SUB-NUCLEAR LOCALIZATION AND TUMORIGENIC FUNCTION OF THE ONCOPROTEIN DEK

by

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FORWARD

As of the submission of this dissertation, Chapter II has been published in modified form.

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CHAPTER I

INTRODUCTION

The human *DEK* gene encodes a structurally unique protein that has been implicated in a diverse set of cellular processes, including regulation of transcription, chromatin architecture, mRNA processing, and cell signaling. The participation of DEK in these disparate processes is most likely regulated through extensive post-translational modification by phosphorylation, acetylation, and poly (ADP)-ribosylation. DEK has been associated with both autoimmunity and cancer, though its contribution to pathogenesis of either disease is unclear. This chapter will address the known structural and functional features of the human DEK protein. The link between DEK and cancer will also be explored in detail.

Structure of DEK

Structurally, DEK is a unique protein that shares little homology with any other known proteins. DEK exhibits high sequence conservation among higher eukaryotes, with ~91% sequence conservation between human and mouse DEK. Notably, there are no known DEK homologs in either *Saccharomyces cerevesiae* or *Caenorhabditis elegans* genomes. The SAP box is the only region of DEK with known homology to other proteins (Figure 1). The SAP box domain is named for three proteins initially found to share this motif: Scaffold Attachment Factors A and B (SAF-A and SAF-B), Acinus, and

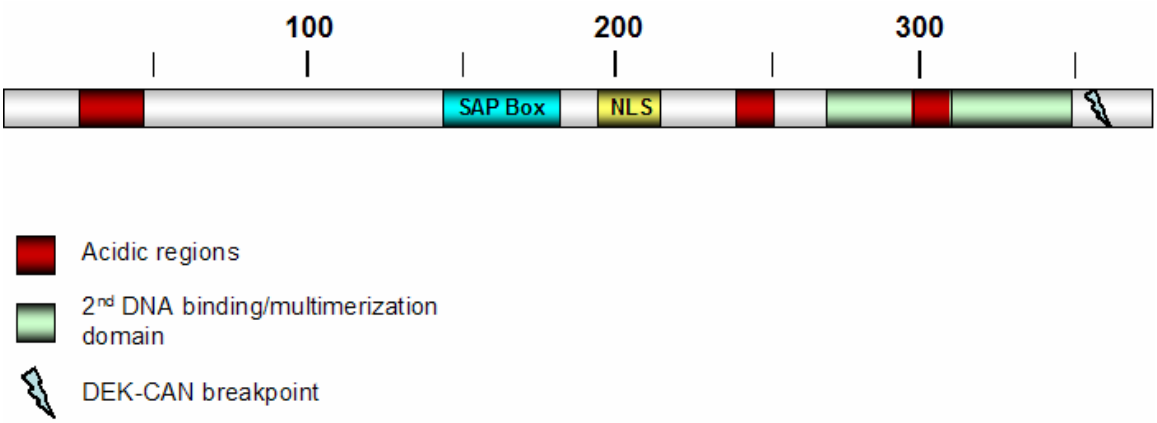

Figure 1. . Structure of the human DEK protein.

PIAS (Protein Inhibitor of Activated Stat) (Aravind and Koonin 2000). The initial search for proteins containing sequence similarity to SAF-A and SAF-B yielded 29 other proteins and identified the SAP box as a 34 residue motif with a number of highly conserved residues. The SAP box functions as a DNA-binding domain and also mediates attachment of SAP-containing proteins to AT-rich chromosomal regions of the nuclear matrix (Romig, Fackelmayer et al. 1992; Bohm, Kappes et al. 2005). Additionally, SAP-containing proteins frequently also possess a separate RNA-binding domain (Aravind and Koonin 2000) and some, like SAF-B, display interactions with both RNA polymerase and mRNA processing factors containing serine-arginine repeats (SR proteins) (Nayler, Stratling et al. 1998), suggesting a possible role as a bridge between transcription and pre-mRNA processing. Finally, several SAP box containing proteins, such as poly(ADP-ribose) polymerase (PARP) and Ku70, have emerged as high profile members of the cellular DNA repair machinery (Schreiber, Dantzer et al. 2006; Morio and Kim 2008).

There exists only one other recognizable motif within the DEK sequence: a bipartite nuclear localization sequence (NLS). The bipartite NLS is composed of two adjacent basic residues followed by a stretch of basic residues 10 amino acids downstream of the initial pair of basic residues (Dingwall and Laskey 1991). The second stretch of basic residues must contain at least 3 basic residues out of five total residues. As the name suggests, the bipartite NLS localizes proteins to the nucleus (Dingwall and Laskey 1991). Although DEK is almost exclusively restricted to the nucleus (exceptions discussed below and in Chapter II), the contribution of the bipartite NLS to its localization has never been experimentally tested.

In fact, it is likely that this bipartite NLS is not necessary for targeting of DEK to the nucleus. *In silico* analysis of the effect of deleting the bipartite NLS reveals no change in the probability of nuclear localization as compared to wild type DEK (Horton, Park et al. 2007). The more significant factor may be its overall basic charge, a characteristic feature of nuclear proteins. The predicted isoelectric point of human DEK is 8.6, and at a pH of 7, DEK would be estimated to have a net charge of +6.3. Strikingly, of the total 375 amino acids of DEK, 82 are strongly basic (~22%). This is offset in large part by three large acidic stretches, one N-terminal and two C-terminal. In total there are 76 strongly acidic residues, and thus 42% of DEK carries a charge, and another 25% of DEK is composed of polar residues.

One of the most remarkable features of the amino acid composition of DEK is that there are 67 lysine residues. In addition to being a basic residue, lysine is also significant in that it is a frequent acceptor of post-translational modifications. These include acetylation, mono-, di-, tri-methylation, Nε-lysine formylation, ubiquitination, SUMOylation, glycation, and ADP-ribosylation (Imamura, Neildez et al. 2004; Krueger and Srivastava 2006; Hunter 2007; Povey, Howard et al. 2008; Wisniewski, Zougman et al. 2008). DEK has long been suspected to be a target of post-translational modification. Indirect evidence of possible post-translational modification was inferred by the discrepancy between DEK's predicted and observed molecular weight. Based on its amino acid composition, DEK is predicted to be a 43 kilodalton protein, yet by SDS-PAGE analysis, DEK is almost invariably observed to migrate as an approximately 50 kilodalton protein (Alexiadis, Waldmann et al. 2000; McGarvey, Rosonina et al. 2000; Faulkner, Hilfinger et al. 2001; Kappes, Burger et al. 2001). Subsequent studies have

confirmed that DEK is substantially post-translationally modified *in vivo*. Radiolabeling with ^{32}P-orthophosphate first demonstrated that DEK was a phosphoprotein (Fornerod, Boer et al. 1995). Detailed quadrupole ion trap mass spectrometry analysis later mapped several phosphorylation sites of human DEK, and CK2 was identified as the primary kinase involved in phosphorylation of DEK (Kappes, Damoc et al. 2004). We and others have additionally found that DEK is a target of poly(ADP)-ribosylation by PARP (Gamble and Fisher 2007; Kappes, Fahrer et al. 2008). Finally, in chapter II of this work, we demonstrate that DEK is also an acetylated protein. Importantly, all three of these described modifications have also been shown to have important implications in the regulation of DNA-binding activity and sub-cellular localization of DEK.

X-ray crystallography structures have not been determined for DEK. However, H. Matsuo and colleagues have provided NMR based structures of two important domains: amino acids 68-226 and amino acids 309-375 (Devany, Kappes et al. 2008). The structure of the 68-226 fragment of DEK revealed that there exists a second domain N-terminal to the SAP box that exhibits structural similarity to the SAP box, despite sharing little sequence similarity. Interestingly, this region had previously been found to be important in enhancing the DNA-binding affinity of the SAP box and also conferred upon DEK DNA-supercoiling activity (discussed below) (Bohm, Kappes et al. 2005). A similar agreement between form and function was observed with the C-terminal fragment of DEK. The NMR structure of this portion of DEK revealed a winged helix DNA-binding motif similar to that of the E2F/DP transcription factor family (Devany, Kotharu et al. 2004), and Kappes et al. (Kappes, Scholten et al. 2004) experimentally determined that this region represents a second, previously unidentified DNA-binding domain of

DEK. In addition to DNA-binding, this region was also shown to be critical to the ability of DEK to form multimers in a phosphorylation-dependent manner (Kappes, Scholten et al. 2004).

Functions of DEK

Transcriptional regulation

The first described functional activity of DEK was as a transcriptional repressor of the HIV-2 virus. DNase footprinting studies had identified a cellular factor that protected a 10 base pair region of the HIV-2 transcriptional enhancer adjacent to a purine-rich ets binding site (termed peri-ets or pets) (Markovitz, Smith et al. 1992). Subsequent studies identified a 43 kilodalton protein capable of protecting this region (Fu and Markovitz 1996). Using a Southwestern screen of a Jurkat T cell phage library, DEK was determined to be the unknown factor (Fu, Grosveld et al. 1997). Its role as a transcriptional repressor was inferred by its presence in transcriptionally inactive protein complexes binding to this site, and its absence in transcriptionally active complexes that are induced upon 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment (Faulkner, Hilfinger et al. 2001).

Several studies have since found evidence supporting a role for DEK as a transcriptional repressor. DEK was found to be a transcriptional co-repressor in a complex composed of the transcriptional repressor Daxx, the core histones H2A, H2B, H3, and H4, and histone deacetylase II (HDAC2) (Hollenbach, McPherson et al. 2002). DEK has also been shown to potently repress NF-κB transcriptional activity via interactions with the p65 subunit (Sammons, Wan et al. 2006). In addition to NF-κB repression, DEK is also capable of repressing activity of the androgen receptor, p53, and

Stat5b (Sammons, Wan et al. 2006; Wise-Draper, Allen et al. 2006). Finally, DEK was described as an element present on chromatin that must be displaced by either SET (a histone chaperone) or PARP1 prior to transcriptional activation (Gamble and Fisher 2007).

The mechanism of transcriptional repression by DEK has not been elucidated, but there are three likely possibilities. First, DEK may act to inhibit histone acetylation at the promoters of target genes. Ko et al. (Ko, Lee et al. 2006) found that DEK can interact with histones and inhibits their acetylation by the histone acetyltransferases p300 and p300/CBP-associated factor (P/CAF). Second, because DEK has been shown to be present in a complex with HDAC2, DEK may act to recruit this histone deacetylase, and possibly others, to target promoters (Hollenbach, McPherson et al. 2002). Both of these activities result in the same outcome, hypoacetylation of promoter-associated histones. Thus, it is very possible that DEK represses transcription by maintaining promoter hypoacetylation. The third possible mechanism of transcriptional repression by DEK may be a consequence of DEK's ability to alter chromatin architecture, a function that will be described later in this chapter.

Conversely, DEK has also displayed characteristics of a transcriptional co-activator. The first example of this ability was a study that identified DEK as an interaction partner for the inducible transcription factor AP-2α (Campillos, Garcia et al. 2003). DEK was found to promote activity of the APOE promoter by enhancing the DNA-binding affinity of AP-2α. Other studies have demonstrated the presence of DEK in transcriptionally active chromatin regions. During active transcription, the WSTF-SNF2h chromatin remodeling complex interacts with several nuclear proteins, including

DEK (Cavellan, Asp et al. 2006). Additionally, DEK was present at promoter-proximal regions of the CD21/complement receptor 2 (CR2) gene in the B cell lymphocytic Ramos cell line that expresses CD21/CR2, but not in another B cell-derived cell line, Nalm-6, that does not express CD21/CR2 (Hu, Illges et al. 2005). By chromatin immunoprecipitation (ChIP) of DEK, DEK was found to be enriched in euchromatic, acetylated histone H4-containing chromatin fractions of HeLa cells (Hu, Scholten et al. 2007). A promoter-proximal element of the actively transcribed human topoisomerase I (TOPI) was enriched in chromatin fractions precipitated with anti-DEK antibodies. When transcription was inhibited with α-amanitin, this region was no longer enriched following ChIP analysis with anti-DEK antibodies, suggesting that DEK was only present at this promoter during active transcription. Thus, DEK may also be capable of promoting gene transcription in certain circumstances.

Modifying chromatin architecture

Although initial findings suggested that DEK's affinity for its DNA targets was sequence dependent (Fu, Grosveld et al. 1997; Adams, Cha et al. 2003), other data have pointed to preferential binding to certain DNA structures. Specifically, DEK has increased affinity for supercoiled and four-way junction DNA (also present in Holliday junctions or cruciform DNA) (Waldmann, Baack et al. 2003; Waldmann, Scholten et al. 2004). Because several other proteins with high affinity for these DNA structures, such as the high mobility group proteins (HMGs) and the linker histone H1, have been shown to play prominent roles in altering chromatin architecture (JR, Norman et al. 1998; Zlatanova and van Holde 1998)(Varga-Weisz, van Holde et al.; Thomas), this has been proposed as a potential function of DEK as well.

Indeed, DEK has also been shown to exert direct effects on chromatin structure. This function is distinct from the roles in chromatin remodeling previously mentioned. Whereas chromatin remodeling is an ATP dependent process that alters access to DNA through nucleosome manipulation (Saha, Wittmeyer et al. 2006), the chromatin modifying activities of DEK do not utilize ATP hydrolysis and do not affect or require nucleosomes. This function of DEK was identified after DEK was purified from nuclear extracts as a factor capable of reducing the number of negative supercoils present in circular SV40 minichromosomes (Alexiadis, Waldmann et al. 2000). Further investigations demonstrated that this activity of DEK was also seen, albeit to a lesser degree, with naked DNA targets lacking histones (Waldmann, Eckerich et al. 2002). Detailed studies revealed that the reduction of negative supercoils was in fact due to the ability of DEK to induce constrained positive supercoiling of both naked and nucleosomal DNA in the presence of topoisomerase (Waldmann, Eckerich et al. 2002). Positive supercoiling activity was observed with a truncated form of DEK (a.a. 68-250) that contained only the SAP box and the regions immediately N-terminal and C-terminal to the SAP box (Bohm, Kappes et al. 2005). Interestingly, a peptide limited only to the SAP box region of DEK (87-137) exhibited negative supercoiling activity.

The implications of this positive supercoiling activity are unclear. Physiological examples of positive supercoiling include DNA unwinding during transcription and replication (Varga-Weisz, van Holde et al. 1993). In these processes, positive supercoiling is induced ahead of the advancing polymerase, and the resulting positive supercoils are generally considered a necessary side effect of DNA unwinding, although recent data suggests that torsional stress caused by these processes may function to

transmit signals through the genome (Kouzine, Sanford et al. 2008). Excessive positive supercoiling can create resistance that can stall advancing polymerases (Wang, Schnitzer et al. 1998), and thus in prokaryotes enzymes such as DNA gyrase exist to dissipate positive supercoils (Duguet 1997). Eukaryotes do not have an equivalent mechanism, but are thought to use DNA/nucleosome interactions to absorb this torsional stress (Bancaud, Conde e Silva et al. 2006). Few proteins capable of promoting positive supercoils have been identified. The best known example of an enzyme with positive supercoiling activity is that of condensin. Supercoiling by condensin during mitosis compacts the mitotic chromosomes and is required for proper chromosome segregation (Hagstrom and Meyer 2003). Recently, positive supercoiling has also been implicated in formation of the complex DNA structure of eukaryotic telomeres. Telomere repeat factor-2 (TRF2) had been shown to promote formation of a lasso-like DNA structure termed the t-loop (Griffith, Comeau et al. 1999). The free guanine-rich DNA end (G-tail) of the telomere can invade this structure and by doing so, protects itself from nuclease degradation and from exposure to DNA strand break machinery (Stansel, de Lange et al. 2001). Although the precise mechanism is still speculative, it was found that TRF2 also possessed positive supercoiling ability (Amiard, Doudeau et al. 2007). Using a panel of TRF2 mutants, the authors found that the ability to induce positive supercoils correlated with the ability to promote t-loop formation and strand invasion.

Recently, we have discovered a role for DEK in global chromatin organization (Kappes, Khodadoust, et al., in preparation). Using minimal and reconstituted chromatin templates and micrococcal nuclease as a tool, we found a significant decrease in chromatin accessibility and altered nucleosomal repeat length upon addition of

recombinant DEK *in vitro*. Furthermore, investigation of lentiviral shRNA-mediated inhibition of DEK in HeLa S3 cells by micrococcal nuclease digestion revealed a more accessible and open chromatin structure accompanied by changes in global histone acetylation and methylation *in vivo*. Furthermore, these cells exhibited reduced nucleosomal repeat length, chromosomal defects, altered cell cycle behavior, and a higher susceptibility to HDAC inhibitors. However, no increased apoptosis or senescence was observed upon DEK depletion. Interestingly, by electron microscopic imaging we found a significant loss of pericentric heterochromatic areas in cells lacking DEK. This observation is further supported by altered localization of HP1-α and aberrant organization of heterochromatic satellite DNA markers in DEKi cells. These results point to a significant function of DEK as an essential factor required for the maintenance of heterochromatin, possibly by tethering HP1-α to pericentric chromatin.

mRNA Processing

Although most cellular DEK is found tightly bound to chromatin, a role for DEK in pre-mRNA processing has also been suggested. Digestion of HeLa nuclei with RNase results in the release of approximately 10% of DEK from the nucleus, implying a direct or indirect association between DEK and RNA (Kappes, Burger et al. 2001). An experiment to discover novel SR-related proteins and associated factors identified DEK in a splicing complex containing these SR proteins (McGarvey, Rosonina et al. 2000). This study utilized a monoclonal antibody specific for a phosphoepitope present within the RS domains (rich in alternating arginines and serine residues) of many SR proteins to purify SR proteins in a multi-step fractionation. In the final SR protein-enriched fraction, the authors found an approximately 50 kilodalton protein. Mass spectrometry analysis

identified this protein as DEK. DEK was found to co-immunoprecipitate with the splicing coactivator SRm160/300. Furthermore, immunoprecipitation of DEK also precipitated splicing complexes. Additionally, it was shown by immunofluorescence that ~15% of HeLa cells exhibited co-localization of DEK and SR proteins. However, in characterizing the interaction between DEK and components of the splicing complex the authors discovered that DEK was not required for splicing, and that DEK did not have direct interactions with RNA splicing substrates.

DEK has also been posited as a member of the exon-exon junction complex. The exon-exon junction complex is a complex of splicing proteins, including SRm160, that remain associated with recently spliced mRNA (Le Hir, Izaurralde et al. 2000). This protein complex was originally identified in an attempt to identify the factors responsible for the protection from RNase of a conserved region 20-24 nucleotides upstream from the site of exon-exon linkage. Immunoprecipitation with DEK-specific antibodies was able to precipitate this complex along with spliced mRNA, but not an identical RNA fragment that had been transcribed from a cDNA template and therefore had not undergone splicing. Some members of the exon-exon junction complex remain associated with mRNA that has been transported to the cytoplasm, however DEK is not found in the cytoplasmic complex (Le Hir, Gatfield et al. 2001).

Since these initial studies, DEK's participation in the exon-exon junction complex has been challenged. Several other investigations focusing on the assembly of the exon-exon junction complex and its interaction with the spliceosome failed to identify DEK as a component of the exon-exon junction complex (Lejeune, Ishigaki et al. 2002; Reichert, Le Hir et al.; Kataoka and Dreyfuss). Furthermore, a detailed proteomic analysis of the

spliceosome C and H complexes failed to identify DEK as a component (Reichert, Le Hir et al. 2002). It has been suggested that the initial observation of DEK associated with the exon-exon junction complex was a spurious result that was attributable to a cross-reactivity of the only available anti-DEK antisera used in those experiments. As evidence for this possibility, one group noted that the anti-DEK antisera reacted with an unidentified 20 kilodalton protein found in the complex (Reichert, Le Hir et al. 2002; Waldmann, Scholten et al. 2004). However, there are several alternative explanations of the inconsistent findings regarding the association of DEK with ribonucleic protein (RNPs) complexes. First, the association of DEK with RNPs may be transient or unstable under experimental conditions. Second, given the array of potential post-translational modifications of DEK, it is possible that the antisera used in early experiments demonstrated limited reactivity to certain modified forms of DEK that preferentially interact with RNPs. The multitude of potential post-translational modifications of DEK also makes its identification by mass spectroscopy technically challenging, and thus may limit its identification in many proteomic-based studies.

The original observations linking DEK with RNPs never found DEK to be required for splicing reactions and never delineated any role for DEK in pre-mRNA processing. However, a breakthrough study revealed a putative role in mRNA splicing. Metazoan 3' splice sites are marked by a pyrimidine-rich tract and a conserved AG, which are recognized by the RNA-binding proteins U2AF65 and U2AF35, respectively (Zamore, Patton et al. 1992; Merendino, Guth et al. 1999; Wu, Romfo et al. 1999; Zorio and Blumenthal 1999). If the polypyrimidine tract is sufficiently long, only U2AF65 is required to initiate the splicing reaction (Reed 1989; Wu, Romfo et al. 1999; Guth, Tange

et al. 2001). However, shorter polypyrimidine tracts require U2AF35 binding to promote this reaction (Wu, Romfo et al. 1999; Guth, Tange et al. 2001). Although previous studies had concluded that U2AF35 was sufficient to recognize the 3' AG signal, experiments by Soares et al. (Soares, Zanier et al. 2006) have demonstrated that DEK can act as a proofreader of the 3' splice site and confers specificity of U2AF35 to the AG signal. Depletion of DEK eliminated this proofreading ability resulting in inability of U2AF35 to discriminate between 3' AG and 3' CG signals. This ability of DEK required its phosphorylation at two specific serine residues. The implications of this proofreading activity are significant, however further work and verification of DEK's role in maintaining 3' splice site fidelity is warranted.

Chemoattraction

DEK has been repeatedly described as a strictly nuclear protein in multiple cell lines and lineages. However, our group had noted that upon TPA stimulation of U937 monocytic cells, DEK is absent from the nucleus (Faulkner, Hilfinger et al. 2001). We subsequently found that DEK migration out of the nucleus could be seen in activated primary human monocyte-derived macrophages (MDMs) (Mor-Vaknin, Punturieri et al. 2006). Activation with 40% human serum resulted in translocation of DEK in to the cytoplasm. Remarkably, we found that after several days of stimulation, DEK could be found extracellularly in MDM cultures. We demonstrated that DEK was actively secreted via a non-classical secretion pathway and was not released as a result of cell death. Extracellular DEK was found in a freely soluble form, but a significant portion of DEK was also found to be contained within exosomes. Exosomes are small membrane-bound vesicles that are released from certain cell types, most notably antigen presenting

cells (Chaput, Flament et al. 2006). These vesicles have been shown to be capable of presenting antigen to cells without requiring cell-cell contact (Raposo, Nijman et al. 1996). Because DEK has been found to be an autoantigen in a number of autoimmune diseases, most prominently juvenile idiopathic arthritis, this finding has important implications in the pathogenesis of those diseases (Szer, Sierakowska et al. 1994; Murray, Szer et al. 1997; Dong, Michelis et al. 1998; Dong, Wang et al. 2000).

We additionally examined whether the freely soluble form of DEK may exhibit pro-inflammatory function. Intriguingly, recombinant DEK was found to be a potent chemoattractant for CD8+ lymphocytes, CD56+ natural killer cells, and neutrophils. Thus, it appears that activated macrophages may secrete DEK to promote inflammation. In support of this possibility, we found anti-inflammatory treatment with dexamethasone and cyclosporine A could block DEK secretion. Notably, inhibitors of classical golgi-secretion pathways, such as brefeldin A and monensin, did not block secretion, but treatment with an inhibitor of the kinase CK2 did inhibit secretion. Therefore, it appears that post-translational modification of DEK may be an important factor in the release of DEK from MDMs. The contributions of the more recently identified post-translational modifications of DEK, namely acetylation and poly(ADP)-ribosylation, to its secretion are currently being investigated.

DEK Expression and Cancer

DEK-NUP214

DEK was initially discovered as part of a chromosomal translocation, t(6;9)(p23;q34), observed in a subset of acute myeloid leukemia (AML) patients (von Lindern, Fornerod et al. 1992; von Lindern, van Baal et al. 1992). Its name derives from

the initials of one such patient: D.K. This translocation event occurs most often in the M2 subset of AML (acute myeloblastic leukemia with maturation), but can occur in other subtypes as well (Soekarman, von Lindern et al. 1992; Lowenberg, Downing et al. 1999). The translocation results in a fusion of the N-terminal 359 amino acids of DEK with the C-terminal 1278 amino acids of the nucleoporin NUP214 (also called CAN) (von Lindern, van Baal et al. 1992). The potential oncogenic function of this fusion protein has been poorly described. However, the fusion of a nuclear factor with a nucleoporin has been seen in other translocation events associated with AML. Notably, NUP214 also forms a similar fusion protein production with SET in acute promyelocytic leukemia (APL) (von Lindern, van Baal et al. 1992). This is of particular interest, given the recently described interaction between SET and DEK (Gamble and Fisher 2007).

DEK overexpression in cancer

DEK has since been found to be overexpressed in a number of malignancies, independent of the t(6;9) translocations. The list of cancers in which DEK is overexpressed continues to grow and currently includes retinoblastoma, hepatocellular carcinoma, bladder carcinoma, glioblastoma, acute myeloid leukemia, lung carcinoma, breast cancer, colon cancer, uterine cervical cancer, and melanoma (Kondoh, Wakatsuki et al. 1999; Grottke, Mantwill et al. 2000; Kroes, Jastrow et al. 2000; Larramendy, Niini et al. 2002; Sanchez-Carbayo, Socci et al. 2003; Lu, Luo et al. 2005; Wu, Li et al. 2008). Overexpression in almost all of these tumors has been evaluated solely by *DEK* mRNA expression. The exceptions are retinoblastoma and lung carcinoma, in which DEK overexpression has been validated by immunoblotting or immunofluorescence (Carro, Spiga et al. 2006; Orlic, Spencer et al. 2006). Overexpression of DEK does not appear to

be ubiquitous in these tumors, but is instead found in a subset of each malignancy. The percentage of these cancers displaying increased DEK expression has not been rigorously examined, but the best estimations have been provided by the work of Carro et al. (Carro, Spiga et al. 2006) Using tissue microarray analysis of 460 total tissue samples, including both tumors and normal tissue counterparts, the authors identified melanoma as the cancer with the greatest proportion of DEK-expressing tumors (72%). Additionally, bladder cell carcinoma and colon cancer showed a high incidence of DEK overexpression (50% of each).

