JOURNAL OF VIROLOGY, Nov. 2005, p. 14309–14317 Vol. 79, No. 22
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.22.14309–14317.2005
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The Human DEK Proto-Oncogene Is a Senescence Inhibitor and an Upregulated Target of High-Risk Human Papillomavirus E7

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Received 2 June 2005/Accepted 19 August 2005

The human DEK proto-oncogene is a nucleic acid binding protein with suspected roles in human carcinogenesis, autoimmune disease, and viral infection. Intracellular DEK functions, however, are poorly understood. In papillomavirus-positive cervical cancer cells, downregulation of viral E6/E7 oncogene expression results in cellular senescence. We report here the specific repression of DEK message and protein levels in senescing human papillomavirus type 16- (HPV16-) and HPV18-positive cancer cell lines as well as in primary cells undergoing replicative senescence. Cervical cancer cell senescence was partially overcome by DEK overexpression, and DEK overexpression was sufficient for extending the life span of primary keratinocytes, supporting critical roles for this molecule as a senescence regulator. In order to determine whether DEK is a bona fide HPV oncogene target in primary cells, DEK expression was monitored in human keratinocytes transduced with HPV E6 and/or E7. The results identify high-risk HPV E7 as a positive DEK regulator, an activity that is not shared by low-risk HPV E7 protein. Experiments in mouse embryo fibroblasts recapitulated the observed E7-mediated DEK induction and demonstrated that both basal and E7-induced regulation of DEK expression are controlled by the retinoblastoma protein family. Taken together, our results suggest that DEK upregulation may be a common event in human carcinogenesis and may reflect its senescence inhibitory function.

Papillomaviruses are a group of small DNA tumor viruses that induce various benign and malignant epithelial lesions in the infected host (24). Classification of the human papillomaviruses (HPVs) into high-risk versus low-risk types is based upon the likelihood that the associated lesions will progress to malignancy or remain benign. High-risk HPV infections are strongly associated with cervical cancer, causing 15% of female cancer mortality worldwide (13). Over 97% of cervical cancers contain high-risk HPV DNA and express the viral oncogenes E6 and E7. A link between HPV infection and carcinogenesis is further supported by the documented in vitro and in vivo immortalizing and transforming activities of the viral oncoproteins (3, 21, 39, 40, 56). Many interactions have been reported between high-risk HPV oncoproteins and host cellular factors, including the targeted degradation of p53 and transcriptional upregulation of telomerase by E6 as well as the interaction with and degradation of retinoblastoma family members by E7 (24).

HPV DNA is maintained episomally in benign, precancerous lesions but is generally found integrated into the cellular genome in malignant carcinomas and cell lines derived from them (12, 42). Sustained expression of the immortalizing viral oncogenes and oftentimes the loss of the viral E2 open reading frame are important attributes of such cell lines. The papillomavirus E2 protein is a regulatory factor with multiple roles in the transcription and replication of the viral DNA. Loss of E2 protein expression is likely important in the progression of HPV-associated carcinogenesis. E2 is known to directly bind and repress the viral E6/E7 promoter of integrated high-risk HPVs (6, 41, 46, 47). Reexpression of E2 in HPV-positive cancer lines results in cellular growth arrest and senescence, which requires E6/E7 promoter repression (14, 17, 18, 54). Under some circumstances, E2 expression can also cause apoptotic cell death in a manner that is independent of the HPV status of the affected cell (7, 8, 52).

The term cellular or replicative senescence defines the finite replicative capacity of most somatic cells in culture, which eventually results in the complete cessation of cellular division. Aside from replicative exhaustion, various forms of cellular stress, such as DNA damage or oncogene expression, can trigger a senescence program. Given that high-risk HPV E6/E7 expression induces cellular immortalization, the HPV oncogenes must therefore inactivate cellular senescence pathways to achieve immortality. Despite the potential biological significance of senescence in human cancer and aging (5, 36), relatively little is known about specific senescence regulators, mediators, or markers. In order to identify senescence-associated genes and gene groups in HeLa cells, we performed a genomic analysis of E2-mediated senescence in HeLa cells (53) and detected a marked downregulation of the human DEK proto-oncogene.

DEK is a 43-kDa nucleic acid-associated mammalian nuclear phosphoprotein that has been associated with a variety of human diseases (45, 51). DEK does not display any significant sequence homology to other known proteins, except for a conserved DNA binding motif, the SAP box domain (2). Although

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first identified as a fusion with the CAN nucleoporin gene in a subset of acute myeloid leukemia (AML) patients (48), transcriptional upregulation of the wild-type DEK gene was discovered in a number of tumors, including AML types which do not exhibit the DEK/CAN translocation (19, 30, 32, 33). In addition to its association with cancer, the DEK protein is often found as a major immunoreactive antigen in patients with autoimmune diseases, such as juvenile rheumatoid arthritis, systemic lupus erythematosus, and sarcoidosis (10, 11, 43, 55). Despite the significant association of the DEK proto-oncogene with human disease, details of its intracellular functions remain poorly understood and its pathophysiological relevance is thus not known.

Our results demonstrate that DEK message and protein levels are specifically repressed during cervical cancer cell senescence when induced either by E2 overexpression or by E6/E7 RNA interference (RNAi). Senescence was partially inhibited by the overexpression of DEK, implicating this molecule functionally as a senescence antagonist. Induction of replicative senescence of primary human keratinocytes and fibroblasts was also associated with DEK repression and, in support of a role for DEK in senescence inhibition, its overexpression was sufficient for extending the life span of primary human keratinocytes. Retroviral transduction of primary cells with the immortalizing HPV16 E6 and E7 oncogenes resulted in marked upregulation of DEK expression and defined high-risk HPV E7 as the relevant regulatory oncogene. Interestingly, DEK upregulation by the high-risk HPV16 E7 protein was not shared by the low-risk HPV6b E7 protein. Our study identifies the human DEK proto-oncogene as an upregulated target of high-risk HPV E7 with implications for HPV-associated carcinogenesis. Experiments using wild-type and retinoblastoma protein-deficient mouse embryo fibroblasts (MEFs) further demonstrate a critical role for the retinoblastoma protein family in the control of basal as well as E7-induced DEK expression. Based on the fact that retinoblastoma pathways are inactivated in the vast majority of human cancers, we propose that DEK may have wide clinical usefulness as a new diagnostic tool and potential drug target.

