Alteration of the Epithelial Environment and Cellular Pathways by HPV type 16 E6 and NFX1-123

Justine Phuong Thuy Levan

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Reading Committee:

Rachel A. Katzenellenbogen, Chair

Stephen J. Polyak

Paul D. Lampe

Program Authorized to Offer Degree:

Pathobiology

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Abstract

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Justine Phuong Thuy Levan

Chair of the Supervisory Committee:

Rachel Adria Katzenellenbogen, M.D.

Department of Pediatrics, Department of Global Health

High-risk human papillomaviruses (HR HPV) are the causative agent of various anogenital and oropharyngeal cancers, accounting for approximately 5% of the global burden of cancer. The highest risk factor for development of these cancers is a persistent infection with HR HPV. Understanding what promotes long-lived HPV infection is critical for predicting and preventing serious disease outcomes. Persistent infection with HR HPV requires successful propagation in stratified squamous epithelium and is therefore driven by how HR HPVs manipulate the host cell to create an environment beneficial to the viral life cycle. The study of HR HPV, their interactions with the host cell, and how these interactions engender cellular changes that may secondarily lead to oncogenesis is the focus of this dissertation. This dissertation summarizes several ways in which a HR HPV oncoprotein, HPV type 16 E6 (16E6), partners with a host

protein, NFX1-123, to manipulate cellular processes and promote its life cycle. Previous work in the laboratory has shown that 16E6 utilizes NFX1-123 to upregulate differentiation markers in keratinocytes; increase levels of Notch1, the master cell fate regulator; and increase expression of telomerase, an enzyme critical to immortalization. The studies presented here further define the cellular processes altered by 16E6 and NFX1-123 to create a cellular milieu beneficial to the viral life cycle.

Evasion of the immune response by HR HPVs has been well-documented, but the many molecular mechanisms and protein partners driving these immune deregulatory functions have not yet been fully defined. The data in Chapter 3 establishes 16E6 and NFX1-123 as one such protein partnership through which HR HPVs interfere with the innate immune response and escape detection within infected epithelial cells. Overexpression of NFX1-123 in 16E6 keratinocytes 1) decreased expression of pro-inflammatory cytokines and interferon-stimulated genes (ISGs) at the mRNA and protein level 2) decreased total levels of TRAF6 and TAB2, two proteins in the signaling cascades that govern induction of these antiviral genes and 3) disturbed the normal localization of these signaling proteins. Initiating the immune response within infected keratinocytes is critical for detection of HPV infection and subsequent clearance; deregulating intracellular immune signaling allows HR HPV to avoid detection and resist immune clearance, a necessity for establishing persistent infection.

The HPV life cycle is tied to the differentiation programming of epithelial cells, with cellular differentiation driving initiation of the late stage of life viral cycle. Chapter 4 identifies for the first time a role for NFX1-123 in regulating keratinocyte differentiation and late events of the HPV life cycle. NFX1-123 augmented JNK signaling in 16E6 HFKs undergoing differentiation through expression of upstream kinase regulators of JNK, MKK4 and MKK7.

Consistent with the connection between the HPV life cycle and differentiation, modulating levels of NFX1-123 in HPV16-positive W12E cells affected late events of the viral life cycle. Altering levels of NFX1-123 resulted in altered mRNA expression levels of L1, the major capsid protein of HPV.

16E6 and NFX1-123 have previously been shown to target the known oncogenic protein telomerase, increasing expression and activity of its catalytic subunit, hTERT. In Chapter 5, we further explore how 16E6 and NFX1-123 targeting of telomerase affects growth and proliferative potential of cells over long term cell culture. The data presented in this chapter demonstrate that NFX1-123 is highly expressed in primary cervical cancers. *In vitro*, cells expressing 16E6 and overexpressing NFX1-123 had extended active growth, decreased senescence marker staining, and more rapid cell cycling compared to 16E6 expressing cells with endogenous amounts of NFX1-123. These effects on cell proliferation were associated with increased telomerase activity and expression of its catalytic subunit, hTERT. HPV 16-positive cervical cancer cell lines with knocked down NFX1-123 had slowed growth and reduced hTERT. These data indicate that 16E6 and NFX1-123 not only extend proliferative potential of cells via telomerase, but also modify active cellular growth, augment hTERT expression, and increase telomerase activity over time.

Altogether, the work in this dissertation describes multiple epithelial cell pathways that HR HPV type 16 E6 and NFX1-123 modify, which subsequently alter the host cell milieu to potentially promote HPV infection and support oncogenic development.

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Chapter 1
Introduction

1.1 Global Burden of Human Papillomavirus-Associated Diseases

The human papillomaviruses (HPV) are a group of over 200 double-stranded DNA viruses whose origins can be traced back millennia. Phylogenetic studies suggest that speciation of HPVs from papillomaviruses of other vertebrate hosts predates the speciation of *Homo sapiens*[1], and the global distribution of the same HPV types, even amongst populations of reproductively isolated Amazonian Indians, provides evidence for HPVs co-evolving with humans for over 100 million years[1–3]. Despite the ancient origins of the human papillomaviruses, their impact is most acutely measured in the modern era. Of the over 200 types of human papillomaviruses that have been identified, a mere fifteen are responsible annually for 569,847 cases of cervical cancer[4], 37,200 cases of oropharyngeal cancer[5], and 68,500 cases of anogenital cancer at other sites[6,7] that altogether account for 4.5% of the global burden of cancer[8–10]. HPV is the most prevalent sexually transmitted infection, affecting an estimated 11% of the world's population[4]. Thus, a significant proportion of the global population is at risk for development of HPV-associated cancers, and this number is rising. In the United States alone, rates of oropharyngeal squamous cell carcinomas increased by 2.7% per year among men and 0.8% per year among women from 1999-2015[11]. Although effective preventive vaccines are available, cumulative coverage of the total population is estimated at a mere 1.4%[12]. Given these statistics, further study about the nexus between HPV infection and development of cancer is still needed to prevent and successfully treat this significant cause of global mortality and morbidity.

The types of HPV that are associated with cancer are termed "high-risk" types of HPV (HR HPVs) and represent a unique subset within the study of human papillomaviruses. Despite their millennia of co-evolution with humans and often asymptomatic, mostly unapparent

infections, persistent infection with HR HPV types may lead to malignant disease. This is in contrast to low-risk HPV types (LR HPVs) which are almost never associated with cancers[13]. What underlies the differences between HR and LR HPVs and how this subsequently dictates the differences in the pathologies they can precipitate has been an intense area of study (reviewed in 14). Although these matters are not fully understood, one clear theme arises from decades of research: the oncogenicity of HR HPVs is intricately linked to their strategies of propagation in stratified squamous epithelium and is therefore driven by how HR HPVs manipulate the host cell to create an environment beneficial to the viral life cycle. The study of HR HPV, their interactions with the host cell, and how these interactions engender cellular changes that may lead to oncogenesis is the focus of this dissertation.

1.2 Human Papillomavirus Life Cycle and Genome Organization

In order to understand how HR HPVs manipulate the host cell, it is necessary to understand the typical life cycle and how the virus genome is organized. These topics will first be described in terms of the features commonly shared by all HPVs— both low- and high-risk— and then the differences that set HR HPVs apart will be highlighted.

1.2.1 HPV Life Cycle

HPV has a unique life cycle that is tied to the differentiation programming of its target cell, the keratinocyte[2,15]. HPV infection begins when the virus accesses the basal layer of stratified squamous epithelium. This is thought to occur through microabrasions, and it is known that infection can only be initiated in basal keratinocytes. Here, it maintains its genome as an episome at a low copy number of approximately 50-200 copies per cell[2,14–17]. In this stage, the early genes (E1, E2, E4, E5, E6, and E7) are expressed at low levels from the early viral promoter[16,18,19]. Viral DNA is replicated alongside cellular DNA as these cells divide, producing

infected daughter cells. Some of these daughter cells are subsequently pushed out of the basal layer and begin terminal differentiation. The second stage of the viral life cycle occurs once an infected cell rises through the epithelial suprabasal layers and begins to differentiate. This migration through the epithelial layers triggers the late stage of the viral life cycle. Events in this stage include vegetative genome amplification, wherein the genome copy number expands to thousands of copies; increased expression of viral replication proteins and expression of transcripts such as the capsid proteins L1 and L2 from a viral late promoter; and ultimately, packaging of the viral genomes and complete virion formation[14,18,20]. Certain late viral life cycle events occur only in specific epithelial layers. For example, expression of L1 and L2 only occurs in the stratum granulosum. There is thus a clear link between cellular differentiation programming of the host cell and the viral life cycle of HPV.

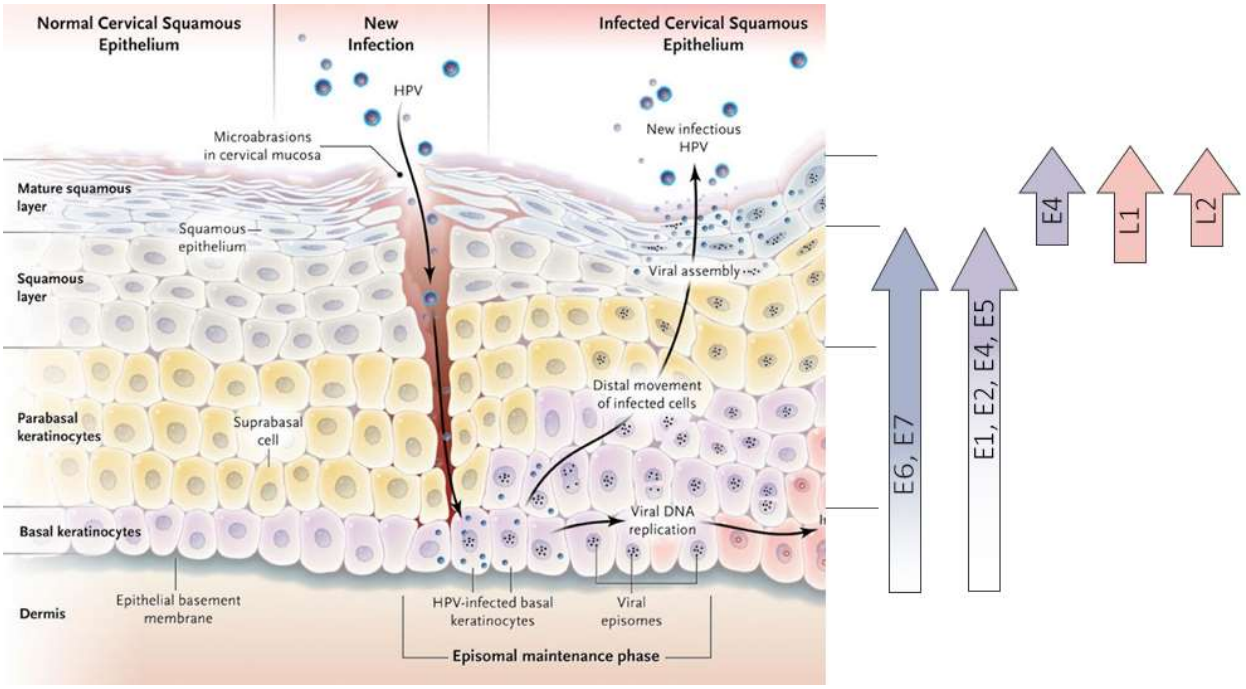


Figure 1.1 The HPV Life Cycle. Adapted from J.A. Khan N Engl J Med. 2009 Jul 16; 361(3):271-8 and J. Doorbar Vaccine. 2012 Nov 20;30 Suppl 5:F55-70. Left, diagram depicting the HPV life cycle in the layers of stratified squamous epithelium. Right, diagram depicting

expression of HPV proteins associated with epithelial layers. Darker coloring indicates increased expression, with E6 and E7 increasing expression in parabasal keratinocytes; E1, E2, E4, and E5 increasing expression in differentiating upper squamous layer (stratum spinosum); and E4, L1, and L2 having highest expression in the mature squamous layer (stratum granulosum).

1.2.2 HPV Genome Organization

HPVs have double-stranded circular genomes approximately 8 kilobases in length. HPVs of the genus *Alphapapillomavirus*, to which all HR and some LR HPVs belong, encode eight viral ORFs[14–16]. These are divided into the early genes, which are involved in viral transcription and replication, and the late genes, which encode the major (L1) and minor (L2) capsid proteins. The early genes are E1, E2, E4, E5, E6, and E7. E1 and E2 are a DNA helicase and transcription factor, respectively[14–16]. Functions of E4 are varied, but it is generally involved in viral genome amplification with additional purported roles in virus release and transmission[21]. E5 encodes a short membrane-associated protein whose role in productive infection is not well understood; the limited information about its functions come from studies in only two papillomavirus types[22]. E6 and E7 are the most widely studied HPV genes, for it is differences in these proteins that largely drive the separation between HR and LR HPVs and the pathologies they can cause. In the broadest terms, E6 and E7 manipulate cellular processes in order to create a replication-competent environment, predominately through binding p53 and Retinoblastoma (Rb) family members[13,14]. E6 can bind p53 and inhibit its transactivation, while E7 destabilizes p130, a member of the Rb family. The differential functions of E6 and E7 in HR HPVs as compared to LR HPVs derive from two arenas: first, associations with cellular partners in addition to those described above and second, the outcome of these associations, which extend beyond just binding. These processes are described in more detail in the following section.

1.3 HIGH-RISK HPV E6 AND E7

Because the cooperative actions of HR E6 and E7 are sufficient to cause cellular immortalization and because they can activate oncogenic pathways that lead to cancer, they are categorized as oncoproteins[15]. Although this cancer is an incidental outcome and not the intention of the virus, it nonetheless underscores the public health importance of understanding the molecular underpinnings of HR HPV dysregulation of and interactions with the host cell, and how these interactions create oncogenic cellular changes.

1.3.1 High-risk E7

The most extensively studied capacity of E7 is its role in promoting cellular proliferation. E7 drives aberrant cell cycle re-entry and cell proliferation predominately through targeting members of the Retinoblastoma (Rb) family, a function that is shared with oncoproteins of other DNA tumor viruses. Indeed, E7 does bear sequence similarity to the SV40 large T antigen and the adenovirus E1A oncoproteins[23]. E7 binds Rb family members and directs their degradation, resulting in constitutive expression of proliferative genes, S phase entry, and DNA synthesis[2,15,24]. Whereas both LR and HR E7 share the ability to bind and degrade Rb family member p130, HR E7 alone is also able to bind and degrade p105 and p107[25]. Degradation of these proteins drives cell cycle re-entry in the upper layers of the stratified squamous epithelium, in cells that would have otherwise ceased proliferating[15,24,25]. This ability of HR E7 to provoke extensive cell division is complemented by HR E6.

1.3.2 High-risk E6

One of the main activities of HR E6 serves to offset the consequences of HR E7 functioning. In normal uninfected epithelial cells, unscheduled proliferation would result in accumulation of the tumor suppressor protein p53 and subsequent apoptosis[26,27]. However, in

cells infected with HR HPV E6 binds p53 and targets it for degradation[15,28–30]. It does so in conjunction with E6 associated protein (E6AP), an E3 ubiquitin ligase[29,31]. In addition to preventing apoptosis, E6 has multiple roles specific to HR HPVs that have been shown to create a cellular environment supportive of viral infection and contribute to development of HPV-associated malignancies. HR E6 directs the degradation of not only p53, but also a class of proteins called PDZ (PSD-95/DLG/ZO-1) domain containing proteins[14,30,32]. The PDZ proteins that are known substrates of HR E6 are involved in cell polarity, adhesion, and signaling. Studies of HR E6 mutants lacking the ability to bind these proteins display decreased transformation phenotypes[32,33], and transgenic mice expressing these mutants did not develop hyperplasia, indicating the importance of the relationship between cellular PDZ proteins and HR E6 in oncogenesis[34]. HR E6 also activates telomerase, an enzyme that drives cellular immortalization and is universally expressed in HPV-associated cancers[35,36]. Telomerase works to extend the linear ends of chromosomes, which are serially eroded with cellular division[37]. When telomeres are critically shortened, growth arrest is triggered[37]. By activating telomerase, HR E6 ensures that the cell avoids senescence, apoptosis, and cellular crisis[35].

1.3.3 High-risk E6 and E7 Drive Oncogenesis

The greatest risk factor for development of HPV-associated cancer is a persistent infection with HR HPV[15,19,38]. In most women, infections with HPV are cleared within a few months to 2 years[15,39–41]. However, some evidence suggests that persistent infection remains below detectable levels, even after clearance of cytological and histological abnormalities[15,42,43]. How persistent HR HPV infection drives oncogenesis is best understood for cervical cancer, but there remain many yet unanswered questions; furthermore, whether or not the same mechanisms apply to oropharyngeal cancers is still unknown. As mentioned earlier, oncogenesis is not a

specific purpose of HR HPVs; rather, it results from the combination of the activities of the HR HPV oncoproteins and unintended consequences that lead to their deregulated expression. E6 and E7 are required for cell immortalization and are found highly expressed in all HPV-associated cancers[15]. Expression of these oncoproteins results in accumulation of cell damage and chromosomal instability, thereby driving cancer progression. This chromosomal instability in the cell is a result both of driving cell proliferation in the absence of normal checkpoints and of activities independent of Rb and p53 degradation[2,24,28,29,44,45]. Apart from those functions, HR E6 and E7 encode additional mechanisms that cause centrosome defects, abnormal nuclei numbers, and anomalous mitosis[46–48]. HPV-associated malignancy is born from the marriage of extended proliferative potential and genetic instability wrought by HR E6 and E7.

HR E6 and E7 are able disrupt a multitude of cell processes in addition to proliferation and apoptosis, including cell adhesion, G protein signaling, and immune evasion, the latter of which will be described in the following section[49,50]. Because the long-term expression of viral oncoproteins and accumulation of genetic errors are key to carcinogenesis, the length of infection with HR HPV is therefore a crucial factor in determining outcome[15,51]. The many ways in which the HPV oncoproteins manipulate the cellular milieu are still being discovered. Uncovering the strategies employed by HR HPVs to promote the viral life cycle will reveal how they engender changes in the cell and unlock what drives development of malignancy.

Notably, HR E6 and E7 lack enzymatic activity and thus rely on partnership with host cellular proteins to achieve any of the functions described above. This reliance on host proteins is exemplified in HR E6's requirement of E6AP and cellular proteasome machinery for degradation of target proteins but is common to all capacities of the HPV oncoproteins. These partnerships, and the cellular processes they disrupt, are critical to promotion of a productive and long-lived

viral life cycle, as well as malignant development and progression. One such partnership between the HR E6 protein of HPV type 16 and the cellular protein NFX1-123 will be discussed in greater detail in this dissertation, and so merits further description.

1.4 Partnership Between HPV Type 16 E6 And NFX1-123

NFX1-123 is one of two splice variants of the *NFX1* gene identified as a binding partner of the HR HPV type 16 E6 (16E6) in a yeast two-hybrid screen and later confirmed via co-immunoprecipitation[52]. The two isoforms of *NFX1*, NFX1-123 and NFX1-91, are named for their relative size in kilodaltons, and although they share a common N terminus and central domain, NFX1-123 has a unique C terminus. NFX1-91 is targeted for degradation by E6 and E6AP and will not be a significant focus of this dissertation[52]. NFX1-123, however, is overexpressed in cervical cancer cell lines[53] and primary cervical cancer samples, and its partnership with 16E6 has been documented. The primarily cytoplasmic NFX1-123 has important conserved protein motifs including: a PAM2 motif, which binds cytoplasmic poly(A) binding proteins (PABPCs), a PHD/RING domain with E3 ubiquitin ligase activity, eight zinc finger-like domains that are putative DNA-binding domains, and an R3H domain with putative single-strand nucleic acid binding ability (Fig 1.2)[54]. These protein motifs define the potential functionalities of NFX1-123. The function of NFX1-123 is also defined by the composition of the other proteins with which it complexes[55,56].

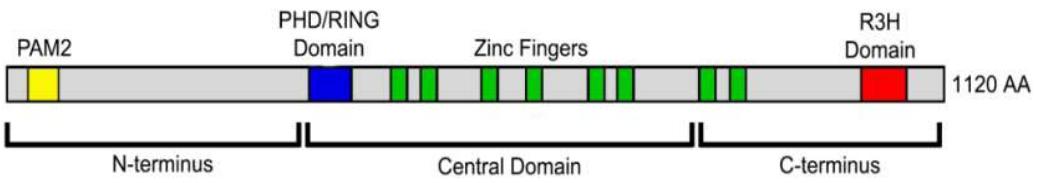


Figure 1.2 Protein motifs of NFX1-123. NFX1-123 contains a PAM2 motif, a recognition site for PABPCs; a PHD/RING domain with E3 ubiquitin ligase function; 8 zinc-like fingers

that are putative DNA binding domains; and an R3H domain with putative single-strand nucleic acid binding ability.

NFX1-123 functions synergistically with 16E6 to post-transcriptionally stabilize the mRNA of hTERT, the catalytic subunit of telomerase, increasing its expression and subsequent telomerase activity[54–56]. This was found to require the R3H and PAM2 motifs and cooperation with PABPCs. In the absence of 16E6, NFX1-123 alone did not have an effect on telomerase, indicating that the partnership between the two proteins was crucial in this particular function[54]. 16E6 and NFX1-123 have also been shown to upregulate Notch1, a regulator of cellular proliferation and differentiation[57–61]. Notch1 and its canonical pathway genes Hes1 and Hes5 are increased by 16E6 and NFX1-123. Differentiation markers Keratin 1 and Keratin 10 were also increased by NFX1-123 through Notch1, as their upregulation was directly associated with Notch1 receptor stimulation; however, blocking Notch1 signaling did not blunt their expression[53]. Altogether, these data establish the multifunctional partnership between HPV type 16E6 and NFX1-123 and set the foundation for investigations into the ways in which they together alter cellular processes and the cellular environment.

1.5 The Immune Response to HPV Infection and Immune Evasion

HPV needs to overcome major barriers in order to successfully complete a life cycle and establish a persistent infection. One of the most significant barriers is host elimination of an infected cell through the immune response. Evading the immune response is yet another arena in which HR HPVs have developed specific mechanisms to manipulate the host cell environment. This, too, contributes to oncogenic potential or HR HPV in the sense that persistent infection with HR HPV is required for cancer development and immune evasion is a necessary condition for a persistent infection.

1.5.1 The immune response to HPV

The majority of HPV infections are cleared within a few months, with only approximately 10% of persons developing a persistent infection[41]. The host innate immune response is the first line of defense during the early stages of an HPV infection. Multiple cells are involved in promoting an immune response against HPV infection, including dendritic cells, Langerhans cells, natural killer T cells, and keratinocytes themselves. Keratinocytes provide robust immune defenses, expressing pathogen recognition receptors (PRRs) that activate signaling cascades and lead to production of soluble immune effector proteins such as cytokines and interferon-stimulated genes[62,63]. These known antiviral genes create a pro-inflammatory environment for viral clearance[40,41,64]. They also provide co-stimulatory signals that, alongside antigen presentation from keratinocytes and professional antigen-presenting cells, orchestrate the T-cell response[64].

Resolution of HPV infection is primarily thought to be a cell-mediated immune response[65], as numerous studies of lesion regression in both animal models and anogenital infections show a clear relationship between T cell activation and infiltration and disease regression[66,67]. Regressing lesions had significantly greater numbers of T cells and macrophages compared to non-regressing controls[65]. Within the cell-mediated response, CD4+ lymphocytes dominate. Reduced CD4+ cell counts correlate inversely with higher grade of neoplasia[68]. The role of the humoral response in natural HPV infection is much less decisive. Although neutralizing antibodies against the L1 and L2 capsid proteins of HPV are protective, thus forming the basis of the vaccine, these are poorly produced in natural HPV infection[40,69,70]. Seroconversion in a natural infection produces much lower antibody titers than in vaccinated individuals, if at all. Studies enumerating the percentage of women that seroconvert after HPV

infection vary in final statistics but concur that it is not 100%[71–73]. Despite most HPV infections being resolved by the immune response, the small proportion of persistent infections nonetheless still occur and clearly indicates the ability of HPVs to evade the immune response.

1.5.2 Immune evasion strategies of high-risk HPV

Indirect evidence for the ability of HPV to evade the immune system can be seen in both in the extended time it takes from initial infection to appearance of a lesion, and then clearance of that lesion—months, even years[14,74–77]. Unlike an acute viral infection such as influenza, where recognition and resolution of infection is rapid, the slow clearance of an HPV infection indicates avoidance of common immune mechanisms. HPV's immune evasion strategies consist of both passive byproducts of its life cycle and active mechanisms.

The tissue tropism and life cycle of HPV preclude many of the normal immune events that would lead to elimination. HPV is extremely tissue-specific, infecting only stratified squamous epithelium and lacking a blood-borne phase[2,14,15,78]. In addition, mature viral particles are shed in a non-cytolytic manner; they are released as the uppermost keratinocytes naturally slough off[14,41]. These factors, in combination with the tight temporal regulation of viral protein expression, contribute to a lack of systemic activation or inflammation and sequester the virus away from most immune defenses.

Many studies have identified the ways in which HPV, and HR HPV especially, are able to actively subvert and hinder immune function. These are predominantly mediated by the E6 and E7 oncoproteins, although some roles for the protein E5 have been demonstrated (reviewed in 79). These mechanisms generally act to prevent production of pro-inflammatory cytokines, interferons, and other antiviral genes or to disrupt antigen presentation. E6 has been shown to dampen interferon signaling by binding and preventing activity of the signaling molecule Tyk2

and the transcription factor IRF3, both members of the interferon signaling pathway[80,81]. E6 also downregulates expression of individual cytokines IL-18 and MCP-1[82,83], and an important keratinocyte surface protein, E-cadherin[84]. E7 inhibits interferon signaling through inactivation of IRF-1, prevents antigen presentation through suppressing expression of MHC Class I family members, and inhibits recognition of cytoplasmic viral nucleic acid[85–87]. While it is evident that E6 and E7 predominate the immune evasion strategies of HPV, the specific molecular mechanisms and protein partners driving these immune deregulatory functions remain largely unknown.

GOALS OF THIS DISSERTATION

Persistent infection with HR HPV is the highest risk factor for cancer development[69,76,88]; understanding what promotes long-lived HPV infection is therefore critical for predicting and preventing serious disease outcomes. Although much of the basic HPV viral life cycle has been described, there are gaps in our knowledge of processes important for HPV replication[14]. Studies in the field have identified the biology of HPV infection and have explored downstream oncogenesis as a result of persistent infection, but the factors influencing the transition from an infection to a persistent infection are not well-defined. The focus of the work presented in this dissertation is to elucidate mechanisms by which HR HPVs alter the cell environment to promote its life cycle and contribute to persistent infection.