The tissue microarray analysis also found that expression of DEK correlated with other tumor characteristics. Importantly, DEK expression in melanoma and colon cancer appeared to correlate with tumor grade (Carro, Spiga et al. 2006). In colonic tissue DEK expression was higher in adenomas than in hyperplastic polyps. Moreover, colon carcinomas expressed much higher levels of DEK than adenomas. Similarly, analysis of melanoma revealed higher expression of DEK in primary melanoma samples than in skin nevi. Additionally, metastatic melanoma tumors had markedly increased DEK expression over primary sites of melanoma formation. In addition to a correlation between DEK expression and tumor grade, the authors also described a direct correlation between expression of DEK and Ki67. Ki67 is a nuclear antigen present in actively dividing cells that is frequently used as a measure of proliferative activity (Scholzen and Gerdes 2000). This association between expression of DEK and proliferation has been seen in normal tissue as well. In cells of hematopoeitic lineage, DEK also appears to be expressed to a higher degree in dividing cells. Bone marrow cells expressing CD34 have greater expression of DEK than CD34-negative cells (Ageberg, Gullberg et al. 2006).

CD34 is a marker that is used in the enrichment of hematopoietic stem cells as well as undifferentiated progenitor cells. Finally, stimulation of lymphocytes with the mitogenic lectin phytohemagglutinin and IL-2 also causes upregulation of DEK (Ageberg, Gullberg et al. 2006).

Chromosome 6p gain

The mechanism responsible for increased DEK expression is not known in the majority of these tumors. However, studies of retinoblastoma and bladder cell carcinoma suggest a likely cause. These cancers, like many others, frequently exhibit gain of the 6p chromosome arm (Squire, Phillips et al. 1984; Bruch, Wohr et al. 1998; Mairal, Pinglier et al. 2000; Evans, Gallie et al. 2004). Though the majority of these gains are a result of whole arm gains (commonly from acquisition of an isochromosome of 6p), comparative genomic hybridization (CGH) and quantitative multiplex polymerase chain reaction (PCR) studies have identified minimal regions of 6p gain and amplification in these two malignancies (Chen, Gallie et al. 2001; Chen, Pajovic et al. 2002; Evans, Gallie et al. 2004; Wu, Hoffmann et al. 2005). These alterations are focused in a narrow region located with the 6p22.3 band that comprised only seven genes. In retinoblastoma, gene dysregulation in this region was examined and only three genes were found to be upregulated: DEK, NUP153, and E2F3 (Orlic, Spencer et al. 2006). A separate group identified DEK and E2F3 as the targets of 6p22.3 gain (Grasemann, Gratias et al. 2005). Thus, it appears likely that chromosomal alterations are at least partially responsible for the overexpression of DEK in these cancers.

Although minimal regions of gain have not been identified in other cancers, 6p gain is frequent chromosomal abnormality found in tumors, including colorectal

carcinoma, hepatocellular carcinoma, breast carcinoma, basal cell carcinoma, Merkel cell carcinoma, osteosarcoma, and several lymphoid tumors (Aalto, Nordling et al. 1999; Al-Mulla, Keith et al. 1999; Ashton, Weinstein et al. 2001; Seute, Sinn et al. 2001; Ozaki, Schaefer et al. 2002; Larramendy, Koljonen et al. 2004; Moinzadeh, Breuhahn et al. 2005; Santos, Zielenska et al. 2007). Interestingly, gain of 6p has been associated with increased tumorigenicity of many of these tumors. For example, 6p22 gain in carcinomas is associated with higher histological grade, greater proliferative activity, and metastases (Prat, Bernues et al. 2001; Tomovska, Richter et al. 2001). Gains of 6p are also associated with transformation from low to high-grade non-Hodgkin's lymphoma (Hough, Goepel et al. 2001). Not surprisingly, 6p gain has also been shown to correlate with poor prognosis as well (Tarkkanen, Elomaa et al. 1999).

Transcriptional upregulation

Although alterations in copy number appear to be a likely cause of DEK overexpression, this does not exclude other possible mechanisms of DEK upregulation. Because overexpression of DEK has been most frequently identified at the mRNA level, deregulation of *DEK* transcription must also be considered. The *DEK* promoter has been characterized and appears to be driven by the transcription factors Yin Yang-1 (YY1) and Nuclear Factor-Y (NF-Y) (Sitwala, Adams et al. 2002). The only other transcription factors that have been shown to directly bind and affect the *DEK* promoter are the E2F family of transcription factors (Carro, Spiga et al. 2006). The activity of the E2F transcription factors is regulated through binding by the pocket protein family members: retinoblastoma tumor suppressor protein (pRb), p107, and p130. Hyperphosphorylation of pRb occurs during the G_1 phase of the cell cycle and results in the release of the E2F

proteins, which in turn are required for progression into S-phase. Interestingly, pRb has also been shown to regulate YY1 activity (Petkova, Romanowski et al. 2001). Thus, inactivation of the pRb tumor suppressor checkpoint would be predicted to result in upregulation of E2F and YY1, and consequently DEK. Indeed, this regulation by pRb has been confirmed experimentally. Using a conditional tetracycline-responsive repressor, it was shown that expression of a constitutively active form of pRb resulted in downregulation of *DEK* transcript (Vernell, Helin et al. 2003).

The pRb tumor suppressor pathway is a major barrier to tumorigenesis. In normal cells, the pRb/E2F pathway is regulated in part by the cyclin dependent kinase (CDK) inhibitor, $p16^{INK4a}$. Through a poorly understood mechanism, repetitive cell division can increase $p16^{INK4a}$ expression, resulting in activation of the pRb checkpoint and thus cell cycle arrest at the G_1 stage. Predictably, this pathway has been found to be frequently inactivated in cancer, with melanoma being a prime example. Through a variety of mechanisms, most commonly gene deletion of $p16^{INK4a}$, the $p16^{INK4a}$/pRb tumor suppressor pathway is almost invariably inactivated in this cancer (Bartkova, Lukas et al. 1996; Walker, Flores et al. 1998). As stated above, this inactivation would be predicted to result in increased DEK expression. Furthermore, since E2F activity is regulated by cell division, it would be expected that rapidly proliferating cells would have increased E2F activity and thus higher DEK expression, potentially explaining the correlation between proliferation and DEK expression. Taken together, a compelling argument can be made that DEK overexpression is secondary to the frequently observed inactivation of the $p16^{INK4a}$/pRb pathway in many cancers. It is also certainly plausible, given the

heterogeneity of most tumors, that both 6p chromosomal gain and transcriptional upregulation are mechanisms of achieving DEK overexpression.

DEK Functions in Cancer

Inhibition of senescence

Based upon its overexpression in cancers, DEK has long been a suspected oncogene. However, the potential cancer-promoting functions of DEK have only recently been examined. The discovery of one such oncogenic property of DEK arose from studies of human papillomavirus infection (HPV). HPV infection is an important cause of carcinogenesis of cervical cancer. Two viral oncogenes, the HPV early genes E6 and E7, can cooperate to immortalize infected cells (Hawley-Nelson, Vousden et al. 1989). The HPV E6 protein binds and promotes degradation of the tumor suppressor p53, whereas HPV E7 binds to and inactivates pRB (Mantovani and Banks 2001; Munger, Basile et al. 2001). However, another HPV gene, E2, can repress the activity of the E6/E7 promoters (Tan, Gloss et al. 1992; Demeret, Desaintes et al. 1997). Loss of E2 expression is frequently observed in malignant carcinomas transformed by HPV infection, and if E2 expression is restored, these cells undergo senescence (Francis, Schmid et al. 2000; Goodwin and DiMaio 2000; Goodwin, Yang et al. 2000; Wells, Francis et al. 2000; Wells, Aronow et al. 2003). Senescence is the irreversible loss of ability to proliferate and is naturally induced in normal somatic cells after completion of a finite number of cell divisions (replicative senescence). In addition to cell death, senescence represents an additional important barrier to the transition of normal cells to cancerous cells.

Gene array analysis of E2-mediated senescent cells, where E2 was reintroduced into HPV positive HeLa cells, revealed that DEK expression was diminished in these cells (Wells, Aronow et al. 2003). Subsequently, it was found that overexpressing DEK in these cells could oppose the senescence response (Wise-Draper, Allen et al. 2005). Furthermore, it was shown that DEK was upregulated by the HPV E7 protein. This upregulation of DEK by E7 required expression of the pRb pocket protein family, suggesting that DEK overexpression is a result of inactivation of pRb and the consequent increase in E2F activity. The control of DEK expression by HPV E7 has also been separately confirmed (Johung, Goodwin et al. 2007). Through microarray studies, 19 E2F gene targets, including DEK, were found to be repressed during E2 induced senescence. To determine if repression of DEK was sufficient to induce senescence, short hairpin RNAs (shRNA) targeting DEK expression were used to knockdown DEK expression in primary HPV-negative human fibroblasts. Notably, even in the absence of HPV infection, interference with DEK expression alone resulted in senescence. It should be noted that interference with expression of another E2F-regulated gene, B-MYB, also induced senescence. In contrast to previous findings showing that overexpression of DEK could delay senescence, this group found that constitutive expression of DEK did not inhibit or delay the induction of senescence in response to E2. Because these data were not shown, they should be considered with some caution. We and others have observed that most methods of enhancing DEK expression result in, at best, modest transient expression of DEK. In our experience, the most effective method of achieving DEK expression is through the use of adenoviral vectors, the method utilized by Wise-

Draper et al. Without data to evaluate, it is reasonable to suspect that the lack of senescence-inhibition observed by Johung et al. was due to insufficient DEK expression.

Replicative senescence represents the ultimate fate of most dividing normal somatic cells. In contrast, tumor cells are capable of undergoing unlimited divisions, and thus appear to possess a means of bypassing the senescence response. In addition to replicative senescence, cancer cells must also overcome another form of senescence, termed oncogene-induced senescence. This refers to the observation that introduction of certain oncogenes into otherwise normal somatic cells results in the rapid onset of senescence. One of the classic examples of this phenomenon is the rapid induction of senescence in fibroblasts following the artificial introduction of an oncogenic mutant form of the *ras* oncogene (Serrano, Lin et al. 1997). Within days these cells cease division and begin to display hallmarks of senescence. If, however, oncogenic *ras* is introduced into rodent cells with defects in either the $p16^{INK4a}$/pRb or ARF/p53 tumor suppressor pathways, cells do not senesce. Interestingly, human cells appear to display an important difference to their rodent counterparts in that both the $p16^{INK4a}$/pRb and ARF/p53 senescence pathways must be inactivated to avoid senescence (Serrano, Lin et al. 1997).

A final important connection between DEK and senescence is the recently discovered link between chromatin structure and senescence. Both replicative and oncogene-induced senescence result in dramatically altered chromatin. Specifically, chromosomes condense and form dense heterochromatin foci, termed senescence-associated heterochromatic foci (SAHF) (Narita, Nunez et al. 2003). It is believed that these foci are responsible for the irreversible nature of senescence. Interestingly, this

heterochromatic change was shown to result in the silencing of E2F target genes (Narita, Nunez et al. 2003). Once heterochromatin foci are formed, ectopic E2F1 expression fails to restore expression of these genes. One hypothesis suggests that pRb and E2F recruit factors to these genes that initiate heterochromatin formation. In this model, these genes act as nucleation sites for the heterochromatization of the entire chromosome (Adams 2007). Importantly, DEK has been described to be downregulated during senescence of human primary keratinocytes and fibroblasts. It is possible, therefore, that the *DEK* gene represents one of the targets of heterochromatin formation and gene silencing in senescencing cells.

The localization of DEK in senescent cells is also of particular interest. We have discovered that interference with DEK expression has the remarkable effect of maintaining proper global chromatin structure (Kappes, Khodadoust, et al., in preparation). Specifically, cells lacking DEK have diminished heterochromatin, and consequently, greater chromatin accessibility. Furthermore, we have identified an interaction between DEK and heterochromatin protein-1α (HP1α). The three HP1 isoforms, HP1α, HP1β, and HP1γ, are components of heterochromatin thought to play an important role in the formation and maintenance of heterochromatin. All three HP1 isoforms were found to co-localize with SAHFs (Narita, Nunez et al. 2003). Surprisingly, the HP1 proteins were not found to be required for SAHF formation (Zhang, Chen et al. 2007). Given DEK's association with HP1α and its role in heterochromatin maintenance, it is plausible that DEK may also localize to SAHF or even may contribute their formation. However, the finding that DEK expression is diminished

in senescing cells argues that DEK may not be associated with SAHFs and may not even be present during SAHF formation.

Apoptosis and DEK

In addition to senescence, apoptosis represents an important barrier to cancer formation. Many known oncogenes that are capable of promoting cell proliferation also simultaneously render cells more susceptible to death by apoptosis (Lowe, Cepero et al. 2004). For example, deregulation of Myc expression results in the activation of the ARF/p53 pathway and ultimately results in the induction of apoptosis (Evan, Wyllie et al. 1992; Harrington, Fanidi et al. 1994). Other oncogenes that display pro-apoptotic potential include E2F1 and Ras (Qin, Livingston et al. 1994; Wu and Levine 1994; Rak, Mitsuhashi et al. 1995; Arber 1999). Thus, it seems the development of pro-apoptotic signals may be inherent in the transformation of a normal cell to a cancerous one.

The failure of tumor cells to undergo apoptosis despite their expression of oncogenes known to induce apoptosis in normal cells intimated the existence of defects in underlying apoptotic pathways. Indeed, a myriad of survival mechanisms utilized by tumor cells has been uncovered (Sharma and Settleman 2007; Letai 2008). A thorough description of these mechanisms is beyond the scope of this discussion, but events leading to the evasion of apoptosis can be crudely divided into two categories: enhancement of pro-survival signals/factors and inhibition of pro-apoptotic signals/factors. Clearly, these same events also contribute to the ability of cancer cells to withstand chemotherapeutic interventions (chemoresistance) (Soengas and Lowe 2003; Eberle, Kurbanov et al. 2007). Because of the clinical implications in overcoming

chemoresistance, identification of these anti-apoptotic alterations has been an extremely active, and fruitful, area of investigation.

One recurring survival mechanism utilized by many malignancies is the upregulation of the Bcl-2 family of anti-apoptotic proteins. Through their binding to two related pro-apoptotic proteins, BAX and BAK, the Bcl-2 protein family protects against mitochondrial depolarization, the initiating event of the intrinsic apoptotic pathway. Initiation of apoptosis is induced through the displacement of the Bcl-2 family proteins from BAX and BAK by another class of pro-apoptotic proteins, the BH3-only proteins (Youle and Strasser 2008). The BH3-only proteins are so-named because they possess a BH3 domain, but not BH1, BH2, or BH4 domains. BH3-only proteins are activated, either transcriptionally or in some cases post-translationally, in response to apoptotic signals. Examples of such signals include DNA damage (induces NOXA and PUMA), growth-factor deprivation (induces BIM and BAD), and endoplasmic reticulum stress (induces BIM) (Zha, Harada et al. 1996; Dijkers, Medema et al. 2000; Oda, Ohki et al. 2000; Nakano and Vousden 2001; Yu, Zhang et al. 2001; Puthalakath, O'Reilly et al. 2007). By overexpressing the Bcl-2 family of proteins, tumor cells can accommodate stress or drug-induced activation of BH3-only proteins without triggering apoptosis. Chapter IV will demonstrate that, in the case of melanoma, increased expression of DEK can augment expression of one such Bcl-2 family member, Mcl-1, and thereby enhance chemoresistance.

The tumor suppressor protein p53 is an important mediator of apoptosis induced by many anti-neoplastic agents, particularly those that induce DNA damage. Because of its critical role in both apoptosis and senescence, p53 is frequently mutated or deleted in

many cancers. Interestingly, DEK has been shown to inhibit the action of p53, and consequently, to also inhibit apoptosis. Using adenoviral delivery of shRNAs, Wise-Draper et al. found that interference with DEK expression in HPV-positive HeLa cervical carcinoma cells resulted in apoptosis (Wise-Draper, Allen et al. 2006). Conversely, adenoviral delivery of DEK inhibited HeLa apoptosis. The anti-apoptotic function of DEK was also observed with primary human keratinocytes, but not with SAOS-2 osteosarcoma cells. Importantly, SAOS-2 cells carry a deletion of the p53 gene. Due to the presence of the HPV E6 protein, HeLa cells do not have detectable levels of p53 despite possessing intact wild type p53 gene. Following interference with DEK expression, HeLa cells and keratinocytes exhibited increased p53 expression. Additionally, the activity of the promoters of several p53 target genes was enhanced. The authors concluded that DEK inhibited p53-mediated basal apoptosis through repression of p53 protein stability and activation.

These data present a remarkable role for DEK in the suppression of p53-mediated apoptosis, however several experimental details complicate the interpretation of these results. First, while the data demonstrate a clear enhancement of apoptosis following shRNA-mediated inhibition of DEK, the empty adenovirus that serves as a control appears to have a significant, but poorly examined effect. In particular, growth curves of infected cells were never exponential, and in some experiments, control-infected cells either did not proliferate for several days or actually diminished in number. Strangely, these control-infected cells displayed a remarkably high rate of basal apoptosis (6-14%) that appears to increase with time after infection, and most of the data is presented as "fold apoptosis," making it difficult to discern the absolute level of background

apoptosis. Therefore, it appears that adenoviral infection itself is a potent apoptotic stimulus in these experiments. Accordingly, this effect demands rigorous assessment of the multiplicity of infection (MOI) for each virus. Although identical MOIs of each vector were used, there is no means of evaluating the degree of infection. Indeed, the sole figure depicting the efficiency of infection appeared to demonstrate different transfection efficiencies for each vector. It is noteworthy that attempts by our group to recapitulate these results using lentiviral vectors in lieu of adenoviral vectors failed to demonstrate the same effects (unpublished results). As a final caveat, estimation of RNAi-mediated interference with the monoclonal antibody used in this study dramatically overestimates the degree of inhibition, again complicating interpretation. An example of this effect will be presented in Chapter III.

DNA Repair

A final putative role for DEK in the promotion of cancer is that of a DNA damage repair protein. Before the DNA damage repair gene *atm* had been identified as the mutated gene in ataxia-telangiectasia patients, a complementation screen identified the C-terminal portion of DEK as being able to complement the phenotypic defects of ataxia-telangiectasia fibroblasts (Meyn, Lu-Kuo et al. 1993). Recently, we demonstrated that DEK promotes repair of DNA damage caused by the radiomimetic neocarzinostatin, the topoisomerase I inhibitor camptothecin, and the topoisomerase II inhibitor etoposide. shRNA interference with DEK expression sensitized HeLa cells to these DNA damage agents. In contrast, knockdown of DEK expression did not affect sensitivity to TRAIL.

Summary

The function of DEK in the context of both normal and malignant cells remains unclear. Conflicting data exist regarding DEK's participation in certain cellular processes, most notably the proposed function of DEK in mRNA processing. Although multiple studies have implicated DEK as a member of mRNA splicing complexes, several others have concluded that DEK is not a prominent component of the cellular splicing machinery. This discrepancy may be a result of differential regulation of DEK in the various model systems examined. As noted above, post-translational modification has emerged as an important regulatory mechanism of DEK activity. We hypothesized that one or more of DEK's 67 lysine residues may be acetylated *in vivo*, and that acetylation of DEK may regulate its interactions with splicing factors and/or RNA. The first aim of this study was to determine whether DEK is a target of acetylation *in vivo*, and to determine if acetylation alters the sub-nuclear localization of DEK. This aim will be addressed in Chapter II.

While there have been many proposed functions of DEK in normal cells, the contribution of increased expression of DEK to the malignant phenotype of cancer cells has not been previously examined. The second aim of this study is to determine whether overexpression of DEK promotes or is required for maintenance of tumor formation. To address this, we focused on the contribution of DEK to the phenotype of malignant melanoma. Gain of 6p has frequently been identified as one of the most common chromosomal alterations in all subtypes of melanoma, yet the target(s) of 6p gain in melanoma have not been identified. We hypothesized that overexpression of DEK may

be a frequent event in melanoma progression and that this overexpression may serve to promote proliferation and/or cell survival in melanoma. This aim will be addressed in Chapters III and IV.

CHAPTER II

P300/CBP-ASSOCIATED FACTOR DRIVES DEK INTO INTERCHROMATIN GRANULE CLUSTERS

Abstract

DEK is a mammalian protein that has been implicated in the pathogenesis of autoimmune diseases and cancer, including acute myeloid leukemia, melanoma, glioblastoma, hepatocellular carcinoma, and bladder cancer. In addition, DEK appears to participate in multiple cellular processes, including transcriptional repression, mRNA processing, and chromatin remodeling. Sub-nuclear distribution of this protein, with the attendant functional ramifications, has remained a controversial topic. Here we report that DEK undergoes acetylation *in vivo*. Furthermore, deacetylase inhibition results in accumulation of DEK within interchromatin granule clusters (IGCs), sub-nuclear structures that contain RNA processing factors. Overexpression of P/CAF acetylase drives DEK into IGCs, and addition of a newly developed, synthetic, cell-permeable P/CAF inhibitor blocks this movement. The acetylation of DEK does not promote *in vivo* interactions with RNA. Finally, transcriptional inhibition results in DEK migration into both IGCs and non-IGC nuclear speckles. To our knowledge, this is the first reported example of acetylation playing a direct role in relocation of a protein to IGCs, and this may explain how DEK can function in multiple pathways that take place in distinct sub-

nuclear compartments. These findings also suggest that DEK-associated malignancies and autoimmune diseases might be amenable to treatment with agents that alter acetylation.

Introduction

The mammalian nuclear protein DEK is associated with the pathogenesis of autoimmune diseases and cancer (Kondoh, Wakatsuki et al. 1999; Kroes, Jastrow et al. 2000; Larramendy, Niini et al. 2002). Autoantibodies specific for DEK are present in patients with juvenile rheumatoid arthritis and other inflammatory diseases (Sierakowska, Williams et al. 1993; Dong, Wang et al. 2000), and fusion of *dek* and *nup214* by translocation of chromosomes 6 and 9 results in acute myeloid leukemia (von Lindern, van Baal et al. 1992). DEK expression is also increased in multiple malignancies, including bladder cancer, hepatocellular carcinoma, glioblastoma, melanoma, T-cell large granular lymphocyte leukemia, and acute myeloid leukemia, independent of the t(6,9) chromosomal translocation (Kondoh, Wakatsuki et al. 1999; Grottke, Mantwill et al. 2000; Kroes, Jastrow et al. 2000; Daibata, Matsuo et al. 2004; Evans, Gallie et al. 2004).

DEK does not belong to any characterized protein family, and sequence similarity with other factors is limited to the SAF (scaffold attachment factor) box (Gohring, Schwab et al. 1997; Kipp, Schwab et al. 2000), also known as the SAP domain (from SAF-A/B, acinus and PIAS) (Aravind and Koonin 2000), a 34-amino acid motif found in nuclear factors that participates in chromatin organization, mRNA processing, and

transcription. Interestingly, DEK has been implicated in all three of these nuclear events. We have previously shown that DEK binds to the TG-rich peri-ets (pets) site from the human immunodeficiency virus type 2 (HIV-2) promoter and that this binding is responsive to cellular signals (Fu, Grosveld et al. 1997; Faulkner, Hilfinger et al. 2001). In the case of HIV-2, DEK appears to function in transcriptional repression (Faulkner, Hilfinger et al. 2001). Subsequently, DEK has also been isolated in a complex containing both Daxx and HDAC2, essential proteins involved in transcriptional repression (Hollenbach, McPherson et al. 2002), lending further support to the role of DEK in this process. In addition, DEK has been shown to play an active role in DNA and chromatin architecture and to bind preferentially to supercoiled and four-way junction DNA (Alexiadis, Waldmann et al. 2000; Waldmann, Baack et al. 2003; Waldmann, Scholten et al. 2004). Moreover, DEK has been found in complexes with a number of mRNA splicing and export factors and spliced transcripts themselves (McGarvey, Rosonina et al. 2000; Le Hir, Gatfield et al. 2001). Although the direct role of DEK in the exon-exon junction complex, as originally proposed, remains controversial (Reichert, Le Hir et al. 2002; Kataoka and Dreyfuss 2004), there is considerable evidence supporting its association with one or more SR proteins that function in pre-mRNA splicing through protein-protein interactions (McGarvey, Rosonina et al. 2000).

There is a growing body of evidence suggesting that separate steps along the pathway of gene expression are integrated (Maniatis and Reed 2002; Proudfoot, Furger et al. 2002). As DEK has defied categorization as a single function protein, it is likely poised at the interface of multiple components of the gene expression pathway; however, this poses the question of how DEK participates in processes known to occur in separate

sub-nuclear compartments. The majority of endogenous DEK is associated with chromatin and DNA, whereas only 10% of DEK is released with RNase treatment (Kappes, Burger et al. 2001). Nonetheless, DEK has been found in association with RNA and RNA-processing factors by separate groups using multiple experimental approaches.

One mechanism by which a single polypeptide can exhibit different properties is through post-translational modifications. Acetylation can modify protein function in a number of ways. The most well known example is that of histone acetylation, which by altering the charge and size of specific lysine residues results in a loosened association with DNA and a subsequent increase in local gene expression (Sterner and Berger 2000). It has recently become clear that many transcription factors are acetylated, which often results in an enhancement of function (Gu and Roeder 1997; Sterner and Berger 2000). Mechanisms mediating this increase in transactivating potential include alterations in DNA-binding affinity (p53, GATA-1, GATA-2, E2F, and c-Myb) (Gu and Roeder 1997; Boyes, Byfield et al. 1998; Martinez-Balbas, Bauer et al. 2000; Marzio, Wagener et al. 2000; Tomita, Towatari et al. 2000; Hayakawa, Towatari et al. 2004), affinity for negative regulators (NF-κB and B-Myb) (Chen, Fischle et al. 2001; Schubert, Horstmann et al. 2004) or positive cofactors (p53) (Barlev, Liu et al. 2001), and localization (CIITA and HNF-4) (Soutoglou, Katrakili et al. 2000; Spilianakis, Papamatheakis et al. 2000). We report that DEK is acetylated in the cell, and *in vitro* it is a substrate for the acetyltransferases CBP (CREB-binding protein), p300, and P/CAF (p300/CBP-associated factor). In addition, we find that acetylation markedly alters the localization of DEK. Inhibition of deacetylase activity triggers redistribution of DEK from a diffusely nuclear to a punctate pattern within the nuclear space. We show that this pattern results from the

accumulation of DEK in structures known as nuclear speckles or interchromatin granule clusters (IGCs), which are well characterized sub-nuclear domains containing RNA-processing and transcription factors. Importantly, DEK can be driven into the IGCs by overexpression of P/CAF, but not CBP/p300, and this movement can be prevented by specifically blocking the activity of P/CAF with a newly developed, synthetic, cell-permeable inhibitor. We further show that inhibition of transcription also causes DEK accumulation in nuclear speckles. Finally, we find that acetylation results in diminished DEK-RNA interactions. Our results indicate that acetylation represents a key regulatory post-translational modification that controls its sub-nuclear localization.

