University of Cincinnati

Date: 3/20/2017

I, Marie C Mattrka, 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: Oncogenic Phenotypes Induced by Overexpression of the DEK Proto-oncogene

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Oncogenic Phenotypes Induced by Overexpression of the DEK Proto-oncogene

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

Doctor of Philosophy (Ph.D.)

in the Department of Cancer and Cell Biology of the College of Medicine March 2017

by

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Abstract

The DEK gene encodes a nuclear protein that binds chromatin and is involved in various fundamental nuclear processes including transcription, RNA splicing, DNA replication and DNA repair. Several cancer types including head and neck squamous cell carcinoma (HNSCC) characteristically overexpress DEK and its overexpression is associated with poor prognosis for certain cancers. Our reported in vitro and in vivo models of SCC have demonstrated that DEK contributes functionally to cellular proliferation and survival and tumor growth. However, the underlying molecular mechanisms remain poorly understood.

An ontology analysis of gene transcripts whose expression patterns are tightly correlated with that of DEK in cancer, revealed an enrichment of genes involved in mitosis. Further investigation of DEK in cell division by immunofluorescence analyses discovered that DEK dissociates from DNA in early prophase and re-associates with DNA during telophase in human keratinocytes. Interestingly, DEK overexpression stimulated its own aberrant association with chromatin throughout mitosis, and was sufficient to increase micronuclei formation. Therefore, the overexpression of DEK and its incomplete removal from mitotic chromosomes can promote the generation of genetically abnormal daughter cells.

HPV oncogenes have also been shown to affect mitosis and cause chromosome instability. This is a relevant topic due to HPV being causal for some HNSCCs and HPV positive HNSCC cell lines are used throughout the following DEK studies. A review of the mechanisms and effects of HPV on cell division are discussed.

Using another global approach to identify DEK functions that relate to oncogenesis, we found DEK expression was necessary for the transcription of several metabolic enzymes based on published RNA sequencing experiments in HNSCC cell lines. To investigate the effects of DEK expression on metabolism we used Seahorse analysis and NMR-based metabolomics approaches in keratinocytes and HNSCC cells. Seahorse analysis revealed that DEK increased baseline and maximum glycolytic rates, and increased the maximum oxidative phosphorylation.
rate. NMR-based metabolomics studies demonstrated that high levels of DEK expression reprogrammed cellular metabolism and altered the presence of amino acids, tricarboxylic acid cycle (TCA) intermediates and the glycolytic end products lactate, alanine and NAD⁺. Taken together, these data support a scenario whereby DEK overexpression reprograms keratinocyte metabolism to fulfill energy and macromolecule demands required to enable and sustain cancer cell growth.

Lastly, we generated a tetracycline responsive Dek transgenic mouse model (Bi-L-Dek) to study the effects of Dek overexpression in vivo for the first time. Dek has been shown to be required for benign and malignant tumor growth in Dek-/− mice but the effects of overexpression have not been reported. Bi-L-Dek mice were bred to mice expressing tetracycline transactivator (tTA) under control of the keratin 5 promoter (K5-tTA). The resulting Bi-L-Dek_K5-tTA offspring express Dek and luciferase in the basal keratinocytes of stratified squamous epithelium and expression is repressed by doxycycline. We have validated the Dek overexpression system and subjected Bi-L-Dek_K5-tTA mice to 4NQO, a carcinogen that induces HNSCC susceptibility. A complete analysis is in progress. The results thus far suggest Dek increases proliferation of basal keratinocytes and the occurrence of gross esophageal tumors.
Preface

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


Acknowledgements

This work is in dedication to my late friend Andy Caress who died at age 24 from melanoma. Andy filled my life with joy and laughter and he was my inspiration for pursing studies in cancer and cell biology. My drive has been to advance cancer research to improve treatments that could save someone else’s Andy.

Many people along the way have helped me tremendously with my dissertation work. First and foremost is my mentor Dr. Susanne Wells (Susa) at the Cincinnati Children’s Hospital Medical Center. Susa has been extremely supportive on both a scientific and personal level. She encouraged me to start new projects and collaborations, and exuded contagious enthusiasm every day. It was her constant support and faith in me that enabled me to be successful. Susa, you have led by example on how to be a good role model, colleague to others, and most of all, a good human being, thank you.

Thanks to my thesis committee members Drs. David Plas, Paul Rosevear, Peter Stambrook, Susan Waltz, and Yi Zheng. My thesis committee has kept me on track and made sure I was focused on relevant projects, experiments, and future directions. Their guidance has been essential throughout my graduate studies.

The people who got me through every day were my co-workers in the Wells’ laboratory. The Wells lab has been a supportive environment full of fun, entertainment, encouragement, meaningful discussions and friendship. Thank you to everyone in the Wells lab who has helped me along the way and made it all worthwhile.

Lastly, thanks to my friends, family, and loved ones. It is in having a strong foundation that has enabled me to get where I am and where I’m going.
Table of Contents:

Oncogenic Phenotypes Induced by Overexpression of the DEK Proto-oncogene .......... 1
   Abstract .................................................................................................................. 2
   Preface .................................................................................................................. 5
   Acknowledgements ............................................................................................... 5

Table of Contents: ...................................................................................................... 7

Chapter 1: An introduction to the versatile DEK oncogene ...................................... 9
   DEK is overexpressed in cancer ............................................................................. 9
   Genetic background and structure: ...................................................................... 9
   DEK is highly post-transcriptionally modified ..................................................... 10
   DEK Functions ...................................................................................................... 12
      DEK binds and regulates DNA and chromatin topology .................................. 12
      DEK positively and negatively regulates gene transcription .............................. 13
      DEK binds to RNA and is involved in RNA splicing and mRNA translation ....... 13
      DEK is located at DNA replication forks .......................................................... 14
      DEK is essential for proper and timely DNA repair .......................................... 14
   Effects of DEK loss and overexpression ............................................................... 15
      DEK loss leads to cell death and decreased tumor growth .............................. 15
      DEK overexpression contributes to transformation and cell growth in human cells ........................................................................................................ 16
   Routes to DEK overexpression in cancer ............................................................... 17
   Molecular pathways affected by DEK overexpression ......................................... 17
   DEK is overexpressed in head and neck squamous cell carcinoma ..................... 19

Chapter 2: DEK overexpression promotes mitotic defects and micronucleus formation ......................................................................................................................... 21
   Abstract .................................................................................................................. 22
   Introduction ............................................................................................................ 23
   Results .................................................................................................................... 26
   Discussion .............................................................................................................. 31
   Methods & Materials ............................................................................................ 33
   Acknowledgements ............................................................................................... 40
   Figures .................................................................................................................... 41
   Supplemental Figures ............................................................................................ 56

Chapter 3: HPV Virology: Cellular Targets of HPV Oncogenes and Transformation: Mitosis and Chromosomal Instability ..................................................... 64
   Mitosis and Chromosomal Instability .................................................................. 64

Chapter 4: Overexpression of the human DEK oncogene reprograms cellular metabolism and promotes glycolysis ................................................................. 69
   Abstract .................................................................................................................. 71
   Introduction ............................................................................................................ 72
   Results .................................................................................................................... 74
   Discussion .............................................................................................................. 80
   Materials and Methods ........................................................................................ 84
   Acknowledgements ............................................................................................... 91
   Figures .................................................................................................................... 92
   Supplemental Figures ............................................................................................ 102
Chapter 5- Generation of a tetracycline responsive *Dek* transgenic mouse model for HNSCC studies. ................................................................. 109
Abstract .............................................................................................................. 110
Introduction ........................................................................................................... 111
Results ................................................................................................................... 112
Discussion .............................................................................................................. 117
Materials and Methods ........................................................................................ 117
Acknowledgements ............................................................................................... 123
Figures .................................................................................................................. 124
Supplemental Figures ............................................................................................ 134

Chapter 6: Conclusions, discussion and future directions .................................... 137
Newly discovered roles for the DEK oncogene in cell division and metabolism ....... 137
Future studies investigating specific metabolic pathways affected by DEK overexpression ................................................................. 139
Future in vivo studies in Dek transgenic mice ...................................................... 142
Targeting Dek in cancer ......................................................................................... 143

References Cited .................................................................................................. 144
Chapter 1: An introduction to the versatile DEK oncogene

DEK is overexpressed in cancer

DEK was originally identified as a fusion gene with NUP214 in a patient with acute myeloid leukemia (AML) (t6:9)(p23;q34) (1). The DEK-NUP214 fusion gene is found in 1% of acute myeloid leukemia and is sufficient to promote cellular proliferation and transformation (2, 3). Since its original discovery, DEK has also been found to be increased in AML types that do not harbor the DEK-NUP214 fusion (4-6). It also is overexpressed in many solid tumors including colon (7, 8), breast (9), ovarian carcinomas (10), bladder cancer (11), retinoblastoma (12), neuroendocrine carcinoma of the lung (13), pancreatic (14), lung (15, 16), neuroendocrine prostate cancer (NEPC) (17), and skin cancer (18, 19). Studies have demonstrated the oncogenic role of DEK through its capacity to promote cellular transformation, survival and proliferation (18, 20-22). The involvement of DEK in carcinogenesis has also been recognized by its ability to inhibit apoptosis, cellular differentiation and senescence (23-26). Recently, different studies have also demonstrated the role of DEK in tumor progression, as the expression of DEK was a factor associated with poor prognosis in patients with hepatocellular carcinoma, gastric adenocarcinoma, small and non-small cell lung carcinoma, colorectal cancer, neuroendocrine prostate cancer, pancreatic cancer, breast cancer and melanoma (9, 14-17, 27-31). The gain-of-function of DEK by its overexpression seemingly provides a selective advantage for tumor development and progression, given it is upregulated in many human cancer types.

Genetic background and structure:

DEK is a highly conserved protein that is found in metazoans and higher eukaryotes. It is a unique, 375 amino acid protein with little homology to other proteins. The human DEK gene is located on chromosome 6p 22.3, contains 12 exons, and is ubiquitously expressed. It has no
enzymatic activity and presumably carries out its function through two conserved DNA binding domains, the SAF/SAP (scaffold attachment factor-box), and the DEK C-terminal DNA binding domain (32-34). While the protein has an overall basic charge, it contains several acidic regions and a putative nuclear localization signal (NLS) (Figure 1.1). The SAP domain is a DNA binding domain that is likely involved in chromatin organization and is also capable of binding RNA. The C-terminal DEK DNA binding domain was first identified in DEK and harbors a DEK multimerization domain enabling DEK to oligomerize with itself (34). Not surprisingly, DEK is predominantly a nuclear protein that binds to both DNA and RNA and other proteins, and participates in numerous nuclear activities and less well defined extra-nuclear functions.

DEK is highly post-transcriptionally modified

DEK contains 67 lysines, 42 serines, 19 threonines, and 5 tyrosines, offering over 100 sites for post-translational modifications. Modifications of these amino acids can affect protein interactions, gene transcription, DEK localization, and its function. DEK has been shown to be phosphorylated, acetylated, ubiquitinated, PARylated, ADP ribosylated and likely SUMOylated. In vitro, phosphorylation decreased the affinity of DEK for DNA and stimulated DEK multimerization with itself (35, 36). It also induced DEK interactions with the FBXW7 (F-box/WD repeat-containing protein 7) and U2AF (U2 Small Nuclear RNA Auxiliary Factor) proteins.

Figure 1.1 Schematic of the DEK oncogene including acidic regions, the NLS, and its two DNA binding domains with their known functions identified below. The numbers above represent the amino acid sequence of DEK.
leading to DEK degradation, and altered RNA splicing respectively (37, 38). The main kinase identified in regulating DEK DNA-binding activity is casein-kinase 2 (CK2), but DEK also harbors consensus phosphorylation sites for PKC, GSK-3, and aurora B kinase (35, 39). DEK can be ubiquitinated by FBXW7 and SPOP (speckle-type pox virus and zinc finger protein) ubiquitin ligases leading to DEK degradation (40). While it is unknown whether DEK is sumolyated it is suspected to be by SUMO1 given that DEK carries a consensus motif recognized by SUMO1 and is found in PML (promylocytic leukemia) nuclear bodies which generally require a SUMO-interacting motif (41, 42). Acetylation of DEK by CBP, p300, and P/CAF also decreases the affinity of DEK for DNA and acetylation can re-localize DEK to interchromatin granules (ICGs), which contain RNA-processing and transcription factors (43, 44). DEK is also PARylated by PARP1, a modification common for DNA repair proteins. PARylation promotes DEK-DEK interactions and causes DEK to enter the cytoplasm (45). ADP ribosylation of DEK is another DNA repair signaling modification and it along with phosphorylation and acetylation, occurs during cell death to release DEK from chromatin and into the extracellular space (46, 47). These modifications alone and in conjunction can lead to the displacement of DEK from DNA although little is known about the precise mechanisms in each case. Interestingly, in patients with juvenile idiopathic arthritis (JIA), extracellular modified DEK is recognized by autoantibodies present in their synovial fluid (48, 49). Furthermore, these autoantibodies preferentially recognized acetylated DEK over the phosphorylated form of DEK. Circulating DEK autoantibodies have been identified in other autoimmune diseases such as lupus erythematosus and sarcoidosis and are thought to alert the immune system. Therefore these modifications can have secondary effects thus deepening DEK’s regulation of cellular processes (50-53). In all, post translational modifications affect the ability of DEK to bind DNA and proteins, and alter the localization and function of DEK. While many modifications have been discovered, it is unclear which modifications are responsible for specific functions of DEK including those involved in oncogenesis.
DEK Functions

*DEK binds and regulates DNA and chromatin topology.*

Evidence suggests that DEK maintains nuclear architecture similar to the high motility group (HMG) proteins that also contain regions enriched in acidic amino acids. Biochemical assays have shown that DEK changes chromatin topology by introducing positive supercoils into SV40 mini-chromosomes and assembles DNA and histones into chromatin (54). DEK can also reduce the super-helical density of chromatin and DNA, which is a chromatin dependent phenotype requiring H2A/H2B and H3/H4 dimers (55). Earlier studies suggested DEK may bind to DNA with some sequence specificity, particularly with a TG rich element found in the HIV-2 enhancer to promote transcription (56). However, more extensive studies revealed DEK can bind DNA independent of sequence and has a preference for supercoiled and cruciform DNA (54). Chromatin immunoprecipitation and DNA sequencing (Chip-seq) analysis revealed DEK preferentially binds open chromatin with a low degree of methylation (57). In the same study, DEK was located at the transcriptional start sites of highly transcribed genes with a similar binding pattern to RNA polymerase II, which transcribes mRNAs. This is in concordance with immunofluorescence imaging and immunoprecipitation data showing accumulation of DEK in regions of transcriptionally active euchromatin and excluded from heterochromatin (58, 59). Conversely, DEK has also been implicated in the preservation of heterochromatin. DEK interacts with HP1 and the loss of DEK reduced the repressive histone mark H3K9me3 (60). Strengthening the role of DEK as a mediator of heterochromatin integrity, in human cells, DEK promotes H3.3 incorporation into chromatin at the telomeric and centromeric regions of chromosomes (42). While these data may seem contradictory, they are in line with diverse roles of DEK in DNA and chromatin organization and accessibility. In general, the evidence suggests that DEK binds all areas of DNA and chromatin and supports distinct functions in transcriptionally active versus dormant loci.
DEK positively and negatively regulates gene transcription.

It is no surprise from the localization and interactions of DEK with DNA and chromatin that it would be involved in the regulation of gene expression. Several studies have reported the ability to DEK to both promote and inhibit transcription. The previously mentioned Chip-seq study found that, while DEK is at the transcription start site of highly transcribed genes, it was able to activate or repress transcription at these sites. In support of this work, previous data found DEK counteracts transcriptional activation by SET, NFκB, P/CAF and p300, and is found in a repression complex with Daxx and HDACII (45, 61-63). DEK also represses hTERT transcription in leukemia, where hTERT is often under-expressed (44). On the other hand, DEK is a coactivator of U2AF and the *Drosophila* ecdysone receptor, and enhances the transcriptional activity of AP-2α, EKLF, and C/EBPα, and it accumulates during transcriptional activation of the CR2 gene (37, 58, 64-67). While DEK likely regulates transcription through its ability to modulate chromatin topology, the underlying mechanisms remain poorly defined.

DEK binds to RNA and is involved in RNA splicing and mRNA translation.

DEK has been shown to form a splicing dependent interaction with exon-product complexes and is involved in proofreading RNA splice sites (68). It does so by interacting with U2AF, an RNA binding protein, when phosphorylated on S19 and S32. This interaction enhances AG dinucleotide discrimination by U2AF, part of a consensus splice site, to enforce specific localization of RNA splice sites (37). In addition to RNA splicing, DEK is enriched at internal ribosome entry sites (IRES), linking DEK to protein translation (69). DEK has been shown to promote the IRES-dependent translation of LEF1, a pro-invasive transcription factor in breast cancer (70). These data suggest DEK may be a central factor in bridging chromatin and mRNA processing with consequences on protein translation.
DEK is located at DNA replication forks.

Another nuclear function of DEK is resolving DNA and/or chromatin structures at replication forks that may inhibit DNA replication or cause DNA double strand breaks (DSB) (71). DEK has been shown to counteract DNA replication stress due to DNA damage by promoting replication fork progression. This activity may give a proliferative advantage to DEK overexpressing cells.

DEK is essential for proper and timely DNA repair.

In addition to transcriptional control of gene expression, one of the most likely, primary endogenous functions of DEK is DNA repair. Post-translation modifications of DEK suggest it is involved in DNA repair and many roles have been identified for DEK in non-homologous end joining (NHEJ), homologous recombination (HR), and protection from genotoxic agents. One study showed that treatment with radiomimetic drugs and topoisomerase poisons led to prolonged DNA damage in Jurkat and HeLa cells with DEK knockdown (47). In another study, DEK loss sensitized human and mouse cells to DNA damage and in Dek knockout mouse embryonic fibroblasts (MEFs), DNA repair by non-homologous end joining (NHEJ) was significantly reduced (72). This was supported by delayed Ku70/80 localization to double-strand breaks and reduced DNA-PK signaling upon DEK loss. Unpublished data from our lab have uncovered that DEK is essential for homologous recombination through DNA repair assays in Dek-/– MEFs and in HeLa cells knocked down for DEK. Lastly, the C-terminus of DEK, amino acids 309-375, was sufficient to partially rescue fibroblasts taken from a patient with ataxia telangiectasia (AT) from three AT phenotypes including mutagen sensitivity, hyper-recombination, and radio-resistant DNA synthesis (73). Together, these data support a role for DEK in DNA repair pathways.
The characteristics of DEK, including its DNA and RNA binding domains, its localization, and its interaction with chromatin, allow it to be involved in the many aforementioned nuclear processes. However, despite significant knowledge derived from its association with multiple cellular functions, a principal biological function for DEK remains elusive.

Effects of DEK loss and overexpression

*DEK loss leads to cell death and decreased tumor growth.*

Numerous studies inhibiting DEK expression have shown a significant increase in cellular apoptosis or senescence through p53-dependent and independent mechanisms in HeLa cells and through transcriptional repression of myeloid cell leukemia 1 (MCL-1) protein in melanoma cells (24, 74, 75). While the mechanism is different in different cell types, DEK expression is important for cell survival. Cell death in response to DEK loss is more pronounced in rapidly proliferating cancer cells compared with normal, differentiated cells. This suggests that dividing cancer cells are dependent upon high levels of DEK expression (22). Interestingly, full body Dek knockout mice (*Dek−/−*) are alive and fertile with no overt phenotype. However, unpublished data suggests these mice are smaller in size and have deregulated hematopoiesis. The causes of these phenotypes are not yet understood. *In vivo* data obtained in the *Dek−/−* mice suggest DEK may be involved in cancer initiation and tumor promotion. Compared to *Dek+/+* mice, *Dek−/−* mice had delayed onset of papilloma formation and decreased number of papillomas when treated with DMPA and TPA a tumor initiator and promoter respectively, suggesting a role for DEK in tumor initiation (22). In another experiment, human papilloma virus (HPV) E7 oncogene transgenic mice, when treated with 4-nitroquinoline 1-oxide (4NQO), a cancer initiator and promoter, developed large oral and esophageal tumors. However, HPV E7_ *Dek−/−* mice only developed microscopic tumors suggesting Dek plays a role in tumor growth (20).
**DEK overexpression contributes to transformation and cell growth in human cells**

DEK is more highly expressed in proliferating cells while non-proliferating differentiated cells have severely diminished or absent DEK expression. Forced overexpression of DEK suppresses quiescence in muscle cells, causes a shift in keratinocytes from a differentiated state to a proliferative state, and suppresses senescence in cancer cells (25, 26). Furthermore, in human keratinocytes, DEK contributes to cellular transformation when expressed along with HRAS and the HPV E6 and E7 oncogenes (22). The cells had increased potential to form colonies in soft agar and form tumors when transplanted into mice. Interestingly, in this study, no differences in colony formation or tumor growth were observed when DEK was expressed with E6 or E7 oncogenes alone. This suggests DEK overexpression is not sufficient to replace the complete inhibition of the Rb family of proteins as E7 does, nor can DEK repress p53 function. Converse to mammalian cell studies, overexpression of human DEK in the drosophila eye caused a rough-eye phenotype due to caspase-9- and -3-mediated apoptosis and was accompanied by histone H3 and H4 hypo-acetylation (76). Therefore, while it is clear that DEK overexpression in normal epithelia promotes transformation at least in human cells, the drosophila study suggests there may be detrimental effects of high DEK expression (13, 21, 22). In support of this, it is difficult to force DEK overexpression above physiological levels, especially in cancer cells (data not shown), where DEK levels are already high. It remains unknown whether this is due to the degradation of excessive DEK mRNA or protein, or apoptosis of the overexpressing cell population (77). No matter the mechanism to prevent high DEK expression, cancer cells find a way to overcome this block and become dependent on high DEK expression.
Routes to DEK overexpression in cancer

The overexpression of DEK in cancer cells occurs through various methods. One such mechanism is amplification of the chromosome 6p22 region where DEK is located. This region is amplified in many tumor types, such as bladder cancer, retinoblastoma, triple negative breast cancer, lung cancer, and osteosarcoma (78-84). There have been reported mutations in ubiquitin ligases responsible for DEK degradation such as FBXW7 in colorectal cancer and SPOP ubiquitin ligase in prostate cancer (38, 40). Mutations in microRNAs (miRNA) responsible for degrading DEK mRNA have also been detected including mir592 in HepG2 hepatocellular carcinoma cells and mir200a in pancreatic ductal carcinoma (85, 86). DEK overexpression can be the effect of increased transcription by upstream regulators such as E2F, NF-Y, YY-1, and ERα all of which are commonly activated in cancers (7, 87, 88). Due to DEK being an E2F target gene, DEK is highly expressed in HPV positive tumors in which the HPV E7 oncogene inhibits all retinoblastoma proteins, releasing E2F to bind the DEK promoter (20, 25). Thus DEK expression in cancer is increased through gene amplification, defects in proteins that regulate its degradation, and transcriptional upregulation.