The goals for this dissertation are to better understand the roles that HR HPV type 16 E6 and NFX1-123 specifically play in altering the host cell milieu to promote HPV infection and secondarily support oncogenic development. These studies will further define the cellular processes altered by this viral oncoprotein-host protein partnership to create a cellular milieu beneficial to the viral life cycle. Chapter 3 outlines how this partnership dysregulates the innate immune response to contribute to viral evasion of immune clearance. Chapter 4 explores the ability of NFX1-123 to regulate keratinocyte differentiation and late events of the HPV life cycle. Chapter 5 demonstrates that 16E6 and NFX1-123 modify active cellular growth and augment hTERT expression and telomerase activity over time. In elucidating how this partnership cooperates to engender cellular changes, this work will uncover possible points of intervention to arrest persistent infection and subsequently decrease disease burden, lower cancer incidence, and prevent cancer mortality.

Chapter 2
Methods

This chapter discusses methods that are common to all of the studies described in Chapters 3, 4, and 5. Additional specific methods and analyses are included in each individual chapter.

2.1 Isolation Of Primary Human Foreskin Keratinocytes

Human keratinocytes were isolated from neonatal foreskins that were anonymous and considered not human subjects by the Seattle Children's Research Institute Institutional Review Board. Foreskins were treated with sterile Dispase type II protease (Gibco, Waltham, MA) for 48 hours at 4°C. After 48 hours, the epidermal and dermal layers were separated, and the dermal layer discarded. The epidermal layer was incubated with 0.05% trypsin, shaking, for 4 minutes. The sample was then centrifuged at 201 x *g*, the trypsin solution discarded, and the cellular pellet resuspended in 10mL of EpiLife medium (Life Technologies, Carlsbad, CA). The resuspended pellet was transferred to a 10cm tissue culture dish and incubated at 37°C. Cultures were fed fresh medium every 48 hours and examined over the subsequent 48-72 hours for presence of primary keratinocyte colonies and the absence of primary fibroblasts.

2.2 Tissue Culture

Monolayer cell culture

Cultures of primary human foreskin keratinocytes (HFKs) were isolated as described above and grown in EpiLife medium (Life Technologies, Carlsbad, CA) supplemented with 60µM calcium chloride, penicillin-streptomycin, and human keratinocyte growth supplement (Life Technologies, Carlsbad, CA)[52]. Human embryonic kidney 293T cells were obtained from ATCC

(CRL-3216) and grown in Dulbeccos' Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. SiHa cervical cancer cells and 3T3 mouse fibroblast cells were received from the laboratory of Dr. Denise Galloway (Seattle, WA) and also grown in DMEM with 10% FBS and penicillin-streptomycin. W12 clone 20863 cells (W12E), cervical epithelial cells containing full copies of the HPV16 genome, were a gift from the laboratory of Dr. Paul Lambert (Madison, WI). W12E cells were maintained in E-media supplemented with 5% fetal bovine serum at sub-confluency on mitomycin C-treated 3T3 feeder cells as published[89]. All cells were grown at 37°C, 5% CO_2.

Organotypic raft cultures

Organotypic raft cultures were grown in E-media containing 5% fetal bovine serum, as published, with 6-well transwells (Corning, Corning, NY) in place of wire mesh[90,91]. Briefly, mitomycin C-treated 3T3 fibroblasts are mixed with collagen I and plated into transwells of a 6-well tissue culture dish. The collagen-fibroblast plug is solidified for 30 minutes at 37°C and subsequently submerged in E medium. After 2-4 days, $1x10^6$ trypsin harvested HFKs or W12E cells are plated on top of the plug. Cells are grown to confluency for 2-4 days, as judged by color change in medium. Once cells are confluent, the rafts are grown at an air-liquid interface, created by feeding cells with medium in the space created between the base of the transwell and the outer well of the 6-well tissue culture dish. Rafts are fed with E medium for 7 days, and then with E medium with 1.2mM calcium chloride for another 14 days.

2.3 PLASMIDS

Expression constructs

pBabe-puro HPV type 16 E6 (16E6)

The cDNA sequence HPV type 16 E6 was inserted into the retroviral vector pBabe-puro BamHI and EcoRI sites through restriction enzyme-based cloning by Dr. Weifeng Luo in the laboratory of Dr. Denise Galloway (Seattle, WA).

FLAG-tagged NFX1-123 (FN123)

The NFX1-123 isoform of the *NFX1* gene was PCR cloned from a HeLa cell cDNA library by Lindy Gewin in the laboratory of Dr. Denise Galloway (Seattle, WA). A FLAG tag was added to the 5' end of the NFX1-123 cDNA, and it was subsequently inserted into the LXSN vector background via the Gateway recombination-based system (Thermo Fisher Scientific, Waltham, MA). Briefly, attB1 and attB2 sites were added 5' and 3', respectively, to the cDNA sequence of FLAG-tagged NFX1-123. This was cloned into the vector pDONR201 vector through a proprietary BP reaction to create the Gateway entry vector. The NFX1-123 gene was subsequently transferred from this entry vector into the LXSN destination vector through the LR reaction, ultimately creating the FLAG-tagged NFX1-123 construct (FN123) containing the NFX1-123 isoform in the LXSN background[52].

Short hairpins NFX1-123 c-FUGW and scrambled short hairpin c-FUGW (sh1, sh2, scr)

Short hairpins were designed specifically for the novel C terminus of NFX1-123. For short hairpin 1 (sh1), the siRNA Finder program formerly by Ambion at http://www.ambion.com/techlib/misc/siRNA_finder.html was used. The scramble short hairpin control (scr) was designed by Dr. Rachel Katzenellenbogen to have equivalent nucleotide distribution. Both sequences were searched using BLAST to confirm that they aligned with only NFX1-123 and no known gene, respectively[54]. Short hairpin 2 (sh2) was designed using Sigma

MISSION and BLAST used to confirm no off-target effects[92]. For all short hairpins, phosphorylated and annealed oligonucleotides with BamHI and EcoRI sticky ends were ligated into the c-FUGW vector BamHI and EcoRI sites. The c-FUGW vector was a gift from the laboratory of Dr. Robert Eisenman (Seattle, WA). See Table 2.1 for oligonucleotide sequences.

Table 2.1 - List of oligonucleotides used to generate short hairpin constructs.

Plasmid	Oligonucleotides (5' – 3')
sh1	**F-** GATCCAACCGAAGCGCAATGTGGTGGTTCAAGAGACCACCACATTGCGCTTCGGTTTTTTTTGG
	R- AATTCCAAAAAAAACCGAAGCGCAATGTGGTGGTCTCTTGAACCACCACATTGCGCTTCGGTTG
sh2	**F-** GATCCGCGTGAATAAGGGAAAGAATTTCAAGAGAATTCTTTCCCTTATTCACGTTTTTTGG
	R- AATTCCAAAAAACGTGAATAAGGGAAAGAATTCTCTTGAAATTCTTTCCCTTATTCACGCG
scr	**F-** GATCCTCGAACGGTAGGACTGCGAGATTCAAGAGATCTCGCAGTCCTACCGTTCGATTTTTTGG
	R- AATTCCAAAAAATCGAACGGTAGGACTGCGAGATCTCTTGAATCTCGCAGTCCTACCGTTCGAG

Retroviral constructs

Vesicular stomatitis virus G glycoprotein CMV (VSV-G pLenti)

This construct was used in the production of lentivirus. It encodes the G glycoprotein of the vesicular stomatitis virus (VSV-G) under control of the CMV immediate-early promoter. VSV-G pLenti was a gift from the laboratory of Dr. Robert Eisenman (Seattle, WA).

Delta 8.9

This construct was used in the production of lentivirus. It encodes the Gag, Pol, Tat and Rev proteins from HIV-1 under control of the CMV immediate-early promoter. Delta 8.9 was a gift from the laboratory of Dr. Robert Eisenman (Seattle, WA).

Tat (CMV-tat)

This construct was used in the production of lentivirus. It encodes the Tat protein from HIV-1 driven by the CMV immediate-early promoter. Required for transactivation of HIV-1 LTR of pJK3. CMV-tat was a gift from the laboratory of Dr. Denise Galloway (Seattle, WA).

pJK3

This construct was used in the production of retrovirus. It encodes the Gag and Pol proteins from Moloney murine leukemia virus driven by the HIV-1 LTR. pJK3 was a gift from the laboratory of Dr. Denise Galloway (Seattle, WA).

LTR vesicular stomatitis virus G glycoprotein (LTR VSV-G)

This construct was used in the production of retrovirus. It encodes VSV-G driven by the HIV-1 LTR. VSV-G was a gift from the laboratory of Dr. Denise Galloway (Seattle, WA).

2.4 Retrovirus Production and Transduction of Primary Cells

Lentivirus and retrovirus were produced in 293T cells by transient vesicular stomatitis virus G-pseudotyped virus (VSV-G) production protocols. Briefly, 293T cells were grown to 50-60% confluency on 15cm tissue culture plates. For lentivirus production, short hairpin NFX1-123 (sh1 or sh2) c-FUGW or scramble c-FUGW (scr) constructs were co-transfected with VSV-G pLenti and Δ8.9 plasmids into 293T cells using FuGENE6 (Roche, Alameda, CA)[93]. Lentivirus was serially collected once daily for four days. For retrovirus production, pBabe-puro HPV type 16 E6 (16E6), FLAG-tagged NFX1-123 (FN123), empty vector pBabe-puro, or empty

vector LXSN were transfected into 293T cells along with CMV-tat, pJK3, and LTR VSV-G plasmids[94]. Retrovirus was serially collected twice daily for 48 hours.

For transduction, virus was concentrated by spinning at 33,900 x *g* in an ultracentrifuge at 4°C for 90 minutes. Supernatant was discarded and the viral pellet mixed with Polybrene (8 μg/mL) (EMD Millipore, Billerica, MA). Resuspended virus was incubated with 50 to 60% confluent cells on 10cm tissue cultures dishes and left on overnight. After 3 to 18 hours, virus-containing medium was aspirated and replaced with fresh medium. All lentivirus infections (scr, sh1 and sh2) were confirmed by green fluorescent protein expression 24-48 hours post transduction. For retroviral transductions (16E6, FN123, LXSN, pBabe-puro) the cells were expanded 24 hours post transduction and placed under neomycin/G418 selection (50 μg/mL) or puromycin selection (0.5 μg/mL) 48 hours post transduction.

2.5 RNA Expression Analyses

RNA extraction and cDNA generation

For analysis of mRNA expression, cells were trypsin harvested and lysed in 1mL of TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated using phenol-chloroform extraction and isopropanol precipitation. RNA pellets were resuspended in nuclease-free water at 100ng – 1μg/μL. To generate cDNA, 2μg of total RNA was treated with DNase I (Thermo Fisher Scientific, Waltham, MA) in a 20μL reaction to ensure elimination of genomic DNA carried over from the extraction process. DNase-treated RNA was then reverse transcribed using random hexamer primers and Superscript IV (Invitrogen, Carlsbad, CA) according to manufacturer directions.

Primer-based quantitative PCR

Quantitative PCR (qPCR) detection of NFX1-123, NFX1-91, hTERT, FLAG-tagged NFX1-123, and 36B4 expression levels was conducted on the ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). Briefly, forward and reverse primers for the gene of interest (400nM final concentration) and 2µL of cDNA were mixed with Power SYBR Green or PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) per 20µL reaction. cDNA was diluted 1:4 prior to usage in the PCR reaction. Cycling conditions were carried out according to manufacturer's instructions, unless otherwise noted in Table 2.2 Standard curves were generated per target gene of interest and each reaction performed in triplicate. The StepOnePlus software was used to calculate Ct thresholds and Microsoft Excel used to calculate quantities using the standard curve. Expression was normalized to 36B4, a ribosomal protein mRNA, and relative fold change calculated as the ratio between the experimental sample compared to the control. Values graphed are the mean fold change in each sample compared to control, and error bars graphed represent 95% confidence intervals.

Table 2.2 List of primers used in qPCR studies

Target	Primer sequence (5' – 3')	Annealing /extension temperature
NFX1-123	**F-** CCACAGCTTCCCTCCCA **R-** CCTGGACGTCAAAATAGTCAA	60°
NFX1-91	**F-** TTACCCTCCAGTTCCCT GTG **R-** CATGCGTGTGCAGGTATCTT	60°
hTERT	**F-** CGAGCTGCTCAGGTCTTTCTTTTATG **R-** CCACGACGTAGTCCATGTTCACAATC	62°

36B4	**F-**	TGCCAGTGTCTGTCTGCAGA	60°
	R-	ACAAAGGCAGATGGATCAGC	
FLAG	**F-**	GGACTACAAAGACGACGAC	60°
	R-	TG CCA AGG TTG ATT CTG AA	

TaqMan probe-based quantitative PCR

Quantitative PCR detection of GAPDH, CXCL1, TNF, OAS1, OAS2, Keratin 1, Keratin 10, Involucrin, and Loricrin expression levels was performed using an ABI StepOne Plus system (Applied Biosystems, Foster City, CA). Amplification was carried out using TaqMan Master Mix and pre-designed TaqMan probes (Applied Biosystems, Foster City, CA). Briefly, probes, master mix, and 9µL of cDNA were mixed in a 20µL reaction. cDNA was diluted 1:20 prior to usage. Cycling conditions were carried out according to manufacturer's instructions. Reactions were performed in triplicate. The StepOnePlus software was used to calculate Ct thresholds and Microsoft Excel used to calculate fold change using the Pfaffl method[95]. Expression levels were normalized to GAPDH mRNA levels within each sample. Values graphed are the mean fold change in each sample compared to control, and error bars graphed represent 95% confidence intervals.

Table 2.3 List of pre-designed TaqMan probes used in qPCR studies

Target	Gene	TaqMan Assay ID
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
CXCL1	Chemokine (C-X-C motif) ligand 1	Hs00236937_m1

TNF	Tumor necrosis factor alpha	Hs00174128_m1
OAS1	2'-5'-Oligoadenylate Synthetase 1	Hs00196324_m1
OAS2	2'-5'-Oligoadenylate Synthetase 2	Hs00942643_m1
K1	Cytokeratin 1	Hs00196158_m1
K10	Cytokeratin 10	Hs00166289_m1
IVL	Involucrin	Hs00846307_s1
LOR	Loricrin	HS01894962_s1

2.6 Protein Expression Analyses

Protein extraction and Western blot

For analysis of protein expression, cells were trypsin harvested and lysed in WE16th lysis buffer [50 mM Tris-HCl at pH 7.5, 250 mM NaCl, 1% NP-40, 0.1% SDS, 20% glycerol, 10 µM zinc chloride, 2 mM dithiothreitol, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a Complete Mini Protease Inhibitor tablet (Roche, Alameda, CA)] or in 2X sodium dodecyl sulfate (SDS) sample buffer [100 mM Tris-HCl pH 6.8, 1% SDS, 40% Glycerol, 20% β-mercaptoethanol]. Lysates were then sonicated and clarified by centrifugation at 20,817 x *g* for 15 minutes. Protein concentrations were determined by using the DC protein assay for lysates in WE16th buffer or the RC DC protein assay for lysates in 2X SDS Sample buffer (Bio-Rad, Hercules, CA). Equal amounts of protein lysate were electrophoresed on 4-12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to Immobilon-FL membranes (Millipore, Burlington, MA). Membranes were blocked in Prometheus OneBlock (Genesee Scientific, San Diego, CA) or 5% nonfat dry milk in TBS-T. Blots were probed using the following antibodies and conditions:

Table 2.4 – List of antibodies used in these studies

Target	Company		Dilution
Keratin 1	Thermo Fisher	PA5-26699	1:500
Keratin 10	Thermo Fisher	DE-K10	1:500
Involucrin	Santa Cruz Technology	SY5	1:1000
Total JNK	Santa Cruz	D-2	1:500
Phospho-JNK/SAPK	Cell Signaling Technology	81E11	1:1000
Total SEK/MKK4	Cell Signaling Technology	9153	1:1000
Total MKK7	Cell Signaling Technology	4172S	1:1000
Total ERK	BioLegend	W15133B	1:1000
p53	Calbiochem	OP03	1:1000
TRAF6	Cell Signaling Technology	D21G3	1:500
TRIF	Cell Signaling Technology	4596	1:1000
TAK1	Cell Signaling Technology	5206	1:500
TAB1	Cell Signaling Technology	3226	1:500
TAB2	Cell Signaling Technology	3745S	1:500
NFX1-91	House made	-	1:1000
NFX1-123	House made	-	1:1000
GAPDH	Abcam	6C5	1:10,000
Actin	Santa Cruz Biotechnology	I-19	1:1000
Rabbit IgG	HRP-linked: Cell Signaling Technology	7074	1:2000
	Alexa Fluor 680-linked: Invitrogen	A27042	1:5,000
	Alexa Fluor 488-linked	A27034	1:500

Mouse IgG	HRP-linked: Cell Signaling Technology	7076	1:2000
	Alexa Fluor 680-linked: Invitrogen	A21057	1:5,000
	Alexa Fluor 546-linked: Invitrogen	A21123	1:500
Rat IgG	Alexa Fluor 680-linked: Invitrogen	A21096	1:5000

The rabbit polyclonal anti-NFX1-123 and anti-NFX1-91 antibodies were generously provided by Dr. Ann Roman[52]. The NFX1-123-specific antibody was raised to a recombinant His-tagged C-terminal fragment of NFX1-123 from amino acids 1102-1120 (SNLQKITKEPIIDYFDVQD). The NFX1-91-specific antibody was raised to a C-terminal peptide from amino acids 813-830 (WASTQKKRSHYMKKIPAH). All films were scanned using Epson Perfection V700 and imported using Adobe Photoshop or blots were scanned using the Li-Cor Odyssey. Densitometry was calculated using ImageJ software.

Chapter 3
Innate immune dysregulation by 16E6 and NFX1-123

The following text is from the article:

Levan J, Vliet-Gregg PA, Robinson KL, Katzenellenbogen RA. Human papillomavirus type 16 E6 and NFX1-123 mislocalize immune signaling proteins and downregulate immune gene expression in keratinocytes. (2017) **PLoS One**. Nov 8;12(11): e0187514. doi: 10.1371/journal.pone.0187514

Figure numbers have been updated to conform to the formatting of this dissertation. The text remains as published with minor editorial changes.

3.1 ABSTRACT

Human papillomavirus (HPV) is the most prevalent sexually transmitted infection, affecting an estimated 11% of the world's population. The high-risk HPV types (HR HPV) account for approximately 5% of the global burden of cancer and thus cause high morbidity and mortality. Although it is known that persistent infection with HR HPV is the greatest risk factor for developing HPV-associated cancer, and that the HPV early proteins E6 and E7 dysregulate immune detection by its host cells, the mechanisms of immune evasion by HR HPV are not well understood. Previous work in the laboratory identified the endogenous cytoplasmic host protein NFX1-123 as a binding partner of the HR HPV type 16 oncoprotein E6 (16E6). Together NFX1-123 and 16E6 affect cellular growth, differentiation, and immortalization genes and pathways. In a whole genome microarray, human foreskin keratinocytes (HFKs) stably expressing 16E6 and overexpressing NFX1-123 showed a diverse set of innate immune genes downregulated two-fold or more when compared to 16E6 cells with endogenous NFX1-123. We demonstrated that 16E6 and NFX1-123 decreased expression of pro-inflammatory cytokines and interferon-stimulated genes (ISGs) in 16E6 HFKs at the mRNA and protein level. Knock down of NFX1-123 in 16E6 HFKs resulted in a derepression of innate immune genes, pointing to the requirement of NFX1-123 for immune regulation in the context of 16E6. Studies using immunofluorescent microscopy revealed that 16E6 and NFX1-123 disturbed the normal localization of signaling proteins involved in initiating the immune response. This chapter identifies NFX1-123 as a critical host protein partner through which 16E6 is able to subvert the immune response and in turn permit a long-lived HR HPV infection.

3.2 INTRODUCTION

As described in the Introduction (Chapter 1), the highest risk factor for development of HPV-associated disease is a persistent infection with high-risk HPV (HR HPV)[15]. Although the majority of HPV infections are cleared by a successful immune response, a persistent infection remains for some people[39,40,42,43]. The factors that contribute to a persistent HR HPV (HR HPV) infection are not fully defined, but it is clear that effective avoidance of the host immune detection and response is integral to this process.

In addition to their structural role, keratinocytes are involved in immune system functioning. During the early stages of an HPV infection, the host innate immune response is the first line of defense against the infection. Multiple cells are involved in promoting an immune response against HPV infection, including dendritic cells, Langerhans cells, natural killer T cells, and keratinocytes. Keratinocytes provide robust immune defenses, expressing pathogen recognition receptors (PRRs) that activate signaling cascades and lead to production of soluble immune effector proteins such as cytokines and interferon-stimulated genes[62]. Despite these defenses, there can be a prolonged period between HPV infection and its clearance—months, even years[74,75,88]. This prolonged infection is a risk factor for development of oncogenic disease. The delay between infection and clearance indicates that HPV, and specifically HR HPVs, have evolved strategies to evade the innate immune responses of keratinocytes. Indeed, upon infection of keratinocytes, HR HPV actively subvert and hinder immune function. These disruptions are predominantly mediated by the HR E6 and E7 oncoproteins (reviewed in [79] and [96]).

Immune evasion mechanisms mediated by E6 and E7 generally act to prevent production of pro-inflammatory cytokines, interferons, and other antiviral genes or to disrupt antigen presentation[40]. E6 has been shown to dampen interferon signaling by binding and preventing

activity of the signaling molecule Tyk2 and the transcription factor IRF3, both members of the interferon signaling pathway[80,97]. E6 also downregulates expression of individual cytokines IL-18 and MCP-1[82,83] and a surface protein important for recruitment of antigen presenting cells, E-cadherin[82–84]. E7 inhibits interferon signaling through inactivation of IRF-1, prevents antigen presentation through suppressing expression of MHC Class I family members, and inhibits recognition of cytoplasmic viral nucleic acid[85–87]. While it is evident that E6 and E7 predominate the immune evasion strategies of HPV, the specific molecular mechanisms and endogenous cellular protein partners that drive these immune deregulatory functions remain largely unknown. Protein partnerships are essential to any E6 and E7 functionality, as the HPV proteins have no enzymatic capacity of their own, and the protein partners utilized are typically host proteins[24,28,29,44,98].

Our laboratory has previously studied the partnership between the E6 protein of HR HPV type 16 (16E6) and the host cytoplasmic protein NFX1-123[54–56]. NFX1-123 is endogenously expressed in human epithelial cells, and is increased in cervical cancer cell lines[53]. NFX1-123 has been shown to bind 16E6[54]. They together target cellular pathways important to the virus and malignant development such as cellular immortalization, growth, and differentiation. We have previously demonstrated that 16E6 and NFX1-123 increase Notch1, a master regulator of cell proliferation and differentiation, and increase hTERT, the catalytic subunit of telomerase[53–56,92]. These studies highlight how the partnership between 16E6 and NFX1-123 modulates cellular genes critical to promoting an ideal environment for HPV infection, as HPV requires its host cells to both grow and differentiate and HPV-associated cancers universally activate telomerase to drive cellular immortalization. By dysregulating these genes and pathways, 16E6 and NFX1-123 may aid in establishing and supporting a long-term HPV infection and potential malignancy.

We were interested in exploring what other pathways are manipulated by 16E6 and NFX1-123 to alter the host cell and engender an environment supportive of persistent HR HPV infection. In this chapter, we establish that 16E6 and NFX1-123 deregulated the innate immune response of keratinocytes, impeding the normal expression of diverse immune genes including cytokines and interferon stimulated genes. The data presented here identified NFX1-123 as a critical host protein partner through which 16E6 was able to subvert the immune response, revealing another cellular process targeted by this protein partnership that could further promote a HR HPV infection.

3.3 Results

3.3.1 Microarray and pathway analyses of genes upregulated in HFKs expressing 16E6 and overexpressing NFX1-123

NFX1-123 is a cytoplasmic protein whose partnership with 16E6 has previously been shown to co-regulate expression of genes such as Notch1, epithelial cell differentiation markers, and hTERT[53–56,92]. To explore what other transcripts and cellular processes might be altered by this partnership, we conducted a gene expression microarray in keratinocytes that stably expressed 16E6 and overexpressed NFX1-123. First, three biologically independent human foreskin keratinocyte cell lines (HFKs 1-3) were stably transduced with 16E6. We confirmed protein expression and function of 16E6 through Western blot of p53, as 16E6 targets p53 for degradation (Fig 3.1A). For each HFK cell line, there was a significant decrease in p53 protein in cells transduced with 16E6 compared to their isogenic, non-transduced control (Fig 3.1A, 16E6/scr and 16E6/FN123 versus HFK). These 16E6 HFKs (1-3) were then expanded and transduced a second time with either a FLAG-tagged NFX1-123 construct (FN123) or with a

scramble short hairpin RNA (control). The scramble short hairpin RNA does not target any known genes by BLAST search, and HFKs transduced with this scramble shRNA display levels of NFX1-123 equal to endogenous levels. Quantitative real-time PCR (qPCR) analysis showed that for each independent 16E6 HFK cell line, there was nearly a two-fold increase in NFX1-123 mRNA expression in FN123 cells versus control (Fig 3.1B) that was also reflected at the protein level (Fig 3.1C).

An Illumina beadchip microarray was performed in the three independent biologic backgrounds for each 16E6/control and 16E6/FN123 (GEO Accession number GSE43082). There were over 200 genes identified whose average expression was decreased by at least two-fold in at least two out of the three independent 16E6/FN123 HFK lines compared to 16E6/control HFKs. There were 26 genes found to be downregulated in all three cell lines (Fig 3.1D, box). These 26 genes are represented in a heat map with unsupervised hierarchical clustering (Fig 3.1E).

A subset of the over 200 genes whose average expression was decreased in at least two of three HFK cell lines are listed in Fig 3.2A. These include proinflammatory cytokines such as IL-8 and IL-23, structural elements such as tubulin, and a proteasome complex protein, PSMB8. Given the large number of genes that were decreased in 16E6 HFKs with increased NFX1-123 expression, we were interested in determining whether there were common nodes or pathways targeted for downregulation. Pathways that were enriched for in these downregulated genes were examined via Gene Ontology analyses, and the results are shown in Fig 3.2B. P-values were adjusted for multiple comparisons and only those pathways with a p-value $\leq$0.001 are shown. Pathways critical to viral clearance and immune detection were returned as highly enriched. Even with these stringent conditions of analyses, a number of different, yet related pathways

involved in the immune response were identified. Crucially, in a parallel microarray comparing HFKs that overexpressed NFX1-123 or had endogenous levels of NFX1-123 but without 16E6 co-expression, none of these same immune pathways were found to be enriched. Thus, it was only in the context of 16E6 that NFX1-123 was involved in regulating the immune response.