Results

DEK is an Acetylated Protein

To determine whether DEK is acetylated *in vivo*, T98G human glioblastoma cells were infected with an adenoviral vector encoding for N-terminally tagged FLAG-DEK. Glioblastoma is one of several tumors that exhibit increased expression of DEK, as compared with its tissue of origin (Kroes, Jastrow et al. 2000). FLAG-DEK was immunoprecipitated with anti-FLAG antibodies and separated by SDS-PAGE. Staining for total protein revealed that one major band is recovered in the immunoprecipitate at 48 kilodalton and a minor protein band at 35 kilodalton (data not shown). N-terminal sequencing has previously demonstrated that the 35-kilodalton band corresponds to a truncated form of DEK (amino acids 70–375) (Sierakowska, Williams et al. 1993). Importantly, immunoprecipitated DEK is reactive with a monoclonal antibody that is specific for acetylated lysine residues (Figure 2A, *lane 4*), suggesting that DEK is acetylated in the cell.

In vitro acetylation assays were used to determine whether DEK could serve as a substrate for the well characterized acetyltransferase proteins CBP, p300, and P/CAF. Figure 2B indicates that all three enzymes acetylate recombinant DEK purified from baculovirus-infected insect cells. In the absence of an acetyltransferase enzyme, DEK remained unlabeled. Similar *in vitro* acetylation reactions using FLAG-DEK immunoprecipitated from T98G cells gave identical results (data not shown). These data suggest that acetylation does not occur solely on the tag, because one recombinant protein is FLAG-tagged while the other is polyhistidine-tagged, and the His-tag contains no lysines. These results also indicate that in mammalian cells there are available CBP/p300 and P/CAF acetylation sites within native DEK, which validates the use of deacetylase inhibitor treatment to shift the equilibrium toward more highly acetylated forms of the protein. To confirm that treatment with a deacetylase inhibitor alters the acetylation state of DEK *in vivo*, T98G glioblastoma cells were treated or mock-treated overnight with the deacetylase inhibitor trichostatin A (TSA). Indeed, FLAG-DEK immunoprecipitated from TSA-treated T98G cells demonstrates considerably more reactivity toward the monoclonal anti-acetylated lysine antibody than similar amounts of FLAG-DEK isolated from untreated cells (Figure 2A, *lane 3 versus lane 4*). These data demonstrate that treatment of DEK-infected cells with the deacetylase inhibitor TSA directly affects the acetylation state of DEK.

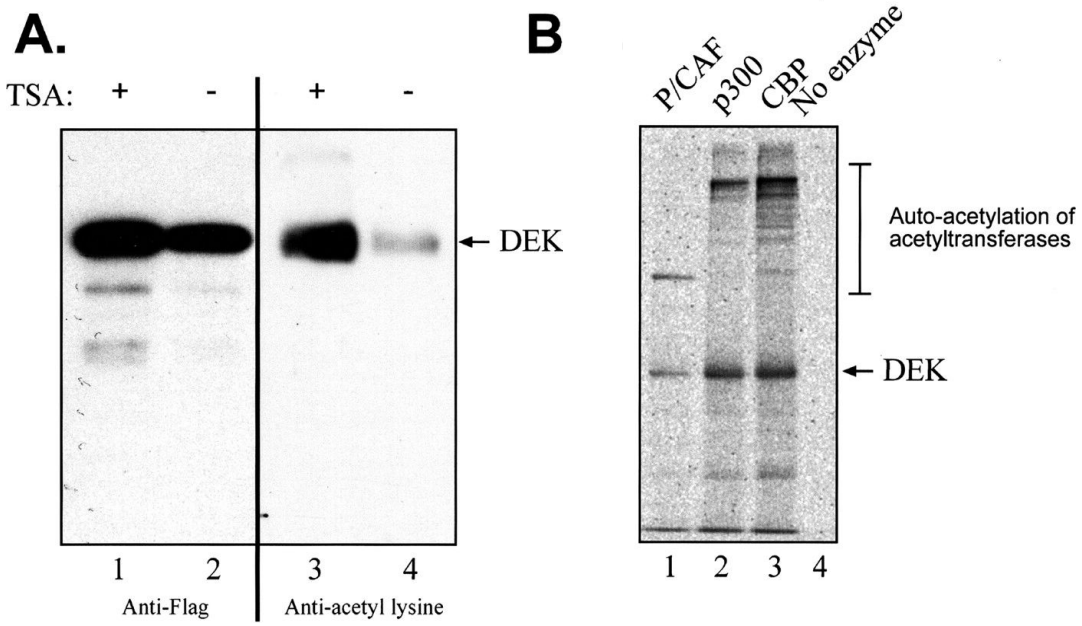

Figure 2. DEK is acetylated in vivo and is acetylated in vitro by CBP, p300, and P/CAF. In *A*, untreated FLAG-tagged DEK (*arrowhead*) immunoprecipitated with anti-FLAG antibodies is detected by anti-FLAG antibody (*lane 2*) or monoclonal anti-acetyl lysine antibody (*lane 4*). FLAG-DEK isolated from cells treated with the histone deacetylase inhibitor TSA shows increased reactivity to anti-acetyl lysine antibody (*lane 3*) with similar reactivity to anti-FLAG antibody (*lane 1*), as compared with FLAG-DEK isolated from untreated cells. In *B*, purified recombinant DEK is incubated *in vitro* with radiolabeled acetyl-CoA and either P/CAF (*lane 1*), p300 (*lane 2*), CBP (*lane 3*), or no acetyltransferase (*lane 4*). An *arrowhead* marks the position of DEK; CBP, p300, and P/CAF, which become auto-acetylated during the reaction, are indicated by the *bracket*.

Deacetylase Inhibitors Alter the Localization of DEK

Recent reports have demonstrated that acetylation alters the localization of certain transcription factors (Soutoglou, Katrakili et al. 2000; Spilianakis, Papamatheakis et al. 2000). Therefore, TSA treatment was used to investigate whether acetylation would change the location of DEK. T98G glioblastoma cells were treated or mock-treated with TSA overnight and fixed for immunocytochemistry. Comparison with 4,6-diaminido-2-phenylindole (DAPI) staining confirmed that, in untreated T98G cells, DEK is distributed diffusely throughout the nucleoplasm as reported previously (Figure 3A). However, the pattern of DEK localization is dramatically altered in TSA-treated cells (Figure 3D): in particular, DEK staining adopts a punctate pattern, suggesting that significant amounts of protein have accumulated in specific sub-nuclear structures in response to deacetylase inhibition. These data also indicate that DEK acetylation is dynamic; in other words, DEK is normally a substrate for deacetylase enzymes as well as acetylases.

DEK Relocates to the Interchromatin Granule Cluster

There are many distinct structures within the nuclear space, a number of which appear as collections of small, round dots when stained (Lamond and Earnshaw 1998). McGarvey *et al.* (McGarvey, Rosonina et al. 2000) have previously demonstrated that in approximately 15% of cells, DEK appears to be enriched in the nuclear speckles, which they speculate to be interchromatin granule clusters (IGCs) based on their observation that DEK associates with splicing factors. In contrast, acetylation of the oncoprotein EVI1 results in its relocalization to punctate structures that are enriched in CBP, which identifies them as promyelocytic leukemia protein oncogenic domains (PODs) (LaMorte,

Dyck et al. 1998; Chakraborty, Senyuk et al. 2001). To determine whether the punctate structures in Figure 3 are either IGCs or PODs, cells were stained with antibodies specific for either SC35 or CBP, respectively. Figures 3B and 3E depict staining of SC35. Figure 3F indicates substantial colocalization between the DEK-containing bodies and the IGC, whereas Figure 3C shows that the colocalization signal does not result simply from the overlap of SC35 staining with diffuse nuclear DEK. Colocalization was not seen with anti-CBP staining of PODs, which revealed a set of nuclear bodies distinct from the DEK-containing structures (Figures 3G-3I). Thus, by preventing deacetylation, TSA shifts the equilibrium toward the more acetylated form of DEK, which causes this protein to accumulate in IGCs. Our data suggest that, in normal cells, the more acetylated fraction of DEK interacts with mRNA processing proteins within IGCs, which are thought to be accumulation sites of transcriptional factors and mRNA processing factors (Spector 1996; Lamond and Spector 2003). Indeed, previous data have supported the association of DEK with spliceosome proteins, most notably in complexes important for the coupling of pre-mRNA splicing and post-splicing events (Kim and Dreyfuss 2001; Le Hir, Gatfield et al. 2001).

Acetylation by P/CAF Drives DEK into IGCs

To investigate which histone acetyltransferase is responsible for the movement of DEK into IGCs, cells were transfected with a vector encoding GFP-DEK and treated with newly developed, specific inhibitors of P/CAF (Table I) or CBP/p300 (Polesskaya, Naguibneva et al. 2001; Guidez, Howell et al. 2005). These inhibitors have a significant advantage over previously employed synthetic histone acetyltransferase inhibitors in that

they are cell-permeable and therefore do not require transfection. Inhibition constants were determined as previously described and are shown in Table I (Lau, Kundu et al. 2000).

Treatment of GFP-DEK-transfected cells with the selective cell-permeable inhibitor of CBP/p300, followed by treatment with TSA, does not block the movement of GFP-DEK to IGCs (Figure 4B). In contrast, treatment of cells with the P/CAF inhibitor H3-CoA-20-Tat, prior to TSA treatment, blocks the movement of GFP-DEK to IGCs (Figure 4C). However, treatment of cells with the control peptide (lacking only the acetyl-CoA functional group), followed by the addition of TSA, has no effect on the localization of DEK (Figure 4D). These data suggest that, although both P/CAF and CBP/p300 can acetylate DEK *in vitro*, it is the specific acetylation by P/CAF that results in the movement of DEK to the IGC.

To further investigate the role of P/CAF and CBP in the sub-nuclear movement of DEK, cells were co-transfected with GFP-DEK and a vector encoding either CBP or P/CAF. DEK remains pan-nuclear with the overexpression of CBP (Figure 5A), whereas overexpression of P/CAF with DEK results in the movement of DEK to IGCs (Figure 5B). This relocalization can be blocked with the addition of the P/CAF-specific inhibitor (Figure 5C), but not the P/CAF inhibitor control molecule (data not shown), following co-transfection of plasmids expressing GFP-DEK and P/CAF. These data support the hypothesis that it is the specific acetylation of DEK, or an associated protein, by P/CAF that causes DEK to move to an alternative location within the nucleus: the IGC. To our

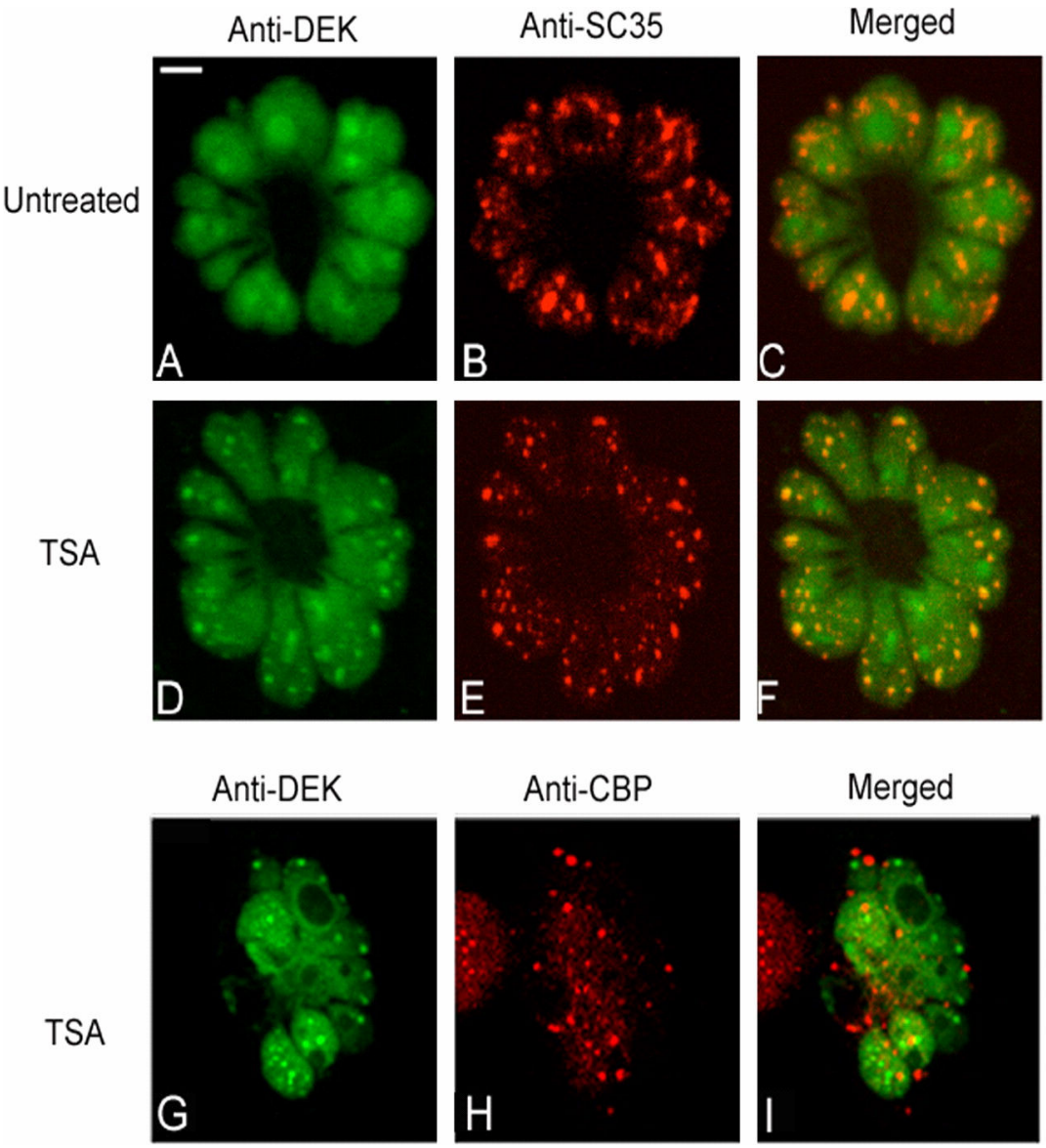

Figure 3. Treatment of T98G cells with TSA results in the concentration of DEK in punctate bodies within the nuclear space, which are IGCs. *A–C* show an untreated cell, while panels *D–I* depict cells treated with TSA. *A, D*, and *G* show staining with polyclonal antibodies to DEK, *B* and *E* show staining with monoclonal antibodies to the splicing factor SC35 (a protein known to be concentrated in IGCs), and *H* shows staining with anti-CBP (a protein known to localize to PODs). *C, F*, and *I* show the merged image, with *yellow* indicating colocalization. Images were captured with a confocal microscope; *bar*, 5 μm.

Compound	IC_{50} for p300 (μM)	IC_{50} for P/CAF (μM)
H3-CoA-20-Tat	12	0.04
H3-(Ac)-20-Tat	NA	3.2

Table 1. Inhibitory properties of H3-CoA-20-Tat and H3-Ac-20-Tat (control) conjugates against p300 and P/CAF. These assays were carried out with recombinant human P/CAF and p300 as described previously (Lau, et al., 2000; Poux, et al., 2002) and contained 10 μM acetyl-CoA and 10 μM peptide substrate (H3-20) for P/CAF, and 20 μM acetyl-CoA and 50 μM peptide substrate (H4-20) for p300. The measurements showed standard error < 20%. H3-CoA-20-Tat was shown to be a linear competitive inhibitor of P/CAF versus acetyl-CoA (150 μM H3-20 substrate) with Ki 2.1±0.7 nM (data not shown).

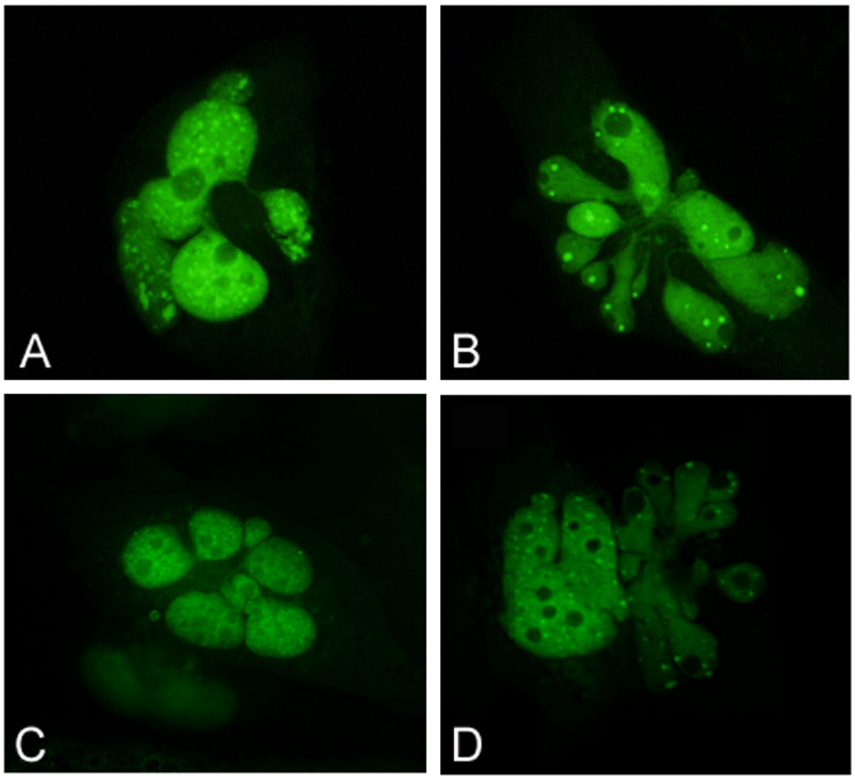

Figure 4. Treatment of T98G cells with the P/CAF-specific inhibitor H3-CoA-20-Tat prevents TSA-mediated accumulation of DEK in IGCs. *A* shows a cell after overnight treatment with TSA. *B* shows a cell pretreated with the CBP/p300 inhibitor Lys-CoA-Tat at a concentration of 50 μM, followed by overnight treatment with TSA. *C* shows a cell pretreated with the P/CAF-specific inhibitor H3-CoA-20-Tat (50 μM), followed by overnight treatment with TSA. *D* depicts a cell pretreated with a control inhibitor, H3-(Ac)-20-Tat (50 μM), followed by overnight treatment with TSA.

knowledge, this is the first demonstration that a specific acetylase can control the movement of a protein into the IGC.

Inhibition of transcription induces DEK accumulation in nuclear speckles

IGCs are nuclear bodies that comprise primarily RNA splicing factors. Given DEK's previously described association with SR proteins, we sought to examine if DEK's migration to the IGC could be induced in a manner similar to other known splicing factors. Classically, SR proteins and splicing factors such as SC35 and U2AF are redirected into IGCs upon treatment with an inhibitor of transcription (Gama-Carvalho, Krauss et al. 1997; Melcak, Cermanova et al. 2000). We tested whether actinomycin D, a transcriptional inhibitor, could similarly drive DEK into IGCs. We found that treatment with actinomycin D did indeed cause DEK to aggregate in punctate bodies (Figure 6A). We next used SC35 co-staining to determine if the DEK aggregates co-localized with IGCs. Interestingly, we found that DEK did co-localize with SC35 in most cells (Figure 6B, *top panels*), but in other cells DEK aggregates only partially coincided with SC35 staining (Figure 6B, *bottom panels*). Additionally, we occasionally identified DEK bodies that appeared to be cytosolic (Figures 6A and 6B, *bottom panels arrowheads*).

We have previously observed DEK movement into the cytosol following phorbol 12-myristate 13-acetate (PMA) treatment of the U937 monocytic cell line (Faulkner, Hilfinger et al. 2001). To confirm the extranuclear localization of DEK after actinomycin D treatment, U937 cells treated with TSA, PMA, or actinomycin D were fractionated into nuclear and cytosolic fractions, and immunoblotted for the presence of DEK. We found

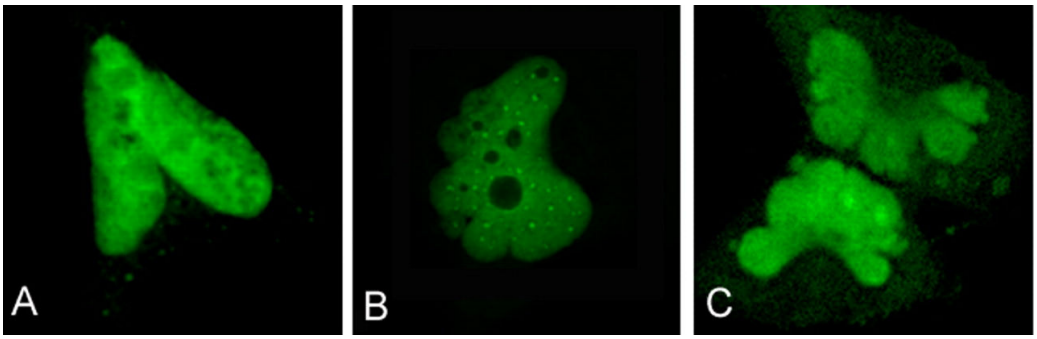

Figure 5. Overexpression of P/CAF, but not CBP, drives DEK into IGCs. *A* shows a cell co-transfected with vectors encoding GFP-DEK and CBP. *B* shows a cell co-transfected with vectors encoding GFP-DEK and P/CAF. *C* shows a cell co-transfected with GFP-DEK and P/CAF-expressing vectors followed by treatment with the P/CAF-specific inhibitor H3-CoA-20-Tat (50 μM).

that TSA, PMA and actinomycin D all induced translocation of a small but significant amount DEK into the cytosol (Figure 6C). Intriguingly, actinomycin D treatment also resulted in the expression in the nucleus of a low-mobility form of DEK (~70 kilodaltons) that was not observed with other treatments (Figure 6C, *lane 4*). This form was recognized by both monoclonal and polyclonal anti-DEK antibodies (data not shown). These data show that, like many mRNA processing factors and SR proteins, DEK translocates to IGCs in response to transcriptional inhibition.

Deacetylation inhibitors do not promote association of DEK with RNA

Taken together with previous associations of DEK with SR protein complexes, the migration of DEK into IGCs in response to transcriptional inhibition suggested a role for DEK in mRNA processing. Because acetylation reduced DEK's DNA-binding affinity and altered its sub-nuclear localization, we hypothesized that acetylation of DEK may also function to regulate the RNA-binding activities of DEK. We tested this possibility by assessing DEK-RNA interactions in the presence of TSA. We digested untreated or TSA-treated HeLa nuclei with either DNase or RNase. We then separated the released fractions and the nuclear retained fractions and assessed the amount of DEK contained in each fraction by immunoblotting. As has been described previously (Kappes, Burger et al. 2001), we found that the vast majority of nuclear DEK was released upon treatment with DNase (Figure 7, *lanes 2 and 5*). This was true for both untreated and TSA-treated HeLa nuclei. In contrast, we observed that RNase treatment resulted in the release of a significant, albeit small, amount of DEK from the nuclei. This

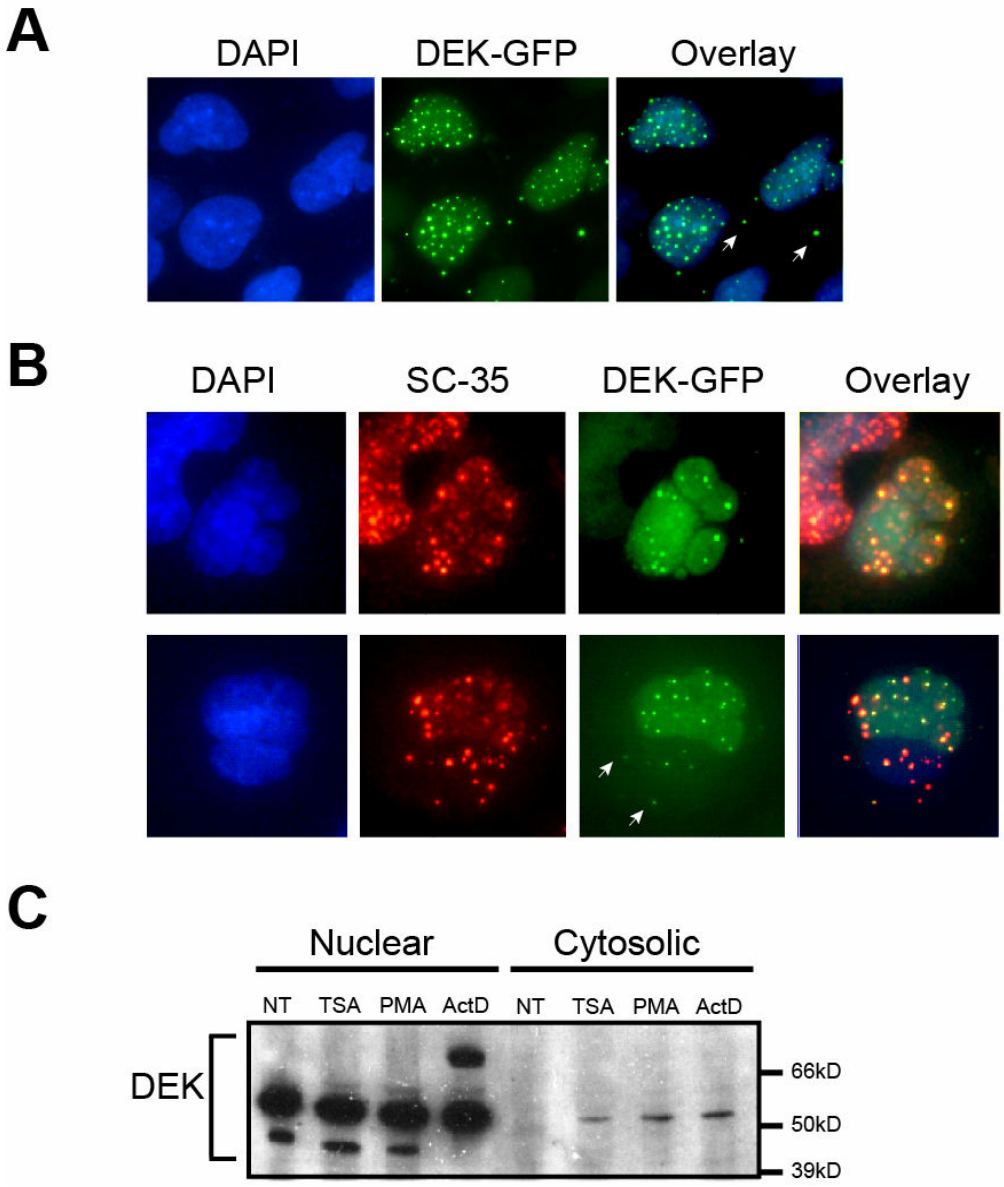

Figure 6. Inhibition of transcription alters DEK sub-nuclear localization. *A* shows a cell transfected with a vector encoding GFP-DEK and treated with 1 µg/ml actinomycin D for 12 hours. *B,* cells were treated as in *A*, and subsequently were stained for SC35. *C,* immunoblotting for DEK expression of nuclear and cytoplasmic fractions of U937 cells treated with either 1 µM trichostatin A (TSA), 32 nM phorbol 12-myristate 13-acetate (PMA), 1 µg/mL actinomycin D (ActD), or no treatment (NT). *(arrows, extranuclear DEK)*

is in agreement with previous observations that estimated approximately 10% of nuclear DEK is released following RNase digestion of HeLa nuclei (Kappes, Burger et al. 2001). However, TSA treatment resulted in a decrease in the amount of DEK released following RNase digestion (Figure 7, *lanes 3 and 4*). This suggests that acetylation of DEK results in reduced DEK-RNA interactions.