MATERIALS AND METHODS

Cell culture. Monolayer HeLa, Caski, C33A, SiHa, U2OS, SAOS-2, and 293 cells as well as primary human fibroblasts were all maintained in Dulbecco's modified Eagle medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics. Primary human foreskin keratinocytes (HFKs) were maintained in keratinocyte growth medium (Cascade Biologics, Portland, OR) and antibiotics. Primary keratinocytes were prepared from human foreskins (IBC protocol CHMC number 02-9-29X). The pRB/p107/p130 triple knockout (TKO) mouse embryo fibroblasts were originally generated by the Te Riele laboratory (5a).

Plasmid and viral constructs. Adenoviral AdE2-TR and AdE2-TA vectors were generated as previously described for AdE2ts virus (53) and using the BPV1 E2-TR and E2-TA open reading frames. The DEK open reading frame was PCR amplified using the following primers: 5′-ATG TCC GCC TCG GCC-3′ and 5′-TCA AGA AAT TAG CTC TTT TAC AG-3′, and a cDNA template provided by the Grosveld laboratory. The amplicon was then cloned into the pGEM-T Easy vector (Promega, Madison, WI), sequenced, excised using NotI, and cloned into the Adtrack cytomegalovirus vector cut with the same enzyme. Individual clones were screened for the correct orientation and were resequenced after recombination into AdEasy1 (22). Viral titers were determined by plaque assays on 293 cells. Producer cell lines for empty LXSN retrovirus as well as retroviruses expressing HPV16 E6, E7, and E6/E7, respectively, were a gift from Denise Galloway, University of Washington, Seattle. The HPV6b E7 producer cell line was purchased from the American Type Culture Collection.

Viral infections. Adenoviral infections were performed on two consecutive days at a multiplicity of infection (MOI) of 10 each unless otherwise indicated. For retroviral transductions, the cells were incubated for 4 hours with viral supernatant containing 2 μg/ml Polybrene and then with fresh medium overnight. The medium was replaced on the next day with medium containing 900 μg/ml of G418 for HeLa cells or 500 μg/ml for MEFs for the remainder of the experiment and 200 μg/ml for HFKs for 36 h.

Northern and Western blot analyses. Both Northern and Western blot analyses were performed as described previously (53). Antibodies used were the p53 monoclonal antibody (Calbiochem, San Diego, CA), polyclonal DEK antiserum (a gift from the Grosveld Laboratory, Memphis, TN) or monoclonal DEK antibody (BD Transduction Laboratories, San Jose, CA), and polyclonal survivin antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected by secondary horseradish peroxidase-conjugated antibody (Amersham, Piscataway, NJ).

Cell cycle analysis. HeLa cells were detached from the plates using 0.1% EDTA in phosphate-buffered saline (PBS) at 37°C. The cells were washed with PBS and fixed in cold 80% ethanol at 4°C overnight. Cells were pelleted by centrifugation, washed twice with 1% bovine serum albumin in PBS, and 1×10^6 cells were resuspended in 800 μl of 1% bovine serum albumin in PBS, 100 μl of 500 μg/ml propidium iodide in 10 mM sodium citrate, pH 7.0, and 100 μl of boiled RNase A (10 mg/ml in 10 mM Tris-HCl, pH 7.5). Incubation was for 30 min at 37°C. Cell cycle profiles were obtained using a flow cytometer (BD Biosciences, San Jose, CA), and analysis was performed using Cell Quest software. For chemically induced cellular arrest in different phases of the cell cycle, HeLa cells were overlaid with medium containing either 400 μM mimosine (G_0/G_1), 2 mM thymidine (S), 400 μM mimosine and 2 mM thymidine (G_1/S interphase), or 0.4 μg/ml nocodazole (G_2/M) and harvested after 16 h.

Senescence assays. HeLa cells were preinfected with Ad versus AdDEK virus at an MOI of 100. Four days postinfection, the cells were split to 5×10^5 cells/10-cm dish and allowed to adhere. The cells were then infected twice with AdE2ts as performed for the microarray experiments previously (53) together with additional Ad or AdDEK virus (MOI = 50) for the respective Ad- or AdDEK-preinfected samples. On the next day, senescence samples were placed at 32°C, whereas control samples were incubated at 39.5°C. Control samples at 39.5°C became confluent rapidly and were split 1:10 prior to morphological examination at 2 weeks post-temperature shift. Staining for perinuclear Senescence-associated-β-galactosidase (SA–β-Gal) activity was performed as previously described (9).

RESULTS

E2-mediated DEK repression is via the HPV oncogenes and correlates with the cellular senescence phenotype. We recently described an experimental approach for the profiling of senescence-associated genes in HeLa cells using an E2-based conditional system (53). One gene that was found markedly repressed in response to adenovirally delivered, temperature-sensitive E2 protein under permissive conditions was the human DEK proto-oncogene. The fully annotated data set is available at http://genet.cchmc.org in the U95Av2 genome under Experiments/Wells_Howley (login as guest). DEK repression was confirmed by Northern blot analysis (data not shown).

In order to examine DEK repression in response to expression of wild-type bovine papillomavirus (BPV) E2 proteins, we measured DEK mRNA and protein levels in HeLa cells after adenoviral expression of full-length BPV1 E2-TA compared to its truncated natural E2-TR variant. Whereas E2-TA efficiently represses the transcription of HPV18 E6/E7 in HeLa cells, E2-TR does not. The resulting ability of E2-TA, but not E2-TR, to cause senescence in HPV-positive cells has been well established (18, 25, 34, 53, 54). HeLa cells were infected with empty Ad, AdE2-TR, and AdE2-TA, and total RNA was subjected to DEK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific Northern blot analysis. DEK mRNA levels were reduced in the presence of E2-TA (Fig. 1A,