3.3.2 Overexpression of NFX1-123 decreased innate immune gene expression in 16E6 HFKs

Following our microarray studies, we further validated the roles of 16E6 and NFX1-123 in co-regulating genes of the immune response at the mRNA and protein levels. We returned to the three 16E6 HFK cell lines in which the microarrays were completed (16E6 HFK 1, 2, and 3) for validation. Fold changes in mRNA expression were quantified by qPCR for a set of genes that were decreased in the screening microarray of 16E6/FN123 cells (Fig 3.3A). Although there were differences based on biologic background, greater NFX1-123 expression in 16E6 HFKs resulted in reduced mRNA expression of the innate immune genes CXCL1, TNF, OAS1, and OAS2 when compared to 16E6/control HFKs with endogenous amounts of NFX1-123 (Fig 3.3A). In all three independent 16E6 HFK lines, CXCL1 had at least a ten-fold decrease in mRNA expression, while TNF had at least a 2.5-fold decrease. Mirroring results from the microarray, OAS1 and OAS2 were decreased in two out of three 16E6 HFK cell lines by at least 2.5-fold. Modulation of innate immune factors at the protein level was also seen. Compared to 16E6/control cells, FN123 cells of 16E6 HFK1 had lowered amounts of IRF7 protein, a transcription factor critical to the interferon response (Fig 3.3B).

3.3.3 Knock down of NFX1-123 led to rebound of innate immune gene expression in 16E6 HFKs

Having found that a diverse set of innate immune genes were downregulated in 16E6 HFKs with overexpression of NFX1-123, we then hypothesized that knock down of the

endogenous amount of NFX1-123 would result in a derepression of these genes. Two additional biologically independent HFK lines were transduced with 16E6 (16E6 HFK 4 and 5), then expanded and transduced with either a short hairpin RNA targeting NFX1-123 (sh1) or a scramble short hairpin control (scr). Again, expression and function of 16E6 were confirmed by p53 Western blot (Fig 3.3C). NFX1-123 expression was quantified five days post transduction. In both 16E6 HFK 4 and 16E6 HFK 5, sh1 reduced NFX1-123 mRNA by approximately 50%. NFX1-123 protein was also reduced (Fig 3.3C). The mRNA expression of CXCL1, TNF, OAS1, and OAS2 was subsequently assessed. In 16E6 HFK 4, both CXCL1 and TNF levels rebounded four- to nearly five-fold when NFX1-123 was decreased compared 16E6/control cells, although there was little to no shift in these two genes in 16E6 HFK 5 (Fig 3.3D). Again, this reflects differences based on biological background of the primary HFKs. Both 16E6 HFK 4 and 16E6 HFK 5 had significant increases in OAS1 and OAS2 upon knock down of NFX1-123. Despite differences in the magnitude of derepression, the interferon-stimulated OAS genes were consistently increased two- to nine-fold in sh1 cells compared to scr (Fig 3.3D). Similarly, decreasing NFX1-123 expression resulted in an increase in the level of IRF7 protein (Fig 3.3E).

3.3.4 NFX1-123 overexpression did not globally decrease levels of innate immune signaling proteins

In normal keratinocytes, the expression of genes such as IL-8, CXCL1, and other genes found to be downregulated in our screening microarray are induced through immune signaling pathways. Input, in the form of pathogen assault or stimulus, is communicated through these signaling pathways; output, in the form of proinflammatory cytokines, interferon-stimulated genes, and other factors, creates the innate immune response. A few common signaling pathways are responsible for induction of hundreds of these immune genes. The broad number of innate

immune genes found modulated in the screening microarray and the lack of alignment with a singular category, such as proinflammatory cytokines, led us to hypothesize that the dysregulation of the immune response genes might be occurring at a level above, or prior to, the direct downregulation of the genes themselves. We therefore next queried whether co-regulation of the immune response by 16E6 and NFX1-123 occurred through disruption of the signaling pathways that govern expression of innate immune genes. A diagram of an immune signaling cascade and the key proteins involved is depicted in Fig 3.4.

The mechanism by which 16E6 and NFX1-123 dysregulate protein signaling complexes could occur in a number of ways, including 1) a decrease in total signaling protein level, 2) sequestration of proteins, or 3) prevention of signaling complex formation. We addressed the first possibility by comparing total quantities of key signaling proteins in normal HFKs, 16E6 HFKs with endogenous levels of NFX1-123, and 16E6 HFKs with overexpressed NFX1-123. As before, HFKs were serially transduced with 16E6, and then either a FLAG-tagged NFX1-123 construct (FN123) or a vector control (control). Whole cell lysates were collected from each, and subsequent protein blots were probed for the immune signaling proteins TRIF, TRAF6, TAK1, TAB1, and TAB2. Experiments were conducted in three independent HFK cell lines with representative data of one cell line shown here (Fig 3.5). Interestingly, there appeared to be largely no difference in total quantities of these proteins across normal HFK, 16E6/control, or 16E6/FN123 cells. There was a slight decrease in levels of TAB2 in 16E6/FN123 cells compared to 16E6/control, but no difference between normal HFKs and 16E6/control cells, nor any significant differences for any of the other signaling proteins examined (Fig 3.5). Thus, altering total levels of upstream signaling proteins does not appear to be a mechanism by which 16E6 and NFX1-123 blunt the immune response.

3.3.5 Subcellular localization of innate immune signaling proteins was altered with increased NFX1-123

Ordered formation of signaling complexes is essential for full functioning of the signaling pathways depicted in Fig 3.4. Often, an event that occurs in one complex results in activation and allows for formation of the next. Sequestration of signaling proteins, and preventing their participation in signaling complexes, would therefore inhibit signaling and induction of immune genes. We employed immunofluorescent microscopy and high-content analysis of images to examine whether 16E6 and NFX1-123 disrupt the subcellular localization of single proteins or co-localization of multiple proteins. Normal HFKs, 16E6/control HFKs, and 16E6/FN123 HFKs were fixed, permeabilized, and stained for the signaling proteins listed in Fig 5 as two groups: TRIF/TRAF6/TAK1 (Fig 3.6A) and TAK1/TAB1/TAB2 (Fig 3.6B). These two groupings represent two signaling complexes whose formation after an immune stimulus is required for completion of the signaling cascade (depicted in Fig 3.4) and subsequent induction of target genes.

The subcellular localization of TRAF6 appeared to change dramatically with overexpression of NFX1-123 (Fig 3.6A, bottom row, 16E6/FN123). Whereas staining of TRAF6 appears diffuse throughout the cytoplasm in normal HFKs and 16E6/control HFKs, increased expression of NFX1-123 in the presence of 16E6 resulted in an altered pattern. With more NFX1-123, TRAF6 appeared to migrate to distinct, perinuclear foci. Additionally, co-localization of TRIF and TRAF6 seemed to increase with expression of 16E6 and even more with NFX1-123 overexpression (Fig 3.6A, top row).

Similar to TRAF6, subcellular localization of TAB2 also appeared to be altered with more NFX1-123 (Fig 3.6B, bottom row). In 16E6/FN123 cells, TAB2 staining gained the

appearance of bright, punctate foci that were localized to the nucleus. Although it is not clear from these data what impact this would have upon TAB2 functioning, it is clear that the normal localization of this important signaling protein is disturbed. Although the total expression levels of these signaling proteins may not be significantly changed, there were alterations to the subcellular localization and co-localization of these proteins. Appropriate localization of these signaling proteins, at the appropriate time, is crucial to their functioning and to subsequent induction of the innate immune response.

Quantitative data was also obtained from the immunofluorescent images. First, the average intensity of staining, representing total amount of innate signaling proteins (Table 3.1), was determined in HFKs, 16E6/control cells, and 16E6/FN123 cells. Images from three independent biologic backgrounds were analyzed and mean fluorescent intensity normalized to HFK values. For TRIF, there was not a significant difference in intensity of staining across the three cell types, indicating that there was not a substantial difference in the total amount of protein present. There was a relative increase in expression of TAK1 and TAB1 that occurred when 16E6 was expressed (16E6/control vs HFK), but with greater expression of NFX1-123, the levels of these proteins were blunted and returned to typical levels seen in normal HFKs. Interestingly, there was a significant decrease in staining intensity of TRAF6 in 16E6/FN123 cells compared to either HFKs or 16E6/control, mirroring the results seen in Western blot analyses. There was a 30% to 40% reduction in intensity of staining for TRAF6 in 16E6/FN123 cells compared to HFK or 16E6/control. Similarly, there was also a striking decrease in TAB2 fluorescence in 16E6/FN123 cells compared to HFK and 16E6/control cells, with approximately a 40% reduction in staining intensity.

Table 3.1 - Whole cell mean fluorescent intensity of innate immune signaling proteins.

	HFK	16E6/control	16E6/FN123
TRIF	100 (99.5-100.5)	116.4 (115.9-116.9)	85.8 (85.4-86.3)
TRAF6	**100 (99.5-100.4)**	**121.4 (120.8-121.9)**	**69.8 (69.4-70.2)** ^, ‡
TAK1	100 (99.7-100.3)	159.2 (158.5-159.6) ¤	104.1 (103.8-104.5)‡
TAB1	100 (99.7-100.5)	190.3 (189.8-190.9) ¤	109.1 (108.7-109.4)‡
TAB2	**100 (99.7-100.5)**	**90.0 (89.7-90.4)**	**35.4 (35.2-35.6)** ^, ‡

Mean fluorescent intensity of the innate immune signaling proteins displayed were normalized to the HFK and averaged across the three biologic cell line backgrounds. 95% confidence intervals are shown in parentheses. Statistical significance determined by one-way ANOVA with Bonferroni post-hoc test. All symbols shown indicate p-value < 0.05: ¤ = HFK compared to 16E6/control; ^ = HFK compared to 16E6/FN123; ‡ = 16E6/control compared to 16E6/FN123. Values for TRAF6 and TAB2 are significantly decreased in 16E6/FN123 cells compared to both HFK cells and 16E6/control cells and are shown in bold.

To quantify the shifts in location of TRAF6 and TAB2 that were seen in HFKs with 16E6 and overexpressed NFX1-123, subcellular localization of these proteins was also quantified (Table 3.2). HFK and 16E6/control cells had a similar ratio of nuclear/perinuclear to cytoplasmic TRAF6 foci, with 26-28% nuclear/perinuclear and 72-74% cytoplasmic. In 16E6/FN123 cells, however, this ratio was reversed, with the higher proportion of TRAF6 found to be nuclear/perinuclear (63%) rather than cytoplasmic (37%). Similarly, although TAB2 was predominately in the cytoplasm for HFK and 16E6/control cells (69-77%), it became highly perinuclear in 16E6/FN123 cells, with the cytoplasmic percentage falling to only 37% and the nuclear/perinuclear increasing to 63%. Thus, not only were total amounts of TRAF6 and TAB2 protein decreased in whole cell extracts and by immunofluorescent staining, their subcellular localization were also shifted in 16E6 HFKs overexpressing NFX1-123.

Table 3.2 - Subcellular localization of innate immune signaling proteins TRAF6 and TAB2.

	HFK		16E6/control		16E6/FN123	
	Nuc/Peri	**Cyto**	**Nuc/Peri**	**Cyto**	**Nuc/Peri**	**Cyto**
TRAF6	26% (21-32%)	74% (68-79%)	28% (22-34%)	72% (66-78%)	63% (56-70%)	37% (30-44%)
TAB2	23% (17-28%)	77% (72-83%)	31% (25-37)%	69% (63-75%)	63% (57-68%)	37% (31-42%)

Subcellular localization of innate immune signaling proteins TRAF6 and TAB2 were determined and averaged across the three biologic cell line backgrounds. 95% confidence intervals are shown in parentheses.

3.4 DISCUSSION

Collectively, the work presented in this chapter establishes a novel role in immune evasion for the protein partners HPV type 16 E6 and NFX1-123. We show that together, 16E6 and NFX1-123 inhibit the typical expression of diverse immune genes in keratinocytes. Greater levels of NFX1-123 resulted in a decrease of a broad range of immune factors such as proinflammatory cytokines and interferon-stimulated genes. The wide diversity of immune functions in the downregulated genes suggests that this may be a broad effect. Crucially, this only occurred when 16E6 was present, revealing the essentiality of partnership between 16E6 and NFX1-123 for this regulation. Furthermore, we demonstrate that NFX1-123 is required for immune dysregulation by 16E6 (Fig 3.3). Knock down of NFX1-123 by half rescued baseline expression of innate immune genes. With less NFX1-123, the ability of 16E6 to decrease immune gene expression was diminished. Interestingly, 16E6 and NFX1-123 appeared to achieve this immune modulation by interfering with intracellular immune signaling, thereby

preventing expression of innate immune genes and dampening the keratinocyte immune response.

The innate immune response is the first line of defense against pathogenic infection; it is therefore not surprising that HPV has evolved mechanisms that target numerous, overlapping points to dismantle these defenses and ensure a long-lived infection. Particularly important in the context of HPV infection is the etiological connection between a persistent infection with HR HPV types and development of anogenital and oropharyngeal cancers. The substantial clinical consequences of successful immune evasion by HR HPV make study of these mechanisms paramount. Both initial development of malignancy and overall progression of oncogenic disease are tied to whether or not there is a successful immune response. Identifying the specific ways in which HR HPV subvert this response may uncover possible points of intervention to circumvent persistent infection, decrease disease burden, and lower cancer incidence.

Our findings complement the many previously identified mechanisms by which 16E6 inhibits the immune response[80–82,99,100]. As indicated earlier, these strategies often overlap in their ultimate effects upon innate immunity—namely, inhibiting the induction of key effector genes such as proinflammatory cytokines, chemokines, and interferon-stimulated genes. It benefits the virus to have redundancy in these tactics, and thus our studies are a logical addition to the expansive repertoire defined by other groups. Universal to the varied immune evasion strategies employed by HPV is the dependence on and partnership with host proteins. Here, we identify NFX1-123 as a novel protein partner involved in innate immune evasion by 16E6. Notably, the host proteins through which HPV enacts its subversion of the immune response do not fall within a single category. Studies have identified interactions with DNA sensors[87,101], transcription factors[81,100], and enzymes involved in post-translational modification, such as UCHL1 and

IFRD1[79,102]. It is evident that the oncoproteins of HR HPV are able to hijack a vast array of host proteins to modify the cellular environment, regardless of the usual function of that endogenous protein. Previous work from our laboratory has demonstrated that 16E6 and NFX1-123 are involved in cellular growth, longevity, and differentiation[53–56,92]. We now define a new role for this partnership in immune evasion.

The data presented here also represent the first documentation of NFX1-123 driving a downregulation of gene expression. NFX1-123 has been previously shown to expression of two cellular genes important in HPV-associated cancers, hTERT and Notch1[55,92]. NFX1-123 posttranscriptionally increases the expression of hTERT through RNA binding and stabilization. Notch1 is a master regulator of cell growth and differentiation across many cell types, and perturbations in Notch signaling can confer either oncogenic or tumor suppressor effects on a cell, depending on its context[61,103]. The new role for NFX1-123 as an inhibitor of immune gene expression suggests that in the context of 16E6, NFX1-123 can be classified more broadly as a gene regulator rather than simply an activator. Taken together, partnership between 16E6 and NFX1-123 changes the cell to engender an environment supportive of HPV infection, both by enhancing pathways important for cellular growth and inhibiting pathways that would lead to viral elimination.

Interestingly, the molecular mechanism employed by 16E6 and NFX1-123 in modulating the immune response is also novel for this particular partnership. Whereas NFX1-123 works together with cytoplasmic poly(A) binding proteins to increase hTERT expression at the posttranscriptional level[55], the effects upon gene expression here seem to be indirectly, through upstream signaling complexes. This is similar to known mechanisms of immune evasion mediated by HR HPV, which frequently target signaling crossroads to prevent the interferon

response and the NFkB pathway. HPV 18 E6, for example, binds Tyk2, prevents phosphorylation of downstream kinases, and inhibits transcription of interferon-stimulated genes[80]. Multiple HR HPV types prevent the expression or function of STAT1, a signaling protein integral to the interferon signaling cascade[96,99,104]. Even amongst mechanisms that target signaling proteins, the specific molecular action by which HR HPV interferes with signaling can differ. Inhibition can be achieved by degradation of the proteins, physical sequestration, and altering post-translational modifications, amongst others. The data presented here indicate that 16E6 and NFX1-123 may employ the second option of the three, disrupting subcellular localization of immune signaling proteins to prevent normal formation of signal transduction complexes. For example, we documented an altered pattern of TRAF6 localization upon overexpression of NFX1-123 (Fig 3.6). TRAF6 migrated from primarily cytoplasmic to perinuclear foci, and this sequestration of TRAF6 likely decreased the probability of TRAF6 interfacing with protein partners. Additionally, co-localization between immune signaling proteins was disrupted, as seen in increased co-localization of TRAF6 and TRIF (Fig 3.6). Such abnormal, forced co-localization of proteins in the absence of immune stimulus may prevent these complexes from forming correctly when a stimulus is present. This would stop the signal cascade at its earliest steps and prevent the expression of immune genes.

Future research will further examine how 16E6 and NFX1-123 deregulate the immune response during an active stimulation. Keratinocytes constitutively express some cytokines, but are also are primed to respond quickly, producing soluble effector proteins to both initiate an anti-pathogenic (antiviral) state and recruit or activate other resident immune cells. This state is largely possible due to constant expression of signaling proteins that, upon stimulation, rapidly transduce a signal and induce gene expression. 16E6 and NFX1-123 appear to disturb the normal

localization and likely function of the signaling proteins. We thus hypothesize that there are functional consequences upon the ability to mount an immune response both at the keratinocyte level and the larger epidermal level. Keratinocytes are the first-line responders to any infection and act as a link to the rest of the immune effector cells in the region[62]. 16E6 and NFX1-123 inhibiting immune signaling has implications not only for whether a viral infection is eliminated, but also whether a malignant growth is detected and cleared.

In conclusion, the studies presented here identify NFX1-12 as a host protein that is co-opted by 16E6 to deregulate the innate immune response of keratinocytes and inhibit the expression of immune genes. This is achieved by perturbing the normal localization of signaling proteins, resulting in a broad inhibition. These data support previous work that elucidates how 16E6 and NFX1-123 contribute to a cellular environment that supports long-lived HPV infection. Persistent infection with HR HPV is the highest risk factor for cancer development; uncovering the mechanisms of immune evasion by HR HPV could advance understanding of how to prevent persistent infection and ultimately cancer.

3.5 METHODS

3.5.1 Microarray

The microarray was performed and analyzed as previously described[92]. Briefly, total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA), purified using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions, and converted to cDNA. cDNA were labeled and hybridized to the HumanHT-12 v4 Expression BeadChip array (Illumina, San Diego, CA). Data are accessible in the Gene Expression Omnibus database under

accession number GSE43082. Array data were analyzed using GeneSpring GX11.5.1 (Agilent Technologies, Santa Clara, CA).

3.5.2 Immunofluorescent staining and microscopy

Immunofluorescence was performed on cells grown on cover slips (#1 1/2 x 18mm). Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, washed with PBS and permeabilized with ice cold methanol/acetone (1:1) for 30 seconds. After blocking with PBS containing 1% TWEEN-20 and 3% bovine serum albumin (BSA) for 1 hour at room temperature, cover slips were incubated overnight at 4°C in blocking buffer with the same primary antibodies used in Western blot analysis above (Abcam, Cambridge, MA), unless otherwise stated: anti-TRAF6 (1:500), anti-TRIF (1:500), anti-TAK1 (1:500), anti-TAB1 (1:500), and anti-TAB2 (1:500). All secondary antibodies were obtained from Life Technologies (Carlsbad, CA) and were as follows: anti-rabbit AlexaFluor 488 (1:500), anti-mouse AlexaFluor 546 (1:500), and anti-goat AlexaFluor 633 (1:500). Secondary antibodies were incubated in the dark for 1 hour at room temperature with Hoechst stain (1:5000, Thermo, Waltham, MA). Cover slips were washed with cold PBS and mounted in glass slides with ProLong reagent (Thermo, Waltham, MA). Confocal images (stacks) were acquired at 0.2 micron spacing with an Olympus 60x oil immersion objective as specified in the figure legends with an Applied Precision DeltaVision RT microscope system (Applied Precision, Issaquah, WA). The exposure times were kept constant for each fluorescence channel within each experiment and antibody used. Stacks were deconvolved using a constrained iterative algorithm with DeltaVision SoftWoRx program, version 4.1.2.

3.5.3 High-content analysis of immunofluorescent microscopy

Total intensity

Previously deconvolved and normalized images of HFK, 16E6/control, and 16E6/FN123 cells grown on coverslips, fixed and co-stained for either TAB1, TAB2 and TAK1 or TRIF, TRAF6 and TAK1 were quantified using the FIJI scientific imaging analysis platform[105]. The fluorescent channels were split into greyscale images and analyzed for mean average intensity. A minimum of 30 cells from each of three separate biological backgrounds were used for quantification.

Subcellular localization

Previously deconvolved and normalized images of HFK, 16E6/control, and 16E6/FN123 cells grown on coverslips, fixed and co-stained for TAB1, TAB2 and TAK1 or TRIF, TRAF6 and TAK1 were split by channels into greyscale images. These were visually sorted according to whether the staining was above background, and whether the staining was in the cytoplasm or had coalesced into distinctive brighter staining perinuclear or nuclear foci. The Hoechst nuclear staining was used as a general mask. A minimum of 60 cells from each of three separate biological backgrounds were used for quantification.

3.6 FIGURES

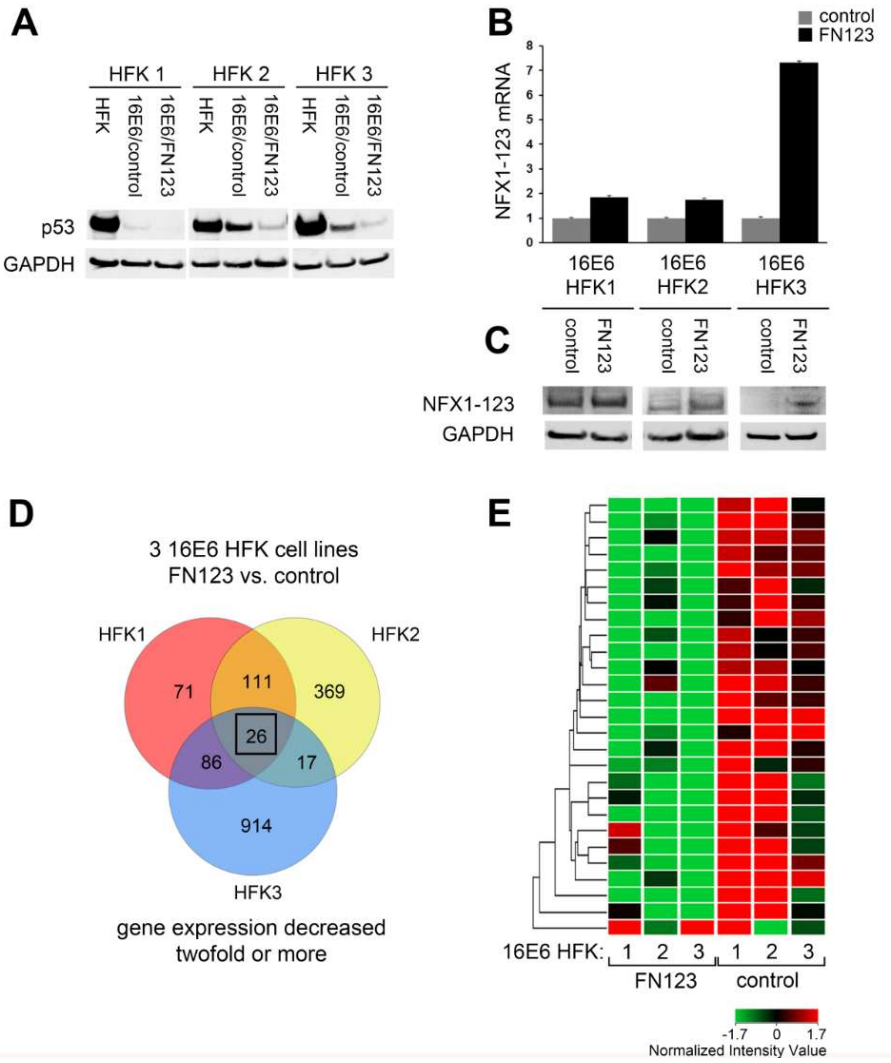


Figure 3.1. Microarray analysis of genes downregulated in 16E6 HFKs with increased NFX1-123. Whole genome expression microarrays were conducted in HFKs stably expressing 16E6 and overexpressing NFX1-123 or with endogenous levels of NFX1-123. (A) Three biologically independent HFKs were transduced with 16E6 and p53 protein levels assessed. (B) 16E6 HFK lines 1, 2, and 3 were transduced with NFX1-123 overexpression construct (FN123) or vector control (control). NFX1-123 mRNA and protein expression levels were quantified and compared. All qPCRs were normalized to the housekeeping gene 36B4, and all error bars represent 95% confidence intervals from the technical replicates shown (n=3). GAPDH = loading control for (A and C). (C) Venn diagram of genes whose average expression was decreased at least two-fold in 16E6/FN123 cells compared to 16E6/control. Box indicates 26 genes represented in (D) (D) Heat map with hierarchical clustering of the 26 genes decreased in 16E6/FN123 cells compared to 16E6/control.

A

Gene	Symbol	Average Fold Change (FN123 vs. control)
Inteferon alpha-inducible protein 6	IFI6	-20.59
Interferon alpha-inducible protein 27	IFI27	-18.86
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	-12.87
2'5'-oligoadenylate synthetase 2	OAS2	-9.72
2'5'-oligoadenylate synthetase 1	OAS1	-9.22
Major histocompatibility complex, class I, B	HLA-B	-9.15
Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	-8.63
Interleukin 8	IL8	-8.53
Bone marrow stromal antigen 2	BST2	-8.34
Signal transducer and activator of transcription 1	STAT1	-6.42
Interferon-induced protein 44-like	IFI44L	-6.34
2'-5'-oligoadenylate synthetase-like (OASL)	OASL	-5.53
Interferon-induced protein 35	IFI35	-5.18
Colony-stimulating factor 2	CSF2	-5.11
Tubulin, beta	TUBB	-4.81
Interleukin 24	IL24	-4.71
Chemokine (C-X-C motif) ligand	CXCL1	-4.52
Interferon-induced protein 44	IFI44	-4.29
12'-5'-oligoadenylate synthetase 3	OAS3	-4.11
Major histocompatibility complex, class I, F	HLA-F	-4.01
Chemokine (C-X-C motif) ligand 10	CXCL10	-3.83
Complement factor B	CFB	-3.73
MHC class I polypeptide-related sequence B	MICB	-3.73
Interferon regulatory factor 7	IRF7	-3.72
Beta-2-microglobulin	B2M	-3.69
Tumor necrosis factor	TNF	-3.43
Tumor necrosis factor, alpha-induced protein 3	TNFAIP3	-3.40
Chemokine (C-C motif) ligand 5	CCL5	-3.27
Interleukin 32	IL32	-3.06
Interleukin 1 receptor, type II	IL1R2	-2.99
Tripartite motif-containing 21	TRIM21	-2.90
Tripartite motif-containing 22	TRIM22	-2.86
Guanylate binding protein 1	GBP1	-2.61
Proteasome subunit, beta type, 8	PSMB8	-2.51
Interleukin 23, alpha	IL23A	-2.50
Apolipoprotein B mrna editing enzyme, catalytic polypeptide-like 3F	APOBEC3F	-2.36
Tripartite motif-containing 5	TRIM5	-2.34
HLA-G histocompatibility antigen, class I, G (HLA-G).	HLA-G	-2.28
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (RIG-I)	DDX58	-2.17
Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	-2.14

B

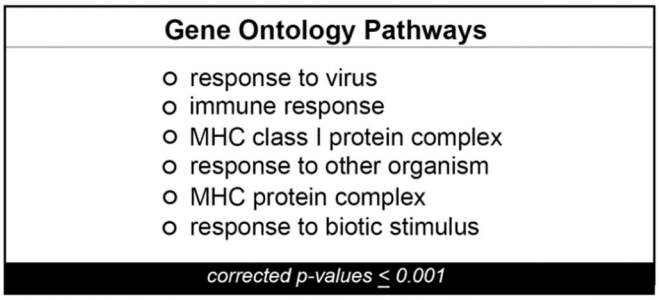


Figure 3.2. Genes and pathways decreased in 16E6/FN123 HFKs. (A) A subset of the over 200 genes that were decreased in 16E6/FN123 cells compared to 16E6/control. Fold change shown is the average fold change over all 16E6 HFK cell lines in which that gene was decreased. (B) Microarray data were analyzed using GeneSpring GX11.5.1, and the significant Gene Ontology pathways for the collection of genes decreased in two out of three 16E6 HFK cell lines were found. Only pathways with a p-value ≤ 0.001 are shown.

