CHARACTERIZATION OF PAPILLOMAVIRUS E6 PROTEINS THAT BIND CELLULAR E6AP

A DISSERTATION

Presented to the faculty of the School of Medicine in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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APPROVAL SHEET

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DEDICATION

I dedicate this work, in its entirety, to my incredible, loving husband Kelly. Here's to exploring as many countries together as we can, having tea every morning, and knowing together we can accomplish anything.

Je t'aime pour toujours mi amor.

There are special moments in a person's life that leave a lasting impression. I have known you for five years now and when I think about these special moments in my life, you are responsible for many of them. Growing up I watched the romantic movies where the girl meets that special guy and they live happily ever after. I thought I knew what love was, but now I realize that I had no idea what true love was until I found you. It's hard to put into words how you make me feel each and every day. How the way you smile or tilt your head can instantly make me feel happier and lighten my mood. You know me better than I know myself. Writing this thesis was one of the most challenging things I have done to-date, and it would not have been completed nearly as well or as efficiently if it were not for you. You know that I do not enjoy writing and I would have much preferred being in lab working at the bench. But instead, I had to lock myself in a room and force myself to attempt what I perceived as an insurmountable task. It was not an enjoyable time. But without you it would have been completely overwhelming. I am so lucky to have such a smart, talented, dedicated, calm, thoughtful person by my side. The completion of this thesis is as much your success as it is mine. I feel privileged each and every day that I get to share this life with you. Thank you for being my sanity, my safe place, my anchor, and my rock. I am forever grateful that you chose me and that I get to love you.

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meeting. I value your opinions and it has truly been an honor to have you on my committee. **Dr. Judy White**, your curiosity is contagious. It has been a pleasure getting to see science through your eyes. You're always so engaging in all of my committee meetings and ask questions that bring forward new and exciting points of view. Thank you for keeping me on my toes and showing me there are many different ways to think about data and scientific problems. I am a better scientist because of you and for that I am eternally grateful. You are a brilliant person and I am honored that I got to have you on my committee before you retire – the academic world is losing a talented scientist and mentor! **Dr. Dan Engel**, thank you for investing your time and energy into my graduate career. You are a gifted scientist and I was lucky to have you as a member of my thesis committee. Although I didn't have the pleasure of interacting with you too much outside of my committee meetings, I greatly appreciated how engaging you were during my talks. You asked very intriguing questions and made me feel confident in my scientific abilities. **Dr. Anne Mills**, thank you for taking a leap of faith and agreeing to serve on my thesis committee. I remember that when I first asked you to be on my committee, I failed at explaining exactly what being a committee member entails. You went the extra mile and reached out to Janet to make sure you understood what you needed to do, which I really respect. Early in my second year of graduate school I met you while doing one of my clinical rotations. You had to look at a cytology slide from a patient in the hallway right outside of the patient's room in order to advise the surgeon on how to proceed. I remember this all so clearly because in that moment, I actually questioned whether or not I made the right choice going to graduate school – I wanted to go to medical school to be like you! Although I didn't end up changing careers, you taught me how important it is to speak clearly and know my audience, both of which are translatable to my future career.

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two are fabulous people and have done more for me in the past few months than most people can say their friends have done for them in years. You are so generous and caring and I wish you both nothing but happiness now and in the future!

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DISSERTATION ABSTRACT

Papillomaviruses infect vertebrate epithelia and induce squamous papillomas in order to replicate. The papillomavirus E6 oncoprotein is important not only in maintenance of the papillomavirus genome but also in disrupting cellular functions to enable viral genome replication. E6 proteins exert their function by interacting with cellular proteins, one of which is the E3 ubiquitin ligase E6AP via its LXXLL motif (LQELL). E6 proteins from high-risk papillomaviruses hijack E6AP ubiquitin ligase activity to target the degradation of cellular p53, along with other cellular proteins. E6 proteins from low-risk papillomaviruses can bind E6AP and stimulate its ubiquitin ligase activity, but cellular degradation targets of the low-risk E6-E6AP complex are unclear. A cellular protein targeted for degradation by both the high and low-risk E6-E6AP complex has yet to be described. Although stimulating the ubiquitin ligase activity of E6AP is an important function of these E6AP-interacting E6 proteins, little is known regarding the precise mechanism(s) responsible. Finally, although both high and low-risk E6 proteins can bind full-length E6AP, only high-risk E6 can bind the short LQELL E6AP peptide. This indicates that there may be other regions within the E6AP protein that are important in mediating the formation of the E6-E6AP complex outside of the well-characterized E6AP LQELL motif.

In this dissertation we describe an E6AP-dependent degradation target common to both high and low-risk E6 proteins: NHERF1. The targeted degradation of NHERF1 by E6AP-binding E6 proteins is not only conserved among high and low-risk E6 proteins, but also among E6 proteins from papillomaviruses discovered in other vertebrate mammals. We identify a novel region within E6 that is required for NHERF1 degradation as well as a domain in NHERF1 that is both necessary and sufficient for E6-mediated degradation. We further find that E6 degradation of NHERF1 augments canonical Wnt/β-catenin, a complex signaling pathway involved in regulating cell proliferation.

In this thesis we also examine regions within E6AP, outside of the LQELL motif, that are important in mediating the E6-E6AP interaction (which we termed E6AP auxiliary regions). We further identify one of these auxiliary regions as being required for both high and low-risk E6 proteins to stimulate E6AP E3 ubiquitin ligase activity to initiate degradation of cellular proteins. Our findings indicate there are two types of E6-E6AP interactions: those that can bind the isolated E6AP LQELL motif (Type I) and those that cannot and instead depend more heavily on E6AP auxiliary regions (Type II). Overall, our work has numerous therapeutic implications as NHERF1 may serve as a viable target for both high and low-risk infections and our identification of E6AP auxiliary regions will inform the design of therapies directed at disrupting the interaction of E6 and E6AP.

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List of Abbreviations

AIG	Anchorage independent growth
APC	Adenomatosis polyposis coli
AZUL	Amino-terminal zinc-finger of Ube3a ligase
BPV1	Bovine papillomavirus type 1
βTrcp	F-box/WD repeat-containing protein 1A
C-terminus	Carboxy terminus
CK1α	Casein kinase 1α
CTFR	Cystic fibrosis transmembrane conductance regulator
Dlg	Drosophila disc large tumor suppressor
Dsh	Dishevelled
EBP50	Ezrin binding protein 50
EB domain	Ezrin-radixin-moesin (ERM)-binding domain
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMBL-EBI MUSCLE	The European Bioinformatics Institute MUltiple Sequence Comparison by Log-Expectation
EMT	Epithelial-mesenchymal transition
ERM	Ezrin-radixin-moesin
E6AP	E6-associated protein
E6APΔHECT	E6AP deleted of the HECT domain
E6AP_Ub⁻	E6AP ubiquitin ligase defective mutant (C843A)
E6C	Carboxy terminal E6 zinc-structured domain
E6N	Amino terminal E6 zinc-structured domain
E6ΔPBM	E6 deleted of the PDZ binding motif
FL	Full-length
FOPFLASH	Mutated TCF Reporter Plasmid
Fzd	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein?
GPCRs	G-protein coupled receptors
GSK3β	Glycogen synthase kinase 3β
HECT	Homologous to the E6AP carboxyl terminus
HPVs	Human papillomaviruses
HPV7	*Homo sapiens* (Human) papillomavirus type 7
HPV11	*Homo sapiens* (Human) papillomavirus type 11
HPV10	*Homo sapiens* (Human) papillomavirus type 10
HPV16	*Homo sapiens* (Human) papillomavirus type 16
HPV18	Human papillomavirus type 18

HPV41	*Homo sapiens* (Human) papillomavirus type 41
IP	Immunoprecipitation
kDa	Kilodalton
MAML1	Mastermind-like protein 1
MaPV1	*Mesocricetus auratus* (golden hamster) papillomavirus type 1
McPV2	*Mastomys coucha* (mouse) papillomavirus type 2
MDCK cells	Madin-Darby Canine Kidney cells
MEFs	Mouse embryonic fibroblasts
MMC	Mitomycin-C
MmPV1	*Macaca mulata* (rhesus monkey) papillomavirus type 1
N-terminus	Amino terminus
NIKS	Normal immortalized keratinocytes
NHE	Na^+/H^+ Exchanger
NHERF1	Na^+/H^+ Exchange Regulatory Factor 1
OcPV1	*Oryctolagus cuniculus* (rabbit) papillomavirus type 1
ORF	Open reading frame
PaVE	Papillomavirus Episteme
PBM	PDZ binding motif
PDB	Protein data bank
PDGFR	Platelet-derived growth factor receptor
PDZ proteins	Post-synaptic density protein 95 (PSD-90)/Drosophila disc large tumor suppressor (Dlg1)/Zona occludens 1 (ZO-1) domain-containing proteins
PEI	Polyethylenimine
PKA	Protein Kinase A
PphPV1	*Phocoena phocoena* (harbor porpoise) papillomavirus type 1
PphPV4	*Phocoena phocoena* (harbor porpoise) papillomavirus type 4
pRb	Retinoblastoma protein
PsPV1	*Phocoena spinipinnis* (Burmeister's porpoise) papillomavirus type 1
PTEN	Phosphatase and tensin homolog
PTPN3	Protein tyrosine phosphatase non-receptor type 3
PTPN14	Protein tyrosine phosphatase non-receptor type 14
PVDF	Polyvinylidene fluoride
PVs	Papillomaviruses
S phase	Synthesis Phase
SfPV1	*Sylvilagus floridanus* (Cottontail rabbit; CRPV1) papillomavirus type 1
shRNA	Short hairpin RNA
SLC9A3	Solute carrier 9 member A3

SLC9A3R1	Solute carrier family 9 Member A3 Regulator 1
SsPV1	*Sus scrofa* (wild boar) papillomavirus type 1
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TAP	Tandem affinity purification
TCF-1B	T cell factor-1B
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor
TOPFLASH	TCF Reporter Plasmid
TtPV5	*Tursiops truncatus* (bottlenose dolphin) papillomavirus type 5
UBE3A	Ubiquitin-protein ligase E3A
UmPV1	*Ursus maritimus* (polar bear) papillomavirus type 1
Wnt	Wingless/integrated
WT	Wild type
XGal	5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside
XL-MS	Chemical cross-linking coupled to mass spectrometry
YAP1	Yes-associated protein 1
YPD	Yeast extract-peptone-dextrose
Y2H assay	Yeast two-hybrid assay
Y3H assay	Yeast three-hybrid assay
16E6	High-risk HPV type 16 E6
11E6	Low-risk HPV type 11 E6
18E6	High-risk HPV type 18 E6

Chapter 1: Introduction

1.1 Papillomaviruses

1.1.1 Papillomaviruses in the Clinic

Papillomaviruses (PVs) are small DNA tumor viruses that infect cutaneous or mucosal epithelia and contain a circular, double-stranded genome. Human papillomaviruses (HPVs) are the most well-studied, though PVs are found throughout nature. The mucosal HPVs are classified into low-risk and high-risk categories, based on their propensity to cause cancer. The high-risk HPV strains are responsible for 5% of all cancers worldwide, specifically anogenital and head and neck cancers [1]. High-risk HPV strains are responsible for more than 99% of cervical cancers, with 70% of these caused by two subtypes: HPV16 and HPV18 [2]. Although cervical cancers account for 85% of the total HPV-associated cancers, both men and women are subject to diseases resulting from HPV infection [3]. Viral infections by low-risk HPVs, although not associated with cancer, can have serious medical consequences. Oropharyngeal papillomatosis results from low-risk HPV infection in the voice box, vocal cords, or air passage from the nose to lungs and can result in potentially life-threatening blockage of the airways if not resected. Currently, there are no effective therapies to cure the disease, and afflicted persons must receive regular surgeries to resect the papillomas [4].

1.1.1.1 Vaccines

In 2006, the first vaccine for HPV was authorized. Branded as Gardasil, the vaccine protected against four different HPV types; two low-risk: HPV6 and

HPV11, and the two most common high-risk: HPV16 and HPV18. Less than ten years later, Gardasil-9, a 9-valent vaccine protecting against HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, and HPV58 (two low-risk HPV, the rest high-risk types) was approved and is currently the only HPV vaccine available in the United States [5]. A comprehensive review published earlier this year by de Oliveira et al. details the journey of the HPV vaccine; from discovery to challenges faced in its implementation [6]. While Gardasil protects against certain HPV types, the low global vaccination rate and overall lack of vaccination of men indicate that HPV-induced cancers will remain a persistent healthcare problem.

1.1.2 Papillomavirus Classification

First identified more than eighty years ago in cottontail rabbits [7], over 350 types of papillomaviruses have been described to date. Frequently referred to by types, PVs are found in the Papillomaviridae family and are classified by sequence similarity within the L1 capsid protein ORF, as it is the most conserved of all PV genes. PVs are classified into taxonomic levels of genus (<60% sequence homology in L1), species (between 60% and 80% sequence homology in L1), and types (between 71% and 89% homology in sequence) [8]. A new type of papillomavirus is recognized if the L1 ORF differs from all other described PVs by more than 10% [8]. To date, there are over 49 described genera of PVs (Fig 1.1), with more expected as the field continues to expand and new PVs are identified (see curated list at Papillomavirus Episteme

(PaVE): https://pave.niaid.nih.gov; [9]). HPVs are found in five of the described genera: Alpha (α), Beta (β), Gamma (γ), Mu (μ), and Nu (ν). The high-risk and low-risk HPVs most frequently discussed in this thesis belong to the Alpha genera.

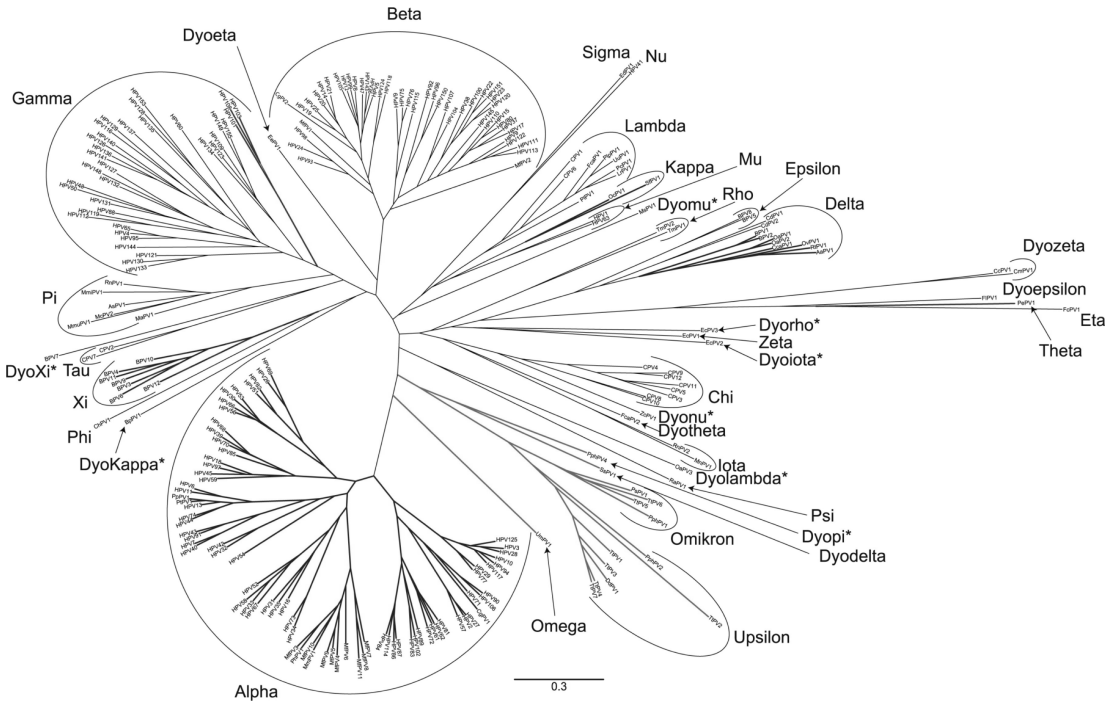


Figure 1.1 Papillomavirus phylogenetic tree.
Different papillomavirus genera are indicated. Human papillomaviruses are classified in five different genera: Alpha, Beta, Gamma, Mu, and Nu. Adapted from Van Doorslaer Virology, 2013 [10].

1.1.3 Life cycle

The life cycle of PVs occurs exclusively in keratinocytes and production of an infectious virion is dependent upon the progression of an infected keratinocyte from the its undifferentiated, stem-like state in the basal layer to its differentiated state in the cornified, top layer of skin. The PV life cycle is complex (Fig 1.2) and has been comprehensively reviewed [11, 12]. In uninfected epithelia, only basal cells progress through the cell cycle and act as stem cells to replenish the skin as dead skin in the cornified layer is continually sloughed off. When the basal cells stratify off the basement membrane, the cells differentiate and exit the cell cycle. An HPV infection begins when a lesion in the cornified layer of cutaneous or mucosal epithelia exposes the basal layer of cells (reviewed in [13]). At the beginning of the HPV life cycle, the viral genome is maintained as an episome at low copy number in the basal layer, and as the cells undergo the cell cycle, the viral genome is replicated in synchrony with the cellular DNA. As the infected keratinocytes are pushed away from the basal layer, a terminal differentiation program initiates, which triggers expression of several viral proteins to aid in the productive phase of the viral life cycle. This phase includes viral genome amplification where viral oncoproteins force differentiating, infected keratinocytes in the spinous layer back into S phase of the cell cycle so that the cellular DNA replicating machinery is not turned off and can still be utilized to replicate the viral genome. At this point, keratinocytes harboring an HPV episome could have

around one hundred thousand copies of the genome per cell. The viral life cycle is completed by the release of mature, infectious virions with the sloughing off of desquamated cells [14].

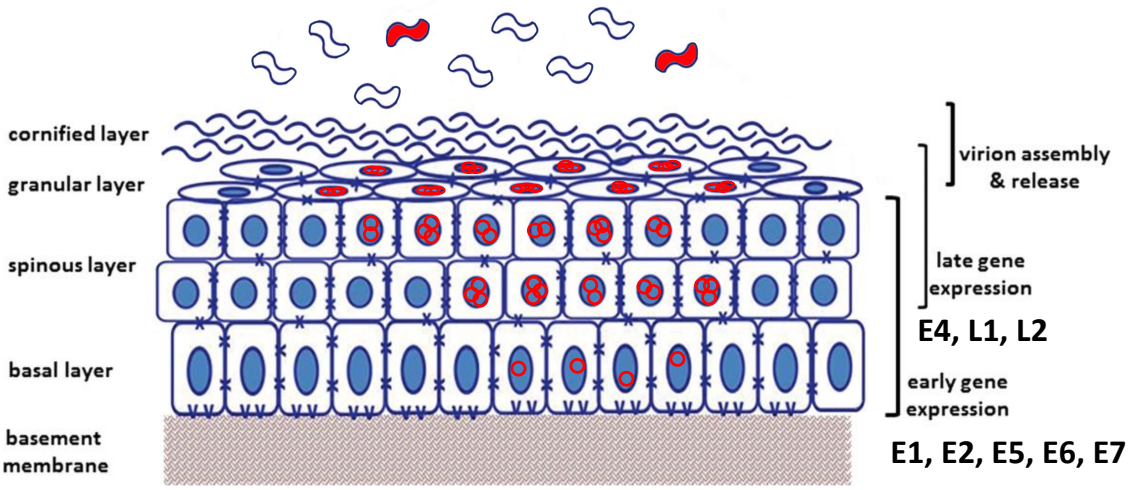


Figure 1.2 The papillomavirus life cycle.
Papillomaviruses infect proliferating cells in the basal layer of epithelia that are exposed by wounding. The viral episome (red circle) is replicated in tandem with the cellular DNA. As basal cells move off the basal layer into the spinous layer they differentiate and exit the cell cycle. However, infected cells remain in the cell cycle due to the action of the papillomavirus oncoproteins E6 and E7 and therefore continue to replicate the viral genome. As infected cells continue to move away from the basal layer, viral capsid proteins are expressed and mature virions are assembled and released with the sloughing off of the cornified layer (red cells). Adapted from Miller, Puricelli, and Stack, Biochem J, 2013 [15].

1.1.4 Papillomavirus Proteins

Ten ORFs have been identified in the papillomavirus genome; eight within the "early" (E) region: E1, E2, E3, E4, E5, E6, E7, E8 and two within the "late" (L) region: L1 and L2. However, to date, E3 is not known to encode a protein, and E8 encodes a fusion protein in only certain PV types . E1, E2, E3, E4, E5, E6, E7, L1, and L2 each play an important role in the papillomavirus life cycle (their expression profile at different stages of the PV life cycle is depicted in Fig 1.2) and will be briefly examined below. The E1 protein is an ATP-dependent DNA helicase and acts to unwind the viral genome to prepare it for replication by cellular DNA replication proteins (reviewed in [16]). E2 is multifunctional: it is a transcriptional regulator of the viral promoters, facilitates the binding of E1 to viral DNA, and tethers the PV genome to cellular chromosomes (reviewed in [17, 18]). Although the nomenclature (E) of E4 implies it is important in early stages of the viral life cycle, the protein is expressed most prominently in the late stages of the viral life cycle. E4 is believed to facilitate virion release as well as genome amplification (reviewed in [19]). E5 is one of the PV oncoproteins, though so far its function has been somewhat elusive. Studies have indicated that E5 may play a role in destabilizing cellular membranes, activating cellular growth signaling pathways, and aiding in evading the host's immune system (reviewed in [20]). E6 and E7 are the most well-studied of the PV oncoproteins as the actions of E6 and E7 result in hyperproliferation and delayed keratinocyte

differentiation ([21, 22], reviewed in [23, 24]). The PV E6 oncoprotein interacts with cellular proteins containing an acidic, alpha-helical LXXLL motif and can alter their normal cellular functions, thereby preventing cellular distress response mechanisms and changing the normal cellular differentiation process (reviewed in [25] and further expanded upon in section 1.2.3). The E7 PV oncoprotein disrupts cell cycle regulation through interaction with various cellular proteins, thereby forcing cells back into their proliferative cell cycle state (reviewed in [26]). Finally, PVs contain two capsid proteins: L1 (the major capsid protein) and L2 (the minor capsid protein) that are expressed in the late stage of the viral life cycle. L1 is responsible for formation of the icosahedral surface of the PV virion and can spontaneously self-assemble into highly-immunogenic pentameric capsomers, making it the foundation of the HPV-vaccines. L1 is also important in mediating PV attachment to host cells (reviewed in [27]). Because every PV identified to date contains a highly-conserved L1 gene, it has been used as the basis of PV taxonomic classification (further expanded upon in section 1.1.2). The L2 minor capsid protein cooperates with L1 to package viral DNA into virions and interacts with numerous cellular proteins throughout the process of a PV infection (reviewed in [28, 29]).

1.2 Papillomavirus E6 Proteins

1.2.1 E6 Structure

E6 is a small protein, and in most mammalian papillomaviruses, it is comprised of around 150 amino acids. It contains a globular amino (E6N) and carboxy (E6C) terminus joined by a linker helix (Fig 1.3), giving it a "bidomain" structure. Each globular domain contains a zinc structured region characterized by a CXXC motif, which is highly conserved among PV E6 proteins. Also conserved is the hydrophobic E6 core, suggesting a conservation of the E6 tertiary structure [30]. E6 has a propensity for self-association making it generally unstable and insoluble [31], but when complexed with an LXXLL peptide (further discussed in section 1.2.3), certain E6 proteins have been successfully crystallized. The bovine papillomavirus type 1 (BPV1) E6 and HPV16 E6 protein have low sequence identity (~30%), but upon superposition of the two crystal structures the E6 proteins appear very similar (Fig 1.4) [32]. This striking similarity between the structure of E6 proteins from a human and bovine papillomavirus suggests E6 proteins may share a similar overall structure, enabling their interaction with cellular LXXLL-containing proteins [33]. Recent proteomic pull-down data on a variety of HPV E6 proteins identified LXXLL motif-containing cellular proteins [34-36], highlighting the likelihood that E6 proteins share a common structure. To date, the bidomain modality has been conserved among described mammalian papillomavirus E6 proteins. Interestingly, both avian [37] and turtle [38]

papillomaviruses have an E6 protein comprising a single, "monodomain" zinc structured region. Because of this, it is likely that the E6-LXXLL recognition pocket, which is formed by the bidomain structure, is an acquired property found only in mammalian papillomaviruses.

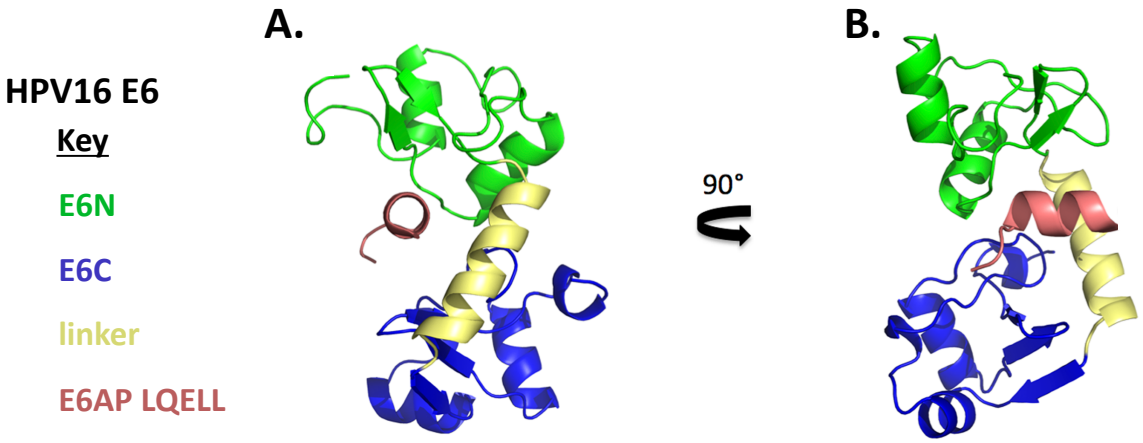


Figure 1.3 Structure of HPV16 E6.
(A) HPV16 E6 structure (PDB ID: 4GIZ) illustrates the E6-amino terminal zinc structured domain (green), the connecting helix (yellow), and the carboxy-terminal zinc structured domain (blue). The E6 protein was crystallized complexed with the E6AP LQELL peptide (light pink). (B) The E6 protein structure depicted in A rotated 90 degrees counterclockwise. Figure adapted from: Vande Pol, S. PLoS Pathogens, 2015 [33].

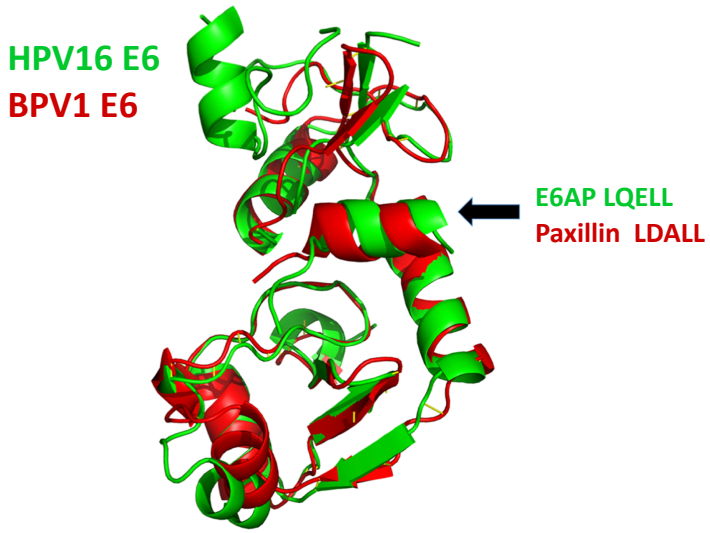


Figure 1.4 BPV1 E6 and HPV16 E6 have very similar folds.
The crystalized BPV1 E6 structure (depicted in red, PDB ID: 3PY7) was superimposed on the structure of HPV16 E6 (depicted in green, PDB ID: 4GIZ). Although the two E6 proteins only share 30% sequence identity, they display a conserved overall structure when complexed with LXXLL peptides. HPV16 E6 was crystalized with the LQELL E6AP peptide, and BPV1 E6 was crystalized in complex with the LDALL peptide from paxillin. Figure adapted from: Vande Pol, S. *PLoS Pathogens,* 2015 [33].

1.2.2 High and Low-Risk PVs

Papillomaviruses that infect mucosal epithelia are found in the Alpha genera and can be subdivided into high-risk and low-risk, based on the likelihood of the initially benign lesions to progress to cancer. High-risk E6 and high-risk E7 proteins are able to more efficiently transform cells than low-risk E6 and low-risk E7. Loss of expression of E6 or E7 results in loss of viral episomal maintenance in both high-risk and low-risk HPV-infected keratinocytes [39, 40]. High-risk HPV genomes that contain point mutations

in hrE6 that disrupt p53 degradation, E6AP binding, or high-risk E6 folding, result in loss of episomal maintenance [40, 41]. The prototypic, and most common, low-risk HPVs are type 6 and type 11 while the prototypic high-risk HPVs include type 16 and type 18.

The carcinogenic potential of a mucosal PV lesion is secondary to intrinsic differences between the high and low-risk E6 and E7 oncoproteins (extensively explored in [12, 42, 43]). Interestingly, both high and low-risk E6 and E7 proteins bind to cellular E3 ubiquitin ligases and hijack their ubiquitin ligase activity to target various cellular proteins for degradation [44]. High and low-risk Alpha genera E6 proteins can both bind the cellular E3 ubiquitin ligase E6AP [45-47], however, only high-risk E6 degradation targets (such as p53) are well established [48, 49]. Although low-risk E6 can bind to E6AP, it cannot target p53 for degradation [47]. To date, no cellular targets of the low-risk E6-E6AP complex have been described.

Another striking difference between high and low-risk E6 proteins is the presence of a PDZ binding motif (PBM) at the extreme carboxy terminus of high-risk E6s [50, 51] (reviewed in [52, 53]). The low-risk E6 proteins lack a PBM. The presence of the PBM enables high-risk E6 proteins to interact with a group of cellular proteins called PDZ proteins. PDZ proteins are a group of signaling proteins that share a common 80-90 amino acid PDZ (PSD-90/Dlg1/ZO-1) homology domain. Although high-risk E6 proteins have the ability to interact with cellular PDZ proteins, if and when high-risk E6 targets

their degradation is controversial. However, comparison of wild type high-risk E6 (E6_WT) and high-risk E6 deleted of the PBM (E6ΔPBM) elucidated numerous phenotypes attributable to the PBM. HPV genomes containing E6ΔPBM mutants are not episomally maintained, although episomal maintenance is rescued when p53 function is disrupted through the addition of shRNA or dominant negative p53 [54]. These results suggest that the high-risk E6 PBM is important in the viral life cycle, and may play a role in the relationship between high-risk E6 and p53.

Not surprisingly, low-risk and high-risk E7 also exhibit different preferences for various cellular targets. The high and low-risk E7 proteins interact with ubiquitin ligases from the cullin and N-end rule families. When complexed with a cellular ubiquitin ligase, high-risk E7 proteins target all three pocket family proteins for degradation: pRb, p107, and p130 as well as the protein tyrosine phosphatase PTPN14 [55-57]. In contrast, low-risk E7 proteins complexed with a cellular ubiquitin ligase only target the degradation of p130 [58].

1.2.3 E6 Binding Partners

While numerous cellular proteins can interact on distinct surfaces of E6 proteins, the primary interaction of E6 with a cellular target protein occurs within a deep E6 pocket, formed upon interaction of E6 with an acidic LXXLL motif (Fig 1.3 and 1.4) found within numerous cellular proteins [32]. These E6-LXXLL peptide interactions stabilize and restructure E6 proteins [59].

Grouping E6 proteins based on their binding preference to different cellular LXXLL proteins identifies three distinct clusters: (1) preferential binding to E6AP, (2) preferential binding to Mastermind-like protein 1 (MAML1), and (3) binding preference currently unknown [60]. MAML1 is a transcriptional coactivator of Notch signaling, which is important in initiating keratinocyte differentiation (reviewed in [61]). The preferential binding of an E6 protein to either E6AP or MAML1 is directly related to the function of that E6 protein. E6 proteins that bind E6AP stimulate E6AP autoubiquitination and its subsequent degradation in an overexpression system [47, 60, 62]. E6 proteins that preferentially associate with MAML1 repressed MAML1 transcriptional activity [60].

Outside of E6 interactions with acidic LXXLL-containing cellular proteins, a subset of E6 proteins (classified as high-risk and further discussed in section 1.2.2) contain a PDZ binding motif (PBM), enabling them to interact with cellular PDZ proteins. PDZ proteins are frequently involved in cell polarity and maintenance of intercellular junctions, and degradation or mislocalization of PDZ proteins could drive cellular proliferation and contribute to tumorigenesis (reviewed in [52, 53]). Cells contain hundreds of PDZ domain-containing proteins, although only a subset of them have been identified interacting with the high-risk E6 PBM. Some PDZ proteins with which high-risk E6 interacts include protein tyrosine phosphatase non-receptor type 3 (PTPN3) [63], discs

large homolog (Dlg) [64, 65], and Scrib [36, 66] (which are involved in epithelial cell polarity).

1.3 E6AP: E6-Associated Protein

1.3.1 E6AP Gene: UBE3A

The E6AP gene (UBE3A) encodes three protein isoforms of E6AP (isoforms I, II, and III) that result from differential splicing [67]. The differences between the three E6AP isoforms is found only within the first 23 amino acids in their extreme N-termini (Fig 1.5) There is not a standard isoform to which E6AP numbering is referenced, so when reading literature in which specific E6AP residues are mentioned, it is important to account for the author's specific reference isoform. All E6AP residue numbers throughout this thesis are in reference to E6AP isoform II, which has a total length of 875 amino acids and is therefore the longest of the E6AP isoforms.

```
E6AP_Isoform_III      ---matackrsgepqsddieasrmkraaakhlieryyhqltegcgneactnefcascptf
E6AP_Isoform_I        ----------------------mkraaakhlieryyhqltegcgneactnefcascptf
E6AP_Isoform_II       meklhqcywksgepqsddieasrmkraaakhlieryyhqltegcgneactnefcascptf
                                            ***********************************
```

Figure 1.5 Human E6AP isoforms differ only in their extreme amino termini.
Human E6AP isoform sequences from the NCBI database were aligned using Clustal Omega.

The UBE3A gene transcript is ubiquitously expressed in humans. Within the brain, the UBE3A gene is imprinted and the paternal allele silenced [68]. Inheritance of a mutated allele resulting in E6AP dysfunction from the mother causes Angelman syndrome [69], a neurodevelopmental disorder characterized by speech impairment, intellectual disability, seizures, motor dysfunction, and frequent smiling [69-71]. Alternatively, duplication or triplication of the maternally inherited UBE3A gene is often associated with autism [72, 73].

1.3.2 E6AP Protein Structure

The 100 kDa E6AP protein is insoluble at high concentrations, making crystalizing the entirety of the protein a formidable task. However, certain well-defined domains of E6AP have been crystalized (Fig 1.6). The crystalized domains include the amino-terminal zinc-finger of Ube3a ligase (AZUL) [74], the motif required for E6 binding [32] (further discussed in section 1.4), and the HECT domain [75].

E6AP is the prototype of a group of cellular E3 ubiquitin ligases termed HECT (**H**omologous to the **E**6AP **C**arboxyl **T**erminus) ligases [76]. The conserved HECT domain is bimodal in structure, with an N-terminal N-lobe that interacts with an E2 ubiquitin-conjugating enzyme and a C-terminal C-lobe containing the active cysteine residue [75]. This active cysteine residue (located at residue 843 within E6AP, highlighted in orange in Fig 1.6) is necessary for interaction with ubiquitin and the transfer of ubiquitin to a substrate. Regions N-terminal to the HECT domain are believed to be

important for substrate specificity, but their structural and functional properties are largely unknown (reviewed in [77]). Importantly, mutation of the active cysteine residue in E6AP results in a mutant E6AP that is defective in ubiquitin ligase activity (E6AP_Ub⁻).

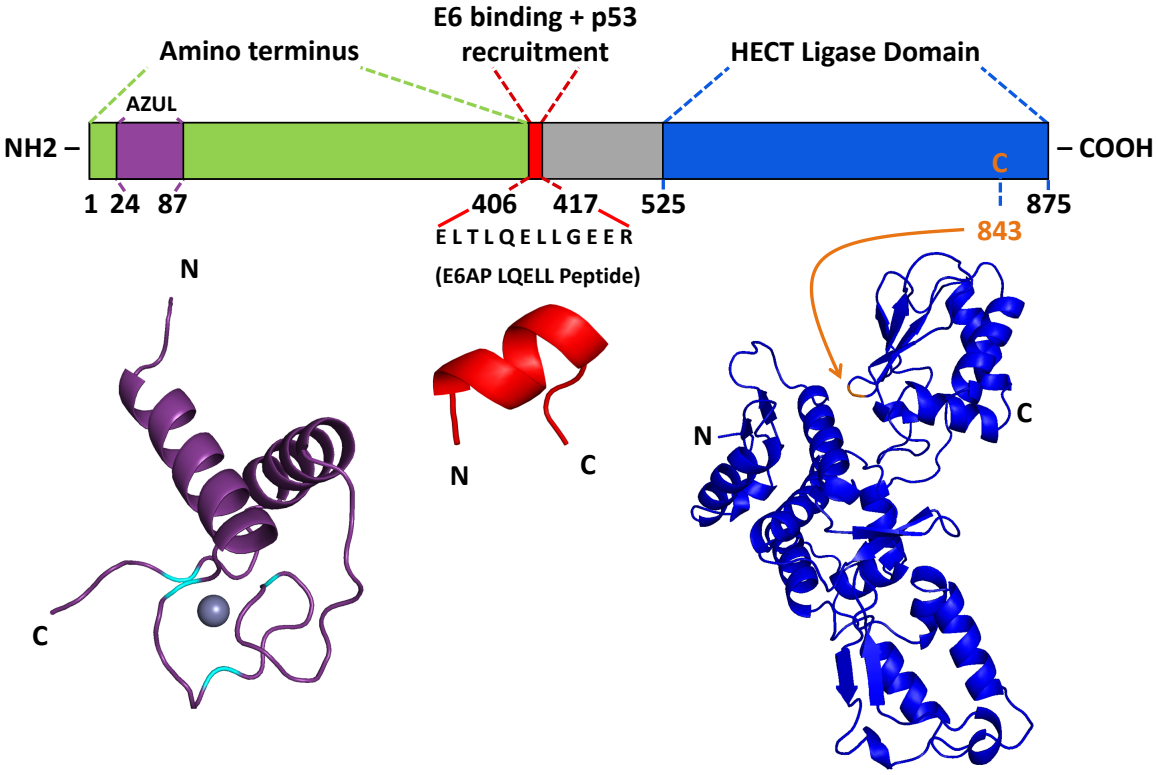


Figure 1.6 General structure of E6AP and crystallized domains.
E6AP is around 100 kDa and contains three characterized domains. (1) In the amino terminus (depicted in green), is the amino-terminal zinc-finger of Ube3a ligase or AZUL (PDB ID: 2KR1). The AZUL domain contains a helix-loop-helix structure with a zinc ion positioned by four cysteine resides (bright blue). (2) The E6 LQELL binding motif of E6AP (red) is centrally located within E6AP. The acidic, alpha-helical peptide has been crystalized in complex with 16E6 (PDB ID: 4XR8) and spans 12 residues. (3) The HECT domain (homologous to E6AP carboxy terminus) is so named because E6AP was the first E3 ubiquitin ligase found with the highly conserved HECT domain, which now characterizes a group of E3 ubiquitin ligases called HECT ligases. The HECT domain (blue) of E6AP contains an active cysteine residue at position 843 (orange; based on E6AP isoform II numbering) that is required for ubiquitin thioester bond formation, thereby linking ubiquitin to the HECT domain (PDB ID: 1C4Z).

1.4 The E6-E6AP Interaction

1.4.1 What is currently known about the E6-E6AP interaction?

Studies utilizing truncation mutants of E6AP to identify regions of E6AP that are required for high-risk E6 binding, high-risk E6-dependent association with p53, and ubiquitination of p53 found that an acidic, alpha-helical peptide of E6AP (which contains an LXXLL motif) is both necessary and sufficient for high-risk E6 binding [59, 78]. The acidic, alpha-helical E6AP peptide consists of twelve amino acids: ELTLQELLGEER; throughout this thesis, this E6AP peptide will be referred to as LQELL.

While high-risk E6 can bind the E6AP LQELL peptide alone, low-risk E6 is unable to bind the isolated peptide, although it is able to find full-length E6AP [47]. Mutagenesis studies of the LQELL region of E6AP ablated the ability of both high and low-risk E6 proteins to interact with E6AP. Therefore, although low-risk E6 is not able to interact with the isolated LQELL peptide, the presence of LQELL is required for both high and low-risk E6 proteins to interact with full-length E6AP, suggesting a common mode of interaction [47]. Additionally, a low-risk E6 chimera to which a high-risk E6 PBM was fused enabled interaction of the low-risk E6 protein with PDZ protein Dlg; and in the presence of E6AP, the low-risk E6 chimera was able to target the degradation of Dlg. This low-risk E6 gain-of-function indicates that low-risk E6 is capable of hijacking the ubiquitin ligase activity of E6AP in a manner similar to high-risk E6 proteins [47].

Replacement of the E6AP LQELL motif with the MAML1 LDDLL motif (E6AP chimera) enabled E6 proteins that preferentially bind MAML1 to bind to this E6AP chimera. Although these MAML1-binding E6 proteins demonstrated gain-of-function binding, they were unable to stimulate autoubiquitination of E6AP [60]. Therefore, the ability of an E6 protein to stimulate the ubiquitin ligase activity of E6AP requires more than just E6 docking on its LQELL motif.

E6AP is necessary for stable expression of high-risk E6 in E6AP null mouse epithelial kidney cells (8B9), among others [59, 79]. Furthermore, the LQELL E6AP peptide is necessary and sufficient for changing the conformation of high-risk E6 to enable p53 binding [59]. Evidence suggests that cellular E6AP does not associate with or target p53 for degradation in the absence of high-risk E6, establishing that the specificity of the high-risk E6-E6AP heterodimer for p53 resides within the E6 protein [48, 80]. The identification of high-risk E6 mutants that lose the ability to bind p53 even though they can still bind E6AP further validates the specificity of high-risk E6 for p53 [48].

1.4.2 The E6-E6AP-p53 heterotrimeric complex

The E6AP LQELL peptide is sufficient to stabilize high-risk E6 *in vivo* and change the conformation of high-risk E6 to enable p53 binding [59]. Additionally, several studies have established that the core DNA binding domain of p53 is required for interaction with the high-risk E6-E6AP heterodimer [59]. These observations enabled the crystallization of high-risk

E6 from HPV type 16 (16E6), the LQELL E6AP peptide, and the p53 core domain [81] (Fig 1.7). This crystal structure provides the framework for our understanding of the contributions of particular residues in 16E6 and p53 that affect p53 binding. Additionally, it enables identification of other 16E6 and p53 residues that may be important in ternary complex formation and illuminates the structure of the 16E6-p53 binding cleft, which could serve as a site for small molecule inhibitors to block the interaction of p53 with the high-risk E6-E6AP heterodimer [81].

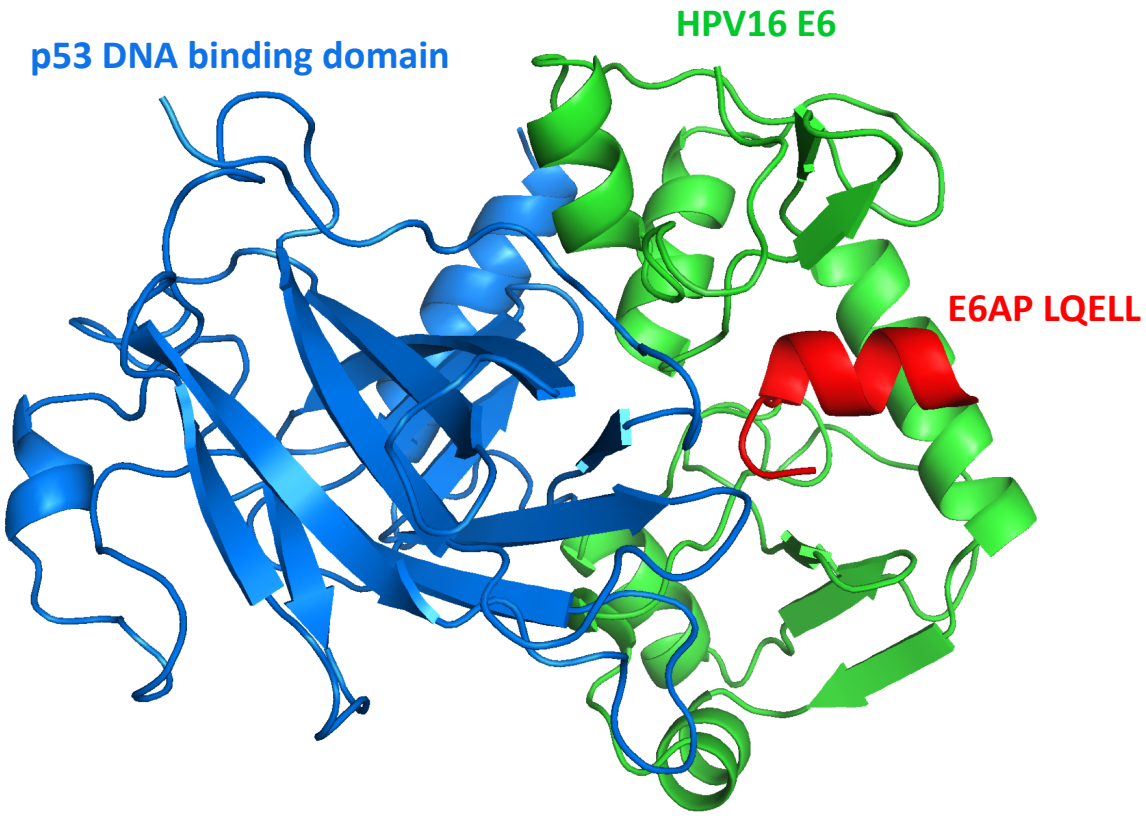


Figure 1.7 The ternary 16E6-E6AP LQELL-p53 complex.
16E6 (green) restructuring by the E6AP LQELL peptide (red) enables recruitment of the p53 DNA binding domain (blue) to the complex [59]. This tertiary complex was crystalized (PDB ID: 4XR8) and provides insight into residues important in mediating the formation of the E6-E6AP LQELL-p53 complex [81].

1.4.3 E6 Binding to E6AP: More than just E6AP LQELL?

Multiple lines of evidence suggest that there are other regions in E6AP, outside of the LQELL motif, that play a role in E6 binding and high-risk E6-mediated p53 degradation [59, 82, 83]. The interactions between E6AP LQELL and residues of E6 observed in the crystal structures have provided insight for structure-based construction of E6 mutants that are unable to interact with the LQELL E6AP peptide: L50A, R102A, and R131A [32]. Further analysis of these E6 mutants indicated that, while important, E6's ability to bind to the isolated E6AP LQELL motif was not always required for E6-mediated degradation of p53 in the presence of full-length E6AP, as E6 R131A was able to initiate p53 degradation when overexpressed [32].

Experimental approaches utilizing a yeast two-hybrid system indicated high-risk E6 displays enhanced affinity for full-length E6AP over the LQELL peptide [59]. Additionally, a quantitative yeast three-hybrid assay found that high-risk E6 bound to the LQELL E6AP peptide was significantly less effective at recruiting p53 than high-risk E6 bound to full-length E6AP [59]. These data corroborate prior measurements of the affinity of high-risk E6 for the E6AP LQELL peptide *in vitro*, which were in the low μM range [82], while the affinities of high-risk E6 for the full-length E6AP protein were in the low nM range [83]. Low-risk HPV E6 proteins cannot bind the isolated E6AP LQELL peptide, but they can bind the peptide in the context of full-length E6AP [47].

These results suggest that other region(s) of E6AP are required for the higher affinity binding of E6 to E6AP.

1.5 NHERF1

1.5.1 NHERF1: A PDZ Protein

NHERF1 (Na^+/H^+ Exchange Regulatory Factor 1), also called EBP50 (ezrin binding protein 50) is a product of the SLC9A3R1 gene and its mRNA is highly expressed in epithelia [84]. The NHERF1 protein contains two PDZ domains, an EB domain (**e**zrin-radixin-moesin (ERM)-**b**inding), and a PBM at its extreme carboxy terminus (Fig 1.8). The PDZ domains enable NHERF1 to interact with proteins containing a PDZ binding motif (PBM), some of which include PTEN, EGFR, and β-catenin. The EB domain is responsible for anchoring NHERF1 to the actin cytoskeleton network via the ERM proteins. NHERF1 acts as a protein scaffold, ensuring the proper subcellular localization of proteins involved in numerous signaling pathways. NHERF1-null mice have reduced lifespans and are prone to hydrocephaly, brittle bone structure, and phosphate wasting due to the mislocalization of proteins with which NHERF1 usually associates [85]. NHERF1 is able to interact with itself via its PBM [86]. This intramolecular interaction between the NHERF1 PBM and PDZ2 results in a "closed state" during which the NHERF1 PDZ domains are blocked from interacting with their usual targets (Fig 1.9). When ERM

proteins bind to the NHERF1 EB domain, the closed state is opened, enabling NHERF1 to once again function as a protein scaffold [86].

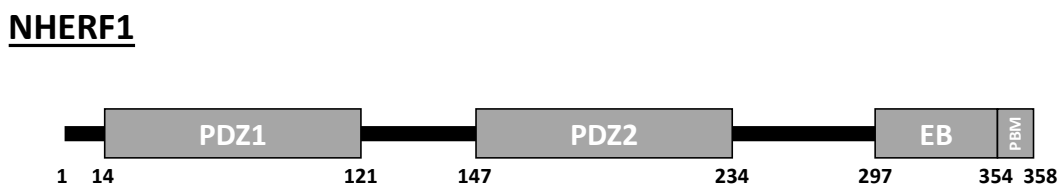


Figure 1.8 NHERF1 protein schematic.
NHERF1 is a PDZ protein that contains two PDZ domains: PDZ1 and PDZ2. These two domains can interact with different cellular proteins that contain a PDZ binding motif (PBM). The NHERF1 EB (ezrin, radixin, moesin (ERM) binding) domain enables NHERF1 to bind to the actin cytoskeleton. NHERF1 also has a PBM at its extreme carboxy terminus.

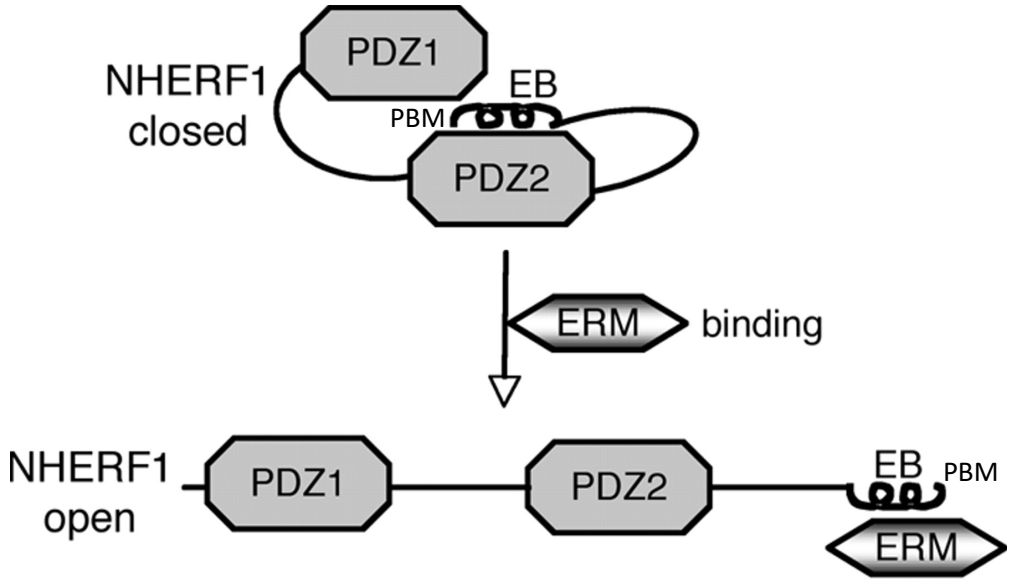


Figure 1.9 Model of NHERF1 intramolecular interaction.
The NHERF1 protein presents a "closed" conformation when its PBM binds to its PDZ2 domain. When in this closed conformation, NHERF1 inhibits other proteins from interaction with its PDZ domains. However, NHERF1 switches to an "open" conformation, enabling access to its PDZ domains, when its EB domain is engaged by ezrin, radixin, or moesin (ERM). Figure adapted from: Morales et al. *Mol Cell Biol,* 2007 [86].

