Characterization of the Ubiquitin-Protein Ligase E6-AP by RNA Interference

Inaugural–Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Michael Aloysius D'silva

aus

Mysore, Indien

Köln, 2005

Referees/Berichterstatter: Prof. Dr. Mats Paulsson

Prof. Dr. Jürgen Dohmen

Tag der letzten mündlichen Prüfung: 22.02.06

Acknowledgements

This thesis would not have been completed, if were not for the scientific guidance, encouragement, and patience of Prof. Dr. Martin Scheffner. Thank you very much for giving me this opportunity.

I thank Prof. Dr. Mats Paulsson, Prof. Dr. Siegfried Roth, and Prof. Dr. Jürgen Dohmen for readily accepting to be on my thesis committee. I also thank Dr. Raimund Wagner for agreeing to be the "Beisitzer".

I specially acknowledge the help, guidance, and contributions of Dr. Arnd Hengstermann to this PhD thesis. Your initial training in cell culture has helped me come to love what I once hated the most.

Special thanks to my lab mate Konstantin Matentzoglo for the help during those "dark days". The continuous scientific discussions, arguments, and critical reading of the thesis are also highly appreciated. I also thank Dr. Petric Kuballa for having critically read and offering his comments on my thesis.

Special thanks to my lab mates, Ulrike Kogel and Steffanie Lang for their help with all the German translations. In addition, it has been pleasant to have you as friends and lab mates. I extend my thanks to my erstwhile lab mates Dr. Sandra Glockzin and Dr. Laëtitia Linares for the initial guidance at getting started with my Ph.D work. It has been fun having you'll as lab mates.

A note of thanks to the rest of my labmates and collegues in Konstanz namely, Hans-Peter Wollscheid, Elvira Weber, Iyappan Saravanankumar, Malte Paulsen, Nicole Richter and Thomas Kapitza. It has been fun working with yourll and thanks for offering a helping hand whenever needed. I also thank Christian Strüh and Iris Andernach for their contributions to this work.

A special word of appreciation and thanks goes to Dr. Brigitte v.Wilcken-Bergmann, Dr. Sebastian Granderath and Mrs. Bettina Lauss, who have been very prompt in helping with all the administrative work.

A PhD can be a long and lonely road to travel, especially if you do not have the right people to give you company. However in my case, my graduate school batch mates especially Bhupendra Shravage, Dr. Palani Murugan, Saline Chakkalakal, Ashwin Kotagiri, Sam Mathew, and Dr. Sunita Shankaran made sure that this was an enjoyable one. It was great having you all as batch mates, right from the time we had to compete for our lab rotations. In addition, I also take this opportunity to specially thank Rajesh Singh, Dr. Neelamegan Dhamodharan, Hemavathi Nandhan, and Phaneeshwara Rao for all the help and support. Your company and friendship during this time will never be forgotten.

I am more than grateful to my Mum, Dad and Sister, who have been a source of motivation and strength. Their sacrifices and hard work has helped me become what I am today.

An acknowledgement page is never complete without thanking the funding agency. Thus I take this opportunity to thank the state of Nord Rhein Westfalia for sponsoring the International Graduate School for genetics and functional genomics at the University of Cologne and the financial assistance to complete my PhD in Germany. I also thank Prof. Dr. Maria Leptin and the faculty of the graduate school, who I have had the opportunity to interact with. In addition, I also acknowledge the financial assistance obtained from the University of Konstanz and DFG.

Time and again during these past four plus years as a graduate student in Germany, a number of people have been a source of inspiration, help, and motivation. Although I would have loved to acknowledge each one of you, space restrictions are the only limitation. Hence, I take this opportunity to offer my sincere thanks to those "Good Samaritans" who at some point of time have been of some help.

The good times with the "Cologne Indian Gang" and the "Konstanz Indian Gang" will always be treasured.

Finally, it has been a pleasure to have done my PhD in two nice cities in Germany (i.e. Köln and Konstanz).

Konstanz, Nov 2005 Michael D'silva

Table of contents

Abbreviations

°C	Degree celcius
A	Ampere
AA	Amino Acid
AS	Angelman Syndrome
ATP	Adenosine Tri-Phosphate
bp	base pairs
cDNA	complementary DNA
C-terminus	Carboxy terminus
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
EDTA	Ethylenediamine-tetraacetic acid
EtOH	Ethanol
FBS	Fetal bovine serum
HA	Haemagglutinin
Hr	hour
Hrs	hours
i.e.	id est or that is
IgG	Immunoglobulin G
IP	immunoprecipitation
kDa	Kilodalton
M	Molar
min	minute
mRNA	messenger-RNA
mu	Murine
n	nano
N	Normal
nm	nanometer
nM	nanoMolar
nt	nucleotide
N-terminus	Amino-terminius
O.D	Optical density
ONPG	Orthonitrophenyl-β-D-galactosidase
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	ribosomal RNA
s	second
S	sedimentation coefficient
siRNA	small-interfering RNA
TAE	Tris-acetated-EDTA
Ub	ubiquitin
V	Volt
wt	wild-type
β-Gal	Beta-Galactosidase
µ	micro

1. Introduction

1.1 Ubiquitination: A functional perspective

Ubiquitination (also referred to as "ubiquitin conjugation" or "ubiquitylation") is the post-translational modification of proteins by the covalent attachment of ubiquitin, a 76 amino-acid protein. "Monoubiquitination", the attachment of a single ubiquitin moiety involves isopeptide bond formation between the α-carboxy group of the C-terminal Glycine residue of ubiquitin and the ε-amino group of a lysine residue in the target protein. In addition to being modified by single ubiquitin moieties, proteins can also be modified by chains of ubiquitin ("polyubiquitination"). Polyubiquitin chain formation takes place by subsequent attachment of ubiquitin moieties linked to one of the lysine residues present in the previously added ubiquitin. Of all the known consequences of ubiquitination, the targeting of proteins for degradation has been best characterized. Substrates tagged with a polyubiquitin chain are selectively degraded by a multi-subunit ATP-dependent protease known as the 26S proteasome (reviewed in Glickman and Ciechanover, 2002).

Of the seven lysine (K) residues present in ubiquitin (Figure 1), K48-linked polyubiquitin chains are recognized and targeted to the proteasome. Covalent attachment of K48-linked tetraubiquitin chain has been shown to be necessary and sufficient for recognition and degradation of a model substrate by the 26S proteasome in vitro (Thrower et al., 2000). In addition, K11 and K29-linked polyubiquitin chains are also reported to target proteins to the proteasome (Johnson *et al.*, 1995; Liu *et al.*, 1996). The progression of cell cycle (reviewed in Koepp et al., 1999), transcriptional regulation (reviewed in Muratani and Tansey, 2003), signal transduction (reviewed in Welchman et al., 2005), and antigen presentation (reviewed in Rock and Goldberg, 1999), are just a few of the many processes regulated by ubiquitin-proteasome-dependent proteolysis. However, degradation by the proteasome is not the only fate possible for ubiquitin tagged proteins. K63-linked polyubiquitin chains can signal nonproteolytic, reversible events such as in DNA repair (Spence et al., 1995; Hofmann and Pickart, 1999; Hoege et al., 2002), the initiation of the inflammatory response (Deng et al., 2000), ribosomal function (Spence et al., 2000), and the regulation of certain transcription factors (Kaiser et al., 2000). K63-linked polyubiquitin chains

might also act as a signal for endocytosis in some cases (Galan and Haguenauer-Tsapis, 1997; Springael et al., 1999).

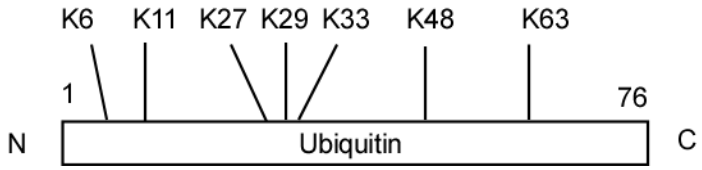

Figure 1: Schematic representation of ubiquitin and position of the lysine residues. Ubiquitin has seven lysine (K) residues, all of which are supposed to be utilized in ubiquitin chain formation. The C- terminal end of ubiquitin has a Glycine residue (G76) through which isopeptide bond formation takes place with the substrate or another ubiquitin moiety.

In contrast to polyubiquitination, monoubiquitination of proteins targets them for endocytosis and lysosomal degradation (reviewed in Hicke and Riezman, 1996; Haglund et al., 2003). In addition, monoubiquitination can also act as a regulatory modification such as in protein sorting, histone function and transcription (reviewed in Hicke, 2001; Katzmann et al., 2002).

No matter what the final outcome is, the whole process of ubiquitination is made possible through a concerted action of at least three enzymes, in a three-step mechanism referred to as the ubiquitin-conjugation cascade (see below). Since ubiquitination regulates a number of cellular pathways, it is unsurprising that deregulation of the ubiquitin-conjugation system has been implicated as a causative factor in cancer and other human diseases (reviewed in Glickman and Ciechanover, 2002; Ciechanover and Brundin, 2003; Pagano and Benmaamar, 2003).

1.2 The ubiquitin-conjugation cascade

As mentioned earlier, ubiquitination of a substrate protein involves the isopeptide bond formation between the α-carboxy group of the C-terminal Glycine residue (G76) of ubiquitin and the ε-amino group of a lysine of a target protein. More rarely, it can also be conjugated to the α-amino group at the N-terminus (Breitschopf *et al.*, 1998; Aviel *et al.*, 2000; Reinstein *et al.*, 2000; Bloom *et al.*, 2003). For ubiquitination to take place the sequential action of three enzymes, namely the ubiquitin-activating enzyme E1 (UBA), ubiquitin-conjugating enzyme E2 (UBC), and ubiquitin-protein ligase E3 is required. Firstly, the E1 adenylates the C-terminus of ubiquitin and then forms a

thioester bond between the C-terminus of ubiquitin and a catalytic E1 cysteine residue (Figure 2).

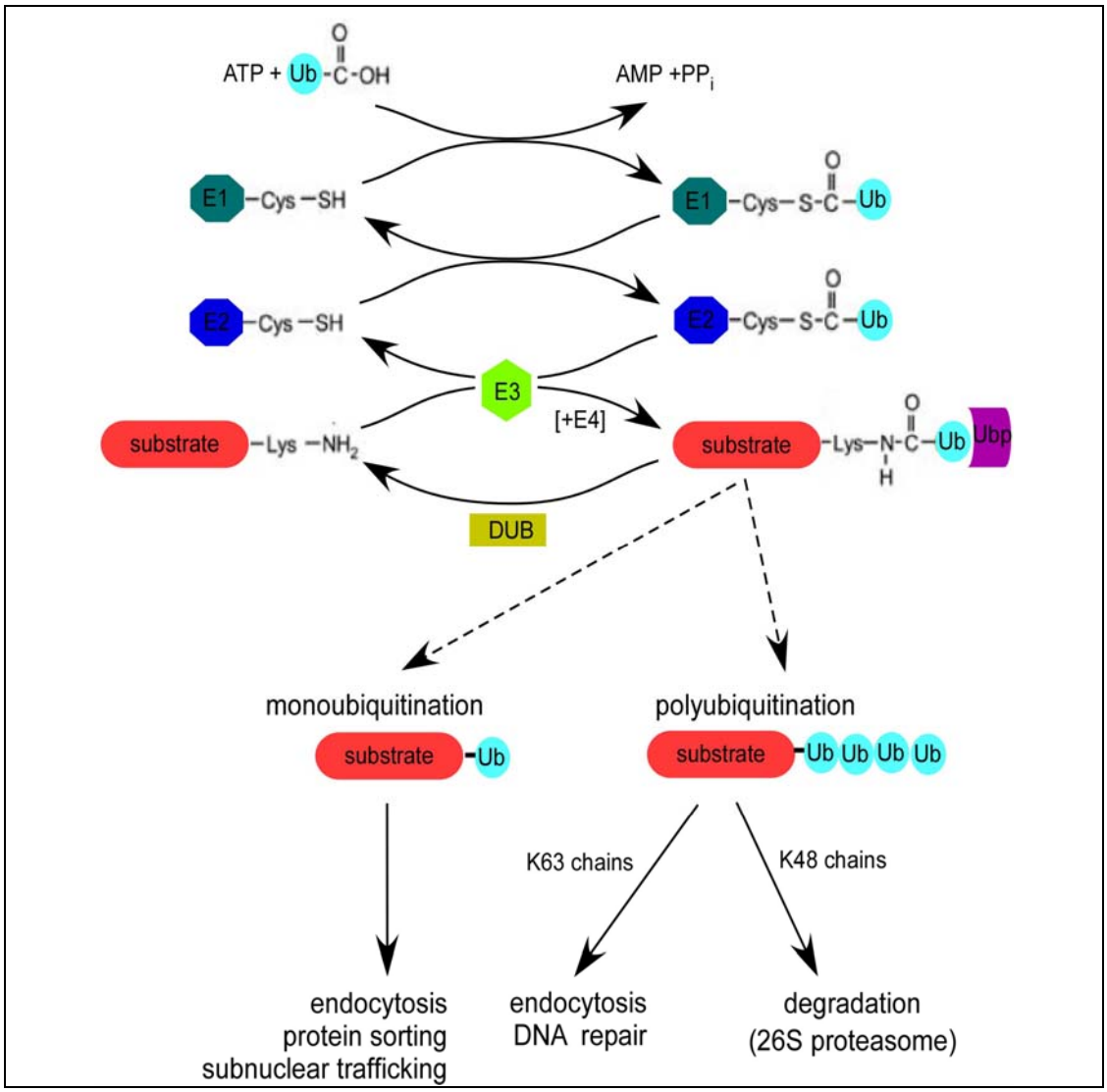

Figure 2: The ubiquitin-conjugation cascade. Free ubiquitin (Ub) is activated in an ATP-dependent manner by the activity of an ubiquitin-activating enzyme (E1), which hydrolyses ATP and forms a thioester bond with ubiquitin. Subsequently, ubiquitin is transferred to one of many distinct ubiquitin-conjugating enzymes (E2). In some reactions, E2s can directly ubiquitinate substrates, whereas others require the help of a ligase (E3). While some E3s have the ability to form ubiquitin thioester during the transfer of ubiquitin to the substrate, others support ubiquitination by recruiting substrates to E2 enzymes. Usually, several ubiquitin molecules, in the form of a polyubiquitin chain, are conjugated to a substrate. In some cases, this requires a specific polyubiquitin chain-assembly factor (E4). Based on the number of ubiquitins (monoubiquitin versus polyubiquitin) and the type of chain linkage (e.g. K48 or K63), the fate of the substrate protein is decided. Ubiquitin modification can simultaneously be removed by deubiquitinating enzymes (DUB). The attachment of ubiquitin to the substrate could also act as signal for the recruitment of ubiquitin-binding proteins (UBP), which could either protect the substrate from deubiquitination and/or act as a bridging factor to transfer polyubiquitinated substrates to the proteasome (Adapted and modified from Passmore and Barford, 2004).

To be fully active, the E1 must non-covalently bind to, and adenylate a second ubiquitin molecule. Secondly, the thioester-linked ubiquitin is transferred from E1 onto the active site cysteine residue of one of a number of E2s, where it is again linked by a thioester bond. Finally, with the help of a third enzyme, the E3 ligase, ubiquitin is transferred from the E2 to a lysine residue of a substrate protein (reviewed in Passmore and Barford, 2004). This final transfer of ubiquitin to the substrate results in an isopeptide bond between a substrate lysine and ubiquitin. In addition, E3s can also catalyze ubiquitin-ubiquitin conjugation to form a polyubiquitin chain. Although the precise mechanisms vary among the different E3s, they all promote the transfer of ubiquitin, either directly or indirectly, from an E2 to a substrate or another ubiquitin. In addition to the aforementioned components of the ubiquitin conjugation cascade, other conjugation factors, or accessory factors have been identified to play a role in either polyubiquitin chain assembly or in delivering ubiquitinated substrates to the proteasome (see below). Furthermore, concurrent to the conjugation of ubiquitin to substrate proteins, the action of deubiquitinating enzymes (DUBs) antagonizes polyubiquitin chain assembly thus adding another layer of selectivity and regulation (reviewed in Wilkinson, 2000).

1.2.1 Ubiquitin-activating enzyme E1

The first evidence towards understanding the physiological role of ubiquitination was demonstrated by using cells expressing a temperature sensitive mutant of E1 (Finley *et al.*, 1984). Cells expressing the temperature sensitive form of E1 underwent cell cycle arrest at the nonpermissive temperature and failed to degrade short-lived proteins. In yeast, E1 has been shown to be an essential enzyme (McGrath *et al.*, 1991). Furthermore, in most eukaryotes, a single E1 activates ubiquitin for all downstream processes. The E1 protein is approximately 100 kDa in size and exists as two isoforms in humans (Cook and Chock, 1992).

1.2.2 Ubiquitin-conjugating enzyme E2

More than thirty E2 enzymes have been identified so far, which mediate transfer of ubiquitin from E1 to E3. While the genome of *S.cerevisiae* encodes 13 E2-like enzymes, there might be up to 96 different E2s in humans (Weissman, 2001; Vaux and Silke, 2005). The family of E2s is structurally well characterized and shares a conserved approximately 150 amino acid core domain through which E2s interact with

E1. In addition, many E2s have a non-conserved amino or carboxy terminal extension, which is supposedly involved in mediating specific interactions with E3s (Pickart, 2001). While most of the E2s identified are less than 35 kDa, there are few exceptions such as the 523 kDa E2 named BRUCE (BIR Repeat containing ubiquitin conjugating enzyme) (Hauser *et al.*, 1998).

1.2.3 Ubiquitin-protein-ligase E3

Of the three principle enzymes involved in ubiquitin conjugation, it is mainly the E3, which mediates substrate recognition. Based on the presence of conserved protein domains and the mechanistic role they play, E3s can be broadly classified into RING E3s and HECT E3s.

1.2.3.1 RING E3

The RING (Really Interesting New Gene) family of E3s is the largest family of E3s identified so far (Borden, 2000). Members of this family contain a RING finger or RING finger-like (see below) domain and are assumed to function predominantly as molecular scaffolds that bring other proteins together rather than as chemical catalysts. The RING finger domain consists of a series of histidine and cysteine residues with a characteristic spacing that allows for the coordination of two zinc ions in a cross brace structure.

RING E3s can further be classified into two subtypes; a) single-subunit RING E3s and b) multi-subunit RING E3s. The single-subunit E3s consist of a single polypeptide that possesses the capacity to recognize the ubiquitination signals in their specific substrates through domains that, in general, are structurally distinct from the RING finger. Single-subunit RING E3s include Mdm2, which ubiquitinates p53 and c-Cbl, which is involved in down-regulation of growth factor receptors (reviewed in Pickart, 2001). In the multi-subunit RING E3s, the substrate recognition and the RING finger are on separate subunits. The SCF (Skp1-Cullin-F-box protein), the APC, and the VCB types belong to this class of multi-subunit RING E3s (reviewed in Deshaies, 1999).

In addition to the RING finger domain two closely related domains, the U-box (Hatakeyama *et al.*, 2001) and the PHD/LAP (plant homeodomain or leukemia-associated protein) domains have shown to confer E3 activity (Boname and Stevenson, 2001). Though these motifs cannot bind zinc, they take up a RING-finger like

conformation and are maintained by salt bridges and hydrogen bonds (Aravind and Koonin, 2000).

1.2.3.2 HECT E3

The family of HECT (Homologous to E6-AP C-Terminus) domain E3s has been named after their founding member E6-AP (further described in section 1.3) (Huibregtse *et al.*, 1995). Members of this family of E3 ligases are characterized by the presence of a conserved catalytically active C-terminal region of approximately 350 amino residues, called the HECT domain. This catalytic activity is mediated by a conserved cysteine residue positioned approximately 35 residues upstream of the C-terminus within the HECT domain, which acts as a site of ubiquitin thioester formation (Huibregtse *et al.*, 1995; Scheffner *et al.*, 1995). HECT domain proteins have a modular structure consisting of the C-terminal HECT domain and a variable N-terminal extension. While the HECT domain is required for interactions with their cognate E2, the N-terminus is presumably involved in determining the substrate specificity of the individual E3. Even though the crystal structure of HECT E3s in complex with their cognate E2s are available (Huang *et al.*, 1999; Verdecia *et al.*, 2003; Zheng, 2003), the mechanism of how exactly ubiquitin is transferred from E2 to E3 and from E3 to the substrate remains ill-defined.

1.2.4 Accessory factors

The conjugation factors labeled as 'E4s' help in polyubiquitin chain assembly by extending nascent chains rather than extending ubiquitin chains already attached to a substrate. This family includes the yeast UFD2 protein that has been shown to display E4 activity by binding to ubiquitin moieties of preformed conjugates and catalyzing polyubiquitination (Koegl *et al.*, 1999). In addition to E4 other accessory factors are involved in recognizing polyubiquitin labeled substrates and delivering them to the proteasome. These include distinct families of chaperones or "shuttle factors" (Hartmann-Petersen *et al.*, 2003).

1.3 E6-AP (E6-Associated Protein)

The cellular ubiquitin-protein ligase E6-AP was originally identified by its ability to interact with the E6 oncoprotein of HPVs associated with cervical cancer and, in complex with E6, to target p53 for ubiquitin-mediated proteasomal degradation

(Huibregtse *et al.*, 1991; Scheffner *et al.*, 1993). As mentioned earlier, it is also the founding member of the HECT family of ubiquitin-protein ligases. The gene encoding E6-AP (*UBE3A*) has been localized to the region q11-q13 of chromosome 15 (Nakao et al., 1994). Five mRNA subtypes of E6-AP encoding three potential isoforms have been identified (Yamamoto et al., 1997). These isoforms are approximately 100 kDa in size and vary at their N-terminal region. Whether these isoforms differ in functionality is not known. In addition to its E3 activity E6-AP has also been reported to serve as a transcriptional coactivator for steroid hormone receptors (Nawaz et al., 1999). Furthermore, these functions have been shown to be separable and independent of one another.

Understanding the physiological role of E6-AP is still of interest because inactivation of *UBE3A* gene has been associated with Angelman Syndrome, a hereditary neurological disorder (see 1.3.4). Moreover, in the case of cervical cancer, the E6/E6-AP complex not only targets p53 for ubiquitin-mediated degradation, but also targets other proteins (see 1.3.3), which is necessary for HPV-induced cervical carcinogenesis.

1.3.1 Human papillomaviruses (HPVs) and cervical cancer

HPVs are small DNA-viruses that belong to the family Papovaviridae. Productive infection with HPVs has long been known to result in benign squamous epithelial lesions, commonly known as warts. More than 130 HPV types have been isolated from epithelial cells of the skin, anogenital, or oropharyngeal mucosa. Of these, some types of HPV have also been found in samples of patients with cancers of the oral cavity, skin cancers of immunosuppressed patients and, most notably cancer of the uterine cervix (cervical cancer) (reviewed in zur Hausen, 1996).

Cervical cancer represents the second most common form of cancer in women. In greater than 90% of the cases, at least one copy of the HPV viral genome has been found integrated into the host-cell genome. Furthermore, continuous expression of the viral E6 (approximately 150 amino acids) and E7 (approximately 100 amino acids) oncoproteins is required for the malignant phenotype of cervical carcinoma cell lines. These oncoproteins have been found to possess cell-transforming and cell-immortalizing potentials in cell culture systems. Taken together, the above evidence has led to the causal association of HPV with cervical cancer (reviewed in zur Hausen, 1996; 2000).

Of around 40 HPV types isolated from the anogenital tract, only few have been associated with malignant lesions. Based on their clinical association with cervical carcinogenesis HPVs can be classified as “high risk” or “low risk” types. High risk HPVs including the type 16, 18, 31, 33, 39, 45, and 52 are associated with malignant lesions. In comparison to high risk HPVs, the low risks HPVs such as type 6 and 11 can be associated with benign lesions such as genital warts (condylomata acuminata).

Studies to elucidate the role of high risk HPVs in cervical carcinogenesis have shown that the E6 protein interacts with the p53 tumour suppressor protein (Werness *et al.*, 1990) and E7 with the retinoblastoma gene product $p110^{RB}$ and the $p110^{RB}$ related proteins p107 and p130 (henceforth collectively referred to as pRB) (Dyson *et al.*, 1989). Subsequently both p53 and pRB have been shown to be targeted for inactivation by ubiquitin-mediated proteasomal degradation (Scheffner *et al.*, 1990; Crook *et al.*, 1991; Dyson *et al.*, 1992; Boyer *et al.*, 1996).

1.3.1.1 E7 and pRB

In normal cells (HPV-negative cells) the pRB family of proteins (also referred to as “pocket proteins”) regulate progression of the cell cycle from G_0/G_1 into S phase, by interacting with the E2F/DP family of transcription factors (referred henceforth collectively as “E2F”). E2F is known to transactivate many genes required for DNA replication. Hence, binding of pRB in its active, growth suppressive form to E2F not only interferes with its transactivation properties, but is also said to convert E2F into a transcriptional repressor (Dyson, 1998), thus limiting the cell from transition into S phase. Cell cycle-dependent phosphorylation of pRB leads to disruption of E2F/pRB complex and reverses the pRB-mediated cell cycle arrest resulting in cell cycle progression. In HPV-positive cells, analogous to the phosphorylation of pRB, binding of E7 to pRB and subsequent degradation results in the release of transcriptionally active E2F complex driving cells into S phase. Since HPVs propagate in differentiated non-dividing cells it is imperative that the infected cells are driven into S phase to allow viral replication (Stubenrauch and Laimins, 1999).

The mechanism of E7 mediated degradation of pRB is unclear. Whether E7 in conjugation with another protein acts as an E3 ligase to target pRB for ubiquitination and degradation is not known. Interestingly, E7 has been shown to directly interact with the S4 ATPase subunit of the 19S regulatory complex of the 26S proteasome

suggesting that E7 could directly target pRB for degradation without prior ubiquitination (Berezutskaya and Bagchi, 1997). However whether this is true remains to be determined.

1.3.1.2 E6 and p53

The p53 tumour suppressor protein is known to play a critical role in cellular responses to various stress signals (Harris and Levine, 2005). In normal cells (HPV-negative cells), p53 is predominantly regulated by a negative feed-back loop with Mdm2, a RING finger ligase that targets p53 for proteasomal degradation (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). However under stress conditions inactivation of the Mdm2 feed-back loop leads to stabilization of p53 levels and increased transcriptional activity, the outcome of which leads to cell-cycle dependent growth arrest, senescence or apoptosis. In contrast to most cancers, wherein the *p53* gene is mutated (greater than 40%), it is rarely mutated in HPV-positive cervical carcinomas (Hainaut *et al.*, 1998). Thus it has been hypothesized that the inactivation of the normal functions of p53 by E6 is similar to the inactivation of p53 by mutation and is a key step in HPV-induced cervical carcinogenesis. In contrast to E7 wherein the biochemical mechanism of pRB degradation remains poorly understood, E6 mediated degradation of p53 has been shown to involve the recruitment of E6-AP (Huibregtse *et al.*, 1991).

1.3.2 The E6-E6-AP-p53 interaction

The biochemical mechanism by which high risk E6s target p53 for inactivation is well characterized (Figure 3). In the initial step, E6 forms a complex with E6-AP. This dimeric complex recognizes p53, binds to it and polyubiquitinates p53 with the help of ubiquitin-conjugating enzymes (UbcH5, UbcH7, or UbcH8). In the final step, p53 is recognized and degraded by the 26S proteasome. It should be noted that E6 or E6-AP alone are not able to enter into a complex with p53 and it is still unclear whether E6 or E6-AP or both actually directly contact p53. In comparison to high risk E6s, the low risk E6s are unable to target p53 for degradation.

Mutational analysis to study the interaction of E6-AP with E6 has identified three functional domains which are required for 1) Binding with E6, 2) E6 dependent association with p53 and 3) E6 dependent ubiquitination of p53 (Figure 4) (Huibregtse *et al.*, 1993). An 18 amino acid region (amino acids 378-395) within the central portion

of E6-AP was found to be necessary and sufficient for binding with E6 of high risk HPVs. The region that directs E6 dependent association with p53 spans approximately 500 amino acids (amino acids 266-768) and includes the E6 binding domain. E6-AP sequences in addition to those required for formation of a stable ternary complex with E6 and p53 are necessary to stimulate the ubiquitination of p53 (amino acids 768-852). These sequences lie within the C-terminal 84 amino acids of E6-AP and encompass the HECT domain. Furthermore, a catalytical cysteine residue (C820) within the HECT domain is required for thioester formation (Huibregtse et al., 1995; Scheffner et al., 1995).

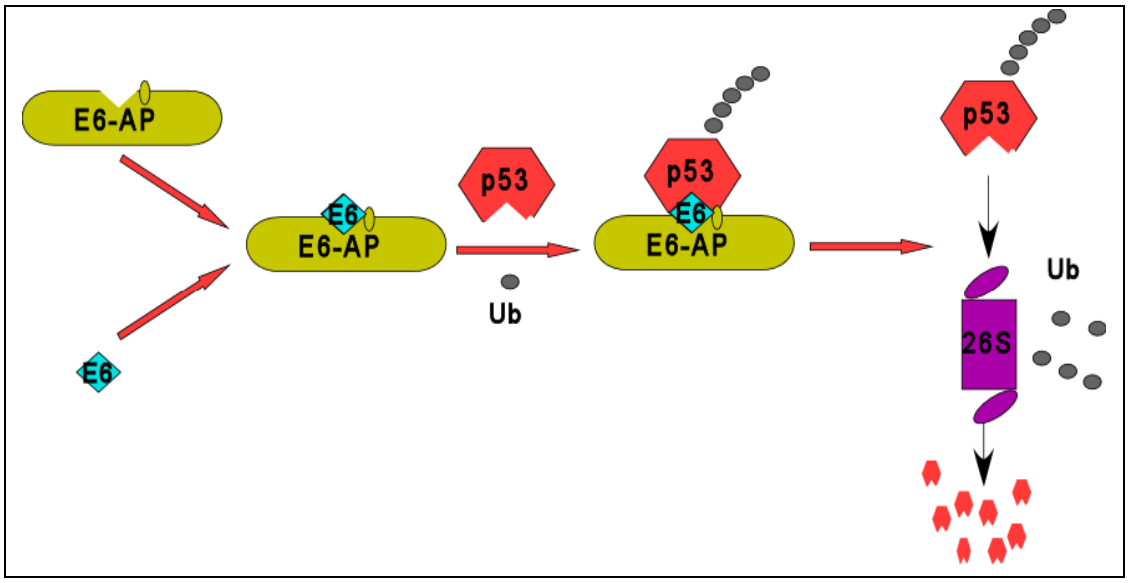

Figure 3: Model for the ubiquitination and degradation of p53 in HPV-positive cells. In HPV-positive cervical cancer cells, the E6 oncoprotein forms a complex with the cellular ubiquitin-ligase E6-AP which then targets p53 for polyubiquitination and subsequent degradation by the 26S proteasome. However in normal cells (HPV-negative), p53 is predominantly targeted for degradation by the RING ligase, Mdm2. Ub- ubiquitin.

While the mechanism of E6/E6-AP targeted ubiquitination of p53 is clear and can be reconstituted *in vitro* using purified enzymes (Scheffner *et al.*, 1993; Rolfe *et al.*, 1995), it remains unclear how exactly polyubiquitinated p53 is delivered to the proteasome for degradation. Recent studies suggest that the human homologue to RAD23 (hHR23) protects polyubiquitinated p53 from deubiquitination and could act as a bridging factor to deliver polyubiquitinated p53 to the proteasome (Glockzin *et al.*, 2003). Interestingly, hHR23 is also known to interact and is a substrate of E6-AP (see section 1.3.5).

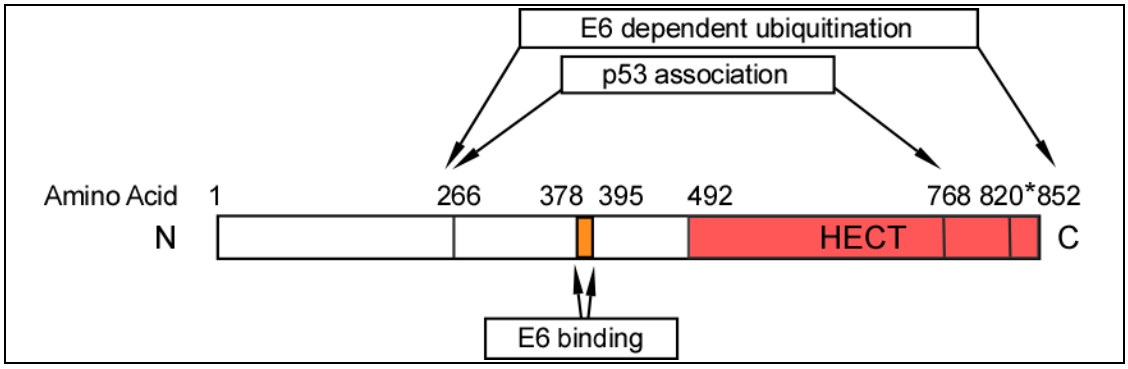

Figure 4: Schematic representation of the regions of E6-AP that direct E6 binding, E6 dependent association with p53, and E6 dependent ubiquitination of p53. Mutational analysis of E6-AP has mapped a central 18 amino acid region that is sufficient for binding to E6 (amino acids 378-395). Amino acid 266-768 is the minimal region required for p53 associated in complex with E6 and includes the E6 binding region. In addition to this, the last 84 amino acids (amino acids 768-852) of the HECT domain is critical for E6 dependent ubiquitination of p53 (Huibregtse *et al.*, 1993).