Molecular pathways affected by DEK overexpression

Through multiple mechanisms, DEK can regulate the activity of various signal transduction pathways that are implicated in tumorigenesis. These include p53 and ΔNp63 to inhibit apoptosis and promote proliferation, Wnt/B-catenin signaling to drive invasion and cellular proliferation, VEGF to promote angiogenesis, Rho signaling to induce migration, mTOR to activate cellular proliferation and NFkB to promote cell survival. Specifically, DEK decreases p53 protein levels and upregulates the epithelial stem cell marker ΔNP63 to promote cell proliferation (20, 24, 25). DEK regulates the Wnt/B-catenin pathway by increasing Wnt10b expression in neuroendocrine carcinomas of the lung and in breast cancer cells (13, 89).
Furthermore DEK expression promotes nuclear localization and activity of B-catenin, driving phenotypes associated with Wnt signaling and cancer such as cellular migration and invasion in breast cancer lines. DEK was also found to activate VEGF transcription, a protein associated with angiogenesis in tumors, through HIF1a dependent and independent mechanisms in breast cancer lines, and promoted cell proliferation, migration and angiogenesis (90). In non-small cell lung cancer DEK depletion reduced active RhoA-GTP levels providing a possible mechanism for the resulting inhibition in cellular migration (91). When the DEK-NUP214 fusion protein found in AML was expressed in myeloid cells, it increased mTORC1 activity leading to increased proliferation that was inhibited with an mTOR inhibitor (92). However, this last study did not determine if overexpression of DEK in place of the fusion protein could cause these same phenotypes.

DEK is also linked to inflammation repression or activation depending on the model system and the level of DEK expression. In support of DEK suppressing immune function, DEK interacts with RelA/p65 and represses NFκB transcriptional activity that can be reversed with TNFα treatment in HeLa cells (62). DEK overexpression has also been shown to repress transcription of NFκB target genes like 1-cys peroxiredoxin in HeLa cells (26, 93). Concurrently, DEK knockdown relieved transcriptional repression of NFκB in HeLa and Caski cervical cancer cells (26). Conversely, in HNSCC cells DEK knockdown decreased IRAK1 transcription, a regulator of inflammatory signaling, and promotor of cell survival (94). In summary, the evidence indicates that DEK can repress NFκB immune signaling in numerous cell types, but is an activator of IRAK1 transcription in HNSCC. Therefore DEK affects inflammatory signaling to possibly mediate cellular proliferation, differentiation, and survival and the migration of immune cells linked to the immune response.
DEK is overexpressed in head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common malignancy worldwide with 600,000 new cases per year, and over 50,000 new cases per year in the United States. The causes for HNSCC are tobacco and alcohol use as well as infection with the human papillomavirus (HPV), which accounts for at least one-quarter of all HNSCCs and is a number on the rise (95). Surgery and high dose chemo-radiation are the standard of care, often causing irreparable facial disfiguration and the loss of the ability to speak, chew or swallow. Although, the 5-year disease free survival rate has increased to ~66% over the past two decades, novel therapeutic targets and biomarkers are necessary to improve patient quality of life and survival rates (96).

A recent publication from our lab showed DEK is frequently overexpressed in HNSCCs. In fact, of 21 primary HNSCC tumor specimens analyzed for DEK-specific immunohistochemistry, all harbored high levels of DEK compared to surrounding normal tissue (20). This data, along with previously mentioned data about Dek expression necessary for overt HNSCC tumor formation in HPV E7+ mice, and DEK loss preferentially killing cancer cells, DEK becomes an attractive therapeutic target for HNSCC.

Studying the effects of DEK overexpression and its advantages for cell growth:

Most studies of DEK have involved its knockdown to identify either endogenous roles of DEK, or effects of DEK loss in cancer cells. DEK knockdown and knockout studies have proven that DEK plays a role in tumorigenesis through cell growth and survival. However few studies have defined tumorigenic effects of DEK via its overexpression. My thesis work has sought to identify how the overexpression of DEK at early and late stages of carcinogenesis gives cells a proliferative advantage. In three separate studies of DEK overexpression, I have uncovered new functions for DEK overexpression in cell division and metabolic regulation, and generated a new mouse model for future studies.
In the second chapter, I discuss how DEK overexpression in immortalized and transformed cells disrupts mitosis, a tightly regulated process involving the alignment and separation of chromosomes to ensure proper cell division. It is not hard to imagine that this process would require a protein like DEK that can interact with DNA, chromatin, and proteins to coordinate this well-choreographed process. We find DEK overexpression leads to its inappropriate retention on the DNA and an increase in micronuclei that represent mitotic abnormalities. In chapter three, I further discuss mitosis and the disruptive effects of HPV viral protein expression on this process.

In chapter four, the effects of DEK overexpression on the metabolism of non-transformed and transformed keratinocytes are described. Previously published RNA sequencing data from head and neck squamous cell carcinoma (HNSCC) cell lines knocked down for DEK revealed that DEK regulates the transcription of multiple metabolic enzymes. No studies have investigated the effects of DEK overexpression on cellular metabolism; therefore we employed NMR-based metabolomics on DEK overexpressing cells compared to control cells. We found DEK increases the rate of glycolysis and can increase the maximum rate of oxidative phosphorylation in non-transformed keratinocytes and in HNSCC cells. Furthermore, DEK increased the production of lactate, alanine, and NAD+, all products of glycolysis. From this study we conclude that DEK overexpression is sufficient to deregulate cellular metabolism.

Lastly, in chapter 5, I discuss the generation of an inducible Dek transgenic mouse model that will greatly benefit the field. Dek overexpression was confirmed and mice were subjected to head and neck cancer development. The data report that Dek overexpression increases the incidence of gross tumor development. In all, my work has identified that DEK overexpression contributes to tumorigenic phenotypes at the earliest stages of cancer through mitotic defects and metabolic deregulation, and contributes to HNSCC tumor growth. Going forward, a Dek transgenic mouse model now exists to investigate the effects of DEK overexpression in tumor development and other diseases.
Chapter 2: DEK overexpression promotes mitotic defects and micronucleus formation

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Keywords:
DEK, mitosis, aneuploidy, micronuclei, mitotic defects, chromosome instability, cancer

Abbreviations:
NIKS: Near-diploid immortalized keratinocytes; SCC:squamous cell carcinoma; CCHMC:Cincinnati Children's Hospital Medical Center; IF: Immunofluorescence; 7AAD: 7-aminoactinomycin D; MSO: Mitotic shake off; NM: Non-mitotic

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Published in Cell Cycle, 2015 May 6:1-15. DOI: 10.1080/15384101.2015.1044177. PMCID: PMC4825741
Abstract

The DEK gene encodes a nuclear protein that binds chromatin and is involved in various fundamental nuclear processes including transcription, RNA splicing, DNA replication and DNA repair. Several cancer types characteristically overexpress DEK at the earliest stages of transformation. In order to explore relevant mechanisms whereby DEK supports oncogenicity, we utilized cancer databases to identify gene transcripts whose expression patterns are tightly correlated with that of DEK. We identified an enrichment of genes involved in mitosis and thus investigated the regulation and possible function of DEK in cell division. Immunofluorescence analyses revealed that DEK dissociates from DNA in early prophase and re-associates with DNA during telophase in human keratinocytes. Mitotic cell populations displayed a sharp reduction in DEK protein levels compared to the corresponding interphase population, suggesting DEK may be degraded or otherwise removed from the cell prior to mitosis. Interestingly, DEK overexpression stimulated its own aberrant association with chromatin throughout mitosis. Furthermore, DEK co-localized with anaphase bridges, chromosome fragments, and micronuclei, suggesting a specific association with mitotically defective chromosomes. We found that DEK overexpression in both non-transformed and transformed cells is sufficient to stimulate micronucleus formation. These data support a model wherein normal chromosomal clearance of DEK is required for maintenance of high fidelity cell division and chromosomal integrity. Therefore, the overexpression of DEK and its incomplete removal from mitotic chromosomes promotes genomic instability through the generation of genetically abnormal daughter cells. Consequently, DEK overexpression may promote the development of oncogenic mutations in cells and cancer initiation.
Introduction

Mitosis is a tightly regulated process to ensure daughter cells obtain a complete, diploid set of chromosomes. Mitotic defects can lead to aneuploidy caused by chromosome segregation errors resulting in a loss or gain of one or more chromosomes (97). Aneuploidy is a remarkably common characteristic of tumor cells and has long been proposed as one of the first events to initiate tumorigenesis (98, 99). Mitotic defects that cause aneuploidy usually lead to cell death; however, some cells continue to divide and pass on genetic abnormalities leading to an unstable karyotype and chromosome instability (100, 101). Over the course of time, this chromosome instability can lead to the creation of an immortalized, proliferative, and highly metastatic population of cells (102). Thus, these mitotic abnormalities potentiate selection for a genetic advantage that supports tumorigenesis (103).

Aneuploidy is caused by mitotic defects including centrosome amplification, a compromised mitotic check point, and abnormal mitotic spindle assembly (104, 105). Daughter cells that arise from these defective mitoses may end up hypodiploid or hyperdiploid, depending on whether they lost or gained a chromosome(s) respectively. Hyperdiploidy is more tolerated in cells and is often caused by problems with cytokinesis. Hypodiploidy, however, is a more common event (106-108) but often results in cell death (109). These malsegregated chromosomes at anaphase may not end up in the nucleus of either daughter cell but remain extra-nuclear. These chromosomes eventually become enclosed by a nuclear membrane to form either nuclear buds or micronuclei. Micronuclei originate from chromosome fragments or whole chromosomes that fail to be incorporated in the daughter nuclei. This can result from lagging chromosomes in anaphase or unrepaired or misrepaired double-stranded DNA breaks (104). Micronuclei are biomarkers of genotoxic events and chromosomal instability. Nuclear buds resemble micronuclei in that they are associated with chromosomal instability, but remain connected to the nucleus by nucleoplasmic material (104). Nuclear buds are thought to represent a mechanism by which cells remove amplified DNA and are therefore considered a
marker of possible gene amplification (110). De-regulation of proteins involved in various stages of mitosis, including chromosome condensation, sister chromatid separation, and spindle assembly checkpoints, are common causes of chromosome instability and carcinogenesis (97).

Multiple studies have shown that proteins that bind to and regulate chromatin structure during interphase also play important roles in mitosis. Examples include the high mobility group (HMG) box containing Structure Specific Recognition Protein (SSRP) family and the heterochromatin protein alpha (HP1α). These proteins are important for microtubule growth, the separation of sister chromatids and the completion of mitosis (111, 112). DEK is a protein similar to the HMG, SSRP and HP1 families in that it functions as a chromatin topology modulator. DEK is a unique, non-histone, chromatin-associated protein that is evolutionarily conserved in higher eukaryotes, and represents the only member of its family. DEK can non-enzymatically bend DNA to introduce positive supercoils (54) and functions as a histone 3.3 chaperone both in Drosophila (113) and human cells (42). It also is involved in the maintenance of heterochromatin by directly interacting with HP1α to enhance its binding to H3K9Me3 (114). DEK is overexpressed in numerous solid tumors through various mechanisms including increased transcription via the Rb/E2F pathway, (7) 6p22.3 amplifications (12, 115, 116), steroid hormone signaling, (117) and mutations in its degradation machinery (38, 118). DEK overexpression can inhibit cell death and differentiation as well as promote proliferation, and invasion in multiple types of cancer (22, 25, 75, 119, 120). During interphase, DEK plays a role in numerous nuclear processes including transcription, RNA processing, chromatin organization, DNA repair, and the maintenance of heterochromatin (34, 62, 64, 114, 121, 122). DEK is a highly modified protein, containing 57 potential phosphorylated sites and over 70 lysine residues with the potential to be poly ADP-ribosylated and ubiquitinated (35, 38, 43, 118, 123). Such modifications alter its ability to bind chromatin, re-localize, and carry out specific biological functions in a manner that remain poorly understood and have been studied primarily in cancer cells wherein DEK is overexpressed (77). For example, DEK is phosphorylated by
CK2 during interphase and phosphorylation levels appear to peak in G1 (35). *In vitro*, CK2 phosphorylation reduces the affinity of DEK for DNA and increases DEK self-multimerization (35).

Previous studies have suggested that DEK associates with mitotic chromatin, potentially in a stage-dependent manner. For instance, using immunofluorescence and metaphase spreads, Kappes et al. 2001 observed that DEK is detectable on HeLa cell chromatin during metaphase and anaphase but appears to be absent in prometaphase. Two other reports confirm DEK association with chromatin in telophase cells (124, 125), with one showing DEK absence from metaphase cells (125). Mass spectrometry of isolated mitotic chromosomes localizes DEK to the chromosome arm (126), but did not distinguish between the various stages of mitosis and may be a result of telophase association. Together, this data suggests that DEK localization may be regulated in cancer cells throughout mitosis. Such regulation, however, has not yet been closely examined in non-transformed cells, and, furthermore, the effect of cancer-related DEK overexpression on mitotic cells remains unknown.

Here we report, and build on, cancer-related ontology analyses of DEK which suggest major associations for DEK expression and mitotic processes. To determine the regulation of DEK during mitosis in non-transformed cells, which harbor relatively low DEK expression levels, we utilized a near-diploid immortalized keratinocyte cell line (NIKS) (127) and murine embryonic fibroblasts (MEFs). We found that endogenous DEK is largely absent from mitotic chromosomes from prophase through anaphase, but re-associates in telophase. Importantly, DEK overexpression leads to aberrant chromosomal retention of DEK during all stages of mitosis, and concomitantly increased the formation of micronuclei and hypodiploid populations. Micronuclei are strongly associated with mitotic errors and are a clinically relevant hallmark of cancer (128-132). We posit that DEK overexpression in normal cell populations contributes to mitotic abnormalities as a first step towards chromosomal instability, aneuploidy, and selection for oncogenic clones.
Results

The nuclear DEK oncogene is absent from mitotic chromosomes.

In order to evaluate the relationship of DEK to other genes and biological processes across a broad series of biological contexts, we carried out gene expression profile analysis to identify genes whose expression was coordinately regulated with that of DEK. To do this we used 2158 tumor biopsy samples that had been subjected to gene expression microarray analysis by the International Genome Consortium Cancer Expression Profile project (Supp. Table 2.I). Somewhat unexpectedly, genes whose expression was very similar to that of DEK (Pearson correlation >0.485; 307 probesets), were highly enriched with respect to functional involvement in the mitotic cell cycle (Fig. 2.1A). This association indicated a potential relationship of DEK function with mitosis. To explore this, we used immunofluorescence to determine DEK localization throughout mitosis in NIKS cells, a near-diploid spontaneously immortalized keratinocyte cell line that harbors low DEK expression levels. (25) While DEK is known to bind chromatin constitutively during interphase, we noted its marked absence from DNA during certain phases of mitosis (Fig. 2.1B-C). Specifically, DEK was not associated with chromatin from prophase through anaphase but was associated during telophase. DEK co-localized with chromosomes (DAPI) in over 95% of cells in telophase but in less than 10% of cells in anaphase (Fig. 2.1D). This was confirmed using three separate DEK antibodies (Supp. Fig. 2.1A-B), a finding which suggests that DEK dissociates from chromatin early in mitosis and re-associates prior to nuclear membrane formation.

DEK protein levels are drastically reduced in mitotic cells.

Since DEK was largely absent from DNA during mitosis, we investigated its regulation at the protein level in cells that were either chemically synchronized or enriched for mitotic cells by mitotic shake-off. Asynchronous NIKS were compared to cells synchronized with mimosine or serum starvation for arrest in G1, with thymidine and aphidicolin for arrest in S, and with
nocodazole for arrest in G2/M. Cells obtained from mitotic shake-off (MSO) were compared to their respective adherent control cells referred to as non-mitotic. Arrest in the predicted phase of the cell cycle was verified by flow cytometry in each case (Fig. 2.2A), with the proportion of cells in G1, S and G2/M quantified in Fig. 2.2B. Interestingly, while DEK protein levels were relatively constant upon G1 and S arrest as previously reported (133), DEK protein levels decreased dramatically in mitotically enriched cells following mitotic shake off (Fig. 2.2C). This was confirmed with three other DEK antibodies (data not shown). G2/M arrest with nocodazole also decreased DEK protein but to a lesser extent as would be expected due to fewer cells arrested in G2/M (Fig. 2.2A-B). It is likely the small amount of DEK remaining in the MSO is from cells in telophase. This data suggests that in early prophase, DEK is degraded or otherwise removed from cells.

**Overexpression of DEK stimulates its aberrant retention on mitotic chromosomes.**

DEK is highly expressed across multiple human tumor types, and expression ontology data in Fig. 2.1A links DEK overexpression to mitosis. We therefore set out to determine the consequences of DEK overexpression on its dynamic chromatin binding pattern during mitosis. To this end, DEK was overexpressed in NIKS using a previously published (25) retroviral vector shown to be functional in vitro (122). DEK was expressed to a level comparable to that observed in cancer cells (Supp. Fig. 2.2A). A DEK protein-DNA co-localization was then monitored by immunofluorescence microscopy. Interestingly, protein overexpression stimulated DEK association with prophase, metaphase and anaphase chromosomes, when compared to the corresponding empty vector transduced cells (Fig. 2.3A-D). DEK overexpression in R780-DEK transduced cells was verified by western blot analysis (Fig. 2.3C). Taken together, DEK overexpression induces abnormal DEK retention on mitotic chromosomes.
A DEK-GFP fusion protein hyper-associates with mitotic chromosomes.

To follow DEK localization during cell division spatially and temporally, we utilized a DEK-GFP fusion protein for live cell imaging (Fig. 2.4A). Expression levels of the DEK-GFP fusion protein are shown in Fig. 2.4B. Interestingly, we found that compared to endogenous DEK protein (Fig. 2.1D), the DEK-GFP fusion protein more frequently associated with DNA throughout mitosis by IF (Fig. 2.4C) and live cell imaging (Fig. 2.4D and Supp. video 2.1). This association was detected both by viewing the DEK-GFP protein directly and using a DEK antibody (Aviva Systems Biology) (Supp. Fig. 2.3A). The observed chromatin retention of DEK-GFP may be due, in part, to the overexpression of the fusion protein. Of interest, we noticed that cells with robust DEK-GFP expression frequently exhibited mitotic defects such as lagging chromosomes and anaphase bridges (Fig. 2.4D, Supp. Fig. 2.3B, and Supp. Movie 2.2 - 2.3). Therefore, the retention of DEK on chromatin throughout mitosis may be detrimental to the fidelity of cell division.

DEK expression that is sufficient to cause aberrant mitotic localization induces micronucleus formation.

Since exogenous expression of DEK stimulates its inappropriate association with mitotic chromosomes it is possible a threshold level of DEK is required for this effect. To determine this, we utilized Dek−/− mouse embryonic fibroblasts either control transduced or complemented retrovirally with low or high levels of human DEK protein. Cells were sorted by low or high IRES-driven GFP expression (Fig. 2.5A), and DEK protein levels were verified by western blot analysis (Fig. 2.5B). Low GFP detection correlated with low DEK expression (DEKLo) and high GFP detection correlated with high DEK expression (DEKHi). As was observed with endogenous DEK, we found DEK in DEKLo expressing MEFs is generally absent from mitotic chromosomes until telophase, and always bound to DNA in interphase (Fig. 2.5C). However, the DEKHi MEFs showed significant increases in DEK association with chromatin in prophase, metaphase and
anaphase compared to DEK\textsuperscript{Lo} MEFs suggesting DEK is not as efficiently removed from the chromatin in these cells (Fig. 2.5E). Furthermore, we observed DEK association with lagging genetic material in the DEK\textsuperscript{Hi} expressing MEFs (Supp. Fig. 2.4A) similar to that observed in NIKS expressing the DEK-GFP fusion protein (Fig. 2.4D). We also consistently observed DEK co-localization with micronuclei and nuclear buds (Fig. 2.5F). This suggested that aberrant retention of DEK on mitotic chromosomes due to high DEK expression may result in mitotic defects. Therefore, we investigated if DEK levels sufficient to cause DEK retention on mitotic chromosomes resulted in the formation of micronuclei. To this end micronuclei were counted by coverslip IF and of significance, DEK\textsuperscript{Hi} expressing cells had an increased incidence of micronuclei compared to empty vector and DEK\textsuperscript{Lo} expressing cells (Fig 2.5G). Together this data supports that high DEK expression promotes aberrant chromatin binding throughout mitosis and leads to mitotic defect that result in micronucleus formation.

Since micronuclei develop from abnormal chromosome segregation during mitosis, the more rounds of cell division, the more chances to develop micronuclei. With regard to DEK, its overexpression is known to increase proliferation in certain scenarios, and this could potentially increase micronuclei indirectly by driving cell division. To consider this scenario, we examined if acute DEK overexpression was sufficient to induce micronuclei formation in the absence of proliferative gains. We utilized a doxycycline inducible GFP-DEK fusion construct (Fig 2.6A). Of note, GFP is fused to the N-terminus of DEK and unlike DEK-GFP has chromatin association patterns similar to that of wild type DEK (Supp. 2.5A). The cells were treated with doxycycline for 48 hours to express GFP-DEK (Fig. 2.6B). This brief period of DEK overexpression was sufficient to increase micronucleus formation by ~1.5 fold as quantified by coverslip IF (Fig. 2.6C) and flow cytometry (Supp. Fig. 2.5B). Importantly, the increase in micronuclei formation occurred in the absence of increased proliferation (Fig. 2.6D) thus ruling out more cell divisions as the cause of micronucleus formation and suggesting a more direct effect by DEK. Lastly, the GFP-DEK protein was detected in a mitotic shake off (Fig. 2.6E) suggesting a portion of this
fusion protein is retained on mitotic chromosomes presumably due to crossing the threshold level of DEK that can be removed from chromosomes prior to prophase.

**DEK overexpression increases the incidence of hypodiploidy and micronuclei in cancer cells.**

Our ontology analysis links DEK expression patterns to mitotic genes across various cancer types. Therefore, to determine the effects of DEK overexpression on mitosis in transformed cells, we used the CCHMC-HNSCC1 (134) cell line which was cultured from a head and neck squamous cell carcinoma and harbors relatively low DEK expression (Supp. Fig 2.2A). DEK overexpression was driven from the R780 vector as before (Fig. 2.7A), and did not alter cell cycle profiles in the respective population (Fig. 2.7B). Next we employed a flow cytometry based assay to quantify micronuclei in R780-DEK versus R780 cells. Viable cells were lysed, releasing nucleated DNA and micronuclei (Fig. 2.7C-D). Nuclei are analyzed for DNA content; thus aneuploidy including hypodiploidy and hyperdiploidy, as well as micronucleus formation can be clearly identified and distinguished from each other. Less than 5% of all events were micronuclei in the empty vector control cells (Fig. 2.7E and G). However, DEK overexpression significantly increased micronucleus formation to ~7% (P value 0.036) (Fig. 2.7F-G), approaching the percentage observed by that of known micronuclei inducing drugs (Supp. Fig 2.6A-B). Interestingly, DEK also increased the hypodiploid population of cells by more than 2 fold (P-value 0.0004) which is comparable to that of colcemid, a known aneugen (Fig. 2.7H and Supp. Fig. 2.6A). Hypodiploidy is a characteristic consequence of aneugen exposure which causes missegregation of chromosomes, often by interfering with the mitotic machinery, and results in aneuploidy. Therefore, DEK overexpression may be acting as an aneugen and increases both the incidence of hypodiploidy and micronucleus formation in cancer cells.
Discussion

The DEK oncogene has been implicated in multiple oncogenic phenotypes including tumor initiation, promotion and progression. However, molecular studies of DEK oncogenicity have mostly focused on roles of DEK in interphase cells, where DEK is constitutively bound to chromatin, and functions in transcription, replication and DNA repair (26, 44, 45, 62, 65, 75, 93, 113). In this study we investigated the regulation of DEK in mitosis and found that endogenous DEK is removed from chromatin in early prophase and re-associates with chromatin in telophase as outlined in Fig. 2.8A. Importantly, DEK overexpression leads to its aberrant retention on chromatin throughout mitosis (Fig. 2.8B) and contributes to mitotic defects and chromosome instability in normal and transformed cells.