1.5.2 The Canonical Wnt/β-catenin Signaling Pathway

Due to its role as a scaffold, NHERF1 in involved in the maintenance of numerous key cellular signaling pathways, one of which is the canonical Wnt/β-catenin pathway. The Wnt pathway in its entirety is extremely complex and has been explored extensively in recent reviews [87-93]. A brief, simplified overview of the pathway follows (Fig 1.10). When in the off-state, β-catenin is bound by the destruction complex, a complex consisting of two scaffold proteins: Axin and adenomatosis polyposis coli (APC), as well as two kinases: glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α). When bound in the destruction complex, β-catenin is phosphorylated by CK1α and GSK3β and then ubiquitinated by the E3 ubiquitin ligase β-Trcp, targeting it for degradation by the proteasome. When in the on-state, an extracellular Wnt ligand binds to the extracellular amino terminus of the GPCR Frizzled (Fzd). The binding of Wnt to Fzd results in the recruitment of Dishevelled (Dsh) to the intracellular carboxy terminus of Fzd. Dsh signals the breakdown of the destruction complex through a series of protein interactions and signaling pathways which sequester the various components of the destruction complex. The reduced number of destruction complexes results in an increase in cytoplasmic β-catenin, which translocates into the nucleus acting as a transcriptional activator of genes important in stimulating cell growth and proliferation.

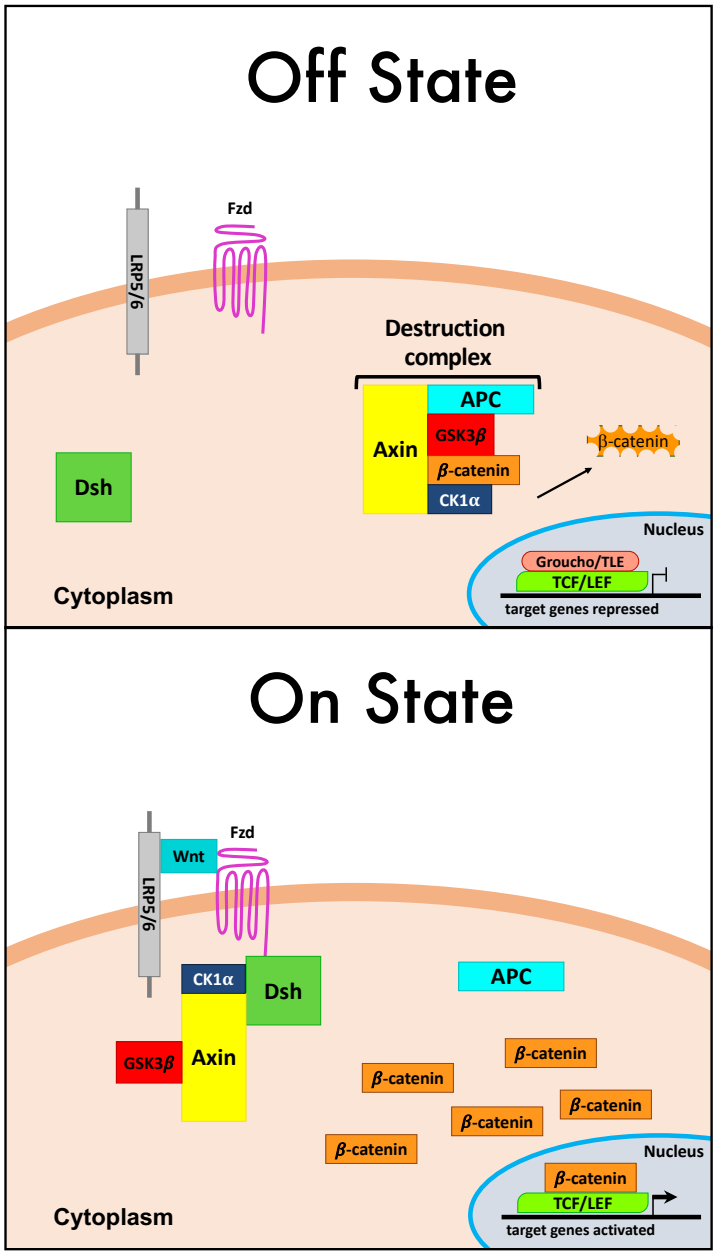


Figure 1.10 Schematic of Canonical Wnt/β-catenin Signaling.
The canonical Wnt pathway is tightly controlled. In the absence of a Wnt ligand (the off-state), cytosolic β-catenin is bound up in the destruction complex (consisting of the protein scaffolds Axin and adenomatous polyposis coli (APC), and the kinases GSK3β and CK1α) and targeted for degradation by the proteasome. In this state, β-catenin target genes are repressed. When a Wnt ligand is present (on state), it binds to the G-protein coupled receptor Frizzled (Fzd) which recruits the transmembrane protein LRP5/6. Dishevelled (Dsh) binds to the intracellular carboxy terminus of Fzd and this signals the breakdown of the destruction complex, releasing β-catenin into the cytosol. The β-catenin translocates into the nucleus and transactivates pro-proliferative target genes.

1.5.2.1 NHERF1 Regulation of the Canonical Wnt/β-catenin Pathway

NHERF1's role in the canonical Wnt/β-catenin pathway is multifaceted and complex. NHERF1 has been shown to directly interact not only with β-catenin [94], but also with the G-protein coupled receptor Fzd [95] (FIG 1.11). There has been evidence indicating NHERF1 can both positively and negatively regulate the Wnt/β-catenin pathway, and these functions are directly related to its subcellular localization. Under normal physiological conditions, NHERF1 is localized to the plasma membrane and is required to maintain both β-catenin and E-cadherin at adherens junctions [96]. The same study demonstrated that NHERF1 is required to suppress anchorage independent growth in NHERF1-null mouse embryonic fibroblasts (MEFs).

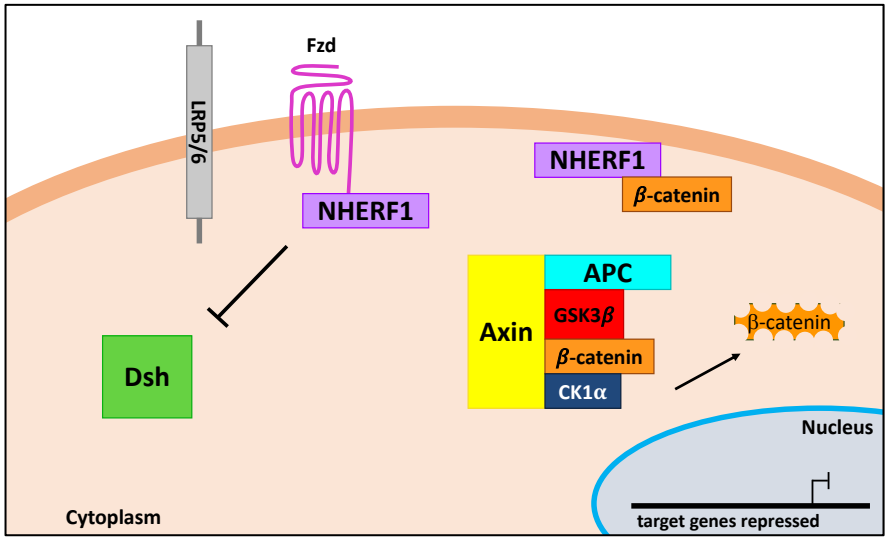


Figure 1.11 NHERF1 acts as an inhibitor of canonical Wnt signaling.
The PDZ2 domain of NHERF1 binds to the PBM at the carboxy terminus of Frizzled (Fzd) and prevents binding of Disheveled proteins (Dsh) [95]. Additionally, NHERF1 can interact directly with β-catenin and localize it to the plasma membrane, assisting in formation of adherens junctions [96].

NHERF1 anchors Fzd to the actin cytoskeleton (Fig 1.11). Because NHERF1 binds to the intracellular carboxy terminus of Fzd receptors, NHERF1 acts to block premature Dsh-Fzd binding thereby negatively regulating canonical Wnt signaling [95]. Comparison of two human breast cancer cell lines (one with low and one with high NHERF1 expression) treated with Wnt proteins demonstrated enhanced Wnt-induced β-catenin activation and cellular proliferation in cells lacking NHERF1 [95]. Loss of NHERF1 localization at the plasma membrane (either by mislocalization or loss of protein expression) has been reported to induce epithelial-mesenchymal transition (EMT) partly due to a reduction of E-cadherin at cell-cell junctions and loss of cell polarity, as well as translocation of β-catenin to the nucleus [97, 98]. There have also been reports that NHERF1 in the nucleus displays a gain-of-function phenotype, acting as a scaffold to bring β-catenin and TCF-1B together to enhance the transactivation of Wnt target genes [94, 99].

1.5.2.2 E6 and Canonical Wnt/β-catenin Signaling

There have been numerous studies indicating that E6 proteins can activate canonical Wnt/β-catenin signaling (reviewed in [100]). Oropharyngeal cells expressing HPV experience an increased accumulation of nuclear β-catenin, whereas in HPV negative cells β-catenin is localized to the plasma membranes. Because E6 is a multifunctional protein and the Wnt/β-catenin pathway is quite complex, there have been multiple lines of evidence suggesting E6 can augment the pathway in numerous ways. The ability of an E6 protein to

increase canonical Wnt signaling is dependent upon the ability of E6 to interact with E6AP [101]. As such, E6s found in the Beta genera (which preferentially associate with MAML1) are unable to augment Wnt signaling [102]. The mechanism by which the E6-E6AP complex stabilizes β-catenin and activates canonical Wnt/β-catenin signaling remains unclear. *In vivo* studies performed in transgenic mice indicated that the E6 PBM is required for prolonged cellular hyperproliferation and activation of Wnt signaling [103]. However, within the same study, *in vitro* experiments demonstrated that E6, as well as E6 deleted of its PBM, are able to stimulate Wnt signaling and do so via an interaction with Dsh [103]. These paradoxical findings reaffirm the complexity of the interplay between E6 and the canonical Wnt/β-catenin pathway and demonstrate the need for continued studies to clarify the mechanism by which E6 augments Wnt signaling.

1.5.3 NHERF1 and Cancer

The role of NHERF1 in tumorigenesis is debated in the literature (comprehensively reviewed in [104, 105]). It has been postulated that NHERF1 may be either a tumor suppressor or an oncogene, depending upon its subcellular localization. The myriad of proteins that have been identified complexed with NHERF1 include PTEN [106], receptor tyrosine kinases [107, 108], ion channels [109], β-catenin [94, 96], Frizzled [95] (among other GPCRs) [110]), transcriptional coactivators [111], and ERM proteins [112]. This collection of identified interaction partners indicates a role for NHERF1 as a

growth regulating adaptor protein, mislocalization of which could have implications for cellular proliferation. While NHERF1 null mice do not spontaneously develop tumors, they do have a shortened life span. Of note, these mice do have elongated intestines, indicating NHERF1 has a growth regulation phenotype. Incorporating an NHERF1 null background into mice that already contain heterozygous deletion of the APC gene resulted in significantly shorter lifespans than their NHERF1-expressing counterparts [113]. Upon histological analysis, this same study found that these heterozygous APC NHERF1 null mice had pronounced tumor burden, not only in tumor number, but also in tumor size when compared to heterozygous APC mice containing at least one WT NHERF1 allele [113]. These data indicate an *in vivo* tumor suppressive role for NHERF1.

1.6 Current Knowledge Gaps

While the field of papillomavirus biology has been studied for over eighty years, there are still numerous gaps in our scientific knowledge. Low and high-risk E6 proteins have been the subject of much research due to their clinical implications, and both have been shown to interact with the cellular E3 ubiquitin ligase E6AP. A well-characterized target of the high-risk E6-E6AP complex is cellular p53. An acidic, alpha-helical LQELL motif in E6AP is both necessary and sufficient to recruit p53 to the E6-E6AP complex. To date, no low-risk E6-E6AP complex degradation targets have been identified, even though low-risk E6 is capable of stimulating E6AP ubiquitin ligase activity and

is responsible for a sizable clinical burden. Additionally, no E6AP-dependent degradation targets common to both low and high-risk E6 proteins have been identified.

Interestingly, while the E6AP LQELL motif can change the conformation of high-risk 16E6 to recruit p53, low-risk 11E6 is unable to interact with the isolated E6AP LQELL peptide, suggesting other auxiliary regions within E6AP may be important in mediating the low-risk 11E6-E6AP interaction. While multiple studies have predicted there may be other regions within E6AP that are important in mediating the E6-E6AP interaction, these potential E6AP auxiliary regions have not yet been characterized. Binding to the E6AP LQELL motif is not sufficient for an E6 protein to stimulate the E6AP ubiquitin ligase activity, suggesting this E6 function may also require E6AP auxiliary binding sites. How an E6 protein stimulates E6AP E3 ubiquitin ligase activity is unclear.

1.7 Overview of dissertation

1.7.1 Aim of Dissertation

The overarching aim of my dissertation is to better understand the actions of papillomavirus E6 proteins. My studies put forth a novel degradation target common to both high and low-risk E6 proteins and demonstrate that the degradation of this target augments an important cellular growth pathway, which may have numerous implications in the viral life cycle. Additionally, my

studies identify previously undescribed regions within E6AP that mediate its interaction with E6 proteins. With our observations, we propose two distinct E6AP-E6 interaction types, with implications for better understanding of how E6 proteins both interact with, and stimulate ubiquitin ligase activity of, cellular E6AP. Understanding this important complex may result in better strategies for HPV antiviral therapies.

1.7.2 Strategy and Summary of Dissertation

Herein, I present my work as it relates to the papillomavirus oncoprotein E6. My thesis took the form of two distinct projects: (1) identification of NHERF1 as a common E6 degradation target and (2) characterization of the E6-E6AP interaction. However, this was not our initial intention. We originally sought to identify what regions in E6AP were required for E6 stimulation of E6AP ubiquitin ligase activity. The literature regarding the interaction of E6 with E6AP in addition to our own observations led us to our hypothesis: the viral oncoprotein E6 requires auxiliary binding sites in the N-terminus of E6AP, in addition to binding to LQELL, to initiate p53 degradation. I utilized a 16E6 mutant (L50A) that was unable to bind E6AP LQELL, but retained its ability to interact with full-length E6AP to investigate other regions within E6AP (outside of LQELL) that are important in E6-E6AP complex interaction and function. 16E6_L50A in combination with amino terminal E6AP truncations identified E6AP residues 310-320 as important in 16E6 targeting the degradation of p53.

We constructed full-length E6AP with the internal deletion of residues 310-320, inclusive (E6APΔ310-320). While 16E6 was unable to target p53 for degradation in the presence of this E6AP deletion mutant, we wondered whether this applied to other cellular degradation targets of 16E6. NHERF1, a PDZ protein, was consistently degraded by 16E6_WT and previous work indicated that it was degraded in a manner dependent on the E6 PDZ binding motif (PBM), located at the extreme carboxy terminus of high-risk E6 proteins. Surprisingly, while 16E6_WT could degrade NHERF1, 18E6_WT could not even though both contain a PBM, albeit not identical in sequence (16E6: …ETQL* and 18E6: …ETQV*). We tested the ability of 16E6_WT to degrade NHERF1 in the presence of the E6APΔ310-320 mutant. As a negative control, we added 16E6ΔPBM, 18E6_WT, and the low-risk 11E6_WT, which does not contain a PBM. To our surprise, we found that in the presence of full-length WT E6AP, not only did 16E6_WT initiate the degradation of NHERF1, but 16E6ΔPBM, 18E6_WT, and 11E6_WT did too. These data were the first, to our knowledge, to identify an E6AP-dependent E6 degradation target shared among both high and low-risk E6 proteins. Additionally, the finding that the PDZ protein NHERF1 could be degraded in a manner independent of the presence of a PBM begged the question: how are these E6 proteins interacting with this PDZ protein if it isn't with their PDZ protein binding motif (PBM)? While our data did show that the E6 proteins were unable to initiate the degradation of NHERF1 in the presence of E6APΔ310-320 and so residues 310-

320 were likely important in the general ability of E6 proteins to stimulate E6AP E3 ubiquitin ligase activity, the focus of my project changed to understanding how and why these E6 proteins target NHERF1 for degradation (**Chapter 3**). Not only did we identify NHERF1 as a degradation target of both high and low-risk human papillomavirus E6 proteins, but we also found it was degraded by a subset of E6 proteins from non-human papillomaviruses which interacted with E6AP. Through the use of 16E6 single amino acid point mutants, we identified a putative novel binding region in the linker helix required for NHERF1 degradation. From the perspective of NHERF1, we determined that the EB domain (located in the NHERF1 carboxy terminus) was both necessary and sufficient for degradation by E6 proteins. Finally, we identified a phenotypic result of E6 degradation of NHERF1: augmentation of the canonical Wnt/β-catenin pathway. Possible implications of E6-mediated activation of this pathway are further discussed in **Chapter 5**.

After spending over two years focusing on NHERF1 in relation to E6 proteins, we returned to our original E6AP project (**Chapter 4**). This endeavor resulted in more than just identifying a region in E6AP required for E6 to stimulate E6AP ubiquitin ligase activity. We propose that among human and non-human papillomavirus E6 proteins, there exist two distinct types of E6 binding to E6AP (Type I and Type II). Type II binding of E6 to E6AP is further broken down into Type IIa and Type IIb. Analyzing E6 interaction with various E6AP truncations identified auxiliary binding regions in E6AP that are

important in mediating formation of the E6-E6AP complex. Our data also suggest that the first eight amino acids of 16E6 are important in mediating E6 interaction with auxiliary regions in E6AP. However, which of the identified E6AP auxiliary regions require the first eight amino acids of 16E6 requires additional study. Taken together, our data led us to further hypothesize that Type IIa E6 proteins require additional auxiliary regions of E6AP in either the N or C-terminus while Type IIb E6 proteins require E6AP auxiliary regions in both the amino and carboxy terminus for successful formation of the E6-E6AP complex. Our thoughts and perspectives on our E6-E6AP binding data, how it relates to the literature and how we propose moving forward are discussed in **Chapter 5**.

Chapter 2: Materials and Methods

Cells and cell culture

E6AP-null 8B9 cells (a gift of Dr. Lawrence banks, ICGEB, Italy) [114] and HPV-negative C33A cervical cancer cells (ATCC) were maintained and transfected using polyethylenimine (PEI) as previously described [59]. Normal immortalized keratinocytes (NIKS, obtained from ATCC) are spontaneously immortalized foreskin keratinocytes [115] that were cocultured with mitomycin C-treated 3T3 feeder cells in F medium as described previously [116]. NIKS were retrovirally transduced with replication-defective murine retroviruses based on pLXSN [117] for E7 expression or pBABEpuro for E6 expression [118]. NIKS were transfected with the HPV16 genome as previously described [54, 116]. Retrovirally transduced NIKS cells and NIKS transfected with the HPV16 genome were counted and seeded at equal confluency in each experiment.

Plasmids

Epitope tagged E6AP, GFP, E6, and NHERF1 were all transiently expressed from the pcDNA3 plasmid. HA-tagged human NHERF1 originated from Vijaya Ramesh's laboratory (from Addgene, plasmid 11635). 16E6 point mutants were created using QuikChange primer design (Agilent Technologies). 16E6ΔPBM was created by mutating the PBM of 16E6 from ETQL* to EL* and E6AP_Ub$^-$ was created by mutating the active cysteine residue at position 843 to an alanine (C843A). The E6AP constructs utilized

express human E6AP isoform III. E6AP and NHERF1 truncations were PCR generated. All NHERF1 truncations used were sequenced.

Immunoprecipitation Assay

12-well plates of transfected E6AP-null 8B9 cells were lysed in 0.5X IGEPAL lysis buffer (1X IGEPAL lysis buffer contains 150 mM NaCl, 50 mM Tris pH 7.5, 0.5 mM Dithiothreitol (DTT), 50 mM NaF, 5 mM NaPPi, 1% IGEPAL, 0.01% phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 1 ug/mL leupeptin/aprotinin) and pelleted at 15,000 xg at 4˚C for 20 minutes. Inputs contain 5% of total supernatant. The remainder of the supernatant was immunoprecipitated with either mouse anti-FLAG M2 antibody coupled beads (Sigma) or mouse anti-HA antibody coupled beads (Thermo Fisher Scientific) by rocking the lysates overnight at 4˚C. Beads were washed three times with 1.0 mL of ice-cold 0.5X IGEPAL lysis buffer. Samples were eluted with SDS, resolved on SDS-PAGE gels, transferred to PVDF membranes, and probed with antibodies.

Antibodies and Western blots

12 well plates of transfected mammalian cells were lysed in 0.5X IGEPAL as described previously [60]. Transduced NIKS were lysed in 1% SDS, 5mM EDTA, and 1 mM sodium vanadate and equilibrated for protein content (Biorad assay kit). All lysates were resolved by SDS-PAGE electrophoresis and transferred to PVDF membranes. Antibodies: anti-HA (Bethyl Laboratories, Inc.), anti-FLAG M2 (Sigma), anti-p53 Ab-8 (ThermoFisher Scientific), anti-

16E6 6G6 (a generous gift from Arbor Vita Corporation), anti-SLC9A3R1 (Sigma), anti-GAPDH (Cell Signaling Technology), and anti-MYC 9B11 (Cell Signaling Technology).

RT-PCR

Retrovirally transduced NIKS were plated at different cell densities and harvested following a TRIzol RNA harvest protocol (Invitrogen). cDNA was generated using random hexamers. Quantitative real-time PCR was performed on the cDNA using iQ™ SYBR® Green Supermix (BioRad #1708880). The primers targeted the SLC9A3R1 gene (BioRad Assay ID: qHsaCEP0050521) and the GAPDH gene (BioRad Assay ID: qHsaCEP0041396). Relative values were analyzed using the $\Delta\Delta C_T$ method (where C_T is the threshold cycle) and GAPDH as a control.

Wnt/β-catenin luciferase reporter assay

C33A cells plated at 70% confluency were transiently transfected with DNA of the TOPFLASH or control FOPFLASH (containing mutated TCF/β-catenin binding sites; 1 ug) plasmid, Renilla luciferase (0.005 ug) plasmid (used to evaluate transfection efficiency), FLAG_E6AP_WT (0.35 ug) plasmid, and the indicated E6 plasmids (0.3 ug). At 18 hrs post-transfection, media was removed and Wnt3A conditioned media was added for 8.5 hours to stimulate the Wnt pathway. Luciferase levels were measured using the Dual-Luciferase® Reporter Assay System (Promega) and a Cytation1 Plate Reader (software version 3.04.17). FOPFLASH luciferase readings were low, and were

subtracted from the paired TOPFLASH readouts. 10% fetal bovine serum Wnt3A conditioned media was generated using L Wnt-3A murine fibroblasts (ATCC, CRL-2647) as previously described [119].

Phylogenetic analysis

Multiple protein sequence files were downloaded from the Papillomavirus Episteme [9] and aligned using the EMBL-EBI MUSCLE (MUltiple Sequence Comparison by Log- Expectation) program [120]. The phylogenetic tree was generated as a neighbor-joining tree without distance corrections within the MUSCLE program [120].

Yeast expression

Modified LexA-based yeast three-hybrid assays were performed as previously described [59]. In brief, YSV1280E yeast (contain an auxotrophic histidine-selected LexA-responsive LacZ promoter) were transformed with the LexA DNA binding domain fused proteins of interest; these yeast strains are referred to as "bait" yeast. In the case of a yeast two-hybrid assay (Y2H) YPH 499 yeast were transformed with transactivator fused (either B42 or Gal4) proteins; called "prey yeast". In yeast three-hybrid assays (Y3H), YPH 499 yeast were transformed not only with a transactivater protein (p53 has intrinsic transactivation activity) but also with another adapter protein; called "prey" yeast. The readout of a Y3H assay is the successful interaction of three proteins at the LexA-responsive promoter to form a blue yeast colony on XGal plates. The bait and prey haploid yeast were mated on YPD (yeast extract-

peptone-dextrose) plates and the diploids replica plated onto glucose selection plates twenty-four hours later. Any auxotrophic markers not provided to the yeast by the transfected plasmids were added to the glucose selection plates before replica plating. Diploid yeast were allowed to grow up on the selection plate for three to four days. These diploids were then patched onto galactose/raffinose XGal plates to develop blue yeast colonies.

Chapter 3: A Degradation Target Common to High and Low-Risk E6 Proteins

Text included in this chapter has been adapted from the following publication:

Drews CM, Case S, Vande Pol SB. E6 proteins from high-risk HPV, low-risk HPV, and animal papillomaviruses activate the Wnt/β-catenin pathway through E6AP-dependent degradation of NHERF1. PLoS Pathog. 2019;15(4):e1007575.

3.1 Abstract

High-risk human papillomavirus E6 proteins associate with the cellular ubiquitin ligase E6AP, and then recruit both p53 and certain cellular PDZ proteins for ubiquitination and degradation by the proteasome. Low-risk HPV E6 proteins also associate with E6AP, yet fail to recruit p53 or PDZ proteins; their E6AP-dependent targets have so far been uncharacterized. We found a cellular PDZ protein called NHERF1 is targeted for degradation by both high and low-risk HPV E6 proteins as well as E6 proteins from diverse non-primate mammalian species. NHERF1 was degraded by E6 in a manner dependent upon E6AP ubiquitin ligase activity but independent of PDZ interactions. A novel structural domain of E6, independent of the p53 recognition domain, was necessary to associate with and degrade NHERF1, and the NHERF1 EB domain was required for E6-mediated degradation. Degradation of NHERF1 by E6 activated canonical Wnt/β-catenin signaling, a key pathway that regulates cell growth and proliferation. Expression levels of NHERF1 increased with increasing cell confluency. This is the first study in which a cellular protein has been identified that is targeted for degradation by both high and low-risk HPV E6 as well as E6 proteins from diverse animal papillomaviruses. This suggests that NHERF1 plays a role in regulating squamous epithelial growth and further suggests that the interaction of E6 proteins with NHERF1 could be a common therapeutic target for multiple papillomavirus types.

3.2 Author summary

Papillomaviruses cause benign squamous epithelial tumors through the action of virally encoded oncoproteins termed E6 and E7, which are classified as either high or low-risk based upon the propensity of the tumor to evolve into cancer. E6 proteins from both high and low-risk HPVs interact with a cellular ubiquitin ligase called E6AP. High-risk E6 proteins hijack E6AP ubiquitin ligase activity to target p53 for degradation. Degradation targets of the low-risk E6 proteins in complex with E6AP have not been described. Here, we describe a protein called NHERF1 that is targeted for degradation by both high and low-risk E6 proteins, as well as E6 proteins from diverse animal species. Degradation of NHERF1 resulted in activation of an oncogenic cellular signaling pathway called Wnt. Identification of NHERF1 as a highly conserved E6 degradation target could inform therapies directed against both low-risk HPVs and cancer-inducing high-risk HPVs.

3.3 Introduction

Human papillomaviruses are small DNA tumor viruses that cause squamous epithelial papillomas in which the virus replicates. The papillomas are initially benign and the host is usually able to clear the underlying HPV infection over time. However, a subset of HPV infections may result in lesions that persist and grow to harmful size or that have a propensity to evolve into

carcinomas [121]. The cancer-causing HPV types are called high-risk and the most commonly occurring high-risk types are HPV16 and HPV18. Worldwide, high-risk HPVs are responsible for 5% of cancers, with cervical cancer being the most common [122]. HPV types that are not associated with malignancies are termed low-risk HPV; although low-risk for malignancies, the size and location of the benign papillomas can render these lesions medically serious [43].

Beyond HPVs, papillomaviruses have been isolated from mammalian species including rodents, primates, bats, cetaceans, and ungulates [123], and are clustered into related genera based upon the divergence of the L1 capsid protein nucleotide sequence (both high and low-risk mucosal HPV types discussed in this study belong to the primate Alpha genera) [8]. Most non-human papillomaviruses encode E6 proteins that are similar in predicted fold to high-risk HPV16 E6 [33]. When diverse mammalian papillomaviruses are clustered based on their E6 sequence similarity, two main groups of papillomaviruses emerge: those that encode E6 proteins that bind to the Notch co-activator MAML1, and those that bind to a cellular E3 ubiquitin ligase called E6AP [60]. An E6 protein that preferentially binds MAML1 suppresses MAML1 transcriptional activation, while an E6 protein that preferentially binds E6AP stimulates E6AP E3 ubiquitin ligase activity to then target additional cellular proteins recruited by E6 to E6AP for ubiquitination and degradation by the proteasome [60].

The difference between the propensity of high and low-risk HPVs to cause cancer is secondary to differences between their respective E6 and E7 oncoproteins [42]. E6 and E7 from both high and low-risk HPVs bind cellular E3 ubiquitin ligases and hijack their ubiquitin ligase activity to perturb certain cellular proteins that are recruited by E6 or E7 [44]. Both high-risk and low-risk E7 proteins interact with ubiquitin ligases of the cullin and N-end rule families and target the degradation of additional cellular proteins recruited to E7 such as pocket-family proteins (pRb, p107, and p130) and PTPN14 [26, 57]. High and low-risk E7 proteins target certain cellular proteins in common (such as the p130 pocket protein) [58]. However only high-risk HPV E7 types interact with and target the degradation of the retinoblastoma pocket proteins [56, 124], which has implications for the carcinogenic properties of high-risk E7. High and low-risk Alpha genera HPV E6 proteins interact with the cellular E3 ubiquitin ligase E6AP [45-47], but only cellular proteins targeted for degradation by the high-risk E6 protein (such as p53) are well established [25, 48].

Another striking difference between high and low-risk E6 is the presence of a PDZ binding motif (PBM) at the extreme carboxy terminus of high-risk E6 proteins [50, 51, 65]. The high-risk PBM enables E6 to interact with a group of cellular proteins termed PDZ proteins, all of which contain PDZ (PSD-90/Dlg1/ZO-1) homology domains [52]. The targeted degradation of cellular proteins that are recruited through interaction with the high-risk E6 PBM has

been controversial, but the E6 PBM functionally promotes retention of the viral DNA plasmid within infected cells [125]; the E6 PBM function can be rescued by disruption of p53 function [54]. Although low-risk E6 proteins bind E6AP, they do not have a PBM at the carboxy-terminus [50], do not interact with p53 [47], and no cellular targets of the low-risk E6+E6AP complex have been described. Such cellular targets would be presumed to be of exceptional interest since they would be common to both high and low-risk E6 proteins, just as p130 is a common target of the high and low-risk HPV E7 protein.

In this study, we identify the PDZ-adapter protein NHERF1 as degraded by both high and low-risk E6 proteins, in a manner dependent upon the ubiquitin ligase activity of E6AP and the proteasome. Other E6 proteins from diverse species where E6 could bind E6AP were also able to initiate NHERF1 degradation, indicating the conservation of this function. Interaction of NHERF1 with E6 required prior association of E6 with E6AP, and we identified a novel interaction domain within 16E6 that is required. Finally, the targeted degradation of NHERF1 by both low and high-risk E6 proteins resulted in the activation of canonical Wnt signaling, connecting the degradation of NHERF1 by E6 to the activation of an oncogenic signaling pathway.

3.4 Results

3.4.1 NHERF1 protein levels are reduced by high and low-risk E6 proteins in the presence of E6AP_WT

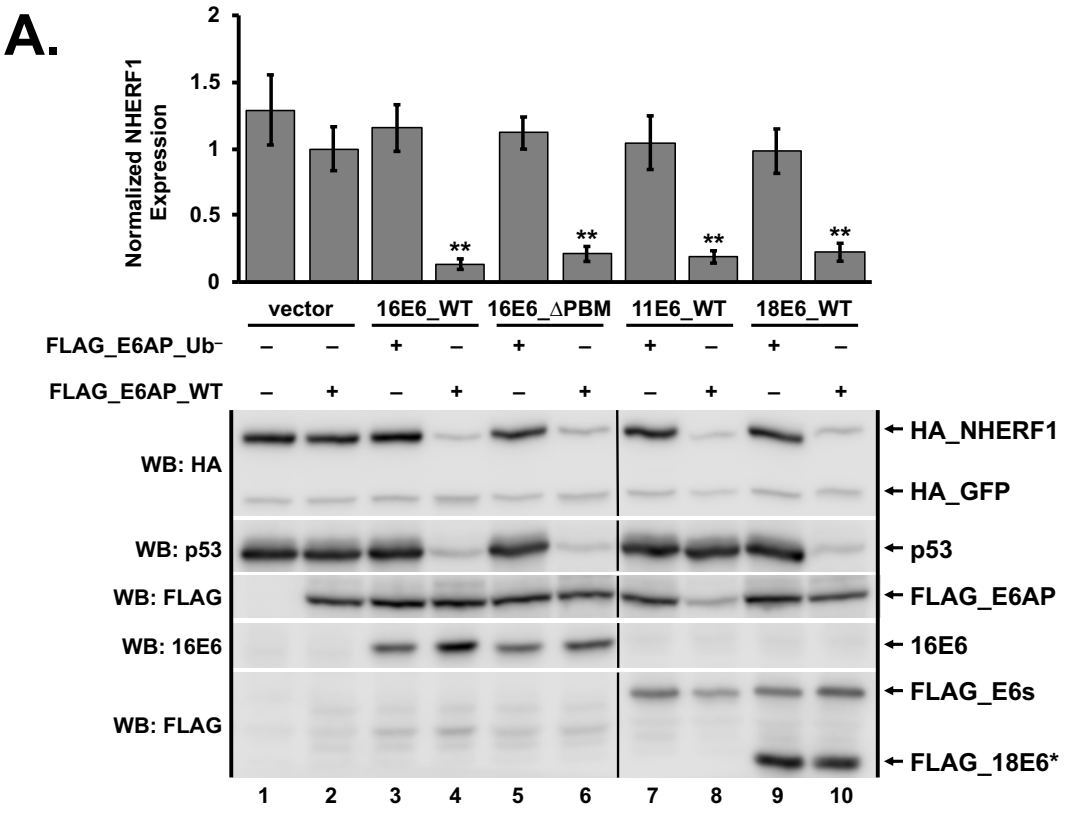
NHERF1 was previously shown to be degraded by HPV16 E6 (16E6) (but not by HPV18 E6 (18E6) or HPV11 E6 (11E6)) through an interaction requiring the PBM of 16E6 [126]. In our proteomic studies of cellular proteins that associate with the 16E6 and 18E6 PBMs [127], we did not identify NHERF1, but in other experiments observed a reduction of NHERF1 protein levels by 16E6, 18E6, and 11E6. In order to characterize the reduction of NHERF1 by these E6 proteins, we performed transient transfections into E6AP-null 8B9 cells reconstituted with either WT E6AP (E6AP_WT) or a mutant E6AP defective in ubiquitin ligase activity (E6AP_Ub$^-$). E6APs were co-transfected with plasmids encoding p53, NHERF1, and 16E6, 16E6 deleted of the PBM (16E6ΔPBM), 11E6, or 18E6. Consistent with published literature, p53 was degraded by high-risk E6 proteins (16E6 and 18E6) independently of a PBM [54] and dependent upon E6AP ubiquitin ligase activity [128] (Fig 3.1). Expression of 11E6 together with E6AP_WT resulted in a lack of p53 degradation by low-risk E6 (11E6), corroborating published findings [47, 129]. However, NHERF1 protein levels were reduced by each observed E6 protein (Fig 3.1A), in contrast to what has been previously published [126].

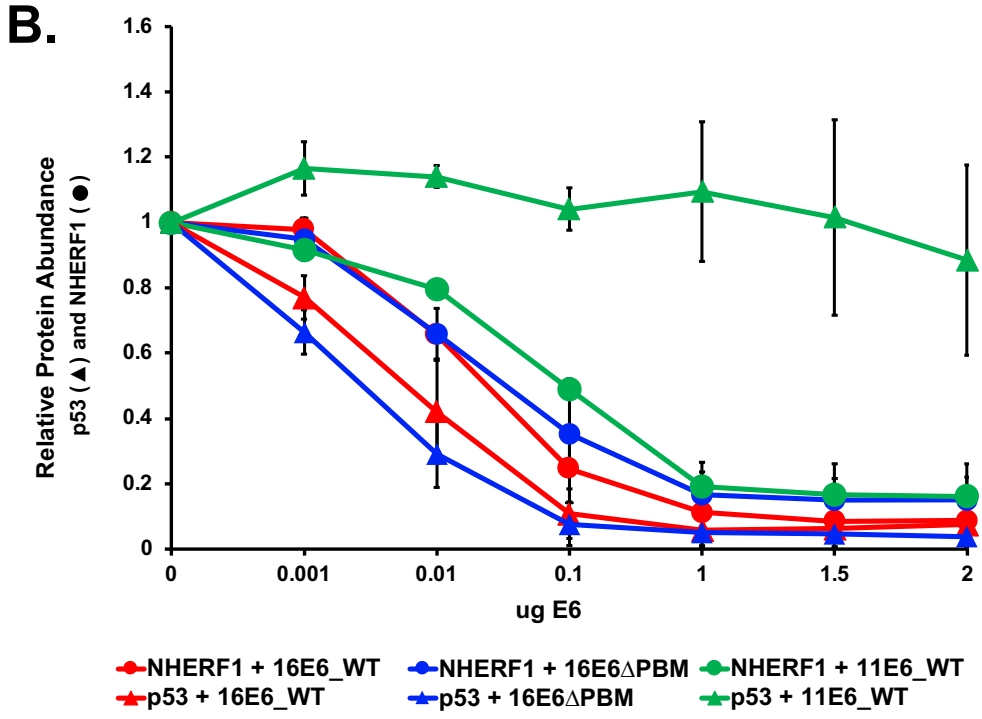
To ensure the reduction of NHERF1 by either high or low-risk E6 proteins was not due to an overexpression artifact, we performed an E6 titration

experiment (Fig 3.1B). Representative western blots from which the quantification in Fig 3.1B was derived are shown in Fig 3.2. Three different E6 proteins were used: 16E6_WT, 16E6ΔPBM, and 11E6_WT. We used p53 as a control for 16E6-mediated degradation, as multiple studies have shown low-risk E6 proteins (11E6) do not degrade p53 [47, 129] while high-risk 16E6 is able to degrade p53 independent of the presence of a PBM [54]. Observing the degradation of p53 in cells expressing variable amounts of E6 provided a guide for physiologically relevant E6 expression levels. NHERF1 and p53 protein levels were similarly reduced by both 16E6_WT and 16E6ΔPBM at the various E6 titrations (Fig 3.1B). 11E6_WT was unable to initiate the degradation of p53 but targeted NHERF1 at levels similar to those required by 16E6. Deletion of the 16E6 PBM did not impact the degradation of p53 or reduction of NHERF1 protein levels by 16E6.

To determine the physiologic relevance of the observed reduction of NHERF1 protein by E6, we seeded keratinocytes either lacking or containing the HPV16 genome at equal confluencies and observed NHERF1 protein levels by western blot (Fig 3.1C). Keratinocytes containing the HPV16 genome not only degraded p53, as expected, but also reduced NHERF1 protein levels by 84%.

** Figure 3.1 panel (C) and legend on next page **





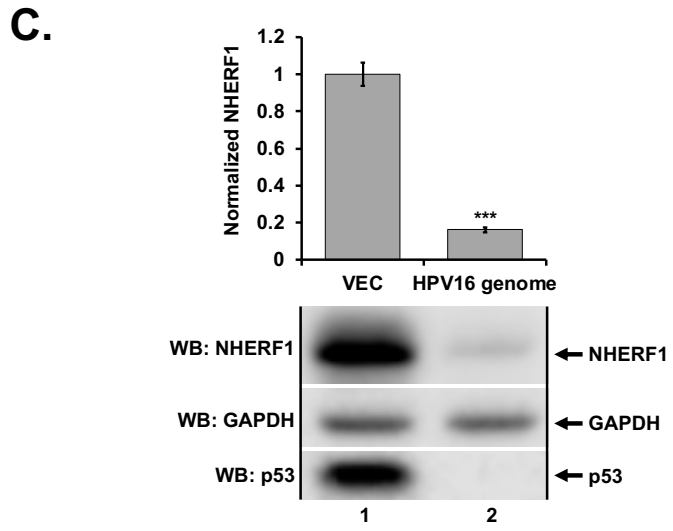


Figure 3.1 NHERF1 protein levels are reduced by both high and low-risk E6 proteins.

(**A**) NHERF1 protein levels are reduced in an E6 and E6AP dependent manner. Plasmids encoding the indicated FLAG_E6AP (1 ug), HA_NHERF1 (0.5 ug), human p53 (0.5 ug), HA_GFP (0.08 ug), and the listed E6 proteins (1 ug) were transiently transfected into E6AP-null 8B9 cells and HA-NHERF1 expression was analyzed by western blot. 2X_FLAG_11E6_WT and 2X_FLAG_18E6_WT constructs were used. Reduction of NHERF1 protein levels by high or low-risk E6 requires ligase active E6AP (E6AP_WT) but does not require the E6 PDZ binding motif (PBM). To disrupt the 16E6 PBM (ΔPBM), we mutated the carboxy terminal PBM from ETQL* to EL*. FLAG_18E6* is a truncated splice isoform of 18E6. E6AP_Ub$^-$ denotes an E6AP mutant defective for ubiquitin ligase activity created by mutating the active cysteine residue at position 843 to an alanine (C843A). Quantitation is the result of three independent experiments (N=3) where NHERF1 levels are normalized to co-transfected HA_GFP. Shown is a single representative blot. Vertical black line in blots represents removal of an irrelevant sample. The means of triplicate independent experiments ± standard error are shown. N=3. *<0.05, **<0.01 by Student's t-test. (**B**) Reduction of NHERF1 protein is not an overexpression artifact. Titrations of the indicated E6 proteins were co-transfected with FLAG_E6AP_WT (1 ug), HA_GFP (0.02 ug), and either HA_NHERF1 (0.5 ug) or p53 (0.5 ug) in murine 8B9 cells. With increased E6 expression, NHERF1 decreased for each E6 protein parallel with p53. As expected, p53 degradation was observed for the high-risk 16E6 proteins (both WT and ΔPBM) but not by low-risk 11E6 protein despite reduction of NHERF1 protein levels by 11E6. The means of triplicate independent experiments ± standard error are shown. (**C**) NHERF1 protein is reduced in keratinocytes containing episomal HPV16. Vector-transfected keratinocytes and keratinocytes transfected with re-circularized HPV16 genomes were seeded at equal confluency. NHERF1 and p53 protein levels were decreased in cells containing episomal HPV16. Quantitation was normalized to cells lacking the HPV16 episome. The means of triplicate independent experiments ± standard error are shown. N=3, ***<0.001 by Student's t-test.

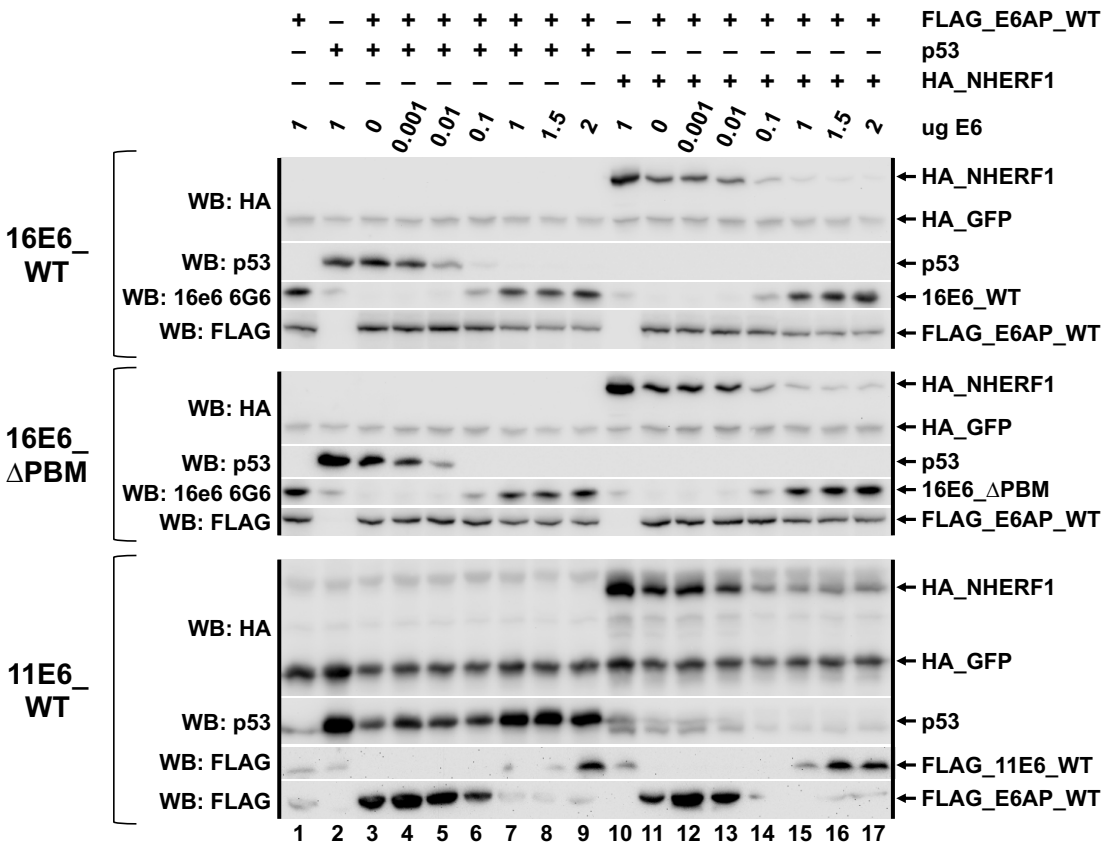


Figure 3.2 Reduction of NHERF1 protein levels is not an overexpression artifact.

Titrations of the indicated three different E6 proteins (16E6_WT, 16E6_ΔPBM, and 11E6_WT) were co-transfected with FLAG_E6AP_WT (1 ug), HA_GFP (0.02 ug), and either HA_NHERF1 (0.5 ug) or p53 (0.5 ug) in E6AP-null 8B9 cells. A representative blot of the triplicate experiments for each E6 protein is shown. Increased E6 expression for 16E6_WT, 16E6ΔPBM, and 11E6_WT resulted in decreased NHERF1 protein levels. Both 16E6_WT and 16E6ΔPBM degrade p53 with increasing E6 expression. Overexpression of 11E6 _WT (>0.1 ug E6) resulted in degradation of co-expressed E6AP_WT.

3.4.2 NHERF1 protein levels are sensitive to cell confluency

We established that NHERF1 protein levels are reduced by E6 in a transient transfection system. To determine whether low levels of stable 16E6-expression could initiate the reduction of NHERF1 protein levels, we retrovirally transduced normal immortalized keratinocytes with either empty vector or 16E6_WT and observed NHERF1 protein levels. Initially, our results were variable. We hypothesized that keratinocyte confluency may affect NHERF1 protein levels. To test this possibility, we seeded vector-transduced and 16E6_WT-transduced keratinocytes at three different cell densities: $5x10^3$ cells/cm^2 (very sub-confluent), $1.3x10^4$ cells/cm^2 (sub-confluent), and $2.6x10^4$ cells/cm^2 (mid-confluent). NHERF1 protein levels increased with an increase in cell density and 16E6_WT consistently reduced NHERF1 (Fig 3.3A). In order to determine if changes in NHERF1 levels with confluency were secondary to changes in NHERF1 RNA levels, we performed qPCR on RNA extracted from keratinocytes retrovirally transduced with vector or 16E6_WT and plated as in Fig 3.3A. Interestingly, NHERF1 RNA levels did not differ between keratinocytes seeded at different densities expressing either empty vector or 16E6_WT (Fig 3.3B).

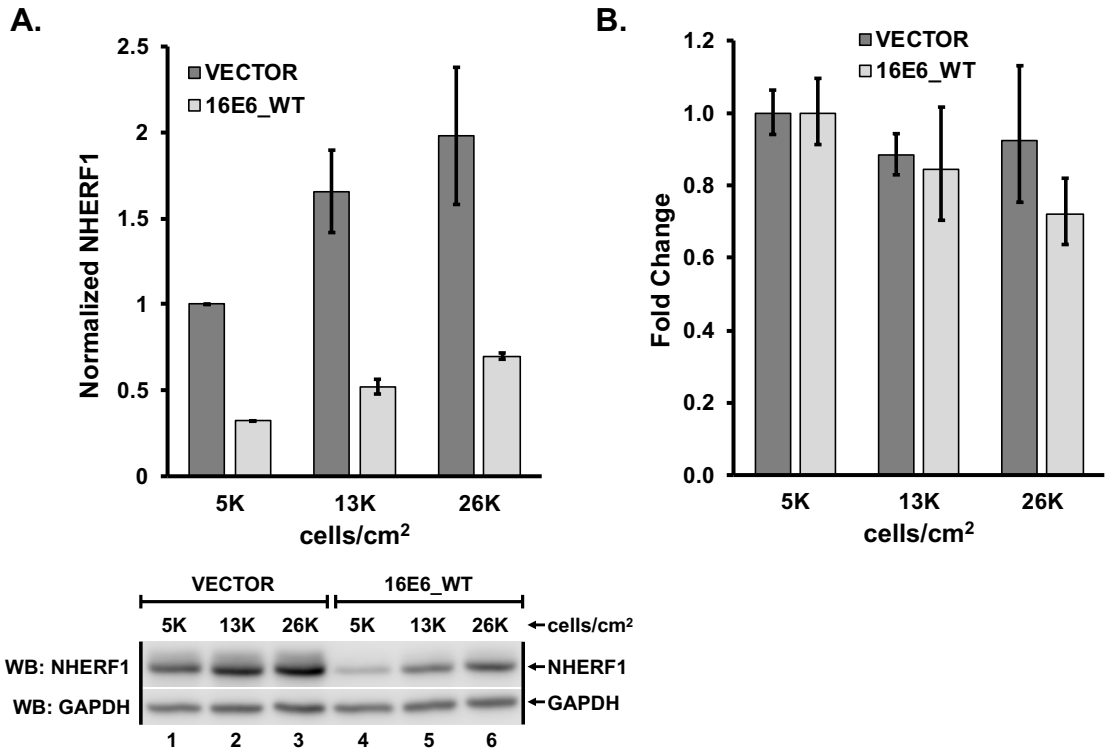


Figure 3.3 NHERF1 protein levels increase with increased cell density.
(**A**) Protein levels of endogenous NHERF1 increase with cell confluency. Keratinocytes retrovirally transduced with either vector or 16E6_WT were counted and plated at the indicated cell densities. As confluency increased, NHERF1 protein levels also increased, though still reduced in the presence of 16E6_WT. The means of triplicate independent experiments ± standard error are shown. (**B**) NHERF1 RNA levels are not changed by cell confluency or by the presence of 16E6_WT. Total RNA was extracted from keratinocytes retrovirally transduced with either vector or 16E6_WT and plated at the indicated cell densities. cDNA was reverse transcribed and NHERF1 RNA levels determined by qPCR. The means of triplicate independent experiments ± standard error are shown.

3.4.3 E6-mediated degradation of NHERF1 occurs via the proteasome

Because the ability of each E6 protein to reduce NHERF1 protein levels was also dependent upon the ubiquitin ligase activity of E6AP (Fig 3.1A), we hypothesized that E6 reduction of NHERF1 levels would be secondary to proteasome activity. We seeded retrovirally transduced keratinocytes expressing either empty vector or 16E6_WT at similar confluency and treated with either DMSO, mitomycin C (MMC) to induce p53 [130], or the proteasome inhibitor MG132 at differing concentrations for 8 hours. As expected, p53 levels increased in vector keratinocytes treated with MMC compared to untreated cells as well as in 16E6_WT cells exposed to increasing concentrations of MG132 [128] (Fig 3.4). NHERF1 protein levels increased significantly in a dose dependent manner upon treatment with MG132 in parallel to that seen with p53. (Fig 3.4, lanes 3-8). This indicated that NHERF1 is degraded through the proteasome by E6 in a manner dependent upon WT E6AP.

