in the context of EGFP-16E2 or EGFP-cE2 (Fig. A2). The reason for this is likely to be that the tertiary or even secondary structure of the C-terminal domain could not be maintained in the truncated proteins resulting in non-functional NLS. As a result of these observations, we decided to generate KHK327AAA mutants which allowed to investigate the role of 327KHK329 sequence in the nuclear import of HPV 16 E2 while allowing to maintain the general folding of the C-terminal domain.
Figure A1: L301A and F305A Mutations Do not Alter the Nuclear Localization of EGFP-16cE2 or EGFP-16E2

A. HeLa cells were transfected with EGFP-C1 (panels A and B), EGFP-16cE2 (panels C and D), or EGFP-16cE2 L301A (panels E and F) plasmids.
B. HeLa cells were transfected with EGFP-C1 (panels A and B), EGFP-16E2 (panels C and D), or EGFP-16E2 L301A (panels E and F) plasmids.
C. HeLa cells were transfected with EGFP-C1 (panels A and B), EGFP-16cE2 (panels C and D), or EGFP-16cE2 F305A (panels E and F) plasmids.
D. HeLa cells were transfected with EGFP-C1 (panels A and B), EGFP-16E2 (panels C and D), or EGFP-16E2 F305A (panels E and F) plasmids.

All cells were examined by fluorescent microscopy 24 hours post transfection. Panels B, D and F represent the fluorescence of EGFP. Panels A, C and E represent the DAPI staining of the nuclei. (Erin Groden, Biology Honors Thesis, 2010.)
Figure A2: V326STOP and A331STOP Mutations Change the Localization of EGFP-16cE2 and EGFP-16E2 from Nuclear to Pancellular

A. HeLa cells were transfected with EGFP-C1 (panels A and B), EGFP-16cE2 (panels C and D), EGFP-16cE2 V326STOP (panels E and F), or EGFP-16cE2 A331STOP (panels G and H) plasmids.

B. HeLa cells were transfected with EGFP-C1 (panels A and B), EGFP-16E2 (panels C and D), EGFP-16E2 V326STOP (panels E and F), or EGFP-16E2 A331STOP (panels G and H) plasmids.

All cells were examined by fluorescent microscopy 24 hours post transfection. Panels B, D, F and H represent the fluorescence of EGFP. Panels A, C, E and G represent the DAPI staining of the nuclei. (Erin Groden, Biology Honors Thesis, 2010.)
Literature Cited