Previous studies of DEK expression and localization in mitosis agree with our findings that DEK co-localizes with DNA during telophase (124, 125, 133). However, we find that DEK is mostly absent from chromosomes in prophase, metaphase and anaphase. This finding is in contrast to a previous report showing DEK bound to metaphase chromosomes (131). This discrepancy may be due to previous results being obtained from cells being treated with nocodazole, compared to our studies carried out in untreated cells. In support of this, nocodazole treated cells harbored increased DEK or GFP-DEK protein levels when compared to cells after mitotic shake off (Fig 2.2D, Fig. 2.6G). Furthermore, we have observed instances wherein endogenous DEK was retained on mitotic chromosomes, and these may also be related to DNA damage. While nocodazole clearly arrests cells in mitosis it is also known to cause DNA damage (135-138), and DEK contributes to DNA damage repair (122). Thus DEK may be retained on chromatin in nocodazole treated cells in order to support the DNA damage response and repair. In fact, the dynamic pattern of DEK localization in mitosis resembles that of many proteins involved in DNA damage repair which are removed from chromatin during mitosis such as Rad51, DNA-PK, FAND2 and Bloom complex components (139-142).
It is important to point out that while the majority of DEK is absent from mitotic chromosomes until telophase, a greater proportion of the fusion proteins, and particularly the C-terminal DEK-GFP fusion, were in fact prominent on mitotic chromosomes. This increased retention is likely due to the overexpression of DEK. However we cannot rule out the possibility that the GFP fusion is more directly involved in causing aberrant retention, or conversely, amplifies the small proportion of DEK that remains on the DNA during mitosis under normal circumstances. Regardless, high levels of wild type DEK protein expression increase its retention on mitotic chromosomes compared to low or endogenous levels, suggesting saturation of unknown mechanisms of DEK dissociation or removal. Perhaps an abundance of DEK causes DEK aggregates which exhibit greater affinity for DNA or precludes appropriate post-translational modification (124). While mitosis-related DEK modifications are unknown, a large number of proteins are phosphorylated during mitosis for subsequent relocalization or degradation (143). DEK is known to be phosphorylated by CK2 in interphase cells and DEK phosphorylation decreases its affinity for DNA. While CK2 phosphorylates some substrates in mitosis, DEK is not known to be one of them (144-147). DEK also carries predicted phosphorylation sites for both aurora kinase A and B as well as polo like kinases which regulate proper entry, progression and exit from mitosis and may be regulating the removal of DEK from chromosomes.

Irrespective of potential regulation by mitotic kinases, DEK overexpression leads to aberrant chromatin retention and increases mitotic defects and the formation of micronuclei (Fig. 2.8b). This occurred in both non-transformed fibroblasts as well as in a head and neck squamous cell carcinoma line, and was independent of cellular proliferative differences. The enhanced capabilities of the flow cytometry micronuclei detection assay also enabled the detection a hypo-diploid population of cells. An increase in hypodiploidy is a characteristic of aneugens which interfere with chromosome segregation resulting in chromosomal gain or loss. Therefore, high DEK expression is expected to contribute to chromosome instability by
ultimately stimulating hypodiploidy, a form of aneuploidy. Critically, this effect was demonstrated to occur upon short term induction of DEK, suggesting DEK overexpression may directly impact chromosome segregation in mitosis. While most hypodiploid cells succumb to cell death, hypodiploidy can potentiate the development of a select subpopulation of cells with enhanced survival capacity and thus aggressive behavior. Taken together, the dynamic DEK association with mitotic chromatin supports functional roles in this process, and DEK overexpression is sufficient to cause mitotic abnormalities that are hallmarks and causes of cellular transformation and carcinogenesis.

**Methods & Materials**

**Cell culture:**

MEFs derived from *Dek+/+* and *Dek−/−* mice were previously described (122). MEFs were maintained in high glucose DMEM (Lifetechnologies) containing 10% FBS, 2mM L-glutamine (Cellgro) 100 mM MEM non-essential amino acids (Lifetechnologies). 0.055mM b-mercaptoethanol (Midwest Scientific) and 10 mg/ml gentamycin (Lifetechnologies). Spontaneously immortalized near-diploid human keratinocyte cell line (NIKS)(127) were maintained in F-media, which is three parts Dulbecco’s modified Eagle’s medium to 1 part Ham’s F12 media (Lifetechnologies) supplemented with the following components: 5% fetal bovine serum, 24ug/ml adenine, 8.4 ng/ml cholera toxin (Millipore) 10ng/ml epidermal growth factor (Sigma), 2.4 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin, 1% penicillin-streptomycin (Lifetechnologies), and 0.2% fungizone (Omegascientific). CCHMC-SCC1 head and neck cancer cells were cultured from a tumor obtained with IRB approval at the time of surgical resection.(134) Cells were placed on irradiated J2-3T3 mouse fibroblasts and were maintained in F-media. Once robust growth was observed they were removed from feeders and adapted to and maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 1% hydrocortisone, 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.2% fungizone.
**EdU incorporation and cell-cycle analysis by flow cytometry:**

NIKS were grown to 70–80% confluency in 6 well plates and pulsed with 10 mM EdU for 2 hours before collection by trypsinization. Cells were prepared using the Click-iT® EdU Alexa Fluor® 647 Imaging Kit (Lifetechnologies) according to manufacturer specifications. DNA content was determined using 7-AAD (BDBiosciences) to stain DNA and cells were analyzed on a BD FACSCanto analyzer (BD Biosciences, San Jose, CA).

**Cell synchronization:**

500,000 cells were plated in 6-well plates and synchronized at various stages of the cell cycle: For G1 arrest, cells were treated with mimosine (Sigma), 400uM, 24 hours, or placed in serum free F-media with no insulin, adenine or EGF for 48 hours. For S phase synchronization cells were place in thymidine (Sigma), 2ug/ml, 12 hours followed by release for 10 hours followed by either another round of thymidine or aphidicolin (Fisher Scientific), 500uM for 12 hours. Aphidicolin was also used alone. For mitotic arrest, cells were treated with nocodazole (Sigma), 2ug/ml, 24 hours or colcemid (Lifetechnologies), 20nM, 16 hours, or subjected to a mitotic shake off.

**Mitotic shake off:**

Cells were grown to 80% confluency on 15 cm plates then washed with PBS. NIKS were treated with 0.05% trypsin (Lifetechnologies) for 1-5 minutes until mitotic cells appeared loosely attached. Cells were rinsed with PBS and the floating cells were collected, centrifuged and lysed as described below for western blot or RNA analysis. The non-mitotic cells, which remained attached to the plate, were trypsinized with 0.25% trypsin (Lifetechnologies), collected and lysed as the control cells. MEFs were treated as above except trypsin exposure was limited to 30 seconds in 0.05% trypsin.(148)
**Plasmid and viral constructs and transductions:**

A retroviral R780 vector was used to overexpress human DEK, and cells were sorted on GFP expression from an IRES signal as previously published.(22) The pLentilox vector is lentiviral and contains full-length DEK with GFP fused to the C-terminus and was a gift from Ferdinand Kappes and previously described (114) The pTRIPZ vector is a doxycycline inducible vector containing full length DEK with GFP fused to the N-terminus. First, the tRFP cassette of the pTRIPZ vector was replaced by a new MCS, where the eGFP cDNA derived from the pEGFP-N1 vector was inserted via the new Xhol/SnaBl sites. Next, the DEK cDNA was cloned into the pTRIPZ-EGFP vector, digested with EcoRI/SnaBl via a three-fragment ligation: DEK bp 1-471 was isolated by restriction digest of the pGEX4T1-DEK vector using EcoRI/NsiI; DEK bp 472-1125 was produced from the pGEX4T1-DEK vector via PCR using the primer pair 5’-GATGCTTAAGCTTCTCGAGCCACCATGGTGAGCAAGGGCGAGGCGAG-3’ and 5’-CCGGTACGTAGCCGCGCTCAAGAAATTAGCTCTTTTACAG-3’ and digestion with NsiI/SnaBl. The resulting pTRIPZ-EGFP-DEK plasmid was analyzed by sequencing. Cells were transduced at 50% confluence. For retroviral infections, cells were incubated with virus for 4 hours in medium containing 2 mg/ml of polybrene (Sigma), then washed and overlaid with fresh medium. Transduced cells were sorted for GFP and expanded as a polyclonal population. Lentiviral infections were conducted in the same way except that the cells were incubated with virus for 16 hours and selected and maintained in 1ug/ml puromycin as previously described.(114) Doxycycline (Sigma) was added to media for the indicated times at 1ug/ml. The MIEG-His-Flag-DEK (GenScript Piscataway, NJ) construct was developed from the pMIEG3 retroviral vector, a kind gift from Dr. David Williams (Children’s Hospital Boston, MA), and has been described earlier.(149) The EcoRI-His(6)-FLAG-DEK-XhoI sequence, CCGGAATTCCGGCGGCCCACCATGCATCACCATCACCATCACATCGACTACAAGGACGAC GATGACAAGATGTCCGCTCGGCCCCTGCTGCGGAGGGGGAGGGAACCCCCACCCAGCC CGCGTCCGAGAAGAACCAGAATGCCCAGGACAGGAGGAGCGAGAGGAAGAGG
ACGAGGACGACGAGGAGGAGGAGGAGGAAAAAGAGAGTCTCATCGTGGAAGGC
AAGAGGAAAAAGAAAAAGTAGAGAGCTTGAATGCAAGTCTTCTTTACAGAGAG
CATTTACAATTGCACAAGGAAGGGGCAGAAAACATTGTGAAATTGAGAGGATACATTTTTT
CTAAGTAAGAAAGAAAAACCAGATGAATTGAAATCTACACAAAAACTGCTTCTCAAACAGGCAG
GCACCTGTGTCCTCATTTAAGAAAGATGTTGGGTCAGTTCTAGGCTTTCCATTTGAAAAGG
AAGTGTCCAAATTAAAAAGGAAGGAAGAATTGTGAAAAAATTTAGAAATGCGATGTTAAAGA
GCATCTGTGAGGTTCTTGGATTTGGAGAGATCAGGTGTAAAATAGTGAACTAGTGGAAGAGGAT
CTTGAAATTTCTTAATGCATCCAAAGCCTTCTGGCAAACACTTGCCGAAATCTAAAAACTT
GTAGCAAAAGGCAGTAAAAAGGAACGGAACAGTTCTGGAATGGCAAGGAAAGGCTAAGCGAA
CCAAATGCTCTGAAATTCTGTGACTGATGAATCTAGTAGTGAAGATGAAAGAAGAAACAGCC
GAAGAGTCTTTGACGATGAAAGATAAAAGAGGAGGAGGCCACAAAAAGAGCCAGCC
AAAAGAGAAAAACCTAAACAGAAAGCTACTTCTTAAAGTAAAGAATCTGTGAAAAAGTGCCAA
TGTTAAGAAAGCGATAGCGACCCACCAAGAAGATCAAAACAGTTCCAAAGAAAGGT
GAGTCTGAGGATAGTTTCAGATGAAACCTTTAATTTAAAAAGTTGAAAGAACCCCTACAGA
TGAAAGCTAAAGGAAAAAATAAAGAAAATTACTGAGCCAGTGCTAACTTTGGAGAGATGCACA
ATGAAACAGATTGCAAAAAAGGTCTATGAAATTATCTCTACTTTATGATTTAATTGAAAGAAAAA
GATTTCATAAAACAACGTAAAAAGACTGTTTTCTTGACTCGAG, was created by flanking
DEK in the R780-DEK vector with primers to introduce EcoRI and in-frame His(6)-FLAG-Stop-
Xhol sequence. The PCR product was produced, cut, and inserted into the EcoRI and Xhol
sites in the pMIEG vector by GenScript.

**Western blot analyses:**

Cells were washed with PBS and whole cell lysates were harvested with RIPA buffer (1% Triton,
1% deoxycholate, 0.1% SDS, 0.16M NaCl, 10 mmol/L Tris pH 7.4, and 5 mmol/L EDTA)
supplemented with a protease inhibitor cocktail (BD Pharmingen- 554779) and analyzed as
described previously (25). Primary antibodies used for DEK were as follows: DEK
(BDBiosciences; 1:1000; DEK-Aviva Biosystems 1:1000; DEK K-877 (1:10,000, a polyclonal antibody; gift from Ferdinand Kappes), a DEK cocktail including the 3 described antibodies and DEK polyclonal rabbit from Proteintech (1:1000;). Other antibodies include alpha tubulin (1:5000, Sigma; pH3-S10 1:1000, Cell Signaling; H3 1:1000, Cell Signaling; pan-actin 1:20,000, a gift from James Lessard, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA). Membranes were exposed to enhanced chemiluminescence reagents (Perkin Elmer).

**Immunofluorescence microscopy:**

Cells were plated onto 100 mg/ml poly-D-lysine coated coverslips, and fixed with 2% paraformaldehyde. Coverslips were incubated in 0.1% Triton X-100 for 3 min, blocked with 5% normal goat serum, and incubated with primary antibody for 1 h at 37˚C. Antibody dilutions were as follows: DEK-BD (1:60; BD Biosciences), DEK-Aviva (1:60 Aviva systems biology), DEK-K-877 (1:500), GFP (1:500; Abcam) and alpha tubulin (1:500; Sigma-Aldrich). Coverslips were incubated for 30 min at 37˚C in secondary anti-mouse or anti-rabbit Alexa Fluor 488 or 568 (1:1000; Lifetechnologies) and mounted onto glass slides with DAPI Vectashield mounting media (VectorLabs). Cell staining was analyzed using a Leica DMI6000 microscope (Leica) and Openlab5 software (Improvision). DEK retention on mitotic chromosomes was quantified in at least 20 cells per phase of mitosis and done in duplicate or triplicate experiments where indicated. Images of varying magnifications were cropped in Microsoft PowerPoint and resized to fit in a specific window and therefore are not representative of size. Micronuclei quantification was analyzed using ImageJ by counting micronuclei and the total number of cells from 20x magnification images. A total of 500 to 1000 cells were analyzed per cell line. The standard error was calculated from the percent of micronuclei per cell across all photos.
Live cell imaging:
Cells were plated in a 35mm-60mm dish. 20mM of HEPES (http://www.lifetechnologies.com/order/catalog/product/15630080) pH 7.4 was added to the media as well as 10uM trolox (Sigma). Media was overlaid with 1.5 mL mineral oil (Sigma) to prevent evaporation. The dish was set inside an incubator attached to the microscope (Zeiss Axiovert 200M) set to 37 degrees Celsius. Pictures were taken every 4 minutes through the FITC channel. Videos were made using ImageJ and converted to AVG files.

Quantification of micronuclei by flow cytometry:
Micronucleus staining was adapted from previously published protocols.(150-152) In brief, CCHMC-SCC1 or MEFs were plated in 6-well or 12-well plates and grown to 50% confluency. At this time cells were either untreated or treated with the indicated drugs: 1ug/ml bleomycin (Millipore), 1uM etoposide (Sigma), or 20nM colcemid (Lifetimes) for 16 hours. Cells were placed on ice for 20 minutes. Media was then aspirated and cells were covered with 0.0125 mg/ml ethidium monoazide bromide (EMA, Biotum) in PBS with 2% fetal bovine serum. EMA was photoactivated for 30 minutes, one foot below a 60 watt bulb on ice. Cells were washed twice with PBS then covered in a non-ionic detergent called “Lysis Solution 1”. “Lysis solution 1” was prepared with deionized water, and consisted of 0.584 mg /ml NaCl (Midwest Science), 1 mg/ml sodium citrate (Amresco), 0.3 μl /ml IGEPAL (Sigma), 0.5 mg/ml RNAse A (Qiagen) and 0.4 μM SYTOX Red (Lifetimes), followed by incubation at 37C for 1 hour in the dark. Then “Lysis Solution 2” was prepared with deionized water, 85.6 mg/ml sucrose (Fisher Scientific), 15 mg/ml citric acid (Sigma Aldrich), and 0.4 μM SYTOX Red. Lysis solution 2 was added to cells for 1 hour in the dark at room temperature. Details regarding the lysis solutions have been published previously.(152) The cell lysis protocol keeps nuclear membranes intact thus enabling the identification of nuclei with <2N, 2N, 4N, and >4N DNA content as well as micronuclei.
Nuclear gating was performed as previously published (152). Samples were analyzed on the BD LSRFortessa with 488 nm, 640 nm and 405 nm lasers in the CCHMC Flow Cytometry core facility. SYTOX-associated fluorescence emission was detected using 640 nm excitation and a 660/20 bandpass filter, and EMA-associated fluorescence was detected using 488 nm excitation and a 610/20 bandpass filter. EMA positive nuclei were gated out prior to the quantification of micronuclei. Bleomycin, etoposide and colcemid were used as positive controls for micronucleus formation. The incidence of cells with micronuclei was determined through the acquisition of at least 10,000 gated nuclei per culture.

**DEK expression module analysis:**

We evaluated DEK expression across the entire series of tumor samples that are profiled in 2158 Affymetrix Gene Chip Hg-133 plus 2.0 microarrays performed by the International Genome Consortium Expression Project for Ontology (ExpO; Gene Expression Omnibus Dataset GSE2109). To do this we used Pearson Correlation Analysis to identify the most strongly correlated probesets to the DEK probeset 200934_at across the RMA-normalized dataset. Using a cutoff of Pearson Correlation 0.485 identified 307 probesets. Geneset enrichment and connectivity analysis was carried out using the ToppGene web server (http://toppgene.cchmc.org/) and ToppGene Suite(153) for gene list enrichment analysis and candidate gene prioritization.

**Statistics:**

Statistical significance was calculated using GraphPad Prism 6 software (La Jolla, CA, USA). The Fisher’s exact test was used to determine significance of DEK localization during mitosis. A t-test was used to determine the significance of micronuclei presence by immunofluorescence and flow cytometry. When samples exceeded more than 2 groups, a two-way anova test was
used to determine significance for micronuclei detection by flow cytometry. Where indicated $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

**Acknowledgements**

This work was supported by the Public Health Service Grant R01 CA116316 from the NIH (SIW). Work in the laboratory of Ferdinand Kappes is supported by the START Program of the Faculty of Medicine, RWTH Aachen and by the German Research Foundation (DFG KA 2799). We are grateful to Dr. James Lessard of Cincinnati Children’s Hospital Medical Center and Seven Hills Bioresearch (Cincinnati, OH) for his gift of the C4 pan-actin monoclonal antibody used in this work. We thank David Williams (Children’s Hospital Boston, MA) for the pMIEG3 retroviral vector. We recognize the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children’s Hospital Medical Center, supported in part by NIH AR-47363, NIH DK78392 and NIH DK90971. We thank Lisa Privette-Vinnedge (CCHMC) for all of her insightful comments and edits to the manuscript as well as Eric Smith for his edits.
Figure 2.1. The nuclear DEK oncogene is absent from mitotic chromosomes. (A) Ontology analysis reveals mitosis is the most highly correlated biological process with DEK expression in tumors. Over 2000 tumor specimens were queried for transcriptional co-expression with the DEK oncogene using microarrays performed by the International Genome Consortium Expression Project for Ontology and connectivity to biological processes was carried out using ToppGene. (B) Immunofluorescence microscopy (IF) of unsynchronized near diploid immortalized keratinocytes (NIKS) shows DEK co-localization with DAPI in a cell in interphase and telophase, but absent from DNA in a cell in prophase. NIKS were stained with DAPI to
detect DNA, along with α tubulin to detect microtubules and the mitotic spindle, and a DEK specific antibody (Aviva Systems Biology). Arrowheads point to cells wherein DEK co-localizes with chromatin (white) or there is no co-localization (yellow). (C) IF was carried out as in (B) with examples of DEK localization throughout mitosis. (D) Quantification of (C) not including prometaphase. Over 140 mitotic cells were counted across 4 cover slips from 3 independent experiments with at least 20 cells counted per mitotic stage. Twenty interphase cells were counted per coverslip.
Figure 2.2. DEK protein is sharply reduced in mitotic cells. NIKS were treated with mimosine or serum starvation to induce cell cycle arrest in the G1 phase, with a combination of thymidine and aphidicolin to induce cell cycle arrest in S phase, and with nocodazole or underwent a mitotic shake off to enrich for cells in G2/M phase. Mitotic shake off occurred after
treatment with colcemid 20 nM for 24 hours. (A) Cellular arrest in the expected phase of the cell cycle was verified by flow cytometry. Cells were pulsed with EdU for 2 hours then stained with 7AAD to determine DNA content. (B) Percent cells from the gated areas of G1, S, and G2/M were quantified from (A). (C) Cells were treated as in (A) and subjected to protein gel blot analysis to detect DEK protein (Aviva Systems Biology) levels throughout the cell cycle.
Figure 2.3. DEK overexpression induces its aberrant retention on mitotic chromosomes. 