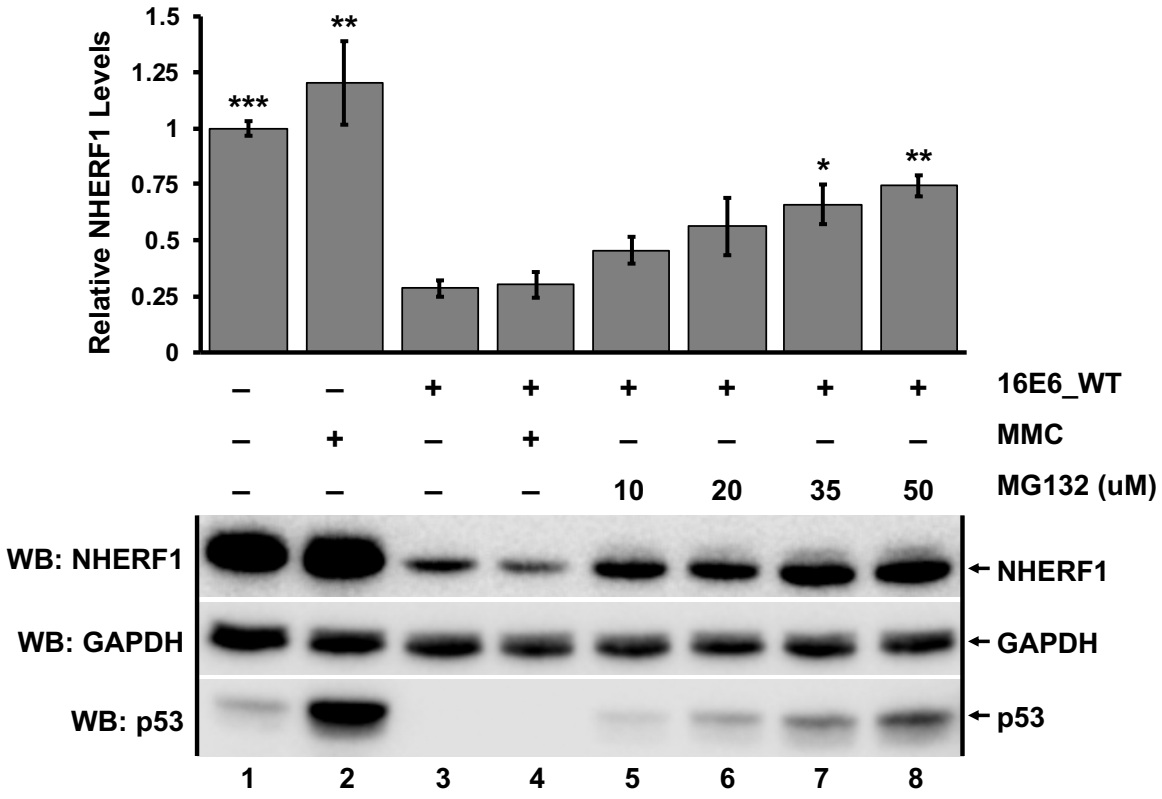


Figure 3.4 Degradation of NHERF1 by 16E6 requires proteasome function. Keratinocytes retrovirally transduced with either vector or 16E6_WT were seeded at equal confluency. Cells were treated with DMSO, mitomycin C (MMC), or the proteasome inhibitor MG132 at varying concentrations for 8 hours as indicated. MG132 significantly rescued NHERF1 protein levels in a dose dependent manner. MMC treatment was used to induce p53 levels, which were observed as a positive control. Quantification was normalized to vector-transduced cells treated with DMSO. The means of triplicate independent experiments ± standard error are shown. N=3, *<0.05, **<0.01, ***<0.001, n.s. = no significance by Student's t-test for samples compared to untreated 16E6 keratinocytes (lane 3).

3.4.4 E6-mediated degradation of NHERF1 is conserved across papillomaviruses from diverse hosts

The observation that NHERF1 was targeted by both high and low-risk HPV E6 proteins suggested that NHERF1 may also be a target of diverse non-primate E6 proteins. We examined the ability of E6 proteins from multiple different genera and different mammalian species to target NHERF1 for degradation (Fig 3.5). E6 proteins that preferentially bind MAML1 were unable to degrade NHERF1 (Fig 3.5A and 3.5B). All of the tested Alpha (primate), Dyodelta (boar), and Dyopi (porpoise) genera E6 proteins that bind E6AP targeted NHERF1. While E6AP-binding was necessary it was not sufficient, as E6 proteins from Omega (polar bear, UmPV1) and Omikron (cetaceans, PphPV1 and TtPV5) did not degrade NHERF1 (Fig 3.5A). Interestingly, E6 proteins that bind E6AP but did not target NHERF1 degradation sequence-clustered separately from E6 proteins that did target NHERF1 degradation, suggesting evolutionary divergence of this function (Fig 3.5B).

** Figure 3.5 legend on next page **

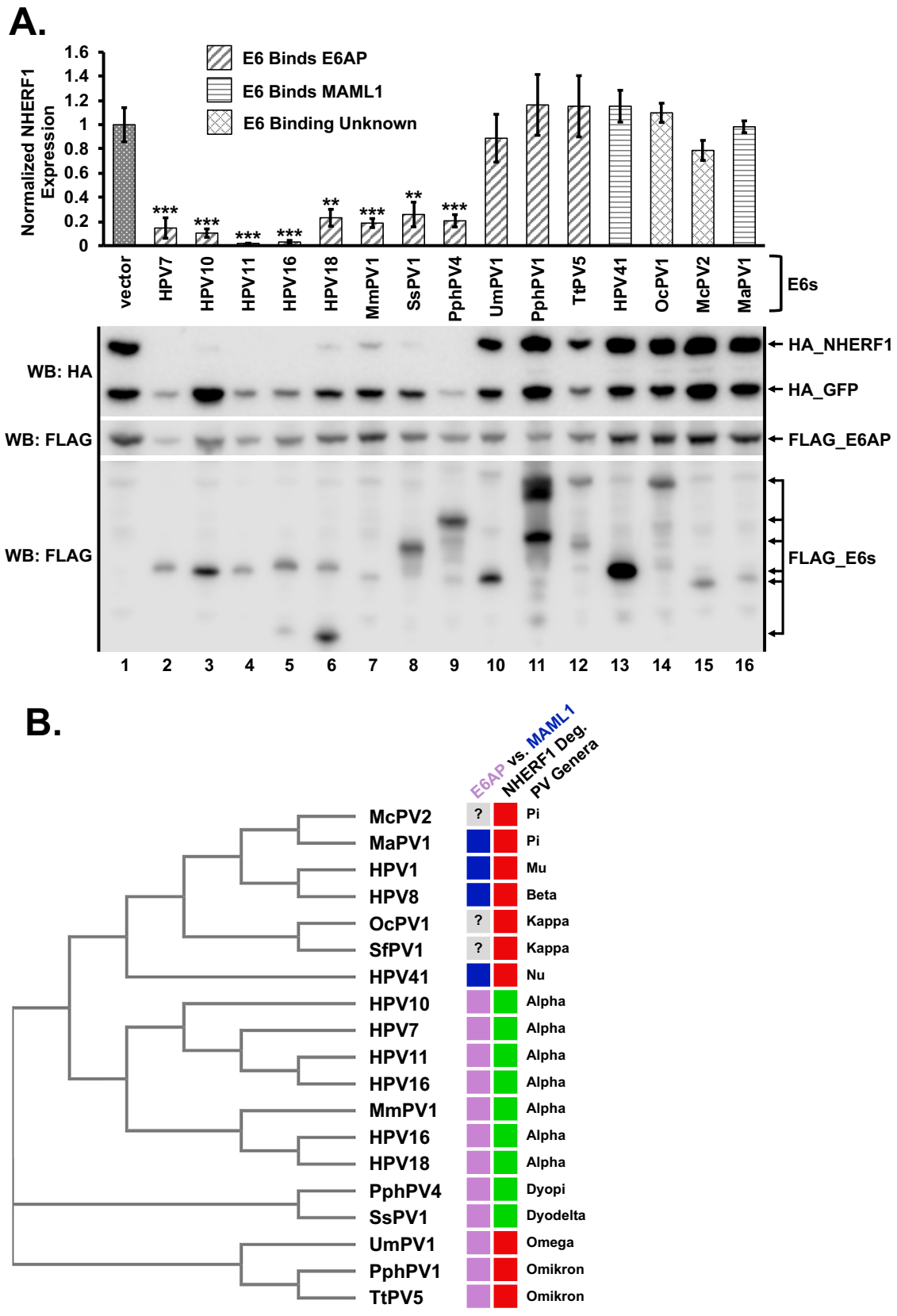


Figure 3.5 E6 proteins from evolutionarily diverse species target NHERF1. (**A**) E6 proteins from divergent animal species degrade NHERF1 via E6AP. HA_NHERF1 (0.4 ug), HA_GFP (0.1 ug), FLAG_E6AP_WT (0.35 ug), and the indicated FLAG_E6 (0.3 ug) plasmids were co-transfected into C33 cells. E6 proteins are classified based on their known preference for binding E6AP or MAML as indicated [60]. NHERF1 was degraded by E6 proteins isolated from numerous different mammalian species. Many, but not all, of the E6 proteins that bind E6AP targeted NHERF1 for degradation, while E6 proteins that bind MAML1 did not. HA_NHERF1 protein levels in the presence of the indicated E6 proteins were normalized to co-transfected HA_GFP as an internal transfection control. A single representative blot and the means of five independent experiments ± standard error are shown. N=5, **<0.01, ***<0.001 by Student's t-test. (**B**) E6 proteins that degrade NHERF1 cluster phylogenetically. The E6 proteins from the listed papillomaviruses were subjected to a multiple sequence alignment and then clustered phylogenetically using the program MUSCLE [66]. For E6 physical association, blue denotes MAML1 and light purple denotes E6AP. The preferential association of three E6 proteins is unknown. Ability to degrade NHERF1 is denoted in green and lack of ability to degrade NHERF1 is indicated by red. Interestingly, E6 proteins that can bind E6AP but not degrade NHERF1 cluster differently from other E6 proteins that cannot degrade NHERF1. The genera of each papillomavirus is listed. Western blot indicating NHERF1 expression in the presence of HPV1 E6, HPV8 E6, and SfPV1 E6 is shown in S3 Fig. H = *Homo sapiens* (human), Mm = *Macaca mulata* (rhesus monkey), Ss = *Sus scrofa* (wild boar), Pph = *Phocoena phocoena* (harbor porpoise), Um = *Ursus maritimus* (polar bear), Tt = *Tursiops truncatus* (bottlenose dolphin), Oc = *Oryctolagus cuniculus* (rabbit), Mc = *Mastomys coucha* (mouse), Ma = *Mesocricetus auratus* (golden hamster), Sf = *Sylvilagus floridanus* (Cottontail rabbit; CRPV1).

3.4.5 A novel 16E6 substrate interaction domain is required for 16E6 degradation of NHERF1

Because the ability of E6 to degrade NHERF1 was not dependent upon the presence of a PBM (Figs 3.1 and 3.5), we attempted to identify which residue(s) of 16E6 were required to mediate degradation of NHERF1. The crystal structure of 16E6 complexed with the E6-binding peptide from E6AP [32] (Fig 1.3) was examined to identify amino acids that were at least 20% exposed, resulting in over eighty candidate residues (Table 3.1). Candidate residues were individually mutated in the context of the 16E6 gene and the resulting point mutants were screened for their ability to degrade NHERF1 in the presence of E6AP_WT in transiently transfected C33A cells. To ensure our point mutants were not functionally defective (i.e. could not fold properly or could not interact with E6AP), we also screened the mutants for ability to degrade p53. A selection of mutants and the results of the screen are shown in Fig 3.6. Four mutants stood out as selectively defective in their ability to degrade NHERF1 (Fig 3.6B) while still being able to degrade p53 (Fig 3.6C): F69A, K72A, F69R and a double mutant: F69A/K72A. As evidenced in the crystal structure of 16E6, the side chains of F69 and K72 (Fig 3.7B) are located along the connecting alpha-helix that links the amino-terminal and carboxy-terminal zinc-structured domains of 16E6. The F69 and K72 side chains are aligned and adjacent on the connecting helix, which is on the opposite side of 16E6 from the p53 interaction surface [81] (Fig 3.7C).

We had identified the 16E6_F69A/K72A mutant in a transient transfection screen. To ensure the identified 16E6_F69A/K72A double mutant was selectively defective for degrading NHERF1 in the context of a stable cell line, keratinocytes retrovirally transduced with empty vector, 16E6_WT, 16E6ΔPBM, 16E6_F69A/K72A, or 11E6_WT were seeded at equal confluency and lysates prepared. Keratinocytes expressing 16E6_WT, 16E6ΔPBM, and 11E6_WT degraded NHERF1 (Fig 3.8, lanes 2, 3, 5). However, keratinocytes expressing 16E6_F69A/K72A were unable to stimulate the degradation of NHERF1 (Fig 3.8, lane 4), indicating a novel substrate interaction domain important for 16E6-mediated degradation of NHERF1.

F2V	E18K	K34A	D49A	Y70C	Y84A	D98A	R124A
Q3A	L19A	Q35A	C51A	K72A	T86A	L100A	R129A
P5R	Q20A	Q36A	R55A	K72E	T87A	P112A	G130A
Q6A	T22A	L38A	D56A	I73A	Q90A	P112S	R131A
E7A	H24A	R39A	D56K	Y76A	Q91A	E113K	R141A
R10A	D25A	R40A	N58A	R77E	Y92A	E113A	S143A
P13L	D25K	F47R	K65A	R77K	N93E	Q116A	T145A
P13S	I27A	F47I	F69A	H78A	K94A	D120A	R146A
Q14R	E29K	F47V	F69A/ K72A	C80A	P95A	K121A	R147T
T17A	E29A	F47L	F69R	Y81A	C97A	K122A	ΔPBM

Table 3.1 16E6 point mutants screened to determine amino acid(s) necessary for NHERF1 degradation.
The 16E6 crystal structure (PDB ID: 4GIZ) was examined for residues that were at least 20% exposed as determined by the Swiss PDB Viewer. Point mutants of these identified amino acids were then screened to identify which residue(s) resulted in an E6 protein that was selectively defective for degrading NHERF1 but retained degradation of p53. Residues of interest are indicated in red.

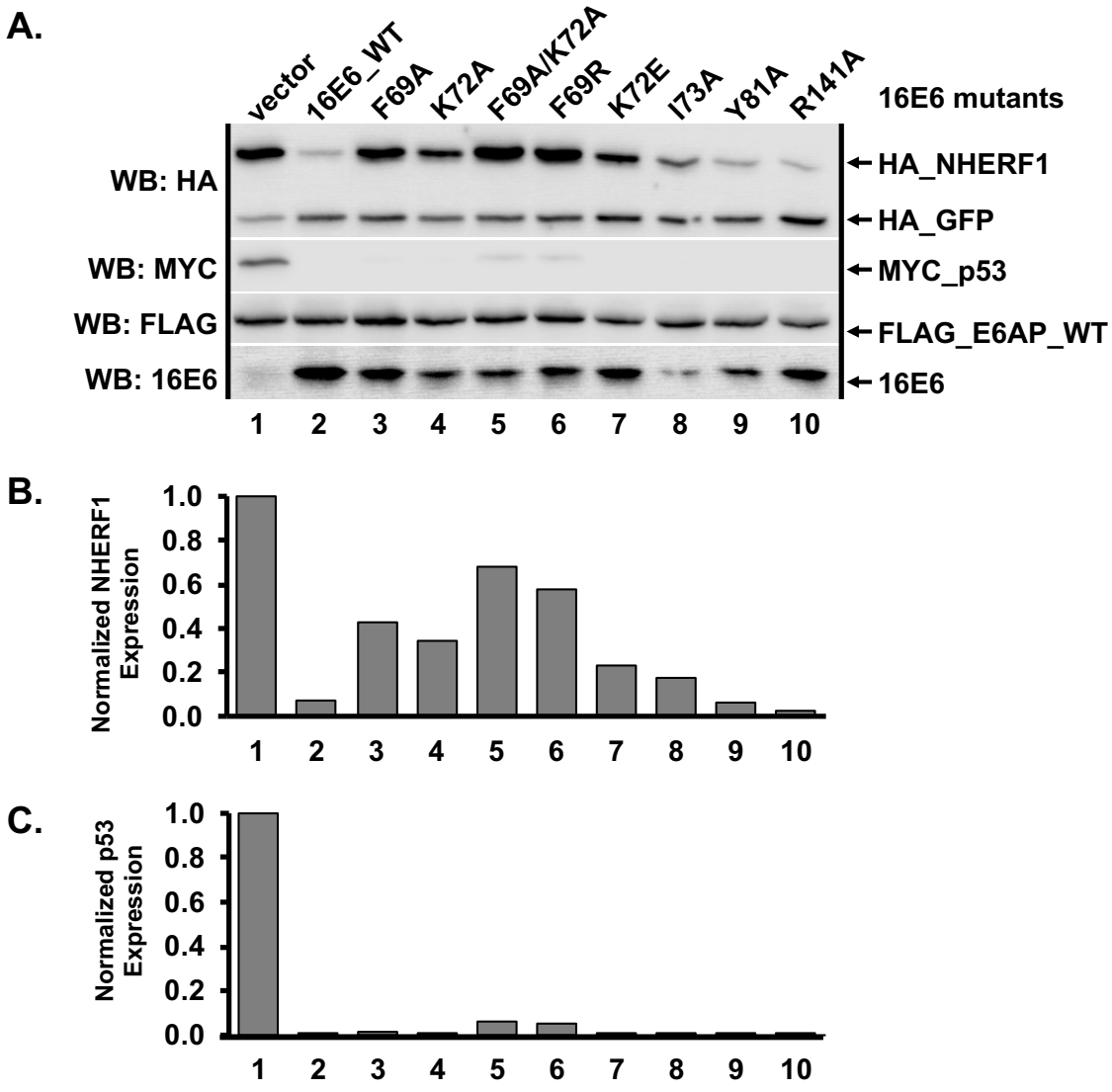


Figure 3.6 16E6 mutagenesis screen identified mutants selectively defective in their ability to degrade NHERF1.

(**A**) Amino acids F69 and K72 are important for degradation of NHERF1 by 16E6. Plasmids encoding untagged 16E6_WT or 16E6 mutants (0.3 ug) were co-transfected with FLAG_E6AP (0.35 ug), HA_NHERF1 (0.4 ug), MYC_p53 (0.25 ug), and HA_GFP (0.08 ug) into C33 cells and HA_NHERF1 levels determined by western blot. Multiple 16E6 proteins were identified that were unable to degrade NHERF1 but were still capable of degrading p53. (**B**) HA_NHERF1 and (**C**) p53 protein levels were quantified and normalized to co-transfected HA_GFP as an internal transfection control.

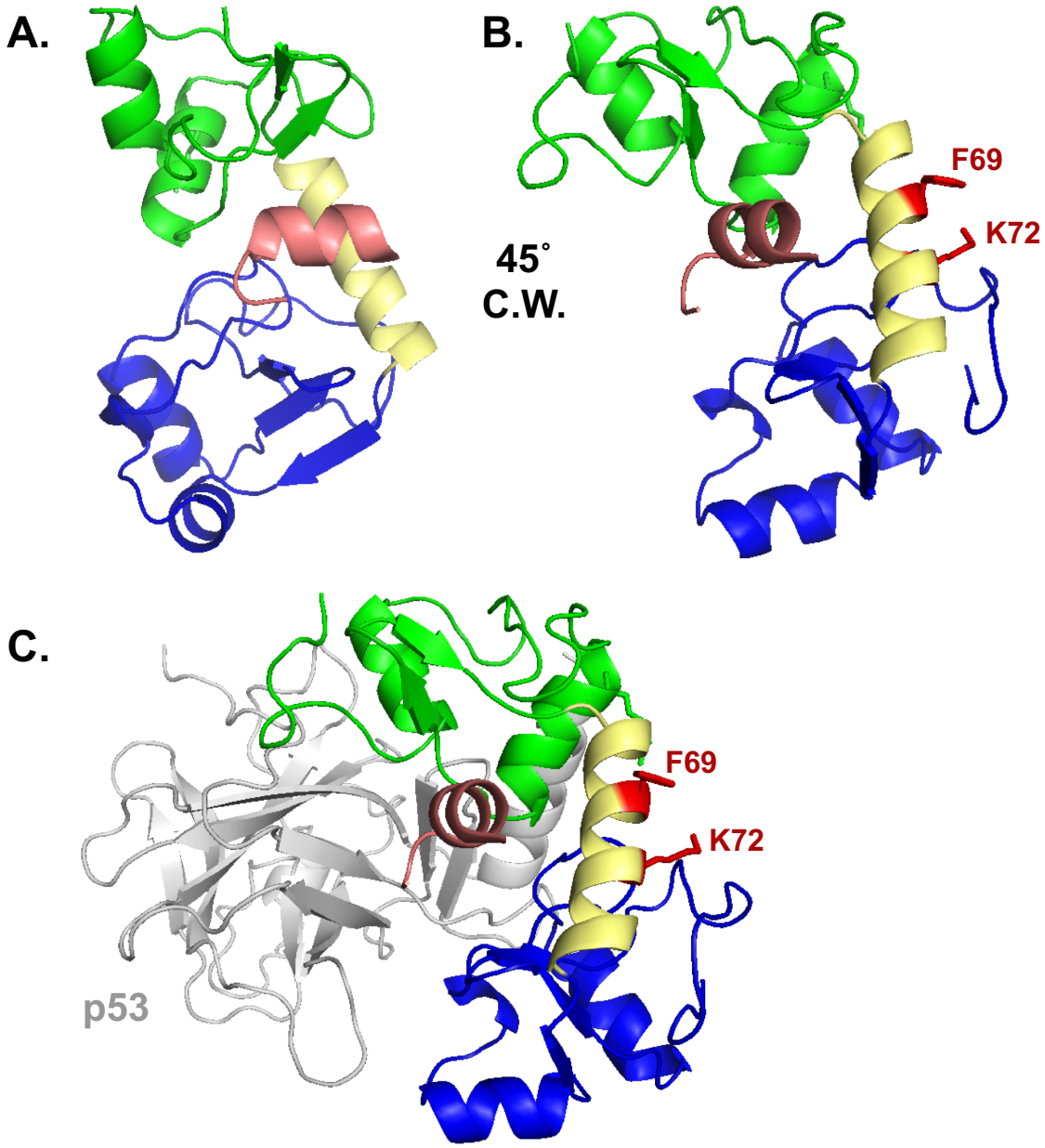


Figure 3.7 Amino acid side chains F69 and K72 define a novel substrate interaction domain on 16E6.
(**A**) HPV16 E6 structure (PDB ID: 4GIZ) showing the amino-terminal zinc-structured domain in green, connecting alpha helix in yellow, and the carboxy-terminal zinc-structured domain in blue. The E6 protein is complexed with the LXXLL peptide of E6AP (pictured in light pink). (**B**) The E6 protein depicted in A is rotated 45˚ clockwise (C.W.) and the F69 and K72 residues and their side chains are highlighted in red. (**C**) A similar view as part B is shown complexed with the core p53 DNA binding domain (grey). The E6 interaction face with p53 is opposite the F69 and K72 residues.

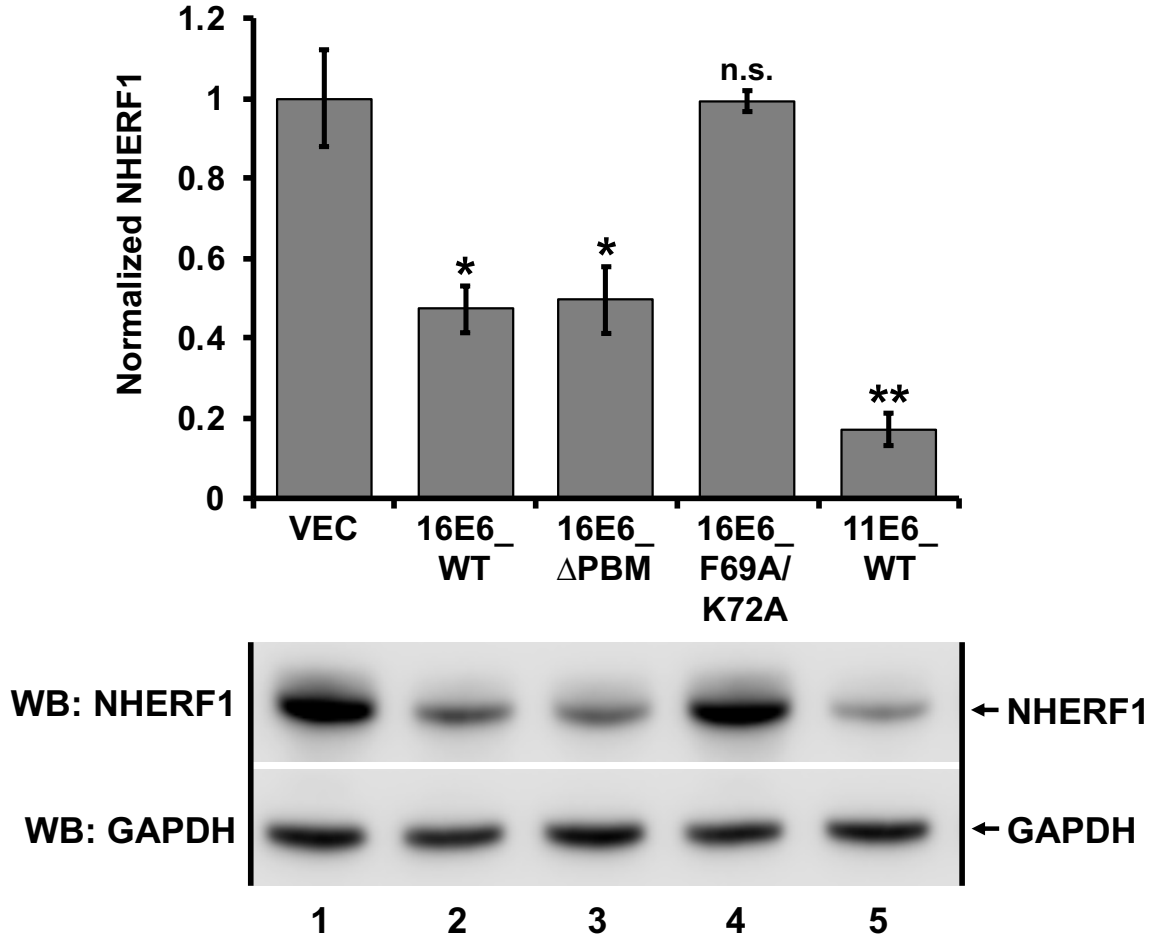


Figure 3.8 NHERF1 degradation by E6 proteins from both high and low-risk papillomaviruses in stable keratinocytes.
Keratinocytes retrovirally transduced with the indicated E6 proteins were seeded at equal confluency and endogenous NHERF1 protein levels were normalized to GAPDH. 16E6_WT, 16E6 deleted of its PBM (ΔPBM), and 11E6_WT all degraded NHERF1. The 16E6_F69A/K72A double mutant did not target NHERF1 for degradation. The means of triplicate independent experiments ± standard error and one representative blot are shown. N=3, *<0.05, **<0.01, n.s. = no significance by Student's t-test.

3.4.6 Degradation of NHERF1 by 16E6 requires the NHERF1 EB domain

Because the PBM of E6 proteins is not required to initiate the degradation of NHERF1 (Figs 3.1, 3.5, and 3.8), we hypothesized that neither of the PDZ domains of NHERF1 would be required for 16E6 to initiate NHERF1 degradation. We truncated NHERF1 and deleted several characterized domains within the protein [86, 131] (Fig 3.9A). E6AP-null 8B9 cells were co-transfected with 16E6_WT, NHERF1 truncations, HA_GFP, and either E6AP_Ub$^-$ or E6AP_WT. NHERF1 protein levels were quantified, and then normalized to the internal transfection control (HA_GFP). The various NHERF1 truncations displayed different expression levels. To account for these variations, levels of NHERF1 truncations in the presence of E6AP_Ub$^-$ were set to 100% and the expression level of the corresponding NHERF1 truncation in the presence of E6AP_WT was normalized accordingly (Fig 3.9B and 3.9C, bar graphs). All NHERF1 truncations containing the EB domain were targeted for degradation by 16E6 in the presence of E6AP_WT (highlighted in green in Fig 3.9A). Truncations of NHERF1 that lacked the EB domain were not targeted for degradation by 16E6 (highlighted in red in Fig 3.9A). In addition, the NHERF1 PBM was not required for 16E6 mediated degradation (Fig 3.9C, lanes 5 vs. 6 and 9 vs. 10).

** Figure 3.9 panels (B) and (C) on next page **

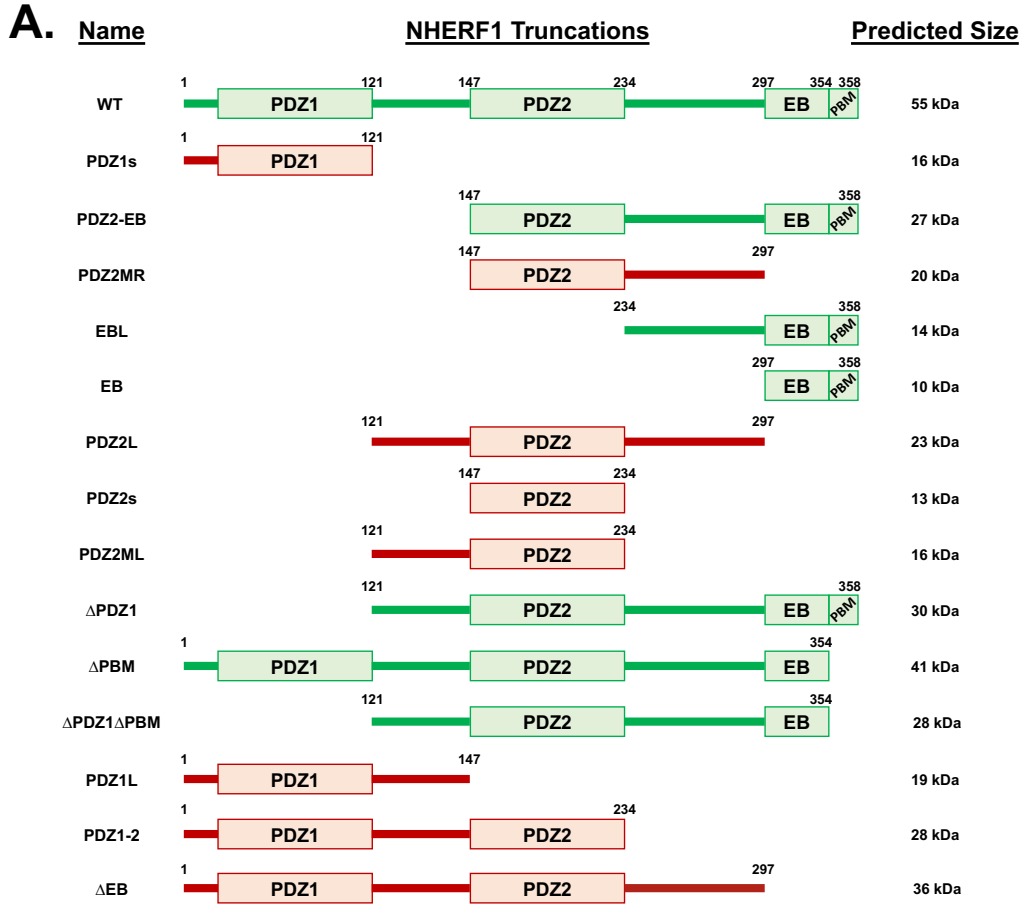
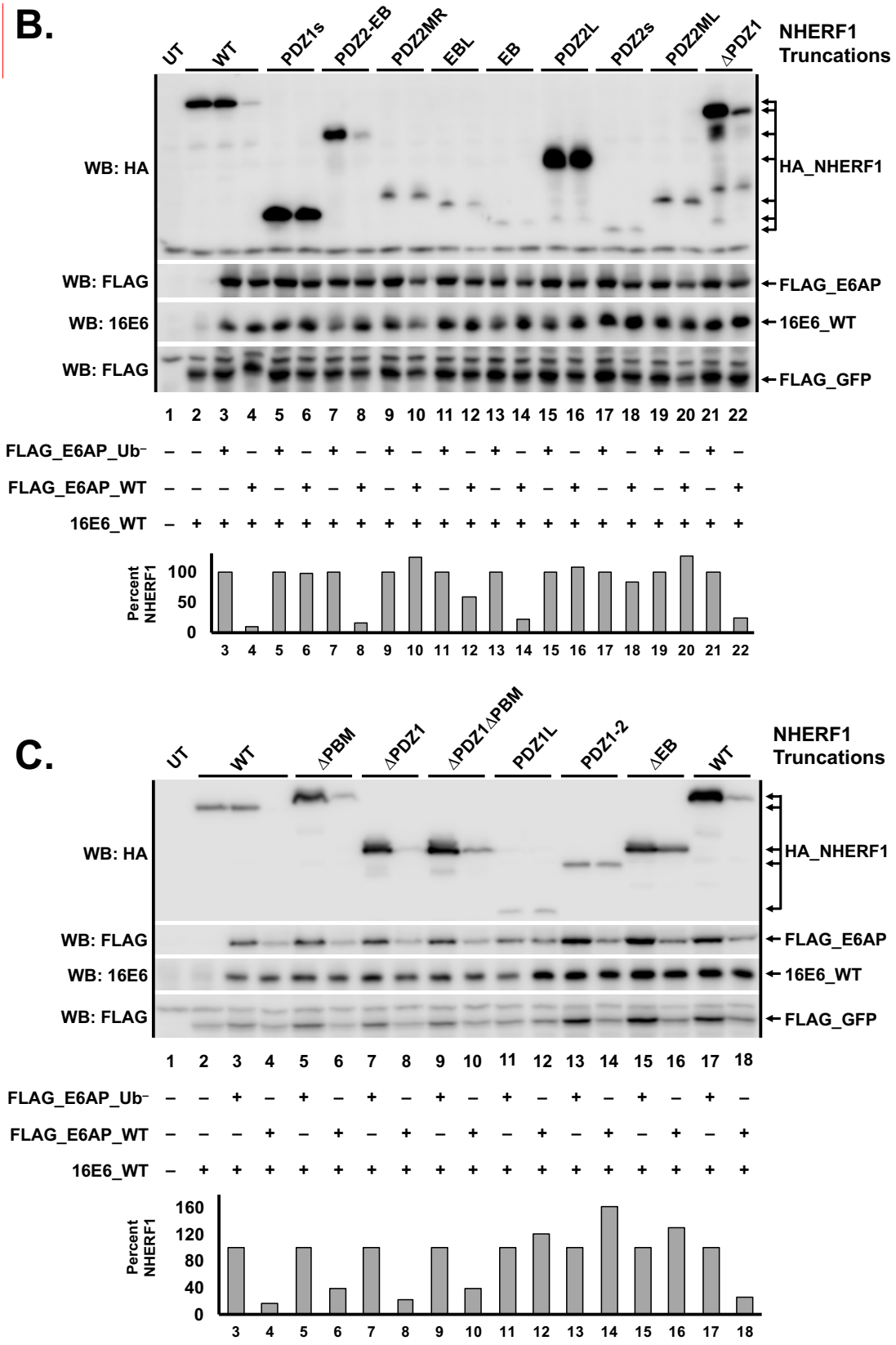


Figure 3.9 NHERF1 truncations identify the EB domain as necessary for NHERF1 degradation by 16E6.

(**A**) Schematic of NHERF1 truncations. NHERF1 proteins that were successfully degraded by 16E6_WT are depicted in green while truncations that were not degraded are depicted in red. (**B** and **C**) NHERF1 truncations containing the EB domain were degraded, while those lacking the EB domain were not. The listed HA_NHERF1 truncations (shown in A in the order loaded in B and C, 0.8 ug), untagged 16E6_WT (1 ug), FLAG_GFP (0.08 ug), and either FLAG_E6AP_WT (1.2 ug) or FLAG_E6AP_Ub$^-$ (1.2 ug, defective for ubiquitin ligase activity) were co-transfected in E6AP-null 8B9 cells. HA_NHERF1 levels were quantified and normalized to FLAG_GFP as an internal transfection control. The bar graph below the blot represents quantification of each listed HA_NHERF1 truncation. In panel C, the WT NHERF1 in lanes 2-4 contains an amino terminal 1X HA tag while the WT NHERF1 in lanes 17 and 18 contains an amino terminal 2X HA tag. All of the NHERF1 truncations contain amino terminal 2X HA tags. Levels of HA_NHERF1 truncations in the presence of FLAG_E6AP_WT were normalized to their corresponding expression in the presence of FLAG_E6AP_Ub$^-$ to account for the differing expression levels. UT = untransfected.

** Figure 3.9 panels (B) and (C) **



We identified the NHERF1 EB domain as a requirement for 16E6 mediated degradation and the importance of 16E6 residues F69 and K72. In order to examine the interactions between the 16E6+E6AP+NHERF1 complex, all three proteins were expressed in a yeast three-hybrid system so as to detect the heterotrimeric complex. We fused 16E6_WT and ubiquitin ligase dead E6AP (E6AP_Ub$^-$) to the LexA DNA binding domain and co-expressed this fusion with either vector, 16E6_WT, or 16E6_F69A/K72A in yeast containing a LexA responsive LacZ reporter. These yeast were then mated to yeast expressing native p53 or Gal4 (G4) transactivator fusions to NHERF1 121-358 (containing the EB domain), NHERF1 121-297 (deleted of the EB domain), 16E6_WT, or the tyrosine phosphatase PTPN3 (a PDZ protein) (Fig 3.10). The LexA_16E6 fusion co-expressed with p53 (in the absence of E6AP) resulted in very weak activation of the LacZ reporter (spot 4B) while co-expression with G4_PTPN3 resulted in strong transactivation (spot 6B), but no interaction with NHERF1 (spots 2B and 3B). We then co-expressed 16E6 and E6AP by using a LexA_E6AP_Ub$^-$ fusion together with native 16E6. When LexA_E6AP_Ub$^-$, untagged 16E6_WT, and p53 were co-expressed, a strong activation of the LacZ reporter was observed (Fig 3.10, spot 4D), illustrating that while p53 has a weak direct interaction with 16E6, it interacts strongly with 16E6 bound to E6AP. This activation was also seen with 16E6_F69A/K72A in the presence of LexA_E6AP_Ub$^-$ and p53 (Fig 3.10, spot 4E), indicating the preserved ability of the 16E6_F69A/K72A double mutant to

bind E6AP and recruit p53. When LexA_E6AP_Ub$^-$, 16E6_WT, and G4_NHERF1 121-358 (contains the EB domain) were co-expressed, we observed activation of the LacZ reporter, indicating the recruitment of NHERF1 to E6AP by 16E6_WT (Fig 3.10, spot 2D). Truncating the EB domain from the G4_NHERF1 (G4_NHERF1 121-297, spot 3D) or the use of the 16E6_F69A/K72A double mutant (spot 2E) ablated the reporter transactivation, indicating the requirement of the EB domain and the importance of 16E6 residues F69 and K72 in the interaction of the 16E6+E6AP+NHERF1 complex.

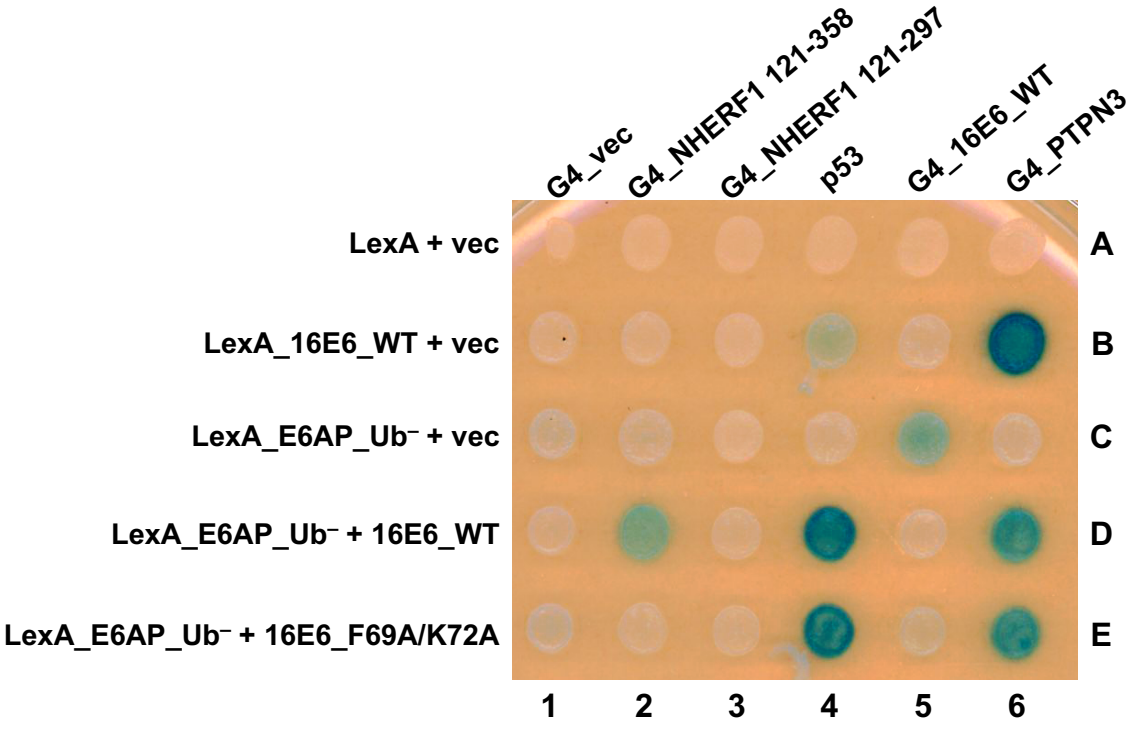


Figure 3.10 The E6-E6AP-NHERF1 complex can be modeled in yeast.
Yeast three-hybrid plasmids expressing the LexA DNA binding domain fused to either 16E6_WT or E6AP_Ub⁻ were co-expressed in yeast (bait) together with either vector, 16E6_WT, or 16E6_F69A/K72A as indicated. The bait yeast were mated to prey yeast expressing Gal4 activation domain (G4), or G4 fused to 16E6_WT, PTPN3, truncations of NHERF1, or native p53 and diploids selected. Positive controls for 16E6 expression included the established interaction of the 16E6 PBM with the PDZ domain of tyrosine phosphatase PTPN3 and 16E6-E6AP complex interaction with p53. 16E6_WT recruited NHERF1, p53, and PTPN3 to LexA_E6AP_Ub⁻. The recruitment of NHERF1 to LexA_E6AP_Ub⁻ by 16E6 was specifically lost upon mutation of residues F69 and K72, however, p53 and PTPN3 recruitment were maintained. 16E6_WT recruitment of NHERF1 was not seen with an NHERF1 truncation lacking the EB domain (G4_NHERF1 121-297).

3.4.7 E6AP-dependent NHERF1 degradation by E6 activates the canonical Wnt/β-catenin pathway

It has been shown that high-risk HPV E6 proteins augment the canonical Wnt/β-catenin signaling pathway [101-103, 132, 133]. Additionally, it has been shown that NHERF1 inhibits the canonical Wnt/β-catenin signaling pathway through multiple mechanisms. NHERF1 forms a complex with β-catenin [96] and can also bind to the intracellular PBM of certain isoforms of Frizzled [95], a G-protein coupled receptor important in the activation of the canonical Wnt signaling pathway. Therefore, we hypothesized E6 degradation of NHERF1 would activate the Wnt/β-catenin signaling pathway in cells expressing E6. To test this possibility, we utilized the TOP/FOP luciferase reporter assay. 16E6, 16E6ΔPBM, 11E6, and 18E6 all stimulated the activity of the Wnt/β-catenin pathway over vector-transfected cells (Fig 3.11). However, cells transfected with 16E6_F69A/K72A were unable to augment the canonical Wnt pathway over vector levels, indicating that the ability of E6 to degrade NHERF1 is required for E6 activation of the canonical Wnt/β-catenin signaling pathway.

The earlier Accardi et al. study proposed that expression of 16E7 sensitized NHERF1 for degradation by the induction of NHERF1 phosphorylation [126]. Our experiments did not show either E7 induction of slow-migrating NHERF1 phosphorylated isoforms or an enhancement of E6-NHERF1 degradation upon co-expression of E7 (Figs 3.12 and 3.13).

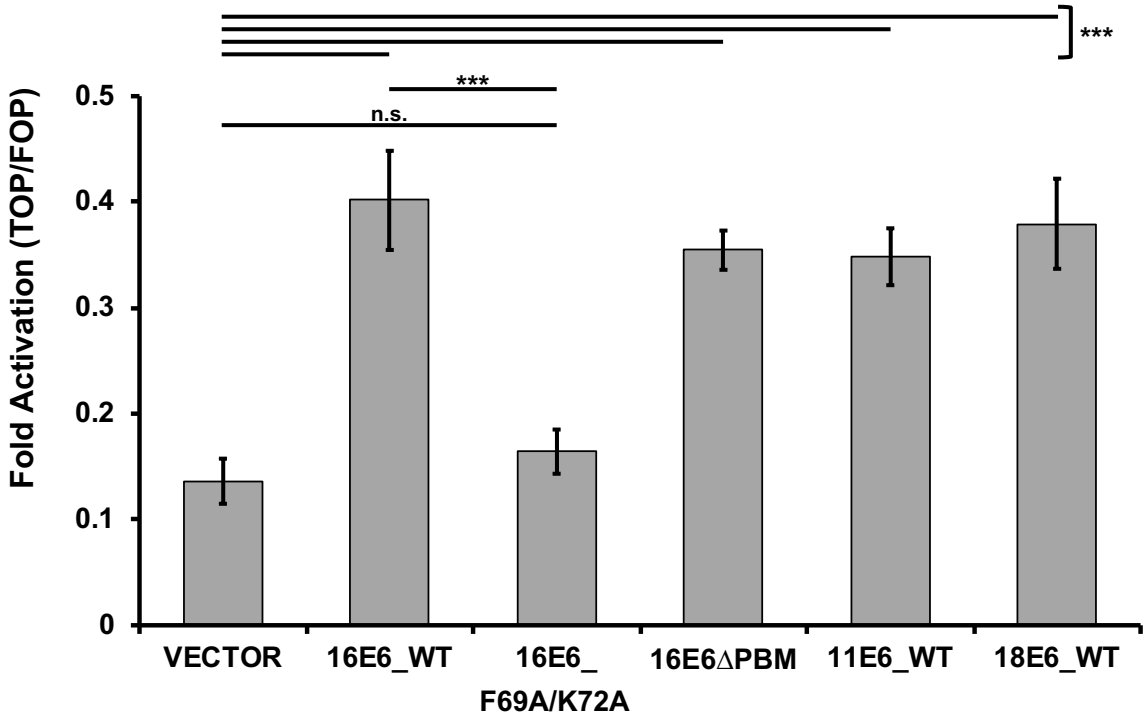


Figure 3.11 Activation of the canonical Wnt/β-catenin pathway is augmented by E6 proteins that can degrade NHERF1.
The listed E6 proteins were co-expressed with FLAG_E6AP_WT, the TOPFLASH or FOPFLASH luciferase reporter, and a renilla luciferase internal transfection control plasmid in C33A cells. Transfected cells were treated with Wnt3A conditioned media for 8.5 hours, lysed in 1X passive lysis buffer (Promega), and measured for luciferase and renilla luminescence. Fold activation was determined by normalizing the TOPFLASH luminescence by the FOPFLASH luminescence. Each E6 protein that could degrade NHERF1 (16E6_WT, 16E6_ΔPBM, 11E6_WT, and 18E6_WT) augmented the canonical Wnt pathway. 16E6_F69A/K72A, which cannot degrade NHERF1, failed to increase Wnt pathway activation over vector levels. Statistical significance was determined from three independent experiments by Student's t-test (***<0.001, n.s. = no significance).

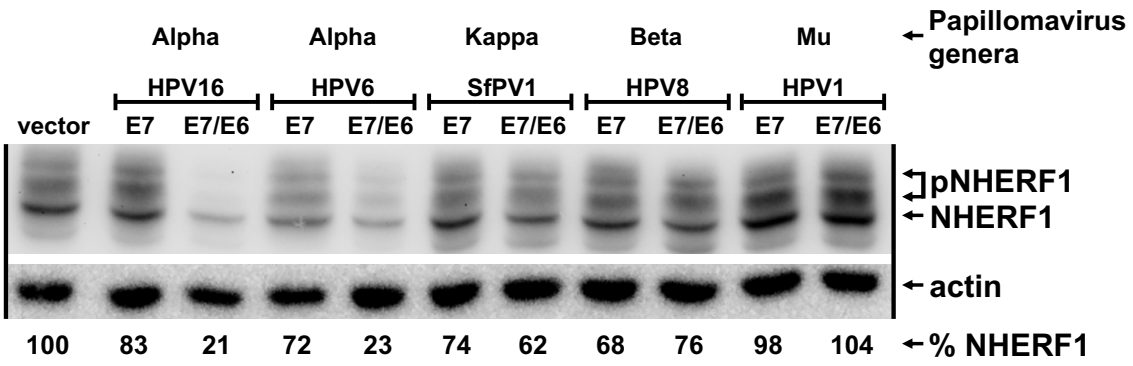


Figure 3.12 The E7 papillomavirus protein does not induce phospho-specific isoforms of NHERF1.

Keratinocytes retrovirally transduced with vector or the indicated E7 and/or E6 proteins were seeded at equal confluency. Levels of endogenous NHERF1 were determined by western blot. Levels of phosphorylated NHERF1 (pNHERF1) were unchanged in keratinocytes expressing empty vector compared to the various E7 proteins. Keratinocytes expressing the E6 protein from high-risk (HPV16) and low-risk (HPV11) degraded NHERF1. H = *Homo sapiens* (human), Sf = *Sylvilagus floridanus* (Cottontail rabbit; CRPV1).

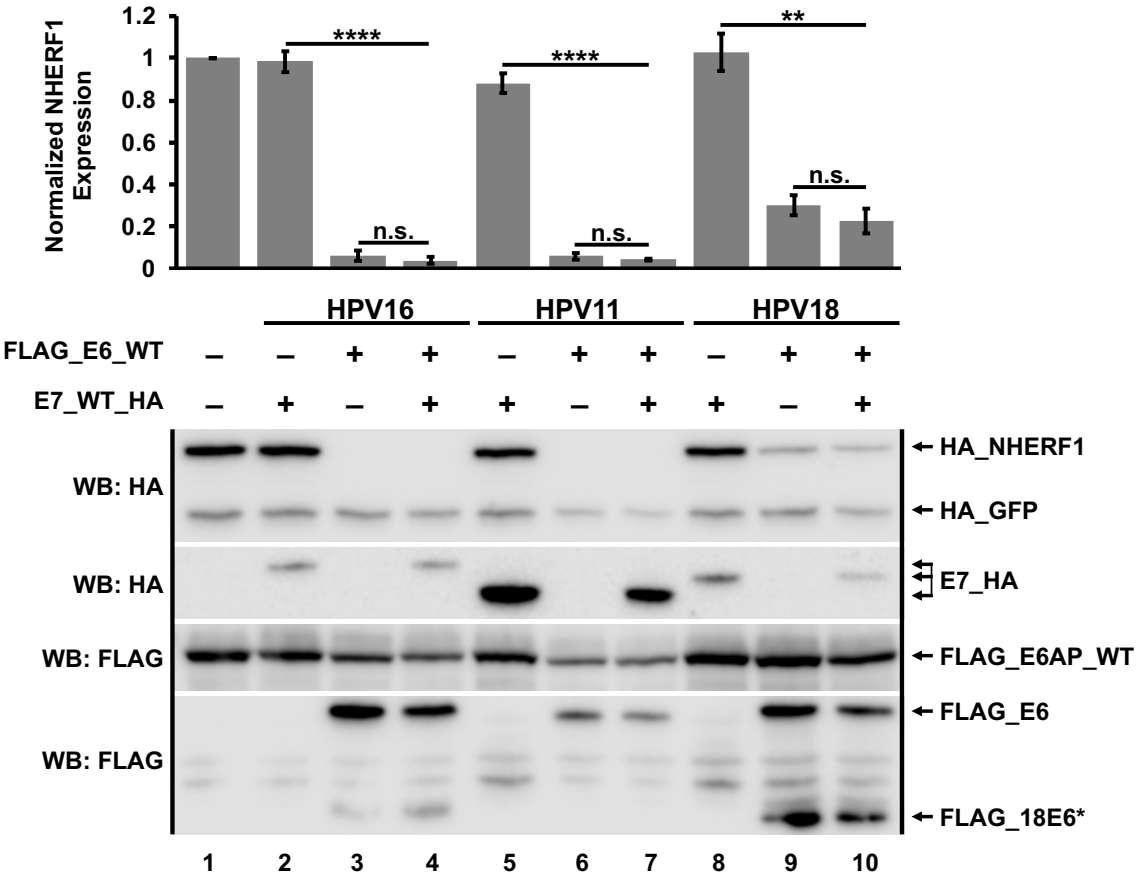


Figure 3.13 The presence of the E7 oncoprotein does not enhance NHERF1 degradation by E6 proteins.