Even though in HPV-positive cancer cells E6-AP targets p53 for degradation (i.e in presence of E6), E6-AP does not appear to regulate p53 stability in HPV-negative cells (Beer-Romero et al., 1997; Talis et al., 1998; Traidej et al., 2000). Investigation of the MDM2-pathway of p53 regulation in HPV-positive cell lines has indicated that the MDM2-mediated degradation pathway is inactive (Hengstermann et al., 2001). Hence, the degradation of p53 is dependent entirely on the E6/E6-AP pathway in HPV-positive cells. Therefore, studies in which E6 mediated degradation of p53 was inhibited by either using peptide aptamers or RNA interference has led to stabilization of p53 which restores its transactivating properties and tumour suppressor functions in HPV-positive cells (Butz et al., 2000; Butz et al., 2003). Similarly interfering with E6-AP expression using antisense oligonucleotides or ribozyme-based gene inactivation leads to p53 stabilization (Beer-Romero et al., 1997; Kim et al., 2003).

Initially it was believed that the anti-apoptotic properties of high risk E6s were attributed to its ability to target p53 for degradation. However, several lines of evidence later suggested that high risk E6s also possess p53-independent anti-apoptotic properties (Pan and Griep, 1995; Spitkovsky et al., 1996). Search for additional cellular targets of E6 that contribute to its anti-apoptotic potential has led to the identification of several proteins which interact with high risk E6 (Table 1) (reviewed in Munger and Howley, 2002; zur Hausen, 2002). Of these, several have been shown to be targeted for degradation either in an E6-AP-independent or E6-AP-dependent manner.

Cellular proteins that interact with E6 (Reference)	Degradation by E6	E6-AP dependent
Bak (Thomas and Banks, 1999)	+	+
E6BP/ERC-55 (Chen *et al.*, 1995)	-	-
E6TP1 (Gao *et al.*, 1999)	+	+
hAda3(Kumar *et al.*, 2002)	+	?
hDlg (Kiyono *et al.*, 1997)	+	+/-
hScrib (Nakagawa and Huibregtse, 2000)	+	+
IRF-3 (Ronco *et al.*, 1998)	-	-
MAGI (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2002)	+	-
MCM7 (Kuhne and Banks, 1998)	+	+
MUPP1 (Lee *et al.*, 2000)	+	-
NFX1-91 (Gewin *et al.*, 2004)	+	+
p300/CBP (Patel *et al.*, 1999)	-	-
PKN (Gao *et al.*, 2000)	-	-
Tyk2 (Li *et al.*, 1999)	-	-
Paxillin (Tong and Howley, 1997)	-	-

Table 1 Cellular proteins that are known to interact with high risk E6s. Proteins that are degraded by E6 (+) in an E6-AP- independent (-) or E6-AP-dependent manner (+) are indicated. (?) Not known. +/- Conflicting evidence.

1.3.3 E6-dependent substrates of E6-AP

As indicated in Table 1, the high risk E6s are known to interact with several proteins that contain PDZ (PSD95/Dlg/ZO-1) domains (i.e., hScrib, MUPP-1, hDlg and MAGI-1) (Mantovani and Banks, 2001). Multi-PDZ domains form modular structures that are involved in protein-protein interaction with membrane or cytoskeletal proteins. Of the PDZ domain-containing proteins that interact with E6, the human homologue to the Drosophila Scribble (Vartul) tumour suppressor protein (hScrib) has been shown to be targeted for degradation by E6 in an E6-AP-dependent manner (Nakagawa and Huibregtse, 2000; Nakagawa *et al.*, 2004). hScrib localizes to basolateral regions of epithelial cells and is down-regulated by E6/E6AP with the progression of normal uterine cervical cancer tissue to invasive cervical cancer. The ability of E6 to interact with, and possibly degrade PDZ domain proteins is said to contribute to the oncogenic potential of E6 (Kiyono *et al.*, 1997; Mantovani and Banks, 2001). Interestingly, the PDZ domain proteins MAGI-1/2/3 and MUPP1 are targeted for degradation by E6 in an E6-AP independent manner, suggesting that an E3 ligase other than E6-AP may be involved (Glaunsinger *et al.*, 2000; Lee *et al.*, 2000; Thomas *et al.*, 2002).

Earlier studies have indicated that the ability of E6 to immortalize epithelial cells is partly dependent on its ability to increase telomerase activity (Klingelhutz *et al.*, 1996) and this is brought about by induction of expression of the catalytic-rate limiting subunit of telomerase called, hTERT (Kiyono *et al.*, 1998; Gewin and Galloway, 2001; Oh *et al.*, 2001; Veldman *et al.*, 2001). More recently, this ability of E6 to induce telomerase activity has been shown to be dependent on E6-AP and involves the degradation of NFX1-91 (nuclear factors that binds to the X1 box), a repressor of hTERT (Gewin *et al.*, 2004; Liu *et al.*, 2005). In addition to degrading p53 and inducing of telomerase activity, the ability of high risk E6 to immortalize certain epithelial cells has been found strongly correlate with its ability to bind to and target E6TP1 (E6 targeted protein 1), a Rap GTPase-activating protein (RapGAP) for degradation (Dalal *et al.*, 1996; Kiyono *et al.*, 1998; Gao *et al.*, 1999; Gao *et al.*, 2001). Degradation of E6TP1 has also been shown to be mediated by E6 in E6-AP dependent manner (Gao *et al.*, 2002).

Thus the presence of E6 redirects the ability of E6-AP to ubiquitinate its normal substrates and thus alters it substrate specificity. In addition, E6 has been found to

promote autoubiquitination and degradation of E6-AP itself thereby reducing its half-life (Kao *et al.*, 2000). Whether dysregulation of E6-AP mediated ubiquitination of its normal substrates also contributes to the transforming function of E6 in HPV-positive cells is unclear.

1.3.4 Angelman syndrome (AS)

Angelman syndrome is a neurological disorder which has an occurrence of 1:15000 births and is characterized by severe mental retardation, 'puppet-like' ataxic gait with jerky arm movements, seizures, EEG abnormalities, hyperactivity, and bouts of inappropriate laughter. (Williams *et al.*, 1995; Kishino *et al.*, 1997; Matsuura *et al.*, 1997). AS is one of the best studied cases of genetic disorders, in which imprinting plays a role. In most tissues, the *UBE3A* gene displays bi-allelic expression but is maternal-specific in certain brain areas due to paternal imprinting (Rougeulle *et al.*, 1997). Thus loss or inactivation of the maternal allele of *UBE3A* in brain causes AS.

Studies conducted on AS patients indicate that approximately 70% involve maternal deletions at chromosome 15q11-q13, 2% involve paternal uniparental disomy (UPD) at this region, and 2-3% result from imprinting mutations that alter the methylation pattern at this region. The remaining 25% of the cases show biparental inheritance without methylation abnormalities or deletions (Kishino *et al.*, 1997). As mentioned earlier, E6-AP has two separable functions, one as an E3 ligase and another as a transcriptional coactivator. To determine the pathogenesis of AS, mutants of E6-AP obtained from AS patients have been studied. In majority of these cases loss of E3 ligase activity of E6-AP has been detected whereas the coactivator function is found to be intact (Nawaz *et al.*, 1999; Cooper *et al.*, 2004), thus suggesting that deregulated degradation of one or more of its substrates could be responsible for the pathogenesis of AS.

In order to study the causal relationship between inactivation of *UBE3A* and Angelman syndrome, mouse models have also been constructed in which *UBE3A* has been inactivated (Jiang *et al.*, 1998; Miura *et al.*, 2002). Mice with maternal deficiency (m-/p+) for *UBE3A* resembles human AS with motor dysfunction, inducible seizures, and a context dependent learning deficit. Mouse models of AS like the human counterparts display imprinted expression in hippocampus and purkinje neurons (Albrecht *et al.*, 1997).

1.3.5 E6-independent substrates of E6-AP

Since loss in E3 ligase activity is supposedly responsible for pathogenesis of AS patients, several labs have tried to identify potential substrates of E6-AP which can be bound and ubiquitinated in an E6-independent manner. Interestingly, E6-AP itself is found to serve as a substrate undergoing autoubiquitination by a mechanism which is predominantly intermolecular (Nuber *et al.*, 1998). In addition, the human homologs of *S. cerevisiae* RAD23, (HHR23A and HHR23B) (Kumar *et al.*, 1999), the Src tyrosine non receptor kinase Blk (B lymphocyte specific receptor kinase) (Oda *et al.*, 1999), and the multicopy maintenance protein (Mcm) 7 subunit of the replication licensing factor-M (Kuhne and Banks, 1998) have been found to be ubiquitinated by E6-AP. However, of the identified substrates of E6-AP none have been correlated to pathogenesis of AS.

1.4 RNA interference (RNAi)

Till recently, the functional characterization of a particular mammalian gene or gene product involved either elimination by gene knock-out strategies in mouse models, or inactivation using ribozymes, antisense or overexpression of a dominant-negative form of the protein product in cell culture systems. The recent discovery of the natural process termed RNA interference (RNAi) offers an alternate tool to functionally characterize a gene product (Fire *et al.*, 1998).

RNAi is an antiviral post-transcriptional gene silencing defense mechanism in animals and plants caused by the introduction of double-stranded RNA homologous to the silenced gene (reviewed in Paddison and Hannon, 2002; Denli and Hannon, 2003). This mechanism of RNAi has been found to be conserved in most organisms with the notable exception of the yeast, *Saccharomyces cerevisiae* (Hutvagner and Zamore, 2002). RNAi involves the cleavage of the double stranded RNA (Figure 5B) by an RNase III enzyme (DICER) into smaller fragments of 21-23 nucleotides (nt) in length with characteristic dinucleotide 3' overhangs referred to as small interfering RNA (siRNA) (Figure 5A) (Zamore *et al.*, 2000). The siRNA is then recruited into a multienzyme complex called RNA induced silencing complex (RISC) which binds specifically to complementary mRNA transcript to target it for cleavage and degradation.

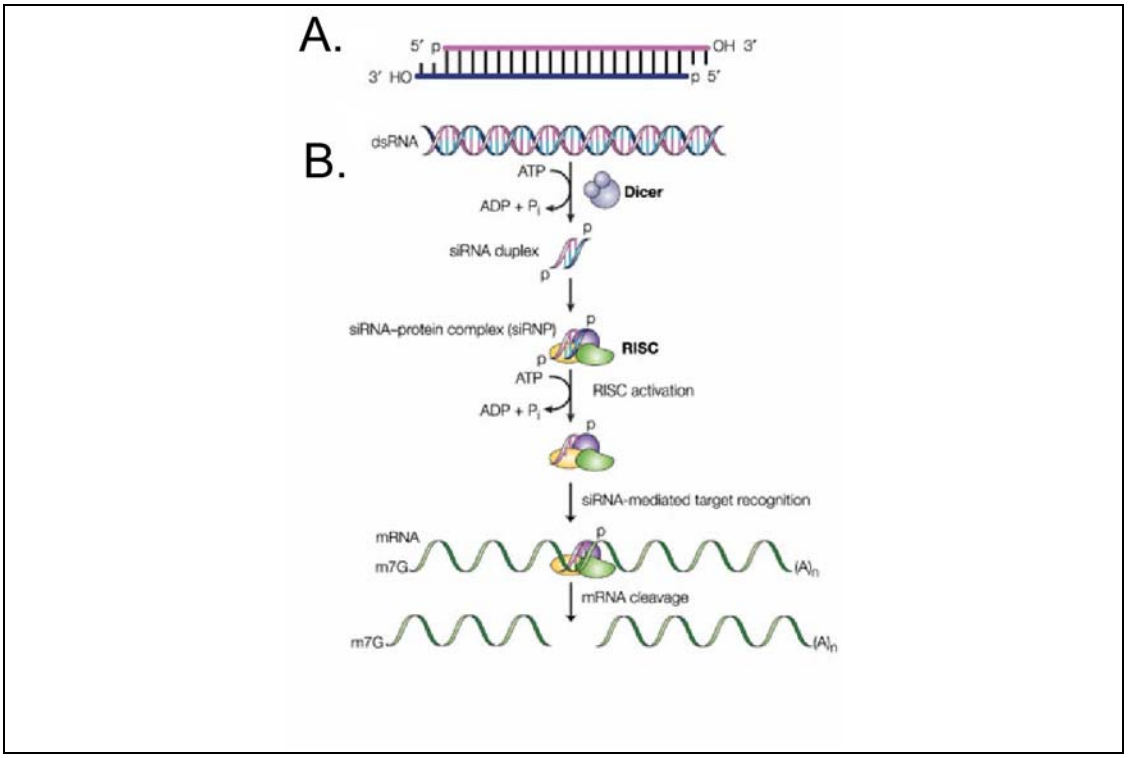

Figure 5: RNA interference. A. Schematic representation of a synthetic 21 nucleotide duplex small interfering RNA (siRNA) with 2 nucleotide overhangs at each 3 prime end. B. Schematic representation of RNAi pathway. Long double stranded siRNA is cleaved into short siRNAs by RNase III enzyme complex (DICER) in an ATP dependent manner. The antisense strand of the siRNA duplex is then recruited into a RNA induced silencing complex (RISC) which specifically recognizes and cleaves the complementary mRNA (adapted from Dykxhoorn et al., 2003)

Initially, RNAi was not used in mammalian studies, because RNA duplexes larger than 30bp are known to trigger generalized cellular responses, predominantly through activation of dsRNA dependent-protein kinases. However, this limitation has been overcome by the use of synthetic double stranded small interfering RNA (siRNA) of 21 nucleotides in length with overhanging 3' dinucleotides (Elbashir *et al.*, 2001). While this approach overcomes the cellular non-specific response to a certain extent, it is limited to transient studies due to the fact that the siRNA gets depleted at each cell division. In order to overcome the transient nature of synthetic siRNA, DNA based vector systems have been created whereby it is possible to bring about stable suppression of gene expression (summarized in Paddison and Hannon, 2002). These vectors utilize RNA polymerase III based promoters (e.g. U6 and HI RNA) to express short hairpin like RNA (shRNA) transcripts with a dinucleotide 3' overhang that can act as siRNA molecules.

Recently, a class of endogenous non-coding RNA molecules called microRNAs (miRNAs) have been found in most organisms (reviewed in Dillon *et al.*, 2005). These miRNAs, like siRNA can also be recruited into RISC, but unlike siRNAs predominantly lead to translational repression by binding to the 3'untranslated region (UTR). In comparison to siRNAs, miRNA have been found to require weak complementation with the target sequence to facilitate translational repression (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). Functionally miRNA play an important role in developmental regulation, and normal physiology. Interestingly, misexpression of miRNAs has been observed in many cancers, suggesting that miRNAs may also have a role to play in carcinogenesis (Calin *et al.*, 2002; Calin *et al.*, 2004). The finding that miRNAs can also function as siRNA suggests that the eventual mechanism of post-transcriptional genes silencing, i.e. translational repression or mRNA degradation, is probably decided by the extent of complementarity between the regulatory RNA and its target (Doench *et al.*, 2003; Zeng *et al.*, 2003).

1.5 Bimolecular fluorescence assay

Recent evidence suggests that localization of substrate and enzyme complexes to distinct organelles or compartments could give another dimesion of regulation and substrate specificity (reviewed in Pines and Lindon, 2005). Even though the biochemical properties of E6-AP have been well characterized, the subcellular localization of individual protein interactions is poorly characterized. Studies to determine the subcellular localization of E6-AP indicated it to be distributed equally in the nucleus and cytoplasm (Hatakeyama *et al.*, 1997). Although a number of potential partners which can interact with E6-AP in an E6 dependent/independent manner have been identified, it is unclear if localization plays a role in substrate preference.

Recently, a new technique called Bimolecular Fluorescent Complementation (BiFC) was reported for visualizing protein complexes in living cells (Hu *et al.*, 2002). This approach involves creating fusion proteins of non-fluorescent N-terminal or C-terminal fragments of the yellow fluorescent protein (YFP) with individual interacting partners of the protein complex to be studied (Figure 6). Co-expression of the N and C –terminal YFP fragments alone within cells does not lead to fluorescence. However, when fused to two separate interacting partners, the interaction between the two proteins leads to fluorescence complementation allowing visualization of the complex. This method has

the advantage of not requiring sophisticated instruments in comparison to other techniques for visualizing protein interactions (e.g. FRET). In addition, BiFC has been used for visualizing multiple protein interactions and to study the relative efficiencies of complex formation of the various interacting partners (Hu and Kerppola, 2003; Grinberg *et al.*, 2004).

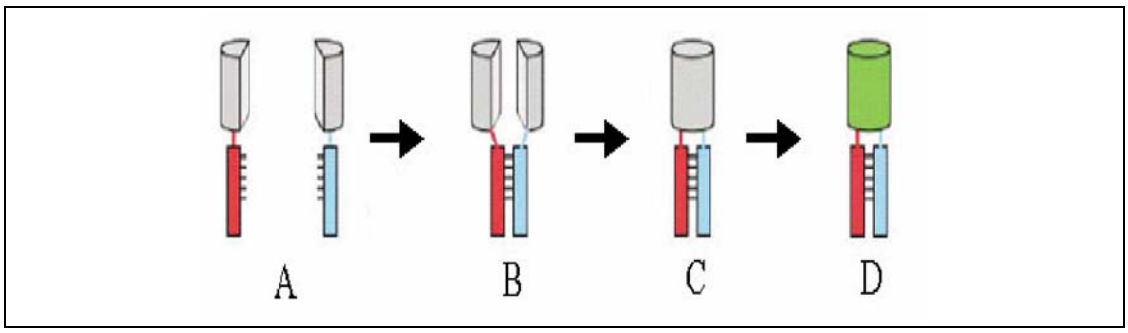

Figure 6: Bimolecular fluorescence assay. Two potential interacting proteins (indicated in Red and blue) are tagged with the two non fluorescent fragment of the yellow fluorescent protein (YFP) (indicated in grey). In order to reconstitute the fluorophore, the two fragments should come into close proximity which is made possible by the interacting proteins. Fluorescence reconstitution can be used as an indicator to study complex formation and localization (adapted from Hu *et al.*, 2002)

2. Aim

E6-AP interacts with the E6 oncoprotein of cancer-associated HPVs and, in complex with E6 targets the tumour suppressor p53 for proteasomal degradation. In contrast to most cancers wherein the p53 gene is mutated or not expressed, it is rarely mutated in HPV-positive cervical carcinomas. This has led to the assumption that targeted inactivation of p53 by the E6/E6-AP complex is functionally equivalent to the inactivation of p53 by mutation. Thus, the aim of this study was to determine the effect of E6-AP inactivation in HPV-positive cervical cancer cells and whether E6-AP contributes to the known anti-apoptotic properties of E6. In order to do so, RNAi was employed to down regulate E6-AP expression in HPV-positive cervical cancer cell lines and its consequences on cell growth were studied.

Loss of E6-AP function has been associated with the development of Angelman Syndrome, a neurological disorder in humans. It has been suggested that deregulated degradation of the substrates of E6-AP could be involved in the pathogenesis of the disease. However, the identities of the substrate(s) are presently unknown. To study the biological importance of E6-AP in HPV-negative cells, E6-AP expression was also down-regulated by RNAi in various HPV-negative tumour cell lines and its essentiality for cell viability was studied.

While E6-AP is biochemically well characterized, little is known about it subcellular localization and/or whether substrate specificity is dependent on localization to specific subcellular organelles/compartments. To determine the subcellular location of the interactions of E6-AP with its substrates and to study the dynamics of these interactions an attempt was made to establish the bimolecular fluorescence complementation assay.

3. Material and Methods

3.1 Materials

3.1.1 Buffers

Buffer Z (β galactosidase assay)	100 mM NaH_2PO_4 (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$, 50 mM β- Mercaptoethanol (storage at -20° C)
DNA stop buffer (10X)	60% Saccharose, 0.25M EDTA, small quantity of Bromophenolblue
Stacking gel buffer	0.5M Tris pH-6.8, 0.4% SDS
Separating gel buffer	1.5M Tris pH-8.8, 0.4% SDS
Laemmlli running buffer (10X)	250 mM Tris-HCl pH 8.4, 2 M Glycine,1% SDS
Laemmlli stop buffer (2X)	62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerin, 100 mM DTT, 0.001 % Bromophenol blue (storage at -20° C)
Phosphate buffer saline (PBS)	137mM NaCl, 2.7mM KCl, 10.1mM NaH_2PO_4, 1.8mM KH_2PO_4, (pH 7.4)
TAE-buffer (50X)	2M Tris-HCl,, 950 mM Acetic acid, 50mM EDTA
TNE-T (wash buffer)	10mM Tris-HCL pH 7.5, 2.5mM EDTA, 50mM NaCl, 0.1% Tween 20
TNN lysis buffer	0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 1% NP-40 (IGEPAL), 1mM DTT, 1µg/ml Aprotinin and Leupeptin, 1mM PefaBloc SC(Roche, Mannheim)
Transfer buffer	12.5mM Tris HCl, 100mM Glycine, pH 8.3
HBS-buffer	20 mM HEPES (pH 7,4), 150 mM NaCl

3.1.2 Solutions and stocks

4% Para formaldehyde fixation solution: 4 g Para formaldehyde was dissolved in 20 ml ddH_2O by stirring at 40°C and adding 3-4 drops of 2M NaOH. After dissolving, the volume was adjusted to 90 ml with distilled water. The pH was adjusted to 6.5 and volume was made upto 100 ml.

Gelvatol: 2.4 g of polyvinyl alcohol (Mw 30,000-70,000; Sigma) was added to 6 g of glycerol in a 50 ml centrifuge tube and mixed by stirring. To the mixture, 6 ml of distilled water was added and the mixture was incubated at room temperature. After several hours of incubation at room temperature, 12 ml of 0.2 M Tris/HCl, pH 8.5, was added and the mixture was heated to 50°C for 10 min with occasional mixing to completely dissolve polyvinyl alcohol. The solution was centrifuged at 5,100 rpm for 15 min. After centrifugation, 2.5% of diazabicyclo-octane (DABCO), an anti-oxidant agent, was added to reduce the bleaching of the fluorescence. The solution was aliquoted in 1.5 ml micro centrifuge tubes and stored at -20°C.

Cycloheximide: 60mg/ml in methanol

Actinomycin D: 1mg/ml in DMSO

DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride): 1µg/ml in PBS

3.1.3 Medium

3.1.3.1 Medium for *E.coli*

Luria Broth medium (LB): 10g/L NaCl, 5g/L yeast extract, 10g/L Bacto-Tryptone (pH-7.5)

SOC-medium: 20g/L Tryptone, 5g/L yeast extract, 20mM glucose, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ (pH 7.5)

3.1.3.2 Medium for mammalian cell culture

For culturing of mammalian cell lines, either RPMI-1640 (Sigma) or DMEM (Sigma) supplemented with 10% of Fetal Bovine Serum (FBS) was utilized.

3.1.4 Cell strains and cell lines

3.1.4.1 Bacterial strains

E. coli DH5α; Genotype: F^- ϕ80d lacZΔM15 Δ(lac) U169 deo R rec A1 hsdR17 ($r_k^-m_k^+$) supE44 λ^- thi-1 gyrA96 rel A1 (Gibco BRL)

E. coli BL 21 (DE3), Genotype: F^- ompT $hsdS_b$ ($r_b^-m_b^-$)βgal dcm (DE3) (Novagen)

3.1.4.2 Cell Lines

-RKO Rectal colon carcinoma, wt p53 (A. Pause)

-U2OS Osteosarcoma, wt p53 (T. Rothmann)

-MCF7 Mammary carcinoma, wt p53 (F. Hoppe-Seyler)

-H1299 Non small lung carcinoma, p53 null (H. Oie)

-SAOS-2 Osteosarcoma, p53-null (M. Scheffner)

-C33A Cervical carcinoma, mutant p53 (R273C), HPV-negative (M. Scheffner)

-SiHa Cervical carcinoma, wt p53, HPV-16 positive (M. Scheffner)

-CaSki Cervical carcinoma, wt p53, HPV-16 positive (M. Scheffner)

-HeLa Cervical carcinoma, wt p53, HPV-18 positive (M. Scheffner)

3.1.5 Mammalian expression vectors

Vector	Characteristics	Reference
pRc/CMV	5.5 kb; Ampicillin resistance; Neomycin (G418, Geneticin) resistance; CMV promoter; for *in vitro* translation and stable protein expression in mammalian cell lines (HPV cell lines exceptional)	Invitrogen
pCDNA3.0.HA	5.4 Kb; Ampicillin resistance; CMV promoter; HA-tag at N-terminal; for *in vitro* translation, transient protein expression in mammalian cell lines	D. Roth, MPI, Frankfurt

pEF/V5- His	6.2 Kb; Ampicillin Resistance; Neomycin (G418,Geneticin) resistance; EF promoter; for *in vitro* expression, transient and stable protein expression in mammalian cell lines	Invitrogen

Table 2: Vectors used for cloning and expression in mammalian cells

3.1.5.1 pRC/CMV constructs:

β-Gal A. Hengstermann

p53 M. Scheffner

HPV-16 E6 M. Scheffner

HPV-18 E6 M. Scheffner

3.1.5.2 Miscellaneous constructs

pd2YFP Clontech

peGFP-C2 Clontech

pHA-CMV.bFos.YC155 Hu *et al,* Mol.Cell, 2002

pFLAG-CMV2.bjun.YN155 Hu *et al,* Mol.Cell, 2000

3.1.5.3 pcDNA3-HA constructs

Protein	Amino acids	Restriction sites 5',3'	Oligonucleotides used
E6-AP	30-852	*Kpn* I, *Apa* I	MD1, MD4
E6-AP ΔE6 *	Δ 378-401	*Kpn* I, *Apa* I	MD1, siRNA2mut sense, MD4
E6-AP *	1-852	*Kpn* I, *Apa* I	siRNA2mut sense, siRNA2mut anti

* contains silent mutations at RNAi binding site of siE6-AP1

HA-E6-AP S. Glockzin

HA-E6-AP C820A S. Glockzin

3.1.5.4 pEF/V5 His constructs

All E6-AP pEF constructs were generated by sub cloning from pcDNA3-HA. Respective HA-E6-AP constructs were digested with *BamHI* and ApaI, klenow filled finally ligated into pEF/V5 His.neo digested with *BamHI-PmeI*.

Protein	Amino acids
E6-AP	1-852
E6-AP	30-852
E6-AP.mut1	1-852 (with silent mutation at si/sh-E6-AP1 target site)
E6-AP.mut2	1-852 (with silent mutation at si/sh-E6-AP2 target site)
E6-AP ΔE6	Δ 378-401

E6-AP ΔE6.mut.pEF/V5 His.hygro K. Martentzoglu and C. Strüh

3.1.6 Molecular weight markers

DNA-Marker:

- λ (Lambda)-DNA Hind III-Marker (Invitrogen):

23130, 9416, 6557, 4361, 2322, 2027, 545, 125 [bp]

- 100 bp ladder (New England Biolabs):

1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 [bp]

Protein marker:

- Prestained Protein Ladder (MBI Fermentas):

180, 130, 100, 73, 54, 48, 35, 24, 16, 10 [kDa]

- MARK12™ Unstained Standard (Invitrogen):

200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6 [kDa]

3.1.7 Antibodies

Antibody	Characteristics	Dilution	Brand
Anti-p53 (IgG Do-1)	Mouse monoclonal	1:1000 (WB) 1:200 (IF)	Dianova
Anti-Hdm2 (SMP14)	Mouse monoclonal	1:1000 (WB)	Santa Cruz
Anti-p53 (1-393)	Rabbit polyclonal	1:1000 (WB)	Santa Cruz
anti-rabbit	Peroxidase conjugated, goat	1:5000 (WB)	Dianova
anti-mouse	peroxidase conjugated, goat	1:5000 (WB)	Dianova
anti-rabbit IgG	AlexaFluor 568, goat	1:1000 (IF)	Molecular probes
anti-mouse IgG	AlexaFluor 568, goat	1:1000 (IF)	Molecular probes
anti-HA IgG	Mouse monoclonal	1:1000 (WB)	Covance

WB-western blotting, IF-immunofluorescence

3.1.8 Primers List

Name	Sequence	5’ or 3’	Protein
siRNA2mut sense	GGCTGTGGAAATGAAGCATGCA CCAATGAGTTTTGTGCTTC	5’	E6-AP
siRNA2mut anti	GAAGCACAAAACTCATTGGTGC ATGCTTCATTTCCACAGCC	3’	E6-AP

si5mute1	TACTACCACCAGTTAACTGAGG GCTGTGGAAATGAAGCATGTAC CAATGAATTCTG	5’	E6-AP
si5mute2	GCGGGATCCATGAAGCGAGCAG CTGCAAAGCATCTAATAGAACG CTACTACCACC	3’	E6-AP
MD1	CGC**GGATCC**ATGTGTGCTTCCT GTCCAAC	5’, *BamH* I	E6-AP
MD2	GG**GGTACC**GTATGGCGTACCCA TACGACGTC	5’, *Kpn* I	HA-tag
MD3	TAAT**GGGCCC**GCAGCATGCCAA ATCCTTTG	3’, *Apa* I	E6-AP
MD4	GACTAGT**GGGCCC**TTACAGCAT GCCAAATCCTTTG	3’, *Apa* I	E6-AP
MD8	ACGC**GTCGAC**CATGAAGCGAGC AGCTGC	5’, *Sal* I	E6-AP
MD9	CGG**GGTACC**CAGCATGCCAAAT CCTTTGG	3’, *Kpn* I	E6-AP
MD10	CGG**GGTACC**AATGAAGCGAGC AGCTGC	5’, *Kpn* I	E6-AP

3.1.9 RNA interference

3.1.9.1 Synthetic siRNA

Synthetic siRNA were ordered either from Dharmacon, USA or MWG-Biotech AG, Germany. siRNAs were ordered as 19-nt RNA duplexes with 2-nt oligo dT 3’ overhangs. In case of siRNA purchased from Dharmacon, individual sense and antisense strands were ordered and annealed according to manufacturers instructions. siRNA from MWG biotech were purchased as annealed oligos and resolubilised in supplied annealing buffer. Stock solutions of 20 µM concentration were stored as aliquots at -20°C. The sequences of the various siRNA are given in Table 3.

Name	Target	mRNA target sequence
si-E6-AP1	E6-AP	TGAAGCCTGCACGAATGAG
si-E6-AP2	E6-AP	AGATGTGACTTACTTAACA
si-Hdm2	Hdm2	CAAGAGACCCUGGUUAGAC
si-Control	Renilla luciferase	AAACAUGCAGAAAAUGCUG

Table 3 Sequence of synthetic siRNA used for transfection into mammalian cells

3.1.9.2 shRNA vectors

Vector	characteristics	Reference
pSUPER	shRNA vector driven by H1 RNA polmerase III promoter. Ampicillin resistance for selection in bacteria. No resistance marker for selection in mammalian cell lines	(Brummelkamp *et al.*, 2002b)
pRetro.SUPER.puro	6.4 kb. derived from the Murine embryonic Stem Cell Virus (pesky). shRNA vector driven by H1 RNA polymerase III promoter. Ampicillin resistance for selection in bacteria. No resistance marker for selection in mammalian cell lines. Can be used to generate virus by transfecting into a suitable packaging cell line or can be transfected into mammalian cell lines.	(Brummelkamp *et al.*, 2002a)

3.1.9.3 Synthetic oligonucleotides used for construction of shRNA expression vectors

Name	Sequence
suprp53fwd	AGCTTTTCCAAAAAGACTCGAGTGGTAATCTACTCTCTTGAAGTAGATTACCACTGGAGTCGGG
suprp53rev	GATCCCCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTTGGAAA
suprE6AP174fwd	GATCCCCACTCTGTGATCCTCATCCCTTCAAGAGAGGGATGAGGATCACAGAGTTTTTTGGAAA
suprE6AP174rev	AGCTTTTCCAAAAAACTCTGTGATCCTCATCCCTCTCTTGAAGGGATGAGGATCACAGAGTGGG
suprE6AP202fwd	GATCCCCGGAGCAAGCTCAGCTTACCTTCAAGAGAGGTAAGCTGAGCTTGCTCCTTTTTGGAAA
suprE6AP202rev	AGCTTTTCCAAAAAGGAGCAAGCTCAGCTTACCTCTCTTGAAGGTAAGCTGAGCTTGCTCCGGG
suprE6AP2421fwd	GATCCCCTGGCCCAGACACAGAAAGGTTCAAGAGACCTTTCTGTGTCTGGGCCATTTTTGGAAA
suprE6AP2421rev	AGCTTTTCCAAAAATGGCCCAGACACAGAAAGGTCTCTTGAACCTTTCTGTGTCTGGGCCAGGG

3.1.9.4 pSUPER constructs (without selection marker)

pSUPER constructs were generated by annealing the respective oligonucleotides (given in the table on the next page) and ligating it into pSUPER vector digested with HindIII–BglII according to manufacturers instructions (Oligoengine).

pSUPER-luc (Renilla luciferase) F. Hoppe seyler

pSUPER-18E6 F. Hoppe seyler

Protein target	mRNA Start-end (nt)	Oligonucleotides used
h-p53	910-928	suprp53fwd, suprp53rev
hE6-AP	69-87	suprE6APfwd, suprE6APrev
hE6-AP	174-192	suprE6AP174fwd, suprE6AP174rev
hE6-AP	2421-2439	suprE6AP2421fwd, suprE6AP2421rev
hE6-AP	300-318	MD41,MD42c

3.1.10 Bimolecular Fluorescence Complementation (BiFC)

BiFC control vectors bFos.pHA.YC155 and bJun.pFLAG.YN155 and the cloning vectors pHA-CMV.YC155 and pFLAG-YN155 were obtained from Hu *et al.*, 2002.