RNA Immunoprecipitation of DEK

We next attempted to identify pre-mRNA and mRNA targets of DEK. RNA immunoprecipitation (RIP) of ribonuclear protein (RNP) complexes is a useful method of dissecting RNA-protein interaction *in vivo* and is particularly useful in identifying biologically relevant RNAs. RIP is technically similar to chromatin immunoprecipitation (ChIP), with the obvious difference being that the target of the former is RNA and the target of the latter is DNA. Like ChIP, RIP requires the use of a reversible crosslinker, since most RNA-protein interactions are not sufficiently stable.

The procedure is outlined in Figure 8A. HeLa cells were infected with either an adenoviral vector expressing Flag-DEK or a control vector expressing GFP. Three days after infection, cells were collected and fixed with formaldehyde. Cells were lysed by sonication, and the lysates were immunoprecipitated with anti-Flag antibodies. HeLa cells infected with the control vector did not express any Flag-tagged proteins, and thus were used a control for non-specific background determination. Following precipitation, beads were washed, crosslinks were reversed and precipitated RNP complexes were processed for protein or RNA analysis. Immunoblotting confirmed successful immunoprecipitation of DEK protein (Figure 8B). RNA analysis of the mock and DEK-

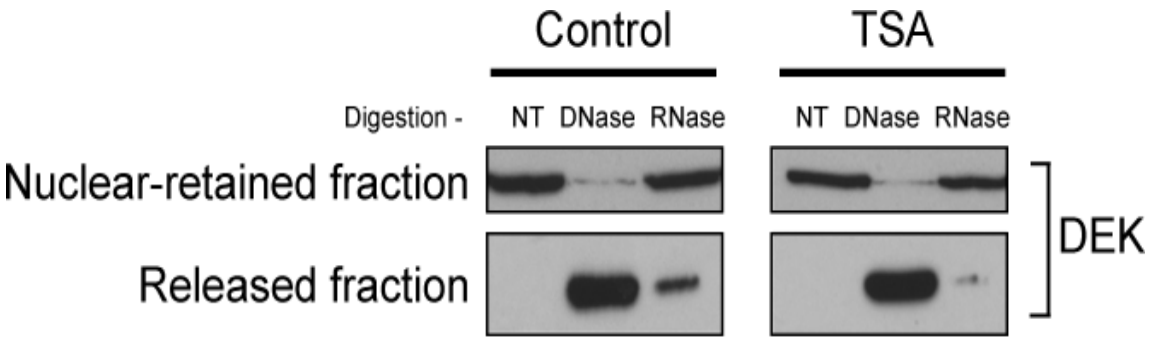

Figure 7. TSA does not enhance DEK association with RNA. Untreated or 1 μM TSA treated nuclei were digested with either DNase or RNase, or received no treatment (NT). Proteins released with treatment, and those retained in the nuclear fraction were immunoblotted for DEK.

immunoprecipitated samples using an Agilent Bioanalyzer 2100 revealed nearly identical electropherograms, indicating similar RNA composition (Figure 8C). Additionally, area under the curve analysis estimated a negligible amount of RNA co-precipitating with DEK (Figure 8D). These results indicate that conventional RIP of DEK does not precipitate DEK-bound RNA targets.

Discussion

We show here that DEK is an acetylated protein whose nuclear localization can be regulated by the acetylase P/CAF. We find that non-specific inhibition of histone deacetylases results in the accumulation of DEK in IGCs. This relocalization of DEK is dependent on the activity of P/CAF, which appears to be both necessary and sufficient to cause migration of DEK to IGCs. We additionally find that DEK accumulates in both IGC and non-IGC nuclear speckles in response to transcriptional inhibition. Finally, we show that acetylation of DEK does not promote interactions with RNA, and direct DEK-RNA interactions cannot be observed with conventional RIP.

We demonstrate for the first time that acetylation alters the sub-nuclear localization of DEK, as deacetylase inhibition results in the redistribution of DEK from being diffusely nuclear to being concentrated into IGCs. To our knowledge, this is the first demonstration that movement of any protein into the IGC is under the control of acetylation changes. We have not strictly shown that acetylation of DEK is the cause, rather than the consequence, of its relocation into the IGC. Attempts to address this issue using protein transfection have proven unrewarding, as we have been unable to transduce DEK into cells. This may be due to the highly charged nature of the individual domains of

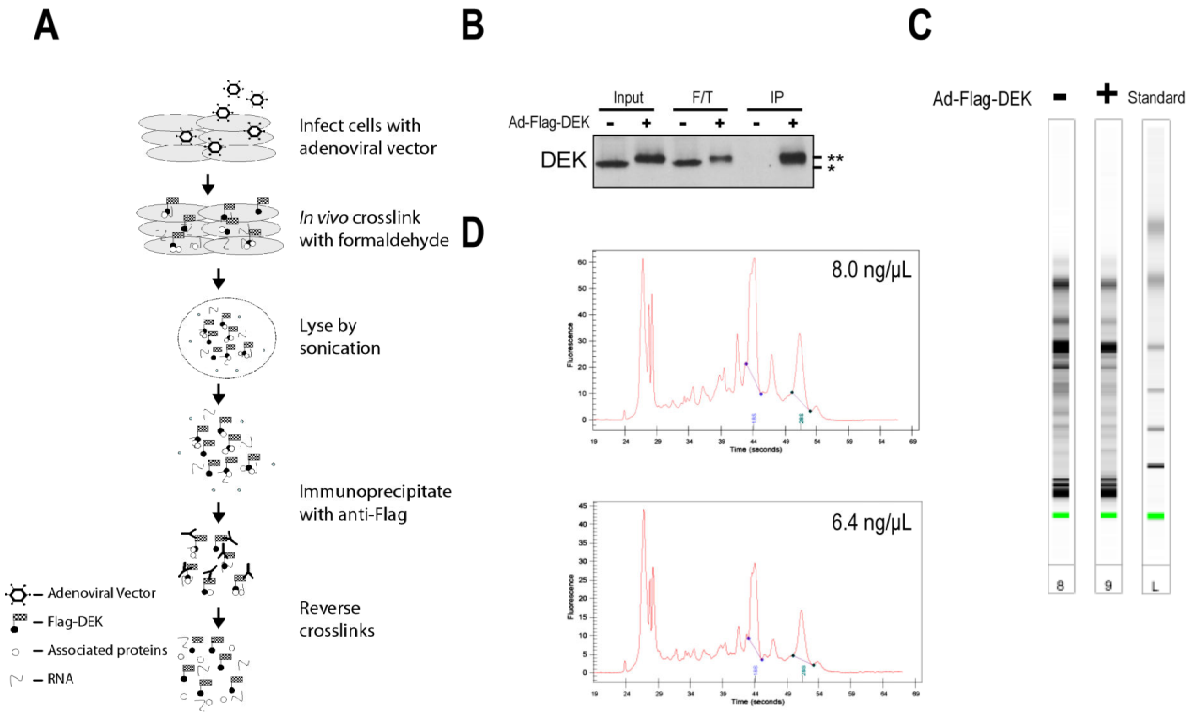

Figure 8. RNA immunoprecipitation of DEK. *A,* experimental setup of RNA immunoprecipitation assay. HeLa S3 cells were transfected with either an adenoviral vector expressing GFP or an adenoviral vector expressing human Flag-tagged DEK. 72 hrs after transfection, cells were then crosslinked with formaldehyde and lysed by sonication. DEK was immunoprecipitated from lysates with anti-FLAG antibodies, washed, and crosslinks were reversed with heat treatment. *B,* immunoprecipitated proteins were immunoblotted to confirm successful immunoprecipitation of DEK in total lysates *(input),* non-precipated *(F/T)*, and precipitated fractions *(IP)* [* - wild type DEK, ** - Flag-tagged DEK] *C* and *D,* RNA was purified from precipitates and analyzed using an Agilent Bioanalyzer. *C,* Agilent Bioanalyzer electropherograms depicting the profile of precipitated RNAs. *D*, Agilent Bioanalyzer histogram profile and quantification of precipitated RNA

DEK, or to the propensity of this protein to multimerize. However, it appears that the most straightforward explanation for the translocation of DEK into the IGC is that this movement follows acetylation. There are several reasons to assume that this is the case. First, when DEK is bound to DNA, it is tightly associated with chromatin, and acetylases are also known to act on chromatin, so this would be a logical place for DEK/acetylase interaction. Further, because it is the addition of deacetylase inhibitors or transfection of a vector expressing P/CAF that is the first step in our experiments, the simplest explanation is that an acetylation event drives DEK into IGCs. Additionally, we show that acetylation reduces the affinity of DEK for DNA, thus it seems likely that acetylation would alter the sub-nuclear localization of this predominately chromatin-bound protein. Therefore, it appears most likely that acetylation events precede the relocalization of DEK into the IGC, rather than DEK first entering IGCs and then undergoing acetylation.

Importantly, DEK is driven into the IGCs by overexpression of P/CAF, but not CBP, even though both histone acetyltransferases can acetylate DEK *in vitro*. In contrast, the majority of studies to date have demonstrated that it is the acetylation by CBP/p300 that has important functional consequences *in vivo*, including DNA binding, chromatin remodeling, and protein-protein recognition (Boyes, Byfield et al. 1998; Marzio, Wagener et al. 2000; Tomita, Towatari et al. 2000; Barlev, Liu et al. 2001; Chen, Fischle et al. 2001; Hayakawa, Towatari et al. 2004; Schubert, Horstmann et al. 2004). Interestingly, one of the few examples of an *in vivo* role for acetylation by P/CAF is for the Class II transactivator protein, CIITA: acetylation results in relocalization of the protein from the cytoplasm to the nucleus (Spilianakis, Papamatheakis et al. 2000). The sub-nuclear movement of DEK can be blocked with a novel P/CAF-specific small

molecule inhibitor, but not by a similar CBP/p300 inhibitor or a control molecule. These compounds and other similar small molecule inhibitors of various acetylases have considerable advantage over previously described acetylase inhibitors, because they are cell-permeable and therefore do not require transfection. We believe these compounds will have significant and broad application in the identification of proteins that are acetylated *in vivo* and further the understanding of the functional consequences of acetylation in controlling gene regulation.

IGCs are dense collections of proteins that have been shown to have a high concentration of pre-mRNA processing factors (Misteli and Spector 1997; Lamond and Spector 2003). Although RNA is found at the periphery of these structures, IGCs generally exclude nucleic acid (Hendzel and Bazett-Jones 1995; Mintz, Patterson et al. 1999). Multiple studies have concluded that mRNA processing does not occur in IGCs, leading to the notion that IGCs represent a storage depository of inactive mRNA processing factors (Fakan, Leser et al. 1984; Zeng, Kim et al. 1997; Cmarko, Verschure et al. 1999). In support of this, treatment with transcriptional inhibitors results in a depletion of pre-mRNA products and causes the subsequent accumulation of mRNA processing factors, such as U2AF and SC35, to the IGCs (Gama-Carvalho, Krauss et al. 1997; Zeng, Kim et al. 1997). Accordingly, DEK's migration into IGCs following transcriptional inhibition suggests a role in pre-mRNA processing. DEK has previously been found to be associated with spliced mRNA and with ribonucleic protein (RNPs) complexes, although a proteomic analysis of the spliceosome did not identify DEK (Zhou, Licklider et al. 2002). We found that acetylation of DEK results not only in migration to IGCs, but also reduced interactions between DEK and RNA. Thus, the DEK

found in IGCs upon acetylation may contain a fraction of DEK that was previously involved in active mRNA processing, in addition to DEK that may be released from chromatin. Our results support a model in which acetylation reduces DEK's affinity for both DNA and RNA interactions, resulting in release of DEK from its normal nucleic acid-bound, sub-nuclear location, and as a result, DEK subsequently accumulates in IGCs. It remains unclear whether accumulation of released DEK in the IGCs contributes to an alternate function, or if the IGCs are merely acting as a storage area for inactive DEK.

Finally, we found that while DEK redistributed from a diffuse to a punctate pattern in response to both transcriptional inhibition and acetylation, there were some significant differences. Notably, the punctate pattern of DEK did not always colocalize with IGCs following transcriptional inhibition. Although we have clearly shown that in response to acetylation DEK migrates into IGCs and not PODs, it is possible that following transcriptional inhibition DEK may additionally enter other sub-nuclear compartments, such as PODs or Cajal bodies. Notably, inhibitors of transcription, such as actinomycin D, have been shown to induce chromatin retraction and the induction of new PML bodies (Eskiw, Dellaire et al. 2004).

We find that, in striking contrast to treatment with deacetylation inhibitors, transcriptional inhibition resulted in the expression of a novel low-mobility form of DEK. As this report and others have shown that DEK is the target of extensive post-translational modifications (Kappes, Damoc et al. 2004; Gamble and Fisher 2007; Kappes, Fahrer et al. 2008), it is very likely that this represents a post-translationally

modified form of DEK. If DEK is indeed redirected to PODs in response to transcriptional inhibition, an interesting candidate for this modification would be sumoylation. Sumoylation is a post-translational modification that involves the covalent attachment of small ubiquitin-like modifier peptides (SUMO) to lysine residues of a target protein. In contrast to ubiquitination, sumoylation tends to enhance protein stability and has been shown to be an important factor in regulating sub-nuclear localization, particularly in targeting of factors to PODs. This SUMO-dependent recruitment to PODs has been best described for the transcriptional co-repressor Daxx (Ishov, Sotnikov et al. 1999; Li, Leo et al. 2000; Lin, Lai et al. 2003). Like Daxx, DEK has multiple putative sumoylation sites (Xue, Zhou et al. 2006), and it is worth noting that Daxx is one of the few verified protein interaction partners of DEK (Hollenbach, McPherson et al. 2002). Taken together, these findings show that in the context of transcriptional inhibition DEK may exhibit both radically altered form and function.

In summary, we identify acetylation as a post-translational modification of DEK that regulates its sub-nuclear localization. Our results suggest a potential mechanism of regulating the participation of DEK in the disparate processes of transcriptional repression, chromatin maintenance, and mRNA processing. Our findings suggest that P/CAF activity may both relieve transcriptional repression by releasing chromatin bound DEK and may also regulate the poorly understood interaction between DEK and RNP complexes. These results support the notion that DEK is a versatile nuclear protein whose location and function is determined by multiple post-translational modifications.

Materials and Methods

Cell Culture and Transfection

T98G and U937 cells were purchased from ATCC and passaged in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. GFP-DEK was constructed as a fusion protein between DEK and an enhanced variant of *Aequorea victoria* green fluorescent protein. Primers with EcoRV (5') and EcoRI (3') restriction sites were designed for PCR-based subcloning of the *dek* coding region into the pGNVL3 mammalian GFP vector (gift of T. Glaser, University of Michigan). Cells were transfected with 2.5 μg of GFP-DEK, P/CAF, or CBP plasmid using Lipofectamine 2000 (Invitrogen). After transfection, cells were treated overnight with 1 μM TSA (Sigma) or left untreated, and fixed for immunocytochemistry on the following day. Alternatively, untransfected cells were treated overnight with 330 nM TSA or left untreated and fixed the following day. Actinomycin D (Sigma) was used at a concentration of 1 μg/mL for 12 hours.

Construction of DEK-encoding Adenovirus

FLAG-tagged DEK was excised from pCMV-Tag1 (Stratagene) using HindIII and BamHI and introduced via those sites into the adenoviral shuttle plasmid pACCMVpLpA(-)loxP-SSP. This vector was linearized with SfiI, and the transgene was recombined into E1A/E1B-deficient adenovirus by the University of Michigan Vector Core. Virus was harvested from E1A/E1B-positive producer cells, purified by cesium chloride density gradient centrifugation, and assayed for plaque-forming unit

concentration. T98G cells were transduced at a multiplicity of infection of 100 by incubation for 48 hours followed by cell harvesting for immunoprecipitation.

Immunoprecipitation and Western Blotting

Immunoprecipitation of FLAG-DEK from transduced T98G cells was performed using agarose-conjugated anti-FLAG M2 monoclonal antibodies (Sigma) by following the manufacturer's instructions with regards to cell lysis, incubation, and washing, and elution by competition with 3xFLAG peptide (Sigma). Fractionation into nuclear and cytosolic components was performed as described previously (Kappes, Burger et al. 2001). For Western blotting, proteins were transferred from the gel to polyvinylidene difluoride membrane, blocked in 5% milk, and probed with either horseradish peroxidase-conjugated anti-FLAG antibody at a dilution of 1:1000 (Sigma), or monoclonal anti-acetylated lysine antibody at a dilution of 1:1000 (Cell Signaling Technologies) or monoclonal anti-DEK antibody (Biosciences), followed by horseradish peroxidase-conjugated anti-mouse IgG (Molecular Probes) for chemiluminescent detection.

Production of Recombinant His-DEK in Baculovirus

DEK coding sequence was subcloned into the pBacPAK-His3 vector for recombination into the baculovirus genome. Recombination, virus production, cell transduction and harvesting, and protein purification were performed as indicated in the manufacturers' instructions for the BacPAK Baculovirus Expression System (Clontech) and HIS-trap nickel-chelating columns (Amersham Biosciences).

In Vitro Protein Acetylation Assay

Recombinant His-DEK was dialyzed overnight at 4 °C into acetylation buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 5% glycerol). DEK was then incubated at 30 °C for 1 hour with 50 μM [^{14}C]acetyl-CoA (Amersham Biosciences), 10 mM sodium butyrate, and 50 nM of either CBP, p300, or P/CAF, which had been purified as previously described (39). Reactions were resolved by SDS-PAGE; gels were stained with Coomassie Blue reagent and dried. The ^{14}C signal was detected using a phosphorimaging screen and FX phosphorimaging device (Bio-Rad).

Immunocytochemistry

Three phosphate-buffered saline (PBS) washes were performed in between each of the following steps; when indicated, PBS washes contained 0.1% saponin. Cells were washed and fixed for 10 min in 4% paraformaldehyde. Fixed cells were washed, blocked for at least 1 hour with 0.2% bovine serum albumin in PBS, rewashed with PBS/saponin, and incubated for 1 hour with mouse monoclonal anti-SC35 (23 μg/ml; Sigma) in PBS/saponin. If cells were not transfected with GFP-DEK, then the incubation included polyclonal anti-DEK serum at a 1:200 dilution (gift of G. Grosveld). Cells were then rewashed in PBS/saponin and reblocked with goat serum in PBS/saponin at a 1:50 dilution for 1 hour, followed by PBS/saponin washing and a 1 hour incubation with Alexa fluor 594-conjugated goat anti-mouse IgG (20 μg/ml; Molecular Probes) in PBS/saponin. If cells were not transfected with GFP-DEK, then incubation included Alexa fluor 488-conjugated goat anti-rabbit IgG (20 μg/ml; Molecular Probes). Cells were washed with

PBS/saponin, refixed for 10 min, rewashed, and incubated with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, rewashed with PBS, and distilled water then dried overnight. Coverslips were mounted with Antifade A reagent (Molecular Probes), and images were captured with a Zeiss Laser Scanning Microscope (LSM 510, version 2.8 SPI). For staining of PODs, the procedure was identical except for the use of polyclonal rabbit anti-CBP as the primary antibody (10 μg/ml, Upstate Biotechnology), and Alexa fluor 594-conjugated goat anti-rabbit IgG as the secondary antibody (20 μg/ml, Molecular Probes).

Preparation of H3-CoA-20-Tat and H3-(Ac)-20-Tat

H3-CoA-20-Tat (Ac-ARTKQTARKSTGGK(CoA)APRKQLYGRKKRRQRRR-OH) is a derivatized version of the synthetic P/CAF inhibitor H3-CoA-20, in which the C-terminal sequence ends in the 12 amino acid residues of the cell permeabilizing Tat sequence. H3-(Ac)-20-Tat, a control compound, differs from H3-CoA-20-Tat in that the CoA moiety is replaced by a hydrogen atom. These compounds were synthesized by the solid phase method on a Rainin PS3 peptide synthesizer using the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) strategy, analogous to the previously described method for H3-CoA-20 (Lau, Kundu et al. 2000; Poux, Cebrat et al. 2002). The epsilon amino group of the lysine residue that corresponds to Lys-14 of histone H3 was protected with the *N*-[1(4,4-dimethyl 2,6-dioxocyclohexylidene)ethyl] (Dde) group whereas other Lys residues were protected with the *t*-butoxycarbonyl group. Following amino acid couplings and N-terminal acetylation, the Dde group was removed by mixing the fully protected peptide resin with 2% hydrazine in dimethylformamide for 3 hours at room temperature. The

peptide resin was then reacted with 5 equivalents of bromoacetic acid and 5 equivalents of diisopropylcarbodiimide for 16 hours at room temperature (or acetic anhydride for 1 hour for the control peptide). Peptides were cleaved from the resin with Reagent K (trifluoroacetic acid:phenol:H_2O: thioanisole:ethanedithiol:triisopropylsilane (81.5:5:5:5:2.5:1)) for 4 h at room temperature and subsequently precipitated with ice-cold diethyl ether. Precipitates were collected by centrifugation (3000 rpm, 5 min), the supernatants discarded, and the pellets washed two times with cold diethyl ether (30 ml). Precipitated peptides were dissolved in 5 ml of water, flash-frozen, lyophilized, and purified by preparative reversed phase (C_{18}) high-performance liquid chromatography using a gradient of H_2O:CH_3CN:0.05% trifluoroacetic acid. The bromoacetylated peptide was conjugated with 2 equivalents of CoASH in a minimal volume of aqueous 0.5 M trimethylammonium bicarbonate (pH 8) for ~16 hours at room temperature, lyophilized, and purified initially by passage over anion exchange chromatography (Dowex 1 x 8–100) to remove excess CoASH followed by reversed phase high-performance liquid chromatography in a gradient of H_2O:CH_3CN:0.05% trifluoroacetic acid. Peptides were confirmed to be >95% pure by high-performance liquid chromatography, and their structural identities were confirmed by mass spectrometry. The inhibitory properties of both peptides against P/CAF and p300 are shown in Table I.

DNase and RNase digestion of nuclei

Release of nuclear DEK following DNase and RNase treatment was performed as described previously with minor modifications (Kappes, Burger et al. 2001). Cells were washed three times with ice cold hypotonic buffer (20 mM HEPES pH 7.4, 20 mM NaCl,

5 mM $MgCl_2$) and lysed by Dounce homogenization. Lysates were incubated for 15 min on ice and nuclei were pelleted by centrifugation (5 min, 600 x g). Nuclei were washed and incubated in elution buffer (20 mM HEPES, pH 7.4, 0.5 mM $MgCl_2$, 1 mM ATP, 0.3 M sucrose, 100 mM NaCl) and were incubated for 30 min at 37 °C with 17 units of RNase-free DNase I (Promega) or 40 units of DNase-free RNase A (Roche). Reactions were stopped on ice, and nuclei were pelleted by centrifugation (5 min, 600 x g). The supernatant and pellet containing the released and retained fractions, respectively, were collected.

RNA Immunoprecipitation

HeLa cells were infected with either a control adenovirus expressing GFP, or a FLAG-tagged DEK encoding adenovirus. Three days after infection, cells were detached with 0.5 mM EDTA and washed with PBS. Cells were fixed with 1% paraformaldehyde for 20 minutes at room temperature. Fixation was quenched by addition of glycine (pH 7.0) to a final concentration of 250 mM. Cells were washed twice with PBS and resuspended in RIPA buffer (50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) containing protease inhibitors (complete, mini, EDTA-free protease inhibitor cocktail tablet, one tablet for 10 mL buffer, Roche) and 40 units/mL RNasin (Promega). Cells were lysed by sonication in 20 second intervals and complete lysis was verified by microscope. Lysates were cleared by centrifugation (10 min, 16,000xg). Aliquots were collected for later RNA and protein analysis. Agarose beads conjugated to anti-FLAG M2 monoclonal antibodies (Sigma) were added, and samples were incubated for two hours at 4 °C with rotation. Beads and

supernatant were collected separately. The beads were washed six times with high-stringency RIPA buffer (50mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 1 M NaCl, 2 M urea, and 0.2 mM phenylmethyl fluoride). Following the washes, beads were resuspended in 50 mM Tris-Cl, pH 7.0, 5 mM EDTA, 10 mM dithiothreitol, and 1% SDS. Lysates were then incubated at 70 °C for 45 min to reverse crosslinks. Supernatants were collected and total RNA was purified using the Trizol method according to manufacturer's instructions (Invitrogen). An aliquot of the supernatant was also kept and analyzed by immunoblotting. RNA yield and composition was determined by Total RNA Pico LabChip assay using an Agilent 2100 Bioanalyzer (Agilent Technologies).

