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Lindy Gewin

Investigation of the mechanism by which the human papillomavirus type-16 E6 oncoprotein induces telomerase in epithelial cells

Lindy Gewin

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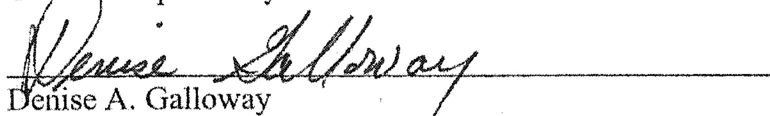

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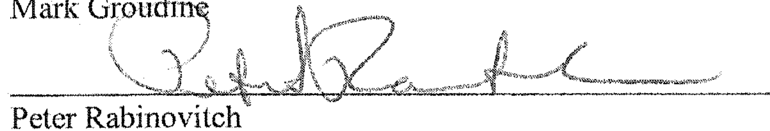

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Abstract

Investigation of the mechanism by which the human papillomavirus type-16 E6 oncoprotein induces telomerase in epithelial cells

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The human papillomavirus (HPV) type-16 E6 oncoprotein activates telomerase in human epithelial cells by inducing expression of *hTERT*, the catalytic and rate-limiting subunit of telomerase. Another well established function of E6 is to bind the cellular E6-associated protein (E6-AP) to form an E3 ubiquitin ligase that targets p53 for ubiquitination and degradation via the proteasome. Telomerase activation, rather than p53 degradation, is essential for the immortalizing activity of E6 in epithelial cells. In our investigation into the mechanism by which E6 induces *hTERT* expression we found that the E boxes (c-Myc/Max binding sites) were required for promoter activation. c-Myc protein levels, however, did not change upon E6 expression and c-Myc protein was localized to the endogenous *hTERT* promoter regardless of promoter activity. Therefore, though c-Myc may be necessary for *hTERT* induction it is not sufficient. Activation of *hTERT* was associated with increased levels of acetylated histones H3 and H4 at the *hTERT* promoter upon E6 expression indicating that chromatin modifications are involved. Furthermore, analysis of several E6 mutant proteins revealed a strong correlation between the ability of E6 to bind E6-AP and its ability to induce *hTERT*. Using shRNAs to reduce *E6-AP* expression, we demonstrated that E6-AP is required for E6-mediated telomerase induction. In a search for new targets of E6/E6-AP involved in telomerase activation, we identified NFX1-91, a transcriptional repressor of the *hTERT* promoter in reporter assays. The stability of NFX1-91 is reduced in cells expressing E6 in a proteasome-dependent manner. Evidence that NFX1-91 repressed the endogenous *hTERT* promoter was found by decreasing expression of *NFX1-91* with shRNAs resulting in derepression of the *hTERT* promoter and elevated levels of telomerase activity in primary human keratinocytes. We propose that the induction of telomerase by HPV-16 E6 requires binding to cellular E6-AP and involves targeting of NFX1-91, a newly identified repressor of telomerase, for ubiquitination and degradation.

TABLE OF CONTENTS

Page

List of Figures ii

List of Tables iii

Chapter 1: Introduction
Immortalization and telomerase 1
Telomerase and telomere structure 4
The senescent response to disrupted telomere structure 5
HPV and Immortalization 8

Chapter 2: E-box dependent induction of hTERT by HPV-16 E6
Introduction 13
Results 14
- Expression levels of c-Myc do not correlate with telomerase activity 14
- Both E6 and c-Myc require intact E boxes to activate the *hTERT* promoter 16
- c-Myc and histone acetylation at the endogenous *hTERT* promoter 18

Discussion 19

Chapter 3: E6-AP is required for induction of telomerase by E6
Introduction 25
Results 26
- Ability of E6 to activate telomerase correlates with ability to bind E6-AP 26
- E6-AP is required for telomerase activation by 16E6 27
- Search for new targets of E6/E6-AP 28

Discussion 30

Chapter 4: Identification of a novel telomerase repressor by virtue of its interaction with the human papillomavirus type-16 E6/E6-AP complex
Introduction 36
Results 38
- Differential repressor and activator functions of the NFX1 isoforms on the *hTERT* promoter 38
- E6 preferentially binds and destabilizes NFX1-91 rather than NFX1-123 40
- NFX1-91 is highly ubiquitinated in the presence of E6 42
- *In vivo* evidence for NFX1-91 as a repressor of *hTERT* 43
- Requirement for E6-AP and role for NFX1-91 in telomerase activity of HeLas 45

Discussion 47

Chapter 5: Conclusions and Future Directions 58

Chapter 6: Materials and Methods 68

Bibliography 77

LIST OF FIGURES

Number Page

1.1 Telomere structure and telomere binding proteins 11

1.2 Organization of the HPV genome 12

2.1 c-Myc expression levels do not correlate with telomerase activity in HFKs 22

2.2 Myc-Max binding sites are required for activation of *hTERT* by either c-Myc or E6 23

2.3 c-Myc and acetylated histones at the endogenous *hTERT* promoter 24

3.1 Induction of *hTERT* expression correlates with the ability of E6 to bind E6-AP.. 33

3.2 E6-AP expression is required for telomerase induction by HPV-16 E6 34

3.3 The E6/E6-AP yeast two-hybrid screen 35

4.1 Alignment of NFX1 homologs from different eukaryotic species 51

4.2 NFX1 isoforms have differential repressive and coactivating functions in hTERT reporter assays 52

4.3 NFX1-91 rather than NFX1-123 is targeted by the E6/E6-AP complex 53

4.4 NFX1-91 is ubiquitinated by E6-independent and E6-dependent means 54

4.5 Knockdown of NFX1-91 expression using shRNAs derepresses the endogenous *hTERT* gene in HFKs 55

4.6 Senescence-associated β-galactosidase activity in late passage HFKs 56

4.7 Telomerase activity of HeLa cells is affected by reduced expression of either *E6-AP* or *NFX1-91* 57

5.1 Models of the *hTERT* promoter in the absence and presence of E6 expression.... 67

LIST OF TABLES

Number Page

3.1 Telomerase activation and E6-AP binding of E6 proteins 32

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CHAPTER 1

INTRODUCTION

Human papillomaviruses (HPV) are small double-stranded (ds) DNA tumor viruses that infect the epithelium of the skin or mucosa. There are nearly 100 different HPV types identified by their DNA sequence. Infection with many of these leads to benign lesions, or warts, which regress upon activation of a cell-mediated immune response. Some types of HPV, however, infect the epithelium of the genital mucosa, establish a latent infection, and cause lesions that may progress to invasive carcinoma. These types are known as the "high risk" HPV types with the two most prevalent being HPV-16 and HPV-18 (66). We are primarily interested in understanding the molecular mechanisms involved in immortalization and transformation of epithelial cells by the "high risk" HPV-16.

Immortalization and telomerase

Immortalization is a key step in the process of transformation (34,53). One of the primary barriers to immortalization arises due to the "end replication problem" (48). As cells with linear chromosomes replicate, the removal of the most 5' RNA primer leaves a 3'overhang that DNA polymerase cannot fill. Over many divisions the chromosome ends will gradually shorten (56,146). To avoid losing critical genetic information, eukaryotic organisms have evolved repetitive, noncoding sequences at the ends of chromosomes known as telomeres. In humans, the telomeric repeats are 10-15 thousand base pairs of TTAGGG sequence that is lost at rate of approximately 50-100 bp per generation (24,56,91,146). The gradual shortening of telomeres is proposed to impose a molecular

clock of cellular lifespan (56,91); originally defined as the Hayflick limit of replicative capacity (59).

Most somatic cells do not need to divide indefinitely; in fact, shortening telomeres are thought to be a natural barrier to the uncontrolled cell growth of tumor cells. Stem cells and germ cells, however, do require a high replicative capacity. These cells express a ribonucleoprotein complex called telomerase which functions as a reverse transcriptase to extend shortened telomeres. Two subunits of telomerase are required for catalytic activity: an RNA template encoded by the *hTR* gene and a catalytic subunit encoded by the *hTERT* gene (74,104,108). Expression of *hTERT* is the primary mode of regulating telomerase activity in cells (22,104,108,109). In fact, exogenous expression of *hTERT* is sufficient to induce telomerase activity in many cell types (13,22,78,145).

Mechanisms regulating the transcriptional activity of the *hTERT* promoter are of significant interest to researchers studying cancer biology and stem cell biology. Though most human somatic cells do not express *hTERT*, it is detectable in stem cells and germ cells (16,75,127). The majority of human tumors also express high levels of telomerase (75,81). Although hTERT can be subject to post-transcriptional and post-translational mechanisms of regulation (reviewed in (1), (155)), it is primarily regulated at the transcriptional level. The *hTERT* promoter region lies within a CpG island, yet promoter methylation does not appear to be the primary mode of silencing *hTERT* expression in telomerase negative cells (28,29). Chromatin conformation, on the other hand, does seem to play a key role in *hTERT* activity. Several studies have demonstrated that inhibition of

histone deactylases by trichostatin A in certain telomerase-negative cells can alter the chromatin structure and induce telomerase expression (19,65,139).

The hTERT promoter contains many proposed transcription factor-binding sites. These include two canonical E boxes (Myc/Max binding sites) in the proximal promoter region, numerous SP1 binding sites, an NF-κB binding site and an estrogen-responsive element located about 2500 base pairs upstream of the transcription start site (20,85,138). Binding of c-Myc/Max to the E boxes of the hTERT promoter has been demonstrated *in vitro* (158) and overexpression of c-Myc in several cell types is sufficient to induce hTERT expression (47,149,158). Additionally, HL60 and U937 cells stimulated to differentiate in culture turn off telomerase expression with a switch from c-Myc/Max bound at the hTERT to the repressive Mad/Max (52,159). Some studies have suggested that p53 may function as a transcriptional repressor at the hTERT promoter as overexpression of p53 has been shown to repress *hTERT* expression in some tumor cell lines (71,160). However, inactivation of p53 is insufficient to induce telomerase (53,115), suggesting that suppression of hTERT by high levels of p53 may be a byproduct of cell cycle arrest rather than direct transcriptional regulation.

A recent screen to identify repressors of *hTERT* discovered three different tumor suppressor/oncogene pathways involved in *hTERT* regulation. These were the previously mentioned c-Myc/Mad1 pathway, the TGF-β pathway mediated by SIP1, and the tumor suppressor Menin. Overexpression of *Mad1*, *SIP1*, or *Menin* decreased *hTERT* expression in HeLa cells (93). Strikingly, reduced expression of any one of these *hTERT* repressors with siRNA was sufficient to induce *hTERT* expression in cells. These data

suggest that the repressors are not redundant; rather, they are independent. Oncogenic stimulation that abrogates any one of them is sufficient to relieve repression.

Though it remains unclear what the critical determinants of *hTERT* transcriptional activity are, it is apparent that this is a highly regulated gene influenced by many pathways in the cell. Future research is likely to find that the activity and responsiveness of the *hTERT* promoter is dynamic and variable among different cell types.

Telomerase and the telomere structure

The maintenance of chromosome length by telomerase is critical to the formation of a proper telomere structure. Beyond the thousands of base pairs of repetitive double-stranded telomeric sequence extends a 3' G-strand overhang of approximately 200 nucleotides (61,101,151). Electron microscopy to visualize telomeres revealed that this G-strand overhang actually folds back and invades the double-stranded telomeric repeats to form a T-loop (Figure 1.1)(49). This functions to protect the single-stranded DNA (ssDNA) from cellular nucleases and to prevent the activation of DNA repair pathways responsive to ssDNA (92). In fact, loss or exposure of the G-strand overhang and disruption of the T-loop structure, or telomeric cap, may be the critical signal of replicative senescence (92,100).

While expression of telomerase is a primary determinant of telomere length, the proper structure of the telomere is also critical. The higher order structure of the telomere provides a scaffold for many telomeric binding proteins, some of which are required for maintenance of the telomeric cap. TRF1 and TRF2 are negative regulators of telomere length that bind to the double stranded (ds) telomeric repeats and stabilize the T-loop

structure (17,134). Recent data suggests that TRF2 binding and maintenance of the T-loop structure protects the 3' overhang from the endonuclease activity of the telomere-associated ERCC1/XPF endonuclease. Also involved in protection of the 3' overhang, hPOT1 (protection of telomeres 1) binds specifically to the single-stranded 3' overhang (Figure 1.1)(9,90,111). The binding of hPOT1 to the telomere is dependent of the amount of ss DNA and the amount of TRF1, which, in turn, is proportional to the amount of ds telomeric DNA. High levels of hPOT1 localized at the telomere are proposed to inhibit further extension of telomeres by telomerase (95).

In addition to telomere-specific binding proteins, a number of DNA repair proteins are also found associated with telomeres. These include the Mre11, Rad50, and Nbs1 (MRN) complex as well as Ku70, Ku86, DNA-PKcs, PARP1 and the Bloom's syndrome (BLM) DNA helicase (reviewed in (45)). Additionally, disruption of progeroid syndrome genes such as the ataxia telangiectasia mutated gene (ATM) or the Werner's syndrome gene (WRN) results in accelerated telomere shortening and telomere dysfunction. Recently, an interaction of WRN, a RecQ helicase, with TRF2 has been proposed to have a role in accurate processing of telomeric structures (99).

The senescent response to disrupted telomere structure

The influence of so many DNA repair proteins on telomere length and structure suggests the paramount importance of maintaining proper telomere structure to the genomic integrity of the cell. Mutation or deletion of many telomere-binding proteins causes chromosomal aberrations that may initiate a senescent or apoptotic response. For example, loss of TRF2 bound to telomeric DNA (using the dominant-negative TRF2 $^{\Delta B \Delta M}$

allele), leads to a rapid induction of apoptosis in some cell types (73) or a senescent phenotype in primary fibroblasts (131). This appears to result from a rapid loss of the 3' G-strand overhang thereby disrupting T-loop formation and subsequently leading to chromosome end-to-end fusions in a DNA ligase IV-dependent manner (131). Such chromosome fusions result in dicentric chromosomes that can cause further genomic instability via the breakage-fusion-bridge cycle (4,144).

A similar senescent phenotype is observed in tissue culture cells after continual passaging. Expression of hTERT in normal human fibroblasts is sufficient to bypass senescence (13). Recent studies confirm that telomere-initiated senescence results from initiation of a DNA damage checkpoint (30,35). As telomeres erode, the telomeric structure is disrupted and cells begin to express markers of a damage response to ds DNA breaks. Nuclear foci of DNA repair and DNA damage checkpoint factors are much more abundant in senescent cells than in proliferating, quiescent, or age-matched proliferating cells expressing telomerase. Chromatin-immunoprecipitations analyzed with whole genome microarrays reveal that these foci predominate at chromosome ends, both in telomere-eroded cells and in TRF2$^{\Delta B\Delta M}$ telomere-disrupted cells (30). The initiated DNA damage checkpoint involves the ATM and ATR pathways that subsequently signal to downstream transducer kinases CHK1 and CHK2. Disruption of this pathway by microinjection of kinase-dead versions of these proteins into senescent cells is sufficient to stimulate S-phase progression of some cells (30). Therefore, activation of the DNA damage checkpoint by disrupted telomere structure appears to be responsible for the senescent arrest of fibroblasts extensively passaged in culture.

Mouse models to study age-related diseases and cancer predisposition further reveal the important roles of telomere integrity and DNA repair pathways to genome maintenance and lifespan. Deletion of either Ku80 or ERCC1, both implicated in telomere structure maintenance, causes a decrease in the lifespan of the mouse and increased cellular senescence (reviewed in (57)). Mice are unlike humans in that the hTERT subunit is constitutively expressed and mice have extremely long telomeres (63,118). Knockout of the telomerase RNA component (mTR) has no apparent phenotype until the third or fourth generation due to the extremely long telomeres present in mice (12). In later generations, as the telomeres have progressively shortened, $mtr^{-/-}$ mice have a decreased lifespan and a premature aging phenotype accompanied by increased cellular senescence. Additionally, these mice have an increased yet latent incidence of lymphoma and teratocarcinoma that may be attributed to higher genomic instability (89,122). Progeny from the $mtr^{-/-}$ mice crossed with p53 heterozygous and homozygous null mice exhibit decreased tumor latency in both cases. Though epithelial carcinomas are rarely observed in mice, the $mtr^{-/-}$, $p53^{+/-}$ mice developed epithelial carcinomas as well as lymphomas and sarcomas (47). As p53 is a downstream target of the ATM DNA damage checkpoint pathway, the effect of ATM deletion in telomerase deficient mice was examined. Interestingly, deletion of the ATM gene in an $mtr^{-/-}$ background exacerbates the aging phenotype (156). In summary, several mouse models of cancer and aging support the idea that intact DNA damage checkpoints are critical to guard against genomic instability initiated by dysfunctional telomeres.

Mouse models, however, do not appear to be the ideal system to study the senescent response to telomere dysfunction. First, the extremely long telomeres present in mice necessitate the use of late generation *mtr*$^{-/-}$ mice to study telomere shortening. Second, mice and humans appear to employ different signaling pathways to initiate senescence in the presence of dysfunctional telomeres (130). In human epithelial cells, telomere-induced senescence is mediated by both the p53 and Rb/p16 pathways as disruption of both pathways is required to bypass the senescent arrest (31,78,130). Mouse cells, however, respond to telomere dysfunction only through the p53 pathway; the Rb/p16 pathway is not initiated (130). Notably, the p53 and Rb/p16 pathways are the primary targets of small double-stranded DNA tumor viruses, such as HPV.

HPV and Immortalization

The HPV genome encodes 7 early genes (E1-E7) and 2 late genes (L1 and L2, see Figure 1.2). The L1 and L2 genes encode the viral capsid proteins while the E1 and E2 genes are involved in efficient DNA replication with E2 being primarily a transcriptional regulator (reviewed in (162). E6 and E7 are the viral oncogenes responsible for the immortalizing activities of HPV-16. Interestingly, the expression of these genes in a productive infection requires a stratified epithelium. The early genes are transcribed in the basal layers of the epithelium and as cells are pushed up into the differentiated epithelial cell layers, the virus stimulates the host cell machinery to replicate the viral genome. The viral capsid proteins are synthesized only in the upper differentiated epithelial cell layers where the genome is packaged and sloughed off with the cornified layers of the epithelium.

The role of E6 and E7 is to stimulate the host replication machinery in the otherwise quiescent suprabasal layers of the epithelium. E7 functions to drive cells into S phase by abrogating the Rb/E2F pathway that normally represses transcription of many S phase genes. E7 disrupts the association of Rb and E2F and targets Rb for degradation via the proteasome (14,60,106), while E6 binds to a cellular protein known as E6-associated protein (E6-AP) to form an E3 ubiquitin ligase which ubiquitinates p53 and targets it for degradation via the proteasome (124,125). Therefore, in order to replicate the viral genome, E6 and E7 allow cells in the suprabasal layers of the epithelium to aberrantly enter S phase without triggering a p53-dependent arrest response.