3.1.10.1 pHA-CMV YC155

In order to see the interaction of E6-AP and HERC2 within cells fusion constructs of E6-AP or RCC1B with the C-terminal fragment of YFP were generated by PCR amplification using the primers as given in the following table. The PCR fragment and pHA-CMV.YC155 vector was digested with the indicated enzymes and then the digested PCR fragment ligated into the digested vector using standard molecular biology protocols. Vectors were sequenced and confirmed for expression of the fusion product within cells.

Protein	Amino acids	Restriction sites (5',3')	Oligonucleotides used
RCC1b	2960-3328	*EcoR* I, *Kpn* I	MD12, MD13
E6-AP	1-852	*Sal* I, *Kpn* I	MD8, MD9

3.1.10.2 pFLAG-CMV2 YN155

Fusion construct of RCC1B (amino acids 2960-3328) of HERC2 with the N-terminal fragment of YFP was generated by PCR amplification using the primers MD14 and MD15. The PCR fragment was digested with *EcoR* I and *Kpn* I and cloned into the pHA-CMV.YN155 vector.

3.1.10.3 pcDNA3.0 YN155 constructs

pcDNA3.0 YN155 vector was generated by PCR amplication of the YN155 fragment from pFLAG.CMV YN155 using primers MD28 and MD37 which contains an additional linker sequence and cloned into pcDNA3.0-HA. This PCR product was digested *Hind* III and *Kpn* I and ligated into the *Hind* III - *Kpn* I sites of pcDNA3.0.HA to generate pcDNA3.0. YN155HA. wt-E6-AP and inactive mutant (C820A) along with an N-terminal HA tag were cloned via *Kpn* I–*Apa* I restriction sites into this vector individually.

3.1.10.4 pcDNA3.0 YC155 constructs

pcDNA3.0 YC155 vector was generated by PCR amplifying the YC155 fragment from pFLAG.CMV.YC155 using primers MD27 and MD36 which contains as additional linker sequence. This PCR product was digested *Hind* III and *Kpn* I and ligated into the *Hind* III - *Kpn* I sites of pcDNA3.0.HA to generate pcDNA3.0 YC155. wt-E6-AP and inactive mutant (C820A) along with an N-terminal HA tag were cloned via *Kpn* I–*Apa* I restriction sites individually.

3.2 Methods

3.2.1 Maintenance of cell lines and bacterial cultures

3.2.1.1 Bacterial cultivation and preparation of glycerol stocks

To maintain glycerol stocks of plasmids, the respective plasmid was transformed into DH5α cells and cultured overnight in LB medium containing respective antibiotic at 37°C with shaking at 220 rpm. Glycerol stocks were prepared by mixing 150 µl of cooled sterile glycerol to 850 µl of overnight culture in a cryovial. The contents were mixed and stored at -80 °C.

3.2.1.2 Cultivation and maintenance of mammalian cell lines

All mammalian cell lines were incubated at 37°C under 95% humidity and 5% Carbon-di oxide. H1299 and RKO cells were cultured in RPMI-1640 (Sigma) medium supplemented with 10% (vol/vol) FBS. HeLa, CaSki, SiHa, MCF-7 were cultured in DMEM (Sigma) medium supplemented with 10% (vol/vol) FBS.

3.2.1.3 Freezing of cell lines in liquid nitrogen

Approximately 70% confluent plate of the particular cell line was trypsinized. The cells were released from the plate with 4 ml medium and centrifuged at 1000 rpm for 2 min at 4° C. The cell pellet resuspended in 2 ml freezing medium (5.9 ml growth medium, 1.7 ml FCS, 1 ml DMSO) was aliquoted out into two cryotubes and then transferred to –80° C freezer. The next day the tubes were transferred to a liquid nitrogen tank.

3.2.2 Cloning and analysis

3.2.2.1 Purification of plasmid DNA from bacteria

For the screening of clones, extraction of plasmid DNA from bacteria by mini-preps (2ml) was performed by alkaline-lysis method of Holmes and Quigley (1981).

Plamid DNA for sequencing and transfection assays was extracted from 100ml bacterial cultures (midi- preps) using Macherey-Nagel AX-100 plasmid extraction kit according to manufacturer's instructions

Plasmid concentration was determined spectrophotometrically using a Bio-Rad Smart-spec UV-spectrophotometer.

3.2.2.2 Polymerase chain reaction

PCR were setup using either Pfu-polymerase (Stratagene) or Triple-Mastermix (Eppendorf) according to manufacturer's instructions.

3.2.2.3 Generation of point mutations by PCR based site directed mutagenesis

Silent point mutations in E6-AP were generated using Pfu polymerase by the quick change site directed mutagenesis method (Stratagene). Complementary primers containing the required mutations were designed and PCR-amplified. The nicked circular PCR product was digested with Dpn I for 1 hr to get rid of the template DNA and 5 µl of the digest was used to transform into *E.coli.*

3.2.2.4 Restriction Digestion

Typically 30-50 µl digestion reactions were set up. All restriction enzymes were obtained from NEB or Invitrogen and the digestions were performed in the buffer systems and temperature conditions according to manufacturers' instructions. Digestion were set up for 2-4hrs.

3.2.2.5 Agarose Gel electrophoresis

Agarose gel electrophoresis was to the method described by Sambrook *et al.* (1989). Electrophoresis was typically performed with 0.8 % (w/v) agarose gels in 1x TAE buffer submerged in a horizontal electrophoresis tank containing 1x TAE buffer at 1-5 V/cm. Only for resolving fragments less than 1,000 bp, 1-2% (w/v) agarose gels in 1x TAE buffer were used. Gels were analyzed under a UV Tran illuminator and photographed using Gel documentation system (from Fuji or MWG biotech)

3.2.2.6 Purification of DNA fragments and PCR products from Agarose gels

DNA fragments from restriction digestion or from PCR reactions were separated by agarose gel electrophoresis. The gel piece containing the desired DNA fragment was carefully excised while observing the ethidium bromide stained gel under a UV Tran illuminator. The DNA fragment was then purified from the excised gel piece using the Qiagen gel extraction kit according to manufacturer's instructions.

3.2.2.7 Ligation

Ligations were set up in 20µl reactions at room temperature for 2hrs. For PCR cloning, the digested PCR product and the appropriate linearized plasmid were mixed in a equimolar ratio of 1:3. T4 DNA ligase (Gibco) was added according to manufacturers instructions. In addition, 1µl of 0.1M DTT was added. For cloning of sh-RNA constructs, dephosporylated vector and annealed and phosphorylated oligonucleotides were used in ligation reactions.

3.2.2.8 Transformation by $CaCl_2$ method

Plasmid DNA (5 µl of a ligase reaction or ~100 ng of a purified plasmid) was mixed with 100-200 µl of $CaCl_2$ -competent *E.coli* cells and incubated on ice for 30 min. The cells were heat-shocked at 42ºC for 60-90s and immediately transferred to ice for 5 minutes. 1 ml of pre-warmed (at 37ºC) SOC medium was added to the above tube and incubated at 37ºC with shaking for 1 hr. Finally, the transformation mix, or an appropriate dilution, was plated onto selection plates and the transformants were allowed to grow overnight at 37ºC.

3.2.2.9 Estimation of DNA concentration

Concentration of purified plasmid DNA was estimated using a UV spectrophotometer. (Biorad Smart spec Plus). Measurements were performed in quartz cuvettes using the standard 1O.D_{280} 1.0 = 50µg/ml DNA. The ratio O.D_{260}/OD_{280} was determined to assess the purity of the sample.

3.2.3 Transfection of mammalian cell lines

Cells were either transfected for transient assays or for generation of stable cell lines using either Lipofectamine 2000 (Invitrogen) (3.2.3.1) or DOTAP (Roche) (3.2.3.2). In transient transfections, transfection efficiency was normalized by co-transfecting 200ng of an expression construct expressing β-galactosidase (pRc/CMV-β gal).

To generate stable cell lines, cells were transfected like transient transfection, but however in the absence of pRC/CMV- β gal construct. For constructs lacking a selection marker, a vector encoding a resistance marker was cotransfected at a ratio of 1:10. 24 hrs after transfection, the cells were trypsinized and plated onto a larger plate (e.g. from 6cm plate to 10cm plate) 24 hrs after transfection. One day later, selection

was started with the respective antibiotic. Prior to this, the amount of antibiotic required for selection was optimized for each cell line. The optimized amount of antibiotic required is tabulated in Table 4 and Table 5.

cell line	concentration (mg/ml)
H1299	1
SiHa	1.6
HeLa	0.7
CaSki	0.7
C33-A	0.7

Table 4: Concentration of Geneticin used for the selection of stable colonies in various cell lines

3.2.3.1 Transfection using Lipofectamine 2000

Cells were transfected by lipofection using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. One day before transfection cells were seeded onto a 6 cm plates such that they were 90% confluent on the day of transfection. For transfections in H1299 cells the total amount of plasmid DNA was maintained at 2µg and was transfected using 4µl of Lipofectamine 2000 reagent. For transfection of HeLa and SiHa cell lines, the amount of Lipofectamine 2000 to DNA ratio was maintained at a ratio of 1:1 (µg/µl).

3.2.3.2 Transfection using DOTAP

Cells were seeded onto a 6cm plate 24 hrs prior to transfection such that they were 50-70% on the day of transfection. For each transfection in a 6cm plate, 2µg of total plasmid DNA was suspended in HBS and was added to 14µl of DOTAP (Roche) and DNA-liposomal complexes were allowed to form by incubating for 10 min. Meanwhile, the medium from the plates was replaced with DMEM without FBS. DNA-Liposomal complexes formed were added to the cells and incubated at standard growth

conditions. The medium was replaced with normal growth medium containing FBS 4 hrs later. Cells were lysed after the indicated time point.

3.2.4 Protein studies

3.2.4.1 Lysis of transfected cells

At indicated time points after transfection, the monolayer of cells was washed thrice with cold PBS. The cells were scraped into 500 µl PBS from the plates using a plastic scraper and pelleted down. The pellet was lysed in 100 µl TNN lysis buffer on ice for 20 min and centrifuged at 13,200 rpm for 30 min to remove cell debris. The supernatant was carefully removed. For transient assay, sample loadings were normalized by β galactosidase assay (3.2.4.3). For stable transfections and siRNA transfection protein loading was normalized by Bradford assay.

3.2.4.2 Normalization of sample loading /Protein Estimation

3.2.4.3 β-galactosidase assay

To a 96 well plate kept on ice, 120µl of buffer Z was added per well. To this 10µl of cell lysate and 5µl of ONPG (Orthonitrophrnyl-β-D-galactopyranoside, concentration: 4mg/ml in 100mM NAH_2PO_4 pH 7.4) was pipetted and mixed. The plate was incubated at 37° C till yellow color developed (approx 20 min) and the absorbance was measured at λ_{410} in an ELISA reader. All estimations were done in duplicates.

3.2.4.4 Bradford assay

Normalization of total protein amounts was performed using Bio-rad Bradford reagent. 5µl of protein lysates was added to 795 µl of milli Q water. 200µl of reagent was added and reading was taken after 5 min at λ_{595} using a Bio-Rad spectrophotometer 3000.

3.2.4.5 SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis was done according to the protocol of Laemmli (1970). For analysis of p53, 10% separating gels were used and for the analysis of Hdm2, E6-AP, 8% separating gels were used. Protein loadings were normalized by β-galactosidase analysis or by Bradford assay. Normalized samples were boiled for 5-10 min at 100° C in Laemmli loading buffer prior to loading onto the gels. The gels were electrophoresed at constant current setting of 44mA (for 1 gel) or 80mA (for 2 gels) for 200min.

3.2.4.6 Immunoprecipitation

Cell lysates were prepared in TNN lysis buffer (3.2.4.1) and protein amounts were normalized by Bradford assay (3.2.4.4). The normalized amounts of cell lysates was precleared with 50 µl of protein A sepharose slurry (1:1 ratio of Protein A sepharose bead: TNN lysis buffer) by incubating at 4°C with rotation for 1 hr. Samples were then centrifuged and to the supernatant 1µl of monoclonal E6-AP antibody was added and incubated for 1hr at 4°C with rotation. 50µl of Protein A sepharose slurry was added and the samples were further incubated at 4°C overnight. The Protein A sepharose beads were washed four times with TNN lysis buffer and to the beads 50µl of 2X Laemmli loading buffer was added. Samples were boiled at 100°C for 5 min, centrifuged and the supernatant was analyzed by SDS-PAGE.

3.2.4.7 Western Blot and ECL

Protein samples were separated by SDS-PAGE and the gel was incubated in Transfer buffer. Required size of PVDF membrane (Millipore) was cut and activated by incubating the membrane in Methanol for 10 sec. The membrane was then incubated in transfer buffer. Western blotting was performed using a wet transfer apparatus (BIO-RAD) for 150 min at 40V. After transfer, the membrane was blocked with 5%(w/v) milk solution in TNE-T for 1hr at RT. The membrane was washed and incubated in Primary antibody for one hour. After washing the membrane with TNE-T for one hour (5 times, 10 min each), the membrane was incubated with secondary antibody conjugated with horse radish peroxidase. The membrane was washed once again for 1 hr with TNE-T the membrane was developed using ECL (Enhanced Chemiluminescence) kit (Amersham) according to manufacturer's instructions.

3.2.5 Bimolecular Flourescence Complementation (BiFC)

Cells were grown in 24 well plates on coverslips and transfected with 500 ng each of the indicated complementing BiFC vectors using lipofectamine 2000 (3.2.3.1). To measure transfection efficiency in parallel 1µg of YFP expressing vector was also transfected. 16-24 hrs later the transfected cells were shifted to 30°c for fluorescence maturation. The cells were initially observed for fluorescence using a Nikon inverted microscope and then fixed using either methanol or 4% Para formaldehyde. Fixed cells were stained with DAPI and mounted onto glass slides using gelvatol (3.2.7.6). Images

were taken using an Olympus IX70 inverse microscope equipped with a 40X LCPlanFI 0.6 objective. Images were captured either with a JAI CV-M10 CCD video camera or a SensiCam cooled CCD video camera. Images were false colored using Adobe Photoshop software.

3.2.6 RNA interference

3.2.6.1 siRNA/shRNA design

siRNA/shRNA targeting sequences were identified using the following RNAi-design software freely available on the internet: Oligoengine ver-1.1(www.oligoengine.com), Dharmacon siRNA design center (www.Dharmacon.com), siDesign tool (www.mwgbiotech.com). All siRNA sequences were BLAST searched in the National Center for Biotechnology Information's (NCBI) ''search for short nearly exact matches'' mode against all human sequences deposited in the GenBank and RefSeq databases. Sequences which did not have significant homology to genes other than the targets were chosen and synthesized at Dharmacon, USA or MWG-Biotech AG, Germany (3.1.9.1).

3.2.6.2 Transfection of synthetic siRNA

One day prior to transfection, cells were seeded onto sterile coverslips (3.2.7.1) such that they were 30-50% confluent at the time of transfection. A final siRNA concentration of 100nM was transfected using Oligofectamine according to the protocol of Tuschl and coworkers (Elbashir *et al.,* 2001). For each transfection per well (24 well cell culture plate), 3µl (60 pmol) of 20µM stock siRNA was diluted in 50 µl of Opti-MEM® I Reduced Serum Medium (Gibco) in a sterile polypropylene tube and mixed gently. In a separate tube, 3µl of Oligofectamine (Invitrogen) was added to 12 µl of Opti-MEM® I Medium, mixed gently and incubated for 5 minutes at room temperature. The diluted siRNA and Oligofectamine were gently mixed together and incubated at room temperature for 20 min. Meanwhile, the adherent cells (on the coverslips) were washed once with Optimem I and replaced with 500µl of Optimem I. The siRNA-Oligofectamine Mixture was added drop wise to the cells and incubated at 37°C. After 4hrs, 300µl of three fold FBS containing medium was added to the cells and incubated for the desired period until analysis.

For comparison of the parental HeLa versus the p53 null clones (A1 and C2 clones), 20µM of E6-AP siRNA was taken while the final concentration of siRNA was maintained at 100nM by diluting with siRNA for renilla luciferase (si-control).

3.2.6.3 Estimation of siRNA transfection efficiency

To estimate the transfection efficiency of synthetic siRNA in various cell lines, cells were seeded onto coverslips in 24 well plates (3.2.7.1). 3µl (stock solution: 30mg/ml in water) of FITC-Dextran (SIGMA) was transfected using oligofectamine in place of siRNA (3.2.6.2). As a negative control, cells were mock transfected with FITC-Dextran minus Oligofectamine. Care was taken to minimize exposure to light to prevent photo bleaching. 24 hrs later, the coverslips were stained by DAPI and mounted onto glass slides (3.2.7.6). Images were taken using and inverse microscope using appropriate filters (3.2.8).

As an alternative to FITC-dextran, labeled siRNA (Negative control siRNA Alexa 555, Qiagen GmBH, Germany) was also used to estimate transfection efficiency.

3.2.7 TUNEL (Tdt mediated dUTP nick end labeling) assay and Immunofluorescence

3.2.7.1 Seeding of cells onto coverslips

12 mm-diameter coverslips were sterilized by soaking in absolute ethanol for 15 minutes, removed, and allowed to dry. Single coverslips were aseptically placed into each well of a 24 well plate (Greiner) and cells seeded according to required confluency and synthetic siRNA was transfected .according as given in 3.2.6.2

3.2.7.2 Fixation using 4% paraformaldehyde

At indicated time points, the coverslips containing the transfected cells were carefully transferred to a new 24 well plate making sure that the adherent cells faced upwards. The coverslips were washed twice with 500µl of PBS containing 100mM of Mgcl2. 500 µl of freshly prepared 4% Para formaldehyde solution was added carefully into each well and incubated at room temperature for 1 hr. The coverslips were washed twice with PBS and further processed for immunofluorescence (3.2.7.3) or were stored in PBS at 4°C.

3.2.7.3 Permeabilization of fixed cells

Prior to immunolabelling and TUNEL reaction the PBS was removed from the fixed cells (3.2.7.2) and permeabilised using 500µl of freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. After permeabilization the coverslips were washed twice with PBS.

3.2.7.4 TUNEL reaction

The permeabilized coverslips containing the fixed cells were transferred from the 24 well plates to a humidified chamber (an opaque humidified box in which parafilm pieces are fixed onto glass slides) making sure that the permeabilized cells face upwards. TUNEL reaction was performed using the in situ cell death detection kit (Roche Diagnostics GmbH, Germany) according to manufacturer's instructions. In brief, 5µl TUNEL–enzyme was mixed with 45 µl of TUNEL-label solution to obtain 50µl TUNEL-reaction mixture per coverslip. The TUNEL-reaction mixture was added carefully onto each coverslip and overlaid with another coverslip to prevent evaporative loss. The humidified chamber was incubated at 37° C in the dark for 1 hr. The overlaying coverslip was removed and the coverslip containing the cell sample was washed three times with PBS and then processed for immunolabelling. During the subsequent steps excessive exposure to light was avoided to prevent photo bleaching of the label.

3.2.7.5 Immunolabelling for p53

To each coverslip containing the fixed cells 180 µl of primary antibody (polyclonal anti-p53 antibody in PBS containing 1% BSA, dilution 1:200) were incubated at room temperature for 1 hr. After incubation, the excess unattached antibody was removed by washing the coverslips 4 times with PBS. Excess PBS was removed and the coverslip was incubated with 180 µl of secondary antibody for 1 hr in dark (Alexa Fluor 568 goat anti rabbit IgG, Molecular probes, dilution 1:1000 in pBS containing 1% BSA). Following this incubation, the coverslip was once again washed 4 times with PBS and processed for DAPI staining.

3.2.7.6 DAPI (4', 6 diamidino-2-phenylindole) staining and mounting onto glass slides

To visualize the nuclei of the cells, coverslips were incubated with 200 µl DAPI (Molecular Probes) at a final concentration of 300 nM in PBS for 15 min. The coverslips were washed with PBS thrice, rinsed with water, ethanol, and air dried. The coverslip was mounted onto a glass slide using Gelvatol. The coverslips was allowed to solidify and observed by fluorescence microscopy (3.2.8).

3.2.8 Microscopy

Immunofluorescence images were taken using an Olympus IX70 inverse microscope equipped with a 40X LCPlanFI 0.6 objective. Images were captured either with a JAI CV-M10 CCD video camera or a SensiCam cooled CCD video camera. Images were false colored using Adobe Photoshop software.

3.2.9 Relative quantitative reverse transcription PCR analysis

3.2.9.1 Isolation of total RNA

Cells were grown in 6cm-diameter plates and transfected with synthetic siRNA (3.2.6.2 48 hrs post-transfection total RNA was isolated by Trizol method using Trizol reagent (Invitrogen) according to manufacturer's instructions. Isolated RNA was dissolved in 10-20µl of RNase free water and quantitated using a UV-spectrophotometer (Bio-Rad).

3.2.9.2 First strand synthesis

First strand cDNA synthesis was performed using random hexamers (Invitrogen) and 5µg of total RNA. Reverse transcription was performed using Superscript II RT (Invitrogen) according to manufacturer's instruction.

3.2.9.3 Quantitation of mRNA knockdown

Relative quantitative RT-PCR analysis was performed using the QuantumRNA^{TM} 18S Internal Standard kit (Ambion, Austin,USA). For amplification of E6-AP specific products, primers MD10 and UK13 were utilized which yields a 750bp product and recognizes all known isoforms of E6-AP. In each amplification reaction, the 18S rRNA PCR primer/competimer pairs were included as an internal standard at a ratio of 1:9. 25µl PCR reactions were set up and separated on a 1.5% agarose gel. The gel was

stained after separation with Ethidium bromide and photographed using a FUJI imaging system (LAS 3000). Signal intensities were quantitated using AIDA imaging analyzer software ver 4.06 and fold change was calculated.

3.2.9.4 Immunoprecipitation and western blot analysis of RNAi treated cells

For IP and western analysis cells were seeded onto 6cm plates the previous day such that they are 30%-50% confluent at the time of transfection. 100nM of siRNA was transfected as in the case for immunofluorescence analysis. Immunoprecipitation was carried out as described under 3.2.4.6.

3.2.10 Generation of stable cell lines

3.2.10.1 Generation of stable cell lines ectopically expressing E6-AP or E6-AP deletion mutants

To generate stable cell lines ectopically expressing E6-AP or E6-AP deletion mutants, cells were transfected using liposomal transfection reagents DOTAP or Lipofectamine 2000. For DOTAP transfection, 2µg of the HA-tagged pEF constructs of Wt E6-AP, ΔN30 or E6-APΔE6 was transfected using 14µl of DOTAP on a 6cm plate as described in 3.2.3.2. For Lipofectamine 2000 transfection 2µg of the respective construct was transfected using 2µl of Lipofectamine 2000 in a 6 cm plate as described in 3.2.3.1. 24 hrs post-transfection, cells were split onto two 10cm plates. The next day, the medium was replaced with selection medium (see Table 4) for amount of G418 antibiotic employed for each cell line). Pooled stable clones were selected for around 4 weeks, and checked for overexpression as soon as sufficient cells were available for analysis. In parallel, aliquots of the pooled stable cell lines were frozen down in liquid nitrogen (3.2.1.3).

3.2.10.2 Generation of stable p53 null single cell clonal cell lines

HeLa and SiHa cell lines were seeded 24 hrs before transfection onto 6 cm –diameter plates such that they were 90% confluent on the day of transfection. Cells were co-transfected with pSUPER-p53 and a vector encoding a neomycin resistance gene (pEF1/V5-His) in a ratio of 1:10 respectively (total DNA transfected 2µg) using

Lipofectamine 2000 (3.2.3.1). The next day cells were trypsinized and plated onto a 10 cm plate. Colonies expressing Neomycin resistance were selected for using Geneticin (G418) for a period of 10-15 days. Cells were trypsinized, counted and plated onto 96 well plates such that each well contained only a single cell. Clones were allowed to grow under selection pressure till sufficient amount of cells were obtained for analysis. Knockdown efficiency was confirmed by western blot analysis (3.2.4.7) and cell lines frozen down.

3.2.11 Colony reduction assay

HeLa and SiHa cells were seeded on a 24 well plate such that they were 90% confluent at the time of transfection. 1µg of indicated pRetroSUPER vector DNA was transfected using 1µl Lipofectamine 2000 reagent (3.2.3.1). The next day cells were trypsinized and plated onto a 6cm cell culture plate. 24 hrs later the medium was replaced with selection medium containing the puromycin (Table 5). Medium was changed every three days. After 15-20 days, the plates were washed twice with PBS and stained with crystal violet for 15 minutes. The plates were washed with deionised water and allowed to dry. Scanned Images of the plates were taken using a Mustek P3600 A3 Pro Scanner.

Cell line	Puromycin final concentration (µg/ml)
HeLa	4.0
SiHa	2.0
H1299	4.0
MCF-7	4.0

Table 5: Concentration of puromycin used for selection of mammalian cells

4. Results

4.1 Identification of potent siRNA/shRNA sequences that target E6-AP mRNA

4.1.1 siRNA/shRNA design

In order to characterize the physiological role of E6-AP by RNA interference, both chemically synthesized small interfering RNA (siRNA) of 21 nucleotides (Elbashir et al., 2001) and DNA-based short hairpin RNA expression vectors (shRNA) were employed (Brummelkamp *et al.*, 2002b). Although a number of computational programs have been developed to identify potent siRNA/shRNA targets, not all siRNA/shRNAs designed against a particular mRNA are functionally efficient. Hence, four different siRNA/shRNA target sequences (Table 6), specifically targeting E6-AP were identified using RNAi-design software (see Materials and Methods, 3.2.6.1). In the course of identifying siRNA/shRNA target sequences, sequences which target all known E6-AP isoforms were chosen. Figure 7A depicts the genomic organization of E6-AP. The isoforms of E6-AP and the regions where the siRNA/shRNAs target the various transcripts are indicated in Figure 7B. The siRNA/shRNA target sequences are numbered based on the first nucleotide of the target sequence (wherein nucleotide 1 corresponds to the first A of the start codon of isoform I).

siRNA/shRNA	Targeting sequence in E6-AP
shRNA-69 (sh-E6-AP1) and si-E6-AP1	UGA AGC CUG CAC GAA UGA G
shRNA-174	ACU CUG UGA UCC UCA UCC C
shRNA-2421	UGG CCC AGA CAC AGA AAG G
shRNA-300 (sh-E6-AP2) and si-E6-AP2	AGA UGU GAC UUA CUU AAC A

Table 6: siRNA/shRNA targeting sequence in E6-AP. To generate shRNA vectors targeting E6-AP, sense and antisense oligonucleotides containing the targeting sequence and a nine nucleotide loop were annealed and cloned. siRNAs against E6-AP were generated by synthesizing sense and antisense strands of the targeting sequence with two nucleotide dT overhang at the 3'end. These were either purchased as annealed oligos or were annealed according to manufacturer's instructions (Material and Methods, 3.1.9.1). Note that the shRNA-69 (sh-E6-AP1) and si-E6-AP1 target the same sequence in E6-AP. Similarly, shRNA-300 (sh-E6-AP2) and si-E6-AP2) target the same sequence (see text for details).

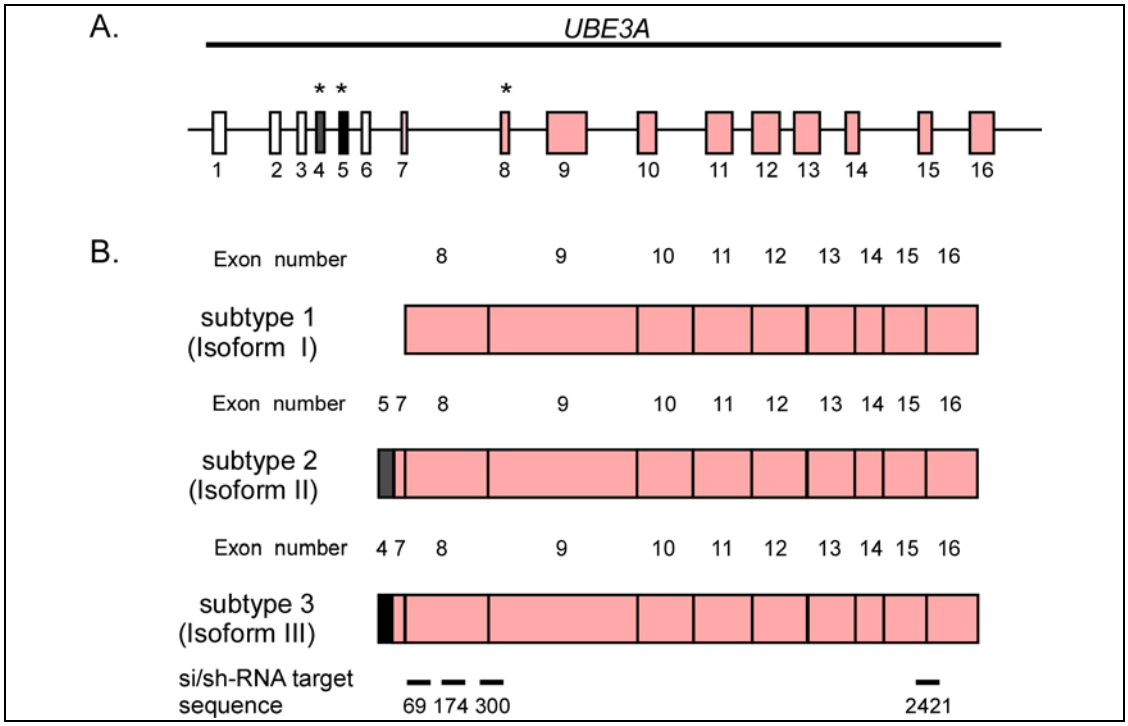

Figure 7: Schematic representation of the splice variants of E6-AP and the location of the siRNA/shRNA targeting sequences. A. *UBE3A* genomic structure. The *UBE3A* gene has the potential to encode five mRNA subtypes coding for three protein isoforms of E6-AP (isoforms I, II and III). Exons are indicated by closed boxes, and the coding regions are indicated by pink boxes. The dark colored boxes correspond to the exons which are present specifically in Isoform II (exon 5) and isoform III (exon 4) respectively (exons are numbered as reported in Kishino and Wagstaff, 1998). The position of translation initiation codon is indicated by asterisks B. Splicing pattern of mRNAs and corresponding location of the siRNA/shRNA targeting sequences. The exons present in subtypes-1, 2, and 3 (representing protein isoforms I, II and III respectively) are indicated (not drawn to scale). Position of siRNA/shRNA targeting sequences in the three isoforms is depicted below as a small dash. siRNA/shRNA target sequences are numbered by the first nucleotide of the target sequence (according to isoform I) . Note: subtype 4 and 5 of E6-AP which encodes isoform I are not shown in figure.

4.1.2 Evaluation of the silencing potency of different shRNAs specific against E6-AP mRNA

To evaluate the silencing potency of the identified shRNA, vectors (pSUPER) with the above target sequences were generated. These vectors (namely shRNA-69, shRNA-174, shRNA-2421, and shRNA-300) were individually co-transfected with an expression vector encoding an HA-tagged form of E6-AP isoform I (HA-E6-AP) and a vector expressing a puromycin resistance gene (pCMV.puro) into H1299 cells. A shRNA vector targeting renilla luciferase was used as negative control (sh-control). To select for transfected cells, the cells were subjected to puromycin selection and

silencing of ectopically expressed E6-AP was determined 96 hr post-transfection by western blot analysis using an anti-HA antibody (Figure 8). Relative comparison of the efficiency of knockdown indicated that the shRNA-69 (hence forth referred to as sh-E6-AP1) and shRNA-300 (hence forth referred to as sh-E6-AP2) gave efficient silencing greater than 55% on the protein level and was used in further experiments.