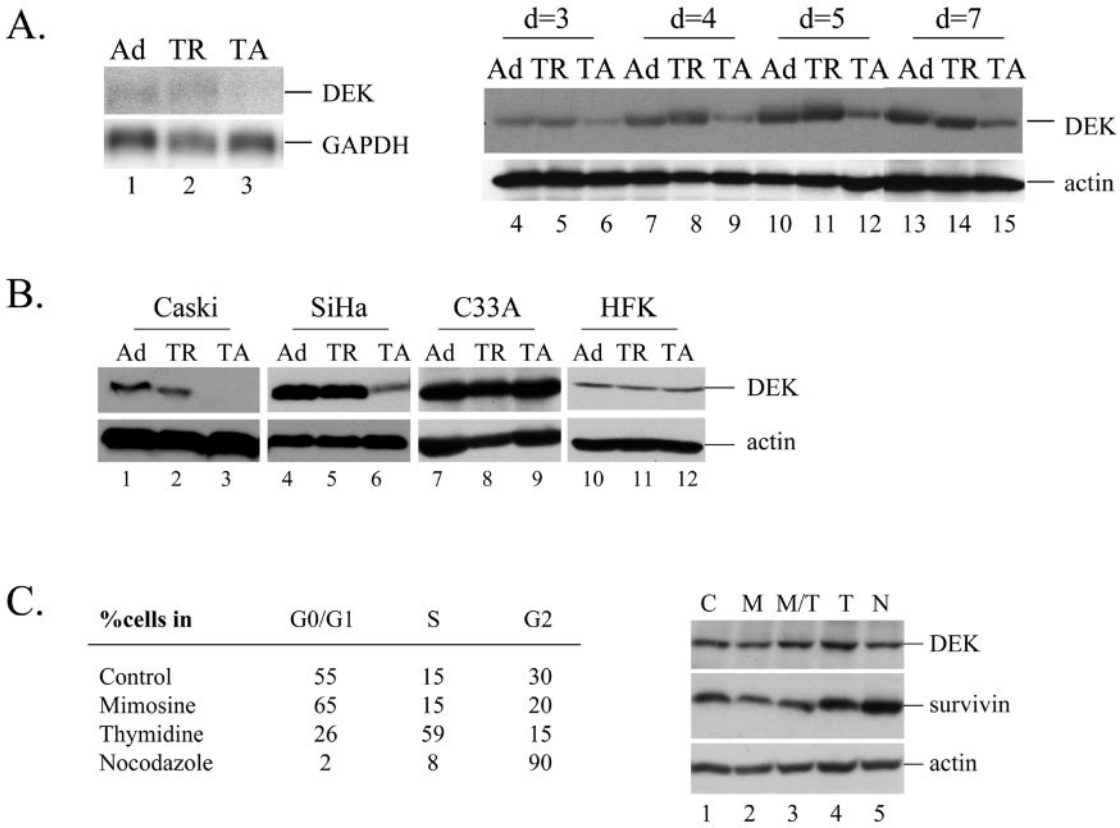

FIG. 1. E2-TA-mediated DEK repression is specific to HPV-positive cells. (A) (Left panel) HPV18-positive HeLa cells were infected with empty adenovirus (Ad) or E2-TR-expressing (TR) or E2-TA-expressing (TA) adenovirus. RNA was harvested after 3 days and subjected to DEK- and GAPDH-specific Northern blot analysis. (Right panel) HeLa cells were infected with either empty Ad (lanes 4, 7, 10, and 13), AdE2-TR (lanes 5, 8, 11, and 14), or AdE2-TA (lanes 6, 9, 12, and 15) for the indicated number of days. Protein lysates were subjected to Western blot analysis using either DEK antiserum or actin antiserum as a loading control. (B) HPV16-positive Caski and SiHa cells, HPV-negative C33A cervical cancer cells, and primary HFKs were infected with empty Ad or E2-TR-expressing (TR) or E2-TA-expressing (TA) adenovirus. Protein lysates were harvested on day 3 postinfection and subjected to DEK- and actin-specific Western blot analysis (lower two panels). (C) HeLa cells were treated with mimosine (M) and/or thymidine (T) or nocodazole (N) as described in Materials and Methods to induce cellular growth arrest in different phases of the cell cycle. The cells were harvested either for cell cycle analysis (table on the left) or for DEK-specific Western blot analysis (right panels). Western blot analysis for a G_2/M-phase-specific marker, survivin (35), was included as a control.

lane 3) compared to E2-TR (lane 2) or empty Ad (lane 1). DEK protein levels were also reduced in response to E2-TA on day 3 (Fig. 1A, compare lane 6 with lanes 4 and 5). The observed repression of DEK was maintained for at least 7 days (lanes 7 to 15).

We next monitored DEK regulation by E2-TA in two HPV16-positive cervical cancer cell lines, SiHa and Caski, as well as in the HPV-negative C33A cervical cancer cell line and in HPV-negative primary human foreskin keratinocytes. The cell lines were infected as above, and a green fluorescent protein (GFP)-expressing adenovirus was used to assess infection efficiency. Over 90% of the respective cell populations was confirmed GFP positive by fluorescence microscopy (data not shown). Similar to our findings in HeLa cells, DEK protein expression was reduced in response to E2-TA in HPV16-positive Caski and SiHa cells (Fig. 1B, lanes 1 to 6). Like HeLa cells, Caski and SiHa cells undergo senescence in response to E2-TA (16, 54). In contrast, reduced DEK expression by E2-TA was not observed in HPV-negative C33A cells or in primary HFKs (Fig. 1B, lanes 7 to 12). DEK was also not repressed by E2-TA in HPV-negative U2OS or SAOS-2 osteosarcoma cells (data not shown). We do not believe that DEK repression during senescence is a simple consequence of cell cycle arrest, since DEK association with chromatin did not change over time following HeLa cell synchronization (26) and since chemically induced cell cycle arrest in different phases of the cell cycle did not affect DEK expression (Fig. 1C).