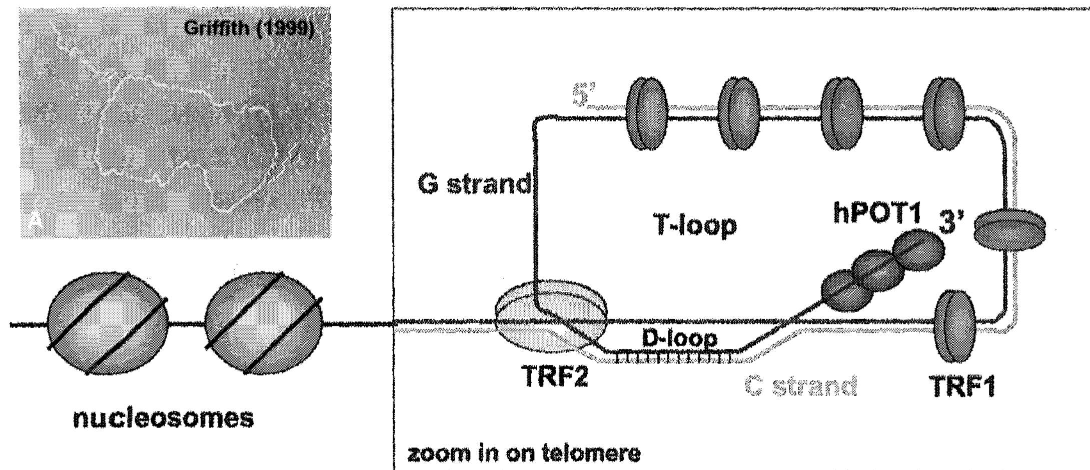
In cervical carcinomas, expression of the E6 and E7 genes are retained whereas most other HPV genes are not. This is generally due to integration of the HPV genome into the host genome. Upon integration, the E2 coding region is usually disrupted (Figure 1.2). The loss of E2 expression causes increased levels of expression of both E6 and E7, normally repressed by E2. In fact, expression of E2 in HPV-positive tumor cell lines represses expression of E6 and E7 and results in a G1 cell cycle arrest and induction of senescence or, in some cases, apoptosis (27,43,87,152).

Many studies have demonstrated that expression of both E6 and E7 is required for efficient immortalization of epithelial cells (58,78). Immortalization requires both disruption of the Rb/p16 pathway and induction of telomerase (31,78). Commonly disrupted in cancers, the Rb/p16 pathway is abrogated in tumor cells by disruption of the Rb/E2F complex (15,60,157) or by loss of p16 expression via methylation of the p16 promoter (40) or via deletions in the CDK2NA/INK4A locus (31). The mechanisms

behind telomerase induction are less well understood, but expression of HPV-16 E6 is sufficient to induce telomerase activity in epithelial cells (80). While telomerase induction is essential, degradation of p53 by E6 is not required for immortalization (78).

Several viruses and bacterial pathogens are proposed to be involved in the development of human cancers. Thus far the clearest relationship between infection and increased risk of cancer is HPV, the initiating factor in the majority of cervical carcinomas. Interestingly, through the combined action of two oncoproteins, E6 and E7, the virus disrupts or activates many of the pathways implicated in tumorigenesis. However, infection with a "high risk" HPV remains only a risk factor for development of cervical cancer. Progression from infection to invasive cervical cancer generally occurs over long latent period of ten to twenty years (66). The latency suggests that HPV is an initiating event for subsequent genetic changes that may eventually give rise to an uncontrolled, rapidly dividing cell. Therefore, study of the molecular targets of the HPV oncoproteins provides insight about critical regulators of cellular growth control.

The goal of this project is to identify the mechanism by which the HPV-16 E6 oncoprotein induces telomerase activity in human epithelial cells.



adapted from Neidle and Parkinson (2003) *Current Opinion in Structural Biology*

Figure 1.1. Telomere structure and telomere binding proteins. The inset shows an electron micrograph of a telomere from a HeLa cell (Griffith 1999). The schematic depicts a model of the telomere structure and where some telomere binding proteins may function. The free 3' G-rich overhang invades the upstream double-strand (ds) telomeric sequence to form a D-loop (displacement loop) and a T-loop. TRF1 binds to the ds telomeric region. TRF2 is required for formation of the T-loop and may interact with WRN, a RecQ DNA helicase, to form the T-loop. hPOT1 is thought to bind to the remaining free 3' overhang or to the displaced G-rich strand of the D-loop and is thought to inhibit access of telomerase to the telomere (Neidle and Parkinson 2003).

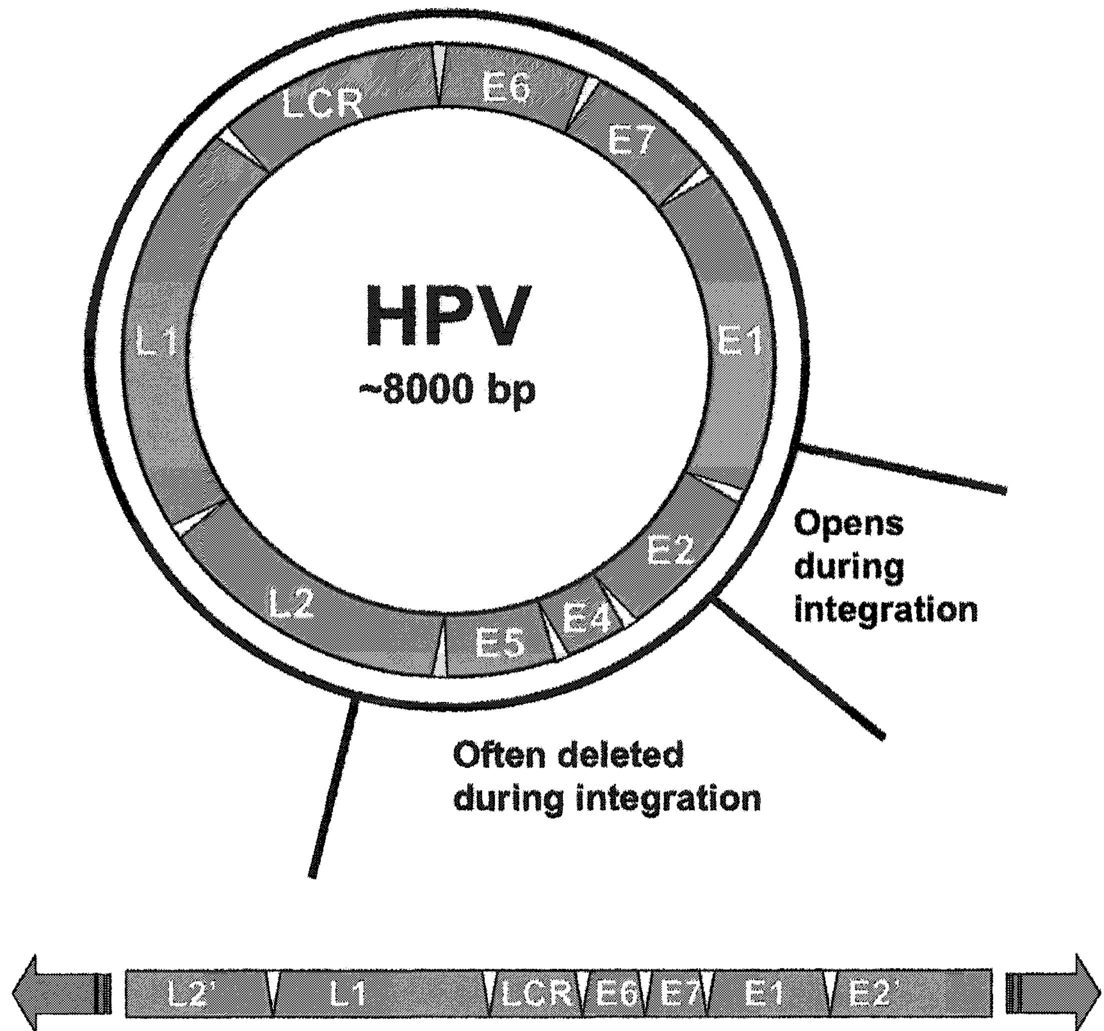


Figure 1.2. Organization of the HPV genome. HPV is a virus with a circular double-stranded (ds) DNA genome encoding eight open reading frames – the early (E) genes are E6, E7, E1, E2, E4, and E5; the late (L) genes are L1 and L2. The LCR, or locus control region, is a transcriptional regulatory region. During a productive infection the virus is maintained as an episome. In cancer cells, however, the viral genome has often integrated into the host genome as depicted in the lower part of the figure. During integration, the genome linearizes by opening in the E1 or E2 coding region and inserts into the host DNA often deleting the E4 and E5 genes and partially deleting the E2 and L2 genes. (Adapted from zur Hausen 2002)

CHAPTER 2

E BOX-DEPENDENT INDUCTION OF hTERT BY HPV-16 E6

INTRODUCTION

Activation of telomerase is a critical step in cellular transformation (34,34,53). Telomerase activity is primarily regulated at the level of expression of the hTERT gene encoding the catalytic subunit of telomerase (22,104,109,151). Ectopic expression of *hTERT* in a number of different telomerase negative cell types has been shown to confer immortality (13,22,22,78,78,145,145). Therefore, much research is now focused on determining the transcriptional regulators of *hTERT*.

The *hTERT* promoter contains a number of putative transcription factor binding sites. Several studies have defined the minimal core promoter as the proximal 200 base pairs upstream of the transcription start site (64,64,138). This core promoter contains numerous SP1 binding sites and two canonical E boxes (Myc-Max binding sites) (20,20,64,64,138,138,154). Previous *in vitro* studies have shown that Myc-Max heterodimers can bind these E boxes in the context of the *hTERT* promoter and can activate *hTERT* reporter constructs (64,64,138,138,158). c-Myc expression has also been shown to induce telomerase activity in post M0 human mammary epithelial cells (HMECs), the fibroblast cell lines IMR90 and WI38 (149), and EBV-immortalized B cells (158). These studies implicate c-Myc as an important transactivator of *hTERT*.

The human papillomavirus (HPV) type-16 E6 oncoprotein can also induce telomerase expression, specifically in epithelial cell types (80). Expression of E6 in

either human foreskin keratinocytes (HFKs) or HMECs induces telomerase activity. Another well-established function of E6 is its association with the cellular E6-associated protein (E6AP) to form an E3 ubiquitin protein ligase that specifically targets p53 for degradation (69,69,70,70,125). Several E6 mutants, such as E6-8S/9A/10T, E6-F2V, and E6-Y54H, are defective for p53 degradation yet retain the ability to activate telomerase, demonstrating that these two functions of E6 are separate and distinct (80,94). Expression of E6 does not induce telomerase in human foreskin fibroblasts (HFFs)(80) or in IMR90s (149). It has been suggested that a cell type-specific ability of E6 to induce c-Myc expression is responsible for this differential telomerase activation (158). In this study, we show that upregulation of c-Myc does not directly correlate with telomerase activation indicating that other regulators of *hTERT* expression are also involved. We also demonstrate that activation of telomerase by E6 does not require upregulation of c-Myc, yet intact E boxes in the *hTERT* promoter are required for E6-mediated transactivation. In addition, c-Myc appears to be present at the endogenous *hTERT* promoter irrespective of the transcriptional status of the promoter. Histone acetylation, on the other hand, is present at the *hTERT* promoter only upon transcriptional activation indicating a chromatin remodeling mechanism for telomerase induction by E6.

RESULTS

Expression levels of c-Myc do not correlate with telomerase activity

To address the mechanism by which E6 activates telomerase in HFKs, we transduced HFKs with retroviruses encoding E6, E6-8S/9A/10T and E6-Δ9-13 (23,38,38,105,105). In addition, the HPV oncogene E7, E6/E7 (55), c-Myc (provided by

R. Eisenman) and vector controls (LXSN and LXSH) were transduced into HFKs. After selection in 50 μg/ml G418 (GIBCO BRL) or 8 μg/ml Hygromycin B (Roche), cells were harvested in parallel for telomeric repeat amplification protocol (TRAP) assay as previously described (78), RT-PCR, and Western blots of nuclear extracts. The transformed cell lines C33A, HeLa, and 293T were also harvested in parallel to serve as positive controls for telomerase activity.

Expression of E6, E6-8S/9A/10T, c-Myc or E6 and E7 activated telomerase as seen in Figure 2.1B. E6 activates telomerase by inducing transcription of the telomerase *hTERT* gene (147) (Figure 2.1C). In each case, induction of telomerase activity was directly correlated with expression of *hTERT* RNA (Figure 2.1C). Overexpression of c-Myc was sufficient to activate telomerase in HFKs. Though c-Myc protein was detected in all cells with active telomerase, the steady-state levels of c-Myc protein in each of these cell lines did not correlate with expression of *hTERT* (Figure 2.1A). Telomerase was activated both with high (lanes 6, 7, 9,10, and 11) and with low (lanes 2 and 3) levels of c-Myc nuclear protein. Conversely, no telomerase activation was detected in the E7-expressing cells, which contain high levels of c-Myc (lane 5). Therefore, activation of telomerase by E6 in HFKs is independent of c-Myc induction. It should be noted that c-Myc expression is elevated in cells actively proliferating and decreased in cells with lower proliferation rates (62). We have observed slightly elevated levels of c-Myc in HFKs expressing E6 and E6 mutants in some experiments (data not shown). However, in those experiments, LXSN-HFKs had undetectable c-Myc expression. Therefore, we

attribute the differences in expression of c-Myc to variation in proliferation rate rather than specific induction of c-Myc by E6.

The induction of c-Myc by E7 was not sufficient to activate telomerase. The increased levels of c-Myc may simply represent a higher proportion of S phase cells in E7-expressing cells. Presumably, E7 induces c-Myc by binding Rb thereby releasing E2F to activate c-Myc in a similar manner as has been demonstrated for SV40 large T antigen (8,33). Given the comparable c-Myc expression levels in lanes 5 and 6 of figure 2.1A, it is perplexing that E7 does not induce *hTERT*. E7 does not inhibit c-Myc-mediated telomerase activation, as shown by the induction of *hTERT* in the E6/E7-expressing cells. Interestingly, in a previous study, Garbe *et al.* found that E7 expression promoted telomerase activation (40). Benzo(a)pyrene treatment of HMECs generated mortal extended life cultures. Subsequent E7 transduction did not immediately activate telomerase, but after 2-4 months of culture, these cells had detectable telomerase activity that gradually increased with further passaging (40). This suggests an epigenetic mechanism of telomerase activation facilitated by E7 (40). Activation of telomerase by E6, on the other hand, is detectable within the first passage after selection, arguing that E6 directly transactivates or affects another regulator of *hTERT* transcription (40,40,80).

Both E6 and c-Myc require intact E boxes to activate the *hTERT* promoter

The *in vivo* expression data suggested that activation of *hTERT* by E6 was independent of c-Myc protein levels. To address whether the c-Myc/Max binding sites are required for activation by E6 and c-Myc, we performed *hTERT* reporter luciferase assays in HFKs. First, two pGL3 luciferase reporter constructs were generated. An

approximately 800 bp region of the *hTERT* promoter (from –710 to +76, the translation start site) was cloned into pGL3-Basic (Promega) from pXP2 constructs provided by K.-J. Wu (158). An identical clone was made with mutations at both proximal E boxes (CACGTG mutated to CACCTG). The constructs were co-transfected with retroviral expression vectors LXSN, LXSN-16E6, and LXSN-c-Myc into HFKs using FuGENE6 (Roche). Cells were harvested 24 hours after transfection and lysates were assayed for luciferase activity (Promega) and total protein concentration (BioRad). As previously published, c-Myc activated the *hTERT* promoter approximately three-fold and activation is dependent on intact E boxes (138,158) (Figure 2.2). Co-expression of E6 activated the *hTERT* promoter two-fold. In comparison to the *in vivo* induction of *hTERT* by E6 seen in Figure 2.1, the rather modest transactivation of this fragment of the *hTERT* promoter by E6 may indicate that the reporter assays do not accurately reflect the endogenous promoter. Nevertheless, E6-mediated activation of the *hTERT* promoter was completely abolished by point mutations within the E box indicating that E6-mediated induction of *hTERT* is also E-box dependent.

Activation of telomerase by E6 is therefore independent of c-Myc expression levels but dependent on intact E boxes within the *hTERT* promoter. This suggests a few possible mechanisms of *hTERT* activation by E6. One, E6 may allow c-Myc to have access to the E boxes either by removing a repressor or by altering the local chromatin structure. Two, E6 may alter the expression of a co-factor that preferentially targets c-Myc to the *hTERT* promoter. Both of these mechanisms may be unnecessary when high levels of c-Myc are present, as suggested by the induction of telomerase by

overexpression of c-Myc. A third possible mechanism involves another unidentified transcription factor that, in the presence of E6, may transactivate the *hTERT* promoter independently of c-Myc.

c-Myc and histone acetylation at the endogenous *hTERT* promoter

The previous data indicate that while the E boxes appear to be required for E6-mediated telomerase induction, the expression levels of c-Myc do not change significantly upon expression of E6. To determine whether c-Myc is differentially bound to the endogenous *hTERT* promoter with E6 expression, chromatin immunoprecipitations were done with HFK/LXSN and HFK/E6 cells. The cells were harvested at equivalent densities to ensure a similar cell cycle profile (as estimated by cyclin A protein levels) and equal expression of c-Myc (data not shown). After crosslinking the proteins to the DNA, immunoprecipitations were performed with a rabbit polyclonal c-Myc antibody or non-specific normal rabbit IgG (Santa Cruz). The DNA bound was extracted and used in radioactive semi-quantitative PCR reactions to detect the *hTERT* promoter and non-specific binding to the *β-globin* gene. As seen in Figure 2.3 (A & B), the amount of c-Myc bound does not differ substantially between HFK/LXSN and HFK/E6 cells. c-Myc is bound to the *hTERT* promoter regardless of E6-expression and promoter activity. The experiment shown is representative of multiple trials with comparable data. Therefore, binding of c-Myc to the *hTERT* promoter is insufficient to induce *hTERT* expression.

Histone acetylation is a common chromatin modification generally associated with regions of active transcription (reviewed in (135)). To determine whether histone acetylation at the hTERT promoter is affected by E6 expression in HFKs, we repeated the

experiments described above with immunoprecipitations of acetylated histones H3 and H4 (Figure 2.3 C & D). Clearly, acetylated histones are enriched at the hTERT promoter in E6-expressing HFKs versus vector control cells. The presence of acetylated histones strongly suggests that E6 expression influences histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity at the hTERT promoter and thereby stimulates transcriptional activity.

DISCUSSION

HPV E6 induces telomerase activity in epithelial cells by activating transcription of the *hTERT* catalytic subunit of telomerase. In *hTERT* expression reporter assays, E6 requires an intact proximal E box in the promoter for activity. Though transcription appears to be E box-dependent, expression of c-Myc does not change significantly upon E6 expression. While c-Myc steady-state levels do not change upon E6 expression, it is possible that this is a result of balanced c-Myc induction (77) and increased degradation (51). E6/E6-AP has been demonstrated to ubiquitinate c-Myc *in vitro* and *in vivo* (51). In fact, Veldman *et al.* find that E6 is bound at the *hTERT* promoter and that E6 can immunoprecipitate c-Myc from cell lysates (148). Though we have been unable to demonstrate an interaction between c-Myc and E6, it remains possible that these proteins do interact in a complex at the hTERT promoter.

Further chromatin immunoprecipitation experiments to determine if c-Myc is preferentially recruited to the *hTERT* promoter indicate that c-Myc is bound to the *hTERT* promoter regardless of promoter activity. Similar experiments by other groups also found that c-Myc is bound to the *hTERT* promoter in E6-expressing cells (6,148).

McMurray and McCance suggest that in E6-expressing cells c-Myc replaces USF1 and USF2 at the *hTERT* promoter, while Veldman *et al.* find that c-Myc is bound to the *hTERT* promoter in both hTERT-negative E7-expressing cells and hTERT-positive E6-expressing cells (103,148). While there are discrepancies among the studies, they suggest that binding of c-Myc to the *hTERT* promoter may be necessary but not sufficient for promoter activity.