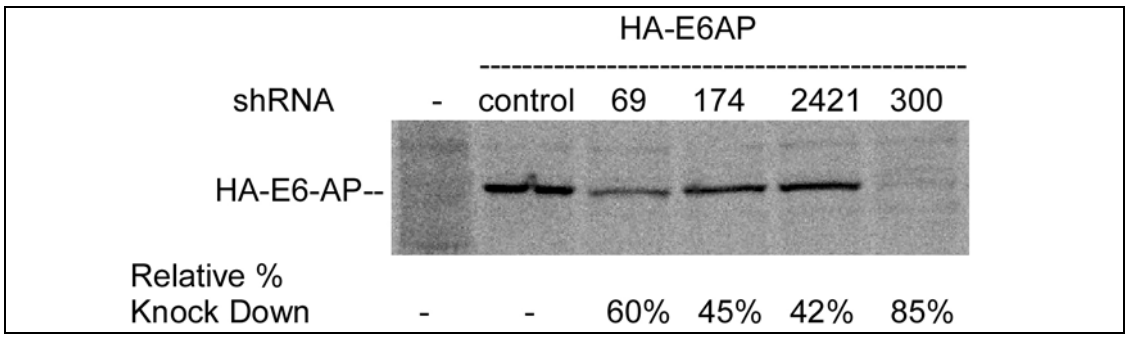

Figure 8: Evaluation of the silencing potency of different shRNAs specifically targeting E6-AP. A. Down-regulation of ectopically expressed E6-AP in H1299 cells. HA tagged wild type E6-AP (HA-E6-AP) and shRNA vectors specifically targeting E6-AP were co-transfected into H1299 cells along with a vector expressing the puromycin resistance gene as indicated. As a negative control shRNA vector targeting renilla luciferase (control) was used. Transfected cells were selected with puromycin and analyzed by western blotting with anti-HA antibody. Relative percentage of knockdown was quantified using AIDA imaging software. Relative knockdown values are the average of 2 independent experiments.

Identical targeting sequences as that of sh-E6-AP1 and sh-E6-AP2 were used for chemical synthesis of siRNA of 21 nts (hence forth referred to as si-E6-AP1 and si-E6-AP2 respectively). As a negative control sequence targeting renilla luciferase (si-control) was also synthesized. In case of synthetic siRNA, transfection efficiencies greater than 90% can be achieved in most cell lines (Figure 24 and data not shown) and hence were used for transient transfection studies.

4.2 Down-regulation of E6-AP in HPV-positive cell lines by RNAi

4.2.1 Down-regulation of E6-AP has a growth-suppressive effect in HPV-positive cell lines

It is known that the E6 oncoprotein recruits the cellular ubiquitin-ligase E6-AP to target p53 for degradation and that continuous expression of E6 is required for the maintenance of the malignant phenotype of HPV-positive cancer cells (zur Hausen, 2000; 2002). Indeed interference with E6 expression or E6 activity results in growth suppression, which is accompanied by significant increase in p53 levels and apoptosis

in HPV-positive cell lines (Butz et al., 2000; Butz et al., 2003). Similarly down-regulation of E6-AP expression by antisense approaches or overexpression of a catalytically inactive E6-AP mutant results in the accumulation of p53 in HPV-positive cells but not in HPV-negative cells (Beer-Romero et al., 1997; Talis et al., 1998). Furthermore, ribozyme-mediated reduction of E6-AP expression enhances the apoptotic response of HeLa cells , an HPV-18-positive cell line, to the DNA damage-inducing drug mitomycin C (Kim et al., 2003).

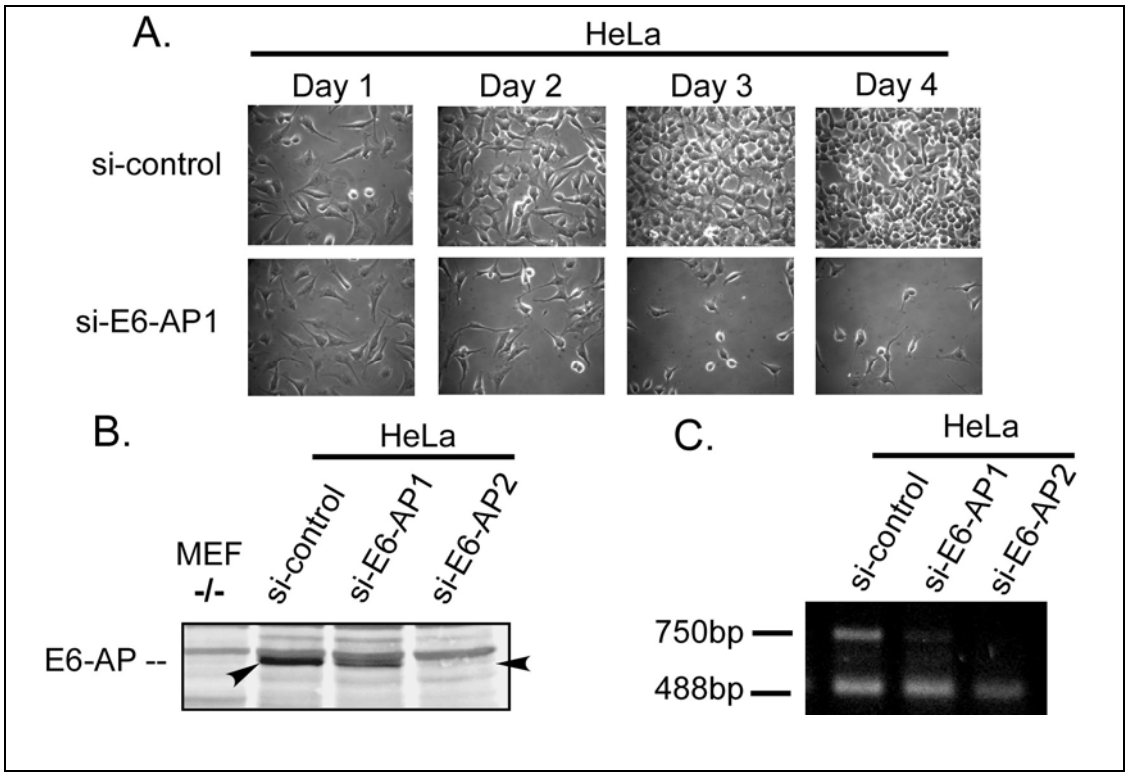

Figure 9: Down-regulation of E6-AP has a growth suppressive effect in HPV-18E6 positive HeLa cell line. A. Phase contrast images of siRNA treated HeLa cells. 100nM synthetic siRNA specific against renilla luciferase (si-control) or E6-AP (si-E6-AP1, siE6-AP2) were transfected into HeLa cells seeded in a 24 well plate and monitored for 4 days. Phase contrast images on day 1 and day 4 post-transfection were taken. B. Western blot analysis of E6-AP down-regulation. HeLa cells were transfected on a 6 well plate with respective siRNA and lysed 48 hrs post-transfection (Material and methods, 3.2.4.1). Western blot analysis was performed using anti-E6-AP monoclonal antibody. Arrow marks indicate the running position of E6-AP. C. Relative quantitative reverse transcription PCR analysis. Silencing of E6-AP mRNA was determined at 48hrs post-transfection by isolating total mRNA from siRNA treated cells. Relative quantitative reverse transcription PCR analysis was performed using E6-AP specific primers which results in a 750 bp amplification product. As an internal standard, 18s rRNA was amplified (488bp product).

To determine if down-regulation of E6-AP expression by RNAi has any effect on cell growth, HPV-18E6 positive HeLa cells were transfected in a 24 well plate with siRNAs

directed either against renilla luciferase (si-control) or E6-AP (si-E6-AP1 and si-E6-AP2) and monitored. In comparison to si-control treated cells, cells treated with si-E6-AP1 showed signs of growth suppression starting from day 2-3 and by day 4, significant differences in cell viability were observed under the conditions used (Figure 9A). While treatment with si-E6-AP1 resulted in growth suppression with only 3-5 percent of cells remaining at day 4, si-E6-AP2 interfered less efficiently with viability with 15-20 percent remaining day 5-6 post-transfection (data not shown and see Discussion, 5.1).

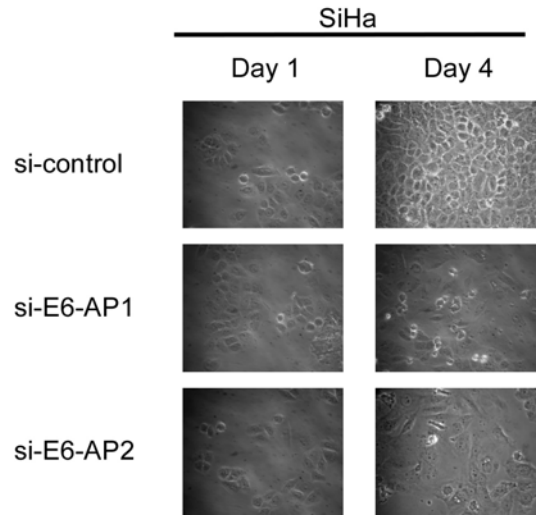

Figure 10: Down-regulation of E6-AP has a growth suppressive effect in HPV-16E6 positive SiHa cell line. Phase contrast microscopy of siRNA treated HeLa cells. 100nM synthetic siRNA specific against renilla luciferase (si-control) or E6-AP (si-E6-AP1, siE6-AP2) were transfected into SiHa cells seeded in a 24 well plate and monitored for 4 days. Phase contrast images were taken on day 1 and day 4 post-transfection.

To obtain evidence that the observed growth suppression correlated with E6-AP silencing, the transfection was scaled up to a 3.5 cm cell culture plate and silencing of E6-AP mRNA and protein expression were assessed. For protein analysis, cells were lysed 48 hrs post-transfection and Western blot analysis was performed using anti-E6-AP antibody (Figure 9B). A 45-70% decrease in E6-AP protein levels was observed with si-E6-AP1 and si-E6-AP2, respectively. For estimating mRNA down-regulation, total RNA was isolated from cells transfected with control or E6-AP specific siRNA 48 hrs post-transfection. A relative quantitative reverse transcription (RT) PCR analysis was performed using E6-AP specific primers (Material and Methods, 3.2.9). As an internal standard, primers against 18S rRNA were used and amplified along with E6-AP. Relative quantification indicated that mRNA levels was down-regulated by approximately 2-3 fold (Figure 9C).

To extrapolate the growth suppressive effect of E6-AP down-regulation, to other high-risk HPV infected cell lines, similar experiments were performed in HPV-16 positive SiHa cells. As shown in Figure 10, cells treated with E6-AP specific siRNA significantly inhibited their growth, too.

4.2.2 Down-regulation of E6-AP expression by RNA interference induces accumulation of p53 and interferes with the viability of HPV-positive cancer cell lines

Activation of the p53 dependent pathway has been linked to the accumulation of the p53 protein and its increased transcriptional activity (Harris and Levine, 2005). As mentioned earlier, down-regulation of E6-AP using antisense, ribozyme or catalytical inactive forms of E6-AP results in accumulation of p53 (Beer-Romero *et al.*, 1997; Talis *et al.*, 1998; Kim *et al.*, 2003). To correlate the growth suppressive effect observed upon down-regulation of E6-AP by RNAi, cellular p53 levels were visualized by immunofluorescence analysis in HeLa cells. siRNAs specifically targeting the expression of either E6-AP (si-E6-AP1 or si-E6-AP2), renilla luciferase (si-control) or Hdm2 (si-Hdm2) were transfected into HeLa cells and immuno-stained for p53 at 24 hrs (day 1) and 96 hrs (day 4) post-transfection (Figure 11A) (Material and methods, 3.2.7.5). Transfection of si-control or si-Hdm2 into HeLa cells did not show any increase in p53 levels or effect on cell viability, supporting the notion that Hdm2 plays no role or only a minor role in p53 degradation in HPV-positive cells (Hengstermann et al., 2001). However, transfection of si-E6-AP1 or si-E6-AP2 showed significantly increased levels of p53. This increase in p53 levels was determined by Western blot analysis (Figure 11B).

Since one of the consequences of p53 stabilization results in induction of apoptosis, TUNEL (TdT-mediated dUTP biotin nick end labelling) analysis was performed at day 1 and day 4 post-transfection (Material and Methods, 3.2.7). Whereas cells treated with control or Hdm2 specific siRNA were negative for TUNEL staining, most of the HeLa cells transfected with si-E6-AP1 were positive for apoptosis at day 4. In comparison to si-E6-AP1 transfected cells, only few of the si-E6-AP2 treated cells showed signs of apoptosis at day 4 (Figure 11B and see Discussion).

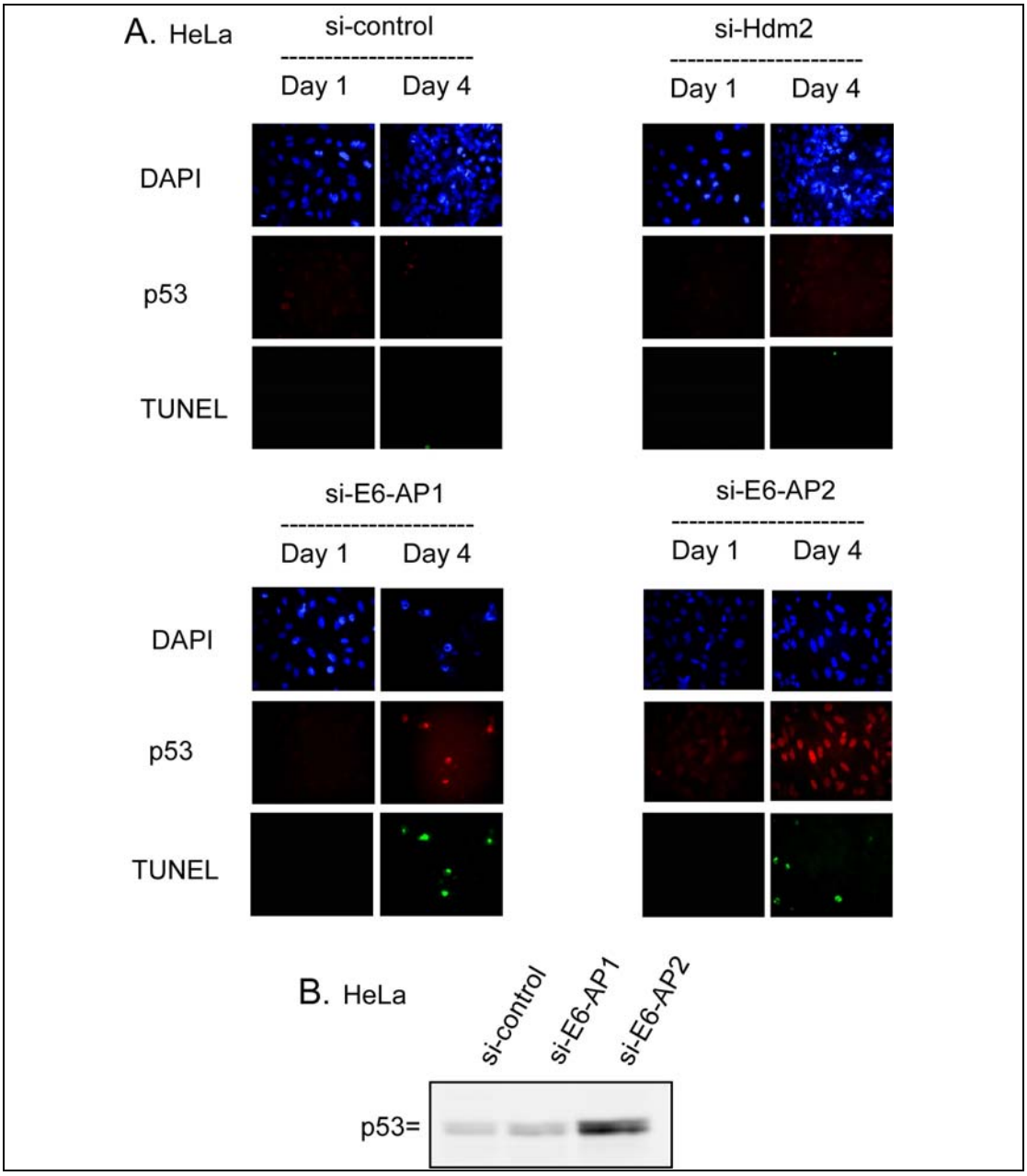

Figure 11: Down-regulation of E6-AP expression by RNAi induces accumulation of p53 and interferes with the viability of HeLa cells. A. Immunofluorescence analysis of HeLa cells treated with siRNA. Synthetic siRNAs specific for E6-AP (si-E6-AP1 and si-E6AP2), Hdm2 (si-Hdm2), and renilla luciferase (si-control) were transfected into HeLa cells seeded on coverslips in 24 well plates. Cells were fixed on day 1 and day 4 post-transfection and levels of p53 (p53) and induction of apoptosis (TUNEL) were determined by immunofluorescence and TUNEL assay (TdT-mediated dUTP-biotin nick end labeling) respectively. In addition, cells were nuclei stained using DAPI (4',6-diamidino-2-phenylindole). B. Silencing of E6-AP expression leads to accumulation of p53. HeLa cells were transfected on a 6 well plate with indicated siRNA and lysed 48 hrs post-transfection. Western blot analysis was performed using anti-p53 DO-1 monoclonal antibody.

A similar experiment was also performed in HPV-16-positive SiHa cell line (Figure 12A). As in the case of HeLa cells, an apoptotic response was also observed in SiHa

cells treated with E6-AP specific siRNA (data not shown for si-E6-AP2), whereas no signs of growth suppression or apoptosis were seen with either si-Hdm2 or si-control treated cells. Parallel to these experiments, the efficiency of silencing was confirmed by western blot analysis (Figure 12B and C).

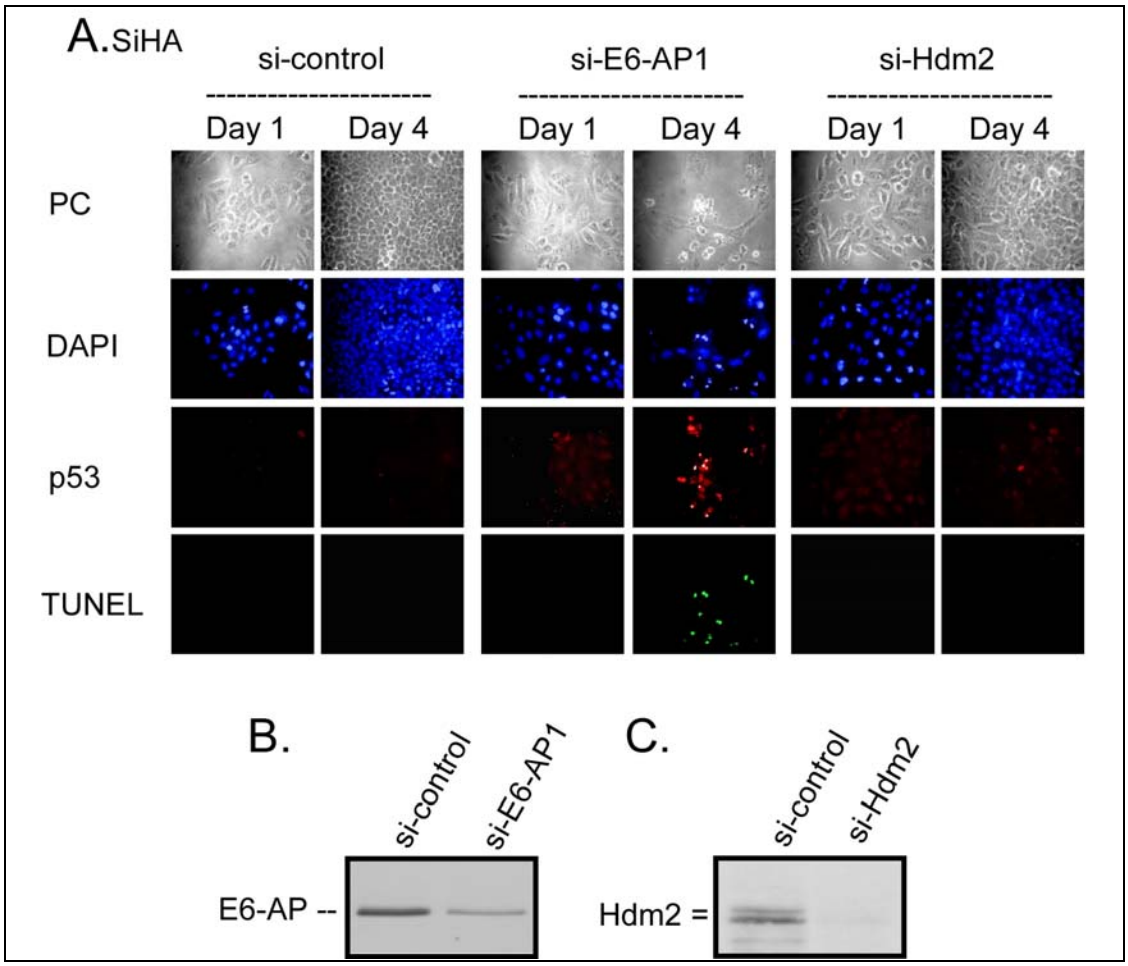

Figure 12: Down-regulation of E6-AP expression by RNAi induces accumulation of p53 and interferes with the viability of SiHa cells. A. Immunofluorescence analysis of SiHa cells treated with siRNA. Synthetic siRNAs specific for E6-AP (si-E6-AP1), Hdm2 (si-Hdm2), or renilla luciferase (si-control) were transfected into SiHa cells seeded on coverslips in a 24 well plate. Cells were fixed on day 1 and day 4 post-transfection and levels of p53 (p53) and induction of apoptosis (TUNEL) were determined by immunofluorescence and TUNEL assay (TdT-mediated dUTP-biotin nick end labeling) respectively. In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei were stained by DAPI (4', 6-diamidino-2-phenylindole). B and C. SiHa cells were transfected with siRNA as indicated siRNA and lysed 48 hrs post-transfection. Western blot analysis was performed using anti-E6-AP monoclonal antibody (B) or anti-Hdm2 monoclonal antibody (C).

To further confirm this growth suppressive phenotype, a shRNA retroviral vector system called pRetroSUPER (pRS) was used. This vector contains the shRNA expression cassette along with a puromycin resistant gene and can be used for short

term and long term assays using DNA transfection or retroviral transduction (Berns *et al.*, 2004). Using this vector system, colony formation assays were performed in HeLa and SiHa cell lines (Materials and Methods, 3.2.6.3). As shown in Figure 13, colony formation assays performed by transfection of pRS-sh-E6-AP1 or pRS-sh-E6-AP2 led to significant decrease in colony numbers in comparison to pRS-sh-control. Of the surviving colonies that appeared on the E6-AP knock down plates showed no significant decrease in E6-AP expression indicating that E6-AP was not down-regulated in these colonies (data not shown).

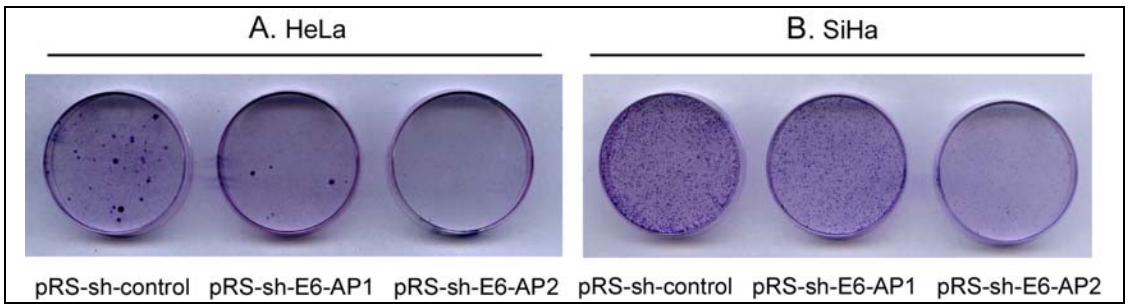

Figure 13: Colony formation assay of E6-AP down regulation in HeLa and SiHa cell lines. HeLa (A.) and SiHa (B.) cell lines were seeded on a 24 well plate and transfected with the indicated vectors targeting renilla luciferase (pRS-sh-control) or E6-AP (pRS-sh-E6-AP1 and pRS-sh-E6-AP2). 24 hrs later, cells were trypsinzed and plated onto a 6cm plate. Colonies were selected for with puromycin and stained after 10-15 days.

The data presented above indicates that a certain threshold level of E6-AP expression is required for viability of HPV-positive cervical cancer cell lines and that down-regulation of E6-AP expression results in p53 accumulation and induction of apoptosis. However, since E6-AP is a cellular protein that is expressed in all cell lines tested regardless of their HPV status, the anti-growth-suppressive properties of E6-AP may not be related to its ability to interact with E6 and target p53 for degradation.

4.3 Down-regulation of E6-AP in HPV-negative cell lines

4.3.1 RNAi induced down-regulation of E6-AP expression has no growth suppressive effect in HPV-negative tumour cell lines under transient conditions

To determine whether down-regulation of E6-AP has a growth suppressive effect in HPV-negative cells, cell lines expressing wild-type p53 (i.e. MCF-7, RKO and U2OS) were transfected with siRNA specific against E6-AP (si-E6-AP1 and si-E6-AP2),

Hdm2 (si-Hdm2), or renilla luciferase (si-control). As reported previously (Linares et al., 2003), down-regulation of Hdm2 resulted in p53 accumulation and induction of apoptosis. However, no indication for p53 accumulation and/or interference with cell viability was observed upon treatment of the cells with E6-AP-specific siRNA (Figure 14 and data not shown).

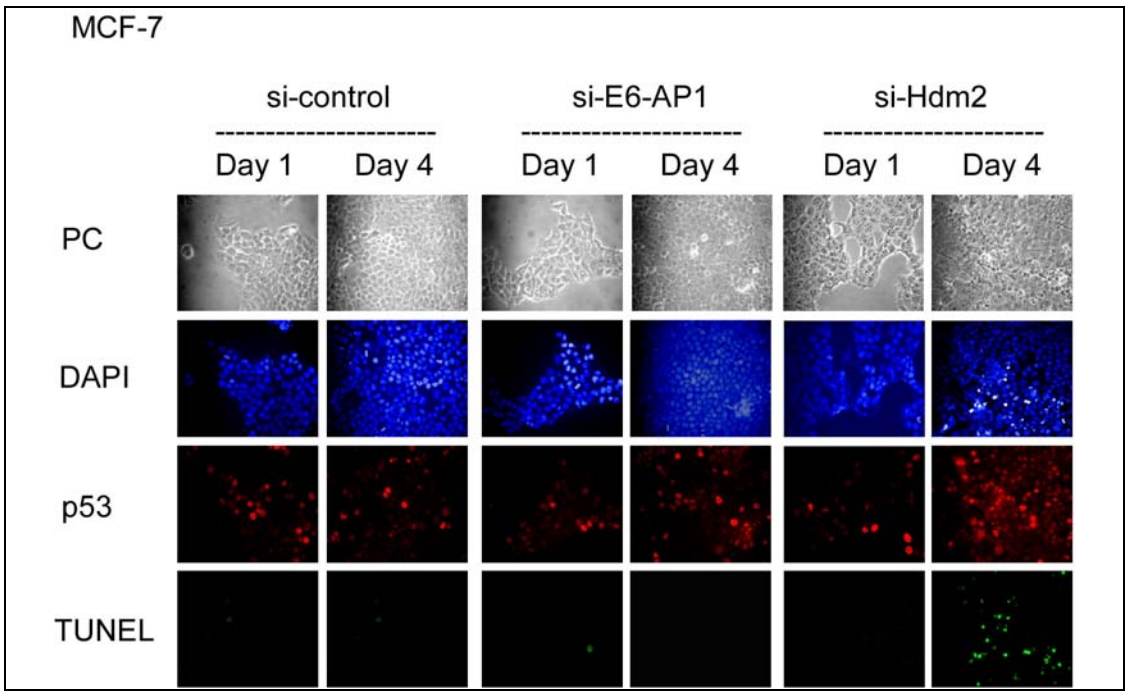

Figure 14: Down-regulation of E6-AP does not affect growth or p53 levels in HPV-negative cell line MCF7. Synthetic siRNAs against E6-AP (si-E6-AP1), renilla luciferase (si-control), and Hdm2 (si-Hdm2) were transfected into HPV-negative cell line, MCF-7. Levels of p53 and induction of apoptosis were determined by immunofluorescence and TUNEL assay (TdT-mediated dUTP-biotin nick end labeling) respectively on day 1 and day 4 post-transfection. In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei by DAPI (4', 6-diamidino-2-phenylindole) staining. Cells were fixed on day 1 and day 4 post-transfection and images were taken using a phase contrast microscope.

To test the efficiency of E6-AP silencing, a Western blot analysis was performed. However, in HPV-negative cell lines E6-AP protein levels were not reduced to equivalent levels as that observed in HPV-negative cell lines within a similar time frame (Figure 15A). However, a relative quantitative RT-PCR analysis revealed that E6-AP mRNA levels were reduced to similar extents (approximately two to three fold) in HPV-positive cells and HPV-negative cells by the siRNA used (compare Figure 9C and Figure 15C). This apparent paradox (similar effect on RNA levels but different effect on protein levels) is possibly explained by the notion that E6-AP has a rather long half-life in HPV-negative cells but is targeted for proteasome-mediated

degradation by the E6 oncoprotein in HPV-positive cells (Kao et al., 2000). Thus, it is expected that transient siRNA treatment affects E6-AP levels in HPV-positive cells more significantly than in HPV-negative cells.

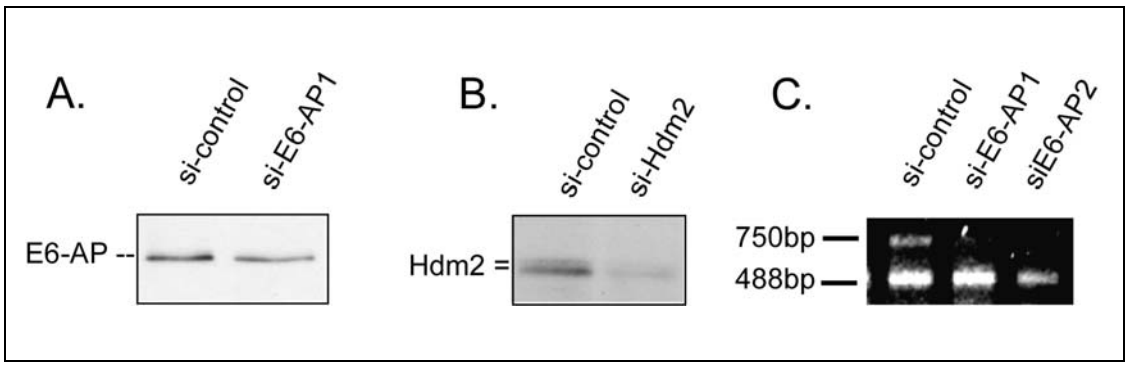

Figure 15: Down-regulation of E6-AP and Hdm2 in MCF7 cells. A. Western blot analysis of E6-AP down-regulation. MCF-7 cell line was transfected with either si-control or si-E6-AP1 siRNA on a 6 well plate. Total protein lysates was prepared 48 hrs post-transfection and analyzed by western blot analysis using anti-E6-AP monoclonal antibody. C. Relative quantitative reverse transcription PCR analysis. Silencing of E6-AP mRNA was determined at 48 hrs post-transfection by isolating total mRNA from siRNA treated cells. Relative quantitative reverse transcription PCR analysis was performed using E6-AP specific primers which results in a 750bp amplification product. As an internal standard, 18s rRNA was amplified (488 bp product).

4.3.2 Stable knockdown of E6-AP expression in a H1299, a HPV-negative cell using shRNA expression vectors

Transient silencing of E6-AP expression using synthetic siRNA had suggested that down-regulation of E6-AP does not have a growth suppressive in HPV-negative cells. However, E6-AP protein levels were not completely abrogated and thus it remained unclear if E6-AP is required for cell viability in HPV-negative cell lines. Therefore, shRNA expression vector targeting E6-AP (pRS-sh-E6-AP1) was transfected into H1299 cells and transfected cells were selected out with puromycin. Cells exhibiting puromycin resistance was used to generate single clonal cell lines stably expressing E6-AP shRNA (Material and Methods, 3.2.10). Cell lysates were prepared from these clonal cell lines and endogenous E6-AP levels were analyzed by Western blot analysis. While some of the single cell clones generated did show decrease in E6-AP levels (knockdown ranging from 30-40%), the efficiency of knockdown was insufficient to rule out the possibility that the available E6-AP is sufficient for viability (data not shown).

Further to this, since sh-E6-AP2 sequence was more efficient than sh-E6-AP1 (Figure 8) attempts were also made to generate stable clones using this sequence. Hence pRS-sh-E6-AP2 was transfected into HeLa and H1299 (HPV-negative) cell lines and clones exhibiting puromycin resistance were picked. In comparison to HeLa cells (HPV-positive cells) wherein down-regulation of E6-AP has a strong growth suppressive effect (see also Figure 13) single cell clones of H1299 were obtained exhibiting significant decrease in E6-AP levels as determined by western blot analysis (Figure 16). This would suggest that in comparison to HPV-positive cell lines, HPV-negative cell lines require lower threshold levels of E6-AP for viability (see also Discussion).