DEK repression during E6/E7 RNA interference mediated senescence. The specific repression of DEK expression in HPV-positive cells (Fig. 1) suggested a possible involvement for the viral oncoproteins in the regulation of DEK, but it did not rule out more direct roles for E2 in this process. In order to determine whether DEK regulation was solely downstream from the viral oncoproteins and independent of other E2 functions, we used RNA interference for the targeted knock-down of HPV18 E6/E7 in the absence of E2 (Fig. 2). To inhibit E6/E7 oncogene expression in HPV-positive HeLa cells, we exploited the fact that transcription of both the HPV18 E6 and E7 oncogenes is directed from the common P_{105} promoter. This results in the synthesis of messages with E6 and E7 intact or with splices in the E6 gene. HeLa cells were either mock transfected or transfected with E6/E7-specific double-stranded

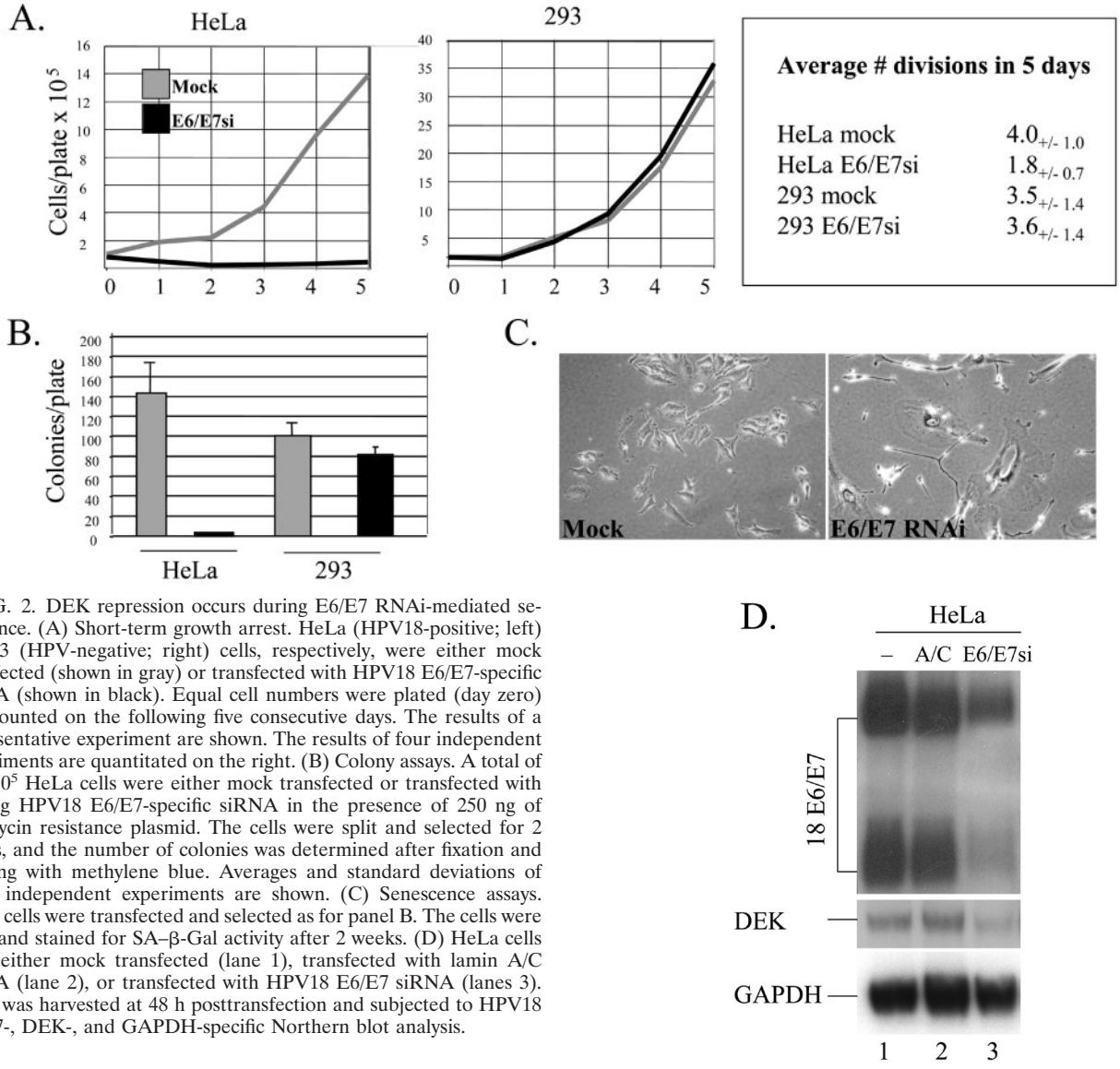

FIG. 2. DEK repression occurs during E6/E7 RNAi-mediated senescence. (A) Short-term growth arrest. HeLa (HPV18-positive; left) or 293 (HPV-negative; right) cells, respectively, were either mock transfected (shown in gray) or transfected with HPV18 E6/E7-specific siRNA (shown in black). Equal cell numbers were plated (day zero) and counted on the following five consecutive days. The results of a representative experiment are shown. The results of four independent experiments are quantitated on the right. (B) Colony assays. A total of 1×10^5 HeLa cells were either mock transfected or transfected with 200 ng HPV18 E6/E7-specific siRNA in the presence of 250 ng of neomycin resistance plasmid. The cells were split and selected for 2 weeks, and the number of colonies was determined after fixation and staining with methylene blue. Averages and standard deviations of three independent experiments are shown. (C) Senescence assays. HeLa cells were transfected and selected as for panel B. The cells were fixed and stained for SA–β-Gal activity after 2 weeks. (D) HeLa cells were either mock transfected (lane 1), transfected with lamin A/C siRNA (lane 2), or transfected with HPV18 E6/E7 siRNA (lanes 3). RNA was harvested at 48 h posttransfection and subjected to HPV18 E6/E7-, DEK-, and GAPDH-specific Northern blot analysis.

small interfering RNA (siRNA) (E7 nucleotides 112 to 132) for repression of HPV E6/E7 mRNA levels.