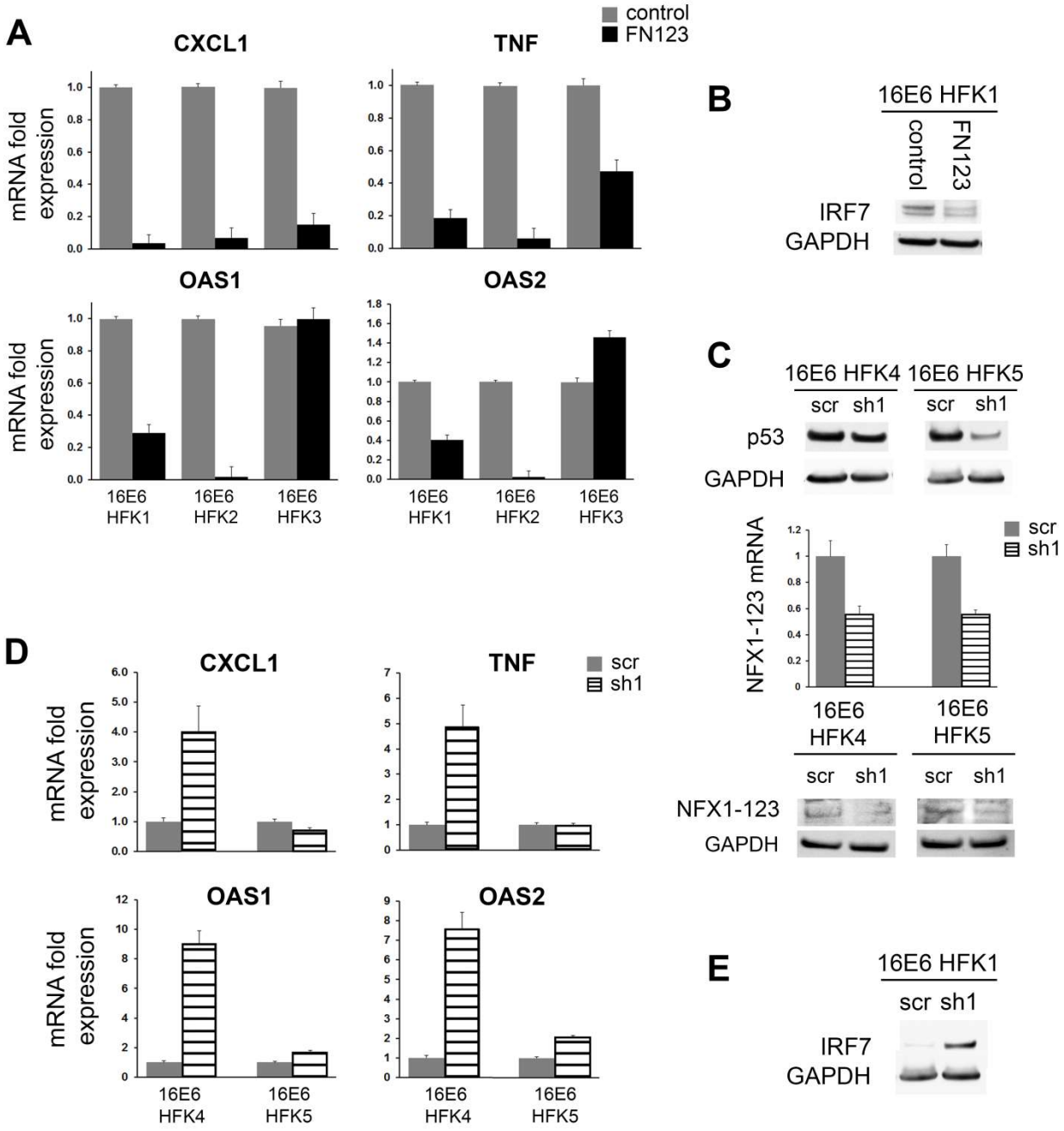


Figure 3.3. Immune gene expression decreased in 16E6 HFKs with more NFX1-123 or increased in 16E6 HFKs with NFX1-123 knockdown. Expression levels of innate immune genes were examined in 16E6 HFKs with increased NFX1-123 compared to endogenous levels, or with NFX1-123 decreased via short hairpin RNA. (A) mRNA levels of CXCL1, TNF, OAS1, and OAS2 were quantified by qPCR in 16E6/FN123 cells compared to 16E6/control. 16E6 HFKs with

increased NFX1-123 showed a decrease in many innate immune genes. (B) Protein levels of IRF7 in 16E6/control cells or 16E6/NFX1-123 cells. 16E6 HFKs overexpressing NFX1-123 had reduced IRF7 protein. (C) Two biologically independent HFKs were transduced with 16E6 and p53 protein levels assessed. Cells were then transduced with a scramble short hairpin construct (scr) or a short hairpin RNA targeting NFX1-123 overexpression construct (sh1). Levels of NFX1-123 mRNA and protein expression levels were quantified and compared. (D) mRNA levels of CXCL1, TNF, OAS1, and OAS2 were quantified by qPCR in 16E6/sh1 cells compared to 16E6/scr. 16E6 HFKs with decreased NFX1-123 showed a rebound in many innate immune genes. (E) Protein levels of IRF7 in 16E6/scr or 16E6/sh1 cells were assessed. 16E6 HFKs with NFX1-123 knocked down had increased IRF7 protein. Innate immune gene qPCRs were normalized to the housekeeping gene GAPDH, while NFX1-123 qPCRs were normalized to the housekeeping gene 36B4. All error bars represent 95% confidence intervals from the technical replicates shown (n=3) GAPDH = loading control.

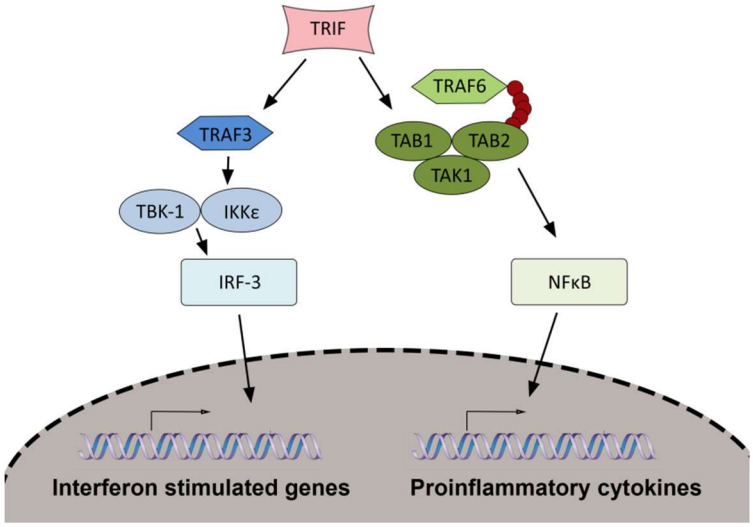


Figure 3.4. Innate immune signal transduction pathways in keratinocytes. An example of an immune signaling cascade downstream of pattern recognition receptors is shown. Stimulus from pathogen infection is sensed by PRRs, communicated through the adaptor protein TRIF, and further communicated through complexes formed by signaling proteins shown. These result in translocation of the transcription factors IRF-3 or NFkB to induce expression of interferon-stimulated genes, or proinflammatory cytokines and other mediators.

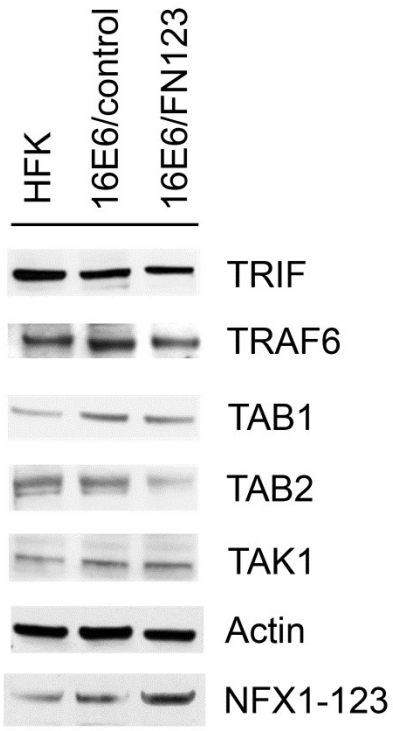


Figure 3.5. Total amounts of innate immune signaling pathway proteins are not globally decreased. Three independent HFK cell lines were serially transduced with 16E6 and then either FN123 or vector control. Whole cell lysates were collected from each, and subsequent protein blots were probed for the immune signaling proteins TRIF, TRAF6, TAK1, TAB1, and TAB2. NFX1-123 overexpression was confirmed in FN123 cells compared to HFK or 16E6/control. Actin = loading control. Data shown are from one representative cell line.

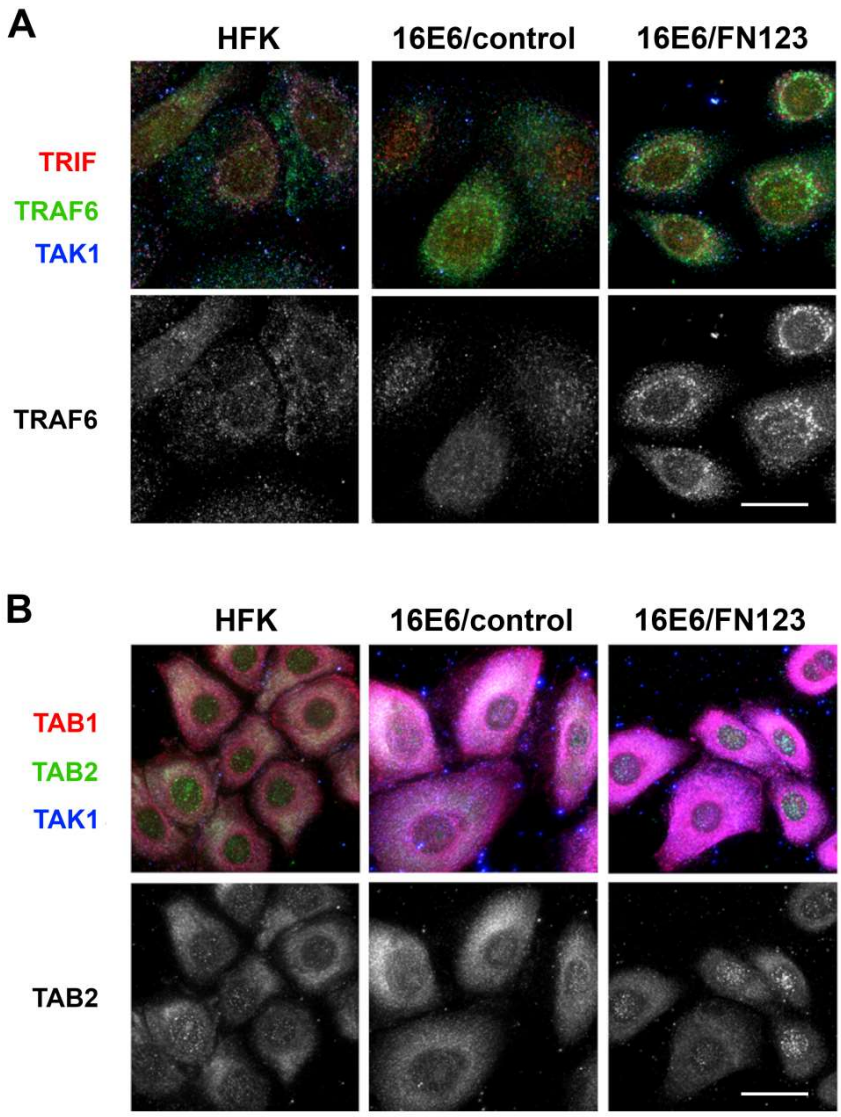


Figure 3.6. Co-localization and subcellular localization of innate immune signaling pathway proteins are altered by 16E6 and NFX1-123. Immunofluorescence was performed on HFKs, 16E6/control HFKs, and 16E6/FN123 HFKs grown on cover slips. Cells were fixed, permeabilized, and probed with primary antibodies. Scale bars = 20 micron. (A) Cells were stained for TRIF (red), TRAF6 (green), and TAK1 (blue) and shown as a merged image (top panel). The green channel representing TRAF6 was isolated and displayed as a greyscale image (bottom panel). 16E6/FN123 cells show strong perinuclear localization of TRAF6 compared to HFKs or 16E6/HFKs. (B) Cells were stained for TAB1 (red), TAB2 (green), and TAK1 (blue) and shown as a merged image (top panel). The green channel representing TAB2 was isolated and displayed as a greyscale image (bottom panel). 16E6/FN123 cells contain bright, punctate staining of TAB2 in the nuclear compared to 16E6/control cells

Chapter 4

Mediation of keratinocyte differentiation and HPV L1 expression by 16E6 and NFX1-123

The following text is from the article:

Levan J, Vliet-Gregg PA, Robinson KL, Matsumoto LR, Katzenellenbogen RA. 16E6 and NFX1-123 Activation of JNK signaling Mediates Keratinocyte Differentiation. (2018) **Virology,** submitted.

Figure numbers have been updated to conform to the formatting of this dissertation. The text remains as published with minor editorial changes.

4.1 ABSTRACT

High-risk human papillomaviruses (HR HPV) cause high morbidity and mortality, accounting for ~ 5% of the global burden of cancer. The HPV life cycle is tied to the differentiation programming of epithelial cells, with cellular differentiation driving initiation of the late stage of life viral cycle. Here, we identify NFX1-123 as a host protein factor that regulates keratinocyte differentiation and late events of the HPV life cycle. We show that NFX1-123 itself increased with differentiation in epithelial cells, and that NFX1-123 augmented JNK signaling in 16E6-expressing human foreskin keratinocytes (16E6 HFKs) undergoing differentiation. NFX1-123 levels affected expression of MKK4 and MKK7, upstream kinase regulators of JNK phosphorylation. Furthermore, we examined consequences upon the differentiation-dependent events of the HPV life cycle. Modulating levels of NFX1-123 in HPV16-positive W12E cells recapitulated the effects on induction of differentiation markers, JNK phosphorylation, and its upstream regulators seen in 16E6 HFKs. With that, mRNA expression levels of L1, the capsid protein of HPV were also affected by NFX1-123 expression. Altogether, these studies define a role for NFX1-123 in epithelial differentiation through the JNK signaling pathway, potentially acting as a signal linking expression of cellular genes and HPV genes during differentiation.

IMPORTANCE: High-risk human papillomaviruses can cause a variety of cancers, including cervical and head and neck, and account for ~ 5% of the global burden of cancer. The greatest clinical risk factor for development of HPV-associated cancer is a persistent, long-lived high-risk HPV infection. Understanding what promotes long-lived HR HPV infection is therefore critical for predicting and preventing serious disease outcomes. Although much of the basic HPV viral life cycle has been described, there are gaps in our knowledge of processes

important for HPV infection. This chapter identifies a new protein and mechanism by which HPV alters the cell environment to promote its life cycle and contribute to persistent infection. In doing so, we will enhance understanding of the ill-defined factors that lead to HPV-associated cancer development.

4.2 Introduction

The human papillomaviruses (HPVs) are small, non-enveloped double-stranded DNA viruses that infect keratinocytes of cutaneous and mucosal stratified squamous epithelium[14,51]. There are over 200 types of HPV, and those that specifically target mucosal epithelium are further categorized as low-risk HPV (LR HPV) or high-risk HPV (HR HPV) based on their epidemiologic association with cancer[78,106,107]. HR HPV cause nearly all cervical cancer, the fourth most common cancer in women, in addition to other anogenital and oropharyngeal cancers[4–6,106,108]. Altogether, cancers caused by HR HPV account for approximately 5% of the global burden of cancer[8].

HPV has a unique life cycle that is tied to the differentiation programming of its target cell, the keratinocyte[2,15]. The viral life cycle can be divided into two stages: in the first, HPV infects keratinocytes in the basal layer of stratified squamous epithelium and maintains its genome as an episome at a low copy number[2,14–17]. In this stage, the early genes (E1, E2, E4, E5, and oncogenes E6 and E7) are expressed from the early viral promoter[16,18,19]. The second stage occurs once an infected cell rises through the epithelial suprabasal layers and begins to differentiate. This triggers the late stage of the viral life cycle, whose events include vegetative genome amplification, expression of the capsid proteins L1 and L2 from a viral late promoter, and ultimately, complete virion formation[14,18,20]. As a keratinocyte differentiates and moves through the stratified squamous epithelial layers, it expresses a successive series of proteins that

commonly serve as differentiation markers. These include cytokeratins 1 and 10, involucrin, loricrin, and filaggrin[109,110]. Similarly, as noted above, HPV expresses genes in a specifically ordered fashion associated with specific epithelial layers. There is thus a clear link between cellular gene expression during differentiation and viral late gene expression. Although it is known that terminal differentiation of epithelial cells is required for the initiation and completion of the late stage viral life cycle, the cellular factors activated during epithelial differentiation, and how these factors signal to induce the appropriate viral promoters, are not fully defined.

Efforts to understand regulation of differentiation-dependent HPV gene transcription have implicated a wide variety of transcription factors and cellular processes. These include transcription factors specific to differentiating keratinocytes, CDP and EPOC-1, as well as ubiquitously expressed transcription factors whose levels may change during epithelial cell differentiation such as AP-1 family members, Oct1, C/EBP, YY1, and others[111–120]. In addition to global changes in levels of transcription factors, mechanisms including shifting ratios of factors (Sp1/Sp3)[121], increased binding to HPV promoters during epithelial differentiation[122], and activation of cellular signaling pathways (protein kinase C)[123] have been associated with HPV transcription. Furthermore, linker scanning mutational studies have mapped *cis* regulatory elements upstream of the HPV promoters that may be important during different stages of the life cycle[124,125].

The understanding of how differentiation-dependent HPV gene transcription is regulated is further complicated by a switch from the early viral promoter to the late viral promoter which does not become active until the host cell rises to upper granular and cornified layers of the stratified squamous epithelium. This further supports the tight linkage between events dictating the expression of cellular genes during differentiation and those dictating expression of HPV

genes. Even after infected cells have begun differentiating, there are additional signals responsible for activating the late promoter as the keratinocytes progress through terminal differentiation. Studies have shown a role for SRp20, a cellular splicing factor, and transcription elongation factors[126,127], but many gaps remain in our knowledge of what controls this transition in HPV transcription. Despite the abundance of transcription factors and complexity of mechanisms found associated with differentiation-dependent HPV transcription, there is not a complete understanding of the process.

We have previously shown that HR HPV type 16 E6 (16E6) and NFX1-123, a known cellular protein partner of 16E6, collaboratively increase levels of Notch1[92]. Notch1 is a master regulator of growth and differentiation[57,61]. Together, 16E6 and NFX1-123 increase Notch1, as well as its downstream canonical pathway genes Hes1 and Hes5[53]. Differentiation markers Keratin 1 and Keratin 10 were also increased by NFX1-123, but their upregulation was indirectly associated with Notch1 receptor stimulation[53]. Blocking Notch1 signaling did not abrogate their expression, pointing to Notch1-independent mechanisms by which 16E6 and NFX1-123 are able to affect keratinocyte differentiation.

In the current chapter, we utilize targeted differentiation stimuli to demonstrate that NFX1-123, a host cellular protein partner of HPV type 16 E6, can regulate differentiation marker expression in HFKs expressing 16E6 and in HPV16-positive cells through JNK signaling and ultimately drive expression of HPV16 L1.

4.3 Results

4.3.1 Expression levels of NFX1-123 increased with epithelial cell differentiation

NFX1-123 is a known cellular protein partner of HR HPV type 16 E6 (16E6) and is endogenously expressed in epithelial cells[52]. Although NFX1-123 is increased in cervical cancer cell lines[53], HPV positive cervical precancerous lesions, and HPV-positive cervical cancers (manuscript under review), the overall expression of NFX1-123 in normal epithelial tissue has not been characterized. To do so, we utilized organotypic raft cultures, which approximate the growth and physiology of stratified squamous epithelium, as well as archived normal cervical epithelial biopsies. Raft cultures of human foreskin keratinocytes expressing 16E6 and either endogenous levels of NFX1-123 (16E6/LXSN HFKs) or overexpressed, FLAG-tagged NFX1-123 (16E6/FN123 HFKs) and normal cervical epithelial samples were stained for NFX1-123 via immunohistochemistry (Fig 4.1A). As expected, there was cytoplasmic staining in the cytoplasm of cells throughout the raft cultures and cervical epithelium, with some perinuclear staining seen in basal layer cells. Notably, when staining intensity was plotted as a function of distance from the basal layer, expression of NFX1-123 in the raft cultures and normal cervical epithelium rose overall with increasing distance from the basal layer (Fig 4.1A, graphs). The greater expression of NFX1-123 with progression through differentiating epithelial layers indicated that NFX1-123 expression increased in in association with epithelial cells undergoing differentiation.

To identify a direct link between cellular differentiation and NFX1-123 expression, we quantified NFX1-123 mRNA and protein in 16E6 HFKs induced to differentiate with high-dose calcium or suspension in methylcellulose. 16E6/LXSN and 16E6/FN123 HFKs were cultured in media containing 1.8mM Ca^{2+} and total mRNA and protein were serially collected. mRNA and protein levels of NFX1-123 were higher after 72 hours of calcium exposure, compared to undifferentiated cells with no calcium exposure, and rose even further after 120 hours (Fig 4.1B). Induction of NFX1-123 with differentiation was observed even in 16E6/FN123 cells that began

with greater expression. With methylcellulose suspension, 16E6/LXSN and 16E6/FN123 cells had rapid and robust increases in levels of NFX1-123 at 24 hours which were slightly increased or maintained at 48 hours (Fig 4.1C). Again, similar to calcium induction, NFX1-123 expression increased during differentiation in methylcellulose suspension in both 16E6/LXSN and 16E6/FN123 cells. Altogether, these data indicated that NFX1-123 itself was increased with keratinocyte differentiation.

4.3.2 NFX1-123 mediated keratinocyte differentiation in both Notch1-dependent and Notch1-independent manners

We have demonstrated previously that greater NFX1-123 affected keratinocyte processes and increased expression of differentiation markers Keratin 1 and Keratin 10[53]. Because these studies had been conducted in non-differentiating conditions, we were interested in interrogating whether NFX1-123 regulates expression of these markers in the context of full keratinocyte differentiation. To do this, the 16E6/LXSN and 16E6/FN123 raft cultures were stained for differentiation markers Keratin 1 and Loricrin (Fig 4.2A). FN123 rafts with greater levels of NFX1-123 had more intense staining of these differentiation markers compared to LXSN rafts. These data confirmed that increased NFX1-123 increased expression of these markers when cells were actively differentiating.

Previous data from our laboratory has shown that NFX1-123 regulated Notch1 expression and affected its canonical pathway targets[53,92]. This effect of NFX1-123 and Notch1 also was linked to keratinocyte differentiation, but that pathway regulation by NFX1-123 was less direct. Specifically, when Notch1 was activated in 16E6 HFKs overexpressing NFX1-123, canonical and differentiation targets were increased; however, when Notch1 activation was blocked in these same cells, the canonical pathway targets of Notch1 rapidly fell while

differentiation pathway targets did not[53]. Therefore, to interrogate the interplay between Notch1, NFX1-123, and keratinocyte differentiation, we selected methods to stimulate differentiation in epithelial cells that would exclude or combine these factors, recognizing that Notch1 pathway activation occurs through cell-to-cell signaling.

First, 16E6/LXSN and 16E6/FN123 HFKs were plated at high confluency, permissive for Notch1 signaling, and treated with high-dose calcium for 72 hours to induce differentiation. 16E6/LXSN HFKs had a robust upregulation of differentiation markers Keratin 1, Involucrin, and Loricrin (Fig 4.2B, gold bars), and there was an even greater induction in the 16E6/FN123 HFKs (Fig 4.2B, gray bars). This was reflected at the protein level as well; in agreement with our previous studies, we saw a greater expression of differentiation markers with more NFX1-123, even in undifferentiated cells (Fig 4.2B). Following differentiation, 16E6/FN123 HFKs had greater levels of Keratin 1 and Involucrin compared to 16E6/LXSN (Fig 4.2B). This confirmed that NFX1-123 played a role in mediating keratinocyte differentiation when Notch1 signaling could occur.

Next, cells were plated at low confluency (~10%), to limit cell-cell contact and therefore Notch1 signaling, and treated with the high dose of calcium to induce differentiation. There was again an upregulation of Keratin 1, Involucrin, and Loricrin, indicating epithelial differentiation was triggered; however there was no difference in the mRNA or protein levels of differentiation markers in 16E6/LXSN cells compared to 16E6/FN123 (Fig 4.2C). Therefore, without the availability of Notch1 signaling, greater expression of NFX1-123 did not augment differentiation activation when 16E6 HFKs were stimulated by high-dose calcium.