CHAPTER III

DEK EXPRESSION INHIBITS SENESCENCE IN MELANOMA

Abstract

The human *DEK* gene is frequently overexpressed in a number of malignancies, though the oncogenic contribution of this overexpression has not been identified. Here, we demonstrate for the first time a putative role for DEK in the progression of malignant melanoma. Expression of DEK protein in metastatic melanoma cell lines was found to be an average of 30 fold higher than in primary foreskin-derived melanocytes. Inhibition of DEK expression by shRNAs was found to inhibit proliferation of a subset of melanoma lines. Staining for senescence-associated β-galactosidase activity revealed that the reduction in cell growth was due to the induction of senescence in these cell lines. DEK expression was required to suppress expression of the $p21^{CIP1}$ in all melanoma lines. In contrast, another senescence-inducing protein, $p16^{INK4a}$, was not affected by knockdown of DEK expression. Oncogene-induced senescence of normal melanocytes induced by introduction of either $BRAF^{V600E}$ or $NRAS^{Q61R}$ did not result in diminished DEK expression. These results suggest that DEK overexpression is a frequent event in melanoma progression and point to a role for DEK in the circumvention of melanoma senescence.

Introduction

Malignant melanoma is the most lethal form of skin cancer, and given its increasing incidence it is likely to remain a major clinical challenge (Jemal, Siegel et al. 2008). Many of the genetic alterations found in melanoma, such as mutation of the oncogenes BRAF and NRAS or activation of the AKT pathway, serve to promote cell proliferation. However, rather than inducing tumor formation, introduction of these alterations alone into normal melanocytes typically results in senescence (Serrano, Lin et al. 1997; Chen, Trotman et al. 2005; Michaloglou, Vredeveld et al. 2005). It is now apparent that multiple tumor suppressive safeguards must be inactivated in addition to oncogene activation before a normal melanocyte progresses to overt melanoma.

Two principal senescence-inducing tumor suppressor pathways have been identified. The first pathway, $p16^{INK4a}$/pRb, has been discovered to be almost invariably inactivated in melanomas, either through deletion, mutation or silencing of $p16^{INK4a}$ itself, or through mutations in downstream pathway components (Bartkova, Lukas et al. 1996; Walker, Flores et al. 1998). Conversely, a second principal senescence-inducing tumor suppressor, p53, is not commonly mutated or deleted in melanomas, despite being a frequent target of inactivation in other neoplasms. Nevertheless, p53 activity does appear to be diminished in melanoma cells through poorly delineated mechanisms (Satyamoorthy, Chehab et al. 2000). It seems likely that melanomas possess heretofore unidentified means of evading this formidable tumor suppression checkpoint.

Cytogenetic analysis of melanoma also points to the involvement of additional oncogenes that may be required for tumor development. Melanomas display remarkable genomic instability that results in a wide array of chromosomal abnormalities. The

frequent gains and losses of specific chromosomes suggest that these regions of instability may harbor additional unidentified oncogenes and tumor suppressor genes. Alteration of chromosome 6 has frequently been identified as the most common cytogenetic change in melanoma (Santos, Zielenska et al. 2007). In particular, gain of the 6p arm is one of the few chromosomal aberrations that is prevalent in all types of melanoma, including cutaneous (both sun-exposed and non-sun-exposed), acral, mucosal, and uveal melanomas (Curtin, Fridlyand et al. 2005). Furthermore, temporal clustering of karyotypic changes in melanoma indicates that 6p gain represents an early event in melanoma evolution (Hoglund, Gisselsson et al. 2004). Although the minimal region of 6p gain has not been described in melanoma, multiple studies in retinoblastoma and bladder cancer have identified a narrow region within 6p22.3 as the minimal region of 6p gain in those carcinomas (Chen, Gallie et al. 2001; Evans, Gallie et al. 2004). This region notably contains the putative oncogene *DEK*.

DEK was originally discovered as a part of a chromosomal translocation event [t(6;9)(p22;q34)] present in a subset of acute myeloid leukemia (von Lindern, van Baal et al. 1992). Subsequent studies have repeatedly identified *DEK* as a frequently overexpressed gene (independent of the t(6;9) translocation) in a number of neoplasms, including retinoblastoma, hepatocellular carcinoma, bladder carcinoma, glioblastoma, acute myeloid leukemia, and melanoma, thus implying that *DEK* may be an oncogene (Kondoh, Wakatsuki et al. 1999; Grottke, Mantwill et al. 2000; Kroes, Jastrow et al. 2000; Larramendy, Niini et al. 2002; Casas, Nagy et al. 2003; Sanchez-Carbayo, Socci et al. 2003; Wu, Hoffmann et al. 2005; Carro, Spiga et al. 2006).

DEK has been implicated in several diverse cellular processes including chromatin architecture maintenance, mRNA splicing, transcriptional repression, DNA damage repair, and cell to cell signaling (Fu, Grosveld et al. 1997; Alexiadis, Waldmann et al. 2000; Kappes, Burger et al. 2001; Waldmann, Eckerich et al. 2002; Kappes, Scholten et al. 2004; Mor-Vaknin, Punturieri et al. 2006; Sammons, Wan et al. 2006; Soares, Zanier et al. 2006; Wise-Draper, Allen et al. 2006; Gamble and Fisher 2007; Kappes, Fahrer et al. 2008). Extensive post-translational modification by phosphorylation, acetylation, and poly-(ADP) ribosylation is thought to regulate its participation in these disparate cellular processes (Kappes, Damoc et al. 2004; Cleary, Sitwala et al. 2005; Gamble and Fisher 2007; Kappes, Fahrer et al. 2008).

Despite the clear association of DEK overexpression with malignancy, its potential contributions to tumorigenesis have yet to be elucidated. One possibility is that DEK may act to inhibit senescence in transformed cells. Two reports have demonstrated that DEK upregulation by the human papillomavirus (HPV) E7 protein is required to inhibit senescence following HPV infection (Wise-Draper, Allen et al. 2005; Johung, Goodwin et al. 2007). The HPV E7 protein enhances DEK expression in a pRB-dependent manner, as mouse embryonic fibroblasts from triple pRB/p107/p130 knockout mice do not upregulate DEK in response to E7. Furthermore, DEK can inhibit senescence even in the absence of HPV infection. RNAi interference with DEK expression was sufficient to induce premature replicative senescence in primary human fibroblasts (Johung, Goodwin et al. 2007).

We sought to examine DEK's oncogenic contribution to melanoma. We find that DEK is overexpressed at the protein level in all metastatic melanoma lines tested. Using

lentiviral shRNA vectors, we demonstrate that interference with DEK expression results in the arrest of cell proliferation and the induction of senescence. Furthermore, we find that the cyclin dependent kinase inhibitor $p21^{CIP1}$ is induced following knockdown of DEK expression in both senescent and non-senescent melanoma lines. Finally, we demonstrate that DEK expression is not diminished following oncogene-induced senescence of primary melanocytes. These results establish DEK as a *bona fide* melanoma oncogene with anti-senescence function.

Results

DEK protein overexpression in metastatic melanoma.

While overexpression of *DEK* mRNA has frequently been identified in numerous malignancies, including melanoma, DEK protein overexpression has been confirmed only in retinoblastoma and lung carcinoma (Carro, Spiga et al. 2006; Orlic, Spencer et al. 2006). To determine DEK protein expression in melanoma, a diverse panel of metastatic melanoma lines, as well as normal melanocytes isolated from two individuals, were immunoblotted for DEK (Figure 9A). DEK was found to be universally overexpressed in all melanoma lines tested by an average of about 30 fold compared to normal melanocytes as estimated by densitometry (Figure 9B). DEK expression did not display any obvious correlation with other known melanoma genetic features (Table 2). These findings suggest that upregulation of DEK may represent a universal event in the development of melanoma.

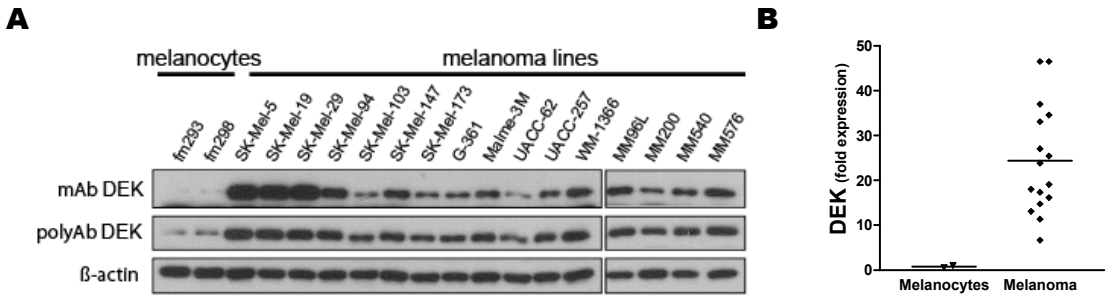

Figure 9. Overexpression of DEK in metastatic melanoma lines. *A,* immunoblotting of a panel of metastatic melanoma lines with monoclonal (mAb) and polyclonal (polyAb) anti-DEK antibodies. Two cultures of primary foreskin-derived melanocytes, fm293 and fm298, were also included. β-actin is shown as a loading control. *B,* Monoclonal anti-DEK band densities were quantitated, and relative DEK expression was determined. β-actin band density was used to adjust for loading, and levels were normalized to fm293 melanocytes. The horizontal line indicates the mean for each group.

NHEM	FM	SK-MEL 5	SK-MEL 19	SK-MEL 29	SK-MEL 28	SK-MEL 94	SK-MEL 103	SK-MEL 147	SK-MEL 173	G-361	Malme 3M	UACC 62	UACC 257	WM 1366	MM 96L	MM 200	MM 540	MM 576
DEK	-/+	+++	+++	+++	+	++	+	++	+	+	++	+	++	++	+++	+	+++	+++
NRAS	wt	wt	wt	ND	wt	wt	Q61R	Q61R	wt	wt	wt	wt	wt	Q61R	wt	wt	wt	wt
BRAF	wt	V600E	V600E	V600E	V600E	V600E	wt	wt	wt	V600E	V600E	V600E	wt	wt	V600E	V600E	V600E	V600E
p53	wt	wt	wt	wt	L145R	wt	wt	wt	wt	wt	wt	wt	wt	ND	wt	wt	S369P	L350P
p14	+	ND	+	+	+	+	-	-	-	-	-	-	+	+	wt	wt	wt	wt
p16	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	wt	-	wt
PTEN	+	+	+	+	-	-	-	-	-	-	+	-	-	-	+	-	+	-

Normal / wild type

Melanoma-associated event

Not determined

Table 2. Melanoma-associated events identified within metastatic melanoma lines

shRNA knockdown of DEK expression in melanoma affects cell proliferation.

The potential contribution of increased DEK expression to the tumorigenicity of melanoma was investigated using lentiviral shRNA vectors targeting DEK expression. Although others have previously demonstrated effective knockdown of DEK expression using RNAi technology, we found that the anti-DEK antibodies utilized in those studies substantially overestimate inhibition of DEK expression (Figure 10A). Due to the extremely high levels of DEK expression in metastatic melanoma, successful RNAi interference with DEK required the expression of two shRNAs targeting distinct regions of the *DEK* transcript (Figure 10B and 10C). A vector that produced two distinct shRNAs was used to disrupt expression of DEK in four melanoma cell lines. Interestingly, shDEK treatment resulted in a reduction of cell proliferation in the SK-MEL-28 and SK-MEL-94 melanoma lines, but not in SK-MEL-19 or SK-MEL-29 lines (Figure 11).

Decreased proliferation of shDEK transduced cells is due to senescence

Cell death was not observed in any of the melanoma lines following treatment with DEK specific shRNA. However, SK-MEL-28 and SK-MEL-94 cells displayed a gradual change in cell morphology. We next investigated whether the defective proliferation and the change in cell morphology were caused by senescence. Senescent cells classically demonstrate β-galactosidase activity that persists in sub-optimal acidic (pH 6.0) conditions (Dimri, Lee et al. 1995). This senescence-associated β-galactosidase activity (SA-β-Gal) is a commonly used biomarker of senescent cells. Staining for SA-β-Gal activity revealed that this decrease in proliferation was indeed the result of the induction of senescence in the SK-MEL-28 and SK-MEL-94 lines (Figure 12A and 12B).

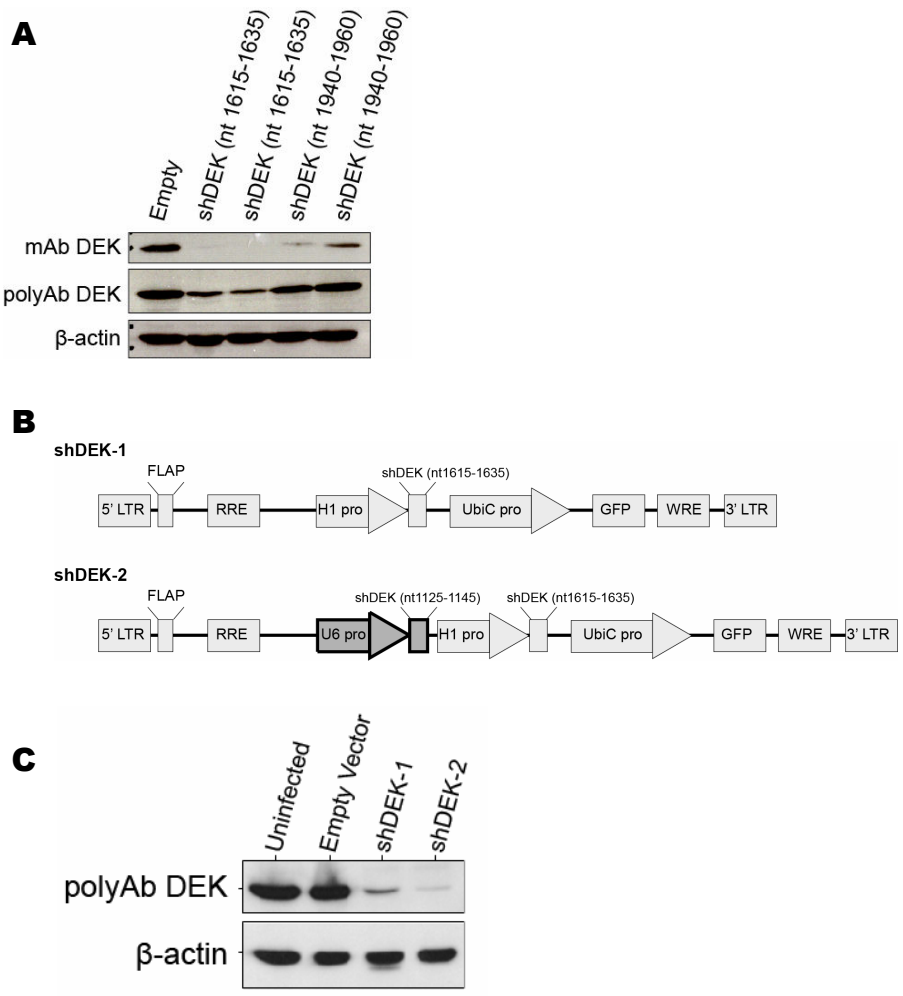

Figure 10. Knockdown of DEK expression requires two distinct shRNAs. *A,* HeLa S3 cells were transduced with the H1-LV lentiviral shRNA vector containing either no shRNA (*empty),* or an shRNA that targets DEK expression (*shDEK)*. DEK expression was determined by immunoblotting with monoclonal (*mAb DEK*) or polyclonal (*polyAb DEK*) antibodies to DEK. *B,* a modified H1-LV lentiviral vector that expressed two distinct shRNAs targeting DEK was contructed by insertion of a second shRNA sequence under the control of the murine U6 promoter *(shDEK-2). C,* DEK expression was detected by immunoblotting following transduction of HeLa S3 cells with a lentiviral shRNA vector expressing no shRNA sequence (*empty),* a single shRNA targeting DEK *(shDEK-1)*, or two distinct shRNAs targeting DEK *(shDEK-2)*.

There was a 13-fold and 9-fold increase in the percentage of SA-β-Gal staining cells in the shDEK treated SK-MEL-28 and SK-MEL-94 lines, respectively. SK-MEL-28 cells additionally displayed classical senescence morphology, marked by flattening and enlargement of cells, while SA-β-Gal positive SK-MEL-94 cells had a less distinct morphology. In contrast, shDEK transduction did not result in any increase in SA-β-Gal staining or cellular morphology in SK-MEL-19 or SK-MEL-29 cells (Figure 12C and 12D).

Knockdown of DEK expression induces $p21^{CIP1}$ in senescing and non-senescing cells

We next examined if inhibition of DEK expression activated either of the two principal senescence pathways, $p16^{INK4a}$/pRB or p53-$p21^{CIP1}$. Although SK-MEL-28 and SK-MEL-94 expressed $p16^{INK4a}$, there was no apparent effect of shDEK treatment on $p16^{INK4a}$ expression (Figure 13A and 13B). Additionally, SK-MEL-28 cells are known to have an R24C mutation in CDK4, effectively inactivating the $p16^{INK4a}$ tumor suppressive pathway (Soengas, Capodieci et al. 2001). Thus, it appears likely that shDEK-induced senescence was not due to $p16^{INK4a}$ activity. In contrast to $p16^{INK4a}$, $p21^{CIP1}$ was induced following knockdown of DEK expression in all four lines. Interestingly, p53 itself was increased in only one of these lines (Figure 13A). This is consistent with $p21^{CIP1}$-mediated senescence in human cells, which characteristically does not display p53 accumulation but does nevertheless exhibit increased p53 activity (Atadja, Wong et al. 1995; Vaziri, West et al. 1997). Notably, the one cell line that did accumulate p53, SK-MEL-28, carries a mutated p53 gene (L145R). SK-MEL-19 and SK-MEL-29 cells also had increased $p21^{CIP1}$ expression, but paradoxically displayed no aberrations in cellular proliferation (Figure 13C and 13D). This may be due to reduced sensitivity or non-

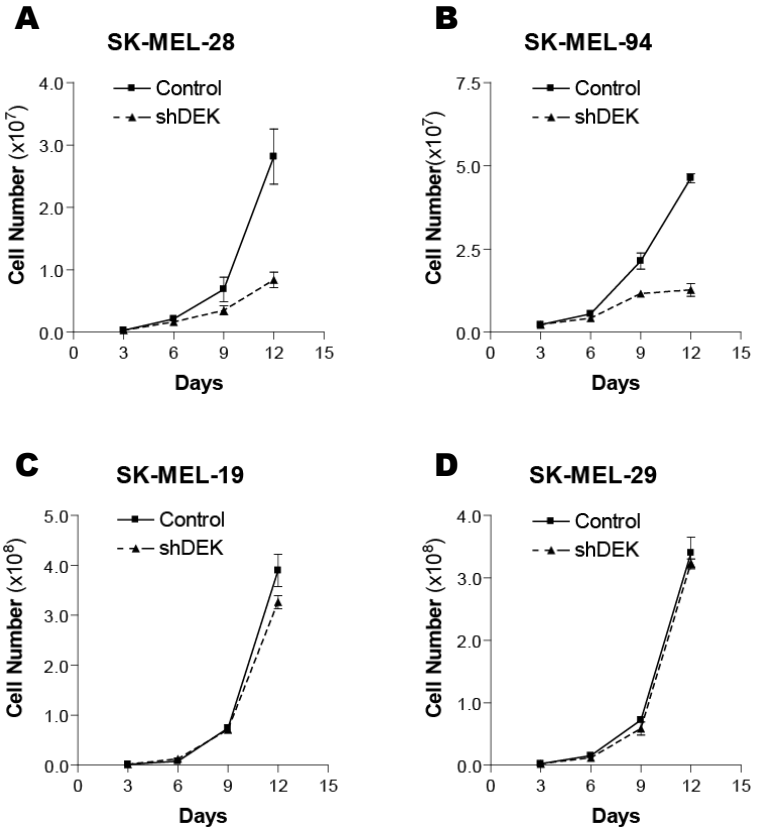

Figure 11. shRNA interference of DEK affects cell proliferation. *A*, SK-MEL-28 , *B,* SK-MEL-94, *C,* SK-MEL-19, and *D,* SK-MEL-29 cells were transduced with either a lentiviral vector expressing a non-specific shRNA (*control*) or a vector expressing two distinct shRNAs targeting DEK (*shDEK)*. Cells were counted every three days. Error bars represent SEM.

responsiveness to p21CIP1 itself. In support of this possibility, SK-MEL-19 cells also demonstrate a failure to arrest or undergo senescence in response to treatment with the p53/MDM2 inhibitor nutlin-3, despite significant expression of p21CIP1 (Verhaegen, personal communication). These findings suggest that DEK expression in melanoma may aid in the circumvention of the p53-p21CIP1 senescence pathway.

DEK expression is stable during oncogene-induced senescence in melanocytes

Expression of DEK has previously been shown to diminish in human keratinocytes and fibroblasts during replicative senescence. We tested whether DEK was similarly downregulated in response to oncogene-induced senescence in primary foreskin-derived melanocytes. Melanomas almost universally express an oncogenic form of either BRAF or NRAS (Brose, Volpe et al. 2002; Maldonado, Fridlyand et al. 2003; Omholt, Platz et al. 2003). However, introduction of these oncogenes into otherwise normal primary melanocytes results in the rapid development of senescence. We used lentiviral vectors to deliver either $BRAF^{V600E}$, $NRAS^{Q61R}$, or an empty control vector to melanocytes. Immunofluorescence was performed seven days after transduction to detect expression of DEK and to assess any changes in localization. $BRAF^{V600E}$ and $NRAS^{Q61R}$ transduced cells exhibited signs of senescence such as flattened morphology and the development of senescence-associated heterochromatic foci (SAHF). Cells transduced with $BRAF^{V600E}$ or $NRAS^{Q61R}$ both continued to express DEK despite the induction of senescence (Figure 14B and 14C). This increase in DEK expression does not appear to be due to non-specific effects of lentiviral transduction, as cells transduced with the empty lentiviral vector did not have enhanced DEK expression (Figure 14A). DEK continued to exhibit a diffuse nuclear staining

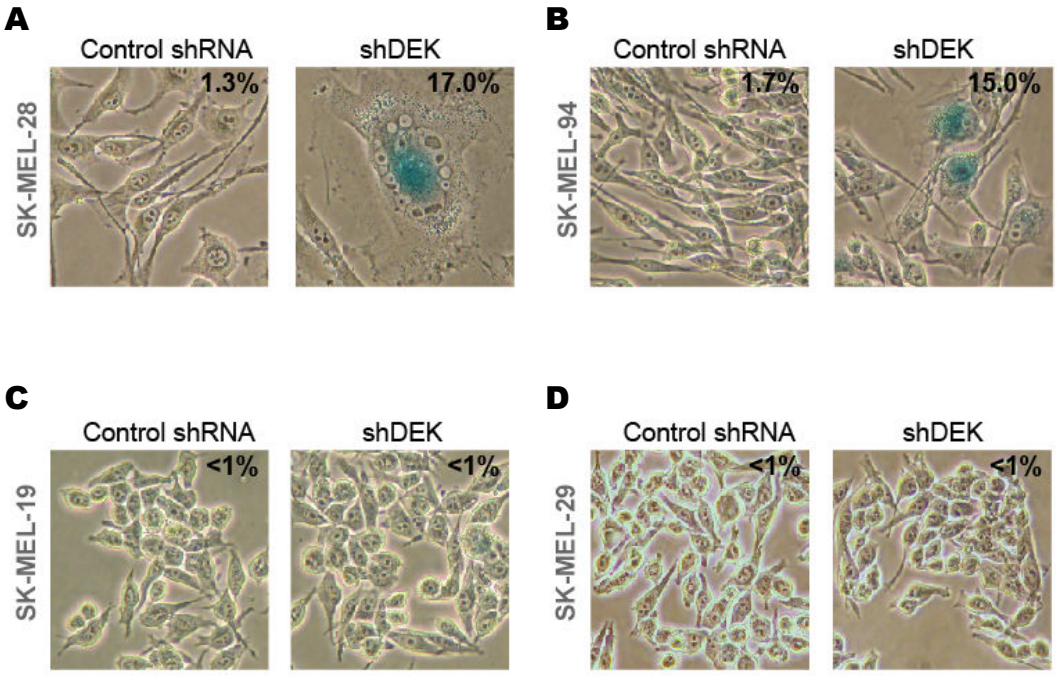

Figure 12. Senescence associated β-galactosidase activity of shDEK-transduced melanoma lines. *A*, SK-MEL-28 , *B,* SK-MEL-94, *C,* SK-MEL-19, and *D,* SK-MEL-29 cells were transduced with either a lentiviral vector expressing a non-specific shRNA (*Control shRNA*) or a vector expressing two distinct shRNAs targeting DEK (*shDEK)*. Fifteen days after transduction, cells were fixed and stained for senescence associated β-galactosidase activity. Phase-contrast micrographs demonstrating changes in cell morphology and SA-β-Gal staining are shown. The number in the upper right corner of each micrograph indicates the percentage of cells that stained positive for senescence associated β-galactosidase activity.

pattern in both $BRAF^{V600E}$ and $NRAS^{Q61R}$ transduced cells (Figure 15). Notably, DEK did not appear to co-localize with senescence-associated heterochromatic foci.

Discussion

Using lentiviral shRNA interference with DEK expression, we identify an important oncogenic function for DEK in melanoma. We show that disruption of DEK expression results in the gradual onset of cellular senescence in a subset of melanomas. Importantly, this is the first demonstration of an anti-senescence function for DEK in an HPV-negative malignancy.