(A and B) NIKS were transduced with a GFP-marked R780 retroviral vector encoding wild-type human DEK. The R780 backbone vector was used as a control. IF images of NIKS in mitosis expressing either control R780 vector (A) or R780-DEK vector (B) reveal DEK overexpression stimulates DEK retention on mitotic chromosomes. (C) Western blot analysis confirms DEK overexpression. (D) Quantification of (A and B). Over 140 mitotic cells were counted (at least 20 per mitotic stage) in 2 independent experiments for the R780 empty vector and over 200 mitotic cells for R780-DEK in 3 independent experiments.
Figure 2.4. A DEK-GFP fusion protein is retained on mitotic chromosomes and associates with lagging chromosomes. (A) Schematic of the DEK-GFP fusion protein expressed from a pLentiLox vector backbone. GFP is fused to the C-terminus of the 375 amino acid protein DEK. (B) NIKS were transduced with DEK-GFP or empty control GFP vector and were sorted for GFP expression. DEK-GFP expression was confirmed by western blot analysis.
using a DEK antibody (BD Biosystems). (C) Quantification of interphase and mitotic cells with chromatin-associated DEK-GFP compared to control GFP protein using a GFP antibody for IF. (D) Mitotic defects were commonly observed in the DEK-GFP expressing NIKS by live cell imaging. Pictures were taken of the same cell in metaphase, anaphase and telophase. DEK-GFP remains bound to the DNA throughout mitosis and is detected on an anaphase bridge that results in a nuclear bud. Arrows point to improperly segregating chromosome(s).
Figure 2.5. High DEK expression, sufficient to cause mitotic retention, stimulates
**micronucleus formation.** (A) Immortalized Dek−/− mouse embryonic fibroblasts (MEFs) were transduced with the R780-DEK or control retroviral vector. The cells were sorted for low vs. high DEK expression by gating on low versus high GFP expression. (B) Western blot analysis of sorted cells verifies low and high DEK protein levels as expected. (C) IF reveals DEKLo expression in Dek−/− MEFs results in DEK localization similar to that of endogenous DEK during mitosis. (D) DEKHi expression in MEFs stimulates abnormal DEK association with mitotic chromosomes throughout mitosis. (E) Quantification of (C and D) as before from 3 independent experiments, comparing DEK localization in DEKLo and DEKHi expressing mitotic cells. (F) DEK associates with extra-nuclear chromosome fragments such as nuclear buds and micronuclei in DEKHi MEFs. Arrowheads point to extra-nuclear DNA. (G) DEKLo and DEKHi MEFs were plated on coverslips and micronuclei were quantified from images using imageJ to count a total of 500–2000 cells.
Figure 2.6. Acute, inducible expression of DEK induces micronuclei formation. *Dek−/−* MEFs were transduced with a doxycycline-inducible GFP-DEK fusion protein (A) encoded by a TRIPZ vector and selected with puromycin. The empty GFP pTRIPZ vector was used as a control. (B) Western blot analysis confirmed fusion protein expression at 48 hours after the addition of doxycycline to GFP-DEK transduced cells. Cells from (B) were fixed on coverslips and micronuclei were quantified by IF as before (C). (D) GFP-DEK expression does not alter cell cycle progression. GFP and GFP-DEK transduced cells, in the presence or absence of dox for 48 hours, were pulsed with EdU for 2 hours and subjected to cell cycle analysis using flow cytometry. Cells in each stage of the cell cycle were quantified from duplicate experiments. (E) The GFP-DEK fusion protein is retained in mitotic cells. Western blot analysis of GFP-DEK cells
with and without doxycycline and arrested in G1 with mimosine or mitosis with nocodazole, or underwent a mitotic shake off (MSO) confirms an increase of GFP-DEK in mitotic cells (NM=non mitotic). A DEK specific antibody was utilized (BD Biosystems) as well as a pH3 antibody to detect cells in mitosis.
Figure 2.7. DEK overexpression increases the incidence of hypodiploidy and micronuclei in cancer cells. CCHMC-SCC1 cells are a squamous cell carcinoma line isolated from a primary tonsillar tumor. **(A)** DEK was overexpressed by R780-DEK compared to control R780 transduction, and expression levels were confirmed by protein gel blot analysis. **(B)** DEK overexpression did not increase proliferation in C-SCC1 cells. Cells were pulsed with EdU for 2 hours, and then stained with 7AAD to determine DNA content. **(C-F)** CCHMC-SCC1 cells treated as in (A) were used for micronuclei detection by a flow cytometry based assay. **(C)** DNA from dead or dying cells was gated out on EMA positivity. **(D)** Remaining DNA that was stained with SYTOX Red was gated on DNA content to reveal G1, G2/M nuclei (labeled nucleated or N), a hypo-diploid population of nuclei (labeled H), and micronuclei (M). **(E,F)** Each population of events was gated in FlowJo to compare empty vector control cells (**E**) with DEK overexpressing cells (**F**). **(G and H)** DEK overexpression increased both micronucleus formation (**G**) and the hypo-diploid population of cells (**H**). Numbers represent 5 replicate cell cultures from 2 independent experiments.
Normal Mitosis

Interphase

Prophase

DNA condensation

Metaphase

Chromosome alignment

Anaphase

Sister chromatid dysjunction

Telophase

Nuclear membrane and midbody formation

Cytokinesis

Separation of daughter cells.

Complete Mitosis

Two diploid cells

DEK Over-Expression

Improper condensation?

Improper spindle attachment?

Non-disjunction/ lagging chromosome

Malsegregated chromosome(s)

Extra-nuclear DNA

Micronucleus

Hypodiploid nucleus

Aneuploidy

Diploid cell
Figure 2.8. Working model: The consequence of DEK overexpression in mitosis. (A) A schematic detailing the various stages of mitosis including the loss of DEK association with chromatin in prophase and re-association in telophase. (B) The overexpression of DEK leads to its aberrant retention on mitotic chromosomes throughout mitosis. The end result is mal-segregated chromosomes that end up as micronuclei in daughter cells.
Supplemental Figure 2.1. Dynamic DEK association with mitotic chromosomes is detectable by multiple DEK antibodies. IF of NIKS undergoing mitosis was performed using DAPI and (A) a polyclonal DEK antibody K-877 and (B) a monoclonal antibody from BD Biosystems. (C) Hela cells were transduced retrovirally with a GFP-marked MIEG vector expressing a His-Flag-tagged DEK protein. Cells were sorted on EGFP expression and subjected to coverslip IF using a Flag antibody.
Supplemental Figure 2.2. DEK protein level in DEK overexpressing NIKS is similar to that in cancer cells. (A) Western blot of NIKS and CCHMC-SCC1 cells transduced with the empty R780 retroviral vector or R780-DEK. CCHMC-SCC1 cells are cells derived from a head and neck squamous cell carcinoma. UM-SCC1 is a head and neck squamous cell carcinoma cell line with high DEK expression levels. All images are from the same western blot.
Supplemental Figure 2.3. DEK-GFP bound to mitotic chromatin is detected by DEK antibodies and on lagging chromosomes. (A and B) NIKS (A) and Hela cells (B) were transduced with pLentiLox containing DEK-GFP and sorted on GFP expression. IF of DEK-GFP transduced NIKS reveals increased association of the fusion protein with chromosomes in metaphase and anaphase compared to endogenous DEK. The DEK antibody (Aviva Systems Biology) detects DEK-GFP association as observed directly by GFP. (B) The DEK-GFP fusion localizes to lagging chromosomes in Hela cells transduced with DEK-GFP and plated on coverslips as in (A). Alpha tubulin and a GFP antibody were used to detect mitotic spindles and the DEK-GFP fusion.
Supplemental Figure 2.4. DEK associates with lagging chromatin in DEK<sup>Hi</sup> expressing Dek<sup>-/-</sup> MEFs (A) IF of DEK<sup>Hi</sup> expressing Dek<sup>-/-</sup> MEFs transduced with an R780 retroviral vector containing full length wild type DEK and sorted on high GFP expression as in Fig. 5. Shown is an example of DEK association with a lagging chromosome during anaphase in DEK<sup>Hi</sup> expressing Dek<sup>-/-</sup> MEFs.
Supplemental Figure 2.5. An inducible GFP-DEK fusion mimics endogenous DEK localization in mitosis and induces micronuclei formation. *Dek−/−* MEFs were transduced with a doxycycline-inducible GFP-DEK fusion protein as in Fig. 6 and placed in dox containing media for 48 hours. (A) Cells were plated on coverslips for IF with DAPI and antibodies for GFP and alpha tubulin. (B) Cells were subjected to a flow cytometric micronuclei detection assay by flow cytometry as described in Fig. 7. The results confirm a 1.5 fold increase in micronuclei observed by IF (Fig. 6C).
Supplemental Figure 2.6 Effects of DEK overexpression compared to that of an aneugen and a clastogen on micronucleus formation. DEK overexpression in CCHMC-SCC1 cells from Fig. 7 was compared to the effects of 1uM bleomycin, a clastogen, and 20nM colcemid, an aneugen, for 16 hours. (A) Flow cytometry data and gating to identify micronuclei (M), hypodiploid nuclei (H), and diploid nuclei (N). (B) Quantification of (A) done in triplicate technical experiments.
The following supplemental table and videos can be found online at:
http://www.tandfonline.com/doi/suppl/10.1080/15384101.2015.1044177

**Supplemental Table 2.1: Ontology analysis of gene transcripts that co-express with DEK in tumors.** In order to characterize patterns of DEK expression across a variety of tumor types, we evaluated its expression across the entire series of tumor samples that are profiled in 2158 Affymetrix Gene Chip Hg-133 plus 2.0 microarrays performed by the International Genome Consortium Expression Project for Ontology (ExpO; Gene Expression Omnibus Dataset GSE2109). To do this we used Pearson Correlation Analysis to identify the most strongly correlated probesets to the DEK probeset 200934_at across the RMA-normalized dataset. Using a cutoff of Pearson Correlation 0.485 identified 307 probesets. Although there was some degree of differential tissue expression (e.g., relatively low expression in kidney, prostate, and some brain tumors), the principle characteristics of genes that are similarly regulated as DEK are virtually entirely involved in cell cycle biology.

**Supplemental Video 2.1. DEK-GFP remains on chromatin throughout mitosis.** Live cell imaging of NIKS transduced with the pLentilox vector containing the DEK-GFP fusion protein. Various cells are observed going through mitosis and DEK-GFP remains bound to DNA and in the cell. Supplemental videos can be found online at:
http://www.tandfonline.com/doi/suppl/10.1080/15384101.2015.1044177

**Supplemental Video 2.2. Mitotic defects observed in NIKS with DEK-GFP fusion.** Live cell imaging of NIKS transduced with the DEK-GFP fusion as before. A cell expressing a high level of DEK-GFP harbors malsegregated chromosomes leading to extranuclear DNA post mitosis.
Supplemental Video 2.3. Formation of nuclear buds in NIKS with the DEK-GFP fusion.

NIKS treated as in supplemental videos 1 and 2 were observed having various mitotic defects. Shown is an example of a cell with chromosome misalignment in metaphase leading to an anaphase bridge and ultimately a nuclear bud.
Mitosis and Chromosomal Instability

Most HPV-associated malignancies have numerous chromosomal imbalances, including gains or losses of whole chromosomes (aneuploidy) and chromosomal rearrangements (154). These chromosomal aberrations are often a result of mitotic defects including spindle polarity defects, chromosomal missegregation, and chromosomal breaks. The accumulation of these chromosome abnormalities happens over time, a process which explains the long latency between initial HPV infection and the development of cancer (155). Importantly, aneuploidy is already detectable in pre-malignant cells suggesting the HPV oncogenes play a causal role in the genetic instability that results in oncogenesis (156).

Some of these chromosomal defects are the result of abnormal multipolar mitoses, with tripolar spindles being characteristic of HPV16 oncogene expression. Such events are associated with abnormal centrosome numbers (157, 158). E6 and E7 cooperate to induce centrosome abnormalities resulting in multipolar spindles, chromosomal missegregation, and aneuploidy (156). Centrosomes are composed of two centrioles that generate the spindle poles during mitosis, and, considering the importance of this organelle to proper cell division, centrosomal duplication is highly controlled and occurs only once prior to each cell division in healthy cells. (159).

The process starts in S phase and is completed in G2 (159). During prophase in mitosis, the centrosomes migrate to opposite poles of the cell and the mitotic spindle forms between them. After cell division, each daughter cell receives one centrosome, containing a pair of
centrioles. Centrioles, cylindrical organelles involved in the organization of the mitotic spindle, can erroneously be duplicated more than once before mitosis. It has been shown that the number of extraneous centrioles correlates with the risk of mitotic defects (160). Centriole amplification can result in two ways. One way is through centrioles being over-duplicated, the other is centriole accumulation in cells that do not properly progress through mitosis. These two defects have different implications. Centrioles are considered over-duplicated when there are one or two maternal centrioles and multiple immature daughter centrioles. Over-duplication is more likely to result in cell division with abnormal chromosomal segregation and aneuploidy. Centriole accumulation is characterized by multiple maternal centrioles and a normal ratio of daughter centrioles. Centriole accumulation is likely the result of aborted mitoses or problems during cytokinesis that are unlikely to result in viable daughter cells or propagate aneuploidy. E6 expression can cause centriole accumulation in cells that are already genomically unstable; however these cells are unlikely to remain in the proliferative pool due to catastrophic DNA damage (159, 161). On the other hand, E7 can cause over-duplication and therefore increase instances of multipolar mitosis and chromosomal abnormalities in daughter cells. E7 is thought to directly cause genomic instability through centriole duplication control as is evidenced by E7 expression introducing abnormal centriole numbers in otherwise normal cells prior to the onset of genomic instability (162). E7 is also capable of causing centrioles to appear rapidly within a single cell division cycle suggesting a direct role (163). These resulting genomic abnormalities and aneuploidy could potentially result in a selective growth advantage that eventually results in carcinogenic transformation.

The mechanism of E7 mediated over-duplication of centrioles enables multiple daughter centrioles to be produced from one maternal centriole. This is partially dependent on high levels of CDK2/cyclin E activity (163), and is thought to happen through both RB dependent and independent mechanisms. Highlighting the role of RB in E7 mediated centriole amplification, an E7 mutant protein that cannot degrade RB is also unable to cause centriole over-duplication.
Interestingly, full length HPV-16 E7 can cause centriole abnormalities in RB/p107/p130-deficient mouse embryo fibroblasts albeit at a much lower incidence. This suggests there is also an RB independent role in E7 induced centriole amplification (164). Several RB independent mechanisms have been identified including the aberrant recruitment of polo-like kinase 4 (PLK4) to maternal centrioles by high levels of CDK2 activity to promote centriole duplication. PLK4 protein levels are rate limiting in centriole multiplication and its overexpression is sufficient to induce centriole multiplication. Along with aberrantly recruiting PLK4 to maternal centrioles, E7 can activate the PLK4 promoter, up-regulating PLK4 mRNA which correlates with the ability of E7 to induce centriole multiplication (165). Typically the PLK4 promoter is bound by the DREAM (DP, RB-like, E2F4 and MuvB) complex which represses PLK4 expression (for a review on DREAM, please refer to Sadasivam and DeCaprio, 2013). E7 however can disrupt the DREAM complex, thus preventing PLK4 repression and causing deregulated centriole duplication (167).

During DNA damage, p53 can activate the DREAM complex to further support the repression of PLK4 and centriole duplication. However, E6 dependent degradation of p53 can further support centriole duplication. Lastly, E7 can directly bind to γ-tubulin, resulting in the removal of γ-tubulin from the mitotic spindles and potentially leading to abnormal centrosome synthesis (168, 169). Together, these studies suggest that aberrant centrosome duplication is an early event caused by E6 and E7 that may drive chromosomal instability.

In addition to centrosome abnormalities, E6 and E7 have been shown to independently bypass mitotic checkpoints resulting in the accumulation of polyploid cells and aneuploidy (170, 171). The HPV oncogenes function individually and in concert to overcome the spindle assembly checkpoint (SAC) in mitosis. The SAC ensures proper alignment and segregation of chromosomes and is activated in response to aberrant microtubule-kinetochore attachments but not necessarily due to the presence of supernumerary centrosome or multiple spindle poles (172, 173). Data suggest that E7 expressing cells exhibiting multipolar spindle poles in metaphase are less likely to complete mitosis. However, the fate of these cells is unknown. Cell
fate could include proceeding through mitosis normally, mitotic dysfunction resulting in apoptosis or mitotic catastrophe, or decondensing of the chromosomes and re-entry into a G1-like state with 4n DNA content. The later can happen upon DNA damage, when E7-expressing cells arrest at the G2 checkpoint and then undergo re-replication. Re-replication is a process of successive rounds of host DNA replication without entering mitosis. This process is thought be a result of Cdt1 up-regulation by E7, Cdt1 being the only known mammalian gene to efficiently trigger re-replication when overexpressed in cancer cells (174-176). E7 can stabilize Cdt1 at the post translational level potentially inducing DNA re-replication and polyploidy causing further genomic instability (177).

E6 and E7 have independently been shown to overcome the SAC and promote the accumulation of polyploidy cell populations (170, 171). HPV-16 E6 and E5 cooperate to allow cells to activate the APC/C to promote the metaphase to anaphase transition. The anaphase-promoting complex/cyclosome (APC/C) is a multi-subunit ubiquitin ligase that must be activated for cells to transition from metaphase to anaphase. The APC/C complex is activated by the binding of Cdc20 in metaphase. The SAC proteins BubR1 and Mad2 can bind to Cdc20 to prevent APC/C activation until all chromosomes are aligned correctly on the mitotic spindle. E6 dependent degradation of p53 can lead to Cdc20 overexpression resulting in a portion of Cdc20 bound to BubR1 and another portion free of BubR1 indicative of an active checkpoint in some cells and not in others. Free Cdc20 can activate APC/C allowing slippage through the mitotic checkpoint (178). E6 can also increase the expression of Ubch10, an E2 ubiquitin-conjugating enzyme that leads to uncontrolled APC/C activity and degradation of cyclin B. Degradation of cyclin B allows cells to exit mitosis despite the presence of unrepaired DNA damage (178). Furthermore, E5 expression correlates with the gradual decreases in expression of Bub1 and Mad2 during cervical cancer progression (179). Exogenous expression of HPV16/18 E5 can decrease both BubR1 and Mad1 mRNA and protein levels potentially though direct interaction
with E5 at least for BubR1. Therefore E5 might also contribute to the weakening of mitotic checkpoints (179).

E5/E6/E7 genes cooperate to promote mitotic progression in the presence of DNA damage, thus further promoting genomic instability and aneuploidy. As malignant progression occurs over the course of many years, it is likely that these mitotic defects occur infrequently and do not often lead to viable progeny. The accumulation of subtle chromosomal alterations may provide a growth advantage to a subclone of HPV-positive cells, resulting in the outgrowth of a cellular population that contributes to viral persistence and, ultimately, malignant progression.

A recent study showed that HPV integration sites in human cancer genomes directly flank genomic structural variations, including focal amplifications, rearrangements, deletions, and/or translocations. These genomic alterations have frequently disrupted the expression and structure of neighboring genes involved in oncogenesis, and correlate with amplification and increased expression of E6 and E7 (180). HPV integration can lead to oncogene amplification and loss of heterozygosity of tumor suppressor genes (Reuter et al., 1998, Schmitz et al., 2012). Homozygous deletion and rearrangements of DIAPH2, a gene whose loss of function promotes chromosomal instability via misalignment of sister chromatids during metaphase, was observed in a HNC cell line after HPV integration (181). This is not surprising as microarray data reveals that the number one cellular process transcriptionally altered in cervical cancer compared to healthy cervical epithelium is the cell cycle and specifically M-phase processes (182).
Chapter 4: Overexpression of the human DEK oncogene reprograms cellular metabolism and promotes glycolysis

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Keywords: DEK, Warburg effect, glycolysis, oncogene, NMR-based metabolomics, Seahorse analysis

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Manuscript submitted to PLOS ONE February, 2017

Abbreviations:

1-MNA 1-methylnicotinamide
2-OIC oxoisocaproate
Ala alanine
αKG alpha ketoglutarate
Asn asparagine
BCAA branched chain amino acids
Cho choline
DMA  dimethylamine
Gln  glutamine
Glu  glutamate
GPC  glycerophosphocholine
GSH  reduced glutathione
HNSCC  Head & Neck Squamous Cell Carcinoma
Myo-Ino  myo-inositol
NAD⁺  nicotinamide adenine dinucleotide
OAA  oxaloacetate
PCA  Principal Components Analysis
P-choline  phosphocholine
Phe  phenylalanine
Poly-Glu  poly glutamate
Pyr  pyruvate
Pyro-Glu  pyroglutamate
Suc  succinate
UDP  uridine diphosphate
Abstract

The DEK oncogene is overexpressed in many human malignancies including at early tumor stages. Our reported in vitro and in vivo models of squamous cell carcinoma have demonstrated that DEK contributes functionally to cellular and tumor survival and proliferation. However, the underlying molecular mechanisms remain poorly understood. Based on recent RNA sequencing experiments, DEK expression was necessary for the transcription of several metabolic enzymes involved in anabolic pathways. This identified a possible mechanism whereby DEK may drive cellular metabolism to enable cellular growth. Functional metabolic Seahorse analysis demonstrated increased baseline and maximum extracellular acidification rates, a readout of glycolysis, in DEK-overexpressing keratinocytes and squamous cell carcinoma cells. DEK overexpression also increased the maximum rate of oxygen consumption. To detect small metabolites that participate in glycolysis and TCA cycle activity, we carried out NMR-based metabolomics studies. We found that high levels of DEK significantly reprogrammed cellular metabolism and altered the presence of amino acids, TCA cycle intermediates and the glycolytic end products lactate, alanine and NAD\(^+\). Taken together, these data support a scenario whereby overexpression of the human DEK oncogene reprograms keratinocyte metabolism to fulfill energy and macromolecule demands required to enable and sustain cancer cell growth.
Introduction

The human DEK proto-oncogene encodes a highly conserved chromatin-associated protein that is overexpressed in a wide range of human malignancies. DEK was originally identified in acute myeloid leukemia as a fusion protein with NUP214 (1), and was subsequently shown to be overexpressed at the mRNA and protein level in various cancer types including squamous cell carcinoma (SCC) (7, 18, 183-186). This oncoprotein modifies the structure of chromatin (55, 121, 124, 133, 187), and has corresponding nuclear functions in transcription (57, 61, 64, 188), epigenetics (43, 61, 188), and mRNA splicing (37, 68). Overexpression in vitro promoted pro-carcinogenic phenotypes, such as cellular life span, proliferation, survival, and motility, depending upon cell types and experimental model systems utilized (18, 21, 22, 24, 25, 91, 189). Keratinocytes comprise 90% of the human epidermis and are the cells of origin for squamous cell carcinoma. We have previously shown that the overexpression of DEK stimulates proliferation and hyperplasia of NIKS, human keratinocytes, once engineered into 3D organotypic rafts that mimic stratified human epidermis (25). Furthermore, such overexpression collaborated with the high-risk human papilloma virus (HPV) E6/E7 and hRas oncogenes to stimulate anchorage independent growth of keratinocytes in vitro and the development of squamous cell carcinoma (SCC) in vivo (22). Finally, Dek knockout mice compared to wild type mice were protected from the growth of chemically induced skin papillomas (22), and head and neck (HN) SCCs in a transgenic HPV16 E7-driven murine tumor model (20). Together, these data clearly demonstrate oncogenic DEK activities at early and late stages of carcinogenesis.