C33A cells were co-transfected with the following plasmids: HA_NHERF1 (0.4 ug), FLAG_E6AP_WT (0.35 ug), HA_GFP (0.08 ug), the indicated E6 protein (0.3 ug), and the indicated E7 protein (0.3 ug). HA_NHERF1 levels were determined by western blot. FLAG_18E6* is a truncated splice isoform of 18E6. Quantitation is derived from three experimental replicates. A representative blot and means of triplicate independent experiments ± standard error are shown. N=3. **<0.01, ***<0.001, ****0.0001, n.s. = no significance by Student's t-test.

3.4 Discussion

E6 proteins from papillomaviruses can be separated into distinct groups: those that bind MAML1 and repress Notch signaling, and those that bind E6AP and hijack its ubiquitin ligase activity [60, 134-136]. E6 proteins from papillomaviruses in the Alpha, DyoDelta, Dyopi, Omega, and Omikron genera all behave similarly in that they bind E6AP and activate its ubiquitin ligase activity [60]. Here, we describe the degradation of NHERF1 by E6 proteins from both high and low-risk HPVs, as well as from papillomaviruses from multiple divergent mammalian species. The ability of these E6s to degrade NHERF1 is dependent upon E6AP (Fig 3.1) and the proteasome (Fig 3.4). In addition, we identify two amino acids in 16E6 (F69 and K72) that are necessary for E6-mediated degradation of NHERF1. These two residues are aligned, and adjacent in the outwardly oriented face of the E6 connecting alpha helix, suggesting a novel interaction domain (Fig 3.7B and 3.7C). Among the E6 proteins that target NHERF1 for degradation there are residues homologous to 16E6 F69, being either phenylalanine or leucine at that position. The residues homologous to 16E6 K72 are less well-conserved. Although many mutants were screened, there may be additional residues in 16E6 that contribute to the interaction. NHERF1 degradation by E6 requires the NHERF1 EB domain, but does not require the PBM at the extreme carboxy terminus of NHERF1 (Fig 3.9B and 3.9C). The ability of E6 proteins to degrade

NHERF1 augments the canonical Wnt/β-catenin signaling (Fig 3.11), an oncogenic pathway frequently active in cancer.

NHERF1 is the product of the SLC9A3R1 gene. SLC9A3R1 mRNA is broadly expressed in epithelia, with the highest mRNA expression in kidney, gut, and esophagus. NHERF1 is not developmentally essential, although mice have considerably reduced lifespans [85]. NHERF1-null mice are prone to phosphate wasting, brittle bone structure, and hydrocephaly [85] due to the mislocalization of proteins with which NHERF1 normally associates [85, 137, 138]. NHERF1 contains two PDZ domains and an EB domain at the carboxy terminus through which it interacts with ezrin, radixin, and moesin to link itself, and proteins to which it is bound, to the actin cytoskeleton network [112]. While the functions of NHERF1 are varied due to its role as a scaffold, multiple studies indicate it regulates cell growth and differentiation, two key cellular functions that papillomaviruses disrupt in the process of viral infection.

Whether NHERF1 is a tumor suppressor or an oncogene has been debated in the literature. There are numerous papers regarding NHERF1 human cancer phenotypes, but they are collectively inconsistent [104, 139]. NHERF1-null mice do not have a direct cancer phenotype, but have lengthened intestines [113], indicating a growth regulatory function of NHERF1. The diminished life span of NHERF1 null mice could limit observation of cancer traits. However, a recent in vivo study provided strong genetic support for NHERF1 as a tumor suppressor. $APC^{Min/+}$ mice bred as either heterozygote or

knockout for NHERF1 experience considerably shorter survival than their NHERF1-expressing counterparts due to increased tumor burden, demonstrating a tumor suppressor phenotype for NHERF1 [113]. Additionally, these $APC^{Min/+}$ mice lacking NHERF1 have greater activation of Wnt/β-catenin signaling, suggesting NHERF1 acts as a negative regulator of this oncogenic pathway. NHERF1-associated proteins that plausibly could regulate cell proliferation are numerous and include β-catenin [96], Frizzled [95], G-protein coupled receptors (β-adrenergic type 2, [110]), receptor tyrosine kinases (PDGFR, [107]), phosphatases (PTEN, [106]), transcriptional coactivators (YAP1, [111]), ion channels (Kir1.1 and CFTR, [109]), phospholipase-C [140], and actin anchoring proteins (ezrin, radixin, and moesin [112]).

Several studies have indicated that HPV E6 proteins can activate canonical Wnt/β-catenin signaling [101-103, 132, 133]. Our work expands and builds upon the scope of these studies. The ability of E6 to degrade NHERF1 and activate Wnt signaling may aid in propagation of papillomaviruses by enhancing the stimulation of cellular proliferation and promoting cell survival. There are numerous cell growth regulatory avenues that E6 could manipulate by degrading NHERF1 and within this study we have explored one possibility: the canonical Wnt/β-catenin signaling pathway (Fig 3.11); other possibilities will be the subject of future studies.

The EB domain of NHERF1 is required for E6-mediated degradation in the presence of E6AP (Fig 3.9B and 3.9C). This domain is responsible for linking

NHERF1 to the actin cytoskeleton network via interaction with ERM proteins [112]. NHERF1 has a PBM at its extreme carboxy terminus and when the EB domain is not bound to ERM proteins, the NHERF1 PBM can self-associate with the NHERF1 PDZ2 domain, resulting in a closed NHERF1 conformation [86]. The head-to-tail closed NHERF1 conformation is not required for E6-mediated degradation, as an NHERF1ΔPBM mutant was still targeted for degradation by E6 in the presence of E6AP_WT (Fig 3.9C). Nor was the 16E6 PBM required for degradation of NHERF1 (Figs 3.1, 3.5, and 3.8), contrary to a prior report [126].

In addition to the requirement of the NHERF1 EB domain, two E6 residues, F69 and K72, are necessary for E6-mediated NHERF1 degradation (Figs 3.6 and 3.8). Crucially, the 16E6_F69A/K72A double mutant can still initiate the degradation of p53, indicating it is still able to bind E6AP, recruit p53 to the complex, and trigger ubiquitination. The F69 and K72 residues are also required to form a tri-molecular complex between E6AP, E6, and NHERF1 in yeast (Fig 3.10, spot 2E vs. 2D). Like the association of E6 with p53, NHERF1 does not interact directly with E6, but requires prior association of E6 with E6AP, indicating that NHERF1 requires an altered conformation of E6 that is secondary to E6 binding to E6AP [59].

As we were testing the ability of E6 proteins to degrade NHERF1 in stable keratinocyte cell lines, we discovered that NHERF1 protein levels are sensitive to cell confluency (Fig 3.3A). As cell confluency increases, so does the

concentration of NHERF1 protein. E6 decreased NHERF1 expression at all confluency states. Thus, misleading interpretations can occur if different samples within an experiment are harvested at non-equivalent confluency. The relationship between NHERF1 and cell confluency may have contributed to the lack of identification of NHERF1 as a degradation target of HPV18 E6 and low-risk E6 proteins in the past, as well as differences between our studies and a prior publication [126]. It is likely that the effect of NHERF1 sensitivity to cell confluency underlies conflicting findings between different laboratories regarding NHERF1 expression levels and cancer associated traits [104, 139]. Future studies of NHERF1 must take into account and carefully control cell densities when performing experiments.

Binding to E6AP is necessary for E6-induced degradation of NHERF1, but it is not sufficient, as three tested E6 proteins that bind E6AP do not target NHERF1 for degradation: UmPV1 E6 (polar bear), PphPV1 E6 (porpoise), and TtPV5 E6 (bottlenose dolphin) (Fig 3.5A). Interestingly, the three E6 proteins that do not degrade NHERF1 cluster together in phylogenetic relatedness (Fig 3.5B). We utilized transfected human NHERF1 throughout our study, so it is possible that the inability of these three E6 proteins to target NHERF1 for degradation may be due to evolutionary divergence in the NHERF1 homologs. Future studies will explore if the lack of degradation of human NHERF1 by UmPV1, PphPV1, and TtPV5 is due to evolutionary divergence of the respective NHERF1 proteins compared to human NHERF1. It would be of

interest to determine if NHERF1 is a “universal” target of E6 proteins that act through association with E6AP.

Discovery of NHERF1 as a novel target for not only high and low-risk mucosal and cutaneous HPV E6 as well as a wide range of E6 proteins across divergent host species indicates a significant and previously undescribed role for NHERF1 in papillomavirus biology. That NHERF1 is a conserved target of papillomavirus E6 proteins further elevates the importance of NHERF1 as a cell growth regulator. Finally, the identification of this highly conserved E6 degradation target may represent a novel avenue for therapeutic intervention against both low and high-risk HPV.

3.6 Acknowledgements

We thank Nicole Brimer for assistance with technical challenges that were encountered throughout this study and Kelly Drews for extensive discussions and for critical reading of the manuscript.

Chapter 4: Auxiliary Regions Involved in the Interaction of Papillomavirus E6 Proteins with Cellular E6AP

4.1 Abstract

Both high-risk and low-risk E6 protein bind the full-length cellular E3 ubiquitin ligase E6AP and can stimulate its ubiquitin ligase activity. High-risk E6 proteins bind to an LXXLL motif within E6AP (LQELL), resulting in a change in E6 conformation and recruitment of cellular p53. While the E6AP LQELL motif is important in mediating the interaction of low-risk E6 proteins with E6AP, low-risk E6 from HPV type 11 is unable to bind the isolated E6AP LQELL motif. We examined a cohort of E6AP-binding E6 proteins from human and non-human papillomaviruses and found that the interaction of different E6 proteins with E6AP have different requirements. Utilizing E6AP truncation mutants, we identified regions in both the amino and carboxy termini of E6AP (which we termed auxiliary regions) that are important for certain E6 proteins to bind E6AP. Additionally, we identified a span of eleven amino acids amino-terminal of the LQELL motif that are required for both high and low-risk E6 stimulation of E6AP ubiquitin ligase activity to degrade both p53 and NHERF1. We propose classification of E6-E6AP interactions into two types: Type I in which E6 interacts with the isolated LQELL and Type II in which E6 proteins fail to interact with the isolated LQELL motif. We further propose that Type II interactions can be subclassified as Type IIa and Type IIb, based on whether the E6 protein requires one or more E6AP auxiliary region(s) to mediate the formation of the E6-E6AP complex. This is the first study describing these E6AP auxiliary binding regions as important for both E6

interaction and E6 stimulation of E6AP ubiquitin ligase activity. This classification of E6-E6AP interaction types and identification of a region in the E6AP amino terminus that is important for E6 stimulation of ubiquitin ligase activity will inform future studies aiming to develop therapeutics to disrupt the formation of the E6-E6AP complex.

4.2 Introduction

Infection with a papillomavirus induces the formation of a papilloma in host vertebrates during the process of creating an environment conducive to the viruses' replication. The papillomavirus oncoprotein E6, in conjunction with the other viral oncoprotein, E7, induces epithelial hyperplasia by inhibiting keratinocyte differentiation [141]. These oncoproteins hijack the activities of cellular proteins, often E3 ubiquitin ligases, and alter the protein landscape within the cell by targeting other proteins for degradation as well as through gain-of-function interactions [44]. Extended infection by mucosal high-risk human papillomaviruses can lead to tumorigenesis of the originally benign papilloma while their counterparts, deemed low-risk, are responsible for genital warts and oropharyngeal papillomatosis [43, 121]. The prototypic high-risk HPV types are 16 and 18, while HPV6 and 11 are prototypic low-risk types. HPV16 and 18 are responsible for over 66% of cervical cancer cases in the United States [142].

Characterization of E6 proteins from both human and non-human papillomaviruses illuminates two distinct groups with two differing functions: E6 proteins that bind the cellular ubiquitin ligase E6-associated protein (E6AP) to stimulate its ubiquitin ligase activity, and E6 proteins that bind the transcriptional co-activator MAML1 and repress activation of Notch signaling [60]. The cellular proteins MAML1 and E6AP both contain an LXXLL motif necessary to mediate E6 interaction. Why and how papillomavirus E6 proteins have diverged to bind either MAML1 or E6AP is not well understood. Both the high-risk and low-risk HPV E6 proteins prefer to bind E6AP and stimulate its ubiquitin ligase activity. The hallmark of high-risk E6 proteins is their ability to initiate degradation of cellular p53 through their interaction with E6AP, a trait not shared by low-risk E6. We recently identified NHERF1 as a cellular degradation target common to both high and low-risk E6 proteins, as well as E6 proteins from non-human papillomaviruses [143]. Furthermore, we determined that the ability of each of these E6 proteins to target the degradation of NHERF1 was mediated by E6AP ubiquitin ligase activity.

E6AP was identified not only as an interaction partner of high-risk E6 proteins, but also as a necessary and sufficient component for E6-mediated degradation of p53 [45, 76, 128, 129]. Knocking down E6AP in human papillomavirus-transformed cells rescues p53 expression and can lead to p53 induced apoptosis [144, 145]. Due to its importance in the oncogenic role of E6 proteins, characterizing the interaction between E6AP and E6 is an important

focus of the papillomavirus field. Huibregtse et al. identified numerous functional domains of E6AP required for 16E6 binding, p53 recruitment, and the targeted degradation of p53 by the 16E6-E6AP complex [78]. They found that amino acids carboxy terminal to E6AP residue 290 were required for E6-dependent ubiquitination and the same region shortened on the carboxy terminus by only 84 amino acids was required for p53 association in the presence of 16E6. The same study identified an 18 amino acid peptide within the E6AP protein (containing an LXXLL motif) as both necessary and sufficient for 16E6 binding to E6AP.

E6AP ubiquitin ligase activity has been mapped to an active cysteine residue in the carboxy terminal HECT domain located at residue 843 [76]. The original 18 amino acid E6AP peptide identified as necessary for 16E6 interaction [78] has been narrowed to just 12 residues encompassing an alpha helical, acidic LQELL sequence [146-148]. Not only can 16E6 interact with the LQELL peptide in the context of E6AP, but it can also interact with peptides containing LXXLL displayed in other cellular proteins [32, 60]. 16E6 can bind this E6AP LQELL motif and recruit p53 [59], requiring only the core p53 DNA binding domain [59] to form a trimolecular complex. This complex has been crystalized [81] (Fig 3.7) and in combination with mutagenesis studies [48] it indicates that the specificity for p53 lies within the 16E6 protein. Without E6 present E6AP neither interacts with, nor degrades, cellular p53 [78, 80]. Full-length E6AP has proven challenging to crystallize as it is insoluble at high

concentrations. Therefore, little is known about how (or if) E6 interacts with other regions of E6AP in addition to LQELL.

While high-risk 16E6 can bind the isolated E6AP LQELL peptide, 11E6 cannot. However, both 16E6 and 11E6 bind full-length E6AP [47, 59], suggesting a possible undescribed E6-E6AP interaction mode. Additionally, high-risk E6 displays enhanced affinity for full-length E6AP over the LQELL peptide and 16E6 bound to LQELL is significantly less effective at recruiting p53 to the complex than 16E6 bound to full-length E6AP [59]. These data corroborate prior measurements of the greater affinity of 16E6 for full-length E6AP (nM range) compared to the LQELL peptide (μM range) *in vitro*. [82], [83]. We hypothesize that there are regions within E6AP other than the LQELL peptide that influence the interaction between E6 and E6AP.

Several observations indicate that the E6-E6AP interaction is more complex than E6 docking upon the LQELL peptide alone. First, low-risk 11E6 does not interact with the LQELL peptide, while interacting robustly with that peptide when expressed in the full-length E6AP [48]. Second, when the LQELL peptide from E6AP is mutated to LQELS, 16E6 cannot bind the isolated E6AP LQELS mutated motif, but interacts robustly with LQELS in the context of full-length E6AP and can initiate p53 degradation [48]. Like the degradation of p53 by 16E6, 11E6 (which fails to bind the isolated E6AP LQELL peptide) can direct the degradation of NHERF1 in an E6AP dependent manner [143]. Conversely, when the E6AP LQELL motif is mutated to reflect the LXXLL

motif found in MAML1 (LDDLL), E6 proteins that bind MAML1 and not E6AP display a gain-of-function binding to the E6AP-LDDLL mutant; although these MAML1 binding E6 proteins can bind E6AP-LDDLL, they are unable to stimulate E6AP ubiquitin ligase activity [60]. Therefore, binding of an E6 protein to the E6AP LQELL motif is not sufficient to initiate E6AP ubiquitin ligase activity. We hypothesize that, in addition to the HECT ubiquitin ligase domain, there are regions in E6AP located amino-terminal to the LQELL motif which are necessary for E6 activation of E6AP ubiquitin ligase activity.

In this study, we characterize regions in both the amino- and carboxy terminus of E6AP that are important in mediating the E6-E6AP interaction, E6 stimulation of E6AP ubiquitin ligase activity, and targeted degradation of p53 and NHERF1 by both high and low-risk E6 proteins. We further found that E6AP-associating E6 proteins isolated from human and non-human hosts require different regions within E6AP to successfully form the E6-E6AP complex. Our observations have led us to classify E6 interactions with E6AP into two distinct types. In Type I, E6 proteins have a direct interaction with the LQELL peptide whereas in Type II, E6 proteins have a facilitated interaction with the LQELL motif in the context of the full-length E6AP protein. We identified a mutant of high-risk 16E6 that is incapable of binding the isolated E6AP LQELL motif, but that retains its ability to interact with the full-length E6AP protein. Using this 16E6 mutant, we identified regions in E6AP outside the LQELL binding-peptide, that enable 16E6 interaction.

Amino-terminal E6AP truncations narrowed down a specific region within the E6AP amino terminus that is required for both high and low-risk E6 proteins to stimulate degradation of cellular substrates in the presence of ubiquitin ligase active E6AP. Therefore, a given E6 protein may or may not display a Type I interaction with E6AP, but to target degradation of a substrate all E6 proteins require a Type II interaction. We provide evidence suggesting the extreme amino terminus of E6 proteins plays a role in enabling high affinity interaction with E6AP. Finally, we find that although the E6-E6AP interaction is conserved among E6 proteins isolated from different vertebrate species, these E6 proteins may subtly differ in their interactions with E6AP.

4.3 Results

4.3.1 Papillomavirus E6 proteins that preferentially bind E6AP require different regions in E6AP to mediate the E6-E6AP interaction

High-risk 16E6 is capable of binding the isolated E6AP LQELL peptide [59], while low-risk HPV11 E6 (11E6) is not [47]. These observations led us to hypothesize that other regions within E6AP, in addition to the alpha-helical structured E6AP LQELL motif may contribute to the E6-E6AP interaction. To test this possibility, we first examined the ability of a cohort of E6AP binding E6 proteins [60] from both human (HPV16, 11, 7, and 10) and non-human (PsPV1, PphPV1, TtPV5, MmPV1, SsPV1, and UmPV1) papillomaviruses to

interact with various E6AP fragments (Fig 4.1A; see figure legend for vertebrate species scientific and common name). We made full-length E6AP with either the WT E6 binding site LQELL, or a mutated LQELS docking site, as well as transactivator fusions to the LQELL peptide or a mutated LQELS peptide and (Fig 4.1A). The full-length E6AP constructs were ubiquitin ligase inactive (designated as Ub^-), containing a mutation of the active cysteine residue to an alanine (C843A). E6AP_Ub^- was utilized to prevent E6-stimulation of ubiquitin ligase activity thereby preventing possible degradation of expressed E6 and E6AP proteins. We expressed the E6 proteins as lexA fusions and various E6AP proteins as transactivator fusions in a yeast two-hybrid system to detect the formation of the heterodimeric complex as a blue XGAL spot (Fig 4.1B). HPV41 E6 interacts with MAML1 and not E6AP [60], and was used as a negative control (Fig 4.1B, row F).

All of the E6 proteins interacted with E6AP_ Ub^- as expected. About half of the E6 proteins (HPV16, HPV7, HPV10, SsPV1, and UmPV1) bound the isolated E6AP LQELL peptide (Fig 4.1B, spots 2B, 2D, 2E, 2G, 2L, 2O, and 2P) and none bound a mutated LQELS the peptide. However, about half of the E6 proteins did not interact with either the isolated LQELL peptide or the mutated LQELS peptide (HPV11, PsPV1, PphPV1, TtPV5, or MmPV1). Among this second group, MmPV1 E6 (Rhesus monkey, high-risk E6) only bound LQELL in the context of the full E6AP (Fig 4.1B, spot 2N), indicating that this phenotype is not exclusive to low-risk E6 proteins (e.g. 11E6). While none of

the E6 proteins interacted with the E6AP LQELS peptide alone (Fig 4.1B, column 3), within the context of Ub– full-length E6AP, all interacted with the exception of TtPV5 E6 and MmPV1 E6 (Fig 4.1B, spot 4J vs. 5J and spot 2N vs. 5N). We concluded that this cohort of E6 proteins, which cannot interact with the isolated LQELS but can interact in the context of full-length E6AP, could be useful to identify additional regions within the E6AP protein that enhance E6-E6AP complex formation.

** Figure 4.1 panel (B) on next page **

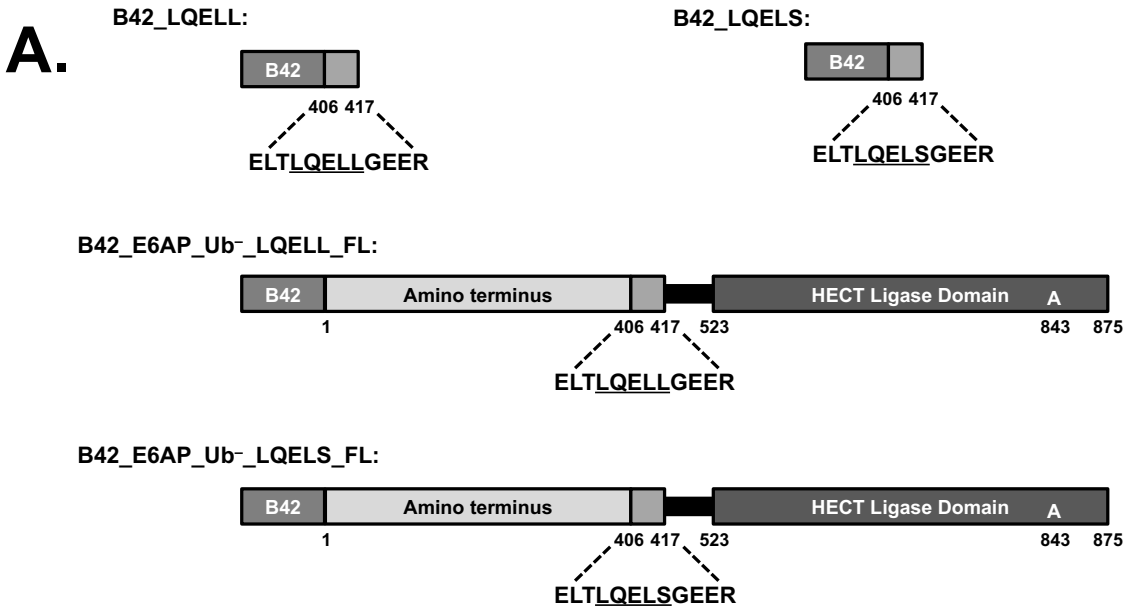
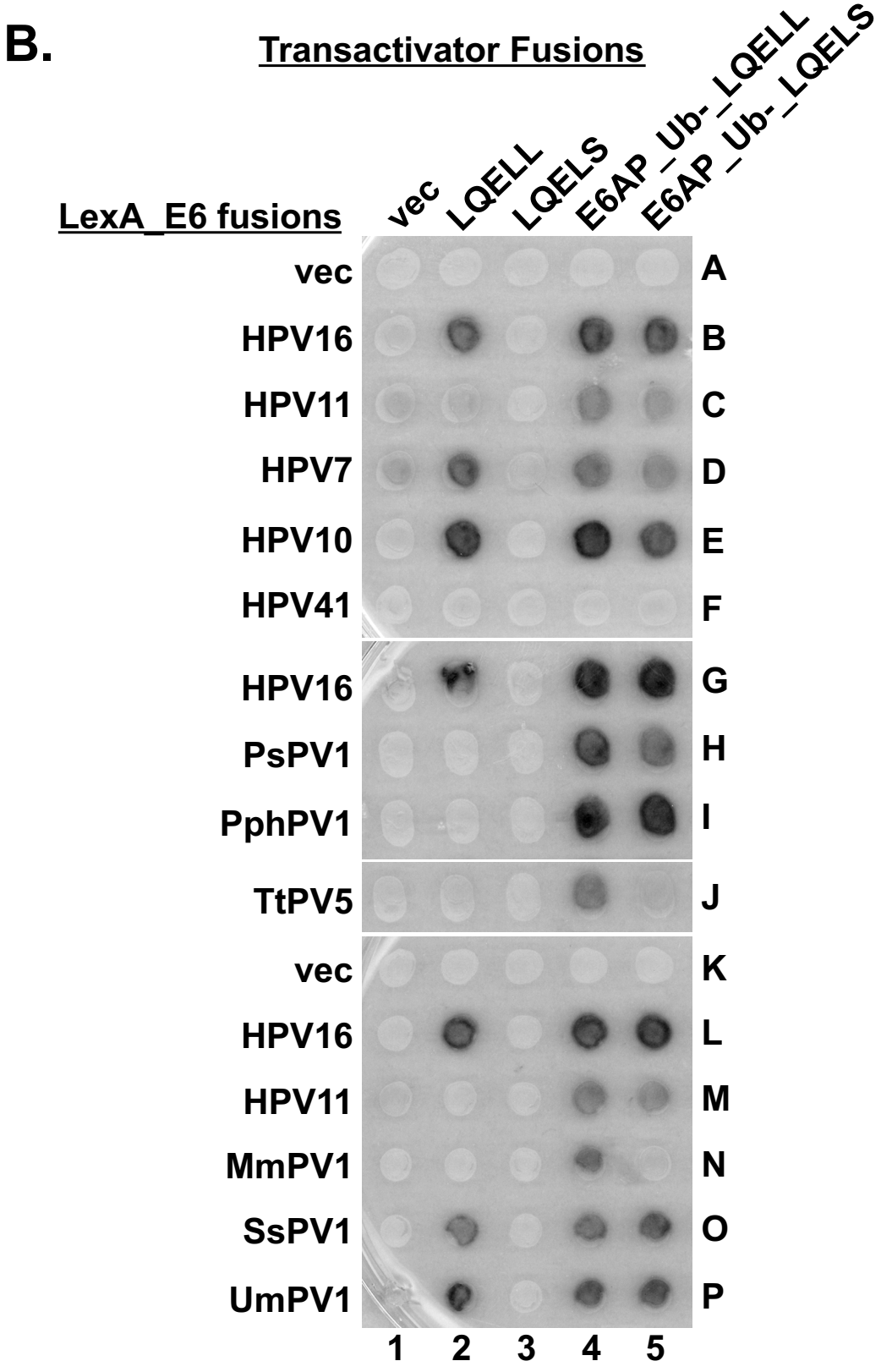


Figure 4.1 E6 proteins that can interact with full-length E6AP display different E6AP interaction profiles.

(**A**) Schematic of B42 transactivator fused E6AP proteins utilized in panel B. B42_LQELL contains E6AP residues 406-417. B42_LQEAS also contains E6AP residues 406-417 but the double Ls (residues 412 and 413) have been mutated to AS. B42_E6AP_Ub–_LQELL_FL consists of ubiquitin ligase dead full-length E6AP. B42_E6AP_Ub–_LQELS_FL is also ubiquitin ligase dead full-length E6AP, but is mutated in the LQELL motif to LQEAS. (**B**) E6 proteins from human and other animal papillomaviruses have different requirements for interaction with E6AP. Bait yeast containing the two-hybrid plasmid expressing the LexA DNA binding domain fused to E6 proteins from the listed papillomaviruses were mated to prey yeast expressing B42 activation domain fused to the wild type E6AP peptide (LQELL), the mutated E6AP peptide (LQELS), the full-length E6AP containing LQELL, or the full-length E6AP containing LQELS. The full-length E6AP proteins were mutated at their active cysteine residue (C843A) resulting in ubiquitin ligase dead E6AP mutants (denoted Ub–). HPV41 E6 prefers to bind MAML1, not E6AP and was used as a negative control for binding. Horizontal white lines indicate development on different plates. H = Homo sapiens (human), Ps = Phocoena spinipinnis (Burmeister's porpoise), Pph = Phocoena phocoena (harbor porpoise), Tt = Tursiops truncatus (bottlenose dolphin), Mm = Macaca mulata (rhesus monkey), Ss = Sus scrofa (wild boar), Um = Ursus maritimus (polar bear).

** Figure 4.1 panel (B) **



4.3.2 16E6 L50A mutation alters E6AP binding profile

As yet, only the 16E6 and BPV1 E6 structures have been solved [32]. We chose to further explore the structural changes that underlie the different interactions between E6 proteins with the LQELL peptide either isolated or in the context of E6AP through the use of 16E6, as the structure has been determined. We hypothesized that impairing the association of HPV16 E6 with the LQELL peptide might make the association of 16E6 resemble the association of 11E6 (or the other E6 species shown in Fig 4.1B) with E6AP. We created a structure-informed mutant of 16E6, 16E6_L50A, predicted to impair binding to the isolated E6AP LQELL peptide. Ideally, we would not need to create LexA fusions to E6 proteins as this could potentially alter their interactions with E6AP, therefore we decided to utilize a yeast-three hybrid (Y3H) system [59] in which E6 is expressed unfused. This system enables observation of trimeric complex formation, where E6AP is fused to LexA, E6 is left in its native state, and the remaining protein has transactivator activity (either via a fusion or intrinsically). Unfortunately this is not always possible as protein(s) that bind to certain E6-E6AP complexes (such as those containing 11E6, 7E6, 10E6, or non-human PV E6s) are not well defined and therefore cannot be utilized in a Y3H assay. However, it is well established that high-risk 16E6 recruits p53 to LexA_E6AP or LexA_E6AP LQELL peptide alone (Fig 4.2A), thereby enabling the use of a Y3H assay. We found that 16E6_L50A could bind full-length E6AP and recruit p53, but failed to recruit p53 to the

E6AP LQELL peptide (Fig 4.2A, compare spot 1C to 2C). The 16E6_L50A mutant's inability to bind E6AP LQELL was previously described [32], however, here we demonstrate it is still able to bind full-length E6AP. The E6 L50 (red) residue is positioned less than 6Å from either of the double leucine resides (numbered 412 and 413) in the E6AP LQELL peptide (light pink) [32] (PDB ID: 4GIZ; Fig 4.2B).

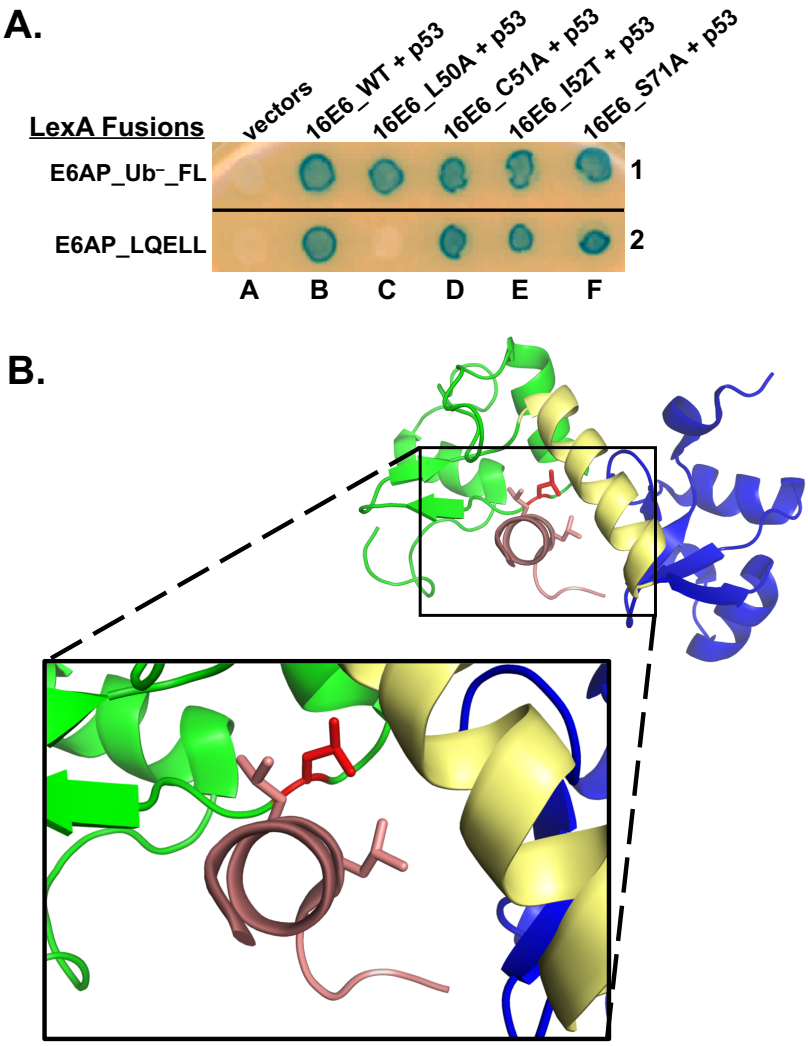


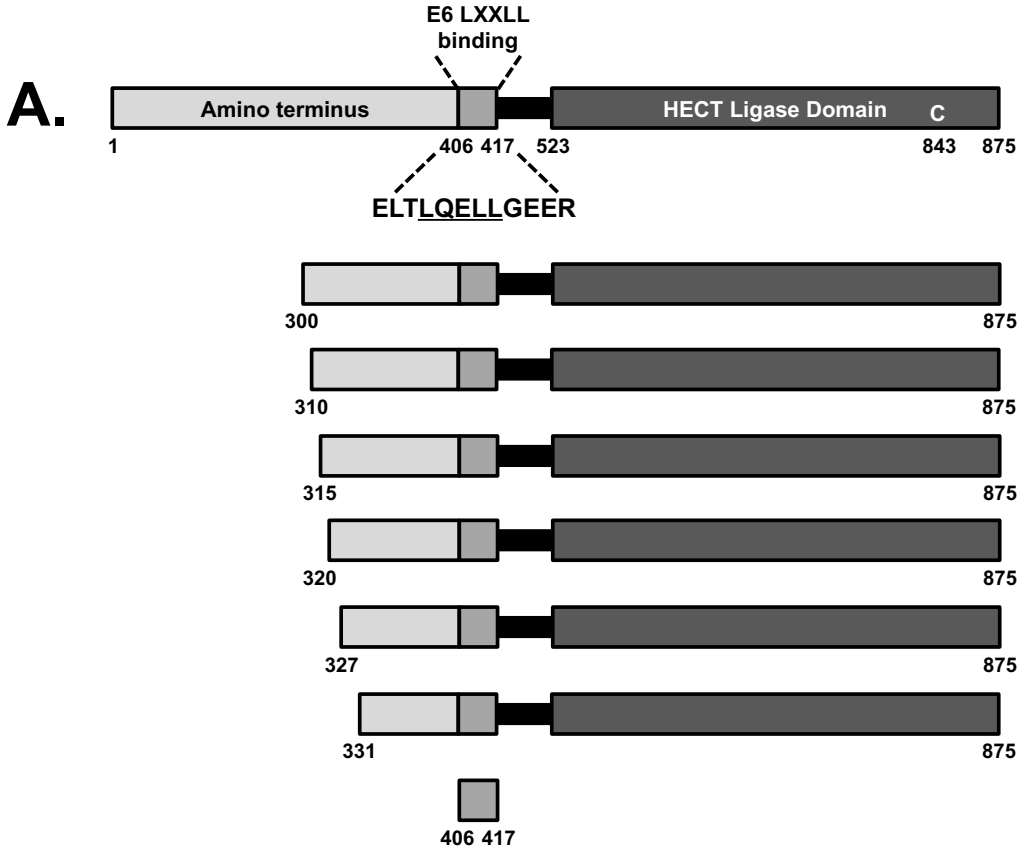
Figure 4.2 16E6 L50A mutant enables characterization of E6-E6AP interaction.

(A) Mutation of 16E6 L50 residue to an A results in a high-risk E6 protein that is unable to bind the E6AP LQELL peptide. Bait yeast expressing LexA fused to either full-length ubiquitin ligase dead E6AP (E6AP_Ub–) or the isolated E6AP LQELL peptide (E6AP_406-417) were mated to prey yeast co-expressing untagged 16E6_WT or 16E6 mutants containing a single amino acid change and p53. The 16E6_L50A mutation, but not other observed 16E6 mutations (C51A, I52T, and S71A) disrupted 16E6 binding to LQELL. (B) The E6 L50 residue is in close proximity to the double L residues in the E6AP LQELL peptide. The HPV16 E6 structure (PDB ID: 4GIZ) is depicted with the amino terminal zinc-structured domain in green, the carboxy terminal zinc-structured domain in blue, and the connecting alpha helix in yellow. The 16E6 protein is shown interacting with the E6AP LQELL peptide (in light pink), and the side chains of the double L residues are shown. The side chain of the E6 L50 residue is highlighted in red. The E6 L50 residue (red) is less than 6 Å from the last two leucine residues (L) in the E6AP LQELL peptide.

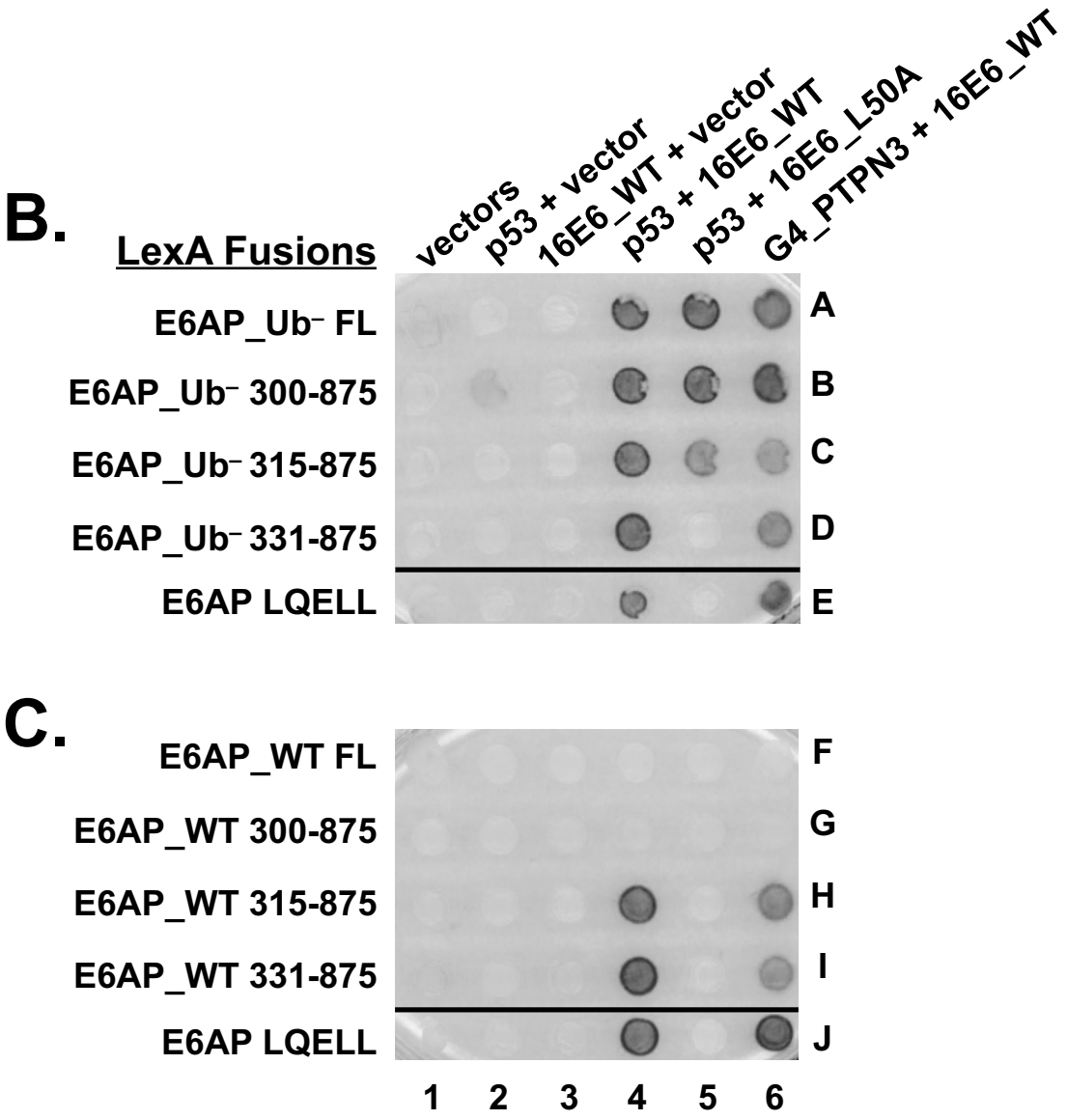
4.3.3 E6AP amino terminal region 310-320 is required for 16E6-mediated degradation of cellular p53

We previously had observed that 16E6 could bind to E6AP truncation 406-875 (containing the LQELL motif and the E6AP HECT domain) and recruit p53. However, this E6-E6AP-p53 complex, although containing the intact HECT domain, did not result in p53 degradation (unpublished results). Therefore, we hypothesized that the region of E6AP located amino-terminal to the LQELL motif (henceforth referred to as amino terminus) is required for 16E6 to initiate degradation of p53. To test this, we created LexA-fused amino-terminal truncations of E6AP (Fig 4.3A) and employed a Y3H assay (Fig 4.3B). Interaction of 16E6_L50A required the E6AP amino terminal region between residues 315 and 331 (Fig 4.3B, compare spots 5C and 5D). Additionally, the ability of 16E6_WT to initiate degradation of p53 was lost in the presence of E6AP_WT truncated to amino acid 315, indicated by the presence of a blue colony (Fig 4.3C, spot 4H). Together, these data suggested that the amino terminal region of E6AP between residues 300 and 320 was involved in mediating the ability of 16E6 to target p53 for degradation.

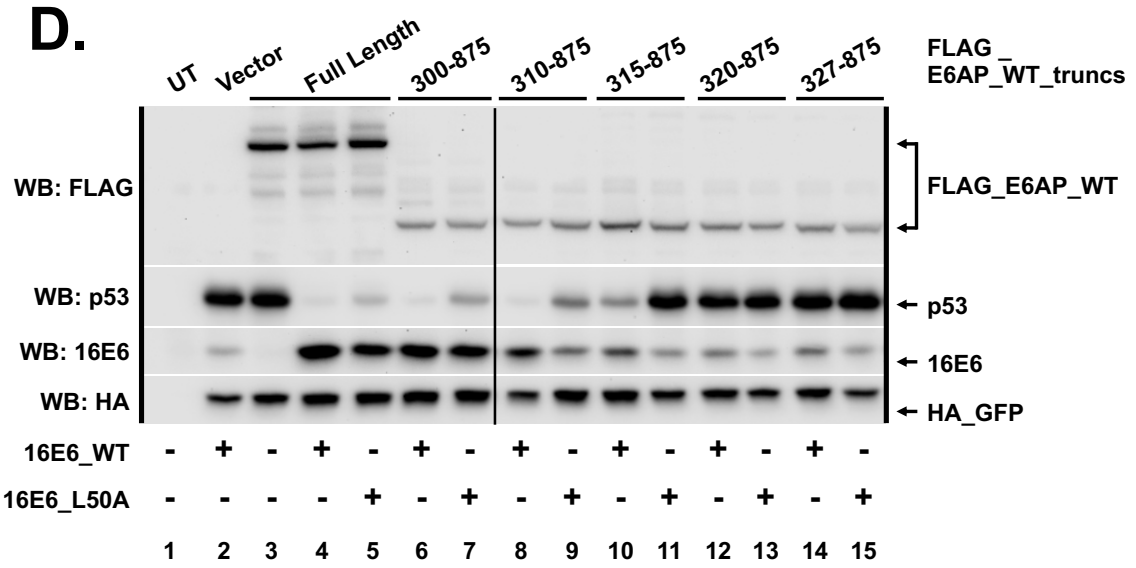
** Figure 4.3 panels (A), (B), (C), (D), and (E) and the figure legend are spread out over the next four pages **



** Figure 4.3 panels (B) and (C) **



** Figure 4.3 panels (D) and (E) **



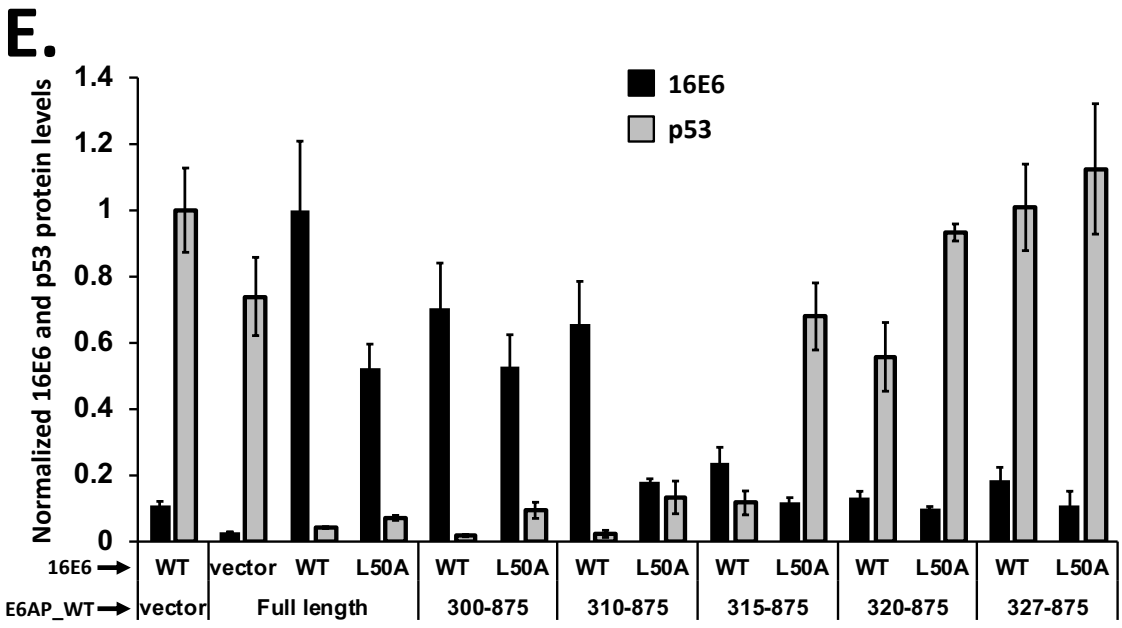


Figure 4.3 The amino terminal region of E6AP is required for 16E6 degradation of p53.

(A) Schematic of E6AP amino terminal truncations. Previously described E6 LQELL binding region is between amino acids 403 and 416, as depicted. Each E6AP amino terminal truncation retains the E6 LQELL binding region and the E6AP carboxy terminal HECT ligase domain. (B) Ability of 16E6_L50A to bind to E6AP requires E6AP residues 315-331. Bait yeast were transfected with the LexA DNA binding domain fused to ubiquitin ligase dead (Ub–) E6AP full-length (FL), amino terminal E6AP truncations, or the isolated E6AP LQELL peptide (406-417). These bait yeast were mated to prey yeast co-expressing p53, 16E6_WT, 16E6_L50A, or transactivator Gal4 (G4) fused to PTPN3 as indicated. Positive controls include 16E6_WT co-expressed with p53 to ensure E6AP expression (as 16E6_WT will interact with an E6AP truncation so long as it contains the LQELL motif) and G4-PTPN3 co-expressed with 16E6_WT to ensure 16E6_WT expression (as 16E6_WT contains a PBM at its extreme carboxy terminus with which PTPN3 will interact). Ability of 16E6 to bind E6AP and recruit p53 is lost when E6AP is truncated from residue 315 to residue 331. Horizontal black line indicates removal of irrelevant samples. (**C**) Ability of E6 to stimulate degradation of p53 and PTPN3 requires E6AP amino acids 310-315. Bait yeast were transfected with the LexA DNA binding domain fused to WT E6AP containing the same truncation endpoints as described in **B**. These bait yeast were mated to the same prey yeast as described in **B**. The ubiquitin ligase active E6AP targeted p53 for degradation in the presence of 16E6. Upon truncation of E6AP from amino acid 300 to 315, the degradation of p53 and PTPN3 by E6 was lost as indicated by the appearance of blue spots. Horizontal black line indicates removal of irrelevant samples. (**D**) Requirement of E6AP amino acids 310-320 for E6 ability to initiate p53 degradation in the presence of E6AP_WT is recapitulated in 8B9 cells. Plasmids encoding the indicated FLAG_E6AP_WT truncations (1.25 ug), human p53 (0.5 ug), HA-GFP (0.01 ug), and either untagged 16E6_WT or 16E6_L50A (2 ug), as indicated, were transiently transfected into E6AP-null 8B9 cells and p53 and E6 expression were analyzed by western blot. 16E6_WT requires E6AP amino acids 315-320 to initiate E6AP-mediated degradation of p53 while 16E6_L50A requires E6AP amino acids 310-315. A single representative blot is shown. Vertical black line indicates removal of an irrelevant sample. UT = untransfected. Quantitation of protein expression from three independent experiments is found in panel **E**. (**E**) E6AP stabilization of 16E6 (black bar) is not required for p53 (gray bar) degradation by the E6-E6AP complex. p53 levels and E6 levels are normalized to co-transfected HA_GFP. HA_GFP normalized E6 expression levels are further normalized to 16E6_WT protein levels in the presence of full-length E6AP (lane 4 in panel D). HA_GFP normalized p53 protein expression levels are normalized to p53 levels in the presence of 16E6_WT with no co-expressed E6AP protein (lane 2 in panel D). The means of triplicate independent experiments ± standard error are shown.

To confirm these results in a mammalian cell line system, we performed transient transfections into E6AP-null 8B9 cells reconstituted with various amino terminal truncations of WT E6AP in the presence of either 16E6_WT or 16E6_L50A (Fig 4.3D). Both WT and L50A 16E6 targeted p53 for degradation in the presence of E6AP 310-875. Upon further amino terminal truncation to residue 315, 16E6_WT still initiated p53 degradation, but 16E6_L50A did not (Fig 4.3D, compare p53 levels in lanes 10 and 11). 16E6-mediated p53 degradation was completely lost by both E6 WT and L50A with E6AP 320-875 (lanes 12 and 13). E6AP 320-875 was unable to coimmunoprecipitate 16E6_WT and p53 in E6AP-null 8B9 cells, while other less extensive E6AP truncations retained this ability (Fig 4.4). We speculated that some of the E6AP truncations may be functionally dead in that their active cysteine residue could not be loaded with ubiquitin. However, this was not the case, as each E6AP truncation coimmunoprecipitated with ubiquitin (Fig 4.5).

