I, Allie K Adams, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Cancer and Cell Biology.

It is entitled:
Targeting the DEK oncogene in head and neck squamous cell carcinoma: functional and transcriptional consequences

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Targeting the DEK oncogene in head and neck squamous cell carcinoma: functional and transcriptional consequences

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements to the degree of

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by

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies worldwide with over 50,000 new cases in the United States each year. For many years tobacco and alcohol use were the main etiological factors; however, it is now widely accepted that human papillomavirus (HPV) infection accounts for at least one-quarter of all HNSCCs. HPV+ and HPV- HNSCCs are studied as separate diseases as their prognosis, treatment, and molecular signatures are distinct. Five-year survival rates of HNSCC hover around 40-50%, and novel therapeutic targets and biomarkers are necessary to improve patient outcomes.

Here, we investigate the DEK oncogene and its function in regulating HNSCC development and signaling. DEK is overexpressed in many cancer types, with roles in molecular processes such as transcription, DNA repair, and replication, as well as phenotypes such as apoptosis, senescence, and proliferation. DEK had never been previously studied in this tumor type; therefore, our studies began with clinical specimens to examine DEK expression patterns in primary HNSCC tissue. We find that DEK is highly overexpressed in all subsets of HNSCC, independent of HPV status, stage, or patient demographics. Furthermore, an established transgenic mouse model, crossed with Dek-/- mice, examined the in vivo importance of DEK in HNSCC development in a 4-NQO model system. Transgenic Dek-/- have improved survival, fewer tumors, and reduced epidermal proliferation compared to Dek+/+ counterparts. Similarly, in vitro systems of HPV+ and HPV- cell lines demonstrated DEK loss reduced cell number and proliferation. Finally, ΔNp63 was identified as a partial mediator of this DEK-dependent cell growth.

We next aimed to identify additional candidates as mediators of DEK-dependent functions. DEK is known to regulate transcription, as either a co-activator or a co-repressor, but the global impact of DEK loss on gene transcription is unknown. Therefore, RNA-Sequencing
was performed on HPV+ and HPV- HNSCC lines, either proficient or deficient for DEK, to assess the transcriptional consequences of DEK loss. From this data IRAK1 was identified as a candidate down-regulated in the absence of DEK. IRAK1 is a driver of inflammatory signaling, downstream of toll-like and interleukin-1 receptors, and activates many well-known signaling cascades including NF-κB and MAPK. These RNA-Seq results were validated by qRT-PCR and western blot analysis. IRAK1 expression was assessed by mining The Cancer Genome Atlas, which revealed IRAK1 is overexpressed in HNSCC. As IRAK1 has never previously been characterized in HNSCC, experiments were designed to identify the function of IRAK1 in this system. IRAK1 loss reduced downstream TRAF6-ubiquitination and signaling and increased apoptosis, with either shRNA or chemical inhibition. Lastly, DEK and IRAK1 cooperated to regulate ERK1/2 signaling, but independently contributed to HNSCC survival. In summary, these findings implicate two novel oncogenes as mediators in HNSCC growth and survival and that individual or combined targeting of DEK and IRAK1 may be therapeutically beneficial.
Preface

The following work in this dissertation is either in preparation for, or previously published, in the following peer-reviewed journals:


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During the course of my dissertation studies, I have been privileged to collaborate with numerous research scientists and clinicians without whom my work would not have been possible. Firstly, I would like to thank my mentor Dr. Susanne (Susa) Wells at Cincinnati Children’s Hospital Medical Center. Susa has provided invaluable advice and guidance over the years, both at a scientific and personal level. She has always allowed me to be highly independent in my work and has encouraged me to pursue the experiments I was the most passionate about. I will always be grateful for all of her support and am thankful she asked me to join the Wells lab.

I would also like to thank my thesis committee, Dr. Keith Casper, Dr. Peter Stambrook, Dr. Ronald Waclaw, Dr. Susan Waltz, and Dr. Kathryn Wikenheiser-Brokamp for their suggestions and feedback on my projects. They have challenged me as a scientist and were an integral part of this work inside and outside of my thesis meetings. Additionally, I thank Dr. Keith Casper and his colleagues at the University of Cincinnati Department of Otolaryngology for collaborating with our lab on the receipt of primary specimens for our studies. I believe without these tissues our work would not have been nearly as impactful--few graduates have this opportunity and I am eternally grateful for the chance to work with you. I also want to acknowledge Dr. Daniel Starczynowski and his lab for helping me with the IRAK1 story. Without their advice (and reagents) we could not have pursued my final project.

To the Wells lab (past and present): thank you for your advice during the highs and lows of research. I have formed lasting friendships with many of you and my time in this lab has been full of entertainment and laughter. Many of you have been secondary mentors (Elizabeth Hoskins and Lisa Privette Vinnedge, specifically) and helped me navigate the world that is science.

I dedicate this work to my parents, Randall and Susan Varner, who have provided unconditional love and support these past twenty-eight years. In times of struggle they have
always been there to listen and have encouraged me to pursue my dreams. My achievements thus far have been possible because of their help, sacrifices, and passion to see their daughter succeed. Thank you for everything!

Finally, to my husband Joshua Adams—words will never be enough to express how thankful I am for all of your emotional support over 5 years of graduate school. You have been my biggest cheerleader and helped me keep the end goal in mind when I struggled with the difficulties of grad school. We met on our first day at Ohio State: two science nerds that have successfully navigated the completion of their degrees, moving to Cincinnati, surviving my qualifying exam, getting married, adopting our pup, Blue, and buying our first home. It has undoubtedly been the greatest journey of my life and I am so glad to have spent it with you.
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Chapter 1: Human Papillomavirus Induced Transformation in Cervical and Head and Neck Cancers

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Abstract

Human papillomavirus (HPV) is one of the most widely publicized and researched pathogenic DNA viruses. For decades, HPV research has focused on transforming viral activities in cervical cancer. During the past 15 years, however, HPV has also emerged as a major etiological agent in cancers of the head and neck, in particular squamous cell carcinoma. Even with significant strides achieved towards the screening and treatment of cervical cancer, and preventive vaccines, cervical cancer remains the leading cause of cancer-associated deaths for women in developing countries. Furthermore, routine screens are not available for those at risk of head and neck cancer. The current expectation is that HPV vaccination will prevent not only cervical, but also head and neck cancers. In order to determine if previous cervical cancer models for HPV infection and transformation are directly applicable to head and neck cancer, clinical and molecular disease aspects must be carefully compared. In this review, we briefly discuss the cervical and head and neck cancer literature to highlight clinical and genomic commonalities. Differences in prognosis, staging and treatment, as well as comparisons of mutational profiles, viral integration patterns, and alterations in gene expression will be addressed.
Introduction

Human papillomaviruses (HPVs) comprise a large family of viruses, 170 of which are now sequenced and are extensively referenced on the Papillomavirus Episteme\(^1\).\(^2\). The high risk (HR) mucosal HPV types, including the HPV16 prototype, are a subgroup of the alpha papillomaviruses that share the ability to cause cancer in their human host\(^3\). These DNA viruses have been extensively investigated for decades for their ability to subvert cellular mechanisms of growth control. In fact, HPV was first implicated in cancer biology approximately forty years ago by Harald zur Hausen, who was awarded the Nobel Prize in Physiology or Medicine in 2008 for the discovery that HPV causes cervical cancer\(^4\).\(^5\). Since then, HPV has also been associated with other anogenital cancers including vulvar, penile, and anal, largely due to sexual transmission. More recently, high risk HPV has been clearly detected in head and neck cancers (HNCs), particularly in the oropharynx, making it an additional etiological factor for a disease previously ascribed most commonly to tobacco and alcohol consumption. Both cervical and head and neck malignancies have poor outcomes when diagnosed at late stages and there is a dire need for clinically effective screening methods for detection of early stage disease and for the identification of novel therapeutic targets.

Cervical cancer in the United States has been on a swift decline due to the general implementation of Papanicolaou (Pap) screening for the detection of pre-malignant lesions of the cervix. However, the expense of treating HPV-related infections is a significant burden to society. Approximately $8 billion was spent for the year 2010, with the majority focused on screening and follow-up visits, and with just over 10% of the total expense on the treatment of cervical cancer\(^6\). Many lives are saved in return on a national level, but cervical cancer remains a major cause of death in developing countries where vaccination and screening programs are unavailable. Furthermore, similar screens are not currently available to detect early HPV positive (or negative) head and neck cancers. Clinicians must rely on surrogate markers, such
as the cyclin-dependent kinase inhibitor p16 (INK4a), to determine HPV status in the tumor. HPV negative (HPV−) HNCs have been steadily declining in correlation with decreased tobacco use, while HPV positive (HPV+) head and neck cancers are on a steep, upward trajectory especially in younger populations, and are estimated to surpass cervical cancer rates in the U.S. by the year 2020. The increasing incidence of HPV+ HNCs is linked to increasing oral HPV exposure via oral sex in younger age groups, and the total number of oral sex and open-mouthed ("French") kissing partners. As for cervical cancer, the risk of HPV infection also increases with greater numbers of sexual partners. Given the prospect of a national epidemic of HPV+ HNCs, it is now critical to test the possibility that protection from cervical cancer through HPV vaccination will also be protective against HNC. For many years, the study of HPV in cancer biology has been focused on cervical cancers. With a large upward shift in the number of HPV positive HNCs it will be imperative to adapt the paradigms of HPV function in cervical cancer to HNC wherever possible.

Almost all cervical and at least one-quarter of head and neck cancers share the presence of HPV genomic DNA and expression of the viral oncogenes. A majority of both cervical and HPV+ HNCs are squamous cell carcinomas (SCCs) due to keratinocyte-specific viral tropism. Advances in the prevention and treatment of cervical cancer may be an excellent starting point for battling HPV positive HNC, but biological distinctions between these cancer types exist and must be clearly defined in order to apply this knowledge more broadly. For example, HPV types are much more restricted in head and neck cancer when compared to cervical cancer, mostly due to HPV-16 infection varying in its distribution (i.e. prevalence) predominantly as a function of HNC site (e.g., tonsillar vs. oral cavity). Furthermore, cervical cancers have shown a dependence on estrogen signaling, whereas hormonal contributions have not been identified in HNC. Here, we discuss the clinical and molecular commonalities and differences between HPV+ cervical and head and neck squamous cell carcinomas (HNSCCs).
HPV biology

Human papillomavirus is an episomal, double-stranded DNA virus. Almost 200 known types have been identified, only some of which contribute to malignancies. The majority of HPV types infect the nonmucosal, cutaneous epithelium. The minority of HPV types that infect mucosal tissues and the genital tract are sub-divided into high risk and low risk categories based on their ability to transform the host cell. HPV16, 18, 31, and 45 are examples of high-risk types and are known to cause cervical neoplasias and tumors, while HPV6 and 11 belong to the low-risk subtypes that induce genital warts and respiratory papillomas. There are also HPV16 variants that are classified based on sequence alterations in E6 and are detected in different regions across the globe and are named correspondingly. These include: European (EUR), Asian American (AA), Asian (As), African-1 (AFR1), and African-2 (AFR2) variants. These variants appear to exhibit differences in their relative contributions to cervical malignancies, with Asian American variants preferentially associated with high-grade CIN lesions and cervical cancer. Research in HNSCC on HPV16 variants has been more limited, although recent studies implicate certain variants, such as R10G, to be more frequent in HNSCC than in cervical cancer. New variants have been identified in HNSCC, but most are shared with those already identified.

The HPV genome is circular with dual promoters that encode two separate groups of viral proteins: the early genes (E1, E2, E4, E5, E6, E7, E8) and the late genes (L1, L2). Some of the early HPV genes are essential for maintaining the viral replicative cycle, while the late HPV genes encode the major (L1) and minor (L2) capsid proteins. E1 and E2 are primarily involved in transcription and replication: E1 functions as the viral DNA helicase and E2 as a transcriptional activator and repressor that also complexes with E1 as a critical component of the HPV replisome. The transforming properties of HPV are a result of E5, E6, and E7 activities, which are discussed in further detail below. The HPV E4 protein is less well...
characterized, but several studies implicate E4 in virion release via its association with keratin filaments\textsuperscript{33, 34}.

The epithelium is comprised of proliferating and differentiated keratinocytes, which are the host cell for HPV infection and replication, respectively. Keratinocytes that transition into malignant cells via immortalization and transformation initiated by the presence of high-risk HPV frequently result in squamous cell carcinoma (SCC). Transmission of HPV relies on microwounds or abrasions in the epidermis for HPV to gain access to stem and/or proliferating cells in the basal epithelium. Once there, it will utilize the host cell replication machinery to initiate viral DNA replication\textsuperscript{35}. Our understanding of the initial HPV infection process including viral attachment and entry remains incomplete, but interactions with heparan sulfate on the cell surface have been implicated\textsuperscript{36-42}. The HPV life cycle is highly organized, geographically as well as functionally, around the cellular differentiation program\textsuperscript{43, 44}. Under normal circumstances, cell division in the basal cell layer regenerates the stem and transit amplifying cell population, with a subset of cells separating to move upwards, exit the cell cycle and differentiate, thus forming the spinous, granular and cornified layers of the epidermis. In the HPV infected environment, expression of early HPV genes, particularly E7, forces re-entry into the cell cycle, thus enabling HPV to take advantage of active host replication machinery to amplify its viral genome\textsuperscript{45, 46}. One mechanism by which HPV regulates viral genome copy number is through an E2 variant, E8\textsuperscript{E2C}. E8\textsuperscript{E2C} is a transcriptional repressor identified in high risk HPV types 31 and 16 that limits viral genome replication and is required for HPV31, but not HPV16\textsuperscript{47-49}. Once HPV infected cells reach the upper layers of the epithelium, late viral genes are expressed for capsid production and virion assembly, and release of infectious particles follows. HPV is shed from the surface of the epidermis in conjunction with squamous flakes in order to maximize local virus concentrations. As such, new infections can occur to repeat the infectious cycle in the same or in a different host.
Over the course of viral replication, HPV is maintained as an extrachromosomal, circular element (episome). Partitioning to daughter cells during mitosis can be attributed, at least in part, to the function of E2. The E2 protein is able to tether the HPV genome to DNA through the binding of cellular DNA-associated factors including, but not limited to, Brd4\(^{50,51}\). Episomal HPV is often detected in non-malignant and pre-malignant tissue, while integrated HPV is detected largely in malignancies, but is not necessarily required for oncogenic progression. This is highlighted by one study examining the frequency of HPV integration in precancerous and cancerous cervical lesions. The three most common high risk HPV types (16, 18, and 45) were integrated more often relative to other high risk HPV types. Furthermore, HPV45 was most frequently integrated in precancerous lesions, while HPV18 was most frequently integrated in cervical cancer. Although HPV was present in all specimens, integration was not ubiquitously detected suggesting integration-independent mechanisms of HPV-induced oncogenesis are at play\(^{52}\). HPV has been reported to integrate in the host DNA at common fragile sites, although the copy number and location varies\(^{53}\). However, other mechanisms of HPV integration have been proposed recently, based on sophisticated genome sequencing technology, in cervical and head and neck cancers. These will be described in further detail below.

Integration events can disrupt E2, allowing for the deregulation of the HPV E6 and E7 oncogenes whose upregulation then further promotes growth advantages and genomic instability through their respective cellular targets\(^{54,55}\). E2 controls E6 and E7 expression in part through promoter binding and repression\(^{56-59}\). Once released from E2-mediated repression, E6 and E7 activities are stimulated. These proteins bind and target p53 and Rb pocket proteins for degradation, respectively. E7 not only binds to Rb, but also to the Rb family members p107 and p130, ensuring cells progress through S-phase in the absence of proper E2F/Rb cell-cycle control, and gain proliferative characteristics\(^{60,61}\). E6 also activates telomerase reverse transcriptase (TERT), which contributes to keratinocyte immortalization\(^{62-64}\). More recent studies of these oncogenes have identified new targets in various signaling pathways. For instance, the
importance of hormone signaling in the development of cervical cancers is clearly appreciated. A requirement for estrogen receptor (ER) was discovered in transgenic K14E7 mouse models for the initiation and maintenance of cervical cancer\textsuperscript{13,16}. Furthermore, microRNAs (miRs) are emerging as an additional mechanism whereby the HPV oncogenes promote SCC\textsuperscript{65-68}. More detailed information on the numerous targets of E6 and E7 can be found in elegant reviews on the biology of HPV \textsuperscript{46,69-71}.

Expression of the HPV E5 oncogene is also believed to contribute to the oncogenicity of HPV, at least in part through signaling from growth factor receptors such as EGFR\textsuperscript{72}. \textit{In vivo} experiments suggest E5 contributes to hyperplasia, aids in the formation of epithelial tumors and synergizes with E6 and E7 to promote more severe disease phenotypes\textsuperscript{73-75}. Recent work also demonstrated E5 regulates growth and invasion in cervical cancer cell lines\textsuperscript{76}. The interplay between HPV E5, E6 and E7, and their downstream targets is critical for enabling HPV to immortalize and transform keratinocytes, eventually leading to SCC formation.

While there is ample data on HPV viral activities and cellular targets in the literature, details on the viral life cycle including mechanisms of uptake, replication and virion production remain to be discovered. Additionally, evolutionarily conserved properties and critical molecular distinctions between high risk and low risk HPVs require further study for an improved understanding of disease etiology\textsuperscript{77,78}. The capacity of low-risk HPV E6 and E7 proteins to bind and degrade p53 and Rb is limited when compared to that of the corresponding high-risk HPV proteins. Low risk HPV E6 proteins also do not harbor PDZ binding domains, which contribute to the transforming ability of E6\textsuperscript{79,80}. Furthermore, the characteristics of basal cells that are initially infected by HPV have remained controversial. The basal compartment of the epidermis is believed to contain bona fide stem cells with low proliferative capacity and highly proliferative transit amplifying (TA) cells with less of a self-renewal capacity. The existence of TA cells has been debated, but it is believed these cells will only proliferate for a limited time and eventually undergo differentiation\textsuperscript{81,82}. It remains to be seen whether and how HPV targets the stem TA
cell population in human epidermis. Finally, HPV has been detected in multiple cancer types including cervical, anogenital and head and neck, but the extent to which tissue specific molecular activities drive cancer phenotypes remains unclear.