A major hurdle in neoplastic transformation is the ability of cells to meet the high bioenergetic and biosynthetic needs necessary to sustain cancer cell growth. It is well established that cancer cells shift to a pro-anabolic metabolism induced by oncogenes, such as c-Myc (190). Most notable is the Warburg effect wherein cancer cells increase glycolysis and lactic fermentation when compared to their non-transformed counterparts (191). An increase in glycolysis provides cancer cells with energy and heightened potential for biomass production
from glycolytic intermediates (192). Several glycolytic intermediates are important precursors for biomass production, including glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and glyceraldehyde 3-phosphate (GAP) via the pentose phosphate pathway (PPP). The PPP generates ribose for nucleotide biosynthesis, and NADPH via the oxidative branch of the PPP. NADPH is used in controlling oxidative stress via the glutathione peroxidase/glutathione reductase system (193). F6P is involved in the synthesis of hexosamines. Dihydroxyacetone phosphate (DHAP) is the precursor of glycerol phosphate for glycerolipid synthesis, and glycerate 3-phosphate (3GP) is the precursor for serine and glycine production used in purine biosynthesis, as well as the production of pyruvate (194-196). Cancer cells may also fuel their growth with glutamine that can be used as an amino acid for protein synthesis, as a carbon source for lipid synthesis and pyrimidine synthesis, and as a primary nitrogen donor for hexosamine and nucleotide synthesis. Glutamine supports anaplerosis by replenishing TCA cycle intermediates used for macromolecule production in actively dividing cells. Metabolic adaptation to effectively support neoplastic proliferation and survival is a hallmark of cancer and involves the deregulation of multiple metabolic pathways through oncogene expression (196-198).

With regards to the human DEK oncogene, our recent transcriptome analyses of DEK-depleted HNSCC cells revealed a decrease in expression of numerous metabolic enzymes (94) (Supp. Fig.1A-B). These enzymes were associated with multiple metabolic pathways including nucleotide synthesis, NAD⁺ metabolism, MTOR signaling, cholesterol synthesis, glycolysis, and glutathione production, all of which support cellular growth. This suggested DEK might regulate cellular metabolism, an activity that has not previously been explored. Therefore, we set out to define DEK-regulated metabolism in HNSCC and non-transformed keratinocytes. DEK was first overexpressed in NIKS, an immortalized keratinocyte cell line and established model for normal human keratinocytes with regards to growth and differentiation characteristics and ability to form stratified epidermis (25, 127). Second, we utilized HNSCC cells that express intermediate levels
of endogenous DEK thus allowing for additional DEK overexpression in malignant cells. We analyzed the resulting isogenic pairs of NIKS and C-SCC1 cells by Seahorse analysis to determine rates of glycolysis and oxidative phosphorylation. DEK overexpression was sufficient to increase baseline and maximum lactic fermentation and maximum oxygen consumption in both cell types. In order to quantify small metabolites that accompany the observed metabolic alterations, we performed nuclear magnetic resonance (NMR)-based metabolomics on extracted metabolites from NIKS and C-SCC1 cells and their respective media. One-dimensional $^1$H (proton) NMR is a powerful tool that reliably and quantitatively detects small metabolites (199). We found DEK-overexpressing NIKS and HNSCC cells harbored an accumulation of glycolytic end products including lactate, NAD$^+$ and alanine. An accumulation of TCA cycle intermediates was detected in HNSCC cells, but not in NIKS, suggesting differential utilization of TCA cycle intermediates for energy and macromolecule production. Taken together, we identify novel DEK functions in driving metabolic pathways that are associated with cancer-related energy and macromolecule production. Significantly, this occurs in keratinocytes and SCC cells, suggesting these effects are not dependent upon a cancer cell specific co-variant but are inherent to DEK overexpression.

**Results**

**DEK overexpression increases cellular metabolism in the absence of proliferative gains.**

Our previous studies showed that DEK oncogene overexpression in NIKS (127) did not stimulate proliferation markers in monolayer cells, but increased proliferation and hyperplasia in 3D epidermis (25). In order to determine whether DEK overexpression could promote metabolic activity, we transduced NIKS with either empty retroviral vector (R780) or with DEK-expressing vector R780-DEK (R-DEK) as previously described (22). DEK overexpression was validated by western blot analysis relative to endogenous DEK levels, and did not affect overall cellular morphology (Fig. 4.1A). Similar data were obtained upon DEK overexpression in a previously
published HNSCC cell line CCHMC-HNSCC1 (C-SCC1) (20, 200) (Fig. 4.1B). These cancer cells harbor moderate levels of DEK expression when compared to most other HNSCC cell lines, thus allowing for DEK upregulation beyond endogenous levels. As expected, DEK did not alter cellular growth as determined by cell counts over the course of 4 days (Fig. 4.1C-D), cell cycle progression measured by EdU incorporation (Fig. 4.1E-F), or apoptosis measured by caspase 3 cleavage (Fig. 4.1G-H), in either NIKS or C-SCC1 cells. However, the tetrazolium-based MTS assay revealed increased oxidation of NADH to the key metabolic coenzyme NAD⁺ in R-DEK cells (Fig. 4.1I-J). The MTS assay is commonly used to measure proliferation; however, here we have shown that DEK does not affect cell growth. Therefore, the data suggests that DEK overexpression stimulates metabolic activity in the absence of proliferative gains.

**DEK overexpression increases the rate of glycolysis and maximum rate of oxidative phosphorylation.**

To define DEK effects on metabolism directly, we measured extracellular acidification rates (ECAR) and oxygen consumption rates (OCR), a readout for glycolysis and oxidative phosphorylation (OxPhos) respectively, using the Seahorse XF24 Extracellular Flux Analyzer (Seahorse Biosciences, Billerica, MA). NIKS and C-SCC1 R780 and R-DEK cells were assayed under baseline conditions and following injection of oligomycin, carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP), and rotenone/antimycin A. Oligomycin is an ATP synthase inhibitor that prevents OxPhos and drives glycolysis to the maximum rate. FCCP is an uncoupling reagent that induces maximum respiration rates and decreases glycolysis to baseline rates. Rotenone and antimycin A are electron transport chain inhibitors that halt OxPhos and have no additional effects on glycolysis. Interestingly, while OCR at baseline was similar between R780 and R-DEK NIKS (Fig. 4.2A) and C-SCC1 cells (Fig. 4.2B), the OCR after FCCP injection was increased with DEK overexpression (Fig. 4.2A-D). Therefore DEK
increases spare OxPhos capacity, i.e. the maximum rate of respiration possible, without affecting ATP production from OxPhos at baseline (Fig 4.2B and D). An increase in the maximum respiration rate with extra OxPhos potential is expected to support high energy demands during cellular growth stimulation. With regards to ECAR, DEK overexpression caused a 60% baseline increase in NIKS (Fig. 4.2E and F), and a 25% baseline increase in C-SCC1 cells (Fig. 4.2G and H). DEK also increased the maximal achievable rate of glycolysis without increasing the glycolytic reserve for each cell type (Fig. 4.2F and H). Overall, DEK increased baseline and maximum achievable glycolytic rates in both cell types and increased spare capacity for OxPhos.

**Metabolic end products of aerobic glycolysis accumulate in DEK overexpressing NIKS.**

The Seahorse data implicated DEK in the regulation of glycolysis and TCA cycle related metabolism. Therefore we utilized NMR-based metabolomics for in depth analyses of DEK-regulated metabolism. R780 and R-DEK NIKS were collected at 80% confluency, together with their respective media, in order to define metabolic profiles by NMR. Principal component analysis (PCA) separated DEK overexpressing cells (Fig. 4.3A) and their respective media (Fig. 4.3B) from the control cells and media. The significant differences spectra (SDS) identified regions of the NMR spectra that differed significantly between R-DEK versus R780 cells and media samples (Supp. Fig. 4.2A-B). Thus, the data revealed high DEK expression is sufficient to alter cellular metabolite profiles. DEK significantly altered 19 intracellular metabolites by fold changes in bucket intensity ranging from 0.6-1.6 (Fig. 4.3C, Supp. Fig. 4.2C). Increased intracellular metabolites included NAD⁺, proline, myo-inositol, alanine, and lactate. Decreased metabolites included glucose, 1-methylnicotinamide (1-MNA), phosphocholine (p-choline), aspartate, UDP-sugars, asparagine, glutamate, choline, phenylalanine, glycerophosphocholine (GPC), tyrosine, glutamine, threonine, and glutathione. We also detected significant changes in metabolite presence in the media of DEK overexpressing NIKS, including 6 metabolites that
were significantly decreased and 2 metabolites that were significantly increased as determined by the SDS (Fig. 4.3D and Supp. Fig. 4.2D). The 6 metabolites decreased were glucose, glycine, glutamate, formate, pyroglutamate (a spontaneous breakdown product of glutamine), and tyrosine. Glucose, glutamate, and tyrosine were lower in both the cells and the media suggesting intracellular consumption and uptake from the media. Two metabolites that were higher in the media were lactate and alanine, which were also increased in the cells suggesting intracellular production and excretion.

In order to better quantify consumption, production, uptake and excretion for each metabolite, we compared bucket intensities present in the conditioned media samples to the bucket intensities detected in F-media alone (unconditioned media). Net uptake into both R780 and R-DEK NIKS was observed for glucose, glycine, tyrosine, glutamate (Fig. 4.3E) and pyroglutamate (Supp. Fig. 4.2E) but with greater uptake into the R-DEK NIKS. Greater uptake along with an intracellular decrease in glucose, glutamate and tyrosine (Fig. 4.3C) suggests higher rates of consumption for these metabolites by R-DEK NIKS. In reverse, lactate and alanine levels were increased in R-DEK cells and increased in the media versus R780 (Fig. 4.3F), thus suggesting higher rates of production and excretion. The metabolites regulated by DEK overexpression are known participants in several metabolic pathways associated with cellular maintenance and growth. These include (1) glutaminolysis through glutamine and glutamate; (2) cell membrane maintenance through myo-inositol, GPC, choline, and p-choline; (3) cellular redox state through glutathione; (4) nucleotide synthesis through glycine, aspartate, glutamine, and formate; (5) protein synthesis through numerous amino acids; (6) one-carbon metabolism through glycine, and 1-MNA; and (7) glycolysis through glucose, lactate, alanine and NAD⁺; (illustrated in Supp. Fig. 4.2F). However, by far the most striking difference in the R-DEK NIKS versus R780 was increased uptake and consumption of glucose, and the concomitant production of lactate, alanine, and NAD⁺. These metabolites include the substrate and end products of aerobic glycolysis demonstrating that DEK promotes the utilization of
glucose-derived pyruvate to yield lactate and alanine (Fig. 4.3G). Therefore, the NMR metabolomics data validated increased ECAR measured by Seahorse analysis, and revealed a Warburg-like phenotype induced by DEK overexpression in immortalized keratinocytes.

**Metabolic end products of aerobic glycolysis accumulate in DEK overexpressing head and neck cancer cells.**

We next determined the effects of DEK overexpression on metabolic profiles in transformed C-SCC1 HNSCC cells. As was observed in the NIKS, high DEK expression was sufficient to alter metabolite presence both intracellularly and in the media based on PCA analysis (Fig. 4.4A-B) and SDS plots (Supp. Fig. 4.3A-B). Fold changes in intracellular metabolite bucket intensities spanned from 0.5 to 2.0 for 20 metabolites identified as significantly changed with DEK overexpression (Fig. 4.4C and Supp. Fig. 4.3C). Most of the 20 intracellular metabolites altered with DEK expression were increased and included the amino acids tyrosine, valine, glutamate, alanine, proline, taurine and asparagine along with other non-amino acid metabolites creatine, p-creatine, myo-inositol, GPC, NAD⁺, fumarate and succinate, with the last two being TCA cycle intermediates. The only metabolites that decreased with DEK overexpression were aspartate, GSH, choline, and p-choline in the C-SCC1 cells. Fold changes in media metabolite bucket intensities were modest and spanned from 0.9 to 1.13 for 19 metabolites (Fig. 4.4D and Supp. Fig. 4.3D). Most metabolites in the media were decreased by DEK overexpression and included mainly amino acids, along with pyruvate, 2-oxoisocaproate, fructose, and succinate (Fig. 4.4D). Seven metabolites in the media were increased by DEK overexpression and included myo-inositol, arginine, alanine, oxypurinol, ethanol, choline, and dimethylamine (DMA) (Fig. 4.4D). Comparison in bucket intensities of significantly changed media metabolites to those in unconditioned media showed that DEK alters the uptake and excretion of various metabolites (Fig. 4.4E-F and Supp. Fig. 4.3E). For instance, bucket intensities of alanine and DMA were higher in the R-DEK and R780 samples compared to
unconditioned media; albeit greater in the R-DEK samples suggesting DEK increases their production and excretion (Fig. 4.4F). As expected from the Seahorse ECAR data, among DEK-induced metabolites in the HNSCC cells were once again lactate, alanine and NAD⁺ (Fig. 4.4C) along with the excretion of alanine (Fig. 4.4F), suggesting increased glycolysis. These data suggest DEK overexpression can further drive aerobic glycolysis and lactate production in already transformed C-SCC cells (Fig. 4.4G).

**DEK overexpression stimulates the accumulation of TCA cycle intermediates in HNSCC cells.**

The most prominent difference in R-DEK compared to empty vector control C-SCC1 cells was an increase in the TCA cycle intermediates fumarate and succinate (Fig. 4.4C), and significant changes in amino acids that can feed into the TCA cycle such as tyrosine, phenylalanine, valine, isoleucine, aspartate and glutamate (Fig. 4.4C and G). R-DEK cells had increased tyrosine and valine intracellularly (Fig. 4.4C) as well as increased uptake from the media (Fig. 4.4E). Isoleucine and phenylalanine were also taken up from the media more so in the R-DEK C-SCC1 cells (Fig. 4.4E) although not altered in presence intracellularly. While the derivation and fate of these metabolites is unknown, they are all capable of converting into or having been converted from TCA cycle intermediates (Fig. 4.4G). Together these metabolite changes are likely reflections of alternative usage of TCA cycle intermediates for energy production and anabolic pathways such as lipid, protein and nucleotide synthesis. They may also be responsible for the large increase in spare capacity of OxPhos in the C-SCC1 cells. A map of DEK-regulated metabolites and the associated metabolic pathway are shown in Supp. Fig 4.3F.

**DEK overexpression affects similar and unique metabolic pathways in NIKS and C-SCC1 cells.**
In order to define metabolic pathways that are shared or unique between NIKS and C-SCC1 cells, we compared fold changes in metabolites induced by DEK overexpression between the cell lines (Fig. 4.5A and Supp. Fig. 4.4A-B). In NIKS and C-SCC1 cells, DEK increased the levels of intracellular lactate, alanine, and NAD’ (Fig. 4.5A), metabolites closely related to increased glycolysis (Fig. 4.5B). Lactate production was further validated in both cell lines by a colorimetric assay (Supp. Fig. 4.4C). DEK expression also consistently decreased choline, p-choline, aspartate, and GSH (Fig. 4.5A), metabolites involved in choline metabolism, protein and nucleotide synthesis, and oxidative stress reduction (Fig. 4.5C). DEK overexpression differentially regulated glutamate, tyrosine, asparagine, glucose, glutamine, valine, succinate, and fumarate between the cell lines (Fig. 4.5A). All were increased in C-SCC1 cells, and decreased in NIKS. While the cause for this differential abundance remains unclear, these metabolites are closely linked to the TCA cycle (Fig. 4.5E) which is vital for energy and macromolecule production. Therefore, DEK uniquely regulated the presence of TCA cycle intermediates in the C-SCC1 cells, consistently decreased GSH, choline, p-choline, and aspartate, and consistently increased glycolytic end products. In conclusion, the presence of TCA cycle intermediates and substantially decreased GSH may be a reflection of the oncogenic effects of DEK specific to cancer cells. In contrast, elevated aerobic glycolysis may be a ubiquitous consequence of DEK overexpression that can promote and sustain uncontrolled cellular growth.

**Discussion**

The Warburg effect is a metabolic hallmark of virtually all cancer cells, characterized by excessive conversion of glucose to lactate even in the presence of oxygen (201, 202). In contrast to an earlier notion that the Warburg effect is accompanied by a decline in OxPhos, more recent studies have shown that mitochondrial function including OxPhos is in general not impaired and in fact required by cancer cells (203-205). In HNSCC, there is plasticity of
metabolic states in which the leading edge of the tumor, with more oxygen availability, relies preferentially on OxPhos while the inner compartment prefers glycolysis (206). The shift and preference of metabolic pathways is often regulated by oncogene expression (207). While most of the metabolic studies of oncogenes have been conducted in cells that were already transformed, the consequences of oncogene expression in normal or immortalized cells are less well understood, particularly in human keratinocytes. Insights into early metabolic responses to oncogene expression offer potential avenues for prophylactic SCC prevention, either through metabolic enzyme modulation or dietary means. Here we overexpressed the human DEK oncogene and compared metabolic consequences in immortalized versus transformed keratinocytes. Interestingly, in both NIKS and C-SCC1 cells, DEK drives glycolysis and increases the maximum potential rate of oxidative phosphorylation.

The activation of cellular oncogenes such as MYC and HIF1α can de-regulate the transcriptional expression of metabolic enzymes like GLUT1/3, HK1/2 and lactate dehydrogenase A (LDHA) which are important drivers of glycolysis (208, 209). Interestingly, DEK knockdown repressed LDHA expression according to a previously published RNA sequencing study (Supp. Fig. 1A-B), and repressed other key metabolic enzymes that drive glucose metabolism in HNSCC such as FASN (210, 211), PDK1 (212), PKM2 (213-215), and HK2 (216). In further support of transcriptional regulation of metabolic enzymes by DEK, we identified a strong increase in DMA in the medium of DEK overexpressing C-SCC1 cells (Fig. 4.4D). DMA is produced by the enzyme dimethylarginine dimethylaminohydrolase (DDAH1), and RNA sequencing studies demonstrate strong repression of DDAH1 gene expression in two HNSCC1 cell lines in response to DEK depletion (Supp. Fig. 4.1). These data suggest DEK promotes the transcription of multiple metabolic enzymes and in this study, we observe aberrant regulation of metabolites known to be controlled by enzymes transcriptionally regulated by DEK.

In NIKS and C-SCC1 cells, NMR-based metabolomics identified shared metabolic signatures related to DEK overexpression. These included an increase in metabolites derived
from glucose including lactate, alanine, NAD$^+$ and myo-inositol (Fig. 4.3C and Fig. 4.4C). With regards to myo-inositol, a previous NMR study revealed significantly increased myo-inositol in HNSCCs from three different anatomical locations relative to normal human oral keratinocytes (217). Furthermore, in HNSCC, the H$^+$-myo-inositol transporter SLC2A13 was consistently increased in sphere-forming cells derived from primary oral SCC specimens suggesting myo-inositol transport is linked to oral SCC stem cells (218). We found DEK overexpression is sufficient to increase myo-inositol, a metabolite that is upregulated in HNSCC and its stem cells. This suggests DEK may regulate cancer stem cell metabolism, in line with data from breast cancer where DEK was required to sustain the growth of a stem cell population (21).

Additional shared metabolites identified by NMR between cell lines are lactate, NAD$^+$ and alanine (Fig. 4.3C-D and Fig. 4.4C-D). The NMR data in conjunction with functional metabolism data based on Seahorse analysis (Fig. 4.2E-H), and lactate assays (Supp. Fig. 4.4C) have established DEK promotes glycolysis. In NIKS, glucose was the most decreased metabolite in cells and in the media of R-DEK relative to empty vector samples, strongly suggesting DEK driven glucose metabolism and glycolysis are responsible for the observed increases in lactate and alanine. However, we cannot rule out the possibility that glutamine and other amino acids sources contribute to the production of lactate and alanine. In addition to driving baseline glycolysis, DEK also increased the maximum glycolytic potential in both NIKS and C-SCC1 cells. An increase in maximum glycolytic rate suggests DEK overexpressing cells are more fit to handle high proliferative rates. It is important to note that glycolysis was measured by ECAR which is not 100% induced by glycolysis. It has been previously shown that glycolysis accounts for $\sim$80% of total ECAR in a number of cancer cells as determined by removing glucose from the assay medium or adding glycolytic pathway inhibitors such as hexokinase inhibitor 2-DG and lactate dehydrogenase (LDH) inhibitor oxamate (219). The remaining 20% of the ECAR can be attributed to other metabolic processes, such as the TCA cycle CO$_2$ evolution (220). The ECAR estimate of glycolysis in our study is likely an underestimation of glycolytic rate since a buildup of alanine suggests
Pyruvate is also being converted to alanine instead of lactate, which does not contribute to ECAR. Increased glycolysis in HNSCC cells has been well documented in the literature (217, 221-224). Here, we note that glycolysis in C-SCC1 cells can be even further stimulated by DEK overexpression (Fig. 4.2G-H). Corresponding decreases in OxPhos were not observed. In fact, DEK overexpression increased the maximum achievable OCR in NIKS (Fig. 4.2A), and in C-SCC1 cells (Fig. 4.2C), thus increasing their potential to produce energy from both lactic fermentation, and OxPhos if required.

**DEK overexpression promoted the consumption of metabolites for nucleotide, protein and/or GSH synthesis.**

Glutamine, glutamate, aspartate, formate and glycine contribute to nucleotide synthesis by donating carbon and/or nitrogen atoms to purine and pyrimidine rings in de novo synthesis. In NIKS, DEK decreased intracellular glutamate, glutamine, and aspartate while promoting glycine uptake and repressing formate export (Supp. Fig. 4.2E). Glycine can also be utilized in one carbon metabolism via methylenetetrahydrofolate reductase (MTHF) and the methylation of proteins, nucleotides and lipids (Supp. Fig. 4.2F). Glutamate and glycine along with cysteine, produces GSH, a key cellular redox regulator, which was decreased with DEK overexpression in NIKS (Fig. 4.3C) and C-SCC1 cells (Fig. 4.4C). The observed decrease may reflect oxidation of GSH by reactive oxygen species, a possible indication of increased ROS in DEK overexpressing cells resulting from increased cellular metabolism. However, oxidized glutathione (GSSG) is not detected by NMR using our cellular extraction protocol, and therefore, GSSG production remains unproven. Interestingly, the decrease in GSH was far greater in the C-SCC1 cells, potentially caused by increases in fumarate in those same cells (Fig. 4.4C). Fumarate can bind GSH and convert it to succinated glutathione, which depletes NADPH levels and increases ROS (225).
Another metabolic consequence of DEK overexpression was decreased intracellular presence of cell membrane components choline and p-choline, along with differentially regulated levels of GCP (Fig. 4.5A and C). The decrease in choline compounds caused by DEK could indicate their increased incorporation into membranes or could be due to increased choline breakdown. While choline compounds are generally increased in other cancer types (226), and are considered biomarkers for breast cancer (227), we have observed a decrease in choline levels in C-SCC1 cells in line with another NMR study of oral SCC (228). Therefore, DEK overexpression is sufficient to decrease choline levels in keratinocytes and SCC cells.

Future studies comparing the effects of DEK overexpression in several isogenic cell lines will enable us to look for consistent changes in the ratios of metabolites that can be more reliable biomarkers of pathway regulation that individual metabolites. Possible metabolite ratios to look for based on published data and the metabolites identified in our samples include GPC:phosphocholine (229), glutamine:glutamate (230), lactate:glucose (201), and NAD⁺:NADH (231).