Lastly, 16E6 HFKs were stimulated to differentiate with single-cell suspension in semisolid methylcellulose medium, which prevents any cell-cell contact and therefore prevents

Notch1 signaling entirely. 16E6/FN123 HFKs had a greater induction of differentiation markers at both the mRNA and protein level than 16E6/LXSN cells, indicating that NFX1-123 was able to mediate keratinocyte differentiation stimulated by suspension (Fig 4.2D). Overexpression of NFX1-123 itself increased induction of differentiation markers, even in the absence of 16E6; however, expression of 16E6 augmented this trend (data not shown). To further confirm the involvement of NFX1-123, 16E6 HFKs expressing short hairpin RNAs targeting NFX1-123 (sh1 and sh2) were differentiated via methylcellulose suspension. Knock down of NFX1-123 reduced induction of differentiation markers at the mRNA level relative to control (scr) (Fig 4.2E). When NFX1-123 was knocked down, this did affect the low levels of Keratin 1 and Involucrin protein in undifferentiated cells (scr vs. sh2). Although the transient knock down of NFX1-123 was not evident in undifferentiated sh1 cells, there was still a dose-dependent association between NFX1-123 expression and differentiation marker expression. After methylcellulose suspension, both sh1 and sh2 cells had decreased levels of NFX1-123 and had reduced induction of Keratin 1 and Involucrin compared to scr (Fig 4.2E). These data collective identified that NFX1-123 itself was increased during differentiation, that with its increased expression, NFX1-123 augmented differentiation stimulated by methylcellulose suspension, and that NFX1-123 could achieve this through Notch1-independent signaling pathways.

4.3.3 Signaling pathways triggered by differentiation stimuli

In addition to their utility for investigating differentiation with or without Notch1 involvement, the stimuli chosen have unique intracellular signaling pathways (Fig 4.3)[128–132]. Because NFX1-123 regulated keratinocyte differentiation stimulated by suspension and by calcium in high confluency cells, but not low confluency, this could occur through a shared

intracellular signaling pathway. MAPK signaling, and specifically ERK and JNK, were therefore likely targets through which 16E6 and NFX1-123 mediate epithelial cell differentiation.

4.3.4 16E6 and NFX1-123 mediate levels of activated JNK through upstream kinases

To remove cross-talk with activated Notch1 pathways, we leveraged suspension in methylcellulose as a differentiation stimulus. To investigate whether 16E6 and NFX1-123 alter MAPK signaling, 16E6 HFKs with endogenous (LXSN) or overexpressed NFX1-123 (FN123) were suspended in methylcellulose and total protein was collected. Upon differentiation, there were no significant differences seen in total protein levels of JNK or ERK, regardless of amount of NFX1-123 (Fig 4.4A). Since these kinases are activated by phosphorylation, we also examined levels of phospho-JNK and phospho-ERK. Suspension-induced differentiation results in decreased phospho-ERK, and the low amounts were difficult to quantify and determine differences due to NFX1-123 (data not shown). However, our results demonstrated that upon methylcellulose-induced differentiation, cells with more NFX1-123 had greater levels of phospho-JNK (Fig 4.4A). JNK can be phosphorylated by a number of MAP kinase kinases (MKKs), but in particular by MKK4, which is specific for JNK and p38, and MKK7, which is solely JNK-specific[133,134]. Intriguingly, 16E6/FN123 HFKs showed greater amounts of MKK4 and MKK7 compared to 16E6/LXSN HFKs both in undifferentiated and differentiated cells (Fig 4.4A).

To confirm the required role of NFX1-123 in JNK phosphorylation and MKK expression, we examined levels of these MAPK signaling proteins in 16E6 HFKs expressing short hairpins against NFX1-123. Again, there were no differences in total levels of ERK or JNK (Fig 4.4B). When NFX1-123 was knocked down by short hairpin RNA, it did, however, result in decreased levels of P-JNK, MKK4, and MKK7 (Fig 4.4B). These data indicate that NFX1-123 was

required for full MKK4 and MKK7 expression and JNK phosphorylation upon suspension-induced differentiation.

4.3.5 16E6 and NFX1-123 regulation of JNK signaling and differentiation in HPV16-positive W12E cells modulates HPV16 L1 expression

Given that NFX1-123 regulated keratinocyte differentiation marker expression, that this regulation by NFX1-123 was augmented by 16E6[53], and that HPV late gene transcription is differentiation-dependent, we wished to assess if NFX1-123 impacted and supported these events of the late HPV life cycle. To explore this, we utilized W12E cells, HPV16-positive cells that harbor the full viral genome. First, we wanted to confirm that NFX1-123 was endogenously expressed in these cells, and that its expression also responded to differentiation in a similar manner as HFKs. Cells were transduced with vector control (LXSN) or NFX1-123 overexpression construct (FN123) and then suspended in methylcellulose for 24 hours to induce differentiation. Comparable to the results observed in 16E6 HFKs, NFX1-123 mRNA was expressed in W12E cells and increased in both W12E LXSN and W12E FN123 cells with 24 hours of methylcellulose suspension (Fig 4.5A). These levels were maintained or further increased at 48 hours (Fig 4.5A). Endogenous protein levels of NFX1-123 in W12E cells appeared to be lower than in HFKs, as NFX1-123 protein could not be detected by immunoblot in either undifferentiated W12E LXSN or W12E FN123 cells. Upon differentiation, however, NFX1-123 increased to detectable amounts, and greater protein could be seen in W12E FN123 cells relative to W12E LXSN cells (Fig 4.5A).

We next examined whether 16E6 and NFX1-123 also regulated expression of differentiation markers in W12E cells. Overexpression of NFX1-123 in W12E cells recapitulated the results seen in 16E6 HFKs, with greater levels of NFX1-123 resulting in increased induction

differentiation markers (Fig 4.5B). As expected, knock down of NFX1-123 resulted in decreased induction of differentiation markers in response to suspension (Fig 4.5C). Analysis of MAPK proteins revealed that modulating levels of NFX1-123 had no effect on total levels of ERK or JNK, but did alter levels of phospho-JNK, MKK4, and MKK7 (Fig 4.5D). Phospho-JNK, MKK4, and MKK7 levels were increased when NFX1-123 expression was increased (Fig 4.5D, FN123 versus LXSN) and were decreased with knock down of NFX1-123 (Fig 4.5D, sh1 and sh2 versus scr). In total, these results support what was observed in HFKs: NFX1-123 was required for full induction of epithelial differentiation marker induction, augmented phosphorylation of JNK, and increased expression of its upstream kinases.

To explore whether this regulation of cellular differentiation also paralleled the transcription of HPV late genes that are triggered during differentiation, we quantified the expression of HPV16 L1, which encodes the HPV major capsid protein and is a common reading frame of all mRNA products expressed from the viral late promoter. As expected, both W12E LXSN and W12E FN123 cells upregulated L1 mRNA following differentiation (Fig 4.5E). However, W12E FN123 cells overexpressing NFX1-123 had an even greater induction of L1 mRNA compared to these cells. Therefore, increased expression of NFX1-123 affected both cellular and viral targets during differentiation.

To determine of NFX1-123 was required for full induction of L1 during differentiation, W12E were transduced with short hairpin RNAs against NFX1-123 (sh1 or sh2) or a control (scr) and then differentiated with methylcellulose suspension. W12E scr cells, with endogenous expression levels of NFX1-123, had a robust upregulation of L1 upon differentiation, while W12E cells with NFX1-123 knocked down had a lowered induction of L1 (Fig 4.5E). This demonstrated that NFX1-123 could augment epithelial cell differentiation pathways in

keratinocytes and in HPV 16-positive cell lines and was required for full activation of cellular differentiation and subsequent differentiation-dependent late gene transcription of HPV16.

4.4 Discussion

This chapter confirms the cellular protein NFX1-123 is increased during differentiation and is a regulator of epithelial cell differentiation. This chapter additionally identifies the intracellular signaling cascade affected by NFX1-123 expression, and subsequently provides insight into the linkage between epithelial differentiation and the HPV life cycle. Previously, we had shown that NFX1-123 was able to increase expression of differentiation markers when Notch1 was stimulated, but also determined that NFX1-123 employed Notch1-independent mechanisms to regulate cellular differentiation markers[53]. Here, we use targeted stimuli and modulation of NFX1-123 expression to explore the Notch1-independent mechanisms by which NFX1-123 regulates epithelial differentiation. Confirming earlier studies, we show that overexpressed NFX1-123 results in increased expression of differentiation markers when cells are stimulated to differentiate in the context of Notch1 signaling, either in raft cultures or in high confluency plating and calcium exposure (Fig 4.2). Cells with more NFX1-123 had an increase in levels of differentiation markers compared to cells with endogenous amounts of NFX1-123. These data extend our previous findings involving NFX1-123 in cells grown in non-differentiating media to those actively undergoing differentiation; these data demonstrate that NFX1-123 is indeed able to regulate induction of these markers during typical physiological conditions. When Notch1 involvement is limited, or eliminated entirely, NFX1-123 plays a direct role in differentiation marker expression, but a role that is more selective. Although NFX1-123 expression is increased during differentiation, it itself does not have a universal effect

on differentiation. Rather, its increased expression affects one differentiation pathway and intracellular signaling cascade.

Previous studies in our laboratory have focused on the post-transcriptional regulatory role NFX1-123 has on gene expression, both with and without 16E6. For the case of telomerase, which is activated by 16E6, NFX1-123 post-transcriptionally stabilized the mRNA of hTERT, the catalytic subunit of telomerase[54–56]. For Notch1, NFX1-123 increased its mRNA and protein expression, and like hTERT, it required its RNA binding and regulatory motifs to do so[92]. However, in our studies of differentiation marker and pathway regulation that are non-Notch1 dependent, NFX1-123 likely does not regulate target protein expression in the same manner. If post-transcriptional stabilization of differentiation genes were the mechanism by which NFX1-123 increased their expression, we would expect NFX1-123 to affect levels of differentiation markers, regardless of what stimulated their expression. We instead identified the MAPK intracellular signaling pathway as one specifically affected by NFX1-123, with greater NFX1-123 expression robustly activating pathway proteins within that cascade.

While keratinocytes differentiating in squamous cell epithelium would have multiple, concurrently active stimuli and pathways, we applied a single stimulus to cells to isolate signaling pathway cascades. Mitogen-activated protein kinase signaling, specifically involving ERK and JNK, is central to triggering suspension-induced epithelial differentiation. Analysis of MAPK pathway proteins demonstrated that after differentiation, NFX1-123 had a significant effect on levels of phosphorylated JNK and its upstream kinases MKK4 and MKK7 (Fig 4.4). Signal amplification in MAPK cascades can be driven by increased abundance of a kinase within any step of the cascade[134]. In HPV-positive W12E cells, this paralleled the expression of L1, a product controlled by the differentiation-dependent late promoter. Elevation of P-JNK levels

during HPV infection has been documented before and, similar to our study, drove differentiation-dependent genome amplification and transcription of HPV16[135]. Our study showing JNK signaling cross-talk with the late viral life cycle joins other studies that document HPV's co-opting and usage of MAPK signaling. MAPK signaling is a central axis around which HPV achieves many of the events required for its life cycle[136]: E1 phosphorylation by ERK and JNK promotes its nuclear accumulation, which is critical for viral DNA replication[137]; activated p38 and JNK enhances E6 targeting of PDZ domain-containing targets[32,138]; and sustained JNK signaling engendered by E7 prolongs the G_2 phase of cells to drive viral DNA replication[139,140]. Thus, our finding that activated JNK influences HPV differentiation-dependent transcription dually strengthens and is strengthened by similar findings across the field. Moreover, this is the first time that NFX1-123 has been identified as a cellular factor exploited by the virus to manipulate JNK signaling. It is interesting to note that in undifferentiated cells, greater NFX1-123 does lead to increased levels of total JNK and total ERK (Fig 4.4). MAPKs are heavily involved in a vast array of cellular processes ranging from cell proliferation to cell differentiation to cell death[134]. A possibility is that NFX1-123 has different modalities depending on the cellular environment; there is one purpose in actively proliferating cells and another in those undergoing differentiation. Indeed, we do see that within proliferating cells, NFX1-123 extends the active growth of keratinocytes expressing 16E6 (P.A. Vliet-Gregg, K.L. Robinson, J. Levan, L. Matsumoto, R.A. Katzenellenbogen, submitted for publication). This is perhaps mediated by increasing total levels of JNK and ERK which may target cellular proliferation, but not differentiation.

Another novel finding was that NFX1-123 expression was itself induced during differentiation, with the highest expression in the upper, more differentiated layers of the

epithelium. This is of interest considering that even within differentiating cells, HPV genes expression is restricted to certain layers of stratified squamous epithelium. What controls this tight linkage between HPV gene expression and progressive epithelial differentiation is ill-defined. NFX1-123 may be a candidate for regulating this aspect. Progressively increasing levels of NFX1-123 could lead to progressively more JNK signaling that, at a certain threshold, initiates the early to late switch in the HPV life cycle. Alternatively, NFX1-123 may function to sequester P-JNK upon reaching a high enough level in the middle to upper layers of the epithelium. Egawa et al. observed P-JNK specifically in the middle and upper layers of HPV-positive raft cultures[135]. Although these studies focused on the role of E4, they were carried out using the full HPV genome, which would include E6 expression and do not eliminate a potential role for NFX1-123.

The chapter presented here extends previous work examining differentiation marker expression indirectly induced by greater NFX1-123 through increased Notch1 expression. Now, we show that NFX1-123 is required for and can augment differentiation marker expression in HFKs and HPV16-positive cells. These data also identify NFX1-123 as a cellular protein partner through which HPV increases MAPK signaling and engenders a cellular milieu favorable to the viral life cycle. Although future studies are required to more fully investigate the molecular underpinnings of how JNK signaling promotes HPV differentiation-dependent transcription, this chapter overall furthers knowledge of the methods through which HPV alters the cell to promote infection and adds insight into the control of differentiation-dependent events of the HPV life cycle.

4.5 METHODS

4.5.1 Immunohistochemistry and histologic analysis

Organotypic HFK raft cultures were formalin fixed and embedded in paraffin (FFPE) following standard procedures. 32 normal cervical epithelium specimen FFPE blocks were obtained from the University of Washington HPV Research Group Specimen Repository. They were deidentified and considered to be not human subjects by the Seattle Children's Research Institute IRB. Sections 4μm thick were stained for NFX1-123 using a rabbit polyclonal anti-NFX1-123 antibody, a gift from Dr. Ann Roman, anti-Loricrin antibody (BioLegend, San Diego, CA), anti-cytokeratin 1 (BioLegend, San Diego, CA), or an isotype control at a 1:1000 dilution. Epitope retrieval was done using citrate for 10 minutes at 100°C. Slides were scanned in brightfield at 20x magnification using the Hamamatsu NanoZoomer Digital Pathology System.

For semiquantitative analysis of NFX1-123, Keratin 1, and Loricrin staining, ImageJ was used. Color deconvolution for hematoxylin and DAB was performed. For NFX1-123, plot profiles of intensity were obtained over a set linear distance from the basal layer upwards through the apical surface of the raft culture or tissue specimen. Per sample, four plots over two sections were averaged and are shown as a function of distance from the basal layer. For Keratin 1 and Loricrin, total intensity of a section were obtained.

4.5.2 Differentiation of keratinocytes

Prior to differentiation, HFKs were trypsin harvested from monolayer cultures. For calcium differentiation, HFKs were grown in EpiLife without human keratinocyte growth supplements, with 1.8mM calcium chloride and penicillin-streptomycin added. After trypsin harvest, cells were counted using the Bio-Rad TC20 Automated Cell Counter (Bio-Rad, Hercules, CA); for low confluency calcium differentiation, $3x10^5$ HFKs were plated on a 10cm tissue culture dish; for high confluency calcium differentiation, $1x10^6$ HFKs were plated. Cells were trypsin harvested after three days' of daily calcium treatment. For differentiation in

semisolid medium, $1x10^6$ HFKs were plated into 25mL of EpiLife containing 1.7% methylcellulose in a non-treated 10cm dish. Cells were harvested using washing in ice-cold PBS and centrifugation as previously described. Untreated controls for differentiation were cells plated into monolayer in normal EpiLife and collected at the same timepoint as differentiated samples.

4.5.3 Differentiation of W12E cells

W12E cells were differentiated in a similar manner to HFKs. W12E cells were trypsin harvested from subconfluent monolayer cultures. $1.5x10^6$ W12E cells were plated into 25mL of semisolid medium, E-media containing 5% FBS and 1.7% methylcellulose in a non-treated 10cm dish. Cells were harvested using washing in ice-cold PBS and centrifugation as above. Untreated controls for differentiation were cells plated into monolayer in E-media containing 5% FBS.

4.5.4 qPCR of HPV16 L1

For qPCR detection of HPV16 L1 expression levels, a modified primer-based protocol was conducted on the ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). Forward and reverse primers for the HPV16 L1 were derived from the laboratory of Dr. Martin Sapp (Shreveport, LA) and are listed below[141]. Primers (400nM final concentration) and 2µL of cDNA were mixed with Power SYBR Green or PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA) per 20µL reaction. cDNA was diluted 1:4 prior to usage in the PCR reaction. For cycling conditions, annealing was carried out for 15 seconds and extension for 45 seconds.

Table 4.1 - List of primers used to amplify HPV16 L1.

Target	Primer sequence (5' – 3')	Annealing /extension temperature
HPV16 L1	**F-** GGTGTTGAGGTAGGTCGTGG **R-** CACACCTGCATTTGCTGCAT	58°/72°

4.6 FIGURES

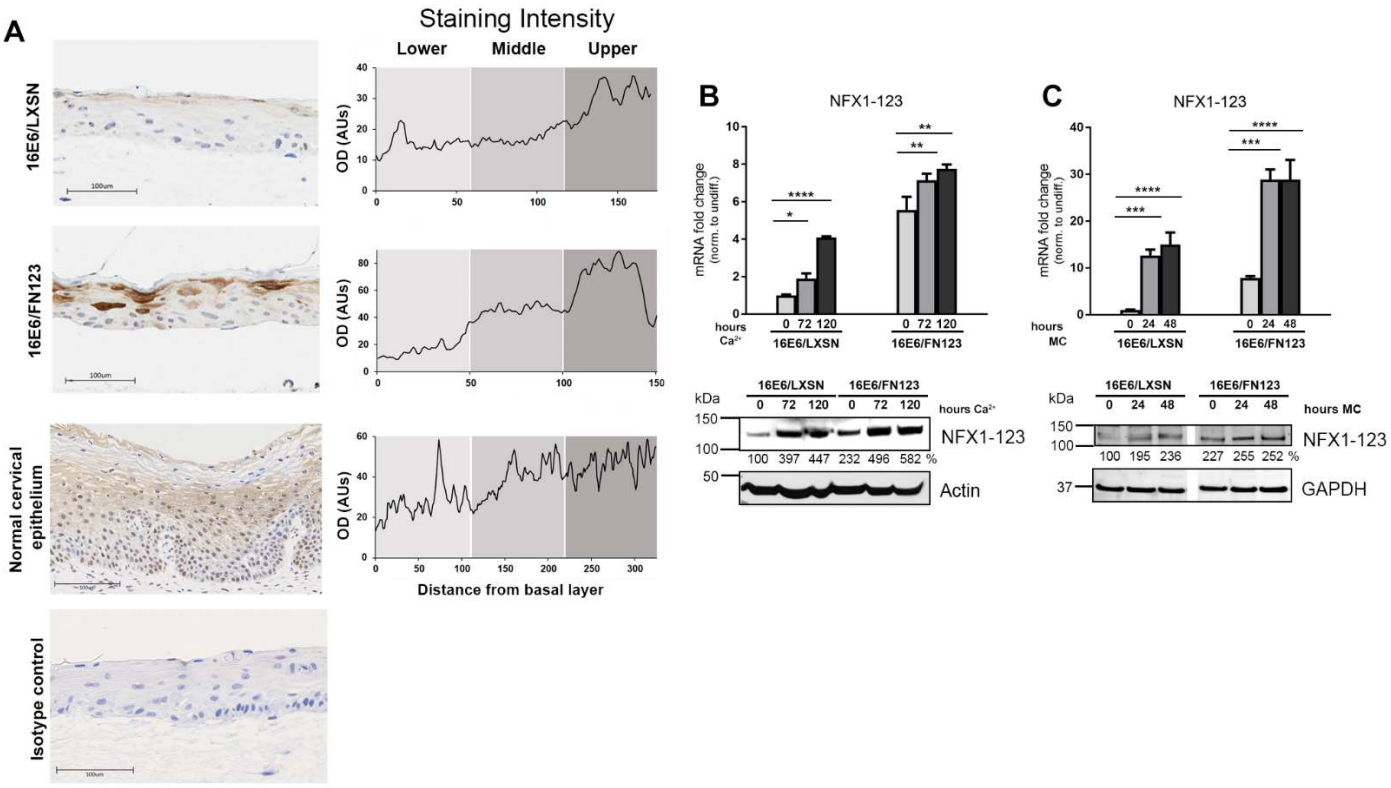


Figure 4.1. NFX1-123 expression increases with keratinocyte differentiation. (A) Organotypic raft cultures grown using HFKs serially transduced with 16E6 and then either an empty vector control (16E6/LXSN) or a FLAG-tagged NFX1-123 overexpression construct (16E6/FN123) and normal cervical epithelial biopsies were stained for NFX1-123 protein via immunohistochemistry. Staining intensity was measured over a linear distance from the basal layer in ImageJ and the average of four plots over two sections is shown. (B) 16E6/LXSN and 16E6/FN123 HFKs were differentiated with 1.8mM Ca^{2+} treatment for 0, 72, or 120 hours, and total mRNA and protein collected. Mean expression of NFX1-123 mRNA was measured by qPCR and normalized to 16E6/LXSN 0 hours. Protein levels of NFX1-123 were measured by Western blot using actin as a loading control. (C) 16E6/LXSN and 16E6/FN123 HFKs were differentiated by suspension in 1.7% methylcellulose medium (MC) for 0, 24, or 48 hours, and total mRNA and protein collected. Mean expression of NFX1-123 mRNA was measured by qPCR and normalized to 16E6/LXSN 0 hours. Protein levels of NFX1-123 were measured by Western blot using GAPDH as a loading control. All qPCRs were normalized to the housekeeping gene 36B4, and all error bars represent 95% confidence intervals from the technical replicates ($n = 3$).

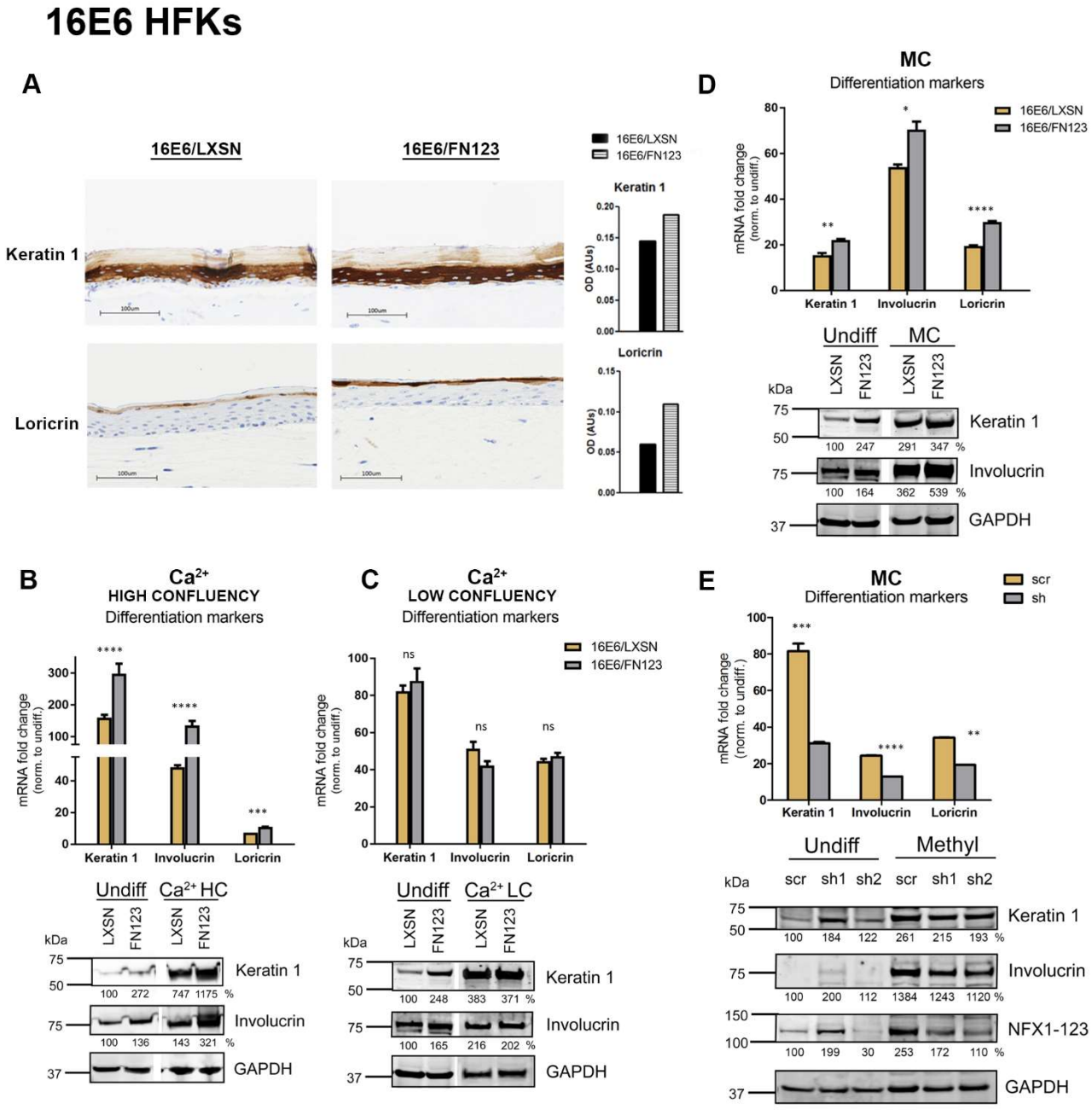


Figure 4.2. NFX1-123 regulates expression of differentiation markers in 16E6 HFKs. (A) 16E6/LXSN and 16E6/FN123 raft cultures were stained for protein expression of differentiation markers Keratin 1 and Loricrin via immunohistochemistry. Total staining intensity for the section area was calculated in ImageJ. (B and C) $1x10^6$ 16E6/LXSN and 16E6/FN123 HFKs (B) or $3x10^5$ 16E6/LXSN and 16E6/FN123 HFKs (C) were plated and treated with 1.8mM Ca^{2+} for 0 or 72 hours, and total mRNA and protein collected. (D) $1x10^6$ 16E6/LXSN and 16E6/FN123 HFKs were suspended in methylcellulose (MC) for 0 or 24 hours, and total mRNA and protein collected. (B,

C, and D) mRNA levels of Keratin 1, Involucrin, and Loricrin were measured by qPCR and normalized to 16E6/LXSN 0 hours. Protein levels of Keratin 1 and Involucrin were assessed by Western blot. GAPDH was used as a loading control. Densitometry analysis was done in ImageJ and normalized to undifferentiated 16E6/LXSN. Statistical significance was calculated using unpaired t-tests. (E) 16E6 HFKs were transduced with short hairpins targeting NFX1-123 (sh1 and sh2) or scramble short hairpin control (scr). $1x10^6$ scr, sh1, and sh2 cells were suspended in methylcellulose (MC) for 0 or 24 hours, and total mRNA and protein collected. mRNA levels of Keratin 1, Involucrin, and Loricrin were measured by qPCR and normalized to scr 0 hours. Statistical significance was calculated using one-way ANOVA with Bonferroni correction. Protein levels of Keratin 1 and Involucrin were assessed by Western blot. Densitometry analysis was done in ImageJ and normalized to undifferentiated scr. (B, C, D, and E) All qPCRs were normalized to the housekeeping gene GAPDH, and all error bars represent 95% confidence intervals from the technical replicates ($n = 3$). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