All metastatic melanomas we examined were found to overexpress DEK protein to varying degrees. There are two likely explanations for the high levels of DEK expression. First, it is possible that DEK expression is augmented by a gain in gene copy number. This has been shown to be true in retinoblastoma and bladder carcinoma, where increased DEK expression appears to be due to genomic gain at the *DEK* locus (6p22.3) (Evans, Gallie et al. 2004; Grasemann, Gratias et al. 2005; Orlic, Spencer et al. 2006). Additionally, in retinoblastoma deregulation of DEK may also be due to 6p chromosomal translocations (Paderova, Orlic-Milacic et al. 2007). The possibility that DEK overexpression is due to chromosomal alterations is supported by the observation that gains in the 6p chromosome represent one of the most frequent chromosomal abnormalities in melanoma, with one study finding that 63% of primary melanomas and 50% of metastatic melanomas possess 6p chromosomal gains (Balazs, Adam et al. 2001; Curtin, Fridlyand et al. 2005; Santos, Zielenska et al. 2007). A second possible mechanism of DEK overexpression is through dysregulation of the E2F family of transcription factors. The *DEK* promoter is a target of the transcription factors NF-Y,

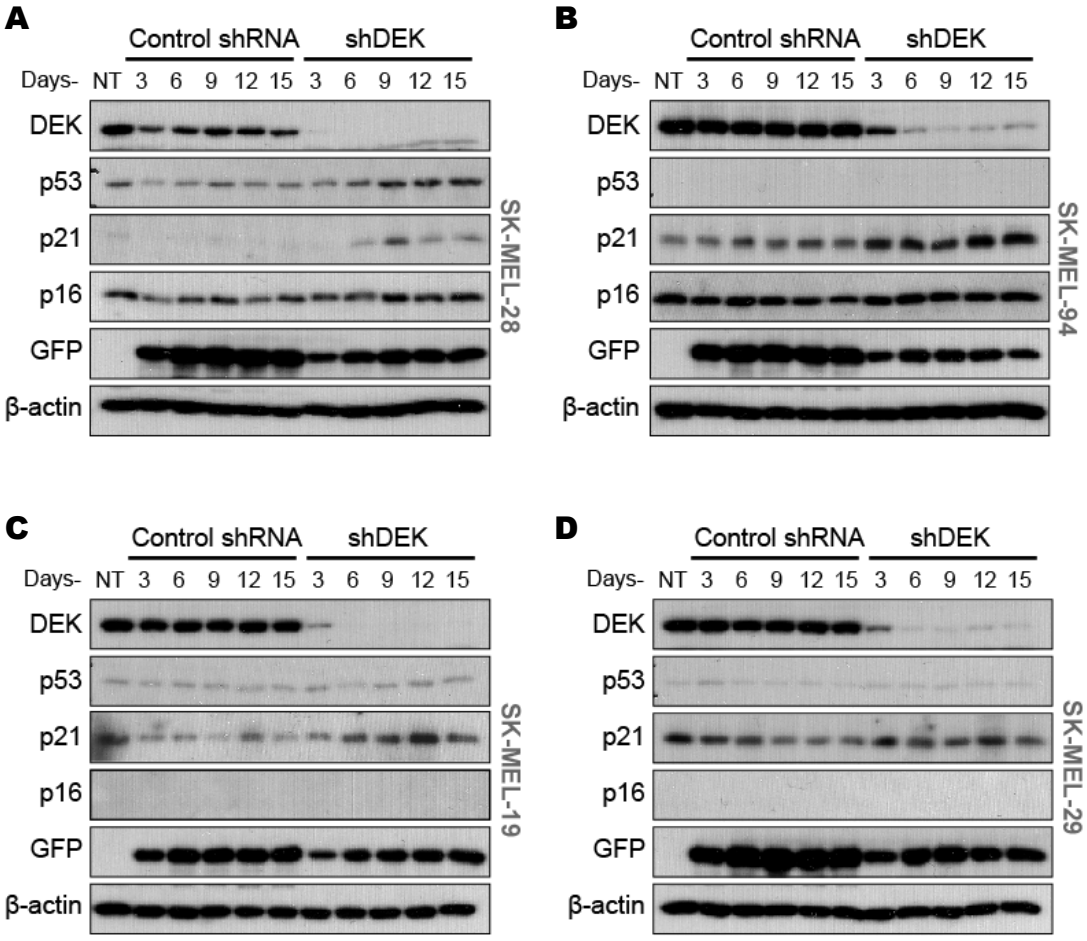

Figure 13. Immunoblotting of shDEK-transduced melanoma lines for senescence markers. *A*, SK-MEL-28 , *B,* SK-MEL-94, *C,* SK-MEL-19, and *D,* SK-MEL-29 cells were transduced with either a lentiviral vector expressing a non-specific shRNA (*Control shRNA*) or a vector expressing two distinct shRNAs targeting DEK (*shDEK)*. Whole cell lysates were collected every third day. Immunoblotting results are shown for DEK, p53, p21CIP1, and p16INK4a. GFP is shown as a marker of viral transduction. β-actin is presented as a loading control. (non-transduced, NT)

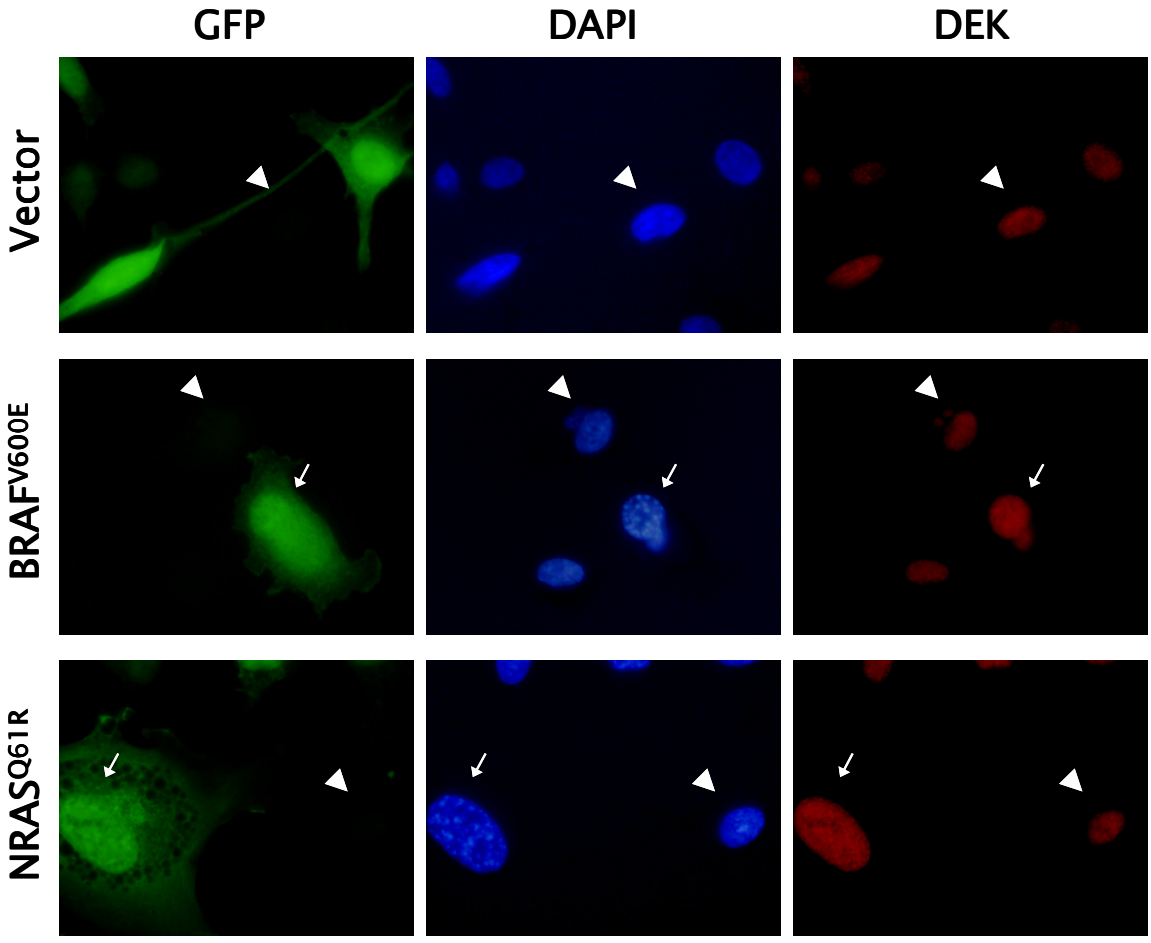

Figure 14. DEK is expressed by melanocytes during oncogene-induced senescence. Primary human melanocytes were isolated and transduced with a lentiviral vector expressing only GFP *(top panels)* or GFP and either BRAFV600E (*middle panels)* or NRASQ61R *(bottom panels)*. Seven days after transduction cells were fixed and DEK expression was visualized by immunofluorescence. (*arrowheads* – Non-transduced cells, *arrows* – senescent cells)

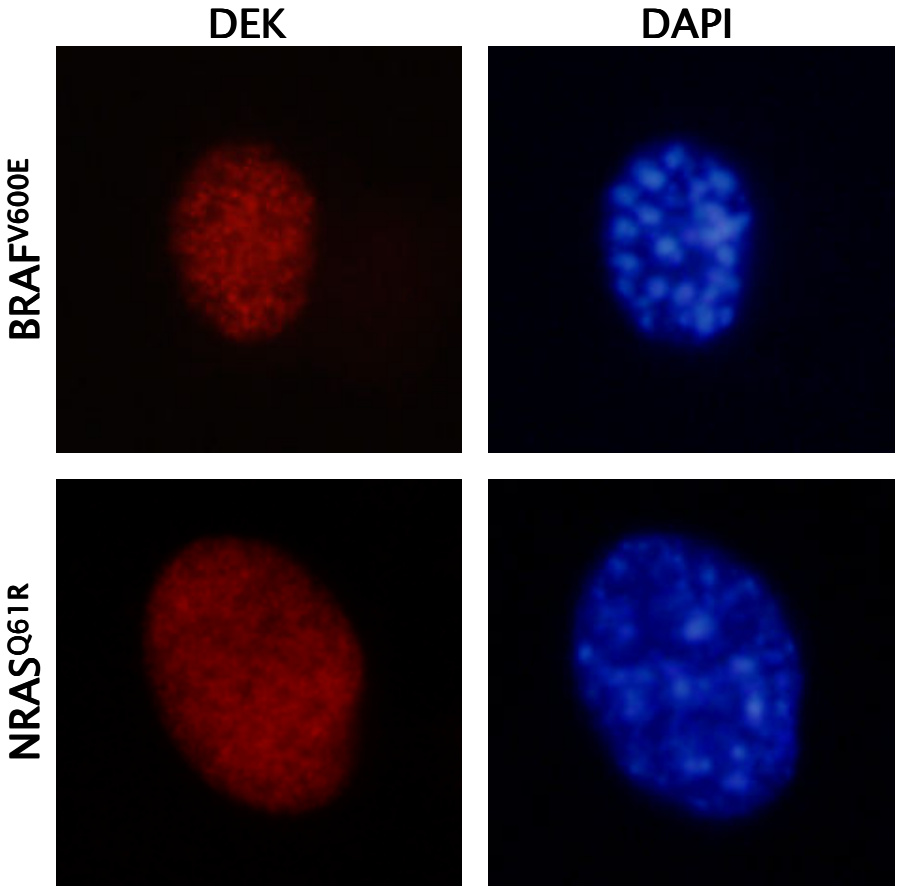

Figure 15. DEK does not co-localize with senescence associated heterochromatin foci. Primary human melanocytes were isolated and transduced with a lentiviral vector expressing either BRAFV600E (*top panels)* or NRASQ61R *(bottom panels)*. Seven days after transduction cells were fixed and DEK expression was visualized by immunofluorescence. Heterochromatic foci were visualized by staining with DAPI.

YY1 and importantly the E2F family of transcription factors (Sitwala, Adams et al. 2002; Carro, Spiga et al. 2006). E2F activity is regulated through its binding to pRb, which is in turn regulated by $p16^{INK4a}$. As mentioned above, melanomas almost invariably have defects in the regulation of this pathway. The inactivation of the $P16^{INK4a}$/pRB pathway in melanoma results in the release of E2F from pRB. Thus, melanomas demonstrate high E2F transcriptional activity (Halaban, Cheng et al. 2000). Thus, the near universal dysregulation of the $p16^{INK4a}$ in melanoma may be the cause of their increased DEK expression. In support of this possibility, it has been shown that ectopic expression of $p16^{INK4a}$ or a constitutively active form of pRB does indeed repress DEK production (Vernell, Helin et al. 2003). Regardless of the mechanism responsible, the frequent overexpression of DEK implies a significant role in melanoma progression.

What could this role be? Our data point to a function in the suppression of senescence. We found that interfering with this abundant DEK expression resulted in the gradual induction of senescence in some melanomas. Consistent with $p21^{CIP1}$-mediated senescence, the onset of senescence corresponded with $p21^{CIP1}$ expression, though not always with an increase in total p53. Replicative senescence of primary human cells does not generally correspond with an accumulation of p53 protein but does critically rely on expression of both p53 and $p21^{CIP1}$ (Vaziri, West et al. 1997; Webley, Bond et al. 2000). This observation is reconciled by findings indicating that although the amount of total p53 is not altered, its activity is substantially increased during replicative senescence (Vaziri, West et al. 1997). This increase in p53 activity can be traced to increased levels of phosphorylation of serine15 and threonine18 (Webley, Bond et al. 2000). Although we did not directly test the requirement of p53 in the development of senescence

following interference with DEK expression, the induction of $p21^{CIP1}$ strongly suggests the involvement of p53. Several groups have proposed p53-independent induction of $p21^{CIP1}$ in various systems, however, most of these claims are based on the erroneous assumptions that mutated p53 forms cannot activate $p21^{CIP1}$ and that p53 activity is always accompanied by accumulation of p53 protein.

One potential explanation of the anti-senescence function of DEK is that DEK may repress basal p53 transcriptional activity. DEK has been shown to repress p53 transcriptional activation of multiple p53-target gene promoters, including $p21^{CIP1}$(Sammons, Wan et al. 2006; Wise-Draper, Allen et al. 2006). Therefore, high levels of DEK in melanoma cells may serve to directly repress transcriptional p53 activity on the $p21^{CIP1}$ promoter in melanoma cells. Interference with DEK expression may release this repression, resulting in a steady accumulation of $p21^{CIP1}$. Another possible explanation for the induction of senescence in cells lacking DEK is that DEK may prevent the activation of p53 in replication-stressed or oncogene-stressed cells by alleviating or masking these stresses. Telomere shortening/uncapping and the accumulation of DNA damage are two major stresses that accumulate with cell division and are prominent triggers of replicative senescence. Both of these stresses trigger a DNA damage response that ultimately results in p53-mediated senescence (Ben-Porath and Weinberg 2005; Collado, Blasco et al. 2007). In addition, recent research has demonstrated that the phenomenon of oncogene-induced senescence is actually a response to DNA damage resulting from stalling of replication forks in rapidly dividing cells (Di Micco, Fumagalli et al. 2006). Thus, the major signals of $p16^{INK4a}$-independent senescence all appear to be involve activation and signaling of the DNA damage repair

machinery and p53. We have recently shown that DEK plays a protective role in repairing DNA damage, raising the possibilty that overexpression of DEK in melanoma cells represses senescence by alleviating DNA damage signals (Kappes, Fahrer et al. 2008).

Our findings have particular significance for understanding the evasion of p53-mediated senescence by melanoma cells. In contrast to $p16^{INK4a}$/pRB senescence, the circumvention of p53-mediated senescence in melanoma is not well understood. p53 is the most frequently mutated or deleted gene in cancer, but it is rarely a target of melanoma mutation. Nonetheless, melanoma displays defective p53 activity (Satyamoorthy, Chehab et al. 2000; Gray-Schopfer, Cheong et al. 2006). Because of the extremely high frequency of genetic inactivation of the p53-regulatory protein ARF, the evasion of p53-mediated senescence is commonly ascribed to the inactivation of ARF. However, attributing significance to the frequent inactivation of ARF is complicated by the fact that the ARF gene shares its locus, CDKN2A, with another important melanoma tumor suppressor, $p16^{INK4a}$. Thus, deletion or methylation of the CDK2NA locus may simply be a reflection of the importance of $p16^{INK4a}$ inactivation in melanoma development. Indeed, the incidence of point mutations affecting only $p16^{INK4a}$ is much greater than those affecting only ARF, suggesting that the primary target of deletion or methylation of CDKN2a is $p16^{INK4a}$ (Bennett 2008). Additionally, while ARF has been shown to play an important role in the development of senescence in rodents, studies of senescence in human cells consistently demonstrate a lesser role for ARF (Wei, Hemmer et al. 2001; Brookes, Rowe et al. 2002; Voorhoeve and Agami 2003).

Surprisingly, we found that in a model of melanocyte oncogene-induced senescence, DEK expression was not diminished concomitantly with the onset of senescence. This is in contrast to previous reports of depletion of DEK in replicative senescence of fibroblasts and keratinocytes. The discrepancy may due to differences between those cell types and melanocytes, or it may reflect a differential role for DEK in oncogene-induced senescence as opposed to replicative senescence. At a minimum, these results demonstrate that cells can undergo senescence in the presence of DEK expression. It is also noteworthy, given our discovery of an interaction between DEK and heterochromatin protein 1α (HP1α), that unlike HP1α, DEK does not colocalize with senescence associated heterochromatic foci (Narita, Nunez et al. 2003; Kappes, Khodadoust et al., in preparation).

These results suggest that overexpression of DEK may be an important mechanism for evasion of senescence in melanoma cells. The frequency of DEK overexpression raises the possibility that this overexpression may be a requisite event in the development of melanoma. Moreover, as DEK is a frequently overexpressed gene in a variety of malignancies, these results may be of significance in the study of other tumor types as well.

Materials and Methods

Cell culture

All melanoma cell lines were cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Human melanocytes were isolated from human neonatal foreskins as described

(Verhaegen, Bauer et al. 2006) and maintained in Medium 254 supplemented with 12-*O*-tetradecanoylphorbol-13-acetate (10 nmol/L) and melanocyte growth factors (Cascade Biologics, Portland, OR).

Extract preparation and protein immunoblots

Total cell lysates were obtained by Laemmli extraction, separated by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). Monoclonal antibodies to DEK were obtained from BD Biosciences (Franklin Lake, NJ), β-actin from Sigma Chemical (St. Louis, MO), and p53 from Novacostra Laboratories (Newcastle upon Tyne, UK). Polyclonal antibodies to DEK were produced as previously described (Kappes, Damoc et al. 2004). Unless otherwise specified, DEK was detected in immunoblots using polyclonal anti-DEK antibodies. Detection was performed using ECL Western blotting substrate from Pierce (Rockford, IL). Protein expression was quantified using Scion image densitometry software (Scion Corporation, Frederick, MD).

Cell proliferation and senescence-associated β-galactosidase staining

Cell cultures were split every three days and counted manually by hemocytometer. Cells were washed in PBS, fixed for 3-5 min (room temperature) in 2% formaldehyde/ 0.2% glutaraldehyde (or 3% formaldehyde), washed, and incubated at 37°C (no CO_2) with fresh senescence associated (3-Gal (SA-,3-Gal) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal) per ml (stock = 20 mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$. Staining was evident in 2-4 hr, and maximal in 12-16 hr.

Lentiviral shRNA constructs

The H1-LV vector was used to produce shRNAs targeting DEK. The shDEK-1 vector used the human H1 promoter to drive expression of an shRNA targeting a sequence in the 3' untranslated region of the human *DEK* transcript (nucleotides 1615-35, GenBank NM_003472.2). The shDEK-2 vector was constructed by inserting a second Pol III promoter and shRNA immediately 5' of the H1 promoter. This insertion contained the murine U6 promoter expressing a second shRNA targeting a coding region of the *DEK* transcript (nucleotides 1118-1141, GenBank NM_003472.2). The construction of $NRAS^{Q61R}$ and $BRAF^{V600E}$ expression lentiviral vectors has been described previously (Denoyelle, Abou-Rjaily et al. 2006). Lentivirus production and infection was performed as described previously (Verhaegen, Bauer et al. 2006). Transduction efficiency of H1-LV and FG-12 derived lentiviral vectors was estimated by detection of GFP expression (driven by the human ubiquitin c promoter) by fluorescence microscopy or flow cytometry and was routinely greater than 95%. Cells transduced with the PLKO.1-DEK832 vector were selected for 3 days in 2 μg/mL puromycin.

Immunofluorescence

Melanocytes were cultured and infected on chamber slides. Immunofluorescence staining for DEK was performed. Three phosphate-buffered saline (PBS) washes were performed in between each of the following steps; when indicated, PBS washes contained 0.1% saponin. Cells were washed and fixed for 10 min in 4% paraformaldehyde. Fixed cells were washed, blocked for at least 1 hour with 0.2% bovine serum albumin in PBS, rewashed with PBS/saponin, and incubated for 1 hour with mouse monoclonal anti-DEK

(BD Biosciences) in PBS/saponin. Cells were then rewashed in PBS/saponin and reblocked with goat serum in PBS/saponin at a 1:50 dilution for 1 hour, followed by PBS/saponin washing and a 1 hour incubation with Alexa fluor 594-conjugated goat anti-mouse IgG (20 μg/ml; Molecular Probes) in PBS/saponin. Slides were washed with PBS/saponin, refixed for 10 min, rewashed with PBS, and distilled water then dried. Coverslips were mounted with Prolong Gold reagent containing DAPI (Molecular Probes), and images were captured with a Zeiss Laser Scanning Microscope (LSM 510, version-2.8SPI).

CHAPTER IV

DEK PROMOTES CHEMORESISTANCE IN MELANOMA THROUGH UPREGULATION OF MCL-1 EXPRESSION

Abstract

Metastatic melanoma has been shown to frequently overexpress the oncogene DEK. We have recently described that DEK expression is required to avoid senescence in a subset of metastatic melanoma cell lines. Here, we address the potential oncogenic contribution of DEK in melanoma lines that express high levels of DEK, but do not senesce in response to inhibition of DEK expression. In these lines, DEK expression confers remarkable chemoresistance to both doxorubicin and TW-37, a BH3 mimetic compound. Interference with DEK expression did not result in aberrations in the p53 response to doxorubicin but did cause reduced expression of the anti-apoptotic protein Mcl-1. Notably, expression of other related proteins, Bcl-2 and Bcl-X_L, were unaffected. Although Mcl-1 protein levels are often regulated at the level of protein stability, diminished Mcl-1 expression in DEK-deficient melanoma cells was not due to changes in Mcl-1 protein stability but instead was a result of defective Mcl-1 production. Quantitative RT-PCR analysis and luciferase reporter assays of the *mcl-1* promoter revealed that inhibition of DEK expression resulted in reduced *mcl-1* promoter activity

and hence reduced levels of *mcl-1* transcript. Finally, Mcl-1 expression was shown to be a critical determinant of doxorubicin resistance in melanoma. These findings describe a second oncogenic function of DEK in melanoma cells, as a promoter of chemoresistance. These results also demonstrate a novel mechanism of Mcl-1 dysregulation in melanoma at the level of transcription.

Introduction

Melanoma is the most lethal form of skin cancer, and its increasing incidence indicates that it will pose a growing clinical challenge (Jemal, Siegel et al. 2008). Although primary melanoma can be successfully treated with surgery at early stages, metastatic melanoma carries a grim prognosis. Metastatic melanoma responds poorly to current therapeutic modalities, include radiotherapy and chemotherapy (Gogas, Kirkwood et al. 2007). The notorious chemoresistance of melanoma to conventional chemotherapies has stimulated research into novel molecularly targeted therapies. Although several newly developed, rationally-designed pharmacologic agents have shown promise, their clinical efficacy has yet to be proven.

Malignant melanoma is one of the most chemoresistant cancers, and a myriad of mechanisms employed by melanoma have been identified that contribute to this phenotype (Soengas and Lowe 2003; Chin, Garraway et al. 2006). These mechanisms can be crudely divided into two categories: suppression of genes that promote apoptosis and enhancement of those that promote survival. Many pro-apoptotic mediators of both the intrinsic and extrinsic pathways of apoptosis, such as p14ARF, Apaf-1, and TRAIL-R, have been shown to be frequently downregulated in melanoma. Conversely, melanomas commonly display enhanced expression of anti-apoptotic proteins, including

Bcl-2 family members (Bcl-2, Bcl-X_L, and Mcl-1), FLIP, and MITF (Irmler, Thome et al. 1997; Selzer, Schlagbauer-Wadl et al. 1998; Chin, Garraway et al. 2006; Eberle, Kurbanov et al. 2007; Wolter, Verhaegen et al. 2007). The dysregulation of some of these genes can be traced to genetic and/or epigenetic alterations. For example, Apaf-1 has been shown to be inactivated by methylation of the gene locus, while amplification of chromosome 3p14 has been implicated in overexpression of MITF (Soengas, Capodieci et al. 2001; Chin, Garraway et al. 2006; Jonsson, Dahl et al. 2007). However, corresponding genetic abnormalities are lacking for most proposed mechanisms of melanoma chemoresistance.

Alteration of chromosome 6 has frequently been identified as the most common cytogenetic change in melanoma (Santos, Zielenska et al. 2007). In particular, gain of the 6p arm is one of the few chromosomal aberrations that is prevalent in all types of melanoma, including cutaneous (both sun-exposed and non-sun-exposed), acral, mucosal, and uveal melanomas (Curtin, Fridlyand et al. 2005). We have previously identified the product of a putative oncogene located in the 6p chromosome arm, DEK, as a frequently overexpressed protein in metastatic melanoma. Interference with DEK expression resulted in the induction of senescence in a subset of metastatic melanoma lines. Additionally, we found a target of p53, $p21^{CIP1}$, to be induced following knockdown of DEK in both senescing and non-senescing lines, suggesting enhanced p53 activity in cells lacking DEK.

Although DEK was overexpressed in all melanoma lines examined, we found the degree of this overexpression to be variable. Surprisingly, we found that interference with DEK expression in the two melanoma lines with the greatest expression of DEK did

not result in senescence or any other abnormalities in cell proliferation. Previously, DEK has been shown to act as an inhibitor of apoptosis in HeLa cervical cancer cells (Wise-Draper, Allen et al. 2006). Depletion of DEK in these cells resulted in spontaneous apoptosis in a p53-dependent manner. DEK expression inhibits p53 transcriptional activity (Sammons, Wan et al. 2006; Wise-Draper, Allen et al. 2006). Thus, in addition to repressing senescence, DEK may also act as an anti-apoptotic factor in DEK-overexpressing cancer cells.

We examined the possibility that DEK expression in melanoma promotes chemoresistance. We found that DEK expression did indeed confer substantial chemoresistance to both the DNA damaging agent doxorubicin and the BH3 mimetic compound TW-37. Interestingly, the ability to inhibit senescence did not involve p53 accumulation or activity. Rather, DEK promoted expression of the anti-apoptotic protein Mcl-1, but not the related proteins Bcl-2 or Bcl-X_L. We show that Mcl-1 protein stability is not affected by DEK expression. Instead, DEK appears to regulate Mcl-1 expression at a transcriptional level by promoting activation of the Mcl-1 promoter. Finally, we demonstrate that Mcl-1 expression, but not Bcl-X_L, is a critical determinate of chemoresistance. Our findings present a novel second oncogenic function for DEK in the setting of melanoma: promotion of chemoresistance. These results also identify a previously unknown regulatory mechanism of Mcl-1 expression that may have implications in dysregulation of Mcl-1 in other cancers beyond melanoma.