Covalent modifications of the DNA or histones within the promoter region of a gene are often determinants of transcriptional activity. Though the *hTERT* promoter region lies within a CpG island, promoter methylation does not appear to be the primary mode of silencing *hTERT* expression in telomerase negative cells (28,29). Chromatin conformation, on the other hand, does seem to play a key role in *hTERT* activity. Several studies have demonstrated that inhibition of histone deactylases by trichostatin A in certain telomerase-negative cells can alter the chromatin structure and induce telomerase expression (19,65,139,150,159). Interestingly, induction of telomerase by E6 also seems to involve an epigenetic mechanism that regulates the extent of *hTERT* induction. Different clones of HFKs transduced with E6 express varying levels of *hTERT* that increase as cells are passaged in culture despite no accompanying increase in E6 expression levels (6,78,80). In preliminary data assessing the histone acetylation status of the hTERT promoter, E6 expression correlates well with significant increases in acetylated histones H3 and H4 associated with the hTERT promoter (Figure 2.3 C & D).

Interestingly, transactivation by c-Myc is proposed to involve acetylation of histones H3 and H4 in the promoters of some c-Myc target genes (2,102). The N-

terminal transactivation domain of c-Myc associates with the transformation-transactivation domain-associated protein (TRRAP), a subunit of the PCAF, GCN5/SAGA and Tip60/NuA4 HAT complexes (reviewed in (2)). In fact, association with TRRAP is required for c-Myc-mediated induction of hTERT in human fibroblasts yet dispensable for activation of several basally expressed genes (113). Our data suggests that c-Myc is constitutively bound to the hTERT promoter in HFKs yet the promoter remains silent in the absence of E6 expression or c-Myc overexpression. Perhaps E6 assists in the recruitment of TRRAP to the hTERT promoter. The adenovirus E1A oncoprotein has been shown to bind to TRRAP and is thought to redirect its HAT activity to cellular and viral promoters thereby repressing some c-Myc target genes (25,86). Future investigations will determine what chromatin remodeling factors or additional transcription co-activators are differentially bound to the hTERT promoter upon E6 expression.

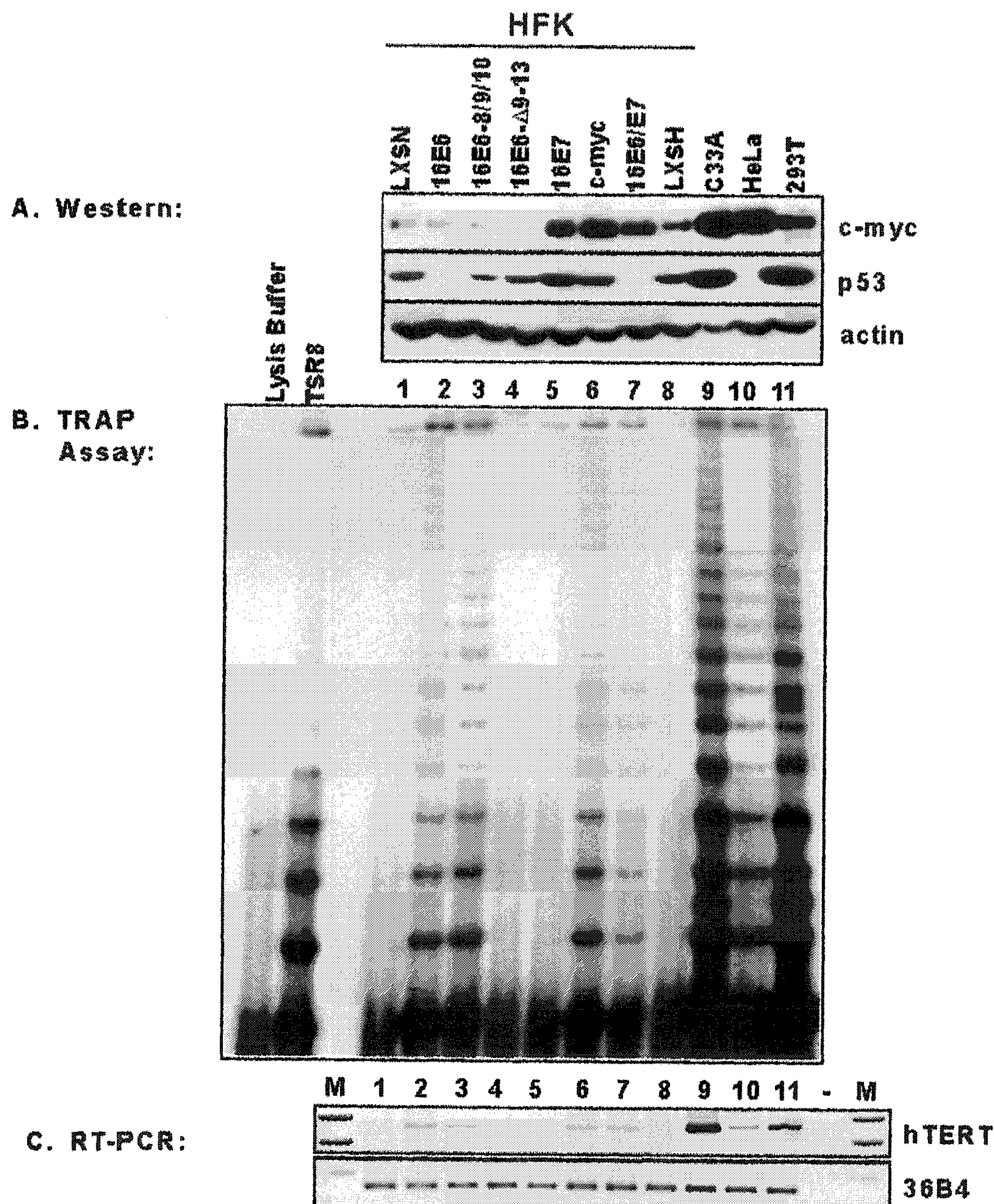


Figure 2.1. c-Myc expression levels do not correlate with telomerase activity in HFKs. Cells were transduced with retrovirus to express the indicated genes. (A) Western blots. 20 μg of nuclear extract were loaded per lane on an 8% SDS-PAGE and transferred to PVDF membrane. Blots were probed with c-Myc mouse monoclonal antibody (C-33, Santa Cruz), p53 mouse monoclonal antibody (Ab-6, Calbiochem), and actin goat polyclonal antibody (Santa Cruz). (B) Telomerase activity. TRAP assays were performed using 5 mg whole cell extract per lane. Lysis buffer was used as a negative control. TSR8 is a synthetic oligo of eight telomeric repeats (Intergen). (C) hTERT RNA expression. RT-PCR was carried out with RNA isolated from the transduced HFKs. 36B4 is a loading control. The negative control (-) is a reaction with no RNA. M is the marker lane.

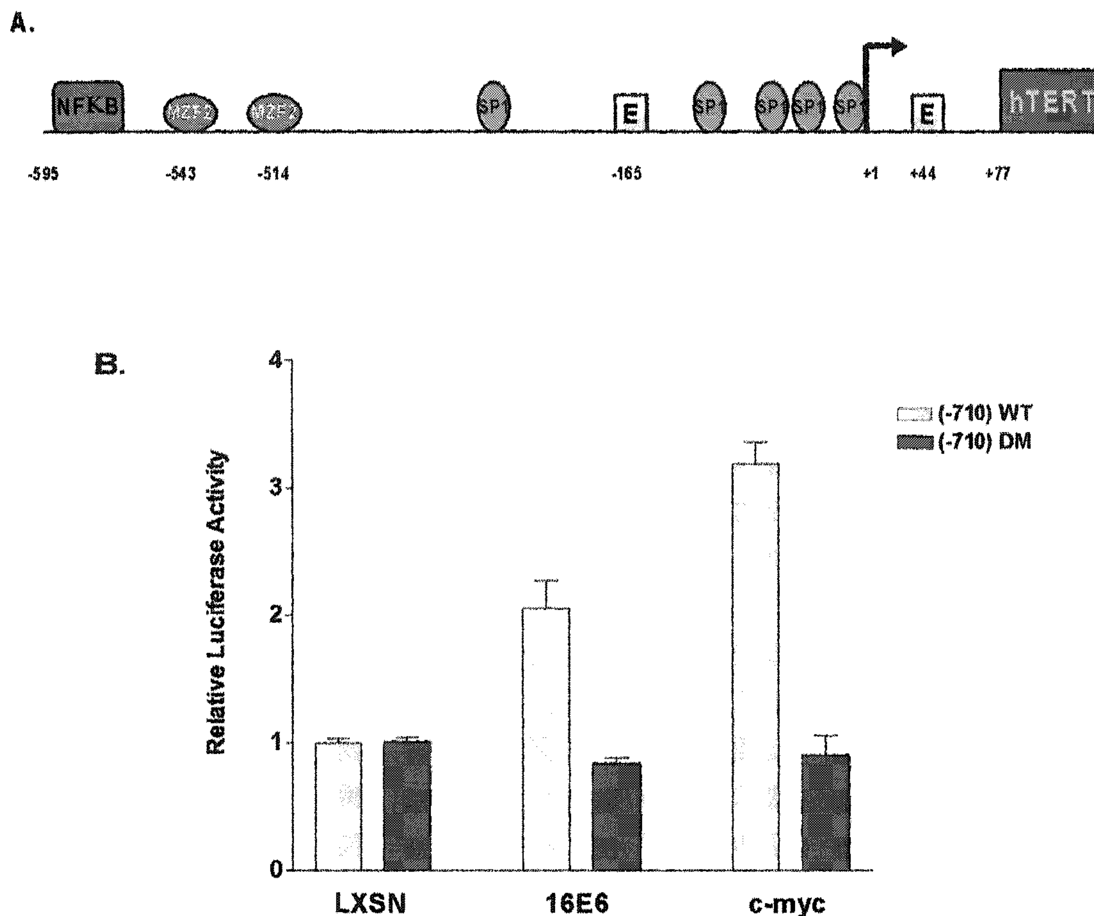


Figure 2.2. Myc-Max binding sites are required for activation of *hTERT* by either c-Myc or E6. (A) Schematic of the *hTERT* promoter. Putative transcription factor binding sites are indicated. This region was fused to the luciferase reporter gene. (B) Luciferase assay. HFKs were co-transfected with the pGL3-hTERT(-710)WT or DM promoter and the indicated retroviral expression construct. The DM construct has mutated versions (CACCTG) of both E boxes. Luciferase values were normalized for protein concentration and are graphed relative to the vector control value for each promoter construct. Graph represents four experiments done either in duplicate or triplicate.

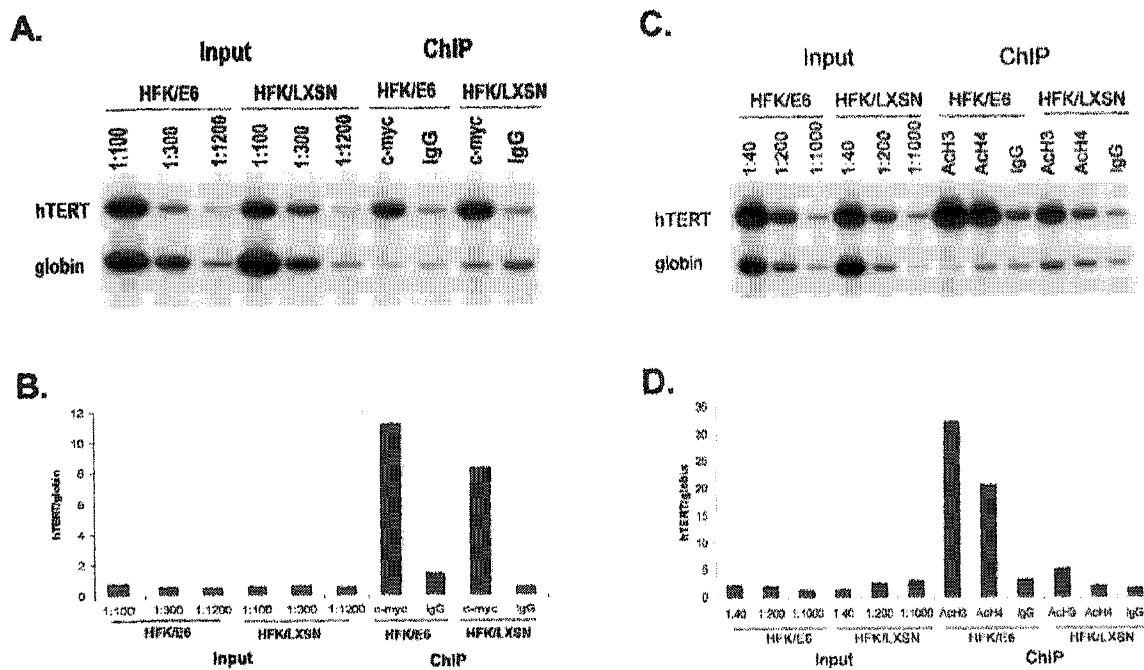


Figure 2.3. c-Myc and acetylated histones at the endogenous *hTERT* promoter.
(A) Chromatin immunoprecipitation (ChIP) of c-Myc at the hTERT promoter. HFK/LXSN and HFK/E6 cells were harvested and proteins were crosslinked to the DNA. Immunoprecipitations of c-Myc (rabbit polyclonal, Santa Cruz, N-262) and negative control rabbit IgG (Santa Cruz) were washed extensively. DNA bound to the proteins was used in radioactive PCR reactions to detect the hTERT promoter or the β-globin locus as an indicator of non-specific DNA-binding. Input DNA dilutions are included for quantitation. (B) Quantitation of the data presented in (A). Each value is presented as a ratio of hTERT signal to β-globin signal for normalization. (C) ChIP of acetylated histones H3 and H4 at the hTERT promoter. Experiments were performed as in (A) except using different antibodies that specifically detect acetylated histones H3 and H4.
(D) Quantitation of the data presented in (C). These experiments were performed by Weifeng Luo.

CHAPTER 3

E6-AP IS REQUIRED FOR INDUCTION OF TELOMERASE BY E6

INTRODUCTION

In epithelial cells the E6 oncoprotein induces telomerase activity and decreases p53 levels (80,125). To target p53 for proteasome-mediated degradation, E6 interacts with a cellular protein known as E6-associated protein (E6-AP) to form an E3 ubiquitin ligase (124). Studies of mutated E6 proteins reveal that induction of telomerase is an independent function of E6 separable from p53 degradation and that telomerase activation rather than p53 degradation is the critical step for immortalization of epithelial cells (78,80).

Several groups, including ours, have found that activation of telomerase by E6 is due to its ability to induce expression of *hTERT*, the catalytic and rate-limiting subunit of telomerase (41,114,147). The molecular details of the transcriptional activation by E6 of *hTERT* are the subject of extensive investigation.

While overexpression of p53 has been shown to repress *hTERT* expression in some tumor cell lines (71,84,160), inactivation of p53 is insufficient to induce telomerase (53,115). We and others have shown that E6-mediated telomerase activation is clearly independent of p53 degradation (80,94). Moreover, there is no correlation between p53 expression levels and *hTERT* expression (Figure 2.1). Both c-Myc-transduced and E6-8S/9A/10T-transduced HFKs induced *hTERT* despite the presence of p53.

The contribution of E6-AP to E6-mediated hTERT induction is investigated in this chapter. Comparison of E6-AP binding activity and ability to induce telomerase expression among several different E6 mutants revealed a strong correlation. The requirement for E6-AP expression in E6-mediated telomerase induction was firmly established using shRNAs to silence expression of the E6-AP gene. The E3 ubiquitin ligase function of the E6/E6-AP complex suggested a model in which E6/E6-AP target a repressor of *hTERT* for ubiquitination and degradation. A yeast two-hybrid screen was conducted in attempt to identify new targets of E6/E6-AP that may be involved in *hTERT* induction.

RESULTS

Ability of E6 to activate telomerase correlates with ability to bind E6AP

hTERT induction and p53 degradation are separable and distinct functions of E6 as indicated by E6 mutants such as F2V, 8S/9A/10T, and Y54H that can activate telomerase but do not target p53 for degradation (72,78,94,161)(Table 3.1). Yet, review of several published studies characterizing mutants of E6 reveals that all of the mutants that retain the ability to target p53 for degradation can also induce *hTERT* expression (Table 3.1). As the E6/E6-AP E3 ubiquitin ligase complex targets p53 for degradation, binding of E6 to E6-AP may also be required for *hTERT* induction. To address this hypothesis, binding assays were performed with several E6 mutants and E6-AP. The E6-8S/9A/10T and E6-Δ9-13 mutants, both having lost the ability to target p53 for degradation, were used in an E6-AP binding assay. Figure 3.1A shows that while both E6 wt and E6-8S/9A/10T bound to E6AP, E6-Δ9-13 does not. Additional E6 binding

assays were performed by incubating GST-E6 proteins with lysates from HFKs transiently transfected with an LXSN-HA-E6AP construct. E6 wt, E6-8S/9A/10T, and E6-F2V interacted strongly with HA-E6AP while E6-Δ9-13 and E6-L50G did not (Figure 3.1B). Though E6 wt, E6-8S/9A/10T, and E6-F2V bound E6AP, only E6 wt interacted with and targeted p53 for degradation (data not shown). Comparison of this binding data with the ability of each mutant to induce *hTERT* expression revealed a strong correlation of E6-mediated telomerase activation with the ability of E6 to bind E6AP.

Other investigators have also found that E6 mutants with decreased binding to E6AP inefficiently immortalized epithelial cells (94). An overview of published data with regard to E6 and E6-AP binding and telomerase induction is presented in Table 3.1. A schematic of the E6 protein demonstrates that mutations disrupting p53 degradation while still allowing *hTERT* induction lay N-terminal of the two zinc fingers found in E6 (Figure 3.1D). This region is required for p53 targeting but not for E6-AP binding. We propose that the E6/E6AP complex targets a repressor of *hTERT* transcription for ubiquitination and degradation.

E6-AP is required for telomerase activation of 16E6

To further support the hypothesis that E6-AP binding is required for E6 to induce *hTERT* expression, we developed short-hairpin RNA (shRNA) retroviral constructs to reduce *E6-AP* expression in primary human foreskin keratinocytes (HFKs). These experiments were performed using constructs to stably express the shRNAs driven by an RNA polymerase III promoter, either U6 or H1(46,116,129). Three different 26-29 nt regions of the *E6-AP* gene were targeted to identify a construct that would give the

greatest decrease in *E6-AP* expression. Figure 3.2A shows shRNA1 (esh1) was the most effective of the three, with *E6-AP* RNA expression reduced to about 29% of that in the vector-control infected cells. The other two hairpins (esh2 and esh3) exhibited intermediate levels of effectiveness. We were unable to detect E6-AP protein with several different commercially available antibodies.

HFK cell lines stably expressing the E6-AP shRNAs were subsequently infected with empty vector or E6 retroviral constructs. Depletion of *E6-AP* abrogated the ability of E6 to target p53 for degradation, with esh1, again, proving to be the most effective of the three shRNA-expressing cell lines (Figure 3.2B). In addition, assays for telomerase activation revealed that *E6-AP* depletion disrupted E6-mediated *hTERT* induction in HFKs (Figures 3.2 C & D). TRAP activity in these cells directly correlated with the level of *hTERT* RNA detected by RT-PCR. In summary, *E6-AP* expression was required for *hTERT* induction by HPV E6.