We hypothesized that 16E6's ability to initiate degradation of p53 was dependent upon its stabilization through E6AP interaction [79]. However, quantitation of p53 and 16E6 levels in the presence of the various E6AP truncation mutants examined in Fig 4.3D demonstrated that both 16E6_WT and 16E6_L50A were able to initiate p53 degradation when 16E6 protein levels were not obviously stabilized. 16E6_L50A protein stabilization by E6AP was lost with E6AP truncation 310-875 and 16E6_WT protein stabilization by

E6AP was lost with E6AP truncation 315-875, but in both cases p53 was still degraded (Fig 4.3E).

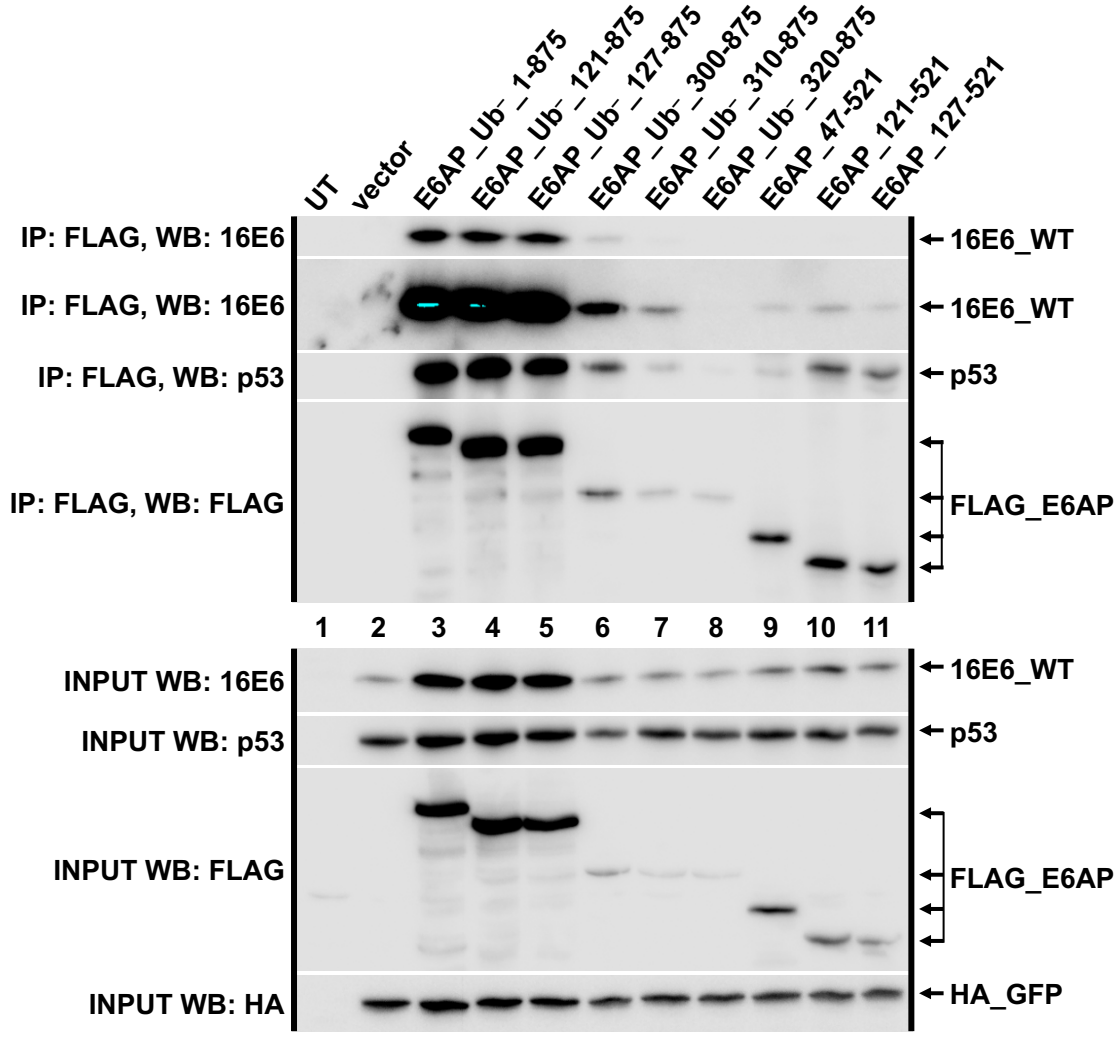


Figure 4.4 All E6AP truncations except 320-875 immunoprecipitate 16E6_WT and human p53.
Untagged 16E6_WT (4 ug), human p53 (2.9 ug), HA_GFP (0.1 ug), and the indicated FLAG_E6AP_Ub⁻ truncations (3 ug) were co-transfected into E6AP-null 8B9 cells and harvested 18 hrs later in 0.5X IGEPAL lysis buffer as described in methods. Western blots of input samples are clustered at the bottom and FLAG-immunoprecipitated (IP) samples are clustered at the top. Input was 5% of the immunoprecipitated sample size. The 16E6_WT IP blot has two exposures, one of which is overexposed in lanes 3 and 4. The overexposure is necessary to see 16E6_WT in lanes 7 and 9-11 (possibly due to lower expression of FLAG_E6AP truncations in those lanes). Ub⁻ indicates a ubiquitin ligase dead E6AP mutant. UT = untransfected.

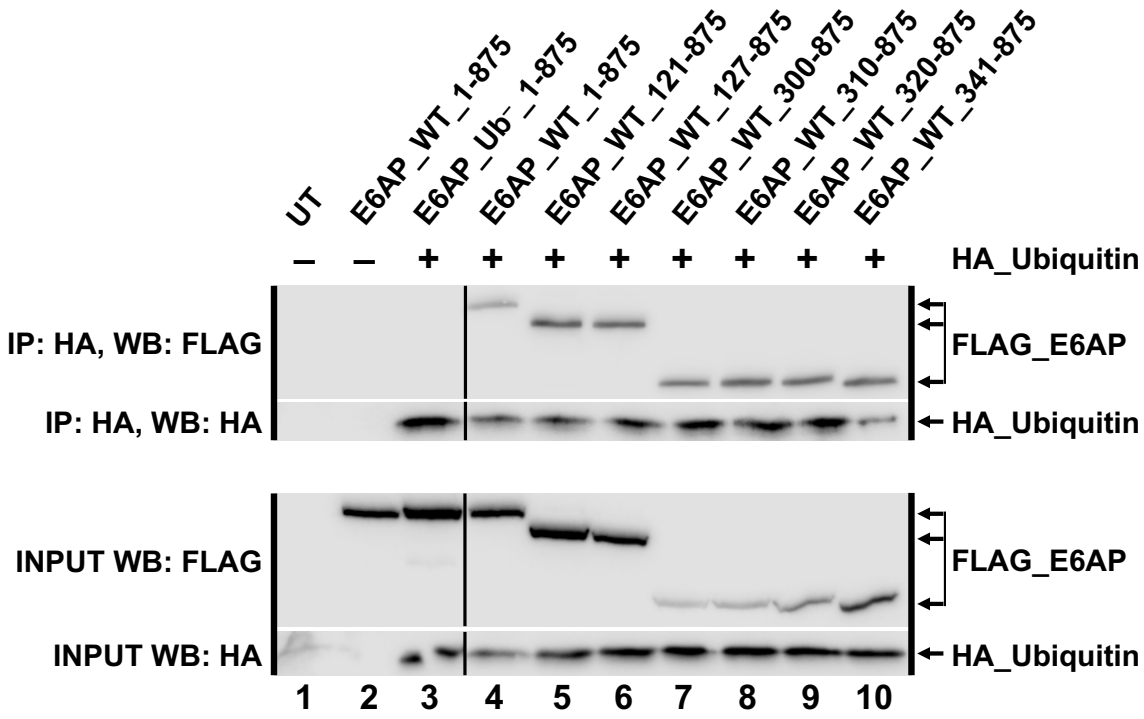


Figure 4.5 Constructed E6AP truncations maintain ability to be loaded with ubiquitin.

HA_Ubiquitin (6 ug) and FLAG_E6AP (3 ug; either ubiquitin ligase dead (Ub^-) or ubiquitin ligase active (WT)) plasmids were co-transfected into E6AP-null 8B9 cells and harvested 18 hrs later in 0.5X IGEPAL lysis buffer as described in the methods. Western blots of input samples are clustered at the bottom and HA-immunoprecipitated samples are clustered at the top. Input was 5% of the immunoprecipitated sample size. E6AP_Ub^- indicates a ubiquitin ligase dead E6AP mutant. Black line indicated removal of an irrelevant sample. UT = untransfected.

To identify the specific residue(s) within E6AP region 310-320 that were required for 16E6-mediated degradation of p53, we constructed single amino acid point mutants within residues 310-320 (inclusive) in the context of full-length E6AP (Fig 4.6A). These E6AP mutants were co-transfected with p53, HA_GFP, and either 16E6_WT or 16E6_L50A into E6AP-null 8B9 cells. None of the observed E6AP single amino acid point mutants prevented 16E6-mediated degradation of cellular p53 (Fig 4.6B). E6AP P317A was not expressed (lanes 17 and 18), likely because it conferred protein instability (Fig 4.6B). We also made an internal deletion of residues 310-320 (inclusive) in the context of full-length E6AP (Δ310-320) and saw that this E6AP mutant, like the E6AP truncation mutant 320-875, resulted in loss of targeted p53 degradation by 16E6 (Fig 4.6B lanes 21-24). Secondary structure analysis predicts the 310-320 region to have alpha-helical secondary structure. Our mutational results suggest that maintenance of the alpha-helicity of this segment of the E6AP 310-320 region is important in enabling high-risk E6 degradation of p53.

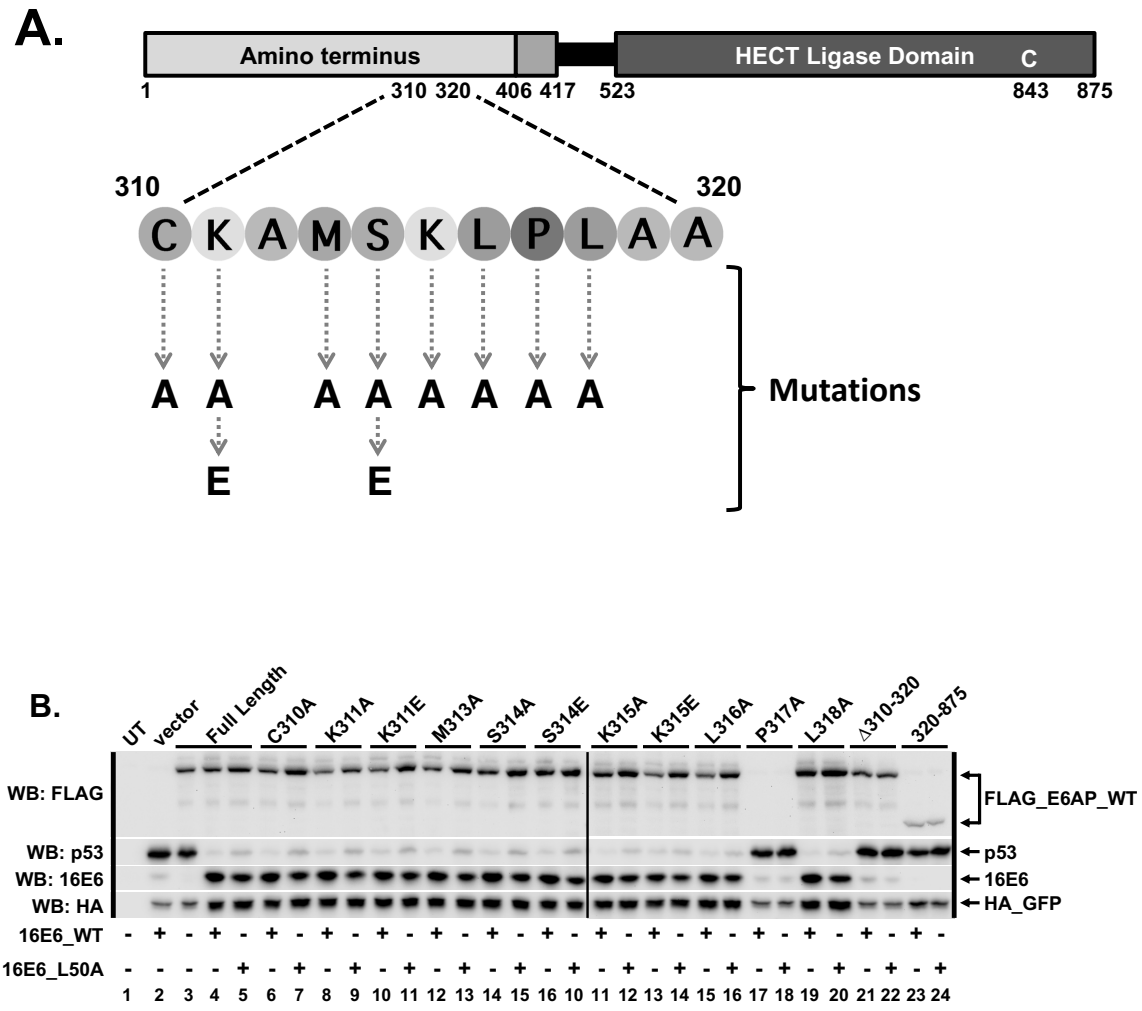


Figure 4.6 No single amino acid point mutation within E6AP region 310-320 prevents 16E6_WT from initiating degradation of p53.

(**A**) Schematic of full-length E6AP protein. The amino acids located between 310 and 320 (inclusive) are depicted, as is the location of the HECT ligase domain and the active cysteine residue (C843), responsible for ubiquitination of p53. (**B**) Full-length E6AP containing single amino acid point mutations within the 310-320 region still degrade p53 in the presence of 16E6_WT. Plasmids encoding the indicated FLAG_E6AP (1.25 ug), human p53 (0.5 ug), HA_GFP (0.01 ug), and either 16E6_WT or 16E6_L50A (2 ug) were co-transfected into E6AP-null 8B9 cells. Δ310-320 is full-length E6AP_WT deleted of residues 310-320 (inclusive). Amino terminal E6AP truncation 320-875 was used as a negative control for p53 degradation in the presence of either 16E6_WT or 16E6_L50A. Vertical black line indicates samples were run on two different western blots. UT = untransfected.

4.3.4 Both high and low-risk HPV E6 proteins require the same amino-terminal E6AP region to target the degradation of cellular NHERF1

We recently reported that both high and low-risk E6 proteins can target the degradation of the PDZ protein NHERF1 in an E6AP-dependent manner [143]. Due to our observed dependence of high-risk 16E6 on the E6AP 310-320 region for initiating p53 degradation (Fig 4.6B), we hypothesized that the same E6AP amino terminal region would also be required for low-risk E6 mediated degradation of NHERF1. To test this possibility, we transiently transfected HA_NHERF1, HA_GFP, FLAG_11E6_WT, and various FLAG_E6AP_WT truncations into E6AP-null 8B9 cells. NHERF1 was degraded by 11E6_WT and amino terminal E6AP truncations until NHERF1 degradation was lost with co-expression of 11E6_WT and E6AP 314-875 (Fig 4.7A). 16E6 was also sensitive to the E6AP 314-875 truncation, as it lost its ability to effectively degrade NHERF1 in the presence of this E6AP truncation (Fig 4.7B). Our observations were recapitulated when the experiments were repeated using E6, NHERF1, and E6AP expressed at more physiologically-relevant levels for both low (Fig 4.8A) and high-risk E6 (Fig 4.8B). Thus, the same region that is required for 16E6 to target p53 degradation is also required for degradation of NHERF1 by both low-risk and high-risk E6 proteins.

** Figure 4.7 legend on next page **

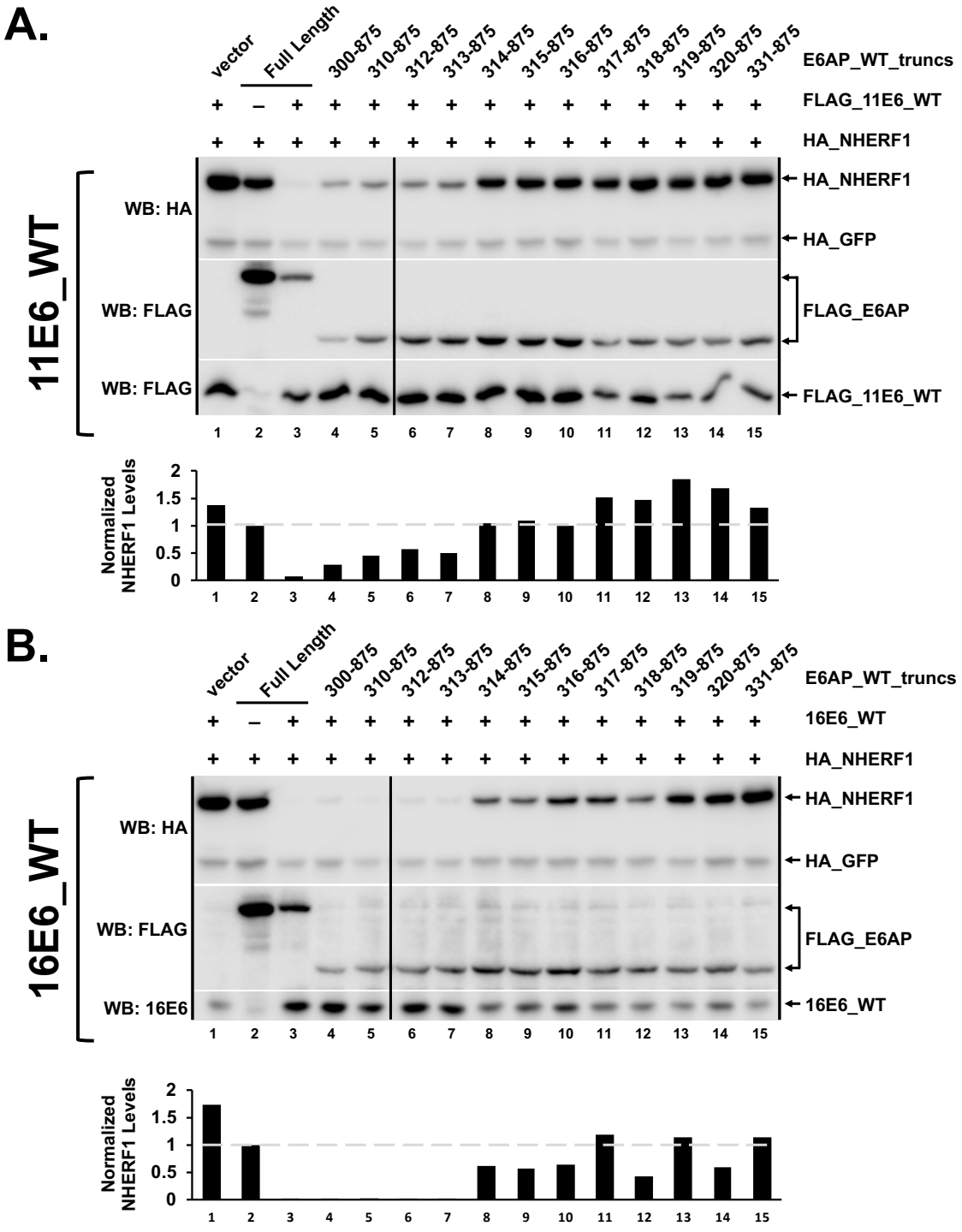


Figure 4.7 Low-risk and high-risk E6 proteins require the same amino terminal region of E6AP to initiate degradation of cellular substrates.
NHERF1 degradation is lost in the presence of a ubiquitin active truncation E6AP 314-875 with both (**A**) low-risk 11E6 and (**B**) high-risk 16E6. The listed FLAG_E6AP_WT truncations were co-transfected with HA_NHERF1 (0.75 ug), HA_GFP (0.08 ug), and FLAG_11E6_WT (2 ug; panel **A**) or 16E6_WT (2 ug; panel **B**) into E6AP-null 8B9 cells and HA_NHERF1 expression analyzed by western blot. HA_GFP was co-transfected as a transfection control. Bar graphs below western blots indicate quantitation of HA_NHERF1 protein levels first normalized to HA_GFP to account for transfection variability and then normalized to HA_NHERF1 protein levels in the presence of full-length E6AP with no co-expressed E6 (lane 2). E6AP amino terminal truncation to residue 314 displays a lack (with 16E6_WT) or loss (with 11E6_WT) of targeted NHERF1 degradation.

** Figure 4.8 legend on next page **

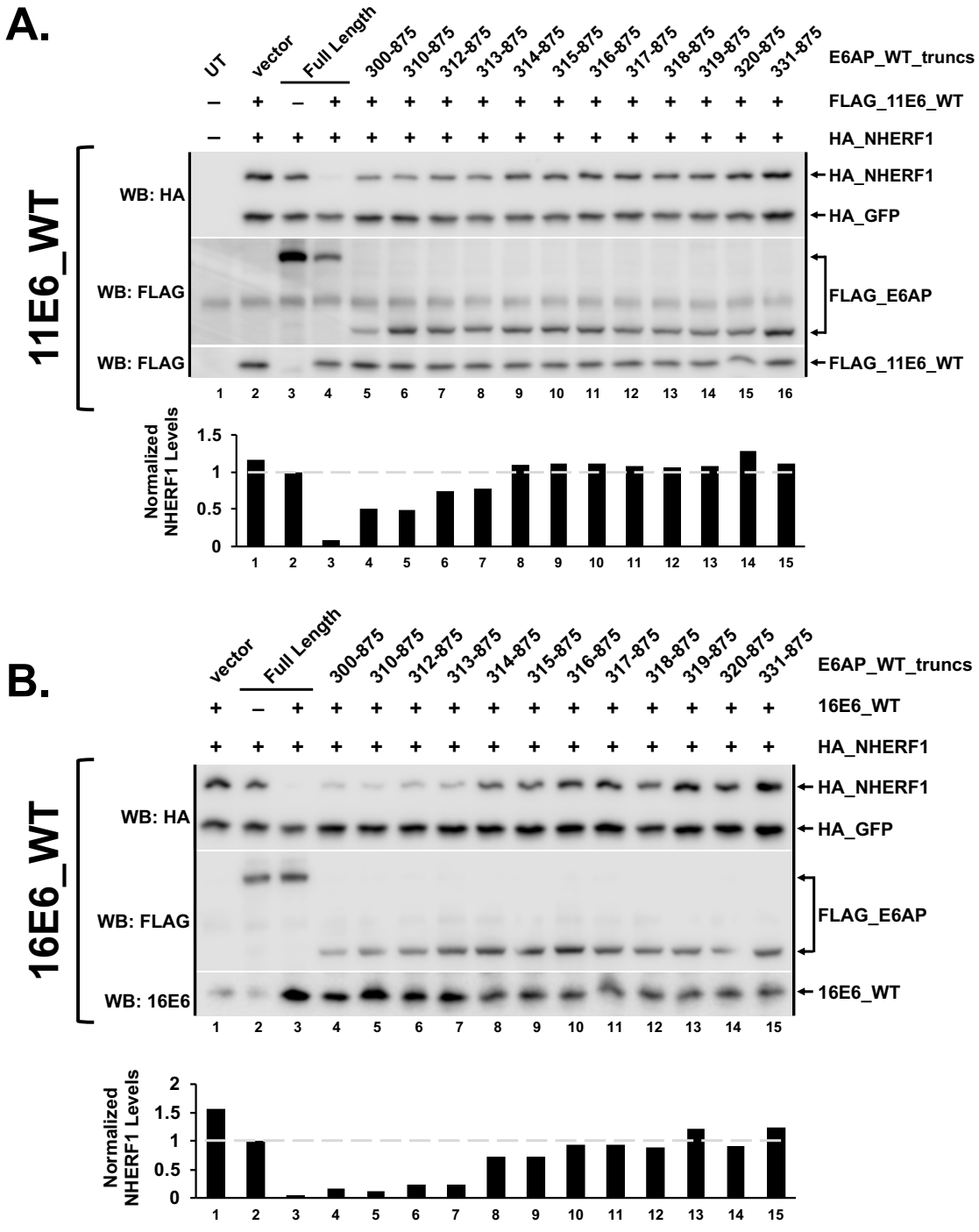


Figure 4.8 Physiologically relevant expression levels of E6 proteins demonstrate lack of NHERF1 degradation in the presence of E6AP_WT 314-875 truncation.

Experiment performed exactly as described in Fig 5, but with different transfected amounts of the expression plasmids: FLAG_E6AP_WT_truncs (0.2 ug), HA_NHERF1 (0.3 ug), HA_GFP (0.1 ug), and 16E6_WT or FLAG_11E6_WT (0.2 ug). These DNA amounts were determined upon performing titration experiments in 8B9 cells for each expression plasmid in order to determine how much plasmid to transfect such that protein was detectible via western blot, but not overexpressed.

4.3.5 The E6AP LQELL motif is important for high and low-risk E6-mediated degradation of cellular targets

High-risk 16E6 can initiate p53 degradation in the presence of E6AP_WT in which the LQELL motif has been mutated to LQELS [48]. To determine whether the E6AP LQELL motif further mutated to LQEAS still enabled E6-mediated degradation of cellular substrates, we performed a transient transfection in E6AP-null 8B9 cells with full-length E6AP (either WT or Ub^-) containing either LQELL or the mutated LQEAS (Fig 4.9). NHERF1 was degraded in the presence of both 16E6 and 11E6 when co-expressed with E6AP_LQELL_WT but not E6AP_LQELL_ Ub^-, reiterating the necessity of E6AP ubiquitin ligase activity for E6-mediated NHERF1 degradation. Although reduced, NHERF1 protein levels when 16E6 or 11E6 were co-expressed with E6AP_LQEAS_WT did not recapitulate NHERF1 levels with E6AP_LQELL_WT and E6 (Fig 4.9, compare lanes 5 and 13 to lanes 3 and 11, respectively). Because NHERF1 was slightly degraded by 16E6 and 11E6 in

the presence of E6AP_LQEAS_WT, it is possible that the E6 interaction with the E6AP LQELL motif aids in E6-mediated NHERF1 degradation, but is not required. Our results further the claim that strong E6-E6AP interactions at the LQELL site are not essential for initiation of E6AP ubiquitin ligase activity [48]. These results were further supported by slight 16E6 degradation of p53 in the presence of E6AP_LQEAS_WT (Fig 4.9, compare lanes 6-9). As expected, 11E6 did not stimulate p53 degradation (lanes 14-17).

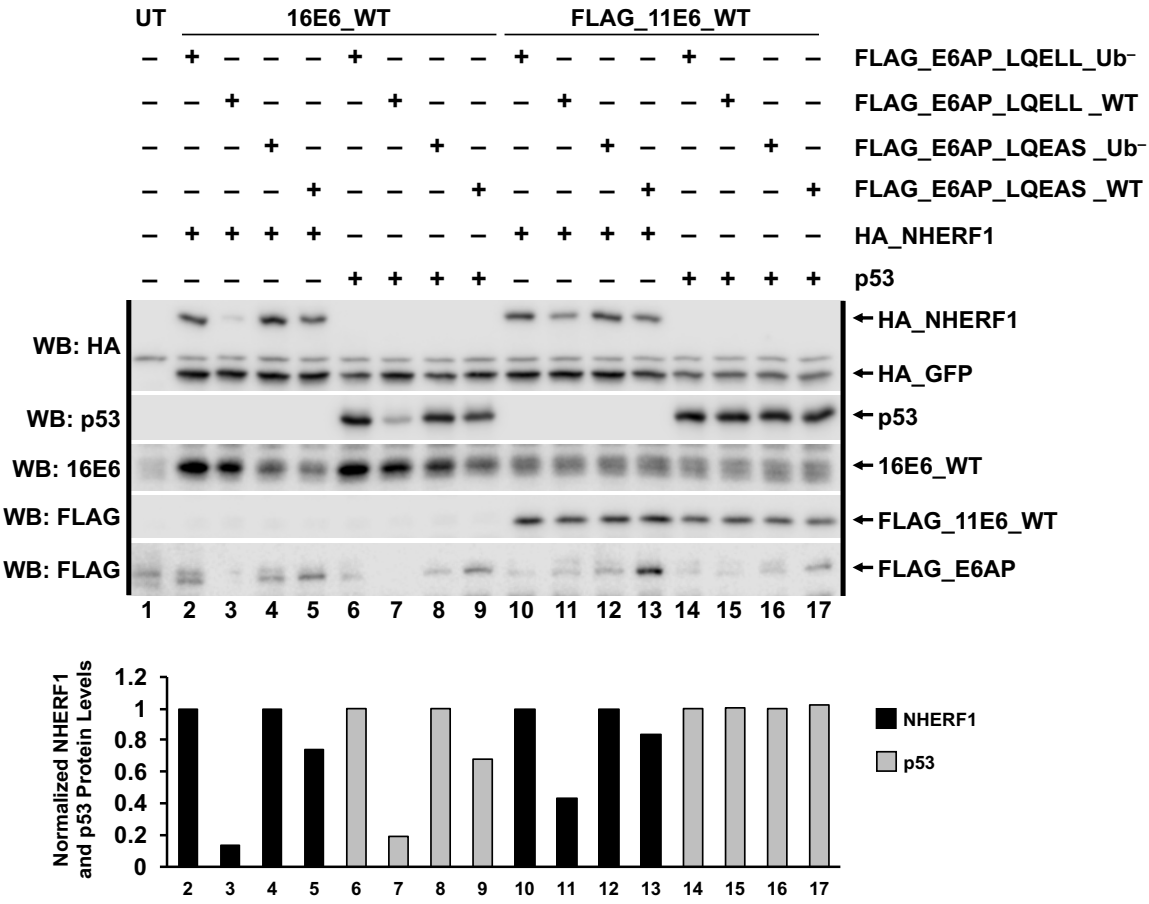


Figure 4.9 E6AP LQELL is important for efficient degradation of cellular substrates by both high and low-risk E6 proteins.
HA_GFP (0.1 ug), human p53 (0.15 ug) or HA_NHERF1 (0.3 ug), and each of the listed FLAG_E6AP variants (0.2 ug) were co-transfected with either untagged 16E6_WT or FLAG_11E6_WT (0.2 ug) in E6AP-null 8B9 cells. E6AP_Ub– indicates a ubiquitin ligase dead E6AP protein that was used as a control. E6AP_LQEAS contains two single amino acid point mutations in the E6 LQELL binding region. Previously reported lack of p53 degradation by 11E6_WT was used as an internal negative control. HA_NHERF1 and p53 protein levels in the presence of the indicated E6 and E6AP proteins were normalized to HA_GFP. The bar graph below the blot represents quantification of either HA_NHERF1 protein levels (black bar) or human p53 protein levels (gray bar) in the presence of the indicated E6 and E6AP proteins. UT = untransfected.

4.3.6 The extreme amino terminus of E6 proteins is important in enabling the enhanced affinity E6AP interaction

16E6 shows higher affinity for full-length E6AP over the isolated E6AP LQELL peptide [59]. Interestingly, full-length E6AP cannot outcompete LQELL bound to 16E6 deleted of the first eight amino acids (16E6_Δ1-8) (unpublished results). Therefore, we postulated that the first eight amino acids in 16E6 may be important in conferring the enhanced binding of E6 to full-length E6AP versus the E6AP LQELL motif. To test this hypothesis, we utilized the E6AP constructs depicted in Fig 4.1A transfected into prey yeast and mated to bait yeast containing LexA DNA binding domain fused E6 proteins. 16E6_L50A and 16E6_Δ1-8 each interacted with E6AP_ Ub^-_LQELS (Fig 4.10, spots 5C and 5D). However, the double mutant 16E6_Δ1-8_L50A lost its ability to interact with full-length E6AP_ Ub^-_LQELS (spot 5E). A multiple sequence alignment indicated that the first eight amino acids of 16E6 were homologous to the first nine amino acids of 11E6. Therefore, we created an 11E6_Δ1-9 mutant to determine whether this amino-terminal E6 region is important in mediating the low-risk E6-E6AP interaction. While 11E6_WT could interact with full-length E6AP_ Ub^-_LQELS, 11E6_Δ1-9 lost this ability (Fig 4.10, compare spots 5F and 5G). Taken together, these data suggest that the initial eight or nine amino acids of 16E6 and 11E6, respectively, play at least a partial role in enabling the more robust interaction between E6 and full-length E6AP compared to the interaction of E6 with LQELL.

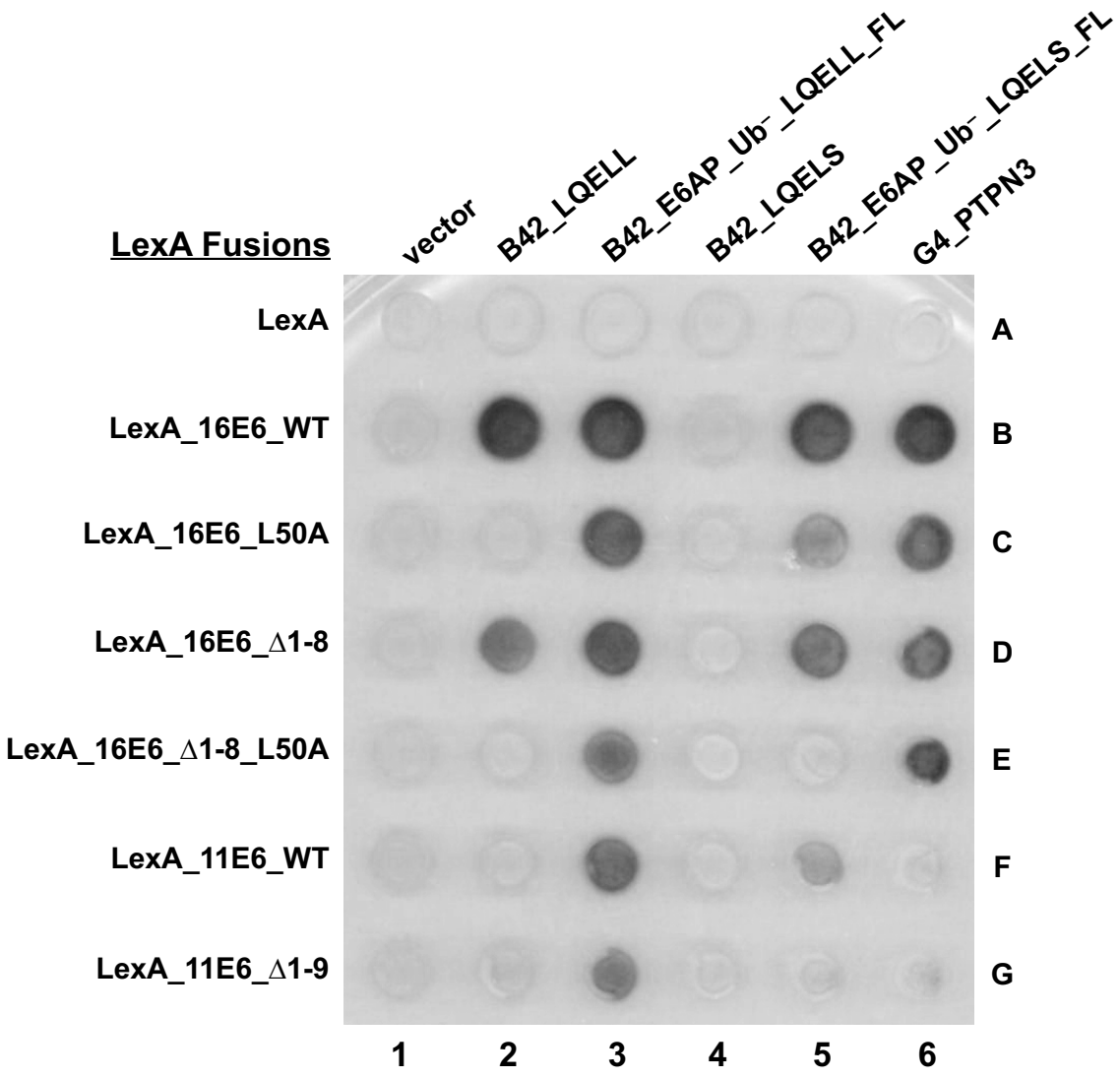


Figure 4.10 The extreme N-terminus of E6 may play a role in E6AP binding. Yeast-two hybrid expression plasmids expressing the LexA DNA binding domain fused to the indicated E6 proteins were transfected into bait yeast. The bait yeast were mated to prey yeast transfected with B42 transactivator fused E6AP constructs depicted in Fig 4.1A or transactivator Gal4 (G4) fused PTPN3. 16E6_WT was used as a positive control for expression of E6AP proteins. 16E6_WT did not interact with B42_LQEAS. G4_PTPN3 was used as a positive control for expression of the high-risk, PDZ binding motif containing, E6 proteins. The low-risk 11E6 protein is unable to interact with PTPN3.

4.3.7 The amino terminus of E6AP is important for E6AP to outcompete LQELL peptide bound to 16E6

We have identified that 16E6_L50A (which cannot bind the isolated E6AP LQELL peptide but can bind full-length E6AP) was able to interact with and initiate p53 degradation in the presence of E6AP 300-875 (Fig 4.3B and 4.3D, respectively). However, both interaction and p53 degradation was lost with 16E6_L50A and E6AP truncated past amino acid 315. Therefore, we hypothesized that the 300-315 region of E6AP contributed to E6 enhanced affinity interaction with E6AP. Previous studies have shown that full-length E6AP can outcompete 16E6_WT LQELL binding in *cis* [59]. To test our hypothesis, we utilized 16E6_WT with an amino terminal fusion of the E6AP LQELL peptide (in *cis*), which was co-transfected with either full-length E6AP_WT or E6AP_WT 300-875 (Fig 4.11). The E6AP LQELL peptide is sufficient to recruit p53 to the complex, but is not sufficient for 16E6-mediated degradation of p53 [59]. Therefore, p53 degradation was only observed if the full-length E6AP or E6AP 300-875 was able to outcompete LQELL for binding to 16E6. As expected, we saw p53 degradation by LQELL_16E6_WT in the presence of full-length E6AP (Fig 4.11, lane 4). However, when co-expressed with E6AP 300-875, we did not see p53 degradation (lane 7), suggesting that other regions within the amino terminus of E6AP upstream of amino acid 300 are important for the observed ability of full-length E6AP to outcompete LQELL binding 16E6 in *cis*.

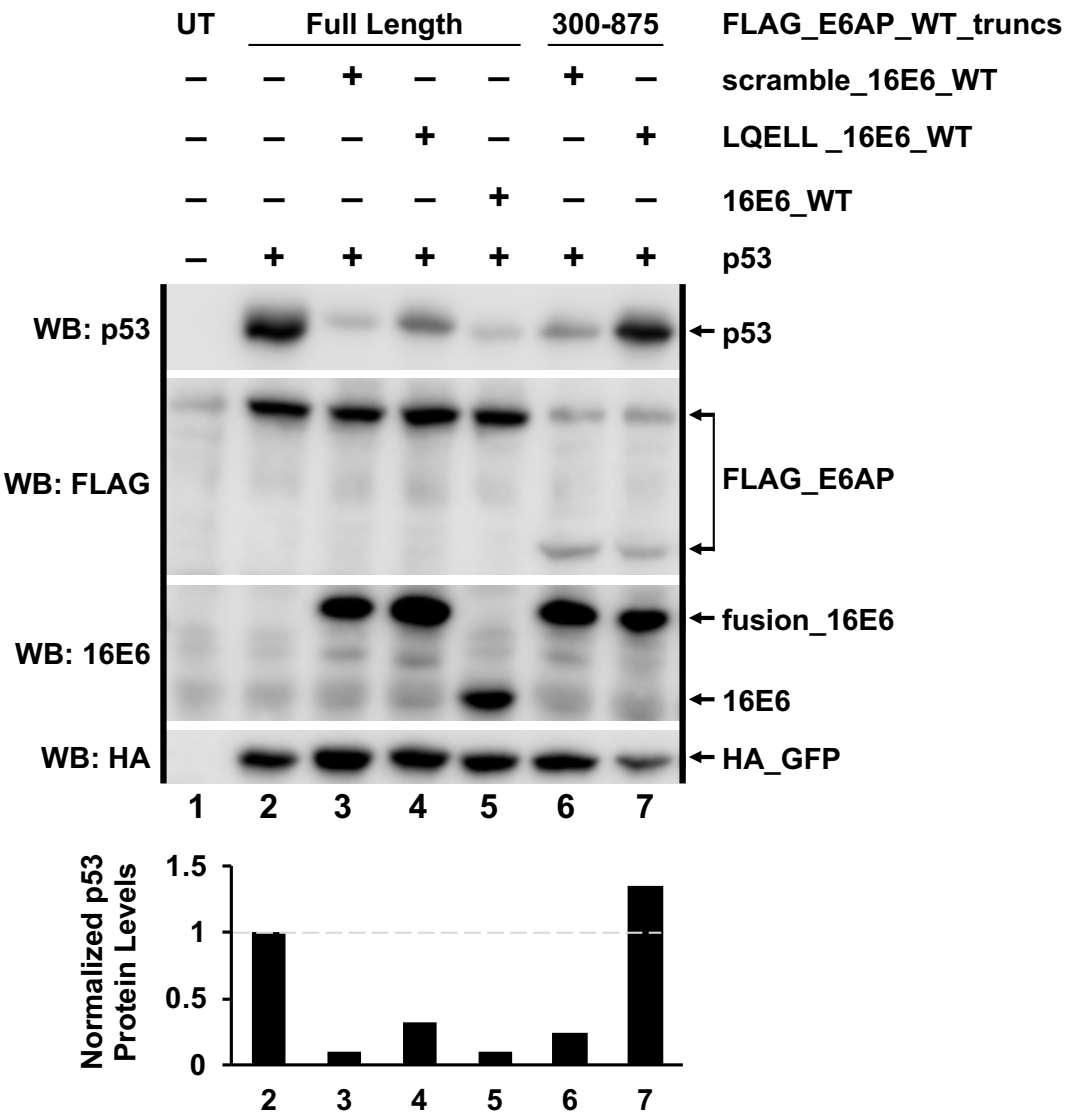


Figure 4.11 Full-length E6AP outcompetes *cis* LQELL binding to 16E6_WT. Plasmids encoding the indicated FLAG_E6AP_WT (0.5 ug), human p53 (0.5 ug), HA_GFP (0.1 ug), and the listed 16E6 proteins (0.5 ug) were transiently transfected into E6AP-null 8B9 cells and p53 protein expression was analyzed by western blot. LQELL_16E6_WT has an intramolecular interaction such that the 16E6 protein binds to the amino terminally fused LQELL E6AP peptide. Scramble_16E6_WT contains an amino terminal fusion with no intramolecular interaction. Unfused 16E6_WT serves as a control to ensure that scramble_16E6_WT degrades p53 as well as 16E6_WT indicating the amino terminal fusion is not disrupting the fold of the E6 protein. Full-length E6AP outcompeted the LQELL_16E6_WT intramolecular interaction resulting in p53 degradation while the amino terminal E6AP truncation 300-875 did not outcompete the LQELL/16E6_WT binding. p53 protein levels were quantitated and normalized to HA_GFP to account for transfection efficiency. For comparison purposes, the HA_GFP normalized p53 levels were then normalized to p53 protein levels in the presence of full-length E6AP with no co-expressed E6 (lane 2). UT = untransfected.

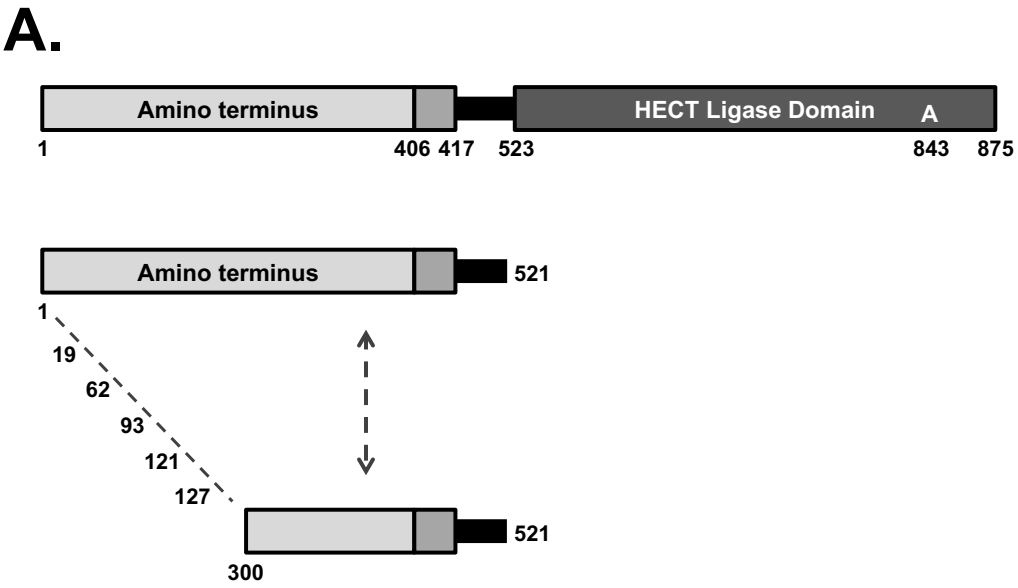
4.3.8 Multiple regions within E6AP 1-521 enable p53 recruitment by 16E6_L50A

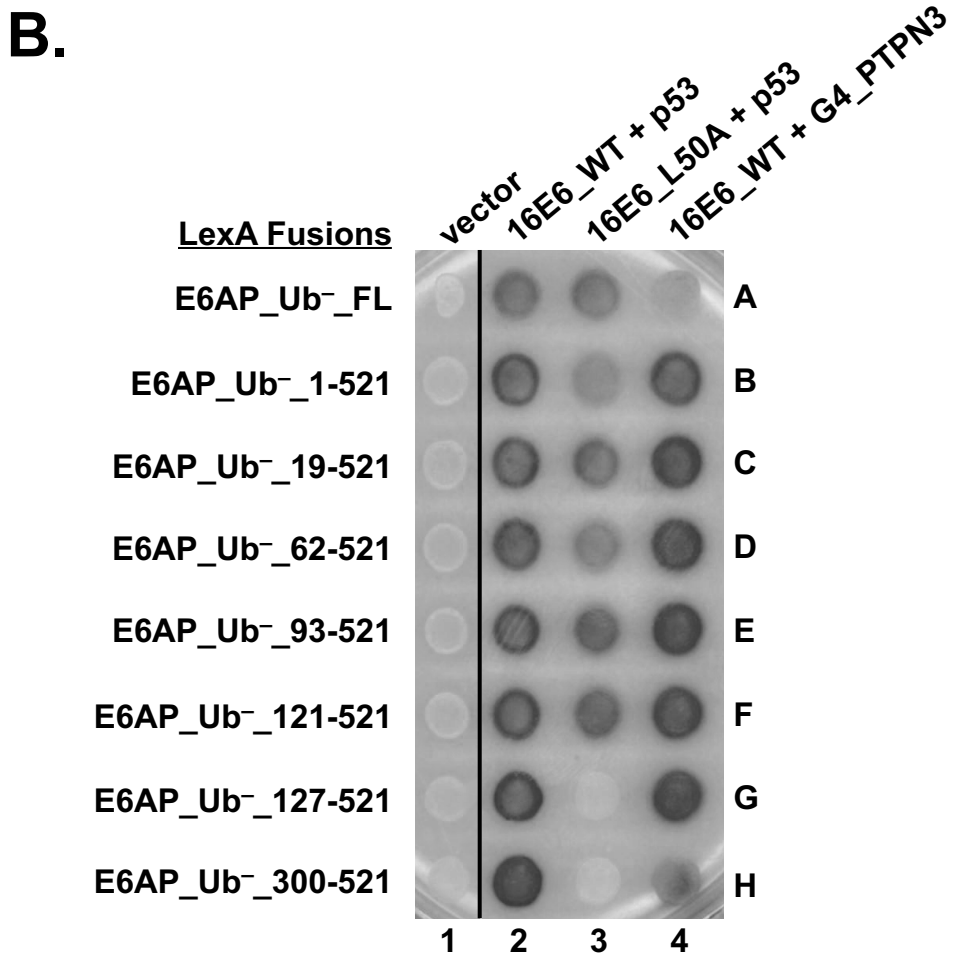
To identify which region(s) within the E6AP amino terminus upstream of residues 300-315 are important in mediating the E6-E6AP interaction, we constructed amino terminal E6AP truncation mutants that lacked the HECT domain (Fig 4.12A). Each truncation retained the E6AP LQELL motif. All of the E6AP truncations enabled 16E6_WT binding and recruitment of p53 (Fig 4.12B, column 2), which was expected since 16E6 WT can bind to the isolated LQELL peptide . However, in the presence of 16E6_L50A, p53 recruitment was lost when E6AP was amino-terminally truncated from amino acid 121 to 127 (spots 3F and 3G), indicating that the first 127 amino acids of E6AP contribute to E6 interaction with E6AP.

We continued to focus on characterizing the region of E6AP amino-terminal to the HECT domain (residues 1-521). Having identified amino acids important in mediating the E6AP-E6 interaction with 521 as the carboxy-terminal endpoint (Fig 4.12B), we constructed carboxy terminal E6AP truncations keeping the amino terminal endpoint at residue 1 (Fig 4.12C). In this way, we could determine whether the region of E6AP between the E6AP LQELL motif and the E6AP HECT domain (residue 417-521) contributed to the E6-E6AP interaction. 16E6_WT successfully recruited p53 to each of the observed E6AP truncations as expected (Fig 4.12D, column 4), indicating that each E6AP was expressed and folded. Upon performing a carboxy terminal truncation of E6AP

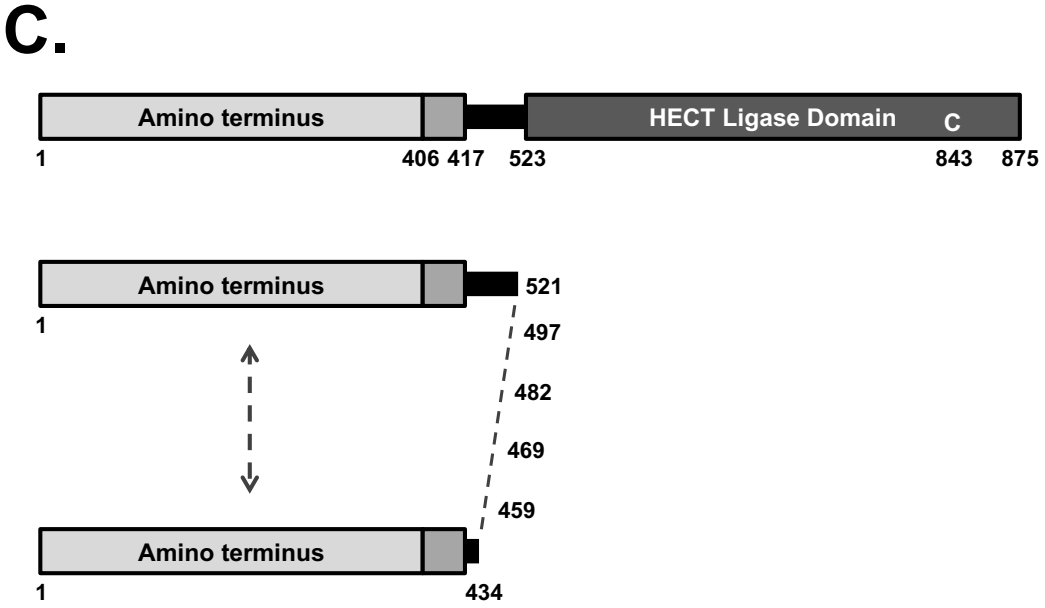
from amino acid 521 to 497, we saw loss of p53 recruitment to the E6AP protein by 16E6_L50A (spots 5B and 5C). This suggests that 16E6 interaction with E6AP is partially mediated by the sequence in E6AP between residues 497 and 521.