Results

Interference with DEK expression increases sensitivity to doxorubicin and the BH3 mimetic TW-37

Previously, we have shown that interference with DEK expression results in senescence in a subset of metastatic melanoma lines. Surprisingly, the two cell lines with the greatest expression of DEK (SK-MEL-19 and SK-MEL-29) did not exhibit any defects in proliferation following shDEK treatment. Because DEK has previously been described as an anti-apoptotic factor in HeLa cells, we hypothesized that DEK may nevertheless contribute to the chemoresistance of these metastatic melanoma lines. Indeed, DEK-knockdown in these lines did result in markedly increased sensitivity to the anthracycline doxorubicin (Figure 16A and 16B).

Because we have recently shown that DEK is required for effective DNA damage repair of damage caused by certain genotoxic agents (Kappes, Fahrer et al. 2008), we sought to determine if the increased sensitivity to apoptotic stimuli in DEK-knockdown cells was limited to DNA damaging agents. To test the sensitivity of DEK knockdown cells to a non-genotoxic apoptotic agent we utilized the recently developed BH3 mimetic TW-37. TW-37 directly stimulates the mitochrondial apoptosis pathway by binding the Bcl-2 family of anti-apoptotic proteins. TW-37, unlike many BH3 mimetics, exhibits a high affinity for Mcl-1, in addition to Bcl-2 and Bcl-X_L (Wang, Nikolovska-Coleska et al. 2006). Cells lacking DEK exhibited greater sensitivity to the TW-37, indicating that chemosensitization in response to interference with DEK expression is not limited to DNA damaging agents (Figure 16C and 16D). It should be noted that although DEK-knockdown cells were more sensitive to TW-37, the difference was not as pronounced as the increased sensitivity to doxorubicin. This may be the result of dual protective effects of DEK in aiding DNA repair as well as repressing the mitochondrial apoptotic pathway through an independent mechanism. These results show that DEK overexpression in

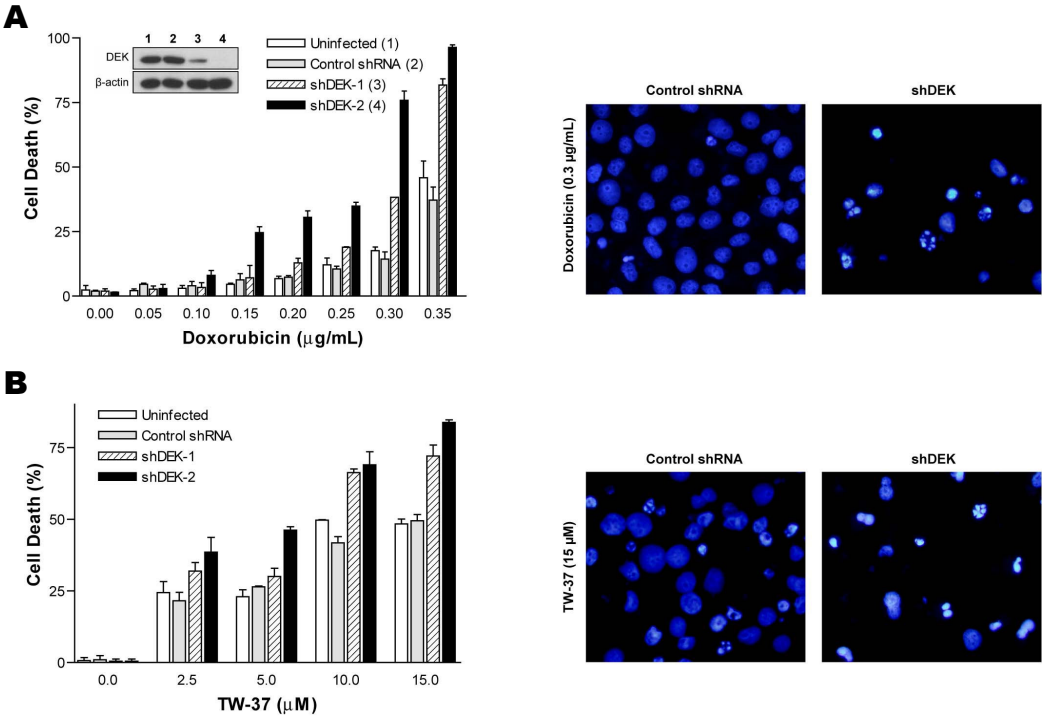

Figure 16. Interference with DEK expression sensitizes SK-MEL-19 metastatic melanoma cells to pro-apoptotic stimuli. *A,* cells were transduced with either a lentiviral vector expressing a non-specific shRNA (*control shRNA*), a vector expressing a single shRNA targeting DEK (*shDEK-1*), or a vector expressing two distinct shRNAs targeting DEK (*shDEK-2*). 72 hours after infection, cells were treated with doxorubicin for 30 hours, and then stained with DAPI. Condensed and fragmented nuclei were counted and divided by total nuclei to determine the percent of dead cells. The inset illustrates the inhibition of DEK expression as determined by immunoblotting. *B,* fluorescence microscopy illustrating cell nuclear morphology after treatment with 0.3 μg/mL doxorubicin for 30 hrs in cells infected with a control shRNA expressing vector (*left*) or a vector expressing two distinct shRNAs targeting DEK (*right*). *C,* sensitivity to the BH3 mimetic compound TW-37 was determined as in *A,* with the exception that cells were treated with TW-37 for 48 hours. *D*, fluorescence microscopy illustrating cell nuclear morphology after treatment with 15μM TW-37 in cells infected with a control shRNA expressing vector (*left*) or a vector expressing two distinct shRNAs targeting DEK (*right*). Error bars show SEM.

melanoma results in broad chemoresistance to genotoxic stress as well as mitochondrial targeted therapies.

Interference with DEK expression does not cause aberrations in the p53 response to doxorubicin

DEK has previously been reported to mediate anti-apoptotic effects through destabilization of p53 protein and inhibition of p53 activity (Sammons, Wan et al. 2006; Wise-Draper, Allen et al. 2006). Therefore, we next examined whether the increased sensitivity in DEK-knockdown cells was due to increased p53 accumulation and activity. Surprisingly, DEK-knockdown SK-MEL-19 cells displayed no detectable aberrations in p53 accumulation nor in activation of p53, as measured by phosphorylation of serine 15 in response to doxorubicin (Figure 17). Additionally, induction of $p21^{CIP1}$ in response to doxorubicin was slightly diminished, not enhanced as would be predicted if DEK were inhibiting p53 activity. Thus, the chemoresistance conferred by DEK does not appear to be related to p53 stability or activity.

DEK-knockdown cells have diminished Mcl-1 expression

The increased susceptibility of DEK-knockdown cells to TW-37 suggested the possible involvement of the Bcl-2 family of anti-apoptotic proteins. Indeed, immunoblotting revealed decreased expression of Mcl-1 in DEK-deficient cells, though not of other closely related members, Bcl-2 and Bcl-X_L (Figure 17). This decrease was most notable approximately 30-36 hours following exposure to doxorubicin, although levels of Mcl-1 were diminished even in non-treated DEK-knockdown cells. Because Mcl-1 is a known target of caspase cleavage (Herrant, Jacquel et al. 2004)(Gomez-Bougie, Wuilleme-Toumi et al. 2007), we tested whether the diminution of Mcl-1

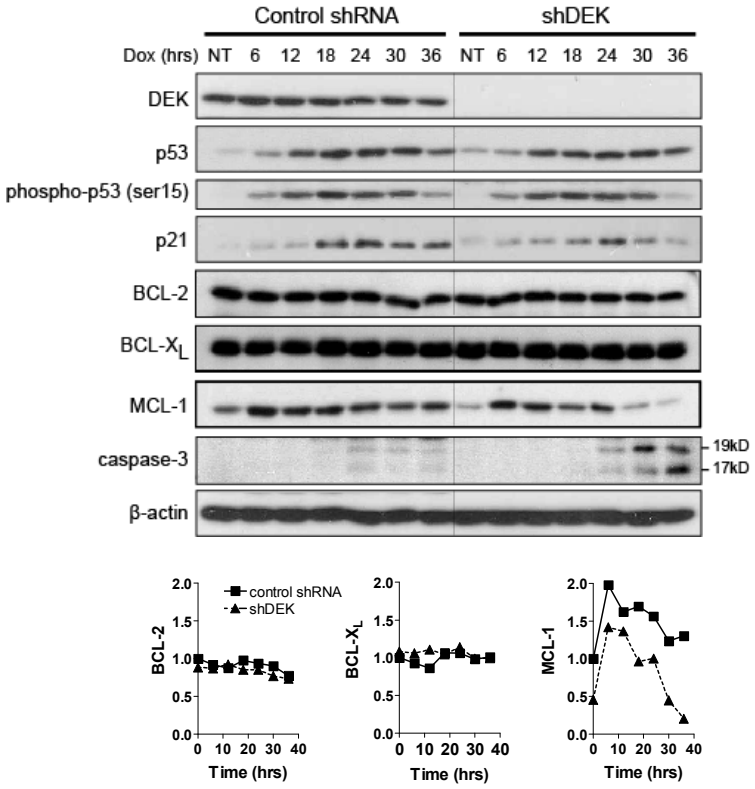

Figure 17. Knockdown of DEK decreases Mcl-1 expression, but does not affect the p53 response to doxorubicin. *A,* SK-MEL-19 cells were transduced with either a lentiviral vector expressing a non-specific shRNA (*control shRNA*) or a vector expressing two distinct shRNAs targeting DEK (*shDEK)*. Cells were treated with 0.2 μg/mL doxorubicin and collected at the indicated times. Expression of p53, phosphorylated p53 (serine 15), p21CIP1, Bcl-2, Bcl-XL, Mcl-1, caspase-3, and β-actin was determined by immunoblotting. The graphs below show the relative expression of Bcl-2 (left panel), Bcl-XL (middle panel) and Mcl-1 (right panel) following doxorubicin treatment as estimated by band densitometry. Expression is shown relative to that of untreated control-transduced cells.

expression in doxorubicin-treated, DEK-knockdown cells was caspase-dependent. Inhibition of caspase activity with the pan-caspase inhibitor z-VAD-fmk eliminated cleavage of caspase-3, but had only a minimal effect on the levels of Mcl-1 in shDEK-treated cells, indicating that the reduction in Mcl-1 is not due to caspase activity (Figure 18). In addition to SK-MEL-19 cells, we found that the reduction of Mcl-1 expression following interference with DEK expression could also be seen in SK-MEL-29 cells (Figure 19). Importantly, the reduction of Mcl-1 was not due to non-specific effects of the lentiviral vector or DEK-targeted shRNA, as the same effects could be reproduced with a third distinct shRNA targeting DEK and expressed in a different vector system (Figure 19).

Decreased Mcl-1 in shDEK treated cells is not due to increased Mcl-1 degradation

We next attempted to determine the cause of the decrease in Mcl-1 expression in cells lacking DEK. Unlike other Bcl-2 family members, Mcl-1 is very labile and rapidly turned over in the cell through ubiquitination and subsequent proteasomal degradation. Thus, a major mechanism of Mcl-1 regulation is the modulation of Mcl-1 protein degradation. To determine if the decrease in Mcl-1 protein in DEK-knockdown cells was due to enhanced proteasomal degradation of Mcl-1, cells were treated with the proteasome inhibitor bortezomib. Bortezomib has been shown to be extremely effective in blocking Mcl-1 degradation, and treatment of melanoma cells with bortezomib has been shown to result in rapid accumulation of Mcl-1 protein (Fernandez, Verhaegen et al. 2005; Qin, Ziffra et al. 2005; Qin, Xin et al. 2006; Wolter, Verhaegen et al. 2007). While Mcl-1 accumulated in both cells containing and cells lacking DEK, the rate of accumulation was greatly diminished in the absence of DEK (Figure 20A). This

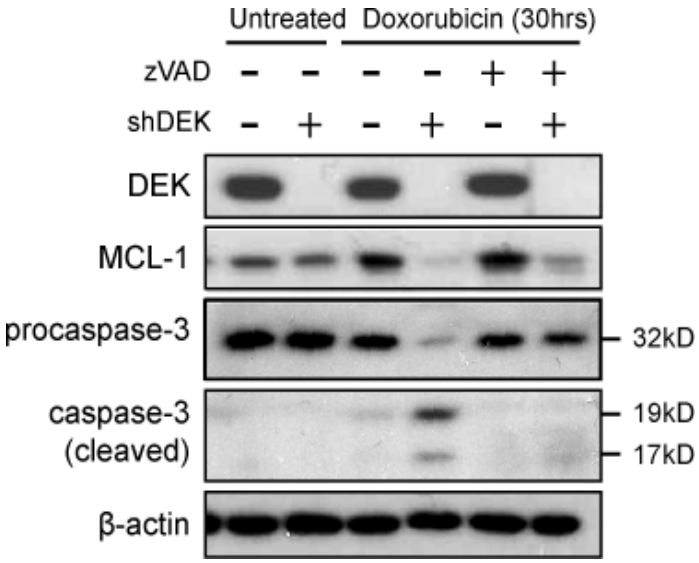

Figure 18. Diminished Mcl-1 expression in doxorubicin shDEK-transduced cells is not caspase-3 dependent. Control and shDEK-transduced SK-MEL-19 cells were treated with 0.2 μg/mL doxorubicin alone or doxorubicin in the presence of 50 μM of the pan-caspase inhibitor z-VAD-fmk. Immunoblotting was used to determine the expression of DEK, Mcl-1, pro-caspase-3, cleaved caspase-3 and β-actin.

suggested that the reduction of Mcl-1 in DEK-knockdown cells was not due to an increase in Mcl-1 degradation. To assess the stability of Mcl-1 protein in DEK-knockdown cells, cycloheximide, a translational inhibitor, was used to inhibit *de novo* Mcl-1 production, and Mcl-1 levels were determined by immunoblotting. To better visualize the decrease in Mcl-1 expression, cells were first pre-treated with bortezomib to augment Mcl-1 levels, then the bortezomib was washed out, and cells were incubated in cycloheximide. Mcl-1 was degraded at a similar rate in both control and shDEK-treated cells, although the initial amount of Mcl-1 was reduced in shDEK-treated cells (Figure 20B). Taken together, these results implied that the presence of DEK does not affect Mcl-1 protein stability but does enhance the production of Mcl-1 protein.

Reduction of Mcl-1 expression is due to decreased Mcl-1 transcription

Since the production but not the stability of Mcl-1 protein appeared to be altered in DEK-knockdown cells, we next examined if these cells had reduced levels of Mcl-1 transcript (Figure 21A). Indeed, quantitative RT-PCR analysis revealed shDEK-transduced cells had an almost 50% reduction of Mcl-1 mRNA as compared to control transduced cells. Luciferase reporter gene assays were next used to determine the activity of the human Mcl-1 promoter in shDEK-transduced cells. We found that shDEK-transduced cells exhibited markedly reduced Mcl-1 promoter activity (Figure 21B). These results show that the decreased Mcl-1 expression in DEK-knockdown cells is likely due to decreased Mcl-1 promoter activity and the consequent decrease of Mcl-1 transcript.

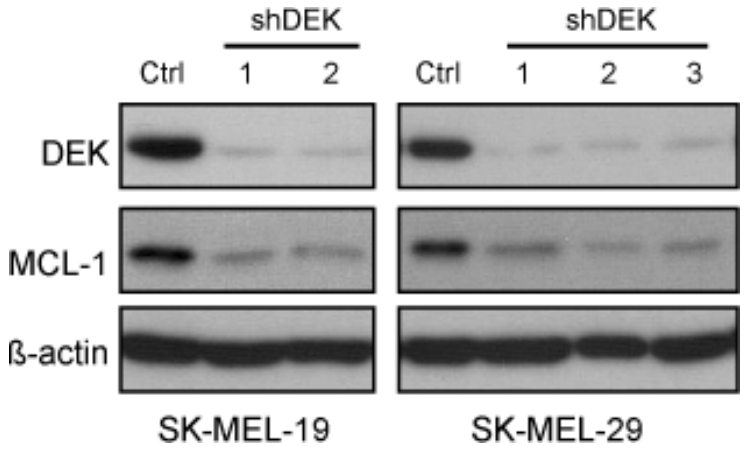

Figure 19. Diminished Mcl-1 expression in shDEK-transduced SK-MEL-19 and SK-MEL-29 cells is not shRNA or vector specific. SK-MEL-19 *(left)* and SK-MEL-29 *(right)* cells were transduced with a lentivirus expressing either a control shRNA sequence, a single shRNA targeting DEK (*shDEK-1)*, or two distinct shRNAs targeting DEK (*shDEK-2*). 96 hours after infection, cells were collected and their lysates were immunoblotted for DEK and Mcl-1 expression. To ensure that the decrease in Mcl-1 expression was not due to non-specific effects of the lentiviral vector, a third DEK-targeting lentivirus (*shDEK-3*) using a different lentiviral vector and expressing a distinct DEK-specific shRNA was also tested *(right panel)*.

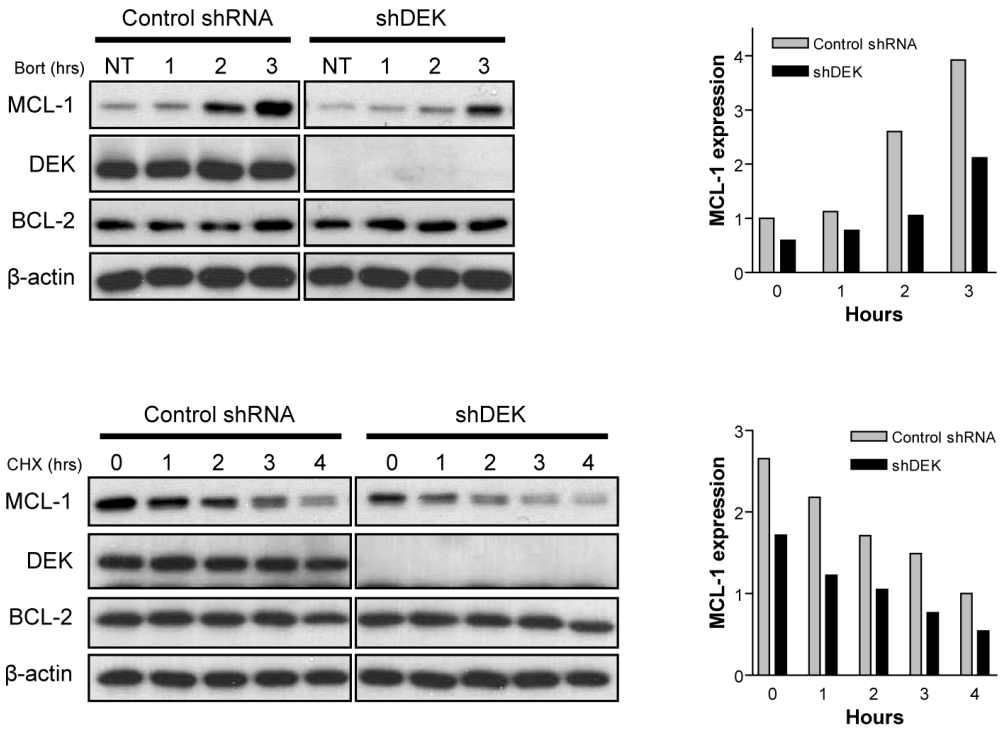

Figure 20. Knockdown of DEK decreases Mcl-1 production, but does not affect Mcl-1 protein stability *A,* Transduced SK-MEL-19 cells were treated for the indicated times with 25 nM bortezomib (*Bort*) Following treatment, cells were collected and lysates were immunoblotted for Mcl-1, DEK, Bcl-2, and β-actin *(left).* Densitometry was used to quantitate Mcl-1 expression (*right).* Expression is shown relative to non-treated control-infected cells. *B,* Transduced SK-MEL-19 cells were treated for 5 hours with 25 nM of the proteasome inhibitor bortezomib. Cells were then washed to remove bortezomib and incubated with cycloheximide (*CHX*) to block protein translation for the indicated times. Following treatment cells were collected and immunoblotted as in *A (left).* Mcl-1 expression was determined by densitometry (*right*).

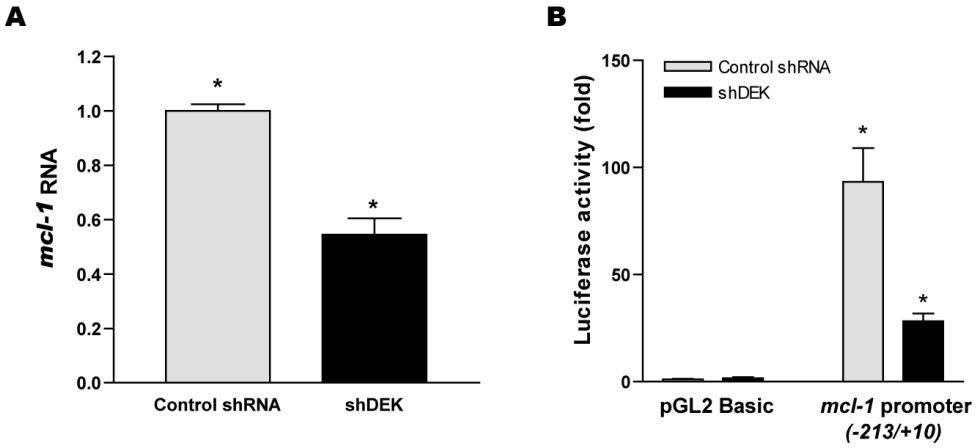

Figure 21. Decreased activity of the mcl-1 promoter in shDEK-transduced cells. *A, mcl-1* mRNA expression of control and shDEK treated SK-MEL-19 cells was determined by quantitative real-time RT-PCR using *mcl-1* specific primers. *mcl-1* mRNA expression was normalized to that of *β–actin*. *B,* control and shDEK transduced SK-MEL-19 cells were transfected with either the the promoterless pGL2 basic plasmid or a plasmid expressing the firefly luciferase gene under the control of the human *mcl-1* promoter (*-213/+10 bp)*. Cells were lysed and luciferase activity was determined 30 hours after transfection. The average luciferase activity of three experiments is shown relative to that of control transduced cells transfected with the pGL2 basic plasmid. Error bars represent SEM. (*, $p < 0.05$).

Mcl-1 expression determines doxorubicin sensitivity

Finally, we investigated whether the increased sensitivity to chemotherapeutic agents of cells lacking DEK could be attributed to their reduction in Mcl-1 expression. Although Mcl-1 has been shown to enhance resistance to several chemotherapeutic agents and apoptotic stimuli, its contribution to doxorubicin resistance in melanoma has not been directly investigated. Interference with Mcl-1 expression resulted in a remarkable increase in sensitivity to doxorubicin in both control transduced and shDEK transduced cells (Figure 22A). In contrast, interference with Bcl-X_L expression resulted in only marginally increased sensitivity (Figure 22B). Thus, Mcl-1 is a particularly potent mediator of doxorubicin resistance. To determine if overexpression of Mcl-1 could rescue DEK-knockdown cells from doxorubicin cytotoxicity, a lentiviral vector expressing human Mcl-1 was constructed. Secondary transduction of DEK-knockdown cells with this vector indeed did restore doxorubicin resistance (Figure 23A). Interestingly, these cells had almost identical total levels of Mcl-1 protein as control transduced cells (Figure 23A, *inset lanes 1 and 4*) and displayed nearly identical sensitivity to doxorubicin (Figure 23A). Additionally, cells with normal DEK expression achieved even greater resistance to doxorubicin upon Mcl-1 overexpression (Figure 23A). Importantly, while shDEK treated cells demonstrated diminished levels of endogenous Mcl-1, expression of Flag-tagged Mcl-1 driven by the lentiviral CMV promoter was not affected (Figure 23A, *inset lanes 3 and 4*). This is further evidence that, as evinced above, cells lacking DEK do not display defects in Mcl-1 protein stability, but rather are deficient in Mcl-1 transcription. Again, Bcl-X_L did not seem to confer equivalent resistance to doxorubicin, as Bcl-X_L overexpression failed to rescue

shDEK cells from doxorubicin cytotoxicity (Figure 23B). These results demonstrate that the marked sensitivity to doxorubicin of melanoma cells lacking DEK is attributable to their reduced Mcl-1 expression.

Discussion

Using shRNA to interfere with DEK expression, we previously demonstrated that continuous DEK expression in a subset of metastatic melanomas is required to avoid the induction of senescence. Here, we show that DEK expression can additionally confer remarkable chemoresistance. Interestingly, the anti-apoptotic function of DEK in melanoma did not appear to be related to the p53-suppressing effect previously seen in HeLa cervical carcinoma cells. Rather, the chemoresistance activity of DEK was attributable to a novel function in the control of Mcl-1 transcription.