Taken together, we have identified a new role for DEK in governing cellular metabolism by increasing the rate of glycolysis and Oxphos potential, with a concomitant regulation of small metabolites involved in multiple anabolic pathways. This suggests that selection for DEK overexpression, a broad and early characteristic of solid tumors, may provide the required gains in energy and macromolecule production to enable uncontrolled cancer growth and progression. Future studies using stable isotope resolved metabolomics will define DEK-driven precursor-product relationships as a function of time, in order to uncover key enzymes which may themselves be targeted for cancer prevention and treatment.

Materials and Methods

Viral constructs and transductions
Human DEK was overexpressed using a retroviral R780 vector (22). Cells were incubated with virus for 4 hours in medium containing 2 μg/mL of polybrene (Sigma Aldrich, product-9268), then washed and overlaid with fresh medium. Transduced cells were sorted for GFP expression on a BD FACS Canto analyzer and expanded as a polyclonal population.

**Cell culture**

The spontaneously immortalized near-diploid human keratinocyte cell line (NIKS) (127) was maintained in F-media, which is 3 parts Dulbecco's modified Eagle's medium to 1 part Ham's F12 media (lifetechnologies, product-1176507) supplemented with the following components: 5% fetal bovine serum, 24 μg/mL adenine, 8.4 ng/mL cholera toxin (Millipore, product-227036), 10 ng/mL epidermal growth factor (Sigma Aldrich, product- e4127), 2.4 μg/mL hydrocortisone (Sigma Aldrich, product-h0888), 5 μg/mL insulin, 1% penicillin-streptomycin (Lifetechnologies, product-15140122), and 0.2% fungizone (Omegascientific). CCHMC-HNSCC1 (C-SCC1) head and neck cancer cells were cultured from an HPV positive, stage IV, tonsillar tumor obtained with IRB approval at the time of surgical resection (20). These cells were used between passages 10-15. Both cell lines were plated on irradiated 3T3-J2 mouse fibroblasts and maintained in F-media. NIKS and C-SCC1 cells were cultured until 80% confluency when few (<5%) feeders remained on the plate.

**Western blot analysis**

Cells were washed with PBS, and whole cell lysates were harvested with RIPA buffer (1% Triton, 1% deoxycholate, 0.1% SDS, 0.16 M NaCl, 10 mmol/L Tris pH 7.4, and 5 mmol/L EDTA) supplemented with a protease inhibitor cocktail (BD PharMingen- 554779) and analyzed as described previously (200). The DEK primary antibody is from BD Biosciences used at a 1:1000 dilution. (BD Biosciences, product 610948), and pan-actin (1:20,000) is a gift from
James Lessard. (Seven Hill Bioreagents, Cincinnati, OH, USA). Membranes were exposed to enhanced chemiluminescence reagents (Perkin Elmer).

**Cell counts**

200,000 NIKS were plated into 6-well plates. Cells were counted every 24 hours using trypan blue and a BioRad cell counter TC-20. C-SCC1 cell counts were determined similarly except 50,000 cells were plated into a 12 well plate. Error bars represent the standard error of the mean (SEM) from triplicate experiments.

**Flow Cytometry for cell cycle and apoptosis analysis**

For cell cycle analysis, NIKS were grown to 70–80% confluency in 6 well plates and pulsed with 10 mM EdU for 2 hours before collection by trypsinization. Cells were prepared using the Click-iT® EdU Alexa Fluor® 647 Imaging Kit (Lifetechnologies, product-C10424) according to manufacturer specifications. DNA content was determined using propidium iodide. Apoptosis was determined using FITC active caspase 3 antibody kit (BD Biosciences, product 550480) using manufacturer instructions. Cells were analyzed on a BD FACS Canto analyzer in biological triplicates (BD Biosciences, San Jose, CA). A t-test was used to determine significance between R780 and R-DEK samples from the independent experiments.

**MTS Assays**

R780 empty vector or R-DEK cells were plated in 10 wells of a 96-well plate at a density of 10,000 cells per well. The cells were allowed to grow for 24 hours. PMS was added to the MTS (Promega product G1112) reagent at a 1:50 dilution. Two hours later the plate was read on a spectrophotometer at an absorbance of 490 nm. Blank media readings were subtracted from the
The data are from three independent experiments and the readings were averaged across all three. Significance was determined by t-test. Error bars represent the SEM from the three independent experiments.

**NMR based metabolomics**

*Media Collection and Cell collection/extraction*

One million cells were plated in a 10 cm plate, media was replaced after 24 and cells were collected 24 hours later. For C-SCC1 cells only, 5 mL of new media was added to cells 4 hours prior to collection. A total of 8 replicates of NIKS and C-SCC1 R780 empty vector control cells and R-DEK overexpression vector cells were collected at 80% confluence. Due to sample loss during the NMR process, C-SCC1 R780 cells and C-SCC1 R-DEK media had 7 replicates. 10 mL of media were collected before cell extraction and centrifuged at 3500 g for 20 minutes at 4 ºC. Three milliliters per sample were collected into two Eppendorf tubes for further processing. Intracellular metabolites were extracted using the methanol-water direct scraping technique as previously described with few modifications (232). Briefly, after removing the growth media, cells were rinsed twice with 5 mL cold PBS on ice, then scraped with 1 mL of ice-cold 2:1 (v/v) methanol: water solution twice and transferred to a 2mL Eppendorf tube. After vortexing, the tubes were incubated on ice for 5 min and centrifuged for 5 min at 6000 xg at 4ºC. The supernatant, i.e. polar extract, was transferred into a pre-weighed new 1.5mL Eppendorf tube. The samples were dried in a SpeedVac centrifuge for 2-4 hrs and stored at -20 ºC. Prior to data collection, the dried polar cell extracts were resuspended in 600 μL of NMR buffer (100 mM potassium phosphate (pH 7.3), 0.1% sodium azide, 1 mM trimethylsilylproprionate (TSP) in 100% D$_2$O).

*Media sample processing*
On the day of the data collection, samples were thawed on ice and centrifuged 4000 xg for 5 min at 4 °C. The 500 µL supernatant of media samples were placed onto pre-washed 3 kDa spin filters (NANOSEP 3K, Pall Life Sciences), and centrifuged at 10000 x g for 90 min at 4 °C. The 400 µL of plasma filtrate was mixed with 200 µL of NMR buffer. In order to monitor the original metabolites supplemented to the cell, unconditioned media samples (F-media) were also prepared in the same manner. For NIKS, three replicates of F-media were prepared and for the C-SCC1s one sample was prepared since the replicates for NIKS showed no variation.

NMR Spectroscopy acquisition and processing

All experiments were conducted using 550 µL samples placed in 103.5 mm x 5 mm NMR tubes (Bruker). One-dimensional ¹H NMR spectra were acquired on a Bruker Avance II 600 MHz spectrometer. All data were collected at a calibrated temperature of 298 K using a three-pulse sequence based on the noesypr1d (or noesygppr1d) pulse sequence in the Bruker pulse sequence library. This pulse sequence provided water-suppression with good baseline characteristics. Experiments were run with 8 dummy scans (DS) and 256 (cell extract) and 128 (media) acquisition scans (NS) with an acquisition time (AQ) of 3.4 s and a relaxation delay (D1) of 2.0 s for a total repetition cycle (AQ+D1) of 5.4 s. The mixing time was 10 ms. The spectral width was 16 ppm, and 64K real data points were collected. Under automation control, each sample analysis took about 10 minutes for setup and 30 minutes for acquisition. During the 10 minute setup time, the temperature was monitored for equilibration. All FIDs were subjected to an exponential line-broadening of 0.3 Hz. Upon Fourier transformation, each spectrum was manually phased, baseline corrected, and referenced to the internal standard TSP at 0.0 ppm for polar samples using Topspin 3.5 software (Bruker Analytik, Rheinstetten, Germany). For two dimensional ¹H-¹H total correlation spectroscopy (TOCSY) data, a relaxation delay equal to 2 s, an isotropic mixing time of 80 ms at a B₁ field strength of 10 kHz were used for 2048 data points with 128 scans per increment were acquired with spectral widths of 14 ppm.
Metabolomics Data Analysis

Multivariate statistical analysis

Principal components analysis (PCA) was performed to look for metabolic differences using AMIX 3.9.15 software (Bruker Analytik, Rheinstetten, Germany). \(^1\)H NMR spectra were processed and analyzed with AMIX for PCA analysis. The spectra from 0.5 to 10.0 ppm, excluding the region of the residual water resonance (4.6 to 5.0 ppm) and methanol (3.36-3.38 ppm), were reduced by uniform binning to 995 buckets 0.01 ppm wide. The spectra were normalized to constant total spectral area. Prior to PCA analysis, the binned spectra were mean-centered with no scaling. PCA scores were exported and scores plots were generated using Microsoft Excel.

Univariate analysis

The spectral bucket intensities tables were further analyzed using a univariate approach, based on bin-by-bin differences between two groups. In order to identify the NMR spectral regions that are significantly different between the groups, \(^1\)H significant difference spectra (SDS) were generated (233, 234). Pairwise differences within each bin were compared using Student’s t-test. The false discovery rate (FDR) was controlled at 0.05 level using the Benjamini-Hochberg method (235). The SDS plot was generated by taking the mean difference of the buckets with significant differences between the two groups. Metabolites were assigned to those significant buckets based on the chemical shifts as described below. Fold changes were calculated by dividing the mean of one group bucket intensity over the mean of another: fold change = \text{mean}(R780-DEK)/\text{mean}(R780).

Spectral analysis/metabolites identification
Metabolites found in cell extract and media were assigned based on 1D $^1$H and 2D $^1$H-$^1$H TOCSY NMR experiments. Peaks were assigned by comparing the chemical shifts and spin-spin couplings with reference spectra found in databases, such as the Human Metabolome Database (HMDB)(236), the Madison metabolomics consortium database (MMCD)(237), the biological magnetic resonance data bank (BMRB)(238), and Chenomx® NMR Suite profiling software (Chenomx Inc. version 8.1)

**Lactic acid assay**

A lactic acid determination kit was purchased from biovision (K607-100) to detect. L(+)-lactate in the media of NIKS and C-SCC1 cells. The manufacturer’s protocol was followed with the following specifications. 250K Cells were plated in a six well dish in triplicate wells overnight. The next day cells were overlaid with 1mL of 1x DMEM with no FBS and media samples were collected after 30 minutes. 20 μl of the media collected was loaded in duplicate in a 96 well plate and measured on a spectrometer at absorbance 570. Significance was determined using a student’s t-test to compare R780 to R-DEK samples from three independent experiments.

**Seahorse XFe96 metabolic flux analysis**

Extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) for NIKS and C-SCC1 R780 and R-DEK were determined using the Seahorse Extracellular Flux (XFe96) analyzer (Seahorse Bioscience, MA, USA). NIKS were seeded at 30,000 cells per well and C-SCC1 at 50,000 cells per well into XFe96 well cell culture plates and incubated for 16 h in F media at 37°C in a 5% CO$_2$ humidified atmosphere. For ECAR, cells were washed in XF assay media. For OCR, cells were washed in XF assay media supplemented with pre-warmed 10 mM glucose, 1 mM Pyruvate, 2 mM L-glutamine adjusted to 7.4 pH). Cells were then kept in 175 μL/well of XF assay media at 37°C, in a non-CO2 incubator for 1 hr. During the cell incubation time, 9 μM oligomycin, 10 μM oligomycin, 9 μM FCCP, 10 μM rotenone, 10 μM antimycin A in
XF assay media were loaded into the injection ports in the XFe96 sensor cartridge. Data sets were analyzed by XFe96 software and GraphPad Prism software, using FDR correction and t-test calculations. The experiment was performed 4 times with 4-6 replicates in each experiment. One experiment was chosen that best represented the trends seen across all experiments.

Acknowledgements

Funding: This work was supported by the National Institutes of Health R01-CA116316 to S.I.W.; the University of Cincinnati Cancer Institute Head & Neck Comprehensive Center Pilot Grant Program 2014; Carmen L. Buck endowment (to ANL) and 1U24DK097215-01A1 to ANL. All flow cytometric data were acquired using the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children’s Hospital Medical Center and supported in part by the Digestive Health Center NIDDK P30 DK078392. All NMR-based metabolomics data was acquired and analyzed within the NMR-Based Metabolomics Core, a Cincinnati Children’s Research Foundation Shared Facility, in the Division of Pathology and Laboratory Medicine at CCHMC. We thank Dr. James Lessard for the monoclonal actin antiserum, and Eric Smith, Lisa Privette-Vinnedge, Allie Adams, and Elizabeth Hoskins for proofreading and editing.
Figure 4.1. DEK overexpression increases cellular metabolic activity in NIKS and C-SCC1 cells in the absence of proliferative gains. (A-B) Western blot analysis validates DEK overexpression in NIKS (A) and C-SCC1 (B) cells with accompanying phase/light microscopy images taken at 10x magnification with a 20x inset of cells transduced with retroviral vector R780 control or R780-DEK (R-DEK). (C-D) Equal numbers of NIKS (C) and C-SCC1 (D) R780
or R-DEK cells were plated and counted over 4 days in triplicate experiments. (E-F) Cell cycle profiles quantified by flow cytometry in NIKS (E) and C-SCC1 (F) R780 and R-DEK cells pulsed with EdU for 2 hours and stained with propidium iodide. The percentage of cells in each phase of the cell cycle was quantified from triplicate wells in three independent experiments. (G-H) Flow cytometry analysis of cleaved caspase 3 for the detection of apoptosis in NIKS (G) and C-SCC1 (H) R780 versus R-DEK cells. (I-J) Fold change in absorbance at 490 nm for MTS assay from 10,000 NIKS (I) or C-SCC1 (J) cells plated in a 96-well plate.
Figure 4.2: DEK overexpression increases glycolysis and the maximum rate of oxidative phosphorylation in NIKS and C-SCC1 cells.
Seahorse XF24 Extracellular Flux Analyzer experiments using the mitochondrial stress test. (A-D) Quantification of oxygen consumption rate (OCR) measurements from 4 replicates of NIKS (A) and C-SCC1 (C) R780 and R-DEK samples taken three times at baseline and after treatment with the following pharmacological inhibitors of metabolism: oligomycin (ATP synthetase inhibitor), FCCP (and uncoupling agent), and rotenone and antimycin A (electron transport chain inhibitors). (B and D) Calculations from the mitochondrial stress test were as follows: non-mitochondrial respiration = oxygen consumed after treatment with electron transport chain inhibitors. Basal OCR = baseline OCR minus non-mitochondrial respiration. ATP production = baseline OCR minus OCR after ATP synthetase inhibitor (oligomycin). Spare capacity = max OCR minus baseline OCR. Proton leak = OCR with oligomycin minus OCR with electron transport chain inhibitors. (E-H) The extracellular acidification rate (ECAR) was quantified for NIKS (E-F) and C-SCC1 (G-H) transduced with R780 or R-DEK. Quantification of glycolysis was calculated for baseline, maximum potential, and reserve potential in NIKS (F) and C-SCC1 (H). Reserve ECAR was calculated by subtracting baseline ECAR from maximum ECAR (oligomycin treated). Error bars represent the SEM of the 4 replicates.
Figure 4.3. DEK overexpression increases the metabolic end products of glycolysis and the utilization of amino acids in keratinocytes. (A-B) Principal components analysis (PCA) scores plots of NIKS generated from normalized bucket intensities for 8 replicates showing separation based on metabolite presence between NIKS R780 (grey) and R-DEK (black) cells (A) and in their respective conditioned media (B). (C-D) Fold change in bucket intensities for each metabolite that was significantly different between R780 and R-DEK cells (C) and in their respective conditioned media (D) is arranged by magnitude of change. Metabolites in red are increased by DEK overexpression and metabolites in blue are decreased by DEK overexpression. (E-F) The bucket intensity of metabolites averaged from triplicate samples of unconditioned media (dashed red line) were compared to R780 (grey) and R-DEK (black) conditioned media samples to identify metabolites that are decreased (E) and increased (F) compared to unconditioned control media. (G) Metabolic pathway schematic highlighting metabolites identified by NMR which were differentially regulated by DEK overexpression. The pathway analysis reveals many of metabolites increased upon DEK expression are products of aerobic glycolysis. Abbreviations: 1-MNA = 1 methylnicotinamide, p = phospho, Asn = asparagine, Phe = phenylalanine, GPC = glycerophosphocholine, NAD⁺ = nicotinamide adenine dinucleotide, Myo-ino = myo-inositol, Glu = glutamate, Gln = glutamine, α-keto = α-ketoglutarate.
Figure 4.4. DEK overexpression in HNSCC cells increases glycolytic end products and TCA cycle intermediates. (A-B) PCA scores plot of C-SCC1 generated from normalized bucket intensities showing separation by metabolite presence between C-SCC1 R780 and R-DEK cells (A) and media (B). (C-D) Fold change in bucket intensities for each significantly changed metabolite between R780 and R-DEK cells (C) and media (D) arranged by magnitude of change. (E-F) The bucket intensity of metabolites in the unconditioned media (dashed red line) were compared to R780 (grey) and R-DEK (black) conditioned media samples to identify metabolites decreased (E) and those increased compared to control unconditioned media (F). (G) Metabolic pathway analysis highlighting metabolites identified by NMR that are differently regulated upon DEK overexpression. The metabolites identified are involved in various metabolic pathways including choline metabolism, protein and nucleotide synthesis, cellular redox state, aerobic glycolysis, and the TCA cycle. Abbreviations: p = phospho, Asn = asparagine, Phe = phenylalanine, GPC = glycerophosphocholine, NAD\(^+\) = nicotinamide adenine dinucleotide, Myo-ino = myo-inositol, Glu = glutamate, Gln = glutamine, α-keto = α-ketoglutarate, 2-OIC = 2-oxoisocaproate, Poly-Glu = polyglutamate, DMA = dimethylamine.
Figure 4.5: DEK overexpression drives glycolytic and glutathione pathways in NIKS and C-SCC1 cells, but uniquely stimulations TCA cycle intermediate accumulation in C-SCC1 cells. (A) Fold change in R-DEK compared to R780 bucket intensities for metabolites identified in NIKS (grey bars) and C-SCC1 cells (black bars). Metabolite names labelled in blue are decreased and those in red are increased in both cell lines. Metabolites labelled in green are either jointly but differentially regulated or only regulated in one cell line; in either case, the metabolite is higher in the C-SCC1 cells and/or lower in the NIKS with DEK overexpression. (B-E) Metabolites regulated in one or both normal/cancer cells are indicated within known
associated metabolic pathways. (B) Metabolite products of aerobic glycolysis are increased in both cell lines (red) with an increase in glucose uptake in the R-DEK NIKS. (C) Glutathione and aspartate are decreased in both cell lines while metabolites surrounding this pathway are decreased in NIKS (green) and increased in C-SCC1 cells. (D) Choline and p-choline are decreased in both cell lines while GPC was differentially regulated. (E) Metabolites in and surrounding the TCA cycle are increased in the C-SCC1 cells (green) and either unchanged or decreased in the NIKS. Abbreviations: p = phospho, GPC = glycerophosphocholine, NAD$^+$ = nicotinamide adenine dinucleotide, α-keto = α-ketoglutarate, OAA = oxaloacetate, ROS = reactive oxygen species.
Supplemental Figures

A

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B

Supplemental Figure 4.1: DEK knockdown in HNSCC cell lines decreases the transcription of metabolic enzymes. (A) The fold change in relative transcript expression of genes encoding metabolic enzymes identified from a previously published RNA sequencing experiment in two HNSCC cell lines (UMSCC1 and UMSCC47) transduced with DEKsh or NTsh control lentiviral vectors (94). The indicated genes were decreased with DEK knockdown in both cell lines and are involved in multiple metabolic pathways. (B) The fold change in mRNA encoding metabolic enzymes from DEKsh compared to NTsh cells represents the average between the two HNSCC cell lines.
Supplemental Figure 4.2: DEK overexpression in NIKS significantly alters metabolite uptake and utilization. (A) Significant differences spectra of metabolites identified in NIKS cells and (B) NIKS media created from normalized bucket intensities with statistically significant differences. Significance is determined by Welch’s test with false discovery rate (FDR) correction. (C) Altered metabolites with corresponding spectral position (bin centers) and their fold changes as well as p-value (t-test) is reported for NIKS and (D) NIKS media. (E) Bucket intensities of metabolites in R780 and R-DEK samples after subtraction of the bucket intensities of metabolites in the unconditioned media. Metabolites in orange are increased, and metabolites in green are decreased in the R-DEK samples compared to unconditioned media. (F) A metabolic pathway schematic of significantly altered metabolites in NIKS with DEK overexpression. Metabolites intracellularly decreased (blue), increased (red), and those taken up from the media (green) or excreted into the media (orange) are identified within their
associated metabolic pathways. DEK overexpression regulates metabolites involved in glutaminolysis, cell signaling and membrane maintenance, cellular redox state, nucleotide synthesis, protein synthesis, methylation, and glycolysis.
Supplemental Figure 4.3: DEK overexpression in C-SCC1 significantly alters metabolite uptake and utilization. (A) Significant differences spectra of metabolites identified in C-SCC1 cells and (B) media created from normalized bucket intensities with statistically significant differences. Significance is determined by Welch’s test with FDR correction. (C) Altered metabolites with corresponding spectral position (bin centers) and their fold changes as well as p-value (t-test) is reported for C-SCC1 cells and (D) media. (E) Bucket intensities of metabolites in R780 and R-DEK samples after subtraction of the bucket intensities for each metabolite in the unconditioned media. Metabolites in orange are increased, and metabolites in green are decreased in the R-DEK samples compared to unconditioned media. (F) A metabolic pathway schematic of significantly altered metabolites in C-SCC1 cells with DEK overexpression. Metabolites intracellularly decreased (blue), increased (red), and those taken up from the media (green) or excreted into the media (orange) are identified within metabolic pathways.
Supplemental Figure 4.4: DEK overexpression affects unique metabolites in NIKS and C-SCC1 cells but consistently increases lactate. (A) Fold change in bucket intensities for metabolites only identified in NIKS and (B) C-SCC1 cells. (C) Colorimetric lactic acid assay comparing lactate production from NIKS and C-SCC1 R780 and R-DEK cells.
Chapter 5- Generation of a tetracycline responsive Dek transgenic mouse model for HNSCC studies.

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Keywords: Dek, inducible transgene expression, 4NQO, head and neck cancer

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 Manuscript in preparation
Abstract

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common malignancy worldwide with a poor survival rate and the need for improved treatments. A potential therapeutic target is the DEK oncogene that is overexpressed in a number of malignancies including HNSCC. DEK is a chromatin-associated protein that functions in several nuclear processes including gene transcription, epigenetics, and DNA repair, and plays an important role in stimulating cellular growth. Importantly, previous data obtained in our laboratory suggested Dek is required for benign papilloma growth and malignant HNSCC tumor growth using a Dek knockout mouse model. However, the contribution of DEK overexpression to tumorigenesis is unknown due to a lack of in vivo models.