Search for new targets of E6/E6-AP

Thus far our studies reveal that E6 must bind to E6-AP but not necessarily to p53 in order to induce hTERT. Other identified targets of E6 include E6-TP1 (a Rap1 GAP protein), paxillin (a focal adhesion protein), E6-BP (a calcium-binding protein), and IRF3 (a transcription factor involved in the interferon response). These proteins, as well as E6-AP, contain an alpha-helical motif known as an LXXLL motif with a hydrophobic patch on one face through which they bind to E6 (10). Mutation of isoleucine 128 to threonine diminished the capacity of E6 to bind to these alpha-helical partners, and expression of this mutant in transgenic mice resulted in lower incidence of hyperplasia and

transformation compared to transgenic mice expressing wild-type E6 (112). Though this data suggests that any of the alpha-helical E6 partners may be critical to transformation by E6, our shRNA data has indicated that E6-AP binding is the critical binding partner for immortalization. The E6/E6-AP complex is proposed to target many different proteins for degradation via the proteasome. Among the other targets of E6/E6-AP are a large family of PDZ domain-containing proteins that are membrane-associated guanylate kinases, or MAGUKs (141). The PDZ binding domain of E6, encompassing the four C-terminal amino acids, has been shown to be required for transformation by E6 (79,88), but is dispensable for immortalization by E6 (78). Therefore it is unlikely that any of the PDZ-containing proteins targeted by E6 are involved in telomerase activation.

In an attempt to identify the target of E6/E6-AP involved in *hTERT* induction, we performed a yeast two-hybrid screen. The bait construct consisted of a catalytically defective E6-AP (C833A) fused to the Gal4 DNA binding domain. To ensure equal dosage of *E6* and *E6-AP,* the plasmid also encoded the E6 oncoprotein. Both fetal brain and HeLa cell cDNA libraries fused to the Gal4 activation domain were screened for interaction with E6/E6-AP (Figure 3.3A). Several known E6/E6-AP interactors were identified, including p53 and HHR23A (human homolog of *rad23*) (82) as well as some novel candidate proteins (Figure 3.3B). One of these was signal-induced proliferation-associated 1 like-2 protein (SIPA1L2, Unigene Hs.406879), a protein highly homologous to E6TP1 (aka. SIPA1L1), a known E6 target (39). We also identified an uncharacterized RING finger protein that is a putative member of the tripartite motif protein family (Hs. 356377). Another new E6/E6-AP target protein was NFX1 (nuclear factor binds to the

X1 box), a transcriptional repressor of MHC class II genes (132). NFX1 was originally identified in a screen for proteins that bound to the X box region of MHC class II genes. It was shown to be induced by interferon γ and is thought to be involved in a feedback loop to limit the immune response following infection (132). Further exploration of a role for NFX1 in E6-mediated telomerase induction will be presented in chapter 4.

DISCUSSION

The induction of *hTERT* expression is dependent on the expression of cellular *E6-AP* and binding of E6 to E6-AP. When *E6-AP* expression was reduced with shRNAs, E6 had a diminished ability to induce expression of *hTERT*. Previous work has demonstrated that antisense oligonucleotides directed against *E6-AP* restore p53 expression in cervical cancer cell lines (143). Our work further demonstrated that *E6-AP* expression is also required for E6 to induce transcription of *hTERT*. While other groups have determined that repression of E6 and E7 by re-expression of the viral E2 gene in HPV-transformed cells causes senescent growth arrest or, in some cases, apoptosis (27,43,87,153,153), we have demonstrated that expression of E6-AP, the cellular partner of E6, is also critical to the immortalization activity of E6.

E6-AP is a dual function protein that can act as an E3 ubiquitin ligase as well as a coactivator for the nuclear hormone receptor superfamily (110). These two functions are apparently separable and distinct in that ubiquitin ligase activity is not required for coactivation function. Interestingly it is the ubiquitination function of E6-AP that appears to be affected in patients with Angelman Syndrome, a genetic neurologic disorder associated with disruption of the maternal allele of *E6-AP*. Because *E6-AP*

expression in the brain is maternally imprinted, disruption of *E6-AP* is associated with severe mental retardation, seizures, ataxia and speech disorders. *E6-AP* is biallelically expressed in other tissues. The crystal structure of E6-AP supports this finding in that several Angelman Syndrome-associated mutations occur in conserved residues in the catalytic cleft of E6-AP. These mutations interfere with ubiquitin-thioester bond formation (67). Though we favor a model in which the E3 ubiquitin ligase activity of E6/E6-AP targets a repressor of telomerase for degradation, the proposed coactivation function of E6-AP may yet play a role in telomerase induction by E6.

To identify a hypothesized E6/E6-AP target involved in hTERT induction, we performed a yeast two-hybrid screen. The screen identified several known targets of E6/E6-AP as well as a few novel interacting proteins. These novel interactors were subsequently screened for activity at the hTERT promoter in luciferase reporter assays (data not shown). The only one observed to affect hTERT transcriptional activity was the NFX1 transcriptional repressor. The possible role of NFX1 in E6-mediated hTERT induction is investigated in the next chapter.

TABLE 3.1. Telomerase activation and E6-AP binding of E6 proteins

HPV 16E6 protein	TRAP Activity	E6-AP Binding	p53 degradation	References
16E6 wt	+	+	+	Klingelhutz 1996, Liu 1999
F2V	+	+	-	Liu 1999, Figure 3.1B
8S/9A/10T	+	+	-	Foster 1994, Klingelhutz 1996, Gewin 2001
Δ9-13	-	-	-	Foster 1994, Gewin 2001
L50G	-	-	-	Zimmermann 1999, Figure 3.1B
Y54H	+ [a]	+	-	Liu 1999
Δ118-122 [b]	-/+ [a]	-/+	-/+	Foster 1994, Dalal 1996, Kiyono 1998
Δ123-127	-	-	-	Foster 1994, Klingelhutz 1996, Liu 1999
Δ146-151	+	NT	+	Foster 1994, Klingelhutz 1996, Kiyono 1998

NT, not tested

[a] TRAP Activity is inferred by the ability of these mutants to immortalize mammary epithelial cells (MECs)

[b] 16E6 Δ118-122 appears to have low level activity in all these functions

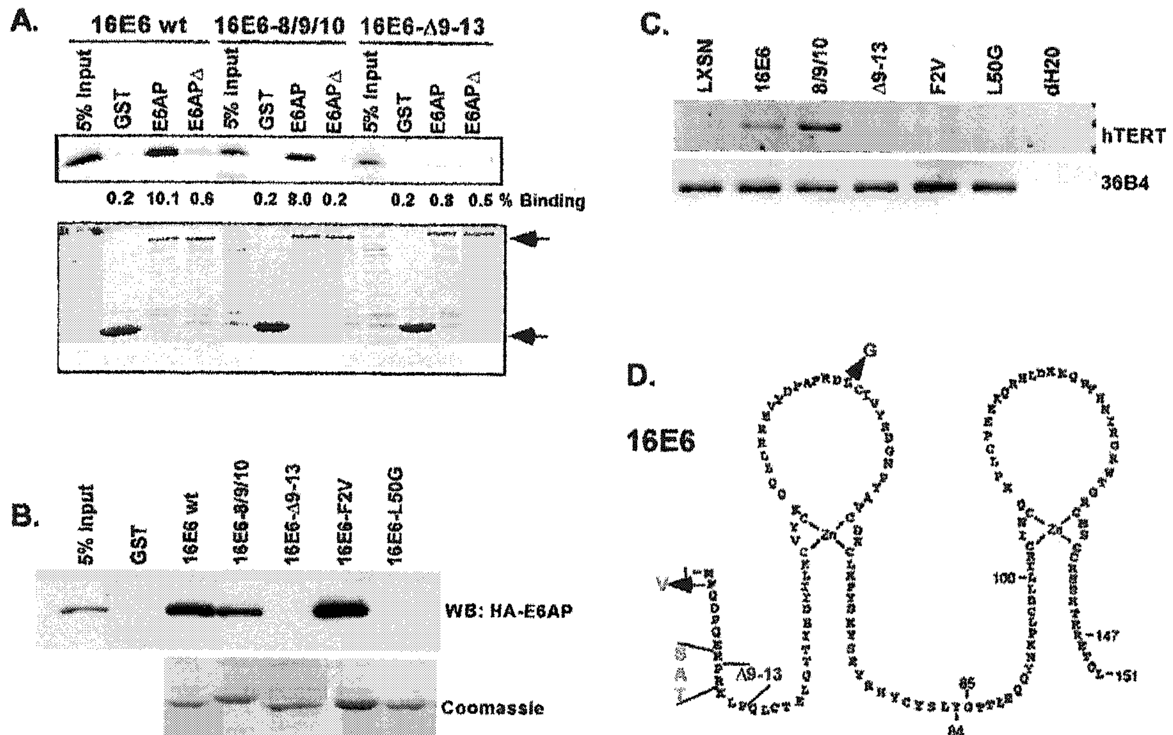


Figure 3.1. Induction of *hTERT* expression correlates with the ability of E6 to bind E6-AP. (A) E6 binding assay. ^{35}S-radiolabeled E6 proteins were incubated with purified GST proteins and precipitated with glutathione sepharose. E6APΔ is a mutant with the E6-binding region (aa 391-408) deleted. Protein complexes were analyzed by SDS-PAGE and autoradiography (upper panel). Binding was subsequently quantitated by phosphorimaging using Image Quant. Percent binding indicates the average percent binding of three independent experiments. Lower panel is a coomassie-stained gel to show loading of GST proteins. Arrows indicate GST-E6AP proteins and GST (B) E6-AP binding assay. HFKs were transiently transfected with HA-E6AP. Cells were lysed in binding buffer (PBS, 1% NP-40, 2mM DTT, 10% glycerol). Lysates were incubated with purified GST proteins and precipitated with glutathione sepharose. Protein complexes were separated by SDS-PAGE, transferred to PVDF, and Western blotted for HA-E6AP and p53. Representative of four independent experiments. (C) RT-PCR. HFKs were stably transduced with the indicated empty vector or E6 gene. RNA was harvested from the cells and analyzed for *hTERT* and *36B4* (loading control) expression. (D) Schematic of 16E6 oncoprotein. The amino acid sequence is drawn illustrating the zinc coordinating cysteines of E6. Amino acid substitutions or deletions that retain the ability to induce *hTERT* expression are shown in green while those that no longer induce telomerase are shown in red.

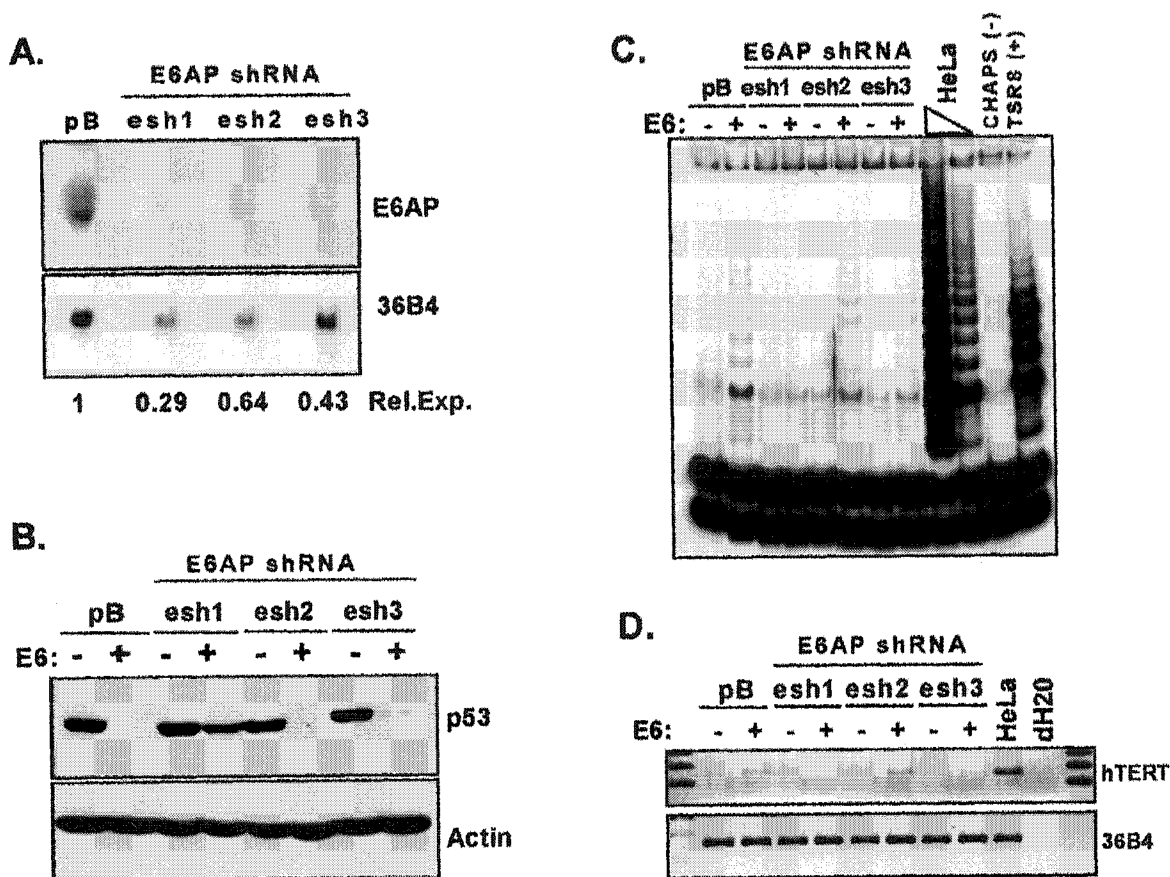


Figure 3.2. ***E6-AP*** **expression is required for telomerase induction by HPV-16 E6.**
(A) Northern blot. Three shRNA constructs targeting *E6-AP* were transduced into HFKs and RNA was harvested to examine levels of *E6-AP* message. *36B4* is a loading control. Relative expression levels are presented normalized to *36B4* and the pB empty vector control.
(B) Western blot. HFKs expressing the *E6-AP* shRNA constructs were subsequently transduced with LXSN empty vector or LXSN-16E6. p53 levels were examined by western blot. Actin is a loading control. (C) TRAP assay. Extracts from the same cells shown in (B) were assayed for telomerase activity. HeLa cells were used as a positive control lysate. CHAPs is a lysis buffer negative control. TSR8 is a synthetic template of 8 telomeric repeats used as a PCR positive control. (D) RT-PCR. Expression of *hTERT* RNA was examined by RT-PCR of RNA extracts. *36B4* is a loading control. DNA fragments were visualized by ethidium bromide staining.

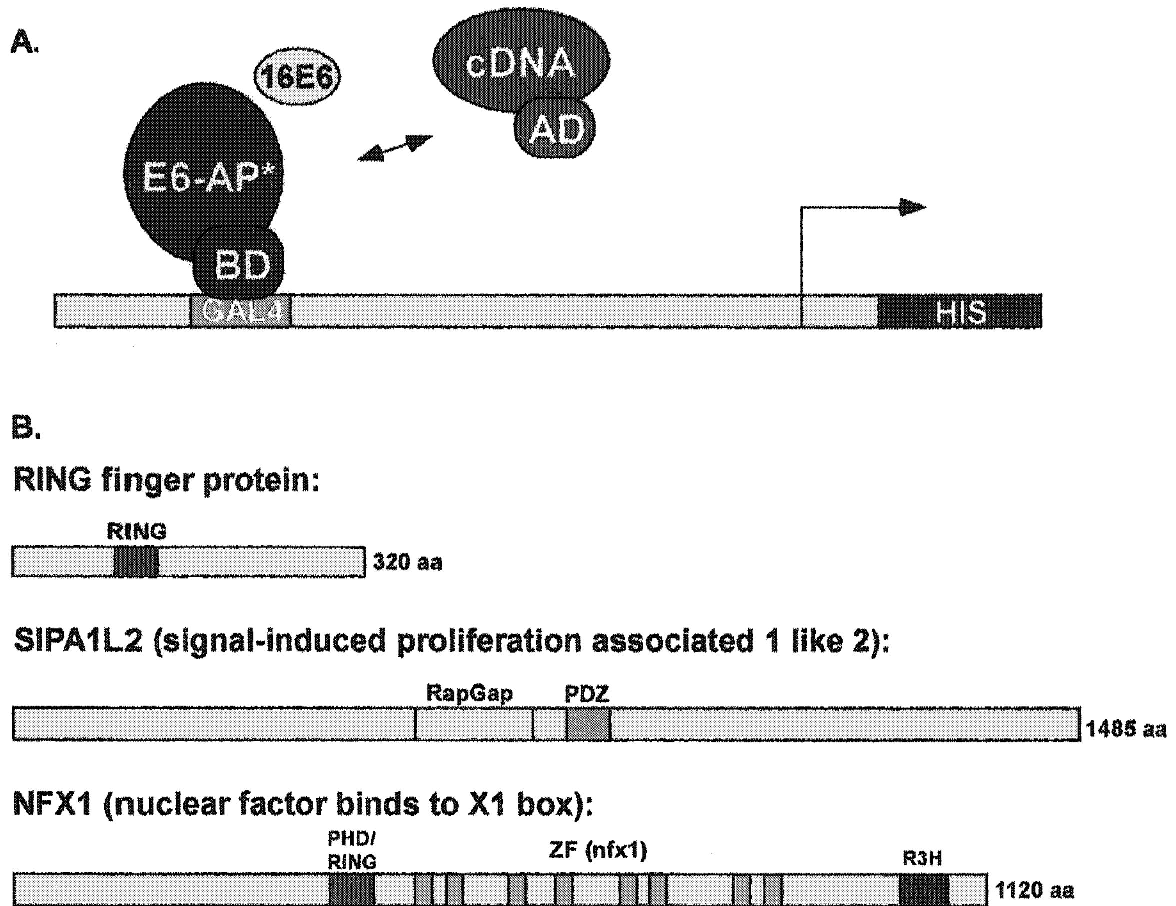


Figure 3.3. The E6/E6-AP yeast two-hybrid screen. (A) Schematic of the yeast two-hybrid screen. A catalytically-defective E6-AP (C833A) was fused to the GAL4 DNA-binding domain. Two cDNA libraries (generated from HeLa cells and fetal brain tissue) fused to the GAL4 activation domain were screened for interaction with the E6/E6-AP complex. (B) Three novel putative interactors with the E6/E6-AP complex. An uncharacterized RING finger protein, a member of the E6TP1 family identified as signal-induced proliferation 1 like 2 (SIPA1L2), and a transcriptional repressor called NFX1 were identified in the screen. Conserved domains of each protein were identified with the Pfam website (http://pfam.wustl.edu/) and are indicated. Schematics are not drawn to scale.

CHAPTER 4

IDENTIFICATION OF A NOVEL TELOMERASE REPRESSOR BY VIRTUE OF ITS INTERACTION WITH THE HUMAN PAPILLOMAVIRUS TYPE-16 E6/E6-AP COMPLEX

INTRODUCTION

Our previous work demonstrated that binding of E6 to its cellular partner E6-AP is required for telomerase induction. A yeast two-hybrid screen investigated possible new targets of the E6/E6-AP complex. In addition to several known targets of E6/E6-AP, the screen identified a transcriptional repressor known as *NFX1*. Originally identified in a screen for proteins that bind to the X box region of MHC class II genes, *NFX1* was shown to be induced by interferon γ and is proposed to repress MHC II genes in a negative feedback loop to limit the immune response. The expression pattern of *NFX1*, however, is not limited to cells of the immune system; it is widely expressed suggestive of a more general transcriptional function.