Mcl-1 has emerged as a particularly potent member of the Bcl-2 family of anti-apoptotic proteins. Previous studies have described Mcl-1 overexpression in melanoma (Selzer, Schlagbauer-Wadl et al. 1998; Tang, Tron et al. 1998; Zhuang, Lee et al. 2007; Wong, Khosravi et al. 2008). The importance of Mcl-1 in melanoma treatment is underscored by the observation that Mcl-1 expression correlates with metastases and poor prognosis whereas Bcl-2 does not (Fecker, Geilen et al. 2006; Zhuang, Lee et al. 2007). The expression of Mcl-1 by melanoma cells importantly has been implicated in resistance to many treatments, including several recently developed targeted therapies. Mcl-1 is a critical determinate of sensitivity in response to proteasome inhibition by bortezomib, and to MEK inhibition by U0126 (Qin, Xin et al. 2006; Nguyen, Marcellus et al. 2007; Wang, Jiang et al. 2007; Wolter, Verhaegen et al. 2007). Additionally, the ability to survive treatment with new BH3 mimetics, such as ABT-737, has been shown to critically

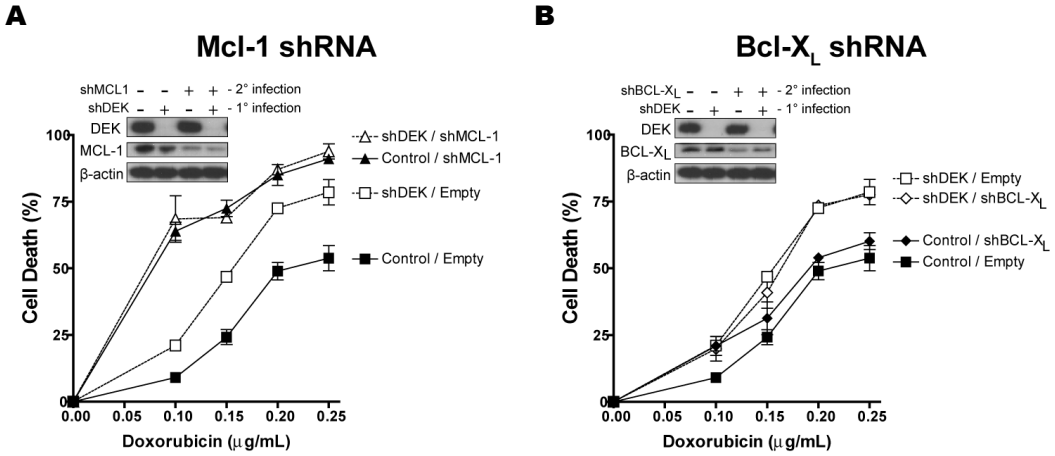

Figure 22. Mcl-1, but not Bcl-X_L, expression confers doxorubicin chemoresistance in melanoma. *A,* SK-MEL-19 cells were transduced with either a control or shDEK lentiviral vector. Cells were subsequently transduced with either an empty lentiviral vector or a vector expressing an Mcl-1 targeting shRNA. 72 hours after the second viral infection, cells were treated with the indicated concentration of doxorubicin for 30 hrs. Following treatment, cells were stained with DAPI and apoptotic cells were counted by fluorescence microscopy. *B,* cells were treated as in *A,* with the exception that the second lentiviral infection was performed with a vector targeting Bcl-X_L instead of Mcl-1.

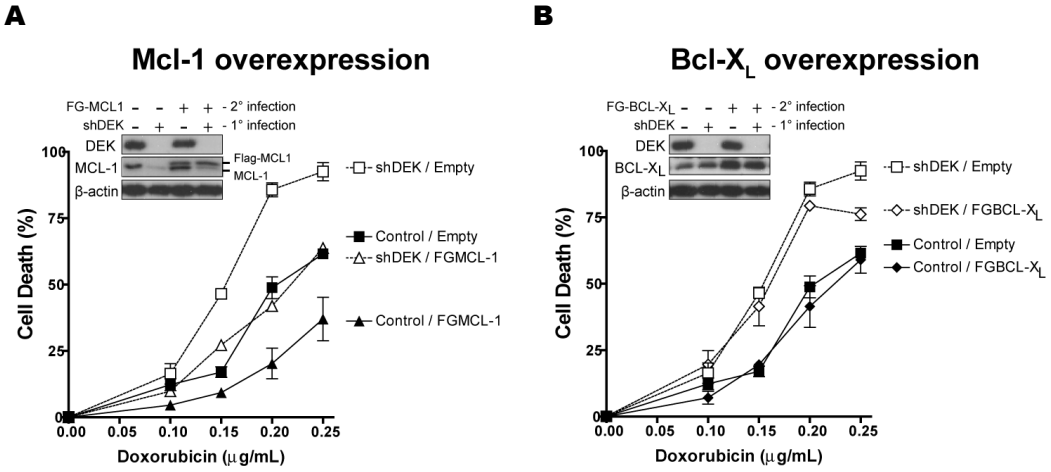

Figure 23. Mcl-1 expression, but not Bcl-X_L, expression restores doxorubicin chemoresistance to shDEK-transduced melanoma cells. *A,* SK-MEL-19 cells were transduced with either a control or shDEK lentiviral vector. Cells were subsequently transduced with either an empty lentiviral vector or a vector expressing Flag-tagged Mcl-1. 72 hours after the second viral infection, cells were treated with the indicated concentration of doxorubicin for 30 hrs. Following treatment, cells were stained with DAPI and apoptotic cells were counted by fluorescence microscopy. *B,* cells were treated as in *A,* with the exception that the second lentiviral infection was performed with a vector expressing Bcl-X_L instead of Mcl-1.

depend on Mcl-1(Nguyen, Marcellus et al. 2007). This is because ABT-737, like many first generation BH3 mimetics, displays selective affinity for Bcl-2 and Bcl-X_L, but not Mcl-1 (Oltersdorf, Elmore et al. 2005). Indeed, Mcl-1 exhibits important differences from these related proteins. Specifically, Mcl-1 has a unique BH3-only protein affinity profile (Youle and Strasser 2008), with one particularly important feature being its ability to bind NOXA but not BAD (Chen, Willis et al. 2005). Another unique feature of Mcl-1 is its lability. Because of its short half life, Mcl-1 is effectively regulated at the post-translational level.

Whereas post-translational regulation of Mcl-1 has been well characterized, studies of transcriptional regulation have given a less clear picture. The *mcl-1* promoter is responsive to STAT signaling, and also can be repressed by two E2F transcription factors, E2F1 and E2F2 (Croxton, Ma et al. 2002; Croxton, Ma et al. 2002). Recently, the *mcl-1* promoter was also identified as a target of p53 repression (Pietrzak and Puzianowska-Kuznicka 2008). Our findings show that DEK promotes activity of the *mcl-1* promoter. Although we do not demonstrate direct binding of DEK to the *mcl-1* promoter, this would appear to be the most likely explanation of our findings. DEK has previously been shown to function as a transcriptional activator (as well as a repressor) and can localize to transcriptionally active areas (Campillos, Garcia et al. 2003; Hu, Illges et al. 2005; Hu, Scholten et al. 2007). It is noteworthy that the regulation of Mcl-1 expression by DEK was observed only in the melanoma lines with the greatest expression of DEK. It may be that this activity of DEK is only observed in cells with extremely abundant DEK expression. Our attempts to address this possibility by stable

overexpression of DEK in melanocytes and melanoma lines were unsuccessful, presumably due a to negative feedback response on endogenous DEK production.

Our previous study of the function of DEK in melanoma demonstrated that only a subset of melanoma cells senesced in response to knockdown of DEK. Interestingly, even melanoma lines that did not senesce in response to interference with DEK nonetheless displayed induction of $p21^{CIP1}$. Because of the universal response of $p21^{CIP1}$ to DEK interference, we speculate that inhibition of $p21^{CIP1}$ may in fact be the primary function of enhanced DEK expression. In this scenario, the upregulation of DEK expression is initially required to bypass $p21^{CIP1}$-mediated cell arrest, and chemoresistance develops as an inherent consequence of the circumvention of the senescence response by overexpression of DEK. One puzzling feature of metastatic melanoma chemoresistance is its intrinsic nature (Soengas and Lowe 2003). In other words, chemoresistance does not develop as a result of acquired alterations during and after exposure to treatment but is rather an inherent characteristic of even untreated melanoma. If overexpression of DEK is indeed a frequently employed mechanism of bypassing the senescence response, the ability of DEK to also regulate Mcl-1 expression may partially explain the remarkable intrinsic chemoresistance of metastatic melanoma.

These results functionally implicate DEK as a melanoma oncogene that displays both anti-senescence and anti-apoptotic activity. Furthermore, we establish DEK, a gene frequently overexpressed in a number of malignancies, as a novel regulator of Mcl-1 transcriptional regulation and hence as a modulator of chemoresitance. Our findings suggest that overexpression of DEK may be a frequent and significant event in the

development of melanoma, and these data warrant the further study of DEK as a potential target in the pursuit of effective treatments for melanoma.

Material and Methods

Cell culture

All melanoma cell lines were cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Human melanocytes were isolated from human neonatal foreskins as described (Verhaegen, Bauer et al. 2006) and maintained in Medium 254 supplemented with 12-*O*-tetradecanoylphorbol-13-acetate (10 nmol/L) and melanocyte growth factors (Cascade Biologics, Portland, OR).

Reagents

Doxorubicin hydrochloride, cycloheximide and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical (St. Louis, MO). Bortezomib was obtained from Millennium Pharmaceuticals (Cambridge, MA). zVAD-fmk was purchased from Calbiochem (Cambridge, MA). TW-37 synthesis has been described previously (Wang, Nikolovska-Coleska et al. 2006).

Extract preparation and protein immunoblots

Adherent and non-adherent cells were collected. Total cell lysates were obtained by Laemmli extraction, separated by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). Antibodies to Bcl-X_L and monoclonal antibodies to DEK were obtained from BD Biosciences (Franklin Lake, NJ), β-actin from Sigma Chemical (St. Louis, MO), p53 from Novacostra Laboratories (Newcastle upon

Tyne, UK), Bcl-2 from DAKO Diagnostics (Glostrup, Denmark), Mcl-1 from Santa Cruz Biotechnology (Santa Cruz, CA), caspase-3 and phospho-p53-ser^{15} from Cell Signaling Technologies (Beverly, MA). Polyclonal antibodies to DEK were produced as previously described (Kappes, Damoc et al. 2004). Unless otherwise specified, DEK was detected in immunoblots using polyclonal anti-DEK antibodies. Detection was performed using ECL Western blotting substrate from Pierce (Rockford, IL). Protein expression was quantified using Scion image densitometry software (Scion Corporation, Frederick, MD).

Cell viability

Adherent and non-adherent cells were collected and pooled at the indicated times after treatment. Cells were washed in PBS, fixed with 3.7% paraformaldehyde, and stained with DAPI. Samples were then mounted unto glass slides, and nuclear condensation and fragmentation were assessed by fluorescence microscopy using an Olympus BX-51 upright microscope. Image capture was performed electronically using an Olympus DP-70 high resolution digital camera.

Lentiviral shRNA constructs

Construction of lentiviral shRNA vectors targeting Bcl-2, Bcl-X_L, and Mcl-1 has been described previously (Verhaegen, Bauer et al. 2006). The H1-LV vector was used to produce shRNAs targeting DEK. The shDEK-1 vector used the human H1 promoter to drive expression of an shRNA targeting a sequence in the 3' untranslated region of the human *DEK* transcript (nucleotides 1615-35, GenBank NM_003472.2). The shDEK-2 vector was constructed by inserting a second Pol III promoter and shRNA immediately 5' of the H1 promoter. This insertion contained the murine U6 promoter expressing a

second shRNA targeting a coding region of the *DEK* transcript (nucleotides 1118-1141, GenBank NM_003472.2). Finally, a third lentiviral vector targeting DEK expression based on the PLKO.1 vector, PLKO.1-DEK832, was purchased from the Sigma Chemicals (St. Louis, MO). This vector targets a third region of the human *DEK* transcript distinct from the shRNA targets listed above. Lentivirus production and infection was performed as described previously (Verhaegen, Bauer et al. 2006). When cells were sequentially infected with two lentiviral vectors, the second infection was performed 72 hours after the initial infection. Transduction efficiency of H1-LV derived lentiviral vectors was estimated by detection of GFP expression (driven by the human ubiquitin c promoter) by fluorescence microscopy or flow cytometry and was routinely greater than 95%. Cells transduced with the PLKO.1-DEK832 vector were selected for 3 days in 2 μg/mL puromycin.

Mcl-1 and Bcl-X_L expression lentivirus

The coding region of human Mcl-1 mRNA was amplified by RT-PCR from SK-MEL-19 cells. An N-terminal FLAG-tag was cloned in frame with the coding region of the cDNA, and this construct was inserted under control of the CMV promoter into the FG12-eGFP lentiviral vector. The FG9EF1a Bcl-X_L vector was a gift from Colin Duckett and expresses Bcl-X_L under the control of the EF1a promoter. Lentivirus production and infection were performed as described previously (Denoyelle, Abou-Rjaily et al. 2006).

Quantitative real-time RT-PCR

Total RNA was purified from cells using the TRIZOL method according to manufacturer's instructions (Invitrogen, Carlsbad, CA). The RNA was further purified using the Qiagen RNeasy mini kit (Qiagen, Chatworth, CA). Quantitative real-time RT-

PCR was performed using the Quantitect Sybr Green RT-PCR kit (Qiagen, Chatworth, CA) using the following primers: *mcl1* forward – ATGCTTCGGAAACTGGACAT; *mcl1* reverse – TCCTGATGCCACCTTCTAGG; *β-actin* forward - CGCCCAGCACGATGAAA ; *β-actin* reverse - CCGCCGATCCACACAGA. Real-time RT-PCR was performed and analyzed on a Bio-Rad iCycler (Bio-Rad, Hercules, CA). Expression of *mcl-1* was normalized to that of β–*actin.* The assay included a no-template control and a standard curve of six dilutions for both *mcl-1* and *β–actin*. Results are given as the average of three experiments ± SE, and each sample was amplified and analyzed in triplicate.

Luciferase reporter gene assays

The human *mcl-1* promoter reporter plasmid -213/+10, which drives expression of firefly luciferase, was a generous gift from Dr. Douglas Cress. Cells were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Cells were co-transfected with the pRL-TK plasmid (Promega Corp., Madison, WI), which drives expression of renilla luciferase, as an internal transfection control. Thirty hours later cells were lysed and the lysates were assayed for firefly and renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega Corp., Madison, WI). Luminescence was detected with a Tecan GENios plate reader (Phenix, Austria). Data are presented as the mean ± SE of three experiments.

Statistics

Statistical analyses of the quantitative real-time RT-PCR and luciferase reporter assay data were done using a paired, two-tailed Student's t-test.

CHAPTER V

Conclusion

DEK and mRNA processing

In Chapter II we demonstrate that the sub-nuclear localization of DEK is modified by acetylation. Our observation that DEK accumulates in introchromatin granule clusters (IGCs) following acetylation suggests that acetylation is a regulatory mechanism of DEK activity. The finding that DEK can enter IGCs, and that its migration into IGCs is enhanced during transcriptional inhibition strongly suggests a role in RNA processing. In fact, the mere presence of a protein in IGCs has been suggested to be diagnostic for interactions with mRNA (Lamond and Spector 2003). However, the significance of DEK migration into IGCs is unclear. Although they are rich in RNA processing factors, IGCs are not an active place of RNA processing (Fakan, Leser et al. 1984; Zeng, Kim et al. 1997; Cmarko, Verschure et al. 1999). In agreement with this, we find that the migration of DEK into IGCs is not accompanied by an increase in interactions with RNA (Chapter II, Figure 7). One hypothesis is that the IGCs represent a storage and assembly area for the formation of splicing complexes (Misteli and Spector 1997). In this model, inactive splicing factors cycle to the IGC where they reassemble splicing complexes components and subsequently "bud" off from the IGC. It is possible that DEK behaves in a similar manner, shuttling to the IGCs and forming complexes with splicing factors.

The link between DEK and RNA processing has been tenuous, highlighted by the fact that the investigators who initially described the association of DEK with splicing factors have themselves since abandoned the notion that DEK is a true member of the splicing machinery (Reichert, Le Hir et al. 2002). Even with their use of a cross-reactive anti-DEK antisera, the original studies implicating DEK with this process never identified a direct interaction between DEK and RNA nor did they find a requirement for DEK in active splicing complexes (Le Hir, Izaurralde et al. 2000; McGarvey, Rosonina et al. 2000). Indeed, we also were unable to identify any *in vivo* RNA targets of DEK by RNA immunoprecipitation (Chapter II, Figure 8). Nevertheless, there are still some data that suggest involvement of DEK in this process. The initial association of DEK with splicing complexes was identified by mass spectrometry in a purification scheme free of the controversial antisera, making it unlikely that its association was simply an artifact. Additionally, we and others have found that a small but consistent fraction of DEK is released upon treatment with RNase (Chapter II, Figure 7) (Kappes, Burger et al. 2001).

The subsequent discovery that DEK could act to proofread 3' splice sites suggests that DEK does have an active function in pre-mRNA processing (Soares, Zanier et al. 2006). However, it is difficult to reconcile this proposed function with the previous findings showing both an absence of DEK in splicing complexes and the ability of U2AF35 to mediate efficient 3' splice site recognition in the absence of DEK (Wu, Romfo et al. 1999; Guth, Tange et al. 2001; Reichert, Le Hir et al. 2002; Kataoka and Dreyfuss 2004). This might be partially explained by the requirement of phosphorylation of DEK for functional activity. Although we demonstrate here that acetylation does not

promote interactions between DEK and RNA (Chapter II, Figure 7), it appears likely that the participation of DEK in RNA processing is regulated through phosphorylation. Thus, only a fraction of total cellular DEK may actually be involved in these RNA interactions increasing the difficulty of detection in many experimental systems. The authors propose that DEK is required for the recognition of the 3' splice site. However, it has previously been demonstrated that the factors required for effective splice site recognition vary depending on the RNA target (Reed 1989; Wu, Romfo et al. 1999; Guth, Tange et al. 2001). It is possible that the involvement of DEK in splice site recognition is limited to only a select subset of pre-mRNA targets, and this could account for the failure to identify a consistent function for DEK in splicing. In summary, there is reasonable evidence linking DEK to RNA processing, but additional work is needed to validate and extend these results before concluding that DEK is a component of the cellular RNA processing machinery.

DEK and Melanoma

Chapters III and IV describe two tumorigenic functions of DEK in the context of melanoma: inhibition of senescence and inhibition of apoptosis. Our finding that DEK was ubiquitously overexpressed in metastatic melanoma lines raises the possibility that DEK may play an essential role in the progression of melanoma (Chapter III, Figure 9).

One of the most puzzling results regarding the function of DEK in melanoma is that interference with DEK expression produced two seemingly mutually exclusive phenotypes. That is, cells that underwent senescence did not display changes in Mcl-1 expression, and cells that downregulated Mcl-1 did not senesce. Why are these two

outcomes exclusive, and what melanoma characteristics are responsible for determining these outcomes?

Melanomas display remarkable heterogeneity, and thus the different responses to inhibition of DEK are not entirely unexpected. These two phenotypes may represent distinct roles for DEK in molecularly disparate melanomas. Considerable effort has been spent in the identification of molecular signatures of melanoma. It has become clear that melanomas tend to target several common pathways for activation or suppression. However, the precise genetic alterations used to achieve the same functional result vary from melanoma to melanoma. For example, the mitogen activated protein kinase (MAPK) pathway is frequently activated in melanoma by mutation of either BRAF or NRAS (Eskandarpour, Hashemi et al. 2003; Smalley and Herlyn 2004). Although these mutations both target the MAPK pathway, they are not equivalent. As a consequence, BRAF mutations tend to associate with specific complementary mutations and NRAS mutations with a different set of alterations (Pavey, Johansson et al. 2004; Curtin, Fridlyand et al. 2005; Haqq, Nosrati et al. 2005; Johansson, Pavey et al. 2007). One of the best described genetic differences between melanomas possessing either BRAF or NRAS mutations is the expression of PTEN (phosphatase and tensin homolog). PTEN is an important negative regulator of another key signaling pathway in melanoma, the AKT pathway. Like the MAPK pathway, the AKT signal transduction pathway is frequently activated in melanoma (Dai, Martinka et al. 2005; Govindarajan, Sligh et al. 2007). Melanomas with mutations in BRAF (which does not affect AKT signaling) frequently also display loss or mutation of PTEN (Tsao, Goel et al. 2004; Goel, Lazar et al. 2006).

In contrast, because active NRAS has a secondary effect of activating AKT signaling, tumors with NRAS mutations do not exhibit mutations or deletions of PTEN.

Interestingly, although the four melanoma lines examined in this work are all known to express the mutant form of BRAF ($BRAF^{V600E}$), they differ in their expression of PTEN (data not shown). Specifically, the lines SK-MEL-19 and SK-MEL-29 both express PTEN, while the SK-MEL-28 and SK-MEL-94 lines do not. As presented in Chapters III and IV, SK-MEL-19 and SK-MEL-29 lines also downregulate Mcl-1 in response to DEK knockdown. Because these lines express functional PTEN, there is no deregulation of AKT signaling, unlike in most melanomas. This is noteworthy because of the prominent role of the AKT pathway in the regulation of Mcl-1 protein stability. In what is thought to be a major Mcl-1 regulatory mechanism, active AKT signaling protects Mcl-1 from ubiquitination and degradation (Zhong, Gao et al. 2005; Maurer, Charvet et al. 2006). The majority of melanomas display constitutive activation of AKT (through either mutation of NRAS or loss of PTEN function), and therefore would be predicted to have increased Mcl-1 protein stability. However, a subset of melanomas that express both $BRAF^{V600E}$ and wild type PTEN do not benefit from active AKT signaling, and thus Mcl-1 protein in these cells would be predicted to have a shorter half-life. Thus, it is possible that the extremely high expression of DEK in this class of melanoma lacking AKT activity evolves as a mechanism to compensate for decreased Mcl-1 half-life.

Because melanoma is such a genetically heterogeneous cancer, the finding that DEK expression is ubiquitous in metastatic melanoma is in itself of interest. The most frequent mutation in melanoma is the inactivation of the $p16^{INK4a}$ gene (Bartkova, Lukas

et al. 1996). Even in cases where this gene is intact and not silenced by methylation, downstream components of the $p16^{INK4a}$/pRB pathway are frequently found to be mutated (Walker, Flores et al. 1998). Thus, it has been postulated that this is a requisite event in the progression of melanoma. The finding that DEK is so frequently overexpressed in melanoma may similarly reflect its requirement for progression, or it may simply be a consequence of the universal inactivation of $p16^{INK4a}$ in this cancer.

To better assess the significance of DEK in melanoma, it will be necessary to also investigate its expression in nevi and primary melanoma lesions, in addition to a larger set of metastatic melanomas. Melanomas typically arise from benign nevi, most commonly in response to chronic exposure to ultraviolet light. Tumor growth is initially radial, but as the melanoma progesses this changes to vertical growth. The transition to vertical growth appears to be accompanied by the potential for metastasis (Clark, Elder et al. 1989; Crotty and Menzies 2004). It is increasingly evident that melanocytes within benign nevi are in a state of oncogene-induced senescence. Melanocytes residing within nevi frequently harbor mutations of either BRAF (80%) or NRAS (5-15%) yet do not proliferate (Daniotti, Oggionni et al. 2004; Thomas, Alexander et al. 2004). This lack of progression is due to $p16^{INK4a}$/pRB mediated senescence. Accordingly, these lesions stain positively for both $p16^{INK4a}$ and senescence-associated β-galactosidase activity (Michaloglou, Vredeveld et al. 2005; Gray-Schopfer, Cheong et al. 2006). The inactivation of $p16^{INK4a}$ through various mechanisms allows progression of melanoma formation. Similarly, the transitions from radial growth to vertical growth to metastatic melanoma are also marked by a number of frequent alterations (Nyormoi and Bar-Eli 2003; Haqq, Nosrati et al. 2005; Govindarajan, Sligh et al. 2007; Magnoni, Tenedini et al.

2007; Sousa and Espreafico 2008). The results presented here demonstrate that in the progression from benign nevus to metastatic melanoma, DEK is upregulated, but they do not indicate at which stage(s) this upregulation occurs. Examination of DEK expression in a well-sized panel of primary and metastatic melanomas, by immunohistochemistry for example, would be of great benefit in further elucidating the contribution of DEK to melanoma pathogenesis. Additionally, confirmation of a cytogenetic correlation of 6p gain with DEK expression would strongly support the notion that DEK expression is a required event for melanoma progression.

While we have demonstrated that the contribution of DEK expression to melanoma chemoresistance is due to regulation of Mcl-1, we did not identify the mechanism through which DEK acts to oppose senescence. Recent data from our group provide some insight into how DEK may function to inhibit senescence. We have discovered that DEK is required for the efficient DNA repair of double strand breaks (DSB) (Kappes, Fahrer et al. 2008). As discussed in Chapter III, DNA damage signaling is involved in the induction of senescence in response to oncogene-induced stress, telomere uncapping, and accumulation of DNA damage. Melanomas appear to exhibit some signs of DNA damage such as γ–H2AX foci and DNA DSBs, suggesting that senescence-inducing signals are present in these cells (Gorgoulis, Vassiliou et al. 2005; Warters, Adamson et al. 2005). However, melanoma cells do not respond with p53-dependent G1 arrest (Gorgoulis, Vassiliou et al. 2005). We hypothesize that the recently discovered DNA repair activity of DEK may be responsible for its apparent function as an inhibitor of senescence. Microarray analysis of 60 primary melanomas revealed that those melanomas that later gave rise to metastasis display increased expression of

components of DNA repair pathways (Kauffmann, Rosselli et al. 2008). The overexpression of DNA repair pathway genes in melanomas with metastatic potential was somewhat counterintuitive because patients with inherited defects in DNA repair proteins, such as xeroderma pigmentosum, have an increased incidence of melanoma (Li, Hu et al. 2006). However, while deficiency in DNA repair genes may promote the genomic instability that initially gives rise to melanoma, functioning DNA repair pathways promote metastatic potential (Sarasin and Kauffmann 2008). This suggests that although a certain degree of genomic instability is necessary for initial transformation, metastasis requires the ability to withstand increased DNA damage stress. By promoting DNA repair, DEK may act to permit transition from primary to metastatic melanoma.

We additionally have found that DEK is an essential factor required for higher-order chromatin structure, potentially by actively participating with HP1 (Kappes, Khodadoust et al., in preparation). The participation of DEK in DNA repair and higher order chromatin structure may be interconnected. Recent discovery of a function of the heterochromatin protein 1β (HP1β) in DNA repair may be of particular relevance to DEK (Ayoub, Jeyasekharan et al. 2008). HP1β is phosphorylated in response to DNA damage. This phosphorylation of HP1β causes its release from methylated histone H3 (H3K9me), and consequently, greater chromatin accessibility. Subsequently, H2AX is phosphorylated, which recruits DNA repair factors to the lesion. Mutant HP1β lacking the essential phosphorylation site remains bound to chromatin and inhibits the phosphorylation of H2AX. Notably, the kinase responsible for HP1β phosphorylation, CK2, is the primary kinase known to phosphorylate DEK. It has been shown that DEK is not recruited to sites of DNA damage suggesting that DEK is not a repair factor *per se,*

but it is interesting to speculate that DEK may be a damage sensing factor analogous to HP1β that promotes repair by altering chromatin in response to double strand breaks (Scholten 2004).

Although the results presented here certainly raise many new questions, they provide a useful starting point for further investigation of the function of DEK in cancer. The continued study of DEK in the context of melanoma promises to contribute to our understanding of this deadly disease, and may even lead to new avenues for treatment.

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