**Clinical characteristics of HPV+ cancers**

Cervical cancer is the second leading cause of female cancer deaths worldwide, but due to national recommendations for screening and vaccination the burden of cervical cancer lies largely outside of the United States. HPV accounts for nearly 100% of all cervical cancers, most of which are squamous cell carcinomas due to infection with high risk HPV16; however, cervical adenocarcinomas are most strongly correlated with infection of high risk HPV18 (Table 1.1). Although the main route of HPV infection results from sexual contact, non-sexual transmission (vertical transmission) can occur from mother to child pre- or perinatally. Cesarean births may eliminate vertical transmission, but the surgical risks may outweigh potential benefits. Papanicolaou (Pap) tests are used to screen the cervix for cellular abnormalities that may be indicative of pre-cancerous or cancerous lesions and these cells can be tested for the presence of HPV DNA through reflex testing by *in vitro* nucleic acid hybridization assays. Reflex testing is performed only if there are abnormal Pap results, at which time HPV testing is requested and detection of high risk or low risk HPV types is possible. Pap tests can detect the early stages of cervical cancer termed cervical intraepithelial neoplasias (CIN). These are pre-malignancies graded on a scale of 1-3, with CIN1 indicating only slight dysplasia while CIN2 and CIN3 indicate moderate to severe disease. Diagnosing CIN lesions prior to progression to cervical cancer drastically improves patient survival and response to treatment.

The peak age of infection for women is under age 25, with the potential for continual infections by either the same or different HPV types over time. Continual HPV infection increases the risk of transformation, as the likelihood of genome instability and oncogenic mutations rises when HPV is persistent and disruptions to the cell cycle and many other
biological processes occur. However, only a small fraction of women will go on to develop cervical cancer. Immune cells clear these infections in most cases, suggesting immune deficiencies, mutations or inherent genomic instability may be contributors to the development of malignancy. Furthermore, whether viral load and disease severity are positively correlated has been controversial in the literature, and may be dependent on the HPV type; however, viral presence and disease risk can now be reliably assessed by E6 and E7 mRNA expression and may be utilized in conjunction with clinical data to more accurately answer this question\textsuperscript{7,90}.

Head and neck squamous cell carcinomas (HNSCCs) account for approximately 50,000 new cancer diagnoses in the United States each year\textsuperscript{92}. These tumors are derived from the epithelial cells that line the mucosal surfaces of the head and neck, which include the oral cavity and oropharynx. Previously, common risk factors were tobacco and alcohol use, but more recently HPV infection has emerged as an additional etiological factor in this disease, particularly in oropharyngeal cancers\textsuperscript{9}. In fact, the number of oropharyngeal cancers is on a steep upwards trajectory, attributable to increased incidence of HPV-associated tumors\textsuperscript{93}. Patients with HPV positive tumors have improved survival rates and respond better to therapies than their HPV negative counterparts\textsuperscript{94}. This had led to the suggestion that HNSCCs may be treated as distinct entities, dependent on the HPV status of the tumor. Treatments for HNSCCs include chemotherapy, radiation, and surgery, all of which can lead to undesirable side effects for patients such as hearing loss, dysphagia, facial disfigurement, and inability to use their natural voice. The combined efforts of clinicians and basic scientists to improve the survival rate of HNSCC have been relatively unsuccessful overall\textsuperscript{95}. Further identification of how HPV positive and HPV negative HNSCCs differ molecularly will be important for identifying specific therapeutic targets in each subset and better understanding the pathogenesis of this disease for improved targeted therapies.

Table 1.1 depicts a summary of clinical characteristics of cervical and head and neck cancers. Almost all HNSCCs that harbor HPV DNA contain HPV16, whereas in cervical cancer,
many more high-risk types of HPV are accountable for the disease. It is not understood why there is such HPV-type specific variability between these cancer types, but perhaps there is a tissue specific or viral exposure preference for HPV16 in the oropharynx over other HPV types. HPV-infected head and neck cancers are mostly localized to the oropharynx, which includes tonsillar and base of tongue cancers, with some evidence that oral cavity cancers have a low prevalence of HPV\textsuperscript{96, 97}. Unlike cervical cancer, there are no available screening tools such as Pap smears to aid in the prevention of HNSCC development. Designing such screens will be difficult due to the feasibility of accessing the appropriate tissue where infection occurs\textsuperscript{98}. Instead, patients must rely on diligently evaluating themselves for persistent symptoms such as sore throats, swollen glands or oral lesions. These seemingly benign symptoms are often overlooked, contributing to the unfortunate fact that HNSCCs are oftentimes not diagnosed until the later stages of disease.
### Table 1.1. Summary of Clinical Characteristics of Cervical and Head and Neck Cancers

<table>
<thead>
<tr>
<th></th>
<th>Cervical SCC</th>
<th>Cervical Adenocarcinoma</th>
<th>HPV+ HNSCC</th>
<th>HPV- HNSCC</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPV Types</strong>&lt;br&gt;(decreasing prevalence)</td>
<td>16 (&gt;50%) 18, 45, 31, 33</td>
<td>18 (38%), 16 (12%), 45, 31</td>
<td>16 (90%), 18, few other HR-HPV types</td>
<td>N/A</td>
<td>[87], [86], [9]</td>
</tr>
<tr>
<td><strong>Prevalence</strong>&lt;br&gt;~73% of all invasive cervical carcinomas Over 99% HPV+</td>
<td>~14% of all invasive cervical carcinomas Over 99% HPV+</td>
<td>&gt;25% of all HNSCC</td>
<td>≤75% of HNSCC</td>
<td></td>
<td>[99], [9]</td>
</tr>
<tr>
<td><strong>Additional Risk Factors</strong>&lt;br&gt;Smoking, HIV infection, Chlamydia, Oral Contraceptives (&gt;5 years)</td>
<td>Tobacco Usage, Alcohol, Paan (Asia), Maté (South America)</td>
<td></td>
<td></td>
<td></td>
<td>[100], [92]</td>
</tr>
<tr>
<td><strong>Incidence</strong>&lt;br&gt;Decreasing USA; High Incidence in Developing Countries</td>
<td>Increasing Incidence</td>
<td>Increasing; younger patients</td>
<td>Decreasing; older patients</td>
<td></td>
<td>[95], [90]</td>
</tr>
</tbody>
</table>
Prognostic comparison and staging

In general, the prognosis of cervical cancer is excellent at early stages, often with surgical resection alone. Cervical cancer screening in developed countries has led to early detection, thus dramatically reducing cervical cancer mortality. Unfortunately, locally advanced cervical cancer and metastatic disease is much more common in developing countries making it the second leading cause of cancer related death in women worldwide\textsuperscript{101}. Women with cervical cancer may present with abnormal or post-coital vaginal bleeding, but most are asymptomatic and therefore, without proper screening detection is delayed. Cervical cancer is the only cancer type that is still staged clinically and criteria are mostly based on local and distant extension of the tumor (Table 1.2)\textsuperscript{102}. Cervical cancer patients with stage I disease have five-year overall survival rates of 80-99%; however, survival is greatly reduced to only 15-16% in those with stage IV disease, even with aggressive treatment\textsuperscript{103}.

HNSCC is a much more heterogeneous group of cancers, and therefore, the prognosis varies with site and stage of disease. Much like cervical cancer, however, many localized HNSCCs (stage I and II) are cured with radiation or surgical resection alone. As in cervical cancer, HNSCC survival decreases with advancing stage. Importantly, the prognosis of oropharyngeal HNSCC has greatly improved over the past several decades (Table 1.2). In fact, for tonsil cancer, five-year survival rates have improved dramatically from the 1980s to 2006 in local disease, locally advanced and distant disease, from 62% to 85.9%, 38.2% to 73%, and 21.2% to 41.5%, respectively (Table 1.2). These improvements are likely reflective of more sophisticated surgical and radiation techniques, increasing HPV prevalence, and better supportive care. However, a more detailed understanding of disease mechanisms will help elucidate strategies to continue to improve these outcomes. In this regard, cooperative group studies investigating treatment paradigms for the earlier and intermediate stage cancers are ongoing. Unfortunately, up to 60% of HNSCCs present locally advanced, and more than 50% of
HNSCC develop locoregional recurrence or distant metastasis even with initially aggressive treatment. Upon recurrence or metastasis, the prognosis is dismal at only 10-15% with a median overall survival of 10-12 months\textsuperscript{104}. These cancers prove the most challenging, and successful treatment will thus require intense investigation.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Cervical Cancer</th>
<th>HNSCC*</th>
<th>Prognosis (5yr OS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Carcinoma confined to cervix</td>
<td>Tumor less than 2cm, no LN involvement</td>
<td>80-93%</td>
</tr>
<tr>
<td>II</td>
<td>Extends beyond cervix, but not pelvic wall</td>
<td>Tumor between 2-4cm, no LN involvement</td>
<td>58-63%</td>
</tr>
<tr>
<td>III</td>
<td>Carcinoma extends to pelvic wall and/or involves lower third of vagina and/or causes hydronephrosis or non-functioning kidney</td>
<td>Tumor &gt;4cm w/o LN involvement; or tumor &lt;4cm with single ipsilateral LN &lt;3cm involvement</td>
<td>32-35%</td>
</tr>
<tr>
<td>IVa</td>
<td>Extends beyond true pelvis or has clinically involved the mucosa of bladder or rectum; IVb: metastatic disease</td>
<td>Invasion of tumor in surrounding structures with or without LN involvement; or any tumor with contralateral or &gt;6cm LN; IVc: metastatic disease</td>
<td>15-16%</td>
</tr>
<tr>
<td>IVa</td>
<td>Combined chemoradiation</td>
<td>IVa: chemoradiation, induction chemotherapy or resection**** +/- adjuvant chemoradiation; IVb: clinical trials vs. chemoradiation; IVc: palliative chemotherapy</td>
<td>41.5% distant IVc</td>
</tr>
</tbody>
</table>

* oropharynx, other sites will vary slightly
** R+/-C: radiation +/- chemotherapy
*** LN: Lymph node
**** + ipsilateral or bilateral neck dissection based on LN involvement required
***** Tonsillar Cancer from 2002-2006
Cervical cancers, whether SCC or adenocarcinoma, have a very high prevalence of HPV. Therefore, it is difficult to determine whether HPV infection per se affects prognosis in these populations. Recently published data suggests HPV is a prognostic factor in anal cancer, predicting both overall and disease-specific survival\(^{106}\). However, there have been reports that cervical cancers positive for HPV18 carry a poor prognosis when compared to those positive for HPV16\(^ {107}\). In contrast, HPV is associated with fewer HNSCC cases, only around 25%, and almost exclusively with the HPV16 subtype and predominantly in the oropharynx. Substantial evidence has recently emerged demonstrating greater survival for HPV positive HNSCC patients; however this protective effect is lessened in those who report tobacco and alcohol use\(^ {94,108}\).

**Treatment variations**

In general, early stage cervical cancer (Stage IA or IB) is treated with resection alone either by conization for those with early stromal invasion or extrafascial hysterectomy for those with lymphovascular invasion. Patients who desire pregnancy may also be candidates for radical trachelectomy with pelvic lymphadenectomy. Modified radical hysterectomy is often offered for patients with stage IA2 or microscopic IB disease. However, stage IB-IIA with high risk pathologic features (positive surgical margin, parametrial invasion, \(\geq 3\) positive pelvic lymph nodes or poorly differentiated tumor) requires radical hysterectomy and adjuvant concurrent radiation and chemotherapy (CRT). Adjuvant radiation is recommended for patients with intermediate risk factors (\(\geq 50\%\) stromal invasion, lymph-vascular invasion, and tumor size \(\geq 4\)cm). Radiation therapy alone is also an acceptable option for early stage disease.

In contrast, locally advanced cervical cancer (Stage IIB-IVA), is treated with radiation alone or CRT. Combined treatment has greatly increased survival in locally advanced disease. The addition of cisplatin treatment was shown to increase overall survival (OS) in several studies including RTOG 90-01 demonstrating 67\% OS in chemotherapy arm versus 41\% in radiation
alone arm at 8 years, GOG-120 with 65% in chemotherapy vs. 47% in radiation alone, and GOG-123 with 83% OS for chemotherapy versus 74% with radiation alone \(^{109-111}\).

Patients that develop recurrent disease often undergo either radiation or surgery depending on previous treatments, although some cases may not be amenable to either option. Therefore, these patients and those with metastatic disease are considered incurable and many opt for palliative chemotherapy. In general, chemotherapy is cisplatin-based but many trials have demonstrated increased response rates for combination therapy combining cisplatin and most commonly, paclitaxel. Unfortunately, despite combinations of chemotherapeutics response rates remain low at 25-35% in recurrent and metastatic disease highlighting a dire need for novel agents\(^{112, 113}\).

Like cervical cancer, HNSCC is often curable with surgical resection or radiation therapy alone for those with early stage disease. However, many of these patients will develop recurrence and oftentimes present with locally advanced disease. Patients with locally advanced disease may undergo surgery, radiation, or CRT with each treatment carrying certain consequences. Surgical resection can result in disfigurement, poor wound healing and at times loss of voice (in the case of laryngeal tumors). However, CRT may result in dysphagia, mucositis, neurotoxicity and renal failure. Despite aggressive treatments, many tumors recur, and patients then undergo the treatment modality not previously offered, or re-resection.

Despite improvements of surgical technique, the rate of relapse for both local and metastatic disease after surgery alone is high, particularly in patients with high-risk features such as perineural invasion, multiple lymph node involvement, extracapsular spread and positive surgical margins. Two pivotal studies demonstrated improved progression-free survival (PFS) and overall survival (OS) in patients with high-risk features treated with adjuvant CRT, albeit with greater toxicity over treatment with radiation alone. However, PFS and OS were only 47% and 53%, respectively at 5 years, suggesting that new treatments are necessary in order to improve outcomes in high-risk patients\(^{114, 115}\).
Combined CRT is frequently offered upfront in locally advanced disease. In 2003, a pivotal phase III trial showed that concurrent chemotherapy and radiation using cisplatin resulted in superior outcomes when compared to radiation alone in unresectable, locally advanced disease. Three year overall survival was 23% in the radiation alone arm compared to 37% in the CRT arm (p=0.014). As in cervical cancer, cisplatin is the most commonly used chemotherapeutic with the highest response rate. This therapy remains a standard of care for patients with unresectable, locally advanced disease and is also offered for organ preservation. Since the original report, many retrospective studies suggest improved survival in these patients with survival rates as high as 90% at 3 years in some instances. Interestingly, the EGFR directed monoclonal antibody, cetuximab, and concurrent radiation also resulted in an increase in survival in HNSCC of 45.6% versus 36.4% for radiation alone; thus, cetuximab is an acceptable agent oftentimes associated with less severe toxicities. Most of the patients in the latter study had oropharyngeal HNSCC, but unfortunately HPV status was unknown. Therefore, it is unclear whether improved survival may have been due to a higher proportion of HPV positive tumors.

Many HNSCC relapses occur locally despite successful initial definitive treatment. Upon local relapse, standard of care is often salvage surgical resection which confers local control rates of 33-50% and a long term overall survival of 20-40%. Many patients, however, are not candidates for resection due to multiple comorbidities. Like cervical cancer, palliative chemotherapy, either single or multiple agent, is regularly offered to these patients as well as to those with metastatic disease. Unfortunately, response rates are poor at 15-36% with increased toxicity and overall survival rates of only 5-11 months.

Our ability to determine which patients (cervical cancer and HNSCC) will recur and which will do well with less intensive therapy and therefore less treatment related toxicity is limited. Active research is ongoing to develop model systems that resemble human HNSCC and that may be useful platforms for the development of alternative treatment strategies. As mentioned
above, it is unclear whether HPV has an influence on cervical cancer survival as almost all of these tumors are HPV positive, but certainly HPV status has an established impact on the prognosis of HNSCC. In fact, a new report suggests that HPV is not only protective against initial disease progression, but also improves survival in patients with recurrent disease\textsuperscript{129}. Patients with HPV positive disease may be able to undergo less intensive therapy, thereby decreasing the incidence of toxicity, at least partly due to their increased sensitivity to radiotherapy \textsuperscript{130, 131}. Several clinical trials are ongoing including Radiation Therapy Oncology Group (RTOG) Trial 1016 comparing cetuximab and radiation versus cisplatin and radiation in locally advanced disease. The benefit of cetuximab in cervical cancer is unknown and actively investigated. However, it is clear that not all HPV positive HNSCC tumors behave equally. Positive smoking history, likely resulting in increased genomic mutations, may explain why some HPV positive tumors do not fare as well with therapy. Accordingly, the improved survival of HPV positive patients is negated by tobacco use\textsuperscript{108}. As the incidence of this group of cancers grows, it is important to further classify these tumors and elucidate optimal targets that are specific to each subset.

**Vaccines offer protection from HPV-associated cancers**

Cancers often arise due to mutations that are either inherited, occur *de novo*, or are acquired from exposure to environmental factors. As cancers are quite heterogeneous in nature, with a multitude of mutations, identifying therapies that work on entire tumor cell populations including tumor-initiating or cancer stem cells has been difficult\textsuperscript{132}. Current therapeutic intervention includes radiation, chemotherapy, and surgery, with the major caveat that many of these treatments cause major disruptions in a patient’s quality of life. The transforming ability of human papillomavirus in the cervix, anogenital tract, and oropharynx suggests that prevention of HPV infection may ultimately prevent HPV-associated cervical and head and neck cancers. Vaccination has been successful at preventing infection of other viruses including influenza,
rotavirus, and varicella, setting the precedent and opportunity for scientists to create a vaccine that prevents infection with HPV and therefore, HPV-associated cancers.

There are currently two vaccines on the market: **Cervarix**® (GlaxoSmithKline) and **Gardasil**® (Merck & Co.). These vaccines cover the most common oncogenic HPV types known to cause cervical cancer, while **Gardasil**® also includes those responsible for genital warts. **Cervarix**® is a bivalent vaccine that protects against high risk HPV16 and 18, while **Gardasil**® is quadrivalent and protects against HPV16 and 18, as well as the low risk HPV6 and 11 types. Additionally, protection for both vaccines has been demonstrated to be ≥5 years\(^{133-135}\). These vaccines are protective against cervical, vaginal and vulvar cancers, as well as genital warts in the case of **Gardasil**®. The current recommendation by the Food and Drug Administration (FDA) is for men and women ages 9-26 years to be vaccinated. This is the age range where HPV infection is most likely to occur. It is imperative to begin the vaccination series at the earliest recommended ages, prior to the start of sexual activity, to offer maximal protection against HPV infection; vaccination after infection does not result in enhanced protection from or clearance of the virus.

Both **Cervarix**® and **Gardasil**® utilize virus-like particles (VLP) formed by self-assembly of the HPV capsid protein L1 to elicit the immune response, as opposed to live or attenuated virus. Antibodies are then produced against the L1-specific to each HPV type included in the vaccine \(^{136}\). Although capsid proteins are highly homologous amongst HPV types, it is unclear how much protection is offered against other HPV types not covered by vaccination; however, a recent study with **Cervarix**® suggests it may also cross-protect against the oncogenic HPV types 31, 33, 45 and 51\(^{137}\). Since the introduction of these vaccines in the mid-2000s, wide spread studies of their efficacy in various populations and countries has been performed. These studies have focused on anogenital cancers and genital warts, but the vaccines are not yet approved as preventives against HPV-infected HNCs, partially impeded by the lack of information on HNCs required by the FDA for future studies\(^{138, 139}\). Unlike cervical cancer where there is still a large prevalence of other high-risk HPV types contributing to disease, over 90% of all head and neck
cancers are HPV16 positive. Even though not yet approved by the FDA for protection against HNCs, these vaccines may be highly efficacious as they cover the HPV type that is overwhelmingly implicated in HNC carcinogenesis.