**Figure 4.12 panels (A), (B), (C), and (D) and the figure legend are spread out over the next three pages **





** Figure 4.12 panels (C) and (D) **



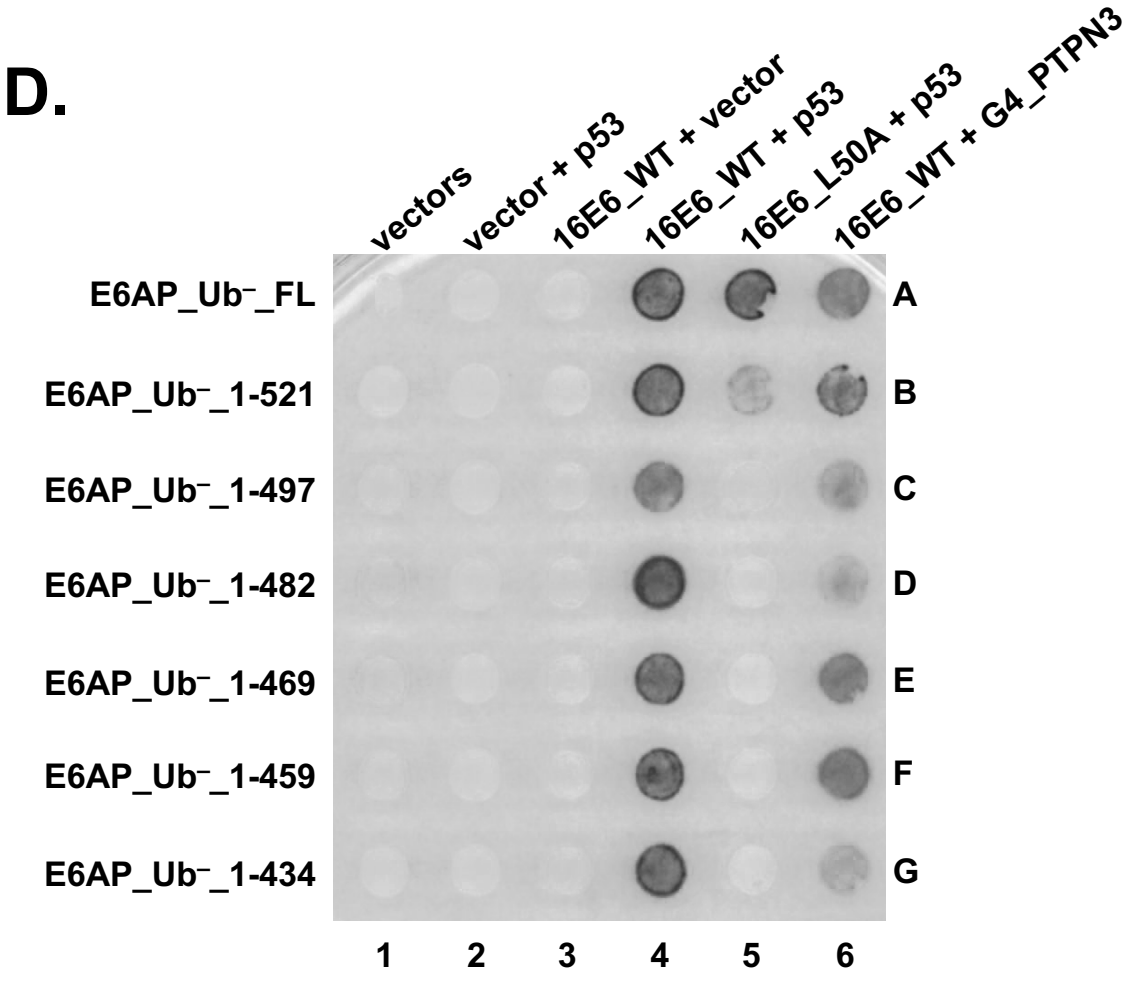


Figure 4.12 Additional E6AP regions in both the amino and carboxy terminus are important in mediating E6 binding.
(**A**) Schematic of E6AP and E6AP truncation mutants examined in panel **B**. E6AP amino terminal truncations lack the HECT ligase domain, but retain the LQELL E6 binding motif. (**B**) E6AP residues 121-127 are important for enabling 16E6_L50A interaction and p53 recruitment. Bait yeast were transfected with LexA DNA binding domain fused E6AP_Ub– (ubiquitin ligase dead) truncation expression plasmids. Bait yeast were mated to prey yeast co-transfected with 16E6_WT, 16E6_L50A, p53, or G4_PTPN3 as indicated. Co-expression of 16E6_WT and p53 with each LexA_E6AP serves as a positive control for E6AP expression and folding. Co-expression of Gal4 (G4) transactivator fused PTPN3 with 16E6_WT and the various LexA_E6AP truncations ensures that 16E6_WT is being expressed and can recruit the PDZ protein PTPN3 to the E6AP protein. Vertical black line indicates removal of irrelevant samples. (**C**) Schematic of E6AP and E6AP truncation mutants examined in panel **D**. E6AP truncations lack the HECT ligase domain but retain the LQELL E6 binding motif. (**D**) Yeast-three hybrid expression plasmids expressing the LexA DNA binding domain fused to E6AP_Ub– truncations were transfected into bait yeast. Those bait yeast were mated to prey yeast containing yeast-three hybrid expression plasmids expressing p53, 16E6_WT, 16E6_L50A, or G4_PTPN3. Co-expression of 16E6_WT and p53 with each LexA_E6AP truncation ensured both expression and proper folding of the E6AP truncation protein. The PDZ protein PTPN3 co-expressed with 16E6_WT and each E6AP truncation demonstrated ability of 16E6 to bind the E6AP protein and recruit PTPN3. Spot A4 in panel (B) appears lighter than other rows in column 4, but is above background (spot A1), indicating full-length 16E6_WT is being expressed.

4.3.9 Low-risk 11E6 requires residues within the HECT domain to interact with E6AP

Because both low-risk 11E6 and high-risk MmPV1E6 are unable to interact with the isolated E6AP LQELL peptide (Fig 4.1B, spots 2C and 2N; Fig 4.13B, spots 8C and 8D), we speculated that these two E6 proteins would display similar E6AP binding profiles. To determine whether the HECT domain played a role in mediating the E6-E6AP interaction for 11E6 and MmPV1 E6, we truncated the entire amino terminal region of E6AP upstream of the E6AP LQELL motif and constructed carboxy-terminal E6AP truncations (Fig 4.13A). Bait yeast expressing a LexA DNA binding domain fused to either 16E6_WT, 11E6_WT, or MmPV1E6_WT were mated to prey yeast expressing the various depicted HECT domain E6AP truncations (Fig 4.13B). Interaction of the E6AP proteins with 16E6_WT indicated each E6AP protein was expressed and folded (row B). While 11E6_WT could interact with E6AP 406-595 (spot 6C), it lost its ability to interact with E6AP 406-561 (spot 7C), indicating the residues between 561 and 595 in the E6AP HECT domain contribute to the interaction of low-risk 11E6 with E6AP. Interestingly, MmPV1E6_WT interacted only with the full-length E6AP and not with any of the E6AP truncations (Fig 4.13B, row C), indicating that the interaction of MmPV1E6 with E6AP requires regions within the E6AP amino terminus. Although neither 11E6 nor MmPV1E6 could interact with the isolated E6AP LQELL peptide (row 8), they display different requirements to mediate their interaction with full-length

E6AP. This may relate to the lack of MmPV1E6 binding to full-length E6AP LQELS, while 11E6_WT could bind this E6AP mutant.

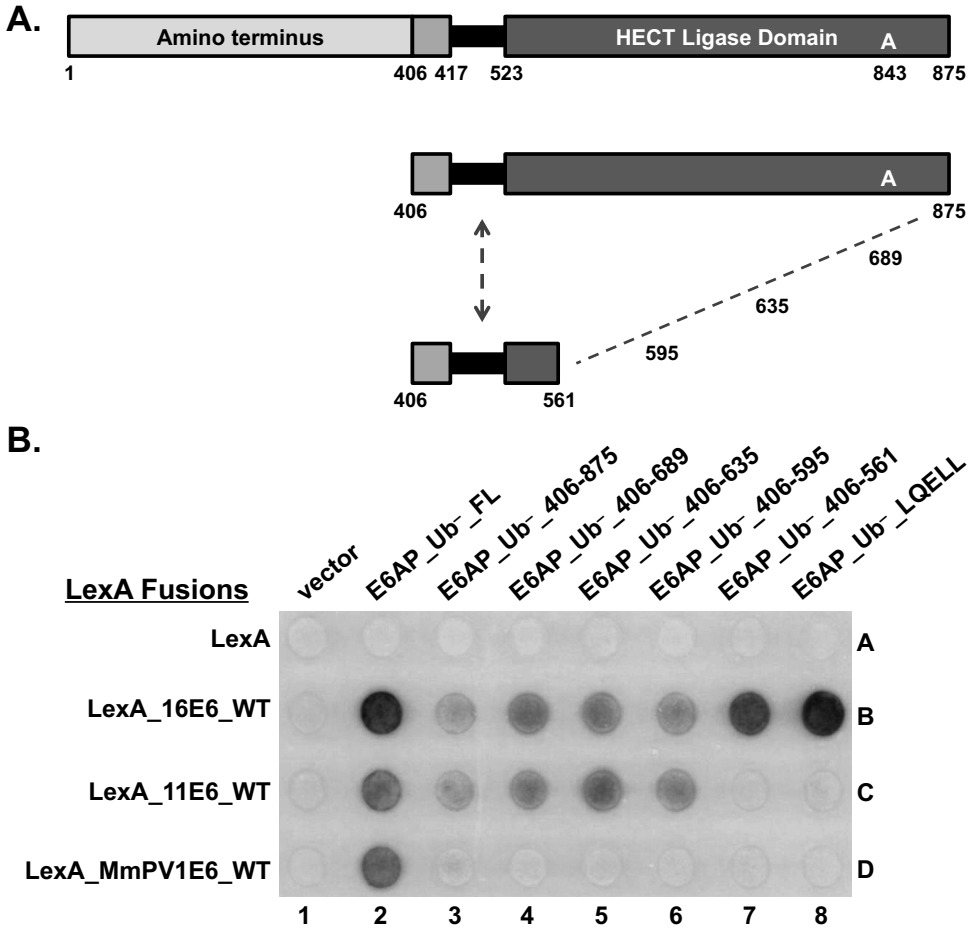


Figure 4.13 The low-risk 11E6 protein binding to E6AP requires E6AP residues located in the HECT ligase domain.
(**A**) Schematic of full-length E6AP and E6AP truncations examined in panel **B**. Each C-terminal E6AP truncation contains the E6AP LQELL E6 binding motif at the amino terminus. (**B**) Bait yeast transfect with plasmids encoding LexA DNA binding domain fused 16E6_WT, 11E6_WT, or MmPV1E6_WT were mated to yeast containing B42 transactivator fused E6AP truncations as indicated. LexA_16E6_WT served as a positive control for E6AP expression and folding as 16E6 should interact with each E6AP truncation because they all contain the LQELL motif. 11E6 requires E6AP residues 561-595 to interact with E6AP. The high-risk E6 protein from *Macaca mulata* (rhesus monkey; MmPV1) can only bind full-length E6AP, none of the truncated E6AP proteins.

4.4 Discussion

The E6-E6AP interaction is critical for E6-induced changes in infected cells [145]. The crystallization of 16E6 bound to the E6AP LQELL-53 complex represented a tremendous leap forward in understanding papillomavirus-host interactions. However, it did not provide insight into how E6 interacts with the full-length E6AP protein, including the potential significance of E6AP regions outside of the LQELL in mediating E6-E6AP interaction or how E6 stimulates the E3 ligase activity of E6AP. Here, we show that E6AP-binding E6 proteins from both human and non-human papillomaviruses require regions of E6AP besides LQELL, and furthermore that these regions differ across the E6s tested. (Figs 4.1B, 4.14B). In addition, we identified a mutant of 16E6 (L50A) capable of binding full-length E6AP, but not the isolated LQELL peptide. Using this mutant we were able to identify a region within the amino terminus of E6AP encompassing residues 310-320 that is important for binding of 16E6_L50A to E6AP as well as for initiation of E6AP ubiquitin ligase activity (Fig 4.3). We demonstrated that this 310-320 E6AP region was not only required for high-risk E6 mediated degradation of cellular substrates, but that it was also required for low-risk E6 to induce degradation of cellular NHERF1 (Fig 4.7). Therefore, although the E6AP auxiliary regions required to mediate the E6-E6AP interaction differ among various E6 proteins, to functionally stimulate E6AP ubiquitin ligase activity we found that Type II binding is required for both 16E6 and 11E6. Through the use of E6AP amino and carboxy

terminal truncations, we identified three regions within E6AP that are important in enabling 16E6_L50A interaction (Fig 4.14): two in the N-terminus (amino terminal to the LQELL motif; Fig 4.3 and 4.12) and one carboxy to the E6 LQELL binding motif (Fig 4.12). Finally, we found that unlike high-risk 16E6, low-risk 11E6 requires a region located in the HECT domain (residues 561-595) in order to interact with E6AP deleted of everything amino terminal to the LQELL motif (deleted of residues 1-405; Fig 4.13).

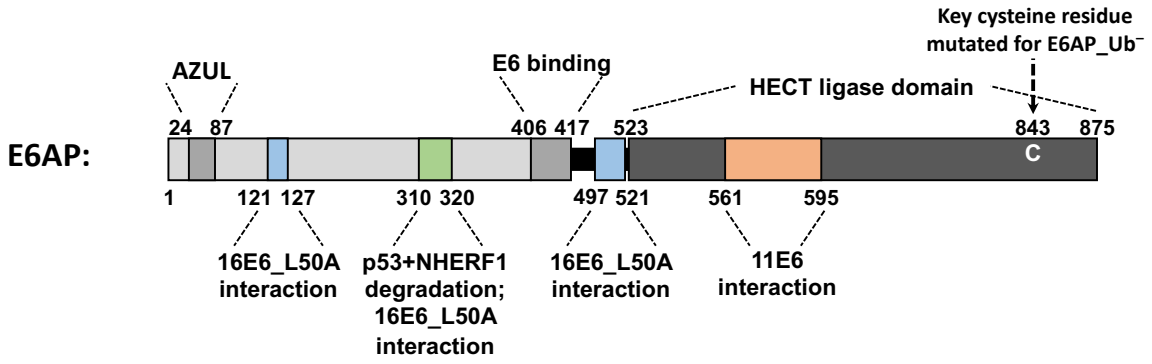


Figure 4.14 Summary schematic: newly identified regions in E6AP important in mediating E6 binding and cellular protein degradation.
The newly identified and described regions are indicated as being important in mediating E6 binding and/or in E6-mediated degradation of cellular proteins. Regions shaded in light blue are required for 16E6_L50A interaction when the HECT domain is deleted, and are involved in Type II interactions. The region shaded in green is important for E6 stimulation of E6AP ubiquitin ligase activity in the context of E6AP 310-875 vs 320-875 and is an E6AP region involved in Type II interactions. The region shaded in light orange is important for binding of 11E6_WT to E6AP deleted of all sequence amino terminal to the E6 LQELL binding region (region amino terminal to residue 406). The orange region is involved in a Type II E6-E6AP interaction. The region shaded in light gray is what is referred to throughout this thesis as the amino terminus of E6AP. The two regions in medium gray: AZUL and E6 binding have been previously described. The AZUL region (residues 24-87) is the **A**mino-terminal **Z**inc-finger of **U**be3a **L**igase. The E6 binding region (residues 406-417) contains the LQELL motif of E6AP and is required for E6 proteins to bind to E6AP. A Type I E6-E6AP interaction occurs when an E6 protein can bind the isolated E6AP LQELL motif (E6 binding region). The dark gray region is the HECT (**H**omologous to **E**6AP **C**arboxy **T**erminus) domain which is required for E6AP ubiquitin ligase activity.

We selected a cohort of E6 proteins that bind E6AP and stimulate its E3 ubiquitin ligase activity [60] and examined their ability to interact with different E6AP proteins: E6AP LQELL, E6AP LQELS, full-length E6AP LQELL, and full-length E6AP LQELS. We found that, while all of these E6 proteins are capable of binding full-length E6AP LQELL, they differ in their ability to bind both the isolated LQELL peptide as well as full-length E6AP LQELS. The ability to bind the isolated E6AP LQELL peptide was first discovered through study of high-risk 16E6 [59, 78, 147]. However, our results indicate that being classified as high-risk is not always sufficient for an E6 protein to interact with E6AP LQELL. MmPV1 is a high-risk virus, and its E6 protein is unable to interact with LQELL alone (Fig 4.1B, spot 2N). Additionally, we found both predominately cutaneous (SsPV1, HPV7, HPV10) and predominately mucosal (HPV16 and UmPV1) E6 proteins can bind E6AP LQELL. Exactly what aspects of the E6 protein contribute to binding to the isolated E6AP LQELL is unclear. Phylogenetic clustering of these E6 proteins does not group them according to their E6AP-binding profile. Interestingly, TtPV5 E6 and MmPV1 E6 failed to interact with full-length E6AP LQELS indicating that mutation of the LQELL motif prevents these E6 proteins from interacting with full-length E6AP. While neither TtPV5 E6 nor MmPV1 E6 could interact with the isolated E6AP LQELL peptide their failure to interact with full-length E6AP LQELS indicates that, in addition to E6AP auxiliary regions, these E6 proteins also require an intact LQELL motif in order to bind

E6AP proteins. Other E6 proteins that are unable to bind E6AP LQELL retained their interaction with full-length E6AP LQELS (E6 proteins from HPV11, PsPV1, and PphPV1).

Through this screen (Fig 4.1B) we propose two distinct types of E6-E6AP interactions: Type I: the E6 protein can bind the isolated E6AP LQELL peptide and Type II: the E6 protein cannot bind isolated E6AP LQELL. We further propose breaking down the Type II interaction into two subtypes: Type IIa and Type IIb. E6 proteins that interact with E6AP via a Type IIa interaction retain their ability to interact with full-length E6AP LQELS whereas Type IIb interactors cannot interact with full-length E6AP LQELS. We speculate that the other regions within E6AP that this study describes (referred to as E6AP auxiliary regions) enable E6 proteins that cannot bind isolated LQELL to bind full-length E6AP. In the instance of Type IIb, the E6 interaction with LQELL in the context of full-length E6AP may be very weak, but the combination of LQELL and the auxiliary regions of E6AP enables E6 binding to full-length E6AP. Whereas within the Type IIa binding classification, E6 interaction with E6AP auxiliary regions is stronger such that mutation of LQELL to LQELS does not ablate E6 interaction with full-length E6AP LQELS.

In order to ascertain the E6AP auxiliary regions important in mediating E6-E6AP interactions, we first required a mutant of 16E6 that was unable to interact with the isolated LQELL peptide while still able to interact with full-length E6AP so that we could employ a Y3H system. Zanier et al. showed

16E6_L50A could not interact with the isolated peptide, but did not demonstrate whether it could still bind full-length E6AP [32]. In our study, we further characterized the 16E6_L50A mutant and demonstrated its preserved ability to not only interact with full-length E6AP, but also to initiate p53 degradation in the presence of E6AP (Fig 4.2A and 4.3). 16E6_WT interacts with isolated E6AP LQELL; therefore any E6AP truncation containing the LQELL motif would bind 16E6_WT. We reasoned that unlike 16E6_WT, if 16E6_L50A could interact with a particular E6AP protein, it would be due to auxiliary regions independent of the E6AP LQELL motif.

E6 is a potent activator of the E3 function of E6AP [149], but the mechanism behind that activation remains unclear. We utilized 16E6_L50A and amino-terminal E6AP truncations in the context of a yeast three hybrid assay (Y3H) to identify E6AP residues 310-320 (located amino-terminal to the E6AP LQELL motif) as required for 16E6_L50A to bind to E6AP. Truncation of E6AP beyond residue 315 prevents 16E6_L50A binding to E6AP and p53 recruitment (Fig 4.3B). Interestingly, the same amino-terminal E6AP truncation endpoints in the context of ubiquitin ligase active E6AP demonstrated that E6AP 310-320 is necessary for E6-mediated degradation of p53 (Fig 4.3C-E). We hypothesize that interaction with auxiliary regions within E6AP is required (illuminated by 16E6_L50A interaction with E6AP) for 16E6 stimulation of E6AP E3 ubiquitin ligase activity. Our Y3H results were corroborated in E6AP-null murine 8B9 cells, the use of which enabled us to measure both p53

and 16E6 protein levels. (Fig 4.3D,E). Because E6 protein is stabilized by E6AP [79] we initially hypothesized that the stability of 16E6 would directly relate to its ability to initiate p53 degradation. However, quantification of both p53 and 16E6 levels in the presence of various E6AP truncations clearly indicated that 16E6 does not have to be stabilized by E6AP in order to target the degradation of p53. Indeed, 16E6_L50A displays a loss of protein stability (mimicking E6 protein levels when there is no E6AP present) in the presence of E6AP 310-875, but p53 is still being degraded (Fig 4.3E). Additionally, 16E6_WT protein levels indicate loss of E6 stability with E6AP 315-875, yet p53 is still degraded. We attempted to identify a single amino acid within 310-320 in the context of full-length E6AP required for mediation of E6-induced p53 degradation (Fig 4.6). No single point mutant recapitulated our 310-320 truncation data, suggesting that a single amino acid in E6AP 310-320 is not solely responsible for enabling E6-mediated degradation. Mutation of multiple amino acids may prove more successful. Alternatively, soft mutations (e.g. mutation to alanine) may be insufficient to disrupt our desired phenotype and strong mutations (e.g. charge reversal) could illuminate a single, necessary residue.

Because 16E6_WT is able to initiate p53 degradation in the presence of E6AP 315-875, while 16E6_L50A requires E6AP 310-875, we hypothesize that that the capacity of 16E6_WT to tightly bind LQELL aids in stimulation of E6AP E3 ligase activity. However, we know that E6 binding to the E6AP

LQELL motif is neither sufficient [60] nor required (Fig 4.9) [47] for E6 to activate E6AP ubiquitin ligase activity. Indeed, 11E6, which is unable to bind the isolated E6AP LQELL peptide, stimulates E6AP ligase activity to target NHERF1 for degradation, but also requires E6AP residues 310-320 (Fig 4.7A). A recent study utilized chemical cross-linking coupled to mass spectrometry (XL-MS) to model the E6-E6AP-p53 trimeric complex and postulated that the interaction results in both E6 and p53 being in close proximity to the E6AP active cysteine residue in the HECT domain [150]. It is possible that this conformational orientation of the E6-E6AP-p53 requires E6AP amino acids 310-320 and that orienting E6 and the cellular substrate close to the E6AP HECT domain activates E6AP ubiquitin ligase activity. Furthermore, previous studies indicate that E6AP-mediated ubiquitin chain formation rely on the N-terminal region of E6AP [149, 150]. If this specifically applies to E6AP residues 310-320, it may be that the processivity of ubiquitin chain formation mediated by E6AP is disrupted with the E6AP 320-875 truncation.

Evidence suggests that 16E6 displays a higher affinity for binding full-length E6AP over the isolated LQELL peptide [59, 82, 83]. We reasoned that because E6AP region 310-320 is important for enhanced affinity of 16E6_L50A binding, it may also be required for the observed higher affinity of E6 for full-length E6AP over LQELL. Surprisingly, we found that the ability of full-length E6AP to outcompete E6 binding to LQELL is lost when E6AP is truncated to residue 300 (Fig 4.11). This suggests that regions in E6AP between residue 1

and 300 must be important in full-length E6AP outcompeting LQELL for E6 binding, although these regions are disposable for 16E6_L50A binding and for E6 initiation of E6AP ubiquitin ligase activity. As stated previously, XL-MS based modeling of the E6-E6AP interaction proposed that E6AP undergoes a conformational rearrangement upon binding to E6, and that the N and C-terminal regions of E6AP are brought into close proximity [150]. This E6AP conformational rearrangement depends on E6AP residues amino-terminal to amino acids 300. We hypothesize that this conformational rearrangement of E6AP is important for higher affinity E6 binding and as a result, E6AP 300-875 (deleted of its N-terminus) is unable to outcompete LQELL for binding to 16E6_WT.

We aimed to identify a region within the N-terminus of E6AP between residues 1 and 300 important for E6 binding and subsequent E6AP conformational rearrangement. We have established that 16E6_L50A can interact with full-length E6AP deleted to residue 315 (Fig 4.3B). In line with modeling of the E6AP-E6 interaction [150], we hypothesized that the N and C-termini of E6AP each played a role mediating the E6 interaction. Therefore, to identify auxiliary binding regions within E6AP located amino-terminal to residue 300, we first truncated off the E6AP HECT domain. By removing the HECT domain, we reasoned that 16E6_L50A would be forced to rely solely on the amino terminus of E6AP for binding. Through this process we identified E6AP amino acids 121-127 as important for 16E6_L50A interaction with E6AP

in the absence of the HECT domain. We hypothesize that the E6AP 121-127 region may be important for enabling the conformational rearrangement of E6AP and serves as the region in the N-terminus that comes into close proximity with the C-terminus suggested by Sailer et al. The XL-MS data localized the N-terminal interaction contact residues as falling mainly between the E6AP residues 83-173, in accordance with our results [150].

To better understand the E6-E6AP interaction we strove to characterize important regions within E6 as well as E6AP. E6 proteins are generally small (around 150 amino acids) and so truncations risk resulting in protein misfolding. However, 16E6 deleted of its first eight amino acids (16E6_Δ1-8) can still bind full-length E6AP, stimulate degradation of E6AP in an overexpression system, and target p53 for degradation [48]. We noticed that full-length E6AP cannot outcompete LQELL bound in *cis* to 16E6_Δ1-8 (unpublished results), suggesting that the first eight residues of 16E6 are important in mediating the higher affinity binding of full-length E6AP to the E6 protein. We hypothesized that there are two main E6-E6AP interaction mechanisms: one which has been described, characterized, and crystalized between the E6AP LQELL motif and E6 L50 (other amino acids are involved, see [32]) and one which requires E6AP auxiliary regions and the first eight amino acids of 16E6. To tease apart the complexity of this proposed dual mode of interaction between E6 and E6AP, we utilized a yeast two hybrid assay with various E6 and E6AP mutants. We found that, like 16E6_WT, 16E6_Δ1-8

interacts with isolated E6AP LQELL, full-length E6AP LQELL, and full-length E6AP LQELS (Fig 4.10, row B vs. row C). Therefore, the deletion of residues 1-8 in 16E6 is not sufficient to prevent interaction with E6AP auxiliary regions and inhibit binding to E6AP LQELS (a mutant which should minimize the E6-LQELL interaction; spot 6D). This observation led us to adjust our hypothesis: there is a dual modality of E6-E6AP interaction that is mediated from both the E6 and E6AP perspective; such that mutating one of the proteins (e.g. E6AP LQELS) does not completely attenuate the corresponding interaction (e.g. E6-E6AP via LQELL). Indeed, the 16E6_Δ1-8_L50A double mutant retains ability to interact with full-length E6AP LQELL, but can no longer interact with full-length E6AP LQELS (Fig 4.10, spot 3E vs. 5E). Further support of our hypothesis stems from examining low-risk 11E6 (rows F and G), which like 16E6_L50A, cannot bind the LQELL peptide alone. 11E6Δ1-9 (homologous to 16E6Δ1-8) could not interact with full-length E6AP LQELS, but did interact with full-length E6AP LQELL (spot 3F vs 3E).

Here we show for the first time that E6AP-binding E6 proteins require different auxiliary binding regions within E6AP in order to form the E6-E6AP complex. Further investigation identified previously undescribed regions within E6AP in both the amino and carboxy terminus (Fig 4.14) that are important in enabling the E6-E6AP interaction. We also describe a region within the amino terminus of E6AP that is required for both high and low-risk

E6 proteins to stimulate E6AP ubiquitin ligase activity. While our approach does not preclude E6 interactions with E6AP regions outside of those we have identified as important, it does highlight regions of E6AP that 16E6_L50A and 11E6 (both unable to interact with LQELL) required to maintain the important interaction with E6AP. Taken together, these data suggest further study is necessary to properly characterize the complexity of this important heterodimer.

Chapter 5: Discussion

5.1 Summary of Results

The major significance of this work is twofold. (1) We have identified a cellular degradation target of papillomavirus E6AP-binding E6 proteins shared by not only high and low-risk HPV E6 proteins, but also by E6 proteins isolated from non-human hosts. Degradation of this target, called NHERF1, by E6 proteins augments the canonical Wnt/β-catenin pathway. (2) We have described multiple auxiliary regions within E6AP that are required for E6 binding. We identified one of these auxiliary E6AP regions as required for both high and low-risk E6 to stimulate the ubiquitin ligase activity of E6AP.

Originally, we set out to find a cellular protein that could be degraded by 16E6_WT, but not degraded by 16E6 deleted of its PDZ binding motif (PBM; 16E6ΔPBM). A literature search identified NHERF1, a PDZ protein that had been shown to be degraded by 16E6_WT in a manner dependent upon the presence of a PBM [126]. Interestingly, within the same study they found that the high-risk E6 protein from HPV18 (18E6), which also contains a PBM, albeit slightly different from 16E6 (…ETQL* in 16E6 vs. …ETQV* in 18E6), was unable to target NHERF1 for degradation. In our hands, we found: (1) both 16E6 and 18E6 could target NHERF1 for degradation, (2) NHERF1 degradation was not dependent upon the presence of the PBM as 16E6ΔPBM also targeted NHERF1 for degradation, and (3) the low-risk 11E6 (which lacks a PBM entirely) also stimulated the degradation of NHERF1. Further examination revealed not only that 16E6_WT, 16E6ΔPBM, and 11E6_WT

targeted NHERF1 for degradation when expressed at physiologically relevant levels (such that the proteins were not overexpressed and instead were just barely detectable on a western blot), but also that the observed NHERF1 degradation phenotype depended upon the ubiquitin ligase activity of E6AP and could be rescued by cellular treatment with the proteasome inhibitor MG132. Binding of the E6 protein to E6AP was necessary, but not sufficient, for degradation of NHERF1 as E6 proteins from UmPV1 (polar bear), PphPV1 (harbor porpoise), and TtPV5 (bottlenose dolphin) bind E6AP but fail to stimulate NHERF1 degradation. Finally, the E6 proteins that target NHERF1 for degradation cluster phylogenetically, indicating this function has been retained over numerous speciation events.

We screened 16E6 single amino acid point mutants to identify E6 proteins that were unable to degrade NHERF1, but retained the ability to degrade p53, ensuring they were still folded correctly and capable of binding to E6AP. Our screen pinpointed 16E6 residues F69 and K72, the side chains of which are aligned and adjacently located, suggesting a novel interaction domain. Within NHERF1, we employed truncation mutations and found that the EB domain was both necessary and sufficient for E6-mediated degradation of NHERF1. We modeled the trimolecular 16E6-E6AP-NHERF1 complex in a yeast three hybrid system. As expected, the 16E6_F69A/K72A mutant was unable to interact with NHERF1, and NHERF1 deleted of the EB domain failed to interact with the 16E6-E6AP complex. We initially had difficulty replicating

our results in the context of a stable keratinocyte cell line and found that NHERF1 protein levels are actually very tightly regulated by cell confluency. After discovering this and adjusting our approach, we found that 16E6_WT, 16E6ΔPBM, and 11E6_WT all targeted NHERF1 for degradation in our stable cell lines, and that 16E6_F69A/K72A failed to do so. Finally, we established a connection between NHERF1 degradation by E6 proteins, and the augmentation of canonical Wnt/β-catenin signaling, a pro-proliferation pathway.

Independently of our NHERF1 project, we characterized E6AP auxiliary regions required for the E6-E6AP interaction. These findings stemmed from the observation that high-risk 16E6_WT could interact with the isolated E6AP LQELL motif and low-risk 11E6_WT could not, although both E6 proteins bind full-length E6AP. Additionally, previous studies showed that 16E6 binds to E6AP with a higher affinity than to the isolated E6AP LQELL peptide. We hypothesized that the binding of low-risk E6 to E6AP must require other regions within E6AP (auxiliary regions) that mediate what we deem enhanced affinity E6-E6AP binding, outside of the well-established E6-E6AP LQELL peptide interaction. We screened E6 proteins that associate with E6AP and identified two distinct E6-E6AP binding types Type I: E6 proteins that could bind the isolated E6AP LQELL peptide and Type II: E6 proteins that were unable to bind the isolated LQELL. We further subdivided Type II E6 proteins into Type IIa, which could interact with full-length E6AP mutated in the

LQELL region to LQELS, and Type IIb, which could not interact with this E6AP LQELS mutant. No E6AP binding profile was specific to a particular type of E6 protein (e.g. high-risk, low-risk, mucosal, or cutaneous).

To tease out potential E6AP auxiliary regions that enable certain E6 proteins to bind to E6AP, we created a mutant of 16E6 (L50A) that resembled low-risk 11E6 in that it does not interact with isolated E6AP LQELL but retains interaction with full-length E6AP. Through the use of this 16E6_L50A mutant we identified a region in the amino terminus of E6AP between residue 310 and 320 that was necessary for 16E6_L50A binding, as well as required for 16E6 stimulation of E6AP ubiquitin ligase activity and degradation of p53. Not only did 16E6 require E6AP 310-320 to stimulate degradation of both p53 and NHERF1, but it was also required for 11E6_WT degradation of NHERF1. Two other regions in E6AP were identified through the use of 16E6_L50A, one N-terminal to E6AP 310-320 (residues 121-127) and one C-terminal to E6AP LQELL (residues 497-521). We did not try creating an 11E6 mutant that displayed Type I binding instead of Type IIa binding as loss-of-function mutations (such as 16E6_L50A) are easier to identify than gain-of-function mutations. As such, we hypothesize that creating an 11E6 mutant in which a leucine residue was incorporated at the 50 position would not be sufficient to alter 11E6 binding from Type IIa to Type I. There are numerous residues within 16E6 that are important in the interaction of 16E6 with LQELL (see

[32]) and it is doubtful that one mutation to make 11E6 more similar to 16E6 would be sufficient to result in gain-of-function Type I 11E6 binding to E6AP.

We identified a region in the E6AP HECT domain required to facilitate the 11E6-E6AP interaction, which differed from the MmPV1E6-E6AP interaction. Together these E6AP auxiliary regions illuminate as yet undescribed modes of interaction between E6 proteins and E6AP. Finally, we found that the first eight amino acids of 16E6 (and the first nine amino acids of 11E6) are important in mediating the enhanced affinity interaction between E6 and E6AP.

5.2 Mechanisms of NHERF1 Degradation

This is the first description of a cellular target than can be degraded by both high- and low-risk E6 proteins in a manner dependent on E6AP. These findings may have broad implications for the papillomavirus life cycle (explored in section 5.3.2) as well as potential therapeutic implications (section 5.7). However, to truly understand the molecular complexity of the trimolecular complex, a more in-depth analysis is needed into the mechanisms through which NHERF1 is targeted for degradation.

5.2.1 The importance of E6 residues F69 and K72

Our mutagenesis studies identified residues F69 and K72 in the 16E6 linker alpha helix as important for initiating NHERF1 degradation (Fig 3.6). However, we have recently found that while K72A enhances the NHERF1

degradation phenotype in the context of F69A, the 16E6 K72A single point mutant failed to alter degradation levels in keratinocytes (Fig 5.1). As such, we hypothesize that F69 may represent the major residue involved in E6 mediated NHERF1 degradation. Indeed, a multiple sequence alignment (Fig 5.2) of E6 proteins we identified as capable of degrading NHERF1 revealed that the F69 position is always hydrophobic (phenylalanine or leucine), while the K72 residue lacked clearly defined homology.

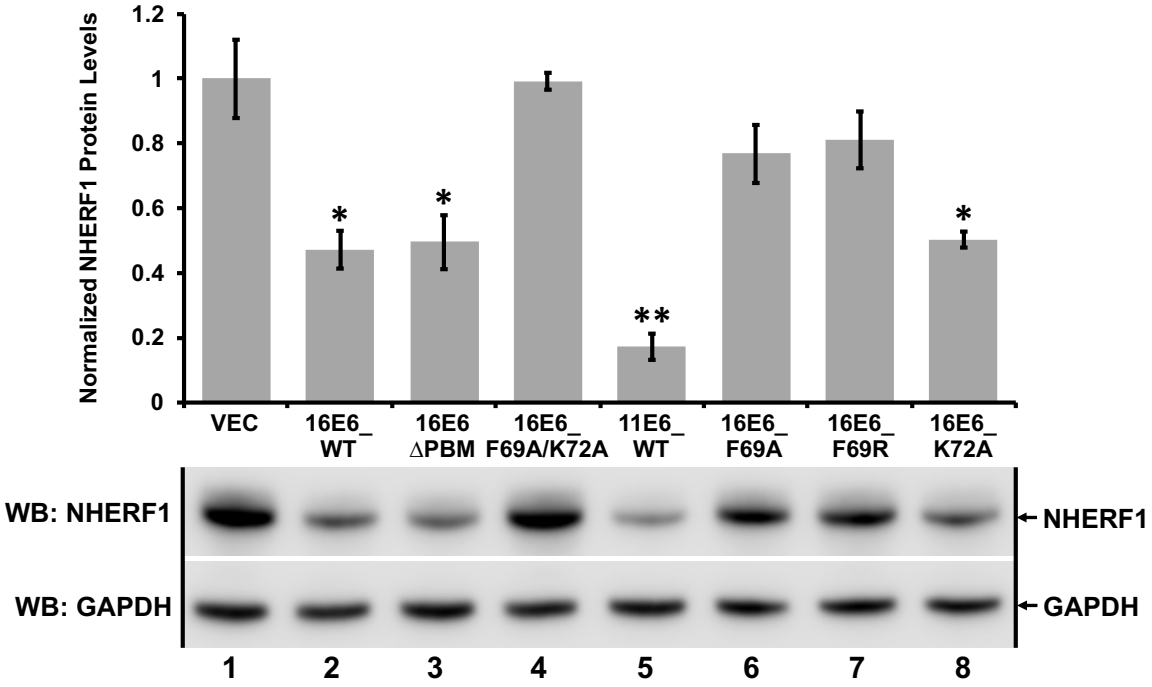


Figure 5.1 16E6_K72A degrades NHERF1 in stable keratinocytes.
Keratinocytes retrovirally transduced with the indicated E6 proteins were seeded at equal confluency and endogenous NHERF1 protein levels were normalized to GAPDH. 16E6_WT, 16E6 deleted of its PBM (ΔPBM), 11E6_WT, and 16E6_K72A all degraded NHERF1. The 16E6_F69A/K72A double mutant and the two 16E6 single mutants F69A and F69R did not target NHERF1 for degradation. The means of triplicate independent experiments ± standard error and one representative blot are shown. N=3, *<0.05, **<0.01, n.s. = no significance by Student's t-test.

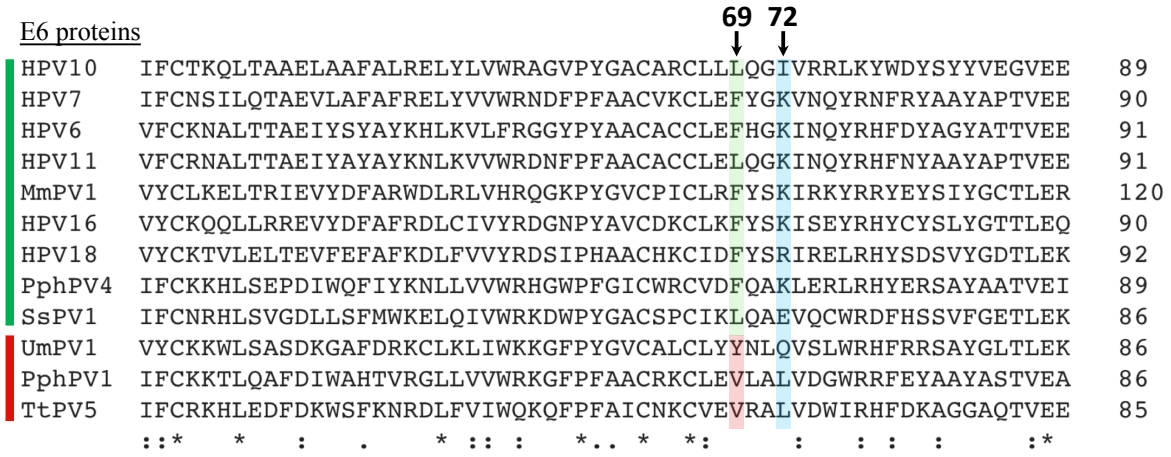


Figure 5.2 16E6 F69 is a conserved, hydrophobic phenylalanine or leucine. We used Clustal Omega to perform a multiple sequence alignment of E6AP-binding E6 proteins examined for ability to degrade NHERF1. Numbers to indicate residue positions use HPV16 E6 as reference. Those E6 proteins that initiate NHERF1 degradation are indicated by a vertical green line next to the papillomavirus names and the three that failed to initiate NHERF1 degradation are indicated by a red vertical line. The E6 proteins that initiated NHERF1 degradation all have a conserved, hydrophobic phenylalanine or leucine at the corresponding F69 position (highlighted in green), while those that did not initiate NHERF1 degradation contain either a smaller, hydrophobic reside (valine) or an amphipathic residue (tyrosine) and are highlighted in red. The K72 position is less well conserved overall (highlighted in blue). Of the nine identified E6 proteins which degrade NHERF1, only six contain a charged lysine. The other three have either a hydrophobic isoleucine or either a charged arginine or glutamic acid. The three E6s which didn't degrade NHERF1 contain either a polar glutamine or a hydrophobic leucine at position 72.

Two non-human papillomaviruses we analyzed were discovered in the same host, the harbor porpoise; PphPV1 and PphPV4. The E6 proteins from these two papillomaviruses can both bind E6AP and stimulate its ubiquitin ligase activity [60], however, we found that only PphPV4 E6 targets NHERF1 for degradation. Comparing the two E6 protein sequences reveal 40.7% shared identity, but they differ from each other at both residues corresponding to 16E6 F69 and K72 (Fig 5.3). The PphPV4 E6 protein has the conserved phenylalanine at residue 69 and a lysine at residue 72 (like 16E6), but PphPV1 E6 instead has a valine at position 69 and a leucine at position 72. We hypothesized that mutation of PphPV1 E6 at these residues such that they instead replicate PphPV4 E6 would result in a gain-of-function for PphPV1 E6, enabling it to target NHERF1 for degradation. We utilized the online RaptorX sequence structure prediction tool to model the potential tertiary structure of PphPV4 E6 and PphPV1 E6, as well as the PphPV1 E6 helix chimera (Fig 5.4). According to overlaying the two structure predictions, replacing the alpha helical region of PphPV1 E6 with that of PphPV4 E6 should not completely disrupt the fold of the protein (Fig 5.5). We created three mutants of PphPV1 E6: V69F, V69F/L72K, and a helix chimera where the entire alpha-helical region of PphPV4 E6 replaced the corresponding alpha-helical region of PphPV1 E6 (this work was performed by UVA undergraduate student, Samuel Case). While none of the three PphPV1 E6 mutants were able to successfully stimulate NHERF1 degradation to the same extent as PphPV4 E6, there was

a reduction in NHERF1 protein levels in the presence of PphPV1 E6_V69F compared to the wild type PphPV1 E6 protein (Fig 5.6; experiments performed by Samuel Case). Because the PphPV1 E6 V69F mutant showed slight change in NHERF1 degradation ability while the other mutants (both of which contain the V69F mutation) did not, further characterization of these PphPV1 E6 mutants is critical in order to demonstrate they are still functional E6 proteins. These preliminary results provide further support that the F69 residue may be more important in mediating the NHERF1 degradation phenotype than the K72 residue. However, lack of complete gain-of-function NHERF1 degradation by the PphPV1 E6_V69F mutant suggests other residues within the E6 protein are also important for mediating NHERF1 degradation.

Our 16E6 mutagenesis screen to identify a mutant that could not target NHERF1 for degradation was extensive but not comprehensive, as we screened around 80 out of a possible 151 residues in 16E6 (Table 3.1). We chose these particular 80 residues to examine, as they were at least 20% exposed as determined by the Swiss PDB Viewer. It would be both interesting and informative to collaborate with a strong bioinformatics laboratory and perform a thorough analysis of E6AP-binding E6 proteins in the context of their ability to degrade NHERF1. We anticipate that a more thorough examination of their sequences may uncover additional residues within the E6 protein that are important in mediating its ability to degrade NHERF1. Finally, screening an E6 random mutagenesis library (which incorporates both single and multiple

mutations into the E6 protein) using a Y3H model would result in an unbiased and thorough approach enabling us to identifying other key residues in 16E6.

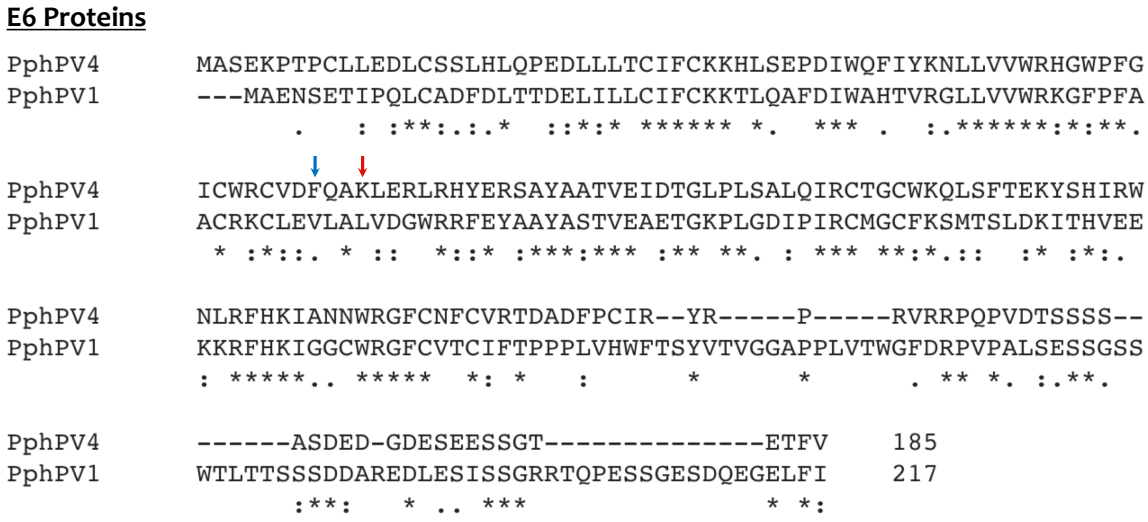


Figure 5.3 PphPV4 and PphPV1 E6 protein sequences are dissimilar.
We used Clustal Omega to perform a multiple sequence alignment of the E6 proteins from the two different papillomaviruses that we examined that both infect the Harbor porpoise. The multiple sequence alignment indicates numerous non-conserved amino acids between the two E6 proteins, including the F69 (blue arrow) and K72 (red arrow); numbering with HPV16 E6 as a reference.

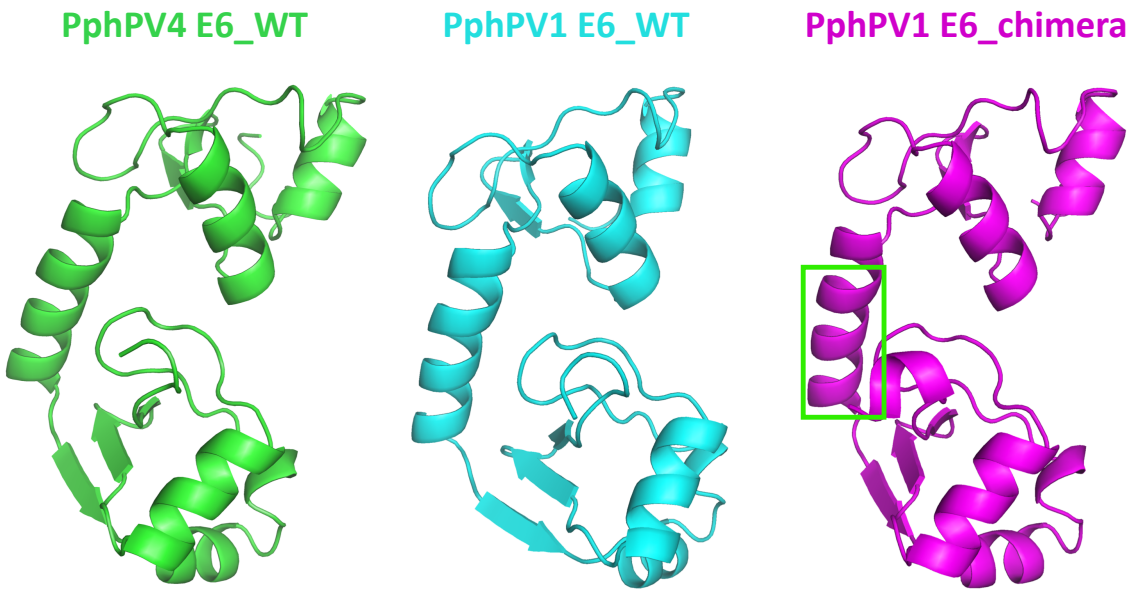


Figure 5.4 The predicted tertiary structure of PphPV4 E6, PphPV1 E6, and our proposed PphPV1 E6 chimera.
We input the sequences of PphPV4 E6, PphPV1 E6 and the PphPV1 E6 chimera into RaptorX, a structure prediction tool. Both PphPV1 and PphPV4 E6 proteins have an extremely long unstructured carboxy tail. For the purposes of clarity, we hid the carboxy tail from our view in PyMol to acquire this figure. The green box around the alpha helical linker in the PphPV1 E6 chimera indicates where we replaced the PphPV1 E6 sequence with that of PphPV4. The sequence change is as follows: EVLALVDGWRR to DFQAKLERLRH. These structures (although modeled on known protein structures) are just predictions.

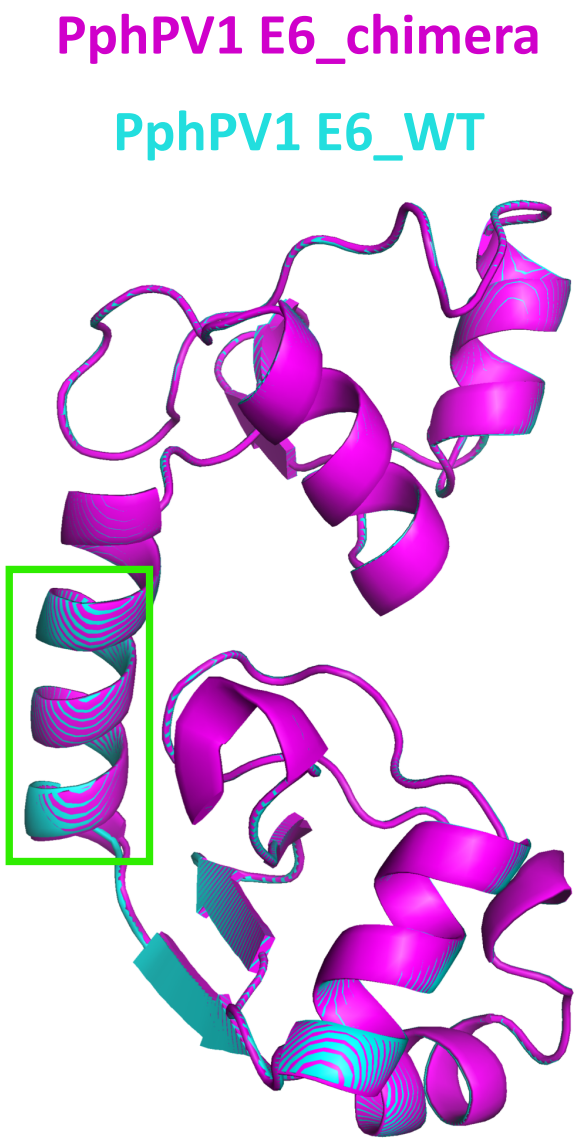


Figure 5.5 PphPV1 E6 and PphPV1 E6 chimera predicted structures are very similar.
In PyMol, we superimposed the predicted tertiary structures of PphPV1 E6 (light blue) and the PphPV1 E6 chimera (purple) as depicted in Fig 5.4. Overall, the two structures are similar, suggesting the replaced PphPV1 E6 alpha-helical sequence (green box) should not completely disrupt the folding of the E6 chimera protein. As these are just predicted tertiary structures from RaptorX, testing must be performed to insure retained protein functionality.