We tested the functional consequences of our E6/E7 RNAi approach using short- and medium-term growth as well as senescence assays. HPV18-positive HeLa cells and HPV-negative 293 cells as controls were mock transfected or transfected with E6/E7 siRNA. Equal cell numbers were plated, and cell counts were performed on five consecutive days. One representative experiment exhibiting significant growth arrest in response to E6/E7 RNAi is shown in Fig. 2A. Average numbers of cell divisions within 5 days are represented on the right for four independent experiments. Whereas control HeLa cells divided 3.6 times within 5 days, the number of E6/E7 siRNA-transfected cells did not increase over the observed time period. No differences in growth rates were observed with HPV-negative 293 cells in the presence versus the absence of siRNA. Colony assays were performed subsequently in order to test medium-term growth effects resulting from E6/E7-specific RNA interference (Fig. 2B). HeLa or 293 cells were transfected with E6/E7-specific siRNA along with a neomycin resistance plasmid, and the cells were split and selected in G418. Control cells were transfected with neomycin plasmid alone. Colonies were fixed, stained with methylene blue, and counted at 2 weeks posttransfection. We observed a dramatic reduction in colony formation in the presence of E6/E7 siRNA compared to a minor reduction in the 293 control cells. For senescence assays (Fig. 2C), HeLa cells were either mock transfected or transfected with E6/E7 siRNA along with the neomycin resistance plasmid. Morphological changes similar to E2-expressing senescent cells were observed as early as 3 days postselection. Positive staining for SA–β-Gal activity was obtained after 2 weeks of selection (Fig. 2C, E6/E7 RNAi) and was not observed in transfections with the neomycin resistance plasmid alone (Fig. 2C, mock) or in the presence of lamin A/C siRNA (data not shown). The above data are in complete agreement with a previous report in which targeting of E7 sequences (nucleotides 142 to 160) via RNAi was shown to silence both

oncogenes and to cause senescence induction in HeLa cells (20).

We next performed Northern blot analyses in order to determine whether DEK expression is affected by HPV18 E6/E7-specific RNA interference (Fig. 2D). HeLa cells were either mock transfected (lane 1) or transfected with lamin A/C control siRNA (lane 2) or E6/E7-specific siRNA (lane 3). Total RNA was harvested at 48 h posttransfection. Equal amounts of RNA were subjected to Northern blot analysis using an HPV18 E6/E7-specific probe. Transfection with E6/E7 siRNA (lane 3) resulted in reduced levels of E6/E7 messages compared with lamin A/C (lane 2) or mock-transfected HeLa cells (lane 1). In order to ask whether direct interference with E6/E7 expression is sufficient for the observed E2-TA-mediated DEK repression, we next monitored DEK message levels following E6/E7 RNAi. DEK mRNA levels were suppressed by E6/E7 siRNA (lane 3), but not by lamin A/C siRNA (lane 2) or in the absence of siRNA (lane 1). E6/E7-specific RNA interference in HPV-negative 293 cells did not result in DEK repression (data not shown). We concluded that DEK expression in HPV-positive cells was dependent upon the functions of E6 and/or E7.

DEK is a cellular senescence inhibitor. In order to determine whether the observed DEK downregulation was important for cervical cancer cell senescence, we performed DEK overexpression experiments in a temperature-sensitive E2 senescence system. HeLa cells infected with the adenoviral AdE2ts virus undergo senescence at the permissive temperature of 32°C, whereas they remain nonsenescent at the restrictive temperature of 39.5°C or 37°C (53) (data not shown). We generated an AdDEK expression vector and observed that the timing of DEK protein expression following AdDEK HeLa cell infection was slow, albeit clearly detectable after 1 week (Fig. 3A, lanes 1 and 2). In order to ensure DEK expression prior to the point of senescence irreversibility (53), HeLa cells were preinfected with AdDEK virus or empty Ad as a control and reinfected with AdE2ts plus additional Ad and AdDEK virus 4 days after the initial infection as described in Materials and Methods. The cells were then subjected to senescence induction by temperature shift to 32°C. Western blot analysis revealed the expected downregulation of DEK expression upon E2 activation at the permissive temperature (Fig. 3A, compare lanes 3 and 4). AdDEK infection resulted in higher DEK protein levels at both temperatures (compare lanes 3 and 5 and lanes 4 and 6). Whereas all of the AdE2ts-infected control cells incubated at the permissive temperature exhibited the characteristically senescent morphology, DEK expression resulted in a mixture of cells with senescent as well as nonsenescent features, indicating partial repression of the E2 senescence phenotype (Fig. 3B, upper panels). No senescent cells were observed at the restrictive temperature due to the lack of E2 activity (Fig. 3B, lower panels). In agreement with these morphological findings, over 70% of the control cell population were positive for the senescence-specific SA–β-Gal marker at 32°C, with a reduction to 15% upon DEK overexpression (Fig. 3C). These results were consistent with increased colony formation upon DEK expression in this experiment (Fig. 3D). Partial inhibition of E2-induced senescence via DEK overexpression supports our model that DEK is a senescence regulator and not simply a marker. We propose that increased DEK transcript levels in human tumors may reflect its senescence inhibitory function.

DEK repression is important during normal replicative senescence. The establishment of a senescent cell phenotype can be in response to replicative exhaustion via telomere shortening or in response to various types of cellular stress. In order to determine whether DEK repression is unique to E2 senescence or whether it is also observed during the replicative senescence of primary cells, we established primary keratinocyte and fibroblast cultures from two different donors and measured DEK protein levels over the course of multiple passages. DEK expression gradually decreased between passage one and five for keratinocytes, at which time the entire population of keratinocytes had reached a senescent state as revealed by SA–β-Gal staining (Fig. 4A, top panels). This decrease in DEK protein levels corresponded to a decrease in DEK mRNA levels as determined by Northern blot analysis (data not shown). DEK levels did not decrease in the corresponding fibroblasts, which unlike keratinocytes showed no sign of senescence at passage five. The onset of fibroblast senescence was first observed at passage 20 with the appearance of flat cells and was complete at passage 25, when cellular growth had ceased completely (Fig. 4A, bottom panels). DEK protein expression was substantially reduced at passage 20 compared to earlier passages and was undetectable at passage 25. Again, SA–β-Gal staining correlated with the senescent phenotype. These data support the notion that DEK repression may be universally associated with cellular senescence. In order to determine whether DEK regulation may be important for replicative senescence, we infected primary keratinocytes with either empty Ad or AdDEK vector and monitored the number of passages achieved (Fig. 4B). Cells were either infected twice at two consecutive passages (experiment 1) or infected at every split (experiment 2). Life span termination at passage 4 following infection with empty Ad in both cases was extended to reach 12 and 10 passages, respectively, in response to AdDEK infection. As expected, DEK protein expression was substantially increased in the AdDEK-infected compared to empty Ad-infected cells over the course of multiple passages (Fig. 4B). These data support the notion that DEK repression is associated with and importantly contributes to the induction of replicative senescence in human cells.