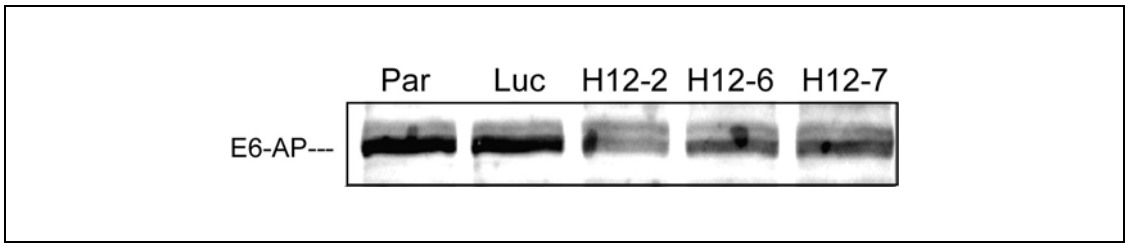

Figure 16: Stable knockdown of E6-AP expression in a H1299, a HPV-negative cell using shRNA expression vectors. H1299 cell line was transfected with either pRS-sh-control or pRS-sh-E6-AP2. Cells were selected for puromycin resistance and single cell clones established. Levels of E6-AP in these single cell clones were determined by western blot analysis using E6-AP specific monoclonal antibody. Of the ten clones picked, three of the clones (H12-2, H12-6, H12-7) showed significant decrease in E6-AP levels. To compare with initial endogenous E6-AP levels, parental H1299 cell lysates (Par) and one of the puromycin resistant clones established from transfection for pRS-control (Luc) is loaded.

Taken together the results described above corroborate previous data indicating that E6-AP is required for both p53 degradation (Beer-Romero et al., 1997; Traidej et al., 2000; Hengstermann et al., 2001; zur Hausen, 2002; Kim et al., 2003; Kelley et al., 2005) and the viability of HPV-positive cells lines. However, since E6-AP is implicated in E6-mediated degradation of proteins other than p53 (e.g., E6TP1 and hscrib) it remains unclear if the apoptosis enhancing effect is directly linked to the ability of E6-AP to target p53 for degradation or whether other yet uncharacterized functions of E6-AP are also involved. One possible way to address this issue is to generate HPV-positive cells, in which expression of endogenous wild-type p53 is abrogated. The rationale for such an approach is the assumption that if the anti-growth-suppressive activity of E6-AP is functionally linked to its ability to degrade p53, p53-null HPV-positive cells should be less sensitive towards siRNA-mediated down-regulation of E6-AP expression.

4.4 Stable suppression of p53

4.4.1 Validation of p53 knockdown construct

In order to bring about stable silencing of p53 expression, DNA-based shRNA expression vector (pSUPER) designed against p53 (sh-p53) was employed (Brummelkamp *et al.*, 2002b). The predicted transcript of sh-p53 is shown in Figure 17A. To test the efficacy of this vector, initial proof of principle experiments were performed.

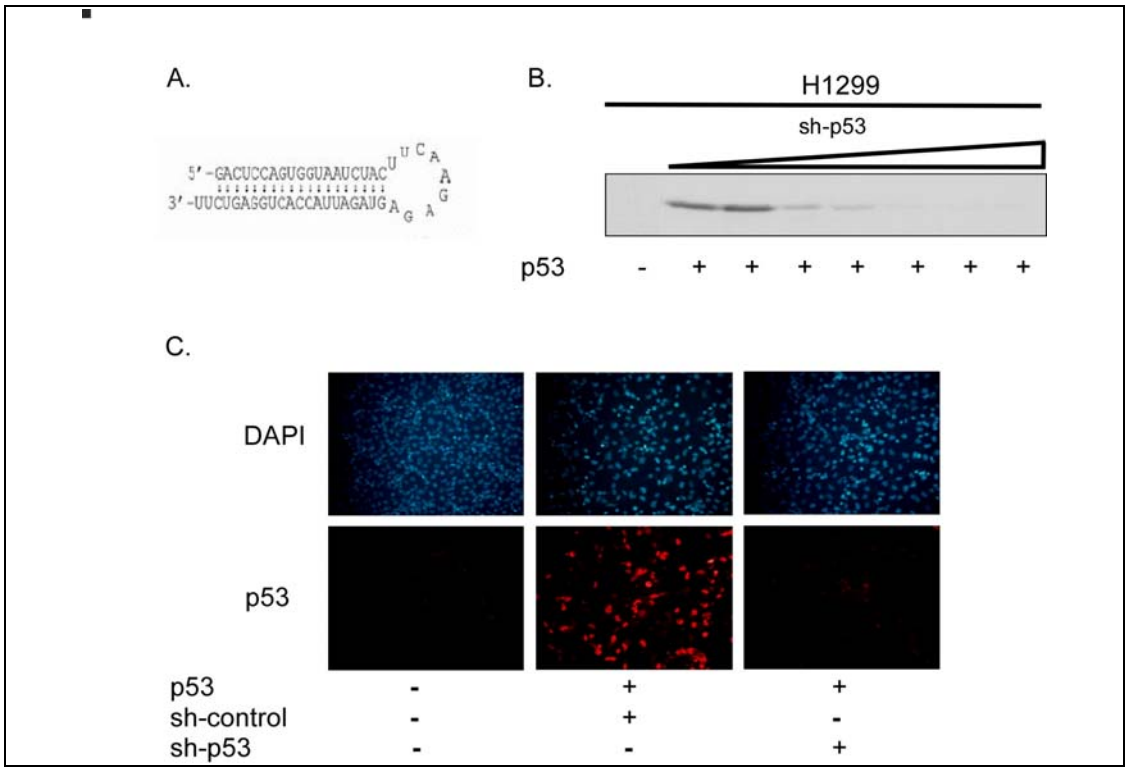

Figure 17: Validation of shRNA vector targeting p53. A. Predicted transcript of sh-p53 (Brummelkamp *et al.*, 2002b). B. Western blot analysis of p53 down-regulation. 25ng of a p53 expression vector was co-transfected with increasing amounts of sh-p53 into H1299 cells (p53 null, lung carcinoma). The total DNA amount was retained the same in each transfection by equalizing with empty vector. Levels of p53 were determined 24 hrs post-transfection by western blot analysis. C. Immunofluorescence analysis. p53 expression vector was co-transfected with a shRNA vector targeting either renilla luciferase (sh-control) or sh-p53 into H1299 cells and levels of p53 expression were determined by immunofluorescence. Cells were simultaneously stained with DAPI for nuclei staining.

A wild-type p53 expressing construct was co-transfected with increasing amounts of sh-p53 into H1299 cells, a p53 null cell line. To normalize for total amounts of DNA transfected, a construct targeting renilla luciferase (sh-control) was used. Protein extracts were prepared 24 hrs post-transfection and p53 levels were determined by

western blot analysis using a p53 specific antibody (Figure 17B). p53 protein levels were significantly reduced with increasing amount of sh-p53 vector transfected indicating an efficient knockdown. These results were also substantiated by immunofluorescence analysis (Figure 17C).

4.4.2 Generation of stable p53 knockdown cell lines

To generate stable p53 knockdown cell lines, HPV-positive (HeLa and SiHa) were co-transfected with sh-p53 and a vector containing a neomycin resistance marker (Materials and Methods, 3.2.10.2). The HPV-negative cell line RKO, which expresses high levels of wild type p53, was also transfected as a control to test the efficiency of the vector system to bring about stable suppression. Transfected cells were selected with G418 and single cell clones stably expressing the neomycin resistance gene were established. Single cell clones obtained were then tested for reduction in p53 levels by western blot analysis. While some of the neomycin resistant clones (marked with asterisks) showed significantly decreased levels of p53 (Figure 18A-C), others did not. This could be due to preferential integration of the resistance vector or due to inactivation of the RNAi construct in these clones.

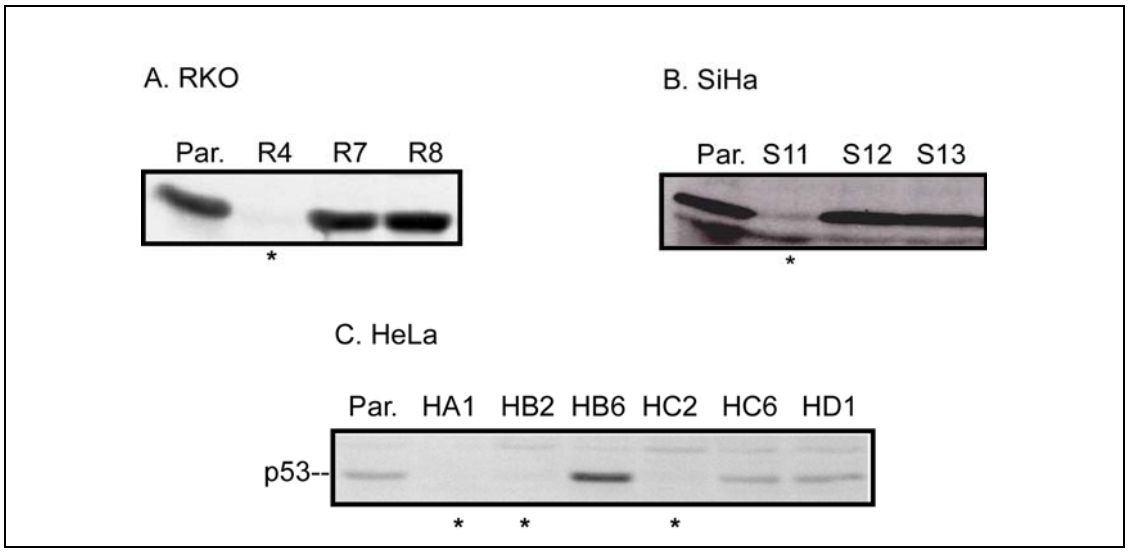

Figure 18: Generation of p53 knockdown HPV-positive and HPV-negative cell lines. HPV-negative cell lines (RKO) and HPV-positive cell lines (HeLa and SiHa) were co-transfected with sh-p53 vector and a vector expressing the neomycin resistance gene. Cells were selected out for neomycin resistance and single cell clones established. Levels of p53 in these single cell clones were determined by western blot analysis using p53 specific antibody. (A to C) are representative western blots of single cell clones. Asterisk marks indicate single cell clones showing stable knockdown of p53.

4.4.3 Stress induced activation of p53 in HeLa and SiHa stable clones down-regulated by pSUPER –p53

Upregulation of p53 levels in response to various stimuli is, at least in part, achieved by significantly attenuated degradation (Balint and Vousden, 2001; Michael and Oren, 2003). Experiments using anti-tumour drugs such as Actinomycin D (AD) have shown that it is a potent activator of p53 and leads to accumulation of p53 levels (Hietanen et al., 2000). Similarly inhibition of proteasomal degradation using proteasomal inhibitors such as MG132 leads to stabilization of p53 levels. Although western blot analysis revealed that p53 was reduced to non-detectable levels in the HeLa and SiHa stable clones (Figure 18B and C), it was necessary to determine if these clones were indeed null with respect to p53 protein levels.

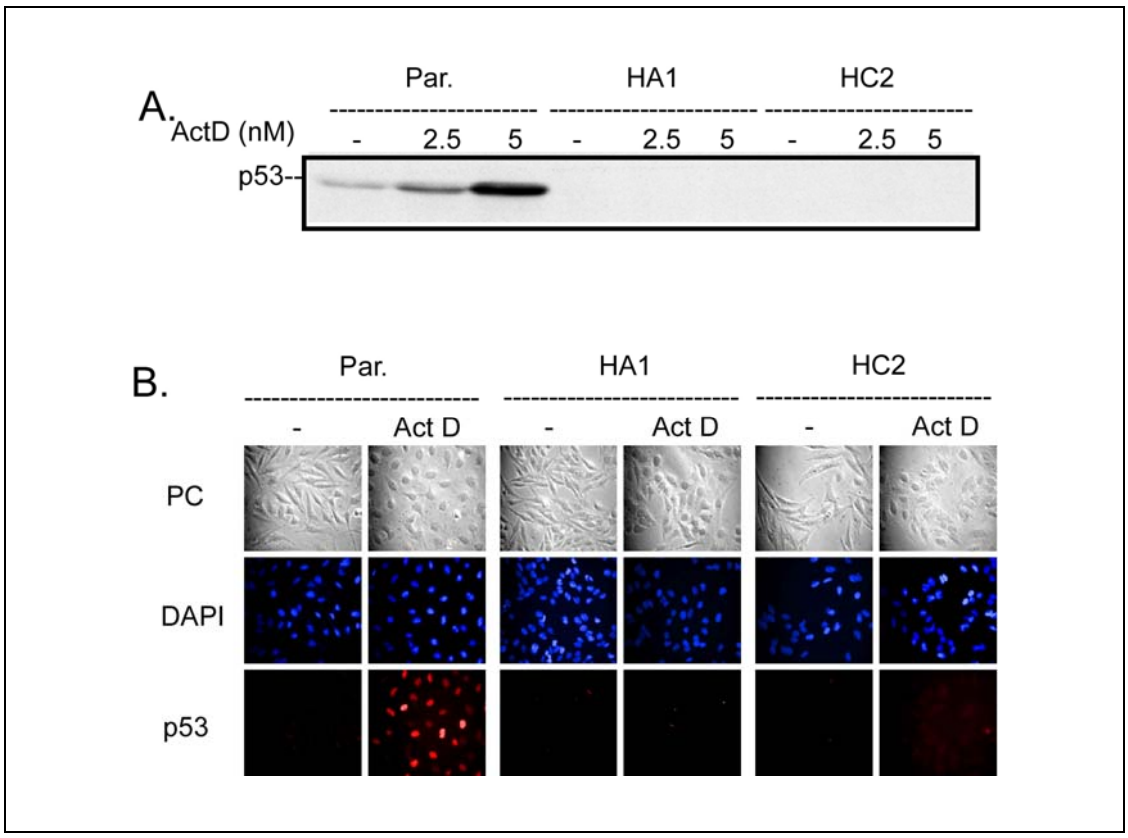

Figure 19: Activation with ActD in down-regulated p53 HeLa clones. A. Parental HeLa cells (Par.) and the clones HA1 and HC2 were treated with for 24hrs with 2.5nM and 5nM Actinomycin D (ActD) as indicated or, as a control, with dimethyl sulfoxide (-). Levels of p53 were determined by Western blot analysis. B. Parental HeLa cells (Par.) and the single cell clones HA1 and HC2 were treated for 24hrs with 5nM Actinomycin D (ActD) or as a control, with dimethyl sulfoxide (-). Levels of p53 were determined by immunofluorescence (p53). In addition cells were visualized by phase contrast microscopy (PC) and nuclei by use of DAPI (DAPI).

4.4.3.1 HeLa

To obtain further evidence that p53 expression was indeed abrogated in these stable clones, p53 levels were determined upon treatment with Actinomycin D. For this, parental HeLa cells and the clones exhibiting down-regulated p53 levels were treated with 2.5nm and 5nm AD. Protein lysates were prepared 24 hrs after treatment and p53 levels determined by western blot analysis. Whereas AD treated parental HeLa cells exhibited significant p53 accumulation, p53 accumulation was not observed in two of the HeLa clones, HA1 and HC2 (Figure 19A), indicating that they were indeed null p53 expression. To substantiate this observation, wild type HeLa cells and the clones HA1 and HC2 were treated with 5 nm of Actinomycin D and p53 levels determined by immunofluorescence analysis (Figure 19B). As in the previous experiment, no detectable levels of p53 stabilization was observed in HA1 and HC2 clones confirming that the clones were indeed p53 null. Furthermore, treatment with the proteasomal inhibitor MG132 also gave the same result (Figure 20).

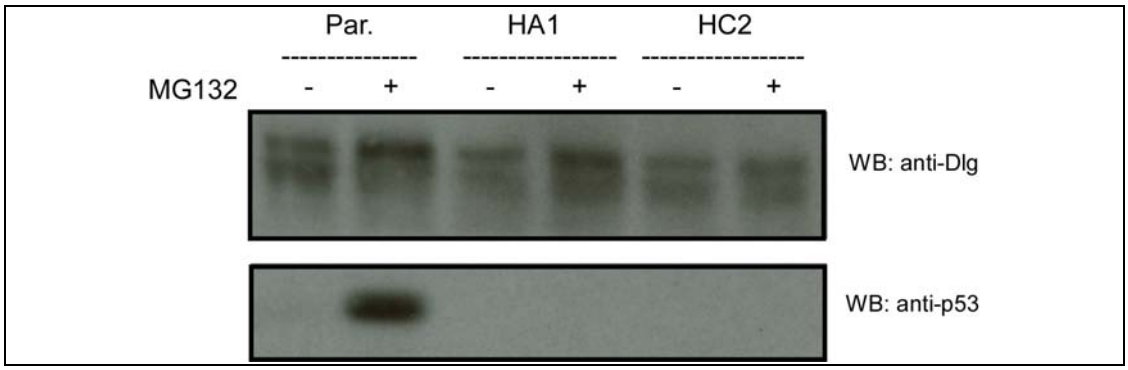

Figure 20: Treatment with of HeLa cell lines with MG132. Parental HeLa cells (Par.) and the clones HA1 and HC2 were treated with 10μM MG132 for 4 hrs as indicated or, as a control, with dimethyl sulfoxide (-). Levels of p53 were determined by Western blot analysis using a p53 monoclonal antibody. As a control for loading and MG132 effect, the blot was also probed with anti-Dlg mouse monoclonal antibody 2D11.

As indicated above, the viral E6 and E7 oncoprotein is continuously expressed in HPV-positive cancer cells lines. To confirm that the stable clones obtained, were indeed derived from HeLa cells, levels of E7 protein in the parental HeLa cells were compared to the two p53 null clones, HA1 and HC2 by western blot analysis. As a negative control, lysates from C33-A, an HPV-negative cervical carcinoma cell line was loaded. As shown in Figure 21 expression of the E7 oncoprotein in the p53-null HeLa cell lines were not affected demonstrating that they were indeed derived from HeLa cells. Taken

together these results indicate that expression of p53 is completely, or almost completely, abrogated in the HeLa clones HA1 and HC2.

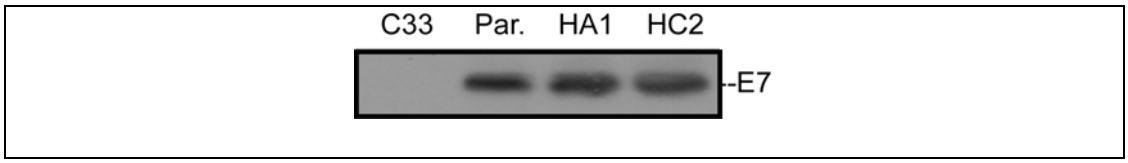

Figure 21: Comparison of E7 levels in parental HeLa and clones HA1 and HC2. Levels of the HPV-18 E7 protein in parental HeLa cells (Par.) and the clones HA1 and HC2 were determined with a polyclonal anti-E7 antibody by western blot analysis. Extracts prepared from HPV-negative C33-A cells (C33) served as a negative control

4.4.3.2 SiHa

Of all the SiHa clones which showed significant down-regulation of p53 two clones showing maximum down-regulation (clones S-10 and S-11) were taken to check whether p53 expression was indeed abrogated. Although p53 levels were significantly lower in these clones than in parental SiHa cells, treatment with Actinomycin D or MG132 led to accumulation of p53 suggesting that p53 was still expressed in these clones though at low levels (Figure 22).

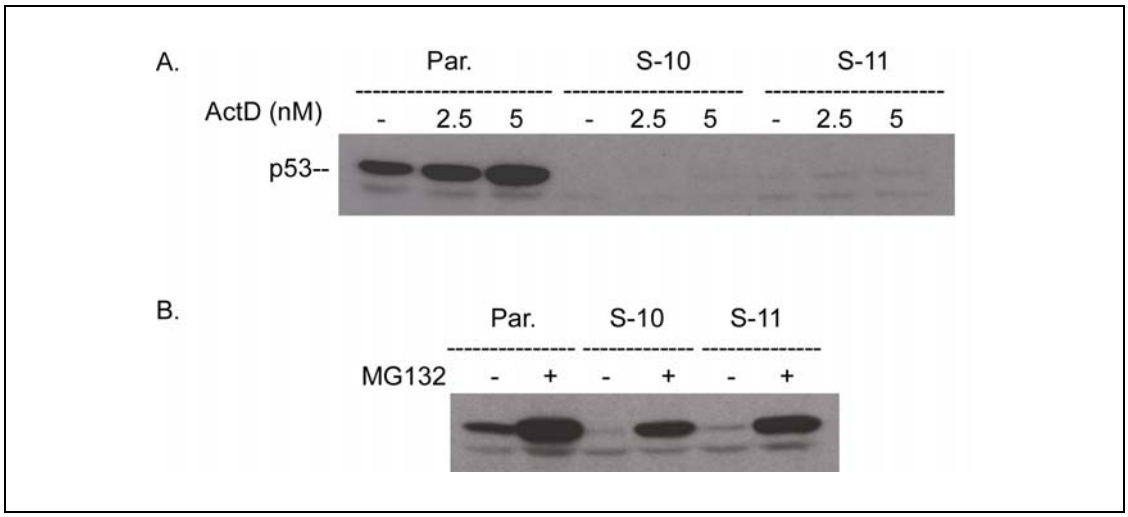

Figure 22: Treatment with Act D and MG132 in SiHa clones. A. Parental SiHa cells (Par.) and the clones Cl-10 and Cl-11 were treated with for 24hrs with 2.5nM and 5nM Actinomycin D (ActD) as indicated or, as a control, with dimethyl sulfoxide (-). Levels of p53 were determined by Western blot analysis. B. Parental SiHa cells (Par.) and the clones Cl-10 and Cl-11 were treated with for 4hrs with 10μM MG132 as indicated or, as a control, with dimethyl sulfoxide (-). Levels of p53 were determined by Western blot analysis.

As an alternative strategy, to obtain SiHa cell lines in which p53 expression was abrogated, a retroviral vector targeting p53 (pRS.sh-p53) was transfected into SiHa

cells and stable clones were selected using puromycin. Although in total more than 100 single cell clones were tested for p53 knockdown, none of the stable cell lines showed complete abrogation of p53 expression (data not shown and see also Discussion 5.4)

4.5 Down-regulation of E6-AP in p53 null clones HA1 and HC2

To determine whether the ability of E6-AP to target p53 for degradation in the presence of E6 is essential for the viability of HPV-positive cancer cells or whether other yet uncharacterized functions of E6-AP are involved, the p53–null clones HA1 and HC2 were transfected with E6-AP specific siRNAs and the effects of E6-AP down-regulation on p53 and on cell viability determined (Figure 23). As expected, p53 was not detectable in the p53-null cells even 4 days after transfection. Remarkably, down-regulation of E6-AP with E6-AP siRNA1 and E6-AP siRNA2 did not interfere with the viability of p53-null HeLa cells. Furthermore, no signs of apoptosis in the p53- null cells were observed under conditions in which apoptosis in parental HeLa cells was significantly induced. Both the transfection efficiencies (Figure 24A and B) and the efficiencies of siRNA mediated down-regulation of E6-AP levels (Figure 24B) were similar for p53-null cells and parental HeLa cells.

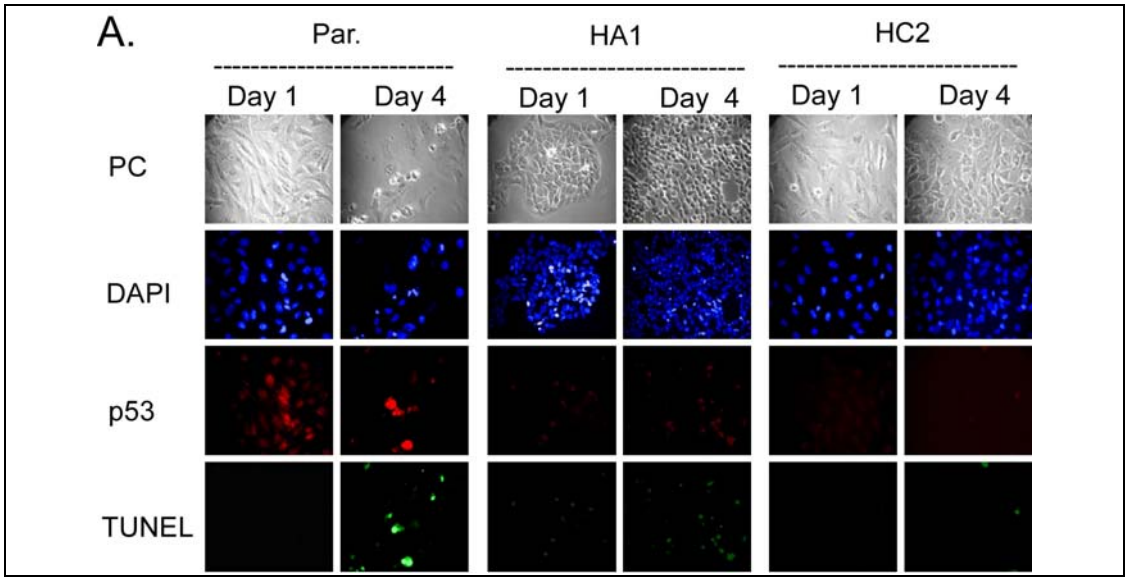

Figure 23: Down-regulation of E6-AP expression does not interfere with the growth of p53-null HeLa cells. E6-AP specific synthetic siRNA were transfected into HeLa cells (Par.) and the p53-null clones HA1 and HC2. Levels of p53 and induction of apoptosis were determined by immunofluorescence and TUNEL assay (TdT-mediated dUTP-biotin nick end labeling) respectively on day 1 (24hrs) and day 4 (96hrs) post-transfection. In addition, cells were visualized by phase-contrast microscopy (PC) and nuclei by DAPI (4', 6'-diamidino-2-phenylindole) staining.

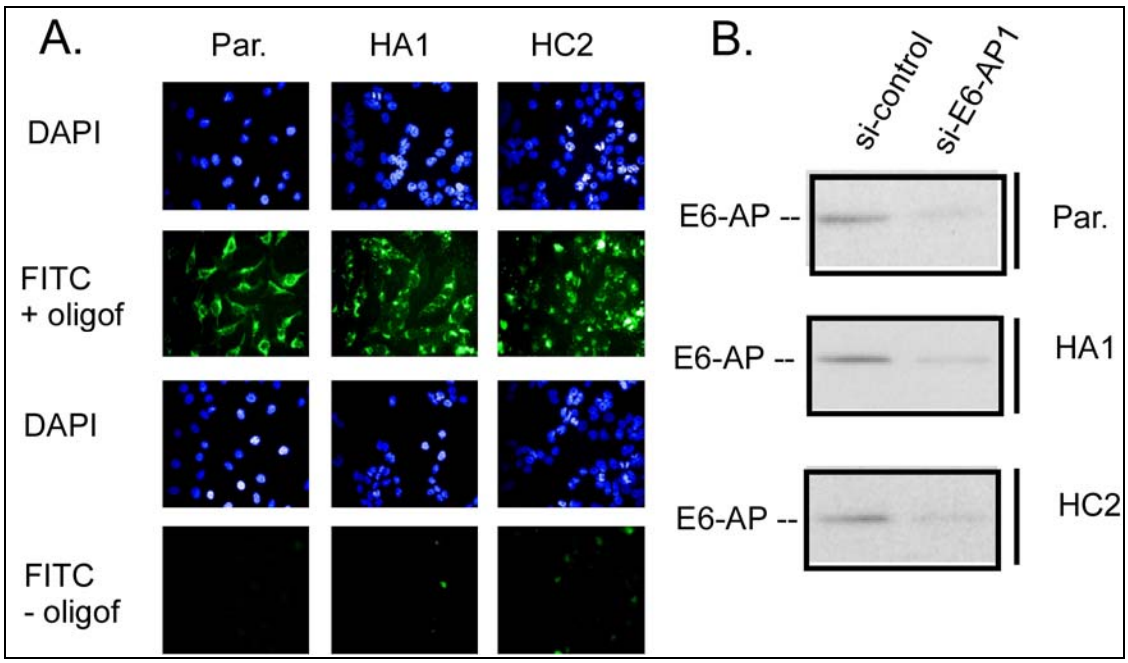

Figure 24: Transfection efficiency and knockdown. A. To compare transfection efficiencies of parental HeLa cells (Par.) and the HeLa derived p53-null clones HA1 and HC2 were incubated with FITC-dextran in the presence (FITC+oligof.) or absence (FITC-oligof.) of Oligofectamine (Invitrogen) as indicated. In addition, nuclei were visualized by use of DAPI. B. E6-AP levels were determined by Western blot analysis using a mouse monoclonal antibody.

4.6 RNAi-rescue assays

The data presented so far indicate that under cell culture conditions the growth suppressive phenotype of E6-AP down-regulation in HPV-positive cell lines, is dependent on p53. Initial reports of gene silencing using RNAi technology suggested it to be highly specific. However, recent reports do indicate "off-target" gene regulation by RNAi in some cases (Jackson et al., 2003; Scacheri et al., 2004; Snove and Holen, 2004). In addition to using two different siRNA/shRNAs targeting sequences against E6-AP to further substantiate the above result an attempt was made to rescue the phenotype. Such a rescue approach could also be exploited to test the ability of different mutant forms of E6-AP to rescue the phenotype, thus performing "quasi genetics" within cells, thereby correlating the biochemical activities of a particular protein such as E6-AP to its biological role within a cell.

4.6.1 Validation of rescue constructs

One possible approach to rescue the phenotype of RNAi-mediated down-regulation of a given protein is by overexpressing an "RNAi–resistant" expression construct of the

target gene. RNAi depends upon perfect complementarity between siRNA/shRNA and the RNAi targeting sequence (within the mRNA). Thus an "RNAi-resistant" rescue construct can be created by introducing silent point mutations at the siRNA/shRNA target site while still maintaining the same amino acid sequence. In contrast, an overexpression construct with perfect complementarity will be "RNAi-susceptible".

To generate an RNAi-resistant rescue constructs of E6-AP, E6-AP expression constructs with two silent point mutations (Figure 25A) at the si/shRNA target site corresponding to either si/sh-E6-AP1 (E6-AP.mut1) or si/sh-E6-AP2 (E6-AP.mut2) were generated in the mammalian expression vector, pEF1/V5-His in which overexpression is driven by a cellular house keeping promoter, the human elongation factor 1α (hEF-1α). In order to differentiate between the endogenous and ectopically expressed E6-AP, an N-terminal haemagglutinin tag (HA) was incorporated into the construct. The logic behind introducing two silent mutations was based on the finding that even a single mismatch at the siRNA-targeting region obliterates silencing (Elbashir et al., 2001). To confirm whether these constructs were indeed resistant to RNAi against E6-AP, the ability of sh-E6-AP1 and sh-E6-AP2 to downregulate E6-AP expression from E6-AP.mut1 or E6-AP.mut2 was tested by transient co-transfection assay (Material and methods, 3.2.3). The ability of sh-E6-AP1 or sh-E6-AP2 to downregulate E6-AP expression from an RNAi susceptible vector (wt-E6-AP) construct was also compared as a control. As shown in Figure 25B, as expected, ectopic E6-AP expression from the wt-E6-AP was down-regulated upon co-transfection with sh-E6-AP2 and not with the sh-control. In comparison, ectopic E6-AP expression from E6-AP.mut2 was not downregulated indicating that this construct was resistant to RNAi. Hence this construct was used in further rescue experiments. In comparison, co-transfection of sh-E6-AP1 with E6-AP.mut1 indicated that E6-AP expression from this vector was silenced nearly as efficiently as an RNAi susceptible E6-AP vector and hence could not be used for a rescue approach (data not shown). As compared to the earlier report of Elbashir et al, wherein RNAi was reported to be highly specific, a recent report has indicated that in some cases, a variable degree of gene silencing can still occur in spite of nucleotide mismatches, depending on the position of the mismatch (Pusch et al., 2003). This could be a reason for silencing of expression even from E6-AP.mut1 vector.