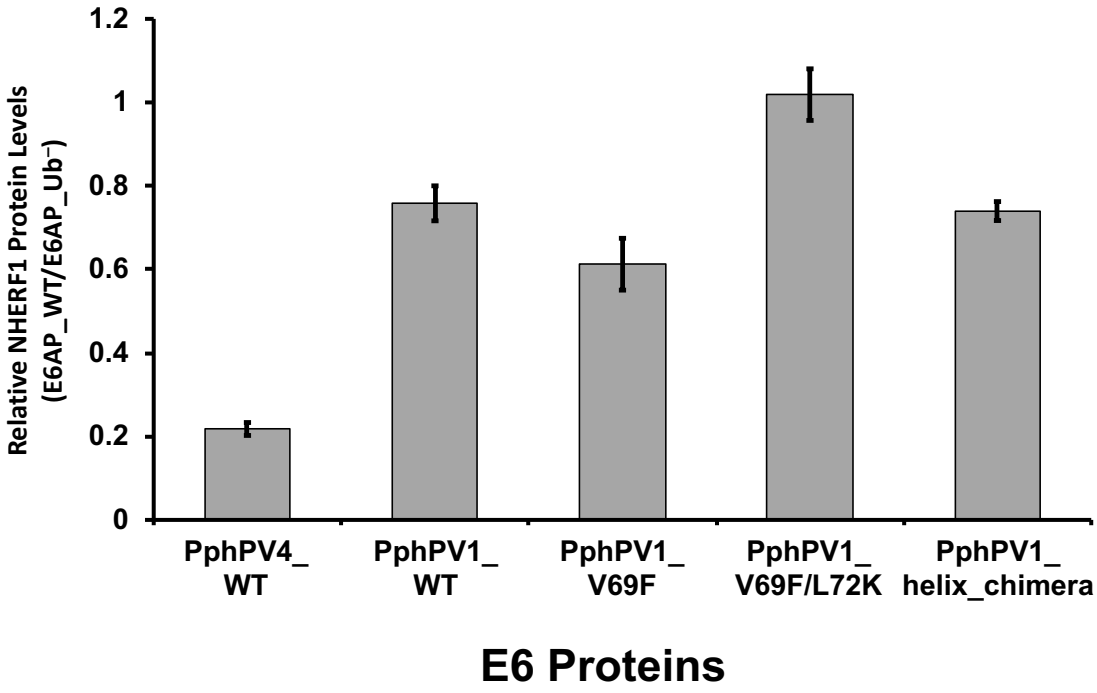


Figure 5.6 PphPV1 E6_V69F displays slight gain-of-function NHERF1 degradation.

Plasmids encoding HA_NHERF1 (0.5 ug), either E6AP_Ub$^-$ or E6AP_WT (0.5 ug), HA_GFP (0.1 ug), or the indicated E6 protein (0.5 ug) were transiently transfected into E6AP-null 8B9 cells and HA-NHERF1 expression analyzed by western blot. HA-NHERF1 expression was normalized to HA-GFP to account for transfection efficiency. We further normalized within each E6 protein such that each bar represents the amount of HA-NHERF1 present when the indicated E6 protein was co-expressed with E6AP_WT/co-expression with E6AP_Ub$^-$. The numbering regarding each E6 protein is used with HPV16 E6 as a reference. N = 3. Experiments performed by Samuel Case.

5.2.2 The role of the NHERF1 EB Domain

Within NHERF1, we pinpointed the EB domain as both necessary and sufficient for degradation by 16E6. The EB domain is a well characterized motif responsible for NHERF1 binding to ezrin-radixin-moesin, enabling linkage of NHERF1 to the cytoskeleton matrix (and any proteins to which NHERF1 is bound) [112]. The EB domain is well conserved among NHERF1 orthologues [84], indicating its evolutionary importance. We hypothesize that there exist several key residues within the EB domain that facilitate E6 mediated degradation of NHERF1.

Our overall hypothesis is that any E6 protein that can bind E6AP and stimulate its ubiquitin ligase activity (as measured by their ability to stimulate E6AP degradation in an over expression assay, see [60]) should be able to target NHERF1 for degradation. However, in our study three tested E6 proteins that bind E6AP failed to target NHERF1 for degradation: UmPV1 E6 (*U. maritimus;* polar bear), PphPV1 E6 (*P. phocoena;* porpoise), and TtPV5 E6 (*T. truncatus;* bottlenose dolphin). One caveat of these findings is that we only examined degradation of human NHERF1 in the presence of human E6AP, instead of the polar bear, porpoise, or bottlenose dolphin orthologues. Of these three animals, the only described NHERF1 sequence to date is for *U. maritimus* (polar bear). Upon examination of the EB domain of polar bear NHERF1 (examination of E6AP ortholog relatedness in Fig 5.14), we found five differing amino acids (Fig 5.7A) as compared to human NHERF1. Therefore,

we hypothesize that mutation of one, all, or a combination of these residues in human NHERF1 to replicate polar bear NHERF1 would enable UmPV1 E6 to degrade the resulting human-polar bear NHERF1 chimera.

In addition, E6 proteins from papillomaviruses discovered in both boar (*S. scrofa*) and rhesus monkeys (*M. mulata*) are able to degrade human NHERF1. Both rhesus monkey and boar NHERF1 sequences have been described [84]. Using these sequences we performed a multiple sequence alignment between human, polar bear, boar, and rhesus monkey NHERF1 EB domains. We proceeded to narrow our focus to the five residues that differ between the human and polar bear NHERF1 EB domain. Next, we reasoned that if these five residues were not completely conserved in human, boar, and rhesus monkey, they would not be important in enabling degradation of NHERF1 by E6 proteins. Through this analysis we found that two polar bear residues within the EB domain of NHERF1 were not conserved among the corresponding boar, human, and rhesus monkey proteins, residues I325 and S340 (numbering is in reference to human NHERF1; Fig 5.7B). We hypothesize that these two residues may be responsible for the lack of human NHERF1 degradation by polar bear E6. We propose to create a human-polar bear NHERF1 chimera that: (i) has all five residues mutated to resemble the polar bear EB domain, and that (ii) has only residue I325 and S340 mutated to represent polar bear EB domain. If our hypothesis is correct, we predict that polar bear E6 will be able to degrade both of these chimeric NHERF1 proteins.

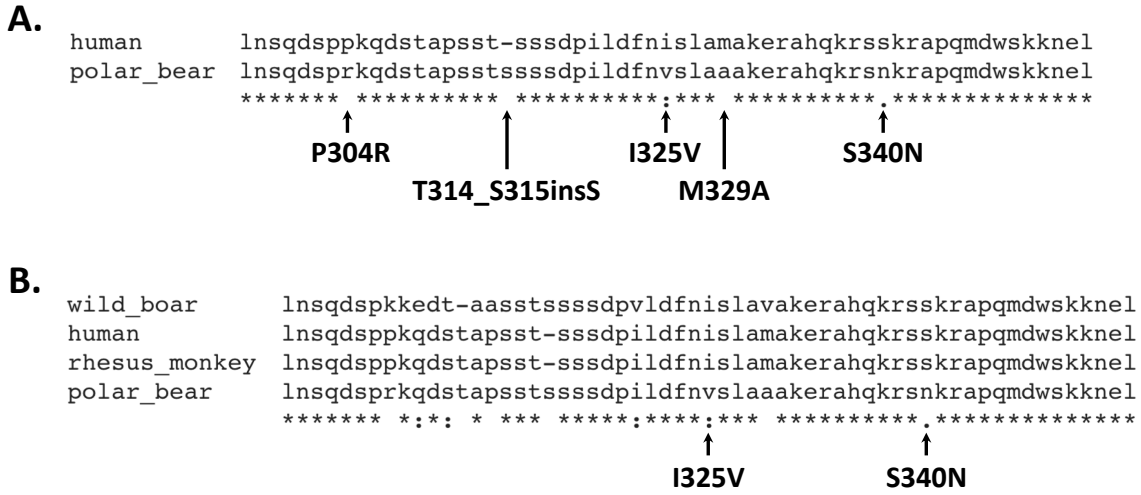


Figure 5.7 Differences between human and polar bear NHERF1 EB domains.

(**A**) Five residues differ between human and polar bear NHERF1 EB domains. A multiple sequence alignment using Clustal Omega was performed with the EB sequences of human (*H. sapiens*) and polar bear (*U. maritimus*) NHERF1 proteins. The five residues indicated differ between the two orthologous proteins. Residue numbering is in reference to human NHERF1. (**B**) Two residues in polar bear NHERF1 EB differ from conserved residues in other NHERF1 EB proteins. A multiple sequence alignment using Clustal Omega was performed with the EB sequences of wild boar (*S. scrofa*), human (*H. sapiens*), rhesus monkey (*M. mulata*), and polar bear (*U. maritimus*) NHERF1 proteins. Because E6 proteins from papillomaviruses that infect wild boars (SsPV1), humans (HPV), and rhesus monkeys (MmPV1) can all degrade human NHERF1, we reasoned that any differences in the polar bear NHERF1 EB domain that are conserved in the other three species may indicate the most important residues in the NHERF1 EB domain that are important for E6-mediated degradation. The two residues that fit this criterion are indicated. Residue numbering is in reference to human NHERF1.

The identification of a single (or multiple) amino acid(s) within the EB domain of NHERF1 that is required for E6-mediated degradation would enable us to construct a full-length NHERF1 protein that is resistant to degradation by E6. Using NHERF1 truncations has been very informative in highlighting the EB domain as both necessary and sufficient for degradation by E6. However, NHERF1 truncations do not retain all of the functions of full-length NHERF1 (e.g. NHERF1ΔEB cannot bind ERM proteins). If the residues in the EB domain identified using polar bear NHERF1 are required for E6 degradation, we could construct an NHERF1 protein that is resistant to degradation by E6 proteins. It is possible that certain NHERF1 functions may be disrupted in the creation of an NHERF1 mutant that is resistant to degradation by E6, especially if E6 relies on highly conserved residues within the NHERF1 EB domain. As the NHERF1 EB domain is extremely important for mediating NHERF1 interaction with ERM proteins, a key component in proper NHERF1 localization in cells, we would confirm proper cellular localization of our identified NHERF1 mutant.

An NHERF1 construct that was resistant to degradation by E6 but that retained other, normal NHERF1 activities would be extremely informative in answering numerous questions we have regarding the role of NHERF1 in the papillomavirus life cycle (see section 5.3.2) as well as why E6 proteins target NHERF1 for degradation (generally see section 5.3). If our analysis of the polar bear NHERF1 EB residues do not determine important amino acids, we will

employ random mutagenesis and gap repair in yeast of the NHERF1 EB domain. These methods will results in an unbiased approach to identify residues within the NHERF1 EB domain that are required for E6-mediated degradation.

Because PphPV4 E6 was able to stimulate the degradation of human NHERF1, we reasoned that PphPV1 E6 should also be able to target human NHERF1 for degradation, if that was a function the two proteins share. However, these two porpoise-specific E6 proteins share only 78% sequence identity, and only PphPV4 was able to degrade human NHERF1. We hypothesize that some of these sequence differences may result in greater specificity of the PphPV1 E6 protein for porpoise NHERF1 whereas the sequence of PphPV4 E6 is less stringent for porpoise-specific NHERF1 and could target human NHERF1 for degradation. Indeed, closely-related types of human papillomaviruses can show distinct pathologies (explored in [151, 152]). The most direct experiment to test our hypothesis is through the use of a porpoise-NHERF1 plasmid. We have been unsuccessful in our search to identify such a clone, and the NCBI does not contain a described *P. phoceona* NHERF1 sequence. Therefore, to obtain both the sequence and the plasmid, RT-PCR of a blood or tissue sample from a porpoise would be required. If we find that the E6 proteins from PphPV1, UmPV1, and TtPV5 do not target NHERF1 for degradation, even after testing NHERF1 from their respective species, this would indicate that these E6 proteins have diverged from other

E6AP binding E6 proteins. It may be that evolutionary pressures resulted in these E6 proteins deregulating cellular growth and differentiation mechanisms through other means, independent of degrading NHERF1. It would be of interest to determine whether any of these E6 proteins (which bind E6AP but fail to degrade NHERF1) are capable of stimulating the canonical Wnt pathway even though they do not degrade NHERF1. If they do augment this important cellular regulatory pathway despite their lack of ability to degrade NHERF1, these E6 proteins must have evolved a different method of deregulating the canonical Wnt pathway. Of course, given that the E6 proteins from UmPV1, TtPV5, and PphPV1 infect non-human cells, testing their ability to augment Wnt signaling in a human cell line may not produce viable results and would need to be carefully controlled.

5.2.3 The physical NHERF1-E6 interaction

Although we were able to model the trimolecular E6-E6AP-NHERF1 complex in yeast, we were unable to recapitulate those results with immunoprecipitation using mammalian cell lysates without the use of crosslinking. Y3H assays are extremely sensitive reporter assays that do not necessarily rely solely on transfected proteins; any yeast proteins present may play a role in formation of the presumed trimolecular complex of E6, E6AP, and NHERF1. Our inability to immunoprecipitate 16E6, E6AP, and NHERF1 suggests that there may exist an as-yet unidentified "Protein X" that is important for mediating the physical interaction of E6-E6AP and NHERF1.

We propose to utilize a tandem affinity purification (TAP) assay comparing 16E6 WT to the 16E6_F69A/K72A to provide an unbiased and thorough examination for proteins that can interact with 16E6_WT but not the double mutant. With this approach, we can determine whether there is a potential "Protein X" that binds to 16E6_WT but fails to bind to 16E6_F69A/K72A.

5.3 Implications for NHERF1 degradation

We identified eight different E6 proteins representing three different genera of papillomaviruses that degrade NHERF1. This conservation of NHERF1 as a degradation target across numerous speciation events implies this is an important function of E6 proteins. Our initial studies indicated that degradation of NHERF1 by E6 proteins augments the canonical Wnt/β-catenin pathway (Fig 3.11), a pathway that is important in regulation of cellular proliferation and is often activated in cancer (reviewed in [87, 88, 92, 93]). However, as a scaffold protein, NHERF1 has been implicated in numerous key cellular growth-regulating pathways. Our current data has explored but one possible outcome of NHERF1 degradation by E6 proteins. Additionally, many questions remain unanswered regarding the role of NHERF1 in the papillomavirus life cycle.

5.3.1 HPV episomal maintenance

Studies of HPV genomes transfected into either primary or immortalized keratinocyte cell lines have demonstrated the importance of the viral E6 and

E7 oncoproteins. Loss of expression of E6 or E7 results in loss of viral episome maintenance in both high-risk and low-risk HPV-infected keratinocytes [39, 40]. High-risk HPV genomes that contain point mutations in the E6 gene that result in disruption of p53 degradation, E6AP binding, or E6 folding result in loss of episomal maintenance [40, 41]. These data indicate a functional E6 is necessary for episomal maintenance in infected cells. High-risk E6 proteins that are unable to interact with E6AP would be unable to degrade both p53 and NHERF1. E6 mutants that are selectively defective for targeting NHERF1 for degradation (such as our identified 16E6_F69A/K72A double mutant) have not yet been examined. Because NHERF1 degradation is conserved by E6 proteins across numerous genera and species, it may serve a previously undescribed role in maintaining the viral episome. To test this possible result of NHERF1 degradation, we will incorporate our 16E6_F69A/K72A mutant into the full HPV16 genome. This 16E6_F69A/K72A containing genome will still be able to bind cellular E6AP and target p53 for degradation, but not NHERF1. Therefore, we will be looking at whether NHERF1 degradation is required for HPV16 genome maintenance. If we successfully identify full-length NHERF1 that is resistant to degradation by E6, we can also address the necessity of NHERF1 degradation by E6 for genome maintenance from the perspective of NHERF1. We would first knock down NHERF1 in keratinocytes using shRNA (Fig 5.8) and then retrovirally transduce our shRNA-containing cells with the NHERF1 E6 degradation-resistant mutant. These NHERF1

escape mutant expressing keratinocytes would be transfected with the wild type HPV16 genome and genome maintenance ability observed. With this approach, we could be certain 16E6 retained all functions other than ability to degrade NHERF1, since we would be observing 16E6_WT in the context of HPV16 genome.

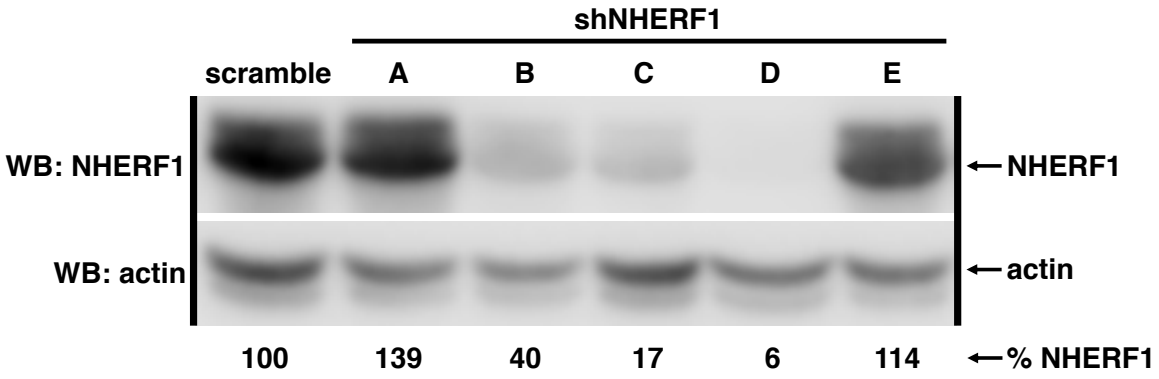


Figure 5.8 NHERF1 can be knocked down in human keratinocytes.
Keratinocytes retrovirally transduced with the indicated shRNAs were seeded at equal confluency. Protein levels were normalized and NHERF1 protein levels resolved via western blot. NHERF1 protein levels were normalized to actin to account for variation in protein loading. Percent NHERF1 (% NHERF1) is shown below each lane with the scramble RNA lane set to 100%. Of the shRNAs we observed, "D" had the best NHERF1 knockdown.

5.3.2 The papillomavirus life cycle

Papillomaviruses infect the basal layer of epithelia and replicate their genomes using cellular machinery. The production of infectious virions depends upon the differentiation of the infected epithelia (reviewed in [152]). Our discovery of NHERF1 as a shared target among the E6 proteins found in mucosal high and low-risk papillomaviruses, as well as papillomaviruses that prefer to infect cutaneous epithelia, suggests that NHERF1 degradation may be intrinsically important to the viral life cycle regardless of any impact on maintenance of the viral genome. As such, further studies of NHERF1 in the context of the full virus life cycle are necessary for a better understanding of why these papillomavirus E6 proteins may target the degradation of NHERF1.

NHERF1 mainly localizes to the apical plasma membrane in human epithelia and is important for proper epithelial morphogenesis [153]. While the functions of NHERF1 are varied due to its role as a protein scaffold, numerous studies have indicated it regulates cell growth and differentiation, which are key cellular functions papillomaviruses disrupt during viral infection. Determining whether NHERF1 is necessary for the viral life cycle and production of infections virions requires the use of organotypic raft cultures, which recapitulate skin formation and are conducive to the entire HPV life cycle [116]. Raft cultures with keratinocytes transfected with the wild type HPV16 genome and raft cultures of keratinocytes with the HPV16 16E6_F69A/K72A genome would be sectioned and compared. If we find that

the HPV16 16E6_F69A/K72A genome is not episomally maintained, this experiment could be performed using the HPV16 genome in the context of regular keratinocytes, or those expressing the NHERF1 E6 degradation escape mutant. It would also be of interest to examine the importance of NHERF1 degradation in the expansion of the spinous layer, as E6 has been implicated in formation of epithelial hyperplasia [154], and NHERF1 is involved in cell polarity and growth regulation [155].

5.3.3 NHERF1 and properties of E6-containing keratinocytes

Recently, our laboratory has been working on characterizing a novel protocol to study the ability of keratinocytes expressing different proteins of interest to compete with each other in mixed cultures. In brief, this cell competition assay enables us to quantitively measure a trait critical to papillomaviruses: the ability of infected cells to compete with normal keratinocytes. A papillomavirus infected cell must be able to outcompete neighboring uninfected cells in order to establish an infection and expand into a papilloma. We will use this assay, which has been extensively optimized by Nicole Brimer, to investigate the necessity of E6-mediated NHERF1 degradation for 16E6_F69A/K72A-transduced cells to outcompete normal keratinocytes, keratinocytes containing 16E6_WT, 16E6ΔPBM, or keratinocytes containing other mutants of 16E6 (such as one that cannot stimulate p53 for degradation). Using this approach, we will gain a much better understanding of exactly why E6 proteins target NHERF1 for

degradation. We hypothesize that because E6-mediated degradation of NHERF1 results in the augmentation of canonical Wnt/β-catenin signaling, keratinocytes expressing a form of E6 that can successfully degrade NHERF1 will have increased proliferation and therefore will outcompete cells expressing 16E6_F69A/K72A. It would also be informative to compare keratinocytes that express the proposed NHERF1 E6 degradation resistant mutant compared to normal keratinocytes, each transduced with 16E6_WT.

Expression of the E6 and E7 oncoproteins is maintained in HPV-induced cancers. Cancer cell lines from HPV-induced tumors in which E6 expression is repressed by means of RNA interference result in growth arrest followed by senescence or apoptosis [144, 156, 157]. Additionally, primary keratinocytes retrovirally transduced with E6 and E7 become transformed and can grow indefinitely [158, 159]. Using our 16E6_F69A/K72A mutant, we will establish the role (if any) of NHERF1 degradation by E6 in the ability of the two oncogenes to immortalize cells and whether NHERF1 degradation contributes to the addiction of HPV-positive cancers to E6 protein expression.

5.3.4 NHERF1 and E-cadherin

We have discovered that NHERF1 protein levels are dependent upon keratinocyte confluency (Fig 3.3). As cell confluency increases, so does the concentration of NHERF1 protein. The keratinocytes utilized in our experiment, called normal immortalized keratinocytes (NIKS), are not transformed and have a near diploid karyotype [115]. Because NHERF1 has

been implicated in adherens junction formation via its interaction with β-catenin [96], we reasoned that NHERF1 protein levels in transformed epithelial cells (having lost adherens junction formation) would not depend on cell confluency. We examined whether NHERF1 protein levels were altered in HPV negative cervical cancer cells (C33A) that were seeded at different confluency and found that regardless of cell density, NHERF1 protein expression remained constant (Fig 5.9). E-cadherin is a major component of adherens junctions [160, 161] and its expression is silenced in C33A cells and is not detectable by western blot [162].

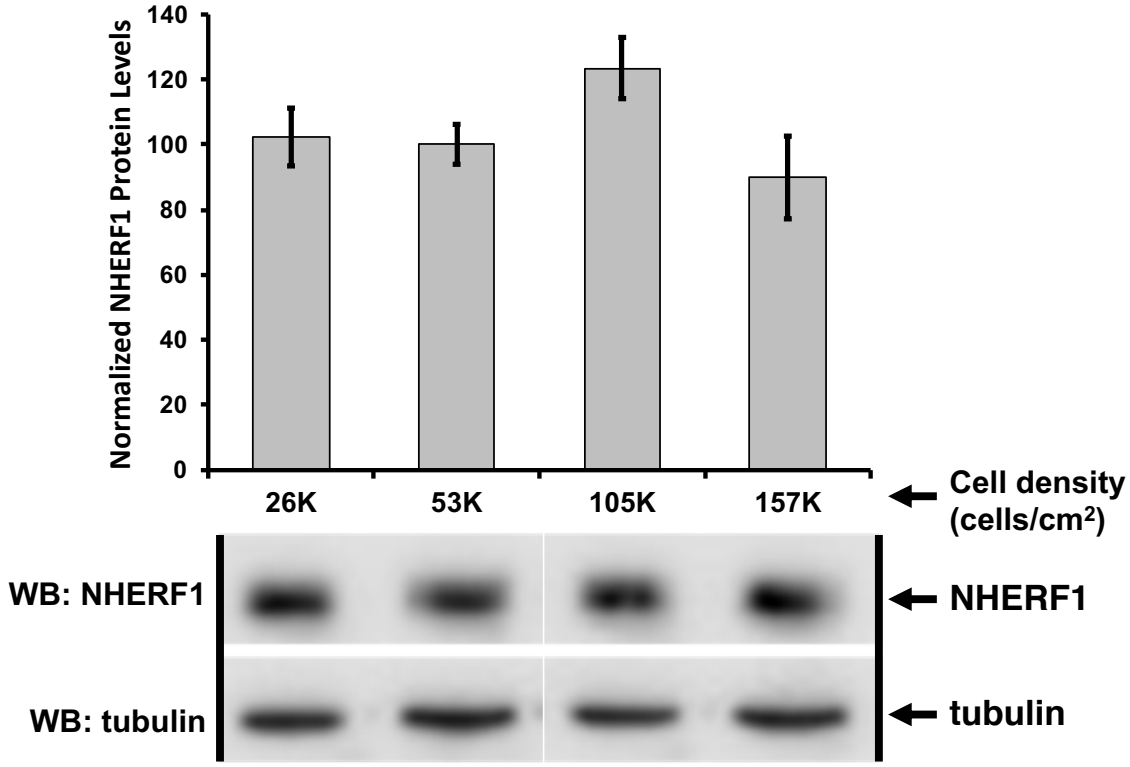


Figure 5.9 NHERF1 is not dependent on cell confluency in HPV-negative C33A cervical cancer cells.

C33A cells were counted and seeded at the indicated cell density. Eighteen hours later, cells were lysed and normalized for total protein. NHERF1 protein levels were determined via western blot and normalized to the loading control tubulin. The NHERF1 protein level in the least confluent (cell density 26K) sample were set to 100%. The vertical white line in the western blots indicates removal of an irrelevant sample. N=3. Experiments performed by Samuel Case.

E-cadherin is an epithelial-specific cell-cell adhesion molecule that is involved not only in adherens junctions, but also in regulating cell migration and mediating intracellular signaling (reviewed in [163]). Down regulation of E-cadherin at adherens junctions is a hallmark of epithelial-mesenchymal transition (reviewed in [164]). Indeed, overexpression of E-cadherin results in a reduction of sarcoma cell anchorage independent growth [165, 166]. A study linking NHERF1 to both anchorage-independent growth and adherens junctions used NHERF1 knockout mouse embryonic fibroblasts to discover that NHERF1 is required to maintain the localization of E-cadherin at adherens junctions through stabilization of the E-cadherin/β-catenin complex [96]. Together, these studies link E-cadherin, adherens junctions, EMT, and NHERF1 (Fig 5.10).

Human papillomaviruses have been shown not only to repress E-cadherin, but also to promote EMT in infected cells [167]. EMT *in vivo* was shown to be correlated with the ability of cells to grow anchorage independent growth (AIG) colonies *in vitro* [168]. As such, AIG serves as a correlated measurement for EMT potential. We (specifically Nicole Brimer) recently completed an AIG assay that indicates the ability of 16E6 to form AIG colonies depends on its ability to degrade NHERF1 (Fig 5.11). Previous work has implicated the E6 PBM as required for AIG, although this study was completed in primary human tonsil epithelial cells with co-expression of both 16E7 and 16E6 [169]. Our results indicate that expression of either 16E6_WT or 16E6ΔPBM in

adherent murine embryo fibroblast (NIH/3T3) cells resulted in the formation of AIG colonies (Fig 5.11). However, NIH/3T3 cells retrovirally transduced with 16E6_F69A/K72A failed to form AIG colonies and resembled vector transduced cells in appearance, indicating E6 degradation of NHERF1 may be important for AIG colony formation. Although, our preliminary data does not exclude the possibility that the 16E6_F69A/K72A expressing NIH/3T3 cells grow more slowly than cells expressing either 16E6_WT or 16E6ΔPBM, which could contribute to the lack of AIG colonies seen with 16E6_F69A/K72A cells. To exclude this possibility, we will perform multiple cell growth assays as well as repeat the AIG assay for an extended period of time. Currently, our data agree with previous findings that NHERF1 is required to suppress AIG in NHERF1-null mouse embryonic fibroblasts [96]. MDCK cells stably expressing 16E6 induced morphological changes: their originally cobblestone-shaped appearance became more spindle-shaped and mesenchyme like [170]. Additionally, cells expressing HPV16E6 have decreased surface and total protein levels of E-cadherin due to repression of the E-cadherin promoter, resulting in fewer E-cadherin transcripts [171]. Therefore, we hypothesize that degradation of NHERF1 by E6 proteins may contribute to formation of AIG colonies and this may occur via repression of E-cadherin (Fig 5.10).

Non-disrupted E-cadherin activity results in maintenance of normal cell differentiation and proliferation [172, 173]. The action of the HPV E6 protein during infection represses E-cadherin, thereby enabling cellular

hyperproliferation and dedifferentiation [171]. The precise mechanism for HPV E6's regulation of E-cadherin remains unclear. As NHERF1 is a positive regulator of E-cadherin activity [96], we propose that the targeted degradation of NHERF1 by E6 proteins contributes to E-cadherin regulation by HPV. The accumulation of our work, and others', has led us to a model for the role of E6 degradation of NHERF1 in promoting cellular proliferation and inhibiting cellular differentiation (Fig 5.10).

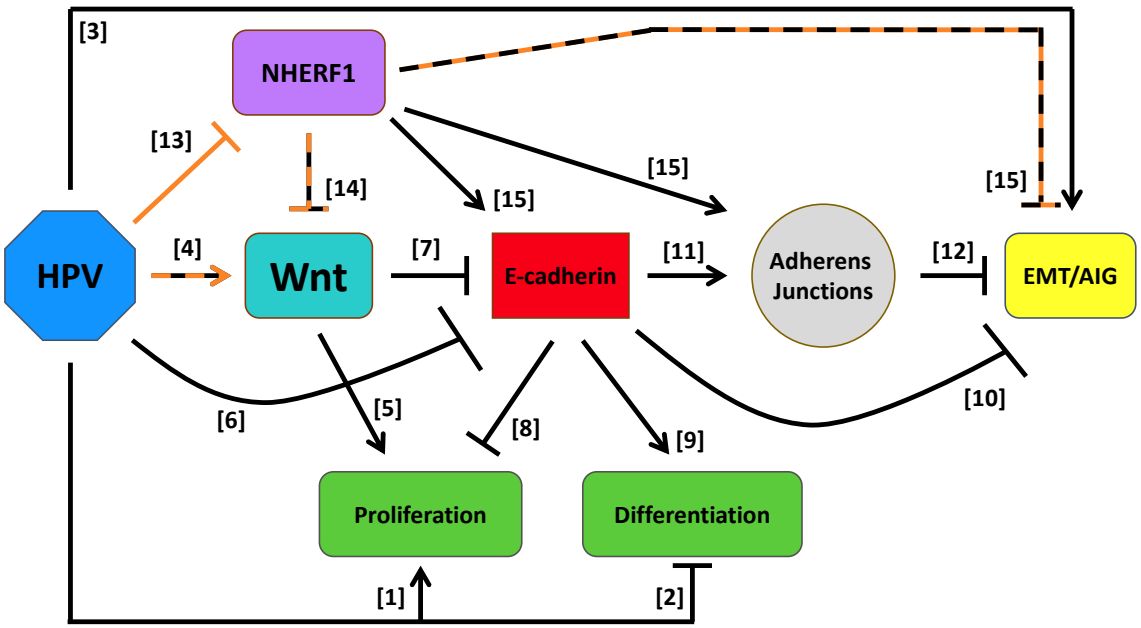


Figure 5.10 Diagram of our hypotheses regarding how HPV E6-mediated degradation of NHERF1 fits into the current literature.
Previous studies established a role for HPV in numerous cellular regulatory mechanisms some of which include: augmenting Wnt signaling, repressing E-cadherin, stimulating cellular proliferation, blocking cellular differentiation, and inducing an epithelial-mesenchymal transition phenotype (EMT). Our finding that HPV E6 targets NHERF1 for degradation suggests that NHERF1 may be one mechanism by which HPV regulates some of these downstream cellular pathways. The data presented in this thesis is indicated by orange lines. Any lines that are dotted orange and black indicate data presented in this thesis that has also been indicated in the literature. Numbers in brackets refer to the following references: 1: reviewed in [23], 2: reviewed in [24], 3: [167, 170], 4: [101-103, 132, 133], 5: reviewed in [174, 175], 6: [171], 7: [176, 177], 8: [172], 9: [173], 10: [165, 166], 11: [160, 161], 12: reviewed in [163], 13: [143], 14: [95], 15: [96].

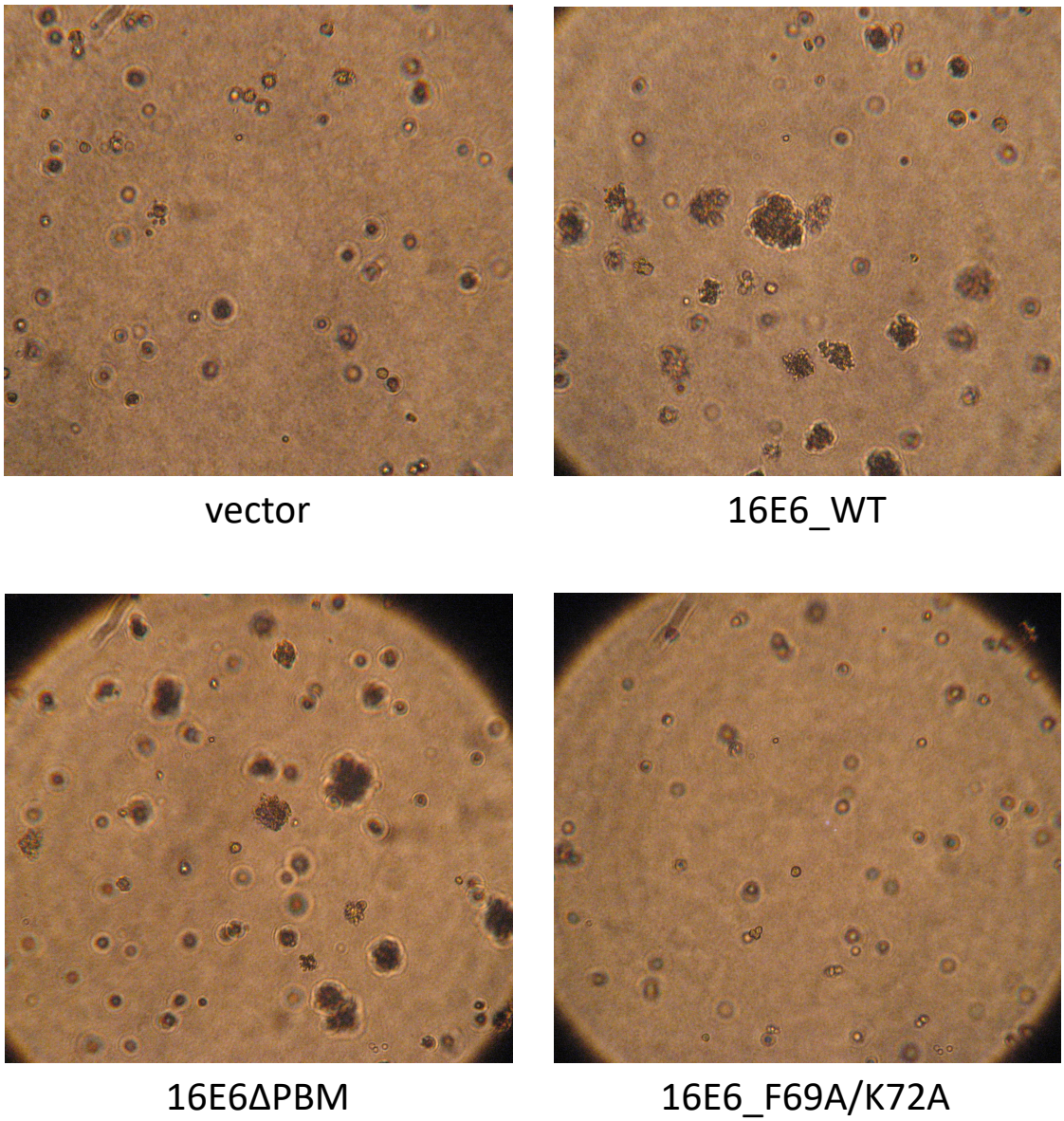


Figure 5.11 16E6_F69A/K72A does not form anchorage independent growth colonies.
NIH/3T3 cells retrovirally transduced with vector, 16E6_WT, 16E6ΔPBM, or 16E6_F69A/K72A were seeded in 0.3% agar media on a 0.5% agar media plug. Colonies were imaged after seven days. N = 1. Experiment performed by Nicole Brimer.

5.3.5 HPV and the canonical Wnt signaling pathway

The Wnt pathway represents a complex and heavily studied cellular signaling pathway, and has been the focus of many recent reviews [87-93]. To date there have been three characterized Wnt signaling pathways, though for the purposes of this thesis we will focus on the canonical Wnt/β-catenin signaling pathway as it relates to HPV and NHERF1. Though HPV E6 has been implicated in Wnt signaling in the past [101-103, 132, 133], to date we are the first to describe the augmentation of Wnt signaling as a function of NHERF1 degradation by both high and low-risk HPV E6 proteins.

We have performed preliminary studies regarding the increase in the canonical Wnt pathway signaling with 16E6 and 11E6. To determine whether the ability of non-human E6 proteins to degrade NHERF1 augments canonical Wnt signaling, we wish to expand these preliminary studies. We utilized the TCF/LEF luciferase reporter assay to specifically measure activation of the canonical Wnt/β-catenin signaling in a transient transfection system. Ideally, repeating these experiments in the context of keratinocyte cell lines with stable expression of the TCF/LEF luciferase reporter and stable expression of various E6 proteins would provide the most physiologically relevant results. However, due to the extreme sensitivity of NHERF1 protein levels on keratinocyte confluency, this would be very technically challenging. Keratinocytes expressing different E6 proteins do not grow at the same rate, nor do they sit down similarly when counted and plated.

Canonical Wnt signaling initiates the transcription of numerous pro-proliferative genes (reviewed in [174, 175]). Therefore, analysis of cellular proliferation in keratinocytes retrovirally transduced with 16E6_WT or 16E6_F69A/K72A may provide further evidence supporting activation of the canonical Wnt pathway. Immunofluorescent staining or western blotting for proteins that are often increased due to the activity of the Wnt/β-catenin pathway (such as c-Myc [178], Slug [179], or Snail [177]) would also be useful in further exploration of the role of NHERF1 degradation by E6 on the activation of Wnt signaling. However, this approach has the pitfall of providing potentially misleading results, as proteins whose expression levels are impacted by the canonical Wnt pathway can also be impacted via other pathways and so may not be a direct result of Wnt signaling. Therefore, suitable controls would be important to enable proper interpretation of the results.

Interestingly, the Wnt/β-catenin pathway can promote EMT via the activation of transcription factors Slug and Snail, which have been shown to repress the E-cadherin promoter [176, 177]. Because 16E6 has been shown to reduce transcripts of E-cadherin in infected cells due to repression of the E-cadherin promoter [171], we hypothesize that degradation of NHERF1 by E6 proteins not only activates the canonical Wnt pathway, but in doing so it also results in repression of the E-cadherin promoter (Fig 5.10).

5.3.6 Regulation of NHE3 by NHERF1

It is now widely accepted that as a scaffold NHERF1 interacts with numerous different proteins, thereby regulating various cellular pathways. However, NHERF1 was originally identified in rabbit kidney epithelia as co-regulator of an Na^+/H^+ exchanger (NHE) [180]. This originally identified function of NHERF1 is its namesake: Na^+/H^+ Exchanger Regulatory Factor 1. There are nine isoforms of integral membrane NHE proteins [181], and NHERF1 was shown to interact specifically with NHE3 [182], which is reflective of their gene names. NHE3 is encoded by the gene SLC9A3 (**Sol**ute **C**arrier Family **9** Member **A3**), whereas NHERF1 is encoded by the gene SLC9A3R1 (SLC9A3 **R**egulator **1**). NHE3 plays a central role in polarized epithelia through the reabsorption of Na^+ and $H(CO3)^-$ along the cellular apical membrane. NHE3 is mainly found in polarized epithelial of the kidney, gallbladder, and gastrointestinal tract [183]. In the epithelia of the skin, there exists only very low levels of detectable NHE3 RNA while the detectible levels of NHERF1 in the skin are much higher (almost four fold) [84]. Therefore, it is likely that the predominant role(s) of NHERF1 in skin epithelia is related more to its function as a scaffold protein and its ability to link itself and proteins to which it is bound to the actin cytoskeleton. Nevertheless, it is possible that the role of NHERF1 as a regulator of NHE3 may be important for papillomaviruses and could therefore provide further insight as to why the degradation of NHERF1 is highly conserved among E6 proteins.

NHERF1 is required for protein kinase A (PKA)-mediated inhibition of NHE3 [180]. Therefore, degradation of NHERF1 by E6 proteins would result in a constitutively active form of NHE3, resulting in an exchange of intracellular protons and extracellular sodium ions. This action would result in an alkaline intracellular pH, which is important for a variety of mechanisms involved in cellular proliferation (reviewed in [184]). Phosphofructokinase, the rate limiting step in glycolysis, functions optimally in an alkaline environment [185] and therefore E6 degradation of NHERF1 could serve to increase glycolysis and enable increased cellular proliferation. Concordant with an increase in intracellular pH, the growth of many tumors is associated with a decrease in extracellular pH ([186], reviewed in [187] and [188]). Interestingly, cells stably transduced with NHE3 were able to proliferate in acidic media, whereas cells lacking NHE expression failed to proliferate [189]. This indicates that NHE3 is important for cellular growth in acidic environments, which often coincides with alkalization of the cell cytosol.

The activity of NHEs is also important for preventing cellular apoptosis. Intracellular alkalization has been linked to the inhibition of endonucleases, acid sphingomyelinase, and caspases and is therefore believed to be anti-apoptotic (reviewed in [190]). The morphology of cells treated with an NHE inhibitor indicated cell shrinkage and condensation of their chromatin structure – events often associated with apoptosis [191]. Therefore the degradation of NHERF1 by E6 proteins may work to prevent cellular apoptosis

of infected cells while also promoting cellular proliferation, both of which are important throughout the course of a papillomavirus infection.

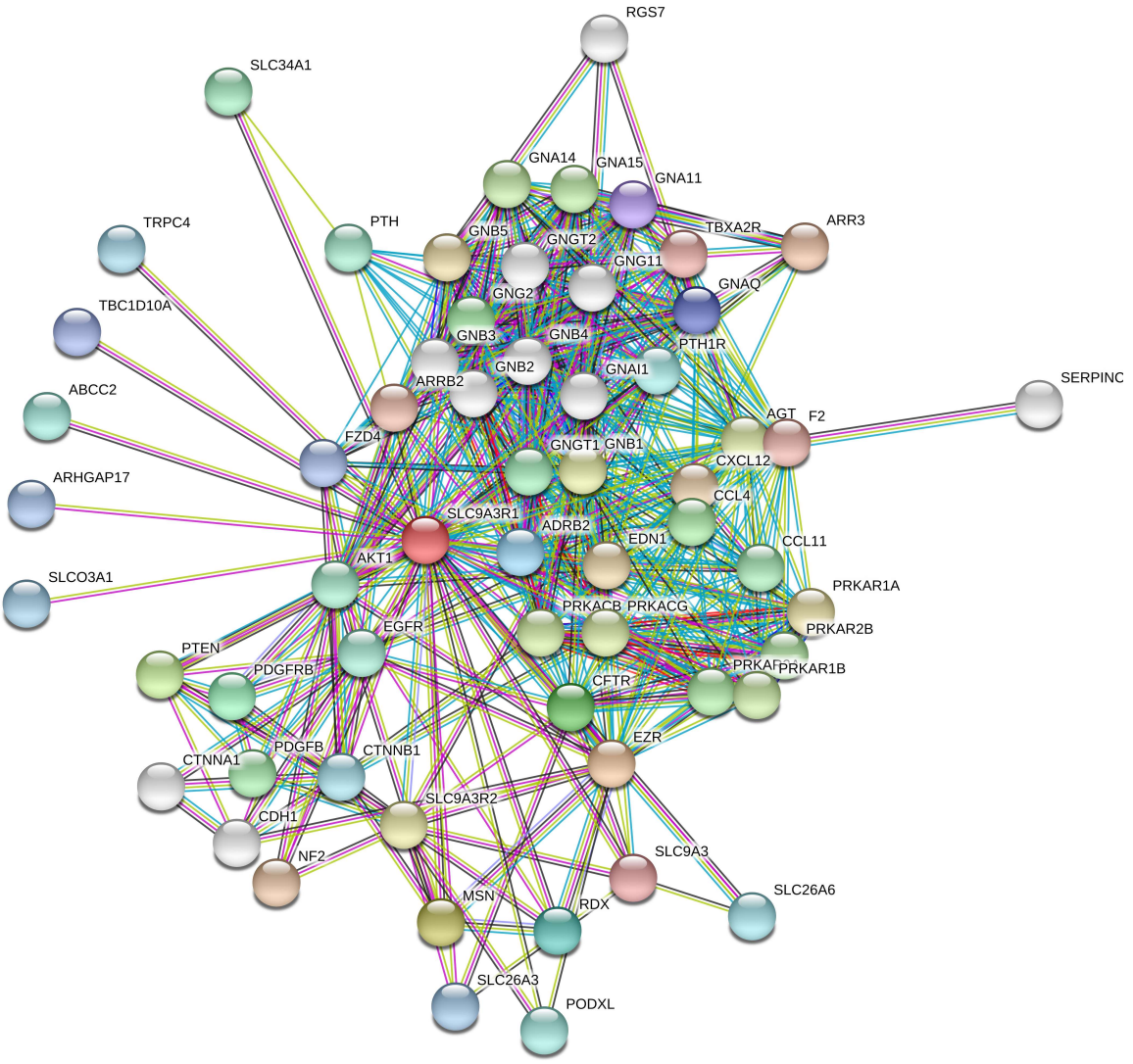
5.3.7 Assessing the interaction of NHERF1 with cellular proteins

Herein we have provided evidence that NHERF1 acts as an inhibitor of the canonical Wnt/β-catenin pathway and found that degradation of NHERF1 by E6 proteins augments this oncogenic signaling pathway. However, due to its role as a scaffold, NHERF1 interacts with a myriad of cellular proteins and therefore has been implicated in regulating numerous cellular pathways. To truly appreciate the implications of E6 targeting NHERF1 for degradation, other pathways involving NHERF1 and the effects of dysregulation of these pathways must be considered.

We examined the NHERF1 protein-protein interaction network through the use of the online STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) but limited our search to only the top 50 interactions with at least a medium confidence score, as determined by the algorithm published by Szklarczyk et al. [192] (Fig 5.12; Table 5.1). The STRING database uses information from curated databases, experimentation, gene neighborhoods, gene fusions, gene co-occurrences, textmining, co-expression, and protein homology (all of which are detailed [193]) to create the protein-protein interaction network. Due to the complexity of our initial protein-protein interaction network (Fig 5.12), we adjusted our parameters to observe NHERF1 protein-protein interactions using only experimentally-

derived evidence and limiting the total number of interactions to 25 (Fig 5.13). Selected NHERF1-protein interactions depicted in Fig 5.13 and potential pathways affected by these interactions are explored in greater detail in Table 5.2. Within Table 5.2 the evidence supporting each NHERF1-protein interaction is highlighted based on my confidence in the data.

** Figure 5.12 legend on next page **



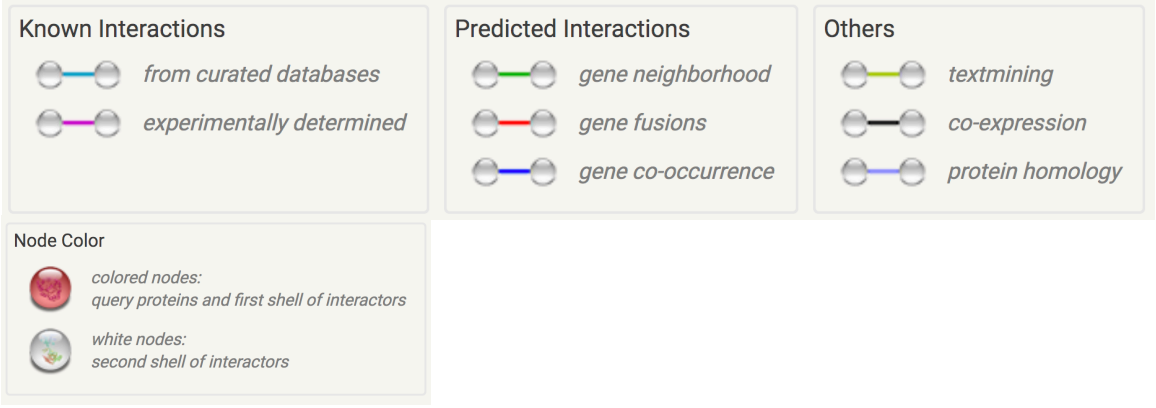


Figure 5.12 A network of NHERF1 protein-protein interactions.
To determine possible protein interactions with NHERF1, we utilized the online STRING database. We searched for NHERF1 by its gene name SLC9A3R1 (red node) and limited the results to fifty proteins that may interact with NHERF1. Each different protein is indicated by a node that is either colored or white. Colored nodes indicate direct interactions with NHERF1 while white nodes indicate proteins that are once removed from interaction with NHERF1 (e.g. they interact with a protein that interacts with NHERF1). For a detailed list of the proteins encoded by the genes in the network above, see Table 5.1. Evidence for interactions between NHERF1 and the listed proteins are indicated by the color of the lines that connect said protein to NHERF1 (see legend). This figure was adapted from [193]. A more thorough description regarding the evidence for each interaction is detailed in the figure legend of Table 5.1.