DEK is transcriptionally activated by high-risk HPV E7 through a pathway dependent upon the retinoblastoma tumor suppressor protein family. The fact that DEK downregulation was observed following the repression of E6 and E7 suggested that DEK may be an upregulated target of the viral immortalizing oncogenes. Expression of the high-risk, but not the low-risk, HPV E6 and E7 proteins allows primary cells to efficiently bypass senescence towards immortality. In order to determine whether DEK expression was induced by E6 and/or E7, we transduced primary human keratinocytes with retroviral vectors expressing E6 and E7 individually or together and monitored DEK mRNA and protein levels. Northern blot analysis revealed increased levels of DEK mRNA in response to E7 and E6/E7 transduction, but not in response to empty vector or E6 transduction alone (Fig. 5A, top panel, lanes 1 to 4). DEK mRNA induction by E7 was associated with upregulated protein levels. Since infection with the E6-expressing retrovirus did not affect DEK expression, we ensured its functionality

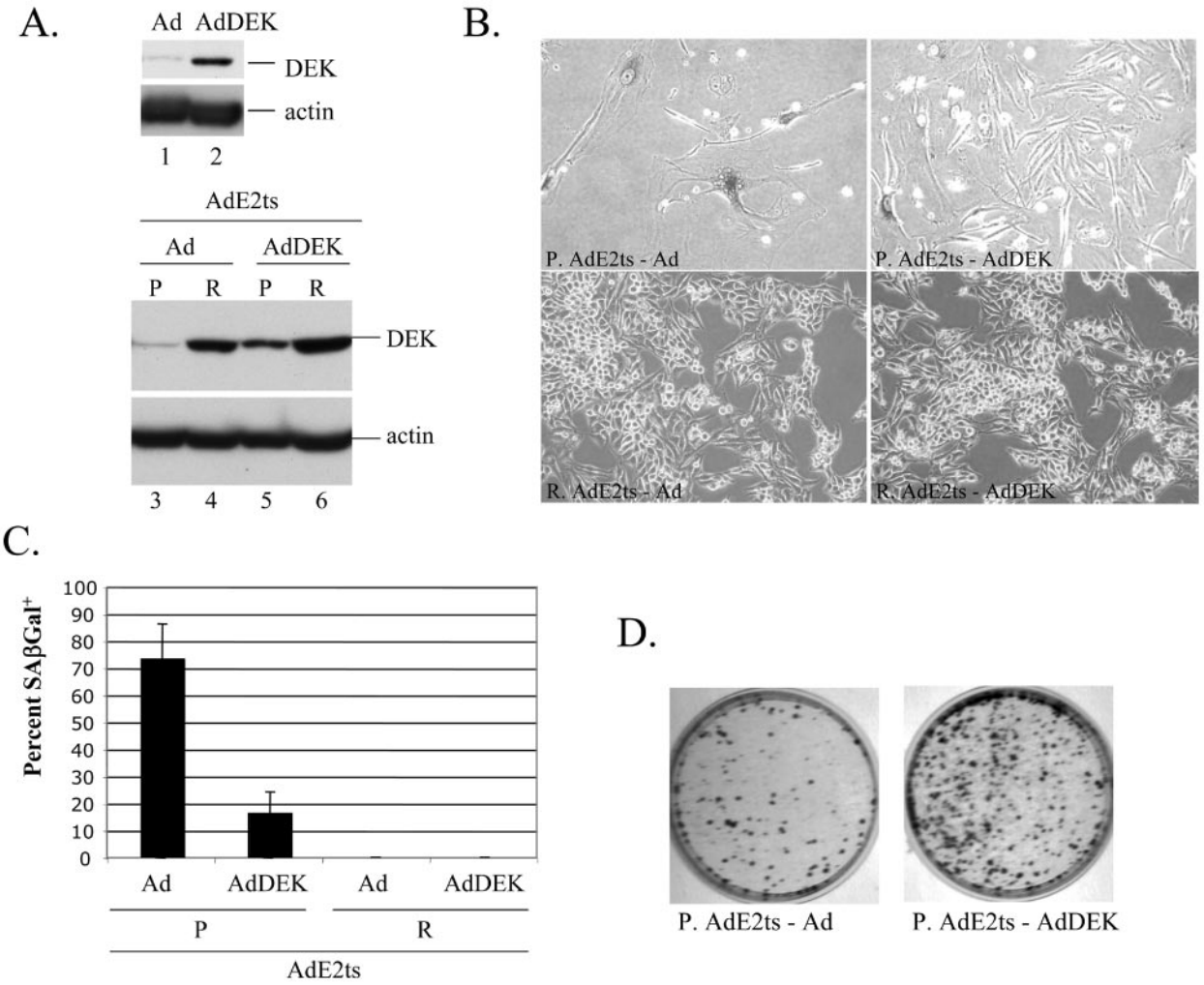

FIG. 3. DEK is a senescence modulator. (A) HeLa cells were infected with empty Ad or AdDEK (lanes 1 and 2). HeLa cells were serially infected with either empty Ad or AdDEK and AdE2ts as described in Materials and Methods (lanes 3 to 6). Senescence was assessed after temperature shift to 32°C, which activates E2. P, permissive temperature; R, restrictive temperature. Protein samples were prepared at 7 days post-temperature shift and subjected to Western blot analysis. (B) Infected cells were photographed at 2 weeks post-temperature shift. (C) SA–β-Gal–positive cells were counted within three independent fields and averaged. (D) Colony reduction assay. HeLa cells were infected with either empty Ad or AdDEK and AdE2ts as above, and the appearance of colonies was assessed by staining with methylene blue at 2 weeks after temperature shift.

using p53 protein levels as a readout. Reduced p53 levels following transduction with E6 or E6/E7, but not with empty vector or E7 alone, demonstrated the functionality of E6 in these experiments. Finally, we examined whether E7 protein encoded by a low-risk HPV could also induce DEK (Fig. 5B). Primary HFKs were transduced with either empty retroviral vector or HPV6b E7- or HPV16 E7-expressing vector, and DEK protein levels were determined by Western blot analysis (upper panels, lanes 1 to 3). DEK induction was only observed in response to HPV16, but not to HPV6b E7 expression. Both genes were efficiently expressed, as verified by Northern blot analysis, following infection of HeLa cells and hybridization with either HPV16 E7 or HPV6b E7 probe (lower panels, lanes 1 to 3).