The *NFX1* gene is highly conserved among eukaryotic species (Figure 4.1; (21)). The *Saccharomyces cerevisiae* homolog of *NFX1* is called *FAP1*, or FKBP-associated protein (83). Overexpression of *FAP1* confers resistance to rapamycin, a potent immunosuppressive drug and anti-fungal agent. Rapamycin binds to FKBP12, an abundant peptidyl-prolyl *cis-trans* isomerase, to form a complex that binds and inhibits the products of the *TOR1* and *TOR2* genes. The TOR proteins are kinases involved in regulating cell growth and protein synthesis pathways in response to nutrient-availability

signals (121,126). Fap1 was shown to compete with rapamycin for binding to FKBP12. Upon rapamycin treatment, Fap1 translocated from the cytoplasm to the nucleus (83). The nuclear localization, putative DNA binding domains, and homology to *NFX1* of *FAP1* suggests a role as a transcription factor. Kunz *et al.* suggest that FKBP12 may regulate the localization and activity of Fap1 (83). Though *FAP1* is not an essential gene, recent data published on the *Saccharomyces* Genome Database (www.yeastgenome.org) describes a moderate growth defect after 20 generations and a severe growth defect after 60 generations in yeast with an insertional mutation of the *FAP1* gene (Dunn *et al.*, in preparation). Based on these data it is interesting to speculate that FAP1 may play a role in the highly linked pathways of nutrient sensing and lifespan regulation.

The *Drosophila melanogaster* homolog of *NFX1* is called *shuttle craft*, or *stc*, and was identified in a screen for single-stranded nucleic acid binding proteins expressed during oogenesis (136). Maternal expression of *stc* is required during embryogenesis but not oogenesis. Germ-line disruption of *stc* causes segmentation defects and abnormalities in the central nervous system, while zygotic expression of *stc* is required for proper motoneuronal axon outgrowth (142). Though STC is localized to the nucleus throughout the central nervous system, its transcriptional targets are unknown (136,142).

Conservation of *NFX1* among eukaryotic species suggests that it contributes to the evolutionary fitness of eukaryotic organisms. The limited research into the molecular functions of *NFX1* homologs indicate that it is a transcription factor that may be involved in signaling pathways as diverse as the nutrient and stress response, nervous system development, and regulation of the immune response.

RESULTS

Differential repressor and activator functions of the NFX1 isoforms on the *hTERT* promoter

Recent advancements in the annotation of the human genome revealed that *NFX1* has two splice variants encoding isoforms with identical amino-termini and variant carboxy-termini (Figure 4.2A, Unigene Hs. 413074). We will specify the longer 1120 amino acid isoform here as NFX1-123, as it is approximately 123 kDa, and the shorter 833 amino acid isoform as NFX1-91 (~91 kDa). Both isoforms have a RING finger/PHD finger domain. This domain has been shown to confer autoubiquitination activity to NFX1 in *in vitro* assays (97). The RING finger domain is followed by several cysteine/histidine-rich sequences identified as NFX1-type zinc fingers by the Pfam database (www.sanger.ac.uk/cgi-bin/Pfam/ getacc?PF01422). These zinc fingers [C-X3-C-X(4-8)-C-G-X(0-4)-H-X-C-X3-CH-X(3-4)-C-X(2-5)-C] are highly homologous to one another but do not exhibit the typical spacing of traditional zinc fingers. Song et al. found that the zinc fingers were the DNA-binding domain of NFX1 (132). NFX1-91 contains the first six zinc fingers followed by a unique lysine-rich stretch of 25 amino acids. NFX1-123 contains all eight zinc fingers as well as a region known as an R3H domain believed to be involved in single-stranded nucleic acid binding (Figure 4.2A)(50).

Activity in *hTERT* luciferase reporter assays was used as a secondary screen for the hits from the yeast two-hybrid screen. The *hTERT* promoter is inactive in primary human foreskin keratinocytes (HFKs). Both c-Myc and E6 can induce an *hTERT*

luciferase reporter construct (Figure 4.2B) two- to five-fold in HFKs. Transfection of the *NFX1* isoforms alone with the reporter construct had very subtle effects, with *NFX1-91* reducing activity to approximately 60% of background levels while *NFX1-123* had virtually no effect (Figure 4.2C). Further cotransfection experiments revealed that these two isoforms have strikingly opposing activities. *NFX1-123* strongly co-activated the *hTERT* promoter when cotransfected with c-Myc whereas *NFX1-91* robustly repressed induction by c-Myc upon cotransfection. Interestingly, co-transfection of c-Myc and E6 cooperatively increased *hTERT* promoter activity. Further, addition of *NFX1-123* to c-Myc and E6 resulted in even greater *hTERT* promoter activity whereas co-transfection of *NFX1-91* with c-Myc and E6 suppressed this activity. These assays indicated that NFX1-91 functioned as a transcriptional repressor whereas NFX1-123 appeared to be a co-activator of transcription.

As NFX1 was originally identified in a screen for proteins that bind the X box of MHC class II genes, the presence of similar sequences in the *hTERT* promoter was investigated. The MHC class II X box sequence is CCTAGCAACAGATG (highly conserved residues are underlined) (132). Sequence scanning of the promoter found two possible X box-like sequences within the *hTERT* proximal promoter (Figure 4.2B). One of these X box-like sequences (CGTGGGAAGCCCTG) overlapped with the proximal E box while the other (CCTGGGAACAGGTG) lay approximately 400 bp upstream of the transcription start site.

In an initial attempt to map the regions of the *hTERT* promoter bound by the NFX1 isoforms, luciferase assays were performed with a truncated version of the *hTERT*

promoter. Activity of the *NFX1* isoforms on an ~ 800-bp region (710 hTERT) of the *hTERT* promoter was compared to that on a ~ 300-bp region (219 hTERT) (Figure 4.2D). Interestingly, whereas the co-activation function of *NFX1-123* was clearly apparent with the longer promoter construct, it was greatly diminished with the minimal 219 hTERT promoter. In contrast, the repressive effects of *NFX1-91* were demonstrated using both promoter constructs. This suggested that the two NFX1 isoforms may have differential binding affinities for specific elements within the *hTERT* promoter or NFX1-123 may require the presence of additional co-factors bound upstream. It should be noted that c-Myc induction of the 219 hTERT construct was significantly lower than that seen with the longer promoter region and this may have impacted the co-activation ability of NFX1-123.

E6 preferentially binds and destabilizes NFX1-91 rather than NFX1-123

Given the repressive effects of NFX1-91 on the *hTERT* promoter in luciferase assays, an attractive hypothesis emerged that E6/E6-AP might target NFX1-91 for degradation thereby relieving repression at the *hTERT* promoter. To investigate this idea, NFX1 expression levels were determined in cell types relevant to HPV infection and in response to E6 expression. RNA was isolated from HFKs expressing E6 or a vector control (LXSN), HeLa (HPV 18-positive cervical carcinoma), C33A (HPV-negative cervical carcinoma), and irrelevant U2OS (osteosarcoma) cells. Because the *NFX1-91* isoform has rather limited unique sequence distinguishing it from *NFX1-1*23, RNA expression was assayed by RT-PCR. As seen in Figure 4.3A, the level of expression of the two isoforms did not vary greatly between cell types, though the telomerase-positive

C33As did have significantly decreased expression of both NFX1 isoforms. Since the E6/E6-AP complex functions primarily post-transcriptionally, it was not surprising that *NFX1* mRNA expression levels were not altered in the presence of E6.

As the E6/E6-AP complex reduces expression of it's targets by ubiquitin-mediated protein degradation, the effect of E6 expression on NFX1 protein levels was determined. A time course of cells treated with cycloheximide revealed substantial differences in the expression level and half-life of NFX1-91 in HFK/E6 cells compared to HFK/LXSN cells. While NFX1-91 had an apparently short half-life in the absence of E6, the stability of NFX1-91 was further decreased in the presence of E6 (Figure 4.3B). In contrast, NFX1-123 seemed to be an abundant, stable protein that was largely unaffected by E6 expression (Figure 4.3B). The reduction of NFX1-91 protein levels in E6-expressing HFKs was proteasome-dependent as treatment with the proteasome inhibitor MG-132 restored NFX1-91 protein levels to that found in vector-transduced HFKs (Figure 4.3C). Though E6 interacted with NFX1-123 in the yeast two-hybrid screen and in *in vitro* binding assays (data not shown), E6 preferentially bound to NFX1-91 and not to NFX1-123 in co-immunoprecipitations from 293Ts transiently-transfected with AU1-tagged E6 (Figure 4.3D). While we were unable to confirm the interaction with the reciprocal co-immunoprecipitation using NFX1 rabbit polyclonal antibody, the sum of the other protein expression data and binding data strongly suggested that NFX1-91 was a novel target of the E6/E6-AP E3 ubiquitin ligase complex.

NFX1-91 is highly ubiquitinated in the presence of E6

As E6 appeared to preferentially bind and destabilize NFX1-91 in a proteasome-dependent manner, we examined the ubiquitination status of NFX1-91 versus NFX1-123 in cell lysates. For these experiments FLAG-tagged versions of the NFX1 isoforms were transiently transfected into 293T cells in order to specifically immunoprecipitate the two different isoforms. Though the CMV promoter drove expression of both isoforms, NFX1-123 was much more highly expressed than NFX1-91 upon transfection, consistent with the differences in protein stability previously observed (Figure 4.4A). In addition, Western blots with a ubiquitin antibody revealed a strong ladder signal only in the FLAG-tagged NFX1-91 immunoprecipitations (Figure 4.4B) indicating NFX1-91 was highly ubiquitinated while NFX1-123 was not, thus explaining their different half-lives. As this experiment was conducted in cells lacking E6 expression, it indicated that NFX1-91 protein levels, much like p53 protein levels, were regulated in a proteasome-dependent manner even in the absence of E6. To determine if E6 expression could influence the degree of NFX1-91 ubiquitination, the experiment was repeated in HFK/LXSN and HFK/E6 cells with endogenous NFX1 protein. We developed specific antibodies to separately immunoprecipitate each isoform of NFX1. As seen in Figure 4.4C, ubiquitinated NFX1-91 was much more prevalent in HFK/E6 cells than in HFK/LXSN cells, and NFX1-123 did not appear to be ubiquitinated. Longer exposures of the ubiquitin Western blot in Figure 4.4C indicated that there is some ubiquitinated NFX1-91 in HFK/LXSN cells (data not shown). Therefore, as we saw in Figure 4.4B with the 293T cells, an E6-independent means of ubiquitinating NFX1-91 seems to exist.

Significantly, the higher levels of ubiquitinated NFX1-91 detected in HFK/E6 cells directly correlated with the decreased stability of NFX1-91 protein in the presence of E6 (shown previously in Figure 4.3B) strongly supporting the hypothesis that NFX1-91 is a target of E6/E6-AP-mediated degradation.

***In vivo* evidence for NFX1-91 as a repressor of hTERT**

Thus far, luciferase reporter assays suggested NFX1-91 functions as a repressor at the *hTERT* promoter while protein expression data indicated that the E6/E6-AP complex could destabilize NFX1-91 protein suggestive of a relief of repression mechanism for E6-mediated *hTERT* induction. To address directly whether NFX1-91 functions as a transcriptional repressor at the endogenous *hTERT* promoter, *NFX1-91* expression was reduced using stable shRNA expression in HFKs. We constructed an shRNA construct targeted to the unique 3' untranslated region (3'UTR) of *NFX1-91*. The NFX1-91 shRNA (n91sh) reduced NFX1-91 protein levels in HFKs while not affecting NFX1-123 protein levels (Figures 4.5B). This was accompanied by derepression of the *hTERT* promoter even in cells lacking E6 expression (Figure 4.5A). The previously characterized E6-AP sh1 (esh1) construct (Figure 3.2) was included as a negative control. When the shRNA-expressing cell lines were subsequently transduced with empty vector or E6, the E6 cells with reduced *NFX1-91* expression had a greater than two-fold increased expression of *hTERT* as demonstrated by RT-PCR and TRAP assay (Figures 4.5C and 4.5D). Furthermore, the reduced telomerase activity in HFK/E6 cells with reduced E6-AP expression (esh1) correlated with increased NFX1-91 protein levels and decreased detection of ubiquitinated NFX1-91 (Figure 4.5E). These data indicated that

NFX1-91 functions as a transcriptional repressor at the endogenous *hTERT* promoter in HFKs and that the activity of the *hTERT* promoter increased in response to decreased NFX1-91 protein expression levels either by shRNA expression or by E6/E6-AP-mediated ubiquitination and degradation.

To further validate the role of NFX1-91 in regulation of telomerase activity, we examined the impact of reduced *NFX1-91* expression on the lifespan of cells in culture. The cells presented in Figure 4.5C were continually passaged splitting 1:3 as needed for approximately two months. As cells divide, the telomeres of cells that do not express telomerase continually shorten and the telomere structure becomes disrupted (54,100). Cells respond to the chromosome ends as though they are a double-stranded DNA break and generally attempt to repair the exposed chromosome ends or initiate a senescent arrest (30,35,137). Senescent cells can be identified by a change in cellular morphology and with a marker for senescent-associated (SA) β-galactosidase activity (32). As expected, there were less senescent cells present in the E6-expressing population compared to the vector control population (Figure 4.6A). Furthermore, about half of the E6 cells with reduced *E6-AP* expression stained positive for SA-β-galactosidase and appeared large and flattened with many vacuoles (Figures 4.6A and 4.6B). In contrast, the n91sh-expressing cells, even in the absence of E6 expression, were more rounded like rapidly dividing cells and displayed reduced SA-β-galactosidase staining. The percent of cells exhibiting SA-β-galactosidase activity (Figure 4.6B) was inversely related to the telomerase activity detected at earlier passages (Figures 4.5C and 4.5D). Additionally, the cells with increased telomerase activity had increased lifespans in culture as indicated by

the number of population doublings (PDLs) each cell line achieved during the same number of days in culture (Figure 4.6A). Therefore, reduction of NFX1-91 protein levels either via reduction of mRNA levels with shRNA or via E6/E6-AP-mediated ubiquitination and degradation was sufficient to induce *hTERT* expression and suppress senescent growth arrest in primary human epithelial cells. Conversely, reduced *E6-AP* expression prohibits E6-mediated telomerase induction and lifespan extension presumably through the stabilization of the *hTERT* transcriptional repressor, NFX1-91.

Requirement for *E6-AP* and role for *NFX1-91* in telomerase activity of HeLas

HeLa cells are a widely used cervical cancer cell line expressing HPV 18 E6 and E7. We wanted to further test the requirement for E6-AP and the role of NFX1-91 in regulation of *hTERT* expression in this transformed cell type. HeLa cells were transduced with shRNA constructs to decrease expression of either *E6-AP* or *NFX1-91*. Immediately after selection, the HeLas expressing esh1 exhibited a strikingly different morphology and noticeably decreased growth rate (Figure 4.7A). This was accompanied by increased p53 protein levels and decreased telomerase activity (Figures 4.7B and 4.7C). We were unable to obtain enough cells at this time point to examine RNA levels by RT-PCR (Figure 4.7D). Approximately one week after selection many small, rapidly dividing cells grew out of the HeLa/esh1 culture. The p53 levels dropped slightly and the telomerase activity increased marginally in the outgrown cells (data not shown). Most likely, the outgrown cells downregulated the p53 or shRNA pathway to escape arrest.

To examine an *E6-AP* dose-response, we also expressed the sh2 *E6-AP* shRNA (esh2) construct that, as shown previously, only slightly decreased *E6-AP* expression

(Figure 3.2A). The esh2-expressing HeLa cells did not change morphology or slow their division. p53 levels in these cells increased minimally in comparison to esh1-expressing HeLa cells, yet *hTERT* expression decreased rather dramatically to an activity about 22% that of vector control HeLa cells (Figure 4.7). One interpretation of this data is that esh2 reduced the levels of E6-AP so that the E6/E6-AP complexes are limited to interactions with only their highest affinity target, p53. But the significant reduction in telomerase activity indicated that the limited amount of E6/E6-AP complexes were insufficient to adequately ubiquitinate and degrade the lower affinity target that functions as a telomerase repressor. Such a model may help explain the variable telomerase activity described for different clones of E6-expressing epithelial cells (78,80).

A direct influence of NFX1-91 on *hTERT* expression in HeLa cells was demonstrated upon reduced expression of *NFX1-91*. Immediately following selection of HeLas expressing the *NFX1-91* shRNA, *hTERT* expression and telomerase activity increased approximately two-fold over that seen in vector-control cells (pB). This suggested that even though *NFX1-91* was targeted for ubiquitination and destabilization in HeLa cells, it could still have some repressive activity at the *hTERT* promoter. Further decreasing its expression at the RNA level resulted in even more robust telomerase activity.

In a parallel set of experiments, we saw no effect on p53 or *hTERT* levels upon expression of the *E6-AP* or *NFX1-91* shRNA constructs in C33A cells (data not shown), where *NFX1-91* expression was reduced at the RNA level by an unknown mechanism (Figure 4.3A). Perhaps the low levels of *NFX1-91* in C33As no longer effectively

repressed *hTERT* expression such that further reduction via shRNA had no appreciable effect. Alternatively, C33As may induce hTERT expression through a completely NFX1-independent mechanism. It should also be noted that the levels of *hTERT* expressed in C33As were significantly higher than that seen in HeLas (Figure 4.3A). C33As should not and did not respond to *E6-AP* reduction, as they do not express E6.

DISCUSSION

Induction of telomerase appears to be a common and requisite event in the transformation of many cell types. In an effort to understand how the HPV type-16 E6 oncoprotein induces telomerase activity in epithelial cells, we have previously shown that E6-AP, the cellular partner of E6, is required for *hTERT* induction. We hypothesized that an unidentified repressor of telomerase was being targeted by the E6/E6-AP ubiquitin ligase for ubiquitination and degradation thereby alleviating repression of *hTERT*. Using a yeast two-hybrid screen we identified a transcriptional repressor called NFX1. The NFX1 gene actually encodes two splice variants, *NFX1-123* and *NFX1-91*, with opposing activities in *hTERT* reporter assays. *NFX1-123* strongly co-activated with *c-Myc* at the *hTERT* promoter while *NFX1-91* repressed the activity of the promoter. Co-expression of E6 with either isoform increased the activity of the promoter suggesting that E6 could cooperatively activate *hTERT* with *NFX1-123* and could decrease the repressive effects of *NFX1-91*. Furthermore, co-immunoprecipitation experiments indicated that the E6/E6-AP complex preferentially interacted with the NFX1-91 isoform and stimulated its ubiquitination and degradation.

To investigate NFX1-91 function as a repressor *in vivo* at the endogenous *hTERT* promoter we used shRNA to decrease *NFX1-91* expression in HFKs. Upon *NFX1-91* reduction we saw a derepression of the *hTERT* promoter and elevated levels of telomerase activity in normal human keratinocytes. Increased *hTERT* expression was also seen in telomerase positive E6-expressing HFKs upon *NFX1-91* downregulation. These data, together with the repressive effect of transient expression of *NFX1-91* in *hTERT* reporter assays, strongly support a role for NFX1-91 as a repressor of *hTERT*. A recent study by Lin and Elledge (2003) found three tumor suppressor/oncogene pathways involved in *hTERT* repression. These were Mad1, menin and SIP1, a transcriptional target of the TGFβ pathway (93). Reduced expression of any one of these *hTERT* repressors was sufficient to induce *hTERT* expression in previously telomerase-negative cells. It is striking that these repressors do not seem redundant; instead they are independent. Oncogenic stimulation that abrogates any one of them is sufficient to relieve repression. Therefore it is not implausible that E6/E6AP specifically target NFX1-91 to induce *hTERT* expression.