** Table 5.1 legend on next page **

Predicted Functional Partners:		Coexpression	Experiments	Databases	Textmining	[Homology]	Score
EZR	Ezrin; Probably involved in connections of major cytoskeletal structures to the plasma membrane. In epithelial cells, requir...		●	●	●		0.998
MSN	Moesin; Probably involved in connections of major cytoskeletal structures to the plasma membrane. May inhibit herpes si...		●		●		0.995
PTEN	Phosphatase and tensin homolog; Tumor suppressor. Acts as a dual-specificity protein phosphatase, dephosphorylating ty...		●	●	●		0.991
CFTR	Cystic fibrosis transmembrane conductance regulator; Epithelial ion channel that plays an important role in the regulation ...		●	●	●		0.984
PDGFRB	Platelet-derived growth factor receptor beta; Tyrosine-protein kinase that acts as cell-surface receptor for homodimeric PD...		●	●	●		0.977
RDX	Radixin; Probably plays a crucial role in the binding of the barbed end of actin filaments to the plasma membrane; Deafnes...		●		●		0.974
ADRB2	Beta-2 adrenergic receptor; Beta-adrenergic receptors mediate the catecholamine- induced activation of adenylate cyclase ...		●	●	●		0.956
GNAQ	Guanine nucleotide-binding protein G(q) subunit alpha; Guanine nucleotide-binding proteins (G proteins) are involved as m...		●	●			0.934
GNA11	Guanine nucleotide-binding protein subunit alpha-11; Guanine nucleotide-binding proteins (G proteins) are involved as mod...		●	●			0.930
SLC9A3	Sodium/hydrogen exchanger 3; Involved in pH regulation to eliminate acids generated by active metabolism or to counter ...		●		●		0.921
TBXA2R	Thromboxane A2 receptor; Receptor for thromboxane A2 (TXA2), a potent stimulator of platelet aggregation. The activity o...			●	●		0.917
F2	Prothrombin; Thrombin, which cleaves bonds after Arg and Lys, converts fibrinogen to fibrin and activates factors V, VII, VIII...			●	●		0.917
ARRB2	Beta-arrestin-2; Functions in regulating agonist-mediated G-protein coupled receptor (GPCR) signaling by mediating both r...			●	●		0.913
ARR3	Arrestin-C; May play a role in an as yet undefined retina-specific signal transduction. Could binds to photoactivated-phosph...			●	●		0.910
NF2	Merlin; Probable regulator of the Hippo/SWH (Sav/Wts/Hpo) signaling pathway, a signaling pathway that plays a pivotal rol...		●		●		0.909
CXCL12	Stromal cell-derived factor 1; Chemoattractant active on T-lymphocytes, monocytes, but not neutrophils. Activates the C-X-...			●			0.906
EDN1	Endothelin-1; Endothelins are endothelium-derived vasoconstrictor peptides; Belongs to the endothelin/sarafotoxin family (...			●			0.906
GNB5	Guanine nucleotide-binding protein subunit beta-5; Enhances GTPase-activating protein (GAP) activity of regulator of G pro...			●			0.906
PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit; Regulatory subunit of the cAMP-dependent protein kinase...			●			0.905
SLC9A3R2	Na(+)/H(+) exchange regulatory cofactor NHE-RF2; Scaffold protein that connects plasma membrane proteins with memb...			●	●	●	0.905
GNB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1; Guanine nucleotide-binding proteins (G proteins) are inv...			●			0.905
AGT	Angiotensinogen; Essential component of the renin-angiotensin system (RAS), a potent regulator of blood pressure, body fl...			●			0.905
PRKACG	cAMP-dependent protein kinase catalytic subunit gamma; Phosphorylates a large number of substrates in the cytoplasm a...			●			0.903
PRKACB	cAMP-dependent protein kinase catalytic subunit beta; Mediates cAMP-dependent signaling triggered by receptor binding t...			●			0.903
PRKAR1B	cAMP-dependent protein kinase type I-beta regulatory subunit; Regulatory subunit of the cAMP-dependent protein kinases ...			●			0.903
GNA14	Guanine nucleotide-binding protein subunit alpha-14; Guanine nucleotide-binding proteins (G proteins) are involved as mod...			●			0.902
GNA15	Guanine nucleotide-binding protein subunit alpha-15; Guanine nucleotide-binding proteins (G proteins) are involved as mod...			●			0.902
PRKAR2B	cAMP-dependent protein kinase type II-beta regulatory subunit; Regulatory subunit of the cAMP-dependent protein kinases...			●			0.901
PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit; Regulatory subunit of the cAMP-dependent protein kinase...			●			0.901
CCL4	C-C motif chemokine 4; Monokine with inflammatory and chemokinetic properties. Binds to CCR5. One of the major HIV-su...			●			0.900
GNG2	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-2; Guanine nucleotide-binding proteins (G proteins) are ...			●			0.900
PDGFB	Platelet-derived growth factor subunit B; Growth factor that plays an essential role in the regulation of embryonic develop...			●			0.900
CCL11	Eotaxin; In response to the presence of allergens, this protein directly promotes the accumulation of eosinophils, a promin...			●			0.900
GNGT1	Guanine nucleotide-binding protein G(T) subunit gamma-T1; Guanine nucleotide-binding proteins (G proteins) are involved ...			●			0.900
SLC34A1	Sodium-dependent phosphate transport protein 2A; Involved in actively transporting phosphate into cells via Na(+) cotrans...		●		●		0.838
AKT1	RAC-alpha serine/threonine-protein kinase; AKT1 is one of 3 closely related serine/threonine- protein kinases (AKT1, AKT2 ...		●		●		0.822
PTH	Parathyroid hormone; PTH elevates calcium level by dissolving the salts in bone and preventing their renal excretion. Stimu...				●		0.754
EGFR	Epidermal growth factor receptor; Receptor tyrosine kinase binding ligands of the EGF family and activating several signali...		●		●		0.748
ABCC2	Canalicular multispecific organic anion transporter 1; Mediates hepatobiliary excretion of numerous organic anions. May f...		●		●		0.739
PODXL	Podocalyxin; Involved in the regulation of both adhesion and cell morphology and cancer progression. Function as an anti-...				●		0.738
PTH1R	Parathyroid hormone/parathyroid hormone-related peptide receptor; Receptor for parathyroid hormone and for parathyroid ...		●		●		0.734
SLC26A6	Solute carrier family 26 member 6; Apical membrane anion-exchanger with wide epithelial distribution that plays a role as ...		●		●		0.729
CTNNB1	Catenin beta-1; Key downstream component of the canonical Wnt signaling pathway. In the absence of Wnt, forms a comp...		●		●		0.724
TRPC4	Short transient receptor potential channel 4; Form a receptor-activated non-selective calcium permeant cation channel. Act...		●		●		0.721
SLCO3A1	Solute carrier organic anion transporter family member 3A1; Mediates the Na(+)-independent transport of organic anions s...		●		●		0.720
SLC26A3	Chloride anion exchanger; Chloride/bicarbonate exchanger. Mediates the efficient absorption of chloride ions in the colon, ...		●		●		0.710
ARHGAP17	Rho GTPase-activating protein 17; Rho GTPase-activating protein involved in the maintenance of tight junction by regulatin...		●		●		0.710
FZD4	Frizzled-4; Receptor for Wnt proteins. Most of frizzled receptors are coupled to the beta-catenin (CTNNB1) canonical signa...		●		●		0.709
TBC1D10A	TBC1 domain family member 10A; Acts as GTPase-activating protein for RAB27A, but not for RAB2A, RAB3A, nor RAB4A (...		●		●		0.702

Table 5.1 Detailed list of NHERF1-interacting proteins depicted in Figure 5.12.

The gene names are listed in column 1 and paired with the colored node as it appears in Figure 5.12. The proteins transcribed from said genes are listed in column 2. The evidence supporting the interaction of NHERF1 with the listed proteins is categorized by coexpression, experiments, databases, textmining, and homology. The intensity of the dot under each evidence column is indicative of how supportive that particular type of evidence is to that particular protein-protein interaction. For a detailed description of each evidence category, please see [193]. They are summarized in brief as follows: "Coexpression" is based on how often NHERF1 is coexpressed with the listed protein in humans and other species. "Experiments" is based on whether the interaction has been experimentally tested. "Databases" denotes that the evidence supporting the interaction of the two proteins was mined from curated databases. Finally, "Textmining" refers to the STRING program searching the scientific literature to identify the frequency in which the two proteins are both mentioned in the same body of text. The score is determined by using a bioinformatics pipeline whose basis rests upon gene expression values arrayed against various statistical analyses. A score value between 0.15 and 0.4 indicates low confidence in the interaction based on the evidence. A score above 0.4 but below 0.7 is medium confidence, above 0.7 but below 0.9 is high confidence, and above 0.9 is highest confidence. The pipeline the online STRING databases utilizes to determine an interaction score value has been published [192]. This table was adapted from [193].

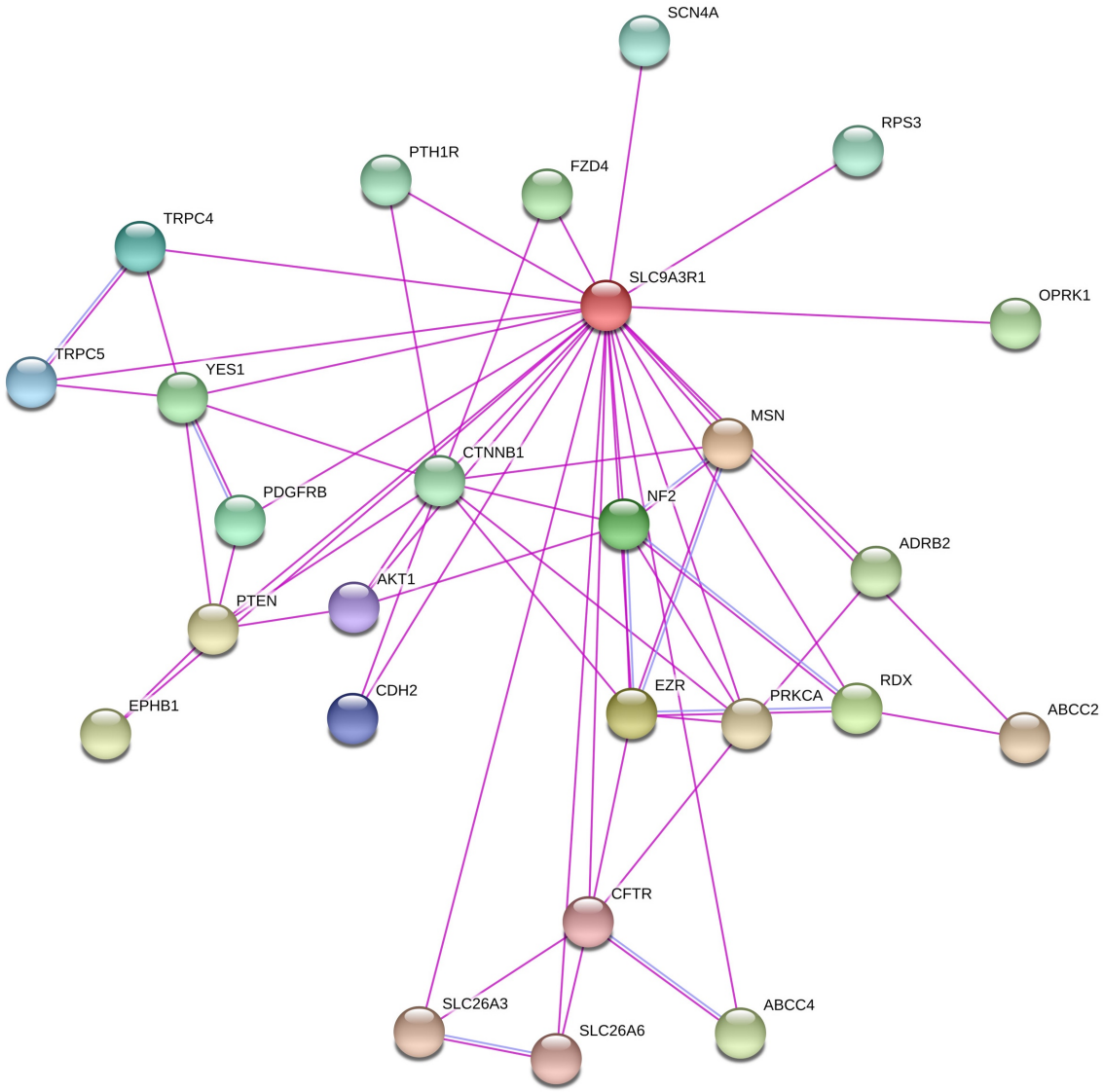


Figure 5.13 Experiment-based NHERF1 centered protein-protein interaction network.
NHERF1 is indicated by its gene name SLC9A3R1 and appears as a red node. All other depicted nodes indicate proteins that have been experimentally shown to interact with NHERF1 and are labeled based on the gene that transcribes said protein. Pink lines connecting nodes indicate evidence for the interaction was experimentally-derived. Light blue lines connect nodes indicate the interaction was also described in a curated database. A subset of these interactions is further analyzed in Table 5.2 which also contains the protein that corresponds to the gene name. Speculation on why E6 proteins may want to disrupt ion channels (many of which are listed here) can be found in section 5.3.6. This figure was adapted from [193].

Gene name	Protein name	Acronym	NHERF1 Interaction Domain	Interaction Evidence	Citation(s)	Potential Cancer-associated Pathways	Why would E6 deregulate?
ADRB2	Beta-2 adrenergic receptor	β2 adrenoreceptor	PDZ1	IP, GST pull-down, mutants, co-localization, K_D affinity studies	Hall 1998a; Hall 1998b		Alters cellular metabolism, differentiation, growth
AKT1	RAC-alpha serine/threonine-protein kinase	Akt	PDZ2	affinity chromatography assay	Song 2015	PI3K/Akt	Regulates cellular metabolism, proliferation, survival, growth, and angiogenesis
CTFR	Cystic fibrosis transmembrane conductance regulator	CFTR	PDZ1	K_D affinity studies, mutagenesis, blot overlay; GST pull-down; crystal	Hall 1998a; Short 1998; Bhattacharya 2013		Protein channel - water and chloride
CTNNB1	Catenin beta-1	β-catenin	PDZ2	293T overexpression IP; co-localization; IP; mutagenesis	Shibata 2003	Canonical Wnt Signaling	Alters cellular growth and proliferation
EZR	Ezrin	ezrin	EB	site directed mutagenesis; affinity chromatography; IP	Reczek 1997		Alter localization of NHERF1-associated proteins
FZD4	Frizzled-4	Fzd4	PDZ2	IP; co-localization; NHERF1 -/- cells; NHERF1 -/- mice; IHC	Wheeler 2011	Canonical Wnt Signaling	Alters cellular growth and proliferation
MSN	Moesin	moesin	EB	crystal; site directed mutagenesis; affinity chromatography; IP	Finnerty 2004; Reczek 1997		Alter localization of NHERF1-associated proteins
NF2	Merlin	merlin	EB	yeast two-hybrid; affinity binding assays; co-localization	Murthy 1998		Merlin is a tumor suppressor located at adherens junctions and is important for contact-mediated growth inhibition
PDGFRB	Platlet-derived growth factor receptor beta	PDGFRβ	PDZ1	endogenous protein IP; NHERF1 -/- cells	Maudsley 2000; Takahashi 2006	PDGFR signaling; PI3K pathway	Cytoskeletal rearrangement; cellular migration; cellular proliferation
PRKCA	Protein kinase C alpha type	PKC	Not determined	NHERF1 -/- cells	Weinman 2010	Diverse cellular signaling pathways	Cell adhesion, cell transformation, cell cycle checkpoint
PTEN	Phosphatase and tensin homolog	PTEN	PDZ1	yeast two-hybrid; GST pull-down; 293T overexpression IP; NHERF1 -/- cells	Takahashi 2006	PDGFR signaling; PI3K pathway	Cytoskeletal rearrangement; cellular migration; cellular proliferation
RDX	Radixin	radixin	EB	crystal; site directed mutagenesis; affinity chromatography; IP	Terawaki 2006; Reczek 1997		Alter localization of NHERF1-associated proteins
YES1	Tyrosine-protein kinase Yes; src family tyrosine kinase	Yes1	PDZ2	GST pull-down; co-localization; mutagenesis with GST pull-down	Mohler 1999	Src family kinase	A proto-oncogene src tyrosine kinase; contrubutes to cellular transformation

** Table 5.2 legend on next page **

Table 5.2 Analyzing the evidence that supports the interaction of NHERF1 with a selected group of proteins.

Listed are a select group of NHEF1-interacting proteins depicted in Figure 5.13. The corresponding protein name and acronym are listed next to the gene name. Evidence supporting the interaction of NHERF1 with the indicated protein is listed and color coded based on my confidence in the data. Dark green indicates high confidence due to the sheer amount of data supporting the interaction and/or due to the interaction being crystalized. Light green indicates some confidence due to the interaction having been tested through numerous different techniques but no crystal structure. Light red indicates hesitation regarding the validity of the interaction due to lack of rigorous testing and/or employing only a few different techniques. The domain of NHERF1 implicated in interacting with each listed protein is indicated. Cancer-associated pathways with which the indicated protein has been associated are listed as well as implications regarding the deregulation of NHERF1 (and thereby proteins with which it interacts) by E6 proteins. References from which the interaction evidence was found include Hall 1998a [194]; Hall 1998b [110]; Song 2015 [195]; Short 1998 [196]; Bhattacharya 2013 [197]; Shibata 2003 [94]; Reczek 1997 [112]; Wheeler 2011 [95]; Finnerty 2004 [198]; Murthy 1998 [199]; Maudsley 2000 [107]; Takahashi 2006 [106]; Weinman 2010 [200]; Terawaki 2006 [201]; and Mohler 1999 [111].

5.4 Insights into the interaction of E6 with E6AP

We have characterized and described multiple novel auxiliary E6AP regions important in the E6-E6AP interaction and complex function. Previous studies have suggested the possibility of additional E6-E6AP interaction modalities outside of the well-characterized E6-E6AP LQELL motif [48, 78] and our extensive analysis supports these postulations.

5.4.1 E6-E6AP Interaction Types

We have previously described a distinct group of E6 proteins from both human and non-human papillomaviruses that bind E6AP and stimulate its ubiquitin ligase activity [60]. E6 proteins from mucosal high and low-risk papillomaviruses, as well as E6 from cutaneous papillomaviruses, can bind to E6AP. Additionally, past work has found that low-risk 11E6 is unable to bind the isolated E6AP LQELL peptide [47], while high-risk 16E6 can [32, 59, 78]. We believe that the ability of low-risk E6 to bind to E6AP despite its inability to bind E6AP LQELL alone, is indicative of other, auxiliary binding regions within E6AP that enable enhanced affinity E6-E6AP binding. Through the analysis of a cohort of E6AP-binding E6 proteins, we found that their interactions with E6AP appear to fall within to main types: (I) the ability to interact or (II) the inability to interact with the isolated E6AP LQELL peptide. Type II could be further subdivided into E6 proteins that are able to interact with E6AP mutated in its LQELL region to LQELS (Type IIa), and those that cannot (Type IIb). Based upon our identification of E6AP auxiliary regions in

both the amino and carboxy termini of E6AP, we hypothesize that the Type IIa E6-E6AP interactions require either N or C-terminal regions while Type IIb E6-E6AP interactions require both N and C-terminal E6AP regions (Table 5.3). In support of this hypothesis, we found that 11E6 (Type IIa) requires E6AP residues 561-595 within the E6AP HECT domain to initiate E6-E6AP complex formation without the presence of the E6AP amino terminus, while MmPV1E6 (Type IIb) is unable to interact with E6AP truncated of its amino terminus, even in the presence of the entire E6AP carboxy terminus (Fig 4.13). Expanding upon our current results, we plan to characterize the ability of other E6 proteins (with Type IIa and Type IIb E6AP interactions) to bind E6AP truncations; this analysis will elucidate a more generalizable E6-E6AP binding pattern.

Type	Bind LQELL	Bind FL E6AP LQELS	E6AP Auxiliary Regions (for degradation)	(for binding)	Examples
Type I	+	+	310-320	Not required*	16E6 7E6 10E6 SsPV1E6 UmPV1E6
Type IIa	—	+	310-320	Amino *or* carboxy	16E6_L50A 11E6 PsPV1E6 PphPV1E6
Type IIb	—	—	n.d.	*Both* amino and carboxy	16E6_Δ1-8_L50A MmPV1E6 TtPV5E6

Table 5.3 Summary of Proposed E6-E6AP Interaction Types.
We propose that there are two general types of E6-E6AP interactions: Type I and Type II. Within Type I interactions, E6 proteins can bind the isolated E6AP LQELL peptide ("Bind LQELL") and well as full-length E6AP with the LQELL motif mutated to LQELS (Bind FL E6AP LQELS). Type II interactors cannot bind the isolated E6AP LQELL peptide. Type II interactions can further be broken down into Type IIa and Type IIb: Type IIa interacts with full-length E6AP LQELS but Type IIb does not. The importance of E6AP auxiliary regions for either degradation of E6 targets or for E6 binding to E6AP also differs between E6-E6AP interaction types, according to our data. A Type I interaction requires the E6AP 310-320 region (in the context of full-length E6AP) as evidenced by 16E6_WT degradation of both p53 and NHERF1. A Type IIa interaction also requires the 310-320 E6AP auxiliary region (in the context of full-length E6AP) as evidenced by 11E6_WT degradation of NHERF1 and 16E6_L50A degradation of p53. We have not yet tested whether 310-320 (or a different E6AP auxiliary region) is required for degradation of E6-mediated targets displaying a Type IIb interaction with E6AP. We hypothesize that Type IIa and Type IIb interactions differ in how much interaction with E6AP (outside of LQELL) is required to mediate the interaction. We believe Type IIa interactions require E6AP auxiliary binding regions in either the amino or carboxy terminus of E6AP while Type IIb interactions require E6AP auxiliary binding regions in *both* the E6AP amino and carboxy terminus. *E6AP auxiliary regions not required for Type I interactions because these E6 proteins can bind the isolated LQELL peptide. However, auxiliary binding regions may enhance the Type I E6-E6AP interaction as 16E6 displays higher affinity binding for full-length E6AP over the isolated LQELL peptide [59].

The observed intrinsic difference between binding of 16E6_L50A and 16E6_WT to E6AP implies that the E6-E6AP complex can have different interaction modalities. The type I E6-E6AP interactions are sufficient to enable recruitment of p53 to the E6-E6AP LQELL complex [59]. However, we have preliminary data suggesting this is not the case for recruitment of NHERF1 to the E6-E6AP LQELL complex (data not shown). In yeast, recruitment of NHERF1 to 16E6 requires full-length E6AP. Therefore, NHERF1 may require a Type II interaction between E6 and E6AP in order to bind to the complex, regardless of whether an E6 protein also binds E6AP via a Type I interaction.

5.4.2 E6 Interaction with E6AP Auxiliary Regions

Our analysis of a mutant of 16E6 (L50A) that lacks the ability to bind the E6AP LQELL motif illuminated two regions within E6AP that were required for successful 16E6_L50A-E6AP interaction when E6AP was deleted of its HECT domain (E6APΔHECT): one located amino terminal to LQELL (residues 121-127) and one located carboxy terminal to LQELL (residues 497-521) (highlighted in blue in Fig 4.14). This observation led us to hypothesize that E6 proteins that cannot interact strongly with the E6AP LQELL motif (Type II), may require auxiliary E6AP regions within the E6AP amino and/or carboxy terminus to enable binding to the E6AP protein. The 16E6_L50A mutant most closely resembles E6 proteins that are unable to bind the isolated E6AP LQELL peptide, but maintain interaction with full-length E6AP LQELS (11E6, PsPV1E6, and PphPV1E6). Using our Y2H assay, we will determine

whether these three E6 proteins require the same amino and carboxy terminal regions of E6APΔHECT to form the E6-E6AP complex.

We performed a multiple sequence alignment of E6AP proteins from the various animals tested in Fig 4.1B. Specifically, we focused on the E6AP auxiliary regions described herein (colored in either blue, green, or light orange in Fig 4.14) to determine whether the sequences are conserved among the different animals. This step is important to establish whether our human E6AP truncations are relevant for comparing the requirement of these non-human E6 proteins for our identified E6AP auxiliary regions. We analyzed rhesus monkey, polar bear, boar, bottlenose dolphin, and finless porpoise E6AP proteins (Fig 5.14, Fig 5.15). While we actually observed papillomavirus E6 proteins from a Harbor porpoise (PphPV1/4) and Burmeister's porpoise (PsPV1), the E6AP proteins for these two porpoise species of have not yet been described. Therefore, we thought it best to examine an E6AP protein from a finless porpoise in lieu of no porpoise at all. Overall, our identified E6AP auxiliary regions were extremely well conserved among the different E6AP orthologs. The only difference observed was in the 121-127 region of polar bear E6AP, with the conservative change of lysine to arginine (K123R). As this is the only difference between human E6AP and the E6APs of the animals tested (within our identified auxiliary regions), we propose that the use of human E6AP with the non-human E6 proteins is a feasible alternative to acquiring and utilizing these non-human E6AP proteins.

** Figure 5.14 legend on next page **

E6AP protein sequences

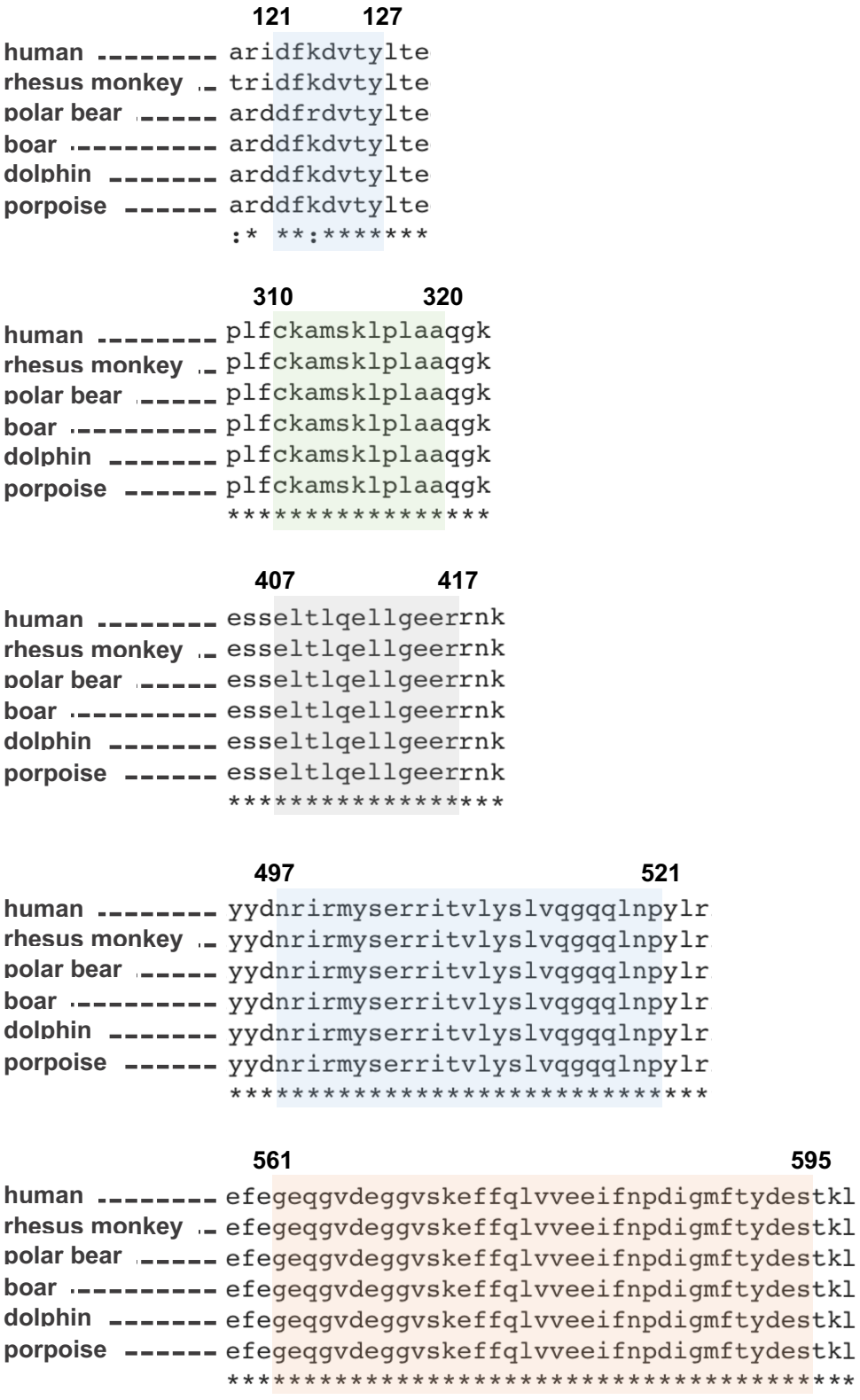


Figure 5.14 Non-human E6AP sequences are conserved in our identified E6AP auxiliary binding regions.

We performed a multiple sequence alignment on E6AP proteins found in humans, rhesus monkeys, polar bears, wild boars, bottlenose dolphins (dolphins in figure), and the finless porpoise (porpoise in figure). We studied papillomavirus E6 proteins from the Harbor porpoise (PphPVs) and the Burmeister's porpoise (PsPV1), but the E6AP sequences from both the Harbor porpoise (*Phocoena phocoena*) and the Burmeister's porpoise (*Phocoena spinipinnis*) have not been described. Therefore, we used the E6AP sequence from the finless porpoise (*Neophocaena asiaeorientalis*) for means of comparison. Overall, sequences were very well conserved among the different species. The only observed difference between the sequences is found in region 121-127 where polar bear has the conservative change of K123R. Sequences shaded in light blue are required for 16E6_L50A interaction when the HECT domain is deleted. Sequence shaded in green is important for E6 stimulation of E6AP ubiquitin ligase activity in the context of E6AP 310-875 vs 320-875. The sequence shaded in gray is the already well-characterized E6AP LQELL motif. The sequence shaded in light orange is important for binding of 11E6_WT to E6AP deleted of all sequence amino terminal to the E6 LQELL binding region. The numbering depicted is with human E6AP isoform II as a reference.

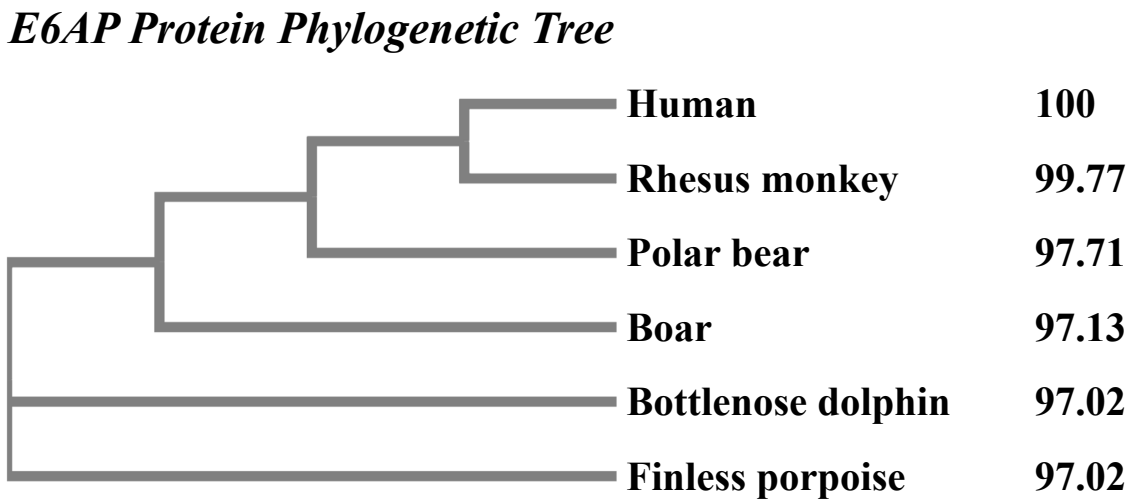


Figure 5.15 Phylogenetic Tree of E6AP Proteins from Different Species. We performed a multiple sequence alignment of E6AP proteins from different species in which we are interested (due to studying E6 proteins from their respective papillomavirus) using Clustal Omega. This data was imported into Clustal Omega Phylogeny to generate this phylogenetic tree. For clarity, results are shown with the cladogram branch length setting. The percent identity matrix values generated by Clustal 2.1 are shown to the right of the animals. We studied papillomavirus E6 proteins from the Harbor porpoise (PphPVs), but the E6AP sequence of Harbor porpoise (*Phocoena phocoena*) has not been described. Therefore, we used the E6AP sequence from the finless porpoise (*Neophocaena asiaeorientalis*) for means of comparison.

While we have identified auxiliary binding regions within E6AP, we sought to truncate the E6 protein in an attempt to identify regions necessary for binding the E6AP auxiliary regions. Although making truncations within such a small protein (only around 18 kDa) can have unforeseen consequences, 16E6 deleted of its first eight amino acids (16E6_Δ1-8) can still bind full-length E6AP and stimulate p53 degradation [48]. We noticed that full-length E6AP cannot outcompete LQELL bound in *cis* to 16E6_Δ1-8 (data not shown). This observation suggests that the first eight residues of 16E6 are important in mediating the higher affinity binding of full-length E6AP to the E6 protein. Based on our E6AP characterization data and this observation, we hypothesized that there are two key E6-E6AP interaction mechanisms: E6-LQELL, and E6AP auxiliary regions in combination with the first eight amino acids of 16E6. Yeast two hybrid analysis revealed that the interaction was not this simple, as 16E6_Δ1-8 could still interact with full-length E6AP LQELS (Fig 4.10, spot 5D). If the E6-E6AP interaction was dependent upon interaction between E6-LQELL and E6_1-8 and the E6AP auxiliary regions, then deletion of 16E6 residues 1-8 in combination with mutagenesis of the LQELL region should prevent the E6-E6AP interaction. Interestingly, when the L50A mutation was add to the 16E6_Δ1-8 protein (16E6_Δ1-8_L50A), this mutant E6 protein lost its ability to interact with full-length E6AP LQELS, but retained its interaction with full-length E6AP LQELL (Fig 4.10, spots 5E and 3E). We therefore adapted our hypothesis: there is a dual modality of E6-E6AP

interaction that is mediated from the perspective of both the E6 and E6AP protein; mutation of one of the proteins does not completely attenuate the corresponding interaction (e.g. mutation of LQELL to LQELS does not entirely block the E6-LQELL interaction). Our hypothesis was supported by data from 11E6_WT, as it could interact with both full-length E6AP LQELL and full-length E6AP LQELS (Fig 4.10, spots 3F and 5F). Because 11E6_WT cannot interact with isolated E6AP LQELL peptide, it is inherently more like 16E6_L50A than like 16E6_WT. Therefore, inability of the 11E6_Δ1-9 mutant (homologous to 16E6_Δ1-8) to interact with full-length E6AP LQELS is comparable to the inability of 16E6_Δ1-8_L50A to interact with full-length E6AP LQELS (Fig 4.10, spots 5G and 5E).

5.5 E6 stimulation of E6AP ubiquitin ligase activity

5.5.1 The 16E6_L50A Mutant

Zanier et al. showed 16E6_L50A could not interact with the isolated peptide, but did not demonstrate whether it could still bind full-length E6AP or stimulate its ubiquitin ligase activity. Instead, the same study characterized 16E6_L50E as being unable to target p53 for degradation in the presence of full-length E6AP [32]. In our study, we further characterize the 16E6_L50A mutant and demonstrate its preserved ability to not only interact with full-length E6AP, but also to initiate p53 degradation in the presence of E6AP (Fig 4.2A and 4.3). We have not tested the 16E6_L50E mutant, but it would be of

interest to examine its interaction with E6AP in a Y2H system as it is more sensitive than GST pull downs. However, examination of the E6-E6AP LQELL crystal structure (Fig 4.2B), provides insight regarding why there's such a difference in phenotype between 16E6_L50A and 16E6_L50E. The 16E6 L50 residue is situated deep in the pocket formed by E6N, E6C, and the E6 alpha helical connecting helix. Altering the non-polar, hydrophobic nature of leucine by mutating it to a negatively charged, polar glutamine could disrupt the ability of the E6 protein to form its deep, hydrophobic pocket in which LQELL binds. Alanine, however, is both non-polar and hydrophobic (like leucine) and its side chain is smaller than that of leucine, so it likely would not disrupt the formation of the E6 deep pocket.

5.5.2 E6 Binding E6AP LQELL is important, but not sufficient for stimulating degradation

The ability of 16E6_L50A to initiate the degradation of p53 in the presence of full-length ubiquitin ligase active E6AP suggested that tight binding of E6 to the E6AP LQELL motif was not sufficient to stimulate E6AP ubiquitin ligase activity. Indeed, E6 proteins that display gain-of-function binding to E6AP LDDLL (containing the MAML1 LXXLL motif) fail to stimulate E6AP ubiquitin ligase activity [60]. Additionally, while bovine papillomavirus type 1 (BPV1) E6 is capable of binding to the isolated E6AP LQELL motif, it is unable to stimulate E6AP ubiquitin ligase activity [48]. Further, upon mutation of the E6AP LQELL motif to LQELS, 16E6_WT is still able to initiate p53

degradation [48]. We expanded upon these results by mutating the E6AP LQELL motif to LQEAS in the context of full-length E6AP; 16E6_WT is unable to interact with this full-length E6AP LQEAS mutant in yeast [59]. We found that both 16E6_WT and 11E6_WT rely at least partially on their interaction with the E6AP LQELL motif in order to induce degradation of NHERF1 (Fig 4.9). Therefore, while a strong E6 interaction with LQELL is not required for degradation of E6-specific cellular substrates, complete ablation of the LQELL region does impact the ability of E6 proteins to stimulate the E3 ligase activity of E6AP.

5.5.3 E6AP Residues 310-320

E6 is a potent activator of E6AP ubiquitin ligase activity [149], although precisely how E6 triggers E6AP ubiquitin ligase activity to stimulate p53 degradation remains unclear. We found that enhanced affinity binding of 16E6_L50A to full-length E6AP was lost when E6AP was amino terminally truncated past residue 315 (Fig 4.3B). Intriguingly, we also found that the ability of high-risk E6 proteins to initiate degradation of p53 is lost in the presence of E6AP wild type amino terminally truncated past residue 315 (Fig 4.3C, D, E). These observations led us to hypothesize that the enhanced affinity binding of E6 proteins to E6AP via an E6AP auxiliary region located N-terminal to the E6AP LQELL enabled E6 proteins to stimulate E3 ubiquitin ligase activity. Our Y3H results were corroborated in E6AP-null murine 8B9 cells, the use of which enabled us to measure protein levels of both transfected

human p53 and HPV 16E6. Interestingly, 16E6_WT was able to initiate p53 degradation in the presence of E6AP 315-875 while 16E6_L50A could not (Fig 4.3D and 4.3E). Initially, we hypothesized that E6AP stabilization of E6 [79] was required for E6-mediated degradation of p53. Because 16E6_L50A cannot bind E6AP LQELL, it requires more of the E6AP amino terminus in order to be stabilized and therefore loses its ability to stimulate E6AP ubiquitin ligase activity sooner than 16E6_WT. However, quantification of both 16E6 and p53 protein levels revealed this was not the case. Although stabilization of 16E6_L50A by E6AP was lost earlier than stabilization of 16E6_WT protein levels, both L50A and WT 16E6 initiated p53 degradation when their protein levels were destabilized; 16E6_L50A with E6AP 310-875 and 16E6_WT with E6AP 315-875. Both 16E6_WT and 16E6_L50A failed to stimulate p53 degradation in the presence of E6AP 320-875 (Fig 4.3D and 4.3E). Additionally, both 16E6_WT and 11E6_WT failed to initiate NHERF1 degradation when co-expressed with E6AP 320-875 (Fig 4.7A, 4.7B, 4.8A, 4.8B). Therefore, our results indicate that E6AP region 310-320 is important in enabling E6 stimulation of E6AP ubiquitin ligase activity.

A recent study utilized chemical cross-linking coupled to mass spectrometry (XL-MS) to model the interaction sites and potential structure of the 16E6-p53-E6AP trimeric complex [150]. Based on their results, they postulated that the interaction of the complex containing full-length E6AP and 16E6 with p53 results in a change in conformation of E6AP such that 16E6 and p53 are

positioned close to the active cysteine residue (at position 843) in the E6AP HECT domain. As both high and low-risk E6 proteins require E6AP 310-320 to target p53 and/or NHERF1 for degradation, we hypothesize that this E6AP auxiliary region may play a role orienting E6 and a cellular substrate close to the active cysteine residue to activate E3 ubiquitin ligase activity. An alternative hypothesis is that E6AP 310-320 is necessary for E6AP-mediated ubiquitin chain formation as previous studies have indicated that the N-terminal region of E6AP plays a role in this function [149, 150]. Further studies are necessary to examine other E6 proteins that target NHERF1 for degradation [143] in the presence of our E6AP amino terminal truncations to determine their reliance on E6AP residues 310-320.

Due to the importance of the E6AP 310-320 region for mediating E6-induced degradation of p53 and/or NHERF1, we sought to identify a single amino acid in the context of full-length E6AP between residues 310 and 320 that would replicate our phenotype. Unfortunately, after performing extensive amino acid mutagenesis studies we were unable to identify a single amino acid in this region that replicated the E6AP 320-875 truncation phenotype that resulted in 16E6 unable to initiate degradation of p53 (Fig 4.6). The lack of identification of a single amino acid in this region suggests that E6 stimulation of E6AP ubiquitin ligase activity may be mediated by more than one residue within E6AP 310-320. Alternatively, our performed soft mutations (e.g. mutation to alanine) may be insufficient to disrupt E6 induction of ubiquitin

ligase activity and strong mutations (e.g. charge reversal) could illuminate a single, necessary residue. We propose to use a non-biased GAP repair in yeast approach in our next attempt to identify a possible residue(s) that is necessary for E6 stimulation of E6AP ubiquitin ligase activity.

5.6 Insights into the E6-E6AP interaction

What we have termed Type I E6-E6AP binding (where E6 can bind the isolated E6AP LQELL peptide) masks any additional E6AP auxiliary binding regions, as those E6 proteins can bind any E6AP protein containing the LQELL motif. However, discovery of the importance of E6AP residues 310-320 for 16E6 stimulation of E6AP ubiquitin ligase activity indicates that E6 proteins that can bind isolated LQELL also depend on at least one E6AP auxiliary binding region. We will expand the breadth of our studies to other E6 proteins that are able to bind E6AP via a Type I interaction (e.g. E6 from HPV7, HPV10, SsPV1, and UmPV1) and determine whether these E6 proteins also rely on the E6AP 310-320 region to stimulate E6AP ubiquitin ligase activity.

Type II E6 proteins display a more complex interaction with E6AP. Although they are unable to interact with isolated E6AP LQELL, they interact with the full-length E6AP suggesting the need for auxiliary E6AP regions to mediate the interaction. We further subclassified E6 Type II proteins into Type IIa and Type IIb, based on whether or not they could interact with full-length E6AP containing a mutated LQELL motif (LQELS). Type IIa interacted with

full-length E6AP LQELS while Type IIb did not. We hypothesize that Type IIa E6s require either an amino or carboxy terminal E6AP auxiliary binding motif whereas Type IIb require both an amino and carboxy terminal E6AP interaction, in addition to the LQELL (Table 5.3). Our hypothesis requires more testing, but is supported by our preliminary results using E6AP truncations and our observations of both Type IIa (11E6) and Type IIb (MmPV1E6) E6 interactions with E6AP. We have only observed interaction of E6AP truncations with one representative of a Type IIa interaction and one representative of a Type IIb interaction; therefore, we will expand upon these results using more Type II E6 proteins to determine whether our generalized hypothesis and categorizing of E6AP binding E6 proteins holds true. Regarding the role of E6AP residues 310-320 in the ability of Type II E6 proteins to initiate E6AP ubiquitin ligase activity, we have only examined Type IIa 11E6, although our results indicate 310-320 is important (Fig 4.7A and 4.8A). MmPV1E6 (Type IIb) can target human p53 for degradation [60] and so we will also determine its dependence upon E6AP residues 310-320.

We hypothesize that E6 proteins all rely on the LQELL motif, but other auxiliary E6AP regions may play a more prominent role in enabling the E6-E6AP interaction of certain E6 proteins (e.g. Type II) than others (e.g. Type I) (though we cannot rule out that E6 proteins actually interact with E6AP in similar ways). While many of these answers would be provided with a crystal structure of E6AP, so far attempts at crystalizing the full-length proteins have

proven fruitless because it is insoluble at high concentrations. Our extensive characterization into the E6-E6AP binding auxiliary regions may provide the insight needed to identify a region within E6AP that is not only physiologically important to formation of the E6-E6AP complex, but also amenable to crystallization.

5.6.1 Measuring binding affinity of E6 and E6AP

Taken together our data indicate there are auxiliary regions in E6 and E6AP that mediate complex formation and may contribute to enhanced binding of E6 proteins to cellular E6AP. There is evidence in the literature that suggests E6 proteins bind to full-length E6AP with greater affinity than to the isolated E6AP LQELL peptide [59, 82, 83]. We hypothesize the auxiliary binding regions we have identified and detailed in this dissertation contribute to the previously described higher affinity E6-E6AP binding. We propose to test this hypothesis by measuring the binding affinity of E6 to various E6AP truncations.

Protein binding affinity may be measured through a variety of different methods including isothermal titration calorimetry and surface plasmon resonance (reviewed in [202, 203]). These techniques rely on measurement of binding affinity between two purified substances - in our case two proteins. In order to utilize these methods it will first be necessary to achieve highly purified E6 and E6AP. Standard methods of protein production include using bacterial, mammalian, or yeast systems followed by isolation and purification

of proteins of interest. For purification purposes, both E6 and E6AP would need to be tagged and so the resulting purified proteins would have to be tested to ensure proper folding and function are retained. In the case of both E6 and E6AP, there are numerous caveats associated with purifying the protein. E6 proteins would have to be expressed in a system in which no endogenous E6AP to ensure they could still bind to our E6AP truncations of interest and were not pre-bound to full-length E6AP. Purification of E6 proteins in the absence of E6AP is challenging as E6 aggregates and addition of detergent will result in misfolded E6 proteins that cannot bind the E6AP LQELL motif [204]. Due to the low-solubility of E6AP, acquiring large amounts of highly purified protein will be technically challenging and require much optimization. Should successful purification of functional, properly folded E6 and E6AP be achieved, we will determine the binding affinities of multiple different E6-E6AP truncation combinations (see Table 5.4).

Table 5.4 is comprehensive and proposes to test the affinities of a multitude of E6 and E6AP protein combinations. However, we would focus our initial testing on certain protein combinations (bolded and italicized in Table 5.4) which we believe would inform numerous aspects of our studies thus far. Initially, we would establish a baseline affinity measurement between 16E6_WT and the full-length E6AP protein and between 16E6_WT and the LQELL peptide. Based on previous studies [59, 82, 83] and our own observations, we hypothesize that the full-length E6AP-16E6_WT interaction

will have a higher binding affinity than the E6AP LQELL peptide-16E6_WT interaction. After establishing these baselines, we will further test our hypothesis that a higher affinity E6-E6AP interaction is required for E6 stimulation of E6AP ubiquitin ligase activity. To do this, we would compare the affinities of E6AP 310-875 and E6AP 320-875 with 16E6_WT. We predict that the 310-875 E6AP truncation will have a binding affinity for 16E6_WT similar to that of full-length E6AP whereas the 320-875 E6AP truncation will have a reduced affinity. To determine whether the E6AP amino terminus (Fig 1.6, E6AP residues 1-406) enables enhanced affinity binding of 16E6_WT to E6AP when compared to the isolated E6AP LQELL peptide (as suggested by 16E6_L50A interaction in yeast: Figs 4.3B and C), we will compare the binding affinities of 16E6_WT to the following E6AP truncations: 1-521, 1-497, 121-521, and 127-521. We predict that 16E6_WT interaction with 1-521 and 121-521 would result in similar binding affinity measurements, but would show higher affinity than 16E6_WT interaction with E6AP truncations 1-497 and 127-521. We predict the binding affinities of 16E6_WT to E6AP 1-497 and 127-521 would mirror the measured affinity of 16E6_WT for E6AP LQELL, as 16E6_L50A (which cannot bind the isolated E6AP LQELL peptide) was unable to interact with these two E6AP truncations (Fig 4.12). Overall, these proposed experiments to measure 16E6_WT binding affinity to the aforementioned E6AP truncations would provide insight into the importance of numerous E6AP auxiliary regions.

Table 5.4 proposes to look at the binding affinities of 16E6_L50A, 11E6_WT, MmPV1E6_WT, and 16E6_Δ1-8 with various E6AP proteins. We believe determining the affinities of these interactions would enable us to provide answers to numerous questions including: *Does 16E6_L50A bind to full-length E6AP with a different affinity than 16E6_WT? Is there a difference in binding affinity between E6 proteins that we have classified as Type IIa and E6 proteins that we have classified as Type IIb?* and *How do the first eight amino acids of 16E6 contribute to 16E6 binding affinities?* Yet however important these question are, we believe they are secondary to our initial studies of 16E6_WT with different E6AP proteins.

** Table 5.4 legend on next page **

E6 protein	E6AP protein	Predicted Binding Affinity
***16E6*_WT**	***Full length LQELL***	High
	Full length LQELS	Medium
	300-875	High
	310-875	High
	320-875	Medium
	331-875	Medium
	1-521	Medium
	1-497	Low
	121-521	Medium
	127-521	Low
	LQELL peptide	Low
16E6_L50A	Full length LQELL	High
	Full length LQELS	Medium
	300-875	High
	310-875	High
	320-875	None
	331-875	None
	1-521	Medium
	1-497	None
	121-521	Medium
	127-521	None
11E6_WT	Full length LQELL	High
	Full length LQELS	Medium
	310-875	High
	320-875	Medium
	406-875	Medium
	406-595	Medium
	406-561	None
MmPV1E6_WT	Full length LQELL	High
	Full length LQELS	None
	310-875	High
	320-875	None
	406-875	None
16E6_Δ1-8	Full length LQELL	High
	Full length LQELS	Low

Table 5.4 Proposed E6 and E6AP protein combinations to measure binding affinity.
Measuring the binding affinities of the listed E6 and E6AP proteins will provide insight as to how much our identified auxiliary binding regions alter the binding of E6 and E6AP. Ideally, we would test each E6 with each listed E6AP protein. However, we highlighted the E6-E6AP combinations (bold and italicized) we believe would be best suited to an initial experiment. In the last column we denote our predicted binding affinity for each protein combination as either high, medium, low, or none. Our predications are based on our data as well as our hypotheses presented within this thesis.

5.7 Clinical Relevance and Therapeutic Implications of this Work

Degradation of NHERF1 has never before been described as a function of low-risk HPV E6 proteins and has only ever been described as a function of the high-risk 16E6 protein [126]. To confirm our observations in the context of HPV-induced lesions, it would be critical to stain patient samples arising from both high and low-risk HPVs. Low-risk HPVs not only cause genital warts, but they can also form papillomas in the oropharyngeal region [4]. HPV16 and HPV18 are the most common types of HPV infection [2], but it would be important to distinguish between the two when staining and cataloging the levels of NHERF1 protein expression to be sure any potential differences (as suggested by [126]) between the two high-risk E6 proteins is observed.

Because NHERF1 serves as a cellular degradation target of both high and low-risk E6 proteins, preventing NHERF1 degradation by these E6 proteins

could be a potential avenue for new therapeutics. As NHERF1 is a highly conserved target of E6 proteins from both human and non-human papillomaviruses, it is reasonable to hypothesize that its degradation is important in the viral life cycle, and/or for E6 activities. Further investigation into these possibilities will illuminate the true importance of NHERF1, and whether its interaction with E6 is a viable therapeutic target. Additionally, exploring the importance NHERF1 degradation for the activation of canonical Wnt signaling (and it's proposed downstream effects, Fig 5.10) within the context of papillomaviruses could indicate that a therapeutic approach combining Wnt pathway inhibitors (reviewed in [205]) with novel NHERF1-E6 or E6AP-E6 inhibitors may more effectively treat HPV-induced lesions.

To date, there are no FDA approved antivirals specifically for HPV proteins [206]. Inhibition of key E6 functions may represent a promising strategy for counteracting the growth of HPV-induced lesions, as evidence by successful induction of senescence or apoptosis by turning off E6 expression in HPV positive cancer-derived cell lines [144, 156, 157]. Numerous studies have focused on blocking the molecular activities of E6 proteins via the means of peptides [207-209] or antibodies [210, 211] and have successfully driven cell growth arrest and/or cellular death. Our identification and preliminary characterization of E6AP auxiliary regions that not only assist in formation of the E6-E6AP complex, but also are important in E6 stimulation of E6AP ubiquitin ligase activity indicate that there may be other targetable

interactions between E6 and E6AP. To truly understand the interactions of E6 and E6AP outside of the established LQELL interaction, we require a crystal structure. A crystal structure would provide invaluable information regarding the best small molecule inhibitors (or possibly antibodies) to inhibit E6-E6AP auxiliary interactions. We hope that our identification of other regions within E6AP will help direct the construction of E6AP truncations that maintain auxiliary interactions, but are more amenable to crystallization than the full-length protein.

Chapter 6: References

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VITA

Camille Michelle Drews (née Lewis) was born in Cottonwood, Idaho in 1992 to mother, Michelle, and biological father, Earl Lewis. Earl passed when Camille was less than three years old. However, her mother remarried to a wonderful man, Joe Tosten, when Camille was seven and he helped raise her and taught her the importance of a good education. Camille graduated valedictorian from Grangeville High School and attended Tulane University in New Orleans. In 2014, she earned her Bachelor's degree in Cell and Molecular Biology, graduating *Summa Cum Laude* with high honors and a minor in Chemistry. While at Tulane Camille worked in the laboratory of Dr. Frank Jones investigating the relationship between tamoxifen resistance in estrogen receptor positive breast cancer and expression of the receptor tyrosine kinase HER4. Upon graduation from Tulane, Camille continued her education at the University of Virginia, pursuing her doctorate degree in the Molecular and Cellular Basis of Disease. Camille's dissertation research focused on the biology of papillomaviruses their and cellular protein targets under the guidance of Dr. Scott Vande Pol. Before beginning graduate school, Camille purchased a house in Charlottesville, VA that she and her husband, Kelly, remodeled and updated while also pursuing their Ph.Ds. Camille currently resides in Durham, NC with Kelly, their two dogs (River and D'Artagnan), and their two cats (Willow and Kinase).