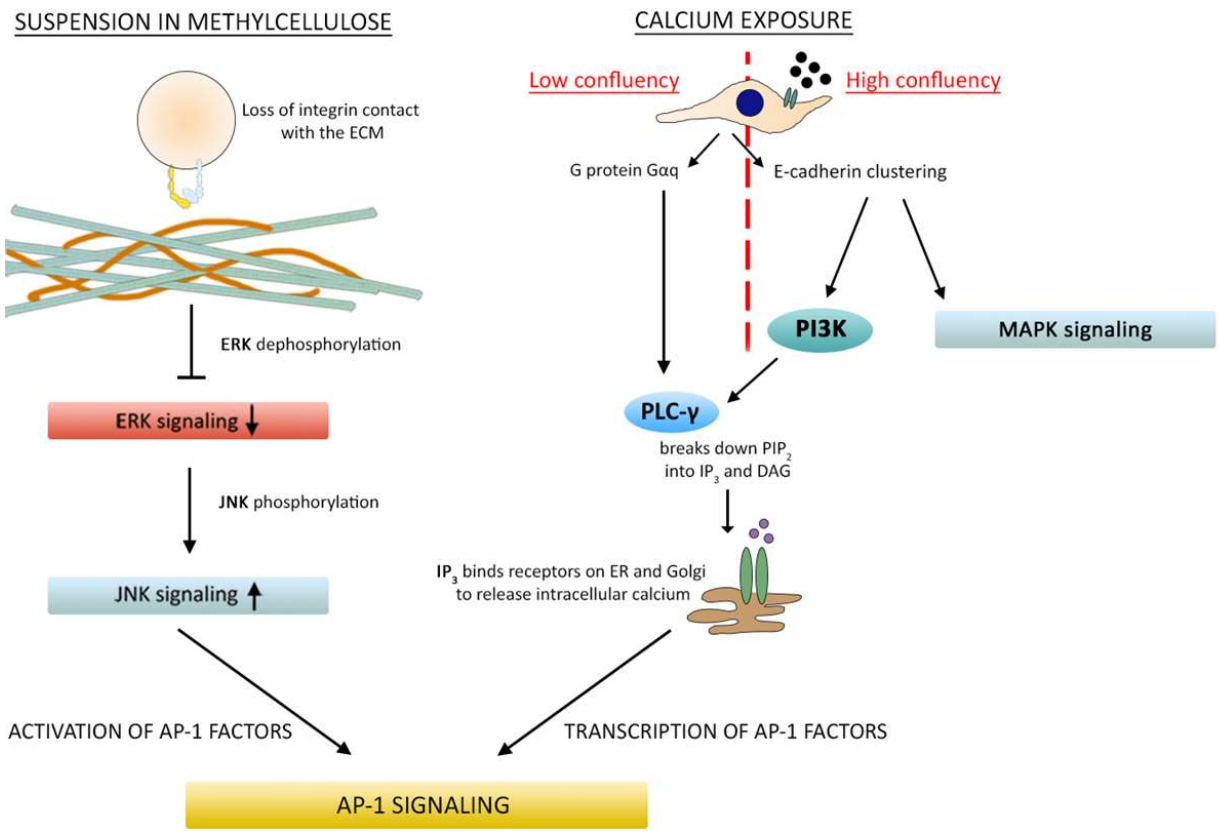


Figure 4.3. Diagram of intracellular signaling pathways activated by differentiation stimuli. Following suspension in methylcellulose, phosphorylation and signaling activity of extracellular signal-regulated kinase (ERK) decreases. Phosphorylation and signaling activity of c-Jun N-terminal kinase (JNK) increases. This leads to activation of AP-1 transcription factors and subsequent transcriptional expression of differentiation markers. For cells plated at low confluency, exposure to extracellular calcium activates the G protein Gαq, which in turn activates phospholipase C gamma (PLC-γ). PLC-γ hydrolyzes the second messenger phosphatidylinositol 4,5-bisphosphate (PIP_2) and activates a series of reactions that lead to the transcription of AP-1 subunits, AP-1 signaling and subsequent transcriptional expression of differentiation markers. For cells plated at high confluency, exposure to extracellular calcium leads to E-cadherin clustering, activating mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) activity. PI3K activates PLC-γ and here, the pathway converges with that of low confluency cells treated with calcium terminating in AP-1 signaling and transcription of differentiation markers.

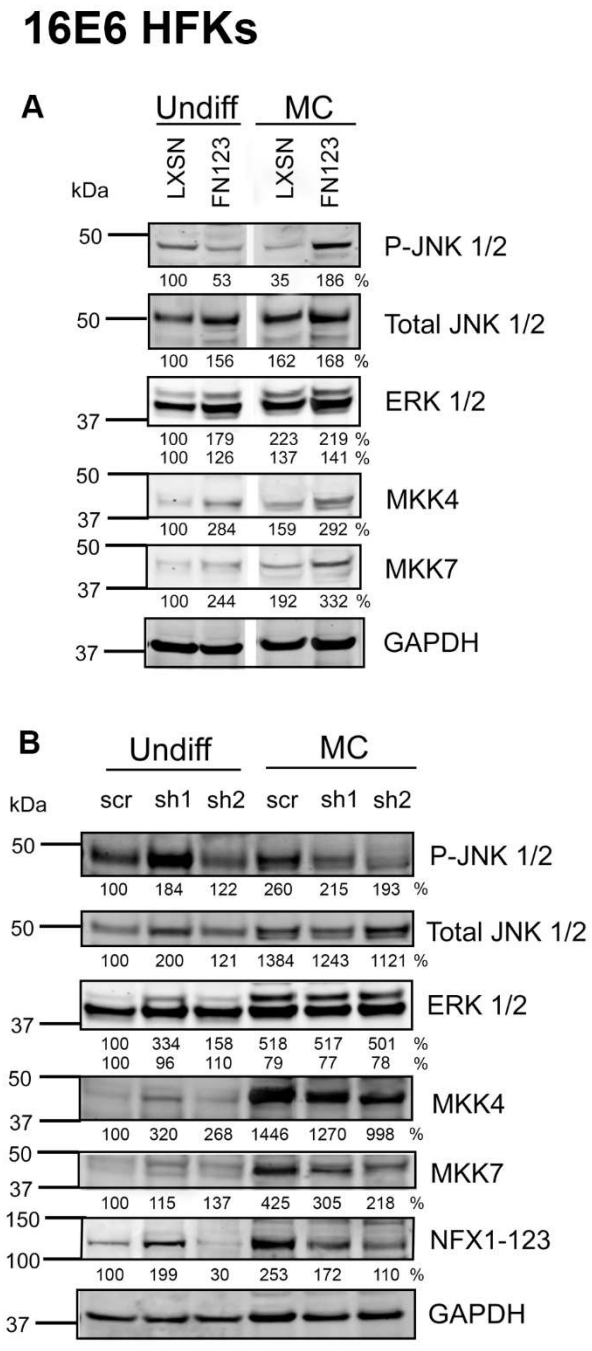


Figure 4.4. NFX1-123 regulates phosphorylation of JNK through MKK4 and MKK7. (A) $1x10^6$ 16E6/LXSN and 16E6/FN123 HFKs were suspended in methylcellulose (MC) for 0 or 24 hours, and total protein collected. Protein levels of phosphorylated JNK (P-JNK), total JNK, total ERK, total MKK4, and total MKK7 were assessed by Western blot. GAPDH was used as a loading control. Densitometry analysis was done in ImageJ and normalized to undifferentiated 16E6/LXSN. (B) 16E6 HFKs were transduced with a short hairpin targeting NFX1-123 (sh1 or sh2) or a scramble short hairpin control (scr). $1x10^6$ scr, sh1, and sh2 cells were suspended in methylcellulose for 0 or 24 hours, and total protein collected. Protein levels of P-JNK, total JNK, total ERK, total MKK4, and total MKK7 were assessed by Western blot. Densitometry analysis was done in ImageJ and normalized to undifferentiated scr.

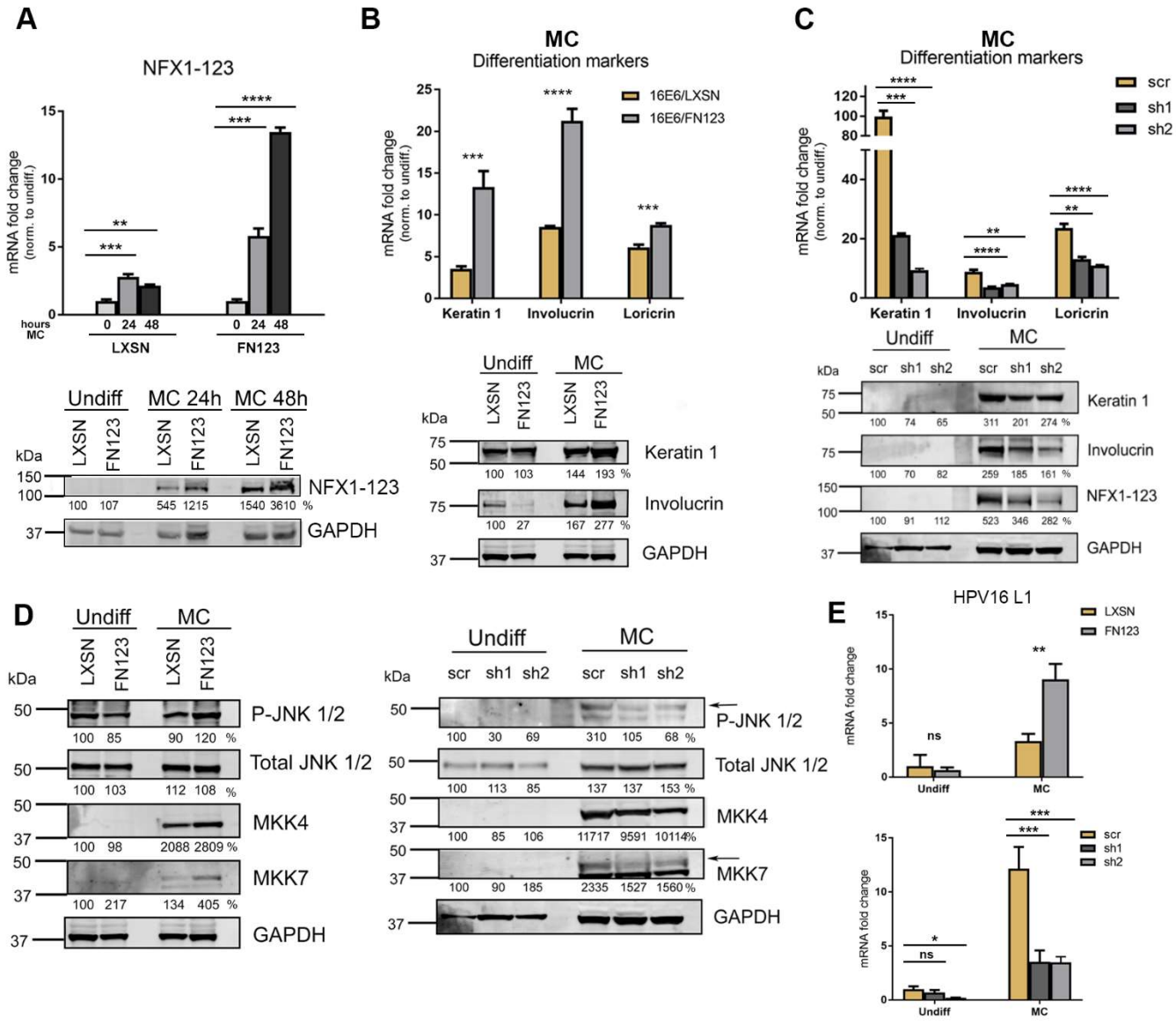


Figure 4.5. NFX1-123 mediates differentiation and L1 expression in HPV16-positive W12E cells. (A) W12E cells were transduced with LXSN control vector (LXSN) or FLAG-tagged NFX1-123 overexpression construct (FN123) and 1x10^6 cells were suspended in methylcellulose (MC) for 0, 24, or 48 hours. Mean expression of NFX1-123 mRNA measured by qPCR and normalized to W12E/LXSN 0 hours. Protein levels of NFX1-123 were measured by Western blot using GAPDH as a loading control. (B) 1.5x10^6 LXSN and FN123 W12E cells were suspended in methylcellulose (MC) for 24 hours and total mRNA and protein collected. mRNA levels of Keratin 1, Involucrin, and Loricrin were measured by qPCR and normalized to W12E/LXSN 0 hours. All qPCRs were normalized to the housekeeping gene GAPDH, and all error bars represent 95%

confidence intervals from the technical replicates (n = 3). Statistical significance was calculated using unpaired t-tests. Protein levels of Keratin 1 and Involucrin were assessed by Western blot. GAPDH was used as a loading control. Densitometry analysis was done in ImageJ and normalized to undifferentiated W12E/LXSN. (C) W12E cells were transduced with a short hairpin targeting NFX1-123 (sh1 or sh2) or a scramble short hairpin control (scr), and $1.5x10^6$ cells differentiated by suspension in methylcellulose for 0 or 24 hours. mRNA expression of Keratin 1, Involucrin, and Loricrin was measured by qPCR and normalized to W12E/scr 0 hours. All qPCRs were normalized to the housekeeping gene 36B4, and all error bars represent 95% confidence intervals from the technical replicates (n = 3). Statistical significance was calculated using one-way ANOVA with Bonferroni correction and p values for the difference in means between scr and sh1 or sh2 are shown. Protein levels of Keratin 1 and Involucrin were measured by Western blot using GAPDH as a loading control. Densitometry analysis was done in ImageJ and normalized to W12E/scr. (D) Protein levels of P-JNK, total JNK, total ERK, total MKK4, and total MKK7 were assessed by Western blot in LXSN and FN123 W12E cells (left) or scr, sh1, and sh2 W12E cells (right). Densitometry analysis was done in ImageJ and normalized to undifferentiated LXSN or scr W12E cells. (E) mRNA expression levels of HPV16 L1 were measured by qPCR and normalized to LXSN or scr W12E cells. All qPCRs were normalized using 36B4 as a housekeeping gene. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

Chapter 5
Extension of active growth and increased telomerase activity by 16E6 greater NFX1-123

The following text is from the article:

Vliet-Gregg PA, Robinson KL, **Levan J,** Matsumoto LR, Katzenellenbogen RA. NFX1-123 is highly expressed in cervical cancer and increases growth and telomerase activity in HPV 16E6 expressing cells. (2018) **Cancer Letters** (under review).

Figure numbers have been updated to conform to the formatting of this dissertation. The text is largely as published with some editorial changes.

5.1 ABSTRACT

Cervical cancer is a significant contributor to women's cancer mortality worldwide, and is caused by persistent infection with high-risk human papillomavirus (HR HPV). The two viral oncoproteins of HR HPV, E6 and E7, partner with host cell proteins to target oncogenic proteins and pathways. Previously, we have shown HR HPV type 16 E6 (16E6) interacts with the host protein NFX1-123 to target telomerase and cellular immortalization, requiring NFX1-123 to fully upregulate telomerase activity. We now report that NFX1-123 is highly expressed in primary cervical cancers. *In vitro*, cells expressing 16E6 and overexpressing NFX1-123 have extended active growth, decreased senescence marker staining, and more rapid cell cycling compared to 16E6 expressing cells with endogenous amounts of NFX1-123. Confirming earlier studies of this protein partnership, these findings were associated with increased telomerase activity and augmented expression of its catalytic subunit, hTERT. In complement, HPV 16 positive cervical cancer cell lines with knocked down NFX1-123 had slowed growth and reduced hTERT. In cells that express HR HPV E6, greater expression of NFX1-123 can modify active cellular growth and augment hTERT expression and telomerase activity over time, potentially supporting the initiation and progression of HPV-associated cancers.

5.2 INTRODUCTION

High risk HPVs (HR HPVs) are the causative agent of cervical cancer, the fourth most common cancer in women, as well as other anogenital and head and neck cancers[4,6,108,142,143]. In total, HR HPV causes 5% of all cancers worldwide[8,143]. HPV-associated cancers universally express the viral oncogenes HR HPV E6 and E7, whose functions activate pathways critical to cancer development. One of the most fundamental of these oncogenic pathways is cellular immortalization, driven by the enzyme telomerase[144].

In normal somatic cells, telomeric DNA at the ends of chromosomes is serially eroded and triggers growth arrest once it becomes critically shortened[145,146]. Telomerase extends the repetitive telomeric DNA such that cells expressing it avoid senescence, apoptosis, and cellular crisis[144,147]. Telomerase is activated in all HPV-associated cancers through increased expression of the catalytic subunit of telomerase, hTERT[148,149]. Expression of hTERT is rate-determining, and HR HPV E6 and E7 have evolved transcriptional, epigenetic, and post-transcriptional mechanisms to increase its expression[35,52,150,151]. HR HPV E6 requires cellular proteins to regulate hTERT and telomerase activity, one of which is NFX1-123[54–56].

NFX1-123 is a cytoplasmic protein that binds HR HPV type 16 E6 (16E6) and is highly expressed in cervical cancer cell lines[55,92]. With 16E6, NFX1-123 increases hTERT and telomerase activity through post-transcriptional stabilization of hTERT mRNA[55,56]. Although it is known that NFX1-123 and 16E6 collaboratively increase hTERT and telomerase, what remains unexplored are the dynamics of NFX1-123 expression over time and its longitudinal effects on hTERT expression, telomerase activity, cellular growth, and longevity. Furthermore, although highly expressed in cervical cancer cell lines, the expression level of NFX1-123 in primary cervical cancers is also unknown.

In the present chapter, we present evidence that NFX1-123 is highly expressed in HPV-positive cervical precancerous lesions and cancers, and that NFX1-123 has a longitudinal and sustained role in cellular growth. Expression of NFX1-123 to levels approaching cervical cancers led to extended active cellular growth, maintained cell cycling, and reduced senescence in 16E6 expressing human foreskin keratinocytes (16E6 HFKs). In parallel with these increases in growth, hTERT expression and telomerase activity were increased in early passages and became further amplified over time. Knock down of the highly expressed endogenous NFX1-123 in the

HPV 16 positive SiHa cervical cancer cell line resulted in slowed growth and reduced hTERT. These results indicate that NFX1-123 is markedly increased in cervical cancer, that its greater expression in 16E6 expressing cells is associated with improved growth and telomerase activity, and that this association is maintained in HPV 16 positive cervical cancer cell lines.

5.3 RESULTS

5.3.1 High NFX1-123 expression in cervical cancer samples

To examine NFX1-123 expression in cervical cancer samples, its typical expression in normal stratified squamous epithelium was first examined as a baseline. NFX1-123 expression in sections of three-dimensional organotypic HFK raft cultures, which mimic stratified squamous epithelium, and normal cervical epithelium was detected by immunohistochemistry. NFX1-123 was detected in raft cultures in the upper, differentiated cells as well as sporadic basal layer cells (Fig 5.1A). This was not significantly changed by 16E6 co-expression (Fig 5.1B). With overexpression, greater NFX1-123 was seen both in the basal and upper differentiated cells (Fig 5.1C). NFX1-123 expression was then examined in 37 HPV 16 positive precancer and cancer specimens. Compared to HFK and 16E6 HFK raft cultures with endogenous NFX1-123, staining of NFX1-123 was more intense, resembling the staining of raft cultures with overexpressed NFX1-123 (Fig 5.1E, CIN2) or with even darker staining (Fig 5.1F, SCC). NFX1-123 protein was also quantified: 11% stained with moderate (Fig 5.1E) and 89% with high intensity (Fig 5.1F).

5.3.2 NFX1-123 increased total population doublings and length of active growth in 16E6 HFKs but not HFKs alone

NFX1-123 is highly expressed in cervical cancer cell lines[53] and primary cervical cancers (Fig 5.1). Given its role in increasing telomerase expression, it was important to determine if

increased NFX1-123 expression in HFKs would affect their growth patterns and longevity over time, and whether changes to growth were dependent on HR HPV E6 co-expression. To quantify growth rate changes mediated by 16E6 and NFX1-123 in HFKs, we developed the following objective definition of active cellular growth: a doubling in cell number within four days' time, at minimum. To identify trends unconnected to the primary cell background, studies were repeated in biologically independent cell lines.

To examine whether effects of increased NFX1-123 on active cellular growth were dependent on 16E6, non-16E6 HFKs were utilized. HFKs transduced with FLAG-tagged NFX1-123 (FN123) had no difference in overall growth, total cell counts, or cumulative population doublings when compared to HFKs transduced with an empty vector control (LXSN) (Fig 5.2A). Next, to explore the role of 16E6 in combination with greater NFX1-123 expression, four HFK cell lines were serially transduced with 16E6 and then with either FN123 (16E6/FN123) or LXSN vector (16E6/LXSN), and their active growth phase assessed. Confirming effects of 16E6 from previous studies, HFKs with 16E6 and endogenous levels of NFX1-123 had a longer period of active growth than HFKs without 16E6[151]. Across all four cell lines, 16E6 HFKs with greater levels of NFX1-123 (16E6/FN123) achieved greater population doublings during active growth at each timepoint compared to 16E6/LXSN HFKs at the same timepoint (Fig 5.2B-E closed squares versus open circles). Although variations in total population doublings and length of time in active growth were observed across biologically independent HFK cell lines, 16E6/FN123 HFKs consistently had a longer period of active growth and supported more population doublings than their isogenic controls (16E6/LXSN HFKs). In fact, after more than 200 days in culture 16E6/FN123 HFK3 and HFK4 cells continue to be actively growing (Fig 5.2D and E,

horizontal arrow). These results indicate that 16E6 HFKs overexpressing NFX1-123 had more robust and longer active growth periods as compared to their matched control cells.

5.3.3 NFX1-123 overexpression was sustained in 16E6/FN123 HFKs

These studies spanned an extended period in cell culture, and NFX1-123 expression over such a period has not been explored before. It was thus important to evaluate if NFX1-123 expression changed during this long term growth. NFX1-123 expression was quantified serially in all 16E6/LXSN and 16E6/FN123 HFK cell lines (Fig 5.3). Results are shown for two lines, HFK3 and HFK4, which encompass the shorter and longer growth potential, respectively, observed across distinct primary cell lines. Timepoints shown include matching shared timepoints, as well as one additional timepoint from the period in which 16E6/FN123 continued growing past the end of active growth for 16E6/LXSN (Fig 5.2, vertical arrows). Overall, NFX1-123 mRNA (Fig 5.3A and E) and protein (Fig 5.3B and F) remained elevated in 16E6/FN123 HFKs when compared to 16E6/LXSN HFKs. These data indicated that ectopic NFX1-123 overexpression was maintained throughout 16E6/FN123 HFK culture, and endogenous NFX1-123 did not significantly change during long-term culture (Fig 5.3B and F, LX). Because the *NFX1* gene expresses two splice variant isoforms in keratinocytes, we wanted to ensure effects were not due to the other isoform, NFX1-91[52–54,152]**.** Indeed, the mRNA and protein expression of NFX1-91 was not affected by overexpression of NFX1-123 (Fig 5.3C, D, G, and H).

5.3.4 16E6/FN123 HFKs had reduced senescence marker expression during long-term active growth

The greater cell numbers consistently achieved by 16E6/FN123 HFKs, as compared to 16E6/LXSN HFKs, could be due to differences in cellular senescence initiation, cell cycle changes, or both. To quantify differences in cellular senescence associated with NFX1-123

amounts, cells were stained for senescence-associated beta-galactosidase (beta-gal) at matching timepoints (Fig 5.2, asterisks). At each timepoint, 16E6/LXSN HFKs had a greater percentage of cells with positive staining for beta-gal compared to 16E6/FN123 HFKs (Fig 5.4). In the shorter growing HFK3 cell line, 89% of 16E6/LXSN HFKs stained positive at the late shared timepoint whereas 16E6/FN123 HFKs maintained a low positivity of 11% (Fig 5.4A). For the longer growing HFK4 cell line, 16E6/LXSN HFK4 had a greater percent of cell staining for beta-gal at both early and late shared passages than 16E6/FN123 HFK4, although they overall did not have as dramatic an increase in beta-gal staining as HFK3. This suggests that the biologic background of 16E6 HFKs can affect the pace of senescence initiation, but in each case greater NFX1-123 levels was consistently linked with reduced senescence associated beta-gal staining.

5.3.5 16E6/FN123 HFKs cycled faster during long-term cell culture

To quantify the rate of cell cycle progression in 16E6/LXSN and 16E6/FN123 HFKs, the cell cycle profiles of density arrested and released cells were determined using BrdU incorporation and FACS analysis. In both HFK3 and HFK4 lines, 16E6/LXSN HFKs had slowed progression through the cell cycle compared to matched 16E6/FN123 HFKs (Fig 5.4C and D). For the shorter growing HFK3 cell line, 16E6/FN123 HFK3 quickly entered S phase after release (41.7% at one hour) whereas most 16E6/LXSN HFK3 did not begin to enter S phase until four hours post-release. By 11 hours, 16E6/LXSN HFK3 had progressed to G2/M while most 16E6/FN123 HFK3 had re-entered G1 and S phases (Fig 5.4C). For the longer growing HFK4 line, 16E6/LXSN HFK4 remained in G2/M even at 11 hours post-release while 16E6/FN123 HFK4 maintained a typical cycling pattern (Fig 5.4D). Therefore, analyses of 16E6/FN123 cells from HFK3 and HFK4 found evidence of both decreased senescence and a faster cell cycle progression late in active phase growth compared to 16E6/LXSN.

5.3.6 16E6/FN123 HFKs had greater hTERT expression and telomerase activity that was amplified over time

We previously have shown that NFX1-123 overexpression in 16E6 HFKs increases hTERT expression and telomerase activity[54,55], and others have shown that increased hTERT expression improves growth and longevity in tissue culture[151,153,154]. We thus hypothesized that the improved active growth seen in 16E6/FN123 could be due to increased telomerase activity. The expression of hTERT was quantified over time in 16E6/LXSN and 16E6/FN123 HFKs. At the early shared timepoint (T1), 16E6/FN123 HFK3 had two fold greater hTERT mRNA (Fig 5.5A) similar to our previously published findings[35,54]. Interestingly, hTERT expression in 16E6/FN123 HFK3 rose over time and remained elevated during late passages (Fig 5.5A – black bars). Dynamics of hTERT expression and telomerase activity over time appeared slightly different for HFK4, but these cells do represent a longer period of growth and the overall trends remain the same. For HFK4, hTERT was three fold greater in 16E6/FN123 cells at T1 (Fig 5.5C – black bars). Both 16E6/LXSN and 16E6/FN123 hTERT levels increased at T2, with 16E6/FN123 HFK4 found to be nearly 25 times the level seen in 16E6/LXSN at T1. Levels did decline at T3 (last shared passage) and T4, but still remained elevated in 16E6/FN123 compared to 16E6/LXSN.