**Mutational analyses**

The advent of whole exome and whole genome sequencing and other “omics” technologies has enabled scientists to identify specific mutations in tumor specimens that may lead to targeted and personalized therapies. This is especially important in cancers such as cervical and head and neck where a diagnosis is often not made until the advanced stages of disease. These late stage tumors are not as treatable and have few targetable molecules identified. Previously, common chromosomal aberrations in both tumor types had been identified in the presence of HPV. These include loss at 13q and 11q22.3-25 and gains at 20q and 3q24-29; albeit unique chromosomal alterations also exist for cervical and HNC\textsuperscript{140}. This indicates the existence of specific genomic alterations which are caused by the presence of high risk HPV independent of anatomical site. Only recently have samples from these two cancer types been analyzed by whole exome sequencing, which revealed profiles of somatic mutations and globally de-regulated pathways.

A recent publication by Ojesina, et.al. compared mutations in cervical squamous cell carcinoma and adenocarcinoma against normal controls to identify novel mutations. In this study the authors identified multiple new mutations in cervical cancer including **MAPK1**, **FBXW7**, and **EP300**\textsuperscript{141}. Additionally, pathway analysis revealed that genes associated with the immune response were most significantly mutated in cervical SCC, while cervical adenocarcinoma had recurrent mutations in the PIK3CA/PTEN pathway.

Similar publications from exome sequencing of HNSCCs were reported in 2011. Only a few somatic mutations match those discovered in the cervical cancers\textsuperscript{142,143}, including **FBXW7** and **PIK3CA**. The head and neck studies were further segmented into HPV+ and HPV- categories,
with far fewer genes mutated in HPV+ as compared to HPV- tumors. Not surprisingly, HPV-
tumors harbored higher global mutation frequencies compared to the HPV+ group, presumably
due to tobacco use and highlighted by $TP53$ mutations in almost all HPV- tumors. Table 1.3
depicts prominent examples of mutated genes in both the cervical and head and neck cancer
studies. A complete list can be obtained from the original research papers. The data suggest
that although HPV can be the etiological cause in both cancer types, associated cellular
mutations are variable. This may be due to other etiological influences that drive mutations
including tobacco use or to the differential anatomical locations of the tumors.
**Table 1.3.** Mutations identified by whole exome sequencing. Mutated genes that are shared between cancer types

<table>
<thead>
<tr>
<th>Cervical SCC(^{141})</th>
<th>HPV+ HNSCC(^{142,143})</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBXW7(^{e})</td>
<td>NOTCH1</td>
</tr>
<tr>
<td>PIK3CA(^{e})</td>
<td>SYNE1</td>
</tr>
<tr>
<td>MAPK1</td>
<td>HRAS</td>
</tr>
<tr>
<td>HLA-B</td>
<td>PIK3CA(^{e})</td>
</tr>
<tr>
<td>STK11</td>
<td>MED1</td>
</tr>
<tr>
<td>EP300</td>
<td>MLL2</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>EZH2</td>
</tr>
<tr>
<td></td>
<td>SYNE2</td>
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<tr>
<td></td>
<td>NOTCH3</td>
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\(\textit{Stransky, et.al.}\)

\(\textit{Agrawal, et.al.}\)
**HPV integration**

HPV integration in cervical cancer can lead to the disruption of E2 as HPV enters common fragile or other sites in the host genome. This is an important event during the progression of cells to a transformed state, albeit not necessarily required, which provides a growth advantage given the control of E6/E7 expression by E2 under normal circumstances. Transformation is continuously dependent upon E6/E7 expression and can be reversed by the reintroduction of E2\textsuperscript{144-146}. Research related to the mechanisms and sites of HPV integration has been limited to cervical cancers, with nascent studies of integration in HNCs. Integration is one of the more poorly understood aspects of HPV biology, with controversy over where integration events occur: either randomly through the genome or at specific “hotspots”. These ‘hotspots’ include cytogenetic bands 3q28, 4q13.3, 8q24.21, 13q22.1, and 17q21.2 accounting for integration sites of 22% of cervical tumors analyzed\textsuperscript{147, 148}. This study also included strong evidence for HPV DNA near various miRNA sites, some of which are already published as known regulators in cervical cancer. On the other hand, recent work demonstrates HPV integrates randomly across the genome, but tends to cluster near sites of structural alterations in the genome\textsuperscript{149}. This report included not only cervical cancer lines, but also HNC cell lines and primary tumor specimens, all exhibiting a similar pattern of HPV integration in regions of genomic instability. An elegant mechanism to explain this phenomenon was proposed, with a viral genome looping model to explain HPV-driven amplifications and rearrangements that occur at sites of integration, which may be continually propagated throughout the genome\textsuperscript{149, 150}. Additionally, the authors identified numerous breakpoints in the integrated HPV genome that are outside of the E2 open reading frame, thus questioning the longstanding model of preferential E2 disruption through integration.

Even with some disparate findings between the above reports, both identify \textit{TP63} as a gene affected by integration events found in both cervical and HNCs. \textit{Schmitz, et.al.} defined the chromosomal hotspot 3q28, where the \textit{TP63} gene is located, and demonstrated \textit{TP63} was
disrupted by HPV integration in multiple cervical tumors. Likewise, Akagi, et.al. identified HPV integration within the TP63 gene, resulting in the loss of normal TP63 gene expression and the creation of a novel protein isoform, along with viral-host fusion transcripts. Furthermore, c-myc was also amplified and overexpressed in both cancer types in the presence of integrated HPV\textsuperscript{151,152}. Although E6 and E7 are sufficient for immortalization in vitro, the accumulation of oncogenic mutations in cellular tumor drivers or suppressors is necessary to achieve neoplastic transformation and cancer development\textsuperscript{46}. In depth studies enabled by next-generation genomics and functional studies will dissect the contribution of HPV integration and genomic rearrangements to carcinogenesis. Elucidating the role of the full complement of human genes whose activation or inactivation initiate and drive transformation will identify new targets and therapies tailored to the mutational landscape of individual tumors and patients.

**Gene expression in HPV\textsuperscript{+} cancers**

It is clear that HPV infection and transformation de-regulates gene expression patterns in keratinocytes. For many years, studies of HPV-driven host gene expression involved either the introduction of the HPV genome or of the HPV E6 and E7 oncogenes in existing cell lines. More recently, studies of primary tumors compared to control specimens have provided additional insights in clinically relevant model systems. To create a database of reliable genomics and transcriptomics information for many cancer types, the National Cancer Institute (NCI) along with the National Human Genome Research Institute (NHGRI) collaborated on the ambitious ‘The Cancer Genome Atlas’ (TCGA) project\textsuperscript{153}. The goal of this project was to create freely available tumor datasets (with appropriately matched normal controls) subjected to genomic studies including exome sequencing, single nucleotide polymorphism (SNP) analysis, and mRNA expression by RNA-Sequencing, together with extensive clinical annotations. Currently, information from over 30 tumor types has been deposited in the TCGA, with a collection target of at least 500 samples for each cancer type\textsuperscript{153}. The TCGA will provide massive datasets for
broad integration with basic and clinical sciences. This includes the opportunity for personalized medicine, where new diagnostics, therapeutic targets, and individualized treatments may eventually be possible. Both cervical squamous cell carcinoma and adenocarcinoma, along with HNSCC are part of the TCGA; however, the cervical data will not be available for data mining until later in 2014. Once these data are available, it will allow for groundbreaking opportunities to compare HPV+ cervical with HPV+ HNCs. Given the large number of specimens already banked for both cancer types, clear trends in gene expression should delineate common molecular and genomic occurrences that are due to HPV activities.

In the absence of published TCGA based comparisons, smaller studies that profile HPV-related gene expression are publically available. One of the first papers published comparing cervical cancer (SCC and adenocarcinoma) to normal cervical keratinocytes by Santin et.al. identified approximately 500 genes up- or down-regulated in the presence of HPV. Up-regulated genes included CDKN2A (p16), TOP2A, E2F1, FOXM1, MCM2/4/5, and PTGES, with a high prevalence of genes involved in cell cycle regulation. Down-regulated genes including TGFβ1, TGFα, and KRT16, were clustered in differentiation and tumor suppressor pathways. This report also demonstrated substantial similarities in expression profiles of cervical SCC and adenocarcinomas, despite the fact that SCCs were HPV-16 positive and adenocarcinomas were HPV-18 positive. This suggested distinct high-risk HPV types evoke similar transcriptome alterations.

Analyses by Mattarocci, et.al. in 2013 utilized a retrospective approach to identify novel genes involved in dysplastic and neoplastic cervical lesions, based on targets previously published in the literature. The authors confirmed earlier results where FOXM1, TOP2A, and E2F1 were not only overexpressed, but where their respective expression levels gradually increased as cervical lesions progressed from low-grade to high-grade lesions and eventually to carcinomas. Other de-regulated genes included CDC25A, TP73, and CCNE2. Conversely,
genes that demonstrated reduced expression in the progression from dysplastic tissue to carcinoma included \textit{PTGS2, BCL2}, and \textit{FOS}\textsuperscript{155}.

A multitude of publications comparing the gene expression profiles of HPV\textsuperscript{+} to HPV\textsuperscript{-} HNSCCs are available. A study by Martinez, \textit{et.al.} compared HPV\textsuperscript{+} vs. HPV\textsuperscript{-} vs. normal oral mucosa and analyzed these specimens in a number of ways. The authors identified many of the same gene expression changes initially reported by Santin \textit{et.al.} including the up-regulation of \textit{CDKN2A, TOP2A, and MCM2/3} in HPV-infected HNCs compared to normal mucosa. Other up-regulated genes reflected pathways such as DNA replication, cell cycle progression, DNA repair and differentiation, while down-regulated genes reflected processes such as proteolysis and chemotaxis. Additional comparisons between HPV\textsuperscript{+} and HPV\textsuperscript{-} HNCs identified up-regulated genes involved in nuclear structure, mitosis, DNA repair and methylation, and down-regulated genes involved in signal transduction and proteolysis\textsuperscript{156}. Finally, Pyeon, \textit{et.al.} directly compared gene expression of HPV\textsuperscript{+} cervical and head and neck cancers and discovered similar trends where cell cycle-related genes are highly up-regulated\textsuperscript{157}. Other datasets confirm these findings where HPV drastically affects gene expression patterns involved in cell cycle, repair and replication\textsuperscript{157-160}. This is not necessarily surprising given the known function of E6 and E7 in the inhibition and degradation of critical tumor suppressors such as p53 and Rb.

p53 and Rb-dependent functions are regulated by p14ARF and p16, respectively, which are encoded by the same gene, \textit{CDKN2A}\textsuperscript{161,162}. This is one of the most frequently overexpressed genes in HPV positive tumors, largely due to regulatory interference by E6 and E7. When E7 inactivates Rb, E2F1 is free to initiate transcription of its targets that includes the \textit{CDKN2A} locus\textsuperscript{163}. p16 is so consistently and highly overexpressed in HPV\textsuperscript{+} cancers that it is used clinically as a surrogate marker for HPV positivity during cancer diagnosis.

Epigenetics is another mechanism whereby gene expression patterns can be altered in normal and transformed cells. This is a complex field that investigates DNA changes that do not affect the inherent nucleotide sequence, but rather processes such as methylation patterns and
histone modifications. The presence of HPV greatly affects methylation signatures across the genome, promoting hyper- and hypomethylation. For example, tumor suppressor genes, such as TERT, exhibit promoter hypermethylation and thus gene silencing in CIN lesions and cervical cancers\textsuperscript{164, 165}. The methyltransferases responsible for methylation, DNMT1, DNMT3A, and DNMT3B, are also highly overexpressed in HPV positive cervical and HNSCC cells\textsuperscript{90, 166, 167}. Conversely, hypomethylation increases during the transition of dysplastic to cancer cells and results in aberrant gene activation, suggesting that hyper- and hypomethylation coordinate for the evolution of an oncogenic state\textsuperscript{168}. Comparisons of HPV positive and negative HNSCC samples also reveal the extent to which HPV alters methylation patterns, research which contributes substantially to the identification of novel target genes\textsuperscript{169}.

Initial studies comparing cervical and HNCs to normal controls suggested HPV elicits similar gene expression alterations between tumor types. Publication and analysis of the TCGA data will likely confirm this trend, but novel details are also expected which should reflect differential tumor origins in various tumor types, data which may be exploited for the targeting of tumor cells by novel agents. The large number of samples combined with thorough bioinformatics analyses and ultimately confirmatory studies will help validate genes of interest shared or unique to cervical and HNCs. Further separation of these groups based on clinical, environmental and demographic data is bound to enrich and surprise the HPV community.

**Conclusions**

Human papillomavirus is the most common sexually transmitted viral pathogen and causes approximately 5% of the global cancer burden\textsuperscript{170}. Even after decades of intense research of the epidemiology and molecular biology of HPV infection and transformation, the aggressive nature of HPV-related late stage cancers and novel molecular targets for new therapies do not meet clinical needs. Cellular events downstream of HPV infection and integration such as the induction of genomic instability and selection for oncogenic mutations must occur in order to
complete transformation process that is initiated by the expression of the E6 and E7 oncogenes. Cervical cancer was the first tumor type attributed to the effects of HPV infection and has since been the standard for studying HPV biology. In fact, one of the most widely used cell lines in cancer biology is the HeLa cell line derived from an HPV18 cervical adenocarcinoma. The discovery that HPV infection is also an underlying cause of head and neck squamous cell carcinomas has opened the door for comparisons that will ultimately inform the treatment of each tumor type towards improved diagnostics, therapeutic options, and patient survival.

Acknowledgements

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Chapter 2: Novel oncogenes in HNSCC: an introduction to DEK and IRAK1
Part 1. The DEK oncogene is a multifunctional protein

Biochemistry of DEK

DEK is a highly conserved and unique protein that has little homology to other proteins, but is most closely related to the HMG family. DEK is 375 amino acids in length with three DNA-binding domains: the pseudo-SAP, the SAF/SAP (scaffold attachment factor-box), and the C-terminal multimerization domain.\textsuperscript{171-173} It also harbors multiple acidic regions and a putative nuclear localization signal (NLS), but has no enzymatic activity (Figure 2.1).\textsuperscript{174} Most of DEK is localized in the nucleus, bound to chromatin, but a small percentage of DEK binds RNA.\textsuperscript{175} Recent work suggests recombinant DEK is internalized by multiple cell types and this is dependent on heparan sulfate proteoglycans (HSPGs). Additionally, DEK is secreted by macrophages and can be taken up by adjacent cells. This supports the role of DEK as an autoantigen in various autoimmune disorders.\textsuperscript{176}

DEK does not bind DNA in a sequence-specific manner, but preferentially binds to particular DNA structures such as supercoiled, helical, and four-way junction DNA.\textsuperscript{177} While the protein prefers associating with negatively supercoiled DNA, it can also introduce positive supercoils into SV40 minichromosomes in cell-free assays.\textsuperscript{178} In mammalian cells there is conflicting data regarding the preference of DEK for heterochromatin or euchromatin. Hu \textit{et al.} reported DEK to be excluded from heterochromatin and concentrated at regions undergoing active transcription.\textsuperscript{179} On the other hand, DEK was found to be essential for heterochromatin integrity and enhanced H3K9me3 foci through binding to heterochromatin protein 1α.\textsuperscript{180} From these findings, it is likely DEK regulates gene expression at least in part through modulating chromatin topology. However, a link between this role for DEK and transcription remains to be established.

DEK undergoes a variety of post-translational modifications that can influence its activity, including phosphorylation, acetylation, and poly(ADP-ribosyl)ation. Phosphorylation decreases
the affinity of DEK for DNA in vitro and stimulates multimerization of DEK with itself. The main kinase identified in regulating DEK DNA-binding activity is Casein-Kinase 2 (CK2), but DEK harbors phosphorylation sites for PKC and GSK-3 phosphorylation as well.\textsuperscript{181} Acetylation by CBP, p300, and P/CAF also decreases the affinity of DEK for DNA and re-localizes DEK within the nucleus to interchromatin granules (ICGs), which contain other RNA-processing and transcription factors.\textsuperscript{182} Interestingly, autoantibodies from the synovial fluid of patients with juvenile idiopathic arthritis preferentially recognize acetylated DEK over the phosphorylated form.\textsuperscript{183} DEK can also be modified with poly(ADP-ribose) (PAR) by PARP-1, which releases DEK into the cytoplasm.\textsuperscript{184, 185} Finally, in cells undergoing apoptosis, DEK is both phosphorylated and PARylated, and excreted into the extracellular space.\textsuperscript{186} Although these modifications are established in the literature, little data is available on the functional importance of these modifications in the DEK-dependent phenotypes observed in various malignancies.

**Dual roles for DEK in transcription**

Given the close relationship of DEK and chromatin, it is not surprising that the extent of DEK expression is important for cellular gene expression, but the role of DEK in transcription is not clearly defined. Paradoxically, DEK has been implicated as either a co-activator or a co-repressor, depending on the system. As mentioned above, DEK is a substrate for PARP-1, and PARP-1 in combination with SET functions to remove DEK from chromatin and permit assembly of the preinitiation complex for transcriptional activation.\textsuperscript{185} Supporting this role for DEK as a transcriptional repressor, DEK represses p300 and P/CAF histone acetyltransferase-dependent transcription in leukemia cells in a manner dependent upon the acidic regions within DEK.\textsuperscript{187} DEK also negatively regulates NF-κB-dependent transcription through interactions with the p65/RelA protein.\textsuperscript{188} On the other hand, DEK has also been described as a transcriptional co-activator, as seen by its interaction with AP-2α and stimulation of transcriptional activity in
glioblastoma, and its co-activation of nuclear receptors in Drosophila.¹⁸⁹, ¹⁹⁰ Lastly, C/EBPα and DEK functionally cooperate to activate genes associated with myeloid differentiation.¹⁹¹

Global profiling of transcriptional networks associated with DEK loss or overexpression in normal or transformed cells has only recently been initiated. When DEK-knockdown lung cancer cells were compared to non-targeting controls, metabolism, nucleosome assembly, and transcription factor expression were significantly altered.¹⁹² Later, chromatin immunoprecipitation combined with microarray analysis in DEK-depleted leukemia cells revealed that DEK represses hTERT transcription in a manner that was dependent on the posttranslational modifications of DEK.¹⁹³ Finally, in myeloid cells DEK preferentially binds open chromatin and is enriched at the promoters of highly transcribed genes. The localization of DEK at these promoters was found to either activate or repress transcription, depending upon the site.¹⁹⁴ Taken together, the relationship between DEK loss and transcriptional regulation remains poorly defined. The effects of DEK overexpression on transcription have yet to be studied and may provide possible explanation for how DEK contributes to the transformation of normal cells.