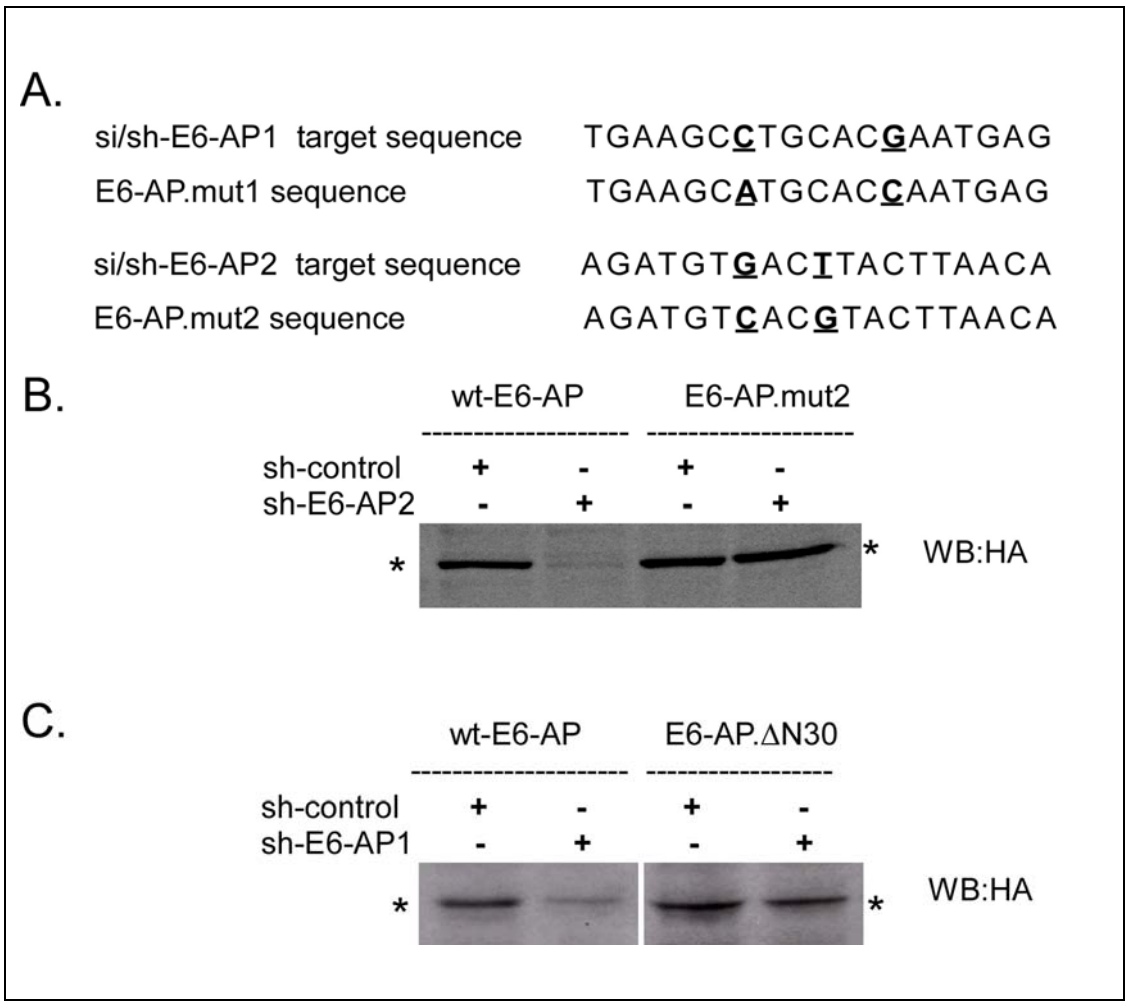

Figure 25: Validation of rescue constructs. A. siRNA/shRNA target sequence in E6-AP and corresponding silent point mutations (indicated in bold and underlined) in the rescue constructs E6-AP.mut1 and E6-AP.mut2. B. pRetroSUPER shRNA construct targeting E6-AP (sh-E6-AP2) or renilla luciferase (sh-control) were co-transfected individually with either a HA tagged wt-E6-AP (RNAi susceptible) or E6-AP.mut2 (RNAi resistant) expression constructs into H1299 cells. 24hrs post-transfection exogenously expressed E6-AP levels were determined by western blot analysis using anti-HA antibody. C. sh-control (targeting renilla luciferase) or sh-E6-AP1 (targeting E6-AP) was co-transfected individually with HA tagged E6-AP.ΔN30 expression construct or HA-tagged wt-E6-AP construct into H1299 cells. 24hrs post-transfection exogenously expressed E6-AP levels were determined by western blot analysis using anti-HA antibody. * indicates the running position of ectopically expressed E6-AP.

Since the initial attempt to generate an RNAi resistant construct against sh-E6-AP1 was unsuccessful, as an alternative approach, a construct of E6-AP having an N-terminal deletion of 30 amino-acids (and thus lacking the sh-E6-AP1 target site) was generated. (E6-AP.ΔN30). E6-AP lacking the N-terminal 30 amino acids has been shown to be sufficient for E6/E6-AP mediated degradation of p53 (Huibregtse *et al.*, 1993). To confirm whether this construct is resistant to RNAi, the ability of sh-E6-AP1 to silence expression of E6-AP from wt-E6-AP or E6-AP.ΔN30 construct were compared by

transient co-transfection. As shown in Figure 25A, while expression from wt-E6-AP was significantly down-regulated, the expression from E6-AP.ΔN30 construct was resistant to silencing and was used for further rescue experiments.

4.6.2 Overexpression of E6-AP in HPV-positive cells and HPV-negative cells

In order to rescue the growth suppressive phenotype of E6-AP in HPV-positive cells, an experimental design as outlined in Figure 26A (strategy 1) was initially tested. This strategy involved co-transfection of a shRNA expression vector (sh-E6-AP1 or 2) and either the overexpression construct of E6-AP which is RNAi susceptible (wt-E6-AP) or a corresponding E6-AP construct which is resistant to RNAi (E6-AP.ΔN30 or E6-AP.mut2). By using colony formation assays (as shown in Figure 13) to compare the number of colonies obtained, a rescue of the phenotype could be determined.

Experiments conducted in HeLa cells using this strategy however did not show any significant rescue of this phenotype (data not shown). To rule out the possibility that the inability to rescue the growth suppressive phenotype was due to the experimental strategy involved, an alternative strategy as depicted in Figure 26B (Strategy 2), was attempted. This strategy involved initially generating stable cell lines overexpressing E6-AP either from the RNAi susceptible or RNAi resistant expression constructs and comparing their ability to rescue the growth suppressive phenotype upon down-regulation of E6-AP by using synthetic siRNA or shRNA vectors.

Hence the E6-AP expressing RNAi resistant or RNAi susceptible constructs were individually transfected into HeLa cells and stable pooled clones exhibiting neomycin resistance were established. However, in the course of these experiments it was unexpectedly observed that that stable ectopic overexpression of E6-AP led to decrease in cell viability as compared to cells transfected with an empty vector (data not shown). Of the clonal population that were neomycin resistant, protein lysates of these pooled clones were prepared and analyzed by IP-Western blot analysis using an anti-HA antibody to detect specifically the overexpressed E6-AP. As shown in Figure 27 B (lane-1), it was not possible to stably overexpress E6-AP either from the RNAi susceptible construct or the RNAi resistant construct (data not shown). This was in spite of the ability to overexpress E6-AP from these constructs in transience (Figure 27A, lane 2 and data not shown). This inability to overexpress E6-AP was also found to

be the case in SiHa and CaSki cell lines which are positive for HPV16 and HPV18 respectively. Similar experiments to stably overexpress E6-AP in HPV-negative cell lines, namely H1299, MCF-7 and RKO exhibited stable overexpression of E6-AP (Figure 27C, lane 2 and data not shown). This would suggest that E6-AP overexpression has a negative effect on cell growth and thus results in selective outgrowth of clones that do not overexpress E6-AP.

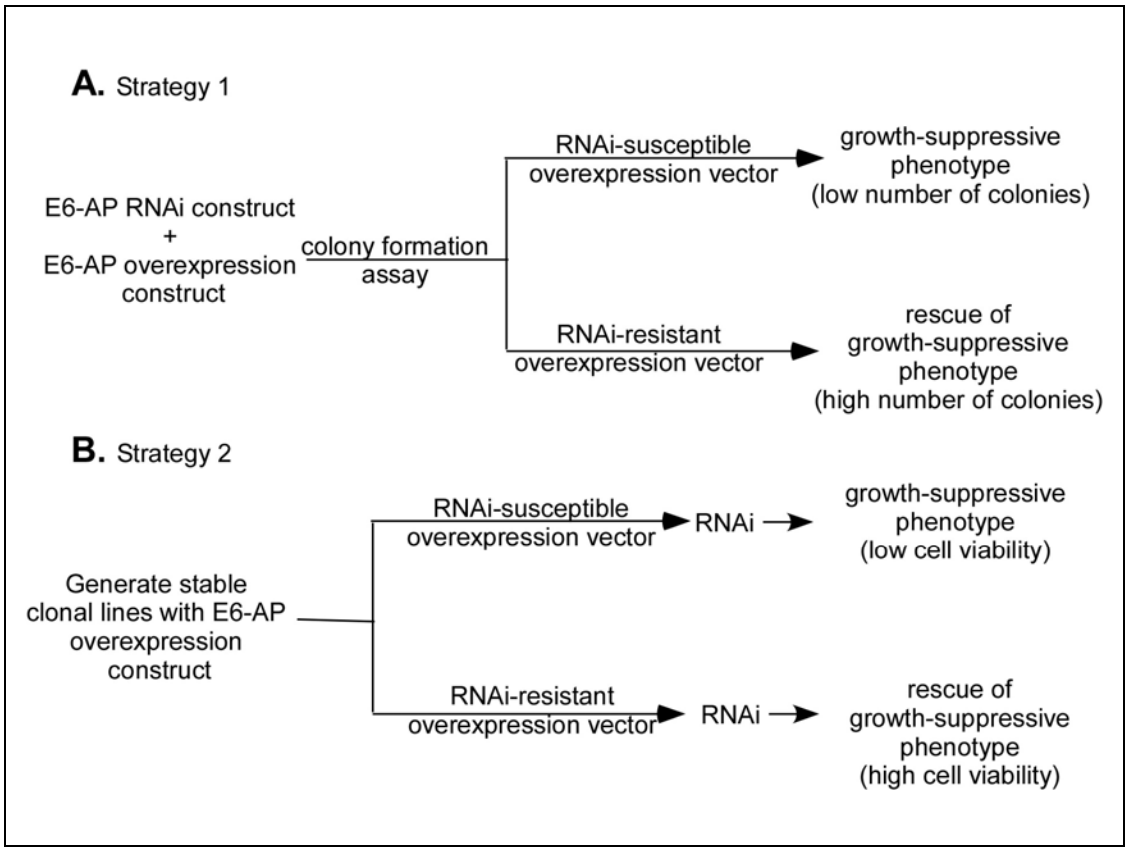

Figure 26: Experimental strategy for RNAi rescue. In strategy 1, the RNAi silencing construct and the overexpression construct are simultaneously transfected and colonies exhibiting resistance for both the constructs are selected. In case of cells transfected with the RNAi susceptible overexpression construct the knockdown of both endogenous and ectopically expressed E6-AP leads to a growth suppressive phenotype in HPV-positive cell lines. However in the case of cells transfected with the RNAi resistant construct, rescue of the growth suppressive phenotype is expected. In strategy 2, stable cell lines expressing the RNAi susceptible/resistant constructs are first generated and then subjected to RNAi to determine rescue of the phenotype.

To determine whether the apparent inability to overexpress E6-AP was dependent on its ability to interact with E6, a deletion construct lacking the E6 binding site (E6-AP.ΔE6) with an N-terminal HA tag was generated. The expression of this construct was tested in a transient transfection experiment in HeLa cells and was found to be expressed at

levels equivalent to wild type E6-AP under these experimental conditions (Figure 27A, lane 3). To determine whether E6-AP.ΔE6 can be stably overexpressed in HeLa cells, stable pool of clones transfected with this vector exhibiting neomycin resistance were established. Cell lysates of these clonal populations were prepared and analyzed by IP-western blot analysis. Indeed, it was possible to overexpress the E6-AP mutant lacking the E6 binding site (Figure 27, lane 2). Taken together the above experiments indicate that the inability to detectably overexpress E6-AP depends on the E6 binding region. Together with the inability to rescue the growth suppressive phenotype by strategy 1, these data suggested that overexpressed E6-AP is toxic to HPV-positive cells lines.

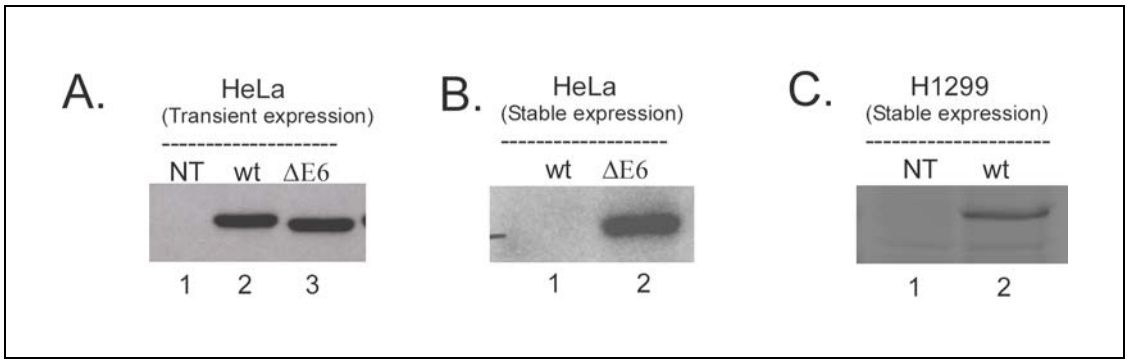

Figure 27 Overexpression of E6-AP in HPV-positive HeLa cell line and HPV-negative H1299 cell line. A. Transient expression of HA tagged wild type E6-AP (WT), E6-AP lacking the 18 amino acid E6 binding region (ΔE6). HeLa cells were transfected with the indicated constructs and expression levels of the various constructs were determined 24 hr post-transfection by western blot analysis and detection with an anti-HA antibody. NT:non-transfected cells. B. Stable overexpression of E6-AP. HeLa cells were transfected with vectors encoding either WT or ΔE6 form of E6-AP. Transfected cells were selected for with G418 for two weeks Overexpressed E6-AP levels were determined in pooled clones by western blotting with an anti-HA antibody. C. H1299 cells were transfected with vector encoding wild type E6-AP (wt) and selected for 2 weeks with G418. Overexpressed E6-AP levels were determined as in B.

4.6.3 Transient rescue assay

Since a rescue of the growth suppressive phenotype of E6-AP in HeLa cells was not possible by colony formation assays, alternatively, to establish a rescue approach transient assays were tested wherein p53 accumulation was monitored as "rescue readout". To this end, pRS-sh-E6-AP2 construct was cotransfected into HeLa cells with the expression vector, wt-E6-AP or E6-AP.mut2. An RNAi resistant construct of E6-AP lacking the E6 binding site (E6-AP ΔE6.mut2) was also similarly transfected in parallel. Likewise, co-transfection of the individual E6-AP vectors (namely, wt-E6-AP, E6-AP.mut2 and E6-AP ΔE6.mut2) with pRS-control served as negative controls.

Transfected cells were selected with puromycin resistance (encoded by the pRS construct). Protein lysates of puromycin selected cells were prepared 96 hrs post-transfection, and a Western blot analysis was performed to detect ectopically expressed E6-AP with an anti-HA antibody. Endogenous p53 levels were also detected with an anti-p53 antibody. As indicated in Figure 28, silencing of endogenous E6-AP in cells by pRS-sh-E6-AP2 led to p53 accumulation (lane 2). While this accumulation was not rescued by overexpression of E6-AP from an RNAi-susceptible expression vector due to down-regulation of the overexpressed E6-AP (lane-4), ectopically expressed E6-AP from the RNAi-resistant construct E6-AP.mut2 rescued p53 accumulation by approximately 80% as determined by quantification of the p53 band (lane 4). As expected, the RNAi-resistant construct E6-APΔE6.mut2 did not rescue p53 accumulation. This indicates that the effect of p53 stabilization is indeed due to the down-regulation of E6-AP and not due to an "off target" effect.

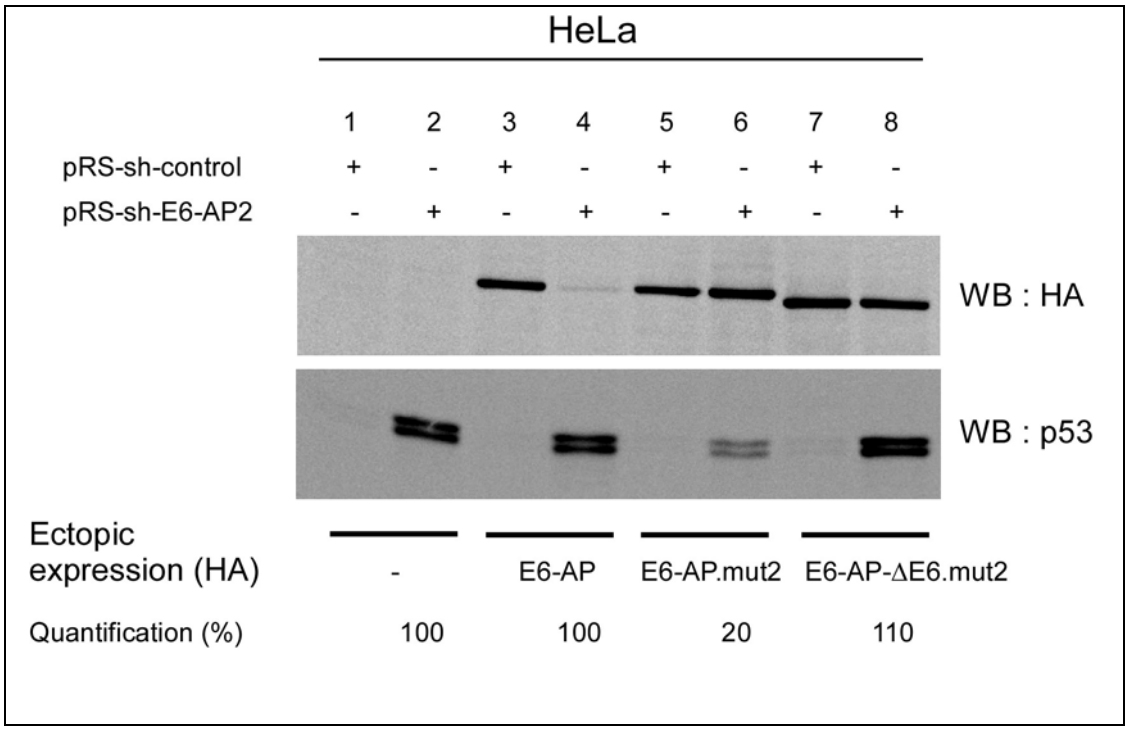

Figure 28 Transient rescue of p53 stabilization. E6-AP RNAi construct pRS-sh-E6-AP2 was co-transfected with either the RNAi-susceptible construct (HA.E6-AP) or the RNAi resistant construct (HA.E6-AP.mut). As negative control the pRS-control vector was also co-transfected with HA.E6-AP or HA.E6-AP.mut2. Cells expressing the RNAi construct were selected for by puromycin selection. 72 hours post-transfection cells were lysed and western blot analysis was performed. Overexpressed E6-AP was detected using anti-HA antibody, while p53 was detected using an anti-p53 DO-1 antibody.

4.7 Visualization of subcellular interactions using Bimolecular fluorescence complementation assay (BiFC)

The BiFC assay has been shown to be a suitable technique to visualize protein-protein interactions in living cells by reconstitution of the yellow fluorescent protein from two fragments attached to two interacting proteins (see Introduction, 1.5). Hence, in order to visualize interactions of E6-AP in living cells, an attempt was made to establish the BiFC assay.

To evaluate the BiFC assay, initial proof of principle experiments were performed using the bZip domains of Fos and Jun tagged to the YFP fragments (Figure 29A) as reported by Hu and coworkers. Therefore, bFosYC and bJunYN expression vectors were co-transfected into H1299 cells seeded on coverslips. As a measure of transfection efficiency, a vector encoding full length YFP was transfected in parallel. As negative controls, bFosYC was co-transfected with the YN vector lacking bJun and likewise bJunYN expression vector was co-transfected with the YC vector lacking bFos. Although BiFC can be directly visualized in living cells, in this particular experiment cells were fixed after 24 hours, nuclei stained with DAPI and visualized by fluorescence microscopy (Materials and Methods, 3.2.7.6). While bFosYC or bjunYN alone did not give any detectable fluorescence complementation (panel-2, 3), co-transfection of both the constructs into H1299 cells gave fluorescence which was nuclear with preferential localization to the nucleoli (Figure 29B, panel-4). This experiment confirmed the utility of BiFC to visualize protein interactions as reported by Hu and coworkers.

A number of potential substrates of E6-AP have been identified to date (see Introduction). To establish the BiFC assay to visualize the interactions of E6-AP with its potential binding partners within cells, a recently identified interacting partner, HERC2 (homologous to E6-AP C-terminus and RCC1), was chosen (Sandra Glockzin, Ulrike Kogel and Martin Scheffner, unpublished results). HERC2 was identified in a yeast two hybrid screen and the RCC1b domain of HERC2 has been shown to be sufficient to interact with E6-AP in vitro and in vivo. Since HERC2 is a large protein of 528 kDa, and the full length cDNA is unavailable, the RCC1b domain of mu-HERC2 was selected for studying its interaction with E6-AP. Hence, fusion constructs of E6-AP with the C-terminal fragment of YFP (E6-AP.YC) and the RCC1b domain (2959-

3327) of mHERC2 with the N-terminal fragments of YFP (RCC1b.YN155) were created (Figure 30A).

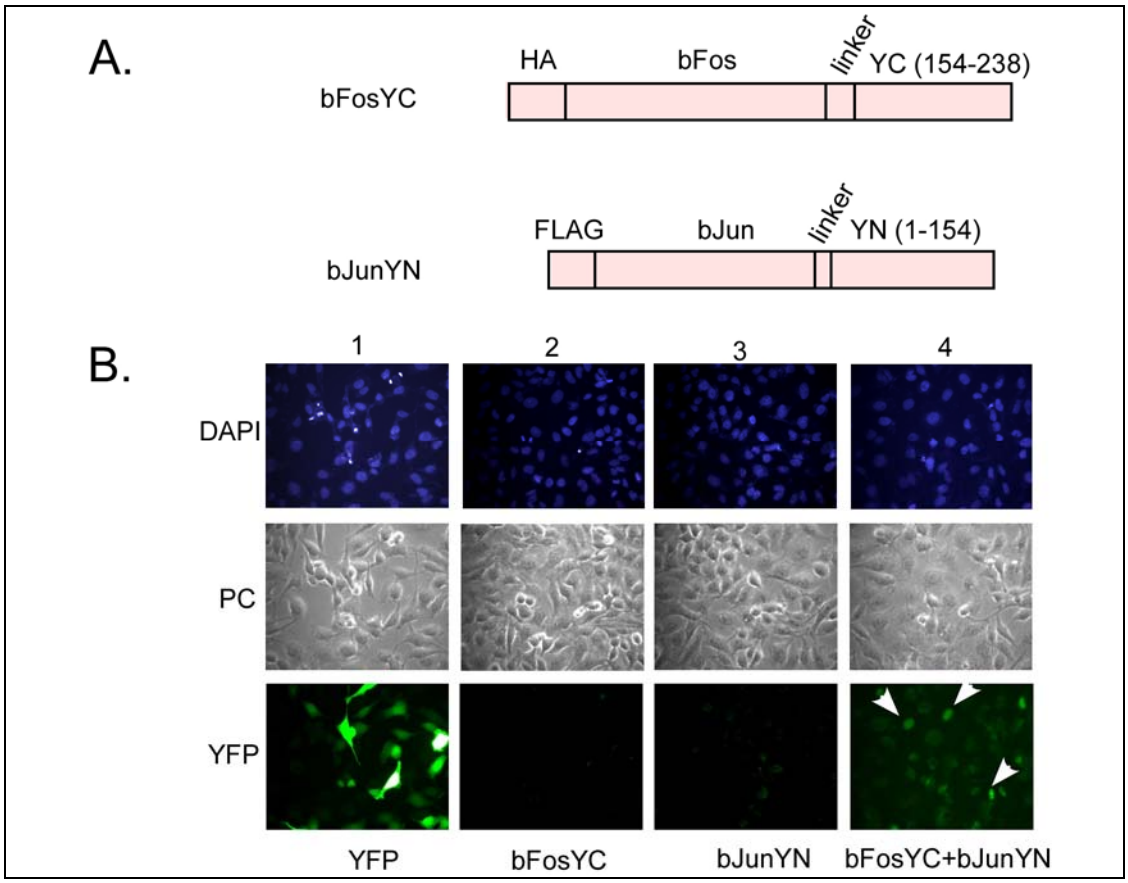

Figure 29: Bimolecular fluorescence complementation (BiFC) of bFosYC155 and bJunYN155. A. Schematic representation of the bFosYC155 and bJunYN155 constructs (Hu *et al.*, 2002). B. BiFC of the bZIP domains of Fos and Jun. The indicated constructs were transfected into H1299 cells seeded on coverslips placed in a 24 well plate. 24 hrs later the cells were fixed and nuclei stained with DAPI. As a positive control for fluorescence full length YFP was also transfected (panel 1). Phase contrast (PC), DAPI, and Fluorescence images (YFP) were taken using an Olympus fluorescence microscope. Arrow marks indicate fluorescence complementation and localization of the bFos and bjun proteins.

To determine whether these fusion constructs interact within cells, co-immunoprecipitation analysis was performed in H1299 cells co-transfected with the indicated constructs as shown in Figure 30B. Co-precipitation analysis revealed that it was possible to immuno-precipitate out HA-E6-AP.YC by RCC1b.YN (lane-3). This was also found to be as efficient as the non-fused fragment of murine HERC2 (2818-3606) encompassing the RCC1b domain (lane-2). This indicated that the two fusion constructs could interact with one another within cells. In order to visualize this interaction of E6-AP and the RCC1b domain, the fusion constructs were transfected

into H1299 or Cos-7 cells and monitored for fluorescence complementation. Even though co-precipitation analysis had indicated that the E6-AP and RCC1B fusion proteins interact within cells, no fluorescent complementation was detected (data not shown). One possibility for the lack of complementation could be that the two YFP fragments as fusion constructs were stearically hindered in fluorophore formation. Another possibility could be that the two YFP fragments were too far apart to reconstitute the fluorophore. Hence as an alternative strategy, the respective YFP fragments were recloned at the N-terminus of E6-AP and RCC1b (Figure 31). The expression of these constructs was tested (data not shown) and their ability to form fluorescent complexes within cells was evaluated. However, no fluorescence complementation was observed even with the new constructs with the YFP fragments at the N-terminus. To rule out that it was a cell line specific problem, these experiments were also performed in HeLa, and Cos-7 cells with similar outcome.

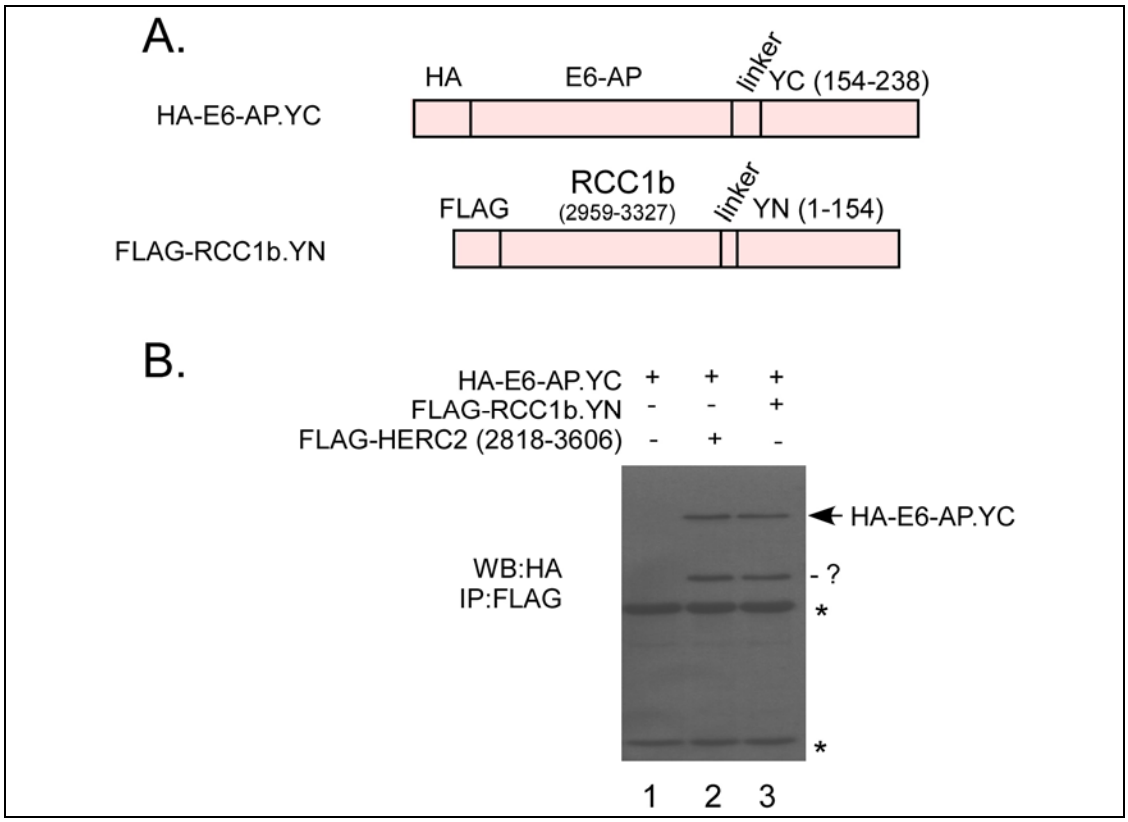

Figure 30: Generation of BiFC constructs of E6-AP and RCC1b domain of HERC2. A. Schematic representation of the cloning strategy used for generation of BiFC constructs with C- terminal YFP fragments. B. Co-immunoprecipitation analysis of E6-AP and RCC1b BiFC constructs. The indicated constructs were co-transfected into H1299 cells and co-immunoprecipitated using HA antibody. Asterisks indicate the running position of the heavy and light chains of the antibody. ? unknown band.

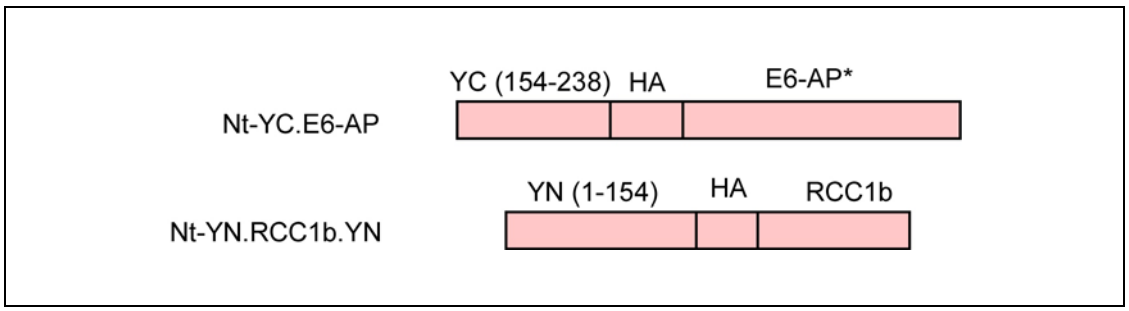

Figure 31: Schematic representation of the cloning strategy used for generation of BiFC constructs with N- terminal YFP fragments. Asterisk mark indicates that a similar construct was prepared by replacing wild type E6-AP with a C820A inactive mutant.

5. Discussion

Recent studies suggest that many of the known functions of E6 are mediated via complex formation with E6-AP (see Introduction, 1.3.3). For example, microarray analyses of HPV-positive cancer cell lines in which either E6 or E6-AP expression was down-regulated by RNAi indicate that the effects of E6 on the transcription program of its host cell are mediated in complex with E6-AP (Kelley *et al.*, 2005). While many studies addressed the role of E6-AP in HPV-positive cells, only little is known about its physiological function in normal cells. Indeed, even though the genetic disorder Angelman syndrome has been linked to the loss of E6-AP E3 ligase activity (Cooper *et al.*, 2004), it is still unclear whether deregulated degradation of E6-AP substrate(s) are responsible for this disorder and if so the respective substrate(s) are yet to be identified. The RNA interference (RNAi) technology provides a powerful tool to study the proposed role(s) of individual genes or gene products within cells and can be used in mammalian cell culture studies. In the present study, to gain insights into the physiological role of E6-AP, RNAi was employed to down-regulate E6-AP expression in HPV-positive and HPV-negative tumour cell lines. The consequences of E6-AP down-regulation on growth and cell viability were studied.

5.1 Knockdown of E6-AP in HPV-positive cervical cancer cell lines results in growth suppression

The E6 and E7 oncoproteins are generally the only viral proteins that are continuously expressed in HPV-positive cervical cancer cells and are necessary for the maintenance of the malignant phenotype of these cells. Indeed, inactivation of E6 by using either peptide aptamers or RNAi leads to activation of the p53-dependent apoptotic pathway in HPV-positive cancer cells (Butz *et al.*, 2000; Butz *et al.*, 2003). Similarly selective silencing of E7 by RNAi has also been reported to lead to apoptotic cell death (Jiang and Milner, 2002). The functional dependency of E6 on E6-AP has previously been studied by using either antisense or ribozyme mediated gene suppression strategies in HPV-positive and HPV-negative cells (Beer-Romero *et al.*, 1997; Kim *et al.*, 2003). However, in these experimental setups, no effects on cell growth or apoptosis were detected, although the cells were more sensitized to DNA damaging agents. In the present study, RNAi mediated down-regulation of E6-AP has further confirmed that

E6-AP is essential for efficient p53 degradation in HPV-positive cancer cell lines. In addition, E6-AP down-regulation resulted in growth suppression and apoptosis. In comparison to the above utilized techniques, the RNAi approach is assumed to be more efficient and has the ability to sustain gene silencing for longer periods of time (Kennerdell and Carthew, 1998; Elbashir *et al.*, 2001). Hence, less efficient interference with E6-AP activity could be the reason for the inability to see the observed phenotype in earlier studies. In any case, the results presented here support the notion that E6-AP has anti-growth suppressive properties in HPV-positive cancer cells.