5.3.7 16E6/FN123 HFKs had greater hTERT expression and telomerase activity that was amplified over time

Telomerase activity was assessed by a modified qRT-PCR-based Telomeric Repeat Amplification Protocol (TRAP) assay. Work done to optimize and modify the protocol are described in the Methods section. Telomerase activity closely paralleled trends seen with hTERT expression. Both 16E6/FN123 HFK3 and HFK4 had increased telomerase activity when

compared to 16E6/LXSN HFKs at early shared timepoints (Fig 5.5B and D). Like hTERT, telomerase activity was amplified over time in 16E6/FN123 HFKs. For 16E6/FN123 cells of both HFK3 and HFK4, telomerase activity remained elevated above initial levels in 16E6/LXSN cells at T1 throughout active growth.

5.3.8 Knock down of NFX1-123 in SiHa cells slowed growth and decreased levels of hTERT

NFX1-123 is highly expressed in cervical cancer cell lines[53] and in primary cervical cancers (Fig 5.1), indicating that increased expression of NFX1-123 may be selected for over the course of oncogenic progression, or that greater expression may promote subsequent development of cancers. To study the role of NFX1-123 in cellular growth and hTERT expression in the context of cancer, NFX1-123 was knocked down in HPV 16 positive SiHa cells. Cells were transduced with two different short hairpin RNAs (sh1 or sh2) targeting NFX1-123, or a scrambled short hairpin RNA (scr) control. Short hairpin 1 (sh1) reduced NFX1-123 protein at both three and six days post transduction (38% and 80%, respectively), when compared to the scramble (scr) control, and NFX1-123 protein was also reduced (40%) in sh2 at day six (Fig 5.6A). NFX1-91 was also measured to assure no off-target effect of the short hairpin constructs (Fig 5.6B).

To quantify change in growth potential associated with a decrease in NFX1-123 in the short hairpin transduced SiHas, a double factor representing cellular growth was calculated. Cells transduced with scr, sh1, or sh2 were counted at day three and day six over a 24 hour period to yield the doubling factor. With knock down of NFX1-123 by sh1 or sh2, there was significantly reduced SiHa cell growth when compared to scr SiHa cells (Fig 5.7C). At day 6, cells with greater knock down of NFX1-123 (sh1) demonstrated a more dramatic reduction in their

doubling factor. In parallel with reduced growth, we found a dramatic 97% decrease in hTERT mRNA when NFX1-123 was decreased at six days post transduction (Fig 5.6D).

5.4 Discussion

NFX1-123 has been shown to be highly expressed in cervical cancer cell lines, and we now demonstrate that NFX1-123 is highly expressed in primary cervical precancers and cancers. 16E6 HFKs that begin with greater NFX1-123 have extended periods of active growth in culture, complete more population doublings at every parallel timepoint, and achieve at least twice the total number of cells in culture. Cells also had faster cell cycling and a relative reduction in senescence. Greater NFX1-123 leads to increased hTERT in 16E6 expressing HFKs[53,92], and accordingly, these effects seen in cells with greater NFX1-123 were associated with increased hTERT and telomerase activity. The effect of NFX1-123 on growth and hTERT is also seen in the HPV 16 positive cervical cancer SiHa cell line, where even transient knock down of NFX1-123 led to slowed growth and reduced hTERT.

NFX1-123 expression was measured across long-term cultures to evaluate whether it shifted over time. *NFX1* gene expression is important for growth maintenance during stress in other systems[155,156], and long-term tissue culture leads to cellular stress[157,158]. The expression of endogenous NFX1-123 may have thus changed over time during long-term culture in response to cellular stress. However, we found 16E6/LXSN HFKs maintained stable NFX1-123 protein levels throughout long term growth. It was also fundamental to confirm that NFX1-123 overexpression was maintained throughout the study; this was especially important as knock down of NFX1-123 is quickly surmounted in tissue culture making reductions only transient (unpublished data). We found NFX1-123 overexpression was consistently increased overall in

16E6/FN123 cells relative to 16E6/LXSN HFKs, and was well within the range seen in cervical cancer cell lines[53].

16E6 HFKs that were transduced with NFX1-123 had greater hTERT and telomerase activity when compared to 16E6 HFKs with endogenous levels of NFX1-123. Interestingly, these greater hTERT levels were further augmented over time – paralleling increased active growth and population doublings in 16E6/FN123 HFKs. Long-term studies of HR HPV expressing HFKs have demonstrated increasing hTERT and telomerase activity[154], and our data in 16E6/LXSN HFKs support that (Fig 5.5A and C, gray bars). Intriguingly, a recent study noted that 16E6 was not the only determinant of hTERT expression over time[159]. The studies in this chapter indicate that during long-term growth, greater NFX1-123 is a determinant of augmented hTERT expression and telomerase activity in 16E6 HFKs (Fig 5.5, black bars). Together NFX1-123, hTERT, and telomerase impact active growth of keratinocytes in the presence of 16E6.

Telomerase is universally expressed in HPV-associated cervical cancers[144,147], and upregulation of hTERT is associated with worse clinical grade of cervical intraepithelial neoplasia and cervical dysplasia[160,161]. As a regulator of hTERT in the context of 16E6 expression, NFX1-123 appears to be an important factor in cervical cancer development. Because NFX1-123 is highly expressed in cervical cancer cell lines and primary cervical cancers, understanding the associative or causal role it plays in supporting and maintaining HPV-associated malignancies will help define factors driving transformation from HR HPV-infected keratinocytes to cancer.

5.5 Methods

5.5.1 Histologic analysis

Organotypic HFK raft cultures were formalin fixed and paraffin embedded (FFPE) following standard procedures. 37 HPV 16+ cervical intraepithelial neoplasia (CIN) 2, CIN3, carcinoma *in situ*, and cervical cancer FFPE blocks were obtained from the University of Washington HPV Research Group Specimen Repository. They were deidentified and not considered human subjects by the Seattle Children's Research Institute IRB. Sections were stained for NFX1-123 using a rabbit polyclonal anti-NFX1-123 antibody gifted by Dr. Ann Roman (1:1000 dilution). To quantify NFX1-123 staining, 3 independent, blinded reviewers scored multiple 20x slide images. Scoring was based on staining intensity of the specimen: none (no stain), low (yellow), moderate (yellow-brown) or high (dark brown).

5.5.2 Growth assay in HFK cells

For growth assays, 4 biologically independent HFK cell lines were produced by transduction and selection with either empty vector LXSN or FN123, or by serial transduction and selection with 16E6 and then either LXSN or FN123. After transduction, $5x10^5$ cells were plated onto 3 or more 10cm tissue culture (TC) dishes and fed every three days. Cells were counted every 4 days. The total cell number of each plate was recorded, averaged, and divided by $5x10^5$ to determine the number of population doublings achieved. After counting, $5x10^5$ HFK cells were replated and the remaining cells were collected for experiments. HFK cell lines were grown until they no longer doubled within 4 days' time, defined as the active growth phase.

5.5.3 Growth assay in SiHa cells

SiHa cervical cancer cells were grown to 70-80% confluency, transduced overnight with scr, sh1 NFX1-123, or sh2 NFX1-123 lentivirus, and then recovered for 24 hours in fresh media. At 48 hours post-transduction (day 2), infection was confirmed by GFP expression, and cells were counted and plated into four 10cm dishes. At 72 hours post-transduction (day 3), cells were

counted again, collected for experiments, and one plate was re-fed with media and kept in culture. This process was repeated again on days 5 and 6. The growth rate over 24 hours (doubling factor) for each cell line (scr, sh1, sh2) was calculated by the cell number on each plate on the day of collection divided by the cell number plated the previous day. At least 3 plates were included in each doubling factor calculation, for each cell line, at each timepoint. Statistical significance (p-values) was calculated using a one-way ANOVA with Bonferroni post hoc correction. The assays were repeated independently 3 times.

5.5.4 Telomeric repeat amplification protocol (TRAP) assay

Telomerase activity was measured using a modified version of the qPCR-based TRAPeze RT kit (Millipore Sigma, Burlington, MA). Trypsin harvested HFKs were counted and lysed in CHAPS lysis buffer, using 200µL per $1x10^6$ cells. In order to quantify telomerase activity, lysates of 293T cells, which are known to express telomerase, were used. To create a telomerase activity standard curve, $1x10^6$ 293T cells were lysed in 200µL of CHAPS lysis buffer and serial 1:10 dilutions prepared. qPCR was performed using an ABI StepOne Plus system (Applied Biosystems, Foster City, CA). Amplification was carried out in replicative triplicates and normalized to T1 16E6/LXSN. Error bars represent 95% confidence intervals.

5.5.5 Fluorescence-activated cell sorting (FACS) analysis

Cells were synchronized by growing to confluency plus 24 more hours before release as previously described[53]. Analysis was performed using a LSR II flow cytometer (BD Biosciences, San Jose, CA) and FlowJo v10 software (Treestar, Ashland, OR).

5.5.6 Senescence Associated Beta Galactosidase (Beta-gal) Assay

Beta-gal assays were completed using the Cellular Senescence Assay Kit (Millipore Sigma, Burlington, MA). Cells were plated onto 6-well plates at $5x10^4$ cells per well, grown to 60-70% confluence, washed with PBS, and fixed for 15 minutes. X-gal detection solution was prepared and added to each well. Cells were incubated in the dark, overnight at 37°C. Images were acquired using a Keyence Imaging Scope (Keyence Corp., Itasca, IL) at 10x and 20x magnification. A minimum of 10 fields were acquired, and total cell number, as well as the number of blue stained cells, were counted using the Keyence cell count program, with further analysis using ImageJ.

5.5.7 Statistical analysis

One-way ANOVA with Bonferroni's multiple comparisons test was used to estimate differences in means as appropriate and p value <0.05 was considered significant. These tests were performed using GraphPad Prism version 7.0, GraphPad Software, USA.

5.6 FIGURES

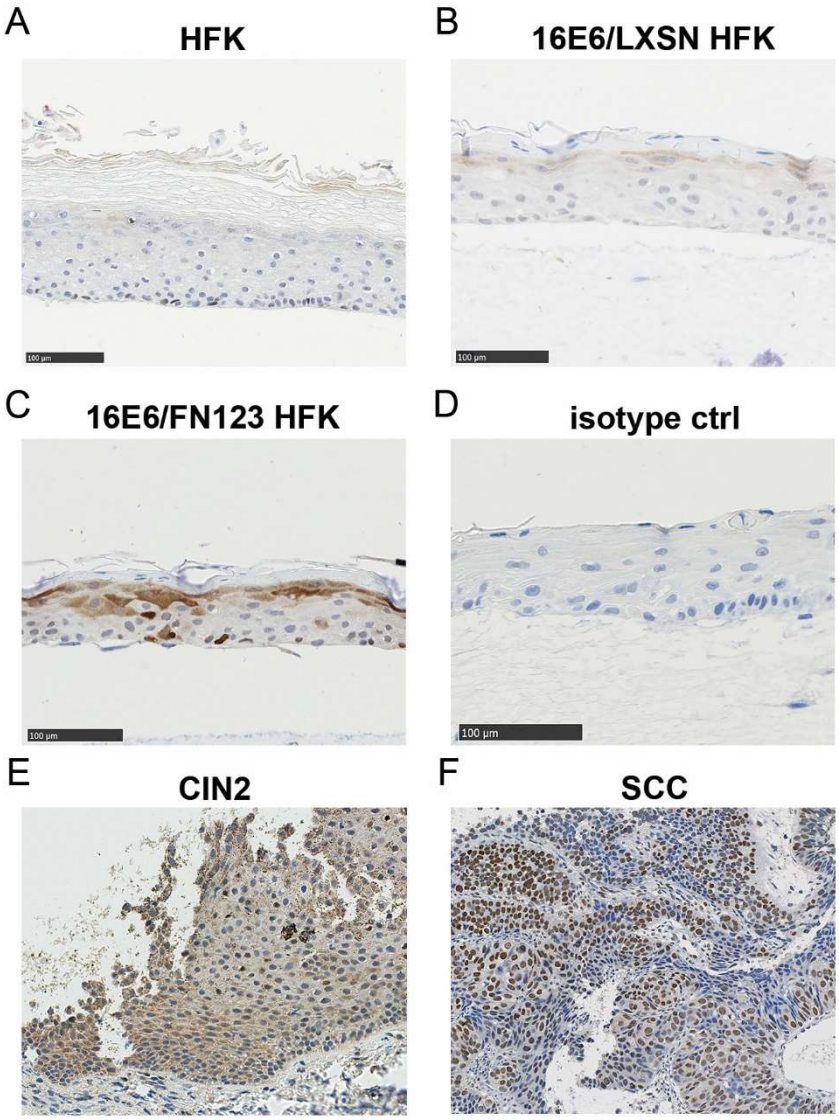


Figure 5.1. NFX1-123 expression in three dimensional raft cultures and in cervical precancerous and cancer samples. (A) HFKs (B) 16E6/LXSN (C) and 16E6/FN123 HFKs were grown in raft cultures and stained for NFX1-123 protein. Overexpression of NFX1-123 in 16E6/FN123 HFKs had increased staining in basal and differentiating keratinocytes. (D) Rabbit IgG isotype control. (E) HPV 16 positive cervical intraepithelial neoplasia 2 (CIN2) stained moderately for NFX1-123. (F) HPV 16 positive squamous cell carcinoma (SCC) stained highly for NFX1-123.

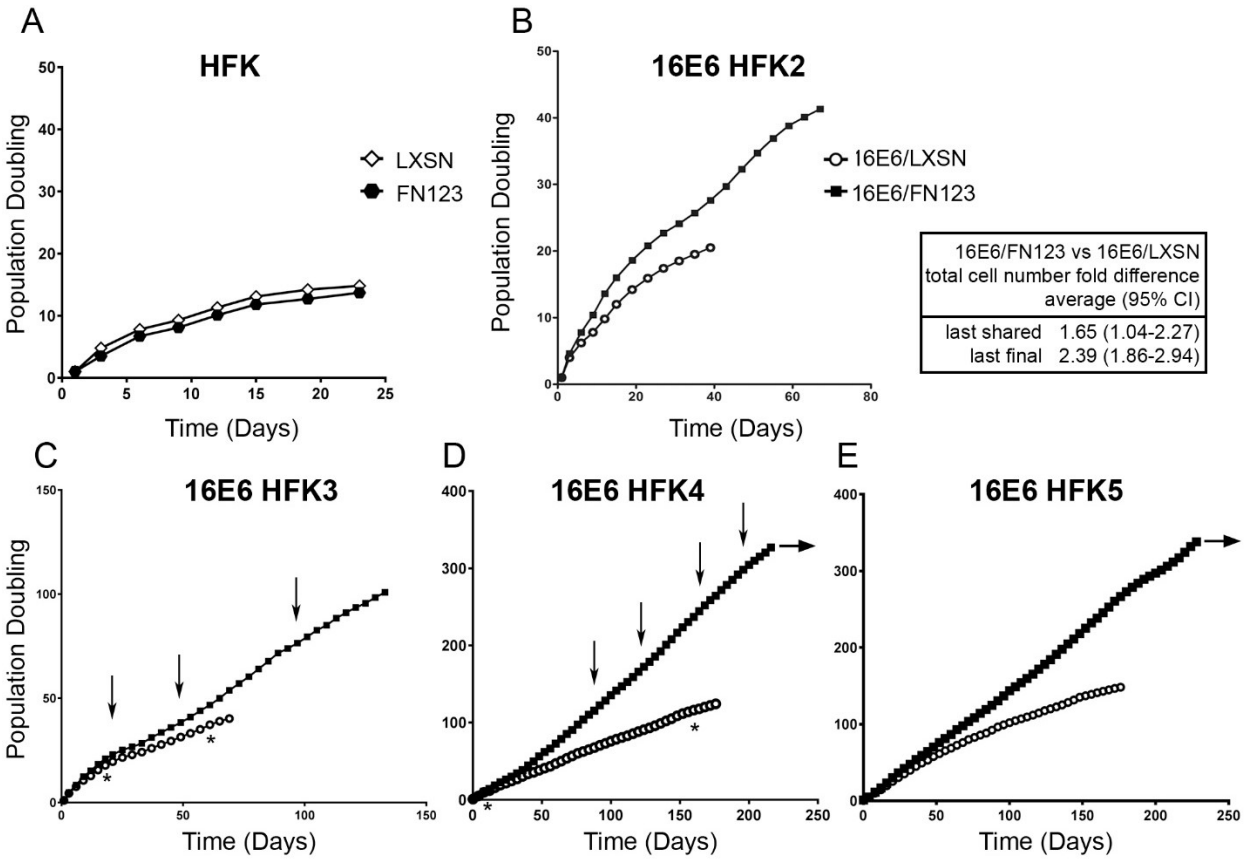


Figure 5.2. NFX1-123 overexpression enhanced active growth and population doublings in 16E6 HFKs. $5x10^5$ cells were plated and grown in culture until they no longer doubled within four days. Cumulative population doublings (PD) were calculated. (A) HFKs transduced with FLAG-tagged NFX1-123 (FN123, black hexagon) or an empty vector (LXSN, open diamond) grew for 14 PDs. (B-E) HFKs (HFK2-5) were transduced with 16E6 and then FN123 (16E6/FN123, black square) or LXSN (16E6/LXSN, open circle). Across 4 independent cell lines, 16E6/FN123 HFKs grew for more PDs and total cell numbers than matched 16E6/LXSN HFKs (average difference and 95% CI shown). Horizonal arrows indicate continued active growth of 16E6/FN123 HFKs in culture. Vertical arrows indicate timepoints T1-T2 in Figures 5.3 and 5.5. Asterisks indicate early and late shared timepoints in Figure 5.4

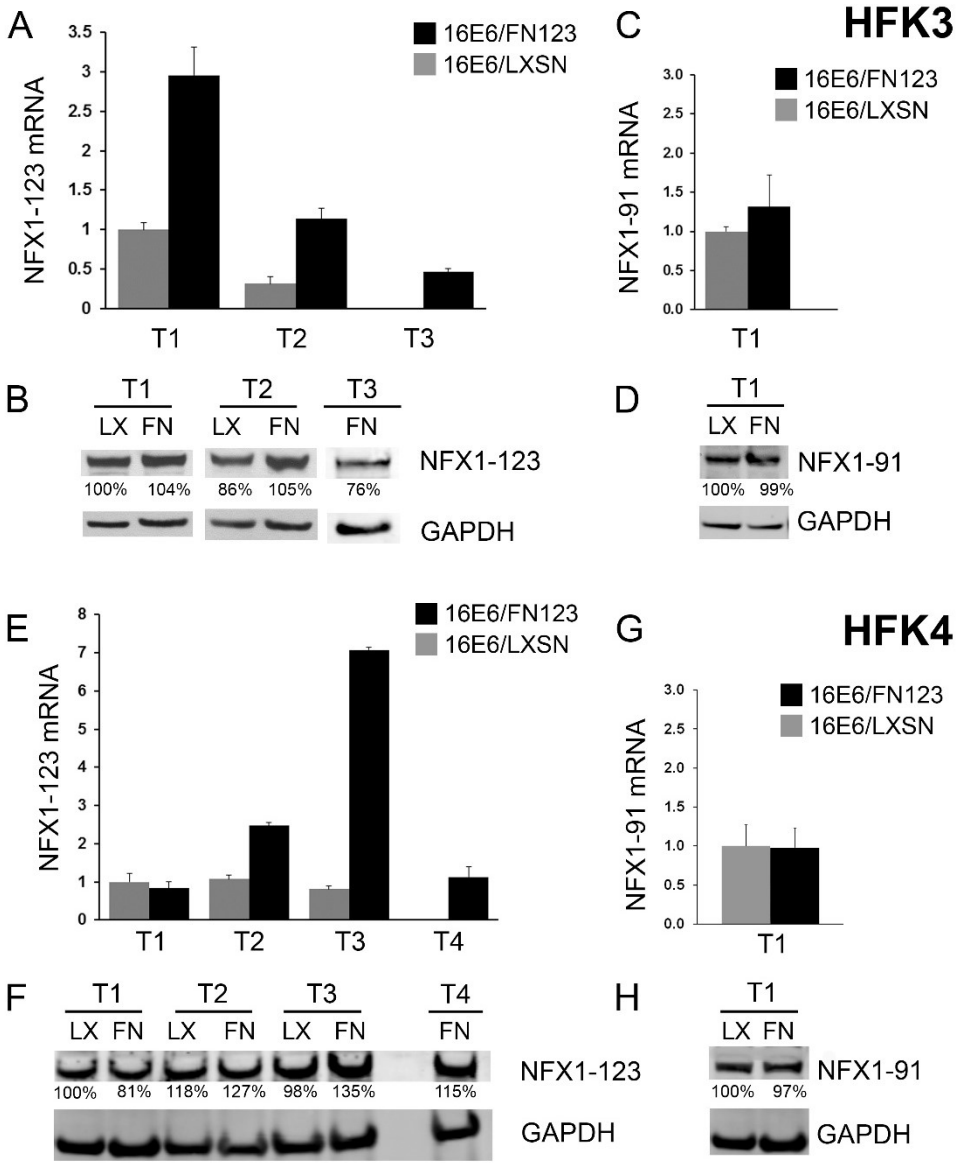
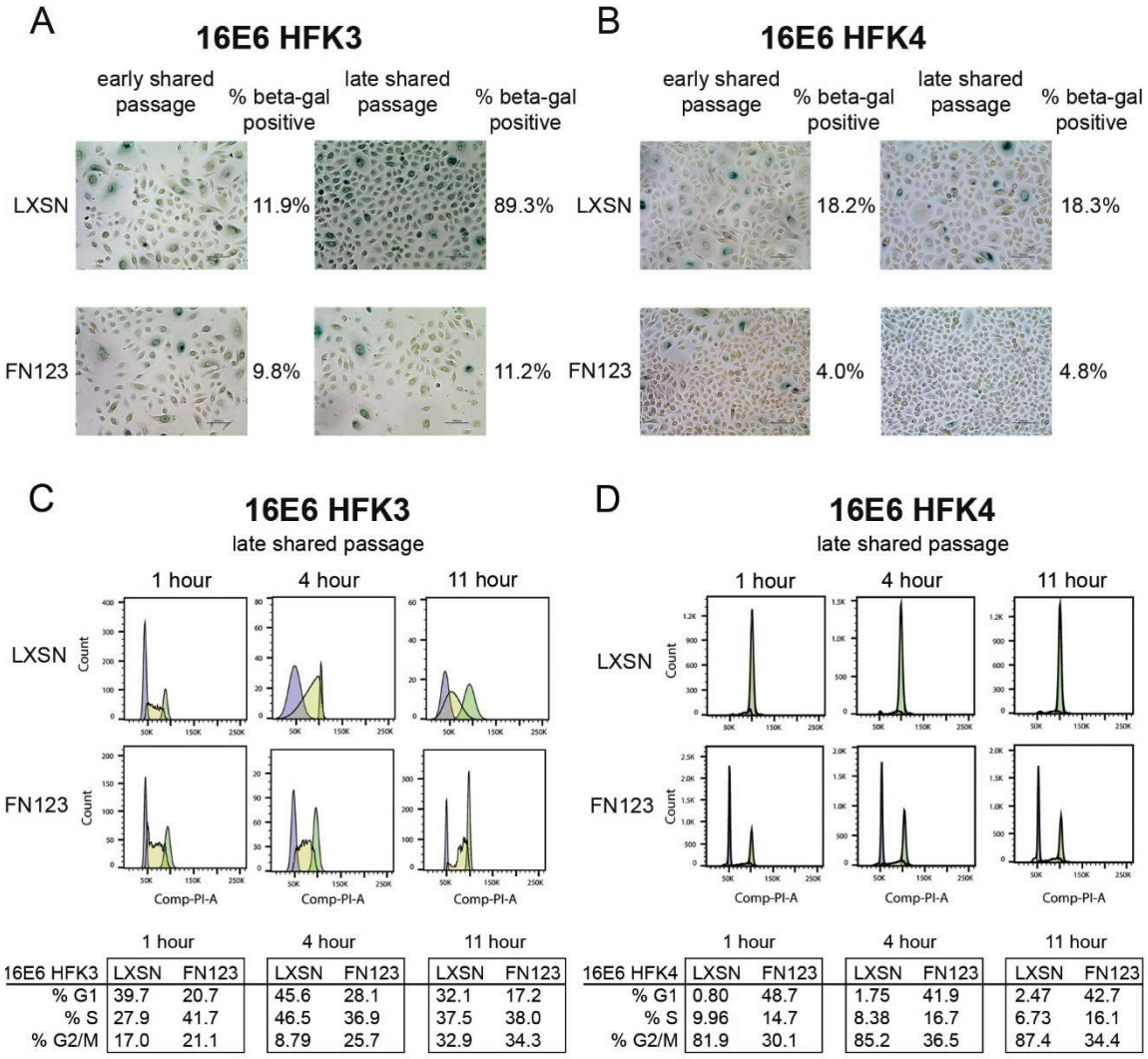


Figure 5.3. NFX1-123 overexpression was sustained in 16E6/FN123 HFKs. (A-D) HFK3; T1 and T2 are matched shared timepoints for 16E6/LXSN and 16E6/FN123. T3 is one timepoint for 16E6/FN123 past the end of active growth for 16E6/LXSN. (E-H) HFK4; T1-3 are matched shared timepoints for 16E6/LXSN and 16E6/FN123. T4 is one timepoint for 16E6/FN123 past the end of active growth for 16E6/LXSN. (A) Mean expression of NFX1-123 mRNA, relative to 16E6/LXSN HFK3 at T1, was quantified. (B) NFX1-123 protein was increased in 16E6/FN123 HFK3 (FN) relative to the 16E6/LXSN HFK3 (LX). (C) Mean expression of NFX1-91 mRNA, relative to 16E6/LXSN HFK3 at T1, was quantified. (D) NFX1-91 protein was equivalent in 16E6/FN123 HFK3 compared to 16E6/LXSN. (E) Mean expression of NFX1-123 mRNA, relative to 16E6/LXSN HFK4 at T1, was quantified. NFX1-123 mRNA was increased in 16E6/FN123 HFK4.

(B) NFX1-123 protein was moderately increased in 16E6/FN123 HFK4 (FN) relative to the 16E6/LXSN HFK4 (LX). (C) Mean expression of NFX1-91 mRNA, relative to 16E6/LXSN HFK4 at T1, was quantified. (D) NFX1-91 protein was equivalent in 16E6/FN123 HFK4 compared to 16E6/LXSN. (A, C, E, and G: all qPCRs were normalized to the housekeeping gene 36B4, and all error bars represent 95% confidence intervals from the technical replicates shown (n = 3). B, D, F and H: GAPDH = loading control.)