DEK as a histone chaperone

Histone modifications and nucleosome assembly play crucial roles in chromatin structure and signaling transcriptional activity; therefore, it is not surprising a chromatin-associated protein like DEK is involved in this process. Histone chaperones are proteins that bind histones and regulate their loading onto DNA. DEK is a histone chaperone and interacts with histones, including H2B.¹⁹⁵ In Drosophila, DEK facilitated the assembly of histone H3.3 into nucleosomes during transcription activation; however, in human cells DEK limits H3.3 incorporation into chromatin to the telomeres, strengthening the role of DEK as a mediator of heterochromatin integrity.¹⁹⁰, ¹⁹⁶ This notion coincides with published reports that H3.3 is found near active transcriptional sites, but also at telomeric and heterochromatic regions.¹⁹⁷ DEK also associates
with Daxx, a transcriptional co-repressor, HDACII, and the core histones (H2A, H2B, H3 and H4) and contributes to transcriptional repression. 198

**DEK loss abrogates the DNA damage response**

In addition to chromatin structure and transcription DEK also is involved in the response to DNA damage, DNA repair, and DNA replication. DEK loss sensitized human and mouse cells to DNA damage and also significantly reduced repair by non-homologous end joining (NHEJ) in Dek knockout mouse embryonic fibroblasts (MEFs). This was supported by delayed Ku70/80 localization to double-strand breaks and reduced DNA-PK signaling upon DEK loss. 186, 199 The sensitivity of melanoma cells to clastogenic chemotherapies could be enhanced by DEK loss, suggesting inhibition of DEK may be a useful clinical target. 200 DEK also counteracts replication stress from stalled replication forks, resulting in fork progression and cellular proliferation, and may be one mechanism for how DEK contributes to cancer progression and chemo-resistance. 201

**DEK functions as an oncogene**

DEK was initially discovered as a DEK-CAN fusion protein in acute myeloid leukemia, and this fusion protein supports leukemia *in vivo* by targeting the long-term hematopoietic stem cell population. 202, 203 Since then, DEK overexpression has been described in most malignancies including breast, melanoma, bladder, and hepatocellular carcinoma. 200, 204-206 DEK was not defined as a bona fide oncogene until 2009, when a report demonstrated that DEK knockout mice exhibited reduced papilloma formation in a chemical carcinogenesis mouse model, and that DEK overexpression could stimulate keratinocyte transformation in soft agar and xenograft assays *in vivo*. 207 Earlier work demonstrated DEK overexpression inhibited normal cellular differentiation of keratinocytes and increased hyperplasia of 3D epidermal models. 208 Additionally, DEK inhibited senescence and apoptosis, the latter being partially attributable to
the destabilization of p53. Together, these observations support a role for DEK as an oncogene.\textsuperscript{209, 210}

The overexpression of DEK in many tumor types may be attributable to its regulation by the E2F family of transcription factors, which are aberrantly activated when Rb is deregulated.\textsuperscript{211} Promoter studies of DEK revealed it also contains YY1 and NF-Y transcription factor binding sites that are implicated in transformation.\textsuperscript{212} In breast cancer, chromatin immunoprecipitation identified DEK as a target of estrogen receptor $\alpha$, which contributes to ER+ breast cancer pathogenesis.\textsuperscript{213} Outside of transcriptional regulation, the E3 ubiquitin ligase, Fbxw7, was identified to target DEK for degradation in intestinal models of carcinogenesis.\textsuperscript{214}

DEK targets multiple proteins and signaling pathways, including, p53, p63, Wnt/$\beta$-catenin signaling, and mTOR which, in part, contribute to DEK-associated phenotypes including proliferation, invasion, and stemness.\textsuperscript{204, 208, 210, 215, 216} In hematopoietic systems, DEK promotes cell survival and is important for hematopoietic stem cell engraftment as measured by competitive bone marrow transplant.\textsuperscript{217, 218} In breast cancer and melanoma, DEK expression levels correlate with disease progression, with lowest DEK expression in benign tumors and with highest expression in advanced disease.\textsuperscript{204, 219, 220} Normal DEK expression is low in primary and differentiated cells and highest in malignant cells, which should be advantageous to patients should a DEK inhibitor be developed or discovered. Because DEK plays important roles in regulating oncogenic phenotypes, and because secreted DEK protein can be detected in urine, it is currently being investigated as a biomarker for bladder cancer and has been described as a potential therapeutic target there.\textsuperscript{221}

Beyond cancer, DEK autoantibodies are found in various autoimmune diseases including juvenile rheumatoid arthritis, juvenile idiopathic arthritis, systemic lupus erythematosus, and sarcoidosis.\textsuperscript{183, 222-226} DEK was demonstrated to be a pro-inflammatory protein and is secreted by macrophages, but DEK also attracts neutrophils, natural killer cells,
and CD8+ T lymphocytes.\textsuperscript{227} This data suggests DEK is involved in multiple aspects of inflammation and has important cellular functions outside of its nuclear localization. However, a defined role for DEK in inflammation has yet to be revealed.
**Figure 2.1. DEK structure and identified domains.** DEK is 375 amino acids in length and contains multiple DNA binding domains: the ψ-SAP, SAP, and the DEK-C. DEK-C is also the multimerization domain. Residues 205-221 harbor a putative nuclear localization signal (NLS). DEK also carries multiple acidic regions, highlighted in pink.
Part 2. IRAK1 is a central, upstream hub of inflammatory signaling

IRAK1 signaling cascade

Interleukin-1 receptor-associated kinase-1 (IRAK1) is part of the IRAK family of kinases that also includes IRAK2, IRAK3, and IRAK4. These kinases are essential for driving inflammatory signaling through the toll-like (TLR) and interleukin-1 receptors (IL-1R). As part of the innate immune system, these receptors recognize foreign pathogens and initiate downstream signaling through IRAK1. This begins with the recruitment of adaptor proteins, such as MyD88, which interact with the N-terminal death-domain of IRAK1.\(^\text{228, 229}\) IRAK4 then complexes with MyD88 and IRAK1, initiating the phosphorylation of IRAK1. Phosphorylation of IRAK1 on Threonine-209 is the most critical event and T209 phosphorylation is used as a marker for activation.\(^\text{230}\) This event then signals IRAK1 auto-phosphorylation and release. Phospho-IRAK1 then binds to TRAF6, an E3 ubiquitin ligase, that autoubiquitinates to enable binding to TAK1 and its two binding partners, TAB1 and TAB2.\(^\text{231-233}\) Together these kinases phosphorylate and activate the IKK complex to initiate NF-κB signaling through the translocation of p50/p65 heterodimer into the nucleus for transcriptional activation.\(^\text{234}\) TAK1 can also phosphorylate MAP3Ks, turning on JNK, ERK1/2, or p38 signaling, driving subsequent translocation of AP-1 into the nucleus.\(^\text{235}\) This cascade is summarized in Figure 2.2.

In addition to phosphorylation, IRAK1 can also be modified by ubiquitination and sumoylation, which alters the localization of IRAK1 within the cell. K48-ubiquitination of IRAK1 results in proteasomal degradation, while K63-ubiquitination recruits the NEMO complex and aids in the activation of NF-κB.\(^\text{236, 237}\) Finally, sumoylation translocates IRAK1 to the nucleus and may activate transcription in monocytes.\(^\text{238}\)

IRAK1 gene structure
The *IRAK1* gene is located on the X chromosome and is 712 amino acids in length. As mentioned previously, IRAK1 contains an N-terminal death domain, a kinase domain, an activation loop, and a C1 and C2 domain at the C-terminus.\textsuperscript{239} Although IRAK family members have similar structures, they only share around 40% homology. Only IRAK1 and IRAK4 have kinase activity and IRAK4 lacks the C-terminal domain that is crucial to the interaction with TRAF6.\textsuperscript{240-242} Additionally, IRAK3 is only expressed in monocytes and macrophages and may negatively regulate IRAK1/IRAK4 signaling.\textsuperscript{243, 244}

**Contributions of IRAK1 to cancer**

The contributions of IRAK1 to the formation of malignancies, particularly hematological disease, have only recently been reported. Overexpression and activation of IRAK1 has been observed in acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and melanoma.\textsuperscript{242, 245, 246} MDS patients are predisposed to the development of AML, and previous work suggested that TRAF6 and IRAK1 are overexpressed in these diseases through loss of miR-146a. miR-146a is deleted in at least 80% of all del(5q)MDS and AML, and since TRAF6 and IRAK1 are both targets of miR-146a, these candidates were examined for their function in MDS and AML.\textsuperscript{247, 248} In particular, IRAK1 was pursued as a novel drug target due to both its overexpression in MDS and central importance in innate immune signaling disease pathology. A small molecular inhibitor of IRAK1, termed IRAK-1/4, blocks kinase activity and as a result, reduced TRAF6 polyubquitination, NF-kB activation, and progenitor cell function and increased apoptosis in MDS.\textsuperscript{245} This inhibitor is a benzimidazole that is highly specific for IRAK1 and IRAK4 and has little effect on other kinases. Likewise, in melanoma IRAK inhibition in combination with vinblastine (a chemotherapy for melanoma treatment) increases apoptosis. Together, this data suggests inhibition of inflammatory signaling, specifically IRAK, may be a novel approach for treating multiple cancer types.
Figure 2.2 IRAK1 is upstream of many oncogenic signaling cascades. Activation of TLR and IL-1R signaling initiates a cascade where MyD88 adaptor proteins complex with IRAK1 and IRAK4. IRAK4 phosphorylates IRAK1 on a critical Thr209 residue, followed by hyperphosphorylation of IRAK1 that releases it from MyD88. pIRAK1 now binds to TRAF6 where ubiquitination enables interaction with the TAK1/TAB complexes. These kinases can now phosphorylate IKK or MAP3K to trigger signaling that will allow the entry of transcription factors into the nucleus.
Chapter 3: DEK promotes HPV positive and negative head and neck cancer cell proliferation

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide, and patient outcomes using current treatments remains poor. Tumor development is etiologically associated with tobacco or alcohol use and/or HPV infection. HPV positive HNSCCs, which frequently harbor wild-type p53, carry a more favorable prognosis and are a biologically distinct subgroup when compared to their HPV negative counterparts. HPV E7 induces expression of the human DEK gene, both in vitro and in vivo. In keratinocytes, DEK overexpression is sufficient for causing oncogenic phenotypes in the absence of E7. Conversely, DEK loss results in cell death in HPV positive cervical cancer cells at least in part through p53 activation, and Dek knockout mice are relatively resistant to the development of chemically induced skin papillomas. Despite the established oncogenic role of DEK in HPV associated cervical cancer cell lines and keratinocytes, a functional role of DEK has not yet been explored in HNSCC. Using an established transgenic mouse model of HPV16 E7 induced HNSCC, we demonstrate that Dek is required for optimal proliferation of E7-transgenic epidermal cells and for the growth of HNSCC tumors. Importantly, these studies also demonstrate that DEK protein is universally up-regulated in both HPV positive and negative human HNSCC tumors relative to adjacent normal tissue. Furthermore, DEK knockdown inhibited the proliferation of HPV positive and negative HNSCC cells, establishing a functional role for DEK in human disease. Mechanistic studies reveal that attenuated HNSCC cell growth in response to DEK loss was associated with reduced expression of the oncogenic p53 family member, ΔNp63. Exogenous ΔNp63 expression rescued the proliferative defect in the absence of DEK, thereby establishing a functional DEK-ΔNp63 oncogenic pathway that promotes HNSCC. Taken together, our data demonstrate that DEK stimulates HNSCC cellular growth and identify ΔNp63 as a novel DEK effector.
Introduction

Head and neck cancer is a devastating disease with approximately 60% of patients presenting with locally advanced disease. Over 600,000 new head and neck cancers are diagnosed each year worldwide, the vast majority representing squamous cell carcinomas (SCCs). HNSCC has traditionally been associated with risk factors such as tobacco and alcohol consumption; however, in recent years human papillomavirus (HPV), a well-known cause of cervical cancer, has emerged as a new etiological pathogen. It is now established that at least 25% of all HNSCCs carry HPV genomic DNA, predominantly of the high risk HPV16 subtype. HPV positive HNSCCs are associated with improved survival for affected patients, and such tumors are more sensitive to available therapies. This increased tumor response is due at least in part to p53 tumor suppressor activation despite the presence of E6, a viral oncogene which targets p53 through physical interactions and degradation. Thus, HPV positive and negative head and neck tumors are biologically distinct subtypes of HNSCC. Regardless of HPV status however, achieving efficacy with radiation and chemotherapy treatments remains notoriously difficult. This is due to the inherent molecular heterogeneity of HNSCC, combined with patients presenting at late stage disease where there is a high likelihood of local recurrence and metastasis. Thus, survival rates for patients with HNSCC have not significantly improved in decades, highlighting the urgent need for novel biomarkers and therapeutic targets. Recent limited analysis of HNSCC tissue arrays by our laboratory had suggested that DEK expression was up-regulated in the majority of HPV negative HNSCCs. Herein we extend these studies by analyzing a panel of primary human tumors to determine DEK expression levels in both HPV positive and negative HNSCCs, and define the functional role of DEK in regulating HNSCC growth in vitro and in vivo.

The human DEK oncogene was first identified as a fusion with the CAN nucleoporin in acute myeloid leukemia, and the DEK protein was subsequently purified based on its ability to
modulate DNA topology. DEK is located primarily in the nucleus where it is bound to chromatin and is rarely amplified or mutated, but frequently transcriptionally up-regulated in a variety of malignancies. Transcriptional induction of DEK can occur through Rb-dependent mechanisms in several tumor types; a finding which is consistent with the original observation that DEK is induced by high risk HPV E7 expression in human keratinocytes and up-regulated in HPV positive cervical cancer cells. DEK knockdown in cervical cancer cells expressing the HPV E6 and E7 oncogenes, results in apoptosis that was at least partially mediated through the stabilization of the p53 tumor suppressor. Conversely, DEK overexpression in near-diploid immortalized keratinocytes that form skin (NIKS) stimulated proliferation and suppressed differentiation in organotypic epithelial rafts through a p53 independent mechanism. Effects of DEK loss beyond those dependent on p53, however, remain undefined in human SCCs.

Dek−/− mice are viable and are relatively resistant to benign papilloma formation in a chemically induced skin carcinogenesis model, thus implicating DEK in tumor initiation. In order to test the requirement for Dek in a malignant SCC model system in vivo, we utilized a transgenic HNSCC mouse model. Therein, HPV E7 is targeted to the stratified squamous epithelium with the keratin 14 promoter (K14E7) and mice develop rapid and highly penetrant tongue and esophageal tumors upon addition of the mutagen 4-nitroquinoline-1-oxide (4-NQO) to the drinking water. To determine whether Dek expression is important for HNSCC development, K14E7 mice in a Dek proficient and deficient background were subjected to 4-NQO treatment. Transgenic K14E7 Dek−/− mice exhibited decreased epidermal cell proliferation and attenuated tumor growth as compared to K14E7 Dek+/+ mice. Importantly, Dek was not required for proliferation in non-transgenic mice, indicating that cellular growth suppression by Dek is specific to the oncogenic stimulus induced by E7. Complementary studies in primary human HNSCC tumor tissue and cells demonstrate that DEK protein expression is universally up-regulated regardless of HPV status and that DEK supports tumor cell proliferation. Finally,
we show for the first time that DEK promotes HNSCC growth through a ΔNp63 dependent mechanism, thus identifying ΔNp63 as a novel downstream effector of DEK function.
Results

Dek knockout mice exhibit attenuated HNSCC development in vivo

High risk E7 protein was previously shown to stimulate DEK expression in vitro and in vivo, and DEK up-regulation was sufficient to stimulate oncogenic phenotypes in cellular models of SCC. To directly determine the functional role of Dek in HPV E7 driven HNSCC, we tested the requirement for Dek in an established K14E7-driven mouse model of HNSCC. K14E7 transgenic mice were crossed into a Dek knockout background to generate K14E7 Dek⁻/⁻ which were compared to K14E7 Dek⁺/⁺ mice. Non-transgenic Dek wild-type and knockout mice were generated as controls. All mice were treated with 4-NQO for 16 weeks followed by normal drinking water for 8 weeks. At this 24 week time point, all mice were sacrificed and analyzed for tumor development. K14E7 Dek⁻/⁻ mice trended towards increased survival compared to the K14E7 Dek⁺/⁺ mice; 82% of the K14E7 Dek⁺/⁺ mice died prior to sacrifice, compared to 43% of the K14E7 Dek⁻/⁻ mice (Figure 3.1a). The cause of premature death for the K14E7 Dek⁺/⁺ and one of three K14E7 Dek⁻/⁻ mice was likely due to tumor burden. The cause of death for the remaining two K14E7 Dek⁻/⁻ mice is unknown. A representative section from a K14E7 Dek⁺/⁺ mouse confirms Dek is highly expressed in the tumor (Figure 3.1b). The results of the pathological analyses for these tumors are summarized in Figure 3.1c. As expected from the literature, all 11 of the K14E7 Dek⁺/⁺ mice developed SCCs of the tongue and esophagus (Figure 3.1c), whereas the non-transgenic mice had no macroscopic tumors at the time of sacrifice. In contrast to K14E7 Dek⁺/⁺ mice, only one of seven K14E7 Dek⁻/⁻ mice developed a visible tumor. K14E7 Dek⁻/⁻ mice did have microscopic tumors indicating that Dek is not required for tumor initiation in this model (Figure 3.1c). Importantly, Dek loss was associated with smaller tumors indicating that Dek promotes HNSCC growth.