Based on a high degree of conservation between the mouse and human DEK genomic structure and putative promoter sequences, we determined whether E7 could upregulate DEK expression in mouse embryo fibroblasts. Wild-type MEFs were infected with either empty LXSN, E6-, E7-, or E6/E7-expressing vectors and selected in G418, and protein extracts were harvested on day 9 postinfection. Western blot analysis of DEK expression is shown in Fig. 5C, with high levels of DEK expression in untreated HeLa cell controls displayed in lane 1 as a marker. Low DEK protein levels in MEFs in the presence of empty or E6-expressing vector (lanes 2 and 3) were significantly increased by E7 expression, either in the absence or in the presence of E6 (lanes 4 and 5). Since one of the distinguishing features of high-risk compared to low-risk HPV E7 is its ability to inhibit the function of retinoblastoma family members by proteasomal degradation, we asked whether the pRB family as a whole was important in the observed regulation of DEK. Triple pRB/p107/p130 knockout MEFs (TKOs) were

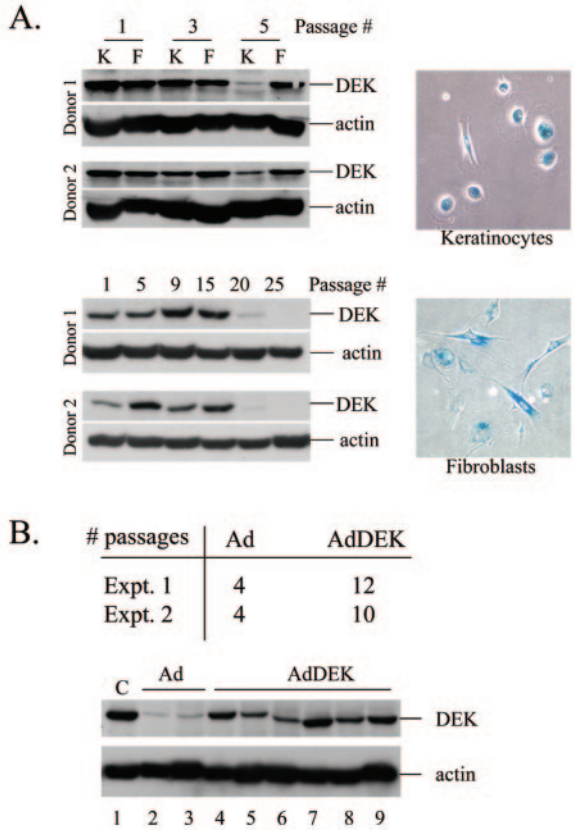

FIG. 4. DEK expression is decreased during replicative senescence of human cells, and ectopic DEK expression extends keratinocyte life span. (A) Keratinocyte (K) and fibroblast (F) cultures were prepared from human foreskin tissue. Protein lysates were prepared at the indicated passage numbers. Equal amounts of total protein were subjected to DEK-specific Western blot analysis. Keratinocytes at passage 5 and fibroblasts at passage 25, respectively, were stained for SA–β-Gal activity and photographed. (B) Keratinocytes were infected with empty Ad or AdDEK at an MOI of 100 and were split when they reached approximately 60 to 80% confluence. For experiment 1, the cells were split 1:4 at every passage and infected twice at passages 3 and 4. For experiment 2, the cells were split to 5×10^5 cells per 10-cm plate at every passage and infected after each split, starting at passage 3. Passage numbers were recorded. Western blot analysis for experiment 2 is shown underneath. A portion of the cells was removed at every split and was subjected to DEK-specific Western blot analysis.

infected with the above retroviral vectors. Empty and E6 vector infection of TKOs compared to E6 infection of control cells revealed strongly upregulated basal DEK expression, which was not significantly upregulated further in response to HPV16 E7 transduction. These findings suggest that both basal and E7-induced regulation of DEK expression is controlled by the pRB protein family. The transduction of wild-type MEFs with a panel of mutant E7 constructs will test our prediction that pocket protein binding-deficient E7 proteins will be unable to upregulate the expression of DEK. In agreement with a potential direct repression of DEK expression by the pRB family is the presence of two E2F transcription factor binding sites near the transcriptional start site that are conserved between the mouse and human DEK gene. We are currently determining whether the presence of these E2F binding sites is required for DEK regulation.

DISCUSSION

Our results identify the human DEK proto-oncogene as a senescence inhibitory molecule and transcriptional target of the high-risk HPV E7 proteins. In vitro studies have implicated DEK as an architectural DNA binding protein with a distinct specificity for supercoiled and cruciform DNA (49). Two independent DNA binding domains exist within the protein, and the ability of DEK to interact with DNA, as well as to multimerize, can be modulated in vitro by phosphorylation (27, 28). DEK can induce positive supercoiling of protein-free as well as chromatinated DNA (50), stimulate the intermolecular joining of linear DNA molecules in the presence of DNA ligase, and convert DNA circles into catenated DNA in the presence of topoisomerase II (49). DEK has been implicated in interactions with viral pathogens, including Kaposi sarcoma-associated herpesvirus (31) and human immunodeficiency virus type 2 (15), and with cellular processes, including transcriptional control (4, 23), chromatin remodeling (1, 50), and mRNA processing (37). We propose that DEK induction during cervical as well as HPV-unrelated carcinogenesis, perhaps via its described association with and modulation of chromatin, contributes to an extended proliferative life span and thus elevated risk of carcinogenic progression over time. It is also possible that DEK overexpression may have direct effects on known positive or negative modulators of keratinocyte immortalization, such as human telomerase or p53 (29, 38). A molecular characterization of DEK-overexpressing keratinocytes together with classical transformation experiments should aid in the identification of specific DEK-regulated oncogenic signaling pathways.