E6 seems to require an intact proximal E box in the *hTERT* promoter for activity in reporter assays (41,148). It is interesting to note that a putative NFX1 binding site overlaps with the proximal E box. Though reporter assays indicated that the NFX1-123 isoform could function as a co-activator of the *hTERT* promoter, this isoform is highly expressed, stable, and apparently unaffected by E6 expression. NFX1-123, with its two additional zinc fingers and R3H domain, most likely exists in a very different conformation than NFX1-91 that is not recognized by the E6/E6-AP complex. As

structure relates to function, this may also influence the DNA-binding affinity and functional consequences of DNA binding. We speculate that NFX1-91 may have a higher affinity for the X box-like sequences in the *hTERT* promoter and that the instability and repressive functions of NFX1-91 may reside in the unique lysine-rich C-terminus. Whether the co-activation function of NFX1-123 is an integral part of E6-mediated *hTERT* induction remains to be tested.

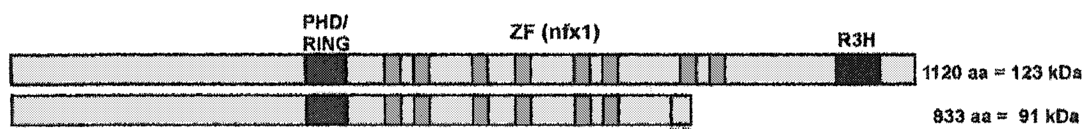
Previous data have indicated that induction of *hTERT* by HPV 16E6 may involve epigenetic phenomenon such as histone acetylation or chromatin remodeling (6,78,80). Many transcriptional regulators function by covalently modifying the histone tails associated with the nearby chromatin. Recently, it has been demonstrated that ubiquitination of histone H2B at lysine 123 may influence the methylation status of histone H3 at lysine 4 and lysine 79 (reviewed in (5)). Methylation at these residues is associated with active transcription. Given that E6/E6-AP and the NFX1 RING finger possess E3 ubiquitin ligase activity, it will be interesting to investigate the ubiquitination status of histone H2B at the *hTERT* promoter in E6-expressing cells. A recent study has found that another *hTERT* repressor, menin, associates with a trithorax family histone methyltransferase complex at homeobox genes (68). Such progressive covalent modifications of histones at the *hTERT* promoter could explain the progressive increases in *hTERT* expression observed with continual passage of E6-expressing cells (6).

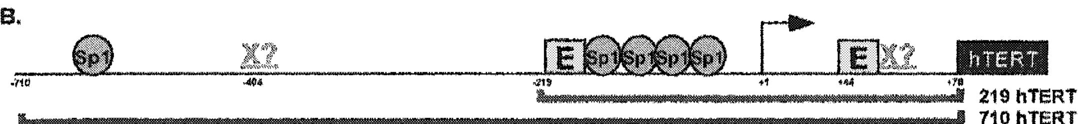
Ubiquitination and transcriptional activation may be intimately linked in a developing theory known as the "suicide model" (5). In this model transcriptional activators bound at the promoter are stimulated by ubiquitination and subsequent

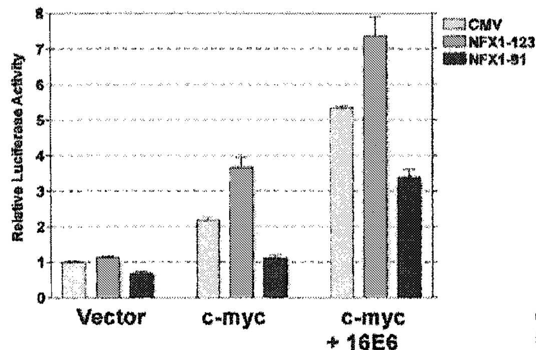
degradation. The activators rely on E3 ligase activity found within co-activators to license their activity. In this way transcriptional activity is tightly linked to increased protein turnover and clearing of the promoter for subsequent rounds of transcription. In fact, the Skp2 ubiquitin ligase is a co-activator of c-Myc at the cyclin D2 promoter and loss of Skp2 can stabilize c-Myc protein levels and reduce transactivation of Myc-responsive promoters (26,76). An attractive synthesis of this model with our data is that E6/E6-AP may function at multiple levels to induce *hTERT*. First, the E6/E6-AP complex may target NFX1-91 for increased turnover to derepress the promoter, and then E6/E6-AP may ubiquitinate and activate the c-Myc bound at the *hTERT* promoter. This theory and the possible role of the E3 ligase activity of the NFX1 isoforms to ubiquitinate either histone H2B or c-Myc remains to be tested.

In summary, we have identified NFX1-91 as a novel cellular repressor of the *hTERT* promoter in primary human epithelial cells. Significantly, interference with its expression is sufficient to induce telomerase expression and extend the lifespan of primary epithelial cells, thus making NFX1-91 an important new target of transformation mechanisms.

Figure 4.1 Alignment of NFX1 homologs from different eukaryotic species. The amino acid sequence of five NFX1 homologs were aligned using the MultAlin program (http://prodes.toulouse.inra.fr/multalin/multalin.html; Corpet 1988). The conserved (open boxes) and identical (black boxes) residues were identified with the ESPript 2.2 program (http://prodes.toulouse.inra.fr/ESPript/ESPript/). The NFX1 homologs from the following species are shown: Human (*Homo sapiens*), Mouse (*Mus musculus*), *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. The RING/PHD finger domain is underlined in blue while the eight NFX1-type zinc finger domains are underlined in green. Zinc finger 5 appears to be truncated and the last two zinc fingers overlap as indicated by the dotted green line. The R3H single-stranded nucleic acid binding domain is underlined in red.







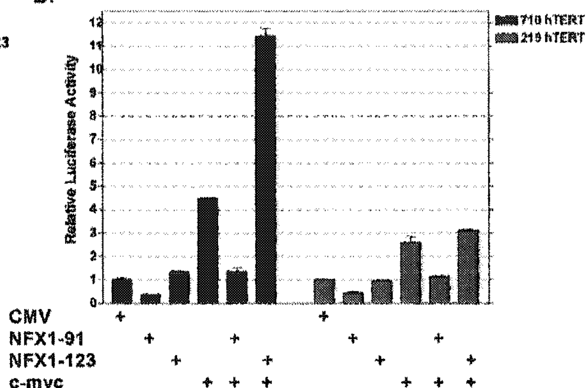


Figure 4.2. NFX1 isoforms have differential repressive and coactivating functions in *hTERT* reporter assays. (A) Schematic of NFX1 isoforms. The blue box represents a PHD/RING finger domain. Multiple green boxes indicate NFX1-type zinc-finger domains. The red box represents an R3H domain present only in NFX1-123. The yellow box indicates the unique lysine-rich C-terminal domain of NFX1-91. (B) Schematic of *hTERT* promoter and the regions included in the luciferase reporter constructs. Several potential SP1 binding sites and E boxes are indicated. Two different regions employed in reporter constructs are indicated. Potential NFX1 binding sites are indicated by X's. (C) *hTERT* reporter assay using the 710 hTERT construct in transient tranfections in HFKS. The indicated genes were co-transfected with CMV, CMV-NFX1-123, or CMV-NFX1-91. The experiment was done in triplicate. (D) *hTERT* reporter assay with two different regions of the promoter in attempt to map the activity of the two NFX1 isoforms. The indicated DNAs were cotransfected with either the 219 hTERT or 710 hTERT reporter construct in HFKs. The data is a representative experiment of 3 trials done in duplicate.

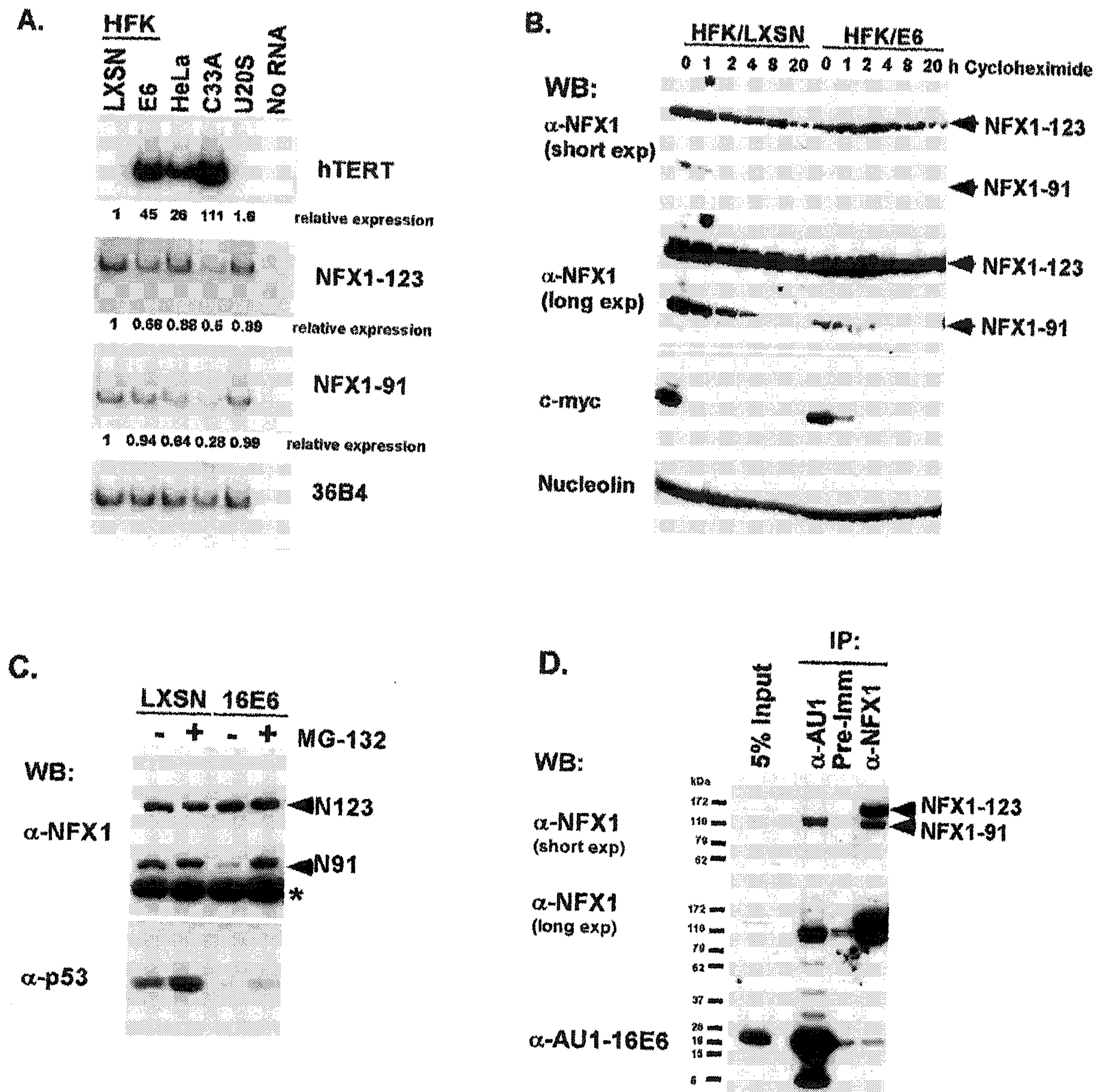


Figure 4.3. NFX1-91 rather than NFX1-123 is targeted by the E6/E6-AP complex. (A) RT-PCR. Expression of the indicated genes were assayed by RT-PCR. Relative expression values were normalized to the *36B4* loading control and are presented relative to HFK/LXSN expression. (B) HFK/LXSN and HFK/E6 cells were treated with 25 ug/ml cycloheximide for the indicated time points. Lysates were assayed for NFX1 expression levels. c-Myc is shown as a positive control for a short-lived protein. Nucleolin is a stable protein used as a loading control. (C) Decreased expression of NFX1-91 is seen in E6-expressing HFKs. Proteasome inhibition with MG-132 restores NFX1-91 protein levels. The asterisk indicates a non-specific background band. p53 expression is shown as a control for proteasome inhibition. (D) Endogenous NFX1-91 co-immunoprecipitates with AU1-tagged 16E6 in transiently transfected 293T cells. A longer exposure of the NFX1 blot shows the presence of a ubiquitin ladder. The reciprocal IP to precipitate AU1-16E6 with NFX1 antibody did not work.

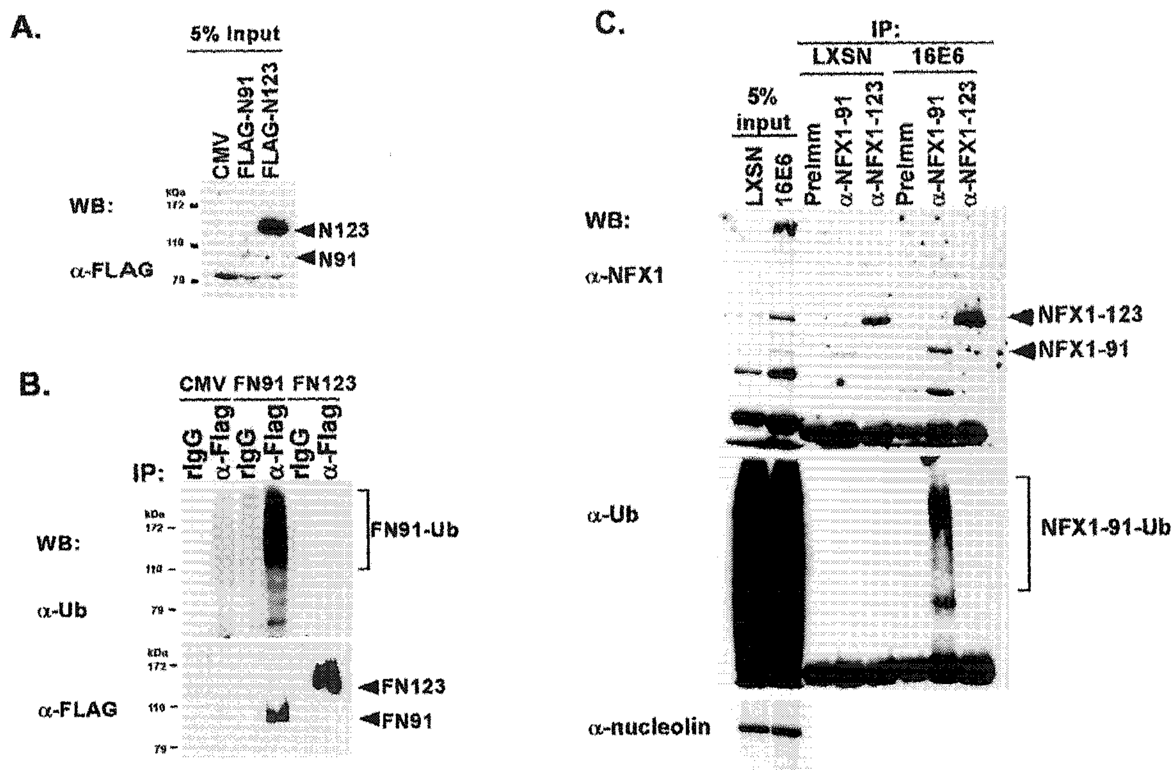


Figure 4.4. NFX1-91 is ubiquitinated by E6-independent and E6-dependent means.
(A) Western blot. 293Ts were transiently transfected with FLAG-tagged forms of NFX1 and cells were treated with MG-132 for 2 hours prior to lysis. 5 % of the lysates used for each IP in (B) was Western blotted with anti-FLAG antibody. (B) The lysates shown in (A) were immunoprecipitated with rabbit IgG (rIgG) or a rabbit anti-FLAG antibody (α-Flag). Western blots with a mouse anti-ubiquitin antibody demonstrated that NFX1-91 is highly ubiquitinated whereas NFX1-123 is not. (C) Endogenous NFX1 isoforms were immunoprecipitated from HFK/LXSN and HFK/E6 cells treated with 20 μM MG-132 with antibodies specific for each isoform or with pre-immune serum. Nucleolin is a loading control for the input lysates.

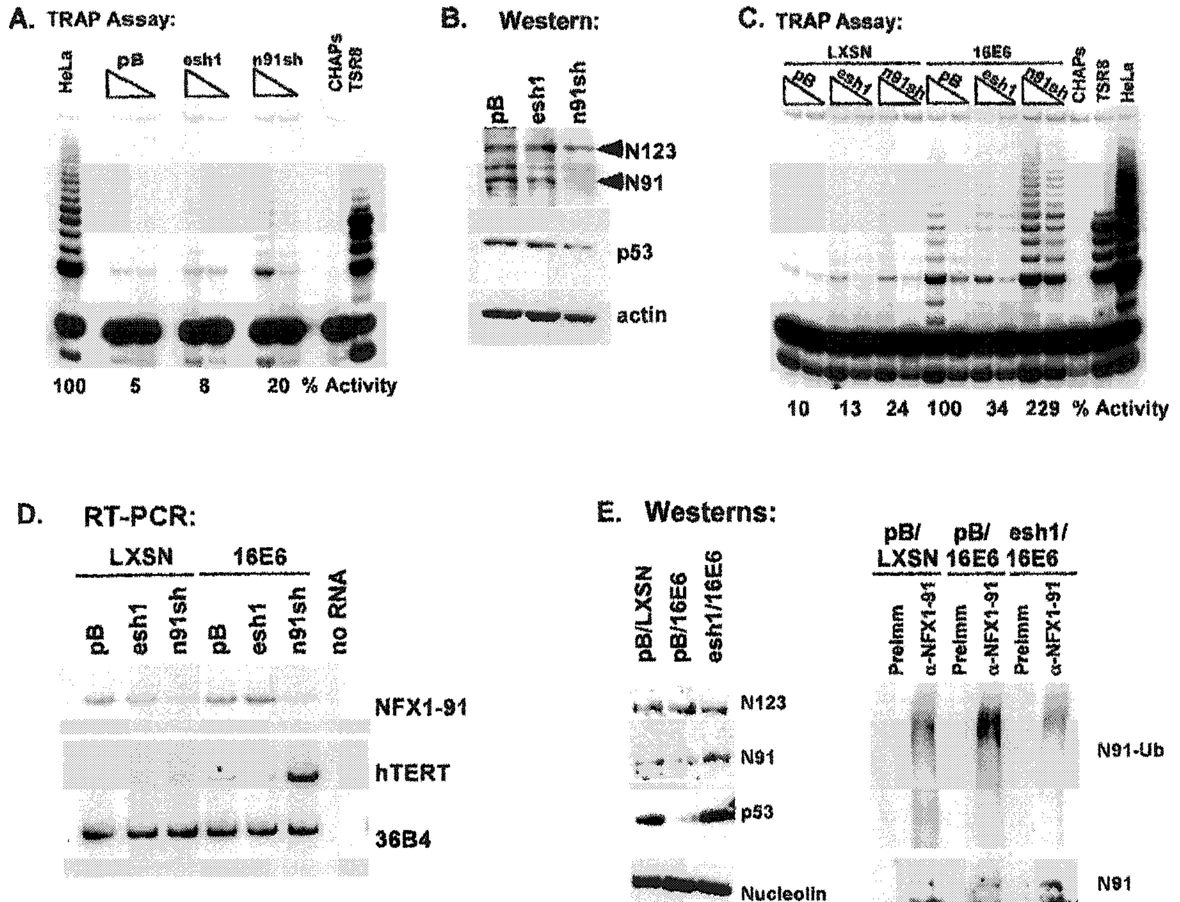


Figure 4.5. Knockdown of *NFX1-91* expression using shRNAs derepresses the endogenous *hTERT* gene in HFKs. (A) TRAP assay. Lysates from HFKs transduced with pB, esh1, or n91sh were analyzed for telomerase activity. 2 ug and 0.5 ug of each lysate were used in the TRAP reactions. HeLa is a positive control lysate (0.2 ug). CHAPs is a lysis buffer negative control. TSR8 is a positive control telomeric template. The percent TRAP activity is presented relative to that in the HeLa lane. (B) Western blot. NFX1-91 protein expression is reduced with expression of n91sh. (C) TRAP assay. The cells shown in (A & B) were subsequently transduced with LXSN or LXSN-16E6. Lysates were examined for telomerase activity. The percent TRAP activity is presented relative to that in the pB/LXSN-16E6 cells. (D) RT-PCR. Expression of *hTERT* and *NFX1-91* was examined in RNA extracts from the cells seen in (C). (E) Western blots. The levels of NFX1-91 protein are reduced in cells expressing E6 but are restored when E6-AP expression is reduced using the esh1 shRNA, as is also observed with p53 protein levels. The right panel is an *in vivo* ubiquitination assay of the lysates shown in the left panel. A stronger NFX1-91-specific ubiquitination signal is seen in the E6-expressing HFKs in comparison to either the vector control cells or the E6-AP-depleted E6-expressing cells. Pre-immune serum was used as a negative control for the immunoprecipitations.