Dek loss suppresses E7-dependent proliferation in the tongue epidermis
We next sought to determine the underlying cellular mechanism responsible for the observed HNSCC growth suppression in $K_{14}E7$ $Dek^{-/-}$ mice. DEK was shown to inhibit apoptosis and stimulate proliferation in vitro, depending on the cellular and experimental context. Accordingly, we assessed for both biological processes in $E7$-transgenic $Dek$ proficient and deficient epidermis. Apoptotic cells were not detected by cleaved caspase-3 detection (data not shown) indicating that increased cellular death did not account for the decrease in tumor growth in Dek knockout mice. As was previously published, $K_{14}E7$ transgene expression was associated with hyperplasia and an increase in BrdU positive cells in both the basal and suprabasal compartments as compared to the non-transgenic controls (Figure 3.2a and 3.2b). Importantly, $K_{14}E7$ $Dek^{-/-}$ mice displayed decreased proliferation when compared to $K_{14}E7$ $Dek^{+/+}$ epidermis, with proliferative rates repressed to baseline levels observed in non-transgenic mice (Figure 3.2b). Dek loss alone was not sufficient to decrease cellular proliferation in the absence of E7 expression. Taken together, these findings support the notion that Dek up-regulation is required for E7 driven epidermal proliferation, and that cellular growth suppression in response to Dek loss in vivo occurs specifically in the oncogenic environment induced by E7.

**DEK protein expression is up-regulated in human HNSCC regardless of HPV status**

DEK mRNA and protein expression is up-regulated in multiple cancer types including breast cancer, melanoma, and hepatocellular carcinoma and is also induced in response to E7 expression. We previously reported DEK up-regulation in high risk HPV positive and HPV negative HNSCC tissue microarrays; however, analyses were limited for HPV positive tumors since only 4/44 (9%) of the examined samples were HPV positive by in situ hybridization. Additionally, potential associations with demographic and clinical information were not examined. The current analysis extends these prior studies by performing rigorous HPV testing with maximal sensitivity for the detection of HPV16 genome sequences. HPV16 is
the most prevalent HPV type in HNSCCs. We also analyzed relevant clinical characteristics including parameters characteristically associated with HPV status. Additionally, optimal tissue preservation was ensured by analyzing human HNSCC specimens that were freshly biopsied or resected with immediate processing and uniform preparation for IHC analysis to detect DEK protein expression. Table 3.1 depicts HPV status as determined by in situ hybridization by two independent institutions, along with highly sensitive RT-PCR for HPV16 sequences, as well as primary tumor site, tumor stage, p16 status, history of cigarette and alcohol consumption, and determination of DEK protein expression. HPV presence and DEK expression are quantified in the table, and a detailed description of the relevant values is provided in the Materials and Methods. Expression of the cyclin-dependent kinase inhibitor p16 is a clinical marker for HPV presence. Chart review indicated that 10/18 (56%) of HNSCCs tested were positive for p16 expression. HPV positive status was detected in 8/18 (44%) specimens by in situ hybridization at Site 1 (Figure 3.3a), which detects multiple high-risk HPV types, with examples of HPV negative and HPV positive specimens shown. Highly sensitive in situ hybridization for HPV16 at Site 2 identified two additional HPV positive specimens (10/18 or (56%)). Finally, RT-PCR identified one additional HPV16 positive specimen, (11/18) resulting in 61% of the tumors being HPV positive. All samples were subjected to IHC for DEK expression (Figure 3.3a). The intensity of DEK protein expression and the proportion of tumor cells positive for DEK were determined relative to adjacent normal tissue using the grading system depicted in Figure 3.3b and summarized in Table 3.1. Interestingly, strong DEK expression was detected in all tumors regardless of HPV status, tumor stage or demographic information. Neither the level of DEK expression nor the DEK-positive proportion of tumor cells correlated with HPV status (Table 3.1, Figure 3.3a, b). Taken together, these data indicate that DEK protein is universally over-expressed in HPV16 positive and negative HNSCCs.

**DEK expression stimulates growth of HPV positive and negative human HNSCC tumor cells**
In order to determine the importance of DEK expression in human HNSCC cells, we next analyzed a panel of HNSCC cell lines that were transduced with non-targeting or DEK knockdown vectors. One HPV positive line, 93VU147T, and two HPV negative lines, UMSCC1 and UMSCC6, were analyzed following DEK knockdown confirmed by western blot analysis (Figure 3.4a-c). DEK knockdown consistently suppressed cellular growth in both HPV positive and HPV negative cell lines (Figures 3.4a-c). Moreover, growth suppression was associated with reduced proliferation in vitro as was observed in the E7-positive epidermis in the in vivo mouse model. BrdU incorporation was significantly reduced in all cell lines after DEK knockdown, indicating decreased progression into S phase in the absence of DEK (Figure 3.4a-c). Together, these data demonstrate that DEK loss in human and murine HNSCC cells attenuates tumor cell growth through suppression of cellular proliferation.

ΔNp63 protein expression is regulated by DEK and is functionally important

Our previous studies uncovered p53 dependent oncogenic function for DEK in HPV positive cervical cancer cells. p53 remains intact, yet is bound and degraded by E6 leading to suppressed p53 activity in HPV positive cells. In line with previous results in HeLa cells, DEK knockdown in HPV positive, primary CCHMC-HNSCC1 cells resulted in the up-regulation of p53 protein levels, as well as increased phosphorylation of Serine15 on p53 indicating p53 activation (Figure 3.5a). In contrast, p53 is mutated in the majority of HPV negative HNSCCs and is not expressed in HPV negative UMSCC1 and UMSCC6 cells (data not shown, and 130, 255). Given that the growth of all three HNSCC cell lines was suppressed by DEK knockdown, p53 activity alone could not account for the proliferative defects associated with DEK depletion. The p63 family of proteins is related to p53 and expressed from two promoters, resulting in two distinct groups of p63 isoforms, TAp63 and ΔNp63, which have opposing functions. TAp63 functions as a tumor suppressor much like p53, while ΔNp63 has oncogenic functions. ΔNp63 is the predominant p63 isoform in keratinocytes and is overexpressed in the majority of HNSCCs. 256
Mechanisms driving ΔNp63 up-regulation are complex and incompletely understood. Our previous data demonstrated that DEK overexpression in human keratinocytes increases ΔNp63 protein expression and the numbers of ΔNp63 positive cells in the basal layer of organotypic epithelial rafts. We therefore determined the effect of DEK loss in HNSCCs on ΔNp63. Lentiviral DEK knockdown in UMSCC1 and 93VU147T cells decreased ΔNp63 protein levels (Figure 3.5b). Furthermore, DEK loss in HPV positive or negative primary HNSCC cell populations also reduced ΔNp63 protein expression (Figure 3.5c) and IHC on consecutive CCHMC-HNSCC1 human tissue demonstrates DEK and ΔNp63 are strongly expressed in similar areas of the tumor (Figure 3.5d). Reduction of ΔNp63 was not associated with decreased mRNA levels indicating that DEK dependent control of ΔNp63 levels is not mediated through transcriptional regulation (Figure 3.5e); a finding which implicates DEK in a post-transcriptional mechanism controlling ΔNp63 levels. To determine whether the observed ΔNp63 repression in DEK-depleted cells was functionally relevant, HA-tagged human ΔNp63 was exogenously expressed in adenovirally DEK-depleted UMSCC1 cells to determine if the proliferation defect in DEK deficient cells could be rescued by restoring ΔNp63 levels (Figure 3.5f). AdDEKsh infection alone resulted in significantly reduced cell growth similar to the effects of lentiviral knockdown in Figure 4 and published data in cervical cancer cells. Expression of HA-ΔNp63 rescued the cellular growth defect in DEK-deficient cells, but did not increase the growth of DEK-proficient cells (Figure 3.5g). These data demonstrate that DEK expression in HNSCC cells is required for maintenance of ΔNp63 protein expression and indicate that DEK-dependent cellular growth in HNSCC cells is at least in part mediated by ΔNp63.
Discussion

HNSCC is a devastating disease causing approximately 350,000 deaths per year.\textsuperscript{259} Thus, development of novel therapies and identification of clinically relevant disease biomarkers and molecular targets is of paramount importance. Current chemotherapeutic strategies result in major toxicity, and surgical resection leads to debilitating physiological side effects and permanent disfigurement. Although there have been promising advancements in the field, it is unlikely that any singular treatment will drastically increase survival, and early tumor detection remains a priority and major challenge. While HPV positive tumors are associated with an improved prognosis over their HPV negative counterparts, outcomes still remain less than optimal. Biological and molecular commonalities and differences between HPV positive and HPV negative HNSCCs are only beginning to emerge.\textsuperscript{95, 130} Thus, studies of molecular pathways that mark and drive HNSCC need to be examined in parallel in these two biologically distinct subtypes. Therefore, our analyses of the expression and role of the human DEK protein were carried out in tumors that were carefully analyzed for the presence of high-risk HPV, with a focus on HPV16, together with associated patient and tumor characteristics. Expression of p16 is considered an excellent clinical marker of HPV positive tumor status. Indeed, positive staining for p16 was in agreement with the detection of HPV sequences by \textit{in situ} hybridization in 70\% (7/10) and 80\% (8/10) of the specimens at two testing sites. However, one of ten p16 positive specimens (CCHMC-HNSCC13) was HPV16 negative by three different analyses, suggesting a need for independent confirmation of HPV status to avoid false positive results. Two specimens of unknown p16 status were additionally identified as HPV positive. All together, 61\% of the HNSCC tumor specimens analyzed were determined to harbor high risk HPV16 DNA sequences. Importantly, 100\% of the tumor samples showed strong DEK over-expression regardless of HPV status. However, it is important to note that our studies were primarily limited to oropharyngeal HNSCC tumors and may not represent HNSCCs as a whole. Comparisons
with published DEK expression studies in breast cancer suggest some notable distinctions in
HNSCCs which need to be investigated further. One report on breast cancer by our laboratory
demonstrated that the proportion of DEK-expressing cells, but not per-cell staining intensity,
increases with disease progression from normal breast epithelium to hyperplastic, and finally to
malignant tumors.\textsuperscript{204} A second report by Liu et al. associated increasing DEK expression levels
with increasing tumor stage.\textsuperscript{219} We note that for stage I-III specimens, the proportion of DEK
positive tumor cells, as well as the staining intensity of the DEK oncogene, were already
maximal (Table 3.1), thus suggesting that DEK may be particularly useful as a biomarker to
detect early stage HNSCCs where identification is most beneficial for the patient. Regarding the
molecular mediators driving DEK protein over-expression, transcriptional up-regulation of DEK
message has been reported for a multitude of human tumors and has been ascribed to distinct
transcriptional mechanisms in different cell and tumor types.\textsuperscript{209, 212, 213} In HPV positive cervical
cancer cells, DEK expression is driven by the high risk HPV E7 oncoprotein through Rb pocket
protein dependent mechanisms. It is likely that in HPV positive HNSCCs, E7-dependent DEK
regulation occurs in a similar fashion, whereas in their HPV negative counterparts, Rb mutations
might be at play.

A requirement for Dek expression in genetic mouse models of cancer, and specifically
during SCC development \textit{in vivo}, has remained unexplored. We utilized an established \textit{E7}-
driven, 4-NQO dependent mouse model of HNSCC and demonstrate that \textit{Dek} expression is
important for the proliferation of epidermal keratinocytes and for sustained tumor cell growth.
The presence of microscopic tumors in the \textit{Dek} knockout tongues shows that \textit{Dek} is not
required for tumor initiation in this 4-NQO-dependent system. The fact that Dek loss attenuated
cellular proliferation specifically in the \textit{E7} oncogene expressing, but not in control epidermis,
indicates the existence of a therapeutic window for targeting DEK. DEK depletion may represent
a reasonably tumor specific approach that can be exploited for tumor prevention and cancer
therapy in HNSCC patient populations and beyond. Along these lines, a degree of specificity for
targeting DEK functions has been supported by earlier in vitro studies, where DEK expression was highest in HPV positive cervical cell lines, lower in normal, primary keratinocytes, and lowest in differentiated keratinocytes.\textsuperscript{208, 209} Broad HNSCC cancer cell addiction to DEK over-expression was observed in both HPV positive and negative HNSCC lines. Previously published data demonstrate that in HeLa cells, DEK loss was associated with apoptosis and senescence by a mechanism that at least partially involved up-regulation and activation of p53.\textsuperscript{210} Similarly, DEK depletion in HPV positive HNSCCs led to p53 up-regulation. However, in light of the fact that HPV negative HNSCCs respond to DEK knockdown, we sought to identify p53-independent DEK effectors.

Expression of the $\Delta$Np63 member of the p53 protein family has been implicated in the maintenance of stem and progenitor cells in various epithelia. In fact, $\Delta$Np63 was recently shown to be essential for reprogramming mouse embryonic fibroblasts as measured by pluripotent stem cell phenotypes such as colony formation by alkaline phosphatase stain and teratoma formation.\textsuperscript{260} Such activities in cellular stemness are supported by the restricted expression of $\Delta$Np63 in the basal cell layer of the epidermis where keratinocyte stem and progenitor cells reside. On the other hand, $\Delta$Np63 has been implicated in triggering terminal differentiation.\textsuperscript{261, 262} The role of $\Delta$Np63 in stem cell functions is therefore complex, and the extent to which $\Delta$Np63 regulates stem cell proliferation, differentiation or a combination of both remains controversial. Apart from pluripotent stem cells, $\Delta$Np63 is now recognized as a somatic stem cell marker in other types of epithelium, highlighting the importance of this protein in multiple developmental contexts.\textsuperscript{263} $p63^{-/-}$ mice are well characterized for their severe developmental defects at birth including the lack of appendages, squamous epithelium, and hair follicles, and not surprisingly, recent work implicates the loss of the $\Delta$Np63 isoform as the largest contributor to these phenotypes.\textsuperscript{264-266} Not surprisingly, maintaining appropriate levels of $\Delta$Np63 is critical for preserving epithelial integrity and suppressing transformation. Indeed,
overexpression of ΔNp63 is considered oncogenic in HNSCC, and p63 amplification and mutation have been reported in a subset of HNSCCs.\textsuperscript{142, 256, 257} Regulation of ΔNp63 expression is complex and can occur at both transcriptional and post-transcriptional levels. Here, we report that DEK controls ΔNp63 expression via a post-transcriptional mechanism. Importantly, exogenous ΔNp63 expression in DEK-depleted cells rescued cancer cell growth demonstrating that DEK-dependent ΔNp63 expression is functionally important for promoting HNSCC growth. DEK activities can thus be placed upstream of ΔNp63, where targeting DEK results in decreased levels of this recognized oncogene that contributes to SCC pathogenesis.\textsuperscript{256} Future work will examine the mechanism(s) whereby DEK regulates ΔNp63. Given that ΔNp63 expression in DEK deficient tumor cells did not completely restore proliferation to the level of control cells, we cannot rule out the possibility that other DEK effectors exist and contribute to tumor cell growth \textit{in vitro} and \textit{in vivo}. Taken together, our results suggest the existence of a new ΔNp63-DEK signaling axis, which promotes proliferation in HNSCC.

**Materials and Methods**

**Cell culture.** HPV negative UMSCC1 and UMSCC6 head and neck cancer cells were maintained in Dulbecco’s modified Eagle medium (DMEM, Gibco, New York, USA) supplemented with 1% hydrocortisone, 10% fetal bovine serum (FBS), antibiotics and antifungals. HPV positive 93VU147T head and neck cancer cell lines were maintained in the same media but without hydrocortisone.

**Lentiviral transduction.** Cells were transduced at 30% confluency with lentiviral pLKO.1 non-targeting control (NTsh) or pLKO.1 DEK832 (DEKsh2) (Sigma-Aldrich Mission shRNA library) as previously described.\textsuperscript{186, 204} A final concentration of 8 µg/mL polybrene was added for each transduction. Cells were selected and carried in 1µg/mL of puromycin for primary cultures or in 1.25 µg/mL for established cell lines, and were independently transduced for each experiment.
Adenoviral transduction. Cells were transduced at 40% confluency with adenoviral control vector (AdGFP) or adenoviral DEK knockdown (AdDEKsh) at 10 infectious units per cell as previously published.\textsuperscript{210} Protein lysates were collected 72 hours post-infection.

Primary human head and neck cancer specimens. Human HNSCC tissues were freshly obtained with IRB approval at the time of surgical resection or biopsy. Chart review was performed to determine patient demographics, risk factors and histopathological characteristics. Tissues were immediately placed in F-media.\textsuperscript{208} A portion of the tissues was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analysis. A second portion of each specimen was also cultured in F-media on irradiated J2-3T3 mouse fibroblasts for primary cell culture as described previously.\textsuperscript{267} Resulting cell populations were considered primary if passaged fewer than 15 times. Tissue sections were subjected to hemotoxylin and eosin (H&E) staining to confirm the presence of squamous cell carcinoma.

HPV detection. For in-house HPV analysis (Site 1), tissue sections were deparaffinized and subjected to \textit{in situ} hybridization using standard DNA probes specific for 12 high risk HPV types including 16 and 18 types provided by Ventana Medical Systems (INFORM HPV III Family 16 Probe (B)). Sections were then incubated with a fluorescein-tagged DNA probe and counterstained using the automated Ventana BenchMark instrument (Ventana Medical System, Tucson, AZ, USA). Further HPV detection by \textit{in situ} hybridization and real-time TaqMAN PCR assays (Site 2) was previously described by the Gillison laboratory.\textsuperscript{268} Briefly, all 21 CCHMC-HNSCC specimens were evaluated for HPV16 using a biotinlyated probe (Genpoint, Dako, Copenhagen, Denmark) and were considered positive based on nuclear staining in tumor cells. Real-time PCR was performed on formalin-fixed, paraffin-embedded specimen (FFPE) extracted DNA, and interrogated for HPV16 sequences. Human endogenous retrovirus-3 (ERV3) was used as the internal control. Threshold for positivity is any value above 1 HPV copy as determined by calculating the ratio of HPV16/ERV3 and accounting for total percentage of tumor present.
Evaluation of patient clinical characteristics. Chart review was performed to extract relevant clinical information for each patient. For Table 3.1, any patient who currently smokes or who smoked previously was designated as a smoker. Alcohol consumption was assigned to patients who self-reported moderate to heavy drinking and not social or occasional drinkers. Patients who answered no to either smoking or drinking were designated as such. Expression of p16, a marker of HPV positivity, was also noted if clinically determined. Quantitation of DEK positive staining by immunohistochemistry was carried out as follows: The number of cells with positive staining for DEK was quantified as: 3= >90% positive tumor cells, 2= 10-50% positive tumor, 1= <10% positive tumor cells. The overall intensity of DEK staining was determined as W (weak), V (variable) or S (strong) as compared to surrounding non-neoplastic squamous epithelium. The overall DEK status is then labeled by a combination of these values. For instance, ‘3S’ in Table 3.1 indicates this tumor specimen is maximally positive for DEK staining intensity and for the proportion of DEK-expressing cells.