Here we generated Dek transgenic mice that express tetracycline responsive Dek and luciferase via a tetracycline response element coupled to two bi-directional mini CMV promoters (Bi-L-Dek mice). Bi-L-Dek mice were crossed with keratin 5 (K5) driven tetracycline transactivator (tTA) mice targeting expression to the basal keratinocytes of stratified squamous epithelium. Dek and luciferase expression are repressible with the administration of tetracycline or its analog doxycycline. We validated Dek overexpression and repression with doxycycline in the squamous epithelium of the tongue, esophagus and skin. To determine the contribution of Dek overexpression to HNSCC development we subjected the mice to a 4NQO-driven HNSCC carcinogenesis protocol followed by assessment of tumor incidence and growth.
Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common malignancy worldwide with 600,000 new cases a year, and little improvement on survival has been made over the past decade (239). HNSCC is associated with alcohol and tobacco use or infection with the human papillomavirus (HPV). Surgery and high dose chemo-radiation remain the standard of care, often causing irreparable facial disfiguration and loss of the ability to speak, chew or swallow (96). In order to enhance survival and improve quality of life, we must identify and exploit new therapeutic targets.

One potential target, the DEK oncogene, is overexpressed in a number of malignancies including HNSCC. In collaboration with head and neck surgeons at the University of Cincinnati, our laboratory discovered that DEK is highly expressed in patient derived head and neck tumor specimens taken from different anatomical origins compared to neighboring normal tissue (20). DEK is a chromatin-associated protein that functions in several nuclear processes. It regulates genome structure, gene transcription, DNA repair and plays an important role in stimulating cellular growth (22, 44, 45, 54, 55, 59, 62, 64, 72, 74, 77, 90, 93, 94, 119, 124). The overexpression of DEK in keratinocytes causes hyperplasia and dysplasia in the context of in vitro organotypic epithelial raft cultures which mimic the layers of skin (25). These and other data suggest DEK overexpression is sufficient to increase cellular growth and inhibit cell differentiation, two key processes in the development of cancer. Conversely, blocking DEK expression in various cancer cells diminishes cellular growth and causes cancer cell death while normal cells are only minimally affected (22). Thus, DEK plays an important role in cancer cell growth, and its targeted inhibition induces selective cancer cell death.

Previous data obtained in our laboratory suggested Dek is required for benign and malignant tumor growth and could potentially be targeted in cancer. In vivo, Dek-/- mice are partially resistant to the formation of benign papillomas when treated with DMBA and TPA a tumor initiator and promoter respectively (22). Xenograft mouse models show that DEK
overexpression in cooperation with classical oncogenes can increase tumor burden and DEK silencing results in apoptosis (22). Furthermore, DEK+/+, HPV E7 transgenic mice when treated with the carcinogen 4NQO, to initiate and promote head and neck tumors, develop large tumors in their esophagus and oral cavity that eventually lead to their death. Impressively, HPV E7_Dek knockout (Dek-/-) mice were protected from gross tumor formation, thus exemplifying the importance of Dek expression for tumor growth (20). Together, this data suggests Dek plays a role in tumor promotion and is needed for uncontrolled cellular growth. However, the effects of DEK overexpression on tumor growth are unknown due to lack of reported transgenic mouse models.

Herein, we generated Bi-L-Dek transgenic mice that harbor a tetracycline-inducible transgene that expresses both Dek and firefly luciferase. When we crossed Bi-L-Dek mice to a K5-tTA transgene-inducing line of mice, which expresses a tetracycline-responsive transactivator selectively in the stratified squamous epithelia, the resulting Bi-L-Dek/K5-tTA bi-transgenic mice expressed Dek and luciferase in the stratified epithelium of skin, tongue and esophagus, and doxycycline repressed this expression (tet-off). The system was validated by qRT-PCR, IVIS, western blot, flow cytometry, and immunohistochemistry and followed by studies of HNSCC in the presence and absence of doxycycline. A 4NQO-driven HNSCC study with Bi-L-Dek_K5-tTA mice has been completed and partial analysis of the results suggests Dek increases proliferation of tongue and esophageal epithelium, as well as increases gross esophageal tumor number and volume.

Results

Generation of tetracycline responsive Dek transgenic mice

In order to overexpress Dek in a tissue specific manner, Dek cDNA was cloned into the pBi-L-Tet expression vector containing a tetracycline response element (TRE) to yield pBi-L-Dek (Fig. 5.1A). The TRE also controls luciferase expression thus allowing for easy transgene
detection and a method to validate the construct *in vitro* and *in vivo*. The pBi-L-Dek expression vector was transfected into mouse keratinocytes which expressed the K5-tTA transgene. PBi-L-Dek transfected keratinocytes overexpressed the Dek protein and Dek overexpression could be repressed with doxycycline (Fig. 5.1B). Similarly, luciferase expression was also detected in these cells that could be repressed with doxycycline in a dose dependent manner (Fig. 5.1C). After confirmation that the Bi-L-Dek plasmid expressed Dek as expected we cut the plasmid with two restriction enzymes, AatII and AseI to linearize the DNA and remove unwanted sequences (Fig. 5.1D). The restriction enzymes cut pBi-L-Dek into the expected sizes and the 5247bp band (Bi-L-Dek) was isolated from the gel. The Bi-L-Dek band contained the DNA sequences for the TRE connected to two bi-directional mini CMV promoters controlling Dek and luciferase expression and the poly A tails on each end (Fig. 5.1E). Bi-L-Dek was randomly inserted into the genome using pronuclear injection. Four Bi-L-Dek founders were confirmed by genotyping (Fig. 5.1F and S5.1) and founder 317 was used for all subsequent experiments based on maximal Dek expression. The Bi-L-Dek transgene copy number of founder 317 stabilized in the F2 generation with roughly 4 transgene insertions (Fig. 5.1G). Therefore, we generated a tetracycline responsive, Dek transgenic mouse model.

**Targeting Dek overexpression to the stratified squamous epithelium**

In order to overexpress the Bi-L-Dek transgene we bred the Bi-L-Dek mice to keratin 5 controlled tetracycline transactivator mice (K5-tTA; Fig. 5.2A). Genotyping confirmed transmission of both transgenes to offspring, producing Bi-L-Dek_K5-tTA mice (Fig. 5.2B). The K5 promoter is endogenously expressed in the basal layer of stratified squamous epithelium (Fig. 5.2C) including that of the tongue, esophagus, and skin. The tTA protein will bind to the TRE in the Bi-L-Dek transgene and turn on Dek and luciferase expression in the basal layer of stratified squamous epithelium from which all suprabasal layers are derived, and expression is repressed with doxycycline (Fig. 5.2C).
Bi-L-Dek_K5-tTA mice overexpress Dek in stratified squamous epithelium and expression is repressed by doxycycline.

In order to confirm Dek overexpression the Bi-L-Dek_K5-tTA mice, we isolated RNA and protein from the skin of the flank of mice. Dek mRNA levels were induced 3.5 fold over endogenous levels and were repressed to endogenous levels with doxycycline (dox) in their food (Fig. 5.3A). Increases in Dek protein expression were also observed in flank skin of Bi-L-Dek_K5-tTA mice (Fig. 5.3B). Immunohistochemistry (IHC) of the tongue sections also showed increases in Dek expression in the Bi-L-Dek_K5-tTA mice over single transgenic control mice and the expected decrease in the corresponding mice on dox chow (Fig. 5.3C). In vivo imaging systems (IVIS) confirmed luciferase expression in the skin of Bi-L-Dek_K5-tTA bi-transgenic mice but not Bi-L-Dek only mice (Fig. 5.3D). Lastly, ex vivo imaging of the flank, ear and esophagus of Bi-L-Dek_K5-tTA mice showed luciferase expression in Bi-L-Dek_K5-tTA (Fig. 5.3E). Altogether, we have shown that Bi-L-Dek_K5-tTA mice overexpress Dek in the skin, tongue, and esophagus and its expression is repressible by doxycycline.

Dek expression in Bi-L-Dek_K5-tTA mice is detected in the absence of endogenous Dek expression

To better quantify the level of Dek overexpression in the Bi-L-Dek_K5-tTA mice, we assessed the extent of exogenous Dek expression in the absence of endogenous Dek expression. Bi-L-Dek and K5-tTA transgenic mice were interbred with Dek-/- mice (Fig. 5.4A) to generate Bi-L-Dek_K5-tTA mice in a Dek-/- background. Exogenous Dek expression in the epidermis was confirmed by qRT-PCR and western blot analysis (Fig. S5.2A; Fig. 5.4B). Dek mRNA in the Dek-/- Bi-L-Dek_K5-tTA mouse was about 4 fold over Dek+/+ mice (Fig. S5.2A). Dek protein expression approximated endogenous Dek levels from Dek+/+ mice (Fig. 5.4B). Detection of exogenous Dek protein was also observed in the esophagus by IHC (Fig. 5.4D).
The *Dek/-* _Bi-L-Dek_K5-tTA_ mice expressed luciferase as expected. We were able to show that Dek mRNA levels are 3-4 fold increased in the _Bi-L-Dek_K5-tTA_ mice and protein levels are up 2-3 fold.

**Further validation of a functioning Bi-L-Dek transgene is confirmed in the brain.**

To confirm the utility for using the _Bi-L-Dek_ transgenic mice in other organ systems, Bi-L-Dek mice were bred to _Dxl5/6-tTA_ mice resulting in tTA driving Dek expression in the brain (Fig. S5.3A-B). As expected, we detected Dek overexpression in the cortex and striatum of post mitotic neurons, further validating the _Bi-L-Dek_ mouse model (Fig. S5.3C).

**Keratinocytes isolated from Bi-L-Dek_K5-tTA_ mice have increased Dek expression and proliferation.**

Next, we characterized mouse keratinocytes isolated from _K5-tTA_, _Bi-L-Dek_, and _Bi-L-Dek_K5-tTA_ mice with and without dox treatment. The keratinocytes have similar morphology despite transgene expression or dox treatment (Fig. 5.5A). Isolated keratinocytes were used for flow cytometry experiments to detect Dek expression, cell cycle profiles, and cleaved caspase 3. Flow cytometry confirmed Dek overexpression in the _Bi-L-Dek_K5-tTA_ isolated keratinocytes and Dek repression in those treated with dox (Fig. 5.5B-C). Dek levels in the control samples were unaffected with dox treatment as expected (Fig. 5.5B). The cell cycle profiles demonstrated increased EdU incorporation in the _Bi-L-Dek_K5-tTA_ keratinocytes suggesting these cells are more proliferative (Fig. 5.5D-E). Dox treated cells had less EdU incorporation compared to their untreated counterparts but higher than the single transgenic controls suggesting dox may not fully repress Dek expression or Dek induced phenotypes. We did not observe any significant changes in apoptosis between _Bi-L-Dek_K5-tTA_ and single transgenic keratinocytes independent of dox treatment (Fig. 5.5F). Lastly we used isolated keratinocytes for immunofluorescence with antibodies for Dek, K5, and DAPI. First, K5 staining confirmed the
cells were basal keratinocytes (Fig. 5.5H). Second, expression of Dek was higher in the Bi-L-Dek_K5-tTA keratinocytes compared to those treated with dox or the single transgenic cells (Fig. 5.5G-H). Lastly, we observed Dek retention on chromosomes during metaphase and anaphase chromosomes undergoing mitosis in the Bi-L-Dek_K5-tTA keratinocytes, a phenotype that was previously associated with high human DEK expression (Fig. 5.5I).

In all, the Bi-L-Dek_K5-tTA mice were validated for the conditional overexpression of Dek in stratified squamous epithelium of skin, tongue, and esophagus (Fig 5.3 B,C,E; Fig. 5.5G-H; and Fig. 5.4B,D). Phenotypes already associated with Dek overexpression in vitro were observed in the Dek overexpressing mice such as increased proliferation and retention on mitotic chromosomes.

**Determining the contribution of Dek overexpression to HNSCC development.**

Keratinocytes are the cell of origin for squamous cell carcinoma, therefore we used our Dek transgenic mouse model to determine the significance of Dek overexpression in HNSCC development. Bi-L-Dek, K5-tTA, and Bi-L-Dek_K5-tTA mice plus minus dox were given 4NQO to initiate oral lesions making them susceptible to HNSCC (Fig. 5.6A). At 6 weeks of age, mice were given 4NQO at 10ug/mL in their drinking water for 16 weeks. After 16 weeks, mice were given normal water and monitored until death or sacrificed at 45 weeks or earlier at the recommendation of veterinary services (Fig. 5.6B). If mice were sacrificed, they were injected with BrdU one hour before death to detect proliferative differences in the mice. Visible tumors were counted, measured with calipers, and embedded along with tongue, esophagus, stomach, and lungs, then sectioned and stained by H&E. A full histological analysis by a pathologist is underway and results thus far suggest the Bi-L-Dek_K5-tTA mice have significantly more gross esophageal tumors (data not shown) than their dox treated counterparts. These data suggest Dek overexpression supports tumor growth. Other phenotypes induced by Dek overexpression will be identified with further analysis.
Discussion

Many *in vitro* studies have proven the importance of DEK overexpression in various cancers and at various stages of the disease. However, it is not known how DEK overexpression contributes toward tumorigenesis *in vivo*. To address this gap in our understanding of DEK, we have generated a much needed mouse model to study the effects of Dek overexpression on cancer and other diseases.

Future efforts in the Wells’ laboratory will go toward completing the analysis of 4NQO treated *Bi-L-Dek_K5-tTA* mice. This involves determining differences in animal survival, tumor incidence, tumor volume, and the existence of metastasis in mice with or without dox chow. Tongue, esophagus, and stomach squamous epithelium will be closely examined for tumors by histological analysis and lungs will be inspected for metastasis. Human DEK overexpression related phenotypes such as increased proliferation and inhibition of apoptosis will be evaluated by IHC using antibodies against BrdU and cleaved caspase 3.

This and future studies will help elucidate fundamental functions of DEK and provide an *in vivo* model system to test *in vitro* DEK overexpression phenotypes. Importantly, Dek overexpression can be repressed in this mouse model offering an opportunity to test the therapeutic effect of Dek loss after tumor formation. The design of the TRE system in the *Bi-L-Dek* mice will allow for a plethora of breeding schemes with tissue specific tTA (or rTA) expression to elucidate the effects of Dek overexpression in disease development and progression.

Materials and Methods

*K5-tTA Mouse Keratinocytes used for pBi-L-Dek expression confirmation studies:*

Keratinocytes were derived from a keratin 5 promoter–driven tet repressor–VP16 transgene (*K5-tTA*) mouse previously published (240). They were grown in E-media with 0.05 mM Ca2 and supplemented with 15% serum as previously published (241).
**Luciferase experiments:**
Luciferase assay was performed using the Dual-Luciferase® Reporter Assay System from Promega and following manufacturer specifications.

**Generation of Bi-L-Dek Transgenic Mice:**
The mouse Dek (mDek) DNA sequence was excised from the previously published R780 retroviral vector (Lisa) by using restriction enzymes Sall and Notl. MDeK was cloned into the pBi-L plasmid from clontech using Sall and Notl. The pBi-L Tet Vector (GenBank Accession No.: U89934. Catalog No. 631005) is a response plasmid used to express Dek and luciferase from a bidirectional tet-responsive promoter. The pBi-L Tet Vector contains the bidirectional promoter Pbi-1 which is responsive to the tTA regulatory protein in the Tet-Off system. The Tet-responsive element (TRE), which consists of seven copies of the 42-bp tet operator sequence (tetO) is between two minimal CMV promoters, which lack the enhancer that is part of the complete CMV promoter. Consequently, gene expression is silent in the absence of binding of the tetracycline tranactivator to the tetO sequences. From the pBi-L-Dek plasmid the DNA insert used to make the Bi-L-Dek transgenic mice was excised using the restriction enzymes AatII and Asell. The resulting 5247bp (Bi-L-Dek) DNA sequence was isolated and purified from the gel. Bi-L-Dek was microinjected into the pronucleus of a fertilized egg and inserted into a pseudopregnant mouse to produce Bi-L-Dek founders. Founders were mated with wild type FVB/N mice to determine the ability of the transgene to pass to offspring. Pups from this F1 generation where then mated with K5-tTA and F2 on mice were further characterized. The mouse Dek sequence cloned into pBi-L-Tet:

ATGTCGGCGGCGGCCGCTGGAGGAGGACGCCCCCGTGGCGCC
TCATCCGAGGAGGAGGAGGAGGAGGAGGAAGTCTAATCGT
GGAAGGCAAGAGAGAGAAGAAGAAAGTAGAGAGAGACTGAGACTGAGTGGCAAGTGTCTTCTTCTTACA
GAGAGAGGCATTTACAGTGACACAAGGGAAGGGTCAGAAACTTTGTGAAATTGAAAGGATA
CATTTCTTTCTGAGTAAGAAAAACCAGATGAACTTAGAAATCTACACAAACTGCTTTACAA
CAGGCCGGGCACAGTGTCTCGTTGAAGAAGAACGTTGCGAGTCAGGTCAGTGGCCTTTCCATT
CGAAAAAGGCAGTACCCAGTATAAAAAAGGAAGAGAAATGGTGGAAAAAGTTTCGAAGATGCC
ATGTTAAGAGCAGGCTGTTACGATTCTTTGATTTAGGAGGTAAGTGGCTTTCCATT
TGAGAGGATCTCTGAACTTTCTTTAAGATGACCATCCAAAGCCTTCTGGCAAAACCATTACAAAGGTCC
AAAAATCTCTCCAGCAAAAGCTAGAAAAAGGAACGAGGTCTCTGGCAAAACAAGAGGAAAT
CAAGCAAACTAAATGCCCCTGAAAATTTGCTGAGATGAGCTTAGCTAGTAGTGATGAAGTGAAGA
GAAAAATAAGGAAGAGTCTTCGGAAGAGTAAGAGAAAAAGTTGGAAGAGGAAGGACACCACC
AAAAAGCASATCTAAAAGAAAAAGCAACAGAAAGCTACTGCTAAAAGTAAAAATCTG
TGAGAGTGCATAATGTTAAGAGGCAGCAGCAGTACCACCAAGAAGAATCAAAAAAGTTC
AAAAAAAAGGTCTCCCAAGATCCCATCCCAGTACCCAGTAAGAAAAGCTACTGCTAAAAAGTCTG
CCAACCTCACAGATGAAGAGCAGCAGTACCACCAAGAAGAATCAAAAAAGTTC
GGAGGAAGTGCAAAATGGAAGGAGAGATTTGCAAGGATATAGAAAATTATCCTGCTTTATGATT
TGACTGAGAGAAAGATTTCCATTAAAAACACTGTAAAAAGAGCAGCTATTTCCTTCTTGA

In Vivo Imaging Systems (IVIS):
Mice were weighed and injected with luciferin at 15ug/mL at 1ul per gram in weight. Mice were allowed to metabolize the luciferin for 5 minutes prior to sedation with isoflurane. Mice were imaged in the Perkin Elmer IVIS Spectrum CT. For ex vivo IVIS, after luciferin injection, mice were allowed to metabolize luciferin for 8 minutes then sacrificed with CO2. Mice were dissected and tissues were placed in PBS containing 300ug/mL of luciferin, kept on ice, and protected from light before immediate analysis by IVIS.
Mouse keratinocyte isolation protocol:

Mouse keratinocytes were isolated following a previously published protocol with modifications (242). Briefly, pups were euthanized within 48 hours of birth. They were washed in 70% ethanol, and placed in PBS. The flank skin was removed, and placed dermis side down in 1mL of dispase (Dispase Gibco/ Invitrogen cat# 17105-041 5g) and 1 mL of DMEM in a 35mm plate, and incubated overnight at 4° Celsius. The epidermis was removed and placed in 1mL of accutase (Sigma product A6964) for 20 minutes with agitation to release keratinocytes. Cells were collected and centrifuged, then plated on irradiated MEFs and overlaid with CnT07 media (CellNtec). Cells were collected and used for experiments either at collection or passage one.

K5-tTA mice:

K5-tTA were obtained internally at CCHMC and have previously been published (243).

Bi-L-Dek copy number determination by qPCR:

Genomic DNA was isolated from the tails of mice that represent varies generations of offspring from either the 317 founder or the 318 founder. Two or more mice were used per generation. DNA concentration was adjusted to 20ng/ul and 60ng of DNA was used for qPCR per sample and done in replicate. Primers were used to identify beta actin and a region in exon 6 of the Dek gene with the following sequences:

Beta actin forward: GATATCGCTGCGCTGGTCGTC
Beta actin reverse: ACCATCACACCCTGGTGCCTAG
Dek Exon 6 forward: AGGTCAGGCGTGAACAGCGA
Dek Exon 6 reverse: TGCCAGAAGGCTTTGGATGCATTA

The delta delta CT values for Dek exon 6 were normalized to actin then compared to wild type FVBN mouse. The delta delta CT values were taken times 2 to identify the number of Dek
genes in each sample. Error bars represent the difference in Dek genes identify between mice from the same generation.

Genotyping:

Ear clips were digested with 25mM NaOH with 0.2mM EDTA at pH of12 by incubating at 95 degrees Celsius for 20 minutes. The reaction was neutralized with 40mM Tris-HCl. For PCR, one ul of digest was added to JumpStart Taq Ready Mix from Invitrogen (product: P2893) using manufacturer’s specifications. Transgenes were detected with the following primers: Bi-L-Dek: Forward: GAAATGTCCGTTCGGTTGGCAGAAGC; Reverse: CCAAAACCGTGATGGAATGGAACAACA. K5-tTA: Forward: GCTGCTTAATGAGGTCGG; Reverse: CTCTGCACCTTGCTGATC.

4-NQO induced HNCC:

All mice were maintained in a hemizygous state for Bi-L-Dek and K5-tTA. All Bi-L-Dek mice in the experiment came from founder 317 and were from generations F3 and F4. Bi-L-Dek mice were bred to K5-tTA mice and bi-transgenic offspring were given 4NQO water for 16 weeks at a dose of 10mg/ml starting at 6 weeks of age. Mice on doxycycline were fed 2mg/kg at the start of 4NQO until sacrifice. After 16 weeks, mice were given normal water until sacrifice at week 45 or veterinary services requested the mice to be sacrificed. At the time of sacrifice, tumors were resected and counted, localization was noted, and tumors were measured by calipers. Tumor volume was measured using the modified ellipsoid formula: volume= (length x width$^2$) / 2 (Euhus DM, Hudd C, LaRegina MC, Johnson FE: Tumor measurement in the nude mouse. J Surg Oncol. 1986, 31: 229-234. 10.1002/jso.2930310402.) (Tomayko MM, Reynolds CP: Determination of subcutaneous tumor size in athymic (nude) mice, Cancer Chemother. Pharmacol. 1989, 24: 148-154.) Resected tumors were fixed and paraffin embedded for IHC and H&E analysis by our collaborating pathologist for morphology, tumor stage and grade.
Flow cytometry:
Each of the following experiments were done with isolated keratinocytes. Once keratinocytes were confluent, cells were split into 6 wells of a 12-well plate and 3 wells were given dox. Cells were collected at 60-80% confluency for flow cytometry experiments.