Of the four identified si/shRNAs targeting the E6-AP ORF, two sequences which gave the most efficient down-regulation of E6-AP were further used for E6-AP characterization. Of the two sequences, the E6-AP2 target sequence either as synthetic siRNA or short hairpin RNA was better than the E6-AP1 target sequence at down-regulating E6-AP, both at the protein and mRNA level (Figure 9B and C). Colony formation assays using RNAi vectors in HeLa and SiHa cells yielded reduced colony numbers with sh-E6-AP1 and sh-E6-AP2 which correlated with the efficiency of knockdown **(**Figure 13). However, although the knockdown efficiency of synthetic si-E6-AP2 was better than that of si-E6-AP1 on both the protein and mRNA level, the efficiency of si-E6AP1 at inducing apoptosis and growth suppression was better in HeLa cells (Figure 11). One possible explanation for this effect could be that in some cases, synthetic siRNA can lead to 'off-target' effects by the recruitment of the sense strand into RISC (Nykanen *et al.*, 2001; Khvorova *et al.*, 2003). While this possibility cannot be ruled out with the present resources, if this would be the case, then these "off target effects" could be minimized in future experiments by utilizing synthetic siRNA which have been modified at the 5' end of the antisense strand of the synthesized siRNA with proprietary chemical modifications which are supposed to minimize "off-target" effects (for instance "ON-TARGET" siRNA from Dharmacon or "Stealth siRNA" from Invitrogen). Despite a difference in killing efficiency, the data clearly show that down-regulation of E6-AP leads to growth suppression in HPV-positive cell lines. A likely mechanism underlying the observed phenotype is illustrated in Figure 32.

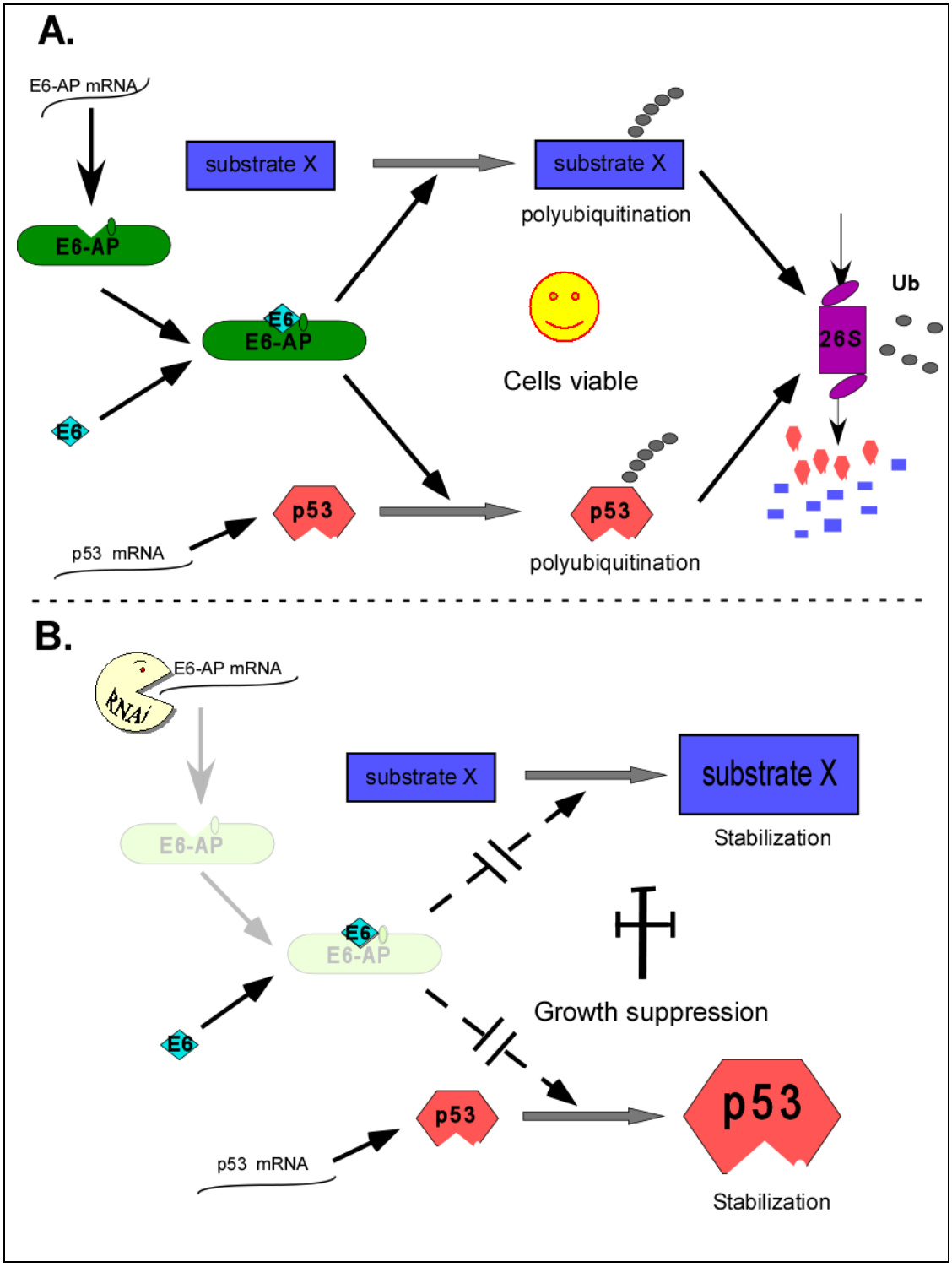

Figure 32: Model for the observed phenotype of HPV-positive cervical cancer cell lines upon E6-AP down-regulation. In case of HPV-positive cervical cancer cell lines, the E6/E6-AP complex targets p53 and other substrates (where X stand for substrates such as E6TP1, Scribble, etc,) for ubiquitination and degradation by the 26S proteasome (A). However, upon down-regulation of E6-AP expression stabilization of these substrates leads to growth suppression (B).

5.2 Transient down-regulation of E6-AP in HPV-negative cell lines does not have a growth-suppressive effect as compared to HPV-positive cell lines

In HPV-negative cell lines, E6-AP does not appear to be involved in p53 degradation (Beer-Romero *et al.*, 1997; Talis *et al.*, 1998; Traidej *et al.*, 2000). Although a few potential E6-independent substrates of E6-AP have been identified (see Introduction, 1.3.5) the physiological significance of these interactions is unclear. Moreover, it is likely that other interacting proteins of E6-AP are yet to be identified. Since it still seems possible that deregulated degradation of one or more substrates of E6-AP could result in a growth suppressive phenotype also in HPV-negative cells (i.e the growth suppressive phenotype of E6-AP down-regulation does not depend on its ability to interact with E6), this was tested by down-regulating E6-AP by RNAi.

Transient down-regulation of E6-AP using synthetic siRNA in various HPV-negative cell lines (i.e. MCF-7, RKO, and U2OS) had no significant effect on p53 stabilization which correlated with the results of earlier publications (Beer-Romero et al., 1997; Talis et al., 1998). Furthermore, no significant effect on viability was observed as compared to HPV-positive cell lines. Although this indicates that transient down-regulation of E6-AP does not have a growth-suppressive effect in HPV-negative cell lines, in this experimental set up it was observed that E6-AP protein levels were not down-regulated to similar extents as compared to HPV-positive cell lines (Figure 9B and Figure 15A). This was in spite of similar knockdown efficiencies at the mRNA level (Figure 9B and Figure 15C, and data not shown). This apparent paradox can possibly be explained by differences in E6-AP protein stability. In the case of HPV-positive cells, E6-AP has been reported to act as a substrate for E6 and hence has a shorter half-life than in HPV-negative cells (Kao et *al.,* 2000).

Transfection of synthetic siRNA is known to bring about transient but not stable knockdown of the mRNA due to the fact that siRNA molecules are not amplified in mammalian cells as compared to other eukaryotes (e.g. fungi, plants and worms) (Zamore, 2002). Thus, the gene silencing is transient since the introduced siRNA gets diluted out at each cell division and also probably due to degradation by cellular enzymes. Therefore, using synthetic siRNA it was not possible to determine if

constitutive down-regulation of E6-AP expression gives a phenotype in HPV-negative cell lines. One approach tested to overcome this limitation was to transfect higher concentrations of synthetic siRNA. Transfection of higher concentration (ranging from 100nM to 500nM) of siRNA resulted in more efficient down-regulation of E6-AP protein levels and also had effects on viability in comparison to cells transfected with similar amounts of control siRNA directed against Renilla luciferase (data not shown). This growth suppressive effect was found to be more pronounced with si-E6-AP1 than si-E6-AP2 and was also observed in H1299 (a p53 null cell line), indicating that the effect was p53-independent. While these data would suggest that E6-AP has anti-growth suppressive effects in HPV-negative cell lines, it should be noted that RNAi has recently been reported to have increased off-target effects at higher siRNA concentrations (Persengiev *et al.*, 2004).

Taken together, the experiments with synthetic siRNA indicate that HPV-negative cell lines are more resistant to transient E6-AP down-regulation than HPV-positive cell lines.

5.3 Stable knockdown of E6-AP in HPV-negative cell lines

In the case of mouse models with maternal deficiency of E6-AP, phenotypic abnormalities similar to Angelman Syndrome patients have been observed (Jiang *et al.*, 1998). Furthermore, while mice with homozygous deletions of E6-AP are viable, the postnatal viability is reduced which is more pronounced when bred within the same genetic background. To determine whether E6-AP is required for the viability of HPV-negative cell lines under cell culture conditions, attempts were made to generate E6-AP null H1299 (HPV-negative) cell lines. Initial attempts with pooled population of cells exhibiting E6-AP down-regulation were found to "loose" the knockdown phenotype over several population doublings in spite of maintaining the cells under selection pressure. While this may be due to partial integration or inactivation of the silencing construct, there could also be the possibility that down-regulation of E6-AP below a certain threshold level gives these cells a growth disadvantage as compared to cells expressing physiological levels of E6-AP. Over a number of population doublings, such a scenario would lead to selective out-growth of cells having above threshold levels of E6-AP. Hence, to overcome this potential hurdle, it was attempted to establish clonal cell lines of H1299 in which E6-AP expression is abrogated. In contrast to HPV-

positive cell lines, it was possible to obtain clonal HPV-negative cell lines in which E6-AP was stably down-regulated by RNAi (Figure 16). This would indicate that under the given conditions, HPV-negative cells are less sensitive to E6-AP down-regulation than HPV-positive cells. Furthermore, preliminary experiments at establishing clonal cell lines of MCF-7 cells in which E6-AP expression is stably down-regulated by RNAi indicate that it is possible (data not shown). This data would suggest that the ability to obtain clones in which E6-AP is significantly down-regulated does not depend on the p53 status in the case of HPV-negative cell lines (since MCF-7 cell lines expresses wild type p53 in comparison to H1299 which are p53 null). However, the inability to obtain clonal cell lines in which E6-AP is completely abrogated supports the hypothesis that certain threshold levels of E6-AP is essential for normal cell viability (Figure 18 and data not shown). However, further characterization would be necessary to determine whether the inability to establish E6-AP "null" cells is due to the inefficiency of the RNAi construct or whether complete knockdown of E6-AP is toxic to cells. In this context it should be noted that it is possible to establish growth of E6-AP null mouse embryonic fibroblasts in culture. However in this case, it cannot be excluded that such cells could have "learnt" to adapt to the lack of E6-AP. Alternatively, the essentiality of E6-AP expression could be cell type specific.

5.4 Growth-suppression induced by down-regulation of E6-AP in the HPV-positive cancer cell line HeLa, depends on p53 expression

While E6-AP down-regulation led to a growth suppressive phenotype in HPV-positive cells with concomitant increase in p53 stability, these observations do not answer the question as to whether p53 is the main or only target of the E6/E6-AP complex that is relevant for growth suppression or whether other E6/E6-AP substrates (e.g. E6TP1, Scribble, MCM7 and NFX1-91) also play a role in the observed phenotype. It should also be noted that, while an earlier report indicated that the PDZ domain containing protein, hDlg (the human homologue of Drosophila disc large protein) was targeted for degradation by E6 in an E6-AP independent manner (Mantovani *et al.*, 2001), recent evidence indicates that hDlg is also degraded via E6-AP mediated ubiquitination (Petric Kuballa and Martin Scheffner, S. Beaudenon and J. Huibregtse, unpublished data).

To answer the question whether the anti-growth-suppressive property of E6-AP was dependent on its ability to degrade p53, HeLa cell lines in which p53 expression was abrogated by RNAi were generated (Figure 18). Down-regulation of E6-AP in these cell lines indicated that indeed the anti-growth suppressive properties of E6-AP depend on p53 degradation. Likewise, the anti-apoptotic activity of E6 was also found to depend on p53 in these cell lines (Arnd Hengstermann and Petric Kuballa, personal communication). From these data, a model is proposed for the cell survival pathway in p53 null HeLa cells in Figure 33.

Although the data presented clearly indicate that, under cell culture conditions, p53 represents the main target for the anti-growth suppressive activities of E6-AP, it cannot be excluded that E6-AP has additional p53-independent anti-apoptotic functions in HPV-positive cells. As shown in Figure 24A, E6-AP levels were significantly but not completely down-regulated upon transfection of siRNA directed against E6-AP in the p53-null HeLa clones (see below). Thus, if p53-independent properties of E6-AP are required for the growth of HeLa cells, it can be postulated that the threshold level of E6-AP required to perform these hypothetical p53-independent functions is lower than the one required for E6-AP mediated degradation of p53. Preliminary attempts to obtain single cell clones of p53 null HeLa clones in which E6-AP expression is completely abrogated suggests that this is not possible. As in the case of HPV-negative cells, certain threshold levels may also be required for the E6- independent functions of E6-AP for long term cell survival. Furthermore, in the case of HPV-positive cells other functions of E6-AP (in association with E6) have been associated with maintenance of the malignant phenotype, for example, increased telomerase activity (Munger et al., 2004). Thus, it is possible that stable down-regulation of E6-AP over a number of population doubling times leads to a secondary growth suppressive effect (other than due to p53 stabilization) due to telomere shortening.

In contrast to HeLa cells, it was not possible to generate p53 null SiHa clones. Furthermore, the number of clones with significant down-regulation of p53 was low in SiHa cells. Of more than 80 clones picked, only 5 of the clones showed close to complete abrogation of p53 levels. However, in these clones p53 levels significantly increased upon treatment with Actinomycin D or MG132 (Figure 22B and data not shown). To exclude the possibility that the RNAi construct per se was not efficient

enough to bring about complete knockdown in SiHa cells, transient transfection assays were performed in SiHa cells, and the efficiency of p53 knockdown was determined. Similar knockdown efficiencies as compared to HeLa cells were observed (data not shown). One reason for the inability to generate p53 null SiHa cell lines could be that a certain threshold amount of p53 is required for proliferation or viability of SiHa cells. In this regard, it is interesting to note that p53 is still functional in HPV-positive cervical cancer cell lines and does possess transactivating activity (Butz *et al.*, 1995; Butz *et al.*, 1999; Hietanen *et al.*, 2000). Therefore, it could be possible that minimal activities of p53 may be required for viability or proliferation. In addition, wild-type p53 is assumed to play an important role in ensuring the integrity of the genome, and thus, its presence may ensure a degree of genomic stability. Another, but not mutually exclusive possibility could be that p53 has been suggested to exert anti-apoptotic effects under certain conditions (reviewed in Oren, 2003). and, thus, cells lacking functional p53 could be more vulnerable to apoptosis. However, additional experiments will be required to address these possibilities.

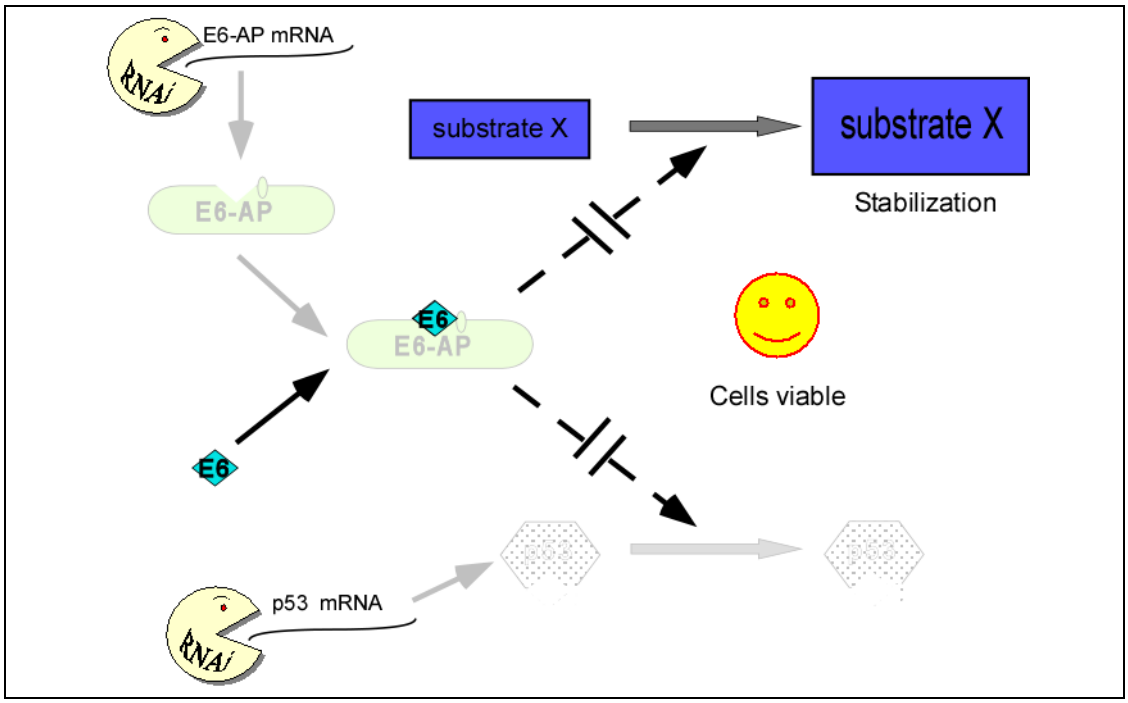

Figure 33: Model of cell survival pathway in p53 null HeLa cells. Knockdown of E6-AP in p53 null HeLa cells although leads to stabilization of other substrates of E6-AP, does not lead to growth suppression.

5.5 RNAi–rescue assays

To substantiate the results obtained by RNAi, an attempt was made to rescue the growth suppressive phenotype of E6-AP down-regulation in parental HeLa cells. The establishment of such a rescue assay has the advantage that it could also be used to perform "quasi-genetics" within cells by down-regulating the endogenous protein and studying the ability of different E6-AP mutants to rescue the phenotype. This approach would help to delineate the various biochemical properties of E6-AP required for its biological function. By identifying specific mutants of E6-AP which would specifically interact with only one or few of its substrates, this system would eventually help to study and characterize the individual E6-AP/substrate interactions within cells.

One approach used for RNAi rescue is to overexpress the target gene in a form that is resistant to RNAi by introducing silent mismatches at the si/shRNA target site. Initial rescue experiments involving simultaneous knockdown of endogenous E6-AP expression and overexpression of E6-AP from a constitutive overexpression vector which is RNAi-resistant and monitoring of rescue by using colony formation assays revealed no rescue of the growth suppressive phenotype of E6-AP in HeLa cells (data not shown). This was not possible since constitutive overexpression of wild type E6-AP appears to be toxic to HeLa cells. In contrast, it was possible to stably overexpress E6-AP in HPV-negative cell lines (namely H1299, RKO, and MCF-7) (Figure 27 and data not shown).

While the reason for the inability to stably overexpress E6-AP in HPV-positive cells is unclear at the moment, it is interesting to note that it was possible to stably overexpress a construct of E6-AP lacking the E6 binding site (E6-AP-ΔE6). One possible reason for this could be that since E6-AP-ΔE6 is not targeted by E6 for degradation it can be overexpressed to high levels whereas overexpressed wild type E6-AP maybe expressed at undetectable levels due to the accelerated degradation by E6 under the experimental conditions used (Kao *et al.*, 2000). However, even if this is the case, it does not answer the question why does the overexpressed wild type E6-AP construct have a negative effect on growth but not the E6-AP-ΔE6 construct. A plausible reason could be that the 18 amino acid E6 binding region on E6-AP is also important for other functions/ interactions of E6-AP. In this regard, it is interesting to note that the E6 binding region encompasses a short sequence motif LXXLL (where L is Leucine and X is any amino

acid) which has been shown to be sufficient for binding to liganded nuclear receptors. Furthermore, mutation of any of the leucines within this motif results in complete loss in ligand binding (Heery *et al.*, 1997). E6-AP is known to act as a coactivator for nuclear hormone receptor independent of E6 (Nawaz *et al.*, 1999). Thus, it can be speculated that overexpressed E6-AP leads to enhanced coactivator function within cells that would lead to its toxicity. Future work to determine which of the known E6-AP activities (i.e E6 binding or receptor coactivation) is involved in this phenotype would require stable overexpression of mutants within this region which can differentiate between the two functions. Mutational analysis of the E6 binding region has indicated that mutation of the LXXLL motif to LXXLS does not have any effect on its ability to mediate p53 degradation (Cooper *et al.*, 2003). Whether this mutant would abrogate the coactivator function of E6-AP is not yet known, but if so, overexpression of such a mutant could lead to insights into the toxicity effects of E6-AP in HeLa cells.

The above arguments could account for the stable overexpression toxicity of E6-AP and require investigation. However, a simpler reason could also be that stable overexpression of E6-AP results in titrating out E6 within cells (as a result of binding to E6) and thus reducing the available free E6 protein required to perform the E6-AP-independent functions of E6 within HPV-positive cells. If this is correct, both the E6-AP-dependent and E6-AP-independent functions of E6 would be required for the viability of HPV-positive cancer cells.

Since a stable phenotypic rescue of the growth suppressive phenotype using colony formation assays as a read out was not possible due to apparent toxicity of E6-AP overexpression, as an alternative strategy, transient rescue assay was tested using p53 accumulation as a read out in HeLa cell line. As shown in Figure 28, while ectopically expressed E6-AP from an RNAi-resistant vector could rescue p53 accumulation brought about by down-regulation of endogenous E6-AP, an E6-AP construct lacking the E6 binding site did not rescue p53 accumulation. These data correlate with the biochemical evidence that E6 binding is required for E6-AP mediated ubiquitination of p53 (ΔE6-E6-AP) (Huibregtse *et al.*, 1993). By using different mutants of E6-AP, future work can employ such rescue experiments to perform “quasi genetics” within cells and study the various E6-AP/substrates interactions. In addition, it may be possible to design “quasi genetic” experiments to bring about a phenotypic rescue if

inducible expression vector systems are employed to regulate ectopically expressed E6-AP expression to overcome the overexpression toxicity.

5.6 E6-AP: A potential target for cervical cancer therapy?

Comparison of HPV-positive and HPV-negative cell lines has indicated that, in case of HPV-positive cell lines, p53 is predominantly targeted for degradation by the E6/E6-AP complex and that the Mdm2 pathway of p53 regulation is not, or only poorly active (Hengstermann *et al.*, 2001). Indeed, studies using peptide aptamers or RNAi technology to target E6 expression have shown to result in the activation of p53-dependent apoptotic pathways in HPV-positive cancer cells lines (Butz *et al.*, 2000; Butz *et al.*, 2003). In the present study, E6-AP was found to contribute to the anti-apoptotic activities of E6 in HPV-positive cancer cells lines. The ability to activate p53-dependent apoptotic pathways by interfering with either E6 or E6-AP expression suggests that these could be attractive targets for cervical cancer therapy.

The ability to knockdown cancer or disease related genes using RNAi has led to the evaluation of the RNAi technology as a therapeutic agent in general. Although a number of obstacles (e.g. mode of delivery, stability) still need to be overcome before it becomes reality, proof of principle experiments in cell culture and animal model systems do show encouraging results that indicate it may prove to be helpful in cancer therapy (reviewed in Milner, 2003; Dorsett and Tuschl, 2004; Dillon *et al.*, 2005; Dykxhoorn and Lieberman, 2005). With respect to using RNAi for cervical cancer therapy targeting of either the E6 or E7 proteins by RNAi has been suggested to be useful targets for therapy of cervical cancer (Jiang and Milner, 2002; Butz *et al.*, 2003). While elimination of the viral oncoproteins would be an ideal target for therapy, the present study suggests that targeting of E6-AP in combination could have a cumulative effect in preferentially eliminating cancerous cells. However, since E6-AP is a cellular protein and, as suggested by the present study, complete elimination could have a negative effect on normal cells, further research would be necessary to determine a threshold level of E6-AP elimination at which minimal toxicity would occur to normal cells. Specific targeting of the RNAi molecules to tumor cells or selecting a suitable RNAi targeting sequence that would reduce E6-AP expression to a level wherein it would be toxic to HPV-positive cervical cancer cells and not normal cells could also

alleviate this problem. As an alternative approach, short term targeting of E6-AP expression could also lead to a preferential elimination of cervical cancer cells.

5.7 BiFC analysis

The BiFC assay was developed to visualize protein-protein interactions in living cells by reconstitution of the yellow fluorescent protein from two fragments attached to two interacting proteins (Hu *et al.*, 2002). In order to determine the subcellular localization of the interaction of E6-AP with its potential binding partners in living cells, an attempt was made to establish the BiFC assay.

To initially test the reproducibility of this technique, experiments were performed using the positive controls of the bZIP domains of Fos and Jun fused to YFP fragments (bFosYC and bJunYN) (Hu *et al.*, 2002). As reported by Hu *et al*, in my hands too, fluorescence complementation was only visualized when both constructs were expressed. To establish this technique for E6-AP, proof of principle experiments were performed using E6-AP and the RCC1b domain of HERC2. The logic behind choosing specifically HERC2 was due to the fact that interaction of HERC2 with E6-AP is well characterized in our lab (unpublished data). However, although interaction of the respective YFP fusions proteins of HERC2 with E6-AP could be detected in-vitro by GST-pull down assays (data not shown) and in-vivo by co-immunoprecipitation assays, this interaction could not be visualized by BiFC. Furthermore, subsequent experiments in our lab to study the binding of E6-AP with E6 by BiFC were also unsuccessful (Kostantin Matentzoglu, personal communication).

The reason for the failure to visualize the interaction of E6-AP with HERC2 could be manyfold. Of these, the following were tested for and did not yield any positive result. To ensure that the two constructs were expressed in sufficient amounts, titration experiments were performed. However, no fluorescence complementation was observed even at rather high expression levels (data not shown). To rule out the possibility that the YFP fragments in the context of the fusion proteins could be spatially too far apart to reconstitute the fluorophore, the YFP fragments were cloned either at the N-terminus or at the C-terminus of E6-AP/RCC1b domain of HERC2. Furthermore, whereas the C-terminal fusion construct of E6-AP is defective for substrate ubiquitination (Salvat *et al.*, 2004), to rule out that the activity of E6-AP *per se* (i.e ubiquitination) could interfere with complementation by targeting the interacting partner for degradation, N-terminal fusion constructs of E6-AP were also generated with a catalytically inactive mutant of E6-AP (C820A). These constructs were

transfected in different combinations (e.g. N-terminal YFP fusion of E6-AP with C-terminal YFP fusion of RCC1b or vice versa) into various cell lines and monitored for interaction. However, no fluorescence complementation was observed.

Further to this, it could be possible that proper folding of the YFP fragment as a fusion with E6-AP/RCC1b domain results in steric hindrance and thus prevents complementation. Since the constructs used possess a linker sequence between the YFP fragment and E6-AP/RCC1b, in order to rule out that this linker sequence is insufficient, in future experiments, one should generate constructs with other linker sequences and check for complementation. Furthermore, additional interacting partners should be tested to determine whether the lack of fluorescence complementation is dependent on the interacting protein.

6. References

Albrecht, U., J. S. Sutcliffe, B. M. Cattanach, C. V. Beechey, D. Armstrong, G. Eichele and A. L. Beaudet (1997). "Imprinted expression of the murine Angelman syndrome gene, Ube3a, in hippocampal and Purkinje neurons." Nat Genet **17**(1): 75-8.

Aravind, L. and E. V. Koonin (2000). "The U box is a modified RING finger - a common domain in ubiquitination." Curr Biol **10**(4): R132-4.

Aviel, S., G. Winberg, M. Massucci and A. Ciechanover (2000). "Degradation of the epstein-barr virus latent membrane protein 1 (LMP1) by the ubiquitin-proteasome pathway. Targeting via ubiquitination of the N-terminal residue." J Biol Chem **275**(31): 23491-9.

Balint, E. E. and K. H. Vousden (2001). "Activation and activities of the p53 tumour suppressor protein." Br J Cancer **85**(12): 1813-23.

Beer-Romero, P., S. Glass and M. Rolfe (1997). "Antisense targeting of E6AP elevates p53 in HPV-infected cells but not in normal cells." Oncogene **14**(5): 595-602.

Berezutskaya, E. and S. Bagchi (1997). "The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26 S proteasome." J Biol Chem **272**(48): 30135-40.

Berns, K., E. M. Hijmans, J. Mullenders, T. R. Brummelkamp, A. Velds, M. Heimerikx, R. M. Kerkhoven, M. Madiredjo, W. Nijkamp, B. Weigelt, R. Agami, W. Ge, G. Cavet, P. S. Linsley, R. L. Beijersbergen and R. Bernards (2004). "A large-scale RNAi screen in human cells identifies new components of the p53 pathway." Nature **428**(6981): 431-7.

Bloom, J., V. Amador, F. Bartolini, G. DeMartino and M. Pagano (2003). "Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation." Cell **115**(1): 71-82.

Boname, J. M. and P. G. Stevenson (2001). "MHC class I ubiquitination by a viral PHD/LAP finger protein." Immunity **15**(4): 627-36.

Borden, K. L. (2000). "RING domains: master builders of molecular scaffolds?" J Mol Biol **295**(5): 1103-12.

Boyer, S. N., D. E. Wazer and V. Band (1996). "E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway." Cancer Res **56**(20): 4620-4.

Breitschopf, K., E. Bengal, T. Ziv, A. Admon and A. Ciechanover (1998). "A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein." Embo J **17**(20): 5964-73.

Brummelkamp, T. R., R. Bernards and R. Agami (2002a). "Stable suppression of tumorigenicity by virus-mediated RNA interference." Cancer Cell **2**(3): 243-7.

Brummelkamp, T. R., R. Bernards and R. Agami (2002b). "A system for stable expression of short interfering RNAs in mammalian cells." Science **296**(5567): 550-3.

Butz, K., C. Denk, A. Ullmann, M. Scheffner and F. Hoppe-Seyler (2000). "Induction of apoptosis in human papillomaviruspositive cancer cells by peptide aptamers targeting the viral E6 oncoprotein." Proc Natl Acad Sci U S A **97**(12): 6693-7.

Butz, K., T. Ristriani, A. Hengstermann, C. Denk, M. Scheffner and F. Hoppe-Seyler (2003). "siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells." Oncogene **22**(38): 5938-45.

Butz, K., L. Shahabeddin, C. Geisen, D. Spitkovsky, A. Ullmann and F. Hoppe-Seyler (1995). "Functional p53 protein in human papillomavirus-positive cancer cells." Oncogene **10**(5): 927-36.

Butz, K., N. Whitaker, C. Denk, A. Ullmann, C. Geisen and F. Hoppe-Seyler (1999). "Induction of the p53-target gene GADD45 in HPV-positive cancer cells." Oncogene **18**(14): 2381-6.

Calin, G. A., C. D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich and C. M. Croce (2002). "Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **99**(24): 15524-9.

Calin, G. A., C. G. Liu, C. Sevignani, M. Ferracin, N. Felli, C. D. Dumitru, M. Shimizu, A. Cimmino, S. Zupo, M. Dono, M. L. Dell'Aquila, H. Alder, L. Rassenti, T. J. Kipps, F. Bullrich, M. Negrini and C. M. Croce (2004). "MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias." Proc Natl Acad Sci U S A **101**(32): 11755-60.

Chen, J. J., C. E. Reid, V. Band and E. J. Androphy (1995). "Interaction of papillomavirus E6 oncoproteins with a putative calcium-binding protein." Science **269**(5223): 529-31.

Ciechanover, A. and P. Brundin (2003). "The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg." Neuron **40**(2): 427-46.

Cook, J. C. and P. B. Chock (1992). "Isoforms of mammalian ubiquitin-activating enzyme." J Biol Chem **267**(34): 24315-21.

Cooper, B., S. Schneider, J. Bohl, Y. Jiang, A. Beaudet and S. Vande Pol (2003). "Requirement of E6AP and the features of human papillomavirus E6 necessary to support degradation of p53." Virology **306**(1): 87-99.