Transgenic and knockout mouse models. The K14E7 head and neck cancer mouse model and Dek⁻/⁻ mouse were previously described. Dek⁻/⁻ mice were backcrossed into an F/VBN background and E7 was maintained in a heterozygous state. For generation of K14E7 Dek⁻/⁻ mice, female Dek⁻/⁻ mice were crossed with K14E7 males. The following primer sets were used for genotyping: Dek wild-type allele (5'-CGA ACT CGT GAA GAG GAT CTT GA-3', 5'-ATG TGT CAG GCT GCA TCT CCA ATG-3'), Dek knockout allele (5'-ATC CAT CAT GGC TGA TGC AAT GCG-3', 5'-TGG AAG GTA AAG AGT GGC CCT TA-3'), E7 transgene (5'-ACT CTA CGC TTC GGT TGT GCG TA-3', 3'-GCA CAC AAT TCC TAG TGTGCC CAT-5'). Mouse use and handling was carried out in accordance with the American Association for Accreditation of Laboratory Animal Care and the Cincinnati Children's Hospital Veterinary Care Facility according to a protocol approved by the Institutional Animal Care and Use Committee to S.I. Wells.
**Head and neck cancer induction.** Eight week old transgenic and non-transgenic mice were administered the chemical carcinogen and tumor initiator 4-NQO in the drinking water at 10 µg/ml as previously published\(^{251}\) for a period of 16 weeks and then returned to normal water for 8 weeks. One hour prior to sacrifice, mice were injected intraperitoneally with 6µg/ gram of mouse weight of bromodeoxyuridine (BrdU) (BD Biosciences, San Jose, CA, USA). Mice were analyzed either upon premature death, or at 24 weeks after timed sacrifice. Sacrifice occurred upon moribund appearance due to the tumor burden which prevented the mice from eating and drinking. In the case of the K14E7 Dek\(^{-/-}\) mice, two of three mice died prematurely from unknown causes. Gross and microscopic tumors were recorded. Tongue and esophagus were collected for histological analyses.

**Histological analysis and immunohistochemistry.** Mouse tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 µm thickness, and fixed onto slides. Routine H&E stained sections were analyzed for histopathology.\(^{207}\) Paraffin sections were deparaffinized in xylene and rehydrated for antigen retrieval in sodium citrate. Sections were then treated with the Mouse on Mouse peroxidase immunostaining kit (Vector Labs, Burlingame, CA, USA). Sections were stained with diaminobenzidine (DAB) and counterstained with Vector Laboratories hematoxylin QS or Nuclear Fast Red (Poly Scientific, Bay Shore, NY, USA) and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA). Staining was detected with a Leica DM2500 microscope and LAS software (Leica Microsystems Inc., Buffalo Grove, IL, USA), and images were captured at the indicated magnifications. Antibody dilutions were used as follows: BrdU (1:100, Invitrogen), p63\(\alpha\) (1:100, Santa Cruz (H-129), Dallas, TX, USA) and DEK (1:50 and 1:100, BD Transduction).

**Western blotting.** Whole cell lysates were harvested with RIPA buffer containing protease and phosphatase inhibitors or Laemmli buffer containing phosphatase inhibitors. 20 µg total protein was analyzed by western blotting as previously described.\(^{210}\) Membranes were probed with DEK (1:1000, BD Biosciences), p53 (1:1000, Cell Signal (1C12), Boston, MA, USA), phospho-
serine15 p53 (1:1000, Cell Signal), ΔNp63 (1:200, Santa Cruz (4A4)) or actin (1:10,000; a gift from James Lessard, Cincinnati Children’s Hospital Medical Center).

**Growth curves.** Following selection, lentivirally transduced cells were plated in triplicate at an equal density of 100,000 cells/well. The cells were then trypsinized, counted on a hemacytometer and total cell number was calculated for each time point. Data represent two independent experiments.

**Flow cytometry.** BrdU incorporation was assessed as described previously. Briefly, HNSCC cells were plated in equal numbers and labeled with 10 µM BrdU for 2 hours. Collected cells were prepared following the BD Pharmingen APC BrdU Flow kit instructions, analyzed on a BD FacsCalibur, and data further analyzed with FlowJo software (Tree Star, Ashland, OR). Samples were analyzed in triplicate and data represents two independent experiments.

**Quantitative RT-PCR.** mRNA was harvested using Trizol reagent (Invitrogen, Carlsbad, CA, USA) from transduced cells immediately following puromycin selection. 1 µg of RNA was reverse transcribed to cDNA using the QuantiTect Reverse Transription kit (Qiagen, Valencia, CA, USA). cDNA expression was detected using SYBR green master mix (Applied Biosystems, Carlsbad, CA, USA) on an ABI7300 Real Time PCR machine (Applied Biosystems). Data was analyzed using the ΔΔCt method. Primers were as follows: (1) ΔNp63 Forward: 5’-GGAAACAATGCCCAGACTC-3’ Reverse: 5’-GTGGAATACGTCCAGGTGC-3’ (2) DEK Forward: 5’-TGT TAAGAAAGCAGATAGCACCACC-3’ Reverse: 5’-ATTAAAGGTTTCATCATCTGAACTATCCTC-3’ (3) GAPDH Forward: 5’-GGTCTCCTCTGACTTCAACA-3’ Reverse: 5’-ATACCAGGAAATGAGCTTGA-3’. ΔNp63 primers were previously published. Cellular proliferation rescue experiments. 400,000 cells were plated and transduced with adenovirus on the following day, as described above. Twenty-four hours post-infection, cells were transfected in duplicate with 4 ug of the control (pcDNA) or ΔNp63 construct (human HA-
ΔNp63), along with 1 ug of dsRed as a transfection efficiency control. DNA was added to Opti-MEM (Gibco), followed by DharmaFECT 1 Transfection Reagent (Fisher) and added drop-wise to the cells. Total cell counts were determined by hemacytometer counts 72 hours post-infection. This represents three independent experiments.

**Statistics.** Statistical significance was calculated using GraphPad Prism 6 software (La Jolla, CA, USA). Student’s two-tailed t-test was used for experiments where noted, with **=p≤.01 and ***=p≤.001.

**Acknowledgements**

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Figure 3.1. Dek loss protects from HNSCC tumor promotion in vivo. (a) Dek loss prolongs survival in a murine model of HNSCC. Nine of eleven K14E7 Dek^{+/+} were sacrificed prematurely due to moribund appearance, whereas only three out of seven K14E7 Dek^{-/-} mice were sacrificed early. (b) Immunohistochemistry depicting a K14E7 Dek^{+/+} mouse tumor that stains positive for DEK, with arrows representing examples of positive cells. (c) Mice were sacrificed at 24 weeks for gross macroscopic and microscopic tongue and esophagus lesion analysis. Tumors grade was determined histologically as previously describe for this 4-NQO-driven HNSCC models and representative images are shown. One K14E7 Dek^{-/-} mouse had microscopic tumors in both the tongue and esophagus, hence the number of microscopic
tumors in this group totals 8 for the 7 mice. Original magnifications are at 200x. p=.0893 as determined by log-rank (Mantel-Cox) test.
Figure 3.2. Dek loss attenuates E7-driven proliferation in tongue epidermis. (a) H&E stained images of Dek-proficient and Dek-deficient E7-trangenic tongue epidermis showing epithelial hyperplasia are shown at a total magnification of 200x. (b) Mice were injected with BrdU prior to sacrifice, and immunohistochemistry done to detect BrdU positive cells in the premalignant tongue epidermis. Representative images with arrows denoting BrdU positive cells are shown. The percentage of epidermal BrdU positive cells was determined by counting >1000 cells in sections from 4 K14E7 Dek+/+, 4 K14E7 Dek−/−, 4 nontransgenic Dek+/+, and 3 nontransgenic Dek−/− mice. Tongues from K14E7 Dek+/+ mice harbored significantly more BrdU positive cells as compared to K14E7 Dek−/− mice. **=p≤0.01 by two-tailed t-test.
Table 3.1 Clinical characteristics of primary human tumor samples

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<th>CCHMC ID</th>
<th>Primary tumor location</th>
<th>Stage</th>
<th>Smoking (Y/N)</th>
<th>Drinking (Y/N)</th>
<th>Clinical p16 status</th>
<th>HPV in situ</th>
<th>RT-PCR</th>
<th>HPV copies per cell</th>
<th>DEK levels</th>
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<td></td>
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<td>−</td>
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<td>Y</td>
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<td>+ +</td>
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<td>Y</td>
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<td>1.61 x 10^4</td>
<td>−</td>
<td>−</td>
<td>3V</td>
</tr>
</tbody>
</table>

Abbreviations: BOT, base of tongue; HPV, human papillomavirus; UK, unknown. CCHMC-HNSCC16 and 19 were removed due to a lack of embedded or malignant tissue, site 1 indicates in situ performed at CCHMC; site 2 indicates in situ performed at Ohio State University. Please refer to the Materials and methods for classifiers used to determine DEK levels.
Figure 3.3. DEK protein expression is universally up-regulated in human HNSCC. (a) Tumor specimens were sectioned, H&E stained for morphology to confirm the diagnosis of HNSCC, and subjected to in situ hybridization for detection of high-risk HPV sequences. Representative HPV positive (Specimen B) and HPV negative (Specimen A) tumors are shown. Strong DEK expression was observed in both HPV positive and negative tumors as compared to adjacent non-neoplastic epithelium. (b) The percentage of DEK positive cells on each section was estimated and staining intensity determined as described in the Materials & Methods. Representative images and scores are shown along with the corresponding H&E image. Original magnification 200x; insets 1000x.
Figure 3.4. DEK knockdown attenuates the proliferation of HPV positive and negative HNSCC cell lines. (a-c, left panels) HPV positive and HPV negative cell lines were lentivirally transduced with control or DEK-specific shRNA, selected in puromycin, and plated in equal cell numbers for cell counts taken over the course of four days. Western blot analysis indicates DEK knockdown is below the level of detection in all cell lines, with actin used as a loading control. DEK loss substantially decreased total cell numbers on day 3 and 4, regardless of HPV status. Data represents the average of two independent experiments with each sample measured in triplicate. Error bars indicate standard deviation (SD). (a-c, right panels) Control and DEK
knockdown cells were incubated with BrdU for 2 hours and BrdU positive cells determined by flow cytometry. Representative contour plots show BrdU positive gating for NTsh and DEKsh cells. Data represents the average of two independent experiments with each sample measured in triplicate. Error bars indicate standard deviation (SD).
Figure 3.5. **DEK promotes cellular growth by up-regulating ΔNp63.** (a) Western blot analysis using CCHMC-HNSCC1 cell lysates shows phospho-ser15 p53 and total p53 up-regulation after DEK knockdown with shRNA as compared to control shRNA. (b) Western blot analysis of UMSCC1 and 93VU147T cells shows reduced ΔNp63 protein levels in DEKshRNA compared to control shRNA lentiviral infected cells and similarly with adenovirally infected UMSCC1 cells. (c) Primary human-derived cell populations CCHMC-HNSCC1 (HPV positive) and CCHMC-HNSCC3 (HPV negative) also show reduced levels of ΔNp63 with DEKshRNA as compared to NTsh controls, with densitometry depicted in the graph to the right for CCHMC-
HNSCC1. **(d)** Immunohistochemistry for DEK and ΔNp63 have similar expression patterns in the CCHMC-HNSCC1 tumor specimen. Dashed boxes indicate expanded area with representative pictures below. Original magnifications at 200x. **(e)** qRT-PCR indicates ΔNp63 mRNA levels are not reduced with DEK loss in either cell line. GAPDH was used as the internal control with relative expression determined by the ΔΔCt method. **(f)** UMSCC1 cells were transduced with AdDEKsh or control AdGFP adenovirus and transfected with control (pcDNA) or human HA-ΔNp63 constructs on the following day. dsRed was used in all samples as a transfection efficiency control. DEK and ΔNp63 expression levels were verified by western blot analysis. **(g)** Viable cells from panel (f) were counted on day 3. Exogenously expressed ΔNp63 increased the total number of cells in the presence of AdDEKsh but not in control cells. Error bars indicate standard error of the mean (SEM) from 3 independent experiments. NS=not significant, **=p≤.01 and ***=p≤.001 as determined by paired two-tail t-test.
Chapter 4: IRAK1 promotes HNSCC survival through a DEK-regulated network

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Abstract

The chromatin-binding DEK protein was recently reported to promote the growth of HPV+ and HPV- head and neck squamous cell carcinomas (HNSCCs). However, the relevant cellular and molecular mechanism(s) remain poorly understood. While DEK is known to activate and repress specific transcriptional targets, the global DEK-dependent transcriptome in HNSCC is unknown. In order to identify DEK transcriptional signatures and functional targets, we carried out RNA-Sequencing (RNA-Seq) in head and neck cancer cell lines that were either proficient or deficient for DEK. Bioinformatic analyses and subsequent validation revealed that the interleukin-1 receptor activated kinase (IRAK1) was significantly repressed upon DEK knockdown. IRAK1 is best known for its role in inflammatory signaling, and was previously shown to have tumor suppressive activities in oral squamous cell carcinoma. Surprisingly, datamining of The Cancer Genome Atlas (TCGA) revealed 14% of HNSCC patients overexpress IRAK1, thus supporting possible oncogenic activities. Furthermore, IRAK1 knockdown in HPV+ and HPV- HNSCC cell lines was sufficient to increase HNSCC cell death by apoptosis and to reduce signaling through downstream pathways such as ERK1/2. Finally, targeting DEK and IRAK1 simultaneously enhanced cell death over the targeting of either. We propose a model wherein IRAK1 stimulates oncogenic signaling and phenotypes both independently and in conjunction with DEK.
Introduction

Head and neck squamous cell carcinoma (HNSCC) is a disease comprised of two distinct entities: human papillomavirus (HPV) positive and HPV negative. HPV− disease is attributable to tobacco and alcohol use, and its declining incidence in the US has been ascribed to the well-publicized health risks of these activities. In stark contrast, HPV+ disease is on the rise, particularly in younger patient populations. While improved response to traditional chemotherapies and thus favorable long-term survival is observed in HPV+ patients, prognoses remain grim for patients with advanced and metastatic tumors. Furthermore, major quality of life issues arise due to treatment-related tissue damage. Therefore, the need for novel therapeutic targets and biomarkers cannot be underestimated. Our previous work highlighted the oncogenic functions of DEK in both HPV+ and HPV− human HNSCCs, wherein DEK was highly overexpressed and required for optimal growth and proliferation. Dek loss of function in mice attenuated the proliferation of HPV16 E7 expressing, but not normal, epidermis and inhibited overt tumor growth in a chemically induced model of HNSCC. In view of the observed specificity of DEK targeting for premalignancies and malignancies, this molecule has been reported as a potential therapeutic target. However, DEK-dependent signaling pathways and molecular mediators of DEK-dependent tumor phenotypes in HNSCC are limited. Our previous work implicated ΔNp63 as a down-stream DEK target that regulated DEK-dependent proliferation. In our studies we aimed to identify additional signaling pathways dependent on DEK expression.

DEK is well published for its importance in various cancer types, including breast and bladder cancer, melanoma, and most recently, HNSCCs. This is a versatile nuclear protein, with functions that range from chromatin modifier and histone chaperone to modulator of DNA repair, replication, and transcription. For example, DEK represses transcription in leukemia cells through inhibition of p300 and P/CAF.
activates transcription via interaction with AP-2α in glioblastoma.\textsuperscript{189} Although DEK has been published as a co-activator or co-repressor of transcription in various systems, transcriptome data to determine the role of DEK in global transcriptional regulation in solid tumors is scarce.\textsuperscript{188, 189, 192}

In this study, we performed transcriptome profiling by RNA-Seq to define the broader DEK-dependent transcriptome in HNSCC cells and to identify novel targets, which may themselves be functionally relevant for the growth of these tumors. We focused on both subsets of HNSCC, HPV− and HPV+, to identify targets that may be beneficial to patients regardless of HPV status. Following gene ontological analysis, biological processes involved in the immune response were strongly implicated. DEK has previously been published as an autoantigen in autoimmune diseases and it can function as a pro-inflammatory protein, suggesting it may regulate inflammatory signaling.\textsuperscript{183, 227} We pursued the IRAK1 serine/threonine kinase, which mediates signaling from the toll-like receptor (TLR) and interleukin-1 receptors (IL1R).\textsuperscript{242} The IRAK1 cascade includes the E3 ubiquitin ligase TRAF6, which engages, among other pathways, NF-κB and MAPK signaling. IRAK1 was recently implicated as a novel therapeutic target in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), but its function in most solid tumors remains unknown.\textsuperscript{245} We first characterized expression patterns of IRAK1 in HNSCC with data from The Cancer Genome Atlas (TCGA) and found it is overexpressed by genomic amplification and transcriptional up-regulation in a portion of tumors. IRAK1 inhibition attenuated downstream signaling through TRAF6 and increased apoptosis, suggesting IRAK1 inhibition may be a new therapeutic avenue for patients with HNSCC. Finally, DEK and IRAK1 contributed to HNSCC survival independently, and targeting them jointly enhanced HNSCC cell death over the targeting of either. Taken together, these data reveal IRAK1 as a component of the DEK transcriptome, and a targetable oncogene in HNSCC.
Results

Profiling the DEK-dependent transcriptome in HNSCC

Little is known about the global impact of DEK loss on gene expression, and relevant transcriptional targets are largely uncharacterized.\(^{192}\) DEK plays dual roles in transcription, either as a co-activator or a co-repressor depending on the cellular context, and was recently published to bind transcriptional start sites of some of the activated or repressed target genes.\(^{194}\) In order to define the consequences of DEK loss on transcription in HNSCC, we used a well-established lentiviral approach previously published in this model system.\(^{272}\) The HPV\(^{-}\) and HPV\(^{+}\) cell lines, UMSCC1 and UMSCC47, respectively, were transduced with DEK versus control knockdown vector and selected. Successful knockdown was confirmed by western blot analysis (Figure 4.1A). Messenger RNA (mRNA) was collected and subjected to RNA-Seq. Independent analyses were performed on UMSCC1 and UMSCC47 RNA-Seq data to identify fold changes for differentially expressed genes. Venn diagrams highlight over 2,000 genes that were differentially expressed upon DEK loss and similarly regulated in these cell lines (Figure 4.1B). Ontology analyses revealed clear trends in altered biological processes. Among the significantly regulated processes identified in this way was the immune response (Figure 4.1C). DEK has previously been implicated in immune system regulation, possibly in part through NFκB signaling, but few DEK targets have been described as mediators of these processes.\(^{227}\) In addition to ontological analysis, we assessed common transcription factor binding sites among the overlapping gene set, and found new DEK targets, along with published ones such as p53 (Supplementary Tables S4.1-4.3).\(^{210}\) To further define those targets, we used network analysis software to highlight nodes of interest. A portion of the down-regulated gene network for UMSCC1 cells is shown (Figure 4.1D), with arrows highlighting two nodes of interest. Expanded versions of this network map, along with others, are found in Supplementary Figures S4.1-4.6. As expected, DEK was significantly down-regulated and connected to many other
genes of interest, including TNFAIP3, IL6, and MAPKs. As these genes are well established for their contribution to inflammation and immune signaling, but are downstream mediators of receptor-initiated signaling, we focused on IRAK1 that lies upstream. Since IRAK1 is a known regulator of toll-like receptor signaling in the innate immune system it may be a potential candidate for DEK-dependent processes.