Although first identified as a fusion protein in a subset of AML types, upregulated transcript levels of the human DEK oncogene have been reported for a variety of human tumors, including hepatocellular carcinoma, glioblastoma, and melanoma, tumors with a generally aggressive phenotype. Regarding the clinical significance of DEK induction in cervical cancer, it will be important to examine levels of DEK expression in human premalignant and malignant HPV-positive tissue samples. It is possible, considering DEK's responsiveness to high-risk HPV E7 expression in primary human keratinocytes, that upregulated levels of DEK may be useful in the diagnosis of high-risk HPV infection. Based on the observed pRB family-mediated repression of DEK expression and on the fact that retinoblastoma tumor suppressor pathways are disrupted in most human cancers, we speculate that upregulated DEK expression may turn out to be a frequent hallmark of human carcinogenesis. Whether DEK targeting strategies may apply to future therapeutic approaches will await studies of the role and requirement for the observed low DEK expression in primary cells.

Little is known regarding the mechanism of transcriptional DEK activation during carcinogenesis, although studies of the proximal 500-nucleotide promoter region have implicated YY1 and NF-Y binding sites in the high constitutive DEK expression in a T-cell lymphoma and glioblastoma cell line (44). Using the respective promoter construct in luciferase assays, we did not observe repression of the 500-bp DEK promoter in response to E2-TA in HeLa cells (data not shown). Distinct mechanisms may be involved in DEK induc-

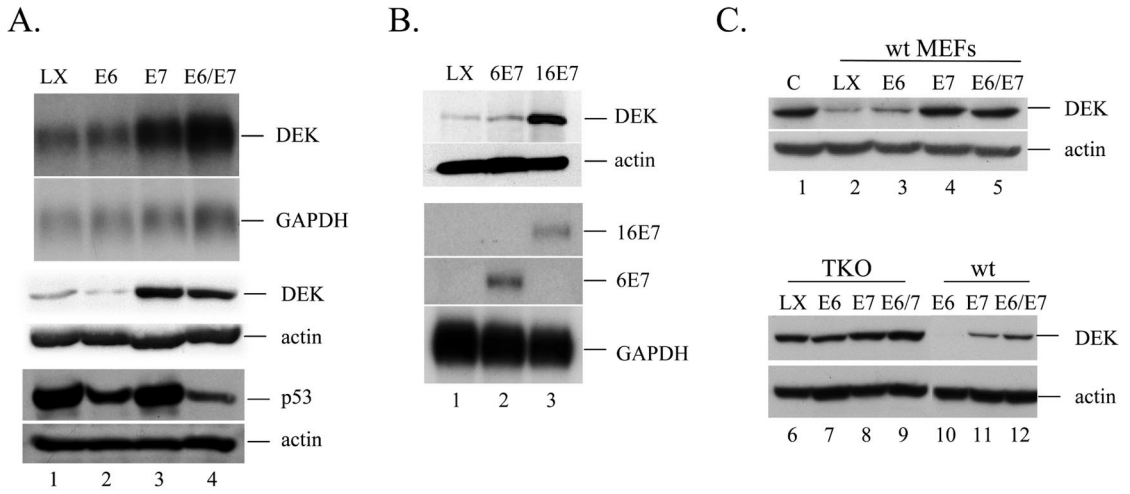

FIG. 5. DEK is an induced target of HPV16 E7 in primary human keratinocytes and is regulated by members of the retinoblastoma protein family. (A) HFKs were transduced with either empty retrovirus (lane 1) or retroviral vectors expressing HPV16 E6 (lane 2), E7 (lane 3), or E6/E7 (lane 4). Cells were selected in G418, and total RNA was harvested after 6 days. Northern blot analysis was performed to analyze DEK mRNA expression. GAPDH expression was analyzed as a loading control. Protein lysates were harvested after 7 days. Western blot analysis was performed using DEK- and p53-specific antibodies, and actin expression is shown as a loading control. (B) HFKs were transduced with either empty retrovirus (lane 1) or HPV6b E7- (lane 2) or HPV16 E7- (lane 3) expressing retroviral vectors. Cells were selected in G418, and protein lysates were harvested after 7 days for DEK- and actin-specific Western blot analysis (top panels). HeLa cells were infected with the above retroviruses and selected in G418 for 10 days. Total RNA was harvested, and 10 μg of each RNA was subjected to either HPV16 E7-, HPV6b E7-, or GAPDH-specific Northern blot analysis (lower panels). (C) Wild-type MEFs were infected with either empty LXSN retroviral vector (lane 2) or HPV16 E6- (lane 3), E7- (lane 4), or E6/E7- (lane 5) expressing retroviruses. The cells were selected in 500 μg/ml G418 for 9 days prior to protein lysate preparation and Western blot analysis. Equal amounts of total protein were subjected to Western blot analysis using either DEK- or actin-specific antibodies. Lane 1 contains HeLa cell protein extract as a positive control. Triple pRB/p107/p130 knockout MEFs in comparison with controls were infected with empty versus oncogene-expressing retroviruses (lanes 6 to 12). The cells were selected, harvested, and subjected to Western blot analysis.

tion in different malignancies or, alternatively, differences may exist in the regulation of the endogenous DEK promoter compared to heterologous promoter plasmids. What might be the molecular mechanism underlying the observed E7-mediated DEK induction? The observed lack of E7-mediated DEK induction in TKO cells strongly suggests that DEK transcription is modulated by at least one member of the retinoblastoma protein family. It is important to emphasize, however, that the lack of regulation of DEK protein expression during cell cycle progression appears to suggest additional levels of control. It will be important to follow DEK mRNA expression during cell cycle progression in a similar fashion in order to identify or exclude regulatory mechanisms that may apply in addition to pocket protein regulation. These studies will be complemented by the identification of the relevant *trans*-acting regulatory factors, which will involve detailed analyses of the respective *cis*-acting sequences within the DEK promoter.

ACKNOWLEDGMENTS

We thank Denise Galloway for the E6/E7 retroviral producer cell lines, Gerard Grosveld for his generous gift of polyclonal DEK antiserum, James Lessard for the monoclonal actin antibody, and David Markovitz for the −496 DEK promoter construct. We thank David Williams, Yi Zheng, Hartmut Geiger, James Mulloy, Patrick Hearing, and Erik Knudsen for many helpful discussions and critical reading of the manuscript.

This research was supported by an American Cancer Society Institutional grant to S.I.W. (92-026-09), by the American Cancer Society Ohio Division Supported Research grant for the year 2002 to S.I.W., by a Trustee grant from the Cincinnati Children's Hospital Research Foundation to S.I.W., and by a grant from the Ohio Cancer Research Associates to S.I.W.

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