16E6 HFK3	1 hour LXSN	1 hour FN123	4 hour LXSN	4 hour FN123	11 hour LXSN	11 hour FN123
% G1	39.7	20.7	45.6	28.1	32.1	17.2
% S	27.9	41.7	46.5	36.9	37.5	38.0
% G2/M	17.0	21.1	8.79	25.7	32.9	34.3

16E6 HFK4	1 hour LXSN	1 hour FN123	4 hour LXSN	4 hour FN123	11 hour LXSN	11 hour FN123
% G1	0.80	48.7	1.75	41.9	2.47	42.7
% S	9.96	14.7	8.38	16.7	6.73	16.1
% G2/M	81.9	30.1	85.2	36.5	87.4	34.4

Figure 5.4. 16E6/FN123 HFKs at shared timepoints had reduced senescent marker expression and maintained cell cycling compared to 16E6/LXSN HFKs. (A) 16E6/LXSN and 16E6/FN123 HFK3 (A) or HFK4 (B) cells were stained for senescence associated beta-galactosidase (beta-gal). (A) At an early shared passage, HFK3 cells were equivalently stained (11.9% vs 9.8%). At a late shared passage, 16E6/LXSN HFK3 had increased staining (89.3% vs 11.2%). (B) At an early shared passage, 16E6/LXSN HFK3 cells had increased staining (18.2% vs 4.0%). At a late shared passage, this was maintained (18.3% vs 4.8%). (C and D) 16E6/LXSN and 16E6/FN123 HFK3 and HFK4 cells were synchronized by density arrest and then released. HFK incorporation of BrdU and propidium iodide were quantified at 1, 4, and 11 hours after release. (C) 16E6/FN123 HFK3 had more than 1½ times as many cells in S phase at 1 hour compared to 16E6/LXSN HFK3, and 16E6/FN123 HFK3 advanced through the cell cycle more rapidly than 16E6/LXSN HFKs at 4 and

11 hours. (D) 16E6/LXSN HFK3 remained in G2/M at 1, 4, and 11 hours after release whereas 16E6/FN123 HFK4 maintained a typical cycling pattern.

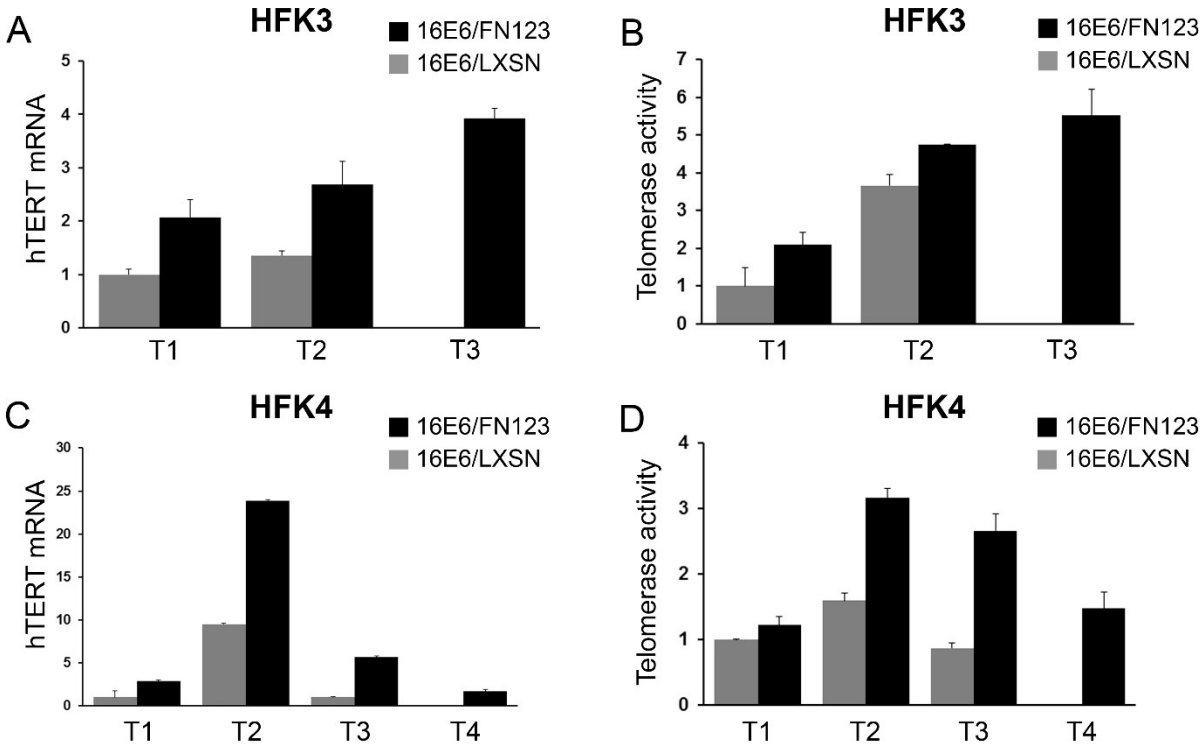


Figure 5.5. hTERT expression and telomerase activity was increased and rose further over time in 16E6/FN123 HFKs. Relative levels of hTERT mRNA and telomerase activity were quantified, and values shown were the mean fold change relative to 16E6/LXSN HFKs at T1. (A) 16E6/FN123 HFK3 (black bars) had 2 to 4 fold greater hTERT. (B) 16E6/FN123 HFK3 (black bars) had 2 to 6 fold greater telomerase activity. (C) 16E6/FN123 HFK4 (black bars) had 2 to 25 fold greater hTERT. (B) 16E6/FN123 HFK4 (black bars) had 1 1/3 to 6 fold greater telomerase activity. (A and C: hTERT qPCR was normalized to the mRNA levels of the housekeeping gene 36B4. A-D: error bars represent 95% confidence intervals from the technical replicates shown (n = 3).)

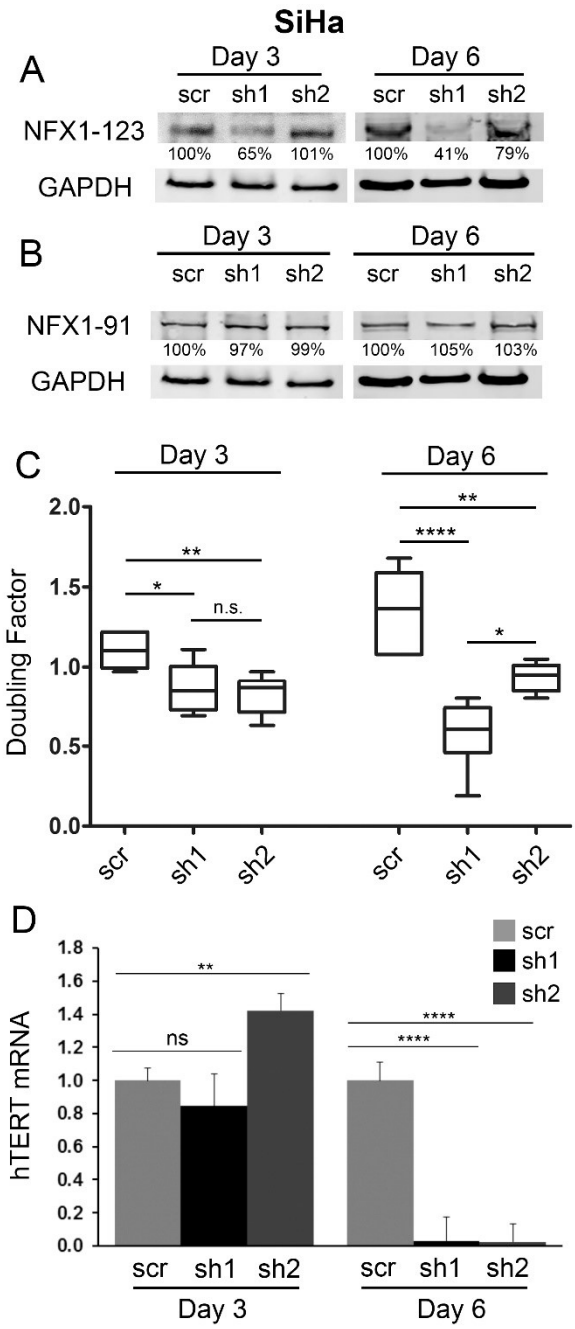


Figure 5.6. Knock down of NFX1-123 in SiHa cells resulted in a slowed growth rate and decreased hTERT. SiHa cells were transduced with either lentivirus containing a short hairpin targeting NFX1-123 (sh1 or sh2) or containing scrambled short hairpin control (scr). (A) NFX1-123 protein levels in sh1 and sh2 cells were decreased compared to scr cells at day 3 and day 6 post-transduction. (B) NFX1-91 protein remained constant after short hairpin transduction. A and B: GAPDH = loading control (C) For each cell line (scr, sh1, sh2), at both day 3 and day 6, scr cells achieved more growth and had a higher doubling factor than either sh1 or sh2. Doubling factor

p values: * ≤ 0.05, ** ≤ 0.01, **** ≤ 0.0001 (D) hTERT mRNA was quantified and values shown were the mean fold change normalized to scr SiHa cells. sh1 and sh2 cells had decreased hTERT compared to scr cells at day 6. hTERT qPCR was normalized to the mRNA levels of the housekeeping gene 36B4, and error bars represent 95% confidence intervals from the technical replicates shown (n = 3).)

Chapter 6
Conclusions and Future Directions

6.1 Summary of Findings

The work presented in this dissertation illuminates several ways in which a high-risk HPV oncoprotein, 16E6, partners with a host protein, NFX1-123, to manipulate cellular processes and promote its life cycle. The data in Chapter 3 establish 16E6 and NFX1-123 as a protein partnership through which HR HPVs interfere with the innate immune response and escape detection within infected epithelial cells. Chapter 4 identifies for the first time a role for NFX1-123 in regulating keratinocyte differentiation and late events of the HPV life cycle. These studies define how NFX1-123 regulates epithelial differentiation and elucidates a mechanism through which 16E6 and NFX1-123 engender a permissive environment for productive HPV infection. In Chapter 5, we further explore how 16E6 and NFX1-123 activation of telomerase affects growth and proliferative potential of cells over long term cell culture. These studies may represent a picture of the consequences of telomerase regulation by 16E6 and NFX1-123 during long term, persistent infection.

Taken as a whole, these chapters attempt to narrow gaps in the understanding of what leads from initial HPV infection to persistent infection and in doing so, helps identify risk factors for long-lived infection and development of HPV-associated cancer. Of course, the inexorable truth regarding scientific discovery, that central impetus driving its perpetual advancement, arises: all scientific advances inevitably beget new questions. Additional discussions of future research are presented below.

6.2 NFX1-123 Partnership with Other High-Risk HPV Types

Fundamental to the studies in this dissertation is the partnership between the E6 protein of HPV type 16 and NFX1-123. NFX1-123 was found to interact with 16E6 and E6AP in a yeast two-hybrid screen[52]. Later co-immunoprecipitation confirmed the interaction and demonstrated that NFX1-123 binding with 16E6 did not depend on E6AP[54]. Although it is known that binding utilizes the central domain of NFX1-123, which domains of E6 are important for interaction are not known[54]. This is an intriguing question because it might yield insight as to whether NFX1-123 can partner with E6 proteins of all HR HPVs or type 16 alone.

The studies presented in the preceding chapters identified roles for 16E6 and NFX1-123 in altering the key cellular processes of immune response (Chapter 3), epithelial differentiation (Chapter 4), and proliferation (Chapter 5). Though HPV16 causes a significant proportion of oropharyngeal cancers[5,10,162], it is responsible for only ~50% of anogenital cancers[143,163,164]. It is therefore important to define whether the multifunctional partnership between 16E6 and NFX1-123 is broadly seen with other HR HPV types. Each of the processes described above contribute to viral propagation in stratified squamous epithelium and the potential for a persistent, long-term infection. Furthermore, though perhaps not oncogenic in and of themselves, these manipulations of the host cell may support the initiation and progression of HPV-associated cancers when combined with the full extent of HR HPV oncoprotein activities. As the second most oncogenic type, studying whether E6 of HPV type 18 interacts with NFX1-123 would be an obvious next step for exploring the partnership of NFX1-123 with additional HR HPVs.

HPV type 16 is a prototype HR HPV type, but there is not sufficient evidence to predict whether NFX1-123 does partner with other HR HPV based on its interactions with 16E6. This is because some central functions of E6 appear to be conserved across HR HPV types while others

are not. Degradation of p53, for example, is common to HR HPV E6 regardless of type[165]. Telomerase activity, as well, has been shown to be universally upregulated by all HR HPV E6[35,166]. In contrast, although all HR HPV E6 can bind PDZ-domain-containing proteins, preferences for which proteins are primarily targeted differ between HPV16 and HPV18, as well as how binding is regulated[167]. What dictates conservation of certain E6 capacities across HR HPV types is not known; however, one theory regarding the two aforementioned conserved functions of E6 is that these were evolved to "neutralize" the activities of E7[14]. Without these, the unscheduled cell proliferation induced by HR E7 would lead to apoptosis or senescence and failed infection. It is perhaps this necessity that led HR HPVs to conserve this function, even under diversifying selection acting upon E6[3,168]. One possibility regarding partnership between NFX1-123 and other HR HPV E6s, is that binding and manipulation of some cellular processes may be conserved, but not all. Telomerase activation during long term growth (Chapter 5) may be a deemed a "necessary" function and therefore conserved, but dysregulation of innate immune signaling proteins (Chapter 3) not. Given the ability of 16E6 and NFX1-123 engender cellular changes that may precede oncogenesis, and the number of cancer cases associated with other HR HPV types, future research in this area would broaden the scope of knowledge regarding development of HPV-associated cancers.

6.3 Defining Molecular Mechanisms of 16E6 and NFX1-123 Activity

The studies presented in this dissertation identified new cellular processes manipulated by the known protein partnership of 16E6 and NFX1-123. Most significantly, these studies also illuminate new cellular protein partners through which HPV manipulates these pathways. In Chapter 3, we show that 16E6, together with NFX1-123, leverages the innate immune signaling proteins TAB2 and TRAF6 to deregulate pro-inflammatory cytokine and interferon stimulated

gene induction. In Chapter 4, we identify the MAP kinase regulators MKK4 and MKK7 and phosphorylation of the kinase JNK as the cellular proteins through which 16E6 and NFX1-123 mediate epithelial differentiation. Future studies are required to more fully investigate the molecular underpinnings of how 16E6 and NFX1-123 interact with these targets. For both innate immune regulation and epithelial differentiation, the mechanism appears to differ from that previously described for regulation of hTERT. Whereas NFX1-123 works together with cytoplasmic poly(A) binding proteins (PABPCs) to increase hTERT expression directly at the posttranscriptional level[54–56], the effects upon pro-inflammatory gene expression in Chapter 3 and differentiation marker expression in Chapter 4 seem to be indirect, through upstream signaling complexes.

6.3.1 Altering subcellular localization as a novel mechanism for NFX1-123

Targeting signaling crossroads is not out of the norm for HR HPV in the context of immune evasion. For example, multiple HR HPV types prevent the expression or function of STAT1, a signaling protein integral to the interferon signaling cascade[96,99,104]. The data presented in Chapter 3 indicate that 16E6 and NFX1-123 may employ physical sequestration of TAB2 and TRAF6 to disrupt subcellular localization of immune signaling proteins and prevent normal formation of signal transduction complexes. With NFX1-123 was overexpressed in 16E6 HFKs, TRAF6 migrated from primarily cytoplasmic to perinuclear foci. However, the association between NFX1-123 and TRAF6 needs to be further examined. This could be tested in a variety of ways, by using immunofluorescent microscopy and co-localization or proximity ligation assay, amongst others. Binding between TRAF6 and a viral protein resulting in perinuclear localization of TRAF6 has been documented before for human cytomegalovirus[169]. Although in this context the interaction ultimately led to immune stimulation, the connection between the

perinuclear localization of TRAF6 and activation of the immune response was not confirmed. Similarly for our study, the consequences of altered subcellular localization on formation of immune signaling complexes must be probed further. Formation of signaling complexes often have a specific outcome that seeds the next step of the cascade, usually a post-translational modification of one of the complex proteins. In the case of TRAF6, K63-linked polyubiquitination is integral to its function as a scaffolding protein in the immune signaling cascade, and this in turn is critical for the necessary phosphorylation of TAK1[170,171]. Examining whether polyubiquitination of TRAF6 or phosphorylation of TAK1 are disrupted in immune-stimulated cells overexpressing NFX1-123 will further confirm the link between its altered subcellular localization and dysregulation of the signaling cascade.

6.3.2 Mechanisms of MKK4 and MKK7 expression and JNK promotion of HPV transcription

The regulation of epithelial differentiation from Chapter 4 suggests a molecular mechanism that may be more akin to what has been already characterized for 16E6 and NFX1-123 in the context of telomerase[54–56]. Much like the study in Chapter 3, a greater cellular signaling pathway is being altered. The data suggest this is achieved through increases in levels of the MAP kinase kinases MKK4 and MKK7. This has characteristics both different from and similar to NFX1-123's role as an activator of telomerase through increasing hTERT expression. As part of the MAP kinase signaling cascade, increasing levels of these proteins alone may have manifold effects; this is thus regulation of a larger pathway and differs from the previously identified role of NFX1-123 wherein it stabilized the hTERT mRNA alone. Still, the exact molecular mechanism of how NFX1-123 modulates expression levels of these kinases remains unanswered. It could in fact be post-transcriptional stabilization, therefore employing the same method used with hTERT. Studies investigating whether these increases of MKK4 and MKK7

are at the transcriptional, post-transcriptional, or protein level are required. Despite post-transcriptional stabilization being the only molecular mechanism documented thus far for 16E6 and NFX1-123, NFX1-123 contains multiple protein domains that could confer potential functions. For example, its RING domain is a putative E3 ubiquitin ligase, and ubiquitination can regulate transcription, protein-protein interactions, and even scaffolding functions[172]. Studies of mRNA induction, mRNA stability, protein stability, and protein degradation are required to fully elucidate the molecular mechanism by which NFX1-123 regulates MKK4 and MKK7.

Another significant, related question that arises from the work in Chapter 4 is how JNK signaling promotes HPV differentiation-dependent transcription. This could be through direct binding of AP-1 factors to the HPV late promoter. Expression of E6 and E7 in differentiating cells is dependent on AP-1[173], and AP-1 family proteins are known to have expression patterns specific to layers of the epithelium that may contribute to the spatial control of gene expression in differentiating keratinocytes[174]. A Panomics TranSignal array conducted by Carson and Khan did demonstrate decreased AP-1 binding to HPV16 sequences post differentiation; however, these studies did not discern between binding to the early versus late promoter[122]. To examine the relationship between P-JNK, AP-1, and induction of the HPV late promoter, levels of L1 mRNA can be assessed in cells expressing phosphomimetic mutants of JNK. Similarly, binding of AP-1 transcription factors to the late HPV promoter can be assessed with electrophoretic mobility shift assays or chromatin immunoprecipitation in these cells. Another possibility is that the contribution of P-JNK to HPV late gene expression is a more indirect result of interaction between P-JNK and other factors. For example, serine/arginine-rich splicing factors (SRSFs), which control HPV oncogene expression and promote capsid protein expression, are activated by phosphorylation[175,176]. Intriguingly, SRSF3 was identified in a tandem affinity pulldown as

interacting with NFX1-123. This is just one possibility, however, amongst an almost limitless catalog of possible cellular proteins. An emerging theme from the studies in this dissertation, as well as other studies from the laboratory, is that 16E6 and NFX1-123 can regulate entire cellular processes downstream of key mediators.

6.3.3 Other cellular protein partners and the role of 16E6

Crucially, mechanisms of 16E6 and NFX1-123 may be defined by the other protein partners with which they complex. In the case of hTERT post-transcriptional stabilization, PABPC1 and PABPC4 were essential partners[56]. As previously mentioned, NFX1-123 has several protein domains. These protein domains not only have diverse putative functions, but also have the ability to direct interactions with a wide repertoire of proteins. Similarly, structural studies of E6 indicate that it has the potential to be in complex with multiple protein partners at a single time[49,177]. The partnership of 16E6 and NFX1-123 may manipulate cellular processes not through enzymatic activity themselves, but through redirecting the substrate spectrum of other proteins. This principle is a familiar one in the biology of HPV: p53 is not targeted by E6AP in the absence of HR HPV E6; rather, E6 redirects E6AP to p53[44,49]. Partnership between 16E6 and NFX1-123 thus has multitudinous possibilities for the cellular processes it targets. Indeed, we see a diversity of processes in this dissertation alone. Comparing what proteins interact with 16E6 alone, NFX1-123 alone, or 16E6 and NFX1-123 together might yield insights into molecular mechanisms of the studies in this dissertation as well as uncover novel processes. Further studies are also required to explore the role of 16E6 in partnering with NFX1-123, especially in the context of epithelial differentiation. The studies in Chapter 4 were conducted in the presence of 16E6 to be able to delineate the role of NFX1-123. Although NFX1-123 appears to play a role in mediating keratinocyte differentiation by itself, data suggest that 16E6 is able to

enhance this. Other studies from the laboratory, involving Notch1 expression, displayed the same relationship as seen with cellular differentiation: NFX1-123 activity being augmented by 16E6. This differs from hTERT stabilization, wherein NFX1-123 alone did not play a role in the absence of 16E6.

The studies in this dissertation reveal new cellular processes and pathways manipulated by 16E6 and NFX1-123, and future studies are required to expand knowledge of exactly how 16E6 and NFX1-123 interact with these targets and what other cellular proteins are relevant players.

6.4 NFX1-123 As A Potential Biomarker

Delineating the ways in which the host protein NFX1-123 manipulates the cellular environment to support HPV infection, and persistent infection as a corollary, sets the foundation for its evaluation as a potential predictive and prognostic biomarker. Risk factors that predispose a person to developing persistent HPV infection are not well characterized. Although screening for cytologic abnormalities and primary testing for HPV DNA have emerged as an effective tools for cancer prevention, they are not sufficient. Ambiguities in interpretation of cytology and the requirement for repeat testing[178] necessitate additional tools to predict the probability of persistent disease or malignancy. A predictive biomarker would lower the burden of costly follow-up testing and monitoring. These goals are especially important in resource-constrained settings, which are plagued by lack of organization, coverage, and quality assurance[179]. These countries unsurprisingly have the highest burden of cervical cancer worldwide[143]. Nor are there currently rigorous measures for predicting the progression of HPV-associated precancers and cancers. In the case of HPV-associated neoplasia, this is complicated by a dynamic pattern of lesion regression and progression during the overall course of malignant development[15,180].

Currently, the biomarkers with commercially available tests undergoing evaluation for cervical and head and neck cancers are HR HPV E6/E7 mRNA, $p16^{ink4a}$, topoisomerase IIA, and MCM2[181,182]. Aspects of the biology of NFX1-123, its interactions with HR HPV, and its role for driving cellular changes highlight its potential as a biomarker.

We see in multiple contexts that greater expression of NFX1-123 can significantly affect cellular processes and engender a milieu beneficial to HR HPV infection. In Chapter 3, greater NFX1-123 expression is linked to dysfunction of innate immune gene expression. Chapter 4 demonstrates that greater NFX1-123 can augment keratinocyte differentiation and expression of the HPV L1 capsid gene, while Chapter 5 further confirms the interplay between NFX1-123 expression and telomerase activity. There is a dose-response relationship between NFX1-123 levels and alterations in these cellular processes, especially in the context of 16E6. It is therefore possible that upon infection with HR HPV, someone who naturally has higher endogenous levels of NFX1-123 will display more acute changes to infected cells and potentially better harbor HPV infection. In this manner, higher endogenous levels of NFX1-123 expression could predict a greater risk for persistence. Alternatively, it may not predict whether someone is at risk for developing persistent infection, but instead predict how likely they are to have progression of lesions once persistent infection has already been established. Because of its cooperation with HR HPV E6 and the changes they subsequently engender, testing levels of NFX1-123 in combination with HPV testing could be a better predictor of disease development than the current paradigms.

Of course, studies examining whether there is a correlation between NFX1-123 expression levels at tissue sites and development of persistent infection or cancer are required first. These could be case control studies or prospective studies examining NFX1-123 levels of

women who developed neoplasia and cancer versus those who cleared infection. Critically, there is evidence that NFX1-123 expression levels vary in primary tissue, which is central to its possibility as a predictive biomarker. We demonstrated for the first time patterns of NFX1-123 expression in normal cervical epithelium (Fig 4.1A). 35 normal cervical epithelial samples total were stained for NFX1-123. While trends in expression, such as predominately cytoplasmic localization and increasing levels with differentiation, were maintained across all the samples, there were differences in average staining intensity between samples. This suggests that some women have higher levels of NFX1-123 in cervical epithelium than others. Supporting this, we have also observed variances in endogenous NFX1-123 mRNA and protein levels in different HFK biological backgrounds. Additionally, we observed that within the basal layer there were sporadic cells with greater expression of NFX1-123 than neighboring cells. If HR HPV infects one of these cells specifically, it would establish infection in a host cell that is better able to evade innate immune signaling (Chapter 3); increases epithelial differentiation pathways required for the viral life cycle (Chapter 4); and has augmented growth and longevity through telomerase (Chapter 5). Contributing to its potential as a prognostic biomarker, we see that NFX1-123 expression levels are upregulated in cervical cancer cell lines[53], primary precancers, and primary cancers (Chapter 5). Future studies examining associations between grade of neoplasia and NFX1-123 expression would further illuminate NFX1-123's culpability in promoting HPV infection and oncogenesis. NFX1-123 is an intriguing candidate that warrants additional investigation into its feasibility as a predictive or prognostic biomarker.

6.5 Final Thoughts

There is some poetic symmetry to the fact that it has been 42 years since Harald zur Hausen first suggested that HPV was the causative agent of cervical cancer, and that women who

are 42 are in the peak age range for diagnosis of cervical cancer. In the relatively short duration since Dr. zur Hausen's hypothesis, the field has made significant leaps in understanding the biology of high-risk HPV infection, persistence, and oncogenesis. There was the first identification of the etiological agent; elucidation of the oncoproteins and oncogenic pathways targeted in the cell; development of three effective preventive vaccines; and recognition of a new epidemic of HPV-associated oropharyngeal cancers. Studies in the field have revealed much of the biology of HPV infection and have explored downstream oncogenesis as a result of persistent infection, but more research is needed. The factors influencing the stage in between an infection and a persistent infection are not well-defined. Given the connection between persistent high-risk HPV infection and cancer, uncovering host protein partners that promote the HR HPV life cycle could help identify risk factors for long-lived infection and ultimately, development of HPV-associated cancer. Several examples of how a cellular host protein, NFX1-123, is co-opted to manipulate the cellular environment are described above, but these do not fully explicate the key questions in the etiology of HPV-associated morbidities: what determines persistence of infection and progression of malignancies? Answering these questions will prevent a substantial proportion of cancer cases before they even occur and, together with effective and universal vaccine uptake, lead to a global future absent of 630,000 annual HPV-associated cancers.

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