**DEK regulates IRAK1 mRNA and protein levels**

IRAK1 is an important mediator of immune signaling, but little is known about its regulation outside of post-translational modifications. *IRAK1* expression was decreased by 1.4- to 3.2-fold upon DEK loss, as determined by RNA-Seq (Figure 4.2A). To validate these findings from the RNA-Seq data, we utilized qRT-PCR in multiple cell lines (Figure 4.2B-4.2D) to confirm that *IRAK1* expression was reduced following DEK depletion. As expected, IRAK1 mRNA levels were reduced in UMSCC1 and UMSCC47 cell lines, as well as an additional HPV- HNSCC cell line. Similarly, DEK knockdown in these cell lines reduced IRAK1 protein expression levels, along with known downstream MAPK signaling (Figure 4.2E). Reduced expression of IRAK1 in the absence of DEK was correlated with reduced expression of downstream pathway components, thus suggesting IRAK1 may a functionally relevant DEK target.

**IRAK1 is over-expressed in primary HNSCC**

Based on the observed IRAK1 transcriptional regulation in HNSCC cell lines, we probed available TCGA databases to determine possible IRAK1 alterations in primary HNSCCs. This data mining revealed *IRAK1* is altered in 14% of HNSCCs, predominantly as a result of gene amplification or mRNA up-regulation (Figure 4.3A). This was observed in HPV+ and HPV- tumor subsets. To confirm that IRAK1 protein is expressed in HNSCC compared to normal tissue, we performed immunohistochemistry for IRAK1 on primary HNSCC tissue samples, which were previously described. Examples of HPV+ and HPV- specimens are shown (Figure 4.3B), with strong IRAK1 protein expression detectable in the cytoplasm as expected, and some additional
nuclear staining. This data suggests IRAK1 is overexpressed in HNSCC in line with possible oncogenic activities.

**IRAK1 loss increases apoptosis in HNSCC**

Having identified IRAK1 as a candidate effector in HNSCC we next sought to characterize its function in this tumor type. Previously, IRAK1 was proposed to have a tumor suppressive role in HNSCCs. Therefore, we aimed to determine the contribution of IRAK1 to HNSCC phenotypes. We utilized a published IRAK1 shRNA construct to deplete IRAK1 levels in both HNSCC cell lines. IRAK1 knockdown resulted in decreased total and activated IRAK1, as measured by phosphorylation of residue Thr209, as well as reduced activation of downstream pathway components. These include a reduction in NF-κB (pIKKα/β) and MAPK (p38 and ERK1/2) signaling (Figure 4.4A), both well-known cascades triggered by IRAK1 activation. Chemical inhibition of IRAK1 was also carried out with the IRAK-1/4 inhibitor which has been shown to increase apoptosis in melanoma *in vitro* and *in vivo* and to inhibit signaling and cell viability in MDS. This inhibitor is a benzimidazole that is selective for IRAK1 and IRAK4 and shows little specificity for other kinases. Similar to these published studies, 10 µM concentrations of IRAK-1/4 attenuated activation of IRAK1 at 24-72 hours post-treatment (Figure 4.4B) in UMSCC1 and UMSCC47 cells. Activated phospho-IRAK1 then complexes with TRAF6, which undergoes ubiquitination, thereby initiating downstream signaling cascades. To verify that signaling effects observed with IRAK1 loss were a result of a reduction in TRAF6-ubiquitination, TRAF6 was immunoprecipitated and subsequently probed for ubiquitin. TRAF6 ubiquitination was decreased in the absence of IRAK1, suggesting that NF-κB and MAPK signaling is mediated through TRAF6 (Figure 4.4C-4.4D). Finally, cellular proliferation and death were quantified upon IRAK1 inhibition in order to probe for a potential functional role in HNSCC cells. Significantly increased apoptosis was observed in the absence of IRAK1, either with shRNA or chemical inhibition (Figure 4E-4G). However, we did not observe any differences in
cell cycle profiles (Supplemental Figure S4.7). These results demonstrate that IRAK1 promotes the survival of HNSCC cells and that IRAK1 inhibition may be a novel therapeutic strategy to enhance cell death in this tumor type.

**IRAK1 and DEK independently regulate HNSCC cellular survival**

To assess if IRAK1 is functionally important downstream of DEK, and if these two proteins coordinate to regulate oncogenic phenotypes, IRAK1 was overexpressed in the presence and absence of DEK in an attempt to rescue deficiencies induced by DEK loss. (Figure 4.5A). Interestingly, IRAK1 overexpression rescued phospho-ERK1/2 signaling (Figure 4.5A), but reconstitution of this pathway was not sufficient to rescue cell death (Figure 4.5B), cell cycle arrest (Figure 4.5C), or total cell number (Figure 4.5D) caused by DEK loss. This suggests DEK and IRAK1 independently contribute to HNSCC cell survival and that targeting both may improve apoptosis efficacy. We used a dual approach of infecting stably transduced IRAK1 knockdown cells with adenovirus to deplete DEK (AdDEKsh). Either DEK or IRAK1 knockdown alone could induce apoptosis as expected, but the combined effect of IRAK1 shRNA with AdDEKsh infection was greater than that of the respective control cells (Figure 4.5E). Taken together, these data support a model wherein DEK and IRAK1 function in parallel pathways that control apoptosis, and highlight an additive relationship that may be beneficial for therapeutic intervention (Figure 4.5F).
Discussion

A majority of patients with head and neck squamous cell carcinoma present at advanced stages of disease, contributing to the poor survival outcomes observed. These tumors also notoriously recur, despite aggressive treatment modalities including surgery, chemotherapy and/or radiation therapy, that have frequent side effects that can dramatically and permanently decrease patient quality of life. This suggests these cells have a high proliferative and survival capacity that is necessary for sustained growth of these tumors. Therefore, understanding the relevant targetable mediators of these phenotypes is of the utmost importance. Here we addressed this clinical need by first profiling the transcriptome of HNSCC cell lines that are dependent on the DEK oncogene. DEK is an important regulator of HNSCC growth, and is up-regulated in >90% of primary HNSCCs tested to date. Although some transcriptional DEK targets have been described, the DEK-dependent transcriptome in squamous cell carcinomas remains unknown. Ontology analysis revealed biological processes significantly altered in the absence of DEK, including adhesion, differentiation, immune regulation, and development. This is in contrast to transcriptional data from neuroendocrine carcinoma of the lung with DEK loss. Shibata et al. revealed in their analysis that steroid metabolism, nucleosome assembly, and lipid synthesis and metabolism were altered most often in the absence of DEK. This suggests the effects of DEK loss in malignancies are cell-type dependent, which is similar to the divergence of gene expression observed in various normal cellular systems.

Here we have identified DEK-dependent gene expression that supports phenotypes previously defined, along with new systems of interest for future studies. Although DEK overexpression is published to promote the migration and invasion of breast and HNSCC cells, alterations in cellular adhesion have not been pursued. Our data suggests this may be one mechanism by which DEK promotes invasion. Furthermore, we have correlated the DEK-dependent transcriptome with common transcription factor binding sites that are associated with
DEK loss (Supplementary Tables S4.1-4.3). Many notable transcription factor sites and known targets of DEK were identified, including p53, CEBP, and p65 validating the importance of DEK in multiple cancer types. For example, control of p53 binding sites was expected given that DEK loss was reported to lead to the stabilization of p53.

This correlated with the up-regulated induction of several p53 regulated genes including BCL11B, KRT15, and PIM1. Herein we focused on the regulation of genes with roles in immune cell signaling. These included NF-κB and MAPK driven genes such as MAPK14, TNFAIP3, IL6, and IRAK1. We chose to probe the role of the IRAK1 serine/threonine kinase, a driver of inflammatory pathways in hematological disease, based on its function as a central signaling hub in the cytoplasm, and it is a targetable molecule in MDS and AML.

The role of IRAK1 in solid tumors has not been explored extensively, but our data suggest oncogenic and potentially targetable activities in HNSCCs. IRAK1 was transcriptionally up-regulated and amplified in a proportion of HNSCCs in the TCGA, in line with a newly discovered functional requirement for maximal survival of HPV positive and negative HNSCC cell lines. Such a role was uncovered through IRAK1 knockdown using stable lentiviral vectors, as well as through chemical inhibition. Downstream IRAK1 signaling was suppressed through TRAF6, attenuating activation of NF-κB and MAPK and stimulated cancer cell death, thus highlighting the potential use of IRAK1 inhibitors in the treatment of HNSCC.

Our data identified IRAK1 as a component of the DEK-dependent transcriptome whose expression in HNSCC contributes to tumor cell survival. IRAK1 drives ERK1/2 signaling, but this alone was not sufficient to rescue cell growth in the absence of DEK. This is not surprising given the large network of genes DEK regulates and suggests multiple genes are required to maintain a proliferative state. It is also possible that the ERK1/2 signaling may be unimportant or may modify the response to DEK loss. Although IRAK1 was not by itself sufficient to rescue DEK-deficiencies, the combined targeting of DEK and IRAK1 demonstrated an additive relationship.
This additive effect emphasizes the large network of signaling hubs through which DEK functions, independent of IRAK1, and which can be further explored from the transcriptional data in future experiments.

Importantly, this work has defined IRAK1 as one functionally important driver of HNSCC survival. Interestingly, tumor suppressive functions of IRAK1 have been proposed in a recent publication in oral squamous cell carcinoma cells (OSCC). Hung et al. described miR-146a, a known regulator of IRAK1, as overexpressed in OSCC. Other publications define miR-146a as tumor suppressive, where its loss hyper-activates IRAK1 and may be one mechanism for IRAK1 overexpression.\textsuperscript{248} In OSCC, exogenous miR-146a expression increased orthotopic tumors and metastasis of SAS cells and reduced IRAK1 protein levels. In these same cells, IRAK1 knockdown combined with TRAF6 knockdown by siRNA increased invasion and tumor volume, but IRAK1 knockdown alone had few effects.\textsuperscript{273} Here, we utilized oropharyngeal HNSCC cell lines wherein IRAK1 surprisingly exhibited oncogenic functions in this tumor type. These opposing findings may be related to the site of origin for each cell line, as their primary focus was oral cancers compared to the more encompassing HNSCC. Additionally, the TCGA data wherein IRAK1 is largely overexpressed in HNSCCs supports the hypothesis that IRAK1 contributes to oncogenic phenotypes.\textsuperscript{273} Many possibilities exist to explain these discrepancies. IRAK1 functions may be anatomically or cell line dependent or the method of inhibition may be important (acute (siRNA) versus stable (shRNA)). Additionally, IRAK1 expression may be a double-edged sword and careful balance of its expression must be maintained. TCGA data in other squamous tumors, such as cervical cancer, identified one patient with homozygous deletion of \textit{IRAK1} and two with truncated mutations. The other twenty cases where IRAK1 was altered were due to copy number amplification and mRNA up-regulation. Even in one HNSCC case from Figure 3, there is a patient with IRAK1 mRNA down-regulated. Overexpression versus loss of IRAK would then be a predictive marker of response to IRAK1 inhibitors and may be a fruitful biomarker for squamous cancers of multiple anatomical origins.
Materials & Methods

Cell culture. HPV negative UMSCC1 and UMSCC6, and HPV positive UMSCC47 head and neck cancer cell lines were cultured in DMEM (Gibco, New York, NY, USA) supplemented with 1% hydrocortisone (HPV' only), 10% fetal bovine serum, antibiotics and antifungals.

Lentiviral transduction. Cell lines were transduced with lentiviral pLKO.1 vectors for either nontargeting control shRNA (NTsh), IRAK1sh (TRCN0000000543, OpenBiosystems, Lafayette, CO, USA), or DEK832 (DEKsh, Sigma-Aldrich Mission shRNA library, St Louis, MO, USA) in the presence of polybrene (8 µg/mL). Cells were selected in puromycin at a final concentration of 1 µg/mL.

Adenoviral transduction. Cells transduced with control (NTsh) and IRAK1 knockdown (IRAK1sh) lentiviruses were plated in equal densities and kept under puromycin selection. 48 hours post-plating cells were transduced with control (AdGFP) or DEK knockdown (AdDEKsh) adenoviral vectors at 10 infectious units per cell as previously published. 210 72 hours post-transduction, cells and media were collected and fixed to analyze for flow cytometry (see below). IRAK1 and DEK knockdown were confirmed by western blot analysis.

IRAK1 overexpression. UMSCC1 cells were lentivirally transduced with control (LeGo-iG2) or IRAK1 overexpression (LeGO-IRAK1) vectors in the presence of 8 ug/mL polybrene. Cells were sorted based on GFP-positivity and expanded for experiments post-sorting. Creation of these vectors has been described previously. 274
cBioPortal Analysis. The results depicted here are in whole or part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov. For IRAK1 expression z-score thresholds were set at 2.0.

RNA-Sequencing. Transduced and selected UMSCC1 and UMSCC47 NTsh and DEKsh cells were collected and processed with a ZR RNA MiniPrep kit (R1064, Zymo Research, Irvine, CA, USA), per kit instructions. A portion of the final RNA isolate for each sample was submitted for quality assurance prior to RNA-Sequencing. RNA-Sequencing was performed by the CCHMC DNA Sequencing and Genotyping Core on an Illumina HiSeq2500 for single-end sequencing. (Will insert GEO accession number once data is uploaded).

GeneSpring NGS Analysis. RNA-Seq files were imported into GeneSpring Multi-Omic Analysis Software V12.6 (Agilent, Santa Clara, CA, USA) and sequences were aligned to the reference genome, hg19GFCh37, which efficiently aligns reads spanning known or novel splice junctions. The reference annotations were produced by the Ensembl project. Aligned reads were filtered on base quality, with a quality threshold >=30. The aligned gene read counts were quantified and used to compute reads per kilobase per million reads (RPKM) for each transcript in each sample. Raw counts were normalized using the DESeq algorithm and threshold set to 1. Subsequent filtrations removed all genes with fewer than 3 reads. Fold change was calculated as DEKsh/NTsh with a cut-off of 1.4. Venn diagrams were created in GeneSpring and entity lists were translated from UMSCC1. Gene lists were submitted to ToppGene (http://toppgene.cchmc.org) for ontological analysis.

Network Analysis: All of the network analyses and visualizations were performed using the NetWalker software and were described in detail previously.
Transcription factor binding site analysis. In order to identify enriched (p value <0.05) putative transcription factor binding sites within the up- and down-regulated genes, we mined the catalog of human, mouse, rat, and dog conserved regulatory motifs in promoters using the ToppGene server.\textsuperscript{278,280}

Quantitative RT-PCR. RNA was collected with Trizol (Invitrogen, Grand Island, NY, USA) and reverse transcribed to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). cDNA expression was detected with TaqMan Gene Expression Master Mix and probes (Applied Biosystems). Data was analyzed using the $\Delta\Delta$Ct method and values calculated relative to GAPDH. TaqMan probes were as follows: DEK (Hs01078267_m1), IRAK1 (Hs0108347_m1), and GAPDH (Hs02758991_g1).

Western blotting. Whole cell lysates were harvested using Laemmli buffer and a total of 20 $\mu$g of protein was analyzed as described previously.\textsuperscript{210} Membranes were probed with DEK (1:1000, BD Biosciences, San Jose, CA, USA), IRAK1 (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA (sc-7883)), IRAK4 (1:1000, Cell Signaling Technologies, Danvers, MA, USA (4363)), TRAF6 (1:1000, Santa Cruz (sc-7221)), phospho-IRAK1 (Thr209, 1:800, Assay Biotechnology Company (A1074), Sunnyvale, CA, USA), phospho-p38 (1:500, Cell Signaling Technologies (4361)), p38 (1:1000, Cell Signaling Technologies (9212)), phospho-ERK1/2 (1:1000, Cell Signaling Technologies (4377)), ERK1/2 (1:1000, Cell Signaling Technologies (4695)), phospho-IKK$\alpha$/\beta (1:500, Cell Signaling Technologies (2697)), IKK$\alpha$ (1:1000, Cell Signaling Technologies (2682)), actin (1:10,000 a gift from James Lessard), and GAPDH (1:1000, Cell Signaling Technologies (5174)).

Flow cytometry for Cleaved Caspase-3. Lentivirally transduced cells were plated at equal densities and collected 48 hours later. Cells were fixed and prepped following the BD FITC
Active Caspase-3 Apoptosis kit protocol (BD Biosciences). Adenovirally transduced cells were prepped with an Alexa-Fluor 647 conjugated cleaved-caspase 3 antibody to account for GFP-positivity (Cell Signaling Technologies (9602)). Analysis was performed on a BD FacsCanto and data analyzed on FlowJo software (Tree Star, Ashland, OR, USA). Experiments were performed 3 times with standard error of the mean (SEM) represented.

**Flow cytometry for cell cycle analysis.** Lentivirally transduced cells were plated in equal numbers and 48 hours later were pulsed with 10 µM BrdU for 45 minutes. Cells were collected and prepped following the BD Pharmigen APC BrdU flow kit and analyzed on a BD FacsCanto. Data was analyzed on FlowJo Software as above. Experiments were performed 3 times and SEM represented.

**IRAK1 inhibitor.** UMSCC1 cells were plated at equal densities and IRAK1 inhibitor (IRAK-1/4 inhibitor, I5409, Sigma-Aldrich, St. Louis, MO, USA) added the same day for caspase-3 flow cytometry experiments. DMSO was added in equal volume for a control. Cells and media were collected 72 hours later and prepared and analyzed as above. For the time-course experiment, cells were plated and inhibitor was added the following day, with protein collected at 24, 48, and 72 hours and analyzed by western blot for IRAK1 inhibition.

**Immunohistochemistry.** Primary human tissue specimens and staining protocol were previously described but with IRAK1 antibody (1:50, sc-7883, Santa Cruz). Images were captured with a Leica DM2500 microscope and LAS software (Leica Microsystems Inc., Buffalo Grove, IL, USA) at the indicated magnifications.

**TRAF6 Immunoprecipitation.** Samples were lysed using RIPA buffer containing protease and phosphatase inhibitors and protein concentration determined using BCA Protein Assay Kit
600 µg of each sample was used to perform TRAF6 immunoprecipitation. Lysates were incubated with A/G beads (sc-2003, Santa Cruz, Dallas, TX, USA) and incubated with TRAF6 antibody (sc-7221, Santa Cruz). Samples were loaded onto a 4-15% gradient Mini-PROTEAN TGX Precast Gel (BioRad, Hercules, CA, USA) and proteins separated by SDS-PAGE electrophoresis. Membranes were probed with ubiquitin primary antibody (sc-8017, Santa Cruz). Protein from the original lysis, prior to immunoprecipitation, was run following the above western blot protocol. Membranes were probed with IRAK1, TRAF6, and GAPDH. Densitometry was performed using ImageJ software.