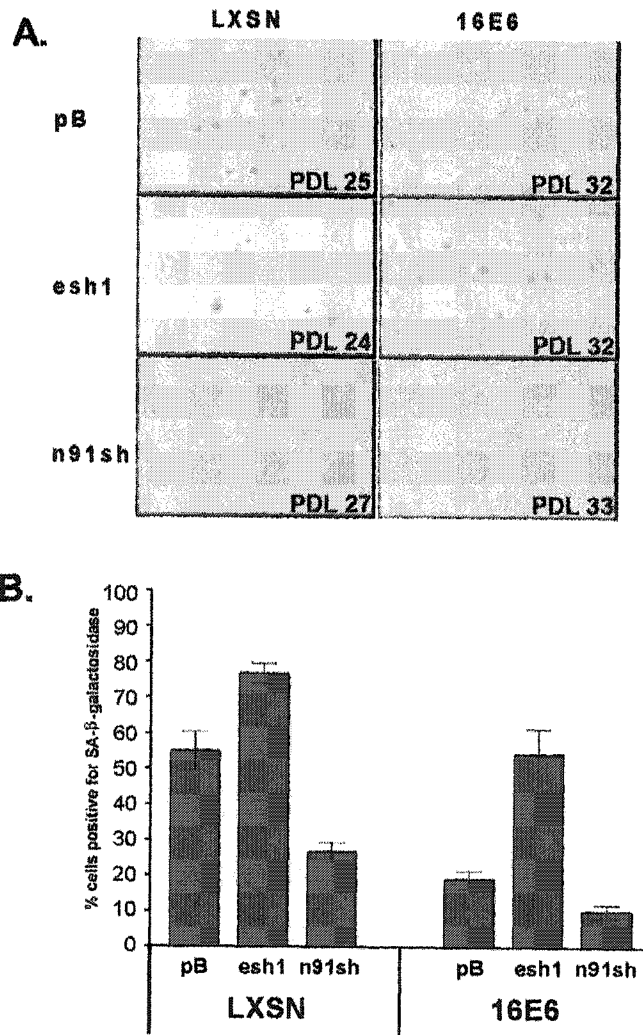


Figure 4.6. Senescence-associated β-galactosidase activity in late passage HFKs.
(A) Micrographs of SA-β-galactosidase staining of late passage HFKs expressing the indicated shRNA and LXSN empty vector or LXSN-16E6. The blue staining indicates senescent cells. PDL (population doubling levels) indicate the relative age of each population of cells.
(B) Quantitation of SA-β-galactosidase staining seen in (A). The data represents the average percent blue cells counted in four different fields at 100 X magnification. The error bars indicate the SEM.

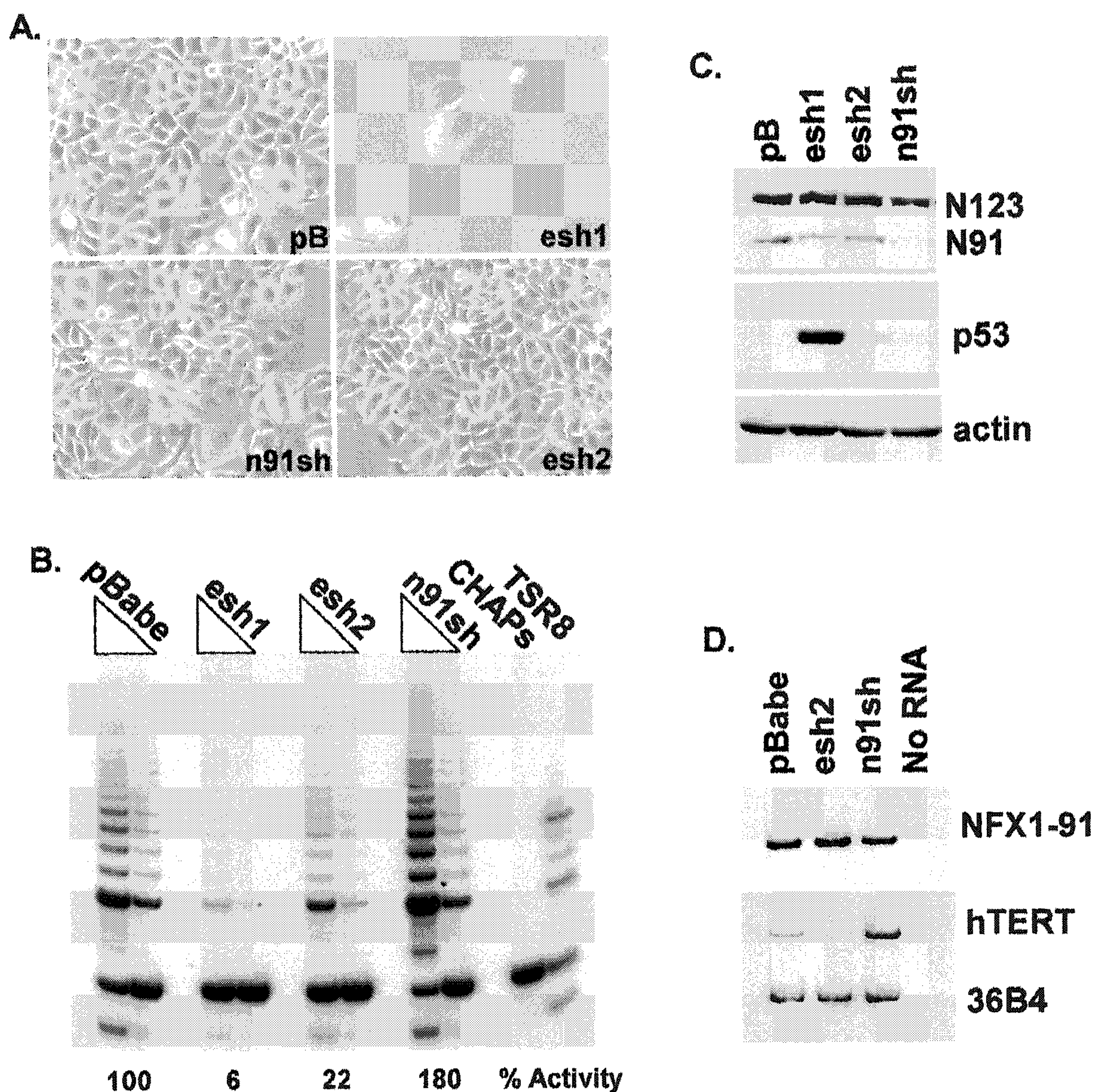


Figure 4.7. Telomerase activity of HeLa cells is affected by reduced expression of either *E6-AP* or *NFX1-91*. (A) Micrographs of HeLa cells expressing the indicated shRNA construct immediately after coming through selection. The esh1 cells were large, flat and arrested for several days and then several small rapidly dividing cells grew out. Cells were harvested for Western blot, TRAP assay, and RT-PCR at the time point pictured in (A). (B) TRAP assay. Extracts from the cells in (A) were assayed for telomerase activity. The percent activity was calculated by phosphorimaging and is normalized to that of the vector control cells. CHAPs is a negative control sample. TSR8 is an artificial template of eight telomeric repeats used as a positive control for the PCR reaction and to quantitate telomerase activity. (C) Western blot. Lysates from the cells in (A) were assayed for NFX1and p53 protein levels. Actin is a loading control. (D) RT-PCR. RNA isolated from the cells in (A) was assayed for *NFX1-91* and *hTERT* expression levels. *36B4* is a loading control.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Induction of telomerase by the human papillomavirus type-16 E6 is critical to the transforming activity of the virus. Though E6 required intact E boxes to induce expression of *hTERT*, the catalytic subunit of telomerase, c-Myc protein levels did not significantly change in response to E6 and c-Myc was associated with the *hTERT* promoter regardless of its transcriptional status. Interestingly, the association of acetylated histones H3 and H4 with the *hTERT* promoter was increased upon E6 expression. Our research further indicated that E6 must bind its cellular partner E6-AP to induce *hTERT*. The function of the E6/E6-AP complex as an E3 ubiquitin ligase prompted the hypothesis that E6/E6-AP may target a repressor of *hTERT* for ubiquitination and degradation. In a search for such possible targets of E6/E6-AP we identified a transcriptional regulator known as NFX1. The *NFX1* gene encodes two splice variants we have termed *NFX1-123* and *NFX1-91*. In *hTERT* luciferase reporter assays, these two isoforms had differential activity; NFX1-123 functioned as a co-activator while NFX1-91 repressed transcription. The E6 oncoprotein preferentially bound the repressive NFX1-91 isoform and targeted it for ubiquitination and decreased stability. Moreover, decreasing *NFX1-91* expression with shRNA was sufficient to relieve repression of the *hTERT* promoter in HFKs. Lower levels of NFX1-91 correlated well with higher telomerase activity and increased lifespan as indicated upon comparison of SA β-galactosidase activity of HFKs passaged for the same time period. Though we have made significant progress toward elucidating the mechanism by which HPV-16 E6

induces telomerase there are still many questions remaining. Many of our findings and additional hypotheses are presented pictorially in Figure 5.1.

Is c-Myc expression required for telomerase induction by E6?

Chromatin immunoprecipitation experiments indicated that c-Myc is bound at the *hTERT* promoter constitutively in HFKs (Figure 2.3 A & B), yet the acetylation status of histones H3 and H4 increased upon expression of E6 (Figure 2.3 C & D). Transactivation of certain genes (including *hTERT*) by c-Myc has been shown to require TRRAP, a subunit of a large HAT complex (113). E6 may be directly involved in recruiting a HAT complex to c-Myc at the *hTERT* promoter as it has been published that E6 can directly bind to c-Myc (148), though we have been unable to confirm this interaction. Induction of *hTERT* by E6 required intact E boxes in the *hTERT* promoter. Whether c-Myc is required for E6-mediated telomerase induction should be investigated in the future either by use of dominant-negative mutants of c-Myc or by silencing of c-Myc with shRNAs. The main caveat of these experiments is that c-Myc function is important for normal cell cycle progression and is involved in protein biosynthesis pathways. Disrupting c-Myc may therefore affect telomerase induction indirectly.

The crystal structure c-Myc/Max heterodimers suggests that oligomerization of c-Myc/Max may be another mechanism of transactivation. The c-Myc/Max heterodimer can form a bivalent heterotetramer in physiological conditions (107). Many c-Myc-responsive promoters contain several E boxes separated by 100-200 nucleotides. If heterodimers bound to E boxes within a promoter heterotetramerize, a DNA loop may form a structure similar to an enhanceosome that then recruits other co-factors to induce

transcription. Evidence of c-Myc/Max oligomerization and DNA looping has been observed by electron microscopy (107). We do not know if the c-Myc we detected at the *hTERT* promoter is bound to one or both of the E boxes or if E6 expression affected E box occupancy, but this may provide an additional layer of transcriptional regulation. It is interesting to note that a proposed NFX1 binding site overlaps with the proximal E box and may therefore play a role in prohibiting c-Myc binding to this site.

Another emerging model of c-Myc-regulated transcription involves recruitment of the ubiquitin/proteasome system to promoters thereby tightly linking active transcription to protein degradation. Skp2, a member of the SCF ubiquitin ligase complex, has been shown to function as a positive co-factor for transactivation by c-Myc. Skp2 recruits the proteasome by ubiquitinating c-Myc and possibly other proteins at the promoter (26,76). Experiments in yeast have shown that proteasome recruitment to promoters may play a non-proteolytic role in stimulating transcription elongation by RNA polymerase II (36). It is currently unknown whether Skp2 may also ubiquitinate histone H2B or play a role in regulating c-Myc's interaction with TRRAP. As many of the proteins we have identified to be involved in E6-mediated *hTERT* induction are associated with the ubiquitin/proteasome system, a more detailed dissection of binding partners and enzymatic activities is required to understand the molecular details of telomerase activation.

Is E6/E6-AP functioning as a co-activator or an E3 ubiquitin ligase?

E6-AP has been shown to function not only as an E3 ubiquitin ligase but also as a coactivator for the steroid receptor family. This coactivation function has been

demonstrated *in vitro* by transient transfection assays (110) and *in vivo* in the E6-AP null mouse (128). Phenotypic characterization of male and female steroid action in the E6-AP null mouse suggests that E6-AP may mediate only a subset of steroid hormone actions (128). We have demonstrated with shRNA silencing that E6-AP expression is required for telomerase induction by E6 (Figure 3.2). Our identification of NFX1-91 as a repressor of *hTERT* targeted for degradation by E6/E6-AP suggests that the E3 ubiquitin ligase function rather than the coactivator function of E6-AP is involved. This issue can be addressed in the future using an E6-AP mutant with an amino acid substitution at the active site cysteine (C833A) that renders the protein inactive in ubiquitin ligation. This mutation is reported to function in a dominant-negative manner with regard to p53 degradation when overexpressed in E6-expressing cells as it titrates E6 away from active E6-AP (140). If this mutant also affects E6-mediated telomerase induction, then the ubiquitin ligase function of E6-AP is required. If not, then E6/E6-AP may be functioning as a coactivator complex at the hTERT promoter.

As described previously for Skp2, the coactivation function of E6-AP may be intimately linked to its ubiquitin ligase activity. E6/E6-AP has been reported to ubiquitinate c-Myc and increase its turnover (129). In this way, silencing of E6-AP may function just as silencing of Skp2 does to decrease transactivation of c-Myc-responsive promoters (129). Interestingly, recent studies have found that estrogen signaling involves a cycling of unliganded and liganded estrogen receptors bound to target promoters that is dependent on the recruitment of E3 ligases and components of the proteasome (96,119). Therefore, the coactivation function of E6-AP may be dependent upon its ubiquitin ligase

activity. However, previous mutational analysis has been able to separate the coactivation and ubiquitin ligase functions of E6-AP, with the majority of mutations seen in patients with Angelman syndrome apparently affecting only the ubiquitin ligase function (110).

Does NFX1 directly bind the *hTERT* promoter?

Using shRNA to reduce expression of *NFX1-91* in telomerase-negative HFKs, we were able to derepress the *hTERT* promoter and stimulate telomerase activity. Though this is strong *in vivo* evidence that NFX1-91 acts to repress the *hTERT* promoter, we have not demonstrated that NFX1-91 directly binds the *hTERT* promoter. In electrophoretic mobility shift assays (EMSA), recombinant NFX1-123 can shift the putative X box regions of the *hTERT* promoter (data not shown). Attempts to repeat these assays with nuclear extracts form cells overexpressing the two NFX1 isoforms, however, were largely unsuccessful (data not shown). These experiments are complicated because, as seen in figure 4.4A, *NFX1-91* does not overexpress well even in transient transfections perhaps due to its apparently short half-life. Two avenues to address NFX1 binding to the hTERT promoter are proposed. First, recombinant NFX1-91 will be generated for use in EMSA. Second, as mentioned in chapter four, we have developed specific antibodies for each of the NFX1 isoforms that can be used in chromatin immunoprecipitation experiments. Whether the NFX1 isoforms are differentially bound to the *hTERT* promoter upon E6 expression will be investigated.

What are the binding partners of the NFX1 isoforms and how do these influence their functions?

Though NFX1-123 could coactivate the *hTERT* promoter in luciferase assays, we have no further evidence of a functional role for NFX1-123 in *hTERT* induction by E6. An amino acid substitution of one of the cysteines in the RING finger domain abolished coactivation by NFX1-123 (data not shown). Furthermore, DNA tethering experiments on an artificial promoter demonstrated that NFX1-123 could generally function as a coactivator (data not shown); that is, this function was not unique to the *hTERT* promoter. Whether NFX1-123 and NFX1-91 interact with each other and/or regulate each other's function remains to be investigated. Since the two isoforms had opposing transcriptional activity at the *hTERT* promoter, it will also be interesting to investigate whether they have associated HAT or HDAC activity. To identify possible binding partners of each NFX1 isoform, we will use immunoprecipitation and mass spectrometry analysis. This will be done using the TAP (tandem affinity purification) tagging system to try to decrease the amount of non-specific protein binding (63,120). Identification of specific NFX1 binding proteins will hopefully provide clues to the biological functions of the proteins.

Though NFX1-91 functioned as a repressor of *hTERT* in luciferase assays, we have not clearly demonstrated that overexpression of *NFX1-91* can repress *hTERT in vivo*. As mentioned above, *NFX1-91* does not overexpress well. The NFX1 isoforms share the same N-terminal sequence and variant C-terminal sequence. Perhaps the instability and repressive functions of NFX1-91 may be attributed to the twenty-five

unique amino acids at its C-terminus. Within those twenty-five amino acids are four lysine residues, or potential ubiquitin ligation sites. Mutational analysis of these residues will indicate whether they regulate NFX1-91 protein stability and repressor function. Perhaps an NFX1-91 stable mutant protein will be a dominant repressor of the *hTERT* promoter. Likewise, mutational analysis of the C-terminus of NFX1-123, encoding a proposed single-stranded nucleic acid binding domain (R3H), may help us determine the function of this protein as well as this domain.

Both isoforms of *NFX1* contain a RING finger domain that appears to be commonly associated with the ubiquitination pathway (97). In *in vitro* experiments using a recombinant segment of NFX1 encompassing the RING finger domain, the NFX1 RING finger was capable of autoubiquitination (97). Whether both isoforms of *NFX1* are able to function as E3 ubiquitin ligases remains to be investigated. This can be addressed using full-length recombinant proteins in an *in vitro* ubiquitin ligation assay with partially purified components of ubiquitin cascade.

Why does HPV induce telomerase?