EdU incorporation: Mouse keratinocytes were grown to 70–80% confluency in 12 well plates and pulsed with 10 mM EdU for 2 hours before collection by trypsinization. Cells were prepared using the Click-it® EdU Alexa Fluor® 647 Imaging Kit (Life Technologies) according to manufacturer specifications. DNA content was determined using propidium iodide to stain DNA and cells were analyzed on a BD FACSCanto analyzer (BD Biosciences, San Jose, CA).

Cleaved caspase 3: Apoptosis was determined using FITC active caspase 3 antibody kit (BD Biosciences, product 550480) using manufacturer instructions. Cells were analyzed on a BD FACS Canto analyzer (BD Biosciences, San Jose, CA).

Dek Expression: Cells were incubated with Dek antibody (Genetex- GTX80509) or (BD Biosciences) at 1:100 dilution overnight at 4° Celsius. The example shown in Fig. 5.4B is with BD-Dek.

Western blot analyses:
Tissues were lysed using mortar and pestal, resuspended in RIPA buffer (1% Triton, 1% deoxycholate, 0.1% SDS, 0.16M NaCl, 10 mmol/L Tris pH 7.4, and 5 mmol/L EDTA), supplemented with a protease inhibitor cocktail (Pharmingen, San Diego, CA), and analyzed as described previously (25). Primary antibodies used for DEK were as follows: DEK (1:1000; BD Biosciences, San Diego, CA, USA), pan-actin (1:20,000; a gift from James Lessard. Membranes were exposed to enhanced chemiluminescence reagents (Perkin Elmer, Boston, MA, USA).
**Immunofluorescence microscopy:**

Keratinocytes were plated onto 100 mg/ml poly-D-lysine coated coverslips, and fixed with 2% paraformaldehyde for 30 minutes. Coverslips were incubated in 0.1% Triton X-100 for 3 min, blocked with 5% normal goat serum, and incubated with primary antibody for 1 h at 37˚C. Antibody dilutions were as follows: DEK-antibody (Abbiotech) 1:300 dilution; keratin 5 antibody (Acris) 1:500; and sealed with a coverslip using Vectashield with DAPI (vector labs).

**Acknowledgements**

Funding: This work was supported by the National Institutes of Health R01-CA116316 to S.I.W. Thanks for Dr. Paul Lambert and the transgenic animal core at CCHMC for help with designing and planning many aspects of the generation of the Bi-L-Dek mice. Thanks to Dr. Jeffrey Whitsett at CCHMC for the K5-tTA mice. Thanks to Ron Waclaw, Diana Nardini, and Lisa Kenney for their help in validating the Bi-L-Dek mouse model. All flow cytometric data were acquired using the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children’s Hospital Medical Center and supported in part by the Digestive Health Center NIDDK P30 DK078392. We thank Dr. James Lessard for the monoclonal actin antiserum.
Figure 5.1: Generating Tet-O Dek transgenic mice. (A) Mouse Dek (Dek) was cloned into the pBi-L-tet plasmid resulting in the 7215 bp plasmid *pBi-L-Dek*. In this plasmid, Dek and luciferase expression are under control of a tetracycline response element (TRE). Restriction enzyme cut sites for AatII and Asel used to linearize the DNA are identified. (B) Western blot for Dek expression in mouse keratinocytes isolated from a *K5-tTA* mouse and transfected with the *pBi-L-DEK* plasmid. Dek expression was repressed with 1ug/ml of dox in the media. (C) Luciferase assay with keratinocytes from B, treated with 0, 0.33 or 1.5 ug/ml of dox show dose dependent luciferase repression. (D) Agarose gel electrophoresis of the *pBi-L-Dek* plasmid linearized with restriction enzymes Asel and AatII and the resulting 5247bp Bi-L-Dek band. (E) The 5247bp Bi-L-Dek band was isolated from the gel, purified, and used for micronuclear injection into the pronucleus of a FVB/N fertilized egg to generate *Bi-L-Dek* founders. (F) Four founders were generated as confirmed by genotyping. (G) Copy number analysis of the *Bi-L-Dek* transgene in
the 317 founder for which characterization data is shown. The number of transgene insertion sites stabilizes at the F2 generation with ~4 insertions (and 2 wild type alleles).
Figure 5.2: Dek overexpression was targeted to the basal layer of stratified squamous epithelium. (A) Bi-L-Dek mice contain a TRE that controls two mini CMV promoters regulating the transcription of Dek and luciferase. Bi-L-Dek mice were bred to K5-tTA mice that have tetracycline transactivator (tTA) expression under control of the keratin 5 promoter. (B) Transgene presence in resulting offspring was confirmed by genotyping. TE is the no DNA control. Wild type (WT) mice are FVB/N controls. (C) Schematic of the layers of stratified squamous epithelium with the basal layer highlighted in blue where K5 driven tTA is expressed and activates Dek and luciferase expression. Addition of doxycycline to food represses tTA binding to the TRE and inhibits Dek and luciferase expression (tet-off system).
Figure 5.3: Dek is overexpressed in the squamous epithelium in Bi-L-Dek_K5-tTA mice.

(A) Quantitative real-time PCR of total Dek levels in skin epithelium taken from the flank. (B) Western blot for Dek in flank skin epithelium showing Dek overexpression in Bi-L-Dek_K5-tTA mice. (C) IHC with DEK antibody in tongue epithelium showing Dek overexpression can be repressed with doxycycline. (D) In vivo imaging systems (IVIS) representative image of single transgenic (Bi-L-Dek) mice and bi-transgenic Bi-L-Dek_K5-tTA mice after injection with luciferin for luciferase detection in the skin epithelium. (E) Ex vivo IVIS of single transgenic (K5-tTA) and
*Bi-L-Dek_K5-tTA* flank, ear, and esophagus tissue after injection of luciferin, sacrifice, and dissection.
Figure 5.4: Detection of Dek expression in Bi-L-Dek_K5-tTA_Dek/- mice.

(A) Mice bred to generate Bi-L-Dek_K5-tTA mice in a Dek/- background. (B) Western blot for Dek protein in the flank demonstrating Dek expression from the Bi-L-Dek transgene. (C) IVIS image of Dek/- _Bi-L-Dek_K5-tTA mice with luciferase expression compared to K5-tTA and Dek/- mice. (D) 10x and 20x images of IHC stained for Dek in the esophagus of Dek/- mice versus Dek/- _Bi-L-Dek_K5-tTA mice.
Figure 5.5 Keratinocytes isolated from *Bi-L-Dek_K5-tTA* mice overexpress Dek and have increased proliferation. (A) Brightfield images of skin keratinocytes isolated and cultured from
new born Bi-L-Dek_K5-tTA pups and their single transgenic littermates. For mice on dox, mothers were placed on dox chow at the time of the mating and keratinocytes were maintained in dox media. (B) Flow cytometry profiles for Dek expression in mouse keratinocytes isolated from the different genotypes. The different color peaks represent triplicate keratinocyte samples from one mouse for each genotype, three untreated and three treated with dox. The Bi-L-Dek_K5-tTA keratinocytes expressed higher levels of Dek that were brought back to normal with the addition of doxycycline in the media at 1ug/mL. (C) Quantification of cells in the high Dek expression gate pictured in B. (D) Cell cycle profiles of cultured keratinocytes using flow cytometry after addition of 10mM EdU for 2 hours and stained with DAPI for DNA content. (E) Percent of cells in each phase of the cell cycle was quantified for each genotype from data in D. (F) Flow cytometry for cleaved caspase 3 in mouse keratinocytes reveals no changes in apoptosis between the genotypes with or without dox. (G) Immunofluorescence pictures of keratinocytes taken at the same exposure and fluorescent intensity probed for Dek, keratin 5 (K5), and stained with DAPI. The mean fluorescent intensity of Dek staining was quantified using imageJ in H. (I) Cropped images of keratinocytes undergoing mitosis from H. Yellow arrow heads point to Dek aberrantly remaining on mitotic chromosomes, a published phenotype observed with Dek overexpression.
Figure 5.6: Experimental plan for HNSCC studies with Bi-L-Dek_K5-tTA mice. (A) A table depicting the groups of mice used in a 4NQO HNSCC study, as well as their purpose and expected levels of Dek expression. (B) HNSCC 4NQO experiment timeline. Mice were given 4NQO water at 10ug/mL at 6 weeks of age for 16 weeks. At this point mice will be given normal water until death, veterinary services requires their sacrifice, or sacrificed at 45 weeks. If sacrificed, mice were injected with 200ul of 15ng/mL of BrdU 1 hour before death. Mice on doxycycline started the dox diet at the start of 4NQO water.
Supplemental Figures

Supplemental Figure S5.1: Luciferase and Dek cDNA incorporation into Bi-L-Dek founders is confirmed. (A) Agarose gel electrophoresis of DNA from Bi-L-Dek founders for detection of the Bi-L-Dek transgene with primers that detect luciferase and exogenous Dek DNA sequences. The Dek primers do not detect endogenous Dek. Primers confirm insertion of the Bi-L-Dek gene. The luciferase primers are: forward: GAAATGTCGTTGTTGGCAGAAGC; reverse: CCAAAACCGTGATGGAATGGAACAACA. The Dek transgene primers are: forward: CAGTGACACAAGGGAAGGTCAGA; reverse: AGCCACTGAACTGACCACCGT.
Supplemental Figure S5.2: Detection of Dek mRNA and protein from the Bi-L-Dek transgene. (A) Quantitative real-time PCR for quantification of Dek transcript in Dek/−, Dek+/+, and Dek/−_Bi-L-Dek_K5-tTA flank tissue. Primers detect a cDNA region present in endogenous and Bi-L-Dek mice. Bars represent duplicate samples from one mouse per genotype.
Supplemental Figure S5.3: Overexpression from the Bi-L-Dek gene in the brain. (A) In collaboration with the Ron Waclaw lab at Cincinnati Children’s Hospital Medical Center, Dlx5/6-tTA mice were crossed with Bi-L-Dek mice. The Dlx5/6 enhancer drives tTA expression largely in the postmitotic neurons of the ventral forebrain. Dlx5/6-tTA is an unpublished transgenic mouse generated by Lisa Ehrman and Kenny Campbell. (B) Genotyping confirms transgene transmission to offspring. (C) IHC for Dek in the brains of pups at embryonic day 18 show Dek overexpression in the Dlx5/6-tTA_Bi-L-Dek mice. Insets are magnified images of the cortex and striatum where Dek is overexpressed. The Dek antibody is from the Proteintech Group at 1:333 dilution; Cat #16448-1-AP.
Chapter 6: Conclusions, discussion and future directions

My thesis work has focused on the effects of DEK overexpression in normal cells and in cancer cells with an emphasis on keratinocytes and HNSCC. Few studies have looked at the effects of DEK overexpression and rather focused on the effects of Dek knockdown. However, it is essential to identify the gain of function(s) of DEK when overexpressed to ascertain the advantages it offers to cancer cells. Identifying these functions will allow us to target affected pathways instead of DEK which has proven hard to target due to no full crystal structure, and no enzymatic activity. Furthermore, if we are ever able to directly inhibit DEK, it is essential we understand the cellular processes that will be affected.

Newly discovered roles for the DEK oncogene in cell division and metabolism

Previous reports from our lab have shown that DEK is highly expressed early on in HNSCC, and multiple other reports have shown DEK is related to poor prognosis and metastasis for other cancers (9, 14-17, 27-31). This suggests DEK may offer different advantages at different stages of cancer and cancer types. We set out to identify early and late stage cancer phenotypes associated with DEK overexpression specifically in HNSCC cells, an HNSCC mouse model, and keratinocytes, the cell of origin for SCC.

The data demonstrate that high DEK expression increases mitotic defects and causes DEK to aberrantly remain on DNA throughout mitosis (Fig. 2.3B and 2.5D). This was the first report suggesting DEK overexpression alone, could have a mutagenic effect. This phenotype was observed not only in HNSCC cells but also in non-transformed, immortalized keratinocytes. Therefore, through mitotic defects, DEK could promote genetic mutations that contribute to cancer initiation or drug resistance. This suggests DEK may be propagating genome instability to promote transformation but it itself is not transformative. In fact, no overexpression studies have shown that DEK overexpression alone is sufficient to transform a cell line. A previous
study by Wise et al. demonstrated that human papillomavirus E6/E7, and hRas, had to be expressed for DEK overexpression to transform keratinocytes in soft agar and for xenograft establishment (22). Interestingly though, DEK did increase tumor incidence and tumor volume in the xenograft model from this same study. This data suggests two things; 1.) DEK is not sufficient to transform normal cells into cancer cells but 2.) offers a growth advantage for tumors once initiated. This dichotomy suggests a different role for DEK in tumor initiation versus tumor progression and maintenance with mitotic defects playing a role at any number of these stages.

In regards to further elucidating roles for DEK overexpression in non-transformed cells and at early cancer stages, we identified a role for DEK in regulating cellular metabolism. Interestingly we found that high levels of DEK are sufficient to alter cellular metabolism in ways that are known to be deregulated in cancer. Specifically, DEK increases lactate, NAD⁺ and alanine production in both non-transformed and transformed keratinocytes, a sign of increased glycolysis (Fig. 4.2E-H; Figure 4.3C-D; Figure 4.4C). Cancer cells are infamously known to produce far more lactate than their non-transformed counterparts due to high rates of glycolysis (191). However, we found DEK promoted an aerobic glycolysis phenotype in non-transformed keratinocytes, a pathway that can support cell growth by increasing metabolite availability for biomass production (192). This supports the notion that DEK alone is able to shift metabolic pathways toward pro-anabolic metabolism without the assistance of other oncogenes. Furthermore, increases in lactate, NAD⁺, and alanine were also observed in the HNSCC cells suggesting DEK can further push metabolism in an already transformed cell line. A possible scenario is that DEK overexpression, in early tumorigenesis, supports cancer cell growth by meeting the metabolic demands necessary to sustain uncontrolled growth. However, DEK overexpression alone is not sufficient to result in cell growth, at least in keratinocytes grown in monolayer, and instead requires the activation of other oncogenes. DEK induced metabolic
phenotypes in conjunction with mitotic defects illustrates that DEK overexpression without other oncogene expression is sufficient to cause cellular changes that support cancer development.

**Future studies investigating specific metabolic pathways affected by DEK overexpression**

Future studies of DEK and metabolism will need to address many questions left unanswered. Such as, how does DEK increase lactate, alanine and NAD+ and is the increase in glycolytic rate observed in the DEK overexpressing cells responsible for any DEK oncogenic phenotypes. It is possible that DEK is binding to the promoters of specific metabolic enzymes such as LDHA1, HK2 or others in the RNA sequencing dataset. DEK could also be transcriptionally regulating drivers of metabolism such as mTOR which activates several anabolic pathways including protein and lipid synthesis. MTOR was also a transcriptional target gene of DEK in the RNA sequencing study (Supp. Fig. 4.1A-B). Furthermore, the DEK-CAN fusion protein in AML has been shown to activate mTOR signaling, although, as mentioned before, this has not been shown for DEK overexpression.

It is important to note that Seahorse assays, NMR studies, MTS assays, and lactate and citrate assays can only measure an end point of metabolism. Metabolic pathway flux analysis will be vital to determining specific pathways affected by DEK expression. To this end, stable isotope resolved metabolomics (SIRM) studies using radio-labelled carbon and nitrogen in glucose and glutamine followed by a metabolic flux analysis (MFA) will indicate specific pathways and enzymes affected by DEK expression and reveal macromolecules produced by those pathways. For this analysis, cells will be given glucose or glutamine with 13C or 15N label that will enter the cell and be distributed among various compounds reflective of the metabolic processes going on in the cell. The result is a series of isotopomers for each intermediate metabolite – a set of different isomers of the same molecule differing in number and position of 13C isotopes that can be deciphered using NMR or mass spectrometry.
Flux analysis, will enable a metabolic pathway map to be constructed for DEK regulated processes including glycolysis, pentose phosphate pathway, TCA cycle and anaplerosis. This entails complex data analysis that calculates enzyme activity based on metabolite isotopomer ratios. Calculated flux values can provide crucial mechanistic information about specific pathways. For example, if flux through the pentose phosphate pathway is high and it is accompanied by high serine synthesis from glycolysis intermediates, such as glycerate-3-phosphate, we will be able to establish a reduced glycolytic flux through the PGAM towards pyruvate and thus lactate synthesis.

In our study, DEK increased both lactate and alanine production, presumably from glucose utilization which could be determined with uniformly labelled $^{13}$C glucose ($U^{13}$C). We would expect the majority of glucose carbons to be incorporated into pyruvate that would then be incorporated into alanine, lactate, and acetyl CoA, as well as TCA cycle intermediates. From our Seahorse analysis, NMR studies, lactate and citrate assays, we would expect the carbons in pyruvate to go more to lactate and alanine than to acetyl CoA with DEK overexpression. Glucose can be incorporated into many metabolic pathways including glycolysis, the TCA cycle, serinolysis, and the pentose phosphate pathway. Thus, while we expect to find many of the glucose carbons incorporated into lactate and alanine, we also expect to see glucose carbons incorporated into many different metabolites in these pathways.

Future studies should also include $U^{13}$C glutamine MFA experiments to look DEK regulated changes in glutamine metabolism. These could include increased incorporation of $^{13}$C into macromolecule precursors for lipid and protein synthesis. Specifically these carbons would be seen in citrate or acetyl-coA precursors for lipid synthesis, or in amino acids such as glutamate, histidine, or asparagine needed for protein synthesis. $^{15}$N labelled glutamine would help determine nitrogen fate such as incorporation into nucleic acids as seen by $^{15}$N incorporation into intermediates of the purine and pyrimidine synthesis pathways. These include 5-phosphoribosyl-1-amine (PRA) and formyglycinamide ribonucleotide (FGAM) as well as in the
nucleotides themselves. $^{15}$N would also be expected to be incorporated into glutathione, a product consisting of glutamate, cysteine, and glycine. DEK overexpression decreased glutathione levels presumably through increased consumption which could be resolved through MFA.

Another consistent metabolic result of DEK overexpression was increased production of NAD$^+$ as shown by NMR and MTS assays. DEK also decreased 1-methylnicotinamine in the NIKS and RNA sequencing data showed a decrease in NNMT, a nicotinamide N-methyl transferase, with Dek knockdown. Together this suggests DEK regulates nicotinamide metabolism. SIRM studies with stable isotope labelled nicotinamide or other NAD$^+$ precursors such as tryptophan and aspartic acid may give insights into the origin and utilization of this key metabolic co-enzyme.

All together, we expect DEK overexpression will cause an increased flux through one or more biosynthetic pathway(s). These SIRM experiments could then lead to labelling other metabolites that may be key to DEK regulated metabolism such as pyruvate, glutamate or alanine which are consistent central metabolites affected by DEK in the NMR studies. In general, we would hypothesize that DEK would increase carbon and nitrogen utilization into the precursors for nucleotide, protein, and/or lipid synthesis. This hypothesis is founded from NMR data suggesting increased consumption of amino acids that are involved in protein and nucleotide synthesis (Supp. Fig. 4.2F and 4.3F), along with previous RNA sequencing data suggesting that DEK increases lipid and steroid pathways (Supp. Fig. 4.1A). These experiments could identify targetable metabolic enzymes that are specifically deregulated by DEK overexpression. Several metabolic inhibitors are already FDA approved such as those used for treating diabetes and high cholesterol. For cancer treatment, many metabolic inhibitors are in clinical trials including those for lactate dehydrogenase, pyruvate dehydrogenase, glucose transporter 1, and PFKFB3 (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3) (244). These inhibitors and others may improve patient outcome in DEK dependent cancers.
Future *in vivo* studies in *Dek* transgenic mice

Newly discovered DEK related phenotypes such as increased glycolysis, DEK localization during mitosis and the presence of micronuclei can now be elucidated *in vivo*, using the *Bi-L-Dek* transgenic mouse model. This mouse model will help determine the over-arching contribution of DEK overexpression in early and late stages of tumorigenesis. Promising observations have already been detected in the *Bi-L-Dek* mouse model including increased proliferation (Fig. 5.5 D-E), Dek aberrantly localizing to mitotic chromosomes (Fig. 5.5I), and increased gross HNSCC tumor formation (data not shown). The results from our HNSCC study are likely to be the first data set showing the *in vivo* effects of Dek overexpression on tumor growth and incidence.

A vital future study will be determining the effects of inhibiting Dek overexpression *in vivo* once tumors have formed; an experiment now feasible with the existence of the tetracycline responsive *Bi-L-Dek* mouse model. These studies would require an alternative approach to address the endogenous Dek overexpression that is likely to occur naturally during tumorigenesis. This problem could be circumvented by breeding *Bi-L-Dek* and tTA transgenes into a *Dek*-/- background in which dox represses the only source of Dek expression. The importance for these studies comes from a magnitude of Dek knockdown experiments showing the importance for DEK expression for cancer cell growth and survival (20, 22, 24, 74, 75). Furthermore, rapidly dividing cells are more sensitive to DEK loss than differentiated and quiescent cells making DEK a good therapeutic target whether late or early stage cancer (22). Lastly, Dek knockout mice are alive and fertile suggesting that targeting DEK in cancer will have far less detrimental effects than standard chemotherapy.
Targeting Dek in cancer

Despite the promising therapeutic potential of inhibiting DEK, no DEK inhibitors exist commercially or have been described in the literature. The major hurdle in generating a Dek inhibitor is that no full length crystal structure for Dek exists. Researchers have attempted to crystallize DEK but several factors have impeded progress including the fact that GST-DEK auto-degrades and kills the bacteria it is grown in, resulting in extremely low yields of DEK (data not published). Furthermore, the domains of DEK responsible for each function are not well characterized. The multiple functions, cellular locations, and post transcriptional modifications of DEK only add to the complexity of resolving structure-function relationships. The use of DEK mutants in some studies have helped tease out the domains necessary for a specific DEK function. These include one study showing the SAF/SAP domain alone is sufficient to rescue Hela cell death when endogenous DEK is knocked down (24). The other study demonstrated that the C-terminus DEK DNA binding domain can rescue ATR phenotypes (73). However, these studies have made little progress toward identifying the main domain(s) responsible for DEK function or the main function for each domain. That being said, the domains most likely to be vital for DEK oncogenic functions are its two DNA binding domains which are very difficult to target with inhibitors. Furthermore, the SAF/SAP domain is common to many proteins and an inhibitor would have effects on many off target proteins.

These obstacles highlight the need to develop ways of targeting DEK that do not involve inhibiting the protein. One such way currently being explored is with the use of nanoparticles. Nanoparticles have been developed that bind to abundant folate receptors on cancer cells and once endocytosed express DEK short hairpin RNA to inhibit DEK translation (245). These molecules are currently being validated with hopes of entering clinical trials in the future. These studies along with previously mentioned metabolism studies will be imperative for finding ways to inhibit DEK directly or pathways responsible for its oncogenic functions. The next vital step in
DEK cancer related research is determining the therapeutic potential of targeting DEK in vivo and developing a way to inhibit DEK expression that can be translated into the clinic.

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