**Growth Curves.** Control and IRAK1 overexpressing cells were plated at equal densities, in triplicate, and total cell number counted over 3 days. Experiments were performed twice with SD represented.

**Statistics.** Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA, USA). Student’s t-test was used to calculate p-values, where *=p≤.05 and **=p≤.01.

**Conflict of Interest.** The authors declare no conflict of interest.

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Figure 4.1. Profiling the DEK-dependent transcriptome in HNSCC. (a) UMSCC1 and UMSCC47 were lentivirally depleted for DEK, as confirmed by western blot analysis, and mRNA from these lines was submitted for RNA-Sequencing (RNA-Seq). (b) GeneSpring NGS analysis was performed on genes differentially expressed 1.4 fold or greater (DEKsh/NTsh). GeneSpring derived Venn diagrams depict genes altered in both cell lines and overlap highlights genes common to both UMSCC1 and UMSCC47. (c) Overlapping genes (from panel B, 2282) from UMSCC1 and UMSCC47 were analyzed on TOPPGene to identify significantly altered biological processes. The top 10 biological processes are represented. (d) A portion of the down-regulated gene networks analyzed on NetWalker highlights nodes containing IRAK1 and DEK in UMSCC1 cells. A full view of view can be found in Supplementary figure S4.2.
Figure 4.2. DEK regulates IRAK1 mRNA and protein levels. (a) Table depicts fold changes for DEK and IRAK1 in UMSCC1 and UMSCC47 from GeneSpring NGS analysis. (b, c, d) IRAK1 mRNA is reduced following DEK depletion. IRAK1 and DEK mRNA levels were confirmed by TaqMan PCR to validate RNA-Seq results in three cell lines: UMSCC1, UMSCC47, and UMSCC6 (HPV negative). Experiments were performed twice and standard deviation (SD) depicted. (e) Western blot analysis confirms IRAK1 protein levels and downstream signaling are also depleted in the absence of DEK.
**Figure 4.3. IRAK1 is overexpressed in primary HNSCC.** (a) cBioPortal analysis of the TCGA HNSCC database reveals IRAK1 alterations occur in 14% of HNSCC. A total of 279 samples were analyzed and were further broken down into HPV− and HPV+ subsets. (b) IRAK1 is highly expressed in primary HNSCC tissues. CCHMC-HNSCC1 (HPV+) and CCHMC-HNSC18 (HPV−) were stained for IRAK1 by immunohistochemistry, with intense staining in both the nucleus and cytoplasm. Images were taken at 5x and 20x magnification.
Figure 4.4. IRAK1 loss increases apoptosis in HNSCC. (a) IRAK1 loss attenuates activation of downstream signaling pathways in HNSCC. UMSCC1 and UMSCC47 were transduced with control (NTsh) or IRAK1 knockdown (IRAK1sh) vectors and protein was collected following selection in puromycin. Whole cell lysates were analyzed by western blot analysis to confirm IRAK1 knockdown, along with reduction in IRAK1 activation (pIRAK1Thr209) and MAPK pathways. GAPDH was used as a loading control. (b) IRAK1-inhibitor reduces IRAK1 activation in HPV- and HPV+ cell lines. UMSCC1 and UMSCC47 cells were plated and DMSO (control) or
IRAK1-inhibitor was added the following day at 1 µM or 10 µM final concentration. Cells were then collected for western blot analysis at indicated time-points. Inhibition of IRAK1 was confirmed by western blot analysis, as measured by phosphorylation of IRAK1. GAPDH was used as a loading control. (c, d) TRAF6 ubiquitination is reduced following IRAK1 loss. Immunoprecipitation was performed on RIPA lysates with the TRAF6 antibody. Western blot was then performed for ubiquitin. Remaining whole cell lysates were analyzed by western blot for TRAF6, IRAK1, and GAPDH. (e, f) IRAK1 loss increases cellular death via apoptosis. Cells were analyzed by flow cytometry for cleaved-caspase 3 conjugated to FITC. Experiments were performed in triplicate with SEM depicted. (g) IRAK1-inhibitor increases apoptosis. UMSCC1 cells were plated and DMSO or IRAK1-inhibitor was added after cells attached. Cells and media were collected 72 hours later and analyzed for cleaved caspase-3 by flow cytometry. Experiment was performed three times, with SEM depicted. (*=p<.05).
Figure 4.5. IRAK1 and DEK depletion cooperate to increase apoptosis. (a) IRAK1 overexpression rescues ERK1/2 signaling. Sorted control or IRAK1 overexpressing cells were transduced with control (NTsh) or DEK knockdown (DEKsh) vector. After selection was complete protein was collected and analyzed by western blot. GAPDH was used as a loading control. Growth curves of iG2 versus IRAK1 overexpressing cells can be found in Supplementary S4.8. (b) IRAK1 and DEK regulate cell growth and viability independently. Cells from (A) were used to analyze apoptosis (b), cellular cycle (c), and cellular proliferation. (d)
IRAK1 overexpression did not rescue the phenotypes observed with DEK loss. Experiments were performed twice and SD is represented. (e) Combined IRAK1 and DEK loss increases cell death. Control and IRAK1 knockdown cells were transduced with control (AdGFP) or DEK knockdown (AdDEKsh) adenovirus. Three days post-adenoviral infection cells were collected and later analyzed for cleaved caspase-3 by flow cytometry. Graph represents fold change compared to NTsh AdGFP samples. Experiments were performed twice and SD represented. (f) Molecular model. IRAK1 is a novel target in HNSCC. DEK is required for efficient expression of IRAK1 and DEK and IRAK1 also independently contribute to HNSCC cell survival and depletion of either promotes apoptosis and this may be a result of ERK1/2, p38 and/or NF-κB signaling.
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**Supplementary Table S4.1.** Transcription factor binding site analysis for genes up-regulated in UMSCC1 and UMSGCC47
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<td>Transcription factor binding site analysis for genes down-regulated in UMSCC1 and UMSCC47</td>
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<td><strong>Supplementary Table S4.2.</strong> Transcription factor binding site analysis for genes down-regulated in UMSCC1 and UMSCC47</td>
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Supplementary Table S4.3. Transcription factor binding site analysis for genes differentially expressed in both UMSCC1 and UMSCC47, but oppositely regulated (up vs. down).

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**Supplementary Figure S4.1.** Network analysis was performed with Netwalker software on UMSCC1 and UMSCC47 RNA-Seq data. Labeled Networks 1-5 can be seen in expanded views in Supplementary figures 2-6.
Supplementary Figure S4.2. Network 1 is represented, with genes down-regulated in UMSCC1, with log fold change represented. This is the expanded view of the network map highlighted in Figure 4.1.
Supplementary Figure S4.3. Network 2 is represented, with genes up-regulated in UMSCC47, with log fold change represented.
Supplementary Figure S4.4. Network 3 is represented, with genes down-regulated in UMSCC1, with log fold change represented.
Supplementary Figure S4.5. Network 4 is represented, with genes up-regulated in UMSCC1, with log fold change represented.
Supplementary Figure S4.6. Network 5 is represented, with genes up-regulated in UMSCC47, with log fold change represented.
Supplementary Figure S4.7. UMSCC1 and UMSCC47 cells with IRAK1 knockdown reveals no alterations in cell cycle profiles. BrdU incorporation was measured by flow cytometry to determine G1, S, and G2/M populations. Experiments were performed in triplicate.
Supplementary Figure S4.8. UMSCC1 IRAK1 overexpression cells (IRAK1) do not exhibit cell growth difference over controls (iG2). Cells were plated at equal densities and counted over three days. Experiments were performed in duplicate.
Chapter 5: Discussion and Future Directions
Functions of DEK and IRAK1 in HNSCC

Identifying novel biomarkers or targets for cancer therapies is of the utmost importance for improving patient outcomes in poorly understood tumor types and advanced stage malignancies. Among these cancer types is head and neck squamous cell carcinoma, where patients often present at advanced stage disease and have an average predicted 5-year survival rate of 40-50%. Additionally, genomic profiling of HNSCC has not fulfilled the promise of many new targetable molecules. Our work aimed to identify new oncogenes and signaling pathways of importance in HNSCC that have the potential to translate into new therapeutic interventions. Based on previous studies and the literature, we hypothesized a protein of interest, DEK, would be functionally implicated in regulating HNSCC phenotypes. Our approach utilized targeting DEK with shRNA to determine the effects of DEK loss in HNSCC. We focused experiments on both HPV+ and HPV- HNSCC subsets to determine if the effects of DEK loss were similar among these subtypes. Although HPV+ patients have improved survival, late stage disease among all HNSCC patients remains a problem. Parallel methods were employed to 1) define the functional role for DEK in HNSCC in vitro and in vivo and 2) determine the global transcriptional effects of DEK loss.

Our work has uncovered two oncogenes, DEK and IRAK1, which are important for the growth and survival of HNSCCs. The first portion of our work demonstrated the in vitro and in vivo consequences of DEK loss in head and neck model systems (Chapter 3). Transgenic K14E7 mice were crossed with Dek/- mice and used in an established mouse model of head and neck cancer. We found K14E7 Dek/- mice had prolonged survival and significantly fewer macroscopic tumors compared to K14E7 Dek+/+ mice. However, all mice still formed microscopic tongue tumors, suggesting Dek loss inhibits tumor progression but not tumor initiation in this chemically and genetically initiated model. It would be interesting to determine in similar 4-NQO mouse experiments, whether tumor development in the Dek/- mice is delayed but
can still occur after the 24-week experimental end-point or whether such tumor development is permanently inhibited. To further define the contributions of DEK to HNSCC tumorigenesis, our laboratory has generated a transgenic mouse model wherein inducible DEK-overexpression was targeted to the epidermis. These mice will be utilized for 4-NQO driven head and neck cancer development in order to determine whether DEK over-expression is sufficient to stimulate tumor growth. In this mouse model, a tetracycline response element regulates two bi-directional CMV promoters that control the expression of DEK and luciferase, respectively. The mice were crossed with tetracycline transactivator (tTa) keratin 5 (K5) mice to drive DEK over-expression to the basal layer of mouse epithelium. Importantly, the over-expression of DEK can be repressed with the addition of doxycycline to either the food or drinking water. This allows for determination of the potential therapeutic impact whereby DEK loss is predicted to cause tumor regression.

We built on previous tumor microarray studies that suggested DEK is overexpressed in HNSCC. To determine whether DEK gene expression translated into protein accumulation, a panel of twenty primary HNSCC specimens, largely oropharyngeal, was evaluated for DEK protein expression. The data was then correlated with HPV status and patient demographics. The results of these studies revealed that DEK is highly overexpressed even at the earliest stages of HNSCC development and that expression levels were independent of HPV status or patient characteristics. We also validated the use of p16 as a surrogate marker for HPV positivity. One limitation of these studies was the exclusive focus on oropharyngeal specimens; therefore, future studies should determine how DEK expression compares in other sites defined as head and neck cancer, including oral cavity, laryngeal and nasopharyngeal.

The final portion of this project utilized in vitro methods to profile DEK knockdown in established and primary head and neck cancer cell lines. DEK loss attenuated cellular proliferation and growth of these cells and ΔNp63 was implicated as a relevant downstream DEK effector. DEK does not appear to regulate ΔNp63 transcription, suggesting post-
transcriptional modes of regulation. DEK may regulate ΔNp63 protein stability, and future work should determine the exact mechanism. We speculate Fbxw7, an E3 ubiquitin ligase that is mutated in some HNSCCs, may be a potential candidate as it induces degradation of both DEK and p63. Furthermore, levels of another p63 variant, TAp63, are increased during apoptosis suggesting DEK may also regulate this functionally antagonistic isoform. Mechanisms of regulation for both isoforms, and the relevance of TAp63 regulation should be evaluated for their contributions to other DEK-dependent phenotypes.

The second approach profiled the impact of DEK loss on transcription in HNSCC cells (Chapter 4). We utilized the same cell lines and knockdown strategies as described above to identify the DEK-dependent transcriptome by RNA-Sequencing. Differentially expressed genes common to the HPV+ and HPV− cell lines were analyzed to identify novel mediators of DEK-dependent phenotypes. These screens revealed gene signatures associated with inflammatory pathways and functional studies focused on an upstream regulator of immune signaling and transcription, IRAK1. This gene was consistently repressed upon DEK knockdown and its function in HNSCC is unknown. IRAK1 drives inflammatory signaling downstream of toll-like and interleukin-1 receptors and can activate multiple signaling cascades. IRAK1 mRNA and protein repression were validated upon DEK loss, with both decreased as predicted. Data mined from The Cancer Genome Atlas found IRAK1 is altered, and predominantly up-regulated, in 14% of HNSCCs. Utilizing the primary human tumor specimens from our earlier work, we confirmed IRAK1 is expressed in human HNSCCs. Beyond melanoma, this is the first report in solid tumors to our knowledge wherein IRAK1 is implicated as an oncogene. To determine the functional role of IRAK1 in HNSCC, knockdown was performed and validated by the observed repression of TRAF6-ubiquitination and attenuated downstream signaling. Importantly, IRAK1 repression by knockdown or chemical inhibition stimulated apoptosis. Lastly, we assessed if IRAK1 overexpression could rescue the effects of DEK loss. Although DEK loss reduced cell number, cell cycle progression, and increased apoptosis, IRAK1 overexpression could not
reverse these phenotypes. This occurred despite a rescue of ERK1/2 activation following IRAK1 overexpression and DEK depletion, thus indicating that ERK1/2 reconstitution is not sufficient for rescue from DEK loss. However, the combined inhibition of IRAK1 and DEK resulted in significant cell killing over the knockdown of either alone. Taken together, our work has identified DEK and IRAK1 as independent contributors to HNSCC growth and survival.

**Functional and therapeutic avenues for future studies**

This dissertation work has established DEK as an important regulator of HNSCC growth and proliferation and has uncovered a new role for IRAK1 in HNSCC survival. Additional follow-up of the DEK knockdown transcriptome results should reveal other functionally important regulators of tumor phenotypes. Many other interesting targets were identified that may also contribute to DEK-dependent phenotypes. These include IL6, a cytokine well published for its roles in inflammation in head and neck cancer.\(^{283}\) Transcriptome profiling of additional cell lines, and particularly primary cell populations, is beneficial to determine global gene expression consequences in response to DEK loss. In targeting of DEK genetically, we have taken the first necessary steps to assess the potential therapeutic value of chemical or genetic DEK inhibition. Whether DEK over-expressing compared to control cells will exhibit inverse regulation remains to be determined.

Although we have established that DEK is required for the expression of IRAK1, it remains unknown if such regulation is direct or indirect. Based on the well accepted role of DEK in transcription (Chapter 2), DEK may be a co-factor at IRAK1 promoters or DEK loss may alter chromatin topology in a way that hinders IRAK1 transcription. There is little published work on the regulation of IRAK1, implicating DEK as a potential transcriptional control mechanism. One well-published regulator of IRAK1 expression is miR-146a. Most often, miR-146a is tumor suppressive and its loss results in hyperactivation of IRAK1. A published report by Hung \textit{et al.} suggests miR-146a functions oppositely in oral carcinoma and is oncogenic, thus suppressing
IRAK1. Since our data demonstrates IRAK1 is oncogenic in HNSCC, future studies should pursue the dual role of miR-146a mediated IRAK1 expression and function in our model system.

Western blot analysis revealed a reduction in NF-κB and MAPK activation upon the loss of IRAK1; however, activation should be measured in more detail and with the use of luciferase reporters. DEK loss also resulted in reduced MAPK signaling, but we have not obtained clear results for how DEK impacts NF-κB. DEK is published to inhibit NF-κB signaling through interactions with p65 in MEFs, but mechanisms whereby DEK affects NF-κB signaling in transformed cells remain uncertain. While IRAK1 rescued ERK1/2 signaling in the absence of DEK, we have yet to define phenotypic consequences of this rescue, if any. We have published DEK overexpression stimulates invasion of breast and HNSCC cells, and this is a potentially relevant and unexplored phenotype that may result as a consequence of DEK and IRAK1 activation.215

Since DEK or IRAK1 loss alone stimulates apoptosis and their dual inhibition increases this further, it is possible they may be useful therapeutic targets. Currently, no DEK inhibitors exist, but IRAK1 inhibitors are available and have proven useful in recapitulating the phenotypes observed with shRNA depletion. IRAK1 has already been published as a treatment strategy for MDS/AML and studies are ongoing to further improve this inhibitor for clinical use. In order to pursue IRAK1 as a candidate therapy in HNSCC, mouse studies will be imperative. Monitoring HNSCC xenograft growth in the presence or absence of the IRAK1 inhibitor will be the first step in determining if turning off this pathway is therapeutically relevant through prevention and/or remission of established tumors.

During the course of patient treatment many drugs are often given simultaneously. Therefore, continuing in vitro cell line studies with IRAK1-inhibitors in combination with the currently used treatment modalities for HNSCC will determine if there is improved killing efficacy
in the absence of IRAK1 signaling. Of critical importance will be defining how normal cells respond to IRAK1 inhibition, as that may indicate undesired consequences of targeting IRAK1.

The pursuit of DEK as a therapeutic target will be more difficult. This molecule has no known enzymatic activity and a complete crystal structure has yet to be reported. In the interim, domains and residues most crucial to DEK-dependent phenotypes in cancer may inform the best targeting strategies. Currently, DEK is a useful biomarker in other cancers. A clinical trial is underway (ID: NCT01563796) to measure DEK protein levels in the urine of patients suspected of having bladder cancer as a potential diagnostic tool. This ELISA technique may prove fruitful for other cancer types and work is ongoing in other laboratories to detect DEK in the serum of breast and head and neck cancer patients. Beyond these clinical considerations of DEK as a biomarker or drug target, the observed complete penetrance of DEK overexpression in HNSCC suggests the oncogene is central to the development of this malignancy. As such, future studies to identify the full complement of DEK activities in HNSCC may identify other targets and thus additional avenues for the development of next-generation therapies.

Summary

This dissertation has defined the consequences of targeting DEK and/or IRAK1 in HNSCCs. We uncovered two proteins important for HNSCC survival that have not previously been reported in the head and neck cancer literature. Our work suggests that co-targeting of these proteins may be a fruitful therapeutic avenue. Future studies should better define the structural properties of DEK in the hope that small molecule inhibitors can be identified for clinical purposes. Further assessment of the contribution of DEK and IRAK1 to HNSCC development will be crucial to better understanding their roles in this malignancy.
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