One of the most interesting questions remaining to be addressed is why did the human papillomavirus evolve the ability to induce telomerase. What evolutionary advantage does telomerase provide? It is difficult to imagine why the virus would need to significantly increase the lifespan of epithelial cells. A normal productive viral infection relies on a balance of proliferation and differentiation for replication and assembly of the virus. The sloughing off of differentiated epithelial cells is required for release of newly synthesized virus, therefore extending the lifespan of epithelial cells

appears unnecessary and even undesirable to the fitness of the virus. One possible explanation is that telomerase induction is an unintentional byproduct of HPV that is merely associated with the shift from a strictly differentiated phenotype to a more proliferative stem cell-like state. Interestingly, expression of E6 in the context of the entire HPV-16 genome does not induce significant levels of telomerase expression (133).

Several recent reports have indicated that telomerase may have additional roles other than the maintenance and extension of telomeres. Telomerase may also play a role in tumorigenesis, cell survival and DNA repair (reviewed in (44)). Several attempts to generate tumor cells with defined genetic alterations have all included introduction of constitutively expressed *hTERT* (34,53). Significantly, an attempt to generate tumor cells that use the recombination-based ALT pathway of telomere maintenance still required introduction of telomerase. An epitope-tagged catalytically active *hTERT* that is defective in telomere maintenance was sufficient for transformation (98). Additionally, overexpression of *mTERT* in the tissues of transgenic mice results in increased wound healing and increased tumor incidence (3,42). These experiments suggest that *hTERT* may be an oncogene.

Telomerase overexpression in several cell types seems to promote cell proliferation and survival. *hTERT*-expressing BJ fibroblasts proliferate faster and have enhanced colony forming activity compared to control cells (37). In epithelial cells, telomerase expression induces growth-promoting genes such as the epidermal growth factor receptor (EGFR) and reduces the growth factor requirements of these cells in culture. Furthermore, silencing of *EGFR* inhibits the enhanced proliferation phenotype

of the cells (129). Similarly, the GM847 cell line expressing *hTERT* has reduced growth factor requirements (98). In unpublished data from our lab, telomerase expression in human foreskin fibroblasts appears to decrease contact inhibition allowing cells to grow to a higher density than control cells in culture (11). Therefore, telomerase appears to have survival- and growth-related functions in addition to and perhaps distinct from its role in telomere maintenance. Induction of telomerase by HPV probably evolved to take advantage of these growth-promoting factors rather than an increased lifespan of epithelial cells.

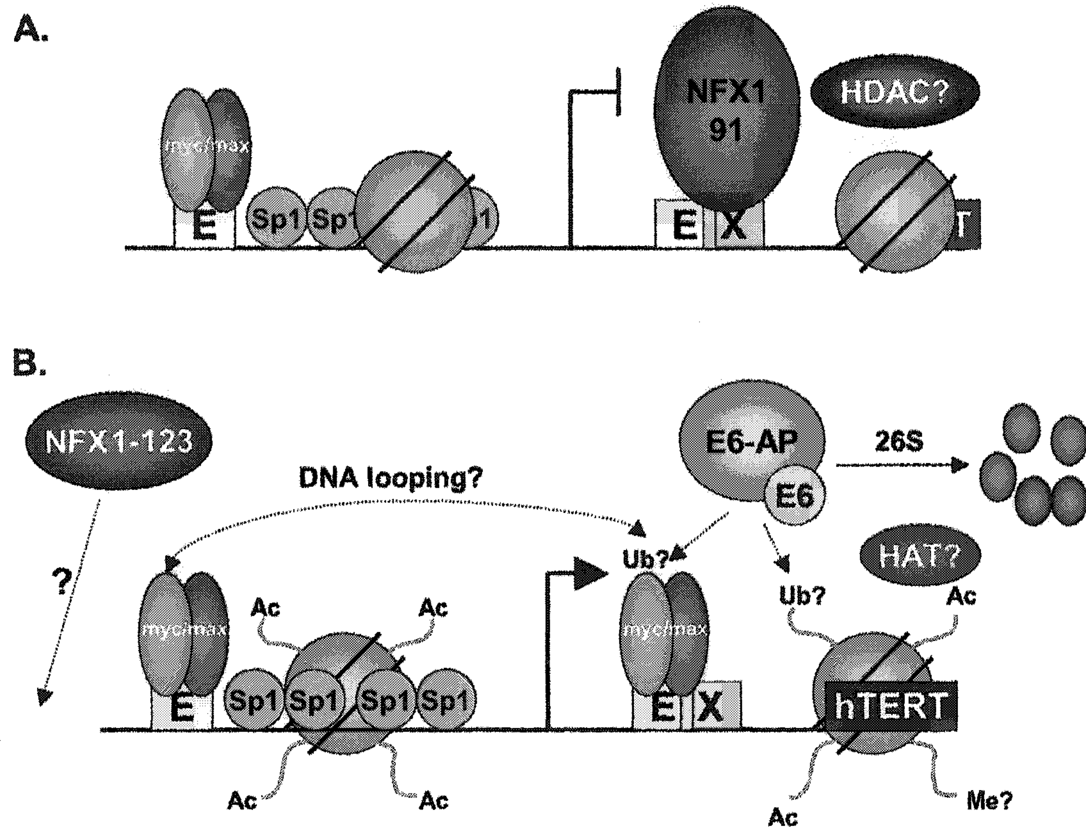


Figure 5.1. Models of the *hTERT* promoter in the absence and presence of E6 expression. (A) In the absence of E6 expression, c-Myc is bound at the *hTERT* promoter with the NFX1-91 transcriptional repressor possibly prohibiting c-Myc binding to the proximal E box. The histones of the associated nucleosomes are deacetylated. (B) In the presence of E6 expression, the E6/E6-AP complex targets NFX1-91 for ubiquitination and degradation via the 26S proteasome. E6/E6-AP may also ubiquitinate c-Myc and/or histone H2B. Ubiquitination of histone H2B is often accompanied by subsequent methylation of histone H3. Chromatin immunoprecipitation data has shown that histones H3 and H4 are acetylated upon E6 expression. c-Myc/Max heterodimers bound to the two E boxes separated by approximately 200 base pairs may heterotetramerize and cause DNA looping to recruit the RNA polymerase II transcriptional machinery and cofactors. Whether NFX1-123 plays a role in E6-mediated *hTERT* induction remains to be tested; luciferase data suggested that co-activation by NFX1-123 requires sequences upstream of the distal E box. All dotted lines and question marks indicate hypotheses that remain to be investigated.

CHAPTER 6

MATERIALS AND METHODS

Cell Culture. Primary human keratinocytes (HFKs) were derived from neonatal foreskins. HFKs were grown in EpiLife medium supplemented with calcium chloride (60 μM) and human keratinocyte growth supplement (Cascade Biologics). 293T, HeLa, C33A, and U20S cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% fetal bovine serum and penicillin-streptomycin. SF9 cells were grown in SF 900 II serum-free medium (Gibco-BRL) containing 5% fetal calf serum and gentamycin.

Plasmids. The E6-AP and NFX1-91 shRNA constructs were generated by previously described methods (46,116,129). Breifly, oligos containing the 26-29 nt shRNA sequence were used in PCR reactions to clone the U6 or H1 RNA polymerase III promoter upstream into a pBabe-puro-based vector. The E6-AP shRNA targeted sequences were: esh1 5'CTAATAGAACGCTACTACCACCAGTTAAC3', esh2 5' AGAGATTGTTGAAGGCCATCACGTATGCC3', and esh3 5'ACAATGAAGAAGATGA TGAAGAGCCCATC3'. The n91sh construct was targeted to the 3'UTR region (5' TGTGGAACCAGCCCAACTGCCCATCAGTCAA3'). The NFX1-123 isoform was PCR cloned from a HeLa cell cDNA library while the NFX1-91 isoform was cloned from a fetal brain cDNA library. These genes (with and without a FLAG-tag) were subsequently inserted by restriction digest or via the

GATEWAY recombination-based system (Invitrogen) into a CMV-based vector for transient transfections. The AU1 tag (DTYRYI) was fused to the N-terminus of 16E6 by PCR and subsequently cloned into a CMV-based vector using the GATEWAY system (Invitrogen). The pBabe-c-myc vector was obtained from Carla Grandori. The pGL3-based *hTERT* luciferase reporter constructs have been previously described (41).

Retroviral Infections. Retroviruses were produced either in established viral producer cell lines (PA317 or PG13) or in 293Ts by a transient VSV-G-pseudotyped virus production protocol as previously described (7). Cells were generally infected at approximately 60% confluence in 6-cm tissue culture plates. Twenty-four hours after infection cells were expanded to 10-cm plates and allowed to adhere to the plate for four to twenty-four hours before adding selective media. HFKs were selected in 0.5 μg/ml puromycin or 50 μg/ml G418. HeLas and C33As were selected in 1.5 μg/ml puromycin. Cells expressing shRNAs were maintained in puromycin-containing media.

Chromatin immunoprecipitations. Proteins are cross-linked to the DNA in formaldehyde solution (50 mM HEPES, pH 8, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, 11% formaldehyde) for 10-20 minutes at room temperature. Fixation is stopped with 1/20 volume of 2.5M glycine and incubated 10 minutes on ice. Cells are scraped from the plate and pelleted. The pellet is washed in Paro Rinse I (10 mM Tris, pH 8, 10 mM EDTA, 10 mM EGTA, 0.25% Triton X-100, 10 mM sodium butyrate, protease inhibitors) 5 minutes on ice. The pellet is washed twice in Paro Rinse II (10 mM Tris, pH

8, 1 mM EDTA, 0.5 mM EGTA, 0.2 M NaCl, 10 mM sodium butyrate, protease inhibitors). The pellet is then resuspended in Paro resuspend (same as Paro Rinse II without the NaCl) and sonicated 6-10 times for 15 seconds in an EtOH ice bath. To solubilize proteins, add sarcosyl to a 1% final concentration and incubate at room temperature for 20 minutes with rotation. Spin the lysate at full speed for 15 minutes in a microcentrifuge and quick-freeze the supernatant or dilute with 2 X RIPA for IP. Immunoprecipitations are washed 6-8 times in wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl) and eluted for 20 minutes at room temperature with rotation in 1% SDS, 0.1 M sodium carbonate. To reverse the crosslink, add NaCl (to 200 mM final) and RNAseA (to 0.2 mg/ml final) and incubate at 55°C for 2 hours. Digest proteins with proteinase K at 55°C overnight. DNA is purified with a phenol/chloroform extraction and ethanol precipitation and then used in PCR reactions.

GST pulldown assays. GST-tagged E6AP and E6 proteins were produced and purified as previously described (70). These proteins were incubated with ^{35}S-radiolabeled E6 proteins (TNT kit, Promega) or lysates from cells transiently transfected with HA-tagged E6-AP for 2 hours in binding buffer (PBS, 1% NP-40, 2mM DTT, protease inhibitors) at 4°C. Complexes were precipitated with glutathione sepharose (Amersham Pharmacia) and analyzed by SDS-PAGE and autoradiography or Western blot.

Northern blotting. Total cellular RNA was isolated using the RNeasy kit (QIAGEN). From 20 to 60 μg of total RNA was electrophoresed on 1% agarose-formaldehyde gels,

transferred to Hybond-N membranes (Amersham), and hybridized to ^{32}P-labeled probes. The E6-AP probe was generated by random primer labeling (Roche) a 440 bp XhoI fragment of the E6-AP gene. The 36B4 loading control probe has been described before (78).

Yeast two-hybrid screen. The yeast two-hybrid screen was performed using the Matchmaker GAL4 two-hybrid system (Clontech). A catalytically defective E6-AP mutant (C833A) was fused to the GAL4 DNA-binding domain in the pGBT9 plasmid. The 16E6 gene was also cloned into this plasmid under the control of the ADH2 promoter. Both a HeLa cell cDNA library and a fetal brain cDNA library were fused to the GAL4 activation domain in the pGAD and pACT2 plasmids respectively. Clones that grew on selective media were subjected to a secondary screen for β-galactosidase activity. Full-length clones were obtained by 5' RACE (rapid amplification of cDNA ends) using the Marathon cDNA amplification kit (Clontech).

Luciferase assays. Luciferase assays were performed as previously described (41). Briefly, HFKs were grown to 50-60% confluence in 6-well plates. They were transfected with a pGL3-based hTERT reporter plasmid (710 hTERT or 219 hTERT) and CMV- or pBabe-based expression constructs. FuGENE6 (Roche) was used for transfections in a 1:3 DNA:FuGENE ratio. Each well was transfected with a total of 2 ug of DNA. Cells were incubated for 24 hours after transfection, rinsed in phosphate-buffered saline and lysed in the well by freeze thawing in 100 μl of reporter lysis buffer (Promega). Cell

debris was removed by centrifugation. Luminescence was quantitated in 10 μl of each lysate upon mixing with luciferase assay buffer (Promega) on a Monolight 2010 luminometer. Each experiment was done in duplicate or triplicate and normalized for total protein concentration.

RT-PCR. RNA was isolated from cells using the RNeasy kit (QIAGEN). cDNA was synthesized using random hexamers and the Superscript II reverse transcriptase system (Invitrogen). To remove remaining RNA, RNase H (2 units) was added to the reaction and incubated for 20 minutes at 37°C. Expression of hTERT, NFX1-123, NFX1-91, and 36B4 was detected by PCR using a touchdown PCR protocol. PCR products were detected either by ethidium bromide staining or by [32P]α-dCTP incorporation and autoradiography. Sequences of hTERT and 36B4 primers are previously described (41). NFX1-123 primers are (F) 5'TCCCTCCCATGAACAGAGAC3' and (R) 5' TTTCAAGCACACCTGTCAGC3'. NFX1-91 primers are (F) 5'TTACCCTCCAGTTCCCTGTG3' and (R) 5'CATGCGTGTGCAGGTATCTT3'.

Telomerase activity. Telomerase activity was detected using the radioisotopic detection method of the TRAPeze telomerase detection kit (Serologicals Corporation, Chemicon International).

Generation of rabbit polyclonal antibodies. Rabbit polyclonal antibodies were generated to recognize both NFX1 isoforms (α-NFX1) and each isoform, NFX1-123 (α-

NFX1-123) and NFX1-91 (α-NFX1-91), individually. Recombinant full-length NFX1-123 was generated in SF9 cells using a baculovirus system. Briefly, NFX1-123 was cloned into the pVIC1 plasmid in which NFX1 is fused to a chitin binding domain with an intervening intein sequence as described previously (18,117). This construct was transfected into SF9 cells using the BaculoGold system (Pharmingen) to generate infectious baculovirus. SF9s infected with baculovirus were harvested and lysed in buffer M (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 500 mM sodium chloride, and COMPLETE protease inhibitor tablet). The SF9 protein lysate was pre-cleared on cellulose resin and subsequently purified with chitin beads. Cleavage of full length NFX1-123 from the chitin-binding domain is done in two volumes of buffer M plus 0.5% Tween-20, 4 mM dithiothreitol, and 5% glycerol incubated at 16°C overnight. This protein was used to generate a rabbit polyclonal antibody to recognize both NFX1 isoforms. The NFX1-123-specific antibody was raised to recombinant His-tagged C-terminal fragment of NFX1-123 (a.a. 932-1120) expressed in and purified from *E. coli*. The NFX1-91-specific antibody was raised to a C-terminal peptide (Ac-CASTQKKRSHYMKKIPAH-amide) generated by BIOSOURCE.

Western blotting. Whole cell lysates were prepared for Western blotting by trypsinizing cells, washing with phosphate-buffered saline, and resuspending in WE16th lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 20% glycerol, 10 μM zinc chloride, 2 mM dithiothreitol, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a complete protease inhibitor tablet [Roche]). Lysates were then sonicated and clarified by

centrifugation. Nuclear and cytoplasmic extracts were prepared as previously described (41). Protein concentrations were determined by using the DC protein assay (Bio-Rad). Protein lysates were electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blots were performed with goat anti-actin (Santa Cruz Biotechnology, I-19), mouse anti-p53 (Oncogene Science, Ab6), mouse anti-c-myc (Santa Cruz Biotechnology, C-33), mouse anti-nucleolin (Santa Cruz Biotechnology, C-23), rabbit anti-AU1 (Bethyl), mouse anti-Flag (Sigma, M2), and mouse anti-ubiquitin (Covance, P4G7). Rabbit polyclonal NFX1, NFX1-123, and NFX1-91 antibodies are described above.

Nuclear extracts. Nuclear extracts were made as previously described (123) except the homogenization step was omitted.

Proteasome inhibition and cycloheximide treatment. In proteasome inhibition experiments, cells were treated with 10-20 μM MG-132 (Calbiochem) or an equal volume of dimethyl sulfoxide (solvent control) for 4 hours at 37°C. For half-life analysis, HFKs were treated with 25 μM cycloheximide (Calbiochem) and harvested in WE16th lysis buffer at the indicated time points. Protein levels were then examined by Western blotting for NFX1 as described above.

Immunoprecipitations. 293Ts at 60% confluence in 15-cm plates were transfected with 20 μg of CMV-AU1-16E6 with FuGENE (Roche). Twenty-four hours later, cells were treated with 10 μM MG-132 (Calbiochem) for 2 hours prior to harvest. 293Ts were

harvested for immunoprecipitation by rinsing with cold phosphate-buffered saline. Cells were pelleted and resuspended in an NP-40 lysis buffer (1 X PBS, 0.5% NP-40, 10% glycerol, 10 μM zinc chloride, 2 mM dithiothreitol, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a COMPLETE protease inhibitor tablet (Roche). Cells were lysed by quick freeze in liquid nitrogen and thaw in a room temperature water bath. Cell debris was pelleted at 14,000 rpm for 15 minutes. Cell lysates were precleared by rotating at 4°C with 50 μl of protein A agarose (Roche). After centrifugation to remove the beads, immunoprecipitations were with the appropriate antibody at 4°C for 1-2 hours and purified by adding protein A agarose and rotating for another hour at 4°C. Immunocomplexes were washed three times with lysis buffer and eluted by heating for 10 minutes at 70°C in 2X sample buffer. Elutions were electrophoresed on NuPAGE 4-12% Tris-Bis gradient gels (Invitrogen) to resolve both AU1-tagged 16E6 and NFX1 isoforms and Western blotted as described above.

***In vivo* ubiquitination assay.** Cells were treated with 20 μM MG-132 for 2 hours prior to harvest. Lysates were prepared by trypsinizing cells and washing with phosphate-buffered saline. Cell pellets were resuspended in 2% SDS in phosphate-buffered saline and boiled for 10 minutes to denature the proteasome and ubiquitin hydrolases. The lysates were sonicated on ice and clarified by centrifugation. Lysates were pre-cleared with protein A agarose at 4°C for 30 minutes. The lysate was subsequently divided for individual immunoprecipitations with the appropriate antibody and incubated at 4°C for 1-2 hours. Pre-immune rabbit serum and normal rabbit IgG (Santa Cruz Biotechnology)

were used as negative controls. To precipitate bound proteins, protein A agarose was added to each immunoprecipitation and rotated at 4°C for 1 hour. Bound proteins were washed three times with phosphate-buffered saline and eluted by boiling for 5 minutes in 2 X sample buffer. Elutions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting as described above.

SA-β-galactosidase staining. Cells were washed three times in phosphate-buffered saline containing 1 mM magnesium chloride and fixed in 3% formaldehyde in phosphate-buffered saline for 5 minutes at room temperature. After three washes in phosphate-buffered saline (pH 6.0), cells were stained in phosphate-buffered saline (pH 6.0) containing 1 mM magnesium chloride, 0.12 mM potassium ferricyanide, 0.12 mM potassium ferrocyanide and 1 mM X-GAL at 37°C overnight.

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