Cooper, E. M., A. W. Hudson, J. Amos, J. Wagstaff and P. M. Howley (2004). "Biochemical analysis of Angelman syndrome-associated mutations in the E3 ubiquitin ligase E6-associated protein." J Biol Chem **279**(39): 41208-17.

Crook, T., J. A. Tidy and K. H. Vousden (1991). "Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation." Cell **67**(3): 547-56.

Dalal, S., Q. Gao, E. J. Androphy and V. Band (1996). "Mutational analysis of human papillomavirus type 16 E6 demonstrates that p53 degradation is necessary for immortalization of mammary epithelial cells." J Virol **70**(2): 683-8.

Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart and Z. J. Chen (2000). "Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain." Cell **103**(2): 351-61.

Deshaies, R. J. (1999). "SCF and Cullin/Ring H2-based ubiquitin ligases." Annu Rev Cell Dev Biol **15**: 435-67.

Dillon, C. P., P. Sandy, A. Nencioni, S. Kissler, D. A. Rubinson and L. Van Parijs (2005). "Rnai as an experimental and therapeutic tool to study and regulate physiological and disease processes." Annu Rev Physiol **67**: 147-73.

Doench, J. G., C. P. Petersen and P. A. Sharp (2003). "siRNAs can function as miRNAs." Genes Dev **17**(4): 438-42.

Dorsett, Y. and T. Tuschl (2004). "siRNAs: applications in functional genomics and potential as therapeutics." Nat Rev Drug Discov **3**(4): 318-29.

Dykxhoorn, D. M. and J. Lieberman (2005). "The silent revolution: RNA interference as basic biology, research tool, and therapeutic." Annu Rev Med **56**: 401-23.

Dykxhoorn, D. M., C. D. Novina and P. A. Sharp (2003). "Killing the messenger: short RNAs that silence gene expression." Nat Rev Mol Cell Biol **4**(6): 457-67.

Dyson, N. (1998). "The regulation of E2F by pRB-family proteins." Genes Dev **12**(15): 2245-62.

Dyson, N., P. Guida, K. Munger and E. Harlow (1992). "Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins." J Virol **66**(12): 6893-902.

Dyson, N., P. M. Howley, K. Munger and E. Harlow (1989). "The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product." Science **243**(4893): 934-7.

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl (2001). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-8.

Finley, D., A. Ciechanover and A. Varshavsky (1984). "Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85." Cell **37**(1): 43-55.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.

Galan, J. M. and R. Haguenauer-Tsapis (1997). "Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein." Embo J **16**(19): 5847-54.

Gao, Q., A. Kumar, L. Singh, J. M. Huibregtse, S. Beaudenon, S. Srinivasan, D. E. Wazer, H. Band and V. Band (2002). "Human papillomavirus E6-induced degradation of E6TP1 is mediated by E6AP ubiquitin ligase." Cancer Res **62**(11): 3315-21.

Gao, Q., A. Kumar, S. Srinivasan, L. Singh, H. Mukai, Y. Ono, D. E. Wazer and V. Band (2000). "PKN binds and phosphorylates human papillomavirus E6 oncoprotein." J Biol Chem **275**(20): 14824-30.

Gao, Q., L. Singh, A. Kumar, S. Srinivasan, D. E. Wazer and V. Band (2001). "Human papillomavirus type 16 E6-induced degradation of E6TP1 correlates with its ability to immortalize human mammary epithelial cells." J Virol **75**(9): 4459-66.

Gao, Q., S. Srinivasan, S. N. Boyer, D. E. Wazer and V. Band (1999). "The E6 oncoproteins of high-risk papillomaviruses bind to a novel putative GAP protein, E6TP1, and target it for degradation." Mol Cell Biol **19**(1): 733-44.

Gewin, L. and D. A. Galloway (2001). "E box-dependent activation of telomerase by human papillomavirus type 16 E6 does not require induction of c-myc." J Virol **75**(15): 7198-201.

Gewin, L., H. Myers, T. Kiyono and D. A. Galloway (2004). "Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex." Genes Dev **18**(18): 2269-82.

Glaunsinger, B. A., S. S. Lee, M. Thomas, L. Banks and R. Javier (2000). "Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins." Oncogene **19**(46): 5270-80.

Glickman, M. H. and A. Ciechanover (2002). "The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction." Physiol Rev **82**(2): 373-428.

Glockzin, S., F. X. Ogi, A. Hengstermann, M. Scheffner and C. Blattner (2003). "Involvement of the DNA repair protein hHR23 in p53 degradation." Mol Cell Biol **23**(24): 8960-9.

Grinberg, A. V., C. D. Hu and T. K. Kerppola (2004). "Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells." Mol Cell Biol **24**(10): 4294-308.

Grishok, A., A. E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D. L. Baillie, A. Fire, G. Ruvkun and C. C. Mello (2001). "Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing." Cell **106**(1): 23-34.

Haglund, K., P. P. Di Fiore and I. Dikic (2003). "Distinct monoubiquitin signals in receptor endocytosis." Trends Biochem Sci **28**(11): 598-603.

Hainaut, P., T. Hernandez, A. Robinson, P. Rodriguez-Tome, T. Flores, M. Hollstein, C. C. Harris and R. Montesano (1998). "IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools." Nucleic Acids Res **26**(1): 205-13.

Harris, S. L. and A. J. Levine (2005). "The p53 pathway: positive and negative feedback loops." Oncogene **24**(17): 2899-908.

Hartmann-Petersen, R., M. Seeger and C. Gordon (2003). "Transferring substrates to the 26S proteasome." Trends Biochem Sci **28**(1): 26-31.

Hatakeyama, S., J. P. Jensen and A. M. Weissman (1997). "Subcellular localization and ubiquitin-conjugating enzyme (E2) interactions of mammalian HECT family ubiquitin protein ligases." J Biol Chem **272**(24): 15085-92.

Hatakeyama, S., M. Yada, M. Matsumoto, N. Ishida and K. I. Nakayama (2001). "U box proteins as a new family of ubiquitin-protein ligases." J Biol Chem **276**(35): 33111-20.

Haupt, Y., R. Maya, A. Kazaz and M. Oren (1997). "Mdm2 promotes the rapid degradation of p53." Nature **387**(6630): 296-9.

Hauser, H. P., M. Bardroff, G. Pyrowolakis and S. Jentsch (1998). "A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors." J Cell Biol **141**(6): 1415-22.

Heery, D. M., E. Kalkhoven, S. Hoare and M. G. Parker (1997). "A signature motif in transcriptional co-activators mediates binding to nuclear receptors." Nature **387**(6634): 733-6.

Hengstermann, A., L. K. Linares, A. Ciechanover, N. J. Whitaker and M. Scheffner (2001). "Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells." Proc Natl Acad Sci U S A **98**(3): 1218-23.

Hicke, L. (2001). "Protein regulation by monoubiquitin." Nat Rev Mol Cell Biol **2**(3): 195-201.

Hicke, L. and H. Riezman (1996). "Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis." Cell **84**(2): 277-87.

Hietanen, S., S. Lain, E. Krausz, C. Blattner and D. P. Lane (2000). "Activation of p53 in cervical carcinoma cells by small molecules." Proc Natl Acad Sci U S A **97**(15): 8501-6.

Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis and S. Jentsch (2002). "RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO." Nature **419**(6903): 135-41.

Hofmann, R. M. and C. M. Pickart (1999). "Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair." Cell **96**(5): 645-53.

Hu, C. D., Y. Chinenov and T. K. Kerppola (2002). "Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation." Mol Cell **9**(4): 789-98.

Hu, C. D. and T. K. Kerppola (2003). "Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis." Nat Biotechnol **21**(5): 539-45.

Huang, L., E. Kinnucan, G. Wang, S. Beaudenon, P. M. Howley, J. M. Huibregtse and N. P. Pavletich (1999). "Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade." Science **286**(5443): 1321-6.

Huibregtse, J. M., M. Scheffner, S. Beaudenon and P. M. Howley (1995). "A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase." Proc Natl Acad Sci U S A **92**(11): 5249.

Huibregtse, J. M., M. Scheffner and P. M. Howley (1991). "A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18." Embo J **10**(13): 4129-35.

Huibregtse, J. M., M. Scheffner and P. M. Howley (1993). "Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins." Mol Cell Biol **13**(8): 4918-27.

Hutvagner, G., J. McLachlan, A. E. Pasquinelli, E. Balint, T. Tuschl and P. D. Zamore (2001). "A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA." Science **293**(5531): 834-8.

Hutvagner, G. and P. D. Zamore (2002). "RNAi: nature abhors a double-strand." Curr Opin Genet Dev **12**(2): 225-32.

Jackson, A. L., S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet and P. S. Linsley (2003). "Expression profiling reveals off-target gene regulation by RNAi." Nat Biotechnol **21**(6): 635-7.

Jiang, M. and J. Milner (2002). "Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference." Oncogene **21**(39): 6041-8.

Jiang, Y. H., D. Armstrong, U. Albrecht, C. M. Atkins, J. L. Noebels, G. Eichele, J. D. Sweatt and A. L. Beaudet (1998). "Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation." Neuron **21**(4): 799-811.

Johnson, E. S., P. C. Ma, I. M. Ota and A. Varshavsky (1995). "A proteolytic pathway that recognizes ubiquitin as a degradation signal." J Biol Chem **270**(29): 17442-56.

Kaiser, P., K. Flick, C. Wittenberg and S. I. Reed (2000). "Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF(Met30)-mediated inactivation of the transcription factor Met4." Cell **102**(3): 303-14.

Kao, W. H., S. L. Beaudenon, A. L. Talis, J. M. Huibregtse and P. M. Howley (2000). "Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase." J Virol **74**(14): 6408-17.

Katzmann, D. J., G. Odorizzi and S. D. Emr (2002). "Receptor downregulation and multivesicular-body sorting." Nat Rev Mol Cell Biol **3**(12): 893-905.

Kelley, M. L., K. E. Keiger, C. J. Lee and J. M. Huibregtse (2005). "The global transcriptional effects of the human papillomavirus E6 protein in cervical carcinoma cell lines are mediated by the E6AP ubiquitin ligase." J Virol **79**(6): 3737-47.

Kennerdell, J. R. and R. W. Carthew (1998). "Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway." Cell **95**(7): 1017-26.

Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon and R. H. Plasterk (2001). "Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans." Genes Dev **15**(20): 2654-9.

Khvorova, A., A. Reynolds and S. D. Jayasena (2003). "Functional siRNAs and miRNAs exhibit strand bias." Cell **115**(2): 209-16.

Kim, Y., M. J. Cairns, R. Marouga and L. Q. Sun (2003). "E6AP gene suppression and characterization with in vitro selected hammerhead ribozymes." Cancer Gene Ther **10**(9): 707-16.

Kishino, T., M. Lalande and J. Wagstaff (1997). "UBE3A/E6-AP mutations cause Angelman syndrome." Nat Genet **15**(1): 70-3.

Kishino, T. and J. Wagstaff (1998). "Genomic organization of the UBE3A/E6-AP gene and related pseudogenes." Genomics **47**(1): 101-7.

Kiyono, T., S. A. Foster, J. I. Koop, J. K. McDougall, D. A. Galloway and A. J. Klingelhutz (1998). "Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells." Nature **396**(6706): 84-8.

Kiyono, T., A. Hiraiwa, M. Fujita, Y. Hayashi, T. Akiyama and M. Ishibashi (1997). "Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein." Proc Natl Acad Sci U S A **94**(21): 11612-6.

Klingelhutz, A. J., S. A. Foster and J. K. McDougall (1996). "Telomerase activation by the E6 gene product of human papillomavirus type 16." Nature **380**(6569): 79-82.

Koegl, M., T. Hoppe, S. Schlenker, H. D. Ulrich, T. U. Mayer and S. Jentsch (1999). "A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly." Cell **96**(5): 635-44.

Koepp, D. M., J. W. Harper and S. J. Elledge (1999). "How the cyclin became a cyclin: regulated proteolysis in the cell cycle." Cell **97**(4): 431-4.

Kubbutat, M. H., S. N. Jones and K. H. Vousden (1997). "Regulation of p53 stability by Mdm2." Nature **387**(6630): 299-303.

Kuhne, C. and L. Banks (1998). "E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box." J Biol Chem **273**(51): 34302-9.

Kumar, A., Y. Zhao, G. Meng, M. Zeng, S. Srinivasan, L. M. Delmolino, Q. Gao, G. Dimri, G. F. Weber, D. E. Wazer, H. Band and V. Band (2002). "Human

papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3." Mol Cell Biol **22**(16): 5801-12.

Kumar, S., A. L. Talis and P. M. Howley (1999). "Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination." J Biol Chem **274**(26): 18785-92.

Lee, S. S., B. Glaunsinger, F. Mantovani, L. Banks and R. T. Javier (2000). "Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins." J Virol **74**(20): 9680-93.

Li, S., S. Labrecque, M. C. Gauzzi, A. R. Cuddihy, A. H. Wong, S. Pellegrini, G. J. Matlashewski and A. E. Koromilas (1999). "The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha." Oncogene **18**(42): 5727-37.

Linares, L. K., A. Hengstermann, A. Ciechanover, S. Muller and M. Scheffner (2003). "HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53." Proc Natl Acad Sci U S A **100**(21): 12009-14.

Liu, X., H. Yuan, B. Fu, G. L. Disbrow, T. Apolinario, V. Tomaic, M. L. Kelley, C. C. Baker, J. Huibregtse and R. Schlegel (2005). "The E6AP ubiquitin ligase is required for transactivation of the hTERT promoter by the human papillomavirus E6 oncoprotein." J Biol Chem **280**(11): 10807-16.

Liu, Z., A. L. Haas, L. A. Diaz, C. A. Conrad and G. J. Giudice (1996). "Characterization of a novel keratinocyte ubiquitin carrier protein." J Biol Chem **271**(5): 2817-22.

Mantovani, F. and L. Banks (2001). "The human papillomavirus E6 protein and its contribution to malignant progression." Oncogene **20**(54): 7874-87.

Mantovani, F., P. Massimi and L. Banks (2001). "Proteasome-mediated regulation of the hDlg tumour suppressor protein." J Cell Sci **114**(Pt 23): 4285-92.

Matsuura, T., J. S. Sutcliffe, P. Fang, R. J. Galjaard, Y. H. Jiang, C. S. Benton, J. M. Rommens and A. L. Beaudet (1997). "De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome." Nat Genet **15**(1): 74-7.

McGrath, J. P., S. Jentsch and A. Varshavsky (1991). "UBA 1: an essential yeast gene encoding ubiquitin-activating enzyme." Embo J **10**(1): 227-36.

Michael, D. and M. Oren (2003). "The p53-Mdm2 module and the ubiquitin system." Semin Cancer Biol **13**(1): 49-58.

Milner, J. (2003). "RNA interference for treating cancers caused by viral infection." Expert Opin Biol Ther **3**(3): 459-67.

Miura, K., T. Kishino, E. Li, H. Webber, P. Dikkes, G. L. Holmes and J. Wagstaff (2002). "Neurobehavioral and electroencephalographic abnormalities in Ube3a maternal-deficient mice." Neurobiol Dis **9**(2): 149-59.

Munger, K., A. Baldwin, K. M. Edwards, H. Hayakawa, C. L. Nguyen, M. Owens, M. Grace and K. Huh (2004). "Mechanisms of human papillomavirus-induced oncogenesis." J Virol **78**(21): 11451-60.

Munger, K. and P. M. Howley (2002). "Human papillomavirus immortalization and transformation functions." Virus Res **89**(2): 213-28.

Muratani, M. and W. P. Tansey (2003). "How the ubiquitin-proteasome system controls transcription." Nat Rev Mol Cell Biol **4**(3): 192-201.

Nakagawa, S. and J. M. Huibregtse (2000). "Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase." Mol Cell Biol **20**(21): 8244-53.

Nakagawa, S., T. Yano, K. Nakagawa, S. Takizawa, Y. Suzuki, T. Yasugi, J. M. Huibregtse and Y. Taketani (2004). "Analysis of the expression and localisation of a LAP protein, human scribble, in the normal and neoplastic epithelium of uterine cervix." Br J Cancer **90**(1): 194-9.

Nakao, M., J. S. Sutcliffe, B. Durtschi, A. Mutirangura, D. H. Ledbetter and A. L. Beaudet (1994). "Imprinting analysis of three genes in the Prader-Willi/Angelman region: SNRPN, E6-associated protein, and PAR-2 (D15S225E)." Hum Mol Genet **3**(2): 309-15.

Nawaz, Z., D. M. Lonard, C. L. Smith, E. Lev-Lehman, S. Y. Tsai, M. J. Tsai and B. W. O'Malley (1999). "The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily." Mol Cell Biol **19**(2): 1182-9.

Nuber, U., S. E. Schwarz and M. Scheffner (1998). "The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate." Eur J Biochem **254**(3): 643-9.

Nykanen, A., B. Haley and P. D. Zamore (2001). "ATP requirements and small interfering RNA structure in the RNA interference pathway." Cell **107**(3): 309-21.

Oda, H., S. Kumar and P. M. Howley (1999). "Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination." Proc Natl Acad Sci U S A **96**(17): 9557-62.

Oh, S. T., S. Kyo and L. A. Laimins (2001). "Telomerase activation by human papillomavirus type 16 E6 protein: induction of human telomerase reverse transcriptase expression through Myc and GC-rich Sp1 binding sites." J Virol **75**(12): 5559-66.

Oren, M. (2003). "Decision making by p53: life, death and cancer." Cell Death Differ **10**(4): 431-42.

Pagano, M. and R. Benmaamar (2003). "When protein destruction runs amok, malignancy is on the loose." Cancer Cell **4**(4): 251-6.

Pan, H. and A. E. Griep (1995). "Temporally distinct patterns of p53-dependent and p53-independent apoptosis during mouse lens development." Genes Dev **9**(17): 2157-69.

Passmore, L. A. and D. Barford (2004). "Getting into position: the catalytic mechanisms of protein ubiquitylation." Biochem J **379**(Pt 3): 513-25.

Patel, D., S. M. Huang, L. A. Baglia and D. J. McCance (1999). "The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300." Embo J **18**(18): 5061-72.

Persengiev, S. P., X. Zhu and M. R. Green (2004). "Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs)." Rna **10**(1): 12-8.

Pickart, C. M. (2001). "Mechanisms underlying ubiquitination." Annu Rev Biochem **70**: 503-33.

Pines, J. and C. Lindon (2005). "Proteolysis: anytime, any place, anywhere?" Nat Cell Biol **7**(8): 731-5.

Pusch, O., D. Boden, R. Silbermann, F. Lee, L. Tucker and B. Ramratnam (2003). "Nucleotide sequence homology requirements of HIV-1-specific short hairpin RNA." Nucleic Acids Res **31**(22): 6444-9.

Reinstein, E., M. Scheffner, M. Oren, A. Ciechanover and A. Schwartz (2000). "Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: targeting via ubiquitination of the N-terminal residue." Oncogene **19**(51): 5944-50.

Rock, K. L. and A. L. Goldberg (1999). "Degradation of cell proteins and the generation of MHC class I-presented peptides." Annu Rev Immunol **17**: 739-79.

Rolfe, M., P. Beer-Romero, S. Glass, J. Eckstein, I. Berdo, A. Theodoras, M. Pagano and G. Draetta (1995). "Reconstitution of p53-ubiquitinylation reactions from purified

components: the role of human ubiquitin-conjugating enzyme UBC4 and E6-associated protein (E6AP)." Proc Natl Acad Sci U S A **92**(8): 3264-8.

Ronco, L. V., A. Y. Karpova, M. Vidal and P. M. Howley (1998). "Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity." Genes Dev **12**(13): 2061-72.

Rougeulle, C., H. Glatt and M. Lalande (1997). "The Angelman syndrome candidate gene, UBE3A/E6-AP, is imprinted in brain." Nat Genet **17**(1): 14-5.

Salvat, C., G. Wang, A. Dastur, N. Lyon and J. M. Huibregtse (2004). "The -4 phenylalanine is required for substrate ubiquitination catalyzed by HECT ubiquitin ligases." J Biol Chem **279**(18): 18935-43.

Scacheri, P. C., O. Rozenblatt-Rosen, N. J. Caplen, T. G. Wolfsberg, L. Umayam, J. C. Lee, C. M. Hughes, K. S. Shanmugam, A. Bhattacharjee, M. Meyerson and F. S. Collins (2004). "Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells." Proc Natl Acad Sci U S A **101**(7): 1892-7.

Scheffner, M., J. M. Huibregtse, R. D. Vierstra and P. M. Howley (1993). "The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53." Cell **75**(3): 495-505.

Scheffner, M., U. Nuber and J. M. Huibregtse (1995). "Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade." Nature **373**(6509): 81-3.

Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine and P. M. Howley (1990). "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53." Cell **63**(6): 1129-36.

Snove, O., Jr. and T. Holen (2004). "Many commonly used siRNAs risk off-target activity." Biochem Biophys Res Commun **319**(1): 256-63.

Spence, J., R. R. Gali, G. Dittmar, F. Sherman, M. Karin and D. Finley (2000). "Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain." Cell **102**(1): 67-76.

Spence, J., S. Sadis, A. L. Haas and D. Finley (1995). "A ubiquitin mutant with specific defects in DNA repair and multiubiquitination." Mol Cell Biol **15**(3): 1265-73.

Spitkovsky, D., F. Aengeneyndt, J. Braspenning and M. von Knebel Doeberitz (1996). "p53-independent growth regulation of cervical cancer cells by the papillomavirus E6 oncogene." Oncogene **13**(5): 1027-35.

Springael, J. Y., J. O. De Craene and B. Andre (1999). "The yeast Npi1/Rsp5 ubiquitin ligase lacking its N-terminal C2 domain is competent for ubiquitination but not for subsequent endocytosis of the gap1 permease." Biochem Biophys Res Commun **257**(2): 561-6.

Stubenrauch, F. and L. A. Laimins (1999). "Human papillomavirus life cycle: active and latent phases." Semin Cancer Biol **9**(6): 379-86.

Talis, A. L., J. M. Huibregtse and P. M. Howley (1998). "The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells." J Biol Chem **273**(11): 6439-45.

Thomas, M. and L. Banks (1999). "Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types." J Gen Virol **80 (Pt 6)**: 1513-7.

Thomas, M., R. Laura, K. Hepner, E. Guccione, C. Sawyers, L. Lasky and L. Banks (2002). "Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation." Oncogene **21**(33): 5088-96.

Thrower, J. S., L. Hoffman, M. Rechsteiner and C. M. Pickart (2000). "Recognition of the polyubiquitin proteolytic signal." Embo J **19**(1): 94-102.

Tong, X. and P. M. Howley (1997). "The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton." Proc Natl Acad Sci U S A **94**(9): 4412-7.

Traidej, M., L. Chen, D. Yu, S. Agrawal and J. Chen (2000). "The roles of E6-AP and MDM2 in p53 regulation in human papillomavirus-positive cervical cancer cells." Antisense Nucleic Acid Drug Dev **10**(1): 17-27.

Vaux, D. L. and J. Silke (2005). "IAPs, RINGs and ubiquitylation." Nat Rev Mol Cell Biol **6**(4): 287-97.

Veldman, T., I. Horikawa, J. C. Barrett and R. Schlegel (2001). "Transcriptional activation of the telomerase hTERT gene by human papillomavirus type 16 E6 oncoprotein." J Virol **75**(9): 4467-72.

Verdecia, M. A., C. A. Joazeiro, N. J. Wells, J. L. Ferrer, M. E. Bowman, T. Hunter and J. P. Noel (2003). "Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase." Mol Cell **11**(1): 249-59.

Weissman, A. M. (2001). "Themes and variations on ubiquitylation." Nat Rev Mol Cell Biol **2**(3): 169-78.

Welchman, R. L., C. Gordon and R. J. Mayer (2005). "Ubiquitin and ubiquitin-like proteins as multifunctional signals." Nat Rev Mol Cell Biol **6**(8): 599-609.

Werness, B. A., A. J. Levine and P. M. Howley (1990). "Association of human papillomavirus types 16 and 18 E6 proteins with p53." Science **248**(4951): 76-9.

Wilkinson, K. D. (2000). "Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome." Semin Cell Dev Biol **11**(3): 141-8.

Williams, C. A., H. Angelman, J. Clayton-Smith, D. J. Driscoll, J. E. Hendrickson, J. H. Knoll, R. E. Magenis, A. Schinzel, J. Wagstaff, E. M. Whidden and et al. (1995). "Angelman syndrome: consensus for diagnostic criteria.Angelman Syndrome Foundation." Am J Med Genet **56**(2): 237-8.

Yamamoto, Y., J. M. Huibregtse and P. M. Howley (1997). "The human E6-AP gene (UBE3A) encodes three potential protein isoforms generated by differential splicing." Genomics **41**(2): 263-6.

Zamore, P. D. (2002). "Ancient pathways programmed by small RNAs." Science **296**(5571): 1265-9.

Zamore, P. D., T. Tuschl, P. A. Sharp and D. P. Bartel (2000). "RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals." Cell **101**(1): 25-33.

Zeng, Y., R. Yi and B. R. Cullen (2003). "MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms." Proc Natl Acad Sci U S A **100**(17): 9779-84.

Zheng, N. (2003). "A closer look of the HECTic ubiquitin ligases." Structure (Camb) **11**(1): 5-6.

zur Hausen, H. (1996). "Papillomavirus infections--a major cause of human cancers." Biochim Biophys Acta **1288**(2): F55-78.

zur Hausen, H. (2000). "Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis." J Natl Cancer Inst **92**(9): 690-8.

zur Hausen, H. (2002). "Papillomaviruses and cancer: from basic studies to clinical application." Nat Rev Cancer **2**(5): 342-50.

7. Zusammenfasung

Die Ubiquitin-Proteinligase E6-AP wurde ursprünglich als ein Protein identifiziert, das mit dem E6-Onkoprotein humaner Papillomaviren (HPVs) interagieren kann. Bestimmte HPVs spielen bei der Entstehung des Cervixcarcinoms eine kausale Rolle. Dabei bindet der E6/E6-AP Komplex an das Tumorsuppressorprotein p53 und führt so zu dessen Ubiquitinierung und anschließendem proteasomalen Abbau. Außer p53 werden weitere zelluläre Proteine E6/E6-AP-abhängig ubiquitiniert und abgebaut. Die Rolle dieser Interaktionen für die Cervixcarcinogenese ist aber noch unklar.

Im Gegensatz zu den meisten Krebsarten, bei denen p53 entweder mutiert vorliegt oder überhaupt nicht exprimiert wird, wird in HPV-positiven Cervixcarcinomen Wildtyp-p53 exprimiert. Dies lässt die Annahme zu, dass eine funktionelle Inaktivierung von p53 durch E6/E6-AP mit einer Inaktivierung durch Mutation gleichgesetzt werden kann. Um die Bedeutung von E6-AP für die Lebensfähigkeit HPV-positiver Krebszellen zu untersuchen, wurde in dieser Arbeit die Methode der RNA-Interferenz angewandt, um die Expression von E6-AP spezifisch abzuschalten. Suppression der E6-AP-Expression resultierte in der Akkumulation von p53 und einer Wachstumsinhibition der behandelten Zellen. Des Weiteren reagierten HeLa-Zellen, in denen die Expression von p53 durch RNA-Interferenz stabil reprimiert ist, signifikant weniger sensitiv auf eine Inhibition der E6-AP-Expression als parentale HeLa Zellen. Diese Daten deuten darauf hin, dass die für das Wachstum HPV-positiver Zellen notwendige Aktivität von E6-AP auf seiner Eigenschaft basiert, im Komplex mit E6 p53 zu ubiquitinieren und somit zu dessen Abbau zu führen.

Mutationen im E6-AP Locus (UBE3A), die in einer funktionellen Inaktivierung von E6-AP resultieren, sind für eine erbliche neurologische Erkrankung, dem Angelman-Syndrom, verantwortlich. Um die Bedeutung von E6-AP für die Lebensfähigkeit von HPV-negativen Zellen zu untersuchen, wurde die E6-AP-Expression durch RNA-Interferenz entweder transient oder stabil reprimiert. Die Tatsache, dass es im Gegensatz zu HPV-positiven Zellen möglich war, die E6-AP-Expression stabil zu reprimieren, lässt den Schluss zu, dass unter den gegebenen Bedingungen HPV-negative Zellen weniger sensitiv auf eine Unterdrückung der E6-AP-Expression reagieren als HPV-positive Zellen. Allerdings war es nicht möglich, klonale Zelllinien zu erhalten, in denen die E6-AP-Expression vollständig abgeschaltet war. Dies unterstützt die Hypothese, dass auch für die Lebensfähigkeit HPV-negativer Zellen eine bestimmte Menge an E6-AP essenziell ist. Nichtsdestotrotz scheint für die Lebensfähigkeit HPV-negativer Zellen ein deutlich niedrigerer Level an E6-AP ausreichend zu sein als für HPV-positive Zellen. Daher mag E6-AP ein potenzielles Zielprotein für die Therapie von HPV-positiven Cervixcarcinomen sein.

8. Abstract

The cellular ubiquitin-protein ligase E6-AP was initially identified as a protein that interacts with the E6 oncoprotein of Human papillomaviruses (HPVs) associated with cervical carcinomas. Subsequently, this E6/E6-AP complex has been shown to bind and target the tumour suppressor protein p53 for ubiquitin-mediated proteasomal degradation. In addition to p53, the E6/E6-AP complex also targets other cellular proteins for ubiquitin-mediated proteasomal degradation. However, the significance of these interactions in cervical carcinogenesis remains unclear.

In contrast to most cancers wherein the p53 gene is mutated or not expressed, it is rarely mutated in HPV-positive cervical carcinomas. This has led to the assumption that functional inactivation of p53 by the E6/E6-AP complex is equivalent to its inactivation by mutation. To understand the significance of E6-AP for the viability of HPV-positive cells, in this study, E6-AP expression was down-regulated by RNA interference (RNAi) in HPV-positive cervical cancer cell lines. This resulted in p53 accumulation and growth suppression. Furthermore, HeLa cells, in which p53 expression was stably suppressed by RNAi, were found to be significantly less sensitive to the down regulation of E6-AP expression with respect to growth suppression than parental HeLa cells. These data indicate that E6-AP has anti-growth-suppressive properties in HPV-positive cells and that these depend on its ability to induce p53 degradation.

Mutations within the E6-AP locus (*UBE3A*) resulting in functional inactivation of E6-AP have been associated with Angelman syndrome, an inherited human neurological disorder. To further elucidate the importance of E6-AP for the viability of HPV-negative cells, E6-AP was down-regulated by RNAi either transiently or stably in HPV-negative tumour cell lines. In contrast to HPV-positive cell lines, it was possible to obtain clonal HPV-negative cell lines in which E6-AP was stably down-regulated by RNAi. This would indicate that under the given conditions, HPV-negative cells are less sensitive to E6-AP down-regulation than HPV-positive cells. However, the inability to obtain clonal cell lines in which E6-AP expression is completely abrogated supports the hypothesis that certain threshold levels of E6-AP expression is essential for normal cell viability. Taken together, these results suggest that in comparison to HPV-positive cells, HPV-negative cells require lower threshold levels of E6-AP expression for viability and thus selective targeting of E6-AP function may have therapeutic potential against HPV-induced cervical carcinogenesis.

9. Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie-abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Mats Paulsson betreut worden.

Köln, Nov 2005 ----------

(Michael D'silva)

Teilpublikationen im Rahmen dieser Arbeit:

Arnd Hengstermann*, Michael A. D'Silva*, Petric Kuballa, Karin Butz, Felix Hoppe-Seyler and Martin Scheffner (2005). "Growth suppression induced by downregulation of E6-AP expression in human papillomavirus-positive cancer cell lines depends on p53." J Virol **79**(14): 9296-300.

10. Lebenslauf

Name und Anschrift	Michael Aloysius D'silva Appt Nr. 529, West I, Werner-Sombart Str.37 78464, Konstanz Telefon: 0179-6837323
Geboren am	09 Juli, 1976
Geburtsort	Mysore, Indien
Staatsangehörigkeit	Indisch

Schulausbildung

1989-1992	**S.S.L.C** (Secondary School Leaving Certificate), Mysore, Indien
1992-1994	**P.U.C** (Pre-University Certificate), Mysore, Indien

Hochschule

1994-1997	**B.Sc** (Bachelor of Science), Universität zu Mysore, Mysore, Indien
1997-1999	M.Sc (Master of Science), Universität zu Mysore, Mysore, Indien
1999-2001	**Project Assistant** in der Arbeitsgruppe von Prof. Dr. H.S Savithri Indian Institute of Science, Bangalore Indien
2001-2005	**Promotion**, Universität zu Köln Thema: "Characterization of the Ubiquitin-Protein Ligase E6-AP by RNA interference" Durchführung der Promotion in der Arbeitsgruppe von Prof. Dr. Martin Scheffner am Institut für Biochemie I, Medizinische Fakultät, Universität zu Köln, Köln und am Fachbereich Biologie, Universität zu Konstanz, Konstanz. Betreuung der Promotion durch Prof. Dr. Mats Paulsson, Institut für Biochemie II, Universität zu Köln, Köln